Characterization of rumen and hindgut development from pre- to post-weaning in Holstein dairy calves

by

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Abstract

In the dairy industry, the development of heifer calves is of high importance as it impacts health, future productivity and farm profitability. Nutrition is one aspect of heifer management that plays a key role in calf development and health. Therefore, the first study (Chapters 2 and 3) investigated the effects of the combination of milk replacer (MR) feeding rate and processing of corn grain in calf starter (CS) fed to calves on growth performance, nutrient digestibility, rumen and hindgut fermentation and inflammatory biomarkers. There were 4 treatments (n = 12 per treatment) with a 2 × 2 factorial arrangement of either: 1) MR feeding rate of 0.749 kg (LO) or 1.498 kg (HI) of MR/d; and 2) whole corn (WC) or flaked corn (FC) in textured CS. All calves were weaned by reducing MR allotment by 50% during wk 6.

Calves fed HI had increased growth pre-weaning. However, these calves also experienced reduced growth compared with calves fed LO during weaning. Moreover, the increased pre-weaning growth advantage was compromised, resulting in similar body weights (BW) between treatments at wk 12. Calves fed HIWC had reduced fecal pH at wk 8, which may be an indication of hindgut acidosis, however, inflammatory biomarkers were unaffected.

The first objective of study 2 (Chapter 4) was to develop a method to collect tissue samples longitudinally from the rumen and colon of calves that can later be used for histological and molecular analysis without sacrificing animals. Collection of rumen biopsy tissue samples was successful using surgical scissors, but not by using an endoscope, while colon biopsy tissue samples were successfully obtained using an endoscope. Both tissue types were of optimum quality for histology and molecular evaluation. Study 2 also characterized rumen fermentation and structural development, and amyloid A (SAA) and LPS binding protein (LBP) concentration changes from pre- to post-weaning (Chapter 5).

Additionally, the colon transcriptome was evaluated for functional changes at the molecular level that may occur from pre- to post-weaning, as well as their relation to colon mucosa thickness, fecal starch, inflammatory markers, SAA and LBP changes (Chapter 6). Six calves were fed MR (150 g/L) at 15% of BW adjusted weekly. Calves were weaned by reducing milk allocation by 50% during wk 6. Ruminal pH was below 5.8 for approximately 936.3 min/d pre-weaning and remained below 5.8 for approximately 388.1 min/d at wk 12, indicating calves underwent ruminal acidosis during weaning. Rumen adaptation may take several wk post-weaning because no structural differences were observed between pre-weaning and 2 wk post-weaning, although ruminal structure had increased at wk 12. SAA and LBP concentrations remained constant pre- and post-weaning, suggesting the depressed ruminal pH did not evoke a systemic inflammatory response. Expression of six genes (*AQP8*, *SLC7A8, SLC13A2, SLC9A3, SLC6A14, SLC40A1*) involved in nutrient transport decreased from pre- to post-weaning and was negatively associated with CS intake and fecal starch concentrations. An upstream regulator gene – *S100A12* – of inflammation and tissue growth also decreased in expression from pre- to post-weaning and was positively associated with colon mucosa thickness and negatively associated with fecal starch and CS intake. Responsive genes identified in this study may be used as gene markers in the future.

In summary, based on findings observed in Chapters 2 and 3, different amounts of MR in combination with CS containing flaked or whole corn can be fed to dairy calves without affecting digestibility, rumen and hindgut fermentation or inflammatory parameters. Furthermore, digestibility and rumen and hindgut fermentation are more responsive to MR feeding rate than the processing of corn in CS.

Additionally, Chapters 5 and 6 enhanced our understanding of the changes that occurs from pre- to post-weaning in the rumen and colon, respectively. Ruminal acidosis experienced by calves pre-

and post-weaning may not be a health concern based on unchanged inflammatory markers and intact papillae structural layers. The colon also needs more investigation based on increased expression of *S100A12* pre-weaning, which suggests that MR feeding may induce colon tissue inflammation.

Preface

This thesis is an original work by Jolet Köhler and consist of five chapters that originated from two studies. Study 1 contributed to chapters 2 and 3 which received research ethics approval from the University of Alberta Animal Care and Use Committee for Livestock (AUP00002303). Study 2 contributed to chapters 4, 5 and 6 which also received research ethics approval from the University of Alberta Animal Care and Use Committee for Livestock (AUP00002010).

A version of Chapter 2 of this thesis has been published as Van Niekerk, J. K., A. J. Fischer-Tlustos, L. L. Deikun, J. D. Quigley, T. S. Dennis, F. X. Suarez-Mena, T. M. Hill, R. L. Schlotterbeck, L. L. Guan, and M. A. Steele. 2020. Effect of amount of milk replacer fed and the processing of corn in starter on growth performance, nutrient digestibility, and rumen and fecal fibrolytic bacteria of dairy calves. J. Dairy Sci. 103:2186-2199. I was responsible for experimental design, data collection, data analysis and manuscript writing. A. J. Fischer-Tlustos, L. L. Deikun and R. L. Schlotterbeck assisted with data collection and J. D. Quigley assisted with experimental design, data collection and manuscript edits. T. S. Dennis and F. X. Suarez-Mena contributed towards manuscript edits, T. M. Hill assisted with experimental design and manuscript edits and L. L. Guan contributed to manuscript writing. M. A. Steele was the corresponding author and assisted with experimental design, data collection and manuscript writing.

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List of abbreviations

ADG - average daily gain

AE - anion exchanger

AQP - aqua porin

BCS - body condition score

BHBA – beta-hydroxy butyric acid

BW - body weight

cDNA - complementary DNA

CLDN - claudin

CPM - counts per million

CS - calf starter

CV – coefficient of variance

DE - differentially expressed

DM - dry matter

DMI - dry matter intake

DRA - downregulated in adenoma

FC - flaked corn

GIT - gastrointestinal tract

H&E - hematoxylin and eosin

HI - high milk replacer

HW - hip width

IGF - insulin-like growth factor

IPA - ingenuity Pathway Analysis

LBP - lipopolysaccharide binding protein

LO - low milk replacer

LPS - lipopolysaccharide

MAPK – mitogen-activated protein kinase

MCT - monocarboxylate transporters

ME - metabolizable energy

MR - milk replacer

mRNA - messenger RNA

NBC - sodium bicarbonate cotransporters

NHE - sodium-hydrogen exchangers

NMR - nuclear magnetic resonance

OCLN - occludens

PANTHER - protein annotation through evolutionary relationship

PAT1 - putative anion transporter 1

PBS - phosphate buffered saline

RIN - RNA integrity number

RT-qPCR - real-time reverse transcriptase quantitative polymerase chain reaction

SAA - serum amyloid A

SARA - sub-acute ruminal acidosis

SCFA - short-chain fatty acids

SI - small intestine

TEM - transmission electron microscopy

TLR - toll like receptor

WC - whole corn

WGCNA - weighted gene co-expression network analysis

1. Literature review

1.1. Introduction

Improving production efficiency and animal welfare is essential for the sustainability of the dairy industry. Calves are the future of the dairy herd but are often neglected. During the pre-weaning period, calves suffer from high rates of morbidity and mortality that mainly due to digestive disorders. Traditionally, calves are fed milk/MR at 10 % of birth body weight (Drackley, 2008) to allow for early weaning, which in turn, reduces feeding cost. This translates to calves being fed ~4 L milk/d or ~ 0.6 kg of dry matter (DM) MR/d. This feeding practice is known as restricted, conventional, or low plane of milk nutrition. Feeding low amounts of milk resulted in poor growth rates with an average daily gain (ADG) of 0.2 to 0.6 kg/d (Jasper and Weary, 2002; Miller-Cushon et al., 2013; Chapman et al., 2016). Additionally, feeding low amounts of milk can influence calf behaviour, including playing and resting, because hungry calves spend more time standing and vocalizing, thereby disrupting normal behaviour (Thomas et al., 2001; Vieira et al., 2008). Overall, it is evident that traditional feeding methods used for the last century, as well as the practice of early weaning, may be limiting calf growth, health, and welfare. Furthermore, low nutrient intake and abnormal behaviour may be a contributing factor to high mortality and morbidity rates observed in calves (Urie et al., 2018; Winder et al., 2018). Thus, improving and optimising current calf feeding regimens is important for our dairy industry to improve production efficiency and animal welfare.

Transition periods, such as weaning, represent a period of dramatic structural and metabolic adaptations of the gastrointestinal tract (**GIT**) and can negatively influence calf growth and compromise health (Khan et al., 2011b). A possible cause of compromised calf health is that during weaning the barrier function of the GIT is compromised (Wood et al., 2015). Ruminal or hindgut acidosis during and after weaning may contribute to a loss in gut barrier function since calves experience reduced ruminal pH (Laarman and Oba, 2011; Kim et al., 2016a) and elevated fecal starch (Eckert et al., 2015; Steele et al., 2017b), suggesting increased fermentation in the hindgut. We know that the effects of early life nutrition have significant economic importance to all dairy producers, as they not only directly impact mortality rate, disease susceptibility and growth rate during the pre-weaning period, but also milk production later in life (Soberon et al., 2012; Soberon and Van Amburgh, 2013; Gelsinger et al., 2016).

Calf nutrition plays a key role in gut development and function and calf performance and health and there is currently great variation in milk and solid feed management of calves in the dairy industry. Therefore, this review will focus on: 1) the GIT of a functional ruminant (section 1.2.1.1-3 forestomach and section 1.2.2.1-3 lower gut); 2) changes that occur in the calf's GIT (especially the rumen and colon) during weaning (section 1.4), and; 3) how different feeding strategies - plane of milk nutrition, transitioning to solid feed and type of solid feed - affect the dairy calf (section 1.5).

1.2. Gastrointestinal tract of a ruminant

Each compartment of the GIT is specialized, with the structure, environment and function unique to that respective region (Church, 1988). This allows each compartment to play a specific role in feed digestion, nutrient absorption and waste elimination. Digestion of feed in the GIT of ruminants is substantially different from that of monogastric animals. For ruminants, the forestomach is comprised of four compartments, namely the reticulum, rumen, omasum and abomasum. The rumen is the largest compartment of the GIT and comprises approximately 70 % of its capacity (Warner et al., 1956). The rumen provides a relatively stable environment for a wide variety of archaea, bacteria, protozoa and fungi that are collectively responsible for breaking down feed particles through fermentation (Dehority, 2003). The epithelium of the rumen is lined by papillae that increase the surface area for absorption of short-chain fatty acids (**SCFA**; Dirksen et al., 1985): by-products of microbial fermentation. Papillae function to increase the absorptive surface area of the rumen and it is well established that diet affects papillae density, width and length (Ishler et al., 1996). However, when the ruminant is born, the rumen is both physically and metabolically non-functional and the animal is considered a "pseudo-monogastric" (Baldwin et al., 2004; Drackley, 2008; Benschop and Cant, 2009). Once solid feed is consumed, ruminal microbial fermentation occurs and SCFA are produced, which stimulate both physical and metabolic development (Sander et al., 1959).

With regards to this literature review, the rumen and hindgut are the focus, and as such, these GIT compartments will be emphasized. The anatomy, structure, environment and function of the forestomach (section 1.2.1.1-3) and lower gut (section 1.2.2.1-3) of an adult ruminant will be discussed below.

1.2.1. Forestomach

As mentioned above, the reticulum, rumen, omasum and abomasum are the forestomach compartments of the ruminant. According to Bath et al. (1978), the epithelium of the reticulum resembles a honeycomb and is only partially separated from the rumen by a partition. The rumen is separated by pillars into four sacs: the dorsal, ventral, caudodorsal and caudoventral. The rumen also has finger-like structures (papillae) that project from the rumen epithelium, increasing the surface area for nutrient absorption (Annison et al., 1957). Despite differences in the gross appearance of the rumen and reticulum, both have stratified squamous epithelium. Digesta flows from the reticulum into the omasum through the omasal orifice and then to the abomasum via the omasal-abomasal orifice (Sherwood et al., 2012). The omasum is non-glandular and possesses a stratified squamous epithelium (Graham and Simmons, 2005). It is compartmentalized with various lamina (book-like structure) that contain papillae which increase the surface area for water, SCFA (Van Soest, Peter J., 1982), amino acid, di- and tripeptides (Gilbert et al., 2008) and mineral absorption (Xu et al., 2014). The abomasum of the ruminant has large spiral folds (Church, 1988) and a columnar epithelium (Masot et al., 2007). Little absorption occurs in the abomasum (Sherwood et al., 2012); instead, its main function is protein denaturation, and partial enzymatic and triglyceride digestion by gastric juices (hydrochloric acid and enzymes). After the reticulorumen and the omasum, the GIT of the ruminant animal functions similar to a monogastric animal. For this thesis, the rumen is the focus for the forestomach and the structure, environment and function will be discussed in greater detail in the following sections.

1.2.1.1. Rumen anatomy and structure

Steven and Marshall (1970) describe the structure of the rumen epithelium as a multilayer complex structure consisting of four cell layers, namely: the stratum corneum, stratum granulosum, stratum spinosum and stratum basale. In the stratum corneum, the cells are flattened and keratinized, with cells containing center cores that have granular material surrounded by peripheral fibrils. The stratum corneum is not metabolically active and is thought to primarily function to protect underlying layers from the abrasive nature of the rumen contents. The cells of the stratum corneum lie parallel to the luminal surface. The number of cell layers in the stratum corneum are greatly influenced by the ruminal environment (pH), which is directly influenced by

diet composition (Tamate et al., 1975; Gäbel et al., 1987; Baldwin and Jesse, 1991; Steele et al., 2016). In contrast to the stratum corneum, the stratum granulosum contains true tight-cell junction protein complexes (Kiddle et al., 1951), which act as a barrier to prevent antigens like lipopolysaccharide (**LPS**; Emmanuel et al., 2007) and histamine (Aschenbach and Gabel, 2000) and bacteria (Owens et al., 1998; Nagaraja et al., 2005) from entering the blood stream.

In the stratum spinosum, the cells are smaller than in the stratum basale. Stratum spinosum cells are cuboidal shaped and have a serrated outline. These cells synthesize keratohylin granules that increase in abundance as cells mature and migrate towards the stratum corneum (Steven and Marshall, 1970). Gap junctions are more prominent in the stratum spinosum than in the stratum granulosum and cells are metabolically active. In the stratum basale, cells are columnar in shape. Mitochondria, free ribosomes and rough endoplasmic reticulum are abundant within these cells. Desmosomes - gap cell junctions - join apical and basal lateral poles of adjacent cells in the stratum basale layer. Graham and Simmons (2005) reported that in the bovine rumen, the Na/K ATPase has the highest density in the stratum basale, with a decrease in density towards the stratum granulosum. A higher density of mitochondria is also found in the stratum basale than in the more apical layers, supporting the high abundance of Na/K ATPase – an energy-demanding transporter. The stratum basale layer plays a key role in whole animal energy metabolism as it has the most metabolic properties (Baldwin and Jesse, 1991). Together the stratum corneum, stratum granulosum, stratum spinosum and stratum basale make up the layers of the rumen papillae.

1.2.1.2. Environment

In general, the reticulo-rumen provides a relatively stable environment with a pH range of 5.8 to 7.0, rumen fluid temperature of approximately 39°C (Kamra, 2005), an anaerobic environment and rumen osmolarity near that of blood to promote microbial fermentation (McDonald et al., 2002). The environment is highly regulated and if any of the aforementioned factors are not adequate, animal production and health may be influenced (Owens et al., 1998; Krause and Oetzel, 2006). An example of such a disruption in the environment is ruminal acidosis, which will be discussed in section 1.2.2.4. Microbial colonization of the GIT occur after birth (Mayer et al., 2012; Li et al., 2012) and the rumen microbial population consists of variouspecies that thrive together to digest various types of feed. The microbial population

remains stable unless the environment in which they occur changes; for instance, ruminal pH reduces populations of total bacteria (Owens et al., 1998; Stefanska et al., 2018) and protozoa (Owens et al., 1998; Dehority, 2005). Characteristics of the feed influence which microbes are present in the rumen. Generally, high forage diets increase cellulolytic microbes, whereas amylolytic microbes increase when high grain or concentrate diets are fed (Tajima et al., 2000). Bacteria are the most abundant group of microbes found in the rumen (Russell and Hespell, 1981) and the dominant phyla are *Firmicutes, Bacteroidetes* and *Proteobacteria* (McCabe et al., 2015; Henderson et al., 2015). Bacteria can be associated with feed particles, fluid or the epithelium (McAllister et al., 1994). The groups predominantly responsible for digesting fibre are called fibrolytic bacteria and can be sub divided into cellulose digesters (*Fibrobacter succinogenes* and *Ruminococcus albus*) and hemicellose digesters (*Butyrivibrio fibrisolvens*, *Prevotella* sp*.,* and *Ruminococcus flavefacien*; Zhou et al., 2015). Other bacterial species groupings based on preferential substrates are amylolytic (*Streptococcus bovis, Succinomonas amylolytica, Ruminobacter amylophilus, Prevotella ruminicola*, and *Selenomonas ruminantium*), sugar-utilizing (*Treponema bryantii,* and *Lactobacillus ruminis*) and lactate-utilizing bacteria (*Megasphaera elsdenii* and *Selenomonas lactilytica*; Ishler et al., 1996; McDonald et al., 2002; Madigan et al., 2003)

Other organisms, such as archaea, protozoa and fungi, also exist within the rumen of ruminants (McDonald et al., 2002) and will not be discussed in great detail. Archaea only account for a small number (0.3 – 3.3 %) of the microbial population within the rumen (Janssen and Kirs, 2008). Archaea methanogens grow using H_2 or formate for energy and use the electrons derived from these products to reduce CO_2 to CH_4. Methanogens work in close association with *entodiniomorphid* protozoa (Kamra, 2005) and receive a constant supply of H_2 from the protozoa (Stumm et al., 1982). Holotrich protozoa are efficient at using starch, pectin and soluble sugars as energy sources because they have amylase, invertase, pectin esterase and polygalacturonase (Mould and Thomas, 1958; Akkada and Howard, 1961; Williams, 1979) enzymes. Protozoa may contribute up to half of the microbial biomass found in the rumen (Kumar et al., 2013), but a low proportion of the microbial biomass flowing out of the rumen (Van Soest, 1994). Rumen fungi are not as diverse as protozoa and bacteria in the rumen (Sniffen and Robinson, 1987), but it appears that fungi have greater cellulase-complexes than most bacteria (Stewart, 1994), which may make them more efficient at breaking down cellulose than certain cellulolytic bacteria.

It should be recognized that microbes not only interact with solid feed, but also with one another and the host. Understanding these interactions is important to increase production and efficiency of ruminants (Firkins and Yu, 2006). Some interactions between microbial species are commensal and symbiotic, while some are competitive and/or pathogenic (Stewart, 1994). Protozoa prey on bacteria, which may partially regulate bacterial density, especially when high concentrate diets are fed (Sniffen and Robinson, 1987; Stewart, 1994). Bacteria also act as a nitrogen source for protozoa protein synthesis (Ishler et al., 1996).

Clearly, the rumen provides an environment for various types of microbes, which are required to function together to digest different components of feed to maximize end products used by the animal for production. As described above, ruminal microbes utilize feed to produce end products, namely microbial crude protein, SCFA (namely acetate, propionate, butyrate), and gasses like carbon dioxide (CO_2) and methane (CH_4; McDonald *et al*., 2002). Short chain fatty acids are the primary source of energy for the ruminant and epithelial tissue (Rémond et al., 1995). Environmental influences, such as SCFA concentrations and rumen pH, can impact the microbial population and rate of SCFA absorption from the rumen through diffusion (Allen, 1997) and SCFA/HCO_3 anion exchange (Aschenbach et al., 2011).

1.2.1.3. Rumen epithelium function

1.2.1.3.1. Absorption, transportation and metabolism

The rumen epithelium facilitates absorption of SCFA (Bergman, 1990; Gäbel et al., 2002), various minerals (Gäbel et al., 2002; Leonhard-Marek et al., 2007; Aschenbach et al., 2009), HCO_3^- (Aschenbach et al., 2009; Penner et al., 2009), glucose (Gabel and Aschenbach, 2002), ammonia (Aschenbach et al., 2011), urea (Gozho and Mutsvangwa, 2008; Doranalli et al., 2011), and peptides and amino acids (Matthews and Webb Jr, 1995; Faix and Faixová, 2001). Even though there are hundreds of molecules absorbed through the rumen epithelium, this thesis will only focus on SCFA. Between 50 to 85 % of SCFA produced in the rumen is absorbed by the rumen epithelium (Bergman, 1990; Rémond et al., 1995; Kristensen et al., 1998) and approximately 10 % reaches the small intestine (**SI**; Harfoot, 1981). Most of the butyrate that is absorbed is metabolized by the rumen epithelial tissue (Stevens and Stettler, 1966; Bergman, 1990), whereas propionate and acetate are metabolized to a much lower extent (Bergman, 1990; Kristensen and Harmon, 2004). When SCFA are metabolized inside the rumen epithelium, it

results in the formation of ketones (beta-hydroxy butyric acid; **BHBA**) and lactate that are transported to the liver for further metabolism (Bergman, 1990).

Short-chain fatty acids can be absorbed by the epithelium either through passive diffusion or facilitated transport (Aschenbach et al., 2011). When SCFA are in an undissociated (HSCFA) form, absorption occurs by passive diffusion because they are lipophilic molecules (Gäbel et al., 2002; Graham et al., 2007; Aschenbach et al., 2009). Passive diffusion thus becomes the predominant absorption method due to the presence of H^+ molecules in the lumen. When SCFA are in a dissociated ($SCFA^-$) form, making them lipophobic, they need to be actively transported into the epithelium (Gäbel et al., 2002). Transporters or exchange proteins namely, monocarboxylate transporters (*MCT*), sodium-hydrogen exchangers (*NHE*), sodium bicarbonate cotransporters (*NBC*), putative anion transporter 1 (*PAT1*), downregulated in adenoma (*DRA*) and anion exchanger (*AE*) exist apically or on the basolateral membrane of the rumen (Bilk et al., 2005; Kirat et al., 2006). The *MCT, NHE, AE* and *NBC* transporters also exist in the omasum, abomasum, SI, and large intestine, but to a much lesser extent than in the rumen (Kirat et al., 2006). There are different isoforms of *MCT* present in the rumen, including *MCT1, MCT2* and *MCT4,* and all are responsible for co-exporting SCFA and H^+ basolaterally (Kirat et al., 2006). The *AE2* imports dissociated SCFA and exports HCO^-. Both SCFA import and HCO^- export methods also play a role in pH regulation. Sodium-hydrogen exchangers 1 and 3 play an important role in maintaining ruminal pH homeostasis by recycling protons back to the rumen and importing sodium (Orlowski and Grinstein, 2004; Laarman et al., 2013; Laarman et al., 2016). Sodium-hydrogen exchanger 2 regulates intracellular sodium concentrations by metabolizing sodium and producing protons (Orlowski and Grinstein, 2004). The *PAT1* and *DRA* exchange proteins commonly transport HCO_3^- into the lumen and chloride into the intra-epithelium, making it possible for SCFA to be absorbed instead of chloride (Kramer et al., 1996). Another pathway independent of HCO_3^- has also been reported, although it is not well understood (Penner et al., 2009; Aschenbach et al., 2009). Absorption of SCFA is influenced by ruminal pH, specifically when pH is < 6 and acetate and propionate absorption is reduced (Annison et al., 1957; Gäbel et al., 1987). The balance between the production rate, absorption, buffering and passage of SCFA and H^+ must occur in order to maintain optimum ruminal pH (Allen et al., 2006). Rumen pH can be partially regulated through SCFA absorption and neutralization (or buffering) of protons (Aschenbach et al., 2011). There are two buffering

systems, namely saliva buffer and bicarbonate secreted by the epithelium (Aschenbach et al., 2011) which will be further discussed in section 1.2.1.4.

1.2.1.3.2. Barrier

In order for a toxin or microbe to enter the blood stream through the rumen epithelium, it has to go through a barrier. The barrier in the rumen is an intrinsic barrier separating the external environment and the host, and this barrier consists of epithelial cells and cell junctions. The keratinization of stratum corneum make this cell layer the first barrier of the rumen epithelium, protecting the underlying cell layers against the rumen digesta (Graham and Simmons, 2005). This specific barrier function is unique to the rumen because the lower gut has only a monolayer structure and not a multilayered structure like the rumen (Graham and Simmons, 2005; Steele et al., 2016). The absorption and transportation of molecules in the epithelium were previously discussed; however, in order for this to occur, a concentration gradient needs to be maintained. Therefore, the epithelium barrier plays an important role in promoting and obstructing molecule movement.

Barrier function is controlled through desmosomes, adherens junctions, tight-cell junctions and gap junctions (Groschwitz and Hogan, 2009). It has been proposed that desmosomes and adherens junctions play a key role in joining adjacent cells (Hartsock and Nelson, 2008), whereas tight cell junctions play an important role in paracellular transport regulation (Van Itallie and Anderson, 2014) and gap junctions play an important role in molecule transport between cells (Bruzzone et al., 1996). Desmosomal cadherins of adjacent cells connect to the intermediate filament cytoskeleton forming junctional complexes (desmosomes; Stahley et al., 2014). The desmosomes are arranged in both the simple and stratified squamous epithelium on the lateral side of the cells. Tight junctions are made from multiple proteins, including claudins (CLDN), occludens (OCLN), zonula occludens (ZO), and gap junctions, (Graham and Simmons, 2005) and bond neighboring cells (Gonzalez-Mariscal et al., 2008). Claudin-1, 4 and 7 have been identified in the ruminal epithelium; *CLDN1* and *4* are found in the stratum granulosum and stratum spinosum, whereas *CLDN7* is found in the stratum corneum (Graham and Simmons, 2005; Stumpff et al., 2011). These tight junctions proteins can also regulate ion movement paracellularly (Gonzalez-Mariscal et al., 2008). Occludens have 4 transmembrane domains (Al-Sadi et al., 2011), are mobile, and can move to different paracellular locations, unlike CLDN that

are immobile (Shen et al., 2008). Occludens form a complex with CLDN proteins to make the epithelial tissue barrier strong (Raleigh et al., 2011), however, when oxidative stress or inflammation occurs (John et al., 2011), the OCLN proteins move from the tight junction into cytoplasmic vesicles and barrier function is compromised (Shen et al., 2008). Another tight junction protein, zonula occluden 1 (ZO1), found in the stratum granulosum (Graham and Simmons, 2005) regulates OCLN attached to CLDN (Raleigh et al., 2011) by offering structural support for CLDN by binding directly to OCLNs (Shen et al., 2008; Gonzalez-Mariscal et al., 2008; Raleigh et al., 2011). Another type of barrier protein is gap junctions. They are between cells, have an hour glass-like shape, and contain multiple protein sub-units with a gated channel in the centre, which allows paracellular passage of small molecules, such as electrolytes (potassium), metabolites (sugars and SCFA), and cyclic nucleotide signalling molecules (Sáez et al., 2003; White, 2003). One of the gap junctions found in the rumen epithelium is connexin 43, which is mainly found on the border of the stratum granulosum/stratum spinosum, as well as on the border of the stratum spinosum/stratum basale – though to a much lesser extent (Graham and Simmons, 2005).

1.2.1.4. Dysfunction

The transition to a high grain diet from a high forage diet can cause rumen dysfunction, most commonly bloat and ruminal acidosis (Meyer and Bryant, 2017). When starch is rapidly fermented in the rumen it can cause bloat, which is most commonly found in feedlot animals (Hironaka et al., 1973; Cheng et al., 1998). Animals can either experience free-gas bloat or frothy bloat. Free-gas bloat occurs when gas accumulates from starch fermentation that cannot be eructated due to obstruction of the esophagus or cardia, or when rumen motility is impaired by the rapid development of ruminal acidosis (Cheng et al., 1998). Frothy bloat occurs when foam accumulates, which contains mucopolysaccharides and macromolecules from bacterial cell lysing that occurs (Cheng et al., 1976) and this foam blocks the cardia and prevents gas from being eructated (Cheng et al., 1998).

Ruminal acidosis is a digestive disturbance of the rumen leading to a reduction in pH and can be categorized as sub-acute (**SARA**) and acute ruminal acidosis depending on the extent of pH depression and organic acid concentration in the rumen (Penner et al., 2007; Aschenbach et al., 2011). When SCFA production exceeds SCFA absorption, ruminal buffering and passage,

ruminal pH decreases (Aschenbach et al., 2011). This is usually the result of increased fermentation due to the consumption of a meal rich in highly fermentable feedstuffs. As the available fermentable material in the rumen decreases, ruminal pH increases. With rapid SCFA production, ruminal pH decreases and results in acidosis, commonly categorized as either subacute ruminal acidosis (pH < 5.8 or 5.5) or acute ruminal acidosis (pH < 5.2 or 5.0; Penner et al., 2007; Aschenbach et al., 2011). Ruminal pH fluctuations occur throughout the day because of the nature of the feed and the frequency of eating (Dehority, 2003). Rumen pH responses can also vary between animals because of differences in dry matter intake (**DMI**), saliva secretion, absorption, and microbial populations, even when a common diet is fed (Brown et al., 2006). Rapidly fermentable carbohydrates increase the risk for ruminal acidosis. Acidosis has the propensity to occur when starch availability in grain has been increased by processing through a reduction in particle size or with heat, moisture, or pressure (Johnson et al., 1974; Britton and Stock, 1987; Brandt Jr et al., 1993). A reduction in forage particle size or proportion of forage in the diet may also reduce rumination time, leading to a decrease in saliva production and therefore rumen pH (Mertens, 1997). Other dietary scenarios that increase risk for acidosis include: diet transition, for example feedlot animals transitioned to a high concentrate diets (Schwartzkopf-Genswein et al., 2003); dairy cows during the transition period (Enemark, 2008); calves during weaning; high levels of feed intake after a period of low feed intake, which is normally associated with animals after transportation (Zhang et al., 2013; Albornoz et al., 2013); or during heat stress (Baumgard and Rhoads Jr, 2013).

