Effect of feeding management on neuroendocrine system and colonisation of gut bacteria in

neonatal dairy calves

by

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Abstract

Neonatal period is challenging for newborn calves as they need to undergo development of immune system and gastrointestinal tract. Stress hormones, glucocorticoids and catecholamines, are elevated in first few days of life and play essential role in neonatal development in newborn calves. In this thesis, two studies were performed to investigate effect of different feeding management on neuroendocrine system and gut bacterial populations. The first study investigated the effect of duration of colostrum feeding management on abundance of active colon and ileum tissue-associated bacteria and expression of neuroendocrine genes in adrenal glands and intestine mucosa. Holstein bull calves were fed colostrum immediately after birth and randomly assigned to different subsequent feeding; whole milk (WM; n=8), mixture of colostrum and milk (CM; n=8) and colostrum feeding (CF; n=8) for 72h with 12h intervals. Samples of adrenal glands, ileum and colon tissues were collected at 75h postpartum. Quantitative RT-PCR was performed to measure expression of neuroendocrine genes in adrenal gland and intestinal tissues as well as abundance of active bacteria associated with intestinal mucosa. The altered expression of these genes in adrenal glands, ileum and colon tissues were observed when calves were under different colostral and milk components feeding strategies. Our results indicate that prolonged colostrum feeding increases the abundance of active tissue-associated *Lactobacillus* spp. and *E. coli* in colon of newborn calves. The second study further assessed the effect of delayed colostrum feeding on fecal cortisol concentration and digesta-associated bacteria in the colon and rectum of neonatal calves. Holstein bull calves (n =23) were fed pooled, pasteurized colostrum either immediately after birth (0 h; n = 7); or at six (6h; n = 8), twelve (12 h; n = 8) hours after birth. Rectum and colon digesta and colon tissue were collected at 51h postpartum. Enzyme immune assay and quantitative PCR were used

to characterise fecal cortisol level and bacterial abundance, respectively. Results indicate that feeding colostrum 12h postpartum decreased ($P < 0.05$) fecal cortisol concentration, compared to non-delayed (0h) and 6h delayed colostrum feeding. This thesis is the first to detect and characterize expression of genes involved in neuroendocrine functions and extra-adrenal production of stress hormone in neonatal dairy calves. The findings suggest that different duration and delayed feeding of colostrum can affect neuroendocrine system and bacterial populations which may influence development of immune system and gastrointestinal tract. Outcomes of this study provide fundamental knowledge to establish effective feeding management in dairy industry, which support development of immune system and gastrointestinal tract.

Preface

This thesis is an original product of the author Jitka Hromadkova with collaborations led by Dr. Lelou Guan at the University of Alberta.

Co-authors for chapter 2 include Jade Pyo and Sarah Pletts of the University of Alberta, who contributed to experimental design and sampling. Co-authors for chapter 3 include Yang Song and Amanda Fischer of the University of Alberta, who contributed to experimental design, calves rearing and sampling. In addition, Dr. Deborah Haines of the Saskatoon Colostrum Company Ltd., who provided pooled colostrum and assisted in manuscript preparation, as well as Dr. Michael Steele of the University of Alberta who contributed to experimental design and manuscript preparation. Additionally, Dr. Michael Steele and Amanda Fischer of the University of Alberta conducted the animal experiment described in chapter 3 and contributed to manuscript preparation.

Animal experiments in chapters 2 and 3 were conducted at the Dairy Research and Technology Centre at the University of Alberta. The research projects conducted in this thesis received ethics approval from the Animal Care and Use Committee for Livestock at the University of Alberta and all procedures performed on animals were conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, ON, Canada, 2009).

Dedication

This thesis is dedicated to my loving parents and sisters.

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I would like to sincerely thank Dr. Leluo Guan for accepting me as her graduate student as well as for her encouragement and support during my studies which helped me to stay on the positive linear trajectory of success. Her trust and great work ethic motivated me to work hard and with her guidance I could improved my critical thinking and writing skills.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACTH   Adrenocorticotropic hormone

CNS   Central nervous system

CRH   Corticotropin-releasing hormone

DAMPs   Damage-associated molecular patterns

GBA   Gut brain axis

GCs   glucocorticoids

GIT   Gastrointestinal tract

GLP-2   Glucagon-like peptide-2

HPA   hypothalamic–pituitary–adrenal axis

LPS   Lypopolysacharides

MAMPs   Microbe-associated molecular patterns

PAMPs   Pathogens associated molecular patterns

PRRs   Pattern recognition receptors

qPCR   Quantitative Polymerase Chain Reaction

SAM   Sympathetic-adrenal-medullary axis

SCFAs   Short chain fatty acids

SDS   Sodium dodecyl sulfate

RT-qPCR   Real time Quantitative Polymerase Chain Reaction

Chapter 1. Literature review

1.0 Introduction

Early life events are critical for neonates and may have long lasting effect on health and performance of animals. Dairy calves are born with an immature immune system and gastrointestinal tract (GIT) and various morphological and functional changes in intestine are vital to adapt to new environmental conditions.

Adequate intake of nutrient and non-nutrient components of milk and colostrum supports neonatal development and establishment of commensal microbiota of in newborn calves. Colostrum feeding in particular is an important management practice that stimulates the development of immune system and GIT of neonatal calves in first few days of life. Bovine colostrum contains high concentrations of nutrient and non-nutrient components, including immunological components (Blum, 2006) which play role in passive transfer of immunoglobulins (Stott et al., 1979). Furthermore, absorption of colostrum components postpartum stimulates formation of mucosal layer in intestine, which helps microbes to colonise GIT (Hulbert & Moisa, 2016). Malmuthuge et al. (2015a) also reported that administration of heat-treated colostrum immediately postpartum promotes colonization of probiotic bacteria such as *Bifidobacterium* and reduces colonisation of *Escherichia coli* in small intestine of newborn calves. In addition, the timing of first meal, quality and quantity of colostrum are the main factors that influence absorption of colostrum components in newborn calves (Hulbert & Moisa, 2016). When the first colostrum meal is delayed, absorption of colostrum components can be affected which may lead to increased rate of mortality and risk enteric diseases (Blum, 2006; Fischer et al., 2018). It has been also suggested that lack of nutrients may affect immune responses, metabolic and neuroendocrine

system, especially in young growing animals with high metabolic demand (Chen et al., 2015; Schaff et al., 2014).

There is increasing evidence that gut microbiota has direct impact on the hypothalamic-pituitary-adrenal (HPA) axis, a major neuroendocrine system involved in regulation of stress and immune responses (Carabotti et al., 2015; Clarke et al., 2014; Freestone & Lyte, 2010; Sandrini et al., 2015; Sarkar et al., 2016). It has been proposed that certain microbes in gut can produce and/or utilize metabolites similar to hormones and neurotransmitters produced by host (e.g. serotonin, dopamine, epinephrine, gamma-aminobutyric acid), which can potentially influence transport these agents across epithelium into effector organs (Clarke et al., 2014).

Previous studies also suggested that stress hormones play a crucial role in fetal and neonatal development and changes occurring during stress affects immune system. As neonates are born with yet immature immune system, their immune responses are regulated by production of stress hormones via HPA axis (Hulbert & Moisa, 2016). However, little is known about how stress and gut microbiota are associated with neonatal development in calves and their effect on immunological and metabolic state of host.

1.1 Neonatal development in mammalians

Neonatal period is challenging for calves due to requirements for development and adaptation to various new environmental factors. Previous studies about mammalian development in neonatal period focused mainly on stress, immune system and their effect on health later in life. There is a considerable evidence that early life event and environmental conditions may cause lasting changes in host physiological state in mammals (Avitsur et al., 2015; Hulbert & Moisa, 2016; Matthews, 2002; Soberon & Van Amburgh, 2017). Passive transfer of immunoglobulins are initiated postpartum due to epitheliochorial placenta of ruminants that prevents contact of uterine

epithelium and maternal blood vessels (Wooding, 1992). Thus, in first few days after birth, innate immune mechanisms play a major role in immune responses of newborn calves due to underdeveloped adaptive immune mechanisms. Given this fact, neonatal calves are more susceptible to infectious diseases. Neonatal morbidity and mortality have been mainly associated with infections and GIT disorders (Blum, 2006; Hulbert & Moisa, 2016; Khan et al., 2017). Although GIT is relatively mature at birth, several morphological and functional changes are needed to adapt changes from parental nutrition supply to enteral feeding (Blum, 2006; Morgan et al., 1987; Steele et al., 2016). Moreover, the initial microbial colonization is triggered during parturition as neonate go through mother's birth canal and colonisation by commensal microbiota contributes to adaptive immune education (Zhao & Elson, 2018). Furthermore, an increasing evidence suggests that gut microbiota may interact with host and contribute to host's physiological and metabolic changes. Mammalian neonatal development is therefore followed by complex interactions of biological, environmental and behavioral factors that can have long lasting systematic effect on health, nutritional status, metabolism and endocrine system.

1.1.1 Factors influencing GIT development in neonatal stage

Right after birth, GIT is exposed to new environmental condition and of morphological and functional changes are required for proper development. Feeding management, nutritional intake, stress hormones, microbial colonization, gene activation and expression are major factors that influence GIT development of neonate (Blum, 2006; Malmuthuge et al., 2015b; Steele et al., 2016). Previous reports indicate that modulation of intestinal differentiation and function as well as immune system regulation can be significantly influence by early nutritional supply (Eckert et al., 2015; Kelly & Coutts, 2000; Steele et al., 2016). In the current dairy industry, feeding newborn calves with colostrum is recommended due to its beneficial role in their gut development. In last

few decades, colostrum composition as well as nutritional requirements of neonate have been well studied and importance of colostrum feeding in neonatal stage was emphasised (Blum, 2006; Godden, 2008; Hammon et al., 2013). Colostrum supplies antibodies to calves which are important for passive immune transfer in first few days after birth. Furthermore, colostrum feeding soon after birth promotes growth of beneficial bacteria and decreases prevalence of potential pathogenic bacteria in neonatal calves (Malmuthuge et al., 2015a), whereas delayed colostrum feeding over 6 and 12 hours significantly decreased concentration of immunoglobulins in newborn calves (Fischer et al., 2018).

As barrier between endogenous and exogenous environment, GI epithelium plays a major role in protection of host against dangerous luminal components (e.g. toxins, pathogenic bacteria) as well as supplementation of nutrient transport. Stress hormones, glucocorticoids in particular, are known to have positive effect on maturation of GIT functions (Blum, 2006; Schaak et al., 2000; Schaff et al., 2014). Maturation of GIT functions and survival rate in newborn premature calves was enhanced by administration of maternal glucocorticoid prior to delivery (Schmidt et al., 2004). However, additional stressors such as group housing or transportation in the first week of life in calves may increase risk of enteric diseases (Hulbert & Moisa, 2016). Review on animals (mainly rodents) and human studies provide evidence that exposure to physical and psychological stress causes intestinal barrier dysfunction which may result in gut inflammation (Soderholm & Perdue, 2001). Stress-induced glucocorticoid exposure causes gut barrier dysfunction which may lead to increased pro-inflammatory cytokine production. These cytokines further reduce integrity of gut barrier as we as blood-brain barrier integrity and facilitate access to potential pathogenic or inflammatory elements through epithelium (Sarkar et al., 2016). To date, few studies have focused on stress responses in neonatal calves, therefore physiological mechanism of stress-induced gut

barrier dysfunction and impact of stress on GIT functions and overall development in neonatal stage need be further investigated.

GIT development is thought to be regulated by changes in gene expression during neonatal period. Gene activation and expression have been also associated with morphological and functional changes of GIT (Steele et al., 2016). For example, glucagon-like peptide-2 (GLP-2) may reduce negative effects of stress conditions on gut and decrease inflammation by supporting gut barrier function and integrity (Connor et al., 2015). Furthermore, GLP-2 supported gut barrier function by increasing epithelial expression of genes encoding tight junction proteins in calves inoculated with pathogenic *Eimeria bovis* (Walker et al., 2015). Liang et al. (2014) suggested regulatory role of mRNAs in cell proliferation and differentiation of gut-associated lymphatic tissue during early life in dairy calves. These findings suggest there is interaction of various exogenous and endogenous factors that play a role in manipulation of GIT development in neonatal period.

1.1.2 Physiological and functional difference in GIT of ruminants and pre-ruminants

Compared to adulthood, pre-ruminants have unique digestion and various physiological and structural changes occur in GIT to establish gut microflora and complete postnatal development. Pre-weaned ruminants are due to dietary requirements and underdeveloped rumen considered as pseudo-monogastric (Blum, 2006; Liang et al., 2014). Although rumen is a major GIT region for digestion in ruminants, food bypass through esophageal groove directly into abomasum during neonatal period which initiates acidic environment and digestion in lower gut leaving rumen yet underdeveloped (Steele et al., 2016). Furthermore, increased intestinal permeability to large molecules such as maternal immunoglobulins which allow immunocompetent newborn ruminants to passive transfer of antibodies and leucocytes during first

12 hours postpartum (Godden, 2008; Liebler-Tenorio et al., 2002). Thus, abomasal and intestinal digestion during preweaning period is crucial for overall development of GIT and immune system of pre-ruminants.

Early life events occurring prior to weaning period can have significant impact on health and performance of adult animals (Eckert et al., 2015). Significant changes in feeding habits occur in weaning period at 1 to 3 months of ruminant's life, which require functional and morphological transformations of GIT. Transition from milk-based to solid-based diet changes nutritional composition of meal, thereby rumen development is required and its capacity increases from 30 to 70% of GIT (Steele et al., 2016). Rumen fermentation by microbiota plays important role in digestion of solid diet and energy supply in form of SCFAs required for growth. Microbial production of SCFAs via fermentation of indigestible carbohydrates in rumen plays important role in development of rumen papillae (Malmuthuge et al., 2015b). These findings suggest that feeding management can affect postnatal microbial colonisation which may have lasting effect on animal performance.

1.2 Gut Microbiota in mammalians

Recent advanced molecular based studies using 16S rRNA gene have enabled characterisation and quantification of gut microbiota in depth. Gut microbiota can be characterized by its high diversity and complex interactions between five main taxonomic groups: bacteria, archaea, fungi, protozoa and viruses.

Previous studies suggest that microbial colonisation occurring during early life may play a key role in establishment of microbial communities, GIT environment and host-microbial interactions that can influence host physiology, immunology and digestion later in life (Malmuthuge et al., 2015b; Sudo et al., 2004; Walker, 2017; Zhao & Elson, 2018). This suggests

that individual variations in overall performance may be associated with establishment of host-specific microbial communities in early stage of life. Similarly, study using germ free mice revealed that autochthonous microbiota from human and rat GIT promoted maturation of immune system, compared to allochthonous gut microbiota (Chung et al., 2012), suggesting the importance of coevolved species-specific microbiota in development of host immune system.

Gut microbiota produce short chain fatty acids (SCFAs) through microbial fermentation, which are then used by host for energy homeostasis and modulation of immune responses (Ang & Ding, 2016). In ruminants, SCFAs produced mainly by ruminal microbiota which account for ~ 70% of host daily energy requirements (Yeoman & White, 2014). Compared with monogastric humans, gut microbiota provides host about 10% daily energy intake (Weickert & Pfeiffer, 2008). Thus, ruminant nutrition with relation to rumen microbiota's capability to produce SCFAs are important aspects in ruminant industry. For example, PCR denaturing gradient gel electrophoresis and analysis of 16S rRNA genes and metatranscriptomics recently revealed the association of composition and activity of rumen microbiota has been linked with feed efficiency in beef cattle (Guan et al., 2008; Li & Guan, 2017b). An increasing number of studies focus on rumen microbial profiling and modulation of early life microbiota to reduce greenhouse gas emissions or improve feed efficiency in ruminant industry. Composition and activity of gut microbiota may also differ due to differences in ruminal and intestinal environment as well as structure of epithelium.

Majority of commensal bacteria in mammalians can be found in lumen and they are represented by three main phyla: Bacteroidetes, Firmicutes and Proteobacteria. The digesta-associated microbiota is capable of producing of various metabolites such as SCFAs or neurotransmitters which can be used for physiological and metabolic pathways by host. Sequencing methods have also revealed the presence of tissue-associated commensal bacteria in

small intestine (Malmuthuge et al., 2015a) which can protect pathogens from crossing epithelial barriers, interact with host immune cells and regulate immune responses (Fung et al., 2014; Goto & Ivanov, 2013). Furthermore, recent evidence suggests association of commensal microbiota with gut-associated and peripheral lymphoid tissues and ability of these microbes to modulate functions of immune cells (Fung et al., 2016; Obata et al., 2010). However, further investigation of interactions between gut microbiota and host in lumen, mucus, epithelium and lymphoid tissue is required for better understanding of gut microbial influence on host physiology.

Neonatal microbial colonization and related events occurring in early life are the most critical determinants that influence perennial composition of gut microbiota. Mammalian GIT is inoculated during parturition from several different sources such as vaginal canal, feces, skin, and saliva (Houghteling & Walker, 2015). Such a transfer of maternal microbes to neonate has been proposed to work as an early inoculation process with implications for the long-term health effect on newborns (Dunn et al., 2017). The most important factors altering gut microbial composition in neonates are diet, mode of delivery, gestational age, antibiotic usage and environmental conditions (Penders et al., 2006). In recent a study about calves (Malmuthuge et al., 2015a), heat-treated and fresh colostrum feeding stimulated bacterial colonisation in small intestine of neonates compared to individuals that were not fed colostrum soon after birth. Similarly, Penders et al. (2006) reported feeding had a major effect on the composition of gut microbiota, where formula-fed diet resulted in predominance of *E. coli*, *Clostridium difficile*, Bacteriodes and Lactobacilli compared to breastfeeding which had stimulating effect on colonisation of Bifidobacteria. Furthermore, caesarean section and administration of antibiotics is associated with decreased numbers of Bifidobacteria and Bacteriodes in infants (Dunn et al., 2017; Penders et al., 2006). In diary industry, however, calves are removed from their dam shortly after birth to minimize the risk

of disease. Thus, this principle of microbial transfer from mother to calf is limited giving by the short exposure of dam to neonates and it remains unknown whether offspring – dam separation can affect physiology, metabolism, development and heath during early stage of life in dairy cows.

Although composition of microbes in GIT is established during early life and remains relatively stable in adulthood, it has been proposed that various environmental factors such as dietary components, antibiotic treatment and inhibitory drugs of gastric proton pumps may affect intestinal microbiota (Kasiraj et al., 2016a). Moreover, alteration of gut microbial composition during adulthood have been also associated with different physiological states during fasting, nutritional deprivation and hibernation in various animal models (Costello et al., 2010; Kasiraj et al., 2016b; Thompson et al., 2008; Zarrinpar et al., 2014). Moreover, intermittent fasting resulted in alteration of gut microbiota composition and increased acetate and lactate products that had positive effect on adipose tissue development and significantly reduced obesity in mice (Li et al., 2017). Caloric restrictions and fasting increased diversity of gut microbiota and stimulated proliferation of mucin-degrading bacteria (Derrien et al., 2004; Remely et al., 2015).

Furthermore, nutritional deprivation or dietary shifts can lead to suppression of host antibacterial defenses due to attenuation of intestinal mucin layer that plays important role in host defensive mechanism (Hanning & Diaz-Sanchez, 2015). Indeed, composition of gut microbiota changes during annual hibernation cycles of hibernating mammals. Carey et al. (2013) reported that hibernation increased relative abundance of mucin-utilizing phyla such as Bacteroidetes and Verrucomicrobia, while relative abundance of bacteria degrading polysaccharides (Firmicutes) decreased. In ruminants, feeding deprivation resulted in alteration of rumen microbiota, increased rumen pH, plasma cortisol concentrations as well as decreased concentration of β-hydroxybutyrate, blood leucocytes counts, reduction of lymphocyte function and antibody

responses (Chen et al., 2015). Moreover, feed withdrawal before animal slaughter may decrease concentration of organic acids in the gut, resulting in increased pH and stimulation of *E. coli* proliferation which affect quality and flavour of animals products (Freestone & Lyte, 2010). These finding suggest that feeding pattern and feed intake changes conditions of luminal environment resulting in altered ecology, abundance and diversity of gut microbiota and physiological and metabolic state of host in adulthood. Thus, establishment of stable gut microbial composition during early life and microbial ecology within gut clearly play an important role in host microbial interactions. Further investigation is needed for comprehensive understanding of these interactions and their impact on host physiology and metabolism.

