Early life gut microbiome in dairy calves and its responses to colostrum feeding strategies

by

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Abstract

The evidence of the role of gastrointestinal microbiota in gut health of neonatal dairy calves is accumulating. However, there is limited understanding of hindgut microbial profiles, and the influence of colostrum management strategies on intestinal microbiome, and host gene expression in neonatal dairy calves. To fill these knowledge gaps, four studies (Chapters 2-5) were performed in this thesis. In chapter 2, the microbial composition and fermentation parameters (short chain fatty acids, SCFAs) in the hindgut (cecum, colon and rectum) of dairy calves during the pre-weaning period were characterized. *Firmicutes, Bacteroidetes* and *Proteobacteria* were the dominant microbial phyla in the hindgut. The significant relationships between the abundance of mucosa-attached carbohydrate utilizing bacteria (*Coprococcus* 1*, Blautia, Lachnospiraceae* NC2004 group), opportunistic pathogenic bacteria (*Escherichia-Shigella* and *Salmonella*) and SCFAs concentrations were identified, suggesting that hindgut microbiota may play an important role in the fermentation and gut health. In chapter 3, the effects of feeding different colostrum (no colostrum: NC; non-heated colostrum: FC; heated colostrum: HC) on the colon microbiota of calves within the first 12 h of life were studied. A higher proportion of *Clostridium* cluster XIVa and *Bifidobacterium,* and a lower abundance of *E. coli* were detected in the colon with colostrum feeding (NC vs. FC). Comparing to FC, HC feeding further reduced the abundance of mucosa-attached *E. coli*, and increased abundance of *Bifidobacterium* in the colon. These results suggest that colostrum feeding, especially HC can benefit calves by shifting colon microbiota to have more beneficial organisms. In chapter 4, the influence of delayed colostrum feeding on the ileal microbiome (composition and function) was studied. The relative abundance of *Enterococcus* was significantly higher in the ileum of the dairy calves when the colostrum feeding was delayed 12 h, suggesting that these calves may

have a higher chance of pathogenic bacterial infection. In total, 116 core KEGG pathways were identified for ileal microbiome, only “Taurine and hypotaurine metabolism” pathway was identified to be higher in the ileum of the dairy calves when the colostrum feeding was delayed 12 h. Further study is needed to understand how such function affects the host. In chapter 5, the effect of delayed colostrum feeding on ileal transcriptome of neonatal calves was investigated. Enriched expression of peptidase inhibitor 3 (*PI3*) gene, which involved in the function of protecting tissue from inflammation, in the ileum of calves with 12 h delayed colostrum feeding, suggests that these calves have a higher capacity to protect themselves in response to the nutritional delay. In addition, the enrichment of expression of genes involved in the function of “Antigen Presentation” was only detected in the calves who were fed colostrum immediately after birth. These suggest that delayed colostrum feeding to 12 h after birth may postpone immune system development in the ileum of neonatal dairy calves. Overall, findings from this thesis have enhanced our understanding on the mucosa and digesta-attached microbial community in the hindgut of dairy calves during pre-weaning period. Additionally, it provides the evidence on how the colostrum feeding strategies affect the intestinal microbial composition and functions, and host gene expression, which form the scientific bases to develop better feeding management strategies to improve gut health of neonatal dairy calves.

Preface

This thesis is an original work by Yang Song. The thesis work includes three animal studies, and they all received ethics approval from the Animal Care and Use Committee for Livestock at the University of Alberta (AUP00001012 and AUP00001595).

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Dedication

This thesis is dedicated to my father and mother!
Sincerely appreciate your support and encouragement all the time!

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AAD: Antibiotic-associated diarrhea

ANS: Autonomic nervous system

APCs: Antigen presenting cells

BCOs: Bovine colostrum oligosaccharides

BMD: Bacitracin methylene disalicylate

CDAD: C. difficile-associated diarrhea

CDI: Clostridium difficile infection

CFU: Colony-forming unit;

CNS: Central nervous system

CPM: Counts per million

C-section: Caesarian section

DAVID: Annotation, Visualization and integrated Discovery

DCs: Dendritic cells

DE: Differential expression

EGF: Epidermal growth factor

ENS: Enteric nervous system

E. coli: *Escherichia coli*

FDR: False Discovery Rate

F. prausnitzii: *Faecalibacterium prausnitzii*

FMT: Fecal microbiota transplant

FC: Non-heated colostrum

GALT: Gut-associated lymphoid tissue

GBA: Gut-brain-axis

GH: Growth hormone

GIT: Gastrointestinal tract

GLP-2: Glucagon-like peptide-2

Gpr41: G protein-coupled receptors 41

HC: Heated colostrum

HPA: Hypothalamic pituitary adrenal axis

IBD: Inflammatory bowel disease

IgA: Immunoglobulin A

IGF-1: Insulin-like growth factor 1

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IL: Interleukin

IPA: Ingenuity Pathway Analysis

KEGG: Kyoto Encyclopedia of Genes and Genomes

LDA: Linear discriminant analysis

LEfse: Linear discriminant analysis effect size

MG-RAST: Metagenomic Rapid Annotations using Subsystems Technology

NEC: Necrotizing enterocolitis

NKs: Natural killer cells

NC: No colostrum

NOD 1: Oligomerization domain 1

PANTHER: Protein Annotation Through Evolutionary Relationship

PCA: Principal Component Analysis

PCoA: Principal-Coordinate Analysis

PYY: Peptide YY

QPCR: Quantitative real-time PCR

SCFA: Short chain fatty acid

TGF: Transforming growth factor

Treg: Regulator T cells

ZO-1: Zonula occludens 1

ZO-2: Zonula occludens 2

ZO-3: Zonula occludens 3

Chapter 1. Literature review

1.1 Introduction

The North American dairy industry has been continuously challenged by high neonatal calf mortality (8.0-10.0%) and morbidity (38.5%), which leads to the economic loss and detrimental impacts on the long-term performance of dairy cattle (Donovan et al., 1998; USDA, 2010). Enteric infection accounts for about 50% of the total death of neonatal dairy calves, which is usually caused by enteric pathogens infection (Cho and Yoon, 2014). Therefore, maintaining and improving gut health during early life is essential for preventing infection and reducing mortality and morbidity of neonatal calves.

Good calf rearing management plays an important role in maintaining calf health and preventing the economic loss. In the modern dairy industry, calves are commonly fed colostrum immediately after birth. Then the calves are raised in an individual hutch/pen or group pen and consume whole milk or milk replacer until weaning. In the calf industry, several issues can pose a threat to the health of dairy calves during early life, including the surrounding environment at birth, time of navel disinfection, time for calf and dam separation, time of colostrum feeding, colostrum quality and quantity. For example, if the calves are left with the dam in the maternal pen for a longer time, it will increase the chances of being infected by pathogenic bacteria, such as *Mycobacterium avium* subspecies paratuberculosis and *Escherichia coli* (*E. coli*) from dam and/or environment, leading to Johne's disease infection or diarrhea (USDA, 2014). However, only 24.2% of the calves are transferred within an hour, and more than 57.5% of calves are removed from the maternal pen during 1.1 to 14 hours after birth (USDA, 2014).

The major concerns in colostrum management are colostrum feeding time and colostrum quality. Delayed colostrum feeding is common for calves born at night due to lower checking frequency of workers (once every 12 h) (Vasseur et al., 2010), which affects the passive immunity of neonatal calves (Fischer et al., 2018). In addition, poor quality colostrum such as high in bacteria (bacteria>10^6 CFU/ml, coliform>10^3 CFU/ml) and low in immunoglobin G (IgG, <50 mg/ml) (Morrill et al., 2012) can also affect the passive immunity and calf health (Thu Hang et al., 2017). Therefore, good calf and colostrum (feeding high-quality colostrum in adequate amounts) management are essential for dairy industry.

1.2 Gut development and immunological status of neonatal calf

The intestine is comprised of the small intestine and the large intestine, which are important in digestion and absorption of feed, and host metabolic and immune functions. The small intestine includes three parts, the duodenum, jejunum and ileum, while the large intestine consists of three sections, the cecum, colon and rectum (Aker and Denbow, 2013). Small and large intestinal regions are different in terms of anatomy structure (Steele et al., 2016) and functions. The ileum is the last part of the small intestine, which ends at the ileocecal junction. It is mainly responsible for absorption of vitamin B12, and other nutrients (e.g., protein, fat) that are not completely absorbed by jejunum. In addition, the ileum includes a variety of immune cells that play an important role in the immune functions. The colon is a part of the large intestine, which is coiled like a wheel in the ruminant (Reece and Rowe, 2013), with primary functions of fermentation, water and electrolyte reabsorption and solid waste excretion. Unlike the large intestine, the ileum possess villi, which are finger-like structures of the mucosa, and are formed by the simple columnar epithelium and cover the whole surface of ileum. However, the

ileum and colon have similarities, such as tight junctions and adherences junctions. These junctions together with the epithelial cells form the intestinal barrier to protect calves from pathogen invasion (Hartsock and Nelson, 2008). Tight junctions are composed of transmembrane proteins occluding and claudin, and cytoplasmic proteins Zonula occludens-1 (ZO-1), Zonula occludens-2 (ZO-2) and Zonula occludens-3 (ZO-3), which are responsible for preventing the combination of the apical and basolateral membrane proteins, and regulate the movement of ions and solutes (Hartsock and Nelson, 2008). Adherences junctions are composed of cadherin superfamily E-cadherin, transmembrane protein catenins, and their main functions are stabilizing cell-cell adhesion, regulating actin cytoskeleton and intracellular signaling (Hartsock and Nelson, 2008).

Calves have an undeveloped gastrointestinal tract (GIT) when they are born. The maturation of the intestine is modulated by many factors, including nutrients and regulatory substances from the colostrum such as growth factors, cytokines and neurotransmitters, colostrum microbes (Blum, 2006). The intestinal tissue grows quickly and the intestinal epithelium are modified after colostrum consumption after birth, including the loss of the ability of macromolecule absorption by the small intestine and secretion of digestive enzymes by the large intestine. In addition, colostrum-derived epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) play a role in the development of the GIT (Xu, 1996). Additionally, host secreted glucagon-like peptide-2 (GLP-2) stimulates nutrient absorption and transportation, and enhances crypt cells proliferation, while reducing crypt and villus apoptosis (Burrin et al., 2005), suggesting that host derived GLP-2 is crucial for gut development.

The immune system (innate and adaptive immune system) of the neonatal dairy calves starts to develop in utero (Chase et al., 2008). The development of the innate immune system

starts before birth, neutrophils and macrophages are released into blood in the middle of gestation, and the bactericidal activity starts to decrease because of the increased cortisol concentration near parturition (Barrington and Parish, 2001). With regards to the adaptive immune system of dairy calves, peripheral blood T cells start to transfer to lymphoid tissues one month before birth, and a small number of B cells (1%-2%) are present before birth (Senogles et al., 1979; Kampen et al., 2006). After birth, neonatal calves have low circulating B lymphocytes (about 30% of the adult level) (Barrington and Parish, 2001), and IgM is the main immunoglobulin during the first few weeks of life. Additionally, IgA and IgG can be detected until 16 to 32 days after birth (Husband and Lascelles, 1975), and in two weeks of life, the cell-mediated adaptive immunity reaches to the level detected in adults (Osburn et al., 1974).

Due to the deficient immunity of dairy calves at birth, they depend on the passive immunity transferred from the maternal colostrum. One of the most important components in the colostrum are the antibodies (e.g., IgG, IgA), which provide immediate protection against the invasion of pathogenic bacteria. In addition, cells in the colostrum can enhance the immune system of neonatal calves. These cells include macrophages, lymphocytes, neutrophils, T cells and B cells, which are suggested to work as an important mediator of adaptive immune response by stimulating the development of antigen-presenting cells (Reber et al., 2008). Langel et al. (2015) reported that feeding colostrum enhanced neonatal calf immunity by increasing peripheral blood CD4+ T cells and CD4+CK62L+CK45RO- T cells than feeding cell-free colostrum. Moreover, cytokines in the colostrum are crucial to the development of the neonatal calf immune system and among them, interleukin 1-beta (IL1-β) and interleukin-6 (IL-6) are related to the recruitment of lymphocytes and B cells growth and differentiation (Hawkes et al., 2002). Therefore, the colostrum feeding is of significant importance to the development of the immune

system of neonatal calves.

1.3 Calf management

1.3.1 Nutritional management during early life

During the first 2 to 3 weeks of life, calves are mainly fed whole milk or milk replacer. During the pre-weaning period, calves depend on calf starter and milk replacer or whole milk for maintenance and growth before the physiological and functional development of rumen (Drackley, 2008). Protein, fat, water are all essential nutrients to neonatal calves. Protein depositions in bone and muscle are important to the growth and fat serves as an extra energy source. Water is also a critical nutritional resource that should be available to calves all the time because of its importance in metabolic activities and positive relationship with starter intake (Kertz et al., 1984). There are two general strategies for feeding dairy calves in commercial industry. The mostly used approach is to provide a certain amount of milk or milk replacer (8% to 10% body weight, usually less than the calves can naturally consume) to the calves, with calf starter being offered together with milk ad libitum from the first week of life. The other feeding method is called intensified nutrition, which allows calves to obtain more nutrients from milk compared to the method mentioned above (12.5% solids and fed at 1.25% of birth BW as daily vs. 15% solids and fed at 1.5% of birth BW as daily during first week) (Stamey et al., 2012).

1.3.2 Calf diarrhea

Calf diarrhea is a serious problem in dairy industry, which accounts for about 50% of the total deaths, and most calves have diarrhea within one month after birth (Cho and Yoon, 2014). Pathogen infections are the main reason for calf diarrhea, including the commonly recognized pathogens *Bovine rotavirus*, *Bovine coronavirus*, *Salmonella* spp, *E. coli*, *Clostridium*

perfringens, and *Cryptosporidium Parvum*. Viral infections by *Bovine rotavirus* and *Bovine coronavirus* could also induce diarrhea in 1-2 weeks old calves (Cho and Yoon, 2014), and the infection leads to intestinal damage (small intestine and colon atrophic) (Schultze et al., 1991). *Salmonella typhimurium* causes acute calf diarrhea, which usually occurs during the first three weeks of life (Mead, 1999). Additionally, *E. coli* infection (e.g., enterotoxigenic *E. coli*) usually happens during the first four days of life (Foster and Smith, 2009). Protozoa (e.g., *Cryptosporidium parvum*) could infect neonatal calves and causes changes in the intestinal structure (Heine et al. 1984), resulting in severe diarrhea (Fayer et al., 1998). The infections from the above pathogens including bacteria, virus and protozoa, affect nutrient absorption and utilization, leading to reduced growth rate of calves and economic loss to the industry.

Timely prevention and effective treatment are very important. The first strategy for reducing calf diarrhea focuses on calving management during peripartum period. The poor management could lead to high rate of dystocia, which is closely related with the susceptibility to environmental pathogens (Larson and Tyler, 2005). In addition, feeding high quality and a high amount of colostrum (usually 3-4L) in a timely manner is another management focus on the improvement of calf immunity to protect the calves from environmental pathogens through passive immune transfer (Barrington and Parish, 2001). Moreover, reducing surrounding environmental stress, such as temperature, moisture, and bacterial load, plays an important role in preventing calf diarrhea (Larson and Tyler, 2005; Carroll and Forsberg, 2007).

1.4 Colostrum feeding

1.4.1 Colostrum

Colostrum is the first fluid produced by female mammals after parturition (Godhia and

Patel, 2013) and contains high concentration of nutritional, immune, and growth factors (Blum and Hammon, 2000). Colostrum is a rich source of immune factors, including immunoglobulins (IgG1, IgG2, IgA, IgM), lactoferrin, polypeptide, oligosaccharides, cytokines, and lysosomes. (Godhia and Patel, 2013). Additionally, colostrum is rich in nutrients such as, protein, fat, vitamins and minerals that are crucial to the calves' survival and growth. As demonstrated in the newborn piglet, fat and carbohydrate in the colostrum are the major energy source that affects the thermoregulation and the gluconeogenesis after birth, (Le Dividich et al., 1994), while proteins are related to muscular and skeleton growth. In addition, colostrum is the primary source for vitamins since some vitamins can not be transferred across the placental barrier to prenatal calves (Quigley and Drewry, 1998). In summary, colostrum are important resource to neonatal mammals.

1.4.2 Bovine colostrum and passive immunity

Cows have a syndesmochorial placenta, which forms a syncytium between the maternal endometrium and the fetal trophectoderm. Therefore, the blood supplies are separated, and the transmission of immunoglobulins is prevented *in utero* (Arthur et al., 1996). After birth, calves are completely dependent on colostrum to get successful passive immunity transfer. The transfer of immunoglobulins from the cow to the newborn calf mainly includes two processes. First, through IgG-Fc receptors mediated transfer, the circulating maternal immunoglobulins enter into the maternal mammary gland and start to accumulate. Next, the colostrum immunoglobulins are absorbed by the newborn calves from the gut lumen through pinocytosis (Pakkanen and Aalto, 1997). The colostrum IgG concentration is ~50 fold higher than that of mature milk (colostrum: 81g/L, mature milk: <2g/L) (Blum and Hammon et al., 2000). Additionally, the concentration of colostrum IgA is more than 20 times higher than that in normal milk (colostrum: 4.50g/L, mature

milk: 0.2g/L) (Porter and Noakes, 1970). Other immune factors, such as lactoferrin and oligosaccharides, can enhance the immunity of neonatal calves by binding pathogenic bacteria and preventing their colonization in the gut lumen (Arnold et al., 1980; Maldonado-Gomez et al., 2015).

The unselective absorption of colostrum macronutrients is time dependent. Previous studies indicated that the intestinal absorption is optimal during the first 4 h postpartum, and starts to decrease 12 h after birth (Stott et al., 1979b; Bush and Staley, 1980), and the unselective absorption stops between 24 and 36 h postpartum (Stott et al., 1979a). Therefore, colostrum should be fed as early as possible after birth to ensure the calf obtains enough passive immunity. There are two indexes usually used by the industry to evaluate whether the calves have successful passive immunity. Serum IgG and serum total protein concentrations, being more than 10 mg/mL (Godden, 2008) and more than 5.2 g/dL, respectively, at 24 h postpartum (Tyler et al., 1996), are defined as successful passive immunity. Failure of passive immunity is associated with a higher mortality rate (Robison et al., 1988) and lower milk production in the first lactation (DeNise et al., 1989). Therefore, ensuring the establishment of successful passive immunity of the calf is of significant importance for their optimal health and performance in later life.

1.4.3 Immune factors in colostrum and bacterial colonization

In general, most of immune factors in the colostrum, are related to bacterial colonization in the gut. In detail, IgA mainly prevents pathogens attaching to intestinal mucosa by agglutinating microbes (Hurley and Theil, 2011). Lactoferrin is a natural antimicrobial protein in the colostrum, which inhibits the growth of a variety of bacteria, including *E. coli,* and *Klebsiella pneumonia* (Arnold et al., 1980) by its iron-chelating ability (Weinberg, 1978). In addition, lactoferrin stimulates the immune system development by promoting neutrophil function (Lakritz

et al., 2000). The oligosaccharides inhibit the attachment of pathogens to the intestinal cells (Maldonado-Gomez et al., 2015) and stimulate the colonization of beneficial *Bifidobacterium*, and *Bacteroidetes longum* ssp. *infantis* (LoCascio et al., 2007; Sela et al., 2011). Sialylated oligosaccharide is the main oligosaccharide in the bovine colostrum (ten Bruggencate et al., 2014). Lysosome is also present in colostrum, which kills bacteria by degrading the peptidoglycan of the cell wall (Nash et al., 2006). Colostrum leukocytes may also enhance the immunity of newborn calf since they are detected in the lymph circulation of neonatal ruminants (Sheldrake and Husband, 1985). All these suggest that colostrum immune factors play important roles in shaping the intestinal microbial colonization.

1.4.4 Functions of growth factors in the colostrum

Besides the nutritional and immunological factors, colostrum also consists of growth factors, including IGF, epidermal growth factor (EGF), growth hormone (GH), transforming growth factors (TGF) and several others, which are involved in cell differentiation and growth (Godhia et al., 2013). Epidermal growth factor (EGF) is important in gut development and health, such as stimulating intestinal cell proliferation, regulating tight junctions (Kaur et al., 2014), enhancing mucins secretion (Clark et al., 2006), and inhibiting pathogens' colonization (Lamb-Rosteski et al., 2008). Epidermal growth factor (EGF) has been reported to stimulate the proliferation of intestinal crypt epithelial cells in the 1-day-old piglet (Jung et al., 2008), suggesting its importance in intestinal cells proliferation of neonatal animals. Bovine colostrum IGF ranges from 289 to 902 ug/L (Baumrucker et al., 1994), and calves with the received milk replacer supplemented with IGF-I increased DNA synthesis in intestinal cells compared to those fed milk replacer plus isolated colostrum-derived globulins or pooled cow colostrum (Baumrucker et al., 1994), indicating that IGF is crucial in promoting the growth of intestinal

tissue in neonatal calves. Moreover, TGF is also important in neonatal intestinal growth and innate immunity development. Colostral TGF-β has been reported to influence gut integrity in the suckling rat (Playford et al., 2000) and the protective effects of TGF-β2 was proven by its enhanced expression when intestinal epithelial cells were challenged with LPS (Nguyen et al., 2015), suggesting that TGF promotes intestinal health.

1.4.5 Heat treatment of colostrum and its beneficial effect

The importance of passive immunity to neonatal calves has been mentioned above. Despite the beneficial effect of colostrum, it has the potential to pass microbial pathogens to newborn calves. Heat treatment of colostrum at 60°C for 30 or 60 min (Godden et al., 2006; Donahue et al., 2012; Malmuthuge et al., 2015) is an effective method for decreasing colostrum bacterial content (Elizondo-Salazar and Heinrichs, 2009). When colostrum is heated at 60°C for 60 min, such process decreases the bacterial counts but maintains the IgG concentration. In addition, pathogens in the colostrum such as *Mycoplasma bovis*, *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella enteritidis* are eliminated after being heated at 60°C for 30 min (Godden et al., 2006). Moreover, higher serum IgG level and lower risk of illness was detected after calves consumed heated colostrum (Godden et al., 2012). Additionally, the beneficial effect of heated colostrum on enhancing the abundance of beneficial bacteria *Bifidobacterium* and inhibiting the abundance of potential pathogenic *E. coli* colonization in the gut of the neonatal calves has been demonstrated (Malmuthuge et al., 2015). Therefore, heat-treated colostrum which contains IgG concentration, and shape intestinal microbial colonization towards a host beneficial way should be encouraged.

1.5 Microbial colonization in the gut during early life

1.5.1 Intestinal microbial establishment in monogastric mammals

Microbial colonization during early life is a dynamic process. Whether microbial colonization starts *in utero* or during birth is still debatable. Studies have indicated that bacteria can be detected in amniotic fluid (Rautava et al., 2012), umbilical cord blood (Jiménez et al., 2005), and placenta tissue (Aagaard et al., 2014). A recent study detected the labeled *Enterococcus faecium* admitted to the pregnant mice in the amniotic fluid and meconium of newborn mice after sterile cesarean section (C-section), suggesting that maternal microbiota could be transmitted to newborn mice in the utero (Jiménez et al., 2005). It is speculated that the mechanism of the maternal-infant transmission is the penetration of dendritic cells to the gut epithelium, taking up the intestinal bacteria from the lumen, and the live bacteria can migrate to the lymphoid organs, then further circulate in the bloodstream (Jiménez et al., 2005). Therefore, based on the above outcomes, the maternal bacteria transmission to the fetal probably starts *in utero*.

Following birth, the infant's intestine is rapidly colonized by a variety of microbes during the first few hours, which mainly originate from microbes inhabiting maternal vaginal tract and feces (Vaishampayan et al., 2010). *Lactobacillus* is detected in the maternal vagina (Levison et al., 1977), and is usually presented in high numbers in the gut of an infant. In addition, facultative anaerobes are among the first colonizers during the first few hours after birth, including *E. coli*, *Streptococci* spp., *Staphylococcus*-, *Enterococcus*- and *Lactobacillus*- like species (Scholtens et al., 2012). These bacteria consume oxygen and provide a comfortable environment for the obligate anaerobes (Orrhage and Nord, 1999), such as *Bacteroides*, *Clostridium* and *Bifidobacterium* spp. Bifidobacteria are abundant in the gut during the first few

months after birth. With the increase of age, the population of *Proteobacteria* and *Actinobacteria* decrease, while the population of *Firmicutes* and *Bacteroidetes* start to increase (Eckburg, 2005; Qin et al., 2010). After the gradual introduction of solid food, the population of butyrate producers, such as *Bacteroides* and *Clostridium* species, are enhanced (Koenig et al., 2010). Finally, the gut microbial profile resembles to that of human adult during 2-5 years after birth (Koenig et al., 2010; Yatsunenko et al., 2012).

Microbial colonization in piglet starts at the moment that fetal membranes are ruptured, and this process can be affected by the surrounding environment. Similar to the human infant, facultative anaerobic *E. coli* and *Streptococcus* spp. are the first colonizers in the gut, and they create an anaerobic environment for *Bacteroides*, *Bifidobacterium* and *Clostridium* colonization (Konstantinov et al., 2006; Petri et al., 2010) by the consumption of oxygen. The anaerobic bacteria replace aerobic and facultative anaerobic bacteria, and become predominant after 48h from birth (Swords et al., 1993). Lactobacilli and Streptococci are the predominant bacteria from one week after birth to weaning (Swords et al., 1993). After weaning, carbohydrate utilizing bacteria, such as *Prevotella*, *Oscillibacter*, *Faecalibacterium*, and *Roseburia* become dominant (Mach et al., 2015). In addition, the microbial profile becomes more diverse with increasing age (Inoue et al., 2005).

Mice are the commonly used experiment models to study intestinal microbial colonization, whose microbial profile is significantly affected by age. *Firmicutes* and *Bacteroidetes* are the predominant phyla in the gut of mice from 3 to 104 weeks. The major genera were *Bacteroides*, *Lactobacillus*, *Blautia*, *Clostridium* XI and *Klebsiella* during the pre-weaning period, and the high relative abundance of *Akkermansia* spp., *Clostridium* XIVa spp., *Lachnospiraceae*, *Alistipes* spp. and *Odoribacter* spp. was observed during the first year of age

after weaning (Flemer et al., 2017). At two years of age, genera *Barnesiella*, *Clostridiales*, *Ruminococcaceae*, *Ruminococcus* and *Clostridium* IV accounted for a higher relative abundance (Flemer et al., 2017). These findings suggest that the gut microbiota is dynamic during early life of monogastric mammals.

1.5.2 Microbiota establishment in ruminants during pre-weaning stage

There are dynamic changes in the intestinal microbiota of ruminants during the pre-weaning period since the pre-ruminants are considered as monogastric before their rumen are developed. There is a conflict about the most predominant phylum in the ruminant gut based on previous publications (Table 1). *Firmicutes* is reported to be the most abundant phylum during the first seven weeks of life in feces of dairy calves (Oikonomou et al., 2013; Foditsch et al., 2015), while others suggest that *Bacteroidetes* is the most predominant phylum in fecal samples of pre-weaned calves (Uyeno et al., 2010; Klein-Jöbstl et al., 2014). Such difference may be due to different breed, calf management strategy, and the sampling method. Malmuthuge et al. (2014) indicated that *Firmicutes* (57.6%) was the predominant phylum in the digesta, while *Bacteroidete*s phylum dominates the mucosa-associated microbiota in the small intestine when studying the microbial community using lumen and tissue samples separately. However, most of studies on gut microbiota in dairy calves are based on fecal samples since sample collection process is not invasive (Uyeno et al., 2010; Oikonomou et al., 2013; Klein-Jöbstl et al., 2014). A few researches have used local intestinal tissue and content samples for microbial profile analysis (Malmuthuge et al., 2012, 2014, 2015). Therefore, based on the inconsistent results from different studies, selecting the representative intestinal samples to explore the microbial profile is encouraged.

A study based on calf meconium indicated that *Citrobacter*, *Lactococcus*, *Leuconostoc*

and *Lactobacillus* are the first gut colonizers (Mayer et al., 2012), which could be obtained from maternal vagina, feces and the surrounding environment during and immediately after birth. Additionally, Mayer et al. (2012) described the microbial colonization process in feces of dairy calves from birth to 42 days of life, suggesting that changing patterns of several bacterial genera. *Citrobacter* spp., appeared in all calves after birth and disappeared after 24 h, and *Clostridium* spp. is one of the dominant bacteria between 24 h and 48 h of life. In addition, *E. coli* is the dominant genus from 24 h to day seven after birth, and its abundance starts to decrease from day 3 to day 7, whereas the population of *Bacteroides* spp. including *B. fragilis* and *B. vulgatus* began to increase at this time (Mayer et al., 2012). This study indicated that gut microbial colonization process of pre-weaned ruminant is similar to human, which was firstly colonized by facultative bacteria, following by obligating anaerobic bacteria (Mayer et al., 2012). Additionally, Malmuthuge et al. (2014) described the intestinal microbiota composition of three-week old dairy calves in terms of using different sample types (Mucosa vs. Digesta). *Bacteroides*, *Prevotella*, *Lactobacillus*, *Sharpea*, *Faecalibacterium* and *Burkholderia* genera are predominant in mucosa-attached bacterial community, whereas, *Bacteroides*, *Prevotella*, *Lactobacillus*, *Clostridium*, *Sharpea* and *Faecalibacterium* are predominant genera in digesta-associated community (Malmuthuge et al., 2014). Moreover, *Bacteroides-Prevotella* and *Clostridium coccoides-Eubacterium rectale* groups have higher relative abundance during the first 12 weeks after birth based on sequence-specific rRNA cleavage analysis (Uyeno et al., 2010).

1.5.3 Importance of early microbiota colonization

Early life microbiota plays an important role in host intestinal barrier function, immune system development, metabolism, and health (Gaboriau-Routhiau et al., 2009; Petersson et al.,

2010; Sommer and Bäckhed, 2013). The cell proliferation rate is lower in germfree animals compared to conventional mice (Nowacki et al., 1993). In addition, the colonic mucosal layer is thinner compared to that of conventional mice, while the mucus thickness became similar as that of conventional mice after administration of microbe-derived lipopolysaccharides and peptidoglycans (Petersson et al., 2010). Moreover, the inoculation of gnotobiotic mice with whole mice microbiota leads to the increase of proinflammatory T helper cells and regulatory T cell responses, and Clostridia-related species could help to coordinate the maturation of the above T cell responses (Gaboriau-Routhiau et al., 2009). Furthermore, postnatal microbial colonization stimulates the development of gut-associated lymphoid tissues (GALT) including Peyer's patches and mesenteric lymph nodes (Renz et al., 2011). All the outcomes mentioned above suggest the importance of early life microbiota on host immune system development.

Microbiota establishment during early life is essential to infant's health. Dysbiosis in infant's gut microbiota has been reported to be associated with neonatal diarrhea and necrotizing enterocolitis (NEC) (Saavedra et al., 1994). In addition, early life intestinal infections with *Shigella*, *Salmonella* and *Campylobacteria* (Saavedra et al., 1994) are associated with acute diarrhea, leading to infant morbidity and mortality. Oral administration of probiotics has been reported to be beneficial for the treatment of diarrhea, which helps to reduce the incidence and duration of the neonatal diarrhea. For example, the duration of diarrhea could be reduced about 50% with the supplement of *Lactobacillus rhamnosus* (Szajewska et al., 2006), and other probiotics such as *Bifidobacterium bifidum* and *Streptococcus thermophiles* reduce the diarrhea rate (Saavedra et al., 1994) in human infants. In addition, preterm infants usually have NEC, which is often detected with disturbed intestinal function. Oral administration of *Lactobacillus acidophilus* (*L. acidophilus*) and *B. infantis* could help to reduce NEC rate to 50% (Lin et al.,

2005). Similarly, administration of a mixture of *B. infantis*, *B. bifidus* and *Streptococcus thermophilus* (*S. thermophiles*) also reduces the incidence of NEC of the preterm infants (Krasan, 2006). However, the mechanisms behind that how the microbiota contribute to host intestinal barrier function, immune system development, metabolism, and health are not well defined.

1.5.4 Effect of early life microbial shift on lifelong health

Microbiota dysbiosis during early life has been reported to affect long-term performance in human, piglet and dairy calves. The disturbance of the early life microbial colonization leads to a variety of disease in adult human, such as food allergies, atopic dermatitis and asthma (Stiemsma and Turvey, 2017). The close relationship between shifts in intestinal microbiota and the development of atopic dermatitis has been found in Sweden and Estonia children. During the first two years of life, the children with atopic dermatitis have lower *Enterococcus* at one month old, lower *Bifidobacterium* at one year old, and higher *Clostridium* at three months of age when compared to healthy ones (Björkstén et al., 2001). In addition, the perturbation of intestinal microbiota related to food allergy is also suggested in the previous study. Children who have food allergies have lower level of *Bacteroides* and *Clostridium* XVIII, higher level of *Clostridium sensu stricto* and *Anaerobacter* during the first year of life (Ling et al., 2014). In piglet, the diversity and composition of postnatal microbiota at day seven is suggested to be an indicator of post-weaning (day 35 after birth) diarrhea. Healthy piglets usually have a higher abundance of *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Lactobacillaceae* compared to those with diarrhea at day seven after birth (Dou et al., 2017). In addition, the higher relative abundance of *Faecalibacterium. prausnitzii* is reported to be related with higher weight gain and lower diarrhea rate in dairy calves at three weeks of life (Oikonomou et al.,

2013). Therefore, the shifts in early life microbiota is important which can affect the long-term performance in human and animals, and those studies support that there is an opportunity in manipulating of early life intestinal microbiota to promote long-term animal health.

1.5.5 Factors affect microbiota colonization during early life

In infants, gut microbiota colonization can be affected by many factors, including delivery mode (cesarean delivery vs. vaginal delivery), feeding method (breast feeding vs. formula feeding), antibiotic usage, the introduction of solid feed (Mayer et al., 2012; Francino et al., 2014), host genetic and geography (De Filippo et al., 2010), prebiotic and probiotics (Gibson et al. 2010). Delivery mode shapes the gut microbiota composition. The maternal vaginal microbiota plays a role in contributing to microbial composition in vaginally delivered infants, while the skin and the surrounding hospital environment are the main contributors to the gut microbiota of babies born with C-section (Scholtens et al.,2012). In the gut of vaginally born infants, *Lactobacillus* spp., *Prevotella* spp., and *Sneathia* spp. usually dominate, whereas in the gut of C-section delivered babies, *Staphylococcus* app., *Corynebacterium* spp., and *Propionibacterium* spp., are predominant bacteria which is more similar to mother's skin microbiota. It has also been shown that the colonization of beneficial *Bifidobacterium* is delayed and less abundant in C-section delivered baby (Biasucci et al., 2008; Huurre et al., 2008). In addition, intestinal microbiota in vaginally born infant is more diverse and has more bacterial numbers than that in C-section delivered infant (Huurre et al., 2008). Another factor affects microbiota colonization is the feeding method (breast feeding vs. formula feeding). Milk is the major source affecting early microbial colonization since it provides the substrates for bacterial proliferation (Le Huërou-Luron et al., 2010). Previous studies have shown higher intestinal *Bifidobacterium* in breast fed babies compared to formula milk fed ones (Rinne et al., 2005;

Fallani et al., 2010; Lee et al., 2015), whereas formula fed infants usually have higher *Bacteroides, Lactobacillus* and *Atopobium* (Jiménez et al., 2008; Fallani et al., 2010; Bezirtzoglou et al., 2011) in the gut. Moreover, dietary effect on microbiota composition has been reported. It is suggested that the introduction of solid food could enhance butyrate producers' colonization in the gut of human infants, such as *Bacteroides* and some *Clostridium* species (Koenig et al., 2010). Additionally, *Bacteroidetes* that helps digesting high-fiber diet, are more abundant in African children compared to children in western countries (De Filippo et al., 2010), suggesting the adaption of intestinal microbiota to the local food. Moreover, host genetics affect gut microbiota composition, which has been proven by that the monozygotic twins have more similar microbiota compared to dizygotic twins (Turnbaugh et al., 2009). Furthermore, antibiotic usage is also a main factor to affect intestinal microbial colonization both in the short term and long term. Clindamycin has been reported to lead to the overgrowth of *Clostridium Difficile*, which causes the high risk of pseudomembranous colitis in human (Bartlett, 2002). In addition, Clindamycin also reduces *Bacteroides* colonization in the short term. Furthermore, the abundance of species (*B. caccae*, *B. distasonis*, *B. thetaiotaomicron*) belong to *Bacteroides* genus is affected by Clindamycin usage in human (Löfmark et al., 2006). Another antibiotics, amoxicillin, commonly used to treat diarrhea, eliminates *Bifidobacterium* in the short term (14 days after treatment) (Young and Schmidt, 2004). Meanwhile, prebiotics could specifically change the composition and function of intestinal microbiota, and provide benefits to host health (Gibson et al., 2010). Compared to formula milk, the formula milk with galacto-oligosaccharides supplement increased *Bifidobacterium* colonization in gut of human babies (Sierra et al., 2015). In addition, the oligosaccharides in the breast milk shape the gut microbiota of infant, leading to the higher number of beneficial *Bifidobacterium longum* subspecies *infantis*. The above studies

suggest that prebiotics play a fundamental role in shaping intestinal microbiota towards a beneficial direction.

Comparing to human, the research on gut microbial colonization in ruminants is scarce. In dairy calves, the supplement of calf starter during weaning period does not affect small intestinal bacterial density and lactic acid bacterial populations, however, it tends to increase the number of bacterial phylotypes (Malmuthuge et al., 2013). Additionally, the effect of antimicrobial bacitracin methylene disalicylate (BMD) on dairy calf intestinal microbial community has been reported. The abundance of potential pathogenic *Escherichia*, *Enterococcus* and *Shigella* increased, while the abundance of beneficial *Roseburia*, *Faecalibacterium* and *Eubacterium* decreased (Xie et al., 2013). In the meantime, neonatal calves fed with milk replacer supplemented with prebiotics tend to have more Lactobacilli in their feces than calves fed with milk replacer and antibiotics (Heinrichs et al., 2009). Moreover, the host genetic effect on gut microbiota in dairy calf has been reported that the rectal microbiota of the calf is more similar with its twin sister compared to other calves who are raised in the same place (Mayer et al., 2012). To date, the factors that affect dairy calf intestinal microbiota are not well studied, and the understanding of how the colostrum management strategies (e.g., heat treatment of colostrum and the time of colostrum feeding) affect gut microbial colonization during early life is very limited.

1.6 Functions of the gut microbiota

There are several hundred bacterial species and around 10^{14} bacterial cells in the human gastrointestinal tract interacting with the host mutually (Round and Mazmanian, 2009; Nicholson et al., 2012). The commensal microbiota has many functions, including polysaccharide digestion,

protecting the host against pathogens, stimulating host immune system development, detoxifying toxic metabolites (Tanaka and Nakayama, 2017), and impacting intestinal barrier development (Petersson et al., 2010). These functions will be discussed respectively in the following sections.

1.6.1 Carbohydrate degradation and short chain fatty acids (SCFA) production

Microbiota plays an important role in nutrient fermentation which produces about 10% of total energy to the host (Bergman, 1990). The major nutrients that are fermented in the large intestine originate from the indigestible or unabsorbed substrates in the small intestine, including the indigestible polysaccharides (resistant starch, non-starch polysaccharides), and some monosaccharides and disaccharides (oligosaccharide, lactose, fructose) (Chassard et al., 2010; Schwab and Gänzle, 2011; Wei et al., 2012; Ze et al., 2012). Therefore, hindgut microbiota plays an important role in the fermentation of carbohydrates. For example, *Bacteroides* is mainly responsible for resistant starch and xylan fermentation, and *Roseburia* utilize resistant starch, xylan and oligosaccharides. Additionally, *Ruminococcus* consume resistant starch and cellulose, and *Bifidobacterium* utilize oligosaccharide (Chassard and Lacroix, 2013). Moreover, short chain fatty acids (SCFAs) are the main microbial fermentation products, mainly including acetate, propionate and butyrate. Butyrate is the major energy source for colon epithelium cells (Rowe and Bayless, 1992). Acetate serves as an energy source to be circulated from the blood to the peripheral tissues, such as the liver, where acetate participates lipogenesis and cholesterol synthesis (Bergman, 1990; Bäckhed et al., 2004). Propionate also circulated into the liver, and is used for gluconeogenesis and cholesterol synthesis regulation (Venter et al., 1990; Reilly and Rombeau, 1993). SCFAs are crucial to the host, not only that they are used as energy source, but also they are related to host metabolism and health. In detail, SCFAs affect energy metabolism through neuroendocrine mechanisms (Samuel et al., 2008; Tolhurst et al., 2012). For example,

SCFAs bind to colonic epithelial cells receptors, G protein-coupled receptors 41 (Gpr41) and 43 (Gpr43), to regulate energy metabolism by releasing peptide YY (PYY) and glucagon-like peptide (GLP) (Samuel et al., 2008; Tolhurst et al., 2012). In addition, butyrate plays an important role in gut integrity by regulating expression of genes encoding tight junction proteins (e.g., claudin-1 and Zonula Occludens-1) (Wang et al., 2012). Furthermore, butyrate is related to host immune function and inflammatory modulation by inhibiting NF-κB activation (Lührs et al., 2002). Therefore, the importance of microbiota in energy metabolism, signal transduction and immune system is reflected via the production of SCFAs.

1.6.2 Intestinal microbiota and immune function development

The intestinal microbiota is important in shaping the development of innate immune system in neonatal animals. Compared to germ free mice, the mice colonized with commensal microbiota have a higher level of mucosal IgA (Benveniste et al., 1971), suggesting that commensal microbiota stimulates the IgA secretion in the gut. In addition, gnotobiotic piglets colonized with *Lactobacillus* spp., *Colostridium, Roseburia Intestinalis* have been reported to have higher concentration of IgA and IgM in the serum compared to germ-free piglets (Laycock et al., 2012). Moreover, the morphology of macrophages in germ-free mice is altered due to lack of SCFAs production (Thaiss et al., 2016). Gut microbiota is also associated with adaptive immune system homeostasis in neonatal animals, mainly related to T and B cells development. T cells mainly include two types of T cells, $CD4^+$ T cells and $CD8^+$ T cells. In addition, $CD4^+$ T cells include four major subtypes: T helper 1 (TH1), Th2, Th17 and regulatory T cells (Treg) that are distinguished by the transcription factors, cytokines, and their different functions. Th1 cells are mainly responsible for intracellular microbial infection, and Th2 cells mainly deal with parasite infections, while Th17 cells mediate protection against extracellular bacterial and fungal

infection and also mediate B cell responses by inducing proliferation. Treg cells are mediators of immune tolerance (Male et al., 2013). Compared to normal mice, the germ free mice have lower number of $CD4^+$ cells in their lamina propria (Macpherson et al., 2002). Previous studies also found that specific bacteria are inducers of T cells when they were introduced to germ-free mice. For example, the introduction of *Bacteroides fragilis* to germ-free mice is related to Th1 response and regulation of the imbalance of TH1 and TH2 cells (Mazmanian et al., 2005). Additionally, the number and function of intestinal $CD8^+$ cells are relatively lower in germ free mice compared to the mice in conventional environment (Imaoka et al., 1996). In addition, most of the gut-associated B cells are IgA secreting plasma cells, and they are usually found in the peyer's patches. Similar to T cells, decreased number of plasma B cells was reported in the gut of germ free animals (Crabbé et al., 1968). All the studies mentioned above imply the importance of commensal microbiota in the immune system development of the animals. However, such aspect has not been well studied in the ruminants.

1.6.3 Impact of microbiota on intestinal barrier development

The intestinal barrier is the location where the host has immediate interaction with intestinal microbiota and the site of pathogen invasion. Therefore, maintaining a healthy intestinal barrier is of significant importance to the host. The intestinal barrier has many defense mechanisms against pathogens, including mucus layer, epithelial integrity, and epithelial cell turnover (Kim et al., 2010). Mucus layer contains two sublayers: an outer loosely attached layer and an inner firmly adherent layer. Mucin is the major component of mucus layer, which is produced by goblet cells, containing digestive enzymes and antimicrobial peptides. Mucin and antimicrobial peptides inhibit bacteria to penetrate into the inner layer (Ashida et al., 2011).

When there is pathogen infection, mucin secretion increases to eliminate the infectious

bacteria. For example, *Pseudomonas aeruginosa* and *Staphylococcus aureus* infection induce the increase of expression of Muc2 gene, leading to more mucin synthesis (Li et al., 1998; Lemjabbar and Basbaum, 2002). Additionally, intestinal integrity is important to inhibit the translocation of pathogens to subepithelial layer. For example, strains of *Bifidobacterium* have been proven to affect gut integrity by strengthening tight junctions *in vitro* (Hsieh et al., 2015). Moreover, intestinal epithelial cells undergo self-renewal (epithelial cell turnover) to maintain homeostasis. When the host is faced with pathogen infection, the self-renewal process accelerates, leading to the fast elimination of damaged cells. For example, *Citrobacter rodentium* infection in mice stimulates stem cells regeneration (Sellin et al., 2009) to replace the damaged cells. In summary, intestinal barrier uses multiple protective mechanisms to maintain the host's health.

The healthy intestinal barrier is of significant important to the neonates, and the breakdown of the barrier function predisposes the gut to the risks from intestinal pathogens and toxins (Bjarnason et al., 1994). Early life microbiota has been proven to be closely related to intestinal barrier development. The immature gut barrier in preterm infants is closely related to necrotizing enterocolitis (NEC) (Grave et al., 2007). Through the mechanism for the NEC is still not clear, the administration of probiotic *Bifidobacterium infantis* protects the intestinal barrier by strengthening the tight junctions in neonatal mice model with NEC (Bergmann et al., 2013), indicating that probiotics may help to reduce NEC in premature infants by improving intestinal barrier function.

1.6.4 Prevention of pathogen invasion by intestinal microbiota

Commensal bacteria serve as a major luminal barrier to compete against the pathogens by the following mechanisms, competing for nutrients, producing metabolites (antibiotics such as

bacteriocins), stimulating host immune defense, and accelerating gut motility to prevent pathogens colonization (Abt and Pamer, 2014). For example, *Bifidobacterium* inhibits Enterohemorrhagic *Escherichia coli* infection and Shiga toxin release by producing acetate in the murine gut (Fukuda et al., 2011). Meanwhile, commensal bacteria drive intestinal epithelial cells to secrete anti-microbial peptides, inhibiting pathogens expansion in mice (Cash et al., 2006). Moreover, commensal *Bifidobacterium breve* stimulates an immunoregulatory response by generating exopolysaccharide, which inhibits *Citrobacter Rodentium* expansion (inducing colonic crypt hyperplasia) in mice (Fanning et al., 2012). Overall, the above findings from mice and human suggest that commensal bacteria are crucial to prevent pathogens colonization in the gut, however, such knowledge on the dairy calf is limited.

