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Investigating microbiomes and developing direct-fed microbials to improve cattle health

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INVESTIGATING MICROBIOMES AND DEVELOPING DIRECT-FED MICROBIALS TO IMPROVE CATTLE HEALTH

by

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Investigating microbiomes and developing direct-fed microbials to improve cattle health

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Over the last decade, global beef production has increased to meet the protein needs of the growing population. As a result, the intensification of beef cattle production systems has resulted in broad spectrum prophylactic use of antibiotics and growth promoting agents. With increased concern around antimicrobial resistance (AMR) and emergence of novel pathogenic strains of bacteria, it is critical to understand disease progression and associated changes in the microbiome to develop novel therapeutic alternatives to reduce antibiotic use and control disease. One such approach that is currently being investigated is the development of novel direct-fed microbial strains to outcompete pathogens by colonization resistance. Studies were conducted to develop a novel direct-fed microbial from the rumen to reduce the occurrence of liver abscesses in beef cattle and to investigate the progression of disease before and after an outbreak of infectious bovine keratoconjunctivitis (IBK) by longitudinal investigation of the bovine ocular microbiome.

To develop direct-fed microbials against liver abscess causing microbes (*Fusobacterium necrophorum necrophorum* and *Streptococcus bovis)* in the rumen, rumen fluid was obtained from fistulated cattle and a high throughput screening approach was implemented to isolate candidate bacterial species that inhibit the growth of *Fusobacterium necrophorum necrophorum* and *Streptococcus bovis.* Following in-vitro testing two candidate strains were identified and further characterized as potential candidates to be used as alternatives to antibiotics. Additionally, a longitudinal study was conducted in a cattle population consisting of 239 calves over 4 time periods to characterize changes in the ocular microbiome by sequencing the V4 region of the 16S rRNA gene. Both alpha and beta diversity analysis demonstrated changes in the bacterial community structure of the ocular microbiome post perturbation suggesting the bovine ocular microbiome is resilient to change. Factors such as age of cattle, time post perturbation, and cattle who were infected with IBK all showed a significant difference in the bovine ocular microbiome community composition ($p < 0.05$). Interestingly, bacteria who were deemed as “core taxa” were composed of opportunistic pathogens such as *Mycoplasma spp.* and *Moraxella spp.*

Dedication

I dedicate this work to my parents, siblings, friends, and those who helped with this project.

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1 Literature Review

1.1 Introduction

Beef has served as a major protein source in human nutrition. Within the last 25 years, global beef production has increased exponentially (FAO, 2008). By 2050, the demand for meat and milk is expected to double (FAO, 2008). As such, to meet this growing demand, production is expected to increase and as a result beef production has migrated to more intensive production systems. This increase in intensification has resulted in more genetic homogeneity and increased density in animal production systems leading to more susceptibility to disease. As a result, such practices have increased prophylactic use of antibiotics and growth promotants to ensure animal health and performance (McEwen and Fedorka-Cray 2002). With the current concerns of antimicrobial resistance and emergence of novel pathogens, new approaches are needed to control pathogen colonization and maintain animal health. The studies described here attempts to develop a novel direct-fed microbial as an alternative to antibiotics to control liver abscesses and characterizes the changes in the ocular microbiome during infectious bovine keraconjunctivitis (IBK) by longitudinal investigation of the bovine ocular microbiome. Below, is a literature review of studies describing the current status of liver abscesses in beef cattle and the bovine ocular microbiome.

1.2 Acidosis and Liver Abscess Introduction

Liver abscesses have become a growing concern within the beef cattle industry (Huebner et al., 2019). Current methods have been successful in decreasing the occurrences of liver abscesses. However, 10- 20% of cattle are still affected, resulting in a huge economic loss to the industry (Nagaraja et al., 2007). Post-harvest, cattle are

scored on a scale ranging from 0, A-, A, and A+ depending on the abundance of abscesses and scaring present. In brief, cattle who receive a score of 0 are animals who have no abscesses or scaring. Whereas, a score of A+ would mean the liver is condemned due to having multitude of liver abscesses present.

Currently, liver abscesses are being treated by a prophylactic, tylosin, which falls in the macrolide antibiotic family (Nagaraja et al., 2007). While tylosin has had great impact on the beef cattle industry, it does have some draw backs. Macrolide antibiotics are widely used in human health and the use in human medicine has increased dramatically in recent years (Hyde et al., 2001). Thus, the overuse of antibiotic classes, that are also used to treat human disease, can lead to antibiotic resistant pathogens. These pathogens can resist antibiotics currently used in the medical industry and thus, prophylactic use of antibiotics in the beef industry has come under great scrutiny (McEwen and Fedorka-Cray 2002).

The current hypothesis of liver abscess formation is described as a dysbiosis of the microbiome due to increase availability of fermentable carbohydrates in high grain diets leading to an increase in abundance of *Streptococcus bovis (S. bovis)* in the rumen (Constable 2015). *S. bovis* is a lactic acid producing organism commonly present within the rumen (Constable 2015). As such, when fermentable carbohydrates are abundant, *S. bovis* increases in abundance and changes its metabolism to homolactic fermentation (Constable 2015), resulting in increased lactic acid production. This shift enables the ruminal epithelium to become compromised, as a result of lactic acidosis to

occur (Constable 2015). Thus, the ruminal pH drops from ~6.5 to ≤5. This condition leads to ruminitis, a condition which dehydrates and “cracks” the epithelial layer of the ruminal wall, allowing for *Fusobacterium necrophorum,* the major pathogen involved in liver abscess formation, to enter into the liver via the portal blood flow (Figure 1) (Nagaraja et al., 2007). There are two biotypes of *Fusobacterium necrophorum* associated with liver abscesses: biotype A- *Fusobacterium necrophorum necrophorum* and biotype B- *Fusobacterium necrophorum funduliforme* (Nagaraja et al., 2007). However, *Fusobacterium necrophorum necrophorum (F. necrophorum)* is the primary organism associated with liver abscesses in cattle due to the leukotoxin produced by this

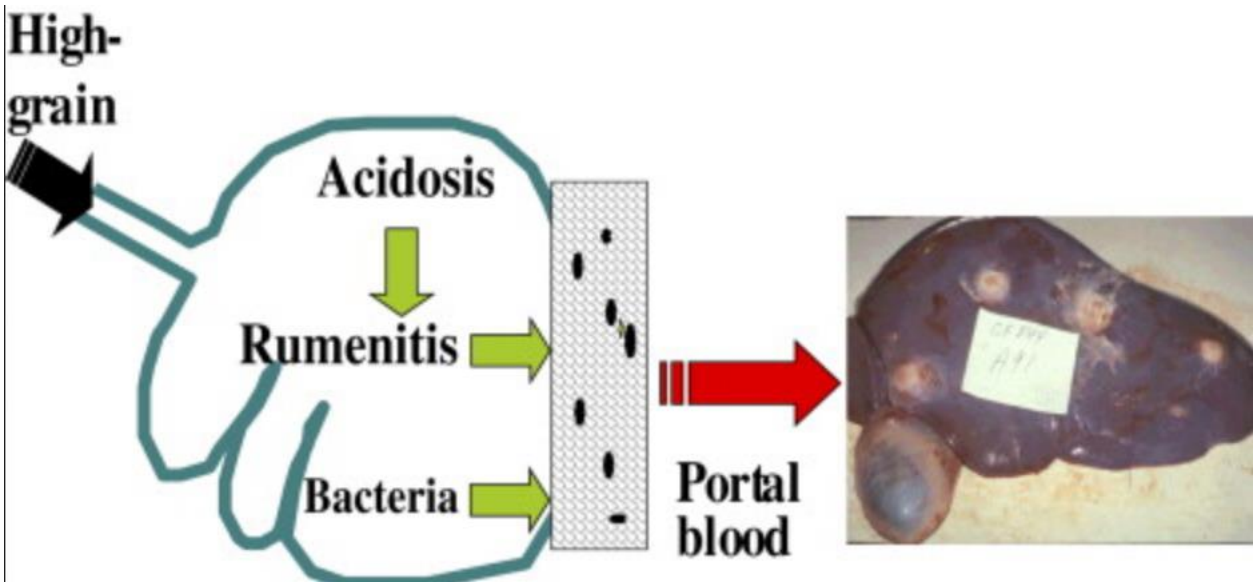

Figure 1. Current hypothesis of acidosis formation in fed cattle (reproduced from Nagaraja et al., 2007).

strain (Nagaraja et al., 2007). In brief, the leukotoxin produced is primarily cytotoxic to neutrophils, macrophages, hepatocytes, and possibly ruminal epithelial cells (Nagaraja et al., 2007). According to Nagaraja et al. (2007), the importance of leukotoxin being a virulence factor is the direct correlation between toxin production and laboratory animals induced with abscesses (Coyle-Dennis et al., 1979). Prior to reaching the liver via the portal blood flow and forming an abscess, *F. necrophorum* utilizes the by-product of *S. bovis* (lactic acid) for proliferation (Nagaraja et al., 2007). Once in the liver, *F. necrophorum* creates an anaerobic environment by creating a "pocket" or abscess.

In brief, virulence factors, as described above, plays a critical role in penetration, colonization and establishment of *F. necrophorum (*Nagaraja et al., 2007). While the liver is extremely vascular and oxygenated as well as full of phagocytic cells, such as leukocytes and Kupffer cells, penetration by *F. necrophorum* is able to occur due to the protease and dermonecrotic activity and the cytotoxic effect of the leukotoxin produced by *F. necrophorum* (Nagaraja et al., 2007). Thus, the leukotoxin is able to protect *F. necrophorum* from the highly oxygenated environment and allows for *F. necrophorum* to establish in the liver (Nagaraja et al., 2007). Additionally, the leukotoxin allows for protection to *F. necrophorum* to survive during phagocytosis while synergistically working with *Acranobacterium pygones (A. pyogenes)* to form an anerobic microenvironment by impairment of oxygen transport to damaged erythrocytes (Nagaraja et al., 2007). *A. pyogenes* is commonly isolated from liver abscesses (Nagaraja et al., 2007). However, *A. pyogenes* is typically seen in high abundance in cattle with condemned livers even when supplemented with tylosin (Nagaraja et al.,

2007). It is assumed that *A. pyogenes* does not facilitate *F. necrophorum* growth but rather intensifies liver abscess formation. However, a synergistic relationship between *F. necrophorum* and *A. pyogenes* has been described by Nagaraja et al. (2007).

1.3 Probiotics and Direct-Fed Microbes (DFM)

Interest in identifying the microbiome's potential as a therapeutic agent is rapidly growing (Fon and Nsahlai 2013). According to Elghandour et al. (2015) probiotics are "a live microbial feed supplement which affects the host animal in a beneficial way by improving intestinal microbial balance". Like probiotics, Direct-Fed Microbes (DFM) are live strains of microorganisms which are commonly utilized to impact and improve the rumen of production animals (Elghandour et al., 2015). Such microorganisms are used to improve the function of livestock productivity (Elghandour et al., 2015).

The two major types of DFMs that are currently used in the rumen include: bacterial or fungal strains (Elghandour et al., 2015). Bacterial DFMs can enhance homeostasis within the rumen or post-ruminal gastrointestinal tract (Elghandour et al., 2015). Various bacterial DFMs have been investigated for their role in maintaining ruminal pH. As such, findings have shown that some bacterial DFMs utilize lactate as a substrate, thus having a positive impact on ruminal pH (Elghandour et al., 2015). Studies have also shown that with certain DFMs, such as *Propionibacterium*, can modify rumen fermentation to increase the amount of propionate present in the rumen (Kung 2006). Thus, allowing increase energy flow to the host animal as propionate is the precursor for glucose synthesis via gluconeogenesis in ruminants (Elghandour et al., 2015). This can

be especially beneficial during early lactation of dairy cows and can be beneficial in improving energetic efficiency and ketosis (Weiss et al., 2008).

Colonization resistance against pathogens (Elghandour et al., 2015) is another avenue that DFMs can benefit the host. DFMs could compete for resources, or attachment to the epithelium against a targeted organism resulting in decreased colonization of the pathogen (Elghandour et al., 2015). Hydrophobic interactions limit the attachment in order to protect the animal against pathogens (Elghandour et al., 2015). DFMs can also secrete antibacterial compounds, such as bacteriocin and hydrogen peroxide, which have probiotic and competitive inhibiting characteristics (Elghandour et al., 2015). Recently, a newer mechanism for DFM has been proposed, where the DFM has the ability to modulate host immune function (Elghandour et al., 2015). This action is thought to occur in immune cells present within the post-ruminal gastrointestinal tract (Elghandour et al., 2015). Briefly, once the DFM is administered, the DFM is taken up by the intestinal epithelial cells via transcytosis (Elghandour et al., 2015), as a result, antigen cells, such as macrophages, engulf the DFM which then stimulates immune responses (Dicks et al., 2010).

Fungal DFMs have been used to act as a buffer in rumen fermentation (Elghandour et al., 2015). As such, these changes in normalizing rumen fermentation have shown to improve cattle performance. The mode of action for enhancements by fungal DFM is described as buffering effects, which keeps the rumen from sharp drops in pH (Elghandour et al., 2015). Fungal DFMs have also been shown to scavenge and

remove oxygen present on freshly ingested feed to increase digestion by the anaerobic microbes which in turn allows the rumen to become more efficient in nutrient utilization (Elghandour et al., 2015). Elghandour et al. (2015) explains that the effects of fungal DFMs, especially in regard to buffering, are typically subtle. Added yeast cannot prevent lactic acidosis and the effect of fungal DFM on rumen VFA production have been inconsistent while the lower gut has not shown any effects with the use of fungal DFMs (Elghandour et al., 2015).

1.4 Use of DFMs in beef cattle

The rumen is a complex environment that has a dense population of microbes. This complex community only has a selected number of microbes which have been investigated for DFM use. The majority of these investigations have been to manipulate fermentation (McAllister et al., 2011), however uses of fungal inoculation have been used to improve fiber digestibility (McAllister et al., 2011). As such, many investigations have implemented the use of lactic acid utilizing bacteria to increase performance (McAllister et al., 2011) by converting lactate to propionate. An example of some of these bacteria include *Megashaera elsdenii.* Additionally, studies have investigated the use of DFMs to change VFA profiles by reducing lactic acid production and increasing other VFAs produced (Elghandour et al., 2015 & McAllister et al., 2011).

With limited success, DFMs have also been utilized to increase fiber digestion by using DFMs such as *Ruminococcus flavefaciens* or *Ruminococcus albus* (McAllister et al., 2011). As described above, DFMs have been developed targeting lactic acid

metabolism (McAllister et al., 2011). Bacteria of such practice include *Megashaera elsdenii*, *Selenomonas ruminantium*, and *Propionibacterium freudenreichii* (McAllister et al., 2011). According to McAllister et al. (2011), lactic acid-producing (LAB) bacteria are present in the majority of DFMs used for cattle production. Current lactic acid-DFM products include representatives from the genera *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Enterococcus* species (McAllister et al., 2011). LAB has become a sought after DFM due to being commonly found in the environment as well as LAB its industrial tolerant. LAB has a high number of mechanisms to alter microbial communities (McAllister et al., 2011). While some of these interactions can disrupt the intracellular pH of bacterial competitors, those bacteria are also known to produce antimicrobials and as such may have beneficial effects by decreasing colonization of pathogens (McAllister et al., 2011).

Additionally, species belonging to genera *Bacillus* and *Bifidobacterium* have been considered as a potential DFM due to their high heat tolerance and stability (McAllister et al., 2011). According to McAllister et al. (2011), *Bacillus* species have been isolated from the rumen. However, they are low in abundancy and play a minor role in cell wall degradation. Similarly, *Bifidobacterium* has been isolated from the rumen but DFM uses has come from other isolation sources (McAllister et al., 2011). The role of *Bifidobacterium* in sugar metabolism and starch digestion is limited (McAllister et al., 2011). Instead, *Bifidobacterium* species are known to colonize the gut epithelium and prevent the occurrence of entero-virulent organisms (McAllister et al., 2011). Yeast cultures such as *Saccharomyces cerevisiae* have been used to enhance the

performance in dairy cows. Such practices have been used to increase dry matter intake, maintain ruminal pH, decrease lactate concentration, and organic matter digestibility (McAllister et. al., 2011).

1.5 Macrolide antibiotics

Macrolide antibiotics have widely been isolated from nature (Ōmura et al., 2002). These antibiotics greatly vary in diversity and can contain 8 to 62-membered lactone rings and carry one or more sugar moieties (Gaynor et al., 2003). Additionally, they are linked to various atoms around the ring (Gaynor et al., 2003). According to Ōmura (2002), macrolides can be simple carbocyclic monolactones as well as complex lactones that contain amino nitrogen, amide nitrogen, an oxazole ring or a thiazole ring within their skeletons. These group of compounds are a class of natural products that are synthesized by bacteria, fungi, and plants (Ōmura et al., 2002). Here, antibiotic production is done through fermentation using microbes (Najafpour 2007).

This family of antibiotics are known to treat infections caused by gram-positive bacteria (Gaynor et al., 2003). Early use of macrolides showed success in fighting infections but also had side effects resulting in increased acid stability. Thus, the antimicrobial activity that came with the use of macrolides, such as tylosin, was thought to be a solution to infections (Gaynor et al., 2003). However, with the extended use of this family of antibiotics, an increase of resistant strains has risen (Gaynor et al., 2003). While the understanding of what gene is expressing the resistance in macrolide antibiotics is not clearly know, it is thought to be within specific short peptides in the cell (Gaynor et al.,

2003). According to Gaynor et al. (2003), this could be a rendering effect to cells resistant to macrolides, suggesting the underlying mechanisms of inducible resistance and the mode of drug-induced inhibition of translation.

The macrolide inhibition of the ribosome is only understood in the most general terms. However, the binding site can be defined by the use of crystallographic structures. Gaynor et al. (2003) suggest RNA constitutes the primary component of the macrolide binding site. Nucleotide residues in domain V of the 23S rRNA interact with the macrolide molecule. Hydrophobic interactions are thought to be what contribute to more than 25% of the free binding energy of this antibiotic family. Sixteen-membered ring macrolides, such as tylosin, are thought to have a double reversible bond with the N6 of A2062 (Gaynor et al., 2003), suggesting a mutation for resistance within the 16-membered ring group. Sugar residues, ketolide binding, and multiple hydrophobic and hydrogen bonds can also account for resistance towards macrolides. Inhibition by macrolides can be described in four ways. According to Gaynor et al (2003), these modes of action include: 1. Inhibition of the progression of the nascent peptide chain during early rounds of translation; 2. Promotion of peptidyl tRNA dissociation from the ribosome; 3. Inhibition of peptide bond formation; and 4. Interference with the 50S subunit assembly.

Bacteria, that are gram-negative, adhere to the surface of the surfaces where they form a biofilm through sequential stages (Shinkai et al., 2008). When gram-negative bacteria are exposed to macrolide antibiotics such as Erythromycin, bacterial abundance is

decreased due to adherence to Type 4 basement membrane collagen (Shinkai et al., 2008). Macrolides inhibit guanosine diphospho-D-mannose dehydrogenase (GMD) production which reduces biofilm formation. However, due to some bacteria being located on the interior of biofilm, the bacteria are simply metabolically slowed rather than depleted. Therefore, the targeted bacterium can be protected from antibiotics. Macrolides given at high concentrations, to show effectiveness in culture, may be deemed as ineffective due to interior location. This gives the belief that gram-negative bacteria are conventionally resistant. (Shinkai et al., 2008).

In general context, antibiotics are known to kill bacteria, however some can be resistant. The two major types of resistant bacteria are intrinsic and acquired resistance. Intrinsic resistance entails bacteria whose cell features are responsible for preventing antibiotic action. Ōmura et al. (2002), describes this example as a cell envelope present in an organism gives rise to low permeability of the cells to many agents, thus making it effective against other bacteria. Acquired resistance occurs from mutations within the chromosomal genes, plasmids, or transposons. This occurs due to selective pressures or by misuse (Ōmura et al., 2002).

Enzymes can cause inactivation of macrolide antibiotics. These described enzyme inactivations occur due to degradation of macrolides or an enzyme that catalyzes the hydrolysis of various bonds such as C-O, C-N, or C-C (Ōmura et al., 2002). Inactivation can also occur with modifications of antibiotics. These occurrences allow an enzyme to

catalyze a phosphate or nucleotidyl group from a donor such as ATP to an antibiotic (Ōmura et al., 2002).

1.6 Bacteriocins

Most bacteriocins are unique as they are only toxic to other bacteria which are taxonomically related (Riley et al., 2002). Thus, making them differ from antibiotics. While little research has been done, close to 99% of all bacteria potentially produce at least one bacteriocin (Riley et al., 2002).

Bacteriocins are unstructured until they are exposed to structure promoting solvents to form a helical structure (Zacharof et al., 2012). They are cationic molecules that contain lysyl and arginyl residues in excess (Zacharof et al., 2012). Bacteriocins from gram-negative, gram-positive bacteria, and low molecular weight organisms showcase various properties. In brief, gram-negative bacteriocins can be characterized using three features: an amino-terminal translocation (T), a central receptor binding domain (R), and a carboxyl-terminal cytotoxic domain (C). The amino terminal translocation transfers via the outer member to the translocator protein. Central receptor-binding binds with a bacterial outer member receptor. The carboxyl-terminal cytotoxic domain simply has antibacterial activity (Cascales et al., 2007). Gram-negative bacteriocins act upon the outer membrane by penetrating the membrane of sensitive bacteria. There are two types of gram-negative bacteriocins. Group A colicins which are small, plasmid encoded, with a lysis gene. Whereas, group B colicins are large plasmid encoded without a lysis gene. Once these two groups enter the target cell, they can act in one of three ways: by formation of pores which promote leakage of cytoplasmic compounds

and result in cell death, nuclease type which digest DNA and RNA, or digest peptidoglycans (Cascales et al., 2007).

Both low molecular weight (microcins) and gram-positive bacteriocins are similar in their properties. As they both are ribosomally synthesized and contain hydrophobic peptides. Microcins contain a N-terminal leader peptide as well as core peptides. These types of bacteriocins are tolerant to heat, extreme pH, and proteases (Rebuffat, 2012). Microcins are classified into two types according to their molecular masses and disulfide bonds. Microcins do not have a lysis gene and are secreted outside of the bacteria using an ATP binding cassette (Duquesne et al., 2007).

Gram-positive bacteriocins are gene encoded bacteriocins with various sizes, structures, and properties. The three major types of gram-positive bacteriocins include: lantibiotics, non-lantibiotics, and large proteins. Lantibiotics contain peptide substances that can be characterized with polycyclic thioether amino acids lanthionine or methyllanthionine as well as unsaturated amino acids dehydroalanine and 2-aminoisobutyric acid (Zacharof et al., 2012). This class of lantibiotics can be further grouped based on their structure (linear vs globular) and mode of action. The linear structure has a membrane mode of action while the globular structure has a cellular enzymatic reaction. According to Zacharof et al. (2012), linear lantibiotic structures are positively charged molecules that have flexibility while the globular lantibiotic structures have either a net negative or no net charge. Both structures contain heat stable peptides that are modified after translation. This results in the formation of thioether

amino acids lanthionine and β-methyllanthionine which ultimately leads to the dehydration of a serine residue. Non-lantibiotics are a positively charged, heat stable molecules that has a non-lanthionine containing membrane of active peptides. Two classes of non-lantibiotics have been identified, those of which possess an N-terminal with a sequence of Try-Gly-Asn-Gly-Val-Xaa-Cys as well as a subclass (II b) that refers to a two-component bacteriocins that requires two peptides to work synergistically in order to generate antimicrobial activity (Zacharof et al., 2012). Large protein bacteriocins are heat stable molecules that have not been widely investigated. According to Nilsen et al. (2003) group A of large protein bacteriocins are bacteriolytic enzymes which kill sensitive strains by lysis of the cell wall. Whereas group B of large proteins are non-lytic.

1.7 Bacteriocins in Bacillus licheniformis

Bacillus licheniformis (B. licheniformis) has been isolated from the mammalian gastrointestinal tract as it is a gut symbiont. As such, this organism is a gram-positive bacterium that has been shown to produce bacteriocin-like peptides (Wang et al., 2017) which has been shown to aid in preservation of food from spoilage (Abriouel et al., 2011). Due to *B. licheniformis* not being widely investigated for its bacteriocin production, there is little information in regard to how and why it is producing bacteriocins. *Bacillus subtilis* (*B. subtilis*), a closely related bacterial species to *B. licheniformis*, has been investigated for its ability to produce bacteriocins and its mode of action. Thus, researchers have used genomic and physiological information from *B.*

subtilis to predict and understand the bacteriocins and their mode of action of *B. licheniformis* (Wang et al., 2017).

