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Investigating the Development of Fecal Bacterial Communities in Growing Dairy Calves

Emily Fowler

INVESTIGATING THE DEVELOPMENT OF FECAL BACTERIAL COMMUNITIES

IN GROWING DAIRY CALVES

BY

EMILY FOWLER

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THESIS ACCEPTANCE PAGE

Emily Fowler

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

ANOVA   Analysis of variance

BLAST   Basic local alignment search tool

BRD   Bovine respiratory disease

CD-HIT   Cluster database at high identity with tolerance

DNA   Deoxyribonucleic acid

GIT   Gastro-intestinal tract

KEGG   Kyoto encyclopedia of genes and genomes

L/d   Liters per day

NCBI   National center for biotechnology information

NCD   Neonatal calf diarrhea

OTU   Operational taxonomic unit

PCoA   Principal coordinate analysis

PCR   Polymerase chain reaction

RDP   Ribosomal database project

SCFAs   Short chain fatty acids

TMR   Total mix ration

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ABSTRACT

INVESTIGATING THE DEVELOPMENT OF FECAL BACTERIAL COMMUNITIES

IN GROWING DAIRY CALVES

EMILY FOWLER

2021

The gut development of young calves is crucial for the producer and the calf's future. Many factors can affect the development of a young calf's gastrointestinal system, and there has been little research into the fecal microbiome development of calves from 0 to 12 weeks of age. To gain further insight into this process, development of the fecal microbiome in 12 dairy calves was investigated. Fecal bacterial composition was determined at four time points (weeks 0, 4, 8 and 12) using the 16S rRNA gene through PCR-amplification of the V1-V3 regions from fecal microbial genomic DNA, followed by Illumina MiSeq 2X300 sequencing. Four highly represented OTUs were found to have a peak of abundance at week 0 which was followed by significantly lower abundance at later time points ($P < 0.05$). Notably, OTU Bt-1063 peaked at week 0 (39.3% $\pm$ 3.6%) then declined at later time points with respective means of 2.3%, 0.1% and 0.05%. Seven other OTUs were found to peak at an intermediate time point ($P < 0.05$), including OTU Bt-1195 which was found in highest abundance at week 4 (4.5% $\pm$ 1.2%) compared to means with a range of 0.001% to 0.01% for the other time points. These results could allow for gut manipulation in the future which could improve the health and productivity of growing dairy calves.

Chapter 1 : Literature Review

1.1. HISTORY OF DAIRY PRODUCTION IN U.S.A

Raising cattle in the United States of America was started by European settlers as early as the 1600s, as a means to provide for the needs of their families. While cattle were bred and raised solely for dairy purposes by the 1800s (USDA, 2021), dairy farming was still limited to providing for the family farm, unless extra product was available to be sold locally (Douphrate et al., 2013b).

As market demand expanded from growing populations and cities, so did dairy farming. Inventions such as electricity and vacuum bucket milking (USDA, 2021) allowed for improved production and shipping of milk and milk-derived products to supply customers in larger cities. During the 20th century, milking pipelines as well as automated milking systems benefited dairy production by decreasing physical workloads (Douphrate et al., 2013b). By the 1950s, advancements in roadways and tanker trucks allowed for the industry to provide milk products throughout the country at lower costs. In the 1970s, milk packaging in plastic or paper containers further reduced costs, ultimately leading to the bulk of retail milk being sold at local stores (Shields, 2010).

Dairy farm outputs progressed in parallel with innovations in packaging, distribution, and marketing. In the last 100 years, advances in genetics, management and automation systems have allowed for greater herd sizes while requiring less labor, allowing to meet the growing demand for dairy products (Douphrate et al., 2013a). In recent years, these advancements have further allowed to increase milk production while reducing the number of farms and cattle populations across the country. For example, the

number of dairy farms fell by 88 % between 1970 and 2006, with a reduction in the

number of dairy cows from 12 million to 9.1 million over the same time period

(MacDonald et al., 2006). Despite this considerable reduction in farms and cows,

production had increased during the course of these 36 years from 116,916 million

pounds in 1970 (SRS, 1971) to 181,839 million pounds in 2006 (NASS, 2007).

Productivity further improved to 192,726 million pounds between 2006 and 2010, while

the number of cows remained stable at 9.1 million (NASS, 2011). In the recent decade,

milk outputs have reached 223,055 million pounds, while the dairy cow population has

only slightly increased to 9.3 million (NASS, 2021).

1.2. MANAGEMENT OF DAIRY CALVES

As farm production grew, improvements in management became essential. One of

the practice structures that has changed over the last 100 years has been the on-farm

management of dairy calves. Surplus calves, which tend to be predominantly males

(95%) but also include females that are not needed as replacements, are sold as soon as

possible, usually for the veal industry (Bolton and von Keyserlingk, 2021). Due to

concerns about animal welfare, handling of surplus dairy calves remains a controversial

topic, as surplus dairy calves have higher rates of mortality and morbidity compared to

heifer calves raised for milk production (Creutzinger et al., 2021). Some of the animal

welfare issues result from the production system, as it requires that surplus calves be

transported to other locations, typically over long distances, thereby increasing their level

of stress and risk of infections (Taylor et al., 2010; González et al., 2012; Creutzinger et

al., 2021).

While dairy heifer calf management practices tend to differ throughout the United States, they typically vary according to size and location of the farms. According to a survey of 113 dairies located in the North Central and Northeastern United States, 50.4% of producers raise their own heifer calves from birth to herd entry, while approximately 30% of producers send their pre-weaning calves to be custom-raised into heifers (Fulwider et al., 2008). A calf is typically separated from its dam within hours after birth. Early separation helps to sever the connection between dam and calf, which reduces their distress responses (Stěhulová et al., 2008; Daros et al., 2014) compared to when they are separated after weaning, which results in higher distress (Loberg et al., 2008).

While this management practice has been widely criticized, with critics pointing out the distress inflicted to dam and calf from their separation (Busch et al., 2017), it provides important benefits. For instance, it facilitates monitoring of calves to ensure that they consume the necessary amount of colostrum and milk for their early development, it decreases the risk of disease transmission, and it allows to maximize milk harvesting from the dam (USDA, 2008).

1.3. CHALLENGES TO RAISING AND DEVELOPING DAIRY CALVES FOR MILK PRODUCTION

In intensive production systems, development of heifer calves starts soon after birth so that they can be bred 13-15 months later to produce their first calf by the time they reach 22-24 months of age, which is when milk production will begin (Hopkins and Whitlow, 1993). While management practices have greatly improved over the years, a number of challenges to developing dairy heifer calves still remain. One of the main difficulties is mitigating the level of stress to which heifer calves are subjected to during

their development. Common stressors include transportation, disbudding, and co-mingling (Hulbert and Moisá, 2016). The response of heifer calves to stressors also varies depending on their age or stage of development, with pre-weaning and weaning representing particularly sensitive periods.

While it can vary depending on management strategies, weaning typically occurs between weeks six and eight in intensive dairy farms. Pre-weaned calves tend to show the highest mortality rate. A survey conducted in 2006, for instance, indicated that the mortality rate for pre-weaned calves was approximately 15% (USDA, 2007), with half of deaths caused by stillbirths. (USDA, 2007). According to that survey, the main causes of death in born-alive calves were digestive problems (56.5%) and respiratory issues (22.5%) (USDA, 2007), which were likely the result of neonatal calf diarrhea (NCD) and bovine respiratory disease (BRD), respectively (Windeyer et al., 2014). While they appear to be independent diseases, NCD-infected calves tend to be at a higher risk of contracting BRD in their first two weeks of life (Windeyer et al., 2014). In contrast, respiratory issues were found to be the main cause of death in weaned calves (46.5%) from the total 1.8% death rate (USDA, 2007).

Mortality in pre-weaned heifer calves is then a concern for the dairy industry. The first step to reducing the risk of pre-weaned calf mortality is to provide high quality colostrum, since calves not provided with colostrum have been reported to be 74X more likely to die within three weeks after birth (Wells et al., 1996). Colostrum is a critical source of maternal antibodies that can be effectively absorbed if provided within 24 hours after birth; thus, timely administration of colostrum decreases the risk of passive immunity failure after birth. While calves produce their own immune cells, they do not

yet express immunoglobulins, and thus lack the ability to effectively fend off pathogens on their own (Kampen et al., 2004) (Hulbert and Moisá, 2016) (USDA, 2008). Maternal antibodies provided by colostrum can protect a calf for up to three weeks; while maternal immunoglobulin levels decrease steadily during this period, calves start to produce their own antibodies during this period as a result of their exposure to pathogens in their environment (Hulbert and Moisá, 2016). Because antibodies cannot be transferred through the placenta from the dam to the calf, the calf must rely on passive immunity, or the immunity received from the colostrum, until its own immune system, referred to as active immunity, is established (Jones and Heinrichs, 2017). The gap between passive and active immunity is when a calf is most vulnerable to infection (Jones and Heinrichs, 2017).

1.4. NUTRITION AND DEVELOPMENT OF THE GASTROINTESTINAL TRACT IN DAIRY CALVES

The gastro-intestinal tract (GIT) of calves undergoes a number of major anatomical and physiological changes as a result of successive transitions in the sources of nutrients that are used, starting from umbilical delivery to gastric and intestinal digestion of colostrum, milk or milk replacer, then to microbial digestion of solid feed (Osorio, 2020). At birth, the gut of young ruminants is underdeveloped, and is functionally similar to the gut of a monogastric animal. Before calves can efficiently digest plant biomass, the rumen compartment of their stomach must be further developed, which involves host tissues such as the ruminal epithelium as well as rumen microbial communities. (Diao et al., 2019).

Colostrum is the first dietary source of nutrients that is offered to calves. It has a very high nutrient content, providing essential fatty acids and amino acids as well as vitamins and minerals for the development of the young animals (Blum, 2006). Compared to whole milk, colostrum has twice the amount of dry matter and minerals, five times the amount of protein, as well as a higher fat content. While high in energy content, colostrum contains less lactose than milk, which has been correlated with a lower risk of diarrhea (Jones and Heinrichs, 2017). These characteristics illustrate how crucial it is for young animals to consume as much colostrum as possible. According to the USDA, they recommend the amount of colostrum fed to a calf should be 10% of its body weight (USDA, 2016).

The first prolonged dietary regimen for dairy calves consists of milk replacer, whole milk and/or waste milk. Since all milk options are considered adequate, selection is usually dependent on farm location, herd size, and cost to each operation. Milk replacer consists of a concentrated powdered formula that is reconstituted in water. Because of its convenience, consistent nutrient content, and favorable biosecurity attributes, milk replacer has become more popular, and is consequently utilized in almost half of the dairies in the USA (Jones and Heinrichs, 2017). However, the main disadvantages of milk replacements are the risks of poor preparations or storage in suboptimal conditions that can result in calf mortality from complications such as Acute Bloat Syndrome (Wellert and Hartschuh, 2020). As protein sources are usually the most expensive ingredient in milk replacer, care must be taken when selecting a particular product to avoid milk replacers that may include inferior quality ingredients (Jones and Heinrichs, 2017; Wellert and Hartschuh, 2020).

Whole milk is obtained from the dam, and it has a higher energy content compared to most milk replacers because it contains a greater amount of fat (Wellert and Hartschuh, 2020). Before the 1950s, i.e. before milk replacer became a more affordable substitute, whole milk was the most widely used option to feed dairy calves. More recently, increases in whey protein prices in the mid-2000s have allowed whole milk to become a more competitive option (Jones and Heinrichs, 2017). Because it is a favorable environment for bacterial growth if it is not stored properly, whole milk poses a higher risk for disease transmission compared to milk replacer (Wellert and Hartschuh, 2020).

Another option to feed pre-weaned calves is waste milk, i.e. milk that is not safe for human consumption because of contamination from antibiotic residues or because it was produced by cows with mastitis or other illnesses (Looper et al., 2001). One of the main advantages of using waste milk is the utilization of a product that would otherwise be discarded. As long as proper guidelines are followed for storage and feeding, there has been no indication that waste milk negatively affects calf growth or increases the incidence of disease (Looper et al., 2001; Jones and Heinrichs, 2017).

Because of its simple nutrient composition, milk is channeled directly to the abomasum for digestion, bypassing the reticulum, rumen and omasum compartments through the rumoreticuler / esophageal groove. The formation of the groove is mainly activated by a reflex response to suckling, and it consists of muscle folds that join together from the reticulorumen (Jones and Heinrichs, 2017). Cattle have evolved this mechanism to delay the development of the rumen in nursing calves until they start to consume solid feed. Accordingly, the reticulorumen, omasum, and abomasum represent 38%, 13%, and 49% of the total stomach weight in newborn calves (Davis and Drackley,

1998). However, since this adaptation is not optimal for intense production in dairy operations, solid feed is made available much earlier to dairy calves as a supplement to milk, in order to accelerate development of the rumen and maximize the efficiency of heifer development. Solid feed provided to dairy calves is commonly in the form of products designated as 'calf starter', which are formulated to be highly palatable and digestible (Klein et al., 1987). The rumen can begin to develop once calf starter is being consumed, which is typically as early as three days after calving in intensive dairy operations (Jones and Heinrichs, 2017). By the second week, the amount of calf starter consumed by calves will typically have greatly increased (Jones and Heinrichs, 2017). As a result of the fermentation of feed that takes place in the developing rumen, the concentrations of short chain fatty acids (SCFAs) increases. SCFAs are microbial end-products that are produced by symbiotic ruminal microorganisms. Of these, butyrate, and to a lesser extent propionate, stimulate the formation of ruminal papillae (Tamate et al., 1962; Jones and Heinrichs, 2017; Bedford and Gong, 2018a). Papillae are finger-like extensions of the rumen tissue that increase the surface area of the rumen, thus permitting better absorption (Jones and Heinrichs, 2017). Rumen epithelial cells, as well as colonocytes, are crucial for the absorption of nutrients, and both cell types use butyrate as their main energy source (Bedford and Gong, 2018b). When comparing calves that were fed only milk to calves that were fed both calf starter and milk, the proportions of the stomach compartments were noticeably different, with the former having smaller rumens and limited papillae development (Jones and Heinrichs, 2017) (Anderson et al., 1987). While solid feed in the form of calf starter pellets is beneficial for rumen development in calves, access to forage should be limited. Indeed, the limited capacity of the rumen at

this stage of development and the higher production of acetate from forage fermentation would not be beneficial for the development of the rumen (Jones and Heinrichs, 2017).

By three weeks of age, sugar digestion has significantly improved, and calves are able to digest higher quantities of carbohydrates, but starch digestion may vary depending on its source and processing methods (Jones and Heinrichs, 2017). To optimize performance, calves need to be eating sufficient amounts of calf starter, which should be occurring by four weeks of age or at least before they are weaned. Depending on management practices, weaning can be implemented at six weeks (early weaning) or at eight to nine weeks of age (later weaning). In 2014, the average weaning age of dairy heifers was nine weeks (USDA, 2016); under this strategy, the reticulorumen, omasum and abomasum were found to represent 61.23%, 13.40% and 25.37% of the total stomach weight, respectively (Diao et al., 2017). As a result of the increased growth and development of the reticulorumen and omasum by this point, the proportion of the abomasum to the total stomach is approximately half of its original value.

