The Fecal Microbiota of Healthy and Diarrheic Calves Before, During and at Recovery From Diarrhea

by

Emma Jessop

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Abstract

Gastrointestinal Microbiota Alteration in Diarrheic Calves

Emma Jessop

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Advisor(s):

Dr. Diego Gomez

The changes associated with the gastrointestinal microbiota before, during and after neonatal calf diarrhea still needs to be fully understood and described. The first chapter of this thesis reviews our current understanding of the composition of a “healthy” fecal microbiome, microbial changes associated with dysbiosis and gastrointestinal disease, etiologic agents associated with diarrhea, and current treatments for calf diarrhea. Throughout the literature review, the author explains the current knowledge gaps regarding the changes in the gastrointestinal microbial communities of calves that experience diarrhea during the preweaning period and provides opinions about future studies that can aid in filling those gaps.

The second chapter reports the findings of a study investigating the changes in the fecal microbiota of neonate calves before, during, and at recovery of diarrhea. This study showed that the bacterial communities of healthy calves and calves that experience diarrhea are not different before or after diarrhea, but the microbiota differed between groups at diarrhea onset. These results suggested that reported changes in bacterial communities during diarrhea appear to be a consequence of diarrhea and gastrointestinal inflammation rather than the predisposing factor for diarrhea development.

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BRoV	Bovine rotavirus
BCoV	Bovine coronavirus
ETEC	Enterotoxigenic *Escherichia coli*
GIT	Gastrointestinal tract
LME	Linear mixed effect model
ANCOM-BC	Analysis of composition of microbiota with bias correction
PERMANOVA	Permutational multivariate analysis of variance
RF	Random forest
ASVs	Amplicon sequence variants

1 Chapter One: Literature Review

Neonatal Calf Diarrhea and Gastrointestinal Microbiota: Etiologic Agents and Microbiota Manipulation for Treatment and Prevention of Diarrhea.

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Emma Jessop[1], Lynna Li[2], David L. Renaud[3], Adronie Verbrugghe[1], Jennifer Macnicol[4], Lisa Gamsjäger[5], Diego E. Gomez[1]

[1]Department of Clinical Studies, University of Guelph, Ontario Veterinary College, 50 Stone Road East, Guelph, ON N1G 2W1, CA

[2]Cornell University, College of Veterinary Medicine, 602 Tower Road, Ithaca, NY 14853, USA

[3]Department of Population Medicine, University of Guelph, Ontario Veterinary College, 50 Stone Road East, Guelph, ON N1G 2W1, CA

[4]Department of Pathobiology, University of Guelph, Ontario Veterinary College, 50 Stone Road East, Guelph, ON N1G 2W1, CA

[5]Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606, USA

Corresponding author: Diego Gomez

[1]Department of Clinical Studies, University of Guelph, Ontario Veterinary College, 50 Stone Road East, Guelph, ON N1G 2W1, CA

E-mail: dgomezni@uoguelph.ca

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1.1 Abstract

Neonatal calf diarrhea is the leading cause of neonatal morbidity and mortality globally. The changes associated with the gastrointestinal microbiota in neonatal calves experiencing diarrhea and its etiology are not fully understood or completely defined in the literature. Several studies demonstrated that the fecal microbiota of calves that experience diarrhea substantially deviates from that of healthy age-matched calves. However, one key question remains; whether the changes observed in the bacterial communities (also known as dysbiosis) are a predisposing factor for, or the consequence of, gastrointestinal inflammation caused by the pathogens associated with calf diarrhea. The first objective of this literature review is to present the current information regarding the changes in the fecal microbiota of diarrheic calves and the impact of the pathogens associated with diarrhea on fecal microbiota. Modulation of the gastrointestinal microbiota using pre- and probiotics, colostrum feeding and fecal microbiota transplantation (FMT) has been used to treat and prevent gastrointestinal diseases in humans and dogs. Although information regarding the use of probiotics for the prevention of diarrhea is available in cattle, little information is available regarding the use of these strategies for treating calf diarrhea and the use of prebiotics or FMT to prevent diarrhea. The second objective of this literature review is to summarize the current knowledge regarding the impact of prebiotics, probiotics, and FMT for the treatment and prevention of calf diarrhea.

KEYWORDS: Dairy cattle, dysbiosis, enteropathogens, gastrointestinal microbiome, neonate

1.2 Introduction

Neonatal calf diarrhea is a major cause of mortality and morbidity, accounting for more than 50% of total deaths in calves (1). This results in substantial economic and productivity losses in the cattle industry (2). Calf diarrhea can be caused by infectious agents, such as viruses, bacteria, and protozoa, with the main causative agents being bovine rotavirus group A (BRoV-A), bovine coronavirus (BCoV), *Salmonella* spp., *Escherichia coli (E. coli)*, and *Clostridium perfringens* type C, and *Cryptosporidium parvum (C. parvum).* Enteropathogens that are commonly implicated in calf diarrhea can also be found in healthy calves, thus their presence does not always indicate disease (3). In most cases, diarrhea is multifactorial, and various enteropathogens can be involved simultaneously in the disease.

A key factor in the health and disease of calves is the gastrointestinal microbiota. The gastrointestinal microbiota is a diverse community of microorganisms, including bacteria, that act symbiotically to maintain the gastrointestinal and host health. Specifically, the microbes in the gastrointestinal tract (GIT) supply nutrients to the host and enterocytes, regulate the local and systemic immune system, and assist in the morphological development of intestines (2,4). The key bacterial groups that compose the GIT of healthy calves have been well studied with inconsistent results. What is known it that the disruption, or dysbiosis, of the gut microbiota is associated with gastrointestinal disease and that the re-establishment of healthy microbiota is essential for recovery (2,4). However, it is still unclear whether dysbiosis occurs before or after pathogenic microorganisms invade the GIT. This narrative review aims to summarize the key bacterial populations of the “healthy” gastrointestinal microbiota in calves and

microbial changes related to calf diarrhea and to describe novel treatment methods for calf diarrhea based on manipulating the gastrointestinal microbiota.

1.3 Development of the gastrointestinal microbiota in healthy calves

Before birth, the GIT of calves is generally considered a sterile environment, and microbial colonization begins immediately post-calving (5,6). During birth, calves are exposed to the dam's vaginal, fecal, skin, and mammary gland microbiotas, which begin colonizing the neonate GIT (6). For instance, meconium samples obtained 6 hours after calving contained bacteria normally present in the udder skin, such as *Leuconostoc* and Citrobacter (7). Within the first hours of the calf's life, colostrum is fed to provide nutrients and, more importantly, immunological factors that protect them from pathogenic microorganisms (8). Beyond that, colostrum appears to modulate the colonization of the GIT with commensal bacteria during early life. Specifically, calves receiving pasteurized colostrum have an increased abundance of bacteria associated with GIT health, such as *Bifidobacterium,* however colostrum-deprived calves have a high abundance of *Lactobacillus* and *E. coli* in the feces, which are related to GIT inflammation and disease (9-10). During the first 3 days after calving *E. coli*, *Clostridium,* and *Bifidobacterium* are the most abundant bacteria detected in calf feces (11-13).

During the first 4 weeks of life the gastrointestinal microbiota undergoes rapid changes with increased abundances of *Bifidobacterium, Bacteroides, Faecalibacterium, Butyricimonas, Clostridium, Eubacterium, and Lactobacillus* (2,14-16). These genera are necessary for calves to properly digest milk and will decrease in abundance as they transition to solid feed (14,16). During the first 4 weeks post-calving the prevalence of

Bacteroides and *Lactobacillus*, two genera associated with the digestion of milk, increase (16). However, as weaning begins, these genera gradually decline in abundance (16).

These findings indicate that the colonization of the GIT of healthy calves is associated with a shift from facultative (e.g., *E. coli*) to obligate anaerobic (e.g., *Bacteroides, Faecalibacterium*, *Clostridium, Bifidobacterium*) bacteria. This shift is also observed in human infants and foals (17-22). This shift is associated with increased butyrate-producing bacteria, which aid in energy storage and gastrointestinal health (17,20). In healthy calves, large populations of anaerobic bacteria and low oxygen tension are observed (13-17). Short-chain fatty acids (SCFA), by-products of bacterial metabolism, aid in maintaining low oxygen tension as well as the concentration of nitrate within the intestinal lumen and mucosa (23, 24). The importance of this low oxygen tension within the intestinal lumen is to create a state of oxygen reduction, which results in an environment that is unfavorable to pathogens (25).

1.4 Etiologic agents and pathophysiology of diarrhea

1.4.1 Bacteria

Bacterial pathogens associated with calf diarrhea include *E. coli, Salmonella* spp., *Clostridium perfringens (C. perfringens), and Clostridiodes difficile*. Enteropathogenic strains of *E. coli* are among the most common causes of diarrhea and mortality in newborn calves (26). There are pathogroups that allow *E. coli* to be classified into enterotoxigenic *E.* coli (ETEC), Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E.* coli (EPEC), attaching and effacing *E. coli* (AEEC), and enterohaemorrhagic *E. coli* (EHEC) (27). Enterotoxigenic *E.* coli is the most common

cause of neonatal calf diarrhea, especially in calves younger than 4 days of age (27). The attachment of ETEC to epithelial cells occurs via the presence of fimbrial antigens, with K99 antigen and heat-stable toxin being the most common (28). Heat-stable toxin-mediated ETEC attaches to the villi and crypts of the small intestine and ultimately leads to the upregulation of chloride secretion into the intestine, which promotes water secretion into the intestinal lumen and leads to the development of diarrhea (27,28). Thus, ETEC does not produce either gross or histopathological changes because the toxins do not damage the intestinal epithelium, and therefore the diarrhea is not hemorrhagic. In older calves, EPEC, STEC, AEEC, and EHEC, can cause diarrhea. Unlike ETEC, these pathogroups cause damage to the intestinal epithelium, and diarrhea can be hemorrhagic. However, a direct link between these pathogroups and calf diarrhea is not well established as laboratories rarely type the *E. coli* detected in the feces of diarrheic calves older than 5 days (29).

