Role of Commensal Microbiota in Neonatal Calf Gut Development

by

Nilusha Malmuthuge

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Animal Science

Agricultural, Food and Nutritional Science

University of Alberta

Abstract

Evidence is accumulating regarding the potential long-term impact of the early gut microbiota on host health. However, our current understanding of the early microbiome of cattle, a domestic livestock species that plays an important role in meeting the increasing demand for high quality protein, is very limited. This thesis investigated the dynamics of microbial colonization within different gut regions (rumen and small intestine) and the impact of the microbiota on the calf gut development from birth to six weeks of life (neonatal period). Study 1 used next-generation sequencing to assess gut microbiota colonization at birth. The calf gut was colonized with an active, dense and diverse bacterial community during the birthing process. However, the small intestinal microbiota composition had diverged significantly from the maternal microbiota (birth canal, rectum). A disparity between the newborn calf gut and maternal microbiota was apparent in the composition of *Bifidobacterium*. *B. longum* subsp. *infantis* dominated the calf small intestine, but only *B. pseudolongum* and *B. longum* were detected in the maternal communities. Study 2 revealed that the abundance of *Bifidobacterium* was significantly increased ($P < 0.01$) in the small intestine epimural community within six hours postpartum, as shown by comparison of calves fed heat-treated colostrum versus calves fed either fresh colostrum or no colostrum. Feeding heat-treated colostrum also decreased *Escherichia coli* colonization in the small intestine. These results suggest that feeding heat-treated colostrum enhances the establishment of beneficial microbiota and prevents colonization by potential pathogens. Study 3 revealed that the small intestinal microbiome of individual calves could be clustered into separate groups based on the abundance of specific bacterial taxa and microbial functions. Taxonomy-based clusters

were differentiated by either a high level of *Lactobacillus* or *Bacteroides*, whereas function-based clusters were differentiated by either a high abundance of protein metabolism-related functions or sulfur metabolism-related functions. Integration of the ileal microbiome and transcriptome revealed that expression of chemokines, which activate Th1 responses, tended to be higher in the *Lactobacillus*-dominant calves compared to the *Bacteroides*-dominant calves. This result suggests that unique bacterial communities within the calf small intestine may be linked to the intestinal immune functions. Study 4 revealed substantial differences in the taxonomic and functional composition of the rumen microbiome when comparing one-week-old calves with three and six-week-old calves. Moreover, the observed changes in the rumen microbiome coincided with significant differences in rumen papillae development and production of volatile fatty acids (VFAs). Network analyses revealed that 3,595 protein coding genes (26.3% of transcriptome) and 169 miRNAs (46.4% of microRNAome) were associated with calf age, concentration of VFAs and development of rumen epithelium (papillae length and width). A three-way interaction among zinc finger protein genes, miRNAs targeting those genes and bacteria suggested a potential role of bacteria-driven transcriptional regulation via miRNAs during early rumen development. In summary, this thesis generated fundamental knowledge regarding bovine gut colonization during birth and the following neonatal period and provided evidence that compositional differences in early gut microbiota may play a significant role in rumen and intestinal development.

Preface

This thesis is an original work by Nilusha Malmuthuge. The research project, of which this thesis is a part, received research ethics approval from the Livestock Care Committee of the University of Alberta (AUP00001012).

A version of chapter 1 of this thesis has been published as N. Malmuthuge, P.J. Griebel and L.L. Guan (2015) "Gut microbiome and its potential role in the development and functions of newborn calf gastrointestinal tract", Frontiers in Veterinary Science, 2:36, doi: 10.3389/fvets.2015.00036. I was responsible for manuscript writing; P.J. Griebel (supervisory committee member) and L.L. Guan (supervisor) contributed to manuscript writing, editing and concept formation.

Chapter 3 of this thesis has been published as N. Malmuthuge, Y. Chen, G. Liang, L.A. Goonewardene, and L.L. Guan (2015) "Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves", Journal of Dairy Science, 98:8044-8053, doi: 10.3168/jds.2015-9607. I was responsible for conducting experiments, data analysis and manuscript writing; Y. Chen performed quantitative real-time PCR; G. Liang involved in animal experiment, L.A. Goonewardene assisted with statistical analysis, L.L. Guan (supervisor) designed experiment, contributed to data analysis and interpretation and finalized the manuscript.

Dedication

To my mom,

For letting me dream and encouraging me to work hard till dreams are realized…

Acknowledgments

This is a combined effort of many great people from all parts of my life. First of all, I would like to give my heartiest gratefulness to my supervisor Dr. Leluo Guan for her excellent guidance and exceptional mentoring that she provided all this time. Thank you for being a role model for my career, a great inspirer for my life and for being there always for me, whether it is a matter of science or life. You have taught me how to enjoy my life, what it means by hard work and how I can truly be a passionate researcher. Many thanks again for guiding me towards greatness.

I have also received a great deal of support from my supervisory committee members, Drs. Philip Griebel and Paul Stothard. Thank you for your time, directions and valuable input towards my research project. Special thank to my examination committee, Drs. Monika Keelan and David Yanez-Ruiz.

I am really grateful to my past and present lab mates (Guanxiang Liang, Mi Zhou, Yanhong Chen, Bibaswan Ghoshal, Fuyong Li, Ou Wang, Rebecca Kong, Yang Song, Xu Sun, Emma Hernandez-Sanabria, Soledad Urrutia, Josue Romao) for their friendship and time. Without your support and sleepless nights at the DRTC while waiting for our calves to be born, and hours long sampling, this research would not be a success.

I must thank my loving parents for bringing me up with the courage and determination as well as molding me into who I am today. My loving husband, Chamila, has been very

understanding, while giving me the supportive shoulder. Thank you very much for loving your always-busy wife.

I would also like to thank Alberta Livestock and Meat Agency and National Science Engineering Research Council for funding this project and Alberta Innovates Doctoral Graduate Student Scholarship for funding my PhD studies.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

CLDN – claudin

CV – coefficient of variance

ERK – extracellular signal-related kinase

FC – fresh colostrum

FPT – failure of passive transfer

GH – glycoside hydrolases

GIT – gastrointestinal tract

HC – heat-treated colostrum

HDAC – histone deacetylase

IgG – immunoglobulin G

MALT – mucosa-associated lymphoid tissue

MAPK – Mitogen-activated protein kinase

miRNA – microRNA

mRNA – messenger RNA

NC – no colostrum

NCD – neonatal calf diarrhea

NGS – next generation sequencing

OS – oligosaccharides

PCR – polymerase chain reaction

PP – Peyer's patches

PPARG – peroxisome proliferator-activated receptor gamma

qPCR – quantitative real-time PCR

SLC – solute carrier family

SRB – sulfur reducing bacteria

Th1 – T helper 1 cell

Th2 – T helper 2 cell

TLR – toll-like receptors

TPC – total plate count

VFA – volatile fatty acids

ZNFs – zinc finger proteins

Chapter 1. Literature Review

1.0 Introduction

Calf management has a major impact on the economic sustainability of dairy cattle operations. However, the North American dairy industry faces a great challenge due to high morbidity and mortality in pre-weaned calves (USDA, 2010). Pre-weaned calf mortality in the United States ranges from 6.5 – 9.1% (USDA, 2010), whereas in Canada it ranges from 7.8 – 11% (OMAFRA, 2011). The major causes of neonatal calf deaths are diarrhea and respiratory diseases that require antibiotic treatments (USDA, 2010). In the United States, 23.9% of pre-weaned heifers are reported to have had diarrhea and 74.5% of them have been treated with antibiotics (USDA, 2010). Likewise, 12.4% of pre-weaned heifers have suffered from respiratory diseases and 66.7% of them have been treated with antibiotics (USDA, 2010). The growing human population combined with limited resources creates an increasing demand for highly productive, yet healthy animals to meet the increasing demand for milk. In this context, strategies to decrease/prevent infectious diseases and deaths in neonatal calves and the replacement herds are fundamental to ensure a viable industry.

Since neonatal calf diarrhea (NCD) is the major cause of calf death (Lorenz et al., 2011; Uetake, 2013), the dairy industry is mainly focused on feeding strategies that minimize NCD incidence in young calves. These recommended practices focus primarily on passive transfer of maternal immunity, which is defined as adequate transfer of maternal/colostral immunoglobulin (Ig) to calves within the first 24 hours of life (Godden, 2008). Failure of passive transfer (FPT) of maternal immunity (less than 10 mg

IgG/ml of serum within 24 hours after birth) is one reason for the high incidence of NCD (Lorenz et al., 2011). Thus, timed feeding (within the first hour after birth) of good quality colostrum (IgG > 50 mg/ml of colostrum) is critical in calf management (Godden, 2008). Although passive transfer of immunity is a key management practice in calf rearing due to the absence of placental transfer of immunoglobulin in ruminants (Godden, 2008), the establishment of a healthy microbiota is also pivotal for the development of the mammalian immune system and health (Hooper, 2004). Human and mouse studies have clearly described the importance of gut microbiota for the development of mucosal and systemic lymphoid tissues (Guarner, 2006). There is also a link between early gut microbiota and susceptibility to enteric infections, metabolic disorders and allergies in children and adults (Arrieta et al., 2014; Subramanian et al., 2015). Knowledge regarding gut microbiome of cattle is limited and has not been a research priority until recently. Understanding of gut microbiota establishment, compositional changes associated with varying calf management practices, and the role of early gut microbiota on gut development is essential to develop effective strategies to minimize enteric infections and neonatal calf deaths. Moreover, this knowledge may be used to develop multidisciplinary approaches, such as microbial manipulation tools and techniques, to further enhance calf health and development.

1.1 Calf rearing and management

The Canadian dairy industry ranks third in the agriculture sector with 6.07 billion CAD total net farm receipt (Canadian Dairy Information Centre). Alberta has the third highest number of dairy farms in Canada, following Québec and Ontario (Canadian Dairy

Information Centre). Canadian research on dairy calf rearing, welfare and diseases is mainly conducted in Québec and Ontario and is very limited in Alberta. One survey performed in the early 1990s reported high calf mortality (15.6%) in Alberta dairy farms within the first week of life (Spicer et al., 1994); however, the present status of calf rearing is not known. A recent study looking at colostrum management practices in central Alberta found 14.1% and 29.4% colostrum samples with < 50 mg IgG/mL, when measured using a colostrometer and radial immunodiffusion assays, respectively (Doepel and Bartier, 2014). A survey conducted in Québec dairy farms revealed that none of the dairy farms checked colostrum quality or checked passive transfer of immunity (Vasseur et al., 2010). These studies suggest that the Canadian dairy industry requires more effective transfer of research knowledge to improve calf management practices that can increase calf health, growth and welfare. Besides affecting pre-weaned calf health, poor calf management practices may also lead to poor post-weaning performance in heifers, affecting parameters such as age at first calving (Waltner-Toews et al., 1986).

1.1.1 Colostrum management and passive transfer of immunity

Ruminants are born agammaglobulinemic due to restricted placental transfer of immunoglobulins (Godden, 2008). Therefore, feeding good quality colostrum soon after the birth is an important management practice in ruminants. Newborn calves absorb colostral immunoglobulin through the small intestinal epithelium; therefore, colostrum quantity and quality is crucial for passive transfer of maternal immunoglobulin successfully. Good quality colostrum contains > 50 mg IgG/mL and is capable of increasing the calf serum IgG levels to > 10 mg/mL within the first 24 hours of life

(Godden, 2008). FPT occurs when the calf serum IgG level is < 10 mg/mL or serum total protein level is < 5.2 g/dL within 24 hours of birth (Doepel and Bartier, 2014). Although FPT has been associated with increased calf mortality (Godden, 2008), the North American dairy industry is still struggling to achieve these basic recommendations for colostrum management (Vasseur et al., 2010; Morril et al., 2012). In the US, 30% of farms feed low quality colostrum (< 50 mg IgG/mL) (Morril et al., 2012), whereas in Canada 15.6% of farms rely on dams to feed colostrum (Vasseur et al., 2010). Besides the availability of adequate immunoglobulin, the presence of pathogenic microbiota is another factor to be considered in colostrum management (Morril et al., 2012). Industrial recommendations for bacterial counts in colostrum are a total plate count (TPC) < 10^5 CFU/mL and coliforms < 10^3 CFU/mL (Morril et al., 2012). However, nearly 43% the US dairy farms had TPC > 10^5 CFU/mL (Morril et al., 2012). Moreover, 16.9% of the farms with a higher TPC than the recommendation had TPC > 10^6 CFU/mL (Morril et al., 2012).

Maximum IgG absorption occurs immediately after the birth and decreases gradually with decreasing intestinal permeability (Quigley, 2002). Thus, feeding good quality colostrum as soon as postpartum is important for a successful transfer of passive immunity (Godden, 2008). A poor colostrum management practice results in FPT (Godden, 2008), which may increase the calf susceptibility to diseases. The prevalence of FPT is extensive in central Alberta, reaching levels of 44.2% and 27.8% based on total serum protein measurement (< 5.2 g/dL) and serum IgG measurements (< 10 mg/mL), respectively (Doepel and Bartier, 2014). FPT also increases calf mortality (Godden, 2008); thus, it is important to maintain records on colostrum quality and passive transfer

of immunity within the first 24 hours after the birth to optimize calf management. Record keeping on colostrum immunoglobulin levels can be used to identify cows with lower IgG in colostrum, and to prevent those dams from directly feeding their calves. Even if calves are allowed to suckle dams, measuring serum IgG within the first 24 hours can be used to monitor that passive transfer of immunity was successful.

Other than the maternal colostrum, the newborn calves may also be fed colostrum replacers (Priestely et al., 2013). Feeding colostrum replacers should be done when there is a lack of available colostrum or when colostrum quality is poor (Priestely et al., 2013). Unlike the maternal colostrum that can vary greatly in IgG concentration (< 1 to 200 mg IgG/mL) (Morril et al., 2012), colostrum replacers ensure homogeneity of the available IgG. Moreover, good hygienic practices during preparation of colostrum replacers can decrease the level of pathogenic bacterial contamination (Priestely et al., 2013). However, Priestely and colleagues (2013) have also shown that successful passive transfer of maternal IgG is higher with good quality maternal colostrum (91.8%) than with colostrum replacers (colostrum-derived colostrum replacer – 49%, plasma-derived colostrum 28.6%). Feeding maternal colostrum improves calf body weight gain and decreases calf morbidity and mortality than colostrum replacer (Priestely et al., 2013). This further implies the importance of feeding an adequate amount of good quality maternal colostrum soon after the birth. Although colostrum management is one of the key factors to prevent NCD (Meganck et al., 2014), researches are concentrating primarily on understanding the effect of immunoglobulin in calf health. Besides immunoglobulin, colostrum contains other bioactive compounds, such as growth factors (insulin-like growth factors), antimicrobial compounds (lactoferrin), and oligosaccharides

(Gopal and Gill, 2000; Tripathi and Vashishtha, 2006) that may play important roles in gut development and colonization, and pathogen control. These other bioactive components, enriched in the maternal colostrum, may also contribute to the low mortality observed in calves fed fresh maternal colostrum versus colostrum replacer (Preistely et al., 2013). However, research has largely ignored the potential impact of these other bioactive compounds on calf health. The impact of human milk oligosaccharides on gut colonization and development has been widely studied (Musilova et al., 2014), but no similar information is available for bovine colostrum/milk. Moreover, how these bioactive components may be involved in controlling enteric infections in pre-weaned calves is of interest. Such knowledge may better reveal the overall importance of colostrum in preventing NCD and improving calf gut health.

1.2 Gut microbial colonization in non-ruminant mammals[1]

The *in utero* sterile mammalian gastrointestinal tract (GIT) is rapidly colonized by an array of microbiota during and after birth. This process of colonization has been described as a co-evolution due to the two-way interaction between host and microbes (van den Abbeele et al., 2011). Host (luminal pH, food retention time in the gut, immune defense mechanisms), microbial factors (adhesion, survival mechanisms under oxygen gradient, mechanisms to obtain nutrients from the host), and external factors, such as maternal microbiota, delivery mode, diet, and antibiotic treatment during early life all combine to influence gut colonization (Fanaro et al., 2003; Penders et al., 2006;

[1] Gut microbial colonization in mammals section is a part of a paper published: Malmuthuge, N., Griebel, P.J., Guan, L.L. (2015) Gut microbiome and its potential role in the development and functions of newborn calf gastrointestinal tract", Frontiers in Veterinary Science, 2:36, doi: 10.3389/fvets.2015.00036.

Adlerberth and Wold, 2009). Although the initial colonizers are aerobic or facultative anaerobic, a rapid establishment of obligate anaerobes (*Bifidobacterium* and *Bacteroides*), which play crucial role in host health, occurs in the GIT (Fanaro et al., 2003; Conroy et al., 2009; Jost et al., 2012). *Bifidobacterium* and *Bacteroides* are two of the main gut bacteria present in the majority of human infants (Penders et al., 2006) that have a beneficial impact on mucosal immune system. The presence of *Bacteroides* in the gut plays a vital role in the development of immunological tolerance to commensal microbiota (Mazmanian et al., 2008), while the composition of *Bifidobacterium* in the infant gut is linked to a reduced incidence of allergy (Sjogren et al., 2009). Therefore, neonatal gut colonization is a crucial period for the developing gut and naïve immune system (Fouhy et al., 2012; Hansen et al., 2012) and may have long-term health effects (Conroy et al., 2009). The composition of the gut microbiota following birth is subsequently shape by various factors such as diet, age, phylogeny, host genetics, gut morphology, and geography (Ley et al., 2008; Yatsunenko et al., 2012; Org et al., 2015), leading to a unique microbiome within each individual.

The central dogma of postnatal GIT microbial colonization was recently challenged by studies reporting the presence of microbiota in the first meconium prior to feeding (Jimenez et al., 2008), in placenta (Aagaard et al., 2014) and amniotic fluid (Jimenez et al., 2005). The meconium of infants that were not fed has been shown to contain *Enterococcus* and *Staphylococcus* (Jimenez et al., 2008), whereas the umbilical cord blood contains *Enterococcus*, *Staphylococcus*, *Streptococcus* and *Propionibacterium* (Jimenez et al., 2005). The same group also reported maternal transfer of *Enterococcus faecium* into amniotic fluid and meconium in mice (Jimenez et al., 2005; Jimenez et al.,

2008). *Escherichia coli* and a few other bacterial species found in the oral cavity, such as *Prevotella tannerae* and *Neissaria* species have been reported as the dominant microbiota present in human placenta (Aagaard et al., 2014). Although it was suggested that translocation from gut to bloodstream was the source of *in utero* bacteria observed in these studies (Funkhouser and Bordenstein, 2013), this still requires experimental validation. Moreover, the lack of details on maintaining tissue sterility during the collection of placenta, duration between amniotic membrane rupturing and collection of the first meconium, and DNA-based identification of microbiota are questions that must be fully addressed before accepting the concept of prenatal microbial colonization of the fetal gut.

1.2.1 Neonatal gut microbial composition in non-ruminants

Neonatal gut microbiota is different from that of adult; however, the gut microbiome stabilizes around three years of life in humans, after introducing solid food (Yatsunenko et al., 2012). Neonatal gut bacteria composition is mainly described using meconium samples obtained within 24 hours after the birth (Hansen et al., 2015). Following the initial acquisition, the neonatal gut is colonized with a less diverse and highly variable microbiome that contains aerobes and facultative anaerobes (Arrieta et al., 2014). These initial colonizers (*Staphylococcus*, *Streptococcus*, *Enterococcus*, *Enterobacteria*) utilize available oxygen to permit the establishment of autochthonous anaerobes in the gut (Fanaro et al., 2003; Jost et al., 2012). However, a recent study using fluorescent in-situ hybridization revealed the presence of anaerobes (*Bifidobacterium* and *Bacteroides-Prevotella*) in meconium of naturally born, healthy full-term human infants

(Hansen et al., 2015). They further reported that bacteria could not be detected in the first meconium of all the infants tested (only 66% of samples were positive for bacteria), while the abundance of the observed bacterial families differed markedly among individuals (Hansen et al., 2015). However, another study using next-generation sequencing of the 16S rRNA gene reported that microbial composition of the newborn meconium was more similar to the mother's birth canal microbiota (Dominguez-Bello et al., 2010). For example, when mothers had a high level of *Lactobacillus* in the birth canal, these bacteria dominated the meconium microbiota of vaginally delivered infants within 24 hours of birth. *Prevotella*-dominated mothers had infants with a high abundance of *Prevotella* in their meconium (Domingeuz-Bello et al., 2010). In contrast to other studies, Domingeuz-Bello et al., (2010) could not detect *Bifidobacterium* in the newborn meconium. This disparity in results may be explained by the primers (27F and 338R) used by Dominguez-Bello and colleagues (2010) for microbial profiling. These primers are not efficient in capturing *Bifidobacterium* species, which may have a few mismatches between the 27F primer and the 16S rRNA gene sequence of genus *Bifidobacterium* (Kim et al., 2013). *Bifidobacterium* rapidly becomes the predominant bacterium identified in feces from one-week-old infants that are breast-fed and two-month-old infants fed formula (Fanaro et al., 2003). A recent study also revealed a higher abundance of *Bifidobacterium* and *Bacteroides* in infants during the early neonatal period (four to six-day-old infants) when using next-generation sequencing of fecal bacteria (Jost et al., 2012). Moreover, the dominance of *Bifidobacterium* in human infants has been reported to occur regardless of geographical location (Yatsunenko et al., 2012).

The presence of *Bifidobacterium*, a probiotic bacterial genus, in human infants has been well studied due to their known roles in the health of the host (Matsuki et al., 1999; Makino et al., 2011; Turroni et al., 2012; Makino et al., 2013). These studies reported vertical transmission of *Bifidobacterium* species from mothers to vaginally delivered infants (Makino et al., 2011; Makino et al., 2013). Strains from *B. adolescentis*, *B. bifidum*, *B. catenulatum*, *B. longum* subsp. *longum* and *B. pseudocatenulatum* transmitted from mothers to infants (Makino et al., 2013). However, these studies did not discuss the colonization of *B. longum* subsp. *infantis*, which is the main *Bifidobacterium* species observed in infant feces (Underwood et al., 2015). *B. longum* subsp. *infantis* successfully utilizes human milk oligosaccharides and intestinal mucin glycans, which enhance their growth in breast milk fed infants (Underwood et al., 2015). Colonization by *Lactobacillus*, another probiotic bacterial genus, is less frequent (Adlerberth and Wold, 2009) and usually less abundant (Jost et al., 2012) in human infants. Despite studies suggesting a vertical transmission of birth canal bacteria to infants (Dominguez-Bello et al., 2010), a study comparing *Lactobacillus* species among infant gut, birth canal and breast milk suggested that *Lactobacillus* species observed in the infant gut were more similar to those in milk than the birth canal (Martin et al., 2007). Composition of *Lactobacillus* in the infant gut varies significantly among individuals (Martin et al., 2007); however, their colonization during the early life has been associated with a reduced risk for allergy (Johansson et al., 2011).

1.2.2 Factors influencing neonatal gut colonization

Infant gut is first colonized by aerobes/facultative anaerobes to create an appropriate environment for obligate anaerobes; however, various factors (host, microbial and environmental) influence the succession and relative abundance of these obligate anaerobes. The available nutrients in the intestine, the ability of microbiota to utilize these available nutrients, and microbial adhesins are major factors during the acquisition and colonization phase (Van den Abbeele et al., 2011). For example, the mucin glycans present in the mucus layer, which provides binding sites and energy sources for microbiota, are one of the mechanisms mediating host selection of gut microbiota (Juge, 2012). Coincidentally, the presence of mucus binding proteins in microbes mediates microbial adhesion to the host, determining microbial fitness for that particular environment (Juge, 2012). These host and microbial factors ultimately regulate gut colonization with a host-specific microbiota and this process may be influenced by other interventions. Thus, environmental factors that can fine-tune gut colonization are of major interest among researchers. These factors include gestational age (full-term versus pre-term), delivery mode (vaginally delivered versus C-section), feeding method (breast-fed versus formula-fed), and early antibiotic treatments (Rodriguez et al., 2015).

Pre-term infants are challenged by various complications during pregnancy, exposing them to *in utero* infections, long-term hospitalization, delayed first feeding, prolonged rupture of amniotic membranes and antibiotics treatments (Groer et al., 2014; Unger et al., 2015). Thus, colonization process and composition of gut microbiota in pre-term infants is markedly different from that of full-term infants (Groer et al., 2014). Pre-term infants are colonized with hospital microbiota (*Staphylococcus epidermidis*,

Klebsiella pneumoniae, *Bacteroides fragilis* and *E. coli*), due to the prolonged stay in the neonatal intensive care units (Brooks et al., 2014). Although there are numerous studies analyzing pre-term gut microbiota, composition reported was heterogeneous among studies due to compounding factors, such as gestation period, antibiotic treatments, milk feeding (breast milk, formula, donor human milk) and enteric disorders (necrotic enterocolitis) (Unger et al., 2015). Regardless a high abundance of *Enterobacteriaceae* in the pre-term gut was reported in all studies (Unger et al., 2015). Moreover, bacterial diversity in the pre-term gut was considerably less than the full-term infant gut (Unger et al., 2015). In contrast to the higher abundance of *Actinobacteria* in full-term infants (Turroni et al., 2012), a higher abundance of species from phylum *Proteobacteria* was observed in pre-term infant fecal samples even with human milk feeding (Gomez et al., 2016). The absence of *Bifidobacterium* colonization in human milk-fed pre-term infants suggests that maternal exposure is required for the early establishment of these beneficial bacteria, which can then increase with milk feeding.

Composition of infant gut microbiota on the first day of life and gut colonization process varies between vaginally delivered and C-section delivered neonates (Fanaro et al., 2003). Gut microbial composition of vaginally delivered infants closely represents that of the mothers' birth canal, whereas the gut microbiota C-section delivered infants was more similar to skin flora (Dominguez-Bello et al., 2010) and flora from the hospital environment (Fanaro et al., 2003). A less diverse bacterial community with delayed colonization by *Bacteroidetes* has been reported in C-section delivered infants when compared to naturally born infants (Jakobsson et al., 2014). This observation suggests that limited exposure to mothers alters the early microbial composition and succession in

newborns. The prevalence of *Actinobacteria* and *Bifidobacterium* in infant gut was the highest around three months of age and then decreased gradually, regardless of the delivery mode or feeding method (Jakobsson et al., 2014). Furthermore, *Firmicutes* dominated the fecal microbiota of vaginally delivered and C-section delivered infants within the first week of life, which then remained predominant throughout the first two years (Jakobsson et al., 2014).

Microbiota observed during the first day of life does not persist in the gut; instead breast-fed infants acquire a *Bifidobacterium*-predominant microbiome within the first week of life (Fanaro et al., 2003; Jost el al., 2012). The abundance of *Bacteroides* in the gut increases gradually with breast-feeding; however, there are some infants with high levels of *Bacteroides* even within the first week of life (Jost et al., 2012). Colonization by other bacterial groups, such as *Clostridium* and *Lactobacillus* was also observed in breast-fed infants within the first week of life, but with a lower prevalence than *Bifidobacterium*, *Bacteroides*, and *Staphylococcus* (Jost et al., 2012). When infants are fed with formula, a higher prevalence of *Firmicutes* and *Proteobacteria* has been observed relative to *Actinobacteria* (Harmsen et al., 2000; Penders et al., 2006; Adlerberth and Wold, 2009). However, the prevalence of *Bifidobacterium* and *Bacteroides* increased gradually and within the first month of life these microbiota became predominant (Harmsen et al., 2000; Fanaro et al., 2003). It is not clear, however, whether breast-feeding/formula-feeding shapes gut colonization differently in vaginally delivered and C-section delivered infants, since these factors are compounded in most of the studies. Formula milk has now been supplemented with prebiotics (oligosaccharides) to increase its similarity to breast milk and feeding prebiotic-supplemented formula has

been successful in achieving microbiota similar to the breast-fed infants (Rinne et al., 2005). Although there are variations in the gut microbial composition and succession with the type of feeding during the early infancy, such differences can no longer be detected in infants following the introduction of solid food (Arrieta et al., 2014). The predominant anaerobe populations in infant gut (*Bifidobacterium* and *Bacteroides*) are also influenced by the oral administration of antibiotics during early life, but not with mothers' antibiotic usage (Penders et al., 2006). Gut microbiota of antibiotic-treated infants contained a higher proportion of *Proteobacteria* and a lower proportion of *Actinobacteria* compared to those not receiving antibiotics (Foughy et al., 2012). Moreover, this *Proteobacteria*-dominant microbiome was observed in infants for a prolonged period following antibiotic treatment (eight weeks after treatments), although the populations of *Bifidobacterium* and *Lactobacillus* progressively recovered (Foughy et al., 2012). This suggests that antibiotic treatment may shift the infant gut microbial composition, creating relatively long lasting dysbiosis.

Neonatal gut microbial composition and the factors influencing gut colonization have been studied primarily in humans, but there is a paucity of such knowledge in other animal species. Based on human studies it is evident that early microbiota play a crucial role in host health and growth (Backhed et al., 2015). Thus, it is essential to study how the early microbiome of livestock species influences growth, health and production.

1.3 Gut microbiota in ruminants[2]

Gut microbes of ruminants, mainly the rumen microbiota, provide 70% of their daily energy requirement via the fermentation of undigestible dietary substrates (Yeoman and White, 2014). Therefore, studies in ruminant gut microbiota have focused mainly on the rumen to understand how this microbiome impacts meat and milk production. Rumen microbiota consists of bacteria, archaea, protozoa and fungi involved in the fermentation of complex carbohydrates and its composition is influenced by a number of factors. For example, distinct microbial populations have been identified for the particle-attached, fluid-associated, and tissue-attached fractions of the rumen (Cho et al., 2006). Rumen microbial composition can also vary significantly depending on the ruminant species, diet, host age, season and geographic region (Tajima et al, 2001). Bacteria dominate the rumen microbiota and contribute mainly to the production of volatile fatty acids (VFAs) and microbial protein (Kim et al., 2011). Despite numerous human and mouse studies reporting the importance of early gut microbiota on host health, there are few attempts to understand the role of early gut/rumen colonization on GIT development or host health in neonatal ruminants. Furthermore, rumen/gut development and establishment of the microbiota have always been studied as separate aspects of ruminant biology and there have been few attempts to understand possible interactions between these two events. Although research focused on understanding gut colonization of mammals has increased dramatically over the last decade (Figure 1.1A & 1.1B), there are still very few studies focused on domestic livestock species, especially ruminants (Figure 1.1C & 1.1D).

[2] Gut microbiota in ruminants section is a part of a paper published: Malmuthuge, N., Griebel, P.J., Guan, L.L. (2015) Gut microbiome and its potential role in the development and functions of newborn calf gastrointestinal tract", Frontiers in Veterinary Science, 2:36, doi: 10.3389/fvets.2015.00036.

Information is extremely limited on ruminant gut colonization, especially when focusing on the role of the microbiota in early development of the GIT during the pre-ruminant period.

1.3.1 Rumen colonization in pre-ruminants

Colonization of pre-ruminant rumen was first studied using light microscopy and Gram-staining to visualize bacteria in the late 1940s (Pounden and Hibbs, 1948; Pounden and Hibbs, 1949). In the 1980s, Gerard Fonty started to investigate the establishment of the rumen microbial community in lambs by using culture-dependent approaches and was the first to report age-dependent changes in the appearance of different microbial populations (Fonty et al., 1987). Anaerobic bacteria dominate in the rumen of neonatal ruminants by the second day of life (10^9 CFU/mL of rumen fluid) and the density of cellulolytic bacteria stabilized (10^7 CFU/mL of rumen fluid) within the first week of life (Fonty et al., 1987). This study revealed that the dominant bacterial species in the neonatal lamb rumen was different from those species colonizing the adult rumen. When the establishment of other rumen microbiota was investigated, their appearance was delayed until after bacteria were established (Fonty et al., 1987). Anaerobic fungi and methanogens appear in the neonatal rumen between 8-10 days postpartum (Fonty et al., 1987), while protozoa appear only after 15 days postpartum (Fonty et al., 1988). Furthermore, comparison of conventionalized lambs with conventionally reared lambs suggested that the establishment of protozoa required a well-established bacterial population (Fonty et al., 1988).

The early rumen microbiota consists of bacterial species from *Propionibacterium*, *Clostridium*, *Peptostreptococcus* and *Bifidobacterium* genera, while *Ruminococcus* species dominated the cellulolytic bacterial population (Fonty et al., 1987). Restricted exposure of lambs to their dams or other animals also delayed the establishment of cellulolytic bacteria, when compared to lambs reared in close contact with their dams during the first few weeks of life (Fonty et al., 1989). This observation revealed the important role of early environmental exposure for the establishment of a host-specific microbiota. Fonty and colleagues have also extended their studies to explore the establishment of tissue-attached (epimural) bacteria in the ovine rumen (Rieu et al., 1990). Similar to the fluid-associated community, the complexity of the epimural community and homogeneity among individuals increased with increasing age (Rieu et al., 1990). A recent study revealed, however, that the rumen epimural bacterial community in pre-weaned calves differs significantly from the content-associated community (Malmuthuge et al., 2014). This observation suggested that host-microbial interactions might play an important role in defining these two distinct microbial communities.

The rumen microbiota has a significant impact on pre-ruminant management, especially during the weaning process, which depends on rumen development and the ability of the microbiome to ferment complex carbohydrates (Heinrichs, 2005). The presence of VFAs (acetate, propionate, butyrate) in the rumen plays an important role in rumen development, especially the development of rumen papillae (Lane and Jesse, 1997). Fermentation of undigestible dietary substrates by rumen microbiota is the major source of VFAs in ruminants (Kim et al., 2011; Yeoman and White, 2014) and it is

generally believed that feeding a solid diet accelerates this process in pre-ruminants (Heinrichs, 2005). Although the establishment of rumen microbiota has long been studied and their importance in the rumen development has been suggested, the mechanisms by which bacteria influence rumen development remain poorly defined. Moreover, culture-based studies can only identify around 10% of the total rumen microbiota, leaving the majority of the microbiome undefined (Weimer, 2015).

Recently, enhanced molecular-based technologies, such as next-generation sequencing (NGS), provide an excellent platform to identify both culturable and non-culturable microbes, as well as characterizing their potential functions (McCann et al., 2014). It is now possible to generate a comprehensive profile of both microbial diversity and functions and explore potential associations between the microbiome and early rumen development. Using NGS, a comparison of the rumen bacteria and metagenome in two-week-old and six-week-old calves, fed a milk replacer diet, revealed a taxonomically and functionally diverse rumen microbiome in pre-ruminant calves with significant age-dependent changes (Li et al., 2012). This study revealed that *Bacteroidetes*, followed by *Firmicutes* and *Proteobacteria* colonized in the rumen content of pre-weaned calves, which displayed age-dependent variations in their relative abundance. For example, the abundance of *Bacteroidetes* increased from 45.7% at two weeks to 74.8% at six weeks of age, despite calves receiving the same diet over time. Such age-related differences were more prominent at the bacterial genera level, where the predominant *Prevotella* (33.1%) at two weeks was replaced by *Bacteroides* (71.4%) at six weeks.

Since the study by Li and colleagues (2012), there have been further studies analyzing changes in the composition of the rumen microbial community from birth to

weaning. Rumen fluid or content was used as a proxy for the rumen microbiome and 16S rRNA amplicon-based sequencing approaches were used to identify and quantify bacteria (Jami et al., 2013; Malmuthuge et al., 2014; Rey et al., 2014; De Barbieri et al., 2015). These studies revealed marked heterogeneity in the rumen bacterial composition of individual animals immediately postpartum, but greater similarity in bacterial composition was observed with increasing age (Li et al., 2012; Jami et al., 2013; Rey et al., 2014; De Barbieri et al., 2015). There were, however, a number of discrepancies in terms of rumen bacterial composition when comparing among studies. For example, Jami and colleagues (2013) reported a higher abundance of *Streptococcus* belonging to the phylum *Firmicutes* in one to three-day-old calves. In contrast, Rey and colleagues (2014) reported a higher abundance of *Proteobacteria* in two-day-old calves. Furthermore, both Jami et al., (2013) and Rey et al. (2014) reported a higher abundance of *Bacteroides* in rumen fluid at two weeks of life, while Li and colleagues (2012) observed a greater abundance of *Prevotella* in rumen content. Targeting different variable regions of 16S rRNA gene (V1/V2 versus V3/V4) for amplicon-based sequencing and differences in the environment in which these calves were raised may have influenced the apparent bacterial composition of rumen fluid.

A study comparing content-associated versus epimural bacterial populations in three-week-old calves revealed that bacterial phylotypes belonging to *Bacteroidetes* (43.8%) and β-*Proteobacteria* (25.1%) dominated the epimural community. In contrast, phylotypes from *Bacteroidetes* (54.8%) and *Firmicutes* (29.6%) dominated the rumen content-associated community (Malmuthuge et al., 2014). Using 16S rRNA amplicon-based sequencing, temporal changes in the epimural bacterial community have also been

reported in goat kids during the first 10 weeks of life (Jiao et al., 2015). The predominant *Proteobacteria* (> 85%) during the first week of life were gradually replaced by an increasing abundance of *Bacteroidetes* (~10%) and *Firmicutes* (> 15%) (Jiao et al., 2015). Similar to previous culture-based approaches, these recent studies have confirmed that dynamic changes occur in the rumen bacterial community during early life, with significant differences between the epimural and content-associated communities in the pre-weaned rumen.

Associated with the age-dependent changes in rumen microbial composition (Figure 1.2), there are also changes in the activity of the rumen microbiota. These functional changes occur in the absence of dietary changes during the first six weeks of life (Li et al., 2012). Currently, this is the only study using a metagenomics approach to assess the metabolic potential of pre-ruminant rumen microbiome. Li and colleagues (2012) revealed that ATP-binding cassette family transporters are more abundant at two weeks than six weeks of age but TonB-dependent receptors are more abundant at six weeks. Glycoside hydrolases (GH2, GH3, GH42, GH92), which breakdown complex carbohydrates, were also detected in the pre-ruminant rumen, even when the diet did not contain complex carbohydrates. These observations suggest that early rumen microbiota has the capacity to ferment dietary fiber prior to being exposed to this material. Moreover, a recent study investigating the activity of the early rumen microbiota revealed that VFA production and xylanase and amylase, enzymes that breakdown complex carbohydrates, were active within two days postpartum (Rey et al., 2012). The observed glycoside hydrolase activity, in conjunction with VFA production, reveals establishment of a metabolically active adult-like microbiome in the neonatal rumen prior to exposure

to appropriate dietary substrates. Thus, the establishment of metabolically active microbiome may occur along with the transfer of microbiota from the dam to newborn calf and the colonization of a species-specific microbiome.

Diet is one of the main factors that influences the composition of gut microbiota and may also play an important role in the observed temporal changes of the rumen microbiota in neonatal calves (Jami et al., 2013; Rey et al., 2014). The rumen content of three-week-old calves fed milk replacer, supplemented with a calf starter ration (20% crude protein, 3% crude fat, 5.7% crude fiber), contained a similar abundance of *Prevotella* (15.1%) and *Bacteroides* (15.8%) (Malmuthuge et al., 2014). Calves that received milk replacer only, however, displayed a shift in the predominant rumen content-associated bacteria from *Prevotella* to *Bacteroides* (Li et al., 2012) within the first six weeks of life. Thus, the observed similar abundance of these two bacterial genera in three-week-old calves fed milk supplemented with calf starter suggests that the age-dependent shift in the dominant bacteria may have been triggered by the dietary supplement that contained fiber. In general, it is believed that introduction of solid diet plays a key role in promoting the establishment of rumen microbiota, as milk bypasses the rumen to enter the abomasum (Heinrichs, 2005). Moreover, pre-weaning diet and feeding methods have been reported to have more pronounced and long-lasting impacts on rumen microbial composition (Abecia et al., 2013; Abecia et al., 2014a; Da Barbieri et al., 2015). Altering feeding practices during the pre-weaning period were reported to significantly alter methanogen composition after weaning (Abecia et al., 2013), as well as the density of bacteria and protozoa in pre-weaned lambs (Abecia et al., 2014a). Therefore, managing pre-weaning feeding may be as importance as managing feeding

during the weaning period in terms of microbiota establishment as well as development of the microbial fermentation capacity of the rumen.

Currently, characterization of the rumen microbiota is based primarily on the sequencing of DNA, which represents both active and dead microbiota. Therefore, the use of RNA-based metatranscriptome approaches may provide a better understanding of the biological activity of the early rumen microbiome. Understanding the activity of the rumen microbiota may help designing multidisciplinary approaches to engineer the early rumen microbiome with the objective of promoting both rumen development and function that better supports the critical transition that occurs when ruminants are weaned.

1.3.2 Intestinal tract colonization in pre-ruminants

Early studies on bacterial colonization of the pre-ruminant intestine focused primarily on pathogenic *E. coli* in calves and described the pathogenesis of neonatal diarrhea (Chanter et al., 1984; Hall et al., 1985; Moxley and Francis, 1986; Janke et al, 1989). Microscopic imaging revealed that pathogenic *E. coli* preferably attached to and effaced the mucosal epithelium in the ileum and large intestine but not the duodenum and jejunum of neonatal calves (Moxley and Francis, 1986). Feeding of probiotic strains isolated from the intestine of calves reduced enteric colonization of pathogenic *E. coli* O157:H7 in pre-weaned calves (Zhao et al., 1998). Furthermore, the administration of *Bifidobacterium* and *Lactobacillus* to newborn calves during the first week of life increased weight gain and the feed conversion ratio, while decreasing diarrhea incidences (Abe et al., 1995). These effects were most pronounced in pre-weaned calves than

weaned calves (Abe et al., 1995), suggesting the probiotic supplements are more effective when the gut microbiota is being established and less effective when the microbiome has stabilized.

Supplementation of *Lactobacillus* in young calves was also reported to increase the total serum IgG concentration (Al-Saiady, 2010), providing evidence of a host-microbial interaction that may influence calf health. More recently, supplementation of newborn calves with prebiotics (galactooligosaccharides) was associated with an increased abundance of *Lactobacillus* and *Bifidobacterium* in the colon of two-week-old calves (Marquez, 2014). However, this effect was less pronounced in four-week-old calves (Marquez, 2014), suggesting that as with probiotics it may be easier to manipulate the microbiome during the early colonization period (Abe et al., 1995). In an attempt to reduce antibiotic usage during the pre-weaning period, studies continue to investigate the impact of probiotics and prebiotics on calf growth and health (Uyeno et al., 2015). The full impact of these approaches on gut microbial colonization and composition throughout the pre-ruminant period has yet to be understood and studies are lacking on how altering the gut microbiota may impact mucosal immune defenses in the GIT.

In 1965, Williams Smith used culture-dependent approaches for the first time to study bacterial colonization in the pre-ruminant GIT, beginning immediately postpartum. He reported colonization by *E. coli* and *Streptococcus* in all gut regions (stomach, small intestine and cecum) of calves within eight hours after birth, while *Lactobacillus* colonization was only observed one-day after birth. *Lactobacillus* then predominated throughout all regions of the GIT tested within the first week (Smith, 1965). *Bacteroides* were observed only in the cecum and feces after the second day of life (Smith, 1965). The

colonization of *Clostridium perfringens*, previously known as *C. welchii*, was also observed in the cecum within eight hours after birth; however, it was not detected in other gut regions until 18 hours after birth (Smith, 1965). Similar to the colonization observed in human (Jost et al., 2012), this study also suggested that the newborn calf GIT was first colonized by facultative anaerobes, which then created the anaerobic conditions required for colonization by obligate anaerobic gut microbiota, such as *Bifidobacterium* and *Bacteroides*.

Subsequent studies have revealed a higher abundance of *Bifidobacterium* and *Lactobacillus* in fecal samples and throughout the GIT of newborn calves (Rada et al., 2006; Vlkova et al., 2006). A higher abundance of *Bifidobacterium* in three- to seven-day-old calves was also associated with a lower abundance of *E. coli* (Rada et al., 2006). More recently, culture-independent approaches have been employed to better understand the diversity and abundance of bacteria throughout the neonatal ruminant GIT (Uyeno et al., 2010; Oikonomou et al., 2013). RNA-based, sequence-specific rRNA cleavage analysis of bacteria throughout the first 12 weeks postpartum revealed a higher abundance of the *Bacteroides-Prevotella* and *Clostridium coccoides-Eubacterium rectale* groups in the feces of dairy calves (Uyeno et al, 2010). *Faecalibacterium* was one of the most abundant bacteria in one-week-old calves (21.7%), but then declined with increasing calf age (Uyeno et al., 2010). *Ruminococcus flavefaciens* and *Fibrobacter*, fibrolytic bacteria, were only observed after five weeks postpartum, while Streptococcus and *Lactococcus* could not be detected after the fifth week (Uyeno et al., 2010). These studies confirmed there were significant age-dependent changes in the composition of the

GIT microbiome and revealed substantial differences between the rumen and lower GIT microbiome.

Regional variations in bacterial phylotypes richness, diversity, density and composition throughout the GIT of newborn calves have described, using both culture-dependent and independent approaches (Vlkova et al., 2006; Collado and Sanz, 2007; Malmuthuge et al., 2012b, Malmuthuge et al., 2014). When bacterial populations throughout the GIT of 20-week-old calves were analyzed, *Bifidobacterium* and *Lactobacillus* displayed a greater survival of stomach passage than coliforms and *E. coli* (Vlkova et al., 2006). The density of these beneficial bacteria was high throughout all GIT regions (rumen, abomasum, duodenum, jejunum, cecum and colon) of the 20-week-old calves (Vlkova et al., 2006). Using culture-independent approaches, higher bacterial phylotype richness was observed in the rumen and large intestinal regions than the small intestinal regions of lambs and calves (Collado and Sanz, 2007; Malmuthuge et al., 2012b, Malmuthuge et al., 2014). Collado and Sanz (2007) reported, however, a similar bacterial richness throughout the GIT, when using a culture-dependent approach. This observation is consistent with there being many more unculturable bacterial species in the rumen and large intestine than the small intestine. A longer retention time, higher availability of nutrients, and reduced scrutiny by the host mucosal immune system have all been suggested to contribute to the increase bacterial diversity and density in the rumen and large intestine of mammals (Van den Abbeele et al., 2011).

When bacterial composition throughout the GIT is explored, the rumen and large intestinal regions consist primarily of *Bacteroidetes* and *Firmicutes*, while > 95% of the bacteria in the small intestine contents are composed of *Firmicutes* (Malmuthuge et al.,

2014). In contrast, the mucosa-associated bacterial community in the small intestine is composed of primarily *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, represented by 17 genera that are unique to this region of the GIT (Malmuthuge et al., 2014). The presence of bacteria unique to the small intestine (Malmuthuge et al., 2014) suggests that fecal sample-based studies do not reveal the true GIT microbiome and may not reveal important regional host-microbial interactions, which was also suggested by a recent study in human infants (Romano-Keeler et al., 2014).

There is increasing evidence that mucosa-attached microbiota are significantly different from those associated with ingesta and present in the intestinal lumen. Collado and Sanz (2007) first studied mucosa-attached bacteria and reported that *Bifidobacterium* and *Lactobacillus* were predominant throughout the GIT (rumen, duodenum, colon) of calves (9 - 11 months) and lambs (6 - 9 months). They did not, however, compare mucosa-associated versus intestinal content communities. Studies by Malmuthuge and colleagues (2012b) compared mucosa-attached and content-associated bacterial communities throughout the GIT of calves and reported that at three weeks of life distinct mucosa-attached bacterial phylotypes had been established. Furthermore, bacterial richness in mucosa-attached communities, especially in the ileum, was higher than the content-associated community (Malmuthuge et al., 2012b). These distinct and richer mucosa-attached bacterial communities were subsequently confirmed by using pyrosequencing of 16S rRNA gene amplicons (Malmuthuge et al., 2014). Although the majority of mucosa-attached bacteria could not be assigned at a genus level, the use of a NGS approach provided a greater understanding of region- (rumen, small intestine, large

intestine) and sample type- (content, mucosa) specific bacteria throughout the GIT of pre-weaned calves (Malmuthuge et al., 2014).

Based on the previously cited studies, it is clear that the composition, diversity and richness of rumen and intestinal microbiota in pre-weaned ruminants can vary depending on factors such as age, diet, feeding method, feed additives, sampling location (content, mucosa, feces) and gut region (rumen, large intestine, small intestine) (Table 1.1, Figure 1.2). Furthermore, variation in microbial composition among individual animals is higher in young than adult ruminants (Jami et al., 2013). The high variation in bacterial diversity and density (Malmuthuge et al., 2012b; Jami et al., 2013) among individual ruminants during early life also suggests that the gut microbiome may be more easily changed at this time of life than in adults. This may explain why probiotics and prebiotics have been reported to have a much greater effect in young animals than older calves (Abe et al., 1995; Marquez, 2014). Of particular interest are the recent studies conducted by Abecia and colleagues (2014b), which revealed long lasting consequences when dietary interventions were used to manipulate the rumen microbiota in young calves. Thus, a much greater understanding of early gut microbial colonization and the factors influencing establishment of microbiota may provide the basis for rational strategies to manipulate the gut microbiome and improve the growth and health of ruminants throughout the entire production cycle.

1.3.3 Factors influencing pre-ruminant rumen/gut microbiota composition

Factors influencing the early GIT microbial composition are well described in human; however, this area is not clearly understood in livestock research. Especially,

there is a lack of knowledge on the early gut microbial composition and the factors shaping the early colonization process in pre-ruminants. However, based on industrial practices it is known that there is a variation in the early feeding management (Vasseur et al., 2010; Morril et al., 2012), use of antibiotics to treat NCD and respiratory diseases (USDA, 2010), exposure to dam (Vasseur, et al., 2010), and calf pen-housing system (group pen-housing *versus* individual pen-housing) (Pereira et al., 2014). In Canada, 15.6% of farms tested relying on dams to provide colostrum to newborn (Vasseur et al., 2010), exposing newborn calves to dams while influencing the first feeding time after birth. Based on human studies it is clear that the exposure to moms and the first feeding impact on early microbial colonization (Unger et al., 2015). Importance of the early exposure for the gut colonization has also reported in piglets via comparing indoor-, outdoor- and isolator-reared piglets (Mulder et al., 2011). Moreover, the colonization of cellulolytic bacteria in pre-ruminant rumen was delayed in the absence of dam or other lambs (Fonty et al., 1987). Thus, the exposure to dams by allowing calves to suck as well as group pen-housing systems may change microbial colonization and composition from that of bottle-fed calves and individual pen-housing systems. Besides, the high morbidity levels in calves observed in North American dairy industry and the heavy use of antibiotics to treat these diseases (USDA, 2010) also suggest a possible variation in the gut colonization of antibiotic-treated calves compared to calves that do not receive treatments. Furthermore, Oikonomou et al., (2013) have also suggested an association of bacterial diversity variations with NCD and pneumonia in calves. The same study reported the importance of early microbial composition on growth and health, thus, it is

important to study these factors in detail to optimize calf gut colonization that may improve growth and health.

1.4 Gut microbiota and neonatal health

While it is apparent that early microbial composition can be varied depending on various environmental factors, it is crucial to know the influence of the early microbiota on host health and how these compositional changes influence the host susceptibility to diseases. Dysbiosis in the early gut microbiome has been shown to mainly associate with the development of allergies, eczema and necrotize enterocolitis in human infants (Francino 2014). The influence of early gut microbiota on mucosal immune system development has been considered as the main reason behind the occurrence of the above-mentioned diseases with gut microbial dysbiosis (Francino, 2014). Furthermore, a recent study reported that early microbiota might influence calf growth and susceptibility to enteric infections (Oikonomou et al., 2013), suggesting their roles in livestock production. Restricted exposure of piglets to sows or to the environment has also delayed the colonization of *Lactobacillus*, leading to distorted immune responses (Mulder et al., 2011).

1.4.1 Impact of microbiota on gut and mucosal immune system development[3]

Gut microbiota are essential for the development and differentiation of the intestinal mucosal epithelium, as well as the mucosal immune system (Sommer and

[3] Impact of microbiota on gut and mucosal immune system development section is a part of a paper published: Malmuthuge, N., Griebel, P.J., Guan, L.L. (2015) Gut microbiome and its potential role in the development and functions of newborn calf gastrointestinal tract", Frontiers in Veterinary Science, 2:36, doi: 10.3389/fvets.2015.00036.

Backhed, 2013). Most of our knowledge regarding host-microbial interactions in the GIT has been obtained from a variety of mouse models. Comparisons between gnotobiotic and conventionally reared mice revealed decreased development of the intestinal epithelium and the mucosal immune system in the absence of gut microbiota. Thickness of the mucus barrier is reduced in germfree mice, but administration of microbe-derived lipopolysaccharides and peptidoglycans to the colonic mucosal surface stimulated mucus production and within 40 minutes restored the thickness of the mucus layer to that of conventional mice (Petersson et al., 2011). This observation supports the conclusion that the gut microbiota is essential for the secretion of intestinal mucus, an important physical barrier throughout the GIT. In addition, the generation rate of epithelial cells in germfree mice is lower than that of the conventionally raised mice (Nowacki, 1993), revealing the importance of gut microbiota for maintaining intestinal epithelial cells proliferation and ensuring recovery of the mucosal barrier following injuries.

The presence of gut microbiota in mice is also necessary for the development of secondary lymphoid structure such as Peyer's patches (PPs), mesenteric lymph nodes and isolated lymphoid follicles (Sommer and Backhed, 2013). The establishment of host-specific microbiota, especially bacterial species belong to phylum *Firmicutes*, is essential for the development of a variety of intestinal immune cells (Chung et al., 2012). For example, when human microbiota colonized the mouse intestine there were low numbers of CD4+ and CD8+ T cells, and fewer proliferating T cells and dendritic cells when compared to mice colonized with mouse microbiota (Chung, et al., 2012). Interestingly, the immune cell profile of human microbiota colonized mice was similar to that of germfree mice (Chung et al., 2012), suggesting the presence of a host-specific microbiota

is fundamental for mucosal immune system development. Thus, host-microbial interactions in the developing gut of newborn animals must be studied within relevant host species to accurately understand the role of early microbiota on gut development.

In ruminants, development of mucosa-associated lymphoid tissues (MALT) in the GIT begins *in utero* and there is active proliferation of B cells in lymphoid follicles of the PP in the complete absence of the gut microbiota (Griebel and Hein, 1996; Yasuda et al. 2004). Furthermore, oral delivery of antigens *in utero* has confirmed that these MALTs are fully functional and can generate specific immune responses with the production of secretory IgA (Gerdts et al. 2000). In the absence of an *in utero* infection, however, the appearance of IgG+ and IgA+ cells in PPs is delayed until after birth (Yasuda et al., 2004). Since immunoglobulin class switching occurs in the germinal centers of PPs (Sommer and Backhed, 2013), this suggests that full development of germinal centers requires exposure to the gut microbiota. However, information regarding the role of the gut microbiota in the early postnatal development of MALT in ruminants is scarce. There is a single report that preventing exposure of the ileal PPs to gut microbiota results in premature involution of lymphoid follicles in the PPs of newborn lambs (Reynolds and Morris, 1984). However, restoration of the gut microbiota at four weeks after birth reversed lymphoid follicle involution in the ileal PPs. Thus, the gut microbiota appears to provide critical signals that maintain the production of the pre-immune B cell repertoire. It remains to be determined whether specific microbial species may influence the selection of this immunoglobulin repertoire or if this interaction is restricted to an interaction with innate immune receptors.

The host uses pattern recognition receptors, such as toll-like receptors (TLRs) to recognize the commensal microbiota and maintain intestinal homeostasis (Rakoff-Nahoum et al., 2004). Activation of TLR signaling by intestinal tissue invading pathogens generally stimulates inflammatory responses. In contrast, commensal microbiota activation of TLR signaling promotes the production of interleukin 6 and tumor necrosis factor that protect intestinal epithelial cells against injuries (Rakoff-Nahoum et al., 2004). Therefore, commensal microbial recognition by mucosal TLRs is crucial for the maintenance of intestinal homeostasis and protection of the gut from injuries. The expression of TLRs in the blood of infants (Teran et al., 2011) was downregulated with increasing age, while memory T cells, such as CD4+ and CD8+, increased in number (Teran et al., 2011). These changes are consistent with a decrease in innate immune responses that is balanced by an increase in adaptive immune responses with increasing age. Downregulation of innate immune responses with increasing age has been suggested as one mechanism by which the host avoids unnecessary inflammatory responses to commensal microbiota (Teran et al., 2011). Similar results have been reported when analyzing the intestinal immune system of calves (Fries et al., 2011; Malmuthuge et al., 2012a). The expression of mucosal TLR genes was downregulated in weaned calves when compared to pre-weaned calves (Malmuthuge et al., 2012a). In contrast, total leukocytes including, CD3+, CD4+ and CD8+ T cells, increased in the jejunal and ileal mucosa of calves with increasing age (Fries et al., 2011). Moreover, a negative correlation was observed between the expression of mucosal TLRs and mucosa-attached bacteria, suggesting a possible link between the gut microbiota and the observed age-related changes in the mucosal immune responses (Malmuthuge et al., 2012a).

However, the mechanism by which gut microbiome colonization affects this shift of mucosal and systemic immune responses from innate to adaptive remains to be defined. There is, however, emerging evidence that microbial colonization is associated with substantial changes in the transcriptome of the bovine intestine during the first week of life (Liang et al. 2014). Transcriptome changes occurred at the level of miRNA and significant correlations were identified between the gut bacteria and these transcriptome changes.

Experiments with the mouse model have clearly demonstrated the importance of gut microbiota in the development of innate and adaptive components of the mucosal immune system as well as development and maintenance of the intestinal epithelial barrier. Increased susceptibility to enteric infections in gnotobiotic and antibiotic-treated mice may also be due to the underdeveloped mucosal immune system and epithelial barrier (Sommer and Backhed, 2013). The immunologically naïve neonatal GIT and the colonizing microbiota undergo a rapid co-evolution during early life and these interactions may be crucial in determining the susceptibility of the neonate to enteric infections. Pre-weaned ruminants are highly susceptible to a variety of viral and bacterial enteric infection within the first few weeks of life (Uetake, 2013). Therefore, a thorough understanding of early gut microbiota and its role in regulating and directing early development of the mucosal immune system is essential to improving the health of young calves and reducing susceptibility to enteric infections.

1.4.2 Commensal microbiota and enteric infections in young ruminants[4]

NCD is the major cause of deaths in pre-weaned calves and accounts for more than 50% of calf deaths in the dairy industry (Uetake, 2013). A recent study reported an association between early gut microbial composition and calf health (neonatal diarrhea, pneumonia) and growth (weight gain) (Oikonomou et al., 2013). Bacterial diversity was lower in calves with pneumonia and neonatal diarrhea when compared to healthy calves (Oikonomou et al., 2013), suggesting a possible link between gut microbiota and host health. The authors speculate that antibiotic treatment may have been one factor influencing the gut microbiome in pneumonic calves. Furthermore, colonization by enteric pathogens may be responsible for the observed dysbiosis in gut microbiota during neonatal diarrhea (Oikonomou et al., 2013). Increased fecal bacteria diversity was also associated with increased weight gain in healthy calves, while a high abundance of *Faecalibacterium* during the first week of life was associated with a lower incidence of diarrhea in calves after the fourth week of life (Oikonomou et al., 2013). Thus, it is difficult to determine if changes in the fecal microbiome were a consequence of prior disease and associated therapeutic interventions or if colonization of the GIT by specific commensal bacteria had a beneficial effect in terms of disease resistance.

Uyeno and colleagues (2010) also reported a high abundance of *Faecalibacterium* in the feces of one-week-old calves and their abundance was higher in the large intestine compared to the small intestine of three-week-old calves (Malmuthuge et al., 2014). *Faecalibacterium prausnitzii*, one of the main butyrate producers in the large intestine,

[4] Commensal microbiome and enteric infections in young ruminants section is a part of a paper published: Malmuthuge, N., Griebel, P.J., Guan, L.L. (2015) Gut microbiome and its potential role in the development and functions of newborn calf gastrointestinal tract", Frontiers in Veterinary Science, 2:36, doi: 10.3389/fvets.2015.00036.

displayed a negative association with calf diarrhea incidences (Oikonomou et al., 2013), suggesting the high prevalence of this species during early life may decrease susceptibility to enteric infections. *F. prausnitzii* also plays a pivotal role in maintaining intestinal homeostasis by promoting anti-inflammatory responses and has been shown to decrease in prevalence in patients with inflammatory bowel disease (Sokol et al., 2009). Inflammatory bowel disease was also associated with a reduced prevalence of *Bifidobacterium* (Sokol et al., 2009), suggesting that these two bacterial groups may have important roles in maintaining intestinal homeostasis and preventing enteric infections. Thus, it will be important to further explore the potential role of such beneficial bacteria in early gut development and their capacity to promote host health.

Poor management of colostrum feeding in newborn calves is one of the main triggers of NCD. Although the importance of timed feeding of high quality colostrum for passive transfer of immunity has been well studied (Godden et al., 2012), the influence of colostrum on gut microbial establishment and susceptibility to enteric infection in young ruminants is not clearly understood. One of the industrial recommended good management practices is to feed calves with heat-treated colostrum that increases passive transfer of immunity (Gelsinger et al., 2015). Moreover, heat treatment (60°C, 60 min) decreases the density of total bacteria including pathogens present in colostrum, one of the reasons behind decreased neonatal diarrhea in calves (Godden et al., 2012). Nonetheless, the impact of feeding heat-treated colostrum on the establishment of gut microbiota is yet to be discovered, especially how this may influence the *Bifidobacterium* dominant calf GIT microbiome (Rada et al., 2006). Establishing a bacterial population dominated by beneficial bacteria may suppress colonization of enteropathogens (Fukuda

et al., 2011) immediately postpartum and provide protection against enteric infections in young ruminants with a naïve immune system. Further investigations are also necessary to understand how a *Bifidobacterium*-dominated early gut microbiome may influence host performances (weight gain, resistance to enteric infections) within the first few weeks and identify the mechanisms by which the commensal microbiome alter enteric health and general physiology. This knowledge is exceptionally important in designing multidisciplinary approaches, such as microbial manipulation tools and techniques, to prevent enteric infections, to increase calf health, to decrease neonatal calf deaths and finally to improve cattle production.