After weaning, calves can continue to consume calf starter for an additional two to three weeks before being transitioned to a grower diet (Phillips et al., 2006) (Fischer et al., 2019), which can consist of a total mixed ration (TMR) that contains high quality forages, a balance of grains and protein sources, as well as vitamins and minerals (Linn, 2021). By 12-16 weeks of age, the reticulorumen, omasum and abomasum compartments typically represent 67%, 18% and 15% of the total stomach weight, respectively (Davis and Drackley, 1998; Diao et al., 2017). The rumen should have reached maturity by then, and heifers should be able to ingest and digest dry food like an adult animal (Teagasc, 2017). Heifers are considered to have reached the 'ruminant stage' when the rumen

makes up approximately 70% of the total stomach weight which is usually obtained by 12 weeks of age (Teagasc, 2017).

1.5. GUT MICROBIOLOGY DEVELOPMENT IN DAIRY CALVES

In mammals, the gut microbiota plays critical roles for their hosts by contributing to a number of functions, including nutrition, physiology, and immunity (Gomez et al., 2017). Many factors influence the development of this complex microbial environment, including diet, host genetics and age, as well as management practices (Khan et al., 2016). In ruminants, gut microbial communities are particularly critical, since they are responsible for the digestion of plant biomass by mature animals. Thus, a gut microbiota must form in growing calves concomitantly with the host's digestive system (Jami et al., 2013).

1.6. COLONIZATION OF THE RUMINANT GASTRO-INTESTINAL TRACT AND DEVELOPMENT OF THE HINDGUT

As a result of the birthing process, a newborn calf is exposed to microorganisms from a number of different sources, including vaginal mucus and feces from the dam, as well as colostrum and the surrounding environment (Meale et al., 2017; Diao et al., 2019). Of the microorganisms that a newborn is exposed to, the species that are best suited for growth in the newborn gut will colonize this environment. In suckling lambs sampled three days after birth, gut microorganisms were for instance found to have originated primarily from the dam's teats and ambient air, whereas gut microorganisms from bottle-fed littermates were derived from the vaginal mucus of the dam, ambient air, and the pen floor (Bi et al., 2019). As the gut environment progressively changes as a result of the metabolic activities (end-products) of resident symbionts and the availability

of substrates provided by the diet of the young animal, the composition and complexity of microbial communities also fluctuate. While the exact timing of this process and the mechanisms involved remain to be fully elucidating, research performed to date has revealed some important insights.

Since they were detected in meconium, as well as in the fecal microbiome of 6- and 12-hour old calves, species affiliated to the genus *Citrobacter* as well as lactic acid producing bacteria such as *Lactococcus*, *Leuconostoc*, and *Lactobacillus* have been reported as first or early gut colonizers in dairy calves (Mayer et al., 2012). Other early colonizers include members of the genera *Bifidobacterium*, *Faecalibacterium*, and *Enterococcus* (Uyeno et al., 2010; Oikonomou et al., 2013; Klein-Jöbstl et al., 2014). *Bifidobacterium* species were notably found to be well represented in intestinal samples of newborn calves within 30 minutes after birth (Malmuthuge et al., 2015a).

Feeding colostrum was shown to be important to maintain bacterial populations for at least 12 hours after birth (Malmuthuge et al., 2015a). Indeed, calves receiving colostrum 12 hours after birth were reported to have a lower intestinal abundance of *Bifidobacterium* and *Lactobacillus* compared to calves that were given colostrum at birth (Fischer et al., 2018). A complex hindgut microbiome continues to develop as microbial diversity was reported to increase after 24 hours (Mayer et al., 2012). Overall, the abundance of early colonizers tends to decline with age. It was reported for instance that the abundance of *Bifidobacterium* decreases as a calf grows, and that it is no longer detectable by 9 weeks (Uyeno et al., 2010). On the other hand, the abundance of Bacteriodetes-affiliated bacterial species has been shown to increase (Uyeno et al., 2010; Oikonomou et al., 2013; Klein-Jöbstl et al., 2014). For instance, by day 8 to day 14,

Bacteriodetes were found to be the most abundant phylum (Klein-Jöbstl et al., 2014) (Malmuthuge et al., 2015a). Microbial composition continues to fluctuate during later stages. Indeed, dramatic changes in the most abundant genera were reported in samples from the colon of calves between two weeks and four-weeks of age; an increase in *Lactobacillus* abundance from 34% to 69% and a decrease in *Streptococcus* from 12% to 4% were for example found between the two time points (Castro et al., 2016).

Development of the gut microbiome in dairy calves is also affected by weaning strategy. In a study investigating the effect of early weaning on microbial composition changes, beta diversity analyses showed that fecal bacterial composition from early-weaned calves experienced greater shifts in composition compared to late-weaned calves, which was attributed to differences in rumen development between the two treatment groups (Meale et al., 2017). The steady change in beta diversity observed in the late weaning group was associated with a gradual increase in starter consumption that resulted in a gradual development of the rumen (Meale et al., 2017). Development of a gut microbiome resembling that of an adult cow has been observed to develop between weaning and one year of age across varying diets (Dill-McFarland et al., 2019).

As calves develop, there is a transition from utilizing simple sugars from milk as main energy substrates to metabolizing polysaccharides into short chain fatty acids, the end products of ruminal fermentation (Quigley et al., 1991). As dairy calves grow, rumen concentrations of SCFAs rise in tandem with solid feed intake. SCFAs can modulate the development of the host digestive tract as well as the composition of its microbiota (Beharka et al., 1998; Govil et al., 2017). While butyrate supplementation benefits the maturation of rumen papillae (Tamate et al., 1962; Govil et al., 2017; Bedford and Gong,

2018a), it does not affect bacterial communities of the rumen (O'Hara et al., 2018).

Intriguingly, however, hindgut bacterial communities were found to respond to butyrate

supplementation (O'Hara et al., 2018).

1.7. RUMINANT HINDGUT MICROBIOTA AND ITS IMPACT ON GUT HEALTH

Dysbiosis, i.e. a state of disruption in the composition of a microbial community,

may be a common step leading to diarrhea or other digestive problems in calves (Gomez

et al., 2017). Consequently, efforts have been devoted to identifying microbial species or

groups that may promote gut health by mitigating the incidence of dysbiosis. Members of

the genus *Faecalibacterium* have been reported as good candidates for this role. They

have, for instance, been associated with lower incidence of diarrhea in calves, as well as

with higher growth performance (Oikonomou et al., 2013). However, one report did

observe an overall lower abundance of *Faecalibacterium* in healthy calves compared to

diarrhetic calves (Gomez et al., 2017). These discrepancies may possibly be the result of

differences in management practices between farms or indicate that other factors are

involved (Barden et al., 2020). Lactic acid producers affiliated to *Bifidobacterium*, and

Lactobacilli are also recognized as beneficial groups of bacteria. It has been proposed

that *Bifidobacterium* can suppress gastro-intestinal illnesses in humans by competing

with pathogenic bacteria (Veiga et al., 2014). In a study by Abe et al. (1995), calves

supplemented with *Bifidobacterium pseudolongum* or with *Lactobacillus acidophilus*

were found to gain more weight compared to the control group.

Since a state of dysbiosis represents an opportunity for pathogens or commensals

to proliferate, an aligned strategy would be to reduce the prominence of microbial groups

with these properties. The phylum Proteobacteria includes a number of groups or species that are pathogenic or suspected of being pathogens (Shin et al., 2015). However, not all uncharacterized species affiliated to Proteobacteria are necessarily a sign of dysbiosis; indeed, sequences affiliated to Enterobacteriaceae were found to be in elevated abundance in healthy calves (Gomez et al., 2017).

1.8. METHODS TO INVESTIGATE MICROBIAL COMMUNITIES

Microbiomes are microbial ecosystems that are found in a wide variety of abiotic environments, such as hot springs and acid waters, as well as biotic habitats, such as the gut of animals where they play essential roles that benefit the physiology and nutrition of their host (Brock and Freeze, 1969; Baker and Banfield, 2003). The most prevalent and diverse organisms in microbiomes are typically prokaryotes, of which bacteria tend to be the most abundant (Whitman et al., 1998; Bilen et al., 2018). Since they consist of microscopic organisms and tend to be very diverse, microbiomes are very challenging environments to study. To this end, two main strategies have been developed: culture-dependent and culture-independent approaches.

1.9. CULTURE-DEPENDENT METHODS

Until the development of DNA sequencing and recombinant DNA techniques, culture-dependent methods were the main tools available for studying microbiomes. This approach consists of culturing or growing microorganisms of interest as a means of determining their nature and abundance in an environment of interest. Since culturing takes place under laboratory conditions, it is critical to know the optimal physical and chemical conditions for growth of the target organisms. Typical ingredients for culture

media would consist of water, a carbon source, a nitrogen source as well as mineral salts (Bonnet et al., 2020). One of the main advantages of culture-based methods over culture-independent approaches is that they can be used to isolate uncharacterized species from microbiomes of interest based on metabolic activities of interest, as well as gain further insights on the biology of microbial isolates of interest.

The main disadvantage of culture-dependent methods is that microbial species can only be effectively cultured if the conditions required for their growth can be mimicked in the laboratory. It has been estimated that up to 99% of microorganisms may be resistant to being cultured under laboratory conditions (Kaeberlein et al., 2002). This assessment does not explicitly imply that "unculturable" microorganisms may never be grown as isolated strains independent of their natural habitat, but acknowledges that our current understanding of their biology is limited at this time (Stewart, 2012b). While simulated environments using bioreactors can be an acceptable compromise to grow microorganisms in their natural environment by culturing a sampled ecosystem in the laboratory, they lack the level of resolution that can be achieved from studying isolates (Stewart, 2012a).

1.10. CULTURE-INDEPENDENT METHODS

Because of the challenges presented by cultivating all bacterial species, technologies such as DNA sequencing and recombinant DNA techniques were adapted for the investigation of microbiomes. Since they are based on analysis of DNA rather than growth, culture-independent methods can be used to identify both characterized ('known') and uncultured ('unknown') microbial species from a given environment. This approach has greatly benefited from advances in DNA sequencing technology in the past

decade to become the most popular method for investigating microbiomes. Great advancements in various aspects of microbiomes were made possible due to these technologies.

All DNA-based culture independent approaches require the effective extraction and purification of genomic DNA from microbial cells. DNA-based culture-independent methods can be divided into two main approaches: use of a genetic/molecular marker or shotgun metagenomics. A typical molecular marker approach consists of using PCR to generate amplicons from a specific chromosome region that is shared amongst all microbial species of the target group of interest. The pool of amplicons that is generated from a sample can then be sequenced, producing a dataset that consists of the various homologs for the amplified genomic region from the microbial species that are present in the environment of interest. The species composition for the microbial group of interest can then be determined from this sequence dataset, providing a microbial census for the sampled environment investigated. In contrast, shotgun metagenomics involves the random sequencing of genomic fragments isolated from the environment of interest, and it does not involve PCR amplification of a molecular marker.

1.10.1. The 16S rRNA gene, a marker for prokaryotes

Since prokaryotes are typically the most abundant and diverse microorganisms in a given environment, most efforts have been dedicated to developing molecular markers for this microbial group. The 16S rRNA gene was one of the first markers to be developed for prokaryotes, and it remains the most widely used because of its characteristics. First, since it is essential as a component of ribosomes, and consequently

for cell survival, it is present in all prokaryotic cells. Secondly, as a structural RNA, its nucleotide sequence is highly conserved and less subject to changes because of the high selective pressure to maintain its structure. In addition, its architecture consisting of alternating conserved and variable regions is well suited for a molecular marker, as the former can be used as targets for PCR amplification (Woese, 1987), while the later can be used to distinguish between different species or subgroups (Sune et al., 2020). Finally, an important benefit of the common use of the 16S rRNA gene as a marker is the large accumulated curated sequence data that is publicly available to researchers in the field.

Advancements in high throughput sequencing technology has greatly benefited culture-independent approaches, because of the greater depth in sequencing data that it provides, permitting more comprehensive surveys of diverse and complex microbial environments such as the mammalian gut. However, one of the main limitations of high throughput sequencing platforms for use of the 16S rRNA gene as a marker is the relatively short length of the nucleotide sequences that they can generate compared to the length of the 16S rRNA gene. Thus, the compromise for use of high throughput sequencing with 16S rRNA is to target a sub-region of the gene. Suitable sub-regions need to have highly conserved nucleotide sequences at both ends so they can be targeted by universal primers for PCR, they need to include one or more hypervariable regions, and the length of the amplicons that are generated need to be within the limits of available platforms so they can be sequenced in their entirety. A number of different sub-regions are available for use as markers, of which V1-V3, V3-V4, V4 or V4-V5 are the most commonly used. However, not all sub-regions exhibit the same level of variability, which affects their resolution when determining taxonomic profiles (Kim et al., 2011).

For instance, the V1-V3 sub-region has been found overall to most closely represent the variability of the full-length 16S rRNA gene compared to other commonly used sub-regions (Kim et al., 2011; Johnson et al., 2019).

1.10.2. Bioinformatics analysis of the 16S rRNA gene

Bioinformatics analysis of sequence data generated from the 16S rRNA gene can be performed using two distinct strategies: taxonomy dependent vs taxonomy independent approaches. The taxonomy dependent strategy relies on available sequence data to assign experimental sequences to taxonomic groups. Taxonomy assignment tools can use a distance-based algorithm to assign 16S rRNA sequences to their corresponding taxonomic groups, such as Ribosomal Database Project (RDP), or they can use an alignment-based strategy, such as Basic Local Alignment Search Tool (BLAST) and Usearch. The taxonomy independent strategy defines groups of sequences based on their level of sequence identity to each other, and thus does not require a reference dataset. Each group is typically referred to as an Operational Taxonomic Unit (OTU), with all sequences within the group sharing a level of sequence identity that is equal or greater to a user-set threshold (Morgan and Huttenhower, 2012) .

1.10.3. Limitations of the 16S rRNA gene

Use of the 16S rRNA gene as a marker for microbial composition has limitations that need to be discussed. One of the more impactful is the variability in the number of copies of the gene across bacterial species, which introduces an important bias in representation or count data when determining the microbial composition of a sample (Chen et al., 2015). 16S rRNA gene sequence data do not by themselves directly provide

information on the function of corresponding microbial species (Mignard and Flandrois, 2006). Since primers targeting 16S rRNA gene sequences are designed to amplify all bacterial DNA, there is a risk of generating false positives due to sample contamination (Sune et al., 2020). Since chloroplast and mitochondrial 16S rRNA genes can also be amplified by this method, they may also be a source of false positives (Hanshew et al., 2013; Sune et al., 2020). Artefacts from PCR and sequencing are also a common source of false positives with this approach. However, it is important to note that a number of tools have been developed to identify the false positives so they can be excluded from further analyses.