Salmonella is a Gram-negative, flagellated, and facultative anaerobe bacillus, with cattle being one of the primary animal hosts (30). The predominant *Salmonella* spp. in calves is *S. typhyimurium,* commonly associated with acute diarrheal disease (27). The virulence of *Salmonella* spp. is attributed to its ability to invade the epithelial cells in the GIT and stimulate the release of inflammatory cytokines, subsequently inducing an inflammatory reaction (30). Furthermore, *Salmonella* spp. multiply in the gastrointestinal lymphoid tissues and evade the host's immune system (27). These responses cause ulceration and destruction of the mucosa resulting in the malabsorption of water and electrolytes and the loss of plasma proteins (30).

Clostridium perfringens is a Gram-positive, spore-forming anaerobic bacterium associated with calf diarrhea, but its role as a main pathogen is still under debate (27). *Clostridium perfringens* induces toxin-mediated cell necrosis resulting in pores in the intestine, which causes an influx of solute and water, leading to malabsorptive and hypersecretory diarrhea (31). First, *C. perfringens* is a commensal organism of the GIT of calves, and all types found have the capability of producing toxins, however Toxin A is most commonly detected in samples (29). Therefore, detecting the bacteria from fecal samples of diseased animals or detecting a toxin gene does not prove that the bacterium is causing the disease. Although some ELISA assays allow the detection of Toxin A in intestinal contents, those assays cannot distinguish among the different types of *C. perfringens* (29).

Clostridium difficile (*C. difficile*) is a Gram-positive, spore-forming, anaerobic bacterium (32). *Clostridium difficile* produces several toxins, such as A, B, and binary toxin, which can result in diarrhea (32). These toxins stimulate an increased secretion of electrolytes and water in the lumen of the intestine and severe ulceration of the intestinal mucosa (33). However, it does not appear to play a pathogenic role in calves, as *C. difficile* has been detected in fecal samples from healthy calves with a prevalence ranging from 2 to 60% depending on the source (29). Furthermore, *C. difficile* has not been found to experimentally induce diarrhea in calves, and no reports linking this bacterium to specific diseases are available (34).

1.4.2 Viruses

The two main viruses implicated in calf diarrhea are bovine coronavirus (BCoV) and bovine rotavirus (BRoV). Bovine coronavirus is an etiological agent involved in the

pathogenesis of neonatal diarrhea, winter dysentery in adult cattle, and respiratory diseases in cows and calves (27). It infects both small and large intestines, leading to the destruction of villi and severe diarrhea (35). Specifically, BCoV infected intestinal epithelial cells die, slough off, and are replaced with immature cells which causes the intestines to lose their capability of absorption and digestive enzyme secretion, causing hyperosmotic and malabsorptive diarrhea (35). The spike proteins found on BCoV allow it to enter the host cells and are crucial to its pathogenesis (27).

Group A BRoV is a major cause of gastrointestinal infections in cattle (27). Bovine rotavirus infects the mature, non-dividing enterocytes of the villi in the small intestine, causing damage and decreasing the small intestines' absorptive capacity (28,36). In addition, BRoV produces enterotoxin NSP4, which interferes with cellular homeostasis by elevating calcium ion influx from the movement of water in the intestine (28). The release of NSP4 alters the movement of nutrients and water across the intestinal epithelium, resulting in hypersecretory diarrhea (27,28).

1.4.3 Parasites

Cryptosporidium spp. is endemic in cattle worldwide, and is one of the most important causes of neonatal calf diarrhea (37). In cattle, the four species of *Cryptosporidium* are *C. parvum, C. bovis, C. ryanae,* and *C. andersoni* (37). There is a relationship between the age of the calves and the species of *Cryptosporidium* causing diseases (37), where *C. parvum* is associated with clinical disease in neonatal calves (< 20 days of age) while *C. andersoni*, *C. bovis,* and *C. ryanae* infect the small intestine of weaned calves (37). *C. parvum* is the most common pathogen that infects neonatal calves worldwide, specifically in the second week of life (37).

Upon ingestion, *C. parvum* oocysts release sporozoites that invade the intestinal epithelium of neonatal calves (37). A specialized feeder organelle allows *C. parvum* to obtain energy and nutrients from the calf without eliciting an immune response, which then allows for sexual and asexual reproduction, finally leading to infection and parasite shedding in the feces (37). *C. parvum* often results in diarrhea because of damage to the intestinal epithelium, which decreases the absorptive capacity of the villi and increases intestinal permeability (38). Specifically, *C. parvum* attaches to the epithelial cell villi, leading to villous atrophy and decreased total absorptive surface area. Ultimately, this will cause malabsorption of fluid, resulting in diarrhea (28). Prostaglandin-mediated anion secretion leading to hypersecretion of water into the lumen of the intestine is also proposed as a mechanism of diarrhea in calves with *C. parvum* infection (28). Depending on the degree of epithelial damage and anion secretion, the severity of the clinical signs can vary from mild diarrhea to severe hemorrhagic diarrhea (28).

1.5 Changes in gut microbiota during calf diarrhea

Regardless of the causative agent responsible for the onset of calf diarrhea, various studies report significant changes in the bacterial communities of the gut microbiota during diarrhea compared to what is considered a “healthy” or “balanced” microbiota (4). During diarrhea, there is a shift from obligate anaerobes to facultative anaerobes in the GIT, resulting in dysbiosis (39). The abundance of *Faecalibacterium prausnitzii, Lachnospiraceae and Ruminococcacea* bacteria associated with gastrointestinal health decreases significantly during calf diarrhea (16). Concurrently, an increase in *Lactobacillus*, *Streptococcus* and Enterobacteriaceae, especially *E. coli*, is

observed (25). The reason for this shift is still not completely understood, but several factors can contribute to these changes. Oxygen tension in the GIT is proposed as a factor associated with the shift from anaerobic to facultative anaerobes (24,25,40). In healthy calves, low oxygen tension creates a favorable environment for obligate anaerobes. However, during gastrointestinal inflammation, an increase of oxygen and reactive oxygen species in the GIT could offer an ecological advantage to the facultative anaerobes (24). In calf diarrhea, an increase in oxygen tension can be linked to the presence of blood and hemoglobin in the mucosa and lumen of the GIT due to inflammation (28,39). Diarrhea caused by pathogens other than ETEC usually results in different degrees of watery intestinal contents, edema, hyperemia, ulceration, or hemorrhage of the intestine (28,39). Thus, the inflammation of the intestine could favor the increase of hemoglobin carrying oxygen to the mucosa and lumen of the GIT, leading to increased oxygen tension (25). The increase in oxygen tension can also be explained by the production of oxygen species by neutrophils associated with respiratory burst (the release of oxygen species) during intestinal inflammation (28,41). Therefore, additional studies are needed to investigate whether changes in oxygen tension within the intestinal mucosa and lumen are indeed associated with the microbial changes observed during calf diarrhea (39).

Alteration in the luminal pH of the intestines can also result in changes in the bacterial communities of the intestinal tract in diarrheic calves as pH affects bacterial growth and metabolism (39). Specifically, during acute diarrhea, the calf's fecal pH decreases as D- and L-lactate increase (39,43). The increase in D- and L- lactate results from increased lactate-producing bacteria, such as *Lactobacillus* (39). This is a

cyclical process, and as luminal pH continues to drop it generates favourable conditions for the continued growth of *Lactobacillus* species and other acid stable bacteria. The continued growth of *Lactobacillus* results in further increased levels of lactate in the GIT that can potentially damage the intestine and reduce its ability to transport electrolytes in the intestines (43), contributing to hyperosmotic and malabsorptive diarrhea.

An increase in the abundance of bacteria from the Enterobacteriaceae family is consistently reported in diarrheic calves (44,45). Dysbiosis associated with inflammation results in the alteration of the metabolites available to and originating from bacteria in the GIT of calves, resulting in an environment that favors the growth of Enterobacteriaceae (2,4). For instance, *Salmonella* spp. and *E. coli* benefit from the production of ethanolamine, lactate, glucarate/galactarate, 1,2, propanediol, succinate and L-serine during dysbiosis (46-49). Metabolomics studies also noted that the amino acid composition in the lumen of the GIT is altered during inflammation. In calves, the concentration of fecal amino acids (e.g., serine, alanine, valine, isoleucine and glycine, leucine and valine) and the genes associated with the metabolism of various vitamins and carbohydrates decrease during diarrhea (4). This change in amino acid availability in the lumen of the GIT could favor some facultative anaerobes to proliferate.

