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Association between commensal bacterial establishment and mucosal innate immune genes expression throughout the gastro-intestinal tract of dairy calves

by

Nilusha Malmuthuge

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Dedication

I dedicate this thesis to my loving parents and husband.

Examination committee

Supervisor	Dr. Leluo Guan
Committee member	Dr. Tom McFadden
External examiner	Dr. Monika Keelan

Abstract

Communication between commensal microbes and host in gastro-intestinal tract (GIT) has been proven to be vital for host immune responses and health maintenance. This study investigated the relationship between bacterial community and mucosal innate immune gene expression throughout the GIT of dairy calves, as well as their potential age related changes. Mucosal tissue and ingesta samples were collected from rumen, jejunum, ileum, cecum and colon of 3 week old (newborn, n = 8) and 6 month old (weaned, n = 8) dairy calves. Analysis of bacterial profiles revealed that predominant mucosa-associated bacteria in calves were distinct from those inhabiting the ingesta, and that bacterial diversity varied significantly among GIT regions. The estimated bacterial populations displayed significant regional differences for bovine mucosa and for ingesta only at 6 months of age, signifying an established segregation of enteric bacterial population through the GIT in weaned calves. Analysis of expression of bovine toll-like receptors (TLR1-10), β-defensin, and peptidoglycan recognition protein1 revealed significant age dependent changes in mucosal innate immune system of dairy calves. TLR10 expression in ileum was significantly higher than that in other gut regions irrespective of calf age; suggesting TLR10 plays a vital role in the recognition of commensal microbes in bovine ileum. Our study provides a fundamental understanding with respect to commensal bacteria establishment and mucosal innate immune gene expression along with their association, in dairy calves. This understanding would be a cutting edge in developing multidisciplinary techniques to improve cattle health.

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Table of Contents

List of tables

List of Figures

List of Abbreviations

AMP	Antimicrobial peptides
APC	Antigen presenting cells
DC	Dendritic cells
DSS	Dextran sulphate sodium
GALT	Gut associated lymphoid tissue
GIT	Gastro-intestinal tract
IRAK-M	IL-1R associated kinase M
LPS	Lipopolysaccharide
LTA	Lipoteichoicacid
MAMPs	Microbes associated molecular patterns
MAP	*Mycobacterium avium paratuberculosis*
NF-κB	Nuclear factor kappa B
NOD	Nucleotide oligomerization domain
PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
PGLYRP	Peptidoglycan recognition protein
PP	Peyer's patches
PRRs	Pattern recognition receptors
qRT-PCR	Quantitative real time- polymerase chain reaction
SCFA	Short chain fatty acids
TLRs	Toll-like receptors

Chapter 1. Literature review

1.0 Introduction

Animal health is one of the key research priorities in the Alberta dairy industry. Health related problems have a major impact on economic losses due to medicinal costs, additional culling in the herd, mortality, low milk quality and production, and reproductive losses. For example, Johne's disease is a prevailing infectious enteric disease in most of the North American dairy farms. This disease is caused by *Mycobacterium avium paratuberculosis* (MAP) and it is reported to cause an annual loss of $49 per cow, in Canada (McKenna et al., 2006; Tiwari et al., 2008). Infectious diseases not only have an impact on animal production but can also be a threat to human health. Therefore, it is important to minimize livestock susceptibility to emerging pathogens by improving natural resistance to pathogenic infections. Host immune systems play a crucial role in preventing infectious diseases; especially the mucosal immune system which is exposed to large numbers of dietary pathogens and commensal microbes. However, our understanding of the development of immune system in cattle is very limited. Commensal microbes inhabiting the gastrointestinal tract of mammalian species are known to play very important roles in host immunity development and functions (Hooper, 2004; Guarner, 2006). Studies conducted using germ-free mice models have supported that development of humoral and cellular immune mechanisms can be influenced by microbial colonization of the gastro-intestinal tract (GIT) (Cebra, 1999) and that perturbations in the commensal consortium can disrupt normal physiological conditions and cause disease (Turnbaugh et al., 2009; Sanz et al., 2010; Sekirov et al., 2010). These findings confirm the

importance of the gut microbiome (community of microbes that reside in the gut) for the development of the immune system and maintenance of host health. However, there have been very few attempts to investigate microbial colonization and its impact on the development of immune mechanisms in dairy cattle.

1.1 Mammalian enteric microflora

The microbial community residing in mammalian GIT consists mainly of bacteria, protozoa and fungi. The enteric microbial community consists of autochthonous microbes that maintain lifelong interactions with host and allocthonous/transient populations which play important roles throughout the host's life (Falk et al., 1998). Many of the normal enteric microbes are commensals (i.e. they benefit from the host while host is unaffected), other groups of enteric flora maintain a mutualism, where both organisms are of benefit to each other (Tortora et al., 1997). Bacteria account for the largest group of enteric microflora. However, most of the bacterial species found in the GIT are obligate anaerobes which cannot be cultured. With the recent developments in culture-independent molecular techniques (Gill et al., 2006), there have been substantial increase in the number of gut microbiome studies in the last decade. To date, around 400 bacterial species have been identified from human gut (Eckburg et al., 2005; Muller et al., 2005). A recent study on feces of 60 mammal species has revealed a total of 17 bacterial phyla, with the majority belonging to *Firmicutes* (65.7%) and *Bacteroidetes* (16.3%) (Ley et al., 2008). Moreover, these studies revealed that an imbalance in the gut microbiome results in various diseases such

as obesity (Ley et al., 2005), inflammatory bowel disease (IBD), and allergies (Sekirov et al., 2010). Prolonged antibiotic treatments in humans have been reported to disrupt the equilibrium of commensal consortiums by allowing overgrowth of pathogens (*Clostridium difficile*) and the development of antibiotic resistant bacteria (Sekirov et al., 2010). It is important to understand bacterial establishment along the GIT in early life because host disease resistance may be improved by manipulating their gut microbiome.

1.1.1 Establishment of commensal microbiota in the GIT

Foetuses are believed to be sterile while in utero. Newborn animals acquire their gut microbes during and after the birth process, first from the mother's vaginal tract and surrounding environment, and later from the diet (Mackie et al., 1999). Furthermore, it has been reported that delivery method, as well as type of feeding, have an important impact on the development of enteric microflora (Mackie et al., 1999). Infants born naturally are reported to have the same bacterial species present in the mother's cervical canal and feces, whereas infants born by caesarean section delivery have mostly microbes from the surrounding environment (Mackie et al., 1999). *Bifidobacterium* spp. were found to be predominant in the feces of 1 week old breast-fed infants, while higher *Clostridia* spp. counts were observed in formula milk-fed infants, suggesting that diet impacts the components of gut microbiota that colonized GIT (Mackie et al., 1999). However, with the increased intake of a solid diet, both breast-fed and

formula milk-fed children gradually obtained adult bacterial profiles (Mackie et al., 1999).

Other than the above external factors, microbial succession in the GIT of newborn animals is also influenced by several internal factors, such as intestinal pH and receptors present for microbial adhesion on the surface of the intestinal epithelium (Mackie and White, 1997; Bourlioux et al., 2003). Intestinal pH varies throughout the GIT depending on the secretions of different regions, which leads to colonization by species that can only survive under those conditions. Gastric secretions drop pH in stomach therefore, it is known that bacterial colonization in stomach is very limited; however, it has been reported that *Helicobacteria pylori* is capable of colonizing on mucosal epithelium of human stomach (Mestecky et al., 2004). Moreover, it has been shown that adhesion receptors present on the surface of the intestinal epithelium of pigs differ with age, which causes a difference in adhesion of *Lactobacillus* spp. among different age groups. Therefore, age is also considered as a factor that has an impact on the colonization of the intestinal epithelium (Blomberg and Conway, 1989). In addition, microbial colonization of the GIT can also be influenced by food retention time, which causes colonization differences between proximal and distal GIT segments (Van et al., 2011). Therefore, the distal GIT, with a longer retention time, tends to colonize heavily compared to the proximal GIT with a shorter retention time. Culture-based studies on bacterial density along the human GIT have reported lower bacterial densities in the small intestine (10^4-10^7 CFU/ml) and higher bacterial densities in the colon (10^{11}-10^{12} CFU/ml), also indicating colonization

differences between proximal and distal GIT, and such differences may be due to luminal pH and retention time variations (O'Hara and Shanahan, 2006). Hence, these potential colonization differences may lead to the development of unique commensal consortiums in different individuals, which would be the result of differences in host-microbe interactions that may be associated with different phenotypes, such as differences in feed efficiency or in disease tolerance.

Colonization of microbes in the mammalian GIT also depends on several microbial factors, such as ability to derive nutrients and survive with the oxygen gradient along the mucus layer, and adhesion capacity to the mucus (van Winsen et al., 2001). Microbial-microbial interactions and communications may also play a role in the colonization of the GIT. Aerobes and facultative anaerobes are the first to colonize new born animals. With the increase in bacterial number they create the anaerobic condition in the gut which is more suitable for obligate anaerobes that colonize later (Falk et al., 1998). It has been shown that neonates are first colonized by *Escherichia coli*, *Staphylococcus* spp., *Streptococcus* spp. and later with *Clostridium* spp., *Lactobacillus* spp., *Bifidobacterium* spp. and *Bacteroides* spp. (Falk et al., 1998; Bourlioux et al., 2003; Round et al., 2010).

Furthermore, the composition of the fecal bacteria community was reported to be more similar within host species than between species, which suggests that host genetics determine gut bacterial colonization (Ley et al., 2008). Other than phylogeny, the composition of the bacterial community may also vary depending on the specific individual, and its age (Zoetendal et al., 1998; Hopkins et al., 2001; Uyeno et al., 2010). Variations in the host (individual genetics), the

environment (physical, chemical and nutritional), and microbial interactions regulate the colonization of GIT by commensal microbes.

1.1.2 Primary functions of commensal microflora

Gnotobiological studies have shown that germ-free animals have low organ weight, decreased serum globulin levels, low numbers of lymphoid nodes, and reduced nutrient uptake when compared to their conventionally reared counterparts (Bourlioux et al., 2003; Backhed et al., 2004; Guarner, 2006). These findings suggest that commensal microflora plays important roles in the development and growth of the host. The functions of commensal microbiota have been categorised into three major activities: metabolism; protection; and trophic functions (Guarner, 2006).

The commensal flora enable the host to ferment non-digestible dietary substrates, and thereby improve the overall nutrient intake for the host by producing short chain fatty acids (SCFA), vitamins, and amino acids. Production of SCFA by microbes contributes up to 10% of the daily energy supply of omnivores and up to 70% in herbivores (Sanz et al., 2010). Moreover, it was reported that microbes can synthesize 16.9 g of crude protein from 100 g of organic matter in the rumen (Stern and Hoover, 1979). Additionally, gut bacteria are a good source of vitamins B and K for the host (Hill, 1997). All these functions, that improve the digestion and nutrient uptake of host, belong to the metabolic functions of gut microbes.

The protective functions of enteric flora include the formation of biofilms and the secretion of antimicrobial substances to prevent pathogenic invasion. Biofilm is a complex aggregation of microbes growing on a solid surface and consists of microbial cells and extracellular polymeric substances (O'Tool et al., 2000). Free-floating microbes first attached to the intestinal epithelium and then start colonization and there by prevent adhesion, expansion and translocation of pathogens in the gut (Kelly and Conway, 2005). Lactic acid bacteria (LAB), as a part of the commensal consortium, are capable of producing a range of bacteriocins against gram-positive bacteria (Cintas et al., 2001). Bactericidal activity of bacteriocins consists of cell lysis by activation of autolytic enzymes and cell membrane disruption (Cintas et al., 2001). The competition between pathogens and commensal flora for nutrients and adhesion receptors on the epithelium surface also prevents pathogen establishment in the GIT of host (Guarner, 2006).

The ability of gut microbes to alter the immune response of the host by regulating epithelial cell proliferation and differentiation, is known as the trophic function, and has been one of the major research areas of the last decade (Shanahan, 2002; Guarner, 2006). Studies using germ-free mice have shown that they lack lymphoid tissue nodes on the gut epithelium surface, and have lower levels of immunoglobulin A in the circulation, than conventional mice (Falk et al., 1998; Beutler, 2004; Macpherson and Slack, 2007). Moreover, the rate of epithelial cell turnover is lower in germ-free animals compared to that of conventional mice (Falk et al., 1998). In addition to the differences addressed

above, germ-free mice are reported to be more susceptible to pathogenic infections such as *Shigella*, and *Leishmenia* (Smith et al., 2007). These interesting findings suggest that gut microbes play a vital role in the immune function of the host. Furthermore, the changes in microbial ecology caused by the weaning of mammals coincide with various changes in the host immune system. For example, in mice, the number of B and T cells in the lamina propia and immunoglobulin A receptors were observed to increase after weaning (Hooper, 2004). This suggests that establishment of commensal gut microbiota may have an impact on the immune development of the host at an early stage of life.

1.2 Host-microbial communications in the gut

The host recognizes microbes by different pattern recognition receptors (PRRs) present on cell membranes (Toll-like receptors TLRs) and in the cytoplasm (nucleotide-binding oligomerization domain NODs) (Abreu et al., 2005). PRRs use specific molecular motifs conserved within microbes, known as microbes associated molecular patterns (MAMPs), to detect non self-molecules that enter into the body of host. For example, TLRs1, 2, 4-6 and 10 recognize bacterial ligands whereas, TLRs3, 7-9 recognize nucleic acids from viruses and bacteria (Chang, 2010; Seabury et al., 2010). Host recognition of MAMPs activates a cascade of immune responses which result in the secretion of cytokines, chemokines and other different molecules (Sandor and Buc, 2005). MAMPs are common to both pathogens and commensal microbes that reside in the GIT (Kelly and Conway, 2005) and the communication between host and

commensal microflora is essential to maintain intestinal homeostasis (Rakoff-Nahoum et al., 2004). Therefore, it is important to understand how the host discriminates commensal microflora from pathogens, in order to avoid unnecessary immune responses.

Until now, research on host-microbial interactions has been conducted using either germ-free or specific pathogen-free animal models, or *in vitro* studies using specific bacteria or their products (Otte et al., 2004; Guarner, 2006). These studies have revealed that initial exposure to microbes plays a vital role in the development of structural and functional properties of GIT as well as the mucosal immune system. Low serum IgA level was restored to normal levels and fucosylated glycan production which represents matured intestinal epithelial cells was started after microbial colonization of germ-free mice (Macpherson and Slack, 2007)). Mulder and colleagues (2009) reported that pigs reared outdoors established a bacterial community dominated by *Lactobacillus* spp. compared to that of indoor-housed and antibiotic-treated pigs, and thus, the expression of genes (type 1 interferon-IFN) in ileal mucosal tissue, which regulate innate immune signalling pathways, were different among these animals. These findings suggest that host-microbial communication not only affects local mucosal immune responses but also adaptive immune responses which act systemically.

1.2.1 Host defence mechanisms against pathogens

The host develops various defence mechanisms for protection against pathogens and any other foreign substances that enter the body. There are

basically two main immune responses: innate immunity; and adaptive immunity. These immune responses are either mediated by immune cells or by soluble chemicals secreted by the cells. Leukocytes (white blood cells) are the main cells involved in cell-mediated immune responses, and cytokines, chemokines, and antibodies secreted by these cells mediate the humoral immune responses.

Innate immunity is the first line non-specific defence mechanism of organisms which is conserved across most species. Immune cells such as macrophages, dendritic cells (DC) and other soluble molecules, such as cytokines and chemokines involved in the innate immune response, are ready to react rapidly to confront infections (Male et al., 2006). Phagocytosis is the prototypical innate defence mechanism where specific receptors (PRRs) present on cell surface recognize and internalize pathogens into cells. Monocytes, immature DC, macrophages, and neutrophils act as professional phagocytes (immune cells), whereas epithelial cells and fibroblasts act as non-professional phagocytes (non-immune cells) (Rabinovitch, 1995). The complement system, which consists of different proteins (~30), mediate immune responses via binding directly to pathogens, or via antibodies and soluble carbohydrates bound pathogens. Complement binding may trigger phagocytosis, inflammatory responses, or damage the cell membrane of the microbes (Male et al., 2006).

Adaptive immunity is the specific systemic response that is stimulated after host exposure to an infection and then adapt to that particular infection. Adaptive immunity is mediated by lymphocytes (B cells and T cells) where B cells mediate humoral immunity using antibodies and T cells mediate cellular

immunity using T helper cells (Male et al., 2006; Abbas et al., 2007). In contrast to the innate immune system which recognizes conserved molecules, the adaptive immune system recognizes specific antigens present on pathogens and produces antibodies and memory B cells (Abbas et al., 2007). These memory cells react quickly and produce more antibodies with secondary exposure to the same antigen (Cooper and Alder, 2006).

Innate and adaptive immune systems are complementary and produce a highly efficient defence against pathogens. Antibodies produced by B cells facilitate pathogen recognition via PRRs by opsonisation (Male et al., 2006; Van et al., 2011). Opsonisation is the process that binds antibodies to pathogens or their toxins which later facilitate phagocytosis. DC; one of the antigen presenting cells (APC), are reported to induce T cells as well as immunoglobulin A class switching in B cells (Iwasaki, 2007). These are two typical examples which represent the complementary action between innate and adaptive immune systems.

1.2.2 Recognition of microbe associated molecular patterns (MAMPs) by Toll-like receptors

Toll-like receptors are a family of PRRs that belong to the innate immune system , and are present on immune cells such as DC, macrophages, mast cells and B cells, as well as in non-immune cells such as epithelial cells, fibroblasts and endothelial cells (Hornung et al., 2002; Sandor and Buc, 2005; Chang, 2010). To date, a total of 13 TLRs with various target ligands have been reported for

mammalian species. Human cells are known to express TLRs1-10 (Table 1.1), whereas mice cells are reported to express TLRs1-9, and 11-13 (Chang, 2010). Some TLRs such as TLR2 form heterodimers to recognize ligands, whereas others (eg.TLR3) act as homodimers (Sandor and Buc, 2005).

