Transcriptomic changes of the gut in dairy calves during pre-weaned period

by

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Abstract

Maintenance of the gut health of calves is vital because enteric infections are associated with high mortality during the pre-weaned period. The small intestine is the primary site of many enteric infections and plays an important role in protecting the host from pathogenic infection through both barrier and mucosal immune functions. However, the molecular mechanisms regulating small intestine development in the pre-weaned calf have not been well characterized. Four studies (Chapters 2, 3, 4 and 5) were performed to investigate the expression profiles of protein-coding genes and microRNAs (miRNAs) in gut tissues of pre-weaned calves, identify their changes in response to gut microbial colonization and enteric infection, and elucidate potential mechanisms of the small intestinal development in the pre-weaned calves. The expression of miRNAs in the gastrointestinal tract had dynamic changes during the pre-weaned period, and their functions were related to the development of intestinal mucosal immune system Moreover, significant associations between miRNA expression and microbial populations in the small intestine provided evidences that miRNAs were involved in mediating host-microbial interactions. Further transcriptomic analysis revealed higher expression levels of genes involved in complement functional pathway, tight junction protein, and IgA complex in the jejunum than those in the ileum, suggesting the roles of the jejunum in the immune and barrier functions in pre-weaned calves. In addition, during the first week after birth, the temporal expression pattern of tight junction protein genes, antimicrobial peptide

genes, NOD-like receptor genes, a regulatory T cell marker gene, and cytokine genes suggested that this is a critical developmental period for intestinal mucosal immune function. An ileal loop model was used to target *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection to the ileal region of the small intestine. Differentially expressed miRNAs and alternatively spliced genes in the infected versus uninfected ileal segment revealed significant changes in endothelial cell proliferation, macrophage maturation and lysosome function as possible mechanisms by which MAP escapes host immune responses. Finally, the same surgical model was used to analyze changes in miRNA expression when the intestinal microbiota was altered by treatment with a dose-dependent exposure to allicin, an antimicrobial compound present in garlic. The observed changes in miRNA expression and their predicted function in lymphocytes development following of the changes in gut bacterial population changes after antimicrobial treatment further supported the conclusion that miRNA expression was associated with microbial colonization and miRNA played a role in regulating the intestinal mucosal immune system of pre-weaned calves. In summary, the transcriptomic analysis revealed that the protein-coding genes and miRNAs are involved in regulating diverse aspects of small intestinal development that are age-dependent and vary among regions of the small intestine. Of particular importance were their functions in the immune system, which were associated with microbial colonization during early life.

Preface

This thesis is an original work by Guanxiang Liang and has been written according to the guidelines for a paper format thesis of the Faculty of Graduate Studies and Research at the University of Alberta. The concept of this thesis originated from Guanxiang Liang's supervisor Dr. Leluo Guan and the research is part of a project founded by Alberta Livestock and Meat Agency Ltd. (ALMA: 2011F129R) and NSERC Discovery grant. All the experimental protocols (Chapters 2 and 3) were reviewed and approved by the Livestock Animal Care committee of the University of Alberta (protocol no. AUP00001012) and all procedures were conducted following the guidelines of Canadian Council on Animal Care. The experimental protocols of the animal studies in Chapter 4 and 5 were reviewed and approved by University of Saskatchewan-University Committee on Animal Care and Supply and all procedures were performed following guidelines approved by the Canadian Council on Animal Care.

The animal studies performed in Chapter 4 and Chapter 5 were completed in collaboration with Dr. P. J. Griebel's group at the University of Saskatchewan.

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contributed to the draft writing on “gut microbiota” section. L. L. Guan and P.J. Griebel were the supervisory authors and wrote the conclusion section.

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Dedication

Dedicated to my beloved family

献给我深爱的家人

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List of abbreviations

ADA: adenosine deaminase

AGO: Argonaute

AMP: Antimicrobial peptide

C3: Complement component 3

CD3: Cluster of differentiation 3

CDH13: Cadherin 13

CLDN: Claudin

CPM: Counts per million reads

Cq: Quantification cycle

DAVID: Database for Annotation, Visualization and Integrated Discovery

DC: Dendritic cell

DDAH1: Dimethylarginine dimethylaminohydrolase 1

DE: Differentially expressed

DEFB: β - defensin

EXP5: Exportin 5

FDR: False discovery rate

FOXP3: Forkhead box P3

FPM: Fragments per million mapped fragments

GIT: Gastrointestinal tract

HOXA5: Homeobox A5

HOXA9: Homeobox A9

HSC: Hematopoietic stem cell

IFN γ: Interferon γ

IGJ: Immunoglobulin J chain

IL: Ileum

IL10: Interleukin 10

IL4R: Interleukin 4 receptor

IPA: Ingenuity pathway analysis

IRAK1: Interleukin-1 receptor-associated kinase 1

ITGA1: Integrin alpha 1

JAM: Junctional adhesion molecule

JD: Johne's disease

JE: Jejunum

KEGG: Kyoto Encyclopedia of Genes and Genomes

lncRNA: Long non-coding RNA

LPS: Lipopolysaccharide

MAMP: Microbial-associated molecular pattern

MAP: Mycobacterium avium subspecies paratuberculosis

MHC Class I: Class I major histocompatibility complex

miRNA: microRNA

MLN: Mesenteric lymph node

MMD: macrophage differentiation-associated

NK cell: Natural killer cell

NLR: NOD-like receptor

OCLN: Occludin

PCA: Principal component analysis

PCR: Polymerase chain reaction

pHLIP: pH-induced transmembrane structure

PIGR: Polymeric immunoglobulin receptor

PP: Peyer's patch

Pre-mRNA: mRNA precursor

Pri-miRNA: Primary miRNA

PSI: Percentage spliced index

REG3 γ: Regenerating islet-derived 3 γ

RISC: RNA-induced silencing complex

RNAP: RNA polymerase

rRNA: Ribosomal RNA

RT-qPCR: Reverse transcription quantitative PCR

RUNX1: Runt-related transcription factor 1

SCFA: Short-chain fatty acid

SEB: Segmented filamentous bacteria

SEEMA6B: Semaphorin 6B

snoRNA: Small nucleolar RNA

snRNA: Small nuclear RNA

snRNP: Ribonucleoprotein

SPARC: Secreted protein, acidic, cysteine-rich

SYK: Spleen tyrosine kinase

Th17: T helper 17 cell

Th2: T helper 2 cell

TJ: Tight junction

TLR: Toll-like receptor

TRAF6: Tumor necrosis factor receptor-associated factor 6

Treg: Regulatory T cell

tRNAs: Transfer RNA

UTR: Untranslated region

VIP: Vasoactive intestinal peptide

ZO: Zonula occluden

Chapter 1. Literature review

1.1 Gut health and the industry problems of dairy calves

The health of calves has a significant effect on the economic viability of the beef and dairy industries, due to the direct costs of calf losses, treatment and the long-term impact on cattle performance (Donovan et al., 1998). It has been reported that 2.3 million calves (6.7% of total calf crop) were lost in 2010 in the United States (USDA, 2010). Among them, the digestive (17.2% of total calf losses) and respiratory problems (29.1% of total calf losses) have been reported to be the two main causes of non-predator calf losses (USDA, 2010). However, when it comes to the calf losses only within the dairy operations, digestive problems represented 30.4% of the total losses (USDA, 2010), indicating that digestive problems have serious influence on the health of dairy calves.

The digestive problems in the calf include ruminal and intestinal diseases, such as bloat, which occurs in the rumen (Jayne-Williams, 1979), and diarrhea, which is usually caused by enteric infection in the intestinal tract (Cho and Yoon, 2014). Diarrhea is one of the major digestive problems in the calf, which has been recognized worldwide as one of the biggest challenges for the dairy industry. The United States Department of Agriculture (USDA) reported that 57% of weaning calf mortality of dairy cattle in the United States was due to diarrhea (USDA, 2007). Besides, high mortality rates for dairy calves due to calf diarrhea has also been reported in many other countries all over the world, such as Korea (Cho and

Yoon, 2014), Norway (Osteras et al., 2007), Ireland (Lorenz et al., 2011), Spain (de la Fuente et al., 1999), and New Zealand (Al Mawly et al., 2015).

Calf diarrhea is commonly reported during the pre-weaned period (newborn to 5-6 weeks) (Cho and Yoon, 2014), and is mainly caused by the infection of enteric pathogens, such as bovine rotavirus, *Salmonella enterica*, *Escherichia coli*, and so on (Cho and Yoon, 2014). Take Typhimurium as an example, calves less than 3 weeks of age are commonly infected (Cho and Yoon, 2014), and the bacteria invade the intestinal epithelium at the terminal small intestine, resulting in exfoliation of epithelial cells and stunting of intestinal villi (Frost et al., 1997). Calves infected experimentally with Typhimurium can develop diarrhea within 48 hours (Rankin and Taylor, 1966).

In addition, some pathogens such as *Mycobacterium avium* subspecies *paratuberculosis* (MAP) may not cause calf diarrhea immediately, but still exert severe impacts on cattle health. MAP always infects the terminal small intestine of pre-weaned calves, and may cause Johne's disease after a long asymptomatic period (Arsenault et al., 2014). Johne's disease is a fatal intestinal disease with clinical signs of rapid weight loss and diarrhea, causing significant economic losses to the worldwide livestock industry. The estimated economic loss due to Johne's disease in the United States cattle industry is between US$200 – 250 million per year (Tiwari et al., 2008).

Collectively, pre-weaned calves are susceptible to a variety of bacterial and viral enteric infections, and the small intestine is always the primary infection site. Although dairy producers have made great improvements with herd

management, animal facilities and care, feeding and nutrition, as well as utility of antibiotics, calf gut problems are still a challenge for the dairy industry.

1.2 Factors that influence gut health and development in calves

The factors affecting gut health and development in young animals can be mainly divided into environmental factors and host factors (Thomson and Keelan, 1986). Two major environmental factors including dietary factors and microbial colonization have been studied in different species of animals. The dietary factor plays a vital role in gut health and development of pre-weaned calves. The colostrum or milk materials are crucial for gut health during the pre-weaned period (Chucri et al., 2010). Maternal antibodies that are contained in the bovine colostrum have the capacity to trigger the development of the immune system in pre-weaned calves within the first 24 hours after birth (Chucri et al., 2010). A recent study showed that solid feed consumption during the pre-weaned stage altered the mRNA expression levels of Toll-like receptors (TLRs), β-defensin, claudin 4, and occludin, which may be related to the intestinal barrier and mucosal immune system to prevent enteric infections (Malmuthuge et al., 2013). The impact of microbial colonization on the gut health and development in neonatal animals will be discussed in the following section.

Genetics is one of the major host factors, which is the information contained in the DNA molecules and controls the biology of specific tissues and cells, affecting gut health in cattle. For example, polymorphisms found in the gene encoding bovine interleukin 10-receptor alpha are associated with the

susceptibility to MAP infection in the gut of cattle (Verschoor et al., 2010). The gene expression process can pass genetic information to functional gene products, such as non-protein coding transcripts and proteins, which can directly affect the animal phenotypes (de Souza, 2013). The altered expression of genes in the gut of neonatal animal, such as the increase of interleukin 10 and TLR genes have been reported to induce the development of intestine mucosal immune system in neonatal mice (Renz et al., 2012). Therefore, gene expression is one of the host factors that affect the gut health. To date, limited studies explored gene expression in the gut of ruminants during the pre-weaned period.

It is worth noting that the above factors do not affect gut development individually; instead, they have close interactions with each other. For instance, switching from a low-fat diet to a high-fat diet in mice resulted in a significant alteration of the composition of microbiota, with an overgrowth of Firmicutes, as well as a reduction of Bacteroides (Turnbaugh et al., 2009). The higher efficiency of Firmicutes in energy harvesting subsequently increased the adiposity in the mice fed the high-fat diet (Brown et al., 2012). A recent study showed that feeding newborn calves heated colostrum can increase the colonization of *Bifidobacterium* spp. in the small intestine (Malmuthuge et al., 2015), and the high density of *Bifidobacterium* spp. can protect the host from pathogens by competitive exclusion and also by the production of antibacterial materials (Matsuzaki and Chin, 2000). These suggest that the diet and the microbial colonization interact with each other, posing impacts on host health afterward. The host-microbial interaction will be reviewed in the following section.

1.3 Host-microbial interaction and gut health

1.3.1 The anatomy of bovine small intestine

The small intestine, which is a specialized tubular structure within the abdominal cavity in continuity with the stomach proximally and the colon distally, plays a role in absorption of nutrients as well as in barrier and immune function to maintain intestinal homeostasis (Kararli, 1995; Santaolalla and Abreu, 2012). In adult cattle, the small intestine normally reaches 40 meters in length and 5 centimeters in width (Umphrey and Staples, 1992). The small intestine is composed of three parts: duodenum, jejunum and ileum (Umphrey and Staples, 1992). The duodenum is the first and shortest section of the small intestine, and is continuous with the abomasum in cattle (Frandson et al., 2009). The jejunum is the midsection and the longest part of the small intestine and is highly coiled. The jejunum is separated from duodenum by the duodenojejunal flexure; however, the separation between the jejunum and ileum is not clear (Frandson et al., 2009). The ileum is the terminal portion of the small intestine and continues from the jejunum, and terminates at the ileocecocolic junction (Frandson et al., 2009).

The wall of the small intestine consists of four layers: mucosa, submucosa, muscularis, and serosa:

Mucosa: The mucosa is the innermost layer of the intestinal tract, and it is directly exposed to the digesta and microbes. It is formed by the epithelium, lamina propria, and muscularis mucosae (Frandson et al., 2009). The luminal surface of the small intestine is covered by villi, which are the extensions of the lamina propria and covered by epithelium (Frandson et al., 2009). The epithelium

of the small intestine mainly contains four cell types: enterocyte (absorptive cell), goblet cell, Paneth cell and enteroendocrine cell (Samuelson, 2007). In addition to these four cell types, intraepithelial cell, stem cell, microfold cell and some other cells have also been observed in the epithelium (Samuelson, 2007). The lamina propria, which supports the epithelium, is a layer of reticular connective tissue. The organized lymphoid tissues, Peyer's patches (PPs), are located in the lamina propria of the mucosa layer in the small intestine, which play vital roles in immune defense (Samuelson, 2007). The muscularis mucosae are composed of a thin layer of smooth muscle at the boundary of the mucosa and submucosa (Frandson et al., 2009).

Submucosa: The submucosa is a fibrous connective tissue layer that contains fibroblasts, mast cells, blood and lymphatic vessels (Samuelson, 2007).

Muscularis: The muscularis, which is responsible for contractility, consists of two layers of smooth muscle: an inner circular coat and an outer longitudinal coat. A prominent nerve fiber plexus called the myenteric plexus is located between these two muscle layers (Frandson et al., 2009).

Serosa: The serosa is the outermost layer of connective tissue (Frandson et al., 2009).

1.3.2 Microbial colonization in gut during early life

The mammal's intestinal tract harbors a diverse and complex microbial community, which is crucial for animal health. It has been estimated that the human gut contains approximately 1000 bacterial species and 100-fold more microbial genes than are found in the human genome (Qin et al., 2010). Bacteria

account for the largest group of the intestinal microbiota, and the Bacteroidetes and Firmicutes are the dominated phyla in different species of mammals (human, mouse, cattle and so on) (Malmuthuge et al., 2014; Nguyen et al., 2015). The density and composition of the gut microbiota stay relatively stable throughout adult life, but can be altered as a result of diet change, antibiotic treatment, and some other environmental factors (Rodriguez et al., 2015).

It is widely accepted that microbial colonization starts during and after birth (Rodriguez et al., 2015). The microbial colonization of the infant gut is known to play a key role on immune function and metabolic pathways of the host. Disruptions of gut microbial colonization have been shown to increase gut disease susceptibility (Fujimura et al., 2010). For example, treatment of mice with antibiotics increased the susceptibility of the intestine to *Salmonella serovar* infection (Lawley et al., 2008). Therefore, the fully established and well maintained gut microbiota throughout life are important for the host health.

Microbial fermentation in the rumen converts fiber materials to short-chain fatty acids (SCFAs), which can provide the host with up to 70% of the total dietary energy (Flint and Bayer, 2008). It has been shown that the rumen microbiota can be affected by diet, antibiotic treatment, farm environment and the age of the host (Olumeyan et al., 1986; Jami and Mizrahi, 2012; Li et al., 2012), and the variation of the rumen microbial ecology may be associated with host feed efficiency and methane emission in cattle (Zhou et al., 2009, 2010). The establishment of the bacterial community in the rumen of calves during early life has been studied. Li et al. (2012b) compared the ruminal microbial communities

of 14-day-old pre-weaned calves and 42-day-old pre-weaned calves, reporting remarkable phylogenetic composition changes (for example, the proportion of Bacteroides increased from 18.08 ± 14.76% (14-day-old) to 71.38 ± 15.15% (42-day-old)) and revealing the existence of bacterial functions (such as fiber digesting function) similar as those of the adult cattle rumen.

Another recent study profiled the bacterial composition of rumen in 1-day-old, 3-day-old and 2-month-old pre-weaned calves, as well as 6-month-old and 2-year-old cows (Jami et al., 2013). This study suggested that the bacterial colonization underwent dramatic and rapid changes involving an increase in the number of anaerobic genera from day 1 to day 3. In addition, the fiber digesting bacterial genes can be detected as early as 1 day after birth (Jami et al., 2013). Moreover, calves or cows that fed with the same diet but at different ages (1-day-old and 3-day-old calves had the same diet; 6-month-old and 2-year-old cows had the same diet) showed distinct bacterial communities, indicating that the rumen bacterial composition can be affected not only by diet but also by host's age (Jami et al., 2013).

Compared to the rumen microbiota, the studies on small intestinal microbiota in cattle, especially in pre-weaned calves, are still limited. A recent study revealed the bacterial prevalence and composition was significantly different among the gastrointestinal tract regions and between mucosa- and digesta-associated microbiota in 3-week-old pre-weaned calves (Malmuthuge et al., 2014). However, the establishment of microbial communities and their

functions on host gut development during the early life of calves are not well studied.

1.3.3 Influence of gut microbiota on host function

The composition and activity of the gut microbiota co-develop with the host from birth. It is important to establish and maintain the beneficial interactions between the host and the microbiota, subsequently to keep the host healthy. The host-microbial interaction can mainly benefit gut health in two functions: metabolic function and immune function (Hooper et al., 2012; Nicholson et al., 2012).

The host gut provides a home and nutrients for the microbes to ferment indigestible carbohydrate to SCFAs (den Besten et al., 2013), mainly acetate, propionate and butyrate. The microbes obtain energy for themselves via this process; meanwhile, the host can absorb SCFAs as nutrients (den Besten et al., 2013). It has been shown that SCFAs have multiple beneficial effects on host energy metabolism (Musso et al., 2011). For example, butyrate can be directly absorbed by colon epithelial cells, which can produce energy in humans (Karaki et al., 2008). In addition, butyrate can promote leptin production in mouse adipocytes (Samuel et al., 2008), and leptin can inhibit food intake and/or regulate energy expenditure to maintain energy homeostasis for the host (Minokoshi et al., 2002). The relationship between gut microbiota and animal metabolic disorders, such as obesity in humans, has been proposed, and the alteration in SCFA concentration is considered to be one of the causes (DiBaise et al., 2012).

The interactions between microbiota and the host immune system begin soon after microbial colonization (Hooper et al., 2012). The microbiota is able to shape the development of the host immune system. Firstly, defects in the development of lymphoid tissues and immune responses have been observed in germ-free mice (Round and Mazmanian, 2009). It has been shown that germ-free mice have fewer and smaller PPs and mesenteric lymph nodes (MLNs) compared with specific pathogen free mice (Round and Mazmanian, 2009). Less secretory IgA production, fewer $CD4^+$ and $CD8^+$ T cells have also been observed in the gut of germ-free mice, suggesting an impaired immune response in germ-free mice (Macpherson and Harris, 2004). Secondly, colonization of bacteria in germ-free mice is able to partially restore the immune function of germ-free mice. For example, colonization of segmented filamentous bacteria (SFB) in the gut can enhance the differentiation or function of T helper 17 (Th17) cells and Regulatory T (Treg) cells in germ-free mice gut, which are important components of the host immune system (Weaver and Hatton, 2009). Transplantation of microbiota from conventionally raised mice to germ-free mice is able to stimulate the release of regenerating islet-derived 3 γ (REG3 γ) by intestinal epithelial cells (Cash et al., 2006). Besides, colonization of a mix of culturable commensal in germ-free mice enhances the production of secretory IgA (Macpherson and Uhr, 2004). Therefore, according to the studies on germ-free mice, microbial colonization promotes the development of immune organs and cells, and maintains the immune responses of the mucosal immune system.

The impacts of microbial colonization in the gut of calves are still not well studied. A previous study reported that the mRNA expression of TLRs, which can recognize microbe-associated molecular patterns and activate host immune responses, revealed regional and temporal difference between 3-week-old pre-weaned calves and 6-month-old weaned calves (Malmuthuge et al., 2012). In addition, strong correlation has been observed between the expression of TLRs and the density of total bacteria and/or *Lactobacillus* spp., suggesting the interaction between TLR gene expression and the microbial community (Malmuthuge et al., 2012). However, the establishment of microbial colonization during the pre-weaned period in calves, and how they affect the host immune system are not well characterized.

1.4 Transcriptomic analysis of gut tissues in calves

Although host-microbial interactions are important, their regulatory molecular mechanisms are not well understood, especially during the early life of ruminants. Recent advanced molecular based technologies have allowed studying the whole transcriptome, which is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding tissue development and disease.

1.4.1 Cell transcriptome

Transcriptomics is the study of the transcriptome, which is the collection of all the RNA molecules (or transcripts) present in the cell (Hoeijmakers et al., 2013). The transcriptome is the product of transcription, which is the first step of

gene expression transcribing the DNA sequence information into RNA molecules, performed by RNA polymerase and transcription factors (Hoeijmakers et al., 2013). All the transcripts can be mainly divided into two types: protein-coding RNAs and non-coding RNAs (Pertea, 2012).

Protein-coding RNAs, or messenger RNAs (mRNAs), are the transcripts of protein-coding genes (Hoeijmakers et al., 2013). Proteins are the main molecules that directly transform the sequence information stored in the DNA to phenotype within the living organisms via a variety of functions, such as structural functions, catalyzing chemical reactions, cell signaling and so on (Rutherford, 2003).

Non-coding RNAs, are the transcripts that will not be translated into protein. It has been shown that they also have essential functions for cell (Morris and Mattick, 2014). Ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) are the two major types of non-coding RNAs (Morris and Mattick, 2014): the rRNAs, being the main component of ribosome, are the most abundant RNAs (Noller, 1991); while the tRNAs transfer the amino acids to the ribosome. Both rRNAs and tRNAs are important for protein synthesis (Ikemura, 1985). In addition, more and more regulatory non-coding RNAs have been found, such as long non-coding RNAs (lncRNA), microRNAs (miRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and so on (Morris and Mattick, 2014). These regulatory non-coding RNAs have been proven to be involved in different biological processes such as cell differentiation and development, metabolism, proliferation, apoptotic cell death, and so on (Morris and Mattick, 2014).

Analyzing the tissue or cell transcriptome enables categorization of different species of transcripts, to determine the transcriptional structure of genes involving splicing patterns and RNA modifications, and to quantify the expression levels of different transcripts under different biological conditions (Wang et al., 2009). Transcriptomic analysis in the tissues or cells will help to better understand the molecular mechanisms during different biological processes.

1.4.2 Gene expression regulation

Regulation of gene expression is important for the cells to enhance or depress a gene to maintain certain cellular functions in response to environmental changes. Gene expression can be regulated at multiple levels, including transcriptional regulation (for example, chromatin remodeling and DNA binding protein (transcription regulator) regulation) (Hübner et al., 2013), post-transcriptional regulation (Glisovic et al., 2008), translational regulation (for example, inhibition of translation initiation) (Sonenberg and Hinnebusch, 2009), and post-translational modification (for example, protein phosphorylation) (Wang et al., 2013).

Here, we focus on the post-transcriptional regulating process. As soon as mRNA precursors (pre-mRNA) are synthesized, they undergo diverse RNA processing reactions including 5′end capping, splicing, editing, 3′end cleavage and polyadenylation to form mature mRNAs (Pertea, 2012). The mature mRNAs will be translated into proteins by ribosomes. The gene regulation occurring during this process is called post-transcriptional regulation (Keene, 2007). The post-transcriptional regulation events mainly include producing isoforms of

mature mRNAs by splicing (alternative splicing event), regulating the stability and translation of mRNAs (such as miRNA-mediated regulation), and regulating the subcellular location of specific mRNAs (Keene, 2007).

Alternative pre-mRNA splicing is an important mechanism for regulating gene expression and for increasing the diversity of the transcriptome and proteome in eukaryotes (Wang et al., 2008). It has been estimated that ~86% of human genes express multiple mRNA isoforms through alternative splicing (Wang et al., 2008). The excision of the introns from a pre-mRNA and the joining of the exons are directed by special sequences at the intron/exon junctions called splice sites (Black, 2003). Splicing is carried out by the spliceosome, a large macromolecular complex that can recognize the splice site (Black, 2003). The spliceosome contains five small nuclear ribonucleoproteins (snRNPs), which are able to assemble onto the intron and catalyze the chemical reactions to ligate the exons and release the introns (Matlin et al., 2005). Changes in the binding sites of the spliceosome will result in alternatively spliced isoforms, and the isoforms may lead to alteration of protein activity or expression level (Matlin et al., 2005). It has been reported that alternative splicing events play roles in different cellular processes, such as the development of muscle (Blencowe, 2006), brain (Modrek et al., 2001) and the immune system (Honey, 2005).

The regulation of gene expression by miRNAs is one of the major post-transcriptional regulation events by decreasing mRNA stability or inhibiting initialization of the translation process, which will be further discussed in the following sections.

1.5 Protein-coding gene expression changes in the gut and relationship to gut health

1.5.1 Protein-coding gene expression alterations in the gut

The intestinal mucosa functions in nutrient absorption and also performs important barrier and immune functions to maintain intestinal homeostasis. The intestinal mucosal immune system includes organized lymphoid tissues, such as PPs, the mucosal epithelial cells, and immune cells located within the epithelial layer and lamina propia (Spahn and Kucharzik, 2004). The mucosal epithelium functions as a physical barrier and releases antimicrobial products (Hooper et al., 2012). For instance, the mucosal epithelium can prevent the passage of microbes through the expression of intercellular tight junction (TJ) proteins (Gunzel and Fromm, 2012). Moreover, mucosal epithelium contains Paneth cells that produce antimicrobial peptides (AMPs) (Porter et al., 2002), which provide the host with a front line of defense against microbes. Studies in mice and rat showed that the expression of genes that are related to immune responses, the expression of TJ protein genes, as well as the expression of AMP genes in the gut undergo dynamic changes after birth due to cellular maturation and bacterial colonization. For example, the mRNA expression of transforming growth factor β and interleukin 1β decreased, while tumor necrosis factor α increased in the small intestine after the weaning of rats (Schaeffer et al., 2000). These cytokines mediate the immune responses in the mucosal immune system, and their dynamic changes suggest that the immune response-related genes in the gut are developmentally regulated during early life. In addition, complex expression

pattern changes of claudin gene mRNAs were observed in the small intestine of neonatal mice (Holmes et al., 2006). The expression of defensin only started two weeks after birth in the mouse intestinal epithelium due to the maturation process of crypt based Paneth cells (Menard et al., 2008).

Although gene expression alterations that are related to intestinal development have been extensively studied in mice and rat, less is known about ruminants. The ruminant mucosal immune system displays several unique developmental features. For example, the small intestine of ruminants contains two distinct types of PPs, which show extensive prenatal development, in different location of the small intestine (Griebel and Hein, 1996). While the continuous PPs in the ileum function primarily as the site for the generation of the pre-immune B cell repertoire, the discreet PPs in the jejunum function as the induction sites for the generation of IgA plasma cells (Mutwiri et al., 1999). Moreover, maternal antibodies are transferred to calf mainly through colostrum feeding during neonatal period, rather than through placenta during the fetal period that normally occurs in humans and mice (Chucri et al., 2010). Thus, the distinct developmental features of intestinal immune system in ruminants may result in gene expression changes that are different from what has been found in mouse or rats. A previous study showed significant temporal changes (comparing 6-month-old with 3-week-old calves) in the mRNA expression of TLRs and AMPs in the small intestine of calves (Malmuthuge et al., 2012). However, the gene expression changes of the small intestine in calves, especially during the pre-weaned period, are not well studied.

1.5.2 Gene expression alterations in response to gut microbial colonization or pathogenic infection

In order to test how host gene expression responds to microbial colonization, germ-free mice are commonly used. A recent study compared the small intestinal whole transcriptome of conventionally raised mice with germ-free mice, revealing approximately 10% of the host's transcriptome was regulated by microbiota (Sommer et al., 2015). These genes were mainly annotated with functions in immunity, cell proliferation, and metabolism (Sommer et al., 2015). In addition, bacterial lipopolysaccharide (LPS) or flagellin can stimulate the expression of REG3γ and interleukin 22 in the gut epithelial cells by altering the expression of TLRs (Vaishnava et al., 2008; Kinnebrew et al., 2010). Colonization of segmented filamentous bacterium (SFB) in germ-free mice results in the accumulation of Th17 cells in the lamina propria of the gut mucosal layer (Ivanov et al., 2009). Th17 cells and their effector cytokines (such as interleukin 22) mediate host defensive mechanisms to various infections (Ouyang et al., 2008). Although the signaling pathways initiated by SFB in host cells is still not clear, it has been hypothesized that SFB can influence epithelial gene expression, such as the expression of cytokines, to pose an effect on the host immune system (Hooper et al., 2012). Besides, *Bacteroides fragilis* are able to induce the expression of interleukin 10 (IL10) in the small intestinal epithelium of mice (Round et al., 2011). IL10 is an anti-inflammatory cytokine that can prevent the expansion of Th17 cells (Round et al., 2011), and suppresses the host response to commensal bacterial colonization (Murray, 2005). Collectively, studies performed

in germ-free animals revealed that microbial colonization in gut can change the host gut gene expression, and these changes are able to shape the host mucosal immune system and maintain the balance between the host gut and commensal microbes.

In addition to the responses to microbial colonization, gene expression in the gut also responds to the pathogenic infections. Upregulated interferon gamma (IFNγ)-regulated genes have been observed in the *Salmonella*-infected small intestine of mice (Rhee et al., 2005). IFNγ is an important activator for phagocytic cells and an inducer of the class I major histocompatibility complex (MHC Class I) molecule expression (Rhee et al., 2005), important to both innate and adaptive immune responses. Microarray analysis revealed that the alterations of gene expression in *Salmonella*-infected cecum of chicks were mainly associated with the nuclear factor kappa B signaling pathway (Higgins et al., 2011), which is one of the main pathways that regulate the immune response. Infection of the human Caco-2 cell line with the parasitic protozoan *Giardia lamblia* resulted in changed gene expression of chemokine genes and stress regulation-related genes (Roxström-Lindquist et al., 2005). These studies displayed that the gene expression of gut tissue or cultured cell lines in different animals can respond to a variety of pathogenic infections, and the majority of gene expression alterations are related to immune functions.

So far, no germ-free calf is available for analyzing the host responses to microbial colonization in ruminants. As we reviewed above, a recent study observed strong correlation between the mRNA expression of TLRs and the total

bacteria in the gut of calves, revealing that host TLR gene expression may respond to microbial colonization to some extent (Malmuthuge et al., 2012). Moreover, studies have been performed to analyze the gene expression alterations of gut that are related to enteric infections in calves. The differentially expressed genes in the early stage of a MAP-infection in the small intestine of calves were associated with host immune tolerance, which may lead to the pathogen survival in the host gut (Khare et al., 2012). Gene expression alterations that are related to defective TLR signaling pathways and subverted mucosal epithelial barrier function were observed in ileum with *Brucella*-infection in calves (Rossetti et al., 2013). Therefore, the gene expression in gut tissue is able to respond to both microbial colonization and pathogenic infection in calves. Understanding the gene expression mechanisms during microbial colonization and pathogenic infection may provide more strategies to protect calves from infection.

1.5.3 Biogenesis, function and regulation of microRNAs

miRNAs are small (~22 nucleotides) single-stranded RNAs that do not encode proteins, and are widely expressed in animals, plants, algae, and viruses (Bartel, 2009). They can regulate gene expression by targeting 3′ UTR of mRNAs, subsequently repressing protein synthesis (Bartel, 2009). More than 60% of the human protein-coding genes contain at least one conserved miRNA-binding site, and, considering that many non-conserved sites also exist, the majority of protein-coding gene expression may be controlled by miRNAs (Ha and Kim, 2014) (Figure 1.1).

a. Biogenesis of miRNAs

miRNAs are processed from independent genes, introns or untranslated regions of protein-coding genes, and introns or exons of non-coding RNAs by RNA polymerase (RNAP) II or III (Lee et al., 2004; Borchert et al., 2006). RNAP produces precursor transcripts from the genome, primary miRNA (pri-miRNA), which later become miRNAs via two catalyzing steps:1) Production of a 70 nucleotide pre-miRNA in the cell nucleus by Drosha; 2) Production of miRNA/miRNA* duplex by Dicer after pre-miRNA being transported to cytoplasm by the RanGTP-dependent nuclear transport receptor exportin-5 (EXP5) (Lee et al., 2003; Kim, 2004). A special case of miRNA biogenesis is the biogenesis of mirtrons in which miRNAs are derived from a short intronic hairpin that bypasses the first catalyzing step by Drosha-DGCR8 to produce pre-miRNAs during the splicing of transcribed mRNA (Okamura et al., 2007). The mature miRNA strand of the double strand is preferentially incorporated into the RNA-induced silencing complex (RISC), which can directly bind with a member of the Argonaute (AGO) protein family (Krol et al., 2010). The other strand of the duplex, miRNA*, is normally degraded but may be loaded into RISC (Okamura et al., 2009). Once the RISC is formed, the complex is guided by miRNA to the target mRNA, which results in the silencing of gene expression (Huntzinger and Izaurralde, 2011).

b. Function of miRNAs

Once miRNAs recognize their target sites that are usually on the 3′ UTR of mRNAs, the RISCs inhibit protein translation by silencing protein synthesis. The gene silencing function of miRNAs is generally explained by one of the three

possible models (Huntzinger and Izaurralde, 2011). According to the first model, a perfect match between miRNAs and target sites results in endonucleolytic cleavage of mRNAs by AGO. However, this mechanism is not as common in animals as plants (Bartel, 2009). The most common gene silencing method observed in animals is a destabilization of target mRNAs (Guo et al., 2010). In this process, one of the proteins in RISCs, GW182, and recruits deadenylation factors following RISCs binding to the target sites. The deadenylation factors remove the polyA tail of target mRNAs and increase their susceptibility to exonucleolytic degradation (Behm-Ansmant et al., 2006). The third model results in reduced protein expression while mRNA levels remain unchanged, suggesting that protein synthesis is blocked by translational repression. Although this model is not well explained, there is evidence that miRNAs directly inhibit translational initiation (Humphreys et al., 2005), elongation (Petersen et al., 2006), and facilitate direct degradation of peptides synthesized from target mRNAs (Nottrott et al., 2006).

The recognition of target mRNAs by miRNAs is based on the complementarity of their sequences. Although the absence of a perfect match between miRNAs and their targets complicates the identification of target genes, pairing between the miRNA SEED region (2–7 nucleotides at the 5′ end of the miRNAs) and the 3′ UTR sequences of target mRNA is widely utilized by numerous computational algorithms to predict the potential targets of detected miRNAs in different animal species (Thomas et al., 2010). Although there is evidence that miRNAs can target the coding region sequence or 5′ UTRs of

mRNAs, targeting efficiency of the 5′ UTR is much lower than for the 3′ UTR (Fang and Rajewsky, 2011; Hausser et al., 2013).

c. Regulation of microRNA expression

Dysregulation of miRNA expression has been associated with many diseases from cancer to heart disease (Nottrott et al., 2006). Thus, it is important to understand how miRNA expression is regulated. Similar to the regulation of protein-coding genes, transcription factors play an important role by either inducing or repressing the recruitment of RNA polymerase to miRNA genes (Davis and Hata, 2009). The proto-oncogene, c-MYC, is a critical regulator of miRNA transcription (O'Donnell et al., 2005). It induces the expression of miR-17-92 cluster, which is highly expressed in tumors (O'Donnell et al., 2005). In addition, c-MYC reduces expression of tumor suppressing miRNAs, such as miR-15a, miR-29, miR-34 (O'Donnell et al., 2005; Chang et al., 2008). Besides, tumor suppressor p53 activates the expression of miR-34, which promotes cell cycle arrest and apoptosis (Davis and Hata, 2009). miRNAs can regulate their own transcription through feedback loops with specific miRNA gene transcription factors. Runt-related transcription factor 1 (RUNX1) promotes the transcription of miR-27a gene; however, miRNA-27a inhibits the expression of RUNX1 by targeting the 3’ UTR of RUNX1 mRNA, which plays a role in hematopoiesis (Ben-Ami et al., 2009). miRNA regulatory feedback is common and crucial for adjusting miRNA expression to an optimal level.

1.6 miRNAs as disease biomarkers or therapy methods

1.6.1 miRNAs regulate gene expression in the gut

More than 400 miRNAs were identified in the intestinal epithelium of mice (McKenna et al., 2010), and targeted removal of Dicer1 in intestinal epithelial cells decreased the number of goblet cells, increased epithelial cell apoptosis, altered mucosal barrier function, and increased intestinal inflammation and leukocyte infiltration (McKenna et al., 2010). This study revealed the multiple roles that miRNAs play in regulating mucosal cell differentiation and maintenance of a functional mucosal barrier. Furthermore, miR-375 specifically inhibited T helper 2 (Th2) cell responses to infection by targeting thymic stromal lymphopoietin gene expression in the gut epithelium (Biton et al., 2011).

In addition to the regulatory function on gut epithelium, miRNAs can regulate host immune responses. For example, LPS treatment decreased let-7 expression and increased TLR4 expression, indicating that the TLR4 signaling pathway may be regulated by the miRNA gene family let-7 (Androulidaki et al., 2009). miR-146 targets IL-1R-associated kinase 1 (IRAK1), IRAK2 and TNFR-associated factor 6 (TRAF6) that activate NF-κB production following TLR signaling (Hou et al., 2009). miR-155-deficient mice showed decreased production of T helper 1 (Th1) and Th17 cells during autoimmune inflammation and reduced antibody production by B cells, implicating this miRNA in T cell-driven inflammation and humoral immune responses (Rodriguez et al., 2007; Dorsett et al., 2008).

Although miRNAs play roles in regulating gut epithelial proliferation as well as innate and humoral immune responses, their roles in regulating the mucosal immune system have not been well explored. Furthermore, less is known about the function of miRNAs in the gut of ruminants.

1.6.2 miRNAs may mediate commensal bacterial colonization or pathogenic infection

Interplay between microRNA expression in gut tissues and the microbiome has been suggested as one possible mechanism to regulate and integrate normal development and function of the mucosal immune system (Masotti, 2012). The expression levels of 4.8% of all cecal miRNAs were reported to be different when comparing germ-free and conventionally raised mice (Singh et al., 2012). The potential targets of the differentially expressed miRNAs included genes involved in regulating the intestinal barrier through encoding junctional and mucous layer proteins (Singh et al., 2012). Furthermore, the miRNAs differentially expressed in germ-free and conventional mice also targeted genes involved in regulating the expression of MHC Class I and II proteins (Singh et al., 2012). This study suggested that modulation of miRNAs by the gut microbiota might play a role in regulating the development of both the mucosal barrier and host mucosal immune system. miR-10a expression is predominant in murine intestinal tissue, including the epithelium and lamina propria (Xue et al., 2011). However, miR-10a expression is significantly higher in the intestine of germ-free mice relative to specific pathogen free (SPF) mice (Xue et al., 2011). Down-regulation of miR-10a expression is also observed when

bone-marrow derived dendritic cells are treated with bacterial ligands recognized by TLRs (Xue et al., 2011). Furthermore, miR-10a expression levels are not changed by the introduction of gut microbes in SPF MyD88 knock-out mice (Xue et al., 2011). Thus, microbial recognition by TLRs appears to be essential for modulation of miR-10a expression. miR-10a is also involved in regulating the production of IL12 and IL23 (Xue et al., 2011), providing a direct link between the microbiome and the regulation of host immune responses and intestinal homeostasis. These studies provide evidence for both direct and indirect interactions between the gut microbiota and intestinal miRNAs, which then impact host mucosal immune responses.

miRNAs are not only involved in gut microbial regulation of mucosal immune system development and function, but also in host interactions with enteric pathogens. There is increasing evidence that miRNAs modulate both innate and adaptive immune responses following bacterial infections. miR-29 expression in human and murine natural killer (NK) cells and T cells influences IFNγ production in response to both *Listeria monocytogenes* and *Mycobacterium bovis* infections (Ma et al., 2011). miR-29 knockout transgenic mice respond with greater Th1 immune responses, which are mediated by increased IFNγ production, relative to wild-type mice (Ma et al., 2011). Expression of miR-146 and miR-155 is associated with infection by a variety of bacterial pathogens, including *Helicobacter pylori*, *Salmonella enterica*, *L. monocytogenes* and *Mycobacterium* spp. (Lawless et al., 2013; Staedel and Darfeuille, 2013). miR-146 is also known to be involved in the establishment and maintenance of oral

tolerance (Staedel and Darfeuille, 2013), suggesting its important role in maintaining host-microbial interactions during gut colonization. However, miR-155 knockout mice failed to develop protective immune responses to *Salmonella* infection and exhibited impaired clearance of *Citrobacter rodentium* from the gut (Staedel and Darfeuille, 2013). Therefore, miRNAs may play an important role in modulating host interactions with both the commensal microbiome and enteric pathogens.

1.6.3 miRNAs can be biomarkers or therapeutic methods

miRNAs are potential biomarkers for diseases. Firstly, the sequences of most miRNAs are conserved among different species (Bartel, 2004), and the conserved miRNAs have been considered to have consistently similar functions (Bartel, 2004). Therefore, biomarker miRNAs that are identified in model animals are transferrable to other animals or even to humans. Secondly, the level of miRNAs can be measured by various approaches, including polymerase chain reaction (PCR) and next-generating sequencing (Pritchard et al., 2012). Moreover, miRNAs are stable in various bodily fluids, such as plasma, serum, urine, and saliva (Etheridge et al., 2011). Collecting these bodily fluids is easy and does not affect animal health. In addition, the expression of some miRNAs is specific to tissues, to different biological stages as well as to diseases, and the changes of several miRNA levels in plasma, serum, urine, and saliva have already been associated with different diseases (Etheridge et al., 2011). For instance, the increased level of miR-141 in the serum was identified as a potential marker to detect advanced prostate cancer (Mitchell et al., 2008); the expression of miR-126

and miR-182 in the urine can be utilized to discriminate patients with bladder cancer from healthy individuals (Hanke et al., 2010); decreased levels of miR-125a and miR-200a in saliva are associated with oral squamous cell carcinoma (Park et al., 2009).

Besides acting as potential biomarkers, miRNAs also provide new therapeutic targets for many diseases. Some diseases may be caused by the loss or reduced expression of a particular miRNA; therefore, increasing the expression of specific miRNAs may be an effective way to control or treat the disease (van Rooij et al., 2012). Conversely, some other diseases may be induced by over-expression of specific miRNA; thus, selecting specific anti-miRNA inhibitors will be useful (van Rooij et al., 2012). One recent successful trial attached the peptide nucleic acid antimiRs (miRNA inhibitor) to a peptide with a low pH-induced transmembrane structure (pHLIP) (Cheng et al., 2015). This novel construct could target the tumor microenvironment, transport antimiRs across plasma membranes, and effectively inhibit the miR-155 oncomiR (overexpression of this miRNA play a causal role in the onset and maintenance of B cell lymphoma) in a mouse model of lymphoma (Cheng et al., 2015). Many miRNA mimics, antimiR oligonucleotides and siRNA therapeutics are in development, and most of these trials are still at the preclinical stage but have shown efficacy in various animal models of disease (Cheng et al., 2015). However, there are still some obstacles that need to be overcome. For example, the effective and safe delivery of miRNAs remains difficult for many tissue types, such as brain and muscle (Li and Rana, 2014). In addition, the specificity of using miRNAs as therapeutic tools or

as therapeutic targets is still not well studied. The off-target effects may have serious influence on animal health (Li and Rana, 2014). Thus, treating diseases with miRNAs requires further trials and modifications.

Specific changes in miRNA expression following enteric infections (reviewed in Section 1.6.2) suggest that miRNAs may be used as diagnostic markers for specific enteric infections. If sufficient miRNA is released from infected tissues and remains stable while circulating in blood, it may be possible to use a non-invasive method to diagnose enteric infections. This may provide a novel method to detect enteric infections that cannot be detected by traditional methods easily, such as MAP infection in cattle. In addition, the therapeutic potential of miRNAs can be used in treatment of clinical diseases of cattle, which may provide new alternative ways to cure some fatal diseases, such as Johne's disease.

