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Novel Approaches to Evaluate and Enhance Neonatal Calf Gastrointestinal Health and Development

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NOVEL APPROACHES TO EVALUATE AND ENHANCE NEONATAL CALF GASTROINTESTINAL HEALTH AND DEVELOPMENT

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DISSERTATION ACCEPTANCE PAGE

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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This Dissertation is dedicated to the memory of my mom Carma Sanguinet Trindade.

Those whom we have loved never really leave us. They live on forever in our hearts and cast their radiant light onto our every shadow - Sylvana Rossetti.

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LIST OF ABBREVIATIONS

ADG	Average daily gain
AMPs	Antimicrobial proteins
BCS	Body condition score
BW	Body weight
cDNA	Complementary DNA synthesis
CLDN	Claudin
CON	Control
d	Days
DNA	Deoxyribonucleic acid
DRTF	Dairy Research and Training Facility
E. coli	*Escherichia coli*
EC	Epithelial cells
FABP2	Fatty acid binding protein 2
FMT	Fecal microbiota transplantation
FPT	Failure passive transfer
GEC	Gastrointestinal epithelial cells
GIT	Gastrointestinal tract
IFNG-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
KRT8	Keratin 8
MPO	Myeloperoxidase
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
OTUs	Operational taxonomic units
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction

PMNL	Polymorphonuclear leukocytes
PRRs	Pattern recognition receptors
RBC	Red blood cells
RNA	Ribonucleic acid
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SDSU	South Dakota State University
TGF-β	Transforming growth factor
TJPs	Tight junction proteins
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRT	Treatment
wk	Week

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ABSTRACT

NOVEL APPROACHES TO EVALUATE AND ENHANCE NEONATAL CALF GASTROINTESTINAL HEALTH AND DEVELOPMENT

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Physiological adaptations of the gastrointestinal tract (GIT) epithelial cells as well as methods to manipulate early life GIT microbiota colonization during the neonatal stage, is of great importance to the dairy industry. The first objective of this research was to optimize a method based on evaluation of bovine transcripts in fecal RNA via RT-qPCR using L-selectin (*SELL*) as a marker for polymorphonuclear leukocytes (PMNL), keratin 8 (*KRT8*) and fatty acid binding protein 2 (*FABP2*) for GIT enterocytes, and tight junctions in fecal RNA and GIT sections in dairy calves. To test the reliability of the fecal RNA method, fecal and PMNL samples from neonatal calves were used. The expression of *KRT8* was greater in fecal RNA than in PMNL. In contrast, a greater *SELL* expression was observed in PMNL. In another study, postmortem GIT and feces samples were collected from healthy calves for total RNA isolation. Overall, the expression of *FABP2* and KRT8 was similar between fecal RNA and the lower GIT. Taken together, these results provide further evidence that the fecal RNA method can potentially be used as a tool to evaluate molecular adaptations of the GIT in dairy calves. The second objective was to evaluate the effects of early life fecal microbiota transplantation (FMT) from healthy adult donors on health and performance of neonatal dairy calves. To test the effects of FMT, newborn calves were subjected to 1×/d inoculations with 25 g of fecal

donor material mixed in the milk replacer from 8 to 12 d of age. Results from this study demonstrated that calves subjected to FMT tended to have greater body weight than control calves. The liver function marker paraoxonase was greater in the blood of FMT calves than control at 3wk of age. These results suggest that FMT in neonatal calves has positive effects not only on growth performance but also in mediating liver function.

INTRODUCTION

Neonatal dairy calves experience several challenges early in life, including adaptation to an extrauterine life, where animals have to adapt from an umbilical-nutrient mode of delivery to ingest milk and food from buckets or bottles, as well as thermoregulate their own body and being exposed to a new environment loaded with pathogens. After birth, the gastrointestinal tract (GIT) undergoes morphological and functional adaptations, which start with the ingestion of colostrum, affecting the gastrointestinal motility as well as the acquisition of passive immunity (Guilloteau et al., 1997, Blum and Hammon, 2000). In addition to the gastrointestinal maturation, the calf has to adapt to a new pathogen-rich environment and has to transition from pre-ruminant to ruminant during the weaning period. Together, these factors can prone calves to develop diarrhea due to the absorption of endotoxins from the GIT or the translocation of microbes (Constable, 2004). Because of these early life challenges neonatal dairy calves have to overcome, enteric infections are the leading cause of mortality in preweaned dairy calves in the US (Berchtold, 2009). Additionally, diarrhea is a major cause of economic loss to dairy producers, including cost of antibiotics, reduced growth, and increased age at first calving (Windeyer et al., 2014). Fecal score systems are commonly used by researchers and dairy producers to keep track and identify animals at risk of developing diarrhea. More sophisticated methods such as microscopy, end-point PCR, and bacterial culturing have been used to identifying specific strains of infectious pathogens. However, these analyses lack information on the actual biological adaptations the GIT undergoes during the onset of diarrhea. The GIT epithelial cells besides the function of absorption of nutrients, also act as a barrier to harmful pathogens, and the exfoliation of these cells has

been associated with an intestinal natural turnover process to preserve tissue structure (Kaeffer, 2011). It has been estimated that in mammals such as humans, 10^{10} cells are shed daily from the small intestine (Donovan et al., 2014). Epithelial cells can be isolated from different body fluids (i.e., urine and feces), which it is a non-invasive technology that can be used instead of performing tissue biopsies and postmortem tissue analysis (Kamra et al., 2005).

The high turnover of cells occurring in the small intestine suggests that at least a small portion of those exfoliated cells are viable for downstream molecular biology applications. A noninvasive mRNA-based method as a highly sensitive technique was originally used for detecting molecular biomarkers of colon cancer in rats (Davidson et al., 1995, Chapkin et al., 1998) and later for intestinal development and function in adult humans (Davidson et al., 2003, Zhao et al., 2009). This method has been used to study nutritional regimens in humans, using stool samples to isolate exfoliated cells from the GIT (Kaeffer et al., 2007), as well as to quantify intestinal gene expression profiles in developing infants (Chapkin et al., 2010). In the latter, Chapkin et al. (2010) were able to differentiate between neonates that were breastfed and formula-fed by their different transcriptomic profiles.

In this context, our group demonstrated that transcriptional changes of the GIT to inflammatory conditions (e.g., diarrhea) could be evaluated by alternative methods such as RNA isolated from feces of neonatal dairy calves (Rosa et al., 2018). In that study, intestinal exfoliated cells in fecal samples of neonatal dairy calves are viable for downstream molecular applications. The expression of genes related to inflammatory pathways as well as cell membrane transporters detected in the fecal RNA was associated

with blood biomarkers of inflammation at the time point where the calves had the greatest fecal score (i.e., scours). However, the possibility to use the above method to investigate intestinal gene expression alterations in dairy calves needed further insights and evaluation.

The GIT microbial colonization and symbiosis are essential for the development and physiology of mammals, including neonatal calves (Alipour et al., 2018). The GIT microbiota has several roles in nutrition, energy metabolism, host defense, and immune system development. Several factors can potentially affect the newborn intestinal microbial complexity and maturation including intrauterine environment (i.e., maternal microbiome), dystocia as well as postnatal factors including environment, diet, antibiotic use, and in the case of neonatal calves colostrum intake and quality (Oikonomou et al., 2013, Perez-Muñoz et al., 2017). Taken together, these several factors suggest that early life interventions on the gut microbiota can be a key factor in manipulating GIT colonization in neonatal calves, and therefore improve their health and growth by reducing the susceptibility to enteric infections. For instance, fecal microbiota transplantation (FMT), which is the transfer of microbiota via inoculation (i.e., oral administration, endoscopy, colonoscopy) present in the fecal material of a healthy-selected donor into a recipient, can potentially restore or shift the GIT microbial composition (de Groot et al., 2017). In human medicine, FMT has been extensively used to treat individuals suffering from different types of infections, including inflammatory bowel disease (Ponte et al., 2015, Khanna et al., 2017). However, in the animal production, there are still many gaps in knowledge when it comes to utilizing FMT in livestock animals, where very few studies have been conducted, primarily in companion animals (Pereira et al., 2018), neonatal piglets (Hu et al., 2018b),

and broiler chickens (Siegerstetter et al., 2018); however, to date, there are no studies that performed FMT in dairy calves.

The two overall goals of this research are to further validate and optimize a non-invasive fecal RNA method to investigate transcriptomic changes in the GIT neonatal calves, and to evaluate the effects of an early life fecal microbiota transplant on growth and health performance of neonatal dairy calves.

CHAPTER 1. LITERATURE REVIEW

Neonatal health and immunity

In dairy calf development, a major focus of research has been the maturation of the rumen (Baldwin et al., 2004). However, there is a critical period prior to the solid feed intake, and consequently, the rumen development and maturation, that is characterized by high susceptibility to disease. In this period, the gastrointestinal tract undergoes several changes such as an increase in permeability and functionality (Steele et al., 2016b). The newborn calf has to adapt its digestive system from an umbilical nutrient delivery during the fetal period to ingestion of bottle- or bucket-fed milk. After birth, the GIT undergoes morphological and functional adaptations, which start with the ingestion of colostrum, affecting the gastrointestinal motility as well as the acquisition of the passive immunity (Guilloteau et al., 1997, Blum and Hammon, 2000). In addition to the gastrointestinal maturation, the calf has to adapt to a new environment, which is loaded with pathogens, thermoregulate its own body, and transition from pre-ruminant to ruminant during the weaning period. After birth, both microbial and gastrointestinal epithelial cells (GEC) maturation are influenced by many factors, including diet, length of gestation, and antibiotic usage. The latter has been closely associated with a fundamental disruption of the normal intestinal flora (i.e., dysbiosis), and consequently, linked with diseases in both humans and animals (Francino, 2015). Besides the several challenges described above that a newborn calf goes through during the first 4 weeks of life, factors affecting early colonization of the neonate microbiome can fundamentally challenge infants and put them at risk for immune and metabolic disorders. In newborn calves, intestinal disorders are common events during the neonatal stage (from birth to weaning), as well as, during

the preweaning and weaning period leading to diarrhea or calf scour, which is the leading cause of mortality in neonatal dairy calves in US during the first 4 weeks of life (Berchtold, 2009). Indeed, according to the National Animal Health Monitoring System (NAHMS), scours or other digestive problems during the pre-weaning stage account for 57% of calf mortality in the US. Additionally, diarrhea is a major cause of economic loss to dairy producers, including the cost of antibiotics, reduced growth, and increased age at first calving (Windeyer et al., 2014). Furthermore, NAHMS also reported high antibiotic usage for the treatment of calf scours.

Neonatal calf scours are frequently observed from 3 to 21 d of age, but its onset and duration are determined by the number of pathogens involved and the immune condition of the animal (Blum, 2006). In mild-diarrhea conditions, loss of extracellular fluids and electrolytes can take place; however, during severe diarrhea conditions, body weight loss due to dehydration and cellular damage of the GIT epithelium due to inflammation can be observed (Blum and Hammon, 2000). The major pathogens that have been identified as contributing to enteric challenge in calves and consequently, to the development of calf diarrhea include *Escherichia coli K99* (*E. coli*), Rotavirus, Coronavirus, *Salmonella* spp., and *Cryptosporidium parvum* (Izzo et al., 2011, Cho and Yoon, 2014).

All these events during the neonatal stage of calves are regulated by an interaction between infectious pathogens, the immune status of the calf, and the environment. Despite major advances in dairy calf rearing, the neonatal development has many gaps in the understanding of the transitional fetal/neonatal physiology, and the transition from pre-ruminant to ruminant. For most dairy operations, calves represent the next generation

of milk producing animals. However, keeping young calves healthy and growing is a complex task. Therefore, understanding these physiological changes and the transcriptional alterations involved within the gastrointestinal tract that might cause or induce inflammation, which can affect the animal systemically can be used as a tool to strategically intervein and manipulate the gastrointestinal tract of dairy calves during the susceptible period from birth to weaning resulting in health improvement and productivity.

Immune status of the neonatal calf and the impact on health

The immune status of neonatal calves can be characterized as dysfunctional. Although all essential immune components are present in neonates at birth, many of the components are not functional until calves are at least 2 to 4 weeks of age and might continue to develop until puberty (Chase et al., 2008).

In bovine, since antibodies don't cross the placenta (Peter, 2013), the immunoglobulins (IgG) transferred from the cow to the calf through the colostrum are essential for the newborn's survival by providing the passive immunity. Poor immunoglobulin transfer is called failure of passive transfer (FPT) and is linked to increased neonatal morbidity and mortality in dairy calves (Torsein et al., 2011). The absorption of intact macromolecules across the intestinal epithelium into the neonatal circulation is possible for approximately 24 hours after birth (Arthington et al., 2000). There is evidence that around 12 h after birth, the intestinal enzyme secretion starts to increase, in addition to the intestinal cell turnover, which reduces the IgG absorption (Quigley and Drewry, 1998). However, there is a gap between the passive immunity provided by colostrum and the acquisition of the neonate active immunity. Considering

that passive immunity acquired from colostrum remains active only up to approximately 21 days of age, the neonatal stage encompasses a wide window of high susceptibility to infections and illness, and consequently immune challenges (Kampen et al., 2006, Ranade et al., 2014).

At birth, neonates have neutrophil and macrophage (innate immune cells) activity reduced, which improves after colostrum ingestion due to the maternal transfer of immunoglobulins, immune cells, and cytokines. On the other hand, maternal immunity interference is a challenge regarding vaccine development for neonatal calves. Several maternal antibodies have a half-life of 28 days (Fulton et al., 2004), therefore the timing for administrating a parenteral vaccine needs to take in consideration the timeframe where the maternal antibody is in a low level to produce an immune response to the vaccine (Cortese, 2009). Additionally, the cow production of estrogen and cortisol prior to parturition can affect the immune response of the newborn calf by suppressing the cell-mediated response (Chase et al., 2008).

Intestinal functionality and integrity

The GIT is a dynamic and complex environment with a microbiologically active ecosystem (i.e., microbiota) and a heightened immune activity comprising non-hematopoietic (e.g., epithelia, Paneth cells, goblet cells, etc.) and hemopoietic cells (e.g., macrophages, dendritic cells, T-cells, etc.) (Maldonado Galdeano et al., 2019).

The small intestine encompasses a dual function: a) provide selective absorption of nutrients; b) provide a barrier against harmful pathogens within the intestinal lumen (Yu et al., 2012). The intestinal microbiota does not interact directly with the intestinal

epithelial cells; however, the feed intake, the host-microbiota, and the host mucosa interact with each other supporting the health of the GIT tract, and consequently the animal growth and performance. Celi et al. (2017) defined the gastrointestinal functionality as a state where diet, effective digestion and absorption, stable microbiota, effective immune system, intestinal mucosa, and neuroendocrine function of the GIT interact in a symbiotic equilibrium maintaining the GIT homeostasis and constraining intestinal dysfunction. Thus, intestinal permeability and GIT barrier play a crucial role in the GIT physiology and overall gut health.

Intestinal structure and immune system

The intestinal epithelium exhibits numerous physical adaptations to separate the host connective tissue from the external environment (Guilloteau et al., 1997). Four important cell types are defined, namely absorptive (enterocytes in the small intestine, and colonocytes in the large intestine), muco-secreting cells (goblet cells), enteroendocrine (function in peptide hormone secretion), and Paneth cells (secretory granules). Under healthy conditions, the renewal of these cells within the GIT occurs every 2-7 days by the GIT stem cells localized in the crypt base of the GIT epithelium, however during tissue damage period, the epithelial shed increases (Brittan and Wright, 2004).

Small intestinal absorptive cells (enterocytes) are columnar epithelial cells, which form crypts and villi to increase surface area for absorption (Steele et al., 2016a). the mucosal epithelium consists in a mucus-glycocalyx layer protecting the enterocytes, which is in continuous contact with commensal and harmful microorganisms, this mucus layer is produced by the goblet cells (mucus-secreting cells), serving as a physical barrier

(Peterson and Artis, 2014). Intestinal epithelial cells are covered with two layers of mucus, the firmly adherent layer that is closest to the epithelium and the loosely adherent layer that is the outer layer (Steele et al., 2016a). The mucus layer is predominantly mucin-rich layer, whereas the mucin 2 (MUC2) produced by the goblet cells is the most abundant mucin in the mammalian intestine (Chen et al., 2015). Intestinal epithelial cells also produce antimicrobial proteins, called AMPs, and secrete them into the mucus layer. The AMPs produced by these cells include the lysozyme, which breaks down cell wall components, lactoferrin, which sequesters iron needed for bacterial growth, and alpha-defensins when exposed to bacterial products. (Gassler, 2017). In addition to the AMPs in the mucus layer, secreted immunoglobulin A (IgA) antibodies produced by B cells in the Peyer's patches and throughout the intestinal lamina propria are actively transported and translocated by polymeric immunoglobulin receptor (pIgR) located on the basolateral membrane of enterocytes. The IgA is then transported through the enterocytes, where the pIgR is converted into the secretory piece and added to the dimeric IgA prior to secretion into the intestinal lumen. The secretory IgA limits the association of pathogens with the intestinal epithelia. The IgA antibody is a major functional component of the humoral adaptive immune system, specifically at mucosal sites. The antibodies are predominantly produced in the intestinal lamina propria by plasma cells following B cell differentiation (Chase and Kaushik, 2019). Also, the commensal microbiota present in the GIT is essential for the mucus layer integrity, including the thickness of the mucosal barrier as well as the mucosal interaction with the host-microbiota, which stimulates a state of inflammatory recognition needed for the maintenance of the intestinal homeostasis (Galdeano and Perdigon, 2004).