Animals with SARA have reduced feed intake, which leads to a reduction in performance while remaining asymptomatic (Owens et al., 1998). Variation in feed intake from day-to-day can be a sign of SARA (Britton and Stock, 1987). Acute acidosis, also known as lactic acidosis, occurs when ruminal animals engorge a large amount of starch or rapidly fermentable carbohydrate causing a decrease in ruminal pH (Krause and Oetzel, 2006) and a reduction in protozoa numbers (Ørskov, 1986; Nocek, 1997; Owens et al., 1998). When ruminants consume large amounts of grain and pH decreases, it promotes excessive growth of lactic acid-producing bacteria in the rumen, like *Streptococcus bovis* and *Lactobacillus* spp (Ahrens, 1967; Allison et al., 1975; Braun et al., 1992), due to their high tolerance to low pH. Additionally, when acidity increases in the rumen, pyruvate dehydrogenase activity improves, leading to greater lactate production (Russell and Hino, 1985). On the other hand, lactic acid-utilizing bacteria decrease

(Nocek, 1997; Owens et al., 1998; Galyean and Rivera, 2003). When lactate production increases, ruminal pH will decrease further because lactate has a lower pKa value and is therefore a stronger acid than SCFA. Ulcers and damage to the rumen epithelium occur and feed conversion rate declines when lactic acidosis occurs (Braun et al., 1992).

The pH drop in sub-acute and acute acidosis is due to the amount of SCFA being produced (Owens et al., 1998; Galyean and Rivera, 2003), with most of the SCFA releasing protons. Sub-acute ruminal acidosis occurs when ruminal pH drops below 5.8 or 5.5: a critical threshold used for dairy cattle and beef cattle (Aschenbach et al., 2011). In SARA, lactic acid levels generally remain low or undetectable and pH depression is not as severe as with acute acidosis (Nocek, 1997; Galyean and Rivera, 2003). High-producing ruminants, like dairy (Nocek, 1997) or feedlot (Smith, 1998) cattle, are prone to SARA because high producing ruminants must consume high amounts of fermentable feed in order to maintain optimal productivity.

Ruminal pH plays a key role in rumen epithelium barrier function. Penner et al. (2010) reported that when pH dropped to 5.8 for 3 h, barrier function stayed intact; however, when pH decreases to 5.2 causes barrier dysfunction. Ruminants transitioned to a high concentrate diet from a high forage diet had rapid sloughing of the stratum corneum (Steele et al., 2009), which is the first barrier of the rumen epithelium. Steele et al. (2011a) also reported that the stratum basal, spinosum, and granulosum layer thicknesses decreased. Furthermore, there were fewer cell layers of the stratum granulosum and tight junction genes were fragmented in animals on the high concentrate diet (Liu et al., 2013), which may demonstrate that junctions between the cells are more relaxed, thus causing barrier dysfunction. The different diets also caused the tight junction proteins to translocate: OCLN was in the cytoplasm of epithelial cells of animals fed the high concentrate diet, whereas CLDN1 and 4 and OCLN were on the epithelial cell surface for the animals fed the high forage diet. The high grain diet resulted in down-regulation of CLDN4, OCLN, and ZO1 expression. When Schurmann et al. (2014) fed calves (approximately 5 to 7 months of age) a 50 % concentrate diet for 3, 7, 14, and 21 d after a high forage diet, permeability increased linearly when measured by tissue conductance and serosal-to-mucosal flux of Na+. In the same study, serosal-to-mucosal mannitol flux was also tested, and a tendency was observed when the diet was changed to a higher concentrate diet (Walpole et al., 2015). Mannitol is a molecule that is generally used to test transcellular permeability, which suggests that the diet change may cause paracellular permeability; however, it was noted that calves did

not experience ruminal acidosis with the 50 % concentrate diet fed for various lengths. Ruminal pH alone is not responsible for ruminal epithelium barrier dysfunction. However, when taken together with high SCFA concentration, the likelihood of compromised barrier function increases significantly (Meissner et al., 2017). When ruminal pH is low (5.1) for several hours, ruminal barrier function is mildly affected; however, when SCFA were present in the same pH measurements, barrier function decreased mRNA expression of tight junction proteins (*CLDN7* and protein expression of *CLDN4* and *7*, and occludin). All the aforementioned conditions are signs that the barrier function could be influenced when animals experience ruminal acidosis or when the diet is switched from a high forage to a high concentrate diet.

When acidosis occurs, gram negative bacteria can release endotoxins (lipopolysaccharides (**LPS**)) during periods when the bacteria lyse or grow rapidly (Mao et al., 2013). When SARA was induced with grain, LPS increased in the blood indicating barrier dysfunction (Khafipour et al., 2009b). However, when SARA was induced with alfalfa pellets, LPS did not increase in the blood (Khafipour et al., 2009a). When endotoxins enter the peripheral blood stream, they can cause localized and systemic inflammatory responses (Khafipour et al., 2009b). Gozho et al. (2006) reported that high grain diets can evoke an inflammatory response that is caused by free endotoxins travelling into the peripheral bloodstream. Furthermore, Emmanuel et al. (2007) reported that free LPS endotoxins increased by five-fold when pH decreased causing SARA. It is important to note that the concentration of endotoxins in rumen digesta or blood is only half the story. The endotoxins released from different bacteria have different toxicity levels. For example, endotoxin released from *E. coli* is much more toxic than endotoxin released from *Megasphaera elsdenii*, *Fibrobacter succinogenes*, *Prevotella* spp., and *Bacteroides* spp (Hurley, 1995). Therefore, not all endotoxin concentrations will evoke an inflammatory response, which may be why there are contradicting results between studies when serum amyloid A (SAA) or haptoglobin were used as markers for systemic inflammation.

There are two main management strategies that can be used to prevent or reduce acidosis: diet composition and additives. As a strategy to limit potential exposure to rumen acidosis, ruminants should be provided a gradual diet transition to allow for epithelial adaptations that maximize absorption when they switch from a roughage-based diet to a high concentrate diet. The intake of starch and rapidly fermented carbohydrates should be monitored by managing the size of the meals and increasing the frequency of meals (Schwartz and Gilchrist, 1975; Mackie et

al., 1978). The occurrence of acidosis may be reduced by adding additives like bicarbonate to buffer the rumen pH (Krause and Oetzel, 2006) and additives that inhibit lactate producing microbes and facilitate lactate using microbes, such as the ionophore antibioitics lasalocid and monensin (Nagaraja et al., 1981). Ionophore antibiotics reduce the populations of *S. bovis* and *Lactobacillus* (Nagaraja et al., 1981; Nagaraja et al., 1987; Thorniley et al., 1998) that may reduce the risk of lactic acidosis.

Ruminal acidosis is associated with high concentrate diets that can cause physiological changes in the rumen, which can lead to several different systemic changes (Nocek, 1997) and other disorders. Among these disorders is diarrhea, which can cause dehydration, reduced rumen motility, poor body condition and depression (Aschenbach et al., 2011). Thus, ruminal acidosis might cause concern from an economic standpoint because it can impact animal production, health and welfare. As seen by this discussion, ruminal dysfunction in adult ruminants has been well characterized. However, knowledge surrounding ruminal dysfunction in calves is lacking (discussed in section 1.4.1).

1.2.2. Lower gut

The forestomach is followed by the SI and the large intestine (also known as the hindgut; Sherwood et al., 2013). The SI consists of three compartments, the duodenum, jejunum, and ileum, while the hindgut consists of the cecum, colon and rectum. In the next sections, the structure, environment and functions of the small and large intestine will be discussed, with major emphasis on the hindgut (colon).

1.2.2.1. Anatomy and structure

The SI has many folds and comprises approximately 80 % of the whole intestinal length in ruminants (Swenson and Dukes, 1977). The SI epithelium has crypts, small finger-like villi that contain hair-like projections called microvilli (Sherwood et al., 2013). These structures contribute to absorption capacity by increasing the surface area (Peterson and Artis, 2014). The microvilli are part of the brush border membrane (Sherwood et al., 2013). The SI epithelium consists of columnar epithelial cells (Liévin-Le Moal and Servin, 2006; Steele et al., 2016), different from that of the rumen, and includes a mixture of cells, such as enterocytes, enteroendocrine, goblet, paneth and stem cells (Sherwood et al., 2013). Cell development begins

in the crypts, and cell proliferation and differentiation occur as the cells migrate up the villi (Sherwood et al., 2013; Eurell and Frappier, 2006).

The cecum is a blind sac where carbohydrates that bypassed SI digestion and absorption are fermented (Gressley et al., 2011). The cecum accounts for 1 % of total lower gut length in cattle (Swenson and Dukes, 1977). The colon is a coiled form in the ruminant (Sherwood et al., 2013) and accounts for 19 % of the lower gut length (Swenson and Dukes, 1977). The colon structure is different from that of the SI because the villi are absent, making the intestinal surface in this compartment more uniform and flattened (Sherwood et al., 2013).

1.2.2.2. Environment

Even though digesta pH in the abomasum is very low (pH 2 - 4), it increases (pH 7 – 9) substantially in the duodenum through gall bladder secretions (alkaline bile) to ensure enzyme survival (Noble, 1981). The hindgut has a similar environment to the rumen and also contains a diverse microbial community that works together to digest feed. However, no protozoa are present in the hindgut (Gressley et al., 2011). Because microbial fermentation also occurs in this compartment, digesta pH will also change depending on the extent of fermentation (Gressley et al., 2011). This pH change can also affect hindgut barrier function (further discussed in section 1.2.2.4).

1.2.2.3. Function

1.2.2.3.1. Digestion, absorption, transportation and metabolism

The main function of the SI in ruminants is the digestion and absorption of nutrients, such as proteins (mainly microbial protein), lipids and some carbohydrates (Sherwood et al., 2013). Amylases, lipases and proteases are the most well-known pancreatic enzymes and are responsible for the digestion of carbohydrates, triglycerides and proteins, respectively. Specialized transporters then transport the monomers (amino acids, fatty acids and monosaccharides) through the intestinal epithelium into the blood stream where they are distributed throughout the body.

Any feed or microbial protein that have not been digested or absorbed by the SI flows into the hindgut where microbial fermentation occurs to breakdown the digesta further. The colon is responsible for water, mineral and SCFA absorption (Sherwood et al., 20123). Hindgut SCFA

production may contribute up to 9 % of metabolizable energy requirements in cattle (Bergman, 1990). When calculated based on the disappearance of starch as a fraction of starch entering the site of fermentation, starch digestion is more efficient in the hindgut than in the rumen (Haimoud et al., 1995; Callison et al., 2001). Hindgut NDF digestibility could also possibly be higher than in the rumen, especially when diet non-forage fibre increases (Firkins, 1997). The passive absorption mechanism of SCFA in the colon is similar than that of the rumen (Argenzio et al., 1975; Sivaprakasam et al., 2011). Unfortunately, no digestion or absorption of microbial proteins occurs in the hindgut and therefore microbial proteins produced in the hindgut are lost in the feces (Lapierre and Lobley, 2001).

1.2.2.3.2. Barrier

The lower gut has three distinct barriers, one that is the same as the rumen (intrinsic; described in section 1.2.1.3.2). In the lower gut, the first barrier consists of the commensal microbial population and luminal secretions. The commensal microbes attached to the epithelium make it difficult for pathogen attachment and secrete substances that can be bactericidal (peptide bacteriocins, hydrogen peroxide, and lactic acid) to impede growth of pathogens (Jutfelt, 2011). The epithelium can secrete mucus through goblet cells (primarily *MUC2*; Jutfelt, 2011), as well as antimicrobial peptides, such as defensins (Jäger et al., 2010) through Paneth cells (Gallo and Hooper, 2012), and immunoglobulin A (IgA; Hooper and Macpherson, 2010). The mucus acts as a barrier between the digesta and the epithelium and also a lubricant and transporter for digesta passing thought the lower gut. There are two types of mucus layers found in the lower gut: the firmly and loosely adherent layers (Kim and Ho, 2010). The loosely adherent layer is generally the layer closest to the digesta and microbes are found within this layer. The thick (firmly adherent) mucus layer is closest to the epithelium. The thickness of the mucus layers depends on the compartment of the lower gut, with the loosely adherent layer being the primary mucus layer in the duodenum and jejunum in rats (Atuma et al., 2001). There is relatively less mucus in the duodenum and jejunum compared with the ileum and colon. Furthermore, mucus thickness increases from the proximal colon towards the rectum (Matsuo et al., 1997). This may be because of the different functions the compartments have and the amount and diversity of microbial populations that are present in these compartments. The role of the mucus layer in the lower gut of ruminants is not well-studied and the majority of knowledge is presumed to be the same as monogastric species.

The second barrier is the intrinsic barrier (separating the external environment and the host); this barrier is made of epithelial cells and cell junctions. Unlike the rumen, which consists of a stratified squamous epithelium, the lower gut consists of a columnar epithelium (Steele et al., 2016) which makes this physical barrier different. The structural differences and the lack of dead keratinized cells in the lower gut may make it more permeable. The lower gut also has desmosomes, adherent junctions, tight-cell junctions and gap junctions that control barrier function as deacribed previously and will not be further discussed in this section. In the SI, high solute permeability increases from the villus to the crypts (Fihn et al., 2000), favouring the crypts as an area of increased permeability. Permeability has been reported to be different in each compartment of the lower gut, yet the SI appears to be more permeable than the colon (Nejdfors et al., 2000) in monogastrics. Penner et al. (2014) reported that in ruminants, the jejunum is most permeable to small particles, followed by the rumen and omasum, with the ileum having very low permeability.

The third barrier consists of an immunology barrier. Material is sampled through endocytosis, phagocytosis, or transcytosis for early detection of harmful substances present, and if necessary, will start an immune response (innate and adaptive) within the epithelium and lamina propria (Jutfelt, 2011). This will also help to build a tolerance against the commensal microbial population present to prevent an unnecessary immune response against commensal microbes (Jutfelt, 2011). Overall barrier function in the lower gut is just as important as in the foregut to maintain healthy animals.

1.2.2.4. Dysfunction

When large quantities of starch (like unprocessed grains) escape the rumen and SI when animals are fed high grain diets (Svihus et al., 2005; Gressley et al., 2011) it causes hindgut dysfunction. Hindgut acidosis, which has received more attention lately (Gressley et al., 2011; Li et al., 2012), occurs when there is high fermentation in the hindgut. One reason why this may occur is low pH in the SI which may influence nutrient absorption as Wheeler et al. (1980) reported, when SI pH is below 6.9, inhibition of pancreatic alpha amylase occurs, thus influencing starch digestion. The cattle in this study fed high energy diets had low fecal pH and increased fecal starch, which could indicate low intestinal pH (SI and LI) and high amounts of unfermented starch reaching the hindgut. Higher passage rate of digesta can also result in lower

SI starch digestion, leading to an increased risk of hindgut acidosis (Gressley et al., 2011). Fecal consistency – diarrhea, frothy feces, and presence of mucin casts – can be an indicator of extensive hindgut fermentation (Hall, 2002; Plaizier et al., 2008). Infusion of corn starch (4 kg/d) into the abomasum caused fecal pH to drop below 5 from 7 (Bissell and Hall, 2010). There was blood and mucin casts in the feces of these animals and fibrinogen (inflammatory marker) was increased in the blood. However, other inflammatory markers – blood haptoglobin and alpha-acid glycoproteins – were unaffected by the starch infusion. The authors reported that the animal response varied a lot between animals and that some animals were not affected by the starch infusion while others had compromised health. When cows were fed either a high forage diet, a high forage diet that contained ground alfalfa, or a high grain diet, it was reported that the high grain diet and the alfalfa diet resulted in similar cecum pH, however, starch content was higher in the high grain diet (Li et al., 2012). The grain diet increased LPS in the cecum digesta, as well as in the feces, which resulted in increased lipopolysaccharide binding protein (LBP) in the blood. The authors concluded that the high grain diet increased the risk of LPS translocation from the hindgut, rather than the alfalfa diet.

When goats were fed a high concentrate diet for 6 wk, they had decreased ruminal pH and increased ruminal LPS concentrations (Tao et al., 2014a). Additionally, hindgut tissue damage was observed and epithelium cell apoptosis was activated and a local inflammatory response occurred. In a similar study, Tao et al. (2014b) reported that digesta from the colon had increased SCFA and starch concentrations when goats were fed a high concentrate diet for 10 wk compared with low concentrate diet. The authors also reported that cell apoptosis was activated, which damaged the barrier function of the colon, similar to their previous study. Clearly, ruminal acidosis or starch that bypasses ruminal fermentation and SI digestion and absorption can cause hindgut acidosis, which can result in compromised animal health. The hindgut has a monolayered epithelium, whereas the rumen has a multilayered epithelium (Graham and Simmons, 2005; Steele et al., 2016), making the hindgut more prone to barrier dysfunction when hindgut acidosis occurs. The hindgut is highly understudied in ruminants and has only recently received attention. One area that needs further investigation is the pH threshold at which barrier function is compromised in the hindgut, because unlike the thresholds used to determine ruminal acidosis, the literature does not dictate any fecal pH thresholds to declare that animals have hindgut acidosis.

1.3. Methods to study gastorintestenal tract changes – biochemical, biomarker, histological and molecular

There are multiple methods that can be used to study changes, be it due to diet or disease, that occur in the gut. Weaning transition, dry cow to fresh cow transition and feedlot animals switching from a high forage to high grain diet are most common diet changes that animals experience. The four main categories under which these methods fall are biochemical, biomarker, histology and molecular, and only the most commonly used methods will be discussed in this section.

Blood is usually the most commonly used sample type for biochemical analysis, as it is more easily obtained than other samples, like GIT digesta. Rumen papillae and hindgut tissue absorb SCFA by passive or facilitated diffusion and acetate and propionate are mostly transported into the animal's bloodstream (Stumpff, 2018). Acetate is utilized for lipogenesis through acetyl-CoA synthesis in adipose tissue and propionate is utilized for synthesizing glucose through gluconeogenesis in the liver (Bergman, 1990). In contrast, most butyrate (85 – 90 %) absorbed into the ruminal epithelium is oxidized to ketones and then transported to the bloodstream (Beck et al., 1984). The most common ketone produced is BHBA, followed by acetoacetate. Butyrate is viewed as an energy source for the epithelium since ATP is synthesized by butyrate oxidation (Bugaut, 1987) which can be used towards epithelial cell proliferation (Sakata and Tamate, 1978). Thus, BHBA maybe used as an indicator of rumen development. Multiple studies have validated that BHBA concentrations are closely related to dry feed consumption (Quigley et al., 1991; Deelen et al., 2016). The BHBA concentration results also have to be interpreted with caution, as shown by (Suarez-Mena et al., 2017), who concluded that time of day, stress, and feed restriction influence BHBA concentrations.

A biomarker is a measurement of biometrics that indicates the biological state of an animal in relation to disease (Aronson, 2005; Orro, 2008). Acute phase protein concentration in the blood can be used as biomarkers of stress (Arthington et al., 2003; Lomborg et al., 2008) or inflammation (Orro, 2008; Khafipour et al., 2009a,b). Of particular interest are SAA and LBP, which can be biomarkers for GIT inflammation (Khafipour et al., 2009a,b). It is important to bear in mind that the specific region of the GIT where inflammation originated cannot be identified.

Histological methods can be used to study changes that occur within the gut. Multiple studies have used the length, width and area of ruminal papillae measured under a microscope to characterize rumen development (Zitnan et al., 2005; Kristensen et al., 2007; Nishihara et al., 2019).When gut tissue are stained with hematoxylin and eosin (**H&E**) the cell nuclei are stained blue by the hematoxylin and extracellular matrixes and cytoplasm are stained pink by the eosin (Chan, 2014). Other structures are stained by different combinations of pink and blue depending on the structure. This method is used to distinguish between the papillae layers (stratum corneum, granulosum, spinosum and basale) of the rumen and to evaluate lesions (Steele et al., 2011; Dieho et al., 2016). Transmission electron microscopy (**TEM**) is a method used to examine ultrastructural changes by using a beam of electrons that transmits through the thinly sliced tissue to form an image (Williams and Carter, 1996). This method is usually used for qualitative assessments of cellular organelles such as cell nucleus mitochondria and tight junctions (Steele et al., 2011; Tao et al., 2014b). The aforementioned methods are only a few that can be used to qualitatively and quantitatively examine GIT changes.

On a molecular level, gene expression can be used to understand the changes that occur in the gut when different diets are fed or during the weaning transition. There are multiple technologies that can be used, each with their own advantages or limitations. Real-time reverse transcriptase quantitative polymerase chain reaction (**RT-qPCR**) is a technique that detects specific genes or regions by using the same method as PCR (denaturation, annealing, and extension), but a fluorescent reporter is added for detection (Fraga et al., 2008). This fluorescent reporter binds to the product formed by amplification and thus the amount of product (genes) is representative of the amount of fluorescent signals received (Bustin, 2002). mRNA is usually used for RT-qPCR and this mRNA strand is converted into a cDNA template. It only takes a few hours to obtain the results using the RT-qPCR, however, only one targeted gene can be quantified within a single run and it can only quantify the expression of known gene sequences. Multiple targeted sequences can also be quantified within a single run using more than one pair of primers, a process called multiples PCR. This method works best if the primers used have similar annealing temperatures (Elnifro et al 2000).

Microarray is a method used to measure gene expression from multiple genes at the same time by oligonucleotides that are attached to glass chip through covalent bonds (Rimm et al., 2001). This method includes the conversion of mRNA from the sample into cDNA,

radiolabeling, and hybridization. Laser scanning or autoradiographic imaging captures the intensity of the signals for each microarray spot and generates data. This method allows for the quantification of more genes at the same time.

High-throughput transcriptome sequencing (RNA-Seq) is a method that involves sequencing, prepared cDNA libraries from fragmented mRNA (Cloonan and Grimmond, 2008). More so, this method gives insight into novel genes. After sequencing, bioinformatic tools are used to align the transcripts to a reference genome (for instance bovine genome) and to gene count (Costa-Silva et al., 2017). Thereafter, bioinformatic tools can be used to predict biological functions, enriched pathways and co-expressed network analysis.

In order to study changes of the GIT using histological or molecular methods, tissue is required, which is normally obtained from animals that have been euthanized. Multiple studies have used serial slaughtering to study GIT changes during weaning (Warner et al., 1956; Lane et al., 2002); however, with this approach temporal responses reported may not be accurate because large variations among animals exist and there is only one sampling timepoint per individual animal. The development of a method to collect GIT tissue over time in the same animal will enable researchers to study GIT changes that may occur in the animal due to weaning or diet changes.

1.4. Gastrointestinal changes during the weaning transition

1.4.1. Forestomach

Many physical changes occur in the rumen during the transition from young pre-weaned calf to an adult ruminant. The size of a mature rumen comprises approximately 70 % of the capacity of the GIT; however, at birth the calf's rumen only comprises 30 % of the GIT capacity (Warner et al., 1956). The authors also reported that at birth, the calf's ruminal epithelium does not have prominent papillae. At wk 4, changes in papillae were observed, but the papillae were still smaller than 2 mm in length. Calves that received milk until wk 16 had no further changes in ruminal development, while calves that received solid feed had heavier rumens (empty dry weight as % of body weight (**BW**)). Kristensen et al. (2007) reported that calves that consumed more concentrate had a heavier reticulorumen and omasum (empty wet weight as % of BW) and the digesta weight in these compartments was increased for calves that consumed more starter at wk 5 of age. Ruminal papillae length of the ventral sac and atrium were not affected by the

amount of concentrate that the calves consumed. The papillae in the atrium lengths ranged from 2.4 to 4.4 mm and the papillae in the ventral sac lengths ranged from 1.7 to 3.5 mm. Zitnan (2005) reported that calves weaned for 1 wk had increased papillae length in the atrium ruminis, and decreased papillae density in the ventral ruminal sac and ventral ruminal blind sac compared to calves that were not yet weaned. Connor et al. (2013) reported that there was no difference in papillae growth between calves that received milk at d 14 vs 42 – in both cases the papillae were ≤0.5 mm in height. Papillae height from calves fed milk and calf stater (**CS**) were 1.5 to 2.0 mm and 2.5 mm at d 56 and 70, respectively. On the other hand, calves fed milk and hay had papillae that were 0.5 and 1.5 mm in height on d 56 and 70, respectively. The authors also noted that the calves fed the milk and CS had pigmentation present in the rumen papillae. Nishihara et al. (2019) reported that weaned calves had higher papillae (1.5 mm) than calves that were not weaned (0.3 mm). This implies again that CS intake is a key factor influencing rumen papillae growth.

Ruminal pH in calves can be influenced by forage inclusion and CS starch amount. Kristensen et al. (2007) sampled rumen fluid every 2 h for a d during wk 2, 3, 4 and 5 and reported that during wk 5 ruminal pH was below 6.2 for more than 12 h/d, regardless of the amount of concentrate intake. Li et al. (2019) reported that when ruminal pH was measured 7 times per d every second wk (wk 6, 8, 10, 12, 14 and 16), calves that received the high starch (42.7 %) diet had lower ruminal pH during all wks compared with calves that received the lower starch (35.3 %) diet. Interestingly, this study does report that calves fed the high starch diet had poor performance (from wk 5 onwards) due to the depression in ruminal pH. Most of the other studies (Kristensen et al., 2007; Laarman and Oba, 2011) did not measure pH for a long enough duration to identify if ruminal pH had an effect on intake and performance. In lactating dairy cows, we do know that depressed ruminal pH impacts performance (Aschenbach et al., 2019). A study conducted by Laarman and Oba (2011) investigating the effect of CS on ruminal pH reported that ruminal pH is not only affected by CS intake, and that other factors, such as rumen epithelium pH regulation, may also play a key role. Laarman et al. (2012a) investigated the effect of CS intake on rumen fermentation and expression of genes involved in rumen epithelium pH regulation and butyrate metabolism. Intake of CS increased total SCFA concentration (Laarman et al., 2012a); however, this did not affect ruminal pH (Laarman and Oba, 2011). Kim et al. (2016a) investigated the effect of forage and CS on ruminal pH post-weaning (weaned at 8 wk)

for 3 wk in dairy calves. The authors reported that ruminal pH was below pH 5.8 for 1050 min/d for calves fed CS and 97.6 min/d for the calves fed CS and hay post weaning for 3 wk (wk 8 to 11). Total ruminal SCFA were not different, indicating that proton regulators might be influenced between the two diets, as well as buffer capacity from saliva. A similar study (Kim et al., 2016b) reported that calves fed hay and CS had higher ruminal pH (>5.8) pre- and post-weaning (wk 7 to 11) than calves fed only CS (pH mean below 5.8). Unfortunately, time spent below pH threshold was not reported and only mean pH was reported. Diurnal changes were averaged by wk and clear changes were observed for calves that were fed hay and CS; however, the calves that received only CS had diurnal ruminal pH changes during the pre-weaning wk and during the third wk post-weaning the diurnal pH change was weak. In this study, total ruminal SCFA was also not different. Based on the Kim et al. (2016a,b) and the Laarman and Oba (2011) studies, the inclusion of hay mitigates the severity of ruminal acidosis. It is clear that ruminal acidosis does occur in calves fed only CS during the weaning transition, however, these four studies (Laarman and Oba, 2011; Laarman et al., 2012a; Kim et al., 2016a,b) are the only studies that elucidate ruminal pH changes using continuous measurements, which are important to capture diurnal changes that may occur. So far, ruminal changes during weaning have only been studied when calves are fed low planes of milk/MR pre-weaning, and since the intake of solid feed is influenced by the amount of milk calves consume, this can influence ruminal pH. Furthermore, there are limited studies that have monitored pH from pre- to at least 3 wk post-weaning (long-term). It is well known that acidosis in adult animals reduces performance, usually by decreased DMI or fibre digestibility (Aschenbach et al., 2019), however, the effects of ruminal acidosis on calf health and performance are lacking.

There are multiple studies that have investigated gene expression in the rumen epithelium in relation to phenotypic trait changes, such as CS intake (Laarman et al., 2012a; Naeem et al., 2012; Connor et al., 2013), papillae growth (Connor et al., 2013; Nishihara et al., 2019), SCFA absorption and pH regulation (Laarman et al., 2012a; Liu et al., 2016). Expression of genes involved in metabolism and synthesis pathways that have been reported to be influenced by CS intake are cholesterol synthesis (Laarman et al., 2012a), beta-oxidation of lipids (Naeem et al., 2012) and ketogenesis (Connor et al., 2013; Liu, et al., 2016). Some of the genes that were studied are related to papillae growth, including insulin-like growth factor binding proteins that are involved in epithelium cell growth (Nishihara et al., 2018; Nishihara et al., 2019),

transforming growth factor (Kim et al., 2016a) involved in cell differentiation and growth (Steele et al., 2015), and *ERBB* receptor feedback inhibitor 1 that is part of the network of cell proliferation and growth (Connor et al., 2013). The genes studied related to SCFA absorption and pH regulation include *NHE1, NHE2, NHE3, DRA PAT1, MCT1* and *MCT4* and the outcomes varied between studies. Liu et al. (2016) reported that ruminal SCFA concentration and genes involved with SCFA transport and pH regulation are influenced by early starter provision in lambs (Liu et al., 2016). The lambs that received starter late had ruminal total SCFA concentrations that remained constant until they received starter. For lambs that received starter early, ruminal total SCFA concentration increased until wk 7 and then remained constant. For the lambs that received starter, their ruminal pH ranged from 5.3 to 6.0 and for the lambs that received starter late, ruminal pH ranged from 5.4 to 6.3 and there was tendency for the pH to be different between treatments. The pH did differ between wk. The higher expression of *MCT1* in the starter fed group does agree with the other studies (Kuzinski et al., 2011; Laarman et al., 2012a; Yan et al., 2014), but also disagrees with additional studies (Steele et al., 2012; Castells et al., 2013). The results for the expression of *NHE2* (Yang et al., 2012; Laarman et al., 2012a), *NHE3* (Laarman et al., 2012a; Yan et al., 2014; Yohe et al., 2015) and *DRA* (Connor et al., 2010; Castells et al., 2013) contrast what other studies have previously reported. Since increased ruminal fermentation and decreased ruminal pH (acidosis) occurs in calves during weaning (discussed earlier in this section), the effect of acidosis on barrier function will not be discussed here again (discussed in section 1.2.1.4).