1.7 Nutritional management and early microbiota in neonatal calves

With the development of next generation sequencing method, we are getting more knowledge about the microbiota composition and functions, as well as the importance of early life microbiota on host life-long health. Therefore, it is necessary to apply practical methods to regulate microbial composition in the dairy production. It has been reported that feeding heat treated (at 60 ℃, 60 min) colostrum enhanced the abundance of small intestinal beneficial bacteria (*Bifidobacterium*) and reduced the abundance of potential pathogenic bacteria (*E. coli*) colonization in calves compared to non-heated colostrum (Malmuthuge et al., 2014), suggesting that feeding heated colostrum shape the bacterial composition towards a "good" direction. Meanwhile, delayed colostrum feeding to 12 h after birth has been shown to reduce the proportion of mucosa-attached *Bifidobacterium* spp., *Lactobacillus* spp., and ileum mucosa-attached *E. coli* in the colon of 2-days old calves, when compared to calves fed colostrum within

one hour after birth (Fischer et al., 2018), implying that delaying colostrum feeding after birth may postpone bacterial colonization in the gut. Moreover, previous study found significant compositional differences at genus level when compared the effect of milk supplemented with antibiotics on fecal microbial profile with milk only, suggesting that antibiotics residues in the milk disrupt the fecal microbiota (Van Vleck Pereira et al., 2016). All the findings above indicate that early life microbial profile can be manipulated through nutritional management strategies, which may affect the long-term health of the dairy calves. Therefore, more researches on the influence of nutritional management on early life microbiota are encouraged.

1.8 Hypotheses and objectives

Based on above literature review, the following knowledge gaps are identified, 1) hindgut microbial composition and fermentation products during the pre-weaning period; 2) how different colostrum feeding strategies affect gut microbial composition and function; and 3) how different colostrum feeding management influence host gene expression. The overall hypotheses are 1) the intestinal microbiota composition and fermentation are affected by age in the hindgut; 2) different first colostrum feeding management (heat treated vs. non-heat treated colostrum, delayed colostrum feeding vs. undelayed colostrum feeding) affect intestinal microbial composition and function, which subsequently affects gut barrier and immune system development. To fill above knowledge gaps, the objectives of the thesis work are: 1) to investigate microbiota composition/population and short chain fatty acids profiles in the hindgut of dairy calves from birth to pre-weaning; 2) to investigate the shift in colon microbiota composition in response to different colostrum feeding (no colostrum, NC; non-heated colostrum, FC; heated colostrum, HC) treatments during first 12 h of life; 3) to characterize the

effect of delayed first colostrum feeding on ileal microbiome (composition and function); 4) to identify the effect of delayed colostrum feeding on ileum gene expression and the relationship between ileum mucosa-attached microbiota and the expression of genes involved in gut barrier and immune system development. In this thesis, four studies were performed to achieve the above objectives. Hindgut regions were selected to study microbiota composition (Chapter 1) and its shift in response to feeding different type of colostrum (Chapter 2) because the small intestinal microbiota of the same animals have been examined previously (Malmuthuge et al., 2015), and by looking at the hindgut microbiota, it will generate the full picture on the gut microbiota of the dairy calves during early life. In chapters 4 and 5, we focused on ileum mainly due to the following reasons: 1) ileum has a moderate pH (7-8) for microbial colonization (10^8 cells/mL) (Walter and Ley, 2011); 2) ileum is rich in immune cells and transcriptome analysis has revealed that ileum is important to neonatal calves' mucosal immune system development during early life (Liang et al., 2016).

Overall, the findings from this thesis will provide fundamental knowledge of hindgut microbial colonization during the pre-weaning period and highlight the potential role of colostrum management in optimisation of intestinal microbiota of calves during early life. The data presented in this thesis can be used to guide producers to improve current nutritional management schemes via implementation of novel colostrum feeding strategies to optimize gut microbiota colonization and improve the intestinal health from microbiology point of view.

1.9 References

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1.10 Table

Table 1.1 Studies on the microbiota composition during pre-weaning period in dairy calves.

Study	Sampling time	Sample	Predominant bacteria
Oikonomou et al., 2013	1-7 week	feces	*Fimicutes*
Foditsch et al., 2015	1-7 week	feces	*Fimicutes*
Uyeno et al., 2010	first 12 weeks	feces	*Bacteroidetes*
	first 12 weeks	feces	*Bacteroides-Prevotella*, *Clostridium coccoides-Eubacterium rectale* groups
Klein-Jöbstl et al., 2014	Within 12 h after birth, 2,3,5-6, before and after weaning	feces	*Bacteroidetes*
Malmuthuge et al., 2014	3 week	Digesta of small intestine	*Fimicutes*
	3 week	Mucosa of small intestine	*Bacteroidete*s
Mayer et al., 2012		meconium	*Citrobacter*, *Lactococcus*, *Leuconostoc*, *Lactobacillus*
	24 h-48 h		*Clostridium* spp
	24 h-7 day		*E. coli*

Chapter 2. Shift of hindgut microbiota and microbial short chain fatty acids profiles in dairy calves from birth to pre-weaning

2.1 Introduction

The dairy industry in the North America has been consistently challenged with high mortality (8% – 10%) and morbidity (~38.5%) rates, which lead to on-farm economic losses and detrimental effects on the later life performance of dairy cattle (Donovan et al., 1998; USDA, 2010). It has also been estimated that about 50% of the pre-weaned calf deaths are caused by enteric infections (USDA, 2010), which is usually caused by pathogenic organisms (Cho and Yoon, 2014). Therefore, the improved gut health is one of the ways to minimize the pathogen colonization and to reduce the prevalence of enteric infections. It is known that microbes colonize the gastrointestinal tract (GIT) of mammals soon after birth and they play important roles in host immune system development, metabolism, and health of human and mouse (Gaboriau-Routhiau et al., 2009; White et al., 2013; Arrieta et al., 2014; Subramanian et al., 2015). Research on the humans and mice hindgut microbiota has revealed that the short chain fatty acids (SCFA) including acetate, propionate and butyrate are the main microbial fermentation products (Topping and Clifton, 2001) that serve as the energy source to peripheral tissue and colonic epithelial cells (Bergman, 1990; Hamer et al., 2009). In addition, butyrate (one of the SCFAs) has been reported to enhance the gut barrier functions (VanHook, 2015). Recent research has also revealed that dysbiosis (imbalance) of the hindgut microbiota is associated with inflammatory bowel disease in human and mouse (Du et al., 2015; Kabeerdoss et al., 2015), further highlighting the importance of the hindgut microbiota contributing to the host functions.

To date, there is limited knowledge on the hindgut microbiota and its microbial fermentation profiles in ruminants, especially in neonatal dairy calves. The GIT of a calf

undergoes rapid anatomical, physiological and functional development before weaning, and pre-weaned ruminants (pre-ruminants) are usually considered functionally similar to monogastric animals (Heinrichs, 2005) due to their underdeveloped rumen. When the rumen is not developed, the plant fiber, oligosaccharide and resistant starch are indigestible by host enzymes and can usually reach the colon, where they are fermented by the gut microbiota (Macfarlane and Englyst, 1986; Saulnier et al., 2009). In addition, the degradation of undigested proteins and fermentation of amino acids in the hindgut can produce branched-chain fatty acids, such as isobutyrate and isovalerate (Jha and Berrocoso, 2016). Therefore, we hypothesized that prior to the complete development of the rumen, hindgut microbial fermentation plays an important role in providing energy to the pre-weaned calves.

Previous studies on the gut microbiota of the pre-weaned calves reported that the microbial composition in the feces and the rumen varied with calf age (Uyeno et al., 2010; Li et al., 2012; Jami et al., 2013; Oikonomou et al., 2013; Klein-Jöbstl et al., 2014) and weaning process (Meale et al., 2016). Another study has revealed regional variations in the microbial composition along the GIT of 3-week-old pre-weaned calves, with cecum and colon microbiota similar to that of rumen (Malmuthuge et al., 2014). Additionally, the activity of xylanases and amylases has been detected in the cecum and colon of 28- day old pre-weaned goats (Jiao et al., 2015), indicating an active microbial fermentation in the hindgut of pre-ruminants. Yet, the understanding of the hindgut microbiota and the fermentation process is very limited in the pre-weaned dairy calves. In this study, we characterized the hindgut microbial composition and fermentation parameters during the pre-weaning period and explored the association between the hindgut microbiota and microbial fermentation from birth to 6 weeks of life.

2.2 Materials and methods

2.2.1 Animal study and sample collection

Animal experiments were conducted at the Dairy Research and Technology Centre, University of Alberta, following the protocols approved by the Livestock Animal Care committee of the University of Alberta (protocol no., AUP00001012). All procedures were conducted following the guidelines of the Canadian Council on Animal Care, and the detailed information on the animal trial has been reported previously (Liang et al., 2014). Briefly, calves were received 4 L of colostrum/day during the first 3 days after birth, and 4L of whole milk/day from the fourth day onwards. Calves had ad libitum access to calf starter (23% crude protein and 4% ether extract, 19.5% neutral detergent fiber, 27.1% starch; Wetaskiwin Co-Op Country Junction, Wetaskiwin, AB, Canada) from day 14 to day 42 postpartum. Calves involved in this study did not have respiratory or enteric diseases, and no antibiotic treatment was given during the experimental period. In the study, 24 Holstein bull calves were humanely sacrificed at four different time points: at birth (NB; n = 6), at day 7 (D7; n = 6), at day 21 (D21; n = 6), and at day 42 (D42; n = 6) to obtain tissue and digesta samples from three different hindgut regions (cecum, colon and rectum). To prevent luminal content flowing out from the GIT following euthanasia, esophagus and rectum were first ligated and then each segment was identified and separated using table ties to prevent the potential cross contamination. The whole cecum, 10-cm-long colon (defined as 30 cm distal to the ileo-cecal junction) and 5-cm-long rectum (proximal to the anus) were collected, snap-frozen in liquid nitrogen and stored at –80℃ until further analysis.

The sampling locations were kept constant for all calves using the predefined anatomical land marks.

2.2.2 DNA isolation

Genomic DNA was extracted from tissue and digesta samples, respectively, using the modified repeated bead-beating and column method (Yu and Morrison, 2004). For newborn samples, the whole tissue was processed due to lack of content. Digesta (~0.5 g) and tissue (0.1–0.2 g) samples were processed from the frozen sample and mixed with 1-mL cell lysis buffer (4% sodium dodecyl sulfate, 500 mL NaCl, 50 mM EDTA and 50 mM Tris-HCl), and were subjected to bead beating at 4800 rpm for 3 min using the BioSpec Mini BeadBeater 8 (BioSpec, Bartlesville, OK). Lysed cells were then incubated at 70◦C for 15 min, and the supernatant was collected for further process. Bead beating and incubation steps were repeated once, and all supernatants were combined. Genomic DNA was precipitated using 10 M ammonium acetate and iso- propanol following by the purification using QIAamp Fast DNA Stool Mini Kit (QIAGEN Inc. CA, USA). Quantity and quality of the extracted DNA were assessed with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.2.3 Estimation of total bacteria and selected bacterial groups in the hindgut of pre-weaned calves using quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed to estimate the copy number of 16S rRNA genes of total bacteria, *Bifidobacterium, Escherichia coli, Faecalibacterium prausnitzii* and *Clostridium* cluster XIVa using universal bacterial primers and group-specific bacterial primers (Table 2.1) with SYBR green chemistry (fast SYBR green master mix; Applied Biosystems, Foster City, CA, USA) on a StepOnePlus real-time PCR system (Applied Biosystems). The standard curves for total bacteria, *Bifidobacterium, E. coli, F. prausnitzii* and *Clostridium* cluster XIVa were generated using plasmid DNA containing the insert of

Butyrivibrio hungatei for total bacteria and the cloned purified PCR products of *B. longum, E. coli K12* (Malmuthuge et al., 2015), *F. prausnitzii* A2-165 and *Roseburia hominis* A2-183, respectively (Ramirez-Farias et al., 2009). The copy number of 16S rRNA gene of mucosa-attached and digesta-associated bacteria (copy number/g sample) was calculated using the equation described by Li et al. (2009). In addition, the proportion of *Bifidobacterium, E. coli, F. prausnitzii* and *Clostridium* cluster XIVa was calculated by dividing the copy number of each bacterial group with the copy number of total bacteria.

2.2.4 Profiling of the hindgut microbiota using amplicon sequencing

Amplification of V1-V3 hypervariable region of the 16S rRNA gene for amplicon sequencing was performed through a nested PCR based approach. Briefly, the total bacterial full length 16S rRNA gene was firstly enriched through PCR amplification with 27F and 1492R primers (27F 5′-AGAGTTTGATCMTGGCTCAG-3′, 1492R: 5′-TACGGYTACCTTGTTACGACTT-3′) (Lane et al., 1991). Then, 10 times diluted PCR products was subjected to a second amplification with 27F and 515R primers (27F-CS1F: ACACTGACGACATGGTTCTACAGAGTTTGATCMTGGCTCAG, 515R-CS2R: TACGGTAGCAGAGACTTGGTCTCCGCGGCKGCTGGCAC) (Kroes et al.,1999) containing pyrotags. The amplicon DNA with targeted size (~ 500 bp) was purified from 1% agarose gel using QIAEX II gel extraction kit (Qiagen Science, MD, USA). The quality and quantity of purified PCR products were evaluated using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE) to ensure that the concentration of DNA from all samples was higher than 25 ng/μL. The amplicons were sequenced at Genome Quebec at McGill University (Montreal, QC, Canada) using 454-sequencing of Roche GS-FLX system with Titanium chemistry.

2.2.5 Taxonomic identification and microbial function prediction

Sequence data was analyzed using QIIME (Quantitative Insight into Microbial Ecology) package, version 1.9 (Caporaso et al., 2010). Firstly, low quality (Phred score < 20) and short reads (< 100 bp) were filtered out from the demultiplexed raw sequences. Then, the chimeric sequences were removed using ChimeraSlayer (Haas et al., 2011) and the remaining sequences were subjected to operational taxonomic units (OTUs) identification based on 97% similarity using closed reference OTU picking function. Taxonomic characterization was performed using the SILVA database (SILVA Release 123, July 2015 release). Alpha diversity indices (Chao 1, Shannon and observed species) and Good's coverage was calculated using alpha rarefaction script within QIIME. Principal coordinate analysis (PCoA) of the microbial profiles was conducted using UniFrac distance metrices. Analysis of similarity (ANOSIM) was used to test the statistical differences among the observed microbial profiles based on sample type, age and region in the hindgut.

The "biom file" generated with assign taxonomy.py was used to predict the functions of the hindgut microbiota with Tax4Fun (A software package that could predict microbial function based on 16S rRNA datasets) (Aßhauer et al., 2015), which is a computational approach to predict the metagenomic function with 16S rRNA marker gene and the reference genomes without using metagenomic and metatranscriptomic techniques (Aßhauer et al., 2015). The functions were summarized at hierarchy level 2 of KEGG pathways.

2.2.6 Measurement of short chain fatty acids (SCFA) concentration in the hindgut of pre-weaned calves

About 0.1 g of digesta sample was weighed and transferred to a 5 mL tube and vortex vigorously until it was fully dissolved in 25% phosphoric acid (4:1; v:v). The concentrations of

acetate, propionate, butyrate, isobutyrate, isovalerate and valerate were measured using gas chromatography, as described by Guan et al. (2008). The SCFA concentrations were presented as μmol/g fresh weight of digesta.

2.2.7 Statistical analyses

Data were analyzed using R version 3.3.1 and SAS 9.4 packages. The effect of age, hindgut region, and sample type on the relative abundance of detected bacterial groups was assessed using nonparametric Kruskal-Wallis test statistical method in R (3.3.1). Effects of the above-mentioned factors on copy number of the 16S rRNA gene of total bacteria, specific bacterial groups, and SCFA concentration were analyzed using PROC MIXED and repeated measure experimental design in SAS (SAS 9.4, SAS Inc, Cary, NC). The repeated measurement was the hindgut region (cecum, colon, rectum) and the experimental unit was individual calf. Compound symmetry covariance structure was selected as the best fit by the Bayesian information criteria. Analysis was performed using the following statistical model; $Y=\mu + T_i + R_j + T_iR_j + e_{ij}$, where, T= age, R=hindgut region, e=residual error, Y=bacterial copy number (total bacteria, *E. coli*, *Bifidobacterium*, *F. prausnitzii* and *Clostridium* cluster XIVa), concentration of SCFA (acetate, propionate, butyrate, isobutyrate, isovalerate, valerate and total SCFA), OTUs, Observed_species, Chao 1 and Shannon index. Differences in the least square means were declared at $P < 0.05$. Bacterial genera with the relative abundance >0.1% and present in more than half number of the total animals at least in one age group were used to perform correlation analysis. Spearman's rank correlations were performed between the relative abundance of mucosa- and digesta-associated bacterial genera and the concentration of SCFAs (acetate, propionate, butyrate and total SCFA) as well as the proportion of 16S rRNA gene copy numbers (*E. coli*, *Bifidobacterium*, *F. prausnitzii*, *Clostridium* cluster XIVa, total bacteria) and SCFAs to

understand the relationships between the hindgut bacteria and fermentation parameter. Significant correlations were declared at $-0.5 > \rho > 0.5$, and P-value < 0.01.

2.2.8 Data submission

All the sequences were deposited at NCBI Sequence Read Archive (SRA) and are publicly accessible under the accession number SRP102324. The files could be found in the following link: https://trace.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi?subid=887915&from=list&action=show:submission.

2.3 Results

2.3.1 The hindgut microbial community of the pre-weaned calves differed among different age groups

Amplicon sequencing of the mucosa-attached microbiota generated 390,726 high quality sequences (5,426±210 for cecum, 5,486±200 for colon, and 5,468±199 for rectum) that were assigned to a total of 37,858 operational taxonomic units (OTUs) based on 97% nucleotide sequence similarity. The Good's coverage ranged between 0.917 and 0.918 (Table 2.2) for the mucosal-attached microbial community. Similarly, 257,722 sequences (5,072±234 for cecum, 5,067±241 for colon, 5,078±252 for rectum) were generated for digesta-associated community that were assigned to a total of 31,300 OTUs. Good's coverage of digesta-associated microbial communities ranged from 0.894 to 0.896 (Table 2.2). PCoA revealed that the bacterial profiles generated from mucosa-attached microbiota tended to separate (ANOSIM R =0.34, P <0.01) from digesta-associated communities, regardless of calf age and hindgut region (Figure 2.1). Therefore, further analyses of the age and the hindgut region effects were performed separately

for each microbial community. Mucosa-attached bacterial profiles formed distinct clusters according to calf age (ANOSIM R =0.64, P <0.01) (Figure 2.2A), in which profiles of NB and D7 separated from those of D21 and D42. While the digesta-associated bacterial profiles tended to separate according to calf age (R=0.22, P <0.01) (Figure 2.2B). However, both microbial profiles did not differ among the hindgut regions (ANOSIM R = -0.03, P =0.98 for mucosa-attached community; R = -0.04, P =0.99 for digesta-associated community) (Figure 2.2C and 2D).

When the diversity indices of the hindgut mucosa-attached microbiota were compared, the lowest number of OTUs, observed species, Chao 1 and Shannon index were observed at D7 when compared to other age groups. In addition, number of OTUs, observed species, Chao 1 and Shannon index were all significantly higher at D21 and D42 than D7 and NB (Table 2.3). For digesta-associated communities, lower observed species, Chao 1, Shannon index and number of OTUs were observed at D7, with no significant differences were observed among other age groups. There were no regional effects observed in the diversity of mucosa- and digesta-associated microbial communities (Table 2.3).

2.3.2 Comparison of the microbial profiles using UniFrac dissimilarity

When the microbial profiles were compared using UniFrac dissimilarity index calculated based on pairwise comparisons of individual microbial profile of different groups (age, region or sample type), the mucosa-attached microbiota had a higher UniFrac dissimilarity among individuals than the digest-associated bacterial community (P< 0.01, Figure 2.3). The UniFrac dissimilarity of the mucosa-attached microbial community was significantly affected by age (P<0.01), in which the highest UniFrac dissimilarity among individuals was observed at D7 (Figure 2.3). The UniFrac dissimilarity among newborn calves was higher than those at D21 and

D42 but was lower than that of D7 (Figure 2.3). When the similarity among individuals was compared for digesta-associated communities, a lower UniFrac dissimilarity was observed at D42 than that at D7 and D21, respectively (Figure 2.3).

2.3.3 Taxonomic composition of the newborn calf hindgut microbiota

In total, 16 bacterial phyla were identified (Supplementary Dataset 2.1a) from NB hindgut microbial communities. Seven out of sixteen phyla were defined as the detected bacterial phyla (the relative abundance >0.1% and present in more than half number of the total animals at least in one age group) in the NB hindgut (Figure 2.4A). *Proteobacteria* (33.85±3.75%), *Firmicutes* (33.32±2.73%) and *Bacteroidetes* (28.34±2.74%) accounted for the majority of the detected bacterial phyla in the hindgut at birth. At family level, 87 families were identified and 24 families were considered as detected using the same cut-off defined above (Figure 2.4B). *Enterobacteriaceae* was the most abundant bacterial family (19.09±2.38%) at birth, followed by *Bacteroidaceae* (18.83±2.04%), *Ruminococcaceae* (13.66±1.01%), *Lachnospiraceae* (10.23±1.28%), *Burkholderiaceae* (6.23±2.32%), *Prevotellaceae* (5.88±0.52%) and *Lactobacillaceae* (5.26±1.25%) (Supplementary Dataset 2.1b). At genus level, 250 genera were identified (Supplementary Dataset 2.1c) and 61 genera were defined as detected genera (Figure 2.4C). Among them, *Bacteroides* (18.83±1.98%) and *Escherichia-Shigella* (13.52±1.66%) were the most abundant bacterial genera in the hindgut of newborn calves.

2.3.4 Taxonomic composition of the hindgut mucosa-attached microbiota and shifts during pre-weaning

In total, 16 phyla were identified from mucosa-attached communities of the hindgut (Supplementary Dataset 2.2a) and seven were considered as detected phyla (Table 2.4) in the hindgut of pre-weaned calves. The three predominant phyla in the hindgut mucosa-attached

microbiota of pre-weaned calves were *Bacteroidetes* (35.96±1.48%), *Firmicutes* (42.24±1.81 %) and *Proteobacteria* (14.92±2.08 %) (Supplementary Dataset 2.2a). The relative abundance of *Proteobacteria* was higher at NB (33.85±2.08%) and D7 (20.52±2.08 %) compared to that at D21 (2.41±2.08 %) and D42 (2.91±2.08%) ($P < 0.01$). In contrast to *Proteobacteria*, *Firmicutes* increased significantly ($P<0.01$) at D21 (55.49±1.81%) and D42 (49.26±1.81%) compared with NB (33.32±1.81%) and D7 (30.89±1.81%). *Bacteroidetes* had the lowest relative abundance at NB (28.34±1.48%), and started to increase after D7. The relative abundance of *Fusobacteria* was numerically higher at D7 (8.95± 1.00 %) compared to that of NB (1.87±1.00%), D21 (3.09±1.00%) and D42 (6.60±1.00%) calves (Table 2.4a).

From 120 identified families (Supplementary Dataset 2.2b), 24 were considered as detected families (Table 2.4b). *Bacteroidaceae* was the predominant family in the hindgut mucosa-attached microbiota, with the highest relative abundance at D7 (26.26±1.43%) compared with NB (18.83±1.43%), D21 (15.65±1.43%) and D42 (9.75±1.43%) ($P<0.01$). *Enterobacteriaceae* had a higher relative abundance at NB (19.09±1.04%) and D7 (13.73±1.04%) calves than that at D21 (1.10±1.04%) and D42 (1.22±1.04%) calves ($P<0.01$). The relative abundance of *Lactobacillaceae* had a similar changing pattern as that of *Enterobacteriaceae*, higher at NB (5.26± 0.08%) and D7 (7.73±0.08%) than those at D21(0.25±0.08%) and D42 (0.28±0.08%) calves ($P<0.01$). On the contrary, the relative abundance of *Ruminococcaceae* was lower at NB (13.66±1.06 %) and D7 (10.86 ±1.06%) compared to that at D21(27.37±1.06%) and D42 (19.63±1.06%) ($P<0.01$). Similarly, *Lanchnospiraceae* had lower relative abundance at NB (10.23±0.89%) and D7 (9.00±0.89%) in comparison to that at D21 (23.46±0.89%) and D42 (18.15±0.89%) ($P<0.01$), respectively. In addition, the relative abundance of *Burkholderiaceae* was the highest at NB (6.23±0.04%)

compared with that at D7 (0.13±0.04%), D21(0.04±0.04%) and D42 (0.37±0.04%) (P<0.01). (Table 2.4b).

At genus level, 349 genera were identified from the mucosa-attached microbial community and 61 genera were considered as detectable. Genera *Bacteroides, Prevotella* 9*, Blautia, Lachnoclostridium, Lachnospiraceae* UCG-004*, Roseburia, Tuzzerella* 4*, Ruminococcus* 2*, Fusobacterium and Escherichia-Shigella* were present in all the animals (Supplementary Dataset 2.2c and Table 2.4c). The relative abundance of *Lactobacillus* was higher at NB (5.26±0.08%) and D7 (7.73±0.08 %) than that at D21 (0.25±0.08%) and D42 (0.28±0.08%) (P<0.01) calves. The relative abundance of *Escherichia-Shigella* was higher in NB (13.52±0.72%) and D7 (9.69±0.72%) calves than in D21 (0.74±0.72%) and D42 (0.92±0.72%) (P<0.01) calves. Similarly, *Salmonella* was higher at NB (2.64±0.09%) and D7 (1.81±0.09%) compared with D21 (0.16±0.09%) and D42 (0.16±0.09%) (P<0.01). *Faecalibacterium, Lachnospiraceae* NC2004 group*, Ruminococcaceae* UCG-014 *and Blautia* had highest relative abundance at D21, comparing with other age groups (Table 2.5).

2.3.5 Taxonomic composition of the hindgut digesta-associated microbiota and shifts during pre-weaning period

In total, 15 phyla were identified from digesta-associated microbiota (Supplementary Dataset 2.2d), with six of them being detected (Table 2.4d). Regardless of the hindgut region, *Firmicutes* was the most predominant phylum detected in digesta-associated microbial community of all ages (D7 - 61.76±1.55%; D21 - 73.75±1.55%; D42 - 73.90±1.55%) (P=0.01). *Bacteroidetes* was the second most abundant phylum in all the age groups (D7 - 20.81±0.89%, D21 - 20.94±0.89%, D42 - 21.36±0.89%). *Proteobacteria* was the third predominant phylum in

digesta-associated microbiota community, and the relative abundance was (7.37±0.66%) at D7, (1.92±0.66%) at D21 and (2.05±0.66%) at D42 (P<0.01), respectively (Table 2.4d).

At family level, 83 families were identified (Supplementary Dataset 2.2e) and 20 of them were considered as detected. Digesta-associated *Lactobacillaceae* was the predominant family (22.36±0.90% at D7; 20.01±1.37% at D21; 21.01±1.37% at D42) (P=0.90). In addition, *Lachnospiraceae*, *Bacteroidaceae* and *Ruminococcaceae* were also the predominant families. Among all the detected families, the relative abundances of *Bacteroidaceae* (9.28±0.59% at D7; 4.64±0.59% at D21; 4.51±0.59% at D42) (P<0.01), *Enterobacteriaceae* (4.99±0.45% at D7; 1.35±0.45% at D21; 1.39±0.45% at D42) (P<0.01) and *Bifidobacteriaceae* (1.06±0.14% at D7; 0.18±0.14% at D21; 0.15±0.14% at D42) (P=0.06) were higher at D7 compared with D21 and D42. While the relative abundances of *Lachnospiraceae* (17.46±1.03% at D7; 25.07±1.03% at D21; 23.85±1.03% at D42) (P<0.01) and *Ruminococcaceae* (15.59±0.84% at D7; 20.10±0.84% at D21; 19.67±0.84% at D42) (P=0.03) were higher at D21 and D42 compared with those at D7 (Table 2.4e).

At genus level, 50 genera were considered as detected out of the 249 identified genera. Genera *Collinsella, Blautia, Lachnoclostridium, Lachnospiraceae* UCG-004*, Lachnospiraceae* UCG-008*, Roseburia, Tyzzerella* 4*, Intestinibacter, Faecalibacterium, Subdoligranulum,* and *Erysipelotrichaceae* UCG-003 were present in all samples (Supplementary Dataset 2.2f and Table 2.4f) with *Lactobacillus* (22.36±1.37% at D7; 20.01±1.37% at D21; 21.01±1.37% at D42) (P=0.90) being the most abundant genus. In addition, the relative abundances of *Bacteroides* (9.28±0.59% at D7; 4.64±0.59% at D21; 4.51±0.59% at D42) (P<0.01), *Megasphaera* (2.72±0.24% at D7; 0.32±0.24% at D21; 0.26±0.24% at D42) (P<0.01), *Escherichia-Shigella* (3.67±0.33% at D7; 0.98±0.33% at D21; 0.98±0.33% at D42) (P<0.01) and *Salmonella*

(0.51±0.05% at D7; 0.13±0.05% at D21; 0.12±0.05% at D42) (P=0.03) were highest at D7. Moreover, the relative abundances of *Blautia* (5.50±0.80% for D7, 13.42±0.80% for D21, 11.87± 0.80% for D42) (P<0.01), *Coprococcus* 1 (0.06±0.02% for D7, 0.18±0.02% for D21, 0.13± 0.02% for D42) (P<0.01), *Lachnospiraceae* NK4A136 group (0.14±0.03% for D7, 0.34±0.03% for D21, 0.29± 0.03% for D42) (P<0.01), *Lachnospiraceae* UCG-008 (0.55±0.04% for D7, 0.71±0.04% for D21, 0.81± 0.04% for D42) (P<0.01), *Pseudobutyrivibrio* (0.29±0.05% for D7, 0.73±0.05% for D21, 0.65± 0.05% for D42) (P<0.01), *Ruminiclostridium* 5 (0.07±0.02% for D7, 0.21±0.02% for D21, 0.18± 0.02% for D42) (P<0.01), *Ruminiclostridium* 6 (0.13±0.06% for D7, 0.36±0.06% for D21, 0.33± 0.06% for D42) (P<0.01) and *Ruminococcus* 1(0.14±0.02% for D7, 0.22±0.02% for D21, 0.27± 0.02% for D42) (P<0.01) (P=0.01) were higher at D21 and D42 than those at D7 (Table 2.6).

2.3.6 Comparison between mucosa- and digesta-associated bacterial communities

Among all the bacterial genera detected in the hindgut, 45 of them were present in both mucosa- and digesta-associated communities. Among the common bacterial genera, 30 genera were significantly different between two communities. Bacterial genera that were highly abundant in mucosa-attached community included *Bacteroides*, *Parabacteroides*, *Alloprevotella*, *Prevotella* 9, *Faecalibacterium*, *Ruminococcus* 2, *Fusobacterium*, *Salmonella* and *Escherichia-Shigella*, while bacterial genera *Atopobium*, *Collinsella*, *Coriobacteriaceae* UCG-002, *Alistipes*, *Lactobacillus*, *Christensenellaceae* R-7 group, *Blautia*, *Dorea*, *Lachnoclostridium*, *Lachnospiraceae* NC2004 group, *Lachnospiraceae* UCG-008, *Pseudobutyrivibrio*, *Intestinibacter*, *Peptoclostridium*, *Romboutsia*, *Ruminiclostridium* 5, *Ruminococcaceae* UCG-005, *Ruminococcaceae* UCG-014, *Subdoligranulum, Erysipelotrichaceae* UCG-003, *Megasphaera* were highly abundant in the digesta-associated community (Table 2.7).

In addition, *Rhodococcus, Moryella, Bifidobacterium, Ruminococcaceae* NK4A214 group and *Akkermansia* were only detected in the digesta-associated microbiota, while *Acidaminococcus, Sutterella, Phascolarctobacterium, Ruminiclostridium* 9*, Streptococcus, Lachnospiraceae* ND3007 group, *Pseudomonas, Ruminococcaceae* UCG-002*, Pantoea, Coprococcus* 3*, Anaerovibrio, Odoribacter, Edaphobacter, Citrobacter, Burkholderia, Prevotella* 7 were only identified in mucosa-attached microbiota community (Table 2.7).

2.3.7 Estimation of bacterial densities in the hindgut of pre-weaned calves

Estimation of selected bacteria using quantitative real-time PCR (qPCR) showed a significant age effect on the proportion of mucosa-attached *E. coli*, *Bifidobacterium*, *Clostridium* cluster XIVa and *F. prausnitzii* (Table 2.8). The proportion of *Bifidobacterium* was the highest at D7 (59.90±4.33%) compared to that of other age groups regardless of the hindgut region. *E. coli* had the highest proportion at D7 (3.57±0.42%) following lower abundance at D21 (1.30±0.37%) and D42 (0.75±0.36%) with no difference observed between D21 and D42. The proportion of *Clostridium* cluster XIVa was the highest at D21 (15.83±1.28%) compared to all other age groups. In the digesta-associated community, effect of age was noted on the proportion of *E. coli* and *Clostridium* cluster XIVa (Table 2.8). Similar to mucosa-attached bacteria, the proportion of *E. coli* was the highest at D7 (0.07±0.01%), while the proportion of *Clostridium* cluster XIVa was higher at D21 (2.90±0.32%) and D42 compared with that at D7 (2.75±0.29%).

2.3.8 Predicted function of the hindgut microbiota in pre-weaned calves using Tax4Fun

Tax4Fun based functional prediction revealed top 10 microbial functions of the mucosa- and digesta-associated hindgut communities. These functions include “metabolism of cofactors and vitamins”, “energy metabolism”, “carbohydrate metabolism”, “amino acid metabolism”, “translation”, “replication and repair”, “nucleotide metabolism”, “signal transduction”,

"metabolism of other amino acids" and "membrane transport" (Figure 2.5A and 2.5B). In the mucosa-attached bacterial community, functions related to "nucleotide metabolism", "translation", "replication and repair" and "amino acid metabolism" was higher at D21 and D42 compared with those at NB and D7. While "signal transduction" and "carbohydrate metabolism" were higher at NB and D7 compared to D21 and D42 (Figure 2.5A). In the digesta-associated bacteria, the functions of "replication and repair", "signal transduction", "translation", "metabolism of other amino acids" were higher at D21 and D42 in comparison to D7. However, "energy metabolism", "metabolism of cofactors and vitamins" and "carbohydrate metabolism" were higher at D7 when compared to D21 and D42 (Figure 2.5B).

2.3.9 Microbial SCFA detected in the hindgut of pre-weaned dairy calves

Concentrations of total SCFA, acetate, propionate, butyrate, isobutyrate and isovalerate were significantly different depending on the calf age (Table 2.9). Concentration of acetate, butyrate and total SCFA increased from D7 onwards, with the highest concentration at D21 regardless of gut region. Propionate and isobutyrate concentration was significantly higher at D21 and D42 compared to those at D7 (Table 2.9). In addition, concentrations of butyrate, isobutyrate, isovalerate and valerate were significantly different among the hindgut regions, with the highest concentration detected in the rectum. Moreover, the molar proportion of acetate, propionate, butyrate, isobutyrate, isovalerate and valerate was also significantly different among the hindgut regions regardless of calf age.

2.3.10 Relationship between bacteria and fermentation parameters

To explore the potential roles of bacteria in the hindgut fermentation, the relationship between SCFA (acetate, propionate, butyrate and total SCFA) concentrations and the relative abundance of mucosa-, digesta-associated bacterial genera was explored using Spearman's rank

correlations. Acetate concentration was positively correlated with the relative abundance of carbohydrate-utilizing bacteria, including *Anaerostipes* (ρ=0.59, *P*<0.01), *Blautia* (ρ=0.68, *P*<0.01), *Coprococcus* 1 (ρ=0.67, *P*<0.01), *Lachnospiraceae* NC2004 group (ρ=0.56, *P*<0.01), *Pseudobutyrivibrio* (ρ=0.63, *P*<0.01), *Ruminiclostridium* 5 (ρ=0.68, *P*<0.01), *Ruminiclostridium* 6 (ρ=0.72, *P*<0.01) and *Ruminiclostridium* 9 (ρ=0.55, *P*<0.01). In addition, propionate concentration was positively correlated with the relative abundance of mucosa-attached *Coprococcus* 1 (ρ=0.56, *P*<0.01), *Lachnospiraceae* NC2004 group (ρ=0.54, *P*<0.01), *Ruminiclostridium* 6 (ρ=0.50, *P*<0.01). Moreover, butyrate concentration was positively correlated with mucosa-attached *Coprococcus* 1 (ρ=0.54, *P*<0.01), *Lachnospiraceae* NC2004 group (ρ=0.63, *P*<0.01), *Faecalibacterium* (ρ=0.63, *P*<0.01) and *Ruminiclostridium* 5 (ρ=0.54, *P*<0.01). Furthermore, total SCFA concentration was positively correlated with the relative abundance of mucosa-attached *Anaerostipes* (ρ=0.53, *P*<0.01), *Blautia* (ρ=0.61, *P*<0.01), *Coprococcus* 1 (ρ=0.61, *P*<0.01), *Lachnospiraceae* NC2004 group (ρ=0.57, *P*<0.01), *Pseudobutyrivibrio* (ρ=0.56, *P*<0.01), *Ruminiclostridium* 5 (ρ=0.62, *P*<0.01), *Ruminiclostridium* 6 (ρ=0.63, *P*<0.01), *Ruminiclostridium* 9 (ρ=0.50, *P*<0.01). Additionally, acetate was negatively correlated with the relative abundance of *Escherichia-Shigella* (ρ= -0.57, *P*<0.01) and *Salmonella* (ρ= -0.53, *P*<0.01). Moreover, we identified negative correlations between mucosa-attached *Bifidobacterium* with acetate (ρ= -0.50, *P*<0.01) and propionate (ρ= -0.57, *P*<0.01) and positive correlations between mucosa-attached *Clostridium* cluster XIVa with acetate (ρ=0.52, *P*<0.01) and total SCFA (ρ=0.50, *P*<0.01) (Figure 2.6).

2.4 Discussion

During early life of ruminants, milk by-passes the undeveloped rumen, nutrients are digested and absorbed in the lower GIT, and the non-digestible dietary substrates are fermented in the hindgut. Therefore, the microbial colonization in the hindgut of dairy calves during the pre-weaned period plays an important role in nutrient and energy harvest. The profiling of the microbiota revealed the colonization of a diverse and dense microbial population at both mucosal surface and in the lumen of hindgut starting from the calves were born.

The present study is the first to report the presence of highly diverse microbiota in the hindgut of NB calves within 30min after birth without any feeding (colostrum consumption). This suggests that the hindgut microbiota begin to colonize possibly during the birth process. The transmission process of maternal microbiota to NB calves may be similar to the findings reported for human NB infant gut microbiota, which could originate from amniotic fluid (DiGiulio, 2012), meconium (Moles et al., 2013) and fetal membranes (van den Berg, 2006). The significant individual variation among the NB calves may be due to the variations in the transmission process from the cow and birth environment (uterus, vaginal canal and fetal membranes).

The predominant identified families including *Bacteroidaceae*, *Lanchnospiraceae*, *Lactobacillaceae*, and *Enterobacteriaceae* in the hindgut of NB calves resembled the dominant families in one-day-old piglets (Frese et al., 2015). In addition, the presence of *Lactobacillus* as a predominant genus and higher relative abundance of facultative anaerobic *Enterobacteriaceae* family at birth and D7 is similar to fecal microbiota of vaginally delivered babies (Matamoros et al., 2013) and in the human infants' gut during early life (Arrieta et al., 2014). The roles of facultative anaerobes, such as *Enterobacteriaceae* spp., are to create the anaerobic environment

by utilizing available oxygen during the immediate neonatal period for the establishment of obligate anaerobes (Favier et al., 2002). In addition, family *Enterobacteriaceae* also contains many potential pathogenic bacteria belong to genera *Escherichia* (Moxley and Francsis, 1986) and *Salmonella* (Zhang et al., 2003). The observed high abundance of mucosa-attached *Escherichia-Shigella* and *Salmonella* during the first week suggests that the calves are more susceptible to infections due to the greater abundance of opportunistic pathogens during this period. On the other hand, the higher abundance of the mucosa-attached *Ruminococcus* at D21 compared with D7 was observed. A recent study has reported that *Ruminococcus gnavus* E1 can modulate the expression of mucins related gene and increase mucin production (Graziani et al., 2016), suggesting that species belong to *Ruminococcus* may play a role in increasing host resistance to pathogenic bacterial invasion through reinforced barrier functions at D21. Moreover, the observed high relative abundance of obligate anaerobic *Bacteroides* in the hindgut of NB calf could be due to the availability of substrates after birth, such as mucus glycans. It has been reported that *Bacteroides* spp. including *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* could utilize host mucus glycans (Marcobal et al., 2011). Based on these findings, it suggests that the hindgut microbiota of NB claves has similar microbial composition to that of the monogastric animals, with the capability to adapt to the anaerobic environment and potentially utilize the available substrates to define their colonization niches.

Similar to adult cattle, the *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* dominated the digesta-associated hindgut microbiota of pre-weaned calves. The relative abundance of these three dominant phyla is in agreement with the reported main phyla in the fecal microbiota within the first seven weeks of life in dairy calves (Oikonomou et al., 2013). This suggests that the microbial composition in the fecal sample is representative of the digesta-associated microbiota

in the hindgut. However, *Bacteroidetes* was dominated in the mucosa-attached community of three-week-old calves (Malmuthuge et al., 2014), whereas *Firmicutes* was found to be the predominant and *Bacteroidetes* was the second most abundant phylum in the hindgut of D21 calves in our study. Such discrepancy may be due to the use of different animals, different farm and management environments, diets and the different primers used to target 16S rRNA gene.

The segregation of mucosa- and digesta-associated bacterial profiles confirmed the previous findings in mouse (Swidsinski et al., 2005), pre-weaned dairy calves (Malmuthuge et al., 2012; Malmuthuge et al., 2014), and dairy cows (Mao et al., 2015; Liu et al., 2016). Previous findings have revealed that mucosa-attached microbiota is more diverse but has a lower microbial density (Malmuthuge et al., 2015; Mao et al., 2015) when compared to digesta-associated microbiota in ruminants, suggesting that the compositional difference in these two microbial communities is a common biological phenomenon. We also observed a high individual variation (higher UniFrac dissimilarity) within the mucosa-attached microbiota than that of the digesta-associated community, suggesting such segregation of these communities occur soon after birth and their ecological niches could be an essential factor that drives the divergence. Therefore, it is vital to study both communities to generate the full understanding of the gut microbiome and their roles in the hindgut during early life.

In addition to different microbial communities between two sample types (mucosa vs. digesta), we also observed compositional changes among age groups. The mucosa-attached bacterial community was significantly affected by the age, which might be influenced by host physiological changes (*e.g.* development of host immune system, epithelial integrity and growth) as well as diet during the pre-weaned period. It is noticeable that the effect of age in the present study is confounded with the dietary changes. The decrease of digesta-associated *Bacteroides* at

D21 and D42 compared to D7, similar to the reported trend in the rumen of pre-weaned calves (Li et al., 2012; Rey et al., 2014), could be due to the increase of age and the increased consumption of calf starter. Similarly, higher relative abundance of digesta-associated carbohydrate-utilizing bacterial genera (*Blautia*, *Ruminococcus, Coprococcus* 1, *Lachnospiraceae* NK4A136 group, *Pseudobutyrivibrio*, *Ruminiclostridium* 5 and *Ruminiclostridium* 6) at D21 compared with D7 are also due to increased intake of starter from D14, which provides available carbohydrates to stimulate the colonization of those bacterial groups.

It is known that genus *Bifidobacterium* is highly abundant in the infants' gut (Turroni et al., 2009; Fanaro et al., 2003) and our previous study revealed that the proportion of *Bifidobacterium* was high in the small intestine during the first 12 h of life, especially when colostrum was fed within the first twelve hours of life (Malmuthuge et al., 2015). In this study, the abundance of mucosa-attached *Bifidobacterium* (detected by qPCR) was higher at D7 than the older calves (D21 and D42), indicating the consumption of milk that is rich in oligosaccharides (Sela et al., 2008; Lozupone et al., 2013) could lead to higher population of this genus. In addition, the *Bifidobacterium* have been reported to form biofilm, which plays an important role in prevention of pathogen invasion and stimulate the host immune functions (Hidalgo-Cantabrana et al., 2013). Moreover, the abundance of *Bifidobacterium* has been reported to be highly correlated with the expression of genes and microRNAs that regulate host immune function in the small intestine of the same calves (Liang et al., 2014). Therefore, it is important to know how the diversity of mucosa-attached *Bifidobacterium* could be impact by the age and how this could influence the host functions. It is noticeable that although high copy number of *Bifidobacterium* 16S rRNA genes were detected in both mucosa and digesta-

associated communities, however, the amplicon sequencing only detected the digesta-associated *Bifidobacterium*. The universal bacterial primers (such as 27F and 1492R used in this study) are usually fail to amplify this genus (Malmuthuge et al., 2014) because the forward primer (27F) has a few mismatches with 16S rRNA gene sequence of *Bifidobacterial* genus (Frank et al., 2008), which leads to a lower amplification of *Bifidobacterial* sequences, resulting in a lower relative abundance. Therefore, future studies are needed to characterize the *Bifidobacterium* in the hindgut of dairy calves using *Bifidobacterium* specific primers.

It has been demonstrated that mucosa-attached bacteria could affect host immune system development, metabolism, and health (Moxley and Francis, 1986; Ivanov et al., 2009). A recent study also reported that SCFA can affect the intestinal cells turnover (Park et al., 2016). We speculate that the shifts in SCFA could also impact on the mucosa-attached bacteria population in addition to their impact on host tissues since the measured SCFA concentration in the lumen is the result of microbial production and host tissue absorption. The observed significant correlation between SCFA concentration and the relative abundance of mucosa-attached bacteria suggests a potential cross-talk between lumen microbial metabolites and mucosa-attached microbiota. For example, the observed negative correlation between the mucosa-attached *Escherichia-Shigella* abundance and acetate concentration (ρ= -0.57, P<0.01) in the study may support our above speculation. The decreased mucosa-attached *Escherichia* from D7 to D21 vs D42 may be caused by the increased acetate concentration in the gut after D7, as acetate has been reported to inhibit the growth of *E. coli* (Fukuda et al., 2011). It was surprising that no significant correlation was found between the relative abundance of digesta-associated bacteria and SCFA concentration in this study, suggesting that future analysis using the quantitative approach is

needed to verify the relationship between lumen microbes and SCFA and to verify whether the lumen SCFA is important in the potential cross-talk between microbes.