1.3 Host-microbial interaction and gut-brain axis

Recent findings on impact of gut microbiota on HPA axis and central nervous system has given rise to gut-brain axis (GBA) theory. It has been proposed that GBA supplies bidirectional communication between gut microbiota and host in form of biochemical signaling throughout neural, endocrine, immune, and humoral pathways. To maintain homeostasis within GIT, apical side of intestinal epithelium is covered with mucus layer that functions as physical and chemical barrier against potential pathogens and other dangerous luminal agents such as bacterial and environmental toxins (Specian & Oliver, 1991). Moreover, immune and epithelial cells of intestinal wall are also equipped with various types of pattern recognition receptors (PRRs) located on membrane and cytoplasm which allow them to recognize microbes based on specific molecular patterns produced by microbes (Abreu, 2010). Recognition of these bacterial products, microbes / pathogens associated molecular patterns (MAMPs or PAMPs), triggers cascades of pro-inflammatory responses which result in production and secretion of cytokines, neurotransmitters and glucocorticoids (Sandor & Buc, 2005). Under certain conditions, PRRs can also recognize

damage associated molecular patterns (DAMPs). Under normal physiological conditions, DAMPs are hidden inside host cells which prevents recognition by PRRs. However, during inflammation, cellular stress or tissue injury, DAMPs are secreted by host cells to allow recognition by PRRs resulting in pro-inflammatory response (Land, 2015).

There is an increasing evidence that gut microbiota may influence host physiology through direct interaction between gut and HPA axis, a major neuroendocrine system that regulates stress and immunity (Carabotti et al., 2015; Clarke et al., 2014; Freestone & Lyte, 2010; Sandrini et al., 2015; Sarkar et al., 2016; Sudo et al., 2004). It has been recently proposed that gut microbiota can contribute to this biochemical signaling by producing hormone like metabolites, such as neurotransmitters, stress hormones and GI metabolites which can be used by host cells in systematic responses (Clarke et al., 2014). Furthermore, some metabolites produced by pathogenic bacteria can dysregulate cytokine production or inhibit lymphocytes proliferation (Sandrini et al., 2015). Moreover, some of these metabolites have been shown to stimulate bacterial proliferation. For example, catecholamines have a positive effect on growth of gram negative bacteria which may potentially lead to increase of their virulence-related factors production (Freestone et al., 2002; Freestone et al., 2000). These finding suggest gut microbiota may contribute to alteration of host physiology throughout gut brain axis (GBA). However, no studies have been done to investigate role of gut microbiota in gut brain axis and relationship of gut microbiota and host stress response in neonatal calves to date.

1.3.1 Role of commensal bacteria and probiotics in GBA

From ecological perspective, commensal microbiota competes with pathogenic bacteria for nutrients and space and protect host from pathogens by antimicrobial substances such as organic acids or bacteriocins (Buffie & Pamer, 2013; Tenaillon et al., 2010). Absence and imbalance in

composition of gut microbiota have been associated with poor functioning of immune system, metabolism and underdevelopment of GIT (Hanning & Diaz-Sanchez, 2015), suggesting importance of microbiota.

The histological and physiological changes of GIT in relation to gut microbiota have been widely studied on germ-free animals. Gordon et al. (1963) reported reduced growth and functions of heart in germ free rats compared to conventional animals. Sudo et al. (2004) reported that exposure to microbes at early stage of life is crucial for postnatal development of HPA axis and neural pathways related to stress responses in mice can be modulated by gut microbiota. Furthermore, study about germ free and gnotobiotic mice suggests microbiota can produce serotonin in gut lumen after recolonization with specific pathogen-free or fecal flora (Hata et al., 2017). Probiotic and prebiotic administration had positive effects on histological and functional properties of GIT and increased growth rate of animals (Hanning & Diaz-Sanchez, 2015). Also, administration of probiotic strains isolated from calf intestine prevented enteric colonisation of pathogenic *Escherichia coli* O157:H7 prior to its exposure (Zhao et al., 1998), suggesting probiotic bacteria can protect host against potential pathogens. Furthermore, administration of probiotic *Bifidobacterium infantis* reduced elevated plasma glucocorticoids concentration in germ free mice, suggesting protective role of *B. infantis* against increased stress hormones under stress condition (Sudo et al., 2004). Moreover, it has been reported that certain strains of lactic acid bacteria can produce serotonin and other neurotransmitters in presence and absence of gram negative bacteria (Ouml et al., 2012). Also, plasma concentration of tryptophan, precursor of serotonin, significantly increased under stress conditions in Bifidobacteria-treated rats (Desbonnet et al., 2008). Reigstad et al. (2015) also reported that indigenous and human derived gut microbiota increased tryptophan hydroxylase (TPH1, enzyme for serotonin biosynthesis) at protein and gene expression level in

mice compared to germ free individuals. Additional *in vitro* experiment from the same study further revealed that microbial production of SCFAs can significantly affect protein concentration and gene expression of TPH1 in human embryonal carcinoma (EC) cells. These results were further supported by a recent study (Bhattarai et al., 2017), which suggested that administration of human-derived gut microbiota results in alteration of expression of epithelial serotonin receptor gene in mice and the gene expression of this receptor changed after acetate treatment in colonoids. All together, these findings suggest a potential role of gut microbiota in production of hormones and neurotransmitters. In ruminants, there were also attempts to create and sustain germ-free cow, however rumen of adult ruminants cannot be fully developed due to lack of symbiotic digestion of indigestible carbohydrates by microbes (Hanning & Diaz-Sanchez, 2015). These findings demonstrate importance of gut microbiota in postnatal development of organs and its crucial role in regulation of host physiology, metabolism and immune system. Future studies are required to investigate neonatal microbial colonisation and its potential role in regulation of host immune system and metabolism.

1.3.2 Effect of pathogenic bacteria on host health and development in GBA

Invasion of pathogenic bacteria increases risk of infectious diseases in GIT that affects overall performance of host and it is becoming increasingly evident gut microbiota can affect host physiology. The complex interactions between host and gut microbiota within healthy gut are mutually beneficial to the host immunology and metabolism. However, disbalance in immune homeostasis within gut may trigger pro-inflammatory responses as a host defensive mechanism against potential pathogens crossing epithelial barriers. Defensive mechanisms of host change depending on various environmental factor such as stress, nutrients intake, overall health and age which all contribute to physiological changes. However, there is a limited knowledge about host-

microbial interactions and a potential role of gut microbiota in regulation of host physiology in ruminants.

Neonatal stage at which development of GIT and immune system occur is associated with increased risk of enteric diseases. Early studies on gut microbiota in neonatal stage of livestock have focused on pathogenesis of *Escherichia coli* revealing its adhesive features to mucosal epithelium of ileum and colon (Chanter et al., 1986; Hall et al., 1985; Janke et al., 1989; Schoonderwoerd et al., 1988). There has been an increasing interest in pathogenesis of *E. coli* and genes involved in virulent factors production associated with diseases of gastrointestinal, endocrine and central nervous system. Diarrheagenic strands of *E. coli* are considered as the most common pediatric diarrhea worldwide in livestock (Nataro & Kaper, 1998).

Previous studies suggested association of pathogenic bacteria with neurotransmitters such as catecholamines and serotonin. It has been proposed that eukaryotic neurotransmitters such as norepinephrine promotes growth and virulence of pathogenic bacteria in intestine (Freestone et al., 2000) which can be regulated either via signaling of adrenergic receptors on basolateral side of intestinal epithelium of host (Green & Brown, 2016) or through bacterial adrenergic receptor, QseC sensor kinase (Clarke et al., 2006). Freestone et al. (2007) reported catecholamines produced by the enteric nervous system as host-derived signals stimulated growth of pathogenic bacteria such as *E. coli, Salmonella enterica* and *Yersinia enterocolitica*. Furthermore, it has been suggested that catecholamines such as epinephrine and norepinephrine can affect n chemotaxis, biofilm formation, motility, gene expression and growth of *E. coli* O157:H7 (Bansal et al., 2007). Pasupuleti et al. (2014) described the principle of chemotaxis at which norepinephrine is converted into 3,4-dihydroxymandelic acid, a strong attractant for *E. coli*. This suggests that norepinephrine may have indirect role in chemotaxis by inducing synthesis of bacterial enzymes that generate 3,4-

dihydroxymandelic from norepinephrine. Moreover, Enteropathogenic *E. coli* infection have been shown to affect function and expression of serotonin transporter (Esmaili et al., 2009). This transporter functions on sodium- and chloride-dependent mechanism and has been localized on apical and basolateral side of intestinal epithelium (Martel et al., 2003). Interestingly, activation of serotonin transporter decreased by 53% on apical side of human intestinal cell infected by enteropathogenic *E. coli* infection, while activity of this transporter on basolateral was less affected (Esmaili et al., 2009). These findings suggest that serotonin is transported via the serotonin transporter either from basal-to-apical or apical-to-basal sides of enteric epithelium and pathogenic *E. coli* can disrupt its transfer mainly by inhibition of serotonin transporter on apical side. Additionally, administration of enteropathogenic *E. coli* resulted in elevation of stress hormones in gnotobiotic mice, while exposure to mutant strain Tir of *E coli* did not affect HPA stress response (Sudo et al., 2004). The mutant strain of enteropathogenic *E. coli* defect translocated intimin receptor (Tir) that allows bacteria internalization to intestine epithelium (Kenny et al., 1997). Thus, it can be concluded that attachment to epithelium layer is required to trigger HPA stress response by enteropathogenic *E. coli*. Furthermore, *in vitro* proliferation of bacterial pathogens stimulated by catecholamines is dosage and type dependent (Freestone et al., 2007). Therefore, *in vitro* studies should be designed in such way that follows dosage and time exposure similar to *in vivo* scenario.

Clearly, there has been considerable evidence that catecholamines can function as potent stimulatory agents of gram negative (notably pathogenic) bacteria. However, Freestone et al. (2000) demonstrated stimulatory effect of catecholamines on commensal *E. coli* in presence of antimicrobial agents such as lactoferrin and transferrin. These iron-binding proteins are present in colostrum (McGrath et al., 2016) and they are thought to have antimicrobial properties by releasing

lipopolysaccharides (LPS) from outer membrane of gram negative bacteria (Ellison et al., 1988). However, catecholamines can stimulate growth of gram negative bacteria in presence by converting the antimicrobial lactoferrin and transferrin into iron-accessible components (Freestone et al., 2000). This suggests that catecholamines may change chemistry of the colostral antimicrobial components and potentially increase risk of some commensal bacteria such as *E. coli*, to become pathogenic. Given the fact that catecholamines are increased in neonatal period in order to regulate metabolism and immune response in intestinal mucosa in pre-weaned calves (Schaak et al., 2000; Schaff et al., 2014), these hormones may play role in adaptive immune education in animals fed colostrum. Tenaillon et al. (2010) suggested that interactions between host immune system and commensal microbiota may vary in each individual which can influence *E. coli* diversity. These findings suggest that stress hormones such as epinephrine and norepinephrine can stimulate bacterial growth and most of the studies focused on pathogenic bacteria, Therefore, further research on catecholamines and their potential stimulatory effect is required.

1.4 Neuroendocrine system in early life

In dairy cows, neuroendocrine hormones released during stress response can affect calf health, food intake and digestion (Freestone & Lyte, 2010). For example, heat stress has been associated with decreased milk productivity and feed intake (West, 2003). Regarding to physiology of neonate, calves born to heat-stressed cows had reduced absorption of colostrum, altered leucocyte function and gene expression (Chen et al., 2015). Transportation, another common stressor in livestock industry, can result in various physiological, immunological and behavioral changes of animal (Earley et al., 2012). Psychological stressors such as social isolation and weaning have impact on animals mainly during neonatal season, which may affect

development of GIT and immune system. Similarly, feed deprivation over longer time can act as stressor, since lack of nutrients was associated with decreased lymphocyte function, antibody responses and altered gut microbiota (Chen et al., 2015). Moreover, it has been also suggested that qualitative and quantitative changes of gut microbiota may have impact on HPA axis responses (Allen et al., 2016; Clarke et al., 2014). Thus, these abnormal physiological responses occurring during stress may be caused by broad range of interactions initiated by multiple stressors, which requires further investigation. It should be noted that stressors may have also different impact on neuroendocrine system of host depending on age by secretion of different stress hormone. For example, social isolation of dams and calves over 24 hours resulted in increased concentrations of norepinephrine and epinephrine in calves and increased level of epinephrine in cows, whereas cortisol level was not affected in animals (Lefcourt & Elsasser, 1995). These reports indicate that exposure to stressors over long time may affect neuroendocrine system in young livestock animals. Future studies should therefore focus on measurement of both, catecholamines and glucocorticoids concentrations, when assessing stress response.

1.4.1 Neuroendocrine hormones production

Production of stress hormones is regulated via neuroendocrine system by activation of two main axis; Hypothalamus-pituitary-adrenal (HPA) axis and sympathetic-adrenal-medullary (SAM) axis (Chen et al., 2015) which result in release of glucocorticoids in adrenal cortex and catecholamines (norepinephrine and epinephrine) in adrenal medulla, respectively. However, human and rodent studies suggest extra-adrenal production of stress hormones such as glucocorticoids (GCs) also occurs in intestinal mucosa and other tissues (Kostadinova et al., 2014; Noti et al., 2009; Talaber et al., 2013).

The process of glucocorticoids production initiates by production of corticotropin-releasing hormone (CRH) and arginine vasopressin in hypothalamus which stimulates production of adrenocorticotropic hormone (ACTH) in pituitary gland (Charmandari et al., 2005; Chen et al., 2015). Once produced, ACTH is sent to adrenal glands to trigger glucocorticoid and adrenergic production in zona fasciculata in adrenal cortex and adrenal medulla, respectively (Charmandari et al., 2005). The hormonal changes modulated by neuroendocrine system have major role in regulation of basal homeostasis and responses to threats (Chrousos, 2009). Appropriate stress response to stressors is key modulator of physiological maintenance and overall performance of host. However, when responsiveness of stress system becomes inappropriate, imbalance or lack of neuroendocrine hormones may have impact on development or even contribute to diseases associated with neuroendocrine and immune dysfunction (Charmandari et al., 2005).

Activation of HPA axis to produce glucocorticoids can be triggered by various environmental stressors and different types of agents such as neurotransmitters, cytokines, damage-associated molecular pattern (DAMPs), and microbe-associated molecular patterns (MAMPs) (Blalock & Smith, 2007; Dantzer et al., 2008; Fleshner, 2013; Matthews, 2002). Glucocorticoid-induced signaling via HPA axis have been shown to play important regulatory role in stress and immune response. Since 1940s, glucocorticoids have been originally described and widely used as medication with anti-inflammatory effects in inflammatory disorders (Clark & Belvisi, 2012a). It has been also suggested that glucocorticoids can act as pro-inflammatory modulators having impact on cell migration, transcription factor activity and production of cytokines (Sorrells & Sapolsky, 2007). The primary anti-inflammatory action of glucocorticoids is regulated via glucocorticoid receptors by repression of pro-inflammatory genes encoding

cytokines, chemokines, cell adhesion molecules, inflammatory enzymes and receptors to restore homeostasis (Clark & Belvisi, 2012b). However, the role of glucocorticoids in regulation immune responses highly depends on basal state of immune system and timing and the pleiotropic effect of glucocorticoids has been described in previous reports. Glucocorticoids have simulative effect on immune response under acute stress as a prevention of inflammation, whereas exposure to chronic stress results in immunosuppressive effect of glucocorticoids (Cruz-Topete & Cidlowski, 2015; Dhabhar & McEwen, 1999; Sorrells & Sapolsky, 2007).

During the first few days of life, glucocorticoids provide homeostatic feedback to support underdeveloped immune system of neonate and prevent infectious diseases (Hulbert & Moisa, 2016). In study on rodents, chronic (6 days) administration of corticosterone resulted in suppression of antigen-specific cell-mediated immunity, compared to acute (4 hours) administration of the same dose of corticosterone which had simulative effect on immune response and enhanced number of lymphocytes in cervical lymph nodes in vivo (Dhabhar & McEwen, 1999). Under normal physiological conditions, glucocorticoids have been shown to have immunoenhancing effect and stimulate development of various organs such as GIT and lungs in neonatal stage (Hulbert & Moisa, 2016). These mechanisms are regulated via glucocorticoid binding to the glucocorticoid receptors inside host cell (Briassoulis et al., 2011). The importance of glucocorticoid receptors for neonatal development has been previously suggested in studies conducted glucocorticoid receptor gene knockout in neonatal mice which caused impaired maturation of lung resulting in respiratory failure and death of neonates (Kadmiel & Cidlowski, 2013).

As mentioned above, part of the stress response also involves production of catecholamine neurotransmitters, norepinephrine and epinephrine, by the fast-acting sympathetic nervous system

via SAM axis. The final release of catecholamines from adrenal medulla leads to automatic responses such as decreased activity of GIT, increased heart rate, blood pressure and respiration rate as part of "fight-or-flight" response associated mainly with acute stressors (Chen et al., 2015; Jafari et al., 2017). It has been suggested that catecholamine-induced signaling can also influence metabolic and immune related function that primarily happens through α- and β-adrenergic receptors (Chen et al., 2015). For example, epinephrine can cause reduction of migratory rates of keratinocytes resulting in inhibition of epithelization and delayed wound healing (Stojadinovic et al., 2012). Similarly, elevated plasma epinephrine has been associated with extended leucocyte recruitment in mice and delayed wound closure (Kim et al., 2014). Mechanism of this action in this study was described by additional experiments. Kim et al. (2014) suggested capability of epinephrine to influence cytokine IL-6 production by regulating its gene expression at the site of wound via β-adrenergic receptors dependent mechanism. Simply, epinephrine and norepinephrine can bind to adrenergic receptors located on surface of immune cells, which leads to cytokine production (Szelenyi & Vizi, 2007). As described above, glucocorticoids and catecholamine can also change host-microbial interactions (Lyte, 2014). Clearly, production of stress hormones during stress response have various effects on host physiology and microbial ecology of gut. Further research is required to enhance our understanding of physiological changes related to immune response and metabolism as well as interactions between host and gut microbiota during stress response. The catecholamines norepinephrine (NE) and epinephrine (EPI) exert their effects by binding to 7 transmembrane spanning G-protein-coupled cell-surface receptors termed adrenoceptors.

1.4.2 Role of neuroendocrine system in neonatal development

There has been increasing interest in regulation of energy metabolism and feed intake by neuroendocrine system in neonatal period. *In vitro* study reported that stimulation of adrenergic receptors with epinephrine induced intestinal secretion of peptides, GLP-1 in rodents (Claustre et al., 1999), which are known to regulated metabolic processes. Connor et al. (2015) suggested that biological actions and properties of glucagon-like peptides in ruminants are similar to those in non-ruminants. Adrenergic and serotonin receptors also regulate secretion of GLP-2 by enteroendocrine L cells, which increases nutrient absorption and intestinal growth (Burrin et al., 2003; Connor et al., 2015; Drucker, 2001). Schaff et al. (2015) reported that binding capacity of glucocorticoid and β2-adrenergic receptors were lower in preterm calves, compared to calves that were born at term, suggesting stress hormone receptors are dependent on stage of maturation in neonatal calves.

Furthermore, importance of stress hormones in prenatal and postnatal development of various organs has been widely studied over last few decades. Glucocorticoids in particular play role in of fetal and postnatal development of brain, lungs and GIT. Their physiological role is to regulate circadian and stress-associated feedback to maintain metabolic and homeostatic functions that are critical for life. However, stress hormones have quite special roles in regulation of immune responses during neonatal period. Thus, mammalian neonates, who are born with lack of immunocompetence, have typically elevated concentration of glucocorticoids in first few days of life (Hulbert & Moisa, 2016). Although mammals are born with developed organs, there is still requirement for postnatal development and maturation of organs. Neonates are equipped with cells of innate immunity such as phagocytic and granulocytic cells, but fully functioning immune system still requires formation of mucosal barriers in GIT and lungs as well as development of

adaptive immunity, which are triggered after birth by dietary components and commensal microbes (Walker, 2014). Elevated glucocorticoids during neonatal stage play role in maturation GIT and respiratory tract by their simulative effect on tight junction and formation of mucus layer (Hulbert & Moisa, 2016).

Similarly, catecholamines were also associated with immune system regulation and their effect may be influence by glucocorticoid treatment. Studies conducted on rodents show that sympathetic innervation involved in catecholamine signaling starts to develop in the second neonatal week and noradrenergic fibers can influence immune system via connection with lymphoid organs (Bakker et al., 2001). Moreover, the prenatal administration of glucocorticoids may have impact on development of the sympathetic pathways and heart rate (Bian et al., 1993). Additional dosage of glucocorticoids during fetal development could interfere with catecholamine signaling possibly leading to altered development of immune system (Bakker et al., 2001). There is a need for comprehensive investigation of effect of stress hormones on neonatal development and their mutual influence on one another.

1.5 Colostrum management

Feeding management and sufficient nutrient intake play a major role in neonatal development that reduces risk of mortality and morbidity (Hulbert & Moisa, 2016). Studies about ruminants suggest that early dietary experiences have a greater and more lasting effect than those occurring later in life (Distel et al., 1994; Eckert et al., 2015; Soberon et al., 2012; Soberon & Van Amburgh, 2017; Yanez-Ruiz et al., 2010). Colostrum, the first milk produced postpartum, contains high concentrations of immunoglobulins and innate immune components which protect neonate against infectious agents (Sears et al., 2017). Supply of colostrum is important for neonate since it contributes to various physiological changes, mainly related to metabolic and endocrine pathways.