1.7 Methods to study gene expression/transcriptome

So far, reverse transcription quantitative PCR (RT-qPCR), microarray, and RNA-Seq, are commonly used to profile the transcriptome. The general concepts, highlighted strengths and limitations of these three methods have been well documented (Pritchard et al., 2012), and therefore will be addressed only briefly here.

1.7.1 Reverse transcription quantitative PCR

This is a major approach that relies on reverse transcription of RNA to cDNA followed by quantitative PCR with real-time monitoring of reaction

product accumulation. RT-qPCR is a well-established, sensitive, and specific method that can be used for absolute quantification (Pritchard et al., 2012). To date, RT-qPCR has been considered as the most accurate method to quantify transcripts, and most of the studies performed by microarray or RNA-Seq need the validation of RT-qPCR (Wang et al., 2014). In order to apply this approach for transcriptome profiling, reactions can be carried out in a high-throughput form (Pritchard et al., 2012). However, the difficulty in performing high-throughput RT-qPCR is that optimal reaction conditions may vary among mRNAs because of the differences in primer annealing temperatures. In addition, RT-qPCR cannot detect novel transcripts, and data output is relatively less when compared with microarray and RNA-Seq.

1.7.2 Microarray

Microarray employs nucleic acid probes, covalently bound to glass slides (Chen et al., 2012). Fluorescence labeled target sequences are then hybridized to the probes and scanned (Chen et al., 2012). The fluorescence signal images are then converted to signal intensities and the data are processed using software specific to the application of the array (Mantione et al., 2014).

Microarray has the advantage of being less expensive than the other profiling methods, and it allows large numbers of parallel measurements (Pritchard et al., 2012). However, it shows lower accuracy than RT-qPCR or RNA-Seq and difficulty in profiling novel transcripts (Pritchard et al., 2012). With the development of next-generation sequencing methods, microarray is

losing its advantage in price, which may restrain it from being widely used in the future.

1.7.3 RNA-Seq

The third major approach to profile the transcriptome is RNA-Seq, which is an approach that begins with preparation of a RNA-Seq cDNA library from the RNA sample, followed by highly parallel sequencing of millions of individual cDNA molecules.

The major advantage of RNA-Seq is the capability of identifying both known and novel mRNAs and providing the expression level of all the transcripts (Mantione et al., 2014). High cost of next-generation sequencing was the primary disadvantage of RNA-Seq, however, as this technology has developed, the price has been much reduced now (Sboner et al., 2011). Recent studies revealed that RNA-Seq performs better in detecting the differential expression of genes with low abundance than microarray, although is still not as accurate as RT-qPCR (Wang et al., 2014). Similar with microarray, RNA-Seq cannot be used for absolute quantification. In addition, it should be noted that RNA-Seq studies may result in a large number of annotated or annotated sequence data, therefore, processing the RNA-Seq data is a challenge.

1.8 Hypotheses and objectives

Enteric infection in pre-weaned calves is one of the major health problems of the dairy industry. Understanding the molecular mechanisms of gut

development and how the gut protects the host from infection are critical for developing more effective strategies to prevent calf disease.

I hypothesize that alterations in the protein-coding gene expression in the gut are related to the gut developmental process and bacterial infection in pre-weaned calves and that miRNAs play an important role in regulating these changes in gene expression. Furthermore, I hypothesize that changes in both protein-coding gene and miRNA expression were associated with commensal bacterial colonization and enteric infection by pathogens.

The long-term goal of this study is to improve an understanding of the mechanisms regulating host-microbial interactions in the gut of ruminants and to discover genomic markers (miRNAs or mRNAs) for enteric diseases of pre-weaned calves. The specific aims included: 1) to identify changes in protein-coding gene and miRNA expression in gut tissues of healthy calves during the pre-weaned period; 2) to evaluate the impacts of MAP infection on the expression of protein-coding genes and miRNAs in the ileum of pre-weaned calves; 3) to analyze the potential regulatory function of miRNAs targeting specific protein-coding genes in healthy and MAP-infected gut tissues of pre-weaned calves; 4) to characterize relationships between microbial colonization and the expression of both protein-coding genes and miRNAs.

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1.10 Figures

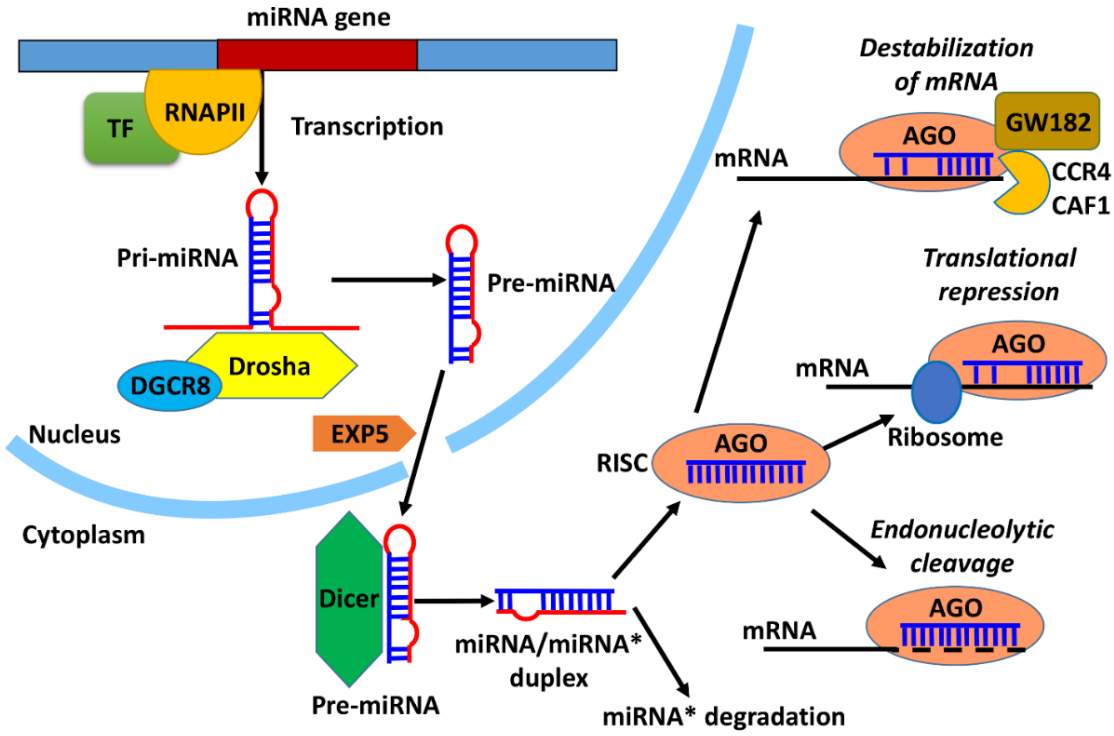

Figure 1.1 miRNA biogenesis and function. miRNA biogenesis undergoes miRNA gene transcription, Drosha and Dicer enzymes catalyzing, and loading into RNA-induced silencing complex (RISC). miRNA function mechanism can be generally divided into three models: destabilization of mRNA, translational repression, and endonucleolytic cleavage. RNAPII: RNA polymerase II; TF: transcription factor; Pri-miRNA: primary miRNA; Pre-miRNA: precursor miRNA; DGCR8: DiGeorge syndrome critical region in gene 8; EXP5: exportin 5; AGO: Argonaute protein; CCR4: C-C chemokine receptor type 4; CAF1: Chromatin assembly factor 1. Both CCR4 and CAF1 are deadenylation factors. Made by Guanxiang Liang (Published as Liang et al., 2015. *Molecular Immunology*, 66: 57–67).

Chapter 2. Potential regulatory role of microRNAs in the development of bovine gastrointestinal tract during early life

2.1 Introduction

The gastrointestinal tract (GIT) of mammals undergoes microbial colonization immediately after birth (Ley et al., 2006) and thereafter is continuously exposed to commensal microbiota, pathogens, and dietary antigens. There is increasing evidence that the gut microbiota plays a vital role in regulating host immune functions (Hooper et al., 2012). The host mucosal immune system prevents bacterial invasion and shapes the gut microbiota, while the gut microbiota influences immune system development (Hooper et al., 2012). Recent studies using mouse models revealed the molecular basis for the complex and dynamic interactions between commensal bacteria and the mucosal immune system (Ayabe et al., 2000; Kobayashi et al., 2005). Toll-like receptors (TLRs) signaling pathway is one of the innate immune responses that recognizes microbial-associated molecular patterns (MAMPs) and activates downstream intracellular signals (Akira et al., 2006). It also maintains host-microbial homeostasis by modulating proliferation of epithelial cells and/or secretion of antimicrobial proteins (Rakoff-Nahoum and Medzhitov, 2007; Vaishnava et al., 2011). Moreover, gut microbial colonization also has profound effects on the differentiation of T cells. For example, bacterial colonization of germ-free mice enhanced the differentiation of T helper 17 cells (Th17) and regulatory T cells

(Tregs) (Ivanov et al., 2009; Olszak et al., 2012). However, the molecular mechanisms involved in regulating these interactions remain largely undefined.

MicroRNAs (miRNAs) are small (~22 nucleotides) endogenous RNAs that regulate gene expression by targeting the 3' untranslated region (3'UTR) (Krutzfeldt and Stoffel, 2006) and/or coding region (Hausser et al., 2013) of mRNAs. They have been reported to be involved in the regulation of many biological processes including embryo development, cell differentiation, apoptosis and metabolism (Song and Tuan, 2006). The expression of miRNAs is ubiquitous among mammalian cells; however, their expression patterns and regulatory roles can be tissue and /or species specific (Lagos-Quintana et al., 2002; Landgraf et al., 2007; Liang et al., 2007). Recent studies reveal that miRNAs regulate genes as part of the complex regulatory networks in the immune system, by playing pivotal roles in the regulation of both immune cell development and effector functions (O'Connell et al., 2010). For example, miR-196 induces the cleavage of homeobox mRNA in hematopoietic stem cells (HSCs) and modulates HSC homeostasis (Yekta et al., 2004; Popovic et al., 2009). The miRNAs also regulate gut homeostasis and mucosal immunity. miR-375 regulates T cell subset amplification in the murine colon (Biton et al., 2011). The expression of miR-155, miR-146 and miR-21 can be induced by TLR activation and target the 3'UTR of mRNAs encoding TLR signaling pathway molecules, like IL-1R-associated kinase 1 and TNFR-associated factor 6 (O'Neill et al., 2011). In addition, recent studies reported that miRNAs may be involved in host-microbial interactions. The up-regulation of miR-27b through TLR4/NF-κB

signaling following a gut protozoan infection was observed and it suppressed expression of KH-type splicing regulatory protein that is vital for antimicrobial activity (Zhou et al., 2012). miR-665, reported to be up-regulated during microbial colonization of germ-free mice, regulates mRNA of ATP-binding cassette, sub-family c, member 3, a protein that mediates the metabolism of xenobiotics and endogenous toxins in intestine (Dalmasso et al., 2011). However, the roles of miRNA in host-microbial interactions during early life have not been reported. We hypothesized that miRNAs coordinate immune system development of the host in response to microbial colonization in the GIT during early life of calves.

This study therefore, 1) analyzed temporal and regional miRNA profiles throughout the GIT during the first 6 weeks after birth; 2) explored the potential roles of miRNAs in mucosal immune system development by identifying predicted target genes, and lastly; 3) investigated whether miRNA expression patterns are temporally and spatially associated with changes in the GIT microbial population in the dairy calves.

2.2 Materials and methods

2.2.1 Animal study and sample collection

Small intestine (mid-jejunum and ileum) and rumen samples were collected from newborn (D0, n = 3), 7-day-old (D7; n = 6), 21-day-old (D21; n = 6) and 42-day-old (D42; n = 6) male Holstein calves at Dairy Research and Technology Center (DRTC), University of Alberta. All experimental protocols

were reviewed and approved by the Livestock Animal Care committee of the University of Alberta (protocol no. Guan 009) and all procedures were conducted following the guidelines of Canadian Council on Animal Care. D0 samples were collected from newborn animals, within 30 min after delivery without feeding colostrum. The D7 calves were fed only whole milk (4 l/day), while D21 and D42 calves received whole milk with *ad libitum* access to calf starter (23% CP and 4% ether extract (EE) as the guaranteed minimum, 19.5% NDF, 27.1% starch - Wetaskiwin Co-Op Country Junction, Wetaskiwin, AB, Canada). All calves were euthanized using captive bolt gun, and samples were collected within 30 min after euthanization. Ileum was defined as 30 cm proximal to ileocecal junction and 10 cm in the middle of the 30 cm segment was collected. Mid-jejunum was defined as 1 m distal to the pyloric sphincter and 10 cm in the middle of the 1 m segment was collected. Rumen tissue samples were collected from the lateral wall (~10 cm^2). Tissue and digesta samples were collected from the same site separately after euthanization, snap-frozen in liquid nitrogen and stored in -80°C. Due to very limited volume of the contents in D0 samples, tissue and content were processed together for D0 samples.

2.2.2 Nucleic acid isolation

Tissue samples were ground into fine powder while immersed in liquid nitrogen prior to nucleic acid extraction. Total RNA was extracted from 80 mg of tissue using mirVana™ miRNA Isolation Kit (Ambion, Carlsbad, CA) following the manufacturer's instructions. The quality and quantity of the RNA were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara,

CA) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), respectively. RNA samples with good quality (integrity number (RIN) > 7.0) were used for further analysis.

Total DNA was extracted from tissue samples (~100 mg) using the bead beating method as described by Li et al (Li et al., 2009). Briefly, samples were subjected to physical disruption in a BioSpec Mini Beads beater 8 (BioSpec, Bartlesville, OK) at 5000 rpm for 3 min, followed by phenol: chloroform: isoamyl alcohol (25: 24: 1) extraction. DNA was then precipitated with cold ethanol and dissolved in nuclease free molecular grade water. The quantity and quality of DNA was measured using ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.2.3 miRNA library construction and sequencing

Total RNA (1.0 µg each) from each sample was used to construct miRNA libraries with an unique index using the TruSeq Small RNA Sample Preparation kit (Illumina, San Diego, CA) according to the manufacturer's instruction. PCR amplification was performed for 11 cycles and libraries with unique indices were purified individually using gel purification. Quantitative real time PCR (qPCR) was performed for library quantification using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and KAPA SYBR Fast ABI Prism qPCR kit (Kapa Biosystems, Woburn, MA).

Individual libraries were then pooled together for sequencing according to Illumina's instruction. The diluted libraries were loaded on cBot (Illumina) for cluster generation using the TruSeq™ SR Cluster kit v3 (Illumina). Sequencing

was performed on the HiScan SQ system (Illumina) using the TruSeq™ SBS kit v3 (50 cycles, Illumina). Real-time analysis and base calling was performed using the HiSeq Control Software Version 1.4.8 (Illumina).

2.2.4 Sequencing data analysis

Low-quality reads were removed from raw data using CASAVA 1.8 based on chastity, and the sequences with good quality were then subjected to 3' adaptor sequence trimming. Then, the sequences of sizes ranging from 18 to 30 nt were mapped to the ncRNA sequences (Rfam) to remove non-miRNA sequences (tRNA, snoRNA, rRNA, and other non-coding RNAs). The known and novel miRNAs were identified using miRDeep2 (Friedlander et al., 2012) based on a probabilistic model of miRNA biogenesis (Friedlander et al., 2008). For the known miRNAs, the filtered sequences were aligned against the corresponding known miRNA precursor sequences (miRBase release version 19) by using the module of quantifier.pl in miRDeep2 with the default parameters to identify known miRNAs. The known miRNAs with total number of reads above 20 were defined as expressed known miRNAs. Novel miRNAs were detected using miRDeep2, with the miRDeep2 score cutoff of 5 and more than 20 mapped reads from all libraries. Each library was processed separately, and the results of each novel miRNA candidate were combined together according to genomic location. The novel miRNAs from different locations within the bovine genome that contain same sequence were reported as single novel miRNA candidate.

The conservation of known miRNAs was analyzed based on the TargetScan definitions for "highly conserved", "conserved", and "poorly

conserved" (Lewis et al., 2003). A highly conserved miRNA means that it is conserved across most vertebrates; a conserved miRNA is conserved across most mammals, but usually not beyond placental mammals; a poorly conserved miRNA is not present in above two groups. In this study, bovine specific miRNAs were defined by using two conditions: (1) miRNAs belong to poorly conserved group; (2) miRNAs with seed region sequences only reported in cattle.

Temporal and regional effects on the miRNA expression were investigated by characterizing differentially expressed (DE) miRNAs using bioinformatics tool edgeR (Robinson et al., 2010), which utilizes a negative binomial distribution to model sequencing data. The expression of miRNAs in each library was normalized to counts per million reads (CPM) by the following method: CPM = (miRNA reads number / total reads number per library) × 1,000,000. For each comparison, miRNAs with CPM > 5 in at least 50% of the samples were subjected to DE analysis. Fold changes were defined as ratios of arithmetic means of CPM within each comparison group. The significant DE miRNAs were determined by an adjusted *P* value (false discovery rate, FDR) < 0.05 based on Benjamini and Hochberg multiple testing correction (Benjamini and Hochberg, 1995) as well as fold change > 1.5.

2.2.5 Estimation of the total bacterial and two beneficial bacterial populations using qPCR

Quantitative PCR (qPCR) was performed using SYBR Green chemistry (Fast SYBR® Green Master Mix; Applied Biosystems) to estimate the tissue-attached total bacterial population using the copy number of 16S rRNA gene.

Total bacterial population was estimated using U2 primers (U2F, 5'-ACTCCTACGGGAGGCAG-3'; U2R, 5'-GACTACCAGGGTATCTAATCC-3') (Stevenson and Weimer, 2007) with StepOnePlus™ Real-Time PCR System (Applied Biosystems). The standard curve was constructed using plasmid DNA containing 16S rRNA gene of *Butyrivibrio hungatei* with a serial dilution of initial concentration of 8.5×10^{10} mol/μl. The populations of *Lactobacillus* (Lac) and *Bifidobacterium* (Bif) spp. were estimated using Lac primers and Bif primers (Lac1: 5'-AGCAGTAGGGAATCTTCCA-3' and Lac2: 5'-ATTTCACCGCTACACATG-3' (Walter et al., 2001); Bif1: 5'-CGTCAAGCTGCTAGGACGC-3' and Bif2: 5'-TACACCGGAATAGCTCCTGG-3') (designed in this study). The standard curves were constructed using serial dilutions of genomic DNA from *Lactobacillus acidophilus* and *Bifidobacterium longum* with serial dilutions of the initial concentration of 5.8×10^8 and 2.05×10^7 mol/μl^{-1}, respectively. The copy number of 16S rRNA gene per gram sample for each targeted bacterial population was calculated using the method reported in a previous study (Malmuthuge et al., 2012): (QM × C × DV)/(S × W), where QM was the quantitative mean of the copy number, C was the DNA concentration of each sample (ng μL^{-1}), DV was the dilution volume of extracted DNA (μl), S was the DNA amount subjected to analysis (ng), and W was the sample weight subjected to DNA extraction (g).

2.2.6 Correlation between miRNA expression and bacterial density

The possible relationships between miRNA expression and microbial colonization in the GIT were explored using Pearson's correlation in R software

and COR function. Normalized miRNA expression (CPM) and $\log_{10}$ copy number of 16S RNA genes of total bacteria, *Lactobacillus* and *Bifidobacterium* spp. were used to define the correlations in each gut region under each time point. In the rumen the correlations were done only between total bacterial density and miRNA expression. The significant correlations were declared at $P < 0.05$, $r^2 > 0.64$.

2.2.7 Experimental validation of miRNA expression using stem-loop RT-qPCR

Expression of regional and temporal DE miRNAs that identified by miRNA-Seq was validated by stem-loop RT-qPCR using TAQMAN miRNA assays following the manufacturer's recommendation (Applied Biosystems). Briefly, cDNAs were reverse transcribed from 10 ng of total RNA using 5 X specific miRNA RT primer and then were amplified using a 20 X TAQMAN miRNA assay. Fluorescence signal was detected with StepOnePlus™ Real-Time PCR System (Applied Biosystems). In this study, U6 snRNA was used as an internal control to calculate the relative expression of target miRNA following the formula: $\Delta Ct_{\text{target miRNA}} = Ct_{\text{target miRNA}} - Ct_{\text{U6}}$. One sample was selected as a calibrator in each comparison group for the relative quantification. The relative expression of miRNA in a sample normalized to internal control and relative to the calibrator was calculated by: Relative expression $_{\text{target miRNA}} = 2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = \Delta Ct_{\text{target miRNA, sample}} - \Delta Ct_{\text{target miRNA, calibrator}}$. Unpaired T-test was used to compare difference between each comparison group (regional DE miRNA: rumen *vs* mid-jejunum, rumen *vs* ileum and mid-jejunum *vs* ileum; temporal DE miRNA: D7 *vs* D0, D21 *vs* D7, D42 *vs* D21). Differences were considered statistically different at $P < 0.05$ and analyses were performed in R using t.test function.

2.2.8 miRNA targets prediction and functional analysis

Target genes for selected miRNAs were predicted by using TargetScan Release 6.0 (http://www.targetscan.org/) (Enright et al., 2003) and miRanda (http://www.microrna.org/microrna). The target genes that were predicted by both TargetScan (default parameters) and miRanda (Total score ≥ 145, Total energy ≤ -10) for each miRNA were further analyzed through IPA (Ingenuity Systems, www.ingenuity.com). The multiple testing corrected *P* value calculated by Benjamini-Hochberg method (FDR) (Benjamini and Hochberg, 1995) was used to determine the significance of the predicted function in IPAs. A threshold of FDR < 0.05 and enriched gene number ≥ 2 were applied to enrich significant biological functions of each miRNA.

2.3 Results

2.3.1 Profiling of miRNAs in rumen and small intestine of dairy calves

The miRNA expression was studied by sequencing 63 small RNA libraries prepared from rumen, mid-jejunal and ileal tissues collected from newborn calves within 30 min after delivery (D0; n = 3), 7-day-old calves fed milk (D7; n = 6), 21-day-old (D21; n = 6) and 42-day-old calves fed with milk supplemented with calf starter (D42; n = 6). A total of 97 million high-quality small RNA reads were obtained, with a median of 1.4 million reads per library (ranged from 0.6 million to 3.8 million). The majority of sequences was 21 nt and 22 nt in size and accounted for 62.7% of total reads (Figure 2.1).

In total, 77 million out of 97 million reads were mapped to the known miRNA database (miRBase release version 19 (Kozomara and Griffiths-Jones, 2011)) and 0.9 million reads were identified as novel miRNAs, resulting in the identification of 383 known miRNAs and 169 putative novel miRNAs from all libraries. Furthermore, 359 known and 53 novel miRNAs in rumen, 360 known and 82 novel miRNAs in mid-jejunum, and 338 known and 65 novel miRNAs in ileum (Figure 2.2A) were identified from all the calves. Among all identified miRNAs, 356 known miRNAs were detected in all three GIT regions (Figure 2.2B). The top 10 expressed miRNAs represented more than 80% of the total mapped reads in each tissue (Figure 2.2C & 2D). The predominant miRNAs in the mid-jejunum were miR-192 (33%), -143 (28%), and -215 (7%), and in ileum were miR-143 (30%), -192 (15%), -10a (12%) and -10b (8%). Only one predominant miRNA, miR-143, which accounted for 60% of total mapped reads, was detected in the rumen (Figure 2.2D). Among the total known miRNAs (383) identified in the calf GIT, 39% (141) belonged to highly conserved, 15% (58) belonged to conserved, and 46% (176) belonged to poorly conserved groups (Figure 2.3). Based on the analysis for each individual gut region, the present study also detected 81 bovine specific miRNAs (Figure 2.3).

2.3.2 Identification of temporally differentially expressed miRNAs

A temporal effect on miRNA expression was examined independently for each tissue by completing the following comparisons: D7 *vs* D0, D21 *vs* D7, and D42 *vs* D21 and the differentially expressed (DE) miRNAs have been deposited in GEO database (GSE52193). Among all comparisons, more DE miRNAs were

detected when comparing D7 *vs* D0 in the mid-jejunum (Figure 2.4B) and ileum (Figure 2.4C) as well as from D21 *vs* D7 in the rumen (Figure 2.4A). The temporal effect is confounded by the effect of a changing diet. To minimize the dietary effect, we selected DE miRNAs present in each tissue in at least two out of three of the temporal comparisons for further functional analysis. The miRNA candidates were defined at family level, since miRNAs belonging to the same family are thought to perform similar functions based on the same seed sequence (Enright et al., 2003). A total of 8 DE miRNAs belonging to 8 miRNA families for rumen, 20 DE miRNAs belong to 16 miRNA families for mid-jejunum, and 6 DE miRNAs belonging to 6 miRNA families for ileum were selected for further functional analysis (Data has been deposited in GEO: GSE52193).

2.3.3 Identification of regionally differentially expressed miRNAs

The miRNA expression was further compared among GIT regions at each developmental stage. As shown in Figure 2.5, more DE miRNAs were identified in rumen *vs* mid-jejunum (Figure 2.5A) and rumen *vs* ileum (Figure 2.5B) compared to ileum *vs* mid-jejunum (Figure 2.5C) at all the time points. First, DE miRNAs that were common to all three developmental stages within a specific GIT region were identified: 42 families (45 DE miRNAs) were from rumen *vs* mid-jejunum, 80 families (87 DE miRNAs) were from rumen *vs* ileum, and 15 families (18 DE miRNAs) were from ileum *vs* mid-jejunum (Data has been deposited in GEO: GSE52193). Then, 5 families (7 DE miRNAs) that had the most significant (FDR < 0.05) DE patterns were subjected to functional analysis (Data has been deposited in GEO: GSE52193). Among these 5 miRNA families,

miR-192/215 and miR-194 were highly expressed in the small intestine (mid-jejunum and ileum), while miR-205 was highly expressed in the rumen but not detected in mid-jejunum (Figure 2.6). The miR-196 family was only detected in ileum; miR-31 had lower expression in the ileum compared to the mid-jejunum and rumen (Figure 2.6).

2.3.4 Association between miRNA expression and gut microbial population

The expression of miRNAs was strongly correlated with the density of total bacteria, *Bifidobacterium* and *Lactobacillus* spp. (Figure 2.7) throughout the GIT at all stages. At D21, there were more miRNAs significantly correlated with bacterial density than other groups (Figure 2.7). Four miRNA families associated with bacterial density were selected for further functional analysis: the miR-129 family, which revealed positive correlation with total bacterial density ($r = 0.90$, $P = 0.01$) in the rumen on D7 and D21; three miRNA families from ileum including the miR-15/16 family (negatively correlated with the density of *Bifidobacterium* spp. on D21; $r = -0.87$, $P = 0.01$), the miR-196 family (positively correlated with the population of both *Bifidobacterium* and *Lactobacillus* spp. on D21, $r = 0.83$, $P = 0.02$), and the miR-29 family (positively correlated with the population of both *Bifidobacterium* and *Lactobacillus* spp. on D42; $r = 0.95$, $P < 0.01$) .

2.3.5 Experimental validation of miRNA expression using stem-loop RT-qPCR

A total of 13 regional and temporal DE miRNAs that identified from miRNA-Seq (regional DE miRNAs: miR-192, miR-194, miR-205, miR-31, and miR-196; temporal DE miRNAs: miR-146b, miR-191, miR-99a, miR-145, miR-211, miR-486, miR-33, miR-7, and miR-196b) were selected for validation using

stem-loop RT-qPCR. The expressions of miR-192 and miR-194 were notably higher in the small intestine than that in the rumen. miR-205 was highly expressed in the rumen, while miR-31 was highly expressed in the rumen and mid-jejunum not in the ileum. The expression of miR-196b could not be identified both in rumen and mid-jejunum by miRNA-Seq and qPCR. Generally, the regional DE miRNAs showed similar trend in the comparison between miRNA-Seq and qPCR (Figure 2.8). Most of temporal DE miRNAs always showed the expression in agreement between miRNA-Seq and RT-qPCR, however three of them (miR-146b, miR-7, and miR-211) revealed opposite trends between miRNA-Seq and RT-qPCR in some comparison groups (Figure 2.9). For example, in mid-jejunum, miR-146b and miR-7 had divergent regulation from D7 to D21; in ileum, miR-211 revealed significant down-regulation from D7 to D21 by RT-qPCR, but there was no significant difference identified by sequencing.

2.3.6 Functional predictions of differentially expressed miRNAs

Based on predicted targets of miRNAs using TargetScan and miRanda as well as functional analysis using IPA, the significantly enriched (FDR < 0.05, enriched gene number ≥ 2) function categories of a total of 33 selected miRNA families were identified. Among these, a total of 9 function categories, include cellular development, cellular growth and proliferation, connective tissue development, digestive system development, hematological system development and function, immune response, inflammatory response, immune cell trafficking, and lymphoid tissue structure and development, were defined to be related to GIT gut development and immune system development. Fifteen out of the 33 selected

miRNA families were predicted to have functions significantly associated with the GIT development or immune functions (Table 2.1). The function of the predominant miR-143 is predicted to be the differentiation of connective tissue cells, which is related to GIT development (Table 2.1). The functions of temporally DE miRNAs in small intestine (miR-146, miR-191, miR-33, miR-7, miR-99/100, miR-486, miR-145, and miR-211) were related to gut epithelial cells development, immune cells development, inflammatory response, and other functions (Table 2.1). However, most of the temporally DE miRNAs in the rumen did not show any significant predicted functions. Two regionally DE miRNA families (miR-194 and miR-192/215) revealed significantly enriched functions related to GIT development and immune system, such as formation of mast cells and differentiation of leukocytes (Table 2.1). In addition, miR-129, miR15/16, and miR-29, which correlated with bacterial populations, may also regulate gut development, immune and digestive functions (Table 2.1). miR-129 was predicted to play a role in rumen development in response to bacterial colonization. In addition, the temporally DE miRNA-196 was only detected in ileum (Table 2.1), and was significantly correlated with bacterial density. This miRNA has known functions related to epithelium differentiation, lymphoid tissue development, and inflammatory response (Table 2.1).

2.4 Discussion

As an important gene expression regulatory mechanism, post-transcriptional regulation by miRNAs plays a much larger role than previously

expected (Pritchard et al., 2012). Although miRNAs have been widely identified from various bovine tissues (Huang et al., 2011), their roles in regulating the development and function of the GIT have not been well studied. To date, 755 miRNAs have been reported in the literature (miRBase release version 19). A recent study compared the role of ruminant-specific miRNAs in shaping divergent mRNA expression between ruminant and non-ruminant species and revealed that at least 3.5% of genes with reduced expression in cattle can be attributed to cattle-specific miRNAs (Bao et al., 2013). Among the detected 383 known miRNAs from calf GIT, nearly half of them were poorly conserved miRNAs, and nearly half of the poorly conserved miRNAs were bovine-specific miRNAs. The number of bovine-specific miRNAs is higher than the number of conserved miRNAs. This suggests that cattle-specific gut miRNAs may contribute to physiological and metabolic variations specific to ruminants. In addition, the detected known miRNAs and their distinct miRNA expression patterns in GIT comparing to those in other bovine tissues (Jin et al., 2009) suggests that these miRNAs may play an important role in the regional and temporal differentiation of GIT development during early life. For example, miR-143 was predominant in three gut regions and it was predicted to regulate genes involved primarily in the differentiation of connective tissue cells. It was reported that miR-143 induced differentiation and proliferation of smooth muscle cells by targeting a group of transcription factors such as serum response factor and kruppel-like factor 4 (Cordes et al., 2009; Wang et al., 2010). Connective tissue and smooth muscle are major components of the gut (Chew and Long, 2008), therefore the observed high expression of

miR-143 throughout GIT may be associated with the rapid development and growth of the GIT in young calves.

The current study also revealed a significant temporal effect on miRNA expression throughout the GIT of calves. Some of the temporal DE miRNAs revealed opposite trends between sequencing and RT-qPCR in some comparison groups, which may be due to their lower sequencing reads (Cristino et al., 2011). During early life, ruminants are considered to be monogastric due to the bypass of milk into abomasum (McLeod et al., 2007). Less DE miRNAs from D0 to D7 and more DE miRNAs from D7 to D21 in the rumen when compared to other GIT regions may indicate slow rumen development during the first week and an accelerated development in response to a dietary change from milk alone to a diet containing solid food after 2 weeks of age. The highest number of DE miRNAs was identified during the first week after birth (D7 *vs* D0) in mid-jejunum and ileum. During this period intestinal tissues develop rapidly with exposure to many cytokines (IL-6; IL-10), growth factors (insulin-like growth factor) from maternal colostrum (Playford et al., 2000), and microbial colonization. It is not surprising that the majority of temporally DE miRNAs found in the small intestine have putative target genes involved in the development of the mucosal immune system. For example, miR-211 has predicted targets involved in IL-6 and IL-17 cytokine signaling pathways as well as differentiation of T lymphocytes. IL-6 stimulates Th17 T cell differentiation (Korn et al., 2008) and colonization of germ-free mice with segmented filamentous bacteria increases the number of Th17 cells (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009), which produce IL-17 and

IL-22 to regulate the microbiota community (Kamada et al., 2013). Therefore, the detected temporal changes of miR-211 expression in mid-jejunum and ileum may be driven by the bacterial colonization and may be a key link between the gut microbiota and mucosal immune system development. Similarly, temporally DE miRNAs, miR-146, miR-191, miR-33, miR-7, miR-99/100, miR-486, and miR-145 may target genes that accelerate gut tissue and immune system development. The functions of miR-7 were predicted to be associated with differentiation of muscle cells, which indicates the fast growth of muscle layers in GIT during the early life of calves. The miR-486 targets genes that mainly regulate development of fibroblast cells, which are the most common cells in the connective tissue in the gut. Functional analysis illustrated that miR-191, miR-33, miR-99/100, and miR-145 were mainly related to differentiation of leukocytes include lymphocytes. A recent study has shown that miR-99/100 targets mammalian target of rapamycin (Nagaraja et al., 2010), and blocks the development and function of Tregs (Liu et al., 2009). Another temporally DE miRNA, miR-146, was predicted to have multiple functions and to be involved in gut connective tissue, epithelial cells development, T cell immune response and the function of dendritic cells. The expression of miR-146 can be induced by TLR activation and it is function was validated to regulate TLR signaling pathways that can recognize MAMPs and play a critical role in innate immune responses (O'Neill et al., 2011). The differential expression of miR-146 may indicate that gut immune system development was triggered by microbial colonization, and miR-146 may be a mediator regulating this process. This evidence supports our hypothesis that

temporally DE miRNAs play a role in regulating the development of the gut mucosal immune system during early life. It is important to mention that the temporal effect in the present study is confounded by calf age/growth, gut microbial colonization, and calf diet. From D0 to D7 GIT tissues were exposed to maternal colostrum, milk, and microbial colonization, while from D14, a calf dietary supplement was introduced. Therefore, future studies that eliminate the confounding effects of diet by feeding animals only milk throughout the first 6 weeks of life, may provide a better understanding of the effect of microbial colonization on miRNA expression in the GIT.

The DE miRNAs detected in different gut regions suggest that miRNAs may play a role in regulating the regional differentiation of the GIT. Therefore, it is important to determine if regional differences in miRNA expression reflect programmed developmental changes or differences in responses to the local microbiota. The miR-192/215 family was highly expressed in the small intestine of calves compared to the rumen during the first 6 weeks. The miR-192/215 family mainly targets mRNA of Runt-related transcription factor 1 (RUNX1) required for the generation of hematopoietic stem cells (HSCs) in mouse embryo, and plays a role in regulating the differentiation of hematopoietic cell lineages in adult mice (North et al., 2004; Growney et al., 2005). However, the role of RUNX1 in gut development is unclear. Functional analysis showed that mir-192/215 may be related to differentiation of leukocytes and the development of lymphoid tissues. The small intestine contains gut-associated lymphoid tissues (GALTs) rich in leukocytes, which are not found in the rumen (Sheldrake and

Husband, 1985). Higher expression levels of miR-192/215 family suggest an active modulation of leukocytes and lymphoid tissue development in mid-jejunum and ileum compared to the rumen. The expression of miR-194, which was predicted to repress induction and formation of mast cells, was also higher in the small intestine compared to that in the rumen. Mast cells may disrupt the intestinal barrier during enteric nematode infection (Groschwitz et al., 2009), and our results suggest that miR-194 may prevent the dysfunction of the mucosal barrier during the early life of calves (De Winter et al., 2012). miR-205, which is highly expressed in the rumen, may regulate proliferation of cells for the development of rumen during early life. miR-196 family was only detected in the ileum, and its expression varied over time. This miRNA family regulates the proliferation of lymphatic endothelial cells by targeting endothelin receptor type B, insulin-like growth factor 1 and sprouty-related EVH1 domain-containing protein 1. Ileum is a major site for GALT development since it contains the continuous Peyer's patches (PPs) (Mebius, 2003). Thus it may not be surprising to observe high and regional-specific expression of miR-196 in the ileum during early life. Further studies are required to determine if miR-196 expression is associated with GALT, such as PPs and mesenteric lymph nodes. Investigations using the bovine fetus will also be important to further define what factors regulate miR-196 expression since GALT development is initiated during the second trimester of fetal development in the absence of the microbiome.

Previous studies revealed that colonization of GF mice with bacteria modulates host gene expression via miRNAs (Dalmasso et al., 2011). The

exposure of newborn mammals to microbes stimulates developmental changes in the host immune system (Jost et al., 2012), and miRNAs may mediate such host microbial interaction (Masotti, 2012). The observed correlations between expression of miRNAs and density of total bacteria, *Lactobacillus* spp., and *Bifidobacterium* spp. suggest that miRNAs may also be involved in host-microbial cross talk through regulation of mRNA expression in response to changes in the number and composition of the microbial population. Higher numbers of miRNAs were identified to correlated with the copy numbers of total bacteria, *Bifidobacterium* and *Lactobacillus* spp. on D21, indicating that microbial population changes in response to the diet may influence the expression of miRNAs. It is known that diet can alter the composition of gut microbiota (Hildebrandt et al., 2009). In our study, calf starter was introduced to the diet from D14 onwards. Therefore, the diet of D21 calves (milk and calf starter) was different from D7 calves (milk only). These changes in diet were associated with an altered density and composition of *Bifidobacterium* and *Lactobacillus* spp. on D21 and D42. Both *Bifidobacterium* and *Lactobacillus* spp. are beneficial commensal bacteria that stimulate host innate immune responses (Macfarlane and Dillon, 2007) and have been widely used as probiotics (van Baarlen et al., 2013). The expression of some miRNAs such as miR-29, miR-196 and miR-15/16 families were significantly correlated with the number of *Bifidobacterium* and *Lactobacillus* spp. in different ages of calves, revealing a potentially fundamental regulatory mechanisms by which probiotics have an effect. The main function of the miR-29 family is related to dendritic cells (DCs) maturation, the miR-196

family is involved in lymphangiogenesis, and the miR-15/16 family is involved in leukocytes differentiation. DCs maturation and leukocytes differentiation were reported to respond to probiotics treatment in the human gut (Hart et al., 2004). Therefore, miRNAs may mediate host-microbial interaction during microbial colonization by responding to changes (density, composition or the presence) in microbiome, including *Bifidobacterium* or *Lactobacillus* spp. Our correlation analysis revealed an association between the expression of miR-129 and the total bacterial population in the rumen. Based on functional analysis, miR-129 was predicted to be involved in development of digestive system, which suggests that miR-129 may regulate rumen development in response to the increasing bacterial population. Further studies on miRNA expression changes in relation to the host transcriptome and gut microbiome (metagenome and metatranscriptome) needs to be done to provide further evidence for the identified miRNAs playing an important role in mediating host-microbial interactions.

2.5 Conclusions

This is the first study to profile miRNA expression throughout the bovine GIT during the immediate postnatal period of dairy calves. The present study revealed temporal and regional differences in miRNA expression. Based on the predicted targets of DE miRNAs, their functions are mainly involved in mucosal immune system development in the small intestine. Some of the abundant miRNAs, temporally DE miRNAs, regionally DE miRNAs, and miRNAs which are associated with bacterial density were predicted to have functions such as gut

tissue development and immune system development through the development of GIT. Significant correlations between miRNAs and the abundance of gut bacteria suggest that miRNAs may provide a mechanism to respond to microbial colonization and regulate the development of the host mucosal immune system. The extensive small RNA sequencing dataset of bovine GIT tissues generated in the present study provides baseline data for future studies on GIT development and host-microbial interactions. Further studies to correlate miRNA expression and transcriptome of the same tissues are in progress and will provide more evidences on how miRNA and mRNA expressions are integrated to mediate host-microbial interactions. Moreover, future studies are required to determine to what extent these changes are directly influenced by factors such as microbiota, age, and diet.

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2.7 Figures and tables

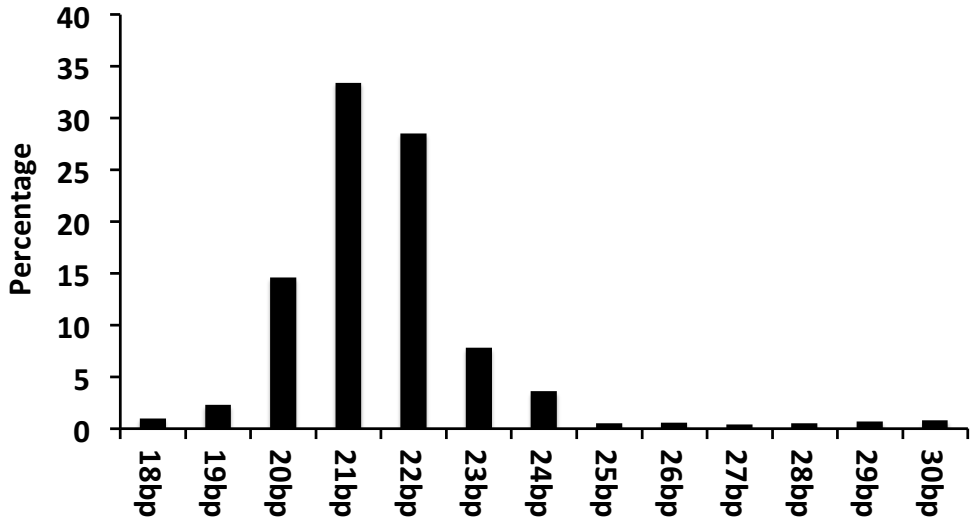

Figure 2.1 Length distribution of small RNA reads.

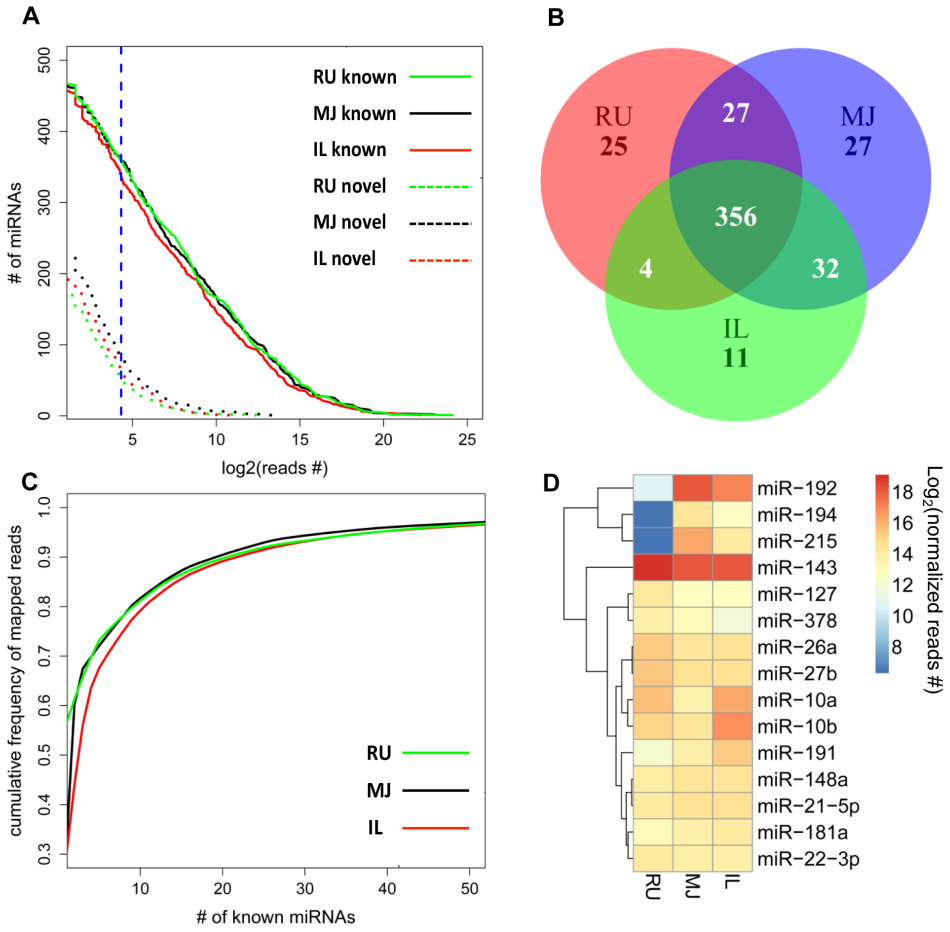

Figure 2.2 Overview of miRNAs detected in the GIT of dairy calves during early life using miRNA sequencing. (**A**). Numbers of known (solid line) and novel miRNAs (dotted line) identified (total reads number for each tissue > 20, blue line). (**B**). Comparison of the number of known miRNAs detected in rumen (RU), mid-jejunum (MJ), and ileum (IL). (**C**). Cumulative frequency of known miRNAs detected in rumen (RU), mid-jejunum (MJ), and ileum (IL). (**D**). Comparative expression of top 10 highly expressed miRNAs in rumen (RU), mid-jejunum (MJ), and ileum (IL) using Heatmap.2 function in R package. Colors represent different normalized sequencing reads number as indicated by the color bar.