The enterocytes are the most abundant epithelial cells type lining the gastrointestinal tract with the primary function of nutrient absorption through active and passive transport and brush border enzyme activity (Peterson and Artis, 2014). However, these cells express toll-like receptors (TLR) in their membrane by which upon pathogen recognition can initiate a pro-inflammatory response in the GIT by producing specific cytokines. In the case of neonates, the intestinal enterocytes are immature at birth and mature within the first week after birth, as indicated by the loss of vacuoles along the proximal-distal axis, which contributes to intestinal barrier closure (Prgomet et al., 2007).

Enteroendocrine cells link central and enteric neuro-endocrine systems through the secretion of peptides hormones that act locally on the nervous system of the intestinal lining. Such secretion regulates food intake, GIT motility, and glucose metabolism (Cani et al., 2013). Beneath the basolateral surface of the GIT epithelium, there is the lamina propria surface, which hosts immune cells such as macrophages, natural killer cells, mast cells, dendritic cells, as well as, B cells and T regulatory cells (Chase and Kaushik, 2019).

Intestinal permeability

The establishment of an epithelial barrier requires the formation of morphologically distinct tight junctions. Tight junctions are adhesive junctional protein complexes between epithelial cells that connect each other, forming a paracellular barrier, which facilitates the passage of ions and solutes through the intercellular space while preventing the translocation of luminal antigens, microorganisms, and toxins (Bischoff et al., 2014). Tight junctions determine the extent and maintain distinct apical and basolateral membrane domains in epithelial cells, thereby tightening and maintaining the permeability capacity of the intestine (Fanning et al., 1998). Tight junctions are crucial

for the integrity of the epithelial barrier function. Among the tight junction proteins families, there are 3 transmembrane proteins: occludin, claudin, and junctional adhesion molecules (JAM). Tight junction protein ZO-1, claudin 1, and 4 are commonly abundant in the epithelial cell layers (Kiuchi-Saishin et al., 2002, Langbein et al., 2002). When the claudin-1 gene was knocked out, the epidermal barrier failed to prevent water loss (Furuse et al., 2002). Pro-inflammatory cytokines such as TNF-α (tumor necrosis factor-α) and IFN-γ (interferon-γ) cause disruption of the tight junction barrier resulting in increased paracellular permeability in intestinal monolayers (Al-Sadi et al., 2009). Anti-inflammatory cytokines such as IL-10 (interleukin-10) and TGF-β (transforming growth factor) maintain tight junction integrity function (Forsyth et al., 2007). Inflammation associated with gastrointestinal disorders is the major inducer of tight junction dysregulation, resulting in increased intestinal permeability (Kvidera et al., 2017).

Pathogenic recognition in the intestinal epithelium and signaling response

The intestinal immune system has the ability to distinguish between inducing either immune responses against harmful antigens or tolerogenic responses towards antigens derived from nutrients and beneficial bacteria (Maynard et al., 2012). However, when the mucosal barrier becomes disrupted, bacteria and/or bacterial products can invade the adjacent tissues and are sensed by antigen-presenting cells, such as dendritic cells and macrophages. These cells migrate to the lymphoid follicles in the Peyer's patches or to gut-draining mesenteric lymph nodes, where they present the antigens to naive T cells leading to their activation and expansion. The resulting effector T cells eventually exit the mesenteric lymph nodes and migrate to the gut interstitium (Antoni et al., 2014). Once in the intestinal lamina propria, they re-encounter their specific antigen

presented by antigen-presenting cells, which initiates a rapid inflammatory response with the production of proinflammatory cytokines.

Although commensal microbiota is essential for immunological tolerance, the immune system needs to differentiate between commensal bacteria and pathogenic bacteria. Therefore, receptors present in the membrane of epithelial and lymphoid cells of the small intestine regulates this differential recognition. These receptors have been named pattern recognition receptors (PRRs), and the structures they sense are termed pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Several classes of PRRs have been described, including toll-like receptors (TLRs) and C-type lectin receptors (CLRs) (Brubaker et al., 2015). Under non-inflammatory conditions, TLR signaling leads to tolerance towards luminal pathogens through the down-regulation of pattern-recognition receptors and promotes mucosal wound healing (Abreu, 2010). When intestinal macrophages and dendritic cells sense PAMPs, pathogen signaling pathways including the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) are activated inducing pro-inflammatory cytokines production such as interleukin 1 (*IL1*), interleukin 6 (*IL6*) and tumor necrosis factor alpha (*TNFA*), and chemokines, such as interleukin 8 (*IL8*) (Ceciliani et al., 2012).

Neonatal calf microbiota

Early neonatal GIT microbiome colonization and development begins with maternal microbiome seeding begins before parturition. The traditional “paradigm” that the fetus develops in a sterile environment has been challenged in the past years (Aagaard et al., 2014). 16S ribosomal RNA sequencing demonstrated that bacterial species from the phyla Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria were

found to exist within the human placental basal plate (an area once thought to be sterile) (Aagaard et al. 2014). In another study, cesarean-born babies deprived of maternal vaginal microbial transfer at birth are predisposed to have health issues (Dominguez-Bello et al., 2010).

GIT microbial colonization in a newborn calf includes the vaginal microbiome and the proximity of the birth canal (Oikonomou et al., 2013). Colostrum and milk microbiota may also influence such colonization. Microbial diversity increases with growth, age, and dietary adaptations in calves (Malmuthuge and Guan 2017).

Lower gastrointestinal tract microbial colonization: Mayer et al. (2012) attempted to investigate first colonizers in newborn calves; however, this study was not able to validate such findings in the meconium samples with placental transfer, but the microbial composition identified was associated with the rectal bacterial flora of the dam. In another study to investigate the initial colonization of GIT of newborn calf (Klein-Jöbstl et al. 2019) demonstrated that *Proteobacteria* were the most abundant in calf feces over time. However, a significant shift in the microbial composition was observed during the first 48 hours after birth, whereas *Actinobacteria* and *Bacteroidetes* decreased within 12 hours after birth, while *Proteobacteria* and *Firmicutes* increased from 24 to 48 hours after birth. Furthermore, the same study showed the presence of operational taxonomic units (OTUs) with *E. coli* in all the fecal samples during all the time points, which is in agreement with previous studies that demonstrated *E. coli* colonization in the calf rectum 24 hours after birth (Alipour et al., 2018).

Most of the intestinal microbiota studies in neonatal calves have focused on identify and classify the fecal microbiota as a representative population of the GIT

microbiota (Meale et al., 2017a). The microbial population in the gastrointestinal tract of neonatal calves is primarily important for the stimulation of the inflammatory mechanism within the intestinal lumen, as discussed previously. The bacteria composition throughout the GIT (i.e., rumen, jejunum, ileum, cecum, and colon) in neonatal bull calves (i.e., 3 wk of age) varied in bacterial composition depending on the region, whereas ruminal samples and large intestine had high levels of *Bacteroidetes* while the small intestine had high levels of *Firmicutes.*

Forestomach microbial colonization: The majority of the microbial investigations on the forestomach of calves have been focused on the rumen maturation rather than omasum and abomasum evaluation. The rumen colonization of calves (pre-ruminant) is mainly colonized by anaerobic bacteria by the second day of life, and the cellulolytic bacteria population reported to be stabilized within the first week of life. The early rumen bacterial species consist of the genera *Propionibacterium, Clostridium, Peptostreptococcus*, and *Bifidobacterium,* while *Ruminococcus* species dominated the cellulolytic bacterial population (Malmuthuge et al., 2015) . Additionally, bacteria that are essential for mature rumen function such as methanogens, fibrolytic bacteria, or *Proteobacteria* have been detected as early as 20 minutes after birth (Guzman et al., 2015), and 24 hours after birth, prior to any ingestion of solid feed (Jami et al., 2013). The findings with rumen microbial colonization in neonatal calves suggest that not only is early microbiota colonization essential for the rumen development but might also play a role in microbial metabolic products involved in the early immune development (Li et al., 2012). This also raised the need to investigate further if such inoculations could be associated with prenatal exposure, including maternal microbiome (Meale et al., 2017a).

Collectively, these studies and findings suggested that microbial diversity throughout the GIT of calves is affected during the preweaning period and can be partially attributed to the increase in solid feed intake. However, the incidence of infectious disease during the first 4 wk of life and the use of antibiotics for treatment of calf diarrhea were also associated with microbial shift (i.e., reduced microbial diversity in calves with scours) (Meale et al., 2016, Meale et al., 2017b).

RATIONALE AND OBJECTIVES

The overall goal of this project was to improve the understanding of the physiological and transcriptional alterations in the gastrointestinal tract (GIT) of dairy calves during the most susceptible period for enteric infections, the neonatal stage. To evaluate calf neonatal overall health status and performance, we attempted to validate and optimized two approaches: a) fecal RNA as a non-invasive technique to study transcriptional changes in the gastrointestinal tract of dairy calves during healthy and inflammatory conditions; b) fecal microbiota transplant (FMT) as a tool for microbiota manipulation in neonatal calves.

Rosa et al. (2018) was the first attempt to utilize the fecal RNA method to evaluate biological adaptations of the gastrointestinal tract of dairy calves through gene expression analysis. However, a limitation in this study is the current lack of data indicating how the transcriptomic profile observed in fecal RNA mirrors that in specific sections of the GIT. Additionally, fecal RNA isolation remains a challenge due to the potential enrichment of bacterial RNA and ribosomal RNA, which can create a dilution effect on the targeted eukaryotic RNA and consequently dampened the sensitivity of the fecal RNA method. Therefore, the objectives of our research on this topic regarding to the fecal RNA as a non-invasive method to investigate GIT adaptations study were: 1) to evaluate the potential enrichment of immune cells and GIT epithelial cells measured through the transcription of target genes for both immune and epithelial cells in RNA isolated from fecal samples compared with RNA isolated from blood polymorphonuclear lymphocytes of neonatal dairy calves; 2) to compare the transcription of gene markers for

GIT epithelial cells, fatty acid binding protein 2 (*FABP2*) and cytokeratin 8 (*KRT8*) in fecal RNA against several GIT sections in dairy calves.

The intestinal microbiota plays an important role in animal health. An imbalance in the GIT microbiota can make animals more prone to develop diarrhea and other diseases. The use of the fecal microbiota transplantation method in a controlled clinical trial was first reported in 2013 to be an effective treatment for *Clostridium difficile* infection in humans (van Nood et al., 2013). To our knowledge, the fecal microbiota transplantation has not been performed or validated in neonatal dairy calves previously. Therefore, we aimed to perform a fecal microbiota transplantation in neonatal dairy calves and to evaluate the effects on health parameters, including inflammatory biomarkers and overall animal growth.

CHAPTER 2. COMPARATIVE GENE EXPRESSION ANALYSIS ON THE ENRICHMENT OF POLYMORPHONUCLEAR LEUKOCYTES AND GASTROINTESTINAL EPITHELIAL CELLS IN FECAL RNA FROM NONDIARRHEIC NEONATAL DAIRY CALVES

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ABSTRACT

Increased understanding of the biology of the gastrointestinal tract (GIT) in neonatal dairy calves during their adaptation to an extrauterine environment will decrease health problems such as diarrhea while increasing feed efficiency and average daily gain in preweaned dairy calves. Within this context, a noninvasive method, based on fecal RNA, to study the GIT in neonatal dairy calves through the isolation of RNA from fecal samples for quantitative reverse-transcription PCR analysis can provide valuable information on GIT biological adaptations during the preweaning period. We aimed to evaluate the potential enrichment of RNA from immune cells or GIT epithelial cells during fecal RNA isolation. Eight neonatal Holstein calves less than 3 wk old (14.9 ± 5.5 d of age at sampling ± SD) and a fecal score of 2.0 ± 0.7 (mean ± standard deviation) were used. During a single sampling, fecal and blood samples were taken simultaneously from each calf before the morning feeding. Fecal samples were immediately frozen in

liquid nitrogen until RNA isolation, whereas polymorphonuclear leukocytes (PMN) were isolated from blood samples before RNA isolation. A quantitative reverse-transcription PCR analysis was performed using a single standard curve composited of equal amounts of all samples including cDNA from fecal and PMN. The genes myeloperoxidase (*MPO*) and L-selectin (*SELL*) were selected for their specific known function in PMN, whereas keratin 8 (*KRT8*) and aquaporin 3 (*AQP3*) have been associated with epithelial enterocytes. Our results showed a contrasting gene expression profile between PMN and fecal RNA; whereas greater mRNA expression of *SELL* was observed in PMN, a greater *KRT8* expression was observed in fecal RNA. The mRNA expression of *AQP3* tended to be greater in PMN than fecal RNA. Additionally, *MPO* was not amplified in fecal RNA. Our findings suggest that under nondiarrheic conditions RNA isolated from stool samples of neonatal dairy calves will have a considerable number of GIT epithelial cells, which confirms the reliability of this method under these conditions. However, further research needs to be done to determine if the same effects are observed during diarrhea or throughout the preweaning period of dairy calves.

Key words: Fecal RNA, gene expression, polymorphonuclear leukocytes, dairy calf

INTRODUCTION

The gastrointestinal tract (GIT) is a complex system with multiple functions including digestion and absorption of nutrients, protecting the host from a constant presence of microorganisms, toxins, and other chemicals in the lumen. In newborn calves, such functions and complexity are well known to be partially influenced by exogenous nutritional and nonnutritional factors (i.e., bioactive compounds) that are ingested with colostrum (Ontsouka et al., 2004). The latter effect is primarily associated

with a direct influence on the maturation of the GIT in early life (Ontsouka et al., 2016). Therefore, understanding the progression of the GIT to a mature state, and determining what bioactive compounds in the colostrum have a predominant influence on this effect, are high priorities to minimize diarrhea and improve welfare among young dairy calves. However, studying early life maturation of the GIT can be challenging if this is done primarily through analysis and characterization of postmortem or surgical collections of GIT specimens. Such a challenge is more evident when repeated samplings over time on the same animals are desired to observe changes over time as well as to minimize animal variation. To overcome such challenges, we have previously proposed a novel technique by isolating RNA from fecal samples of newborn dairy calves with the aim of obtaining biological information on the GIT over time and using the same animals (Rosa et al., 2018).

Results from that study suggested that isolating RNA from fecal samples for subsequent quantitative reverse-transcription PCR analysis is a promising technique, where we observed biological adaptations of the young ruminant GIT over time to a mild diarrhea event, and these data were confirmed through blood biomarkers for inflammation and oxidative stress (Rosa et al., 2018). Although this technique is promising, several optimizations and validation steps are required to improve accuracy and robustness. For instance, the migration of PMN across the intestinal epithelium is a common pathological event of many mucosal inflammatory diseases (Brazil et al., 2010). Because the fecal RNA method described by Rosa et al. (2018) discriminated primarily against prokaryotic RNA (i.e., microbial RNA) but not immune cells, it is important to understand the potential influence immune cells such as PMN might have during fecal

RNA analysis. Additionally, the fecal RNA method is based on the premise that stool samples contain a considerable number of epithelial cells (Nishizawa et al., 2005). Consequently, evaluating the RNA enrichment from immune cells during fecal RNA isolation is essential to understand the reliability of this method. Therefore, the objective of this study was to evaluate the potential enrichment of RNA from PMN or GIT epithelial cells during RNA isolation from fecal samples of neonatal dairy calves.

MATERIALS AND METHODS

Experimental design

The Institutional Animal Care and Use Committee of South Dakota State University approved all procedures for this study (protocol no. A3958–01). During a single sampling, fecal and blood samples were taken simultaneously from 8 neonatal Holstein calves less than 3 wk old (14.9 ± 5.5 d of age at sampling ± SD) with a mean fecal score [scale 1–4, 1: firm, well-formed (not hard); 4: liquid, splatters (Osorio et al., 2012b)] of 2.0 ± 0.7 (mean ± SD) at sampling.