The expression of TLRs varies depending on cell type. Eosinophils express TLRs1, 2, 4, 6, 7, 9 and 10; M cells express TLRs1, 2 and 4 at high levels; small intestinal epithelial cells express TLRs1-5 and 9; and B cells are reported to express TLRs1, 6, 9 and 10 at high levels (Sandor and Buc, 2005; Cashman and Morgan, 2009; Abreu, 2010). The presence of TLRs both on innate immune and adaptive immune cells (B and T cells) suggests that they are capable of regulating both the innate and adaptive immune responses. The expression of TLRs can be either intracellular or on the cell surface, based on the specificity of MAMPs. TLRs1, 2, 4, and 6 recognize conserved molecules from extracellular pathogens, and are expressed on cell surfaces, while TLRs3, 7, 8 and 9 recognize intracellular pathogens and are expressed inside cells (Chang, 2010).

Most TLR studies have focused on TLRs2 and 4 *in vivo* and *in vitro*. The aim was to understand their responses to target ligands secreted by Gram negative and positive bacteria, respectively, their signalling pathways, and their expression differences in healthy and diseased animals (Takeuchi et al., 1999; Nomura et al., 2000; Abreu et al., 2001; Rakoff-Nahoum et al., 2004; Devaraj et al., 2008). Ligands for TLR10 were unknown until recently Guan and colleagues (2010) reported that TLR10 shares common mechanisms with TLR1 to sense ligands (Table 1.1). Lately, there has been research to determine whether

TLR ligands can be used as adjuvants in vaccine formulations for farm animals, in order to increase efficiency (Coffey and Werling, 2011). Vaccines that are usually developed to stimulate the adaptive immune response of the host however, if TLR ligands use as adjuvants it would also stimulate the host innate immune system.

Recognition of ligands by TLRs activates signalling pathways and generates an effective immune response. There are two main signalling pathways that have been described in literature: MyD88 (myeloid differentiation factor)-dependent pathway (all the TLRs except TLR3); and transcript factor interferon regulatory factor (TRIF)-dependent pathway (Uematsu and Akira, 2007). The MyD88-dependent pathway signals via nuclear factor kappa B (NF-κB) and produces inflammatory cytokines, whereas the TRIF-dependent pathway signals via interferon regulatory factor 3 (IRF3) and produces type 1 interferon (IFN) (Sandor and Buc, 2005; Uematsu and Akira, 2007).

Both commensal microbes and pathogens have MAMPs and thus, how exactly TLRs discriminate against these two groups, and why, under normal physiological conditions, commensal bacteria do not induce inflammatory responses, are still pertinent questions. Polarized expression of TLRs has been described as one of the mechanisms developed to distinguish pathogens from commensal microbes. TLR5, which recognizes flagellin, was reported to be expressed only on basolateral surfaces of the intestinal epithelium cells (Gewirtz et al., 2001). Thus, only flagellin that breaches the epithelial barrier could activate pro-inflammatory genes. Under normal physiological conditions, commensal bacteria do not breach the epithelial cell barrier, leading the host to distinguish

pathogens and commensal bacteria which contain flagellin. Furthermore, Lee and colleagues (2006) reported that TLR9 expressed on the basolateral surface induced NF-κB pathway, whereas TLR9 expressed on the apical surface were unable to induce the NF-κB pathway, suggesting that inflammatory responses are produced only against microbes that breach the epithelial barrier. The same study also revealed that apical stimulation of TLR9 showed tolerance to subsequent exposure to ligands, and slowly lost the IL-8 response, suggesting that TLRs are capable of regulating inflammatory responses. Moreover, TLRs2 and 4 and other molecules such as MD-2 and CD14 involve in their signalling pathways were reported to express at low levels on epithelial cells to avoid unnecessary detection of commensal bacteria (Cario and Podolsky, 2000).

1.2.3 Role of TLRs in maintaining intestinal homeostasis

The recognition of MAMPs produced by commensal microbes, not only regulates the immune responses, but also provides protection against intestinal epithelium injuries and maintains intestinal homeostasis (Rakoff-Nahoum et al., 2004). Administration of 2% (wt/vol) dextran sulphate sodium (DSS- toxic to colonic epithelium) to TLR2, TLR4 or MyD88 deficient mice and antibiotic treated wild type mice caused severe mortality while wild type mice without antibiotic treatments after the same treatment were able to recover from injuries (Rakoff-Nahoum et al., 2004), suggesting the importance of interactions between commensal microbes and host. Recognition of microbial ligands by TLRs present in Paneth cells was also reported to be crucial in maintaining intestinal

homeostasis (Vaishnava et al., 2008). Moreover, Rakoff-Nahoum and colleagues, (2004) reported that oral administration of lipopolysaccharide (LPS) or lipoteichoicacid (LTA) prior to DSS administration was able to recover antibiotic treated wild type mice from DSS-induced tissue injuries. Recently, it was reported that commensal microbes induced expression of IL-1R associated kinase M (IRAK-M; a negative regulator of TLRs signalling), and negative regulation of TLRs signalling by IRAK-M prevent colitis in IL-10 deficient mice (Biswas et al., 2011). This suggests that commensal bacteria are capable of regulating the expression of negative regulatory factors to maintain intestinal homeostasis (Shibolet and Podolsky, 2007; Biswas et al., 2011). TLR9 deficient mice showed severe DSS-induce colitis compare to wild type and TLR2 deficient mice (Lee et al., 2008), signifying that polarized expression of TLRs is also one of the factors which regulate intestinal homeostasis. Stimulation of apical TLR9 regulates genes involve in antimicrobial activity instead of inflammatory genes. However, TLR9 deficient mice shows a down-regulation of genes involve in antimicrobial activity that regulate by the apical expressed TLR9, which leads to severe DSS induced colonic tissue damage (Lee et al., 2008).

1.3 Commensal bacteria in GIT of cattle

Most of the studies have been conducted to investigate symbiotic microbes present in the rumen. Microbial fermentation in the rumen converts fibre materials to volatile fatty acids and produces microbial proteins, and vitamins which are essential to host energy metabolism. The microbial diversity in the rumen have

been reported to be impacted by many factors such as diet, antibiotic use, and age (Jones et al., 1994; Stewart et al., 1997) and recent studies have shown that the variation of the microbial diversity in the rumen may be associated with host feed efficiency and methane emission in beef cattle (Guan et al., 2008; Zhou et al., 2009; Hernandez-Sanabria et al., 2010). A recent study showed that bacterial communities different between rumen and feces were different in cattle, suggesting probable regional differences in the commensal consortium along the GIT (Michelland et al., 2009).

To date, there has been very limited study on GIT commensal bacteria and their impact on host performance in cattle due to limited access to gut samples. A pyrosequencing study conducted using cattle fecal samples reported that fecal bacteria were dominated by *Clostridium* spp. followed by *Bacteroides* spp. (Dowd et al., 2008). A recent study demonstrated that fecal bacteria composition undergoes drastic changes during the first 3 months of life, when most of the metabolic and physiological changes occur in the GIT of dairy calves (Uyeno et al., 2010). However, there hasn't been any attempt to study the regional and environmental (mucosal tissue/ingesta) differences in bacterial communities along the GIT and possible age-related changes in bacterial diversity and density.

1.3.1 Host-microbial interactions in cattle

Studies on bovine innate immunity such as TLR expression along the GIT are limited. So far, 10 functional TLRs (TLR1-10) have been identified from the bovine genome (Seabury et al., 2010). Mutations in genes encoding TLRs have

been reported to be associated with increased susceptibility of individuals to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in dairy cows (Mucha et al., 2009). MAP is the causal microorganism of Johne's disease in cattle, which causes significant economic losses to the dairy industry. In addition to TLRs, the antimicrobial peptides (AMPs) and peptidoglycan recognition proteins (PGLYRPs) which also belong to the innate immune system have been identified from bovine genome and are considered to have potential microbicidal activity (Fjell et al., 2008; Seabury et al., 2010). A variety of host defense peptides or AMPs were reported in eukaryotes and β-defensin has been reported to present in cattle (Ganz, 2002; Fjell et al., 2008). β-defensin is capable of disrupting bacterial cell walls rich in phospholipids and killing phagocytised pathogens (Ganz, 2002). Mammals express four PGLYRPs (*PGLYRP*1-4) and bovine *PGLYRP*1 has been reported to recognize a variety of microbes (Tydell et al., 2006). However, up to now there is no study on the expression pattern of these genes in the GIT of dairy calves.

There are limited studies on bacterial diversity and density as well as basal expression level of innate immune genes (TLRs, β-defensin, *PGLYRP*1), throughout the GIT of dairy calves and it is not clear whether age has an impact on related changes. Moreover, the interactions between bacterial density and innate immune genes expression throughout the GIT are not well understood.

1.4 Use of molecular techniques to study gut commensal microbes

In the past, microbial studies were mainly relied on culture-based techniques and our understanding on intestinal microbes, host-microbe interactions, and microbial-microbial interactions was limited. The discovery of microbial identification by their small subunit ribosomal RNA (SS rRNA) and the use of 16S rRNA gene-based techniques have helped to explore a wide range of unculturable microbes (Muyzer, 1999b).

Molecular fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), and terminal restriction fragment length polymorphism (TRFLP) have been widely used for microbial profiling (Muyzer et al., 1993; Muyzer, 1999a; Muyzer, 1999b; Frey et al., 2010). Each of these fingerprinting techniques has their own pros and cons (Table 1.2) (Muyzer, 1999b; Enwall and Hallin, 2009). Among the molecular finger printing techniques, DGGE is widely used due to its fast and economical detection of 99% of predominant bacterial species in environmental samples (Muyzer et al., 1993).

Cloning and sequencing of PCR-amplified 16S rRNA gene has been used to identify bacterial species and has allowed identification of most of the unculturable bacterial species that reside in the GIT (Amann et al., 1995). Although, construction of 16S rRNA gene libraries allowed comparing different microbial communities, the process was laborious and the identified community can be biased based on the numbers of clones sequenced. The recent invention of next generation sequencing techniques, such as pyrosequencing, provides fast,

accurate, and high throughput identification of microbes at a taxonomic level (Ronaghi, 2001).

Metagenomics is the culture-independent study of whole microbial genomes from environment samples (Handelsman, 2004) and does not suffer from the shortcomings, such as species abundance biasness observed in other sequencing studies (Gill et al., 2006; Arumugam et al., 2011). Moreover, metagenomics studies have been able to reveal interactions between less abundant microbial species and the host (Arumugam et al., 2011). A study on human gut microbes reported that low abundance *Escherichia* accounts for over 90% of two proteins associated with bacterial pili present in feces, revealing that metagenomics allows studying less abundant species in the gut microbiome (Arumugam et al., 2011). Thus, metagenomics studies are important to understand the complexity of the gut microbiome and its interactions with the host.

1.5 Summary

To date, a large number of studies have reported on the importance of bacterial colonization for the development of host immunity. However, there have been very limited attempts to apply this knowledge in dairy cattle. With the increasing infectious disease outbreaks reported in last decades (Jones et al., 2008), it has become necessary to develop multidisciplinary techniques to improve livestock health. Gut microbes are crucial in determining host immunity development and health. Therefore, if the goal is to improve host health via manipulating gut microbes, a better understanding of microbial establishment in

the GIT is a must. However, due to the limited access to GIT samples, previous studies on cattle gut bacteria depended largely on feces as the representative sample, which did not allow the study of regional and environmental variations along GIT and how these impact microbial colonization and alter host immune responses.

Thus, our knowledge of regional and environmental differences in bacterial communities and their impact on innate immune gene expression in dairy calves is still very limited. We hypothesized that variations in the establishment of gut bacteria in the early stages of life can contribute to differences in the development of immunity in dairy cattle. It is important to study the expression patterns of host innate immunity related genes, such as TLRs, throughout the GIT in order to understand the basal expression, and possible alterations with perturbations in commensal flora in the early stages of life. Therefore, in this study, we aimed to investigate the commensal bacterial community and the expression of innate immune genes in the gut of newborn and weaned claves.

1.6 Objectives

The key objective of this project was to investigate the relationship between diversity and density of the gut microflora with the development of the immune system in dairy calves using culture-independent methods.

The specific objectives of this study were:

1) To investigate the bacterial diversity associated with mucosal tissue and ingesta along the GIT of 3 week and 6 month old dairy calves, using PCR-DGGE
2) To study the expression of ten TLRs, β-defensin and PGLYRP1 along the GIT of dairy calves
3) To explore the effect of dairy calf age on the commensal bacterial community and the expression of genes associated with the innate immunity
4) To explore the relationship between the establishment of gut bacteria and host immunity

The long term goal of this study is to determine the relationship between gut microflora and the development of the host immune system, and to identify the possible influence of host genetics in this relationship. This information may also be applied to identify possible gene markers associated with improved host health and disease resistance traits, which could be used in animal selection programs.

Table 1.1 Mammalian TLRs and examples of their microbial ligands

TLRs	Ligands	Reference
TLR1/2	Triacyl lipopeptides (B)	(Wells et al., 2011)
TLR2/6	Diacyl lipopeptides, glycolipids, lipoproteins, lipoteichoic acid (B)	(Wells et al., 2011)
TLR 2	Cell wall components from gram positive bacteria (B), zymosan (F), heat shock proteins/HSP (H)	(Wells et al., 2011)
TLR3	dsRNA (V)	(Akira and Takeda, 2004)
TLR4	Cell wall components from gram negative bacteria (B), HSP (H)	(Akira and Takeda, 2004)
TLR5	Flagellin (B)	(Akira and Takeda, 2004)
TLR7	ssRNA (V)	(Honey, 2004)
TLR8	ssRNA (V)	(Honey, 2004)
TLR9	CpG containing DNA (B)	(Akira and Takeda, 2004)
TLR10	Triacylated lipopeptides and wide variety of other ligands shared by TLR 1 (B)	(Guan et al., 2010)
TLR11	Profiling from Toxoplasma gondii (P)	(Bird, 2005)
TLR12	Unknown	
TLR13	Unknown	

B – bacteria, F – fungi, H – host, P – protozoa, V – virus

Table 1.2 Pros and cons of widely used DNA fingerprinting techniques

Technique	**Strengths**	**Weaknesses**
DGGE/TGGE	High discrimination among community members	Can only use shorter fragments, gel-to gel comparison biasness
T-RFLP	High throughput, direct quantification of fragments	No phylogenetic information obtained, possible appearance of false TRF
RAPD	No special primers required	Low reproducibility, no phylogenetic information obtained

Adapted from Muyzer (1999).

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Chapter 2. Distinct commensal bacteria associated with ingesta and mucosal epithelium throughout the gastro-intestinal tract of dairy calves[1]

2.0 Introduction

The mammalian gastrointestinal tract (GIT) contains dynamic microbial populations and is rapidly colonized during and after birth. The newborns acquire GIT microbes from their mother, the surrounding environment, and with their diet (Mackie *et al.*, 1999). The enteric microbial community consists of autochthonous (lifelong interaction with host) and allochthonous (transient) populations which both play important roles through the host's life (Mackie & White, 1997).

Research on GIT microbes has been limited in the past by the complexity of the microbiota and inability to culture many GIT bacterial species which are obligate anaerobes (Fakhry *et al.*, 2009; Walker, 2010). With the development of molecular biological tools, there has been a substantial increase in research to analyze the gut microbiome and establish specific associations with host biological functions. Metagenomics (sequencing analysis of total DNA from environmental samples) is one technique recently applied to characterize gut microbiota and study its predicted functions (Handelsman, 2004). These analyses have revealed the complexity of such microbial communities, and established correlations between imbalances in the gut microbiome and perturbations of host health, such as obesity and other related metabolic disorders (Ley *et al.*, 2006; Turnbaugh *et al.*, 2009; Ley, 2010; Sanz *et al.*, 2010). Most of these studies used

[1] A version of this chapter has been submitted for publication. FEMS Microbiology Ecology.

feces as representative samples, due to limited access to the GIT (Gill *et al.*, 2006; Ley *et al.*, 2008; Walker, 2010). It is known that conditions within the GIT differ depending on region and sample type (mucosal tissue/ingesta) (Van *et al.*, 2011) and these differences may result in site-specific microbial communities. For example, the number and diversity of lactic acid bacterial species are different when comparing stomach, ileal and cecal contents in pigs (van Winsen *et al.*, 2001). Recent studies have also reported that mucosa-associated microbiota in the colon were distinct from the predominant bacteria found in feces and such microbiota influenced TLR2 and TLR4 expression on epithelial cells in mice (Zoetendal *et al.*, 2002; Wang *et al.*, 2010). However, our understanding of microbiota segregation between the mucosa and ingesta is still very limited with no knowledge to what extent regional differences are established throughout the GIT.

Infectious diseases in domestic species significantly impact animal production and health and may be a source of zoonotic infections or foodborne illness. Thus, it is important to minimize livestock susceptibility to emerging pathogens by improving natural resistance to pathogenic infections. Based on recent studies in humans and mice, the GIT microbiota play a determinant role in health. It is therefore, important to understand if we can improve the health of domestic species through manipulation of the GIT microbiota. There is, however, limited knowledge on the commensal GIT microbiota diversity in different animal species and how age and other factors impact microbiome diversity.