Manipulation of gut microbiota either by feeding microbes, probiotics and prebiotics has been widely studied in livestock animals as a strategy to improve production and health through altered rumen fermentation and preventing pathogen colonization (Uyeno et al., 2015; Weimer, 2015). Direct fed microbials have been shown to decrease rumen acidosis in cattle, increase milk production in cows and decrease fecal shedding of *E. coli* in calves (Krehbiel et al., 2003). These direct fed microbials may prevent enteropathogen colonization of the gut by either competing for nutrients and space in the gut environment, or producing antimicrobial substances (Krehbiel et al., 2003). *Megasphaera elsdenii* modifies ruminal fermentation and decreases ruminal acidosis by utilizing lactic acid produced in the rumen (Krehbiel et al., 2003). However, most of these outcomes are limited to a relatively short interval following feeding (Weimer, 2015) or are effective only in pre-weaned calves (Abe et al., 1995), suggesting that these manipulations are either temporary or need to be instituted within a defined developmental period. Moreover, it is essential to know how the autochthones gut

microbial population responds to these dietary manipulations and how their compositional changes influence overall gut metabolic and immune functions. It may also be important to determine if developing probiotics or direct fed microbials, based on *Faecalibacterium* and *Bifidobacterium* that have already been linked to calf health, provides a more effective or long-lasting effect. The establishment of host-specific bacteria is crucial for the development of mucosal immune system, especially for the differentiation and proliferation of T cell populations (Chung et al., 2012). Thus, there would be substantial value in isolating and testing bacteria within the same host species that might provide the basis for the developing microbial manipulation techniques.

1.5 Methods to study gut microbiota

Composition of gut microbiota and their functions have long been studied using culture-dependent techniques. Staining techniques, microscopy and liquid or agar culture media were used to identify species, whereas pH-based reactions and fermentation capacity (different sugars, protein, lipids) were used to study functions of culturable bacteria (Lagier et al., 2015). However, it is known that only less than 10% of gut microbiota can be studied using the available anaerobic culture methods (Yeoman and White, 2014). Thus, the culture-independent sequencing techniques, which allowed rapid identification of bacteria largely based on 16S rRNA gene sequence (Tringe and Hugenholtz, 2008; Lagier et al 2015), were soon adopted in the gut microbial research. 16S rRNA gene is the most conserved molecule generated from 30S rRNA precursor molecule (Rajendhran and Gunasekaran, 2011), which contains conserved and variable regions. The hypervariable regions (69-99 (V1) 137-242 (V2), 433-497 (V3), 576-682

(V4), 822-879 (V5), 986-1043 (V6), 1117-1173 (V7), 1243-1294 (V8) and 1435-1465 (V9)) in 16S rRNA gene are used in differentiating bacterial species; however, different hypervariable regions are efficient in distinguishing different bacterial groups (Chakravorty et al., 2007). For example, the analysis of pathogenic bacterial 16S rRNA gene sequences revealed that V2 region could distinguish most of pathogens, except species from *Enterobacteriaceae* family (Chakravorty et al., 2007). Although 16S rRNA gene is widely used, there are few limitations with 16S rRNA gene-based bacterial identification. The bacterial species with higher variations in their genome can still be highly similar in their 16S rRNA gene sequences, making it difficult to differentiate such species via 16S rRNA gene sequencing (Rajendhran and Gunasekaran, 2011). Moreover, if there is a horizontal gene transfer within 16S rRNA gene, leading to mosaicism, it can also influence the taxonomic classification of bacteria (Rajendhran and Gunasekaran, 2011). It has also been suggested that sequences of multiple copies of 16S rRNA may not be identical within a species (Rajendhran and Gunasekaran, 2011). Therefore, few other marker genes with a single gene copy in the bacterial genome were used for taxonomic classification of bacteria, such as RNA polymerase beta subunit-encoding gene (rpoB), DNA gyrase (gyrB), GroEL chaperonin, and heat shock protein (dnaK) (Rajendhran and Gunasekaran, 2011). For example, the use of rpoB is more powerful in discriminating *Enterobacteriaceae* family than 16S rRNA gene (Mollet et al., 1997). However, 16S rRNA gene is still the main marker gene used in most of the microbial studies conducted up to date.

The first generation of sequencing was mainly based on Sanger sequencing, which used a termination-based chemistry (Shendure and Ji, 2008). Desired region of 16S

rRNA gene is first amplified using either universal bacterial primers or specific primers and then cloned using plasmid, before sequencing (Chakravorty et al., 2007; Tringe and Hugenholtz, 2008). The main limitation of Sanger sequencing is low throughput, which is overcome by the introduction of NGS approaches (Shendure and Ji, 2008). NGS approaches generate high throughput data via sequencing large number of samples pooled together, after the addition of identifiers/barcodes (Tringe and Hugenholtz, 2008). Use of NGS approaches not only allowed sequencing large number of samples at once, but also drastically decreased the cost of sequencing (Tringe and Hugenholtz, 2008). The two main NGS approaches used by the gut microbiologists are 454 sequencing and Illumina sequencing (Arrieta et al., 2014).

Other than 16S amplicon-based sequencing, NGS can be used to sequence genomic DNA and messenger RNA to study metagenome and metatranscriptome of a gut microbiome, respectively (collective microbial gene content – Yeoman and White, 2014). These two approaches allow studying the functions of microbial communities, while generating taxonomic composition data. Metagenomics approaches provide information on microbial gene composition/functional abundance, whereas metatranscriptomics approaches provide details on the activity of microbial community in terms of gene expression (Hugenholtz and Tyson, 2008). Metagenomics-based study of gut microbiome revealed a highly diverse human gut microbiome that contained over 1000 bacterial species and 3.3 million non-redundant microbial genes (Qin et al., 2010). Moreover, one of these studies broadly categorized human population into three groups, known as enterotypes (*Bacteroides* – enterotype 1, *Prevotella* - enterotype 2, *Ruminococcus* - enterotype 3), based on the gut bacterial composition (Arumugam et al., 2011). These

different enterotypes were enriched with distinctive microbial functions; for example, enterotype 1 microbial functions were enriched with carbohydrate (galactosidases) and protein (proteases) metabolism (Arumugam et al., 2011). This study further revealed that low abundant bacterial taxa might contribute largely to the microbial functions within an ecosystem, which might also represent their survival strategies in a complex environment. This further suggests the importance of low abundance taxa in terms of functions within a microbiome, which is generally overlooked by 16S amplicon-based approaches. Although metagenomics approaches provide in-depth understanding of gut mcirobiome functionality, metatranscriptomics approaches suggest that metagenome can misinterpret the activity of gut microbiota (Fransoza et al., 2014). A comparison between metagenome and metatranscriptome of human gut revealed that 22% of microbial gene families are over or under expressed, when comparing to their abundance (Fransoza, et al., 2014). This indicates that highly abundant microbial genes may not be the highly active genes or *vice versa* in a microbiome. Therefore, combination of 16S amplicon-based approaches along with omics approaches can improve our understanding on gut microbiome as well as its associations with host.

1.6 Knowledge gap

Although there is an increasing interest in studying the calf gut microbiota, these studies still focus primarily on either the rumen (Li et al., 2012; Jami et al., 2013; Rey et al., 2014) or fecal microbiota (Uyeno et al., 2010; Oikonomou et al., 2013). Composition of gut microbiota (Malmuthuge et al., 2012b; Malmuthuge et al., 2014; Romano-Keeler et al., 2014) and host-microbial interactions (Sommer et al., 2015) can be gut region- or

sample type- (mucosa versus digesta) specific, thus studying fecal microbiota may fail to fully explain the role of microbiota on gut health. Therefore, this thesis focuses on the establishment of early microbiota in the calf gut (small intestine and rumen), management practices-associated changes in the colonization process and host-microbial interactions during the early development of calf gut using region-specific (small intestine, rumen) samples.

1.7 Hypothesis and objectives

The hypothesis for the current research project was that region-specific microbiota establishes in the neonatal calf gut during birth and varying calf management practices can shape microbial composition during the succession process. The present study is the first step in understanding the role of early microbiota in calf gut development and health. This knowledge may then be used to identify microbial markers to define healthier calves as well as to design feeding strategies that optimize gut colonization to ensure gut health during early life.

The specific objectives of this research were: 1) to analyze calf small intestinal microbiota composition at birth and to investigate possible associations among the calf intestinal, maternal and birth environment microbiota (Chapter 2); 2) to elucidate short-term (within the first 12 hours) effects of colostrum feeding immediately after birth on microbiota colonization (Chapter 3); 3) to analyze postnatal evolution of the gut microbiome (taxonomy and functions) in the calf small intestine within the first six weeks of life (Chapter 4); and 4) to analyze pre-ruminant rumen colonization and to identify

potential host-microbial interactions that influence the development of rumen (Chapter 5).

1.8 References

Aagaard, K., Ma, J., Antony, K.M., Ganu, R., Petrosino, J., Versalovic, J. (2014). The placenta harbors a unique microbiome. Sci. Transl. Med. 6, 237ra65. doi: 10.1126/scitranslmed.3008599.

Abe, F., Ishibashi, N., Shimamura, S. (1995). Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Dairy Sci. 73, 2838-2846.

Abecia, L., Martin-Garcia, A.I., Martinez-Fernandez, G., Newbold, C.J., Yanez-Ruiz, D.R. (2013) Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. J. Anim. Sci. 91, 4832-4840. doi: 10.2527/jas.2012-6142.

Abecia, L., Waddams, K.E., Martinez-Fernandez, G., Martin-Garcia, A.I., Ramos-Morales, E., Newbold, C.J., Yanez-Ruiz, D.R. (2014a). An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by archaea. Archaea. 2014, Article ID 841463. doi: http://dx.doi.org/10.1155/2014/841463.

Abecia, L., Ramos-Morales, E., Martinez-Fernandez, G., Arco, A., Martin-Garcia, A.I., Newbold, C.J. et al., (2014b) Feeding management in early life influences microbial colonization and fermentation in the rumen of newborn goat kids. Animal Production Science 54, 1449-1454. http://dx.doi.org/10.1071/AN14337.

Adlerberth, I. and Wold, A.E. (2009). Establishment of the gut microbiota in Western infants. Acta Paediatr. 98, 229-238. doi: 10.1111/j.1651-2227.2008.01060.

Al-Saidy, M.Y. (2010). Effect of probiotic bacteria on immunoglobulin G concentration and other blood components of newborn calves. Journal of Animal and veterinary Advances. 9, 604-609. doi:10.3923/java.2010.604.609.

Arrieta, M.C., Stiemsma, L.T., Amenyogbe, N., Brown, E.M., Finlay, B. (2014) The intestinal microbiome in early life: health and disease. Front. Immunol. 5, 427. doi: 10.3389/fimmu.2014.00427.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R. et al., (2011). Enterotypes of the human gut microbiome. Nature. 473, 174-180. doi: 10.1038/nature09944.

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al., (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbe.17, 852. doi: 10.1016/j.chom.2015.05.012.

Brooks, B., Firek, B.A., Miller, C.S., Sharon, I., Thomas, B.C., Baker, R., et al., (2014). Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants. Microbiome, 2, 1. 10.1186/2049-2618-2-1.

Chakravorty, S., Helb, D., Burday, M., Connell, N., Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J. Microbiol. Methods. 69, 330-339.

Chanter, N., Hall, G.A. Bland, A.P., Hayle, A.J., Parsons, K.R. (1984). Dysentery in calves caused by an atypical strain of *Escherichia coli* (S102-9). Vet. Microbiol. 12, 241- 253. doi:10.1016/0378-1135(86)90053-2.

Cho, S.J., Kim, H., Yun, H.D., Cho, K.M., Shin, E.C., Lim, W.J. et al., (2006). 16S rDNA analysis of bacterial diversity in three fractions of cow rumen. J. Microbiol. 16, 92-101.

Chung, H., Pamp, S.J., Hill, J.A., Surana, N.K., Edelman, S.M., Troy, E.B., et al., (2012). Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 149, 1578-1593. doi: 10.1016/j.cell.2012.04.037.

Collado, M.C. and Sanz, Y. (2007). Quantification of mucosa-adhered microbiota of lambs and calves by the use of culture methods and fluorescent in situ hybridization coupled with flow cytometry techniques. Vet. Microbiol. 121, 299-306.

Conroy, M.E., Shi, H.N., Walker, W.A. (2009). The long-term health effects of neonatal microbial flora. Curr. Opin. Allergy Clin. Immunol. 9, 197-201. doi: 10.1097/ACI.0b013e32832b3f1d.

De Barbieri, I., Hegarty, R.S., Silveira, C., Gulino, L.M., Oddy, V.H., Gilbert, R.A. et al., (2015). Programming rumen bacterial communities in newborn Merino lambs. Small Ruminant Research. 129, 48-59. doi:10.1016/j.smallrumres.2015.05.015.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., et al., (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. U S A. 107, 11971-11975. doi: 10.1073/pnas.1002601107.

Doepel, L., Bartier, A. (2014). Colostrum management and factors related to poor calf immunity. WCDS Advances in Dairy Technology. 26, 137- 149.

Fanaro, S., Chierici, R., Guerrini, O., Vigi, V. (2003). Intestinal microflora in early infancy: Composition and development. Acta Paediatr. Suppl. 91, 48-55.

Fonty, G., Gouet, P., Jounay, J.P., Senaud, J. (1987). Establishment of the microflora and anaerobic fungi in the rumen of lambs. Microbiology. 133, 1835-1843, doi: 10.1099/00221287-133-7-1835.

Fonty, G., Senaud, J., Jouany, J.P., Gouet, P. (1988). Establishment of ciliate protozoa in the rumen of conventional and conventionalized lambs: influence of diet and management conditions. Can. J. Microbiol. 34, 235-241.

Fonty, G., Gouet, P., Nebout, J.M. (1989). Development of the cellulolytic microflora in the rumen of lambs transferred into sterile isolators a few days after birth. Can. J. Microbiol. 35, 416-422.

Foughy, F., Guinane, C.M., Hussey, S., Wall, R., Ryan, C.A., Dempsey, E.M. et al., (2012). High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob. Agents Chemother. 56, 5811-5820. doi: 10.1128/AAC.00789-12.

Francino, M.P. (2014). Early development of the gut microbiota and immune health. Pathogens. 3, 769-790; doi:10.3390/pathogens3030769.

Franzosa, E.A., Morgan, X.C., Segata, N., Waldron, L., Reyes, J., Earl, A.M., (2014). Relating the metatranscriptome and metagenome of the human gut. Proc. Natl. Acad. Sci. U S A. 111, E2329-E2338. doi: 10.1073/pnas.1319284111.

Fries, P., Popwych, Y.I., Guan, L.L., Griebel, P.J. (2011). Age-related changes in the distribution and frequency of myeloid and T cell populations in the small intestine of calves. Cell. Immunol. 271, 428-437. doi:10.1016/j.cellimm.2011.08.012.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al., (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 469, 543-547. doi: 10.1038/nature09646.

Funkhouser, L.J., Bordenstein, S.R. (2013). Mom knows best: the universality of maternal microbial transmission. PLoS Biol. 11, e1001631. doi: 10.1371/journal.pbio.1001631.

Gelsinger, S.L., Jones, C.M., Heinrichs, A.J. (2015). Effect of colostrum heat treatment and bacterial population on immunoglobulin G absorption and health of neonatal calves. J. Dairy Sci. 98, 4640-4645. doi: 10.3168/jds.2014-8790.

Gerdts, V., Babiuk, L.A., van Drunen Littel-van den Hurk, S., Griebel, P.J. (2000) Fetal immunization by a DNA vaccine delivered orally into the amniotic fluid. Nat. Medicine. 6, 929-932.

Godden, S. (2008) Colostrum management for dairy calves. Vet. Clin. North Am. Food. Anim. Pract. 24,19-39. doi: 10.1016/j.cvfa.2007.10.005.

Godden, S.M., Smolenski, D.J., Donahue, M., Oakes, J.M., Bey, R., Wells, S. et al., (2012). Heat-treated colostrum and reduced morbidity in preweaned dairy calves: Results of a randomized trial and examination of mechanisms of effectiveness. J. Dairy Sci. 95, 4029-4040. doi: 10.3168/jds.2011-5275.

Gomez, M. Moles, L., Melgar, A., Ureta, N., Bustos, G., Fernandez, L., et al., (2016). Early gut colonization of preterm infants: effect of enteral feeding tubes. J. Pediatr. Gastroenterol. Nutr. 62, 893-900. doi: 10.1097/MPG.0000000000001104.

Gopal, P.K., Gill, H.S. (2000). Oligosaccharides and glycoconjugates in bovine milk and colostrum. Br. J. Nutr. 84, S69- S74.

Groer, M.W., Luciano, A.A., Dishaw, L.J., Ashmeade, T.L., Miller, E., Gilbert, J.A. (2014). Development of the preterm infant gut microbiome: a research priority. Microbiome 2, 38. doi: 10.1186/2049-2618-2-38.

Griebel, P.J., Hein, W.R. (1996). Expanding the role of Peyer's patches in B-cell ontogeny. Immunol. Today. 17, 30-38.

Guarner, F. (2006) Enteric flora in health and disease. Digestion. 73, 5-12.

Hall G.A., Reynold, D.J., Chanter, N., Morgan, J.H., Parsons, K.R., Debney, T.A., et al., (1985). Dysentery caused by *Escherichia coli* (S102-9) in calves: natural and experimental disease. Vet. Pathol. 22, 156-163. doi: 10.1177/030098588502200210.

Hansen, C.H.F., Nielsen, D.S., Kverka, M., Zakostelska, Z., Klimesova, K., Hudcovic, T. et al., (2012). Patterns of early gut colonization shape future immune responses of the host. PLoS One. 7, e34043. doi:10.1371/journal.pone.0034043.

Hansen, R., Scott, K.P., Khan, S., Martin, J.C., Berry, S.H., Stevenson, M., et al., (2015). First-pass meconium samples from healthy term vaginally delivered neonates: an analysis of the microbiota. PLoS One. 10, e0133320. doi: 10.1371/journal.pone.0133320.

Harmsen, H.J., Wildeboer-Veloo, A.C., Raangs, G.C., Wagendorp, A.A., Klijn, N., Bindels, J.G., et al., (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J. Pediatr. Gastroenterol. Nutr. 30, 61-67.

Hart, A.L., Lammers, K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., et al., (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut. 53, 1602-1609.

Heinrichs, J. (2005). Rumen development in the dairy calf. Advances in Dairy Technology. 17, 179-187.

Hooper, L.V. (2004) Bacterial contributions to mammalian gut development. Trends Microbiol. 12, 129-134.

Jakobsson, H.E., Abrahamsson, T.R., Jenmalm, M.C., Harris, K., Quince, C., Jernberg, C., et al., (2014). Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut. 63, 559-566. doi: 10.1136/gutjnl-2012-303249.

Jami, E., Israel, A., Kotser, A., Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7, 1069-1079. doi: 10.1038/ismej.2013.2.

Janke, B.H., Francis, D.H., Collins, J.E., Libal, M.C., Zeman, D.H., Johnson, D.D. (1989). Attaching and effacing *Escherichia coli* infections in calves, pigs, lambs, and dogs. J. Vet. Diagn. Invest. 1, 6-11.

Jiao, J., Huang, J., Zhou, C., Tan, Z. (2015). Taxonomic identification of ruminal epithelial bacterial diversity during rumen development in goats. Appl. Environ. Microbiol. 81, 3502–3509. doi:10.1128/AEM.00203-15.

Jimenez, E., Fernandez, L., Marin, M.L., Martin, R., Odriozola, J.M., Nueno-Palop, C., et al., (2005). Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. Curr. Microbiol. 51, 270-274.

Jimenez, E., Marin, M.L., Martin, R., Odriozola, J.M., Olivares, M., Xaus, J., et al. (2008). Is meconium from healthy newborns actually sterile? Res. Microbiol. 159, 187-193. doi: 10.1016/j.resmic.2007.12.007.

Johansson, M.A., Sjogren, Y.M., Persson, J.O., Nilsson, C., Sverremark-Ekstrom, E. (2011). Early colonization with a group of *Lactobacilli* decreases the risk for allergy at five years of age despite allergic heredity. PLoS One. 6, e23031. doi: 10.1371/journal.pone.0023031.

Jost, T., Lacroix, C., Braegger, C.P., Chassard, C. (2012). New insights in gut microbiota establishment in healthy breast fed neonates. PLoS One, 7, e44595. doi: 10.1371/journal.pone.0044595.

Juge, N. (2012). Microbial adhesins to gastrointestinal mucus. Trends Microbiol. 20, 30-39.

Kim, M., Morrison, M., Yu, Z. (2011). Status of the phylogenetic diversity census of ruminal microbiomes. FEMS Microbiol. Ecol. 76, 49-63. doi: http://dx.doi.org/10.1111/j.1574-6941.2010.01029.

Kim, S-W., Suda, W. Kim, S., Oshima, K., Fukuda, S., Ohno, H., et al., (2013). Robustness of gut microbiota of healthy adults in response to probiotic

intervention revealed by high-throughput pyrosequencing. DNA Res. 20, 241–253. doi:10.1093/dnares/dst006

Krehbiel, C.R., Rust, S.R., Zhang, G., Gilliland, S.E. (2003). Bacterial direct-fed microbials in ruminant diets: Performance response and mode of action. J. Anim. Sci. 81, E120-E132.

Kryzer, A.A., Godden, S.M., Schell, R. (2015). Heat-treated (in single aliquot or batch) colostrum outperforms non-heat-treated colostrum in terms of quality and transfer of immunoglobulin G in neonatal Jersey calves. J. Dairy Sci. 98, 1870-1877. doi: 10.3168/jds.2014-8387.

Lagier, J-C., Hugon, P., Khelaifia, S., Fournier, P-E., Scola, B.L., Raoult, D. (2015). The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. Clin. Microbiol. Rev. 28, 237-264. doi: 10.1128/CMR.00014-14

Lane, B.A. and Jesse, B.W. (1997). Effect of volatile fatty acid infusion on development of the rumen epithelium in neonatal sheep. J. Dairy Sci. 80, 740-746.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., Gordon, J.I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. 6, 776-788.

Li, R.W., Connor, E.E., Baldwin, R.L., Sparks, M.L. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environ. Microbiol. 14, 129-139. doi: 10.1111/j.1462-2920.2011.02543.

Liang, G., Malmuthuge, N., Bao, H., McFadden, T., Stothard, P., Griebel, P.J., et al., (2014). Potential regulatory role of microRNAs in the development of bovine

gastrointestinal tract during early life. PLoS One. 9, e92592. doi: 10.1371/journal.pone.0092592.

Lorenz, I., Fagan, J., More, S.J. (2011). Calf health from birth to weaning. II. Management of diarrhoea in pre-weaned calves. Ir. Vet. J. 64, 9. doi: 10.1186/2046-0481-64-9.

Makino, H., Kushiro, A., Ishikawa, E., Muylaert, D., Kubota, H., Sakai, T., et al., (2011). Transmission of intestinal *Bifidobacterium longum* subsp. *longum* strains from mother to infant, determined by multilocus sequencing typing and amplified fragment length polymorphism. Appl. Environ. Microbiol. 77, 6788-6793. doi: 10.1128/AEM.05346-11.

Makino, H., Kushiro, A., Ishikawa, E., Kubota, H., Gawad, A., Sakai, T., et al., (2013). Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota. PLoS One. 8, e78331. doi: 10.1371/journal.pone.0078331.

Malmuthuge, N., Fries, P., Griebel, P.J., Guan, L.L. (2012a). Regional and age dependent changes in gene expression of toll-like receptors and key antimicrobial defence molecules throughout the gastrointestinal tract of dairy calves. Vet. Immunol. Immunopathol. 146, 18-26. doi:10.1016/j.vetimm.2012.01.010.

Malmuthuge, N., Li, M., Chen, Y., Fries, P., Griebel, P.J., Baurhoo, B. et al., (2012b). Distinct commensal bacteria associated with ingesta and mucosal epithelium in the gastrointestinal tracts of calves and chickens. FEMS Microbiol. Ecol. 79, 337-347. doi: 10.1111/j.1574-6941.2011.01220.

Malmuthuge, N., Griebel, P.J, Guan, L.L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of pre-weaned calves. Appl. Environ. Microbiol. 80, 2012-2028. doi: 10.1128/AEM.03864-13.

Martin, R., Heilig, G.H., Zoetendal, E.G., Smidt, H., Rodriguez, J.M. (2007). Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. J. Appl. Microbiol. 103, 2638-2644.

Marquez, C.J. (2014). Calf intestinal health: Assessment and dietary interventions for its improvement. PhD Thesis. University of Illinois. Urbana-Champaign, USA.

Matsuki, T., Watanabe, K., Tanaka, R., Fukuda, M., Oyaizu, H. (1999). Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. Appl. Environ. Microbiol. 65, 4506-4512.

Mazmanian, S.K., Round, J.L., Kasper, D.L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 453, 620-625. doi: 10.1038/nature07008.

McCann, J.C., Wickersham, T.A., Loor, J.J. (2014). High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. Bioinformatics and Biology Insights. 8, 109-125. doi: 10.4137/BBI.S15389.

Meganck, V., Hoflack, G., Opsomer, G. (2014). Advances in prevention and therapy of neonatal dairy calf diarrhoea: a systematical review with emphasis on colostrum management and fluid therapy. Acta. Vet. Scand. 56, 75. doi: 10.1186/s13028-014-0075-x.

Mollet, C., Drancourt, M., Raoult, D. (1997). rpoB sequence analysis as a novel basis for bacterial identification. Mol. Microbiol. 26, 1005-1011.

Morrill, K.M., Conrad, E., Lago, A., Campbell, J., Quigley, J., Tyler, H. (2012). Nationwide evaluation of quality and composition of colostrum on dairy farms in the United States. J. Dairy Sci. 95, 3997-4005. doi: 10.3168/jds.2011-5174.

Moxley, R.A. and Francis, D.H. (1986). Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect. Immunol. 53, 336-349.

Mulder, I., Schmidt, B., Lewis, M., Delday, M., Stokes, C.R., Bailey, M., et al., (2011). Restricting microbial exposure in early life negates the immune benefits associated with gut colonization in environments of high microbial diversity. PLoS One 6, e28279. doi: 10.1371/journal.pone.0028279.

Musilova, S., Rada, V., Vlkova, E., Bunesova, V. (2014). Beneficial effects of human milk oligosaccharides on gut microbiota. Benef. Microbes. 5, 273-283. doi: 10.3920/BM2013.0080.

Nowacki, M.R. (1993). Cell proliferation in colonic crypts of germ-free and conventional mice - preliminary report. Folia Histochem. Cytobiol. 31, 77–81.

Oikonomou, G., Teixeira, A.G., Foditsch, C., Bichalho, M.L., Machado, V.S., Bicalho, R.C. (2013). Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. PLOS One. 8, e63157. doi: 10.1371/journal.pone.0063157.

OMFARA (2011). Optimizing calf survival at birth. Ontario Ministry of Agriculture, Food and Rural Affairs. Last accessed 2016 June 14. http://www.omafra.gov.on.ca/english/livestock/dairy/facts/optbirth.htm.

Org, E., Parks, B.W., Joo, J.W.J., Emert, B., Schwartzman, W., Kang, E.Y., et al., (2015). Genetic and environmental control of host-gut microbiota interactions. Genome Res. 25, 1558–1569. doi: 10.1101/gr.194118.115

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, S. et al., (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics. 118, 511-521.

Pereira, R.V., Siler, J.D., Ng, J.C., Davis, M.A., Warnick, L.D. (2014). Effect of preweaned dairy calf housing system on antimicrobial resistance in commensal *Escherichia coli*. J. Dairy Sci. 97, 7633-7643. doi: 10.3168/jds.2014-8588.

Petersson, J., Schreiber, O., Hansson, G.C., Gendler, S.J., Velcich, A., Lundberg, J.O., et al., (2011). Importance and regulation of the colonic mucus barrier in a mouse model of colitis. Am. J. Physiol. Gastrointest. Liver Physiol. 300, G327-333. doi: 10.1152/ajpgi.00422.2010.

Pounden, W.D. and Hibbs, J.W. (1948). The influence of ration and rumen inoculation on the establishment of certain microorganisms in the rumens of young calves. J. Dairy Sci. 31, 1041-1050.

Pounden, W.D. and Hibbs, J.W. (1949). The influence of pasture and rumen inoculation on the establishment of certain microorganisms in the rumen of young dairy calves. J. Dairy Sci. 32, 1025-1032. doi: http://dx.doi.org/10.3168/jds.S0022-0302(49)92157-8.

Priestley, D., Bittar, J.H., Ibarbia, L., Risco, C.A,, Galvao, K.N. (2013). Effect of feeding maternal colostrum or plasma-derived or colostrum-derived colostrum replacer on passive transfer of immunity, health, and performance of preweaning heifer calves. J. Dairy Sci. 96, 3247-3256. doi: 10.3168/jds.2012-6339.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., et al., (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 464, 59-65. doi:10.1038/nature08821.

Quigley, J.D. (2002). Passive immunity in newborn calves. Advances in Dairy Technology. 14, 273-292.

Rada, V., Vlkova, E., Nevoral, J., Trojanov, I. (2006). Comparison of bacterial flora and enzymatic activity in faeces of infants and calves. FEMS Microbiol. Lett. 258, 25-28. doi:10.1111/j.1574-6968.2006.00207.

Rajendhran, J. and Gunasekaran, P. (2011). Microbial phylogeny and diversity: small subunit ribosomal RNA sequence analysis and beyond. Microbiol. Res. 166, 99-110. doi: 10.1016/j.micres.2010.02.003.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 118, 229-241.

Rey, M., Enjalbert, F., Monteils, V. (2012). Establishment of ruminal enzymatic activities and fermentation capacity in dairy calves from birth through weaning. J. Dairy Sci. 95, 1500-1512. http://dx.doi.org/ 10.3168/jds.2011-4902.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. J. Appl. Microbiol. 116, 245-257. doi: 10.1111/jam.12405.

Reynolds, J.D., Morris, B. (1984). The effect of antigen on the development of Peyer's patches in sheep. Eur. J. Immunol. 14, 1-6.

Rieu, F., Fonty, G., Gaillard, B., Gouet, P. (1990). Electron microscopy study of the bacteria adherent to the rumen wall in young conventional lambs. Can. J. Microbiol. 36, 140-144.

Rinne, M.M., Gueimonde, M., Kalliomaki, M., Hoppu, U., Salminen, S.J., Isolauri, E., et al., (2005). Similar bifidogenic effects of prebiotic-supplemented partially hydrolyzed infant formula and breastfeeding on infant gut microbiota. FEMS Immunol. Med. Microbiol. 43, 59-65.

Rodriguez, J.M., Murphy, K., Stanton, C., Ross, R.P., Kober, O.I., Juge, N., et al., (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. Microb. Ecol. Health Dis. 26, 26050. 10.3402/mehd.v26.26050.

Romano-Keeler, J., Moore, D.J., Wang, C., Brucker, R.M., Fonnesbeck, C., Slaughter, J.C. et al., (2014). Early life establishment of site-specific microbial communities in the gut. Gut Microbes. 5, 192-201. doi: 10.4161/gmic.28442.

Shendure, J. and Ji, H. (2008). Next-generation DNA sequencing. Nat. Biotechnol. 26, 1135 – 1145. doi:10.1038/nbt1486

Sjogren, Y.M., Jenmalm, M.C., Bottcher, M.F., Bjorksten, B., Sverremark-Ekstrom, E. (2009). Altered early infant gut microbiota in children developing allergy up to 5

years of age. Clin. Exp. Allergy. 39, 518-526. doi: 10.1111/j.1365-2222.2008.03156.

Smith, H.W. (1965). The development of the flora of the alimentary tract in young animals. The Journal of Pathology and Bacteriology. 90, 495-513. doi: 10.1002/path.1700900218.

Sokol, H., Seksik, P., Furet, J.P., Fermisse, O., Nion-Larmurier, I., Beaugerie, L. et al., (2009). Low counts of *Faecalibacterium prausnitzii* in colitis microbiota. Inflammatory Bowel Diseases. 15, 1183-1189. doi: 10.1002/ibd.20903.

Sommer, F. and Backhed, F. (2013). The gut microbiota - masters of host development and physiology. Nat. Rev. Microbiol. 11, 227-238. doi: 10.1038/nrmicro2974.

Sommer, F., Nookaew, I., Sommer, N., Fogelstrand, P., Backhed, F. (2015). Site-specific programming of the host epithelial transcriptome by the gut microbiota. Genome Biol. 16, 62. doi: 10.1186/s13059-015-0614-4.

Spicer, H.M., Goonewardene, L.A., McNeil, A.O., Slack, W.L. (1994). Alberta dairy farm survey response. J. Dairy Sci. 77, 3460-3472.

Subramanian, S., Blanton, L.V., Frese, S.A., Charbonneau, M., Mills, D.A., Gordon, J.I. (2015). Cultivating healthy growth and nutrition through the gut microbiota. Cell. 161, 36-48.

Tajima, K., Aminov, R.I., Nagamine, T., Matsui, H., Nakamura, M., Benno, Y. (2001). Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Enivorn. Microbiol. 66, 2766-2774. doi: 10.1128/AEM.67.6.2766-2774.2001.

Teran, R., Mitre, E., Vaca, M., Erazo, S., Oviedo, G., Hubner, M.P. et al., (2011). Immune system development during early childhood in tropical Latin America: evidence for the age-dependent down regulation of the innate immune response. Clin. Immunol. 138, 299-310.

Tringe, S.G. and Hugenholtz, P. (2008). A renaissance for the pioneering 16S rRNA gene. Curr. Opin. Microbiol. 11, 442-446. doi:10.1016/j.mib.2008.09.011

Tripathi, V., and Vashishtha, B. (2006). Bioactive compounds of colostrum and its application. Food Reviews International. 22, 225-244. doi: 10.1080/87559120600694606

Turroni, F., Peano, C., Pass, D.A., Foroni, E., Severgnini, M., Claesson, M.J., et al., (2012). Diversity of bifidobacteria within the infant gut microbiota. PLoS One. 7, e36957. doi: 10.1371/journal.pone.0036957.

Uetake, K. (2013). Newborn calf welfare: A review focusing on mortality rates. Anim. Sci. J. 84,101-105. doi: 10.1111/asj.12019.

Underwood, M.A., German, J.B., Lebrilla, C.B., Mills, D.A. (2015). *Bifidobacterium longum* subspecies *infantis*: champion colonizer of the infant gut. Pediatr Res. 77, 229-235. doi: 10.1038/pr.2014.156.

Unger, S., Stintzi, A., Shah, P., Mack, D., O'Connor, D.L. (2015). Gut microbiota of the very-low-birth-weight infant. Pediatr Res. 77, 205-213. doi: 10.1038/pr.2014.162.

USDA (2010). Dairy 2007, Heifer calf health and management practices on US dairy operations. pp 122-131. USDA:APHIS:VS, CEAH. Fort Collins, CO.

Uyeno, Y., Sekiguchi, Y., Kamagata, Y. (2010). rRNA-based analysis to monitor succession of fecal bacterial communities of Holstein calves. Lett. Appl. Microbiol. 51, 570-577. doi: 10.1111/j.1472-765X.2010.02937.

Uyeno, Y., Shigemori, S., Shimosato, T. (2015). Effect of probiotics/prebiotics on cattle health and productivity. Microbes. Environ. 30, 126-132. doi: 10.1264/jsme2.ME14176.

Van den Abbeele, P., Van de Wiele, T., Verstraete, W., Possemiers, S. (2011). The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiol. Rev. 35, 681-704. doi: 10.1111/j.1574-6976.2011.00270.

Vasseur, E., Borderas, F., Cue, R.I., Lefebvre, D., Pellerin, D., Rushen, J., et al., (2010). A survey of dairy calf management practices in Canada that affect animal welfare. J. Dairy Sci. 93, 1307-1315. doi: 10.3168/jds.2009-2429.

Vlkova, E., Nevoral, J., Rada, V. (2006). Distribution of bifidobacteria in the gastrointestinal tract of calves. Folia Microbiol. 51, 325-328.

Waltner-Toews D., Martin, S.W., Meek, A.H. (1986). The effect of early calfhood health status on survivorship and age at first calving. Can. J. Vet. Res. 50, 314-317.

Weimer, P.J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. Front. Microbiol. 6, 296. doi: http://dx.doi.org/10.3389/fmicb.2015.00296.

Yasuda, M., Fujino, M., Nasu, T., Murakami, T. (2004). Histological studies on the ontogeny of bovine gut-associated lymphoid tissue: appearance of T cells and

development of IgG+ and IgA+ cells in lymphoid follicles. Dev. Comp. Immunol. 28, 357-369.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., et al (2012). Human gut microbiome viewed across age and geography. Nature. 486, 222-227. doi: 10.1038/nature11053.

Yeoman, C.J., White, B.A. (2014). Gastrointestinal tract microbiota and probiotics in production animals. Ann. Rev. Anim. Biosci. 2, 469-486. doi: 10.1146/annurev-animal-022513-114149.

Zhao, T., Doyle, M.P., Harmon, B.G., Brown, C.A., Mueller, P.O.E., Parks, A.H. (1998). Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol. 36, 641-647.

1.9 Tables and Figures

Table 1.1 Factors influencing pre-weaned calf rumen/gut microbiota.

Factor	Study
Age	Fonty et al., 1987, Uyeno et al., 2010, Li et al., 2012, Malmuthuge et al., 2012, Jami et al., 2013, Oikonomou et al., 2013, Rey et al., 2014, Jiao et al., 2015
Diet (colostrum, calf starter)	Uyeno et al., 2010, Abecia et al., 2013, Malmuthuge et al., 2013, Abecia et al, 2014a, Rey et al., 2014, De Barbieri et al., 2015
Feeding method (suckling, bottle feeding)	Abecia et al., 2014b
Probiotics, prebiotics	Abe et al., 1995, Marquez, 2014
Exposure to dam	Fonty et al., 1989, Abecia et al., 2014b
Sample site	Smith, 1965, Collado and Sanz, 2007, Malmuthuge et al., 2012, Malmuthuge et al., 2014
Sample type (fluid, content, mucosa)	Reiu et al., 1990, Malmuthuge et al., 2012, Malmuthuge et al., 2014
Host (individuality)	Jami et al., 2013
Infections	Oikonomou et al., 2013

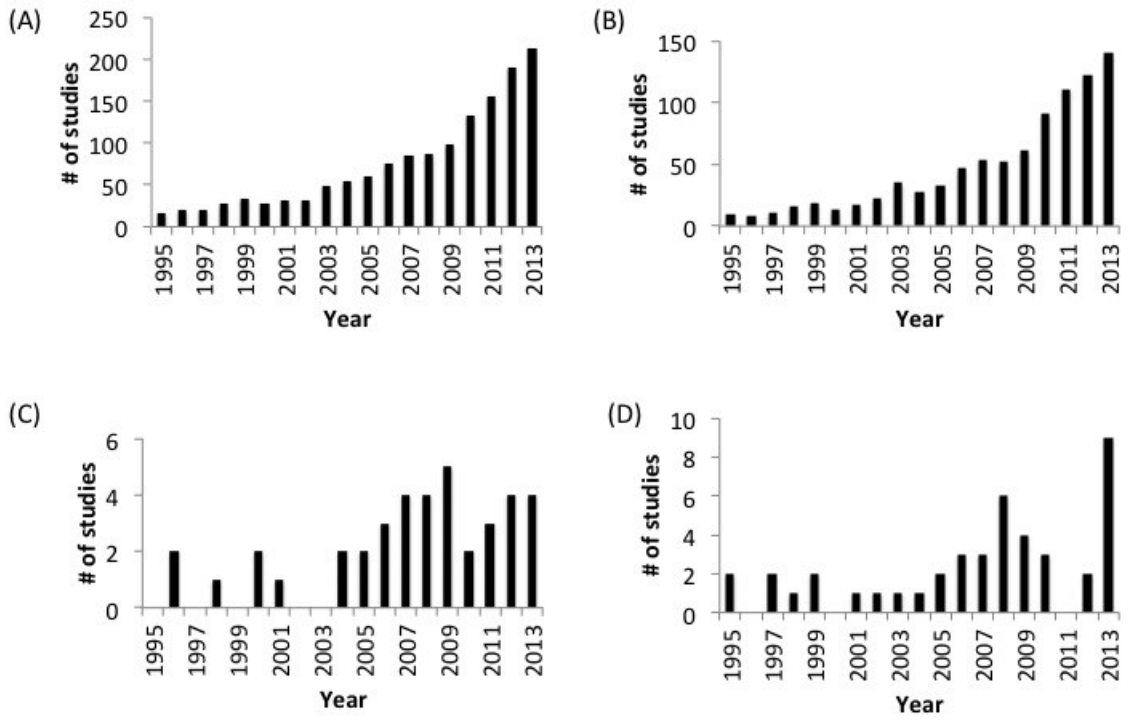

Figure 1.1 Number of publication entries in Medline (PubMed) trend* from 1995 – 2013. (A) Publication entries searched with query "gut colonization" (B) Publication entries searched with query "gut colonization and human" (C) Publication entries searched with query "gut colonization and ruminant" (D) Publication entries searched with query "rumen colonization." * Medline Trend, URL:http://dan.corlan.net/medline-trend.html.

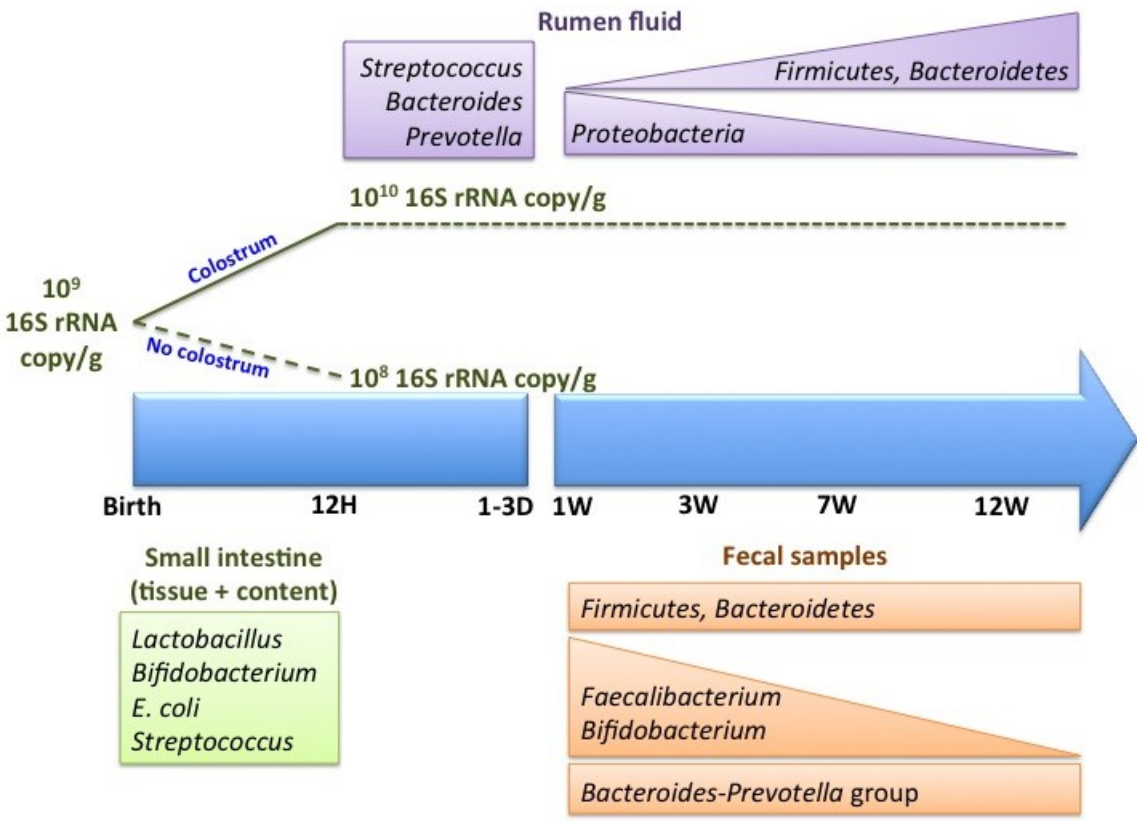

Figure 1.2. Colonization of neonatal calf rumen/gut, immediately postpartum and within the first 12 weeks of life.

Chapter 2. Assessment of the initial gut microbiota at birth revealed the establishment of region-specific microbiomes in neonatal ruminants

Abstract

Initial microbial colonization plays an important role in neonatal gut health. However, studies examining the gut microbial composition at birth are challenging due to limited access for accurate sampling. The present study characterized the jejunal and ileal bacterial composition (epimural and luminal) of neonatal calves within five minutes after birth and compared the calf microbiota with the maternal (birth canal and rectum) and birth environment microbiota. RNA-based quantification along with 454 pyrosequencing revealed the colonization the newborn calf small intestine by active, dense (1.1 – 9.4E08 16S rRNA copy/g of sample) and diverse bacterial populations. *Pseudomonadaceae* and *Propionibacteriaceae* dominated the epimural communities, whereas *Propionibacteriaceae*, *Prevotellaceae*, *Ruminococcaceae* and *Lachnospiraceae* dominated the luminal communities. The calf gut bacterial composition at birth was significantly different from the birth canal and rectal bacteria, especially for *Bifidobacterium* species. *Bifidobacterium longum* subsp. *infantis* dominated the calf gut, but only *B. pseudolongum* and *B. longum* were detected in the birth canal and rectum. The bacterial composition of the calf body habitat was similar to that in the birth environment, which was again divergent from the gut microbiota. This study suggested the establishment of a small intestinal-specific microbiota during birth that deviated substantially from the maternal microbiota.

2.1 Introduction

A dynamic microbial population colonizes the mammalian gut after birth, and this process is modulated by the host, microbes and environmental factors (Van den Abbeele et al., 2011). The environmental factors may include the maternal microbiota, the birthing process, diet and antibiotic treatments (Fanaro et al., 2003; Adlerberth and Wold, 2009; Dominguez-Bello et al., 2010). The importance of microbial colonization in the subsequent development of the mucosal immune system has been thoroughly described in human and mouse (Hooper, 2004). Furthermore, recent evidence has highlighted the impact of the microbiota on the health of children (Subramanian et al., 2015). Moreover, the presence of "age-discriminatory taxa" fine-tunes the establishment of a healthy gut microbiome, which can serve a role in preventing colonization by enteropathogens (Subramanian et al., 2015). For example, *Ruminococcus obeum* has been reported to be associated with healthy gut microbial establishment in children and to restrict colonization by *Vibrio cholerae* in humanized mice (Subramanian et al., 2015). Thus, it is evident that the early gut microbiota may have long-term impacts on host health and the establishment of autochthonous microbes, although the composition does not stabilize until the introduction of a solid diet (Yatsunenko et al., 2012). Furthermore, alterations in the microbial succession pattern, such as the delayed colonization of *Bacteroidetes* in the gut, were associated with reduced Th1 responses (Jakobsson et al., 2014), suggesting that the succession process is as important as early microbiota composition.

Due to its immense importance to the host health, the early gut microbiome (composition, colonization process) has been well studied in humans and mouse models. However, studies on the gut microbiota at birth are challenging due to limitations in

sampling, and the first pass meconium is often used as a proxy. The collection time of the first pass meconium can vary from a few hours to a day after the birth of human infants (Jimenez et al., 2008; Hansen et al., 2015), and this sample may not be representative of the gut microbiome at birth. In addition, establishment of gut region-specific microbiota may regulate intestinal epithelium development in a region-specific manner (Sommer et al., 2015), suggesting the importance of understanding the local establishment of microbiota.

To date, our understanding of the initial colonizers in the gut, especially the small intestine of ruminants, is very limited. Gut health during early life is an emerging research area that aims to reduce the impact of infectious diseases and antibiotics usage in the cattle industry. Therefore, this study investigated small intestinal bacterial composition at birth and compared it with the calf body habitat, maternal environment (birth canal and rectum) and birthing environment (calving pen floor). This analysis provided information regarding initial microbial composition and the source of the inoculum, which could provide a basis for the future improvement of gut health in young ruminants.

2.2 Materials and Methods

2.2.1 Animal experiments and sampling

All experimental protocols were approved by the Livestock Care Committee of the University of Alberta (AUP00001012) and were conducted following the guidelines of the Canadian Council on Animal Care. All calves and dams were obtained from the Dairy Research and Technology Center (DRTC), University of Alberta (Edmonton, AB).

Dams with male fetuses were transferred into calving pens a week before their predicted due dates and were closely monitored through cameras. The newborn calves (n = 6) were removed from dams soon after birth using clean plastic containers to prevent the calves from contacting the floor, immediately transferred to a surgery room, and humanely euthanized within five minutes. Small intestinal tissue and contents samples were collected as closed segments at pre-determined regions within 30 minutes after euthanization. All samples were snap frozen in liquid nitrogen and stored at -80°C. Briefly, the esophagus and rectum were first ligated to occlude the lumen and prevent environmental contamination of the intestine. The proximal jejunum was defined as 100 cm distal to the pylorus sphincter, the distal jejunum was defined as 30 cm proximal to the collateral branch of the cranial mesenteric artery and the ileum was defined as 30 cm proximal to the ileo-cecal junction. Ten centimeter-long intestinal segments were collected in the middle of each segment aligned with the above-mentioned measurements.

The rectal and birth canal swab samples from respective dams were collected using BD Screw Cap Single SWUBE™ Applicators (polyester) (Becton, Dickinson and Company, NJ, USA) immediately following calving. A similar approach was used to collect calf body habitat swabs (nose, mouth, skin) before euthanasia. Litter materials/shavings from the calving pens were also collected immediately following calving.

2.2.2 Nucleic acid isolation

Total genomic DNA was extracted from the small intestinal tissue and contents samples using the repeated bead-beating plus column method (Yu and Morrison, 2004).

Total DNA from the swab samples and litter material was extracted using the GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, MA, USA), following the manufacturer's instructions for Gram-positive bacteria genomic DNA purification. DNA quality and quantity were measured using an ND 1000 spectrophotometer (NanoDrop Technologies, DE, USA). The intestinal epithelium was scraped along with its contents to extract total RNA for the estimation of active/live bacteria using quantitative real-time PCR. Total RNA was extracted from the intestinal epithelial tissue and contents together using a mirVana™ miRNA isolation kit (Ambion, Carlsbad, CA), following the manufacturer's instructions.

2.2.3 Diversity and phylogenetic analyses[5]

The diluted total DNA (25 ng/µl) from all samples was used to amplify the V1-V3 region using universal bacterial primers (9F - 5'-ACACTGACGACATGGTTCTACAGAGTTTGATCMTGGCTCAG-3' and 519R - 5'-TACGGTAGCCAGAGACTTGGTCTCCGCGGCKGCTGGCAC-3') with added tags. PCR products (~500 bp) were purified using a 1% agarose gel and the QIAEX II gel extraction kit (QIAGEN Science, MD, USA) following the manufacturer's instructions. Barcodes were added to the purified PCR products, and they were subjected to 454 sequencing using the Roche GS-FLX System with titanium chemistry at Genome Quebec, McGill University (Quebec, Canada). Phylogenetic assignments and the analysis of bacterial diversity (α- and β-diversity) were performed using the Quantitative Insights Into Microbial Ecology (QIIME) software version 1.8.0 (Caporaso et al., 2010). Briefly,

[5]Generation of PCR amplicons for 454 sequencing was done by the laboratory technician Yanhong Chen

the raw sequences were first filtered through a quality control pipeline to retain sequences longer than 200 bp and bases with a Phred quality score > 25. Then, the chimeric sequences were filtered out using usearch61 within the QIIME platform, and the remaining high quality sequences were used for taxonomic assignments using the Greengenes database 13_8 (2013 July release). Raw sequences were deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRP072997.

2.2.4 Co-occurrence analysis of identified bacterial families

A co-occurrence analysis was performed using the relative abundance of bacterial families and Spearman rank correlation analysis to explore the interactions among initial bacteria colonizing the calf small intestine. A positive correlation ($\rho > 0.5$, $P < 0.05$) between two families was considered a co-occurrence incidence, and the distance was calculated using the Spearman rank correlation value (distance $=1 - \rho$). The distance values were used to generate co-occurrence networks using Cytoscape 3.3.0 (Shannon et al., 2003).

2.2.5 Quantification of bacterial density[6]

Diluted DNA and RNA were used to estimate total bacteria, *Lactobacillus* and *Bifidobacterium* densities using SYBR green chemistry (Fast SYBR® Green Master Mix, Applied Biosystems) with a StepOnePlus real-time PCR system (Applied Biosystems) employing universal bacterial primers and genus-specific primers (Liang et al., 2014). The standard curves for total bacteria, *Lactobacillus* and *Bifidobacterium* were generated

[6] qPCR was performed by laboratory technician Yanhong Chen

using purified 16S rRNA genes of *Butyrivibrio hungateii*, *Lactobacillus acidophilus* ATCC4356 and *Bifidobacterium longum,* respectively. Bacterial density (DNA and RNA-based) was then calculated using the equation described by Li et al., (2009).

2.2.6 Taxonomic identification of the ileal *Bifidobacterium* population[7]

The extracted total DNA was used to amplify 16S rRNA genes with primers targeting the genus *Bifidobacterium* (BifF- 5'-CTCCTGGAAACGGGTGG-3' and BifR-5'-GGTGTTCTTCCCGATATCTACA-3') (Matsuki et al., 2002). 16S clone libraries were prepared using the purified PCR products (Malmuthuge et al., 2012) to further explore the *Bifidobacterium* composition in the calf ileum at birth. Briefly, the purified PCR products from intestinal tissue and content samples of individual calves were first pooled and cloned into a TOP10 vector (TOPOTA cloning kit; Invitrogen, Carlsbad, CA, USA) using chemical transformation. Plasmid DNA extraction, sequencing and taxonomic assignment were performed according to the method described by Malmuthuge et al., 2012. Sequence data were deposited in the NCBI database under the accession numbers KF828998 – KF829902.

2.2.7 Statistical analysis

Effects of sample type (tissue versus content) and small intestinal region were evaluated using group_significance.py function and Kruskal-wallis non-parametric ANOVA with P value corrected by the Benjamini-Hochberg FDR procedure for multiple comparisons within QIIME. Significant comparisons were declared at FDR < 0.05. Beta

[7] Preparation of clone libraries and sequencing was performed by laboratory technician Yanhong Chen

diversity was computed using beta_diversity.py and the weighted UniFrac distance matrices were used to perform analysis of similarity (ANOSIM) using compare_category.py function to declare bacterial communities (intestinal, body habitat, maternal and birth environmental communities) different from each other (ANOSIM R 0.5-1, $P < 0.05$).

Bacterial population data (total bacteria density, density/proportion of *Bifidobacterium*, density/proportion of *Lactobacillus*) were analyzed using SAS version 9.4 (SAS Inc., Cary, NC) and analysis of variance. A repeated-measures experimental design was used with small intestinal region as the repeated measurement and individual animal as the experimental unit. Compound symmetry covariance structure was selected as the best fit by the lowest Bayesian information criteria (BIC) and the following statistical model was fitted to test the effect of small intestinal region and sample type on bacterial populations: $Y_{ijk} = \mu + R_i + S_j + TS_{(ij)} + e_{ijk}$, where Y = bacterial density/proportion (total bacteria, *Lactobacillus*, *Bifidobacterium*); μ = mean; R = small intestinal region; S = sample type (tissue, content); and e = residual error. Differences in least square means were declared at $P < 0.05$ using the PDIFF option in SAS when applicable.

2.3 Results

2.3.1 Active and dense bacterial population at birth

DNA and RNA-based estimations revealed dense and active bacterial colonization in the calf small intestine at birth (Figure 2.1). Live/active bacterial densities detected using RNA-based approach were lower than the densities detected using DNA-based

approach that estimates dead and active bacteria (Figure 2.1). Although the bacterial densities (total, *Bifidobacterium*, *Lactobacillus*) were not different among intestinal regions, the proportion of *Bifidobacterium* was higher in the ileum than in the proximal and distal jejunum (Figure 2.1). When the composition of *Bifidobacterium* in the ileum was further explored, more than half of *Bifidobacterium* (69.8%) could not be identified at the species level. However, *B. longum* subsp. *infantis* (23.9%) accounted for the majority of the identified species in the calf ileum at birth. The other *Bifidobacterium* species identified in the calf small intestine at birth were *B. pseudolongum*, *B. pseudolongum* subsp. *pseudolongum* and *B. boum* (Figure 2.2).

2.3.2 Taxonomic assessment of the gut microbiota at birth

The small intestine epimural (tissue-attached) and luminal (content-associated) communities of the calves at birth were already colonized with a diverse bacterial population comprised of 12 phyla (Figure 2.3A). *Proteobacteria* (40.4±4.9%), followed by *Firmicutes* (23.2±3.0%), *Actinobacteria* (20.5±3.7%) and *Bacteroidetes* (10.7±1.8%) dominated the calf small intestine, regardless of the sample type (epimura versus lumen). In total, 89 bacterial families and 122 genera were identified from all of the small intestinal regions. Among the detected families, *Pseudomonadaceae*, *Propionibacteriaceae* and *Ruminococcaceae* dominated the calf gut at birth, while *Pseudomonas*, *Propionibacterium, Prevotella* and *Bacillus* were predominant at the genus level of the taxonomic hierarchy (Figure 2.3B). However, the abundance of these detected bacterial genera varied greatly among individuals. For example, the abundance

of *Pseudomonas* ranged from 0.3% to 83.8%, *Propionibacterium* ranged from 0 to 86.4% and *Prevotella* ranged from 0 to 20.8% in six newborn calves.

The luminal bacteria were dominated by *Firmicutes* (28.6±4.2%), followed by *Proteobacteria* (27.4±5.3%), *Actinobacteria* (20.2±5.1%) and *Bacteroidetes* (15.9±2.8%), but their abundances were numerically different among the small intestinal regions. For example, *Firmicutes* dominated the ileal lumen (36.7±7.4%), while *Actinobacteria* dominated the distal jejunal lumen (37.3±11.3%). Although *Firmicutes* was abundant in all of the luminal communities, the majority of them were only identified at either the bacterial order or the family levels of the taxonomic hierarchy. For example, most of the *Firmicutes* were only assigned to order *Clostridiales* (6.8±2.0%) and families *Ruminococcaceae* (3.5±1.1%) and *Lachnospiraceae* (4.9±1.9%). The abundance of the dominant bacterial genera was also numerically different among the three small intestinal regions. The abundance of *Prevotella* was higher in the lumen of the ileum (14.2±1.4%) than the jejunal communities (proximal jejunum - 6.9±3.5%; distal jejunum - 5.0±1.7%), whereas the abundance of *Pseudomonas* was lower in the lumen of the ileum (1.4±0.7%) than the jejunal communities (proximal jejunum - 15.8±13.5%; distal jejunum - 9.9±4.3%).

The epimural bacteria were dominated by *Proteobacteria* (53.4±7.2%), *Firmicutes* (17.8±3.9%), *Actinobacteria* (20.8±5.6%) and *Bacteroidetes* (5.6±1.7%); however, *Actinobacteria* was the second most predominant phylum in the jejunal epimural communities (proximal jejunum - 34.0±14.9%; distal jejunum - 20.8±4.2%). Similar to the luminal bacteria, the abundance of the observed dominant bacterial genera was numerically different among the three small intestinal regions. *Pseudomonas* was the

most abundant genus in the epimura of the distal jejunum (32.4±12.3%) and ileum (39.2±12.9%); however, *Propionibacterium* (30.6±15.8%) dominated the proximal jejunum. Moreover, the abundance of *Bacillus* was higher in the distal jejunum (11.9±7.3%) than the proximal jejunum (2.5±1.9%) and ileum (1.8±1.7%).

When the bacterial composition was compared between the lumen and epimural communities, abundances of detected bacterial phylotypes were not statistically different. A higher prevalence of *Bacteroidetes* was observed in the small intestinal lumen (proximal jejunum - 11.7±5.0%; distal jejunum - 9.4±3.4%; ileum - 26.6±2.7%), than the corresponding epimural communities (proximal jejunum - 0.6±0.2%; distal jejunum - 6.6±2.6%; ileum - 9.5±3.8%). At the genus level, *Propionibacterium* (17.8±5.0%), *Pseudomonas* (9.0±4.7%), *Prevotella* (8.7±1.6%), *Ralstonia* (3.1±1.7%), *Ruminococcus* (2.8±1.2%), *Lactobacillus* (1.8±0.6%), *Bacillus* (1.6±1.2%), *Corynebacterium* (1.3±1.0%), *Succinivibrio* (1.1±0.6%) and *Butyrivibrio* (1.0±0.6%) were abundant (> 1% relative abundance) in the luminal communities. Additionally, *Pseudomonas* (33.8±7.6%), *Propionibacterium* (19.6±5.6%), *Bacillus* (5.4±2.7%), *Prevotella* (3.6±1.4%), *Ralstonia* (2.3±0.9%), *Lysinibacillus* (3.5±1.3%), *Akkermansia* (1.7±1.7%) and *Burkholderia* (1.5±0.9%) were also predominant in the epimura. Among the observed genera, *Burkholderia* and *Akkermansia* were not identified in any of the luminal communities, while *Coprococcus*, *Succinivibrio*, *Staphylococcus* and *Clostridium* were absent in the epimural communities.

In addition to the composition, bacterial richness (number of families/genera) was different among the small intestinal regions, between the epimural and luminal communities and among individual calves (Table 2.1). The number of identified families

and genera in the ileal lumen was numerically higher than the jejunal communities (Table 2.1). Similarly, a significantly higher bacterial diversity was observed in the ileum (phylogenetic distance, observed species, Shannon index) when comparing two jejunal regions, regardless of the gut environment (epimura versus lumen) (Table 2.1). However, the bacterial diversity and richness were lower in the small intestinal epimural communities at birth compared to the luminal communities (Table 2.1).

2.3.3 *Firmicutes* and *Bacteroidetes* families co-occur at birth

When the co-occurrence of bacteria at birth was explored, families from *Firmicutes* and *Bacteroidetes* phyla were mainly found as the hubs of the co-occurrence network. The main *Firmicutes* families detected in the network hubs were *Lachnospiraceae* (14 positive correlations), *Clostridiaceae* (8 positive correlations), *Ruminococcaceae* (5 positive correlations) and an unidentified family belonging to the order *Clostridiales* (13 positive correlations). *Prevotellaceae* (10 positive correlations) and *Bacteroidaceae* belonging to the order *Bacteroidetes* were also among the main hubs identified in the co-occurrence network. The only *Proteobacteria* family observed among the main hubs was *Succinivibrionaceae*, with 10 positive correlations identified from all three small intestinal regions (Figure 2.4).