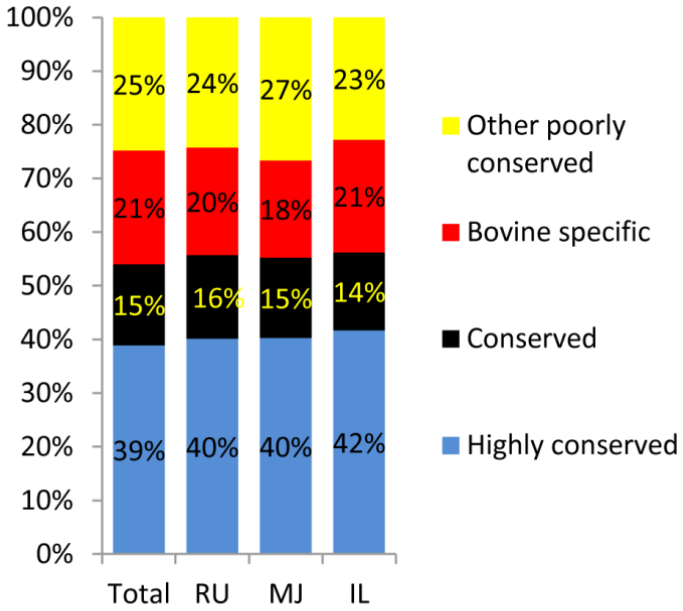

Figure 2.3 Conservation analysis of known miRNAs detected in calves' GIT. Proportion of different miRNAs conservation categories in total identified known miRNAs (Total), in rumen (RU), in mid-jejunum (MJ), and in ileum (IL). Highly conserved (blue bar): conserved across most vertebrates; Conserved (black bar): conserved across most mammals, but usually not beyond placental mammals; Poorly conserved: miRNAs that do not belong to the above two groups, including bovine specific (red bar, belong to poorly conserved group, and seed region sequence only reported in cattle) and other poorly conserved miRNAs (yellow bar).

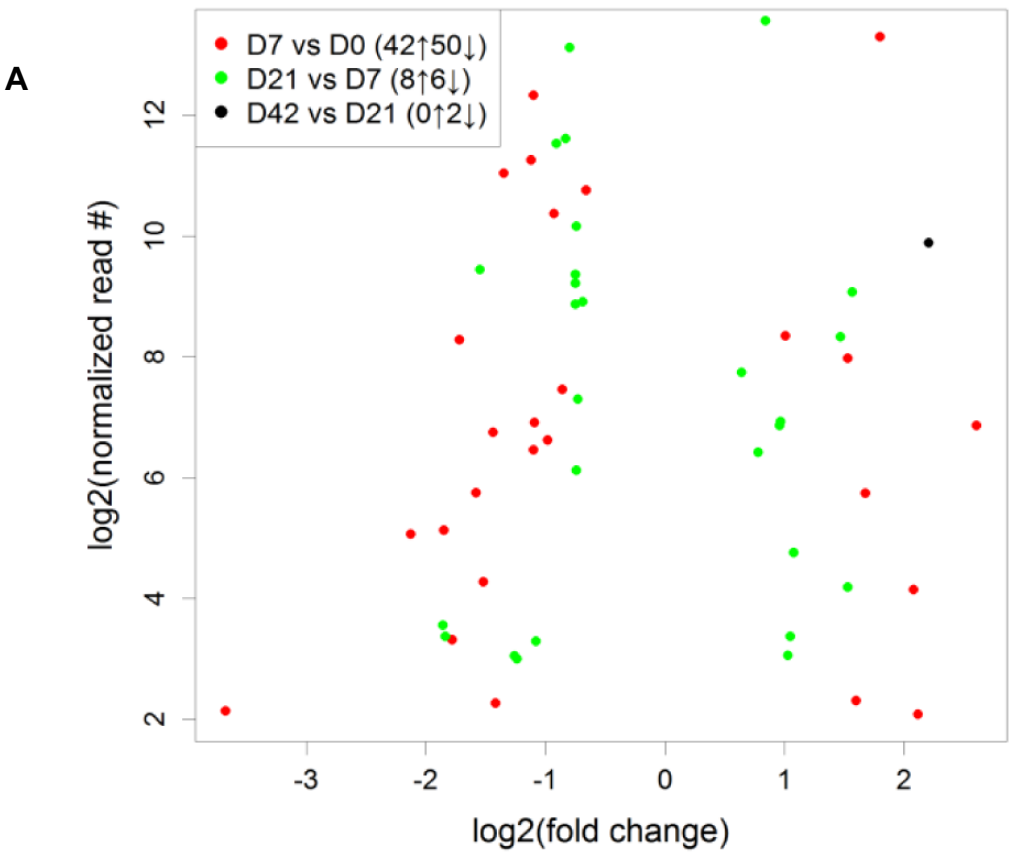

Figure 2.4 Temporally DE miRNAs. Dots represent DE miRNAs in D7 vs. D0 (red), D21 vs. D7 (green), and D42 vs. D21 (black). The X and Y-axes show log2 (fold change) and log2 (normalized reads number) of each DE miRNA, respectively. (**A**). DE miRNAs detected in rumen (RU) tissue. (**B**). DE miRNAs identified in tissue collected from mid-jejunum (MJ). (**C**). DE miRNAs detected in tissue collected from ileum (IL). "↑": the number of up-regulated miRNAs; "↓": the number of down-regulated miRNA.

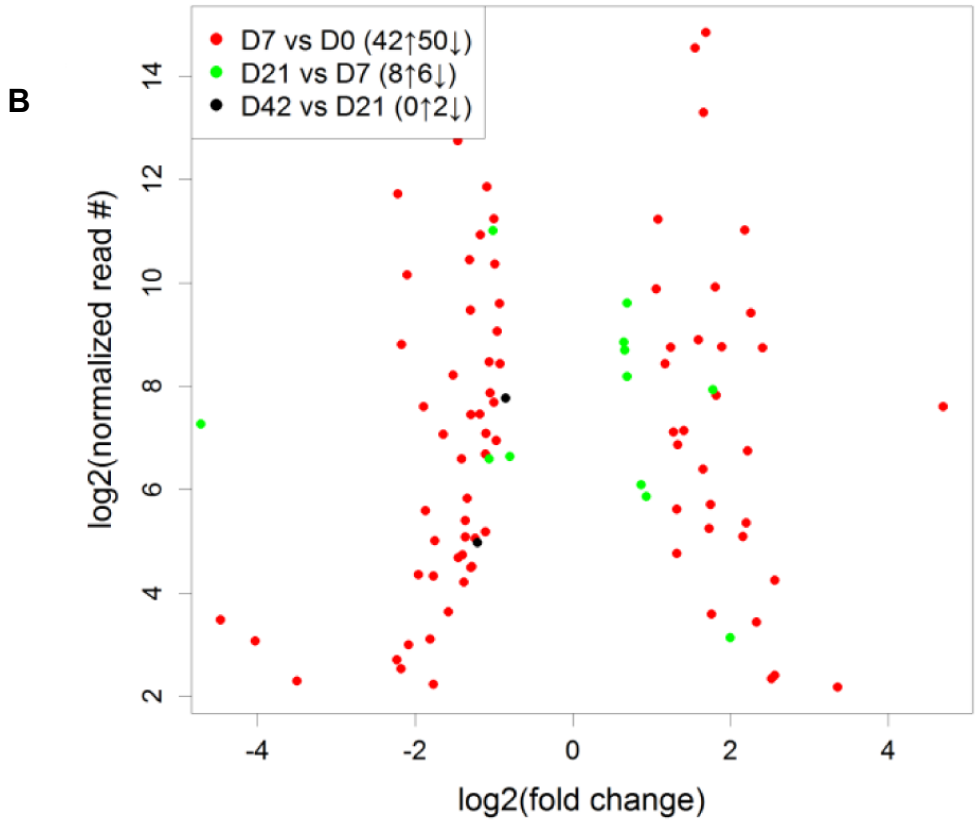

Figure 2.4 (Continued) Temporally DE miRNAs. Dots represent DE miRNAs in D7 vs. D0 (red), D21 vs. D7 (green), and D42 vs. D21 (black). The X and Y-axes show log2 (fold change) and log2 (normalized reads number) of each DE miRNA, respectively. (**A**). DE miRNAs detected in rumen (RU) tissue. (**B**). DE miRNAs identified in tissue collected from mid-jejunum (MJ). (**C**). DE miRNAs detected in tissue collected from ileum (IL). "↑": the number of up-regulated miRNAs; "↓": the number of down-regulated miRNA.

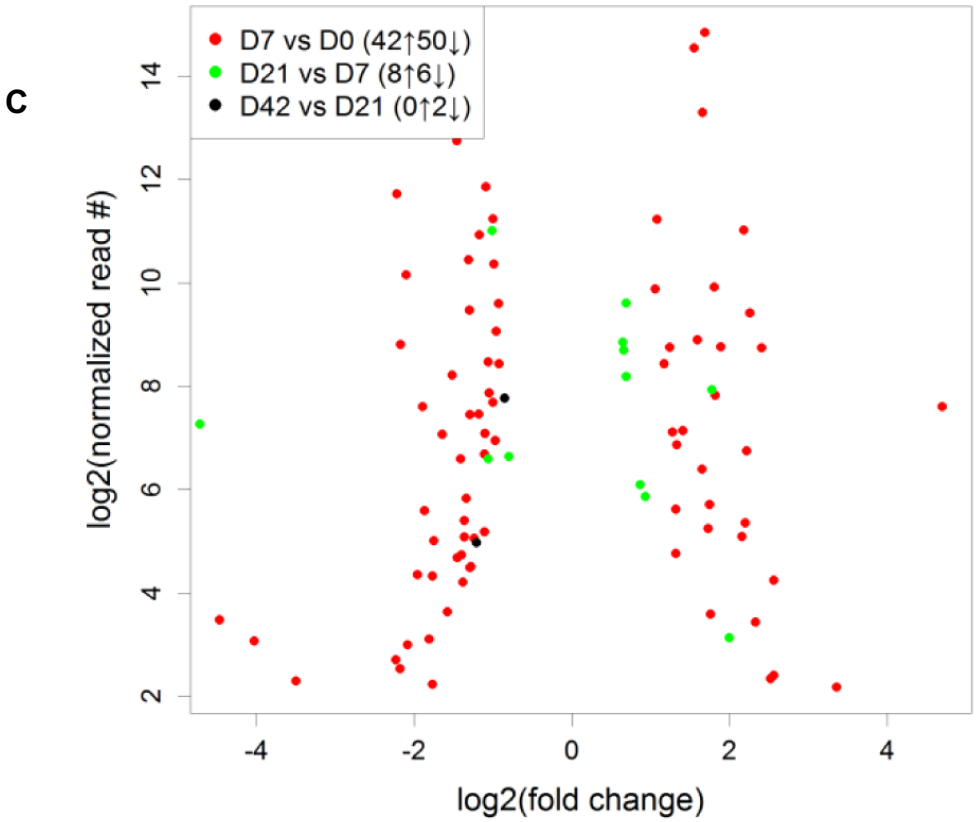

Figure 2.4 (Continued) Temporally DE miRNAs. Dots represent DE miRNAs in D7 vs. D0 (red), D21 vs. D7 (green), and D42 vs. D21 (black). The X and Y-axes show log2 (fold change) and log2 (normalized reads number) of each DE miRNA, respectively. (**A**). DE miRNAs detected in rumen (RU) tissue. (**B**). DE miRNAs identified in tissue collected from mid-jejunum (MJ). (**C**). DE miRNAs detected in tissue collected from ileum (IL). "↑": the number of up-regulated miRNAs; "↓": the number of down-regulated miRNA.

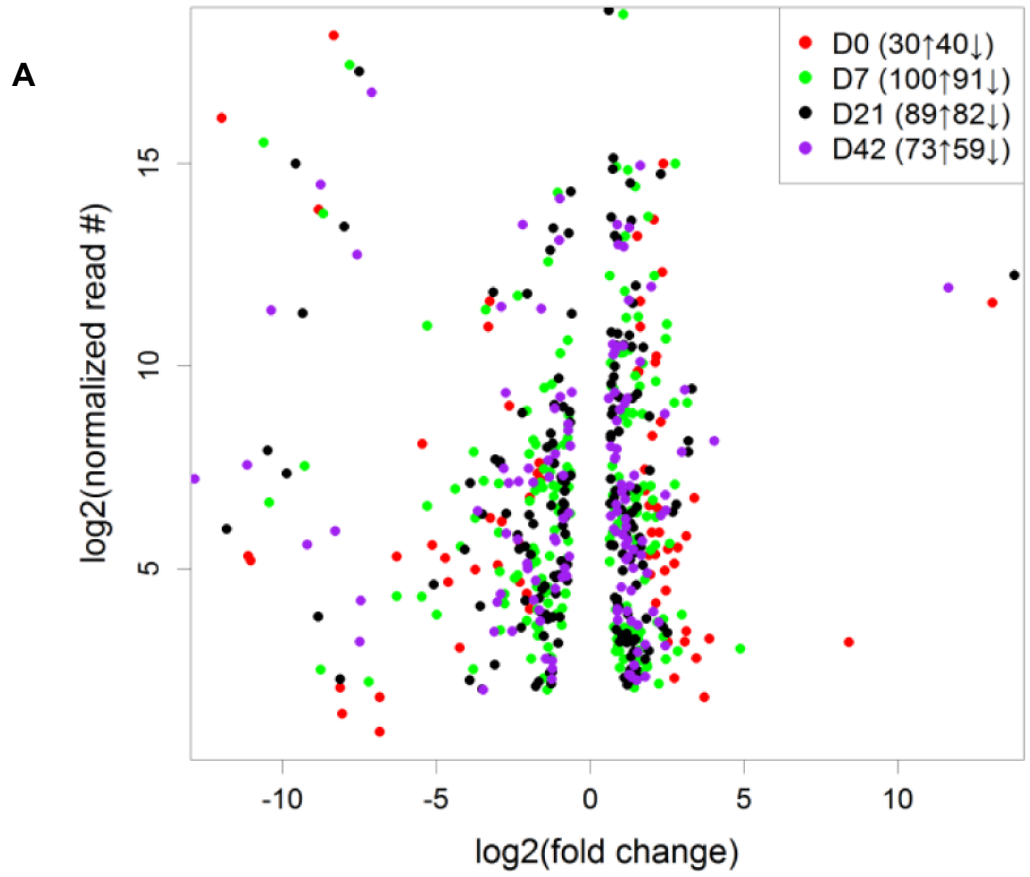

Figure 2.5 Regionally DE miRNA. Dots represent DE miRNAs between different tissues at D0 (red), D7 (green), D21 (black), and D42 (purple). (**A**). Detected DE miRNAs when comparing rumen (RU) *vs.* mid-jejunum (MJ). (**B**). Detected DE miRNAs when comparing rumen (RU) *vs.* ileum (IL). (**C**). Detected DE miRNAs when comparing mid-jejunum (MJ) *vs.* ileum (IL). "↑": the number of up-regulated miRNAs; "↓": the number of down-regulated miRNA.

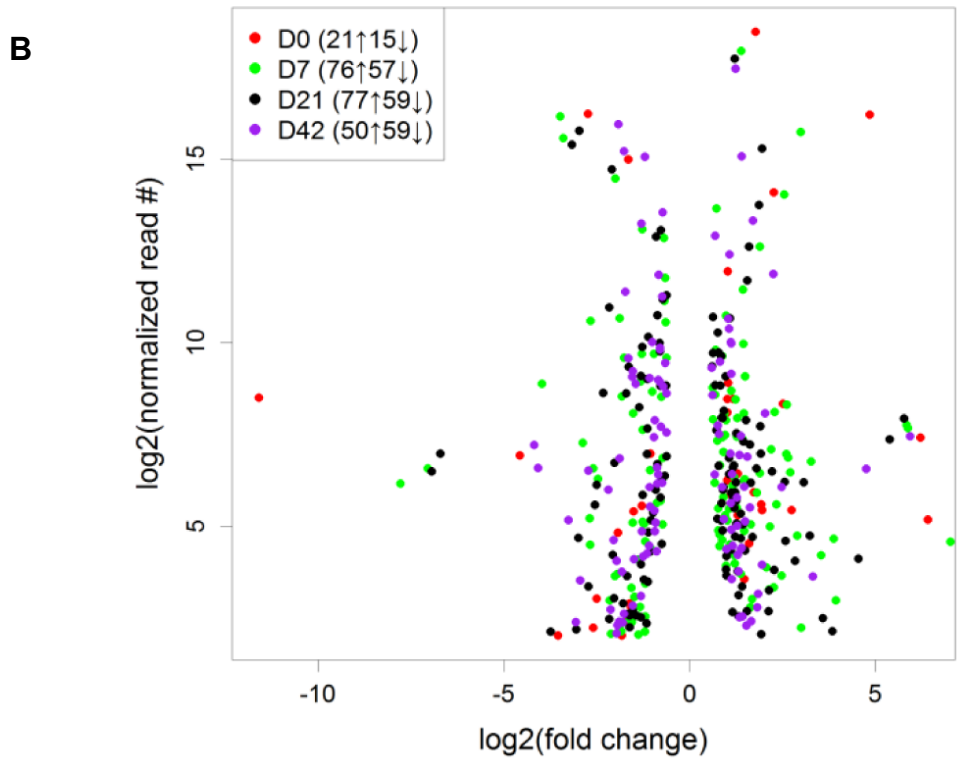

Figure 2.5 (Continued) Regionally DE miRNA. Dots represent DE miRNAs between different tissues at D0 (red), D7 (green), D21 (black), and D42 (purple). (**A**). Detected DE miRNAs when comparing rumen (RU) *vs.* mid-jejunum (MJ). (**B**). Detected DE miRNAs when comparing rumen (RU) *vs.* ileum (IL). (**C**). Detected DE miRNAs when comparing mid-jejunum (MJ) *vs.* ileum (IL). "↑": the number of up-regulated miRNAs; "↓": the number of down-regulated miRNA.

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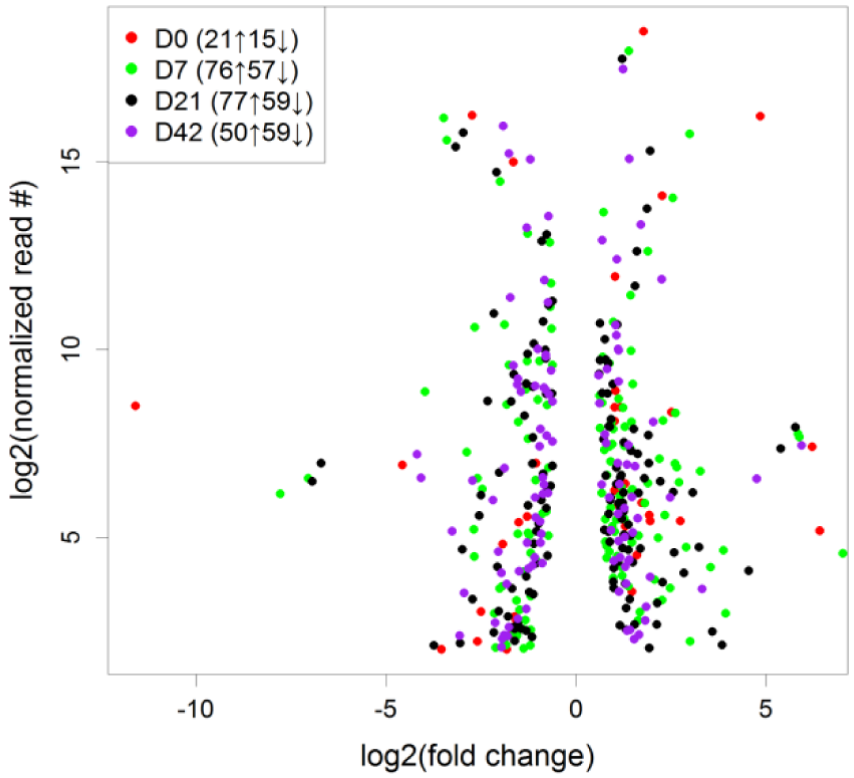

Figure 2.5 (Continued) Regionally DE miRNA. Dots represent DE miRNAs between different tissues at D0 (red), D7 (green), D21 (black), and D42 (purple). (**A**). Detected DE miRNAs when comparing rumen (RU) *vs.* mid-jejunum (MJ). (**B**). Detected DE miRNAs when comparing rumen (RU) *vs.* ileum (IL). (**C**). Detected DE miRNAs when comparing mid-jejunum (MJ) *vs.* ileum (IL). "↑": the number of up-regulated miRNAs; "↓": the number of down-regulated miRNA.

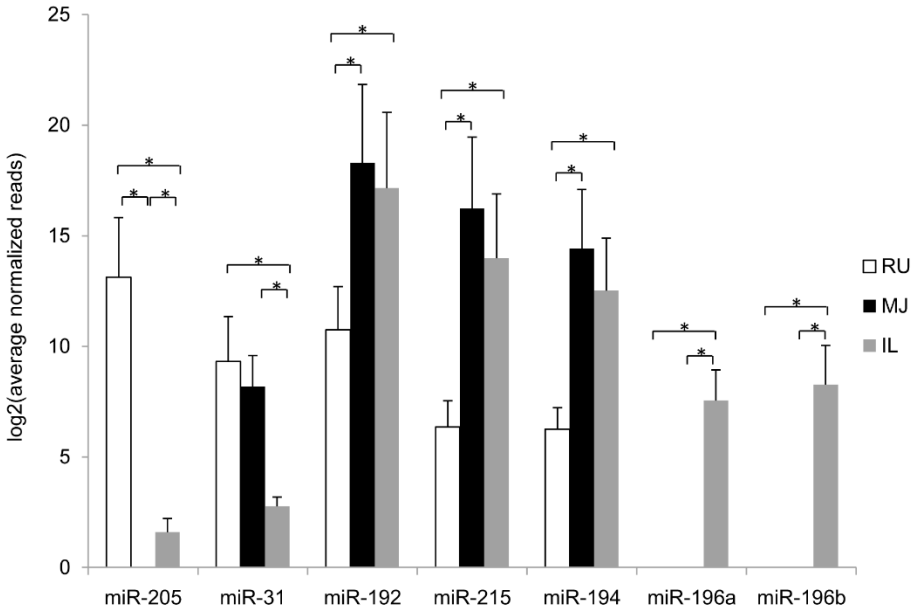

Figure 2.6 The most significantly regionally DE miRNAs. The open bars mean expression levels in rumen (RU), mid-jejunum (MJ, solid black bar) and ileum (IL, solid grey bar). "*" represents miRNA differentially expressed between two tissues (FDR < 0.05, fold change >1.5). No bar is shown when miRNA expression was not detected.

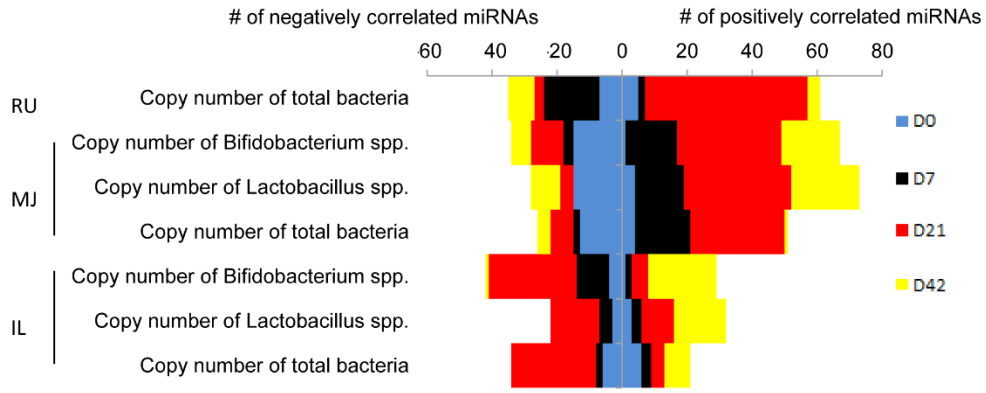

Figure 2.7 Numbers of miRNAs with significant correlations between their expression and the copy number of bacterial 16S rRNA gene as determined by qPCR. The correlation analysis was performed between normalized miRNAs expression and log copy number of total bacteria, *Lactobacillus* and *Bifidobacterium* spp. in each location (RU: rumen; MJ: mid-jejunum; IL: ileum) and at each time point (D0: blue bar; D7: black bar; D21: red bar; D42: yellow bar). The horizontal axis shows the number of miRNAs correlated with microbial density. "# of negatively correlated miRNAs" means the number of miRNAs with a negative correlation (P < 0.05, r <- 0.8) with microbial density. "# of positively correlated miRNAs" means the number of miRNAs with a positive correlation (P < 0.05, r > 0.8) with microbial density.

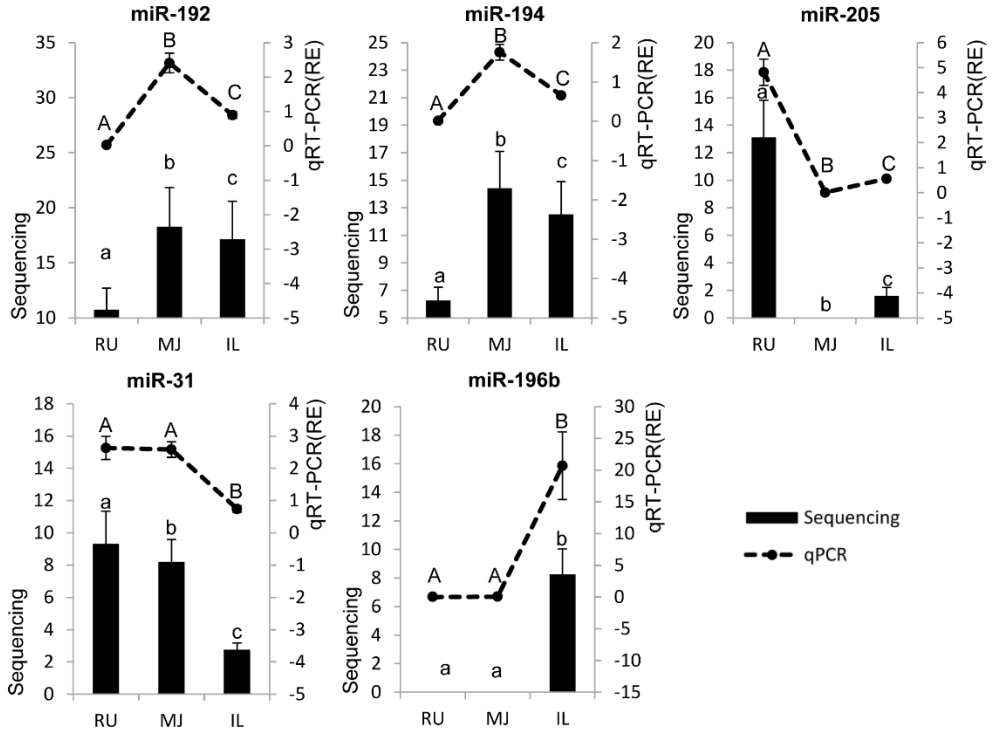

Figure 2.8 Expression of regionally DE miRNAs by RT-qPCR and miRNA-Seq. miRNA expression from RT-qPCR represented by lines on the top and values are shown on the right vertical axis as relative expression (RE). miRNA expression from miRNA-Seq represented by bars on the bottom and values are shown on the left vertical axis as $\log_2$ (normalized reads number). Different letters (A, B, C, a, b, c) on the top of lines or bars indicate significant ($P < 0.05$ or FDR < 0.05) difference between each group. Data are presented as Mean ± Standard deviation. The X-axis shows the names of tissue: RU (rumen), MJ (mid-jejunum), and IL (ileum).

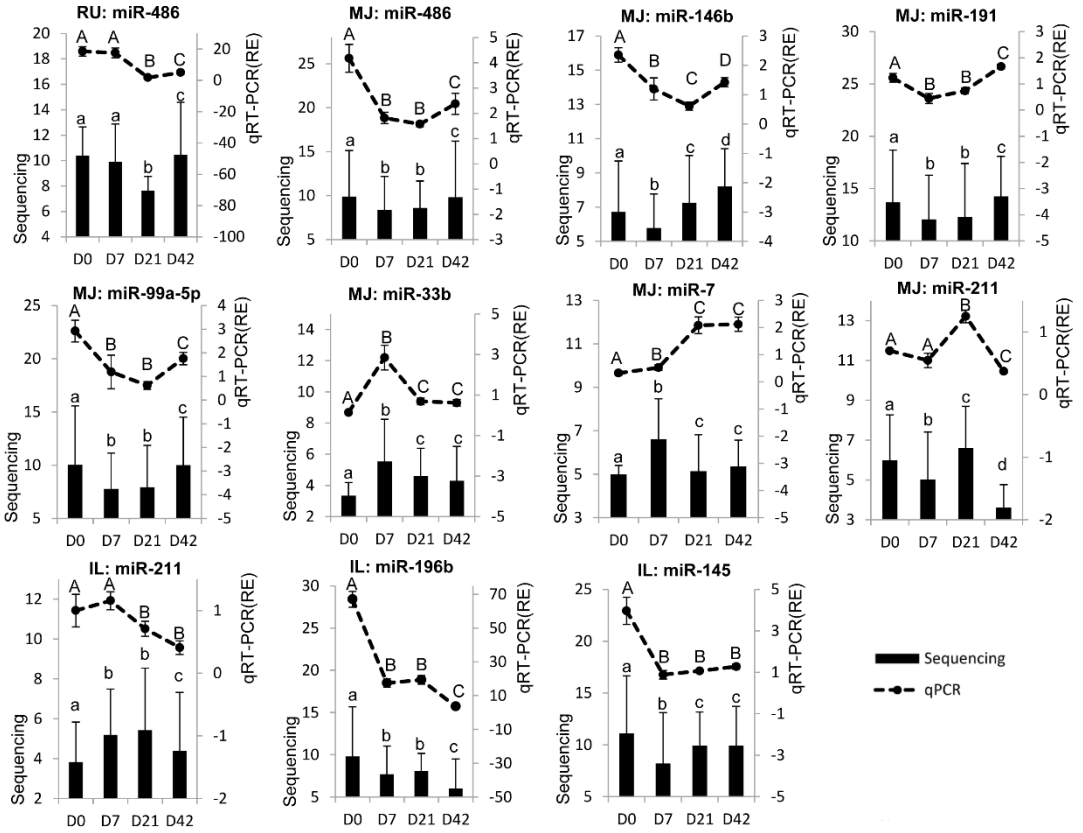

Figure 2.9 Expression of temporally DE miRNAs by RT-qPCR and miRNA-Seq. miRNA expression from RT-qPCR represented by lines on the top and values are shown on the right vertical axis as relative expression (RE). miRNA expression from miRNA-Seq represented by bars on the bottom and values are shown on the left vertical axis as $\log_2$ (normalized reads number). Different letters (A, B, C, D, a, b, c, d) on the top of lines or bars indicate significant ($P < 0.05$ or FDR < 0.05) difference between adjacent groups (D7 *vs* D0, D21 *vs* D7, D42 *vs* D21). Data are presented as Mean ± Standard deviation. The X-axis shows the names of time points. RU: rumen, MJ: mid-jejunum, and IL: ileum.

Table 2.1 Enriched gut development-related and immune-related functions of selected miRNAs.

miRNA families	Functional Category	Functional Annotation	FDR	Molecules #	miRNA Category
miR-143	Cellular Development	Differentiation of Connective Tissue Cells	4.98E-02	3	Predominant miRNA expressed in GIT
miR-146	Cellular Development	proliferation of enterocytes	3.67E-02	2	Temporal DE miRNA in MJ
	Cellular Growth and Proliferation	proliferation of cells	4.47E-02	8	
	Digestive System Development and Function	quantity of Paneth cells	3.67E-02	2	
	Inflammatory Response	TH1 immune response	2.66E-02	3	
	Inflammatory Response	phagocytosis by dendritic cells	3.03E-02	2	
miR-191	Digestive System Development and Function	morphology of intestinal villus	1.44E-02	2	Temporal DE miRNA in MJ
miR-33	Cellular Development	differentiation of cells	4.60E-02	5	Temporal DE miRNA in MJ
	Hematological System Development and Function	accumulation of neutrophils	3.43E-02	2	
	Inflammatory Response	phagocytosis of cells	2.54E-02	4	
miR-7	Cellular Development	Differentiation of Muscle Cells	4.99E-02	2	Temporal DE miRNA in MJ
miR-99/100	Cellular Development	Differentiation of Cells	3.35E-02	6	Temporal DE miRNA in MJ
	Hematological System Development and Function	Differentiation of T Lymphocytes	4.66E-02	2	
miR-486	Connective Tissue Development and Function	Proliferation of Fibroblast Cell Lines	4.53E-02	4	Temporal DE miRNA in RU and MJ
miR-145	Hematological System Development and Function	Differentiation of Leukocytes	4.92E-02	20	Temporal DE miRNA in IL
miR-211	Hematological System Development and Function	Differentiation of T Lymphocytes	3.64E-03	17	Temporal DE miRNA in MJ and IL
	Inflammatory Response	Il-6 Signaling	2.45E-02	3	
	Inflammatory Response	Il-17 Signaling	1.58E-02	2	
miR-194	Hematological System Development and Function	Colony Formation of Mast Cells	2.41E-02	2	Regional DE miRNA (RU vs IL; RU vs MJ)
miR-192/215	Hematological System Development and Function	Differentiation of Leukocytes	4.71E-02	8	
	Hematological System Development and Function	Expansion of Leukocytes	4.62E-02	5	Temporal DE miRNA in MJ; Regional DE miRNA (RU vs IL; RU vs MJ)
	Lymphoid Tissue Structure and Development	Proliferation of Lymphatic System Cells	4.42E-02	6	
	Hematological System Development and Function	Expansion of Leukocytes	4.62E-02	5	

	Lymphoid Tissue Structure and Development	Organogenesis of Lymphatic System Component	4.62E-02	2	
miR-129	Digestive System Development and Function	Development of Digestive System	1.21E-02	2	Positive correlated with total bacterial density in RU
miR-15/16	Cellular Growth and Proliferation	Proliferation of Cells	2.45E-02	16	
	Hematological System Development and Function	Development of Leukocytes	4.39E-02	12	
	Hematological System Development and Function	Development of Lymphocytes	2.72E-02	10	Negatively correlated with the density of *Bifidobacterium* species on D21 in IL
	Hematological System Development and Function	T Cell Development	3.71E-02	11	
	Cellular Development	Differentiation of Cells	2.45E-02	6	
	Hematological System Development and Function	Development of B Lymphocytes	1.58E-02	7	
miR-29	Cellular Development	Differentiation of Epithelial Cells	2.29E-02	7	
	Connective Tissue Development and Function	Proliferation of Fibroblasts	2.59E-02	7	Positively correlated with the density of both *Bifidobacterium* and *Lactobacillus* species on D42
	Connective Tissue Development and Function	Quantity of Connective Tissue Cells	2.29E-02	6	
	Hematological System Development and Function	Maturation of Dendritic Cells	4.75E-02	2	
miR-196	Cellular Growth and Proliferation	Proliferation of Epithelial Cells	3.84E-02	4	Regional DE miRNA (RU vs IL; IL vs MJ); Temporal DE miRNA in IL; positively correlated with the density of both *Bifidobacterium* and *Lactobacillus* species on D21
	Lymphoid Tissue Structure and Development	Proliferation of Lymphatic Endothelial Cells	4.63E-02	2	
	Inflammatory Response	Morphology of Phagocytes	4.86E-02	2	

Chapter 3. Transcriptome analysis reveals regional and temporal difference of mucosal immune functions in the neonatal bovine small intestine

3.1 Introduction

Calves are susceptible to a variety of bacterial and viral enteric infections during the neonatal period, due to their undeveloped immune system and lacking of passive immunity during fetal period (Cho and Yoon, 2014). The intestinal mucosal immune system comprises various organized lymphoid tissues and immune cells, such as Peyer's patches (PPs), T and B cells, and is one of the first lines to protect the host from enteric infection by providing physical barriers, activating innate and adaptive immune responses (McGhee and Fujihashi, 2012). After birth, neonatal intestinal mucosal immune system undergoes rapid development in response to the microbial colonization, and environmental factors including toxins and dietary antigens and so on (Tourneur and Chassin, 2013). A well-developed intestinal mucosal immune system can tolerate commensal microbes, and can recognize the pathogenic microbes to protect the host from being infected (Tourneur and Chassin, 2013), which is vital for maintaining the health of neonatal animals. Although intestinal mucosal immune system development has been extensively studied in mice and humans (Renz et al., 2012), substantially less is known about postnatal development of intestinal mucosal immunity in calves. Understanding the development of mucosal immune

function in the intestine of newborn calves is critical for developing more effective strategies to improve their gut health.

Compared to monogastric animals, the ruminant intestinal mucosal immune system displays several unique developmental features. For example, it contains two distinct types of PPs in different locations of the small intestine (Griebel and Hein, 1996). While the continuous PPs in the ileum (IL) function primarily as a site for the generation of the pre-immune B cell, the discreet PPs in the jejunum (JE) function as induction sites of the generation of IgA plasma cells (Mutwiri et al., 1999). Such regional differences can lead to the different capacities for jejunum and ileum to respond to microbes. Ileum has been the mostly studied and considered as the primary site of most enteric infections during neonatal period of dairy calves, such as *Salmonella* infection (Tsolis et al., 1999) and *Mycobacterium avium* subspecies *Paratuberculosis* infection (Ponnusamy et al., 2013). However, the role of jejunum in enteric infection is not well studied. In addition, maternal antibodies are transferred to calf mainly through colostrum feeding during the first 24 hours of life rather than through the placenta during fetal period that normally occurs in humans and mice (Chucri et al., 2010), suggesting that the postnatal developmental process of the intestinal immune system in calves may be different from other animals. Collectively, knowledge on the regional and temporal difference of intestinal immune system in neonatal calves is important for understanding their immune competence. It has been suggested that the full complement of the mucosal immune system includes

innate and adaptive immune system as well as physical barriers (Taschuk and Griebel, 2012).

Previous studies have shown significant temporal changes in the number and distribution of mucosal leukocyte populations (6-month-old vs 3- to 5-week-old) (Fries et al., 2011), as well as in the mRNA expression of Toll-like receptors and antimicrobial peptides (6-month-old vs 3-week-old) (Malmuthuge et al., 2012) when comparing weaned versus pre-weaned ones. In addition, a recent study described significantly dynamic changes in intestinal microRNAs (miRNAs) expression in calves during the postnatal period and their involvement in the regulation of mucosal immune system development (Liang et al., 2014). However, the molecular mechanisms involved in the regulation of the regional and temporal differences of the intestinal mucosal immune system in neonatal calves (especially within the first few weeks of life) are still unclear. Identifying regulatory relationships between miRNAs and their corresponding mRNA targets is critical for interpreting the development processes of small intestine in neonatal calves.

Therefore, in this study, we aimed to characterize the regional and temporal changes of intestinal function of neonatal calves by analyzing global changes of gene expression (transcriptome) in jejunum and ileum from birth till 42 days postpartum with a specific focus on genes related to the mucosal immune system. Furthermore, an integrated analysis of expression of miRNAs and mRNAs were performed to determine the potential regulatory roles of miRNAs during this process.

3.2 Materials and methods

3.2.1 Animals and tissue sample collection

Tissue samples from the jejunum and ileum were collected from male Holstein calves at 30 min after birth (D0; n = 3), 7 days postpartum (D7; n = 5), 21 days postpartum (D21; n = 5) and 42 days postpartum (D42; n = 5) (Dairy Research and Technology Center, University of Alberta). All experimental protocols were reviewed and approved by the Livestock Animal Care Committee of the University of Alberta (protocol no. AUP00001012) and all procedures were conducted following the guidelines of the Canadian Council on Animal Care. All samples were collected within 30 min after calves were euthanized using captive bolt gun. The D0 samples were collected from newborn calves within 30 min after delivery and without ingestion of colostrum. All the other calves were fed with 2 L of colostrum within 2 hours after birth, and fed with another 2 L of colostrum at 12 hours after birth. The D7 calves were fed 4 L milk/day and D21 and D42 calves were fed 4 L milk/day with ad libitum access to calf starter (23% CP and 4% ether extract as the guaranteed minimum, 19.5% NDF, 27.1% starch; Wetaskiwin Co-Op Country Junction, Wetaskiwin, AB, Canada). The jejunum was defined as 100 cm distal to the pyloric sphincter and a 10 cm segment of intestine was collected. The ileum was defined as the 30 cm segment proximal to the ileocecal fold and a 10 cm segment of tissue was collected. All tissue samples, with the exception of D0 samples, were rinsed three times with sterile phosphate buffered saline (PBS, pH = 7.0) buffer to remove ingesta. Tissues were snap-frozen in liquid nitrogen and stored in -80°C.

3.2.2 RNA isolation

Tissue samples were ground into powder while immersed in liquid nitrogen in frozen mortar prior to RNA extraction. Total RNA was extracted from 80 mg of tissue powder using mirVana™ miRNA Isolation Kit (Ambion, Carlsbad, CA) following the manufacturer's instructions. The quality and quantity of the RNA were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), respectively. RNA samples with good quality (integrity number (RIN) > 7.0) were used for further analysis.

3.2.3 RNA-Seq library construction and sequencing

Total RNA (1.0 μg) from each sample was used to construct RNA-Seq libraries with an unique index using the TruSeq mRNA Sample Preparation kit (Illumina, San Diego, CA) according to the manufacturer's instruction. Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) was performed for library quantification. cDNA libraries were sequenced at Génome Québec (Montréal, Canada) using the Illumina HiSeq 2000 system (Illumina). Sequencing was performed as 100 bp paired-end reads. All reads were demultiplexed according to their index sequences with CASAVA version 1.8 (Illumina) and reads that did not pass the Illumina chastity filter were discarded.

3.2.4 RNA-Seq reads mapping and annotation

RNA-Seq reads were aligned to the bovine genome (UMD 3.1) using Tophat 2.0.10 with default parameters (Kim et al., 2013). The number of reads mapped to each gene was counted by htseq-count (http://www-

huber.embl.de/users/anders/HTSeq/) based on the annotation from ENSEMBL (http://uswest.ensembl.org/) bovine gene annotation v75.30. The expression levels of mRNAs in each sample was calculated by normalizing reads number to counts per million reads (CPM) by the following formula: CPM = (gene reads number / total mapped reads number per library) × 1,000,000.

3.2.5 Identification of differentially expressed genes

Identification of differentially expressed (DE) genes was performed using edgeR (Robinson et al., 2010). Regionally DE genes were identified by comparing mRNA expression of the jejunum with the mRNA expression of ileum at each time point (D0, D7, D21, and D42). Temporally DE genes were identified via comparing any two adjacent developmental stages (D7 *vs* D0; D21 *vs* D7; D42 *vs* D21). The DE mRNAs were identified with a false discovery rate (FDR) < 0.05 based on Benjamini and Hochberg multiple testing correction (Benjamini and Hochberg, 1995) as well as fold change ≥ 2.

3.2.6 Analysis of genes related to immune system and barrier function

The immune-related genes list was obtained from ImmPort database (Bhattacharya et al., 2014). Genes encoding the subunits of Cluster of Differentiation 3 (CD3D, CD3E and CD3G) and CD247 were selected as lineage-specific markers for T cells (Risueno et al., 2006; Byers et al., 2008), while genes encoding CD79A and CD79B were selected as lineage markers for B cells (Luger et al., 2013). The gene encoding forkhead box P3 (FOXP3) was selected as a lineage-specific marker for regulatory T cells (Tregs) (Chen and Oppenheim, 2011). Genes that were belonged to tight junction (TJ) protein families were

selected for further analysis, such as claudin (CLDN) family, zonula occluden (ZO) family, and junctional adhesion molecule (JAM) family. Genes coding β-defensin (DEFB) and regenerating islet-derived 3 γ (REG3G) were selected to analyze antimicrobial peptides (AMPs) gene expression pattern (Bevins and Salzman, 2011). The genes coding Toll-like receptors (TLRs) and NOD-like receptors (NLRs) were selected to analyze the innate immune sensing-related genes. The cytokine list was obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation "Cytokine-cytokine receptor interaction".