1.10.4. 'Shotgun' Metagenomics

In contrast to the use of a marker gene where sequencing data is generated from the same genomic region in all organisms in a sample, metagenomics uses sequence data that is generated at random from DNA extracted from a sample of interest, a strategy commonly referred to as 'shotgun sequencing'. As it is not restricted to a specific genomic region, metagenomics can reveal information on any possible region of a chromosome, including genes encoding protein sequences, from which insights on metabolic functions can be inferred.

1.10.5. Bioinformatics analysis for 'Shotgun' Metagenomics

A number of different strategies and tools are available for analysis of shotgun sequence data. A simple and strait forward approach is to align nucleotide sequence data to reference genomes (Lee and Behr, 2016), as this method does not require assembly. It is typically very effective for screening for the presence of host sequences in

experimental datasets. Its main disadvantage for analysis of microbial environment is that its effectiveness is dependent on the availability of genome sequences for the microbial species present in the environment of interest.

A more common strategy is to predict coding sequences from metagenomics datasets, and find their closest match to known or candidate proteins in available databases. A number of software programs such as Blast (Altschul et al., 1997), Usearch (Edgar, 2010) or CD-HIT (Fu et al., 2012), as well as online tools such as MG-RAST (Meyer et al., 2008) / RAST (Aziz et al., 2008) can be used to assign sequence reads from experimental datasets to particular enzymes or protein families. This information can then be used for determining taxonomic affiliation and / or elucidating potential metabolic functions with the help of other tools such as KEGG (Kanehisa and Goto, 2000).

1.10.6. Limitations of 'Shotgun' Metagenomics

Metagenomics is a very effective approach to uncover the metabolic potential of the various species in a microbial community. However, it is not always well suited to provide an accurate estimate of microbial community composition, particularly for microbial environments that consist of uncharacterized or unknown species. False negatives and false positives may be more prevalent compared to the use of 16S rRNA. The former are more likely because many sequence reads cannot be reliability assigned to a match since they correspond to unknown species. For the latter, homologs from different species may have a high level of sequence homology at the amino acid level, while the DNA sequences of the coding regions do not show a significant match; assigning using highly conserved proteins to available database entries may be the closest match, but not the real match (Lind and Pollard, 2021). If the goal of a study is to find

"rare" or less prevalent bacterial species, this strategy may not be optimal unless sequencing depth is greatly increased.

1.11. RATIONALE FOR THESIS RESEARCH

Dairy calves can be subjected to a variety of stressors during their development, including separation from their dam at birth, transportation, as well as acclimation to new surroundings and co-mingling. Since development has not yet been completed in these young animals, certain systems such as immunity and the gastro-intestinal tract may be greatly affected by stress response, resulting in higher susceptibility to disease. Hence, morbidity and mortality rates in dairy calves tend to be high and remain a cause of concern in the dairy industry.

A great deal of effort has been dedicated to determining how nutrition can alleviate mortality and morbidity in dairy calves. This includes, for instance, investigating the timing of colostrum intake or determining the optimal plane of nutrition and timing when transitioning between diets. Other aspects, such as development of the symbiotic microbial communities of the gut, remain to be further investigated.

Due to the importance of rumen development for ruminant production, elucidating the development of ruminal microbial communities has been a major research focus (Li et al., 2012; Jami et al., 2013; Dias et al., 2017; Malmuthuge et al., 2019; Furman et al., 2020). However, in comparison to the rumen, development of the hindgut microbiota in young ruminants remains mostly uncharacterized.

While they are not as involved in the digestion of feed as their ruminal counterparts, microbial communities of the hindgut are critical for maintaining gut health. As dairy calves are very likely to experience fluctuations in gut microbial composition because of their rapidly developing physiology, varying intake of milk and solid feed, as well as exposure to stress (Uyeno et al., 2010), they may consequently be at a high risk for dysbiosis. Dysbiosis is a state of imbalance in the microbiome that can provide favorable conditions for the proliferation of opportunistic pathogens if it persists. These can result in enteric infections that can jeopardize the life of the young ruminant and / or impact performance later during the productive stage of their life.

1.12. HYPOTHESIS

We hypothesized that the hindgut bacterial communities of neonatal calves undergo a series of compositional changes starting from after birth until weaning

1.13. RESEARCH OBJECTIVES

The objectives of the research presented in this thesis were:

1) Determine the composition of fecal bacterial communities from dairy calves at four monthly time points, starting from soon after birth until after weaning

2) Identify fecal bacterial groups and / or species that fluctuate in abundance across time points.

Chapter 2 : Investigating the Development of the Fecal Microbiome of Dairy Calves

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2.1. ABSTRACT

Development of the gut microbiome in young animals is critical for maximizing productivity in adults through beneficial functional contributions of symbiotic microbial communities to the health and nutrition of their host. To gain further insight into this process, development of the fecal microbiome in 12 dairy calves was investigated. Fecal bacterial composition was determined at four time points (weeks 0, 4, 8 and 12) using the 16S rRNA gene through PCR-amplification of the V1-V3 regions from fecal microbial genomic DNA, followed by Illumina MiSeq 2X300 sequencing. A comparative analysis of the most highly represented Operational Taxonomic Units (OTU) using the non-parametric Kruskal-Wallis sum-rank test and Wilcoxon pairwise test identified both known and uncharacterized fecal bacterial species whose abundance fluctuated during development of the calves. Four highly represented OTUs were found to have a peak of abundance at week 0 which was followed by significantly lower abundance at later time points ($P < 0.05$). Notably, OTU Bt-1063 peaked at week 0 (39.3% ± 3.6%) then declined at later time points with respective means of 2.3%, 0.1% and 0.05%. Seven other OTUs were found to peak at an intermediate time point ($P < 0.05$), including OTU Bt-1195 which was found in highest abundance at week 4 (4.5% ± 1.2%) compared to means with

a range of 0.001% to 0.01% for the other time points. In contrast, another set of well

represented OTUs were found to increase in abundance with time, which included OTU

Bt-1204 whose abundance was highest at week 12 (1.4% $\pm$ 0.3%) ($P < 0.05$). These

results are indicative of microbial succession in the gastro-intestinal tract of dairy calves

and highlight candidate bacterial species whose function could be manipulated towards

improving the health and productivity of growing dairy calves.

Key words: Neonatal dairy calves, hindgut, microbiome

2.2. INTRODUCTION

In light of the ever-increasing global human population and the growing demand for animal protein, the dairy industry represents an important contributor to the global food supply. Indeed, steady yearly production increases of approximately 2% have been reported between 2000 and 2012 (Federation, 2013), and this rate has remained consistent in recent years (FAO, 2021). In 2020, global production had reached almost 906 million tons, with Asia and Europe continuing to be the top producing regions (FAO, 2021). Within the United States, production has increased as well over the years, as shown by a comparison between production in 2010 (192,726 million pounds) (NASS, 2011) and production in 2020 (223, 220 million pounds) (NASS, 2021).

The economic sustainability of dairy operations relies on the continuous development of replacement heifers. Replacement heifers do not generate a profit for a dairy farm until they produce their first calf, which is generally at 24 months of age, so they represent an important investment by producers. As with most livestock industries, maintaining the health of young animals remains a major priority and a challenge for the dairy sector. To mature into adult ruminants, heifer calves must overcome two key challenges: high susceptibility to enteric infections during their first few weeks of life, and optimal development of their rumen to efficiently digest plant biomass and absorb microbial end-products.

To this end, a typical practice in intensive dairy operations is to separate calves from their dams immediately after birth. This benefits the health and safety of the calves by ensuring that they receive a sufficient amount of colostrum shortly after birth, i.e. during a period when absorption is optimal. Colostrum is not only an important source of

nutrients, but it also supplies immunoglobulins that provide passive immunity against pathogens until the immune system of the young animal matures (Blum, 2006; Steinhoff-Wagner et al., 2014; Kertz et al., 2017).

Another important system undergoing development in calves is their gut microbiota. While the calf gut environment is considered mostly devoid of microorganisms at birth, it is rapidly colonized within hours after calving by microbial species present in the vaginal mucus of the dam and in the colostrum, as well as from exposure to co-habitating calves and to the surrounding environment (Malmuthuge and Guan, 2017; Hang et al., 2021). Bacterial colonization of the gastrointestinal tract in pre-weaned calves is critical for their health and their future performance. Indeed, since calves essentially function as monogastrics after they are born, their rumen compartment needs to develop into an organ that can digest plant biomass and absorb microbial end-products. Development and maturation of the rumen is influenced by a number of factors, including the developmental stage of the young ruminant, the amount of time spent suckling, as well as diet composition (Blum, 2006). A common practice in intensive production systems to accelerate development of rumen microbial communities is then to limit suckling by feeding milk or milk replacer using buckets, and to provide solid feed in the form of calf starter pellets. Using this system, the consumption of solid feed by calves increases while the intake of milk replacer decreases, promoting development of the rumen before weaning. It is critical for calves to consume a sufficient amount of starter, since calves that do not meet the target starter quantity run the risk of having a consistently lower weight throughout weaning and post-weaning (Benetton et al., 2019).

Depending on farm management practices, the period between birth and full weaning can range between six to eight weeks (Uyeno et al., 2010).

Due to the importance of rumen development for dairy production, a great deal of efforts has been dedicated to elucidating the development of ruminal microbial communities in pre-weaning calves. In comparison, development of the hindgut microbiota in young ruminants remains mostly uncharacterized (Uyeno et al., 2010; Malmuthuge et al., 2015b; Malmuthuge and Guan, 2017). Since they were detected in the meconium, as well as in the fecal microbiome of six- and twelve-hour calves, species affiliated to the genus *Citrobacter* as well as lactic acid producing bacteria such as *Lactococcus*, *Leuconostoc*, and *Lactobacillus,* have been reported as first or early gut colonizers in dairy calves (Mayer et al., 2012). A complex hindgut microbiome continues to develop, as microbial diversity was reported to increase after 24 hours (Mayer et al., 2012). Overall, the respective abundances of early colonizers such as *Bifidobacterium*, *Lactobacillus*, *Faecalibacterium*, and *Enterococcus* tend to decline with age, whereas the abundance of Bacteriodetes-affiliated species increases (Uyeno et al., 2010; Oikonomou et al., 2013; Klein-Jöbstl et al., 2014). Notably, *Faecalibacterium* have been linked to higher growth and a reduction in the incidence of diarrhea (Oikonomou et al., 2013).

While they are not as involved in the digestion of feed as their ruminal counterparts, microbial communities of the hindgut are critical for maintaining gut health. As dairy calves are very likely to experience fluctuations in gut microbial composition because of their rapidly developing anatomy and physiology, varying intake of milk and solid feed, as well as exposure to stress (Uyeno et al., 2010), they may consequently be at a high risk for dysbiosis. Dysbiosis is a state of imbalance in the microbiome that can

provide favorable conditions for the proliferation of opportunistic pathogens if it persists. These can result in enteric infections that can jeopardize the life of the young ruminant and / or impact performance later during the productive stage of their life.

Reports to date on the hindgut microbiome of dairy calves has been in the context of particular conditions such as management practices or the use of antibiotics. Overall, there is very limited information on the development of the hindgut microbiome during the weaning period. In this context, this report presents an analysis of the fecal microbiome of dairy calves raised under a typical management regimen for the industry (in an intense production setting) for the first 12 weeks following calving. Most of the changes in bacterial composition were observed before weaning, after which bacterial composition appeared to remain stable for the remainder of the study.

2.3. METHODS AND MATERIALS

2.3.1. Animal trial and sample collection

For this study, 12 Holstein dairy calves were raised under standard industry practices from calving until 12 wks of age. Throughout this period, calves were housed in individual hutches, and offered calf starter pellets and drinking water ad libitum. For the first 5 weeks, calves were fed pasteurized milk twice every day, for a total of 5.6 L/d. Week 6 was a transition period, during which the amount of pasteurized milk was reduced by half (1X 2.6 L /d). From wk 7 to wk 12, calves were only offered calf starter pellets. Fecal samples were collected from all calves under study at wk 0, wk 4, wk 8 and wk 12.

2.3.2. Microbial DNA isolation, PCR amplification, and Next Generation Sequencing

Microbial DNA was isolated from fecal samples using the repeated bead beating plus column method (Yu and Morrison, 2004), which included the use of the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The V1-V3 region of the bacterial 16S rRNA gene was targeted using the 27F forward (Edwards et al., 1989) and 519R reverse (Lane et al., 1985) primer pair. Generation of V1-V3 16S rRNA gene amplicons and Next Generation Sequencing were performed by the South Dakota State University Genomic Sequencing Facility.

2.3.3. Computational analysis of PCR generated 16S rRNA amplicon sequences

Unless specified, sequence data analysis was performed using custom written Perl scripts. Raw bacterial 16S rRNA gene V1-V3 amplicon sequences were provided by Molecular Research DNA as assembled contigs from overlapping MiSeq 2x300 paired-end reads from the same flow cell clusters. Reads were then selected to meet the following criteria: presence of both intact 27F (forward) and 519R (reverse) primer nucleotide sequences, length between 400 and 580 nt, and a minimal quality threshold of no more than 1% of nucleotides with a Phred quality score lower than 15 (Opdahl et al., 2018).

Following quality screens, sequence reads were aligned, then clustered into Operational Taxonomic Units (OTUs) at a genetic distance cutoff of 4% sequence dissimilarity (Opdahl et al., 2018; Poudel et al., 2020). OTUs were screened for DNA sequence artifacts using the following methods. Chimeric sequences were first identified

with the 'chimera.uchime' (Edgar et al., 2011) and 'chimera.slayer' (Haas et al., 2011)

commands from the MOTHUR 1.44.1 open source software package (Schloss et al.,

2009). Secondly, the integrity of the 5' and 3' ends of OTUs was evaluated using a

database alignment search-based approach; when compared to their closest match of

equal or longer sequence length from the NCBI 'nt' database, as determined by BLAST

(Altschul et al., 1997) , OTUs with more than five nucleotides missing from the 5' or 3'

end of their respective alignments were discarded as artifacts. Single read OTUs were

subjected to an additional screen, where only sequences that had a perfect or near perfect

match to a sequence in the NCBI 'nt' database were kept for analysis, i.e. that the

alignment had to span the entire sequence of the OTU, and a maximum of 1% of

dissimilar nucleotides was tolerated.