We hypothesized that mucosa-associated bacteria are a distinct community from bacteria inhabiting the ingesta and such variation exists among different regions throughout the GIT. We also speculated that the age of animals can be one of the factors to impact on bacterial population in the GIT. The bacterial diversity and populations were detected from the mucosal tissue and ingesta along the GIT of dairy calves and determined if there were age-dependent effects on bacterial population throughout the GIT. Bacterial communities were compared on the basis of diversity generated from PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and bacterial density was estimated by quantification of bacterial 16S rRNA copy numbers.

2.1 Materials and methods

2.1.1 Animal experiment

One week old, castrated male, Holstein calves were purchased from a commercial dairy farm and housed at the Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan. All experimental protocols were reviewed and approved by the University Committee on Animal Care (University of Saskatchewan) and all procedures were performed following guidelines approved by the Canadian Council on Animal Care. Calves were reared at VIDO and fed fresh, non-pasteurized whole milk and Blue Medollian calf supplement for the first 12 weeks. Calves were then fed alfalfa hay and one kg oats/day supplemented with trace minerals, for the next 4 months.

2.1.2 Sampling of GIT mucosal tissue and ingesta

Mucosal tissue and ingesta were collected from 3 week old (n = 8) and 6 month old (n = 8) male Holstein calves. Calves were humanely euthanized with an intravenous injection of Euthanyl® (240mg/ml; Bimeda-MTC Animal Health Inc., Cambridge, ON) and intestinal tissue was immediately collected. GIT tissues from each site were rinsed three times with phosphate buffered saline (PBS) to remove ingesta, cut into 4-5 mm^2 segments, and immersed in 5X the volume of RNAlater (Applied Biosystems, Foster City, CA, USA) and stored at -80°C. Ingesta were collected from each mucosal tissue collection site and 200 µl of ingesta was mixed in 1 ml RNAlater and stored at -80°C until further analysis.

2.1.3 DNA extraction

Total DNA was extracted from mucosal tissue and ingesta samples (~100 mg, all samples were weighted before DNA extraction) using the bead beating method as described by Li *et al*., (2011). Briefly, samples were subjected to physical disruption in a BioSpec Mini Beads beater 8 (BioSpec, Bartlesville, OK, USA) at 4800 rpm for 3 min, followed by phenol: chloroform: isoamyl alcohol (25:24:1) extraction of DNA. DNA was precipitated with cold ethanol and dissolved in nuclease-free water (30 µl and 40 µl for ingesta and mucosal tissue, respectively). The quantity and quality of DNA was measured using ND1000 spectrophotometer (NanoDrop Technologies, Wilmington) and stored at 20°C.

2.1.4 PCR-Denaturing gradient gel electrophoresis (PCR-DGGE) analysis

Total DNA extracted from ingesta samples was diluted to a final concentration of 50 ng/µl and total DNA from mucosal tissue samples was diluted to 25 ng/µl. Nested PCR was performed for DNA extracted from mucosal tissue samples using 27F and 1492R bacterial primers (27F, 5'-AGAGTTTGA TCMTGGCTCAG-3'; 1492R, 5'-TACGGYTACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991) with the following program: 95°C for 5 min followed by 35 cycles of 94°C for 30 s; 58°C for 30 s and 72°C for 90 s and 72°C for 7 min elongation period to enrich bacterial 16S rRNA full-length genes (~1. 4 kb). V2-V3 region of amplified 16S rRNA was then amplified by HDA1-GC and HDA2 primers (HDA1-GC, 5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCA CGGGGGGACTCCTACGGGAGGCAGCAGT-3'; HDA2, 5'-GTATTACCG CGGCTGCTGCTGGC AC-3') (Walter *et al.*, 2000) with the following program: 95°C for 5 min, followed by 35 cycles of 94°C for 30 s; 53°C for 30 s and 68°C for 30 s and 68°C for 7 min elongation period. Conventional PCR was performed for DNA extracted from ingesta samples using HDA1-GC and HDA2 primers. PCR products (200bp) were run on a 6% acrylamide gel with 30-55% gradient using Bio-Rad DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 130 V, 60 °C for 4 h. Gels were stained with 1% (vol/vol) ethidium bromide, and viewed with FluorChem SP imaging system (Alpha Innotech. San Leandro, CA).

Analysis of PCR-DGGE band patterns was conducted using Bionumerics® software package version 6.0 (Applied Maths, Austin, TX, USA).

All gels were normalized using a reference ladder of PCR products prepared with 12 different plasmid DNAs containing the known sequences from a rumen tissue 16S rDNA clone library constructed in an another study (Li *et al.*, 2011) which was run on each gel. This reference lane was used to normalize the positions when DGGE profiles generated from multiple gels were compared. The comparison between mucosal tissue and ingesta for each GIT sample site was performed using the Dice's similarity coefficient (D_{sc}) with individual animals analyzed separately. Dendrograms were generated using unweighted pair group with mathematical averages (UPGMA) at 1% position tolerance.

The total number of bands was determined based on the best fit Gaussian curve for each band, following the method described by Hernandaz-Sanabria *et al*., (2010). Each band belongs to one particular category was considered as one bacterial phylotype. Bacterial phylotype richness for a particular region was defined as the total number of PCR-DGGE bands present in each mucosal tissue and ingesta sample. To calculate the relative abundance of common bacterial phylotypes, shared bands between mucosal tissue and ingesta were counted for each individual. The shared band count was then divided by the total number of bands present in both mucosal tissue and ingesta for each GIT region.

2.1.5 Analysis of 16S rRNA gene libraries

Total DNA extracted from ileum mucosal tissue and ingesta of individual animals was diluted to 50 ng/µl concentration. The 16S rRNA gene was amplified using 27F and 1492R primers from individual animals and verified on an agarose

(1.0%) gel. Then the amplicons were pooled for tissue and ingesta, respectively and a ~1.4 kb DNA fragment was extracted from 1.0% agarose gel using QIAEX II Gel Extraction Kit (QIAGEN Sciences, MD, USA) following the manufacturer's instructions. Briefly, excised gel slices were re-suspended in a buffer containing high concentration of salt to solubilize agarose gel and dissociate DNA from gel. DNA was bound to QIAEX II (silica particles) by incubating gel slices in the buffer. Silica bound DNA was then washed with high salt buffer followed by ethanol containing buffer to remove residual agarose and salt, respectively. Finally, DNA was eluted by 20 µl of nuclease-free water. The purified DNA was then cloned into a TOP10 vector (TOPO TA cloning kit, Invitrogen, Carlsbad, CA, USA) using chemical transformation. Seven hundred colonies were randomly selected from S-Gal medium (Sigma, St. Louis, MO, USA), and plasmid DNA was extracted using Millipore Plasmid Extraction Kit (Millipore, Billerica, MA, USA). Sequence reaction was performed in 10 µl total volume, containing 0.5 µl of BigDye, 3.2 pmol of M13 Forward primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') for TOP10 vector (TOPO TA cloning kit, Invitrogen), 2.0 µl of 5x sequencing buffer, 100 ng of plasmid DNA as the template with ABI 3730 sequencing system using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) to generate partial 16S rRNA gene sequences (~800 bp).

The sequences were subjected to chimera sequence check using the Bellerophon program (Huber *et al.*, 2004) and removed chimera sequences prior to data analysis. The sequences with good quality (600 bp) of each colony was

compared using the RDP Classifier online tool (http://rdp.cme.msu.edu/) (Wang *et al.*, 2007) and were aligned using ClustalW program (http://www.ebi.ac.uk /Tools /clustalw2/). The obtained sequences were then analyzed using Mothur program (http://schloss.micro.umass.edu/mother/Main_Page) by comparing generated operational taxonomic units (OTUs – entity use for phylogenetic studies) based on 97% similarity of the sequences as described by Zhou *et al*., (2009) to compare the similarity (Shannon index), diversity, richness, between mucosal tissue and ingesta bacterial communities. All the sequences were submitted to NCBI database under the accession numbers JN575780- JN576426.

2.1.6 qRT-PCR analysis of total bacterial populations

qRT-PCR was performed using SYBR Green chemistry (Fast SYBR® Green Master Mix, Applied Biosystems) and U2 primers (U2F, 5'-ACTCCTACGG GAGGCAG-3'; U2R, 5'- GACTACCAGGGTATCTAATCC-3') (Stevenson & Weimer, 2007) with a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The fast cycle and melt curve method was applied with the following program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s and 62 °C for 1 min. A standard curve was constructed using serial dilutions of plasmid DNA containing the 16S rRNA sequence of *Butyrivibrio hungatei*. Copy numbers for each standard curve were calculated based on the following equation: $(NL \times A \times 10^{-9})/ (660 \times n)$, where NL was the Avogadro constant (6.02×10^{23}), A was the molecular weight of DNA molecules (ng), and n was the length of amplicon (bp). The copy number of 16S rRNA genes for total bacteria per gram

of sample was calculated using the following equation as previously reported (Li *et al.*, 2009): (QM x C x DV)/ (S x W), where QM was the quantitative mean of the copy number, C was the DNA concentration of each sample (ng/µl), 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).

2.1.7 Statistical Analysis

All detected PCR-DGGE bands were categorized based on the best fit Gaussian curve as described by Hernandaz-Sanabria *et al.*, (2010). Then, the frequency of each band per category (sample type (mucosal tissue vs. ingesta); gut region) was obtained using PROC FREQ and results were plotted. The interaction between sample type and bands was analyzed using PROC CATMOD (version 9.2, SAS Institute Inc., Cary, NC). Total bacterial population data, phylotype richness, relative abundance of common phylotypes and D_{sc} were analysed using MIXED procedure in SAS. The statistical model included gut region/sample site and age as fixed effects. Differences in least square means among locations were declared at $P < 0.05$ using the PDIFF option in SAS. Comparisons between mucosal tissue and ingesta were used to analyse sample type effect, comparisons among different GIT regions were used to analyse regional effect and comparisons between 3 week old and 6 month old calves were used to analyse host age effect on bacterial density, phylotype richness and relative abundance of common phylotypes in dairy calves.

2.2 Results

2.2.1 Comparison of detectable PCR-DGGE profiles from mucosa and ingesta as well as among different regions through GIT of calves.

In this study we used PCR-DGGE profiling to study the predominant bacterial diversity differences throughout the GIT. Firstly, the PCR-DGGE profiles generated from different sample types (mucosal tissue vs. ingesta) were compared within each region throughout the gut (Figure 2.1 A & B). The PCR-DGGE profiles generated from ingesta of all sample sites including rumen, jejunum, ileum, cecum and colon were different from those generated from the matched mucosal tissues (data not shown). When index of D_{sc} was used to compare mucosal tissue versus ingesta PCR-DGGE profiles for each GIT region, D_{sc} was significantly lower ($P < 0.01$) for mucosal tissue versus ingesta bacterial diversity of jejunum and ileum when compared to cecum, colon and rumen in 3 week old calves (Figure 2.2). However, no significant difference ($P = 0.75$) in mucosal tissue versus ingesta bacterial diversity was observed for 6 month old calves.

2.2.2 Comparison of bacterial phylotype richness and relative abundance between mucosal tissue and ingesta as well as among different regions through GIT of calves.

To verify above observed bacterial diversity difference, we further compared the bacterial phylotype richness and relative abundance of the bacterial phylotypes between sample types and among gut regions. Based on the best-fit

Gaussian curve for each band, 76 and 75 bands were obtained for 3 week and 6 month old calves (Figure 2.3 A & B), respectively and 80 bacterial phylotypes (band categories) were defined based on both 3 week and 6 month old calves DGGE band patterns.

When the richness of the bacterial community in 3 week old calves was compared, a regional effect was not detected for either ingesta or mucosa-associated communities (Table 2.1). However, a significant sample type (mucosa vs. ingesta) effect was observed for ileum (P = 0.04), where ileal mucosa-associated bacterial community phylotype richness was higher than that of ingesta. A similar trend (P = 0.07) was observed for rumen and cecum (Table 2.1). In contrast to 3 week old calves, 6 month old calves displayed a significant regional effect on ingesta associated bacterial phylotype richness, but there was no sample type effect. The richness of the bacterial community in 6 month old calves was lower in jejunal and ileal ingesta when compared to other regions, but no significant differences were detected for the mucosal-associated communities (Table 2.1). When the age effect on richness of bacterial communities was analyzed, a significantly higher richness was observed for 3 week mucosal-associated bacterial communities in the rumen, jejunum, and ileum and the richness of ingesta-associated communities tended (P = 0.08) to be higher in rumen and jejunum of 3 week old calves than 6 month old calves (Table 2.1).

Further analysis of the relative abundance of common phylotypes revealed that jejunum and ileum mucosa and ingesta shared a significantly lower number of predominant phylotypes when compare to rumen, cecum and colon of 3 week

calves (Table 2.2). Although there was no significant regional effect on the relative abundance of common phylotypes, jejunum and ileum of 6 month old calves also shared fewer phylotypes compare to other gut regions (Table 2.2). However, the relative abundance of shared phylotypes in rumen, cecum and colon of 6 month calves was reduced significantly when compare to 3 week calves (Table 2.2).

2.2.3 Analysis of association between bacterial phylotypes and sample types as well as regions.

To further define how bacterial communities differ between mucosal tissue and ingesta, the associations between PCR-DGGE bands and sample types (mucosa vs. ingesta), as well as and sample sites (region) were analysed for each age category of dairy calves. Eight out of 76 bands from 3 week and 6 out of 75 bands from 6 month calves were associated with sample site (Table 2.3). Significantly higher frequency of the presence of these bands detected from mucosal community revealed that all of these bands were more likely to be associated with mucosal tissue than ingesta. The same analysis was conducted for individual GIT regions to study band distribution regionally between mucosal tissue and ingesta. This analysis revealed that mucosa-specific bacteria phylotypes distribution varied throughout the GIT (Table 2.3). For example, the segregation of band 44 between mucosal tissue and ingesta varied greatly among the four GIT regions analysed and regardless gut region, band 44 was tended to be associated with the mucosal tissue.

2.2.4 16S rRNA library analysis of ileal mucosal tissue and ingesta associated bacterial communities.

To verify the above observed difference between the mucosal tissue and ingesta associated bacterial diversity, a sequence analysis of partial 16S rRNA gene libraries was conducted for ileum of 3 week old calves. The ileum was selected for its direct involvement in host immune functions. In total, 145 and 69 OTUs were identified for mucosal tissue and ingesta libraries, representing 85% and 67.5% of each bacterial community, respectively. As shown in Table 2.4, Shannon index, richness and diversity of the mucosa-associated bacterial community was significantly different than those of ingesta. When the two communities were compared at phylogenic levels, the mucosa-associated bacterial community was dominated by significantly higher *Firmicutes* (42.4%) followed by *Bacteroidetes* (40.4%) whereas, the ingesta-associated community was solely dominated by *Firmicutes* (93.4%) (Figure 2.4).

2.2.5 Comparison of total bacterial populations in the GIT of calves.

The total bacterial population at different GIT regions was estimated with qRT-PCR analysis by measuring the total copy number of bacterial 16S rRNA genes. It is known that bacteria can possess multiple copies of 16S rRNA which may lead to an overestimation of population data (Dahllof *et al.*, 2000). No region and sample type effect was observed on the total bacterial population in ingesta

and mucosa-associated communities of 3 week old calves (Table 2.5), except a significant sample type effect was observed for cecum ($P = 0.01$).

However, both GIT region and sample type had a significant impact on bacterial density of 6 month calves (Table 2.5). Higher mucosa-associated bacterial numbers were observed in the rumen ($P = 0.05$) and higher ingesta-associated bacterial numbers in the rumen, cecum and colon ($P = 0.03$) of 6 month old calves (Table 2.5). Moreover, the mucosa-associated bacterial density was significantly lower than that of ingesta (Table 2.5).

A significant age effect was observed only with colon ingesta-associated ($P = 0.02$) and rumen mucosa-associated bacterial densities ($P = 0.02$).

2.3 Discussion

This study is the first to analyze sample type (mucosa vs. ingesta) and gut region dependent bacterial segregation as well as age-dependent bacterial diversity and density throughout the GIT of dairy calves. Despite the limitations associated with low resolution DNA fingerprinting method (PCR-DGGE), small sample size for tissue and ingesta, and the small number of animals sampled, the present observations on the detectable predominant bacterial profiles supported the hypothesis that mucosa-specific bacteria colonize the GIT of cattle, and were distinct than those inhabit in ingesta. This observation is consistent with a recent report for human (Zoetendal *et al.*, 2002) which further supports the conclusion that mucosa-associated commensal microbiota is a common phenomenon in a wide variety of species. Fraune and Bosch (2010) recently reported that *Hydra*

epithelium grown under identical conditions for 20 years was colonized by a complex and dynamic microbial community, and that host selection of microbes was genetically encoded in the epithelium. Different *Hydra* species cultured under same conditions in the lab were reported to colonize with different microbes, while same *Hydra* species that were grown in the lab and natural habitats colonized with similar microbes (Fraune & Bosch, 2010). Moreover, Bourlioux *et al*., (2003) reported that bacterial adhesion to the intestinal mucosa is genetically controlled by the host through the expression of different carbohydrates on the epithelium. Together with our results, these suggest that mucosal associated microbial population in calves are also regulated by the host. Future studies on such population may improve our understanding of commensal bacteria and their roles in gut functions.