1.4.2. Lower gut

The effect of CS intake or weaning on barrier function in the lower gut is an area that has received more attention lately. Wood et al. (2015) reported that GIT barrier function (tested using Cr-EDTA) improves as the calf ages. However, during weaning the permeability of the gut becomes compromised. Weaning had no impact on barrier function when Ussing chambers were used to quantify barrier function in the same study. However, there were compartment differences, with the rumen being the most permeable to mannitol, followed by the jejunum, cecum and duodenum. When inulin was used, the rumen and omasum were the most permeable, followed by the jejunum, cecum and duodenum. The authors explained that the data differs between the two different methods used and that the different size of molecules used to test permeability could have had an impact. Molecule size can play a big role as tissue may be more

permeable to smaller molecules than larger molecules. Also, the contrast in results may be indicative of the limitations of employing the Ussing chamber technique, which only tests a small amount of tissue from each compartment. As such, this may not be representative of the whole compartment and does not consider each compartment's permeability as a whole. Tight junction protein and/or gene expression in the gut may be the reason why a decrease in permeability is observed in the aforementioned study. Claudin 4 expression was the lowest in the ileum compared with the jejunum, cecum and colon in calves that received starter in the diet compared with the MR only (Malmuthuge et al., 2013). The inclusion of starter downregulated *CLDN4* expression in the ileum and jejunum and occludin in the jejunum, ileum and cecum compared with only a MR diet. Based on these results, it appears that the addition of CS caused a decrease in the expression of tight junction genes involved in barrier function.

Barrier function might be compromised through energy depletion when hindgut acidosis occurs. When SCFA production increased in the colon, cellular ATP reserves were depleted because of higher mitochondrial gene and protein expression, which led to higher increased mitochondrial proton pump activity in rats (Rodenburg et al., 2008). Dickman et al. (2000) reported that when Caco-2 cells were infected with rotavirus, it led to ATP depletion, which as a result caused *ZO-1* to be located in the cytoplasm, thus reducing transepithelial barrier function. The effect of hindgut acidosis on barrier function was already discussed in section 1.2.2.4 and will not be further discussed in this section.

The genes involved in the three different barriers (luminal secretions, intrinsic and immunological) in the lower gut have been studied in relation to CS intake and weaning. Expression of the β-defensin gene tended to be lower in calves that received starter compared with just MR (Malmuthuge et al., 2013). Peptidoglycan recognition protein 1 (*PGLYRP1*) were higher in calves fed MR compared with MR and CS. In addition, expression of *PGLYRP1* were higher in the cecum and colon. *β-Defensins* are part of the first line defense system promoting proper barrier function of the lower gut; peptidoglycans are also part of the barrier function. However, in order for peptidoglycans to be activated, microbes or molecules have to reach inside the epithelium, because *PGLYRP1* is expressed in polymorphonuclear leukocytes (Dziarski and Gupta, 2006). *β-Defensin* has antimicrobial activity that acts on bacteria (gram-negative and -positive), fungi, and viruses (Luenser and Ludwig, 2005), whereas *PGLYRP1* has antimicrobial

activity that acts on gram-positive bacteria, fungi and certain molecules (lipoteichoic acid and LPS; Dziarski and Gupta, 2006).

Intrinsic and immunology barrier genes play a critical role in keeping the animal healthy by preventing unwanted molecules from entering the systemic circulation. Upregulation of *IL1β, IL6,* and *TNF-α* expression was reported when piglets were weaned, possibly indicating inflammation in the intestinal tract (Pié et al., 2004). The expression of tight junction proteins decreased during weaning in piglets, leading to reduced barrier function (Bruewer et al., 2003). Moeser et al. (2007) reported that barrier function was compromised in the mid-jejunum and ascending colon when piglets were weaned early. Moreover, Hu et al. (2013) reported that weaning negatively influences intestinal morphology and permeability in piglets by reducing villus height, villus height to crypt depth ratio and paracellular barrier function. This response lasted for almost 2 wk before the intestines recovered morphologically and more than 2 wk before barrier function returned to the same as pre-weaning. It is possible that when cytokines, such as IL1β and IL6, are upregulated during weaning, it caused a decrease in tight junction proteins and therefore compromised barrier function. Bach et al. (2017) reported that no expression changes occurred in immune response genes (*IL1B, IL10, TNFA, DEFB1, TLR4*) in the colon of cows transitioned from a dry cow diet to a fresh cow diet. In the rumen, *TLR4* and *IL1B* had reduced expression when cows were on the fresh cow diet compared with the dry cow diet. This might indicate that the colon is not as responsive to diet changes as the rumen. Calves fed MR and CS had an upregulation of *TLR6* gene expression and downregulation of *TLR10* gene expression in the jejunum, ileum, cecum and colon compared to calves fed only MR (Malmuthuge et al., 2013). In the colon, calves fed MR and CS had higher expression of *TLR2* than the MR fed calves. In the jejunum and cecum, the expression of *TLR2* was lower compared with the ileum and colon in calves fed MR and CS. The expression of *TLR9* and *TLR10* was highest in the ileum compared with the jejunum, cecum and colon. It is clear that the expression of some TLR genes are gut and diet dependent. Colon tissue from lambs fed starter had decreased gene expression of *TLR4*, *TNF-a* and *IFN-g* (Liu et al., 2017). The TLR genes are part of the immunology barrier of the GIT. When lambs were weaned early, gene expression of *TLR1 TLR2, TLR3*, *CLDN1* and *4* increased on d 42 and on d 84, and *TLR2, TLR 4 TLR5* and occludin were increased in the ileum (Li et al., 2018). Age of the lambs also influenced gene expression, with *TLR1, TLR2* and occludin expression increasing with age, possibly indicating that weaning

has a longer lasting impact on barrier function in the ileum. The TLR recognize bacteria by-products and activate an innate immune response (Abreu, 2010); however, each TLR has its own distinct way of recognizing molecules. The following TLR binds to the specific molecules: *TLR1* to triacyl lipopeptides, *TLR2* to peptidoglycans and lipoproteins, *TLR3* to double-stranded RNA, *TLR4* to lipopolysaccharide and *TLR5* to flagellin (Li et al., 2018). The effect of weaning or the inclusion of starter in the diet might not be the only factors impacting the immune system of the lower gut, for as Hammon et al. (2018) reported, genes involved in the jejunum immune system were activated in calves fed a high plane of milk compared with calves fed a low plane of milk. The authors speculated that butyrate might have caused a direct or indirect effect on the immune response and this might have influenced glucose metabolism.

Based on the data available, expression of lower gut genes that are involved in the immune response are influenced during weaning or when solid feed is introduced. It is important to note that gene expression data only indicates what genes are present and not which genes are activated. There are a large number of studies investigating the effect of weaning on the rumen; however, the effect of weaning on the lower gut, especially the hindgut, remains scarce. The majority of the available data investigating the lower gut during weaning uses monogastrics (piglet and rat models), so it remains unclear how this data relates to the calf during weaning.

1.5. How liquid and solid feed impact dairy calf nutrition, development and health.

1.5.1. Liquid feed

Recently, there has been interest in feeding dairy calves more milk or MR pre-weaning than the conventional 10 % of birth BW due to observed advantages during the pre-weaning phase. These feeding strategies are considered intensified, elevated or high plane of milk nutrition because the amount of milk/MR fed is double (20 % of the calf's birth BW) that of conventional feeding. This translates to calves being fed >8 L milk/d or >0.88 kg of DM MR/d. Feeding higher amounts of milk/MR results in higher ADG (0.6 – 1.2 kg/d) during the pre-weaning period (Jasper and Weary, 2002; Hill et al., 2010; Miller-Cushon et al., 2013). However, post-weaning ADG did not differ between low or high plane of milk/MR nutrition (Jasper and Weary, 2002; Hill et al., 2010; Miller-Cushon et al., 2013), though the growth advantage obtained during the pre-weaning period could be maintained in some cases (Jasper and Weary, 2002; Khan et al., 2007a,b; Miller-Cushon et al., 2013), due to a smoother transition.

When calves are fed high planes of milk/MR nutrition pre-weaning, this negatively influences solid feed intake (Hill et al., 2016; Dennis et al., 2018a and 2019) because the milk/MR provides enough nutrients to sustain satiety and therefore calves do not feel the need to consume solid feed. Such decrease in solid feed intake negatively influences rumen development, since minimal solid feed intake leads to minimal microbial fermentation products (SCFA). Results reported on neutral detergent fibre (**NDF**) and acid detergent fibre (**ADF**) digestibility have been consistent with calves fed high planes of milk having lower NDF and ADF digestibility at wk 7 (Dennis et al., 2018a), wk 8 (Chapman et al., 2016), wk 11 and 16 (Hill et al., 2016). This may be indicative of impaired rumen development due to decreased CS intake pre-weaning, which explains the reduced feed efficiency reported during the weaning transition and thereafter (Hill et al., 2006).

Calves fed elevated planes of milk only for the first 4 wk of life, followed by a conventional plane of milk until wk 7, had heavier forestomach weights and increased ruminal wall thickness, papillae length, width and density at wk 9 compared to calves fed a conventional plane of milk (Khan et al., 2007a). Calves fed high amounts of milk had lighter reticulo-rumen and omasum (as % of BW) compared to calves fed lower amount of milk at d 38 and 56 (Kosiorowska et al., 2011). These calves also had lower atrium epithelial dry weight (g), but no difference was reported in papillae length or width. The SI length (m/kg empty BW) was shorter for calves that received the high milk allowance. They also reported that the number of villi and crypts (n/mm) and the ratio of goblet cells (area/villus area %) in the jejunum decreased when calves received more milk. Furthermore, no other differences were reported in the duodenum or ileum. Another study (Zhang et al., 2018) reported that the higher amount of milk fed to calves led to lighter reticulo-rumen and SI weights (as full weight and % of body weight) on d 60 (before weaning). Small intestine length was increased by low milk allowance, which possibly contributed to the increase in weight. The high milk allowance increased papillae length in the rumen anterior ventral blind sac and increased villi width in the jejunum, but reduced papillae length in the rumen posterior ventral sac. The difference in rumen papillae length might be due to different pH in these areas. In Kosiorowska et al. (201) and Zhang et al. (2018), the high milk allowance was only 6 L/d, which would not be considered elevated milk nutrition and may have influenced the reported responses.

It is believed that feeding more milk causes scours (Andrews et al., 2004). However, previous research has largely found no effect of feeding high amounts of milk/MR on the incidence of diarrhea and the length of diarrheic bouts (Jasper and Weary, 2002; Borderas et al., 2009; Rosenberger et al., 2017). McCorquodale et al. (2013) reported that there are lower risks of mortality for calves that are heavier (> 44 kg) during the first wk of life because more resources are available to fight disease (McCorquodale et al., 2013). This is not directly linked to the amount of milk calves are fed but supplying more nutrients by feeding higher amounts of milk or more nutrient dense milk will result in higher BW.

When raising heifer calves, the most important aspects to producers are efficiency and cost effectiveness over the non-productive. Simply stating the growth, behaviour and welfare benefits associated with feeding more milk to calves is sometimes not as important as the cost the producer puts in to supply the extra nutrients. Although the producers may not have a direct financial benefit from feeding more milk to calves during the pre-weaning period, there may be long-term financial benefits. Soberon and Van Amburgh (2013) reported that first lactation production increases by 155 kg for every extra 0.1 kg of pre-weaning ADG based on a meta-analysis conducted to evaluate the effects of milk/MR nutrient intake on ADG and milk production during first lactation. It is important to note that solid feed intake could also have contributed to the increase in ADG pre-weaning, but solid feed intake was not measured, so it is difficult to separate which contributed to the higher ADG. Another meta-analysis reported a similar response of 130 kg increase in production during first lactation for every extra 0.1 kg ADG during the pre-weaning period, however, the increase in milk production was only observed when ADG were higher than 0.5 kg/d (Gelsinger et al., 2016).

1.5.2. Solid feed

There is a tremendous amount of research investigating the optimal solid feed program for young calves. Solid feed differs in chemical composition, resulting in different end products from rumen microbial fermentation. The different microbial by-products have different influences on epithelial development, as discussed in section 1.4.1. The only consensus throughout the years is that solid feed that includes grain more readily enhances rumen development than if only hay is fed during the pre-weaning phase (Kertz et al., 2017). The

effects of CS form, starch content, forage inclusion and particle size on intake, performance, GIT development and digestibility will be discussed below.

Readily fermentable carbohydrates in grain are more nutrient dense compared to forages and allow for greater energy for growth in young calves. Corn is one of the ingredients most commonly found in CS. Calves consumed more CS that contained corn compared with barley, oats, or wheat pre- and post-weaning (Khan et al., 2007b). In addition, hay, NDF, CP and starch intake were also higher in calves fed corn-based CS. This resulted in higher performance (BW and body measurements) in these calves. Corn- and wheat-fed calves had higher ruminal total SCFA than oat- and barley-fed calves, however, corn-fed calves had higher ruminal pH compared with the other treatments (Khan et al., 2008). Empty rumen-reticulum, omasum and abomasum weights were reported for calves fed corn and wheat compared with barley and oats. Ruminal papillae length and width were greatest in corn fed calves, and papillae concentrations were greatest in both corn and wheat fed calves compared with barley and oat fed calves. There was no difference reported in this study for total tract nutrient (DM, CP, NDF and starch) apparent digestibility. Overall, these studies concluded that corn or wheat as the main ingredient in CS resulted in heavier calves with heavier forestomaches and more developed (longer, wider and denser) rumens.

Calf starter can come in many different kinds of physical forms, including texturized, pelleted and mashed. A few studies have investigated how the different physical form may impact intake and performance. Calves consumed more textured and pellet starters than mash starter, which resulted in higher ADG (Franklin et al., 2003; Hill et al., 2012; Nejad et al., 2012). It is important to note that when such comparisons are made, the different forms need to be equal in nutrient content and that the same ingredients in the same proportions need to be used in order to truly compare the different physical forms. Otherwise, it could potentially have confounding effects between treatments. Based on all these studies, it is evident that the physical form of CS does influence intake and performance, and that texturized or pelleted starter is preferred over a CS containing finer particles like a mash.

Alteration (processing) of grains (e.g., grinding, steam- or dry-rolling and steam flaking) in CS enhances surface area for degradation by fractioning the seed pericarp and damaging the starch-protein matrix (Huntington, 1997). This allows for improved ruminal fermentation rate,

passage rate and total-tract starch digestibility in mature animals. An abundance of studies (Owens et al., 1997; Firkins et al., 2001), have investigated the effects of processing grain (especially corn) on mature animals (dairy cows and feedlot animals mostly), however, there are far fewer studies investigating the effect of processing grain on dairy calves.

Processing of grain in CS may influence calf performance and rumen fermentation. Coverdale et al. (2004) reported that although intake was similar between treatments, calves fed a texturized starter containing whole oats had increased ADG and feed efficiency (kg gain /kg DM) compared with calves fed a texturized starter containing ground oats post-weaning. Bateman II et al. (2009) reported that corn processing in CS did not have an effect on CS intake, BW, ADG, body measurements, and feed efficiency in calves during the pre- and post-weaning stage. However, Lesmeister and Heinrichs (2004) reported that during the post-weaning stage (wk 5 and 6), DMI was greater when calves were fed whole corn compared with steam flaked corn, resulting in higher total CS intake. Consequently, ADG tended to be higher post-weaning in calves fed whole corn compared with the steam flaked corn, but did not result in different final BW (wk 6). Additionally, Terré et al. (2016) reported that CS intake increased when whole grains (corn and barley) were fed to calves compared to steam rolled grains. Such increase in CS intake during the pre-weaning phase did not result in performance or efficiency changes. All three of these studies (Lesmeister and Heinrichs, 2004; Bateman II et al., 2009; Terré et al., 2016) investigated the effects of processing grain on calves while feeding a low plane of MR (<6 L); thus, the effects of this combination when high planes of milk/MR are fed is unknown. By investigating the effects of processing grain in CS on rumen fermentation characteristics, Lesmeister and Heinrichs (2004) reported that dairy calf total rumen SCFA, acetate and propionate concentrations were greater in calves fed the steam flaked compared with the whole corn diet. They also report that butyrate concentration tended to increase during wk 6 for calves fed whole corn compared with steam flaked corn. Coverdale et al. (2004) reported that total ruminal SCFA concentrations were greater in calves fed ground oats starter compared with whole oat starter. Total ruminal SCFA, acetate, propionate and butyrate concentrations were unaffected when calves were fed either whole corn or steam rolled corn (Terré et al., 2016). In lactating cows, Crocker et al. (1998) also reported that steam flaking increased ruminal propionate. However, Murphy et al. (1994) reported that ruminal propionate concentrations increase when whole corn was fed compared with rolled corn. Decreased ruminal pH has been

reported when dry-rolled corn or steamed rolled corn were fed to calves instead of whole corn (Lesmeister and Heinrichs, 2004; Terré et al., 2016). Similarly, when calves were fed diets containing either ground corn and barley or rolled corn and barley, ruminal pH was decreased when corn was more processed (ground; Rezapour et al., 2016). Overall, the effect of processing grain on ruminal SCFA is variable between calf studies (Lesmeister and Heinrichs, 2004), as well as in mature ruminant studies (Murphy et al., 1994; Crocker et al., 1998). Furthermore, processed grains can decrease ruminal pH compared with whole grains (Owens et al., 1997; Firkins et al., 2001).

Readily fermentable carbohydrates supplied in CS are important in promoting rumen developments described in section 1.4.1 (Flatt et al., 1958; Sander et al., 1959). However, the optimum amount of readily fermentable carbohydrates in CS is unknown. The starch content in CS is not always known as it is not required to be on commercial feed labels and therefore is not reported in many studies unless the amount of starch is specifically being investigated. Additionally, when weaning method or physical form of diet are investigated, it is hard to compare studies if the starch content is not known. Common non-forage fibre added to the diet to decrease the starch content include wheat middlings, soybean hulls, distillers dried grains and beet pulp. Multiple studies (Hill et al., 2008; Dennis et al., 2018b) have investigated various amounts of starch in CS and its effect on performance and intake. The relationship between CS starch content and growth performance were examined using 6 studies containing 18 different dietary treatments with 372 calves from 0 to 8 wk and 26 different dietary treatments with 660 calves from 8 to 16 wk (Hu et al., 2018). The starch content of the diets ranged from 10.1 to 53.3 %. The authors reported that as starch in CS increased, calf intake, ADG and hip width change increased linearly during the first 8 wk. Furthermore, during wk 8 to 16, ADG, hip width change, and feed efficiency (ADG/DM intake) increased linearly with increased starch content in the diet. However, DM intake and DM intake/body weight were unaffected. Additionally, DM and CP digestibility were greater with increased starch content in the diet. They predicted that ADG and hip width change may increase by 5.8 and 5.0 %, respectively, for the first 8 wk and by 9.6 and 11.2 %, respectively for wk 8 to 16 when diet starch content increased from 23 to 43 % (DM basis). These effects on calf intake and performance prove the importance of starch content in CS. In the mixed-model analysis study, some of the studies included had different physical form of diet between the low starch diet and the high starch diet. Generally, the low starch diet

consisted of pellet whereas the high starch diet consisted of a texturized diet. This introduces bias because, as described previously, physical form of diet may influence the observed effects on calf intake, performance and digestibility.

A concern with feeding calves high starch diets is that rapid rumen fermentation will occur, resulting in reduced ruminal pH (Kristensen et al., 2007). This low pH can lead to ruminal acidosis (described in section 1.2.1.4) and therefore forage is provided in addition to CS to increase ruminal pH (Laarman et al., 2012b; Kim et al., 2016b). However, there is a concern that the forage will dilute the energy content of the diet and affect rumen development, and thus calf performance. Khan et al (2011) reported that the inclusion of forage (chopped orchard grass-hay) increased total DMI when calves were fed an elevated plane of milk nutrition; however, ADG was not affected (Khan et al., 2011). On the other hand, Terre et al. (2015) reported no difference in DMI or growth when chopped rye grass-straw was included in texturized or pelleted CS pre- and post-weaning when calves were fed an elevated plane of milk nutrition (Terré et al., 2015). Increased rumen empty weight was reported when calves consumed forage in combination with CS (Khan et al., 2011). Ruminal pH was higher for the calves fed forage in combination with CS (Khan et al., 2011; Terré et al., 2015), which may have resulted from higher saliva production due to more chewing. In a review about the effect of forage provision on feed intake in dairy calves, the authors reported that the need for forage provision in calves can be affected by age, stage of life (pre- or post-weaning) and the nutrient composition and physical form of both the CS and the forage (Suarez-Mena et al., 2016). Briefly, calves may benefit from forage provision when a pelleted diet that is readily fermented in the rumen is provided. This will allow for rumen buffering through saliva, which will decrease the risk of acidosis and increase DMI. On the other hand, when forage is provided with a texturized CS, the risk of low pH is reduced due to the slower ruminal starch fermentation. Inasmuch, the forage may inhibit CS intake due to rumen fill. Smaller amounts of chopped forage provided may lead to increased DMI over longer piece forage. Overall, there is no definite conclusion on the inclusion of forage in the calf diet.

In this section, the starch content, processing of grain and physical form of CS and the inclusion of forage in a calf diet were discussed. There are large amounts of studies investigating these effects to improve calf performance and health. However, this is not a one size fits all situation, because the liquid feed (amount of milk, milk composition), age and weaning method

can all contribute to the different outcomes. Therefore, it is important to keep researching different nutritional ways to improve calf performance and health.

1.5.3. Liquid to solid feed transition

Weaning represents a period of dramatic structural and metabolic adaptations in a calf's life as noted in section 1.4 (Baldwin et al., 2004). Briefly, during pre-weaning the GIT functions similar to that of a monogastric GIT. The calf relies on milk as the primary nutrient source that is digested in the abomasum and the SI, with glucose being the primary energy source. As the calf starts to consume solid feed, rumen development begins, and fermentation occurs. Post-weaning, the rumen will be the main site of fermentation and SCFA (fermentation product) will account for 80 % of the calf's energy sources (Bergman, 1990). With the recent interest in feeding higher amounts of milk/MR (section 1.5.1) to dairy calves, it became evident that the traditional weaning system (early and abrupt) needs improvement.

1.5.3.1. Method

With offering high planes of milk/MR during the first wk of life, one can take advantage of the fact that calves consume minimal concentrate during this period and mostly rely on milk/MR for their nutrients. Warner et al. (1956) reported that concentrate consumption increased during wk 4, though not by a large amount until the milk offered was decreased (Hill et al., 2012; Eckert et al., 2015). This is the critical period in which we need to start thinking about rumen development, especially when feeding high planes of milk/MR that will reduce/delay solid feed intake.

One-step weaning is better for calves than abrupt weaning. A study by Steele et al. (2017) reported that a one-step (decrease by 50 %) weaning protocol provided energy balance benefits compared to an abrupt weaning protocol when calves were fed high planes of MR (1.35 kg /d). The authors reported higher CS intake and ADG and lower fecal starch post-weaning (d 54) for calves on the one-step protocol compared with abruptly weaned calves. However, gut compartment and rumen morphology measurements did not differ between weaning method and higher fecal starch in abrupt weaned calves indicated that rumen fermentation was impaired, leading to higher amounts of starch reaching the hindgut. These higher amounts of starch reaching the hindgut could lead to hindgut acidosis.

Researchers have combined the high plane of milk nutrition followed by low plane of milk to ease the weaning transition of calves. Khan et al. (2007a,b) and Schäff et al. (2016, 2018) used a feeding scheme to take advantage of feeding high planes of milk during the first wk of life and then tried to ease the weaning transition by feeding low planes of milk for the remaining wk until weaning. Khan et al. (2007a,b) conducted two studies investigating how adding a step-down process to calves fed high planes of MR would impact the calves. In general, the STEP calves were fed high plane of MR (20 % of BW) for approximately 24 d then gradually reduced (STEP) over 5 d so that the calves received a low plane of MR (10 % of BW). The calves were fed this amount until approximately d 44, then gradually weaned over 5 d. The conventional calves received 10 % of BW of MR and were weaned at the same time (d 45-50). The authors reported that the STEP calves had higher BW gain and solid feed intake post-weaning than conventional fed calves (Khan et al., 2007a,b). The STEP calves also had higher total ruminal SCFA concentrations, heavier forestomach weights and increased rumen papillae lengths, widths and densities post-weaning compared with conventional calves (Khan et al., 2007a). In Schäff et al. (2016, 2018), STEP calves were fed MR ad libitum for 5 wk, then gradually reduced (wk 6 and 7) to restricted (6 L/d), where they were kept until d 60. The restricted calves were fed MR at 6 L/d until d 60. In the first paper, the authors investigated the growth, metabolic adaptation, health, and immune status (Schäff et al., 2016) of calves. Carcass weight, perirenal fat and muscle mass were greater in the STEP calves compared with the restricted calves, which demonstrates that the increased milk fed to calves, even for a short period, can have growth benefits. An increase in blood insulin-like growth factor (IGF)-1 and a slight increase in grain intake in the STEP calves at d 60 compared with restricted calves indicates that these calves had improved anabolic metabolism. However, treatment did not impact immune or health status in these calves. In the second paper, the authors investigated rumen and SI growth (Schäff et al., 2018) and reported that treatment did not impact forestomach or SI weight. There was also no difference in ruminal papillae length, width, and surface in the atrium, ventral, and ventral blind sac. This might be because there was only a slight increase in CS intake during wk 9. Papillae density was higher for the restricted fed calves in the atrium and ventral sac compared to the STEP calves. There was also no treatment effect on SI epithelium measurements. The Mid-jejunum villi circumference, distal jejunum villus surface area and the villus height/crypt depth ratio of the distal jejunum were greater in the STEP calves compared with the restricted calves.

However, the distal jejunum crypt depth was smaller in the STEP calves compared to the restricted calves. It is important to note that this study only followed the calves during pre-weaning and weaning. The findings from these studies indicate that by feeding an elevated plane of nutrition with a step-down protocol after 4 or 5 wk of life, it is possible to promote similar CS intake to calves on restricted protocols, resulting in no negative impact on GIT development/growth and greater growth performance.

A few authors have considered different gradual weaning methods (Sweeney et al., 2010; Dennis et al., 2018a; Welboren et al., 2019) to ease the transition from liquid feed to solid feed intake. A study compared an abrupt weaning strategy and three gradual weaning strategies with one another (Sweeney et al., 2010); all calves were fed 12 L/d until their respective weaning method. The abrupt calves were abruptly weaned on d 41, and the three gradually weaned groups were weaned gradually over 4, 10 or 22 d by decreasing the amount of milk fed each d (3, 1.2 and 0.55 L/d respectively). The calves in the gradually weaned groups were also fully weaned on d 41. The results show that gradual weaning over 10 and 22 d increased CS intake and BW gains post-weaning. The best weaning strategy from this study was gradual weaning over 10 d because it allowed calves to gain weight pre-weaning from milk and promote CS intake during the weaning process, easing the transition and allowing for greater CS intake and BW gain after weaning. Dennis et al. (2018a) evaluated the effect of weaning method (one-step vs gradual) on performance and digestion. In this study, one group of calves was subjected to a step-down (STEP; 50 % of previously offered) at wk 8 and the other group was gradually weaned during wk 7 and 8. The STEP calves had lower OM and ADF digestibility during wk 12 compared with the gradually weaned calves, but during wk 16, digestibility of nutrients was similar among treatments and there was no effect on performance. Welboren et al. (2019) compared step-down (3 steps with fixed volumes), linear (1 step and then milk was slowly reduced daily) and dynamic (3 steps, 75%, 50% and 25% based on actual consumption) weaning strategies. The authors reported that the weaning strategy used should be as gradual as possible because higher growth rates can be obtained post-weaning.

Another strategy is to wean calves based on solid feed consumption, which is similar to what would happen when the calf is reared with the dam. Roth et al. (2008) reported that calves weaned based on solid feed consumption had higher growth rates than calves weaned conventionally. Another study also reported that calves weaned based on solid feed consumption

were weaned earlier (d 76) compared with conventional calves (d 84; Roth et al., 2009). In this study, there were largely no differences in weight gain and rumen papillae length (1 area measured had a tendency out of 8) between treatments. A recent study used the combination of step-down and weaning by solid feed consumption to investigate impacts on performance (Benetton et al., 2019). The STEP calves were fed 12 L/d for 30 d, then gradually reduced (STEP) over 5 d to 6 L/d, fed at this rate until d 63, and then gradually reduced again until d 70. The combination group (STEP and solid feed consumption calves) were fed 12 L/d for 30 d then reduced to 75 % of previous intake. For the remainder of the time, the milk allocated to calves was reduced by 25 % when the following solid feed intake targets were met: 225, 675, and 1,300 g/d. Six out of 16 calves failed to reach the final solid feed intake target by d 63 and were weaned gradually over 7 d. Calves on the STEP method consumed more milk and less CS than calves in the combination system, resulting in similar BW at the end. This study also indicates that some calves are less capable of transitioning to solid feed, even over 9 wk. The Roth et al. (2008, 2009) studies only fed 6 L/d and the conventional calves were weaned gradually from wk 8 to 12 (Roth et al., 2008, 2009). Traditionally, calves fed low planes of milk were weaned around 5 wk of age to limit feed cost leading to increase solid feed intake, which could have affected rumen development. Therefore, the delay in weaning could have masked growth and rumen development differences between treatments. This strategy is based on the calf's individual needs, allowing each calf to transition from liquid to solid feed at their own pace.

In review, the goal is to have a smooth transition that supports calf health and efficient costs for farmers. Yet, no such strategy has been found because there are multiple factors that can have an influence, such as age, which will be discussed in the next section. In summary, calves do transition better when weaning is more gradual. However, this is not always feasible on big farms where calves are individually housed and fed by bucket or bottle. Thus, further research about how to increase starter intake pre-weaning to facilitate weaning is required when calves are fed high planes of milk nutrition.

1.5.3.2. Age

In order to decrease feed cost, the dairy industry traditionally weaned calves early (4-6 wk of life (Kertz and Loften, 2013). Now that we have almost doubled the amount of milk/MR offered to calves, we can no longer successfully wean calves early because the rumen is

underdeveloped due to limited starter intake and therefore, calves struggle during weaning and post-weaning (Cowles et al., 2006). This section will discuss how the age at weaning influences calf growth, development and health.