In summary, the main microbial alteration occurring in diarrheic calves is the shift from obligated anaerobes to facultative anaerobes. The exact cause for this shift is still to be determined. However, changes in oxygen tension in the lumen of the intestines and nutrient availability appear to play a key role in facilitating some taxa to proliferate more than others. Longitudinal studies investigating the changes in fecal microbiota before diarrhea are warranted to understand which microbial changes predispose

calves to diarrhea. Similarly, studies investigating the fecal microbiota of calves during the recovery of disease can contribute to identifying the microorganisms that play an important role in the resolution of gastrointestinal inflammation. Studies investigating microbial and metabolic changes in diarrheic calves could advance the understanding of the mechanisms responsible for dysbiosis in calves.

1.6 Microbiota changes in calves infected with specific etiologic agents

Few studies have addressed the question of microbial alterations in calves infected with specific pathogens, and most of the available information originates from outbreaks rather than well-controlled experimental studies.

1.6.1 Bovine rotavirus

The effects of BRoV on the gastrointestinal microbiota were previously studied in a small number of diarrheic calves (50). Infection with BRoV reduces the richness and evenness of the fecal microbiota and alters the bacterial membership (taxa present in each community) and structure (the abundance of each taxon in a community) of the fecal microbiota (50). Calves with rotaviral diarrhea had a lower relative abundance of Firmicutes and Bacteroidetes and a high abundance of Proteobacteria compared to their healthy counterparts. At the genus level, the genera *Escherichia, Clostridium,* and *Streptococcus* increased in BRoV-infected calves, while *Blautia, Bacteroides, Lactobacillus,* and *Coprococcus* decreased (50). One limitation of this study was the small sample size of five calves in both the healthy and rotavirus groups (50). More importantly, the study lacked the inclusion of a group of calves with naturally occurring diarrhea associated to other pathogens to determine if the specific changes in the

bacterial communities were caused by BRoV or to another pathogen associated with the development of diarrhea. Furthermore, it is still unknown whether the changes in the microbiota observed in calves with BRoV-induced diarrhea are the cause or consequence of the BRoV infection. Thus, longitudinal studies assessing the gastrointestinal microbiota before, during and after recovery of diarrhea are warranted.

1.6.2 Enterotoxigenic *Escherichia coli*

Studies evaluating the effect of ETEC on the microbiota of calves are lacking. In pigs, ETEC-induced diarrhea is associated with a decrease in the Bacteroidetes:Firmicutes ratio, which aligns with the changes observed in other animal species such as dogs (51). ETEC-induced diarrhea in piglets decreases the microbial diversity in the jejunum and feces and lowers the abundance of *Prevotella* compared to healthy counterparts (51). ETEC in piglets is also associated with an increased abundance of *Lactococcus* in the jejunum and *Escherichia-Shigella* in the feces. Of interest, oral inoculation of piglets with ETEC failed to induce diarrhea in six piglets, suggesting that specific conditions including pre-existing microbiota may aid in facilitation or prevention ETEC infection in piglets (51).

1.6.3 *Cryptosporidium parvum*

C. parvum is one of the main agents causing diarrhea in calves, however little is known regarding the effect of this protozoal organism on the gastrointestinal microbiota (52). Infection with *C. parvum* in calves results in reduction of the microbial diversity and this reduction is proportional to the amount of oocytes detected in feces (53). Furthermore, an increase in fecal abundance of *Fusobacterium* is reported in diarrheic calves infected with *C. parvum* compared to uninfected calves or calves with BRoV

diarrhea (52,53). A high abundance of *C. parvum* and *Fusobacterium* is associated with the severity of diarrhea in calves with both microorganisms (53). These results suggest that proliferation of *Fusobacterium* could play an important role in the pathophysiology of diarrhea due to *C. parvum* in calves. However, experimental studies are required to determine the cause and effect of this association and its effect on calf health.

1.7 Treatment approaches to restore the gastrointestinal microbiota of diarrheic calves

Currently, there are many proposed methods to restore healthy microbiota in different species, including the use of prebiotics, probiotics, and synbiotics, colostrum supplementation, and fecal microbiota transplantation (FMT). However, studies assessing the potential benefit of some of those approaches (i.e., colostrum supplementation, FMT) are limited in food-producing animals.

1.7.1 Prebiotics, probiotics, and synbiotics

Administration of prebiotics, probiotics, and synbiotics has been proposed as a method to treat dysbiosis by restoring microbial diversity and altering the disturbed intestinal microbiota in many digestive disorders, such as diarrhea, irritable bowel syndrome, inflammatory bowel disease, and ulcerative colitis in both calves and human neonates (26,54). Prebiotics contain non-digestible nutrients that promote the growth of beneficial microbiota and aid in protecting the gut from potentially harmful pathogens (55). Probiotics are groups of live, beneficial microorganisms that, when administered to patients sufficiently, will lead to overall health benefits for the host (55). However, dead bacteria and their components can also exhibit probiotic properties (55). Synbiotics

contain a mixture of both pre- and probiotics that are deemed to be advantageous to the host, to promote the growth and metabolism of beneficial bacteria (56).

Many prebiotics contain oligosaccharides, most commonly mannan and fructo-oligosaccharides, as they could deter harmful pathogens from colonizing as well as aid in reducing the severity of disease and diarrhea (60). The most used prebiotics are oligosaccharides, which encompass various prebiotics, including β-glucans (63). These oligosaccharides contain sugars that prevent Enterobacteriaceae*, E. coli,* and *Salmonella* from adhering and colonizing in the intestinal epithelium (60). However, the effect of prebiotics on the gastrointestinal microbiota and their associated mechanisms are poorly understood (55).

Probiotics are thought to competitively exclude pathogens, modulate enzymatic activities related to the metabolism of toxic substances, and aid in the production of fatty acids, which helps maintain energy homeostasis in peripheral tissues (57,58). The mechanism by which probiotics improve gut health is still not exactly known, but it is believed that probiotics produce inhibitory substances, organic acids, hydrogen peroxides, and biofilms that reduce pathogen proliferation and promote healing of the intestinal lining (26,55,57). Most importantly, in neonates, probiotics are associated with enhanced digestive and immune system development by increasing microbial diversity and species richness (26,59,60).

Studies have investigated the effect of probiotics as preventative measures for calf diarrhea. A meta-analysis assessing the effect of probiotics on the incidence of diarrhea and intestinal microbial balance showed that the administration of probiotics

containing lactic acid-producing bacteria reduced the incidence of diarrhea in calves fed whole milk (61). However, this effect was only observed when a multistrain lactic acid bacteria probiotic was administered (61).

For diarrhea treatment, a single randomized clinical trial evaluated a multispecies probiotic containing *Pediococcus acidilactici*, *Enterococcus faecium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum* as a supportive treatment for calf diarrhea (62). A total of 148 calves were enrolled in the trial, and all calves received the probiotic at the onset of diarrhea and for three additional consecutive days. The mean time to diarrhea resolution was 5.1 and 5.9 days in the probiotic and control groups, respectively (62). Although this study showed that a multispecies probiotic administered to diarrheic calves reduced the duration of diarrhea, the clinical relevance of this reduction is likely irrelevant, as no statistically significant difference were found (62,63).

One of the most common probiotics to administer to calves is live yeast, specifically *Sacchromyces cerevisiae.* In calves, the administration of probiotics containing yeast increases grain consumption. This may be due to its ability to facilitate fiber digestion, which aids in gut development (64,65). Yeast of *Saccharomyces cerevisiae* (SCY) origin is used in different food-producing animals to improve performance and health conditions (65). *In vitro* studies demonstrated the ability of yeast metabolites to inhibit pathogenic organisms and promote the growth of commensal microbiota essential for producing volatile fatty acids (66). In calves, SCY increases bacterial diversity and the abundance of bacteria from the family *Ruminococcaceae,* a butyrate-producing microorganism (67). These findings highlight

the potential benefit of SCY in modifying the gastrointestinal microbiota and the potential to accelerate the recovery from diarrhea. However, the clinical and economic benefits of administering SCY to diarrheic calves is yet to be examined.

1.7.2 Colostrum feeding

Appropriate administration of good quality and sufficient quantity of colostrum to neonatal calves decreases the incidence of diarrhea in the first weeks of the calf's life (68). Bovine colostrum, which is the milk produced by the dam immediately after parturition, has been found to contain many beneficial compounds such as nutrients, hormones, growth factors, and, most importantly, antibodies and immune factors essential in the first hours of life (69). Administration of natural bovine colostrum after the first day of life has been shown to have beneficial effects on calves. For example, calves receiving natural colostrum for up to 14 days postpartum have less diarrhea and fewer antimicrobial treatments than control calves (70).

The benefits of spray-dried maternal-derived bovine colostrum replacer at the onset of diarrhea on calf growth and duration and severity of the disease in preweaned dairy calves has only been investigated in one study. This study showed that the administration of colostrum to diarrheic calves accelerates the recovery of diarrhea by 0.75 days and increased their average daily gain by 100g/ day compared to the control group (70). Of interest, the administration of colostrum to diarrheic calves failed to alter the bacterial communities of the GIT (69). However, in another study, daily supplementation with a high-quality bovine colostrum product ameliorated the clinical signs of calves experimentally infected with *C. parvum* and modulated the gastrointestinal immune response and microbiota to a pattern more similar to that of

healthy unchallenged calves (71). Based on these results, supplementation of bovine colostrum appears to have beneficial effects both economically as well as physiologically for neonate calves. However, additional studies are required to further determine the effect of colostrum on the gastrointestinal immune system and gastrointestinal microbiota of calves.