RNA isolation, cDNA synthesis, primer design and evaluation, and quantitative PCR

The fecal RNA isolation was conduct using the same protocol as explained in Rosa et al. (2018). Briefly, ~200 mg of fecal sample was used for RNA isolation using a Trizol (catalog no. 15596018, Ambion, Carlsbad, CA) based method along with the RNeasy Plus Mini Kit (Qiagen, catalog no. 74134), following the manufacturer's instructions with some modifications. The RNA was eluted in 50 µL of RNase-free water, and the quantity for all fecal samples was 378.8 ± 192.3 ng/µL and purity (260/280 ratio) was 2.0 ± 0.1 determined via Nanodrop. In the case of PMN, the complete PMN isolation protocol is presented in the Supplemental File (https://doi.org/

10.3168/jds.2018-16074), and this protocol has been previously described by Osorio et al. (2013). Briefly, 100 mL of blood was collected into evacuated tubes (10 mL, BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing solution A of trisodium citrate, citric acid, and dextrose. Samples were centrifuged at 600 × g for 30 min at 4°C. The plasma, buffy coat, and approximately one-third of the red blood cells were discarded. The remaining sample was purified through a series of washing and centrifugation steps using deionized water, 5 × PBS, and 1 × PBS. An aliquot (20 µL) obtained during the isolation process was used for PMN quantification and viability using a granulocyte primary antibody (CH138A, Veterinary Microbiology and Pathology, Washington State University, Pullman, WA) followed by a second antibody (Goat Anti-Mouse IgM, Human ads-PE, Southern Biotech, Birmingham, AL). All samples harvested and used for analysis contained more than 80% PMN and had at least 90% viability. After PMN isolation, RNA was extracted using the Trizol-based method along with the RNeasy Plus Mini Kit. The RNA was eluted in 50 µL of RNase-free water, the quantity for all PMN samples was 55.6 ± 32.7 ng/µL, and the purity was 1.9 ± 0.1. The RNA integrity was assessed using the TapeStation (Agilent Technologies, Santa Clara, CA), and the final RNA integrity number was 5.0 ± 2.6.

Details regarding primer sequences information and sources are presented in Supplemental Table 2.1 (https://doi .org/10.3168/jds.2018-16074). The cDNA synthesis and quantitative PCR were performed according to the protocol described by Rosa et al. (2018) with some modifications. Each cDNA was synthesized by reverse transcription using 500 and 100 ng of RNA for fecal and PMN samples, respectively. The quantitative PCR reaction was performed in a QuantStudio 6 Flex Real Time PCR System (Applied

Biosystems, Waltham, MA) in a MicroAmp Optical 384-well Reaction Plate (Applied Biosystems). Relative mRNA expression of each gene was calculated based on a 6-point relative standard curve plus the nontemplate control. Such a standard curve was composited from all samples including cDNA from fecal and PMN. The internal control genes (ICG) used in this experiment were golgin subfamily A, member 5 (*GOLGA5*), oxysterol-binding protein-like 2 (*OSBPL2*), single-strand-selective monofunctional uracil-DNA glycosylase 1 (*SMUG1*), β-2microglobulin (*B2M*), β-actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and ribosomal protein 9 (*RPS9*). Additionally, the ribosomal protein S15A (*RPS15A*) and peptidylprolyl isomerase A (*PPIA*) were evaluated as potential ICG, but they were deemed to have low stability across samples. Therefore, the geometric mean of the 7 ICG was used to normalize the expression of the target genes on both types of samples (i.e., fecal and PMN). The stability of the ICG was assessed using the geNorm software (Vandesompele et al., 2002), with a favorable final pairwise variation of 0.19. The ribosomal protein S15A (*RPS15A*) and peptidylprolyl isomerase A (*PPIA*) were evaluated as potential ICG, but they were deemed to be unsuitable due to low stability across samples. Normalized gene expression data were log-transformed before statistical analysis. The target genes were selected based on their known specific association with either PMN or GIT epithelial cells. In the case of PMN, the genes selected were myeloperoxidase (*MPO*) and selectin-L (*SELL*), which are well-documented genes specific for PMN (Bionaz and Loor, 2008, Moyes et al., 2009, Batistel et al., 2017), whereas aquaporin 3 (*AQP3*) (Ricanek et al., 2015) and keratin 8 (*KRT8*) (Nishizawa et al., 2005) have been associated with epithelial enterocytes.

Statistical analysis

Data were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). The model included type (PMN or GIT) as fixed effect and calf as random. Statistical significance and tendencies were declared at $P \leq 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

RESULTS AND DISCUSSION

Keratins are the largest family of cellular intermediate filament proteins and are predominant in epithelial cells (Iyer et al., 2013), and among these, keratin 8 has been associated with mammary epithelial cells and small intestinal mucosa (Moll et al., 1982, Moll et al., 2008). In fact, the antibody against keratin 8 has been commonly used in ruminants for cell purification of mammary epithelial cells from total milk cells (Boutinaud et al., 2015). In contrast to epithelial cells, to the authors' knowledge, the expression of keratin 8 has not been reported in immune cells, and specifically PMN. Accordingly, our results showed a substantially lower ($P < 0.01$) mRNA expression of *KRT8* in PMN in comparison to fecal RNA (Table 2.1), which suggests that the fecal RNA samples contained a greater number of GIT epithelial cells. Interestingly, this is supported by *KRT8* having the greatest mRNA abundance in fecal RNA (Table 2.2), in contrast to PMN, where *SELL* occupied the greatest mRNA abundance.

The genes *MPO* and *SELL* encode the enzyme myeloperoxidase and the cell membrane L-selectin, respectively, and are essential for the normal cellular functions associated with PMN cells in bovine (Paape et al., 2003). Myeloperoxidase is required by PMN to produce reactive oxygen species responsible for the oxidative burst and consequently killing capacity of PMN, whereas L-selectin allows PMN to recognize sites

of infection or inflammation (Paape et al., 2003). Our results showed a lower ($P = 0.01$) mRNA expression of *SELL* and a lack of amplification of *MPO* in fecal RNA samples in comparison to the PMN samples (Table 2.1). Therefore, it is conceivable that the fecal RNA samples contained small amounts of PMN mRNA transcripts, which is in agreement with the greater expression of *KRT8* in fecal RNA. Among the target genes in this study, *MPO* had a greater efficiency than normal (>3; Table 2.2), which could be associated with PCR amplification issues and indicates that the amplification, unrealistically, had more than a 1-fold increase during every cycle amplification. The authors associated this with the complete lack of amplification of MPO in fecal samples, which might have skewed the slope and consequently the amplification efficiency. Aquaporins are important cell transporters of water across biological membranes, and aquaglyceroporins, a subtype of aquaporins, are capable of transport water, glycerol, urea, and other small uncharged solutes across cell membranes (Rojek et al., 2008). Aquaporin 3 (encoded by *AQP3*) is an aquaglyceroporin with a wide tissue distribution including kidney, skin, esophagus, spleen, and small intestine among others (Rojek et al., 2008). However, *AQP3* is seldom reported to be expressed in PMN, and this aquaglyceroporin has been suggested to play a role in the uptake of microbes during phagocytosis (Marchini et al., 2003). In contrast to PMN, more data are available confirming the expression of *AQP3* in enterocytes and, furthermore, describing a role for *AQP3* during bacterial-induced diarrhea events in humans (Ricanek et al., 2015) and rats (Zhao et al., 2014). We recently demonstrated the amplification of *AQP3* in fecal RNA in neonatal dairy calves, which also demonstrated a downregulation during a mild diarrhea event (Rosa et al., 2018). Interestingly, the current results demonstrate a trend ($P = 0.06$)

for a greater expression of *AQP3* in PMN in comparison to fecal RNA. Due to the lack of functional data on *AQP3* in PMN, the latter effect remains unclear; however, the expression of other aquaglyceroporins such as *AQP9* have been reported in peripheral leukocytes (Ishibashi et al., 1998).

Taking these data together, we observed a contrasting gene expression profile between PMN and fecal RNA; whereas the greater mRNA expression of *SELL* in PMN was expected, the greater mRNA expression of *KRT8* in fecal RNA suggests that a considerable amount of RNA originated from epithelial cells in fecal RNA. These data provide new evidence on the reliability of the fecal RNA method; however, the data are not conclusive and further research needs to be done to improve the accuracy and robustness of this method while determining its limitations.

CONCLUSIONS

The data presented here are nuances in the refinement of the fecal RNA method to study biological adaptations of the GIT of neonatal dairy calves through analysis of transcriptional alterations. Our findings suggest that under nondiarrheic conditions RNA isolated from stool samples of neonatal dairy calves will have a considerable number of GIT epithelial cells, which confirms the reliability of this method under these conditions. However, further research needs to be done to determine if the same effects are observed during diarrhea or throughout the preweaning period of dairy calves.

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Table 2.1 Messenger RNA expression for specific gene markers for PMNL and GIT epithelial cells in non-diarrheic neonatal dairy calves.

	Sample Type			
Gene	Fecal	PMNL	SEM[1]	P-value
KRT8	2.43	-9.08	0.52	< 0.01
SELL	-5.58	-2.57	0.70	0.01
MPO[2]	--	-0.36	2.31	--
AQP3	-4.25	-2.45	0.70	0.06

[1]Largest standard error of the mean.
[2]Standard deviation for MPO in PMNL.

Table 2.2 Slope, coefficient of determination of the standard curve (R^2), efficiency of amplification, and median cycle threshold (Ct) of quantitative reverse transcription PCR.

Gene	Slope[1]	R^2	E[2]	Ct[3]	mRNA abundance (%)[4]
All samples					
SELL	-3.459	0.98	1.95	21.90	99.38
AQP3	-3.171	0.99	2.07	29.93	0.48
KRT8	-2.902	0.99	2.21	32.41	0.13
MPO	-2.076	0.95	3.03	32.19	0.00
PMNL samples					
SELL	-3.459	0.98	1.95	21.46	99.55
AQP3	-3.171	0.99	2.07	29.05	0.44
KRT8	-2.902	0.99	2.21	33.12	0.01
MPO	-2.076	0.95	3.03	31.87	0.00
Fecal samples					
KRT8	-2.902	0.99	2.21	27.03	75.15
SELL	-3.459	0.98	1.95	29.91	24.74
AQP3	-3.171	0.99	2.07	35.60	0.10
MPO	-2.076	0.95	3.03	N/A	0.00

[1]Slope of the 6-point standard curve.

[2]Efficiency of amplification [$E=10^{(-1/slope)}$].

[3]Ct = median cycle threshold which is defined as the number of cycles required for the fluorescent signal to cross the threshold (i. e. exceed background level), and the amount of target nucleic acid in the sample is inversely correlated to Ct cycles (i. e., the greater the amount of target nucleic acid the lower the Ct cycles will be required).

[4]mRNA abundance is calculated as a percentage of ($1/E^{\Delta Ct}$) specific gene in the sum ($1/E^{\Delta Ct}$) all genes.

Supplemental Table 2.1 Features of used primers for qPCR analysis. Hybridization position, sequence, and amplicon size of primers for *Bos taurus* used to analyze gene expression.

Gene Symbol	Primer[1]	Primers (5'-3')	bp[2]	Source
GOLGA5	F.1370	GAGCTACAGCAGCAAGTCAAAGTG	103	Moyes et al. (2009)
	R.1472	CTTTAGACTGGAGTATTCGAGTAGCTTTT		
OSBPL2	F.198	TGCCGTCACAGGCTTTGAC	100	Moyes et al. (2009)
	R.297	CCATTACTTGCTGGTGTCCACAT		
SMUG1	F.258	CAGCTACGTGACCCGCTACTG	117	Moyes et al. (2009)
	R.374	CGGACTACACTCACTTCACCAAAG		
GAPDH	F.908	TTGTCTCCTGCGACTTCAACA	103	Kadegowda et al. (2009)
	R.1010	TCGTACCAGGAAATGAGCTTGAC		
ACTB	F.624	GCGTGGCTACAGCTTCACC	54	Kadegowda et al. (2009)
	R.677	TTGATGTCACGGACGATTTC		
B2M	F.99	TCCAGCGTCCTCCAAAGATT	86	Kadegowda et al. (2009)
	R.184	CCCATACACATAGCAGTTCAGGTAA		
RPS9	F.192	CCTCGACCAAGAGCTGAAG	54	Bionaz and Loor (2007)
	R.254	CCTCCAGACCTCACGTTTGTTC		
RPS15A	F.405	GCAGCTTATGAGCAAGGTCGT	151	Bionaz and Loor (2007)
	R.555	GCTCATCAGCAGATAGCGCTT		
PPIA	F.125	TCTGAGCACTGGAGAGAAAGGATTTG	88	Rosa et al. (2018)
	R.212	GAAGTCACCACCCTGGCACATA		
AQP3	F.398	GGGTTGTATTACGATGCGATCTG	81	Rosa et al. (2018)
	R.478	AAAGATGCCAGCTGTGCCATTG		
MPO	F.1311	AGCCATGGTCCAGATCATCAC	105	Batistel et al. (2017)
	R.1415	ACCGAGTCGTTGTAGGAGCAGTA		
SELL	F.588	CTCTGCTACACAGCTTCTTGTAAACC	104	Li et al. (2016)
	R.691	CCGTAGTACCCCAAATCACAGTT		
KRT8	F. 1352	AGTGGCTACGCAGGTGGACT	181	Boutinaud et al. (2008)
	R. 1532	CCGCAAGAGCCTTTCACTTG		

[1]Primer direction (F – forward; R – reverse) and hybridization on the sequence.
[2]Amplicon size in base pair (bp).

CHAPTER 3. COMPARATIVE GENE EXPRESSION ANALYSIS OF EPITHELIAL CELL MARKERS ACROSS GASTROINTESTINAL TISSUES AND FECAL RNA ISOLATED FROM DAIRY CALVES

ABSTRACT

Our objective in this study was to compare the transcription of gene markers for gastrointestinal tract (GIT) epithelial cells, fatty acid binding protein 2 (*FABP2*) and cytokeratin 8 (*KRT8*), and tight junction complex genes (*TJP1*, *CLDN1*, *CLDN4*) in fecal RNA against several GIT sections in dairy calves. Postmortem samples were collected from ruminal epithelium, cecum, large intestine, duodenum, jejunum, ileum, and feces from six healthy male Jersey calves (5 wk of age) for total RNA isolation. Normalized gene expression data were log-transformed prior to statistical analysis using the Proc Mixed of SAS. The expression of *FABP2* was greater ($P < 0.01$) in the duodenum tissue than in the GIT section associated with fermentation (i.e., rumen, large intestine, and cecum). Within the small intestine, the mRNA expression of *FABP2* was greater ($P = 0.01$) in duodenum than in jejunum, but not different than ileum. In fecal RNA, the *FABP2* expression was greater ($P \leq 0.03$) than in the GIT section related to fermentation. However, *FABP2* was similar ($P = 0.3$) between fecal RNA and ileum. The expression of *KRT8* was greater ($P \leq 0.02$) in cecum and large intestine than in rumen and jejunum. Among the small intestine sections *KRT8* was greater ($P = 0.03$) expressed in duodenum than in jejunum. The fecal RNA had greater ($P \leq 0.02$) expression of *KRT8* than jejunum and ileum. In contrast, the *KRT8* expression in fecal was not different from the transcripts observed in cecum and large intestine. Fecal RNA showed the greatest expression of the

3 tight junctions proteins measured ($P < 0.01$) compared with the GIT tissues. However, the expression of *CLDN1* was higher in the ruminal tissue than in the fecal RNA ($P = 0.03$). The tight junctions *TJP1*, *CLDN1*, and *CLDN4* were ubiquitously expressed ($P \geq 0.5$) through duodenum, jejunum, and ileum. Since the transcription of the genes specific for GIT epithelial cells were significantly observed in the RNA isolated from feces, as well as the genes encoding the tight junctions proteins, these preliminary data further confirm that fecal RNA has a potential to be used as a tool to evaluate molecular adaptations in the GIT of dairy calves.