2.3.4 Comparison of maternal, environmental and calf body habitat microbiota

The microbial compositions of the maternal communities (rectum and birth canal of dams), the calving pen floor/birth environment, and the calf body habitats (mouth, nose, skin) were explored to understand the impact of exposure to the dam and

environment on early gut bacterial composition. Ten phyla were identified from the rectal and birth canal communities of dams, and the dominant phylum was *Firmicutes* (Figure 2.5). In total, there were 44 bacterial families identified from the birth canal environment. However, the relative abundances of eight dominant families (*Streptococcaceae*, *Aerococcaceae*, *Ruminococcaceae*, *Clostridiaceae*, *Peptostreptococcaceae*, *Lachnospiraceae*, *Bacteroidaceae*, and *Corynebacteriaceae*) accounted for 77.2% of total detected bacterial families. There were seven dominant families (*Ruminococcaceae*, *Clostridiaceae*, *Peptostreptococcaceae*, *Lachnospiraceae*, *Bacteroidaceae*, *Veillonellaceae* and *Streptococcaceae*) in the rectum that accounted for 59.2% of the 44 detected bacterial families. The main bacterial genera identified from the maternal communities were *Streptococcus, Facklamia*, *Clostridium* and *Ruminococcus* (Table 2.2). The calf body habitat (10 phyla) and the birth environment (9 phyla) bacteria mainly consisted of *Firmicutes* and *Proteobacteria* at the phylum level (Supplementary Figure 2.5). The main families identified from these communities were *Aerococcaceae*, *Staphylococcaceae* and *Xanthomonadaceae*, whereas the main genera observed were *Jeotgalicoccus* and *Facklamia* (Table 2.2).

The operational taxonomic units (OTUs) generated from the environmental, calf body habitat and maternal communities were compared with the calf small intestinal communities using weighted UniFrac distance matrices (Figure 2.6) and analysis of similarity (ANOSIM) (Table 2.3) in QIIME. The bacterial communities of the calf small intestine at birth were significantly different from those of the maternal, calf body habitat and birth environments (Table 2.3, Figure 2.6A, 2.6B, 2.6C). Although *Firmicutes* was one of the main phyla in all of these communities, its composition and abundance were

different at lower levels of the taxonomical hierarchy. For example, *Ruminococcus* was mainly found in the calf gut, whereas *Streptococcus*, *Facklamia*, and *Clostridium* dominated the maternal communities (Table 2.2). Although the calf body habitat communities were different from maternal communities, they were not different from those in the birth environment (Table 2.3, Figure 2.6D). The microbiota observed in the calf body habitat at birth were similar to the birth environment (calving pen floor) microbiota, which were dominated by facultative anaerobes (*Jeotgalicoccus*, *Facklamia*) and aerobes (*Corynebacterium*). Additionally, the birth environment could not be differentiated from the rectal microbiota (ANOSIM R = 0.0741, $P = 0.50$) but was different from that of the birth canal (ANOSIM R = 0.4012, $P < 0.01$).

2.4 Discussion

The early microbiome, which plays a crucial role in the development of the mucosal immune system, subsequent microbial succession and host health, has been well studied in human and mouse models; however, knowledge in ruminants is very limited. This is the first attempt to report the colonization of the small intestinal epimural and luminal communities with an active bacterial population soon after birth and to evaluate their link to the maternal, birth environment and calf body habitat microbiota. Use of next-generation sequencing along with RNA-based quantification of bacteria residing the small intestinal tissue and content samples revealed that newborn calf gut colonized with a dynamic microbial population, which mainly consisted of *Proteobacteria*. This observation suggests that gut microbial establishment may have started during the birthing process. The process of calving (from rupturing of amniotic membranes and

leaking of amniotic fluid till delivering the calf) extended nearly around an hour to three hours, which may allow dense and diverse microbial colonization in calf gut. The bacterial composition of the calf gut at birth differed from that of three-week-old calves (Malmuthuge et al., 2014) as well as the fecal microbiota of one-week-old calves (Oikonomou et al., 2013). *Firmicutes* (> 97%) dominated the jejunal and ileal digesta-associated bacteria of three-week-old calves fed milk and calf starter (Malmuthuge et al., 2014). *Bacteroidetes* were predominant in the jejunal (23.6%) and ileal (33.7%) tissue-attached communities at three weeks (Malmuthuge et al., 2014). Studies on fecal bacterial communities have also reported a higher abundance of *Firmicutes* in one-week-old calves (Uyeno et al., 2010; Oikonomou et al., 2013). However, the present study revealed a lack (< 10%) of these obligate anaerobes in the calf gut at birth. Thus, these studies indicate that obligate anaerobes belonging to *Firmicutes* and *Bacteroidetes*, which are dominant in the gut microbiomes (Ley et al., 2008), establish gradually and become the predominant bacteria with increasing calf age. In addition to the observed differences among the fecal or small intestinal bacteria of older calves, the calf gut microbiota also differed from the meconium bacterial composition of human infants. The gut microbiota of human infants is dominated by *Lactobacillus*, *Bifidobacterium, Enterobacteriaceae,* and *Enterococcaceae* during the first 24 hours (Dominguez-Bello et al., 2010; Hansen et al., 2015). In contrast, the present study revealed a higher abundance of *Propionibacterium*, *Pseudomonas, Prevotella*, *Bacillus* and *Ralstonia* in the calf small intestine at birth. However, it is not clear whether these differences represent species specificity or variations in sampling time and location. *Actinobacteria* dominates the three- to four-month-old infant fecal microbiota (Turroni et al., 2012; Azad et al., 2013), whereas

Firmicutes dominates the calf fecal microbiota within the first seven weeks of life (Oikonomou et al., 2013). Thus, these observed differences at birth might indicate establishment of a species-specific microbiome from birth.

In addition to the differences in the abundance of detected bacteria, the diversity of bacterial communities at birth also differed from that of three-week- and six-month-old calves (Malmuthuge et al., 2012). In contrast to the higher diversity and richness observed in the small intestine epimural communities compared to the respective luminal communities in older calves (Malmuthuge et al., 2012; Malmuthuge et al., 2014), this study revealed a less diverse bacterial community in the small intestinal epimura at birth. The low bacterial diversity in the epimura might be attributable to the higher abundance of *Pseudomonas*, an aerobic bacterial genus, observed in all epimural communities when compared to the luminal communities. *Pseudomonas* as a transient species may play a role in creating an anaerobic environment for the autochthonous gut bacteria through tissue oxygen scavenging, which is similar to the roles of *Enterococcus* and *Streptococcus* observed in the infant gut (Fanaro et al., 2003; Jost et al., 2012). Despite the higher abundance, *Psuedomonadaceae* (only 2 positive correlations) was not among the main hubs in the co-occurrence networks, which may also be due to its role as a transient bacterium. Although the roles of aerobes and facultative anaerobes during gut colonization have been defined, the role of anaerobic bacteria present at birth on subsequent microbial colonization is not yet understood. *Prevotellaceae*, *Ruminococcaceae* and *Lachnospiraceae* families, the main hubs of the co-occurrence network, were also predominant in the calf small intestine at birth. These anaerobic bacterial families belonging to *Firmicutes* and *Bacteroidetes*, the two main phyla found in

the small intestinal contents and tissue communities of pre-weaned calves (Malmuthuge et al., 2014), may be the inoculum for obligate-anaerobic gut microbiota. The composition and abundance of *Firmicutes* and *Bacteroidetes* have been shown to be influenced by intestinal inflammation (Hansen et al., 2012; Dillon et al., 2014), indicating their responses towards changes in the mucosal immune system. Therefore, the roles of these co-occurring families in the gut colonization process and their interactions with the host need to be investigated further to understand the establishment of region-specific microbiota in depth.

DNA- and RNA-based estimations revealed a higher abundance of *Bifidobacterium* in the calf small intestinal communities, especially in the epimura. This beneficial bacterium has been reported to be one of the main bacteria to first colonize the neonatal calf gut (three to seven-day-old calves) and remains predominant until six to nine months of age (Rada et al., 2006; Vlkova et al., 2006). Although our pyrosequencing primers failed to detect *Bifidobacterium* species, 16S clone libraries revealed that the majority of *Bifidobacterium* in the calf ileum belonged to *B. longum infantis*. The presence of conserved solute binding proteins in *Bifidobacterium* facilitates their binding to mucin-glycans present on the intestinal epithelium (LoCascio et al., 2010). This may also enhance the density of *Bifidobacterium* in the calf gut (Rada et al., 2006; Vlkova et al., 2006; Uyeno et al., 2010) and also their preferable rapid colonization of the intestinal tissue (Malmuthuge et al., 2015). Moreover, the highly conserved milk oligosaccharide utilization genes in *B. longum infantis* enable the effective utilization of sialylated oligosaccharides (LoCascio et al., 2010), which are the major oligosaccharide in the bovine colostrum and milk (ten Bruggencate et al., 2014). The present study revealed an

increase in the abundance of *B. longum infantis* (50.2%) in the ileum of one-week-old calves compared to the abundance at birth. These calves were fed with colostrum during the first three days of life, followed by exclusive milk feeding until sample collection. Sialylated oligosaccharides present in the bovine colostrum and milk (ten Bruggencate et al., 2014) may have boosted the population of *B. longum infantis* in the ileum within the first week of life. However, the abundance of *B. longum infantis* later decreased to 0.4% in three-week-old calves and could not be detected in six-week-old calves, when calves were supplemented with calf starter. Alternatively, the proportion of *B. pseudolongum* subsp. *globosum*, a bovine-specific *Bifidobacterium* (Balleste and Blanch, 2011), was increased in three- and six-week-old calves. Human and mouse studies have reported that *Bifidobacterium* species play a vital role in the development of the mucosal immune system and also prevent pathogen colonization (Hart et al., 2004; Hidalgo-Cantabrana et al., 2014). Thus, future studies are necessary to understand the roles of *Bifidobacterium* in calf gut development and health to prevent diarrhea, which is the major cause of neonatal calf death.

Human studies have suggested the vertical transmission of *Bifidobacterium* species from mother to infant (Makino et al., 2013). DNA-based estimation in the present study revealed the presence of *Bifidobacterium* in the birth canal (16.8±13.2%) and the rectum (13.9±5.8%) of dams, which could be the inoculum for the newborn calf gut. However, only *B. longum* and *B. pseudolongum* (PCR amplification using species-specific primers; Figure 2.7) were detected in the maternal communities, not *B. longum* subsp. *infantis*. A past study also reported that *B. longum* subsp. *infantis* was not detectable in the fecal samples of adult humans or other animal species (Lamendella et

al., 2008). Moreover, when the vertical transmission of *Bifidobacterium* species was explored, *B. longum* subsp. *infantis* was not listed among the species transmitted from mother to infant (Makino et al., 2013). The reason for these observations may be the low abundance of *B. longum* subsp. *infantis* in the adult gut (Lamendella et al., 2008), which is below the detection limit of PCR. Nonetheless, I speculated that *B. longum* subsp. *infantis* transmitted from the dam might become predominant in the calf gut at birth and during early life due its ability to adapt well in the neonatal gut environment, which is enriched with mucin-glycans and milk oligosaccharides.

In humans, the fecal microbiota of vaginally delivered infants highly resembles the maternal microbiota (Dominguez-Bello et al., 2010; Backhed et al., 2015). In contrast to humans, our data revealed the establishment of a notably different bacterial community in the calf small intestine at birth compared to the maternal bacteria (birth canal and rectum) in naturally born calves (Figure 2.8). Although bacterial genera detected in the maternal communities (birth canal and rectum), such as *Prevotella*, *Ruminococcus*, *Clostridium*, *Streptococcus*, *Butyrivibrio* and *Staphylococcus* were present in the calf small intestine, relative abundances were different among these communities. Similar to *Bifidobacterium*, these bacterial genera may also be acquired from maternal microbiota; yet, their colonization may depend on the available nutrition and adhesion mechanisms leading to a unique microbial community in newborn calf gut. Jami and colleagues (2013) reported that *Streptococcus* predominated the rumen fluid of one-day-old calves, which was also the predominant genus observed in the birth canal of dams used in the present study. This suggests that newborn calf rumen fluid at birth may be more similar to maternal communities than the small intestine. The rumen (content and tissue together)

of the same calves used in the present study were colonized with bacterial genera, such as *Veillonella* (23.3±14.1%), *Prevotella* (23.0±4.0%), *Bacteroides* (21.6±5.6%), and *Eubacterium* (7.0±0.7%) (Malmuthuge et al., unpublished data). Whereas the small intestine content-associated bacteria were colonized with *Propionibacterium*, *Psuedomonas*, *Prevotella*, *Ruminococcus*, *Corynebacterium*, *Ralstonia* and *Bacillus* at birth. Our previous studies (Malmuthuge et al., 2012; Malmuthuge et al., 2014) and a recent study in humans (Romano-Keeler et al., 2014) revealed region-specific bacterial composition in the gut that could not be detected in the large intestinal regions or in the feces. Thus, the observed variations between the small intestinal and ruminal bacterial composition within a few minutes after birth also suggest that the gut region-specific bacteria are established from the birth.

The small intestinal bacterial community was also lacked a core microbiome (no single bacterial genus was observed in all of the samples). The absence of a core microbiome among individual calves implies that the impact of the host on the establishment of the small intestinal microbiota may be stronger than that of the environment. The ruminant small intestine, the major site of the mucosal immune system, contains Peyer's patches (PP) that develop prenatally (Griebel et al., 1996; Yasuda et al., 2004). The microbiota colonizing the small intestine largely undergoes comprehensive scrutiny by the host immune system (Hansen et al., 2012; Van den Abbeele et al., 2011). This may result in a higher impact of the host on the small intestinal microbiota than environmental factors. The observed differences between the luminal and epimural communities also suggest a segregation of microbial colonization in these communities from birth. Although the activity levels of gut-associated lymphoid tissues or secretary

immunoglobulin in calves at birth are not clearly understood, the establishment of a unique microbial population suggests that calves may have an active immune system that can influence the microbiota colonization process.

2.5 Conclusion

The present study revealed the colonization of the calf small intestine with a dense, diverse and active microbiota at birth. However, the small intestinal bacterial composition within a few minutes after birth was different from that of the maternal microbiota (birth canal and rectum). This suggests that small intestine-specific bacterial establishment begins during the birthing process and may be influenced by mucosal immune components. Although aerobic bacteria (*Pseudomonadaceae*) were the most abundant, a high abundance of anaerobic bacterial families (*Prevotellaceae*, *Ruminococcaceae* and *Lachnospiraceae*) was also observed at birth, which were generally present in the adult gut. These anaerobic families were the hubs of the co-occurrence networks; however, their roles in the succession of region-specific microbiota have yet to be elucidated. Although a high prevalence of *Bifidobacterium* in the maternal and calf small intestinal microbiomes was observed, the composition was significantly different between the calves and dams, in that *B. longum infantis* dominated the ileum within the first week of life. The favorable conditions (mucin-glycans, milk oligosaccharides) for *B. longum infantis* in the newborn gut may have enhanced their population immediately postnatal. The microbial colonization process in the small intestine must be carefully moderated during early life, as newborn gut permeability is high at birth to absorb colostrum components. Such meticulous modulation prevents

unnecessary inflammatory responses directed at the establishing commensal microbiome and will not exert stress on the naïve immune system. Thus, studies to explore variations in microbial colonization immediately postnatal with varying management practices (different colostrum/milk feeding methods) may be valuable to understand the role of the initial microbiota in subsequent succession and gut development. This will also reveal a means to manipulate the microbial colonization process by modifying early management practices for dairy calves.

2.6 References

Adlerberth, I., Wold, A.E. (2009). Establishment of the gut microbiota in western infants. Acta. Paediatr. 98, 229-238. doi: 10.1111/j.1651-2227.2008.01060.

Azad, M.B., Konya, T., Maughan, H., Guttman, D.S., Field, C.J., Chari, R.S., et al., (2013). Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. CMAJ. 185, 385-394. doi: 10.1503/cmaj.121189.

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al., (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbes.17, 852. doi: 10.1016/j.chom.2015.05.012.

Balleste, E., Blanch, A.R. (2011). *Bifidobacterium* diversity and the development of new microbial source tracking indicators. Appl. Environ. Microbiol. 77, 3518-3525.

Benjamini, Y., Hochberg, y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological). 57, 289-300.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al., (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7, 335-336. doi: 10.1038/nmeth.f.303.

Dillon, S.M., Lee, E.J., Kotter, C.V., Austin, G.L., Dong, Z., Hecht, D.K., et al., (2014). An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. Mucosal Immunol. 7, 983-994. doi: 10.1038/mi.2013.116.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., et al., (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. U S A. 107, 11971-11975. doi: 10.1073/pnas.1002601107.

Fanaro, S., Chierici, R., Guerrini, O., Vigi, V. (2003). Intestinal microflora in early infancy: Composition and development. Acta. Paediatr. Suppl. 91, 48-55.

Griebel, P.J., Kugelberg, B., Ferrari, G. (1996). Two distinct pathways of B cell development in Peyer's patches. Dev. Immunol. 4, 263-277.

Hansen, C.H.F., Nielsen, D.S., Kverka, M., Zakostelska, Z., Klimesova, K., Hudcovic, T., et al., (2012). Patterns of early gut colonization shape future immune responses of the host. PLoS One. 7, e34043. doi:10.1371/journal.pone.0034043.

Hansen, R., Scott, K.P., Khan, S., Martin, J.C., Berry, S.H., Stevenson, M., et al., (2015). First-pass meconium samples from healthy term vaginally-delivered neonates: an analysis of the microbiota. PLoS One. 10, e0133320. doi: 10.1371/journal.pone.0133320.

Hart, A.L., Lammers, K., Brigidi. P., Vitali. B., Rizzello, F., Gionchetti, P., et al., (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut. 53, 1602-1609.

Hidalgo-Cantabrana, C., Sanchez, B., Milani, C., Ventura, M., Margolles, A., Ruas-Madiedo, P. (2014). Genomic overview and biological functions of exopolysaccharide biosynthesis in *Bifidobacterium* spp. Appl. Environ. Microbiol. 80, 9-18. doi: 10.1128/AEM.02977-13.

Jakobsson, H.E., Abrahamsson, T.R., Jenmalm, M.C., Harris, K., Quince, C., Jernberg, C., et al., (2014). Decreased gut microbiota diversity, delayed *Bacteroidetes* colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut. 63, 559-566. doi: 10.1136/gutjnl-2012-303249.

Jami, E., Israel, A., Kotser, A., Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7, 1069-1079. doi: 10.1038/ismej.2013.2.

Jimenez, E., Marin, M.L., Martin, R., Odriozola, J.M., Olivares, M., Xaus, J., et al. (2008). Is meconium from healthy newborns actually sterile? Res. Microbiol. 159, 187-193. doi: 10.1016/j.resmic.2007.12.007.

Jost, T., Lacroix, C., Braegger, C.P., Chassard, C. (2012). New insights in gut microbiota establishment in healthy breast fed neonates. PLoS One. 7, e44595. doi: 10.1371/journal.pone.0044595.

Lamendella, R., Domingo, J.W.S., Kelty, C., Oerther, D.B. (2008). Bifidobacteria in feces and environmental waters. Appl. Environ. Microbiol. 74, 575-584. doi: 10.1128/AEM.01221-07.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., Gordon, J.I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. 6, 776-788. doi: 10.1038/nrmicro1978.

Li, M., Penner, G.B., Hernandez-Sanabria, E., Oba, M., Guan, L.L. (2009). Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107, 1924-1934.

Liang, G., Malmuthuge, N., Bao, H., McFadden, T., Stothard, P., Griebel, P.J., et al., (2014). Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life. PLoS One. 9, e92592. doi: 10.1371/journal.pone.0092592.

LoCascio, R.G., Ninonuveo, M.R., Freeman, S.L., Sela, D.A., Grimm, R., Lebrilla, C.B., et al., (2007). Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J. Agric. Food Chem. 55, 8914-8919.

Makino, H., Kushiro, A., Ishikawa, E., Kubota, H., Gawad, A., Sakai, T., et al., (2013). Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota. PLoS One. 8, e78331. doi: 10.1371/journal.pone.0078331.

Malmuthuge, N., Li, M., Chen, Y., Fries, P., Griebel, P.J., Baurhoo, B., et al., (2012). Distinct commensal bacteria associated with ingesta and mucosal epithelium in

the gastrointestinal tracts of calves and chickens. FEMS Microbiol. Ecol. 79, 337-347. doi: 10.1111/j.1574-6941.2011.01220.

Malmuthuge, N., Griebel, P.J., Guan, L.L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of pre-weaned calves. Appl. Environ. Microbiol. 80, 2012-2028. doi: 10.1128/AEM.03864-13.

Malmuthuge, N., Chen, Y., Liang, G., Goonewardene, L.A., Guan, L.L. (2015). Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves. J. Dairy Sci. 98, 8044-8053. doi: 10.3168/jds.2015-9607.

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., et al., (2002). Development of 16S rRNA-gene-targeted group specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. 68, 5445-5451.

Oikonomou, G., Teixeira, A.G., Foditsch, C., Bichalho, M.L., Machado, V.S., Bicalho, R.C. (2013). Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. PLOS One. 8, e63157. doi: 10.1371/journal.pone.0063157.

Rada, V., Vlkova, E., Nevoral, J., Trojanov, I. (2006). Comparison of bacterial flora and enzymatic activity in faeces of infants and calves. FEMS Microbiol. Lett. 258, 25-28. doi:10.1111/j.1574-6968.2006.00207.

Romano-Keeler, J., Moore, D.J., Wang, C., Brucker, R.M., Fonnesbeck, C., Slaughter, J.C., et al., (2014). Early life establishment of site-specific microbial communities in the gut. Gut Microbes. 5, 192-201. doi: 10.4161/gmic.28442.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., et al., (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498-2504.

Sommer, F., Nookaew, I., Sommer, N., Fogelstrand, P., Backhed, F. (2015). Site-specific programming of the host epithelial transcriptome by the gut microbiota. Genome Biol. 16, 62. doi: 10.1186/s13059-015-0614-4.

Subramanian, S., Blanton, L.V., Frese, S.A., Charbonneau, M., Mills, D.A., Gordon, J.I. (2015). Cultivating healthy growth and nutrition through the gut microbiota. Cell. 161, 36-48. doi: http://dx.doi.org/10.1016/j.cell.2015.03.013.

ten Bruggencate, S.J., Bovee-Oudenhoven, I.M., Feitsma, A.L., van Hoffen, E., Schoterman, M.H. (2014). Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. Nutr. Rev. 72, 377-389. doi: 10.1111/nure.12106.

Turroni, F., Peano, C., Pass, D.A., Foroni, E., Severgnini, M., Claesson, M.J., et al., Diversity of bifidobacteria within the infant gut microbiota. PLoS One. 7, e36957. doi: 10.1371/journal.pone.0036957.

Uyeno, Y., Sekiguchi, Y., Kamagata, Y. (2010). rRNA-based analysis to monitor succession of fecal bacterial communities of Holstein calves. Lett. Appl. Microbiol. 51, 570-577. doi: 10.1111/j.1472-765X.2010.02937.

Van den Abbeele, P., van de Wiele, T., Verstraete, W., Possemiers, S. (2011). The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiol. Rev. 35, 681-704. doi: 10.1111/j.1574-6976.2011.00270.

Vlkova, E., Nevoral, J., Rada, V. (2006). Distribution of bifidobacteria in the gastrointestinal tract of calves. Folia Microbiol. 51, 325-328.

Yasuda, M., Fujino, M., Nasu, T., Murakami, T. (2004). Histological studies on the ontogeny of bovine gut-associated lymphoid tissue: appearance of T cells and development of IgG+ and IgA+ cells in lymphoid follicles. Dev. Comp. Immunol. 28, 357-369.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., et al (2012). Human gut microbiome viewed across age and geography. Nature. 486, 222-227. doi: 10.1038/nature11053.

Yu, Z., Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 36, 808-812.

2.7 Tables and Figures

Table 2.1 Bacterial diversity and richness in calf small intestine at birth.

	All regions		**Proximal jejunum**		**Distal jejunum**		**Ileum**	
	Tissue	**Content**	**Tissue**	**Content**	**Tissue**	**Content**	**Tissue**	**Content**
OTUs (97% similarity)	4233±508	5271±827	4564±1223	6840±1539	3455±587	2813±274	4679±782	6160±1636
Assigned genera	20±2	33±4	12±1	12±2	9±1	11±1	9±1	20±4
Unassigned sequences genus level (%)	15.7±3.2	33.6±4.1	14.3±4.8	30.1±6.9	16.0±7.5	27.5±8.8	17.6±5.0	43.2±4.2
Assigned families	19±1	33±3	12±1	14±2	9±1	13±2	10±1	22±3
Unassigned sequences family level (%)	1.0±0.5	7.1±2.0	0.1±0.05	2.6±1.8	1.5±1.3	4.1±2.6	1.4±0.5	14.5±3.6
Good's coverage	0.98±0.002	0.96±0.004	0.97±0.004	0.97±0.01	0.98±0.002	0.97±0.002	0.97±0.003	0.94±0.01
Shannon index*	3.4±0.1	4.3±0.2	3.2±0.5	3.8±0.6	3.4±0.2	3.7±0.5	3.8±0.1	5.6±0.6
Chao 1*	80.8±6.4	125.4±10.7	79.4±9.3	97.6±29.4	77.6±7.7	103.2±8.0	82.2±8.7	175.3±43.2
Observed species*	47.1±2.2	81.2±8.8	46.3±20.0	58.7±3.9	44.8±2.1	61.8±5.9	50.2±4.1	123.3±30.7
Phylogenetic distance*	4.7±0.3	9.3±0.8	4.7±0.4	7.4±2.1	4.5±0.3	6.9±1.0	5.0±0.3	13.5±2.8

*Phylogenetic distance, Observed species, Shannon index – Ileum > Proximal jejunum, Distal jejunum at $P < 0.05$

*Phylogenetic distance, Observed species, Shannon index, Chao1 – Content-associated community > tissue-associated community at $P < 0.01$

Table 2.2 Maternal, calf body habitats and birth environment bacteria.

	Birth canal	**Rectum**	**Mouth**	**Nose**	**Skin**	**Floor**
OTUs (97% similarity)	5533±1450	4655±2331	4587±1176	7548±1654	4042±294	3308±1342
Assigned genera	21±3	26±4	33±8	31±4	29±6	31±9
Unassigned sequences genus level (%)	44.3±9.6	72.5±3.5	35.1±8.2	47.6±10.3	24.7±9.3	44.1±9.6
Assigned families	19±3	29±4	28±5	27±5	29±4	28±8
Unassigned sequences family level (%)	17.9±4.9	32.6±4.0	4.8±2.8	7.7±4.9	10.4±5.6	14.3±8.3
Main families (%)	*Streptococcaceae* (25.5±13.2) *Aerococcaceae* (16.7±13.1) *Ruminococcaceae* (11.4±4.6) *Clostridiaceae* (9.6±3.4)	*Ruminococcaceae* (24.6±1.1) *Clostridiaceae* (13.3±2.7) *Peptostreptococcaceae* (6.6±2.6) *Lachnospiraceae* (6.4±0.6)	*Staphylococcaceae* (17.9±10.6) *Aerococcaceae* (16.2±8.5) *Xanthomonadaceae* (11.7±6.4) *Caulobacteraceae* (10.6±6.5)	*Xanthomonadaceae* (14.3±14.2) *Staphylococcaceae* (12.3±11.1) *Aerococcaceae* (8.4±7.3)	*Aerococcaceae* (32.6±10.5) *Staphylococcaceae* (12.5±12.3) *Methylobacteriaceae* (11.8±4.8)	*Ruminococcaceae* (16.9±13.9) *Aerococcaceae* (16.2±10.8) *Staphylococcaceae* (13.6±13.0) *Xanthomonadaceae* (7.9±7.3)
Main genera (%)	*Streptococcus* (22.5±13.2) *Facklamia* (11.6±10.6) *Corynebacterium* (2.9±1.2) *Ruminococcus* (2.4±0.7)	*Clostridium* (5.3±0.4) *Ruminococcus* (4.2±3.2) *5-7N15* (3.7±2.1) *Dorea* (2.2±0.3)	*Jeotgalicoccus* (17.8±10.6) *Facklamia* (9.6±5.6) *Streptococcus* (3.8±3.7) *Brevundimonas* (2.8±1.7)	*Jeotgalicoccus* (12.2±11.2) *Facklamia* (4.2±4.1) *Corynebacterium* (3.9±3.0) *Bacillus* (2.9±2.3)	*Facklamia* (19.8±9.1) *Methylobacterium* (12.3±12.2) *Jeotgalicoccus* (10.7±4.4) *Aerococcus* (6.5±5.9)	*Facklamia* (12.1±7.6) *Jeotgalicoccus* (11.8±11.3) *Stenotrophomonas* (5.3±5.0) *Corynebacterium* (3.5±3.1)

Table 2.3 Comparison of calf, dam and birth environment bacterial communities.

Comparison	**ANOSIM R**	***P*-value**
Calf gut *vs.* Dam	0.8893	< 0.01
Calf gut *vs.* Birth canal	0.9049	< 0.01
Calf gut *vs.* Rectum	0.9130	< 0.01
Calf gut content *vs.* Birth canal	0.7187	< 0.01
Calf gut content *vs.* Rectum	0.6788	< 0.01
Calf gut tissue *vs.* Birth canal	0.7574	< 0.01
Calf gut tissue *vs.* Rectum	0.6133	< 0.01
Calf gut *vs.* Body habitats	0.7090	< 0.01
Calf gut *vs.* Floor	0.7942	< 0.01
Content *vs.* Tissue	0.2652	< 0.01
Body habitats *vs.* Floor	0.0595	0.29
Body habitats *vs.* Dam	0.6557	< 0.01
Floor *vs.* Dam	0.0984	0.24
Floor *vs.* Rectum	0.0741	0.50
Floor *vs.* Birth canal	0.4012	0.04

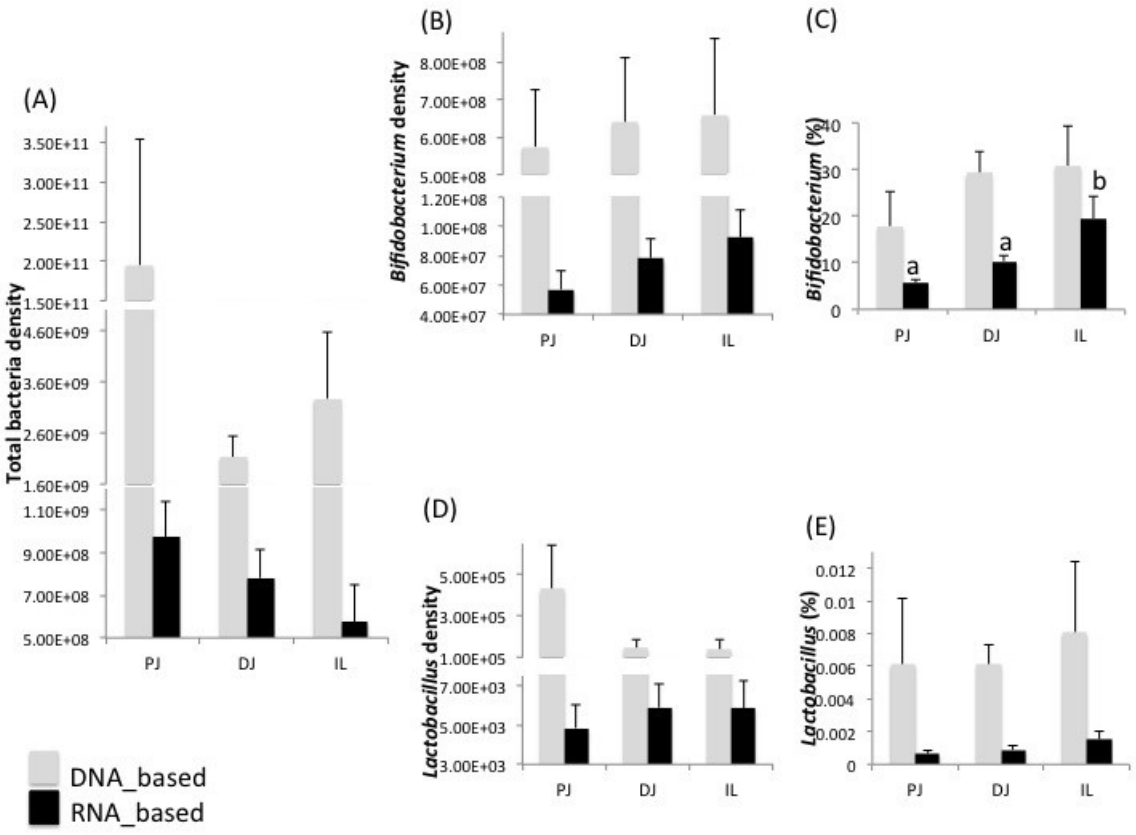

Figure 2.1 Estimation of small intestinal bacteria at birth using DNA (16S rRNA gene copy/g of fresh sample) and RNA (16S rRNA copy/g of fresh sample) extracted from small intestinal tissue and content samples. PJ – proximal jejunum, DJ – distal jejunum, IL – ileum.

(A) Density of total bacteria (B) Density of *Bifidobacterium* (C) Proportion of *Bifidobacterium* [(density of *Bifidobacterium* / density of total bacteria)*100]. a,b represents mean proportions that are different among gut regions at $P < 0.05$ (D) Density of *Lactobacillus* (E) Proportion of *Lactobacillus* [(density of *Lactobacillus* / density of total bacteria)*100]

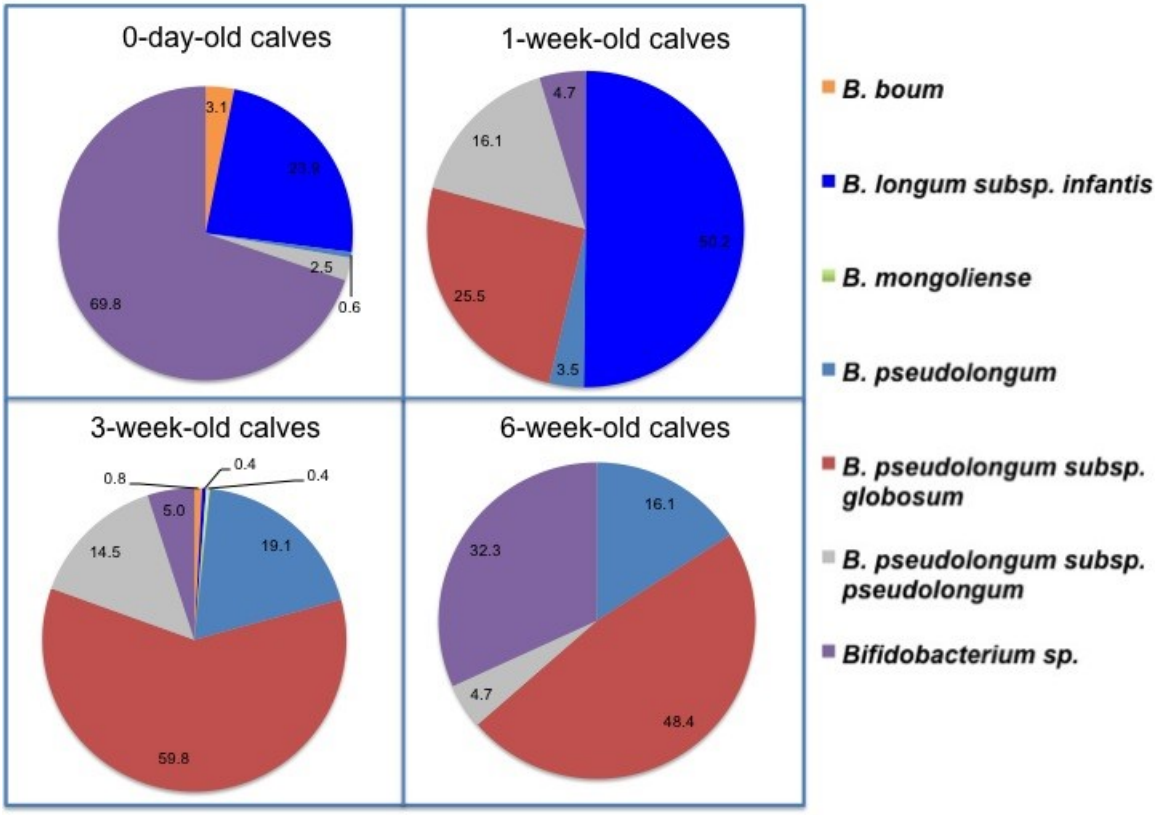

Figure 2.2 Ileal (tissue and content together) bifidobacterial composition at birth obtained through sequencing of 16S clone libraries.

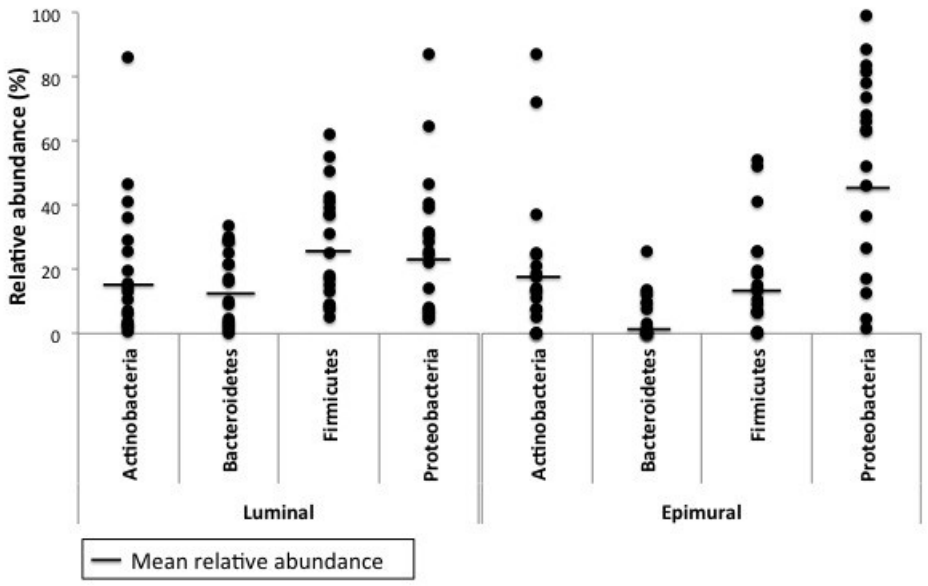

Figure 2.3A Bacterial composition in epimural and luminal communities at phylum level (454 sequencing of V1-V3 region of 16S rRNA gene). Relative abundances of main four phyla in all three small intestinal regions are presented together and each data point represents an individual calf.

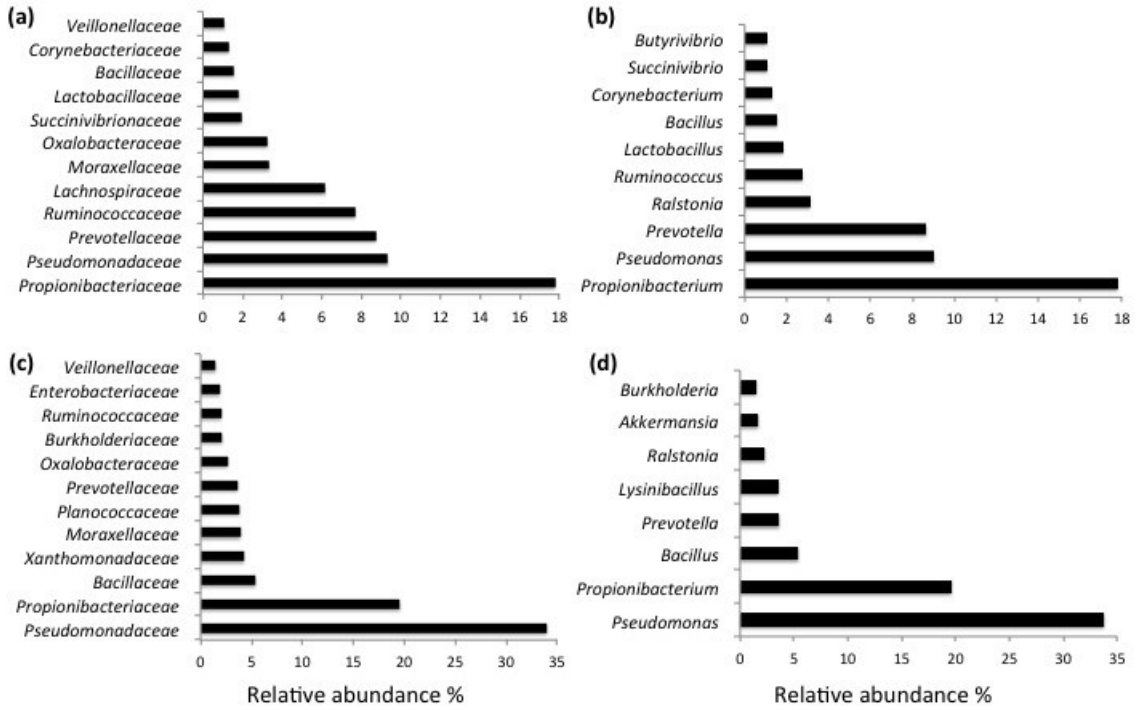

Figure 2.3B Relative abundance of predominant (relative abundance > 1% in at least one intestinal sample) bacterial families and genera.

(a) Predominant bacterial families in luminal communities (b) Predominant bacterial genera in luminal communities (c) Predominant bacterial families in epimural communities (d) Predominant bacterial genera in epimural communities. Bars represent mean relative abundance of bacterial families and genera.

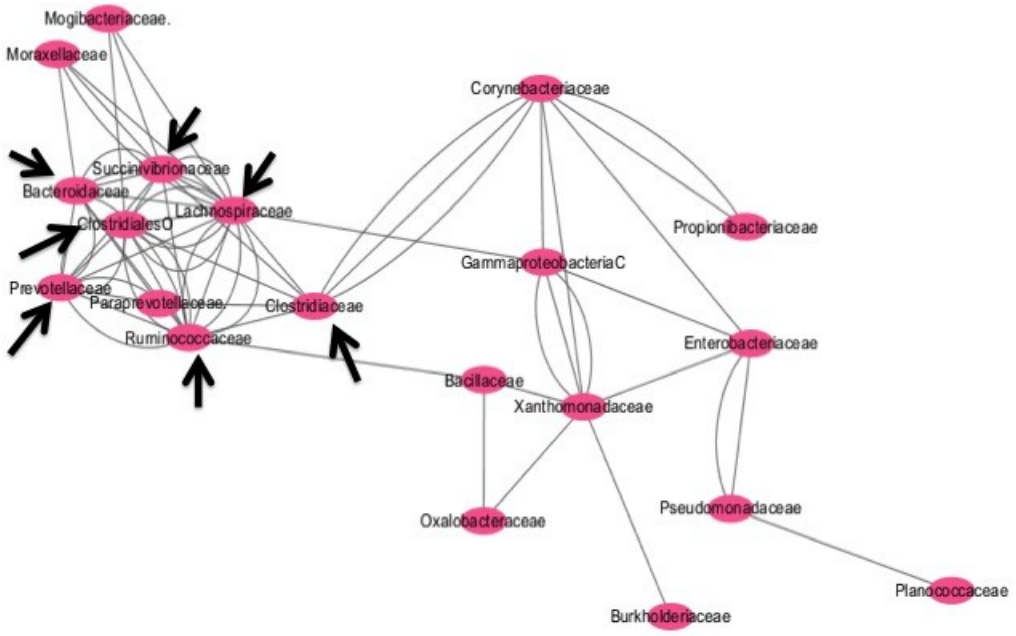

Figure 2.4 Co-occurrence network of bacterial families detected from calf small intestine. Positive correlations ($\rho > 0.5$, $P < 0.05$) among bacterial families are defined as co-occurred families and distance between two families are calculated using Spearman correlation co-efficient (distance = $1 - \rho$). Lower distance represents higher correlation and *vice versa*. All the co-occurrence incidences in all three-gut regions were plotted in one network using Cytoscape software platform. Black arrows represent hubs of the co-occurrence network.

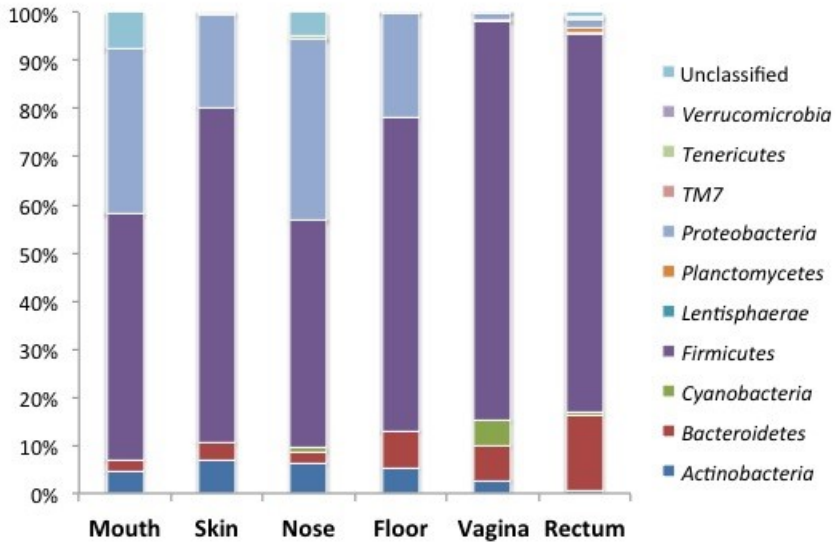

Figure 2.5 Bacterial compositions in maternal (vagina/birth canal, rectum), body habitat (mouth, nose, skin) and birth environment (pen floor) communities at phylum level. Mean relative abundances of detected bacterial phyla are presented in bars.

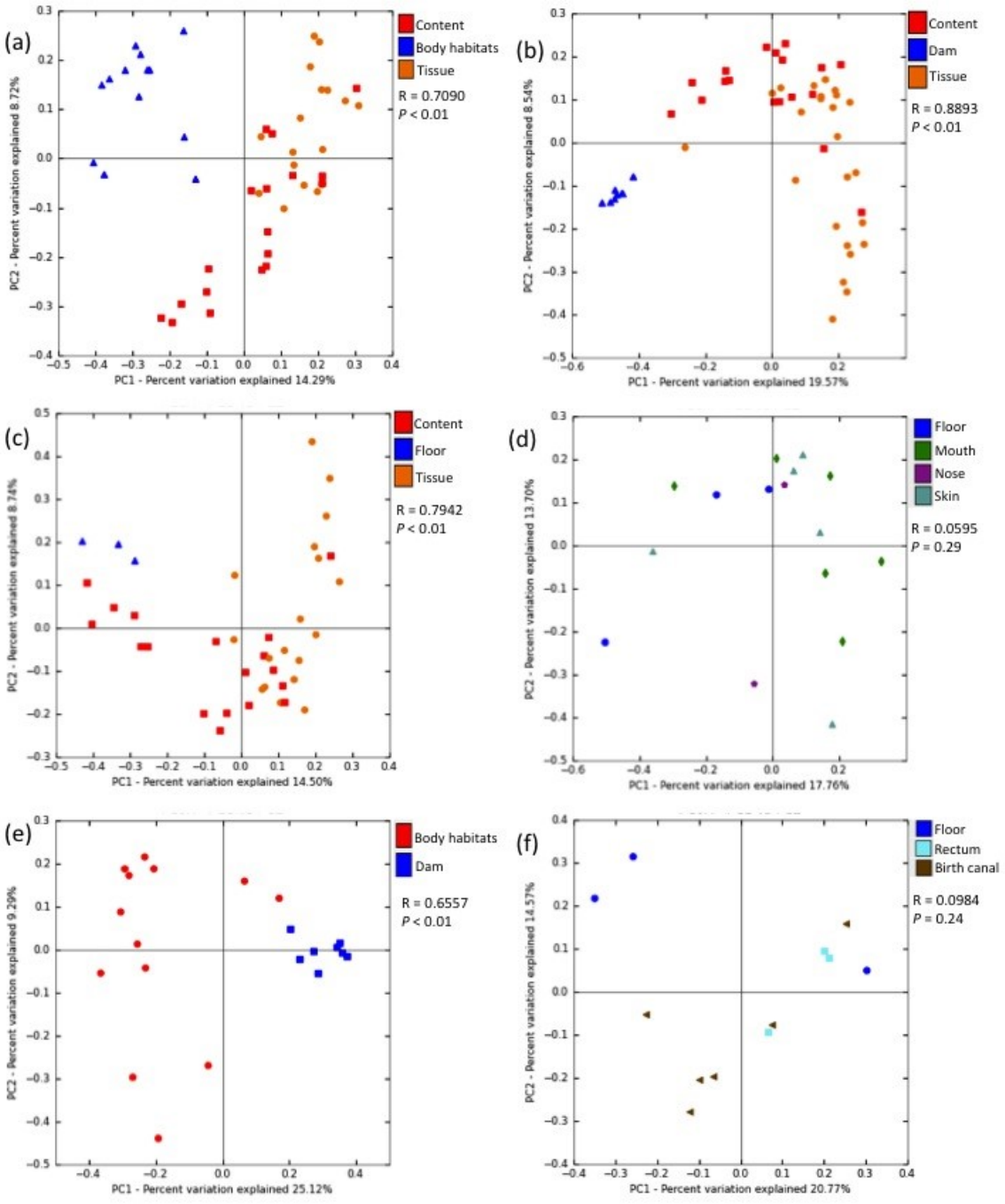

Figure 2.6 Comparison of calf gut, calf body habitats, maternal and birth environment bacterial communities.

OTU (97% level) profiles generated from each samples are compared using beta-diversity function within QIIME platform to generate distance matrices. Weighted UniFrac

distance matrices are used to perform principle co-ordinate analysis (PCoA) and analysis of similarity (ANOSIM). R represents ANOSIM R that ranges from 0 to1, 0 representing microbial communities that are not separated from each other and 1 representing microbial communities that are separated from each other. P value represent significance level of ANOSIM R value.

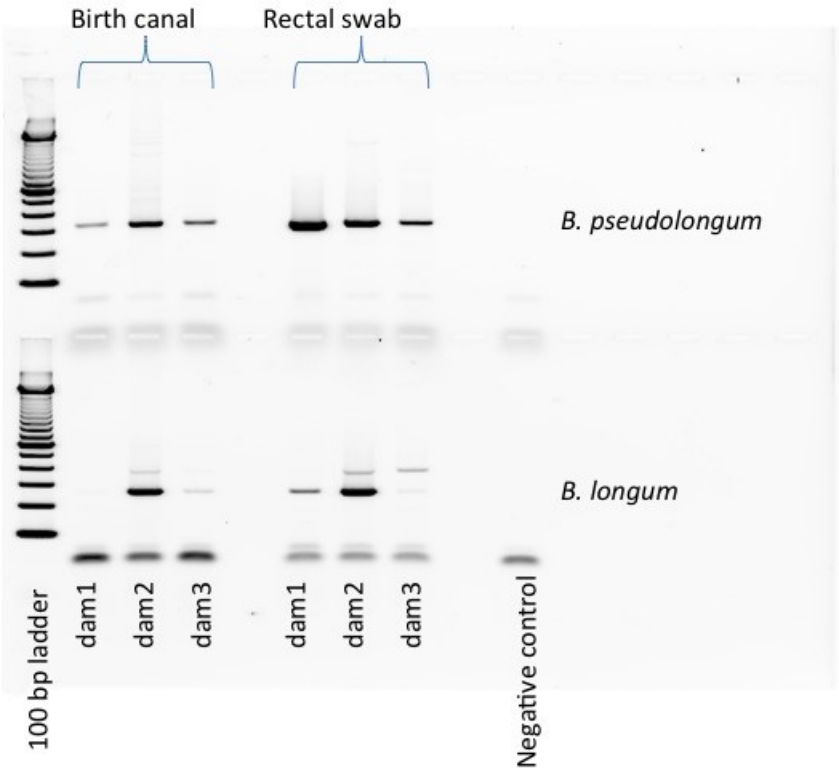

Figure 2.7 PCR-detection of *Bifidobacterium* species in maternal communities (birth canal and rectum).

Dam1, dam2 and dam3 represent individual IDs of dams. PCR primers target groEL chaperone gene of the *Bifidobacterium* genome (Junick and Blaut, Appl. Environ. Microbiol. 2012, 78: 2613–2622).

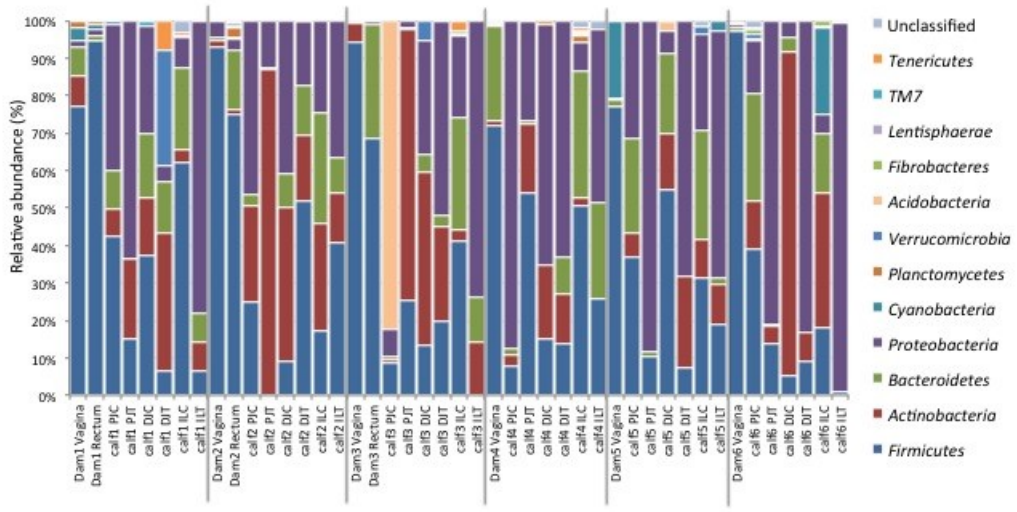

Figure 2.8 Comparison of bacterial compositions (phylum level) among maternal communities (birth canal/vagina and rectum) and calf small intestinal communities. PJC – proximal jejunum content, PJT – proximal jejunum tissue, DJC – distal jejunum content, DJT – distal jejunum tissue, ILC – ileum content, ILT – ileum tissue.

Chapter 3. Feeding heat-treated colostrum promotes beneficial bacteria colonization in the small intestine of neonatal calves[8]

Abstract

The present study investigated the impact of feeding heat-treated colostrum on bacterial colonization in the small intestine of neonatal calves, within the first 12 hours of life. Newborn Holstein bull calves (n = 32) were assigned to three treatment groups and fed either fresh colostrum (FC, n = 12) or heat-treated colostrum (60°C, 60 min.; HC, n = 12) soon after birth, while the control (NC, n = 8) group did not receive colostrum or water. Small intestinal tissues and contents were collected from proximal jejunum, distal jejunum and ileum at 6 and 12 hours after birth, following euthanasia. Quantitative real-time PCR was used to explore the colonization of total bacteria, *Lactobacillus, Bifidobacterium* and *E. coli*. Feeding colostrum soon after birth increased the colonization of total bacteria in calf gut within the first 12 hours compared to NC. In contrast, the prevalence of *Lactobacillus* was lower in HC and FC, comparing to NC. Remarkable changes in the prevalence of small intestinal tissue-attached *Bifidobacterium* were observed with feeding HC, but not in contents. The prevalence of *Bifidobacterium* was 3.2-fold and 5.2-fold higher in HC than FC and NC, respectively, at 6 hours. Although feeding FC did not enhance the prevalence of tissue-attached *Bifidobacterium* at 6 hours compared to NC, it displayed a gradual increase over the time that was higher than NC, but similar to HC at 12 hours. Moreover, the colonization of *E. coli* was

[8] A version of this chapter has been published as, Malmuthuge, N., Chen, Y., Liang, G., Goonewardene, L.A., Guan, L.L. (2015). Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves. J. Dairy Sci. 98, 8044-8053. doi: 10.3168/jds.2015-9607.

drastically reduced in HC calves compared to FC and NC. Thus, the present study suggests that feeding HC enhances the colonization of *Bifidobacterium*, but lessens *E. coli*, in the calf small intestine immediately postpartum compared to FC and NC. The increased colonization of beneficial bacteria along with the decreased colonization of potential pathogens in calf gut may also diminish the neonatal calf diarrhea, when they fed heat-treated colostrum soon after birth.

3.1 Introduction

Colostrum management and feeding is crucial for passive immune transfer in calf management. Calves are immune-deficient at birth, as there is no placental transferring of immunoglobulin into the fetuses in cattle (Godden, 2008). Thus, calves are solely depending on the absorption of immunoglobulin present in colostrum until the development of their own immune system (Godden, 2008). Feeding high quality colostrum (immunoglobulin G (IgG) > 50 mg/mL) soon after birth plays a vital role in the passive transfer of immunity (Jaster, 2005, Chigerwe et al. 2008), which decreases calf mortality and morbidity and increases calf weaning weight and body weight gain (Priestely et al., 2013). Despite the industrial recommendations, the North American dairy industry is still having concerns regarding the colostrum management (Vasseur et al., 2010; Morril et al., 2012). Feeding of calves with contaminated (high bacterial count), and low quality colostrum (< 50 IgG mg/mL) (Morril et al., 2012) as well as low surveillance of calf birth at night and relying on dams to feed colostrum (Vasseur et al., 2010) are some of the major concerns observed in the current North American dairy industry.

Feeding heat-treated colostrum is one of the management practices introduced recently to the dairy industry, aiming to decrease bacterial contaminations and increase passive immune transfer (Donahue et al., 2012; Godden et al., 2012; Teixeira et al., 2013; Gelsinger et al., 2014). Heat treatments successfully (60°C, 60 min.) decrease total bacterial count including pathogenic bacteria while maintaining concentration of IgG (Donahue et al., 2012). Additionally, feeding heat-treated colostrum increases serum colostrum concentration (Godden et al., 2012; Teixeira et al., 2013) and reduces the risk for illnesses and treatment for scours in dairy farms, when comparing to fresh colostrum (Godden et al., 2012). These observations suggest a decrease in disease transmission among the calves in dairy herds, when they receive heat-treated colostrum soon after birth. Although the decreased total bacteria including pathogens in colostrum, is one of the possibilities of reduced enteric infections in calves fed heat-treated colostrum (Godden et al., 2012), the impact of heat-treated colostrum on gut colonization is not well studied. The present study hypothesized that feeding heat-treated colostrum influences bacterial colonization in the calf intestine and facilitates the colonization of beneficial bacteria. Here, we investigated the impact of colostrum feeding (heat-treated and fresh) soon after birth on the colonization of total bacteria, *Lactobacillus*, *Bifidobacterium* and *E. coli* in the calf small intestine within the first 12 hours of life.

3.2 Materials and methods

3.2.1 Colostrum preparation and animal experiments

Prior to the animal experiment, first-milking colostrum containing ≥ 50 IgG mg/mL was collected from cows raised at the Dairy Research and Technology Center

(DRTC), University of Alberta, Edmonton, Canada and immediately laid flat on wire racks and frozen at -20°C. Once ~ 48 L of colostrum was collected, all the samples were thawed slowly for 24 hours in 4°C cold room and mixed thoroughly to obtain the pool of colostrum that will be using during the entire animal experiment. Half of the colostrum (24 L) was pasteurized (60 minutes at 60°C) using commercial a batch pasteurizer DT 10G (Dairy Tech Inc., Greeley, CO, USA). Colostrum was held at 60°C for 60 minutes apart from the time (~30 minutes) taken to reach 60°C, followed by rapid cooling. The heat-treated colostrum and the remaining half of fresh colostrum were aliquoted into 1-liter plastic freezer bags and stored at -20°C.

Animal experiment was conducted at the DRTC, University of Alberta, following the guidelines of the Canadian Council on Animal Care. The Livestock Care Committee of the University of Alberta approved all the protocols (AUP00001012) prior to beginning the experiment. Near parturition, Holstein cows predicted to have bull calves were transferred into individual maternity pens and monitored closely with video cameras. Calves were removed from the dams soon after birth, before exposing to the dams, and transferred into individual calf units with fresh wood shavings followed by navel dipping in 7% (v/v) iodine solution. Frozen colostrum (2 L/calf) was thawed to 37 - 38°C using a water bath and bottle-fed to calves within an hour after birth. Calves (n = 32) were randomly allocated into three treatment groups; fresh colostrum-fed calves (FC, n = 12), heat-treated colostrum-fed calves (HC, n = 12), and control calves that did not receive either colostrum or water during the experimental period (NC, n = 8).

3.2.2 Intestinal sample collection

Intestinal samples from all calves were collected at 6 hours (HC, n = 6; FC, n = 6; NC, n = 4) and 12 hours (HC, n = 6; FC, n = 6; NC, n = 4) after birth. All calves were euthanized following captive bolt gun stunning and small intestinal tissues and contents (proximal jejunum, distal jejunum, ileum) were collected together as closed gut sections within 30 minutes after euthanization. The esophagus and rectum were first ligated to occlude the lumen and prevent environmental contamination of the intestine. Then, 10 cm long closed intestinal segments were collected in the middle of pre-defined gut regions. Ileum was defined as 30 cm proximal to the ileo-cecal junction, distal jejunum was defined as 30 cm proximal to the collateral branch of the cranial mesenteric artery and proximal jejunum was defined as 100 cm distal to the pylorus sphincter. All samples were snap-frozen and transferred into a -80°C freezer until further processing.

3.2.3 Intestinal sample collection from newborn calves

Dams predicted to have bull calves were transferred into calving pens three days before the predicted due date and monitored via remote video cameras. Newborn calves (n = 6) were separated from dams soon after birth to make sure there were no interactions between calves and dams. Then, the calves were transferred into the surgical room at the DRTC and humanely euthanized immediately following captive bolt gun stunning (within five minutes after birth). The collection of small intestinal segments from the newborn calves was completed within 30 minutes after the calf birth, similar to the 6- and 12-hour calves used in the colostrum feeding trial.

3.2.4 DNA extraction from tissue and digesta samples[9]

A portion of frozen intestinal section was thawed on ice and separated content from tissue. Then, genomic DNA from tissues and contents was extracted separately using the repeated bead-beating plus column (RBB+C) method (Yu and Morrison, 2004). Briefly, content (0.5g) and tissue (0.5g) samples were subjected to physical disruption with a cell lysis buffer containing 4% SDS using the BioSpec Mini Beads beater 8 (BioSpec, OK, USA) at 4800 rpm for three minutes. Then, the tubes containing lysed cells were incubated at 70ºC for 15 minutes and separated the supernatant. The bead-beating and incubation steps were repeated once more. Any remaining impurities and SDS from supernatants were removed with 10M-ammonium acetate and DNA was precipitated using isopropanol. The genomic DNA was then further purified using the QIAmp fast DNA stool mini kit (QIAmp fast DNA stool mini kit, QIAGEN Inc. CA, USA). The DNA quantity was measured using an ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington) and stored at -20ºC.