1.7.3 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is a procedure where fecal matter from a healthy donor is administered into the intestinal tract of the recipient to directly change the recipient's gastrointestinal microbiota (72). The goal is to restore the "healthy" or "balanced" microbial communities within the recipient's GIT to alleviate and facilitate recovery from disease (38). The process of FMT was first described in human medicine in fourth-century China to treat diarrhea and gastrointestinal diseases (72). Fecal transplantation is used to treat *C. difficile* infections in humans, with a success rate of 90% (73,74). While the success of FMT in human medicine is mainly reported in cases of *C. difficile*, FMT has shown promising benefits for human patients suffering from irritable bowel disease, ulcerative colitis, chronic fatigue syndrome, and metabolic and cardiovascular disorders (74). In dogs, FMT, in addition to standard treatment, accelerates the resolution of diarrhea in puppies suffering from parvoviral diarrhea compared with those receiving only standard treatment (38). Additionally, FMT also offers protection against diarrhea in young pigs and prevents necrotizing enterocolitis in premature pigs (75,76).

In calves, little is known about the potential role of FMT for the prevention and treatment of neonatal diarrhea. Fecal transplantation from healthy donor calves to

recipient diarrheic calves, regardless of the cause of diarrhea, decreases the water content in the feces (77). Of interest, the fecal microbiota of calves receiving the FMT resembles that of the healthy donor following treatment, suggesting that transplanted bacterial communities are able to colonize the GIT and therefore facilitate recovery from diarrhea (77). Recovery of diarrhea after FMT is associated with an increased abundance of *Porphyromonadaceae and Prevotellaceae, an increased concentration of SCFA and a decreased fecal concentration of amino acids (*alanine, leucine, valine, isoleucine, glycine, arginine, ornithine, and glutamic acid) (77,78)*. These findings suggest that bacterial metabolism of amino acids to synthesize proteins and other metabolites may be particularly important for the recovery from diarrhea in calves.* However, additional randomized clinical trials involving a larger number of calves from different farms are needed to establish the beneficial effect of FMT on calf diarrhea. Similarly, there is still need for further research to identify beneficial microorganisms in the feces that promote health and the criteria it takes to be considered a recipient or a donor calf (78).

1.8 Conclusions

Although many etiologic agents are involved, and several pathophysiological mechanisms of diarrhea have been identified, the effect of the gastrointestinal microbiota in health and disease of neonatal calves remains to be validated in multiple studies. While there are many etiological agents and causes of diarrhea, the overall impact on the microbiota and the role of the GI microbial community on disease is unclear. Several changes in bacterial composition occur during diarrhea, where there is a shift from strict anaerobes to facultative anaerobes and an increase in lactate-

producing bacteria. An increase in lactic acid producing bacteria, appeared to reduced pH, and increase in oxygen tension. Studies investigating these effects have been limited by number of those enrolled in the study, single time point sampling, and lack of etiology as to the cause of diarrhea.

Overall, determining whether the changes identified in calves with diarrhea are the cause or the consequence of diarrhea, or whether those changes are pathogen-specific is not possible without proper diagnostic testing. Furthermore, treatments for calf diarrhea which focuses on modulating the microbiota holds many benefits and needs to be further explored. Longitudinal studies evaluating the gastrointestinal microbiota before, during and at recovery of diarrhea are warranted. Similarly, studies characterizing the gastrointestinal microbiota in calves with diarrhea due to specific pathogens are needed.

1.9 Objectives and hypothesis

1.9.1 Objectives

1. Investigate the fecal microbiota of calves before, during, and after recovering from neonatal calf diarrhea of unknown origin to identify taxa related to the development and recovery from disease.

1.9.2 Hypothesis

1. Healthy calves and calves experiencing diarrhea will have dissimilar microbial communities.
2. Calves that recover from diarrhea by the end of the study will have the same microbiota present as the calves that remained healthy.

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2 Chapter 2: Dynamics of fecal microbiota in diarrheic calves: Before, during, and after recovering from disease

Emma Jessop[1], David L. Renaud[2], Aoife McMahon[1], Lynna Li[1], Jenniffer Macnicol[3], Dasiel O. Alvarez[4], Lisa Gamsjäger[5], Adronie Verbrugghe[1], Diego E. Gomez[1]

[1]Department of Clinical Studies, University of Guelph, Ontario Veterinary College, 50 Stone Road East, Guelph, ON N1G 2W1, CA

[2]Department of Population Medicine, University of Guelph, Ontario Veterinary College, 50 Stone Road East, Guelph, ON N1G 2W1, CA

[3]Department of Pathobiology, University of Guelph, Ontario Veterinary College, 50 Stone Road East, Guelph, ON N1G 2W1, CA

[4]School of Environmental Sciences, University of Guelph, 50 Stone Road East, Guelph, ON N1G 2W1, CA

[5]Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA

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Sensu Stricto, Lactobacillus

Corresponding author: dgomezni@uoguelph.ca

2.1 Abstract

Background: It is unknown if the changes in the bacterial communities observed in the feces of diarrheic calves are associated with the development of disease or if they are a consequence of gastrointestinal inflammation. It is also unknown whether the recovery of diarrhea coincides with the return of bacterial communities to similar stages of age-matched healthy calves.

Objectives: Describe the fecal microbiota of calves before, during, and after recovering from diarrhea to identify changes associated with the development and recovery of disease.

Animals: 15 female Holstein calves, followed from 0 to 21 days old, with sampling beginning at 3 days of age, divided into calves that remained healthy throughout the study (n = 7) and calves that developed diarrhea at 14 days of age (n = 8).

Methods: Longitudinal cohort study. Microbiota composition was characterized by amplifying the V4 region of the 16s rRNA gene. Alpha and beta diversity measurements and the relative abundance of the different taxa at different levels were compared between groups.

Results: Diversity (Shannon entropy) tended to increase with age in all calves examined from day 3 to 21 of age. Calves that experienced diarrhea had a lower diversity on the day that diarrhea was first observed (day 14), compared to healthy age-matched calves. By day 21, the diversity increased in calves that recovered from diarrhea and were not significantly different from their healthy counterparts ($P > 0.05$). Weighted UniFrac distance showed significant differences of the fecal microbiota between diarrheic and

healthy calves at day 14 of age, when diarrhea occurred (PERMANOVA, $P < 0.05$), but not before (days 3, 5 and 10 of age) or after diarrhea (21 days of age) (PERMANOVA, $P > 0.05$). Random forest analyses found that *Phascolarctobacterium, Ruminoccocus, Roseburia, Odoribacter* and *Prevotella* were differentially abundant in calves that remained healthy during the study period, while *Lactobacillus*, *Clostridium Sensu Stricto 1* and *Collinsella* were differentially abundant in calves that developed diarrhea only on day 14 ($P < 0.05$).

Conclusion and clinical importance: The dysbiosis observed in the diarrheic calves could be linked to gastrointestinal disturbance rather than being a predisposing factor for diarrhea. However, the enrichment of *Lactobacillus*, *Clostridium Sensu Stricto 1* and *Collinsella* before diarrhea onset could have contributed to the development of diarrhea.

2.2 Introduction

Neonatal calf diarrhea accounts for more than 50% of calf morbidity and mortality worldwide and greatly affects the economy and productivity of the dairy sector (1,2). Neonatal calf diarrhea is a multifactorial disease and infectious and non-infectious factors are associated with disease development (3,4). The most frequently reported pathogens identified in diarrheic calves during the first 21 days of the calf's life are bovine rotavirus (BRoV), bovine coronavirus (BCoV), enterotoxigenic *Escherichia coli (E. coli)*, and *Cryptosporidium parvum* (1,3).

The calves' gastrointestinal tract (GIT) is considered sterile before birth and the process of microbial colonization begins once it is exposed to the cow's vaginal microbiota (5). During the first weeks of life, the calf is exposed to different factors that facilitate the colonization of the GIT bacteria, including consumption of colostrum, milk or milk replacer, environmental bacteria, antimicrobial drugs, and pre- and pro-biotics (1,6-8). Bacterial colonization of the calves' GIT follows the classic pattern of neonates in several species, which includes early colonization by facultative anaerobes (e.g., *Lactobacillus*, *E. coli* and Enterobacteriaceae) that exhaust oxygen concentrations, facilitating the colonization by strictly anaerobic bacteria (*Clostridium* spp, *Bifidobacterium* spp, *Eubacterium* spp, and *Bacteroides* spp) (14-18). This colonization process results in an anaerobic milieu in the GIT (24). The change in those bacterial communities is associated with increased richness and diversity of the GIT microbiota during the first weeks after calving (1-4). Of interest, a high diversity of the calf GIT microbiota and enrichment of specific taxa (e.g., Lachnospiraceae, Ruminoccocaceae, Faecalibacterium and Prevotellaceae) are consistent with GIT health, while low diversity

is consistently identified in calves with diarrhea (25-28). However, most of the studies investigating changes in the fecal microbiota of diarrheic calves are limited by sampling calves on the day of diarrhea and comparing them with age-matched healthy calves (29,30).

Although some studies have assessed the longitudinal development of the fecal microbiota in healthy and diarrheic calves, the time frame between sampling is long and prevents capturing changes occurring shortly before diarrhea onset (2,28,31). These sampling approaches also prevent researchers from determining whether the changes in alpha and beta diversity are associated with the development of diarrhea or whether they are consequences of the GIT inflammation. Furthermore, information regarding how the GIT microbiota recovers after diarrhea is also lacking (2,28,31). Therefore, the objective of this study is to describe the fecal microbiota of calves before, during, and after recovering from diarrhea to identify changes related to the development and recovery of disease. We hypothesize that the fecal microbiota of healthy and diarrheic calves differs significantly at onset of diarrhea, but not before diarrhea. We also hypothesize that the resolution of diarrhea coincides with the return of the microbiota to a balanced stage similar to the one observed in healthy age-matched calves.