According to Stein et al (Wang et al., 2017), *B. subtilis* is able to produce more than two dozen bacteriocins which contain a variety of different structures. *B. subtilis* bacteriocin synthesis is activated by phosphorylation which then initiates the sporulation process (Wang et al., 2017). According to Wang et al. (2017), this phosphorylation is referred as Spo0A-P. Spo0A is the primary component of the transcriptional activator and repressor, which controls close to 120 genes. This gene transcription is the regulator for the start of development. Since Spo0A-P is the regulator of other genes, it activates the abrB gene which is thought to be coded for starvation-induced processes. This process is involved in sporulation and the production of antibiotics as well as degradative enzymes (Robertson et al., 1989). This transcriptional activator also helps activate more than 100 genes directly as well as indirectly. Since the abrB gene is part of the growth development stage, it is only found among cells during the exponential growth. The spo0A-P gene mediates the abrB gene throughout the stationary stage. Both spo0A-P and abrB genes are speculated to play an important role in *Bacillus* species during bacteriocin synthesis. Thus, insinuating that both spo0A-P and abrB genes are active in *B. licheniformis* (Figure 2).

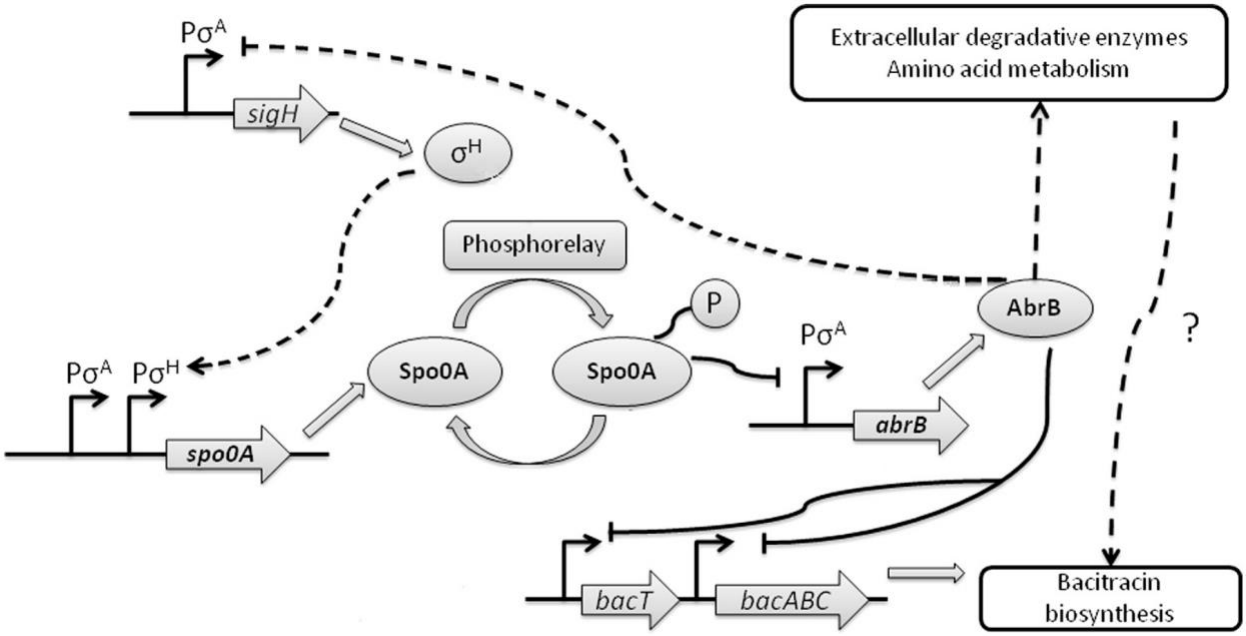

Figure2. Proposed spo0A-P and abrB gene activation. Reproduced from Wang et al., 2017.

Nisin is a commonly used bacteriocin within the industry and has been investigated in *B. licheniformis* (Zacharof et al., 2012). Nisin's mode of action includes rapidly binding to anionic liposomes (Zacharof et al., 2012). This allows nisin to form pores which interacts with the lipid membrane and peptidoglycan precursor lipid II. Which then depolarizes the transmembrane electric potential (Zacharof et al., 2012). According to Zacharof et al. (2012), the presence of lipid II disrupts the lipid bilayer. Nisin is able to form pores during this step and induces transmembrane movement. Nisin can insert the C-terminus and its mode of action has been described using a wedge like model. Zacharof et al. (2012), describes the wedge like model as follows: the induced pores involve a proton motive force driven by co-insertion of lipids and nisin domains. The nisin molecule may

allow bending of the C-terminal and then insert into the membrane. This then leads to disruption of the lipid protein pores. Hydrophobic forces occur and are the driving force of rearrangements among the lipid bilayer.

Tests conducted by Cladera-Olivera et al. (2004), demonstrated that *B. licheniformis* did have antimicrobial inhibitors as it was resistant to proteolytic treatments. This suggest that *B. licheniformis* contains unusual amino acids which makes the bacteria more resistant to proteases. Moreover, *B. licheniformis* was sensitive to pronase E and lost activity with Trichloroacetic acid treatment. According to Cladera-Olivera et al. (2004), this suggest that *B. licheniformis* is proteinaceous.

In recent years, Wang et al. (2017), conducted a similar test in which *B. licheniformis* growth was quantified using real-time PCR. Results showed that growth trends were similar in the exponential growth stage and minimal growth during stationary stage was achieved. During this study, transcription regulators were also tested in hopes to better understand bacteriocin production in *B. licheniformis*. These transcription regulators showed significant decreases in the cells during growth (Wang et al., 2017).

Previous studies have also shown that amino acids are essential part of the backbone of bacitracin. According to Haavik (1981), 25% of bacitracin molecule branched chains are composed of amino acids. In addition to the amino acids, soybean meal added to the media confirmed that nitrogen was a main source for bacitracin biosynthesis.

Similarly, Anthony et al. (2008), looked at the influence of medium components and fermentation condition on the production of bacteriocin by *B. licheniformis*. Among the parameters tested, *B. licheniformis* showed that it was induced by the production of yeast extract. As such, yeast extract supplement showed an increased bacitracin production of *B. licheniformis* when higher concentrations were added to media. According to Anthony et al. (2008), this could be due to the availability of amino acids. These results concluded the optimal fermentation conditions for *B. licheniformis* is dependent on pH, temperature, and incubation. Optimal conditions were determined in this study to be pH of 6.5, growth at 43°C for 24 hours with an increase in yeast extract concentration (45g/l) (Thangamani et al., 2008).

Alvarez-Ordóñez et al. (2013), investigated the antimicrobial activity of *B. licheniformis* in strains isolated from retail powered infant milk. These findings showed that *B. licheniformis* possesses a bacteriocin like compound called lichenin which inhibits pathogens (Alvarez-Ordóñez et al. 2013).

1.8 Bacteriocins in *Bacillus pumilus*

The *Bacillus* group, *sensu lato*, are considered good producers of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins (Abriouel et al., 2011; Stein 2005). With the presence of antimicrobial production in *Bacillus* strains, survival among habitats has been said to double. *Bacillus* species can be used as a food preservative due to their ability to produce antimicrobial peptides. One of the species that is of particular interest is *Bacillus pumilus* (*B. pumilus).*

Antimicrobial peptides that are ribosomally synthesized are commonly referred to as bacteriocins (Abriouel et al., 2011), and are a heterologous group of proteinaceous substances that are produced by every major bacterial lineage (Abriouel et al., 2011; Riley & Wertz, 2002a, b). According to Abriouel et al. (2011), bacteriocins produced by *Bacillus* species may be the second most important group of bacteriocins behind lactic acid bacteria (LAB) as *Bacillus species* produce an array of antimicrobial peptides with different chemical structures (Gebhardt et al., 2002; Stein 2005). While LAB bacteriocins has been classified, no classification scheme to date has been made for bacteriocin classification in Bacillus species (Abriouel et al., 2011). The *Bacilli* produce a vast diversity of peptides and proteins, thus, little information on the bacteriocin amino acid sequences have been described (Abriouel et al., 2011). *Bacillus* species can be classified in both Class I and II of LAB bacteriocins. However, the main classification is type A or type B. Type A includes elongated and positively charged lantibiotics whereas type B includes globular and noncharged lantibiotics.

B. pumilus is not well characterized for its bacteriocin-like peptides (Abriouel et al., 2011), however *B. pumilus* produces a plasmid-encoded peptide called pumilicins (Abriouel et al., 2011). Pumilicins are heat stable, up to 121°C for 15 minutes, and active within a broad pH range of 3-9 (Abriouel et al., 2011). Additionally, Abriouel et al. 2011 states that *B. pumilus* shows antimicrobial activity against MRSA, vancomycin-resistant, *E. faecalis*, and other gram-positive bacteria (Aunpad & Na-Bangchang, 2007).

A study conducted by Hill et al. (2009), demonstrated the inhibitory response of a novel strain of *B. pumilus* against marine pathogens. Shrimp aquaculture routinely uses antibiotics as a prophylactic and as therapeutic agent to reduce disease. However, the overuse of antibiotics causes both the pond and shrimp microbiota to change (Hill et al., 2009). Thus, Hill et al. (2009), investigated an alternative approach by looking for an isolate from the midgut of the microbiome shrimp. By looking at physiological and morphological characteristics, 16S rRNA analysis, and inhibition assays, the investigators concluded *B. pumilus* to be a viable alternative to the use of antibiotics. According to Hill et al. (2009), *B. pumilus* shows results of even being marginally inhibitory to itself, as well as other *Bacilli* species such as *B. licheniformis* and *B. subtilis*. While *in vitro* this investigation demonstrated promising results, Hill et al. (2009), still suggested an *in vivo* study is needed to convincingly demonstrate the inhibitory effect *B. pumilus* on marine pathogens.

1.9 *Bacillus* species in Livestock Applications

Bacillus strains have been used as probiotics among livestock animals to improve body weight and to inhibit colonization of pathogenic bacteria (Abriouel et al., 2011). *B. licheniformis* has been reported to inhibit both *S. bovis* and *Eubacterium ruminantium* due to its lichenin properties (Abriouel et al., 2011; Pattniak et al., 2001).

In a study conducted by Rojo et al. (2005), *B. licheniformis* was used in an assay to compare amylolytic activity of exogenous alpha-amylase. In addition, *B. licheniformis*

was investigated *in vitro* to analyze enzyme degradation and digestibility. Results demonstrated that amylolytic activity of *B. licheniformis* to be optimal at a pH range of 5.0 to 8.0 at 76°C. This explains the increased activity of *B. licheniformis* in the rumen under standard conditions (Rojo et al., 2005).

In vitro studies demonstrated a response to ruminal starch digestion with amylase from *B. licheniformis* suggesting its ability to increase in dry matter digestibility (Rojo et al., 2005). As such, this suggested increased digestibility of *B. licheniformis* increased ruminal digestion and could be considered as an alternative in ruminants fed high grain diets.

A recent study found *B. licheniformis* to be isolated from the rumen of buffalo showed inhibition against multiple bacterial species (Pattnaik et al., 2001). With further peptide analysis, Pattnaik et al. (2001), found lichenin to be the bacteriocin associated with this particular strain of *B. licheniformis*. Lichenin is a twelve base pair peptide which is produced anaerobically during the late logarithmic growth phase. Additionally, lichenin is stable under high heat conditions and a broad range of pH. However, the crystallography structure of lichenin found among *B. licheniformis* has not yet been identified.

Ultimately, bacteriocins, derived from Bacillus strains, may have a wide array of uses and needs further investigations. According to Abriouel et al. (2011), use of *Bacillus* strains in the food industry could be beneficial as Bacilli could solve many of the

limitations found in LAB bacteriocins. Additionally, this bacterium has the potential to reduce bacterial pathogens identified in the human health sector, improve animal health, and promote plant growth (Abriouel et al., 2011).

1.10 Introduction to investigation of infectious bovine keratoconjunctivitis

With the importance of the role of the microbiome in animal health and productivity emerging (Song et al., 2019), more and more studies are developing DFMs to improve animal health and performance. However, before using DFMs to manipulate the microbiome it is important to understand microbial community changes and how dysbiosis of the microbiome affect animal health and productivity. Therefore, studies are needed to investigate the microbiome over time to evaluate baseline microbiomes and how microbiomes change or adapt due to environmental changes and pathogen colonization. Below we summarize studies related to microbial changes during infectious bovine keratoconjunctivitis and how longitudinal microbiome studies can help better understand microbiome changes and adaptations to pathogens and environmental changes.

1.11 Infectious Bovine Keratoconjunctivitis

A commonly found disease among beef cattle is infectious bovine keratoconjunctivitis (IBK), or better known as “pinkeye”. Cattle infected with IBK cost the beef cattle industry close to 150 million dollars annually (Whittier et al., 2009). This economic loss stems from decreased average daily gain, decreased milk production, as well as treatment costs. A study conducted by Whittier et al. (2009), reported that fed cattle with IBK had

a decrease in weaning weight of 19.6 pounds when compared to healthy calves at weaning. Additionally, IBK is the most common condition affecting breeding age beef heifers where, most cattle who are affected by IBK in both eyes are more prone to death due to starvation or death by an accident (Whittier et al., 2009).

According to Whittier et al. (2009), IBK is a highly contagious disease that causes inflammation of the cornea (clear outer layer of the eye), and inflammation of the conjunctiva (the pink membrane lining of the eyelids). Ulcerations occur within the eye and form what looks like a hole or depression within the cornea. IBK is reported to be caused by bacteria, *Moraxella* and *Mycoplasma*, but eye irritation must occur first in order for infection to become an issue as both opportunistic pathogens are common residents among the microflora in the eye. As such, *Moraxella* and *Mycoplasma* are known as a pre-disposer of the infection. Eye irritation can occur from face flies, which feed around the eyes of cattle, or other sources such as tall grass or feed from feed bunks (Whittier et al., 2009). Additionally, it is reported that UV light from the sun can make cattle with lighter pigment more susceptible to pinkeye as it increases their sensitivity and decreases their immune system (Whittier et al., 2009). Examples of light pigmented beef cattle breeds are Hereford, Charolais, as well as F1 Baldie crosses. Angelos (2010), reported that cattle with more periocular pigmentation, such as Brahmans, are less susceptible to pinkeye.

The eye, nose, or vagina can all be infected *Moraxella* or *Mycoplasma*, which can be secreted and transmitted by contact. Face flies are the primary transmitter of *Moraxella*

or *Mycoplasma* as they feed around the eyes and nostrils (Whittier et al., 2009). Animals could be asymptomatic for *Moraxella* or *Mycoplasma* infection (Whittier et al., 2009). Asymptomatic carrier animals shed *Moraxella* or *Mycoplasma* for long periods of time, thus infection among healthy animals can occur during the winter months (Whittier et al., 2009).

IBK can be treated but has the potential to progress through four stages if left untreated (Whittier et al., 2009). “During stage one of IBK, cattle will have excessive tearing and increased sensitivity to light. Cattle will frequently blink while having redness along the eyelids. Additionally, cattle will often seek shade, decrease feed intake/grazing time, and a small ulcer will progress in the center of the cornea. This ulcer will appear as a small white spot within the cornea” (Whittier et al., 2009). Stage two consists of an ulcer further spread across the cornea as well as the signs described in stage one. During this stage, the cornea begins to become cloudy and blood vessels begin to become enlarged to help recover the cornea. Thus, the cornea appears pink, giving the disease its name (Whittier et al., 2009). Once the ulcer covers most of the cornea, inflammation continues into the inner eye, (Whittier et al., 2009), suggesting the progression of IBK is in stage three. The eye color changes from its natural brown color to a yellow shade as fibrin, a pus like substance, fills the eye. In the last stage (stage four), the ulcer is completely through the cornea and the iris may protrude the ulcer. After healing, the iris is stuck in the cornea, resulting in blindness (Whitter et al., 2009). Depending on the severity, the eye may stay a blue cloudy to white color once the blood vessels recede.

Treatment of IBK is crucial in prevention of shedding of *Moraxella* or *Mycoplasma* further into herds. According to Cullen et al. (2017), prevention of IBK is preferred. However, antibiotic treatment is effective in reducing the duration of IBK. Treatments for eliminating the duration of IBK can be done as followed: stage one and two can be treated with long-acting tetracyclines such as LA200®. According to Whittier et al. (2009), the recommended dose is 4.5cc per hundred weight subcutaneously (SQ). Additionally, a second injection 48-72 hours later increases the percentage of cattle which responds to treatment (Whittier et al., 2009). Veterinarians perform “bulbar conjunctival injections” (Whittier et al., 2009), which is an injection of penicillin and dexamethasone in the bulbar conjunctiva of the eye. This causes swelling and a bulge to form. Stage three and four can be treated as stated above with the addition of an eye patch or suturing the third eyelid over the eye. Benefits of suturing the third eyelid include: reducing irritation which reduces tearing and shedding of *Moraxella* or *Mycoplasma* as well as supporting the cornea (Whittier et al., 2009).

Currently, prevention for IBK has been limited. Proper management practices can reduce the occurrence of outbreaks; however, this practice is still limited. According to Whittier et al. (2009), controlling flies are essential. Within a given day of moderate to heavy fly infestation, cattle can have ten to 20 flies around their eyes at any time (Whittier et al., 2009). Prevention methods can include fly tags, insecticide pour-ons, back rubbers, and knock-down sprays (Whittier et al., 2009). Manure management, through feed additives or dung beetles, can impact decreasing the prevalence of flies. Pesticide resistance among flies can increase if the class of drug is not switched from

year to year or if flag tags are not removed in the fall. Grazing management and keeping pastures clipped also impact the prevalence of IBK. According to Whittier et al. (2009), preventing seed head development in pastures, reduces irritation to the eyes of cattle as well as reducing the resting areas for flies. Additionally, a good vaccination and nutrition program improve the overall fitness of cattle and can reduce the occurrence of IBK. Commonly, operations that have nutritional imbalances, including protein, energy, vitamin, and mineral deficiencies, have higher outbreak rates of IBK comparatively.

1.12 Moraxella

1.12.1 Moraxella bovis

Moraxella bovis (*M. bovis*), a gram-negative coccobacillus bacterium, and is one of the primary implicator of ocular disease in cattle (Angelos et al., 2004). This hemolytic and nonhemolytic pathogen is commonly found in nature (Angelos et al., 2004). However, according to Angelos et al. (2004), pathogenicity has been found to be associated with the expression of pilin proteins and a hemolytic cytolytic toxin (cytotoxin). While *M. bovis* has hydrolytic enzymatic expression, pilin and cytotoxin protein (MbxA). According to Angelos et al. (2007), MbxA is encoded with a classical RTX operon which is comprised of *mbx*CABS*tol*C. These cytotoxins cause corneal ulceration due to the bacterium being RTX (repeats in the structural toxin) which is lytic for bovine neutrophils, erythrocytes, lymphocytes, and corneal epithelial cells (Angelos et al., 2004).

1.12.2 Moraxella bovoculi

Similarly, *Moraxella bovoculi* plays a critical role in IBK, however, the pathogenic role of *Moraxella bovoculi* is unknown (Angelos et al., 2011 & Dickey et al., 2016). According to

Angelos et al. (2011), *Moraxella bovoculi* has a RXT operon that encodes an RTX toxin designed to MbvA, which is 83% similar to *Moraxella bovis* amino acid sequence identity to MbxA.

1.13 Mycoplasma

1.13.1 Mycoplasma bovis

Mycoplasma bovis plays a critical role in chronic infections. With no vaccine to date (Bürki et al., 2015), no prevention can occur. Thus, the use of antibiotics has become scarcely efficient. While more common occurrences of *Mycoplasma bovis* is found among cattle diagnosed with bovine respiratory disease, mastitis, or genital disorders, the organism has still been found among IBK infections.

According to Bürki et al. (2015), *Mycoplasma bovis* infection begins with adhesion, thus making the interaction with the host cells crucial for survival. *Mycoplasma bovis* lack genes and have a small genome, therefore they acquire essential substances for biosynthetic pathways such as amino acids, nucleotides, and lipids (Bürki et al., 2015). Additionally, *Mycoplasma bovis* has different cell invasion interactions. Bürki et al. (2015), states depending on the cell type and time of invasion, *Mycoplasma bovis* can be associated with cytosolic side of the cell membrane, with vacuole like structures as well as diffuse distribution. Biofilm formation has been observed in *Mycoplasma bovis* strains that are persistent (Bürki et al., 2015). These biofilms have been seen to increase reactive oxygen species as well as reactive nitrogen species due to the attraction of phagocytes.

1.14 IBK intervention studies

Previously, other researchers have tested the applicability of developing vaccines using pilin as a target and have seen pilus-based vaccines to be the most effective in reducing IBK (Angelos et al., 2004). Differences in the pilin gene in different serogroups and the inversions of the pilin gene has resulted genetic variability across strains, thus, causing variability in responses to the pilus-based vaccine when used against other *M. bovis* serogroups. However, *M. bovis* cytotoxin appears to be more conserved against different strains as hemolysin neutralizing antibodies develop in cattle with IBK (Angelos et al., 2004) have shown more consistent results.

A study conducted by Angelos et al. (2004), tested the prevalence of IBK when treated with a recombinant *M. bovis* cytotoxin-ISCOM matrix adjuvanted vaccine. Ulcer area measurements were taken, and tear and serum were collected at weeks zero and seven. Results found that the recombinant vaccine group showed fewer ulcerated calves comparatively to the saline control group up to week 12. By week 20, the differences between the two treatments was not significant among the groups ($p = 0.131$), but still was the lowest among the recombinant group (Angelos et al., 2004). Tear and serum IgA ratios were significantly different between the recombinant vaccine nonulcerated calves compared to both the saline and adjuvant nonulcerated calves. Age did seem to play a role as calves significantly older in the saline group showed less ulcer development by week seven. Calves among the adjuvant and recombinant vaccine groups showed no age difference. Ultimately, results suggest that further studies need to be conducted. However, *M. bovis* cytotoxin based vaccines show promising results in prevention of IBK (Angelos et al., 2004).

Angelos et al. (2010), conducted a second study using a recombinant *Moraxella bovoculi* (*M. bovoculi*) cytotoxin-ISCOM matrix adjuvanted vaccine to reduce IBK. Novel specie of the Moraxella family, *M. bovoculi*, was isolated from ulcerated cattle. This *M. bovoculi* strain had a similar RTX which encodes an RTX toxin that is designated MbvA which had 83% deduced amino acid sequence to MbxA, also known as the *M. bovis* cytotoxin (Angelos et al., 2011). Ulcer surface area measurements and blood serum were taken. Results showed no difference among the recombinant *M. bovoculi* and the adjuvant vaccine. However, bacterial isolates from ulcers deemed a larger portion showing dominance of *M. bovis*. According to Angelos et al. (2010), it is possible that the *M. bovoculi* cytotoxin vaccine may be more effective among herds which have a majority of IBK cases stemming from bacterium related to *M. bovoculi*. It is still unsure if *M. bovoculi* is the primary pathogen among IBK cases. Thus, investigating the resident microflora of healthy cattle eyes and IBK infected cattle are necessary to establish mechanisms of infection and to develop more effective prevention strategies.

In a study conducted by O'Connor et al. (2011), with the primary hypothesis to test *M. bovis* autogenous vaccine did not associate with the incidence of IBK. A secondary hypothesis was also implemented in looking at the correlation between receiving the vaccination with *M. bovis* and weight at the end of the study. Both null hypotheses were not rejected as no significant difference was seen. Conclusions stated that *M. bovis* autogenous vaccines consistently fail to offer protection. However, there may have been some bias in this study as the herd could have developed an immunity to the vaccine.

Still, other studies suggest that the autogenous *M. bovis* vaccines are not universally effective (O'Connor et al., 2011).

1.15 Microbiome Analysis in IBK

With limited investigations on the ocular microbiota and the incidence of IBK, Cullen et al. (2017), conducted a study comparing bacterial communities of calves who developed IBK in comparison to calves who did not to assess potential causation of infection. The main objective of this study was to identify bacterial agents which can potentially enhance the occurrence of IBK. To date, this study is the first to look at the ocular microbiota in cattle (Cullen et al., 2017).