Keywords: dairy calves; feces; RNA; intestinal health

INTRODUCTION

The gastrointestinal tract (GIT) or "gut" functionality is crucial for optimal animal performance, including growth and milk yield in dairy cattle. Gut health has been associated with animal welfare involving physiological aspects such as nutrient absorption and energy metabolism, barrier function via mucosal secretion, interaction with the gut microbiome (Kogut and Arsenault, 2016). The GIT has multiple functions beyond the digestion and absorption of nutrients, for instance, regulation of the physiological homeostasis that protects the host against exogenous stressors such as harmful pathogens present in the lumen (Leaphart and Tepas, 2007). The GIT consists of a symbiotic system which at equilibrium, involves bacteria-host interaction that regulates intestinal barrier by a network of immune mechanisms including mucosal, pro- and anti-inflammatory response (Hooper and Macpherson, 2010). Together, all these mechanisms in the intestinal epithelium are the key to maintain the GIT equilibrium state, while alterations in the gut barrier such as mucus degradation, and increased intestinal

permeability can cause pro-inflammatory reactions and translocation of pathogens, leading to gastrointestinal inflammatory conditions and possibly systemic inflammation (Bischoff et al., 2014). Additionally, the GIT of neonatal animals undergoes morphological and functional adaptation after birth, which is associated with overall health. Regarding dairy calves, the neonatal stage is the period of high susceptibility to infections, including pathogens that target the mucosal epithelium of the small intestine (Malmuthuge et al., 2019). Therefore, understanding the interactions between these diverse physiological features by gut health and how they affect the host can potentially regulate animal production. Several experimental methods, including histology and immunochemical staining, are used to investigate GIT physiology and pathology. However, tissue preparation for histology requires invasive techniques, such as biopsy or euthanasia (Bischoff et al., 2014). Besides the histological assays, molecular-based tools (i.e., PCR and next-generation sequencing) have been used to investigate the biological mechanisms underlying the gastrointestinal epithelium and barrier function early in life of dairy calves (Liang et al., 2016, Malmuthuge and Guan, 2017), however the initial acquire of samples relies on invasive method of tissue collections. A noninvasive method, based on fecal RNA, to study the GIT in neonatal dairy calves through the isolation of host-RNA from fecal samples for quantitative reverse-transcription PCR was described by (Rosa et al., 2018). This study demonstrated that inflammatory transcripts present in fecal RNA were associated with blood biomarkers of inflammation during the neonatal stage of dairy calves undergoing a mild-diarrhea condition. Further improvements of this method quantified genes in the feces of dairy calves associated with transcripts of epithelial cells origin rather than from immune cells (Rosa and Osorio, 2019a). Taking

together, these two approaches demonstrated that fecal RNA has the potential to be used as a non-invasive tool to provide valuable information on GIT biological adaptations during the preweaning period of dairy calves. Currently, no biomarkers have been described as tools to evaluate GIT inflammatory condition or intestinal barrier function through a non-invasive method such as feces analysis in dairy calves. Therefore, the objective of this study was to evaluate well-known epithelial cell markers [i.e., fatty acid binding protein 2 (*FABP2*); cytokeratin 8 [*KRT8*]), as well as regulatory proteins of the intestinal permeability (i.e., tight junction proteins complex) across gastrointestinal tissues and in the feces isolated from preweaned dairy calves.

MATERIALS AND METHODS

Animals and experimental design

All the protocols for this study (protocol no. 4747) were approved by the Institutional Animal Care and Use Committee. Details for the original experimental design have been published previously (Rosa et al., 2018). Briefly, 8 healthy male Jersey neonatal calves were enrolled in a completely randomized design from birth to 5 wk of age (i.e., preweaned period). Calves were housed in individual pens and fed twice daily with pasteurized whole cow milk. Calves had ad libitum access to water and a starter grain throughout the experiment.

Euthanasia and tissue collection

At the end of the trial, 6 preweaned calves (5 wk old) were euthanized by rapid intravenous injection of a barbiturate (Somnasol euthanasia solution, Henry Schein) to harvest gastrointestinal tissues (i.e., ruminal tissue, duodenum, jejunum, ileum, large

intestine, and cecum) for gene expression analysis. Tissues were harvested using sterile scalpel and forceps and were immediately cleaned with RNase decontamination solution (RNaseZap®, AM9780, Thermo Fisher Scientific), quickly blotted with sterile gauze to remove residual blood and snap-frozen in liquid nitrogen. Additionally, prior to the euthanasia procedure, a fresh fecal sample was collected from each calf through rectum stimulation into a 2 mL cryogenic vial (Corning®, Cat. # 430488), and immediately snap-frozen in liquid nitrogen. All samples were then stored at -80°C until RNA extraction.

RNA extraction and real-time quantitative PCR

The fecal RNA isolation was conduct using the same protocol, as described in Rosa et al. (2018). Briefly, ~200 mg of fecal sample was utilized for RNA isolation using a Trizol (Ambion, Cat. No. 15596018) based method along with the RNeasy Plus Mini Kit (Qiagen, Cat. No. 74134), following the manufacturer's instructions with some modifications. The overall RNA quantity for all fecal samples was 428.38 ± 37.92 ng/µL, and purity (260/280 ratio) was 1.97 ± 0.08 determined via Nanodrop. For the rumen and gastrointestinal samples, 100mg of tissue was used for RNA isolation. And the RNA quantity and purity determined via nanodrop was 1,868.53 ± 61.23 ng/µL and 2.09 ± 0.02, respectively.

Details regarding primer sequences information and sources are presented in Tables 3.1 and 3.2. The complementary DNA (cDNA) synthesis and quantitative PCR were performed according to the protocol described by Rosa et al. (2018) with some modifications. Each cDNA was synthesized by reverse transcription using 500 ng and 100 ng of RNA for fecal and GIT tissues, respectively. The cDNA was then diluted 1:3

(vol:vol) with DNase/RNase-free water. The qPCR reaction was performed in a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, USA) in a MicroAmp® Optical 384-well Reaction Plate (Applied Biosystems, USA). Relative mRNA expression of each gene was calculated based on a six-point relative standard curve plus the non-template control, which was composed of all cDNA samples from fecal and GIT tissues. The internal control genes (ICG) used in this experiment were β-2-microglobulin (*B2M*), β-actin (*ACTB*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), ribosomal protein 9 (*RPS9*), ribosomal protein S15A (*RPS15A*), and peptidylprolyl isomerase A (*PPIA*). The stability of the ICG was assessed using the geNorm software (Vandesompele et al., 2002), with a favorable final pairwise variation of 0.21. Therefore, the geometric mean of the six ICGs was used to normalize the expression of the target genes. The target genes evaluated in this study were the intestinal epithelial cell marker *FABP2*, the keratin epithelial cell marker (*KRT8)*, the tight junction protein ZO-1 (*TJP1)*, and the claudins 1, and 4, members of the tight junction complex (*CLDN1* and *CLDN4*, respectively).

Statistical analysis

Data were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). The model included type of sample (fecal or GIT tissues) as a fixed effect and calf as random. Statistical significance and tendencies were declared at $P \leq 0.05$ and $0.05 \leq P \leq 0.10$, respectively.

RESULTS AND DISCUSSION

The GIT is considered to be the largest organ to play a key role in the immune system, encompassing an intensive communication between the host and the gut microbiome and digesta. The GIT represents a structural barrier, including vascular endothelium, the epithelial cell lining, and the mucus layer, as well as, a functional barrier, including immune molecules (i.e., immune cells, IgA secretion), antimicrobial peptides secretion, inflammatory mediators, and cytokines production (Vighi et al., 2008). The epithelial layer of the GIT presents a physical barrier that protects againts exogenous pathogens and toxins entering the host. And such an epithelial layer consists of cells connected by tight junction complexes. The tight junction protein complex is an intercellular junction located in the apical membrane of the epithelial cells that connected each cell, excluding the influx of pathogens and large molecules through the paracellular pathway while allowing the diffusion of ions, water, and small molecules (Wells et al., 2017). Tight junctions proteins include transmembrane proteins such as occludins and claudins and intracellular proteins such as zonula occludens (ZO) proteins. In the intestinal epithelium, the tight junctions proteins have been reported to decrease the paracellular permeability (Angelow and Yu, 2009). Then, it is conceivable that such tight junction proteins are essential during the development of gut-related diseases such as celiac disease due to hyperpermeability of the intestinal epithelium (Bischoff et al., 2014). Thus, tight junctions proteins are directly associated with intestinal epithelium permeability (Van Itallie et al., 2009, Yu et al., 2014). In this study, we observed that the expression of *TJP1* is similar ($P > 0.05$) among the small intestine sections. *TJP1* expression did not differ between the sections of the small intestine when compared to

the cecum and large intestine (Figure 3.1). The *TJP1* expression was similar ($P = 0.12$) between the duodenum and ruminal tissue. However, the fecal samples had the greatest ($P < 0.01$) expression of the *TJP1* when compared with all the other small intestine sections, cecum, large intestine, or ruminal tissue (Figure 3.1). The ruminal tissue had greater ($P \leq 0.04$) expression of the *TJP1* than cecum, large intestine, jejunum, and ileum. The claudins gene expression (*CLDN1* and *CLDN4*) were similarly expressed ($P > 0.05$) in the duodenum, jejunum, or ileum (Figure 3.1). The *CLDN1* expression was greater ($P = 0.03$) in the ruminal tissue than in the fecal samples; however, the *CLDN4* expression was greater ($P < 0.01$) in the fecal samples rather than in the ruminal tissue. In fecal and ruminal samples, both *CLDN1* and *CLDN4* expression was greater ($P \leq 0.02$) when compared to cecum, large intestine, and the small intestine sections (Figure 3.1). These findings are similar with other reports, where the expression of *CLDN1* did not differ among gastrointestinal regions of dairy calves during weaning transition from milk to solid feed (Malmuthuge et al., 2013), as well as in dairy calves of 4 wk old (Walker et al., 2015). Also, studies conducted in mice demonstrated that among the claudins (from claudin-1 to- 19), the *CLDN1*, *CLDN4*, *CLDN5*, *CLDN9*, and *CLDN10* were the lowest expressed in the gastrointestinal regions (i.e., duodenum, jejunum, ileum, cecum, and colon). Additionally, this study showed that there was no difference in the expression of those claudins throughout the gut regions (Holmes et al., 2006). Several studies demonstrated that tight junction complexes could be disrupted in the GIT due to pathogenic infections either in humans, piglets, or calves, reducing the barrier function of the epithelium (Guttman and Finlay, 2009, Jacobi et al., 2013, Walker et al., 2015). A study conducted in neonatal lambs demonstrated that *CLDN1* and *CLDN4* expression in

the ileum was altered due to early weaning (Li et al., 2018), which can be considered a stressor triggering inflammation and intestinal barrier dysfunction. Inflammatory responses including the production of pro-inflammatory cytokines (i.e., IFN-γ, TNF-α, and IL-1β) can cause a dysregulation of tight junction proteins by altering their expression, leading to increased paracellular permeability (Adams et al., 1993, Ma et al., 2004, Al-Sadi and Ma, 2007). The tight junction proteins, *TJP1* and *CLDN,1* were expressed at higher levels in the jejunum mucosal of heated stressed cows than control (i.e., healthy) cows, which can be related with the relationship between inflammation and GIT permeability (Koch et al. 2019). In the current study, the time of tissue collection used for the RNA isolation was during the pre-weaning phase, when the dairy calves were under the transition of being weaned from milk 1× d to an exclusively solid feed diet, which is a stressor for dairy calves (Klotz and Heitmann, 2006).

The high tight junction expression in the ruminal tissue might be associated with the morphological structure of the ruminal epithelium in contrast to the intestinal epithelium. The rumen epithelium consists of stratified cells with leaf-like papillae that increase the absorptive surface area. It is well established that the transport of nutrients across the rumen epithelium is mediated by a gradient concentration difference (Sehested et al., 1999, Shen et al., 2004). This rumen epithelium forms a permeability barrier including tight junction proteins facing the ruminal lumen at the apical membrane of the stratum granulosum. In contrast to the multilayered stratified squamous epithelium found in the rumen, the small intestine, cecum, and large intestine consist of a simple columnar epithelium that encompasses mucus-secreting cells resulting in a dense mucus layer (Steele et al., 2016a). This mucus layer results in an extra layer of protection to the host within GIT

regions, which does occur in the rumen (Graham and Simmons, 2005). The high abundance of the tight junctions related genes (Table 3.3) within the ruminal samples can be attributed to the morphological and physiological difference between the rumen and small intestine epithelium. However, the high level of *TJP1*, *CLDN1*, and *CLDN4* mRNA expression in the fecal of preweaned calves observed in the current study` might be associated with epithelium permeability of the rumen and lower gut. Moreover, these findings can be related with the greater expression of inflammatory-related genes *TNFA*, *NFKB1*, *IL1B*, and *TLR4* in the feces of dairy calves at 4 and 5wk of age (Rosa et al. 2018), In that study, the calves were not undergoing diarrhea (i.e., formed solid feces) during the time of the euthanasia and tissue collection.

Keratins are the largest family of cellular intermediate filament proteins and are predominant in epithelial cells (Iyer et al., 2013), and among the keratin family, keratin 8 has been associated with mammary epithelial cells and small intestinal mucosa (Moll et al., 1982, Moll et al., 2008). Anti-keratin 8 antibody has been commonly used in ruminants for cell purification of mammary epithelial cells from total milk cells (Boutinaud et al., 2015). Keratin 8 has been used to measure exfoliated epithelial cells in the feces of preterm infants with the aim to use fecal samples instead of invasive methods to investigate GIT physiology (Kaeffer et al. 2007). Additionally, *KRT8* expression was used as a marker for intestinal epithelial cells in dairy cows (Koch et al., 2019). In the current study, *KRT8* expression was similar between the duodenum and fecal samples ($P = 0.18$), as well as, *KRT8* was similarly expressed in the cecum and large intestine when compared to the feces mRNA ($P = 0.30$). However, the expression of *KRT8* was greater ($P \leq 0.02$) in feces than in the jejunum and ileum tissues (Figure 3.2).

Fatty acid binding proteins (FABP), which are small cytosolic proteins found in enterocytes of both the small and large intestine are involved in the transportation and in the metabolism of long-chain fatty acids, and such proteins have been used as a potential marker for intestinal epithelial cells, since they are primarily produced by epithelial cells of the small intestine and their release in blood circulation depends upon disease state of the GIT (Funaoka et al., 2010, Bottasso Arias et al., 2015). Among the nine FABP identified, the mRNA expression of *FABP2* is highly expressed in the small intestine, and it has been identified as a specific marker for the relative amount of epithelium in humans (Levy et al., 2001). In livestock production, the *FABP2* expression in the small intestine of pigs was associated with intestinal permeability (Schroyen et al., 2012), as well as, the loss of enterocytes was associated with a decrease in the *FABP2* expression in the jejunal mucosa of broiler chickens (Chen et al., 2015). In the present study (Figure 3.2), the similar expression of *FABP2* in the feces of calves compared to the expression in the jejunum ($P = 0.58$) and ileum ($P = 0.36$), suggests that *FABP2* gene can be used as a biomarker of enterocytes when analyzed in feces.

Taken together, our findings demonstrated that there might be a difference in the level of tight junction proteins between the rumen epithelium and intestinal epithelium of dairy calves, and that can be attributed to the differential cell layers structure of the epithelia. Regarding the specific epithelial cell markers, the low abundance of *KRT8* and *FABP2* mRNA within ruminal samples (Table 3.3) is an indication that these markers are highly specific to intestinal epithelial cells in ruminants, as previously demonstrated in humans (Wiercinska-Drapalo et al., 2008, Gajda and Storch, 2015). Thus, this is in

accordance with the greatest mRNA abundance of the *FABP2* within the small intestine samples evaluated (Table 3.3).

CONCLUSION

The similar mRNA expression of *FABP2* and *KRT8*, known intestinal tissue biomarkers, between fecal RNA and the lower GIT tissues indicates that to some extent, the fecal RNA is a suitable proxy for lower GIT gene expression. The greater expression of tight junctions genes (*CLDN1*, *CLDN4*, and *TJP1*) observed in the fecal transcripts in comparison to GIT tissues was unexpected and warranted further exploration of this effect in future research on fecal RNA. Based on the paramount importance of gastrointestinal functionality to animal health and performance, having identified biomarkers of GIT function and permeability in fecal RNA provide further evidence that this technique can be a valuable asset to livestock research in gut health in the future.

ACKNOWLEDGMENTS

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Table 3.1 Gene symbol, hybridization position, sequence, and amplicon size of primers for Bos Taurus used to analyze gene expression.

Gene symbol	Primers[1]	Primers (5' – 3')	bp[2]	Source
GAPDH	F.908	TTGTCTCCTGCGACTTCAACA	103	(Kadegowda et
	R. 1010	TCGTACCAGGAAATGAGCTTGAC		al., 2009)
ACTB	F.624	GCGTGGCTACAGCTTCACC	54	(Kadegowda et
	R.677	TTGATGTCACGGACGATTTC		al., 2009)
B2M	F.99	TCCAGCGTCCTCCAAAGATT	86	(Kadegowda et
	R.184	CCCATACACATAGCAGTTCAGGTAA		al., 2009)
RPS9	F.192	CCTCGACCAAGAGCTGAAG	54	(Kadegowda et
	R.254	CCTCCAGACCTCACGTTTGTTC		al., 2009)
RPS15A	F.405	GCAGCTTATGAGCAAGGTCGT	151	(Bionaz and Loor,
	R.555	GCTCATCAGCAGATAGCGCTT		2007)
PPIA	F.125	TCTGAGCACTGGAGAGAAAGGATTTG	88	(Rosa et al., 2018)
	R.212	GAAGTCACCACCCTGGCACATA		
KRT8	F.1352	AGTGGCTACGCAGGTGGACT	181	(Boutinaud et al.,
	R.1532	CCGCAAGAGCCTTTCACTTG		2008)
FABP2	F.371	GTGGCGAGATGGTCCAGACT	101	This manuscript
	R.471	TCTGTGTTCTGGGCAATGCTC		
CLDN1	F.480	GGCATCCTGCTGGGACTAATAG	100	(Minuti et al.,
	R.579	CAGCCATCCGCATCTTCTGT		2015)
CLDN4	F.695	CCCCAGCCAGCAACTACGT	103	(Minuti et al.,
	R.797	TCACAGATTGCAGTGAGCTCAGT		2015)
TJP1	F.3748	GCACATAGGATCCCTGAACCA	107	(Minuti et al.,
	R.3854	TGCTTCCGGTAGTACTCCTCATC		2015)

[1]Primer direction (F – forward; R – reverse) and hybridization on the sequence.
[2]Amplicon size in base pair (bp).