3.2.7 Functional analysis

The functional analysis of temporal DE genes was performed by Ingenuity pathway analysis (IPA, Ingenuity Systems, www.ingenuity.com). A threshold of $P < 0.01$ was applied to enrich significant biological functions. All the GO terms and KEGG pathways enrichment were performed using Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov) (Huang da et al., 2009). Each analysis was performed using the functional annotation clustering option, and significant GO terms and KEGG pathways were selected at $P < 0.05$ and enriched gene number ≥ 2.

3.2.8 Integrated analysis of miRNA and mRNA expression

All the results for miRNAs were obtained from Chapter 2, which utilized the same samples to profile miRNA expression (Liang et al., 2014). The DE miRNAs and DE mRNAs in D7 *vs* D0 were used in this study. The regulatory relationship between DE miRNAs and DE mRNAs was identified based on two criteria: computational target prediction and the DE miRNAs have opposite

expression patterns with their predicted targets. Target genes of miRNAs were predicted by TargetScan Release 6.0 (default parameters, http://www.targetscan.org/) and miRanda (total score ≥ 145, total energy ≤ -10 kcal/mol, http://www.microrna.org/microrna). Furthermore, the miRTarBase was used to identify the experimentally validated miRNA-mRNA regulatory pairs (Hsu et al., 2011).

3.2.9 Experimental validation of mRNA expression by reverse transcription quantitative PCR

A total of 12 genes were selected to validate their regionally and temporally DE patterns. Claudin 1 (CLDN1), CLDN4 and OCLN from TJ protein genes; TLR2, TLR4, TLR6 and TLR10 from TLRs gene family; FOXP3 as Treg marker; Interleukin 8 (IL8), IL10, transforming growth factor β (TGFB) and tumor necrosis factor α (TNFA) from cytokines; were selected for reverse transcription quantitative PCR (RT-qPCR) expression analysis. All the primer information was summarized in Table 3.1. All protocols were performed as described in a previous study (Malmuthuge et al., 2012). The qPCR performed using SYBR Green (Fast SYBR® Green Master Mix; Applied Biosystems) to detect mRNA relative expression. Fluorescence signal was detected with StepOnePlus™ Real-Time PCR System (Applied Biosystems). Relative gene expression (ΔCq value) was calculated based on quantification cycle (Cq) of reference gene (β-actin) and target gene ($\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{reference gene}}$).

3.2.10 Correlation analysis between host gene expression and total bacterial number

The ΔCq values obtained from qPCR results and the 16S rRNA gene copy numbers of total bacteria in the small intestinal content and tissue obtained from a previous study (Liang et al., 2014) was subjected to correlation analysis. Pearson's correlations were performed between host gene expression and total bacterial number in the content as well as between host gene expression and total bacterial number in the tissue at different ages. R software (version3.0) was used to calculate the correlation coefficient (r) and *P* value.

3.3 Results

3.3.1 Small intestinal transcriptomes of calves

A total of 1,350 million high-quality 100 bp paired-end reads were obtained from 36 libraries (jejunum and ileum samples from male Holstein calves at birth (D0; n = 3), 7 days postpartum (D7; n = 5), 21 days postpartum (D21; n = 5) and 42 days postpartum (D42; n = 5)), with an average of 38.2 ± 8.3 million reads per library. Of all the reads, ~81.5% from the jejunum and ~82.5% from the ileum, were mapped to the bovine genome (UMD 3.1). Based on the normalized data, expression of 15,362 and 15,644 genes were detected (CPM (counts per million mapped reads) > 1 in at least 50% of samples) in the jejunum and ileum, respectively. Among these genes, 15,007 genes were commonly detected in both jejunum and ileum (Figure 3.1A). The most relevant gene ontology (GO) terms of commonly expressed genes were "metabolic process" and "protein transport".

3.3.2 The regionally and temporally differentially expressed genes in the small intestines of calves

The generated transcriptome profiles revealed a clear clustering pattern depending on the small intestinal region regardless of calf age (Figure 3.1B). When the regional difference was further explored within each age category using edgeR (Robinson et al., 2010) (fold change > 2, false discovery rate (FDR) < 0.05), a total of 479 genes showed higher expression in the jejunum than that in the ileum (JE-enriched, Figure 3.1C), whereas 343 genes had higher expression in the ileum than that in the jejunum (IL-enriched, Figure 3.1D) throughout the four age groups. Functional classification showed that 30% and 14% of the JE-enriched genes were related to "metabolic process" and "transport" (Figure 3.1C), while fewer IL-enriched genes (21% and 7%) was related to these two functions (Figure 3.1D). Further, 4% and 6% of JE-enriched and IL-enriched genes, respectively, were related to "immune system process".

In addition to the regional variations observed, the transcriptome profiles displayed rapid temporal changes within small intestinal regions during the first six weeks of life. These temporal changes were investigated by comparing the expression of genes between two adjacent ages (D7 *vs* D0; D21 *vs* D7; D42 *vs* D21). All of the temporally differentially expressed genes were categorized into different expression patterns depending on their expression during the first six weeks of life (Figure 3.2). The expression patterns of jejunal DE genes were categorized into 26 patterns (Figure 3.2A), while 23 patterns were observed for that of ileal DE genes (Figure 3.2B) with "U", "D" and "N" representing the

genes upregulated, downregulated, and not differentially expressed, respectively. The order of the combination of them represented the expression pattern change following the comparisons D7 *vs* D0, D21 *vs* D7 and D42 *vs* D21. For example, genes that were categorized into expression pattern "UND" means that these genes were upregulated in D7 *vs* D0, unchanged in D21 *vs* D7, and downregulated in D42 *vs* D21. From all the patterns observed, three patterns made up of 96% of genes for both tissues. The largest numbers of genes had unchanged expression (pattern "NNN") during the first six weeks with 80% of total expressed genes in the jejunum (Figure 3.3) and 86% of total expressed genes in the ileum (Figure 3.3). The next largest number of genes belonged to patterns UNN" and "DNN" and displayed changed expression only during the first week of life with 8% each of total expressed genes in the jejunum and 3% and 7% of total expressed genes in the ileum (Figure 3.3).

3.3.3 Systematic analysis of regional and temporal difference in the expression of immune-related genes

To understand the regionality and temporality of mucosal immune system development in the calf small intestine, a total of 3,314 and 3,306 expressed immune-related genes (CPM > 1 in at least 50% samples) in the jejunum and ileum, respectively, were subjected to further analysis. Similar to the whole transcriptome profiles (Figure 3.1B), the expression of immune-related genes also displayed a clear separation between jejunum and ileum based on PCA analysis (Figure 3.4A). When the expression of these genes was compared between the two regions, 214 genes (105 JE-enriched and 109 IL-enriched) were identified as

regional DE immune-related genes. The KEGG pathway analysis showed that the JE-enriched immune-related genes were mainly related to “complement and coagulation cascades”, whereas the IL-enriched immune-related genes were mainly relevant to “B cell receptor signaling pathway”.

The above expressed immune-related genes within each small intestinal region were then compared among four ages to understand their temporal expression variations. The expression of immune-related genes at birth was different from three other developmental stages in both jejunum (Figure 3.4B) and ileum (Figure 3.4C) as highlighted by the orange circle. Further analysis of the expression patterns of temporally DE immune-related genes showed that 867 were DE and 2,431 were unchanged in the jejunum, while 605 were DE and 2,689 were unchanged in the ileum during three postnatal periods (D7 *vs* D0, D21 *vs* D7 and D42 *vs* D21). The KEGG pathway analysis showed that the temporally DE genes in the jejunum were mainly related to “complement and coagulation cascades” and “cytokine-cytokine receptor interaction”, while the temporally DE genes in the ileum were mainly related “cytokine-cytokine receptor interaction”, “ECM-receptor interaction” and “focal adhesion”.

3.3.4 Expression changes in genes encoding tight junction proteins and antimicrobial peptides in the calf small intestine

In total, the expression of 16 genes encoding TJ proteins including claudin family (CLDN1, CLDN2, CLDN3, CLDN4, CLDN5, CLDN7, CLDN11, CLDN12, CLDN15, and CLDN23), occludin (OCLN), zonula occluden family (ZO1, ZO2, and ZO3) and the junctional adhesion molecule family (JAM2 and

JAM3) were detected in calf jejunum and ileum. The expression of these TJ protein genes were clustered by the small intestinal regions, due to the higher expression levels in the jejunum compared to those in the ileum (Figure 3.5A). The DE TJ protein genes analysis also revealed that CLDN2, CLDN3, CLDN4, CLDN7, CLDN15, CLDN19, CLDN23, OCLN, ZO1, ZO2 and ZO3 were JE-enriched genes, while no IL-enriched genes were identified (Figure 3.5B). Among these DE genes, CLDN15 was highly expressed in jejunum throughout all the ages (Figure 3.5B). In addition, the expression of CLDN3, CLDN4, CLDN5 and CLDN15 was upregulated at D7 when compared to D0 in the jejunum but no further change after D7 (Figure 3.5C), while none of these genes were temporally DE in the ileum.

For genes encoding antimicrobial peptides (AMPs), the expression of β-defensin (DEFB, including DEFB1, DEFB4A, DEFB5, DEFB7, DEFB117, DEFB405) and REG3G were detected by RNA-Seq (Figure 3.5D). Between two regions, DEFB405, DEFB5 and DEFB7 were IL-enriched genes, while expression of REG3G was JE-enriched throughout the four age groups (Figure 3.5E). Besides, REG3G was upregulated from D0 to D7 but no further increase after D7 in both the jejunum and the ileum (Figure 3.5F). No DEFB genes were identified as temporally DE genes.

3.3.5 Toll-like receptor and NOD-like receptor gene expression patterns

The expression of TLR family genes including TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR9, and TLR10, and NLR family genes including NOD1, NOD2, NLRC4, NLRC5, NLRP1, NLRP3, NLRP6, and NLRX1 were detected

by RNA-Seq (Figure 3.6A). Between two regions, NLRP6 was a JE-enriched gene, while TLR2, TLR4, TLR6, TLR9, TLR10, NLRP3, NLRP13, NOD1 and NOD2 were IL-enriched at different ages (Figure 3.6B). When the expression was compared under different ages, NLRP3 was temporally DE in both jejunum and ileum and was significantly higher at D7 comparing to D0 with no further change after D7 (Figure 3.6C). No TLR genes were identified as temporally DE genes.

3.3.6 Expression patterns of T and B cell lineage-specific genes and IgA production-related genes

Among the detected T cell lineage-specific markers, no temporal or regional effects were observed for the expression of CD3D, CD3E, CD3G and CD247 (Figure 3.7A), while a significant increase was found for FOXP3 in both jejunum and ileum only during the first week of life (Figure 3.7B). For the expression of detected B cell lineage-specific genes, CD79A and CD79B were IL-enriched genes (Figure 3.7C) and their expression increased significantly with the increased ages in the jejunum (Figure 3.7C), and no temporal changes were observed for ileum (Figure 3.7C).

For genes encoding immunoglobulins, the expression of immunoglobulin J chain (IGJ) and polymeric immunoglobulin receptor (PIGR) was detected by RNA-Seq in both jejunum and ileum. IGJ showed higher expression in the ileum at D0 when compared to the jejunum, and then showed higher expression in the jejunum since D21 (Figure 3.7D). Moreover, PIGR was a JE-enriched gene since D7 (Figure 3.7D). Both IGJ and PIGR showed remarkably increased expression

when compared D7 to D0, and the expression of IGJ continued to increase until D21 in the jejunum (Figure 3.7E) with none of them temporally DE in ileum.

3.3.7 Altered expression pattern of cytokine genes

The expression of IL10, IL8, IL18, IL7, IL15, TNFA and TGFB were detected by RNA-Seq in the small intestine of calves. IL8, IL10, TGFB and TNFA were identified as IL-enriched genes, whereas, IL15 was a JE-enriched gene (Figure 3.7F). The expression of IL8, IL18 and TNFA increased within the first week in the jejunum but no further increase from D7 (Figure 3.7G). Similarly, the expression of IL8 increased within the first week and no changes was detected after D7 in the ileum (Figure 3.7H).

3.3.8 Integrated analysis of miRNA and mRNA expression

From our previous study, most of temporally DE miRNAs were identified in D7 vs D0 comparison from the same samples (Liang et al., 2014). In this study, we only focused on the regulatory roles of miRNAs during the first week due to the dynamic changes of gene expression at the same time period. The regulatory relationship between DE miRNAs (obtained from our previously published paper (Liang et al., 2014)) and DE mRNAs was identified based on two criteria: computational target prediction and the DE miRNAs have opposite expression patterns with their predicted targets. In total, 3206 miRNA-mRNA (83 miRNAs and 1180 mRNAs) pairs and 1367 miRNA-mRNA (73 miRNAs and 570 mRNAs) pairs were identified in the jejunum and ileum, respectively. The miRTarBase was used to further identify the experimentally validated miRNA-mRNA regulatory pairs (Hsu et al., 2011). In total, 36 miRNA-mRNA (10

miRNAs and 36 mRNAs) pairs and 10 miRNA-mRNA (4 miRNAs and 10 mRNAs) pairs haven been experimentally validated in the jejunum and ileum, respectively (Table 3.2). CLDN1, CLDN4, IL8 (the targets of miR-335) and NLRP3 (the target of miR-100) were related to the barrier and mucosal immune functions (Table 3.2). When the expression was compared, the expression of miR-335 was downregulated (CPM from 66.8 ± 11.9 to 22.3 ± 8.9), and its targets, CLDN1 (CPM from 2.1 ± 0.8 to 6.1 ± 1.3) and CLDN4 (CPM from 44.2 ± 1.9 to 88.2 ± 12.6), showed upregulation in ileum within the first week. Meanwhile, miR-335 was also predicted to target IL8 (CPM from 6.0 ± 0.2 to 18.0 ± 4.9), which was upregulated in the ileum within the first week. In addition, miR-100 (CPM from 1600.8 ± 61.1 to 745.9 ± 57.0) was downregulated while its predicted target gene, NLRP3 (CPM from 6.5 ± 2.3 to 27.2 ± 7.5), was upregulated in the jejunum.

3.3.9 Reverse transcription quantitative real-time PCR validation of differentially expressed mRNAs

CLDN1 and CLDN4 from TJ proteins; TLR2, TLR4, TLR6 and TLR10 from TLRs gene family; FOXP3 as Treg marker; IL8, IL10, TGFB, TNFA from cytokines were selected for reverse transcription quantitative real-time PCR (RT-qPCR) expression analysis. The expression of TLR2, TLR4, TLR6, TLR10 and IL10 was significant higher ($P < 0.05$) in the ileum than that in the jejunum, which was consistent with the RNA-Seq results (Figure 3.8). Although CLDN4 and OCLN were identified as JE-enriched genes by RNA-Seq, no significant regional differences were detected by RT-qPCR (Figure 3.8). The RNA-Seq

showed that the expression of CLDN4, FOXP3 and IL8 increased from D0 to D7 in both jejunum and ileum, and they were validated by RT-qPCR (Figure 3.8). In addition, RT-qPCR results revealed temporally DE genes within the first week that were not detected by RNA-Seq. For example, the expression CLDN1 and OCLN were upregulated within the first week in both jejunum and ileum; and IL10 and TLR6 showed increased expression in the jejunum and in the ileum, respectively, from D0 to D7 (Figure 3.8). Moreover, RT-qPCR also detected temporally DE genes after the first week, which was not identified by RNA-Seq. For example, the expression of CLDN1 and CLDN4 was downregulated when comparing D21 to D7; while TGFB was downregulated from D7 to D21 in both regions (Figure 3.8).

3.3.10 Associations between content- and tissue-associated total bacteria population and host gene expression

In order to test the relationship between host genes and the bacterial colonization during the early life, the Pearson's correlation analysis was performed between host gene expressions (12 genes with RT-qPCR data) and the 16S rRNA gene copy numbers of the content-associated and tissue-associated total bacteria in the small intestine (Table 3.3). The content-associated total bacterial population was negatively correlated with TLR6 and TNFA at D7, with TLR4, OCLN and IL8 at D42 in the jejunum (Table 3.3). No significant correlations were observed between content-associated total bacterial population and host genes in the ileum (Table 3.3). The same analysis for the tissue-associated total bacterial population revealed that negative correlations with

TLR4, TLR6, TLR10 and OCLN at D21, TLR6 and TLR10 at D42 in the jejunum (Table 3.3), and positive correlations with TLR2, TLR4, CLDN1 and OCLN at D7 in the ileum (Table 3.3).

3.4 Discussion

Before weaning, the small intestine of ruminants plays a vital role in nutrient absorption and immune function development similar as the monogastric animals. In the present study, the whole transcriptome analysis was applied to study the global gene expression patterns of two regions (jejunum and ileum) in the small intestine during the neonatal period. Understanding the whole transcriptome is essential for revealing the breadth of molecular events within cells and tissues, and also for understanding complex developmental processes (Wang et al., 2009). Our in-depth analysis revealed significantly regional and temporal differences in whole transcriptomes of the calves' small intestine. Since our main objective is to explore the host mucosal immune system development during the early life, we mainly focused on the enriched immune-related genes. The observed regional expression differences in immune related genes and their functions suggest the important roles of jejunum in the mucosal immune system. In addition, the observed temporal differences in the immune related genes of small intestine suggest that the first week after birth is a critical period for the small intestinal development in neonatal calves, and these changes could be associated with microbial colonization and miRNAs regulation. Therefore, the following discussion will be focused on 1) regional differences between the two

regions and the differences of temporal changes between the two regions; 2) the temporal changes that were related to microbial colonization and miRNAs regulation.

Systematic analysis of the immune-related genes (including barrier function related-genes) revealed significant regional difference in the small intestine. Our results identified the IL-enriched immune-related genes with function related to “B cell activation”. Ileum has been reported to be an abundant mucosa-associated lymphoid tissue that develops in utero and functions as a primary B cell lymphoid organ in young calves (Landsverk, 1984). Thus, the systematic functional analysis of IL-enriched immune-related genes reflects the functional specialization of the lymphoid tissue in the ileum. Comparing to the ileum, higher expression of complement functional pathway related genes in the jejunum was found. For example, higher mRNAs expression of complement component 3 (C3) (fold change = 2.5) and complement component 5a (C5a) (fold change = 4.8) were observed in the jejunum than that in the ileum. C3 plays a key role in the activation of complement system, and C5a is crucial in the downstream functions of complement cascade, which can recruit complements and phagocytic cells at the infection sites (Tomlinson, 1993). The complement system plays a role in the immune system against microorganisms through the cooperation with phagocytic cells (Tomlinson, 1993), which is one of the earliest systems to be fully established in mucosal tissues during the neonatal period in human (Dekaris, 1998). The functions of complement system in the serum are mainly related bacteriolysis and enhancement of phagocytosis (Ogundele, 2001). Our findings on

the higher expression of complement functional pathway related genes suggest that the jejunum may be more competent in innate immune responses during neonatal period. However, the specific functions of the complements in mucosal immune system are still not clear, therefore, further studies are necessary to explore the function of complement system in the small intestinal mucosal immune system of calves.

We also observed regional expression differences of the T and B cell lineage specific genes. It is not surprising to observe higher expression of CD79A and CD79B genes (B cell markers) in the ileum than that in the jejunum at all ages due to the abundance of B cell lymphoid follicles in the ileal submucosa of newborn calves (Landsverk, 1984). However, the B cell abundance does not represent the adaptive immune competence at this stage since Ileal PPs have been suggested as primary lymphoid tissues for B cell development, which contain large amount of precursors of plasma cells that cannot produce antibodies, and limited adaptive immune responses were detected in the ileal tissue of ruminants (Mutwiri et al., 1999). The adaptive humoral immune defense at mucosal surface is mainly mediated by IgA antibody (Holmgren and Czerkinsky, 2005), which is secreted by IgA-producing plasma cells. Our results showed that the expression of IgA complex mRNA was higher in the jejunum than that in the ileum (IGJ with fold change = 2.5; PIGR with fold change = 3.2), and this is consistent with previous report that the jejunal PPs function as induction sites for humoral immune responses (Mutwiri et al., 1999). Furthermore, we observed gradually increasing expression of genes involved in B cell number in the jejunum but not

in the ileum, suggesting the important adaptive immune function of jejunum during the postnatal period.

The RNA-Seq results revealed higher expression of TJ protein genes, such as CLDN3, CLDN4, OCLN, ZO1, ZO2 and ZO3, in the jejunum compared to those in the ileum. All these genes were related to the intestinal permeability (Liu et al., 2005), and higher expression of these genes could lead to reduce intestinal permeability and increased the intestinal integrity, suggesting higher gut integrity in the jejunum than that in the ileum, which may provide better protection for jejunum from invasion of the pathogenic bacteria (Ulluwishewa et al., 2011). However, the RT-qPCR analysis on CLDN4 and OCLN in this study did not confirm their higher expression in the jejunum, further studies will be necessary to compare the TJ protein expression between the jejunum and ileum. In addition, the most of the TLRs mRNA expression were higher in the ileum than that in the jejunum, such as TLR2, TLR4, TLR9 and TLR10. TLR2 and TLR4 recognize components contained in the cell wall of bacteria (Lavelle et al., 2010), TLR9 recognizes bacterial DNA (Lavelle et al., 2010), and TLR10 was reported to recognize viral infection (Lee et al., 2014). All these TLRs could promote downstream immune response to invasive pathogen via stimulating the expression cytokines in the mucosal immune system (Lavelle et al., 2010). The lower expression levels of TLRs in the jejunum suggest that the jejunum may have lower capacity to respond to the gut microbes. As discussed above, the transcriptome analysis revealed the regional gene expression difference of the jejunum and ileum. These differences indicate that the jejunum is different with

ileum in terms of innate and adaptive immune responses, bacterial sensing and barrier functions.

In addition, we observed significant differences in the expression levels of immune-related genes during the first week in both jejunum and ileum. This reveals a rapid development of immune system during the first week after birth, when they are exposed to the microbial colonization, the colostrum feeding, or the ontogenetic development of small intestine. The increased expression of TJ protein genes CLDN1, CLDN4 and OCLN were observed in both jejunum and ileum, when comparing D7 versus D0. These TJ protein genes have been reported to decrease the gut permeability (Ulluwishewa et al., 2011), suggesting that the integrity of small intestine increased within one week postpartum, which is similar what have been reported in the jejunum of mice (Holmes et al., 2006). Furthermore, the strong correlation observed between mRNA expression of TJ proteins and total bacterial number at D7 (OCLN was positively correlated with ileal tissue-associated total bacterial density) suggests that the increase the expression of these TJ protein genes may be triggered by increased bacterial colonization.

Similarly, increased expression of REG3G was identified within the first week in both jejunum and ileum, which binds peptidoglycan and is bactericidal against Gram‑positive bacteria (Bevins and Salzman, 2011). In the meantime, the total bacterial density also increased within the first week, which was in accordance with the REG3G expression. It has been reported that the production of REG3G rely on the microbial colonization in the small intestine of human

(Sanos et al., 2009), and the expression of REG3G increases after the microbial colonization. Our results were consistent with the previous findings in human, and provide new insights in the relationship between REG3G and microbial colonization in the small intestine of newborn calves. There was no age-related change in the expression of lineage-specific genes used to monitor total T cell number in either jejunum or ileum during the 42 days post-partum. It is known, however, that there is an increased number of mucosal T cells in the small intestine of calves when comparing 2-3 week old calves and 6-month-old calves (Fries et al., 2011). This suggests that the number of T cells increases after the first 6 weeks (after weaning period). It was surprising to observe a significant increase in the expression of gene (FOXP3) related to Tregs from D0 to D7. There are increasing evidences that Tregs play a significant role in suppressing immune responses to commensal bacteria (Sakaguchi et al., 2008). The number of Tregs in the colon increased following bacterial colonization in mice (Round and Mazmanian, 2010) and circulating Tregs increase rapidly in newborn infants during the first days of life (Grindebacke et al., 2009). The significant increase in Tregs in both the ileum and jejunum of newborn calves during the first week of life provide a probable adaptive response to maintain the gut immune homeostasis following microbial colonization.

In addition to above adaptive immune response, the expression of IL8 and IL10 was upregulated in the small intestine within the first week. IL10 has been well studied as an anti-inflammatory cytokine (Mosser and Zhang, 2008) and the colonization of commensal bacteria, such as *Bifidobacterium sp.*, can stimulate

the expression of IL10 (Hart et al., 2004). The upregulation of IL10 within the first week suggests the repression the host inflammatory response during the microbial colonization. In contrast, IL8, a pro-inflammatory cytokine, can stimulate the migration of neutrophils from intravascular to interstitial sites and directly activate neutrophils (Joshi-Barve et al., 2007). The co-upregulation of IL8 and IL10 at one week postpartum indicates that the host may have reached a balance in terms of inflammatory response, which is crucial for the immune homeostasis in the small intestine. Similarly, the expression of genes encoding molecular pathogen recognition patterns such as TLR6 and NLRP3 increased significantly in both jejunum and ileum within the first week. TLR6 forms a heterodimer with TLR2, recognizing diacylated lipopeptide from bacterial cell wall (Hajjar et al., 2001) through the stimulation on dendritic cells resulted in the development of regulatory T cells (Tregs), and the development of Treg (Depaolo et al., 2008). The significant correlation between the expression of TLR6 and total bacterial number (TLR6 and jejunal content-associated total bacteria at D7, $r = -0.85$, $P < 0.05$; TLR6 and jejunal tissue-associated total bacteria at D21 and D42, $r = -0.94$ and $r = -0.87$, $P < 0.05$) in the jejunum, suggesting its role in regulating host responses to the dynamic changes of bacterial population. In addition to the membrane-bound TLRs, the increased expression of NLRP3 was also observed in both jejunum and ileum within the first week. NLRP3 forms an inflammasome complex in cytoplasm, which can be activated by commensal bacteria-produced adenosine triphosphate, leading to downstream host responses such as the differentiation of T helper 17 cells (Th17) (Lavelle et al., 2010). The upregulation

of NLRP3 within the first week indicates its potential roles in responding to the microbial colonization and modulating the host response in the small intestine of calves during the postnatal period. However, the functions of NLRs are still limited in the mucosal immune system of ruminants, and more studies will be needed in the future to elucidate its role in host-microbial interactions in the gut of neonatal calves.

As described above, we reported the alterations of gene expression that may be vital for the development of mucosal immune system in the ruminants during first week of life which could be directly associated with commensal bacterial colonization. Although it has been long proposed that microbial population and community shift can impact on host gene expression through the microbial metabolites such as LPS (Renz et al., 2012). We have recently proposed that miRNAs, the non-coding RNAs that regulate gene expression, could also be one of the mechanisms to regulate the host-microbial interactions. Similar as the host mRNAs, the most significant changes of miRNA expression in small intestine was also observed during the first week (Liang et al., 2015). In this study, we further identified the mRNA-miRNA regulatory pairs, which could be associated with microbial colonization and host immune functions. For example, the expression of miR-335 was downregulated during the first week in the ileum (Liang et al., 2014) and the overexpression of miR-335 can repress the expression of CLDN1, CLDN4 and OCLN (Tavazoie et al., 2008). Indeed, the increased expression of CLDN1, CLDN4 and OCLN suggested that the downregulation of miR-335 together with dynamics in gut microbiota might play a role in regulating

alterations of TJ proteins during first week. Besides, miR-335 has been proven to repress the expression of IL8 (Tavazoie et al., 2008). Therefore, the upregulation of IL8 and downregulation of miR-335 within the first week manifested the potential role of miR-335 on the expression of IL8 during the postnatal period. Besides, miR-100 was downregulated, while its experimentally validated target, NLRP3 (Helwak et al., 2013), was up during the first week, suggesting that miR-100 may have regulatory function on the induction of NLRP3. All the results suggest the roles of miRNAs in host-microbial interactions during early life through regulating expression of genes involved in gut permeability, cytokine expression, and pathogen sensing.

3.5 Conclusions

This is the first study to analyze the whole transcriptome in the small intestine of calves during the early postnatal period. We reported that the JE-enriched genes were mainly related complement functional pathway. In addition, the expression of TJ protein genes and IgA complex genes were higher in the jejunum than that in the ileum, while the expression of Toll-like receptor and NOD-like receptor genes were lower in the in the jejunum than that in the ileum. The regional differences indicate important roles of jejunum in the mucosal immune system and provide potential mechanism to explain why the enteric infection occurs primarily in the ileum rather than in the jejunum in neonatal calves. Besides, the expression of genes that are related to tight junction proteins, antimicrobial peptides, NOD-like receptors, regulatory T cell marker, and

cytokines underwent dynamic changes when comparing 7-day-old calves with newborn calves. Thus, we proposed that the first week postpartum is a critical developmental period for both the intestinal epithelial barrier and the mucosal immune system development. Furthermore, there were strong correlations between mucosal immune-related genes and total bacterial population in different regions at different ages, suggesting microbial colonization plays roles in modulating host gene expression. Moreover, we reported that miRNAs, such as miR-335 and miR-100, may also manipulate the host gene expression that are related to intestine mucosal immune system in the small intestine of the neonatal calves.

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3.7 Figures and tables

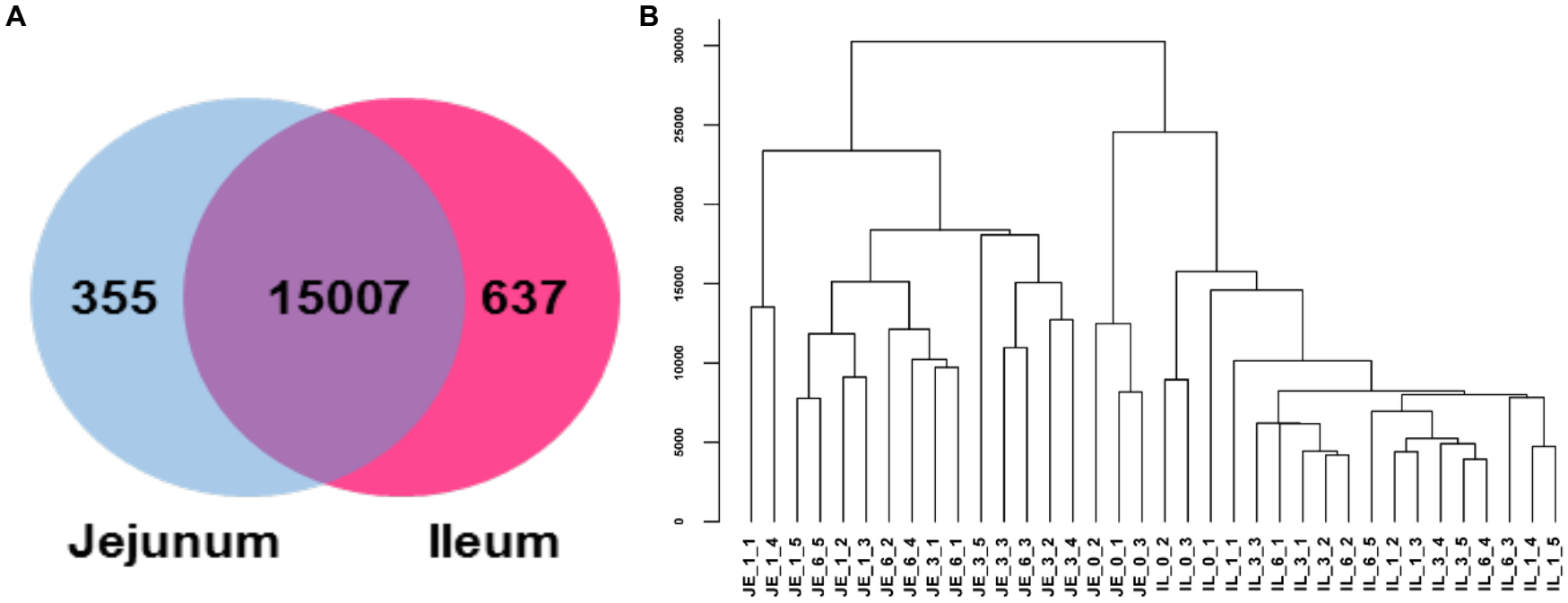


Figure 3.1 Regional differences of the transcriptome profiles between the jejunum (JE) and ileum (IL). (**A**) Venn diagram of expressed genes. (**B**) Hierarchical cluster of mRNA expression. Each ID represents one animal. "JE_0_1" means the jejunum sample from D0 animal No.1 (D0). (**C**) Functional classification of JE-enriched genes. (**D**) Functional classification of IL-enriched genes.

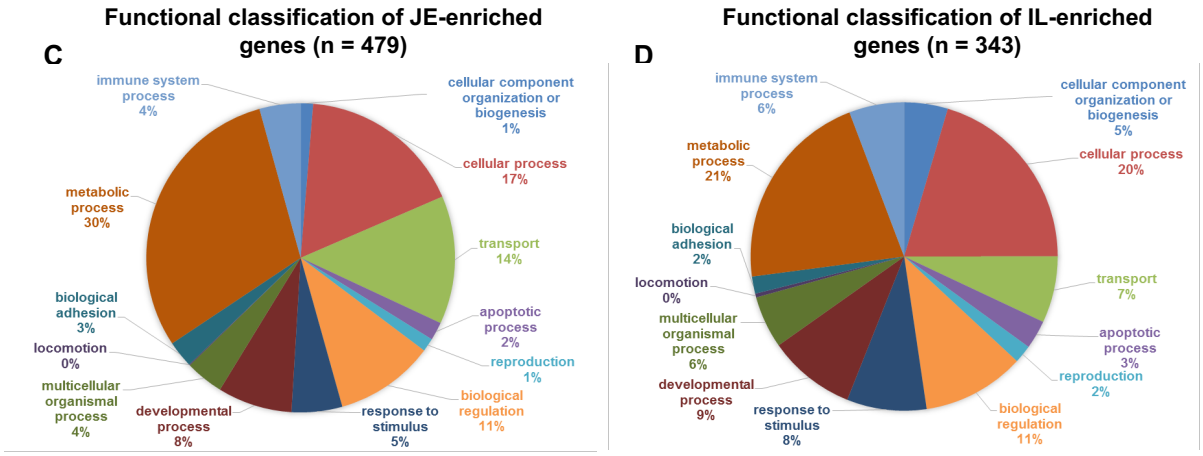


Figure 3.1 (Continued) Regional differences of the transcriptome profiles between the jejunum (JE) and ileum (IL). (**A**) Venn diagram of expressed genes. (**B**) Hierarchical cluster of mRNA expression. Each ID represents one animal. "JE_0_1" means the jejunum sample from D0 animal No.1 (D0). (**C**) Functional classification of JE-enriched genes. (**D**) Functional classification of IL-enriched genes.

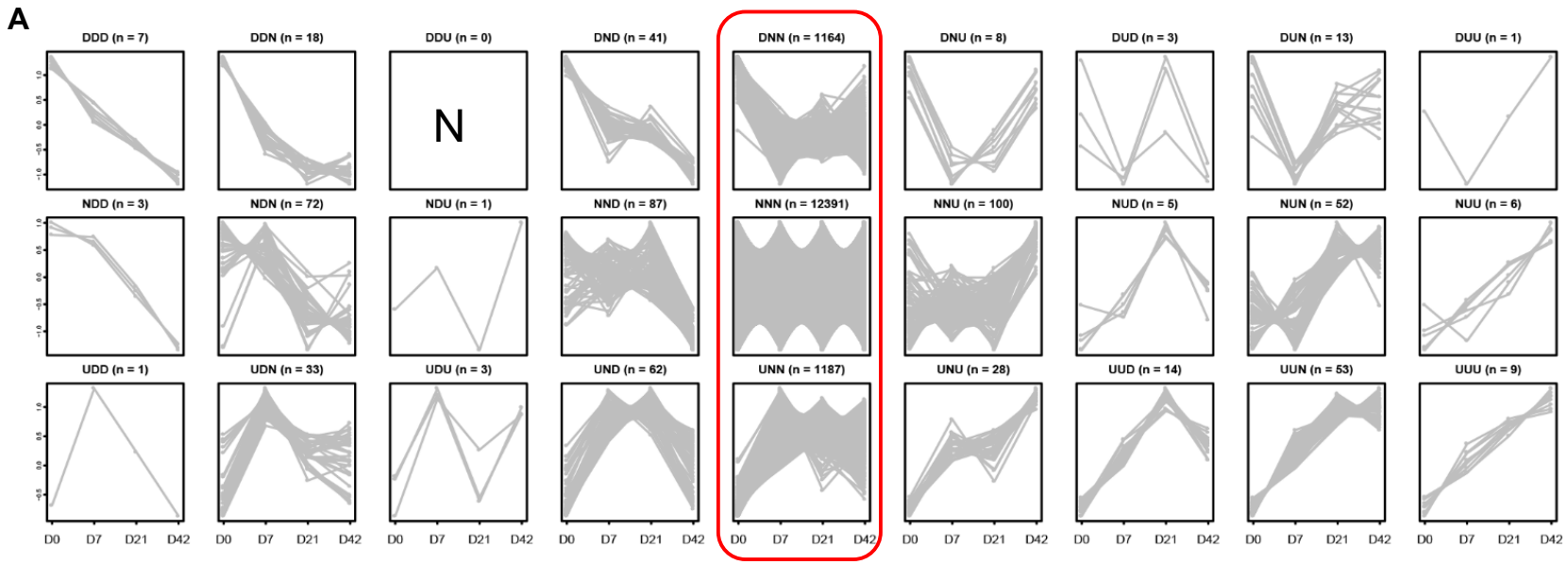


Figure 3.2 Temporally DE gene expression patterns for jejunum (A) and ileum (B). All the expressed genes were categorized into 27 expression patterns based on the temporally DE analysis (fold change > 2 and FDR < 0.05). "U" means the genes were DE and upregulated; "N" means the genes were not DE; and "D" means the genes were DE and downregulated. The order of each pattern follows the comparison between D7 vs D0, D21 vs D7, and D42 vs D21. X-axis depicts four ages and Y-axis depicts the fold change of each gene. "NA" in the square means no genes were categorized into that pattern.

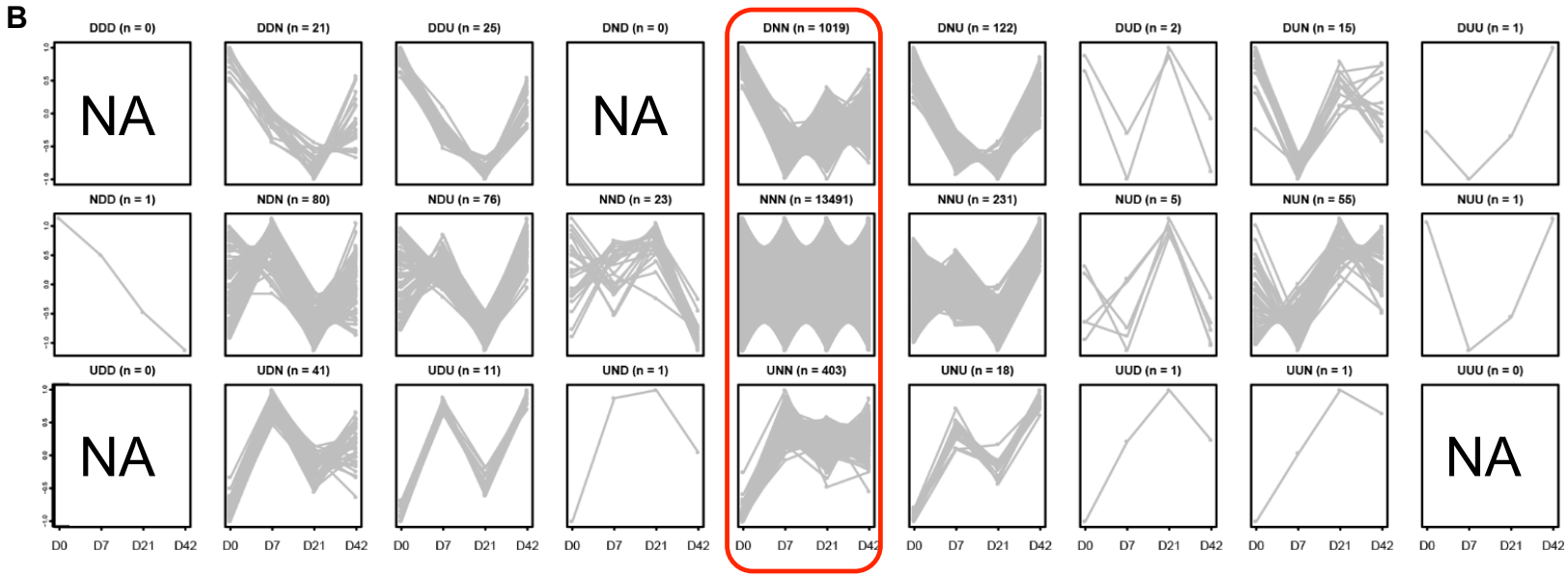


Figure 3.2 (Continued) Temporally DE gene expression patterns for jejunum (A) and ileum (B). All the expressed genes were categorized into 27 expression patterns based on the temporally DE analysis (fold change > 2 and FDR < 0.05). "U" means the genes were DE and upregulated; "N" means the genes were not DE; and "D" means the genes were DE and downregulated. The order of each pattern follows the comparison between D7 vs D0, D21 vs D7, and D42 vs D21. X-axis depicts four ages and Y-axis depicts the fold change of each gene. "NA" in the square means no genes were categorized into that pattern.

JE	IL		JE	IL
0.05	0.00	DDD	Transport	NA
0.12	0.13	DDN	Nitrogen compound metabolic process	Cholesterol homeostasis
0.00	0.16	DDU	NA	Tissue development
0.27	0.00	DND	Transport	NA
7.58	6.51	DNN	Developmental process	Developmental process
0.05	0.78	DNU	Phosphorylation	Metabolic process
0.02	0.01	DUD	Transport	NA
0.08	0.10	DUN	Proteolysis	Translation
0.01	0.01	DUU	Carbon dioxide enzyme	Chemokine
0.02	0.01	NDD	Protein-lipid complex remodeling	Epithelial cell development
0.47	0.51	NDN	Transport	Transport
0.01	0.49	NDU	Structural molecule	Cell adhesion
0.57	0.15	NND	Metabolic process	Immune system process
80.66	86.24	NNN	Metabolic process	Metabolic process
0.65	1.48	NNU	Cellular morphogenesis	Muscle contraction
0.03	0.03	NUD	Lipid transport	Defense response
0.34	0.35	NUN	Immune system process	Defense response
0.04	0.01	NUU	Response to bacterium	Structural molecule
0.01	0.00	UDD	Transport	NA
0.21	0.26	UDN	Immune response	NA
0.02	0.07	UDU	Cell fraction	Cell development
0.40	0.01	UND	Immune response	NA
7.73	2.58	UNN	Immune system process	Immune system process
0.18	0.12	UNU	Immune system process	Cytokine production
0.09	0.01	UUD	Intrinsic to membrane	Cell death
0.35	0.01	UUN	Immune response	Transcription factor
0.06	0.00	UUU	Intrinsic to membrane	NA

Figure 3.3 The percentage of genes within each expression pattern and GO term enrichment for each expression pattern in jejunum (JE) and ileum (IL). "NA" means no significant functions were enriched.

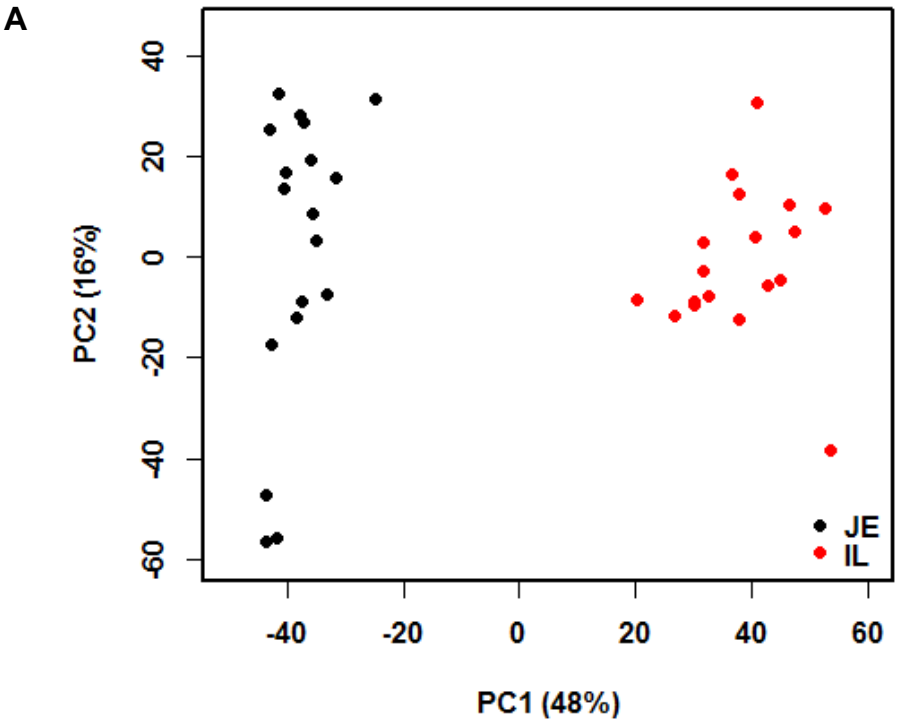


Figure 3.4 PCA plot of all the immune-related genes. The X and Y-axis represent the first two principle components. The percentage value in the bracket represents the percentage of variance explained by that principle component. (**A**) PCA plot of immune-related genes for the jejunum (JE, black dots) and ileum (IL, red dots). (**B**) PCA plot of immune-related genes for the jejunum at different ages (D0, D7, D21 and D42). (**C**) PCA plot of immune-related genes for the ileum at different ages (D0, D7, D21 and D42).