The higher SCFA concentrations at D21 compared with D42 and D7 (including acetate, butyrate and total SCFA) in the hindgut indicate the stronger fermentation ability of hindgut microbiota at D21 compared with D7 and D42. This could be explained by the increased development of rumen from D21 to D42 with increased solid feed intake (Malmuthuge et al., 2016), leading to less substance available to the hindgut microbiota fermentation. The lower SCFA concentration at D7 compared with D21 and D42 also indicates the less developed and/or functional gut at this stage. However, our limitation is that the dairy starter intake was not recorded during this study which was suggested to be recorded in the future study. Butyrate plays important roles in regulation gut physiology, immune system, and inflammatory response (Wang et al., 2012; Arpaia et al., 2013; Nastasi et al., 2015). It can enhance intestinal barrier function by increasing the expression of tight junction protein related genes (claudion-1, Zonula, Occludens-1) (Wang et al., 2012). Moreover, butyrate and propionate have been reported to regulate T cells production and function (Arpaia et al., 2013), as well as inhibiting lipopolysaccharide-induced expression of proinflammatory cytokines IL-6 and IL-12p40 (Nastasi et al., 2015). Therefore, the lower concentration of SCFA in the lower gut at D7 suggests potential lower immune function, and the importance to enhance gut health at this stage.

Microbial amino acid metabolism, carbohydrate metabolism and energy metabolism are crucial functions in the hindgut, which provide energy to the host (McNeil, 1984). The higher microbial energy metabolism at D7 indicates that microbiota during early life tend to harvest more energy from the lumen substance for their own growth and proliferation. In addition, the significant increase in amino acid metabolism of mucosa-attached bacteria at D21 and D42

suggests that bacteria tended to derive more energy from amino acid fermentation with increasing age of calves. The observed temporal variations in the predicted microbial functions of the hindgut bacteria suggests potential temporal variations in the energy harvesting mechanisms with the changes associated with host diet. It is noticeable that the functional prediction based on 16s rRNA gene is biased and future metagenomics and metatranscriptomics are needed to assess the hindgut microbiome. However, the predicted function could provide preliminary information of the hindgut microbial functions of pre-weaned calves.

2.5 Conclusion

This is the first study to explore the mucosa-attached and digesta-associated microbial composition along the cecum, colon and rectum using 16S rRNA sequencing during the pre-weaning period of dairy calves. The results showed effect of age on both communities, while no regional effect was detected. It is important to note that calf age is confounded by the changes in dietary regimes (*e.g*. colostrum, whole milk and calf starter feeding), the management (*e.g*. housing) and the potential rumen development, which can also influence the microbial composition in the hindgut of dairy calves during pre-weaned period. The changing pattern of the relative abundance of SCFA-producing bacterial genera, including *Christensenellaceae R-7 group*, *Blautia*, *Coprococcus* 1, *Lachnospiraceae* NK4A136 group, *Lachnospiraceae* UCG-008, *Pseudobutyrivibrio*, *Ruminiclostridium* 5, *Ruminiclostridium* 6 and *Ruminococcus* 1 with the increase of age was accompanied by the variation of SCFA concentration in the gut, indicating the importance of hindgut microbiota on energy harvest. The higher relative abundance of potential pathogenic bacteria *Escherichia-Shigella* and *Salmonella* during the first week may indicate that calves are more susceptible to intestinal infections. However, further studies are

needed to explore the functional roles of hindgut microbiota through metagenomics or metatranstriptomics-based approaches. Such knowledge may provide a comprehensive understanding of the importance of hindgut microbiota and microbial manipulation methods in industry. Overall, this preliminary study provided fundamental knowledge on hindgut microbial profile of pre-weaned calves under the regular management practice, which is a stepping stone for future nutritional intervention and disease challenge studies to define the role of hindgut microbiota in animal production and health.

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2.7 Tables

Table 2.1 Primers used in the present study for qPCR analysis.

Bacterial group	Primers	Product size	Annealing temperature	Reference
Total Bacteria	F: 5'-actcctacgggaggcag-3' R: 5'-gactaccagggtatctaatcc-3'	467bp	62°C	Stevenson & Weimer, 2007
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
Clostridium **cluster XIVa**	F:5'-cggtacctgactaagaagc-3' R:5'-agtttyattcttgcgaacg-3'	429bp	60°C	Ramirez-Farias et al., 2009
Faecalibacterium. Prausnitzii	F:5'-ggaggaagaaggtcttcgg-3' R:5'-aattccgcctacctctgcact-3'	248bp	60°C	Ramirez-Farias et al., 2009

Table 2.2 Operational taxonomic units (OTUs), Good's coverage, bacterial diversity and richness of all the samples along the hindgut.

Region	sample	OTUs	Sequences	Good's coverage
Cecum	Mucosa	518±21	5426±210	0.918±0.004
Colon	Mucosa	523±26	5486±200	0.917±0.004
Rectum	Mucosa	536±19	5468±199	0.918±0.004
Cecum	Digesta	591±38	5072±234	0.896±0.005
Colon	Digesta	627±26	5067±241	0.894±0.005
Rectum	Digesta	625±25	5078±252	0.894±0.005

Table 2.3 Operational taxonomic units (OTUs), bacterial diversity and richness along the hindgut of the pre-weaned calves.

	Diversity metrix	Age					Hindgut Region				*P*-value	
		NB	D7	D21	D42	SEM	Cecum	Colon	Rectum	SEM	Age	Region
Mucosa-attached bacteria	OTUs	475[b]	429[a]	602[c]	597[c]	13	518	523	536	12	<0.01	0.58
	Observed_species	316[b]	254[a]	385[c]	376[c]	13	334	330	335	11	<0.01	0.95
	Chao 1	607[b]	503[a]	741[c]	707[c]	26	638	623	656	22	<0.01	0.58
	Shannon index	6.06[b]	5.31[a]	6.60[b]	6.42[b]	0.15	6.07	6.06	6.16	0.13	<0.01	0.86
Digesta-associated bacteria	OTUs	NA	527[a]	624[ab]	687[b]	18	591	627	625	15	<0.01	0.68
	Observed_species	NA	334[a]	383[ab]	429[b]	11	369	395	388	11	<0.01	0.56
	Chao 1	NA	666[a]	769[ab]	864[b]	23	730	805	773	24	<0.01	0.40
	Shannon index	NA	6.16[a]	6.51[ab]	6.91[b]	0.10	6.39	6.65	6.59	0.10	<0.01	0.49

NA - not applicable (not enough DNA was extracted from digesta samples of newborn animals to perform amplicon sequencing)
[a,b,c] means with different superscripts are significantly different at $P < 0.01$

Table 2.4a Mucosa-attached bacterial colonization at phylum level.

Phylum	Age				SEM	*P*-value
	NB	D7	D21	D42		
Proteobacteria	33.85[a]	20.52[b]	2.41[c]	2.91[c]	2.08	<0.01
Firmicutes	33.32[a]	30.89[a]	55.49[b]	49.26[b]	1.81	<0.01
Bacteroidetes	28.34[a]	38.89[b]	37.94[b]	38.69[b]	1.48	0.01
Fusobacteria	1.87[a]	8.95[b]	3.09[a]	6.60[b]	1.00	0.02
Actinobacteria	1.02[a]	0.43[b]	0.56[b]	0.96[a]	0.10	<0.01
Acidobacteria	1.24	0.15	0.11	0.12	0.11	0.77
Cyanobacteria	0.29[a]	0.13[a]	0.33[a]	1.38[b]	0.13	<0.01
Others	0.09	0.06	0.06	0.08	0.01	0.60

P<0.01 means there is significant difference among different age groups.
P<0.05 means there is difference among different age groups.

Table 2.4b Mucosa-attached bacterial colonization at family level.

Family	Age				SEM	*P*-value
	NB	D7	D21	D42		
Acidobacteriaceae	1.24[a]	0.15[b]	0.11[b]	0.12[b]	0.11	<0.01
Coriobacteriaceae	0.76[ab]	0.32[a]	0.51[ab]	0.86[b]	0.09	<0.01
Bacteroidaceae	18.83[a]	26.26[b]	15.65[a]	9.75[c]	1.43	<0.01
Bacteroidales S24-7 group	2.13[a]	2.09[a]	0.78[a]	10.29[b]	0.76	<0.01
Porphyromonadaceae	0.65[a]	1.14[a]	3.50[b]	2.21[c]	0.18	<0.01
Prevotellaceae	5.88	8.64	9.16	9.36	0.59	0.06
Rikenellaceae	0.76[a]	0.72[a]	8.83[b]	6.97[b]	0.56	<0.01
Lactobacillaceae	5.26[a]	7.73[a]	0.25[b]	0.28[b]	0.08	<0.01
Streptococcaceae	0.27[a]	0.14[b]	0.01[c]	0.01[c]	0.01	<0.01
Christensenellaceae	0.29[a]	0.08[a]	0.94[b]	1.26[b]	0.07	<0.01
Clostridiaceae 1	0.62[a]	0.59[a]	0.80[a]	2.61[b]	0.23	<0.01
Clostridiales Family XIII	0.04[a]	0.02[a]	0.12[a]	0.38[b]	0.02	<0.01
Lachnospiraceae	10.23[a]	9.00[a]	23.46[b]	18.15[c]	0.89	<0.01
Peptostreptococcaceae	0.40[a]	0.08[b]	0.50[a]	1.52[c]	0.07	<0.01
Ruminococcaceae	13.66[a]	10.86[a]	27.37[b]	19.63[c]	1.06	<0.01
Erysipelotrichaceae	0.84[ab]	0.58[a]	1.11[b]	0.73[ab]	0.10	<0.01
Acidaminococcaceae	0.28	0.31	0.24	0.31	0.04	0.54
Veillonellaceae	1.21[a]	1.34[a]	0.52[a]	4.19[b]	0.46	<0.01
CFT112H7	0.68	2.34	0.39	0.48	0.27	0.12
Fusobacteriaceae	1.18	6.55	2.67	6.08	0.84	0.21
Alcaligenaceae	0.20[a]	0.52[b]	0.56[b]	0.53[b]	0.07	<0.01
Burkholderiaceae	6.23[a]	0.13[b]	0.04[b]	0.37[b]	0.04	<0.01
Enterobacteriaceae	19.09[a]	13.73[b]	1.10[c]	1.22[c]	1.04	<0.01
Pseudomonadaceae	0.39[a]	0.06[b]	0.06[b]	0.04[b]	0.03	<0.01
Others	8.90[a]	6.62[a]	1.32[b]	2.68[b]	0.60	<0.01

P<0.01 means there is significant difference among different age groups.
P<0.05 means there is difference among different age groups.

Table 2.4c Relative abundance of mucosa-attached bacteria at genus level.

Phylum	Genus	Age				SEM	*P*-value
		NB	D7	D21	D42		
Acidobacteria	*Edaphobacter*	1.19^{a}	0.15^{b}	0.10^{b}	0.12^{b}	0.08	<0.01
Actinobacteria	*Atopobium*	0.21^{a}	0.01^{b}	0.24^{a}	0.54^{c}	0.01	<0.01
	Collinsella	0.38^{a}	0.29^{a}	0.04^{b}	0.03^{b}	0.03	<0.01
	Coriobacteriaceae UCG-002	0.15^{ab}	0.02^{a}	0.22^{b}	0.10^{ab}	0.02	<0.01
Bacteroidetes	*Bacteroides*	18.83^{a}	26.26^{b}	15.65^{a}	9.75^{c}	1.43	<0.01
	Odoribacter	0.04^{a}	0.03^{a}	0.20^{b}	0.16^{b}	0.02	<0.01
	Parabacteroides	0.49^{a}	0.92^{a}	3.04^{b}	1.86^{c}	0.16	<0.01
	Alloprevotella	4.58^{ab}	7.05^{a}	6.50^{a}	3.54^{b}	0.51	<0.01
	Prevotella 7	0.23^{a}	0.35^{a}	0.08^{b}	0.10^{b}	0.04	<0.01
	Prevotella 9	0.84^{a}	1.15^{a}	2.19^{b}	2.89^{b}	0.21	<0.01
	Alistipes	0.04^{a}	0.03^{a}	0.07^{a}	0.20^{b}	0.01	<0.01
	Rikenellaceae RC9 gut group	0.72^{a}	0.70^{a}	8.76^{b}	6.77^{b}	0.56	<0.01
Firmicutes	*Lactobacillus*	5.26^{a}	7.73^{a}	0.25^{b}	0.28^{b}	0.08	<0.01
	Streptococcus	0.26^{a}	0.14^{b}	0.01^{c}	0.01^{c}	0.01	<0.01
	Christensenellaceae R-7 group	0.29^{a}	0.08^{b}	0.93^{c}	1.25^{c}	0.07	<0.01
	Clostridium sensu stricto 1	0.56^{a}	0.35^{a}	0.76^{a}	2.54^{b}	0.22	<0.01
	Anaerostipes	0.09^{ab}	0.03^{a}	0.13^{b}	0.18^{b}	0.02	<0.01
	Blautia	2.66^{a}	1.31^{a}	10.55^{b}	6.00^{c}	0.51	<0.01
	Coprococcus 1	0.05^{a}	0.08^{a}	0.50^{b}	0.22^{c}	0.03	<0.01
	Coprococcus 3	0.00^{a}	0.01^{a}	0.04^{a}	0.31^{b}	0.00	<0.01
	Dorea	0.37^{a}	0.71^{b}	0.21^{a}	0.12^{a}	0.06	<0.01
	Lachnoclostridium	2.76^{a}	2.32^{a}	2.36^{a}	1.65^{b}	0.14	<0.01
	Lachnospiraceae NC2004 group	0.04^{a}	0.04^{a}	0.19^{b}	0.12^{c}	0.01	<0.01
	Lachnospiraceae ND3007 group	0.02^{a}	0.00^{a}	0.02^{a}	0.15^{b}	0.00	<0.01
	Lachnospiraceae NK4A136 group	0.13^{a}	0.08^{a}	0.64^{b}	1.09^{c}	0.08	<0.01
	Lachnospiraceae UCG-004	0.59	0.66	0.72	0.54	0.05	0.12
	Lachnospiraceae UCG-008	0.19^{a}	0.17^{a}	0.65^{b}	0.68^{b}	0.06	<0.01
	Pseudobutyrivibrio	0.13^{a}	0.07^{a}	0.57^{b}	0.43^{c}	0.03	<0.01
	Roseburia	0.98^{a}	0.68^{a}	1.82^{b}	2.23^{b}	0.19	<0.01
	Tyzzerella	0.08^{a}	0.13^{a}	0.23^{ab}	0.42^{b}	0.05	<0.01

	Tyzzerella 4	1.37[a]	2.39[b]	2.46[b]	0.77[a]	0.22	<0.01
	Intestinibacter	0.11[a]	0.03[a]	0.09[a]	0.39[b]	0.03	<0.01
	Peptoclostridium	0.19[a]	0.04[a]	0.31[a]	0.90[b]	0.04	<0.01
	Romboutsia	0.11[a]	0.01[b]	0.10[a]	0.23[c]	0.01	<0.01
	Anaerotruncus	0.15[a]	0.04[b]	1.26[c]	1.45[c]	0.03	<0.01
	Faecalibacterium	6.01[a]	4.54[a]	14.56[b]	7.68[a]	0.83	<0.01
	Ruminiclostridium 5	0.04[a]	0.01[a]	0.18[b]	0.15[b]	0.01	<0.01
	Ruminiclostridium 6	0.03[a]	0.01[a]	0.41[b]	0.44[b]	0.01	<0.01
	Ruminiclostridium 9	0.00[a]	0.00[a]	0.11[b]	0.10[b]	0.00	<0.01
	Ruminococcaceae UCG-002	0.03[a]	0.03[a]	0.14[b]	0.11[b]	0.01	<0.01
	Ruminococcaceae UCG-005	0.49[a]	0.06[b]	4.31[c]	4.95[c]	0.06	<0.01
	Ruminococcaceae UCG-010	0.02[a]	0.05[a]	0.10[a]	0.21[b]	0.02	<0.01
	Ruminococcaceae UCG-014	0.40[a]	0.31[a]	1.59[b]	0.85[c]	0.11	<0.01
	Ruminococcus 1	0.18[a]	0.19[a]	0.57[b]	1.00[c]	0.08	<0.01
	Ruminococcus 2	4.61[a]	2.89[b]	1.53[c]	0.74[c]	0.27	<0.01
	Subdoligranulum	1.30[a]	2.18[b]	0.78[a]	0.50[a]	0.30	0.01
	Erysipelatoclostridium	0.34[a]	0.48[a]	0.03[b]	0.04[b]	0.03	<0.01
	Erysipelotrichaceae UCG-003	0.39[a]	0.07[b]	0.98[c]	0.49[a]	0.06	<0.01
	Acidaminococcus	0.22[a]	0.27[a]	0.08[b]	0.02[b]	0.02	<0.01
	Phascolarctobacterium	0.02[a]	0.01[a]	0.11[b]	0.27[c]	0.01	<0.01
	Anaerovibrio	0.03[a]	0.03[a]	0.32[b]	4.06[c]	0.03	<0.01
	Megasphaera	0.75[a]	0.47[a]	0.17[b]	0.08[b]	0.06	<0.01
Fusobacteria	*Fusobacterium*	1.18[a]	6.55[b]	2.67[a]	6.08[b]	0.84	0.21
Proteobacteria	*Sutterella*	0.19[a]	0.49[b]	0.53[b]	0.47[b]	0.07	<0.01
	Burkholderia	6.17[a]	0.06[b]	0.01[b]	0.34[c]	0.01	<0.01
	Citrobacter	0.23[a]	0.18[a]	0.05[b]	0.02[b]	0.02	<0.01
	Enterobacter	2.41[a]	1.84[a]	0.12[b]	0.15[b]	0.11	<0.01
	Escherichia-Shigella	13.52[a]	9.69[b]	0.74[c]	0.92[c]	0.72	<0.01
	Pantoea	0.20[a]	0.15[a]	0.01[b]	0.02[b]	0.01	<0.01
	Salmonella	2.64[a]	1.81[b]	0.16[c]	0.10[c]	0.09	<0.01
	Pseudomonas	0.38[a]	0.06[b]	0.06[b]	0.04[b]	0.03	<0.01
Others	Others	14.07[a]	13.55[a]	7.79[b]	22.14[c]	0.13	<0.01

$P<0.01$ means there is significant difference among different age groups.

Table 2.4d Digesta-associated bacterial colonization at phylum level.

Phylum	Age			SEM	*P*-value
	D7	D21	D42		
Actinobacteria	4.81	2.43	1.79	0.48	0.39
Bacteroidetes	20.81	20.94	21.36	0.89	0.86
Firmicutes	61.76[a]	73.75[b]	73.90[b]	1.55	0.01
Fusobacteria	4.58	0.48	0.44	0.91	0.59
Proteobacteria	7.37[a]	1.92[b]	2.05[b]	0.66	<0.01
Verrucomicrobia	0.91	0.25	0.38	0.23	0.14
Others	0.04[a]	0.22[b]	0.08[a]	0.04	0.02

P<0.01 means there is significant difference among different age groups.
P<0.05 means there is difference among different age groups.

Table 2.4e Digesta-associated bacterial colonization at family level.

Family	Age			SEM	*P*-value
	D7	D21	D42		
Bifidobacteriaceae	1.06[a]	0.18[b]	0.15[b]	0.14	0.06
Nocardiaceae	0.12[a]	0.04[b]	0.04[b]	0.01	<0.01
Coriobacteriaceae	3.62	2.21	1.59	0.37	0.57
Bacteroidaceae	9.28[a]	4.64[b]	4.51[b]	0.59	<0.01
Bacteroidales S24-7 group	6.69[a]	6.42[a]	9.05[b]	0.49	<0.01
Porphyromonadaceae	0.63[a]	1.36[b]	1.12[ab]	0.12	0.02
Prevotellaceae	3.07	5.61	3.04	0.80	0.73
Rikenellaceae	1.14[a]	2.90[b]	3.62[b]	0.25	<0.01
Lactobacillaceae	22.36	20.01	21.01	1.37	0.90
Christensenellaceae	0.43[a]	2.04[b]	2.33[b]	0.25	<0.01
Clostridiaceae 1	0.95[ab]	0.66[a]	1.21[b]	0.20	<0.01
Clostridiales Family XIII	0.06[a]	0.18[b]	0.26[b]	0.02	<0.01
Lachnospiraceae	17.46[a]	25.07[b]	23.85[b]	1.03	<0.01
Peptostreptococcaceae	0.72[a]	2.42[b]	3.17[b]	0.32	<0.01
Ruminococcaceae	15.59[a]	20.10[b]	19.67[b]	0.84	0.03
Erysipelotrichaceae	0.96[a]	2.58[b]	1.78[ab]	0.24	<0.01
Veillonellaceae	2.96[a]	0.39[b]	0.32[b]	0.40	0.04
Fusobacteriaceae	3.22[a]	0.44[b]	0.39[b]	0.39	0.44
Enterobacteriaceae	4.99[a]	1.35[b]	1.39[b]	0.45	<0.01
Verrucomicrobiaceae	0.91	0.25	0.38	0.23	0.14
Others	3.80[a]	1.16[b]	1.13[b]	0.42	0.01

P<0.01 means there is significant difference among different age groups.
P<0.05 means there is difference among different age groups.

Table 2.4f Relative abundance of digesta-associated bacteria at genus level.

Phylum	Genus	Age			SEM	*P*-value
		D7	D21	D42		
Actinobacteria	*Bifidobacterium*	1.06[a]	0.17[b]	0.14[b]	0.14	0.04
	Rhodococcus	0.12[a]	0.04[b]	0.04[b]	0.01	<0.01
	Atopobium	0.24[a]	0.75[b]	0.61[b]	0.09	<0.01
	Collinsella	3.17	0.67	0.48	0.37	0.08
	Coriobacteriaceae UCG-002	0.16[a]	0.70[b]	0.41[ab]	0.06	<0.01
Bacteroidetes	*Bacteroides*	9.28[a]	4.64[b]	4.51[b]	0.59	<0.01
	Parabacteroides	0.51[a]	1.22[b]	1.01[b]	0.11	0.02
	Alloprevotella	2.78[a]	5.34[b]	2.29[a]	0.80	0.05
	Prevotella 9	0.21	0.15	0.13	0.02	0.28
	Alistipes	0.09	0.20	0.17	0.03	0.06
	Rikenellaceae RC9 gut group	1.05[a]	2.70[b]	3.45[b]	0.24	<0.01
Firmicutes	*Lactobacillus*	22.36	20.01	21.01	1.37	0.90
	Christensenellaceae R-7 group	0.42[a]	2.03[b]	2.32[b]	0.25	<0.01
	Clostridium sensu stricto 1	0.32[a]	0.60[a]	1.13[b]	0.09	<0.01
	Anaerostipes	0.12	0.14	0.14	0.01	0.38
	Blautia	5.50[a]	13.42[b]	11.87[b]	0.80	<0.01
	Coprococcus 1	0.06[a]	0.18[b]	0.13[b]	0.02	<0.01
	Dorea	1.40[a]	0.58[b]	0.61[b]	0.13	<0.01
	Lachnoclostridium	3.62	3.18	3.11	0.19	0.73
	Lachnospiraceae NC2004 group	0.15	0.19	0.16	0.02	0.41
	Lachnospiraceae NK4A136 group	0.14[a]	0.34[b]	0.29[b]	0.03	<0.01
	Lachnospiraceae UCG-004	0.53	0.51	0.53	0.05	0.39
	Lachnospiraceae UCG-008	0.55[a]	0.71[ab]	0.81[b]	0.04	<0.01
	Moryella	0.03[a]	0.11[b]	0.12[b]	0.01	<0.01
	Pseudobutyrivibrio	0.29[a]	0.73[b]	0.65[b]	0.05	<0.01
	Roseburia	1.45	0.83	1.08	0.13	0.31
	Tyzzerella	0.20	0.13	0.15	0.04	0.16
	Tyzzerella 4	2.34	1.79	2.15	0.29	0.20

	Intestinibacter	0.29[a]	0.63[b]	0.82[b]	0.07	<0.01
	Peptoclostridium	0.35[a]	1.39[b]	1.82[b]	0.19	<0.01
	Romboutsia	0.09[a]	0.40[b]	0.51[b]	0.06	<0.01
	Anaerotruncus	0.22[a]	0.47[b]	0.65[c]	0.04	<0.01
	Faecalibacterium	5.97	3.79	3.53	0.47	0.46
	Ruminiclostridium 5	0.07[a]	0.21[b]	0.18[b]	0.02	<0.01
	Ruminiclostridium 6	0.13[a]	0.36[b]	0.33[b]	0.06	<0.01
	Ruminococcaceae NK4A214 group	0.14	0.08	0.14	0.01	0.06
	Ruminococcaceae UCG-005	1.16[a]	4.76[ab]	6.66[b]	0.79	<0.01
	Ruminococcaceae UCG-010	0.13	0.12	0.10	0.02	0.26
	Ruminococcaceae UCG-014	1.14[a]	4.30[b]	2.69[ab]	0.53	<0.01
	Ruminococcus 1	0.14[a]	0.22[b]	0.27[b]	0.02	0.01
	Ruminococcus 2	1.52	1.21	0.80	0.15	0.44
	Subdoligranulum	4.20	3.28	3.01	0.58	0.47
	Erysipelatoclostridium	0.44[a]	0.17[b]	0.13[b]	0.06	0.02
	Erysipelotrichaceae UCG-003	0.46[a]	2.23[b]	1.48[ab]	0.24	<0.01
	Megasphaera	2.72[a]	0.32[b]	0.26[b]	0.24	<0.01
Fusobacteria	*Fusobacterium*	3.22	0.44	0.39	0.37	0.41
Proteobacteria	*Enterobacter*	0.66[a]	0.19[b]	0.21[b]	0.06	<0.01
	Escherichia-Shigella	3.67[a]	0.98[b]	0.98[b]	0.33	<0.01
	Salmonella	0.51[a]	0.13[b]	0.12[b]	0.05	0.03
	Akkermansia	0.91	0.25	0.38	0.23	0.14
Others	Others	13.68[ab]	12.00[a]	15.04[b]	0.67	0.05

P<0.05 means there is difference among different age groups.
P<0.01 means there is significant difference among different age groups.

Table 2.5 Mucosa-attached carbohydrate-utilizing and intestinal health-related bacterial genera.

Phylum	Genus	Age				SEM	*P*-value
		NB	D7	D21	D42		
Bacteroidetes	*Bacteroides*	18.83[a]	26.26[b]	15.65[a]	9.75[c]	1.43	<0.01
Firmicutes	*Lactobacillus*	5.26[a]	7.73[a]	0.25[b]	0.28[b]	0.08	<0.01
	Anaerostipes	0.09[ab]	0.03[a]	0.13[b]	0.18[b]	0.02	<0.01
	Blautia	2.66[a]	1.31[a]	10.55[b]	6.00[c]	0.51	<0.01
	Coprococcus 1	0.05[a]	0.08[a]	0.50[b]	0.22[c]	0.03	<0.01
	Coprococcus 3	0.00[a]	0.01[a]	0.04[a]	0.31[b]	0.00	<0.01
	Lachnoclostridium	2.76[a]	2.32[a]	2.36[a]	1.65[b]	0.14	<0.01
	Lachnospiraceae NC2004 *group*	0.04[a]	0.04[a]	0.19[b]	0.12[c]	0.01	<0.01
	Lachnospiraceae ND3007 *group*	0.02[a]	0.00[a]	0.02[a]	0.15[b]	0.00	<0.01
	Lachnospiraceae NK4A136 *group*	0.13[a]	0.08[a]	0.64[b]	1.09[c]	0.08	<0.01
	Lachnospiraceae UCG-004	0.59	0.66	0.72	0.54	0.05	0.12
	Lachnospiraceae UCG-008	0.19[a]	0.17[a]	0.65[b]	0.68[b]	0.06	<0.01
	Pseudobutyrivibrio	0.13[a]	0.07[a]	0.57[b]	0.43[c]	0.03	<0.01
	Roseburia	0.98[a]	0.68[a]	1.82[b]	2.23[b]	0.19	<0.01
	Faecalibacterium	6.01[a]	4.54[a]	14.56[b]	7.68[a]	0.83	<0.01
	Ruminiclostridium 5	0.04[a]	0.01[a]	0.18[b]	0.15[b]	0.01	<0.01
	Ruminiclostridium 6	0.03[a]	0.01[a]	0.41[b]	0.44[b]	0.01	<0.01
	Ruminiclostridium 9	0.00[a]	0.00[a]	0.11[b]	0.10[b]	0.00	<0.01
	Ruminococcaceae UCG-002	0.03[a]	0.03[a]	0.14[b]	0.11[b]	0.01	<0.01
	Ruminococcaceae UCG-005	0.49[a]	0.06[b]	4.31[c]	4.95[c]	0.06	<0.01
	Ruminococcaceae UCG-010	0.02[a]	0.05[a]	0.10[a]	0.21[b]	0.02	<0.01
	Ruminococcaceae UCG-014	0.40[a]	0.31[a]	1.59[b]	0.85[c]	0.11	<0.01
	Ruminococcus 1	0.18[a]	0.19[a]	0.57[b]	1.00[c]	0.08	<0.01
	Ruminococcus 2	4.61[a]	2.89[b]	1.53[c]	0.74[c]	0.27	<0.01
	Erysipelatoclostridium	0.34[a]	0.48[a]	0.03[b]	0.04[b]	0.03	<0.01
	Erysipelotrichaceae UCG-003	0.39[a]	0.07[b]	0.98[c]	0.49[a]	0.06	<0.01
	Megasphaera	0.75[a]	0.47[a]	0.17[b]	0.08[b]	0.06	<0.01
Proteobacteria	*Escherichia-Shigella*	13.52[a]	9.69[b]	0.74[c]	0.92[c]	0.72	<0.01
	Salmonella	2.64[a]	1.81[b]	0.16[c]	0.10[c]	0.09	<0.01

P<0.01 means there is significant difference among different age groups. [1] Values represents mean of three hindgut regions.

Table 2.6 Digesta-associated carbohydrate-utilizing and intestinal health-related bacterial genera.

Phylum	Genus	Age			SEM	*P*-value
		D7	D21	D42		
Actinobacteria	*Bifidobacterium*	1.06[a]	0.17[b]	0.14[b]	0.14	0.04
Bacteroidetes	*Bacteroides*	9.28[a]	4.64[b]	4.51[b]	0.59	<0.01
Firmicutes	*Lactobacillus*	22.36	20.01	21.01	1.37	0.90
	Anaerostipes	0.12	0.14	0.14	0.01	0.38
	Blautia	5.50[a]	13.42[b]	11.87[b]	0.80	<0.01
	Coprococcus 1	0.06[a]	0.18[b]	0.13[b]	0.02	<0.01
	Lachnoclostridium	3.62	3.18	3.11	0.19	0.73
	Lachnospiraceae NC2004 *group*	0.15	0.19	0.16	0.02	0.41
	Lachnospiraceae NK4A136 *group*	0.14[a]	0.34[b]	0.29[b]	0.03	<0.01
	Lachnospiraceae UCG-004	0.53	0.51	0.53	0.05	0.39
	Lachnospiraceae UCG-008	0.55[a]	0.71[ab]	0.81[b]	0.04	<0.01
	Pseudobutyrivibrio	0.29[a]	0.73[b]	0.65[b]	0.05	<0.01
	Roseburia	1.45	0.83	1.08	0.13	0.31
	Faecalibacterium	5.97	3.79	3.53	0.47	0.46
	Ruminiclostridium 5	0.07[a]	0.21[b]	0.18[b]	0.02	<0.01
	Ruminiclostridium 6	0.13[a]	0.36[b]	0.33[b]	0.06	<0.01
	Ruminococcaceae NK4A214 *group*	0.14	0.08	0.14	0.01	0.06
	Ruminococcaceae UCG-005	1.16[a]	4.76[ab]	6.66[b]	0.79	<0.01
	Ruminococcaceae UCG-010	0.13	0.12	0.10	0.02	0.26
	Ruminococcaceae UCG-014	1.14[a]	4.30[b]	2.69[ab]	0.53	<0.01
	Ruminococcus 1	0.14[a]	0.22[b]	0.27[b]	0.02	0.01
	Ruminococcus 2	1.52	1.21	0.80	0.15	0.44
	Erysipelatoclostridium	0.44[a]	0.17[b]	0.13[b]	0.06	0.02
	Erysipelotrichaceae UCG-003	0.46[a]	2.23[b]	1.48[ab]	0.24	<0.01
	Megasphaera	2.72[a]	0.32[b]	0.26[b]	0.24	<0.01
Proteobacteria	*Escherichia-Shigella*	3.67[a]	0.98[b]	0.98[b]	0.33	<0.01
	Salmonella	0.51[a]	0.13[b]	0.12[b]	0.05	0.03

P<0.05 means there is difference among different age groups. [1] Values represents mean of three hindgut regions.

Table 2.7 Comparison of mucosa and digesta-associated bacteria.

Item		Sample type		*P*-value
		Mucosa	**Digesta**	
Present in Mucosa	*Acidaminococcus*	0.15±0.03	NA	
	Sutterella	0.42±0.07	NA	
	Phascolarctobacterium	0.10±0.02	NA	
	Ruminiclostridium 9	0.06±0.01	NA	
	Streptococcus	0.11±0.03	NA	
	Lachnospiraceae ND3007 group	0.05±0.01	NA	
	Pseudomonas	0.14±0.03	NA	
	Ruminococcaceae UCG-002	0.08±0.01	NA	
	Pantoea	0.09±0.01	NA	
	Coprococcus 3	0.09±0.03	NA	
	Anaerovibrio	1.11±0.46	NA	
	Odoribacter	0.11±0.02	NA	
	Edaphobacter	0.39±0.13	NA	
	Citrobacter	0.12±0.02	NA	
	Burkholderia	1.65±0.65	NA	
	Prevotella 7	0.19±0.04	NA	
Present in digesta	*Rhodococcus*	NA	0.07±0.01	
	Moryella	NA	0.09±0.01	
	Bifidobacterium	NA	0.46±0.14	
	Ruminococcaceae NK4A214 group	NA	0.12±0.01	
	Akkermansia	NA	0.51±0.23	
Present in mucosa and digesta	*Coriobacteriaceae* UCG-002	0.12±0.03	0.42±0.06	<0.01
	Roseburia	1.43±0.19	1.13±0.13	0.91
	Prevotella 9	1.77±0.21	0.16±0.02	<0.01
	Ruminococcus 1	0.48±0.08	0.21±0.02	0.24
	Alistipes	0.09±0.01	0.15±0.03	<0.01

Romboutsia	0.11±0.02	0.33±0.06	<0.01
Lachnospiraceae NC2004 group	0.10±0.01	0.17±0.02	<0.01
Lachnospiraceae NK4A136 group	0.48±0.08	0.26±0.03	0.32
Clostridium sensu stricto 1	1.05±0.22	0.69±0.09	0.25
Rikenellaceae RC9 gut group	4.24±0.56	2.41±0.24	0.62
Peptoclostridium	0.36±0.07	1.20±0.19	<0.01
Collinsella	0.18±0.04	1.44±0.37	<0.01
Lachnoclostridium	2.27±0.14	3.31±0.19	<0.01
Atopobium	0.25±0.05	0.53±0.09	<0.01
Erysipelatoclostridium	0.22±0.06	0.25±0.06	0.61
Christensenellaceae R-7 group	0.64±0.09	1.60±0.25	<0.01
Ruminococcus 2	2.44±0.27	1.17±0.15	<0.01
Fusobacterium	4.12±0.84	1.35±0.69	<0.01
Ruminiclostridium 6	0.22±0.05	0.27±0.06	0.31
Anaerotruncus	0.73±0.09	0.45±0.04	0.62
Lactobacillus	3.38±0.80	21.14±1.37	<0.01
Bacteroides	17.62±1.43	6.14±0.59	<0.01
Anaerostipes	0.11±0.02	0.13±0.01	0.51
Ruminococcaceae UCG-014	0.79±0.11	2.68±0.53	<0.01
Tyzzerella	0.21±0.05	0.16±0.04	0.47
Lachnospiraceae UCG-004	0.63±0.05	0.52±0.05	0.11
Pseudobutyrivibrio	0.30±0.03	0.55±0.05	<0.01
Enterobacter	1.13±0.18	0.35±0.06	0.27
Ruminiclostridium 5	0.10±0.01	0.15±0.02	<0.01
Subdoligranulum	1.19±0.30	3.49±0.58	<0.01
Salmonella	1.18±0.19	0.26±0.05	<0.01
Ruminococcaceae UCG-005	2.45±0.52	4.23±0.79	<0.01
Coprococcus 1	0.21±0.03	0.12±0.02	0.32
Tyzzerella 4	1.75±0.22	2.10±0.29	0.05
Intestinibacter	0.16±0.03	0.58±0.07	<0.01
Megasphaera	0.37±0.06	1.10±0.40	0.04
Erysipelotrichaceae UCG-003	0.48±0.08	1.38±0.24	<0.01

	Ruminococcaceae UCG-010	0.10±0.02	0.12±0.02	0.51
	Dorea	0.35±0.06	0.86±0.13	<0.01
	Escherichia-Shigella	6.22±0.95	1.88±0.33	0.02
	Blautia	5.13±0.51	10.23±0.80	<0.01
	Faecalibacterium	8.20±0.83	4.42±0.47	<0.01
	Parabacteroides	1.58±0.16	0.91±0.11	<0.01
	Lachnospiraceae UCG-008	0.42±0.06	0.69±0.04	<0.01
	Alloprevotella	5.42±0.51	3.41±0.80	0.03

P<0.05 means there is difference between different sample type.
P<0.01 means there is significant difference between different sample type.

Table 2.8 Quantification of five bacterial groups in the hindgut during pre-weaned period.

	Bacterial groups	Age					Hindgut Region				*P*-value	
		NB	D7	D21	D42	SEM	Cecum	Colon	Rectum	SEM	Age	Region
Mucosa-	Total bacteria	2.43×10^{9}	1.21×10^{10}	1.58×10^{10}	5.49×10^{10}	1.93×10^{9}	1.10×10^{10}	4.47×10^{10}	8.30×10^{9}	1.67×10^{9}	0.24	0.24
attached	*E. coli*[x]	2.02^{a}	3.57^{b}	1.30^{ac}	0.75^{c}	0.42	2.11	1.70	1.92	0.37	<0.01	0.73
bacteria	*Bifidobacterium*[x]	36.29^{a}	59.90^{b}	35.16^{a}	10.62^{c}	4.33	40.00	32.80	33.69	3.75	<0.01	0.34
	Clostridium cluster *XIVa*[x]	0.92^{a}	4.28^{ab}	15.83^{c}	9.36^{d}	1.28	7.42	6.38	8.99	1.11	<0.01	0.25
	Faecalibacterium Prausnitzii[x]	0.41^{a}	1.09^{ab}	1.76^{b}	0.93^{a}	0.24	1.12	0.76	1.25	0.21	<0.01	0.23
Digesta-	Total bacteria	NA	2.02×10^{13}	1.21×10^{13}	1.91×10^{13}	5.51×10^{12}	1.31×10^{13}	1.53×10^{13}	2.31×10^{13}	5.51×10^{12}	0.53	0.39
Associated	*E. coli*[x]	NA	0.07^{a}	0.02^{b}	0.01^{b}	0.01	0.04	0.02	0.03	0.01	0.02	0.69
bacteria	*Bifidobacterium*[x]	NA	1.51	1.85	2.51	0.33	2.20	1.88	1.79	0.32	0.08	0.62
	Clostridium cluster XIVa[x]	NA	1.64^{a}	2.90^{b}	2.75^{b}	0.35	2.67	2.16	2.47	0.35	0.02	0.56
	Faecalibacterium Prausnitzii[x]	NA	0.11	0.06	0.06	0.05	0.08	0.09	0.07	0.67	0.07	0.80

NA – not applicable

[x] means bacterial groups were expressed as ratio, ratio= (16S rRNA gene copy number of each bacterial groups/total bacterial 16S rRNA gene copy number) *100

[a,b,c] means with different superscripts are significantly different at $P<0.05$.

Table 2.9 Short chain fatty acid (SCFA) concentration (μmol/g) and molar proportion in the hindgut of pre-weaned dairy calves.

SCFA	Age				Region				*P*-value	
	D7	D21	D42	SEM	Cecum	Colon	Rectum	SEM	Age	Region
Acetate	18.22^{a}	56.71^{b}	44.39^{c}	4.18	40.13	40.58	38.62	4.05	<0.01	0.93
Propionate	5.47^{a}	13.87^{b}	12.47^{b}	1.31	9.33	9.72	12.77	1.26	<0.01	0.09
Butyrate	3.45^{a}	8.46^{b}	5.95^{c}	0.92	5.00^{x}	5.01^{x}	7.84^{y}	0.89	<0.01	0.03
Isobutyrate	0.30^{a}	1.12^{b}	0.82^{b}	0.15	0.51^{x}	0.45^{x}	1.27^{y}	0.15	<0.01	<0.01
Isovalerate	0.71^{a}	1.67^{ab}	0.88^{b}	0.27	0.67^{x}	0.69^{x}	1.89^{y}	0.26	0.02	<0.01
Valerate	0.42	0.94	1.06	0.21	0.61^{x}	0.58^{x}	1.23^{y}	0.20	0.06	0.03
Total SCFA	28.57^{a}	82.77^{b}	65.57^{c}	6.25	56.25	57.03	63.62	6.08	<0.01	0.59
Acetate	0.67	0.69	0.67	0.02	0.70^{x}	0.70^{x}	0.61^{y}	0.02	0.81	<0.01
Propionate	0.17	0.17	0.19	0.01	0.17^{x}	0.17^{x}	0.20^{y}	0.01	0.77	0.03
Butyrate	0.10	0.10	0.09	0.01	0.09^{x}	0.08^{xy}	0.11^{y}	0.01	0.62	0.01
Isobutyrate	0.01	0.01	0.01	0.00	0.01^{x}	0.01^{x}	0.02^{y}	0.00	0.22	<0.01
Isovalerate	0.03^{a}	0.02^{b}	0.01^{c}	0.00	0.02^{x}	0.01^{y}	0.03^{z}	0.00	0.03	0.01
Valerate	0.01	0.01	0.02	0.00	0.01^{x}	0.01^{x}	0.02^{y}	0.00	0.08	0.02

^{a,b,c} means with different superscripts are significantly different among age groups at $P<0.05$.

^{x,y} means with different superscripts are significantly different among regions at $P<0.05$.

2.8 Figures

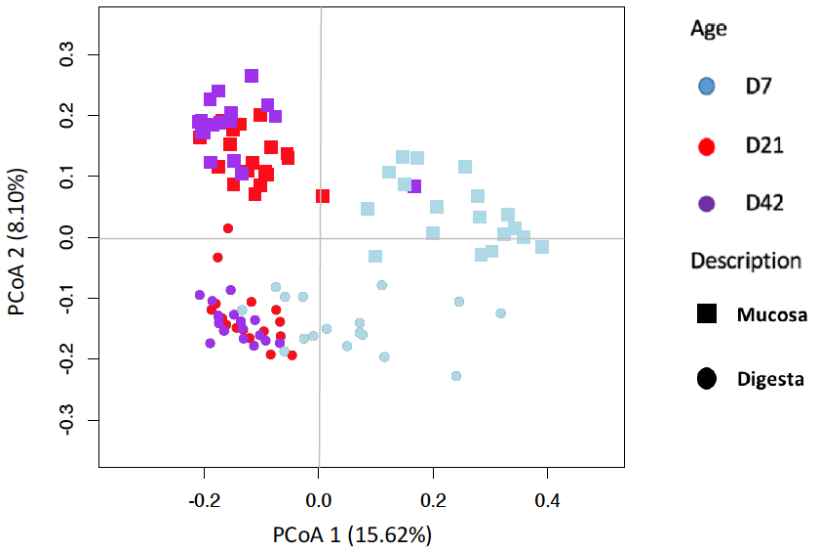


Figure 2.1 Comparison of mucosa-attached and digesta-associated bacterial profiles with Principal Coordinate Analysis (PCoA). PCoA plot was generated using unweighted UniFrac for D7, D21 and D42 old calves. Mucosa-attached bacteria (square) and digesta-associated bacteria (dot) are plotted along the first two principal component axis (PC1 and PC2), with blue, red and purple representing different age groups. The two components explained 15.62% and 8.10% of the variance.