Bovine colostrum supply calf several components important for development of immunity and GIT. The effect and composition of colostral components has been described by Blum (2006). Colostrum contains nutritional components that consist of minerals, trace elements, vitamins, essential fatty and amino acids, and non-nutrient components including immunoglobulins and eukaryotic cells such as lactocytes, leucocytes and erythrocytes (Blum, 2006). Colostrum provide the innate immune cells which can secrete various immune related components such as cytokines, antimicrobial proteins and peptides (e.g. lactoferrin, defensins and tranferrin) as well as passively acquired maternal immunoglobulins which are important for host-defensive mechanisms against potential pathogens (Stelwagen et al., 2009).

Numberous studies have reported that feeding colostrum have a positive effect on postnatal development. It has been suggested colostrum feeding supports development of GIT and growth of commensal bacteria. For example, enhanced nutrient intake and feeding management in pre-weaned calves are the major environmental factors altering epigenetic programing that had positive effect on lactation milk yield (Soberon et al., 2012). The effects of ingested colostrum depend on the supply of its nutrients and non-nutrient components (Blum, 2006). It has been suggested that prolonged colostrum feeding can influence number of binding sites of insulin receptors in intestinal mucosa of neonatal calves (Hammon & Blum, 2002). Besides the positive colostral effect on host, colostrum may contribute to proper colonisation of gut microbiota in small intestine. It has been suggested heat treated colostrum increases intestinal colonization of *Bifidobacterium* and reduces *E. coli* proliferation in newborn calves (Malmuthuge et al., 2015a). Also, increased mortality within the first 3 weeks of life was reported in calves when colostrum was not provided (Hulbert & Moisa, 2016), suggesting the importance of colostrum feeding.

Altogether, these finding show colostrum have simulative effect on maturation and function of GIT and immune system of neonate.

1.6 Summary of literature review in relation to research hypotheses and objectives

To date, a considerable number of studies about neuroendocrine system and its association with host physiology have reported that hormones produced by neuroendocrine cells play a role in neonatal development in various animal models, mainly rodents. Similarly, previous mainly *in-vitro* and culture-based studies suggest the existence of gut-brain axis in which gut microbiota contributes to changes of host physiology via direct communication with host. However, there has been limited attempt to study the neuroendocrine system and host microbial interactions using *in-vivo* techniques to validate the relationship between microbes and host within ecological complexity of the gut. Previous findings on calves reported that colostrum feeding increased binding capacity of glucocorticoid and adrenergic receptors in liver (Schaff et al., 2014). However, it is currently unknown whether feeding management can affect the neuroendocrine system in adrenal glands and intestine of newborn calves. Also, accessibility to collect samples of different GI regions is sometimes limited. Thus, previous *in-vivo* studies conducted on gut bacteria depended mainly on identification and quantification of microbes from feces, which did not allow investigation of the environmental differences among GI regions. The comparison of gut bacteria from different regions of GIT may improve our understanding of gut ecology at regional level.

The aim of this project was to study the effect of delaying and duration of colostrum feeding on expression of genes involved in neuroendocrine functions and bacterial population. We hypothesised that different feeding managements alter bacterial proliferation as well as expression of neuroendocrine genes in tissue from different GI region (ileum, colon) and consequently lead to changes in fecal cortisol concentration. The main objectives of this thesis were: 1) to investigate

effect of duration of colostrum feeding on changes in expression of neuroendocrine genes and abundance of active tissue-associated bacteria in ileum and colon; 2) to investigate the effect of delayed colostrum feeding on fecal cortisol concentration and abundance of digesta-associated gut bacteria in rectum and colon. To our knowledge, this is the first study to investigate effect of duration and delayed colostrum feeding on expression of adrenal and extra-adrenal neuroendocrine genes and fecal cortisol, respectively.

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Chapter 2. Effect of colostrum feeding management on expression of neuroendocrine gene and gut bacterial population in neonatal male Holstein calves

2.0 Introduction

Adequate intake of nutrient and non-nutrient components of colostrum and milk during first days of life is required during the first a few days of life to support neonatal calf development and adaption to extra-uterine life. Postnatal development of the gastrointestinal tract is vital to adapt changes from maternal nutrition supply to enteral feeding (Blum, 2006). Colostrum feeding to newborn calves is a common practice in dairy industry and good colostrum management is important for neonatal development. Colostrum contains hormones and growth factors which can bind to host receptors on the surface of the gut mucosa to stimulate maturation of gastrointestinal tract (GIT) (Hammon et al., 2013). Moreover, since newborn calves lack immunocompetence (Hulbert & Moisa, 2016), their development of passive immunity relies on transfer of immunoglobulins (mainly IgG) from colostrum (Hammon et al., 2013). Thus, management strategies in feeding neonatal calves is important to ensure adequate intake of nutrients, passive immunity, and bioactive components.

Commonly, calves are fed with milk or milk replacer after the first colostrum feeding on farm. Although concentration of colostral and milk components might differ based on quality (Boudry & Thewis, 2009), milk generally contains higher concentration of lactose than colostrum (Ontsouka et al., 2003). Beside its nutrient components, colostrum contains high concentrations of immunoglobulins, growth factors, bioactive and immunological components (Kuhne et al., 2000) and plays important immunomodulatory role in first few days of calf life. For example, colostral immunoglobulins account for up to 80% of total proteins, compared to the proportion of immunoglobulins (1% of total protein) in milk (McGrath et al., 2016). Previous

reports suggest colostrum management can have an effect on metabolic processes and energy supply in pre-weaned calves. For instance, calves fed colostrum on day 1 had higher concentration of plasma glucose, bilirubin, insulin and insulin-like growth factors, compared to those fed colostrum on day 2, suggesting that colostrum intake stimulates metabolic processes (Hadorn et al., 1997). Similarly, prolonged feed restriction of colostrum and milk in pre-weaned calves resulted in increased concentrations of cholesterol, albumin and cortisol and reduced insulin in plasma (Hammon et al., 2002). These findings suggest that different strategies of colostrum feeding can affect metabolic processes and level of neuroendocrine hormones in neonatal calves. To date, most studies have been focused on the effect of colostrum feeding on metabolic and endocrine changes at systemic level from blood or at local level in liver in newborn calves. However, little is known about the effect of colostrum feeding management on the expression of genes involved in neuroendocrine function in adrenal glands and intestine in newborn calves.

Neuroendocrine system can be defined as set of cells present in various organs which are capable of hormone/neurotransmitters production (Toni, 2004). Hypothalamic-pituitary-adrenal (HPA) axis, a major neuroendocrine system that regulates stress response (Farzi et al., 2018), is underdeveloped at birth and develops together with the immune system to make calves immunocompetent and resilient (Hulbert & Moisa, 2016). Stress hormones are primarily produced via HPA axis and sympathetic-adrenal-medullary (SAM) axes in adrenal glands (Chen et al., 2015). However, emerging evidence suggests extra-adrenal production of stress hormones such as glucocorticoids (GCs) also occurs in intestinal mucosa in rodents and human tissue (Bouguen et al., 2015; Cima et al., 2004; Kostadinova et al., 2014; Sidler et al., 2011). The local synthesis of GCs is thought to play a role in regulating local immune, metabolic and homeostatic processes

(Noti et al., 2009; Talaber et al., 2013). However, whether GCs are produced in intestinal mucosa in neonatal calves is currently unknown.

Microbial colonisation during neonatal development may also play a role in establishment of immune tolerance of host to commensal bacteria (Zhao & Elson, 2018). As reported previously, colostrum feeding has an impact on microbial colonisation in intestine of neonatal calves. For instance, Malmuthuge et al. (2015a) reported that feeding colostrum within first 12 hours of life increased proliferation of *Bifidobacterium* spp. and reduced *E. coli* colonisation in the small intestine of newborn calves. Similarly, delaying colostrum feeding resulted in lower abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. in large intestine of newborn calves, compared to that in calves fed with colostrum soon after birth (Fischer et al., 2018). It has been suggested that gut microbial colonisation in early life may influence neuroendocrine responses to stress (Toni, 2004). However, it is currently unknown whether prolonged colostrum feeding influences microbial colonisation.

The present study aimed to investigate effect of duration of colostrum feeding management on abundance of tissue-associated active bacteria and expression of neuroendocrine genes in adrenal glands and intestine mucosa. We hypothesized that different compositions of nutrient and non-nutrient components from colostrum and milk influence expression of genes related to the neuroendocrine system at systematic and local level and abundance of active gut microbiota is associated with the expression of these genes in ileum and colon tissue. Therefore, the objectives of this study were 1) evaluate effect of different management feeding on neuroendocrine gene expression and its relationship with the abundance of tissue-associated bacteria.; 2) to design primers targeting genes that are involved in regulation of GCs production and receptors that binds to GCs and neurotransmitters such as catecholamines, serotonin and γ-Aminobutyric; 3) to detect

expression of neuroendocrine genes in adrenal glands, ileum and colon in newborn calves. Outcomes of this study contribute to better understanding of changes in neonatal neuroendocrine and intestinal microbiota in newborn calves, which play an essential role in postpartum development of the immune system and GIT.

2.1 Materials and methods

2.1.1 Experimental design

The animal study was performed at Dairy Research and Technology centre of the University of Alberta from January to August of 2018. Experimental protocol (AUP00001595) was reviewed and approved by the Animal Care and Use Committee (University of Alberta) and all procedures were performed following guidelines approved by the Canadian Council on Animal Care. All calves were separated from the dam immediately after birth and kept in individual pens that were disinfected with Virkon (1% solution- 2 tables in 1L of water) and rinsed off with water. Calves were dried with towels and stimulated for 20 min. Calibrated electronic scale (Digi-Star SW300, Digi-Star L.L.C., Fort Atkinson, WI) was used to measure birth weight. Heat treated colostrum was provided by the Saskatoon Colostrum Company Ltd. (SCCL, Saskatoon, SK, Canada). Whole milk was pursued from Dairy Research Technical Centre (DRTC). Colostrum and whole milk were stored at -20°C and thawed in water bath until temperature 39°C was reached before feeding.

Holstein bull calves (n= 24) with average body weight (42.5 ± 0.97 kg) were fed first colostrum meal (fed at 7.5% body weight) through nipple bottle within one hour of life. Followed with second feeding, calves were randomly assigned into 1 of 3 feeding managements with the amount of meal being at 5% of body weight. Depending on treatment, calves received milk (whole milk, WM; n=8), mixture of 50% colostrum and 50% whole milk (mixture of colostrum and milk,

CM; n=8) or colostrum (colostrum feeding only, CF; n=8) 10 hours following the first meal and every subsequent meal 12 hour following that. All animals were euthanized at 75 hours after birth with intravenous injection of pentobarbital sodium (Euthanol, Vetoquinol, Lavaltrie, Quebec, Canada) at 0.125 ml per kg body weight. Ileum, colon, right and left adrenal glands tissues were collected within less than 30 min after euthanization using sterile dissection tools following procedure as reported previously (Liang et al., 2014). Samples were washed three times with phosphate buffered saline (PBS), feezed with liquid nitrogen and stored in sterile bags at -80°C.

2.1.2 RNA extraction

Tissue samples were ground into fine powder under liquid nitrogen condition prior to RNA isolation. Total RNA from adrenal glands, ileum and colon tissue (~ 0.1 g) was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's procedures. Assessment of RNA integrity and quantity was done using Agilent 2200 Tape station (Agilent Technologies, Santa Clara, CA) and Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA). RNA samples exhibiting integrity values of more than 7.0 was used for further analysis. Total RNA was reversely transcribed using iScript reverse transcription Supermix for RT-qPCR (Bio-Rad, California, USA) to generate strands of complementary DNA (cDNA). Quantitative RT-PCR was performed with ViiA^{TM} 7 Real Time PCR and QuantStudio^{TM} 6 Real Time PCR (Applied Biosystems, USA) using the SYBR green chemistry with specific primers targeting each bacterial group (table 2-1) and host neuroendocrine genes (Table2) to obtain bacterial data.

2.1.3 Primers design and reference gene validation

Sequences of mRNA for targeted genes of *Bos Taurus* species was obtained through National Centre of Biotechnology Information (NCBI) website, www.ncbi.nlm.nih.gov/. Primer Express Software was used to design primers targeting 11 genes related to neuroendocrine system

(Table 2-2). Among these selected genes, two genes are directly involved in glucocorticoid production; Steroidogenic acute regulatory protein (*StAR*) and Cytochrome P450, subfamily XI B, polypeptide 1 (*CYP11B1*), which encode key enzymes in steroids and glucocorticoid production, respectively (Liu et al., 2013). In addition, eight genes encoding receptors for glucocorticoids, catecholamines (epinephrine and norepinephrine), adrenocorticotropic hormone (ACTH), serotonin and γ-aminobutyric acid were selected since hormones of neuroendocrine system exert their effect via binding to the receptors. Specifically, genes encoding stress hormones receptors, nuclear receptor subfamily 3 group C member 1 (*NR3C1*), which is encoding glucocorticoid receptor (Juszczak & Stankiewicz, 2018); melanocortin 2 receptor (*MC2R*) encoding ACTH receptor (Proudnikov et al., 2008) and two genes encoding adrenergic receptors for catecholamines; Adrenoceptor beta 2 (*ADRB2*) and adrenoceptor alpha 1A (*ADRA1A*) (Li et al., 2014). Also, gene encoding serotonin transporter, Solute carrier family 6 member 4 (*SLC6A4*) and two genes encoding serotonin receptors; 5-hydroxytryptamine receptor 2B (*HTR2B*) and 5-hydroxytryptamine receptor 4 (*HTR4*) (Corominas et al., 2010) were selected. Additionally, genes encoding receptor for *neurotransmitter* γ-aminobutyric acid gamma-aminobutyric acid type A receptor beta2 subunit (*GABRB2*) and Gamma-aminobutyric acid type B receptor subunit 1 (*GABBR1*) (Zhao et al., 2007) were also included.

In silico validation of designed primers was performed using Primer-BLAST followed by primer testing using PCR to confirm product length. The expression of *β-actin* and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) reference genes in ileum, colon and adrenal glands was tested using NormFinder algorithm and PROC MIXED procedure of SAS (v9.2; SAS Institute Inc., Cary, NC). Since *GAPDH* had more stable expression between and within tissues compared to *β-actin*, the further analysis was performed using *GAPDH* as an

endogenous house keep gene. For each sample, the expression of targeted gene was presented as ΔCt calculated by subtracting the average Ct (Cycle threshold) of the *GAPDH* from the average Ct of targeted gene. Differences in gene expression among adrenal glands, ileum and colon tissue were evaluated by calculating fold change using $2^{-\Delta\Delta CT}$ method.

2.1.4 Statistical analysis

The effect of treatment on expression of gene related to neuroendocrine system and bacterial population was evaluated using non-parametric Kruskal Wallis test followed by Dunn's Multiple Comparison test in R software. Benjamini-Hochberg method was used to adjust P values and significant differences were declared at P < 0.05 or P< 0.01 and tendencies of differences at $0.05 < P < 0.10$. Gene expression delta Ct (ΔCt) and bacterial data (Copy number / g) were presented in least square means (LSM) with standard error of mean (SEM). Pearson's correlation in R software using function Rcorr was used to investigate a potential relationship between neuroendocrine gene expression and gut bacteria. Level of significance for correlation coefficients was declared at r > 0.5 or r < - 0.5 with P < 0.05.

2.2 Results

2.2.1 Expression of genes involved neuroendocrine functions in adrenal glands, ileum and colon tissues

The expression of 11 selected genes was detected in all three tissues, including adrenal glands, ileum and colon (Figure 2-1.). Expression of *CYP11B1* (-2.22 ± 0.14 ΔCt mean ± SEM) was higher in adrenal glands, compared to expression levels in ileum (7.14 ± 0.53) and colon (5.61 ± 0.64) tissue (P< 0.01 with fold change 244.04 and 101.34), respectively. Similarly, *StAR* had higher expression in adrenal glands (-3.11 ± 0.09; P< 0.01 with fold change 872.62 and 652), when compared to its expression in ileum and colon tissue (6.66 ± 0.15 and 6.24 ± 0.49), respectively.

Expression of *MC2R* in adrenal glands (2.56 ± 0.09) was also higher (P < 0.01 with fold change 9.24 and 6.89), compared to that in ileum and colon (5.77 ± 0.28 and 5.34 ± 0.67), respectively.

In contrast, expression of *HTR4* was higher in ileum (0.16 ± 0.15; P< 0.01 with fold change 191.46) and colon tissue (-1.05 ± 0.28; P< 0.01 with fold change 442.9), compared to its expression level in adrenal glands (7.74 ± 0.28). There was also higher expression of *ADRA1A* and *ADRB2* in ileum (7.37 ± 0.43 and 3.9 ± 0.12; P<0.01 with fold change 4.86 and 4.31) and colon (5.61 ± 0.64 and 3.71 ± 0.23; P<0.01 with fold change 16.55 and 4.92), compared to their expression levels in adrenal gland (9.66 ± 0.56), respectively.

2.2.2 Treatment effect on expression of genes related to neuroendocrine system

Different feeding management affected (P < 0.05) the expression of *ADRA1A* and *StAR* in adrenal glands (Figure 2-2). Calves that were fed colostrum (CF) had higher (P < 0.05) expression of *ADRA1A* (7.17 ± 0.84 ΔCt mean ± SEM) in adrenal glands, when compared to its expression level in calves that received milk (WM; 10.97 ± 0.59) and mixture of colostrum and milk (CM; 11.04 ± 0.41). Expression of *StAR* in adrenal glands was higher (P < 0.05) in CM (-2.76 ± 0.15), compared to that in WM (-3.26 ± 0.12) and CF (-3.26 ± 0.14).

In addition, different feeding had significant effect (P < 0.05) on expression of *ADRA1A* and *NR3C1* (Figure 2-3) and tended to affect (P<0.1) expression of *SLC6A4* and *HTR4* in ileum. Expression of *NR3C1* in ileum was significantly lower (P < 0.05) in CF (1.14 ± 0.17), compared to its expression in CM (0.52 ± 0.12) and WM (0.5 ± 0.12). Conversely, expression of *ADRA1A* in ileum was significantly higher (P < 0.05) in CF (5.72 ± 0.3) compared to its expression in CM (8.4 ± 0.38). There was tendency for difference in ileal *ADRA1A* expression between CF and WM (7.68 ± 0.84). Expression of *SLC6A4* in ileum tended to be lower (P <0.05 and P < 0.1) in WM (8.7 ± 0.35), compare to CM (6.34 ± 0.96) and CF (6.05 ± 1.38), respectively. Ileal expression of

HTR4 tended to be lower (P < 0.01) in WM (0.43 ± 0.35), compared to CF (-0.21 ± 0.19). However, there was not significant difference (P ≥ 0.1) in expression of *HTR4* in ileum between CF and CM (0.26 ± 0.21).

In colon tissue, feeding management had significant effect (P < 0.05) on expression of *MC2R* and *ADRA1A* (Figure 2-4). Expression of *MC2R* in colon was significantly lower (P < 0.05) in CF (7.46 ± 0.43), compared to WM (5.15 ± 0.89) and CM (3.27 ± 1.52). Colonic expression of *ADRA1A* was significantly higher (P < 0.05) in WM (4.7 ± 0.76), compared to its expression in CM (7.87 ± 0.42). However, there was not a significant difference (P ≥ 0.1) in the expression of *ADRA1A* between WM and CF (6.86 ± 0.81).

2.2.3 Treatment effect on tissue-associated active bacteria

Feeding management affected the abundance of active tissue-associated *Lactobacillus* spp. in colon (P < 0.05) and ileum (P < 0.1) (Figure 2-5A) but did not affect (P ≥ 0.1) the abundance of active *Bifidobacterium* spp. associated with ileum and colon tissues (Figure 2-5B). Different feeding management influenced (P < 0.05) the abundance of active tissue-associated *E. coli* in the colon (Figure 2-6), while it did not affect (P ≥ 0.1) the abundance of active tissue-associated *E. coli* in the ileum.

Calves that received only colostrum (CF) tended to have higher (P < 0.05 and P < 0.1) abundance of active tissue-associated *Lactobacillus* spp. (4.19 ± 1.66 x 10^8 16S rRNA gene copy/g of sample ± SEM) in the colon, compared to calves that were subsequently fed WM (5.77 ± 2.66 x 10^7 16S rRNA gene copy/g of sample ± SEM) and CM (2.15 ± 1.34 x 10^8 16S rRNA gene copy/g of sample ± SEM). Abundance of active tissue-associated *E. coli* (35.6 ± 5.73 x 10^7 16S rRNA gene copy/g of sample ± SEM) in CF was higher (P < 0.05 and P < 0.1) in the colon, compared to

that under management feeding CM (13.5 ± 5.83 x $10^7$16S rRNA gene copy/g of sample $\pm$ SEM)

and WM (15.0 ± 4.63 x 10^7 16S rRNA gene copy/g of sample $\pm$ SEM), respectively.