Cullen et al. (2017), set eligibility requirements for calves to have no ocular lesions at enrollment of the study and evaluated the microbiome in IBK using a randomized controlled trial. All calves were monitored from June of 2015 to November of 2015 while still on the dam. The trial consisted of three cohorts: A, B, and C. This division was due to the already implanted separation of cattle herds at Iowa State University (Cullen et al., 2017). Group A consists of first calving heifers and their parity whereas group B and C are mature cows with second or greater parity. Initially, calves from all three cohorts were enrolled within the trial as long as they had not shown any IBK infection prior to the start of the study. Calves enrolled had eye swabs taken prior to the study and after as well as if lesions were present during. Additionally, calves enrolled in the study had weights taken at the start and end of the study.

Due to the vaccination that was given to the control group being ineffective, cattle were intermingled irrespective of if they received a vaccination or not. In both the control and enrolled group of calves, eight swabs were selected from each group at random, giving 16 samples per management groups (Cullen et al., 2017). Calves who had incidences of IBK were treated according to manufactures recommended dosage of antibiotics florfenicol (Nuflor, Merk Animal Health) or oxytetracycline (Liquamycin LA-200, Zoetis) (Cullen et al., 2017). When calves were treated, as a result of IBK, swabs were collected from the control group at the same time for microbiome analysis. Equal number of swabs from both the enrolled and control group were used for DNA extraction and were used for 16S rRNA sequencing using Illumina MiSeq platform. A total of 48 samples were analyzed to evaluate microbiome changes during IBK. According to Cullen et al. (2017), negative controls were not included into sequencing. However, Cullen et al. (2017), made several efforts to reduce experimental errors which could cause contamination.

Raw reads were analyzed using the QIIME (v1.9) pipeline (Kuczynski et al., 2011). After assembling and quality filtering reads, the dataset included 6,789,231 high quality contigs, with 31,963 unique contigs. According to Cullen et al. (2017), alpha diversity showed no significant difference in species richness between both the enrolled groups and control calves. Additionally, OTU specie richness did not have a significant difference among the groups although higher microbial diversity was seen among the enrolled group.

Microbial composition showed 56.51% of phyla came from *Tenericutes*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. Cullen et al. (2017), states the genera frequently associated with IBK, *Mycoplasma* and *Moraxella*, were identified as one of the top ten most abundant genera found within the samples. Among the control group, *Mycoplasma* and *Moraxella* were more prevalent compared to the enrolled group.

Among the three groups, alpha and beta diversity showed significant differences within species richness. within groups B and C in comparison to group A when using Cohen's d=1.9 (Cullen et al., 2017). Observed OTUs were significantly different in species richness when comparing groups B and C when using Cohen's d=0.88. (Cohen's d is used to indicate the standard size difference between two means). However, no significant differences were reported when comparing the study groups when using Faith's Phylogenetic Diversity analysis. Faiths Phylogenetic diversity can be described as the sum of all branch lengths connecting all species to a targeted lineage (Faith 1992). However, there was a clear separation among enrolled groups when looking at beta diversity (Cullen et al., 2017). While the microbial composition consisted of five abundant phyla including *Tenericutes, Proteobacteria, Firmicutes, Bacteroidetes,* and *Actinobacteria*, the most abundant genera was *Moraxella* and *Mycoplasma*. Both *Moraxella* and *Mycoplasma* were the most abundant in group B.

Conclusions from Cullen et al., 2017 showed that there were no large-scale bacterial community differences between the groups at the genera level. However, Faith's PD, results demonstrated that there was a greater diversity between the enrolled calves

and the controls. Additionally, Cullen et al. (2017), concludes that results show *Moraxella* was not significantly different among the controls and the enrolled cattle, even though it was one of the more abundant genera. Other studies have shown vaccines against both *M. bovis* and *M. bovoculi* are not effective in control outbreaks, which Cullen et al. (2017), confirmed. Limitations in this study include, dam parity, management and organization, timing among collecting samples, and limited characterization of the cattle eye microbiota (Cullen et al., 2017). Cullen et al. (2017), states that it is difficult to find the correct “window” in identifying a predictive microbiota thus, establishing a prediction of when disease may occur is insufficient and the samples end up being a measure of the difference in the microbiotas. Therefore, we believe longitudinal studies are needed to better understand microbiome changes and the effect of the ocular microbiome change on disease occurrence and progression.

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2 Developing Direct fed microbials to reduce liver abscesses

2.1 Abstract

An increase in beef production to meet future food demand has led to an intensified production system. This has led producers to implement the use of antibiotics as prophylactics in order to ensure animal health and productivity. As a result, the antimicrobial resistance (AMR) is at an all-time high and the beef industry has come under great scrutiny. Thus, an alternative approach to prophylactic use of antibiotics is greatly needed. Probiotics of bacterial origin have been utilized as a natural method to replace antibiotics and has shown to improve animal health. Currently, several bacterial species have been isolated from the rumen showing promising antimicrobial properties in controlling the growth of acidosis forming bacteria *Streptococcus bovis* (*S. bovis*) and liver abscess forming bacteria *Fusobacterium necrophorum necrophorum* (*F. necrophorum necrophorum*). In this study, bacterial strains were isolated using various isolation strategies and the resulting isolates were subjected to high throughput screening to identify bacterial strains with the ability to inhibit both *S. bovis* and *F. necrophorum necrophorum*. Positive cultures, showing consistent inhibition, were then characterized by 16S rDNA sequencing and whole genome sequencing. Of the isolates, Generally Recognized as Safe (GRAS) strains were used for further *in vitro* experiments to identify optimal growth conditions and their effects on rumen digestibility. These strains are currently being utilized in an ongoing *in vivo* experiment. This study demonstrates the potential of using probiotics as an alternative to antibiotics to control acidosis and liver abscesses.

Keywords: probiotics, Generally Recognized as Safe, liver abscesses

2.2 Introduction

By 2050, the global demand for beef as a major protein source is projected to double, thus an intensified production system is needed to meet the growing population demand (FOA, 2006). As a consequence, the increased prophylactic use of antibiotics to manage animal health has resulted in the rise of antibiotic resistant bacteria (Threlfall et al., 2000). Thus, novel methods are needed to reduce the use of antimicrobials while still maintaining efficient and healthy cattle. One such approach is targeting pathogenic and opportunistic pathogenic microbes such as *Streptococcus bovis (S. bovis)* and *Fusobacterium necrophorum necrophorum (F. necrophorum necrophorum)* using bacterial probiotic strains to outcompete the target organism using competitive exclusion to replace the use of antibiotics while still improving animal health and productivity (McEwen and Fedorka-Cray 2002)

Liver abscesses continue to be a concern among the beef cattle industry (Huebner et al., 2019). While current methods have shown to be effective in reducing the occurrence of liver abscesses, 10 to 20% of fed cattle are still affected, causing an economic loss to the industry (Nagaraja et al., 2007). Depending on the number of liver abscesses and scarring present, cattle are given a score post-harvest ranging from 0 to A+. Cattle who are deemed a score of A+ are condemned and ultimately cause a huge economic loss in terms of hot carcass weight. A prophylactic antibiotic, tylosin, is being used to treat liver abscesses and has been shown to reduce the occurrence of liver abscessed up to 70% (Nagaraja et al., 2007). However, tylosin has fallen under great scrutiny as it is from the same Macrolide antibiotic family that is widely used in human health (Nagaraja et al., 2007). Thus, causing concern that the overuse of tylosin could

increase antibiotic resistant bacteria making current antibiotics for human health ineffective (Hyde et al., 2001).

The current hypothesis of liver abscess formation is described as a dysbiosis of the microbiome due to increase availability of fermentable carbohydrates in high grain diets leading to an increase in abundance of *Streptococcus bovis (S. bovis)* in the rumen (Constable 2015). As such, when fermentable carbohydrates are abundant *S. bovis* increases in abundance and changes its metabolism to homolactic fermentation (Constable 2015), resulting in increased lactic acid production which compromises the ruminal epithelium and causes ruminitis (Constable 2015). Ruminitis is characterized as "inflammation of the rumen by a way of carbohydrate engorgement" (Constable 2015). Once the rumen epithelial layer is compromised, *F. necrophorum necrophorum* can enter into the liver via the portal blood flow (Nagaraja et al., 2007). Once *F. necrophorum* reaches the liver, *F. necrophorum necrophorum* begins to create lesions which in turn generates abscesses.

Probiotics have gained interest as having potential to increase health and to provide beneficial effect in the gut (Hemarajata et al., 2013). According to Elghandour et al. 2015, probiotics are a live microbial supplement, which effects the host in a beneficial way by improving the intestinal microbial balance. Similarly, directly fed microbials (DFM) are live strains of microorganisms or their fermentative products which are commonly found within the digestive tract and are used to improve livestock health and productivity.

This study was designed to isolate beneficial bacteria from the rumen with the capabilities to inhibited *S. bovis* and *F. necrophorum necrophorum* to reduce the occurrence of liver abscesses in fed cattle. To this end, we isolated a large library (n=4,692) of strains using various isolation techniques and performed high throughput functional screening to identify potential probiotic strains and characterized the identified isolates using 16S rRNA sequencing and whole genome sequencing. Finally, we performed *in vitro* and *in vivo* testing to evaluate the ability of the identified strains to reduce *S. bovis* and *F. necrophorum* populations and the applicability of the strains as direct-fed microbials.

2.3 Materials and Methods

2.3.1 Rumen fluid collection and preparation of isolation

All animal-related intervention protocols and husbandry practices were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska-Lincoln and US Meat Animal Research Center under separate IACUC protocols. Rumen fluid was collected from fistulated cattle, through the rumen fistula, in animals housed within the metabolized area of the University of Nebraska-Lincoln. Rumen samples were collected from six different animals from an ongoing study that were fed six different diets (Table 1) All collected rumen samples were individually processed individually by straining 100mL of rumen fluid through four layers of cheese cloth into a thermos to make a total collection volume of 50mL per individual. Each rumen fluid sample was prepared individually by mixing 15mL of 1X sterile phosphate buffered saline (PBS) with 30mL of collected rumen fluid. The mixture was agitated for five

minutes and incubated in a 70°C water bath for one hour. After incubation, each rumen fluid sample was serially diluted and 50ul inoculants were spiral plated using the Eddy Jet spiral plater in duplicate on BD BBL™ ISP1, BD BBL™ ISP2, BD BBL™ ISP4, BD BBL™ Brain Heart Infusion (BHI), and Casein Starch media (USBiological Life Sciences). Media was prepared according to manufactures directions with the modification of adding Cycloheximide (VWR International Solon, OH) at a concentration of 50 ug/ml and Nalidixic Acid (ACROS Organics, NY) at a concentration of 20 ug/ml prior to pouring of plates to kill vegetative cells.

The plates were incubated both aerobically and anaerobically for up to 96 hours at 37°C. Colonies were picked every 24 hours and placed in 150ul of BHI broth in a 96 well round bottom plate. Picked colonies were grown for an additional 24 hours and 25% glycerol was added to each well at a volume of 37.5ul prior to -80°C storage.

2.3.2 Functional screening of isolates

High throughput functional screening of isolates was conducted against *Streptococcus bovis ATCC 33317* and *Fusobacterium necrophorum necrophorum ATCC 25286* on bioassay Dish Low Profile plates (Thermo Scientific Rochester, NY) using BHI agar medium. In brief, using a sterilized 96 well plate replicator, frozen glycerol stocks of isolates in 96 well format were replicated on a pathogen lawn on bioassay plates. Between each replication, the plate replicator was sterilized by dipping in 100% ethanol and flaming. Six, 96 well plates were replicated on each bioassay plate thus screening 576 isolates in each plate. The inoculated plates were placed in a 37°C incubator (both

aerobically and anaerobically) with observation of clearing zones at 24, 36, and 48 hours. Isolates which demonstrated a clearing zone were identified and used for a second round of screening. Secondary screenings were conducted against *Streptococcus bovis ATCC 33317* and *Fusobacterium necrophorum necrophorum ATCC 25286* on Mueller Hinton Agar with 5% Sheep Blood plates (Remel Lenexa, KS) and grown at 37°C for up to 48 hours anaerobically.

2.3.3 DNA extraction and PCR amplification

Candidate isolates that showed inhibition on both *Streptococcus bovis ATCC 33317* and *Fusobacterium necrophorum necrophorum ATCC 25286* during high throughput and secondary screening were grown in 10ml of BHI media at 37°C for 24 hours. The resulting cultures were used for DNA extracting using the Lucigen Quick Extract (Lucigen Corporation Middleton, WI) according to manufactures extraction protocol. Extracted DNA was used for PCR amplification of the 16S bacterial rRNA gene in 25µl volumes containing 10-25ng of DNA, 0.4 uM of 27F/1492R primer (Baker et al., 2003) (27 forward: 5'-AGAGTTTGATCMTGGCTCAG-3' & 1492 reverse: 5'-TACGGYTACCTTGTTACGACTT-3') (Integrated DNA Technologies, Coralville, IA), 0.75 Units of Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc, Mountain View, CA), 1X Terra PCR Direct Buffer (Clontech Laboratories Inc, Mountain View, CA). Amplifications were performed on a Veriti 96-well thermocycler (Life Technologies, Carlsbad, CA). Cycling conditions include 98℃ for 2 minutes followed by 30 cycles of 98°C for 30s, 58°C for 30 seconds, 68°C for 45s, with a final extension of 68°C for 4 minutes. Post amplification, 5µl of the PCR product was subjected to gel electrophoresis

on a 1.5% agarose gel at 120V for 45 minutes using 6X Blue Bromophenol dye and the 100 bp ladder to ensure that the correct fragment size was amplified with no mis-binding. Negative controls with no template was used to detect contamination. No contamination was observed.

2.3.4 Sequencing the 16S rRNA bacterial gene for taxonomic identification

The PCR product was purified to remove unused primers using Shrimp Alkaline Phosphatase and Exonuclease I (rSAP & EXO I) (New England BioLabs Ipswich, MA) according to manufactures protocol. The cleaned PCR product was then diluted accordingly with nuclease-free water to obtain a concentration ranging from 40ng/ul-60ng/ul. Diluted PCR product was mixed with 4µl of 10uM of primer to make a total of 12ul that was sent to Eurofins Genomics for sequencing. Resulting data was quality filtered and aligned to NCBI non-redundant database using Blastn to identify the bacterial species.

2.3.5 Analytical Profile Index (API) testing

Selected cultures were grown in Brain Heart Infusion medium at 37°C, shaking at 150 rpm, for 24 hours. The resulting cultures were then utilized for API50 CHB/E (bioMérieux, USA) and API 20E (bioMérieux, USA) testing according to manufactures protocol for biochemical characterization of the strains. The API test kit included 48 biochemical tests and the test strip results were recoded after 24 and 48 hours of anaerobic incubation. The test results after 48 hrs of incubation was analyzed using the api*web* interface available at https://apiweb.biomerieux.com.

2.3.6 DNA extraction and whole genome sequencing

DNA was extracted from 1 ml aliquots of overnight cultures (~10_8 cells) of two selected candidate strains using Epicentre's QuickExtract Bacteria DNA Extraction Kit (Epicentre Biosciences, Chicago IL) per the manufacturer's instructions. Total DNA for each sample was quantified on a DeNovix QFX fluorimeter with a DeNovix dsDNA Ultra High Sensitivity assay (DeNovix inc. Wilmington DE). A total of 600ng of DNA per sample in a 50ul volume was used for Shearing. Nucleic acids were sheared to ~750bp using a Diagenode Bioruptor (Denville, NJ), with the power setting on low and cycle conditions at 30 seconds on/90 seconds off for 6 minutes. Sheared DNA was used to prepare sequencing libraries using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs inc., Ipswich MA) per the manufacturer's instructions. After quality control analysis, libraries were sequenced on the Illumina MiSeq platform using V2 500 cycle kit using 250bp paired-end sequencing strategy.

2.3.6.1 Sequencing Quality Control, Genome Assembly and Annotation

Raw reads from the MiSeq sequencing run underwent quality control using Sickle software (Joshi and Fass 2011) using default parameters. The sliding window was 0.1% of the total read length, and low-quality bases were trimmed from both the 5' and 3' ends of the read. Quality controlled reads were then assembled using SPAdes (Bankevich et al., 2012) (v.3.12.0) using additional options "-k 21,33,55,77,99,127" "--careful", to optimize assembly. Genome assemblies were then passed through additional quality control software QUAST (v.4.0) (Gurevich et al., 2013) to visualize

parameters, and then contamination was assessed using CheckM (v.1.0.18) (Parks et al., 2015) software to search for genome completeness and contamination. Assembled genome sequences were then searched for open reading frames and genes and annotated using Prokka (Seemann 2014). The resulting genome assemblies were screened against Bagel4 (van Heel et al., 2018) and bacitbase (Hammami et al.,2010) to identify potential bacteriocins produced to gain insight into how these DFM strains may inhibit *F. necrophorum* and *S. bovis*.

2.3.7 Harvesting of isolate for *in vivo* study

Large batch growth and harvesting of the selected isolate(s) (or direct fed microbial (DFM)) was performed by growing the DFM in BHI at 37°C, shaking, for 48 hours in 1L volumes. At the end of 48 hrs of growth, Invitrogen's Live/Dead Assay (Life Technologies Corporation Eugene, Oregon) was used to estimate bacterial cell concentrations in the medium using flow cytometry. Flow cytometry was performed at the UNL Flow Cytometry Facility. Once concentration was determined, calculations were made to contain a minimum cell concentration of five billion cells (5×10^9) per mL and 20% of sterile glycerol was added to culture for storage at -80°C until fed.

2.3.7.1 Flow Cytometry

Invitrogen Live/Dead Assay (Life Technologies Corporation Eugene, Oregon) was used to estimate cell concentration as an alternative to estimating colony forming units (CFUs). Spherotech 5.14 μm beads (Sphero ACFP-50-5 Spherotech Inc. Lake Forest, IL) were used as a concentration reference while conducting the flow cytometry in order

to determine how many particles were drawn within each pressurized flow cytometry analysis run. Stained cells were counted using the FACS Aria II instrument at the University of Nebraska-Lincolns Nebraska Center for Biotechnology flow cytometry facility. Calculations for exact number of cells per milliliter was performed using the equation described below:

$$\left[\left(\frac{\text{Bacterial Cell Count}}{\text{Bead Count}}\right) \times \left(\frac{\text{Bead Constant}}{\text{Dilution Volume}}\right) \times (\text{Dilution of cell volume}) \times (\text{Dilution of stain})\right] \times 1000$$

2.3.7.2 in vivo study

A 179 day preliminary trial was conducted at United States Meat Animal Research Center (USMARC) to evaluate the impact of the DFM developed to reduce liver abscesses. Briefly, a cohort of 296 steers on a finishing, high concentrate diet (Table 8) with no tylosin feeding were used in this study with a starting average weight of 869 lb. The 296 animals were randomly distributed into 10 pens. The study contained two treatment groups which included a DFM group (n=148, 5 pens) which was provided the test GRAS approved DFM at a minimum concentration of five billion cells/head/day. For the first 89 days of the study, *Bacillus pumilus* (*B. pumilus)* was top dressed by spraying the DFM. From day 90 onwards a cocktail DFM containing *B. pumilus* and *Bacillus licheniformis* (*B. licheniformis*) was fed due to synergistic effects observed *in vitro* against *S. bovis* and *F. necrophorum necrophorum*. Additionally, a control group of cattle was fed the base diet with no DFM supplementation. The control group contained 148 animals within 5 pens. By day 179, only 288 animals remained on trial due to death. Thus, only 288 were analyze with n=146 Control and n=142 DFM. Deaths that occurred

during the trial was due to lameness and not due to the feeding of DFM. During harvest, liver scores were collected at the packing facility to evaluate impact of DFM on liver abscesses.

2.3.7.3 Collection of rumen fluid from cattle on experimental trial

All animal-related intervention protocols and husbandry practices were approved by the Institutional Animal Care and Use Committee (IACUC) at the United States of Meat Animal Research Center. Rumen samples were collected from each animal using esophageal tubing as described previously (Paz et al., 2016). Forty mL of rumen fluid was collected and placed into 15mL conical tubes and were snap-frozen in liquid nitrogen and stored in a -20°C until used for DNA extraction and realtime-PCR analysis. Rumen samples were collected on day 89 before feeding the DFM cocktail. Simultaneously, similar set of samples were collected from the control animals on day 89. Additionally, 4 days prior to cattle harvest, rumen samples were collected from both cohorts to evaluate the effect of the DFM cocktail on *F. necrophorum* abundance.

2.3.7.3.1 DNA extraction of obtained rumen fluid

DNA was extracted from both time periods using the Mag-Bind ® Soil DNA 96 Kit (Omega Bio-tek Inc., Norcross, GA, USA) according to the manufacturers' protocol with the following modifications. During the cell lysis step, a bead-beating step was added using a TissueLyser (Qiagen Inc., Valencia, CA, USA) for 10 minutes at a frequency of 20 Hz. Additionally, the tubes were incubated at 95°C for 5 min in a water bath. After the removal of PCR inhibitors, nucleic acids were precipitated similar to the procedure

described by Yu and Morrison (2004) and Paz et al. (2018) and then subjected to magnetic bead-based purification as described in the kit.

2.3.7.3.2 Quantitative PCR (qPCR)

Extracted DNA was quantified using Denovix Fluorescence High Sensitivity Assay (Denovix Inc., Wilmington, DE, USA). All samples were diluted to a concentration of 10ng/ul and was used for quantitative PCR (qPCR) analysis using the Faststart Universal SYBR Green Master Mix (Rox) (Roche Basel, Switzerland) according to the manufacture's protocol. *Fusobacterium necrophorum* specific rpoB gene primers were previous described by Jensen et al. (2007) and was used to quantify *F. necrophorum* copy numbers. Forward primer 5'- TCTCTACGTATGCCTCACGGATC-3' (position 171-193) and reserve primer 5'-CGATATTCATCCGAGAGGGTCTC-3' (positions 447-424) was used in the qPCR assay for *F. necrophorum*. The reaction conditions included 95°C for 10 minutes, followed by 95 °C for 10s and 58°C for 30s for 55 cycles. A melt curve was performed from 65°C to 95°C in 0.05°C increments to ensure the amplification of a single product. Additionally, the single copy universal rpoB gene was used to normalize for total bacterial abundance as previously described by Santos et al., 2004. Forward primer for the universal rpoB consisted of 5′-AACATCGGTTTGATCAAC-3' and reverse 5′-CGTTGCATGTTGGTACCCAT-3′ with conditions of 95°C for 10 minutes, followed by 95°C for 10s, 53°C for 30s, and 60°C for 60s with 55 cycles as reaction conditions. A melt curve was performed as described above.

2.3.8 Digestibility study

The effect of the DFM on total digestibility in the rumen was evaluated for one of the selected DFM strains (*B. licheniformis*). Feed rations used for testing included dry rolled corn, meadow hay, a total mixed ration (TMR), and bran. *E. coli* was grown and used as a control to ensure that increased digestibility is not due to increased cell density of the DFM. A modified Tilley and Terry (1963) method was used where rumen fluid and McDougalls buffer was mixed at 1:1 ratio after cells of the *B. licheniformis,* or *E. coli,* and rumen fluid (control) cells were estimated using Flow Cytometry.

B. licheniformis was grown on BHI media shaking at 150 rpm at 37°C for 48 hours prior to inoculation of rumen fluid used for the *in vitro* digestibility study. Culture was prepped for live/dead cell count using Invitrogen Live/Dead Assay (Life Technologies Corporation Eugene, Oregon) according to manufacture protocol and was measured using the counted on the 'FACSAria II' instrument available at the Nebraska Center for Biotechnology Flow Cytometry Facility in Lincoln, Nebraska. Additionally, *E. coli* was grown for 24 hours at 37℃ and cells were counted as described above to ensure adequate testing of the effect of *B. licheniformis* on digestibility.

Briefly, rumen fluid was obtained from a fistulated steer located in the metabolizable area at the University of Nebraska-Lincoln and strained through four layers of cheese cloth prior to being prepared for a live/dead cell count as described above. Samples that included *B. licheniformis* or *E. coli* were calculated to bring total cell concentration equal to the rumen fluid control. Thus, 63.66ul of *B. licheniformis* and 66.41ul of *E. coli* was

added to respective samples on a per tube basis. Each treatment was performed in triplicate.