Up to date, the evidence of the segregation of bacterial diversity between mucosal tissue and ingesta bacterial community throughout the GIT is very limited. The observed distinct PCR-DGGE profiles, variation at bacterial phylotype richness and relative abundance between mucosal tissue and ingesta bacterial communities from both 3 week and 6 month calves in the present study strongly supports the conclusion that mucosal bacterial community is significantly different than that of ingesta since mucosal epithelium has been reported to determine to attachment of particular bacterial species which results in the segregation of bacterial species between ingesta and mucosa-associated communities (Bourlioux *et al.*, 2003; Fraune & Bosch, 2010). The observed significantly higher number of OTUs, Shannon index, diversity and richness in

ileal mucosa-attached bacterial community than ingesta confirmed a highly diverse mucosa-associated bacterial community in ileum. Mucosa-associated bacteria can influence intestinal cell proliferation and differentiation via short chain fatty acids (SCFA) production (Shanahan, 2002a; Shanahan, 2002b). Thus, the observed diversity of mucosa-associated bacteria implies possible biological differences among individuals that may lead to different phenotypes such as disease resistance.

It is known that gut environment vary due to the differences of chemical component such as, pH, SCFA, hormone secretion, and physical conditions such as surface structure of each region, ingesta particles size (Van *et al.*, 2011). Zoetendal and colleagues (2002) reported that predominant colon mucosa-associated bacteria in humans were host specific and different from those in feces, suggesting the dynamics of the bacterial communities throughout the GIT. Our results on different diversity of proximal region versus distal region as well as regional effect on bacterial diversity and population also revealed region specific dominant bacterial species throughout the GIT of dairy calves, suggesting that further segregation of bacterial communities is a mechanism along the whole GIT, which provide strong evidence that fecal microbiome may represent only a partial gut microbiome. Therefore, when fecal samples are accessed, in particular, the bacterial species belonging to the mucosa-associated population of the small intestine will not be detected. These mucosa-associated populations and their potential interactions with ingesta-associated populations may be critical for understanding the diversity of the gut microbiome and their roles in both host-

microbe interactions and the contribution of the microbiome to a range of diseases (Ley *et al.*, 2006; Turnbaugh *et al.*, 2009; Ley, 2010; Sanz *et al.*, 2010).

In agreement with previous studies conducted in humans and cattle, our study also revealed age-related changes in the predominant bacterial phylotypes (Table 2.1). Uyeno *et al*., (2010) reported that the fecal bacterial profile of dairy calves changed with age and diet. These changes included both extinction of abundant species present with a milk diet during the first few weeks of life and the emergence of new species following the transition to a solid diet. For example, *Ruminococcus flavefaciens* was only observed in weaned dairy calves (Uyeno *et al.*, 2010). Moreover, Hopkins and colleagues (2001) reported that bacterial composition of feces from children was different than that of adult feces which was dominated by facultative anaerobes. Microbes entering the GIT of a young animal are more likely to colonize than bacteria entering the GIT of an adult animal which has a stable established enteric flora (Mackie *et al*., 1999). This difference in colonization potential might contribute to the differences in predominant bacterial species observed when comparing 3 week and 6 month calves. Following bacterial colonization, changes occur in the GIT, such as reduced available energy and oxygen for microbes and altered expression of adhesion receptors on mucosal epithelium (Willemsen & de Graaf, 1992; Hopkins *et al.*, 2001). In addition, higher phylotype richness was specifically observed in the jejunum and ileum mucosa of 3 week calves than that of 6 month calves (Table 2.1). The bovine small intestine serves as an important immune induction site through the activity of the organized lymphoid tissue within the Peyer's

patches (Griebel & Hein, 1996). A significantly higher richness in the ileal mucosa-associated bacterial community based on with both DGGE and 16S rRNA gene library sequence analysis and a distinct pattern of mucosal lymphocytes and leukocytes distribution between jejunum and ileum in the same 3 week calves (Fries *et al.*, 2011), suggest this site may be essential for host immunity development. The recognition of both commensal microbiota and pathogens in early life of cattle by these mucosal immune cells may play a key role in both innate and adaptive immunity development. Further studies are required to determine if commensal microbiota have a direct impact on immune cells development and function under different ages. Studies with combined sequencing analysis of GIT bacterial communities and an analysis of mucosal leukocyte and lymphocyte populations in newborn and weaned calves may answer these questions in greater detail.

The present observations on the detectable predominant bacterial profiles through the GIT of dairy calves suggest that segregation of bacterial community between mucosa and ingesta, among different regions and under different ages. Further work is required to confirm whether the mucosa-associated community is more likely to be autochthonous and less responsive to external changes such as diet. Future studies comparing groups of animals fed different diets may provide direct evidence for specific factors that alter mucosa- and ingesta-associated bacterial diversity.

Besides the diversity of the bacterial communities, it has been known that different population of bacteria inhabit various regions throughout the GIT in

human, mice and pigs (van Winsen *et al.*, 2001; O'Hara & Shanahan, 2006). This study is the first to estimate the population in the mucosa-associated communities in GIT of calves. There was no significant regional effect on total bacterial population in ingesta and mucosa-associated communities in 3 week calves. However, in agreement with previous studies we observed high individual variation in total bacterial population and species richness among individual 3 week old calves and a more stable enteric bacterial population and community among weaned calves (Mackie *et al.*, 1999; Uyeno *et al.*, 2010) suggesting that early exposure of the newborn to microbes might play a crucial role in commensal gut flora establishment and relevant host functions. The observed difference in total bacterial populations in the proximal and distal GIT, regardless of age, may be due to the short transit time in the small intestine which does not allow for bacterial growth and also may be determined by distribution differences in host adhesion receptors for bacteria (Mestecky *et al.*, 2005). Studies of culturable bacteria had reported that bacterial density in the small intestine is relatively low (10^4-10^7 cfu/ml) when compared to the large intestine (10^{11}-10^{12} cfu/ml) (O'Hara & Shanahan, 2006). However, our analysis using qRT-PCR did not reveal a significant difference in bacterial density when comparing the proximal and distal GIT of dairy calves. The estimated total bacterial numbers by qRT-PCR revealed a much higher population in the small intestinal tract of calves, suggesting that more unculturable species reside in small intestine.

In addition, the present study provided evidence for a large population of bacteria reside in the rumen of 3 week old calves. The bovine rumen, as a key

fermentation site supplying nutrients for the production of volatile fatty acids, amino acids and vitamins, has been widely studied since 1960 (Marshall *et al.*, 1992; Martin & Nisbet, 1992; Robinson *et al.*, 1998; Penner *et al.*, 2009). However, establishment of rumen microbial community has not been well studied and it was surprising to identify similar rumen bacterial populations and complicated bacterial profiles for both ingesta and mucosa samples, suggesting that colonization of the rumen epithelium-attached bacteria begins early in life. This raises a very interesting question: Is this initial population important for the development of later rumen bacterial populations? For example, the rumen epithelial absorption of fermented volatile fatty acids in adult cattle is important both for nutrient transportation and to maintain a balanced pH rumen environment, in order to prevent acidosis or subacute acidosis (Owens *et al.*, 1998). The early colonization of bacterial populations on the surface of the ruminal epithelial wall may help the animals to develop the capability to adapt to the solid feed particles as well as to other microbes needed after weaning. The ingesta-associated bacterial community is influenced by dietary changes and this may be the reason for the high bacterial population in the rumen at 6 months. Recent studies have shown the distinct bacterial taxonomy for epithelial attached community in adult cows (Cho *et al.*, 2006) and its adaptation to the dietary changes (Chen *et al.*, 2011), future studies which sample epithelium-associated bacteria from the same individual from newborn to adults may further define adaptation responses of the bacterial community in this organ which will supply fundamental understanding of their functions in the life span of ruminants.

2.4 Conclusions

Our study on regional and sample type (mucosa vs. ingesta) influence on bacterial establishment is the first to reveal the predominant bacterial profiles in GIT of diary calves and how they differed among different regions as well as between mucosa and ingesta. The analysis of generated bacterial profiles revealed the presence of a mucosa-associated bacterial community in which the species were different from those associated with the ingesta. Our results suggested that previous knowledge on microbial communities, based on the analysis of fecal samples, may have been biased due to the segregation among different regions of gut. Future studies to use more representative samples including multiple sampling sites within each region and large number of animals and apply the next generation sequencing technology will further verify the regional and sample site variations in the GIT bacterial community observed in our study. Furthermore, the age-dependent mucosa and ingesta associated bacterial diversity was also observed in this study. It is important to mention that the observed age effect on mucosal associated bacterial diversity in this study is the result of interaction between age and diet, the two confounding factors. The effect of the diet on mucosal associated bacterial community and whether the DIVERSITY alteration in ingesta associated community influences the mucosal community has not been studied. Future research comparing animals under different diets will help to understand ingesta associated bacterial population, their interactions with mucosal attached community, and with the host.

Table 2.1 Richness of predominant bacterial PCR-DGGE bands throughout the GIT of dairy calves

Gut region	**3 week old calves**		***P*-value** [1]	**6 month old calves**		***P*-value** [2]	***P*-value** [3]	***P*-value** [4]
	Mucosal tissue	Ingesta		Mucosal tissue	Ingesta			
Rumen	39±1.6	33±2.2	0.07	31±1.1	27±2.9 [a]	0.17	0.04	0.08
Jejunum	33±4.6	27±2.9	0.34	25±1.9	20±2.8 [b]	0.13	0.04	0.08
Ileum	32±2.4	24±2.9	0.04	25±2.7	19±2.7 [b]	0.15	0.05	0.25
Cecum	36±2.8	27±3.1	0.07	27±1.8	28±0.5 [a]	0.72	0.02	0.97
Colon	34±3.2	26±3.4	0.12	28±1.8	28±2.2 [a]	0.98	0.15	0.57
P-value [5]	0.59	0.22		0.16	0.02			

[1] environment effect on richness of predominant bacteria throughout the GIT of 3 week old claves

[2] environment effect on richness of predominant bacteria throughout the GIT of 6 month old claves

[3] age effect on richness of mucosa-associated predominant bacteria throughout the GIT

[4] age effect on richness of ingesta-associated predominant bacteria throughout the GIT

[5] gut region effect on richness of predominant bacteria throughout the GIT

[a,b] means with same superscript within the same column are not significantly different at $P < 0.05$

Table 2.2 Relative abundance of predominant species common to mucosal tissue and ingesta throughout the GIT of dairy calves

Gut region	**3 week old calves**	**6 month old calves**	***P*-value** [1]
Rumen	0.51±0.02 [a]	0.39±0.03	0.05
Jejunum	0.33±0.07 [b]	0.28±0.06	0.48
Ileum	0.36±0.04 [b]	0.26±0.05	0.12
Cecum	0.48±0.05 [a]	0.33±0.02	0.02
Colon	0.47±0.06 [a]	0.33±0.02	0.02
P-value [2]	0.05	0.24	

[1] age effect on relative abundance of predominant species common to mucosal tissue and ingesta throughout the GIT of dairy calves

[2] gut region effect on relative abundance of predominant species common to mucosal tissue and ingesta throughout the GIT of dairy calves

[a,b] means with same superscript within the same column are not significantly different at $P < 0.05$

Table 2.3 Regional dependent or independent distribution of PCR-DGGE bands associated with mucosal tissue in the GIT of dairy calves

Band	Age	Region[1]	Mucosal tissue (%)	Ingesta (%)	*P*- Value
12	3 week	Independent[1]	55	24	<0.01
		Jejunum[2]	75	0	<0.01
20	3 week	Independent	68	34	<0.01
		Rumen	87.5	25	0.02
25	3 week	Independent	55	26	0.01
		Cecum	62.5	0	<0.01
33	3 week	Independent	70	42	0.01
44	3 week	Independent	70	18	<0.01
		Jejunum	50	0	0.03
		Ileum	75	14	0.03
		Cecum	87.5	0	<0.01
47	3 week	Independent	65	29	<0.01
50	3 week	Independent	55	32	0.03
		Colon	75	12.5	0.02
60	3 week	Independent	45	24	0.02
		Ileum	50	0	0.03
5	6 month	Independent	48	23	0.02
		Ileum	62.5	0	<0.01
32	6 month	Independent	73	45	0.01
		Independent	65	43	0.04
42	6 month	Independent	70	40	<0.01
43	6 month	Independent	73	50	0.04
53	6 month	Independent	73	50	0.04

[1] Mucosa specific bands distribution gut region independent

[2] Mucosa specific bands distribution within particular gut region

Table 2.4 Comparison of structure diversity of ileum mucosal and ingesta 16S gene libraries[a]

Library	No.of Sequences	No.of OTUs	Shannon index	Diversity[b]	Richness[c]	Coverage (%)[d]	*P*-value
Mucosa	314	145	3.7	0.03	189	85	<0.01
Ingesta	330	69	2.5	0.12	128	67.5	

[a]Estimates of Shannon index, diversity and richness are all based on 3% differences in nucleic acid sequence alignments.

[b]Sample size-independent estimate of diversity based on natural log transformation of Simpson index values as calculated by Mothur program.

[c]Chao 1 values, a nonparametric estimate of species richness.

[d]Coverage values for a distance of 0.01, as calculated by Mothur program.

Table 2.5 Total bacterial density throughout the GIT of dairy calves*

	3 week old calves			6 month old calves		
	Mucosal tissue	Ingesta	*P*- value[1]	Mucosal tissue	Ingesta	*P*-value[2]
Rumen	$(4.23\pm3.16)\times10^{10}$	$(6.81\pm3.48)\times10^{10}$	0.52	$(4.39\pm1.13)\times10^{9\,a}$	$(3.73\pm0.98)\times10^{10\,a}$	<0.01
Jejunum	$(2.65\pm1.62)\times10^{9}$	$(9.34\pm5.61)\times10^{9}$	0.24	$(1.21\pm0.30)\times10^{9\,b}$	$(8.86\pm3.63)\times10^{9\,b}$	0.04
Ileum	$(1.91\pm0.52)\times10^{9}$	$(7.60\pm3.23)\times10^{9}$	0.37	$(1.83\pm0.49)\times10^{9\,b}$	$(8.25\pm2.58)\times10^{9\,b}$	0.01
Cecum	$(3.17\pm0.87)\times10^{9}$	$(6.23\pm1.99)\times10^{10}$	0.01	$(2.84\pm0.92)\times10^{9\,b}$	$(4.23\pm1.35)\times10^{10\,a}$	<0.01
Colon	$(2.43\pm1.51)\times10^{10}$	$(1.22\pm0.59)\times10^{11}$	0.13	$(2.14\pm0.59)\times10^{9\,b}$	$(4.46\pm1.38)\times10^{10\,a}$	<0.01
P –value[3]	0.32	0.12		0.05	0.03	

* Copy number of 16S rRNA gene (copy/g)

[a,b]means with same superscript within same column are not significantly different at $P < 0.05$

[1]environmental effect on bacterial density throughout the GIT of 3 week old calves

[2] environmental effect on bacterial density throughout the GIT of 6 month old calves

[3] regional effect on bacterial density throughout the GIT of dairy calves

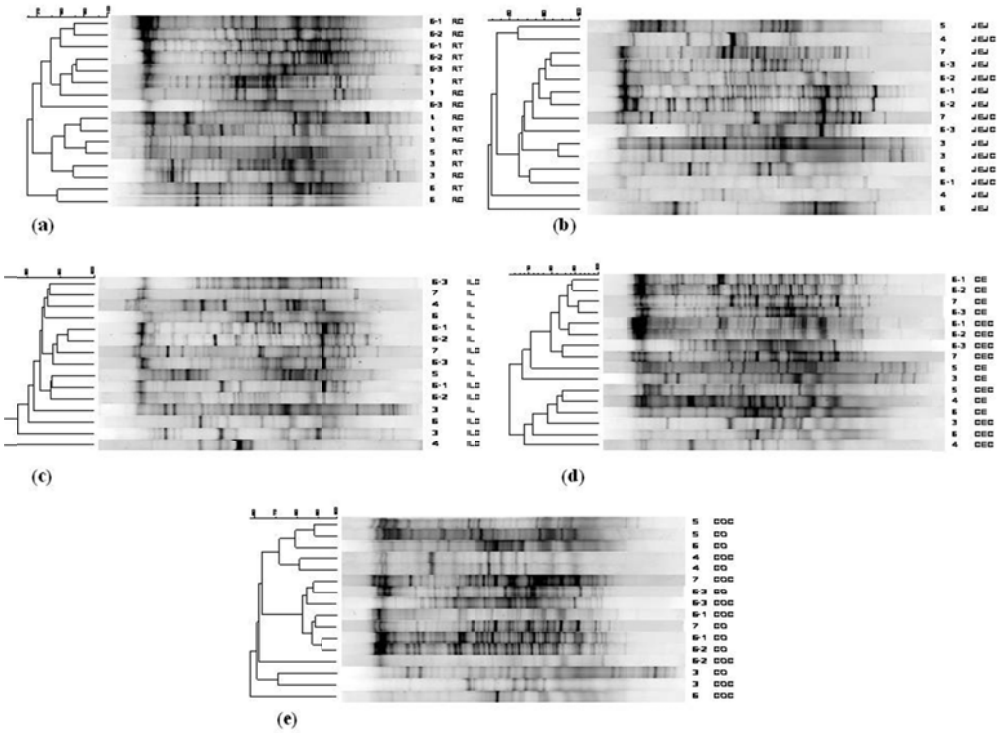

Figure 2.1 (A) PCR-DGGE profiles generated from tissue and ingesta DNA from 3 week old calves (30-55% DGGE). (a) rumen (b) jejunum (c) ileum (d) cecum (e) colon