   After removal of sequence chimeras and artifacts, taxonomic assignment of valid

OTUs was performed. All OTUs were classified at the levels of Phylum and Family

using RDP Classifier (Wang et al., 2007). For select OTUs, BLAST queries were also

used to identify their respective closest valid relatives (Altschul et al., 1997). Alpha

diversity indices (Observed OTUs, Chao, Ace, Shannon, and Simpson) were determined

using the 'summary.single' command from MOTHUR 1.44.1 (Schloss et al., 2009) on a

dataset rarified to 5,000 reads for each sample. Principle Coordinate Analysis (PCoA) for

beta diversity was performed using the same rarefied dataset, by first determining Bray

Curtis distances with the 'summary.shared' command, followed by the 'pcoa' command

in MOTHUR 1.44.1 (Schloss et al., 2009).

2.3.4. Statistical Analysis

Comparisons of abundance for bacterial taxonomic groups and OTUs amongst different time points were performed in R (Version R-3.6.2), first using the non-parametric test Kruskal-Wallis, then the Wilcoxon test for multiple pairwise comparisons. For comparison of alpha diversity indices across age groups, a one-way analysis of variance (ANOVA) and Tukey's range test for multiple comparison were conducted using R (Version R-3.6.2). Statistical significance was set at $P \leq 0.05$.

2.4. RESULTS

2.4.1. Taxonomic composition analysis

Fecal samples were used as a proxy to investigate the development of gut bacterial communities in neonatal dairy calves. A total of 2,152,504 high quality-filtered sequence reads from V1-V3 amplicons of the 16S rRNA gene were used for bacterial composition analysis from 12 Holstein calves at four-week intervals over a period of 12 weeks.

Firmicutes, Bacteroidetes and Proteobacteria were identified as the most prominent phyla in this study. Firmicutes showed an increase in abundance from wk 0 to wk 12, with divergent composition dynamics observed at the family level (Figure 2.1, Figure 2.2). Ruminococcaceae, Lachnospiraceae and Erysipelotrichaceae followed the overall trend of their phylum, displaying their lowest abundance at wk 0, then higher representation at later time points. Ruminococcaceae and Lachnospiraceae were the most highly represented families in wk 8 and wk 12 samples, together representing 57.7% and

58.4% of sequences at these time points, respectively (Table 2.1). Other Firmicutes families showed an opposite pattern of abundance. For instance, Clostridiaceae 1, Enterococcacea and Streptococcacea were most highly represented at wk 0, then their respective abundances were dramatically lower at the later time points ($P < 0.05$). Lactobacillaceae showed a similar trend, but with highest representation maintained within the same range at wk 0 and wk 4 (8.99% - 8.84%), followed by significantly reduced abundances at wk 8 and wk 12 by a factor of 899X and 88,400X, respectively ($P < 0.05$). Notably, sequences affiliated to Enterococcacea, Streptococcacea and Lactobacillaceae were at levels just above detection in wk 12 samples.

For Bacteroidetes, an increase of approximately two-fold was observed between wk 0 and wk 4, then their abundance was maintained within a narrow range (19.35 - 21.50%) from wk 4 to wk 12. Prevotellaceae displayed the highest and most representative change in abundance for this phylum, with 146.5X - 177.5X greater representation in samples from wk 4 to wk 12 compared to wk 0. While not as prominent, the levels of Porphyromonadaceae varied across time points, with lowest representation of affiliated sequences observed at wk 0, intermediate at wk 4, then highest at wk 8 and wk 12 ($P < 0.05$). Differences across time points in the abundance of Bacteroidaceae, the most highly represented Bacteroidetes family in this study, were not found to be statistically significant.

In contrast to Firmicutes and Bacteroidetes, Proteobacteria were at their peak levels in wk 0 samples. Enterobacteriaceae were identified as the main family for this phylum as they represented 41.2% of observed sequences at the first time point; their abundance was progressively reduced in later collections by a factor of 17.5X (wk 4),

343.5X (wk 8) and 1,030.5X (wk 12). While detected at much lower levels compared to the main phyla, Verrucomicrobia, Actinobacteria and Spirochaetae were found to fluctuate in abundance across the four time points.

2.4.2. OTU composition analysis

To gain further insight, OTU clustering was performed to investigate changes in bacterial composition at a higher resolution. This analysis identified 6,539 OTUs across all 48 samples. The alpha diversity indices Observed OTUs, Ace and Chao were found to be different amongst the different groups analyzed, showing progressively higher values from wk 0 to wk 12 ($P < 0.05$) (Figure 2.3 – 2.7, Table 2.2) . Beta diversity analysis by PCoA revealed that samples clustered into three distinct groups according to their respective time of collection: wk 0, wk 4 and wk 8 – wk 12 (Figure 2.8). Variation in taxonomic composition patterns, increases in OTU numbers as well as differential clustering of samples by PCoA indicated that OTU composition changed across time points. To gain further insight, the most highly represented OTUs, defined as having a group mean of at least 1% for at least one group, were further investigated. Of the 35 most abundant OTUs identified, 34 OTUs were found to vary across the time points investigated ($P < 0.05$). These OTUs could be divided into separate categories based on their composition pattern.

Six OTUs (Bt-1021, Bt-1063, Bt-1075, Bt-1192, Bt-1200 and Bt-1202) were in highest abundance at wk 0, which included the most highly represented OTUs identified in this study (Bt-1063 and Bt-1192). Following their initial peak at the first time point, these six OTUs were found in much lower abundance in samples from the next collection

period, with differences ranging between 11.4X and 737.5X when comparing

representation at wk 0 and wk 4 (Figure 2.9). Seven other OTUs (Bt-1052, Bt-1067, Bt-

1068, Bt-1194, Bt-1195, Bt-1198, Bt-1201) showed a different composition pattern, with

a peak in abundance at wk 4 (Figure 2.10 - 2.11). Notably, five of these OTUs were at

much higher levels in wk 4 samples (1.7% - 7.3%) compared to wk 0 (<0.01%).

Six OTUs affiliated to Bacteroidetes (Bt-1053, Bt-1070, Bt-1073, Bt-1193, Bt-

1199, Bt-1208) showed highest abundance in samples from wk 4 to wk 12 collections,

with comparatively very low abundance at wk 0 (<0.01). Similarly, for Firmicutes, three

OTUs affiliated to Ruminococcaceae (Bt-1013, Bt-1050, Bt-1203) and two OTUs

affiliated to Lachnospiraceae (Bt-1001 and Bt-1051) were at their lowest levels at the wk

0 and wk 4 time points (<0.01 - 0.20%), while they were significantly higher in the

samples from the wk 8 and 12wk collections (0.77 - 2.4%) (Figure 2.12 – 2.14).

Of the 35 most abundant OTUs analyzed, 17 OTUs showed high nucleotide

sequence identity to validly characterized bacterial species (>97% similarity). Thirteen of

these OTUs were found in highest abundance in samples from early collections (wk 0 -

wk 4). Eleven of the remaining abundant OTUs showed only limited sequence identity to

their respective closest valid relatives (86.1 – 93.2%), with 9 of these OTUs being most

highly represented in samples from wk 8 and wk 12 time points (Table 2.3).

2.5. DISCUSSION

Nutritional, physiological, and immunological functions of the gut are all

impacted by the gut microbiota (Gomez et al., 2017). A more in depth understanding of

the dynamics of gut microbiota development in neonatal dairy calves would lead to the

development of interventions that could minimize the risks of dysbiosis, and mitigate its effect on the growth of calves. In comparison to the rumen, our knowledge of the development of the hindgut microbiota in ruminants is more limited. Considering the challenge that enteric diseases pose to neonatal calves, the present study aimed to yield more insight on this process.

Increases in OTU numbers, PCoA clustering of samples into distinct groups, as well as variation in OTU composition patterns amongst time points were indicative of microbial succession. Microbial succession in the gut microbial communities of dairy calves has previously been reported (Uyeno et al., 2010; Edrington et al., 2012; Furman et al., 2020; Hang et al., 2021). In this study, the most dramatic changes were observed between wk 0 and wk 4, then between wk 4 and wk 8. Comparatively fewer differences found at later stages between wk 8 and wk 12.

Members of the genera *Enterococcus* and *Streptococcus* have been recognized as early gut colonizers, where they help to render the environment anoxic by consuming residual oxygen. In humans, both genera have been reported as being highly abundant in the first days after birth (Conroy et al., 2009; Jost et al., 2012), as a result of inoculation of newborns through ingestion of vaginal mucus during birth. In this study, OTUs Bt-1202 and Bt-1021 were identified as likely candidate strains of *Enterococcus lactis* and *Streptococcus macedonicus*, respectively. Their respective composition pattern was consistent with a potential role as transient colonizers, as their abundances were found to be much lower at wk 4 compared to wk 0 by a factor of 737.5X (Bt-1202) and 11.4X (Bt-1021), respectively. By wk 12, these OTUs were only detected at very low levels (Bt-1202: 0.004%; Bt-1021: 0%).

Members of the genera *Lactobacillus*, *Faecalibacterium* and *Butyricicoccus* have also been reported as prevalent in the gastrointestinal tract of neo-natal calves during the first weeks of life. As their respective abundances have been found to decrease as young animals grow (Uyeno et al., 2010; Alipour et al., 2018; Dias et al., 2018; Dill-McFarland et al., 2019), they also represented early colonizers of the ruminant gut. Accordingly, OTUs assigned to these genera were found in highest abundance in samples from wk 0 and / or wk 4 collections (*Lactobacillus*: Bt-1066, Bt-1195 and Bt-1200; *Faecalibacterium*: Bt-1194; *Butyricicoccus*: Bt-1197). Notably, only Bt-1194 from this group of OTUs was detected at levels greater than 0.01% in samples collected at wk 8 and wk 12. Members of these genera have been reported to have beneficial effects for the gut of young animals. In particular, *Faecalibacterium prausnitzii* and *Butyricicoccus spp.* have been found to contribute to weight gain during the pre-weaning stage, by decreasing the occurrence of diarrhea, and potentially through their ability to produce butyrate (Hang et al., 2021). Butyrate is a critical SCFA for the development of the ruminal mucosa and the longitudinal growth of papillae (Mentschel et al., 2001); accordingly, higher butyrate levels in the rumen have been associated with increased feed efficiency in steers (Guan et al., 2008). By reducing the incidence of severe diarrhea and promoting increased body weight, *F. prausnitzii* has thus shown probiotic properties when provided as an oral supplement to dairy calves (Foditsch et al., 2015).

Intriguingly, the two most highly represented OTUs at wk 0 were very closely related to potential bacterial pathogens. Bt-1063, which was responsible for the predominance of Proteobacteria at the earliest time point, was very closely related to *Shigella sonnei* (99.81%). This species has long been considered to be an agent of

shigellosis in humans, which has a higher prevalence in children (Hawkey et al., 2021). In this study, Bt-1063 was found in high abundance in all calves at wk 0, ranging from 14.9% to 59.4%; at wk 4, however, abundances were distributed between low (n=9; 0.01%- 0.68%) and high (n=3; 1.66% - 20.38%). OTU Bt-1192 was most closely related to *Clostridium perfringens*, a bacterial species found to be the most common cause of foodborne illness. This species is known to produce toxins that can cause gastro-intestinal diseases in animals, such as bloody scours in lambs or inflammation of the small intestine in poultry (Mehdizadeh Gohari et al., 2021). These potential pathogens could have the ability to destabilize the gut microbiome's balance, resulting in dysbiosis in neonatal calves, i.e. a state in which the microbial communities in the gut are out of balance and less resistant to change (Messer and Chang, 2018).

The gut bacterial composition in neo-natal calves during the first few weeks of life can be attributed in large part to their diet consisting of milk or milk replacer (Hang et al., 2021). As these contain high concentrations of simple sugars, such as lactose, they favor the growth of genera such as *Lactobacillus* and *Streptococcus*. As dairy calves further develop, their diet includes a higher proportion of complex carbohydrates, such as starch. Gut conditions then become more favorable for polysaccharide utilizers, concomitantly resulting in lower abundance of bacterial species that metabolize simple sugars as their main substrates (Gänzle and Follador, 2012). For instance, Bt-1071, an OTU related to *Prevotella shahii,* increased throughout the study with its highest abundance at wk 8 (0.94%) and wk 12 (1.44%), compared to its lowest abundance at wk 0 (<0.01%). Many members of the genus *Prevotella* are known to metabolize polysaccharides and produce SCFA (Precup and Vodnar, 2019). While a limited number

of reports are available on *P. shahii*, this species was found to be more abundant in the rumen of Jersey steers compared to Holstein steers, when individuals from both breeds were fed the same diet (Islam et al., 2021)

In contrast to OTUs that were prevalent at the wk 0 and wk 4 time points, the majority of OTUs that were highly represented at wk 8 and all OTUs that were most abundant at wk 12 were designated as unknown or uncharacterized, because they showed only limited sequence identity to their respective closest valid relatives. Thus, their metabolic potential could not be predicted with high confidence based on the characteristics of currently known bacterial species. Based on the developmental stage of the calves (post-weaning) and their diet (calf starter only), these OTUs are likely involved in the digestion of polysaccharides that escaped ruminal digestion. Further investigations will be required to determine their respective metabolic potential and their roles in the hindgut of weaned calves.