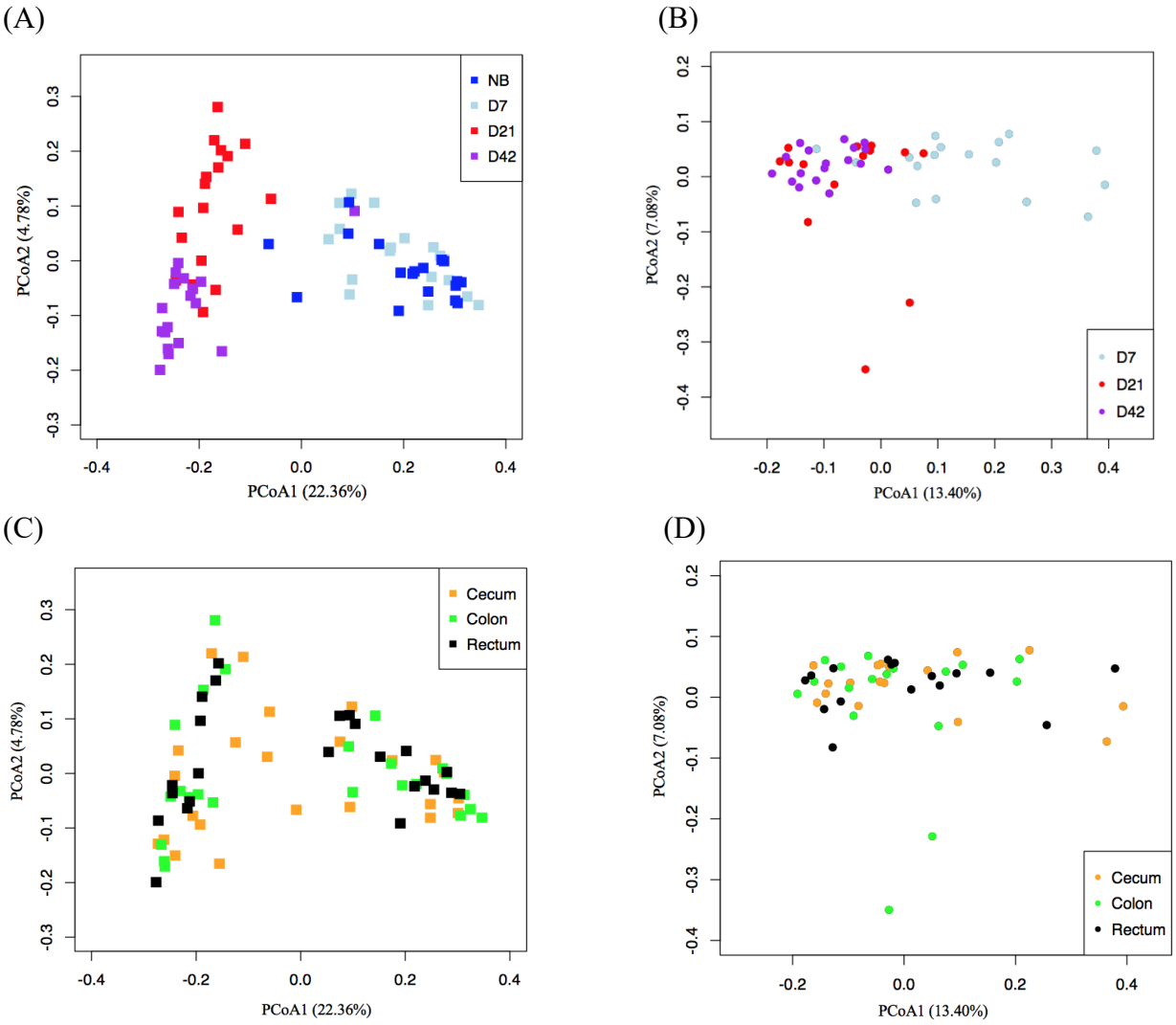


Figure 2.2 Comparison of mucosa-attached and digesta-associated bacterial profiles among different age groups and hindgut regions with Principal Coordinate Analysis (PCoA). (A) Mucosa-attached bacterial community among different age groups are plotted along the first two principal component axis (PC1 and PC2), with blue, light blue, red and purple representing different age groups. The two components explained 22.36% and 4.78% of the variance. (B) Digesta-associated bacterial community among different age groups are plotted along the first two principal component axis (PC1 and PC2), with blue, red and purple representing different age groups. The two components explained 13.40% and 7.08% of the variance. (C) Mucosa-attached bacterial community among different hindgut regions are plotted along the first two principal component axis (PC1 and PC2), with orange, green and black representing different hindgut regions. The two components explained 22.36% and 4.78% of the variance. (D) Digesta-associated bacterial community among different hindgut regions are plotted along the first two principal component axis (PC1 and PC2), with with orange, green and black representing different hindgut regions. The two components explained 13.40% and 7.08% of the variance.

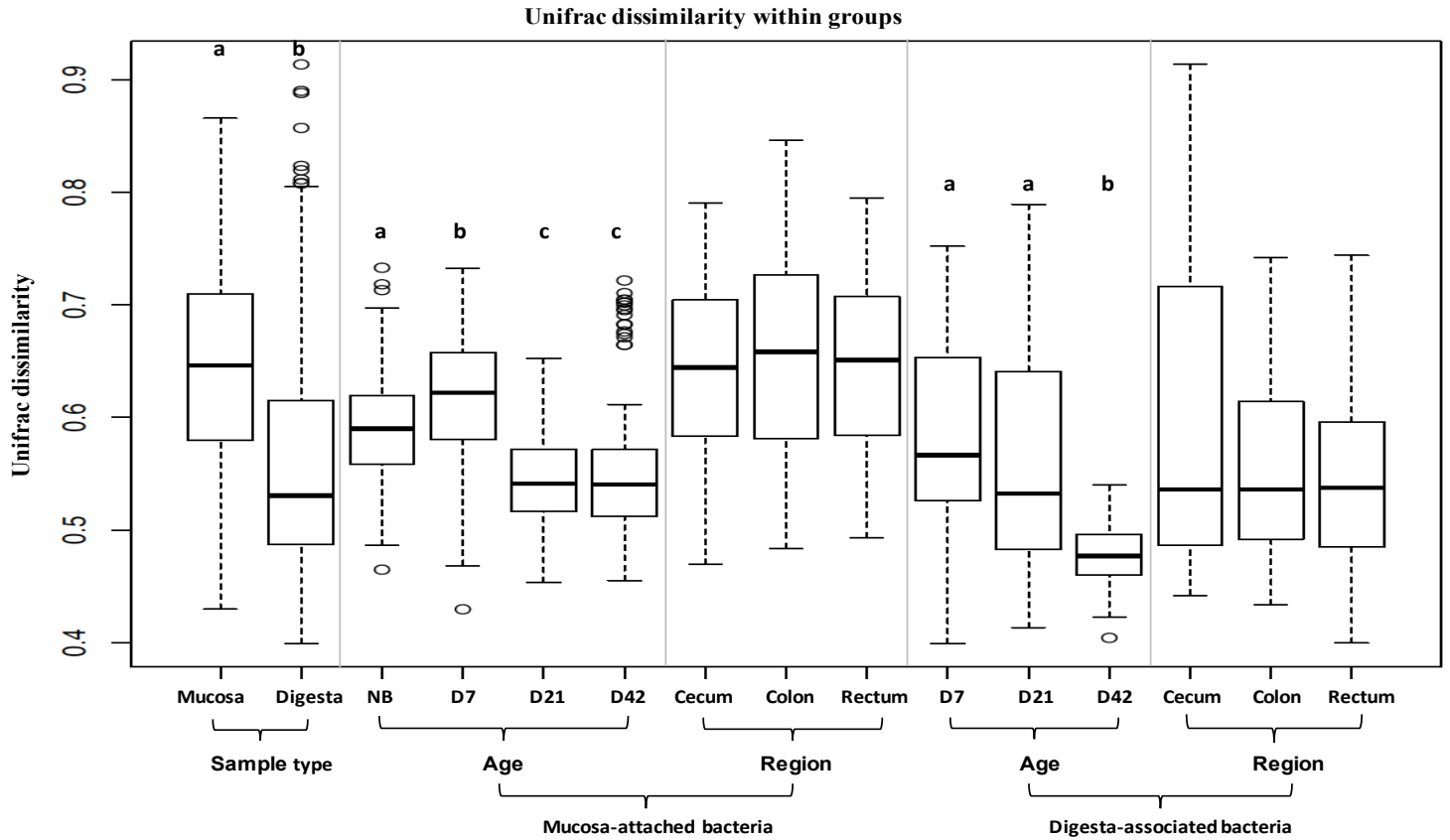
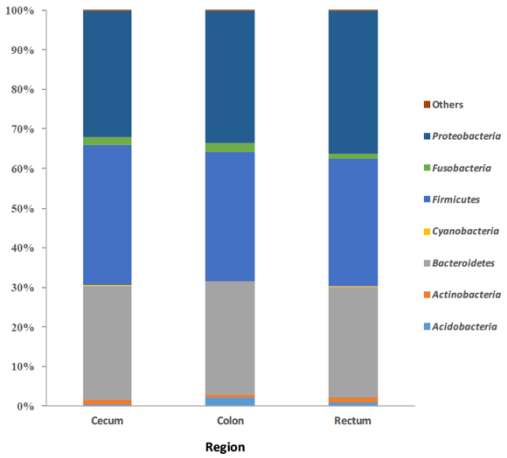


Figure 2.3 Microbial uniFrac dissimilarity within groups of pre-weaned calves. Box plot showing within-group similarity, and this value was calculated based on the average of the pairwise dissimilarity between each paired sample within different groups (sample type, age and region) using unweighted UniFrac metric. The X-axis indicates different groups (sample type, age or region), and Y-axis represents the degree of uniFrac dissimilarity. The boxes represent the interquartile range (IQR) between the first and third quartiles and (25th and 75th percentage, respectively), and the vertical line inside the box is the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively. Samples with the dissimilarity value exceeding the range are represented as the circle besides the box. Different letters (a, b and c) represent uniFrac dissimilarity values that are different between groups at $P < 0.05$ using t-test analysis within sample type and age.

(A)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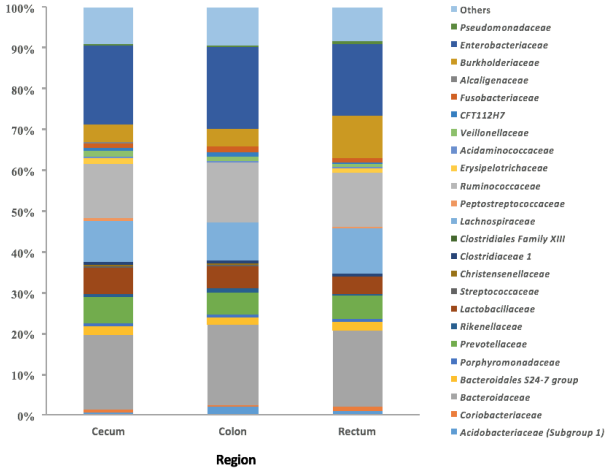
Cecum
Colon
Rectum
Region
Others
Proteobacteria
Fusobacteria
Firmicutes
Cyanobacteria
Bacteroidetes
Actinobacteria
Acidobacteria

(B)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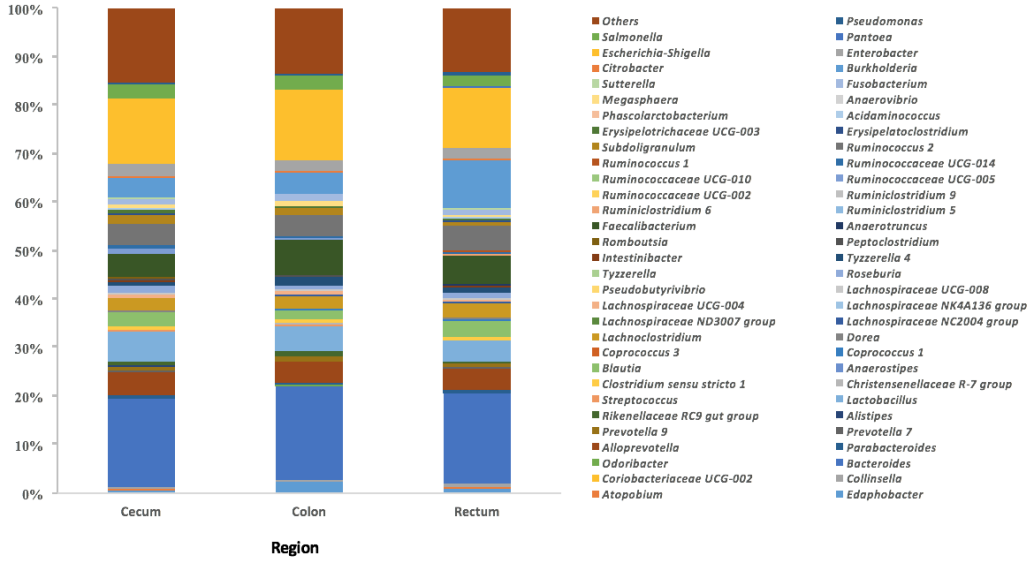
Enterobacteriaceae
Burkholderiaceae
Alcaligenaceae
Fusobacteriaceae
CF112H7
Veillonellaceae
Acidaminococcaceae
Erysipelotrichaceae
Ruminococcaceae
Peptostreptococcaceae
Lachnospiraceae
Clostridiales Family XIII
Clostridiaceae 1
Christensenellaceae
Streptococcaceae
Lactobacillaceae
Rikenellaceae
Prevotellaceae
Porphyromonadaceae
Bacteroidales S24-7 group
Bacteroidaceae
Coriobacteriaceae
Acidobacteriaceae (Subgroup 1)

(C)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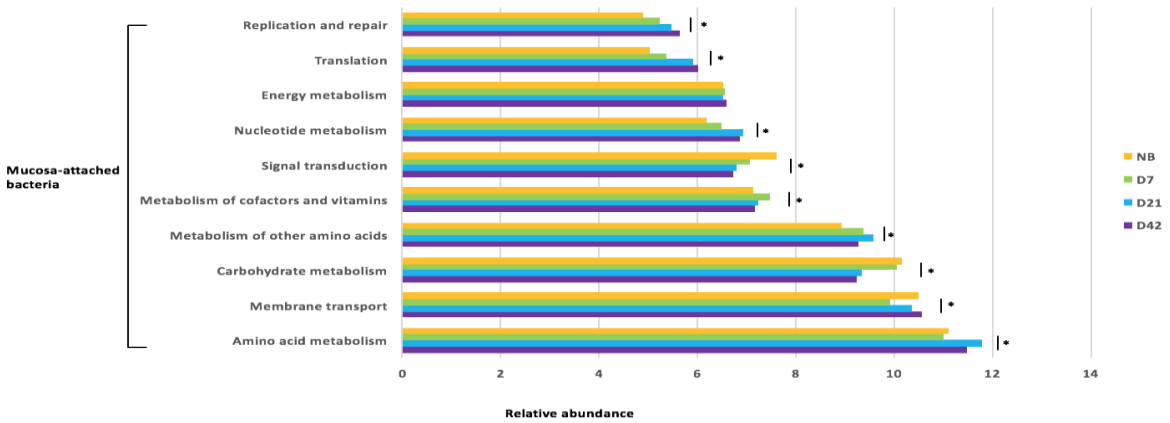
Cecum
Colon
Rectum
Region
Others
Salmonella
Escherichia-Shigella
Citrobacter
Sutterella
Megasphaera
Phascolarctobacterium
Erysipelotrichaceae UCG-003
Subdoligranulum
Ruminococcus 1
Ruminococcaceae UCG-010
Ruminococcaceae UCG-002
Ruminiclostridium 6
Faecalibacterium
Romboutsia
Intestinibacter
Tyzzerella
Pseudobutyrivibrio
Lachnospiraceae UCG-004
Lachnospiraceae ND3007 group
Lachnoclostridium
Coprococcus 3
Blautia
Clostridium sensu stricto 1
Streptococcus
Rikenellaceae RC9 gut group
Prevotella 9
Alloprevotella
Odoribacter
Coriobacteriaceae UCG-002
Atopobium
Pseudomonas
Pantoea
Enterobacter
Burkholderia
Fusobacterium
Anaerovibrio
Acidaminococcus
Erysipelatoclostridium
Ruminococcus 2
Ruminococcaceae UCG-014
Ruminococcaceae UCG-005
Ruminiclostridium 9
Ruminiclostridium 5
Anaerotruncus
Peptoclostridium
Tyzzerella 4
Roseburia
Lachnospiraceae UCG-008
Lachnospiraceae NK4A136 group
Lachnospiraceae NC2004 group
Dorea
Coprococcus 1
Anaerostipes
Christensenellaceae R-7 group
Lactobacillus
Alistipes
Prevotella 7
Parabacteroides
Bacteroides
Collinsella
Edaphobacter

Figure 2.4 Microbial composition in the hindgut of newborn calves (NB). (A) Microbial composition of NB calf at phylum level. Bars represent the relative abundance of the identified bacterial phyla (the relative abundance >0.1% and present in more than half number of the total animals at least in one age group) in different regions (cecum, colon and rectum) of hindgut. (B) Microbial composition of NB calf at family level. Bars represent the relative abundance of detectable bacterial family (the average relative abundance of the family >0.1%, and presented in at least half of the animals) in different regions (cecum, colon and rectum) of hindgut. (C) Microbial composition of NB calf at genus level. Bars represent the relative abundance of detectable bacterial genera (the average relative abundance of the genus >0.1%, and presented in at least half of the animals) in different regions (cecum, colon and rectum) of hindgut.

(A)



(B)

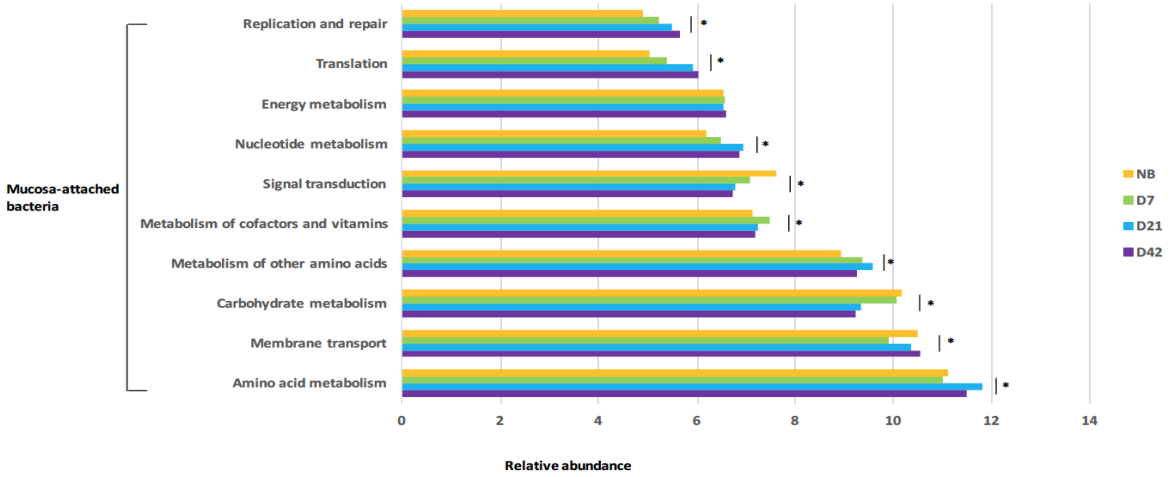


Figure 2.5 Predicted microbial functions using Tax4fun. (A) Comparisons of the top 10 predicted microbial predominant gene pathways for mucosa-attached bacterial community among different age groups in the hindgut. (B) Comparisons of the 10 predicted

microbial predominant gene pathways for digesta-associated bacterial community among different age groups in the hindgut. Star means significant difference among different age groups.

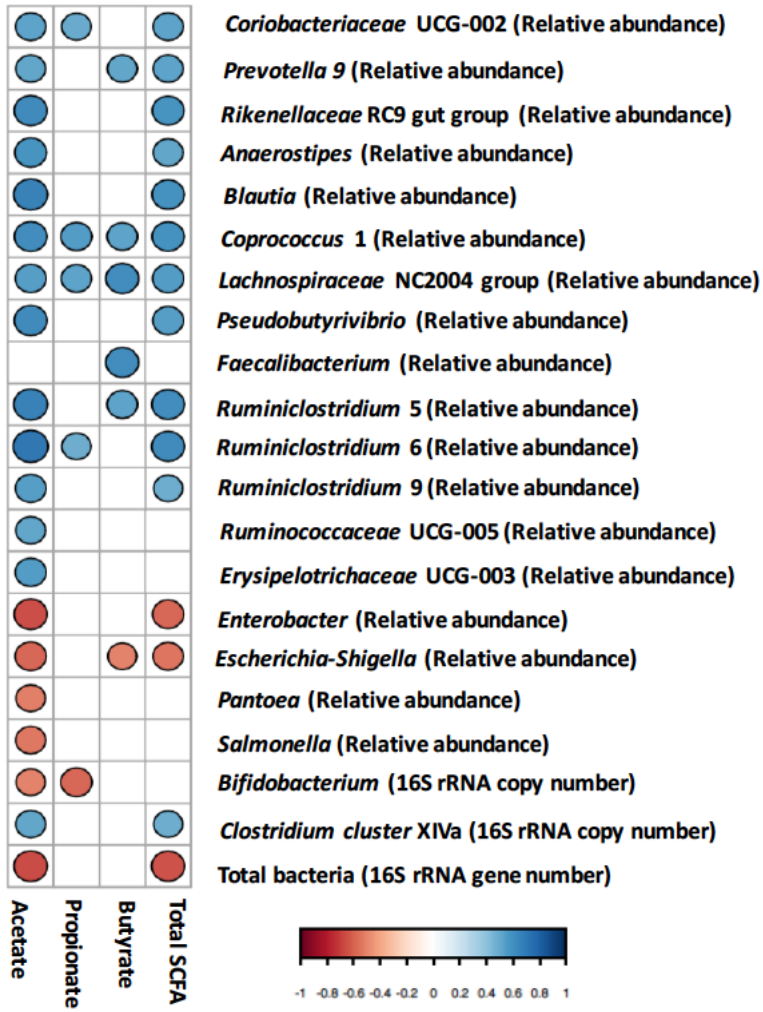


Figure 2.6 Relationship between mucosa-attached bacteria and SCFA concentration. Significant correlations were found between SCFA concentrations and the relative abundance of mucosa-attached *Coriobacteriaceae* UCG-002, *Prevotella* 9, *Rikenellaceae* RC9 gut group, *Anaerostipes, Blautia, Coprococcus* 1, *Lachnospiraceae* NC2004 group, *Pseudobutyrivibrio, Faecalibacterium, Ruminiclostridium* 5, *Ruminiclostridium* 6, *Ruminiclostridium* 9, *Ruminococcaceae* UCG-005 and *Erysipelotrichaceae* UCG-003 and the density of mucosa-attached *Bifidobacterium, Clostridium* cluster XIVa and total bacteria by Spearman's rank correlation.

Chapter 3. Colostrum feeding shapes the hindgut microbiota of dairy calves during the first 12 h of life

3.1 Introduction

Recent publications show that microbial colonization during early life is a dynamic process in mammals, which plays important roles in host immune system development (Wang et al., 2010; Mulder et al., 2011), health (Francino, 2014) and gut maturity (Yu et al., 2016). However, research on gut microbiota of neonatal ruminants is still scarce. The gastrointestinal tract bacterial composition differs between mucosa and digesta communities as well as among gut regions of neonatal calves (Malmuthuge et al., 2014). Meanwhile, our recent study revealed a diversely colonized bacterial community in the hindgut of calves at birth and its compositional changes with age and diet (Song et al., 2018). It has also been reported that fecal microbiota of calves is age and growth dependent (Uyeno et al., 2010; Klein-Jöbstl et al., 2014) and its composition is associated with pre-weaned calf health (Oikonomou et al., 2013). However, how the colostrum management affects the neonatal dairy calves' gut microbiota during early life is unknown.

Colostrum administration to newborn calves immediately after birth is recommended in all the North America dairy farms due to its abundant nutritional, and immune components (Foley and Otterby, 1978; Godhia and Patel, 2013). Calves are born agammaglobulinemic (Godden et al., 2008), and therefore passive transfer of immunoglobulin G (IgG) via colostrum prevents calves from the development of sepsis (Besser and Gay, 1985). In addition, feeding colostrum inhibits colonization of pathogens (Corley et al., 1977) and promotes colonization of beneficial bacteria in the gut (Malmuthuge et al., 2015), weight gain (Swanson and Gorman,

1967; Hammon et al., 2002) and intestinal development (e.g., villus circumference, height and epithelial cell proliferation) (Roffler et al., 2003) of neonatal calves. Poor hygiene practices during colostrum handling increase microbial contamination, leading to increased intake of pathogens by neonatal calves (Morrill et al., 2012). Thus, feeding heat-treated colostrum has become popular recently as a method to prevent microbial contamination in the fresh colostrum. Heat treatment (usually at 60℃ for 30 min) not only eliminates the potential pathogens such as *Mycoplasma bovis*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella. enteritidis* in colostrum (Godden et al., 2006) but also increases the transfer of passive immunity (Johnson et al., 2007). Additionally, feeding heat-treated colostrum enhanced *Bifidobacterium* and inhibited *E. coli* colonization in the ileum of calves during the first 12 hours of life (Malmuthuge et al., 2015). Enhanced colonic *Bifidobacterium* colonization is due to the more available oligosaccharides, the substrate for their growth, in heat-treated colostrum compared to non-heated colostrum (Fischer et al., 2018). However, the effect of colostrum (either non-heated or heated) on the colon microbial colonization during early life has not been studied.

Colon, as part of large intestine, is an important gut region where harbours a higher density of specific bacteria and total bacteria in neonatal calves (Smith and Williams Smith, 1965; Song et al., 2018). Moreover, the colon microbiota plays an important role in the hindgut fermentation (Song et al., 2018), immune system development (Malmuthuge et al., 2012) and health (Chanter et al., 1986; Moxley and Francis, 1986) of calves. Therefore, in this study, we aimed to identify the effect of differing colostrum feeding strategies (no colostrum, NC; non-heated colostrum, FC; heated colostrum, HC) on the colon microbiota of dairy calves within 12 h of life.

3.2 Materials and methods

3.2.1 Preparing colostrum

The procedures for colostrum collection and the animal trial were described by Malmuthuge et al. (2015). Briefly, before the animal trial, ~ 48 L colostrum with IgG concentration ≥ 50 mg/mL (measured at room temperature with hydrometer) were collected from 16 Holstein dairy cows at Dairy Research and Technology Center (DRTC), University of Alberta (Edmonton, Canada). The colostrum collection lasted for three months and it was stored at -20°C immediately after each collection. The frozen colostrum (~ 48 L) were then thawed completely at 4°C for ~ 24 h, pooled and mixed thoroughly. Half of the colostrum (24 L) was pasteurized (60 °C for 60 min) using pasteurizer DT 10G (Dairy Tech Inc., Greeley, CO) to obtain heat-treated colostrum. The heat-treated colostrum (~ 24L) and the remaining half (~ 24 L) of non-heated colostrum were aliquoted into 1L plastic freezer bags and stored at – 20 °C prior to the animal trial.

3.2.2 Animal experiment and sample collection

The animal experiment was conducted at the DRTC, University of Alberta following the approved protocols of the experiment by Livestock Care Committee of the University of Alberta (AUP00001012). The neonatal Holstein bull calves were separated from dams immediately after birth, and transferred to individual pen, following navel disinfection with 7% iodine. In total, thirty-two Holstein bull calves with birth weight (42.79±0.56 kg) were randomly divided into three treatment groups: non-heated colostrum (FC, n=12), heated colostrum (HC, n=12) and no colostrum (NC, n=8). Colostrum (FC or HC) were thawed at ~37°C using a water bath and the calves were fed 2 L colostrum within 1 h after birth using bottle feeding. Calves were humanely

euthanized at either 6 h (NC, n=4; FC, n=6; HC, n=6) or 12 h (NC, n=4; FC, n=6; HC, n=6) after birth.

Colon tissue and digesta samples were collected within 30 min after euthanasia following the procedures reported by Song et al. (2018). Briefly, colon was defined as 30 cm distal to the ileo-cecal junction, and 10-cm long colon segment was identified and ligated using table ties to prevent the flow of luminal content. Colon tissue and its contents were snap-frozen together (without separation) in the liquid nitrogen and stored at -80°C for future analysis.

3.2.3 DNA isolation

After the colon sample was thawed, the tissue and contents were separated on a petri dish kept on the ice. DNA extraction was performed using the modified repeated bead-beating and column method (Yu and Morrison, 2004). Briefly, digesta (~0.5 g) and tissue (0.1~0.2 g) were mixed with 1 mL lysis buffer and were physically disrupted using BioSpec Mini Beads beater 8 (BioSpec, Bartlesville, OK). Following beads beating, samples were incubated at 70ºC for 15 minutes and the supernatant was collected through centrifugation, and this step was repeated once. Genomic DNA was then precipitated using 10 M ammonium acetate and isopropanol following the purification using QIAamp fast DNA stool mini kit (QIAGEN Inc. CA, USA). The quantity and quality of the extracted DNA was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.2.4 Estimation the abundance of total bacteria and selected bacterial groups using quantitative real-time PCR

The abundance of mucosa- and digesta-associated total bacteria, *Bifidobacterium*, *Escherichia coli*, *Faecalibacterium prausnitzii*, and *Clostridium* cluster XIVa were quantified through estimating the copy number of the respective targeted 16S rRNA genes using

quantitative real-time PCR (qPCR) with SYBR green chemistry (fast SYBR green master mix, Applied Biosystems) and group specific primers (Table 3.1). The copy number of 16S rRNA gene of mucosa-attached and digesta-associated bacteria per gram (copy number/g sample) was calculated using the equation described by Li et al. (2009). In addition, the proportion of each bacterial group was calculated by dividing the copy number of each bacterial group with the copy number of total bacteria. Four bacterial groups including *Bifidobacterium*, *Clostridium* cluster XIVa, *F. prausnitzii* and *E. coli* were plotted under different colostrum treatments (NC, FC and HC) and time points (6 h and 12 h) using PCA biplot diagram in R (R package 3.3.1).

3.2.5 Profiling of the colon microbiota using amplicon-sequencing

Amplicons for sequencing were generated with universal primers 27F and 1492R (27F 5′-AGAGTTTGATCMTGGCTCAG-3′, 1492R: 5′-TACGGYTACCTTGTTACGACTT-3′) (Weisburg et al., 1991) to amplify the full length 16S rRNA gene first, and then followed by a second PCR amplification using primer 27F and 515R (Bac9F-CS1F: ACACTGACGACATGGTTCTACAGAGTTTGATCMTGGCTCAG, 515R-CS2R: TACGGTAGCAGAGACTTGGTCTCCGCGGCKGCTGGCAC) (Kroes, Lepp and Relman 1999) containing pyrotags, targeting V1-V3 hypervariable region of 16S rRNA gene. The PCR products were purified with QIAEX II gel extraction kit (Qiagen Science, MD, USA). The quality and quantity of purified amplicon was evaluated using NanoDrop 1000 (NanoDrop Technologies, Wilmington) and Picofluor Handheld Fluorometer conjunct with picogreen (Quant-iT™ PicoGreen™ dsDNA Reagent). The amplicon sequencing with paired end was carried out at the Genome Quebec Innovation Centre, McGill University (Montreal, Quebec, Canada) using the MiSeq platform (Illumina, 2 × 300bp).

3.2.6 Sequencing data analysis

Sequence data was analyzed using QIIME 1 (Quantitative Insight into Microbial Ecology) (Caporaso et al., 2010) following the pipeline published by Li et al. (2016). Briefly, forward and reverse sequences were joined for each sample, and the joined reads were subjected to quality filtering to remove low quality (Phred score < 25) and short reads (< 400 bp). Then, the chimeric sequences were removed using USEARCH (Edgar, 2010) within QIIME. The operational taxonomic units (OTUs) picking was performed using the *de novo* protocol with usearch61 at 97% similarity, followed by the taxonomic assignment using SILVA 128 database (September 2016 release, Quast et al., 2013). Alpha diversity indices (Chao1, Shannon and Observed species) and Good's coverage were obtained using alpha rarefaction script. Principal coordinate analysis (PCoA) of mucosa- and digesta-associated microbial profiles was conducted based on the Bray-Curtis dissimilarity matrix.

3.2.7 Statistical analyses

For the bacterial taxonomy, only taxa with the relative abundance >0.05% (> 10 reads per sample) and present in more than half of the total animals at least within one treatment group (colostrum treatment or age group) were defined as detectable and were used for comparison analysis. The effect of colostrum treatment on the relative abundance of the detected bacterial taxa (amplicon sequencing) was compared using nonparametric Wilcoxon rank-sum test method in R (3.3.1). Additionally, the density of four bacterial groups (based on qpcr) were first transformed to obtain a normal distribution using arcsine square root transformation and then the transformed data were analysed using ANOVA. Changes in bacterial taxa (amplicon sequencing) among different time point under different colostrum treatments (NC, FC and HC) were tested using nonparametric Kruskal-Wallis test statistical method in R (3.3.1). The significant

difference among two different age groups was tested with Dunn's test in R (3.3.1). The *P*-value (both for amplicon sequencing and qpcr data) was adjusted with Benjamini-Hochberg approach for false discovery rate (FDR) with statistical significances declared at $P < 0.05$ and tendencies at $0.05 \leq P < 0.10$.

3.2.8 Data submission

The sequences in this study were deposited at NCBI sequence read archive (SRA) under the accession number SRP102324 and SRP102363.

3.3 Results

3.3.1 General sequencing stats

Amplicon sequencing generated 447,481 high quality sequences in total. There were 12,430±1,104 sequences per mucosal sample with the Good's coverage ranging from 0.99 - 1; and there were 20,304±1,233 sequences per digesta sample with Good's coverage ranging from 0.994 to 0.999.

3.3.2 Diversity of the colon microbiota during the first 12 h of life

When the diversity indices of colon microbiota were compared, Chao 1, Shannon and number of observed species tended to be higher in digesta-associated bacterial community at 12 h in the colon of FC fed calves than that of NC calves, with no significant difference found for mucosa-attached bacterial communities (Table 3.2). Similarly, Chao 1 index and number of observed species of both mucosa and digesta were tentatively higher in the colon digesta of HC fed calves compared to that in NC treatment at 12 h after birth (Table 3.2). Moreover, no difference was found in the diversity indices (Chao 1, Shannon and number of observed species) between FC and HC fed calves. When the bacterial profiles were compared, PCoA plot showed

no clear separation in both mucosa and digesta-associated bacterial community among three colostrum treatments (NC, FC and HC) (Figure 3.1). Meanwhile, no separation was found when comparing the mucosa and digesta associated microbial profiles at different time points (6 h and 12 h) (Figure 3.2).

3.3.3 Taxonomic assessment of neonatal calves during the first 12 h of life

In total, 10 bacterial phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, *Saccharibacteria* and *Spirochaetae*) were identified from the mucosa-attached bacterial community at 6 h of life regardless of colostrum treatment. However, only six phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes* and *Proteobacteria*) were identified at 12 h (Supplementary Dataset 3.1). In addition, 54 and 32 bacterial families were identified at 6 h and 12 h (Figure 3.3A and 3.3B), respectively, with *Burkholderiaceae, Enterobacteriaceae* and *Acidobacteriaceae* being the top three families at 6 h, and *Enterobacteriaceae, Clostridiaceae* 1 and *Enterococcaceae* being the top three families at 12 h (Supplementary Dataset 3.2). Moreover, 105 and 72 genera were identified at 6 h and 12 h respectively, with *Burkholderia-Paraburkholderia* and *Escherichia-Shigella* being the predominant genera at 6 h, and *Escherichia-Shigella*, *Clostridium sensu stricto* 1 being the dominant genera at 12 h (Supplementary Dataset 3.3).

For digesta-associated bacteria, nine phyla (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Chloroflexi*, *Fibrobacteres*, *Firmicutes*, *Proteobacteria* and *Spirochaetae*) were identified at 6 h after birth while only eight phyla excluding *Bacteroidetes* were identified at 12 h, regardless of colostrum treatment (Supplementary Dataset 3.4). There were 35 families identified at 6 h (Figure 3.3C), with *Enterobacteriaceae, Burkholderiaceae* and *Clostridiaceae* 1

being the predominant families (Supplementary Dataset 3.5) and 25 bacterial families were identified at 12 h (Figure 3.3D), with *Enterobacteriaceae, Clostridiaceae* 1 and *Lachnospiraceae* being the top three families (Supplementary Dataset 3.5). In total, 72 and 54 genera were identified at 6 h and 12 h separately, with *Escherichia-Shigella* and *Burkholderia-Paraburkholderia* being the top two genera at 6 h, and *Escherichia-Shigella, Clostridium sensu stricto* 1 were the predominant bacteria at 12 h (Supplementary Dataset 3.6).

3.3.4 Effect of non-heated colostrum feeding (FC vs. NC) on taxonomic composition of colon microbiota during the first 12 h of life

When the colon mucosa-attached bacteria were compared between FC and NC at both 6 h and 12 h separately after birth, FC did not affect the bacterial community at both family and genus level (Table 3.3; Table 3.4). For digesta-associated microbiota, the relative abundance of an unclassified genus from *Peptostreptococcaceae* family tended to be lower under in colon of FC fed claves compared to that in NC calves (P=0.09) (Table 3.4) at 6 h after birth and the relative abundances of *Lactococcus* (P=0.01), *Citrobacter* (P=0.01), *Enterobacter* (P=0.01), *Pantoea* (P=0.01), *Salmonella* (P=0.01) and *Pseudomonas* (P=0.01) were higher in the colon of FC fed calves than those with NC feeding at 12 h after birth, (Table 3.4).

3.3.5 Effect of heat-treated colostrum feeding (HC vs. NC) on taxonomic composition of colon microbiota during the first 12 h of life

For the mucosa-attached bacteria, the relative abundances of *Clostridiaceae* 1 (P=0.08) and *Enterococcaceae* (P=0.05) families and *Clostridium sensu stricto* 1 (P=0.06) and *Enterococcus* (P=0.06) genera were tentatively lower in the colon of HC fed calves compared to those in NC calves at 6 h after birth (Table 3.3; Table 3.4). When the bacterial abundance at 12 h of life were compared, the relative abundances of *Burkholderiaceae* (P=0.05) and

Xanthomonadaceae (*P*=0.05) families, and the relative abundances of *Clostridium sensu stricto* 1 (*P*=0.08), *Peptoclostridium* (*P*=0.08), *Lactococcus* (*P*=0.08) and *Streptococcus* (*P*=0.07), *Burkholderia-Paraburkholderia* (*P*=0.06), *Cupriavidus* (*P*=0.06), and *Stenotrophomonas* (*P*=0.06) genera tended to be higher, while the relative abundances of *Enterobacteriaceae* (*P*=0.05) and *Escherichia-Shigella* (*P*=0.06) tended to be lower in the colon of HC fed calves (Table 3.3; Table 3.4).

For digesta-associated bacterial community, the relative abundance of *Fibrobacteraceae* family (*P*=0.05) was tentatively lower and *Fibrobacter* genera (*P*=0.03) was lower at 6 h after birth, while the relative abundances of *Pseudomonadaceae* (*P*=0.05) family and *Epulopiscium* (*P*=0.06), *Lactococus* (*P*=0.06) genera were tentatively higher*, and Lachnospiraceae* UCG-009 (*P*=0.02)*, Citrobacter* (*P*=0.02)*, Enterobacter* (*P*=0.02)*, Pantoea* (*P*=0.02)*, Salmonella* (*P*=0.02) and *Pseudomonas* (*P*=0.02) genera were significantly higher at 12 h after birth in the colon of HC fed calves compared to those in NC calves (Table 3.3; Table 3.4).

3.3.6 Effect of colostrum feeding treatment (HC vs. FC) on taxonomic composition of colon microbiota during the first 12 h of life

When the bacterial abundance at 6 h of life were compared between HC and FC feeding, the relative abundance of *Enterococcus* (*P*=0.06) in mucosa-attached community and the relative abundances of *Fibrobacter* (*P*=0.08) and *Salmonella* (*P*=0.08) were tentatively lower in the digesta-associated community in the colon of HC fed claves than those fed FC (Table 3.4). At 12 h after birth, there was no colostrum treatment effect on the mucosa-attached bacteria at both family and genus level (Table 3.3; Table 3.4), while the relative abundances of *Enterobacter* (*P*=0.09) and *Salmonella* (*P*=0.09) were tentatively lower in the digesta-associated community in the colon of calves fed HC compared to those fed FC (Table 3.4).

3.3.7 Variations in the bacterial abundances in response to colostrum feeding strategies detected by quantitative PCR

PCA plot based on qPCR analysis showed a clear separation of four selected bacterial groups among treatments in colon mucosa (Figure 3.4A and 3.4C) and digesta (Figure 3.4B and 3.4D) at 6 h and at 12 h. The length of the vectors in PCA plot indicated that *Bifidobacterium* and *Clostridium* cluster XIVa were the predominant bacterial groups, followed by *F. prausnitzii* and *E. coli*, in both colon mucosa and digesta communities. When the bacterial abundances were compared, the proportion of *Bifidobacterium* and *Clostridium* cluster XIVa were higher, while the proportion of *E. coli* was lower in the colon of calves fed FC than those fed NC during the first 12 h after birth (Table 3.5). Similarly, The proportion of mucosa and digesta-attached *Bifidobacterium* and *Clostridium* cluster XIVa was higher, while the proportion of *E. coli* was lower with HC feeding at both 6 h and 12 h after birth in comparison to NC (Table 3.5). In addition, the proportion of mucosa-attached *Bifidobacterium* and *Clostridium* cluster XIVa were higher at 6 h after birth and the proportion of mucosa-attached *Bifidobacterium* was higher at 12 h after birth, while the proportion of mucosa and digesta-attached and *E. coli* and total bacterial copy numbers were lower at 12 h after birth in the colon of HC fed calves when compared to those fed FC (Table 3.5).

3.3.8 Variations in the bacterial abundances in response to colostrum feeding strategies detected by quantitative PCR

To further identify how the microbiota shifted during the first 12 h of life and how the colostrum feeding affected such shift, we compared the changing pattern of 16S rRNA gene copy number of total bacteria, the relative abundance of major bacterial phylum and genera using data from newborn calves (0h, obtained from Song et al., 2018), 6 h, and 12 h. Total bacterial

population had different changing patterns under NC compared to those under FC and HC, but it was similar between FC and HC fed calves (Figure 3A). In addition, the relative abundance of *Bacteroidetes* phylum changed in the same way from 0h to 12 h under FC and HC treatment. However, the shift pattern of the relative abundances of predominant phyla *Firmicutes* and *Proteobacteria* varied differently during the first 12 h after birth under different colostrum treatment (NC, FC and HC) (Figure 3.5B). Moreover, the relative abundance of the most predominant bacterial genera *Escherichia-Shigella* numerically decreased first from 0 h to 6 h, and increased significantly from 6 h to 12 h in the colon of both FC and HC fed calves, while it kept increasing from 0 h to 12 h in the colon of NC calves (Figure 3.5C).

3.4 Discussion

Early life microbiota affects the host in both short term and long term. It has been reported that postnatal microbial colonization stimulates the development of Peyer's patches and mesenteric lymph nodes in monogastric animals (Renz et al., 2011). In the long term, the higher relative abundance of *F. prausnitzii* at first week of life has been reported to be related to higher weight gain and lower diarrhea rate of dairy calves at three weeks of life (Oikonomou et al., 2013). Based on the potential importance of initial microbiota colonization during the first few hours of life and the colostrum feeding can directly affect the initial colonization, our study was performed to obtain a complete understanding of how the colostrum feeding affects the microbiota colonization in the large intestine during the first 12 h after birth.

This study is the first to assess the shift of the bacterial population and the relative abundance of the major bacterial genera in the colon of neonatal calves during the first 12 h of life, which is more dynamic than those at the older ages during the pre-weaning period

(Oikonomou et al., 2013, Song et al., 2018). The rapid changes of bacterial composition may be related to the intestinal environment (e.g., existence of oxygen, pH), the time of the availability of nutrients and host adaptation. Mucosa-attached *Bacteroidetes* is one of the predominant phylum (28.34±2.74%) in the colon of the newborn calves (Song et al., 2018), but it was less than 1% at 6 h and 12 h. The significant lower mucosa-attached *Bacteroidetes* may be due to microbial adaptation to the surrounding environment and nutrients, as well as the competition with other phyla (e.g., high abundance of *Proteobacteria*) for the colonization niche. Additionally, *Enterobacteriaceae* was the most predominant family at all time points (0 h, 6 h, 12 h; the present study for 6 h and 12 h, and Song et al., 2018 for 0 h). The higher relative abundance of *Enterobacteriaceae* during early life is related to the important function of the members belong to this family. The colonization of facultative anaerobic *Enterobacteriaceae* spp. has been reported to utilize the intestinal oxygen during early life, which could provide an environment for the obligate anaerobic bacteria to grow (Favier et al., 2002). In addition to the well-known bacterial phyla (*Bacteroidetes*, *Firmicutes* and *Proteobacteria*), *Cyanobacteria,* the phylum that usually achieves their energy through photosynthesis, was detected in the colon of neonatal calves. In the past decade, many papers have reported the presence of this phylum in the gut of human (Ley et al., 2005, Di Rienzi et al., 2013) and rumen of cattle (Neves et al., 2017; Schären et al., 2017). The "gut *Cyanobacteria*" was hypothesised to be a non-photosynthetic descendants of *Cyanobacteria*, which was adapted to the gut environment (Ley et al., 2005). The genomic analysis revealed that gut *Cyanobacteria* are highly conserved but they do differ from the photosynthetic *Cyanobacteria*, whose members are lack of photosynthetic capability but are capable of fermenting a range of sugars (e.g., glucose, fructose, sorbitol) into acetate and butyrate under anaerobic conditions (Soo et al., 2014). The authors also prosed a new candidate

class called *Melainabacteria* within *Cyanobacteria* phylum. From our dataset, part of the sequences identified as *Cyanobacteria* were belonged to *Melainabacteria* class (Supplementary Dataset 3.2), suggesting that non-photosynthesizing *Cyanobacteria* may be the natural colonizers as seen in human gut which they could remove oxygen from gut environment and to provide energy for the neonatal calves. The source of this coloniser could be from the contact with maternal faeces in the anus area during the birthing process. However, whether these taxa colonize the ruminant gut through the life time as well as what are their roles in the gut warrant further investigations.

To gain a better understanding on the bacterial colonization in the gut during the first 12 h of life, we firstly compared the bacterial population in the colon detected in this study with those detected in the small intestine of the same animals as reported by Malmuthuge et al. (2015). Significant higher total bacterial population (estimated by 16S rRNA gene copy number) in the colon were found when compared to small intestine (mucosa-attached colon bacteria - $1.71\pm0.61\times10^{12}$, digesta-associated colon bacteria - $2.90\pm0.82\times10^{12}$; mucosa-attached small intestinal bacteria - $8.20\pm2.40\times10^{9}$, digesta-associated small intestinal bacteria - $1.50\pm0.80\times10^{10}$), which is similar with the findings in a human study (Walter and Ley, 2011), suggesting that large intestinal environment is more suitable for microbial colonization compared to small intestinal regions during the first 12 h of life. In addition, the observed higher mucosa and digesta-associated total bacterial density at 12 h of life in the ileum and colon of FC fed calves than NC calves indicate that colostrum could enhance microbial colonization in the gut. In addition, bacterial population is related to mucosal and systemic immune responses in pups (Lamousé-Smith et al., 2011), suggesting that the immune system development in the calves could be strengthened by colostrum via enhancing the "trained immunity" (Netea et al., 2011)

through the responses to the higher number of colonized bacteria. Moreover, the tendency of higher digesta-associated bacterial diversity (Chao 1, Shannon and observed species) at 12 h in the colon of FC fed calves than those NC calves, suggests that FC may benefit immune system development in the colon of neonatal calves because the higher bacterial diversity may be responsible for modulating normal immune system balance (Cahenzli et al., 2013).