2.3 Material and methods

2.3.1 Ethics statement

The animals used to obtain fecal samples for this study were approved by the University of Guelph Animal Care Committee (Animal Use Protocol #4565).

2.3.2 Study design

Prospective longitudinal cohort study.

2.3.3 Calves, housing, and feeding

Dairy heifer calves from a commercial dairy farm in Guelph, Ontario, Canada, were enrolled in this study from 3 to 21 days of life. This dairy farm was selected based on its proximity to the University of Guelph facilities and their willingness to participate in this study. The dairy farm milks Holstein-Friesen cows, with approximately 700 calving's a year. Each dam was vaccinated against *Clostridium perfringens* types C & D, and *E. coli* type K99. BCoV and BRoV at 8 weeks and 4 weeks pre-calving.

Upon birth, the calves were separated from their dams and received 1 oral bolus containing antibodies against enterotoxigenic *E. coli* and BCoV. Calves were fed 4L of colostrum within the first 4 hrs after birth. Calves were then housed in group pens (20 calves per pen) containing sawdust bedding. For the following 8 to 12 weeks, the calves were fed pasteurized milk at 14% of their body weight via an automated milk feeder. Texturized calf starter, containing 20% crude protein and coccidiostat (decoquinate) was also offered to all calves from day 5 on.

2.3.4 Data collection and outcomes

All treatments administered to the calves were recorded. Any calf that received antimicrobial drugs at any time point was excluded from the study. Calves were also excluded from the study if they had any disease, including pneumonia or an umbilical abscess. As antimicrobials can alter the GIT, and medical diagnoses often require immediate intervention.

Fecal consistency was evaluated on the sampling day by one of the researchers (DG) on the day of sampling. Each calf was given a score from 0 to 3, where a fecal score of 0 = normal consistency; 1 = semi-formed or pasty; 2 = loose feces; 3 = watery feces. A calf was defined as diarrheic if it had a fecal score of 2 or 3 at two consecutive timepoints. Fecal samples collected from the rectum were collected via swabbing on days 3, 5, 10, 14, and 21 after calving. Once the fecal swabs were collected, they were placed on ice and transported to the University of Guelph within 2 hours, where samples were stored at -20°C until analysis.

2.3.5 Sample processing

Once all the fecal sample swabs were thawed, they were prepared for DNA extraction using the Qiagen PowerSoil Pro Kit (Qiagen, NRW, GER) and QIAshredder columns (QIAGEN) following manufacturer instructions with the following modifications. Swab tips were cut and added into initial power bead tubes with buffer. Tubes with swab tips were incubated at 70°C for a total of 10 min and briefly vortexed at 5 min. Tubes with swab tips were then attached to a horizontal vortex and vortexed for 10 min on high-speed setting. Swab tips were then transferred to shredder columns. Both shredder columns and power bead tubes were centrifuged for 1 min. Supernatant from power bead tubes was then combined with flow through from shredder columns and the Qiagen PowerSoil protocol was continued. DNA was eluted into 30µl. The V4 region of the 16s rRNA gene was amplified using the modified primers 515-F and 806-R, as described by Li et al (32). Purification of the PCR products was done using Mag Bind RXNPure Plus Beads (Omega Bio-TEK, GA, USA) (33). Illumina indexing primers were added to the 16S PCR products and the purification step was repeated. The final DNA

concentration was confirmed on a NanoDrop spectrophotometer and samples were sent for sequencing on an Illumina MiSeq at the Agri-Food Labs (University of Guelph).

2.3.6 Bioinformatic and statistical analysis

All sequence processing and statistical analyses were executed within the QIIME2 (Quantitative Insights Into Microbial Ecology 2, v. 2021.8) framework (34). Demultiplexed sequences underwent denoising, read-merging, and chimera filtering through the 'q2-dada2' implementation of DADA2 (35). The resulting amplicon sequence variants, ASVs, were aligned using the MAFFT method with 'q2-alignment' tool (36). The alignments were then used to construct the phylogeny using the FastTree2 method (37), via 'q2-phylogeny'. The taxonomic classification of ASVs was done using the Classify-Sklearn Naive Bayes method via the q2-feature-classifier tool (38). A pre-trained classifier (99%) established on 16S rRNA full-length sequences from the SILVA database v.138 was then used (39).

Several phylogenetic and non-phylogenetic metrics were calculated from rarefied feature tables at the ASVs level. However, Shannon entropy and weighted UniFrac distance were selected to explore alpha and beta diversity, respectively (40,41). Comparisons between groups of alpha diversity metrics were performed using the Kruskal–Wallis test. For beta diversity, multiple comparisons were done using the PERMANOVA test. To account for the variability of each sample over time, Shannon entropy and weighted UniFrac were also analyzed using longitudinal approaches, or repeated measures. These analyses were carried out via the algorithms included in the 'q2-longitudinal' tool (38). Between-group comparisons were performed for: *i*) first differences (i.e., differences in Shannon entropy from the same sample between two time

points), and *ii*) first distances (i.e., differences in weighted UniFrac distance between an individual's microbial composition at two separate sample time points).

To discern the combined and synergistic effects of temporal dynamic and diarrheal occurrences on the microbial diversity in calf microbiota, we conducted a mixed-effects model analysis of the Shannon index and weighted UniFrac distances. The analyses utilized the Linear Mixed Effects (LME) modeling approach implemented through the 'q2-longitudinal' tool (38). The model incorporated two fixed effects (Days and Diarrhea) and one random effect to capture individual variability among animals (b(animal)). The LME model is formalized as follows: Shannon = β0 + β1*Days* + *β2*Diarrhea + b(animal) + ε, where: β0 is the intercept, β1 and β2 are the coefficients associated with the fixed effects, b(animal) represents the random effect for individual animals, and ε represents the error term.

The ANCOM-BC (Analysis of Compositions of Microbiomes with Bias Correction) method (42) was used to identify taxa with significantly different abundances between healthy and diarrheic calves, while controlling for multiple testing. This was done in a mixed-effects model that accounted for the fixed effects of days and diarrhea, and their interaction. The analyses were conducted within the QIIME 2 using the 'composition' tool. The model was represented by the equation: Y = β0 + β1Days + β2Diarrhea + β3Days*Diarrhea + b(animal) + ε, where Y represents the taxonomic profile data of bacterial communities, β0 is the intercept, β1 and β2 are the coefficients associated with the fixed effects of days and diarrhea, respectively, β3 is the coefficient associated with the interaction effect between days and diarrhea, b(animal) represents the random effect for individual animals, and ε represents the error term. The interaction effect between

days and diarrhea is included to capture any synergistic or antagonistic effects of the two factors on the abundance of different taxa. The random effect for individual animals is included to account for the variability among animals that the fixed effects cannot explain. We report the taxa with a false discovery rate (FDR) adjusted P-value ($P < 0.05$) below the significance threshold (e.g., FDR < 0.05) and a minimum fold-change threshold (e.g., fold-change >1).

Subsequent to the LME analysis, Random Forests (RF) modeling was utilized to ascertain the predominant taxa with differential abundance within the fecal microbiomes of calves. This comparative analysis between healthy and diarrheic calves was particularly focused on samples collected on day 10 and day 14 of age. The rationale for concentrating on these specific time points was derived from previous analytical findings. The RF is an ensemble machine learning approach, and is esteemed for its efficacy in identifying and ranking variables according to their significance in enhancing the predictive capacity of the model (43). This is achieved by assessing the mean decrease in model accuracy upon the permutation of each variable. The analysis was conducted via the MicrobiomeAnalyst platform (44).

2.4 Results

2.4.1 Calves

Fifteen female Holstein-Friesen calves were included in this study. Of the 15 calves, 7 were healthy and 8 were diarrheic. Eight of the calves developed diarrhea on day 14 +/- 1. By day 21, 6 of the 8 diarrheic calves recovered from diarrhea. Therefore, only samples from those 6 diarrheic calves on day 21 were included in the analysis.

2.4.2 Sequence analysis

A total of 14,419,778 raw sequences were available, and approximately 83% were retained (yielding 12,060,805 sequences after denoising and merging paired-end reads). The average number of sequences per sample was 129,686.07 (SD = 20,185), with a median frequency of 128,417 sequences. In total, 2,656 ASVs were detected across the 144 samples. The rarefaction curves indicated that the sequence counts captured a substantial portion of the microbial diversity present in all samples.

2.4.3 Impact of diarrhea on microbial diversity in the gut microbiome of calves

Diversity (Shannon index) showed a general trend of increasing with age in all calves examined from day 3 to 21 (Figure 1). There were no differences between healthy and diarrheic groups before the onset of diarrhea, at days 3 ($P = 0.620$), day 5 ($P = 0.695$) and day 10 ($P = 0.335$). However, calves that experienced diarrhea had a lower diversity on the day of diarrhea (day 14, $P = 0.028$) than their healthy counterparts. By day 21, Diversity increased in calves that recovered from diarrhea, and while there was no statistical significance ($P = 0.393$), it remained lower compared to the healthy age matched calves (Figure 1a). To confirm these trends, we used the random forest classification in the q2-longitudinal pipeline to compare the variation of Shannon diversity for each calf at different ages in relation to day 3 and compared between groups. This analysis confirmed the significant differences ($P = 0.035$) between group means in Shannon variation at day 14 in relation to day 3 (Figure 1b). Similar patterns were observed for beta diversity variation, measured by weighted UniFrac distance, which showed significant differences ($P = 0.022$) between healthy and diarrheic calves on day

14 (Figure 1c). Healthy animals exhibited a consistent microbiota diversification from day 3 to 21, whereas this diversification was reduced in calves that developed diarrhea at day 14 (P = 0.006).