When calves are offered high amounts of milk, delaying weaning from 47 to 89 d of age increases the time those calves would have growth advantages because the reduction in weight gain during weaning is less due to minimal impact on digestible energy (DE) intake during weaning (de Passillé et al., 2011). The later weaned calves also showed fewer signs of hunger after weaning. Eckert et al. (2015) investigated the effect of weaning calves 2 wk apart (wk 6 vs. 8) and reported that calves weaned later tended to have an 11 kg growth advantage compared with early weaned calves (wk 22). Fecal starch one wk post-weaning was also higher in calves weaned early, indicating that GIT function may be impaired and unable to utilize the drastic increase in starch. Another study compared weaning at wk 8, 10 and 12 and reported that ADG still decreased at weaning compared to the ADG calves had the wk before weaning (Meale et al., 2015). Yet, ADG in the later weaned calves did not decrease as much during the weaning wk, compared with the early weaned calves. Dennis et al. (2018a) also evaluated the effect of weaning age (wk 6 vs. 8) on performance and digestion. Starter intake was higher during wk 6 to 8 for calves weaned early (wk 6), leading to an increase in CS intake during the first 8 wk of life. The early weaned calves were lighter during wk 7 to 12, probably because during wk 7 and 8, late weaned (wk 8) calves were still consuming milk, which is highly digestible, and the early weaned calves did not consume enough energy from CS to compensate for the loss of energy from MR. The calves weaned early had higher ADF digestibility during wk 12 and lower BCS at wk 8 compared with late weaned calves. Overall, by wk 16 weaning age had no effect on performance or digestibility.

Later on, de Passillé et al. (2016) conducted a similar study, investigating the effects of delaying weaning from d 47 to 89. In this study though, they also included a group that was weaned based on CS consumption. This group of calves were offered less milk when starter intake reached goals of 200, 600 and 1,000 g/d. Once the calves consumed 1,400 g/d of CS, the calves were weaned. The authors reported that by using the CS weaning strategy, the calves were weaned earlier than the late weaned group without compromising growth during the weaning process. In the second experiment of Benetton et al. (2019), they only used the combination weaning strategy (see section 1.5.3.1), but calves were allowed to meet the target solid feed

intake by d 84. Thirty-one calves were weaned before d 63 (early) and 12 calves were weaned on d 63 or later (late). The calves that were weaned late consumed more milk and less CS, which resulted in reduced ADG and final BW. This indicates that some calves transition onto solid feed much slower than other calves and weaning calves on an individual basis would allow for sufficient transitioning.

In conclusion, preparing calves for weaning by extending the age of weaning and utilizing the step-down protocol method allows for a smoother weaning transition. However, this is not always practical on farm, nor economically feasible for farmers. Therefore, more research is required to determine the interaction between weaning age and weaning method, especially for developing protocols that reduce the growth slump during weaning.

1.6. Knowledge gaps, hypotheses and objectives

There is plenty of research on calf performance and ruminal characteristics when calves are fed low planes of milk, as outlined in the review above. Several knowledge gaps were identified in the review above that still require further investigation, however this thesis only address four knowledge gaps. Firstly, there are multiple studies that report the short- (Jasper and Weary, 2002; Khan et al., 2011; Rosenberger et al., 2017) and long-term (Soberon et al., 2012; Soberon and Van Amburgh, 2013; Gelsinger et al., 2016) benefits of feeding calves high MR feeding rates however, there are also multiple studies that have reported reduced CS intake pre-weaning (Hill et al., 2016; Dennis et al., 2018, 2019) which impede rumen development (Khan et al., 2016) and thereby place the calf at a disadvantage shortly post-weaning because it can not utilize the nutrients as efficiently (Hill et al., 2016b; Dennis et al., 2018) as calves that were fed low MR feeding rate. When taking into account that MR feeding rate negatively influences CS intake, there is the possibility the calves fed high MR feeding rate have rumens that are not equipped to handle the sudden increase in CS intake during weaning which will lead to increased starch reaching the hindgut or be excreted in the feces (Eckert et al., 2015; Steele et al., 2017). More so, increased hindgut fermentation may also occur when calves are fed a texturized CS containing whole corn, which may bypass the rumen and be fermented in the hindgut (Svihus et al., 2005). Feeding calves high MR feeding rates may become more popular because of the long-term benefits. Studies have reported that CS containing corn lead to increased CS consumption and BW compared with barley, oats, or wheat (Khan et al., 2007b) therefore, CS manufacturing

companies supply texturized starter containing either flaked corn or whole corn. Thus, it is important to investigate the combination of MR feeding rates and corn processing in CS because feeding high MR feeding rates in combination with texturized CS containing whole corn may lead to excessive amounts of starch that reach the hindgut that may potentially reduce calf health by evoking an inflammatory response.

The second knowledge gap that will be addressed in this thesis is monitoring ruminal pH from pre- to post-weaning (long term). Multiple studies report that ruminal pH is influenced by diet or weaning in calves and that calves experience SARA during weaning (Laarman et al., 2012b; Kim et al 2016a,b, Li et al., 2019). However, far fewer studies have investigated the effect of weaning on ruminal pH using continuous measurements. Moreover, these changes have not been investigated long-term: at least 3 wk post-weaning. The effects of reduced ruminal pH during weaning on calf health have also not been investigated. Therefore, the third knowledge gap addressed in this thesis is whether calf health, measured by acute phase proteins, is influenced by ruminal acidosis. It is important to address these knowledge gaps because SARA is of big concern in mature ruminants because it effects animal health, performance and economic return (Abdela, 2016).. Ultimately, if calf health is compromised by the reduced ruminal pH during weaning, we may have to investigate different mitigation feeding strategies.

The fourth knowledge gap is the effects of weaning on the hindgut, especially the colon. The colon is a part of the GIT that is not well understood in young or weaned calves. It is possible that the colon undergoes changes during weaning in order to adapt to increased nutrients, like starch, reaching the hindgut. If increased hindgut fermentation occurs during weaning there is a possibility of barrier dysfunction and inflammation that may occur in the colon tissue that may impact animal health, thus by addressing this knowledge gap, we would gain knowledge on how the colon is influenced during weaning.

It is hypothesized that: 1) feeding dairy calves at a high MR feeding rate pre-weaning and whole corn in CS will result in increased hindgut fermentation post-weaning, leading to increased fecal free LPS which will activate an inflammatory response; 2) that calves will experience reduced ruminal pH during weaning, and as such, will experience an acute inflammatory response; and 3) gene expression profile changes in the colon, from pre- to post-weaning, will occur in calves. Therefore, the objectives of this thesis were: 1) to determine how milk replacer feeding rate (low

vs. high) and processing of corn (whole vs. flaked) alter performance, nutrient digestibility, rumen and fecal fibrolytic bacteria, rumen and hindgut fermentation and inflammatory biomarkers of dairy calves; 2) to develop a methodology for biopsy of the rumen and colon of young dairy calves and to determine whether the samples are suitable for microscopic and gene expression analysis; and 3) to characterize ruminal structural development, fermentation characteristics, colon transcriptome changes and acute phase proteins from pre- to post-weaning in calves.

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2. Impact of amount of milk replacer fed and the processing of corn in starter on growth performance, nutrient digestibility and rumen and fecal fibrolytic bacteria of dairy calves

2.1. Introduction

Recently, there has been interest in feeding dairy calves a higher volume of whole milk or milk replacer (**MR**) pre-weaning than the conventional rate of 10 % of birth BW due to observed advantages during the pre-weaning phase, including improved weight gain (Jasper and Weary, 2002; Khan et al., 2011a; Rosenberger et al., 2017) and improved feed efficiency (Khan et al., 2007b; Moallem et al., 2010; Davis-Rincker et al., 2011). In addition, there is potential for heifer calves with increased pre-weaning ADG to have increased milk production later in life (Soberon et al., 2012; Soberon and Van Amburgh, 2013; Gelsinger et al., 2016). However, negative results of such feeding regimens have also been reported, such as poor performance (Cowles et al., 2006) and reduced feed efficiency during the weaning transition (liquid to solid feed) and thereafter (Hill et al., 2006). This is likely because pre-weaning starter intake is negatively influenced by a high MR feeding rate (Jasper and Weary, 2002; Jensen, 2006; Kristensen et al., 2007; Hill et al., 2009), resulting in diminished intake of calf starter (**CS**; Hill et al., 2006), leading to a delay in rumen development (Khan et al., 2016) that causes a reduction in nutrient digestibility - especially fibre - post-weaning (Terré et al., 2007; Hill et al., 2010, 2016b). Inadequate rumen microbial fermentation caused by decreased CS intake when calves are fed higher volumes of milk or MR may result in a reduction in fibre digestion; however, the exact cause by which fibre digestion is decreased when calves are fed higher volumes of milk or MR is unknown. Therefore, further investigation regarding fibrolytic bacterial communities present in the rumen and feces is warranted.

The importance of supplying readily fermentable carbohydrates in CS is well-established for its role in butyrate and propionate production in the rumen (Flatt et al., 1958; Sander et al., 1959). Corn is one of the ingredients most commonly used in CS and substituting corn with barley, oats, or wheat in CS resulted in reduced solid feed intake and weight gain in dairy calves up to 12 wk old (Khan et al., 2007a). Alteration (processing) of grains (e.g., grinding, steam- or dry-rolling and steam-flaking) enhances surface area for degradation, which leads to improved ruminal fermentation rate, passage rate and total-tract starch digestibility in mature animals

(Huntington, 1997). However, CS intake was increased when whole grains (corn and barley) were fed to calves compared to steam rolled grains (Bateman et al., 2009; Terré et al., 2016). To date, most studies (Owens, et al., 1997; Firkins et al., 2001) have investigated effects of processing corn on mature animals (mainly dairy cows and feedlot animals) and research regarding the effect of feeding processed corn to dairy calves in CS is limited.

The objective of this study was to determine how amount of MR and processing of corn in CS can affect performance, nutrient digestibility and the fibrolytic bacterial community in dairy calves. We hypothesized that feeding an elevated amount of MR with CS with whole corn would reduce nutrient digestion, resulting in poor performance.

2.2. Materials and methods

2.2.1. Animals, housing and environment

A total of 48 Holstein bull calves (± 3 d of age) were obtained from a single farm. Calves received 2 L of fresh colostrum within one hour of birth followed by 2 additional feedings at 6 and 12 h. Thereafter, calves were fed 2 L of pasteurized whole milk twice daily until transported (3.5 h) to the Nurture Research Center (New Paris, OH). In this study, calves were cared for and handled in accordance with the Canadian Council on Animal Care (CCAC, 2009) regulations, Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010) and the institutional Animal Care and Use Committee (University of Alberta, AB; AUP00002303).

This study consisted of two phases. In the first phase (nursery; wk 1 to 8), calves were housed in a barn with side curtains and natural ventilation in individual pens (1.2 m × 2.4 m) bedded with wheat straw and given access to the experimental textured CS and fresh water *ad libitum*. Individual animal was the experimental unit. This phase consisted of two periods, pre- and post-weaning at wk 1-6 and wk 7-8, respectively. In phase two (grower; wk 9 to 16), calves were randomly assigned within nursery treatment to group pens (n = 4 calves/pen) that provided 6.5 m^2 per calf outside with inside shelter (1.35 m^2 space per calf) bedded with wheat straw. Pen (n = 3/treatment) was experimental unit during the grower phase. During this phase, calves had access to a TMR consisting of CS and chopped grass hay (Table 2.7-1) in a 95:5 on an as-fed ratio. Calves were dehorned and castrated at d 36. Average temperature was 24ºC (range from 12 – 38ºC) and 19ºC (range from 2 – 33ºC) for the nursery and grower phases, respectively.

Average relative humidity was 79 % (range from 24 to 100 %) and 81 % (range from 24 to 100 %) for the nursery and grower phases, respectively.

2.2.2. Experimental design and dietary treatments

The experiment was a completely randomized design with a 2 × 2 factorial arrangement of 0.749 kg of MR powder/d (**LO**) or up to 1.498 kg of MR powder/d (**HI**), and whole (**WC**) or flaked **(FC**) corn in a textured CS (37 % corn, 35 % supplement pellet, 25 % whole oats, and 3 % molasses; Table 2.7-1). Milk replacer (Table 2.7-1) contained 25.2 % CP, 17.9 % fat on a DM basis and was reconstituted to 14 % solids. Corn for whole and flaked corn treatments was obtained from the same batch of corn to ensure similar composition between starch sources. Calves fed LO (n = 24) received 0.749 kg/d (DM basis) of MR for 6 wk, followed by 0.375 kg/d of MR for the last wk. Calves fed HI received 0.749 kg/d of MR during wk 1, 0.851 kg/d during wk 2, 1.135 kg/d during wk 3, 1.498 kg/d during wk 4 and 5 and then 0.749 kg/d of MR for the last wk. Amounts of MR fed to calves were divided into two equal feedings (0700 and 1530 h) until wk 6, when MR was only fed once (AM). All calves were weaned at the end of wk 6.

2.2.3. Data and sample collection

Intake and Growth Performance. During the pre- and post-weaning period, individual MR and CS intakes were recorded daily and during the grower phase group pen intakes were recorded daily. Samples of MR and CS were taken from every second bag, whereas samples of hay were taken from every bale. Samples were composited by type and stored (4°C) prior to chemical analysis. Individual BW was measured wkly (wk 1 to 8) pre- and post-weaning and at wk 10, 12 and 16 during the grower phase. Body condition score (**BCS**), adapted from Wildman et al. (1982), and hip width (**HW**) were measured every 2 wk. Hip height, heart girth and paunch girth were measured at wk 0, 8 and 16. Efficiency was estimated by calculating total metabolizable energy (**ME**) intake divided by BW gain.

Apparent Total-Tract Nutrient Digestibility. Fecal samples were collected over 5 d, as described previously (Hill et al., 2016b) to estimate apparent total tract digestibility. During wk 5 and 8, six calves from each treatment (total n = 24) were randomly selected for fecal sample collection. Samples were pooled by calf (5 d) by timepoint. During wk 11 to 12, fecal samples were collected from the pen and were pooled (5 d) by pen. All fecal samples were stored at -20˚C prior to chemical analysis.

Fibrolytic bacteria in rumen and feces. Ruminal fluid was sampled 4 h after AM feeding from the subset (n = 24) of calves at wk 5 and 8 using a Geishauser probe (adapted for calves; Geishauser, 1993) fitted to a 60 ml syringe. Ruminal contents were squeezed through 4 layers of cheesecloth and 8 mL of ruminal fluid was snap frozen (dry ice and ethanol mixture). Spot fecal samples were collected 3 h after the AM feeding from the same subset (n = 24) of calves at wk 5 and 8 and snap frozen. Both samples (rumen and fecal) were stored at -20˚C before the quantification of the fibrolytic bacterial community.

2.2.4. Sample analyses

Feed and Fecal Composition. Milk replacer, CS, hay and fecal samples were analyzed (AOAC International, 2000) for DM (oven method 930.15), ash (oven method 942.05), CP (Kjeldahl method 988.05), fat (MR only; alkaline treatment with Roese-Gottlieb method 932.06) and acid-insoluble ash (Van Keulen and Young, 1977). Calf starter, hay and fecal samples were analyzed for ADF (Robertson and Van Soest, 1981), NDF (Van Soest et al., 1991) without sodium sulfite or α-amylase, ether extract (**EE**; method 2003.05), sugar (colormetric method; Dubois et al., 1956) and starch (α-amylase method; Hall, 2009) Acid-insoluble ash measured in MR, CS, hay and fecal samples was used as an internal digestibility marker (Van Keulen and Young, 1977). The ratio of acid-insoluble ash and nutrients in the feed and fecal matter were used to estimate apparent total tract nutrient digestibility.

Quantification of fibrolytic bacteria in rumen and feces. DNA was extracted from rumen fluid and fecal samples using repeated bead beating plus column method (Yu and Morrison, 2004). Briefly, samples were treated with cell lysing buffer (containing 4 % SDS) followed by physical disruption (2,160 x *g* for 3 min using Biospec Mini Beads Beater 16, BioSpec, Bartlesville, OK). Then, DNA was isolated using 10 M ammonium acetate and precipitated using isopropanol followed by further purification using a QIAmp fast DNA stool mini kit (Qiagen Inc., Germantown, MD). Quantity and purity of the DNA were evaluated using NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and DNA was stored at −20°C until further analysis.

Real-Time PCR was used to estimate 16S rRNA gene copy numbers of total bacteria, cellulose digesters: *Ruminococcus albus* (Zhou et al., 2015), *Faecalibacterium prausnitzii* (Kopečný et al., 2004), *Fibrobacter succinogenes S85* (Zhou et al., 2015); hemicellulose digesters: *Butyrivibrio fibrisolvens* (Zhou et al., 2015), *Ruminococcus flavefaciens* (Zhou et al.,

2015), *Prevotella sp.* (Zhou et al., 2015); and the fibre digester *Clostridium cluster IV* (Zeng et al., 2015). Primers (Table 2) specific to each bacteria and SYBR Green chemistry (Fast SYBR Green Master Mix, Applied Biosystems, Foster City, CA) were used for PCR. Bacterial populations were estimated using high throughput Viia 7 Real-Time PCR System (Thermo-Fisher Scientific). Purified 16S rRNA genes of *Butyrivibrio hungatei, Ruminococcus albus 7, Clostridium cluster IV, Butyrivibrio fibrisolvens*, *Faecalibacterium prausnitzii A2-165*, *Ruminococcus flavefaciens* (ATCC 49949), *Prevotella ruminicola* (ATCC 19189) and *Fibrobacter succinogenes* (ATCC 19189) were used as standards for each bacterial community, respectively.

The copy number of 16S rRNA genes per unit of sample for total bacteria and other communities was calculated using the equation as described by Li et al. (2009):

$$\text{copy number} = \frac{\left[\frac{\text{copy number obtained} \times \text{total DNA amount per unit of sample (ng)}}{\text{20 ng (amount of template used for real- time reaction)}}\right]}{\text{amount of sample used (g)}}$$

Proportion (% of total bacteria) of each community was also calculated:

$$\text{proportion} = \frac{\text{copy number of a community}}{\text{total bacterial copy number per unit of sample}} \times 100$$

2.2.5. Statistical analyses

Data were analyzed as a completely randomized 2 × 2 factorial design using the mixed model procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). The statistical model used was: $Y = \mu + MR + CS + (MR \times CS) + e$, where Y is the dependent variable, μ is the mean, MR is the effect of MR feeding rate, CS is the effect of corn processing, MR × CS is the interaction of MR and corn processing in CS, and e is the error term. Data collected over time were summarized by wk and repeated measures were used. Tukey test was used to separate the least squares means. At least 3 variance–covariance structures (auto-regressive type 1, Toeplitz and compound symmetry) were tested and the covariance structure that minimized the Schwarz's Bayesian information criterion was chosen. Significance was declared at $P < 0.05$ and tendencies at $0.05 \leq P < 0.10$.

2.3. Results

Intake, Growth Performance and Efficiency. Milk replacer intake was affected by wk × treatment interaction (Figure 2.7-1A; $P < 0.0001$) due to the step-up protocol followed by calves

on the HI diet. Calf starter intake, BW and ADG were influenced by wk (Figure 2.7-1B, C and D; $P < 0.0001$). Calves fed HI had greater (Figure 2.7-1A; $P < 0.001$) MR intake at wk 2 to 6 compared with calves fed LO which led to a 48.6 % increase in total MR intake (Table 2.7-3; $P < 0.0001$) for calves fed HI compared to calves fed LO. Calves fed HI had 3.6 kg greater MR refusals than calves fed LO. Calf starter intake was greater at wk 4 to 7 (Figure 2.7-1B; $P \leq 0.0005$) and had a tendency ($P \leq 0.10$) to be greater at wk 8 in calves fed LO vs. HI. Total CS intake was higher (Table 2.7-3; $P \leq 0.0083$) during the pre- and post-weaning period for calves fed LO vs. HI, which resulted in total CS intake for the overall period being 33 % greater ($P = 0.001$) for calves fed LO vs. HI. Calves fed HI were heavier from wk 3 to 8 compared to calves fed LO (Figure 2.7-1C; $P \leq 0.022$). Calves fed HI had greater ADG at wk 2, 3, 4 and 5 (Figure 2.7-1D; $P = 0.01$), but lower ADG at wk 7 (the wk after weaning) compared to calves fed LO. Body weight gain during the pre-weaning period was higher (Table 2.7-3; $P < 0.0001$) and lower ($P = 0.0008$) during the post-weaning period for calves fed HI compared with LO. Overall BW gain for the pre- and post-weaning period were higher ($P = 0.01$) in calves fed HI compared with LO. Other initial and wk 8 measurements (BW, hip height and width and heart and paunch girth) were similar among dietary treatments. Pre- and post-weaning and overall (pre- and post-weaning period) energy efficiency did not differ among dietary treatments. Milk replacer and CS intake, BW and ADG were unaffected by corn processing in CS (Figure 2.7-1). This resulted in no change in total MR and CS intake and performance measurements during the pre- and post-weaning and overall (pre- and post-weaning) period (Table 2.7-3). There was also no effect of the combination of MR feeding rate and corn processing on intake, performance and efficiency during the pre- and post-weaning and overall (pre- and post-weaning period) period (Figure 2.7-1 and Table 2.7-3).

Total DM intake was unaffected by dietary treatment during the grower phase (Table2.7-4). Final BW and other body measurements were unaffected by dietary treatment. Calves fed LO had greater ($P < 0.0001$) energy efficiency during the grower phase than calves fed HI which resulted in LO calves having higher ($P = 0.018$) BW gain during this period. Hip width tended ($P = 0.06$) to be higher for calves fed LO compared with HI. There was no effect of processing of corn or the combination of MR feeding rate and corn processing on intake, performance and efficiency during the grower phase.

Apparent Total-Tract Nutrient Digestibility. Digestibility of DM and OM were lower ($P \leq 0.02$) in calves fed LO compared with HI during wk 5, but during wk 8 and 12 calves fed LO had higher ($P \leq 0.02$) DM and OM digestibility compared with calves fed HI (Table 2.7-5). Calves fed FC had higher ($P \leq 0.05$) DM, OM and fat digestibility and tended to have higher ($P = 0.07$) CP digestibility and lower ($P = 0.08$) NDF digestibility during wk 12 compared with calves fed WC. Calves fed LO had higher ($P = 0.039$) CP digestibility during wk 12 compared with calves fed HI. During wk 5, 8 and 12, calves fed LO had 5.6, 1.3, and 1.3-fold higher ($P \leq 0.001$) NDF digestibility, respectively, compared with calves fed HI. Digestibility of ADF had a similar pattern to NDF, where calves fed LO had 7.9, 1.8, and 1.3-fold higher ($P \leq 0.004$) digestibility for wk 5, 8 and 12, respectively, compared with calves fed HI. There was an interaction between MR level and processing of corn in CS for sugar digestibility during wk 5. Calves fed HIWC had the lowest ($P = 0.028$) digestibility. Calves fed LO tended to have higher ($P = 0.10$) sugar digestibility during wk 5 and higher ($P = 0.042$) fat digestibility during wk 12 compared to calves fed HI.

Fibrolytic Bacteria Quantification. Density of total bacteria and fibrolytic bacterial community in rumen fluid were unaffected by dietary treatment at wk 5 and 8 (Table 2.7-6). No effect was observed for ruminal total bacteria at wk 5 and 8. At wk 5, *Butyrivibrio fibrisolvens* density tended to be higher ($P = 0.05$) in calves fed LO compared with HI. Proportion (% of total bacteria) of *Clostridium cluster IV* and *Butyrivibrio fibrisolvens* tended ($P \leq 0.07$) to be 2-fold higher in rumen fluid when calves were fed LO compared with HI at wk 5. At wk 8, proportion of *Ruminococcus albus* was higher ($P = 0.045$) in rumen fluid of calves fed LOWC compared to calves fed LOFC and HIWC. In fecal matter, total bacteria was unaffected by diet at wk 5 and 8 (Table 2.7-7). *Clostridium cluster IV*, *Faecalibacterium sp.*, and *Prevotella sp.* densities were higher ($P \leq 0.003$) and *Butyrivibrio fibrisolvens* density tended to be higher ($P = 0.09$) in calves fed LO compared with HI at wk 5. *Faecalibacterium sp.* density also tended to be higher ($P = 0.07$) at wk 8 in calves fed LO compared with HI. In contrast, *Ruminococcus flavefaciens* density was higher ($P < 0.0001$) in calves fed HI compared with LO during wk 5. At wk 5, proportion of *Clostridium cluster IV*, *Faecalibacterium sp.* and *Prevotella sp.* were higher ($P \leq 0.043$) and *Ruminococcus flavefaciens* tended to be lower ($P = 0.05$) in calves fed LO compared with HI. There were no dietary treatment effects on proportion of any bacterial community at wk 8.

2.4. Discussion

The goal of this study was to determine the impact of amount of MR fed and the processing method of corn in CS on calf performance, digestibility and rumen and fecal bacterial communities. Based on our findings, MR treatment had a greater impact on performance, fibre digestibility and rumen and fecal fibrolytic bacterial community in dairy calves than corn processing in CS treatment.

2.4.1. Effect of MR feeding rate

Similar findings to the present study regarding MR feeding rate (LO vs. HI) and its effect on pre-weaning CS intake (Hill et al., 2016b; Dennis et al., 2018, 2019), BW (Jasper and Weary, 2002; Khan et al., 2011a; Rosenberger et al., 2017) and fibre apparent total-tract digestibility (Hill et al., 2016b; Dennis et al., 2018) have been reported. Feeding high rates of MR to calves should provide enough nutrients to sustain satiety largely on MR alone, thus decreasing CS intake compared with calves fed MR at a low rate, as was shown in the present study and previous reports (Hill et al., 2016b; Dennis et al., 2018, 2019). The higher rate of MR feeding also resulted in higher BW until wk 7 from the greater ME intake compared to calves fed MR at a lower rate (Rosenberger et al., 2017). In addition, MR is more digestible than CS, therefore contributing more energy towards growth compared to CS. Studies reporting NDF and ADF digestibility have consistently noted that calves fed higher rates of MR have lower NDF and ADF digestibility at various wk of life (Chapman et al., 2016; Dennis et al., 2018, 2019). In our study, poor fibre apparent total-tract digestibility was carried over into the grower period (6 wk after weaning) by calves fed HI, as reported previously (Hill et al., 2016b). This was thought to be indicative of inadequate rumen microbial fermentation due to reduced CS intake when calves are fed a high MR feeding rate pre-weaning (Hill et al., 2016b). Calves fed LO tended to have increased *Butyrivibrio fibrisolvens* density and *Butyrivibrio fibrisolvens* and *Clostridium cluster IV* proportions during wk 5 in the rumen, possibly from the increase in CS intake, which may support greater fibre digestion observed in LO vs. HI calves. The proportion of *Clostridium cluster IV* and *Faecalibacterium sp.* increased in the fecal matter of LO calves at wk 5, which suggests that more fibre digestion may occur in the hindgut of calves fed LO vs. HI. The extent and biological significance of fibre digestion post-ruminally in calves is largely unknown, however Gressley et al. (2011) summarized a variety of studies from mature ruminants and

reported that NDF digestion can range from 3.7 to 23.2 % and ADF digestion from 3.3 to 6.5 % in the large intestine. In the present study, only a select number of fibrolytic bacteria were identified using qPCR and their activity was not evaluated. Few differences were observed regarding the effect of MR treatment on the proportion of bacteria. We postulate that this may be due to the high variation (CV range: 10.0 to 240.7 % in rumen fluid, 40.3 to 214.1 % in fecal matter) between calves within the same dietary treatment for the proportion of fibrolytic bacterial communities.

Different dietary sources (MR vs. CS) have different digestibilities and the ratio of MR to CS intake in calves differed at wk 5, which resulted in calves fed HI having higher DM and OM digestibility compared with calves fed LO. Digestibility of DM and OM may influence intake and calves fed LO had higher DM and OM digestibility when only consuming solid feed, as well as higher CS intake post-weaning. However, during the grower phase, intakes were unaffected by dietary treatment.

Calves fed LO tended to have a higher proportion of ruminal fibrolytic bacteria (*Butyrivibrio fibrisolvens* and *Clostridium cluster IV*) and a higher proportion of fecal fibrolytic bacteria *(Faecalibacterium sp., Clostridium cluster IV* and *Prevotella sp.*) than calves fed HI during wk 5. Additionally, calves fed LO had greater digestion of OM and fibre than calves fed HI. Greater growth rates in both BW and structure during wk 9 to 16 for calves fed LO vs. HI was likely attributed to better development of the digestive system and transition to a dry feed diet. These digestibility and performance measures are consistent with several other studies (Hill et al., 2016b; Dennis et al., 2018). The CS in the present study contained a high amount of starch (41.5 %), with ADF and NDF being low at 7.2 and 15.4 %, respectively; therefore, additional factors contributing to fibre digestion may play a role and resulting in a growth depression. It is possible that HI calves, when weaned at wk 6 with a one wk step-down, create greater gut health challenges for the wk post-weaning, causing more energy to be partitioned to the gut to adapt and recover from the more dramatic weaning transition (Steele et al., 2016).

Overall, the poor DM, OM and fibre digestibility observed in our study during the post-weaning stage for calves fed the HI diet resulted in fewer nutrients available for utilization towards growth. During the grower phase, calves fed the LO diet were more efficient at utilizing feed, resulting in calves fed the HI diet to lose their pre-weaning growth advantage by wk 16. However, it is important to note that these calves were bedded with long wheat straw and

consumption was not accounted for. Yet, Hill et al. (2019) reported that digestion estimates were not affected when calves were bedded on long straw compared to no straw. Thus, we speculate that bedding consumption would have had minimal impact in the current study.