Table 3.2 Sequencing results obtained from qPCR of Bos Taurus specific primers.

Gene	Sequence
FABP2	CATAGGTGTCACCAGATGATTTCCAAAAGGACGAGCACTGTTCCTGGAGCATCCCAAT CACACAGATTTTTTGGGGCTCCTTGGTGGCCCTGTTTTGGTGAAGCTGAAGTATTCATT GATGCTGATGCCTGACAAAGAGGTATGGGTTAACTGGGATTGTTTGGGAAGGAAATCG GTTGGC

Table 3.3 Slope, efficiency (E) of amplification, coefficient of determination of the standard curve (R2), median cycle threshold (Ct) of quantitative reverse-transcription PCR, and relative mRNA abundance.

Gene	Slope[1]	E[2]	R[3]	Median Ct[4]	mRNA abundance %[5]
All Samples					
FABP2	-3.006	2.15	0.99	21.84	66.54
KRT8	-2.888	2.22	0.98	22.77	18.45
CLDN1	-3.290	2.01	0.99	25.30	1.79
CLDN4	-2.920	2.20	0.98	24.09	3.39
TJP1	-3.258	2.03	0.99	22.77	9.83
Fecal samples					
FABP2	-3.006	2.15	0.99	26.02	21.70
KRT8	-2.888	2.22	0.98	26.37	8.83
CLDN1	-3.290	2.01	0.99	29.23	1.23
CLDN4	-2.920	2.20	0.98	23.77	63.13
TJP1	-3.258	2.03	0.99	27.11	5.11
Small Intestine samples					
FABP2	-3.006	2.15	0.99	17.86	95.25
KRT8	-2.888	2.22	0.98	22.18	2.07
CLDN1	-3.290	2.01	0.99	24.92	0.39
CLDN4	-2.920	2.20	0.98	24.70	0.28
TJP1	-3.258	2.03	0.99	22.89	2.00
Rumen samples					
FABP2	-3.006	2.15	0.99	32.76	0.01
KRT8	-2.888	2.22	0.98	25.05	1.32
CLDN1	-3.290	2.01	0.99	21.12	37.24
CLDN4	-2.920	2.20	0.98	21.05	40.89
TJP1	-3.258	2.03	0.99	22.01	20.54

[1]Slope of the 6-point standard curve.

[2]Efficiency of amplification [$E=10^{(-1/slope)}$].

[3] coefficient of determination of the standard curve (R^2)

[4]Ct = median cycle threshold which is defined as the number of cycles required for the fluorescent signal to cross the threshold (i. e. exceed background level), and the amount of target nucleic acid in the sample is inversely correlated to Ct cycles (i. e., the greater the amount of target nucleic acid the lower the Ct cycles will be required).

[5]mRNA abundance is calculated as a percentage of ($1/E^{\Delta Ct}$) specific gene in the sum ($1/E^{\Delta Ct}$) all genes.

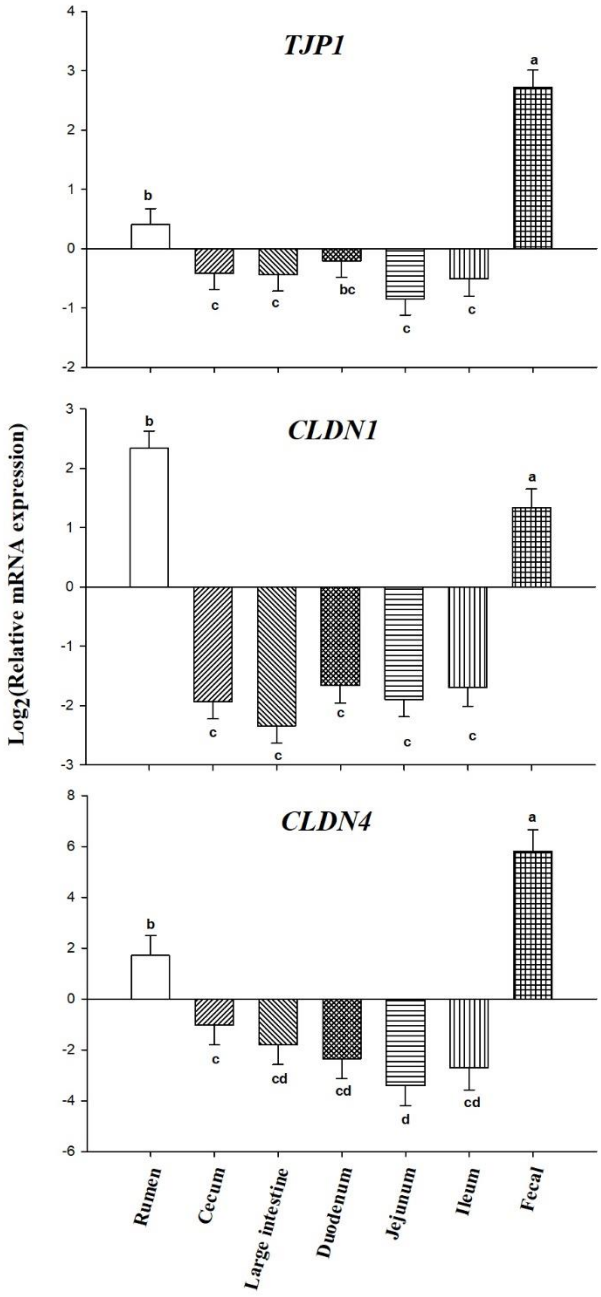

Figure 3.1 Tight junction protein ZO-1 (*TJP1*), claudin 1 (*CLDN1*), and claudin 4 (*CLDN4*) relative mRNA expression (log2 scale) in rumen, gastrointestinal tract tissues, and in total fecal RNA isolated from Jersey calves with 5 wk of age.
[a,b,c,d]means without a common superscript differ ($P \leq 0.05$).

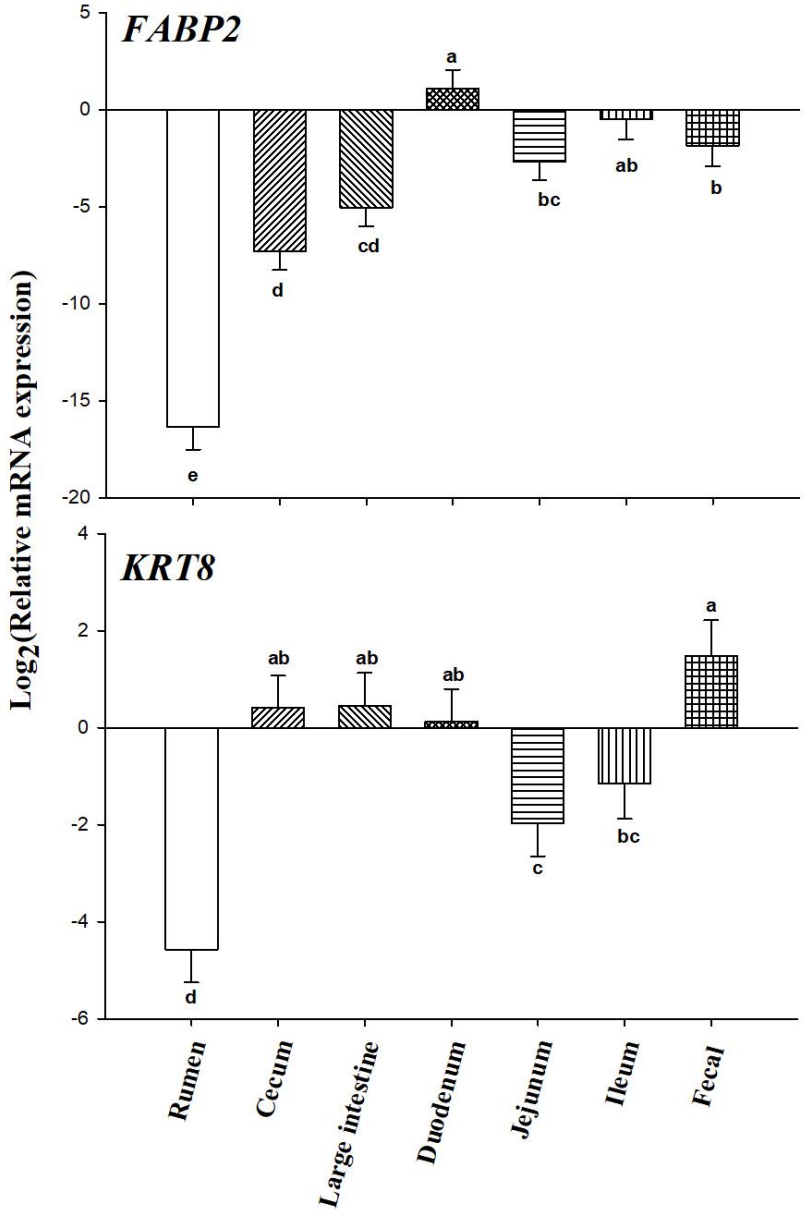

Figure 3.2 Fatty acid binding protein 2 (*FABP2*) and keratin 8 (*KRT8*) relative mRNA expression (log2 scale) in rumen, gastrointestinal tract tissues, and in total fecal RNA isolated from Jersey calves with 5 wk of age.
[a,b,c,d]means without a common superscript differ ($P \leq 0.05$).

CHAPTER 4. EARLY LIFE FECAL MICROBIOTA TRANSPLANTATION AFFECTS GROWTH PERFORMANCE, CALF SYSTEMIC, AND POLYMORPHONUCLEAR LEUKOCYTES MRNA INDICATORS OF INFLAMMATION AND LIVER FUNCTION IN NEONATAL DAIRY CALVES

ABSTRACT

The objective of this study was to evaluate the effects of early life fecal microbial transplantation (FMT) on health and performance of neonatal dairy calves. The adult donor was selected based on health and production records as well as fecal samples testing negative for infectious pathogens. Sixteen healthy newborn Holstein calves (n=8/trt) were used in a randomized complete block design and housed in individual hutches from birth to 7 wk of age. Calves were fed 2.8 L/d of antibiotic-free milk replacer 2×/d during wk 1 to 5, 1×/d on wk 6, and weaned at wk 7. Antibiotic-free starter and water were fed ad libitum. Calves were assigned to either a baseline nutritional program (CON) or 1×/d inoculations with 25 g of fecal donor material (FMT) mixed in the milk replacer from 8 to 12 d of age. Blood samples were collected weekly for immunometabolic profiling and polymorphonuclear leukocytes (PMNL) isolation for gene expression analysis. Data were analyzed using the MIXED procedure of SAS. There was a trend ($P = 0.09$) for greater body weight (50.8 vs 52.7 kg±0.7) in FMT calves. A TRT × Wk ($P = 0.02$) interaction was observed in haptoglobin, that was reflected in a positive quadratic effect ($P = 0.04$) in FMT calves but not in CON. A trend for a TRT × Wk ($P = 0.06$) interaction was observed in the liver function biomarker paraoxonase, which resulted in greater ($P < 0.01$) paraoxonase in FMT calves than CON at 3 wk of age. In albumin, the trend ($P = 0.14$) in TRT × Wk

resulted in a trend ($P \leq 0.09$) for lower albumin in FMT calves than CON at wk 1 and 2, but albumin in FMT calves reached a similar concentration than CON by wk 3. A trend ($P = 0.07$) for a TRT × Wk was observed in IL1B, which resulted in a contrasting positive quadratic effect ($P = 0.07$) over time in FMT, while a linear increase ($P = 0.07$) was observed in CON. The TRT × Wk ($P = 0.09$) in NFKB1 mRNA expression in PMNL was associated with an upregulation ($P = 0.03$) in FMT calves than CON at wk 1 of age. These results suggest that early life FMT in neonatal calves has positive effects in their neonatal stage not only on growth performance but also in mediating the inflammatory response and liver function.

Keywords: calves, fecal inoculation, microbiota

INTRODUCTION

The paradigm that the fetus develops within a sterile environment, born bacteria-free, and its first contact with bacteria only occurs after birth has been fundamentally challenged (Elgin et al., 2016); the presence of microbiota was detected in newborn human meconium (Jiménez et al., 2008), amniotic fluid and in the umbilical cord blood (Jimenez et al., 2005), as well as in placental membrane (Aagaard et al., 2014, Collado et al., 2016). Similar to humans, bacteria were detected in newborn calf meconium (Mayer et al., 2012) and bovine uterus (Karstrup et al., 2017). Early life neonatal gut colonization can be affected by prenatal factors including maternal microbiota, delivery mode, and by postnatal factors such as colostrum intake, immune defense mechanisms, microbes adhesion, and survival mechanisms as well as host luminal pH, diet, and antibiotic treatment (Malmuthuge and Guan, 2017). Factors affecting the early life microbiome colonization of the neonate gut can fundamentally challenge neonates and put them at risk for immune and

metabolic disorders. It has been demonstrated that cesarean-born infants are deprived of the vaginal microbiota inoculation as they pass through the birth canal and this can predisposed them to long-term health consequences (Dominguez-Bello et al., 2016). Similarly, other maternal microbiota in colostrum and milk have invaluable benefits in the health and immunity of neonates across mammalian species.

In addition to the microbial colonization after birth, there is a gradual maturation of gastrointestinal epithelial cells (GEC) that can be influenced by diet, length of gestation, and antibiotic usage. The latter has been closely associated with a fundamental disruption of the normal intestinal flora (i.e., dysbiosis), and consequently, linked with diseases in both humans and animals (Francino, 2015). Therefore, early life gut colonization is a crucial period for the adequate development of the neonate gut and immune system (Hansen et al., 2012) and may have long-term health effects. Manipulation of gut microbiota by feeding either probiotics or prebiotics has been studied in livestock animals as strategies to improve production and health (Herfel et al., 2011, Vieira et al., 2013).

Enteric infections are the major causes of calf death during the first 4 wk of life (Cho and Yoon, 2014). Developing methods to minimize calf diarrhea and improve gut health during the preweaning period using alternative approaches to antibiotics is essential.

In humans, fecal microbial transplantation (FMT), which is the transfer of fecal material from a healthy donor into the gastrointestinal tract (GIT) of a recipient with an enteric condition (de Groot et al., 2017) has been used. FMT has been highly successful in curing enteric diseases such as irritable bowel syndrome and Crohn's disease in humans (Ponte et al., 2015, Suskind et al., 2015). FMT has the capacity to shift microbial taxa and to restore gut microbiota in humans. (Kelly et al., 2014). Currently, there are very few FMT

studies in the animal science field, including companion animals (Pereira et al., 2018) and livestock (Hu et al., 2018a) .

From a health standpoint, the host immune system and the commensal bacteria present in the GIT need to be in equilibrium; otherwise, disruption of this balance may cause dysbiosis, and an inflammatory response in the host might be triggered (Kamada et al., 2013). However, the understanding of microbial communities and their interactions with the host in neonatal calves during the preweaning in terms of gut health and development is very limited. Therefore, we hypothesized that neonatal gut inoculation with a healthy adult gut microbiota could improve GIT development and maturation early in life. Thus, the objective of this study was to evaluate the effects of performing fecal microbiota transplantation in neonatal dairy calves on growth and health performance, and in the immune status.

MATERIALS AND METHODS

Experimental design

The Institutional Animal Care and Use Committee (IACUC) of the South Dakota State University (SDSU) approved all procedures for this study (protocol#A3958-01). This study comprised of a pre-trial phase and a trial phase. The fecal material from the selected cow donor was used in both phases of the study.

Donor Selection and fecal preparation

A group of 5 lactating Holstein cows was pre-selected from the herd at SDSU Dairy Research and Training facility as potential donors under the following criteria: 1) high milk yield production; 2) multiparous cows over 3 lactations ; 3) no clinical history of

any disease (e.g., retained placenta, mastitis, etc.) during any lactation. Fecal samples were collected from each cow, immediately kept on ice, and sent to the Animal Disease Research and Diagnostic Laboratory at SDSU for analysis of infectious pathogens and other risk factors. The stool testing included aerobic culture, fecal float for ova, and PCR detection for the presence of *Mycobacterium paratuberculosis*, *Salmonella*, and *Cryptosporidium*. From this group of pre-selected cows, one cow was negative for all the fecal pathogens measured and had no clinical history of any disease during 5 lactations at SDSU; therefore, this cow was designated as the donor. The final fecal material from the donor was collected via rectum stimulation, immediately frozen in liquid nitrogen, and stored at -80°C until the inoculation day.