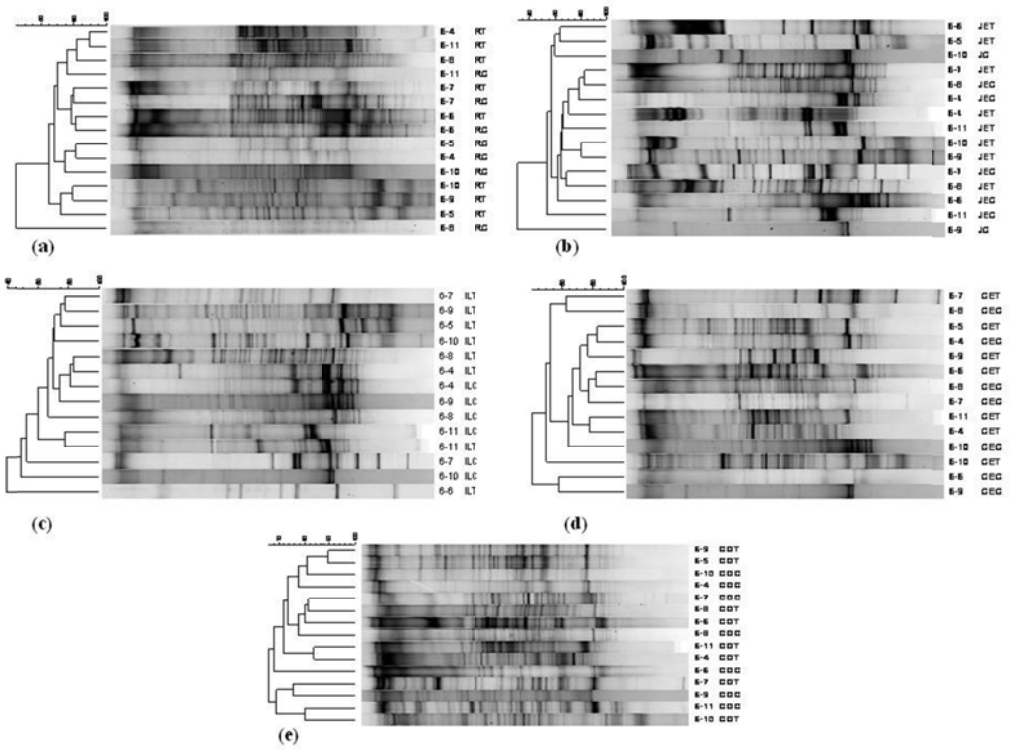

Figure 2.1 (B) PCR-DGGE profiles generated from tissue and ingesta DNA from 6 month old calves (30-55% DGGE). (a) rumen (b) jejunum (c) ileum (d) cecum (e) colon

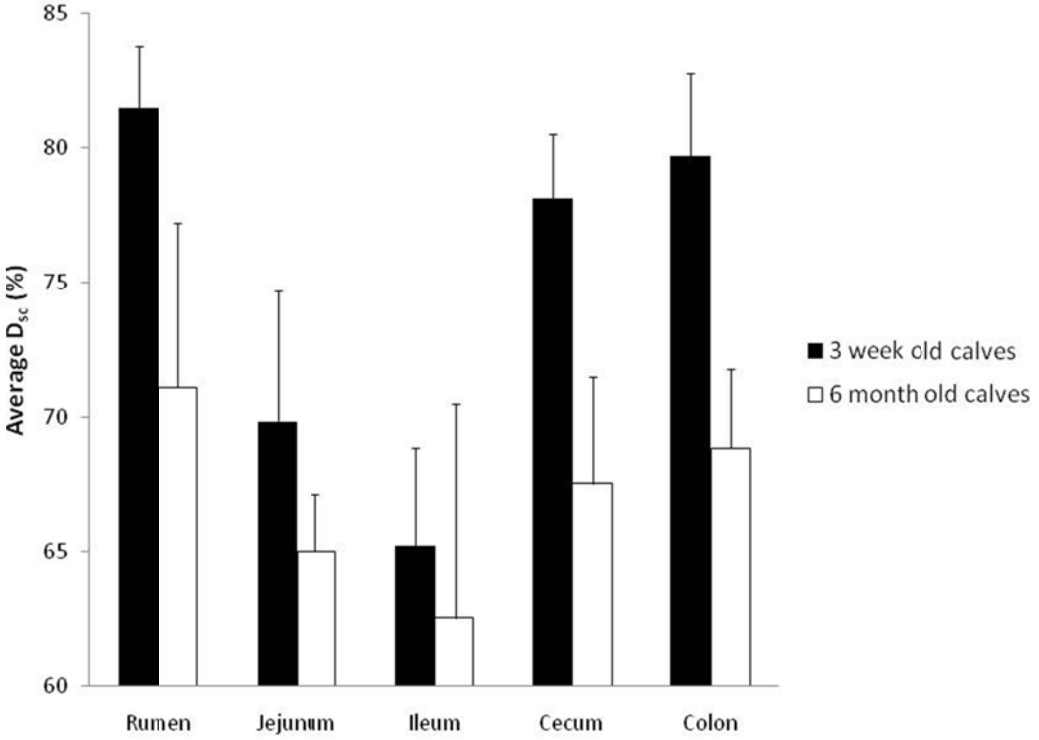

Figure 2.2 Comparison of mucosal tissue versus ingesta bacterial PCR-DGGE profiles from each gut region of 3 week and 6 month calves

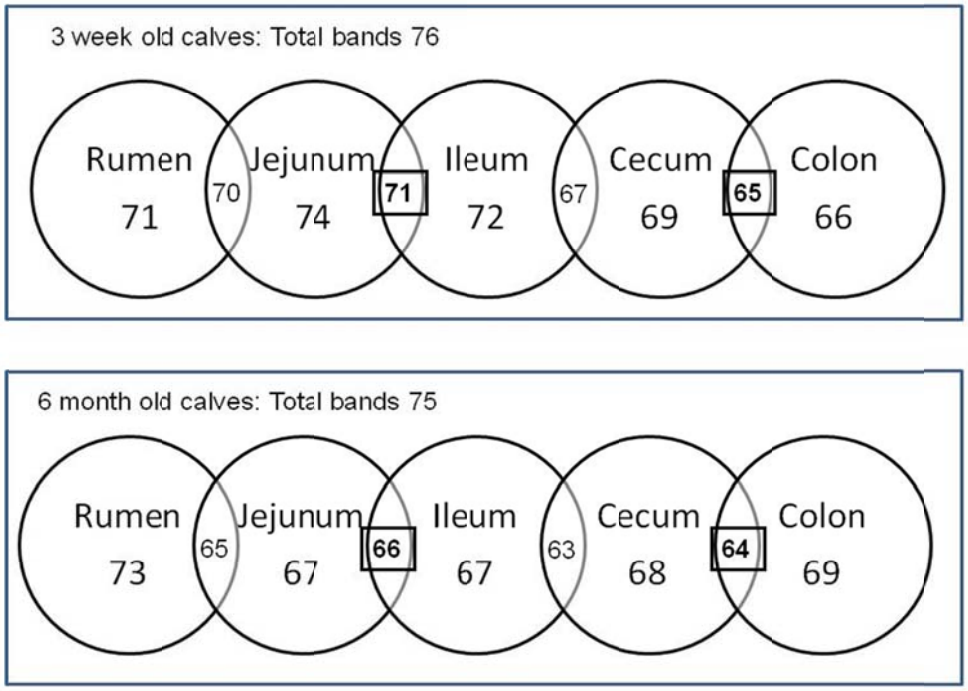

Figure 2.3 (A) Detected PCR-DGGE bands distribution throughout the GIT including rumen of 3 week (n=8) and 6 month (n=8) old calves

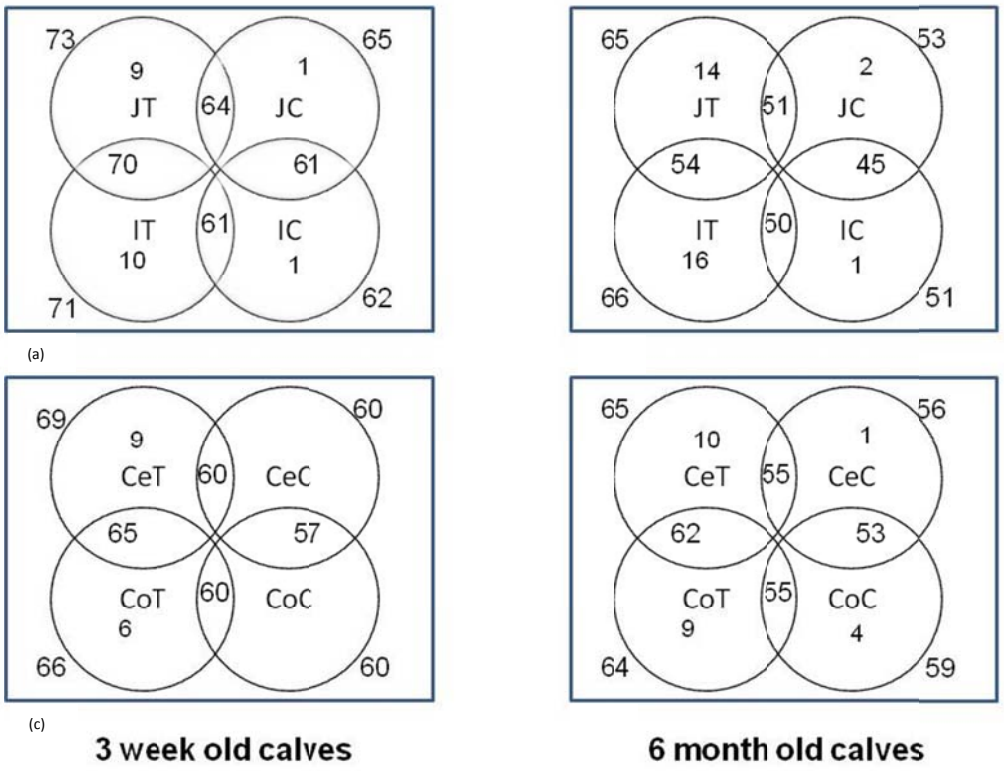

Figure 2.3 (B) Distribution of detected PCR-DGGE bands between mucosal tissue and ingesta through GIT of dairy calves (a) jejunum and ileum of 3 week old calves (b) jejunum and ileum of 6 month old calves (c) cecum and colon of 3 week old calves (d) cecum and colon of 6 month old calves.

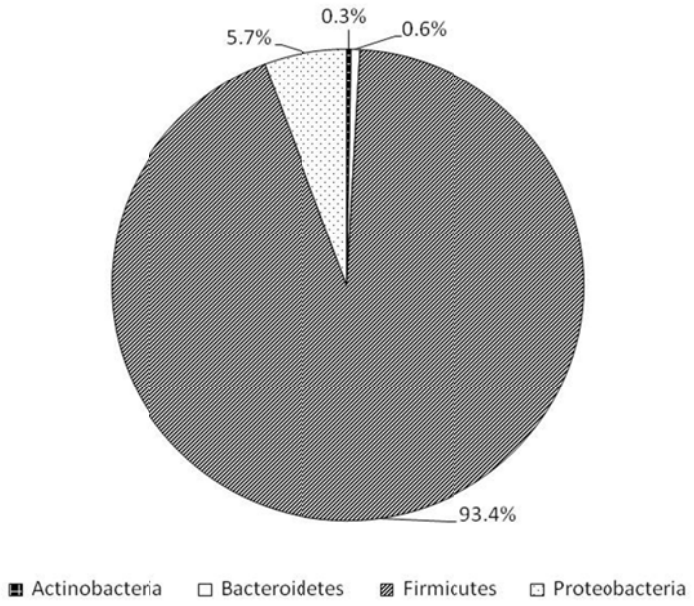

Figure 2.4 (A) Ileum ingesta-associated bacterial community of 3 week old dairy calves at phylum level based on obtained OTUs based on 97% similarity

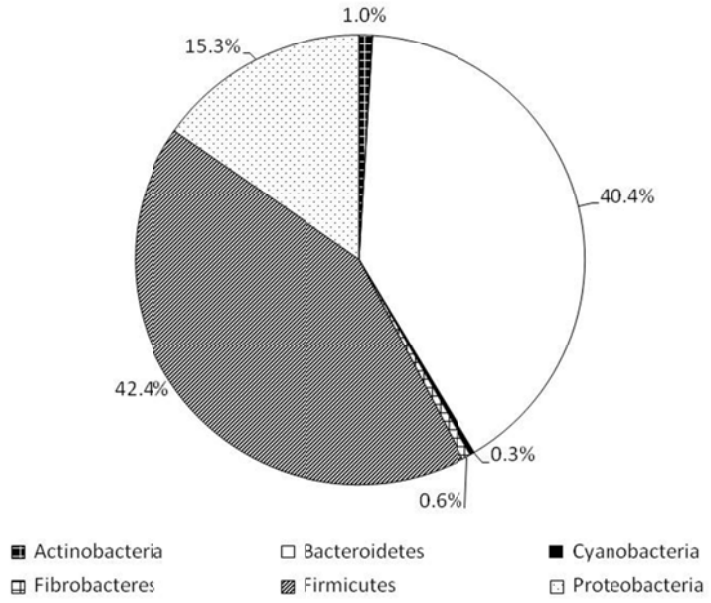

Figure 2.4 (B) Ileal mucosa-associated bacterial community of 3 week old dairy calves at phylum level based on obtained OTUs based on 97% similarity

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Chapter 3. Regional and age dependent changes in expression of Toll-like receptors and key antimicrobial defence molecules through gastro intestinal tract of dairy calves[1]

3.0 Introduction

The gastro-intestinal tract (GIT) harbours a diverse and abundant bacterial population and the intestinal epithelium is continuously exposed to commensal microbiota, pathogens and dietary antigens. The mucosal epithelium provides a critical barrier between the host and the gut environment and it has become increasingly apparent that epithelial cells play a key role in recognizing the GIT microbiome. Toll-like receptors (TLRs); a group of genetically encoded pattern recognition receptors (PRRs), peptidoglycan recognition proteins (*PGLYRP*1), and antimicrobial peptides (β-defensin) have been reported to interact with microbes to maintain intestinal homeostasis (Hooper, 2004, Rakoff-Nahoum et al., 2004, Fjell et al., 2008, Seabury et al., 2010). Toll-like receptors are present on a wide range of cells and can detect conserved molecular products of microorganisms (Rakoff-Nahoum et al., 2004, Abreu et al., 2005, Kelly and Conway, 2005, Abreu, 2010, Van et al., 2011). Currently, a total of 13 mammalian TLRs have been identified and human cells express TLRs1-10, whereas mice cells express TLRs1-9 and 11-13 (Sandor and Buc, 2005, Chang, 2010). Specific TLRs are expressed either on the cell surface (TLRs1, 2, 4-6, 10) or intracellular compartments (TLRs3, 7-9) (Chang, 2010). Previous

[1] A version of this chapter has been submitted for publication. Veterinary Immunology and Immunopathology.

investigations have elucidated that TLRs1, 2, 4-6, and 10 recognize bacterial surface-associated molecular patterns and TLRs3, 7-9 recognize nucleic acids from viruses and bacteria (Chang, 2010, Guan et al., 2010).

Recently, it has been reported that TLRs have the ability to discriminate between pathogens and non-pathogenic commensal microbiota (Kelly and Conway, 2005). From a host perspective it is important to avoid inflammatory responses to commensal microflora yet recognize pathogens. Rakoff-Nahoum and colleagues (2004) reported that recognition of commensal flora by TLRs is essential to maintain intestinal homeostasis. Reduced intestinal epithelial cells proliferation in germ-free mice, MyD88 (adaptor protein essential for signal transduction of TLRs) and TLR4 deficient mice suggest that interactions between commensal microflora and TLRs are necessary for maintenance of healthy intestinal epithelium in host (Rakoff-Nahoum et al., 2004, Guarner, 2006, Abreu, 2010). Moreover, it was reported that expression of TLR2 and 4 in proximal and distal colon of germ free mice was significantly lower than conventional mice and transplantation of microbiota from specific pathogen free mice was able to increase expression of these TLRs (Wang et al., 2010). These findings suggest that interactions between commensal microbiota and TLRs are necessary to maintain the mucosal epithelial barrier and innate immune responses. Recently identified TLR10 expression in the B cell lineage, Th1 cell activation by TLR2, and TLRs1, 2, 4 and 9 expression in CD4+ and CD8+ T cells suggest that TLRs also regulate host adaptive immune responses (Chang, 2008, Guan et al., 2010). Therefore, knowledge on the basal expression level of TLRs which regulate both

innate and adaptive immune responses, is very important for understanding immune competence and how this may change with the lifespan of the animals. In addition, host defence peptides or antimicrobial peptides (AMPs) and peptidoglycan recognition proteins are also a part of the innate immune system that possess microbicidal effects and were recently reported to present in bovine genome (Tydell et al., 2006, Fjell et al., 2008).

Knowledge regarding the expression of TLRs, β-defensin, and peptidoglycan recognition protein 1 (*PGLYRP*1) throughout the GIT of cattle is very limited. Our recent studies revealed significant age-related changes in the number and distribution of mucosal leukocyte populations (Fries et al., 2011) and distinct segregation of commensal bacterial populations among GIT regions when comparing 3 week and 6 month calves (Malmuthuge et al., unpublished data). These observations suggest that either the mucosal immune system may influence microbiome changes or alternatively the microbiome influences mucosal immune development. Therefore, this study analyzed the expression of 10 bovine TLRs, β-defensin and *PGLYRP*1 throughout the GIT of 3 week and 6 month calves, their association with total bacterial population and lactic acid bacterial population, and investigated whether the regional expression patterns of these genes change with increasing age.

3.1 Materials and methods

3.1.1 Animal experiment

Castrated male Holstein calves (1 week old) were purchased from a commercial dairy farm and housed at the Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan. All experimental protocols were reviewed and approved by the University Committee on Animal Care (University of Saskatchewan) and all procedures were performed following guidelines approved by the Canadian Council on Animal Care. Calves were fed with fresh, non-pasteurized whole milk and Blue Medollian calf supplement for the first 12 weeks and then fed with alfalfa hay and 1 kg oats/day, supplemented with trace minerals for the next 4 months.