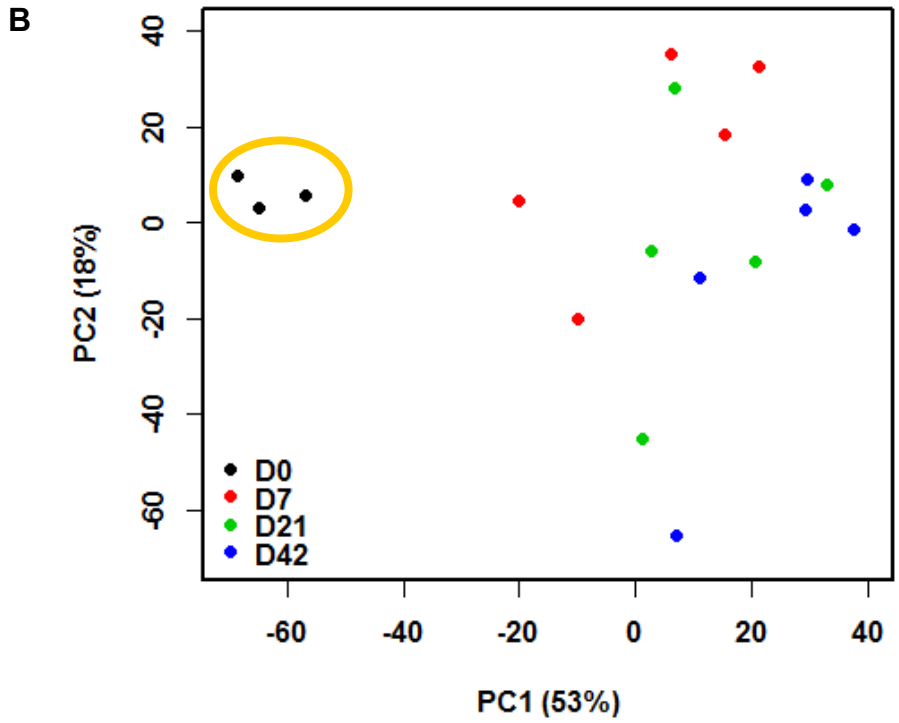
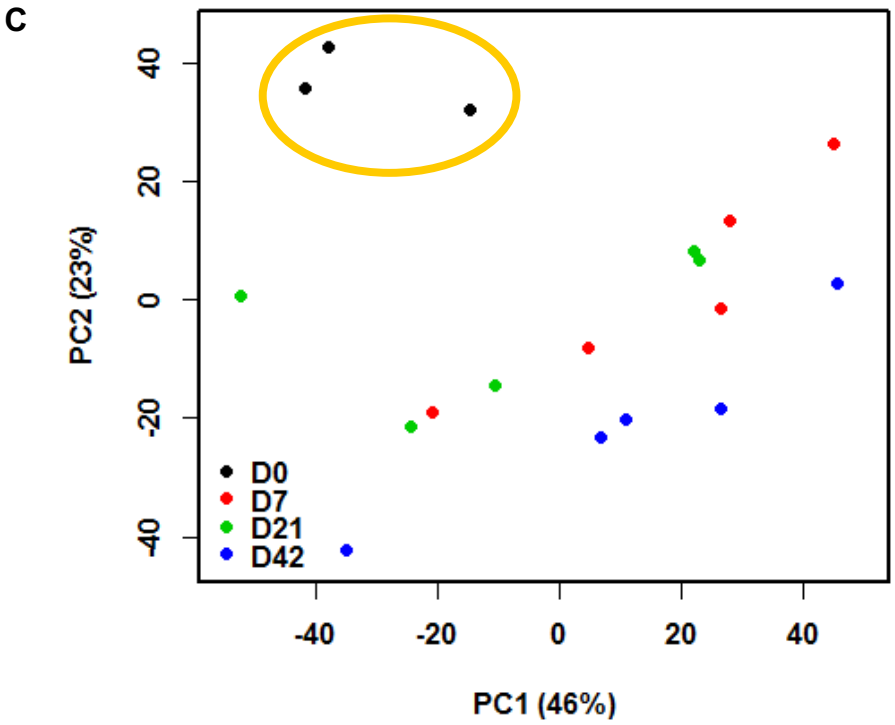


Figure 3.4 (Continued) PCA plot of all the immune-related genes. The X and Y-axis represent the first two principle components. The percentage value in the bracket represents the percentage of variance explained by that principle component. (**A**) PCA plot of immune-related genes for the jejunum (JE, black dots) and ileum (IL, red dots). (**B**) PCA plot of immune-related genes for the jejunum at different ages (D0, D7, D21 and D42). (**C**) PCA plot of immune-related genes for the ileum at different ages (D0, D7, D21 and D42).



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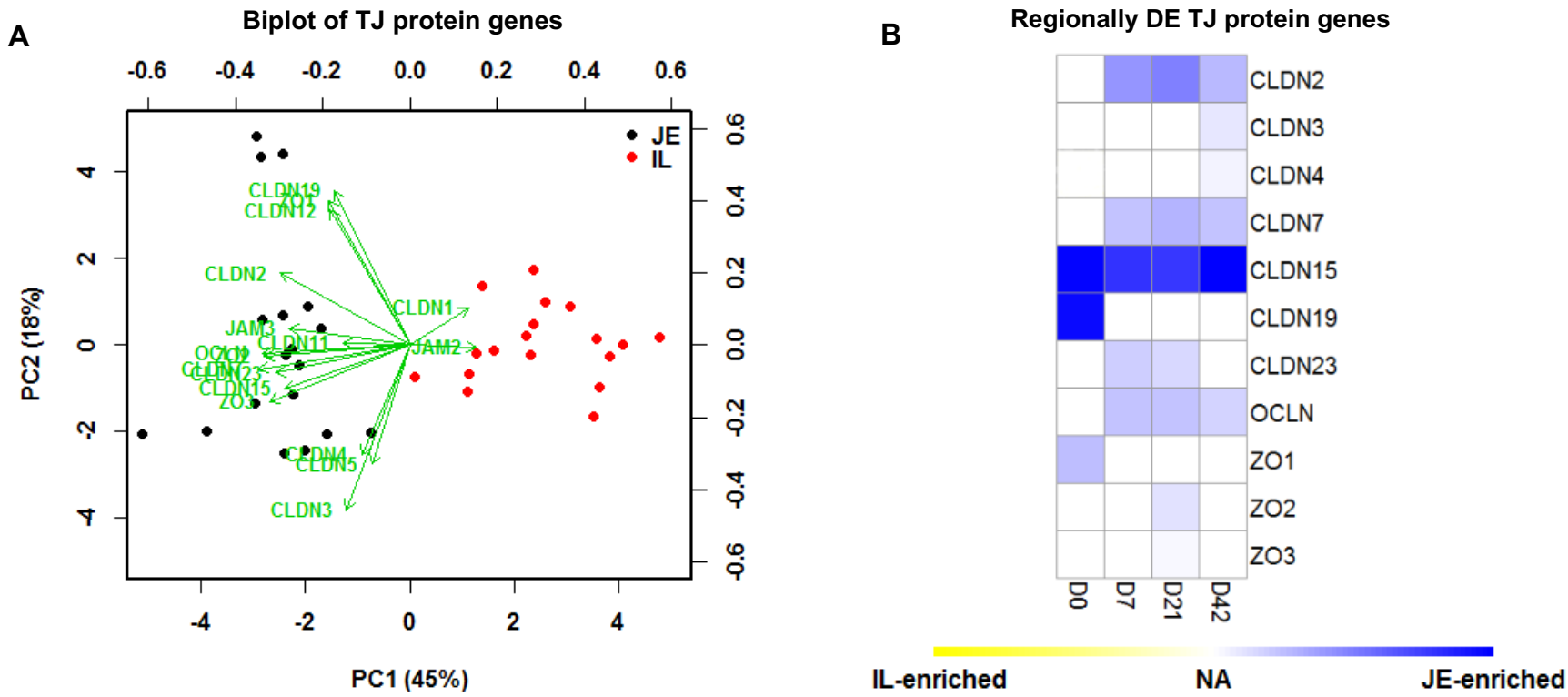


Figure 3.5 The expression patterns of TJ protein genes. (**A**) Biplot of TJ protein genes. The X and Y-axis represent the first two principle components. The green arrow points to the samples with higher expression of the gene. (**B**) Regionally DE analysis of TJ protein genes. Blue means highly expressed in the jejunum and yellow means highly expressed in the ileum. (**C**) Temporally DE analysis of TJ protein genes. Y-axis depicts the gene expression level (log2 (CPM)). "*" means significantly difference identified by temporally DE analysis. (**D**) Biplot of AMP genes. (**E**) Regionally DE analysis of AMP genes. "*" means significantly difference identified by regionally DE analysis. (**F**) The expression pattern of REG3G in both jejunum and ileum. "*" means significantly difference identified by temporally DE analysis.

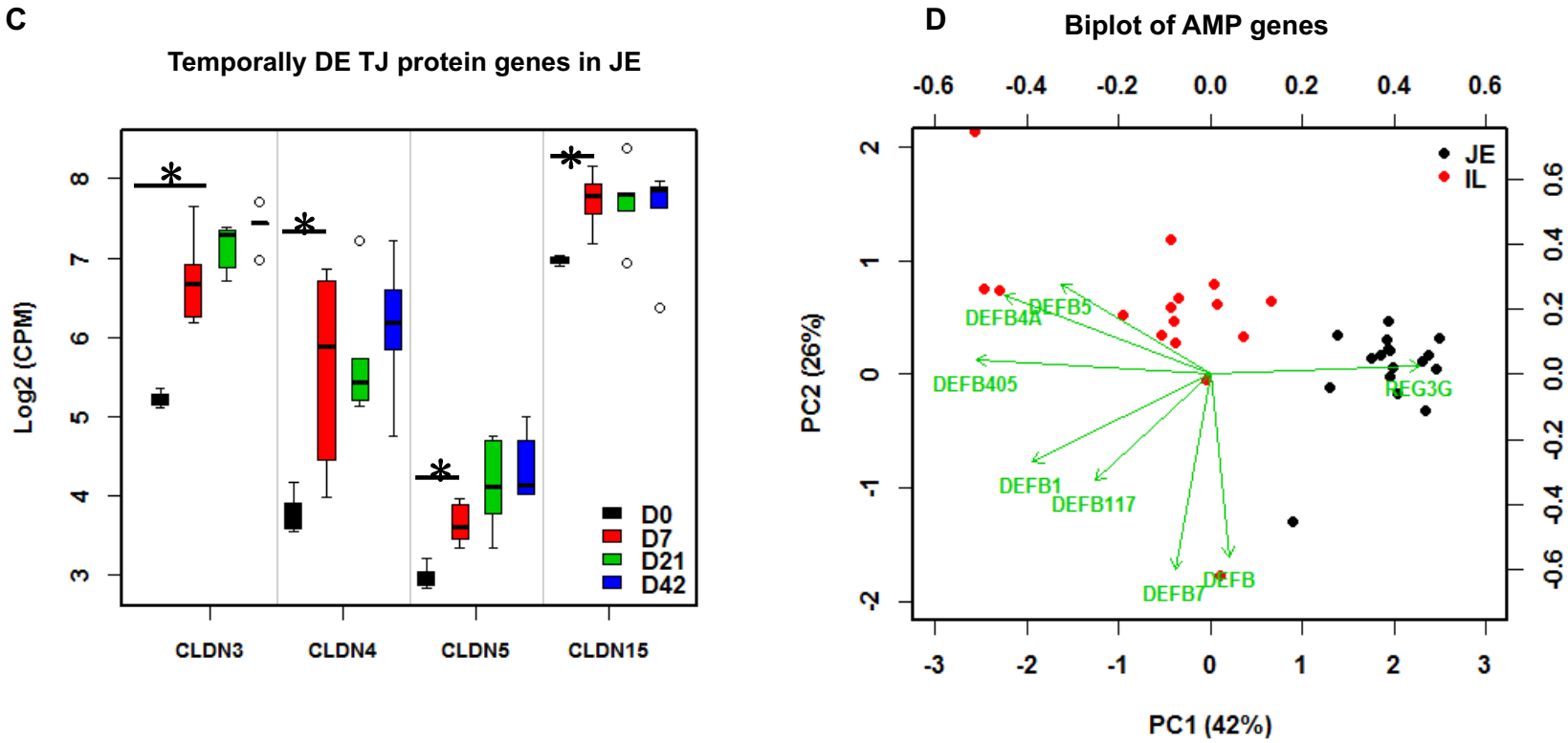
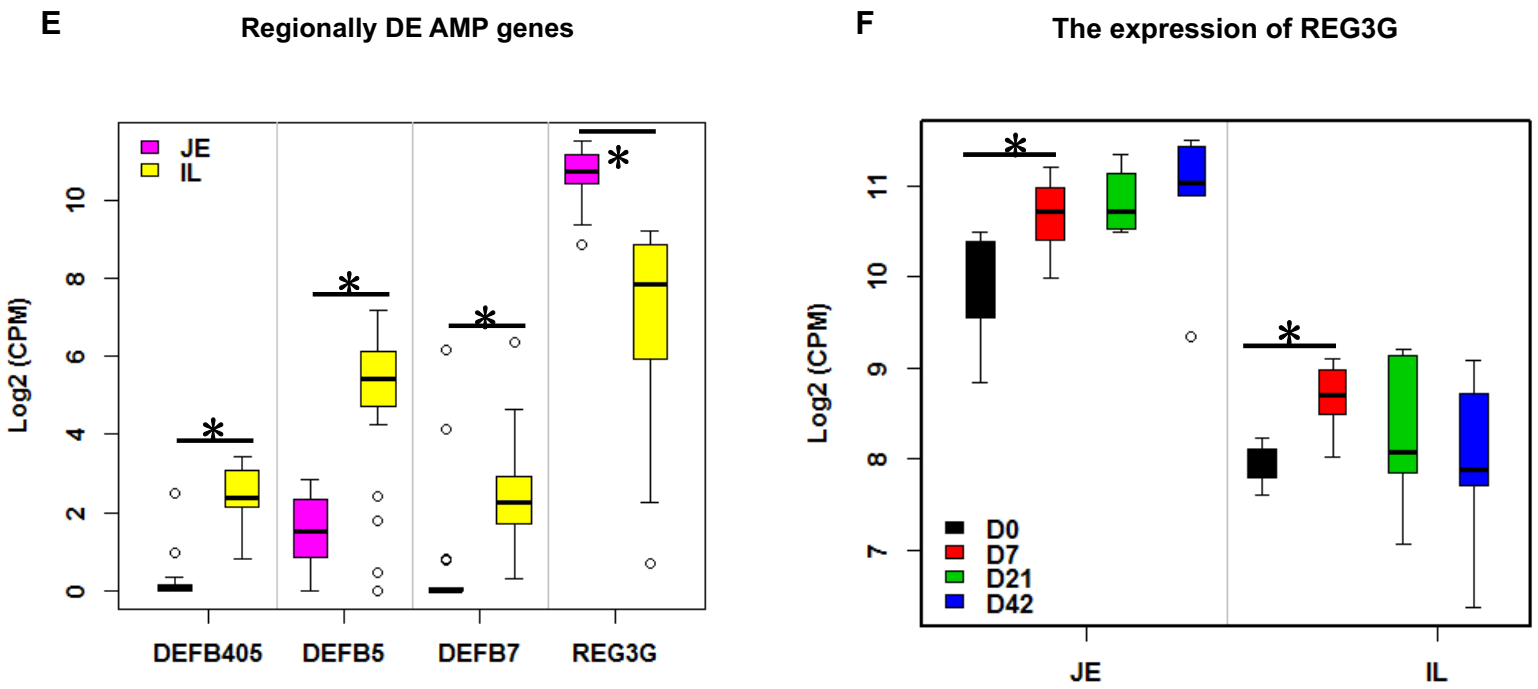


Figure 3.5 (Continued) The expression patterns of TJ protein genes. (**A**) Biplot of TJ protein genes. The X and Y-axis represent the first two principle components. The green arrow points to the samples with higher expression of the gene. (**B**) Regionally DE analysis of TJ protein genes. Blue means highly expressed in the jejunum and yellow means highly expressed in the ileum. (**C**) Temporally DE analysis of TJ protein genes. Y-axis depicts the gene expression level (log2 (CPM)). "*" means significantly difference identified by temporally DE analysis. (**D**) Biplot of AMP genes. (**E**) Regionally DE analysis of AMP genes. "*" means significantly difference identified by regionally DE analysis. (**F**) The expression pattern of REG3G in both jejunum and ileum. "*" means significantly difference identified by temporally DE analysis.



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A

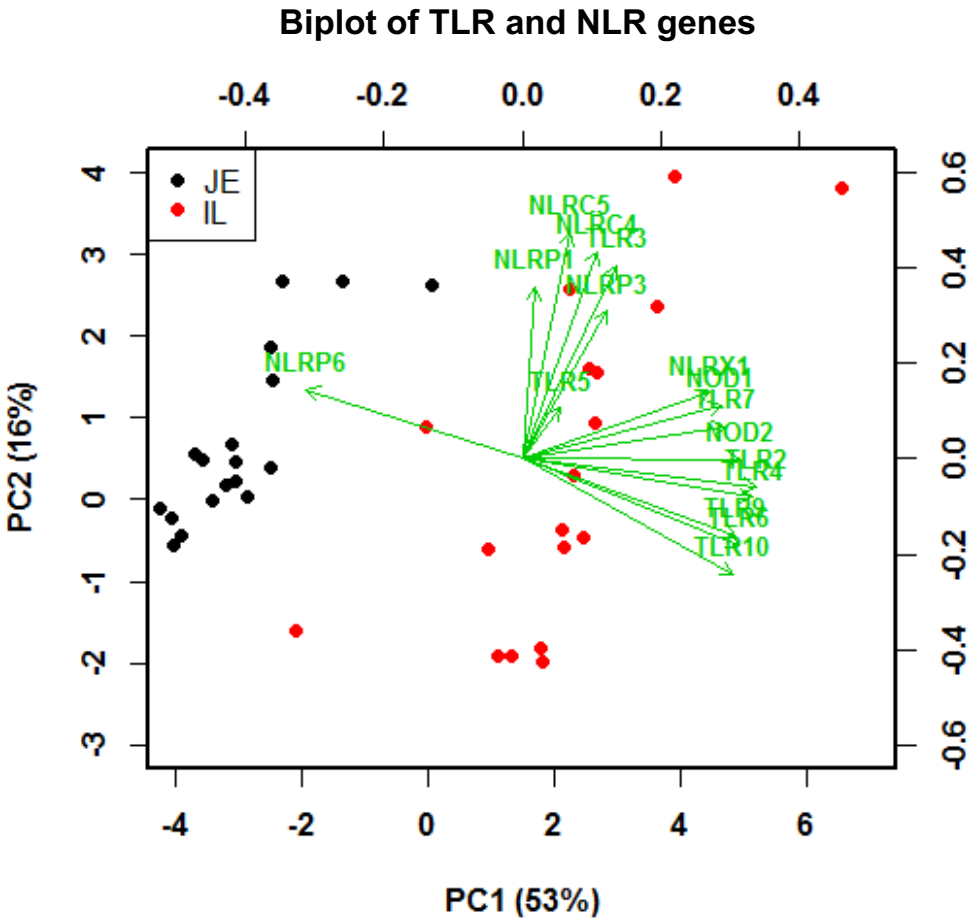


Figure 3.6 The expression patterns of TLR and NLR genes. (**A**) Biplot of TLR and NLR genes. The X and Y-axis represent the first two principle components. The green arrow points to the samples with higher expression of the gene. (**B**) Regionally DE analysis of TLR and NLR genes. Blue means highly expressed in the jejunum and yellow means highly expressed in the ileum. (**C**) Boxplot of expression pattern of NLRP3 in the jejunum and ileum. Y-axis depicts the gene expression level (log2 (CPM)). "*" means significantly difference identified by temporally DE analysis.

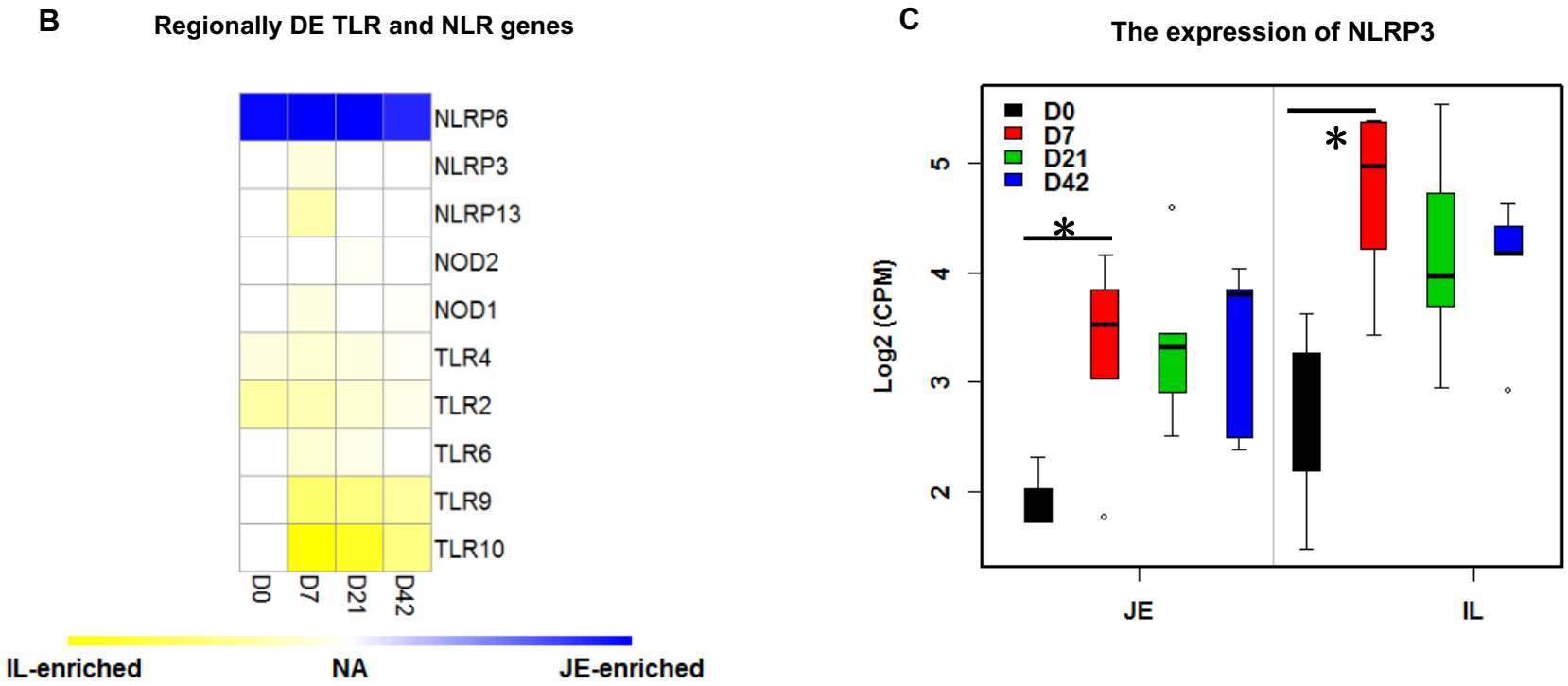


Figure 3.6 (Continued) The expression patterns of TLR and NLR genes. (**A**) Biplot of TLR and NLR genes. The X and Y-axis represent the first two principle components. The green arrow points to the samples with higher expression of the gene. (**B**) Regionally DE analysis of TLR and NLR genes. Blue means highly expressed in the jejunum and yellow means highly expressed in the ileum. (**C**) Boxplot of expression pattern of NLRP3 in the jejunum and ileum. Y-axis depicts the gene expression level (log2 (CPM)). "*" means significantly difference identified by temporally DE analysis.

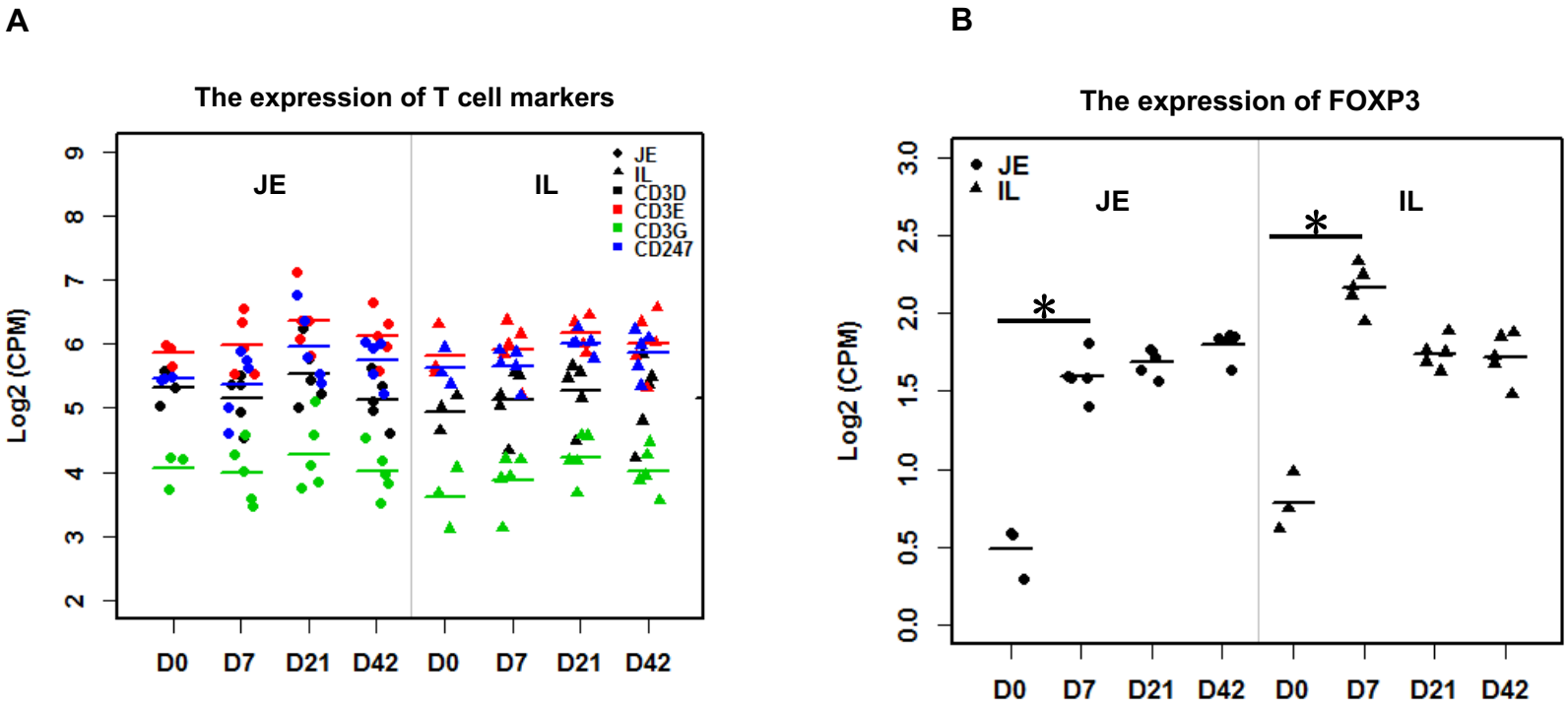
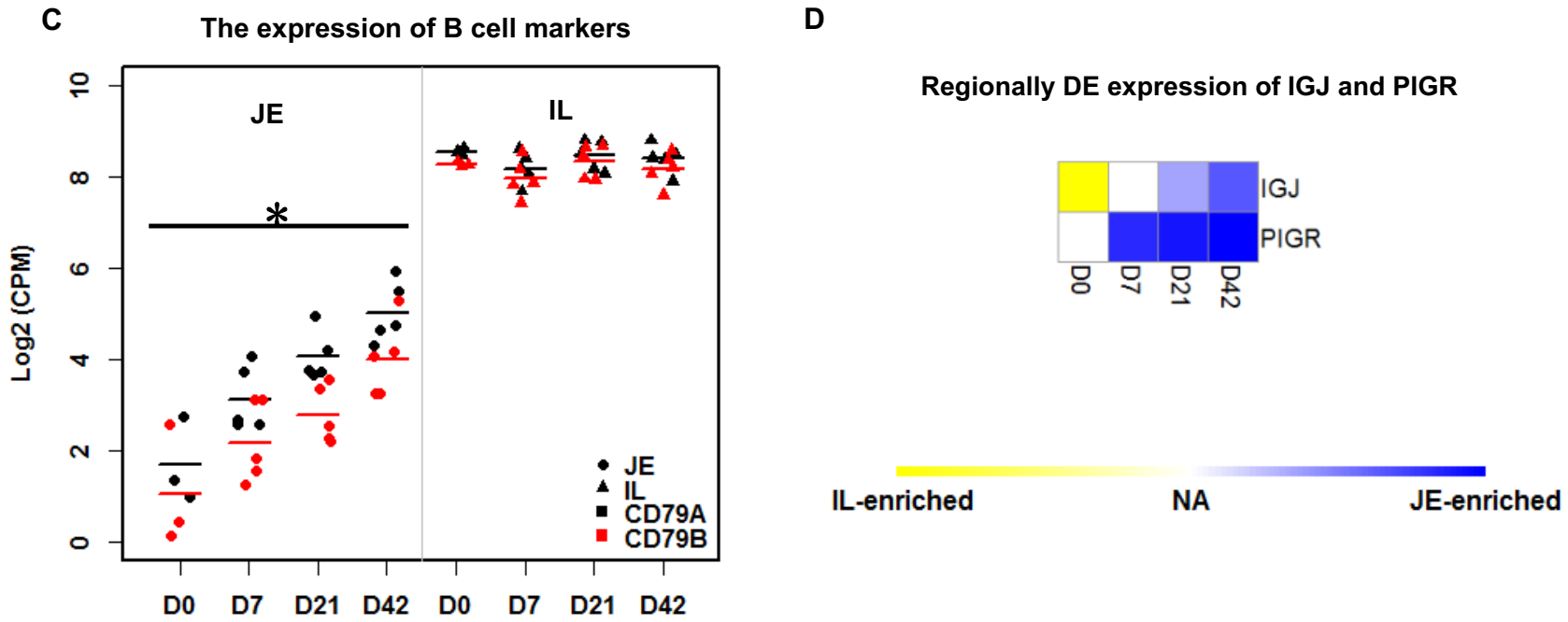


Figure 3.7 The expression patterns of adaptive immune-related genes and cytokine genes. (**A**) Dot plot of the expression of T cell markers (CD3 subunits and CD247). Each dot represents the gene expression each sample. Y-axis represents the gene expression level (log2 (CPM)). Y-axis represents the ages. (**B**) Dot plot of the expression of Treg marker (FOXP3). "*" means significantly difference identified by temporally DE analysis. (**C**) Dot plot of the expression of B cell markers (CD79 subunits). (**D**) Regionally DE analysis of IGJ and PIGR. Blue means highly expressed in the jejunum and yellow means highly expressed in the ileum. (**E**) Temporally DE analysis of IGJ and PIGR in the jejunum. (**F**) Regionally DE analysis of cytokine genes. (**G**) Temporally DE analysis of cytokine genes in the jejunum. (**H**) Temporally DE analysis of cytokine genes in the ileum.



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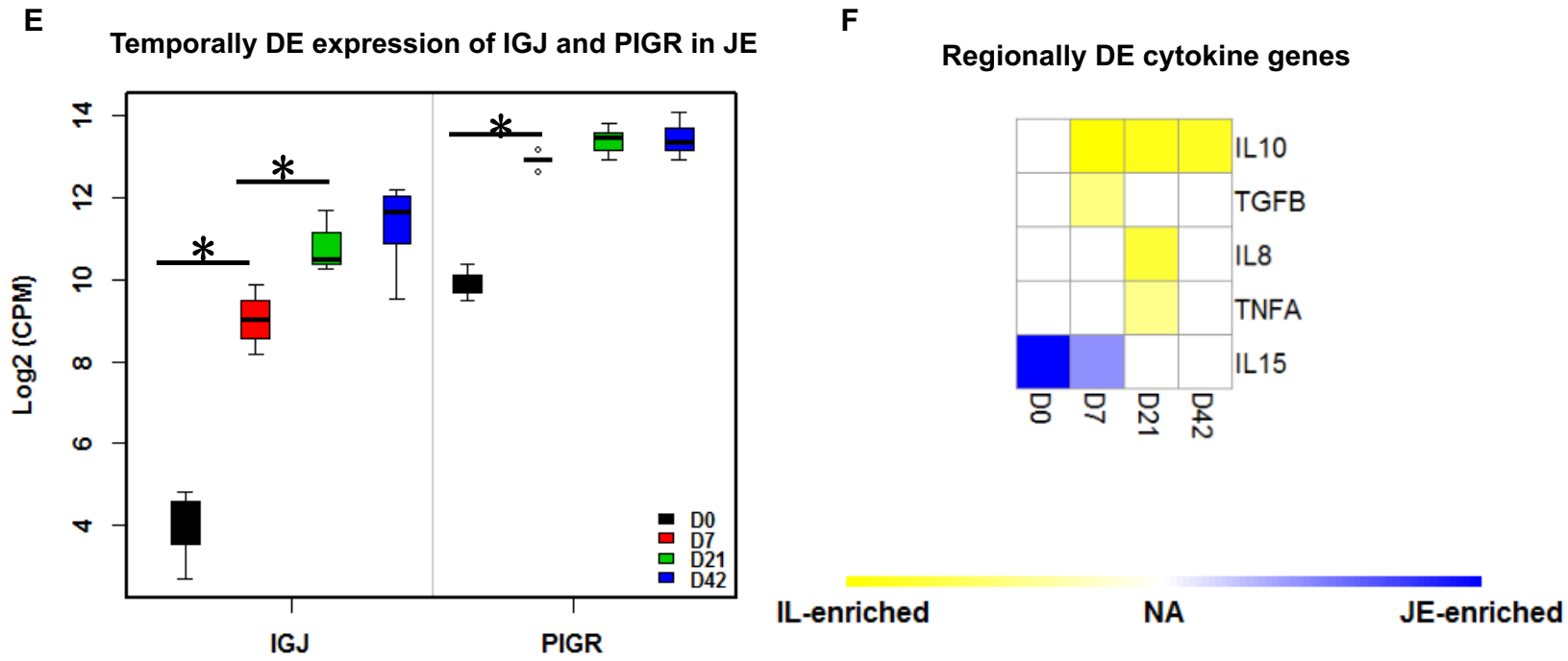

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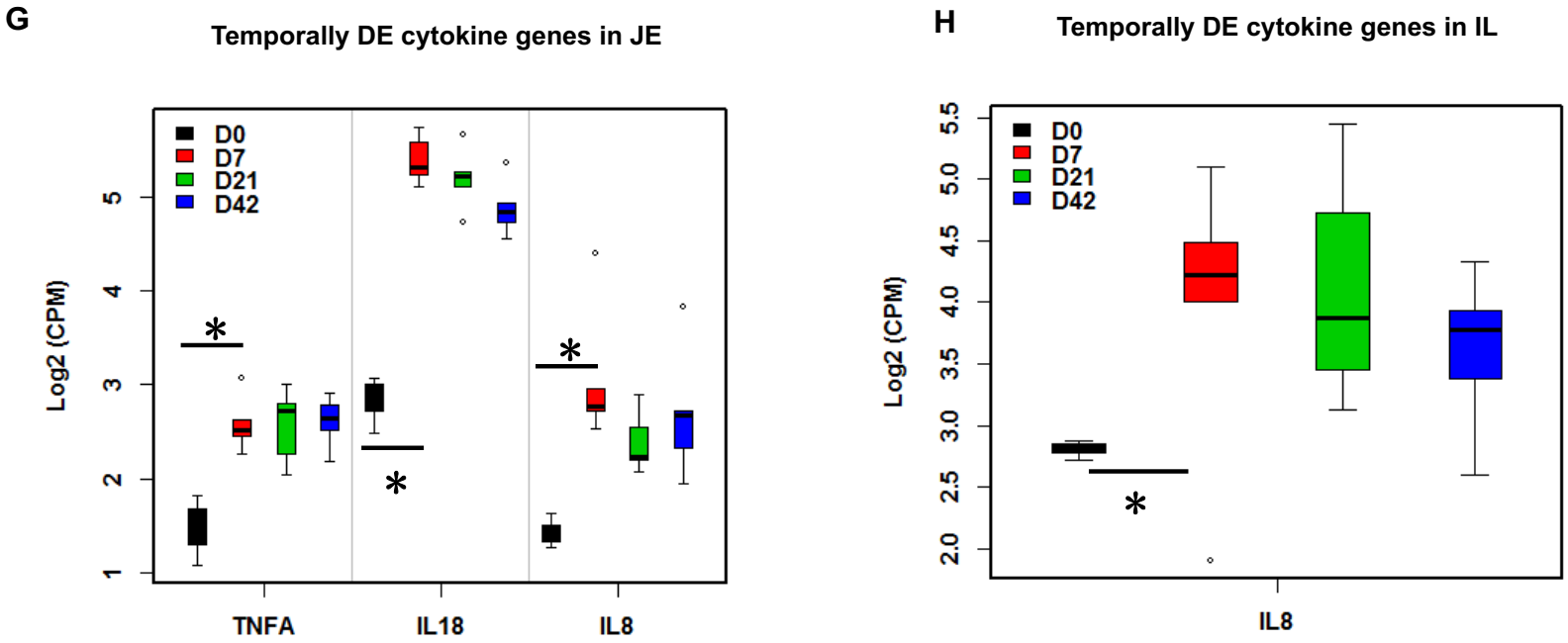

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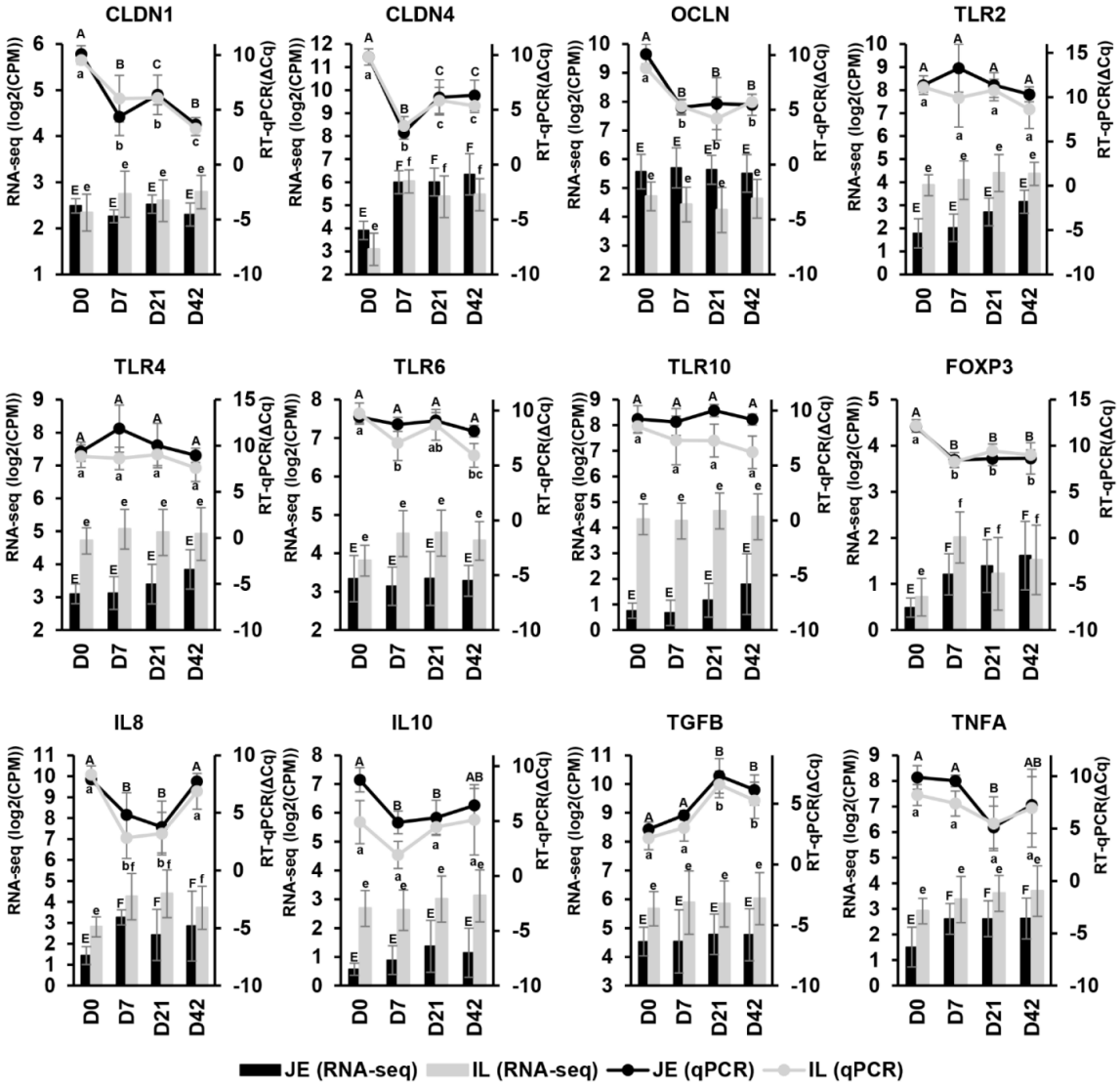

Figure 3.8 RT-qPCR validation of selected DE genes identified by RNA-Seq. The gene expressions detected by RT-qPCR are shown by line graphs (black: jejunum; grey: ileum) on the top and values are shown on the right Y-axis as relative expression (ΔCq). Lower ΔCq values represent higher gene expression levels and vice versa. The gene expressions detected by RNA-Seq are shown by bar graphs (black: jejunum; grey: ileum) on the bottom and values are shown on the left Y-axis as log2 (CPM). A, B, C - indicate significant expression difference detected via RT-qPCR between different ages in the jejunum. a, b, c - indicate significant expression difference detected via RT-qPCR between different ages in the ileum. E, F - indicate significant expression difference detected via RNA-Seq between different ages in the jejunum. e, f - indicate significant expression difference detected via RNA-Seq between different ages in the ileum. Data are presented as Mean ± Standard deviation.

Table 3.1 Primers sequences used for RT-qPCR.