2.4.2. Effect of corn processing

Processing grain can maximize starch digestibility, yet processing can also change the site of digestion (Owens, 2005). Processing corn may be beneficial to young calves because starch digestion in the rumen stimulates rumen papillae development (Flatt et al., 1958; Sander et al., 1959). Based on unpublished data, dietary treatment had no influence ($P \geq 0.12$) on total ruminal short chain fatty acid (SCFA) concentration during wk 5 or 8, which may indicate that the corn was fermented to the same extent in the rumen in both treatments. The SCFA concentration did increase from wk 5 to wk 8, which is reflective of increased starter intake. The total SCFA averages were as follows: 47.3 ± 8.3, 36.9 ± 5.7, 31.4 ± 1.1 and 36.8 ± 6.4 μmol/ml for LOWC, HIFC, HIWC and HIFC, respectively, during wk 5 and 62.6 ± 6.0, 62.5 ± 6.3, 61.5 ± 4.2 and 72.5 ± 8.3 μmol/ml for LOWC, HIFC, HIWC and HIFC, respectively, during wk 8. It has been demonstrated that total tract digestibility of steam-rolled (or flaked) corn was 97 % compared to 84 % for whole corn in mature ruminants (Owens, 2005). Thus, starch digestibility in the current study was expected to be different among CS treatments based on mature ruminant data; however, no differences were observed (> 95 % for both treatments wk 5, 8 and 12). Owens (2005) reported that site and extent of digestion and passage rate of corn (whole or processed) can be altered by dietary fibre levels in the diet. We speculate that the low levels of fibre in our diet compared to feedlot or lactating cow diets may have resulted in equal digestion between calf starter treatments. It is possible that passage rate of corn is different in calves than that of adult animals because of different particle distribution in the rumen and calves may also have the ability to utilize whole corn kernels more efficiently than cows by chewing their feed more thoroughly (Owens, 2005), which would impact starch digestion. Thus, further investigation into corn processing and digestion when forage is included in the diet pre-weaning is warranted. Specifically, future research should investigate rumination times in young calves fed differentially processed corn in order to provide further insight into the mechanisms responsible for the comparable digestion of whole corn and steam flaked corn evidenced in the present study.

Calves fed WC had decreased DM and OM digestibility during wk 12 compared to calves fed FC. The OM difference is most likely due to the observed lower CP and fat digestion in WC calves. If the WC diet was digested in the hindgut, it may have led to higher microbial protein in the feces; therefore, CP digestibility tended to be lower in the calves fed WC compared with FC. Consistent with previous studies (pre-weaning: Lesmeister and Heinrichs, 2004; pre- and post-weaning: Bateman et al., 2009), the processing of corn in CS did not influence intake, and thus, calf performance. In contrast, previous studies have shown that CS intake increased when whole corn was fed to calves compared to steam flaked (post-weaning: Lesmeister and Heinrichs, 2004) and steam-rolled (during weaning: Terré et al., 2016) corn. Although digestibility was not measured in these studies, the increase in CS intake did not result in increased BW, suggesting that calves were unable to utilize the additional nutrients towards growth. Lesmeister and Heinrichs (2004) reported that total SCFA concentrations were lower in the whole corn-fed calves compared to the steam flaked corn-fed calves, indicating that whole corn was not digested in the rumen to the same extent as steam-flaked, and that a certain amount must have by-passed the rumen. Starch digestion in the rumen is beneficial for ruminal development and thus further investigation on site of digestion of whole corn in calves is warranted – especially given the high cost of corn processing. All of the above-mentioned studies (Lesmeister and Heinrichs, 2004; Bateman et al., 2009; Terré et al., 2016) only investigated the effects of grain processing when calves were fed a low MR feeding rate (< 6 L) and therefore the interaction with higher MR feeding rate is unknown.

2.4.3. Interaction of MR feeding rate with processing of corn in calf starter

Effect of milk/MR feeding rate on intake, growth performance and total-tract nutrient digestibility has been investigated in numerous studies (Terré et al., 2007; Hill et al., 2010, 2016b; Chapman et al., 2016) in relation to weaning method/age (Dennis et al., 2018), amount of starch in CS (Quigley et al., 2018), and additives (Hill et al., 2016a; Dennis et al., 2018), but not in relation to the processing of corn in CS. To our knowledge, this is the first study that evaluated the combination of MR feeding rate with processing of corn in CS (whole vs. steam-flaked corn) in calves. We thought that feeding a higher rate of MR would delay rumen development and WC fed calves would have reduced nutrient digestibility. When digestibility is reduced, and CS intake remains the same between the WC and FC, calf performance would be reduced. However, we found that the combination of MR feeding rate and corn processing in CS did not have an

effect on digestibility, as hypothesized, although CS intakes were similar between the WC and FC. However, during wk 5, sugar digestibility was lower for calves fed the HIWC diet compared with the other dietary treatments, which could have resulted in numerically lower starch digestion for this treatment. As expected, body weight, ADG, body measurements, and feed efficiency throughout the study were not affected by dietary treatment, since there was no change in CS intake or digestibility. As described above, fibre content in the diet could influence corn digestibility, yet minimal amounts of fibre were included in these diets. Therefore, the addition of fibre or low starch diets on the digestibility of corn in calves fed high rates of MR needs to be explored further.

2.5. Conclusion

Our results show that a high MR feeding rate reduced fibre digestibility, both pre- and post-weaning and during the grower phase (wk 5, 8, and 12). Pre-weaning ADG and hip width measurements were higher in HI vs. LO MR feeding rate, but reduced post-weaning, resulting in similar BW and hip width measurements by 16 wk of age. Calves fed LO had a higher proportion of fibrolytic bacteria in both the rumen and feces than calves fed HI. When considering the combination of MR feeding rate and the processing of corn feeding, low and high MR feeding rate can be combined with either flaked corn or whole corn in the CS because corn processing had little to no impact on digestibility, intake and growth of calves.

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2.7. Tables and figures

Table 2.7-1 Chemical composition of milk replacer (MR), starter and hay fed to dairy calves.

Item	MR	Starter[1]	Hay
Dry matter, % as fed	93.1 ± 1.13	87.8 ± 0.66	86.2 ± 2.1
DM basis, %			
Ash	5.9 ± 0.33	6.1 ± 0.67	8.3 ± 1.14
CP	25.2 ± 0.57	20.3 ± 0.45	6.6 ± 0.91
Fat	17.9 ± 0.01	3.6 ± 0.22	1.9 ± 0.45
ADF	-	7.2 ± 0.50	40.9 ± 0.65
NDF	-	15.4 ± 1.14	63.3 ± 0.85
Starch	-	41.5 ± 0.86	1.4 ± 0.43
Sugar	-	7.3 ± 1.62	8.8 ± 1.49
ME[2], Mcal/kg	4.75	3.26	2.05

[1]37 % corn (either whole or steam-flaked), 35 % protein/mineral pellet, 25 % whole oats, and 3 % liquid molasses.

[2]Calculated using NRC (2001

Table 2.7-2 . Bacterial primers used to determine the copy number of 16S rRNA genes in rumen fluid and fecal matter of dairy calves.

Bacteria	Primer	Sequence (5' to 3')	Product size	Annealing temperature (°C)	Reference
Total bacteria	Forward	ACTCCTACGGGAGGCAG	467	60	Stevenson and Weimer, 2007
	Reverse	GACTACCAGGGTATCTAATCC			
Ruminococcus albus	Forward	TGTTAACAGAGGGAAGCAAAGCA	75	60	Stevenson and Weimer, 2007
	Reverse	TGCAGCCTACAATCCGAACTAA			
Faecalibacterium prausnitzii	Forward	GGAGGAAGAAGGTCTTCGG	248	60	Vital et al., 2013
	Reverse	AATTCCGCCTACCTCTGCACT			
Butyrivibrio fibrisolvens	Forward	GGTGAGTAACGCGTGGGTAA	189	60	Present study
	Reverse	CAGGTCGGCTACTGATCGTC			
Clostridium cluster IV	Forward	TTAACACAATAAGTWATCCACCTGG	314	60	Ramirez-Farias et al., 2009
	Reverse	ACCTTCCTCCGTTTTGTCAAC			
Ruminococcus flavefaciens	Forward	TGGCGGACGGGTGAGTAA	71	60	Stevenson and Weimer, 2007
	Reverse	TTACCATCCGTTTCCAGAAGCT			
Prevotella sp.	Forward	GGTTCTGAGAGGAAGGTCCCC	121	60	Stevenson and Weimer, 2007
	Reverse	TCCTGCACGCTACTTGGCTG			
Fibrobacter succinogenes S85	Forward	GCGGGTAGCAAACAGGATTAGA	77	60	Stevenson and Weimer, 2007
	Reverse	CCCCCGGACACCCAGTAT			

Table 2.7-3. Effect of treatment[1] on intake, growth performance and efficiency in dairy calves (0 to 8 wk).

	Treatments					Contrast, *P* value		
Item	LOWC	LOFC	HIWC	HIFC	SEM	LO vs. HI	FC vs. WC	Interaction
Pre-weaning (week 1-6)								
Milk replacer offered, kg	28.84	28.84	46.11	46.11	-	-	-	-
Milk replacer intake, kg	28.30	28.09	41.27	42.54	0.796	<0.0001	0.51	0.36
Starter intake, kg	7.89	5.85	3.00	3.51	0.796	<0.0001	0.34	0.12
ME intake, Mcal	160.14	152.47	205.84	213.47	4.774	<0.0001	0.99	0.12
BW gain, kg	21.41	19.85	26.17	29.63	1.172	<0.0001	0.42	0.038
Energy efficiency, Mcal/kg	7.68	7.95	7.96	7.27	0.333	0.55	0.53	0.16
Post-weaning (week 7-8)								
Starter intake, kg	25.10	21.87	20.01	19.12	1.416	0.0083	0.15	0.41
ME intake, Mcal	81.83	71.29	65.24	62.34	4.62	0.0083	0.15	0.41
BW gain, kg	10.46	9.64	8.10	5.70	0.87	0.0008	0.07	0.37
Energy efficiency, Mcal/kg	7.91	7.58	9.15	12.40	2.674	0.26	0.59	0.51
Overall (week 1-8)								
Starter intake, kg	33.00	27.71	23.01	22.63	2.064	0.001	0.18	0.24
ME intake, Mcal	241.97	223.75	271.08	275.81	8.042	<0.0001	0.41	0.16
BW gain, kg	31.87	29.49	34.28	35.33	1.533	0.01	0.67	0.27
Energy efficiency, Mcal/kg	7.66	7.78	7.95	7.88	0.237	0.41	0.90	0.69
Initial								
BW, kg	40.4	39.7	40.7	40.6	1.06	0.58	0.71	0.76
Hip height, cm	70.1	70.6	69.6	71.8	0.88	0.71	0.14	0.33
Heart girth, cm	80.4	78.4	78.9	78.7	0.98	0.53	0.26	0.38
Paunch girth, cm	83.1	81.6	82.4	81.4	0.99	0.68	0.21	0.80
Hip width, cm	17.1	17.1	16.8	17.0	0.23	0.45	0.82	0.62
BCS	2.1	2.0	2.1	2.1	0.03	0.07	0.54	0.22
Week 6								
BW, kg	61.8	59.6	66.9	70.2	1.60	<0.0001	0.73	0.08
Hip width, cm	19.5	19.6	20.0	20.2	0.21	0.013	0.47	0.59
BCS	2.3	2.3	2.4	2.5	0.04	0.001	0.32	0.14
Week 8								
BW, kg	72.3	69.2	75.0	76.0	1.99	0.022	0.6	0.31
Hip height, cm	81.3	81.0	81.6	81.7	1.09	0.62	0.91	0.85
Heart girth, cm	97.3	95.8	97.2	97.9	1.09	0.35	0.73	0.31
Paunch girth, cm	111.6	110.7	113.0	110.8	1.42	0.60	0.27	0.64
Hip width, cm	20.7	20.7	20.9	21.0	0.23	0.22	0.22	0.82
BCS	2.4	2.3	2.4	2.4	0.04	0.08	1.00	0.08

[1] Treatment: LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d; WC = whole corn and FC = steam-flaked corn in textured calf starter.

Table 2.7-4. Effect of treatment[1] on intake, growth performance and efficiency from wk 9 to 16 in dairy calves.

	Treatments					Contrast, *P* value		
Item	LOWC	LOFC	HIWC	HIFC	SEM	LO vs. HI	FC vs. WC	Interaction
Week 9-16								
DM intake kg	173.71	159.64	163.39	162.71	8.340	0.68	0.40	0.45
ME intake, Mcal	555.87	510.86	522.85	520.66	26.689	0.68	0.40	0.45
BW gain, kg	61.37	60.18	52.21	52.69	1.067	<0.0001	0.75	0.46
Energy efficiency, Mcal/kg	9.01	8.48	10.02	9.87	0.401	0.018	0.39	0.61
Week 16								
BW, kg	133.9	129.6	127.4	128.9	2.86	0.24	0.64	0.34
Hip height, cm	92.7	91.9	92.3	91.9	1.04	0.84	0.55	0.87
Heart girth, cm	116.6	113.8	114.5	115.5	1.43	0.91	0.53	0.19
Paunch girth, cm	145.9	142.0	144.0	143.3	1.72	0.87	0.19	0.35
Hip width, cm	25.9	25.4	25.1	25.3	0.24	0.06	0.73	0.15
BCS	2.7	2.6	2.6	2.7	0.08	1.00	1.00	0.59

[1] Treatment: LO = 0.749 kg/d of milk replacer (MR) for 35 d, then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d; WC = whole corn and FC = steam flaked corn in textured calf starter.

Table 2.7-5. Effect of treatment[1] on apparent total tract digestibility during 3 different time points (wk 5, 8 and 12) in dairy calves.

		Treatments					Contrast, *P* value		
Digestion, %	n	LOWC	LOFC	HIWC	HIFC	SEM	LO vs. HI	FC vs. WC	Interaction
DM									
Wk 5	6	94.6	95.1	96.4	96.0	0.46	0.010	0.87	0.30
Wk 8	6	75.5	76.6	74.1	73.1	0.94	0.018	0.29	0.95
Wk 12	3	78.8	79.7	75.7	76.6	0.30	<0.0001	0.016	0.92
OM									
Wk 5	6	94.9	95.5	96.5	96.2	0.46	0.021	0.75	0.31
Wk 8	6	77.1	77.1	74.4	75.6	0.98	0.017	0.30	0.90
Wk 12	3	80.8	82.2	77.3	79.2	0.71	0.002	0.05	0.77
CP									
Wk 5	6	92.0	93.1	93.1	91.9	0.72	0.93	0.94	0.14
Wk 8	6	72.6	74.7	73.7	70.8	1.86	0.45	0.84	0.20
Wk 12	3	80.1	82.0	77.7	79.7	0.96	0.039	0.07	0.95
NDF									
Wk 5	6	43.4	35.8	7.3	6.7	4.32	<0.0001	0.35	0.43
Wk 8	6	42.4	35.7	30.2	28.2	2.60	0.001	0.11	0.37
Wk 12	3	53.5	49.1	41.5	40.2	1.41	<0.0001	0.08	0.29
ADF									
Wk 5	6	22.4	21.2	1.8	3.7	3.65	<0.0001	0.92	0.67
Wk 8	6	29.1	29.4	14.5	18.0	2.29	<0.0001	0.42	0.49
Wk 12	3	44.0	41.1	33.1	30.3	2.66	0.004	0.31	0.98
Starch									
Wk 5	6	99.3	99.3	94.1	98.9	1.67	0.11	0.17	0.17
Wk 8	6	95.9	96.3	94.1	95.4	1.19	0.25	0.49	0.72
Wk 12	3	99.5	99.4	99.3	99.4	0.06	0.20	0.62	0.23
Sugar									
Wk 5	6	87.8	81.2	26.2	52.0	6.83	<0.0001	0.17	0.028
Wk 8	6	88.5	89.1	86.6	87.9	0.91	0.10	0.29	0.69
Wk 12	3	78.5	71.6	68.7	72.1	3.97	0.28	0.67	0.23
Fat									
Wk 5	6	98.4	98.1	98.3	98.2	0.21	0.89	0.41	0.76
Wk 8	6	75.5	78.4	76.0	74.6	1.97	0.42	0.68	0.29
Wk 12	3	77.6	81.9	71.3	78.6	1.99	0.042	0.020	0.48

[1] Treatment: LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d; WC = whole corn and FC = steam-flaked corn in textured calf starter.

Table 2.7-6. Effect of treatment[1] on density and proportion (% of total bacteria) of fibrolytic bacteria in rumen fluid of dairy calves.

	Treatments					Contrast, *P* value		
Item	LOWC	LOFC	HIWC	HIFC	SEM	LO vs. HI	FC vs. WC	Interaction
Wk 5								
Copy number, log 10/ml								
Total bacteria	11.47	11.50	11.51	11.59	0.083	0.48	0.50	0.78
Ruminococcus albus	5.13	5.72	5.61	5.62	0.248	0.44	0.24	0.26
Clostridium cluster IV	7.77	7.80	7.41	7.77	0.226	0.41	0.40	0.47
Butyrivibrio fibrisolvens	9.44	9.27	9.04	9.21	0.100	0.05	0.74	0.18
Ruminococcus flavefaciens	7.40	7.30	7.17	7.19	0.152	0.28	0.78	0.69
Prevotella sp	8.39	8.40	8.57	8.69	0.161	0.17	0.68	0.76
Fibrobacter succinogenes S85	7.77	7.51	7.50	7.56	0.153	0.47	0.54	0.33
Wk 5								
% of total bacteria								
Ruminococcus albus	5.35E-05	4.00E-04	2.46E-04	2.45E-04	0.000173	0.92	0.33	0.33
Clostridium cluster IV	0.032	0.028	0.014	0.017	0.0076	0.07	1.00	0.65
Butyrivibrio fibrisolvens	1.01	0.65	0.43	0.57	0.160	0.05	0.49	0.13
Ruminococcus flavefaciens	0.0117	0.0081	0.0059	0.0049	0.00273	0.13	0.42	0.64
Prevotella sp	0.106	0.102	0.119	0.127	0.0184	0.33	0.90	0.74
Fibrobacter succinogenes S85	0.025	0.015	0.014	0.012	0.0054	0.21	0.29	0.47
Wk 8								
Copy number, log 10/ml								
Total bacteria	11.19	11.25	11.40	11.26	0.11	0.35	0.72	0.39
Ruminococcus albus	5.44	4.73	5.07	5.10	0.28	0.99	0.25	0.20
Clostridium cluster IV	7.11	7.03	7.14	7.18	0.17	0.60	0.91	0.74
Butyrivibrio fibrisolvens	9.32	9.42	9.34	9.60	0.13	0.45	0.19	0.52
Ruminococcus flavefaciens	6.84	6.79	6.72	7.09	0.15	0.57	0.28	0.16
Prevotella sp	7.84	8.01	8.19	7.57	0.25	0.85	0.37	0.13
Fibrobacter succinogenes S85	7.44	7.67	7.43	7.70	0.391	0.99	0.53	0.96
Wk 8								
% of total bacteria								
Ruminococcus albus	3.24E-04	4.33E-05	7.18E-05	2.03E-04	9.60E-05	0.64	0.45	0.045
Clostridium cluster IV	0.0113	0.0067	0.0057	0.0125	0.00352	0.98	0.77	0.12
Butyrivibrio fibrisolvens	1.55	1.57	1.36	2.34	0.339	0.40	0.16	0.17
Ruminococcus flavefaciens	0.0063	0.0040	0.0043	0.0076	0.00178	0.66	0.78	0.13
Prevotella sp	0.060	0.102	0.113	0.028	0.0354	0.76	0.55	0.08
Fibrobacter succinogenes S85	0.043	0.283	0.019	0.046	0.1456	0.38	0.37	0.47

[1]Treatment: LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d; WC = whole corn and FC = steam-flaked corn in textured calf starter.

Table 2.7-7. Effect of treatment[1] on density and proportion (% of total bacteria) of fibrolytic bacteria in fecal matter of dairy calves.

	Treatments					Contrast, *P* value		
Item	LOWC	LOFC	HIWC	HIFC	SEM	LO vs. HI	FC vs. WC	Interaction
Wk 5								
Copy number, log 10/ml								
Total bacteria	11.14	11.23	11.13	11.14	0.134	0.67	0.72	0.76
Ruminococcus albus	4.38	4.24	4.37	3.98	0.250	0.59	0.31	0.61
Clostridium cluster IV	8.30	8.38	7.64	7.61	0.187	0.001	0.91	0.78
Faecalibacterium sp	7.95	8.16	7.44	7.55	0.186	0.007	0.41	0.78
Butyrivibrio fibrisolvens	9.28	9.25	9.06	9.00	0.134	0.09	0.75	0.90
Ruminococcus flavefaciens	7.04	6.64	7.56	7.79	0.199	0.0006	0.68	0.14
Prevotella sp	8.72	8.65	7.94	7.98	0.217	0.0031	0.93	0.81
Fibrobacter succinogenes S85	7.87	8.13	7.74	7.79	0.184	0.22	0.41	0.60
Wk 5								
% of total bacteria								
Ruminococcus albus	2.48E-05	1.54E-05	8.45E-05	1.01E-05	3.70E-05	0.48	0.28	0.40
Clostridium cluster IV	0.164	0.204	0.038	0.035	0.0478	0.006	0.70	0.66
Faecalibacterium sp	0.083	0.143	0.023	0.028	0.0405	0.043	0.44	0.51
Butyrivibrio fibrisolvens	1.49	1.48	0.95	0.82	0.382	0.13	0.85	0.88
Ruminococcus flavefaciens	0.011	0.004	0.038	0.084	0.0251	0.05	0.46	0.32
Prevotella sp	0.530	0.314	0.103	0.088	0.0888	0.0015	0.21	0.27
Fibrobacter succinogenes S85	0.079	0.111	0.056	0.063	0.0300	0.25	0.53	0.69
Wk 8								
Copy number, log 10/ml								
Total bacteria	11.76	11.77	11.49	11.86	0.160	0.61	0.25	0.28
Ruminococcus albus	6.22	6.71	6.28	6.66	0.306	0.98	0.17	0.87
Clostridium cluster IV	8.33	8.25	7.90	8.22	0.165	0.18	0.46	0.24
Faecalibacterium sp	6.99	6.87	6.61	6.61	0.164	0.07	0.73	0.71
Butyrivibrio fibrisolvens	9.35	9.17	9.21	9.19	0.123	0.64	0.43	0.51
Ruminococcus flavefaciens	7.59	7.56	7.54	7.98	0.169	0.28	0.25	0.18
Prevotella sp	8.74	8.57	8.08	8.32	0.280	0.12	0.90	0.47
Fibrobacter succinogenes S85	8.11	8.06	8.17	7.99	0.161	0.95	0.50	0.70
Wk 8								
% of total bacteria								
Ruminococcus albus	7.21E-04	1.07E-03	1.11E-03	1.61E-03	6.05E-04	0.45	0.49	0.91
Clostridium cluster IV	0.049	0.044	0.041	0.028	0.0142	0.39	0.53	0.78
Faecalibacterium sp	0.049	0.044	0.041	0.028	0.0020	0.58	0.16	0.30
Butyrivibrio fibrisolvens	0.53	0.39	1.19	0.29	0.408	0.50	0.22	0.36
Ruminococcus flavefaciens	0.008	0.010	0.022	0.016	0.0060	0.14	0.52	0.71
Prevotella sp	0.141	0.093	0.110	0.058	0.0397	0.41	0.22	0.96
Fibrobacter succinogenes S85	0.061	0.022	0.085	0.019	0.0289	0.73	0.09	0.65

[1] Treatment: LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d; WC = whole corn and FC = steam flaked corn in textured calf starter.

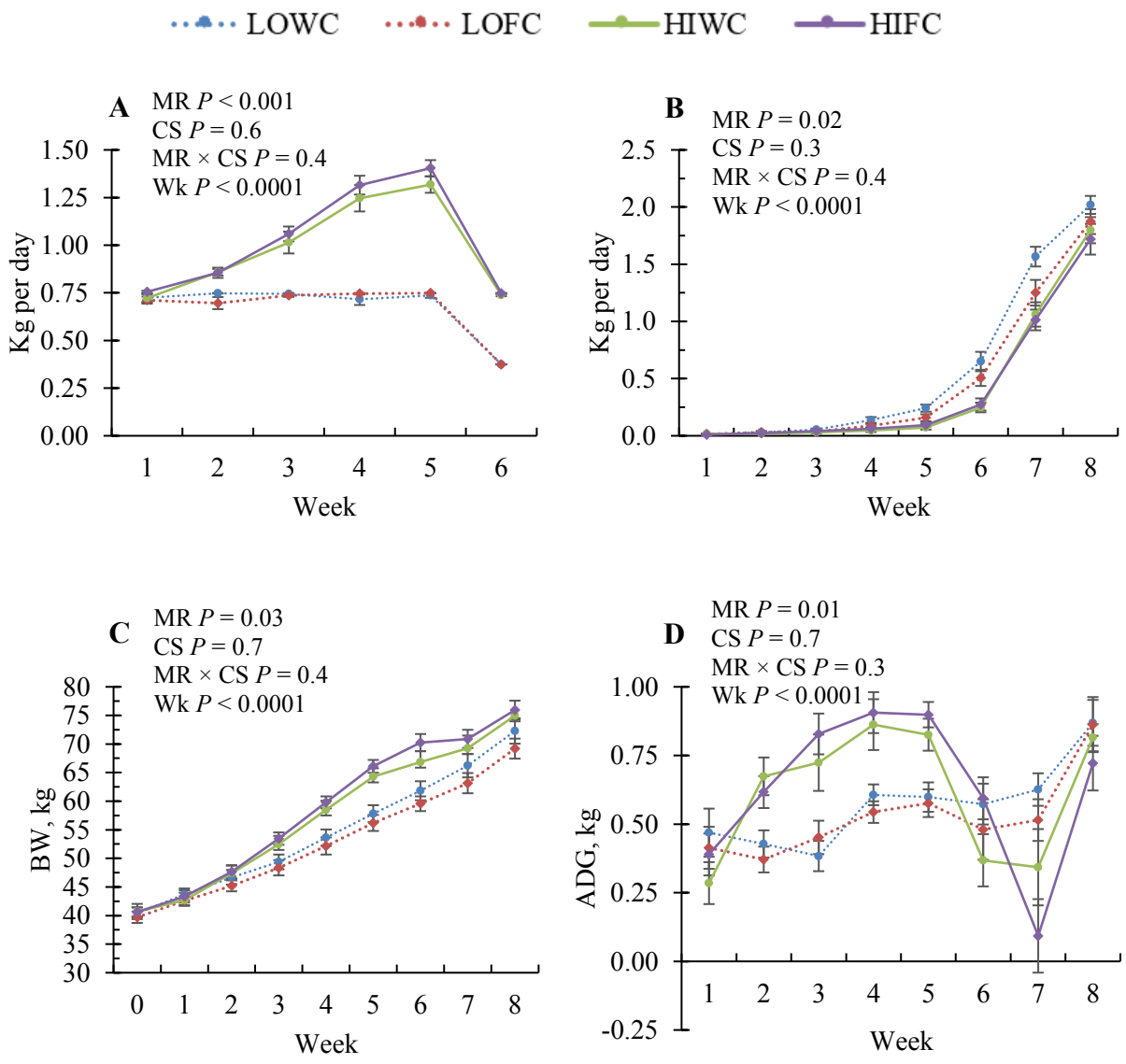

Figure 2.7-1. Mean weekly (A) milk replacer (MR) intake, (B) calf starter (CS) intake, (C) body weight, and (D) average daily gain for calves fed a low (LO) or high (HI) MR feeding rate and CS containing whole (WC) or flaked (FC) corn (wk 1 to 8). Milk replacer was fed at LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d.

3. Impact of amount of milk replacer fed and the processing of corn in starter on rumen and hindgut fermentation and inflammatory biomarkers of dairy calves.

3.1. Introduction

During the early wk of life, calves rely on milk to support their maintenance and growth because they are born without a functional rumen (Baldwin et al., 2004; Drackley, 2008). Once solid feed is consumed, microbial fermentation occurs and short-chain fatty acids (**SCFA**) are produced in the rumen, which stimulate rumen papillae development (Sander et al., 1959). It is well known that carbohydrate diets, especially cereal grains, are beneficial for rumen development due to propionate and butyrate production that are important for stimulating papillae growth (Baldwin and McLeod, 2000; Baldwin et al., 2004).

When comparing starch content in grains, corn has the highest amount (72 – 78 %) on a DM basis compared to sorghum, barley and oats (Herrera-Saldana et al., 1990; Huntington, 1997), making it a suitable ingredient to include in calf starter (**CS**) to promote rumen development. Corn grain is the preferred cereal grain to add to CS because solid feed intake and performance in dairy calves up to 12 wk old were negatively affected when corn was replaced by barley, oats, or wheat in CS (Khan et al., 2007). Surface area of grain can be increased by different processing methods (grinding, steam- or dry-rolling and steam flaking) which may enhance rumen fermentability, increase passage rate and improve total-tract starch digestibility (Huntington, 1997). Processed grain (steam rolled corn and barley) caused CS intake to decrease compared with whole grains (corn and barley) in calves (Terré et al., 2016). In a companion paper, Van Niekerk et al. (2020; Chapter 2) reported no difference in CS intake, weight gain, body measurements and digestibility in calves fed a texturized CS containing either whole or flaked corn. Thus, whole corn and flaked corn were equally utilized by the calves, although the site and extent of fermentation is unknown. Ruminal pH was reduced when processed grains were fed to calves (Lesmeister and Heinrichs, 2004; Terré et al., 2016) and mature animals, dairy cows and feedlot animals (Owens et al., 1997; Firkins et al., 2001). To our knowledge, Lesmeister and Heinrichs (2004) and Terré et al. (2016) are the only studies that have investigated the effects of processed corn in CS on rumen fermentation in dairy calves, however these studies limit fed MR.