The mixed linear regression (LME) analysis results, aimed at discerning the impacts of age, diarrheal episodes, and their potential interplay on microbial diversity, revealed age as a significant determinant of alpha and beta diversity metrics (Figure 2). Notably, age emerged as the sole factor with a statistically significant influence on the Shannon entropy (P = 0.000) index and weighted UniFrac metrics (P = 0.001). In contrast, neither the occurrence of diarrhea nor the interactive effects of diarrhea and age manifested any significant impact on these diversity metrics (Table 1).

2.4.4 Differences in the taxonomic composition of calf gut microbiota before and during diarrhea

Exploring the impact of diarrhea on the developing gut microbiota, our study documented significant compositional changes over a monitored period of 21 days. These changes suggest an interplay between the natural maturation process of the microbiome and its perturbation due to diarrheal events. Among the 150 genera detected across 144 samples, 50 genera (33%) were differentially abundant between healthy and diarrheic calves throughout the study (Figure 3). Preceding onset of diarrhea an increase in genera such as *Bifidobacterium*, *Eggerthella*, and *Tyzzerella* by day 5. Other genera such as *Enterococcus*, *Escherichia-Shigella*, *Lachnoclostridium*, and *Anaerostipes* were more abundant after day 10, suggesting a possible adaptive or opportunistic shift in the gut microbiota before diarrheal events.

In contrast, genera like *Coprobacter*, *Campylobacter*, and *Alloprevotella* were notably reduced in diarrheic calves by day 10, with others, including *Intestinimonas*, *Faecalicoccus*, and *Pseudoflavonifractor,* becoming affected by day 14. This pattern indicates an initial disruption of the microbiome associated with diarrheic illnesses. Overall, these fluctuations illustrate the microbial dynamics within the gastrointestinal tract and highlight selective pressures exerted by diarrheal stress, offering insights into the gut microbiota's resilience and adaptative response.

Random forest analysis confirmed distinct bacteria associated with health and diarrheal states in calves (Figure 4). On day 10, genera such *Phascolarctobacterium, Ruminoccocus, Roseburia, Odoribacter* and *Prevotella* were differentially abundant in calves that remained healthy during the study period, while *Lactobacillus*, *Clostridium Sensu Stricto 1* and *Collinsella* were indicator taxa on day 10 in calves that developed diarrhea. In contrast, on day 14, bacteria associated with health included *Slackia*, *Pyramidobacter*, *Blautia*, and *Anaerococcus*, while diarrheic calves had an increased abundance in *Butyricimonas*, *Olsenella*, *Acinetobacter*, *Trueperella*, and *Escherichia_Shigella*.

While distinct bacterial genera were associated with health and diarrhea on days 10 and 14, the variability between these time points indicates a lack of consistent indicator taxa for health or disease. This suggests that the relationship between calf fecal microbiota and disease states may be transient and influenced by various dynamic factors. Further research with more extensive temporal sampling is necessary to elucidate stable microbial markers and to understand their role in calf health and disease resilience.

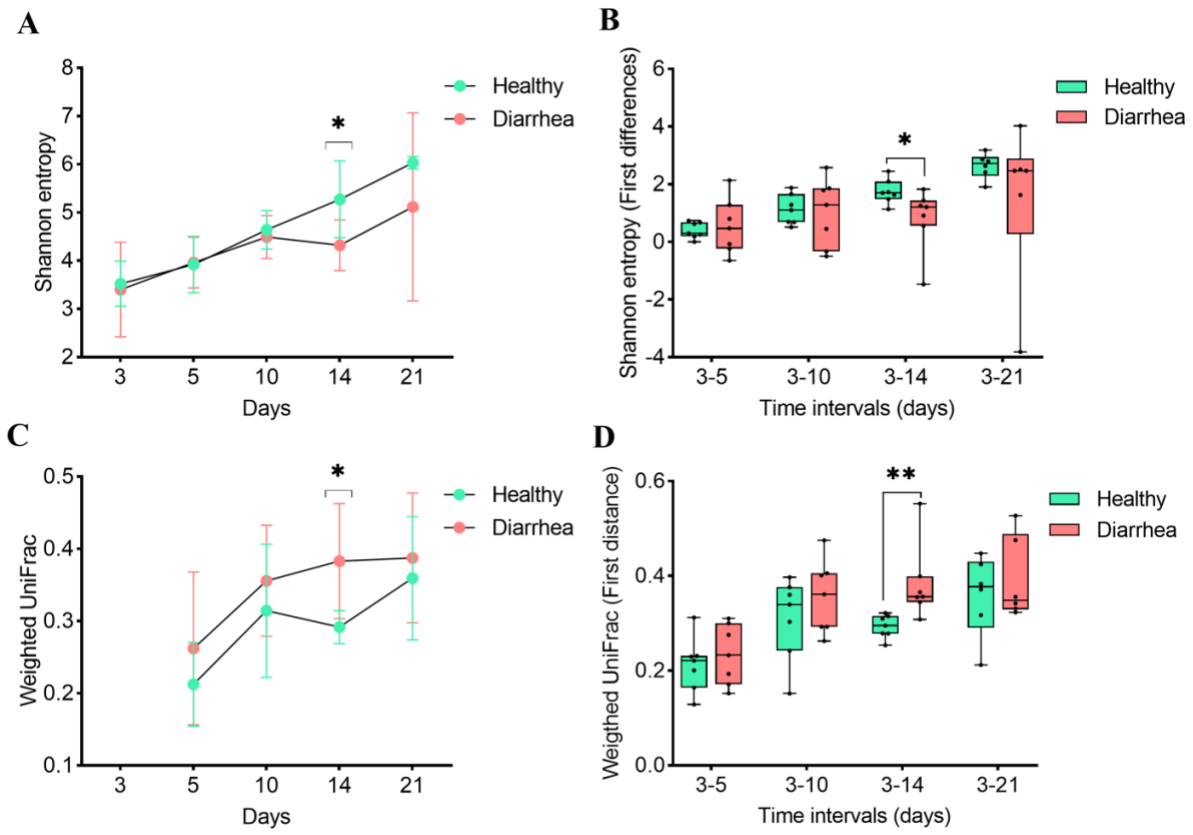

Figure 2.1: Longitudinal analysis of alpha and beta diversity in healthy calves and those with diarrhea. (A) The trend in Shannon diversity is shown for each group (mean and standard deviation), including pairwise comparisons on each sampling day. (B) Pairwise comparisons on the "first differences," representing the variation in the Shannon index for each calf at different sampling days relative to their initial status on day 3. (C) Comparisons of weighted UniFrac distance between groups of calves on different sampling days. (D) Pairwise comparisons on the "UniFrac distance first distance," which indicates the variability in microbiota composition for the same animals between days 3 to 5, 3 to 10 days, and so on. Asterisks indicate significant group differences (*P < 0.05, **P<0.01, ***P<0.001).

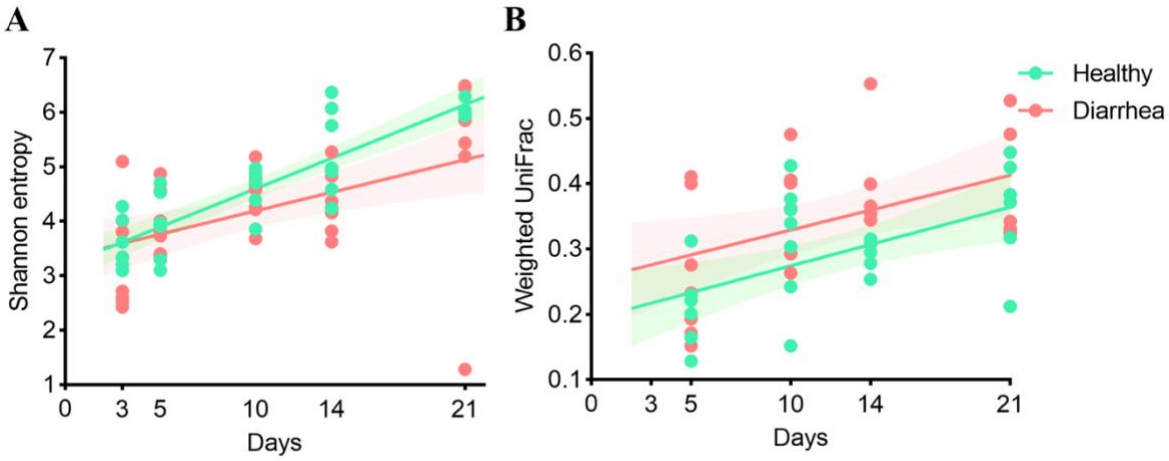

Figure 2.2: Regression scatterplots depicting (A) Shannon entropy and (B) weighted UniFrac distance for the fecal microbiota of healthy and diarrhea-affected calves as a function of time elapsed between 3 to 21 days after birth.