As previously mentioned, rumen fluid containing *B. licheniformis, or E. coli,* and the rumen fluid control was mixed at a 1:1 ratio with McDougalls buffer (McDougall, 1948). Thirty milliliters of the above mixture was added to plastic tubes containing diet and was capped with rubber stoppers to maintain anaerobic conditions. The tubes were incubated in a water bath at 39°C for 48 hours. To quench the reaction, and stop fermentation, 6ml of 20% hydrochloric acid and 2ml of 5% pepsin solution was added per tube and then placed back in the 39°C water bath for 24 hours. Tubes were removed and placed in freezer until subsequent analysis.

Tubes were thawed in a 39°C water bath for 15 minutes and filtered through Watman 541 filter paper (pore size = 0.22uM). The filtered samples were dried for 24 hours in a 100°C oven and weighed to determine dry matter digestibility. Additionally, blanks of each feed ration were included to adjust for any feed particles that may have originated from the rumen inoculum. Filters were placed in crucibles and placed in a mutle ferna to determine ash content (Table 3).

2.4 Results

2.4.1 DFM library isolates

A library consisting of 4,692 isolates were generated using different culture methods from the rumen samples from the 6 animals. The breakdown of the isolates based on

the media used included 424 isolates from Brain Heart Infusion, 707 from ISP1 medium, 1088 from ISP2 medium, 1076 from ISP4 medium, and 1397 from Casein Starch. The resulting bacterial isolates were screened against *Streptococcus bovis ATCC 33317* and *Fusobacterium necrophorum necrophorum ATCC 25286* using a high throughput approach where the cultures were grown in high density on a pathogen background to identify candidate species with the ability to inhibit pathogen growth. Inhibition was identified as clearing zones (Figure 1). Our screening strategy allowed us to screen 6 X 96 isolates (n= 576) in one plate. Of the 4,692 isolates, 81 demonstrated inhibition after secondary screening and was further analyzed to characterize taxonomy by sequencing the 16S rRNA gene. The 81 isolates that were sequenced (Table 1) were used for further in vitro analysis and were prioritized based on their classification of Generally Recognized as Safe (GRAS) by the FDA. From the 81 isolates identified, 56 isolates were classified as GRAS approved. These isolates can be further classified into families, with the predominant family being *Bacillus spp*.. Two isolates of *Bacillus spp*. with GRAS status were identified as the top candidates based on greatest inhibition (Figure 2).

Bacillus species are gram positive, rod shaped bacteria which are spore formers (Voigt et al., 2009). They can be grown both anaerobically or aerobically and exhibit a wide range of physiological traits (Voigt et al., 2009). Thus, allowing them to flourish in a wide variety of environments. Due to *Bacillus spp.* being spore formers, they have a high heat tolerance and can remain stable over a long period of time making them suitable for a DFMs as they can undergo thermal processing as feed additives. Additionally,

Abriouel et al. (2011) explains how Bacillus species are used to prevent food spoilage due to properties such as production of bacteriocin-like peptides (Wang et al., 2017). McAllister et. al. (2011) discussed the probability of using *Bacillus spp.* as DFMs due to their ability to form spores to be stable over harsh conditions and their ability to produce bacteriocins. However, relatively, *Bacillus spp.* are low in abundance within the rumen and play a minor role in cell wall degradation, from a digestion point of view (McAllister et. al, 2011).

The *Bacillus spp*. identified were tested for optimal growth conditions using elevated growth temperatures, starvation, and incubation time. Growth temperatures at 37°C, 42°C, 45°C, and 50°C showed that 37°C was the optimal growth condition. M9 Minimal Salts 5X (Sigma-Aldrich, St. Louis, MO) was used in comparison to BHI media to evaluate if starvation would induce antimicrobial production in the DFM strains identified. Clearing zones showed that BHI medium enabled the best inhibition for both species. Lastly, both species were grown for 12, 16, 24, 36, 48, 72, and 96 h to identify if the stage of the growth curve effects (exponential vs stationary) expression of inhibitory products. Optimal growth time for viable cells were at 48 h. All testing for each stress factor was performed in duplicate using both shaken (150 rpm) and non- shaken cultures. For each stress factor, shaken cultures showed the greatest inhibition. Once optimal growth conditions were confirmed, both *Bacillus spp*. were grown separately and in co-culture to see if they worked synergistically. Results showed that both species should be grown separately for 48 h but when combined together in a 1:1 ratio and screened together, showed the greatest inhibition.

2.4.1.1 Biochemical characteristics of the identified DFMs

To evaluate the biochemical characteristics of the selected DFM isolates, we performed Analytical Profile Index (API) testing for each isolate to identify unique characteristics present in each isolate compared to know *B. licheniformis* and *B. pumilus* strains. The API test results for each isolate is shown in Table 5. Interestingly, our *B. licheniformis* (12F) was able to utilize melibiose whereas Logan et al. (1984) did not see the same result in the 81 strains of *B. licheniformis* that they screened. Additionally, our *B. pumilus* strain utilized similar substrates as described by Logan et al. (1984). However, substrates such as maltose, sorbitol, starch, glycogen, xylitol and D-turanose showed different responses in the *B. pumilus* strain in comparison to Logan et al. (1984) (Table 5).

2.4.1.2 Genome Sequencing

Genome sequencing yielded ~2.69 million high quality 250 bp read-pairs for *B. licheniformis* and ~1.35 million high quality 250 bp read-pairs from *B. pumilus* strains after read QC with Sickle (Joshi and Fass 2011). Sample assemblies showed N50 values of 152,937bp and 447,432bp for *B. licheniformis* and *B. pumilus* strains, respectively with the largest contig sizes for each genome being 440,068 and 447,432bp for *B. licheniformis* and *B. pumilus* respectively. A summary for the assemblies are shown in Table 6. The *B. pumilus* draft genome was shown to be 99.59% complete with 0.83% contamination, with a genome length of 3,760,977bp, and would be considered a high quality draft genome. Average nucleotide identity (ANI)

comparison to available genomes demonstrated the closest genome to be *Bacillus sp. WP8 (B. pumilus)* with total query coverage and template coverage of 80.98% and 83.22% respectively. Suggesting this *B. pumilus* genome to be significantly different from other *B. pumilus* genomes (Table. 7a).

B. licheniformis genome length was predicted to be 3,677,812bp. ANI comparison revealed the closest genome to be *Bacillus sp. H15-1* with total query coverage of 87.05% and total template coverage of 96.71%, suggesting that this genome is larger than other B. licheniformis genomes (Table 7b).

To identify unique genomic features that may provide insight into how these isolates may inhibit *F. necrophorum* and *S. bovis*, we screened the genomes to identify bacteriocins present within each genome. Briefly, both strains were aligned to Bagel4 database (van Heel et al., 2018) to search for any bacteriocins associated with either *B. licheniformis* or *B. pumilus.* Forty-seven contigs were analyzed for *B. licheniformis* and five areas of interests were found. These areas include the class of sactipeptides, sonorensin, bottromycin, lichenicidin, and lasso_peptide. Additionally, 14 contigs were analyzed for *B. pumilus* with four areas of interest found including, UViB, UviB, BmbF, and amylocyclicin. A secondary genome screening against the bacitbase database (Hammami et al., 2010) confirmed the bacteriocin results obtained by Bagel4 database (van Heel et al., 2018).

2.4.2 Digestibility study

A digestibility study was conducted to evaluate if the DFM would inhibit beneficial strains and thereby reduce nutrient digestion in the rumen. Using two controls consisting of rumen fluid and rumen fluid + *Escherichia coli*. A DFM added rumen fluid sample consisting of *Bacillus licheniformis* ("12F") was used to test if there was negative effects on digestibility. Feed samples consisted of dried rolled corn, a total mixed ration, meadow hay, and bran. Resulting data showed that the rumen fluid with the DFM numerically increased dry matter and organic matter digestibility on diets consisting of high starch levels (Table 3). As such this data demonstrates that no negative impact on digestibility in the rumen due to the addition of the DFM.

2.4.3 An alternative approach for determining cell concentrations

As an alternative to performing Colony Forming Units (CFUs) we determined cell concentrations using live/dead staining and flow cytometry (Figure 3). The isolate grew rapidly to acceptable concentrations as such, all batch growths performed met the requirement of having at least one billion cells (10_9) per mL. Thus, in a single growing we were able to harvest cells enough for feeding at least 28 days of feeding 148 head.

2.4.4 *in vivo* study and qPCR analysis

Realtime PCR analysis was performed on rumen samples collected from DFM fed cattle and cattle with no DFM supplementation to evaluate the abundance of *F. necrophorum* in the rumen. Cattle on trial were fed for 179 d with a DFM intervention fed to half of the cattle (n=148). Both cohorts did not receive a tylosin prophylactic throughout the entire duration of the trial. The DFM cohort was fed a minimum of five billion cells per head per

day, with 148 head of cattle divided among five pens. Eight cattle were removed from the trial due to death from lameness which resulted in a total of 288 cattle. Rumen sampling was performed at day 89 for quantification PCR and day 175. Quantitative PCR analysis showed that the presence of *Fusobacterium necrophorum necrophorum* was significantly decreased 100-fold ($p < 0.032$) compared to control group not receiving the DFM during the first sampling (Figure 4). However, after second sampling (4 days prior to harvest), there was no significant difference between the two treatments ($p < 0.382$) suggesting that an intervention may need to be fed in higher abundances (Figure 4). Additionally, liver scores were given to all cattle on trial post-harvest with the percent of abscesses present for DFM and control cohort as follows 21.8% and 18.4% (Table 3).

2.5 Discussion

The growing concern of antibiotic resistant bacteria has brought light on the use of probiotics and the additional benefits from this unique approach. Probiotics are live supplements which are consumed to enhance the hosts intestinal microbial community. Likewise, DFMs are live strains, which can be commonly found within the rumen, that have the potential to promote animal health while having additional fermentation properties (Krehbiel et al., 2003). Elghandour et al. (2015) demonstrated how DFMs can inhibit or prevent the establishment of unwanted pathogens that adhere to the intestinal mucosa. Additionally, Elghandour et al. (2015) and Dicks et al. (2010) suggest that DFM administered could be used within a hosts immune system by macrophages engulfing the DFM and causing an immune response. As such, the use of DFMs could benefit the host in many ways. Here we show the potential of identifying new bacterial species with

beneficial properties that could be used to improve animal health and productivity while reducing the burden on antimicrobial compounds.

2.5.1 Isolating and growth performance in DFM

A novel approach to antibiotics has become increasingly important with the overuse of antibiotics over the past several decades. While this overuse has stemmed from various uses, the use of antibiotics as prophylactics in production livestock, has come under great scrutiny (McEwen and Fedorka-Cray 2002). Within this study, isolation of DFMs as an alternative approach to antibiotics, more specifically tylosin, is demonstrated.

In this study, we isolated 4,692 isolates, from the rumen of healthy fistulated cattle on different diets and screened for candidate bacterial species with antimicrobial properties. Using targeted inhibition against *S. bovis* and *F. necrophorum* we identified 81 isolates to be effective at inhibiting these two species at varying efficiencies. Demonstrating that rumen may be a hotspot for isolating novel DFMs. The 81 isolates identified were predominated by the genus Bacillus and belong to 9 different species, which included *Bacillus pumilus, Bacillus licheniformis, Bacillus gylcinifermentans, Bacillus altitudinis, Bacillus safensis, Bacillus clausii, Bacillus circulans*, *Bacillus borstelensis,* and *Bacillus sororensin*. Fifty-six isolates fell into the category of Generally Recognized as Safe (GRAS) by the FDA. Interestingly, out of the 56 isolates, the predominant family was Bacillaceae. Based on the zones of inhibition among the GRAS approved isolates we identified isolate *ISP4 P1A 11E* (11E) *B. pumilus* and *ISP4 P2A 12F* (12F) *B. licheniformis* as the top two candidates for further testing. Although, the

rest of the discussion will be focused on these two isolates that were the top candidates for inhibition of *S. bovis* and *F. necrophorum,* it is noteworthy to mention that our bacterial library would contain a broad range of bacterial species with unique metabolic functions that need to be further explored. Some functions of interest would be increased digestibility, increased VFA production, ionophore production, inhibition of other pathogens and increase feed efficiency. As such the library generated in this study is an invaluable resource for future DFM screening and development studies. Additionally, we identified more potent strains of bacteria with the capability to inhibit *S. bovis* and *F. necrophorum* however, these strains were less attractive to the industry as they needed to go through rigorous evaluations to obtain GRAS status. As such the GRAS approved strains were used for subsequent testing.

The isolates identified were classified as *Bacillus spp.* and are able to exhibit industrial tolerant properties such as changes in pH, growth temperatures, and variability in substrates for growth. Abriouel et al. (2011) explains how *B. pumilus* is not well characterized for its bacteriocin-like peptides. Instead, *B. pumilus* is able to produce a plasmid-encoded peptide called pumilicin which are heat stable and are active within a broad pH range (3-9). Thus, demonstrating the vast opportunities available to use Bacillus strains as DFMs.

It is well documented that bacterial isolates/strains are host/environment specific (Foster et al., 2017) and as such develop unique features to survive in unique environments. As such, the isolates we have obtained from the rumen are assumed to have developed

unique features and therefore has a greater potential as being successful DFMs for ruminants and is supported by genomic features identified.

In addition to identifying potential strains of DFM to decrease *S. bovis* and *F. necrophorum* population abundance, we have optimized growth condition to increase growth and probiotic potential. Optimization experiments using elevated temperatures, starvation, and incubation times, we identified both isolates to produce the most antimicrobials at 37°C, shaking at 150 rpm for 48 hours in BHI medium. The plasticity of the isolates under different pH, temperature and starvation condition also lends information into the stability of the DFM developed. This observation coincides with the conclusion of Abriouel et al. (2011) which also reported the metabolic plasticity of Bacillus strains.

2.5.2 The DFM developed is capable of reducing the abundance of *F. necrophorum* in the rumen

The *in vivo* study, conducted at USMARC (Clay Center, NE), using 296 fed cattle in a 179 day feeding trial receiving *B. pumilus* for 89 days demonstrated a significant decrease in *F. necrophorum* abundance in the DFM fed cattle compared to control animals. The 100 fold decrease in *F. necrophorum,* we saw within the first rumen sampling, suggest the current DFM is effective in helping decrease *F. necrophorum* in beef cattle. However, the cocktail we fed during the second part of the feeding trial was not effective as realtime PCR results for that period did not indicate a significant decrease in F. necrophorum abundance similar to the first half of the trial. Additionally, liver abscess scores recorded at harvest did not show a significant effect of the DFM on

liver abscesses. We believe the decrease effectiveness could be due to several reasons; 1) the concentration of DFM fed may have been too low to affect function in the rumen where the volume and concentration of other microbial species is high and have significant competition for resources, 2) the DFM cocktail would have been less effective than feeding just *B. pumilus*, and finally, 3) the cattle may have not been in an environment where it needed to compete for a niche or fed a high enough concentrate diet to induce liver abscesses. As such, the occurrence of liver abscesses even in the control pens were 18.44% and are similar to the level of liver abscessed observed in the industry with feeding of Tylan (McKeith et al., 2012). It is well documented that liver abscesses could occur early or late in the feeding period (Nagaraja et al., 2007). Within this study, we only sampled at two time periods in order to try to capture both the early and late effects that we were hoping to see. Therefore, with our results we believe more frequent sampling is needed to understand the relationship between *F. necrophorum* abundance and liver abscesses. Additionally, we believe measuring host immune responses, longitudinally, may also provide insight into when liver abscess formation mostly occurs and what is the best time for intervention. As such we believe, looking at white blood cell counts, could be an indicator for when *F. necrophorum* influx occurs and give a “in time” indication of the effectiveness of a DFM intervention. Macdonald et al. (2017) demonstrated the use of blood collection in comparison to determine cattle with liver abscesses prior to harvest. This group suggested cattle with elevated levels of blood cortisol and AST as well as lower levels of blood albumin and cholesterol indicated a higher chance of having abscesses. Further investigation on the precise

concentration of DFM needed to have optimal effect within the rumen is another factor that needs to be investigated.

2.5.3 Genomic and metabolic features of the DFMs isolated

Logan et al. (1984) used an API identification testing for identification of Bacillus species. In this study, Logan et al. (1984) evaluated 81 strains of *B. licheniformis* and 63 strains of *B. pumilus* and identified core metabolism present within each species. The API testing of the DFM strains in this study was tested against 48 substrates and was compared to the results described by Logan et al. (1984). The *B. licheniformis* (12F) displayed similar substrate utilization patterns to Logan et al. (1984) (Table 5). However, 12F utilized melibiose whereas Logan et al. (1984) did not see the utilization of this substrate by the strains tested. Melibiose is a disaccharide consisting of one galactose and glucose moiety in an alpha (1-6) linkage and is suggested to be only metabolized and utilized by gut microflora (PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/D-Melibiose).

B. pumilus (11E) demonstrated larger differences in fermentation results compared to Logan et al. (1984) (Table 5) where, maltose and D-turanose were utilized by 11E but not in the strains from Logan et al. (1984). D-turanose is composed of fatty acyl glycosides mono- and disaccharides (https://hmdb.ca/metabolites/HMDB0011740). Maltose is the result from the breakdown of starches. Thus, leading us to hypothesize that 11E would be able to survive under high starch conditions in the rumen.

Additionally, sorbitol, glycogen, and xylitol were not utilized by strains analyzed in Logan et al. (1984) study but was utilized by strain 11E.

Multiple bacteriocins were found during genome scanning for bacteriocins using the BAGEL4 database. The results demonstrated that four bacteriocins were identified in the *B. pumilus* genome and five for *B. licheniformis*. Interestingly, among the bacteriocins found in *B. pumilus,* UviB gene was identified from both BAGEL4 and bactibase searches. UviB is reported to be expressed by UV irradiation (https://www.uniprot.org/uniprot/P15936). Searching the NCBI database using Blastn search parameters, the UviB gene sequence was identified in *Bacillus* species such as *B. safensis* and *B. altitudinis*. Additionally, UviB has been seen in a *Brevibacillus laterosporus* species and is thought to help *Brevibacillus laterosporus* inhibit gram-positive and gram-negative multi- drug resistant pathogens (Mijkovic et al., 2019). Amylocyclicin was another bacteriocin found within the *B. pumilus* genome. Scholz et al. (2014) describe this bacteriocin as a ribosomally synthesized circular bacteriocin which is capable of inhibiting gram-positive bacteria. Interestingly, Abriouel et al. (2011) found *B. pumilus* to produce a bacteriocin called pumilicin however, we did not see this bacteriocin within our “11E” specific strain.

We identified 5 potential bacteriocins within the *B. licheniformis genome.* Sororensin that was identified is thought to be a bacitracin capable of inhibiting biofilms and act as a food bioperservative. As such, Chopra et al. (2015) demonstrates the effects of bacteria with sororensin production to inhibit opportunistic biofilm pathogens. This

suggests that *B. licheniformis* may be able to inhibit biofilm forming pathogens much like what was seen in Chorpra et al. (2015) study. Additionally, *B. licheniformis* genome also contained three different Lichenicidin genes which are commonly associated with various *B. licheniformis* species. As such, this type of bacteriocins are known as a two-component lantibiotics that inhibits the growth of pathogens such as *Staphylococcus aureus* (MRSA) (Caetano et al., 2011).

Both *B. pumilus* and *B. licheniformis* possess the antimicrobial gene BmbF. However, little information was found on the mode of action for this gene. BmbF falls within the class of Sactipeptides. According to Grove et al. (2017) this peptide class is a typically co-localized in operons with members of radical S-adenosyl-L-methionine (SAM) enzyme of which is required for biosynthesis. As such, the bacteriocins identified in both *B. pumilus* and *B. licheniformis* could provide clues into how these strains could inhibit *F. necrophorum* and *S. bovis*. Still, further investigation and complete genome analysis is needed. Finally, the *in vitro* digestibility study did not show any negative effects of the DFM on animal performance or digestibility. As such the DFMs developed in this study has great potential to be used as a DFM. However, further studies are needed to estimate dosage and evaluate colonization and persistence of the DFM in the rumen.

2.6 Conclusions

Direct fed microbials offer an array of applicable practices that may not only improve health but also livestock performance. Adjusting host microbiomes to allow establishment may propose implications for the use of DFM. However, isolation

methods from hosts may be one option to avoid this problem. Additionally, increasing the samplings and pinpointing the exact time peak influx of *Fusobacterium necrophorum necrophorum* occurs, may be more indicative for finding the correct concentration to feed DFM.

2.7 Future Directions

An *in vivo* study is currently being investigated at the University of Nebraska-Lincoln Individual Barn in Mead, Nebraska. There are 58 head of cattle on trial with two cohort groups of control and DFM. Of which, the DFM cohort (n=29) is being fed a cocktail of *Bacillus pumilus* and *Bacillus licheniformis* at 81 billion cells per head per day for roughly 182 days. Five jugular blood samplings will be taken and analyzed for white blood cell counts to estimate acidosis and liver abscess formation prior to harvest. Cattle will be liver scored post-harvest.

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2.9 Appendix I Tables

Table 1. Diet information for the six animals from which rumen fluid was collected to generate the for isolation library.

Table 1a.

[1] Supplement included 0.74% Fine Ground Corn, 1.73% Limestone, 0.16% Tallow, 1.52% Urea, 0.47% Potassium Chloride, 1.50% Corn Gluten Meal, 0.30% Salt, 0.05% Beef Trace Mineral, 0.02% Vitamin A-D-E, 0.02% Rumensin-90, and 0.01% Tylan-40.

Ingredients	% Diet Dry Matter
Dry Rolled Corn	43.25
High Moisture Corn	43.25
Alfalfa	3.00
Sorghum Silage	4.00
Supplement[1]	6.50

Table 1b.

Ingredients	% Diet Dry Matter
Dry Rolled Corn	24.00
High Moisture Corn	24.00
MDGS Deoiled	40.00
Alfalfa	3.00
Sorghum Silage	4.00
Supplement[2]	5.00

[2] Supplement included 2.79% Fine Ground Corn, 1.70% Limestone, 0.13% Tallow, 0.30% Salt, 0.05% Beef Trace Mineral, 0.02% Vitamin A-D-E, 0.02% Rumensin-90, and 0.01% Tylan-40.

Table 1c.

Ingredients	% Diet Dry Matter
Dry Rolled Corn	24.00
High Moisture Corn	24.00
MDGS Deoiled	38.00
Corn Oil	2.00
Alfalfa	3.00
Sorghum Silage	4.00
Supplement[2]	5.00

[2] Supplement included 2.79% Fine Ground Corn, 1.70% Limestone, 0.13% Tallow, 0.30% Salt, 0.05% Beef Trace Mineral, 0.02% Vitamin A-D-E, 0.02% Rumensin-90, and 0.01% Tylan-40.

Table 1d.

Ingredients	% Diet Dry Matter
Dry Rolled Corn	24.00
High Moisture Corn	24.00
MDGS Full Fat	40.00

Alfalfa	3.00
Sorghum Silage	4.00
Supplement[2]	5.00

[2] Supplement included 2.79% Fine Ground Corn, 1.70% Limestone, 0.13% Tallow, 0.30% Salt, 0.05% Beef Trace Mineral, 0.02% Vitamin A-D-E, 0.02% Rumensin-90, and 0.01% Tylan-40.

Table 1e.

Ingredients	**% Diet Dry Matter**
Dry Rolled Corn	33.75
High Moisture Corn	33.75
Alfalfa	7.500
WDGS	20.00
Supplement[3]	5.00

[3] Supplement included 2.66% Fine Ground Corn, 1.35% Limestone, 0.13% Tallow, 0.50% Urea, 0.30% Salt, 0.05% Beef Trace Mineral, and 0.02% Vitamin A-D-E.

Table 1f.

Ingredients	**% Diet Dry Matter**
Corn Silage	95.00
Supplement[3]	5.00

[3] Supplement included 2.66% Fine Ground Corn, 1.35% Limestone, 0.13% Tallow, 0.50% Urea, 0.30% Salt, 0.05% Beef Trace Mineral, and 0.02% Vitamin A-D-E.

Table 2. Protentional Isolates characterized using full length 16S rDNA sequencing. The pathogens inhibited by each isolate is shown below.