In regards to bacteria associated with ileum tissue, calves fed colostrum formula (CF) tended to have higher (P < 0.1) abundance of active *Lactobacillus* spp. (8.95 ± 6.29 x 10^7 16S rRNA gene copy/g of sample $\pm$ SEM), compared to calves that were fed colostrum as first meal and received whole milk (WM) as subsequent meal (28.7 ± 4.99 x 10^4 16S rRNA gene copy/g of sample $\pm$ SEM). However, prolonged feeding of colostrum (CF) did not have significant effect (P ≥ 0.1) on abundance of active *E. coli* in ileum (24.2 ± 9.28 x 10^5 16S rRNA gene copy/g of sample $\pm$ SEM), compared to abundance of *E. coli* associated with ileum tissue (3.58 ± 1.46 x 10^5 16S rRNA gene copy/g of sample $\pm$ SEM) in calves that were subsequently fed milk (WM).

2.2.4 Correlation analysis

Pearson's correlation analysis revealed a positive correlation (r = 0.76; P = 0.000019) between abundance of active tissue-associated *Lactobacillus* spp. and *E. coli* in colon tissue, suggesting a potential association of these bacterial groups (Figure 2-7). We also observed positive correlation between gene expression of *HTR4* and *HTR2B* and both bacterial groups, *Lactobacillus* spp. (r=0.57, P=0.0036 and 0.64 P=0.00072) and *E. coli* (r=0.51, P=0.01 and r=0.77, P=0.0000089) in colon tissue (Figure 2-8), respectively. Additionally, there was a positive correlation (r=0.52, P= 0.0094) between abundance of active tissue-associated *E. coli* and gene expression of *ADRB2* in colon (Figure 2-9). However, no significant correlations between gut bacteria and host gene expression of neurotransmitter receptors were observed in ileum.

2.3 Discussion

Concentration of stress hormones, glucocorticoids and catecholamines are elevated at birth in neonatal calves which are needed to support neonatal development of immune system and GIT

(Hulbert & Moisa, 2016; Schaff et al., 2014). Production of stress hormones primarily happens in adrenal glands, however, other organs such as brain, thymus, skin, vascular system, lungs and intestine are also capable of producing GCs and catecholamines locally (Eisenhofer et al., 1997; Noti et al., 2009; Pacak, 2011). The extra-adrenal production of stress hormones in other tissues have been reported in rodents and human tissues (Cima et al., 2004; Eisenhofer et al., 1997; Kostadinova et al., 2014; Pacak, 2011; Sidler et al., 2011; Talaber et al., 2013), however, it is currently unknown whether stress hormones are also produced by intestinal mucosa in neonatal calves.

In the present study, expression of genes involved in regulation of GCs production and receptor for GCs, catecholamines and serotonin was detected in adrenal glands, ileum and colon tissues of 3-day old calves. In particular, the expression of *StAR* and *CYP11B1* genes that encode key enzymes in steroid and glucocorticoid production, was detected in ileum and colon tissue, suggesting that intestinal mucosa can potentially synthesize GCs in neonatal calves. Moreover, expression of *MC2R* (ACTH receptor) was also detected in ileum and colon. *MC2R* is important in the stress response, since it encodes a protein involved in regulation of cortisol secretion (Proudnikov et al., 2008). These results correspond with previous findings about capability of intestinal mucosa to synthesized GCs in rodents and human intestinal tissue (Cima et al., 2004; Eisenhofer et al., 1997; Kostadinova et al., 2014; Noti et al., 2009; Sidler et al., 2011), suggesting that ruminant gut may have similar function as that reported in monogastric animals. However, the expression of *StAR*, *CYP11B1* and *MC2R* was significantly higher in adrenal glands, compared to intestinal tract of the claves, suggesting that extra-adrenal production of stress hormones may not have a great impact on endocrine at systematic level, but rather regulates various physiological processes including immune system and metabolism at local level (Kostadinova et al., 2014). On

the other hand, *ADRA1A*, *ADRB2* and *HTR4* had higher expression in the mucosa of ileum and colon, compared to that in adrenal glands. *ADRA1A* and *ADRB2* encode α1- and α2- adrenergic receptors for epinephrine and norepinephrine, respectively, and *HTR4* encodes receptor for serotonin (5-hydroxytryptamine). The higher expression of these genes in the gut may indicate that the stimulation of internal signal transduction by these neurotransmitters exert physiological changes in regulation of immune system, food intake, postnatal energy balance (Hodge et al., 2013; Santulli et al., 2012; Scanzano & Cosentino, 2015). Since calves are born with immature immune system and gastrointestinal tract (Blum, 2006; Hulbert & Moisa, 2016), the stimulation of signal transduction via these neurotransmitters may play a role in adaptation to environmental changes in neonatal development. However, further studies are required for better understanding of the effect of catecholamines and serotonin receptors on intestinal mucosa in neonatal calves by measuring the protein level of these receptors.

Our study also found that feeding different composition of colostrum and milk components affected expression of neuroendocrine genes in adrenal glands and intestine of newborn calves. Calves fed mixture of colostrum and milk (CM) had higher expression of *StAR* in adrenal glands, compared to calves fed whole milk (WM) and colostrum (CM), suggesting potential changes in steroidogenesis when calves under different feeding management. However, in a recent review, Miller (2017) suggested that low level of steroidogenesis can also occur in the absence of *StAR*. Furthermore, the expression of *CYP11B1* encoding enzyme that converts progesterone into cortisol in adrenal glands was not affected by feeding treatment, suggesting that treatment did not affect expression of genes related to production of glucocorticoid in adrenal glands. Thus, CM feeding may have minor effect on steroid production and these changes might be associated with production of different steroids than glucocorticoid. Schaff et al. (2014) reported that calves fed

colostrum tend to have higher binding capacity of glucocorticoid and α1- adrenergic receptors in the liver, compared to calves that received milk-based formula. It has been proposed that such a stimulation of adrenergic receptors via catecholamines binding in calf liver is involved in modulating processes related to glucose production (Carron et al., 2005; Schaff et al., 2014). However, whether colostrum affects adrenergic receptors in adrenal glands, a main location of catecholamines production, is currently unknown. Our results showed that calves fed only colostrum (CF) had higher expression of *ADRA1A* in adrenal gland, suggesting colostral feeding may stimulate adrenal α1-adrenergic receptors in calf adrenal glands, which needs further study to verify this speculation to measure the receptors at protein level.

Compared to changes in gene expression induced by feeding treatments in adrenal glands, different colostrum feeding caused more changes in the expression of neuroendocrine genes in the intestinal tract of the calves, notably genes for GCs, epinephrine and serotonin receptors. Prolonged feeding of colostrum (CF) tended to induce higher expression of *ADRA1A* in ileum, which corresponds to the similar effect in adrenal glands. However, expression of *ADRA1A* in the colon was higher in calves that were subsequently fed whole milk (WM). These results suggest that colostrum and milk-derived components stimulate α1-adrenergic receptors in ileum and colon, respectively. In addition, calves fed only colostrum (CF) tended to have increased expression of serotonin receptor (*HTR4*) and transporter (*SLC6A4*) in ileum, suggesting prolonged ingestion of colostrum may have effect on serotonin-mediated signaling in ileum. Similar to adrenergic receptors, stimulation of serotonin receptors has been suggested to play a role in modulation of immune response and metabolism (Keszthelyi et al., 2009; Shajib & Khan, 2015). Previous reports also suggest a potential interaction of catecholamines and serotonin due to overlapping nerve fiber of these neurotransmitters (Hensler et al., 2013; Kim & Camilleri, 2000), suggesting that

serotonin-modulated signaling in ileum could interplay with signaling induced by α1-adrenergic receptor to regulate local immune and metabolic processes. However, colostrum feeding did not induce changes in expression of *HRT2B* in ileum, and the expression of *HTR4*, another serotonin receptor and *SLC6A4* in colon, suggesting that different colostrum feeding may not exert a great effect on metabolic and immune changes mediated by serotonin receptors in intestine. In addition, expression of *NR3C1* and of *MC2R* was higher in ileum and colon respectively under WM and CM feeding, compared to that under CF. This suggests that ingestion of milk-derived components can have effect on expression of glucocorticoid and adrenocorticotropic receptors in ileum and colon, respectively. Interestingly, Schaff et al. (2014) reported that hepatic expression of *NR3C1* was not affected by colostrum and milk feeding, suggesting colostrum and milk feeding may have different effect on hepatic and intestinal expression of glucocorticoid receptor. Since colostrum contains higher concentration of cortisol than milk (Shutt & Fell, 1985), we speculate that decrease in expression of *NR3C1* and *MC2R* in ileum and colon induced by prolonged colostrum feeding (CF) could reduce systemic transport of glucocorticoids in intestine. Further studies are needed to investigated possible interactions of signaling mediated by serotonin and adrenergic receptors in intestine.

The differences in expression of genes encoding α1-adrenergic and serotonin receptors among ileum and colon induced by feeding treatment can be explained by the fact that ileum and colon have different functions (Schottstedt et al., 2005) and slightly different structural and physiological properties of epithelium (Bergmann, 2017; Kim & Ho, 2010). Given these difference in structure, the small intestine is known to have higher absorptive capacity than large intestine (Schottstedt et al., 2005). Moreover, ileum has greater number of immune cells compared to colon, which is richer in mucous cells (Kim & Ho, 2010). Previous reports indicate that stimulation of

neurotransmitter receptors induces changes in physiological processes related to metabolism, immune system and tissue development (Hodge et al., 2013; Scanzano & Cosentino, 2015; Schaak et al., 2000; Schaff et al., 2014; Szelenyi & Vizi, 2007). This evidence can be explained by fact that adrenergic nerve fibers are present in Peyer's patches and in non-follicular mucosa in close proximity to immune cells, including dendritic cells and mast cells of mucosa (Lyte et al., 2011). Furthermore, adrenergic and serotonin receptors regulate secretion of GLP-2 by enteroendocrine L cells, which increases nutrient absorption and intestinal growth (Burrin et al., 2003; Connor et al., 2015; Drucker, 2001), suggesting a role of neurotransmitters in regulation of metabolic processes. Thus, we speculate that colostrum/milk-derived component may have different effect on expression of neuroendocrine genes among GIT regions depending on histological and functional features of epithelium. Milk-derived components such as minerals and lactose may exert effect on metabolic pathways related to fermentation and nutrient absorptions in colon, whereas bioactive and immune components from colostrum may also play role in immune response mediated via α1- adrenergic and serotonin receptors in ileal lymphoid tissue. However, further studies are required for better understanding of molecular mechanisms behind these processes and interaction of serotonin and α1- adrenergic receptors in modulation of metabolic and immune processes in intestine.

It has been recently suggested by Malmuthuge et al. (2015a) that ingestion of heat-treated and fresh colostrum soon after birth enhances the abundance of total bacterial (copy number of 16S rRNA gene/g of sample) in small intestine of calves within 12 hr of life, compared to that in calves that did not receive colostrum. In the same study, calves fed heat-treated colostrum soon after birth had higher prevalence of tissue-associated *Bifidobacterium* spp. and lower prevalence of tissue-associated *E. coli* in small intestine. Similarly, delayed colostrum feeding tended to

reduce prevalence of tissue-associated *Bifidobacterium* and *Lactobacillus* spp. in colon at 51 hr of life, compared to prevalence of the probiotics in large intestine of calves that received colostrum soon after birth (Fischer et al., 2018). These studies suggest that colostrum accelerate microbial colonization, especially colonization of beneficial microbiota. In the present study, different feeding management did not affect the abundance of tissue attached active *Bifidobacterium* spp. in ileum and colon, suggesting that the changes of the population at 75 hr of life is not affected by the nutrients as observed in calves at 12 hr and 51 hr of life. Further studies are required for better understanding of colostrum feeding effect on abundance of active *Bifidobacterium* spp. in intestine of newborn calves. Fischer et al. (2018) also reported that colostrum feeding increases prevalence of tissue-associated *Lactobacillus* spp. in large intestine of calves fed colostrum soon after birth, compared to calves that received colostrum 6h or 12h after birth. Our results show that prolonged colostrum feeding (CF) significantly increased the abundance of active tissue-associated *Lactobacillus* spp. in ileum and colon of newborn calves, compared to calves that were subsequently fed milk, suggesting that colostrum feeding accelerate colonisation of active tissue-associated probiotic *Lactobacillus* spp. in calf intestine. Colostrum generally contains higher concentration of casein and lactoferrin than milk (McGrath et al., 2016), which have been shown to promote growth of *Lactobacillus* species (Artym & Zimecki, 2005; Carvalho et al., 2011). Furthermore, our results indicate prolonged colostrum feeding (CF) significantly increased abundance of active tissue-associated *E. coli* in calf large intestine, compared to calves that received milk (WM) or mixture of milk and colostrum (CM) as subsequent meal, suggesting that prolonged colostrum feeding stimulates *E. coli* growth in large intestine of newborn calves. Furthermore, we identified a strong positive correlation ($r = 0.76$; $P = 0.000019$) between abundance of *Lactobacillus* spp. and *E. coli*, suggesting a potential association of these bacterial

groups. In vertebrates, *E. coli* is predominant gram-negative bacteria that inhabits GIT and generally lives in symbiosis with host (Tenaillon et al., 2010). For example, commensal *E. coli* has been reported to prevent growth of competitive and pathogenic bacteria and supply host vitamin K2 (Fischer et al., 2018; Tenaillon et al., 2010).

Colostral oligosaccharides are important energy source of probiotic bacteria, which can prevent adhesion of pathogens to intestinal epithelium to prevent infection (Malmuthuge et al., 2015a). Compared to colostrum, however, milk has higher concentration of lactose (McGrath et al., 2016) which can be further converted to lactose-derived oligosaccharides by probiotic enzymes (Gänzle, 2012). Furthermore, the lactose-derived oligosaccharides can also inhibit adhesion of enteropathogenic *E. coli* to GIT epithelial cells in vitro (Shoaf et al., 2007). Therefore, calves that received mixture of colostrum and milk may be provided with diverse types of oligosaccharides that have been previously shown to prevent infection by reduced adhesion of pathogens to host epithelial cells. Also, lactoferrin and casein, that are generally present in high amounts in colostrum, are known as protein-derived peptide of tryptophan, a serotonin precursor (Nongonierma & FitzGerald, 2015; Slominski et al., 2002). Moreover, Several different *Lactobacillus* spp. have been reported to produce serotonin, dopamine and other hormone-like metabolites *in vitro* and this ability was enhanced in presence of gram positive bacteria, including *E.coli* (Ouml et al., 2012). Similarly, recent study on germ free and gnotobiotic mice provides evidence that commensal bacteria can produce serotonin in the gut lumen *in vivo* (Hata et al., 2017). Furthermore, we observed positive correlation between gene expression of *HTR4* and *HTR2B* and both bacterial groups, *Lactobacillus* spp. (r=0.57, P=0.0036 and 0.64 P=0.00072) and *E. coli* (r=0.51, P=0.01 and r=0.77, P=0.0000089) in colon tissue, suggesting a potential relationship of these gut bacteria with serotonin receptors in colon mucosa of newborn calves.

Thus, previously described mechanism of serotonin production by *Lactobacillus* spp. together with our results suggest increased *Lactobacillus* spp. and *E. coli* could be associated with increased expression of serotonin receptor and transporter genes observed in ileum. These findings suggest a potential ecological interaction between active tissue-associated *E. coli* and *Lactobacillus* spp. and their association with host neurotransmitter receptors.

Since colostrum is rich in various immunological components, including immunoglobulins, cytokines and leucocytes (Blum, 2006) this could be one of mechanisms that helps calves to develop immune tolerance to commensal microbiota in neonatal period (Tenaillon et al., 2010; Zhao & Elson, 2018). This also emphasis the importance of good strategies of colostrum management in calves rearing which is known to stimulate postnatal development of immune system and colonisation of commensal bacteria in newborn calves (Hammon et al., 2013; Hulbert & Moisa, 2016). Moreover, Bergmann (2017) reported that hindgut of ruminants is inhabited by greater number of microbial biomass, which is due to the environmental conditions such as lower pH in small intestine. Also, Kim and Ho (2010) reported that gut microbiota are mainly associated with outer loose mucus layer of intestinal epithelium, which is thicker in colon, compared to intestinal mucosa in ileum. Since there were no significant correlation observed between gut bacteria and host neurotransmitter receptors in ileum, we propose that the interactions between gut bacteria and host receptors may differ depending on environmental and structural properties of GIT regions. However, future research is required for better understanding of the host-microbial interactions and their region-dependent changes.

2.4 Conclusion

To our knowledge, this is the first study that detected and characterized expression of genes involved in neuroendocrine functions in the adrenal gland and intestinal tissues of neonatal dairy

calves. In addition, the altered expression of these genes in adrenal glands, ileum and colon tissues were also observed when calves were under different colostral and milk components feeding strategies. Different colostrum management induced changes in the expression of neuroendocrine gene expression mainly in the intestine, when compared to that in adrenal glands. Prolonged Colostrum feeding increased expression of serotonin receptor and transporter genes (*HTR4, SLC6A4*) and α1- adrenergic receptor (*ADRA1A*), decreased glucocorticoid receptor gene (*NR3C1*) in the ileum. Our results further indicate that colostrum feeding may exert different effect on expression of α1- adrenergic receptor gene (ADRA1A) among GIT regions. Future studies should therefore focus on metabolic and immune processes mediated by receptors of these neurotransmitters in different regions of GIT. Furthermore, colostrum feeding increased the abundance of tissue-associated *Lactobacillus* spp. in small and large intestine, compared to calves that received milk as subsequent meal. Similarly, abundance of tissue-associated active *E. coli* was higher in large intestine of calves fed colostrum for prolonged time. Our results suggest that growth of gut bacteria and neuroendocrine gene expression are both diet-dependent and the changes in neuroendocrine system and bacterial colonisation may have effect on metabolic processes and development of immune system in newborn calves. Future studies are required for better understanding of specific colostrum and milk-derived components that can influence microbial colonization and its interaction with expression of genes involved in neuroendocrine functions in newborn calves.