2.6. CONCLUDING REMARKS

The results presented in this report described changes in bacterial composition of the hindgut of dairy calves that were raised under conditions that would be similar to those of an intensive dairy production system. Dramatic fluctuations in abundance were observed, particularly in pre-weaned calves, which are consistent with microbial succession events that would be expected to take place in young animals. Under the conditions of our study, fecal bacterial communities of pre-weaned calves included species that were predicted to be beneficial, as well as species that could represent potential pathogens. This observation is also consistent with pre-weaned calves being more susceptible to enteric diseases by being more prone to dysbiosis events. In contrast,

fecal bacterial composition in post-weaned calves was found to be more complex but also more stable. Thus, this study provided candidate bacterial species that could be further explored as potential targets for intervention to benefit gut health in pre-weaned dairy calves.
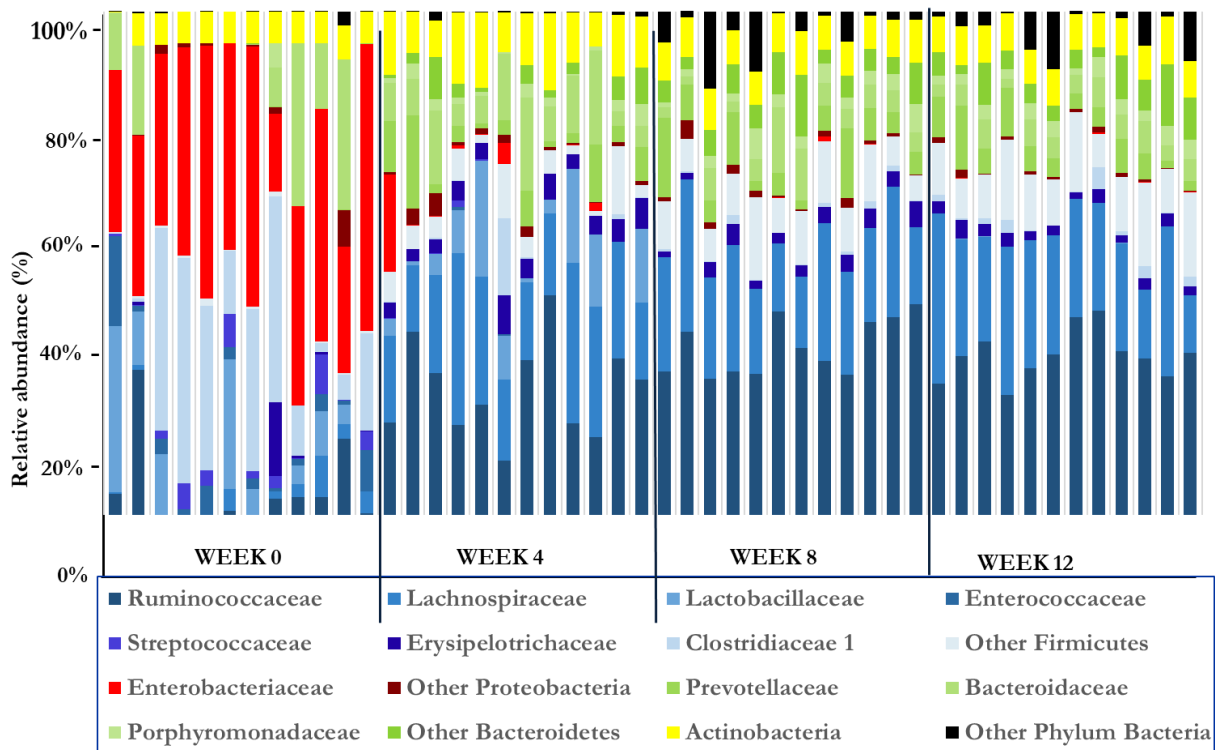
Figure 2.1. Individual samples: Phylum and Family level taxonomic composition of fecal bacterial communities in neonatal dairy calves. Families belonging to the same phylum are represented by different shades of the same color: Bacteroidetes (green), Firmicutes (blue), Proteobacteria (red).
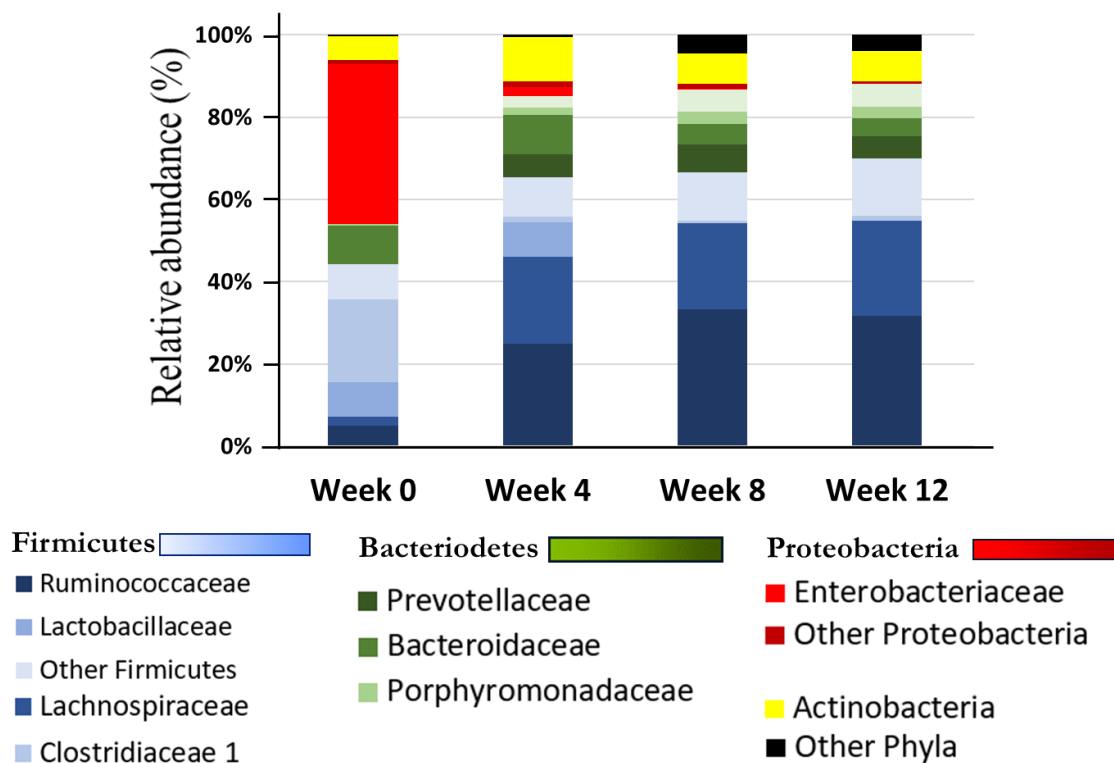
Figure 2.2. Means: Phylum and Family level taxonomic composition of fecal bacterial communities in neonatal dairy calves. Families belonging to the same phylum are represented by different shades of the same color: Bacteroidetes (green), Firmicutes (blue), Proteobacteria (red).
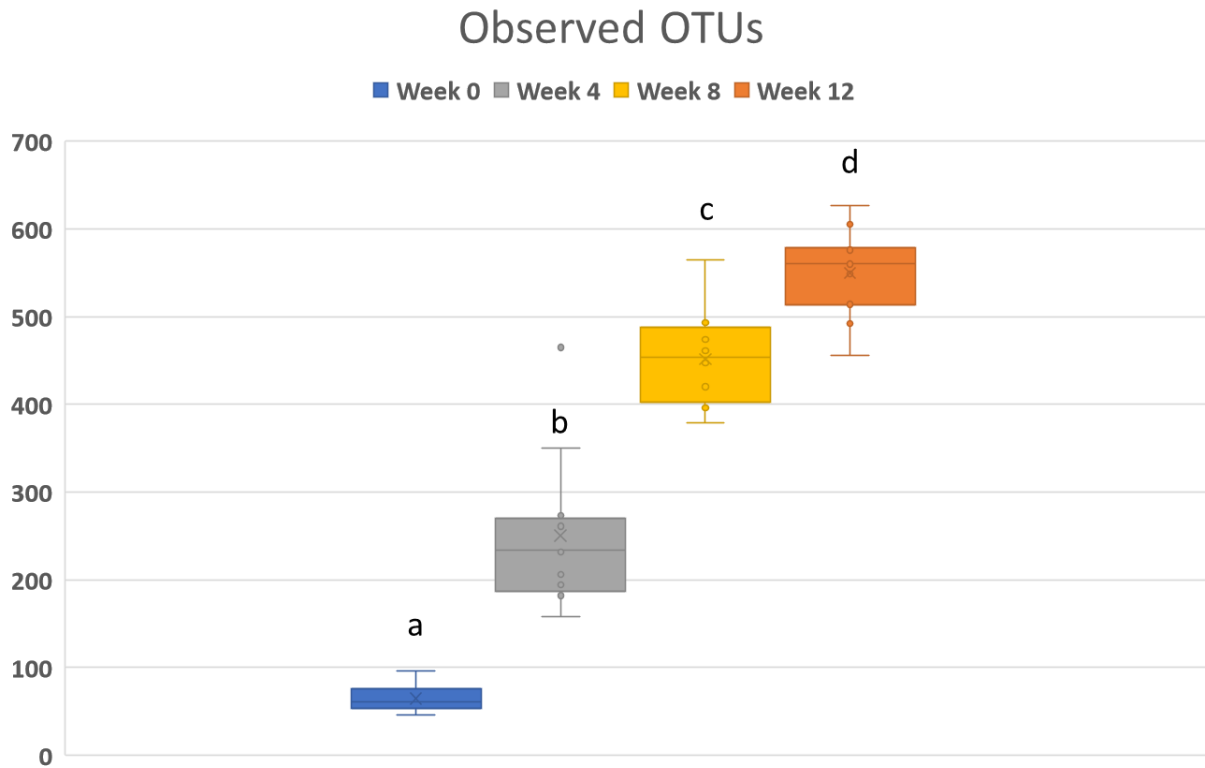
Figure 2.3. Boxplot showing the distribution in observed OTUs (alpha diversity index) across time points. Different superscripts indicate that groups are significantly different by the Tukey's range test for multiple pairwise comparison.
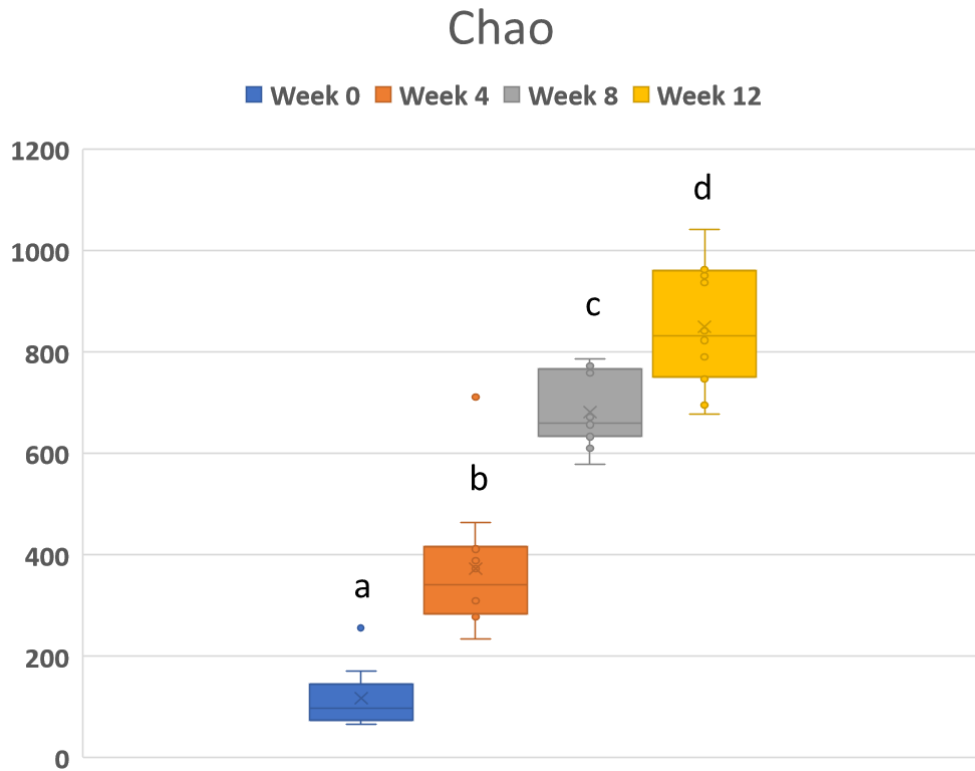
Figure 2.4. Boxplot showing the distribution in Chao (alpha diversity index) across time points. Different superscripts indicate that groups are significantly different by the Tukey's range test for multiple pairwise comparison.
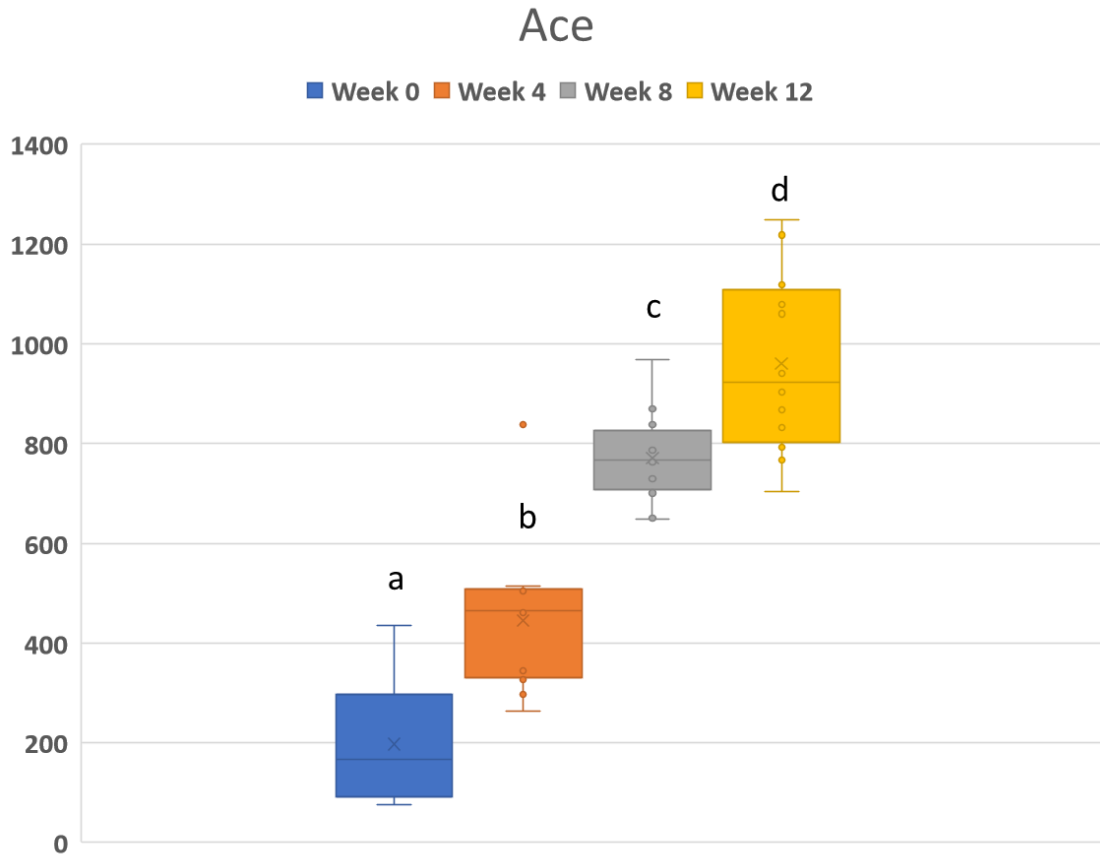
Figure 2.5. Boxplot showing the distribution in Ace (alpha diversity index) across time points. Different superscripts indicate that groups are significantly different by the Tukey's range test for multiple pairwise comparison.