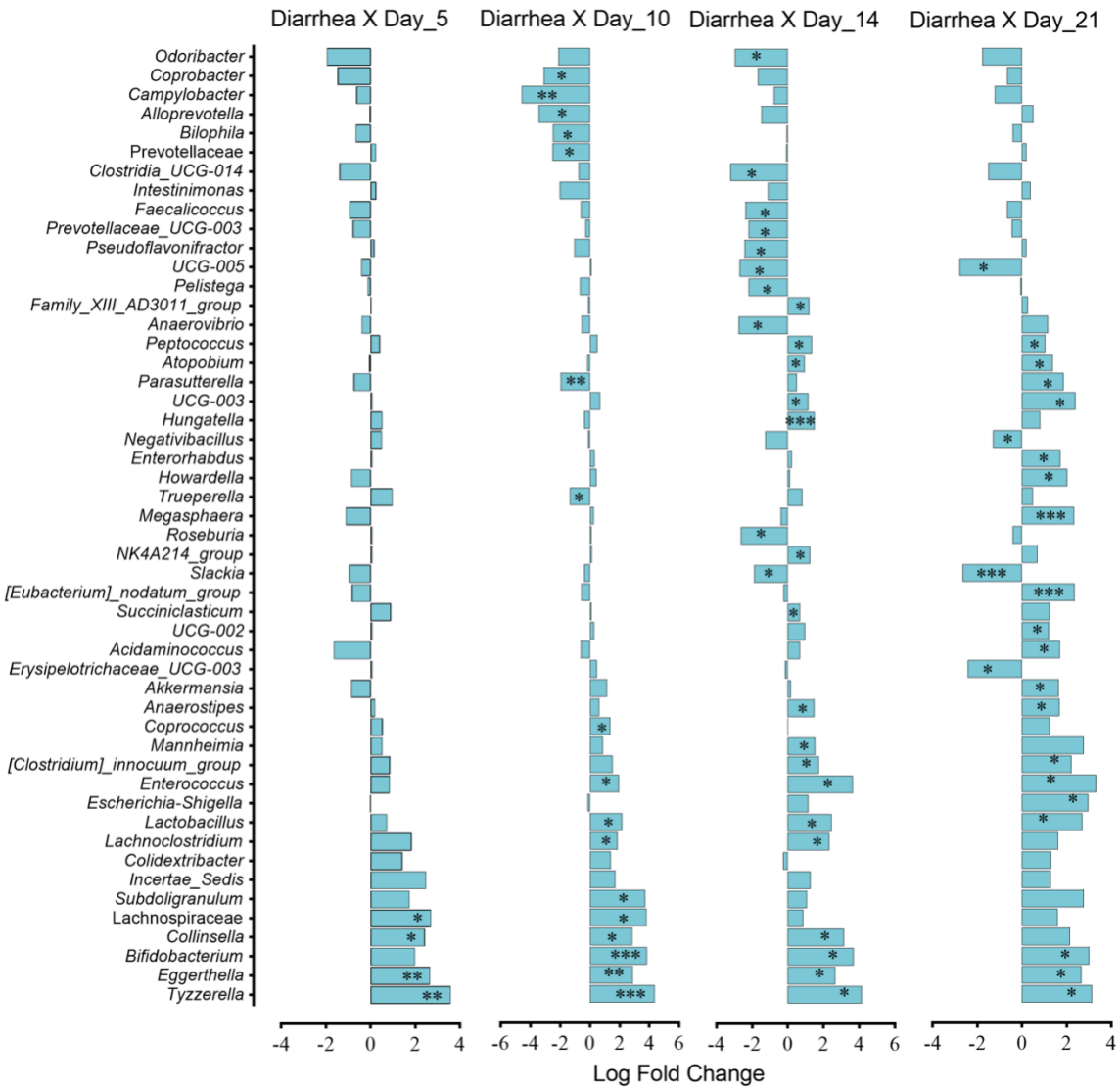

Figure 2.3: Differential abundance of taxa at the genus level detected between healthy calves and those that experienced diarrhea, determined using the mixed-effects model in the ANCOM-BC method. Healthy calves and day 3 status were used as reference values, so differences are indicated according to the results in the diarrhea-affected animals at different sampling days. Only taxa that were statistically significant on at least one of the sampling days are shown. Asterisks denote statistically significant differences (* P<0.05; ** P<0.01; *** P<0.001).

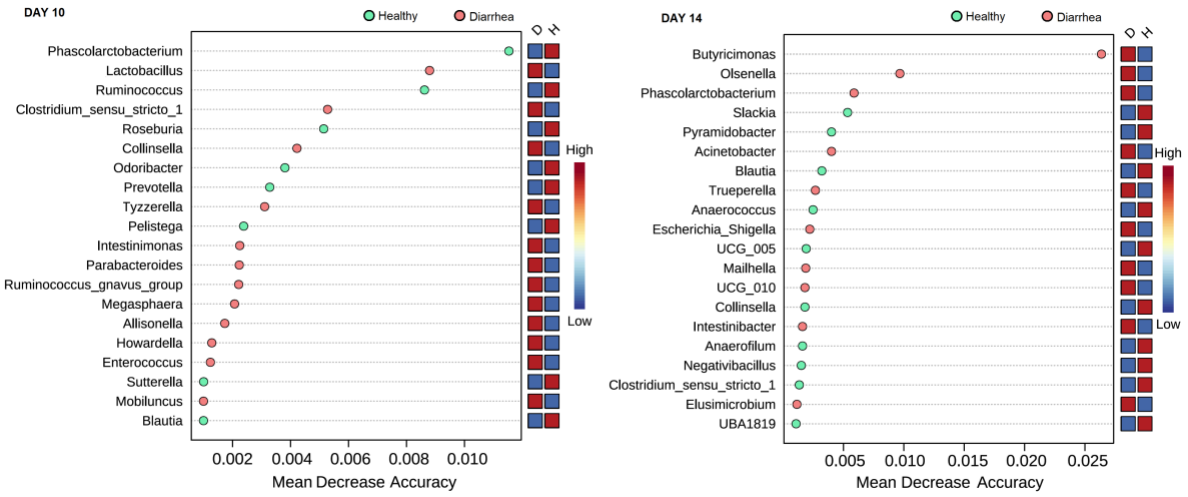

Figure 2.4: Comparative analysis of fecal microbiota in healthy and diarrheic calves using random forest. These plots highlight the top 20 distinctive taxa at days 10 and 14, focusing on their relative importance (measured as mean decrease in accuracy) and abundance. It illustrates the dynamic changes in the microbiota over time in both healthy calves and those with diarrhea on day 14.

Table 0.1: Linear mixed effect model (LME) of the impact of diarrhea in calves during the first three weeks after birth on Shannon entropy and weighted UniFrac diversity measurement from calves' fecal microbiota.

MixedLM 1: Shannon Entropy	**Coef.**	**Std. Err.**	**P-value**
Intercept	2.672	0.377	**0.000**
Diarrhea	-0.535	0.550	0.331
Days	0.391	0.092	**0.000**
Days: Diarrhea	0.244	0.133	0.067
MixedLM 2: Weighted -UniFrac	**Coef.**	**Std. Err.**	**P-value**
Intercept	0.163	0.058	**0.005**
Diarrhea	-0.051	0.081	0.528
Days	0.041	0.012	**0.001**
Days: Diarrhea	-0.001	0.017	0.968

2.5 Discussion

Our study showed that the richness and diversity of the fecal microbiota of healthy calves increases during the first 21 days after calving. Increasing richness and diversity of the fecal microbiota of calves during the first weeks after calving are consistently reported in the literature (2,14,18). A continuous development occurred from day 3 to day 21 in healthy calves in the current study. This development was similar in diarrheic calves, except for an interruption at day 14; the time of diarrhea. The bacterial colonization of the calf's GIT tract begins immediately after calving through the colonization of few facultative anaerobes, followed by strictly anaerobic bacteria once oxygen concentrations are exhausted and changes in the luminal pH occur (42-45). We observed that the bacterial composition of feces of healthy and diarrheic calves evolved similarly during the first 10 days of age but differed significantly on day 14, which agrees with previous studies (5,6,24). In addition, differences in the relative abundance of specific taxa were present on day 10 in both healthy and diarrheic calves, and appeared to predict the development of diarrhea on day 14.

Additionally, the development and colonization of the newborn calf's GIT are influenced by internal and external factors, such as exposure to the dam's vaginal and mammary gland, consumption of colostrum, diet, maturity and functionality of the systemic and local immune system, intestinal pH and oxygen concentration, intestinal motility, bile secretion, bacterial mucosal receptors, antimicrobial drug use, and the use of pre- and pro-biotics (49-51). The process of development and establishment of the microbiota occurs until 6 to 7 weeks of age, when the microbiota becomes stable (28,29). Among all the aforementioned factors, diet likely plays a major role in this dynamic change

in fecal microbiota, as soon after birth, calves are fed milk before transitioning to increased amounts of starter concentrate (16).