Bifidobacterium is a commonly recognized probiotic, which plays an important role in host (humans and mice) immune system development (Paineau et al., 2008; Hougee et al., 2010), antimicrobial activities (Servin, 2004). Malmuthuge et al. (2015) reported that *Bifidobacterium* was not detected in the colostrum which was used to feed the same animals of current study, indicating the observed higher *Bifidobacterium* in the colon of FC fed calves is facilitated by non-heated colostrum feeding. Oligosaccharide, one of the bioactive components in bovine colostrum, has been reported to stimulate the growth of *Bifidobacterium* (Ward et al., 2007), and this may explain the higher prevalence of *Bifidobacterium* in the colon of FC fed calves. Additionally, sialylated oligosaccharide, the main oligosaccharide of bovine colostrum, can adhere the intestinal epithelial cells (Kavanaugh et al., 2013), supporting that there is a higher prevalence of mucosa-attached *Bifidobacterium* in the colon of colostrum fed calves. In addition, *Clostridium* cluster XIVa includes many butyrate producing organisms, such as *Clostridium, Dorea, Lachnospira, Roseburia, Butyrivibrio* (Lopetuso et al., 2013). Butyrate can regulate gut barrier functions (Wang et al., 2012) and immune system development (Arpaia et al., 2013), meanwhile, it is one of the major energy sources for the growth of colonic cells (Bergman, 1990). Therefore, the higher abundance of *Clostridium* cluster XIVa in the colon of FC fed calves suggests potential higher butyrate production in the colon, which can be considered as another beneficial effect of colostrum feeding. In addition, *Clostridium* cluster XIVa was

reported to promote Treg cells accumulation in the colon of mice (Atarashi et al., 2011), therefore, the higher abundance of *Clostridium* cluster XIVa in the colon after FC and HC further indicates that colostrum feeding, especially feeding heat-treated colostrum could motivate colon immune system development and barrier function. Future studies to measure the butyrate concentration and gut immune and barrier functions are needed to verify our above speculations.

Benefits of feeding heat-treated colostrum in reducing total bacterial counts (Donahue et al., 2012; Gelsinger et al., 2014), eliminating pathogens (Godden et al., 2006) and increasing IgG absorption (Godden et al., 2012) have been reported. Moreover, heat-treated colostrum was suggested to optimize microbiota colonization in the small intestine (Malmuthuge et al., 2015). The lower relative abundance of *Enterobacteriaceae* family in the colon of HC fed calves than those in NC animals probably suggests a lower prevalence of colonization of opportunistic pathogens when heat-treated colostrum is fed. *Enterobacteriaceae* family includes members that belong to pathogenic species, including *Salmonella* (Zhang et al., 2003) and *Escherichia coli S102-9* (Chanter et al., 1986), which could cause diarrhea. Moreover, *Escherichia-shigella* genus consists of many *Escherichia. coli* strains (e.g. *Escherichia coli* O157:H7) that are related to calf and pig diarrhea (Chanter, et al., 1986; Francis et al., 1986). Therefore, the lower abundance of this genus in the colon of HC fed calves compared with NC fed calves also suggests that calves fed with HC may have less prevalence of diarrhea caused by these pathogens compare to those fed no colostrum. However, both *Enterobacteriaceae* and *Escherichia-shigella* include commensal and pathogenic bacterial species, it is necessary to research if the decrease in *Enterobacteriaceae* and *E. coli* density represents the changes in pathogenesis. Future studies to evaluate the shifts in the proportion of pathogenic bacterial specie/strains (e.g., *E. coli* O157:H7, *E. coli S102-9*) within the them and/pathogenicity associated genes can provide more direct

evidence on the role of this family and genus in calf health. Additionally, the higher increased fold change of mucosa-attached *Bifidobacterium* and *Clostridium* cluster XIVa at 6 h and 12 h (*Bifidobacterium*-6 h: FC/NC - 2.00, HC/NC -2.44; *Bifidobacterium*-12 h: FC/NC – 2.26, HC/NC – 2.77; *Clostridium* cluster XIVa – 6 h: FC/NC – 7.84, HC/NC – 18.64; *Clostridium* cluster XIVa – 12 h: FC/NC – 25.13, HC/NC – 32.45) in the colon of HC fed calves further highlight the importance of HC feeding in protecting the intestine against pathogens, as well as immune function development when compared to FC feeding (Table 3.6). In the future, it is necessary to include the measurement of immune functions related parameters in the colon tissue (e.g., T, B cells density, toll like receptors family gene expression), and more phenotypic data for more convincing interpretations.

In addition, heat treatment was hypothesized to denature the oligosaccharide-protein bond structure, resulting in enriched free oligosaccharide (Neeser et al., 1991). Indeed, higher bovine colostrum oligosaccharides (bCOs) was detected in heat-treated colostrum (3511.6 μg/g) than non-heated colostrum (1329.9 μg/g) (Fischer et al., 2018), which was used in this study. As bCOs serve as substrates for the growth of *Bifidobacterium* spp. (Ward et al., 2007), the lower concentration of bCOs in the colon of HC fed calves (25.60±13.1 μg/g) compared with that of FC fed calves (267.04±125.81 μg/g) (Fischer et al., 2018) of the same animals may explain why the higher prevalence of mucosa and digesta-attached *Bifidobacterium* in the HC compared with FC at 6 h of life. Together, these findings indicate that HC could enhance colonic beneficial *Bifidobacterium* colonization in comparison to FC by increasing the free bCOs in the colostrum. Lastly, the different shift patterns of *Escherichia-Shigella* genus from birth to 12 h after life under different colostrum feeding groups (NC, FC and HC) (Figure 3.5C) indicate that colostrum feeding, especially HC feeding shapes the initial microbial colonization towards a host benefiting

way. Therefore, based on our above findings, colostrum feeding, especially heat-treated colostrum (60 ℃ for 60 min) feeding is suggested to be used in dairy farms.

3.5 Conclusions

This study showed that non-heated colostrum feeding (FC vs. NC) stimulated the colonization of beneficial bacterial groups (e.g., *Clostridium* cluster XIVa and *Bifidobacterium*), and inhibited the colonization of opportunistic pathogenic bacteria, including *E. coli*. Comparing the bacterial communities of calves fed HC with those of FC revealed that feeding HC fortifies the beneficial effects of colostrum via further reducing the relative abundance of mucosa-attached *Enterobacteriaceae* and *E. coli*, and enhancing the abundance of mucosa-attached *Bifidobacterium* at 12 h of life, which may affect the colon microbiota in both short and long terms. In the practical setting, it is difficult to control potentially pathogenic contamination of colostrum during collection, storage and transportation. Therefore, the heat treatment (60 ℃, 60 min) is a valid management to reduce the potential pathogen but enhance the beneficial bacteria colonization in the colon, which can improve calf gut health and potentially overall health. Overall, our results suggest that non-heated colostrum feeding shapes the colon microbiota to have higher beneficial and lower chance of potentially pathogenic organisms, which may play an important role in enhancing gut health of dairy calves, with heat treatment having more significant benefit.

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3.7 Tables

Table 3.1 Primers used in the present study for qPCR analysis.

Bacterial group	Primers	Product size	Annealing temperature	Reference
Total Bacteria	F: 5'-actcctacgggaggcag-3' R: 5'-gactaccagggtatctaatcc-3'	467 bp	62 °C	Stevenson & Weimer, 2007
Bifidobacterium	F: 5'-tacaccggaatagctcctgg-3' R: 5'-cgtcaagctgataggacgc-3'	115 bp	64 °C	Liang et al., 2014
E. coli	F: 5'-ggaagaagcttgcttctttgctgac-3' R: 5'-agcccggggatttcacatctgactta-3'	544 bp	62 °C	Sabat et al., 2000
***Clostridium* cluster XIVa**	F:5'-cggtacctgactaagaagc-3' R:5'-agtttyattcttgcgaacg-3'	429 bp	60 °C	Ramirez-Farias et al., 2009
Faecalibacterium. Prausnitzii	F:5'-ggaggaagaaggtcttcgg-3' R:5'-aattccgcctacctctgcact-3'	248 bp	60 °C	Ramirez-Farias et al., 2009

Table 3.2 Effect of non-heated colostrum feeding and heat treatment on bacterial diversity and richness of colon during the first 12 h[1] of life (mean ± SEM).

Sample type	Time	Items	Treatment[1]			*P*-value		
			NC	FC	HC	NC vs. FC	FC vs. HC	NC vs. HC
Mucosa-attached bacteria	6 h	Chao 1	219.65±16.21	179.48±22.66	157.28±10.77	0.36	0.73	0.08
		Shannon	3.82±0.30	3.66±0.53	3.33±0.23	0.89	0.73	0.38
		Observed species	167.25±17.11	131.2±12.50	119.33±9.94	0.36	0.73	0.11
	12 h	Chao 1	105.61±11.60	133.11±12.05	173.36±12.40	0.36	0.36	0.07
		Shannon	2.62±0.18	3.25±0.35	3.50±0.28	0.36	0.73	0.10
		Observed species	85.00±8.40	101.17±11.26	136.80±11.44	0.50	0.36	0.07
Digesta-associated bacteria	6 h	Chao 1	235.32±13.94	246.18±38.26	183.85±21.30	0.89	0.63	0.31
		Shannon	4.05±0.13	3.92±0.27	3.59±0.36	0.89	0.73	0.48
		Observed species	183.75±9.89	181.20±28.93	133.50±21.28	0.94	0.63	0.23
	12 h	Chao 1	133.94±11.05	216.44±22.61	219.53±18.21	0.08	0.92	0.07
		Shannon	2.85±0.32	4.39±0.35	4.03±0.16	0.08	0.73	0.07
		Observed species	100.50±9.51	173.67±16.25	178.00±10.93	0.06	0.92	0.07

$P<0.05$ means there is significant difference between treatment; $0.05<P<0.1$ means there is a tendency to be significant difference between treatment.

[1]Treatments: FC = non-heated colostrum; HC= heated colostrum; NC = no colostrum.

Table 3.3 Effect of non-heated colostrum feeding and heat-treated colostrum on the relative abundance of colon bacterial taxa at family level within the first 12 h of life[1] .

Time	Item	Phylum	Order	Family	Treatment			Adjusted *P*-values		
					NC	FC	HC	NC vs. FC	FC vs. HC	NC vs. HC
6 h	Mucosa	*Firmicutes*	*Clostridiales*	*Clostridiaceae* 1	2.81±1.28	3.39±3.32	0.02±0.01	0.63	0.10	0.08
			Lactobacillales	*Enterococcaceae*	0.23±0.11	0.48±0.28	0.00±0.00	1.00	0.10	0.05
	Digesta	*Fibrobacteres*	*Fibrobacterales*	*Fibrobacteraceae*	0.05±0.01	0.08±0.03	0.01±0.00	0.85	0.10	0.05
12 h	Mucosa	*Proteobacteria*	*Burkholderiales*	*Burkholderiaceae*	0.55±0.20	1.89±0.90	5.06±0.84	0.36	0.11	0.05
			Enterobacteriales	*Enterobacteriaceae*	90.44±5.88	63.80±8.07	40.25±7.59	0.11	0.10	0.05
			Xanthomonadales	*Xanthomonadaceae*	0.00±0.00	0.03±0.03	0.39±0.20	0.98	0.10	0.05
	Digesta	*Proteobacteria*	*Pseudomonadaceae*	*Pseudomonadaceae*	0.01±0.00	1.05±0.24	0.49±0.18	0.11	0.21	0.05

P<0.05 means there is significant difference between treatment; *P*<0.1 means there is a tendency to be significant between treatment.
[1]Treatments: FC = non-heated colostrum; HC= heated colostrum; NC = no colostrum.

Table 3.4 The effect of non-heated colostrum and heat-treated colostrum feeding on bacterial genera.

	Sample type	Phylum	Family	Genus				*P-value*		
					NC	FC	HC	NC vs. FC	FC vs. HC	NC vs. HC
6 h	Mucosa	*Firmicutes*	*Clostridiaceae* 1	*Clostridium sensu stricto* 1	2.24±1.04	3.34±3.28	0.01±0.00	0.67	0.10	0.06
			Enterococcaceae	*Enterococcus*	0.23±0.11	0.48±0.28	0.00±0.00	1.00	0.06	0.06
	Digesta	*Fibrobacteres*	*Fibrobacteraceae*	*Fibrobacter*	0.04±0.01	0.08±0.03	0.00±0.00	0.73	0.08	0.03
		Firmicutes	*Peptostreptococcaceae*	Unclassified	0.39±0.27	0.01±0.00	0.02±0.01	0.09	0.18	0.11
		Proteobacteria	*Enterobacteriaceae*	*Salmonella*	0.51±0.16	0.84±0.23	0.29±0.15	0.62	0.08	0.35
12 h	Mucosa	*Firmicutes*	*Clostridiaceae* 1	*Clostridium sensu stricto* 1	5.82±5.81	23.55±10.03	25.35±9.98	0.12	1.00	0.08
			Peptostreptococcaceae	Unclassified	0.06±0.03	0.00±0.00	0.00±0.00	0.12	1.00	0.08
				Peptoclostridium	0.01±0.01	0.28±0.16	0.31±0.19	0.12	1.00	0.08
			Streptococcaceae	*Lactococcus*	0.00±0.00	2.24±1.34	0.04±0.01	0.12	0.50	0.08
				Streptococcus	0.11±0.08	0.44±0.25	3.43±1.39	0.58	0.15	0.07
		Proteobacteria	*Burkholderiaceae*	*Burkholderia-Paraburkholderia*	0.30±0.17	1.29±0.72	3.29±0.65	0.39	0.15	0.06
				Cupriavidus	0.25±0.09	0.60±0.20	1.76±0.45	0.47	0.15	0.06
				Escherichia-Shigella	89.10±5.66	58.46±8.39	39.12±7.35	0.12	0.16	0.06
			Xanthomonadaceae	*Stenotrophomonas*	0.00±0.00	0.03±0.03	0.39±0.20	0.91	0.15	0.06
	Digesta	*Firmicutes*	*Lachnospiraceae*	*Epulopiscium*	0.07±0.07	0.47±0.18	0.36±0.09	0.08	1.00	0.06
				Lachnospiraceae UCG-009	0.01±0.00	0.10±0.08	0.53±0.29	0.11	0.12	0.02
			Streptococcaceae	*Lactococcus*	0.00±0.00	1.40±0.56	0.03±0.02	0.01	0.20	0.06
		Proteobacteria	*Enterobacteriaceae*	*Citrobacter*	0.00±0.00	0.58±0.21	0.09±0.05	0.01	0.11	0.02
				Enterobacter	0.02±0.01	3.71±0.83	0.95±0.43	0.01	0.09	0.02
				Pantoea	0.00±0.00	0.32±0.07	0.10±0.08	0.01	0.12	0.02
				Salmonella	0.01±0.00	1.47±0.31	0.52±0.18	0.01	0.09	0.02
			Pseudomonadaceae	*Pseudomonas*	0.01±0.00	1.05±0.24	0.49±0.18	0.01	0.20	0.02

$P<0.05$ means there is significant difference between treatment; $0.05<P<0.1$ means there is a tendency to be significant difference between treatment.

[1]Treatments: FC = non-heated colostrum; HC= heated colostrum; NC = no colostrum.

Table 3.5 Effect of non-heated colostrum feeding and heat-treated colostrum on quantification of five bacterial groups in the colon during the first 12 h after birth[1].

Time	Sample type	Bacterial group	Treatment[1]			*P*-value		
			NC	FC	HC	NC vs. FC	FC vs. HC	NC vs. HC
6 h	Mucosa-attached bacteria	Total bacteria[2]	$2.43\times10^{9}\pm2.31\times10^{9}$	$9.83\times10^{10}\pm7.60\times10^{10}$	$1.56\times10^{11}\pm1.83\times10^{11}$	0.28	0.47	0.18
		Bifidobacterium[3]	16.50±1.75	33.01±4.69	40.25±4.13	<0.01	0.03	0.02
		Clostridium cluster XIVa[3]	0.45±0.38	3.53±2.91	8.39±3.58	0.03	0.03	0.02
		E. coli[3]	0.06±0.02	0.02±0.01	0.01±0.00	<0.01	0.13	0.02
		F. prausnitzii[3]	0.72±0.04	0.06±0.02	0.54±0.16	0.03	0.05	0.36
	Digesta-associated bacteria	Total bacteria[2]	$2.76\times10^{10}\pm4.44\times10^{10}$	$4.04\times10^{11}\pm3.61\times10^{11}$	$1.34\times10^{11}\pm0.98\times10^{11}$	0.08	0.25	0.74
		Bifidobacterium[3]	2.17±0.68	10.33±1.52	12.02±2.54	<0.01	0.25	<0.01
		Clostridium cluster XIVa[3]	0.71±0.31	8.35±5.04	12.41±8.10	0.01	0.28	<0.01
		E. coli[3]	0.12±0.02	0.03±0.01	0.02±0.01	<0.01	0.25	<0.01
		F. prausnitzii[3]	0.23±0.05	0.38±0.17	0.12±0.07	0.79	0.25	0.21
12 h	Mucosa-attached bacteria	Total bacteria[2]	$1.02\times10^{10}\pm0.55\times10^{10}$	$4.69\times10^{12}\pm4.15\times10^{12}$	$1.61\times10^{12}\pm1.09\times10^{12}$	0.03	0.15	0.03
		Bifidobacterium[3]	20.71±1.28	46.88±6.59	57.40±4.16	<0.01	<0.01	<0.01
		Clostridium cluster XIVa[3]	0.60±0.59	15.08±5.75	19.47±8.88	<0.01	0.39	0.01
		E. coli[3]	0.28±0.05	0.02±0.01	0.01±0.00	<0.01	<0.01	<0.01
		F. prausnitzii[3]	0.22±0.17	0.84±0.50	2.12±1.45	0.43	0.43	0.22
	Digesta-associated bacteria	Total bacteria[2]	$3.14\times10^{10}\pm1.41\times10^{10}$	$7.59\times10^{12}\pm3.66\times10^{12}$	$3.30\times10^{12}\pm2.75\times10^{12}$	<0.01	0.08	0.06
		Bifidobacterium[3]	2.32±0.46	14.88±5.89	11.73±5.79	<0.01	0.38	0.03
		Clostridium cluster XIVa[3]	1.70±2.15	19.71±5.51	23.95±5.95	<0.01	0.35	<0.01
		E. coli[3]	0.13±0.05	0.04±0.03	0.01±0.00	<0.01	0.08	0.01
		F. prausnitzii[3]	0.07±0.02	1.36±0.54	2.49±1.33	0.13	0.58	0.10

P<0.05 means there is significant difference between treatment; *P*<0.1 means there is a tendency to be significant between treatment

[1]Treatments: FC = non-heated colostrum; HC= heated colostrum; NC = no colostrum.
[2]Copy number of 16S rRNA gene/g of fresh sample.
[3]Prevalence of *Bifidobacterium*, *Clostridium* cluster XIVa, *E. coli* and *F. prausnitzii* as a % of total bacteria.

Table 3.6 Effect of non-heated colostrum feeding and heat-treated colostrum on the fold change of five bacterial groups in the colon during the first 12 h after birth[1].

Time	Sample type	Bacterial group	Treatment[1]	
			FC/NC	HC/NC
6 h	Mucosa-attached bacteria	Total bacteria[2]	40.45	64.20
		Bifidobacterium[3]	2.00	2.44
		Clostridium cluster XIVa[3]	7.84	18.64
		E. coli[3]	0.33	0.17
		F. prausnitzii[3]	12.5	1.33
	Digesta-associated bacteria	Total bacteria[2]	14.64	4.86
		Bifidobacterium[3]	4.76	5.54
		Clostridium cluster XIVa[3]	11.76	17.48
		E. coli[3]	0.25	0.17
		F. prausnitzii[3]	1.65	0.52
12 h	Mucosa-attached bacteria	Total bacteria[2]	459.80	157.84
		Bifidobacterium[3]	2.26	2.77
		Clostridium cluster XIVa[3]	25.13	32.45
		E. coli[3]	0.07	0.04
		F. prausnitzii[3]	3.82	9.64
	Digesta-associated bacteria	Total bacteria[2]	241.72	105.10
		Bifidobacterium[3]	6.41	5.06
		Clostridium cluster XIVa[3]	11.59	14.09
		E. coli[3]	0.31	0.08
		F. prausnitzii[3]	19.43	35.57

[1]Treatments: FC = non-heated colostrum; HC= heated colostrum; NC = no colostrum.
[2]Copy number of 16S rRNA gene/g of fresh sample.
[3]Prevalence of *Bifidobacterium*, *Clostridium* cluster XIVa, *E. coli* and *F. prausnitzii* as a % of total bacteria.

3.8 Figures

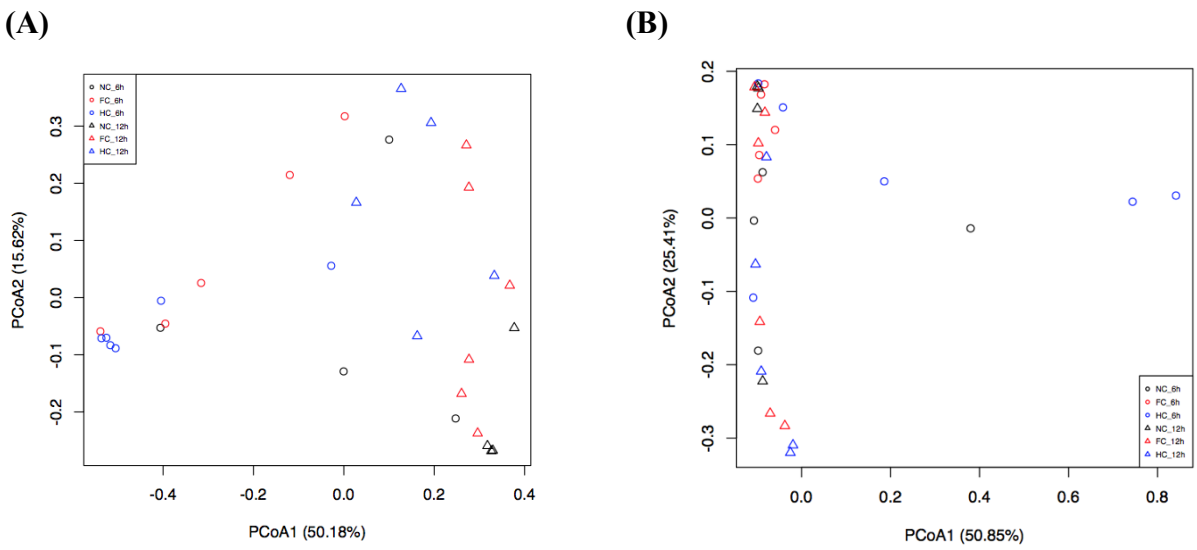

Figure 3.1 Comparison of bacterial profiles among different age groups and treatments with Principal Coordinate Analysis (PCoA). (A) Mucosa-attached bacterial community among different age groups and treatments are plotted along the first two principal component axis (PC1 and PC2). The two components explained 50.18% and 15.62% of the variance. (B) Digesta-associated bacterial community among different age groups and treatments are plotted along the first two principal component axis (PC1 and PC2). The two components explained 50.85% and 25.41% of the variance.

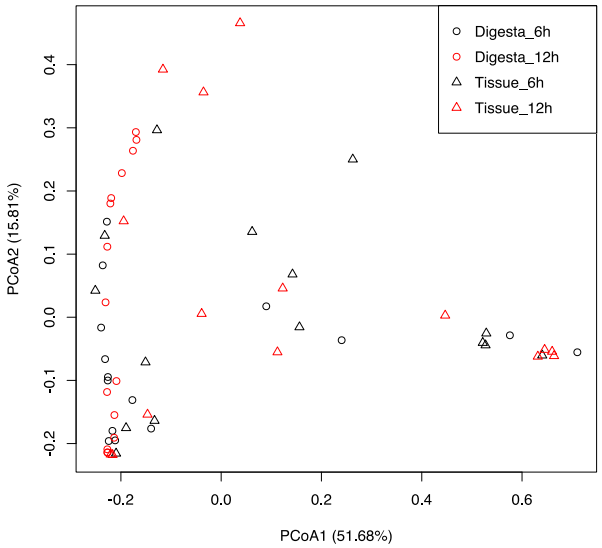

Figure 3.2 Comparison of digesta-associated and mucosa-attached and bacteria at different time point with PCOA plot. The two components PC1 and PC2 explained 51.68% and 15.81% of the variance.

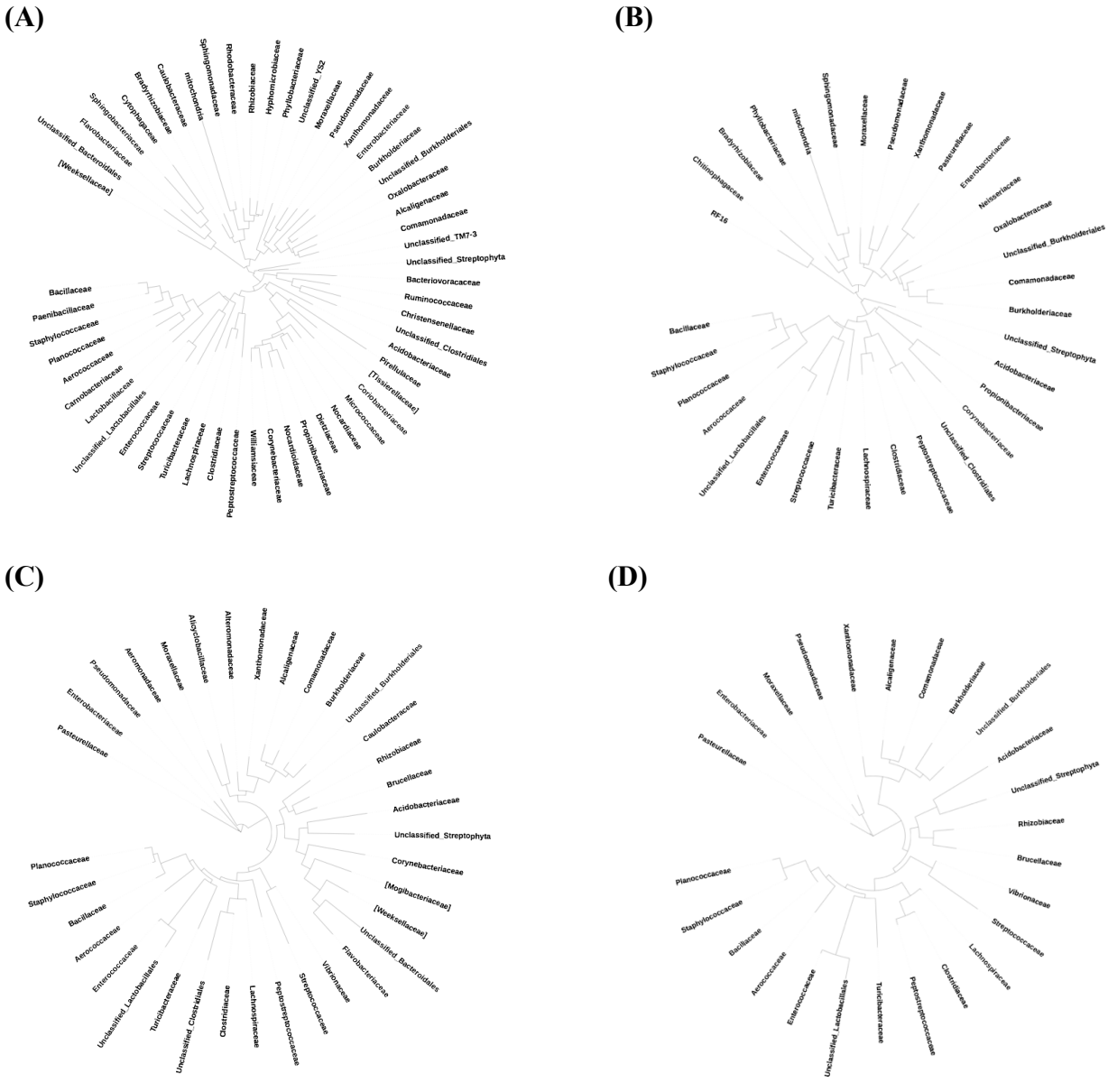

Figure 3.3 Bacterial composition of mucosa and digesta-attached microbiota at family level in the colon of neonatal calves during the first 12 h of life. (a) Mucosa-attached bacterial families at 6 h. (b) Mucosa-attached bacterial families at 12 h. (c) Digesta-associated bacterial families at 6 h. (d) Digesta-associated bacterial family at 12 h.

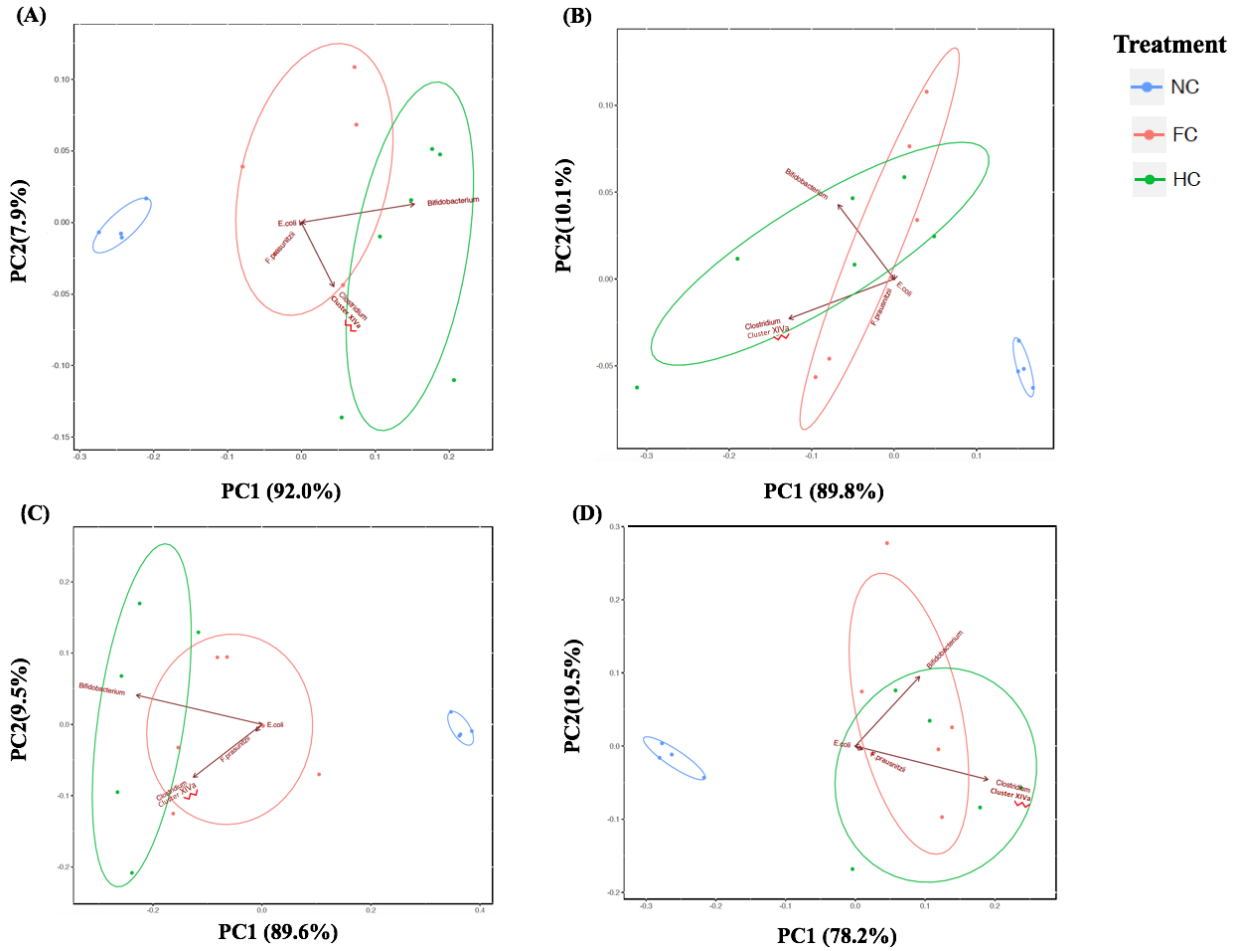

Figure 3.4 PCA biplot diagram showing the distribution of the relative abundance of four bacterial groups *Bifidobacterium*, *E. coli*, *Clostridium* cluster XIVa and *F. prausnitzii* under three colostrum treatments (NC, FC and HC). The animals belonging to each treatment are depicted using different colors. Blue circle means NC, red circle means FC and green circle means HC. The direction and length of the arrows indicate how each bacterial group contributes to the first two components in the biplot. (a) The biplot of mucosa-attached bacterial groups at 6 h, and these two components explained 92.0% and 7.9% of the variance, respectively. (b) The biplot of digesta-associated bacterial groups at 6 h, and these two components explained 89.8% and 10.1% of the variance, respectively. (c) The biplot of mucosa-attached bacterial groups at 12 h, and these two components explained 89.6% and 9.5% of the variance, respectively. (d) The biplot of digesta-associated bacterial groups at 12 h, and these two components explained 78.2% and 19.5% of the variance, respectively.

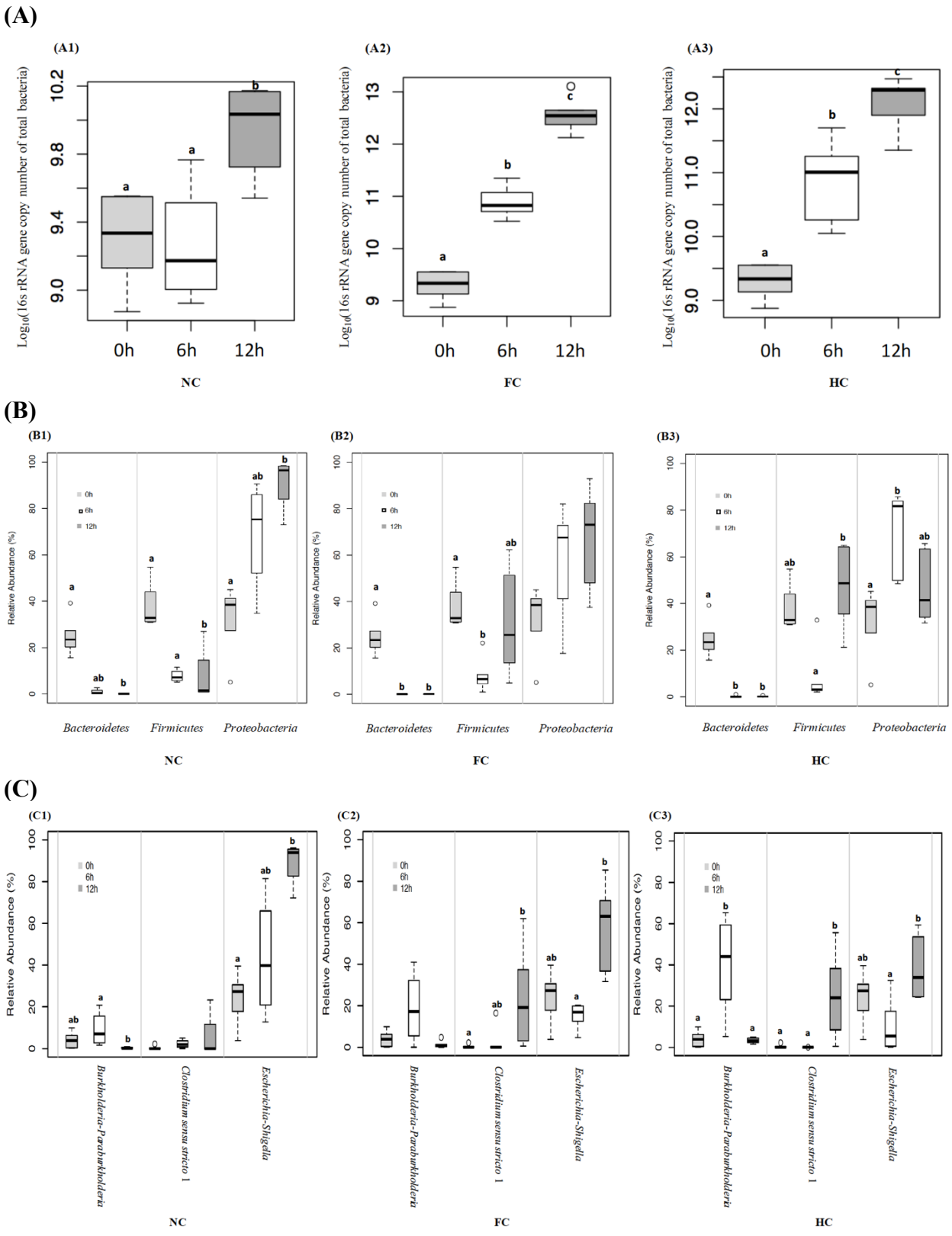

Figure 3.5 The changing pattern of colon microbiota among different time points under different colostrum treatment. (A) Box-plot showing the shift pattern of $\log_{10}$ 16S rRNA gene

copy number of total bacteria among different time points under different colostrum treatments. (B) The changing pattern of the relative abundance of predominant bacterial phylum among different time points under different colostrum treatments. (C) The changing pattern of the relative abundance of predominant bacterial genus among different time points under different colostrum treatments.

Chapter 4. Metagenomic analysis revealed the shift in ileal microbiome of neonatal calves in respond to delayed colostrum feeding

4.1 Introduction

Colostrum, as the first meal to dairy calves, has been proven to be extremely important to the newborn calves for obtaining passive immunity (Godden, 2008). Colostrum is rich in nutritional, immunological (mainly immunoglobulin G (IgG)), and growth factors (Godhia and Patel, 2013), which provides adequate nutrients and immune protection to the newborn calves. However, the absorption of colostrum macronutrients after birth is affected by the time of feeding. Previous studies have indicated that the best time for the absorption of IgG is during the first 4h after birth, and the complete gut closure (usually happens at 24-36h after birth) leads to inability of the unselective absorption of macromolecules (Stott et al., 1979; Weaver et al., 2000). However, delayed colostrum feeding is common, especially when calves are born at night (Vasseur et al., 2010).

Gut microbiota undergoes a succession of changes during early life, which plays important roles in host health (Arrieta et al., 2014), immune system development (Mulder et al., 2011), nutrient metabolism (Krajmalnik-Brown et al., 2012), and physiological changes (Yu et al., 2016). It was suggested that not feeding colostrum for 12 hours after birth reduced the bacterial population in the ileum lumen (Malmuthuge et al., 2015), and delayed *E. coli* colonization in the ileum mucosa (Fischer et al., 2018). Ileum has a moderate pH (7-8) for microbial colonization (10^8 bacterial cells/mL) (Walter and Ley, 2011) and the shifts in commensal microbiome during early life has been reported to alter the gut immune function in infants which have life-long impact on host health (Arrieta et al., 2014).

However, whether or how colostrum feeding strategies affect ileal microbiome at composition and metabolic levels are not well defined. We hypothesized that delayed colostrum feeding enhanced pathogenic bacteria colonization in the ileum which could alter the commensal microbiome. Therefore, our objectives were to characterize the taxonomic composition and functions of the ileal microbiome during early life, and how they changed in response to delayed colostrum feeding using whole genome based metagenomics.

4.2 Materials and methods

4.2.1 Colostrum and calf management

The animal experiment was conducted at the Dairy Research and Technology Centre, University of Alberta, following the guidelines of the Canadian Council of Animal Care. The protocol was approved by the Livestock Care Committee of the University of Alberta (AUP00001595). Detailed information on the animal trial has been described by Fischer et al. (2018). Briefly, male Holstein calves (n=27) were randomly assigned to three feeding treatments: fed colostrum within 1h (TRT0h, n=9), at 6h (TRT6h, n=9), and at 12h (TRT12h, n=9) after birth, respectively. The colostrum was purchased from Saskatoon Colostrum Company Ltd. (Saskatoon, SK, Canada), with an IgG concentration 62g/L. The calves were fed with colostrum based on their 7.5% of birth body weight (BW) (ranged from 2.63L to 3.79L), followed by feeding the first milk replacer (Excel Pro-Gro Calf Milk Replacer, Grober Nutrition, Cambridge, ON, Canada) at 2.5% birth BW twelve hours after colostrum feeding. The milk replacer was provided to the calves every 6h after the first milk replacer feeding.

4.2.2 Ileal digesta sample collection

All calves were euthanatized at 51h after birth and the digesta sampling was followed the procedures as described by Malmuthuge et al. (2015). Briefly, after exsanguination, the whole gastrointestinal tract was removed from animal and moved onto a sterilized table surface. After tied the esophagus and rectum being ligated with table ties, the ileum section was defined as the region with ~10cm in length starting at 30 cm proximal to the ileo-cecal junction. Table ties were used to tie the starting and ending points to prevent the contamination from other gut regions before the collection. After cut between two table ties at each end point, the ileal digesta was squeezed into a 50 mL falcon tube. The tube was snap frozen using liquid nitrogen immediately and transferred to a -80 ℃ freezer.

4.2.3 DNA extraction and metagenomic sequencing

Genomic DNA was extracted from ileal digesta samples using the modified repeated bead-beating and column method (Yu and Morrison, 2004). The quantity and quality of the extracted DNA was assessed using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Due to the lack of ileal digesta for some calves, DNA was only able to be extracted from seven TRT0h calves, eight TRT6h calves, and seven TRT12h calves. DNA libraries for metagenomic sequencing were prepared using a Truseq DNA PCR-free library preparation kit (Illumina, CA, USA). Briefly, the genomic DNA was normalized with a resuspension buffer to the final volume of 55 μL, with a 20 ng/μL concentration, and then the solution was transferred into a Covaris microTUBE (Covaris Inc., Massachusetts, USA) for fragmentation to 350bp using a Covaris S2 focused-ultrasonicator (Covaris Inc., MA, USA). The metagenomic library for each sample was quantified using a Qubit 2.0 Fluorometer

(ThermoFisher Scientific, MA, USA), and sequenced at Génome Québec (Montréal, Canada) using an Illumina HiSeq 4000 (Illumina, USA) to generate the paired end reads (100 x 2) .

4.2.4 Analysis of ileum metagenomes

Functional annotation of the ileal microbiome was performed using the pipeline developed by Li and Guan. (2017). Briefly, the quality control of raw sequences was performed using fastq-mcf (Aronesty, 2013) and the qualified sequences were then filtered to remove host DNA sequences through the alignment with bovine genome (UMD 3.1) using TopHat 2.0.9 (Kim et al., 2013). The filtered DNA sequence of each sample was assembled using MetaVelvet (Namiki et al., 2012) with a kmer of 51 as suggested by Wallace et al. (2015). Then the assembled contigs from each sample were pooled together, and the identical contigs were binned to construct a set of unique contigs. To identify protein coding sequences and/or open reading frames, gene prediction was conducted for obtained unique contigs using the program Prodigal (Hyatt et al., 2010). Unique predicted genes with minimum length of 100 bp were then kept for the downstream analysis. The contigs were annotated against the KEGG database (Kanehisa et al., 2012) using UBLAST program in USEARCH (Edgar, 2010) with E value of $\leq$ 1e-5, bit score of $\geq$ 60, and sequence identity of $\geq$ 30% as the cutoff. Filtered metagenomic reads from each sample were then mapped to the annotated contigs using the UBLAST program with an E value of 1e-5 as the cutoff to define the functions of each ileal microbiome. Then HUMAnN2 program was applied to calculate the relative abundance of the KEGG pathways within each sample (Abubucker et al., 2012).

The filtered sequences were also subjected to microbial taxonomy analysis using Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) version 3.3.9 (Meyer et al., 2008). The taxonomic assignments at phylum, family and genus levels were

performed using Refseq database within MG-RAST, with e-value ⩽1e-5, identity ⩾ 60%, alignment ⩾ 50bp (Meyer et al., 2008). In addition, the 16S rRNA gene copy number of total bacteria and the proportion of *Faecalibacterium prausnitzii* (*F. prausnitzii*), *Clostridium* cluster XIVa, and *E. coli* was obtained from Fischer et al. (2018).

4.2.5 Short chain fatty acids (SCFAs) measurement

Ileal digesta sample (~0.1g) and phosphoric acid were mixed thoroughly (1:4; v:v) in a 5 mL tube. Then the concentrations of acetate, propionate, butyrate, isobutyrate, isovalerate and valerate were measured using gas chromatography following the methods described by Guan et al. (2008). The SCFA concentrations were presented with μmol/g fresh weight of digesta sample.

4.2.6 Statistical analysis

Only taxa with the relative abundance >0.05% and presented in more than 50% of the total animals in each treatment (TRT0h, TRT6h or TRT12h) were defined as detected microbial taxa. In addition, the metabolic pathways with counts per million of reads (CPM) >5, in at least 50% of the animals within each one treatment group were considered as detected microbial functions. Only the detected microbial taxa and functions were used for the following analysis. To identify the differences in microbial composition (bacteria and archaea) among three treatments, the relative abundance of the detectable microbial taxa was transformed with arcsine square root (Franzosa et al., 2014), and then the transformed data were subjected to ANOVA test using in R (3.3.1) (R Core Team, 2014). The significant difference between any two treatments was tested with the Turkey test, and the *P*-value was adjusted with false discovery rate (FDR) using the Benjamin Hochberg method (Benjamini and Hochberg et al., 1995), with $P \leq 0.05$ declared as significant difference and $0.05 < P < 0.10$ as tendency to be significantly different. The comparisons of ileal microbiome function between any two treatments (TRT0h vs. TRT6h,

TRT6h vs. TRT 12h, TRT0h vs. TRT 12h) was performed using linear discriminant analysis (LDA) effect size (LEfse) (Segata et al., 2011), with an LDA score of >2 and *P*-value <0.05 considered as significant (Mottawea et al., 2016). Principal-coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity matrices was performed for bacterial and archaeal communities at the genus level among three treatments. In addition, principal component analysis (PCA) was performed for microbial metabolic functions among three treatments. Spearman's rank correlation was performed between the 16S rRNA gene copy of total bacteria, the proportion of *F. prausnitzii*, *Clostridium cluster* XIVa, *E. coli* (obtained from Fischer et al., 2018), and the second category of KEGG pathways, and significances were declared at $|\rho| > 0.5$, and *P*-value < 0.01.

4.2.7 Data submission

All sequences were deposited in MG-RAST and are publicly accessible with the following link https://www.mg-rast.org/linkin.cgi?project=mgp82820.