Figure 2.6. Boxplot showing the distribution in Shannon (alpha diversity index) across time points. Different superscripts indicate that groups are significantly different by the Tukey's range test for multiple pairwise comparison.
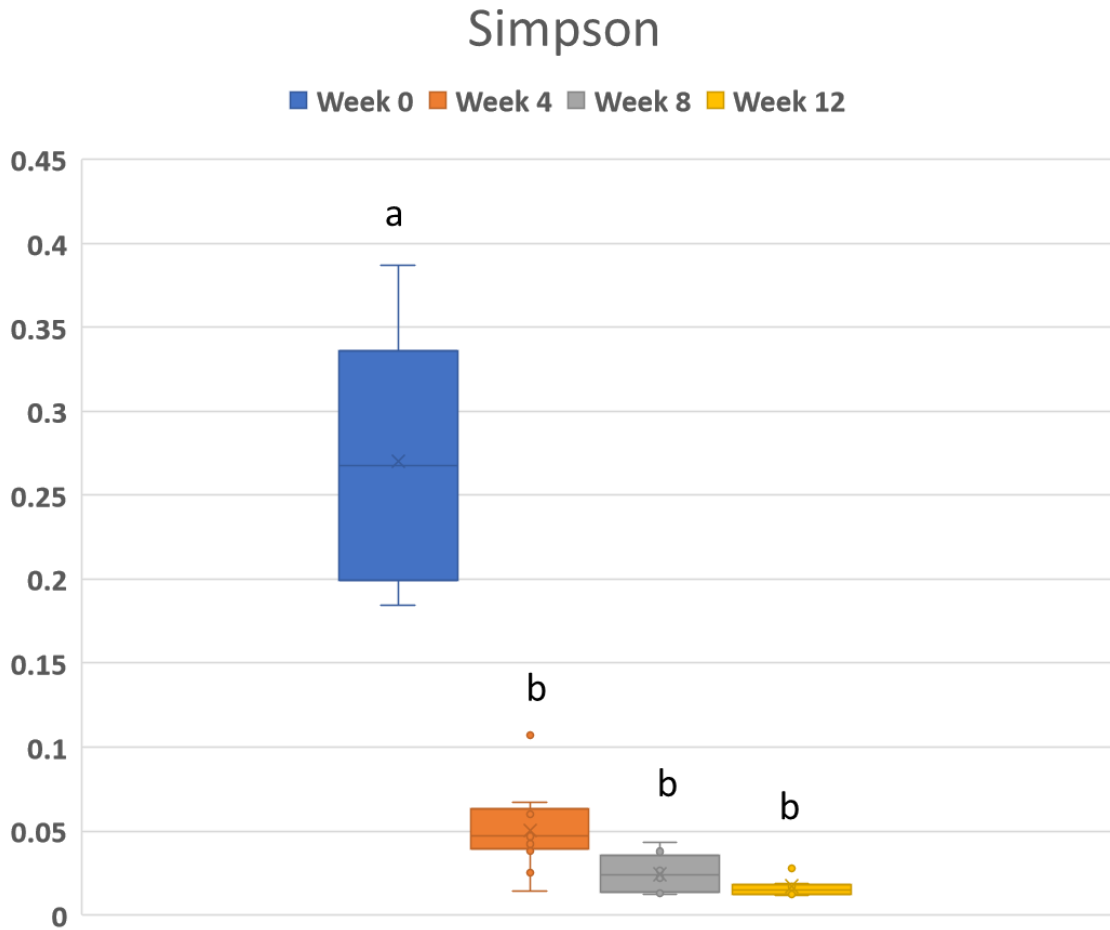
Figure 2.7. Boxplot showing the distribution in Simpson (alpha diversity index) across time points. Different superscripts indicate that groups are significantly different by the Tukey's range test for multiple pairwise comparison.
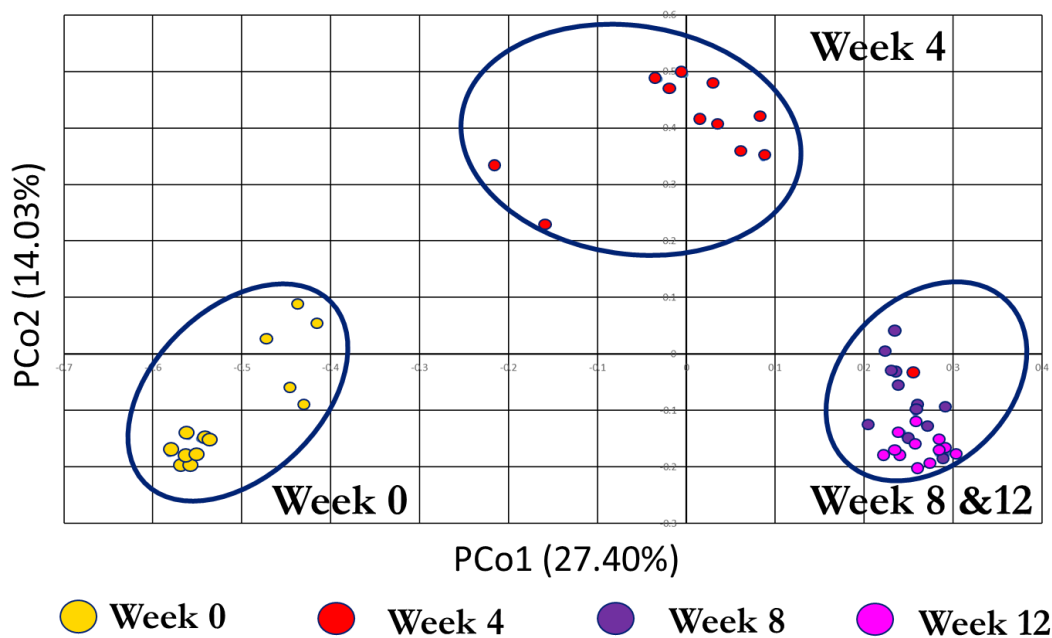
Figure 2.8. Comparison of fecal bacterial communities from neonatal dairy calves using Principal Coordinate Analysis (PCoA). The x and y axes correspond to Principal Component 1 (PCo1) and Principal Component 2 (PCo2), which explain the highest (41.43%) level of variation.

Figure 2.9. OTU abundances of fecal bacterial communities in neonatal dairy calves. OTUs are represented by different colors across four different time points (0wk, 4wk, 8wk, 12wk). Statistically significant differences (p < 0.05) across all 4 time points based on the Kruskal-Wallis sum-rank test. Different superscripts indicate that groups are significantly different.

* Indicates likely strains of known bacterial species
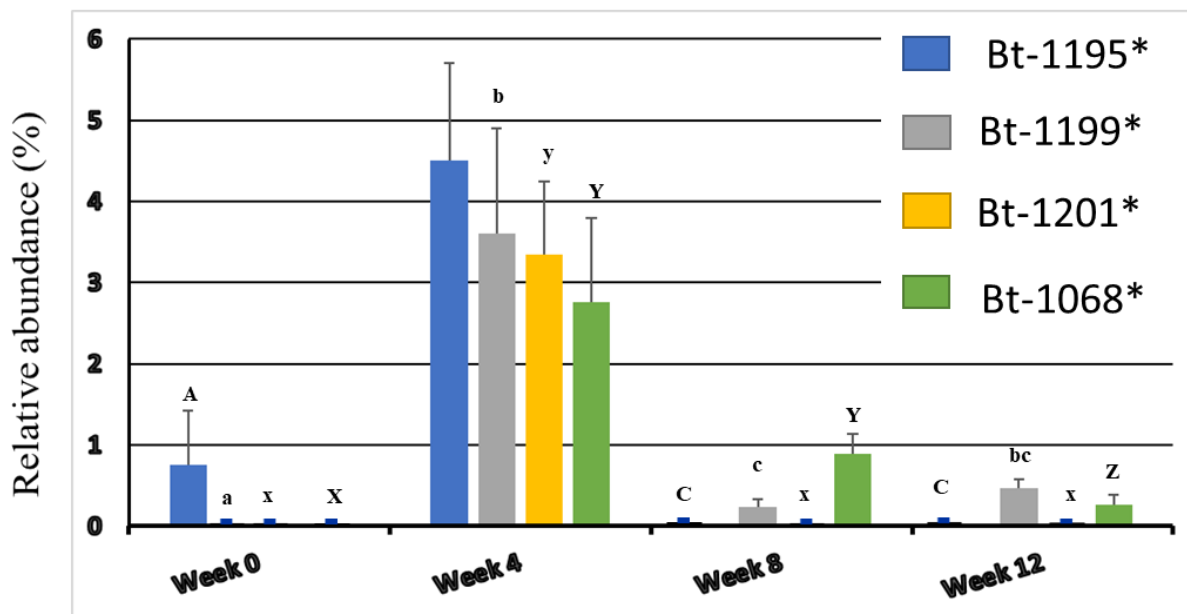
Figure 2.10. OTU abundances of fecal bacterial communities in neonatal dairy calves. OTUs are represented by different colors across four different time points (0wk, 4wk, 8wk, 12wk). Statistically significant differences (p < 0.05) across all 4 time points based on the Kruskal-Wallis sum-rank test. Different superscripts indicate that groups are significantly different.

* Indicates likely strains of known bacterial species
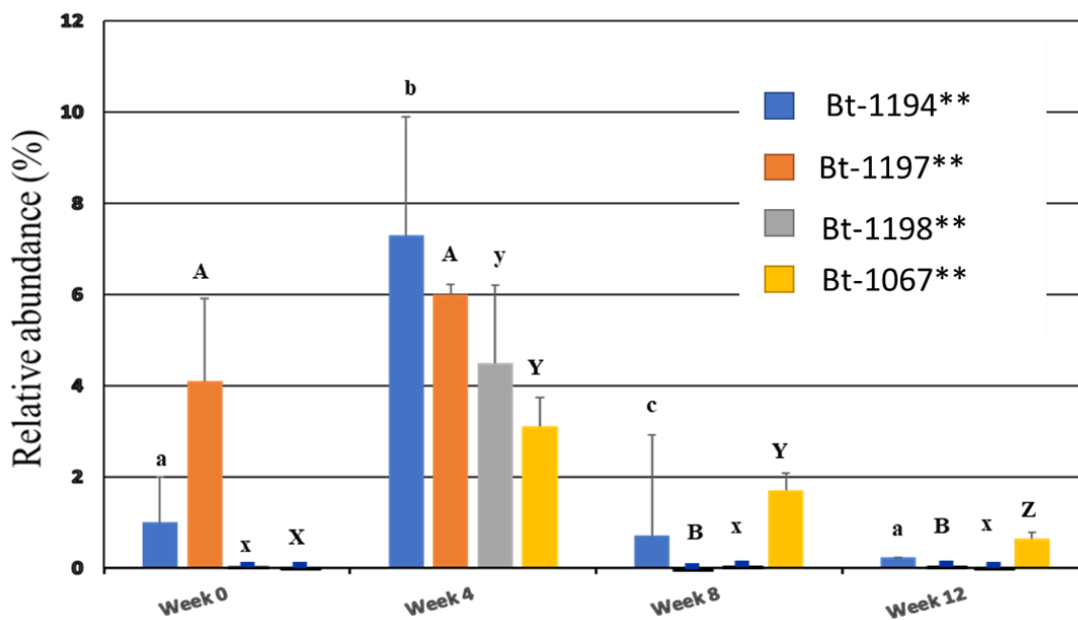
Figure 2.11. OTU abundances of fecal bacterial communities in neonatal dairy calves. OTUs are represented by different colors across four different time points (0wk, 4wk, 8wk, 12wk). Statistically significant differences ($p < 0.05$) across all 4 time points based on the Kruskal-Wallis sum-rank test. Different superscripts indicate that groups are significantly different.

** Indicates likely strains of unknown bacterial species
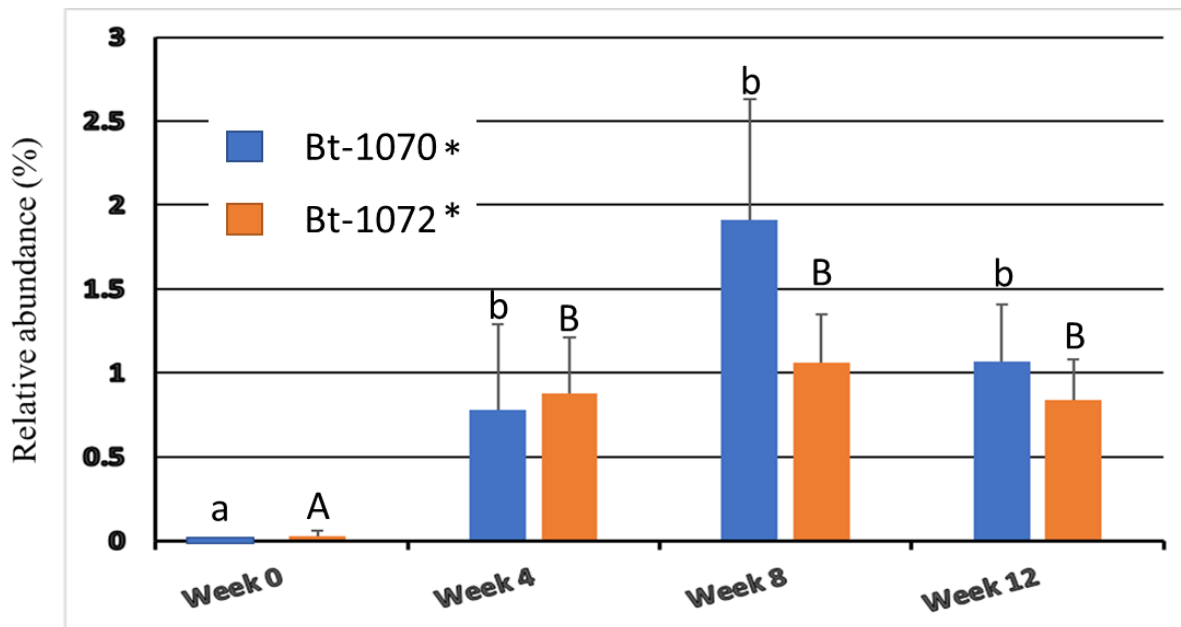
Figure 2.12. OTU abundances of fecal bacterial communities in neonatal dairy calves. OTUs are represented by different colors across four different time points (0wk, 4wk, 8wk, 12wk). Statistically significant differences (p < 0.05) across all 4 time points based on the Kruskal-Wallis sum-rank test. Different superscripts indicate that groups are significantly different.