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2.7 Tables and Figures

Table 2- 1: Primers used to estimate abundance of bacterial groups (copy number of 16S rRNA gene)

Target	Product size (bp)	Annealing temp. (°C)		Oligo sequence (5' to 3')	Reference
Lactobacillus	120	62	F:	GAGGCAGCAGTAGGGAATCTTC	Delroisse et al. (2008)
			R:	GGCCAGTTACTACCTCTATCCTTCTTC	
Bifidobacterium	196	66	F:	ATCTTCGGACCBGAYGAGAC	Cleusix et al. (2010)
			R:	CGATVACGTGVACGAAGGAC	
Escherichia coli	544	62	F:	GGAAGAAGCTTGCTTCTTTGCTGAC	Sabat et al. (2000)
			R:	AGCCCGGGGATTTCACATCTGACTTA	

Table 2- 2: Primers used for gene expression of host neuroendocrine

Name of gene	Product length (bp)	Gene ID		Oligo sequence (5' to 3')
Cytochrome P450, subfamily XI B, polypeptide 1	208	*CYP11B1*	F:	TCACGGACATCGTGGAGACA
			R:	CAGACTCGGGACCGCAATAA
gamma-aminobutyric acid type A receptor beta2 subunit	150	*GABRB2*	F:	TACGATGGACCCCCATGAGA
			R:	AACCCGGCTTTCCGATACTG
5-hydroxytryptamine receptor 2B	156	*HTR2B*	F:	CGAGCCCCCACATCAGTAAA
			R:	TGAACCTCGGAGCCTCATTG
Melanocortin 2 receptor	199	*MC2R*	F:	TGTGGTGGACTCCCTGTTCA
			R:	ATGGGAGAAGGTCACGATGGT
Nuclear receptor subfamily 3 group C member 1	280	*NR3C1*	F:	GAGAGGGGAAATGTGATGGA
			R:	CAGAGGAAAGGCTGATTTGG
Steroidogenic acute regulatory protein	166	*StAR*	F:	CTGCCGAAGACCATCATCAA
			R:	CTCCTCGTGAGCCCTCAAAC
5-hydroxytryptamine receptor 4	265	*HTR4*	F:	AGCCATCAGCTACCTCGAAA
			R:	ATCAAACCACAGCCAAGACC
Adrenoceptor beta 2	265	*ADRB2*	F:	GGATTGCCTTCCAGGAGCTT
			R:	CGGCATTACAGCAGTGAGTCA
Solute carrier family 6 member 4	288	*SLC6A4*	F:	GGCAGTACCACCGAAATGGA
			R:	CGTGCCGCGTGTAAAATTC
Gamma-aminobutyric acid type B receptor subunit 1	212	*GABBR1*	F:	GCTGTGCCCGTCAAAAACC
			R:	GCCTCGGTCATCTCGTCAACT
Adrenoceptor alpha 1A	294	*ADRA1A*	F:	CTCAAATTTTCCCGCGAGAA
			R:	CGTGTAGCCCAGGGTGTGTT
Glyceraldehyde-3-phosphate dehydrogenase (reference gene)	119	*GAPDH*	F:	GGCGTGAACCACGAGAAGTATAA
			R:	CCCTCCACGATGCCAAAGT

Table 2- 3: Effect of treatment on gene expression in Adrenal glands

Gene ID	Treatment (ΔCt mean ± SEM)			*P*-value
	WM	CM	CF	
ADRA1A	10.97 ± 0.59^a	11.04 ± 0.41^a	7.17 ± 0.84^b	0.02
ADRB2	5.83 ± 0.25	6.23 ± 0.17	5.90 ± 0.42	0.66
CYP11B1	-2.12 ± 0.18	-2.02 ± 0.24	-2.50 ± 0.30	0.30
GABBR1	3.03 ± 0.17	3.15 ± 0.16	2.93 ± 0.26	0.99
GABRB2	5.68 ± 0.18	5.51 ± 0.22	5.23 ± 0.28	0.40
HTR2B	1.33 ± 0.49	1.83 ± 0.22	1.20 ± 0.30	0.47
HTR4	7.62 ± 0.41	7.78 ± 0.41	7.83 ± 0.66	0.91
MC2R	2.47 ± 0.21	2.64 ± 0.11	2.58 ± 0.12	0.94
NR3C1	1.90 ± 0.12	1.97 ± 0.12	2.16 ± 0.21	0.28
SLC6A4	5.92 ± 0.22	6.45 ± 0.14	6.16 ± 0.27	0.42
STAR	-3.26 ± 0.12^a	-2.76 ± 0.15^b	-3.26 ± 0.14^a	0.02

Table 2- 4: Effect of treatment on gene expression in ileum tissue

Gene ID	Treatment (ΔCt mean ± SEM)			*P*-value
	WM	CM	CF	
ADRA1A	7.68 ± 0.84[a]	8.40 ± 0.38[a]	5.72 ± 0.30[b]	0.03
ADRB2	3.97 ± 0.12	3.74 ± 0.24	3.98 ± 0.24	0.65
CYP11B1	6.88 ± 0.57	5.59 ± 0.92	4.66 ± 1.14	0.24
GABBR1	3.12 ± 0.21	2.70 ± 0.18	2.99 ± 0.20	0.26
GABRB2	6.08 ± 0.92	4.80 ± 0.69	6.57 ± 0.51	0.10
HTR2B	1.70 ± 0.35	1.51 ± 0.19	1.75 ± 0.22	0.50
HTR4	0.43 ± 0.35[a]	0.26 ± 0.21	-0.21 ± 0.19[b]	0.09
MC2R	5.53 ± 0.53	5.62 ± 0.28	6.50 ± 0.67	0.36
NR3C1	0.50 ± 0.12[a]	0.52 ± 0.12[a]	1.14 ± 0.17[b]	0.01
SLC6A4	8.70 ± 0.35[a]	6.34 ± 0.96[b]	6.05 ± 1.38[b]	0.04
STAR	6.68 ± 0.23	6.58 ± 0.18	6.72 ± 0.36	0.87

Table 2- 5: Effect of treatment on gene expression in colon tissue

Gene ID	Treatment (ΔCt mean ± SEM)			*P*-value
	WM	CM	CF	
ADRA1A	4.70 ± 0.76[a]	7.87 ± 0.42	6.86 ± 0.81[b]	0.03
ADRB2	3.34 ± 0.35	3.41 ± 0.45	4.37 ± 0.29	0.12
CYP11B1	5.85 ± 0.99	2.35 ± 1.15	5.12 ± 0.91	0.10
GABBR1	2.82 ± 0.22	3.52 ± 0.44	2.98 ± 0.24	0.67
GABRB2	5.97 ± 0.58	3.77 ± 0.81	6.07 ± 0.57	0.10
HTR2B	0.72 ± 0.53	-0.38 ± 0.35	1.02 ± 0.39	0.11
HTR4	-1.44 ± 0.56	-1.13 ± 0.29	-0.58 ± 0.57	0.40
MC2R	5.15 ± 0.89[ab]	3.27 ± 1.52[b]	7.47 ± 0.43[b]	0.02
NR3C1	2.12 ± 0.16	2.02 ± 0.97	2.49 ± 0.13	0.13
SLC6A4	7.11 ± 0.71	4.99 ± 1.13	7.63 ± 0.58	0.23
STAR	6.47 ± 0.80	5.05 ± 1.08	7.20 ± 0.47	0.40

Table 2- 6: Effect of treatment on abundance (Mean ± SEM of 16S rRNA gene copy/g of tissue) of active tissue-associated bacteria

Region	Ileum			Colon		
Treatment	WM	CM	CF	WM	CM	CF
Bifidobacterium	$4.9 \pm 3.3 \times 10^3$	$8.3 \pm 4.3 \times 10^3$	$7.5 \pm 3.5 \times 10^3$	$29.9 \pm 7.1 \times 10^7$	$18.1 \pm 5.8 \times 10^7$	$18.7 \pm 5.7 \times 10^7$
P-value		0.59			0.41	
Lactobacillus	$28.7 \pm 5.0 \times 10^{4\ a}$	$3.5 \pm 3.1 \times 10^7$	$9.0 \pm 6.3 \times 10^{7\ b}$	$5.8 \pm 2.7 \times 10^{7\ a}$	$2.2 \pm 1.3 \times 10^{8\ ab}$	$4.2 \pm 1.7 \times 10^{8\ b}$
P-value		0.08			0.02	
Total *E. coli*	$3.6 \pm 1.5 \times 10^5$	$8.5 \pm 3.4 \times 10^5$	$24.2 \pm 9.3 \times 10^5$	$15.0 \pm 4.6 \times 10^{7\ a}$	$13.5 \pm 5.8 \times 10^{7\ a}$	$4.2 \pm 1.7 \times 10^{8\ b}$
P-value		0.14			0.03	
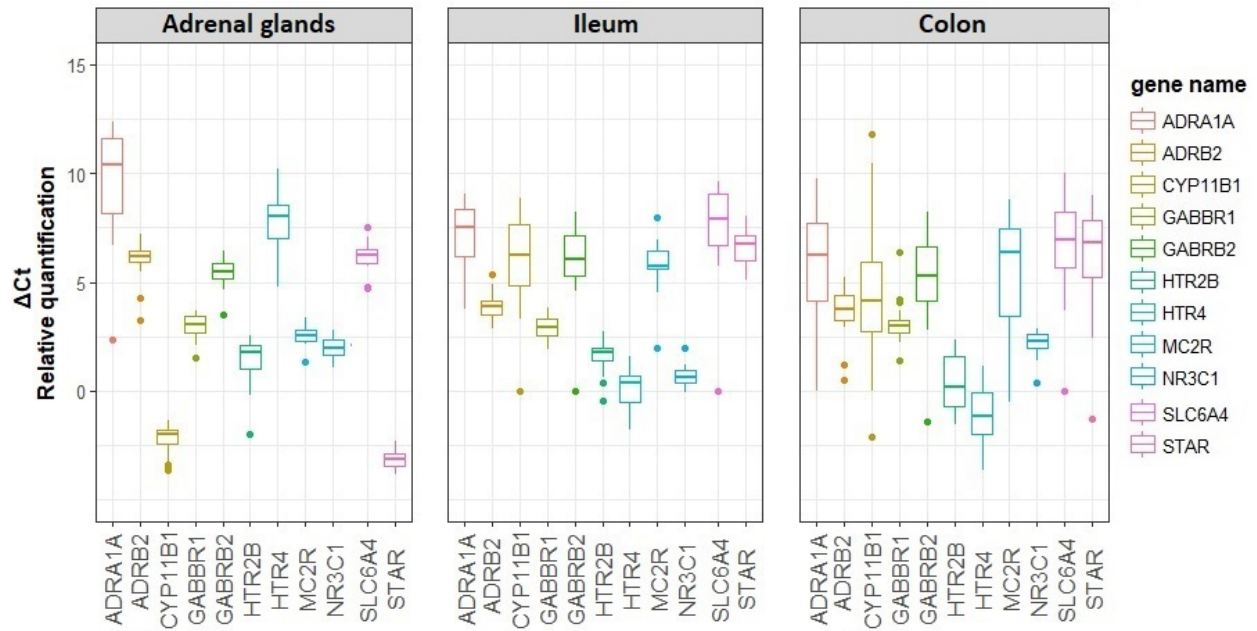
Figure 2- 1: Whisker diagram displays expression of targeted genes in Adrenal glands, Ileum and colon tissues.

Figure 2- 2: Effect of management feeding (WM = whole milk; CM = mixture of colostrum and milk; CF = colostrum feeding), on abundance of active probiotic bacteria in ileum and colon. A) Abundance active of *Lactobacillus* spp. associated with intestinal tissue. B) Abundance of active *Bifidobacterium* spp. associated with intestinal tissue. Data is presented as mean ± SEM with significant levels declared at **P < 0.05 and tendencies *P < 0.1.
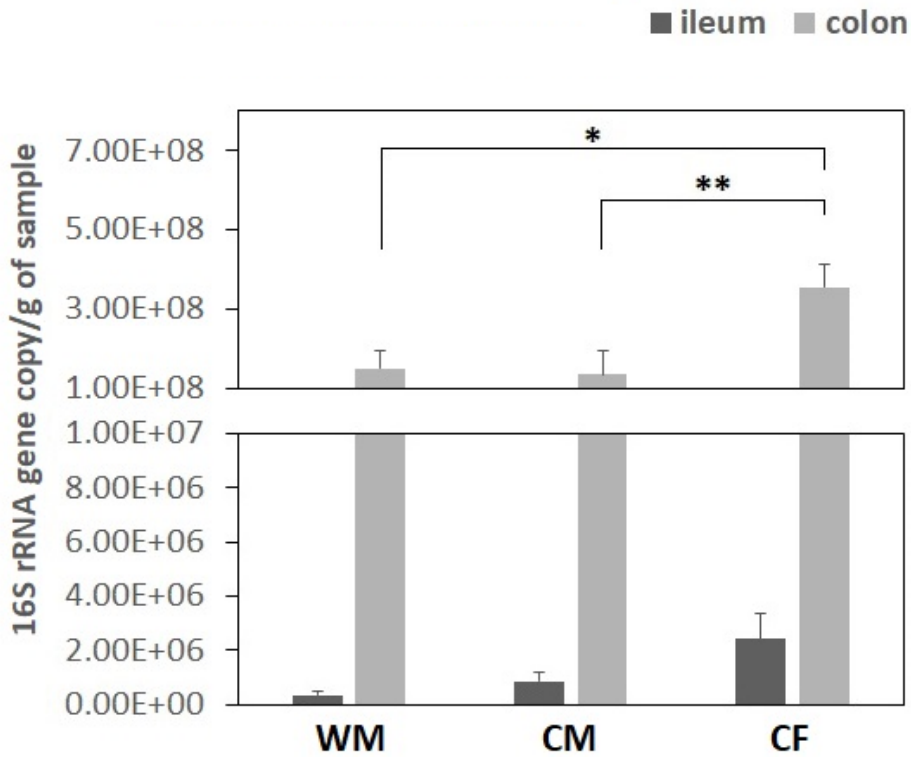
Figure 2- 3: Effect of management feeding (WM = whole milk; CM = mixture of colostrum and milk; CF = colostrum feeding), on abundan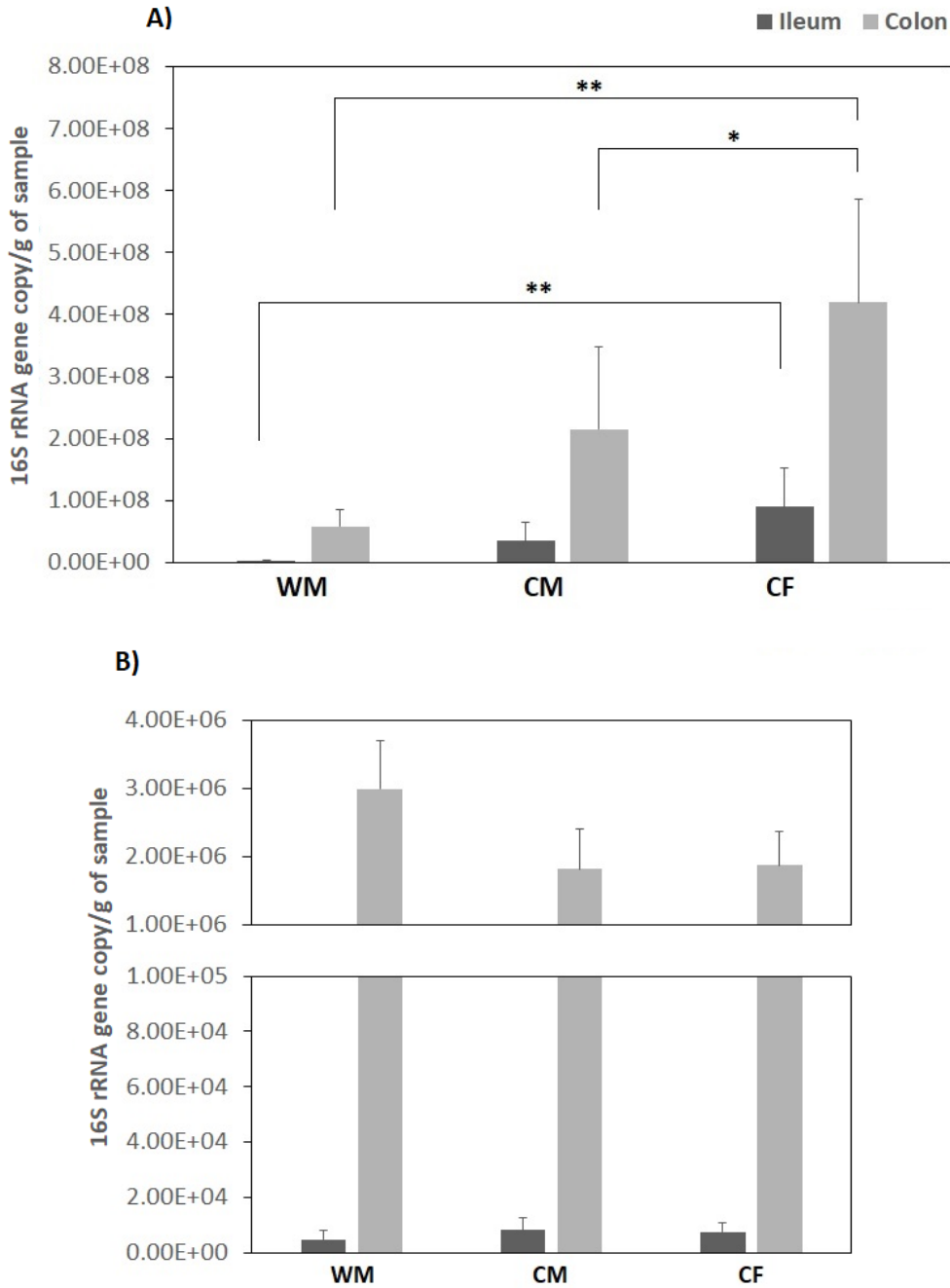ce of active *E. coli* associated with ileum and colon tissue. Data is presented as mean ± SEM with significant levels declared at **$P < 0.05$ and tendencies *$P < 0.1$.
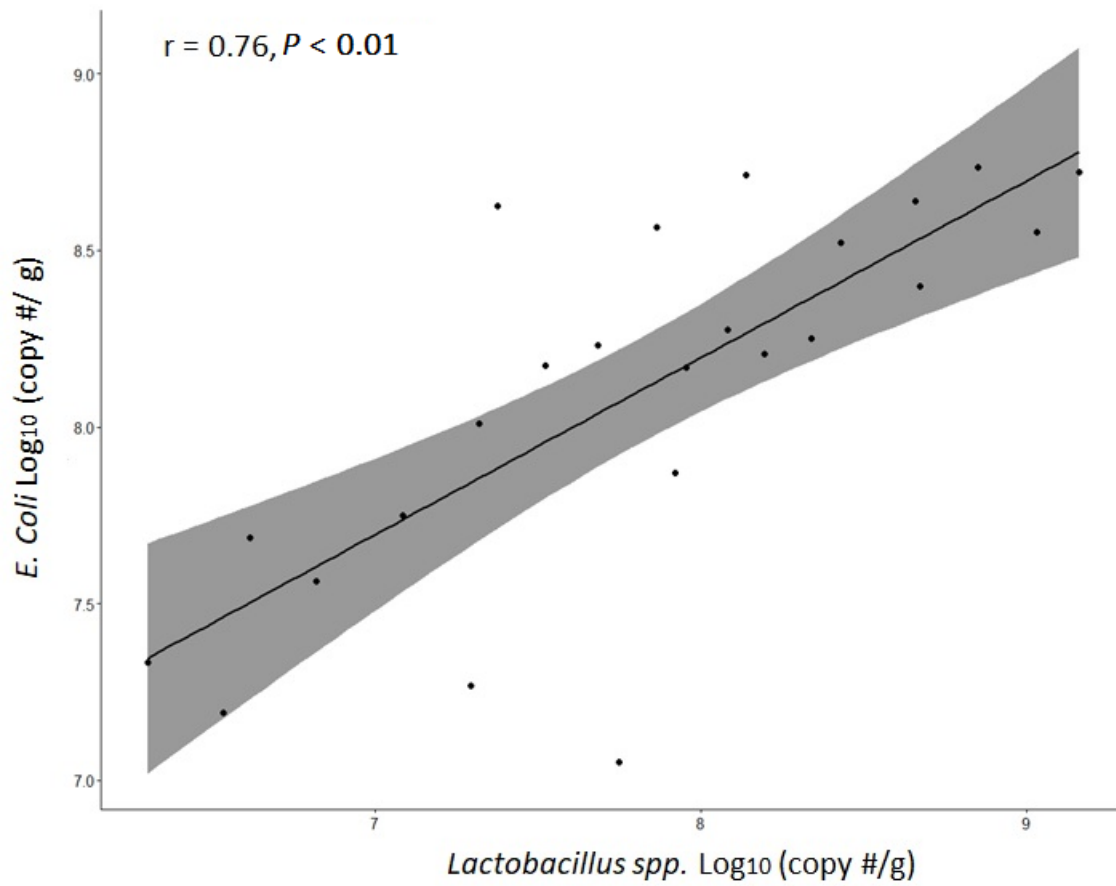
Figure 2- 4: Pearson correlation between log transformed abundance of active tissue-associated

E. coli and *Lactobacillus* spp. in calf colon. Correlation coefficient and level of significance (r

and P).

Figure 2- 5: Pearson correlation between log transformed abundance of active tissue-associated gut bacteria and gene expression of serotonin receptors in calf colon. Correlation coefficient and level of significance (r and P).

Figure 2- 6: Pearson correlation between log transformed abundance of active tissue-associated *E. coli* and gene expression of adrenergic receptors in calf colon. Correlation coefficient and level of significance (r and P).