4.3 Results

4.3.1 Ileal digesta metagenomes of neonatal dairy calves

In total, 845,924,103 reads were generated for the ileal microbiome of all animals, with 38,451,095±2,676,171 reads (mean ± SEM) per sample. After quality control and removing host DNA, 548,962,590 reads (24,952,845±1,875,326 reads per sample) were kept for the downstream analysis. After *de novo* assembly, a total of 209,118 unique contigs were generated. After gene prediction, a total of 224,443 unique genes with a minimum length of 100bp were obtained, which were then subjected to the KEGG database for annotation. Among them, 132,717 genes were successfully annotated, which accounted for 59.13% of the total number of

genes. These genes were then used as the reference to define function of each calf ileum metagenome. After the filtered reads of each sample were mapped to the above reference, 43.80±3.64% (mean ± S.E.M) metagenomic reads per sample were mapped back for each sample. The detailed sequences information is presented in Supplementary Dataset 4.1.

4.3.2 Ileum microbial metabolic functions

In total, 175 KEGG pathways were identified from calf ileum metagenomes. After removing the exogenous pathways, 136 KEGG pathways were considered as microbial functions. Among them, 116 pathways (the metabolic pathways with CPM of >5, in at least 50% of the animals within each treatment group) were detected and used for downstream analysis. The 116 core KEGG pathways represented 79.59%±5.10% of mapped DNA sequences, which belonged to four first-level functional categories and 20 second-level functional categories (Supplementary Dataset 4.2). The four first-level category functions were "metabolism" (61.04±4.61%), "cellular processes" (2.97±0.21%), "environmental information processing" (4.41±0.25%) and "genetic information processing " (11.97±0.30%) (Figure 4.2 and Supplementary Dataset 4.2). The top five second-level KEGG functions were "carbohydrate metabolism" (13.28±1.06%), "amino acid metabolism" (9.68±0.76%), "metabolism of cofactors and vitamins" (9.51±0.68%), "metabolism of other amino acids" (5.79±0.45%), and "metabolism of terpenoids and polyketides" (4.97±0.41%) (Supplementary Dataset 4.2). The top ten KEGG pathways were "Biosynthesis of vancomycin group antibiotics" (2.00±0.18%), "Biosynthesis of ansamycins" (1.75±0.15%), "Valine, leucine and isoleucine biosynthesis" (1.64±0.13%), "Ribosome"(1.63±0.12%), "D-Alanine metabolism" (1.59±0.11%), "Fructose and mannose metabolism" (1.39±0.11%), "Pantothenate and CoA biosynthesis" (1.38±0.10%), "C5-Branched dibasic acid metabolism" (1.36±0.13%), "D-Glutamine and D-glutamate metabolism"

(1.34±0.11%), and "Pentose phosphate pathway" (1.33±0.10%) (Figure 4.2A and Supplementary Dataset 4.2).

4.3.3 Composition of neonatal calves' ileal microbiome

The ileal microbiome of two-days old dairy calves consisted of four domains, including archaea, bacteria, eukaryota and viruses. Bacteria were the predominant domain in the microbiomes of all samples (TRT0h – 94.28±2.37%; TRT6h – 87.23±4.78 %; TRT12h – 97.16 ±1.28 %). Eukaryota was the second predominant microbial group, with 2.62±1.69% for TRT0h, 8.57±3.90% for TRT6h, and 0.63±0.25% for TRT12h, respectively. Viruses and archaea were less abundant in the ileal microbiome, with 2.85±1.26% for TRT0h, 3.40±1.32% for TRT6h, and 1.98±1.27% for TRT12h as virus, and 0.03±0.01% for TRT0h, 0.06±0.02% for TRT6h, and 0.02±0.01% for TRT12h as archaea (Figure 4.1A). Moreover, the abundances of archaea, bacteria, eukaryote and viruses were not different among three colostrum feeding treatments.

Regarding to the bacterial community, six phyla were detected (with average relative abundance > 0.05% and presented in each treatment group), including *Actinobacteria*, *Bacteroidetes*, *Chlorobi*, *Firmicutes*, *Fusobacteria* and *Proteobacteria*. *Firmicutes* (49.83±5.54%) and *Proteobacteria* (49.38±5.59%) were the two predominant phyla in the ileal microbiome of all animals (Supplementary Dataset 4.3). In total, 28 families were detected, with *Enterobacteriaceae* (47.06±5.77%), *Streptococcaceae* (22.88±2.78%), *Lactobacillaceae* (16.66±3.90%), *Enterococcaceae* (6.96±1.36%) and *Clostridiaceae* (1.20±0.41%) being the top five abundant bacterial families (Supplementary Dataset 4.4). In addition, 37 bacterial genera were detected (Figure 4.1B), and *Escherichia* (35.26±4.57%), *Streptococcus* (20.07±2.52%), *Lactobacillus* (16.57±3.89%), *Enterococcus* (6.95±1.36%), *Shigella* (5.59±0.73%) were the top five abundant bacterial genera (Supplementary Dataset 4.5). At species level, the top five

bacterial species were *Escherichia coli* (33.40±4.34%), *Streptococcus thermophiles* (10.60±1.60%), *Enterococcus faecium* (4.12±0.94%), *Lactobacillus reuteri* (3.10±1.10%) and *Lactococcus lactis* (2.78±0.48%) (Supplementary Dataset 4.6).

In total, three archaeal phyla *Euryarchaeota* (91.35±1.56%), *Crenarchaeota* (7.85±1.51%), and *Thaumarchaeota* (0.36±0.13%) were detected in ileal microbiome (Supplementary Dataset 4.7) consisting of 48 genera. After removing the "non-gut archaeal taxa" based on previous studies (Zhou et al., 2014; Nkamga et al., 2017), 20 genera were detected as calf ileal archaea (Figure 4.1C) with *Methanosarcina* (10.19±1.64%), *Methanobrevibacter* (8.23±1.34%), *Methanocorpusculum* (3.09±0.61%), *Methanocaldococcus* (3.07±0.70%) and *Thermoplasma* (2.26±0.45%) being the top five abundant archaeal genera (Supplementary Dataset 4.8). For archaeal species, *Methanosarcina barkeri* (2.26±0.45%), *Methanospirillum hungatei* (1.92±0.51%), *Methanobrevibacter smithii* (0.06±0.01%), *Methanosarcina acetivorans* (0.04±0.01%) and *Methanocorpusculum labreanum* (0.03±0.01%) were the top five abundant in the ileal digesta (Supplementary Dataset 4.9). Additionally, only one unclassified virus phylum was detected. Meanwhile, 82.45%, 85.84% and 82.77% of Eukaryota phylum, family and genus belong to others groups (including less abundant, unclassified and non-gut Eukaryota), which were not included in further taxonomic comparison analysis.

4.3.4 Effect of delayed colostrum feeding on ileum microbial composition

When the bacterial and archaeal communities were compared, the Bray-Curtis dissimilarity matrices based principal-coordinate analysis (PCoA) did not show clear separations of ileal microbiota among the three colostrum feeding time treatments (Figure 4.3). In addition, the diversity indices of ileal microbiota, Shannon and Simpson indices, were not significantly different among the treatments (Table 4.1). As shown in Table 4.2, the relative abundance of

Enterococcus was significantly higher in the ileum of TRT12h calves (12.67±2.48%) compared to that in TRT0h (5.48±2.31%) and TRT6h (3.23±0.53%) calves (P<0.01), respectively. Additionally, the relative abundances of *Achromobacter, Bordetella*, *Ruminococcus* and *Burkholderia* were tentatively higher in in the ileum of TRT6h calves (0.31±0.13%, 0.17±0.06%, 0.37±0.16%, 0.52±0.16%) compared to those in TRT0h (0.09±0.04%, 0.05±0.02%, 0.07±0.02%, 0.21±0.09%) and TRT12h (0.05±0.01%, 0.03±0.01%, 0.05 ±0.01%, 0.16±0.04%) calves (P=0.07). For archaea genera, *Aciduliprofundum, Desulfurococcus, Haloferax, Halorubrum* tended to have higher relative abundance in the ileum of TRT12h calves (1.00±0.33%, 0.76±0.41%, 1.35±0.50, 0.49±0.16%) compared to calves in TRT0h (0.14±0.09%, 0.09±0.08%, 0.33±0.20%, 0.17±0.16%) and TRT6h calves (0.13±0.13%, 0.03±0.03%, 0.15±0.13%, 0.03±0.03%) (P=0.07). Furthermore, *Methanococcoides, Methanoplanus, Methanospirillum* and *Methanothermobacter* had highest relative abundance in the ileum of TRT0h calves (2.60±0.40%, 1.75±0.45%, 2.90±1.20%, 2.65±0.59%) compared to calves in TRT6h (1.28±0.79%, 0.61±0.48%, 0.90±0.46%, 1.36±0.45%) and TRT12h (1.98±0.45%, 1.26±0.45%, 2.43±1.16%, 0.81±0.28%) (Table 4.2).

4.3.5 Functional difference of ileal microbiome in response to colostrum feeding strategies

The overall KEGG pathway functions was not affected by the colostrum feeding strategies based on PCA plot (Figure 4.2A). However, the functional profiles of six animals were clearly separated (Group A: Two animals from TRT0h and four animals from TRT6h, Group B: the rest of animals) (Figure 4.2B). When the relative abundance of the individual KEGG pathway was compared, “Ko00430: Taurine and hypotaurine metabolism” function was found to be enriched in ileal microbiome of TRT12h (0.81±0.03%) animals, when compared to that in TRT6h (0.48±0.13%) and TRT0h (0.61±0.08%) (P<0.01) calves (Figure 4.2C).

4.3.6 SCFA concentration among different delayed colostrum treatment groups

Acetate and propionate showed the highest concentration in the ileal digesta of two-days old calves. In addition, the SCFA concentration and molar proportion were not affected by colostrum feeding treatments (Table 4.3).

4.3.7 Relationship between microbial functions and SCFA

The Spearman's rank correlation analysis revealed that acetate concentration was negatively correlated with "transcription" ($\rho = -0.58$, $P = 0.02$). In addition, there was significant correlations between propionate concentration with "Xenobiotics biodegradation and metabolism" ($\rho = 0.54$, $P = 0.03$) and "transcription" ($\rho = -0.55$, $P = 0.03$). Moreover, total SCFA concentration was significantly correlated with "Metabolism of cofactors and vitamins" ($\rho = 0.52$, $P = 0.04$), "Xenobiotics biodegradation and metabolism" ($\rho = 0.54$, $P = 0.03$), "Lipid metabolism" ($\rho = 0.51$, $P = 0.04$), and "transcription" ($\rho = -0.66$, $P = 0.01$) (Figure 4.4).

4.3.8 Individualized ileal microbiome during early life

Although the overall microbial functions, SCFA concentrations were not affected by colostrum feeding treatments, the ileal microbiome showed two patterns as described above (Group A and B). We speculated that the microbiomes of some animals are more resistant to the nutrient deficiency and/or more susceptible to early life stress comparing to others. The further comparison of the relative abundances of bacterial and archaeal genera as well as microbial functions showed that ileum of group B animals had higher relative abundances of bacterial genera *Escherichia, Photorhabdus and Salmonella* and archaeal genera *Methanobrevibacter, Methanosarcina* and *Thermoplasma,* whereas ileal microbiome of group A animals had higher relative abundances of bacterial genera *Bifidobacterium, Chlorobium, Clostridium, Eubacterium, Lactobacillus, Ruminococcus, Achromobacter, Bordetella, Burholderia,* and *Neisseria,* (Table

4.4). When all the KEGG second category functions were compared between two groups of animals, 15 functions were found to be significantly different between the two types of microbiomes (Figure 4.5). Among them, the relative abundance of top abundant functions "Carbohydrate metabolism" (Group A - 5.83±1.17%, Group B – 16.08±0.26%) (P<0.05), "Amino acid metabolism" (Group A - 4.36±0.85%, Group B – 11.68±0.18%) (P<0.05), "Metabolism of cofactors and vitamins" (Group A - 4.74±0.70%, Group B - 11.29±0.20%) (P<0.05), "Metabolism of other amino acids" (Group A - 2.71±0.41%, Group B – 6.94±0.21%) (P<0.05), "Metabolism of terpenoids and polyketides" (Group A - 2.25±0.61%, Group B – 5.99±0.14%) (P<0.05) were significantly higher in Group B ileal microbiome (Figure 4.5). Meanwhile, when the 16S rRNA gene copy number of total bacteria, the proportion of *F. prausnitzii*, *Clostridium* cluster XIVa, and *E. coli*, were further compared, higher 16S rRNA gene copy numbers of total bacteria, and higher proportions of *E. coli* 16S rRNA gene copy number were observed in the ileum of Group B animals (Table 4.4).

4.3.9 Relationships between ileal microbiome and functional changes within the "patterned" groups

Further we explored the relationships between the bacterial and archaeal genera and the functions between Group A and Group B microbiome (Figure 4.6). In total, 11 bacterial genera within Group A was found to be significantly correlated with the 15 (Figure 4.6A), and 36 bacteria within Group B were significantly correlated with the 15 functions (Figure 4.6B). Additionally, four archaeal genera in Group A was significantly correlated with the varied functions (Figure 4.6C), while 13 archaeal genera within Group B was found to be significantly correlated with the varied functions (Figure 4.6D).

4.4 Discussion

This study is the first to report the ileal digesta microbiome of two-days old neonatal calves and how it can be affected by different colostrum feeding time treatments using metagenomics. The whole microbial community and functions did not form differ among treatments, suggesting that may be other factors such as environmental microbiota, transmission of maternal microbiota, together with the first feeding affecting the early life ileal microbiome establishment. In addition, bacteria, archaea, eukaryotes and viruses were detected in the ileal microbiome of two-days old calves with bacteria being the most abundant, which is in consistent with the findings in human infant gut microbiome (Palmer et al., 2007; Lim et al., 2015). In consistent with the findings of ileal digesta microbiota of one-day old Sika deer and newborn Liu yang black goat (Li et al., 2018), *Proteobacteria* and *Firmicutes* comprised the two predominant phyla in both studies. However, the difference was detected at genus level between Li et al. (2018) and our study, that the most predominant genera *Halomonas* (35.7%) in the Sika deer (Li et al., 2018) was not detected in our study. In the meantime, less number of bacterial phyla (6 vs.10) were detected the ileum of neonatal calves (this study) compared to that in the neonatal Liu yang black goat soon after birth (Jiao et al., 2016), and the top three abundant genera *including Escherichia* (35.26%), *Streptococcus* (20.07%) and *Lactobacillus* (16.57%) of the neonatal dairy calves were significantly different from those varied with in the Liu yang black goat at D0, which were *Lactobacillu*s (32.81%), *Enterococcus* (30.94%) and *Escherichia* (2.03%) (Jiao et al., 2016). Such discrepancy may be caused by multiple factors, including the animal species (calf, goat), the feeding management (colostrum and milk replacer, no feeding and suckled from the mom), and taxa assignment method (16S rRNA gene amplicon sequencing vs. metagenomic).

This study is also the first to report the archaeal community in the ileum of two-days old dairy calves. The major archaeal phyla *Euryarchaeota* (91.35%), *Crenarchaeota* (7.85%), and *Thaumarchaeota* (0.36%) of neonatal calves were similar to those found in the human intestinal tract (Nkamga et al., 2017). The predominant archaea are methanogen, which could utilize H_2 and CO_2 to produce methane during methanogenesis in the ruminant (Yáñez-Ruiz et al., 2010). Methanogens were found to be established in the rumen of lambs and calves during the first 3 days after birth (Fonty et al., 1987; Minato et al., 1992; Morvan et al., 1994). Meanwhile, *Methanobrevibacter* was suggested to be the main taxon in the ileum of 3-4 weeks old dairy calves based on cloning libraries and PCR-DGGE method (Zhou et al., 2014), while this genus was presented at a lower relative abundance (8.23%) in the ileal digesta in our study. The different method of studying archaeal taxa (16s rRNA gene vs. whole genome), calf age (3-4 weeks vs. two-days old), diet (whole milk, and calf starter vs. milk replacer) could lead to the detection difference. However, this study has provided the evidence that methanogen may start to colonize the intestinal tract at Day 2. In addition, archaea also play important roles in carbohydrate degradation, acting as probiotics (e.g., *Methanomassiliicoccales*), and related to inflammatory bowel disease (*M. stadtmanae*) (Nkamga et al., 2017). Currently, the standard database for the "real intestinal archaea" is still lacking. In total, 59.90% archaea achieved in our study belong to environmental archaea, and the most abundant environmental genus was *Halogeometricum* (14.09%), which is a halophilic archaeon (Montalvo-Rodriguez et al., 1998). Therefore, further studies are needed to establish a complete intestinal archaeal database.

In the gut, the identified positive correlations between bacteria and methanogens, such as *Lactococcus* and *Methanoplanus* (ρ =0.76, and P <0.01), *Leuconostoc* and *Methanoplanus* (ρ =0.71, and P <0.01), *Oenococcus* and *Methanospirillum* (ρ =0.64, and P <0.01) (Supplementary

Dataset 4.10) suggest the potential interactions and symbiotic relationship between ileum bacteria and archaea. *Lactococcus* and *Leuconostoc* are lactate producing bacteria (Taniguchi et al., 1994, Wagner et al., 2005), which could utilize lactose in the dairy products (e.g., colostrum or milk replacer) to produce lactate (Taniguchi et al., 1994). In addition, *Oenococcus* oeni can ferment milk lactose to produce pyruvate (Starrenburg and Hugenholtz, 1991) for lactate production (Wagner et al., 2005). H_2 could be produced during lactate fermentation (Ohnishi et al., 2012), which can then be utilized by intestinal methanogens as an energy source (Janssen and Kirs, 2008). Therefore, the identified relationship between bacterial and archaeal genera indicate their potential interactions in the hydrogen production and utilization.

Considering the effect of delayed colostrum feeding on the bacterial profiles, higher relative abundance of *Enterococcus* found in the ileum of TRT12h animals may be related to the delayed provision of colostrum. In general, *Enterococcus* genus consists of commensal and pathogenic species. Several pathogenic *Enterococcus* species have been reported to be related to intestinal inflammation, as they adhere to the intestinal epithelium, forming biofilms and impeding antioxidant defense ability (Popović et al., 2018). Previous study have suggested that *Enterococcus faecalis* was an opportunistic pathogen, which was enriched in patients with inflammatory bowel disease (IBD) (Zhou et al., 2016) and was able to induce bowel disease in germ-free interleukin-10 knockout mice (Balish and Warner, 2002). Therefore, higher relative abundance of *Enterococcus* may lead to higher chance of intestinal inflammation for the calves when colostrum feeding is delayed for 12h. However, further studies on measuring the specific pathogenic *Enterococcus* species as well as expression of genes involved in inflammation using qPCR are needed to validate our speculations.

In addition, the explored gene annotated KEGG pathway functions in this study provide fundamental knowledge about the ileal microbiome function of dairy calves. Interestingly, the top two KEGG pathways "Biosynthesis of vancomycin group antibiotics", "Biosynthesis of ansamycins" were both related to antibiotics production, suggesting that early ileal microbiota plays an important role in creating a competitive intestinal environment for later colonized organisms. The enriched genes related to KEGG pathway "Taurine and hypotaurine metabolism" (KO00430) in the ileum of TRT12h calves were probably a self-defense mechanism in response to the higher level of stress (lack of nutrients) through regulating adrenaline release (Nakagawa and Kuriyama, 1975) by the ileum microbiota. This conclusion was inferred from a previous study (Nakagawa and Kuriyama, 1975), which showed that oral administration of taurine (4-7g/kg/day for 3 days) could prevent the reduction of adrenaline in the adrenal gland, hence suppressing the increase of blood sugar of rats in response to the cold stress. However, further researches are needed to verify the stress response indicators including concentration of stress hormones, blood cortisol, catecholamine, adrenaline in all calves.

Interestingly, six animals (Group A) and the other animals (Group B) were clearly separated in the PCA plot (Figure 4.2B) regardless of different colostrum feeding time treatment. The difference in the relative abundances of microbial functions (Figure 4.5) may be the determining factor behind. In general, the microbiota of neonatal calves is affected by external and internal factors (Gomez et al., 2017). External factors include environmental microbiota, colostrum treatment including the type of colostrum or milk (fresh vs. pasteurized) (Malmuthuge et al., 2015) and delayed colostrum feeding (Fischer et al., 2018), prebiotics (Nyangale et al., 2014) and probiotics (Gargari et al., 2016). Internal factors include the movement of the intestine, intestinal pH, the immune status of the host, and host microbial interactions (Mackie et

al., 1999), genetics (Goodrich et al., 2014), maternal environment before birth (DiGiulio, 2012), which are more individual dependent. In our study, the difference in the ileum microbial composition (Supplementary Table 3) and function (Figure 4.8) at the same treatment group (four animals from TRT6h as A1 group and four animals from TRT6h as B1 group) suggests that host influence may be stronger than the colostrum feeing time treatment on the two-days old dairy calves. Moreover, the functional difference between ileal microbiome of Group A and Group B may be resulted from different microbial taxa, because different significant correlations were found between microbial functions and certain bacterial and archaeal groups (Figure 4.6). In the meantime, the varied functions were determined by fewer number of bacteria and archaea in Group A (Figure 4.6A and 4.6C) compared to Group B (Figure 4.6B and 4.6D), suggesting that ileal digesta-associated microbiota were more resilient in Group B compared with Group A. Moreover, the enriched functions related to transcription, transport and catabolism were detected in group A, and the highly abundant genes involved in metabolic related functions (carbohydrate metabolism, amino acid metabolism, energy metabolism, lipid metabolism) were found in group B, which suggest that the microbiome of the animals from group A maybe more likely to have fundamental survival functions, while animals in group B are more active in biological functions. These results suggest that host, host microbial interaction can both shape microbial composition and functions during early life under different colostrum feeding time strategy. Therefore, further work on exploring the individual difference at phenotypic and molecular level, as well as the relationship between host and microbiota interaction are needed.

4.5 Conclusion

In the study, we found that the ileal microbiota consisted of bacteria, archaea, viruses and eukaryotes. Different colostrum feeding time on microbial composition and functions, had no effect on overall ileal microbiome at both taxonomic and functional levels. However, segregation in ileal microbiome of the calves within the same delayed and non-delayed treatment groups, implying that host plays and important role in shaping them in the neonatal calves. Moreover, significant higher relative abundance of *Enterococcus* was detected and the genes related to the KEGG pathway "Taurine and hypotaurine metabolism" were enriched in the ileum of 12h delayed calves, suggesting that these calves' ileal microbiome may have developed a self-defense mechanism of regulating adrenaline release in response to stress of delayed nutrient supply and higher prevalence of potential pathogenic species. Therefore, this study provides suggestions to the dairy industry that feeding colostrum earlier (better within 6h after birth) is better to the neonatal calves from the microbiological point of view.

4.6 References

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4.7 Tables

Table 4.1 Effect of delayed colostrum feeding on ileum bacterial diversity.

Diversity matrix	**Treatment**			***P*-value[a]**
	TRT0h	TRT6h	TRT12h	
Shannon	1.87±0.20	1.90±0.14	1.85±0.08	0.24
Simpson	0.68±0.06	0.70±0.04	0.73±0.02	0.66

[a]*P* values among the three colostrum treatment groups were obtained using the one-way anova test, and the comparison between any two treatments was analyzed with Turkey's test.

Table 4.2 Comparison of the relative abundance of bacterial and archaeal genera among delayed colostrum treatments.

Phylum	Genus	Treatment			*P*-value[b]
		TRT0h	TRT6h	TRT12h	
Bacteria[a]					
Firmicutes	*Enterococcus*	5.48±2.31[a]	3.23±0.53[a]	12.67±2.48[b]	0.01
	Ruminococcus	0.07±0.02	0.37±0.16	0.05±0.01	0.07
Proteobacteria	*Achromobacter*	0.09±0.04	0.31±0.13	0.05±0.01	0.07
	Bordetella	0.05±0.02	0.17±0.06	0.03±0.01	0.07
	Burkholderia	0.21±0.09	0.52±0.16	0.16±0.04	0.07
Archaea[a]					
Crenarchaeota	*Desulfurococcus*	0.09±0.08	0.03±0.03	0.76±0.41	0.07
Euryarchaeota	*Aciduliprofundum*	0.14±0.09	0.13±0.13	1.00±0.33	0.07
	Haloferax	0.33±0.20	0.15±0.13	1.35±0.50	0.07
	Halorubrum	0.17±0.16	0.03±0.03	0.49±0.16	0.07
	Methanococcoides	2.60±0.40	1.28±0.79	1.98±0.45	0.09
	Methanoplanus	1.75±0.45	0.61±0.48	1.26±0.45	0.09
	Methanospirillum	2.90±1.20	0.90±0.46	2.43±1.16	0.07
	Methanothermobacter	2.65±0.59	1.36±0.45	0.81±0.28	0.07

[a]Bacterial and archaeal communities were analyzed separately.

[b]*P* values among the three colostrum treatment groups were obtained using the one-way anova test based on the arcsine square root-transformed relative abundance values, and the comparison between any two treatments was analyzed with Turkey's test. The *P*-value was adjusted with Benjamin Hochberg method. $P<0.05$ means there is significant difference among the treatments, and $0.05<P<0.10$ means there is a tendency to be significant different among the treatments.

Table 4.3 Short chain fatty acid (SCFA) concentration (umol/g) and molar proportion among three treatments.

SCFA	Treatment			*P*-value
	TRT0h	TRT6h	TRT12h	
Acetate[a]	21.33±8.25	14.45±2.33	14.52±2.91	0.56
Propionate[a]	3.10±1.19	2.49±0.31	2.68±0.56	0.86
Total SCFA[a]	26.31±9.19	18.93±3.50	18.43±3.47	0.59
Acetate[b]	0.87±0.03	0.84±0.02	0.84±0.01	0.93
Propionate[b]	0.11±0.02	0.16±0.02	0.15±0.01	0.24

[a] means the SCFA concentration, and [b] means the molar proportion of the SCFA.

Table 4.4 Comparison of the 16S rRNA gene copy number of certain bacterial groups (qPCR), the relative abundance of bacterial and archaeal genera (metagenomic) between two "patterned" groups*.

Microbe	"Patterned" Groups		*P*-value
	A(six animals)	B(other animals)	
Certain bacterial groups (qpcr)			
Total bacteria	$2.40\times10^{9}\pm0.42\times10^{9}$	$3.0\times10^{10}\pm0.47\times10^{10}$	<0.01
F. prausnitzii	0.04±0.01	0.01±0.00	<0.01
Clostridium cluster XIVa	0.05±0.02	0.01±0.00	<0.01
E. coli	4.76±2.49	20.86±3.76	<0.01
Bacterial genera (metagenomic)			
Bifidobacterium	0.09±0.01	0.05±0.02	<0.01
Chlorobium	0.44±0.15	0.01±0.00	<0.01
Clostridium	2.04±0.58	0.86±0.51	0.01
Eubacterium	0.38±0.10	0.05±0.01	<0.01
Lactobacillus	27.51±9.43	12.46±3.74	0.08
Ruminococcus	0.50±0.19	0.05±0.01	<0.01
Achromobacter	0.41±0.15	0.06±0.01	<0.01
Bordetella	0.23±0.07	0.03±0.01	<0.01
Burkholderia	0.72±0.18	0.15±0.02	<0.01
Escherichia	21.60±6.11	40.38±5.39	0.08
Neisseria	0.58±0.46	0.02±0.00	<0.01
Photorhabdus	0.03±0.01	0.06±0.01	0.02
Salmonella	1.67±0.39	2.78±0.33	0.08
Archaea genera (metagenomic)			
Methanobrevibacter	3.73±2.43	9.92±11.43	0.02
Methanosarcina	1.58±0.72	13.42±1.61	<0.01
Thermoplasma	0.43±0.43	2.95±0.50	0.01

*Group A: Two animals from TRT0h and four animals from TRT6h of the PCA plot (Figure 4.2B) clearly separated with most of the other animals (Group B).
P<0.05 means there is significant difference between the "patterned" groups; *P*<0.1 means there is a tendency to be significant between the "patterned" groups.

Table 4.5 Comparison of the 16s rRNA gene copy number of certain bacterial groups (qPCR), the relative abundance of bacterial and archaeal genera (metagenomic) between Group A1 and Group B1*.

Microbe	Groups		*P*-value
	A1 (four animals from TRT6h)	B2 (four animals from TRT6h)	
Certain bacterial groups (qpcr)			
Total bacteria	$1.98\times10^{9}\pm0.33\times10^{9}$	$2.67\times10^{10}\pm0.47\times10^{10}$	0.03
F. prausnitzii	0.06±0.02	0.002±0.000	0.03
Clostridium cluster XIVa	0.07±0.03	0.001±0.000	0.03
E. coli	6.00±3.69	28.73±9.32	0.06
Bacterial genera (metagenomic)			
Chlorobium	0.56±0.21	0.01±0.00	0.03
Clostridium	1.92±0.72	0.38±0.07	0.06
Eubacterium	0.45±0.14	0.04±0.01	0.03
Ruminococcus	0.69±0.22	0.04±0.02	0.06
Achromobacter	0.53±0.21	0.07±0.04	0.03
Bordetella	0.29±0.09	0.04±0.02	0.03
Burkholderia	0.84±0.23	0.16±0.07	0.03
Neisseria	0.12±0.03	0.01±0.00	0.03
Archaea genera (metagenomic)			
Methanobrevibacter	1.75±1.01	11.41±3.32	0.03
Methanosarcina	0.89±0.89	16.30±5.59	0.03

*Group A1: four animals in TRT6h treatment of the PCA plot (Figure 4.2B) clearly separated with the other four animals in TRT6h treatment (Group B1).
$P<0.05$ means there is significant difference between groups; $P<0.1$ means there is a tendency to be significant between groups.

4.8 Figures

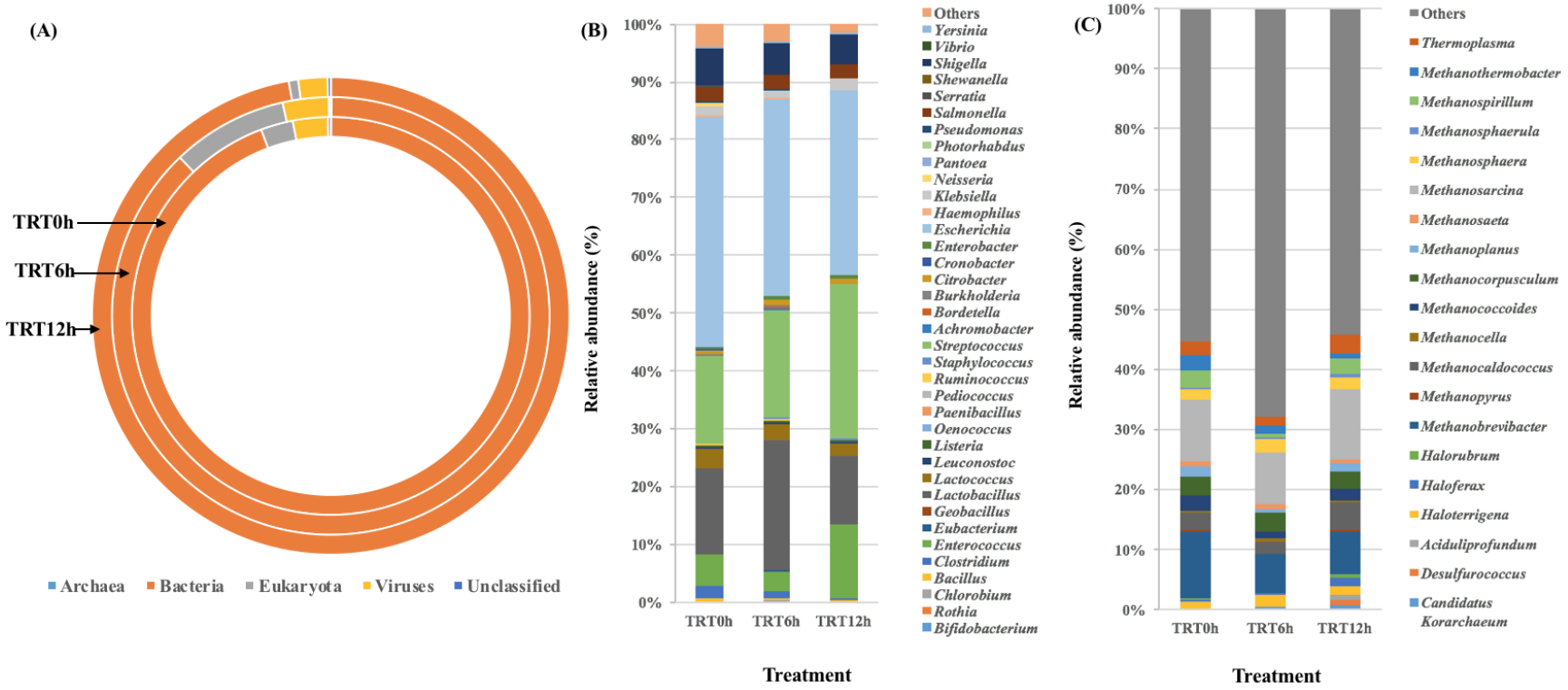


Figure 4.1 The composition of ileal digesta microbiome community under three colostrum treatments obtained through whole genome sequencing. (A) Each data series present mean relative abundance (%) of ileum microbiota per treatment. TRT0h means colostrum feeding within 1 hour, TRT6h means colostrum feeding at 6h after birth, and TRT12h means colostrum feeding at 12h after birth. **(B)** Bacterial composition in the ileal digesta under three treatments of neonatal dairy calves. Bars represent the relative abundance of the identified bacterial genera (the relative abundance >0.05% and present in more than half number of the total animals at least in one treatment) **(C)** Archaeal composition in the ileum digestea under three treatments of neonatal dairy calves. Bars represent the relative abundance of the identified archaeal genera (the relative abundance >0.05% and present in more than half number of the total animals at least in one treatment).

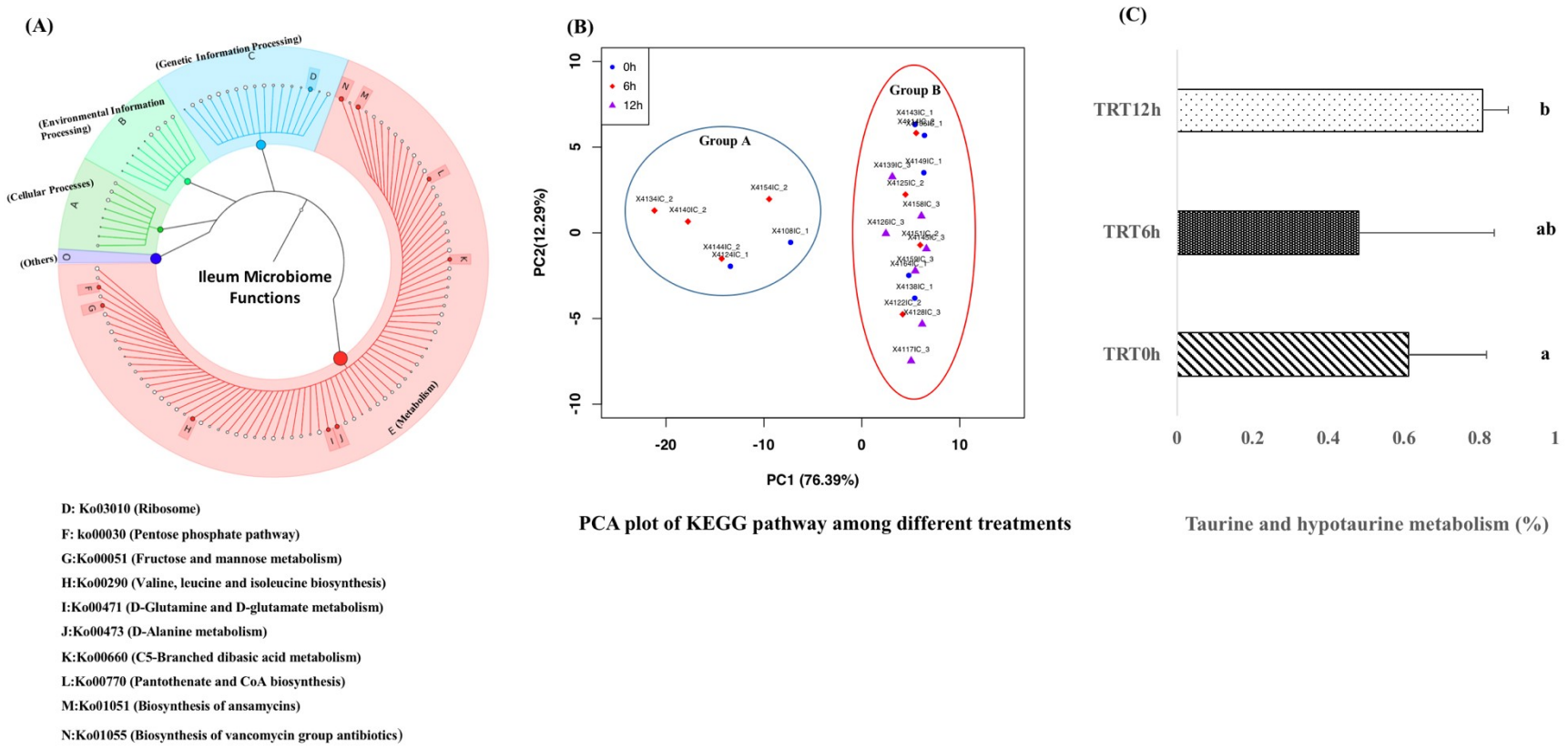


Figure 4.2 Ileal microbiome functions of neonatal dairy calves. (A) Microbial metabolic pathways at first and KEGG pathway level was annotated in the figure. The top 10 KEGG pathways were listed with the legend. **(B)** Principal component analysis (PCA) plots of the ileum microbial metabolic KEGG pathways among three treatments in neonatal dairy calves. TRT0h means colostrum feeding within 1 hour, TRT6h means colostrum feeding at 6h after birth, and TRT12h means colostrum feeding at 12 h after birth. **(C)** The significant change KEGG pathway among different colostrum feeding time treatment. Pathway with an LDA score > 2 and *P* value < 0.05 was considered significantly differential features.

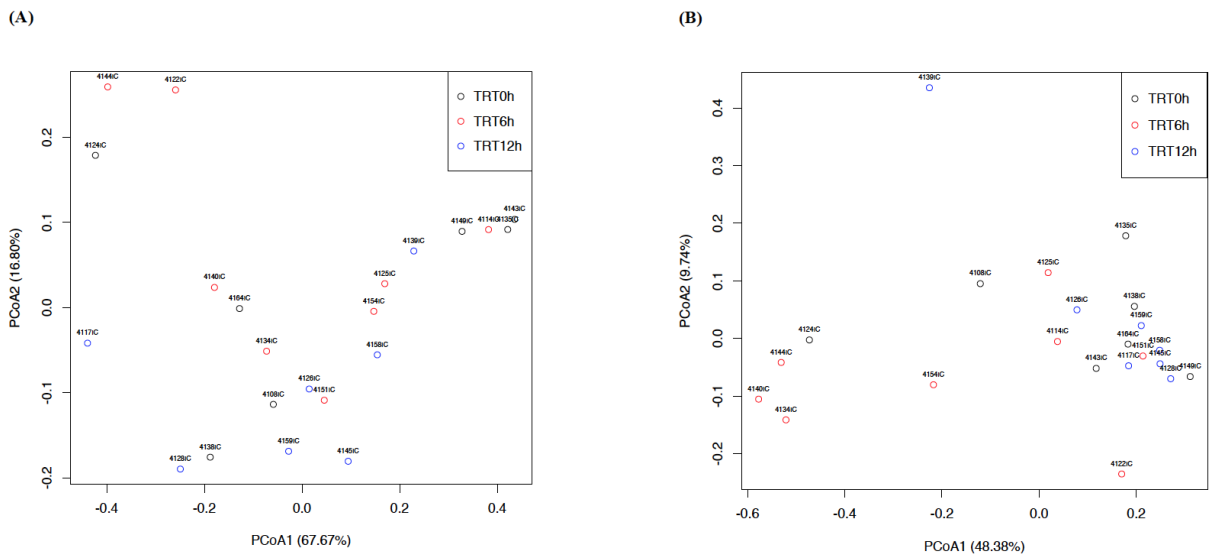


Figure 4.3 Principal-coordinate analysis (PCoA) of microbial composition profiles among different colostrum feeding time treatments. The first two PCoAs were plotted, and they were calculated based on the Bray-Curtis dissimilarity matrices. (A) PCoA plot of bacterial genera composition profiles among three treatments. The two components explained 67.67% and 16.80% of the variance. (B) PCoA plot of archaeal genera composition profiles among three treatments. The two components explained 48.38% and 9.74% of the variance. TRT0h means colostrum feeding within 1 hour, TRT6h means colostrum feeding at 6h after birth, and TRT12h means colostrum feeding at 12 h after birth.

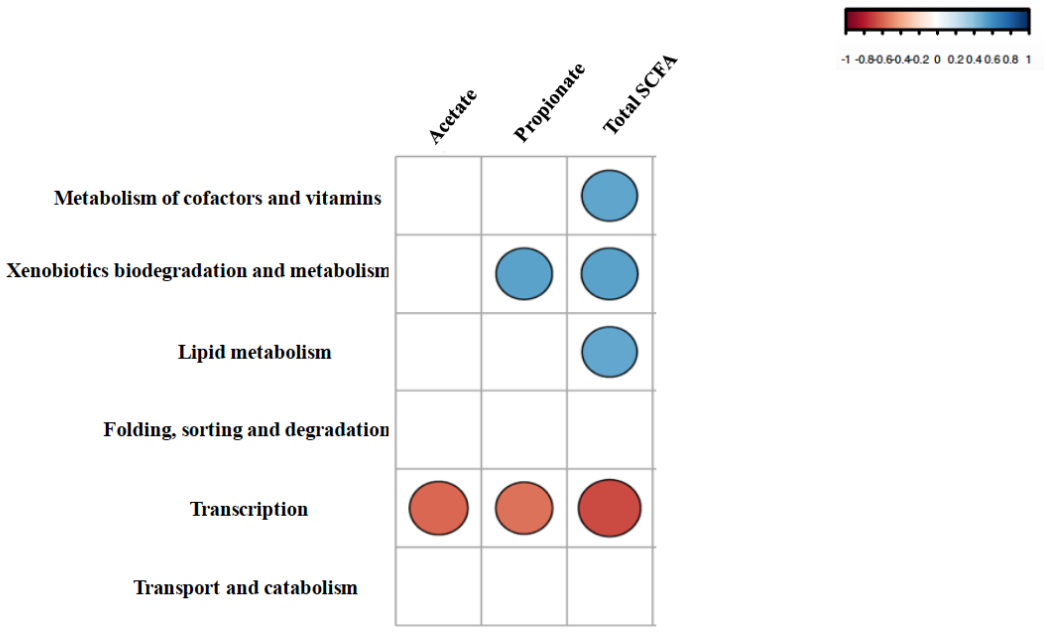


Figure 4.4 Relationship between ileal digesta microbiome functions and SCFA.
The correlation was analyzed with Spearman's rank correlation. And the one with $0.5 < |\rho|$, and *P*-value < 0.05 were plotted.

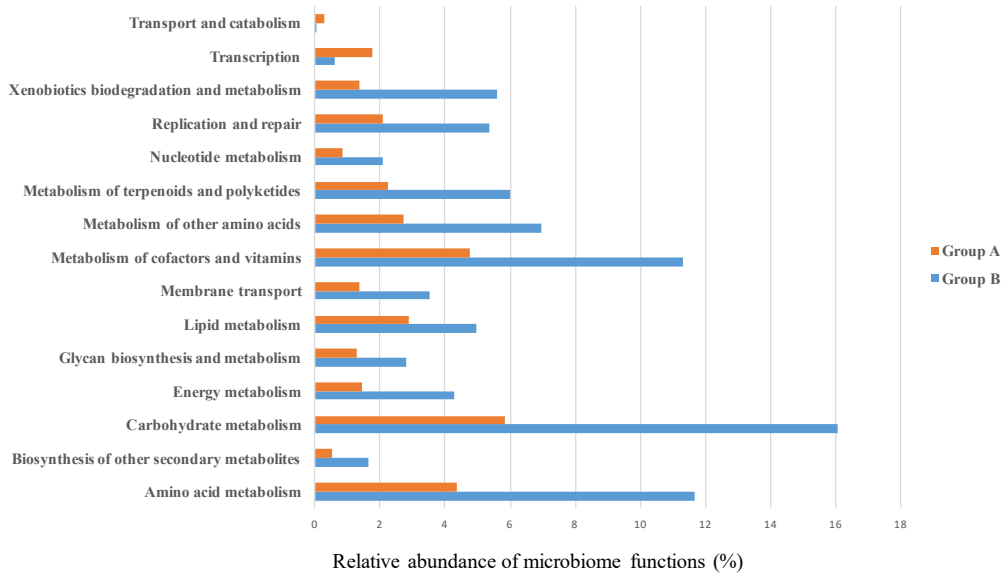
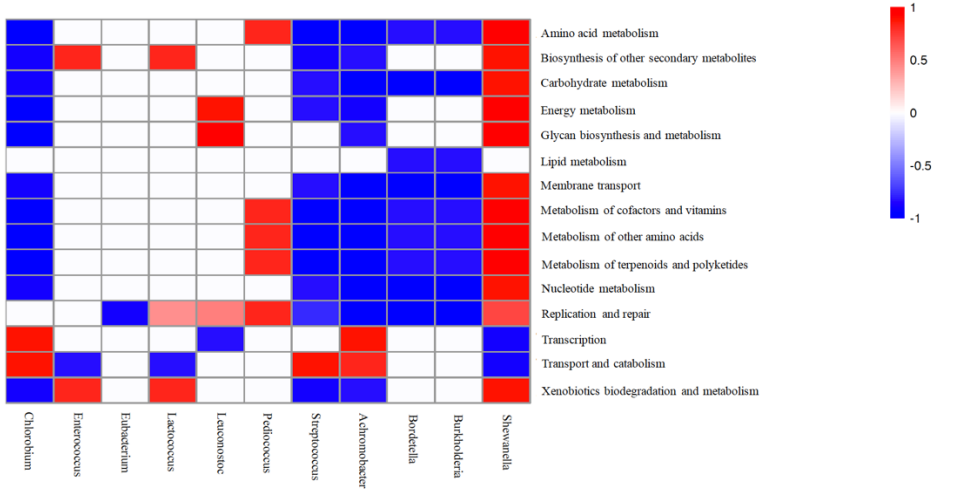
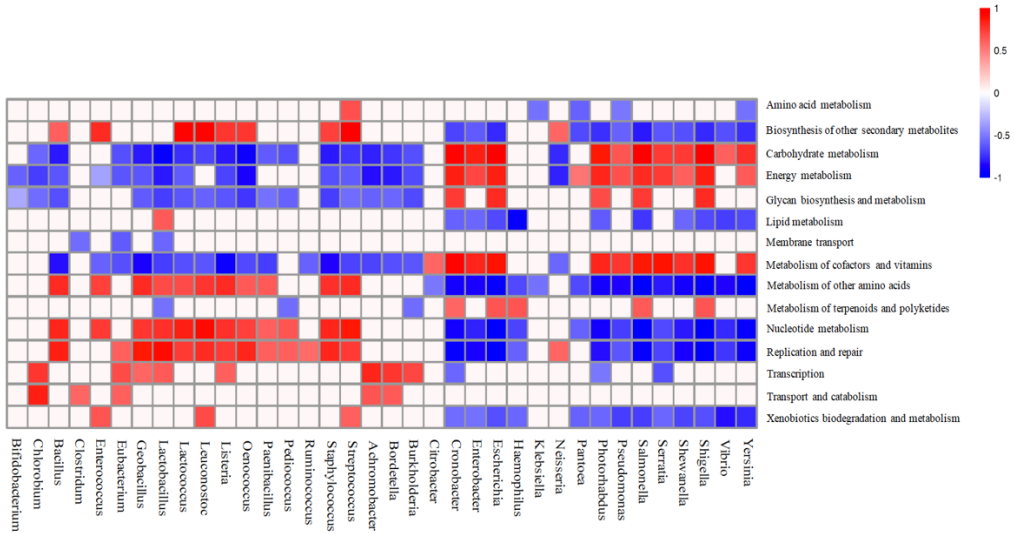


Figure 4.5 The relative abundance of varied KEGG second category functions between the "patterned" groups. Group A are comprised of the six animals that separate with other animals in PCA plot (Figure 4.2B), and group B are comprised of the other animals regardless of colostrum feeding treatment effect.