3.1.2 Sampling of GIT Tissue

Mucosal tissue samples from rumen, jejunum, ileum, cecum and colon were collected from 3 week old (n = 8) and 6 month old (n = 8) calves, following humane euthanization with an intravenous injection of Euthanyl® (240mg/ml; Bimeda-MTC Animal Health Inc., Cambridge, ON). Mucosal tissues from rumen, jejunum, ileum, cecum and colon were rinsed three times with phosphate buffered saline (PBS) to remove ingesta, cut into 4-5 mm^2 fragments, and immersed in 5X the volume of RNAlater (Applied Biosystems, Foster City, CA, USA) and stored at -80°C. Ingesta samples were collected from each mucosal tissue collection site and 200 mg of ingesta was mixed in 1 ml RNAlater and stored at -80°C.

3.1.3 RNA extraction

Total RNA was extracted from tissue samples using a physical disruption method. Briefly, samples were transferred into CK-14 Precellys® lysine kits and homogenised with added TRIzol (Invitrogen, CA, USA) using Precellys® 24 homogenizer (2 cycles at 5500 rpm for 30 s; 10 s pause between cycles) (Bertin Technologies, Montigny, France). After incubation with chloroform, isopropanol and high salt solution (1.2 M NaAc, 0.8 M NaCl), RNA was precipitated using cold ethanol and dissolved in 200 µl of nuclease free water. The quantity and quality of RNA was measured using a ND1000 spectrophotometer (NanoDrop Technologies, Wilmington) and extracted RNA was stored at -80°C.

3.1.4 Expression of 10 bovine TLRs, β-defensin and peptidoglycan recognition protein 1

qRT-PCR was performed to analyse the expression of 10 TLRs, β-defensin and *PGLYRP*1 relative to β-actin using gene specific primers (Table 3.1) with the StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the fast cycle and the following program: 95°C for 15 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. All PCR products were subjected to sequencing analysis to confirm their specific target to each gene and a melting curve was generated to ensure only the desired product was being amplified. Amplified RNA (aRNA) was generated from the total RNA extracted from each tissue sample using Ambion Allyl Message Amp II a RNA kit (#1753). Briefly, mRNA was purified from the extracted total RNA and synthesised first

and second strand cDNA. Then, purified cDNA was eluted using nuclease-free water and used to synthesis aRNA by *in-vitro* transcription. One µl of 50 ng/µl aRNA was used as the template for qRT-PCR reaction. Gene expression (ΔC_T value) was calculated based on threshold cycle (C_T) of endogenous gene (β- actin) and target gene. ($\Delta C_T = C_{T\ target\ gene} - C_{T\ endogenous\ gene}$). Up- or down-regulation of TLRs in 6 month old calves ($\Delta\Delta C_T$ value) were calculated in relative to 3 week old calves to analyze age related changes in gene expression and these values were expressed as $\Delta\Delta C_T$ which was calculated as $\Delta C_{T3week} - \Delta C_{T6month}$.

3.1.5 Analysis of total bacteria and lactic acid bacteria (LAB) population in GIT of dairy calves

Total DNA from matched ingesta and mucosal tissue samples were extracted using the bead beating method described Walter et al., (2000). One µl of 50 ng/µl DNA was used as template and qRT-PCR was performed using SYBR Green chemistry (Fast SYBR® Green Master Mix, Applied Biosystems) with StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Copy number of total bacterial population was estimated using U2 primers (U2F, 5'-ACTCCTACGGGAGGCAG-3'; U2R, 5'- GACTACCAGGGTATCTAATC C-3') (Stevenson and Weimer, 2007) using fast cycle method with following program; 95°C for 10 min, followed by 40 cycles of 95°C for 20 s and 62°C for 1 min. A standard curve was constructed using serial dilutions of plasmid DNA containing 16S rRNA insertion of *Butyrivibrio hungatei*.

LAB population was estimated using universal LAB primers Lac1 (5'-AGCAGTAGGGAATCTTCCA-3') and Lac2 (5'-ATTTCACCGCTACACATG -3') (Walter et al., 2001) with the following program: 95°C for 10 min, followed by 40 cycles of 95°C for 20 s and 62°C for 45 s. A standard curve was constructed using serial dilutions from genomic DNA of *Lactobacillis acidophilus*. Copy numbers for each standard curve was calculated based on the following equation: (NL x A x 10^9)/ (660 x n), where NL was the Avogadro constant (6.02 x 10^{23}), A was the molecular weight of DNA molecules, and n was the length of amplicon (bp). The copy number of 16S rRNA genes for total bacteria and LAB per gram of sample were calculated using the following equation: (QM x C x DV)/ (S x W), where QM was the quantitative mean of the copy number, C was the DNA concentration of each sample, DV was the dilution volume of extracted DNA, S was the DNA amount subjected to analysis and W was the sample weight subjected to DNA extraction (Li et al., 2009).

3.1.6 Statistical Analysis

Gene expression data (ΔC_T) and age-related changes in gene expression ($\Delta\Delta C_T$) were analysed using MIXED procedure in SAS (version 9.2; SAS Institute Inc., Cary, NC). The statistical model included GIT location and age as fixed effects. Differences in least square means among locations were declared at $P < 0.05$ using the PDIFF option in SAS. Correlation between bacterial population and TLRs expression was analysed using CORR procedure in SAS.

3.2 Results

3.2.1 TLRs expression throughout the GIT of dairy calves

In this study, expression of bovine TLRs was measured at mRNA level using qRT- PCR in relative to β-actin expression level. β-actin was selected as the reference gene in the current study as it showed the minimum expression variation among all samples tested (C_T value = 22.5 ± 0.14, mean ± SE). Expression of the 10 bovine TLRs was detected throughout the GIT, including the rumen of 3 week old calves. Expression level of most TLRs was low in the rumen with significantly lower expression of TLRs1, 6, 8-10 in 3 week old calves when compared to other GIT regions (Table 3.2). Moreover, several TLRs were expressed at the highest level in the ileum followed by the jejunum. In particular, TLR9 and 10 expression was significantly ($P < 0.01$) higher in the ileum than all other GIT regions of 3 week old calves (Table 3.2, Figure 3.1).

Expression of all the bovine TLRs was also detected throughout the GIT of 6 month old calves with significantly lower expression of all TLRs in the rumen than that of other gut regions, except TLR5 and 9 (Table 3.3). TLR10 expression was highest in the ileum of 6 months old calves ($P < 0.01$) and TLR2 and 4 were significantly higher in cecum and colon than the other GIT regions ($P < 0.01$) (Figure 3.2).

A comparison of TLR expression throughout the GIT of 3 week versus 6 month old calves revealed that most TLRs, with the exception of TLR1 and 3 were significantly down-regulated with increasing age (Figure 3.3). Expression of all TLRs was significantly down-regulated in the rumen of 6 month old calves

when compare to that of 3 week old calves (Figure 3.4), except that TLR4 expression was significantly up-regulated in the cecum of 6 week calves.

3.2.2 β-defensin and *PGLYRP*1 expression

β-defensin and *PGLYRP*1 expression was not detectable throughout the GIT of 3 week old calves (C_T value – undetermined). However, both genes were expressed at detectable levels in all regions throughout the GIT of 6 month old calves (Figure 3.5). While β-defensin expression was not significantly different among the GIT locations, level of *PGLYRP*1 expression was significantly lower in the rumen than the other GIT locations analyzed ($P < 0.02$). However, the expression level of *PGLYRP*1 was relatively low throughout the GIT compared to that of β-defensin.

3.2.3 Associations between mucosa-associated and ingesta total bacteria, lactic acid bacteria (LAB) populations and TLR expression

Correlation analysis between the total bacterial population in the ingesta and expression of TLRs involved in bacterial ligand recognition revealed a negative correlation for TLR1, 2 and 9 in colon, jejunum and rumen, respectively, while TLR6 revealed a negative correlation in ileum and cecum of 3 week old calves (Table 3.4). In contrast, there was a positive correlation between TLR4 expression and the total bacterial population in the rumen of 3 week old calves (Table 3.4). The same analysis for the total mucosa-associated bacterial

population revealed a negative correlation with TLR2, 6 and 9 expression in jejunum, cecum, and rumen, respectively (Table 3.4).

In 6 month old calves, the ingesta bacterial population showed a positive correlation with TLR6 and 10 expression in colon and ileum, respectively whereas the mucosa-associated population showed a negative correlation with TLRs1 and 6 in colon and jejunum, respectively (Tables 3.4). Moreover, TLRs5 and 9 displayed a negative correlation with rumen mucosa-associated bacterial density (Table 3.4). However, TLR6 and 10 expression was positively correlated with the mucosal bacterial population in the ileum of 6 month old calves (Table 3.4). A consistent correlation pattern between TLR expression and either total ingesta or mucosa-associated bacterial population was not observed with restricted regional associations that changed significantly with age.

Interactions between the innate mucosal immune system and the commensal microbiome are very complex. Therefore, we investigated whether a significant correlation may exist between a specific bacterial group and targeted TLRs expression. Analysis of lactic acid bacteria (LAB) population and expression of TLRs revealed that TLR6 was negatively correlated with the LAB population in the cecum ingesta and TLR2 was negatively correlated with jejunal mucosa-associated LAB population of 3 week old calves (Tables 3.5). TLR1 and 6 expression was negatively correlated to the mucosal-associated LAB populations in colon and jejunum, respectively, whereas TLR6 expression was positively correlated with the mucosal-associated LAB population in the ileum (Table 3.5).

3.4 Discussion

There is increasing evidence that the recognition of commensal microbiota plays an important role in the development of mucosal epithelial barrier and mucosal immune system. Understanding interactions between the host and the commensal microbiome is critical for a complete understanding of both health and disease. Our study is the first to report the expression of 10 bovine TLRs, β-defensin and *PGLYRP*1 expression throughout the GIT of dairy calves and analyze age-related expression changes under normal physiological conditions. The GIT samples collected in this study represent both mucosal epithelium cells as well as other mucosal-associated immune cells (Fries et al., 2011) which also express TLRs. Therefore, unlike research conducted using either only intestinal epithelium or one particular leukocyte population (Cashman and Morgan, 2009, Abreu, 2010) our analysis provides an overall value for TLR expression levels throughout the GIT of dairy calves. The present study revealed significant regional differences in the expression level of TLRs along the GIT. At a tissue level it is difficult to interpret these results since it may represent differences in the abundance of specific cell types with restricted TLRs expression patterns such as M cells (Menzies and Ingham, 2006) or regional differences in TLR expression within the same cell type, such as mucosal epithelium such as epithelial cells (Abreu, 2010). In our previous study we reported that bacterial diversity also varied throughout the GIT as well as by sample type (mucosal tissue/ingesta) (Malmuthuge et al., unpublished data). Therefore, the variations in bacterial

population along the GIT may represent variations in bacterial ligands which could modulate regional TLRs expression.

Significantly increased TLR10 expression in the ileum, irrespective of animal age, suggests that this TLR plays a unique role in the ileum which may be vital for the host immune system in dairy calves. TLR10 is reported to be highly express on B cells when compared to other TLRs (Hornung et al., 2002). Cattle ileal Peyer's patches (PP) function as a primary site for B cell proliferation, and B cells constitute over 97% of the cells in PP lymphoid follicles (Griebel et al., 1996a, Griebel et al., 1996b, Yasuda et al., 2006). Ileal PP B cell development begins during foetal development (Guan et al., 2010) and it is therefore, not surprising to observe high TLR10 expression in ileum of 3 week old dairy calves since the ileum tissue used for this study did contain PP (Fries et al., 2011). Furthermore, B cell development in ileal PP continues throughout the first year of life, and thus, this may be the reason for higher expression of TLR10 in 6 month old calves ileal tissue than other gut region. Further studies on TLR10 expression in ileal tissue with and without PP may explain the observed higher expression in bovine ileum and its role in B cell development. Taylor & colleagues (2008) reported that TLRs9 and 10 were not detected in jejunal and ileal tissues of *Mycobacterium paratuberculosis* infected, exposed or non-exposed sheep, while TLRs1-8 expressed abundantly with an up-regulation of TLRs2-5 and 8 in infected and exposed sheep. In contrast, Menzies and Ingham (2006) reported low levels of TLR9 and 10 expression in sheep jejunal tissue with an abundant expression of TLR10 in jejunal PP. In the present study we observed both TLR9

and 10 expression in bovine jejunal and ileal tissue with significantly higher expression in ileum. These observations suggest that the level of TLR9 and 10 expression is influenced by the site of tissue sampling, the inclusion of PP in the sample, and also the host species being analyzed.

Interestingly, an age-related down-regulation of the expression level for most TLRs was a common phenomenon throughout the GIT of dairy calves. In-vitro studies have shown that prolonged exposure to lipopolysaccharide (LPS) and lipoteichoicacid (LTA) results in hyporesponsiveness in intestinal epithelium cells by down regulating surface TLRs expression (Abreu et al., 2001, Otte et al., 2004). Continuous exposure to large amount of microbial ligands derived from commensal flora, even in the absence of pathogens might down-regulate TLR expression on the mucosal epithelium and provide a mechanism to minimize unnecessary inflammatory responses. In young mammals, weaning coincides with a drastic change in the enteric microflora as well as morphological and functional changes in GIT (Hooper, 2004). Therefore, the observed down regulation in TLRs expression in 6 week calves may represent a normal developmental program that occurs during the establishment of the adult immune system. TLR5 and 6 expression was reported to elevate significantly following the removal of ingesta and commensal microflora from the ileum of 3 week old calves signifying that presence of commensal microflora may play role in regulating their expression (Charavaryamath et al., 2011).

Commensal bacterial density is an indirect measurement for bacterial ligand concentration in the GIT. Therefore, we analyzed the correlation between

total bacterial density in ingesta and the mucosa-associated communities with expression of TLR1, 2, 4-6, 9 and 10 which recognize bacterial products. Although, a consistent pattern did not emerge in terms of either TLR or GIT region, the observed negative correlations were consistent with the expected development of TLR hyporesponsiveness with increased age (Nomura et al., 2000, Abreu et al., 2001, Otte et al., 2004). The bacterial population density in ingesta and the mucosa-associated community also revealed distinct correlation patterns with TLRs expression. Previous studies conducted with matched samples revealed that the ileal ingesta and mucosa-associated bacterial communities are significantly different in 3 week old calves (Malmuthuge et al., unpublished data). These observations support the conclusion that distinct bacterial ligands may play an important role in modulating TLR expression and innate mucosal immune responses. Recent study has shown that the *Lactobocillus plantarum* can stimulate the TLR2 expression *in vivo* and *in vitro* which may regulate the gut epithelium tight junctions (Karczewski et al., 2010). Earlier, we reported that 3 week old calf ileum was dominated by *Lactobacillus* spp. (Malmuthuge et al., unpublished data). Higher proportion of *Lactobacillus* spp. during early life may crucial for the epithelial tight junctions as well as to intestinal homeostasis in dairy calves. The different associations observed between TLR expression and digesta or mucosa associated with LAB density suggest that the regulation of expression of TLRs may be regional dependent as well as determined by their populations in either mucosa surface or the ingesta. Since TLRs expression is affected by various factors in the GIT and therefore, defined interactions between LAB and TLRs

expression in this study is very limited and may not be witnessed clearly *in vivo*. However, the observed difference in the association of TLRs expression and ingesta LAB density between 3 week and 6 month old claves, suggests that LAB may play an important role in the host immunity development in early life of dairy calves.

We also observed significantly lower TLR expression in the rumen when compared to the other GIT locations. As noted previously, this may reflect either the relative paucity of mucosal-associated leukocytes in the lamina propria of the rumen or the lack of TLR expression in the stratified squamous epithelium of the rumen. These differences, however, confirm the importance of the GIT as a component in host immune system. Rumen epithelium and ingesta harbour a very high density bacterial population in adult ruminants which facilitates digestion and provides a rich source of nutrients. Thus, it may be necessary to minimize unnecessary responses to these symbiotic populations. The negative correlation between TLR5 and 6 expression and rumen epithelial-associated bacterial population in 6 month old calves supports the speculation that down-regulation of TLR expression may be an important mechanism to prevent unnecessary inflammatory responses to microbes.

Defensins are a family of antimicrobial peptides capable of killing gram-negative bacteria by disrupting membranes and these antimicrobial peptides are produced by neutrophils, Paneth cells and epithelial cells (Ganz, 2002, Fjell et al., 2008). Cattle are reported to express only β-defensin, and in this study, we observed that expression of β-defensin in the GIT was age-dependent, being

undetectable in 3 week old calves. Furthermore, β- defensin expression was independent of the GIT region since it was detected throughout the GIT of 6 month calves. Unlike TLRs, which can discriminate between pathogens and commensal microbes, β-defensin kills all phagocytised bacteria and thus, it may be advantageous for the host to express β-defensin throughout the GIT. Meyerholz and colleagues (2004) reported that sheep expressed β-defensin throughout the GIT tissue in foetuses, neonates and adults. Therefore, our observations suggest that there is a species-specific, developmental regulation of β-defensin expression in the bovine GIT.

PGLYRPs have been isolated from bovine blood granulocytes and also reported to have very low expression in M cells and epithelial cells (Dziarski and Gupta, 2006, Tydell et al., 2006). However, we observed a very low level of *PGLYRP*1 mRNA expression in rumen tissue as well as through GIT of 6 month dairy calves compared to that of β-defensin. *PGLYRP*1 is capable of recognizing ligands from gram positive bacteria and is involved in killing a wide variety of bacteria (Seabury et al., 2010). The restricted expression of both β-defensin and *PGLYRP*1 prior to weaning in calves once again confirmed that significant developmental changes occur in the mucosal immune system of cattle. Dairy calves may depend on TLRs as a primary innate immune mechanism to monitor commensal microflora and pathogens prior to weaning but with increasing age it appears that other innate immune effecter mechanisms may become more active in providing host defences and minimizing harmful inflammatory responses.