Isolate	**Specie**	***Streptococcus bovis***	***Fusobacterium necrophorum necrophorum***	***H. somni***	***Manhemmnia***
6	*B. pumilus*	+	+	-	-
7	*B. licheniformis*	+	+	-	+
ISP4 P2A 12F	*B. licheniformis*	+	+	-	+
ISP4 P1A 11E	*B. pumilus*	+	+	-	+
11	*B. pumilus*	+	+	-	-
ISP4 P2A 12H	*B. pumilus*	+	+	-	-
P16 B4 SR	*B. licheniformis*	+	+	-	-

P5A C1 SR	*B. licheniformis*	-	-	-	-
P5A E1 SR	Bacillus	-	-	-	-
ISP2 P8 D1	*B. licheniformis*	-	+	-	-
ISP2 P8 D2	*B. licheniformis*	-	+	-	-
ISP2 P8 F1	*B. licheniformis*	-	+	-	-
ISP2 P8 H2	*B. licheniformis*	-	+	-	-
ISP2 P8 G2	*B. pumilus*	-	+	-	-
ISP2 P15 C3	*B. licheniformis*	+	-	-	-
ISP2 P16 D6	*B. licheniformis*	+	-	-	-
ISP2 P16 E6	*B. licheniformis*	+	-	-	-
ISP2 P16 G2	*B. licheniformis*	+	+	-	-
ISP2 P16 B4	*B. licheniformis*	+	+	-	-
ISP4 P2A F12	N/A	+	-	-	-
ISP4 P3A B7	*B. licheniformis*	+	-	-	-
C/S P8 D5	*B. licheniformis*	-	+	+	-
ISP2 P8 G2	N/A	-	+	-	-
ISP2 P14 A3	*B. pumilus*	-	+	-	-
ISP2 P14 A4	*B. pumilus*	-	+	-	-

ISP2 P14 B2	*B. pumilus*	-	+	-	-
ISP2 P14 D9	*B. pumilus*	-	+	-	-
ISP2 P14 E5	*B. pumilus*	-	+	-	-
ISP4 P2 A10	*B. pumilus*	-	+	-	-
ISP4 P2 H10	N/A	-	-	-	-
ISP4 P3 A4	*B. pumilus*	-	+	-	-
ISP4 P3 B9	*B. pumilus*	-	+	-	-
ISP4 P3 B11	*B. pumilus*	-	+	-	-
ISP4 P3 C4	*B. pumilus*	-	+	-	-
ISP4 P3 G7	*B. pumilus*	-	+	-	-
ISP4 P1A B5	*B. pumilus*	-	+	-	-
ISP4 P1A C4	*B. pumilus*	-	+	-	-
ISP4 P1A D2	*B. pumilus*	-	+	-	-
ISP4 P1A E1	*B. pumilus*	-	+	-	-
ISP4 P1A E4	*B. pumilus*	-	+	-	-
ISP4 P1A B2	*B. pumilus*	-	+	+	-

ISP4 P2A B1	*B. pumilus*	-	+	-	-
ISP4 P2A C2	*B. pumilus*	-	+	-	-
ISP4 P2A C7	*B. pumilus*	-	+	-	-
ISP4 P2A D6	*B. pumilus*	-	+	-	-
ISP4 P2A E6	*B. pumilus*	-	+	-	-
ISP4 P2A F6	*B. pumilus*	-	+	-	-
ISP4 P2A H8	*B. pumilus*	+	-	-	-
ISP4 P2A H12	N/A	-	-	-	-
ISP4 P6A G1	*B. pumilus*	+	-	-	-
C/S P7 A6	*B. pumilus*	-	+	-	-
C/S P5A B1	*B. pumilus*	+	-	-	-
C/S P5A C6	*B. pumilus*	-	+	-	-
C/S P5A G1	*B. pumilus*	+	-	-	-
C/S P5A H2	*B. pumilus*	-	+	-	-
C/S P6A H1	*B. pumilus*	+	-	-	-
C/S 7A 5A	*B. pumilus*	+	-	-	-

C/S 7A C3	*B. pumilus*	+	-	-	-
ISP4 P5A 9C	*B. pumilus*	+	-	-	-
ISP2 P8 A2	*B. gylcinifermentans*	+	-	-	-
ISP2 P14 D1	*B. altitudnis*	-	+	-	-
ISP2 P14 H7	*B. safensis*	-	+	-	-
ISP2 P15 B2	*A. aneurinilyticus*	-	+	-	-
ISP2 P15 C9	*A. aneurinilyticus*	+	+	-	-
ISP2 P16 C6	*B. gylcinifermentans*	+	-	-	-
ISP2 P16 F4	Brevibacilus	+	+	-	-
ISP4 P2 D1	*B. ooleronius*	-	+	-	-
ISP4 P2 E2	*A. migulans*	-	+	-	-
ISP4 P2 A9	*B. clausii*	-	+	-	-
ISP4 P2 B11	*B. clausii*	-	+	-	-
ISP4 P3 E7	*B. altitudnis*	-	+	-	-
ISP4 P1A D3	*B. altitudnis*	-	+	-	-
ISP4 P1A F9	*B. circulans*	-	+	-	-
ISP4 P1A G9	*B. safensis*	-	+	-	-

ISP4 P2A A7	*B. clausii*	-	+	-	-
ISP4 P2A B6	*B. borstelensis*	-	+	-	-
C/S P7 A7	*B. subtilis*	-	+	-	-
C/S P7 G4	*A. migulans*	-	+	-	-
C/S P5A G1	*B. pumilus*	-	+	-	-
C/S P5A F1	*B. sonorensis*	+	+	-	-

Table 3. Results from in-vitro digestibility studies evaluating the effect of DFM on rumen digestibility.

Table 3A.

in vitro Dry Matter Digestibility

Ingredients	**Control**	**DFM**	**E Coli**
Dry Rolled Corn	90.4%	93.8%	90.7%
Total Mixed Ration	76.7%	80.9%	79.2%
Meadow Hay	52.8%	51.8%	56.1%
Bran	79.3%	74.6%	75.4%

Table 3B.

in vitro Organic Matter Digestibility

Ingredients	**Control**	**DFM**	**E Coli**
Dry Rolled Corn	90.8%	91.1%	89.6%
Total Mixed Ration	77.4%	82.3%	78.9%
Meadow Hay	58.6%	58.1%	62.9%
Bran	79.1%	75.5%	75.2%

Table 4. Liver abscess data collected post-harvest.

Treatment	**% Abscesses**
Control	18.4
DFM	21.8

Table 5. API results from *B. licheniformis* (12F) and *B. pumilus* (11E) after 24 hours of anaerobic incubation. (+ positive, - negative, + partially positive)

Substrate	***B. licheniformis***	***B. pumilus***
Glycerol	+	+
Erythritol		
D-Arabinose		
L-Arabinose	+	+
Ribose	+	+
D-Xylose	+	+
L-Xylose		
Adonitol		
β-Methylxyloside		
Galactose	+	+
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	+
L-Sorbose		
Rhamnose	+	
Dulcitol		
Inositol		
Mannitol	+	+
Sorbitol	+	+
α-Methyl-D-mannoside		
α-Methyl-D-glucoside		+
N-Acetylglucosamine	+	+
Amygdalin	+	+
Arbutin		+
Aesculin	+	+
Salicin	+	+
Cellobiose	+	+
Maltose	+	+
Lactose	+	+
Melibiose	+	
Sucrose	+	+
Trehalose		+
Inulin		+
Melezitose		+
D-Raffinose		+
Starch		+
Glycogen		+
Xylitol		+
β-Gentiobiose		+
D-Turanose	+	
D-Lyxose		
D-Fucose		
L-Fucose		
D-Arabitol		

L-Arabitol		+
Gluconate		
2-Ketagluconate		+
5-Ketogluconate		

Table 6. Genome assembly statistics for *B. pumilus* and *B. licheniformis.*

	B. pumilus	***B. licheniformis***
# contigs (> = 0 bp)	245	215
# contigs (> = 1000 bp)	21	21
# contigs (> = 5000 bp)	13	13
# contigs (> = 10000 bp)	11	11
# contigs (> = 25000 bp)	9	9
Total length (> = 50000 bp)	9	9
Total length (> = 0 bp)	3830136	3817708
Total length (> = 1000 bp)	3738374	3738922
Total length (> = 5000 bp)	3725698	3724640
Total length (> = 10000 bp)	3710277	3709219
Total length (> = 25000 bp)	3671113	3670055
Total length (> = 50000 bp)	3671113	3670055
# contigs	21	55
Largest contig	962803	962803
Total length	3738374	3760977
N50	478599	477432

Table 7. ANI results for *B. licheniformis* and *B. pumilus.*

Table 7a.

Template	**p_value**	**Total query coverage**	**Total template coverage**	**Total dept**	**Description**	**TaxID**	**Species**
GCF_001896025.1_ASM189602v1	1.01E-22	87.05	96.71	0.97	NZ_CP018249.1	1856406	Bacillus sp. H15-1
GCF_003253815.1_ASM325381v1	1.01E-22	86.88	95.89	0.96	NZ_CP021970.1 1402	1402	Bacillus licheniformis CBA7132
GCF_002074075.1_ASM207407v1	1.01E-04	86.66	94.11	0.94	NZ_CP014795.1	1402	Bacillus licheniformis
GCF_002236895.1_ASM223689v1	1.01E-22	86.34	93.65	0.94	NZ_CP022477.1	1402	Bacillus licheniformis
GCF_001726125.1_ASM172612v1	1.01E-22	85.74	91.79	0.91	NZ_CP017247.1	1402	Bacillus licheniformis

Table 7b.

Template	p_value	Total query coverage	Total template coverage	Total dept	Description	TaxID	Species
GCF_000800825.1_ASM80082v1	1.01E-22	80.98	83.22	0.83	NZ_CP010075.1	756828	Bacillus sp. WP8 (Bacillus pumilus)
GCF_001938705.1_ASM193870v1	1.01E-22	68.66	70.24	0.70	NZ_CP015611.1	561879	Bacillus safensis
GCF_002077215.1_ASM207721v1	1.01E-22	55.96	57.03	0.57	NZ_CP018100.1	561879	Bacillus safensis
GCF_001895885.1_ASM189588v1	1.01E-17	56.72	56.04	0.56	NZ_CP018197.1	561879	Bacillus safensis
GCF_001938665.1_ASM193866v1	1.01E-22	55.63	55.05	0.54	NZ_CP015607.1	561879	Bacillus safensis

Table 8. Diets for *in vivo* feeding experimental trial conducted at USMARC.

Table 8a. Growing diet for cattle on feeding experimental trial.

Ingredients	% Dry Matter
Corn	39.00%
Corn Silage	29.00%
Wet Distillars Grains and Soluables	28.50%
Urea	0.00%
Balance Pellet	3.50%

Table 8b. Finishing diet for cattle on feeding experimental trial.

Ingredients	% Dry Matter
Corn	71.75%
Corn Silage	9.00%
Wet Distillars Grains and Soluables	15.00%
Urea	0.75%
Balance Pellet	3.50%

Appendix II Figures

Figure 1. A representative high throughput functional screening plate against *F. necrophorum* used to identify candidates from the isolate library for further testing.

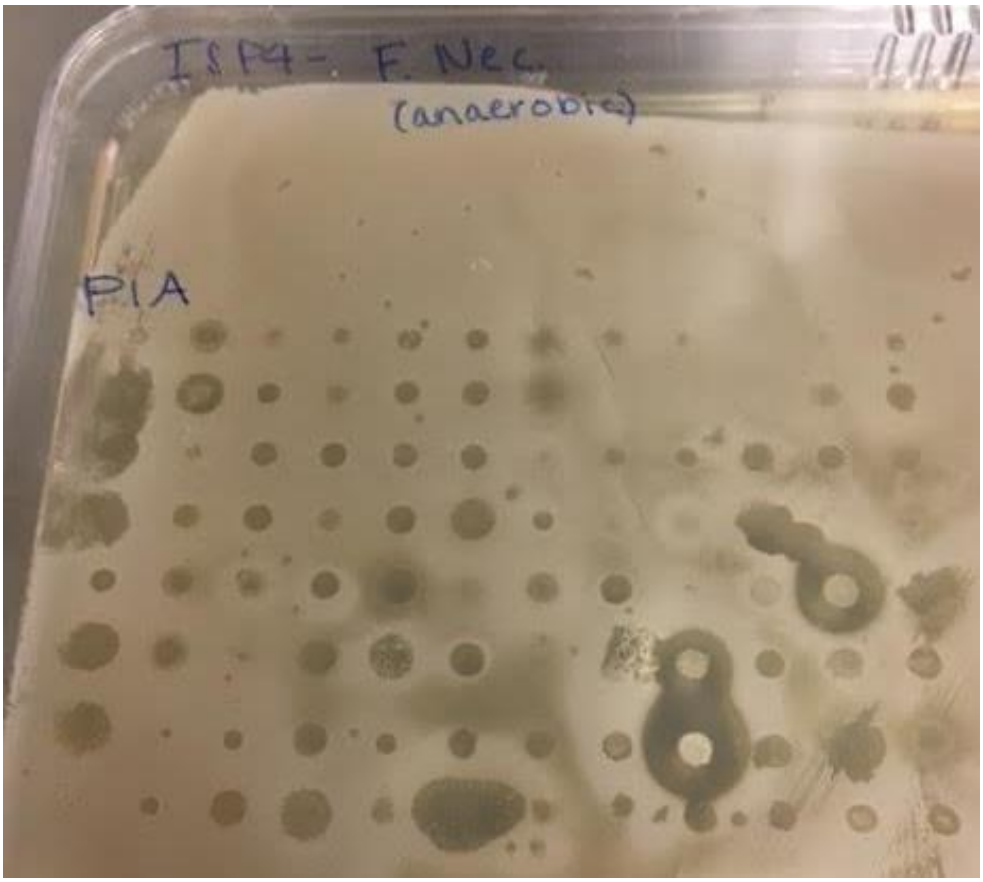


Figure 2. Secondary inhibition test conducted for isolates “11E” and “12F” against *Streptococcus bovis ATCC 33317* and *Fusobacterium necrophorum necrophorum ATCC 25286.* **a.** Isolate *ISP4 P1A 11E* inhibiting *Streptococcus bovis ATCC 33317.* ***b.*** Isolate *ISP4 P2A 12F* inhibiting *Streptococcus bovis ATCC 33317* ***c.*** Isolate *ISP4 P1A 11E* inhibiting *Fusobacterium necrophorum necrophorum ATCC 25286* ***d.*** Isolate *ISP4 P2A 12F* inhibiting *Fusobacterium necrophorum necrophorum ATCC 25286.* All tests were performed on BHI agar plates and inhibition was observed after 24hrs of growth under anaerobic conditions. The cells of interest are circled in red.

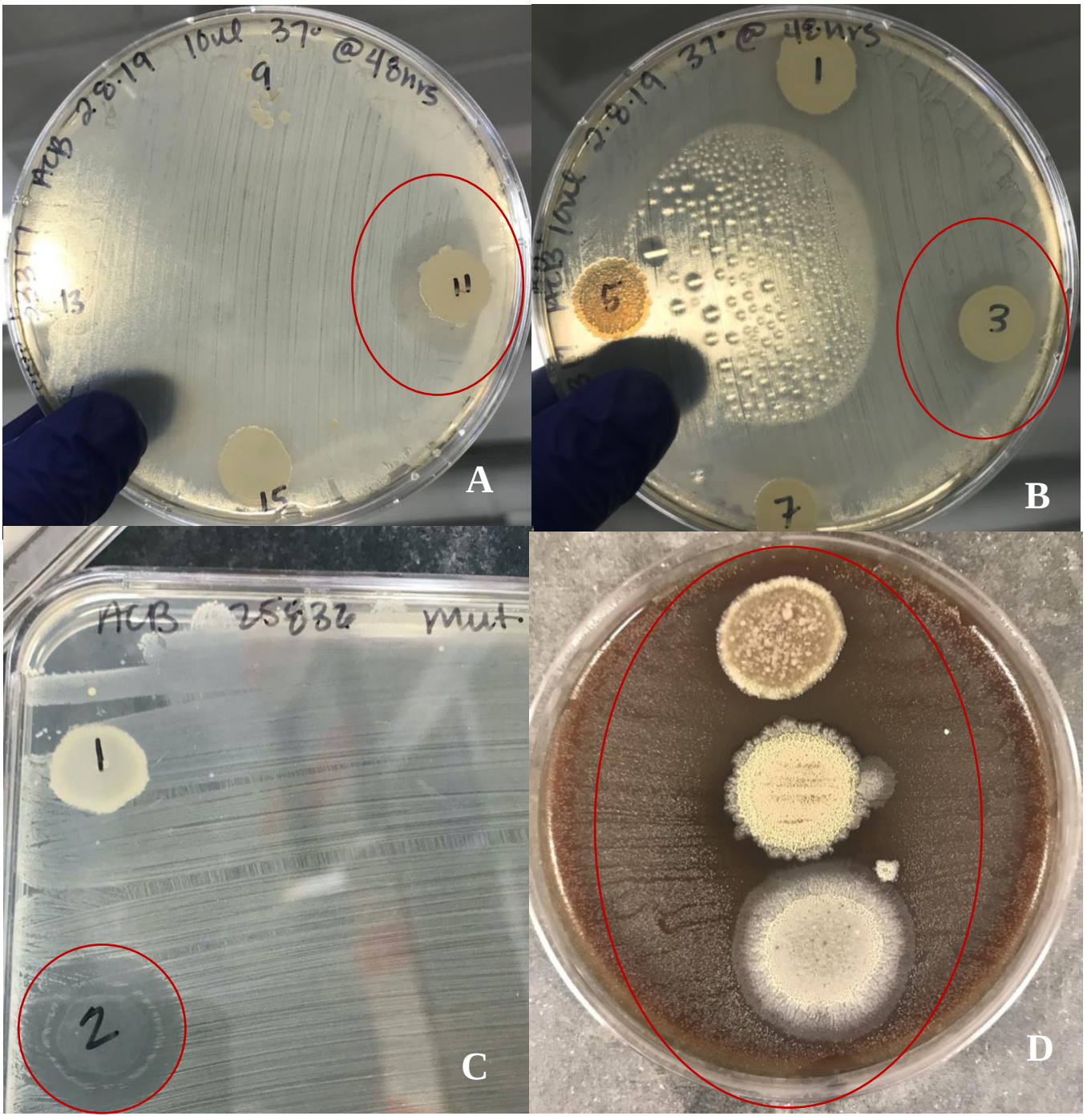


Figure 3. Representative results from flow cytometry analysis after live/dead staining. The cell counts were used to determine the amount of cells present on cultured to determine feeding concentrations. Panel A shows the gate set for counting cells, panel B shows the live, dead, damaged bacterial cell populations identified. Panel C shows

absolute cell counts and distribution. Panels D & E shows the gate and the population of control beads identified by cell cytometry.