Low ruminal pH can be the result of a sudden increase in rapid fermentable carbohydrate intake through CS after weaning, which may lead to SCFA accumulation in the rumen (Aschenbach et al., 2011). In mature animals this low ruminal pH may result in decreased digestion (Cerrato-Sanchez et al., 2007), which may negatively impact ruminal health (Kleen et al., 2004) and cause a systemic inflammatory response (Khafipour et al., 2009a) – all of which are unfavorable because they lead to different diseases and disorders (Plaizier et al., 2018). Feeding unprocessed grains has been shown to reduce rumen fermentability and increased bypass starch to the hindgut (Svihus et al., 2005) which may lead to possible hindgut acidosis and damage to the gastrointestinal tract (**GIT**) epithelium (Gressley et al., 2011). The effects on calves, however, remain unknown. It is known that ruminal acidosis (Aschenbach and Gabel, 2000; Khafipour et al., 2009b; Steele et al., 2011) and hindgut acidosis (Gressley et al., 2011; Tao et al., 2014a,b) can influence the integrity of the respective epithelial tissues. The GIT epithelia play an important role by acting as a barrier, preventing lipopolysaccharide (LPS), histamine, bacteria and other harmful molecules from translocating into the portal circulation (Penner et al., 2011). Compromised GIT barrier function may be one of the factors that contributes to calf mortality and morbidity early in life. Research shows that the hindgut plays an important role in animal nutrition and health, but how the hindgut is influenced by weaning and diet composition has largely been overlooked.

Due to improved BW gain (Jasper and Weary, 2002; Khan et al., 2011; Rosenberger et al., 2017) and feed efficiency (Khan et al., 2007; Moallem et al., 2010; Davis-Rincker et al., 2011), feeding higher rates (> 10 % of birth BW) of milk or milk replacer (**MR**) preweaning has become of interest to the industry. When calves gain more weight during the pre-weaning period there is also the potential for them to produce more milk as adults (Soberon et al., 2012; Soberon and Van Amburgh, 2013; Gelsinger et al., 2016). Feeding calves high MR feeding rates have delayed rumen development (Khan et al., 2016) due to reduced CS intake pre-weaning (Hill et al., 2016; Dennis et al., 2018, 2019). This may lead to increased starch reaching the hindgut post-weaning when CS intake increases rapidly, and the rumen may not be capable of adapting to the increase in CS intake. It has been reported that fecal starch increases at weaning (Eckert et al., 2015; Steele et al., 2017), suggesting increased starch reaching the hindgut. Since MR feeding rate influences CS intake, it is important to investigate the interaction between the MR feeding rate and the processing of corn in the CS, because feeding calves a high MR feeding rate and CS

containing whole corn may increase the amount of starch that reaches the hindgut, and when combined may negatively influence calf health. The objective of this study was to determine how amount of MR and processing of corn in CS affects rumen and hindgut fermentation, free LPS concentration, *Escherichia coli* numbers, and acute phase proteins in dairy calves. We hypothesized that feeding an elevated amount of MR with CS containing whole corn would increase bypass starch during weaning, causing hindgut acidosis, which in turn would increase fecal LPS concentration and induce a systemic inflammatory response.

3.2. Materials and methods

3.2.1. Animals, housing, experimental design, and dietary treatments

Animals, housing and environment, experimental design, and dietary treatments were described in a companion paper (Van Niekerk et al., 2020; Chapter 2). All experimental procedures were reviewed and approved by the institutional Animal Care and Use committee (University of Alberta, AB; AUP00002303) and the calves were cared for and handled according to the Canadian Council on Animal Care (CCAC, 2009) regulations and the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 2010).

Briefly, 48 Holstein bull calves (± 3 d of age) were enrolled in a completely randomized design with a 2 × 2 factorial arrangement study. The treatments were MR (25:17, CP:fat on a DM basis and reconstituted to 14 % solids) fed at a rate of 0.749 kg of powder/d (**LO**) or up to 1.498 kg of powder/d (**HI**), and whole (**WC**) or flaked **(FC**; density 296.1 to 321.8 kg/m^3) corn in a textured CS (37 % corn, 35 % supplement pellet, 25 % whole oats, and 3 % molasses; 41.5 % starch; 21.3 % CP, 3.6 % crude fat, 7.2 % ADF, 15.4 % NDF). Calves assigned to LO MR (n = 24) received MR at the following rate (DM basis): 0.749 kg/d for 5 wk, followed by 0.375 kg/d during wk 6. Calves assigned HI (n = 24) received MR at the following rate: 0.749 kg/d during wk 1, 0.851 kg/d during wk 2, 1.135 kg/d during wk 3, 1.498 kg/d during wk 4 and 5 and 0.749 kg/d during wk 6. All the calves (LO and HI) were weaned at the end of wk 6. Experimental textured CS and fresh water were offered *ad libitum* daily.

3.2.2. Data and sample collection

Ruminal Fermentation Characteristics. All 48 calves were used to delineate the dietary effects on ruminal fermentation. Ruminal fluid was sampled 5 hours after the AM feeding, at the

end of wk 5, 6, 7 and 8 using a Geishauser probe (adapted for calves; Geishauser, 1993) fitted to a 60 ml syringe. Ruminal contents were squeezed through 4 layers of cheesecloth. Ruminal pH was measured immediately using a hand-held pH meter (Apera Instruments, AI209, PH20) and an aliquot of 8 mL and 2 ml of ruminal fluid were snap frozen (dry ice and ethanol mixture) and stored at -20˚C until SCFA (n = 48) and *E. coli* (n = 24) analysis, respectively. In addition, 10 mL extra rumen fluid (n = 24) was collected at wk 5, 7 and 8 for LPS analysis. Samples were subjected to initial processing right after collection (Gozho et al., 2005). Briefly, at collection, samples were put on ice, then centrifuge for 30 min at 10,000 × *g*. The supernatant was filtered using a disposable 0.22-μm sterile, pyrogen-free filter (Millex, Millipore Corporation, Bedford, MA). The filtered fluid was then heated at 100°C for 30 min, cooled and stored at −20°C until LPS analysis.

Hindgut Fermentation Markers. To determine dietary effects on hindgut fermentation markers, spot fecal samples were collected from a subset (n = 24) of calves every 4 hrs for 24 hrs at the end of wk 5, 6, 7 and 8. Fecal pH was immediately measured (Apera Instruments, AI209, PH20) and 1.5 g of fecal matter was snap frozen (dry ice and ethanol mixture) and stored at -20˚C for SCFA analysis (wk 5, 6, 7 and 8) and quantification of *E.coli* analysis. Additional 6 g fecal samples were taken on wk 5, 7 and 8, and processed for LPS quantification by mixing 6 ml of 0.9 % physiological saline with the feces. Samples were then further processed, similar to the rumen fluid samples. The rest of the fecal matter was composited over the 24 hrs and stored at -20˚C pending starch analyses.

Serum acute phase proteins. Blood samples were collected weekly at the end of wk 5 to 8, 2.5 hours after the morning feeding, via jugular venipuncture into a vacutainer tube (silica-coated clot-activating vacutainer tube; Becton Dickinson, Franklin Lakes, NJ) from all 48 calves. The tube was inverted for 30 s and stored until clotted (± 30 min) and centrifuged at 1,500 x g, RT for 15 min. The serum was then transferred into microcentrifuge tubes and stored at −20°C for later analysis of serum amyloid A (**SAA**) and LPS binding protein (**LBP**).

3.2.3. Sample analyses

Quantification of E. coli bacteria in rumen and feces. DNA was extracted from rumen fluid and fecal samples taken at wk 6 and 7, as described in the companion paper (Van Niekerek et al., 2020). Real-Time PCR was used to estimate 16S rRNA gene copy numbers from wk 6 and 7

samples of total bacteria, also described in the companion paper. The DNA obtained were used for Real-Time PCR to estimate 16S rRNA gene copy numbers of *E. coli* with specific primers (forward: GGAAGAAGCTTGCTTCTTTGCTGAC and reverse: AGCCCGGGGATTTCACATCTGACTTA); melting temperature 62 °C ; product size 544; (Sabat et al., 2000) and by using SYBR Green chemistry (Fast SYBR Green Master Mix, Applied Biosystems, Foster City, CA) for PCR. Bacterial populations were estimated (Viia 7 Real-Time PCR System; Thermo-Fisher Scientific) and purified *E. coli* K12 16S rRNA gene was used as standard. The copy number of 16S rRNA genes per unit of sample for total bacteria or *E. coli* and the proportion of *E. coli* was obtained by using the same calculation described in the companion paper (Van Niekerk et al., 2020; Chapter 2).

Ruminal and Fecal Fermentation Characteristics. The composited 24 h fecal sample that was used as a marker of hindgut fermentation was analyzed (AOAC International, 2000) for DM (oven method 930.15) and starch (α-amylase method; Hall, 2009). To preserve ruminal fluid and fecal SCFA, 2 mL and 4 mL 25 % H_2PO_4 were added to frozen ruminal fluid and fecal matter, respectively. Samples were slowly thawed at RT, centrifuged (18,000 × g, 15 min, 4 °C) and 1 mL of supernatant was transferred to a clean tube. After adding 0.2 mL isocaproic acid, ruminal and fecal SCFA were quantified by gas chromatography (Khorasani et al., 1996). Ruminal and fecal free LPS concentrations were determined using Limulus amebocyte lysate end-point assay kits (QCL-1000, Lonza Group Ltd., Basel, Switzerland; Gozho et al., 2005) and the reaction times were monitored using a microplate reader (Cytation 5 microplate reader; BioTek Instruments, Winooski, VT). The intra-assay coefficient of variation (**CV**) values were 1.1 and 0.8 % for the rumen fluid and fecal samples, respectively.

Serum acute phase proteins. Serum amyloid A concentrations were determined using ELISA kits TP-802 (Tri-Delta Diagnostics Inc., Morris Plains, NJ) and LBP concentrations were determined using ELISA kit HK503 (HyCult Biotechnology, Uden, Netherlands). The samples were diluted according to the suggested manufacturer guidelines (SAA X500 and LBP X100) and for each duplicate sample the absorbance values were read at 450 nm (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA). The intra-assay CV values were 3.16 and 6.10 % for SAA and LBP, respectively, and the inter-assay CV values were 2.98 and 6.64 % for SAA and LBP, respectively.

3.2.4. Statistical analyses

The mixed model procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC) was used to analyze the data as a completely randomized 2 × 2 factorial design. The statistical model used was: $Y = \mu + MR + CS + (MR \times CS) + e$, where Y is the dependent variable, μ is the mean, MR is the effect of MR rate, CS is the effect of corn processing, MR × CS is the interaction of MR and corn processing in CS and e is the error term. Data were also analyzed as repeated measures. For free ruminal and fecal LPS concentrations the data were $\log_{10}$ transformed and used for statistical analysis, however actual concentrations (EU/mL) were reported. A Tukey test was used to separate the least squares means and at least 3 variance–covariance structures (auto-regressive type 1, Toeplitz and compound symmetry) were tested and the covariance structure that minimized the Schwarz's Bayesian information criterion was chosen. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Calf starter, ruminal and fecal fermentation characteristics, and acute phase proteins were used for pair relationships and estimated through Pearson correlation co-efficient using R software (3.6.1).

3.3. Results

Ruminal Fermentation Characteristics. Overall total SCFA concentration was the greatest ($P = 0.001$) at wk 7 and 8 compared to earlier wk. Total ruminal SCFA concentration was highest ($P \leq 0.02$) at wk 5 and 6, and tended to be highest ($P = 0.10$) at wk 7 in calves fed LO compared to HI (Table 3.7-1). Ruminal acetate proportions were lower in calves fed LO compared with HI diet at wk 5, 6 and 7 ($P \leq 0.032$). Calves fed LO diet had higher ($P = 0.001$) propionate proportions at wk 5 compared with calved fed HI diet. Calves fed LO diet had higher ($P \leq 0.048$) butyrate proportions at wk 6 and 7 compared with calved fed HI diet. Calves fed FC diet tended ($P = 0.088$) to have a higher acetate proportion at wk 7. Calves fed FC diet had higher ($P \leq 0.02$) propionate proportions at wk 5 and 6 compared to calves fed WC diet. Total SCFA were influenced ($P = 0.008$) by feeding rate of MR and corn processing at wk 6, with total SCFA being the highest in LOWC fed calves and the lowest in HIFC fed calves. Butyrate proportions were the highest ($P = 0.04$) in LOWC fed calves and the lowest in HIFC fed calves at wk 6. Ruminal pH was influenced by wk, with wk 5 (6.63 ± 0.10) having the highest and wk 7 having the lowest ruminal mean pH (5.57 ± 0.100); dietary treatments had no effect on ruminal mean pH.

Rumen *E. coli* density was higher ($P < 0.0001$) in rumen fluid at wk 5 than the other wk, and proportion (% of total bacteria) tended to be greater ($P = 0.051$) at wk 5 than wk 8. At wk 5, *E. coli* density in the rumen fluid tended to be higher ($P = 0.08$) and at wk 6 it was higher ($P = 0.004$) in calves fed HI compared with LO (Table 3.7-3). Proportion of *E. coli* in rumen fluid was 3-fold higher ($P = 0.031$) when calves were fed HI compared with LO at wk 6. Corn processing had no effect on *E. coli* density and proportion in rumen fluid. At wk 5, *E. coli* density in rumen fluid tended to be lower in the LOWC than in the HIWC. Ruminal fluid free LPS was unaffected by wk and dietary treatments (Figure 3.7-3).

Hindgut Fermentation Characteristics. Dietary treatment did not influence fecal DM (Table 3.7-2). Total fecal SCFA and the major SCFA, acetate, propionate and butyrate, were largely unaffected by dietary treatment. Total fecal SCFA were unaffected by wk. Total fecal SCFA tended ($P = 0.09$) to be higher at wk 6 in calves fed LO diet compared with HI. At wk 7, calves fed LO had lower ($P = 0.0022$) acetate proportions than calves fed HI. There was a wk ($P < 0.0001$) effect on fecal pH and fecal starch. Fecal pH decreased over time, whereas fecal starch increased over time (Figure 3.7-1A and B). At wk 5, fecal pH was higher ($P = 0.041$) for the calves fed LO diet compared to the calves fed HI diet (Figure 3.7-1A). At wk 6, it was the opposite, with calves fed HI diet having higher ($P = 0.039$) fecal pH compared to calves fed LO diet. Fecal pH was affected by feeding rate of MR, processing of corn in CS, and the interaction between the two at wk 8. Calves fed LO or FC diet had higher ($P \leq 0.002$) fecal pH compared to calves fed HI or WC diet, respectively. Calves fed HIWC diet had the lowest ($P = 0.02$) fecal pH (6.6 ± 0.09) compared to the calves fed other diets. At wk 6, fecal pH was lower ($P = 0.002$) at the 14 and 18 h sampling points compared to the 22 and 2 h sampling points (Figure 3.7-2B). At wk 7, fecal pH was lower ($P = 0.007$) at the 14 h sampling point compared to the 10, 22 and 2 h sampling points (Figure 3.7-2C). Fecal pH was lower ($P < 0.0001$) at the 14 h compared with the 10, 22 and 2 h sampling points at wk 8 (Figure 3.7-2D). The 6, and 18 h sampling points were also lower than the 22 and 2 h. At wk 8, calves fed HI diet tended to have higher ($P = 0.086$) fecal starch compared with calves fed LO diet (Figure 3.7-1B; 10.9 ± 2.60 vs. 6.8 ± 1.37 %). Corn processing or the interaction between MR rate and corn processing were unaffected for fecal starch.

Fecal *E. coli* density increased by wk, with wk 5 proving lower ($P = 0.03$) than 8 (6.9 ± 0.34 vs. 8.2 ± 0.64 log 10/g). Density of *E. coli* in fecal matter at wk 5 was higher ($P = 0.02$) in

the LO fed calves than HI fed calves. At wk 7, calves fed FC tended to have higher ($P = 0.08$) *E. coli* density in fecal matter than calves fed WC. Calves fed HIFC had higher ($P = 0.007$) density and tended to have higher ($P = 0.09$) proportion of fecal *E. coli* at wk 8 compared with calves fed HIWC. Fecal matter free LPS tended to be higher at wk 8 compared with wk 5 and dietary treatments did not affect free fecal LPS (Figure 3.7-3).

Acute phase proteins. Week 5 tended to have lower ($P = 0.09$) LBP concentration (Figure 3.7-4A) than wk 8, and wk 5 had higher ($P = 0.03$) SAA concentration than wk 7 (Figure 3.7-4B). Dietary treatments had no effect on LBP concentrations (Figure 3.7-4A). At wk 8, calves fed LO tended to have higher ($P = 0.10$) SAA concentrations than calves fed HI (39.1 ± 5.55 vs. 30.1 ± 4.37 ug/ml). At wk 7, calves fed WC tended to have higher ($P = 0.06$) SAA concentrations than calves fed FC (37.6 ± 5.22 vs. 28.4 ± 3.93 ug/ml; Figure 3.7-4B).

Relationship between intake, rumen and hindgut fermentation and inflammatory biomarkers. The analysis of correlation showed that total ruminal SCFA had a very strong negative correlation with ruminal pH (r = -0.79, $P < 0.001$); Figure 3.7-5). Calf starter intake had a moderate positive correlation with total ruminal SCFA (r = 0.57, $P < 0.001$) and fecal starch (r = 0.56, $P < 0.001$). A moderately positive correlation was also observed between total ruminal SCFA and fecal starch (r = 0.44, $P < 0.001$) and LBP and SAA (r = 0.45, $P < 0.001$). Weak positive correlations existed between total SCFA and fecal *E.coli* (r = 0.21, $P = 0.049$), fecal starch and fecal *E.coli* (r = 0.22, $P = 0.037$), ruminal pH and ruminal LPS (r = 0.26, $P = 0.034$), CS intake and fecal LPS (r = 0.35, $P = 0.003$), and ruminal pH and fecal pH (r = 0.36, $P = 0.0004$). Weak negative correlations also existed between total ruminal SCFA and fecal pH (r = -0.37, $P = 0.0002$), CS intake and ruminal pH (r = -0.32, $P = 0.002$) , CS intake and fecal pH (r = -0.31, $P = 0.002$), ruminal pH and fecal starch (r = -0.31, $P = 0.002$), fecal starch and fecal pH (r = -0.30, $P = 0.003$), total ruminal SCFA and ruminal *E.coli* (r = -0.28, $P = 0.006$), CS intake and *E.coli* (r = -0.28, $P = 0.006$), fecal pH and fecal LPS (r = -0.26, $P = 0.03$), and ruminal pH and fecal *E.coli* (r = -0.25, $P = 0.02$).

3.4. Discussion

The goal of this study was to determine the impact of amount of MR fed and the processing method of corn in CS on rumen and hindgut fermentation, LPS concentrations and *E. coli* numbers, and acute phase proteins in dairy calves. Based on our findings, there are limited

interactions, and as such, this section will also focus on the effect of MR feeding rate and the processing of corn in CS separately.

3.4.1. Interaction of MR feeding rate with processing of corn in calf starter

The total ruminal SCFA at wk 6 was lower for calves fed the HIWC compared with the other dietary treatments, which may indicate that calves fed the WC CS had lower rumen fermentability and starch bypassed to the hindgut, as CS intakes were similar for the HIFC fed calves (Van Niekerk et al., 2020; Chapter 2). It is unclear whether this resulted in increased hindgut fermentation, since dietary treatment did not influence total fecal SCFA or fecal starch.

At wk 8, calves fed the HIWC diet had lower fecal pH. The decreased fecal pH in calves fed the HIWC diet is likely a response to the sudden increase in CS after weaning (Van Niekerk et al., 2020; Chapter 2), which increases passage rate of starch leading to greater amounts of starch being fermented in the hindgut (Depenbusch et al., 2008). When large quantities of starch escape the rumen and small intestine when animals are fed high grain diets, it leads to increased acidity in the hindgut that can damage integrity and leave the animal at greater risk of inflammation (Tao et al., 2014a,b). When hindgut acidosis was induced in adult ruminants by infusing 4 kg/d of corn starch into the abomasum, fecal pH decreased from 7 to below 5 (Bissell and Hall, 2010). Fecal pH is not the only indicator of possible hindgut acidosis. Other symptoms include fecal fluidity and the presence of mucin casts or tissue in feces (Bissell and Hall, 2010), although fecal DM was not affected in our study. Unlike the thresholds used to determine ruminal acidosis, the literature does not dictate any fecal pH thresholds to declare hindgut acidosis. Fecal *E. coli* density was higher in calves fed HIFC and had slightly higher proportions than calves fed HIWC at wk 8. This is the opposite of what was expected. We hypothesized that the HIWC diet would increase the amount of starch bypassing the rumen, which would increase hindgut fermentation and lead to increased *E. coli* proportions, and possibly even increased free fecal LPS. Thus, based on the fecal pH we cannot conclude if calves fed the HIWC diet experienced hindgut acidosis. However, since fecal *E. coli,* LPS, SAA and LBP were unaffected during this time, the decrease in fecal pH did not affect inflammatory biomarkers. We speculate that hindgut acidosis may not have occurred in the calves fed HIWC because calves may have the ability to utilize whole corn kernels more effectively than cows by chewing their feed more

thoroughly (Owens, 2005). Thus, the WC may have also been fermented in the rumen like the FC.

3.4.2. Effect of MR feeding rate

Total ruminal SCFA in our study was greatest at wk 7 and 8; this increase in total SCFA production was due to increased CS intake over time (Van Niekerk et al., 2020; Chapter 2), indicated by the positive correlation between total SCFA and CS intake. Calves fed the LO diet had higher total SCFA concentration at wk 5, 6 and 7, which reflects more microbial fermentation as a result of higher CS intake. This is in agreement with various studies reporting that increased CS intake results in higher total ruminal SCFA concentration pre-weaning and immediately after weaning (Suarez-Mena et al., 2015; Steele et al., 2017). When concentrate diets are fed to animals, it is likely that ruminal propionate and butyrate concentrations will increase as a result of increased starch digesting bacteria present in the rumen that favor the production of these SCFA (Sander et al., 1959). Our results reinforced these findings, as calves fed the LO diet had increased CS intake, which resulted in lower acetate proportions at wk 5, 6 and 7 compared with calves fed the HI diet. Butyrate production is critical in early life for rumen development (Sander et al., 1959; Mentschel et al., 2001). Therefore, the minimal CS intake resulting from high MR feeding rate in our study could be associated with a decrease in microbial fermentation products, which suggests a delay in rumen development.

We anticipated that the observed increase in CS intake (Van Niekerk et al., 2020; Chapter 2) would result in increasing total SCFA in the rumen and lowering ruminal pH of calves fed the LO diet; however, no difference was observed in the current study. Ruminal pH was higher than 5.8 during wk 5, 6 and 8, but at wk 7 it was below 5.8, suggesting that the animal could experience sub-acute ruminal acidosis (Schwartzkopf-Genswein et al., 2003). Even though there is a strong negative correlation between ruminal pH and total ruminal SCFA, and weak negative correlation between ruminal pH and CS intake, ruminal pH recovered at wk 8 – despite increased started intake – suggesting that the combination of buffering through rumination and absorption of SCFA became more effective over time. The calves in the currents study were bedded on wheat straw, which may have been consumed, and thus may have influenced ruminal pH. The sampling techniques may have also influenced the data, as a Geishauser probe was used to obtain the spot ruminal fluid samples and saliva contamination could have possibly influenced the

SCFA concentration and pH values observed in the current study (Duffield et al., 2004). Only one sample was taken per calf per timepoint in the current study, which does not account for diurnal changes caused by time of intake and extent of fermentation (Kim et al., 2016). Therefore, the ruminal fermentation parameters reported in the current study only provide a small snapshot.

Gram negative bacteria, like *E. coli,* stimulate a host inflammatory response by producing endotoxins like LPS (Erridge et al., 2002). An increase of *E. coli* in ruminal fluid has been reported in adult animals fed high grain diets that induced SARA (Diez-Gonzalez et al., 1998; Nagaraja and Titgemeyer, 2007). This is in disagreement with the current study, where *E. coli* density was higher earlier in life when calves did not consume as much CS (Van Niekerk et al., 2020; Chapter 2). Moreover, it disagrees with our finding that CS intake is affected by MR feeding rate (Van Niekerk et al., 2020; Chapter 2), where calves fed HI MR feeding rate had lower CS intake pre-weaning and during weaning and higher ruminal fluid *E. coli* density and proportion at these times. This might be because SARA was not induced during this time – for, as Khafipour et al. (2009c) reported, *E. coli* density and SARA severity are highly correlated. Taking this into consideration, the fact that ruminal fluid LPS was unchanged for wk or MR feeding rate is likely because pH was not low enough to cause bacteria lysing.

Ruminal LPS concentration and its association with SARA is well studied in adult ruminants, however in young ruminants more knowledge is needed. Plaizier et al. (2012) summarized free LPS concentrations in rumen fluid from 12 studies where SARA was induced by feeding high concentrate diets in mature animals. In some studies (Gozho et al., 2005; Gozho et al., 2006; Emmanuel et al., 2008) the LPS concentrations observed during SARA were well within range (up to 31,749 EU/mL) observed in the current study. In other studies though (Khafipour et al., 2009a,b; Li et al., 2012), observed free LPS concentrations during SARA challenge were much higher (up to 145,593 EU/mL) than those in the current study. It is important to note that the severity of SARA (time spend below pH 5.6/d) was also different in all of these studies. The starch amounts ranged from 22.4 to 33.7 % in the studies that reported high free ruminal LPS concentrations (Khafipour et al., 2009a,b; Li et al., 2012), which is much lower than the 41.5 % starch in the CS fed to calves in the current study. Based on these mature animal studies, SARA clearly played a key role in free LPS concentration in the rumen. Based on the aforementioned mature animal studies, SARA plays a key role in free LPS concentration in the

rumen; however, ruminal pH was only measured at one timepoint each wk and these timepoints did not indicate that the calves experienced SARA during wk 5, 6 and 8 even though the starch in the CS was high. This could explain why rumen LPS was within average range. The weak positive correlation between ruminal pH and LPS does indicate that there is a relationship between ruminal pH and LPS, even though there was no difference between wk for ruminal LPS.

When nutrients, such as starch, bypass the rumen, they can still be fermented post-ruminally. Pancreatic amylase in the small intestine can digest starch, however, only up to 60 % of starch entering the small intestine is likely to be digested due to enzymatic resistance; ultimately, this leads to starch reaching the large intestine (Harmon et al., 2004). Fecal starch concentration increased over time in our study, suggesting that as CS intake increased over time (Van Niekerk et al., 2020; Chapter 2), the undeveloped GIT was not able to utilize all the starch in the CS. There were wide variations in fecal starch, as noted in Figure 3.7-1B, among calves within the same dietary treatment. The tendency of higher fecal starch when calves were fed the HI diet compared with the LO diet could also be an indication that these calves' GITs were not capable of digesting the starch in the calf starter, even though CS intake tended to be lower. When calves were weaned 2 wk apart (wk 6 and 8) and fecal starch was evaluated, calves fed elevated levels of milk (1.2 kg/d of MR) and weaned earlier had the most drastic fecal starch changes after weaning (Eckert et al., 2015). This might indicate that starch digestion in the GIT depends on calf age, regardless of MR intake. Early data also indicates that amylase activity increases with calf age (Huber et al., 1961; Guilloteau et al., 1985) which could impact starch digestion. The CS starch level in our study was higher than in the Eckert et al. study though, which may have influenced the tendency of fecal starch between the feeding rates of MR. Steele et al. (2017) investigated the impact of gradual vs. abrupt weaning in calves fed an elevated level of milk (1.35 kg/d of MR) and reported that fecal starch increased over time for both groups, but that abruptly weaned calves had higher fecal starch than gradually weaned calves. Thus, age and weaning method could play a big role in the amount of fecal starch that is digested.

Fecal starch is used in dairy cows to determine efficiency, with < 5 % as ideal (Firkins et al., 2001). Independent of dietary treatment, fecal starch in our study was >5 % at wk 7 and 8, which is similar to abruptly weaned calves (Steele et al., 2017) and calves fed a 39.5 % starch texturized CS (Quigley et al., 2019). With the increase in starch passing through the hindgut, increased hindgut fermentation is expected, but we only observed a slight increase in total fecal

SCFA at wk 6 when calves were fed the LO diet compared with the HI diet. In addition, fecal pH increased at wk 5 and decreased at wk 6 for calves fed the LO diet compared to the HI diet. Similarly, fecal starch concentration increased, whereas fecal pH decreased, as animals consumed more grain, which was also reported in young calves (Suarez-Mena et al., 2015; Suarez-Mena et al., 2016), lambs, older calves (Wheeler and Noller, 1977), yearlings (Depenbusch et al., 2008) and steers (Wheeler and Noller, 1977). When calves consume more CS (wk 6, 7 and 8) it causes diurnal fecal pH changes, with fecal pH reaching a low at 1400 h and a high at 2200 and 0200 h, which indicate the importance of these changes when hindgut fermentation is studied. Fecal fluidity is an indicator of intestinal starch fermentation (Gressley et al., 2011), however in the current study, fecal dry matter did not differ by wk or dietary treatment. With increased starch reaching the hindgut, there was a possibility that fecal *E. coli* density would be higher, which proved true in the current study.