3.2.4 Quantification of total bacteria, *Lactobacillus* and *Bifidobacterium* in calf small intestine

Quantitative real-time PCR (qPCR) was performed to estimate densities of total bacteria, *Lactobacillus*, *Bifidobacterium* and *E. coli* using SYBR green chemistry (Fast SYBR® Green Master Mix, Applied Biosystems) with a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) and bacterial primers (Table 3.1). Standard curves for total bacteria, *Lactobacillus*, *Bifidobacterium* and *E. coli* were

[9] DNA extraction and qPCR was performed by laboratory technician Yanhong Chen

generated using purified 16S rRNA genes of *Butyrivibrio hungateii*, *Lactobacillus acidophilus* ATCC4356, *Bifidobacterium longum* and *Escherichia coli* K12, respectively. The copy number of 16S rRNA gene/g of fresh tissue or content was then calculated using the equation described by Li et al., (2009). The prevalence of *Lactobacillus*, *Bifidobacterium* and *E. coli* was calculated by dividing the copy number of 16S rRNA gene of each genus/species by the copy number of total bacteria.

3.2.5 Statistical analysis

All bacterial density data were analyzed using MIXED procedure in SAS (SAS 9.4, SAS Inc., Cary, NC) with small intestinal region as the repeated measurement and animal as the experimental unit. Compound symmetry covariance structure was selected as the best fit by the Bayesian information criteria (BIC). The following statistical model was fitted to test the effect of colostrum treatment, time point, gut region and sample type on bacterial density: $Y_{ijklm} = \mu + C_i + T_j + R_k + S_l + CT_{(ij)} + CR_{(ik)} + CS_{(il)} + TR_{(jk)} + TS_{(jl)} + RS_{(kl)} + CTR_{(ijk)} + CTS_{(ijl)} + CRS_{(ikl)} + TRS_{(jkl)} + e_{ijklm}$, where Y = bacterial density (total bacteria, *Lactobacillus*, *Bifidobacterium*, *E. coli*); μ = mean; C = colostrum treatment; T = time point; R = small intestinal region; S = sample type (tissue, content); and e = residual error. A similar model was fitted when comparing newborn calves against 6 and 12-hour-old calves under each diet, after removing colostrum treatment effect from the model ($Y_{ijkl} = \mu + T_i + R_j + S_k + TR_{(ij)} + TS_{(ik)} + RS_{(jk)} + TRS_{(ijk)} + e_{ijkl}$). Differences in LSM were declared at $P < 0.05$ using the PDIFF option in SAS when applicable.

3.3 Results

3.3.1 Impact of colostrum feeding on the colonization of newborn calf gut within the first 12 hours of life

Feeding of colostrum (heat-treated or fresh) accelerated bacterial colonization in the calf small intestine, compared to the calves that did not receive colostrum soon after birth. Density of total bacteria was significantly higher ($P < 0.01$) in the small intestine of HC ($9.77\pm3.44\times10^{9}$ 16S copy/g of sample) and FC ($1.37\pm0.73\times10^{10}$ 16S copy/g of sample) calves, when comparing to NC calves ($5.26\pm2.17\times10^{8}$ 16S copy/g of sample), regardless of time point and small intestinal region (Figure 3.1A). Besides the effect of colostrum treatment, effect of type by time interaction on total bacterial density was also significant ($P < 0.01$). The content-associated total bacterial density was higher at 12 hours comparing to that of 6 hours as well as tissue-attached bacteria at 12 hours (Figure 3.1B). When the prevalence of beneficial bacteria in the calf small intestine was explored, the prevalence of *Lactobacillus* was higher ($P < 0.01$) in NC calves compared to the colostrum fed calves (Figure 3.2A). In contrast, the prevalence of *Bifidobacterium* was higher ($P < 0.01$) in tissue-attached community of HC (28.6±10.3%) calves than FC (8.9±2.5%) and NC (5.5±1.5%) calves at 6 hours (Figure 3.2B). Moreover, the prevalence of *Bifidobacterium* was higher ($P < 0.01$) in proximal jejunum (19.0±8.0%) of the calves, when comparing to distal jejunum (11.1±4.5%) and ileum (10.4±3.7%), regardless of colostrum treatment, time point and sample type. In contrast, feeding colostrum significantly decreased the colonization of *E. coli* in the calf small intestine within the first 12 hours of life (Figure 3.3). The prevalence of *E. coli* was higher in the tissue and content-associated small intestinal communities of NC calves (tissue –

0.17±0.03%, content – 0.16±0.01%) compared to FC (tissue – 0.019±0.005%, content – 0.069±0.061%) and HC (tissue – 0.001±0.0004%, content – 0.004±0.002%).

3.3.2 Impact of colostrum feeding on the bacterial colonization process

Bacterial densities of 6- and 12-hour-old calves were also compared with the newborn calves obtained within 5 minutes after birth to explore the changes in the initial bacterial densities within the first 12 hours of life and how this process influence by feeding colostrum (Figure 3.4). Total bacterial density observed at birth ($1.21\pm0.56\times10^{9}$ 16S copy/g of sample) was significantly increased ($P < 0.01$) within the first 12 hours of life, when the calves were fed heat-treated colostrum ($1.25\pm0.50\times10^{10}$ 16S copy/g of sample) or fresh colostrum ($2.03\pm1.13\times10^{9}$ 16S copy/g of sample) soon after birth. Although the prevalence of *Lactobacillus* was lower ($P = 0.01$) in HC calves at 6 (0.008±0.002%) and 12 (0.006±0.003%) hours after birth compared to the newborn calves (0.046±0.024%), there were no such changes ($P = 0.12$) observed in FC calves within the first 12 hours (6hr-0.018±0.010%; 12hr-0.010±0.006%). The prevalence of *Bifidobacterium* in the small intestinal tissue-attached community was significantly ($P = 0.03$) lower at 6 hours in FC (8.9±2.5%) calves compared to newborn (31.2±9.0%); however, there were no such differences ($P = 0.51$) observed in HC calves (28.6±10.3%). When the colonization of *E. coli* within the first 12 hours of life was compared, there were no differences ($P = 0.32$) observed between the newborn and FC calves. However, the HC group had lower ($P < 0.01$) colonization of *E. coli* in the small intestinal tissue-associated communities at 6 (0.0004±0.0002%) and 12 hours (0.0008±0.0003%) after birth compared to the newborn calves (0.054±0.001%). There were no differences

observed when comparing total bacteria, *Lactobacillus* and *Bifidobacterium* of NC calves with the newborn calves. However, the prevalence of *E. coli* in the small intestinal tissue of NC increased ($P < 0.01$) at 12 hours (0.13±0.02%) compared to the newborn calves.

3.3.3 Effect of feeding heat-treated colostrum on small intestinal bacteria within the first 12 hours of life

Feeding heat-treated colostrum to calves soon after birth did not significantly influence ($P = 0.22$) the total bacteria density, when comparing to fresh colostrum; however, there was a reduction in the number of bacteria colonized in the small intestinal regions (Table 3.2). When the prevalence of *Lactobacillus* was compared, it was 4 times higher ($P < 0.01$) in the small intestinal content-associated community (0.023±0.015%) of FC calves compared to the tissue community (0.005±0.002%), regardless of the time point. In general, feeding heat-treated colostrum decreased *Lactobacillus* prevalence in the small intestine of calves compared to fresh colostrum (Table 3.2). Feeding heat-treated colostrum, however, had remarkable impact on the prevalence of *Bifidobacterium* in the small intestine tissue-attached community. The prevalence of *Bifidobacterium* was 3.2-fold higher in HC calves than FC calves, at 6 hours after birth ($P < 0.01$). In contrast, feeding fresh colostrum gradually increased the colonization of *Bifidobacterium* in the small intestine and there was no effect of colostrum treatment on the prevalence of *Bifidobacterium* at 12 hours after birth (Figure 3.2B). When the colonization of *E. coli* in the calf small intestine was compared between two colostrum-feeding methods, FC calves had higher ($P < 0.01$) density of *E. coli*, but not proportions compared to HC calves (Table 3.2).

3.4 Discussion

Neonatal diarrhea is responsible for ~ 50% of the calf deaths occur in dairy industry (Uetake, 2013). A good colostrum management is one of the most important preventive measures of neonatal calf diarrhea, because colostrum transfers passive immunity to the newborn calves. Besides, the bioactive compounds, such as oligosaccharides (OS), present in bovine colostrum also inhibit the adherence of pathogens to the intestinal epithelial cells and prevent infections (Maldonado-Gomez et al., 2015). Feeding heat-treated colostrum to dairy calves is one of the industrial suggested good practices to increase passive transfer of immunity (Godden et al., 2012; Teixeira et al., 2013; Gelsinger et al., 2014) and to minimize calf mortality and morbidity. Heat treatments (60°C, 60 min) decrease total bacterial count and eliminate pathogens, such as *Mycobacterium avium* subsp *paratuberculosis* present in colostrum, while maintaining IgG concentration (Godden et al., 2006; Johnson et al., 2007). Although the effect of feeding heat-treated colostrum on passive immune transfer has been widely studied, there is a very limited knowledge regarding its impact on gut microbial colonization. Gut microbiota plays a key role in host health and susceptibility to diseases (Round et al, 2009). Besides, the importance of early gut microbiota on immune system development has been well described in humans (Russell et al., 2013; Jakobsson et al., 2014).

The present study revealed that feeding colostrum (either fresh or heat-treated) facilitated the gut microbial colonization, allowing the bacterial numbers to reach 10^{10} 16S rRNA gene/g in fresh samples within the first 12 hours of life. In contrast, the calves that did not receive colostrum soon after birth had very low number of total bacteria,

which was similar to that of newborn calves, suggesting a slower bacterial colonization in the absence of colostrum. A lower total bacterial density in mouse pups has been shown to associate with differed mucosal and systemic immune responses compared to the pups with normal bacterial densities (Lamouse-Smith et al., 2011). Here, the authors speculate that the timed feeding of colostrum is important for the establishment of stable gut microbiome that plays a crucial role in the development of naïve newborn calf immune system. Therefore, besides the efficient and effective absorption of immunoglobulin, the timed feeding of colostrum also plays a vital role in calf health via promoting gut microbial colonization.

The calf small intestine was already colonized with a higher level of *Bifidobacterium* within five minutes after birth. However, only the heat-treated colostrum was capable of maintaining the higher level of *Bifidobacterium* throughout the first 12 hours of life. *Bifidobacterium* is widely studied due to its use as a probiotics, and it can modulate host immune responses and immune cell phenotypes *in vitro* (Hart et al., 2004). Moreover, *Bifidobacterium* protects host against enteropathogenic infections via the production of acetate (Fukuda et al., 2011). The elevated *Bifidobacterium* colonization, especially in the tissue-attached community immediately postpartum may play an important role in the development of calf immune system and gut barrier. Besides the improved IgG absorption (Godden et al., 2012), increased *Bifidobacterium* population may be another reason for the reduction of enteric infections in the newborn calves fed heat-treated colostrum. Feeding heat-treated colostrum soon after birth was also associated with the reduced colonization of *E. coli*, within the first 12 hours of life. The prevalence of *E. coli* in the calf small intestine was 58-fold and 15-fold higher in NC and

FC calves, respectively compared to HC calves. *E. coli* is one of the most commonly associated pathogens in neonatal calf diarrhea (Foster and Smith, 2009). Thus, the reduced potential pathogenic bacteria and increased beneficial bacteria colonization in the small intestine can be considered as one of the advantages of feeding heat-treated colostrum to calves soon after birth.

Estimation of total bacteria in colostrum using qPCR revealed that detectable bacteria were only present in fresh colostrum (1.8×10^3 16S rRNA gene copy/ml of colostrum), but not in the heat-treated colostrum (not detected) used in the present study. Besides, 70.6% (1.31×10^3 16S rRNA gene copy/ml of colostrum) of these detectable bacteria were consisted of *E. coli*, and neither *Lactobacillus* nor *Bifidobacterium* was detected in fresh colostrum samples used in the present study. Thus, the increased colonization of *Bifidobacterium* observed in HC calves was not due to the introduction of this bacterium via colostrum, rather this increase might have been promoted by the bioactive components. OS are one of the main energy sources for *Bifidobacterium* and *B. longum* subsp. *infantis* grows robustly on human milk oligosaccharides, due to their ability to breakdown sialylated OS (LoCascio et al., 2007; LoCascio et al., 2010; Sela et al., 2011). Moreover, sialylated OS promotes adhesion of *Bifidobacterium* to epithelial cells *in vitro* (Kavanaugh et al., 2013). Sialylated OS, the main oligosaccharides present in bovine colostrum (ten Bruggencate et al., 2014), is mainly bound to milk proteins (Nesser et al., 1991). Moreover, the availability of sialylated OS in heat-treated bovine milk is higher than fresh milk (Nesser et al., 1991). Thus, the authors propose that the increased availability of sialylated OS in heat-treated colostrum retains the higher level of *Bifidobacterium* observed in the newborn small intestine, when they received heat-treated

colostrum soon after birth.

In conclusion, feeding heat-treated colostrum soon after birth enhanced the colonization of *Bifidobacterium* and reduced the colonization of *E. coli* in the calf small intestine, immediately postpartum. This may be one of the reasons for observed lower prevalence of enteric infections in calves fed heat-treated colostrum compared to calves fed fresh colostrum (Godden et al., 2012). However, the present study only explored the microbial colonization immediately postpartum (with the first 12 hours), therefore, the long-term effects of feeding heat-treated colostrum on the gut microbial colonization and succession is not clearly defined. Moreover, the impact of enhanced colonization of *Bifidobacterium* on calf performances (body weight gain, resistance to enteric infections, weaning weight) needs to be studied in detail. Lastly, to the authors' knowledge this is the first study to understand the influence of heat-treated colostrum on calf gut colonization.

2.5 References

Chigerwe, M., Tyler, J.W., Schultz, L.G., Middleton, J.R., Steevens, B.J., Spain, J.N. (2008). Effect of colostrum administration by use of oroesophageal intubation on serum IgG concentrations in Holstein bull calves. Am. J. Vet. Res. 69, 1158–1163. doi: 10.2460/ajvr.69.9.1158.

Delroisse, J-M., Boulvin, A-L., Parmentier, I., Dauphin, R.D., Vandenbol, M., Portetelle, D. (2008). Quantification of *Bifidobacterium* spp. and *Lactobacillus* spp. in rat fecal samples by real-time PCR. Microbiol. Res. 163, 663-670.

Donahue, M., Godden, S.M., Bey, R., Wells, S., Oakes, J.M., Sreevatsan, S., et al.,

(2012). Heat treatment of colostrum on commercial dairy farms decreases colostrum microbial counts while maintaining colostrum immunoglobulin G concentrations. J. Dairy Sci. 95, 2697–2702. doi: 10.3168/jds.2011-5220.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al., (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 469, 543-547. doi: 10.1038/nature09646.

Gelsinger, S.L., Jones, C.M., Heinrichs, A.J. (2015). Effect of colostrum heat treatment and bacterial population on immunoglobulin G absorption and health of neonatal calves. J. Dairy Sci. 98, 4640-4645. doi: 10.3168/jds.2014-8790.

Godden, S. (2008) Colostrum management for dairy calves. Vet. Clin. North Am. Food. Anim. Pract. 24, 19-39. doi: 10.1016/j.cvfa.2007.10.005.

Godden, S.M., Smolenski, D.J., Donahue, M., Oakes, J.M., Bey, R., Wells, S. et al., (2012). Heat-treated colostrum and reduced morbidity in preweaned dairy calves: Results of a randomized trial and examination of mechanisms of effectiveness. J. Dairy Sci. 95, 4029-4040. doi: 10.3168/jds.2011-5275.

Godden, S., McMartin, S., Feirtag, J., Stabel, J.R., Bey, R., Goyal, S., et al., (2006). Heat treatment of bovine colostrum II: Effects of heating duration on pathogen viability and immunoglobulin G. J. Dairy Sci. 89, 3476–3483.

Hart, A.L., Lammers, K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., et al., (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut. 53:1602-1609.

Jakobsson, H.E., Abrahamsson, T.R., Jenmalm, M.C., Harris, K., Quince, C., Jernberg, C., et al., (2014). Decreased gut microbiota diversity, delayed *Bacteroidetes*

colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut. 63, 559-566. doi: 10.1136/gutjnl-2012-303249.

Jaster, E.H. (2005). Evaluation of quality, quantity, and timing of colostrum feeding on immunoglobulin G1 absorption in Jersey calves. J. Dairy Sci. 88, 296-302.

Johnson, J.L., Godden, S.M., Molitor, T., Ames, T., Hagman, D. (2007). Effects of feeding heat-treated colostrum on passive transfer of immune and nutritional parameters in neonatal dairy calves. J. Dairy Sci. 90, 5189–5198

Kavanaugh, D.W., O'Callaghan, J., Butto, L.F., Slattery, H., Lane, J. Clyne, M., et al., (2013). Exposure of *Bifidobacterium longum* subsp. *infantis* to milk oligosaccharides increases adhesion to epithelial cells and induces a substantial transcriptional response. PLoS One 8, e67224. doi: 10.1371/journal.pone.0067224.

Lamouse-Smith, E.S., Tzeng, A., Starnbach, M.N. (2011). The intestinal flora is required to support antibody responses to systemic immunization in infants and germfree mice. PLoS One 6, e27662. doi:10.1371/journal.pone.0027662.

Li, M., Penner, G.B., Hernandez-Sanabria, E., Oba, M., Guan, L.L. (2009). Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107, 1924-1934. doi: 10.1111/j.1365-2672.2009.04376.

Liang, G., Malmuthuge, N., McFadden, T.B., Bao, H., Griebel, P.J., Stothard, P., et al., (2014). Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life. PLoS One. 9, e92592. doi:10.1371/journal.pone.0092592.

LoCascio, R.G., Ninonuveo, M.R., Freeman, S.L., Sela, D.A., Grimm, R., Lebrilla, C.B., et al., (2007). Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J. Agric. Food Chem. 55, 8914–8919

LoCascio, R.G., Desai, P., Sela, D.A., Weimer, B., Mills, D.A. (2010). Broad conservation of milk utilization genes in *Bifidobacterium longum* subsp. *infantis* as revealed by comparative genomic hybridization. Appl. Environ. Microbiol. 76, 7373-7381. doi: 10.1128/AEM.00675-10.

Maldonado-Gomez, M.X., Lee, H., Barile, D., Lu, M. Hutkins, R.W. (2015). Adherence inhibition of enteric pathogens to epithelial cells by bovine colostrum fractions. Int. Dairy J. 40, 24-32. doi: 10.1016/j.idairyj.2014.08.014.

Morrill, K.M., Conrad, E., Lago, A., Campbell, J., Quigley, J., Tyler, H. (2012). Nationwide evaluation of quality and composition of colostrum on dairy farms in the United States. J. Dairy Sci. 95, 3997-4005. doi: 10.3168/jds.2011-5174.

Nesser, J-R., Golliard, M., Vedovo, S.D. (1991). Quantitative determination of complex carbohydrates in bovine milk and milk-based infant formulas. J. Dairy Sci. 74, 2860-2871.

Priestley, D., Bittar, J.H., Ibarbia, L., Risco, C.A., Galvao, K.N. (2013). Effect of feeding maternal colostrum or plasma-derived or colostrum-derived colostrum replacer on passive transfer of immunity, health, and performance of preweaning heifer calves. J. Dairy Sci. 96, 3247–3256. doi: 10.3168/jds.2012-6339.

Round, J.L., Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. 9, 313-323. doi: 10.1038/nri2515.

Russell, S.L., Gold, M.J., Willing, B.P., Thorson, L., McNagny, K.M., Finlay, B.B. (2013). Perinatal antibiotic treatment affects murine microbiota, immune responses and allergic asthma. Gut Microbes. 4, 158-164. doi: 10.4161/gmic.23567.

Sabat, G., Rose, P., Hickey, W.J., Harkin, J.M. (2000). Selective and sensitive method for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. Appl. Environ. Microbiol. 66, 844-849.

Sela, D.A., Li, Y., Lerno, L., Wu, S., Marcobal, A.M., German, J.B., ET AL., (2011). An infant-associated bacterial commensal utilizes breast milk sialyloligosaccharides. J. Biol. Chem. 286, 11909-11918. doi: 10.1074/jbc.M110.193359.

Stevenson, D.M., Weimer, P.J. (2007). Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl. Microbiol. Biotechnol. 75, 165–174.

Teixeira, A.V.G., Bicalho, M.L.S., Machado, V.S., Oikonomou, G., Kacar, C., Foditsch, C., et al., (2013). Heat and ultraviolet light treatment of colostrum and hospital milk: Effects on colostrum and hospital milk characteristics and calf health and growth parameters. Vet. J. 197, 175-181. doi: 10.1016/j.tvjl.2013.03.032.

ten Bruggencate, S.J., Bovee-Oudenhoven, I.M, Feitsma, A.L., van Hoffen, E., Schoterman, M.H. (2014). Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. Nutr. Rev. 72, 377-389. doi:

10.1111/nure.12106.

Uetake K. (2013). Newborn calf welfare: a review focusing on mortality rates. Anim. Sci. J. 84, 101-105. doi: 10.1111/asj.12019.

Vasseur, E., Borderas, F., Cue, R.I., Lefebvre, D., Pellerin, D., Rushen, J., et al., (2010). A survey of dairy calf management practices in Canada that affect animal welfare. J. Dairy Sci. 93, 1307-1315. doi: 10.3168/jds.2009-2429.

Yu Z., Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTechniques. 36, 808-812.

3.6 Tables and Figures

Table 3.1 Bacterial primers used to estimate the copy number of 16S rRNA gene in the calf small intestine

Bacterial group	Primers	Product size	Annealing temperature	Reference
Total bacteria	F: 5'-actcctacgggaggcag-3' R: 5'-gactaccagggtatctaatcc-3'	467bp	62°C	Stevenson & Weimer, 2007
Lactobacillus	F: 5'-gaggcagcagtagggaatcttc-3' R: 5'-ggccagttactacctctatccttcttc-3'	120bp	62°C	Delroisse et al., 2008
Bifidobacterium	F: 5'-tacaccggaatagctcctgg-3' R: 5'-cgtcaagctgataggacgc-3'	115bp	64°C	Liang et al., 2014
E. coli	F: 5'-ggaagaagcttgcttctttgctgac-3' R: 5'-agcccggggatttcacatctgactta-3'	544bp	62°C	Sabat et al., 2000

Table 3.2 Impact of heat-treated colostrum feeding on small intestinal bacterial densities within the first 12 hours

	Colostrum treatment		**Time point**		**Small intestinal region**			**Sample type**	
	FC	**HC**	**6hr**	**12hr**	**PJ**	**DJ**	**IL**	**Tissue**	**Content**
Total bacteria[1]	$1.3\pm0.3\times10^{10}$	$8.4\pm1.4\times10^{9}$	$6.3\pm0.7\times10^{9a}$	$1.5\pm0.4\times10^{10b}$	$5.8\pm1.1\times10^{9}$	$1.3\pm0.4\times10^{10}$	$1.7\pm0.5\times10^{10}$	$8.2\pm2.4\times10^{9a}$	$1.5\pm0.8\times10^{10b}$
P - value	0.22		< 0.01		0.14			0.03	
Lactobacillus[1]	$4.4\pm2.6\times10^{5}$	$3.7\pm1.6\times10^{5}$	$3.6\pm1.6\times10^{5}$	$4.5\pm2.6\times10^{5}$	$3.5\pm1.9\times10^{5}$	$3.7\pm1.8\times10^{5}$	$4.7\pm2.6\times10^{5}$	$3.3\pm1.3\times10^{5}$	$5.1\pm3.2\times10^{5}$
P - value	0.56		0.44		0.61			0.25	
Bifidobacterium[1]	$6.0\pm2.1\times10^{8}$	$9.4\pm3.2\times10^{8}$	$8.3\pm2.9\times10^{8}$	$7.2\pm2.4\times10^{8}$	$6.3\pm2.2\times10^{8}$	$8.4\pm3.0\times10^{8}$	$8.5\pm2.9\times10^{8}$	$1.2\pm0.4\times10^{9a}$	$3.7\pm1.6\times10^{8b}$
P - value	0.16		0.65		0.26			< 0.01	
E. coli[1]	$7.9\pm3.4\times10^{5}$	$7.8\pm4.3\times10^{4}$	$1.3\pm0.5\times10^{5}$	$7.4\pm3.3\times10^{5}$	$1.5\pm0.6\times10^{5}$	$5.9\pm2.7\times10^{5}$	$5.6\pm2.4\times10^{5}$	$2.6\pm0.9\times10^{5}$	$5.7\pm2.8\times10^{5}$
P - value	< 0.01		< 0.01		0.18			0.07	
Lactobacillus[2]	0.01±0.003	0.007±0.001	0.01±0.003	0.008±0.002	0.02±0.005	0.01±0.002	0.01±0.002	0.005±0.002[a]	0.016±0.010[b]
P - value	0.28		0.20		0.12			< 0.01	
Bifidobacterium[2]	14.3±2.0	16.2±2.0	15.0±1.8	15.5±2.2	19.7±3.4	12.7±2.2	12.6±2.4	21.2±8.1[a]	7.8±2.3[b]
P - value	0.79		0.95		0.07			< 0.01	
E. coli[2]	0.022±0.02	0.001±0.0008	0.004±0.002	0.019±0.015	0.004±0.002	0.03±0.02	0.004±0.001	0.005±0.001	0.017±0.014
P - value	0.17		0.28		0.37			0.35	

[1] copy number of 16S rRNA gene/g of fresh sample; [2] prevalence of *Lactobacillus*, *Bifidobacterium* and *E. coli* as a % of total bacteria

[a,b] means with different superscript within a raw are significantly different at $P < 0.05$ (mean ± SEM)

FC – fresh colostrum, HC – heat-treated colostrum, PJ – proximal jejunum, DJ – distal jejunum, IL - ileum

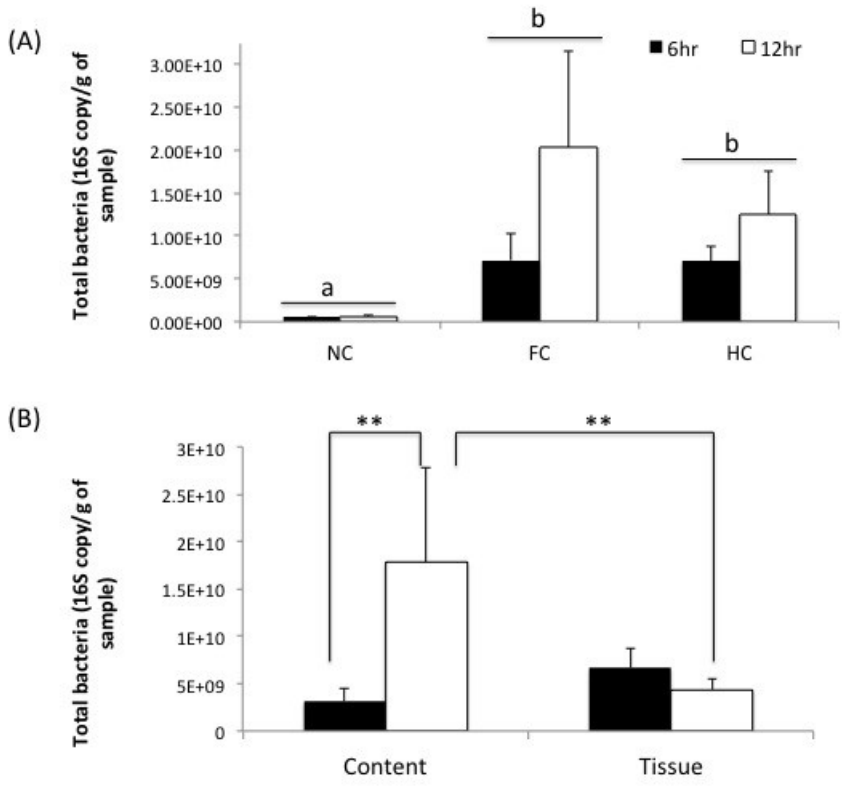

Figure 3.1 Effect of colostrum feeding on small intestinal total bacterial density of neonatal calves

(A) Total bacterial density in the calf small intestine within the first 12 hours of life with differing colostrum feeding methods (B) Total bacterial density in tissue and content-associated communities within the first 12 hours postpartum (NC- no colostrum; FC – fresh colostrum; HC – heat-treated colostrum). (Bars represent mean ± SEM; a,b – means with different letters are different at $P < 0.05$; ** - mean densities of total bacteria within content- or tissue-associated communities are different between 6 and 12 hours at $P < 0.05$)

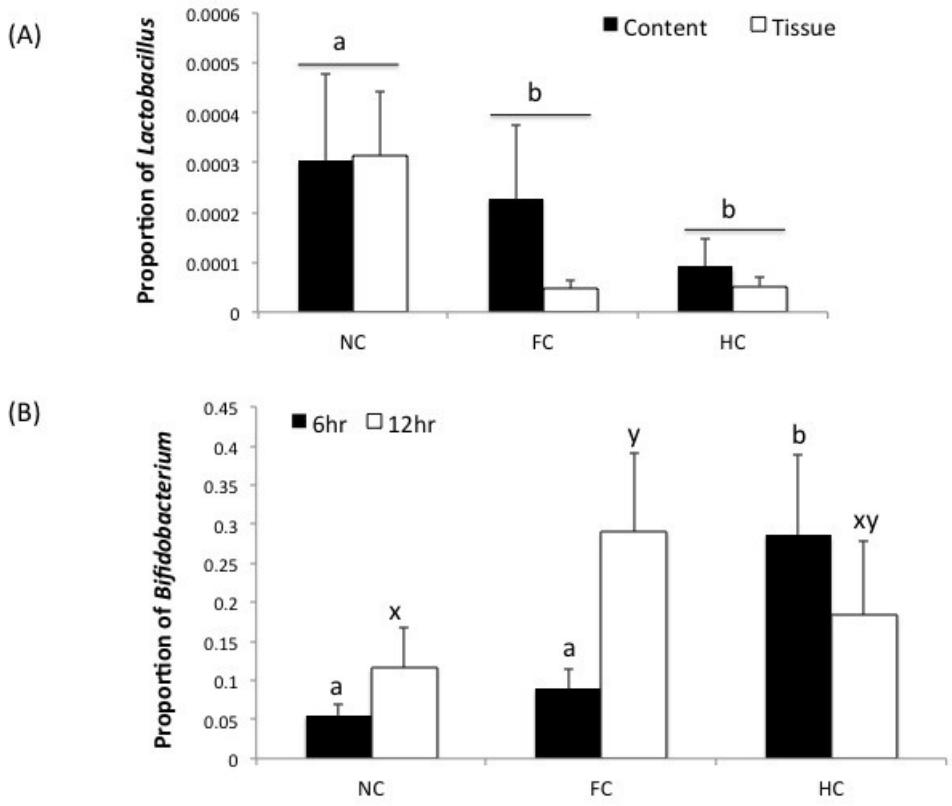

Figure 3.2. Impact of colostrum feeding on beneficial bacterial establishment in calf small intestine

(A) Prevalence of *Lactobacillus* in the calf small intestine content and tissue-associated communities with differing colostrum feeding methods (B) Prevalence of *Bifidobacterium* in the small intestinal tissue-attached community within the first 12 hours of life with differing colostrum feeding methods. (Bars represent mean ± SEM; a,b; x, y – means with different letters are different at $P < 0.05$)

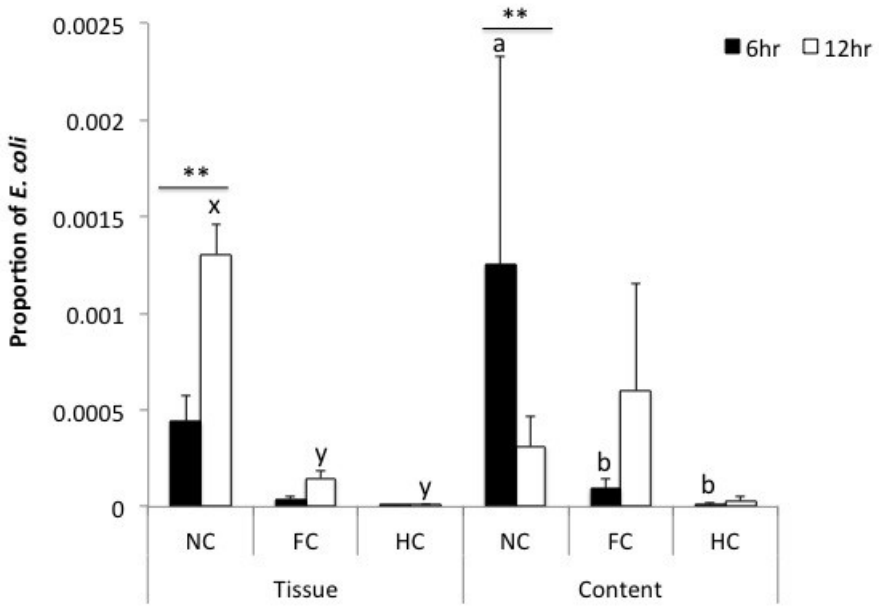

Figure 3.3 Impact of colostrum feeding on the colonization of *E. coli* in the calf small intestine.

a,b – mean *E. coli* prevalence in the small intestinal contents is different among the colostrum feeding methods at $P < 0.05$, x,y – mean *E. coli* prevalence is different among the colostrum feeding methods at $P < 0.05$, ** - mean *E. coli* prevalence within NC is different between 6 and 12 hours after birth at $P < 0.05$. (Bars represent mean ± SEM)

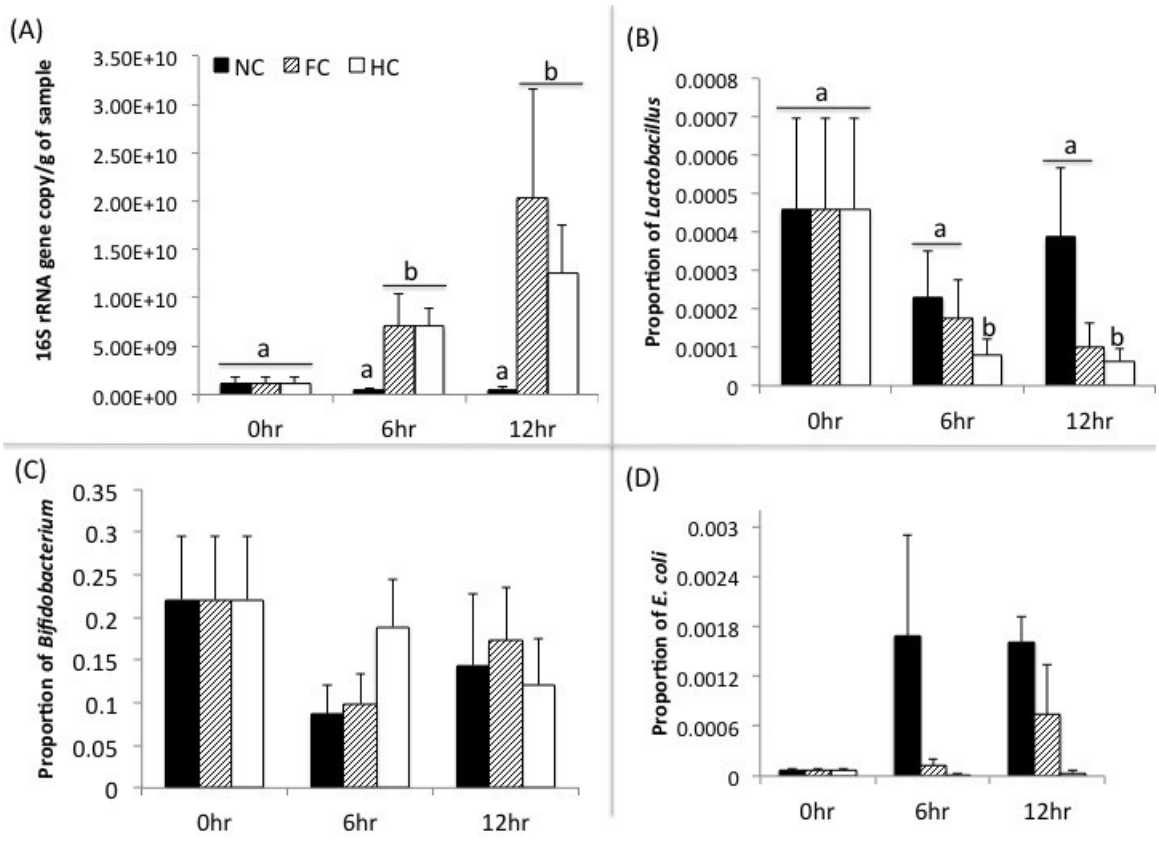

Figure 3.4 Bacterial colonization within the first 12 hours of life when calves fed with differing colostrum feeding methods.

(A) Total bacterial density in the calf small intestine (B) The prevalence of *Lactobacillus* in the calf small intestine (C) The prevalence of *Bifidobacterium* in the calf small intestine (D) The prevalence of *E. coli* in the calf small intestine (data present as mean ± SEM, a,b – means with different letters are different at $P < 0.05$)

Chapter 4. Taxonomic and functional composition of the small intestinal microbiome in newborn calves provides a novel framework to analyze the evolution of pioneer species

Abstract

This study analyzed the taxonomic and functional composition of the small intestinal microbiome of newborn calves to understand the dynamics of microbial establishment and to elucidate potential functional differences in the microbiomes of individual animals. Despite highly individualized microbial communities in each animal, I identified two distinct taxonomic-based clusters of microbial profiles that comprised of either a high level of *Lactobacillus* or *Bacteroides*. Furthermore, *Bacteroides*, *Prevotella*, *Roseburia*, *Ruminococcus*, and *Veillonella* were of low abundance (≤ 0.1%) or were absent in the ileum of *Lactobacillus*-dominant calves. Microbiome profiles also revealed two distinct function-based clusters that related to either high protein metabolism or sulfur metabolism. The bacterial composition of these two function-based clusters suggests an antagonistic relationship between sulfur reducing bacteria and *Bifidobacterium*, which compete for the sulfide amino acid cysteine. The present study identified novel microbial markers that can be used to broadly categorize the microbiome in newborn calves, despite highly individualized early microbiomes. These distinct taxonomic and functional clusters may provide a novel framework to further analyze interactions between the intestinal microbiome and the immune function, health, and growth of newborn calves.

4.1 Introduction

Microbial functions associated with the human microbiome include three broad categories: housekeeping processes universal to microbes, biosynthesis processes conserved across all body sites, and body site-specific pathways (Shafquat et al., 2014). The gut microbiome consists of millions of non-redundant genes that are necessary for microbial survival in the gastrointestinal tract environment, and the majority of these microbial genes (~99%) are of bacterial origin (Qin et al., 2010). The biosynthetic processes of the gut microbiota are now being used to explain the mechanisms underlying host-microbial interactions in humans (Turnbaugh et al., 2009). For example, the *Firmicutes*: *Bacteroidetes* ratio is high in obese individuals, and the high-energy harvesting capacity of *Firmicutes* relative to *Bacteroidetes* has been implicated as a causal factor contributing to the obese phenotype in humans (Turnbaugh et al., 2009). Thus, it is important to understand both the microbial functions and the taxonomic composition when elucidating the mechanisms underlying host-microbial interactions.

Metagenome (genetic content of a microbial community) sequencing has been widely used to explore human microbiomes (The Human Microbiome Project Consortium, 2012). Metagenomic analyses provide information on gene composition and abundance, which can then be used to predict microbial functions and to profile taxonomic composition (Petrosino et al., 2009). Few metagenomic studies have focused specifically on cattle, an economically important livestock species. Furthermore, these existing studies have focused primarily on functional characterizations of the microbiomes in adult cattle (Denman and McSweeney, 2015), including a recent study of antibiotic resistance genes present in cattle feces (Chambers et al., 2015).

Metagenomic-based studies in newborn calves have been limited to a single publication reporting the presence of glycoside hydrolases in the rumen microbiota of two-week-old calves prior to the ingestion of a solid diet (Li et al., 2012). Links between early gut microbial composition and calf health and growth were first suggested in a study characterizing the fecal microbiota of pre-weaned calves (Oikonomou et al., 2013). This type of study fails, however, to reveal whether changes in the microbiome are a cause or the result of enteric infections. The small intestine of newborn dairy calves harbors region-specific microbiota (Malmuthuge et al., 2014), and a recent study in mice demonstrated region-specific regulation of the intestinal epithelium by gut microbes (Sommer et al., 2015). There is no information on microbial functions in the intestinal tracts of newborn calves, a developmental period characterized by diverse pioneer species (Malmuthuge et al. 2014). Neonatal dairy calves are highly susceptible to infection by a variety of enteric pathogens that target the mucosal epithelium in the small intestine (Cho et al., 2014). Therefore, the taxonomic diversity and functions of the early intestinal microbiome must be characterized before the mechanisms by which the microbiome may alter mucosal immune and barrier function can be elucidated.

In this study, whole genome sequencing of the metagenome was used to characterize the taxonomic and functional composition of the microbiome in the small intestine of newborn calves. The present study generated a 296.6 Gb dataset (26,090,126±1,062,327 reads) via metagenomic sequencing of small intestinal digesta-associated communities collected from one-, three- and six-week-old healthy calves. In addition, we sequenced the V1-V3 region of the 16S rRNA genes from mucosa-attached bacteria (0.21 Gb dataset; 22,176±1,171 reads) to better define colonization by epimural

bacteria. The results from these analyses provide the first characterization of functions associated with the intestinal microbiome and provide a novel framework for investigating the functional consequences of microbial dysbiosis during the neonatal period.

4.2 Materials and methods

4.2.1 Animal experiments and sampling

All experimental protocols were approved by the Livestock Care Committee of the University of Alberta (AUP00001012) and were conducted following the guidelines of the Canadian Council on Animal Care. Holstein bull calves (n = 18) were obtained from the Dairy Research and Technology Center (DRTC), University of Alberta (Edmonton, AB), and reared under the DRTC standard management practices. Calves received 4 L of colostrum/day during the first three days postpartum and 4 L of whole milk/day from the fourth day onwards. Calves had *ad libitum* access to calf starter (29.5% crude protein, 4.0% crude fat, 9.0% crude fiber, Wetaskwin Co-Op Country Junction, Wetaskwin, Alberta) from the second to the sixth week postpartum. Calves used in the present study had no record of respiratory or enteric diseases.

Mucosal tissue and digesta samples (proximal jejunum, distal jejunum and ileum) were collected from calves at week one (1W; n = 6), week three (3W; n = 6) and week six (6W; n=6) after birth, snap-frozen and stored in a -80°C freezer. Sampling sites were consistent among all animals. Ileal samples were collected 30 cm proximal to the ileo-cecal junction, distal jejunal samples were collected 30 cm proximal to the collateral branch of the cranial mesenteric artery and proximal jejunal samples were collected 100

cm distal to the pylorus sphincter. Ten-centimeter intestinal segments were collected from each site in the middle of each segment aligned with the above-mentioned measurements.

4.2.2 Analysis of the small intestinal microbiome[10]

Total DNA was extracted from tissue and digesta samples (n = 5/age group) using the repeated bead-beating plus column (RBB+C) method (Yu and Morrison, 2004). DNA libraries were prepared for whole-genome sequencing using the Truseq DNA PCR-free library preparation kit (Illumina, CA, USA) following the manufacturer's instructions. The genomic DNA was first normalized with a resuspension buffer to a final volume of 55 µL at 20 ng/µL, and 50 µL of the solution was transferred into a Covaris microTUBE (Covaris Inc., Massachusetts, USA) for fragmentation using a Covaris S2 focused-ultrasonicator (Covaris Inc., MA, USA). Then, the cleaned up fragmented DNA was subjected to end repair and size selection, followed by the adenylation of the 3' ends and ligation of the adaptor index. Each metagenomic library was then quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, MA, USA) and was subjected to whole genome sequencing using an Illumina Hiseq 2000 at Génome Québec (Montréal, Canada).

Data were analyzed using the MG-RAST metagenomic analysis server, version 3.3.9 (Meyer et al., 2008) to explore the functional and taxonomical composition of the small intestinal digesta-associated microbiome. The subsystems approach (set of related functions that implement a biological process or structural complex; Overbeek et al.,

[10] Laboratory technicians Xu Sun and Yanhong Chen assisted with library preparation and qPCR

2005) in the SEED hierarchy was used to assign microbial functions to the small intestinal microbiomes. The taxonomic assignments were performed using the M5 non-redundant protein database (M5NR) within MG-RAST. All metagenome sequence data were deposited in MG-RAST and are publicly accessible at http://metagenomics.anl.gov/linkin.cgi?project=6020.

DNA extracted from tissue samples (n = 3/age group) was amplified with the 27F and 338R primers containing pyrotags (Malmuthuge et al., 2014), and purified PCR products (~400 bp) were subjected to 454 sequencing (Roche GS-FLX Titanium) at Génome Québec (Montréal, Canada). Data were analyzed using the Quantitative Insight into Microbial Ecology (QIIME) tool kit (Caparaso et al., 2010), following the removal of low quality (Phred score < 25, length < 100 bp) and chimeric sequences. All sequences were deposited at the NCBI Sequence Read Archive (SRR1697258 – SRR1697304).

4.2.3 Estimation of small intestinal bacterial densities using quantitative real-time PCR

Total bacteria, *Lactobacillus* and *Bifidobacterium* densities were estimated using SYBR green chemistry (Fast SYBR® Green Master Mix, Applied Biosystems) with a StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using universal bacterial primers (U2F - 5'-ACTCCTACGGGAGGCAG-3', U2R - 5'-GACTACCAGGGTATCTAATCC-3'; Stevenson and Weimer, 2007) for total bacteria as well as genus-specific primers for *Lactobacillus* (LacF - 5'-GAGGCAGCAGTAGGGAATCTTC-3', LacR - 5'-GGCCAGTTACTACCTCTATCCTTCTTC-3'; Delroisse et al., 2008) and

Bifidobacterium (BifF - 5'-TACACCGGAATAGCTCCTGG-3', BifR - 5'-CGTCAAGCTGATAGGACGC-3'; Liang et al., 2014). Standard curves for total bacteria, *Lactobacillus* and *Bifidobacterium* were generated using purified 16S rRNA genes of *Butyrivibrio hungateii*, *Lactobacillus acidophilus* ATCC4356 and *Bifidobacterium longum,* respectively. The copy number of 16S rRNA gene/g of fresh tissue or digesta was then calculated using the equation described by Li et al., (2009).

4.2.4 Data analysis

Taxonomic and functional abundances of digesta-associated communities obtained from the MG-RAST platform were subjected to pairwise comparisons (1W versus 3W; 1W versus 6W; 3W versus 6W) using metastats (White et al., 2009) to explore the postnatal changes in microbial taxonomy and functions. Regional effects on the gut microbiota were evaluated using pairwise comparisons between gut regions within an age group (proximal jejunum versus distal jejunum; proximal jejunum versus ileum, distal jejunum versus ileum). Multiple test correction was performed according to the method described in Benjamini and Hochberg (1995) and the significant comparisons were declared at FDR < 0.05. All bacterial density data were analyzed using the MIXED procedure in SAS (SAS 9.4, SAS Inc., Cary, NC). Repeated measurement experimental design (experimental unit – animal; repeated measure – gut region) with a compound symmetry covariance structure was selected as the best fit according to the Bayesian information criteria (BIC), and the following statistical model was fitted to evaluate postnatal changes in bacterial density. $Y_{ijkl} = \mu + A_i + R_j + S_k + (AR)_{ij} + (AS)_{ik} + (RS)_{jk} + (ARS)_{ijk} + e_{ijkl}$, where Y = bacterial density/proportion (total bacteria, *Lactobacillus*,

Bifidobacterium); μ = mean; A = calf age; R = small intestinal region; S = sample type (tissue, content); and e = residual error.

4.3 Results

4.3.1 Postnatal changes in the taxonomic composition of small intestinal microbial communities

4.3.1.1 Digesta-associated microbiota

The small intestinal digesta microbiome of newborn calves was dominated by bacteria (Figure 4.1) that consisted of 21 phyla, 46 families and 510 genera. Four bacterial phyla accounted for nearly 93% of all identified bacteria; however, there was substantial variation in phyla abundance among individual calves (*Firmicutes* - 11-80%; *Bacteroidetes* - 0.5-75%; *Proteobacteria* - 0-35%; and *Actinobacteria* - 1-85%) (Figure 4.2). These four main phyla were observed in all intestinal regions of all calves (n = 15), with the exception of *Proteobacteria*, which was absent from the proximal jejunum of one 6W calf. Individual animal variation in bacterial composition was highest within the lower taxonomic hierarchy, and no single bacterial family or genus was detected in the intestinal samples collected from all animals. The most frequently detected families (> 40 out of 45 samples) were *Clostridiaceae*, *Eubacteriaceae*, *Lachnospiraceae*, *Bacillaceae*, *Bacteroidaceae*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Streptococcaceae* and *Veillonellaceae*.

Among the bacterial genera detected in newborn calf intestinal samples, *Clostridium* and *Eubacterium* were present in all jejunal samples, whereas *Prevotella* and *Bacteroides* were observed in all ileal samples. *Bifidobacterium*, *Prevotella*,

Lactobacillus, *Clostridium*, *Bacteroides*, *Streptococcus*, and *Eubacterium* were present in all calves sampled, although they were not detected in all intestinal regions surveyed. For example, *Eubacterium* was not detected in the ileum sample collected from one of the 1W calves. Moreover, the relative abundance of the bacteria detected differed when comparing among the three age groups (Table 4.1) but not when comparing the three small intestinal regions. These differences were most apparent when 1W calves were compared to either 3W or 6W calves, but not when 3W and 6W calves were compared (Table 4.1). There were increasing and decreasing trends in the abundance of individual bacterial genera as calves aged (Figure 4.3A, 1B). For example, the mean abundance of *Bifidobacterium* and *Ruminococcus* increased with calf age (Figure 4.3A), whereas the abundance of *Bacteroides* and *Lactobacillus* decreased with calf age (Figure 4.3B).

4.3.1.2 Tissue-attached microbiota

Sequencing of the V1-V3 region of 16S rRNA genes revealed that the small intestine epimural bacterial community was comprised of 6 phyla, 38 families and 104 genera. Similar to the digesta-associated bacteria, the relative abundance of phyla varied greatly among individual calves (*Actinobacteria* - 0-93.3%, *Firmicutes* - 0-94.4%, *Proteobacteria* - 0-99.9% and *Bacteroidetes* - 0-35.3%) (Figure 4.4). No single bacterial family was identified in all epimural samples; however, *Lachnospiraceae*, *Clostridiaceae*, *Prevotellaceae*, *Coriobacteriaceae* and *Ruminococcaceae* were observed in at least one intestinal region of all calves sampled. Similarly, no single bacterial genus was observed in all tissue samples anlayzed. The predominant epimural bacteria observed were *Propionibacterium* (7.3±4.0%), *Prevotella* (7.0±2.9%), and *Lactobacillus*

(4.8±2.0%) in 1W samples, *Propionibacterium* (12.2±8.9%) and *Prevotella* (6.6±3.3%) in 3W samples, and *Bifidobacterium* (8.5±4.2%), *Sharpea* (4.6±3.8%) and *Prevotella* (3.0±1.4%) in 6W samples. *Prevotella* was detected in at least one intestinal region of all calves sampled. The mean abundances of *Sharpea* (1W – 0%, 3W – 0.4±0.2%, 6W – 4.6±4.2%) and *Faecalibacterium* (1W – 0.2±0.2%, 3W – 0.4±0.2%, 6W – 0.7±0.6%) increased with age, while the abundances of *Prevotella* (1W – 6.8±2.3%, 3W – 6.7±3.5%, 6W – 3.3±2.0%), *Bifidobacterium* (1W – 6.0±4.8%, 3W – 3.4±1.2%, 6W – 2.3±2.1%) and *Succiniclasticum* (1W – 2.4±2.3%, 3W – 0.4±0.2%, 6W – 0.2±0.2%) decreased with increasing calf age. *Candidatus Arthromitus* (segmented filamentous bacteria) was observed in the ileum of only one calf at 1W but with a high abundance (40.8%).

4.3.2 Diversity and richness of calf small intestinal microbiota

The digesta-associated gut microbiome increased in diversity with age and dietary changes, but this increase was not significantly different when comparing among age groups (Table 4.2). Although the total number of bacterial genera detected was not different among age groups, there was a significantly (P = 0.02) lower number of predominant genera (relative abundance > 1% in at least one sample) in the ileal digesta of 3W and 6W calves than in the age-matched jejunal communities (Table 4.2). When bacterial diversity and richness (number of detected genera) were compared for tissue-attached bacteria, they were not significantly different among either intestinal regions or age groups (Table 4.2).

4.3.3 Small intestinal bacterial densities of pre-weaned calves

DNA-based estimation revealed that the density of total bacteria and the proportion of *Lactobacillus* were significantly lower ($P < 0.01$) in epimural versus digesta communities, regardless of calf age or intestinal region (Table 4.3). There was a high individual animal variation in the estimated total bacterial density, which ranged from 2.63×10^{8} – 3.22×10^{11} 16S rRNA gene copies/g of sample. Similarly, the density of *Lactobacillus* varied greatly between 1.49×10^{4} – 3.72×10^{9} 16S rRNA gene copies/g of sample. The proportion of *Bifidobacterium* was significantly high ($P = 0.02$) in 1W proximal jejunal digesta compared to that of 3W and 6W digesta as well as 1W proximal jejunal epimura (Table 4.3). Similar to total bacteria and *Lactobacillus*, the density of *Bifidobacterium* varied greatly among individual animals (1.19×10^{7} – 2.21×10^{10} 16S rRNA gene copies/g of samples). However, the coefficient of variation (CV) of *Bifidobacterium* (6.5%) was lower than that of total bacteria (18.5%) and *Lactobacillus* (18.8%).

4.3.4 Small intestinal metagenome of pre-weaned calves

Functional assignment using MG-RAST revealed 29 subsystems/level 1 functions in the SEED hierarchy for all the combined small intestine metagenomes. The metagenome was dominated by genes involved in the metabolism of carbohydrate and proteins for all age groups (Figure 4.5A). Subsystem “respiration” was observed in all samples, and it contained enzymes involved in fermentation, such as anaerobic glycerol-3-phosphate dehydrogenase (EC 1.1.5.3) and formate hydrogenlyase. Although other subsystems were not observed in all samples, they were detected in more than 70% of the

samples. Small intestine metagenomes (functional abundance) did not differ significantly by age group or gut region sampled (Figure 4.5B). Thus, for further analysis of postnatal changes in microbial functions, we grouped all of the sampled intestinal regions. The top subsystems identified from the collective small intestine metagenomes were "carbohydrate metabolism" (range = 0-13.7%; mean±sem = 10.5±0.6%), "protein metabolism" (range = 0-19.5%; mean±sem = 9.7±0.6%), "amino acids and derivatives" (range = 0-12.6%; mean±sem = 6.7±0.4%), "phages, prophages, transposable elements, plasmids" (range = 0-95.4%; mean±sem = 9.3±3.0%), "DNA metabolism" (range = 0-9.6%; mean = 4.8±0.3%), "RNA metabolism" (range = 0-13.7%; mean±sem = 4.8±0.3%), and "cofactors, vitamins, prosthetic groups, pigments" (range = 0-8.1%; mean±sem = 4.3±0.3%).

When the collective intestinal metagenomes were compared among different age groups, the subsystems could be categorized into three patterns depending on age-dependent trends in their abundances (Figure 4.5C, 4.5D, 4.5E). Essential functions for all microbiota, such as "protein metabolism", "carbohydrate metabolism", and "DNA and RNA metabolism" as well as biosynthesis processes ("cofactors, vitamins, prosthetic groups, pigments"), were among the functions that increased in abundance from the first to the third week and then remained stable from the third to the sixth week (Figure 4.5C). In contrast, the abundance of "phages, prophages, transposable elements, plasmids", "membrane transport", "virulence, disease and defense", "iron acquisition", "respiration" and "sulfur and potassium metabolism" decreased from the first to the third week and then remained stable from the third to the sixth week (Figure 4.5D). However, the abundances of "motility and chemotaxis", "cell wall and capsule", "regulation and cell

signaling", "secondary metabolism", "phosphorus metabolism" and "stress response" remained stable throughout the first six weeks of life (Figure 4.5E).

In total, 185 level 2 functions in the SEED hierarchy were detected within the small intestinal communities (proximal jejunum – 179, distal jejunum – 144, ileum – 183). However, there was no single level 2 function observed in all of the samples analyzed. We identified 16 and 5 level 2 functions present in all distal jejunum and ileum samples, respectively, but no level 2 functions were conserved among all of the proximal jejunum samples. Microbial functions related to "protein biosynthesis", "folate and pterines", "DNA repair", "lysine, threonine, methionine, cysteine", and "RNA processing and modification" were detected in all of the distal jejunum and ileal samples. Among the detected level 2 functions, the abundances of "DNA repair", "di and oligosaccharides", "carbohydrates", "transcription", "alanine, serine and glycine", as well as "pyridoxine (vitamin B6)" increased numerically with age (Figure 4.5F). In contrast, the abundances of "electron donating reactions", "protein biosynthesis", "cell division and cell cycle", "central carbohydrate metabolism", "lysine, threonine, methionine and cysteine", and "ABC transporters" decreased numerically with age (Figure 4.5G).

4.3.5 Small intestinal microbiome variation among individual animals

The composition of small intestinal microbiomes exhibited substantial variation among individual animals. Therefore, the taxonomic and functional compositions of digesta-associated microbiomes were further analyzed together as a whole, regardless of the calf age and intestinal region, to understand the individual variations. The CV within an individual animal was compared to that of the relevant age group to define the level of

individuality. The results of this analysis revealed a lower intra-individual variation (variation among three intestinal regions) than the inter-individual variation (variation among individuals within an age group) for the digesta-associated bacterial taxonomic abundance of all age groups (Figure 4.6). Therefore, the bacterial genera (27) present in at least half of the digesta samples (≥ 22 out of 45 samples) were used to perform a Spearman rank correlation analysis to understand the similarities/dissimilarities among samples. This analysis revealed that the majority of individual bacterial profiles (27 out of 45 samples) were not correlated with each other based on their taxonomic composition, while the rest of the samples separated into two clusters (Figure 4.7A). One cluster had a higher abundance of *Lactobacillus* [ileal profiles of 4 calves: 1W – calf5 (72.4%), 3W – calf7 (86.4%) and calf8 (85.8%), 6W – calf12 (56.4%); jejunal profiles of six calves: proximal jejunum: 1W – calf2 (40.4%), calf3 (23.0%), and calf5 (36.2%), 6W – calf12 (28.0%); distal jejunum; 1W – calf1 (38.7%), calf3 (14.9%), and calf5 (58.1%), 3W – calf8 (14.9%), 6W – calf12 (31.2%)], when compared to the other small intestinal samples. Another cluster contained a higher abundance of *Bacteroides* compared to other samples (0.01 – 5.5%) and included all three small intestinal regions of one 1W calf (calf4 - proximal jejunum – 23.7%; distal jejunum – 41.7%; ileum – 56.3%), the distal jejunum of one 1W calf (calf2 – 22.2%) and one 6W calf (calf11 – 16.4%), and the ileum of two 1W calves (calf2 – 45.8%; calf3 – 33.9%) as well as one 3W calf (calf6 – 17.9%). Furthermore, *Bacteroides*, *Prevotella*, *Roseburia*, *Ruminococcus*, and *Veillonella* were either less prevalent (≤ 0.1%) or absent in the four-ileal bacterial profiles of *Lactobacillus*-dominant calves compared to the ileum of *Bacteroides*-dominant calves.

A comparison of the CVs of microbial functions revealed a lower inter-individual variation in the abundance of functions compared to that of taxonomic abundance (Figure 4.6). Moreover, the individual variations of subsystems containing essential functions (protein and carbohydrate metabolism, RNA and DNA metabolism) were lower than other identified subsystems. Functions related to "phages, prophages, transposable elements, plasmids" had the highest CV when compared to all other functions, especially during the first week of life. Spearman rank correlation analysis further confirmed a higher correlation ($R^2 \geq 0.9$, $P < 0.01$) among small intestine functional composition (34 out of 45 samples) than taxonomic composition, regardless of calf age or gut region (Figure 4.7B). Within the highly correlated samples, one cluster contained samples from 3W and 6W calves (cluster 1), whereas the other cluster contained samples from 1W and 3W calves (cluster 2). Comparison of these two clusters revealed that functions related to "protein metabolism" (cluster 1- 12.3±0.5%, cluster 2 - 9.0±0.5%; $P < 0.01$), "amino acids and derivatives" (cluster 1- 7.9±0.4%, cluster 2 - 6.4±0.6%; $P = 0.06$) and "nucleosides and nucleotides" (cluster 1- 5.8±0.3%; cluster 2 - 3.8±0.3%; $P < 0.01$) were significantly enriched in cluster 1 compared to cluster 2. In contrast, functions related to "cell wall and capsule" (cluster 1- 3.1±0.3%; cluster 2 - 4.4±0.3%; $P = 0.01$) and "sulfur metabolism" (cluster 1- 0.3±0.05%; cluster 2 - 0.8±0.2%; $P = 0.02$) were enriched in cluster 2 when compared to cluster 1. Comparison of taxonomic composition revealed differences in the abundances of bacterial genera detected in these two function-based clusters (Figure 4.7B; Table 4.4). The abundances of the predominant genera *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Lactococcus*, and *Corynebacterium* were relatively higher in cluster 1, while the abundance of sulfur reducing bacteria

(*Desulfarculus, Dethiosulfovibrio, Desulfatibacillum, Desulfuromonas, Desulfurispirillum, Desulfotalea*) was relatively higher in cluster 2. Furthermore, there were 19 and 16 additional other genera that were relatively enriched in cluster 1 and cluster 2, respectively (Table 4.4).

4.3.6 Linking identified ileal bacterial taxonomic-based clusters to the ileal transcriptome

Ileal transcriptome data generated from the same calves (Liang et al., In Press) were used to explore whether the variations identified in microbial composition may be associated with the expression of protein coding genes of the host. Although there were no significantly different protein-coding genes between the two groups, expressions of the pro-inflammatory chemokines *CXCL9*, *CXCL10*, *CXCL11* and *CCL2* were numerically higher (> 2-fold change) in *Lactobacillus*-dominant calves than in *Bacteroides*-dominant calves (Figure 4.7C). These genes exhibited an enrichment in functions related to "leukocytes and lymphocytes chemotaxis" as well as "cytokine/chemokine-mediated signaling pathway" (Figure 4.7D). In contrast to *Lactobacillus*-dominant calves, the *Bacteroides*-dominant calves had a numerically higher (> 2-fold change) expression of genes involved in the mitogen-activated protein kinase (MAPK) pathway (*PLCE1*, *CCL22* and *EGFR*) (Figure 4.7C). These genes revealed an enrichment of processes related to "cell adhesion", "response to stimulus" and "regulation of MAPK cascades" (Figure 4.7E).