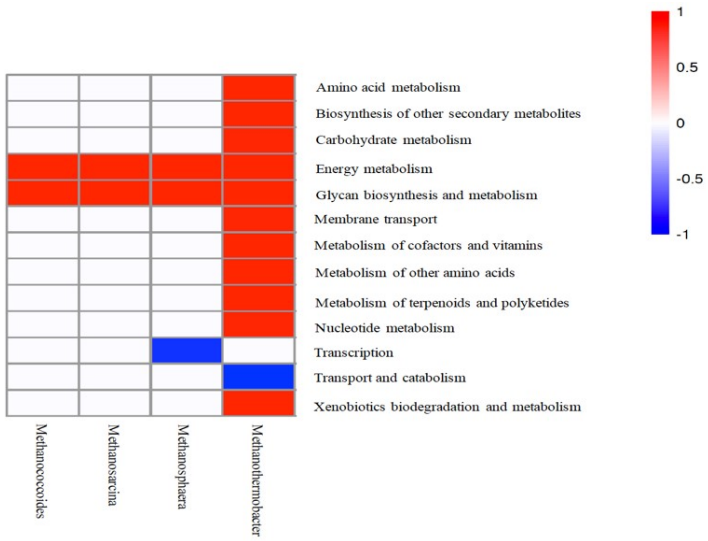
(A)


Amino acid metabolism
Biosynthesis of other secondary metabolites
Carbohydrate metabolism
Energy metabolism
Glycan biosynthesis and metabolism
Lipid metabolism
Membrane transport
Metabolism of cofactors and vitamins
Metabolism of other amino acids
Metabolism of terpenoids and polyketides
Nucleotide metabolism
Replication and repair
Transcription
Transport and catabolism
Xenobiotics biodegradation and metabolism
Chlorobium
Enterococcus
Eubacterium
Lactococcus
Leuconostoc
Pediococcus
Streptococcus
Achromobacter
Bordetella
Burkholderia
Shewanella
1
0.5
0
-0.5
-1

(B)


Amino acid metabolism
Biosynthesis of other secondary metabolites
Carbohydrate metabolism
Energy metabolism
Glycan biosynthesis and metabolism
Lipid metabolism
Membrane transport
Metabolism of cofactors and vitamins
Metabolism of other amino acids
Metabolism of terpenoids and polyketides
Nucleotide metabolism
Replication and repair
Transcription
Transport and catabolism
Xenobiotics biodegradation and metabolism
Bifidobacterium
Chlorobium
Bacillus
Clostridium
Enterococcus
Eubacterium
Geobacillus
Lactobacillus
Lactococcus
Leuconostoc
Listeria
Oenococcus
Paenibacillus
Pediococcus
Ruminococcus
Staphylococcus
Streptococcus
Achromobacter
Bordetella
Burkholderia
Citrobacter
Cronobacter
Enterobacter
Escherichia
Haemophilus
Klebsiella
Neisseria
Pantoea
Photorhabdus
Pseudomonas
Salmonella
Serratia
Shewanella
Shigella
Vibrio
Yersinia
1
0.5
0
-0.5
-1

(C)



（D）

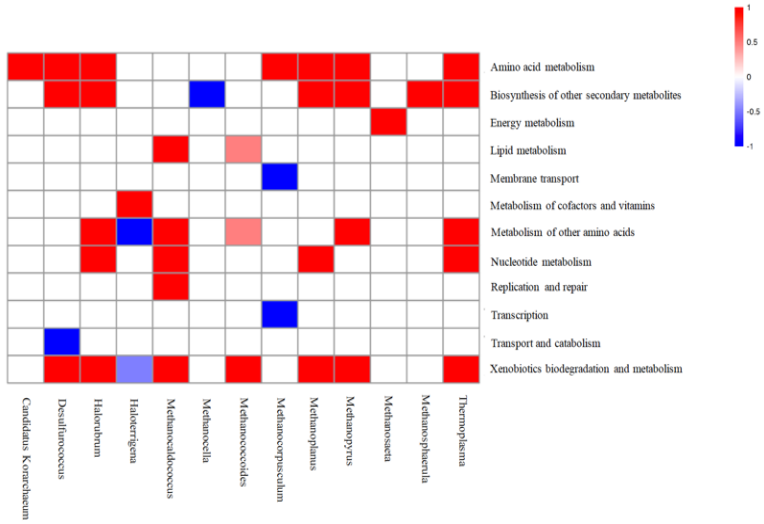


Figure 4.6 Relationship between the relative abundance of ileal digesta-associated bacterial and archaeal genera with the varied second category KEGG functions within each "patterned" group. The correlation was analyzed with Spearman's rank correlation. And the one with ρ >|0.5|, and *P*-value < 0.05 were plotted. (A) Relationship between the relative abundance of ileal digesta-associated bacterial genera in Group A with the varied second category KEGG functions. (B) Relationship between the relative abundance of ileal digesta-associated archaeal genera in Group A with the varied second category KEGG functions. (C) Relationship between the relative abundance of ileal digesta-associated bacterial genera in Group B with the varied second category KEGG functions. (D) Relationship between the relative abundance of ileal digesta-associated archaeal genera in Group B with the varied second category KEGG functions.

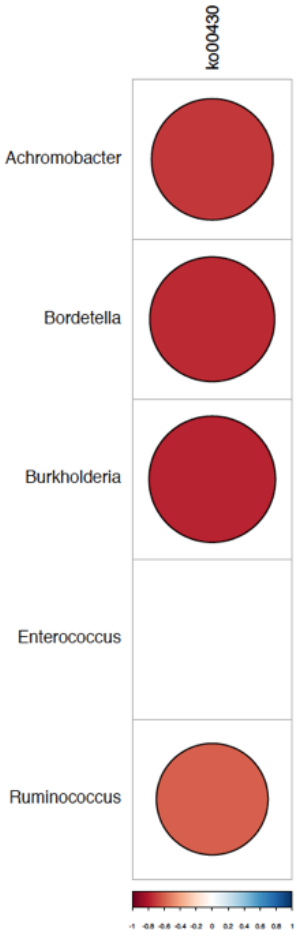


Figure 4.7 Relationship between the significant change bacterial genera and KEGG pathway function. The correlation was analyzed with Spearman's rank correlation. And the one with $|\rho| > 0.5$, and *P*-value < 0.01 were plotted.

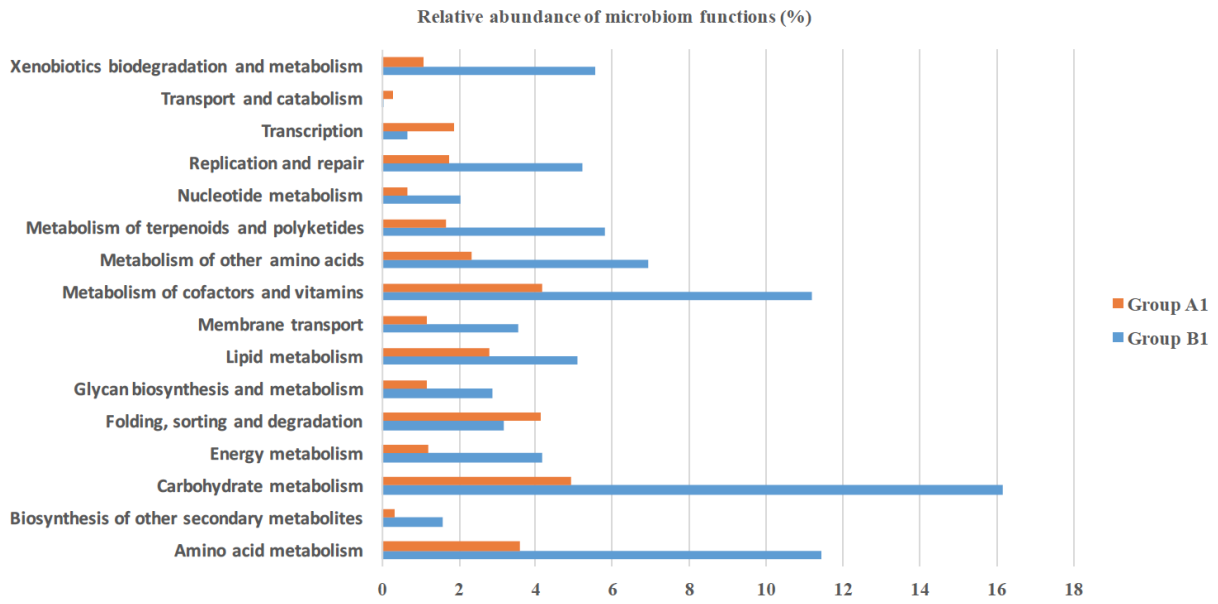


Figure 4.8 The relative abundance of varied KEGG second category functions between Group A1 and Group B1. Group A1 are comprised of the four animals that belong to TRT6h treatment within Group A that separate with other animals in PCA plot (Figure 4.2B), and group B are comprised of the other four animals belong to TRT6h treatment within Group B.

Chapter 5. Transcriptome analysis revealed that delayed colostrum feeding postponed ileal immune system development of neonatal calves

5.1 Introduction

Scours, diarrhea or other digestive problems account for 56.5% of the total mortality of the dairy calves in north America (USDA, 2010). The intestinal tract functional immaturity, undeveloped immune system, as well as the exposure to the intestinal microbiota and pathogenic organisms during early life may be the reasons for the higher risk of intestinal disease during early life. Small intestine is responsible for nutrients digestion and absorption, and also serves as a physical and immunological barrier to intestinal antigens, including microbes (Pluske et al., 1997; McGhee and Fujihashi, 2012). In addition, it has been reported that ileum has active mucosa immunity and gut barrier function during the first week of life (Liang et al., 2016), and also a favorable region for pathogenic bacteria (e.g., *E. coli* and *Salmonella* spp.) colonization in neonatal calves (Moxley and Francis, 1986, Tsolis et al., 1999). However, how the feeding management, especially the first feeding, affects such functions at the molecular level is largely unknown.

It is a common practice in North America to feed colostrum to neonatal calves immediately after birth to obtain the immunity through passive transfer (Immunoglobin G) and other nutritional and growth factors. Previous studies have indicated that colostrum feeding regulates intestinal structure and function development in human infants (Commare and Tappenden, 2007), piglet (Wang and Xu, 1996), and dairy calves (Blum and Hammon, 2000). Delayed colostrum feeding is common in dairy industry due to the lower surveillance rate when calves are born during night (Vasseur et al., 2010). When colostrum feeding is delayed, it can

lead to delayed provision of nutrients, immunoglobulin and growth factors (Godhia and Patel, 2013).

It has been known that various factors affect the gut development in neonatal calves, including host genetics, nutrition, microbes, and management. Yang et al. (2015) has shown that calves received colostrum right after birth had better developed villus, crypt and mucosal thickness in the duodenum, jejunum, and ileum compared to those who received transition milk. In addition, Liang et al. (2014) suggested that host-microbial interaction may play an important role in gut development of the dairy calves. However, how the delayed colostrum feeding influences the molecular mechanism regulating the ileal immune and intestinal barrier functions are still lacking. Therefore, we hypothesized that delayed colostrum feeding inhibited the development of intestinal immune system and barrier function of neonatal calves. Our study aimed to identify how the different first colostrum feeding time on genome wide gene expression and their related functions, as well as host-microbial interaction in the ileum of two-days old dairy calves.

5.2 Materials and methods

5.2.1 Calf management and sample collection

The animal study was conducted at the Dairy Research and Technology Center (the University of Alberta) with the approved animal protocol by the Livestock Animal Care Committee of the University of Alberta (AUP00001595) as detailed by in Fischer et al., (2018). Briefly, male Holstein calves (n=27) were randomly assigned to three colostrum feeding strategies: fed colostrum within one hour after birth (TRT0h, n=9), at 6 h (TRT6h, n=9), and at 12 h (TRT12h, n=9) after birth. The colostrum used in the experiment was purchased from

Saskatoon Colostrum Company Ltd. (Saskatoon, SK, Canada), with IgG concentration being 62 g/L and was fed 7.5% of birth body weight. First milk replacer (2.5% of birth body weight) was provided 12 h after colostrum feeding for each treatment group, following the first milk replacer feeding, other milk replacer was fed to calves every 6 h until 48 h after birth.

Ileal tissues were collected after euthanasia, using the methods as described by Malmuthuge et al., (2015). Briefly, to prevent environmental contamination, the whole gastrointestinal tract was sealed with table ties at esophagus and rectum sections. Ileum section was defined as 30 cm length proximal to the ileo-cecal junction and 15 cm proximal to this point was collected. The ileal tissue was rinsed with sterile phosphate buffered saline (PBS, pH = 7.0) buffer for three times after the contents were completely squeezed out. Then ileal tissues were snap frozen in liquid nitrogen immediately and stored in -80 °C.

5.2.2 RNA isolation, RNA-seq library construction and sequencing

The liquid nitrogen grounded ileal tissue powder (100 mg) was used for RNA extraction using a mirVana total RNA Isolation Kit (Ambion, Carlsbad, CA, USA) following the manufacturer's instructions. The quantity of RNA was measured with Qubit 2.0 Fluorimeter (Invitrogen, Carlsbad, CA, USA), and the integrity of the RNA was assessed using the Agilent 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Only samples with integrity number (RIN) >7 were used for the following RNA library construction.

Total RNA (1.0 µg) from each sample and TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA, USA) was used for RNA-Seq library construction. The RNA library construction was followed the procedures based on manufacturer's instruction. After quality of cDNAs was examined, they were pooled together for sequencing at Génome Québec (Montréal, Canada) using the Illumina HiSeq 4000 system to obtain 100bp paired-end reads.

5.2.3 Analysis of ileal transcriptome and differentially expressed genes

RNA-Seq data was analyzed following the pipeline described by Wang et al. (2016). Briefly, after the quality check of raw sequences using fastq-mcf, adapter and low quality sequences were removed (Aronesty, 2013). The remaining reads were aligned to the reference bovine genome (UMD 3.1) using Tophat 2.1.1 (Kim et al., 2013). Then HtSeq-count (Anders et al., 2015) was applied to obtain the gene counts of each sample using the Ensembl bovine gene annotation. The counts of each gene were then normalized to counts per million (CPM) based on the following formula: CPM = (gene read counts/total number of reads mapped to all annotated genes) × 10^6. Genes with CPM > 1 in at least half number of all the animals in each treatment (≥ 5 animals per treatment) were defined as expressed under each treatment group and were subjected to differential expression (DE) analysis in R (using package edge R). DE genes between different treatments were defined with false discovery rate (FDR) < 0.1, and $|\log_2 \text{fold change}| > 0$. Core transcriptome was defined for those presenting in all samples with CPM > 1.

5.2.4 Temporally changing pattern of neonatal calf ileum core transcriptome during the first week of life

The ileal transcriptome of two-days (D2) old calves from all treatment groups were combined together to be considered as D2 transcriptome. They were compared with the ileal transcriptomes of D0 (n=3) and D7 (n=5) old calves from Liang et al. (2016) to determine the changing pattern of ileal transcriptomes of neonatal calves during the first week of life (D0-D2-D7). The genes with fold changes of CPM value between any two treatments (D2 vs. D0 and D7 vs. D2) higher than 2, and with FDR < 0.05 were considered as upregulated, those with fold changes less than 0.5 as well as FDR < 0.05, were considered as downregulated, and the ones with fold changes between 0.5 and 2, were considered as unchanged. “U”, “D”, and “N”

represented the upregulated, downregulated, and not differentially (unchanged) expressed genes respectively. The DE genes that were detected at least in D2 vs. D0 or D7 vs. D2 were selected for the downstream analysis. Therefore, the variation of genes was categorized into eight expression patterns based on the temporal fold changes between two treatments (D2 vs. D0 and D7 vs. D2), including "UU", "DD", "UD", "DU", "NU", "ND", "UN", "UD".

5.2.5 Analysis of commonly and uniquely expressed genes, and genes related to immune system and intestinal barrier development

The ensemble ID of expressed genes in each treatment (TRT0h, TRT6h or TRT12h) were plotted together with Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/), and the number of commonly and uniquely expressed genes in the three treatments were described in the diagram.

Immune system and gut barrier development are closely related to intestinal health, therefore, the list of commonly expressed genes involved in immune and intestinal barrier development functions were obtained from ImmPort database (Bhattacharya et al., 2014) and Liang et al. (2016). And the genes related to immune system and intestinal barrier development among three treatments were plotted based on Principal Component Analysis (PCA) analysis separately.

5.2.6 Weighted Gene Co-expression Network Analysis (WGCNA)

To explore the relationship between ileal transcriptome and mucosa-attached microbiota, the 16S rRNA gene copy numbers of total bacteria, *Escherichia coli* (*E. coli*), *Bifidobacterium*, *Clostridium* cluster XIVa and *Faecalibacterium prausnitzii* (*F. pruasnitzii*) obtained from Fischer et al., (2018), as well as the relative abundance of bacterial genera based on 16S rRNA gene sequencing (unpublished data), were used to correlate with ileal transcriptome using

WGCNA R software package (WGCNA; v1.49) (Langfelder and Horvath, 2008). The commonly expressed genes (the expressed genes shared among three treatments) were normalized using (Log2(CPM + 1)) transformation. In total, 13,879 commonly expressed genes were subjected to WGCNA for weighted gene co-expression network analysis. The automatic one-step function, blockwise module was conducted for unsigned, weighted correlation network construction and module detection. The correlations between the resulting modules and the relative abundance of mucosa-attached bacterial genera (unpublished data), the 16S rRNA gene copy numbers of total bacteria, *Lactobacillus*, *F. prausnitzii, E. coli*, *Bifidobacterium* and *Clostridium* cluster XIVa (Fischer et al., 2018), as well as blood parameters (glucagon-like peptide-1 concentration at 48 h, glucagon-like peptide-2 concentration at 48 h, Insulin (obtained from Inabu et al., 2018), IgG concentration at 24 h and IgG concentration at 48 h (obtained from Fischer et al., 2018)), and growth parameters (body weight, the weight and length of the ileum after dissection) were calculated using Pearson's correlation. Modules with |correlation| > 0.50 and $P \leq 0.05$ were defined as significant, and were then used for downstream functional analysis.

5.2.7 Functional analysis

Annotation, Visualization and integrated Discovery (DAVID), Ingenuity Pathway Analysis (IPA; QIAGEN, Redwood City, CA, United States) and Protein ANnotation THrough Evolutionary Relationship (PANTHER) were applied for functional annotation. The core transcriptome among three treatments and genes involved in all the changing patterns from D0 to D7 were subjected to PANTHER for gene ontology (GO) terms annotation and enrichment analysis (Mi et al., 2015). The function of the uniquely expressed genes in each treatment was analyzed using IPA to find out functional terms (molecular functions) and enriched canonical pathways. Gene modules that were significantly correlated with mucosa-attached bacteria using

WGCNA analysis were subjected to DAVID for GO terms annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis, with enrichment score > 1, and *P* value < 0.05 defined as significant.

5.2.8 Data submission

All the RNA-Seq data were deposited in the publicly available NCBI's Gene Expression Omnibus Database. The data are accessible through GEO Series accession number GSE113331.

5.3 Results

5.3.1 Profiles and functions of ileal transcriptome in neonatal dairy calves

In total, 929,227,981 paired reads were generated from 27 ileal tissue samples, with 34,415,851±4,328,780 raw sequences per sample. On average, 85.69%±0.87% of total reads were mapped to the bovine reference genome (UMD 3.1). The number of expressed genes was 14,530±90 in the ileum regardless of treatment (Supplementary Dataset 5.1), with 12,599 of them expressed (CPM > 1) in all animals, which were defined as core transcriptome (Supplementary Dataset 5.2). The functional analysis of the core transcriptome using PANTHER revealed 12 biological processes in total, with "cellular process" (30.7%) and "metabolic process" (24.0%) being the predominant biological processes (Figure 5.1A). In addition, IPA analysis of top 8,000 genes of the core transcriptome identified 14 physiological functions with "hematological system development and function", "tissue morphology", "hematopoiesis", "lymphoid tissue structure and development", and "tissue development" being the top five functions (Figure 5.1B).

5.3.2 The temporal change of ileal core transcriptome during the first week of life

To further identify the ileal transcriptome changing patterns during the first week, we compared the temporal change of core ileal transcriptome among D0 (Liang et al., 2016), D2 (this study) and D7 (Liang et al., 2016). For this purpose, core ileal transcriptomes of D2 old calves in TRT0h treatment were used because these calves were raised under the same way. In total, the expression of 2969 genes was not different among three time points. Among 3,343 DE genes, six genes had continuously decreased (DD) expression from D0 to D7, and their main functions were "response to stimulus", "cellular process" and "biological regulation". There were 250 and 748 DE genes following "DU" and "UD" patterns, separately, and, 230, 73, 981 and 880 DE genes belonged to "ND", "NU", "DN" and "UN" patterns. "Cellular process" and "metabolic process" were the top two predominant functions enriched with the genes in all the patterns except for "DD" (Table 5.1; Figure 5.2). However, no DE genes were detected belong to "UU" pattern.

5.3.3 Commonly and uniquely expressed genes in the ileal tissue under different colostrum feeding time

When the whole transcriptome profiles were compared using PCA, they were not separated according to different treatments (TRT0h, TRT6h, and TRT12h) (Figure 5.3). Venn Diagram analysis revealed that 13,879 genes were expressed in the ileum under all the treatments, and 80, 286 and 129 genes were uniquely expressed in the ileum of TRT0h, TRT6h and TRT12h calves, respectively. In addition, 132, 123 and 223 genes were differentially expressed between two treatments, being TRT0h vs. TRT6h, TRT0h vs. TRT12h, and TRT6h vs. TRT12h, respectively (Figure 5.4A; Supplementary Dataset 5.3).

IPA analysis of uniquely expressed genes identified their molecular functions and canonical pathways (Figure 5.4B-D). Regardless of being uniquely expressed genes, four molecular functions were identified from them, including "Cellular Movement", "Cell Death and Survival", "Cell-to-Cell Signaling and Interaction" and "Cellular Function and Maintenance". However, functions of "Nucleic Acid Metabolism", "Antigen Presentation" and "Carbohydrate Metabolism" were only identified from the uniquely expressed genes in the ileum of the calves from TRT0h treatment, with functions of "Molecular Transport" and "Cellular Assembly and Organization" functions only detected TRT6h calves, and functions of "Amino Acid Metabolism", "Energy production" and "Cell Cycle" being only found in the ileum of the calves from TRT12h treatment (Figure 5.4B-D). In addition, the enriched top five canonical pathways included "CD40 Signaling", "Dendritic Cell Maturation", and "IL-6 Signaling" belonged to cellular immune response, were enriched for the uniquely expressed genes in the ileum of from TRT0h calves. Additionally, functions of "Granulocyte Adhesion and Diapedesis" and "Agranulocyte Adhesion and Diapedesis" belonged to cellular immune response were only enriched for the uniquely expressed genes in the ileum of TRT6h calves (Table 5.2). Moreover, functions "Melatonin Degradation III", "Glutamate Receptor Signaling" and "Phosphatidylcholine Biosynthesis I" were the top three enriched canonical pathways for the uniquely expressed genes in the ileum of TRT12h calves (Table 5.2).

5.3.4 Differential expression of genes affected by different colostrum feeding time

In total, six DE genes (*bta-mir-2904-3, SEPRINH1, PI3, ATP5F1E, CYP2C18*, ENSBTAG00000044212) were detected among different colostrum feeding time treatments, with log_2CPM values for *Bta-mir-2904-3* (TRT0h: 2.27±0.47; TRT6h: 3.31±0.47; TRT12h: 3.96 ± 0.50), *SEPRINH1* (TRT0h: 6.86±0.07; TRT6h: 7.20±0.07; TRT12h: 7.41±0.04), *PI3* (TRT0h:

3.71±0.18; TRT6h: 4.07±0.18; TRT12h: 4.83±0.20), *ATP5F1E* (TRT0h: 1.89±0.15; TRT6h: 2.22±0.14; TRT12h: 2.86±0.15), *CYP2C18* (TRT0h: 5.34±0.30; TRT6h: 5.14 ±0.30; TRT12h: 4.14±0.28) and ENSBTAG00000044212 (novel gene) (TRT0h: 1.89±0.22; TRT6h: 2.60±0.22; TRT12: 1.60±0.21) higher than 2.0 and FDR < 0.05 in at least 2 groups (Figure 5.5).

5.3.5 Genes involved in immune and intestinal barrier functions of two-days old dairy calves

In total, 720 immune function related genes were expressed in the ileum of two-days old calves, including antigen processing and presentation (60 genes), antimicrobials (224 genes), B cell receptor signaling interactive pathway (53 genes), chemokines (35 genes), chemokine receptors (28 genes), cytokines (102 genes), cytokine receptors (107 genes), interleukins (1 gene), interleukins receptor (24 genes), natural killer cell cytotoxicity (23 genes), T cell receptor signaling interactive pathway (33 genes), transforming growth factor beta family member (2 genes), transforming growth factor beta family member receptor (10 genes), Tumor necrosis factor members (8 genes), and tumor necrosis factor family members receptors (10 genes) (Supplementary Dataset 5.4). In addition, 12 intestinal barrier function related genes were expressed including claudin (9 genes), occluding (1 gene) and junctional adhesion molecule family (2 genes) (Supplementary Dataset 5.4). When the expression of all these genes were compared using PCA, no difference was found among three treatment groups (Figure 5.6).

5.3.6 Relationships between ileal transcriptome, mucosa-attached bacteria as well as phenotypes

Hierarchical clustering dendrogram of ileal transcriptomes (TRT0h, TRT6h, TRT12h) and measurements for ileal microbiota (16S rRNA gene copy number of total bacteria, *E. coli*, *Bifidobacterium*, *Clostridium* cluster XIVa and *F. pruasnitzii* and the relative abundance of

bacterial genera based on 16S rRNA gene sequencing) showed no clear grouping among different colostrum feeding time treatments (Figure 5.7). Further WGCNA based co-expression analysis identified 13 gene modules. No significant correlations were identified between the gene modules and the blood parameters (glucagon-like peptide-1_48h, glucagon-like peptide-2_48h, Insulin (data obtained from Inabu et al., 2018), IgG_24h and IgG_48h (data obtained from Fischer et al., 2018)), and growth parameters (body weight, the weight and length of the ileum, data not shown). However, WGCNA co-expression analysis identified 13 co-expressed gene modules correlated with various measurements of gut microbiota. For example, Turquoise gene module consisting of 2,206 genes was significantly correlated with 16S rRNA gene copy number of total bacteria (r = -0.59, $P < 0.01$), *F. prausnitzii* (r = -0.70, $P < 0.01$) and *Bifidobacterium* (r = -0.55, $P < 0.01$) (Figure 5.8A). Magenta gene module consisted of 252 genes was significantly correlated with the relative abundance of *Butyricicoccus* (r = -0.63, $P < 0.01$), and the co-expressed genes in Pink gene module (286 genes) were significantly correlated with the relative abundance of *Blautia* (r = 0.54, $P < 0.01$) and *Brevibacterium* (r = 0.57, $P < 0.01$). In addition, the co-expressed genes in Black gene module (303 genes) were significantly correlated with the relative abundance of *Enterobacter* (r = -0.59, $P < 0.01$) (Figure 5.8B).

5.3.7 Functional analysis of gene modules associated with mucosa-attached bacteria

Further GO term functional analysis revealed 11, 10 and 7 GO terms significantly enriched in Turquoise, Pink, and Magenta gene modules respectively, but no significant enriched go terms for black gene module (Table 5.3). The enriched GO term functions for Turquoise gene module included “Mitochondrial inner membrane” (48 genes), “Mitochondrial translational initiation” (18 genes), “Mitochondrial translational elongation” (18 genes), “Mitochondrial translation” (11 genes), and “Cadherin binding involved in cell-cell adhesion” (14 genes), “Cell-

cell adherences junction" (7 genes). In addition, the enriched go terms functions for pink gene module were "Inflammatory response" (19 genes), "Chemokine-mediated signaling pathway" (7 genes), "Chemokine activity" (6 genes), "Immune response" (12 genes), "Positive regulation of neutrophil chemotaxis" (3 genes), "Chemokine activity" (6 genes), and "Cellular response to interleukin-1" (5 genes). Moreover, the Go term functions enriched in Magenta gene module were "Cytosolic small ribosomal subunit" (7 genes), "Translation" (11 genes), "Structural constituent of ribosome" (10 genes), and "Mitochondrial respiratory chain complex IV" (3 genes) (Table 5.3).

KEGG pathways analysis showed that several pathways were enriched for genes included in turquoise, black, pink and magenta gene modules respectively (Table 5.4). "Oxidative phosphorylation", "T cell receptor signaling pathway", "B cell receptor signaling pathway", "Osteoclast differentiation", and "TNF signaling pathway" were enriched in turquoise gene module. Four pathways including "TNF signaling pathway", "NOD-like receptor signaling pathway", "Toll-like receptor signaling pathway" and "RIG-I-like receptor signaling pathway" were enriched in pink gene module. "Oxidative phosphorylation", "Metabolic pathways" and "Cardiac muscle contraction" pathways were enriched in magenta gene module. "RNA polymerase" pathway was enriched in black gene module.

5.4 Discussion

In this study, the ileal transcriptome profiles of two-days old dairy calves and how it can be affected by different colostrum feeding time (TRT0h, TRT6h and TRT12h) was characterized. This study was the first to provide the molecular bases behind ileum physiological and functional development. The number of expressed genes (13,879) in the ileum of D2 old

calves was similar to those in the ileum at D0 (13,858) and D7 (13,994). The identified dominant biological function of "cellular process" in the ileum of D2 old calves, suggesting that cell proliferation, growth, movement were the most active in the ileum of the neonatal calves. While the predominant function "cellular process" is different from the previous reported function "Metabolic process" in the pre-weaned calves (Liang et al., 2016), and this could be contributed to the difference of functional annotation method (PANTHER vs. DAVID) and the different calves' age. Moreover, "cellular process" function was also found to shift to have different patterns ("DD", "DU", "UD", "ND", "NU", "DN", "UN") from D0 to D7 further suggests the importance of cellular process related functions (cell proliferation, growth, movement) to neonatal dairy calves. In addition, the highly enriched "metabolic process" (e.g., energy metabolism, carbohydrate metabolic process, amino acid metabolic process) was in accordance with that calves need nutrients and energy to survive and growth after birth.

Ileum has been chosen as the target region for transcriptome analysis of neonatal livestock species (piglets and calves) due to its importance in immune response (Inoue et al., 2015; Liang et al., 2016; Saraf et al., 2017). Regarding to the enriched physiological functions in our study, immune system development related functions, including lymphoid tissue structure and development, cell-mediated immune response, immune cells trafficking and humoral immune response were enriched at D2, which was in accordance with the previous finding (Liang et al., 2016). From 720 genes involved in immune functions were identified in the ileum of two-days old calves in our study, 601 of them was also detected at D0 (Liang et al., 2016), implying that immune function development started during early life. Additionally, mucosal dendritic cells, which are linked to innate and adaptive immune system, were populated in the

small intestine of neonatal calves, further indicating that immune functions development during early life at cellular level as suggested by Fries et al. (2011).

It was not surprising to identify the uniquely expressed genes in the ileum under each treatment. Only unique genes expressed in TRT0h group were enriched for "Antigen Presentation" function, which was related to immune system response. This suggests that feeding colostrum within one hour may have promoted immune system function compared to feeding colostrum at 6 h and 12 h after birth. In addition, the enriched functions of "Nucleic Acid Metabolism" and "Carbohydrate Metabolism" in TRT0h calves may be related to the early provision of colostrum, suggesting that exposure to the colostrum nutrients earlier could stimulate the development of corresponding functions. Interestingly, the enriched "Energy production" function in the ileum of TRT12h calves indicate that when the first colostrum feeding is delayed, the calves may enhance to expression of genes involved in energy metabolism to obtain enough energy. In addition, the maturation of DCs is the fundamental step for interactions with T cells and induce immune response (Moser and Murphy, 2000). Therefore, the enriched "Dendritic cell maturation" pathway in ileum of TRT0h calves suggest that feeding colostrum without delay may stimulate the cellular immune response of neonatal calves. In addition, the enriched IL-6 signaling and CD 40 signaling pathways further indicate that calves fed with colostrum soon after birth (TRT0h group) has better developed immune system at day 2 of life. Previous studies have indicated that IL-6 stimulates naïve CD4+ T cells differentiation, TH17 cells differentiation (Korn et al., 2009), and $CD8^+$ T cells into cytotoxic T cells (Okada et al., 1998), highlighting its various effects in modulating immune response. Similar as IL6, CD 40 stimulates DCs maturation and further induces T-cell activation and differentiation (Quezada et al., 2004). In addition, CD40 signaling of B cells promoted immunoglobulin (Ig) isotype

switching, germinal center formation (Danese et al., 2004). Therefore, CD 40 is also actively engaged in immune function development. Additionally, immune related pathways Agranulocyte/granulocyte adhesion and diapedesis are the first lines of host defense against infection, which are important in protecting the host from injury by recruiting agranulocytes and granulocytes to the specific intestinal region (Xing et al., 2017). The enriched function of "Granulocyte Adhesion and Diapedesis" and "Agranulocyte Adhesion and Diapedesis" in the ileum of TRT6h calves suggests that the delay 6 h of feeding of colostrum may not affect the functions in terms of host protect themselves from intestinal antigens.

In general, no clear separation in the ileal transcriptome among the different treatments (TRT0h, TRT6h, TRT12h) may be due to the high individual variation within each treatment group. However, six DE genes were identified in the ileum under different colostrum feeding treatments. Among them, *PI3* is related to neutrophil serine proteinase inhibitors, which play a role in antimicrobial activity and innate immune system and protecting the tissue from inflammation (Chowdhury et al., 2006). Therefore, the higher expression of *PI3* gene in the ileum of TRT12h calves suggests that these calves are likely to be a self-protection against the potential pathogenic organism invasion in the ileum due to the higher chance of infection. Indeed, the opportunistic pathogens *Enterococcus* was higher in the lumen of the same animals (Chapter 4). In addition, *ATP5F* is responsible for the synthesis of ATP synthase (Weber and Senior, 2000), which plays an important role in the ATP synthesis (Weber and Senior, 1997). Hence, the higher expression of *ATP5F1E* gene in the ileum of TRT12h calves suggest that delaying the colostrum feeding for 12 hours may enhance the ATP synthesis compared to those receiving colostrum without delay and or with 6 hr delay. This may be the host self-regulatory defense mechanism to maintain the growth when nutrient is deficient. Moreover, higher

expression of *SERPINH1* gene in the ileum of the TRT12h calves suggests that delayed colostrum feeding for 12 h may postpone intestinal structural and functional development since *SERPINH1* gene is associated with preterm birth (Wang et al., 2006) as preterm infants higher expression of *SERPINH1* but less maturity of intestinal tract compared to full-term infants (Neu et al., 2007).

Intestinal microbiota has profound effects on host physiology by regulating host intestinal gene expression such as in piglets (Zhang et al., 2017), mice (Larsson et al., 2012), and dairy calves (Malmuthuge et al., 2013, Liang et al., 2014). This study is the first to identify the co-expressed genes associated with mucosa-attached microbiota in the ileum. Turquoise module, consists of 2,206 co-expressed genes, has the enriched function of "Mitochondrial inner membrane" and "oxidative Phosphorylation" KEGG pathway, highlighting their main functions in the production of ATP through oxidative phosphorylation (Goodlad, 1981). Their significant associations between mucosa-associated total bacteria, *F. prausnitzii*, *Bifidobacterium* suggesting that colonization of bacteria plays an important role in host energy metabolism. In accordance with previous findings, the enriched GO term functions "cadherin binding involved in cell-cell adhesion" and "cell-cell adherences junction" in Turquoise gene module may be related to its closely relationship with *F. prausnitzii* and *Bifidobacterium* (*F. prausnitzii* ($r = -0.70$, $P < 0.01$) and *Bifidobacterium* ($r = -0.55$, $P < 0.01$)). Supplement of *F. prausnitzii* has been reported to enhance intestinal barrier function by affecting paracellular permeability in mice (Carlsson et al., 2013). *Bifidobacterium* is known to protect intestinal barrier against barrier dysfunction by improving intestinal tight junction integrity in mice (Ling et al., 2016). Furthermore, *Bifidobacterium* was reported to be involved in triggering host immune response, and its admission improved B and T cell responses following rotavirus vaccination in piglets

(Kandasamy et al., 2014; Ishizuka et al., 2016). Higher *Bifidobacterium* abundance was observed in the ileum mucosa-attached bacterial community, which may play a role in the enriched KEGG pathways “T cell receptor signaling pathway” and “B cell receptor signaling pathway”.

The intestine of dairy calves was exposed to intensive microbes after birth. The ten enriched immune related GO term functions for genes in pink gene module which were positively correlated with the relative abundance of *Blautia* ($r = 0.54$, $P < 0.01$) and *Brevibacterium* ($r = 0.57$, $P < 0.01$)), suggesting that *Blautia* and *Brevibacterium* may influence host immune response. In addition, the activation of “NOD-like receptor signaling pathway”, “Toll-like receptor signaling pathway” and “RIG-I-like receptor signaling pathway” KEGG pathways were also identified for the genes enriched in this gene module. NOD-like receptor, Toll-like receptor and RIG-I-like receptors are all pattern-recognition receptors, which contribute to the first step of host innate immune system for detecting the intestinal microbes and inducing the innate immune responses (Kawasaki and Kawai, 2014). These receptors also play a key role in intestinal barrier integrity and mucosal immune system maturation (Lavelle et al., 2010). However, more researches are needed to study whether and how these two bacteria genera affect immune system development in the neonatal dairy calves.

In addition, the significant negative correlation was detected between the abundance of *Butyricicoccus* and the “Oxidative phosphorylation” KEGG pathway enriched for genes in Magenta gene module ($r = -0.63$, $P < 0.01$). *Butyricicoccus* genus consists of butyrate producing species, such as *Butyricicoccus pullicaecorum*, which could utilize the intestinal substrates and produce butyrate to serve as the energy source to peripheral tissue and intestinal epithelial cells (Bergman, 1990; Hamer et al., 2009). In addition, butyrate was reported to regulate energy metabolism (e.g., promoting oxidative metabolism) in the colon of mice (Donohoe et al., 2011).

Therefore, the identified relationship between *Butyricicoccus* and Magenta genes module (r = -0.63, P <0.01) suggests the key roles of this genus in the ileum of neonatal dairy calves which also warrant further validations. However, six DE genes were not detected in any of the gene modules (turquoise module, black module, pink module and magenta module) that related to the mucosa-attached bacteria. We also did not observed many differentially abundant mucosa-attached bacteria among different colostrum feeding treatments. These suggest that the delayed colostrum feeding up to 12 h may not affect the host-microbial interactions at day two due to the host's self-protective activity.

5.5 Conclusion

This is the first study to explore the ileal transcriptome profiles of two-days old calves under different colostrum feeding time treatments (TRT0h, TRT6h and TRT12). The enriched innate immune system related function "Antigen Presentation" in the ileum of TRT0h calves indicate that feeding colostrum within one hour after birth may have stimulated immune system development compared to those had delayed colostrum feeding. Moreover, the enriched expression of *PI3* gene in the ileum of TRT12h calves suggest that delaying feeding colostrum for 12 hours may lead to higher inflammatory responses. Significant correlations between mucosa-attached total bacteria, *F. prausnitzii*, *Bifidobacterium* and co-expressed genes in the turquoise gene module, with enriched functions of "Cadherin binding involved in cell-cell adhesion" and "Cell-cell adherences junction" GO term functions, and "Oxidative Phosphorylation" KEGG pathway, suggesting that ileal mucosa-attached bacteria may impact the host energy metabolism and gut barrier development. Additionally, the significant correlation between the relative abundance of *Blautia*, *Brevibacterium* and co-expressed genes in the pink

gene module (286 genes), with enriched of immune Go term functions and innate immune system related KEGG pathways “NOD-like receptor”, “Toll-like receptor” and “RIG-I-like receptors”, indicating that these two bacteria may play a role in ileal immune system development. The findings from this study provide the fundamental information on host-microbial interactions in the ileum of neonatal calves, further studies on isolating these organisms and/or using epithelial cells are needed to verify our speculations.

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5.7 Tables

Table 5.1 Major functions of the different changing patterns of dairy calves during the first week.

Pattern	Genes No.	Top functions	Top functions (%)
DD	6	response to stimulus (GO:0050896)	28.60%
		cellular process (GO:0009987)	28.60%
		biological regulation (GO:0065007)	28.60%
DU	250	cellular process (GO:0009987)	32.50%
		metabolic process (GO:0008152)	26.70%
UD	748	cellular process (GO:0009987)	29.80%
		metabolic process (GO:0008152)	20.80%
ND	218	cellular process (GO:0009987)	29.70%
		metabolic process (GO:0008152)	22.00%
NU	71	cellular process (GO:0009987)	25.40%
		metabolic process (GO:0008152)	23.70%
DN	1163	cellular process (GO:0009987)	33.00%
		metabolic process (GO:0008152)	26.30%
UN	887	cellular process (GO:0009987)	28.70%
		metabolic process (GO:0008152)	21.30%

Table 5.2 The top 5 ingenuity canonical pathways in different colostrum feeding time treatment of neonatal calves.

Treatment	Ingenuity canonical pathways	Category	-log(p-value)	Genes No.
TRT0h	Renin-Angiotensin Signaling	growth factor signaling	3.67	4
	CD40 Signaling	cellular immune response	3.03	3
	Dendritic Cell Maturation	cellular immune response	2.91	4
	Adrenomedullin signaling pathway	cellular stress and injury	2.91	4
	IL-6 Signaling	cellular immune response	2.44	3
TRT6h	Calcium Signaling	Intracellular and second messenger signaling	2.74	7
	Phototransduction Pathway	Neurotransmitters and other nervous system signaling	2	3
	Granulocyte Adhesion and Diapedesis	Cellular immune response	1.73	5
	Agranulocyte Adhesion and Diapedesis	Cellular immune response	1.63	5
	1,25-dihydroxyvitamin D3 Biosynthesis	Biosynthesis	1.6	1
TRT12h	Melatonin Degradation III	Degradation/Utilization/Assimilation	2.43	1
	Glutamate Receptor Signaling	Neurotransmitters and other nervous system signaling	1.72	2
	Phosphatidylcholine Biosynthesis I	Biosynthesis	1.59	1
	Glycoaminoglycan-protein Linkage Region Biosynthesis	Biosynthesis	1.59	1
	Eicosanoid Signaling	Intracellular and second messenger signaling	1.58	2

Table 5.3 The enriched Go term functions in different gene modules with DAVID analysis.

Module	Term	*P*-value	Genes No.
Turquoise	**Enrichment score: 1.78 (Cluster 1)**		
(2206 genes)	Mitochondrial translational initiation	0.01	18
	Mitochondrial translational elongation	0.01	18
	Mitochondrial translation	0.02	11
	Mitochondrial small ribosomal subunit	0.02	8
	Mitochondrial large ribosomal subunit	0.04	10
	Enrichment score: 1.54 (Cluster 2)		
	NuA4 histone acetyltransferase complex	<0.01	7
	Enrichment score: 1.30 (Cluster 3)		
	Cadherin binding involved in cell-cell adhesion	0.02	14
	Cell-cell adherences junction	0.04	17
	Enrichment score: 1.17 (Cluster 4)		
	COPI vesicle coat	0.03	5
	Retrograde vesicle-mediated transport, Golgi to ER	0.04	8
Pink	**Enrichment score: 3.01 (Cluster 1)**		
(286 genes)	Inflammatory response	<0.01	19
	Chemokine-mediated signaling pathway	<0.01	7
	Chemokine activity	<0.01	6
	Immune response	<0.01	12
	CXCR chemokine receptor binding	0.01	3
	Positive regulation of neutrophil chemotaxis	0.03	3
	Response to lipopolysaccharide	0.04	5
	Enrichment score:2.32 (Cluster 2)		
	Chemokine activity	<0.01	6
	Neutrophil chemotaxis	0.01	5

	Cellular response to interleukin-1	0.01	5
Magenta	**Enrichment score 5.46 (Cluster 1)**		
(252 genes)	Cytosolic small ribosomal subunit	<0.01	7
	Translation	<0.01	11
	Structural constituent of ribosome	<0.01	10
	Enrichment score 3.81 (Cluster 2)		
	Hydrogen ion transmembrane transport	<0.01	6
	Cytochrome-c oxidase activity	<0.01	5
	Respiratory chain complex IV	<0.01	4
	Mitochondrial respiratory chain complex IV	0.01	3

Table 5.4 The enriched KEGG pathways in different gene modules with DAVID analysis.