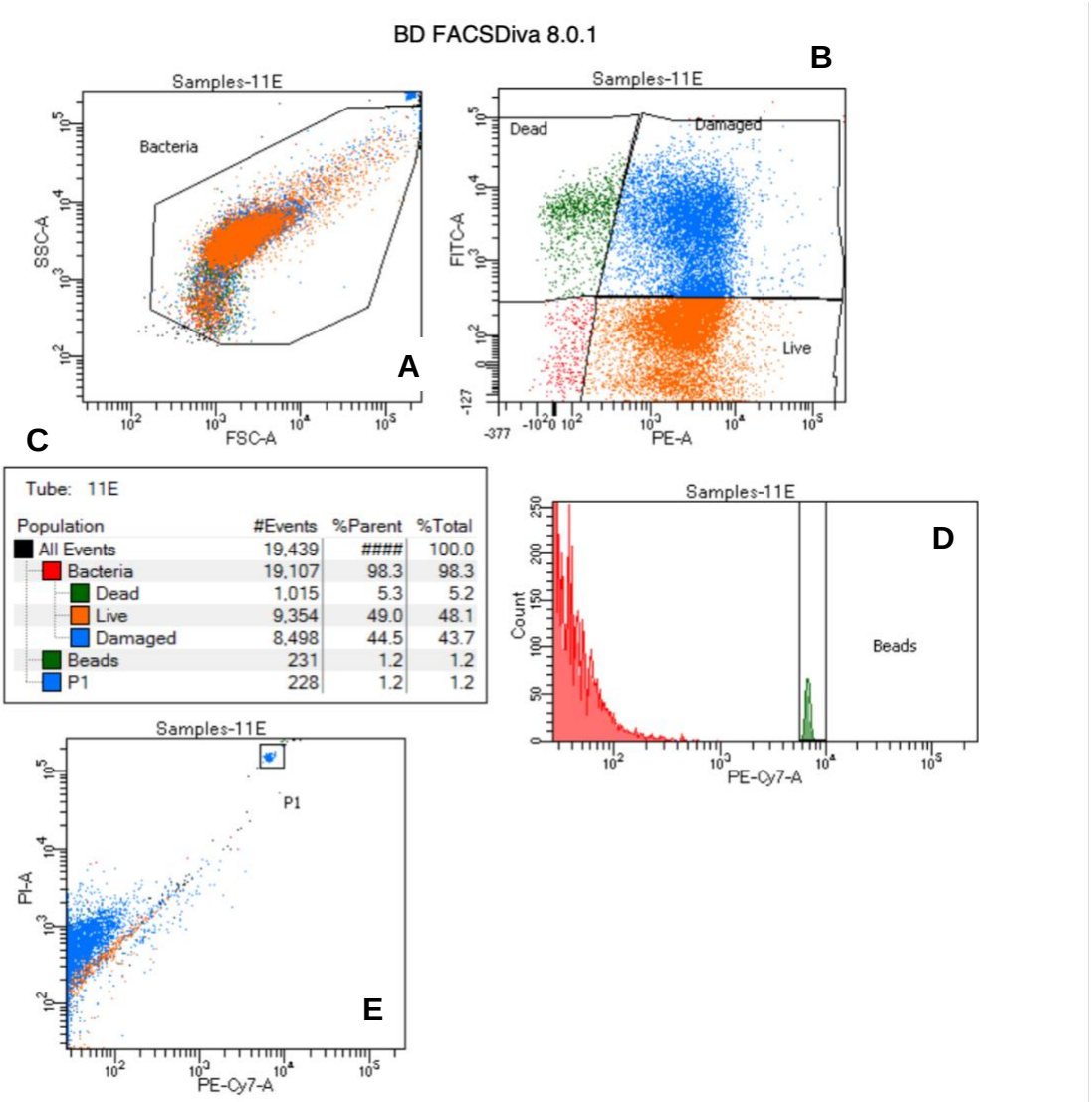
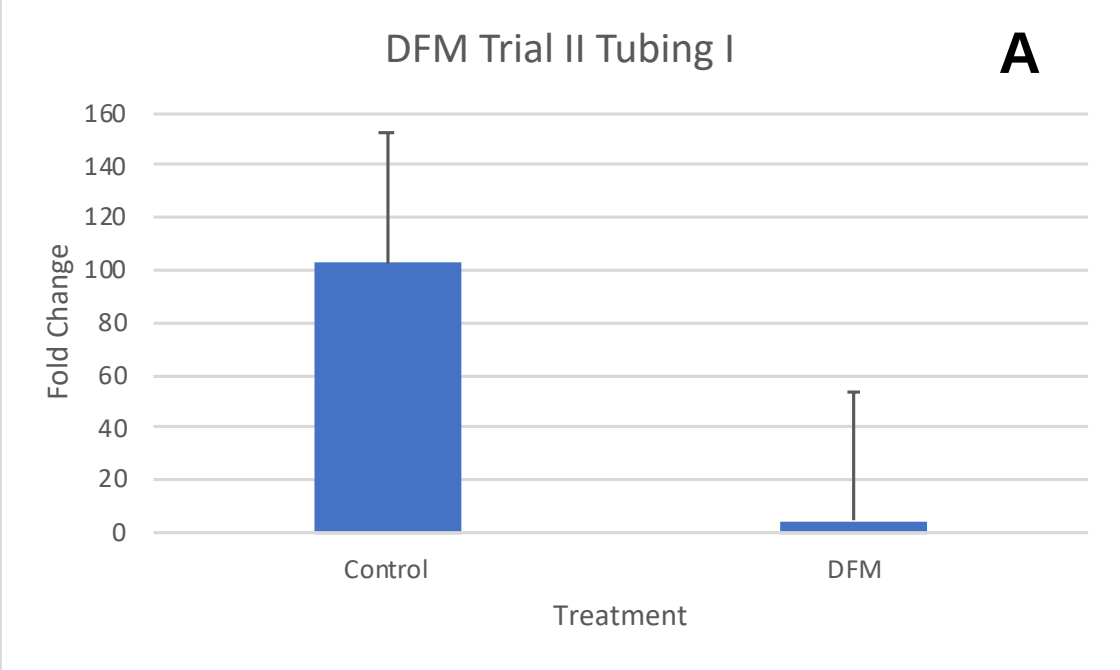
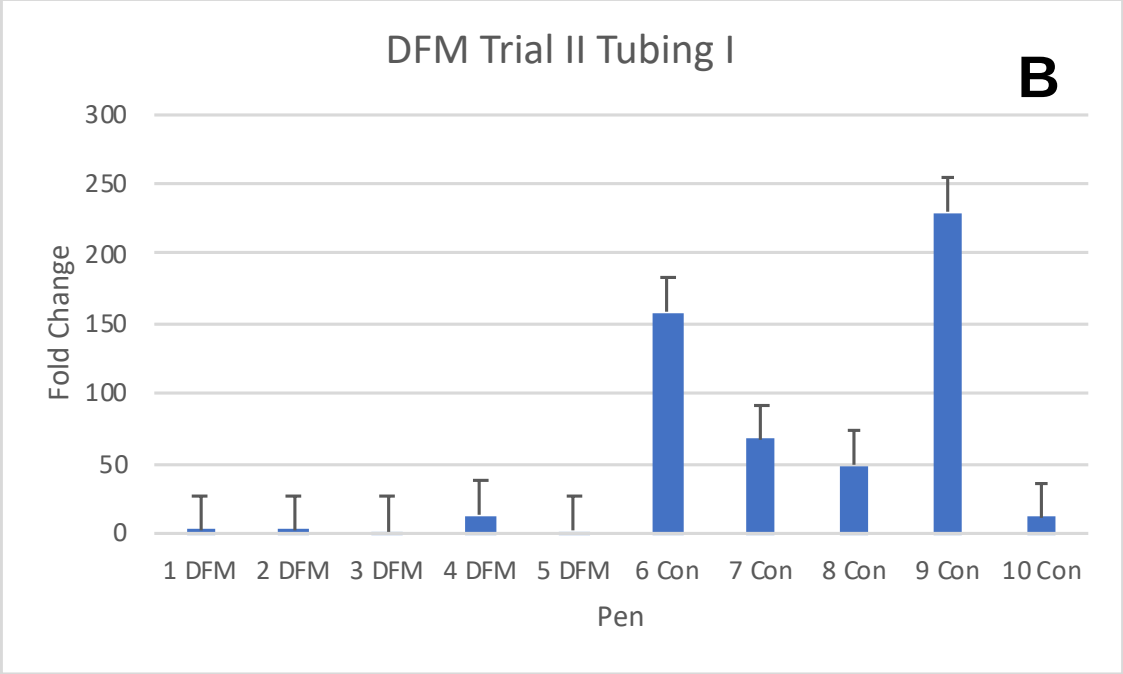
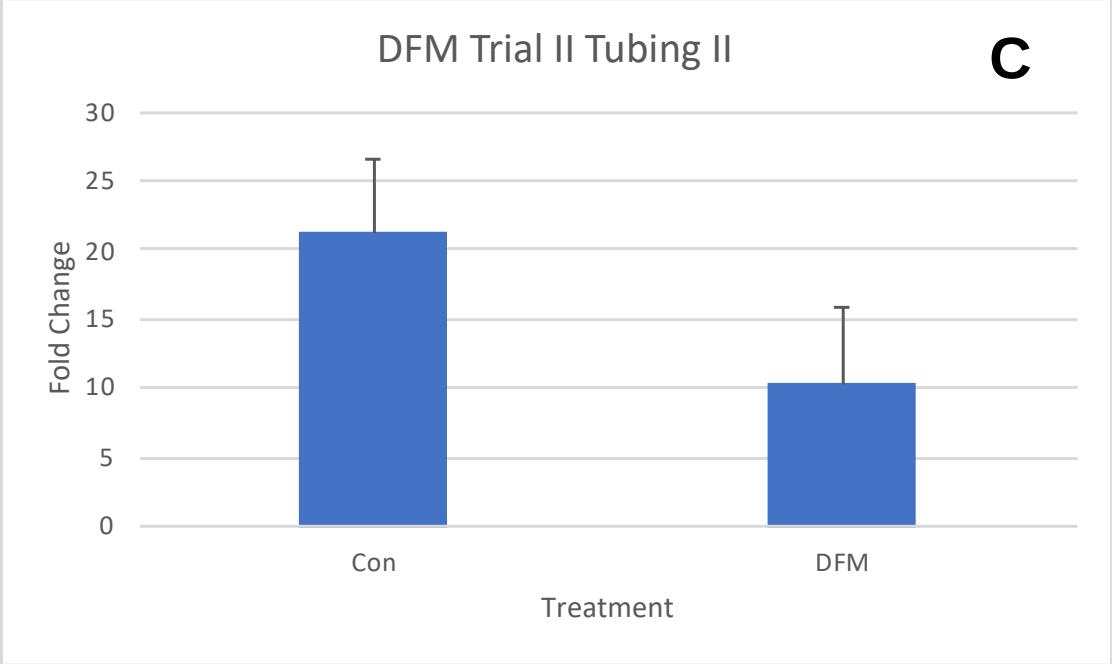
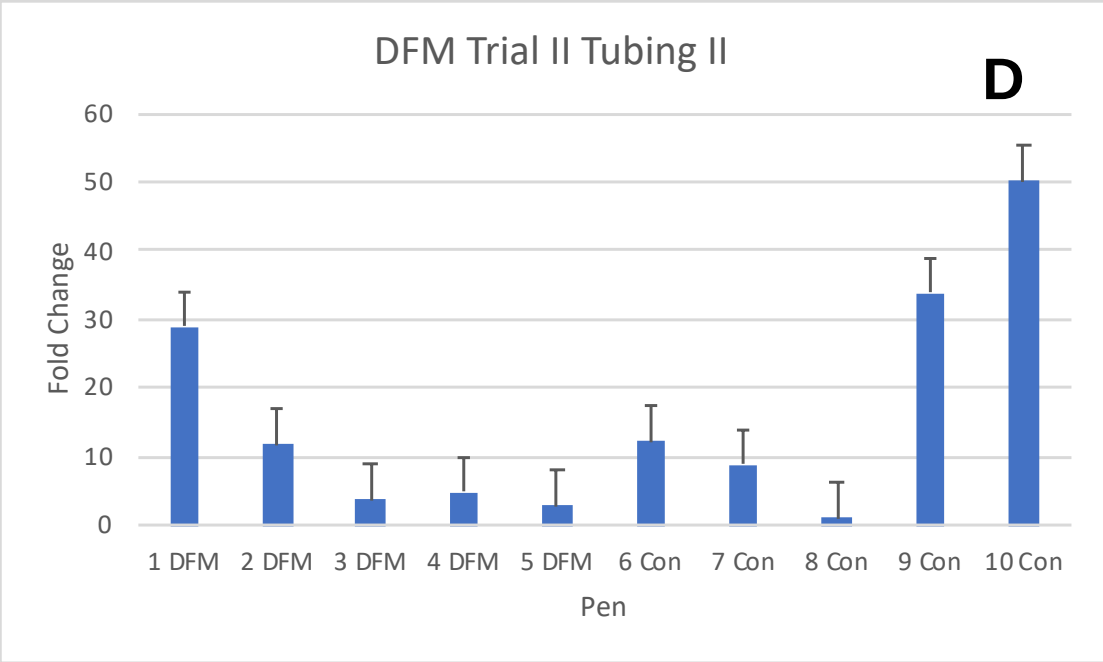


Figure 4.
Realtime PCR analysis results for abundance of *F. necrophorum* in rumen samples collected from the in-vivo trial conducted at United States Meat Animal Research Center (USMARC) to investigate the efficacy of the DFM to reduce *F. necrophorum* abundance and in turn liver abscesses in cattle. Panel 4a and 4b shows realtime PCR results for rumen samples collected on day 89 after feeding *B. pumilus* (*11E*) overall abundance and on pen-basis respectively. Panel 4c and 4d shows realtime PCR results for rumen samples collected on day 171 after feeding a cocktail of *B. pumilus* (11E) and *B. licheniformis* (12E) overall abundance and pen-basis respectively. Pens 1-5 received a DFM whereas pens 6-10 were control pens. There was a significant difference in *F. necrophorum* abundance detected among treatments for the first tubing ($p < 0.038$) however, no difference was seen at second tubing ($p = 0.322$).




DFM Trial II Tubing I
B
300
250
200
150
100
50
0
Fold Change
1 DFM
2 DFM
3 DFM
4 DFM
5 DFM
6 Con
7 Con
8 Con
9 Con
10 Con
Pen


DFM Trial II Tubing II
C
30
25
20
15
10
5
0
Fold Change
Con
DFM
Treatment


DFM Trial II Tubing II
D
60
50
40
30
20
10
0
Fold Change
1 DFM
2 DFM
3 DFM
4 DFM
5 DFM
6 Con
7 Con
8 Con
9 Con
10 Con
Pen

3 Longitudinal assessment of the bovine ocular microbiome

3.1 ABSTRACT

A commonly found disease in the beef cattle industry is Infectious Bovine Keratoconjunctivitis (IBK). Better known as pinkeye, this disease has an impact of close to 150 million dollars annually to the beef cattle industry. However, intervention strategies to prevent IBK is fairly limited, with most cases resulting in treatment with antibiotics once the disease has developed. This is partly, due to the lack of studies evaluating establishment of the ocular microbiota to identify critical times of IBK outbreak. The understanding of the establishment and composition of the ocular microbiome in cattle are limited. The few studies that have investigated the ocular microbiome in cattle suggest that colonization by opportunistic pathogens lead to IBK outbreaks. In an attempt to characterize the establishment and colonization patterns of the bovine ocular microbiome, we conducted a longitudinal study consisting of 239 calves using three different preventative treatments (Autogenous, Commercial, and Placebo) and evaluated the microbiome composition over time using 16S rDNA. A significant change in bacterial community composition was observed across time periods ($p < 0.001$). Where, the community reverted back to the original composition post perturbation and vaccination after 139 days of the initial perturbation demonstrating the resilience of the ocular microbiome to change once established. Additionally, dynamic changes in opportunistic pathogens *Mycoplasma spp.* and *Moraxella spp.* were observed. This study characterizes the diversity of the ocular microbiome in calves and demonstrates the plasticity of the ocular microbiome to change. Characterizing the dynamic nature of the ocular microbiome provides novel opportunities to develop potential probiotic intervention to reduce IBK outbreaks in cattle.

Key words: Infectious Bovine Keratoconjunctivitis, 16S rDNA, microbiome, *Moraxella*, *Mycoplasma,* probiotic

3.2 Introduction

One of the most limiting diseases found among the beef cattle industry is Infectious Bovine Keratoconjunctivitis (IBK), commonly known as pinkeye (Cullen et al. 2017, Dickey et al., 2016). IBK impacts the beef industry with costs close to 150 million dollars annually, due to increased treatment cost, decreased weaning weight and decreased milk production (Whittier et al., 2009). The symptoms of IBK are variable and cattle with IBK exhibit corneal ulceration and edema. Additionally, this disease causes ocular pain and varying degrees of corneal scarring, corneal rupture and permanent blindness depending on the severity of the IBK infection (Angelos et al., 2004, Angelos et al., 2010). Whittier et al., (2009), reported that cattle with IBK on average weighed 19.6 lb less at weaning than did healthy calves. Likewise, IBK is commonly thought of as the most problematic disease in breeding age beef heifers, where cattle infected are more prone die due to starvation or due to an accident.

While there are many factors that contribute to an IBK infection, *Moraxella bovis* and *Mycoplasma bovoculi,* are thought to predispose the animal to disease. It is suggested that *Moraxella* and *Mycoplasma* enter the eye via face flies or as an effect of secretion after irritation of the eye due to cuts from tall grass, feed, or UV light from the sun. Asymptomatic animals can also affect IBK as eyes, nose, and vagina are locations that commonly harbor *Moraxella* and *Mycoplasma* and can shed pathogens to the environment that can lead to infection (Whittier et al., 2009).

Currently, prevention for IBK has been limited to the use of vaccines (Whittier et al., 2009, Angelos 2010, Angelos et al., 2010, & O'Connor et al., 2011). However, the use of vaccines has shown variable outcomes. Thus, producers are utilizing fly tags, back rubbers, pour-ons and pasture management to reduce the occurrence of IBK outbreaks. Partly, one reason for the lack of intervention strategies for IBK control is our limited understanding of the ocular microbiome in cattle and how the ocular microbiome is established and reacts to perturbation. Additionally, what changes in the ocular microbiome that leads to increase in opportunistic pathogen colonization is poorly understood. This is mainly due to the lack of studies investigating the ocular microbiome. In a recent study, Cullen et al. (2017), utilized high-throughput sequencing of the 16S rDNA gene to compare bacterial communities of calves who developed IBK to calves who did not show IBK symptoms. This first study of the bovine ocular microbiome demonstrated no large-scale community differences between cattle infected with IBK and cattle with no IBK symptoms. Still, Faith's Phylogenetic Diversity showed that there was a greater diversity found among cattle infected with IBK than the control cohort. Additionally, Cullen et al. (2017), concluded that there was no variation in *Moraxella* sp. abundance among the two cohorts even though *Moraxella* was identified as the most abundant genera. With many studies describing the dynamic nature of the microbiome (Greber 2014), it is critical to monitor the microbiome over time to identify the stable phenotype of the microbiome and to identify "windows of opportunity" that increase disease susceptibility and for intervention. Therefore, in this study, to better assess bacterial composition within the bovine eye, we monitored bacterial community changes over time. Thus, we utilized a longitudinal study to assess the bacterial

community which included before and after perturbation of the ocular microbiome using 16S rDNA sequencing to identify ocular microbiome changes over time and changes that occur during ocular perturbation.

3.3 Materials and Methods

3.3.1 Animals

All animal-related procedures and interventions implemented in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska-Lincoln. The experimental design consisted of 239 calves (n=239) with three treatments of Autogenous, Commercial, and Placebo given at day zero and a booster at day 21. The longitudinal study consisted of 139 days with four sampling at day 0, 21, 41 and 139. Months for the duration of the study spanned from May 16th to October 2nd. The cohort of calves were composed of both male and female, red and black hide color, and breeds of angus or Simmental composites. Average age of calves on trial began at 65 and at weaning (day 139 or time 4) the average age of calves were 204 days old.

Flocked nylon swabs were used to sample the inferior ocular conjunctival surface and the resulting swabs were placed in modified liquid amies transport media (Eswab Transport System, Copan). Media was kept on ice and transported to the laboratory where an aliquot was frozen -80 °C until used for microbial community analysis.

3.3.2 DNA Extraction and Sequencing

DNA was extracted using Lucigen Quick Extract kit (Lucigen Corporation Middleton, WI) according to the manufactures protocol with a modification of adding a bead beating step using a TissueLyser (Qiagen Inc., Valencia, CA, USA) at a frequency of 20 Hz for 15 minutes prior to water bath incubations at 65for six minutes and 98°C for five minutes. Isolated DNA was stored at -20°C until used for bacterial community analysis. Amplification of the V4 region of the 16S rDNA gene and sequencing using multiplexed barcodes was performed using the MiSeq 250 bp paired ends sequencing strategy as described previously (Kozich et al. 2013). Briefly, barcoded universal primers specific to the V4 region were used to amplify the 16S rDNA gene in 25 μl PCR reactions. Library preparation PCR reaction contained 0.75 Units of Terra PCR Direct Polymerase Mix (Clontech Laboratories Inc., Mountain View, CA, USA), 1X Terra PCR Direct Buffer (Clontech Laboratories Inc., Mountain View, CA, USA), 0.4 uM indexed primers, and 20-50ng of DNA. Amplifications were performed on a Veriti 96-Well Thermocycler (Life Technologies, Carlsbad, CA, USA) with conditions of 98°C for 2 minutes followed by 25 cycles of 98°C for 30s, 58°C for 30 seconds, 68°C for 45s, with a final extension of 68°C for 4 minutes. The resulting amplicons were normalized and sequenced using the 250bp paired end sequencing strategy using the Illumina MiSeq platform with V2 500 cycle sequencing kit as described by the manufacturer (Illumina, San Diego, CA, USA).

3.3.3 Protein Quantification and ELISA preparation

A subset of animals were identified based on microbial community differences that reflect the largest changes in community structure across the time points sampled and

were used to evaluate bovine immunoglobulin A levels (IgA). Briefly, protein levels in the eye swabs were quantified using Qubit™ Protein Assay Kit (Invitrogen Carlsbad, CA, USA) according to the manufactures protocol and normalized protein amounts (19-24 mg) were assayed using the Bovine IgA ELISA kit (BioMatik Corporation Cambridge ON, Canada) according to the manufactures protocol to evaluate bovine IgA amounts.

3.4 Data Processing

Detailed information regarding the bioinformatic analysis used including the mapping file, scripts, and commands can be found in the GitHub page of the Fernando Lab (https://github.com/FernandoLab/IBK-Year1-). The Dada2 pipeline (Callahan et al., 2016) was used for subsequent analysis. Briefly, analysis steps were performed using R (R Core Team, 2019) within the phyloseq package (McMurdie and Holmes, 2013) following the workflow described in https://www.bioconductor.org/help/course-marterials/2017/BioC2017/Day1/Workshops/Mircobiome/MicrobiomeWorkflowII.html.

In short, low quality reads were filtered (Q score of ≥20) and reads were trimmed to 230 bp length. “Unique sequences” were identified by combining identical sequences and error rates were estimated to evaluate read quality. Forward and reverse reads were assembled to generate contigs for the V4 region. Furthermore, quality filtering was performed to remove sequences with ambiguous bases, incorrect contig length and assembly, or chimeras. SILVA reference alignment database v123 was used to assign taxonomy and a phylogenetic tree was generated using MOTHUR (v.1.42.1). The

resulting phylip.tree was used to generate a phyloseq object which was used for further analysis.

Any potential contaminant ASVs arising from reagent contamination were removed using the decontam package as described previously (Davis et al., 2018). Additionally, any ASV with a prevalence of only one sample were removed. All data generated in this project have been deposited in the NCBI sort read archive under accession number PRJNA600014.

3.5 Statistics

Statistical analyses were performed using the R package ‘vegan’ (Dixon et al., 2003) using the adonis function. The PERMANOVA analysis was performed to identify factors effecting microbial community structure using factors of time, age, treatment, hide color, breed, ulcer_postive, and sex. Additionally, a pairwise Wilcox test was used in R to look at differences between times of sampling. Significant differences in *F. necrophorum* abundance in qPCR analysis was performed using ANOVA. All significance was determined at $p < 0.05$.

3.6 Results

3.6.1 Changes in the bovine ocular microbiome over time

Following quality filtering and removal of reads classified as Archaea and Eukaryota, the resulting data contained 862 samples with 23,647,648 reads with an average of 26,620 reads per sample. These reads were binned into 5,275 ASVs. To evaluate bacterial community changes during establishment and perturbation of the ocular microbiome,

reads were rarefied to an even depth and was used for subsequent analysis. Changes in bacterial community diversity was evaluated using alpha diversity metric of observed ASVs and Chao1 estimates. Alpha diversity using both observed ASVs and Chao1 estimates displayed significant differences in bacterial diversity between all-time points demonstrating the dynamic and rapid changes in the bovine ocular microbiome. The least diversity was identified 3 weeks after the initial perturbation of the eye and at subsequent sampling the diversity increased over time reaching diversity levels greater than before perturbation of the eye (Figure 1).

To further evaluate global changes in the ocular bacterial community over time, a principal component analysis was performed on the normalized data using a weighted UniFrac distance matrix (Lozupone et al., 2011). The PERMANOVA analysis reveal significant differences in bacterial community composition between time period sampled ($p < 0.001$), days old ($p = 0.011$), and cattle who showed positive signs of ocular ulcers ($p = 0.$ 0.032). However, treatment ($p = 0.491$), hide color ($p = 0.383$), sex ($p = 0.407$), and breed ($p = 0.162$) did not show significant effect (Table 1, Figure 2) on bacterial community composition. Interestingly, time points 1 & 4 and 2 & 3 displayed similar bacterial community to each other respectively but were significantly different from each other (1&4 vs 2&3). This suggests the bacterial community is changing after perturbation and is reaching a community similar to before perturbation over time. To further evaluate this observation, we performed a hierarchical clustering of the bacterial communities. This analysis also displayed a similar result of bacterial communities changing after perturbation to establish the community before perturbation.

3.6.2 Core measurable microbiome in the bovine ocular microbiome

It is well documented that the microbiome varies between individuals (Gerber 2014). As such, to look beyond variation in the microbiome and to identify the core bacterial community present within the ocular microbiome, we identified the core measurable microbiome (CMM) by identifying ASVs that were present in at least 80% of total samples collected. This analysis only identified three ASVs to be part of the CMM. These core ASVs accounted for 81% of total reads in the dataset. The ASVs represented genera of *Preveotella*, *Mycoplasma*, and family *Weeksellacea*. The distribution of the core ASVs across time points is shown in Figure 3. To further evaluate the clustering of bacterial communities shown in the principal component analysis, we identified core microbial ASVs present in each time point using a criteria of ASVs represented in 75% of the animals in each time point. For time period one 25 ASVs were identified, accounting for 39% of the total reads. Similarly, 3, 5, and 23 ASVs accounting for 81%, 81%, 85% of the total reads for each time point was identified for time periods two, three, and four respectively. Hierarchical clustering of this core ASVs based on abundance displayed clear clustering based on time point where, time periods 2 and 3 clustered closely together whereas, timepoints 1 and 4 clustered together away from time points 2 and 3 (Figure 4). The major genera driving this difference included *Preveotella, Mycoplasma,* and *Moraxella. Preveotella* and *Mycoplasma* which appear to be in inverse of each other. Additionally, *Preveotella* was higher in abundance during time periods 2&3 and *Mycoplasma* as well as *Moraxella* were in higher abundance during time periods 1&4.

This analysis further confirmed the structuring of bacterial communities by time and also suggested that in time points 2 and 3 that the community is changing rapidly with loss of diversity as the few core ASVs identified represented 81% and 81% of the total reads sequenced.

3.6.3 Relationship between bacterial ASVs and IBK outbreak

During the study period, a few cattle were identified with IBK and were treated. Cattle treated for IBK during the study were given an ulcer score. Of the 239 animals, only 20 animals were infected with IBK. We evaluated the bacterial community composition of the animals treated for IBK using the sampling time points collected before IBK infection and after IBK infection using a weighted UniFrac distance matrix. The PCoA plot displayed clustering based on if the sample was collected before or after IBK infection (Figure 5). PERMANOVA analysis did detect a significant change ($p < 0.035$) in the overall structure of the bacterial community suggesting a composition shift during pink eye infection. Further analysis of the differential ASVs identified before and after IBK infection displayed differences in abundances of differential ASVs over time. More specifically, those ASVs belonged to Family of *Ruminococcaceae* and *Lachnospiraceae* with an increase in abundance post infection. (Figure 6).

3.6.4 Abundance of known IBK predisposers in the bovine ocular microbiome

Previous studies have reported *Mycoplasma spp.* and *Moraxella spp.* to be the major pathogens that predispose cattle to IBK. Therefore we evaluated the abundance of

Mycoplasma spp. and *Moraxella spp..* The mean abundance of these taxa are shown in Figure 7. Although the relative abundance was low, *Moraxella spp.* were identified on all time points, with the highest abundance during the first time period. However, *Mycoplasma* abundance was much greater than the abundance of *Moraxella* with the highest abundance of almost 50% in time period four.

3.6.5 Effect of Immunoglobulin A (IgA) on structuring of the bovine ocular bacterial community

Previous reports in the human ocular microbiome has suggested IgA levels within the eye has an effect on the ocular microbiome (Ozkan and Wilcox 2019). To evaluate if the effect of IgA on the ocular microbiome was enhanced post perturbation, we evaluated IgA levels in animals with significantly different ocular bacterial community structure. IgA concentrations did not differ in animals with different bacterial community composition suggesting IgA concentration was not a driver of the ocular microbiome in this study (Figure 8). which showed no significant difference among time periods of IgA concentration when ocular swabs were taken.