3.5 Conclusions

Our study revealed that bovine innate immune genes expression varies significantly throughout the GIT and with a significant age-dependent reduction in TLR expression levels and an age-dependent increase in β-defensin and *PGLYRP1* expression. We are the first to report the higher expression of TLR10 in the bovine ileum irrespective calf age, which might play a very important role in maintaining intestinal homeostasis through the interactions with commensal flora. Moreover, significant correlations were found between ingesta and mucosa-associated bacterial communities which suggest that specific bacterial molecules may play an important role in modulating the development of the innate mucosal immune system. Understanding the expression pattern of highly conserved bovine innate immune genes will contribute to our understanding of the interaction between the commensal microflora and the host immune system which is critical for optimizing animal health and host responses to pathogens.

Table 3.1 Primer sequences for bovine TLRs, β-defensin, *PGLYRP*1 and β-actin used for qRT-PCR

Gene	Forward primer	Reverse primer	Product Size[a]	Source
TLR1	5'ctgcccatatgccaagagtt3'	5'ggcatcttctctttccccat3'	159	1
TLR2	5'ctgtgtgcgtcttcctcaga3'	5'tcagggagcagagtaaccaga3'	228	1
TLR3	5'tcttttcgggactgttgacc3'	5'aaatcccccatccaaggtag3'	224	1
TLR4	5'ggtttccacaaaagccgtaa3'	5'aggacgatgaagatgatgcc3'	137	1
TLR5	5'tcaatgggagccagattttc3'	5'ccttcagctcctggagtgtc3'	198	1
TLR6	5'cgacattgaaggcactgaaa3'	5'tcctgaggacaaagcatgtg3'	148	1
TLR7	5'tctccaaggtgctttccagt3'	5'ccaccagacaaaccacacag3'	166	1
TLR8	5'tcacacgggtaacgaatgaa3'	5'tttgaggttgagaaatgccc3'	143	1
TLR9	5'ctctccttggactgctttgg3'	5'cactgcactctgcaccttgt3'	204	1
TLR10	5'tcacctgacatctttgcgag3'	5'tcggaatggatttcttcctg3'	187	1
β-defensin	5'ggtcacaagtggcagaggat3'	5'tggttgaagaacttcagggc3'	152	1
*PGLYRP*1	5'tccagccccggccctcatac3'	5'actgcggcagcatcgtgtcc3'	249	2
β-actin	5'ctaggcaccagggcgtaatg3'	5'ccacacggagctcgttgtag3'	177	1

[a] amplicon length (bp)

1 Charavaryamath et al., 2011

2 This study

Table 3.2 TLR expression throughout the GIT of 3 week old calves*

TLR	Rumen	Jejunum	Ileum	Cecum	Colon	*P*-value
TLR1	9.3±0.37[a]	8.3±0.46[a]	7.4±0.31[b]	8.4±0.44[a]	8.5±0.34[a]	0.02
TLR2	9.9±0.85	10.7±0.61	8.7±0.58	9.7±0.64	9.4±0.62	0.35
TLR3	9.1±0.31	8.5±0.34	8.7±0.22	8.6±0.36	8.6±0.41	0.72
TLR4	8.4±0.42	8.9±0.20	8.6±0.09	8.74±0.25	8.3±0.18	0.48
TLR5	9.1±0.18	8.8±0.28	9.2±0.22	9.5±0.11	9.3±0.12	0.21
TLR6	10.3±0.46[a]	9.1±0.40[b]	8.3±0.34[b]	8.6±0.68[b]	9.4±0.35[ab]	0.04
TLR7	7.1±0.24	6.2±0.38	6.3±0.31	6.6±0.34	6.2±0.19	0.22
TLR8	10.3±0.92[a]	6.5±0.56[b]	6.2±0.38[b]	7.3±0.72[b]	7.2±0.27[b]	<0.01
TLR9	10.2±0.49[a]	9.2±0.29[a]	7.7±0.41[b]	9.5±0.42[a]	9.8±0.23[a]	<0.01
TLR10	9.8±0.47[a]	8.2±0.52[a]	5.3±0.62[b]	8.3±0.86[a]	7.6±0.48[a]	<0.01

[a,b] Means with same superscript within the same row are not significantly different at $P < 0.05$

*TLRs expression data presented as $\Delta C_T \pm$ SE

Table 3.3 TLR expression throughout the GIT of 6 month old calves*

TLRs	Rumen	Jejunum	Ileum	Cecum	Colon	P value
TLR1	10.8±0.3[a]	8.5±0.3[b]	7.8±0.2[b]	8.1±0.4[b]	8.8±0.1[b]	<0.01
TLR2	13.8±0.5[a]	12.2±0.3[b]	12.1±0.1[b]	9.9±0.7[c]	10.7±0.2[c]	<0.01
TLR3	10.6±0.6[a]	8.0±0.6[b]	9.3±0.6[ab]	7.4±0.6[cb]	8.0±0.6[b]	<0.01
TLR4	8.3±0.5[a]	9.3±0.3[b]	9.2±0.2[b]	7.1±0.6[a]	7.9±0.1[a]	<0.01
TLR5	10.7±0.7	10.8±0.4	12.2±0.3	11.1±0.5	11.3±0.3	0.18
TLR6	13.2±0.5[a]	10.8±0.3[b]	10.4±0.4[b]	9.9±0.6[b]	10.8±0.4[b]	<0.01
TLR7	11.8±0.4[a]	7.1±1.0[b]	9.9±0.3[b]	8.5±0.7[b]	9.8±0.3[b]	<0.01
TLR8	14.8±0.7[a]	9.9±0.7[b]	9.9±0.3[b]	11.0±0.6[b]	11.0±0.3[b]	<0.01
TLR9	10.8±0.9	10.3±0.9	8.4±1.0	9.2±0.9	10.3±0.9	0.39
TLR10	14.2±0.5[a]	12.2±0.4[b]	8.9±0.6[c]	9.5±1.0[c]	11.8±0.4[b]	<0.01

[a,b] Means with same superscript within the same row are not significantly different at $P < 0.05$

*TLRs expression data presented as $\Delta C_T \pm$ SE

Table 3.4 Correlation between total bacterial population in mucosa and ingesta and TLR expression in 3 week and 6 month old calves

	Ingesta					**Mucosal tissue**				
	Rumen	Jejunum	Ileum	Cecum	Colon	Rumen	Jejunum	Ileum	Cecum	Colon
TLR1	NS	NS	NS	NS	-0.9^{**1}	NS	NS	NS	NS	-0.8^{*2}
TLR2	NS	-0.9^{**1}	NS	NS	NS	NS	-0.8^{*1}	NS	NS	NS
TLR4	0.8^{*1}	NS	NS	NS	NS	NS	NS	NS	NS	NS
TLR5	NS	NS	NS	NS	NS	0.8^{*2}	NS	NS	NS	NS
TLR6	NS	NS	-0.9^{**1}	-0.9^{**1}	0.8^{*2}	0.8^{*2}	-0.8^{**2}	0.8^{*2}	0.8^{*1}	NS
TLR 9	-0.9^{**1}	NS	NS	NS	NS	-0.7^{*1}	NS	-0.8^{*2}	NS	NS
TLR10	NS	NS	0.8^{*2}	NS	NS	NS	NS	0.8^{*2}	NS	NS

$^{**}P < 0.01$, $^{*}P < 0.05$, NS- not significant, ^{1} 3 week old calves, ^{2} 6 month old calves

Table 3.5 Correlation between LAB population in mucosa and ingesta and TLRs expression in 3 week and 6 month old calves

	Ingesta					**Mucosal tissue**				
	Rumen	Jejunum	Ileum	Cecum	Colon	Rumen	Jejunum	Ileum	Cecum	Colon
TLR1	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.9**[2]
TLR2	NS	NS	NS	NS	NS	NS	-0.8**[1]	NS	NS	NS
TLR6	NS	NS	NS	-0.8**[1]	NS	NS	-0.7*[2]	0.9**[2]	NS	NS
TLR10	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

**$P < 0.01$, * $P < 0.05$, NS-not significant, [1] 3 week old calves, [2] 6 month old calves

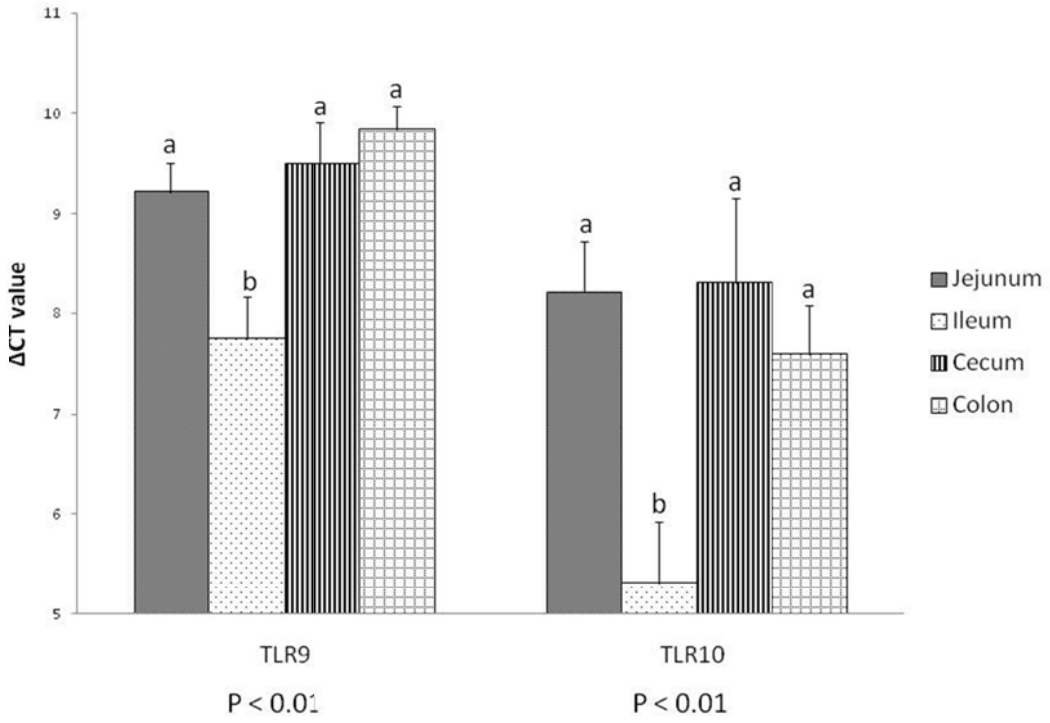

Figure 3.1 TLRs differentially expressed throughout the GIT of 3 week old calves. Bars with same letter in each TLR are not significantly difference at $P < 0.05$. $\Delta C_T = C_{T(TLRs)} - C_{T(\beta\text{-actin})}$, lower ΔC_T represent higher mRNA abundance level and higher ΔC_T represent lower mRNA abundance level.

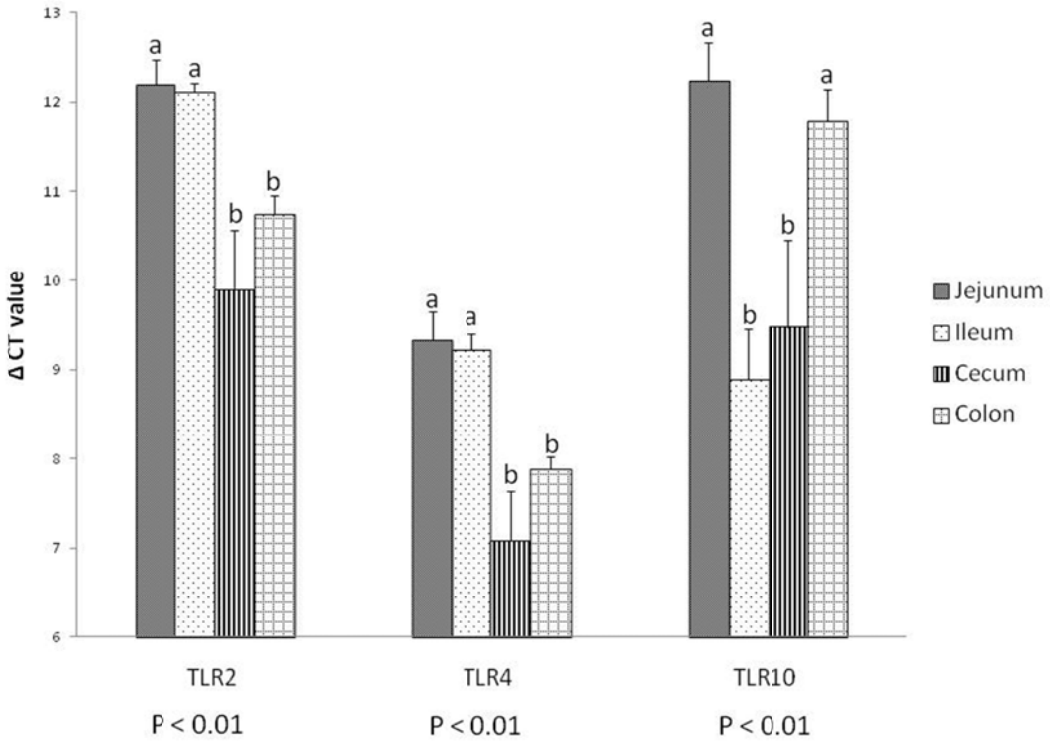

Figure 3.2 TLRs differentially expressed throughout the GIT of 6 month old calves. Bars with same letter in each TLR are not significantly difference at $P < 0.05$. $\Delta C_T = C_{T(TLRs)} - C_{T(\beta\text{-actin})}$, lower ΔC_T represent higher mRNA abundance level and higher ΔC_T represent lower mRNA abundance level.

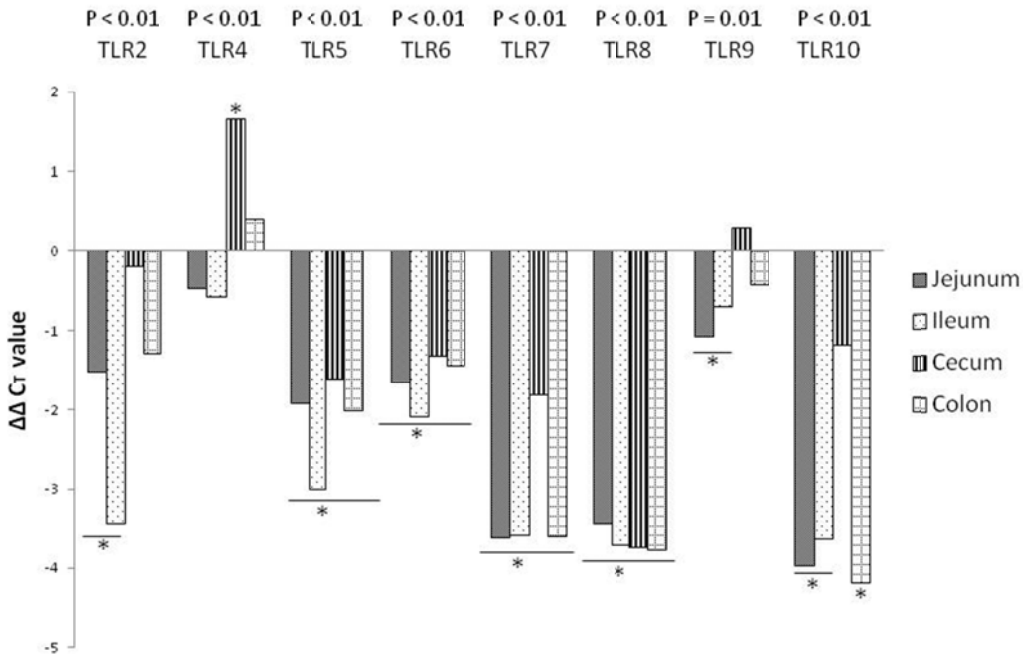

Figure 3.3 Age-dependent differential expression of TLRs throughout the GIT of dairy calves with increasing age. * and—— represents significantly up or down-regulated TLRs. $\Delta\Delta C_T = \Delta C_{T3week} - \Delta C_{T6month}$, negative $\Delta\Delta C_T$ value represents down-regulation in TLR expression and positive $\Delta\Delta C_T$ value represents up-regulation in TLR.

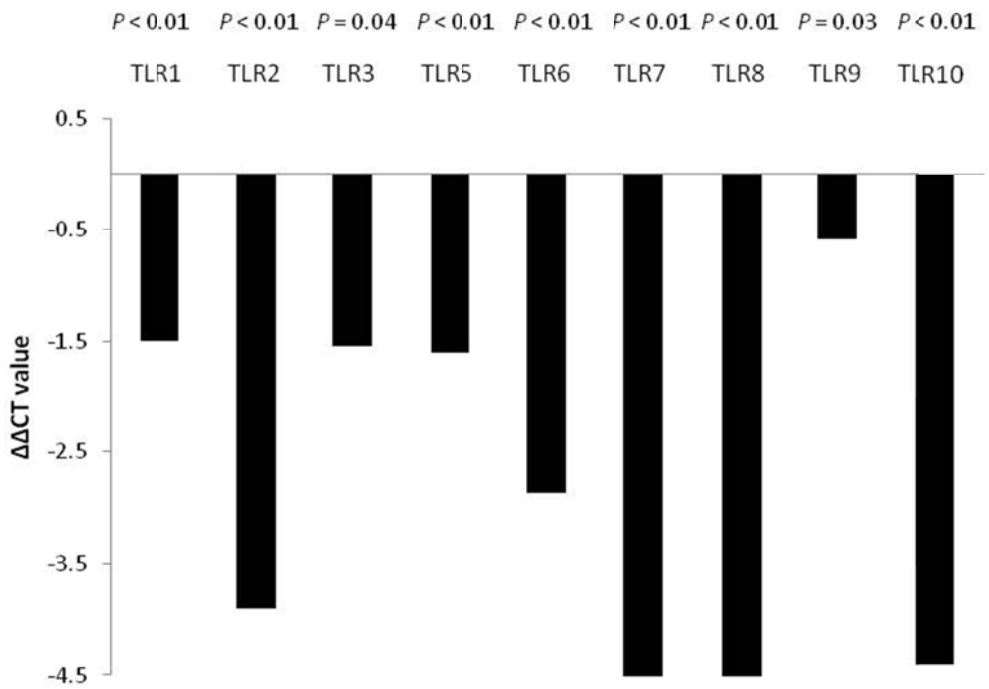

Figure 3.4 Age-dependent differential expression of TLRs in the rumen of dairy calves with increasing age. $\Delta\Delta C_T = \Delta C_{T3week} - \Delta C_{T6month}$, negative $\Delta\Delta C_T$ value represents down-regulation in TLR.