Pre-trial phase: experimental design

Four healthy newborn Holstein calves from the SDSU Dairy Research and Training facility were randomly selected if they met the following criteria: 1) between 2 and 4 weeks of age; 2) fecal score ≤ 2 [scale from 1 – 4, 1:well-formed-solid feces; 2:soft, pudding-like; 3: runny, pancake batter; 4: liquid, splatters; (Osorio et al., 2012a)]. Calves received one FMT inoculation via milk replacer in the morning feeding, and health records were collected for 5 consecutive days. Calves received 25 g of fecal material from the donor. The proposed 25 g of fecal material was derived from previous FMT performed in humans [~70.55 kg BW of adult (Walpole et al., 2012)]. On the inoculation day, fecal material was thawed in a warm (37°C) water bath as described by other studies (Hu et al., 2018a, Wang et al., 2019). After the inoculation day, health checks were measured daily, including fecal score (scale from 1 – 4), rectal temperature, and respiratory score [scale 1 –

5, 1: normal; 2: runny nose; 3: heavy breathing; 4: cough moist; 5: cough dry; (Osorio et al., 2012)]. There was no adverse effect on milk replacer intake, calf scours did not increase, and the maximum rectal temperature observed was 39°C (Figure 4.1), whereas there was no need to use anti-inflammatory medication for fever (not higher than 39°C). Thus, no adverse effects were detected during the pre-trial phase, we proceeded with the trial phase of this study.

Trial phase: experimental design and fecal microbiota inoculations

Sixteen healthy newborn Holstein calves (n = 8/TRT) from the SDSU Dairy Research and Training facility were used for the trial phase in a completely randomized design (CRD) experiment from birth to 7 weeks of age. Calves were enrolled in the experiment if they fit the following criteria: calving difficulty < 3, single calf, and calf birth weight ≥ 36 kg were. Calves were processed within 4 hours after birth; calf handling included weighing, vaccinated with CALF-GUARD® (Bovine Rota-Coronavirus vaccine, Zoetis, MI), navel disinfected with 7% tincture of iodine solution (First priority Inc., Elgin, IL), and received 3.8 L of high-quality colostrum (> 60 mg/L) colostrum. Calves were housed in individual hutches bedded with straw and had ad libitum access to water and antibiotic-free starter grain (Calf 20 Nonmed®; 20% CP, Purina Animal Nutrition, MN) throughout the experiment. Calves were randomly assigned to a control treatment (CON) receiving 2.8 L of a commercial antibiotic-free milk replacer (Herd Maker®PB; Land O'Lakes Animal Milk products, MN) twice daily, while calves subjected to the FMT received the same regimen of milk replacer; however, during 8 to 12 d of age the morning milk replacer was inoculated with 25 g fecal material from the donor. On the first inoculation day, fecal material was thawed in a warm (37°C) water bath as described by

other studies (Hu et al., 2018a, Wang et al., 2019), and subsequent homogenous aliquots were kept at -20°C for the following 4 consecutive inoculations. After the 5th FMT inoculation, calves received the same feeding program and management as CON calves. During the 6 wk of age, all calves received milk replacer once a day and were completed weaned at 7 wk of age.

Animal measurements

Growth performance, including body weight (BW) and withers height, were recorded weekly before morning feeding. Health evaluations, including fecal score [scale 1 – 4], rectal temperature, and respiratory score [scale 1–5] were recorded daily throughout the experiment. Individual intakes of milk replacer, starter grain, and water were measured and recorded daily throughout the trial.

Blood sample collection

Blood samples were collected prior to morning feeding from the jugular vein using 20-gauge BD vacutainer needles (Becton Dickinson, Franklin Lakes, NJ). Samples were collected into evacuated tubes (10 mL, BD Vacutainer®) containing either serum clot activator or lithium heparin. The first blood sample was collected at 24 h after birth (i.e., 0 wk), and subsequent samples were taken weekly until 7 wk. After blood collection, tubes with lithium heparin were placed on ice (4 °C), and tubes with clot activator were kept at 21°C (~30 min) until centrifugation. Serum and plasma were obtained by centrifugation of clot activator and lithium heparin tubes, respectively, at 1,300 × g for 15 min at 4 °C. Aliquots of serum and plasma were frozen (-80°C) until further analysis.

Blood biomarkers

Blood samples were analyzed for energy metabolites [i.e., glucose, β-hydroxybutyric acid (BHB)], muscle mass catabolism (i.e., urea and creatinine), liver function [i.e., albumin, cholesterol, total bilirubin, gamma-glutamyl transferase (GGT), paraoxonase (PON), glutamic-oxaloacetic transaminase (GOT)], inflammation [i.e., haptoglobin, ceruloplasmin, Interleukin (IL)-6, and IL-1β], and oxidative stress [i.e., reactive oxygen metabolites (ROM), ferric reducing antioxidant power (FRAP)] using kits purchased from Instrumentation Laboratory (Lexington, MA) following the procedures described previously (Trevisi et al., 2012, Batistel et al., 2016, Jacometo et al., 2016).

Polymorphonuclear leukocytes (PMNL) isolation

The PMNL were isolated based on procedures described by Rosa and Osorio (2019b). Briefly, blood samples (~100 mL) were collected from the jugular vein prior to morning feeding at 0, 1, 2, and 3 wk of age using a scalp vein butterfly (Excel International, CAT# 26702) into evacuated tubes containing ACD solution A (BD, Cat# 268426) and mixed well by inversion and placed on ice until isolation. Samples were centrifuged at 600 × g for 30 min at 4 °C. The plasma, buffy coat, and ~ one-third of the red blood cells were discarded. The remaining sample was poured into a 50-mL conical tube (Fisher Scientific, Pittsburgh, PA). Twenty-five milliliters of deionized water at 4 °C was added to lyse the red blood cells (RBC), followed by addition of 5 mL of 5 × PBS at 4 °C to restore an iso-osmotic environment. Samples were centrifuged at 900 × for 10 min at 4°C, the supernatants were decanted, and the cell pellet was washed twice with 10 mL of 1 × PBS at 4°C. The cell suspension was centrifuged at 900 × for 5 min at 4°C, and the

supernatants were discarded. The remaining RBC were lysed with 8 mL of ice-cold deionized water, homogenized gently by inversion and 2 mL of 5 × PBS at 4°C was added. The samples were centrifuged at 900 × for 5 min at 4°C, and the supernatant was discarded. Subsequently, samples were washed twice using 10 mL of 1 × PBS at 4°C were performed, followed by centrifugation 900 × for 5 min at 4°C. The PMNL pellet was homogenized with 1.5 mL of 1 × PBS at 4°C, transferred to a 2-mL RNase-DNase-free microcentrifuge tube and centrifuged at 6,000 × g (Sorvall Legend Microcentrifuge 21R) for 5 min at 4°C. The final PMNL pellet was homogenized in 1 mL of Trizol reagent (Ambion, Carlsbad, CA) and stored at -80 °C until further gene expression analysis.

Viability analysis of PMNL through flow cytometry

During the PMNL isolation process, an aliquot containing at least 500,000 cells [counted via an automated cell counter (Countess II FL Cell counter from Life Technologies)] was used for PMNL quantification and viability. The PMNL aliquots were incubated for 30 min at 4°C with a primary antibody for granulocytes (CH138A, Veterinary Microbiology and Pathology, Washington State University) followed by another 30 min incubation at 4°C with a secondary antibody (Goat Anti-Mouse IgM, human ads-PE, Southern Biotech). Cells were fixed with 150 µL of 4% paraformaldehyde (Sigma-Aldrich) and preserved at 4°C until analysis. Flow cytometry data were acquired with the Attune NxT cytometer (Attune® NxT Flow Cytometer, Invitrogen), and the panel with the cell population of interest was analyzed using the Attune NxT cytometer software.

RNA isolation, cDNA synthesis, and Quantitative PCR (qPCR)

Total RNA was extracted from blood PMNL using Trizol (Invitrogen, Cat# 15-596-026) reagent in combination with the RNeasy® Plus Mini Kit (Qiagen, Cat#74134), following the manufacturer's instructions with some modifications. Briefly, the cell pellet immersed in TRIzol was transferred to a 2-mL RNase-DNase-free O-ring tube, containing one stainless steel bead, 5 mm (Qiagen, Cat#69989) and homogenized in a Beadbeater (BioSpec Products, Cat#BSP74540) for 30s. After homogenization, the lysate was transferred to a 2-mL RNase-DNase-free microtube and 200µL of Phenol:chloroform (Invitrogen™, Cat#AM9730) at 4 °C was added in order to isolate the RNA from the organic phase. After centrifugation at 13,000 x g for 15 min at 4 °C, the upper phase supernatant was transferred into a new 2-mL RNase-DNase-free microtube. The total RNA was purified RNeasy® Plus Mini Kit and eluted in 50 µL of RNase-free water. The RNA quantity (111.13 ± 102.23 ng/µl; mean ± SD) and purity as 260/280 ratio (1.85 ± 0.15) were determined using Nanodrop.

The complementary DNA (cDNA) synthesis was performed according to Bionaz and Loor (2007). The cDNA was then diluted 1:4 with RNase-DNase-free water (HyClone, UltraPure™, Cat#10977015). The qPCR was performed in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems, Grand Island, NY). Within each well, 4µL of diluted cDNA combined with 6µL of a mixture composed of 5 µL 1×SYBR Green master mix (Applied Biosystems, Woolston Warrington, UK), 0.4µL each of 10µM forward and reverse primers, and 0.2 µL of DNase/RNase-free water. Three replicates were run for each sample and a non-template control was run for each gene analyzed. The qPCR reaction was conducted in QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems)

following the conditions: 2 min at 50°C, 10 min at 95°C, and 40 cycles with 15s at 95°C followed by 1 min at 60°C. A dissociation curve was performed (gradient from 95C to 60°C to 95°C for 15s) to check for amplicon quality.

For this study, relative mRNA expression of each gene was calculated based on a six-point relative standard curve and the internal control genes (ICG) used were golgin subfamily A, member 5 (*GOLGA5)*, oxysterol-binding protein-like 2 (*OSBPL2)*, and single-strand-selective monofunctional uracil-DNA glycosylase 1 (*SMUG1)*, which have been previously used to normalize PMNL gene expression data (Moyes et al., 2010). The geometric mean of the ICG was used to normalize the expression of the target genes. The stability of the ICG was assessed using the geNorm software (Vandesompele et al., 2002) with a favorable final pairwise variation of 0.20. The target genes measured within the PMNL samples in this study are described in Table 4.1.

Statistical analysis

Data were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Institute, Inc., Cary, NC, USA). The model included treatment (TRT; CON and FMT), time (day or week), and their interaction as fixed effects, and calf as a random effect. The autoregressive (1) covariance structure was used for repeated measures for all parameters analyzed. Blood metabolites and gene expression results were log-scale transformed if needed, in order to comply with a normal distribution of residuals. Baseline samples taken at 24 h after colostrum intake or 0 wk were used as a covariate in the model if they were significant ($P < 0.05$). A priori orthogonal contrasts were used to determine linear and quadratic effects over time whenever at least a trend ($P \leq 0.10$) for a treatment × week (TRT × Week) was

observed. Statistical significance and tendencies were declared at $P \leq 0.05$ and $P \leq 0.15$, respectively.

RESULTS

Growth performance and health

Main effects of TRT, time, and their interaction for growth performance and health parameters are presented in Figure 4.2. There was a trend ($P = 0.09$) for greater BW (50.8 vs 52.7 kg±0.7) in FMT calves in comparison to CON. Similarly, there was a trend ($P = 0.13$) for greater WH in FMT (82.6 vs 83.8 kg ± 0.49) calves in comparison to CON. Starter intake and average daily gain (ADG) were not affected by treatment effects; however, they increased over time ($P < 0.01$) (Figure 4.3). Fecal scores and rectal temperature were not affected ($P \leq 0.65$) by FMT inoculation (Figure 4.4).

Blood biomarkers

Main effects of treatment, time, and their interaction for blood biomarkers of energy metabolism, inflammation, liver function, and oxidative stress are presented in Table 4.2.

Energy metabolism. The blood concentrations of glucose, BHBA, and creatinine were not affected ($P \geq 0.20$) by treatment (Table 4.2). A trend ($P = 0.07$) for a TRT × Week was observed for urea.

Inflammation. There was a TRT × Week ($P = 0.02$) for haptoglobin, and this was reflected by a positive quadratic ($P = 0.04$) effect observed on FMT treatment only, and this was particularly evident at 2 wk, where FMT calves had greater ($P = 0.01$)

concentration of haptoglobin when compared with CON calves (Figure 4.2). Similarly, a trend for a TRT × Week ($P = 0.07$) was observed for IL-1β in blood (Figure 4.5), which was reflected in a trend ($P = 0.07$) for a positive quadratic effect in FMT calves only, while IL-1β I in CON tended ($P = 0.07$) to have a linear increase over time (Figure 4.5). Ceruloplasmin was not affected ($P = 0.51$) by treatment effects. Blood IL-6 decrease ($P < 0.01$) gradually regardless of treatment was observed in blood IL6 over time (Table 4.2).

Liver function. There was a TRT × Week ($P \leq 0.14$) for albumin, paraoxonase, and total bilirubin. In the case of albumin, FMT calves tended ($P \leq 0.10$) to have lower albumin than CON at 1 and 2 wk of age; however, albumin was similar ($P = 0.89$) in both groups by 3 wk (Figure 4.5). The latter was reflected in a greater rate of increase in albumin concentration from wk 1 to 3 in FMT calves than CON (6.2% vs. 3.5%, respectively). The TRT × Week ($P = 0.06$) in paraoxonase, which was explained by greater ($P < 0.01$) paraoxonase in FMT calves than CON at 3 wk of age (Figure 4.5). There was a trend for a TRT × week ($P = 0.13$) effect on total bilirubin, which can be explained by a trend ($P = 0.06$) for lower total bilirubin in FMT calves at 1 wk of age when compared to CON. Cholesterol, GOT, and GGT were not affected ($P \geq 0.25$) by treatment effects. Cholesterol and GOT increased ($P < 0.01$) over time, while GGT decreased ($P < 0.01$; Table 4.2).

Oxidative stress. No effects ($P \geq 0.20$) were observed for ROM and FRAP (Table 4.2).

PMNL gene expression

Pro-inflammatory signaling and cell adhesion genes. Main effects of treatment, time, and their interaction on mRNA expression in PMNL are reported in figure 4.6. Overall, FMT calves had greater ($P = 0.03$) expression of *ZBP1* than CON, and to a lesser extent, FMT calves tended ($P = 0.12$) to have greater *NFKB1* than CON. The expression of *STAT3* and *SELL* was not affected by treatment.

Pathogen recognition and cytokines. Main effects of treatment, time, and their interaction on mRNA expression in PMNL are reported in figure 4.7. A trend for a TRT × week ($P = 0.11$) was observed for the *TLR2* expression, which was reflected on a trend ($P = 0.11$) for greater *TLR2* in FMT calves than CON at 2 wk of age. Overall, *IL8* expression decreased ($P = 0.01$) over time in all animals. The expression of *TLR4* and *IL1B* was not affected by treatment or time.

DISCUSSION

Animal Performance

We reported for the first time the use of FMT in neonatal dairy calves. The procedure proved to be safe with no adverse effects, including no milk refusal, no increase in rectal temperature, or respiratory score in FMT animals. These results are in agreement with other FMT studies in swine and companion animals (Xiao et al., 2017, Pereira et al., 2018). Regarding gastrointestinal signs, no severe diarrhea (FS = 4) was observed during the trial, and the fecal score was not affected by FMT, suggesting that FMT can be a safe procedure. However, further studies are needed to confirm this lack of effect on fecal scores.

To our knowledge, our study is the first experiment in dairy calves that attempted to evaluate the effects of performing FMT on calf performance (i.e., body conformation and feed intake). The tendency for greater BW and WH observed in the treated calves suggest that FMT can enhance growth performance. In contrast to BW and WH, the lack of treatment effects on starter intake suggests that FMT might improve feed efficiency and animal performance. Our findings regarding animal performance are in accordance with other studies that performed FMT in young livestock animals. For instance, Hu et al. (2018b) orally inoculated newborn pigs from day 1 to day 11 of life with a fecal suspension from a healthy donor, and the recipient piglets had greater average daily gain than the control piglets. Also, the incidence of diarrhea decreased in the FMT group when compared with the control piglets. Additionally, fecal inoculations from a highly feed-efficient donor chicken into broiler chickens early in life tended to increase feed intake and body weight in the FMT recipients chickens (Siegerstetter et al. 2018).