4.4 Discussion

Although functions of the human gut microbiome are being studied extensively through metagenomics approaches, such knowledge is very limited for ruminants. The present study profiled the small intestine metagenomes of healthy newborn calves to better understand the temporal dynamics of compositional and functional variation associated with the establishment of the gut microbiome. Small intestinal microbiome was mainly colonized by bacteria; however, the metagenomic sequencing revealed the presence of archaea, fungi, protozoa and virus in pre-weaned calf gut. Genus *Methanobrevibacter* dominated small intestine archaeal population, whereas family *Microviridae* dominated virus (phage) population of pre-weaned calves. These observations are in agreement with human studies reporting a higher abundance of *Methanobrevibacter* (Lozupone et al., 2012) and *Microviridae* (Minot et al., 2013) in fecal microbiomes. Presence of fungi phyla *Ascomycota* and *Basidiomycota*, the most abundant fungi identified in the present study, have also reported in a metagenomic-based study conducted using fecal samples of healthy human (Hoffmann et al., 2013). The main protozoa observed in the pre-weaned calf small intestine was genus *Cryptosporidium*. *C. parvum* is the main parasitic protozoa that contributes to neonatal calf diarrhea; however, calves may harbor *Cryptospiridium* even at the absence of clinical signs or symptoms (De Waele et al., 2013). The present study explored the bacterial population of pre-weaned calves in greater depth.

The increased diversity and richness in small intestinal bacterial communities with increasing age suggests a progressive establishment of a complex microbiome during the first six weeks of life. Furthermore, total bacterial densities throughout the

small intestines of newborn calves reached levels similar to those of weaned calves (Malmuthuge et al., 2012). The calves used in this study were fed whole milk during the first week postpartum and whole milk with *ad libitum* calf starter supplementation thereafter. Thus, observed gut microbiome changes in this study were both age- and diet-driven. The dietary changes may have contributed substantially to the significant differences in microbial composition observed when comparing 1W versus 3W and 6W calves. The higher similarity between 3W and 6W calves further suggests that the gut microbiome may have stabilized once solid feed was introduced. Regardless, a high individual animal variation in the composition and density of bacteria was evident throughout the neonatal period. In humans, the fecal microbiome begins to stabilize and increase in similarity among individuals around three years of age (Yatsunenko et al., 2012). This process has been associated with the intake of a stable solid diet. Similarly, high individual animal variation in rumen microbial composition has been reported to decrease with increasing age in cattle (Jami et al., 2013). Thus, high individual variation in the intestinal microbiome may be a conserved trait in the neonate of different species.

Host genetics are one factor known to shape the gut microbiome, leading to individualized-microbial composition in eight-week-old mice (Benson et al., 2010). However, the use of metagenome sequencing of the fecal microbiome categorized the human population into three enterotypes based on microbial taxonomic profiles (Arumugam et al., 2011). A higher individual variation in the microbial composition was also evident in the small intestinal digesta-associated microbiomes of newborn calves. Moreover, these microbial profiles could not be clustered depending on calf age or intestinal region. Therefore, the present study compared the individual microbial profiles

generated from three small intestinal regions of 1W, 3W and 6W calves together to understand individuality of the newborn calf intestinal microbiota in depth. Despite the small number of samples, the clustering of samples based on microbial taxonomic composition revealed individual calves with either high *Lactobacillus* or high *Bacteroides* in the ileum, regardless of calf age. Comparison of the intestinal transcriptomes of these calves suggested a potential link between the individualized gut microbiome and the host immune system.

Lactobacillus-dominant calves exhibited higher expression of *CXCL9*, *CXCL10*, and *CXCL11* in the ileal tissue than *Bacteroides*-dominant calves. These pro-inflammatory chemokines have been shown to be upregulated in dendritic cells and human intestinal epithelial cells following exposure to *Lactobacillus acidophilus* (Weiss et al., 2010; O'Flaherty and Klaenhammer, 2012). The upregulation of pro-inflammatory genes in intestinal epithelial cells quickly returned to baseline levels one-hour after exposure to *L. acidophilus* (O'Flaherty and Klaenhammer, 2012). These observations suggest a potential priming of immune responses by *Lactobacillus* during the first encounter. The priming effect of early *Lactobacillus* exposure may prepare the host for subsequent anti-inflammatory regulatory responses, which may be a crucial process in neonatal calves with a naïve mucosal immune system. In contrast to these studies, infectious models (colitis and pneumovirus infection in mouse) have demonstrated that *Lactobacillus* exerts an anti-inflammatory effect through the downregulation of *CXCL10* and *CXCL11* (Gabryszewski et al., 2011; von Schillde et al., 2012). Therefore, these studies further suggest that the beneficial effects of *Lactobacillus* may vary depending on

the context, with a priming of immune responses upon initial encounter but a reduction of inflammatory responses during infections.

MAPK cascade-related genes are involved in the regulation of T-cell differentiation (Rincon et al., 2001) and increased expression of these genes in *Bacteroides*-dominant calves may be consistent with an increased capacity for T-cell-dependent responses. Although our transcriptome data from calves with either a higher abundance of *Lactobacillus* or *Bacteroides* in the ileum revealed differential activation of the mucosal immune system, further investigations are required to determine how these changes in mucosal immune function may alter the host responses to enteric infections. The expression of enriched-genes in each cluster was not statistically different. Therefore, validation of these enriched-genes in a larger population and/or using challenged models with phenotypic measurements will be required to determine if *Lactobacillus* and *Bacteroides* define enterotypes in the calf small intestine that are associated with specific immune functions and health outcomes.

Although taxonomic profiles of the microbiome in individual calves were markedly different, such differences were not directly reflected in functional profiles. This may be due to the observed higher abundance of universal microbial functions that are important for microbial survival, such as “protein biosynthesis”, “RNA processing and modification”, “DNA replication”, “central carbohydrate metabolism”, and “cell cycle”. Nevertheless, two distinct clusters based on functional abundance were observed within the small intestinal microbiome. Cluster 1 was enriched with functions related to microbial protein metabolism, which has been suggested to play an important role in host health through the regulation of amino acid homeostasis in the gut (Neis et al., 2015).

Moreover, the bacteria of calves in cluster 1 had a higher abundance (69-fold) of *Bifidobacterium* than calves in cluster 2. A recent study has shown that *Bifidobacterium* utilize milk protein effectively and require cysteine, a sulfide amino acid, for growth (Ferrario et al., 2015). Sulfur reducing bacteria (SRB), which were mainly found in cluster 2, also utilize cysteine as their energy source (Carbonero et al., 2012). Thus, the observed low abundance or absence of *Bifidobacterium* in cluster 2 calves may be due to the lack of cysteine for their growth. Dissimilatory sulfur reduction and cysteine degradation by SRB produce H_2S that can have detrimental effects on intestinal epithelial cells (Carbonero et al., 2012). An accumulation of H_2S in the colon inhibits the activity of acyl-CoA dehydrogenase, which catalyzes the oxidation of butyrate in colonocytes and compromises epithelial barrier permeability (Babidge et al., 1998). *Bifidobacterium* has been reported to promote intestinal epithelial barrier functions (Ulluwishewa et al., 2011; Hsieh et al., 2015), while SRB have been reported to have a negative impact on the epithelial barrier (Babidge et al., 1998). Although these observed differences in bacterial composition might be consistent with potential differences in epithelial barrier functions between cluster 1 and cluster 2, transcriptome data (Liang et al., In Press) revealed no differences in the expression of tight junction genes (< 2-fold change in the expression of genes belonging to the claudin family, occludin, zonula occludens and junctional adhesion molecules) when comparing animals in cluster1 and cluster2. Intestinal barrier integrity loss can be measured using changes in the expression of tight junction proteins, epithelial cell damage or the translocation of bacterial products (Grootjans et al., 2010). Therefore, assessing barrier function by using these alternative indicators may provide

more sensitive measurement of how composition and functional variations in the gut microbiota impact epithelial barrier function.

The small intestine bacterial taxonomic composition observed through sequencing of the digesta-associated metagenome in the present study was similar to previous studies reporting a higher abundance of *Firmicutes* in the small intestine (Malmuthuge et al., 2014) and the fecal microbiome (Oikonomou et al., 2013) of young calves. However, there were significant differences within the lower taxonomic hierarchy that could be attributable to differences in the microbial profiling tools (whole genome sequencing versus 16S amplicon sequencing), calf age, and sample type (fecal versus regional intestine). Sequencing the V1-V3 region of 16S rRNA genes revealed a higher abundance of *Lactobacillus* in the ileum and *Sharpea* in the proximal jejunum at three weeks of life (Malmuthuge et al. 2014). However, the use of metagenomic profiling in the present study revealed a higher abundance of *Prevotella* (17.9±12.8%) in the ileum and *Streptococcus* (12.0±6.1%) in the proximal jejunum of three-week-old calves. Moreover, an analysis of postnatal changes in the fecal microbiome reported a decrease in *Bifidobacterium* abundance with increasing calf age (Uyeno et al., 2010; Oikonomou et al., 2013), but the present study revealed that abundance of *Bifidobacterium* in the small intestine increased with increasing calf age. As the present study used different animals at each time point to analyze the patterns in postnatal changes, caution is required when comparing these results with previous studies that sampled the same animals repeatedly overtime. In contrast to digesta communities, the epimural communities differed from those reported in a previous study, even at a higher level of the taxonomic hierarchy (Malmuthuge et al., 2014). In contrast to our previous study (Malmuthuge et al., 2014),

the present study revealed a higher abundance of *Actinobacteria* (41.2±15.1%), followed by *Firmicutes* (26.8±9.6%), *Proteobacteria* (20.1±15.5%) and *Bacteroidetes* (11.8±6.1%) in the ileal tissues of three-week-old calves, and a higher abundance of *Proteobacteria* (66.5±23.2%), followed by *Firmicutes* (14.0±8.1%), *Actinobacteria* (11.0±10.5%) and *Bacteroidetes* (8.0±4.2%) in the jejunal tissues. The use of animals from different herds and feeding calves different starter rations may explain some of the observed differences between these two studies. Additionally, the small intestinal epimural bacterial community is closely scrutinized by the host mucosal immune system (Hansen et al., 2012), which may also contribute to taxonomic variation in the microbial communities among different animals.

Despite the extensive use of preventative health management strategies, including vaccination of pregnant cows and management of colostrum feeding (Smith, 2015), a high rate of diarrhea-related neonatal deaths remains a major health problem in the cattle industry (USDA, 2010). Neonatal calf diarrhea is often treated with antibiotics, but the unrestricted use of antibiotics in the livestock industry is a controversial issue (Smith, 2015), especially given that antibiotic treatment of calf diarrhea has a low efficacy (Berge et al., 2009). One reason for the use of antibiotics as a treatment for calf diarrhea is to prevent pathogenic *E. coli* colonization of the small intestine (Smith, 2015). However, with increasing restrictions on the prophylactic use of antibiotics (Smith, 2015), it is important to explore other microbial manipulation techniques that can limit or prevent pathogen colonization of the calf small intestine. Feeding probiotics has been shown to reduce the frequency of pathogenic *E. coli* in the calf gastrointestinal tract (Zhao et al., 1998), suggesting that promoting the colonization of beneficial bacteria may exclude

pathogens. Limited knowledge of the calf microbiome, especially in the small intestine, is a barrier to developing such strategies to prevent or treat diarrhea. Thus, the present study is a first step in understanding the dynamics of commensal microbiota establishment and the function of the microbiome in the small intestine of newborn calves.

4.5 Conclusion

The present study analyzed the taxonomic and functional evolution of the microbiome in the small intestine of newborn calves during the first six weeks of life. During this developmental period, age- and diet-related increases in microbial diversity, richness and density were observed; however, the taxonomic composition varied widely among individual animals. The comparison of individual animal taxonomic profiles of the ileal digesta classified calves as either *Lactobacillus*-dominant or *Bacteroides*-dominant calves based on the relative abundance of the detected bacterial genera. A comparison of functional profiles also classified calves into two distinct clusters, revealing an antagonism between SRB and *Bifidobacterium*. This is the first study to generate a detailed analysis of the taxonomical and functional development of the small intestinal microbiome in healthy pre-weaned calves. This analysis provides a novel framework to further develop strategies to manipulate the microbiome and improve calf health.

4.6 References

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R. et al., (2011). Enterotypes of the human gut microbiome. Nature. 473, 174-180. doi: 10.1038/nature09944.

Babidge, W., Millard, S., Roediger, W. (1998). Sulfides impair short chain fatty acid beta-oxidation at acyl-CoA dehydrogenase level in colonocytes: implications for ulcerative colitis. Mol. Cell Biochem. 181, 117-124.

Benjamini, Y., Hochberg, y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological). 57, 289-300.

Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim, J., et al., (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc. Natl. Acad. Sci. U S A. 107, 18933-18938. doi: 10.1073/pnas.1007028107.

Berge, A.C.B., Moore, D.A., Besser, T.E., Sischo, W.M. (2009). Targeting therapy to minimize antimicrobial use in preweaned calves: Effects on health, growth, and treatment costs. J. Dairy Sci. 92, 4707-4714. doi: 10.3168/jds.2009-2199.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al., (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7:335-336. doi: 10.1038/nmeth.f.303.

Carbonero, F., Benefiel, A.C., Alizadeh-Ghamsari, A.H., Gaskins, H.R. (2012). Microbial pathways in colonic sulfur metabolism and links with health and disease. Front. Physiol. 3, 448. doi: 10.3389/fphys.2012.00448.

Chambers, L., Yang, Y., Littier, H., Ray, P., Zhang, T., Pruden, A. et al., (2015). Metagenomic analysis of antibiotic resistance genes in dairy cow feces following therapeutic administration of third generation cephalosporin. PLoS One. 10, e0133764. doi: 10.1371/journal.pone.0133764.

Delroisse, J-M., Boulvin, A-L., Parmentier, I., Dauphin, R.D., Vandenbol, M., Portetelle, D. (2008). Quantification of *Bifidobacterium* spp. and *Lactobacillus* spp. in rat fecal samples by real-time PCR. Microbiol. Res. 163, 663-670.

Denman, S.E., McSweeney, C.S. (2015). The early impact of genomics and metagenomics on ruminal microbiology. Annu. Rev. Anim. Biosci. 3, 447-465. doi: 10.1146/annurev-animal-022114-110705.

De Waele, V., Van den Broeck, F., Huyse, T., McGrath, G., Higgins, I., Speybroeck, N., et al., (2013). Panmictic structure of the Cryptosporidium parvum population in Irish calves: influence of prevalence and host movement. Appl. Environ. Microbiol. 79, 2534-2541. doi: 10.1128/AEM.03613-12.

Ferrario, C., Duranti, S., Milani, C., Mancabelli, L., Lugli, GA., Turroni, F., et al., (2015). Exploring amino acid auxotrophy in *Bifidobacterium bifidum* PRL2010. Front. Microbiol. 6, 1331. doi: 10.3389/fmicb.2015.01331.

Gabryszewski, S.J., Bachar, O., Dyer, K.D., Percopo, C.M., Killoran, K.E., Domachowske, J.B., et al., (2011). *Lactobacillus*-mediated priming of the respiratory mucosa protects against lethal pneumovirus infection. J. Immunol. 186, 1151-1161. doi: 10.4049/jimmunol.1001751.

Grootjans, J., Thuijls, G., Verdam, F., Derikx, J.P.M., Lenaerts, K., and Buurman, W.A. (2010). Non-invasive assessment of barrier integrity and function of the human gut. World J. Gastrointest. Surg. 2: 61-69. doi: 10.4240/wjgs.v2.i3.61.

Hansen, C.H.F., Nielsen, D.S., Kverka, M., Zakostelska, Z., Klimesova, K., Hudcovic, T., et al., (2012). Patterns of early gut colonization shape future immune responses of the host. PLoS One. 7, e34043. doi:10.1371/journal.pone.0034043.

Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G.D., et al., (2013). Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. PLoS One. 17, 8:e66019. doi: 10.1371/journal.pone.0066019.

Hsieh, C.Y., Osaka, T., Moriyama, E., Date, Y., Kikuchi, J., Tsuneda, S. (2015). Strengthening of the intestinal epithelial tight junction by *Bifidobacterium bifidum*. Physiol. Rep. 3, e12327. doi: 10.14814/phy2.12327.

Jami, E., Israel, A., Kotser, A., Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7, 1069-1079. doi: 10.1038/ismej.2013.2.

Li, M., Penner, G.B., Hernandez-Sanabria, E., Oba, M., Guan, L.L. (2009). Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107:1924-1934. doi: 10.1111/j.1365-2672.2009.04376.x.

Li, R.W., Connor, E.E., Baldwin, R.L., Sparks, M.L. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environ. Microbiol. 14, 129-139. doi: 10.1111/j.1462-2920.2011.02543.

Liang, G., Malmuthuge, N., Bao, H., McFadden, T., Stothard, P., Griebel, P.J., et al., (2014) Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life. PLoS One. 9, e92592. doi: 10.1371/journal.pone.0092592.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature. 489, 220-230. doi: 10.1038/nature11550.

Malmuthuge, N., Griebel, P.J, Guan, L.L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of pre-weaned calves. Appl. Environ. Microbiol. 80, 2012-2028. doi: 10.1128/AEM.03864-13.

Malmuthuge, N., Li, M., Chen, Y., Fries, P., Griebel, P.J., Baurhoo, B. et al., (2012). Distinct commensal bacteria associated with ingesta and mucosal epithelium in the gastrointestinal tracts of calves and chickens. FEMS Microbiol. Ecol. 79, 337-347. doi: 10.1111/j.1574-6941.2011.01220.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., et al., (2008). The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics. 9, 386. doi: 10.1186/1471-2105-9-386.

Minot, S., Bryson, A., Chehoud, C., Wu, G.D., Lewis, J.D., Bushman, F.D. (2013). Rapid evolution of the human gut virome. Proc. Natl. Acad. Sci. U S A. 110, 12450-12455. doi: 10.1073/pnas.1300833110.

Neis, E.P., Dejong, C.H., Rensen, S.S. (2015). The role of microbial amino acid metabolism in host metabolism. Nutrients. 7, 2930-2946. doi: 10.3390/nu7042930.

O'Flaherty, S., Klaenhammer, T.R. (2012). Influence of exposure time on gene expression by human intestinal epithelial cells exposed to *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 78, 5028-5032. doi: 10.1128/AEM.00504-12.

Oikonomou, G., Teixeira, A.G., Foditsch, C., Bichalho, M.L., Machado, V.S., Bicalho, R.C. (2013). Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium* species with health and growth. PLOS One. 8, e63157. doi: 10.1371/journal.pone.0063157.

Petrosino, J.F., Highlander, S., Luna, R.A., Gibbs, R.A., Versalovic, J. (2009). Metagenomic pyrosequencing and microbial identification. Clin. Chem. 55, 856-866. doi: 10.1373/clinchem.2008.107565.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., et al., (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 464, 59-65. doi: 10.1038/nature08821.

Rincon, M., Flavell, R.A., Davis, R.J. (2001). Signal transduction by MAP kinases in T lymphocytes. Oncogene. 20, 2490-2497.

Shafquat, A., Joice, R., Simmons, S.L., Huttenhower, C. (2014). Functional and phylogenetic assembly of microbial communities in the human microbiome. Trends Microbiol. 22, 261-266. doi: 10.1016/j.tim.2014.01.011.

Smith, G. (2015). Antimicrobial decision making for enteric diseases of cattle. Vet. Clin. North Am. Food Anim. Pract. 31, 47-60. doi: 10.1016/j.cvfa.2014.11.004.

Sommer, F., Nookaew, I., Sommer, N., Fogelstrand, P., Backhed, F. (2015). Site-specific programming of the host epithelial transcriptome by the gut microbiota. Genome Biol. 16, 62. doi: 10.1186/s13059-015-0614-4.

Stevenson, D.M., Weimer, P.J. (2007). Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl. Microbiol. Biotechnol. 75, 165–174.

The Human Microbiome Project Consortium. (2012). A framework for human microbiome research. Nature. 486, 215–221. doi:10.1038/nature11209.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., et al., (2009). A core gut microbiome in obese and lean twins. Nature. 457, 480-484. doi: 10.1038/nature07540.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., Roy, N.C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. J. Nutr. 141, 769-776. doi: 10.3945/jn.110.135657.

USDA (2010). Dairy 2007, Heifer calf health and management practices on US dairy operations. pp 122-131. USDA:APHIS:VS, CEAH. Fort Collins, CO.

Uyeno, Y., Shigemori, S., Shimosato, T. (2015). Effect of probiotics/prebiotics on cattle health and productivity. Microbes. Environ. 30, 126-132. doi: 10.1264/jsme2.ME14176.

von Schillde, M.A., Hormannsperger, G., Weiher, M., Alpert, C.A., Hahne, H., Bauerl, C., et al., (2012). Lactocepin secreted by *Lactobacillus* exerts anti-inflammatory

effects by selectively degrading proinflammatory chemokines. Cell Host Microbe. 11, 387-396. doi: 10.1016/j.chom.2012.02.006.

Weiss, G., Rasmussen, S., Nielsen Fink, L., Jarmer, H., Nohr Nielsen, B., Frokiaer, H. (2010). *Bifidobacterium bifidum* actively changes the gene expression profile induced by *Lactobacillus acidophilus* in murine dendritic cells. PLoS One. 5, e11065. doi: 10.1371/journal.pone.0011065.

White, J.R., Nagarajan, N., Pop, M. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput. Biol. 5, e1000352. doi: 10.1371/journal.pcbi.1000352.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., et al., (2012). Human gut microbiome viewed across age and geography. Nature. 486, 222-227. doi: 10.1038/nature11053.

Yu Z, Morrison M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 36, 808-812.

Zhao, T., Doyle, M.P., Harmon, B.G., Brown, C.A., Mueller, P.O.E., Parks, A.H. (1998). Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol. 36, 641-647.

4.7 Tables and Figures

Table 4.1 Prevalence of bacterial phyla and genera observed in the small intestinal digesta communities

	Proximal jejunum				Distal jejunum				Ileum				Significant comparisons		
	1W	**3W**	**6W**	**SEM**	**1W**	**3W**	**6W**	**SEM**	**1W**	**3W**	**6W**	**SEM**	**Proximal jejunum**	**Distal jejunum**	**Ileum**
Phylum[1]															
Actinobacteria	6.1	22.7	32.1	5.2	2.3	18.7	10.1	0.5	2.7	2.8	28.8	5.5	1W vs. 3W, 6W	1W vs. 3W, 6W	
Bacteroidetes	18.4	14.3	25.0	3.9	26.7	5.8	9.8	2.0	35.4	7.3	9.2	3.8		1W vs. 3W, 6W	1W vs. 3W, 6W
Firmicutes	53.9	50.6	31.8	3.6	43.2	48.0	59.5	4.0	47.4	63.8	36.7	9.6			
Proteobacteria	19.4	7.6	6.9	1.1	26.6	20.6	12.7	2.2	11.2	23.3	16.6	3.8	1W vs. 3W, 6W	3W vs. 6W	
Genus[1]															
Bifidobacterium	4.3	6.0	23.5	5.2	1.1	8.1	3.6	0.2	1.4	1.1	2.2	2.4		1W vs. 3W	
Prevotella	7.9	8.0	20.7	6.1	10.6	2.3	3.8	0.5	5.4	61.7	6.2	0.2		1W vs. 3W, 6W	
Lactobacillus	23.6	3.9	7.4	2.6	24.9	5.4	9.3	2.4	17.1	44.4	11.5	2.5	1W vs. 3W, 6W	1W vs. 3W, 6W	
Clostridium	5.4	7.1	3.6	2.5	3.7	2.8	9.5	0.3	9.8	2.7	6.6	1.7			1W vs. 3W
Ruminococcus	0.7	3.6	3.4	1.3	0.6	1.0	1.9	0.0	1.2	0.7	1.8	0.0	1W vs. 3W, 6W		
Bacteroides	9.0	4.3	3.3	1.8	15.1	2.6	4.9	1.2	27.9	4.7	2.2	0.1			1W vs. 3W, 6W
Streptococcus	3.0	5.7	2.9	2.0	1.6	13.4	7.2	0.8	1.0	4.8	2.3	0.3		1W vs. 3W, 6W	
Eubacterium	4.0	4.0	1.7	0.2	2.1	2.1	4.0	0.1	2.0	2.7	0.9	0.0			

[1] Mean relative abundance as a % of total bacterial phyla or genera observed. 1W- 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves

Table 4.2 Small intestinal bacterial diversity and richness

	Proximal jejunum			Distal jejunum			Ileum		
	1W	**3W**	**6W**	**1W**	**3W**	**6W**	**1W**	**3W**	**6W**
Digesta-associated bacteria (whole genome sequencing)									
Shannon index	1.8±0.8	1.9±1.6	2.2±1.6	1.6±1.0	2.1±1.5	2.2±1.4	1.7±1.2	2.1±1.7	2.0±1.5
# of genera	100±21	66±16	98±21	96±23	96±12	109±12	79±16	78±24	89±19
Predominant genera[1]	15±2	16±1	13±4	11±2	21±1	17±2	13±3	8±3	9±3
Tissue-attached bacteria (16S amplicon sequencing of V1-V3)									
Shannon index	4.2±0.4	4.8±0.7	4.7±0.5	4.2±0.7	3.3±0.6	4.1±0.5	3.7±0.6	3.8±0.9	4.0±0.4
# of genera	23±8	36±7	32±3	23±7	19±9	30±7	22±6	28±9	25±6
Predominant genera[1]	11±2	14±4	8±2	13±4	8±4	8±3	11±3	7±3	11±4

[1] relative abundance > 1% in at least one sample. 1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

Data presented as mean±SEM.

Table 4.3 Bacterial densities in the small intestinal communities of pre-weaned calves

Gut region	Sample type	Age of calves		
		1W	3W	6W
Total bacteria (16S rRNA gene copy/g of fresh sample)[1]				
Proximal jejunum	Tissue	$1.1\pm0.4\times10^{9}$	$1.3\pm0.3\times10^{9}$	$3.5\pm0.9\times10^{9}$
	Digesta	$1.6\pm1.2\times10^{10}$	$2.0\pm0.3\times10^{10}$	$8.5\pm6.1\times10^{10}$
Distal jejunum	Tissue	$1.1\pm0.3\times10^{9}$	$0.8\pm0.2\times10^{9}$	$2.5\pm0.9\times10^{9}$
	Digesta	$2.6\pm2.1\times10^{10}$	$9.4\pm6.6\times10^{9}$	$2.0\pm1.1\times10^{10}$
Ileum	Tissue	$1.0\pm0.2\times10^{9}$	$1.0\pm0.2\times10^{9}$	$3.2\pm1.6\times10^{9}$
	Digesta	$1.5\pm0.8\times10^{10}$	$3.7\pm2.2\times10^{9}$	$9.0\pm7.4\times10^{9}$
***Lactobacillus* (%)[2]**				
Proximal jejunum	Tissue	0.02±0.01	0.001±0.0001	0.012±0.01
	Digesta	3.3±1.7	9.0±8.3	0.80±0.79
Distal jejunum	Tissue	0.03±0.02	0.003±0.001	0.001±0.0001
	Digesta	11.1±6.8	6.1±4.1	0.08±0.07
Ileum	Tissue	0.01±0.0001	0.001±0.0001	0.001±0.0001
	Digesta	6.7±6.1	2.1±1.4	0.56±0.55
***Bifidobacterium* (%)[3]**				
Proximal jejunum	Tissue	5.6±1.9	12.6±0.9	18.7±12.5
	Digesta	25.7±6.4	9.9±6.5	6.2±2.4
Distal jejunum	Tissue	3.9±0.7	9.0±2.2	7.5±2.2
	Digesta	7.8±2.7	17.5±4.5	11.4±3.4
Ileum	Tissue	5.0±0.9	6.0±0.5	2.6±0.4
	Digesta	11.4±3.6	9.6±4.3	14.4±3.3

[1] total bacterial density differed between tissue and digesta, regardless of age and gut region ($P < 0.01$)

[2] prevalence of *Lactobacillus* differed between tissue and digesta, regardless of age and gut region ($P < 0.01$)

[3] prevalence of *Bifidobacterium* was high; in 1W proximal jejunum digesta than 3W and 6W; in 1W proximal jejunum digesta than 1W proximal jejunum tissue; in 1W ileum digesta than 1W ileum tissue ($P = 0.02$)

1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

Table 4.4 Differentially abundant bacterial genera of function-based clusters identified in the calf gut

Genus	**Cluster1**	**Cluster2**	***P* – value[1]**
Aquifex	0.009±0.0	0.10±0.0	2.56456E-10
Alicycliphilus	0	0.18±0.002	6.16124E-10
Desulfarculus	0.003±0.0	0.08±0.0	4.72058E-09
Asticcacaulis	0.003±0.0	0.16±0.001	1.51907E-06
Lysinibacillus	0.033±0.0	0.04±0.0	4.36666E-05
Dethiosulfovibrio	0.053±0.0	0.30±0.01	9.53096E-05
Erwinia	0.001±0.0	0.14±0.0	0.0002
Desulfatibacillum	0	0.74±0.05	0.0001
Alkaliphilus	0	0.05±0.0	0.0001
Eggerthella	0	0.05±0.0	0.0001
Leeuwenhoekiella	0	0.60±0.02	0.0001
Klebsiella	0.22±0.001	0	0.0001
Mycobacterium	0.17±0.0	0.007±0.0	0.0001
Campylobacter	0.39±0.002	0.02±0.0	0.0001
Streptococcus	6.8±1.3	0.21±0.003	0.0001
Bacillus	1.1±0.02	0.08±0.0	0.0001
Bifidobacterium	12.6±5.7	0.18±0.002	0.0001
Allochromatium	0.018±0.0	0.06±0.0	0.001
Desulfuromonas	0.033±0.0	0.05±0.0	0.002
Halanaerobium	0.15±0.001	0	0.003
Lactococcus	3.5±1.0	0.01±0.0	0.003
Acinetobacter	0.34±0.004	0.02±0.0	0.003
Desulfurispirillum	0.02±0.0	0.09±0.001	0.003
Rickettsia	0.04±0.0	0	0.005
Mycoplasma	0.47±0.005	0.01±0.0	0.005
Corynebacterium	1.48±0.10	0.04±0.0	0.006
Leuconostoc	0.58±0.01	0.01±0.0	0.007
Proteus	0.04±0.0	0.002±0.0	0.01
Anabaena	0.01±0.0	0.001±0.0	0.01
Desulfotalea	0.006±0.0	0.05±0.0	0.01
Erysipelothrix	0.008±0.0	0.002±0.0	0.01
Ammonifex	0.06±0.0	0.07±0.0	0.01
Rothia	0.06±0.0	0.04±0.0	0.01
Megasphaera	0.16±0.002	0	0.01
Pedobacter	0.04±0.0	0.02±0.0	0.02
Vibrio	0.26±0.001	0.03±0.0	0.02
Pelodictyon	0.02±0.0	0.05±0.0	0.02
Comamonas	0.23±0.002	6.1±0.8	0.02
Brucella	0.13±0.0	6.8±1.5	0.02
Alicyclobacillus	0.12±0.0	5.4±1.3	0.03

Francisella	0.07±0.0	2.07±0.2	0.03
Myxococcus	0.14±0.002	0	0.03
Geobacillus	0.29±0.002	0.021±0.0	0.04
Exiguobacterium	0.12±0.0	1.504±0.06	0.04
Haemophilus	0.22±0.001	0.026±0.0	0.04
Aeromonas	0.17±0.002	0.014±0.0	0.05

Data presented as mean±SEM.

[1]Mulitple testing correction is performed using Benjamini and Hochberg, 1995

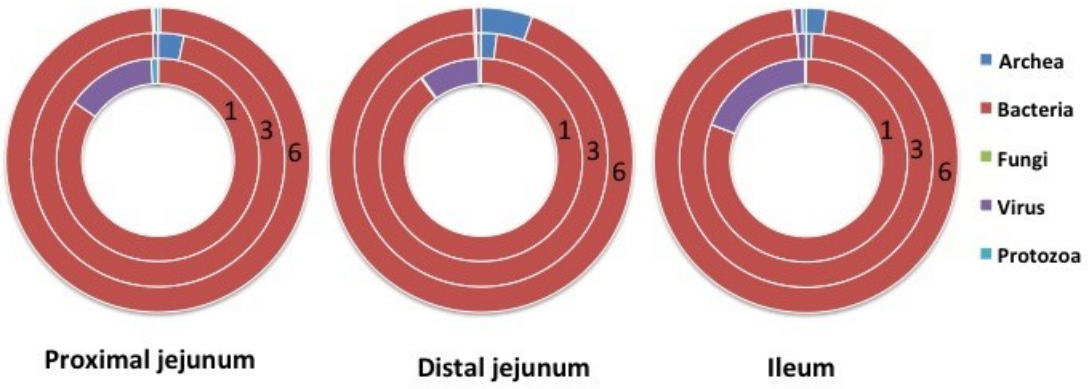

Figure 4.1 Composition of small intestinal microbiomes obtained through whole genome sequencing of digesta communities.

Each data series present mean relative abundance (%) of small intestinal microbiota per age category. 1 – 1-week-old calves, 3 – 3-week-old calves, 6 – 6-week-old calves.

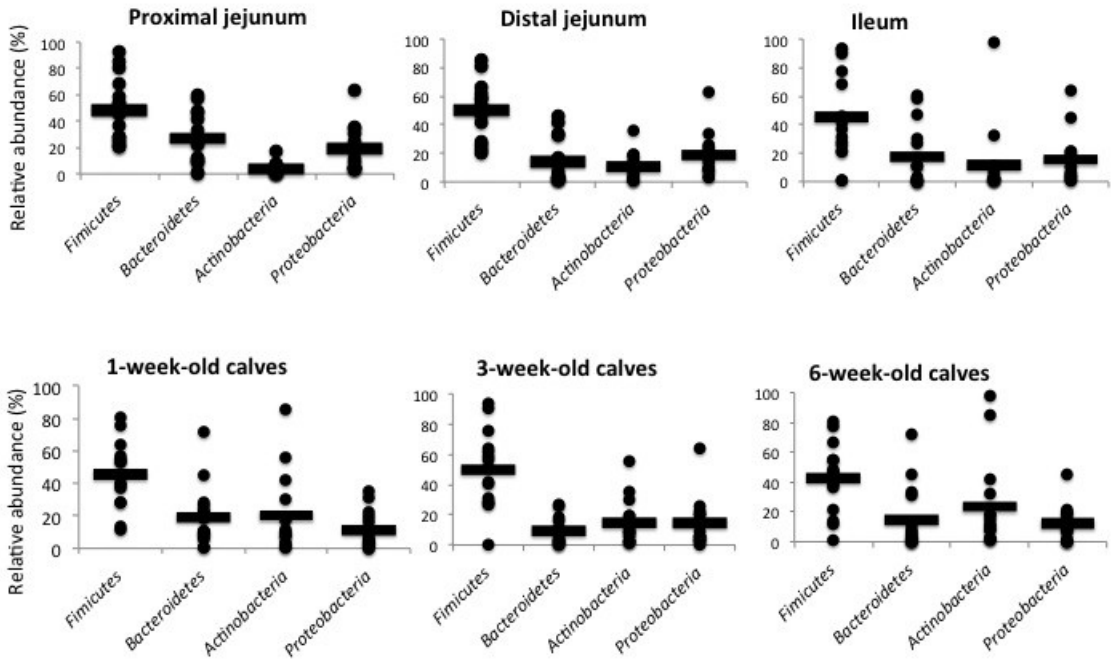

Figure 4.2 Individual variations in the relative abundance of four main bacterial phyla detected in small intestinal digesta communities.

Each data point represents an individual calf and bar represents mean relative abundance of a small intestinal region or age group. Upper panel presents all three age groups within a small intestinal region (15 data point/ phylum) and lower panel presents all three small intestinal regions within a age category.

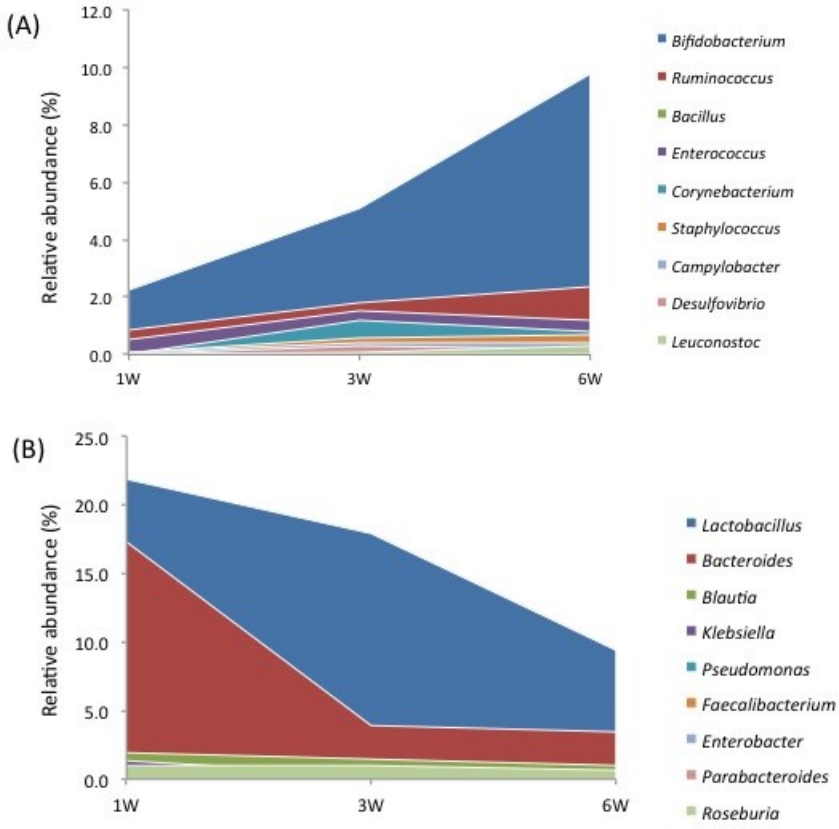

Figure 4.3 Bacterial genera with mean relative abundance displaying increasing (A) and decreasing (B) trends with increasing calf age.

1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

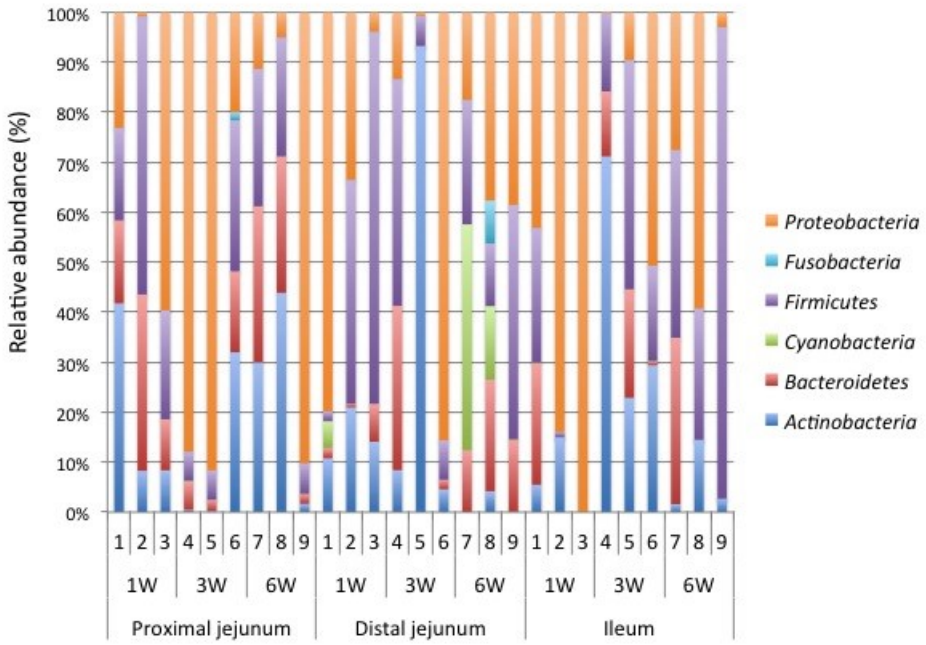

Figure 4.4 Phylum-level composition of tissue-attached bacteria generated through 454 sequencing of V1-V3 region of 16S rRNA gene.

Numerical values represent individual calf IDs. 1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves. Each data series represent relative abundance of detected bacterial phyla.

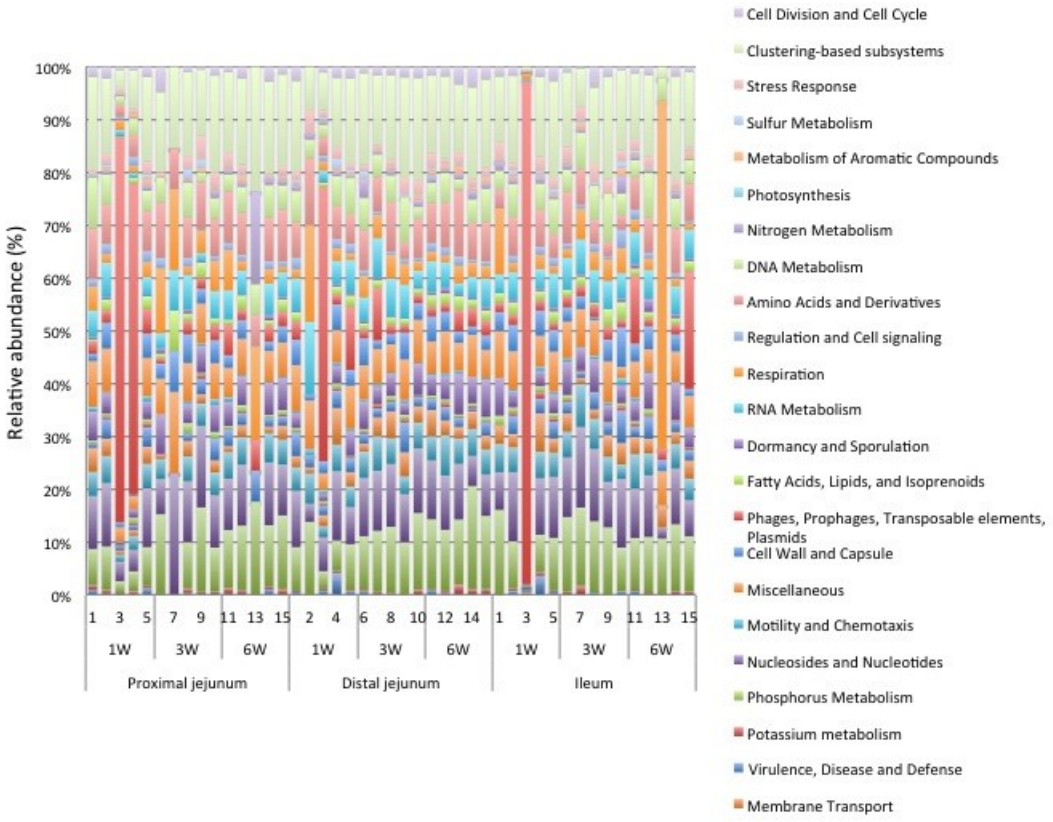

Figure 4.5 Small intestinal microbial functions.

(A) Abundance of the subsystems (level 1 functions of the SEED hierarchy) detected in the calf small intestine digesta-associated microbial communities. Numerical values represent individual calf IDs. 1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves. Each data series represent relative abundance of detected subsystems.

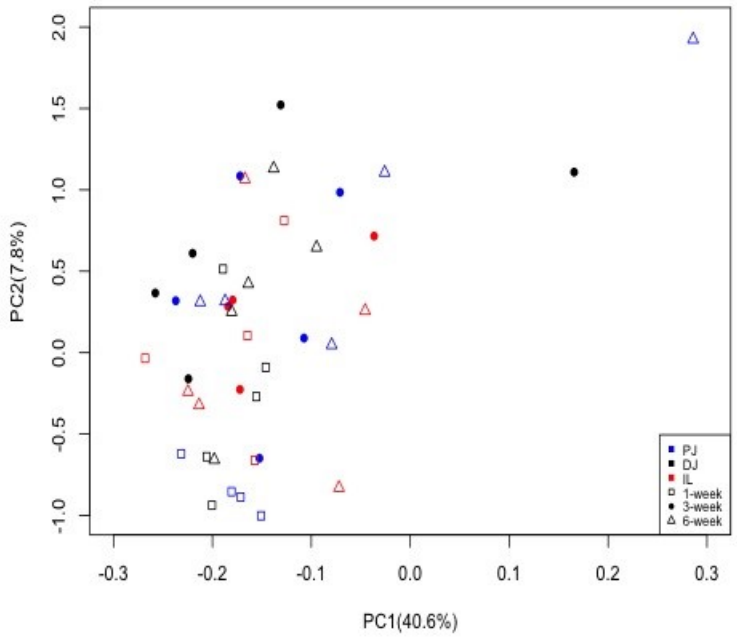

Figure 4.5B PCoA plot for functional composition.

PC1 and PC2 values of the individual microbial profiles obtain within MG-RAST platform using operational taxonomic units (OTUs) identified at gene level (level 4 functions of the SEED hierarchy) and plot is generated using R package (plot function).

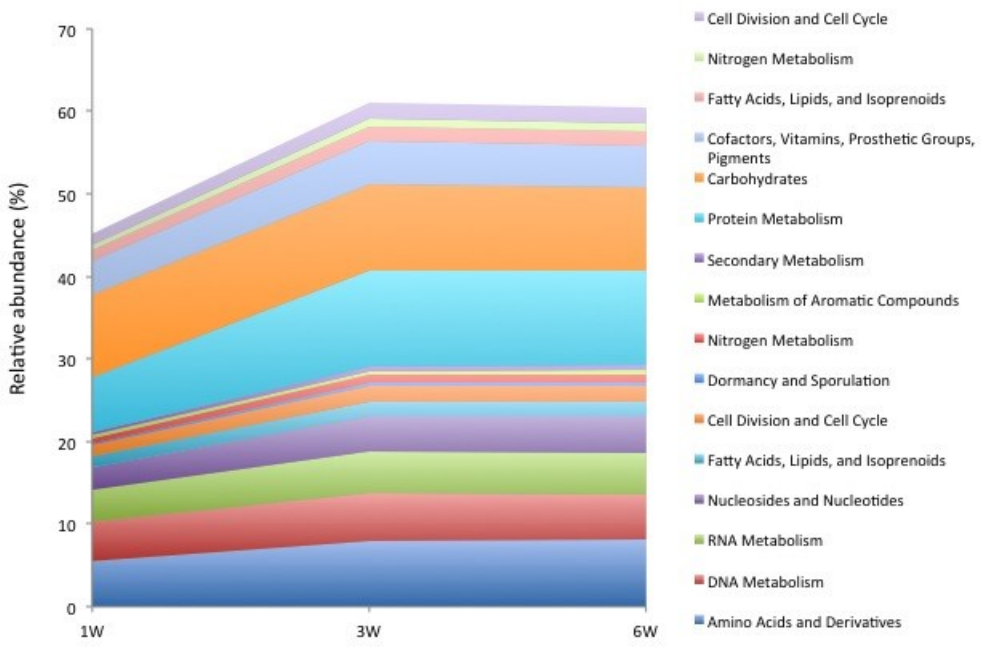

Figure 4.5C Subsystems with mean relative abundance displaying increasing and stabilizing trends with increasing calf age.

1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

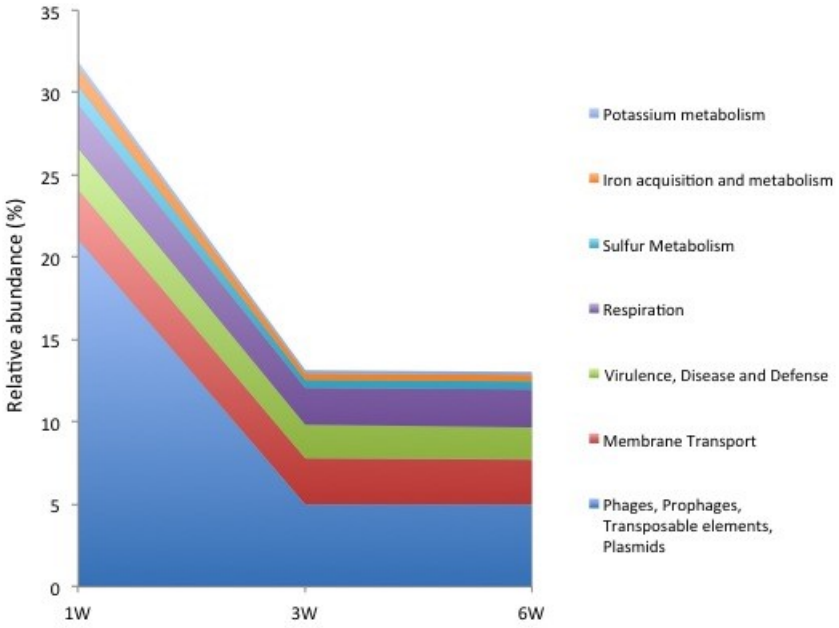

Figure 4.5D Subsystems with mean relative abundance displaying decreasing and stabilizing trends with increasing calf age.

1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

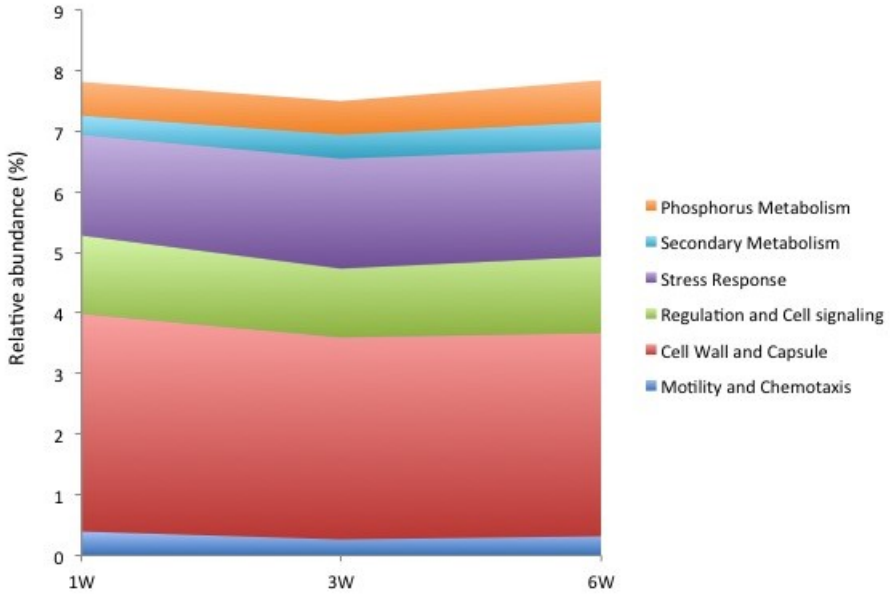

Figure 4.5E Subsystems with stable mean relative abundance throughout the neonatal period (1-week to 6-week).

1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

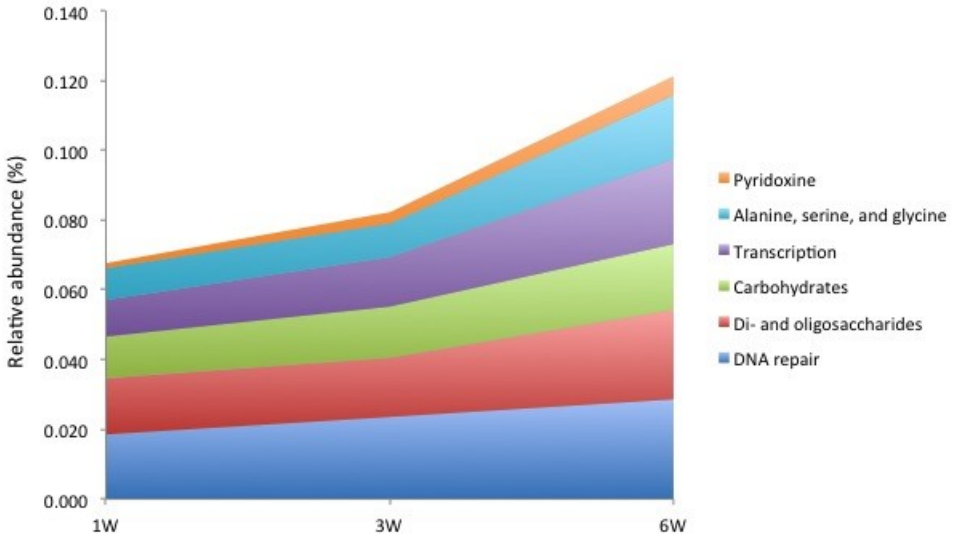

Figure 4.5F Level 2 functions with mean relative abundance displaying increasing trends with increasing calf age.

1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

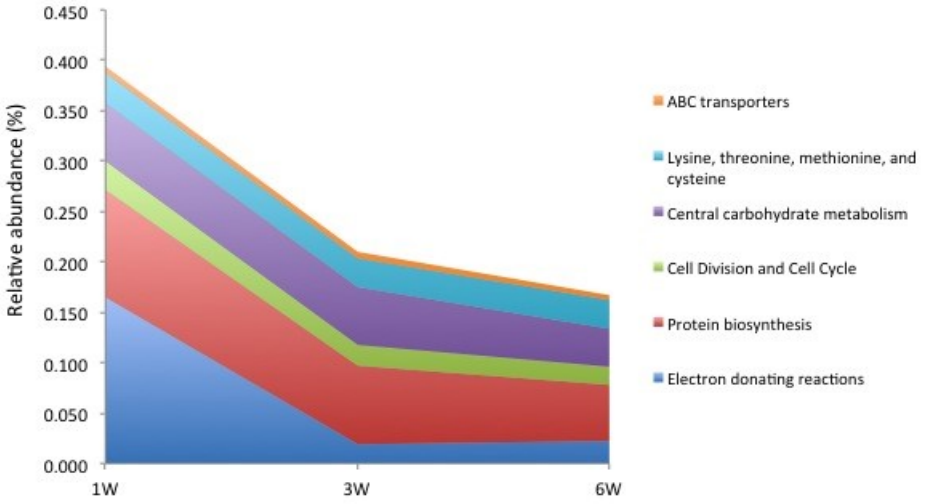

Figure 4.5G Level 2 functions with mean relative abundance displaying decreasing trends with increasing calf age.

1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

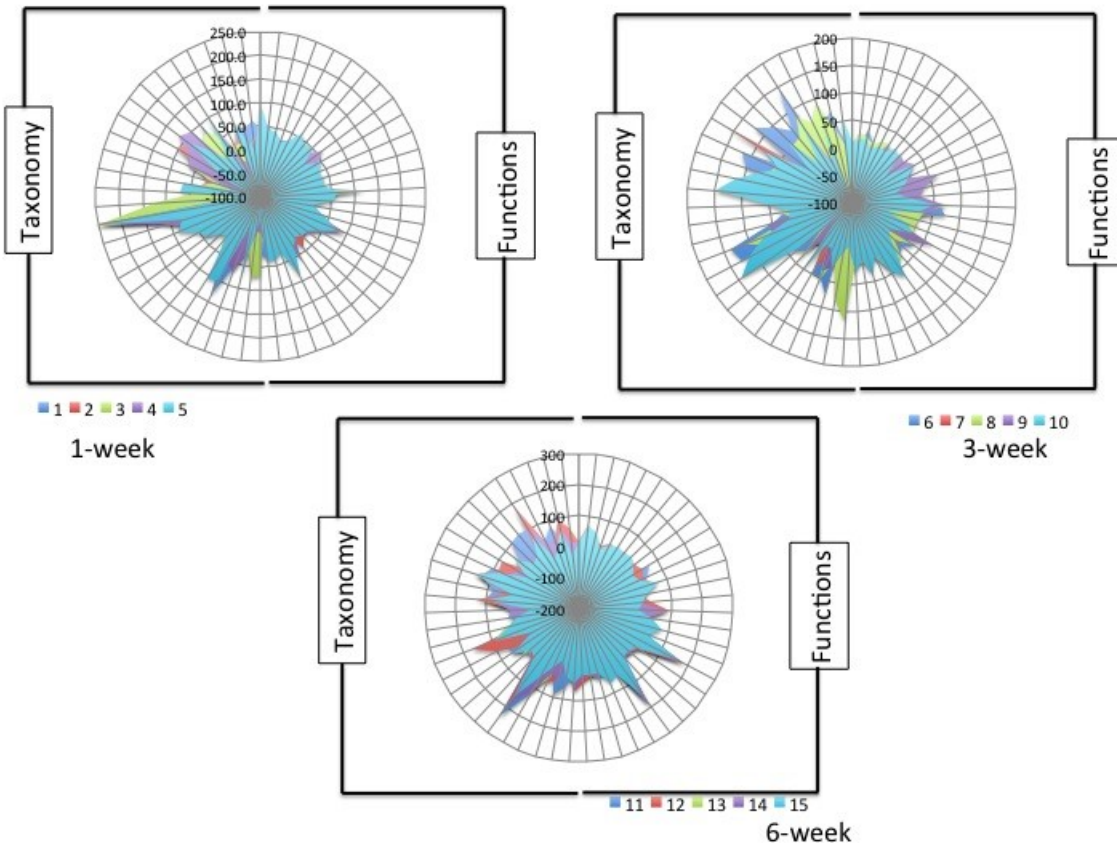

Figure 4.6 Coefficient of variations (ΔCV) of the relative abundance of the subsystems and bacterial genera (present in > 50% of the samples).

Axis represent ΔCV value of each genera/subsystem and $\Delta CV = CV_{population} - CV_{individual\ animal}$. Positive ΔCV represents higher inter-individual and lower intra-individual variation within an age group. Negative ΔCV represents lower inter-individual and higher intra-individual variation within an age group. Numerical values represent individual calf IDs.

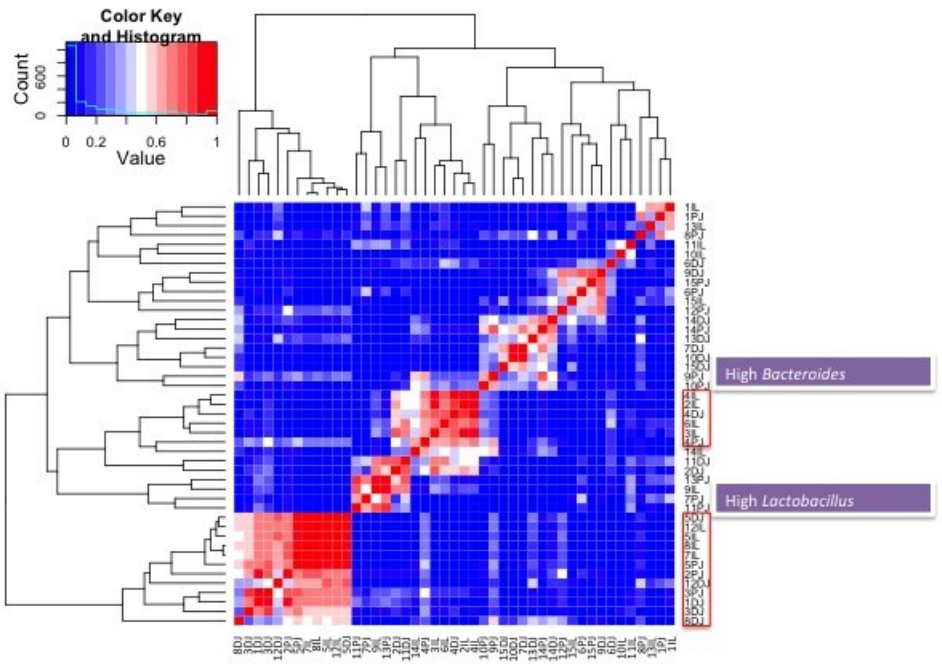

Figure 4.7 Clustering of microbial profiles based on taxonomic/functional similarities. Figure 4.7A Clustering of calves based on microbial taxonomic composition. Clustering is based on the Spearman rank correlation coefficient between two samples generated based on the relative abundance of 27 bacterial genera identified in at least 50% of samples. Each raw and column represents an individual microbial profile generated from small intestinal samples (45 microbial profiles).

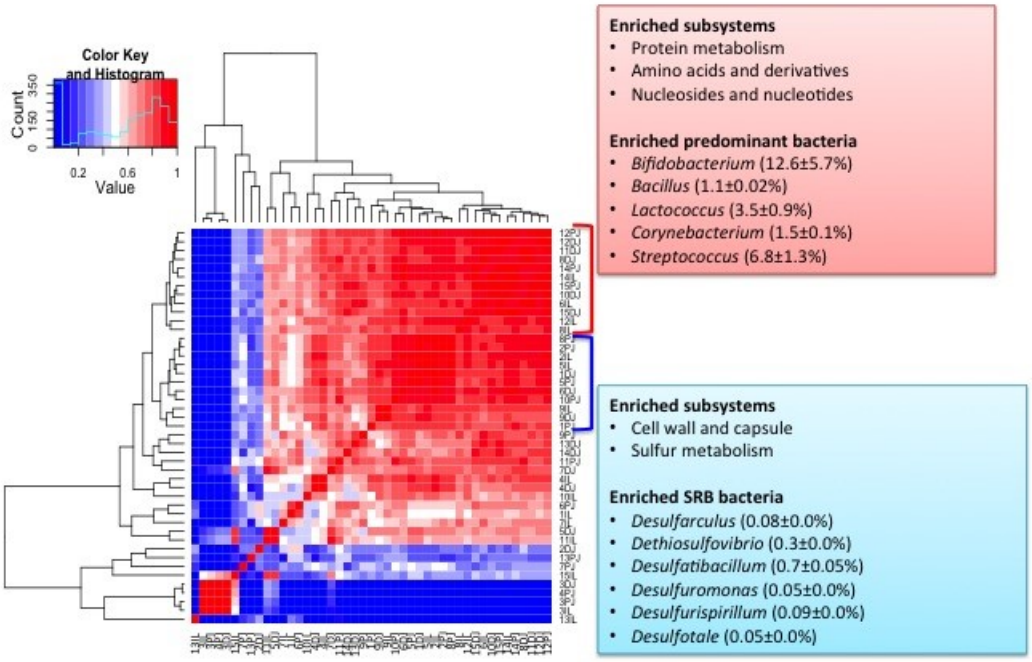

Figure 4.7 B Clustering of calves based on microbial functional abundance.

Clustering is based on the Spearman rank correlation coefficient between two samples generated based on the relative abundance of identified Subsystems. Abundances of bacterial genera between two clusters are compared using metastats and data presented as mean±SEM. Each raw and column represent an individual microbial profile generated from small intestinal samples (45 microbial profiles).