Most studies evaluating the alterations in fecal microbiota indices of diarrheic calves collect samples at the onset of disease and compare them with healthy age-matched animals (28,29). Unfortunately, this common sampling prevents determination of whether alteration in fecal microbial communities predispose the host to disease or if it is a consequence of the disease itself. In our study, both groups' richness and diversity and weighted UniFrac distances were not different on day 3 and 10, before diarrhea onset, suggesting that dysbiosis could be a predisposing factor for diarrhea development. Studies report contradictory results regarding the alterations in fecal microbiota diversity before diarrhea. Although some studies show no differences in microbial diversity within a week before diarrhea onset, particularly 5 days and 3 days before onset, respectively (31,53), others report a lower fecal microbiota diversity in dairy calves one week before calf diarrhea (25,28). The reason for these contradictory results is not apparent; however, other factors could be at play, including prolonged sampling times and the administration of antimicrobial drugs. In calves the frequency of diarrhea is increased in calves that receive antimicrobial therapy compared to those that do not (55). Furthermore, treatment of mice with antimicrobial drugs before experimental infection with *Cryptosporidium* spp. facilitates pathogen infection, resulting in a more severe infection than infected animals not receiving antimicrobial drugs (56). These findings indicate that reduced microbial richness and diversity can increase the susceptibility of calves to diarrhea. This may suggest that pathogen exposure plays a role in calf diarrhea, however we could not conclude this as pathogen testing was not performed on the calves in our study.

In calves suffering from undifferentiated neonatal diarrhea (1,2), diarrhea associated with the detection of bovine rotavirus (58-60), coronavirus (60), or *Cryptosporidium spp.* (61,62), a lower richness and diversity of the fecal microbiota at the onset of diarrhea is reported. However, samples in our study were taken on days 3, 5, 10, 14, and 21, thus we may have missed key differences in bacteria between healthy and diarrheic calves shortly before the onset of diarrhea as changes may have developed in the four day window between day 10 and 14. In some cases of calf diarrhea, pathogens can induce diarrhea even in the presence of well-balanced microbial communities (62). This was found in our study, where a low richness and diversity of the fecal microbiota is detected only at the onset of the calf diarrhea. These findings imply that the dysbiosis (i.e., variations in bacterial diversity and Unifrac distances) observed in the diarrheic calves within our study were likely linked to the pathogen-induced damage to intestinal cells, immune responses, interactions between the pathogen and commensal bacterial communities, as well as alterations in the intestinal environment, such as shifts in luminal and mucosal pH, and oxygen tension (62-66).

The fecal microbial diversity and Unifrac distances of diarrheic calves included in this study returned to levels comparable to those of healthy calves seven days after the onset of diarrhea, with statistically insignificant residual alterations. ANCOM-BC analysis, used to identify taxa with significantly different abundances between healthy and diarrheic calves, showed that changes that occurred in microbial taxonomy at the onset of diarrhea (day 14) recovered by day 21. A similar trend is reported in calves receiving antimicrobial treatment. Administration of danofloxacin (67) and enrofloxacin (68) leads to dysbiosis characterized by alteration in the diversity and bacterial composition, but they return to

basal levels soon after the therapy ceases. These findings underscore the remarkable resilience of calf gastrointestinal microbial communities, which is pivotal in preserving the gastrointestinal tract and the host's health.

The random forest classification analysis revealed that *Phascolarctobacterium, Ruminococcus, Roseburia, Odoribacter,* and *Prevotella* were enriched in day 10 fecal samples of calves that remained healthy throughout the study compared to calves that developed diarrhea on day 14. In addition, *Lactobacillus, Clostridium Sensu Stricto 1,* and *Collinsella* were enriched in day 10 samples calves that developed diarrhea on day 14. Enrichment of obligated anaerobes taxa such as Ruminococcaceae (*Ruminococcus*), Lachnospiraceae (*Roseburia*), and the genera *Odoribacter* and *Prevotella* are associated with GIT health in calves (27,32). In contrast, an increased abundance of obligated anaerobes such as *Collinsella*, *Clostridium Sensu Stricto,* and facultative anaerobes such as *Lactoballius* are associated with diarrhea (57,69). The proliferation of facultative anaerobes in the GIT of laboratory animals resulted in a proinflammatory mucosal immune response linked to the development of GIT inflammation (66,70,71). This facilitates further growth of additional facultative anaerobes, such as Enterobacteriaceae, which can elicit an inflammatory response (72). *Lactobacillus* and *Collinsela* are lactate-producing bacteria (73), and therefore the proliferation of these bacteria could have contributed to an increase in luminal concentrations of lactate and a reduction in the pH (27). Alteration in the luminal pH causes selective pressure on bacterial growth and metabolism, resulting in dysbiosis (74). In horses with grain overload and cattle with ruminal acidosis, an increased abundance of lactic acid-producing bacteria leads to cecal and ruminal mucosal

damage, eliciting GIT inflammation (75-78). In addition, *Collinsella* can contribute to GIT inflammation by reducing the expression of tight junction proteins and inducing the expression of proinflammatory cytokines (i.e., IL-17) (79). These findings highlight the importance of the GIT microbiota on maintaining gastrointestinal health and predisposing to diarrhea in calves. However, as pathogen testing was not done, the occurrence of diarrhea and the association between the GIT microbiota and the pathogens associated with diarrhea is unknown.

In summary, the dysbiosis observed in the diarrheic calves could be linked to pathogen-induced damage of the gastrointestinal tract rather than be a predisposing factor for diarrhea. Although the only changes we detected were in the presence of diarrhea (day 14), the enrichment of *Lactobacillus*, *Clostridium Sensu Stricto 1* and *Collinsella* before diarrhea onset could have contributed to the gastrointestinal modulation that can result in disease. Future studies should aim to pathogen test study samples to determine specific etiology as this may provide deeper insight to microbial changes observed and allow for the development of targeted preventative and treatment measures.

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3 Chapter Three: Thesis conclusions

Neonatal calf diarrhea has a detrimental impact on the dairy industry, accounting for many deaths on an annual basis. Several studies have described the development and establishment of the GIT microbiota of newborn calves until weaning. In humans, microbial dysbiosis occurs during diarrhea, and the reestablishment of balanced bacterial groups using FMT is associated with recovery from gastrointestinal diseases. However, it is unknown whether the changes in the bacterial communities observed in diarrheic calves are associated with the development of diarrhea or whether they result from GI inflammation. It is also unknown whether the recovery of calf diarrhea coincides with the return of the balanced microbial communities. Thus, this thesis aimed to describe the fecal microbiota of calves before, during, and after recovering from diarrhea to identify changes related to the development and recovery of disease. This study hypothesized that the development of GI microbiota of healthy and diarrheic calves differs significantly, particularly in the alpha and beta diversity indices, and that the resolution of diarrhea coincides with the return of a balanced microbiota similar to what was observed in healthy age-matched calves. Understanding the changes that occur before, during and after diarrhea could facilitate the development of new preventative strategies and targeted treatment for calves with diarrhea.

The study's results reported in Chapter 2 of this thesis showed that bacterial richness and diversity increase in calves during the first 3 weeks after calving, but calves that experienced diarrhea had a lower diversity on the day of diarrhea onset. During diarrhea, the bacterial richness and diversity is lower in diarrheic calves compared to their healthy counterparts, but return to levels that are slightly lower, but

not significantly different from healthy calves. Weighted UniFrac distance showed significant differences between groups on the day of diarrhea onset, but not before or after diarrhea. Indicating that in the calves included in our study, dysbiosis is a consequence of diarrhea and the inflammatory process occurring in the GIT, which is likely associated with pathogen invasion, rather than a predisposing factor for the development of diarrhea. It is important to highlight that although the overall microbiota is not different between healthy and diarrheic calves before the onset of diarrhea, specific taxa including Lactobacillus, *Clostridium Sensu Stricto* 1 and *Collinsella* were enriched. This is of interest because these bacteria could modify the intestinal environment by producing metabolites such as lactate that can alter the luminal pH. These changes in luminal pH could predispose calves to gastrointestinal inflammation and the proliferation of bacteria that can facilitate colonization of the GIT.

The long intervals between sampling limited the results of our study as fecal samples were taken on days 3, 5, 10, 14, and 21 after calving. Thus, we could have missed key differences in microbial communities between healthy and diarrheic calves occurring shortly before or after the onset of diarrhea. By having more frequent sampling times, researchers may observe bacterial changes that have not been previously observed. This may create an opportunity to understand how the microbiota is associated with different stages of the calf's life and how these changes may influence susceptibility to disease. The results of our investigation can help clinicians, veterinarians, and commercial farm owners develop targeted treatment for the resolution of diarrhea and aid in preventative measures to decrease outbreaks and diarrhea severity.

Another limitation of our study was that we did not test the feces for pathogens asccocaited with the development of diarrhea. Therefore, the occurrence of diarrhea observed and the associated changes in the GIT microbiota cannot be linked to specific pathogens. Future studies may benefit from testing fecal samples for etiologic agents associated with diarrhea to determine specific etiology and determine whether changes in the fecal microbiota occur prior to or after an pathogen invasion. This will also allow us to determine if the microbial changes observed are pathogen specific, or if they are similar across all pathogens. This will help to develop targeted strategies to modulate the GIT microbiota during diarrhea, such as the development of pre and probiotics or FMT treatments. Therefore, studies assessing daily changes in fecal microbiota could further aid in understanding whether dysbiosis predisposes calves to diarrhea, or, similarly to the results reported in this thesis; if dysbiosis is the consequence of gastrointestinal inflammation by the pathogen invasion.

Future studies may benefit from determining how beneficial bacteria, such as *Slackia* and *Anaerococcus*, observed in our study, impact the GIT of calves during the first 21 days of life. We know that during the preweaning period calves are especially susceptible to dysbiosis and disease as their GIT are going through dynamic changes. Such studies may offer further guidance in affordable and economic preventative measures for farmers and producers, to keep their young calves healthy and ultimately reduce the presence of diarrhea on farm