Gene	Forward primer	Reverse primer	Source
TLR2	5′-CTGTGTGCGTCTTCCTCAGA-3′	5′-TCAGGGAGCAGAGTAACCAGA-3′	Charavaryamath et al. (2011)
TLR4	5′-GGTTTCCACAAAAGCCGTAA-3′	5′-AGGACGATGAAGATGATGCC-3′	Charavaryamath et al. (2011)
TLR6	5′-CGACATTGAAGGCACTGAAA-3′	5′-TCCTGAGGACAAAGCATGTG-3′	Charavaryamath et al. (2011)
TLR10	5′-TCACCTGACATCTTTGCGAG-3′	5′-TCGGAATGGATTTCTTCCTG-3′	Charavaryamath et al. (2011)
β-Actin	5′-CTAGGCACCAGGGCGTAATG-3′	5′-CCACACGGAGCTCGTTGTAG-3′	Charavaryamath et al. (2011)
FOXP3	5′-CACAACCTGAGCCTGCACAA-3′	5’-TCTTGCGGAACTCAAACTCATC-3′	Hoek et al., (2008)
TNFA	5′-GCCCCCAGGGCTCCAGAAGT-3’	5’-AGCGTGGTGGCTCCTGCAAC-3’	This study
TGFB	5’-TGCTTCAGCTCCACAGAAAAGA-3’	5’-AGGCAGAAATTGGCGTGGT-3’	Meade et al., 2008
IL8	5’-GCTGGACAGCAGAGCTCACA-3’	5’-TGCCAAGAGAGCAACAGCCAGC-3’	This study
IL10	5’-GCCGAGATGCGAGCACCCTG-3’	5’-TCGGAGCTCCCGCAGCATGT-3’	This study
CLDN1	5’-GCGCTGCCCCAGTGGAAAGT-3’	5’-GGATCTGCCCGGTGCTCTGC-3’	Malmuthuge et al., 2013
CLDN4	5’-CCCGCGCCCTCATCGTCATC-3’	5’-GTTGGCCGACCAGGACACCG-3’	Malmuthuge et al., 2013
OCLN	5’-ACGCAGGAAGTGCCTTTGGTAGC-3’	5’-GCAGCCATGGCCAGCAGGAA-3’	Malmuthuge et al., 2013

Table 3.2 Experimentally validated miRNA-mRNA regulatory pairs.

miRNA	miRNA-fold change (D7/D0)	Target gene	Gene-fold change (D7/D0)	Tissue
miR-21-5p	2.35	FBXL2	0.26	JE
miR-17-5p	1.53	CAMK2N1	0.31	JE
miR-17-5p	1.53	TNFRSF21	0.31	JE
miR-17-5p	1.53	RAPGEFL1	0.32	JE
miR-17-5p	1.53	ASH1L	0.33	JE
miR-21-5p	2.35	PDCD4	0.36	JE
miR-21-5p	2.35	TRIM2	0.40	JE
miR-107	1.80	GNS	0.42	JE
miR-17-5p	1.53	TOR1AIP2	0.42	JE
miR-17-5p	1.53	KCTD7	0.46	JE
miR-142-3p	1.52	TSPAN6	0.46	JE
miR-17-5p	1.53	FCHO2	0.47	JE
miR-21-5p	2.35	LCORL	0.50	JE
miR-21-5p	2.35	JMY	0.46	JE
miR-21-5p	2.35	SLC31A1	0.46	JE
miR-21-5p	2.35	RAB22A	0.48	JE
miR-21-5p	2.35	TIMP3	0.47	JE
miR-21-5p	2.35	RMND5A	0.46	JE
miR-17-5p	1.53	UBE3C	0.48	JE
miR-375	0.53	SLC7A6	2.06	JE
miR-24-3p	0.65	PTPLAD1	2.06	JE
miR-423-5p	0.57	ARHGDIA	2.04	JE
miR-375	0.53	HN1	2.16	JE
miR-24-3p	0.65	PIM2	2.04	JE
miR-24-3p	0.65	TOMM34	2.03	JE
miR-375	0.53	F3	2.13	JE
miR-24-3p	0.65	CSK	2.16	JE
miR-455-3p	0.62	SRM	2.03	JE
miR-423-5p	0.57	MYBL2	2.01	JE
miR-24-3p	0.65	DHCR24	2.01	JE
miR-375	0.53	ADIPOR2	2.04	JE
miR-375	0.53	CTGF	2.13	JE
miR-24-3p	0.65	SLC11A2	2.14	JE
miR-362-5p	0.25	SEMA4F	2.66	JE
miR-375	0.53	BIRC3	4.33	JE
miR-100	0.47	NLRP3	4.59	JE
miR-17-5p	2.43	RAPGEFL1	0.25	IL
miR-17-5p	2.43	CAV1	0.35	IL
miR-17-5p	2.43	PFN2	0.35	IL

miR-142-3p	3.71	TSPAN6	0.37	IL
miR-21-5p	2.93	RHOB	0.45	IL
miR-17-5p	2.43	LAPTM4A	0.49	IL
miR-17-5p	2.43	MAPRE3	0.46	IL
miR-335	0.23	CLDN4	2.15	IL
miR-335	0.23	CLDN1	3.20	IL
miR-335	0.23	IL8	3.78	IL

Table 3.3 Correlation between content- and tissue-associated total bacteria population and host gene expression.

	Content associated						Tissue associated					
	JE			IL			JE			IL		
	D7	**D21**	**D42**	**D7**	**D21**	**D42**	**D7**	**D21**	**D42**	**D7**	**D21**	**D42**
TLR2	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.87*	NS	NS
TLR4	NS	NS	-0.84*	NS	NS	NS	NS	-0.89*	NS	0.82*	NS	NS
TLR6	-0.85*	NS	NS	NS	NS	NS	NS	-0.94**	-0.87	NS	NS	NS
TLR10	NS	NS	NS	NS	NS	NS	NS	-0.84*	-0.86	NS	NS	NS
CLDN1	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.85*	NS	NS
CLDN4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
OCLN	NS	NS	-0.81*	NS	NS	NS	NS	-0.87*	NS	0.83*	NS	NS
IL8	NS	NS	-0.98**	NS	NS	NS	NS	NS	NS	NS	NS	NS
IL10	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
TGFB	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
TNFA	-0.84*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
FOXP3	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

NS: Not significant

*: $P < 0.05$

**: $P < 0.01$

Chapter 4. Altered microRNA expression and pre-mRNA splicing events reveal new mechanisms associated with early stage *Mycobacterium avium* subspecies *paratuberculosis* infection

4.1 Introduction

Johne's disease (JD) is chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Arsenault et al., 2014). Clinical symptoms of JD in cattle include persistent diarrhea, progressive weight loss, decreased production and death (Tiwari et al., 2006). Although only 10-15% of MAP-infected cattle may develop clinical disease (Ott et al., 1999; Tiwari et al., 2008), the carriers shed MAP into feces and milk (Pradhan et al., 2011), which are the main sources of infection for other animals and possibly a zoonotic threat to humans (Pierce, 2009). To date, vaccines for JD are capable of controlling MAP shedding and clinical disease, but are not effective in preventing MAP infection (Rosseels and Huygen, 2008). In addition, animals infected by MAP usually undergo a long asymptomatic period and the diagnosis of MAP infection during the early subclinical stage remains challenging (Nielsen and Toft, 2008; Timms et al., 2011).

MAP infects the gastrointestinal tract primarily through the ileum or distal small intestine during the first few months after a calf is born (Khare et al., 2009; Sweeney, 2011). Bacteria enter via M-cells overlying lymphoid follicles in the ileal Peyer's patches (PPs), and establish a persistent infection in submucosal macrophages (Arsenault et al., 2014). Many studies have focused on the

mechanisms of MAP infection by characterizing host innate and adaptive immune responses *in vitro* (macrophage cell line) and *in vivo* (ileal tissue) during the subclinical period, revealing a pronounced effect on immune cells, the systemic immune system, and the mucosal immune system (Coussens, 2004; Charavaryamath et al., 2013). Recently, gene expression changes in MAP-infected macrophages and whole blood of MAP-infected calves have been reported (Machugh et al., 2012; David et al., 2014). However, little is known about transcriptome alterations and the molecular mechanisms regulating the host response to MAP at the site of infection during the subclinical stage of disease.

A previous study reported the existence of MAP in ileal tissues and MAP-specific immune responses (such as interferon gamma responses) in calves one month after MAP infection, suggesting that a persistent infection was established within one month post-infection (Maattanen et al., 2013). Moreover, the post-transcriptional regulation by microRNAs (miRNAs) and alternative splicing can play a role in host responses to pathogenic bacteria (Eulalio et al., 2012; Rodrigues et al., 2013). Thus, we hypothesized that the regulatory mechanisms of miRNAs and alternative splicing of pre-mRNAs may be associated with host responses during persistent MAP infection. This study used an *in vivo* model to localize MAP infection to the terminal small intestine and studied gene expression and post-transcriptional regulation (miRNA expression and pre-mRNA splicing) at the site of infection one-month post-infection. These transcriptional and post-transcriptional changes provide new insights into the mechanisms by which MAP effectively evades host immune responses and establishes a persistent infection.

4.2 Materials and methods

4.2.1 Animal study and tissue collection

The materials for this study were collected during a previous study (Maattanen et al., 2013) and all experimental protocols were performed following the guidelines approved by the Canadian Council on Animal Care. Protocols for animal housing, anesthesia, surgery, MAP infection, and postsurgical care were performed as previously described (Maattanen et al., 2013). Five male, Holstein calves that were 10-14 days old were inoculated with MAP using surgically isolated intestinal segments. Briefly, a 30- to 35-cm segment of intestine was surgically isolated, proximal to the ileocecal fold, and subdivided into three equal compartments using silk ligatures. The distal compartments were injected with 1-3×10^8 CFU of MAP strain K10 in a final volume of 5 ml phosphate-buffered saline (PBS). The proximal compartment of the intestinal segment was injected with 5 ml PBS. After surgery, calves were treated with 1.1 mg/kg flunixin (Banamine; Schering Plough Canada Inc., Pointe Claire, Quebec, Canada) for 3 days and with 3 to 4 mg/kg enrofloxacin (Baytril; Bayer Inc.) for 5 days. Calves were fed a whole-milk diet for 4 weeks. One-month post-infection, tissues from the distal (infected) and proximal (control) compartments were collected immediately after euthanizing calves (Figure 4.1). Each intestinal compartment was opened longitudinally, the contents removed, and longitudinal strips of intestinal tissue measuring 0.5 cm by 3 cm were collected into cryovials, and snap-frozen in liquid nitrogen prior to storage at -80°C.

4.2.2 RNA extraction

All samples were processed in a level 2-biosecurity lab at University of Alberta and tissue samples were ground into a fine powder in a frozen mortar, while immersed in liquid nitrogen. Total RNA was then extracted from 80 mg of tissue powder using mirVana™ miRNA Isolation Kit (Ambion, Carlsbad, CA) following the manufacturer's instructions. The quality and quantity of the RNA were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), respectively. RNA samples with good quality (integrity number (RIN) > 7.0) were used for further analysis.

4.2.3 RNA-Seq library construction and sequencing

Total RNA (1.0 μg) from each sample was used to construct RNA-Seq libraries using the TruSeq mRNA Sample Preparation kit (Illumina, San Diego, CA) following the manufacturer's instruction. Individual libraries were then pooled for sequencing according to Illumina's instruction and sequenced at Génome Québec (Montréal, Canada) using the Illumina HiSeq 2000 system (Illumina, San Diego, CA). Sequencing was performed as 100 bp paired-end reads. All reads were demultiplexed according to their index sequences with CASAVA version 1.8 (Illumina) and reads that did not pass the Illumina chastity filter were discarded.

4.2.4 RNA-Seq reads mapping and annotation

RNA-Seq reads were aligned to the bovine genome (UMD 3.1) using Tophat 2.0.11 with default parameters (Kim et al., 2013). HTSeq was used

(version 0.6.1, http://www-huber.embl.de/users/anders/HTSeq/) to count the number of reads that were mapped to each gene. The expression level of mRNAs in each library was obtained by normalizing reads number to fragments per million reads (FPM) by the following method: FPM = (gene fragments number / total mapped fragments number per library) × 1,000,000. edgeR was used to identify significantly differentially expressed (DE) mRNAs (Robinson et al., 2010), and the significances were declared at fold change (FC) > 1.5 and $P < 0.05$.

4.2.5 Identification and annotation of alternative splicing events

As suggested by a previous study (Ding et al., 2014), 25 million properly paired reads were randomly selected from each RNA-Seq library for further analysis to confirm that the comparison of alternative splicing events was performed without any bias. Tophat 2.0.11 was used to predict the splice junctions. Splicing analysis was performed for events that had at least 20 total RNA-Seq reads (Wang et al., 2008). JuncBASE (Brooks et al., 2011) was used to annotate the entire alternative splicing events (cassette exons, alternative 5' splice site, alternative 3' splice site, mutually exclusive exons, coordinate cassette exons, alternative first exons, alternative last exons, and intron retention). Values for Percentage Spliced Index (PSI) were calculated using the formulas provided by a previous study (McManus et al., 2014). Fisher's exact tests were performed on raw read counts from 2x2 tables of exclusion and inclusion read counts for each animal to test the different alternative splicing event, as suggested by a previous

study (Wang et al., 2008). The different alternative splicing events were declared at adjusted $P < 0.05$, and $\Delta PSI > 10\%$.

4.2.6 Construction and analysis of small RNA libraries from ileal tissue

Total RNA (1 μg) from each sample was used to construct a small RNA library using the TruSeq Small RNA Sample Preparation kit (Illumina, San Diego, CA) and following the manufacturer's instruction. Individual libraries were then pooled for sequencing according to Illumina's instruction and sequenced at Génome Québec (Montréal, Canada) using the Illumina HiSeq 2000 system (Illumina, San Diego, CA). Sequencing was performed as 50 bp single reads. All reads were demultiplexed according to their index sequences with CASAVA version 1.8 (Illumina, San Diego, CA) and reads that did not pass the Illumina chastity filter were discarded.

All the small RNA data processing was conducted according to the method described in a previous study (Liang et al., 2014). Briefly, sequences with good quality were processed to be short tags by removing the 3'adapter using a perl script provide by miRDeep2 (Friedlander et al., 2012). After trimming the 3' adaptor sequence, all identical sequences of sizes ranging from 18 to 25nt were mapped to the ncRNA sequences (Rfam) to remove non-miRNA small RNA sequences. Then, all the sequences were aligned against the corresponding known miRNA precursor sequences (miRBase release version 20) by using the module of quantifier.pl in miRDeep2 with the default parameters to identify known miRNAs. Novel miRNAs was detected using miRDeep2, with the miRDeep2 score cutoff of 5 and more than 20 mapped reads in all samples. The expression of

miRNAs in each library was normalized to reads per million total mapped reads (RPM) by the following method: RPM = (miRNA reads number/total mapped reads per library) × 1000000. edgeR was used to identify significantly differentially expressed miRNAs (Robinson et al., 2010), and the significances were declared at fold change > 1.5 and $P < 0.05$.

4.2.7 Functional analysis

Ingenuity pathway analysis (IPA, Ingenuity Systems, www.ingenuity.com) was used to identify the functions of DE mRNAs. A threshold of $P < 0.01$ was applied to enrich significant biological functions. The IPA regulation z-score algorithm was used to predict the direction of change for a given function (increase or decrease) according to the FC of DE genes. A z-score > 0 means that a function is increased, whereas a z-score < 0 indicates a significantly decreased function.

The putative target genes for miRNAs were predicted by miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/) as described previously (Liang et al., 2014). The targets predicted by both algorithms were used for further functional analysis. The target genes of DE miRNAs were uploaded into PANTHER for functional analysis of DE miRNAs (Mi et al., 2013). Each analysis was performed using the statistical overrepresentation test option, and significant GO terms were selected at $P < 0.05$ and molecule number > 2.

4.2.8 RT-qPCR validation of differential alternative splicing events

The differential alternative splicing of monocyte to macrophage differentiation-associated (*MMD*) and adenosine deaminase (*ADA*) was validated by duplex RT-qPCR using customized primers and probes. Primers and TaqMan® probes (Life Technologies, Carlsbad, CA) were designed based on each event using Custom TaqMan® Assay Design Tool (https://www.thermofisher.com/). The primers and probes are presented as followed:

Isoform 1 of *MMD*: Forward primer: 5′-AATGGCCGCTACAAGCCAAC-3′; Reverse primer: 5′-CATCAGAGAGCCGGTGAAGG-3′; Probe: 5′FAM-AATGGCCGGAACAATGAGGAATGC-NFQ-MGB3′;

Isoform 2 of *MMD*: Forward primer: 5′-CAGTGCTGATCCTATCTGGAAGA-3′; Reverse primer: 5′-CATCAGAGAGCCGGTGAAGG-3′; Probe: 5′VIC-AGTTTGCATTCTTTCCTCATTGTTCCGG-NFQ-MGB3′;

Isoform 1 of *ADA*: Forward primer: 5′-TGTGGAGATGAAGGCCAAGG-3′; Reverse primer: 5′-CCAGTGACACCACCTCATCC-3′; Probe: 5′FAM-AGCCGATCCCCTGGAACCAGGCTGAAGGGG-NFQ-MGB3′;

Isoform 2 of *ADA*: Forward primer: 5′-CTCCTTCCTCTCTCTCCTACC-3′; Reverse primer: 5′-GATGAGGTGGTGTCACTGG-3′; Probe: 5′VIC-TTCCCCACACACAGAGGGGACCTCACCCCG-NFQ-MGB3′.

Total RNA (1 μg) was treated with DNAase I (Invitrogen, Carlsbad, CA), and reverse-transcribed to cDNA by using a standard protocol (SuperScript II

reverse transcriptase, Invitrogen). A total of 100 ng cDNA, 10.0 µL TaqMan® Fast Advanced Master Mix, primers (final concentrations was 900 nM) and probe (final concentration was 250 nM) were used in a 20 µl reaction, which was suggested by TaqMan® Gene Expression Assays Protocol. Primers and probes for two isoforms of *MMD* or *ADA* were added to one reaction. The fluorescence signal was detected with StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA) with the following cycling times and temperatures: 50°C for 2 mins, 95°C for 20 s and 40 cycles of 94°C for 1 s, 60°C for 20 s. The alternative splicing events were detected by evaluating the ratio between the expressions of two isoforms: $\text{Expression}_{\text{isoform 1}} / \text{Expression}_{\text{isoform 2}} = 2^{(\text{Ct isoform 2} - \text{Ct isoform 1})}$.

4.2.9 Experimental validation of miRNA expression using stem-loop RT-qPCR

Expression of DE miRNAs that were identified by RNA-Seq was validated using stem-loop RT-qPCR with TAQMAN miRNA assays (Applied Biosystems, Carlsbad, CA). Briefly, cDNAs were reverse-transcribed from 10 ng of total RNA using specific miRNA RT primer (Applied Biosystems, Carlsbad, CA) and then were amplified using a TAQMAN miRNA assay. Fluorescence signal was detected with StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). U6 snRNA (Applied Biosystems, Carlsbad, CA) was used as an internal control and the relative expression of miRNA was calculated by ΔΔCt method. Students' t-test was used to compare miRNA expression between infected and control tissues. Differences were considered statistically different at $P < 0.05$ and analyses were performed in R using t.test function.

4.3 Results

4.3.1 RNA-Seq profiling of MAP-infected and control compartments of ileum tissues

The surgically isolated ileum in calves (10 to 14 days old) was subdivided into two compartments: MAP-infected (infected) and non-infected (control). Intestinal tissues from each ileal compartment, including the PPs, were collected from all animals (n = 5) at one-month post-infection (age = 40-44 days) and prepared for transcriptome profiling using RNA-Seq. A total of 300,676,396 paired-end sequence reads were obtained from the 10 samples. On average, ~ 89% of these reads were mapped to the bovine reference genome (UMD3.1). The quality of RNA-Seq data was evaluated via the genomic regions of reads, the RNA-Seq 3'/5' bias and the sequencing depth. Approximately 78% of the reads were derived from exonic regions and intronic, gene upstream and downstream regions, whereas 22% were derived from intergenic regions (Figure 4.2A, B). In addition, the coverage of reads along each transcript revealed no noticeable 3'/5' bias, confirming the good quality of sequencing data (Figure 4.2C). The number of detected transcripts increased along with increasing sequencing reads and the transcript number eventually plateaued, revealing that the most expressed transcripts were detected by RNA-Seq (Figure 4.2D).

A total of 17,789,335 small RNA reads were obtained from the same 10 samples and 58.94% of the total reads were 21 nt and 22 nt in length (Figure 4.2E). The reads (15,207,875) with 18-25 nt lengths were used for further miRNA expression analysis. Among these reads, 14,062,510 reads were mapped to a

known miRNA database (miRBase version 21), which resulted 3,802 reads representing 55 putative novel miRNAs (Data has been deposited in GEO: GSE68274). The rest of 1,137,761 reads that were not identified as miRNAs belonged to other small noncoding RNAs (tRNA, snoRNA, snRNA and others). Cumulative frequency analysis revealed that the 20 most highly expressed miRNAs accounted for around 90% of the sequenced reads, representing the majority of expressed miRNAs (Figure 4.2F).

4.3.2 Transcriptome, alternative splicing events, and miRNA expression changes after MAP infection

An average of 14,444 ± 187 (mean ± SD) and 14,430 ± 87 genes were identified (fragments per million mapped fragments (FPM) > 1) in control and infected ileal compartments, respectively. In total, 13,046 transcripts were commonly expressed in all 10 samples. Functions of the 3000 most highly expressed transcripts were related to “metabolic process” and “protein synthesis”. Principal component analysis (PCA) and hierarchical cluster analysis showed no clear separation between infected and control samples (Figure 4.3A, F). The transcriptome profile of animal #5 control compartment was an outlier from all control samples of other animals (Figure 4.3A), thus this animal was subsequently removed from further analysis.

An average of 20,036 ± 3,163 alternative splicing events were detected in the control compartment tissues, whereas 21,005 ± 870 were detected in infected tissues. There were no significant differences between the two groups in terms of the number of alternative splicing events. However, the number of alternative

splicing events differed greatly between control and infected compartments (14,598 *vs.* 19,661) of animal #5, which was also an outlier (Figure 4.3B). These differences led us to exclude animal #5 from further pre-mRNAs splicing analyses. The remaining four animals revealed 26,045 alternative splicing events (5,903 alternative acceptor, 3,535 alternative donor, 1,262 alternative first exon, 324 alternative last exon, 4,344 cassette, 705 coordinate cassette, 9,918 intron retention, and 54 mutually exclusive events) from 6,695 genes expressed in the ileal transcriptome (46.2% of total expressed genes) (Figure 4.3D, E).

The number of detected miRNAs (reads per million mapped reads (RPM) > 1) was 375 ± 26 in infected compartments and 375 ± 25 in control compartments with 280 miRNAs commonly expressed in all 10 samples. The most highly expressed miRNA was miR-143. Although PCA did not show a clear separation (Figure 4.3C), hierarchical cluster displayed a separation between the miRNA expression patterns of control and infected tissues (Figure 4.3G). Unlike mRNA profile and alternative splicing events, the control compartment of animal #5 was not an outlier (Figure 4.3C); however, to be consistent, this animal was excluded from further miRNAs differential expression analyses.

4.3.3 Altered expression of mRNAs in MAP-infected ileal compartments

The analysis of differentially expressed (DE) mRNAs between infected (n = 4) and control compartments (n = 4) using edgeR (Robinson et al., 2010) revealed 81 DE transcripts (14 down-regulated and 67 up-regulated in infected compartments; $P < 0.05$, fold change > 1.5). PCA plots revealed a clear clustering of these DE transcripts based on the ileal compartments (infected vs. control)

(Figure 4.4A). The most relevant function of DE genes estimated by the Ingenuity Pathway Analysis (IPA) was “proliferation of endothelial cells”, which showed an activated trend in infected tissues (z-score = 1.01) (Figure 4.4B). Moreover, the IPA revealed that the DE genes were significantly related to “glucose metabolism disorder” and down-regulated “proliferation of muscle cells” (z-score = -1.67) (Figure 4.4B).

To evaluate the impact of MAP infection on host innate and adaptive immune responses, genes related to innate (793 ± 6, annotated by GO: 0045087) or adaptive immune responses (220 ± 3, annotated by GO: 0002250) were enriched. Although some genes in certain individual animals revealed fold change more than 1.5 (Figure 4.4C, D), neither innate nor adaptive immune-related gene expression showed statistical differences between two compartments (fold change > 1.5, $P < 0.05$, paired t-test).

4.3.4 Altered alternative splicing events of pre-mRNA in MAP-infected ileal compartments

To test the differences in alternative splicing events between control and infected compartments, Fisher's exact tests were performed on raw read counts from 2 × 2 tables of exclusion and inclusion read counts for each animal as suggested by a previous study (Wang et al., 2008). The differential alternative splicing events were identified in each animal (adjusted $P < 0.05$, Fisher’s exact test, ΔPSI >10%). There were 16 genes, which displayed significantly different alternative splicing events between control and infected compartments of all four animals. Among them, an alternative first exon event was detected in the mRNA

of monocyte to macrophage differentiation-associated (*MMD*) (Figure 4.5A). An average of 113 ± 12 and 16 ± 6 reads were mapped to exon 1A (the first annotated exon in bovine genome UMD 3.1) and exon 1B (the alternatively spliced exon for the first exon), respectively in control tissues, whereas 36 ± 9 and 95 ± 20 reads were mapped to exon 1A and exon 1B, respectively in infected tissues (Figure 4.5A). In addition, an intron retention event was detected in Adenosine deaminase (*ADA*) transcript (Figure 4.5B). A lower number (14 ± 8) of reads was mapped to intron 4 (intron region between exon 4 and exon 5 of the *ADA* mRNA) in control tissues, compared to that of infected tissues (56 ± 15) (Fisher's exact test, $P < 0.0001$) (Figure 4.5B). Subsequently, further protein sequence analysis (translate RNA sequence to protein sequence) on the above alternative spliced forms of *MMD* and *ADA* mRNAs in infected tissues revealed the potential introduction of stop codons, when the detected alternative splicing events happen (Figure 4.6). The multiplex reverse transcription quantitative PCR (RT-qPCR) was used to further verify the differences between MAP-infected and control tissues in alternative spliced forms of above two genes. The primers and probes were designed to target the identical isoforms that were detected by RNA-Seq. Using the primers and probes designed, only two isoforms were detected for both genes. The ratio between the expression of isoform 1 (exon 1A + exon 2) and isoform 2 (exon 1B + exon 2) for *MMD* decreased significantly ($P < 0.01$, paired t-test) in infected tissues (0.21 ± 0.05) when compared to control tissues (0.10 ± 0.05) (Figure 4.5C). Similarly, the ratio between the expression of isoform 1 (exon 4 + exon 5) and isoform 2 (exon 4 + intron 4 + exon 5) for *ADA* decreased

significantly ($P < 0.01$, paired t-test) in infected tissues (8.26 ± 4.02) when compared to control tissues (1.48 ± 0.48) (Figure 4.5D). RT-qPCR confirmed that the expression of isoform 2 for both genes was upregulated in the infected tissues *vs*. control tissues.

4.3.5 Altered miRNA expression in MAP-infected ileal compartments

A total of 14 DE miRNAs were identified when comparing infected (n =4) and control (n = 4) compartments of the ileum ($P < 0.05$, edgeR paired group comparison, fold change > 1.5). The expression of bta-miR-105a, bta-novel-53, bta-miR-433, bta-miR-2400, bta-miR-137, bta-miR-424-3p, and bta-miR-138 was down-regulated in the infected compartment, when compared to the control (Figure 4.7A). In contrast, the expression of bta-miR-146b, bta-miR-196b, bta-miR-2483-5p, bta-miR-133b, bta-miR-1247-5p, bta-miR-184, and bta-miR-202 was up-regulated in the infected compartment, when compared to the control (Figure 4.7A). Stem-loop RT-qPCR results confirmed differential expression patterns of 9 miRNAs (bta-miR-105a, bta-miR-133b, bta-miR-137, bta-miR-146b, bta-miR-184, bta-miR-196b, bta-miR-202, bta-miR-433, and bta-miR-1247-5p) revealed by RNA-Seq (Figure 4.7B).

4.3.6 Integrated analysis of miRNA and mRNA networks

The integrated analysis of miRNA and mRNAs was performed using a two-step method. First step included the computational prediction of target genes of DE miRNAs using miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/) and the selection of targets predicted by both algorithms to use in further analyses. Then, the Pearson's correlation analysis was

performed between the expression of DE miRNAs and their computationally predicted targets detected by RNA-Seq. The significantly correlated miRNA-mRNA pairs ($r < -0.5$, $P < 0.05$) were selected for further functional analysis. Functional analysis of the miRNA-mRNA pairs revealed that the DE miRNAs were involved in "lymphocyte activation" (miR-133b, miR-146b, and miR-196b), "inflammatory response activation" (miR-146b, miR-184, and miR-1247), "muscle tissue and epithelium development" (miR-146b and miR-137), as well as "proliferation of endothelial cells" (miR-137, miR-196b, miR-433, and miR-1247) (Figure 4.7C).

4.4 Discussion

It is well established that MAP inhibits macrophage bactericidal activity by blocking phagolysosome formation, a primary effector function of macrophage following intracellular infection (Souza et al., 2008). Our analysis revealed that alternative splicing of pre-mRNAs may be a potential mechanism by which MAP evades macrophage killing by altering macrophage maturation and lysosome functions. Especially, *MMD*, a marker gene of macrophage maturation (Rehli et al., 1995), displayed an increased frequency for an abnormal first exon that could disrupt protein synthesis in the infected tissues, when compared to control tissues. MMD is an ion channel protein and decreased synthesis of this protein may block macrophage maturation (Liu et al., 2012). Furthermore, retention of the fourth intron in *ADA* mRNA can produce a truncated protein, resulting in defective lysosome functions (Lindley and Pisoni, 1993). Together, these two splice

variants can result in a failure of macrophage maturation and lysosome function, reducing MAP clearance from the site of infection during the early stage of infection. Changes in alternative splicing events following bacterial (*Escherichia coli*, *in vitro*), viral (*Sendai* virus, *in vitro*), and parasite (*Crithidia bombi*, in insect) infections have been reported previously, but the impacts of splice variants on protein expression levels are not well studied (Huang et al., 1997; Rodrigues et al., 2013; Chen et al., 2014; Riddell et al., 2014). Thus, it is necessary to confirm that these two alternative splicing events observed in the present study impact on macrophage protein expression levels during MAP infection, *in vivo*. However, this will be a challenge to study *in vitro,* when MAP infects only a subpopulation of macrophages during the early stage of infection.

In addition to the influence on macrophages, MAP infection also may have altered toll-like receptor (TLRs) signaling pathway and downstream inflammatory responses by altering miRNA expression. The expression of miR-146b, which inhibits the activity of intermediate molecules of the TLR-pathway, such as interleukin-1 receptor-associated kinase 1 (IRAK1), interleukin-1 receptor-associated kinase 2 (IRAK2) and TNF receptor-associated factor 6 (TRAF6) was significantly increased in infected tissue (Hou et al., 2009). The up-regulation (FC = 3.22) of miR-146b in infected ileum may be one of the mechanisms by which MAP disrupts TLR signaling, following the infection. This is consistent with a previous *in vivo* study that reported inhibition of the TLR signaling pathway at 12 hours after MAP infection in the ileum (Khare et al., 2012). Our results suggest that subversion of TLR signaling may be sustained

throughout a persistent MAP infection and increased production of miR-146b may play a key role in this process. There were no significant differences in the expression of TLR signaling molecules (IRAK1, IRAK2 and TRAF6) at the mRNA level, when comparing infected and control compartments. It is still possible; however, that protein expression of these molecules may have been reduced due to the post-transcriptional regulation by miR-146b. Future studies using antibodies to detect TLR signaling molecules (miR-146b target genes) in MAP-infected tissues, may provide in depth understanding on the role of miR-146b during early infection. Moreover, miR-146b was negatively correlated with its predicted target genes, interleukin 4-receptor (*IL4R*) ($r = -0.67$, $P < 0.01$) and spleen tyrosine kinase (SYK) ($r = -0.59$, $P < 0.01$). SYK activates the NF-κB-mediated transcription of cytokines (Kashiwada et al., 2001; Fleischer et al., 2014). Their negative correlations with miR-146b indicate that inflammatory responses triggered via TLR-signaling pathway may also be inhibited by miR-146b. These observations suggest that miR-146b may be able to regulate host responses to MAP infection at multiple stages. Overall, the miRNA results provide further supporting evidence to a previous study reporting the inhibition of TLR-signaling pathway and expression of pro-inflammatory cytokines following MAP infection (Arsenault et al., 2014).

The RNA-Seq data also suggested that the most relevant function of DE genes in infected ileum was related to “endothelial cell proliferation”. The up-regulation of genes, such as cadherin 13 (CDH13), semaphorin 6B (SEMA6B), dimethylarginine dimethylaminohydrolase 1 (DDAH1), vasoactive intestinal

peptide (VIP) and integrin alpha 1 (ITGA1), are consistent with increased proliferation of endothelial cells in the infected tissues (Pozzi et al., 2000; Ivanov et al., 2004; Yang et al., 2009; Kigel et al., 2011; Zhang et al., 2011). Activation of endothelial cell proliferation was also suggested by the detected changes in miRNA expression. We identified four DE miRNAs, including miR-1247, miR-137, miR-196b, and miR-433, with target genes involved in "proliferation of endothelial cells". For example, increased expression of miR-196b, an ileum-specific miRNA in young calves (Liang et al., 2014), has been reported to increase endothelial cell proliferation in cancer (How et al., 2013). It is intriguing to consider the possibility that the tissue specificity of this miRNA in the gastrointestinal tract of young calves may contribute to the proclivity for MAP infections to persist in the ileal region of the small intestine. Furthermore, the negative correlation between miR-196b and secreted protein, acidic, cysteine-rich (SPARC) ($r = -0.65$, $P < 0.05$), an inhibitor of the proliferation of endothelial cells (Sage et al., 1995), suggests that upregulation of miR-196b in infected tissues may increase the proliferation of endothelial cells by inhibiting SPARC expression. Proliferation of endothelial cells after MAP infection is one of the causes of granuloma formation (Pierce, 2009), which is a significant feature of JD in ruminants (Arsenault et al., 2014). Granulomas mainly consist of MAP-infected macrophages and provide an organized and protected microenvironment within which MAP can establish a persistent infection in the host (Silva Miranda et al., 2012). Although, no granulomas were histologically visible at one-month post-infection in our study (), a previous study reported that granulomas were

observed at 3 to 4 weeks after mycobacteria infection (Co et al., 2004). Nevertheless, this study revealed that MAP infection promotes endothelial cell proliferation by changing both mRNA and miRNA expression at the site of infection and these transcriptional changes may support granuloma formation during a persistent infection of MAP.

In the present study, transcriptional changes of immune genes observed at one-month post infection were all related to innate immune response functions but not the induction of adaptive immune responses. This observation is consistent with a previous study that reported the absence of detectable immune responses during the subclinical stage of MAP infection in ileum (Weiss et al., 2006). The proliferation of regulatory T cells was proposed as a potential mechanism for the suppression of an adaptive immune response (Weiss et al., 2006). However, similar to Roussey and colleagues (Roussey et al., 2014), our analysis did not reveal any significant transcriptional changes related to T cell function at one-month post-infection. More detailed studies are required to determine whether there is either the induction of adaptive immune responses in the ileal PPs or recruitment of mucosal effector cells to the site of MAP infection during the subclinical stage of disease.

4.5 Conclusions

In conclusion, we identified DE miRNAs and alternative splicing events that may contribute to molecular regulatory mechanisms that permit persistent MAP infection during early stage infection. The surgically isolated intestinal

segment model system enabled identification of significant changes between infected and non-infected ileal tissues within the same animal. The overall effect of MAP infection on mRNA expression was limited at one month post-infection, and DE genes were primarily related to "endothelial cell proliferation". Furthermore, two genes related to macrophage functions (*MMD*, *ADA*) were confirmed to be differentially spliced in MAP-infected compartments, suggesting a possible mechanism by which MAP escapes the host innate immune response. Profiling miRNA expression revealed that miRNAs might be more responsive than mRNA during the early stage of MAP infection. The integration of DE miRNA with DE mRNAs revealed potential miRNA-mRNA regulatory pairs (miR-196b and *SPARC*; miR-146b and *IL4R*; miR-146b and *SYK*) that were related to MAP-associated changes in "endothelial cell proliferation", "bacteria recognition", and "activation of inflammatory responses". These miRNAs, especially if they are released into circulation system from infected tissues, may provide potential biomarkers to diagnose subclinical-stage MAP infection.

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4.7 Figures and tables

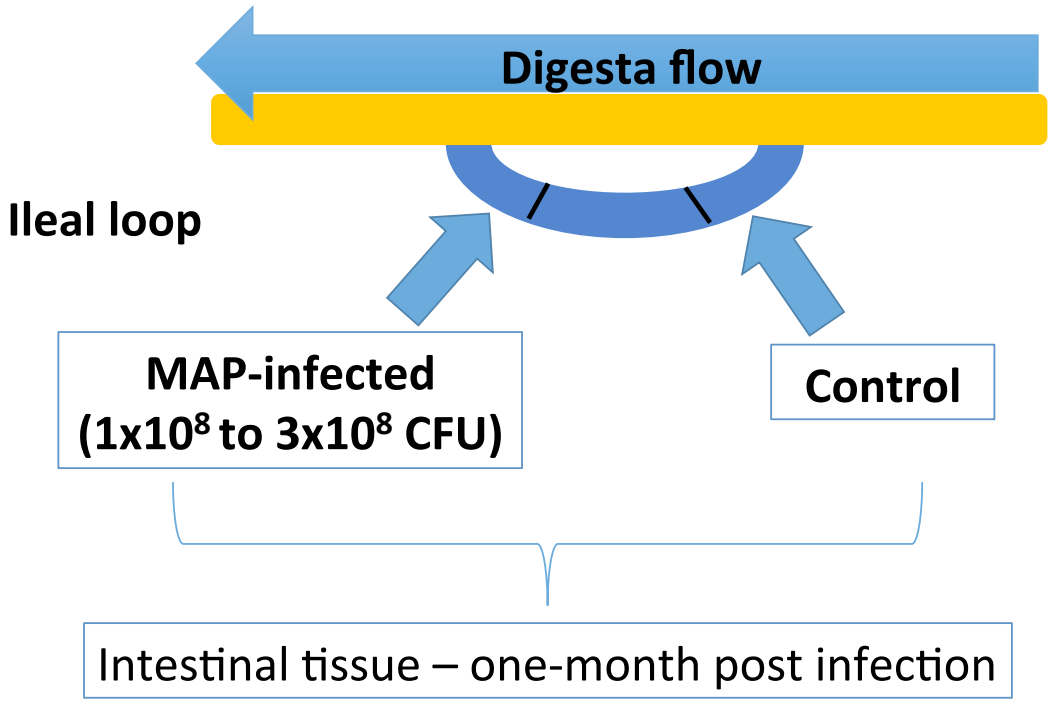


Figure 4.1 Diagram of the ileal loop model. The light blue arrow represents direction of digesta flow; the Yellow bar represents the ileal tissue; the dark blue circle represents the isolated ileal loop tissues. The isolated ileal loop tissues were divided into three segments by silk. The distal and proximal terminus were treated by MAP and used as control tissues.

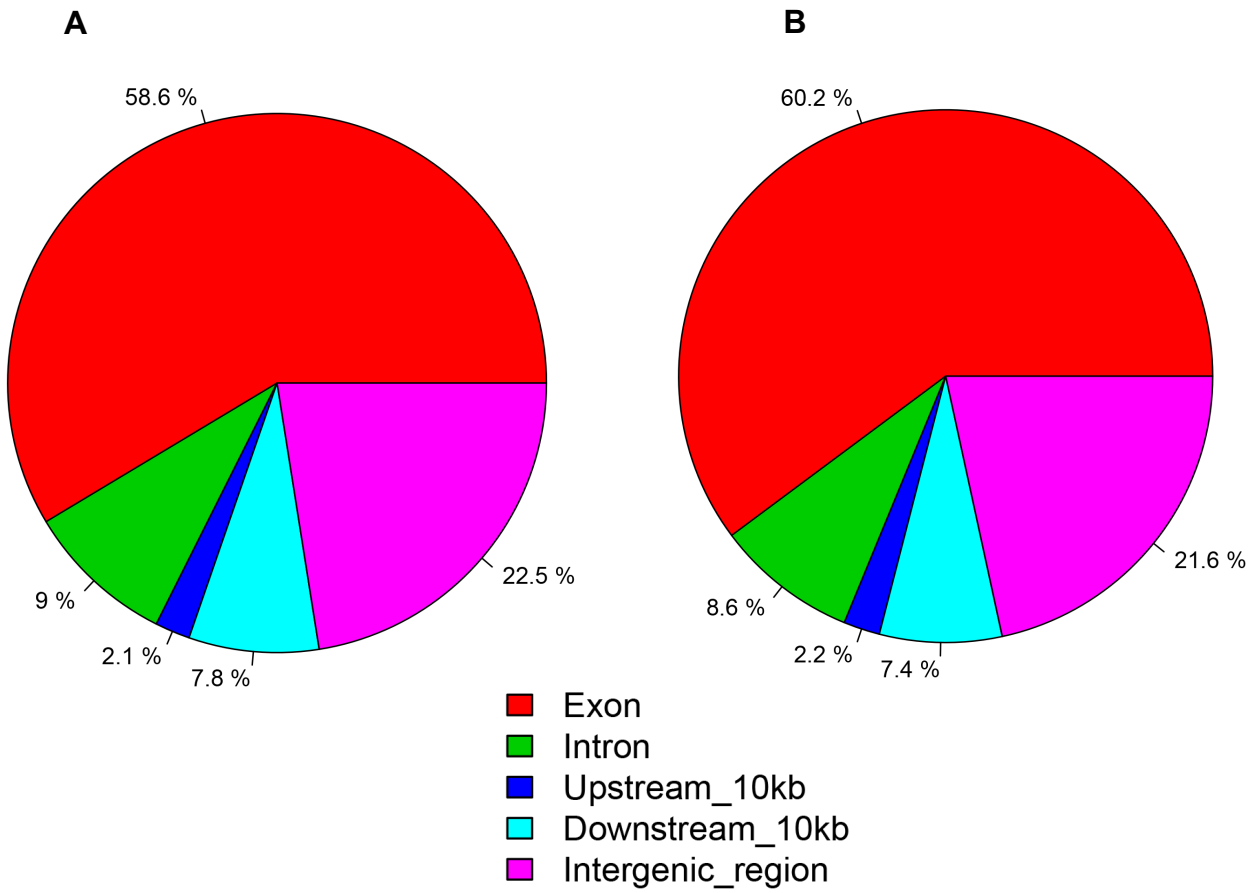


Figure 4.2 Quality control of RNA-Seq dataset. (A) The distribution of genomic locations of RNA-Seq reads in control compartment. **(B)** The distribution of genomic locations of RNA-Seq reads in infected compartment. **(C)** Saturation curve for gene number detection; X-axis - number of the mapped reads; Y-axis - number of the expressed genes (FPM > 1). **(D)** The plot of RNA-Seq coverage of gene body. **(E)** Length distribution of small RNA reads. **(F)** Cumulative frequency of detected miRNAs.