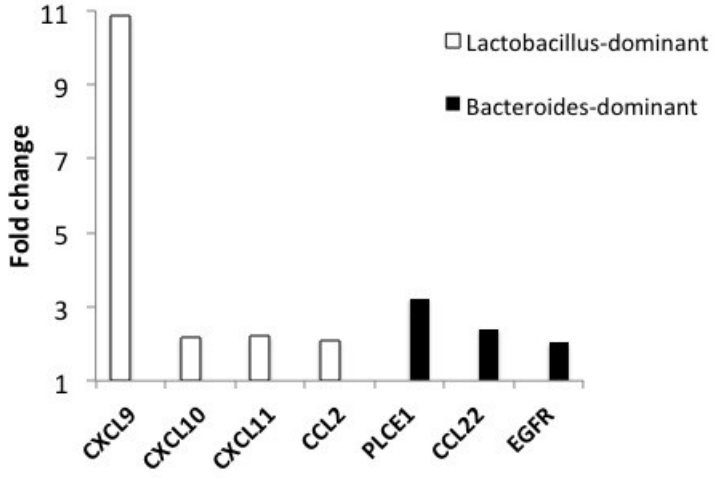

Figure 4.7C Genes enriched in the ileum of *Lactobacillus*-dominant and *Bacteroides*-dominant calves.

Fold change is calculated by dividing expression of genes (mean CPM) in *Lactobacillus*-dominant calves by expression of genes (mean CPM) in *Bacteroides*-dominant calves and *vice versa*. *CXCL9* – chemokine ligand 9, *CXCL10* – chemokine ligand 10, *CXCL11* – chemokine ligand 11, *PLCE1* - Phospholipase C Epsilon 1, *CCL22* - C-C Motif Chemokine Ligand 22, *EGFR* - Epidermal Growth Factor Receptor.

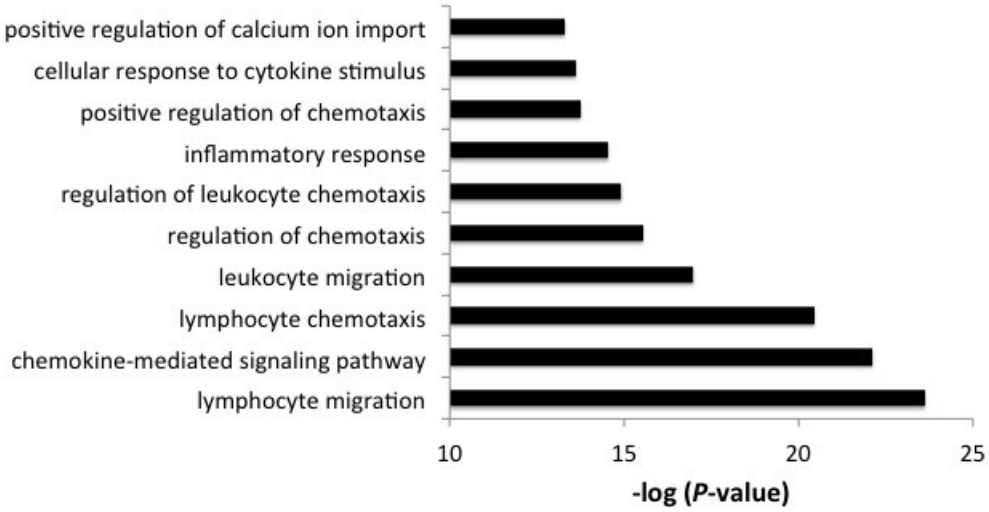

Figure 4.7D Functions of the genes highly expressed in the ileum of the *Lactobacillus*-dominant calves.

Functional enrichment is performed using GO enrichment analysis in Gene Ontology Consortium. *P* – value - significance for the enrichment in the dataset of the listed GO identifier. A *P* – value close to zero (a higher –log (*P* - value) means that the group of genes associated with the particular GO term is more significant and is less likely the observed annotation of the particular GO term to a group of genes occurs by chance.

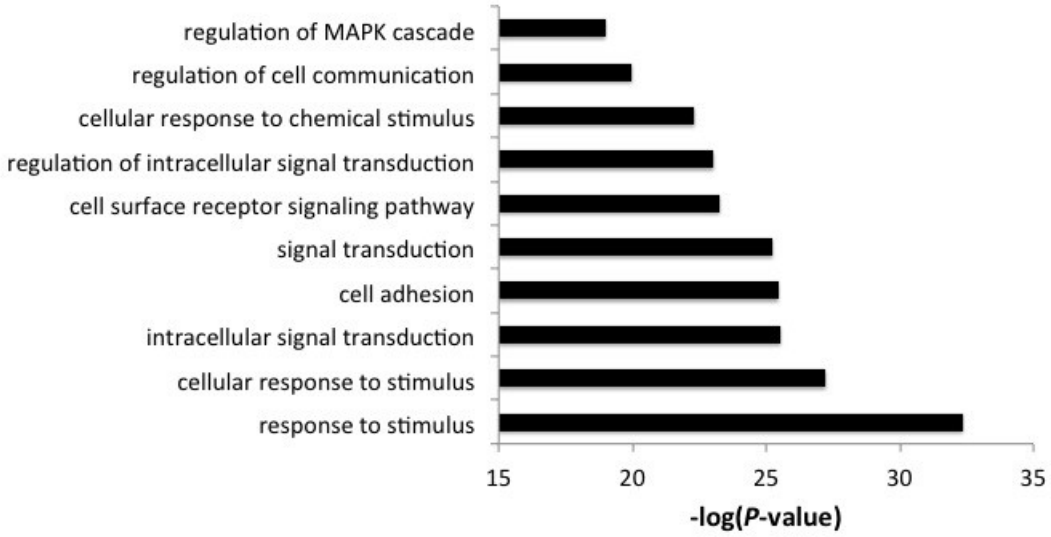

Figure 4.7E Functions of the genes highly expressed in the ileum of the *Bacteroides*-dominant calves.

Functional enrichment is performed using GO enrichment analysis in Gene Ontology Consortium. *P* – value - significance for the enrichment in the dataset of the listed GO identifier. A *P* – value close to zero (a higher –log (*P* - value) means that the group of genes associated with the particular GO term is more significant and is less likely the observed annotation of the particular GO term to a group of genes occurs by chance.

Chapter 5. A three-way systematic regulation of rumen development through microbial metagenomes and host transcriptome-microRNAomes during early life

Abstract

The development of the rumen and the mechanisms regulating this process in pre-ruminants are largely unknown. This study investigated the rumen microbial establishment process and their impact on the rumen development through profiling and integrating rumen microbiome (taxonomic and functional composition, production of volatile fatty acids) and tissue transcriptome (expression of mRNA and miRNA) during the first six weeks of life. An active microbial community was established in the rumen during the birthing process that increased diversity and density significantly within a week of life. The detection of active archaea during the first week of life suggests that the mechanisms to maintain H_2 pressure, which accelerate rumen microbial fermentation, also begin before the introduction of a solid diet. The integrated network analysis revealed the active involvement of microbial metabolites/volatile fatty acids in the rumen tissue metabolism and in epithelium development. Only 3,595 genes (26.3% of the transcriptome) and 169 miRNAs (46.4% of the microRNAome) were responsive to the microbial metabolites, while others were ontogenic. This finding signifies that miRNA may be the main responder to the microbial colonization that influences the rumen development through its interactions with the microbiome and transcriptome. Furthermore, a three-way interaction among zinc finger protein genes, miRNAs targeting those genes and bacteria suggests a potential role of bacteria-driven transcriptional regulation via miRNAs during early rumen development. The observed associations in

the present study suggests that miRNAs may coordinate the host-microbial interactions during early rumen development in neonatal calves and this phenomenon may also be applicable to early gut development in other mammalian species.

5.1 Introduction

A dynamic microbial population rapidly colonizes the *in utero* sterile mammalian gut during and after birth. The impact of microbiota on gut development has mainly been studied using germfree animal models, reporting structural and functional (mucosal immune system) changes in the small intestine in their absence (Sommer and Backhed, 2013). A recent study revealed that the colonization of gut region-specific microbiota, which cannot be detected via fecal sampling, regulates the gut development in a region-specific manner through its interactions with the host transcriptome (Sommer et al., 2015). Furthermore, the altered composition of the early microbiota has been linked to the development of chronic metabolic disorders (obesity) (Subramanian et al., 2015), revealing long-lasting impacts of the early microbiota on the host health.

The rumen is the unique organ of ruminants that converts low-quality forage into high-quality animal protein through fermentation. Rumen fermentation is a complex process conducted by the symbiotic microbiota, which produces 70% of the ruminant's daily energy in the form of volatile fatty acids (VFAs) (Yeoman and White, 2014). Ruminants are considered functionally monogastric animals before weaning (Heinrichs, 2005), and the development and function of the rumen have only been studied during weaning itself. The proper development of the rumen facilitates a smooth weaning transition from pre-ruminant to ruminant (Heinrichs, 2005), which is influenced by the

calf's diet (Gorka et al., 2011; Khan et al., 2011), feeding methods (Khan et al., 2007) and the microbial colonization (Li et al., 2011). Recently, an increasing number of studies have explored the molecular mechanisms underlying rumen development during the weaning transition (Connor et al., 2013; Naeem et al., 2014) as well as the rumen microbiota in pre-ruminants (Li et al., 2012; Jami et al., 2013; Malmuthuge et al., 2014; Rey et al., 2014). Rumen microbial colonization begins as early as the first day of life (Jami et al., 2013), and the pre-weaning diet alters its composition and the production of VFAs (Abecia et al., 2014a), suggesting the importance and the potential of pre-weaning feeding strategies to manipulate the early rumen microbiota to alter rumen development. Manipulation of the rumen microbiota is one of the potential approaches to enhancing rumen fermentation (Eisler et al., 2014). However, the current understanding of the establishment of the rumen microbiome and its importance for rumen development remains very limited, which is a barrier to achieving such improvement.

Our previous studies revealed the establishment of rumen-specific bacteria (Malmuthuge et al., 2014) as well as the presence of a rumen-specific microRNA (miRNA) profile associated with the bacterial densities (Liang et al., 2014) of pre-weaned calves. Thus, this study hypothesized that the early microbiome is actively involved in rumen development through its interaction with the host transcriptome and employed next-generation sequencing of the rumen metagenomes and tissue transcriptomes with an integrated bioinformatics approach to explore host-microbial interactions in the pre-ruminant rumen. A detailed understanding of early rumen development (functions, morphology, colonization) may provide a means to manipulate its functions in the future

to improve the productivity and health of ruminants and to meet global food production demands.

5.2 Materials and Methods

5.2.1 Animal experiments and sampling

All the experimental protocols were approved by the Livestock Care Committee of the University of Alberta (AUP00001012) and were conducted following the guidelines of the Canadian Council on Animal Care. Holstein bull calves at day 0 (n = 6, within five minutes after birth), week 1 (1W, n = 6), week 3 (3W, n = 6) and week 6 (6W, n = 6) were obtained from the Dairy Research and Technology Center, University of Alberta (Edmonton, AB). Dams with male fetuses were transferred into calving pens a week before the predicted due dates and closely monitored by camera. Newborn calves (n = 6) were removed from the dams soon after birth, transferred to a surgery room immediately, and humanely euthanized within few minutes. The whole rumen of each of these newborn calves was collected as a closed section to avoid environmental contamination. The remaining calves (n = 18) used in the study were also removed from the dams soon after birth and fed with 2 L of colostrum within one hour. Calves were fed with 4 L of colostrum/day during the first three days postpartum, followed by 4 L of whole milk/day from the fourth day onward throughout the experimental period. From the second week onward, the calves were supplemented with 23% accelerated calf starter (23.0% crude protein, 4.0% crude fat, 9.0% crude fiber, Wetaskiwin Co-op. Association, Wetaskiwin, Alberta, Canada) *ad libitum* along with 4 L of milk/day. Rumen samples (tissue and content separately) were collected from the pre-weaned calves at week 1,

week 3 and week 6 within 30 minutes after euthanization. Tissue and content samples were collected at the bottom of ventral sac and the site of sampling kept constant for all the animals.

5.2.2 Analysis of content-associated rumen microbiome[11]

Total DNA was extracted from the rumen content sample using the repeated bead-beating plus column method (Yu and Morrison, 2004). However, DNA extraction was performed for tissue and contents together for day 0 calves. DNA libraries were prepared for whole-genome sequencing using the Truseq DNA PCR-free library preparation kit (Illumina, CA, USA) following the manufacturer's instructions. Briefly, the genomic DNA was first normalized with a resuspension buffer to a final volume of 55 µL at 20 ng/µL. Then, 50 µL of the buffer containing genomic DNA was transferred into a Covaris microTUBE (Covaris Inc., MA, USA) for fragmentation using a Covaris S2 focused-ultrasonicator (Covaris Inc., MA, USA). The cleaned up fragmented DNA was then subjected to end repair and size selection, followed by the adenylation of the 3' ends and ligation of the adaptor index. Each metagenomic library was quantified using a Qubit 2.0 Fluorometer (ThermoFisher Scientific, MA, USA), and sequencing was performed at Génome Québec (Montréal, Canada) using the HiSeq 2000 system (Illumina, CA, USA).

The demultiplexed (CASAVA version 1.8, Illumina) 100 bp paired-end reads (82.9 Gb) were uploaded into the MG-RAST metagenomic analysis server, version 3.3.9, and paired-ends were joined for each sample before submitting for processing (Meyer et al., 2008). Artificial replicates, host (bovine) DNA and low-quality (Phred score < 25)

[11] Laboratory technicians Xu Sun and Yanhong Chen assisted with library preparation and qPCR

sequences were removed from the raw data, and the remaining good-quality sequences were used to assign the taxonomy and functions. The data were deposited in MG-RAST and are publicly accessible at http://metagenomics.anl.gov/linkin.cgi?project=6020. The taxonomic abundance was analyzed using the best-hit classification method and the M5NR annotation source within the MG-RAST platform. The functional abundance of the rumen microbiome was analyzed using the hierarchical classification and the subsystems annotation source in the SEED hierarchy. A maximum cut-off e-value of 1e-10, maximum identity of 70% and maximum alignment length of 50 were used as data selection criteria for both the taxonomy and function abundance analyses. The taxonomic and functional abundances were then subjected to pairwise comparisons (0-day versus 1-week; 1-week versus 3-week; 1-week versus 6-week; 3-week versus 6-week) using metastats (White et al., 2009) to explore the rumen microbiome changes throughout calf growth. Multiple test correction was performed using Benjamini and Hochberg (1995) and significant comparisons were declared at FDR < 0.05.

5.2.3 Estimation of bacterial/archaeal density using quantitative real-time PCR

DNA- and RNA-based quantitative real-time PCR (qPCR) was performed to estimate the bacterial (total bacteria, *Ruminococcus flavefaciens*, *R. albus*, *Eubacterium ruminantium, Prevotella ruminicola*) and total archaea density using SYBR green chemistry (Fast SYBR® Green Master Mix, Applied Biosystems) with the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) and group-specific primers (Table 5.1). The bacterial densities were calculated using the equation described by Li et al., (2009).

5.2.4 Measurement of rumen papillae and volatile fatty acids

Rumen tissue sections (~1 cm^2) adjacent to the sample collected for RNA and DNA extraction were collected into cassettes and then fixed in 10% formalin. After 24 hours of fixing in formalin, the cassettes were stored in 70% ethanol until further processing. The rumen tissue samples were embedded in paraffin blocks, and 4-5 µm sections were stained with hematoxylin and eosin at Li Ka Shing Centre for Health Research Innovation (Edmonton, AB, Canada). The height and width of the rumen papillae (20 papillae/calf) were measured using the Axiovision software (Zeiss, Oberkochen, Germany).

Concentration of ruminal VFAs was quantified using a Varian 430-gas chromatograph (Varian, Walnut creek, CA) with a Stabilax® -DA column (Restek Corp., Bellefonte, PA). The concentrations of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate were calculated according to the method described in Guan et al., (2008).

5.2.5 Transcriptome profiling and integration with rumen microbiome and calf phenotypic traits[12]

5.2.5.1 Transcriptome profiling

Total RNA was extracted from the rumen tissue samples using the mirVana™ miRNA isolation kit (Ambion, CA, USA), and 100 bp paired-end RNA libraries were prepared using the TrueSeq RNA Sample Preparation Kit v2 (Illumina, CA, USA). The total RNA was sequenced at Génome Québec (Montréal, Canada) using the HiSeq 2000

[12] Guanxiang Liang (former PhD student worked on the same research) performed laboratory works and data generation

system (Illumina, CA, USA). Demultiplexed reads (CASAVA version 1.8, Illumina) were aligned to the bovine genome (UMD 3.1) using Tophat 2.0.10 with the default parameters (Kim et al., 2013), and gene expression levels were calculated by normalizing the reads number to counts per million reads (CPM) using the following equation: CPM = (reads number of a gene / total mapped reads number per library) × 1,000,000. Sequencing data were deposited in the publicly available NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE65017 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65017).

5.2.5.2 Network analysis

The interactions among the protein coding genes, miRNAs (Liang et al., 2014) and metagenomes were explored through network analysis and correlation analysis. Weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008) was performed to understand the link between the host transcriptome/miRNAome (profiles generated from same calves) and the calf phenotypic traits (calf age, concentration of acetate, propionate, butyrate, valerate, isobutyrate, isovalerate and total VFAs, papillae length and width). The associations between the host transcriptome and the rumen bacteria were explored using the genes co-expressed in the M10 module of the mRNA network, the miRNAs co-expressed in the R7 module of the miRNA network and the relative abundance of the identified rumen bacterial genera. The associations between the host transcriptome and the microbial functions were explored using the microbial level 2 functions in the SEED hierarchy and GO terms enriched under “host carbohydrate metabolism” (GO: 0005975, 20 genes), “tight junction protein genes” (GO: 0005923, 14

genes), “membrane transportation” (GO: 0008643, 14 genes) and “epithelial development” (GO: 0060429, 34 genes).

5.2.6 Statistical analyses

The DNA- and RNA-based bacterial/archaeal density, concentration of VFAs, and papillae length and width were analyzed using mixed procedure in SAS (SAS 9.4, SAS Inc., Cary, NC) and one-way analysis of variance. The following statistical model was fitted to test the effect of calf age on bacterial/archaeal densities, papillae length and width as well as the concentration and molar proportion of VFAs: $Y_{ij} = \mu + A_i + e_{ij}$, where Y = bacterial/archaeal density (total bacteria, *R. flavefaciens*, *R. albus*, *E. ruminantium P. ruminicola*, total archaea), VFA concentration/molar proportion, papillae length or width; μ = mean; A = calf age; and e = residual error. Correlations among the concentration of VFAs, bacterial densities and papillae length and width were identified using PROC CORR in SAS. Differences in LSM were declared at $P < 0.05$ using the PDIFF option in SAS when applicable.

5.3 Results

5.3.1 Active and functional microbiota establishes at birth

We used a metagenomics-based approach together with DNA- and RNA-based quantification of microbiota to explore the calf rumen colonization from birth up to six weeks of life. Use of metagenomic-based sequencing revealed that rumen was mainly colonized with bacteria (99.9±0.5%) at birth (Figure 5.1A), which was dense ($9.1\pm3.1\times10^8$ 16S rRNA gene copy/g) (Figure 5.1B), diverse (83 genera), and active

(1.9±0.4×10^8 16S rRNA copy/g) (Figure 5.1B). *Veillonella*, followed by *Prevotella*, *Bacteroides*, *Eubacterium*, *Streptococcus*, *Acidaminococcus*, *Clostridium*, *Bifidobacterium* and *Ruminococcus,* were predominant in the calf rumen at birth. The abundance of the other identified 72 genera accounted for only 11.3% of the rumen bacteria. No archaea and protozoa were detected in the calf rumen at birth, while fungi and virus together accounted for 0.1% of total identified rumen microbiota (Figure 5.1A). Metagenomics profiling of the rumen microbiome revealed 27 level 1 (subsystems) and 116 level 2 functions in the SEED hierarchy along with 543 microbial genes at birth. The predominant subsystems identified in the calf rumen were "respiration" and "protein metabolism", whereas "folate and pterines" (11.2±2.3%) and "electron donating (9.1±0.5%) and accepting" (5.3±0.6%) functions were prevalent among the level 2 functions. The predominant microbial genes identified at birth were "decarboxylase" (8.6±7.7%) and "NADH dehydrogenase" (4.7±4.3%).

5.3.2 Rumen microbiome undergoes rapid changes during early life

Relative abundance of other detected microbial groups in rumen increased in pre-weaned calves, when comparing to newborn calves; however, bacteria remained as the predominant microbial group in calf rumen (Figure 5.1A). The bacterial density in the calf rumen increased 438-fold (RNA-based) and 7829-fold (DNA-based) within the first week of life (Figure 5.1B). These bacteria belonged to 14 different phyla, dominated by *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* (Figure 5.1C). A total of 167 genera were identified, with 9.3±2.2% unassigned sequences, 63 of which were predominant bacterial genera (abundance > 1% in at least one sample). Among the

detected genera, *Prevotella, Bifidobacterium*, *Corynebacterium*, *Streptococcus*, *Lactobacillus*, *Clostridium*, *Staphylococcus*, *Bacillus*, *Campylobacter*, *Pseudomonas*, *Yersinia*, *Neisseria*, *Campylobacter*, *Haemophilus*, *Burkholderia*, *Vibrio* and *Brucella* were present in all the pre-weaned calves. The prevalence of the identified bacterial genera varied with the calf age, with substantial differences observed when comparing week 1 (1W) against weeks 3 (3W) and 6 (6W). For example, the abundance of *Prevotella* was higher in 1W than in 3W and 6W; however, the density of active *P. ruminicola* increased numerically with calf age (Table 5.2). A higher prevalence of *Ruminococcus* was observed from the first week of life, with active *R. flavefaciens* and *R. albus* increasing over time (Table 5.2). The prevalence of *Eubacterium* and *Roseburia* also increased over time, with the introduction of solid feed. For example, the abundance of *Eubacterium* and *Roseburia* increased by 12- and 86-fold, respectively, from 1W to 6W. However, there were no significant temporal changes in the active *E. ruminantium* density (Table 5.1).

In total, 28 level 1 and 168 level 2 functions in the SEED hierarchy were observed in the pre-weaned calf (1W to 6W) rumen microbiomes, dominated by the "protein and carbohydrate metabolism" subsystems (Figure 5.1D). "Protein metabolism" mainly consisted of functions related to "protein biosynthesis", while "carbohydrate metabolism" comprised functions related to "central carbohydrate metabolism" at level 2 of the SEED hierarchy. The differentially abundant microbial functions were mainly identified when comparing 1W calves against 3W and 6W calves (Table 5.3). In total, 3,443 microbial genes were identified from all the pre-weaned calves, but with a high inter-individual variation. The majority of differentially abundant genes were observed between 1W and

6W (396), followed by 1W versus 3W (134) and 3W versus 6W (59). Nineteen glycoside hydrolases (GHs) were identified in the pre-weaned rumen with varying relative abundances over calf age (Table 5.4). The abundances of α-galactosidase, α-glucosidase SusB, α-L-arabinofuranosidase II precursor, α-N-acetylglucosaminidase, α-N-arabinofuranosidase 2, β-galactosidase large subunit, glucan 1,6-alpha-glucosidase, and maltose-6'-phosphate glucosidase were higher in 6W than in 1W and 3W (Table 5.4).

5.3.4 Active archaea population is established in neonatal calves from the first week of life

Quantification of 16S rRNA gene using RNA-based real-time PCR revealed the colonization of active archaea from the first week of life (Figure 5.1E), while the archaeal density was 10,000-fold lower in 1W compared to 3W and 6W (Figure 5.1E). Similarly, metagenomics-based sequencing revealed archaeal colonization from the first week of life (0.03±0.01%) that increased relative abundance by 41- and 54-fold in 3W and 6W calves, respectively (Figure 5.1A). Regardless the presence of archaea from the first week, methyl coenzyme M reductase gene (mcrA) was only detected in the metagenomic profiles of 3W (0.2±0.0003%) and 6W (0.2±0.0001%) calves. A higher abundance of archaeal-specific glycolysis enzymes (glucose-6-phosphate-isomerase, fructose-biphosphate aldolase, 2,3-biphosphate-independent phosphoglycerate mutase and non-phosphorylating glyceraldehyde-3- phosphate dehydrogenase) was observed in 1W compared to 3W and 6W (Table 5.4). Metagenomics profiling further revealed that the pre-ruminant ruminal archaea mainly consisted of the families *Methanomicrobiaceae*, *Methanobacteriaceae* and *Methanococcaceae* (Figure 5.1F). The prevalence of

Methanobacteriaceae in the metagenomic profiles was higher (P = 0.01) in 3W (39.0±9.8%) and 6W (36.1±14.3%) than in 1W (9.6±6.0%). Although no single genus was present in all the calves, *Methanobrevibacter, Methanothermobacter, Methanobacterium,* and *Methanoplanus* were observed in 60% of six-week-old calves.

5.3.5 Rumen epithelium development and volatile fatty acid profile in pre-weaned calves

The rumen epithelium at birth displayed a unique structure compared to pre-weaned calves (Figure 5.1G). There were no separated protruding papillae or stratified squamous epithelium in the calf rumen soon after birth; however, developing papillae were noticeable (Figure 5.1G). The rumen epithelium of newborn calves consisted of a large number of nucleated squamous cells with a thickness of 279.9±7.6 μm that developed into 678.1±41.1 μm length papillae within six weeks. The increase in the length and width of the rumen papillae was significantly different among the three age groups (Table 5.2, Figure 5.1G).

The concentrations of total VFA, acetate, butyrate, propionate, valerate, isobutyrate and isovalerate increased with increasing age and dietary changes (Table 5.2). However, only the molar proportions of acetate and valerate displayed age-related variations, while the molar proportion of butyrate ranged from 13-16% of total VFA during the first six weeks of life (Table 5.2). In addition, the concentration of VFAs was positively correlated with *R. flavefaciens* density and the rumen papillae development (Table 5.5).

5.3.6 Microbiome-transcriptome interactions may influence rumen epithelial development and tissue metabolism

We investigated the associations between the rumen development and the microbiome by examining the rumen tissue transcriptome in association with the papillae measurements, the concentrations of VFAs and the microbial composition and functions. In total, the calf rumen tissue consisted of 13,676±399 genes (CPM > 1). Weighted gene co-expression network analysis (WGCNA) clustered the common genes (11,772) detected among all calves into 29 gene modules (M1-M29) that displayed varying associations with the phenotypic measurements (Figure 5.2A). The expression of genes in the M2 module (2,313 genes) and M18 module (212 genes) was negatively correlated and the expression of genes in the M10 module (1,070 genes) positively correlated with the calf phenotypic traits and the microbial metabolites (Figure 5.2A). The genes co-expressed in the M2 module were related to "transcription", "splicing", "ribonucleoprotein complex biogenesis", and "RNA metabolic process". The genes in the M18 module were enriched with functions related to "chromatin organization", "histone modification" and "transcription". There were nine genes involved in "chromatin organization", such as histone genes (*H1F0*, *H1FX*), and histone deacetylase coding genes (*HDAC3*). M10, the largest gene module (22.5% of all the sequencing reads), was related to "tissue metabolism", and the largest proportion of these genes (38 genes, 7.65% of total reads) related to "respiratory electron transport chain". They consisted of "mitochondrial respiratory chain complex proteins", such as "cytochrome c oxidase subunits" (*COX1, COX3*, and *COII*), "NADH dehydrogenase subunits" (*ND2, ND5*),

"succinate dehydrogenase subunits", "ubiquinol-cytochrome c reductase subunits" and "ATP synthase subunits".

The use of the M10 module to understand the role of bacteria in rumen development revealed six bacterial clusters based on their associations with the expression of genes (Figure 5.2B). A cluster (cluster 1) consisting of *Prevotella*, *Bacteroides*, *Ruminococcus*, *Klebsiella* and *Propionibacterium* was positively correlated with 49 genes involved in "ion binding", "regulation of cell cycle, catalytic activity, molecular functions" and "transcription regulatory activity" (Figure 5.2B, 5.2C). The majority of "ion binding" genes (8/13) were related to zinc finger proteins (*ZNF*s) (LIM and calponin homology domain 1, *ZNF238*, *ZNF445*, *ZNF397*, bromodomain adjacent to zinc finger domain 1B, ADAM metallopeptidase with thrombospondin type 1 motif 10, deltex 1 E3 ubiquitin ligase, ash2 (absent, small, or homeotic)-like). Another cluster (cluster 6) containing genera mainly from *Firmicutes* and *Proteobacteria* was negatively correlated with the same sets of genes (Figure 5.2B, 5.2C).

Among the level 2 microbial functions, "microbial carbohydrate metabolism" was mainly linked to the rumen tissue transcriptome. The genes associated with these microbial functions included 19 of 34 genes related to "rumen epithelium development" (Figure 5.3A), genes involved in "rumen tissue carbohydrate metabolism" (Figure 5.3B) and "membrane transportation" (solute carrier family 35 and monocarboxylate transporters - *SLC16A3/MCT3, SLC16A9/MCT9, SLC16A11/MCT11, SLC16A13/MCT13*) (Figure 5.3C), and 8 of 14 "tight junction protein genes" (TJs) (Figure 5.3D). Some of these correlated host genes were co-expressed in the M10 module, such as *FUCA1, GANC,* and *GALC* from "rumen tissue carbohydrate

metabolism" (Figure 5.3B); *SLC35A3* from "membrane transportation" (Figure 5.3C); *CLDN23* from TJs (Figure 5.3D); and *PPARG, GSTK1, SULT1B1*, and *GJA1* from "rumen epithelial development" (Figure 5.3A).

5.3.7 microRNAome coordinates microbiome-host transcriptome crosstalk

To identify the potential regulatory mechanisms of host-microbial interactions, microRNAome data (364±17 miRNAs) generated using the same animals in a previous study (Liang et al., 2014) were analyzed using WGCNA. The rumen microRNAome was clustered into nine modules (R1-R9) based on the expression of miRNAs. The R7 module (129 miRNAs) was negatively correlated with the calf phenotypic traits and the microbial metabolites, except for isovalerate (Figure 5.4A). The target prediction analyses (TargetScan and mirBase) revealed 3,847 genes that were targeted by the R7 module, 3,710 of which (~96%) were identified in the calf rumen tissue transcriptome. Moreover, 258 of the detected 3710 genes were co-expressed in the M10 gene module. The R7 member miR-375, which was downregulated with calf age (Figure 5.4B), was associated with "rumen epithelial morphogenesis" and "blood vessel development". The R8 module (40 miRNAs) was also negatively correlated with the calf phenotypic traits, propionate and isobutyrate (Figure 5.4A). The miRNAs co-expressed in the R8 module had 2,751 target genes, and 2,649 (~96%) of these genes were observed in the calf rumen tissue transcriptome. Functional analysis revealed that miRNAs co-expressed in the R8 module were involved in "protein localization and transportation" and "cell motility". However, only R7 miRNAs had their targets co-expressed in the M10 gene module.

The roles of miRNAs in regulating host-microbial interactions were further evaluated by exploring the relationships among the expression of R7 miRNAs and M10 genes and the relative abundance of bacterial genera. Nearly 37% (55/147) of the M10 genes associated with bacterial cluster 1 and cluster 6 (Figure 5.2D, 5.2E) were targeted by 28 miRNAs co-expressed in R7 (Figure 5.4D). Among these miRNAs, bta-miR-2904, bta-miR-199b, bta-miR-541, bta-miR-574 and bta-miR-423-5p were associated with a bacterial cluster comprising *Prevotella, Bacteroides, Ruminococcus, Propionibacterium, Klebsiella* (cluster 1 from Figure 5.2D) and *Megasphaera* (Figure 5.4E). Furthermore, these five miRNAs targeted 65 different genes related to *ZNF*s.

5.4 Discussion

The microbiota that rapidly colonizes the *in utero* sterile mammalian gut during and after birth constantly interacts with the host to maintain the mammal's metabolism and health. The early gut microbiome has been suggested to have long-term impacts on human health (Subramanian et al., 2015). Despite the accumulating knowledge on the diversity of the rumen microbiome during early life (Fonty et al., 1987; Li et al., 2012; Jami et al., 2013; Malmuthuge et al., 2014; Rey et al., 2014), the importance of rumen colonization for tissue development and the regulatory mechanisms of host-microbial interactions in pre-ruminants are largely unknown.

This study, using metagenomic and RNA-based approaches, is the first to reveal the establishment of a dynamic, dense and active microbiome in the pre-ruminant rumen at birth that undergoes rapid changes during the first six weeks of life. The gut microbiota has been widely studied in mammalian species using DNA-based approaches; however, it

is evident that such evaluation may over-estimate both the organisms and their activities. The RNA-based quantification used in this study revealed the colonization of active bacteria within a few minutes of birth, indicating that the process might have started during the birthing process, which extended from an hour to three hours. Exploring the dam birth canal (*Streptococcus* - 23.3±13.3%, *Ruminococcaceae* - 12.6±4.6%) and rectal bacteria (*Ruminococcaceae* - 18.9±1.8%) following birth (data not shown) suggested that the vaginal/fecal bacteria of dams were the main inoculum of the calf rumen bacteria at birth. Our findings also confirmed previous studies claiming the establishment of fibrolytic bacteria within the first week of life (Fonty et al., 1987), a higher prevalence of *Prevotella* (Li et al., 2012; Rey et al., 2014) and the presence of GHs in the absence of proper substrates (Li et al., 2012). We revealed colonization with active *R. flavefaciens*, *R. albus*, *E. ruminantium* and *P. ruminicola*, the classical rumen bacteria that degrade plant polysaccharides (cellulose, hemicellulose, xylan and glycan) (Koike and Kobayashi, 2009; Purushe et al., 2010), from the first week of life, when calves were fed solely with milk. The increasing density of these species coincided with elevated concentrations of VFAs as well as increased papillae length and width in 3W and 6W calves fed with starter and milk. This finding suggests that the introduction of a solid diet stimulates the rapid growth of the rumen papillae by influencing the rumen microbial composition and functions. Traditionally, solid feed is considered the major driver of rumen development, which stimulates microbial fermentation (Heinrichs, 2005; Connor et al., 2013). However, the appearance of cellulolytic bacteria (Fonty et al., 1987) and the activity of xylanase and amylase (Rey et al., 2012) can be detected from the second day of life.

Thus, we propose that the presence of active microbiota as early as the first week calls for a detailed understanding of their roles in the development of the rumen.

The removal of H_2 from the rumen, which has inhibitory effects on microbial fermentation, increases the rate of fermentation (Janssen and Kirs, 2008) and can be considered as one of the features of rumen development. The presence of mcrA gene in the rumen metagenome of 3W and 6W calves, but not in 1W calves, suggests the activation of methanogenesis process in calf rumen after the introduction of a solid diet. A recent study has reported that lambs fed only milk replacer+cream produced 84% less methane than lambs fed hay (Haque et al., 2014). Moreover, the production of methane increased by 15.9-fold within four days of introducing hay to these milk-replacer+cream fed lambs (Haque et al., 2014). Therefore, these observations suggest that the introduction of a solid diet to pre-ruminants may activate the methanogenesis to effectively decrease the H_2 pressure in rumen with increasing microbial fermentation. The composition of archaea and the production of methane in lambs have already been manipulated in the long-term via manipulating pre-weaned diet (Abecia et al., 2013; Abecia et al., 2014b). The high heterogeneity and low richness observed in the present study represent an establishing and unstable archaeal community in the pre-weaned calves, which can easily be altered via diet. Thus, the alteration of rumen methanogens during early life through pre-weaned calf feeding strategies can be used to enhance microbial fermentation and to decrease methanogenesis in the rumen.

The rumen transcriptome has widely been studied in cattle to understand the molecular changes associated with weaning, age, diet and metabolic disorders (Penner et al., 2011; Connor et al., 2013). However, this study is the first to explore the postnatal

changes in the rumen transcriptome and the molecular mechanisms behind the host-microbial interactions during the rumen development process. Integrated analysis of the host transcriptome and the microbiome revealed potential molecular mechanisms behind the early rumen development, which could be divided into microbial-driven and ontogenic mechanisms (Figure 5.5). Only three gene modules (3,595 genes) and two miRNA modules (169 miRNAs) were associated with the concentration of VFAs and the development of papillae, indicating that only a portion of the rumen transcriptome was microbial-driven, while the majority was ontogenic (Figure 5.5). These ontogenic miRNA and gene modules revealed three mRNA-miRNA pairs (miR-25 and fatty acid binding protein 7 (*FABP7*); miR-30 and integrin-linked kinase (*ILK*); and miR29a and platelet-derived growth factor α polypeptide (*PDGFa*)) involved in the rumen development (Figure 5). *FABP7* is involved in "fatty acid uptake, transport and metabolism" (Liu et al., 2010); *ILK*-mediated signal transduction in "cytoskeletal organization" (Longhurst and Jennings, 1998); and *PDGFa* involved in intestinal villus morphogenesis (Betsholtz et al., 2001). The ontogenic control of the calf rumen development has been suggested previously (Baldwin et al., 2004); however, this study mainly focuses on the microbial-driven molecular mechanisms, as they are the black box of rumen development.

The identified genes in M10 and the predicted target genes of R7 provide a common ground to identify host-microbial interactions and their potential regulatory mechanisms in the developing rumen (Figure 5.5). Approximately 22% of the M10 genes (235/1,070) were similar to the differentially expressed genes identified in a previous study examining the rumen epithelial gene expression changes when calves were weaned

from milk replacer (42 days) to hay/grain (56 – 72 days) (Connor et al., 2013). These 235 common genes were differentially expressed in the rumen epithelial transcriptome when calves were weaned from a milk replacer-based diet (42 days) to a hay/grain-based diet (56 – 72 days), but not with calf age while they received milk replacer from day 14 – 42 (Connor et al., 2013). In this study, 87 of these 235 genes were differentially expressed when 1W was compared against 3W and 6W, after the introduction of solid diet. The strong positive correlations between these genes and the microbial metabolites (VFAs) suggest that they may be responsive to diet-driven changes in the microbial metabolites and may facilitate the early rumen development. Connor et al., (2013) also identified peroxisome proliferator-activated receptor-α (*PPARA*) as an important molecular mechanism of the rumen epithelial development during the weaning process. Although *PPARA* was expressed in all the pre-weaned calves in this study, it did not display a temporal expression pattern with calf age. However, the expression of *PPARG,* which co-expressed in the M10 module and was correlated with "microbial carbohydrate metabolism", was upregulated with the calf age. Similarly to adult cattle (Bionaz et al., 2013), the expression of *PPARG* in the calf rumen tissue was higher than the expression of *PPARA*. *PPARG* is widely studied in ruminants, and its expression level in the rumen is second only to its expression in the bovine adipose tissue (Bionaz et al., 2013). It induces epithelial cell proliferation in the colon (Su et al., 2007), upregulates the barrier functions within nasal epithelial cells (Ogasawara et al., 2010) and is one of the regulators of intestinal inflammation (Annese et al., 2012) stimulated via butyrate (Kinoshita et al., 2002). Butyrate has been shown to upregulate *PPARG* epigenetically via the inhibition of *HDAC* (Paparo et al., 2014). The observed negative correlations of

HDAC3 (M18 module) with the rumen papillae length and width and the butyrate concentration further reinforces the positive impact of butyrate on early rumen development through the modulation of host transcriptome. Therefore, I speculate that in addition to influencing cell apoptosis (Mentschel et al., 2001), butyrate may also be involved in rumen development via the activation of *PPARG*. The observed positive associations between the expression of *PPARG* and the concentration of VFAs as well as the functions related to " microbial carbohydrate metabolism" suggest its involvement in the overall rumen tissue development in response to microbial fermentation.

*ZNF*s are transcriptional factors that regulate a wide array of functions, including "recognition of DNA", "packaging of RNA", "activation of transcription", "protein folding and assembly", and "regulation of apoptosis" (Laity et al., 2001). The absorption of zinc, a major component of *ZNF*s, also plays an important role in the early rumen papillae development and keratinization in goat kids (Cernik et al., 2013). The present study revealed that five R7 miRNAs and eight M10 genes related to *ZNFs* were correlated with the abundance of the same bacterial genera (*Prevotella*, *Bacteroides*, *Propionibacterium*, *Ruminococcus*), suggesting that the early microbiota may influence the rumen development through zinc absorption, and this interaction may be regulated via miRNAs (Figure 5.5). The supplementation of cattle diets with zinc has long been studied to understand its impact on milk production and calf health (Spears, 1996); however, its role in the early rumen development and the microbial modulation of this process are yet to be understood.

Direct (abundance of bacteria) and indirect (microbial metabolites) associations between the expression of miRNAs and the early microbiota were evident in this study. A

higher proportion of miRNAs (169/364 or 46.4% of microRNAome) than of protein coding genes (3,595/13,676 or 26.3% of transcriptome) was associated with the concentration of VFAs, further corroborating our previous findings and speculations on interactions between miRNAs and microbes (Liang et al., 2014). A VFA-associated miRNA from R7, miR-375, inhibits the alveolar epithelial cell differentiation via the Wnt/β-catenin pathway, which participates in "tissue differentiation" and "organogenesis" in rats (Wang et al., 2013). The temporal downregulation of miR-375 and its negative associations with the concentration of VFAs and the development of papillae indicate one of the miRNA regulatory mechanisms that can be initiated by microbial metabolites. Thus, the M10 and R7 modules are indeed biologically important during rumen development and may serve as potential candidates to explore the host-microbial interactions and their regulatory mechanisms (Figure 5.5).

5.5 Conclusions

This study demonstrated that rumen colonization began during the birthing process and the pre-ruminant rumen microbiota was active and ready to ferment a solid diet even from the first week of life. The VFAs produced by the early microbiome were associated with the rumen tissue metabolism and the development of the epithelium via interacting with the host transcriptome and microRNAome (Figure 5.5). I therefore propose that early feeding management has a similar importance to the weaning period and may enhance the rumen development and facilitate the weaning transition. This study urges to understand host-microbial interactions in the developing rumen and lower gut in depth that may allow developing multidisciplinary approaches to improve the production

and health of livestock ruminants through early-life manipulations of rumen and/or gut microbiota.

5.6 References

Abecia, L., Martin-Garcia, A.I., Martinez-Fernandez, G., Newbold, C.J., Yanez-Ruiz, D.R. (2013) Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. J. Anim. Sci. 91, 4832-4840. doi: 10.2527/jas.2012-6142.

Abecia, L., Waddams, K.E., Martinez-Fernandez, G., Martin-Garcia, A.I., Ramos-Morales, E., Newbold, C.J., Yanez-Ruiz, D.R. (2014a). An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by archaea. Archaea. 2014, Article ID 841463. doi: http://dx.doi.org/10.1155/2014/841463.

Abecia. L., Ramos-Morales, E., Martinez-Fernandez, G., Arco, A., Martin-Garcia, A.I., Newbold, C.J. et al., (2014b) Feeding management in early life influences microbial colonization and fermentation in the rumen of newborn goat kids. Animal Production Science. 54, 1449-1454. http://dx.doi.org/10.1071/AN14337.

Annese, V., Rogai, F., Settesoldi, A., Bagnoli, S. (2012). PPARγ in inflammatory bowel disease. PPAR Res. 2012, Article ID 620839. http,//dx.doi.org/10.1155/2012/620839.

Baldwin, R.L., McLeod, K.R., Klotz, J.L., Heitmann, R.N. (2004). Rumen development, intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. J. Dairy Sci. 87, E55-E65.

Benjamini, Y., Hochberg, y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological). 57, 289-300.

Betsholtz, C., Karlson, L., Lindhal, P. (2001). Developmental roles of platelet-derived growth factors. BioEssays. 23,494-507.

Bionaz, M., Chen, S., Khan, M.J., Loor, J.J. (2013). Functional role of PPARs in ruminants, Potential targets for fine-tuning metabolism during growth and lactation. PPAR Res. 2013, Article ID 684159. doi: http,//dx.doi.org/10.1155/2013/684159.

Cernik, J., Pavlata, L., Misurova, L., Jokverova, O., Lunacek, J., Halouzka, R. (2013). Effect of peroral supplementation of zinc on the ruminal mucosa of goat kids - a morphometric study. Acta. Vet. Brno. 82, 399-403. doi: http://dx.doi.org/10.2754/avb201382040399.

Connor, E.E., Baldwin, R.L., Li, C., Li, R.W., Chung, H. (2013). Gene expression in bovine rumen epithelium during weaning identifies molecular regulators of rumen development and growth. Funct. Integr. Genomics. 13, 133-142. doi: 10.1007/s10142-012-0308-x.

Fonty, G., Gouet, P., Jouany, J.P., Senaud, J. (1987). Establishment of the microflora and aerobic fungi in the rumen of lambs. J. General Microbiol. 133, 1835-1843.

Gorka, P., Kowalski, Z.M., Pietrzak, P., Kotunia, A., Jagusiak, W., Zabielski, R. (2011). Is rumen development in newborn calves affected by different liquid feeds and small intestine development? J. Dairy Sci. 94, 3002-3013. doi: 10.3168/jds.2010-3499.

Heinrichs, A.J. (2005). Rumen development in the dairy calf. Adv. Dairy Tech. 17, 179-187.

Jami, E., Israel, A., Kotser, A., Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7, 1069-1079. doi: 10.1038/ismej.2013.2.

Khan, M.A., Lee, H.J., Lee, W.S., Kim, H.S., Ki, K.S., Hur, T.Y., et al., (2007). Structural growth, rumen development and metabolic and immune responses of Holstein male calves fed milk through step-down and conventional methods. J. Dairy Sci. 90:3376-3387.

Khan, M.A., Weary, D.M., von Keyserlingk, M.A.G. (2011). Hay intake improves performances and rumen development of calves fed higher quantities of milk. J. Dairy Sci. 94, 3547-3553. doi: 10.3168/jds.2010-3871.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L. (2013). TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36. doi: 10.1186/gb-2013-14-4-r36.

Kinoshita, M., Suzuki, Y., Saito, Y. (2002). Butyrate reduces colonic paracellular permeability by enhancing PPARgamma activation. Biochem. Biophys. Res. Commun. 293, 827-831.

Koike, S., Kobayashi, Y. (2009). Fibrolytic rumen bacteria: Their ecology and functions. Asian-Aust. J. Anim. Sci. 22, 131-138. doi: http://dx.doi.org/10.5713/ajas.2009.r.01.

Laity, J.H., Lee, B.M., Wright, P.E. (2001). Zinc finger protein, new insight into structural and functional diversity. Curr. Opin. Struc. Biol. 11, 39-46.

Langfelder, P., Horvath, S. (2008). WGCNA, An R package for weighted correlation network analysis. BMC Bioinf. 9, 559. doi,10.1186/1471-2105-9-559.

Li, R.W., Sparks, M.E., Connor, E.E. (2011). Dynamics of the rumen microbiota. In Metagenomics and its applications in agriculture, biomedicine and environmental studies R.W. Li, ed, (Nova Science Publishers, New York, USA) pp.135-164.

Li, R.W., Connor, E.E., Baldwin, R.L., Sparks, M.L. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environ. Microbiol. 14, 129-139. doi: 10.1111/j.1462-2920.2011.02543.

Li, M., Penner, G.B., Hernandez-Sanabria, E., Oba, M., Guan, L.L. (2009). Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107, 1924-1934. doi: 10.1111/j.1365-2672.2009.04376.x.

Liang, G., Malmuthuge, N., McFadden, T.B., Bao, H., Griebel, P.J., Stothard, P., et al., (2014). Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life. PLoS One. 9, e92592. doi,10.1371/journal.pone.0092592.

Liu, R.Z., Mita, R., Beauliu, M., Gao, Z., Godbout, R. (2010). Fatty acid binding proteins in brain development and disease. Int. J. Dev. Biol. 54, 1229-1239. doi: 10.1387/ijdb.092976rl.

Longhurst, C.M., Jennings, L.K. (1998). Integrin-mediated cellular transduction. Cell Mol. Life Sci. 54, 514-526.

Malmuthuge, N., Griebel, P.J, Guan, L.L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the

gastrointestinal tracts of pre-weaned calves. Appl. Environ. Microbiol. 80, 2012-2028. doi: 10.1128/AEM.03864-13.

Mentschel, J., Leiser, R., Mulling, C., Pfarrer, C., Claus, R. (2001). Butyric acid stimulates rumen mucosa development in the calf mainly by a reduction of apoptosis. Arch. Anim. Nutr. 55, 85-102.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., et al., (2008). The metagenomics RAST server- a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinf. 9, 386. doi,10.1186/1471-2105-9-386. doi: 10.1186/1471-2105-9-386.

Ogasawara, N., Kojima, T., Go, M., Ohkuni, T., Koizumi, J., Kamekura, R., et al., (2010). PPARgamma agonists upregulate the barrier function of tight junctions via a PKC pathway in human nasal epithelial cells. Pharmacol Res. 61, 489-498. doi: 10.1016/j.phrs.2010.03.002.

Paparo, L., di Costanzo, M., di Scala, C., Cosenza, L., Leone, L., Nocerino, R., et al., (2014). The influence of early life nutrition on epigentic regulatory mechanisms of the immune system. Nutrition. 6, 4706-4719. doi: 10.3390/nu6114706.

Penner, G.B., Steele, M.A., Aschenbach, J.R., McBride, B.W. (2011). Ruminant nutrition symposium, Molecular adaptation of ruminal epithelia to highly fermentable diets. J. Anim. Sci. 89, 1108-1119. doi: 10.2527/jas.2010-3378.

Purushe, J., Fouts, D.E., Morrison, M., White, B.A., Mackie, R.I. (2010). Comparative genome analysis of *Prevotella ruminicola* and *Prevotella bruantii*, Insights into their environmental niche. Microb. Ecol. 60, 721-729. doi: 10.1007/s00248-010-9692-8.

Rey, M., Enjalbert, F., Monteils, V. (2012). Establishment of ruminal enzymatic activities and fermentation capacity in dairy calves from birth through weaning. J. Dairy Sci. 95, 1500-1512. http://dx.doi.org/ 10.3168/jds.2011-4902.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. J. Appl. Microbiol. 116, 245-257. doi: 10.1111/jam.12405.

Sommer, F. and Backhed, F. (2013). The gut microbiota - masters of host development and physiology. Nat. Rev. Microbiol. 11, 227-238. doi: 10.1038/nrmicro2974.

Sommer, F., Nookaew, I., Sommer, N., Fogelstrand, P., Backhed, F. (2015). Site-specific programming of the host epithelial transcriptome by the gut microbiota. Genome Biol. 16, 62. doi: 10.1186/s13059-015-0614-4.

Spears, J.W. (1996). Organic trace minerals in ruminant nutrition. Animal Feed Science Technology. 58, 151-163.

Stevenson, D.M., Weimer, P.J. (2007). Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl. Microbiol. Biotechnol. 75, 165-174.

Su, W., Bush, C.R., Necela, B.M., Calcagno, S.R., Murray, N.R., Fields, A.P., et al., (2007). Differential expression, distribution, and function of PPAR-gamma in the proximal and distal colon. Physiol. Genomics. 30, 342-353.

Subramanian, S., Blanton, L.V., Frese, S.A., Charbonneau, M., Mills, D.A., Gordon, J.I. (2015). Cultivating healthy growth and nutrition through the gut microbiota. Cell. 161, 36-48. doi:10.1016/j.cell.2015.03.013.

Wang, Y., Huang, C., Chintagari, N.R., Bhaskaran, M., Weng, T., Guo, Y., et al., (2013). miR375 regulates rat alveolar epithelial cell trans-differentiation by inhibiting Wnt/β catenin pathway. Nucleic Acids Res. 41, 3833-3844. doi: 10.1093/nar/gks1460.

White, J.R., Nagarajan, N., Pop, M. (2009). Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput. Biol. 5, e1000352. doi: 10.1371/journal.pcbi.1000352.

Yeoman, C.J., White, B.A. (2014). Gastrointestinal tract microbiota and probiotics in production animals. Annu. Rev. Anim. Biosci. 2, 469-486. doi: 10.1146/annurev-animal-022513-114149.

Yu, Z., Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTechniques. 36, 808-812.

Zhou, M., Hernandez-Sanabria, E., Guan, L.L. (2010). Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Appl. Microbiol. Ecol. 76, 3776-3786. doi: 10.1128/AEM.00010-10.

5.7 Tables and Figures

Table 5.1 Primers used in qPCR to estimate bacterial/archaeal densities

	Forward primer (5'-3')	Reverse primer (5'-3')	Source
Total bacteria	actcctacgggaggcag	gactaccagggtatctaatcc	Stevenson & Weimer, 2007
E. ruminantium	ctcccgagactgaggaagcttg	gtccatctcacaccaccgga	Stevenson & Weimer, 2007
P. ruminicola	gaaagtcggattaatgctctatgttg	catcctatagcggtaaacctttgg	Stevenson & Weimer, 2007
R. albus	gttaacagagggaagcaaagca	tgcagcctacaatccgaactaa	Stevenson & Weimer, 2007
R. flavefaciens	tggcggacgggtgagtaa	ttaccatccgtttccagaagct	Stevenson & Weimer, 2007
Total archaea	ccggagatggaacctgagac	cggtcttgcccagctcttattc	Zhou et al., 2010

Table 5.2 Postnatal changes in active rumen bacteria, rumen morphology and metabolites in pre-weaned calves

	Calf age			*P*- value
	1-week	3-week	6-week	
Active rumen bacteria				
R. flavefaciens[1]	3.8±1.9E08[a]	1.6±0.4E09[b]	1.3±0.5E09[b]	0.03
R. albus	1.4±0.5E05	6.3±6.2E06	2.2±1.7E07	0.34
P. ruminicola	6.4±2.7E04	4.4±3.0E05	7.6±7.4E07	0.37
E. ruminantium	7.8±3.8E05	1.5±0.6E06	1.6±0.6E06	0.48
Rumen papillae				
Length (μm)	317.8±7.6[a]	413.0±13.6[b]	678.1±41.1[c]	< 0.01
Width (μm)	155.5±2.7[a]	224.0±6.3[b]	275.8±9.0[c]	< 0.01
Rumen fermentation parameters				
Acetate[2]	21.1±2.2[a]	37.0±2.8[b]	50.3±3.8[c]	< 0.01
Propionate	10.6±2.0[a]	25.8±4.3[b]	36.3±1.8[c]	< 0.01
Butyrate	5.6±1.8[a]	11.8±2.7[b]	17.8±2.2[b]	< 0.01
Isobutyrate	0.3±0.04[a]	0.8±0.1[b]	1.3±0.1[c]	< 0.01
Valerate	1.0±0.4[a]	3.7±0.7[b]	5.1±0.8[b]	< 0.01
Isovalerate	0.3±0.07[a]	0.8±0.1[a]	1.3±0.3[b]	< 0.01
Total	39.6±6.2[a]	80.5±9.0[b]	113.9±6.9[c]	< 0.01
Acetate[3]	55.3±2.7[a]	47.0±2.4[b]	44.2±1.9[b]	0.01
Propionate	26.7±1.4	31.3±2.9	32.1±1.4	NS
Butyrate	13.0±2.0	14.4±2.5	15.4±1.4	NS
Isobutyrate	0.8±0.1	1.0±0.1	1.2±0.1	NS
Valerate	2.1±0.5[a]	4.6±0.6[b]	4.5±0.6[b]	0.01
Isovalerate	0.8±0.2	1.0±0.2	1.2±0.2	NS

[a, b, c] – means with different superscript within a raw are significantly different at $P < 0.05$.

[1]Density of active rumen bacteria (16S rRNA copy/ g of rumen content).

[2]VFA concentration (mM/ml of rumen fluid).

[3]Molar proportion of VFA (%).

Table 5.3 Differentially abundant functions of the rumen microbiome

Functions	0D	1W	3W	6W	Significant comparisons[2]
Subsystems[1]					
Amino acids and derivatives	5.6	11	8.3	7.9	1W vs. 3W, 6W
Carbohydrates	9.4	12.4	14.5	11	3W vs. 6W
Cofactors, vitamins, prosthetic groups, pigments	5.8	5.4	4.7	4.8	1W vs. 3W
Dormancy and sporulation	0.02	0.2	0.3	0.4	0D vs. 1W, 3W, 6W
Metabolism of Aromatic Compounds	0.1	0.5	0.7	0.5	0D vs. 1W
Fatty acids, lipids, isoprenoids	0.6	1.7	1.1	2.1	3W vs. 6W
Motility and chemotaxis	0	0.3	1.1	0.3	0D vs. 1W, 1W vs. 3W, 3W vs. 6W
Nitrogen metabolism	1.1	1.6	0.6	1.4	0D vs. 3W
Nucleosides and Nucleotides	1.6	3.8	3.8	4.4	0D vs. 1W, 3W, 6W
Phages, prophages, transposable elements, plasmids	1.1	4.4	7	6	0D vs. 1W, 3W, 6W
Potassium metabolism	0.08	0.2	0.01	0.05	1W vs. 3W
Virulence, disease and defense	1.2	3	2.2	2.9	0D vs. 1W
Respiration	20.5	3.2	4.5	2.8	0D vs. 1W, 3W, 6W
Level 2 functions in SEED hierarchy[1]					
Adhesion	0	0.05	0.04	0.02	0D vs. 1W; 1W vs. 6W
ATP synthases	0.2	0.5	1.6	0.8	1W vs. 3W
Biologically active compounds in metazoan cell defence and differentiation	0.1	0.1	0	0.5	1W vs. 3W
Biosynthesis of galactoglycans and related lipopolysacharides	0	0.03	4.00E-05	0.03	0D vs. 1W
Biotin	0.2	0.2	0	0.06	1W vs. 3W
Branched-chain amino acids	0.2	2.3	2.9	1.8	0D vs. 1W
Carotenoid biosynthesis	0.01	0	0.003	0	0D vs. 1W

Cell Wall and Capsule	1.6	1.3	0.6	0.6	1W vs. 3W; 1W vs. 6W
Cell wall of Mycobacteria	0	0.1	0	0.001	0D vs. 1W; 1W vs. 3W
Central carbohydrate metabolism	2.8	6.4	10.5	9.2	1W vs. 6W
Choline bitartrate degradation, putative	0	0.1	0.2	0.2	0D vs. 1W
Coenzyme A	0.04	0.3	0	0.3	0D vs. 1W; 1W vs. 3W
Coenzyme F420	0	0.001	0.06	0.03	1W vs. 6W
Cofactors, Vitamins, Prosthetic Groups, Pigments	0.05	0.4	0.4	0.2	0D vs. 1W
Cold shock	0	0	0.01	0	1W vs. 3W
DNA recombination	0.01	0.1	0.1	0.2	0D vs. 1W
Dormancy and Sporulation	0.01	0.2	0.2	0.3	0D vs. 1W
Fatty acids	0.1	0.4	0.4	2.5	0D vs. 1W
Fatty Acids, Lipids, and Isoprenoids	0	0.1	0.2	0.1	0D vs. 1W
Flagellar motility in Prokaryota	0	0.3	1.3	0.4	0D vs. 1W; 1W vs. 3W; 3w vs. 6W
Glycoside hydrolases	0.01	0.1	0	0.03	0D vs. 1W
Heat shock	0.2	0.5	1	1.2	1W vs. 6W
Histidine Metabolism	0.1	0.5	0.3	0.2	0D vs. 1W
Lysine, threonine, methionine, and cysteine	1.9	3.1	2.3	1.9	1W vs. 6W
Metabolism of Aromatic Compounds	0	0.03	0.01	0.02	0D vs. 1W
Methylamine utilization	0.1	0.7	0.1	0.5	1W vs. 3W
Motility and Chemotaxis	0	0.03	0.3	0.1	1W vs. 3W
Nucleosides and Nucleotides	0.03	0.6	0.5	0.2	0D vs. 1W; 1W vs. 6W; 3W vs. 6W
Organic acids	0.8	1	0.7	0.9	1W vs. 3W; 3W vs. 6W
Osmotic stress	0	0.06	0.01	0.03	0D vs. 1W; 1W vs. 3W
Pathogenicity islands	0.2	0.3	0.5	0.8	1W vs. 6W
Peripheral pathways for catabolism of aromatic compounds	0.01	0.3	0.001	0.3	0D vs. 1W
Periplasmic Stress	0.03	0.04	0.01	0.01	1W vs. 6W
Phages, Prophages	0.2	0.7	2.2	3.9	1W vs. 3W
Phospholipids	0.3	0.7	0.22	0.5	1W vs. 3W

Plant-Prokaryote DOE project	11.7	7.1	6.4	4.6	1W vs. 6W
Potassium metabolism	0.05	0.2	0.01	0.07	1W vs. 3W
Programmed Cell Death and Toxin-antitoxin Systems	0.01	0.05	0.01	0	1W vs. 3W; 1W vs. 6W
Proteasome related clusters	0.4	0	0.04	0.02	1W vs. 3W
Protein biosynthesis	0.01	0	0.03	0.05	0D vs. 1W
Protein folding	0.05	0.6	1	1.9	1W vs. 6W
Protein secretion system, Type II	0	0.034	0.08	0.03	0D vs. 1W
Proteolytic pathway	0.2	0	0	0	0D vs. 1W
Putrescine/GABA utilization cluster-temporal	0	0.05	0.004	0.008	1W vs. 3W
Pyridoxine	0.1	0.5	0.3	0.4	0D vs. 1W
Pyrimidines	0.5	1.2	0.7	1.8	0D vs. 1W; 1W vs. 3W; 3w vs. 6W
Pyruvate kinase associated cluster	0.03	0	0	0	0D vs. 1W
Quinone cofactors	0.1	0.3	0.1	0.03	0D vs. 1W; 1W vs. 3W; 1W vs. 6W; 3W vs. 6W
Resistance to antibiotics and toxic compounds	0.7	2.4	1.4	1.9	0D vs. 1W
Reverse electron transport	0.03	0.04	0.3	0.3	1W vs. 6W
Riboflavin, FMN, FAD	0.04	0.3	0.2	0.2	0D vs. 1W
Ribosome-related cluster	0.1	0.2	0.7	0.4	1W vs. 3W
Selenoproteins	0	0.1	0.5	0.4	0D vs. 1W
Transcription	0.5	1.6	1.4	2.2	0D vs. 1W
Translation	0.1	0.2	0.1	0.5	1W vs. 6W; 3W vs. 6W
Two related proteases	0	0.3	0.3	0.4	0D vs. 1W

[1]relative abundance as % of total detected subsystems or level 2 functions in the SEED hierarchy.

0D – at birth/newborn, 1W – week-old calves, 3W – 3-week-old calves, 6W – 6-week-old calves.