3.7 Discussion

IBK outbreaks have continued to have economic consequences to the beef cattle industry. With the lack of effective preventative methods to control IBK, the cattle industry is faced with the challenge of developing novel methods to control and mitigate IBK outbreaks. There has been an increasing emphasis on microbiome investigations to harness the therapeutic potential of the microbiome. As such, novel opportunities may be available to develop novel methods to control and monitor IBK outbreaks in cattle. In

this study we investigated the bovine ocular microbiome over time to evaluate the bacterial compositional changes in the ocular microbiome before and after subsequent perturbations to investigate the dynamic and resilience nature of the ocular microbiome. We phenotyped the microbiome using 16S rDNA based sequencing of the V4 region using the Illumina MiSeq platform using 250 bp paired-end sequencing strategy.

3.7.1 The window for re-establishment of the ocular microbiome is long

The bovine ocular microbiome changed significantly over the 4 time periods sampled, which was during peak IBK periods. Where, the diversity of the microbiome dramatically decreased after swabbing and was very low when sampled 21 days after the first swabbing. At 41 days post swabbing (after the second swabbing) the bacterial diversity slightly increased relative to the first swabbing but remained significantly low compared to the initial swabbing. However, with time the bacterial diversity increased and re-established at diversity levels similar to initial swabbing after 139 days post swabbing (Figure 1). This demonstrates that when a change in the bovine ocular microbiome occurs, it takes a long time for the microbiome to re-establish to stable levels prior to the perturbation. As such, this suggests that it is possible that perturbations of the ocular microbiome due to environmental factors such as cuts from tall grass, feed, or UV light from the sun (Whittier et al., 2009) can lead to changes in the ocular microbiome that provide a window of opportunity for opportunistic pathogens such as *Moraxella spp*. and *Mycoplasma spp*. to establish and lead to IBK.

Further evaluation of factors that influence the ocular bacterial community in cattle using PERMANOVA analysis revealed time ($p < 0.001$), days old ($p = 0.011$), and cattle who showed positive signs of ocular ulcers ($p = 0.032$) to be significant drivers of the bacterial community. Previous studies have reported the human ocular microbiome to be low in both bacterial diversity and abundance (Ozkan et al., 2017).This observation was consistent with the bovine ocular microbiome where diversity estimates were lower than the estimates reported from the rumen and fecal microbiota in cattle (Figure 1) (Ozkan et al., 2017). Additionally, we observed a greater decrease in the microbiome with perturbation of the eye. Many of the studies investigating the ocular microbiome has performed single point analysis and therefore have not investigated the changes over time (Cullen et al., 2017). As such, to our knowledge this is the first report of the changes and re-establishment of the ocular bacterial community over time as a result of perturbation. Interestingly, the bovine ocular microbiome has a higher represented population of bacteria in comparison to what has been published in human literature (Ozkan and Wilcox 2019, Ozkan et al., 2017).

3.7.2 Core taxa present within the bovine ocular bacterial community

The core microbiome analysis identified 3 ASVs to be part of the CMM that accounted for a major proportion of the reads sequenced (Figure 3). These core ASVs included three opportunistic pathogens *Preveotella*, *Mycoplasma*, and *Weeksellacea.* In a study conducted by Haung et al. (2016), the conjunctiva of adult humans were sampled. The results from this study found that genera related to *Corynebacterium* and

Pseudomonads were the primary bacteria found among samples (n=31) and concluded that bacteria found among the eye, are typically deemed as opportunistic pathogens. In addition to identifying core bacterial taxa present within all-time points to identify autochthonous bacterial species within the ocular microbiome, we identified core bacterial taxa present within each time period sampled. This analysis identified 36 unique ASVs across the 4 sampling periods. Interestingly, 31 of these ASVs belonged to Phylum *Firmicutes*, *Bacteroidetes*, or *Proteobacteria*, which constituted for 86% of the core group. Of the three major phyla making up this core group, two-thirds belong to opportunistic pathogen groups with phylum *Bacteroidetes* being the only group with health benefits suggesting that the core bacterial community within the eye may harbor a major proportion of opportunistic pathogens which may respond to environmental changes and physical damages that lead to IBK and other eye related diseases. This observation of low diversity and predominance of *Firmicutes*, coincides with observations reported in the human ocular microbiome where genus *Staphylococcus* has been reported to predominate (Ozkan and Wilcox 2019 and Ozkan et al., 2017). Analysis of core ASVs share across the different time points revealed that out of the 25 core ASVs were identified during baseline sampling, only 2,4, and 13 of these ASVs overlapped with bacterial communities identified in time points 2,3 and 4. Similarly, 3, 5, and 23 ASVs accounting for 81%, 81%, 85% of the total reads for each time point was identified for time periods two, three, and four respectively. Hierarchical clustering of this core ASVs based on abundance displayed clear clustering based on time pointy where, time periods 2 and 3 clustered closely together whereas, timepoints 1 and 4

clustered together away from time points 2 and 3 (Figure 4). The major families driving this difference included *Preveotella, Mycoplasma,* and *Moraxella*.

3.7.3 Shifts in the ocular bacterial community in response to IBK

The study was designed to evaluate microbiome composition over time in a cattle cohort. However, we identified a small subgroup of cattle that were infected with IBK and underwent treatment. The ulcer scores recorded for cattle with IBK infection, when analyzed with respect to the weighted UniFrac distance matrix, scores displayed a negative /positive correlation where the higher/lower UniFrac scores were significantly correlated with increased ulcer size. This suggests changes in the bacterial community is associated with size of ulcer and in-turn disease severity. Additionally, the PCoA plot displayed some clustering based on, if the sample was collected before or after IBK infection (Figure 5). Suggesting a microbial community shift during IBK infection. Due to small number of animals being infected in this study we were unable to further pursue this question as to which features in the microbiome may be indicators of IBK infection or may predispose animals to IBK.

3.7.4 *Mycoplasma* and *Moraxella* are common inhabitants of the bovine ocular microbiome

Current literature suggests *Moraxella* and *Mycoplasma* to be the major predisposers of IBK which then allows for other opportunistic pathogens to cause infection (Angelos 2010, Loy and Brodersen 2014, & Zheng et al. 2019). We identified both *Moraxella spp.* and *Mycoplasma spp.* during all time points sampled, suggesting that these two genera may be commensals of the bovine ocular microbiome. Similar to this study, Cullen et al.

(2017) observed *Moraxella* to be one of the most abundant genera in the bovine ocular microbiome. Additionally, the correlation analysis displayed a somewhat negative correlation between the two genera suggesting these two genera may be competing for similar resources and niches within the ocular microbiome.

Previous reports in the human ocular microbiome has suggested the IgA levels in the eye to effect structuring of the ocular microbiome (Ozkan et al., 2017). We investigated the effect of IgA on ocular microbiome using ocular swab samples from animals with significantly different ocular bacterial communities. IgA concentrations did not differ in animals with different bacterial community composition suggesting IgA concentration was not a driver of the ocular microbiome in this study (Figure 8).

The cattle used in this study was not the same age at sampling (Supplementary Figure S1). However, the ocular microbiome establishes over time. Therefore, some of the variation in the microbiome that we are observing can be a result of the age of the animal. This was also clear from the significant effect of age seen in the beta-diversity analysis. Our observation is consistent with a study performed by Wen et al. (2017) as this group reported that the human ocular microbiome changes with age. As such, this study also lends information into the establishment of the ocular microbiome with age. Data from this study suggests the ocular microbiome establishes quickly, but recovery after perturbation is slow. As such future investigations of the microbiome need to be longitudinal. Additionally, this study also demonstrates that since the bovine ocular microbiome is susceptible to change, microbiome manipulation could be used to introduce beneficial microbes to establish a more stable and resilient microbiota.

3.8 Conclusion

The understanding of the establishment and composition of the ocular microbiome in cattle is limited. In this study to better assess bacterial composition within the bovine eye, we performed a longitudinal study to evaluate bacterial community changes before and after perturbation of the ocular microbiome using 16S rDNA sequencing. Our results demonstrate that the bovine ocular microbiome has higher diversity than the human ocular microbiome and is slow to recover and reach stable levels after a perturbation event. Additionally, many factors including age, time, and ulcer infection effect ocular microbiome composition.

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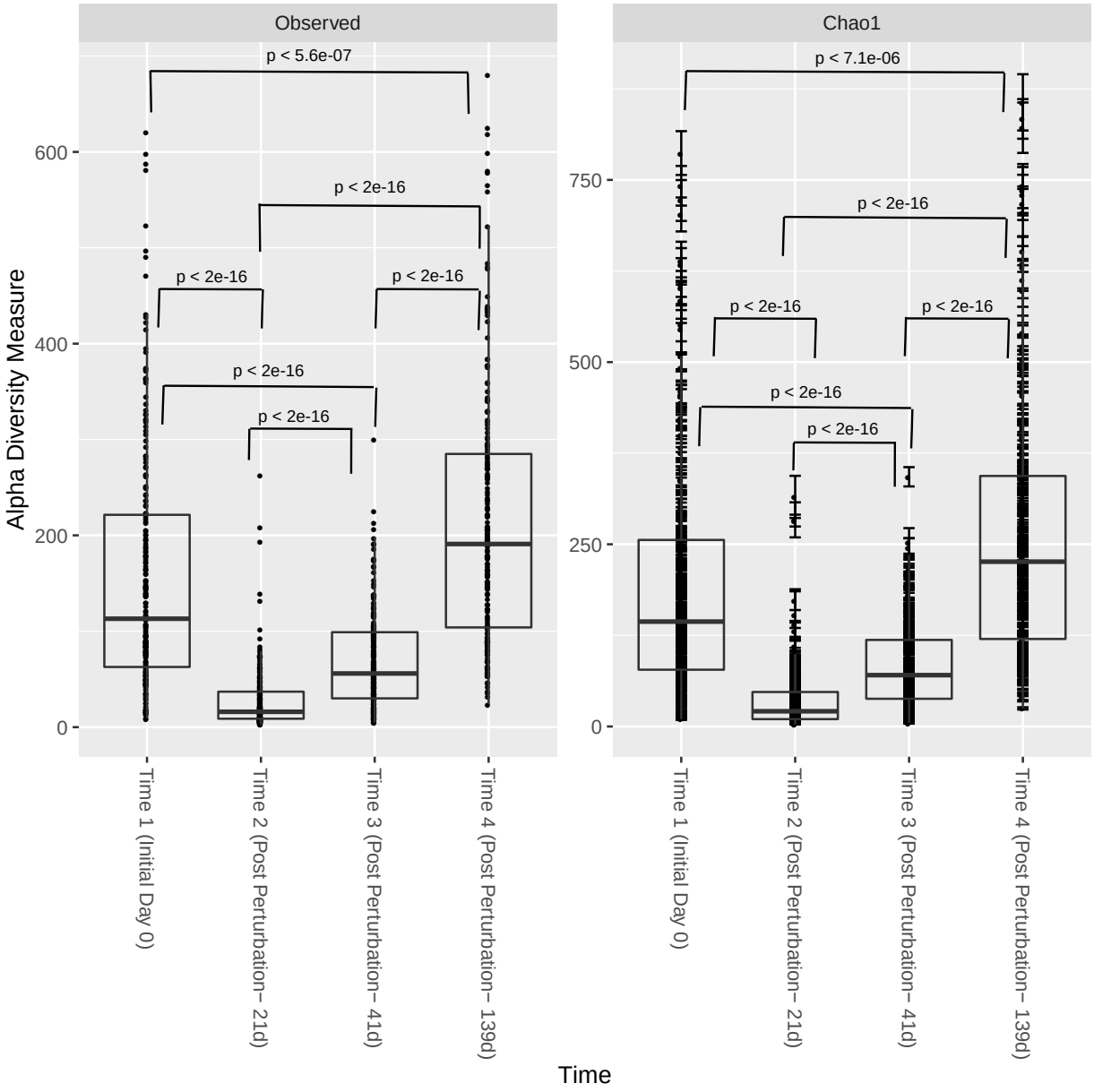
3.10 Appendix II

Figure 1. Changes in alpha diversity over time periods sampled. Observed ASVs (A) and Chao1 estimates (B). Significant differences in alpha-diversity were identified between the sampling periods where the greatest diversity was found in time period 4.



After initial swabbing for baseline community, the diversity significantly decreased and recovered over time in subsequent samplings.

Figure 2. Principal Coordinate analysis (PCoA) demonstrating between sample variations in beta-diversity. PCoA plot was generated using a weighted UniFrac distance matrix. PERMANOVA analysis demonstrated microbial communities to be significantly different based on time of sampling ($p < 0.05$) and is apparent in clustering pattern. Time points 1 and 4 demonstrated similar community structure that was different from time points 2 and 3. Microbial community composition and abundance in time points 2 and 3 were similar to each other. The major drivers of microbial community composition included, time of sampling, age, and ulcer positive. Color scheme; Red – time point 1, Green - time point 2, Teal – time point 3, and Purple – time point 4.

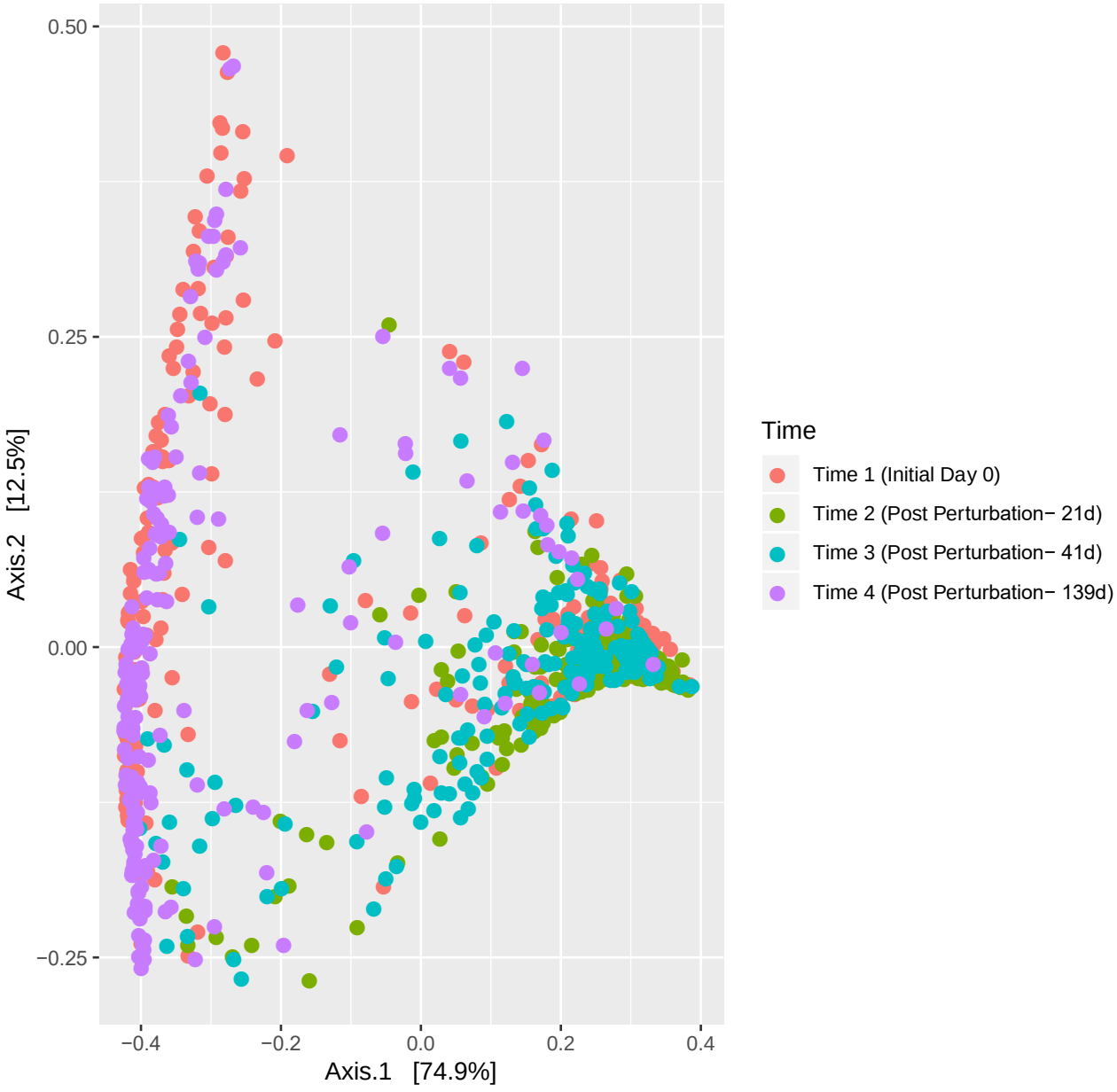


Figure 3. Distribution of “Core” ASVs across sampling time-points demonstrating predominance of opportunistic pathogens in the bovine ocular microbiome. Core bacterial ASVs were identified based on the presence of an ASV in at least 80% of all

samples. Only 3 core ASVs were identified that fell into this criteria. The core ASVs include; *Preveotella spp*. (Blue), *Mycoplasma sp.* (Orange) and *Weeksellacea sp.* (Grey).

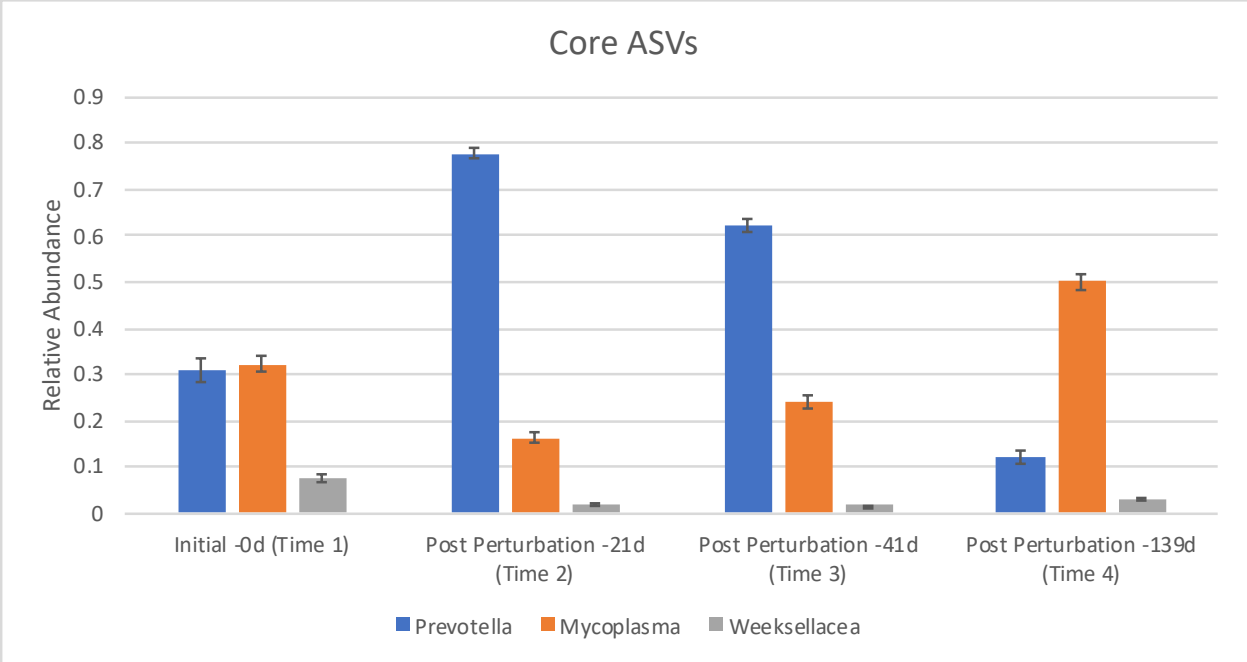
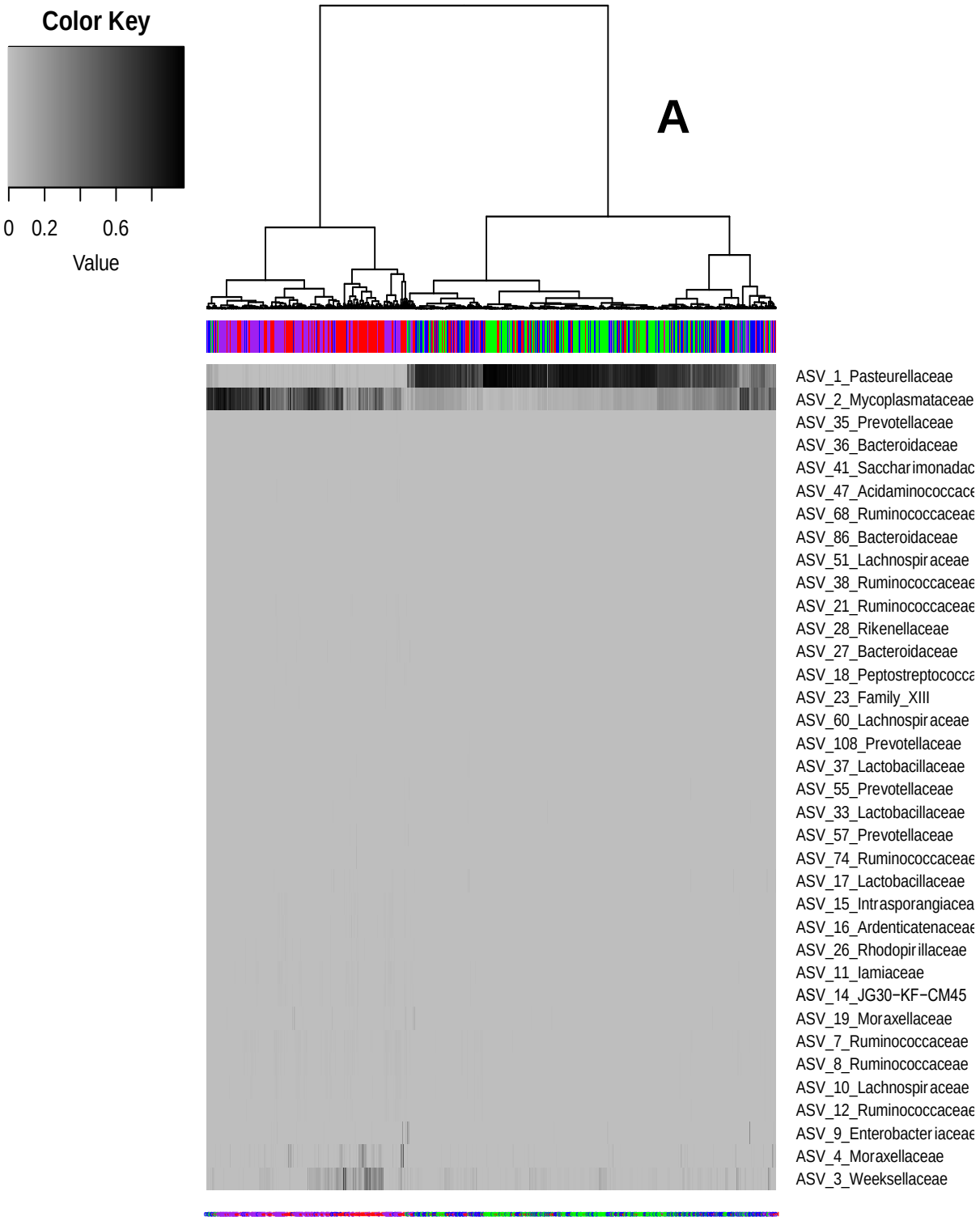


Figure 4. Heat-map showing the distribution of "Core" ASVs representing each time period. Hierarchical clustering of ASV abundance based on sampling time period clearly shows clustering based on sampling period. Red and purple represent sampling periods 1 and 4 and green and blue represent time periods 2 and 3. Samples were individually clustered using hierarchical clustering based on time period. The top 4 most abundant ASVs (4A) and the remaining top 32 ASVs (4B) are shown. Figures A and B are drawn using different scales due to differences in abundance. The top 4 ASVs include

ASV_1_Prevotella, ASV_2_Mycoplasma, ASV_3_Weeksellaea, and ASV_7_Ruminococcacaea.