KEGG Pathways	First level category	Second level category	Genes No.	*P*-value
Turquoise module (2206 genes)	**Enrichment score: 3.65 (Cluster 1)**			
Oxidative phosphorylation	Metabolism	Energy metabolism	28	<0.01
	Enrichment score: 1.58 (Cluster 2)			
T cell receptor signaling pathway	Organismal Systems	Immune system	19	0.01
B cell receptor signaling pathway	Organismal Systems	Immune system	14	0.01
	Enrichment score: 1.56 (Cluster 3)			
Osteoclast differentiation	Organismal Systems	Development	23	0.01
TNF signaling pathway	Environmental Information Processing	Signal transduction	19	0.01
Black module (303 genes)	**Enrichment score: 1.41**			
RNA polymerase	Genetic Information Processing	Transcription	3	0.05
Pink module (386 genes)	**Enrichment score: 2.07**			
TNF signaling pathway	Environmental Information Processing	Signal transduction	11	<0.01
NOD-like receptor signaling pathway	Organismal Systems	Immune system	7	<0.01
Toll-like receptor signaling pathway	Organismal Systems	Immune system	8	<0.01
RIG-I-like receptor signaling pathway	Organismal Systems	Immune system	5	0.03
Magenta module (256 genes)	**Enrichment score:6.87**			
Oxidative phosphorylation	Metabolism	Energy metabolism	16	<0.01
Metabolic pathways	Metabolism	Global and overview maps	23	<0.01
Cardiac muscle contraction	Organismal Systems	Circulatory system	8	<0.01

5.8 Figures

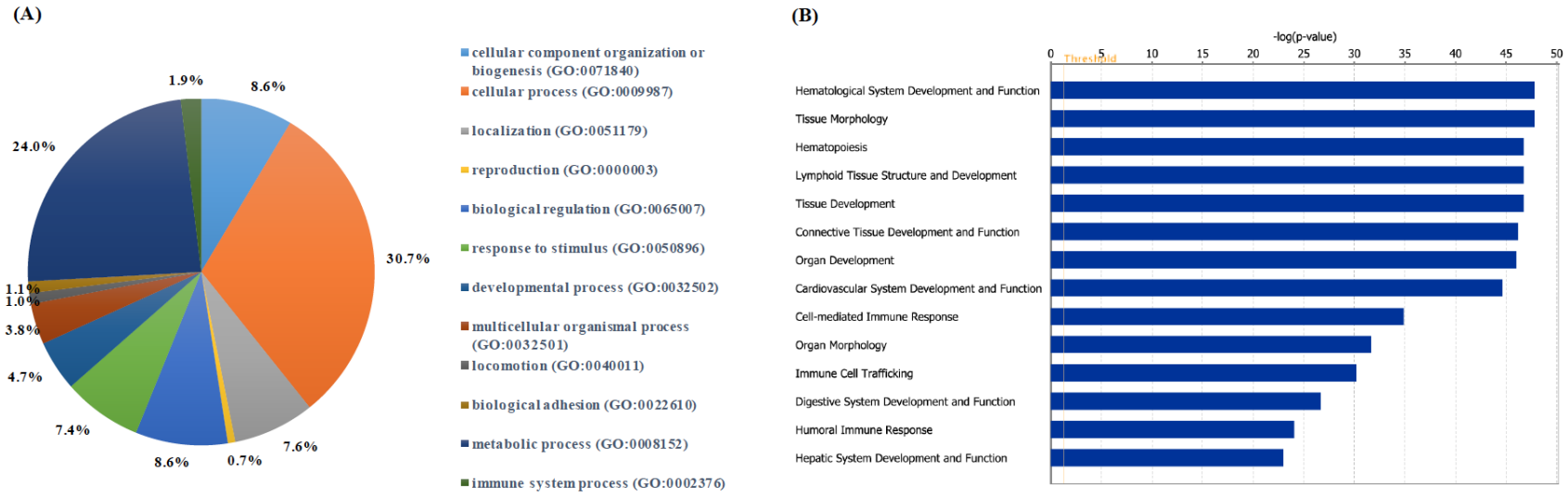


Figure 5.1 Functional classification of the core genes in the ileum of neonatal dairy calves with PANTHER and IPA. (A) Biological processes of core genes with PANTHER. (B) The enriched physiological pathways of core genes with IPA.

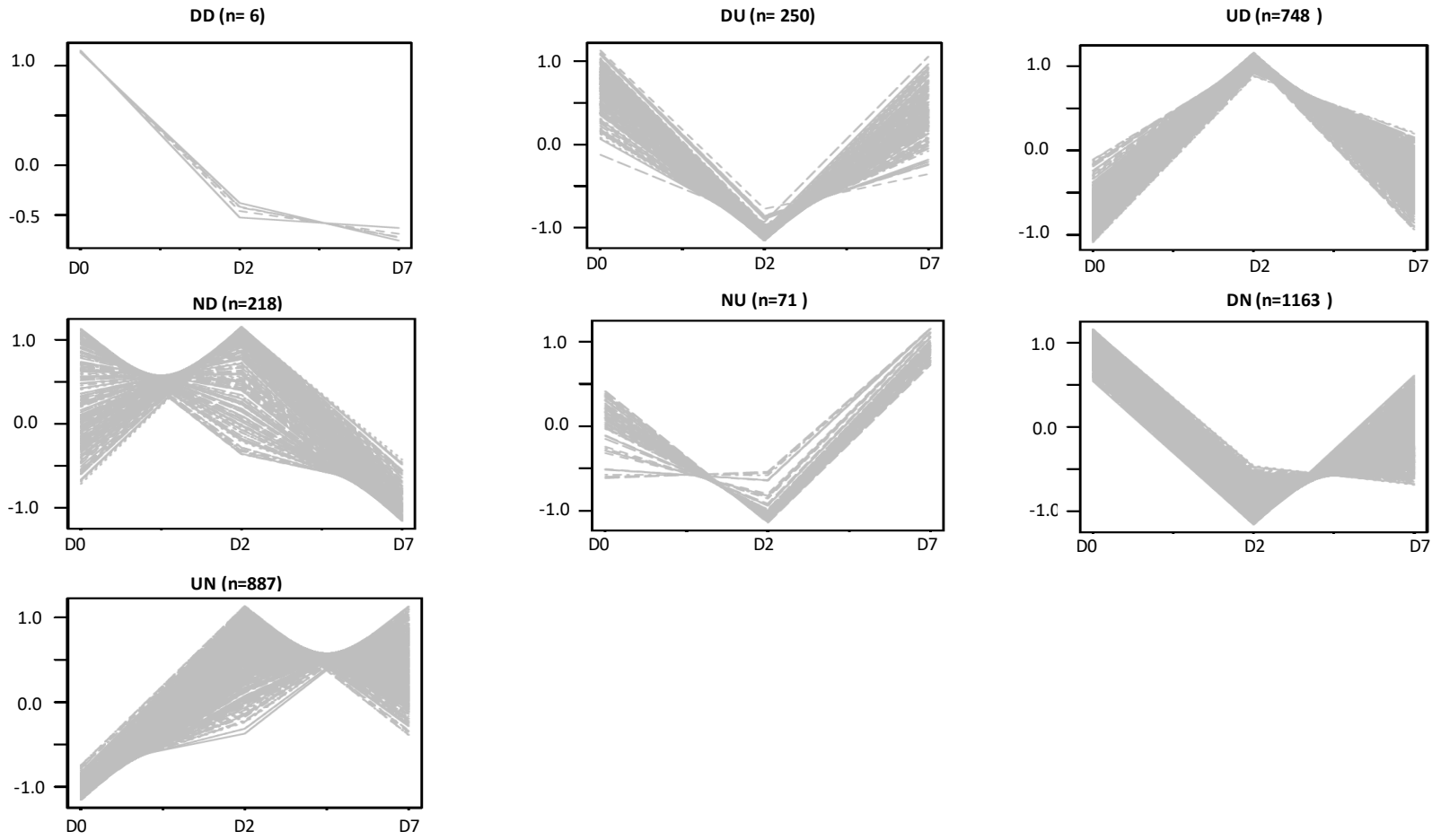


Figure 5.2 Temporally changing pattern of ileal core transcriptome of neonatal calves during the first week after birth. All the DE genes were categorized into 7 expression patterns based on the temporally fold change between two treatments. "U" means the genes were upregulated (fold change > 2), "D" means the genes were downregulated (fold change < 1/2), and "N" means the genes were not changed (1/2 < fold change < 2). The order of each pattern follows the comparison between D2 vs D0 and D7 vs D2. X-axis depicts three age points (D0, D2 and D7) and Y-axis depicts the normalized CPM value of each gene.

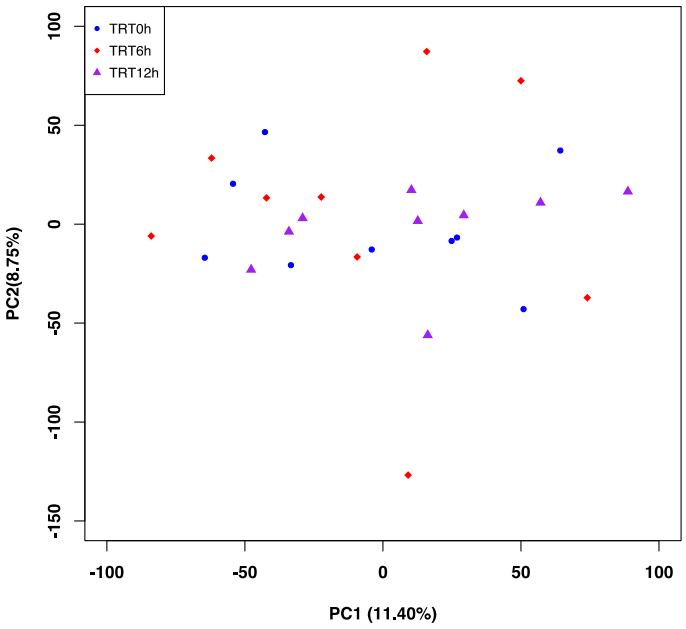


Figure 5.3 PCA plot of ileal transcriptome profiles among different colostrum feeding time treatments. TRT0h means feeding colostrum within one hour after birth. TRT6h means feeding colostrum at 6 h after birth. TRT12h means feeding colostrum at 12 h after birth.

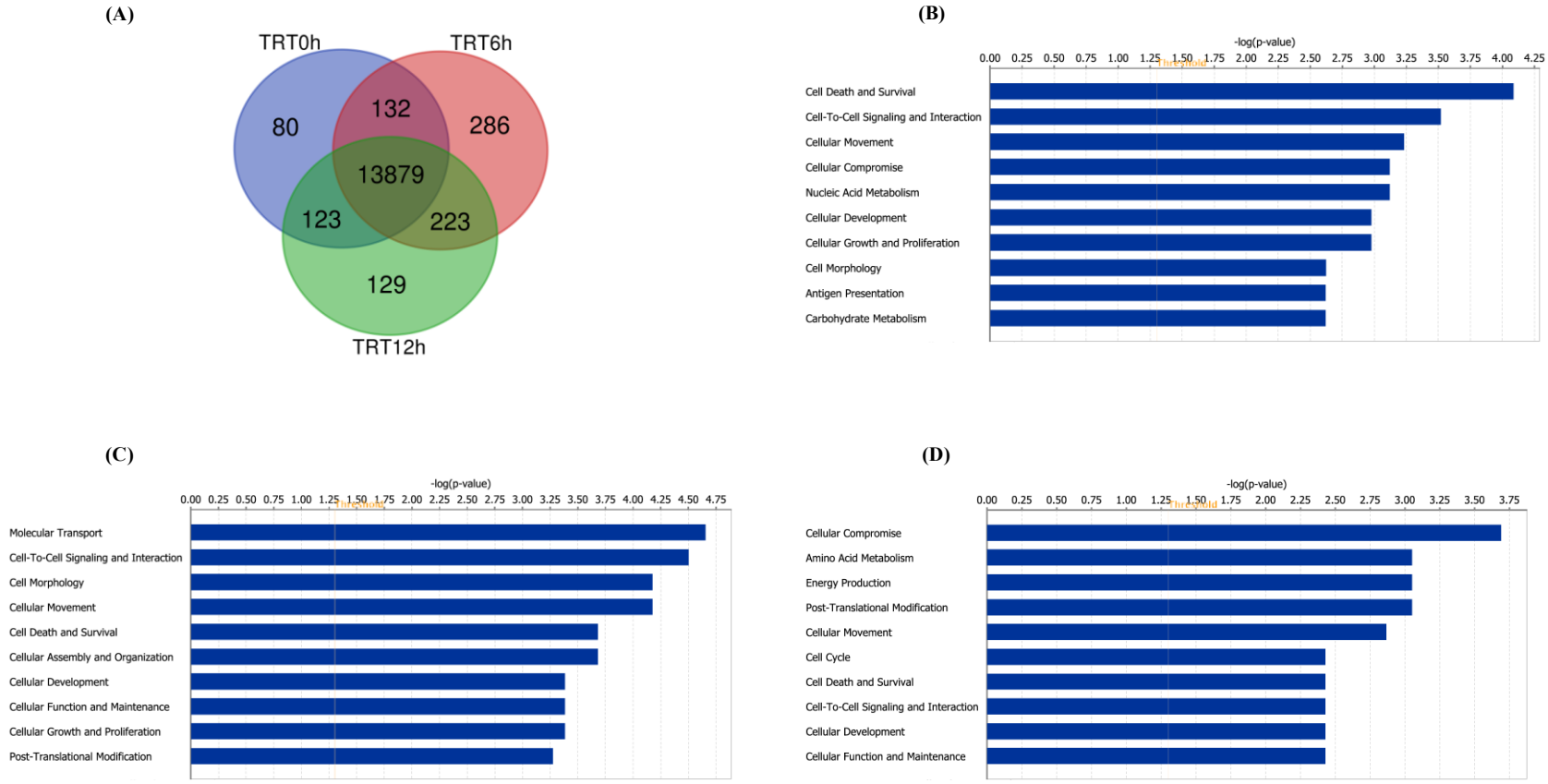


Figure 5.4 Ileal transcriptome profiles among different colostrum feeding time treatment. TRT0h means feeding colostrum within one hour after birth. TRT6h means feeding colostrum at 6 h after birth, TRT12h means feeding colostrum at 12 h after birth. (A). Venn diagram of unique and commonly expressed genes among different treatments. (B) The top 10 molecular functions enriched by the uniquely expressed genes in TRT0h treatment. (C) The top 10 molecular functions enriched by the uniquely expressed genes in TRT6h treatment. (D) The top 10 molecular functions enriched by the uniquely expressed genes in TRT12h treatment.

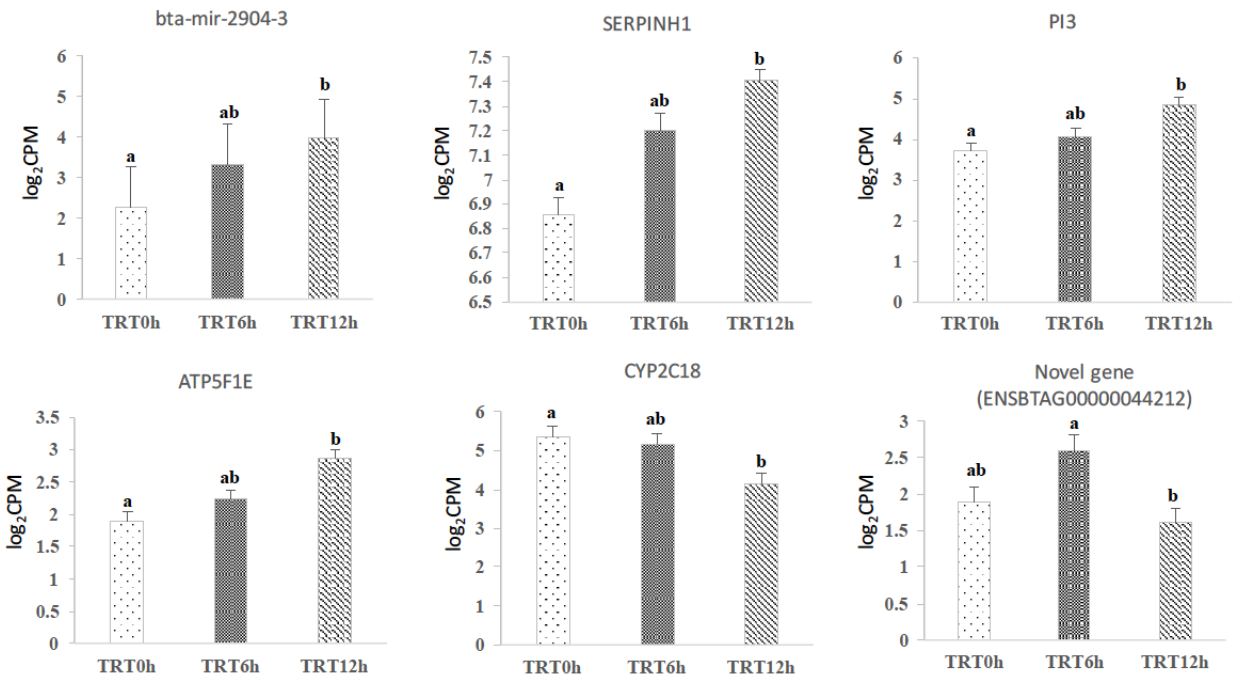


Figure 5.5 The expression level of differential expression genes among different colostrum feeding time treatments of neonatal calves. TRT0h means feeding colostrum within one hour after birth. TRT6h means feeding colostrum at 6h after birth, TRT12h means feeding colostrum at 12 h after birth, with a, b means with different superscripts are significantly different among treatment.

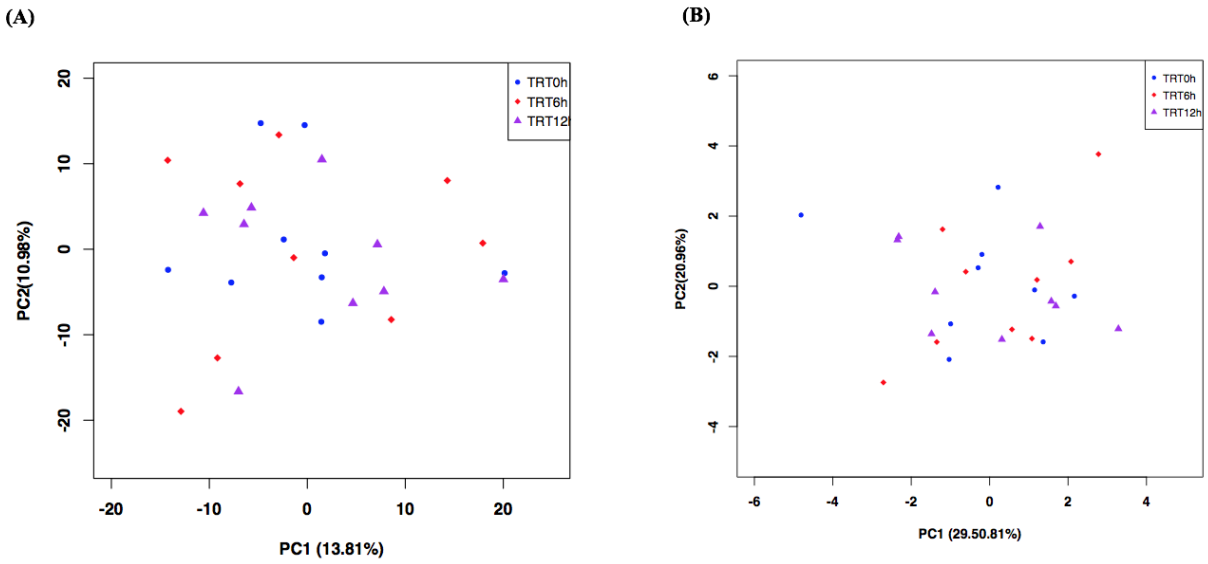
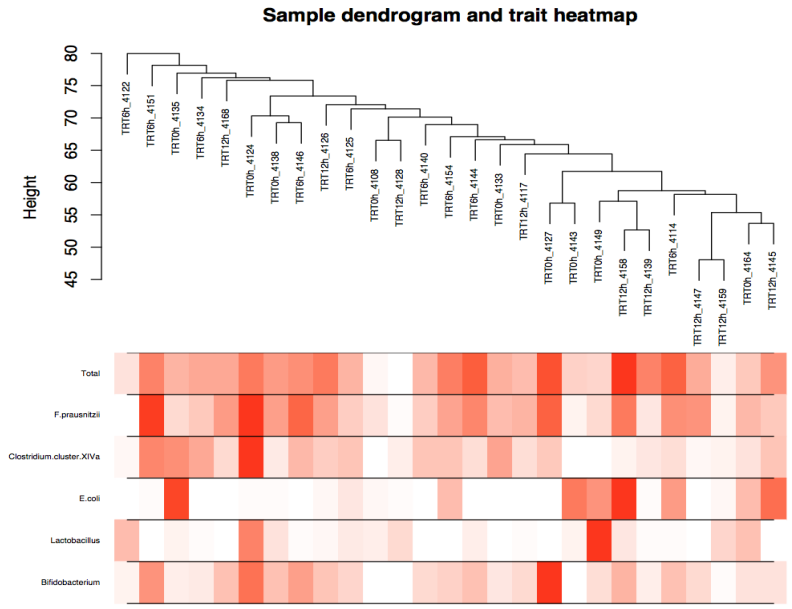


Figure 5.6 PCA plot of transcriptome profiles among different colostrum feeding time treatments. TRT0h means feeding colostrum within one hour after birth. TRT6h means feeding colostrum at 6 h after birth. TRT12h means feeding colostrum at 12 h after birth. (A) PCA plot of the immune-related genes in the ileum. (B) PCA plot of ileum barrier related genes.

(A)



(B)

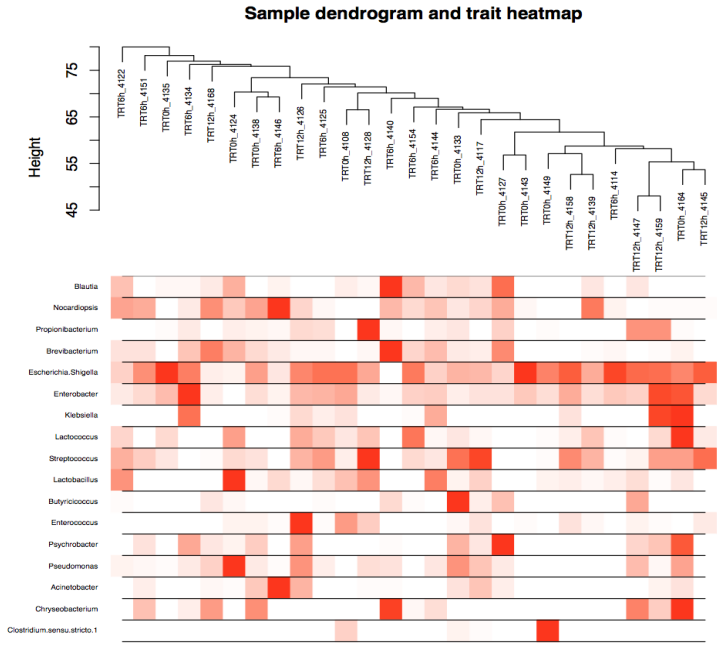
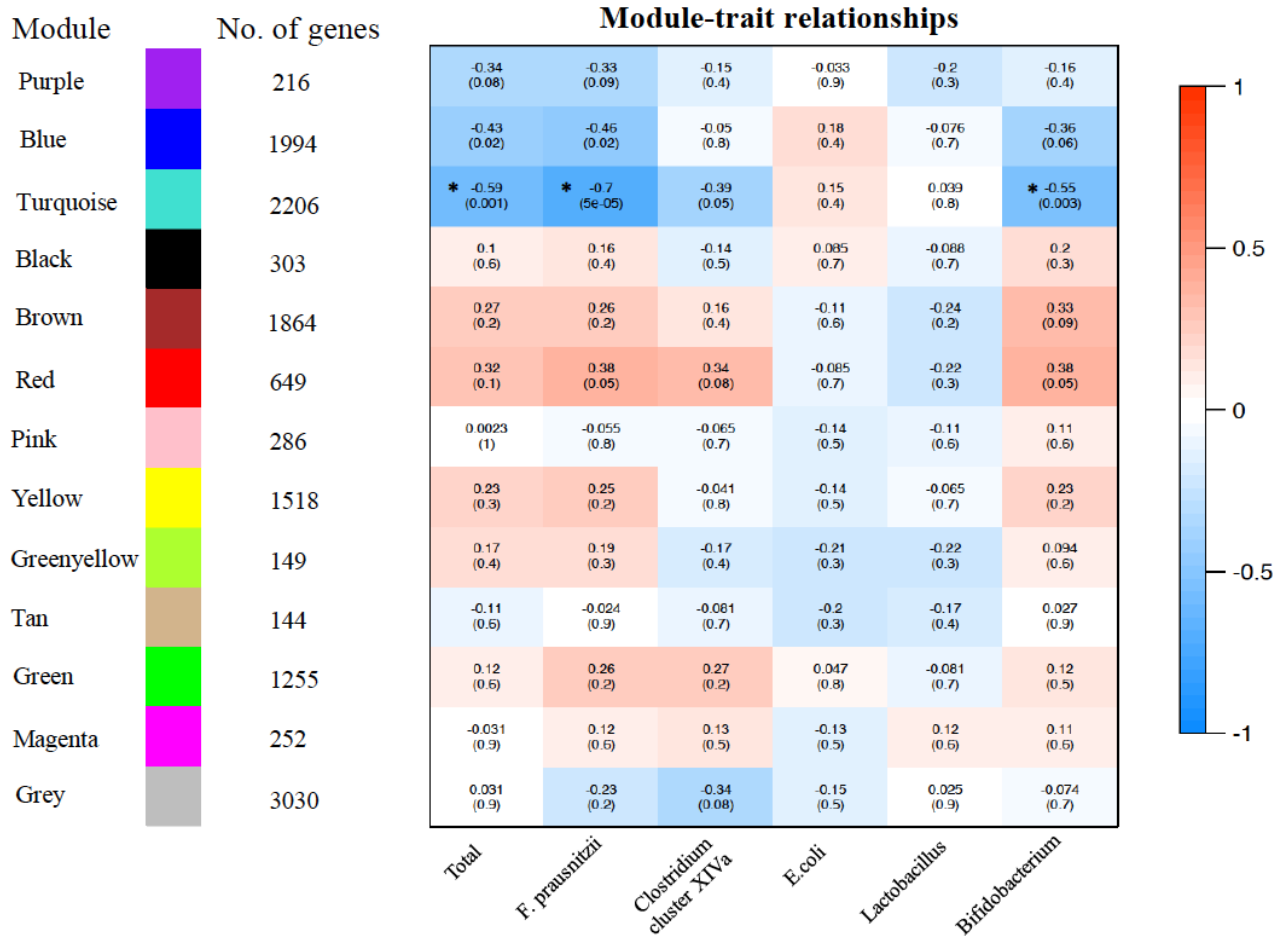


Figure 5.7 Hierarchical clustering dendrogram of ileal tissue transcriptomes (TRT0h, TRT6h and TRT12h) and trait (mucosa-attached bacteria) heat map. (A) Traits examined were the 16S rRNA gene copy number of total bacteria, *Lactobacillus*, *F. prausnitzii, E. coli*, *Bifidobacterium* and *Clostridium* cluster XIVa (B) Traits examined were the relative abundance of mucosa-attached bacterial genera (unpublished data).

(A)


Module
No. of genes
Purple 216
Blue 1994
Turquoise 2206
Black 303
Brown 1864
Red 649
Pink 286
Yellow 1518
Greenyellow 149
Tan 144
Green 1255
Magenta 252
Grey 3030
Module-trait relationships
-0.34 (0.08) -0.33 (0.09) -0.15 (0.4) -0.033 (0.9) -0.2 (0.3) -0.16 (0.4)
-0.43 (0.02) -0.46 (0.02) -0.05 (0.8) 0.18 (0.4) -0.076 (0.7) -0.36 (0.06)
* -0.59 (0.001) * -0.7 (5e-05) -0.39 (0.05) 0.15 (0.4) 0.039 (0.8) * -0.55 (0.003)
0.1 (0.6) 0.16 (0.4) -0.14 (0.5) 0.085 (0.7) -0.088 (0.7) 0.2 (0.3)
0.27 (0.2) 0.26 (0.2) 0.16 (0.4) -0.11 (0.6) -0.24 (0.2) 0.33 (0.09)
0.32 (0.1) 0.38 (0.05) 0.34 (0.08) -0.085 (0.7) -0.22 (0.3) 0.38 (0.05)
0.0023 (1) -0.055 (0.8) -0.065 (0.7) -0.14 (0.5) -0.11 (0.6) 0.11 (0.6)
0.23 (0.3) 0.25 (0.2) -0.041 (0.8) -0.14 (0.5) -0.065 (0.7) 0.23 (0.2)
0.17 (0.4) 0.19 (0.3) -0.17 (0.4) -0.21 (0.3) -0.22 (0.3) 0.094 (0.6)
-0.11 (0.6) -0.024 (0.9) -0.081 (0.7) -0.2 (0.3) -0.17 (0.4) 0.027 (0.9)
0.12 (0.6) 0.26 (0.2) 0.27 (0.2) 0.047 (0.8) -0.081 (0.7) 0.12 (0.5)
-0.031 (0.9) 0.12 (0.6) 0.13 (0.5) -0.13 (0.5) 0.12 (0.6) 0.11 (0.6)
0.031 (0.9) -0.23 (0.2) -0.34 (0.08) -0.15 (0.5) 0.025 (0.9) -0.074 (0.7)
Total
F. prausnitzii
Clostridium cluster XIVa
E.coli
Lactobacillus
Bifidobacterium
1
0.5
0
-0.5
-1

(B)

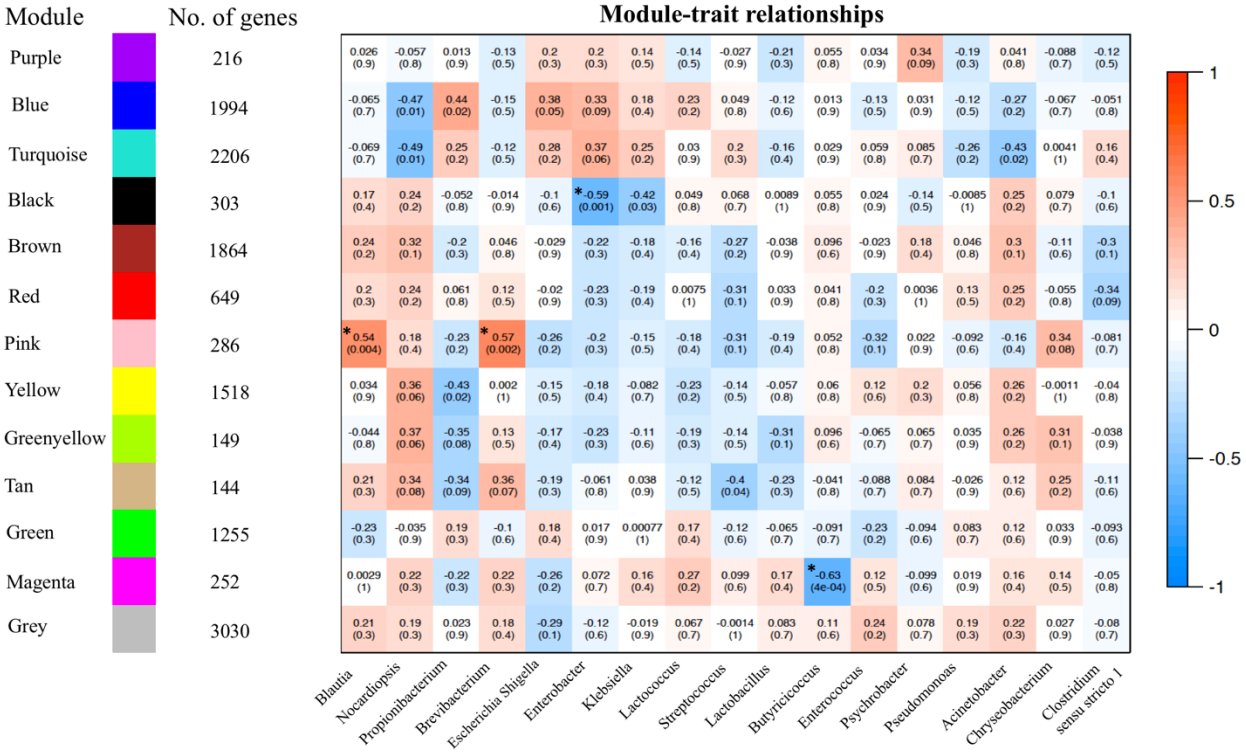


Figure 5.8 WGCNA identification of ileal gene modules correlated with mucosa-attached bacteria. The significant correlations were described with asterisks, with |r|>0.5 and p < 0.05. (A) The correlation of ileum gene modules with 16S rRNA gene copy number of mucosa-attached total bacteria, *Lactobacillus*, *Faecalibacterium prausnitzii, E. coli*, *Bifidobacterium* and *Clostridium* cluster XIVa (qpcr data) (B) The correlation of ileum gene modules with mocusa-attached bacterial genera (Miseq data).

Chapter 6. General Discussion

Maintaining the intestinal health and reducing diarrhea in neonatal dairy calves is one of the urgent tasks for dairy industry. A healthy intestinal tract encompasses effective digestion of food and absorption of nutrient, lack of gastrointestinal illness, effective immune status, status of well-being, normal and stable intestinal microbiota (Bischoff, 2011). In this thesis, the focus was on the gut microbiota during early life and its impact on host functions in neonatal calves. In total, four studies were performed to fill the knowledge gaps on the microbial composition in the hindgut of neonatal dairy calves, and the effect of colostrum management strategies on the neonatal calves' gut microbiome and host gene expression. Mainly, the hindgut microbial profiles of dairy bull calves during pre-weaning period was identified, and the effects of colostrum feeding strategies commonly used in the industry (heated colostrum feeding vs. non-heated colostrum feeding; delayed colostrum feeding vs. undelayed colostrum feeding) on small intestinal microbiome and transcriptomes.

Firstly, the establishment of hindgut microbiota of neonatal dairy calves at birth (Chapter 2), during the first 12 h after birth (Chapter 3), and pre-weaned period (Chapter 2) were identified. I found that microbiota establishment is dynamic during early life, especially at birth and during the first week of life. The first colostrum meal can significantly affect the bacterial colonization in the hindgut during the first 12 h of life, which could provide the information on what are the initial colonizers in the colon and how the early life nutrition could affect the initial colonization. Moreover, the gut microbial function were characterized and how they could be affected by different colostrum feeding strategies, highlighting the understanding of the functionality of gut microbiome is needed in addition to the microbiota taxonomic assessment. Lastly, the identified host gene expression in response to microbial shift driven by colostrum

feeding further provides the fundamental understanding on host-microbial interaction can be impacted by the first colostrum feeding strategies, and such knowledge will provide scientific bases and clues for improving calves' gut health during early life.

6.1 Advanced understanding of the hindgut microbiome in neonatal dairy calves

To my knowledge, it is the first study to report the hindgut microbiota of dairy calves at birth and its shift through pre-weaning period (Chapter 2). These results have added more information to the same calves which had their small intestinal microbiota assessed (Malmuthuge, 2016). At birth, the calf's small intestinal microbiota consisted of *Pseudomonadaceae, Propionibacteriaceae, Ruminococcaceae*, *Clostridiaceae, Bacteroidaceae* and *Lachnospiraceae* families, while *Enterobacteriaceae*, *Bacteroidaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Burkholderiaceae*, *Prevotellaceae* and *Lactobacillaceae* were the main bacterial families in their hindgut, including the cecum, colon and rectum. Such difference further highlights that to study the intestinal microbiota, regional effect should be taken into account and feces may not represent gut microbiota completely.

Intestinal microbial composition is segregated by different sample types (mucosa vs digesta) (Malmuthuge et al., 2012, Malmuthuge et al., 2015). Comparing to digesta-associated microbial community, mucosa-attached microbial community has a lower abundance, but more diverse during the first 12 h after birth in neonatal calves (Malmuthuge et al., 2015). In addition, the discrepancy on the microbial composition between these two communities was detected through the gut (rumen, jejunum, ileum, cecum, colon) in the three-weeks old neonatal calves in Malmuthuge et al. (2014). To generate a full understanding of the gut microbiota during early life and how it can be affected by the nutritional changes, it is necessary to study both

communities separately and at the same time. Therefore, findings of hindgut microbiota composition during the first 12 h (Chapter 3) and pre-weaned period (Chapter 2) were based on tissue mucosa and digesta separately, which provides more comprehensive understanding of the hindgut microbiota.

Although more and more studies on the intestinal microbiota of dairy calves during early life are emerging (Oikonomou et al., 2013; Malmuthuge et al., 2014), all studies have just focused on the composition of intestinal microbiota but the functional aspects of them are lacking. Based on my knowledge, this research is the first to explore functions of neonatal dairy calf gut microbiome based on 16S rRNA gene using Tax4Fun package (Chapter 2) and metagenomics (Chapter 4), which has provided the fundamental knowledge on the functions of hindgut microbiota including amino acid metabolism, carbohydrate metabolism, and energy metabolism. Together with the measured concentration of SCFA in the hindgut, it is evident that during early life, microbiota plays a key role in hindgut fermentation which could provide energy to the host and also affect gut immune system development. For example, butyrate has been reported to inhibit lipopolysaccharide-induced expression of proinflammatory cytokines IL-6 and IL-12p40 (Nastasi et al., 2015), and regulate T cells production and function (Arpaia et al., 2013). Overall, the outcomes of studying neonatal calves' gut microbiome provide fundamental knowledge on their taxonomic composition and functions, which are vital to study host-microbial interactions and host that can be affected by the early life nutrition.

6.2 Understanding of the effect of colostrum feeding strategy (heated colostrum vs. non-heated colostrum; delayed colostrum feeding vs. undelayed colostrum feeding) on intestinal microbiota of neonatal calves

The study of hindgut microbial profile during pre-weaning period suggests that there are dynamic microbiota colonized immediately after birth, which raises the question on what factors can affect early life microbiota colonization, especially the first feeding. From chapters 3 to 5, I focused on studying how the colostrum feeding strategies influence gut microbiota and host function.

Considering the role of hindgut microbiota in host health, metabolism of neonatal calves (Chapter 2), the effected of non-heated colostrum and heated colostrum on its shift was examined (Chapter 3). Both mucosa and digesta-associated large intestinal bacterial profiles were not affected by different colostrum treatments (no colostrum, non-heated colostrum and heated colostrum), which may be due to the short-term of surviving time of neonatal calves (euthanized at 6 h or 12 h after birth) after colostrum treatment, that colonic microbes were not completely affected by the different colostrum feeding. Meanwhile, the findings from chapter 3 suggests that heated colostrum (60℃ for 60 min) enhanced the colonization of beneficial bacteria and inhibited the colonization of opportunistic pathogenic bacteria in the hindgut during the first 12 h of life in dairy calves. The increased relative abundance of *Bifidobacterium* in the colon after feeding heated colostrum could be due to higher amount of oligosaccharides, which promotes beneficial bacteria colonization (Zivkovic and Barile, 2011). Furthermore, colostrum IgG level was not affected by the heat treatment (FC - 74.49±7.9 g/L and HC - 69.05±8.6 g/L) (Kent-Dennis, 2014). Therefore, our findings suggest that heated colostrum has more advanced than non-heat treated colostrum from gut microbiota point of view. In addition to colostrum

oligosaccharide and IgG, other bioactive components, such as lactoferrin, IgA, EGF, GH should be studied about their effects on colon microbiota.

In chapter 4, I further studied how delayed colostrum feeding affected intestinal microbiota. Furthermore, the previous two studies (Chapters 2 and 3) only revealed the taxonomic changes of microbiota, and such changes may not be related to the functional shifts. Delayed colostrum feeding affected serum IgG concentration and the initial colonization of several bacterial groups, such as *Escherichia coli*, *Lactobacillus* and *Bifidobacterium* (Fischer et al., 2018), who used the samples originated from the same animal study). However, this study only focused on five bacterial groups, and how the delayed colostrum feeding affected the whole microbial community composition and function was not studied. My research (Chapter 4) studied the changes in the whole microbial community and the functions in the ileum in response to the delay of colostrum feeding for 6 and 12 hours, respectively. Surprisingly, no difference was found for the entire microbial composition and function among different treatments. The subtle differences on microbial taxonomy and function among treatments may be related to the high individual variation in the ileal microbiome of neonatal calves at birth as the previous study mentioned above (Malmuthuge et al., 2016), hence increasing the number of animals per treatment are encouraged when studying early life microbiota of neonatal calves. Another reason contributing to the subtle difference among different colostrum feeding time treatment may be related to the sequencing method used in the study. Although metagenomics is an useful tool for studying microbial taxonomy and functions, it is based on DNA, which could not distinguish if the microbes are alive and active (Gaidos et al., 2010). Additionally, the short-term period of living (51 h after birth) before sacrifice may be another reason contributing to the subtle difference. During the first 51 h of life, the microbiota may still in the process to adapt to the

intestinal environment (e.g., pH and oxygen), and the stable microbiota may not be established. Therefore, reducing the individual variation of neonatal calves and extending the period of the experiment are suggested to identify whether the delay in colostrum feeding can affect the ileal microbiome.

6.3 Understanding of the effect of delayed colostrum feeding strategy on ileal transcriptome profiles and host-microbiota interactions

A well-developed intestine during early life is a determinant factor for the health of neonatal calves. Ileum has been reported to be an important intestinal region in mucosal immune and gut barrier functional development (Liang et al., 2016), as well as a favorable region for pathogenic bacteria colonization (Moxley and Francis, 1986, Tsolis et al., 1999) in neonatal calves. Hence, the ileum was chosen as the research target in the chapter 5. Previous studies indicated that nutrition and microbes influence intestinal gene expression in neonatal calves (Blum and Hammon, 2000, Yang et al., 2015), however, no research has performed to study the effect of colostrum feeding strategy (delayed colostrum feeding) on small intestinal transcriptome. To my knowledge, the research presented in chapter 5 is the first to explore the relationship between delaying colostrum feeding and intestinal genome wide transcriptomes. We found that the enriched physiological functions were closely related to immune system development, including lymphoid tissue structure and development, cell-mediated immune response, immune cells trafficking and humoral immune response in the ileum of the two-days old calf, which is in accordance with previous finding (Liang et al., 2016), suggesting that immune system development is crucial to the neonatal calves, especially during early life. In addition, the outcomes suggest that feeding colostrum within one hour after birth stimulated

innate immune system development. Moreover, significant correlations between mucosa-attached bacteria and host ileal tissue gene modules (with enriched functions related to host immune system, energy metabolism and intestine barrier development) were detected, highlighting that manipulation of intestinal microbiota may be able to fortify the intestinal immune system response during early life.

6.4 Limitations and future directions

In chapter 2, the limitations are that calf starter intake, as well as the nutritional composition of the diet is not recorded. It is known that diet can affect gut microbiota (De Filippo et al., 2010), therefore, future studies should measure the calf feed intake for more precise assessment of the shifts in hindgut microbiota. Meanwhile, in chapters 4 and 5, the animal experiment was conducted in the small-scale research dairy farm, and the collection of samples from all the animals lasted for eight months (from February, 2018 to September 2018), which means that the animals involved in the experiment experienced four seasons. The animals euthanized in winter probably had different microbial profile compared with animals sacrificed in summer, considering the effect of seasonal and dietary effect on human intestinal microbiota composition (Davenport et al., 2014). Moreover, several people participated in the animal trial and sample collection, which could be another factor that affects the results due to the inconsistency in sample collection process. Therefore, reduction of the sampling effect on the microbial profile of the neonatal calves, and using animals in a large-scale farm are encouraged in the future.

In chapters 4 and 5, we did not find much difference in microbial taxonomy and function, as well as host transcriptome profiles under different colostrum feeding time treatment. Large

individual variation among individual animals have been observed that could be due to the difference in host genetics and may have led to the non-statistical significance. To reduce the individual variation, cloning technology or twins could be used to reduce the genetic variation. Moreover, the short surviving time of the calves through thesis work, could be another limitation since this does not allow us to detect long-term effect. Since the establishment of the first stable microbial community probably needs more than 7 days (Arrieta et al., 2014), extending the period for the experiment should be considered in the future studies.

For the ileal transcriptome profiles (Chapter 5), quantitative real-time PCR should be performed to validate the differential expressed genes. In addition, further experiments on investigating the effect of direct fed microbes to intestinal immune and gut barrier development of the neonatal calves should be performed to verify the significant correlations found among bacterial groups, such as *Bifidobacterium*, *F. prausnitzii* and the gene modules that are enriched with immune and gut barrier related functions (Chapter 5).

6.5 Implications

The identified mucosa and digesta-attached microbial community in the hindgut from birth to pre-weaning, allows us to have a deeper understanding of the whole gut microbial community in the neonatal calves during early life. The similarity of the microbial community among different hindgut regions (cecum, colon and rectum) suggests that the hindgut microbiota could be studied as a whole or any of them can be representative for hindgut microbiota. Additionally, this is the first study to highlight that the changes of SCFAs concentration in the gut can influence mucosa-attached microbiota during pre-weaning stage. Additionally, negative correlation was detected between SCFAs concentration and opportunistic pathogens, suggesting

the increase of SCFAs may inhibit pathogens (e.g., *E. coli*) colonization. The finding indicated the importance of microbial fermentation products SCFAs in microbial colonization and intestinal health of the neonatal calves. Understanding the hindgut microbial profile is fundamental for early life nutritional intervention and disease challenge studies in the future.

In addition to the novel scientific knowledge on the neonatal claves' microbiota, the findings from this thesis can also be applied to the dairy industry. It was implied that heated colostrum enhanced the beneficial bacteria (*Bifidobacterium* and *Clostridium* cluster XIVa) colonization and inhibited the colonization of opportunistic pathogenic bacteria (*E. coli*) in the colon during the first 12 h of life. Delaying colostrum feeding to 12 h may lead to more stress and postponed immune system development of the calves. Therefore, our studies suggest that two colostrum management strategies, including feeding heated colostrum (60 ℃ for 60 min) and feeding colostrum earlier (within one hour) after birth are encouraged to be used in the industry. Additionally, the significant correlations between *Bifidobacterium*, *F. prausnitzii* and the gene modules that are enriched with immune system and gut barrier development related functions suggest that microbial manipulation (e.g., supplement of *Bifidobacterium*, *Colostridium* spp. in the milk replacer) during early life improve the gut health of neonatal calves.

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