* Indicates likely strains of known bacterial species
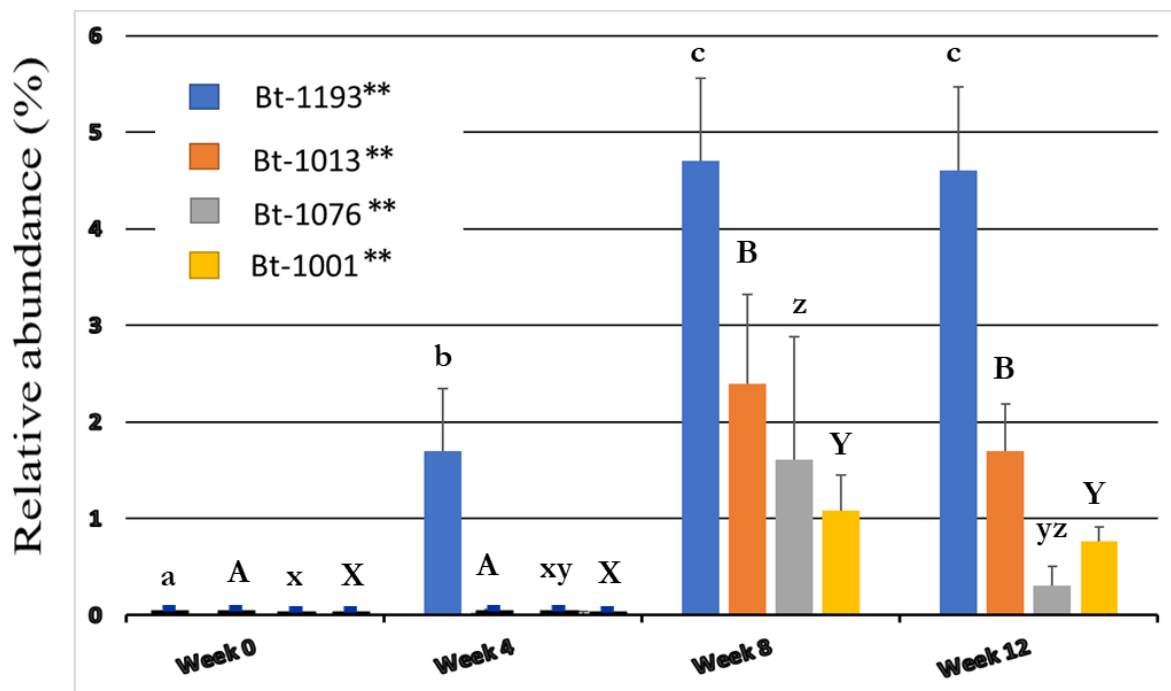
Figure 2.13. OTU abundances of fecal bacterial communities in neonatal dairy calves. OTUs are represented by different colors across four different time points (0wk, 4wk, 8wk, 12wk). Statistically significant differences ($p < 0.05$) across all 4 time points based on the Kruskal-Wallis sum-rank test. Different superscripts indicate that groups are significantly different.

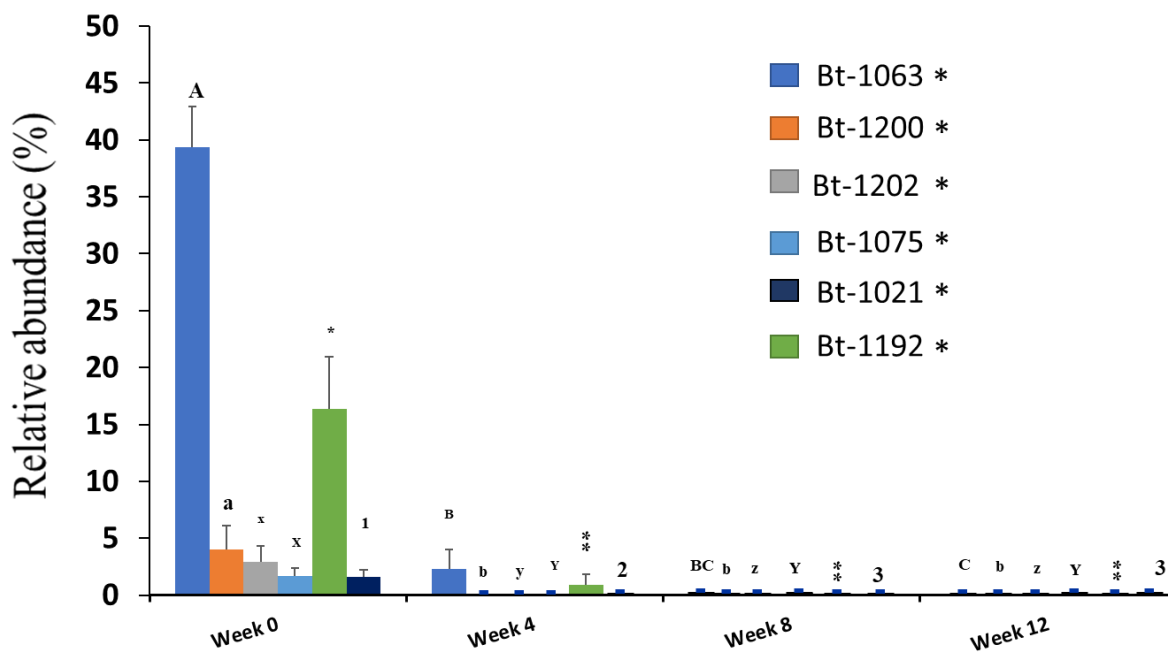**** Indicates likely strains of unknown bacterial species**

Figure 2.14. OTU abundances of fecal bacterial communities in neonatal dairy calves. OTUs are represented by different colors across four different time points (0wk, 4wk, 8wk, 12wk). Statistically significant differences ($p < 0.05$) across all 4 time points based on the Kruskal-Wallis sum-rank test. Different superscripts indicate that groups are significantly different.

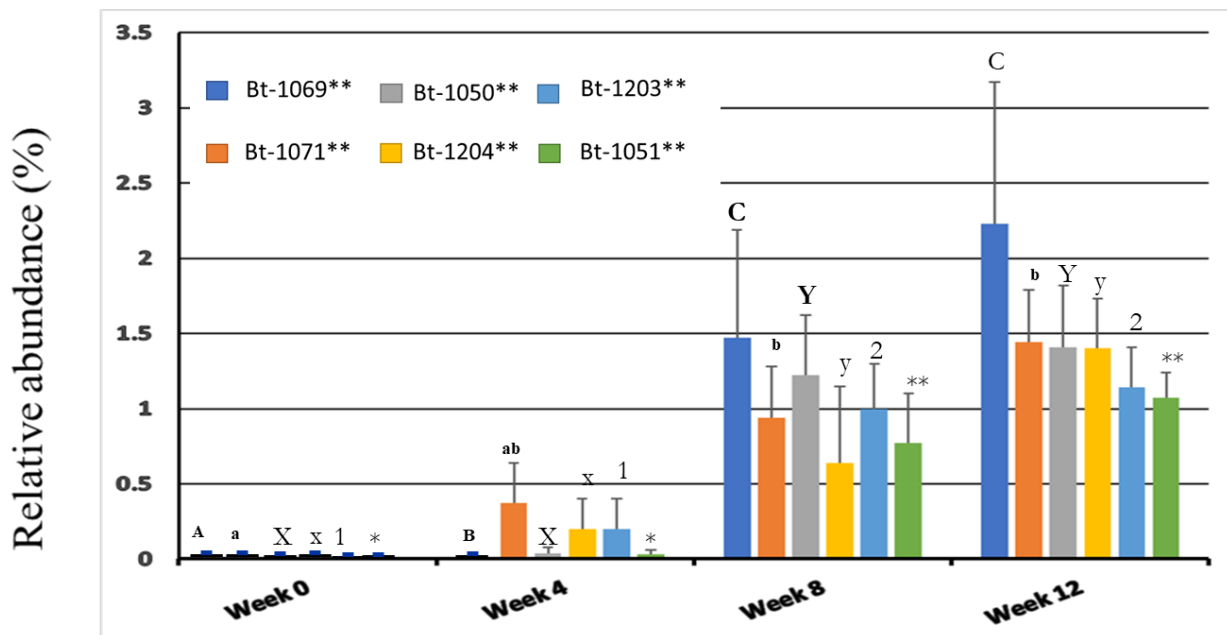**** Indicates likely strains of unknown bacterial species**

Table 2.1. Major Taxonomic groups identified in the hindgut of neonatal dairy calves

Taxonomic Affiliation	Week 0	Week 4	Week 8	Week 12
Firmicutes[#]	**46.89[a] ± 3.82**	**69.51[b] ± 2.74**	**70.90[b] ± 1.97**	**74.48[b] ± 1.49**
Clostridiaceae 1[#]	21.30[a] ± 5.30	1.51[b] ± 1.34	0.60[bc] ± 0.17	1.35[c] ± 0.40
Ruminococcaceae[#]	5.31[a] ± 2.65	26.48[b] ± 2.92	35.48[c] ± 1.72	33.78[c] ± 1.51
Lactobacillaceae[#]	8.99[a] ± 3.16	8.84[a] ± 2.37	0.01[b] ± < 0.01	< 0.01[b]
Lachnospiraceae[#]	2.25[a] ± 0.78	22.43[b] ± 2.13	22.19[b] ± 1.68	24.65[b] ± 1.95
Enterococcaceae[#]	4.13[a] ± 1.43	0.09[b] ± 0.05	< 0.01[c]	< 0.01[c]
Erysipelotrichaceae[#]	1.48[a] ± 1.29	4.38[b] ± 0.48	3.06[bc] ± 0.37	2.42[c] ± 0.21
Streptococcaceae[#]	2.89[a] ± 0.82	0.16[b] ± 0.11	< 0.01[c]	0[c] [$]
Other Firmicutes[&]	0.53 ± 0.11	5.42 ± 1.16	9.51 ± 1.00	12.25 ± 1.15
Bacteriodetes[#]	**10.39[a] ± 3.66**	**21.29[b] ± 2.67**	**21.50[b] ± 1.49**	**19.35[b] ± 1.02**
Porphyromonadaceae[#]	0.44[a] ± 0.43	1.88[b] ± 0.38	3.15[c] ± 0.48	2.94[c] ± 0.28
Bacteroidaceae	9.90 ± 3.65	10.10 ± 1.68	5.39 ± 1.04	4.63 ± 0.45
Prevotellaceae[#]	0.04[a] ± < 0.01	6.10[b] ± 1.65	7.10[b] ± 1.37	5.86[b] ± 1.01
Other Bacteroidetes[&]	0.02 ± < 0.01	2.99 ± 0.83	5.86 ± 0.95	5.91 ± 1.05
Proteobacteria[#]	**42.30[a] ± 3.43**	**3.93[b] ± 1.64**	**1.62[bc] ± 0.33**	**0.91[c] ± 0.16**
Enterobacteriaceae[#]	41.22[a] ± 3.68	2.35[b] ± 1.69	0.12[bc] ± 0.08	0.05[c] ± 0.03
Other Proteobacteria[&]	1.08 ± 0.63	1.44 ± 0.40	1.25 ± 0.30	0.76 ± 0.14
Verrucomicrobia[#]	**0.24[a] ± 0.23**	**< 0.01[b]**	**1.50[c] ± 0.72**	**2.24[ac] ± 0.94**
Actinobacteria[#]	**0.09[a] ± 0.04**	**4.81[b] ± 0.93**	**1.26[c] ± 0.24**	**1.17[c] ± 0.22**
Coriobacteriales	0.02 ± 0.01	4.75 ± 0.92	1.24 ± 0.24	1.14 ± 0.22
Spirochaetae[#]	**< 0.01[a]**	**0.12[b] ± 0.08**	**2.30[c] ± 1.23**	**1.11[c] ± 0.31**
Other Bacteria[&$]	**0.09 ± 0.04**	**0.34 ± 0.14**	**0.93 ± 0.35**	**0.74 ± 0.20**

Note: Mean relative abundance of taxonomic groups is presented as a percentage (%) of the total number of analyzed reads per sample.

[#] Taxa showing a statistically significant difference by the Kruskal-Wallis sum rank test ($p <$ 0.05). Different superscripts in the same row indicate that groups are significantly different by the Wilcoxon test for multiple pairwise comparison.

[&] Statistical test not performed because of group heterogeneity.

[$] Other bacteria include: Lentisphaerae, Fibrobacteres, Elusimicrobia, Candidatus Saccharibacteria, Acidobacteria, Planctomycetes, Tenericutes, Fusobacteria, Synergistetes, Chloroflexi as well as unclassified bacteria.

Table 2.2. Observed OTUs and α-diversity indices of neonatal dairy calves hindgut microbiome

Index	Week 0	Week 4	Week 8	Week 12
Ace[#]	$197.50^a \pm 33.81$	$445.20^b \pm 44.21$	$770.73^c \pm 26.40$	$960.77^d \pm 52.13$
Chao[#]	$117.39^a \pm 16.18$	$372.50^b \pm 36.64$	$682.15^c \pm 20.45$	$850.26^d \pm 34.80$
Shannon[#]	$1.83^a \pm 0.06$	$3.89^b \pm 0.12$	$4.76^c \pm .082$	$5.05^c \pm 0.05$
Simpson[#]	$0.27^a \pm 0.02$	$0.05^b \pm 0.01$	$0.02^b \pm 0.003$	$0.02^b \pm 0.001$
Sobs[#]	$64.42^a \pm 4.08$	$250.33^b \pm 24.61$	$451.58^c \pm 15.31$	$550^d \pm 13.98$

Note: Values are presented as means.

[#] Taxa showing a statistically significant difference by ANOVA ($p < 0.05$). Different superscripts in the same row indicate that groups are significantly different by the Tukey's range test for multiple pairwise comparison.

Table 2.3. Mean relative abundance of the main bacterial OTUs found in the hindgut of neonatal dairy calves. Abundance is presented as a percentage (%) of the total number of analyzed reads per sample.