Color Key
0
0.2
0.6
Value
A
ASV_1_Pasteurellaceae
ASV_2_Mycoplasmataceae
ASV_35_Prevotellaceae
ASV_36_Bacteroidaceae
ASV_41_Saccharimonadac
ASV_47_Acidaminococcace
ASV_68_Ruminococcaceae
ASV_86_Bacteroidaceae
ASV_51_Lachnospiraceae
ASV_38_Ruminococcaceae
ASV_21_Ruminococcaceae
ASV_28_Rikenellaceae
ASV_27_Bacteroidaceae
ASV_18_Peptostreptococca
ASV_23_Family_XIII
ASV_60_Lachnospiraceae
ASV_108_Prevotellaceae
ASV_37_Lactobacillaceae
ASV_55_Prevotellaceae
ASV_33_Lactobacillaceae
ASV_57_Prevotellaceae
ASV_74_Ruminococcaceae
ASV_17_Lactobacillaceae
ASV_15_Intrasporangiacea
ASV_16_Ardenticatenaceae
ASV_26_Rhodopirillaceae
ASV_11_Iamiaceae
ASV_14_JG30−KF−CM45
ASV_19_Moraxellaceae
ASV_7_Ruminococcaceae
ASV_8_Ruminococcaceae
ASV_10_Lachnospiraceae
ASV_12_Ruminococcaceae
ASV_9_Enterobacteriaceae
ASV_4_Moraxellaceae
ASV_3_Weeksellaceae

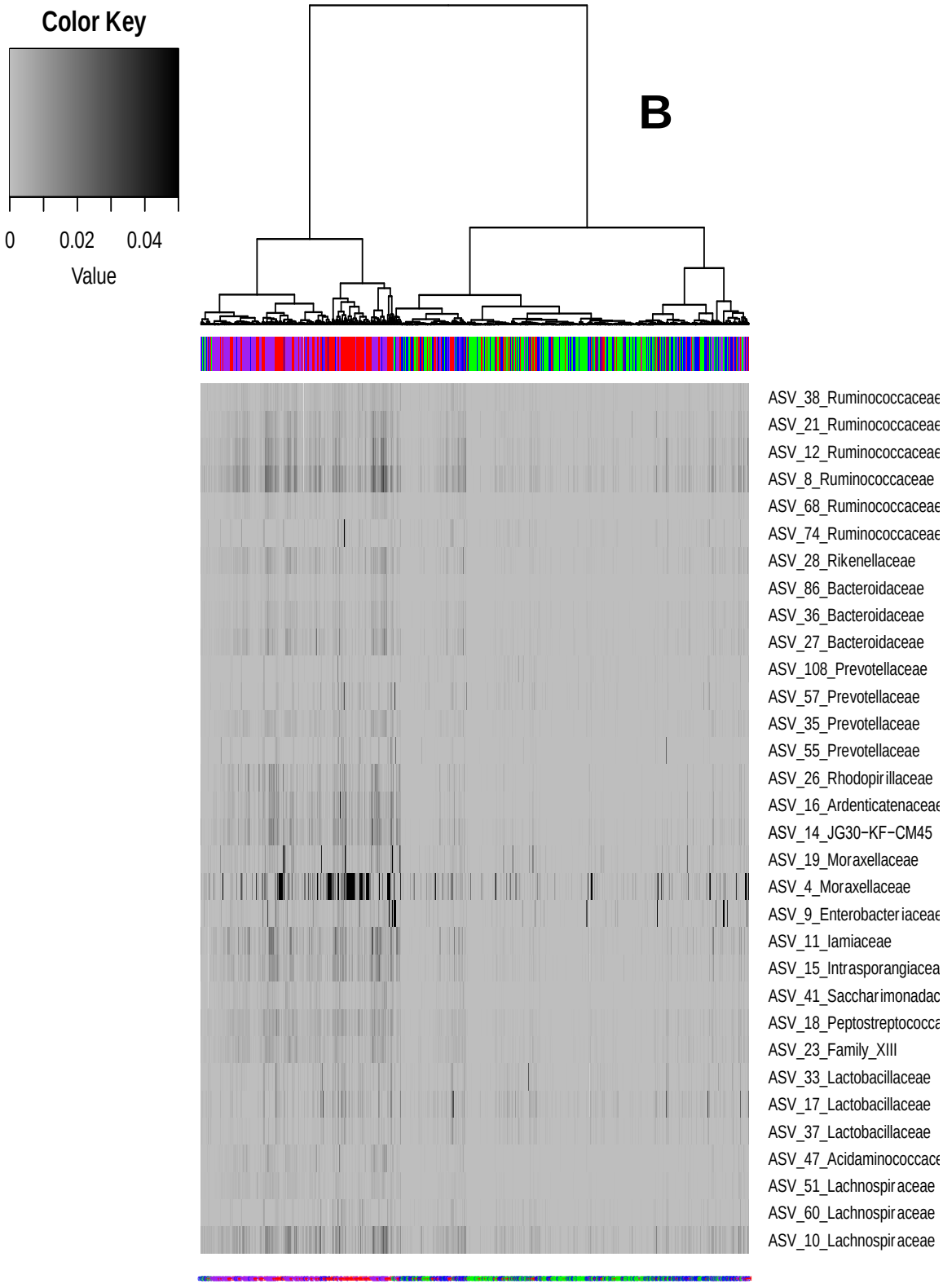

Color Key
0
0.02
0.04
Value
B
ASV_38_Ruminococcaceae
ASV_21_Ruminococcaceae
ASV_12_Ruminococcaceae
ASV_8_Ruminococcaceae
ASV_68_Ruminococcaceae
ASV_74_Ruminococcaceae
ASV_28_Rikenellaceae
ASV_86_Bacteroidaceae
ASV_36_Bacteroidaceae
ASV_27_Bacteroidaceae
ASV_108_Prevotellaceae
ASV_57_Prevotellaceae
ASV_35_Prevotellaceae
ASV_55_Prevotellaceae
ASV_26_Rhodopirillaceae
ASV_16_Ardenticatenaceae
ASV_14_JG30−KF−CM45
ASV_19_Moraxellaceae
ASV_4_Moraxellaceae
ASV_9_Enterobacteriaceae
ASV_11_Iamiaceae
ASV_15_Intrasporangiacea
ASV_41_Saccharimonadac
ASV_18_Peptostreptococca
ASV_23_Family_XIII
ASV_33_Lactobacillaceae
ASV_17_Lactobacillaceae
ASV_37_Lactobacillaceae
ASV_47_Acidaminococcace
ASV_51_Lachnospiraceae
ASV_60_Lachnospiraceae
ASV_10_Lachnospiraceae

Figure 5. Principal Coordinate analysis (PCoA) demonstrating microbial community differences before and after ulcer formation. A significant difference in community composition was detected when samples before infection and after ulcer formation was analyzed. The PCoA was performed using a weighted UniFrac distance matrix. Color scheme; Teal –before ulcer and Red- after ulcer.

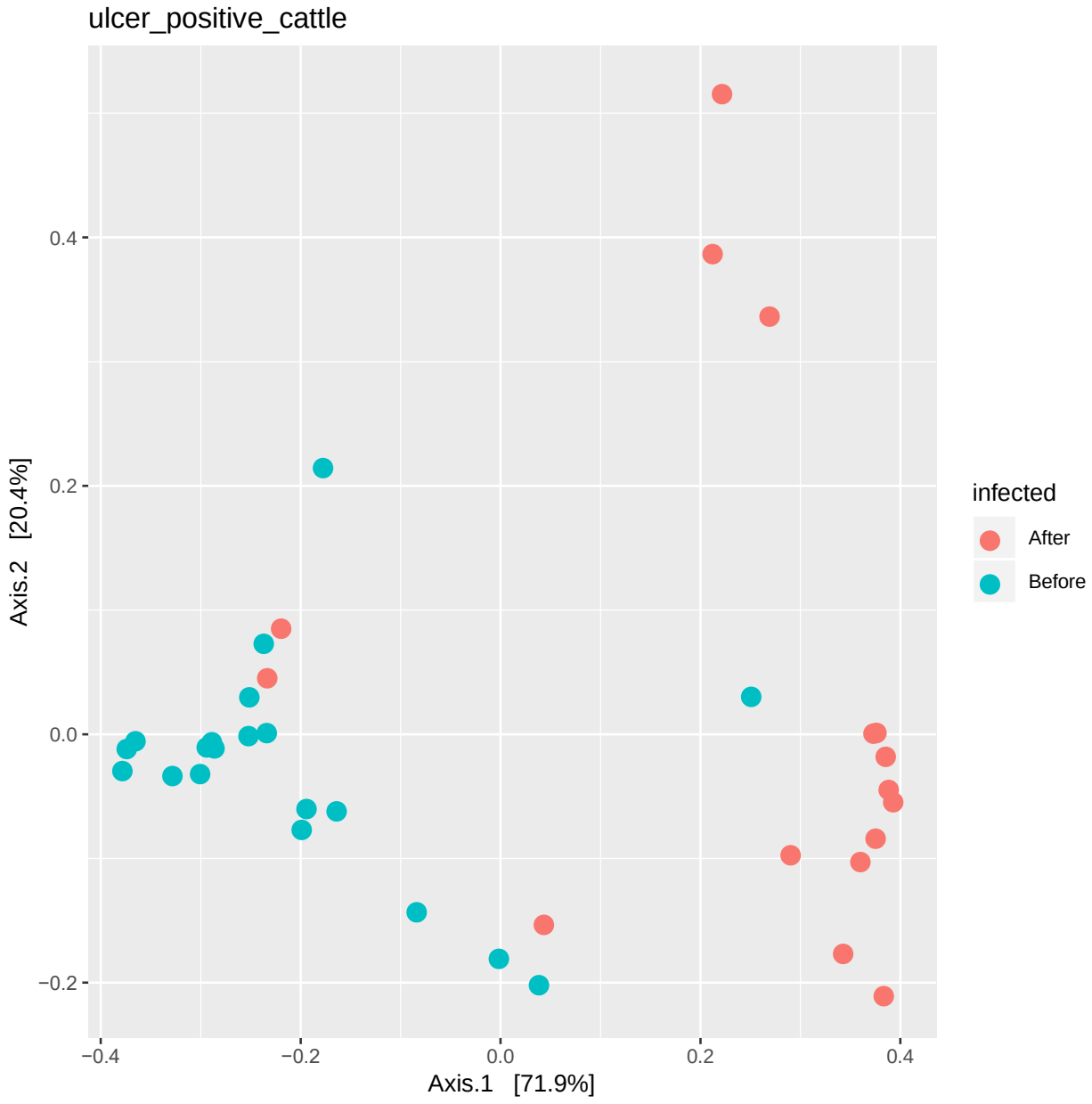


Figure 6. Heat-map showing differential ASVs identified within the bacterial community of ulcer positive animals before and after IBK infection. The major differences in community structure was driven by ASVs belonging to family *Lachnospiraceae* and *Ruminococcaceae.*

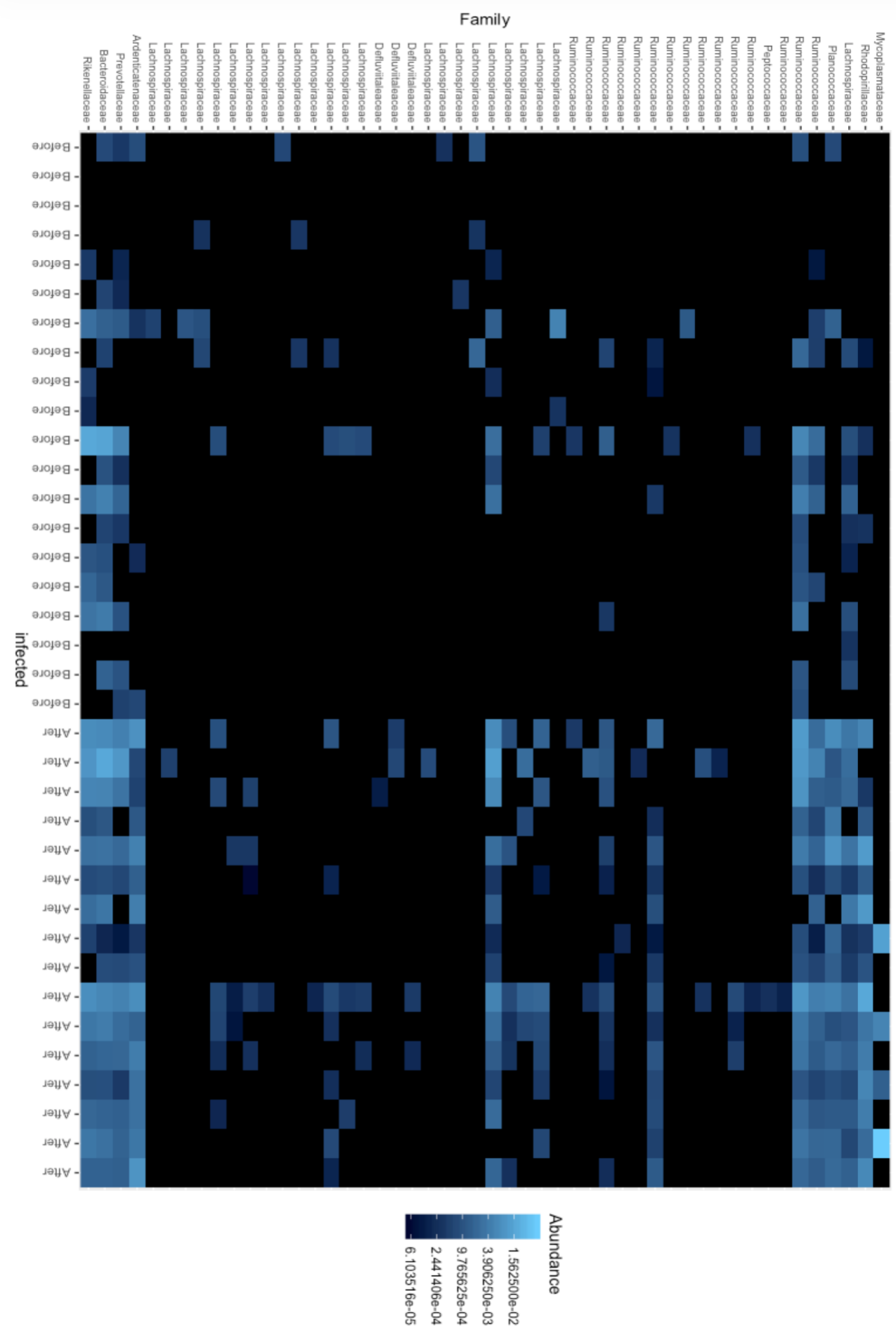
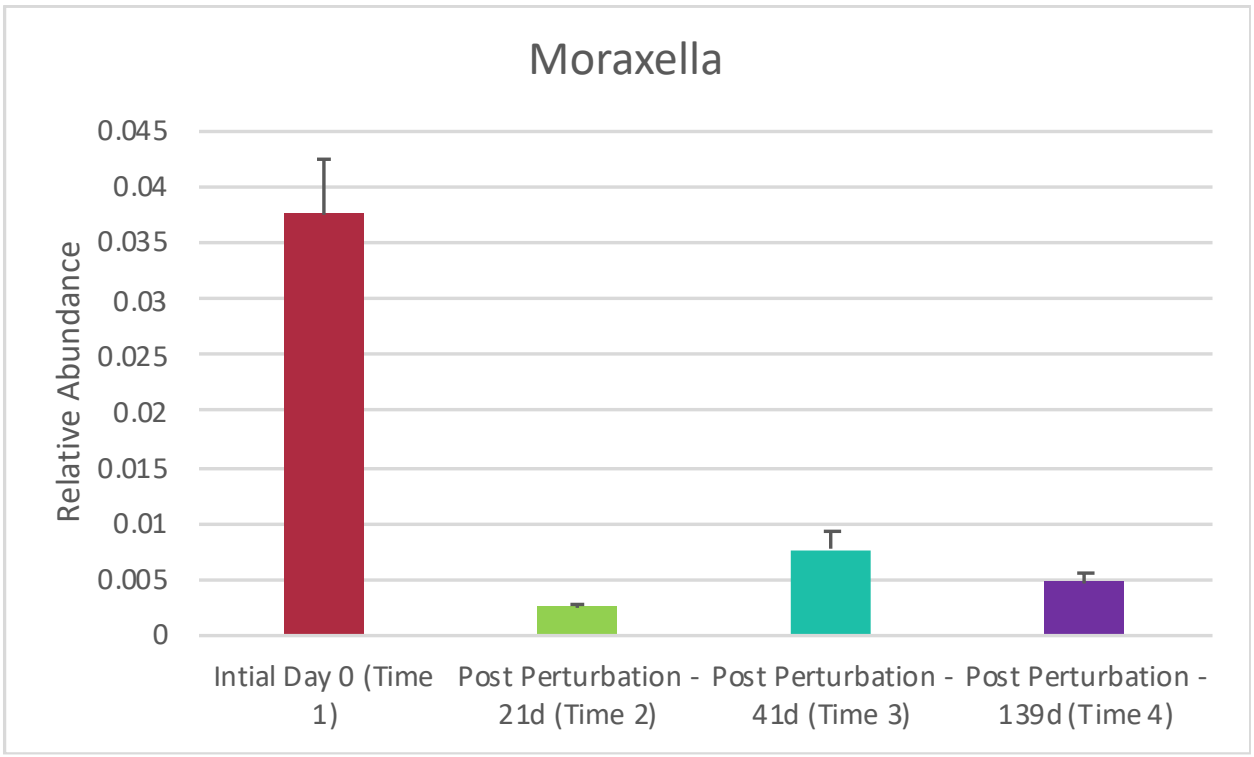

Family
Mycoplasmataceae
Rhodopirillaceae
Lachnospiraceae
Planococcaceae
Ruminococcaceae
Peptococcaceae
Defluviitaleaceae
Ardenticatenaceae
Prevotellaceae
Bacteroidaceae
Rikenellaceae
infected
Before
After
Abundance
1.562500e-02
3.906250e-03
9.765625e-04
2.441406e-04
6.103516e-05

Figure 7. Distribution of opportunistic pathogens *Moraxella spp.* and *Mycoplasma spp.* across the 4 sampling time points. *Moraxella spp.* and *Mycoplasma spp.* are considered as opportunistic pathogens that predispose animals to IBK infection.


Moraxella
Relative Abundance
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Intial Day 0 (Time 1)
Post Perturbation - 21d (Time 2)
Post Perturbation - 41d (Time 3)
Post Perturbation - 139d (Time 4)

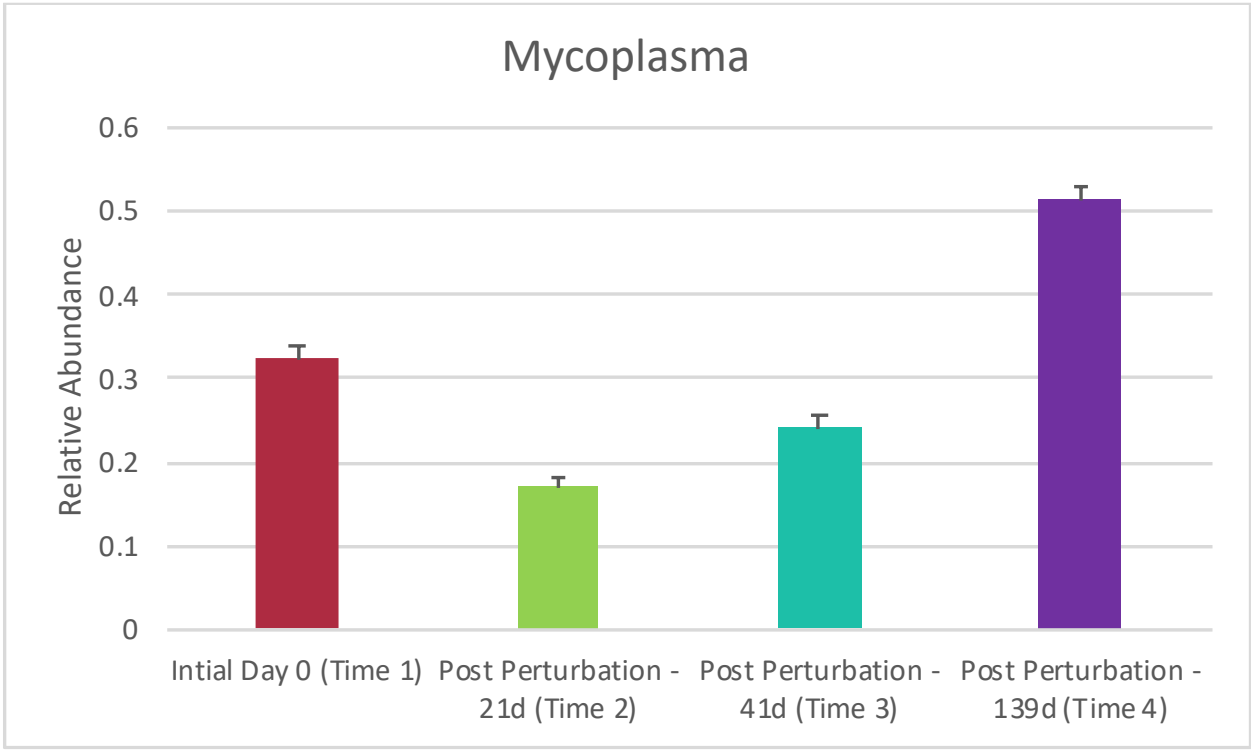
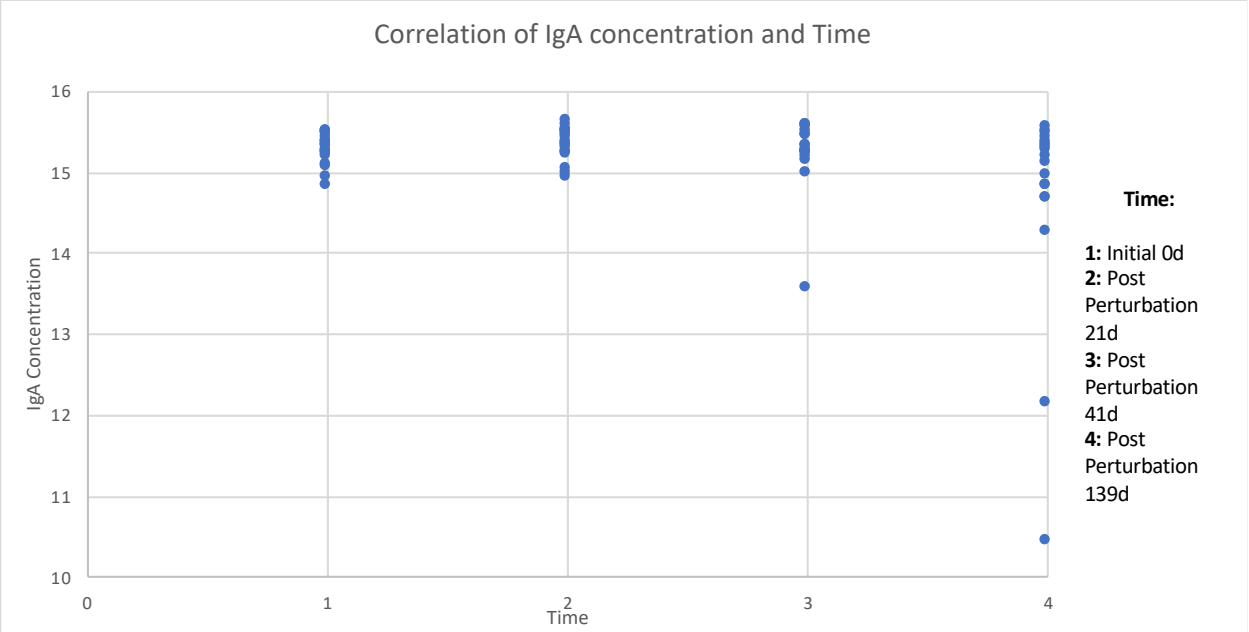

Mycoplasma
Relative Abundance
0.6
0.5
0.4
0.3
0.2
0.1
0
Intial Day 0 (Time 1)
Post Perturbation - 21d (Time 2)
Post Perturbation - 41d (Time 3)
Post Perturbation - 139d (Time 4)

Figure 8. Association between IgA concentration and time the ocular swab was taken. No change in IgA concentration was detected in animals overtime or age.



Supplementary Material

Supplementary S1. Distribution of the calves age throughout trial.