Biomarkers of inflammation and liver function

The acute phase response is triggered by an inflammatory condition, and it is regulated by several cytokines such as IL1-β which is produced by immune cells (i.e., dendritic cells and macrophages) present in the inflamed tissue (Murata et al., 2004). In the small intestine, epithelial cells in the inner surface (i.e., enterocytes) can release cytokines (e.g., IL-6) upon inflammation, which stimulates the production of the acute phase proteins (APPs) by hepatocytes and other tissues (Pritts et al., 2002). In the neonatal liver, the synthesis of APPs can be related to liver maturation rather than a disease-related process (Orro et al., 2008), which makes the interpretation of APPs in neonates cumbersome. Regardless of treatment, the high IL-6 concentration after birth

could be attributed to an activation of the immune system due to colostrum ingestion and adaptation to an extrauterine environment. Then, the decline in IL-6 over time could be related to the consolidation of the active immunity in the newborn calves, which agrees with the results observed by Jacometo et al. (2016). In the current study, the greater haptoglobin in FMT calves at 2 wk of age confirms that fecal inoculations between 8 and 12 d of age challenged the host to some extent. This is in agreement with Bertoni et al. (2009), where they reported that haptoglobin greater than 0.2 g/L in blood of neonatal calves is associated with an inflammatory condition. However, the haptoglobin concentration in FMT calves was modest when compared with other studies, where dairy calves were challenged with an infection (Haptoglobin = 1.23g/L) or undergoing a mild diarrhea (Haptoglobin = 0.35g/L) (Silva et al., 2011, Rosa et al., 2018). The fact that IL-1β in FMT calves have a similar pattern as haptoglobin at 2 wk of age concomitantly with the lack of FMT effect on IL-6 over time, is an indicative of a local inflammatory condition rather than systemic inflammation.

The anti-inflammatory enzyme paraoxonase (PON) blood levels increased over time healthy neonatal calves; in calves of 7 d of age average concentration of PON in blood was 8.9±5.2 U/mL (mean±SD) and in calves with 28 d of age the concentration was 42.8±14.8 U/mL (Giordano et al. (2013). However, when calves demonstrated a degree of inflammation (i.e., diarrhea or respiratory disease), the average PON observed in the blood of sick calves was 3.7±1.4 U/mL at 7 d of age and 24.5±13.4 U/mL at 28 d of age. Thus, there is a significant difference in PON concentration in healthy vs. sick (i.e., inflammatory condition) neonatal calves.

The hepatic metabolic activity in neonatal calves is lower than the adult ruminant (Baldwin et al. 2004). This can be partially explained by the alterations in the metabolizable substrates by the liver from pre-ruminant to ruminant (i.e., glucose and fatty acids vs. short-chain fatty acids [SCFA] due to rumen development). In this context, the shift from glucose intestinal absorption during pre-weaning period to SCFA, ketones, and microbial protein absorption in an adult ruminant lead to changes in the hepatic function and energy metabolism during the transition from pre- to- ruminant, including the reduction in enzyme activity of glucose oxidation in the liver (Baldwin et al., 2004). Thereby, the synthesis of proteins, including the acute phase (i.e., albumin, paraoxonase) in the liver during the neonatal stage can be associated with liver development. This can be attributed to metabolic adaptations and immune stimulation leading to maturational changes of the hepatic organ during the pre-weaning phase (Orro et al., 2008, Giordano et al., 2013).

There is also an increase in blood albumin and paraoxonase from birth to weaning (i.e., 5wk of age) of dairy calves (Jacometo et al., 2016, Rosa et al., 2018). Others have reported that decreased liver function can be associated with albumin less than 30 g/L in blood (Bertoni et al., 2009). Therefore, the greater paraoxonase concentration in FMT calves suggests a positive effect on the liver maturation. Similarly, the greater rate of increase for albumin in FMT calves suggests a positive effect of fecal microbial inoculations in liver function.

PMNL gene expression

The ZBP1 gene encodes the protein Z-DNA binding protein 1, which plays a role in sensing cell death and microbial pathogens, including viruses that are not recognized by TLR2 or TLR4 (Kuriakose and Kanneganti, 2018). Overall, the increase in *ZBP1* expression between birth and 7 d of age could have been linked with a transient inflammatory response triggered by colostrum intake and other conditions such as environmental pathogens exposure during the first wk of life. However, the decreased in its expression after FMT (8-12 d of age) can be attributed to the regulation of the innate immune response, since the release of pro-inflammatory cytokines upon TLR pathway is initiated is essential for neutrophil pathogen recognition and activation (Sabroe et al., 2005, Takaoka et al., 2007), which can be associated with the lack of acute or systemic inflammation (i.e., unresponsive IL-6 to FMT effect) during the trial.

Although calves in the FMT group tended to have greater *NFKB1* expression, that response was not associated with an upregulation of pro-inflammatory pathways as indicated by the lack of response to FMT in *STAT3*, *SELL*, *TLR4*, *IL1B*, and *IL8* expression. Taken together, these results seem to support that fecal inoculations starting at 8 d of age triggered a mild to moderate inflammatory response that allowed FMT calves to maintain a higher expression of the activators of the immune system and further upregulation of pro-inflammatory signaling genes was not warranted. Similarly, lower expression of pro-inflammatory mediators, including *NFKB1* and *MYD88*, low *IL1B* expression, and lack of change in the expression of *TNFA* in PMNL isolated was seen in neonatal dairy calves in response mineral supplementation (Jacometo et al. 2015).

CONCLUSIONS

This study demonstrated that early life fecal microbiota transplantation into neonatal dairy calves is a relatively safe method and can influence calf growth and development. Taken together, these data demonstrated that FMT performed using fecal material from a selected donor (elite cow of the herd) improved the hepatic maturation due to the increase in acute phase protein synthesis during calf neonatal stage. Based on the pattern of the biomarkers of inflammation in calves subjected to FMT, there is evidence that FMT is less likely to cause an acute or systemic inflammatory response, and perhaps it can modulate the transition from a passive immunity (i.e., colostrum) to an active immunity in neonatal calves. The precise biological mechanisms being altered by fecal microbiota inoculations in neonatal calves remains to be determined; however, the evidence for such alterations is underpinned by the seemly improved growth in FMT calves. Additional research in this area is needed to confirm our results and shed light on putative biological mechanisms on the FMT response observed in the current study.

ACKNOWLEDGMENTS

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Table 4.1 Genes selected for transcript profiling in bovine polymorphonuclear leukocytes (PMNL).

Gene Name	Symbol	Function
Nuclear Factor of Kappa Light Polypeptide Gene Enhancer In B-Cells 1	NF-KB1	NF-kappa-B is a pleiotropic transcription factor present in almost all cell types and is the endpoint of a series of signal transduction events that are initiated by a vast array of stimuli related to many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis.
Signal Transducer and Activator of Transcription 3 (Acute-Phase Response Factor)	STAT3	This protein is activated through phosphorylation in response to various cytokines and growth factors including IFNs, EGF, IL5, IL6, HGF, LIF, and BMP2. This protein mediates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis.
Leukocyte-Endothelial Cell Adhesion Molecule 1	SELL	This gene encodes a cell surface adhesion molecule that belongs to a family of adhesion/homing receptors. The gene product is required for binding and subsequent rolling of leucocytes on endothelial cells, facilitating their migration into secondary lymphoid organs and inflammation sites.
Interleukin 1 B	IL1B	Produced by activated macrophages, IL-1 stimulates thymocyte proliferation by inducing IL-2 release, B-cell maturation and proliferation, and fibroblast growth factor activity. Potent proinflammatory cytokine.
Z-DNA Binding Protein 1	ZBP1	This gene encodes a Z-DNA binding protein. The encoded protein plays a role in the innate immune response by binding to foreign DNA and inducing type-I interferon production.
Toll-Like Receptor 2	TLR2	The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity.
Toll-Like Receptor 4	TLR4	The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. This receptor has been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in most gram-negative bacteria. Mutations in this gene have been associated with differences in LPS responsiveness
Interleukin 8	IL8	Also, referred to as CXCL8 (C-X-C Motif Chemokine Ligand 8). It is a chemotactic factor that attracts neutrophils, basophils, and T-cells, but not monocytes. It is also involved in neutrophil activation. It is released from several cell types in response to an inflammatory stimulus.

Table 4.2 Blood immunometabolic biomarkers during neonatal period in Holstein dairy calves under a conventional nutritional program (CTR) or subjected to five days of fecal microbiota inoculations (FMT) within antibiotic-free milk replacer.

	Group[2]			*P*-value[4]		
Parameter[1]	CTR	FMT	SEM[3]	TRT	Week	TRT × week
Energy metabolism						
Glucose, mmol/L	6.23	5.69	0.34	0.33	0.04	0.28
BHBA[5], mmol/L	0.09	0.07	0.01	0.20	0.36	0.28
Creatinine, μmol/L	94.65	91.75	4.41	0.65	0.03	0.72
Urea, mmol/L	2.98	2.74	0.16	0.29	0.01	0.07
Inflammation						
Haptoglobin, g/L	0.191	0.212	0.01	0.24	0.06	0.02
Ceruloplasmin, μmol/L	1.82	1.95	0.13	0.51	0.38	0.29
IL1-β, (Log_2) pg/mL	3.69	3.67	0.39	0.98	0.26	0.07
IL-6, (Log_2) pg/mL	8.17	7.96	0.20	0.48	< 0.01	0.68
Liver function						
Albumin, g/L	30.61	30.03	0.29	0.18	< 0.01	0.14
Paraoxonase, U/mL	34.50	40.56	2.19	0.07	< 0.01	0.06
Total bilirubin, μmol/L	4.67	6.30	0.97	0.26	< 0.01	0.13
Cholesterol, mmol/L	2.68	2.40	0.17	0.25	< 0.01	0.31
GOT[6] (Log2), U/L	5.76	5.89	0.11	0.42	< 0.01	0.71
GGT[7], U/L	458.42	418.14	87.38	0.76	< 0.01	0.16
Oxidative Stress						
ROM[8], mg H_2O_2/100 mL	12.06	12.09	0.60	0.98	0.23	0.20
FRAP[9], mmol/L	160.45	149.64	7.79	0.36	0.46	0.34

[1]Metabolites and biomarkers were analyzed from 1 to 3 week of age. [2]CTR = control; FMT = fecal microbial transplantation; [3]Largest SEM is shown; [4]*P*-value for treatment (TRT), week, or either interaction (TRT × week); [5]BHBA = β-hydroxybutyric acid; [6]GOT = glutamic-oxaloacetic transaminase; [7]GGT = γ-glutamyltransferase; [8]ROMt = reactive oxygen metabolites; [9]FRAP = ferric reducing antioxidant power.

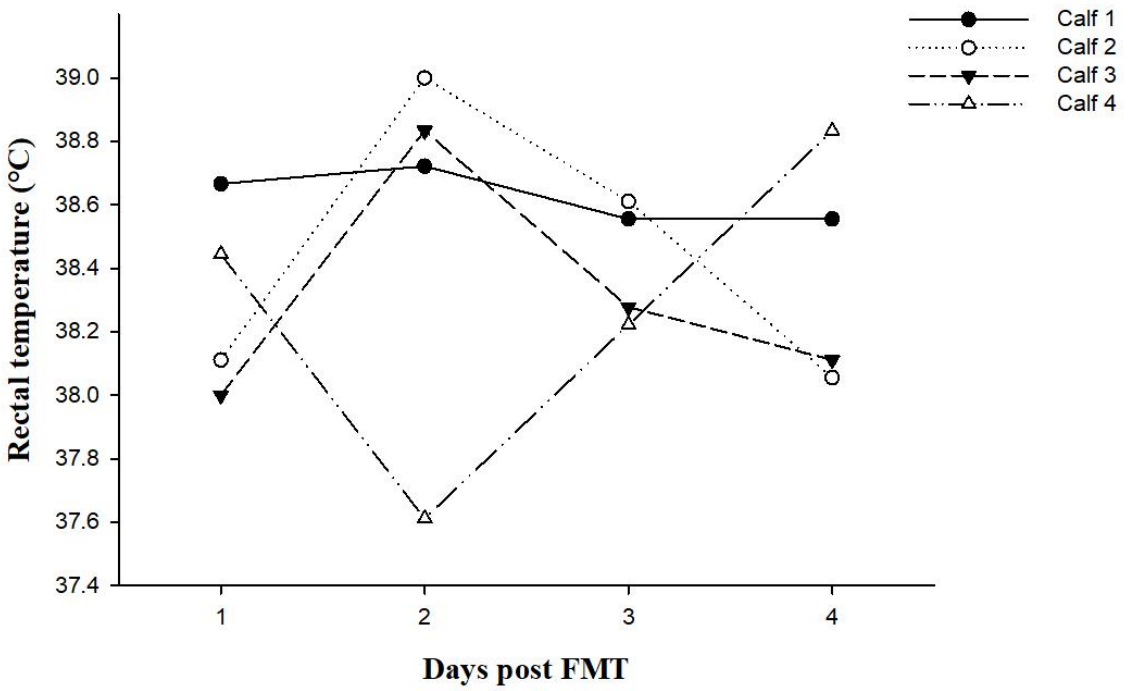

Figure 4.1 Rectal temperature (°C) after a fecal microbiota transplantation (FMT) within antibiotic-free milk replacer into neonatal Holstein calves during a pre-trial phase of 4 days.

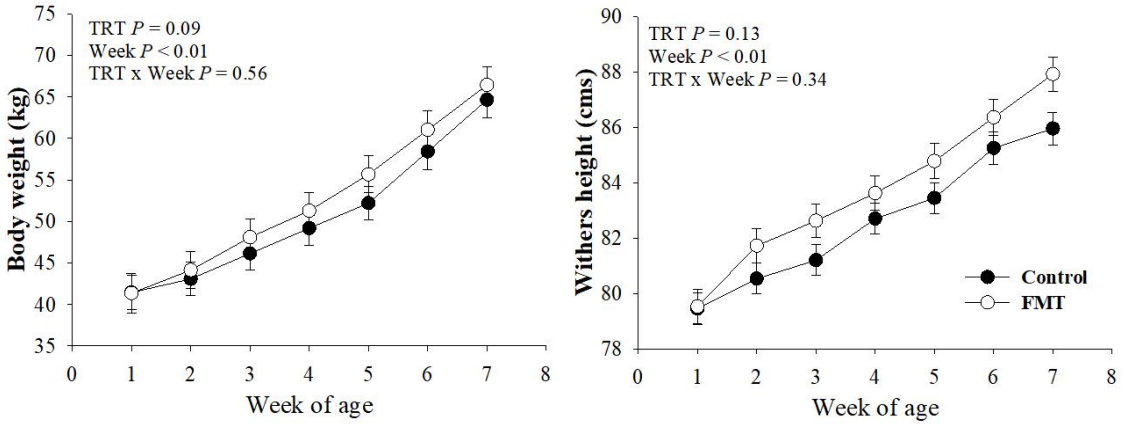

Figure 4.2 Growth performance from birth (week 1 f age) to weaning (7 weeks of age) of Holstein dairy calves under a conventional nutritional program (Control) or subjected to five days of fecal microbiota inoculations (FMT) within antibiotic-free milk replacer from 8 d to 12 days of age. The *P*-values for main effects of treatment (TRT) and week, and their interaction (TRT x Week) are shown in each plot. Values are means with standard errors represented by vertical bars.

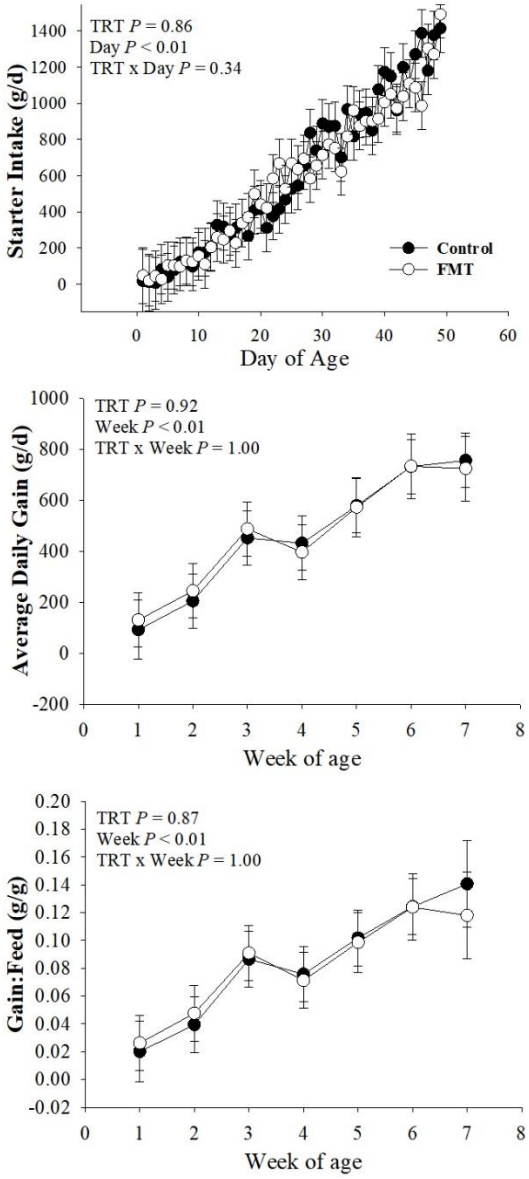

Figure 4.3 Performance parameters from birth to weaning of Holstein dairy calves under a conventional nutritional program (Control) or subjected to five days of fecal microbiota inoculations (FMT) within antibiotic-free milk replacer from 8 d to 12 days of age. The *P*-values for main effects of treatment (TRT) and week or day, and their interaction (TRT x Week; TRT x Day) are shown in each plot. Values are means with standard errors represented by vertical bars.