Table 5.4 Relative abundance of glycoside hydrolases and archaeal-specific glycolysis enzymes in calf rumen

Enzyme	1-week	3-week	6-week	GH family*
Glycosides Hydrolases*				
6-phospho-beta-glucosidase ascB (EC 3.2.1.86)	10.0±5.0	10.0±5.0	0.2±0.001	1, 4
Alpha-amylase (Neopullulanase) SusA (EC 3.2.1.135)	21.5±19.3	22.8±19.3	5.0±1.2	13
Alpha-galactosidase (EC 3.2.1.22)	5.0±1.2	5.0±1.2	11.7±6.9	4, 21, 31, 36, 57, 97, 110
Alpha-glucosidase (EC 3.2.1.20)	3.6±0.7	4.0±0.7	0	4, 13, 31, 63, 97, 122
Alpha-glucosidase SusB (EC 3.2.1.20)	12.3±5.5	12.0±5.5	36.1±17.0	4, 13, 31, 63, 97, 122
Alpha-L-arabinofuranosidase II precursor (EC 3.2.1.55)	0	0	2.2±0.2	2, 3, 10, 43, 54, 62
Alpha-mannosidase (EC 3.2.1.24)	7.7±3.0	8.0±2.9	0	31, 38, 92
Alpha-N-acetylglucosaminidase (EC 3.2.1.50)	0	0	1.8±0.2	89
Alpha-N-arabinofuranosidase 2 (EC 3.2.1.55)	2.1±0.2	2.1±0.2	11.8±7.0	2, 3, 10, 43, 54, 62
Beta-galactosidase large subunit (EC 3.2.1.23)	0.2±0.001	0.02±0.001	11.1±0.06	1, 2, 3, 35, 49, 50, 52
Endo-beta-1,3-1,4 glucanase (Licheninase) (EC 3.2.1.73)	10.0±5.0	10.0±5.0	0	5, 7, 8, 9, 11, 12, 16, 17, 26
Glucan 1,6-alpha-glucosidase (EC 3.2.1.70)	0	0	17.4±10.6	13, 15
Glycogen debranching enzyme (EC 3.2.1.-)	14.6±5.5	14.6±5.5	0	1-5, 8-10, 13, 16-20, 26, 28, 30, 32, 33, 35, 38, 42-44, 48, 51, 55
Maltose-6'-phosphate glucosidase (EC 3.2.1.122)	1.4±0.1	1.4±0.1	8.8±1.5	4
Periplasmic beta-glucosidase (EC 3.2.1.21)	0	0	3.4±0.6	1, 3, 5, 9, 30, 116
Polygalacturonase (EC 3.2.1.15)	10.6±0.05	1.1±0.06	0	28
Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL (EC 3.2.1.-)	0.2±0.001	0.15±0.001	0	2, 3, 10, 43, 54, 62
Trehalase (EC 3.2.1.28)	8.9±2.8	8.9±2.8	0.3±0.005	13, 15, 37, 65
Trehalose-6-phosphate hydrolase (EC 3.2.1.93)	1.5±0.1	1.5±0.1	0	13

Archaeal-specific glycolysis enzymes			
Glucose-6-phosphate isomerase, archaeal (EC 5.3.1.9)	0.00012±0.00	0	0
Fructose-bisphosphate aldolase, archaeal class I (EC 4.1.2.13)	0.01±0.00	0	0
2,3-bisphosphoglycerate-independent phosphoglycerate mutase, archaeal type (EC 5.4.2.1)	0.2±0.00	0.006±0.00	0.1±0.00
Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (NADP) (EC 1.2.1.9)	0.03±0.00	0.0008±0.00	0.0003±0.00

* http://www.cazy.org/Glycoside-Hydrolases.html. Data presented as mean±SEM.

Table 5.5 Association between rumen papillae development, concentration of VFAs, and cellulolytic bacteria

	Acetate	**Propionate**	**Butyrate**	**Valerate**	**Isobutyrate**	**Isovalerate**
Papillae length[1]	0.7***	0.6***	0.7***	0.6**	0.7***	0.6***
Papillae width	0.8***	0.8***	0.7***	0.7***	0.8***	0.7***
R. flavefaciens	0.6***	0.4	0.6***	0.4*	0.4*	0.3

[1] Pearson correlation coefficient.

* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$.

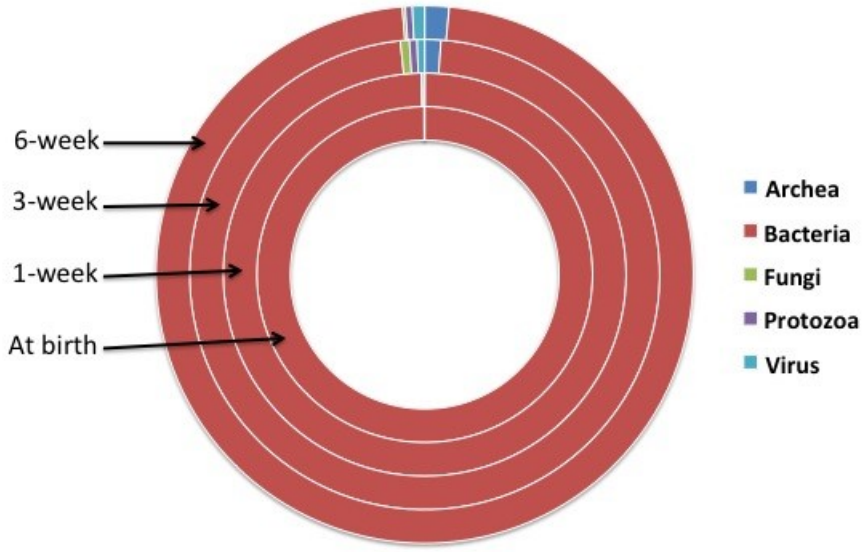

Figure 5.1 Establishment of rumen microbiome from birth up to the first six weeks of life and the development of rumen papillae.

(A) Composition of rumen microbiome in calves from birth (at birth) up to six weeks of life. Each data series represent mean relative abundance of microbial group as a % of total detected microbiota.

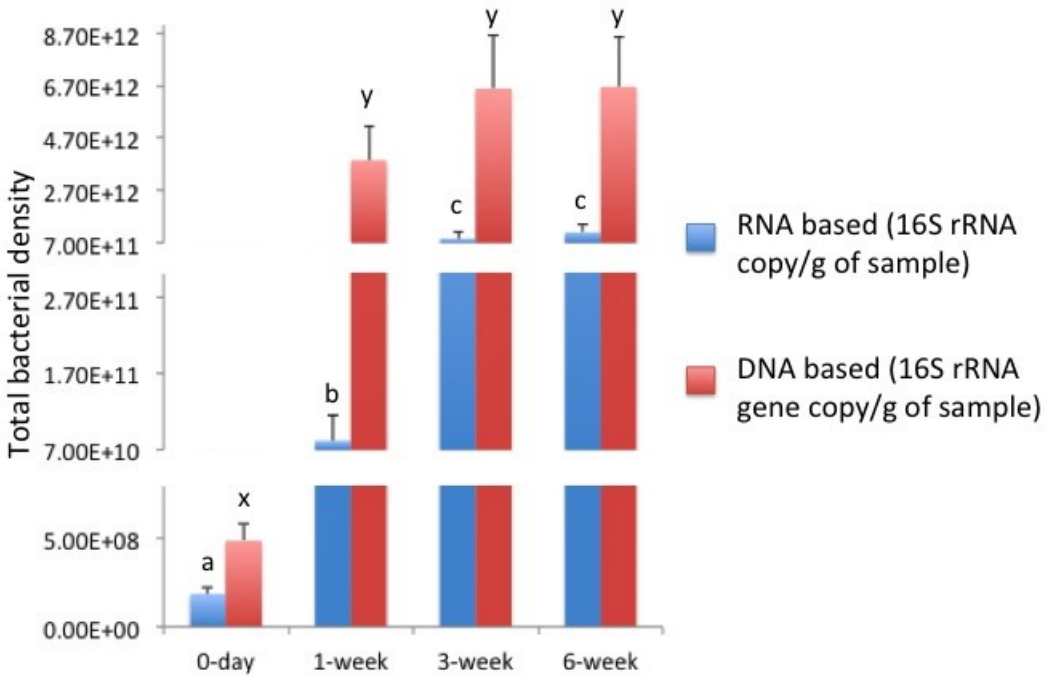

Figure 5.1B Estimated total bacterial density (DNA-based (16S rRNA gene copy/g of sample) and RNA-based (16S rRNA copy/ g of sample) in calf rumen during the first six weeks of life ($P = 0.02$).

Bars represent mean bacterial densities and error bars represent SEM. a,b – represents mean RNA-based bacterial densities different at $P < 0.05$. x,y – represents mean DNA-based bacterial densities different at $P < 0.05$.

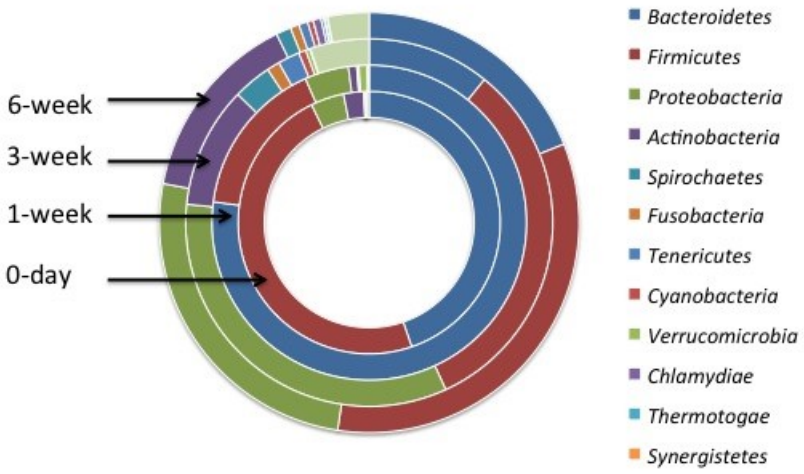

Figure 5.1C Composition of rumen content-associated bacteria (mean relative abundance) at phylum level.

Each data series represent mean relative abundance of bacterial phylum as a % of total detected bacteria.

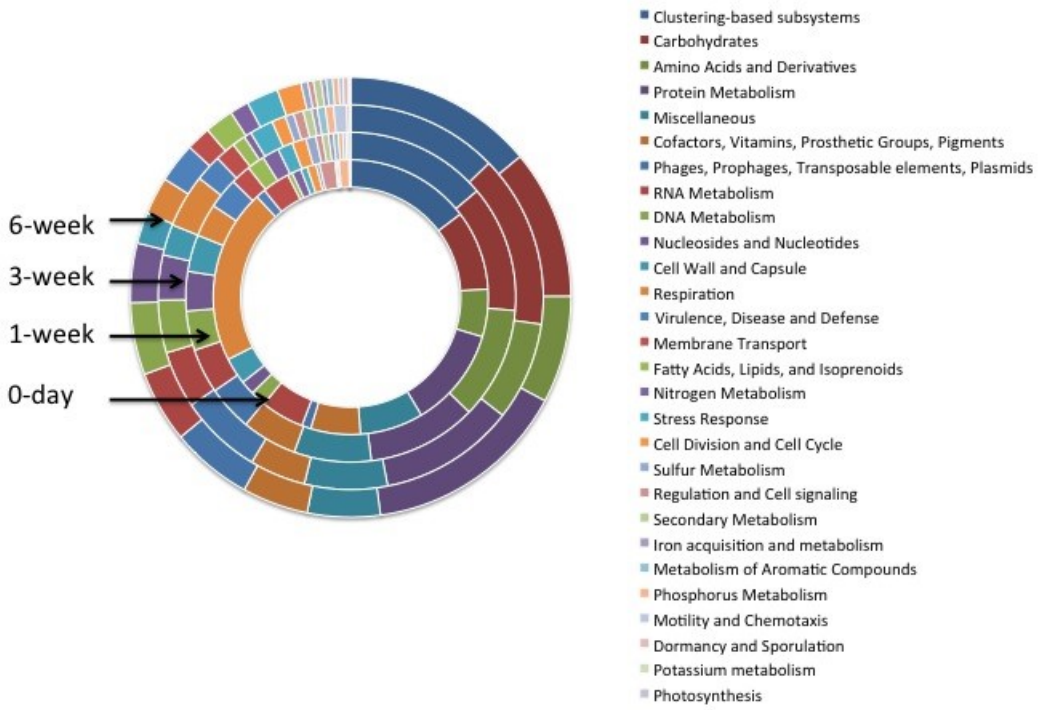

Figure 5.1D Functional composition of rumen content-associated bacteria at level 1 SEED hierarchy/subsystems.

Each data series represent mean relative abundance of microbial function as a % of total detected subsystems.

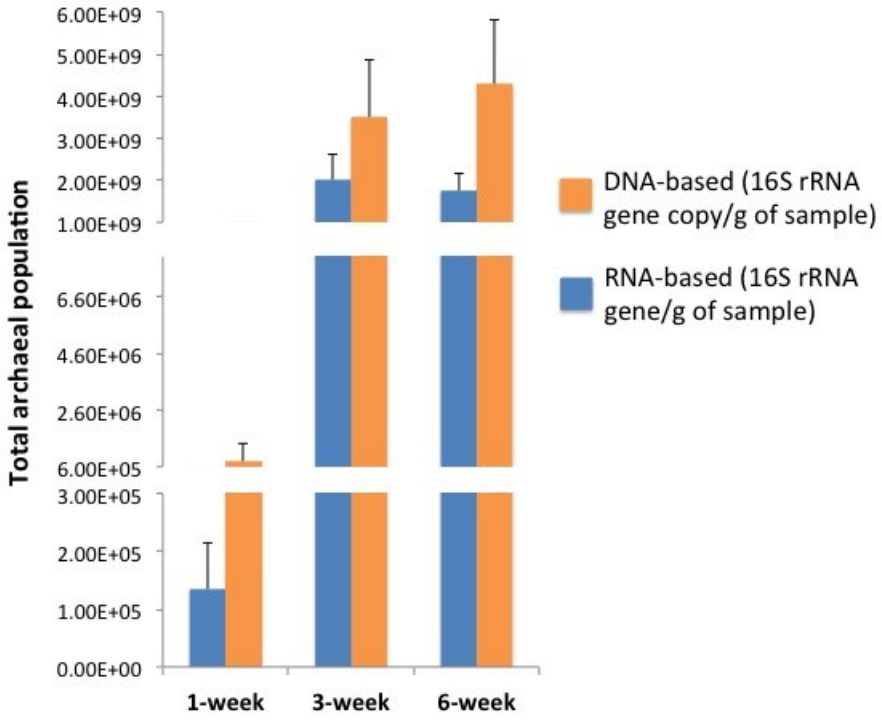

Figure 5.1E Estimated total archaea density using DNA-based (16S rRNA gene copy/g of sample) and RNA-based (16S rRNA copy/ g of sample) quantifications.

Bars represent mean bacterial densities and error bars represent SEM.

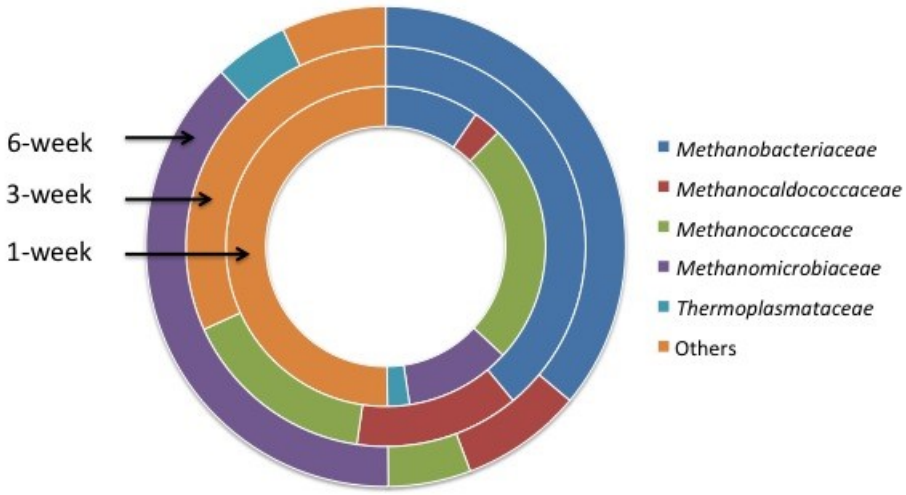

Figure 5.1F Rumen content-associated archaeal composition at family level.

Each data series represent mean relative abundance of archaeal family as a % of total detected archaea.

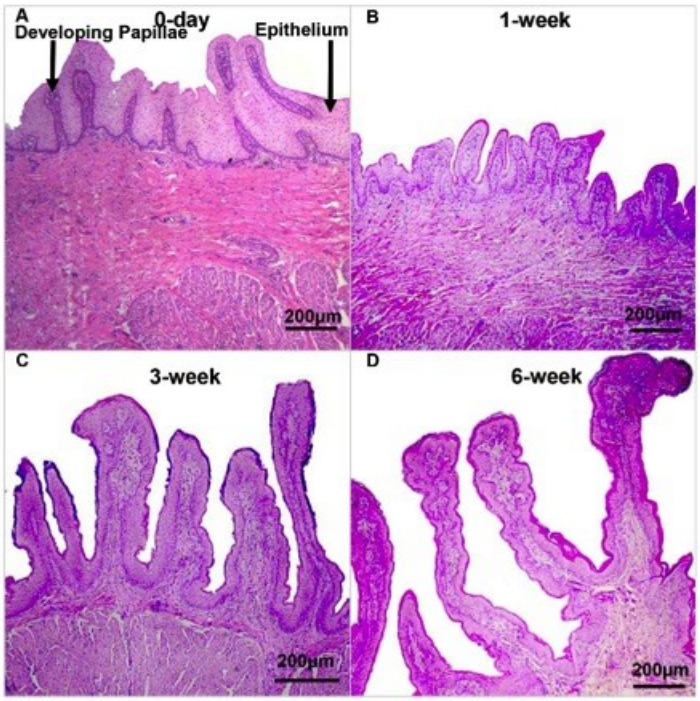

Figure 5.1G Rumen papillae development in calves within the first six weeks of life. Images are obtained through light micrograph of rumen tissue at a magnification of 10X objective lens (bar = 200 µm).

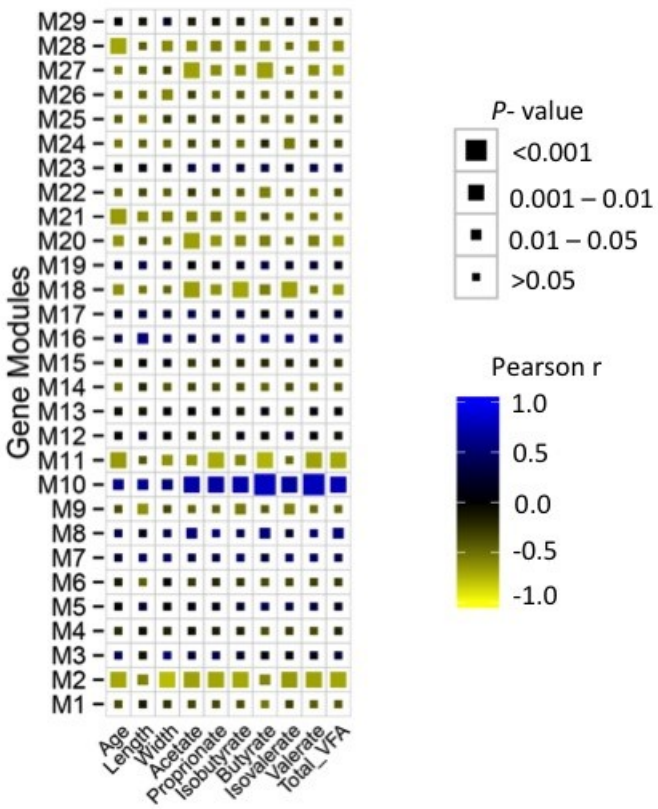

Figure 5.2 Associations among transcriptome networks (gene modules), calf phenotypic traits (concentration of VFAs, papillae length and width, calf age) and bacterial composition (taxonomy – genus level).

Figure 5.2A Relationship between gene modules (M1 – M29) and calf phenotypic traits. Gene modules obtained using weighted gene co-expression network analysis and eigengene/PC1 value of each gene module is correlated with the calf phenotypic traits.

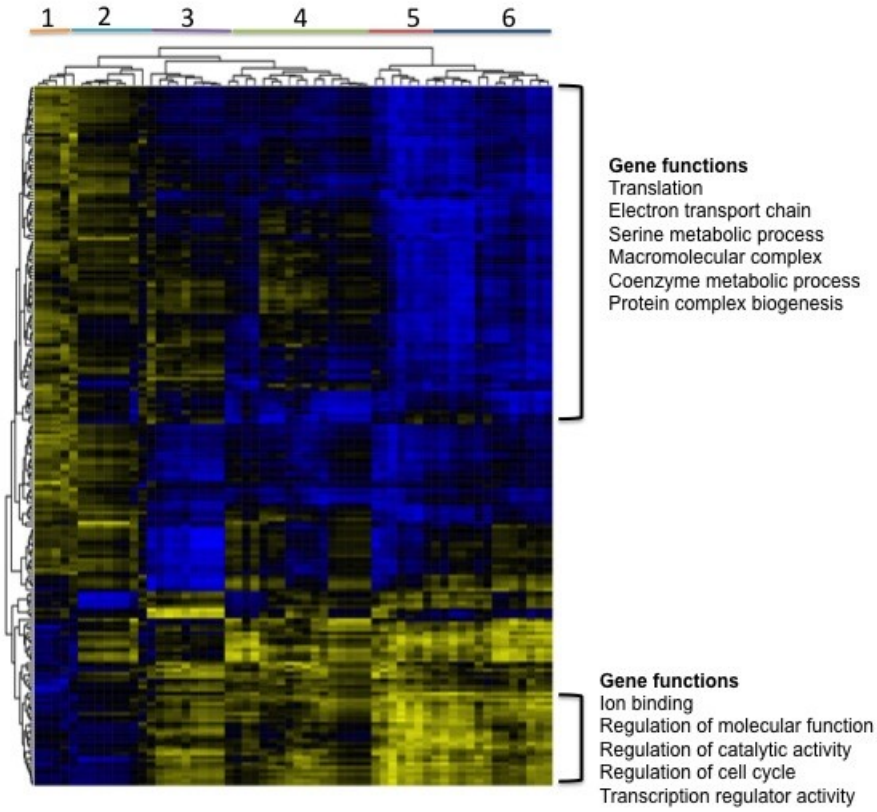

Figure 5.2B Association between the genes co-expressed in the M10 module and content-associated bacterial genera relative abundance.

Heatmap is generated using Pearson correlation value between the expression of a gene and the relative abundance of a bacterial genus. Blue represents positive correlations, whereas yellow represents negative correlations. Numerical values represents the identified bacterial clusters based on their associations with the expression of genes.

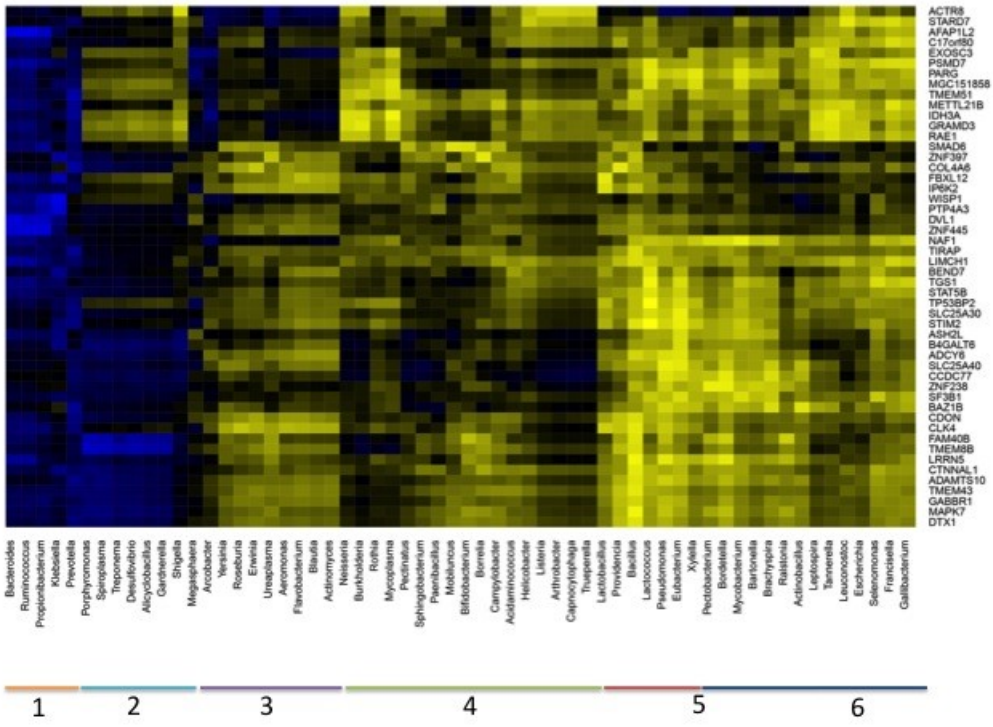

Figure 5.2C Bacterial clusters associated with ion binding related genes co-expressed in the M10 module.

Cluster 1 (*Bacteroides, Ruminococcus, Propionibacterium, Klebsiella, Prevotella*) positively correlates to the expression of the ion binding related genes ($P < 0.05$, $r \geq 0.5$). Cluster 6 (*Pectobacterium, Bordetella, Mycobacterium, Bartonella, Brachyspira, Ralstonia, Actinobacillus, Leptospira, Tannerella, Leuconostoc, Escherichia, Selenomonas, Francisella, Gallibacterium*) negatively correlates to the expression of the ion binding related genes ($P < 0.05$, $r \leq -0.5$).

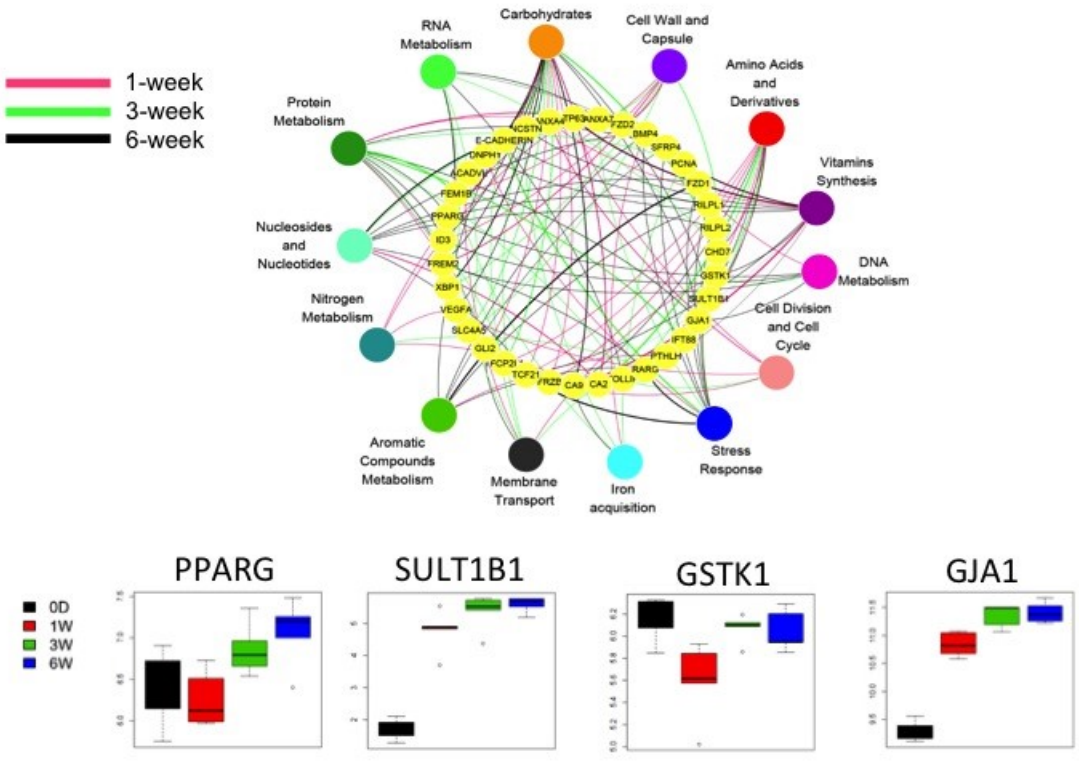

Figure 5.3 Level 2 microbial functions associated with host protein coding genes expression.

Figure 5.3A Level 2 microbial functions associated with ($P < 0.01$, $r^2 \geq 0.98$) host genes involved in rumen epithelial tissue development (GO: 0060429, 34 genes). Lower panel represents genes co-expressed in M10 gene module. PPARG – peroxisome proliferator activated receptor gamma; SULT1B1 – sulfotranferase family 1B member 1; GSTK1 – glutathione S-transferase kappa 1; GJA1 – gap junction protein alpha 1. 0D – at birth, 1W – 1-week-old calves, 3W – 3-week-old calves, 6W – 6-week-old claves.

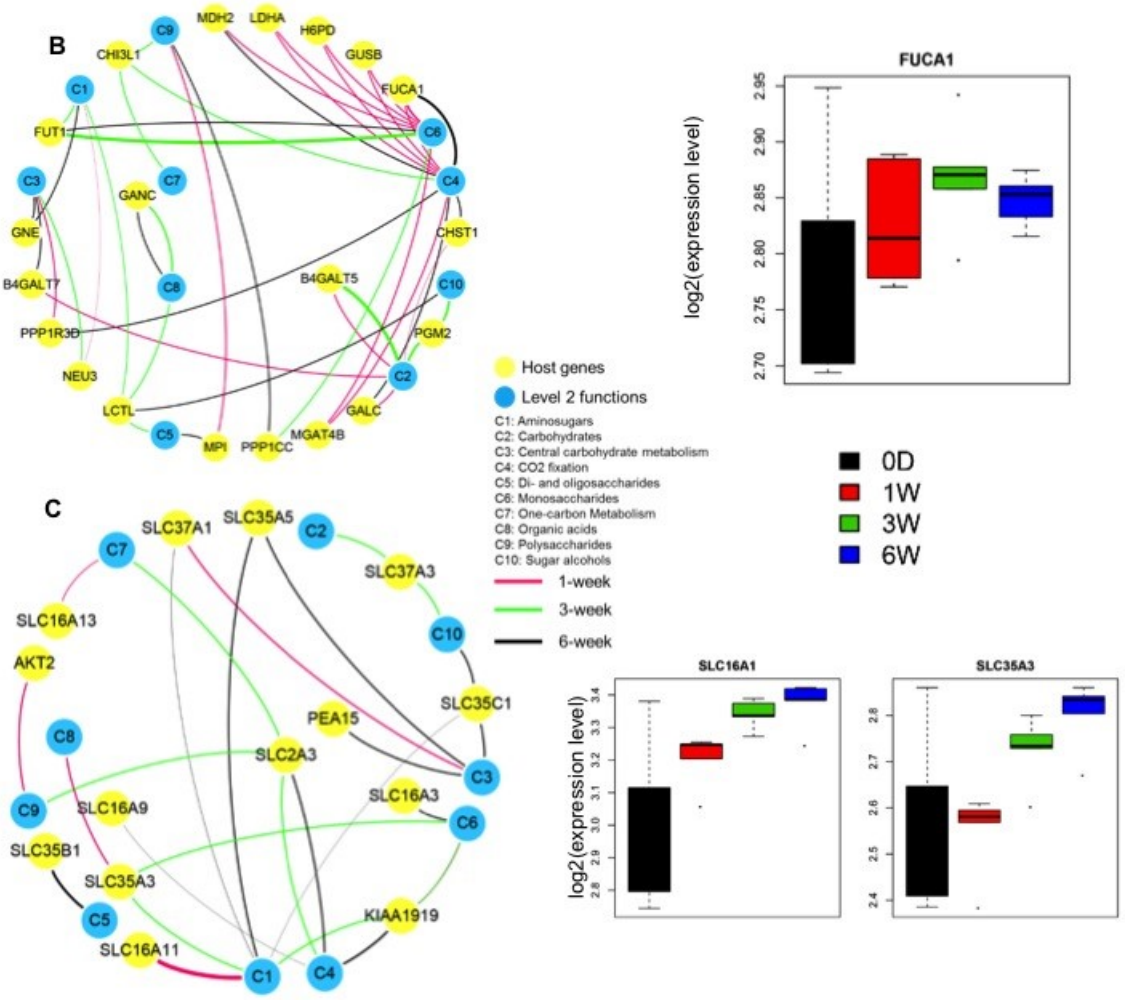

Figure 5.3B Level 2 microbial functions associated with ($P < 0.01$, $r^2 \geq 0.98$) host genes involved in rumen tissue carbohydrate metabolism (GO: 0005975, 20 genes).

Figure 5.3C Level 2 microbial functions associated with ($P < 0.01$, $r^2 \geq 0.98$) host genes involved in membrane transportation (GO: 0008643, 14 genes).

Right side panel represents genes co-expressed in M10 gene module. FUCA1 – fucosidase alpha L-1; SLC16A1 – solute carrier family 16 member 1; SLC35A3 - solute carrier family 35 member 3.

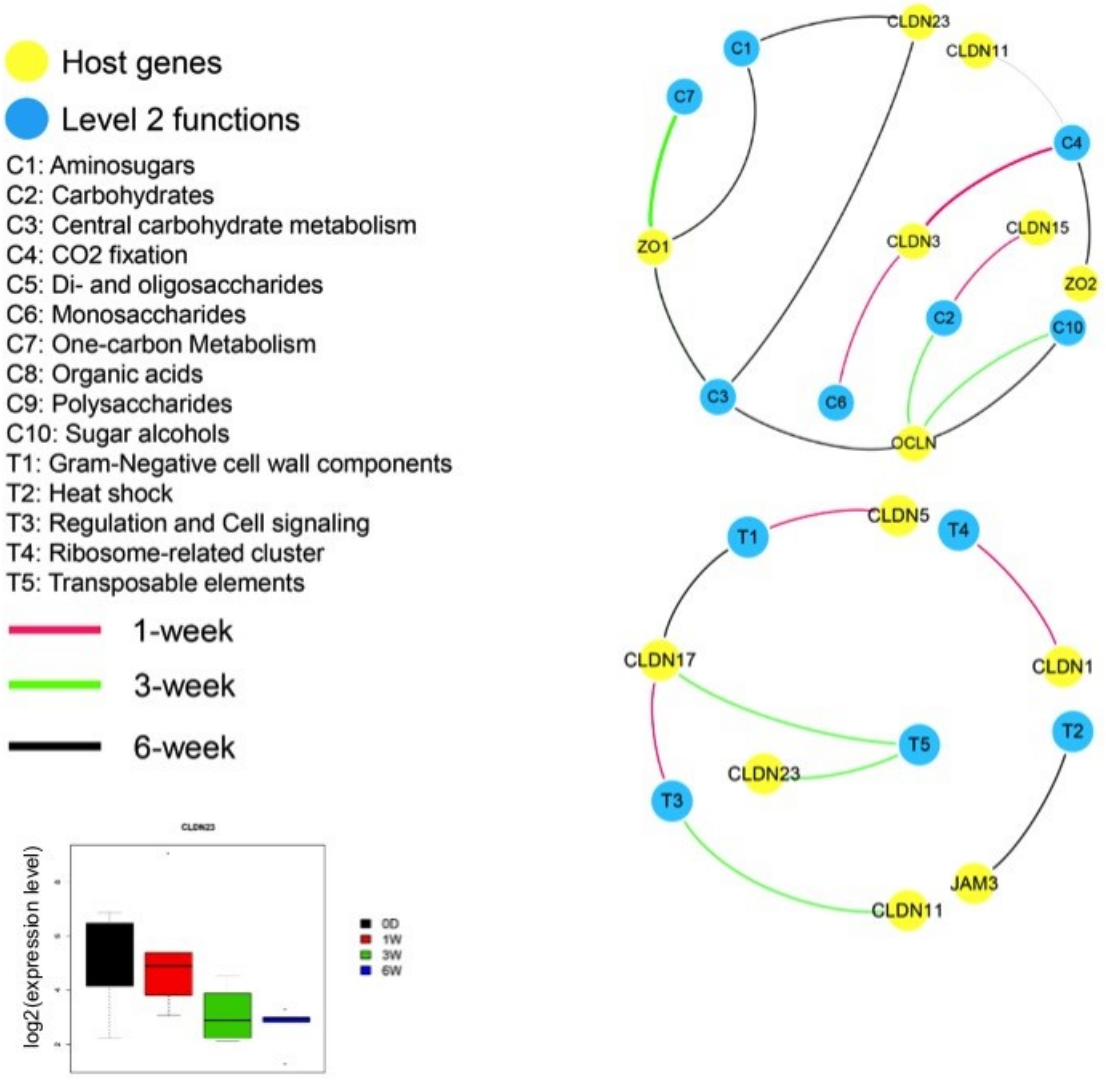

Figure 5.3D Level 2 microbial functions associated with ($P < 0.01$, $r^2 \geq 0.98$) host genes involved in tight junction protein genes (TJs) (GO: 0005923, 14 genes).

Upper network represents TJs correlated with microbial carbohydrate metabolism. Lower network represents TJs correlated with other microbial functions. Left side panel represents genes co-expressed in M10 gene module. CLDN23 – claudin 23.

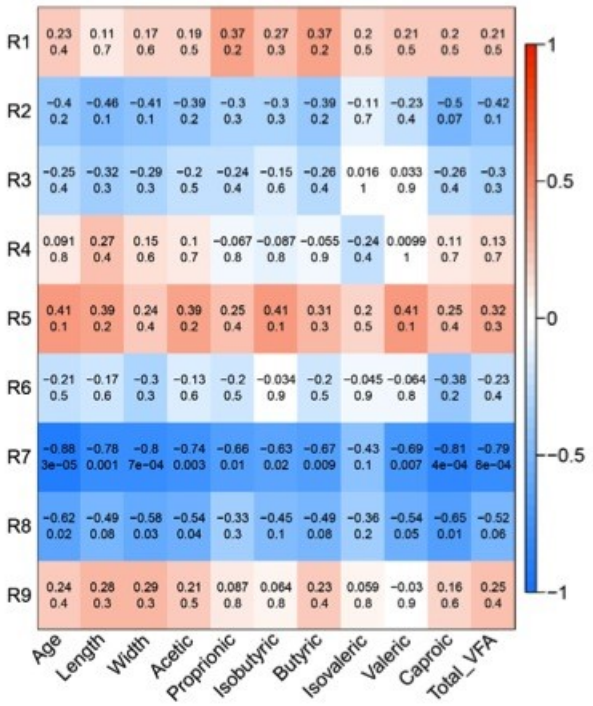

Figure 5.4 Association between rumen miRNA profile (expression of miRNA) and rumen microbiota (bacterial genera, concentration of VFAs).

Figure 5.4A Relationship between miRNA modules (R1-R9) and calf phenotypic traits. miRNA modules are generated using WGCNA and eigengene/PC1 values of each modules is correlated with calf phenotypic traits. Numerical values within a square represent Pearson correlation (upper value) and *P* value (lower value). Color bar represents Pearson correlation from -1 to 1.

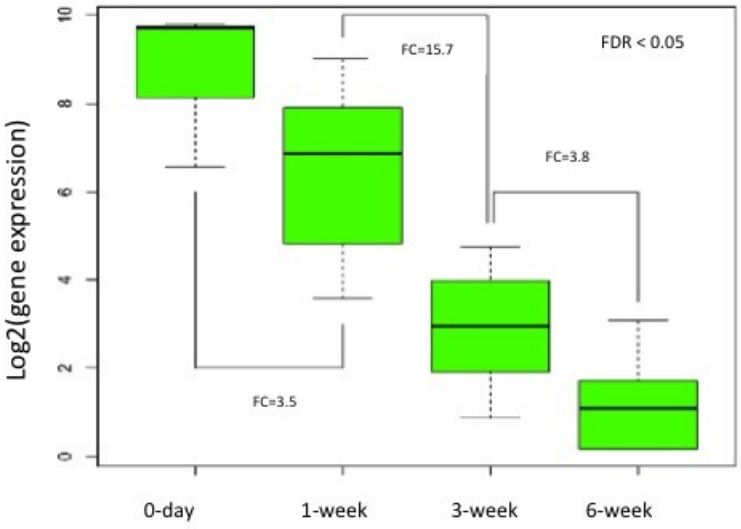

Figure 5.4B Temporal changes in the expression (CPM) of miR-375 in calf rumen (day 0 - 605.1±40.3; week 1 - 171.5±15.6; week 3 - 10.9±3.8; week 6 - 2.9±1.2; $P < 0.01$).

FC (fold change) is the expression ratio between two adjacent age groups.

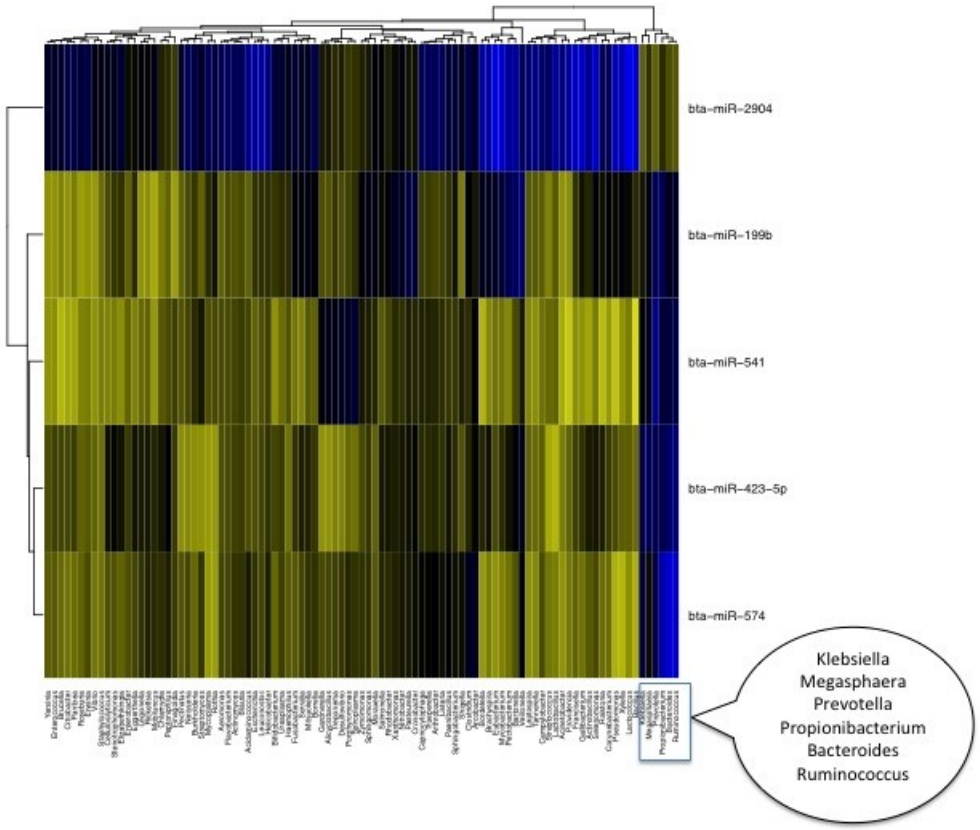

Figure 5.4C Association between rumen bacterial taxonomy and miRNAs co-expressed in R7 miRNA module.

Heatmap is generated using Pearson correlation value between the expression of a miRNA and the relative abundance of a bacterial genus. Blue represents positive correlations, whereas yellow represents negative correlations. A bacterial cluster consists of *Klebsiella, Megasphaera, Prevotella, Propionibacterium, Bacteroides* and *Ruminococcus* displayed strong correlation with the expression of bta-miR-574, bta-miR-423-5p, bta-miR-5441, bta-miR-199b and bta-miR-2904 ($-0.9 > R > 0.9$, $P < 0.05$).

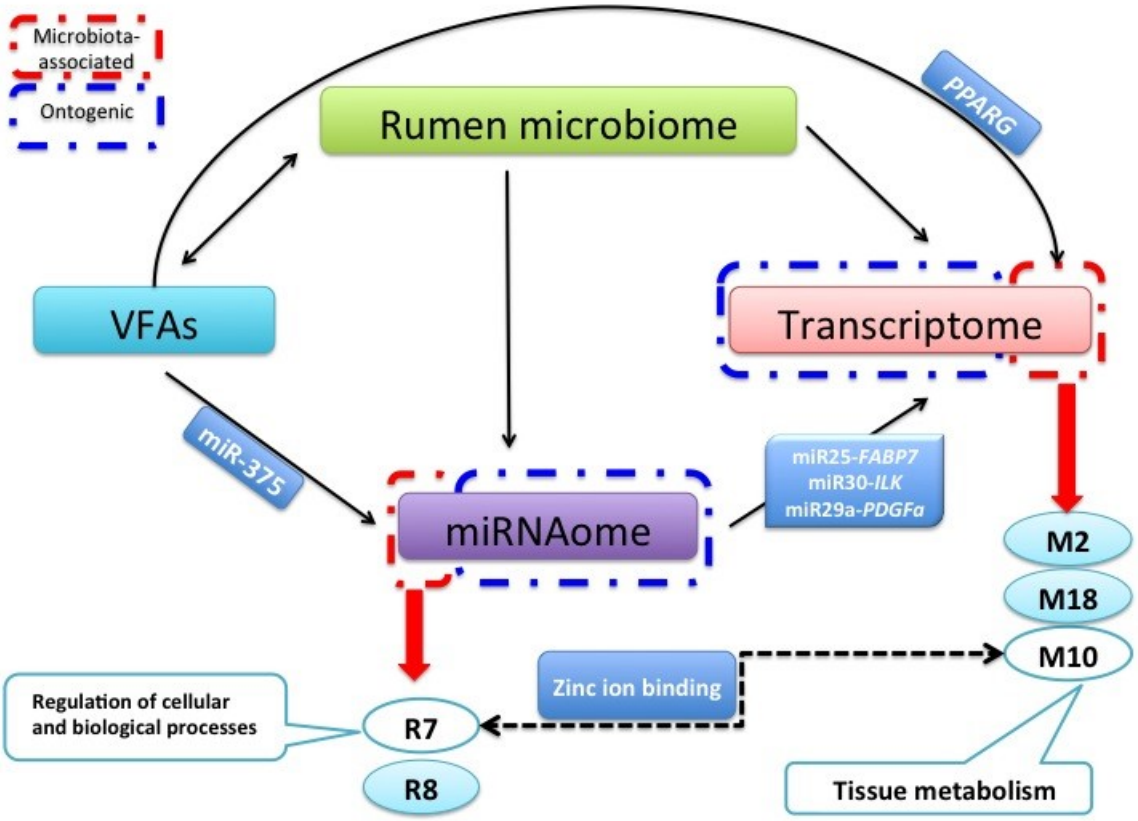

Figure 5.5 Proposed host-microbial interactions and their regulatory mechanisms in the developing rumen.

Early rumen microbiota alters rumen development via direct and indirect (miRNAs) interactions with the rumen tissue transcriptome. Microbial-derived VFAs are associated with genes involved in ruminal tissue metabolism (M10 gene module), non-coding RNA processing (M2 gene module) and epigenetic modifications (M18 gene module) as well as miRNAs regulating epithelial morphogenesis (R7 miRNA module). miRNAs regulate the host transcriptome either in response to microbial metabolites/ rumen microbiota or directly during the early rumen development.

Chapter 6. General Discussion

6.1 Significance of the study

Evidence is accumulating regarding the immense importance of early gut microbiota on the postnatal development and adult health of humans (Arrieta et al., 2014). These findings suggest that a "healthy gut microbiome" may prevent a wide variety of potential gut microbiota-associated diseases (Subramanian et al., 2015). Microbial composition has long been linked with obesity (metabolic disorder) in adults (Turnbaugh et al., 2009), and the early microbial establishment process has now been identified as a potential factor that leads to obesity later in life (Dogra et al., 2015). Children with a high abundance of *Streptococcus*, an initial colonizer of the infant gut (Fanaro et al., 2003), at six months of life have a greater tendency toward adiposity than other infants (Dogra et al., 2015). Moreover, the risk of adiposity is higher in cesarean-delivered infants, who have a different initial colonization from breast-fed infants (Mesquita et al., 2013). Although colonization by host-specific gut microbiota is widely studied in humans and mice, such knowledge is limited for ruminants.

The findings in this thesis add new information regarding the pre-ruminant gut microbiome. Further, this thesis generates fundamental knowledge regarding the microbial establishment process in the rumen and small intestine, which may be crucial for cattle gut health. Recent studies have demonstrated the existence of region-specific microbiota throughout the gastrointestinal tract (Malmuthuge et al., 2014) that cannot be reflected by fecal microbiome profiling (Romano-Keeler et al., 2014). The region-specific microbiota modulate the intestinal transcriptome in a region-specific manner

(Sommer et al., 2015). Hence, studying host-microbial interactions demands the use of regional sampling relevant to the host phenotype. Thus, this study has advantages over studies of early microbial colonization in human infants, which are based mainly on the analysis of fecal microbiota. In contrast to human infants, the calf small intestine is observed to be colonized with a unique microbial population that differs from the maternal and environmental microbiota at birth (chapter 2). Moreover, the feeding of heat-treated colostrum within an hour after birth facilitated rapid colonization of the small intestine by *Bifidobacterium* within the first six hours of life (chapter 3). Thus, these findings suggest that good management practices are critical for optimal gut microbiota establishment during early life. The observed postnatal variations in the small intestinal microbiota revealed the establishment of an individualized microbiome in each animal; however, the calves could be categorized based on the relative abundances of microbial taxonomy and functions (chapter 4). In contrast to the small intestinal microbiota, the pre-ruminant rumen microbiota consisted of bacterial genera found primarily in the maternal (birth canal and rectum) environments (chapter 5). However, the relative abundance of phylotypes was different between the calf rumen and maternal communities (birth canal and rectum). The postnatal changes in the rumen microbial taxonomy and functions revealed involvement in rumen tissue metabolism and epithelial development. Thus, this thesis addressed a major knowledge gap on the pre-ruminant gut microbiome by revealing important details on gut microbial acquisition and succession in newborn dairy calves. This knowledge provides a basis for future studies and the development of microbial manipulation tools and techniques to improve cattle production and health.

6.2 Understanding gut microbial composition at birth

The *in utero* sterility of the gastrointestinal tract is generally accepted, despite reports that microbiota are present in the umbilical cord blood of mice (Jimenez et al., 2005) and the human placenta (Aagaard et al., 2014). Thus, colonization by gut microbiota is believed to begin during and after the birthing process. The gut microbial composition at birth has, however, been studied primarily using first-pass meconium in human infants. However, the first passing of meconium can vary between 0 hours and one day after birth (Hansen et al., 2015). Therefore, the meconium may not be representative of the gut microbiota at birth. Moreover, evidence is accumulating regarding site-specific microbial colonization throughout the gastrointestinal tract of mammals (Malmuthuge et al., 2014; Romano-Keeler et al., 2014; Sommer et al., 2015). Thus, studying the gut microbiota at birth is challenging, and existing knowledge based on fecal samples may be of limited relevance. This thesis (chapter 2) characterized the small intestinal microbiome using luminal and epimural samples collected from calves soon (less than five minutes) after birth. These calves were separated from their dams immediately and were not allowed to interact with the dam or calving pen environment. Hence, the samples used in this research are representative of microbiota colonization during the birthing process.

In contrast to human studies reporting similarities between the mother and infant gut microbiomes at birth using fecal samples (Dominguez-Bello et al., 2010; Backhead et al., 2015), this research (chapter 2) revealed that the calf small intestine was colonized by a unique microbial population that differed from the maternal microbiota (birth canal and rectal communities). The maternal microbial communities contained primarily bacterial

families such as *Streptococcaceae*, *Ruminococcaceae*, *Clostridiaceae*, *Lachnospiraceae*, *Peptostreptococcaceae* and *Bacteroidaceae*. However, the small intestinal communities of newborn calves consisted predominantly of *Pseudomonadaceae*, *Propionibacteriaceae* and *Ruminococcaceae. Clostridiaceae*, *Bacteroidaceae* and *Lachnospiraceae* families were also observed in the calf gut at birth, but their abundance was less than those observed in the maternal communities.

Understanding of *Bifidobacterium* as potential probiotic bacteria for use to prevent neonatal calf diarrhea (Abe et al., 1995) is limited. However, *Bifidobacterium* has been reported to be predominant throughout the calf gut (Rada et al., 2006; Vlkova et al., 2006). Therefore, in addition to the profiling of intestinal microbiota using 454 sequencing, this research sequenced 16S clone libraries to analyze the composition of *Bifidobacterium* in the calf ileum at birth. Although the majority of *Bifidobacterium* could not be identified at the species level, *B. longum* subsp. *infantis* dominated the calf ileum at birth. When the *Bifidobacterium* species in the maternal microbiomes were compared to the calf microbiomes using species-specific primers, the identified species differed between the dams and calves. The maternal communities were positive for *B. pseudolongum* and *B. longum* but not *B. longum* subsp. *infantis*. An absence of *B. longum* subsp. *infantis* in the adult gut has also been reported in other mammals, including humans (Lamendella et al., 2008), possibly due to a population density below the detection limit of PCR. I speculate that this strictly anaerobic bacterium may be vertically transmitted from dam to calf, and the favorable growth conditions in the calf intestine (mucin-glycans) may preferentially enhance their growth relative to the other detected *Bifidobacterium* species. Thus, the developmental differences between the newborn calf

and the maternal gastrointestinal tract may contribute to the observed microbial composition variations at birth. This difference may be very important when microbial manipulation techniques and tools are implemented to alter early colonization.

6.3 Understanding the effect of colostrum feeding practices on gut microbiota

Colostrum management is one of the most important aspects of early calf management (Godden, 2008). Bovine colostrum is rich in immunoglobulin G (IgG) (Korhonen et al., 2000), which transfers passive immunity to the newborn calves (Godden, 2008). Previous research on colostrum feeding has focused primarily on the successful transfer of maternal immunity. The findings of this thesis (chapter 3) revealed that colostrum might also be important for gut microbial colonization. The feeding of colostrum (fresh or heat-treated) soon after birth (within the first hour of life) increased the bacterial population density in the calf small intestine within 12 hours of birth, compared to calves not receiving colostrum. This suggests that the timed feeding of colostrum is also crucial for the colonization of microbiota. Heat-treated colostrum feeding has recently been introduced as a good management practice by the industry to improve the successful transfer of IgG from dam to calf (Godden et al., 2012; Gelsinger et al., 2014). Heat-treated colostrum has been shown to decrease neonatal calf diarrhea (Godden et al., 2012); however, the mechanism of this reduction is not clear. The feeding of heat-treated colostrum enhanced colonization by *Bifidobacterium* within six hours after birth compared to the feeding of fresh colostrum. Moreover, it decreased colonization by potential pathogens (*Escherichia coli*) in the small intestine during the first 12 hours post-partum. The increased *Bifidobacterium* population in calves fed heat-

treated colostrum might have prevented colonization by potential enteropathogens. This study only evaluated the effect of colostrum feeding on bacterial densities. Therefore, future studies are necessary to understand the impact of colostrum on microbial composition. Moreover, future studies may provide in-depth understanding of the mechanism(s) mediating changes in the epimural *Bifidobacterium* population under different regimens of colostrum feeding.

6.4 Understanding postnatal changes in small intestinal microbiome

The small intestinal microbiota, which is different from the large intestinal (Malmuthuge et al., 2014; Sommer et al., 2015) and fecal microbiomes (Romano-Keeler et al., 2014), maintains close interactions with the host mucosal immune system and regulates the epithelial transcriptome in a site-specific manner (Sommer et al., 2015). However, our current understanding of the calf small intestinal microbiota is very limited. Profiling the small intestinal microbial composition of newborn calves in this thesis revealed the establishment of a microbial composition that was unique to each animal during the first six weeks of life. Despite the substantial variation observed in taxonomic composition, some of the newborn calves could be grouped into two types based on the abundance and composition of the bacterial genera. One group was dominated by *Lactobacillus* (*Lactobacillus*-dominant calves) and the other by *Bacteroides* (*Bacteroides*-dominant calves). The ileal transcriptome of the *Lactobacillus*-dominant calves was enriched with pro-inflammatory chemokines (CXCL9, CXCL10, CXCL11) compared to *Bacteroides*-dominant calves. These chemokines are known to stimulate Th1 responses in epithelial cell lines upon exposure to *L. acidophilus* (Weiss et al., 2010;

O'Flaherty and Klaenhammer, 2012). *L. acidophilus* downregulates the expression of CXCL10 and CXCL11 in infectious mouse models (Gabryszewski et al., 2011; von Schillde et al., 2012). Thus, these findings may suggest that during microbial establishment, *Lactobacillus* can prime the mucosal immune responses and prepare the mucosal immune system to better respond to pathogenic infections during the first six weeks of life. This process may be a crucial step in mucosal immune system development and may play a role in preventing enteric infections in neonatal calves. Thus, in future work, comparing healthy and diseased calves and linking them to host phenotypes (weight gain, fecal scores, fecal shedding of enteropathogens) may help to determine whether *Lactobacillus* can serve as a microbial marker/enterotype.

The *Bacteroides*-dominant calves displayed enrichment in MAPK cascades, especially the ERK1/ERK2 (extracellular signal-related kinases) pathway. The ERK1/ERK2 pathway has been associated with increased Th2 cell differentiation (Yamashita et al., 1999), which is associated with allergic reactions and the recognition of extracellular pathogens (Wu and Wu, 2012). A study on the human gut metagenome has also suggested that *Bacteroides* species predominate in the gut metagenome of low-gene-count individuals (Le Chatelier et al., 2013). The microbial functions of these low-gene-count individuals were biased toward handling oxidative stress, compared to high-gene-count individuals dominated by *Bifidobacterium*, *Lactobacillus*, and *Faecalibacterium* (Le Chatelier et al., 2013). For example, the dissimilatory nitrate reduction-related microbial functions were higher in low-gene-count individuals than in high-gene-count individuals. In this study, *Bacteroides*-dominant calves had a higher abundance of functions related to "phages, prophages", "nitrogen metabolism", and "iron

acquisition" than *Lactobacillus*-dominant calves. Furthermore, a lower number of microbial functions (level 2 functions in the SEED hierarchy) were detected in the ileum (114±2) of *Bacteroides*-dominant calves than *Lactobacillus*-dominant calves (129±4). Therefore, future studies are necessary to understand the reasons behind the enrichment of virulence-associated microbial functions in *Bacteroides*-dominant gut communities and whether *Bacteroides* occur with other inflammation-associated microbiota.

The clusters based on the microbial functions revealed an antagonism between *Bifidobacterium* and SRB colonization in the calf small intestine. This antagonistic relationship between *Bifidobacterium* and SRB suggested potential differences in the intestinal barrier functions. *Bifidobacterium* and SRB compete for cysteine (Carbonero et al., 2012; Ferrario et al., 2015), and the absence of cysteine inhibits the growth of *Bifidobacterium* (Ferrario et al., 2015). *Bifidobacterium* has been shown to strengthen the epithelial barrier (Ulluwishewa et al., 2011; Hsieh et al., 2015). In contrast, the use of cysteine by SRB produces H_2S, which interrupts the butyrate utilization pathway in epithelial cells (Carbonero et al., 2012), resulting in disruption of the epithelial barrier. However, the transcriptome data did not reveal a difference in the expression of tight junction genes between the two function-based clusters. Thus, future research is necessary to validate our speculation on the changes in epithelial barrier functions by measuring the barrier integrity.

6.5 Understanding the role of microbiota in rumen development in neonatal calves

Recently, several studies analyzed the pre-ruminant rumen microbial composition and the postnatal colonization process in the rumen (Li et al., 2012; Jami et al., 2013;

Abecia et al., 2014; Rey et al., 2014). Dietary manipulations of the early rumen microbiota have also been shown to influence post-weaning rumen functions (Abecia et al., 2013), suggesting a long-term effect by changing the pre-ruminant rumen microbiota. The adult rumen microbiota is highly resistant to manipulation and quickly resumes its original status following any manipulation (Weimer, 2015). In contrast, the unstable and evolving pre-ruminant rumen microbiota may be more affected by dietary interventions. Thus, it has been suggested that the pre-weaning period may offer a better window of opportunity for microbial manipulations to improve rumen fermentation (Yanez-Ruiz et al., 2015). However, our understanding of the host-microbial interactions in the pre-ruminant rumen remains limited. The integrated analysis of microbial metagenomes with host transcriptomes (mRNA and miRNA profiles) in this thesis (chapter 5) revealed that early rumen microbiota were involved in rumen tissue metabolism and papillae development through interactions with the transcriptome and microRNAome. Sommer et al., (2015) reported that only 10% of the small intestinal transcriptome was regulated by its microbiota in mice. This thesis also revealed that a minority of host genes (26.3%) was modulated by the rumen microbiota, and the majority of transcriptomic changes were ontogenic. However, the proportion of microbiota-driven miRNAs (46.4%) was higher than the proportion of microbiota-driven mRNAs, suggesting that miRNAs may be more responsive to the early microbiota. This research is the first to reveal the three-way interactions among the microbiome, transcriptome and microRNAome of neonatal calves. One of the identified three-way interactions suggested bacteria-driven transcriptional regulation (zinc finger protein related functions) via miRNAs during the early rumen development. Zinc plays an important role in the rumen papillae development and

keratinization in goat kids (Cernik et al., 2013). Therefore, the role of the early microbiome in modulating rumen development through zinc absorption needs to be further investigated. In addition to these three-way interactions, I also identified microbial metabolites (VFAs) associated with the expression of host protein coding genes. The increasing concentration of butyrate with increasing calf age was associated with an upregulation of *PPARG*, which modulates epithelial barrier functions (Ogasawara et al., 2010) and inflammation (Annese et al., 2012). Therefore, this observation suggests that the early microbiome may also play a role in the development of the rumen epithelial barrier in newborn calves. Epithelial barrier functions are important in pre-weaned calves during the weaning transition, as the intake of concentrate diet can affect the rumen epithelium through decreased ruminal pH (Aschenbach et al., 2000). A higher individual animal variation in rumen microbial composition has been reported during early life compared to the variation reported for adults (Jami et al., 2013). However, this thesis used different individuals to understand the microbial regulation of the rumen development during the first six weeks of life. Thus, the repeated sampling of the rumen microbiome and epithelium throughout the pre-weaning period of calves may provide a better understanding of the interactions between the host and the rumen microbiota. Moreover, it is well known that solid feed intake is crucial for the development of the rumen epithelium (Heinrichs, 2005). In this study, calf feed intake was not monitored, which limits the analysis of the role of this potentially important factor. The high individual animal variation in the microbial profiles of the 3W and 6W calves may be due in part to the variation in calf starter intake.