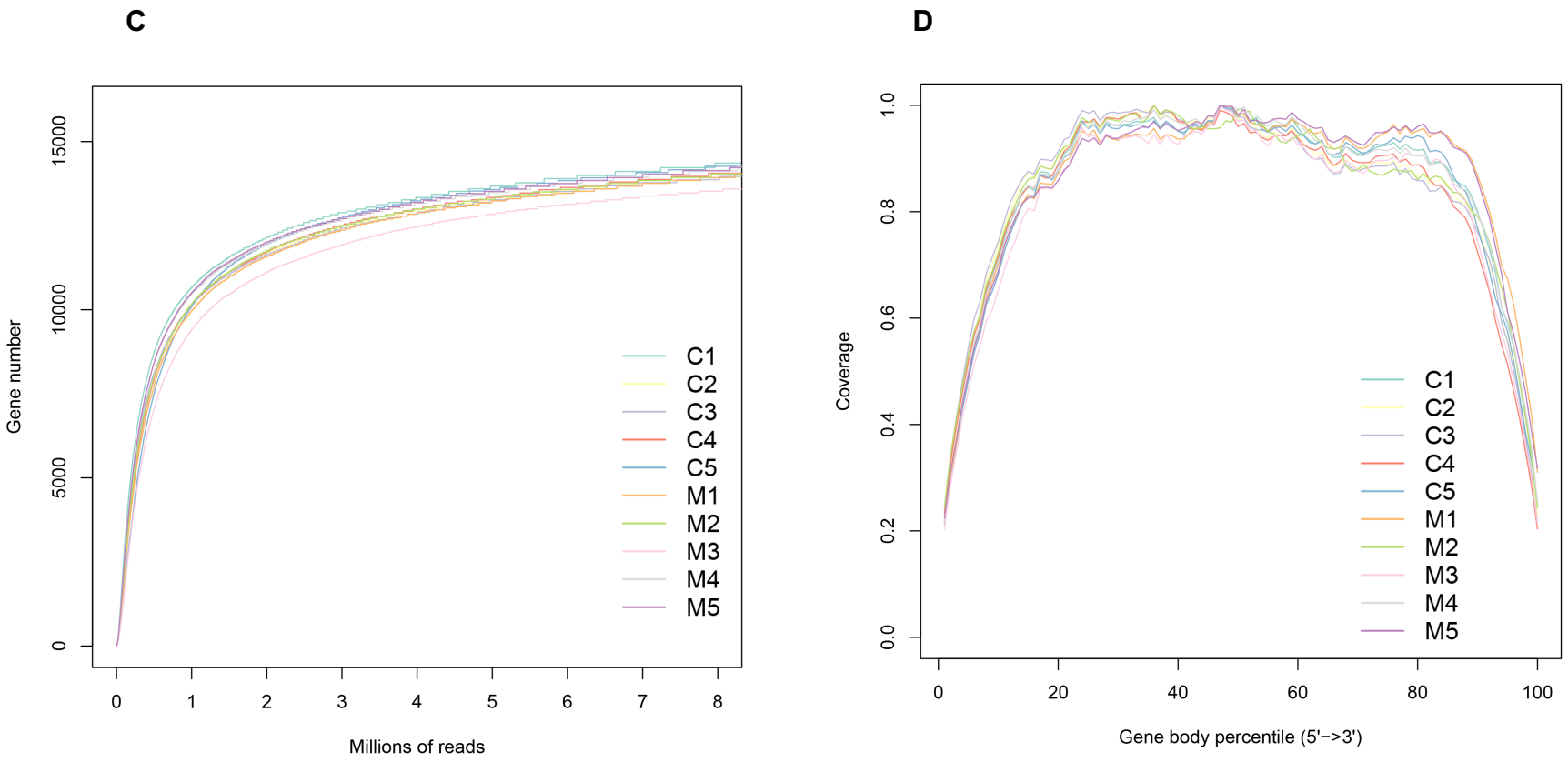
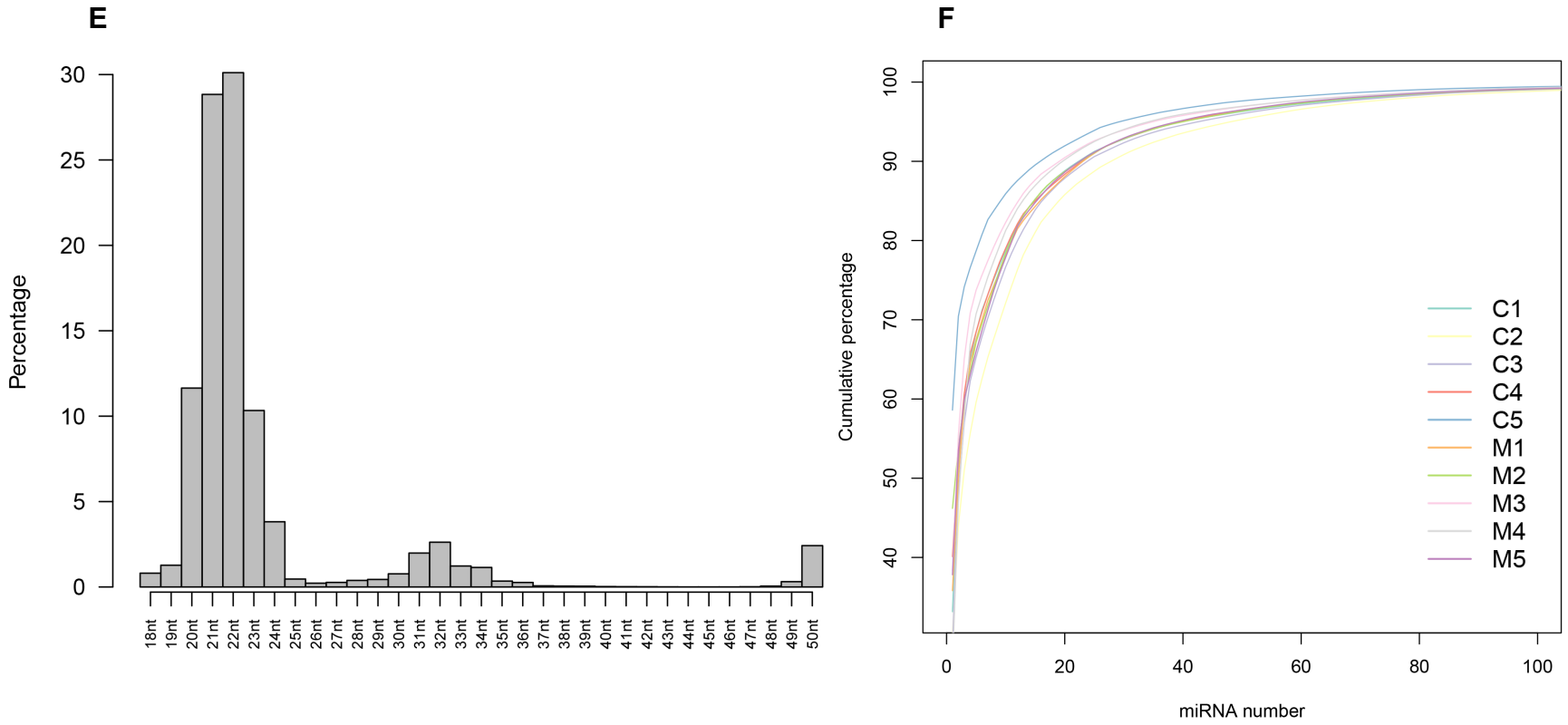


Figure 4.2 (Continued) Quality control of RNA-Seq dataset. (A) The distribution of genomic locations of RNA-Seq reads in control compartment. **(B)** The distribution of genomic locations of RNA-Seq reads in infected compartment. **(C)** Saturation curve for gene number detection; X-axis - number of the mapped reads; Y-axis - number of the expressed genes (FPM > 1). **(D)** The plot of RNA-Seq coverage of gene body. **(E)** Length distribution of small RNA reads. **(F)** Cumulative frequency of detected miRNAs.



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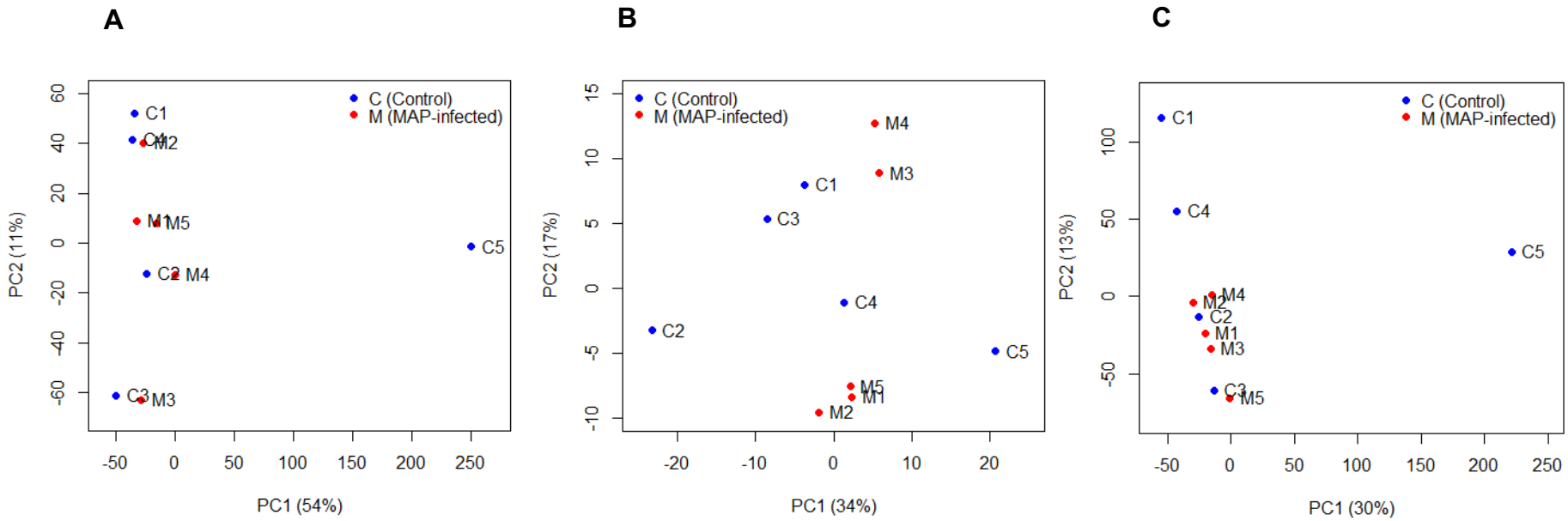


Figure 4.3 Profiling of mRNA expression, alternative splicing events and miRNA expression. (A) PCA plot of mRNA expression. **(B)** PCA plot of alternative splicing events. **(C)** PCA plot of miRNAs expression. **(D)** Categories of alternative splicing events. **(E)** The numbers of different alternative splicing events. **(F)** Hierarchical cluster of mRNA expression. **(G)** Hierarchical cluster of miRNAs expression. The red and green frames indicate two clusters based on miRNAs expression.

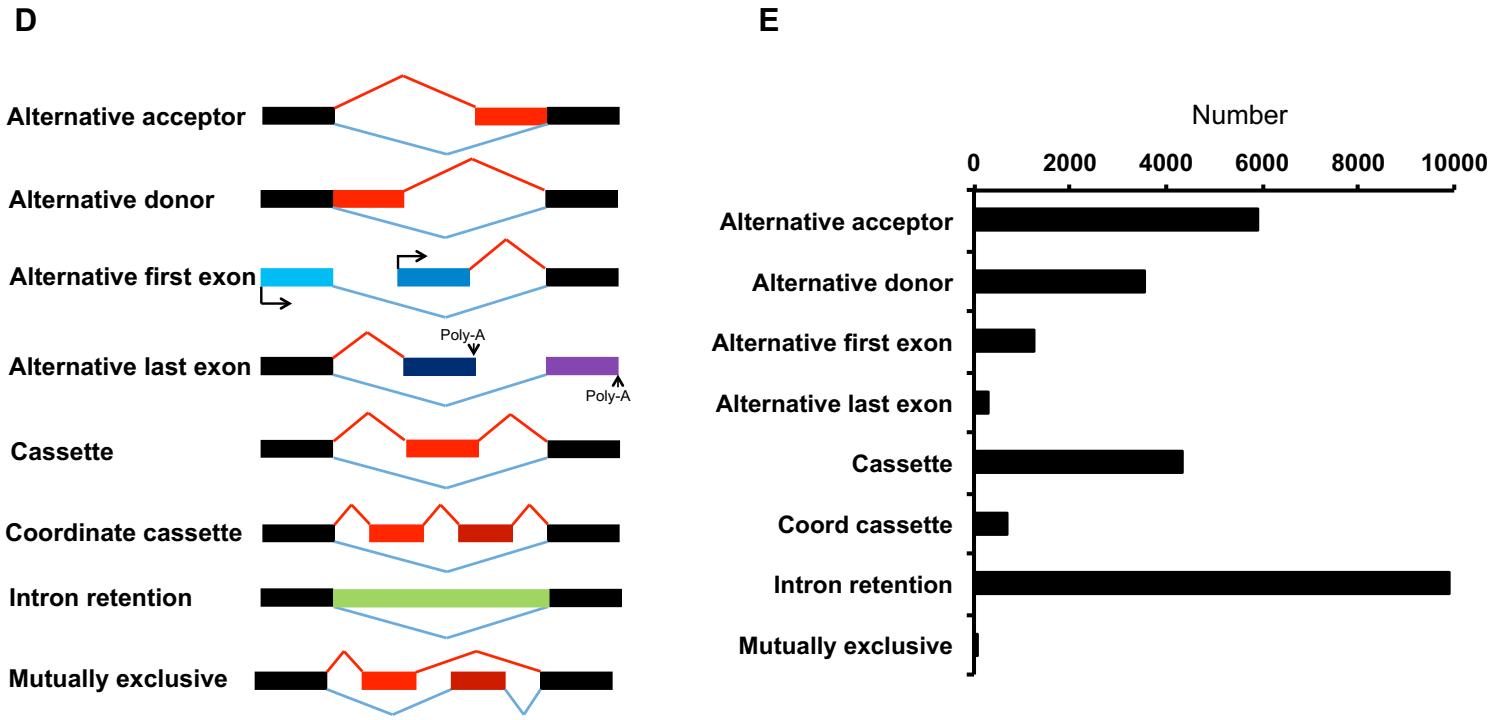
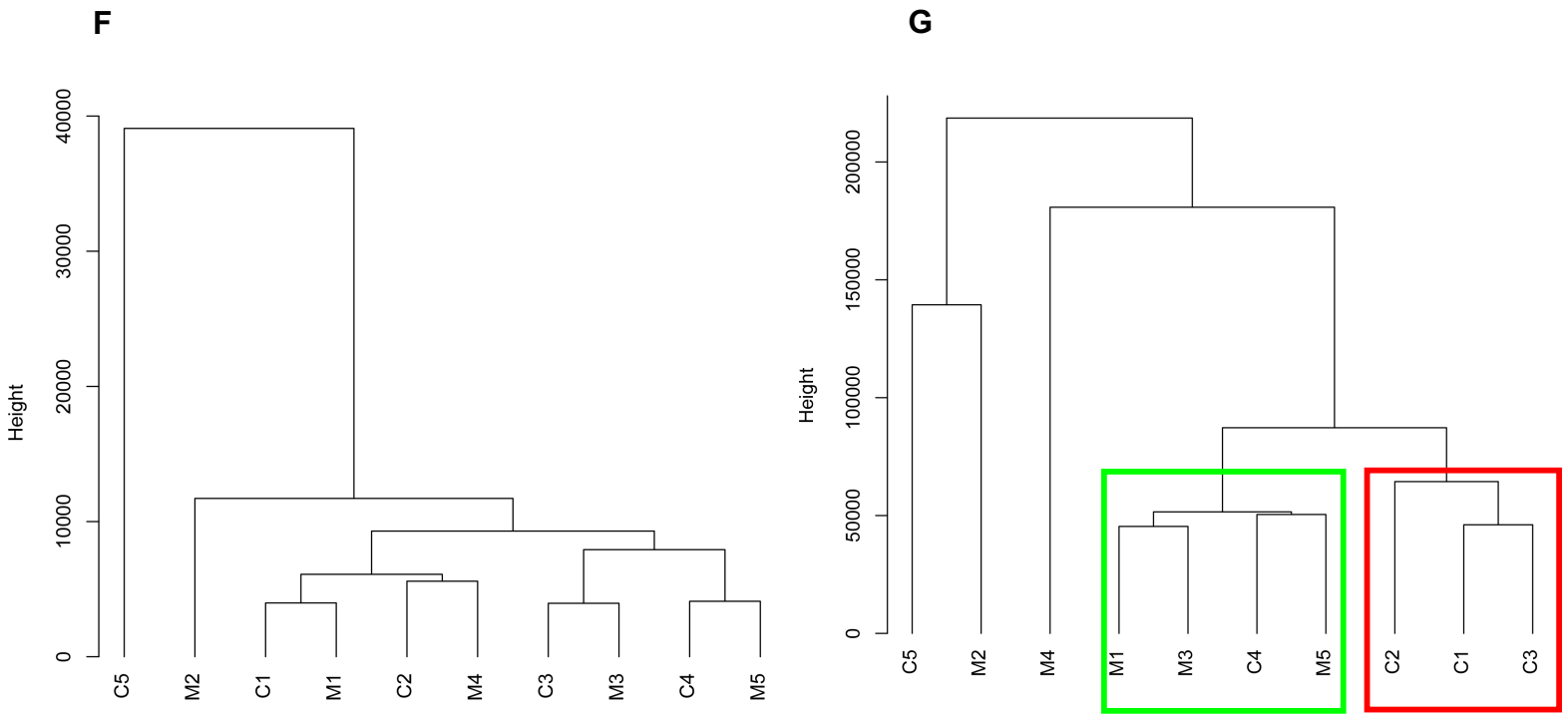


Figure 4.3 (Continued) Profiling of mRNA expression, alternative splicing events and miRNA expression. **(A)** PCA plot of mRNA expression. **(B)** PCA plot of alternative splicing events. **(C)** PCA plot of miRNAs expression. **(D)** Categories of alternative splicing events. **(E)** The numbers of different alternative splicing events. **(F)** Hierarchical cluster of mRNA expression. **(G)** Hierarchical cluster of miRNAs expression. The red and green frames indicate two clusters based on miRNAs expression.



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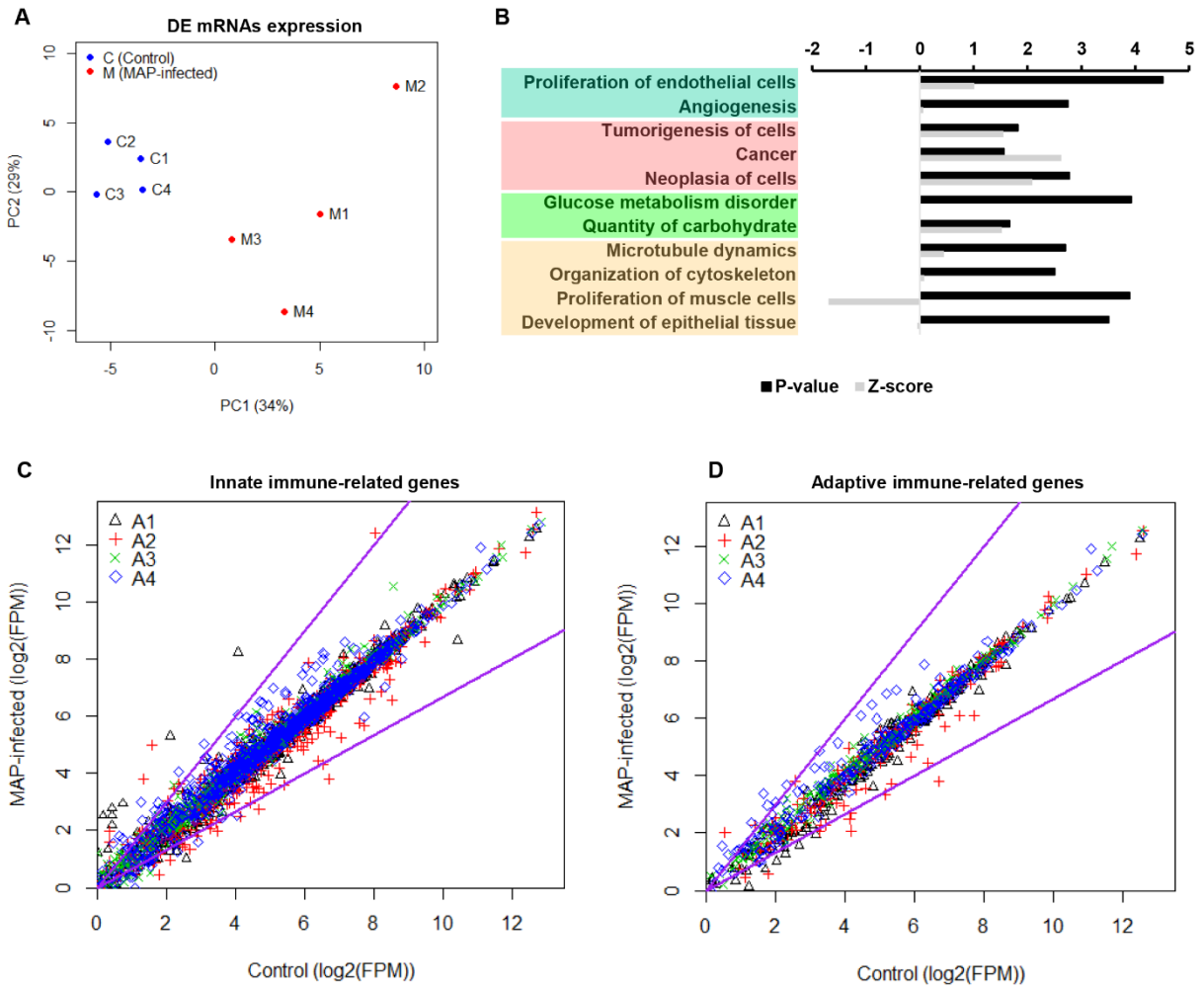

Figure 4.4 Analysis of differentially expressed mRNAs. (A) PCA plot of differentially expressed mRNAs. **(B)** Barplot of GO terms enrichment analysis. The X-axis represented the –log (*P*-value), with longer bars indicating more relevant functions. **(C)** Fold change of innate immune-related genes when comparing control versus infected tissues. Each dot denotes a single gene. The X-axis represents the log2 (FPM) value for each gene in control tissue, while Y-axis represents the log2 (FPM) value for each gene in infected tissues. Dots beyond the purple lines indicate fold change > 1.5. A1, A2, A3 and A4 represent four individuals. **(D)** Fold change of adaptive immune-related genes when comparing infected versus control tissues with data presented as described above. A1, A2, A3 and A4 represent four individuals.

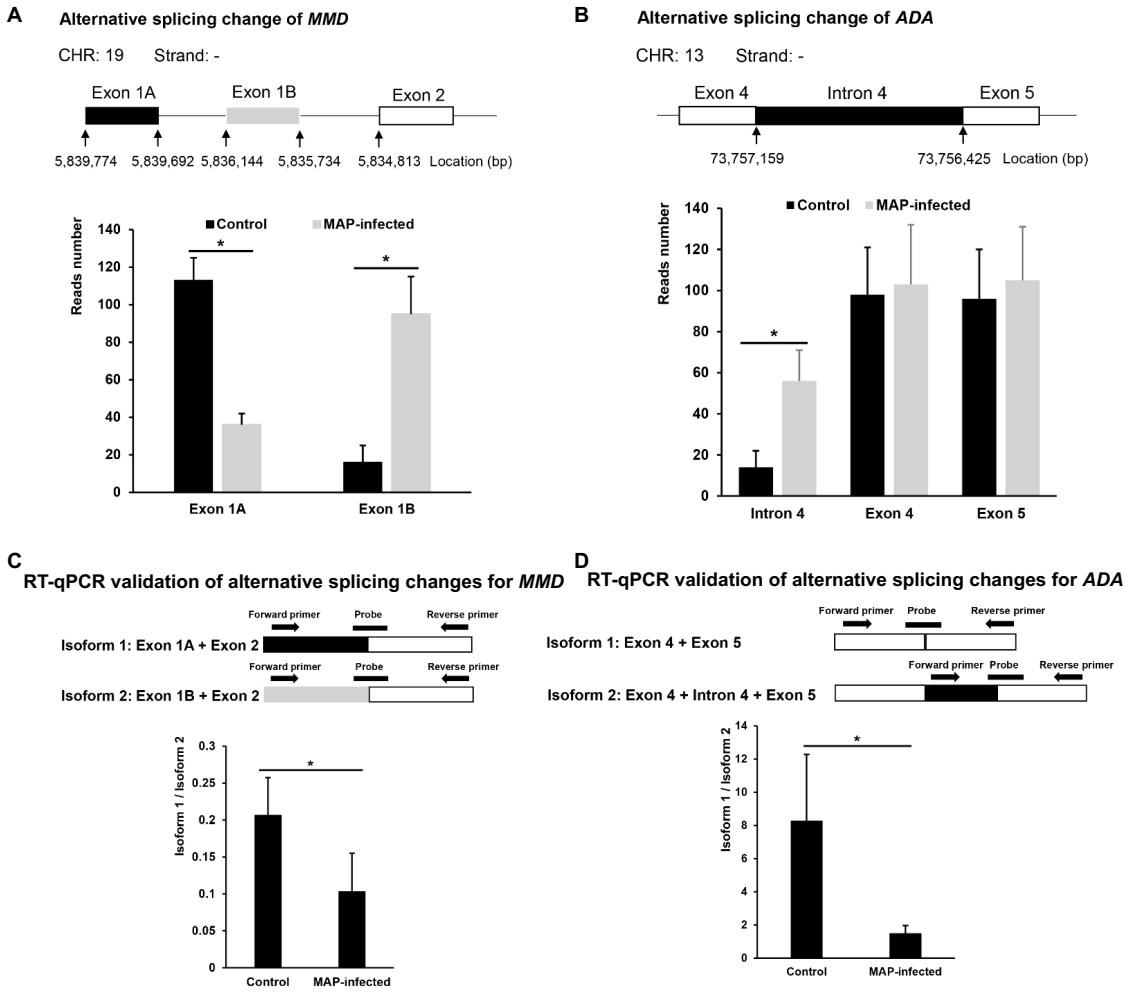

Figure 4.5 Analysis of alternative splicing events. (A) The alternative first exon event of MMD. The figure shows the genomic location of this event, and Exon 1A and Exon 1B were alternatively spliced as the first exon of MMD mRNA. The Y-axis represents the read number mapped to Exon 1A or Exon 1B. **(B)** The intron retention event for ADA. The figure shows the genomic location of this event and Intron 4 was alternatively spliced. The Y-axis represented the read number mapped to Exon 4, Exon 5 or Intron 4. RT-qPCR validation of alternative splicing changes for MMD **(C)** and ADA **(D)**. Primers and probes were designed based on each event. Y-axis of the bar plot represented the ratio between the expressions of two isoforms: isoform 1 / isoform 2. *significant difference at $P < 0.05$.

Protein sequence of Isoform 1 for *MMD*:

Met N H R A P A N G R Y K P T C Y E H A A N C Y T H A F L I V P A I V G S A L
L H R L S D D C W E K I T A W I Y G Met G L C A L F I V S T V F H I V A W K K S
H L R T V E H W F H Met C D R Met V I Y F F I A A S Y A P W L N L R E L G P L
A S H Met R W F I W L Met A A G G T I Y V F L Y H E K Y K V I E L F F Y L T Met
G F S P A L V V T S Met N N T D G L H E L A C G G L I Y C L G V V F F K S D G I
I P F A H A I W H L F V A T A A A V H Y Y A I W K Y L Y R S P T D F Met R H L
Stop

Protein sequence of Isoform 2 for *MMD*:

G T L I Stop

Protein sequence of Isoform 1 for *ADA*:

Met A Q T P A F N K P K V E L H V H L D G A I K P E T I L Y Y G R K R G I A L P
A D T P E E L Q N I I G Met D K P L S L P E F L A K F D Y Y Met P A I A G C R E A
V K R I A Y E F V E Met K A K D G V V Y Y V E V R Y S P H L L A N S K V E P I P
W N Q A E G D L T P D E V V S L V N Q G L Q E G E R D F G V K V R S I L C C
Met R H Q P S W S S E V V E L C K K Y R E Q T V V A I D L A G D E T I E G S S L
F P G H V Q A Y A E A V K S G V H R T V H A G E V G S A N V V K E A V D T L
K T E R L G H G Y H T L E D T T L Y N R L R Q E N Met H F E V C P W S S Y L T
G A W K P D T E H P V V R F K N D Q V N Y S L N T D D P L I F K S T L D T D Y
Q Met T K N E Met G F T E E E F K R L N I N A A K S S F L P E D E K K E L L D L
L Y K A Y G Met P S P A S A E Q C L Stop

Protein sequence of Isoform 2 for *ADA*:

Met A Q T P A F N K P K V E L H V H L D G A I K P E T I L Y Y G R K R G I A L P
A D T P E E L Q N I I G Met D K P L S L P E F L A K F D Y Y Met P A I A G C R E A
V K R I A Y E F V E Met K A K D G V V Y Y V E V R Y S P H L L A N S K V E P I P
W N Q A E Stop

Figure 4.6 Protein sequence analysis of alternative splicing events

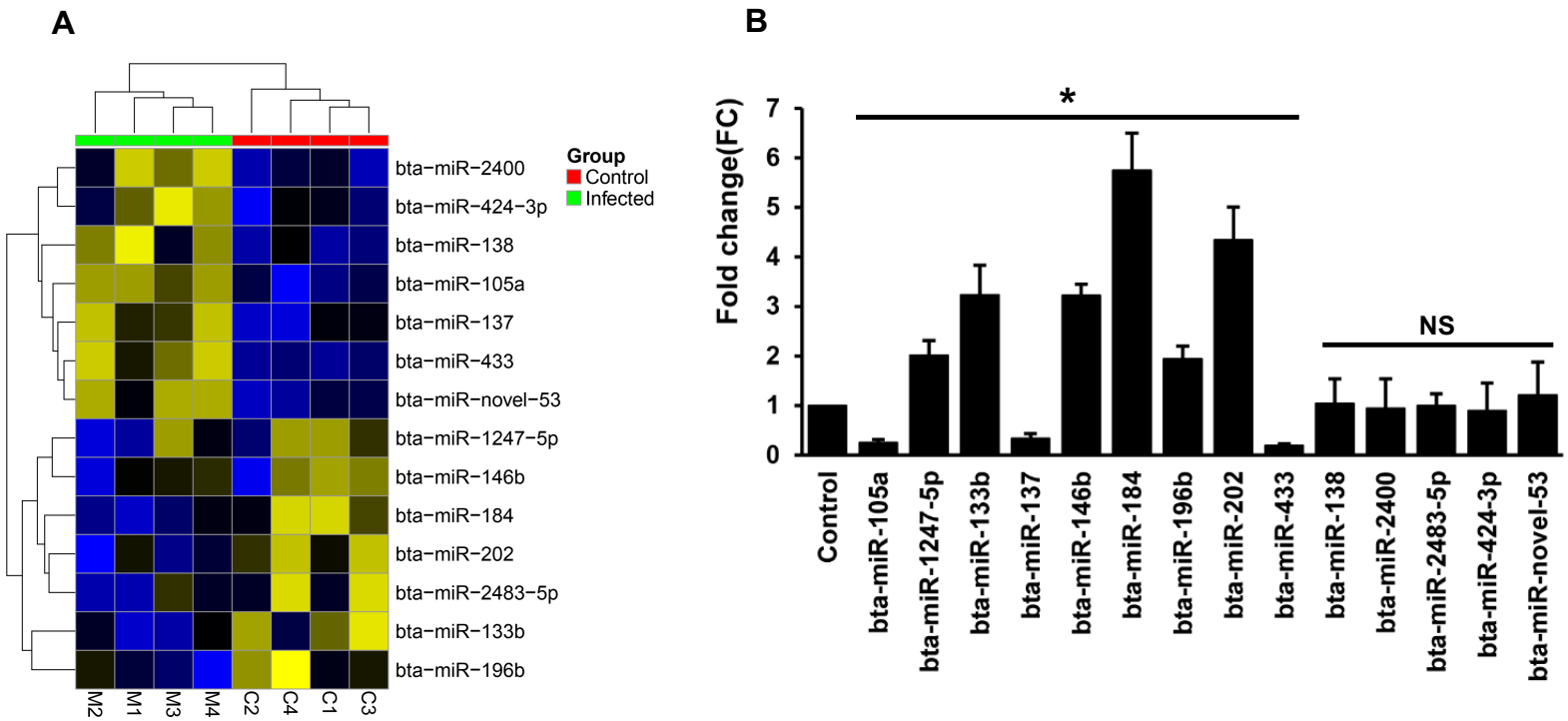

Figure 4.7 Analysis of differentially expressed miRNAs. (A) Heatmap of differentially expressed miRNAs based on RNA-Seq expression data. Blue indicates a higher expression level, whereas yellow represents decreased expression levels. **(B)** Validation of miRNA expression using stem-loop RT-qPCR. Y-axis presents fold change (FC) of miRNA when infected tissue was compared to control tissues. *significant difference at $P < 0.05$; NS: no significant differences. Data presented as mean ± standard error of the mean (SEM). **(C)** GO term enrichment of miRNA targeted genes. Targeted genes were identified on the basis of two criteria: computational prediction and negative correlation analysis, r < -0.5, $P < 0.05$, Pearson's correlation analysis. Black squares represent functions with significant enrichment ($P < 0.05$) that were identified by PANTHER. Red/green arrows indicate miRNAs were down-/up-regulated, respectively, in infected compartment.

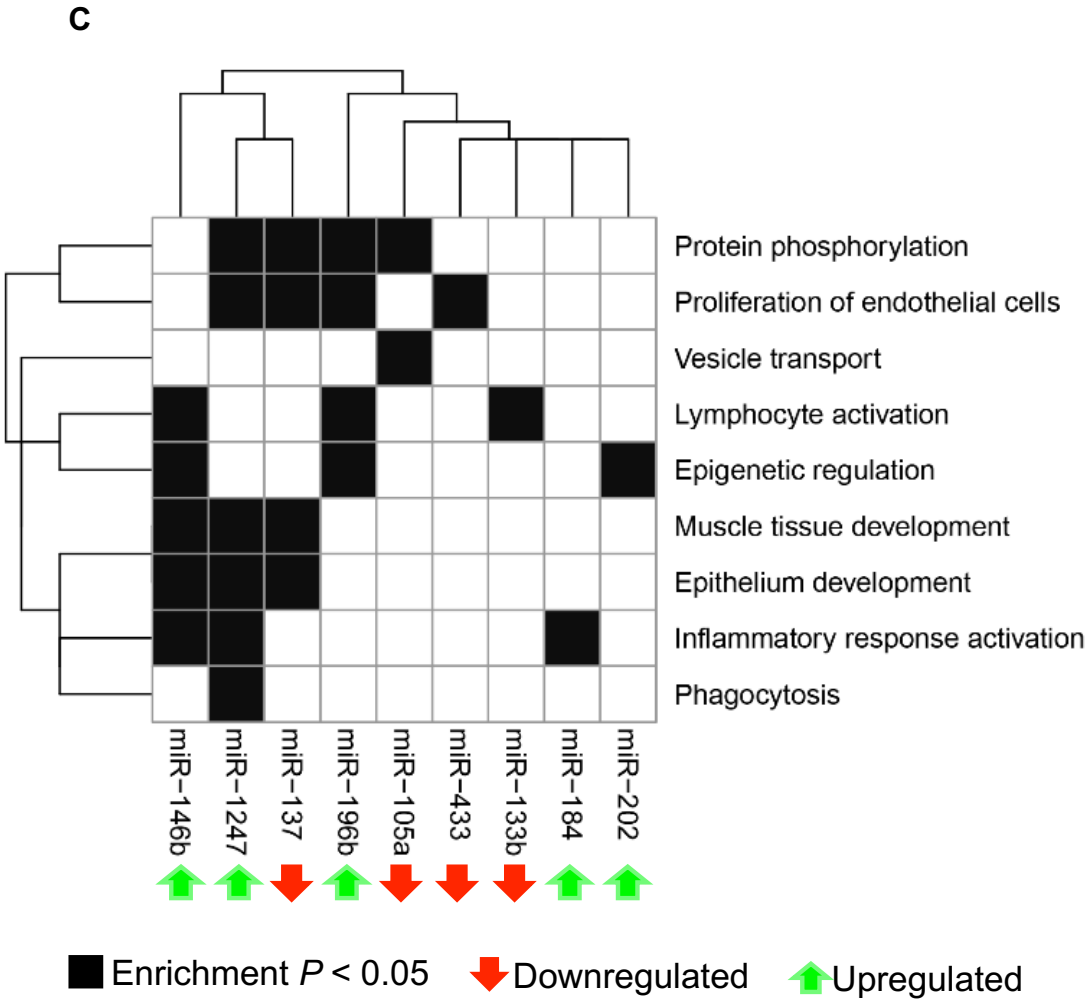

Figure 4.7 (Continued) Analysis of differentially expressed miRNAs. (A) Heatmap of differentially expressed miRNAs based on RNA-Seq expression data. Blue indicates a higher expression level, whereas yellow represents decreased expression levels. **(B)** Validation of miRNA expression using stem-loop RT-qPCR. Y-axis presents fold change (FC) of miRNA when infected tissue was compared to control tissues. *significant difference at $P < 0.05$; NS: no significant differences. Data presented as mean ± standard error of the mean (SEM). **(C)** GO term enrichment of miRNA targeted genes. Targeted genes were identified on the basis of two criteria: computational prediction and negative correlation analysis, $r < -0.5$, $P < 0.05$, Pearson's correlation analysis. Black squares represent functions with significant enrichment ($P < 0.05$) that were identified by PANTHER. Red/green arrows indicate miRNAs were down-/up-regulated, respectively, in infected compartment.

Chapter 5. Altered microRNA expression in allicin-treated ileal tissues revealed their potential roles in regulating host responses to antimicrobials

5.1 Introduction

Antibiotics and antimicrobials have been widely used in livestock production since 1970, due to their involvement in growth promotion and disease prevention (Hao et al., 2014). For example, dairy calves are susceptible to many pathogens during the pre-weaned period, and adding antibiotics to pre-weaned calf diets has been reported to decrease the incidence of enteric infection and calf mortality (Smith, 2015). However, the misuse of antibiotics may increase antibiotic resistance of microorganisms, which is an emerging public health concern (Davies and Davies, 2010). Allicin, a natural antimicrobial phytochemical, has been recently studied for its potential use as a feed additive in the livestock industry and potential alternative to antibiotics (Ghosh et al., 2011). Allicin is naturally present in garlic and can repress the growth of a wide range of bacteria by inhibiting sulfur-containing enzymes in the microorganisms (Rabinkov et al., 1998). For example, allicin has been shown to have bactericidal activity against *Escherichia coli* and *Staphylococcus aureus*, that are common enteric pathogens of pre-weaned calves (Ruddock et al., 2005; Ruiz et al., 2010). Besides the bactericidal effect, supplementation of diets with allicin has been shown to have effects on growth similar to that of antibiotics in swine, poultry, and cattle (Donovan et al., 2002; Huang et al., 2011; Velkers et al., 2011),

suggesting that allicin can be an alternative for antibiotics that promote calf growth while preventing infections.

It has been recently reported that antibiotics may disrupt the existing gut microbiota or have direct/indirect toxic impacts on the host gut tissue (Keeney et al., 2014; Morgun et al., 2015), however, the effects of allicin on the host gut tissue have not been well studied. It has been reported that allicin reduced the secretion of TNF-α, and exerted an inhibitory immunomodulatory effect on intestinal epithelial cells (Lang et al., 2004). However, the majority of studies have been only performed in animal cell lines, and the influence of allicin on the host gut tissue is not known.

The expression of microRNAs (miRNAs) in gut tissues has been suggested to be the modulators of host-microbial interaction (Masotti, 2012). For example, the expression of miR-665 in the gut can be induced by microbial colonization, which inhibits the expression of ATP-binding cassette, sub-family c, member 3, a protein that mediates the metabolism of xenobiotics and endogenous toxins in intestine (Dalmasso et al., 2011). A previous study revealed that the expression of miRNAs showed dynamic changes in the gut of pre-weaned calves, and some miRNAs, such as miR-196b, were significantly correlated with the tissue-associated commensal bacterial density (Liang et al., 2014). These results indicated that miRNA-gut microbiota interactions may regulate genes involved in the mucosal immune system development. Thus, we hypothesized that allicin can affect the expression of miRNA in gut tissue of calves, and the changes in miRNA expression are related to the alterations of bacterial community.

High individual variations in the expression patterns of Toll-like receptors (TLRs) of the gut tissues were observed in the calves with same ages (Malmuthuge et al., 2012). TLRs are pattern recognition receptors, which can sense the microbial colonization or pathogen infection (Hajjar et al., 2001), and this result suggested that the gene expression in the gut showed highly variable responses to microbial colonization among individuals. In this study, in vivo ileal loop models were used to minimize the individual variation in 2-week-old pre-weaned calves, and different doses of allicin were used to treat the ileal loops. The changes in the expression levels of miRNAs of the ileal loops and their linkages with tissue-associated total bacterial density were studied after 1-day, 3-day, and 12-day treatment.

5.2 Materials and methods

5.2.1 Animal study and tissue collection

Animal experiments including animal housing, anesthesia, surgery, and postsurgical care were performed following the guidelines approved by the Canadian Council on Animal Care and all procedures were conducted in accordance with a previously described, approved protocol (Maattanen et al., 2013). Nine male, Holstein calves (10-14 days old) were treated with allicin using surgically isolated intestinal segments. Briefly, a 30- to 35-cm segment of intestine was surgically isolated, proximal to the ileocecal fold, and subdivided into five equal segments using silk ligatures. All the digesta in the ileal segment was removed and the emptied ileal segments were flushed with saline and infused

with a mixture of antibiotics (250 mg metronidazole (Hospira Healthcare Corp., Montreal, QC) and 200 mg enrofloxacin (Baytril, Bayer Inc., Toronto, ON Canada)) for 30 minutes. The five segments of tissues were further treated with allicin 0 mg (C), 0.5 mg (T0.5), 5 mg (T5), 50 mg (T50), and 500 mg (T500). The animals were euthanized at 1 day (D1, n = 3), 3 days (D3, n = 3) and 12 days (D12, n = 3) after allicin treatment. The tissue samples belonged to 15 treatment groups were collected into cryovials, and snap-frozen in liquid nitrogen prior to storage at -80°C.

5.2.2 RNA extraction

The tissue samples were ground to a fine powder while immersed in liquid nitrogen in a frozen mortar. Total RNA was then extracted from 80 mg of tissue powder using mirVana™ miRNA Isolation Kit (Ambion, Carlsbad, CA) following the manufacturer's instructions. The quality and quantity of the RNA were determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA), respectively. RNA samples with good quality (integrity number (RIN) > 7.0) were used for further analysis.

5.2.3 Construction and analysis of small RNA libraries

Total RNA (1.0 μg) from each sample was used to construct a small RNA library using the TruSeq Small RNA Sample Preparation kit (Illumina, San Diego, CA) following the manufacturer's instruction. The libraries were sequenced at Génome Québec (Montréal, Canada) using the Illumina HiSeq 2000 system (Illumina) to generate 50 bp single reads. All reads were demultiplexed

according to their index sequences using CASAVA version 1.8 (Illumina) and reads that did not pass the Illumina quality filter were discarded.

The processing of small RNA sequencing data was conducted according to the method described in a previous study (Liang et al., 2014). Briefly, sequences with acceptable quality were processed to be short tags by removing the 3'adapter using a perl script provide by miRDeep2 (Friedländer et al., 2012). After trimming the 3' adaptor sequence, all identical sequences with sizes ranging from 18 to 25 nt were mapped to the ncRNA sequences (Rfam) to remove non-miRNA small RNA sequences. Then, all the sequences were aligned against the corresponding known miRNA precursor sequences (miRBase release version 21) by using the module of quantifier.pl in miRDeep2 with the default parameters to identify known miRNAs. The expression of miRNAs in each library was normalized to reads per million total mapped reads (RPM) by the following method: RPM = (miRNA reads number/total mapped reads per library) × 1,000,000. RPM data matrix was used for further expression analysis.

5.2.4 Construction and analysis of RNA-Seq library

Total RNA (1.0 μg) from each sample was used to construct RNA-Seq libraries using the TruSeq mRNA Sample Preparation kit (Illumina, San Diego, CA) following the manufacturer's instruction. Individual libraries were then pooled for sequencing according to Illumina's instruction and sequenced at Génome Québec (Montréal, Canada) using the Illumina HiSeq 2000 system (Illumina). Sequencing was performed as 100 bp paired-end reads. All reads were

demultiplexed according to their index sequences with CASAVA version 1.8 (Illumina) and reads that did not pass the Illumina chastity filter were discarded. RNA-Seq reads were aligned to the bovine genome (UMD 3.1) using Tophat 2.0.11 with default parameters (Kim et al., 2013). HTSeq was used (version 0.6.1, http://www-huber.embl.de/users/anders/HTSeq/) to count the number of reads that were mapped to each gene. The expression level of mRNAs in each library was obtained by normalizing reads number to fragments per million reads (FPM) by the following method: FPM = (gene fragments number / total mapped fragments number per library) × 1,000,000.

5.2.5 Effect of allicin on expression of selected miRNAs

The impacts of allicin on the expression of 16 miRNAs were analyzed (Table 5.1). These 16 miRNAs were selected according to the results from Chapter 2, which were differentially expressed in the ileum during pre-weaned period and related to the development of mucosal immune system. The changes of miRNAs expression in allicin treated samples were analyzed within each individual using fold change calculation: fold change = miRNA expression in treated tissues (T0.5, T5, T50, and T500) / miRNA expression in Control tissues (C).

5.2.6 Prediction of miRNAs target genes

The putative target genes for miRNAs were predicted using miRanda (http://www.microrna.org/) and TargetScan (http://www.targetscan.org/) as described previously (Liang et al., 2014). The targets predicted by both algorithms were used for further functional analysis.

5.2.7 Estimation of the total active bacterial population using RT-qPCR

To investigate the effect of allicin on the density of the tissue-associated microbiota, the copy number of 16S rRNA was estimated using RT-qPCR. Total RNA (1 μg) was reverse transcribed to cDNA using SuperScript® II Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Total bacterial population was estimated using U2 primers (U2F, 5'-ACTCCTACGGGAGGCAG-3'; U2R, 5'-GACTACCAGGGTATCTAATCC-3') (Stevenson and Weimer, 2007) and SYBR Green chemistry (Fast SYBR® Green Master Mix; Applied Biosystems) with StepOnePlus™ Real-Time PCR System (Applied Biosystems). The standard curve was constructed using plasmid DNA containing 16S rRNA gene of *Butyrivibrio hungatei* with a serial dilution of initial concentration of 8.5×10^{10} molecules μl^{-1}. The range of the copy numbers in the standard curve was from 8.5×10^{3} to 8.5×10^{8} molecules μl^{-1}. The copy number of 16S rRNA per gram sample was calculated using the method described in a previous study (Malmuthuge et al., 2012): (QM × C × DV)/(S × W), where QM was the quantitative mean of the copy number, C was the RNA concentration of each sample (ng μl^{-1}), DV was the dilution volume of extracted RNA (μl), S was the RNA amount subjected to analysis (ng), and W was the sample weight subjected to RNA extraction (g).

5.2.8 Statistical analysis

Pearson's correlation analysis was performed between miRNA expression values (RPM) and miRNA target gene expression (FPM) (obtained from another study, Liang et al., unpublished data) to identify the relationship between

miRNAs expression and expression of their putative target genes. Similarly, Pearson's correlation analysis was also performed between miRNA expression values (RPM) and total bacteria density to define the association between miRNAs and tissue-associated gut microbiota. The significant correlation was declared at $P < 0.05$, and correlation coefficient (r) < - 0.5 or > 0.5.

5.3 Results

5.3.1 Profiling of miRNAs in isolated ileal loop tissues of pre-weaned calves

In total, 53,759,336 small RNA reads with 1,194,652 ± 411,768 (mean ± SD) reads for each sample was obtained from all 45 libraries. A total of 47,695,994 reads with 18-25 nt lengths were used for miRNA expression analysis. Among these reads, 90.1% (43,380,509 out of 47,695,994) reads were mapped to the miRNA database (miRBase version 21). The remaining 9.9% (4,338,051 out of 47,695,994) reads belonged to novel miRNAs or other small noncoding RNAs (tRNA, snoRNA, snRNA and others). An average of 366 ± 33 (mean ± SD) miRNAs were detected by RNA-Seq, and no statistical significances were observed among different treatments in terms of the number of detected miRNAs.