OTUs	Week 0	Week 4	Week 8	Week 12	Closest valid taxon (id%)
Firmicutes					
Bt-1192[#]	16.4[a]	.90[b]	< 0.01[b]	< 0.01[b]	*Cl. perfrongens* (98.73%)
Bt-1194[#]	1.0[a]	7.3[b]	.72[c]	0.23[a]	*F. prausnitzii* (96.57%)
Bt-1066[#]	3.3[a]	4.0[a]	< 0.01[b]	< 0.01[b]	*Lactobacillus johnsonii* (99.63%)
Bt-1067[#]	< 0.01[a]	3.1[b]	1.7[b]	0.64[c]	*Blautia wexlerae* (96.59%)
Bt-1195[#]	0.76[a]	4.5[b]	< 0.01[c]	< 0.01[c]	*Lactobacillus reuteri* (99.26%)
Bt-1197[#]	4.1[a]	6.0[a]	< 0.01[b]	0[b]	*Bu. pullicaecorum* (95.19%)
Bt-1198[#]	0.01[a]	4.5[b]	0.03[a]	0.01[a]	*Blautia stercori* (96.18%)
Bt-1013[#]	< 0.01[a]	0.03[a]	2.4[b]	1.7[b]	*Oscillibacter ruminantium* (90%)
Bt-1200[#]	4.0[a]	0.04[b]	< 0.01[b]	< 0.01[b]	*Lactobacillus murinus* (100%)
Bt-1068[#]	< 0.01[a]	2.76[b]	0.89[b]	0.26[c]	*Blautia caecimuris* (97.09%)
Bt-1202[#]	2.95[a]	< 0.01[b]	< 0.01[c]	< 0.01[c]	*Enterococcus lactis* (99.36%)
Bt-1050[#]	< 0.01[a]	0.04[a]	1.22[b]	1.41[b]	*Os. valericigenes* (90.28%)
Bt-1203[#]	< 0.01[a]	0.20[a]	1.00[b]	1.14[b]	*Oscillibacter ruminantium* (89.02%)
Bt-1204[#]	< 0.01[ab]	0.20[a]	0.64[b]	1.4[c]	*Oscillibacter ruminantium* (89.21%)
Bt-1074[#]	< 0.01[a]	1.80[b]	0.31[c]	0.02[a]	*Faecalicatena orotica* (96.89%)
Bt-1206	1.7	0.22	0	0	*Ruminococcus gnavus* (98.61%)
Bt-1001[#]	< 0.01[a]	0.02	1.08[b]	0.77[b]	*Clostridium bolteae* (96.64%)
Bt-1051[#]	< 0.01[a]	0.03[a]	0.77[b]	1.07[b]	*Cuneatibacter caecimuris* (93.02%)
Bt-1207[#]	< 0.01[a]	1.14[b]	0.67[b]	0.01[a]	*Blautia wexlerae* (98.06%)
Bt-1075[#]	1.68[a]	0.01[b]	0.09[b]	< 0.01[b]	*Cl. paraputrificum* (99.80%)
Bt-1021[#]	1.60[a]	0.14[b]	< 0.01[c]	0[c]	*S. macedonicus* (99.25%)
Bt-1052[#]	< 0.01[a]	1.70[b]	0.04[a]	< 0.01[a]	*Su. variabile* (98.57%)
Bacteriodetes					
Bt-1193[#]	< 0.01[a]	1.7[b]	4.7	4.6[c]	*Muribaculum intestinale* (89.39%)
Bt-1065[#]	2.0[a]	3.3[b]	1.0[b]	1.3[b]	*Bacteroides vulgatus* (99.62%)
Bt-1199[#]	< 0.01[a]	3.6[b]	0.24[c]	0.47[bc]	*Bacteroides coprophilus* (96.77%)
Bt-1070[#]	< 0.01[a]	0.78[b]	1.91[b]	1.07[b]	*Prevotella copri* (99.24%)
Bt-1072[#]	0.03[a]	0.88[b]	1.06[b]	0.84[b]	*Bacteroides uniformis* (99.81%)
Bt-1071[#]	< 0.01[a]	0.37[ab]	0.94[b]	1.44[b]	*Prevotella shahii* (90.53%)
Bt-1073[#]	< 0.01[a]	1.71[b]	0.78[b]	0.25[b]	*P. timonensis* (89.09%)
Bt-1053[#]	< 0.01[a]	1.0[b]	0.33[c]	0.57[c]	*Ercella succinigenes* (88.89%)
Bt-1208[#]	< 0.01[a]	1.22[b]	0.22[b]	0.42[b]	*Prevotella stercorea* (99.05%)
Proteobacteria					
Bt-1063[#]	39.33[a]	2.3[b]	0.12[bc]	0.05[c]	*Shigella sonnei* (99.81%)
Verrucomicrobia					
Bt-1069[#]	0.01[a]	< 0.01[b]	1.47[c]	2.23[c]	*M. fagopyrum* (91.43%)
Actinobacteria					
Bt-1201[#]	< 0.01[a]	3.34[b]	< 0.01[a]	< 0.01[a]	*Collinsella aerofaciens* (98.38%)
Spirochaetae					
Bt-1076[#]	0[a]	< 0.01[ab]	1.61[c]	0.31[bc]	*T. pectinovorum* (86.15%)

Note: Statistically significant differences ($p < 0.05$) across all 4 time points based on the Kruskal-Wallis sum-rank test. ***** no reads detected in any of the samples for this group.

[#] OTUs showing a statistically significant difference by the Kruskal-Wallis sum rank test ($p < 0.05$). Different superscripts in the same row indicate that groups are significantly different by the Wilcoxon test for multiple pairwise comparison.

Chapter 3 : Future Research and Impact

3.1. INTRODUCTION

Increases in dairy production are expected to continue in order to meet the market needs of an ever-growing global population that is becoming more urbanized. While this represents an opportunity for the U.S. dairy sector to grow, it also raises concerns over already existing challenges faced by the industry, such as environmental and economic sustainability as well as animal welfare. Calf welfare continues to be an important issue, as producers are still in need of novel or improved solutions to mitigate current high mortality and morbidity rates in young animals. Indeed, pre-weaning and weaning remain the most sensitive stages during development of a calf, with high susceptibility to enteric and pulmonary diseases.

Young animals are more sensitive to diseases in large part because their immune system has yet to reach maturity. Indeed, innate and passive immunity represent the main lines of defense in young animals, because adaptive immunity requires exposure to pathogens in order to develop. While feeding colostrum soon after birth provides antibodies from the dam that give a neonate passive immunity until it can produce its own immunoglobulins, it only provides a temporary line of defense.

Another important system that is still in development in neonates consists of the communities of symbiotic microorganisms, referred to as microbiomes, that live in association with the various epithelial surfaces of an animal's body. One of the benefits

that they provide to their host is acting as a line of defense against pathogens. When perturbations, such as stress or a change in diet for instance, alter conditions of these microbial habitats, they can result in changes in microbiome composition. If severe enough that they overcome the resistance of the microbial community, these changes can result in a state of dysbiosis, i.e. an imbalance of the microbiome. If prolonged, dysbiosis can provide opportunities for pathogens to proliferate, resulting in disease. If the microbial communities are resilient enough, they can recover and return to their original composition prior to when the perturbation occurred.

As immature ruminants, calves need to develop two important microbiomes: the rumen microbiome and the hindgut microbiome. The former is essential for digestion of plant biomass by mature animals, while the latter plays a more critical role for maintaining gut health than contributing to the host's nutritional needs. Because of the importance of the rumen microbiome for ruminant production, more efforts have been dedicated to elucidating its development in dairy calves compared to the hindgut microbiome. However, a better understanding of the development of the hindgut microbiome would likely yield great insight towards disease prevention by benefiting mitigation of dysbiosis.

In this context, the study presented in this thesis aimed at providing improved insight on the development of the hindgut microbiome in dairy calves. The aim of this chapter is to provide a perspective on the findings from this study, as well as possible future lines of research that would be of interest. Applications that could be developed from the findings of this study are also discussed.

3.2. PERSPECTIVE ON MAIN STUDY FINDINGS

Together, the results from this study describe changes in microbial abundance in dairy calves from early post-natal life to post-weaning that are consistent with microbial succession. The dramatic changes in composition between the wk 0 and wk 4 time points can be attributed at least in part to the consumption of milk, while the differences between wk 4 and wk 8 can be attributed to weaning, i.e. the removal of milk from the diet so that calves were only fed calf starter pellets. Consistent with this interpretation is the observation that there were fewer observed differences in composition between wk 8 and wk 12, two post-weaning time points when calves were fed only starter pellets, compared to the earlier time points. This is in contrast to Meale et al. (2017), who reported that late-weaning calves had a more stable gut microbiome than early weaning calves, because they benefited from gradual changes in the bacterial communities, which ultimately would reduce the risks of detrimental effects on the calf.

3.3. ANALYSIS BY METAGENOMICS

As the main families and genera whose abundance fluctuated in this study were the same as published in other reports, our results corroborated conclusions from previous investigations (Conroy et al., 2009; Uyeno et al., 2010; Dill-McFarland et al., 2019; Hang et al., 2021). However, our analysis of main OTUs provided a higher degree of resolution, with the identification of candidate bacterial species that could potentially be beneficial or pathogenic to dairy calves at this age (Hawkey et al., 2021; Islam et al., 2021; Mehdizadeh Gohari et al., 2021). To gain further insight, metagenomics would be a logical next step in this research, as it would reveal the metabolic potential of the bacterial species corresponding to main OTUs through assembly of shotgun sequencing

data into genomic contigs. One of the benefits of performing 16S rRNA gene-based composition analysis before metagenomics is that it provides an estimate of the number of different bacterial species and their taxonomic affiliation in each sample. Considering that it is unlikely that metagenomics sequence data generated from complex microbial communities can be assembled into whole genomes, the 16S rRNA-generated information can be of great help when determining whether contigs with similar taxonomic affiliations should be assigned to the same bacterial species or to different bacterial species.

Metagenomics can provide valuable insights for OTUs, whether they showed very high or very low sequence identity to their closest valid relative. For instance, Bt-1063 was found to be 99.81% identical to *Shigella sonnei*, a bacterial species reported as a pathogen in humans, particularly in children (Hawkey et al., 2021). However, the effect of this species in livestock as yet to be fully investigated. Bt-1063 was detected at its highest representation at the earliest time point (wk 0), then declined by 17-fold by the fourth week. This could be interpreted as a potential opportunistic pathogen whose abundance is reduced in healthy neonates as they grow, but it could also indicate that OTU Bt-1063 is simply an early and transient gut colonizer. Assembly of genomic contigs for this OTU from shotgun sequencing data would then allow to confirm whether it should represent a strain of S. sonnei, and whether it possesses genes potentially involved in causing disease. If the OTU is confirmed to be a pathogenic strain, then this information could be used to implement screens to assess its distribution in herds, as well as develop strategies to minimize its abundance as early as possible.

Another OTU of interest would be Bt-1194, as it was found to be 96.57%
identical to *Faecalibacterium prausnitzii,* a bacterial species considered beneficial to
calves because it has been linked to increased growth and reduced incidence of diarrhea
(Oikonomou et al., 2013). Butyrate production is considered its main benefits (Mentschel
et al., 2001), but perhaps determination of its metabolic potential through metagenomics
analysis would reveal other benefits to growing dairy calves.

Finally, metagenomics would provide valuable insights in elucidating the
functions of OTUs that show very limited sequence identity to validly characterized
bacterial species. In some cases, these OTUs may represent novel phylogenetic lineages
that could be capable of metabolic activities of great interest. Bt-1196, for example,
showed only 87.05 % identity to *Sporobacter termitidis*, and could only be reliably
classified as a member of the family Ruminococcaceae. Overall, this OTU was found in
highest abundance at later time points. Its representation in the first four weeks ranged
from 0 % to 0.08 % in almost all samples, with only one sample from these sets showing
a high abundance (5.44 % at wk 4). At the wk 8 and wk 12 time points, samples from
four calves had Bt-1196 abundances ranging from 9.30% - 17.95 %. Since this OTU was
in highest abundance at time points when calf starter was the only source of feed, it could
potentially encode novel glycoside hydrolases for the digestion of structural
polysaccharides that would benefit young ruminants as they mature.

3.4. POTENTIAL APPLICATIONS

While there are typically very few objections to using antibiotics to treat bacterial
infections, it is their prophylactic use that is more generally a concern to the public
(Mathur and Singh, 2005). While antibiotic use in the livestock industry varies across

species, a previous survey focused on pre-weaned heifers in the dairy sector revealed that

57.5% of dairies utilized medicated milk replacer for disease prevention and growth

promotion, in comparison to 17.9% use for treatment of disease (USDA and VS, 2008).

In addition to reducing the incidence of disease, which is beneficial by minimizing labor

as well as the costs of veterinary services and treatments, the other benefit from the

prophylactic use of antibiotics is the resulting increase in production, and thus in

producer revenues (Sneeringer et al., 2015).

However, public concerns over food safety as well as the alarming increase in the

incidence of antibiotic-resistance affecting human health have led to stricter regulations

for antibiotic use. (Salyers et al., 2004). With the intent to decrease the use of antibiotics

for the purpose of improving feed efficiency and performance, the FDA enacted the VFD

or Veterinary Feed Directive in 2017, restricting antibiotic use for only therapeutic

purposes. A survey released after the enactment of the VFD found that a third of

producers had changed their practices, with a small portion that transitioned to use

alternatives to antibiotics (Okello et al., 2021).

Pre- and probiotics have been developed as alternatives to antibiotics to benefit

animal performance, resulting in many commercial products that are available for

humans and food animals. As these dietary supplements are not regulated in the U.S,

there is currently little incentive for adequate labeling. Certain studies have revealed that

a number of products do not include the strains that are advertised (Mattarelli et al., 2002;

Huff, 2004; Wannaprasat et al., 2009). Notably, a study conducted on thirteen products

used for animal consumption that claimed inclusion of *Lactobacillus* and *Bacillus* in their

formulation revealed that one product did not include either genus, none of the products

displayed the correct names of the species, and all products had more species than what was labeled (Wannaprasat et al., 2009). From a personal online search for milk substitutes, I found many companies claiming that their products included probiotics, but they did not specify which microorganisms were included in their products, and simply referred to them as 'beneficial microorganisms'. As the industry works toward increasing the use of these additives, additional regulation and quality assurance will be required.

When available, data so far on the benefits provided by probiotic products tend to be conflicting, (Gaggìa et al., 2010), but the potential of probiotics to mitigate the incidence of diarrhea in neonatal calves has shown some promise. When yeast, lactic acid bacteria or *Lactobacillus acidophilus* were added to milk replacer, the incidence of diarrhea was found to be reduced compared to control calves (Agarwal et al., 2002). Similarly, the use of a probiotic consisting of *E. coli* strain Nissle 1917 was associated with a decrease in the incidence of diarrhea, as well as a reduction in its duration or reduced severity of the symptoms (Von Buenau et al., 2005). When investigating means for compensating for failure in the transfer of passive immunity, calves fed a yeast supplement (*Saccharomyces cerevisiae*) showed increased intake of grain and weight gain during pre-weaning; although supplementation with yeast did not prevent diarrhea, its duration was shorter than in the control calves (Galvão et al., 2005).

Most probiotic-based supplements that are available for use in the dairy industry include bacterial species and strains that are not common residents of the bovine gut, which may explain why their effectiveness seems to vary from study to study. If developed as probiotics, however, the bacterial species corresponding to the OTUs identified from this research would be more likely to have an impact because they are

members of bovine gut microbial communities. Determining their metabolic potential,

through metagenomics analysis as described above, would provide insights on their

potential benefits. As residents of the bovine gut, they would be expected to survive and

function if provided as supplements to dairy calves. Another strategy could be to use the

information on metabolic potential of the OTU candidates to identify a substrate or a

metabolic pathway that could be targeted by a prebiotic, thus manipulating the growth or

activities of indigenous gut bacterial populations. While the development of supplements

using this approach would require time and efforts, it is likely to become more common

because of its higher chance of success and effectiveness.

Another potential future application of this research would be to improve or

accelerate the development of the gut microbiome in dairy calves. Indeed, as formulation

of both the weaning and the pre-weaning diets have been reported to be associated with

growth and productivity in mature animals (Dill-McFarland et al., 2019), it can be

hypothesized that one effect of the optimal diets was modulating the development of the

gut microbiome in growing calves. This would then provide long-term benefits once the

animals have reached the productive stage of their lives. Similarly, identifying the

optimal microbial composition at each stage could allow for individual monitoring of

calves during their development, allowing to adjust the time of weaning for each calf

based on its individual gut microbial composition. Ultimately, characterization of an

optimal gut bacterial composition at weaning could allow for the development of pre- or

probiotics to accelerate gut microbiome maturation. For instance, a study conducted on

45 Holstein dairy calves revealed that it took between weaning (eight weeks) and one

year for all animals to develop an adult-like gut microbiome (Dill-McFarland et al.,

2019). Further elucidating the required core gut microbial species and how their

respective abundance is controlled could be applied to accelerating the development of an

adult gut microbiome in a more uniform fashion across individual calves.

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