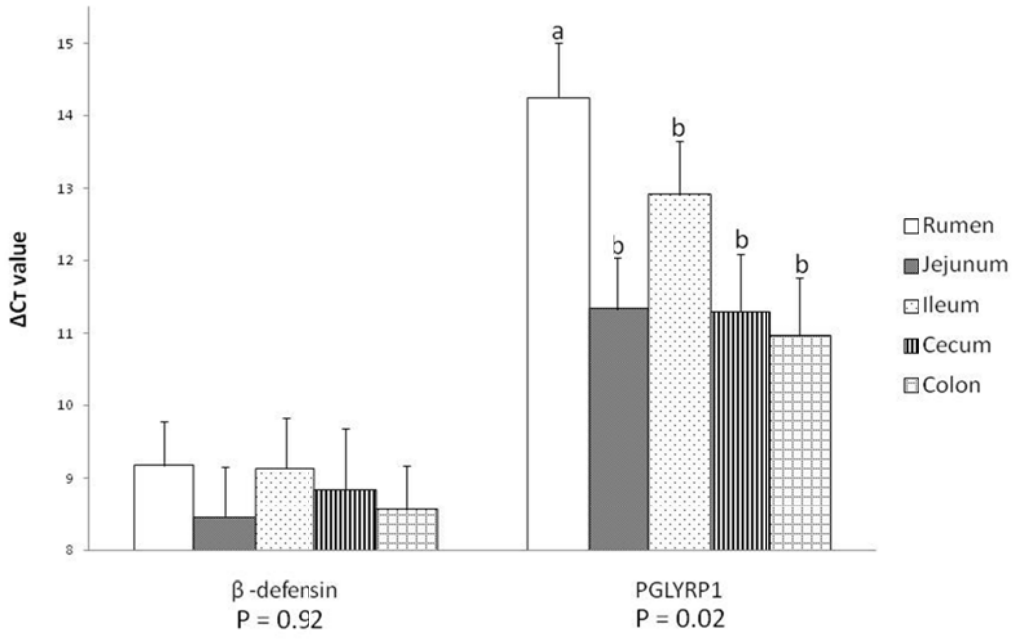

Figure 3.5 β- defensin and *PGLYRP*1 expression throughout the GIT of 6 month old calves. Bars with same letter are not significantly difference at $P < 0.05$. $\Delta C_T = C_{T(TLRs)} - C_{T(\beta\text{-actin})}$, lower ΔC_T represent higher mRNA abundance level and higher ΔC_T represent lower mRNA abundance level.

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Chapter 4. General Discussion

4.1 Significance of the study

Health management is one of the crucial aspects in livestock production. In the past few decades, improvements in beef and dairy cattle management were mainly focused on production traits and thus, animal health traits were left behind. In recent years, direct and indirect production losses due to increasing infectious diseases have been causing significant losses to the industry. Therefore, the aim is now to maintain productive and profitable, yet healthy animals. These new industrial goals emphasise the importance of multidisciplinary techniques that can be used to improve host disease resistance ability. Gnotobiological studies conducted using mice have been shown the importance of gut microbes in host immune system development, suggesting that alterations in the gut microbiome can be used to improve host health (Hooper, 2004; Guarner, 2006). However, little is known about gut microbial establishment and their interactions with the host in dairy cattle. Therefore as the first step, we investigated variations and correlations between two key factors; gut bacteria and mucosal innate immune genes that regulate host immune responses using newborn and weaned calves. Our study found that there were significant changes in gut bacterial diversity and density depending on gut region, age of the calf, and environment (mucosal tissue/ ingesta). Expression of mucosal innate immune genes also revealed significant differences throughout the GIT as well as with increasing age. Moreover, this study revealed a correlation between bacterial density and TLRs expression in dairy calves under normal physiological conditions, suggesting that

the host innate immune functions are related to the colonization of the gut microbes.

4.1.1 Achievement of understanding on gut microbial community in GIT of calves

Our understanding of the gut microbiome has been heavily based on studies using fecal samples (Gill et al., 2006; Uyeno et al., 2010; Walker, 2010) due to the limitations of obtaining direct samples from the gut. It is known that the GIT environment varies its chemical and physiological conditions such as pH, buffering and epithelial surfaces (Van et al., 2011) along its length. However, how these regional and environmental changes impact bacterial colonization density and diversity is not well understood. To date, most studies have been focused on humans, mice and pigs; but knowledge regarding the bovine gut microbiome was scarce. Our study on regional and environmental influences on bacterial establishment, using PCR-DGGE molecular typing technique, was the first to reveal the predominant bacterial profiles in the GIT of diary calves and how they differ among different regions as well as environments. The analysis of generated bacterial profiles revealed the presence of a mucosa-associated bacterial community in which the species were different from those associated with the ingesta. Our results suggested that previous knowledge of microbial communities, based on the analysis of fecal samples, may have been biased due to the segregation among different regions of gut.

To date, three main factors have been considered to impact gut microbial diversity: host genetics, environment, and microbial-microbial interactions. Both host and microbial factors regulate bacterial colonization and it is therefore, debatable whether the host selects its gut microbiome or microbes choose their habitats. The mucosa-associated predominant bacterial species observed in our study supported the hypothesis that the host may determine the microbial colonization on mucosal tissue, which results in colonization differences between mucosal and ingesta bacterial communities. Since mucosal-attached bacteria are in direct contact with the host, the observed mucosa-associated bacterial species might be playing a significant role in mucosal immunity development of dairy calves.

Enteric infectious diseases of livestock species greatly affect both animal production and health. Some of these diseases pose a risk to human health as well, due to their possible zoonotic condition. Consumer concerns regarding the use of antibiotics in the livestock industry, lead to the importance of finding alternative ways to aid the animals in building resistance against pathogens in order to maintain health and production levels. Therefore, it is important for researchers to understand how the host develops natural resistance to pathogens. We found that dairy calves establish mucosa-specific bacterial community at a very early stage, what may be a key factor in assisting the host to develop its immune function in the early life. This also suggests that gut bacterial manipulation could possibly be a way to alter host immunity development.

Fecal-associated bacteria diversity of dairy calves was reported to change with age (Uyeno et al., 2010). This indicated that diet plays a vital role in determining the bacterial composition in ingesta. However, the effect of diet on the mucosa-associated bacterial community and whether the diversity alteration in ingesta-associated community influences the mucosal community has not been studied. Future research comparing animals under different diets will help to understand ingesta associated bacterial population, their interactions with mucosal attached community, and with the host.

In this study we also constructed 16S rRNA gene libraries for the ileum, to further verify the differences in the gut microbial community at a taxonomical level. This section of the small intestine was selected for its direct involvement in host immune functions. Ileum tissue used in this study had Peyer's patches, which is gut-associated lymphoid tissue (GALT) that contains various immune cells such as dendritic cells, B cells, and macrophages. The Peyer's patches of germ-free mice had been reported to be undeveloped (Guarner, 2006), suggesting that the colonization of gut microbial population is vital for the development and function of PP and GALT. Our results on ileum ingesta and mucosal tissue of 3 week old calves revealed that mucosa-associated bacterial community richness was significantly higher than in the ingesta community. Moreover, the mucosa-associated bacterial community structure of the ileum was significantly different from that of ingesta. These findings strongly support our hypothesis that mucosa is colonized with specific bacterial species that are distinct from those that colonized the ingesta, emphasizing the importance of accurate sampling in gut

microbiome studies, if the aim is to understand host-microbial interactions. Additionally, an investigation on numbers of mucosal lymphoid (T cells, natural killer cells) and myeloid cell (DC, macrophages) populations in the ileum of 3 week and 6 month old calves that was carried out as a collaborative project with Dr. Griebel's group at the University of Saskatchewan, revealed significant age related changes (Fries et al., 2011). This suggests that, among the investigated populations, the variation of mucosal bacteria among different individuals may be associated with the diversification of host immune cells.

In the present study we discovered that total bacterial population density was affected by gut region, environment, and age in dairy calves. In human infants, their fecal bacteria population was reported to fluctuate significantly as compared to that of adults (Mackie et al., 1999). The total bacterial population present in the gut of 3 week old calves also had very high individual variation. In contrast to 3 week old calves, there was less individual variation and significant regional effect on total bacterial population in the gut of 6 month old calves. This more stable gut bacterial population observed after weaning in dairy calves may represent an adult cattle gut. The data obtained in this study suggests that the gut microbiome of calves during weaning may play an essential role in determining adult gut microbiome composition as well as host-microbial interactions.

In addition to the GIT bacterial community, we also investigated the rumen ingesta and mucosa-associated bacterial communities which help adult cattle with their fermentation of feed particles and nutrients uptake. The microbial establishment in the rumen has been considered to occur after weaning, but

knowledge regarding the rumen during the early life of cattle is lacking. Our study was the first effort to investigate bacterial colonization in the rumen. Newborn dairy calves pass milk directly into the abomasum, therefore they are considered as functional monogastrics. The observation that the high bacterial density in rumen tissue and ingesta of newborns is similar to weaned calves in our study, suggests that rumen colonize with a dynamic bacterial population soon after birth. This raises a very interesting question: Is this initial population important for the development of later rumen bacterial populations? For example, the rumen epithelial absorption of fermented volatile fatty acids in adult cattle is important both for nutrient transportation and to maintain a balanced pH rumen environment, in order to prevent acidosis or subacute acidosis (Owens et al., 1998). The early colonization of bacterial populations on the surface of the ruminal epithelial wall may help the animals to develop the capability to adapt to the solid feed particles, as well as to other microbes needed after weaning. Future studies, in which samples from the same individual animal are obtained over time, may explain bacterial establishment in the rumen mucosa and ingesta, as well as the bacterial community changes with increasing age.

4.1.2. Understanding of host innate immune functions in GIT of calves

In the present study, we also found that the expression of 10 bovine TLRs, β-defensin and PGLYRP1 expression through GIT of dairy calves, as well as age- and region-dependent expression, changes under normal physiological conditions. We also attempted to link the expression of these innate immune related genes

expression to gut bacterial density. TLR9 and 10 were differentially expressed in 3 week old calves while TLR2, 4 and 10 were differentially expressed in 6 month old claves. These differentially expressed TLRs among gut regions are known to recognize conserved bacterial molecules. TLR9 recognizes CpG containing DNA and TLR10 recognizes triacylated lipopeptides (Akira and Takeda, 2004; Guan et al., 2010). TLR2 and 4 recognize cell wall components of Gram positive and Gram negative bacteria, respectively (Akira and Takeda, 2004). In addition, our findings indicate that TLR expression is not only region-dependent but also age-dependent. Weaning in young mammals is associated with drastic functional and physiological changes in the GIT and host immune system (Hooper, 2004). Therefore, the observed down-regulation in TLRs expression may also a part of the mucosal developmental program.

The higher expression of TLR10 in bovine ileum regardless of the calf age was one of the most interesting outcomes of this study. This receptor has been reported to be highly expressed on B cells and thus linking adaptive and innate immune systems of the host (Guan et al., 2010). Since cattle ileal PP are considered to be a primary B cell proliferation site, it is not surprising to observe significantly higher expression of TLR10 in the bovine ileum (Griebel and Hein, 1996). Our results with respect to TLR10 suggest that this TLR may play very important roles in the bovine ileum in terms of host immune responses and mucosal immune system maturation. However, the biological significance of TLR10 in bovine ileum and its role in immune responses of dairy calves is still not well understood.

Windsor and Whittington (2010) reported that exposure to MAP induced Johne's disease lesions in 75% of calves <6 months of age, 50% of calves in 6-12 months of age, and 20% of >12 months of age, suggesting that disease susceptibility decreases with increasing age. Therefore, we decided to investigate the expression pattern of antimicrobial defence molecules involved in bacterial recognition and elimination. The expression of antimicrobial defence molecules (β-defensin and *PGLYRP*1) in the GIT of dairy calves was age-dependent, and expressed only after weaning. The absence of β-defensin and *PGLYRP*1 in newborn calves confirmed the weaning-associated developmental changes in host mucosal immune system. Less inflammatory or other mechanisms less harmful to the host, such as β-defensin, might replace the abundant TLRs functions in newborn calves. β-defensin expression was not region-dependent while *PGLYRP*1 revealed a significantly lower expression in the rumen as compared to the rest of the GIT. As β-defensin is involved in the direct killing of pathogens, an even expression throughout the GIT may be necessary. The changes in mucosal innate immune gene expression observed in the present study indicated that in dairy cattle, increasing age may be associated with age-related disease tolerance ability.

Interactions between commensal microbes and TLR expression have been reported to play a crucial role in maintaining intestinal homeostasis as well as immune responses in mice (Rakoff-Nahoum et al., 2004; Kelly and Conway, 2005). In our exploration of the interactions and correlations between commensal bacterial density and TLR expression that recognize bacterial ligands, we found that, although the correlation patterns were not consistent, a negative correlation

between bacterial density and TLR expression for several gut regions. This was the first attempt to correlate total bacterial density and LAB density with mucosal tissue TLR expression in dairy calves *in vivo*. Environment and host-microbial interactions in the GIT are very complex; however, even under such a complex situation, the expression of TLRs still displayed a negative correlation confirming their hyporesponsive reactions towards commensal bacteria. Observed bacterial diversity variations throughout the GIT and between mucosal tissue and ingesta communities may result in inconsistent correlation between bacterial density and TLRs expression. Moreover, TLR expression observed in this study may be considered to represent TLRs present on both mucosal epithelial cells as well as other mucosa-associated immune cells. Thus, unlike the *in vitro* studies conducted using pure bacterial molecules and epithelial cell cultures, our study can be considered to successfully explain the interaction between commensal bacterial products and TLRs expression in this complex real scenario.

4.2 Future directions

Our study contributed fundamental knowledge regarding commensal bacterial segregation between mucosal tissue and ingesta, as well as mucosal innate immune gene expressions along the GIT of dairy calves. However, there are a few limitations of the present study:

1. The molecular fingerprinting method used in this study, PCR-DGGE, has low resolution. For human the GIT, more than 500 bacterial species have been reported (Eckburg et al., 2005; Muller et al., 2005), therefore the 80 PCR-DGGE

bands identified in this study may only represent a small part of the population. Future studies utilizing the recently developed next generation sequencing technology may supply more solid and direct evidence of the species present in dairy cattle.

2. Sampling location limitations. For this study, only a small section of gut was collected and investigated, therefore it represented only a small proportion of each gut region. Future studies using larger samples of multiple locations from each gut region may provide a higher coverage of the bacterial species present and a better understanding of the innate immune gene expressions in each region.

3. Small sample number of animals. Only a relatively small number of animals were available for bacterial profiling, and this may affect the bacterial diversity detected, since it could only partially represent the GIT bacterial diversity in calves.

4. The gene expression study. The mucosal tissue samples represented a mixture of cells (epithelial cells and other immune cells). In the future, the use of single cell populations will provide a better understanding of TLR expression and correlations between bacterial density and TLRs expression. Furthermore, the use of single cell populations may further clarify the variations in TLRs and antimicrobial defence molecules expression throughout the GIT, as well the changes with increasing age. When high throughput gene expression assays such as microarray or RNA-seq using next generation sequencing technology, are used to investigate the genes involved in signalling pathways, we can expect a better

overall understanding of host immune responses towards its commensal consortium.

The current understanding on host selection of mucosa-associated gut bacteria that directly interact with the host seems to suggest that genetics can affect the commensal microbial establishment, and thereby regulate the host immune responses. The future identification of specific bacterial species associated with better immune-related phenotypes, such as disease resistance, could be used as microbial markers for animal selection. Microbial marker-based selection could also be used as a technique for any other host trait related to commensal microflora, such as feed efficiency. Microbial markers could become a fast and easy tool for field animal selection. Future studies to investigate the influence of initial exposure to microbes, subsequent gut colonization, and immune development in dairy calves, may provide the necessary knowledge to develop microbial feed additives that could be used during the early life of the animal to manipulate the gut microflora establishment, and there by the development of the host immune system.

The bovine TLRs and the antimicrobial defence molecules studied in the present study belong to the innate immune system, which is a well-conserved system across species. The genes implicated in this system are often considered to be candidate genes for animal selection in order to improve host health. In the future, a comparison of the genetic variation among these genes with respect to disease tolerance, disease resistance and susceptibility, may lead to the discovery of genetic markers for selection of dairy and beef cattle. The improved

understanding of the interactions between commensal microflora and host immune responses generated by this study could be cutting-edge in the development of multidisciplinary techniques and tools that improve host health and prevent zoonotic diseases.

4.3 References

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