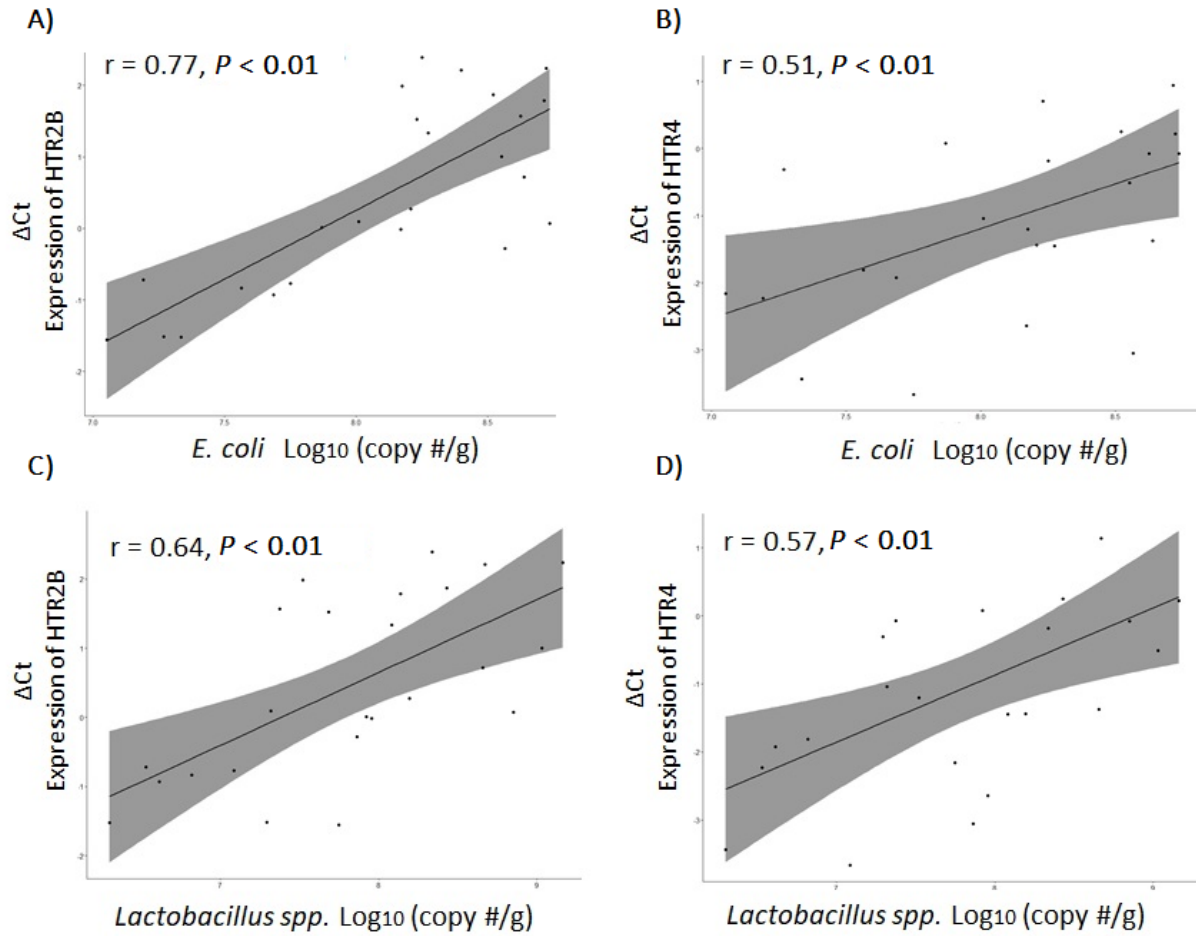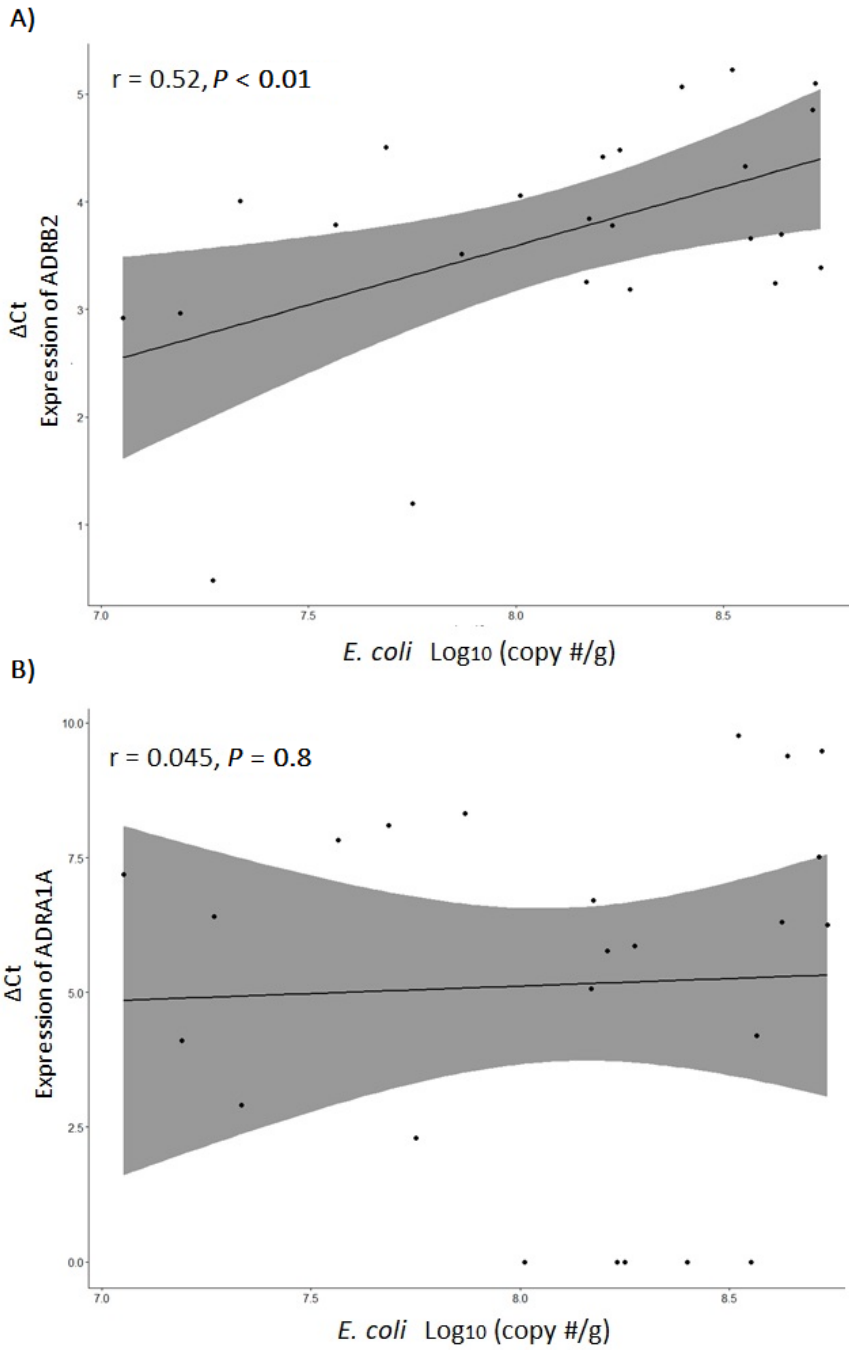**Chapter 3. Effect of delayed colostrum feeding on fecal cortisol concentration and gut bacterial population in neonatal male Holstein calves**

3.0 Introduction

Colostrum management and feeding is an important practice in dairy industry. Bovine colostrum contains high concentration of essential and beneficial components such as nutrients, immunoglobulins, hormones, growth factors, cytokines, enzymes and even viable immune cells that support development of immune system and gastrointestinal tract of calves during first few days of life (Blum, 2006). To date, positive effect of colostrum on development of immunity and gastrointestinal tract in neonatal dairy calves has been well described. For instance, feeding colostrum soon after birth stimulates absorption of immunoglobulins, growth of intestinal epithelial surface, enzyme activity and cell proliferation resulting in increased surface of intestine (Besser et al., 1985; Blättler et al., 2001; Fischer et al., 2018). Also, colostrum ingestion within 12 h after birth accelerated bacterial colonization in small intestine of neonatal calves, compared to calves that did not receive colostrum (Malmuthuge et al., 2015a). These findings suggest that colostrum derived components can support development of adaptive immunity and gastrointestinal tract (GIT) in neonatal calves.

Since calves are born with lack of immunocompetence and immature GIT, colostrum intake soon after birth helps neonate to adapt and undergo the changes occurring during neonatal development (Hulbert & Moisa, 2016). However, the permeability of intestinal epithelium is increased within first a few hours of calf life (Steele et al., 2016), therefore timing of the first colostrum meal and its quality and quantity are main factors that influence absorption of IgG and its concentration in serum (Morin et al., 1997). In a recent study, it has been reported that delaying first colostrum meal ingestion by 6 h or 12 h after birth resulted in decreased passive transfer of

IgG and lower abundance of tissue-associated *Bifidobacterium* spp. and *Lactobacillus* spp. in large intestine, compared to calves fed colostrum soon after birth (Fischer et al., 2018). Similarly, feeding colostrum within first 12 hours of life increased prevalence of *Bifidobacterium* spp. and reduced colonisation of *E. coli* in calf small intestine, compared to calves that did not receive colostrum (Malmuthuge et al., 2015a). These findings suggest that delaying colostrum feeding may affect physiological events and microbial colonisation in neonatal development of newborn calves.

It has been suggested that immature immune system of neonate is primarily modulated via hypothalamic pituitary adrenal (HPA) axis by production of stress hormones (Hulbert & Moisa, 2016). Concentration of stress hormones such as cortisol and catecholamines is therefore elevated at birth due to their role in regulation of physiological processes related to postnatal development (Schaff et al., 2014). Cortisol, one of the components of bovine colostrum (Shutt & Fell, 1985), is produced in adrenal cortex via HPA axis during stress (Chen et al., 2015). Beside regulation of immune responses, cortisol together with other glucocorticoids play a role in prenatal and postnatal development of organs, especially lungs, brain and GIT (Cintra et al., 1993; Gould et al., 1991; Hulbert & Moisa, 2016; Kadmiel & Cidlowski, 2013). Furthermore, previous studies on pre-weaned calves suggested stress hormones a play role in regulation of metabolic processes (Hadorn et al., 1997; Schaff et al., 2014). For instance, prolonged colostrum and milk restriction resulted in higher level of plasma cholesterol, albumin and cortisol in pre-weaned calves, compared to calves that had unlimited access to feed (Hammon et al., 2002). Also, Schaff et al. (2015) reported that binding capacity of glucocorticoid and β2-adrenergic receptors in liver were lower in preterm calves, compared to calves that were born at term, suggesting stress hormone receptors are dependent on stage of maturation in neonatal calves. However, it is currently unknown whether

delayed colostrum feeding affect on fecal cortisol concentration and its relationship with shift in gut microbiota of neonate in early stage of life. Therefore, in this study the effect of delaying feeding of first colostrum meal on level of fecal cortisol and its relationship with microbial colonisation in large intestine was investigated.

3.1 Materials and methods

3.1.1 Animal trial and sample collection

The animal study was performed at Dairy Research and Technology Centre of the University of Alberta from February to September of 2016. Experimental protocol (AUP00001595) was reviewed and approved by the Animal Care and Use Committee (University of Alberta) and all procedures were performed following guidelines approved by the Canadian Council on Animal Care. Holstein heifers and cows were transferred to maternity pens where were provided with fresh shavings daily approx. 3-10 days before parturition. The pens were disinfected and cleaned with 1% iodine solution during calving and a disinfected iVET birth monitor device (iVET, Papenburg, Germany) was inserted into the vagina. All calves were separated from the dam immediately after birth and kept in individual pens where were disinfected with Virkon and lime and bedded with shavings and fresh straw. Calves were dried with towels for 10 min and calves' navels were dipped with 7% iodine. Calibrated electronic scale (Digi-Star SW300, Digi-Star L.L.C., Fort Atkinson, WI) was used to measure birth weight.

Holstein bull calves (n= 23) with a body weight between 35-55 kg were randomly divided into 3 treatment groups: calves fed colostrum within 45 mins after birth (0h, n=7); 6 hours after birth (delayed feeding 6h, n=8); and 12 hours after birth (delayed feeding 12h, n=8). Heat treated colostrum (pasteurization at 60°C for 60 min) was provided by the Saskatoon Colostrum Company Ltd. (SCCL, Saskatoon, SK, Canada) and fed to calves in one batch at 7.5% of birth body weight

according to respective feeding times. Colostrum was heated to 39°C and kept in consistent temperature using water bath. Prepared colostrum meal was provided to calves in 2 L esophageal tubing bottles. Twelve hours after colostrum meal feeding, calves were fed milk replacer (Excel Pro-Gro Calf Milk Replacer, Grober Nutrition, Cambridge, Ontario, Canada) every six hours at 2.5% of birth body weight per meal. Milk replacer meal was prepared by mixing 150 g of milk replacer powder per 1 L of water in a clean bucket and heated in the water bath to 39°C. All calves were humanely euthanized at 51 hours after birth (3 hours after last meal) with intravenous injection of pentobarbital sodium (Euthanyl, Vetoquinol, Lavaltrie, Quebec, Canada) at 0.125 ml per kg body weight. Digesta and intestinal tissue were collected within less than 30 min using sterile dissection tools following the procedures as reported previously (Liang et al., 2014). Content of each GIT regions was separated from tissue using tweezers and stored in 50 mL Falcon tube. Tissues samples were rinsed three times with phosphate buffered saline (PBS) and placed in sterile bags. All samples were frozen with liquid nitrogen and stored at -80°C freezer immediately after collection. Colon digesta- and tissue-associated bacterial data were obtained from recent publication which used the same animal trial (Fischer et al., 2018).

3.1.2 Fecal cortisol measurement

Extraction and measurement of fecal cortisol concentration were conducted using CORTISOL Enzyme immune assay (EIA) Kit (Arbour Assays, Michigan, USA), following manufacturer's instructions. Briefly, prior to extraction, rectum content samples were dried at 50°C in an oven over 4 days. Dried rectum content samples (~ 0.1 g) was then supplemented with 1ml of ethanol and vortexed over 30 minutes. Samples were then centrifuged at 5,000 rpm and 1 ml of supernatant solution was dried in new tubes using SpeedVac concentrator with Universal Vacuum system (Thermo ELECTRON CORPORATION, Milford USA). Dried pellets were sealed with

parafilm and were stored at -20°C. Dried pellets were dissolved in 100 µl and 400 µl Assay Buffer. Samples were further diluted 1:5 with Assay Buffer due to high concentration of fecal cortisol. Reactions were performed in duplicates with standard curve constructed with serial dilutions of EIA Cortisol stock solution. To reduce variation associated with differences in fecal matter consistency, the concentrations of fecal cortisol (pg/mL) obtained from EIA measurement were converted into dry matter basis (ng/g) based on the weight of raw fecal matter.

3.1.3 DNA extraction and quantitative PCR (qPCR)

Total DNA was isolated from grounded colon tissue (~ 0.1 g) and rectum and colon contents (~ 0.5 g each) using the repeated bead beating method as previously described by Li et al. (2009). Briefly, frozen samples were washed twice with Tris EDTA (TE) buffer and physically disturbed with cell lysis buffer containing 4% sodium dodecyl sulfate (SDS) using BioSpec Mini Beads beater 8 (BioSpec, Bartlesville, OK) at 5000 rpm for 3 min. Samples were then incubated at 70°C for 15 mins and centrifuged at 4°C and 146 00 g speed for 5 min. Extraction of nucleic acid was performed using phenol:chloroform:isoamyl (25:24:1), followed by DNA precipitation with cold ethanol and final dissolution in Nuclease-free water. Concentration and quality of DNA was measured using ND1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

Quantitative real time-PCR was performed to evaluate densities of total bacteria, *Bifidobacterium* spp. and *E. coli* using SYBR green chemistry (Fast SYBR green Master Mix, Applied Biosystems Foster City, CA) with StepOnePlus real-time PCR system (Applied Biosystems). The primers used in this study are listed in Table 3-1. All reactions for total bacteria, *Bifidobacterium* spp. *and E. coli* were conducted with standard curve constructed out of 16S DNA of *Bytuvibrio bungatei, Bifidobacterium longum* and *Escherichia coli* K12, respectively. The copy

number of 16S DNA per gram of tissue or content was calculated with equation previously described by Li et al. (2009). Relative abundance of *Bifidobacterium* spp. and *E. coli* was calculated by dividing the copy number of 16S DNA of genus/species by copy number of total bacteria. *Bifidobacterium* spp. and *E. coli* log transformed data were calculated as Log 10 of copy number of 16S DNA per gram.

3.1.4 Statistical analysis

To determine the effect of delayed colostrum feeding, fecal cortisol and bacterial data were analysed using one-way analysis of variance (ANOVA) test followed by Tukey's test to analyse the pairwise difference in R software. Digesta-associated bacterial groups were compared between GIT regions (rectum and colon) using dependent sample t-test. Significant differences in least square means (LSM) were declared at P < 0.05 and tendencies of differences in LSM at $0.05 < P < 0.10$. Similarities between rectum and colon digesta-associated bacterial entities were explore by analysis of Principal component (PCA) using package MixOmics in R software. Bacterial data for rectum and colon digesta (log10 copy number of 16S RNA genes and proportion of E. coli and *Bifidobacterium* spp.) were plotted were plotted along the first two principal components axis (PC1 and PC2).

3.2 Results and discussion

Previous studies have focused on effect of colostrum feeding on changes of plasma cortisol levels and these findings suggest that increased concentration of plasma cortisol at birth generally decreased after first feed intake in colostrum-fed calves (Hammon & Blum, 1998; Rauprich et al., 2000; Steinhoff-Wagner et al., 2011). In the present study, we examined the effect of delayed colostrum feeding on level of cortisol in feces. Our results revealed that fecal cortisol concentration was the highest (25.33 ± 2.99 ng/g of dry fecal matter; mean ± SEM) in calves that received

colostrum soon after birth (0 h delayed colostrum feeding), when compared to calves with 6 h and 12 h delayed colostrum feeding. This suggests that colostrum feeding soon after birth increases concentration of fecal cortisol in large intestine of neonatal calves. Delayed colostrum feeding over 6 h after birth did not affect level of fecal cortisol (23.50 ± 3.17 ng/g of dry fecal matter), when compared to 0 h delayed feeding, suggesting that 6h delayed colostrum feeding does not significantly affect concentration of fecal cortisol. Calves fed at 12 h of life (12 hr delayed colostrum feeding) had significantly lower ($P < 0.01$ and $P < 0.05$) level of fecal cortisol (12.03 ± 2.05 ng/g of dry fecal matter), compared to 0 h and 6 h calves, respectively (Figure 3-1), suggesting that 12h delayed colostrum feeding reduces fecal cortisol concentration in neonatal calves. Although cortisol is primarily produced in adrenal cortex via HPA axis (Chen et al., 2015), increasing evidences suggest extra-adrenal production of glucocorticoid in various organs including brain, thymus, skin, vascular system, lungs and intestine (Eisenhofer et al., 1997; Noti et al., 2009; Pacak, 2011). The local synthesis of glucocorticoids (GCs) is thought to play a role in regulation of local immune, metabolic and homeostatic processes (Noti et al., 2009; Talaber et al., 2013). Therefore, we propose that these changes in fecal cortisol concentration in calf large intestine may be caused by altered systemic transportation or local production of glucocorticoids in the gut. Cortisol, as one of the products from neuroendocrine system, is involved in regulation of metabolic process, notably glucose production that helps newborn calves to adapt extrauterine life during neonatal development (Schaff et al., 2015; Steinhoff-Wagner et al., 2011). Furthermore, it has been suggested that underdeveloped immune system is regulated by stress hormones and help calves to become immunocompetent and resilient (Hulbert & Moisa, 2016) and contribute to development of GIT (Schaak et al., 2000; Schaff et al., 2015). Thus, the observed different cortisol level suggest that different colostrum management may alter neuroendocrine traits which may lead

to indirect impact on neonatal development of immune system and GIT in newborn calves. Further studies are needed to investigate effect of colostrum feeding on cortisol concentration at systemic and local level in newborn calves to measure its abundance in the gut and blood.

It has been suggested that the gut microbiota is an endocrine organ, which can produce hormone-like metabolites and contribute to physiological changes of host (Clarke et al., 2014). Therefore, we speculate that the altered the fecal cortisol in 12h delayed calves may be resulted from the altered the hindgut microbiota. Fischer et al. (2018) reported that delaying colostrum feeding 6h postpartum tended to decrease abundance of tissue-associated total bacteria in small intestine, compared to non-delayed colostrum feeding and 12h delayed feeding. In this study, the bacterial population in the rectum digesta was measured and abundance of rectum digesta-associated bacteria was: total bacteria (20.4 ± 5.3 x 10^{10} copy number of 16S rRNA gene/g digesta), *Bifidobaterium* spp. (4.9 ± 2.6 x 10^8) and *E. coli* (7.6 ± 3.1 x 10^{10}). Delayed colostrum feeding did not affect ($P \geq 0.1$) the abundance of colon and rectum digesta and colon tissue-associated total bacteria (Figure 3-2). Similarly, populations of rectum and colon digesta-associated *E. coli* and *Bifidobacterium* spp. were not affected ($P \geq 0.1$) by 6 h and 12 h delayed colostrum feeding (Figure 3-3 and 3-4). These results suggest that changes in diet and delaying first colostrum meal may affect microbial colonisation in small intestine.

As previously published by Fischer et al. (2018), non-delayed colostrum feeding tended to increase ($P < 0.05$ and $P < 0.1$) abundance of colon tissue-associated *Bifidobacterium* spp. (5.61 ± 2.70 x 10^{06} 16S rRNA gene copy/ g of sample; mean $\pm$ SEM), compared to calves that receive colostrum 6 h and 12 h after birth (4.40 ± 0.98 x 10^{05} and 9.60 ± 2.88 x 10^{05} 16S rRNA gene copy/ g of sample), respectively (Figure 3-5A). However, delayed colostrum feeding did not have significant effect ($P = 0.1$) on relative abundance of tissue-associated *Bifidobacterium* spp., when

comparing prevalence of *Bifidobacterium* spp. among treatments. Additionally, absolute and relative abundance of tissue-associated *E. coli* in large intestine was not affected ($P \geq 0.1$) by delayed colostrum feeding (Figure 3-6). This suggests that delayed feeding may have a minor effect on the abundance of tissue attached *Bifidobacterium* spp. in colon of neonatal calves. Furthermore, preventing the immediate initiation of growth and establishment of commensal bacterial niches may have lasting effects on microbial ecology in large intestine and affect susceptibility to enteric diseases such as neonatal calf diarrhea (Oikonomou et al., 2013). Thus, we propose that colostrum ingestion at 6h and 12h after birth could lead to delayed establishment of stable ecological niches of probiotic bacteria associated with colon tissue and these changes could potentially alter host-microbial interactions in GIT. Further studies are required to investigate effect of colostrum management on microbial colonisation in different GIT regions and at different ages in neonatal calves.