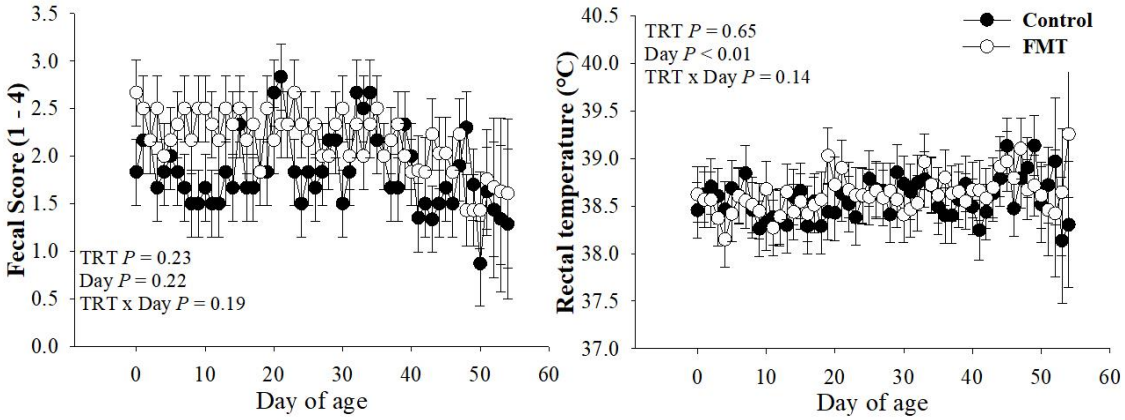

Figure 4.4 Health parameters from birth to weaning of Holstein dairy calves under a conventional nutritional program (Control) or subjected to five days of fecal microbiota inoculations (FMT) within antibiotic-free milk replacer from 8 d to 12 days of age. The *P*-values for main effects of treatment (TRT) and day, and their interaction (TRT x Day) are shown in each plot. Values are means with standard errors represented by vertical bars.

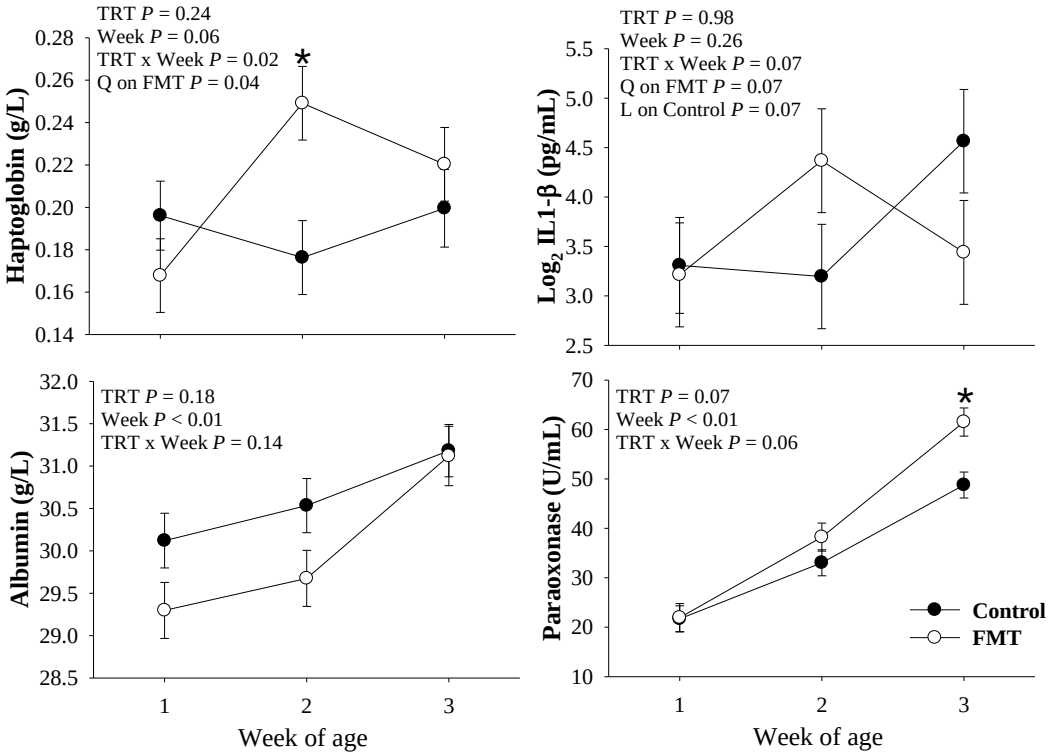

Figure 4.5 Inflammatory [Haptoglobin and interleukin 1 beta (IL1-β)] and liver function biomarkers [albumin and paraoxonase) during the neonatal period in Holstein dairy calves under a conventional nutritional program (Control) or subjected to five days of fecal microbiota inoculations (FMT) within antibiotic-free milk replacer from 8 d to 12 days of age. The *P*-values for main effects of treatment (TRT) and week, and their interaction (TRT x Week) are shown in each plot. The TRT x Week effect ($P \leq 0.10$) were further analyzed through orthogonal contrast in order to determine linear (L) and quadratic (Q) effects. A significant difference ($P < 0.05$) between treatments in a specific time point was denoted by an asterisk (*). Values are means, with standard errors represented by vertical bars.

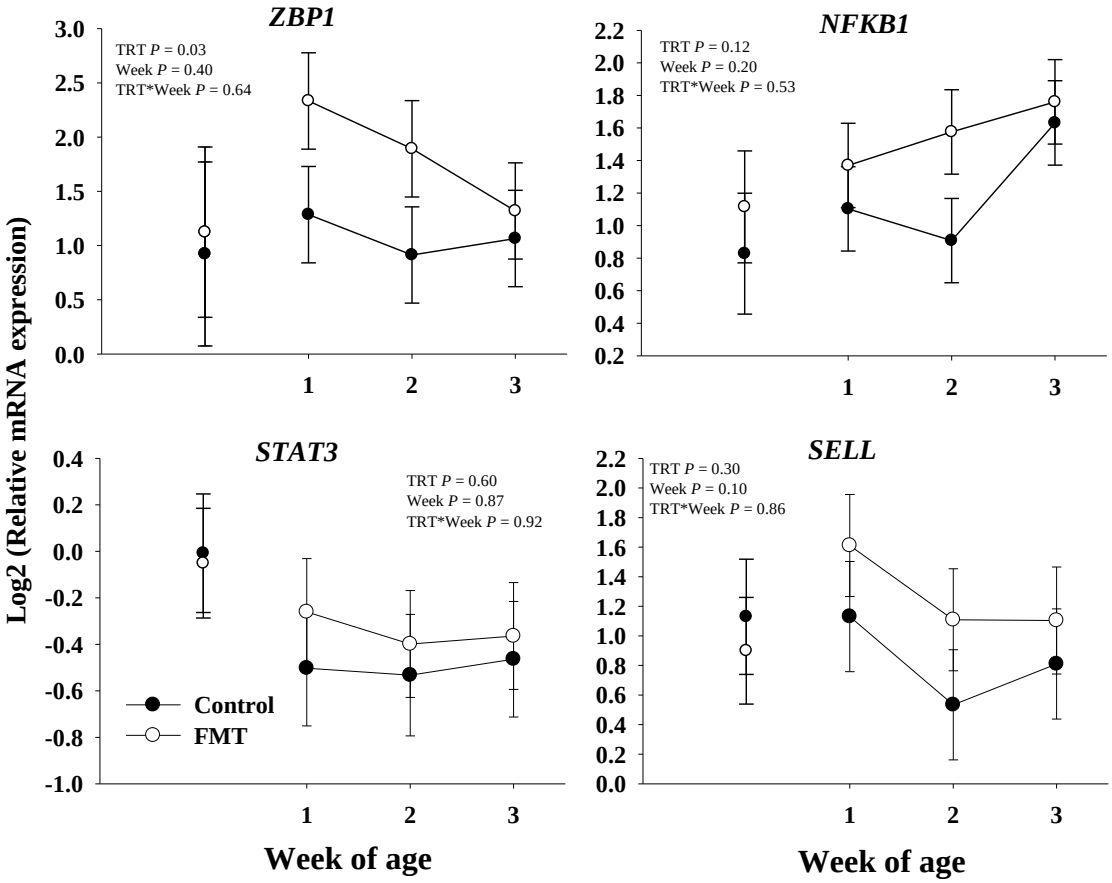

Figure 4.6 Expression of genes related to pro-inflammatory signaling cascade (*ZBP1*, *NFKB1*, *STAT3*) and cell adhesion (*SELL*) in polymorphonuclear leukocytes (PMNL) of dairy calves under a conventional nutritional program (Control) or subjected to five days of fecal microbiota inoculations (FMT) within antibiotic-free milk replacer from 8 d to 12 days of age. The *P*-values for main effect of treatment (TRT) and week and TRT*Week are shown. Data before week 1 of age are from samples 24 hours after birth (baseline).

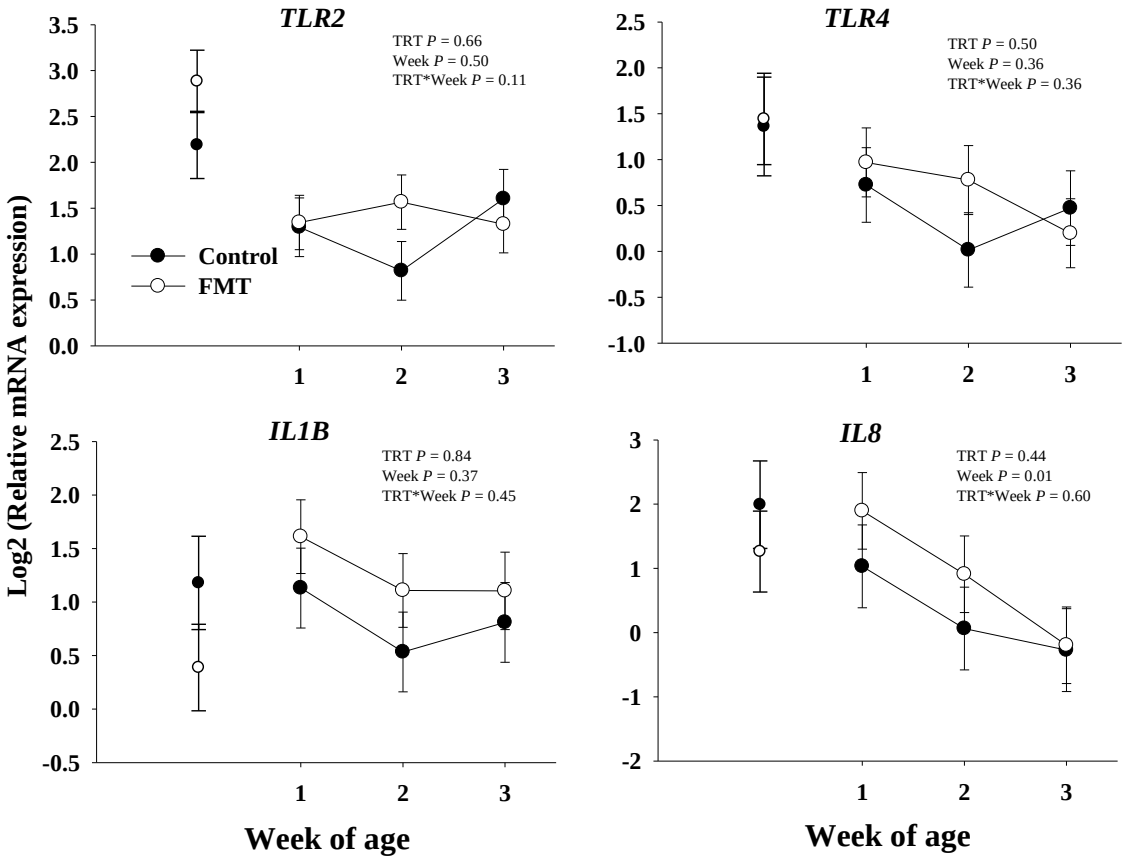

Figure 4.7 Expression of genes associated with pathogen recognition (*TLR2* and *TLR4*) and pro-inflammatory cytokines (*IL1B* and *IL8*) in polymorphonuclear leukocytes (PMNL) of dairy calves under a conventional nutritional program (Control) or subjected to five days of fecal microbiota inoculations (FMT) within antibiotic-free milk replacer from 8 d to 12 days of age. The *P*-values for main effect of treatment (TRT) and week and TRT*Week are shown. Data before week 1 of age are from samples 24 hours after birth (baseline).

OVERALL SUMMARY AND CONCLUSION

The research presented accomplished the overall objective, which was: a) to validate and optimize a non-invasive method to investigate transcriptomic changes in the gastrointestinal tract of neonatal dairy calves; b) to evaluate the effects of an early life fecal microbiota transplant on growth and health performance on neonatal dairy calves. The results in Chapter 2 indicated that RNA isolated from fecal samples contain a low number of immune cells such as PMNL and further confirms the signal observed in genes related to inflammation in fecal samples. Interestingly, the mRNA abundance of 99.55% detected for the gene marker for immune cells *SELL* within the PMNL samples, in contrast to the 75.15% abundance of *KRT8* gene marker for epithelial cells within fecal samples provided new insights on the fecal RNA reliability.

The results in Chapter 3 indicated that the tight junction complex might affect the rumen epithelia in a different manner than the intestinal epithelia based on the contrasting expression between ruminal tissue and intestinal tissues for the genes *TJP1*, *CLDN1*, and *CLDN4*. Based on the greater expression of genes markers of epithelial cells, *FABP2* and *KRT8* observed in the fecal samples together with the high abundance of *CLDN4* mRNA in feces further confirm the reliability of transcripts identified in feces with the host. The gene expression performed across studies at different fecal scores levels suggests that fecal RNA could be a robust technique that can be utilized to investigate physiological alterations in non-diarrheic and diarrheic animals.

Regarding the FMT study, the findings from Chapter 4, demonstrated that early life inoculations of fecal material from selected donors into neonatal calves did not induce scouring or produced a strong inflammatory condition. Transient and mild

increases in pro-inflammatory blood biomarkers, haptoglobin, and IL-1B, suggests that fecal inoculations stimulate the innate immune response in neonatal calves. In addition, the greater rates of increase in albumin and paraoxonase over time in FMT calves provide evidence for an improved hepatic maturation effect on calves subjected to FMT.

The gene expression in the PMNL samples in Chapter 4 indicated that all animals, including control and FMT calves, experienced an inflammatory condition at birth, which dwindled over time. Moreover, the lack of effect of FMT on PMNL gene expression confirms that such fecal inoculations did not produce an adverse inflammatory condition in FMT calves.

The linear increase in starter (grain pellets) intake over time indicated that FMT did not present an adverse effect on solid feed intake. The trend observed on BW and WH for greater overall growth on FMT calves provides further evidence for the beneficial effects of altering the gut microbiota of neonatal dairy calves.

Overall, our studies provided new insights on the utilization of fecal material to not only improve our understanding of the GIT maturation in neonatal dairy calves but perhaps its modification through FMT to enhance growth and welfare. Although our results on the early life fecal microbiota transplantation into neonatal dairy calves demonstrated positive effects on calf growth and performance, further research is needed to investigate the mechanisms through which the immature GIT in neonates interacts with the newly colonizing microbiota and correlate these effects with neonatal immunity and performance.

An approach such as FMT can be used as a method to manipulate the early life GIT microbiota colonization of neonates to prime a more desirable host-microbiota development and interaction so that neonates can better face the challenges during the first weeks of life. For instance, within a given herd, dairy cows could be selected as elite donors, and based on fecal screening and production records (similar to our study), each farm can have fecal samples stored for further inoculations into the newborn calves. In this way, neonates would acquire adapted and targeted microbiota (i.e., from the same environment) from high producing and healthy cows, priming the GIT colonization and development during the neonatal stage.

The inability to take sequential GIT samples over time using a traditional postmortem sample collection approach is a limitation to account for the inherent animal variation and may restrict the accurate development of models and mechanisms occurring over time during enteric infections. Like any other biological process, enteric infections such as diarrhea are a dynamic process where time is an important factor. Over time, infections in the GIT can lead to the disruption of tight junctions between enterocytes in the epithelial barrier, compromising the electrolytes balance, thus, increasing the flux of these into the intestinal lumen. The molecular changes underpinning this phenomenon occur in the early phases leading to diarrhea. In our research, RNA isolated from feces of neonatal dairy calves is proposed as a proxy for GIT gene expression to investigate the molecular adaptations to pathogenic infections of the GIT, which could lead to uncover key regulatory mechanisms employed by pathogens, which can, in turn, can become therapeutic targets based on the new knowledge generate from the fecal RNA method.

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