5.3.2 Effects of allicin on ileal miRNA expression

According to the results from Chapter 2, a total of 16 miRNAs were selected to analyze the impacts of allicin on miRNAs expression (Table 5.1). The analysis of miRNAs expression at D1 showed that the expression level of miR-196b was upregulated ($\log_2$ (fold change) > 2) in A2 and A4 under T0.5, T5 and T50 treatment, while downregulated ($\log_2$ (fold change) < -1.5) in A2 and A7

under T500 treatment (Figure 5.1A). Similar analysis was performed for the samples at D3, and the expression of miR-196b was upregulated ($\log_2$ (fold change) > 2.5) in A6 and A9 under T5 and T50 treatment, while downregulated ($\log_2$ (fold change) < -2.5) in A8 and A9 under T500 treatment (Figure 5.1B). Besides, at D12, miR-196b was increased ($\log_2$ (fold change) > 1) in A3 under T5 and T50 treatment, and increased ($\log_2$ (fold change) > 1) in A5 and A10 under T0.5, T5 and T50 treatment (Figure 5.1C). miR-196b was downregulated ($\log_2$ (fold change) < -5) in A3 and A10 under T500 treatment (Figure 5.1C).

5.3.3 The association among miR-196b, predicted targets of miR-196b and tissue-associated total bacterial density

A total of 223 genes were computationally predicted as the targets of miR-196b in bovine. According to RNA-Seq analysis, 192 out of 223 genes showed normalized expression level larger than 1 (FPM > 1) in at least 50% of samples. Pearson correlation analysis was performed between the expression miR-196b and 192 putative target genes at different post-treatment time. The expression of miR-196b was positively correlated with homeobox A5 (HOXA5) (r = 0.57 at D1, r = 0.76 at D3, and r = 0.76 at D12; $P < 0.05$ at all three time points) and homeobox A9 (HOXA9) (r = 0.72 at D1, r = 0.88 at D3, and r = 0.89 at D12; $P < 0.05$ at all three time points) at all post-treatment time points.

The tissue-associated total bacterial density was estimated by quantifying the 16S rRNA copy numbers. The Pearson's correlation analysis was performed between the expression level of miR-196b and the tissue-associated total bacterial density. The expression of miR-196b was negatively correlated with the total

bacterial density at D1 (r = -0.63, $P < 0.05$) and D12 (r = -0.64, $P < 0.05$). A negative correlation was also detected between miR-196b expression and total bacterial density at D3 (r = -0.33, $P = 0.11$), however; no statistical significance was observed.

The expression of miR-196b, HOXA5, HOXA9 and total bacterial density were profiled individually (Figure 5.2). The co-expression of miR-196b, HOXA5 and HOXA9 was observed in all the animals (Figure 5.2). The increase of total bacterial density and the decreased expression of miR-196b, HOXA5 and HOXA9 were detected in all the animals (Figure 5.2).

5.4 Discussion

This is the first study to analyze the impacts of allicin on miRNA expression in the gut tissues using RNA-Seq. The expression of miR-196b was upregulated under the treatment of low doses allicin (0.5 mg, 5 mg and 50 mg), coinciding with the upregulation of HOXA5 and HOXA9 in the current study. miR-196b is an ileum-specific miRNA, which has been predicted to be involved in the lymphatic endothelial cell development (Liang et al., 2014). The expression level of miR-196b was upregulated in the ileum within one week postpartum in the calves, which may be crucial for the mucosal lymphoid tissue development during the early life of calves (Liang et al., 2014). The upregulation of miR-196b observed in this study suggest that the low dose allicin treatment might have positive effects on the gut mucosal immune system.

HOXA5 and HOXA9 belonged to the Hox A gene family (from HOXA1 to HOXA10), which are clustered together in the bovine genome (Chromosome 4, UCSC Genome Browser), and encode transcription factors that are involved in the establishment of regional identities along the anteroposterior body axis (Krumlauf, 1994). It has been reported that Hox genes affect mesodermal components of the gut during the perinatal period of the mice. For example, over-expression of HOXA4 led to abnormal mesodermal development, resulting in the megacolon in the mice (Wolgemuth et al., 1989). However, the roles of Hox genes in the postnatal development of gut tissues were not well studied. The number of B cells in the lymphoid tissues was significantly reduced in the HOXA9 knockout mice, indicating the roles of HOXA9 in B cell development (Gwin et al., 2010). Ileum has been suggested to be the primary site for the generation of the pre-immune B cells during the early life to ruminants (Mutwiri et al., 1999). The upregulation of HOXA9 under lower dose of allicin treatment suggests that the allicin treatment might promote the gut mucosal adaptive immune immunity by inducing the B cell development.

However, over-expression of HOXA9 is related to the infinite proliferation of B cells, resulting in mixed lineage leukaemia in humans (Li et al., 2012). miR-196b is located in the cluster of HOXA genes on the bovine genome (UCSC Genome Browser), and is predicted to target HOXA5 and HOXA9. In addition, the regulatory roles of miR-196b on HOXA genes have been experimentally validated in human (Popovic et al., 2009). The co-expression of miR-196b and HOXA genes observed in the B cells from the mixed lineage

leukaemia patients has suggested the inhibition role of the over-expression of HOXA9 by miR-196b (Li et al., 2012). Therefore, we speculated that the co-upregulation of miR-196b and HOXA genes under the treatment of low dose allicin is the mechanism to constrain the over-expression of HOXA genes, which may be able to prevent the over-proliferation of B cells. On the other hand, a significant reduction of miR-196b, HOXA5 and HOXA9 expression was observed under the treatment of high dose (500 mg) allicin. As discussed above, lacking of HOXA9 gene may inhibit the B cell development in the lymphoid tissues (Gwin et al., 2010), thus, the reduced expression of miR-196b, HOXA5 and HOXA9 indicat that high dose of allicin may pose negative effects on the mucosal immune system.

There were negative correlations between miR-196b expression and tissue-associated total bacterial density after the allicin treatment. A previous study revealed the positive correlations between miR-196b expression and tissue-associated beneficial bacterial density, such as *Bifidobacterium* and *Lactobacillus*, in the ileum of pre-weaned calves, suggesting that miR-196b might respond to the beneficial bacterial colonization, which may be crucial for the mucosal immune system development (Liang et al., 2014). In the current study, the negative correlations of miR-196b with tissue-associated total bacterial density further support the specualted relationship between miR-196b and tissue-associated bacteria. Although, the casual effect on this relationship is not known, we speculate that the changes in bacterial community may alter the expression of miR-196b. As one of antimicrobial, Allicin may change the bacterial community

that attached to the gut tissues, and subsequently, influence the expression of miR-196b. On the other hand, allicin can be directly uptaken by host cells and alter host gene expression (Borlinghaus et al., 2014). As observed in this study, high dose of allicin treatment reduced the expression of miR-196b and HOXA genes which could inhibit the mucosal immune system that may lead to the increased tissue-associated bacterial density. Future studies, such as analyzing the alterations of tissue-associated bacterial composition in response to allicin treatment or measuring the abundance of B cells in the ileum that is treated with allicin, can further validate these speculations.

5.5 Conclusions

Low dose of allicin treatment increased the expression of miR-196b, and its target genes, HOXA5 and HOXA9, which may promote the development of gut mucosal immune system, while high dose of allicin treatment decreased their expression. A negative correlation between miR-196b and tissue-associated bacterial density further indicate the relationship between miR-196 and bacterial colonization. Further studies on the changes of host transcriptome as well as the composition of tissue-associated bacteria may provide more information on the impacts of allicin on host gene expression and bacterial community as well as their relationship.

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5.7 Figures and tables

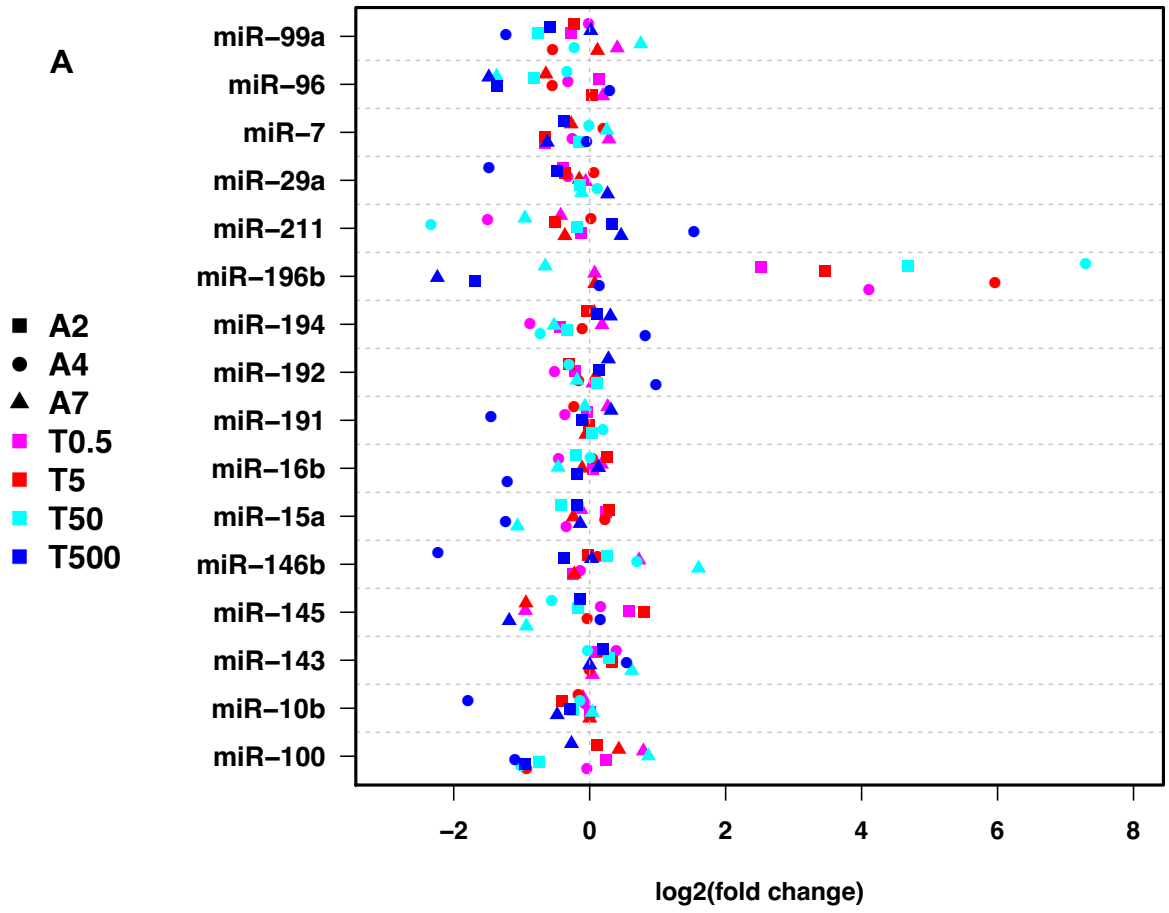

Figure 5.1 The fold change of 16 miRNAs expression under allicin treatment at D1 (A), D3 (B), and D12 (C). The X-axis represents the log2 (fold change) of miRNA expression. The Y-axis represents the selected 16 miRNAs. Different color of spots indicates different dose of allicin treatment (T0.5, T5, T50, and T500). Different shape of spots indicates different individuals. A2, A4 and A7 are animals with 1-day post-treatment of allicin. A6, A8 and A9 are animals with 3-day post-treatment of allicin. A3, A5 and A10 are animals with 12-day post-treatment of allicin.

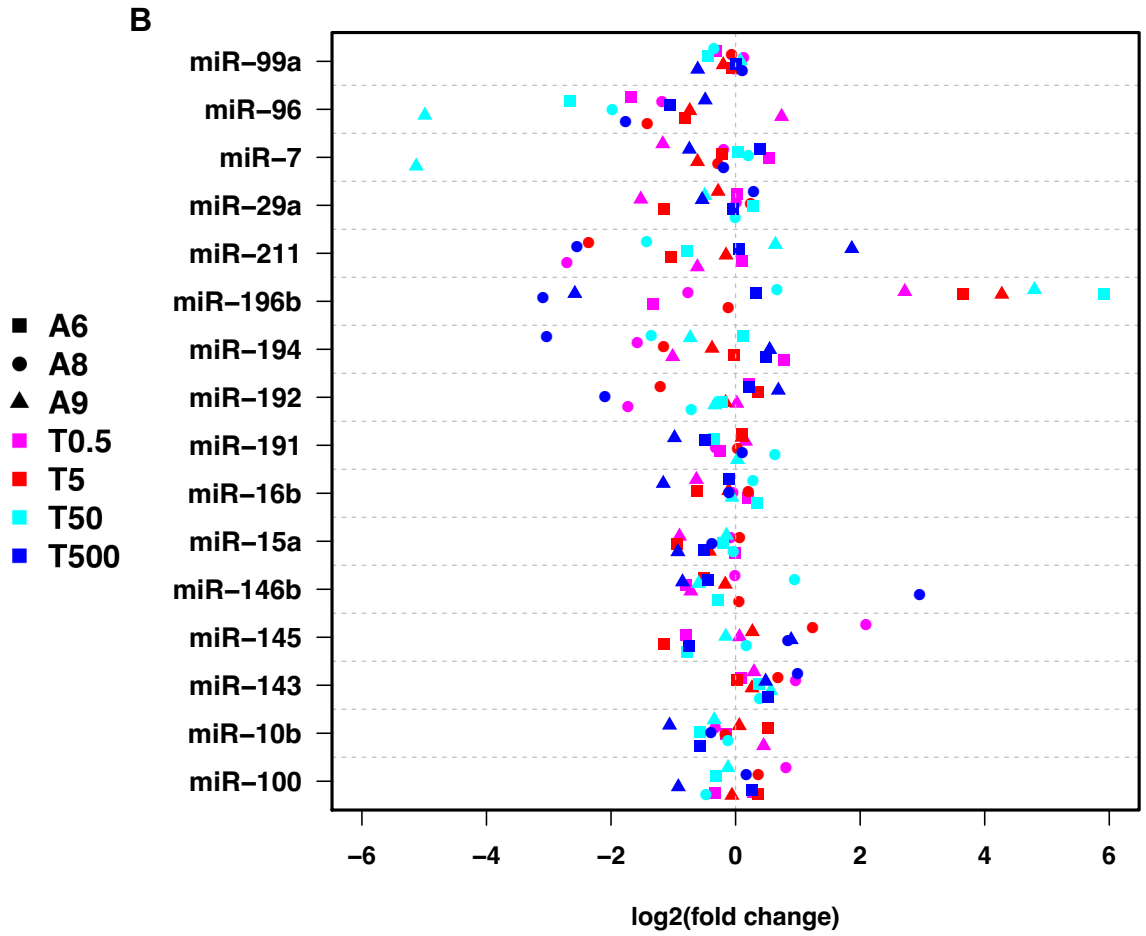

Figure 5.1 (Continued) The fold change of 16 miRNAs expression under allicin treatment at D1 (A), D3 (B), and D12 (C). The X-axis represents the log2 (fold change) of miRNA expression. The Y-axis represents the selected 16 miRNAs. Different color of spots indicates different dose of allicin treatment (T0.5, T5, T50, and T500). Different shape of spots indicates different individuals. A2, A4 and A7 are animals with 1-day post-treatment of allicin. A6, A8 and A9 are animals with 3-day post-treatment of allicin. A3, A5 and A10 are animals with 12-day post-treatment of allicin.

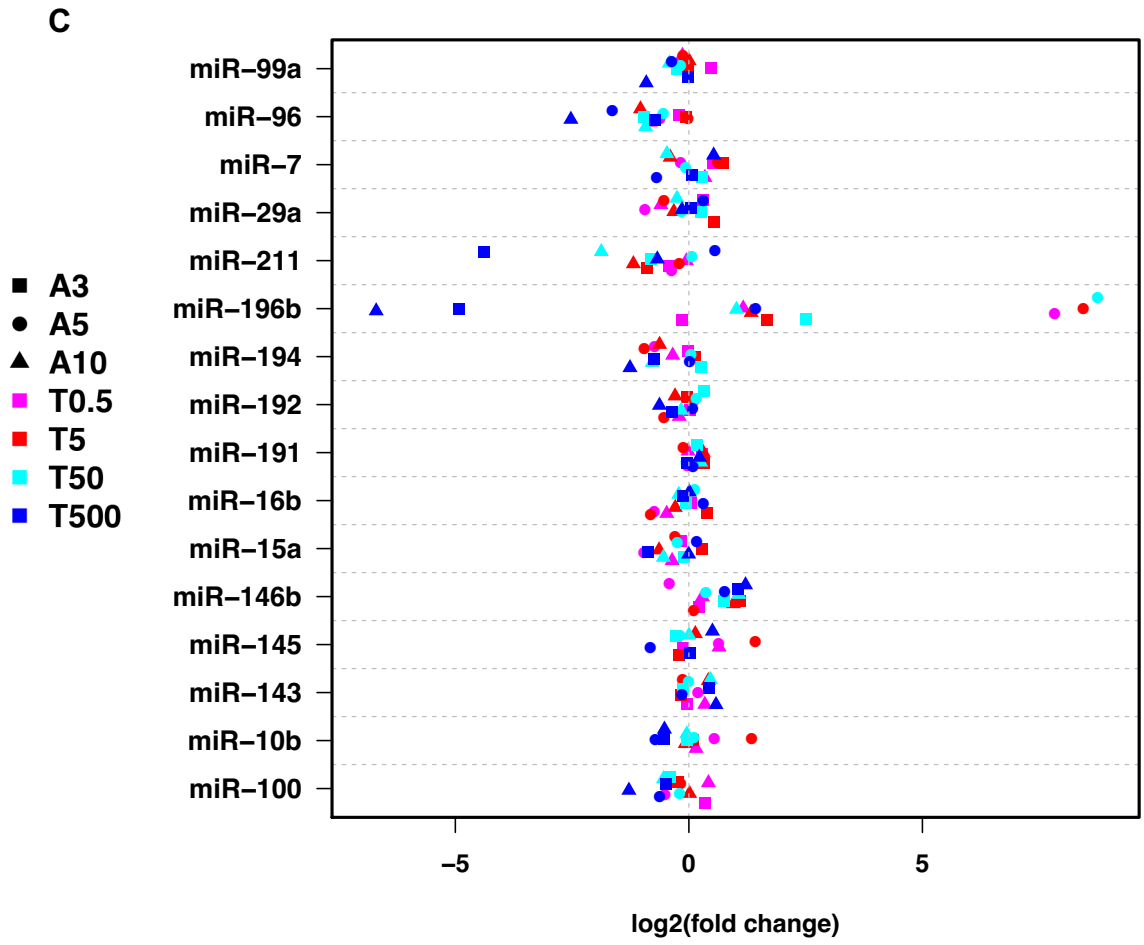

Figure 5.1 (Continued) The fold change of 16 miRNAs expression under allicin treatment at D1 (A), D3 (B), and D12 (C). The X-axis represents the log2 (fold change) of miRNA expression. The Y-axis represents the selected 16 miRNAs. Different color of spots indicates different doses of allicin treatment (T0.5, T5, T50, and T500). Different shape of spots indicates different individuals. A2, A4 and A7 are animals with 1-day post-treatment of allicin. A6, A8 and A9 are animals with 3-day post-treatment of allicin. A3, A5 and A10 are animals with 12-day post-treatment of allicin.

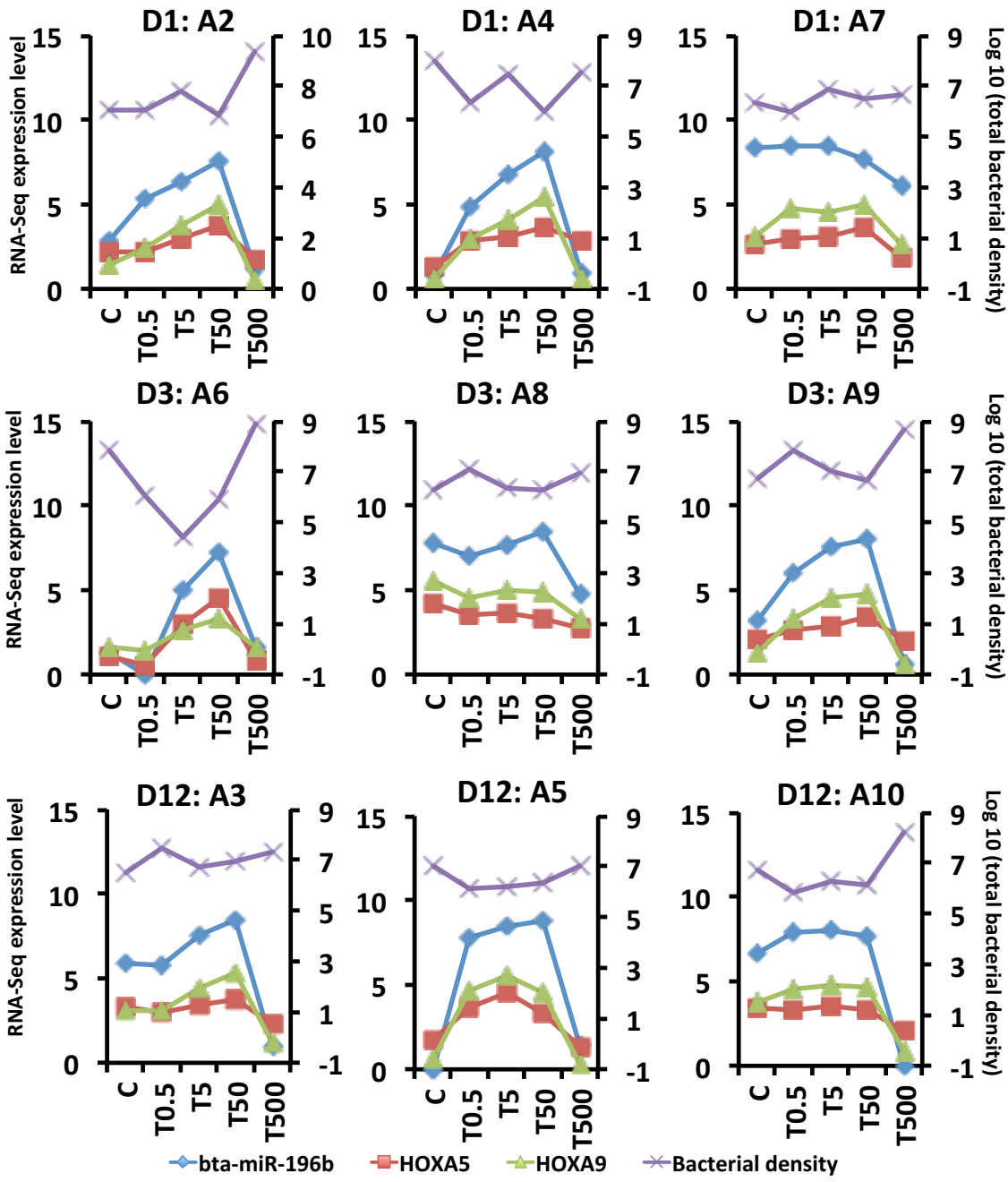

Figure 5.2 Relationship among the expression of miR-196b, HOXA genes and total bacterial density. The left Y-axis represents the normalized expression levels (RNA-Seq) of miR-196b, HOXA5 and HOXA9. The right Y-axis represents the log 10 (total bacterial density). X-axis represents different doses of allicin treatment (T0.5, T5, T50, and T500). A2, A4 and A7 are animals with 1-day (D1) post-treatment of allicin. A6, A8 and A9 are animals with 3-day (D3) post-treatment of allicin. A3, A5 and A10 are animals with 12-day (D12) post-treatment of allicin.

Table 5.1 The selected miRNAs based on their expression changes in the ileum of preweaned calves as reported in Chapter 2.

miRNA	**Annotation**
bta-miR-100	Upregulated in D7 vs D0 and D21 vs D7
bta-miR-10b	Upregulated in D7 vs D0
bta-miR-143	Predominant miRNA
bta-miR-145	Downregulated in D7 vs D0 and D21 vs D7
bta-miR-146b	Upregulated in D7 vs D0 and D21 vs D7
bta-miR-15a	Upregulated in D7 vs D0
bta-miR-16b	Upregulated in D7 vs D0
bta-miR-191	Downregulated in D7 vs D0 and D21 vs D7
bta-miR-192	Upregulated in D7 vs D0
bta-miR-194	Downregulated in D7 vs D0
bta-miR-196b	Upregulated in D7 vs D0
bta-miR-211	Upregulated in D7 vs D0 and, downregulated in D21 vs D7
bta-miR-29a	Upregulated in D7 vs D0
bta-miR-7	Downregulated in D7 vs D0
bta-miR-96	Upregulated in D7 vs D0 and D21 vs D7
bta-miR-99a	Upregulated in D7 vs D0

Table 5.2 The total bacterial density in each sample.

Animal ID	Post-treatment time	Dose	16S Copy number
A2	D1	C	1.14E+07
A4	D1	C	9.99E+07
A7	D1	C	2.39E+06
A2	D1	T0.5	1.20E+07
A4	D1	T0.5	2.32E+06
A7	D1	T0.5	8.96E+05
A2	D1	T5	5.92E+07
A4	D1	T5	3.12E+07
A7	D1	T5	8.21E+06
A2	D1	T50	7.17E+06
A4	D1	T50	9.99E+05
A7	D1	T50	3.02E+06
A2	D1	T500	2.50E+09
A4	D1	T500	3.48E+07
A7	D1	T500	4.46E+06
A3	D12	C	3.07E+06
A5	D12	C	1.00E+07
A10	D12	C	5.09E+06
A3	D12	T0.5	2.77E+07
A5	D12	T0.5	1.30E+06
A10	D12	T0.5	6.29E+05
A3	D12	T5	5.85E+06
A5	D12	T5	1.61E+06
A10	D12	T5	1.96E+06
A3	D12	T50	8.53E+06
A5	D12	T50	2.27E+06
A10	D12	T50	1.33E+06
A3	D12	T500	2.04E+07
A5	D12	T500	1.04E+07
A10	D12	T500	1.70E+08
A6	D3	C	7.70E+07
A8	D3	C	1.90E+06
A9	D3	C	5.75E+06
A6	D3	T0.5	1.21E+06
A8	D3	T0.5	1.38E+07
A9	D3	T0.5	6.89E+07
A6	D3	T5	2.63E+04
A8	D3	T5	2.43E+06
A9	D3	T5	1.14E+07
A6	D3	T50	8.07E+05
A8	D3	T50	1.77E+06
A9	D3	T50	4.53E+06
A6	D3	T500	7.98E+08
A8	D3	T500	9.81E+06
A9	D3	T500	4.62E+08

Chapter 6. General discussion

Pre-weaned calves are more susceptible to a variety of bacterial and viral enteric infections compared to adult cattle, and the development of the small intestine is critical for the health of animals during this period, as well as in later life (Cho and Yoon, 2014). Many factors could impact on gut health such as diet, microbial colonization, host genetics, management and so on (Cho and Yoon, 2014). To date, the molecular mechanism at the transcriptome level of gut development in calves during pre-weaned period has not been well studied. I hypothesized that the gut developmental process and enteric infection are associated with the changes of protein-coding gene expression in the gut of pre-weaned calves, which could be regulated by microRNAs (miRNAs). Furthermore, the altered expression of protein-coding genes and miRNAs are related to microbial colonization. In order to test the hypotheses, four studies were performed in this project. The following sections will discuss the main findings resulting from four studies as described in Chapters 2 to 5.

6.1 Gene expression alterations in healthy and pathogen-infected gut of pre-weaned calves

The gene expression process encodes proteins that dictate the cell functions (Maston et al., 2006). According to the abundance of RNA transcripts in cells or tissues, we are able to determine whether the expression of a gene is promoted or repressed at the transcription level (Maston et al., 2006). Based on

the transcriptomic analysis of the gut tissues collected from pre-weaned calved, the altered expression of protein-coding genes (mRNAs expression) in the small intestine of healthy as well as *Mycobacterium avium* subspecies *Paratuberculosis* (MAP)-challenged pre-weaned calves were detected in Chapter 3 and Chapter 4, respectively. These two chapters provided fundamental understanding on the developmental process of small intestine and host responses to enteric pathogenic infection at the molecular level.

The terminal ileum has been considered as the primary site of numerous enteric infections in calves, such as *Salmonella enterica serovar typhimurium* infection (Tsolis et al., 1999), MAP infection (Arsenault et al., 2014), and Rotavirus infection (Pospischil et al., 1986). It has been shown that M cells are the antigen-sampling cells, which cover the domed villi of the Peyer's patch (PPs) (Frost et al., 1997). More PPs can be found in the ileum than that in the jejunum (10 fold) (Griebel and Hein, 1996), indicating that the ileum has more capacity to uptake pathogens than the jejunum. Besides, in Chapter 3, comparative analysis of the transcriptome between the jejunum and ileum revealed more regional differences in the small intestine of pre-weaned calves at the transcriptional level. The expression levels of complement function pathway related genes were higher in the jejunum than in the ileum. The complement system plays a role in preventing the immune system from being infected by microorganisms through cooperation with phagocytic cells (Tomlinson, 1993). The higher expression of complement function-related genes suggests the roles of the jejunum in innate immune responses during pre-weaning period. Moreover, the expression levels of

IgA complex genes were higher in the jejunum than that in the ileum. The humoral immune defense at mucosal surface is mainly mediated by IgA antibody (Holmgren and Czerkinsky, 2005), which is secreted by IgA-producing plasma cells. The results indicate the roles of the jejunum in adaptive immune responses in young calves. The expressions of Toll-like receptor (TLR) genes were lower in the jejunum when compared to the ileum, and the TLRs play roles in recognizing pathogen-associated molecular patterns (Lavelle et al., 2010). These results suggest that the jejunum may have less pathogen sensing receptors. Higher expression of tight junction (TJ) protein genes in the jejunum indicated higher gut permeability in the jejunum than the ileum (Liu et al., 2005). Based on my limited knowledge, this is one of the few studies to define the function of jejunum at the molecular level. Previous studies mainly focused on the different types of PPs in the ileum and jejunum to compare their different functions in the immune system (Griebel and Hein, 1996). We found that the gene expression levels that are related to complement function, IgA complex and gut permeability are greater, and genes that are associated with pathogen recognizing are lower in the jejunum than in the ileum. Future studies are necessary to understand the expression at the protein level, to further validate these transcripts differences between the jejunum and ileum.

In addition, the temporally differentially expressed genes in the small intestine of the pre-weaned calves (Chapter 3) revealed the molecular mechanism on mucosal immune system development and suggested such development is more critical during the first week of life. Based on the results, the expression of

tight junction genes (such as claudin 1 and claudin 4), an antimicrobial peptide gene (regenerating islet-derived 3 γ) and a cytokine gene (interleukin 8) increased in the small intestine within the first week postpartum. Upregulation of claudin 1 and claudin 4 can decrease gut permeability (Liu et al., 2005). The regenerating islet-derived 3 γ (REG3γ) protein is able to bind peptidoglycan and is bactericidal against gram positive bacteria (Bevins and Salzman, 2011), while interleukin 8 (IL8) is a pro-inflammatory cytokine, which can induce host inflammatory response to pathogens (Joshi-Barve et al., 2007). All of these findings indicate that the intestinal mucosal immune system developed rapidly to protect the host from infection during the first week of life. This is the first study to report the temporal changes of gene expression in the gut during early life, which provides potential molecular mechanism on mucosal immune system develops in the ruminants. Understanding of the mucosal immune system develops in the ruminants will help to generate more effective strategies to protect the calves from enteric infections.

In addition to the observations on altered gene expression in the small intestine of healthy calves, our study also reported that the expression changes of the protein-coding genes after enteric infection. As reported in Chapter 4, the most relevant function of differentially expressed genes in the MAP-infected ileum was related to the promotion of endothelial cell proliferation. The increased proliferation of endothelial cells after MAP infection was speculated as one of the causes for granuloma formation (Pierce, 2009) and the granuloma can provide an organized and protected microenvironment for MAP to establish a persistent

infection in the host (Pierce, 2009). Therefore, the results provide the evidences at molecular level for the theory that increased endothelial cells proliferation is associated with MAP survival during early infection stage. In addition, the transcriptomic analysis identified differential alternative splicing events after MAP infection, which is a novel finding for the host responses to MAP infection. The alternative splicing forms of their pre-mRNAs for two genes (Monocyte to macrophage differentiation-associated (MMD) and adenosine deaminase (ADA)) were identified and further verified using RT-qPCR. MMD protein is a marker gene of macrophages maturation (Liu et al., 2012), and ADA is an enzyme functions in lysosome (Lindley and Pisoni, 1993). The isoform mRNAs of these two genes were highly expressed in the infected tissues, which can result in a failure of macrophage maturation and lysosome function. To my knowledge, the changes of alternative splicing events in the MAP-infected tissue are novel findings in this area, and all the alterations of the transcriptome reported in Chapter 4 displayed potential new mechanisms that MAP evade host immune response and establish persistent infection in the small intestine of calves. Future studies analyzing the expression levels of proteins that are related to the changes at transcriptome level after MAP infection will be needed.

6.2 The relationship between host protein-coding gene expression and commensal bacterial colonization

The host-microbial interaction is important for maintaining gut health in mammals (Sun and Chang, 2014). However, the studies on such aspects in

ruminants are scarce. This thesis is one of the first efforts to link the host gene expression to microbial colonization in the gut of pre-weaned calves. Without the availability of germ-free calves, correlation analysis can be a way to analyze the relationship between host gene expression and microbial colonization in the gut of pre-weaned calves. In Chapter 3, we reported that the mRNA expression of occludin were significantly correlated with the total number of either content- or tissue-associated bacteria at different ages of pre-weaned calves, suggesting that the alterations of this TJ protein gene may be related to bacterial colonization. It has been reported that the introduction of *Lactobacillus* can stimulate the TJ formation in newborn piglets (Yang et al., 2015). Our data on the association between TJ gene expression and Lactobacillus copy numbers suggest that this could also be the case in the gut of ruminants. Future studies studies on identifying whether beneficial bacteria such as *Lactobacillus* or *Bifidobacteria* can modulate the TJ proteins expression could be valuable since these are the probiotics, which can be applied to improve, calve gut health. Similarly, the increased expression of REG3γ, which is an antimicrobial peptide, was identified within the first week in both jejunum and ileum (van Ampting et al., 2012). In the meantime, the total bacterial density also increased within the first week, which was in line with the expression of REG3γ. It has been reported that the production of REG3γ is dependent on the microbial colonization in the small intestine of mice (Hooper et al., 2012). The findings in this thesis are consistent with the previous findings in mice, which provide new insights in the relationship between REG3γ and microbial colonization in the small intestine of newborn calves.

Chapter 3 reports the connection between microbial colonization and host gene expression that related to gut permeability and antimicrobial activity in the pre-weaned calves. Further studies exploring whether beneficial bacteria exert these effects on host genes can offer more evidences to apply probiotics in cattle industry.

6.3 The potential regulatory roles of miRNAs in the healthy and pathogen-infected gut of pre-weaned calves

This research is the first to demonstrate that as important post-transcriptional regulators, microRNAs (miRNAs) manipulate gene expression in the gut tissues of pre-weaned calves. As described in Chapter 2, the expression of miR-146 family, miR-191, miR-33, miR-7, miR-99/100 family, miR-145 and miR-211 were temporally differentially expressed during the pre-weaned period and their predicted function is mainly related to gut tissue and mucosal immune system development. For example, the target genes of miR-486 mainly regulate development of fibroblast cells, which are the most common cells in the connective tissue in the gut (Göke et al., 1998). Functional analysis illustrated that miR-191, miR-33, miR-99/100, and miR-145 were mainly related to differentiation of lymphocytes. Another temporally differentially expressed miRNA, miR-146, has multiple functions that are implicated in the development of gut epithelial cells development, T cell development and TLR signaling pathway (Schulte et al., 2012). Based on the integrated analysis (miRNA-mRNA regulatory pairs identification) (Chapter 3), the expression of miR-335 was

downregulated and its experimentally validated targets (Tavazoie et al., 2008), the mRNA expression levels of claudin 1, claudin 4, occludin and IL8 were upregulated in the ileum within the first week after birth. Similarly, expression of miR-100 showed downregulated pattern in the jejunum, while NOD-like receptor P3 (NLRP3), the target of miR-100 (Helwak et al., 2013), was upregulated during the first week.

In addition, we reported that miRNAs can also respond to enteric infection in the small intestine (Chapter 4). A total of 9 miRNAs were differentially expressed after MAP infection and among them miR-1247, miR-137, miR-196b, and miR-433 were also related to "endothelial cell proliferation" which was the most relevant function of the differentially expressed mRNAs. For example, our results suggested that upregulation of miR-196b in MAP-infected tissues may promote the proliferation of endothelial cells. Moreover, miR-146b, which has been proven to inhibit the TLR signaling pathway (Schulte et al., 2012), was upregulated in infected tissues.

All the results suggest that together with mRNA, miRNA play important roles in regulating the functions in gut permeability, cytokine expression, and pathogen recognition. This is the first study to reveal the expression alterations of miRNAs in the healthy and bacterial infected gut and elucidate their potential functions in regulating gut tissue development as well as immune system in pre-weaned calves. The miRNAs that identified in this study can be used as markers to indicate the gut health status or diagnostic markers for MAP pathogenesis in the calves.

6.4 The relationship between miRNAs and commensal bacteria in the gut of pre-weaned calves

Interplay between miRNA expression in gut tissues and the microbiota has been proposed as one possible mechanism to regulate and integrate normal development and function of the mucosal immune system in mice (Masotti, 2012). We also reported potential interplay between miRNA expression and commensal bacteria in the gut of pre-weaned calves. We found that the expression of miRNAs was correlated with the copy numbers of total bacteria as well as two beneficial bacteria *Bifidobacterium* and *Lactobacillus* spp. and such relationship could be impacted by the diet. As reported in Chapter 2, higher numbers of miRNAs correlated with bacterial density at day 21 after birth (changing the diet to starter) indicated that microbial population changes in response to the diet might influence the expression of miRNAs. It is known that diet can alter the composition of gut microbiota significantly (Scott et al., 2013). Specifically, the expression levels of miR-196 and miR-15/16 families were significantly correlated with the density of *Bifidobacterium* and *Lactobacillus* spp. in small intestine during pre-weaned period. The functions of miR-196 family were mainly related to endothelial cell proliferation and B cell development (Popovic et al., 2009). The predicted functions of miR-15/16 family were involved in T cells and dendritic cells differentiation. These results showed that miRNAs may regulate host immune functions by responding to changes (density, composition or the presence) in microbiota, including *Bifidobacterium* or *Lactobacillus* spp. This is the first study to pinpoint the potential crosstalk between miRNAs and beneficial

commensal bacteria, which supply the fundamental understanding on the host-microbial interactions in ruminants. Such knowledge could be valuable to other mammalian species.

This relationship was verified in the gut treated with allicin (one of antimicrobials) using an ileal loop as a study model (Chapter 5). The expression miR-196b was negatively correlated with the total bacterial density in allicin treated ileum tissue (Chapter 5). It was also positively correlated with density of *Bifidobacterium* and *Lactobacillus* spp. in small intestine of pre-weaned calves at different ages (Chapter 2) and upregulated after MAP infection (Chapter 4). Studies in mice showed that the existence of miR-196b is necessary for B cell development (Popovic et al., 2009), and the upregulation of miR-196b can induce endothelial cell proliferation (How et al., 2013). Our results suggest the potential crucial roles of miR-196b in responding to the gut microbiota of pre-weaned calves; however, the specific functions of miR-196b in ruminants need further studies.

In summary, this thesis provided evidence on the interaction between miRNAs and gut microbiota of pre-weaned calves. This will contribute to the researches on molecular regulatory mechanisms of host responses to microbial colonization in the gut of calves.

6.5 Future directions

To my knowledge, this is the first study to perform transcriptomic analysis (including both protein-coding genes and miRNAs analysis) using RNA-Seq in

the gut of pre-weaned calves. Although it has provided expression profiles of genome wide protein-coding genes and miRNAs, and predicted the potential functions of their differential expression in the gut during the pre-weaned development and enteric infection, there are limitations of the present study.

The functional analysis of the alterations of gene expression was mainly based on bioinformatics analysis. The changes in mRNAs expression can only represent ~40% of the changes in protein expression (Schwanhausser et al., 2011). Therefore, the transcriptomic changes may not fully explain the biological processes. The functions of selected miRNAs are also mainly based on bioinformatics prediction. More studies *in vitro* or *in vivo* are necessary to identify the specific biological functions of these miRNAs. When analyzing the relationship between transcriptome and microbiome, the total bacterial or selected beneficial bacterial density was used to correlate with gene or miRNA expression levels. In addition to the density, the composition of the bacteria is also important for the host function (Rodriguez et al., 2015). However, the changes in bacterial composition were not tested in this study. In addition, the ileal loop models have been used to analyze the host responses to MAP infection and allicin treatment. The intestinal digesta cannot pass through the isolated ileal loop tissues; therefore, this model cannot reflect the host responses to the microbial colonization or infection under the normal physiological conditions.

As such, the future directions are listed below:

System biology based technology can be used to profile the proteome or metabolites in the gut of the pre-weaned calves. Profiling both proteome and

metabolites will provide better understanding on the biological processes in the gut of the pre-weaned calves.

To understand the specific functions of miRNAs, “loss of function” or “gain of function” in the cell lines or model animals can be used. The over-expression of miRNAs can be performed using miRNA mimics or using miRNA gene-containing vector *in vitro*. The inhibition of miRNAs can be performed by using anti-miRNA oligo or knocking out miRNA genes *in vitro*. All these studies can provide direct evidences on miRNA functions.

To apply miRNAs as disease biomarkers or therapy methods, animal studies will be needed. For example, we identified that miR-196b was upregulated in the MAP-infected tissues. The blood samples from cattle at subclinical period or clinical period of Johne's disease can be collected. Then the expression of miR-196b can be evaluated to verify and/or validate whether miR-196b can be a diagnostic marker for Johne's disease.

Metagenomic tools can be used to analyze the gut microbiota in the pre-weaned calves, which will provide microbial taxonomy and genes functional analysis. The integration between metagenome and host transcriptome will lead to a clearer relationship between host and gut microbiota.

6.6 Applications for the dairy industry

This study will help the dairy industry in different ways. First, modifying the diet of the pre-weaned dairy calves can be a potential way to alter the expression levels of protein-coding genes and miRNAs to benefit the gut health.

In addition, the genomic loci of the genes or miRNAs that are important for the gut health can be used for the genetic selection markers. Moreover, miRNAs have been suggested as potential markers, therapeutic targets or even medicine for different diseases (Etheridge et al., 2011). For example, miR-196b can be a potential marker for MAP infection, and it can also be served as a therapeutic target of the medication to treat cattle infected by MAP.

6.7 References

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Appendix

The list of seven research articles that I contributed as a co-author:

1. Guan Y[§], **Liang G**[§] (**Co-first Author**), Hawken PAR, Malecki IA, Cozens G, Vercoe PE, Martin GB, Guan LL (2015) Roles of small RNAs in the effects of nutrition on apoptosis and spermatogenesis in the adult testis. *Nature Scientific report* 5: 10372-10384.
2. Majumder K, **Liang G**, Chen Y, Guan LL, Wu J (2015) Oral Administration of Egg-derived Tri-peptide IRW Affects the Gene Expression on Vascular Tissues in Spontaneously Hypertensive Rats. *Molecular Nutrition and Food Research* doi: 10.1002/mnfr.201500050.
3. Malmuthuge N, Chen Y, **Liang G**, Goonewardene LA, and Guan LL (2015) Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves. *Journal of Dairy Science* doi: 10.3168/jds.2015-9607.
4. Guan Y, **Liang G**, Hawken PAR, Meachem S, Malecki IA, Ham S, Stewart T, Guan LL, Martin, G.B. (2015). Sertoli cell function and proliferation in sexually mature male sheep – responses to nutrition. *Reproduction, Fertility and Development* doi: 10.1071/RD14368.
5. Jin W, Ibeagha-Awemu EM, **Liang G**, Beaudoin F, Zhao X, Guan LL. (2015) Transcriptome microRNA profiling of bovine mammary epithelial cells challenged with Escherichia coli or Staphylococcus aureus bacteria

reveals pathogen directed microRNA expression profiles. *BMC Genomics* 15: 181-192.

6. Bao H[§], Kommadath A[§] (Co-first Author), **Liang G**, Sun X, Arantes AS, Tuggle CK, Bearson S, Plastow GS, Stothard P, Guan LL (2015) miR-214 and miR-331 regulate critical genes associated with Salmonella pathogenesis. *Nature-Scientific report* 5:12620.
7. Liang GY, Qin R, Li J, **Liang G**, Guan Y, Gao ZH**.** (2011) Optimal level of 25-(OH)D in children in Nanjing (32°N Lat) during winter. *Pediatrics International* 53: 541-545.