6.6 Implications and future directions

The findings of this thesis provide new knowledge regarding calf gut microbial establishment from birth to six weeks of life. The negative impact of *Bifidobacterium* on the colonization of potentially pathogenic *E. coli* suggests the use of these bacteria as a probiotic in newborn calves to prevent pathogen colonization. The use of regional samples revealed that the establishment of region-specific microbiota began during birth. This information may be crucial for developing effective microbial manipulation techniques to improve rumen fermentation and/or gut health later in life. The microbial markers identified in chapter 4, which are associated with the differential expression of genes related to mucosal immunity, may provide a new framework for the identification of calves resistant to neonatal calf diarrhea or other enteric infections. However, these taxonomic and function-based groupings require further validation in larger populations and by comparing infected and healthy calves. Our study highlights the importance of the early rumen microbiome for rumen development, which may then facilitate the transition from pre-ruminant to ruminant. The current observations indicate that further research is required to understand the role of VFA production in the pre-ruminant rumen in terms of calf nutrition and gut development. Such understanding will support the design of new calf management strategies to improve the weaning transition. Furthermore, I used RNA-based quantitative real-time PCR, a novel approach to quantifying alive/active microbiota, instead of the widely used DNA-based quantification. Our RNA-based density estimations revealed that past studies may have over-estimated microbial densities through DNA-based approaches that amplified both dead and alive microbiota. Therefore, our approach adds a new dimension to existing microbial quantification

methods that can be used in future work to provide more accurate information on microbial populations. In addition to fundamental knowledge on the calf gut microbiome, the information generated by our study suggests a new appreciation for the importance of colostrum feeding in newborn calves, which is of direct relevance to the industry. Our study on the effects of colostrum feeding suggested that the feeding of heat-treated colostrum, a suggested good management practice to improve the passive transfer of immunity (Godden et al., 2012), also has a major impact on early microbial colonization. The probiotic effect of colostrum requires further investigation to identify potential long-term benefits.

Comparing the newborn gut microbiome (rumen and small intestine) to the microbiome of one-week-old calves, as well as the significant changes in bacterial densities observed within the first 12 hours of life, suggests that the first week of life is a very dynamic time for microbial establishment. There are significant differences in the microRNAome (Liang et al., 2014) and transcriptome (Liang et al., In Press) when comparing newborn and one-week-old calves. Therefore, future studies to understand the host-microbial interactions during this critical period of life may provide a greater understanding of the role of early colonizers in gut development. This study used 16S amplicon sequencing and metagenomic approaches to explore the gut microbiome; however, these approaches may not provide a full understanding of the postnatal dynamics of alive/active microbiota and the functionality of the rumen microbiome. Thus, a metatranscriptomics-based approach would be a more appropriate tool to study the gut microbiome in future studies. Finally, as addressed throughout this chapter, future studies using challenged and/or infectious disease models may be useful for validating

the newly identified microbial markers and monitoring the effects of microbial manipulation techniques intended to improve ruminant production and health.

6.7 References

Aagaard, K., Ma, J., Antony, K.M., Ganu, R., Petrosino, J., Versalovic, J. (2014). The placenta harbors a unique microbiome. Sci. Transl. Med. 6, 237ra65. doi: 10.1126/scitranslmed.3008599.

Abe, F., Ishibashi, N., Shimamura, S. (1995). Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Dairy Sci. 73, 2838-2846.

Abecia, L., Martin-Garcia, A.I., Martinez-Fernandez, G., Newbold, C.J., Yanez-Ruiz, D.R. (2013) Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. J. Anim. Sci. 91, 4832-4840. doi: 10.2527/jas.2012-6142.

Abecia. L., Ramos-Morales, E., Martinez-Fernandez, G., Arco, A., Martin-Garcia, A.I., Newbold, C.J. et al., (2014) Feeding management in early life influences microbial colonization and fermentation in the rumen of newborn goat kids. Animal Production Science. 54, 1449-1454. http://dx.doi.org/10.1071/AN14337.

Annese, V., Rogai, F., Settesoldi, A., Bagnoli, S. (2012). PPARγ in inflammatory bowel disease. PPAR Res. 2012, Article ID 620839. http//dx.doi.org/10.1155/2012/620839.

Arrieta, M.C., Stiemsma, L.T., Amenyogbe, N., Brown, E.M., Finlay, B. (2014) The intestinal microbiome in early life: health and disease. Front. Immunol. 5, 427. doi: 10.3389/fimmu.2014.00427.

Aschenbach, J. R., Gabel, G. (2000). Effect and absorption of histamine in sheep rumen: significance of acidotic epithelial damage. J. Anim. Sci. 78, 464-470.

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al., (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbe.17, 852. doi: 10.1016/j.chom.2015.05.012.

Carbonero, F., Benefiel, A.C., Alizadeh-Ghamsari, A.H., and Gaskins, H.R. (2012) Microbial pathways in colonic sulfur metabolism and links with health and disease Front Physiol. 3:448. doi: 10.3389/fphys.2012.00448.

Cernik, J., Pavlata, L., Misurova, L., Jokverova, O., Lunacek, J., Halouzka, R. (2013). Effect of peroral supplementation of zinc on the ruminal mucosa of goat kids - a morphometric study. Acta. Vet. Brno. 82, 399-403.

Chung, H., Pamp, S.J., Hill, J.A., Surana, N.K., Edelman, S.M., Troy, E.B., et al., (2012). Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 149, 1578-1593. doi: 10.1016/j.cell.2012.04.037.

Dogra, S., Sakwinska, O., Soh, S-E., Ngom-Bru, C., Bruck, W.M., Berger, B., et al., (2014). Dynamics of infant gut microbiota are influenced by delivery mode and gestational duration and are associated with subsequent adiposity. mBio. 6, e02419-14doi: 10.1128/mBio.02419-14.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., et al., (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A. 107, 11971-11975. doi: 10.1073/pnas.1002601107.

Fanaro, S., Chierici, R., Guerrini, O., Vigi, V. (2003). Intestinal microflora in early infancy: Composition and development. Acta Paediatr. Suppl. 91, 48-55.

Ferrario, C., Duranti, S., Milani, C., Mancabelli, L., Lugli, GA., Turroni, F., et al., (2015) Exploring amino acid auxotrophy in *Bifidobacterium bifidum* PRL2010. Front Microbiol 6:1331. doi: 10.3389/fmicb.2015.01331.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al., (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 469, 543-547. doi: 10.1038/nature09646.

Gelsinger, S.L., Jones, C.M., Heinrichs, A.J. (2015). Effect of colostrum heat treatment and bacterial population on immunoglobulin G absorption and health of neonatal calves. J Dairy Sci. 98, 4640-4645. doi: 10.3168/jds.2014-8790.

Godden, S. (2008) Colostrum management for dairy calves. Vet. Clin. North Am. Food. Anim. Pract. 24,19-39. doi: 10.1016/j.cvfa.2007.10.005.

Godden, S.M., Smolenski, D.J., Donahue, M., Oakes, J.M., Bey, R., Wells, S. et al., (2012). Heat-treated colostrum and reduced morbidity in preweaned dairy calves: Results of a randomized trial and examination of mechanisms of effectiveness. J. Dairy Sci. 95, 4029-4040. doi: 10.3168/jds.2011-5275.

Hansen, R., Scott, K.P., Khan, S., Martin, J.C., Berry, S.H., Stevenson, M., et al., (2015). First-pass meconium samples from healthy term vaginally-delivered neonates: an analysis of the microbiota. PLoS One. 10, e0133320. doi: 10.1371/journal.pone.0133320.

Heinrichs, J. (2005). Rumen development in the dairy calf. Advances in Dairy Technology. 17, 179-187.

Hsieh, C.Y., Osaka, T., Moriyama, E., Date, Y., Kikuchi, J., and Tsuneda, S. (2015) Strengthening of the intestinal epithelial tight junction by *Bifidobacterium bifidum*. Physiol Rep 3:e12327. doi: 10.14814/phy2.12327.

Jami, E., Israel, A., Kotser, A., Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7, 1069-1079. doi: 10.1038/ismej.2013.2.

Jimenez, E., Fernandez, L., Marin, M.L., Martin, R., Odriozola, J.M., Nueno-Palop, C., et al., (2005). Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. Curr Microbiol. 51, 270-274.

Korhonen, H., Marnila, P., Gill, H.S. (2000). Milk immunoglobulins and complement factors. B. J. Nutr. 84, 75-80. doi: http://dx.doi.org/10.1017/S0007114500002282.

Lamendella, R., Domingo, J.W.S., Kelty, C., Oerther, D.B. (2008). Bifidobacteria in feces and environmental waters. Appl. Environ. Microbiol. 74, 575-584. doi: 10.1128/AEM.01221-07.

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., et al., (2013). Richness of human gut microbiome correlates with metabolic markers. Nature. 500, 541-546. doi: 10.1038/nature12506.

Li, R.W., Connor, E.E., Baldwin, R.L., Sparks, M.L. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environ. Microbiol. 14, 129-139. doi: 10.1111/j.1462-2920.2011.02543.

Liang, G., Malmuthuge, N., Bao, H., McFadden, T., Stothard, P., Griebel, P.J., et al., (2014) Potential regulatory role of microRNAs in the development of bovine

gastrointestinal tract during early life. PLoS One. 9, e92592. doi: 10.1371/journal.pone.0092592.

Malmuthuge, N., Griebel, P.J, Guan, L.L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of pre-weaned calves. Appl. Environ. Microbiol. 80, 2012-2028. doi: 10.1128/AEM.03864-13.

Mazmanian, S.K., Liu, C.H., Tzianabos, A.O., Kasper, D.L. (2005). An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 122, 107-118.

Mesquita, D.N., Barbieri, M.A., Goldani, H.A.S., Cardoso, V.C., Goldani, M.Z., Kac, G. et al., (2013). Cesarean section is associated with increased peripheral and central adiposity in young adulthood: cohort study. PLoS One. 8, e66827. doi: 10.1371/journal.pone.0066827.

O'Flaherty, S., and Klaenhammer, T.R. (2012). Influence of exposure time on gene expression by human intestinal epithelial cells exposed to *Lactobacillus acidophilus*. Appl Environ Microbiol 78:5028-5032. doi: 10.1128/AEM.00504-12.

Ogasawara, N., Kojima, T., Go, M., Ohkuni, T., Koizumi, J., Kamekura R, et al., (2010). PPARgamma agonists upregulate the barrier function of tight junctions via a PKC pathway in human nasal epithelial cells. Pharmacol Res. 61, 489-498.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. J. Appl. Microbiol. 116, 245-257. doi: 10.1111/jam.12405.

Romano-Keeler, J., Moore, D.J., Wang, C., Brucker, R.M., Fonnesbeck, C., Slaughter, J.C. et al., (2014). Early life establishment of site-specific microbial communities in the gut. Gut Microbes. 5, 192-201. doi: 10.4161/gmic.28442.

Sommer, F., Nookaew, I., Sommer, N., Fogelstrand, P., Backhed, F. (2015). Site-specific programming of the host epithelial transcriptome by the gut microbiota. Genome Biol. 16, 62. doi: 10.1186/s13059-015-0614-4.

Subramanian, S., Blanton, L.V., Frese, S.A., Charbonneau, M., Mills, D.A., Gordon, J.I. (2015). Cultivating healthy growth and nutrition through the gut microbiota. Cell. 161, 36-48.

ten Bruggencate, S.J., Bovee-Oudenhoven, I.M., Feitsma, A.L., van Hoffen, E., Schoterman, M.H. (2014). Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. Nutr. Rev. 72, 377-389. doi: 10.1111/nure.12106.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., et al., (2009). A core gut microbiome in obese and lean twins. Nature 457:480-484. doi: 10.1038/nature07540.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., and Roy, N.C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr 141:769-776. doi: 10.3945/jn.110.135657.

Underwood, M.A., German, J.B., Lebrilla, C.B., Mills, D.A. (2015). *Bifidobacterium longum* subspecies *infantis*: champion colonizer of the infant gut. Pediatr Res. 77, 229-235. doi: 10.1038/pr.2014.156.

von Schillde, M.A., Hormannsperger, G., Weiher, M., Alpert, C.A., Hahne, H., Bauerl, C., et al., (2012). Lactocepin secreted by *Lactobacillus* exerts anti-inflammatory effects by selectively degrading proinflammatory chemokines. Cell Host Microbe 11:387-396. doi: 10.1016/j.chom.2012.02.006.

Weimer, P.J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. Front. Microbiol. 6, 296. doi: http://dx.doi.org/10.3389/fmicb.2015.00296.

Weiss, G., Rasmussen, S., Nielsen Fink, L., Jarmer, H., Nohr Nielsen, B., and Frokiaer, H. (2010). *Bifidobacterium bifidum* actively changes the gene expression profile induced by *Lactobacillus acidophilus* in murine dendritic cells. PLoS One 5:e11065. doi: 10.1371/journal.pone.0011065.

Wu, H-J., Wu, E. (2012). The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes. 3, 4-14. doi: 10.4161/gmic.19320.

Yanez-Ruiz, D.R., Abecia, L., Newbold, C.J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: a review Front Microbiol. 6, 1133. doi: 10.3389/fmicb.2015.01133.

Bibliography

Aagaard, K., Ma, J., Antony, K.M., Ganu, R., Petrosino, J., Versalovic, J. (2014). The placenta harbors a unique microbiome. Sci. Transl. Med. 6, 237ra65. doi: 10.1126/scitranslmed.3008599.

Abe, F., Ishibashi, N., Shimamura, S. (1995). Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Dairy Sci. 73, 2838-2846.

Abecia, L., Martin-Garcia, A.I., Martinez-Fernandez, G., Newbold, C.J., Yanez-Ruiz, D.R. (2013) Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. J. Anim. Sci. 91, 4832-4840. doi: 10.2527/jas.2012-6142.

Abecia, L., Waddams, K.E., Martinez-Fernandez, G., Martin-Garcia, A.I., Ramos-Morales, E., Newbold, C.J., Yanez-Ruiz, D.R. (2014a). An antimethanogenic nutritional intervention in early life of ruminants modifies ruminal colonization by archaea. Archaea. 2014, Article ID 841463. doi: http://dx.doi.org/10.1155/2014/841463.

Abecia. L., Ramos-Morales, E., Martinez-Fernandez, G., Arco, A., Martin-Garcia, A.I., Newbold, C.J. et al., (2014b) Feeding management in early life influences microbial colonization and fermentation in the rumen of newborn goat kids. Animal Production Science 54, 1449-1454. http://dx.doi.org/10.1071/AN14337.

Adlerberth, I., Wold, A.E. (2009). Establishment of the gut microbiota in western infants. Acta. Paediatr. 98, 229-238. doi: 10.1111/j.1651-2227.2008.01060.

Al-Saidy, M.Y. (2010). Effect of probiotic bacteria on immunoglobulin G concentration and other blood components of newborn calves. Journal of Animal and Veterinary Advances. 9, 604-609. doi:10.3923/java.2010.604.609.

Annese, V., Rogai, F., Settesoldi, A., Bagnoli, S. (2012). PPARγ in inflammatory bowel disease. PPAR Res. 2012, Article ID 620839. http//dx.doi.org/10.1155/2012/620839.

Arrieta, M.C., Stiemsma, L.T., Amenyogbe, N., Brown, E.M., Finlay, B. (2014) The intestinal microbiome in early life: health and disease. Front. Immunol. 5, 427. doi: 10.3389/fimmu.2014.00427.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R. et al., (2011). Enterotypes of the human gut microbiome. Nature. 473, 174-180. doi: 10.1038/nature09944.

Aschenbach, J. R., Gabel, G. (2000). Effect and absorption of histamine in sheep rumen: significance of acidotic epithelial damage. J. Anim. Sci. 78, 464-470.

Azad, M.B., Konya, T., Maughan, H., Guttman, D.S., Field, C.J., Chari, R.S., et al., (2013). Gut microbiota of healthy Canadian infants: profiles by mode of delivery and infant diet at 4 months. CMAJ. 185, 385-394. doi: 10.1503/cmaj.121189.

Babidge, W., Millard, S., Roediger, W. (1998). Sulfides impair short chain fatty acid beta-oxidation at acyl-CoA dehydrogenase level in colonocytes: implications for ulcerative colitis. Mol. Cell Biochem. 181, 117-124.

Backhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P., et al., (2015). Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbe.17, 852. doi: 10.1016/j.chom.2015.05.012.

Baldwin, R.L., McLeod, K.R., Klotz, J.L., Heitmann, R.N. (2004). Rumen development, intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. J. Dairy Sci. 87, E55-E65.

Balleste, E., Blanch, A.R. (2011). *Bifidobacterium* diversity and the development of new microbial source tracking indicators. Appl. Environ. Microbiol. 77, 3518-3525.

Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim, J., et al., (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proc. Natl. Acad. Sci. U S A. 107, 18933-18938. doi: 10.1073/pnas.1007028107.

Benjamini, Y., Hochberg, y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological). 57, 289-300.

Berge, A.C.B., Moore, D.A., Besser, T.E., Sischo, W.M. (2009). Targeting therapy to minimize antimicrobial use in preweaned calves: Effects on health, growth, and treatment costs. J. Dairy Sci. 92, 4707-4714. doi:10.3168/jds.2009-2199.

Betsholtz, C., Karlson, L., Lindhal, P. (2001). Developmental roles of platelet-derived growth factors. BioEssays. 23,494-507.

Bionaz, M., Chen, S., Khan, M.J., Loor, J.J. (2013). Functional role of PPARs in ruminants, Potential targets for fine-tuning metabolism during growth and lactation. PPAR Res 2013, Article ID 684159. http,//dx.doi.org/10.1155/2013/684159.

Brooks, B., Firek, B.A., Miller, C.S., Sharon, I., Thomas, B.C., Baker, R., et al., (2014). Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants. Microbiome. 2, 1. 10.1186/2049-2618-2-1.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., et al., (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7, 335-336. doi: 10.1038/nmeth.f.303.

Carbonero, F., Benefiel, A.C., Alizadeh-Ghamsari, A.H., Gaskins, H.R. (2012). Microbial pathways in colonic sulfur metabolism and links with health and disease. Front. Physiol. 3, 448. doi: 10.3389/fphys.2012.00448.

Cernik, J., Pavlata, L., Misurova, L., Jokverova, O., Lunacek, J., Halouzka, R. (2013). Effect of peroral supplementation of zinc on the ruminal mucosa of goat kids - a morphometric study. Acta. Vet. Brno. 82, 399-403. http://dx.doi.org/10.2754/avb201382040399.

Chakravorty, S., Helb, D., Burday, M., Connell, N., Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J. Microbiol. Methods. 69, 330-339.

Chambers, L., Yang, Y., Littier, H., Ray, P., Zhang, T., Pruden, A. et al., (2015). Metagenomic analysis of antibiotic resistance genes in dairy cow feces following therapeutic administration of third generation cephalosporin. PLoS One. 10, e0133764. doi: 10.1371/journal.pone.0133764.

Chanter, N., Hall, G.A. Bland, A.P., Hayle, A.J., Parsons, K.R. (1984). Dysentery in calves caused by an atypical strain of *Escherichia coli* (S102-9). Vet. Microbiol. 12, 241- 253. doi:10.1016/0378-1135(86)90053-2.

Chigerwe, M., Tyler, J.W., Schultz, L.G., Middleton, J.R., Steevens, B.J., Spain, J.N. (2008). Effect of colostrum administration by use of oroesophageal intubation on serum IgG concentrations in Holstein bull calves. Am. J. Vet. Res. 69, 1158–1163. doi: 10.2460/ajvr.69.9.1158.

Cho, S.J., Kim, H., Yun, H.D., Cho, K.M., Shin, E.C., Lim, W.J. et al., (2006). 16S rDNA analysis of bacterial diversity in three fractions of cow rumen. J. Microbiol. 16, 92-101.

Chung, H., Pamp, S.J., Hill, J.A., Surana, N.K., Edelman, S.M., Troy, E.B., et al., (2012). Gut immune maturation depends on colonization with a host-specific microbiota. Cell. 149, 1578-1593. doi: 10.1016/j.cell.2012.04.037.

Collado, M.C. and Sanz, Y. (2007). Quantification of mucosa-adhered microbiota of lambs and calves by the use of culture methods and fluorescent in situ hybridization coupled with flow cytometry techniques. Vet. Microbiol. 121, 299-306.

Connor, E.E., Baldwin, R.L., Li, C., Li, R.W., Chung, H. (2013). Gene expression in bovine rumen epithelium during weaning identifies molecular regulators of rumen development and growth. Funct. Integr. Genomics 13, 133-142. doi: 10.1007/s10142-012-0308-x.

Conroy, M.E., Shi, H.N., Walker, W.A. (2009). The long-term health effects of neonatal microbial flora. Curr. Opin. Allergy Clin. Immunol. 9, 197-201. doi: 10.1097/ACI.0b013e32832b3f1d.

De Barbieri, I., Hegarty, R.S., Silveira, C., Gulino, L.M., Oddy, V.H., Gilbert, R.A. et al., (2015). Programming rumen bacterial communities in newborn Merino lambs. Small Ruminant Research. 129, 48-59. doi:10.1016/j.smallrumres.2015.05.015.

De Waele, V., Van den Broeck, F., Huyse, T., McGrath, G., Higgins, I., Speybroeck, N., et al., (2013). Panmictic structure of the Cryptosporidium parvum population in Irish calves: influence of prevalence and host movement. Appl. Environ. Microbiol. 79, 2534-2541. doi: 10.1128/AEM.03613-12.

Delroisse, J-M., Boulvin, A-L., Parmentier, I., Dauphin, R.D., Vandenbol, M., Portetelle, D. (2008). Quantification of *Bifidobacterium* spp. and *Lactobacillus* spp. in rat fecal samples by real-time PCR. Microbiol. Res. 163, 663-670.

Denman, S.E., McSweeney, C.S. (2015). The early impact of genomics and metagenomics on ruminal microbiology. Annu. Rev. Anim. Biosci. 3, 447-465. doi: 10.1146/annurev-animal-022114-110705.

Dillon, S.M., Lee, E.J., Kotter, C.V., Austin, G.L., Dong, Z., Hecht, D.K., et al., (2014). An altered intestinal mucosal microbiome in HIV-1 infection is associated with mucosal and systemic immune activation and endotoxemia. Mucosal Immunol. 7, 983-994. doi: 10.1038/mi.2013.116.

Doepel, L., Bartier, A. (2014). Colostrum management and factors related to poor calf immunity. WCDS Advances in Dairy Technology. 26, 137- 149.

Dogra, S., Sakwinska, O., Soh, S-E., Ngom-Bru, C., Bruck, W.M., Berger, B., et al., (2014). Dynamics of infant gut microbiota are influenced by delivery mode and gestational duration and are associated with subsequent adiposity. mBio. 6, e02419-14doi: 10.1128/mBio.02419-14.

Dominguez-Bello, M.G., Costello, E.K., Contreras, M., Magris, M., Hidalgo, G., Fierer, N., et al., (2010). Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc. Natl. Acad. Sci. U S A. 107, 11971-11975. doi: 10.1073/pnas.1002601107.

Donahue, M., Godden, S.M., Bey, R., Wells, S., Oakes, J.M., Sreevatsan, S., et al., (2012). Heat treatment of colostrum on commercial dairy farms decreases colostrum microbial counts while maintaining colostrum immunoglobulin G concentrations. J. Dairy Sci. 95, 2697–2702. doi: 10.3168/jds.2011-5220.

Fanaro, S., Chierici, R., Guerrini, O., Vigi, V. (2003). Intestinal microflora in early infancy: Composition and development. Acta Paediatr. Suppl. 91, 48-55.

Ferrario, C., Duranti, S., Milani, C., Mancabelli, L., Lugli, GA., Turroni, F., et al., (2015) Exploring amino acid auxotrophy in *Bifidobacterium bifidum* PRL2010. Front. Microbiol. 6:1331. doi: 10.3389/fmicb.2015.01331.

Fonty, G., Gouet, P., Jouany, J.P., Senaud, J. (1987). Establishment of the microflora and aerobic fungi in the rumen of lambs. J. General Microbiol. 133, 1835-1843.

Fonty, G., Gouet, P., Nebout, J.M. (1989). Development of the cellulolytic microflora in the rumen of lambs transferred into sterile isolators a few days after birth. Can. J. Microbiol. 35, 416-422.

Fonty, G., Senaud, J., Jouany, J.P., Gouet, P. (1988). Establishment of ciliate protozoa in the rumen of conventional and conventionalized lambs: influence of diet and management conditions. Can. J. Microbiol. 34, 235-241.

Foughy, F., Guinane, C.M., Hussey, S., Wall, R., Ryan, C.A., Dempsey, E.M. et al., (2012). High-throughput sequencing reveals the incomplete, short-term recovery

of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob. Agents Chemother. 56, 5811-5820. doi: 10.1128/AAC.00789-12.

Francino, M.P. (2014). Early development of the gut microbiota and immune health. Pathogens. 3, 769-790; doi:10.3390/pathogens3030769.

Franzosa, E.A., Morgan, X.C., Segata, N., Waldron, L., Reyes, J., Earl, A.M., (2014). Relating the metatranscriptome and metagenome of the human gut. Proc. Natl. Acad. Sci. U S A. 111, E2329-E2338. doi: 10.1073/pnas.1319284111.

Fries, P., Popwych, Y.I., Guan, L.L., Griebel, P.J. (2011). Age-related changes in the distribution and frequency of myeloid and T cell populations in the small intestine of calves. Cell. Immunol. 271, 428-437. doi:10.1016/j.cellimm.2011.08.012.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., et al., (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature. 469, 543-547. doi: 10.1038/nature09646.

Funkhouser, L.J., Bordenstein, S.R. (2013). Mom knows best: the universality of maternal microbial transmission. PLoS Biol. 11, e1001631. doi: 10.1371/journal.pbio.1001631.

Gabryszewski, S.J., Bachar, O., Dyer, K.D., Percopo, C.M., Killoran, K.E., Domachowske, J.B., et al., (2011). *Lactobacillus*-mediated priming of the respiratory mucosa protects against lethal pneumovirus infection. J. Immunol. 186, 1151-1161. doi: 10.4049/jimmunol.1001751.

Gelsinger, S.L., Jones, C.M., Heinrichs, A.J. (2015). Effect of colostrum heat treatment and bacterial population on immunoglobulin G absorption and health of neonatal calves. J. Dairy Sci. 98, 4640-4645. doi: 10.3168/jds.2014-8790.

Gerdts, V., Babiuk, L.A., van Drunen Littel-van den Hurk, S., Griebel, P.J. (2000) Fetal immunization by a DNA vaccine delivered orally into the amniotic fluid. Nature Medicine. 6, 929-932.

Godden, S., McMartin, S., Feirtag, J., Stabel, J.R., Bey, R., Goyal, S., et al., (2006). Heat treatment of bovine colostrum II: Effects of heating duration on pathogen viability and immunoglobulin G. J. Dairy Sci. 89, 3476–3483.

Godden, S. (2008) Colostrum management for dairy calves. Vet. Clin. North Am. Food. Anim. Pract. 24,19-39. doi: 10.1016/j.cvfa.2007.10.005.

Godden, S.M., Smolenski, D.J., Donahue, M., Oakes, J.M., Bey, R., Wells, S. et al., (2012). Heat-treated colostrum and reduced morbidity in preweaned dairy calves: Results of a randomized trial and examination of mechanisms of effectiveness. J. Dairy Sci. 95, 4029-4040. doi: 10.3168/jds.2011-5275.

Gomez, M. Moles, L., Melgar, A., Ureta, N., Bustos, G., Fernandez, L., et al., (2016). Early gut colonization of preterm infants: effect of enteral feeding tubes. J. Pediatr. Gastroenterol. Nutr. 62, 893-900. doi: 10.1097/MPG.0000000000001104.

Gopal, P.K., Gill, H.S. (2000). Oligosaccharides and glycoconjugates in bovine milk and colostrum. Br. J. Nutr. 84, S69- S74.

Gorka, P., Kowalski, Z.M., Pietrzak, P., Kotunia, A., Jagusiak, W., Zabielski, R. (2011). Is rumen development in newborn calves affected by different liquid feeds and

small intestine development? J. Dairy Sci. 94, 3002-3013. doi: 10.3168/jds.2010-3499.

Griebel, P.J., Hein, W.R. (1996). Expanding the role of Peyer's patches in B-cell ontogeny. Immunol. Today. 17, 30-38.

Griebel, P.J., Kugelberg, B., Ferrari, G. (1996). Two distinct pathways of B cell development in Peyer's patches. Dev. Immunol. 4, 263-277.

Groer, M.W., Luciano, A.A., Dishaw, L.J., Ashmeade, T.L., Miller, E., Gilbert, J.A. (2014). Development of the preterm infant gut microbiome: a research priority. Microbiome. 2, 38. doi: 10.1186/2049-2618-2-38.

Grootjans, J., Thuijls, G., Verdam, F., Derikx, J.P.M., Lenaerts, K., and Buurman, W.A. (2010). Non-invasive assessment of barrier integrity and function of the human gut. World J. Gastrointest. Surg. 2: 61-69. doi: 10.4240/wjgs.v2.i3.61.

Guarner, F. (2006) Enteric flora in health and disease. Digestion. 73, 5-12.

Hall G.A., Reynold, D.J., Chanter, N., Morgan, J.H., Parsons, K.R., Debney, T.A., et al., (1985). Dysentery caused by *Escherichia coli* (S102-9) in calves: natural and experimental disease. Vet. Pathol. 22, 156-163. doi: 10.1177/030098588502200210.

Hansen, C.H.F., Nielsen, D.S., Kverka, M., Zakostelska, Z., Klimesova, K., Hudcovic, T. et al., (2012). Patterns of early gut colonization shape future immune responses of the host. PLoS One. 7, e34043. doi:10.1371/journal.pone.0034043.

Hansen, R., Scott, K.P., Khan, S., Martin, J.C., Berry, S.H., Stevenson, M., et al., (2015). First-pass meconium samples from healthy term vaginally-delivered neonates: an

analysis of the microbiota. PLoS One. 10, e0133320. doi: 10.1371/journal.pone.0133320.

Harmsen, H.J., Wildeboer-Veloo, A.C., Raangs, G.C., Wagendorp, A.A., Klijn, N., Bindels, J.G., et al., (2000). Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J. Pediatr. Gastroenterol. Nutr. 30, 61-67.

Hart, A.L., Lammers, K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., et al., (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. Gut. 53, 1602-1609.

Heinrichs, A.J. (2005). Rumen development in the dairy calf. Adv. Dairy Tech. 17, 179-187.

Hidalgo-Cantabrana, C., Sanchez, B., Milani, C., Ventura, M., Margolles, A., Ruas-Madiedo, P. (2014). Genomic overview and biological functions of exopolysaccharide biosynthesis in *Bifidobacterium* spp. Appl. Environ. Microbiol. 80, 9-18. doi: 10.1128/AEM.02977-13.

Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G.D., et al., (2013). Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. PLoS One. 17, 8:e66019. doi: 10.1371/journal.pone.0066019.

Hooper, L.V. (2004) Bacterial contributions to mammalian gut development. Trends Microbiol. 12, 129-134.

Hsieh, C.Y., Osaka, T., Moriyama, E., Date, Y., Kikuchi, J., and Tsuneda, S. (2015) Strengthening of the intestinal epithelial tight junction by *Bifidobacterium bifidum*. Physiol. Rep. 3, e12327. doi: 10.14814/phy2.12327.

Jakobsson, H.E., Abrahamsson, T.R., Jenmalm, M.C., Harris, K., Quince, C., Jernberg, C., et al., (2014). Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut. 63, 559-566. doi: 10.1136/gutjnl-2012-303249.

Jami, E., Israel, A., Kotser, A., Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7, 1069-1079. doi: 10.1038/ismej.2013.2.

Janke, B.H., Francis, D.H., Collins, J.E., Libal, M.C., Zeman, D.H., Johnson, D.D. (1989). Attaching and effacing *Escherichia coli* infections in calves, pigs, lambs, and dogs. J. Vet. Diagn. Invest. 1, 6-11.

Jaster, E.H. (2005). Evaluation of quality, quantity, and timing of colostrum feeding on immunoglobulin G1 absorption in Jersey calves. J. Dairy Sci. 88, 296-302.

Jiao, J., Huang, J., Zhou, C., Tan, Z. (2015). Taxonomic identification of ruminal epithelial bacterial diversity during rumen development in goats. Appl. Environ. Microbiol. 81, 3502–3509. doi:10.1128/AEM.00203-15.

Jimenez, E., Fernandez, L., Marin, M.L., Martin, R., Odriozola, J.M., Nueno-Palop, C., et al., (2005). Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. Curr. Microbiol. 51, 270-274.

Jimenez, E., Marin, M.L., Martin, R., Odriozola, J.M., Olivares, M., Xaus, J., et al. (2008). Is meconium from healthy newborns actually sterile? Res. Microbiol. 159, 187-193. doi: 10.1016/j.resmic.2007.12.007.

Johansson, M.A., Sjogren, Y.M., Persson, J.O., Nilsson, C., Sverremark-Ekstrom, E. (2011). Early colonization with a group of *Lactobacilli* decreases the risk for

allergy at five years of age despite allergic heredity. PLoS One. 6, e23031. doi: 10.1371/journal.pone.0023031.

Johnson, J.L., Godden, S.M., Molitor, T., Ames, T., Hagman, D. (2007). Effects of feeding heat-treated colostrum on passive transfer of immune and nutritional parameters in neonatal dairy calves. J. Dairy Sci. 90, 5189–5198

Jost, T., Lacroix, C., Braegger, C.P., Chassard, C. (2012). New insights in gut microbiota establishment in healthy breast fed neonates. PLoS One. 7, e44595. doi: 10.1371/journal.pone.0044595.

Juge, N. (2012). Microbial adhesins to gastrointestinal mucus. Trends Microbiol. 20, 30-39.

Kavanaugh, D.W., O'Callaghan, J., Butto, L.F., Slattery, H., Lane, J. Clyne, M., et al., (2013). Exposure of *Bifidobacterium longum* subsp. *infantis* to milk oligosaccharides increases adhesion to epithelial cells and induces a substantial transcriptional response. PLoS One. 8, e67224. doi: 10.1371/journal.pone.0067224.

Khan MA, Lee HJ, Lee WS, Kim HS, Ki KS, Hur TY, et al., (2007). Structural growth, rumen development and metabolic and immune responses of Holstein male calves fed milk through step-down and conventional methods. J. Dairy Sci. 90:3376-3387.

Khan, M.A., Weary, D.M., von Keyserlingk, M.A.G. (2011). Hay intake improves performances and rumen development of calves fed higher quantities of milk. J. Dairy Sci. 94, 3547-3553. doi: 10.3168/jds.2010-3871.

Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L. (2013). TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14, R36. doi: 10.1186/gb-2013-14-4-r36.

Kim, M., Morrison, M., Yu, Z. (2011). Status of the phylogenetic diversity census of ruminal microbiomes. FEMS Microbiol. Ecol. 76, 49-63. doi: http://dx.doi.org/10.1111/j.1574-6941.2010.01029.

Kim, S-W., Suda, W. Kim, S., Oshima, K., Fukuda, S., Ohno, H., et al., (2013). Robustness of gut microbiota of healthy adults in response to probiotic intervention revealed by high-throughput pyrosequencing. DNA Res. 20, 241–253. doi:10.1093/dnares/dst006

Kinoshita, M., Suzuki, Y., Saito, Y. (2002). Butyrate reduces colonic paracellular permeability by enhancing PPARgamma activation. Biochem. Biophys. Res. Commun. 293, 827-831.

Koike, S., Kobayashi, Y. (2009). Fibrolytic rumen bacteria: Their ecology and functions. Asian-Aust. J. Anim. Sci. 22, 131-138. doi: http://dx.doi.org/10.5713/ajas.2009.r.01.

Korhonen, H., Marnila, P., Gill, H.S. (2000). Milk immunoglobulins and complement factors. B. J. Nutr. 84, 75-80. doi: http://dx.doi.org/10.1017/S0007114500002282.

Krehbiel, C.R., Rust, S.R., Zhang, G., Gilliland, S.E. (2003). Bacterial direct-fed microbials in ruminant diets: Performance response and mode of action. J. Anim. Sci. 81, E120-E132.

Kryzer, A.A., Godden, S.M., Schell, R. (2015). Heat-treated (in single aliquot or batch) colostrum outperforms non-heat-treated colostrum in terms of quality and transfer of immunoglobulin G in neonatal Jersey calves. J Dairy Sci. 98, 1870-1877. doi: 10.3168/jds.2014-8387.

Lagier, J-C., Hugon, P., Khelaifia, S., Fournier, P-E., Scola, B.L., Raoult, D. (2015). The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. Clin. Microbiol. Rev. 28, 237-264. doi: 10.1128/CMR.00014-14

Laity, J.H., Lee, B.M., Wright, P.E. (2001). Zinc finger protein, new insight into structural and functional diversity. Curr. Opin. Struc. Biol. 11, 39-46.

Lamendella, R., Domingo, J.W.S., Kelty, C., Oerther, D.B. (2008). Bifidobacteria in feces and environmental waters. Appl. Environ. Microbiol. 74, 575-584. doi: 10.1128/AEM.01221-07.

Lamouse-Smith, E.S., Tzeng, A., Starnbach, M.N. (2011). The intestinal flora is required to support antibody responses to systemic immunization in infants and germfree mice. PLoS One. 6, e27662. doi:10.1371/journal.pone.0027662.

Lane, B.A. and Jesse, B.W. (1997). Effect of volatile fatty acid infusion on development of the rumen epithelium in neonatal sheep. J. Dairy Sci. 80, 740-746.

Langfelder, P., Horvath, S. (2008). WGCNA, An R package for weighted correlation network analysis. BMC Bioinf. 9, 559. doi,10.1186/1471-2105-9-559.

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., et al., (2013). Richness of human gut microbiome correlates with metabolic markers. Nature. 500, 541-546. doi: 10.1038/nature12506.

Ley, R.E., Lozupone, C.A., Hamady, M., Knight, R., Gordon, J.I. (2008). Worlds within worlds: evolution of the vertebrate gut microbiota. Nat. Rev. Microbiol. 6, 776-788.

Li, M., Penner, G.B., Hernandez-Sanabria, E., Oba, M., Guan, L.L. (2009). Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen. J. Appl. Microbiol. 107, 1924-1934. doi: 10.1111/j.1365-2672.2009.04376.

Li, R.W., Connor, E.E., Baldwin, R.L., Sparks, M.L. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environ. Microbiol. 14, 129-139. doi: 10.1111/j.1462-2920.2011.02543.

Li, R.W., Sparks, M.E., Connor, E.E. (2011). Dynamics of the rumen microbiota. In Metagenomics and its applications in agriculture, biomedicine and environmental studies R.W. Li, ed, (Nova Science Publishers, New York, USA) pp.135-164.

Liang, G., Bao, H., McFadden, T., Stothard, P., Griebel, P.J., Guan, L.L. (2014) Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life. PLoS One. 9, e92592. doi: 10.1371/journal.pone.0092592.

Liu, R.Z., Mita, R., Beauliu, M., Gao, Z., Godbout, R. (2010). Fatty acid binding proteins in brain development and disease. Int. J. Dev. Biol. 54, 1229-1239. doi: 10.1387/ijdb.092976rl.

LoCascio, R.G., Desai, P., Sela, D.A., Weimer, B., Mills, D.A. (2010). Broad conservation of milk utilization genes in *Bifidobacterium longum* subsp. *infantis* as revealed by comparative genomic hybridization. Appl. Environ. Microbiol. 76, 7373-7381. doi: 10.1128/AEM.00675-10.

LoCascio, R.G., Ninonuveo, M.R., Freeman, S.L., Sela, D.A., Grimm, R., Lebrilla, C.B., et al., (2007). Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. J. Agric. Food Chem. 55, 8914-8919.

Longhurst, C.M., Jennings, L.K. (1998). Intergrin-mediated cellular transduction. Cell Mol. Life Sci. 54, 514-526.

Lorenz, I., Fagan, J., More, S.J. (2011). Calf health from birth to weaning. II. Management of diarrhoea in pre-weaned calves. Ir. Vet. J. 64, 9. doi: 10.1186/2046-0481-64-9.

Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature. 489, 220-230. doi: 10.1038/nature11550.

Makino, H., Kushiro, A., Ishikawa, E., Muylaert, D., Kubota, H., Sakai, T., et al., (2011). Transmission of intestinal *Bifidobacterium longum* subsp. *longum* strains from mother to infant, determined by multilocus sequencing typing and amplified fragment length polymorphism. Appl. Environ. Microbiol. 77, 6788-6793. doi: 10.1128/AEM.05346-11.

Makino, H., Kushiro, A., Ishikawa, E., Kubota, H., Gawad, A., Sakai, T., et al., (2013). Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota. PLoS One. 8, e78331. doi: 10.1371/journal.pone.0078331.

Maldonado-Gomez, M.X., Lee, H., Barile, D., Lu, M. Hutkins, R.W. (2015). Adherence

inhibition of enteric pathogens to epithelial cells by bovine colostrum fractions. Int. Dairy J. 40, 24-32. doi:10.1016/j.idairyj.2014.08.014.

Malmuthuge, N., Fries, P., Griebel, P.J., Guan, L.L. (2012a). Regional and age dependent changes in gene expression of toll-like receptors and key antimicrobial defence molecules throughout the gastrointestinal tract of dairy calves. Vet. Immunol. Immunopathol. 146, 18-26. doi:10.1016/j.vetimm.2012.01.010.

Malmuthuge, N., Li, M., Chen, Y., Fries, P., Griebel, P.J., Baurhoo, B. et al., (2012b). Distinct commensal bacteria associated with ingesta and mucosal epithelium in the gastrointestinal tracts of calves and chickens. FEMS Microbiol. Ecol. 79, 337-347. doi: 10.1111/j.1574-6941.2011.01220.

Malmuthuge, N., Griebel, P.J., Guan, L.L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of pre-weaned calves. Appl. Environ. Microbiol. 80, 2012-2028. doi: 10.1128/AEM.03864-13.

Malmuthuge, N., Chen, Y., Liang, G., Goonewardene, L.A., Guan, L.L. (2015). Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves. J. Dairy Sci. 98, 8044-8053. doi: 10.3168/jds.2015-9607.

Marquez, C.J. (2014). Calf intestinal health: Assessment and dietary interventions for its improvement. PhD Thesis. University of Illinois. Urbana-Champaign, USA.

Martin, R., Heilig, G.H., Zoetendal, E.G., Smidt, H., Rodriguez, J.M. (2007). Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. J. Appl. Microbiol. 103, 2638-2644.

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., et al., (2002). Development of 16S rRNA-gene-targeted group specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. 68, 5445-5451.

Mazmanian, S.K., Liu, C.H., Tzianabos, A.O., Kasper, D.L. (2005). An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 122, 107-118.

Mazmanian, S.K., Round, J.L., Kasper, D.L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 453, 620-625. doi: 10.1038/nature07008.

McCann, J.C., Wickersham, T.A., Loor, J.J. (2014). High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. Bioinformatics and Biology Insights. 8, 109-125. doi: 10.4137/BBI.S15389.

Meganck, V., Hoflack, G., Opsomer, G. (2014). Advances in prevention and therapy of neonatal dairy calf diarrhoea: a systematical review with emphasis on colostrum management and fluid therapy. Acta. Vet. Scand. 56, 75. doi: 10.1186/s13028-014-0075-x.

Mentschel, J., Leiser, R., Mulling, C., Pfarrer, C., Claus, R. (2001). Butyric acid stimulates rumen mucosa development in the calf mainly by a reduction of apoptosis. Arch. Anim. Nutr. 55, 85-102

Mesquita, D.N., Barbieri, M.A., Goldani, H.A.S., Cardoso, V.C., Goldani, M.Z., Kac, G. et al., (2013). Cesarean section is associated with increased peripheral and central

adiposity in young adulthood: cohort study. PLoS One. 8, e66827. doi: 10.1371/journal.pone.0066827.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E.M., Kubal, M., et al., (2008). The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics. 9, 386 doi: 10.1186/1471-2105-9-386.

Minot, S., Bryson, A., Chehoud, C., Wu, G.D., Lewis, J.D., Bushman, F.D. (2013). Rapid evolution of the human gut virome. Proc. Natl. Acad. Sci. U S A. 110, 12450-12455. doi: 10.1073/pnas.1300833110.

Mollet, C., Drancourt, M., Raoult, D. (1997). rpoB sequence analysis as a novel basis for bacterial identification. Mol Microbiol. 26, 1005-1011.

Morrill, K.M., Conrad, E., Lago, A., Campbell, J., Quigley, J., Tyler, H. (2012). Nationwide evaluation of quality and composition of colostrum on dairy farms in the United States. J. Dairy Sci. 95, 3997-4005. doi: 10.3168/jds.2011-5174.

Moxley, R.A. and Francis, D.H. (1986). Natural and experimental infection with an attaching and effacing strain of *Escherichia coli* in calves. Infect. Immunol. 53, 336-349.

Mulder, I., Schmidt, B., Lewis, M., Delday, M., Stokes, C.R., Bailey, M., et al., (2011). Restricting microbial exposure in early life negates the immune benefits associated with gut colonization in environments of high microbial diversity. PLoS One. 6, e28279. doi: 10.1371/journal.pone.0028279.

Musilova, S., Rada, V., Vlkova, E., Bunesova, V. (2014). Beneficial effects of human milk oligosaccharides on gut microbiota. Benef. Microbes. 5, 273-283. doi: 10.3920/BM2013.0080.

Neis, E.P., Dejong, C.H., Rensen, S.S. (2015). The role of microbial amino acid metabolism in host metabolism. Nutrients. 7, 2930-2946. doi: 10.3390/nu7042930.

Nesser, J-R., Golliard, M., Vedovo, S.D. (1991). Quantitative determination of complex carbohydrates in bovine milk and milk-based infant formulas. J. Dairy Sci. 74, 2860-2871.

Nowacki, M.R. (1993). Cell proliferation in colonic crypts of germ-free and conventional mice - preliminary report. Folia Histochem. Cytobiol. 31, 77–81.

O'Flaherty, S., and Klaenhammer, T.R. (2012). Influence of exposure time on gene expression by human intestinal epithelial cells exposed to *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 78, 5028-5032. doi: 10.1128/AEM.00504-12.

Ogasawara, N., Kojima, T., Go, M., Ohkuni, T., Koizumi, J., Kamekura, R., et al., (2010). PPARgamma agonists upregulate the barrier function of tight junctions via a PKC pathway in human nasal epithelial cells. Pharmacol Res. 61, 489-498. doi: 10.1016/j.phrs.2010.03.002.

Oikonomou, G., Teixeira, A.G., Foditsch, C., Bichalho, M.L., Machado, V.S., Bicalho, R.C. (2013). Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of *Faecalibacterium*

species with health and growth. PLOS One. 8, e63157. doi: 10.1371/journal.pone.0063157.

OMFARA (2011). Optimizing calf survival at birth. Ontario Ministry of Agriculture, Food and Rural Affairs. http://www.omafra.gov.on.ca/english/livestock/dairy/facts/optbirth.htm

Org, E., Parks, B.W., Joo, J.W.J., Emert, B., Schwartzman, W., Kang, e.Y., et al., (2015). Genetic and environmental control of host-gut microbiota interactions. Genome Res. 25, 1558–1569. doi: 10.1101/gr.194118.115

Paparo, L., di Costanzo, M., di Scala, C., Cosenza, L., Leone, L., Nocerino, R., et al., (2014). The influence of early life nutrition on epigentic regulatory mechanisms of the immune system. Nutrition. 6, 4706-4719. doi: 10.3390/nu6114706.

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, S. et al., (2006). Factors influencing the composition of the intestinal microbiota in early infancy. Pediatrics. 118, 511-521.

Penner, G.B., Steele, M.A., Aschenbach, J.R., McBride, B.W. (2011). Ruminant nutrition symposium, Molecular adaptation of ruminal epithelia to highly fermentable diets. J. Anim. Sci. 89, 1108-1119. doi: 10.2527/jas.2010-3378.

Pereira, R.V., Siler, J.D., Ng, J.C., Davis, M.A., Warnick, L.D. (2014). Effect of preweaned dairy calf housing system on antimicrobial resistance in commensal *Escherichia coli*. J Dairy Sci. 97, 7633-7643. doi: 10.3168/jds.2014-8588.

Petersson, J., Schreiber, O., Hansson, G.C., Gendler, S.J., Velcich, A., Lundberg, J.O., et al., (2011). Importance and regulation of the colonic mucus barrier in a mouse

model of colitis. Am. J. Physiol. Gastrointest. Liver Physiol. 300, G327-333. doi: 10.1152/ajpgi.00422.2010.

Petrosino, J.F., Highlander, S., Luna, R.A., Gibbs, R.A., Versalovic, J. (2009). Metagenomic pyrosequencing and microbial identification. Clin. Chem. 55, 856-866. doi: 10.1373/clinchem.2008.107565.

Pounden, W.D. and Hibbs, J.W. (1948). The influence of ration and rumen inoculation on the establishment of certain microorganisms in the rumens of young calves. J. Dairy Sci. 31, 1041-1050.

Pounden, W.D. and Hibbs, J.W. (1949). The influence of pasture and rumen inoculation on the establishment of certain microorganisms in the rumen of young dairy calves. J. Dairy Sci. 32, 1025-1032. doi: http://dx.doi.org/10.3168/jds.S0022-0302(49)92157-8.

Priestley, D., Bittar, J.H., Ibarbia, L., Risco, C.A,, Galvao, K.N. (2013). Effect of feeding maternal colostrum or plasma-derived or colostrum-derived colostrum replacer on passive transfer of immunity, health, and performance of preweaning heifer calves. J. Dairy Sci. 96, 3247-3256. doi: 10.3168/jds.2012-6339.

Purushe, J., Fouts, D.E., Morrison, M., White, B.A., Mackie, R.I. (2010). Comparative genome analysis of *Prevotella ruminicola* and *Prevotella bruantii*, Insights into their environmental niche. Microbial Ecology. 60, 721-729. doi: 10.1007/s00248-010-9692-8.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., et al., (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 464, 59-65. doi:10.1038/nature08821.

Quigley, J.D. (2002). Passive immunity in newborn calves. Advances in Dairy Technology. 14, 273-292.

Rajendhran, J. and Gunasekaran, P. (2011). Microbial phylogeny and diversity: small subunit ribosomal RNA sequence analysis and beyond. Microbiol. Res. 166, 99-110. doi: 10.1016/j.micres.2010.02.003.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 118, 229-241.

Rey, M., Enjalbert, F., Monteils, V. (2012). Establishment of ruminal enzymatic activities and fermentation capacity in dairy calves from birth through weaning. J. Dairy Sci. 95, 1500-1512. http://dx.doi.org/ 10.3168/jds.2011-4902.

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. J. Appl. Microbiol. 116, 245-257. doi: 10.1111/jam.12405.

Reynolds, J.D., Morris, B. (1984). The effect of antigen on the development of Peyer's patches in sheep. Eur. J. Immunol. 14, 1-6.

Rieu, F., Fonty, G., Gaillard, B., Gouet, P. (1990). Electron microscopy study of the bacteria adherent to the rumen wall in young conventional lambs. Can. J. Microbiol. 36, 140-144.

Rincon, M., Flavell, R.A., Davis, R.J. (2001). Signal transduction by MAP kinases in T lymphocytes. Oncogene. 20, 2490-2497.

Rinne, M.M., Gueimonde, M., Kalliomaki, M., Hoppu, U., Salminen, S.J., Isolauri, E., et al., (2005). Similar bifidogenic effects of prebiotic-supplemented partially

hydrolyzed infant formula and breastfeeding on infant gut microbiota. FEMS Immunol. Med. Microbiol. 43, 59-65.

Rodriguez, J.M., Murphy, K., Stanton, C., Ross, R.P., Kober, O.I., Juge, N., et al., (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. Microb. Ecol. Health Dis. 26, 26050. 10.3402/mehd.v26.26050.

Romano-Keeler, J., Moore, D.J., Wang, C., Brucker, R.M., Fonnesbeck, C., Slaughter, J.C. et al., (2014). Early life establishment of site-specific microbial communities in the gut. Gut Microbes. 5, 192-201. doi: 10.4161/gmic.28442.

Round, J.L., Mazmanian, S.K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. 9, 313-323. doi: 10.1038/nri2515.

Russell, S.L., Gold, M.J., Willing, B.P., Thorson, L., McNagny, K.M., Finlay, B.B. (2013). Perinatal antibiotic treatment affects murine microbiota, immune responses and allergic asthma. Gut Microbes. 4, 158-164. doi: 10.4161/gmic.23567.

Sabat, G., Rose, P., Hickey, W.J., Harkin, J.M. (2000). Selective and sensitive method for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. Appl. Environ. Microbiol. 66, 844-849.

Sela, D.A., Li, Y., Lerno, L., Wu, S., Marcobal, A.M., German, J.B., ET AL., (2011). An infant-associated bacterial commensal utilizes breast milk sialyloligosaccharides. J. Biol. Chem. 286, 11909-11918. doi: 10.1074/jbc.M110.193359.

Shafquat, A., Joice, R., Simmons, S.L., Huttenhower, C. (2014). Functional and phylogenetic assembly of microbial communities in the human microbiome. Trends Microbiol. 22, 261-266. doi: 10.1016/j.tim.2014.01.011.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., et al., (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498-2504.

Shendure, J. and Ji, H. (2008). Next-generation DNA sequencing. Nat. Biotechnol. 26, 1135 – 1145. doi:10.1038/nbt1486.

Sjogren, Y.M., Jenmalm, M.C., Bottcher, M.F., Bjorksten, B., Sverremark-Ekstrom, E. (2009). Altered early infant gut microbiota in children developing allergy up to 5 years of age. Clin. Exp. Allergy. 39, 518-526. doi: 10.1111/j.1365-2222.2008.03156.

Smith, G. (2015). Antimicrobial decision making for enteric diseases of cattle. Vet. Clin. North Am. Food Anim. Pract. 31, 47-60. doi: 10.1016/j.cvfa.2014.11.004.

Smith, H.W. (1965). The development of the flora of the alimentary tract in young animals. The Journal of Pathology and Bacteriology. 90, 495-513. doi: 10.1002/path.1700900218.

Sokol, H., Seksik, P., Furet, J.P., Fermisse, O., Nion-Larmurier, I., Beaugerie, L. et al., (2009). Low counts of *Faecalibacterium prausnitzii* in colitis microbiota. Inflammatory Bowel Diseases. 15, 1183-1189. doi: 10.1002/ibd.20903.

Sommer, F. and Backhed, F. (2013). The gut microbiota - masters of host development and physiology. Nat. Rev. Microbiol. 11, 227-238. doi: 10.1038/nrmicro2974.

Sommer, F., Nookaew, I., Sommer, N., Fogelstrand, P., Backhed, F. (2015). Site-specific programming of the host epithelial transcriptome by the gut microbiota. Genome Biol. 16, 62. doi: 10.1186/s13059-015-0614-4.

Spears, J.W. (1996). Organic trace minerals in ruminant nutrition. Animal Feed Science Technology. 58, 151-163.

Spicer, H.M., Goonewardene, L.A., McNeil, A.O., Slack, W.L. (1994). Alberta dairy farm survey response. J. Dairy Sci. 77, 3460-3472.

Stevenson, D.M., Weimer, P.J. (2007). Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Appl. Microbiol. Biotechnol. 75, 165–174.

Su, W., Bush, C.R., Necela, B.M., Calcagno, S.R., Murray, N.R., Fields, A.P., et al., (2007). Differential expression, distribution, and function of PPAR-gamma in the proximal and distal colon. Physiol. Genomics 30, 342-353.

Subramanian, S., Blanton, L.V., Frese, S.A., Charbonneau, M., Mills, D.A., Gordon, J.I. (2015). Cultivating healthy growth and nutrition through the gut microbiota. Cell. 161, 36-48. doi:10.1016/j.cell.2015.03.013.

Tajima, K., Aminov, R.I., Nagamine, T., Matsui, H., Nakamura, M., Benno, Y. (2001). Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Enivorn. Microbiol. 66, 2766-2774. doi: 10.1128/AEM.67.6.2766-2774.2001.

Teixeira, A.V.G., Bicalho, M.L.S., Machado, V.S., Oikonomou, G., Kacar, C., Foditsch, C., et al., (2013). Heat and ultraviolet light treatment of colostrum and hospital milk: Effects on colostrum and hospital milk characteristics and calf health and

growth parameters. Vet. J. 197, 175-181. doi: 10.1016/j.tvjl.2013.03.032.

ten Bruggencate, S.J., Bovee-Oudenhoven, I.M., Feitsma, A.L., van Hoffen, E., Schoterman, M.H. (2014). Functional role and mechanisms of sialyllactose and other sialylated milk oligosaccharides. Nutr. Rev. 72, 377-389. doi: 10.1111/nure.12106.

Teran, R., Mitre, E., Vaca, M., Erazo, S., Oviedo, G., Hubner, M.P. et al., (2011). Immune system development during early childhood in tropical Latin America: evidence for the age-dependent down regulation of the innate immune response. Clin. Immunol. 138, 299-310.

The Human Microbiome Project Consortium. (2012). A framework for human microbiome research. Nature. 486, 215–221. doi:10.1038/nature11209.

Tringe, S.G. and Hugenholtz, P. (2008). A renaissance for the pioneering 16S rRNA gene. Curr. Opin. Microbiol. 11, 442-446. doi:10.1016/j.mib.2008.09.011

Tripathi, V., and Vashishtha, B. (2006). Bioactive compounds of colostrum and its application. Food Reviews International. 22, 225-244. doi: 10.1080/87559120600694606

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., et al., (2009). A core gut microbiome in obese and lean twins. Nature. 457, 480-484. doi: 10.1038/nature07540.

Turroni, F., Peano, C., Pass, D.A., Foroni, E., Severgnini, M., Claesson, M.J., et al., (2012). Diversity of bifidobacteria within the infant gut microbiota. PLoS One. 7, e36957. doi: 10.1371/journal.pone.0036957.

Uetake K. (2013). Newborn calf welfare: a review focusing on mortality rates. Anim. Sci. J. 84, 101-105. doi: 10.1111/asj.12019.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., and Roy, N.C. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. J. Nutr. 141, 769-776. doi: 10.3945/jn.110.135657.

Underwood, M.A., German, J.B., Lebrilla, C.B., Mills, D.A. (2015). *Bifidobacterium longum* subspecies *infantis*: champion colonizer of the infant gut. Pediatr. Res. 77, 229-235. doi: 10.1038/pr.2014.156.

Unger, S., Stintzi, A., Shah, P., Mack, D., O'Connor, D.L. (2015). Gut microbiota of the very-low-birth-weight infant. Pediatr. Res. 77, 205-213. doi: 10.1038/pr.2014.162.

USDA (2010). Dairy 2007, Heifer calf health and management practices on US dairy operations. pp 122-131. USDA:APHIS:VS, CEAH. Fort Collins, CO.

Uyeno, Y., Sekiguchi, Y., Kamagata, Y. (2010). rRNA-based analysis to monitor succession of fecal bacterial communities of Holstein calves. Lett. Appl. Microbiol. 51, 570-577. doi: 10.1111/j.1472-765X.2010.02937.

Uyeno, Y., Shigemori, S., Shimosato, T. (2015). Effect of probiotics/prebiotics on cattle health and productivity. Microbes. Environ. 30, 126-132. doi: 10.1264/jsme2.ME14176.

Van den Abbeele, P., van de Wiele, T., Verstraete, W., Possemiers, S. (2011). The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiol. Rev. 35, 681-704. doi: 10.1111/j.1574-6976.2011.00270.

Vasseur, E., Borderas, F., Cue, R.I., Lefebvre, D., Pellerin, D., Rushen, J., et al., (2010). A survey of dairy calf management practices in Canada that affect animal welfare. J. Dairy Sci. 93, 1307-1315. doi: 10.3168/jds.2009-2429.

Vlkova, E., Nevoral, J., Rada, V. (2006). Distribution of bifidobacteria in the gastrointestinal tract of calves. Folia Microbiol. 51, 325-328.

von Schillde, M.A., Hormannsperger, G., Weiher, M., Alpert, C.A., Hahne, H., Bauerl, C., et al., (2012). Lactocepin secreted by *Lactobacillus* exerts anti-inflammatory effects by selectively degrading proinflammatory chemokines. Cell Host Microbe. 11, 387-396. doi: 10.1016/j.chom.2012.02.006.

Waltner-Toews D., Martin, S.W., Meek, A.H. (1986). The effect of early calfhood health status on survivorship and age at first calving. Can. J. Vet. Res. 50, 314-317.

Wang, Y., Huang, C., Chintagari, N.R., Bhaskaran, M., Weng, T., Guo, Y., et al., (2013). miR375 regulates rat alveolar epithelial cell trans-differentiation by inhibiting Wnt/β catenin pathway. Nucleic Acids Res. 41, 3833-3844. doi: 10.1093/nar/gks1460.

Weimer, P.J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. Front. Microbiol. 6, 296. doi: http://dx.doi.org/10.3389/fmicb.2015.00296.

Weiss, G., Rasmussen, S., Nielsen Fink, L., Jarmer, H., Nohr Nielsen, B., Frokiaer, H. (2010). *Bifidobacterium bifidum* actively changes the gene expression profile induced by *Lactobacillus acidophilus* in murine dendritic cells. PLoS One. 5, e11065. doi: 10.1371/journal.pone.0011065.

White, J.R., Nagarajan, N., Pop, M. (2009). Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput. Biol. 5, e1000352. doi: 10.1371/journal.pcbi.1000352.

Wu, H-J., Wu, E. (2012). The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes. 3, 4-14. doi: 10.4161/gmic.19320.

Yanez-Ruiz, D.R., Abecia, L., Newbold, C.J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: a review Front Microbiol. 6, 1133. doi: 10.3389/fmicb.2015.01133.

Yasuda, M., Fujino, M., Nasu, T., Murakami, T. (2004). Histological studies on the ontogeny of bovine gut-associated lymphoid tissue: appearance of T cells and development of IgG+ and IgA+ cells in lymphoid follicles. Dev. Comp. Immunol. 28, 357-369.

Yatsunenko, T., Rey, F.E., Manary, M.J., Trehan, I., Dominguez-Bello, M.G., Contreras, M., et al., (2012). Human gut microbiome viewed across age and geography. Nature. 486, 222-227. doi: 10.1038/nature11053.

Yeoman, C.J., White, B.A. (2014). Gastrointestinal tract microbiota and probiotics in production animals. Ann. Rev. Anim. Biosci. 2, 469-486. doi: 10.1146/annurev-animal-022513-114149.

Yu, Z., Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques. 36, 808-812.

Zhao, T., Doyle, M.P., Harmon, B.G., Brown, C.A., Mueller, P.O.E., Parks, A.H. (1998). Reduction of carriage of enterohemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. J. Clin. Microbiol. 36, 641-647.

Zhou, M., Hernandez-Sanabria, E., Guan, L.L. (2009). Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Appl. Microbiol. Ecol. 76, 3776-3786. doi: 10.1128/AEM.00010-10.