Higher fecal *E. coli* led to a slight increase in free fecal LPS concentrations. In the current study, thefree LPS concentrations from the fecal matter were higher than those in the rumen. On 10 surveyed dairy farms, fecal LPS concentrations ranged from 3,514 to 252, 345 EU/g of wet feces (Li et al., 2010). The values in the current study were closer to the upper limit of this survey. The animals' diet may contribute to the variation observed for fecal LPS in the surveyed study. When grain induced SARA was compared with alfalfa pellet induced SARA, it was noted that fecal LPS was not affected by the alfalfa pellet diet, which means that LPS in the hindgut is more affected by starch bypassing the rumen (Khafipour et al., 2019b). The site of fermentation is important because the hindgut has a monolayer epithelial structure, which may be more susceptible to pH changes caused by excessive fermentation, unlike the rumen which has a multilayered epithelial structure (Steele et al., 2016). The SARA induced by the alfalfa pellet did not cause an inflammatory response whereas the grain induced SARA did, meaning that increased fermentation in the hindgut plays a bigger role in animal acute phase protein inflammatory response. It may be possible that the endotoxic activity from the LPS in the feces were low and therefore no inflammatory was response observed. However, to our knowledge, measuring endotoxic activity is a complicated undertaking and future studies are required. In the current study, fecal starch increased over time, but fecal LPS only increased slightly over time

The fecal starch values (>5 %) might be associated with GIT inflammation (Li et al., 2010) caused by increased LPS. It has been reported that when fecal starch is higher in dairy

cows, it can be linked to LPS production, which can cause systemic inflammation of the GIT (Khafipour et al., 2009c; Li et al., 2012). The acute phase protein LBP tended to be higher during wk 8, which may indicate that LPS translocation to the blood is higher during this time. There was a moderate negative correlation between CS intake and SAA, which indicates that the increase in CS did not evoke an inflammatory response in the current study, unlike in cows that were fed high-grain diets where SAA and LBP in the blood were increased (Khafipour et al., 2009c), indicating inflammatory response activation. Similarly, SAA concentrations were increased in cows fed high-grain, but other inflammatory markers (e.g. haptoglobin and fibrinogen) were unchanged (Gozho et al., 2007). Gozho et al. (2005) reported that SAA and haptoglobin increased 1 and 3 d respectively after grain was introduced to steers. In all three previously mentioned studies, the animals experienced SARA due to the high-grain diet. It has been reported that in healthy calves, acute phase proteins peak at 1 wk of life (up to 300 μg/m) (Seppä-Lassila et al., 2013), and thereafter decrease (Orro, 2008; Seppä-Lassila et al., 2013; Tóthová et al., 2015) to concentrations of 80 to 200 μg/mL (Seppä-Lassila et al., 2013) for SAA, which are considered healthy levels for calves. The basal range for LBP is from 10.8 to 37.8 μg/mL (Bannerman et al., 2003; Suojala et al., 2008). Based on these levels, the calves in the current study did not experience an inflammatory response before, during, or after weaning. Kim et al. (2011) reported that 3 and 5 d post-weaning SAA concentrations (49.67 ± 9.99 μg/mL and 47.26 ± 11.45 μg/mL, respectively) were elevated in calves compared with pre-weaning SAA concentrations (20.97 ± 4.09 μg/mL). The authors attributed the increase in SAA to weaning stress. It is possible that the calves in the current study also experienced weaning stress, however, due to sampling schedule (every 7 d) it may have not been reflected because SAA has a short half-life (Jacobsen and Andersen, 2007; Wells et al., 2013). In the Kim et al. (2011) study, samples were not taken after 5 d post-weaning so it is unclear if SAA concentrations would still be elevated at that time due to weaning stress. Overall, in the current study, CS intake played a role in total ruminal and fecal SCFA and fecal starch, pH and *E. coli* density. However, CS intake had no influence on LPS or acute phase proteins concentrations.

3.4.3. Effect of corn processing

Readily fermentable carbohydrates are commonly fed to calves, as they are more nutrient dense compared to forages and offer greater energy allowance for growth in young calves. Readily fermentable carbohydrates are also important to promote rumen development (Flatt et

al., 1958; Sander et al., 1959). Grain is processed to maximize starch digestibility, but this processing can change the site of digestion (Owens, 2005). In mature ruminants, total tract digestibility of whole corn was 84 %, whereas steam rolled (or flaked) was 97 % (Owens, 2005). Since the effect of processing of corn did not influence rumen or hindgut fermentation markers (SCFA and pH), it could indicate that the WC and FC diets were digested in a similar manner among sites (rumen and hindgut). Processing of grain has been shown to reduce ruminal pH (Lesmeister and Heinrichs, 2004; Terré et al., 2016), but this was not observed in our study. It is possible that the long wheat straw bedding used in the current study may have influenced ruminal pH – especially given the contrast with Lesmeister and Heinrichs (2004) and Terré et al. (2016), who bedded with sawdust. In feedlot animals, fecal starch was much higher (25.9 %) when the animals were fed whole corn compared with flaked corn (1.8 %), which is in disagreement with the current study, where processing of corn had no influence on fecal starch (Corona et al., 2006). We expected WC would bypass rumen fermentation and instead be fermented in the hindgut, increasing the likelihood of greater *E. coli* proportions, which in turn would lead to higher LPS production. However, fecal *E. coli* density and free LPS were unaffected because there were no differences in fecal starch or pH. Overall, the processing of corn in CS did not have an effect on intake, ruminal and fecal SCFA, pH, LPS and *E. coli* and fecal starch. As a result, performance (Van Niekerk et al., 2020; Chapter 2) was also not influenced.

3.5. Conclusion

In this study, feeding HI rate of MR combined with WC CS resulted in a decrease in fecal pH at wk 8. However, there is no evidence to suggest that hindgut acidosis occurred, as inflammatory biomarkers were not affected in this treatment group. There may be other factors that contributed to our findings: for instance calves may have the capability of chewing the feed more thoroughly, impacting the amount of starch that reached the hindgut. When considering the combination of feeding rate of MR and the processing of corn feed, low and high feeding rate of MR can be combined with either flaked corn or whole corn in the CS.

3.6. Literature cited

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3.7. Tables and figures

Table 3.7-1. Effect of treatment[1] on rumen fermentation wk 5 to 8 in dairy calves.

	Treatments					Contrast, *P* value		
Item	LOWC	LOFC	HIWC	HIFC	SEM	LO vs. HI	FC vs. WC	Interaction
Total SCFA, µmol/ml								
Week 5	55.36	45.63	39.64	40.29	4.372	0.013	0.33	0.4
Week 6	74.52	61.31	45.35	62.95	5.562	0.017	0.70	0.008
Week 7	97.63	91.04	88.97	84.44	4.519	0.10	0.23	0.82
Week 8	79.88	86.75	80.51	86.15	5.584	1.00	0.27	0.91
Acetate, mol/100mol								
Week 5	56.02	56.66	63.63	59.20	1.700	0.005	0.27	0.14
Week 6	47.22	47.17	52.61	49.05	1.507	0.020	0.24	0.25
Week 7	48.11	47.18	50.73	48.53	0.896	0.032	0.088	0.48
Week 8	48.35	47.55	48.61	48.04	0.751	0.62	0.37	0.88
Propionate, mol/100mol								
Week 5	29.61	30.76	23.22	27.95	1.222	0.001	0.02	0.15
Week 6	30.20	36.12	30.83	36.19	1.305	0.79	<0.0001	0.83
Week 7	36.89	39.86	38.45	38.48	1.162	0.94	0.20	0.21
Week 8	39.81	41.03	39.98	40.20	0.802	0.68	0.37	0.53
Butyrate, mol/100mol								
Week 5	8.89	7.63	7.69	7.32	0.968	0.44	0.41	0.64
Week 6	16.75	11.00	10.51	10.17	1.285	0.009	0.023	0.041
Week 7	10.20	8.39	6.94	7.91	0.915	0.048	0.65	0.14
Week 8	7.19	6.81	6.92	7.16	0.587	0.94	0.90	0.60
Isobutyrate, mol/100mol								
Week 5	1.25	1.31	1.50	1.58	0.172	0.14	0.71	0.95
Week 6	0.91	0.88	1.25	0.67	0.122	0.62	0.016	0.030
Week 7	0.70	0.62	0.57	0.73	0.113	0.92	0.72	0.30
Week 8	0.83	0.76	0.81	0.85	0.129	0.80	0.93	0.65
Valerate, mol/100mol								
Week 5	2.61	1.92	1.90	1.84	2.092	0.14	0.16	0.23
Week 6	3.76	3.63	3.02	3.04	0.285	0.025	0.84	0.79
Week 7	3.16	3.11	2.56	3.36	0.320	0.59	0.25	0.19
Week 8	2.69	2.80	2.63	2.63	0.246	0.64	0.84	0.81
Isovalerate, mol/100mol								
Week 5	1.62	1.72	2.06	2.10	0.251	0.11	0.78	0.91
Week 6	1.16	1.20	1.79	0.88	0.172	0.37	0.016	0.009
Week 7	0.95	0.84	0.76	1.00	0.171	0.90	0.71	0.31
Week 8	1.13	1.05	1.05	1.12	0.185	0.96	0.98	0.69
Caproate, mol/100mol								
Week 5	0.40	0.24	0.32	0.19	0.299	0.29	0.030	0.73
Week 6	0.84	0.50	0.42	0.32	0.096	0.003	0.028	0.22
Week 7	0.49	0.53	0.39	0.32	0.081	0.063	0.80	0.50
Week 8	0.87	0.81	0.90	0.89	0.109	0.64	0.72	0.81
Ruminal pH								
Week 5	6.54	6.57	6.68	6.73	0.095	0.12	0.68	0.98
Week 6	6.09	6.22	6.32	5.99	0.159	1.00	0.53	0.16
Week 7	5.53	5.63	5.48	5.63	0.100	0.84	0.24	0.84
Week 8	6.11	6.19	6.05	5.99	0.146	0.38	0.93	0.63

[1] Treatment: LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d; WC = whole corn and FC = steam-flaked corn in textured calf starter.

Table 3.7-2. Effect of treatment[1] on fecal dyr matter (DM) and short chain fatty acids (SCFA) in dairy calves.

	Treatments					Contrast, *P* value		
Item	LOWC	LOFC	HIWC	HIFC	SEM	LO vs. HI	FC vs. WC	Interaction
DM, %								
Week 5	22.50	20.35	19.47	19.82	1.37	0.21	0.52	0.37
Week 6	19.75	17.37	19.42	20.23	1.57	0.43	0.62	0.32
Week 7	18.83	21.20	15.80	19.02	1.72	0.14	0.12	0.81
Week 8	20.12	19.88	19.20	19.60	1.92	0.76	0.97	0.87
Total SCFA, µmol/g								
Week 5	107.27	90.52	98.17	100.23	15.55	0.98	0.64	0.55
Week 6	121.36	98.47	97.22	86.12	10.17	0.09	0.11	0.57
Week 7	94.02	88.75	86.63	103.90	12.97	0.77	0.65	0.40
Week 8	114.84	88.29	95.19	93.50	14.01	0.61	0.33	0.39
Acetate, mol/100mol								
Week 5	59.42	59.28	59.11	62.91	2.64	0.54	0.49	0.46
Week 6	65.09	64.16	62.51	64.90	1.66	0.59	0.67	0.33
Week 7	68.41	68.33	72.59	71.54	1.48	0.022	0.71	0.75
Week 8	71.98	71.69	71.66	72.94	1.23	0.71	0.69	0.54
Propionate, mol/100mol								
Week 5	23.60	23.48	24.20	21.75	1.25	0.65	0.31	0.36
Week 6	22.63	22.79	22.57	22.98	1.10	0.95	0.80	0.91
Week 7	19.53	19.08	16.63	17.50	1.34	0.11	0.88	0.63
Week 8	17.83	17.04	18.67	16.89	1.11	0.76	0.26	0.66
Butyrate, mol/100mol								
Week 5	9.21	8.98	9.60	8.37	0.82	0.89	0.38	0.55
Week 6	7.92	7.99	7.93	7.41	0.70	1.00	1.00	0.99
Week 7	7.61	7.55	6.69	7.17	0.62	0.31	0.74	0.66
Week 8	6.27	7.17	6.51	5.86	0.49	0.29	0.81	0.13
Isobutyrate, mol/100mol								
Week 5	3.15	2.95	2.54	2.23	0.49	0.19	0.61	0.91
Week 6	1.33	1.82	2.56	1.90	0.41	0.12	0.84	0.17
Week 7	1.32	1.67	1.45	1.18	0.27	0.51	0.89	0.26
Week 8	1.37	1.49	1.10	1.52	0.16	0.46	0.098	0.35
Valerate, mol/100mol								
Week 5	0.58	1.31	0.59	1.36	0.36	0.93	0.048	0.95
Week 6	1.49	1.05	1.19	0.30	0.37	0.18	0.091	0.57
Week 7	1.77	1.61	1.17	1.43	0.45	0.40	0.92	0.65
Week 8	1.20	1.25	1.08	1.46	0.14	0.74	0.14	0.25
Isovalerate, mol/100mol								
Week 5	3.70	3.61	3.57	2.80	0.72	0.51	0.55	0.64
Week 6	1.32	1.94	3.00	2.16	0.55	0.099	0.84	0.20
Week 7	1.15	1.55	1.31	0.98	0.29	0.48	0.91	0.22
Week 8	1.13	1.15	0.79	1.14	0.14	0.22	0.19	0.25
Caproate, mol/100mol								
Week 5	0.35	0.39	0.39	0.59	0.06	0.074	0.079	0.23
Week 6	0.22	0.25	0.24	0.34	0.05	0.29	0.17	0.44
Week 7	0.20	0.21	0.16	0.20	0.03	0.38	0.50	0.70
Week 8	0.22	0.20	0.19	0.18	0.02	0.27	0.48	0.94

[1] Treatment: LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d; WC = whole corn and FC = steam-flaked corn in textured calf starter.

Table 3.7- 3. The influence of 4 feeding regimens[1] on ruminal and fecal *E.coli* in dairy calves.

	Treatments					Contrast, *P* value		
Item	LOWC	LOFC	HIWC	HIFC	SEM	LO vs. HI	FC vs. WC	Interaction
Rumen								
Copy number/ml (log10)								
Week 5	6.90	7.34	7.69	7.34	0.215	0.080	0.77	0.10
Week 6	6.41	6.56	6.99	7.12	0.178	0.004	0.42	0.97
Week 7	6.35	6.74	6.52	6.70	0.242	0.79	0.27	0.68
Week 8	6.53	6.32	6.59	6.56	0.215	0.51	0.57	0.68
% of total bacteria								
Week 5	0.0038	0.0114	0.0392	0.0071	0.00357	0.27	0.39	0.17
Week 6	0.0019	0.0042	0.0089	0.0099	0.00272	0.031	0.56	0.80
Week 7	0.0020	0.0059	0.0053	0.0071	0.00315	0.5	0.39	0.75
Week 8	0.0025	0.0020	0.0045	0.0024	0.00190	0.5	0.48	0.69
Fecal matter								
Copy number/g (log10)								
Week 5	7.24	7.36	6.29	6.52	0.335	0.02	0.61	0.87
Week 6	7.31	6.98	8.03	7.87	0.700	0.28	0.74	0.91
Week 7	7.34	7.89	7.96	8.74	0.775	0.36	0.41	0.89
Week 8	8.33	7.60	6.96	9.98	0.638	0.42	0.080	0.007
% of total bacteria								
Week 5	0.032	0.034	0.007	0.028	0.0180	0.40	0.54	0.62
Week 6	0.154	0.188	1.61	1.22	0.734	0.11	0.82	0.78
Week 7	0.172	3.16	1.07	1.76	1.315	0.86	0.19	0.40
Week 8	1.68	0.22	0.02	4.79	3.277	0.42	0.36	0.09

[1] Treatment: LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d; WC = whole corn and FC = steam-flaked corn in textured calf starter

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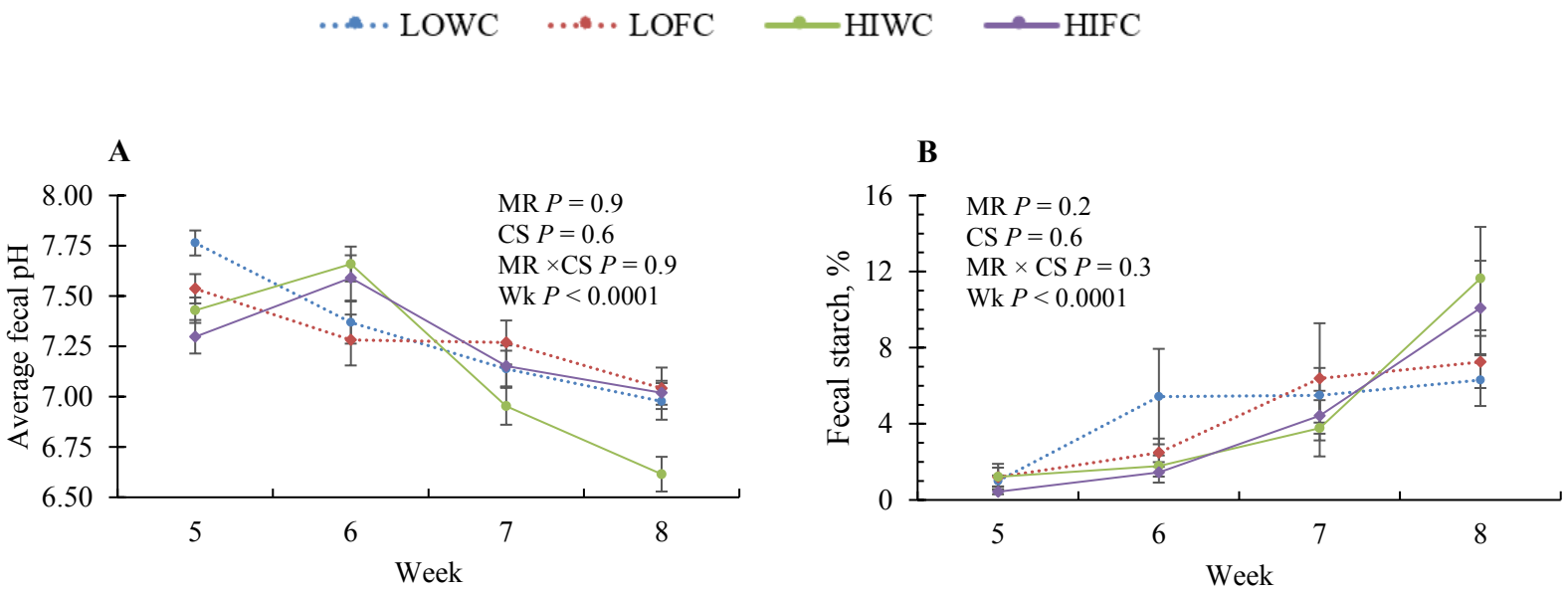

Figure 3.7- 1. Mean weekly (A) fecal pH and (B) fecal starch for calves fed a low (LO) or high (HI) milk replacer (MR) feeding rate and calf stater (CS) containing whole (WC) or flaked (FC) corn (wk 1 to 8). Error bars represent SEM for each treatment × wk interaction. Milk replacer was fed at LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d. At wk 5 and 6, calves fed LO diet had higher ($P = 0.041$) and lower ($P = 0.039$) fecal pH respectively, than calves fed HI diet (A). Fecal pH was affected by MR rate, processing of CS, and the interaction between the two at wk 8. Calves fed LO or FC diet had higher ($P \leq 0.002$) fecal pH compared to calves fed HI or WC diet, respectively. Calves fed HIWC diet had the lowest ($P = 0.02$) fecal pH (6.6) compared to the calves fed other diets. Fecal starch tended to be higher ($P = 0.086$) in the calves fed HI diet compared with calves fed LO diet at wk 8 (B).

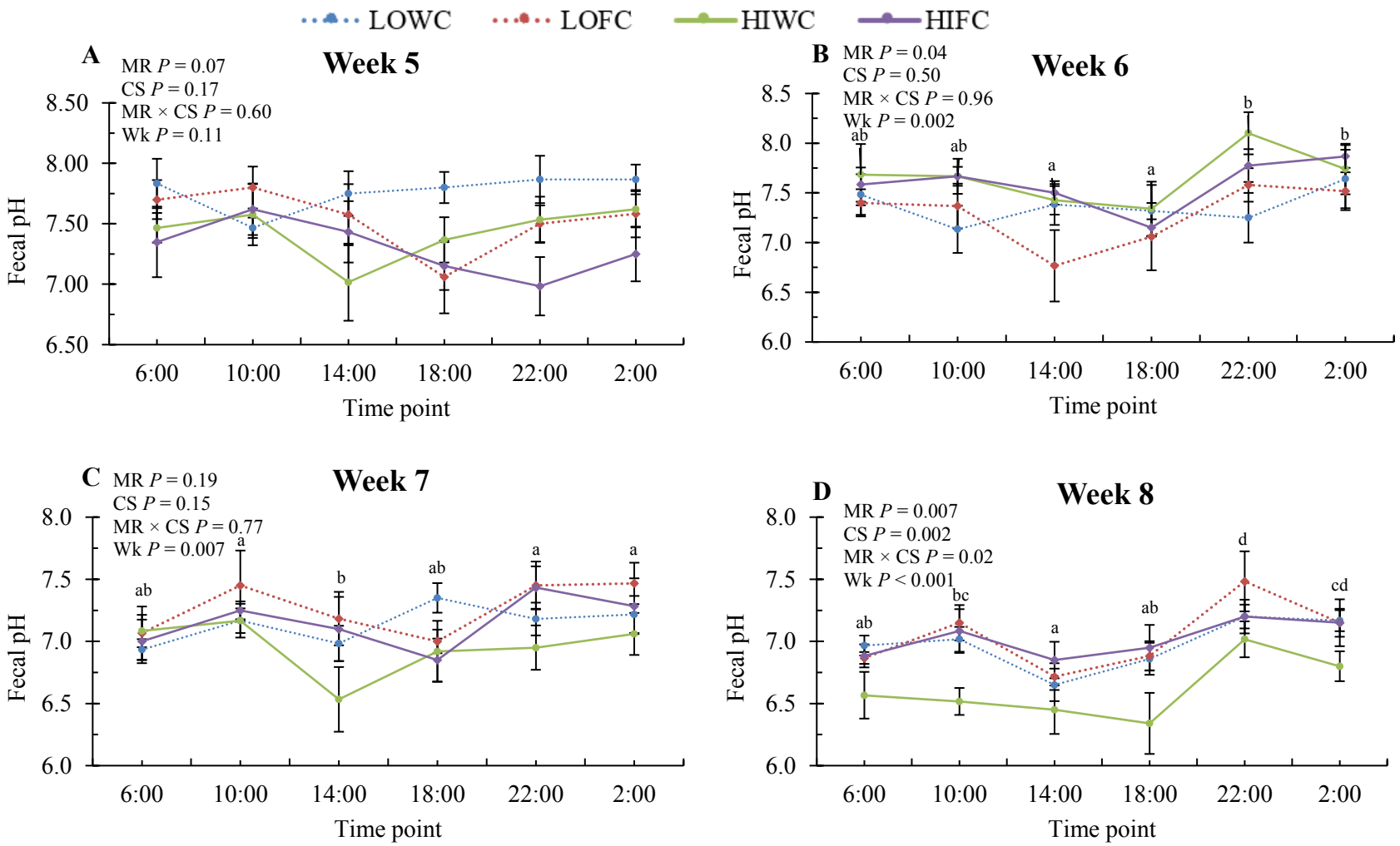

Figure 3.7- 2 Fecal pH measured every 4 hours for a day during week 5 (A), 6 (B), 7 (C) and 8 (D) from calves fed a low (LO) or high (HI) milk replacer (MR) feeding rate and calf stater (CS) containing whole (WC) or flaked (FC) corn. Error bars represent SEM for each treatment × time point interaction. Milk replacer was fed at LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d. Different letters represent a significant difference ($P < 0.05$) between time points.

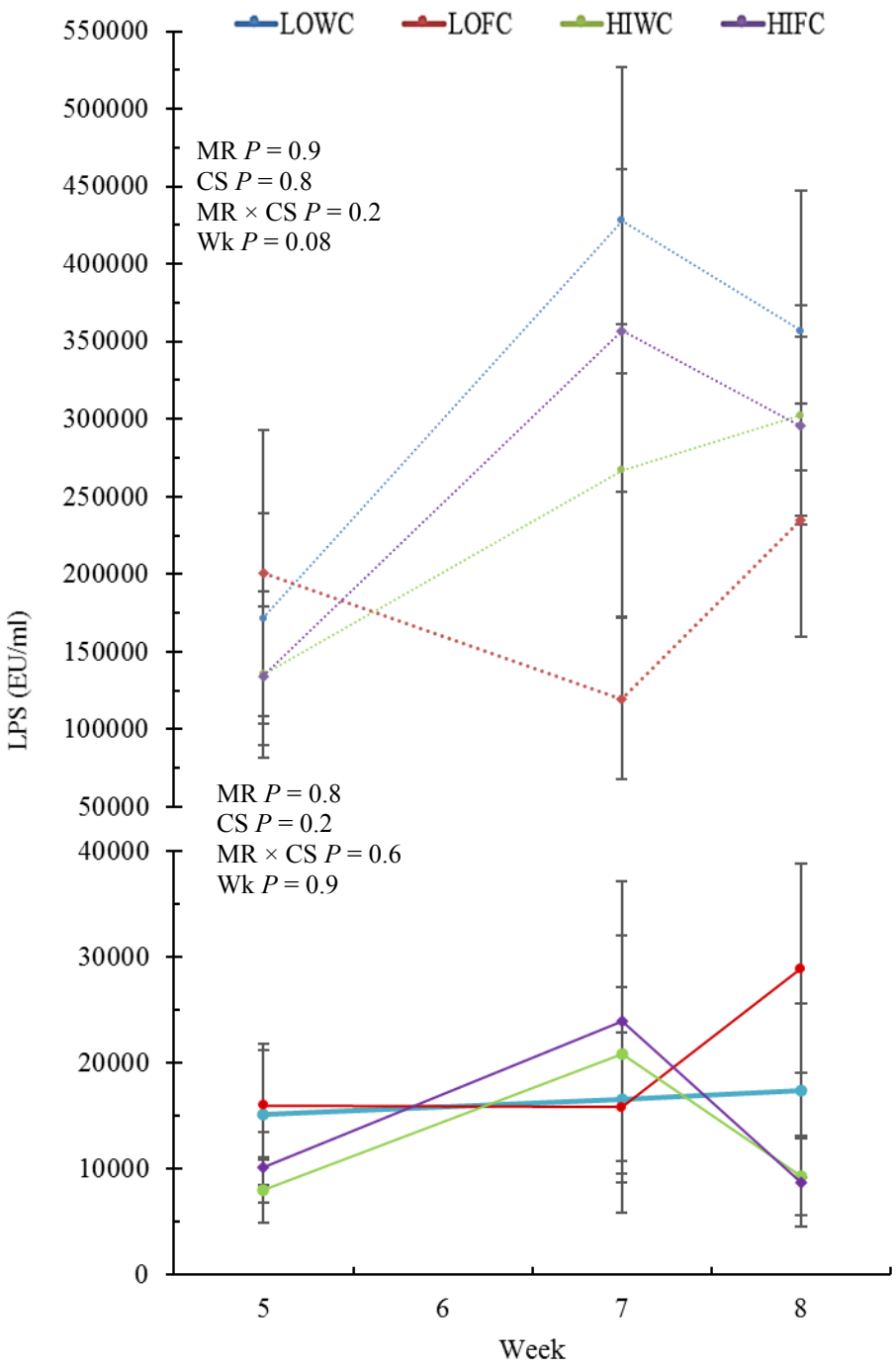

Figure 3.7- 3. Mean weekly rumen fluid LPS (solid line) and fecal LPS (dotted line) for calves fed a low (LO) or high (HI) milk replacer (MR) feeding rate and calf stater (CS) containing whole (WC) or flaked (FC) corn (wk 1 to 8). Error bars represent SEM for each treatment × week interaction. Milk replacer was fed at LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d. Fecal free LPS were higher in wk 8 than in wk 5.

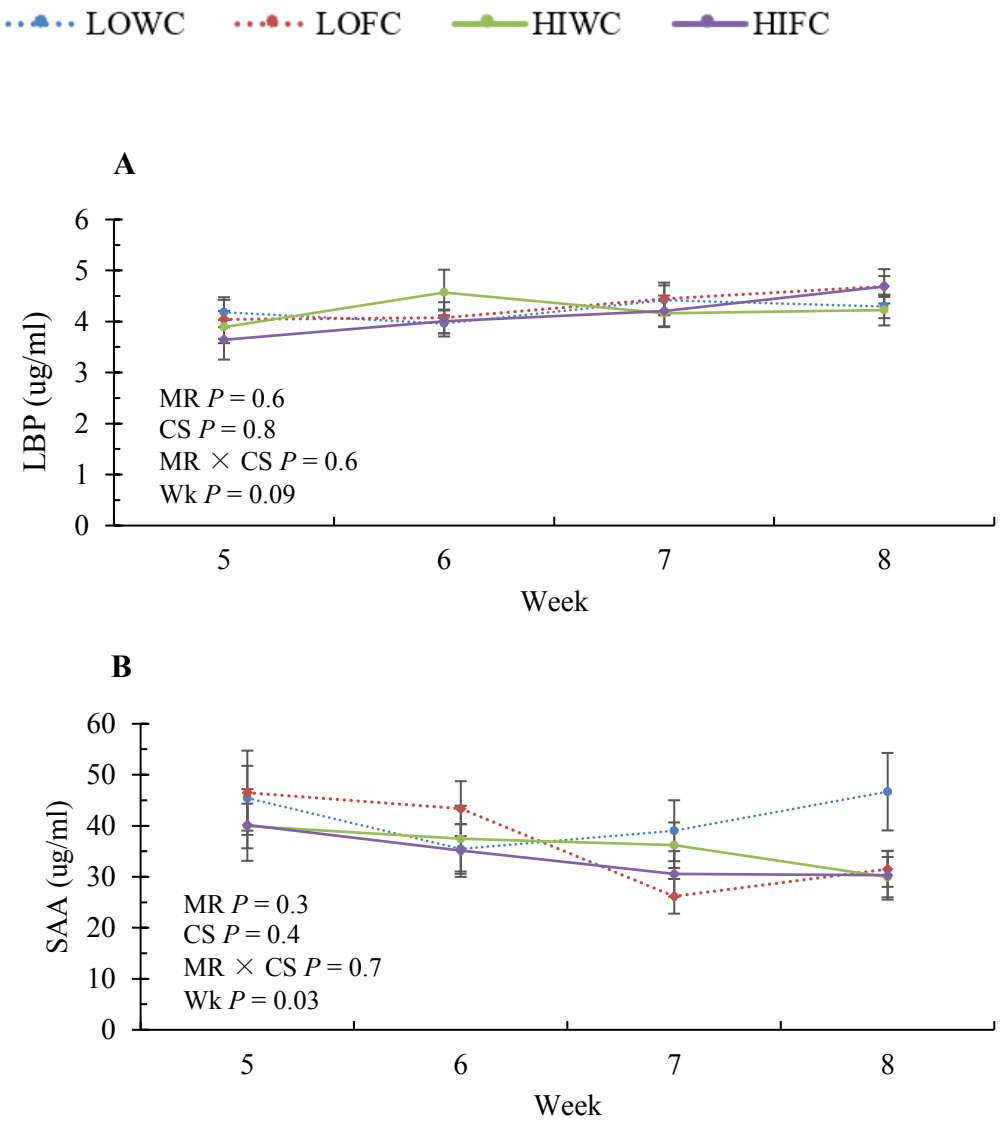

Figure 3.7- 4. Mean weekly (A) lipopolysaccharide binding protein fecal pH and (B) serum amyloid A for calves fed a low (LO) or high (HI) milk replacer (MR) feeding rate and calf stater (CS) containing whole (WC) or flaked (FC) corn (wk 1 to 8). Error bars represent SEM for each treatment × week interaction. Milk replacer was fed at LO = 0.749 kg/d of milk replacer (MR) for 35 d then 0.375 kg/d for last 7 d; HI = 0.749 kg/d of MR for 6 d, 0.851 kg/d for 7 d, 1.135 kg/d for 7 d, 1.498 kg/d for 15 d, then 0.749 kg/d for the last 7 d. Concentration of LBP tended to be lower ($P = 0.09$) LBP at wk 5 (Figure 3.7-3A) than wk 8. Concentration of SAA were higher ($P = 0.03$) wk 5 than wk 7 (Figure 3.7-3 B). At wk 8, calves fed LO tended to have higher ($P = 0.1$) SAA concentrations than calves fed HI (39.1 vs. 30.1 ug/ml). and at wk 7 calves fed WC tended to have higher ($P = 0.06$) SAA concentrations than calves fed FC (37.6 vs. 28.4 ug/ml; Figure 3.7-3B).