Recently, analysis of gut microbiota among different regions of GIT in young and adult ruminants revealed spatial heterogeneity in composition, diversity and abundance of gut microbiota (Malmuthuge et al., 2014; Mao et al., 2015). Furthermore, 15,771 operational taxonomic units (OTUs) were identified in 3weeks old pre-weaned calves GIT, which showed various distribution across GIT regions (Malmuthuge et al., 2014). In the present study, we performed principal component analysis to compare bacterial populations of colon and rectum digesta in newborn calves. The PCA plot (Figure 3-7) showed not separation between rectum and colon digesta-associated bacteria. However, PCA analysis of digesta-associated *E. coli* showed that rectum and colon E. coli populations fall in two different clusters (Figure 3-8A), suggesting that *E. coli* colonisation in colon and rectum are independent of one another. In contrast, there was no separation of digesta-associated *Bifidobacterium* spp. between colon and rectal regions (Figure

3-8B), suggesting continuity in colonisation of probiotic *Bifidobacterium* spp. in colon and rectum digesta. Furthermore, when comparing mean of digesta-associated bacteria in two different regions of GIT (rectum and colon), relative abundance of *E. coli* was significantly lower ($P < 0.001$) in rectum ($0.3 \pm 0.04\%$) compared to that in colon ($8.6 \pm 2.0\%$) (data not shown). Comparison of rectum and colon digesta-associated *E. coli* within each treatment group showed the difference in relative abundance of *E. coli* was significant ($P < 0.05$) in calves fed at 12 h life (Figure 3-3B), suggesting that 12h delayed colostrum feeding affects prevalence of *E. coli* in colon and rectum, which may be the results of altered composition of gut microbial. Non-delayed colostrum feeding calves tended to have higher ($P < 0.05$) absolute abundance of rectum digesta-associated *E. coli* compared to colon digesta-associated *E. coli* (Figure 3-3A), but no such difference was observed in calves fed at 6 h and 12 h of life ($P \geq 0.1$). This suggests that the differences in abundance of digesta-associated *E. coli* between rectum and colon may be induced by colostrum feeding and could be associated with feed digestion. However, when comparing mean of absolute and relative abundance, rectum and colon digesta-associated Bifidobacteria did not differ ($P \geq 0.1$; data not shown), suggesting that delayed feeding did not alter *Bifidobacterium* spp. population in colon and rectum. Also, treatment did not induce changes between rectum and colon relative abundance of *Bifidobacterium* spp. ($P \geq 0.1$; Figure 3-4B) , suggesting that delayed feeding did not induce significant changes between colonic and rectal abundance of digesta-associated *Bifidobacteium* spp. in newborn calves. Mao et al. (2015) reported that volatile fatty acids (VFA) concentrations and pH values vary among GIT regions. Thus, the differences in bacterial population along GIT may be due to different environmental conditions in each location. Furthermore, due to initiation of microbial colonisation in early life, these changes can vary at different age. The calves were slaughtered at 51 hr of age, and we speculate the changes in microbiota may be more significant

within first 24 hr of life. Future studies should therefore investigate changes in bacterial populations at multiple timepoints, for instance at 12h or 24h postpartum, and in different GIT regions.

3.3 Conclusion

In conclusion, the present study is the first to investigate fecal cortisol concentration changes upon delayed colostrum feeding. Our results suggest that 6h delayed colostrum feeding does not affect level of fecal cortisol whereas 12h delayed colostrum feeding results in decrease concentration of fecal cortisol, compared to calves that were fed colostrum immediately after birth. Decrease in concentration of fecal cortisol may be due to changes in systemic transportation or local glucocorticoid production in large intestine. Future studies are required to investigate effect of delayed colostrum feeding on neuroendocrine traits at systemic and local level in neonatal calves. Furthermore, delayed colostrum feeding (6h and 12h) tended to reduce tissue-associated Bifidobacteria in colon. Additionally, regional differences were observed between prevalence of *E. coli* in rectum and colon digesta, regardless of treatment. This suggests regional differences between *E. coli* population in rectum and colon in newborn calves. It should be noted that present study examined effect of delayed colostrum feeding on fecal cortisol level and microbial populations in rectum and colon only 51 hours postpartum. Thus, further studies are still required to evaluate long-term effect of delayed feeding on neuroendocrine traits and microbial populations.

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3.6 Tables and Figures

Table 3 - 1: Primers used to determine the copy number of 16S rDNA of selected bacterial groups in rectum and colon tissues and digesta.

Bacterial Group	Primer	Product Size(bp)	Annealing Temp. (°C)	Reference
Total Bacteria	F: 5'-actcctacgggaggcag-3' R: 5'-gactaccagggtatctaatcc-3'	467	62	Stevenson and Weimer (2007)
Bifidobacterium	F: 5'-atcttcggaccbgaygagac-3' R: 5'-cgatvacgtgvacgaaggac-3'	196	66	Cleusix et al. (2010)
Escherichia coli	F: 5'-ggaagaagcttgcttctttgctgac-3' R: 5'-agcccggggatttcacatctgactta-3'	544	62	Sabat et al. (2000)

Table 3 - 2: Effect of treatment on absolute abundance of bacteria (Mean ± SEM of 16S rRNA gene copy/g of digesta) and fecal cortisol (Mean ± SEM of ng/g of dry fecal matter) in rectum and colon digesta

| Region	Colon digesta			Rectum digesta		
Treatment	0 h	6 h	12 h	0 h	6 h	12 h
Total bacteria	$9.8 \pm 2.2 \times 10^{10}$	$16.0 \pm 9.4 \times 10^{10}$	$16.4 \pm 7.8 \times 10^{10}$	$15.9 \pm 3.7 \times 10^{10}$	$3.5 \pm 1.4 \times 10^{11}$	$10.0 \pm 2.4 \times 10^{10}$
P-value		0.59			0.13	
Bifidobacterium	$3.5 \pm 2.7 \times 10^{9}$	$2.6 \pm 1.4 \times 10^{8}$	$2.7 \pm 2.1 \times 10^{8}$	$9.6 \pm 8.1 \times 10^{8}$	$4.3 \pm 2.8 \times 10^{8}$	$1.0 \pm 1.1 \times 10^{8}$
P-value		0.22			0.34	
Total *E. coli*	$4.0 \pm 1.9 \times 10^{9}$	$3.0 \pm 2.6 \times 10^{10}$	$2.8 \pm 1.6 \times 10^{10}$	$6.0 \pm 1.6 \times 10^{10}$	$14.2 \pm 8.8 \times 10^{10}$	$23.8 \pm 5.7 \times 10^{9}$
P-value		0.35			0.29	
Fecal Cortisol	-	-	-	25.3 ± 3.0 [a]	23.5 ± 3.2 [a]	12.0 ± 2.0 [b]
P-value		-			0.005	
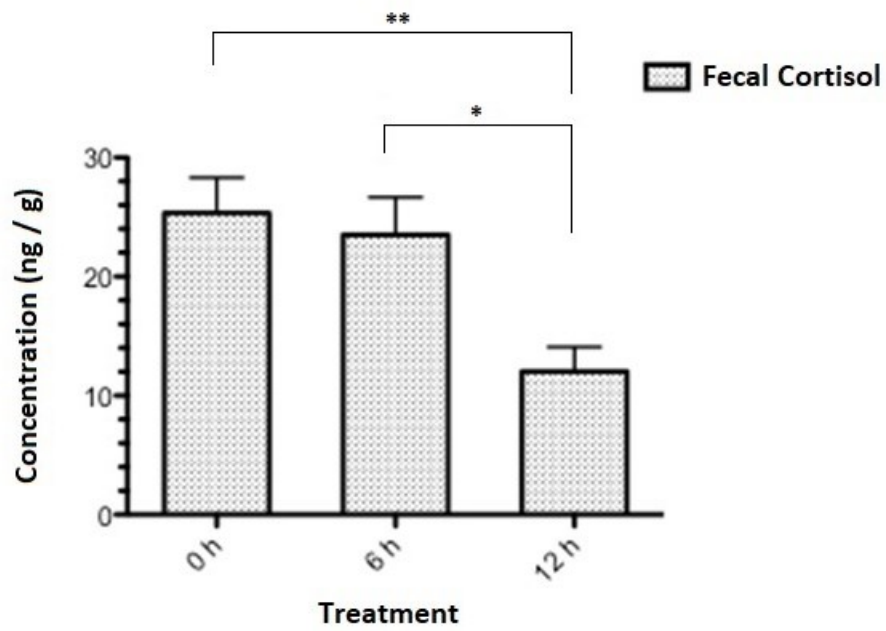
Figure 3- 1: Effect of delayed colostrum feeding on fecal cortisol concentration (ng / g). Bars represent mean + SEM; level of significance (**P < 0.05 and *P < 0.1).
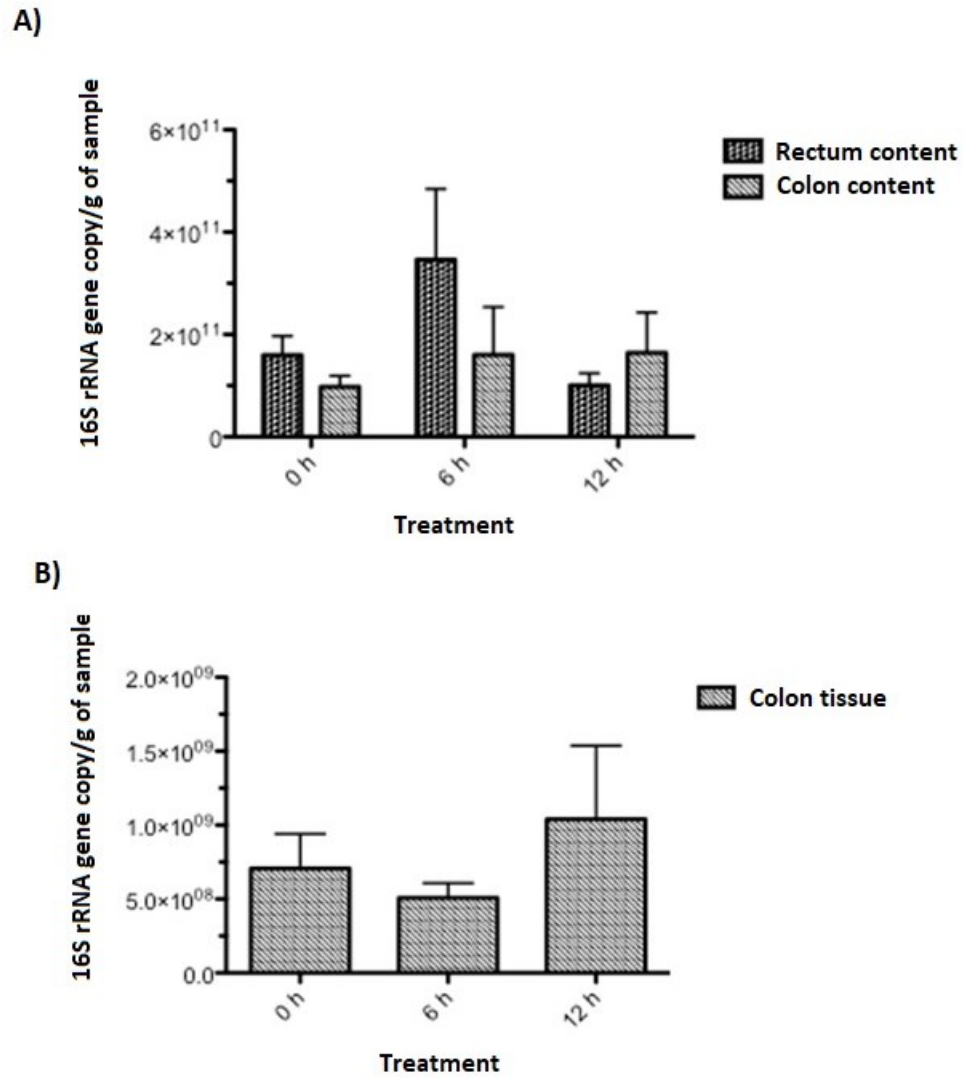
Figure 3- 2: Delayed feeding did not have an effect on absolute abundance of digesta and tissue-associated total bacteria ($P \geq 0.1$).

Figure 3- 3: Comparison of Rectum and Colon digesta-associated *Escherichia coli* and effect of delayed colostrum feeding on abundance (A) and proportion (B) of *E. coli* (% of total bacteria) associated with rectum and colon digesta. Bars represent mean + SEM; level of significance (**P < 0.05 and *P < 0.1).

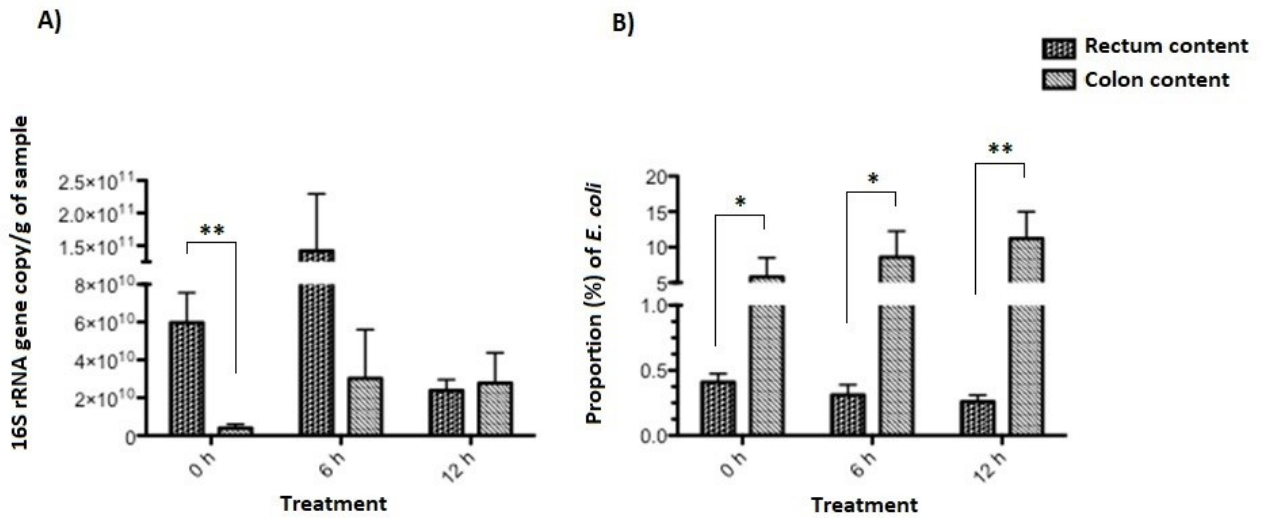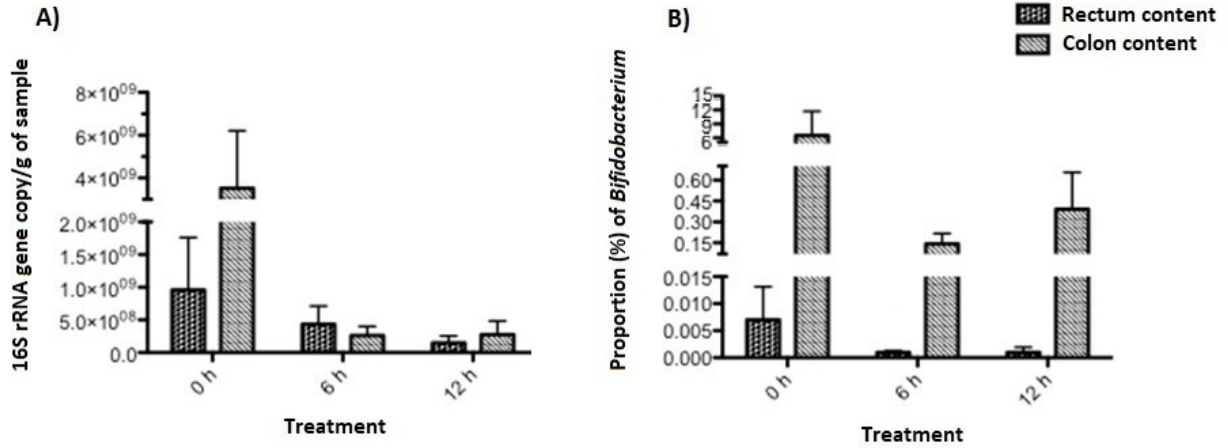**Figure 3- 4:** Comparison of Rectum and Colon digesta-associated *Bifidobacterium* and effect of delayed colostrum feeding on abundance (A) and proportion (B) of *Bifidobacterium* (% of total bacteria) associated with rectum and colon digesta.
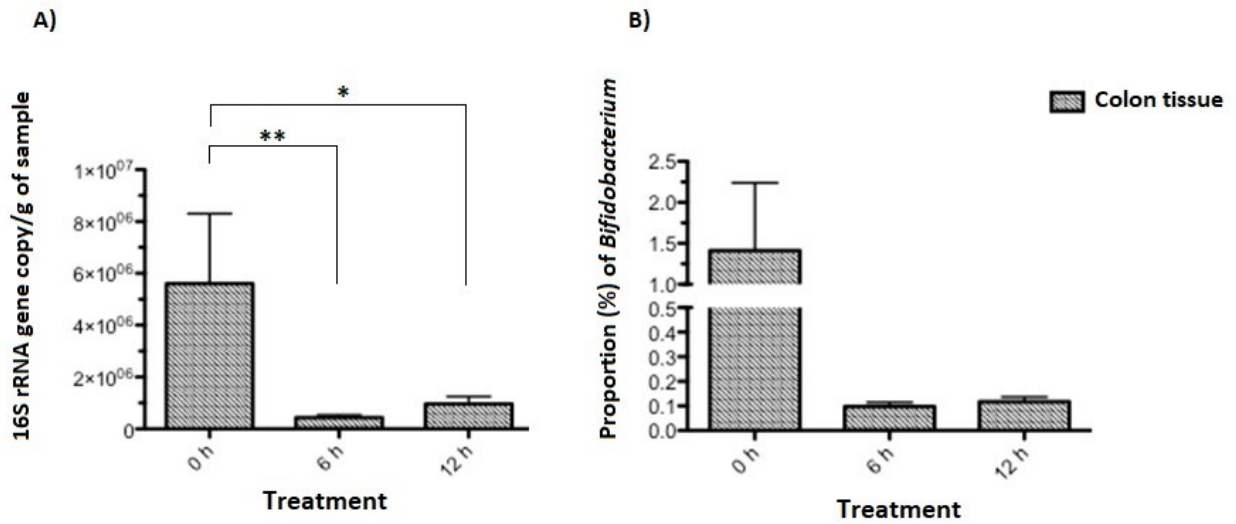
Figure 3- 5: Effect of delayed colostrum feeding on abundance (A) and proportion (B) of tissue-associated *Bifidobacterium*. Bars represent mean + SEM; level of significance (**P < 0.05 and *P < 0.1).
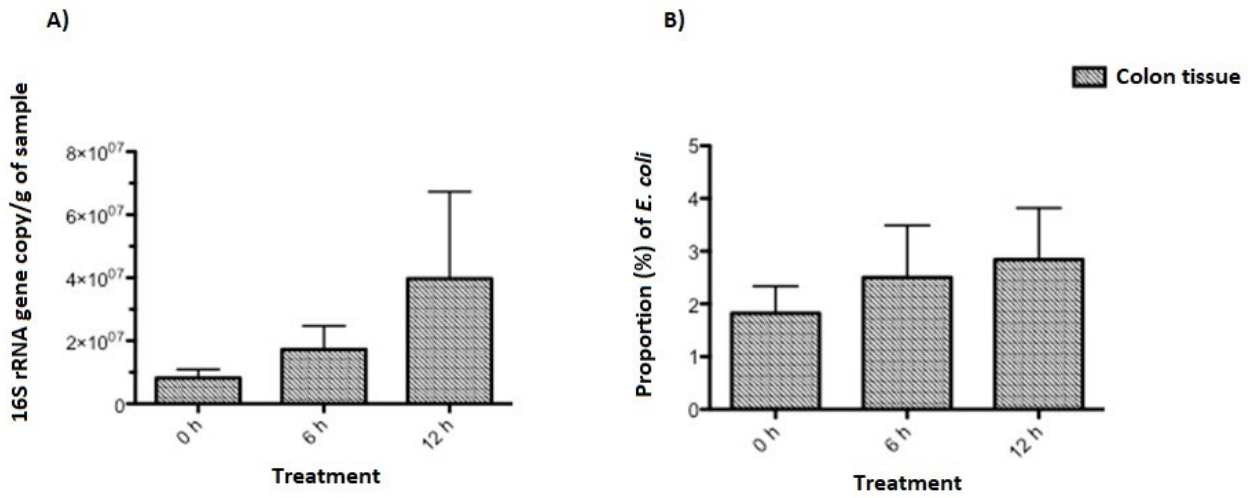
Figure 3- 6: Effect of Delayed colostrum feeding on abundance (A) and proportion (B) of tissue-associated *Escherichia coli*. Bars represent mean + SEM.
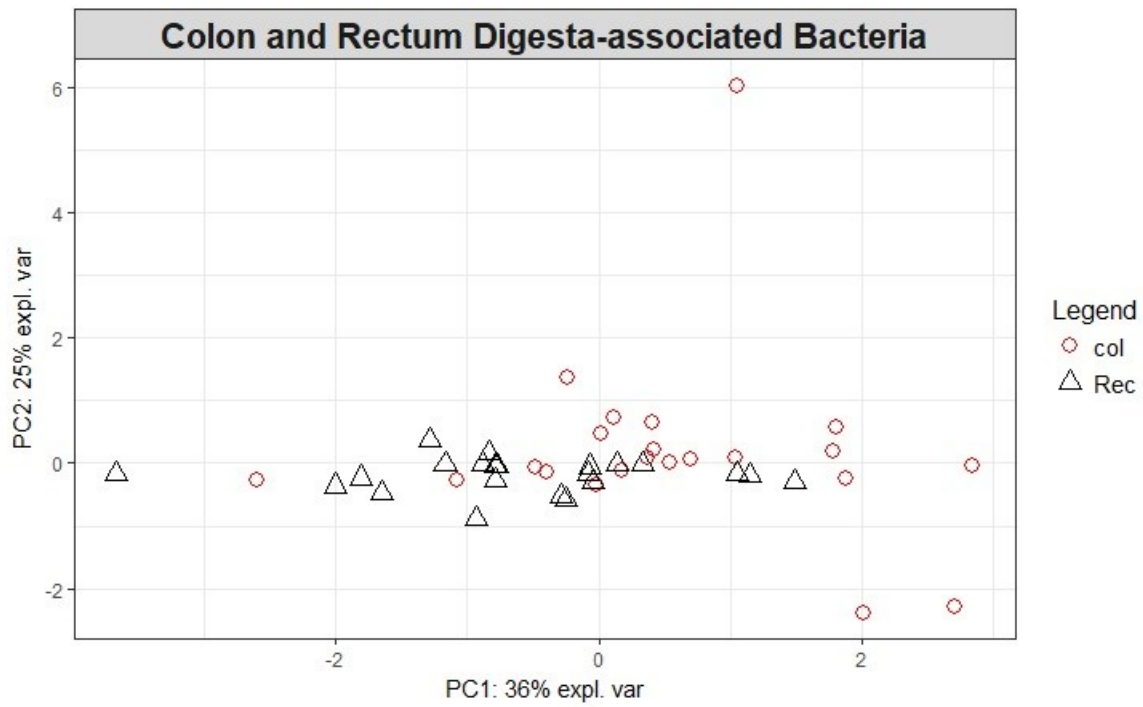
Figure 3- 7: PCA plot of the digesta-associated bacteria from colon (col; n = 22) and rectum (Rec; n = 22) content samples are plotted along the first two principal component axis (PC1 and PC2).
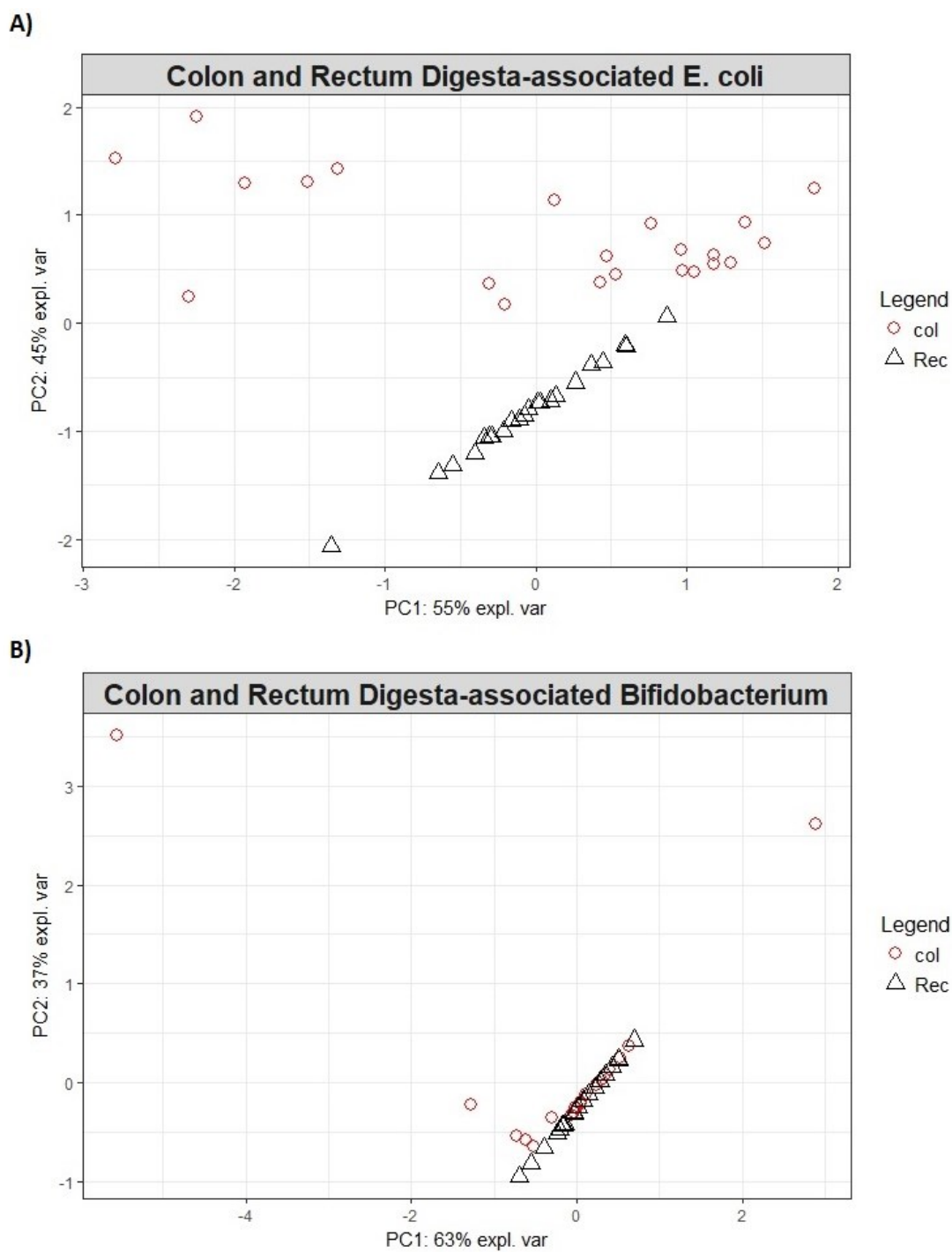
Figure 3- 8: PCA plot of the digesta-associated *E. coli* (A) and Bifidobacterium (B) from colon (col; n = 22) and rectum (Rec; n = 22) content samples are plotted along the first two principal component axis (PC1 and PC2).

Chapter 4. General Discussion

Good feeding management is one of the most important aspects in rearing dairy calves that supports development of neonate and improve gut health, which reduces risks of enteric diseases. Over the last decades, bovine colostrum and milk have been compared in terms of their composition and effect on neonates. For example, colostrum ingestion soon after birth accelerated microbial colonisation in small intestine of newborn calves (Malmuthuge et al., 2015a) and increased prevalence of probiotic *Bifidobacterium* spp. (Fischer et al., 2018; Malmuthuge et al., 2015a) and *Lactobacillus* spp. (Malmuthuge et al., 2015a) were observed in calves that received colostrum immediately after birth. Moreover, Liang et al. (2014) revealed correlation between miRNAs and the abundance of gut bacteria in GIT of newborn calves, suggesting that microbial colonisation may be associated with development of intestinal immune system regulated by miRNAs during neonatal period. Findings from this thesis (chapter 2.) indicate that prolonged colostrum feeding increases abundance of active tissue-associated *Lactobacillus* spp. and total *E. coli* in intestine of newborn calves. Thus, we propose that prolonged colostrum feeding may have stimulative effect of on microbial colonisation in calves GIT.

This thesis is the first to reveal the expression of genes involved in neuroendocrine functions of dairy calves. There has been an increasing interest in neuroendocrine functions and its role in regulation of energy metabolism and feed intake in ruminants. Neuroendocrine system is underdeveloped at birth and its development can be affected by birth conditions, diet and maturity in pre-weaned calves (Carron et al., 2005; Schaff et al., 2015). For example, A significant differences in abundance of mRNAs and binding sites of hepatic β-adrenergic receptors was increasing with age in pre-term, full-term and veal calves (Carron et al., 2005), suggesting that the neuroendocrine system is immature at birth and develops postpartum. Also, Schaff et al. (2014)

reported that colostrum feeding increased binding capacity of alpha adrenergic and glucocorticoid receptors in liver of neonatal calves, compared to that in milk formula fed calves, suggesting that different feeding can influence development of neuroendocrine receptors. These findings have focused on effect of neuroendocrine system on neonatal regulation of glucose metabolism in liver. However, there have been no studies done on the effect of feeding management on the expression of genes involved in neuroendocrine system in adrenal glands and intestine in newborn calves. The results in Chapter 2 revealed that ingestion of different amount of colostrum induced changes in expression of neuroendocrine gene in adrenal glands, ileum and colon of neonatal calves. In particular, prolonged colostrum feeding increased expression of *ADRA1A* in adrenal glands and ileum. However, colonic expression of *ADRA1A* was the highest in calves that received whole milk as subsequent diet. Thus, we speculate that different ingestion of colostrum and milk derived components can exert effect on neuroendocrine gene expression depending on functional and structural properties among GIT regions. Furthermore, the expression of *StAR* and *CYP11B1* in ileum and colon of newborn bull calves was identified. As these two genes encode a key enzymes involved in production of steroids and glucocorticoids (Liu et al., 2013), we propose that intestinal epithelium of newborn calves may produce these hormones. In the second study (chapter 3.) delayed colostrum feeding over 12 hours resulted in reduced concentration of fecal cortisol 51 hours postpartum, suggesting that feeding management may affect concentration of cortisol present in the lumen of large intestine. Cortisol is primarily produced in adrenal glands (Chen et al., 2015). However, increasing evidence suggest there may be local production of cortisol in various organs in the body including intestinal mucosa (Cima et al., 2004; Kostadinova et al., 2014; Sidler et al., 2011; Talaber et al., 2013), which is thought to play a role in regulation of immune,

metabolic and homeostatic processes (Kostadinova et al., 2014). Thus, future studies are required to evaluate the changes in cortisol concentration at systemic and local level.

Over last few decades, a considerable number of reports have proposed a bidirectional communication between gut microbiota and host. For instance, microbial production of short chain fatty acids, a main energy source for host, can stimulate host sympathetic nervous system (Kimura et al., 2011) and release of serotonin (Grider & Piland, 2007). Ouml et al. (2012) further showed capability of *Lactobacillus* spp. to produce serotonin, dopamine and other hormone-like metabolites *in vitro* in presence of gram positive bacteria, including *E. coli*. In the present study (chapter 2.), we observed significant positive correlation between growth of *E. coli* and *Lactobacillus* spp. and both of these bacterial groups were also correlated with expression of *HTR4* and *HTR2B* which encode serotonin receptor in colon. However, correlations of gut bacteria and gene expression of serotonin receptors was not significant in the ileum. This suggests there may be a potential association of *Lactobacillus* spp. and *E. coli* and modulation serotonin receptors in intestinal mucosa of colon. However, weak correlation in ileum suggests that there may be regional differences in the host-bacterial interactions. Moreover, eukaryotic catecholamines have been proposed to stimulate proliferation of commensal and pathogenic gram-negative bacteria (Freestone et al., 2000), suggesting that stress hormones such as epinephrine and norepinephrine can stimulate bacterial growth. Our results (chapter 2) indicate there was moderate positive correlation between abundance of active tissue-associated *E. coli* and expression of *ADRB2* in colon tissue. However, no such correlation was observed in ileum and *ADRA1A* expression was not correlated with abundance of active *E. coli* in both intestinal regions, ileum and colon. The *ADRB2* and *ADRA1A* genes encode β2-adrenergic receptors and α1-adrenergic receptors, which mediate effect of norepinephrine and epinephrine (Schaff et al., 2014), suggesting a potential

relationship of E. coli with β2-adrenergic receptors. Due to the low pH level in small intestine, abundance of gut microbiota is higher in hindgut (Bergmann, 2017). Therefore, we propose that these potential relationships may differ depending on environmental, functional and structural properties of GIT regions and the host-microbial interactions may be more intended in the regions of GIT with higher microbial biomass. Further studies are required to assess host-microbial interaction in neonatal period of newborn calves which may play an important role in establishment of immune tolerance of host to commensal microbiota. For example, investigation of other genes related to neuroendocrine functions which were not involved in this study may further help to better understanding of neuroendocrine changes in newborn animals. Up to date, most of the studies focused on simulative effect of catecholamines on pathogenic bacteria (Freestone et al., 2007) and there is clearly limited knowledge of association of catecholamines with growth of commensal bacteria. We observed positive correlation of *ADRB2* expression and active tissue-associated *E. coli* in colon, whereas gene encoding α adrenergic receptor (*ADRA1A*) was not correlated with *E. coli* growth. The effect of norepinephrine and epinephrine is mediated by β and α adrenergic receptors, respectively (Schaff et al., 2014) and these neurotransmitters play role in regulation of immunity and metabolism (Szelenyi & Vizi, 2007). Thus, we speculate that *E. coli* growth may be associated with β adrenergic receptors that transport norepinephrine. Further research is required to investigate association of catecholamines with growth of commensal bacteria which may contribute to better understanding of host-microbial interaction and establishment of immune-tolerance towards commensal microbiota in early life. Increasing number of studies about host-microbial interactions gathered over last few decades has given rise to a new field "microbial endocrinology" and gut brain axis theory. In particular, *in vitro* experiments as well as studies on germ free and gnotobiotic mice provide evidence about host-

microbial interaction using specific experimental design (Freestone et al., 2000; Ouml et al., 2012; Reigstad et al., 2015; Sudo et al., 2004). These finding suggest there is interactions between host and gut microbiota in human and mice. However, there is limited number of *in vivo* studies conducted on other animal models focusing on host-microbial interaction to confirm these mechanisms exist within the ecological complexity in GIT. Therefore, further studies about microbial neuroendocrinology are required to investigate interaction between level of endocrine hormones and microbial populations at different GIT regions.

Furthermore, lactoferrin is iron-binding protein that has been shown to inhibit adhesion of diarrheagenic *E. coli* to intestinal mucosa, which demonstrates another mechanism of colostral-derived components to benefit underdeveloped immunity of intestinal mucosa (Pereira & Giugliano, 2013). However, considerable evidence suggests that eukaryotic catecholamines can increase growth of commensal and pathogenic *E. coli* (Freestone et al., 2007; Freestone et al., 2002; Hendrickson et al., 1999; Lyte et al., 2011). Freestone et al. (2000) reported that norepinephrine stimulated growth of commensal *E. coli* in presence of lactoferrin by converting the antimicrobial lactoferrin into a nutritional iron source that enhanced *E. coli* growth. Therefore, we speculate that increased concentration of catecholamines together with colostral lactoferrin could have simulative effect on *E. coli* growth. Furthermore, we observed positive correlation (r=0.52, P= 0.0094) between abundance of *E. coli* and gene expression of *ADRB2* encoding β2 adrenergic receptor, which is present in all types of immune cells and play role in modulation of immunity (Madden, 2003).

Given the increasing evidences about host-microbial interactions over last few decades, several previous reports proposed gut-brain axis theory (Carabotti et al., 2015; Clarke et al., 2014; Freestone & Lyte, 2010; Sandrini et al., 2015; Sarkar et al., 2016; Sudo et al., 2004). Gut-brain

axis is a complex network of interactions between molecular processes in gastrointestinal tract and central nervous system of host (Sudo et al., 2004) that involves bidirectional communication between gut microbiota and host (Clarke et al., 2014; Sarkar et al., 2016). It has been suggested that microbiota can produce various hormone-like metabolites (serotonin, norepinephrine and γ-Aminobutyric acid) to regulate hormonal output and potentially respond to hormonal secretion by host (Clarke et al., 2014). Also, changes in composition of gut microbiota and diet can influence gut-brain axis in neonatal development (Sherman et al., 2014). Thus, the postnatal host-microbial interactions may play role in modulation of neonatal gut-brain axis and establishment of host immune tolerance to commensal gut microbiota during first days of life, which warrant future research.

It is noted that the microbial population was measured at two different levels, rRNA (chapter 2) and rDNA (chapter 3). Up to date, most of the studies about gut bacterial population have done DNA-based analysis, however RNA-based analysis may reveal fundamental linkage between active gut microbiota and host physiology. In the recent report about active rumen microbiome and feed efficiency, cattle with different residual feed intake had different compositional a functional characteristics of rumen microbiota (Li & Guan, 2017a). Similarly, the findings in this thesis (chapter 2) indicate that enteric bacteria may be associated with host neurotransmitter receptors in newborn calves. Therefore, future studies should focus on association of active gut microbiota with host physiology which will contribute to better understanding of host-microbial interactions.

There were several limitations of the research conducted for this thesis, which can provide directions for future studies focusing on postnatal development of neuroendocrine system and colonisation of gut bacteria in neonatal calves. Although concentrations of proteins generally

correlate with their corresponding mRNAs concentrations (Vogel & Marcotte, 2012), a Pearson correlation coefficient between mRNA and protein level can be as low as 0.45 (Maier et al., 2009), suggesting that there are variations between mRNA and protein levels. Thus, the missing data at protein levels to validate expression of neuroendocrine genes are one of the limitations in our study (chapter 2.) and level of endocrine hormones should be measured in blood and tissue samples. Also, Carron et al. (2005) revealed age-dependent differences in abundance of mRNAs and binding sites of hepatic β-adrenergic receptors in calves, suggesting that there is postpartum development of neuroendocrine system. However, the present studies (Chapter 2. and 3.) measured neuroendocrine gene expression and level of fecal cortisol and gut bacterial population only at 75 and 51 hours after birth. In previous reports about mammals, development of cardiac sympathetic nerve fiber was investigated up to 6 weeks in neonatal dogs (Kralios & Millar, 1978) and postpartum development of hepatic adrenergic receptors up to age of 159 days has been studied in calves (Carron et al., 2005). However, no studies have focused on postpartum development of neuroendocrine system in adrenal glands and intestine of newborn calves. Therefore, further studies are required to assess age-dependent changes in neuroendocrine system during early life in intestine, adrenal glands and other tissues which will contribute to a better understand of the postnatal development of neuroendocrine system. Furthermore, a fact that different age-related changes were observed during development of neuroendocrine system between young female and male rats (Leidy et al., 1993; Martinez et al., 1981) suggest there may also be gender differences in development of neuroendocrine system. As the research in this thesis focused on effect of feeding management only on neonatal bull calves, the feeding management could have a different effect on neuroendocrine system in neonatal female calves. Future research is therefore required to assess sex-related difference during postnatal development of neuroendocrine system in

newborn female and male calves. Additionally, substantial evidence suggest that stimulation of neuroendocrine receptors play a role in regulation of immune system and tissue development in human tissue and rodents (Claustre et al., 1999; Scanzano & Cosentino, 2015; Szelenyi & Vizi, 2007). However, no studies have been done to evaluate the role of neuroendocrine system on development of immune system and GIT in neonatal calves. Thus, future studies should investigate the role of neuroendocrine system in regulation of neonatal development of immune system and GIT in calves.

In regards to gut bacterial colonisation, the results of present studies (chapter 2. and 3.) suggest that feeding management may exert different effect on tissue and digesta-associated gut bacterial population in only in two locations of calf intestine. Furthermore, sample collection in the present studies of this thesis was performed at 75h (chapter 2.) and 52h (chapter 3.) postpartum. In previous report on calves, RNA-based analysis of fecal bacterial profile of dairy calves revealed age and diet dependent changes throughout 12-week period (Uyeno et al., 2010), suggesting that there are changes in composition of fecal bacteria during early life. Furthermore, Malmuthuge et al. (2015c) revealed a heterogeneity in terms of composition, diversity and abundance of gut microbiota during early life among different regions of GIT in calves. Thus, future research is needed to perform a complete RNA-based analysis of gut microbiota in different regions of GIT to evaluate regional changes in composition and diversity of active bacteria.

Findings in this thesis contribute to better understanding of neonatal neuroendocrine changes in newborn calves, which play an essential role in postpartum development of immune system and GIT. We conclude that different feeding strategies have effect on neuroendocrine system and gut bacterial colonisation during early life in neonatal calves, which can further help to establish feeding strategies to support neonatal development.

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