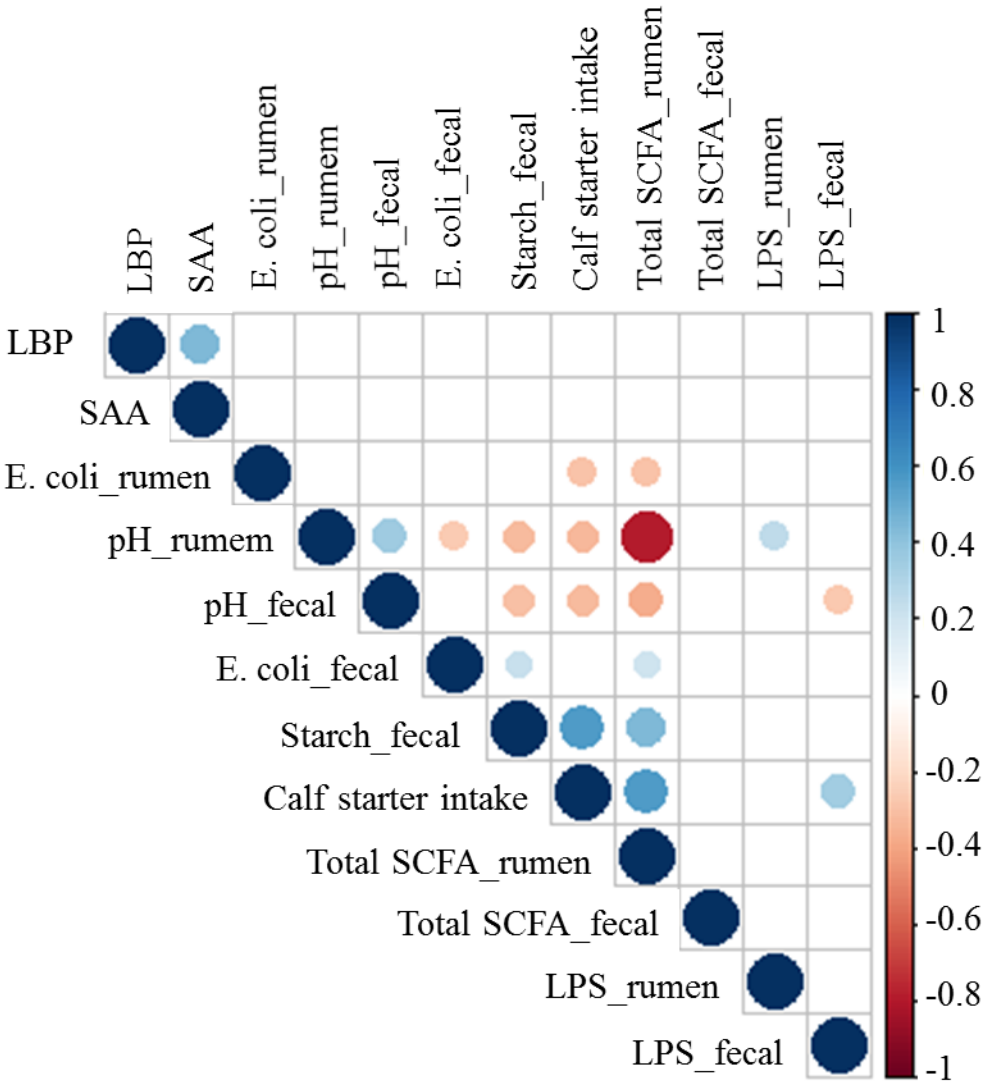

Figure 3.7- 5. A heatmap illustrating Pearson's correlation coefficients between calf starter intake rumen and fecal fermentation characterizes, lipopolysaccharide binding protein (LBP) and serum amyloid A (SAA). Positive correlations in blue and negative correlations in red. Circle size indicates significant correlations $P \leq 0.05$, the bigger the circle the higher the significance.

4. The development of a methodology for ruminal and colon tissue biopsying of young Holstein dairy calves.

4.1. Technical Note

The gastrointestinal tract (**GIT**) is of great importance in dairy production systems, as it is involved in many biological processes and its function and health can be easily influenced by both internal and external factors. The GIT has multiple roles that include, but are not limited to, absorption, metabolism, nutrient delivery and barrier function (Steele et al., 2016). Young calves experience dramatic structural and metabolic adaptations of the GIT. It has been reported that weaning increases GIT permeability (Wood et al., 2015) and negatively influences cell-mediated and humoral immunity of calves (Mackenzie et al., 1997; Hickey et al., 2003), and therefore may lead to increased health risks for calves. The impact of weaning on the rumen has been researched extensively, but little is known about its impact on the lower gut.

Gastrointestinal tract development of the calf has been reported in multiple publications using serial slaughtering (Warner et al., 1956; Lane et al., 2002), however this approach is not ideal to study temporal responses because the sampling time point for the animal is limited to one. Large variations also exist among animals and repeated measurements can increase statistical power, reduce animal numbers, and reduce the time it takes to conduct the research given that fewer animals need to be recruited (Dell et al., 2002). Developing a method to collect GIT tissue in the same animal throughout time using an endoscope will enable researchers to investigate the impact of weaning regimens, feed rations and age on the GIT post-weaning, as well as long-term responses.

Ruminal cannulation remains the best approach to access the rumen because it is minimally invasive and allows for long-term, sequential sampling (Hecker 1969). However, cannulation can be a limitation when large numbers of animals are required. Ruminal tissue is normally obtained by partially evacuating the ventral sac, retracting the ventral sac to the cannula, then clipping of the ruminal tissue using surgical scissors (Steele et al., 2011). As an alternative approach, McRae et al. (2016) introduced an endoscope through the oesophagus of sedated, non-cannulated sheep, in a dorsally recumbent position at 45 degrees with the head upright. They were able to biopsy the anterio-ventral region of the rumen using single-use biopsy forceps in sheep fasted for 4 or 24 hours. Furthermore, Saskikala et al. (2017) reported that when

endoscopic biopsying of the reticulum was attempted via the nasal route in non-sedated but restrained cattle, the cattle tolerated the procedure well and biopsies were successful. However, they also reported that it was only possible to visualize the reticular mucosal surface after 36 hours of fasting, which is a limitation given that it may influence performance and other metabolic processes the study may be evaluating. Ultimately, both methods (McRae et al., 2016; Saskikala et al., 2017) have disadvantages when access to the ruminal epithelium for long-term sequential sampling is desired due to either sedation and/or fasting.

Endoscopic biopsy of the colon is commonly performed in humans to diagnose intestinal diseases like intestinal tuberculosis (Kim et al., 1998) and cancer (e.g. Lieberman et al., 2000; Schoenfeld et al., 2005), as well as in companion animals (cats, dogs, etc.) to diagnose intestinal diseases (Washabau et al., 2010). Histology is also used to examine the tissue samples obtained via endoscopy because it allows for a more precise diagnosis – especially in inflammatory and neoplastic diseases in humans (Geboes et al., 2013) and companion animals (Washabau et al., 2010). However, currently no data or literature exists around endoscopic biopsying of the colon of ruminants to investigate development throughout time. Thus, the primary objective of this study was to develop a methodology for biopsying the rumen and colon of young dairy calves. The secondary objective was to determine if the samples were suitable for microscopic and gene expression analysis.

To meet our objectives, six Holstein dairy bull calves (45 ± 1.5 kg birth weight) were obtained from a commercial dairy farm and housed in individual pens at the Metabolic Unit, University of Alberta, Edmonton, Canada. The calves were cared for and handled in accordance with the Canadian Council on Animal Care (CCAC, 2009) regulations and the institutional Animal Care and Use committee approved all experimental procedures (UCACS Protocol No. 00002010). Calves received colostrum replacer the first d of life, and thereafter, 3 L of milk replacer (MR) solution twice daily for the first wk of life. From the second wk onwards, MR was fed according to 15 % of bodyweight per d in two equal volumes twice daily. During weaning, calves were restricted to 50 % of milk for wk 6 and fully weaned at the end of wk 6. Texturized calf starter (23.4 % CP, 4.5 % crude fat, 15.1 % NDF, 9.3 % ADF), straw (4.1 % CP, 1.0 % crude fat, 75.7 % NDF, 53.9 % ADF) and water was offered ad libitum at 0700 h and when required at 1900 h. In the second wk of life all calves were fitted with rubber ruminal cannulas (2.8 cm diameter: Lesmeister and Heinrichs, 2004).

Ruminal and colon tissue biopsies were taken at the end of wk 5, 6, 7, 8 and 12. The calves were not sedated, but were restrained in a calf chute during sampling. For ruminal tissue sampling, the distal tip of the endoscope (100 cm length, 9.8 mm diameter, GIF-Q140, Olympus, Tokyo, Japan) connected to a light source and processor (CLV-U40 and CV-140, Olympus, Tokyo, Japan) was introduced into the rumen through the cannula. The biopsy was attempted at the blind sac (Figure 4.3-1A) using Captura® hot biopsy forceps (Figure 4.3-2B; 2.4 mm diameter, HDBF-2.4-230-S, Cook Medical, Bloomington, IN, USA). The blind sac was the chosen site of sampling because it could be visualized with the endoscope; the other sites were submerged with digesta. Sampling without visualization of the biopsy site was not attempted as the pressure of the endoscope or forcep against the tissue risked puncturing through the tissue. With 5 attempts using the Captura® hot biopsy forceps, samples could only be collected 15 % of the time. Alternatively, alligator jaws with needle biopsy forceps (2.4 mm diameter, DBF-2.4-230SP-S, Cook Medical, Bloomington, IN, USA) were used to attempt sampling the blind sac. Five sampling attempts were made per calf per forcep type. The outside of the endoscope was washed with 4 % chlorohexidine and rinsed with water between calves. The instrument channel was washed with double distilled water and 70 % ethanol between calves. Both endoscopic biopsy forceps were only able to excise rumen epithelium 15 % of the time, which we suspect is due to strong connective tissue. Even though the success rate was low using endoscopic biopsying in the rumen, it still can be used as a means of tissue sampling in live animals (MacRae et al., 2016; Saskikala et al., 2017). Franz et al. (2006) used an endoscope for visualization of the rumen in calves that were fasted for 12 hours via a rumen cannula and through the oesophagus. The authors reported that when the endoscope was introduced through the oesophagus in non-sedated calves, it failed in 3 of 9 calves because of the calves moving too much. In addition, even though the calves were fasted for 12 hours, visualization of the ventral sac and the caudoventral blind sac were not possible due to rumen fill when the endoscope was introduced through the oesophagus. Thus, biopsying of the ventral sac and the caudoventral blind sac would not be possible due to compromised visualization and the increased possibility of tissue damage. More recently, it was shown that animals may need to be fasted (Saskikala et al., 2017) or sedated and positioned in a dorsally recumbent position (MacRae et al., 2016) in order to obtain a visual of the area that would be sampled. However, this process is not ideal in studies

where long term and frequent sampling is required given that fasting animals can influence performance and other metabolic processes (Keogh et al., 2015).

After using the endoscope, it was determined that an alternative methodology to harvest ruminal tissue should be considered. To meet this objective, the cannula was removed from the animal and OB Caesarean Forceps (30 cm, Jorvet, Loveland, CO, USA) were used to attempt retraction of the ventral sac of the rumen through the cannula. During wk 5 and 6 the OB Caesarean Forceps could grip onto the ventral sac, but once retraction was attempted the grip was lost. It also was not possible to grip onto the ventral sac in wk 7, 8 and 12. Alternatively, the dorsal coronary pillar (Figure 4.3-1A) was retrieved through the cannula opening using Allis clamps (15 cm, Jorvet, Loveland CO, USA; Figure 4.3-1B) exposing the caudodorsal blind sac for sampling. By wk 12 (6 wk after weaning) it became necessary to evacuate half of the rumen contents with a vacuum due to rumen fill, which made the rumen too heavy to retract through the cannula. All rumen contents were replaced after sampling. Four biopsy tissue samples (16.1 – 136.3 mg wet weight) were collected from the caudodorsal blind sac with surgical scissors. Ruminal tissue sample weight increased as the calves got older because the papillae increased in size. The ruminal tissue samples were washed in phosphate buffered saline (**PBS**) once obtained. Two ruminal tissue samples were submerged in 10 % formalin solution and stored at room temperature for histological analysis. An additional two ruminal tissue samples were stored in RNA stabilization fluid (RNALater^{TM}, ThermoFisher Scientific, Burlington, ON, Canada) at room temperature for 24 hours and then frozen at -20 °C for later extraction of RNA. Rumen papillae dimensions were obtained by retrieving the rumen tissue from the fixative (formalin) and submerging them in PBS. When necessary, a row of subsequent papillae or individual papillae (depending on size) were dissected. For histological measurements, ruminal tissue samples were dehydrated overnight, submerged in paraffin wax, sectioned in 4 μm thick slices, and stained with hematoxylin and eosin (Steele et al., 2011). The papillae were imaged with a magnification of x 10 and x 20 using a light microscope (Zeiss Axio Scope.A1, Oberkochen, Germany) and optronics digital camera (PictureFrame Ver, 2.3; Figure 4.3-1C). These images were of suitable quality to measure the thickness of the epithelium, corneum, granulosum, spinosum, and basal layer, as well as the number of cells per layer using ImageJ (National Institutes of Health, Bethesda, MD, USA). Ruminal tissue samples were also prepared for transmission electron microscopy (TEM) and examined using TEM Morgagni 268 (Philips FEI,

Hillsboro, OR, USA; Figure 4.3-1D). Our success with microscopic analysis is in agreement with Saskikala et al. (2017) who reported that biopsy tissue from the reticulum revealed muscularis mucosae, which indicates that endoscopic biopsy tissue from the reticulum can be used for histological measurements.

The total RNA was isolated from each sample (average total RNA harvest was 12958 ± 1013 ng) using an RNeasy mini kit (QIAGEN Inc, Germantown, MD, USA) and assessed for RNA quality. The mean RNA integrity number (**RIN**; Agilent 2200 TapeStation, Agilent Technologies, Santa Clara, CA, USA) for the ruminal tissue samples was 8.9 ± 0.13. The reported RIN is of similar or higher quality than when ruminal tissue was obtained through slaughtering (RIN 8.4 ± 0.7, Conner et al., 2013) and when samples were obtained from the rumen using endoscopic biopsying (RIN 6.3–8.2, McRae et al., 2016). Total RNA was used to construct the library with a unique index and the individual indexed libraries were then pooled and sequenced on the IlluminaHiSeq 4000 system (Illumina, Toronto, Canada). Sequencing reads were aligned to the reference bovine genome (UMD 3.1) and read counts were obtained based on the annotation from ENSEMBL bovine gene annotation. Total detectable genes expressed in the rumen tissue samples were 14,636, 14,556 and 14,737 in wk 5, 7 and 12, respectively, which is similar to other reports from rumen tissue collected from 11-wk-old calves (Kim et al., 2016).

Collecting ruminal tissue through retraction of the rumen and sampling with surgical scissors was more successful than using the endoscopic biopsy method. However, differences between regions of the rumen were found in previous studies (Roth et al., 2009) and may be a limitation when endoscopic biopsying is used and visualization is impaired. In non-cannulated animals, sedation and fasting (MacRae et al., 2016; Saskikala et al., 2017) may aid in endoscopic biopsying but frequent sedation and fasting might influence performance and other metabolic processes, thereby hindering accurate results (Keogh et al., 2015). In small animals, like calves, the cannula is small (2.8 cm diameter), thus limiting the use of instruments to retract the ventral sac to sample other regions of the rumen. Using a cannula allows the rumen to be emptied, making endoscopic biopsying through the cannula a more effective option because different regions can be sampled, and the rumen contents can be returned to the rumen. It might also be possible to collect density measurements of rumen papillae in live animals that have been ruminally cannulated. Nevertheless, collecting ruminal tissue through retraction of the rumen and

sampling with surgical scissors or biopsying tissue with the endoscope can enable repeated measures, resulting in fewer animals required for studies (Dell et al., 2002).

The next objective of our study was to obtain colon tissue samples using the same endoscope, light source and processor as previously described. The lubricated distal tip of the endoscope was gradually inserted into the calf's anus. Six colon tissue samples (12.6 ± 0.74 mg per sample) were collected per calf per time point from the distal colon (30-40 cm from the calf's anus; Figure 4.3-2A) with endoscopic biopsy forceps (Figure 4.3-2B; Captura® hot biopsy forceps, HDBF-2.4-230-S, Cook Medical, Bloomington, IN, USA), which were inserted through the instrument channel. The distal part of the colon was the targeted site of sampling, although it is possible to sample various parts of the lower GIT. For example, in human medicine, sampling of the terminal ileum is frequently utilized to provide information regarding inflammatory diarrhoea in patients (Geboes et al., 2013). The instrument channel and endoscope were washed as described above. Two samples were taken for microscopic analysis and four samples for extraction of RNA (average total RNA harvest was 9961 ± 662 ng per sample) per calf per time point. The colon tissue samples were processed for hisotology and ultrastructure analysis using the same approach as the ruminal tissue samples (Figure 4.3-2C and 2D).

The colon tissue samples had a mean RIN of 8.7 ± 0.09. Total detectable genes expressed in the colon tissue samples were 15,173, 15,085 and 15,149 at wk 5, 7 and 12, respectively. From the colon tissue samples obtained, 45 out of 60 contained muscularis mucosa, which indicates that samples were viable for histological measurements of the outer mucosa (Figure 4.3-2C). In human medicine, the average diameter of forceps used are 2.4 mm and 60 % of these samples contain muscularis mucosa (and upper submucosa; Geboes et al., 2013). Additionally, the larger forceps have a 3.4 mm diameter, meaning that the tissues obtained are larger, but usually do not contain increased submucosa. Moreover, the larger forcep boast a higher risk of complications compared to the smaller forcep used in this study.

It was thought that the amount of tissue sampled in each biopsy attempt would not be sufficient for histological and ultrastructural analysis. Based on the micrographs collected from our samples (Figure 4.3-2 C and 2D) it is feasible to use this technique to investigate the impact of weaning regimens, feed rations and age on the GIT post-weaning, as well as long-term effects on colon tissue structure and function. When attempting tissue biopsying of the colon there were

some initial concern around the damage caused by taking a large number of samples. In human medicine, Magro et al. (2013) reported that when ulcerative colitis is suspected, biopsies should be obtained at 10 cm intervals and 6 to 10 samples are required from different sites. Rubin et al. (1992) removed 68 shallow biopsy samples (5mm) with spiked "jumbo" forceps throughout the intestines of patients with dysplasia and concluded that 56 biopsy samples are needed in order to ensure a 95 % confidence level. After investigating routine procedures in human medicine, it was determined that the number of samples taken in the current study are within a very conservative number range.

Collecting colon tissue through endoscopic biopsying in the same animal throughout time means that less animals are required for studies (Dell et al., 2002). Sampling the distal colon in non-sedated but restrained animals was successful and would allow other parts of the small intestine to be sampled through the rectum. However, these animals may require sedation for this procedure, which could influence performance and other metabolic processes. Alternatively, full body restraint of the animals may provide a suitable substitute to sedation.

In summary, it was found that collecting ruminal tissue by retracting the rumen through the cannula and sampling with surgical scissors was more successful than using the endoscopic biopsy method in young calves. Endoscopic biopsying from the colon in young, non-sedated and restrained calves is possible. The ruminal tissue samples that were excised with surgical scissors and the colon tissue samples obtained via endoscopic biopsy both resulted in high quality samples suitable for microscopic and gene expression analysis.

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4.3. Tables and figures

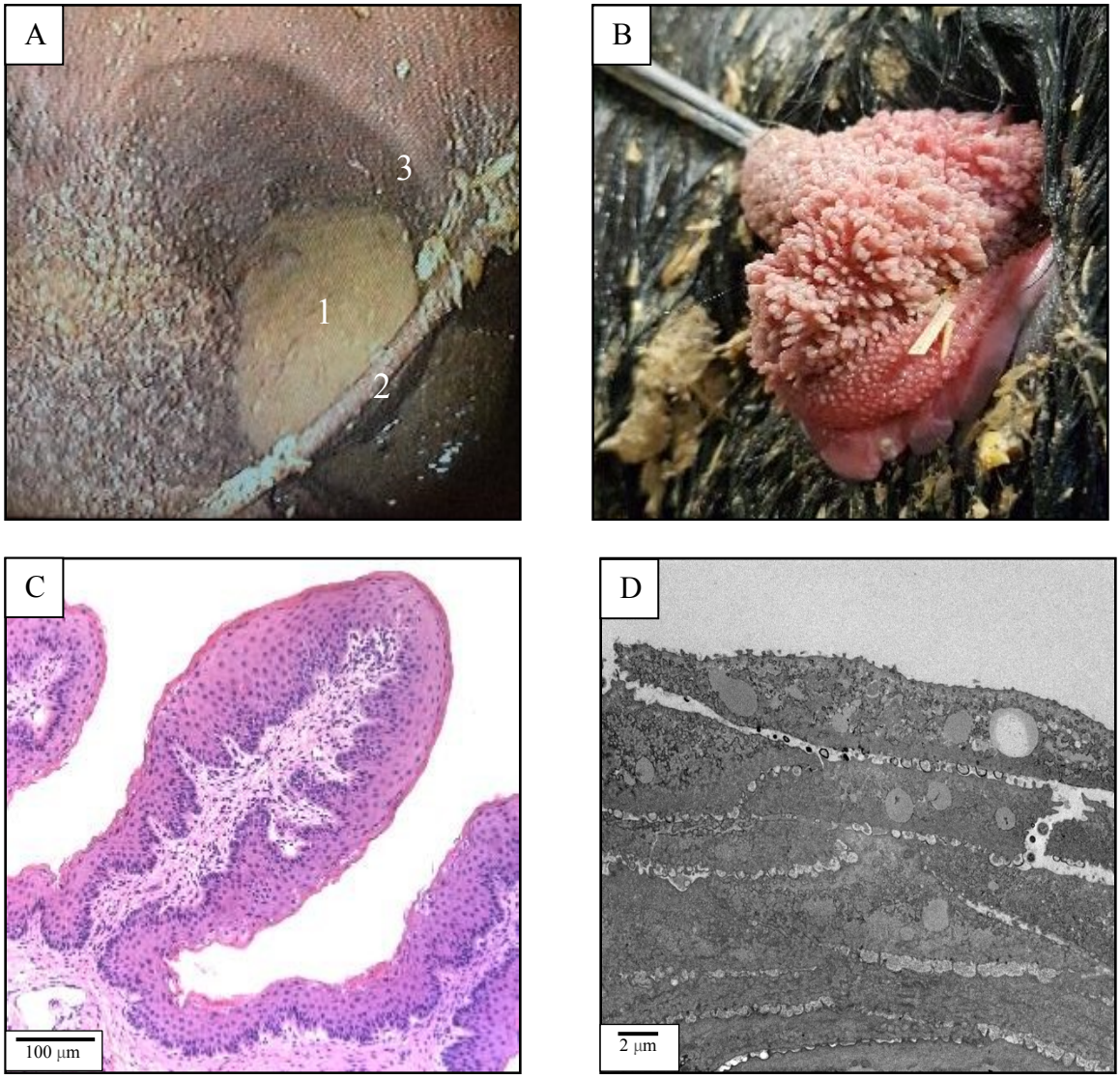

Figure 4.3- 1 (A) A photograph of the caudodorsal blind sac (1), caudal pillar (2) and dorsal coronary pillar (3) of the rumen. (B) the dorsal coronary pillar retrieved through the cannula hole using Allis clamps. (C) Image from rumen papillae at week 7 under the light microscope with a X 20 magnification. (D) Image of strata in rumen papillae under the transmission electron microscope.

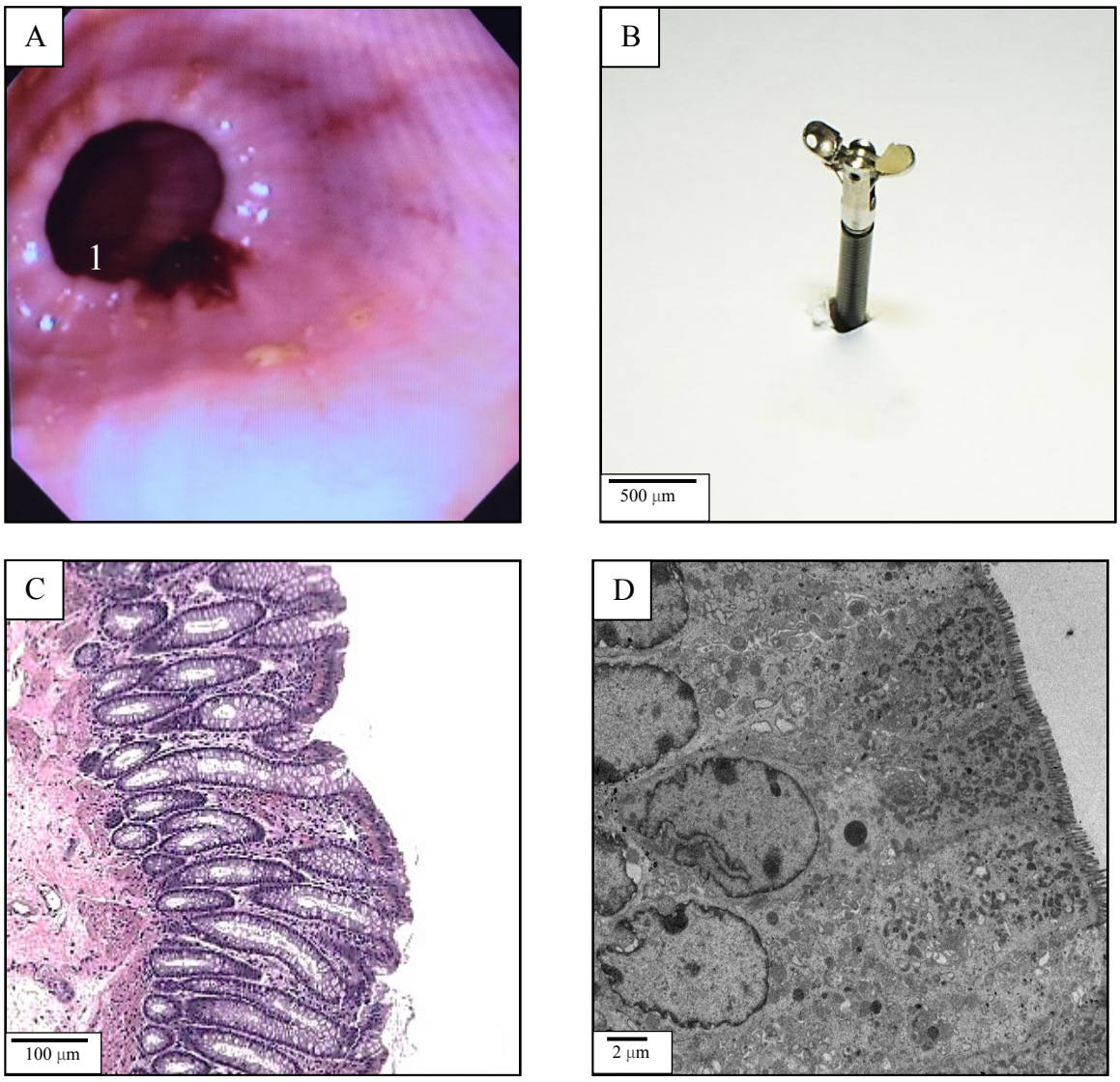

Figure 4.3- 2 (A) Photograph of the colon 40 cm from the calf's anus. As shown, sampling resulted in minimal bleeding (1). (B) Image of colon tissue sample in the biopsy forcep.(C) Image of colon mucosa under the light microscope. (D) Image of the colon under the transmission electron microscope.

5. Rumen papillae structural growth, ruminal fermentation characteristic and acute phase proteins from pre- to post-weaning in calves fed a high MR feeding rate.

5.1. Introduction

During the pre-weaning period, the calf's gastrointestinal tract (**GIT**) functions similarly to that of a monogastric, where milk/milk replacer (**MR**) is digested and absorbed in the abomasum and small intestine to supply adequate nutrients (carbohydrate and protein) for maintenance and growth (Baldwin VI et al., 2004; Drackley, 2008; Benschop and Cant, 2009). When calves start to consume solid feed post-weaning, the rumen is the major site of fermentation, providing short chain fatty acids (**SCFA**) that account for up to 80 % of the ruminant's energy (Bergman, 1990). Thus, the calf GIT experiences dramatic morphological and metabolic adaptations during weaning through the transition from milk to solid feed (Baldwin VI et al., 2004) in order to support the energy requirements of the calf.

When calf starter (**CS**) intake increases during and after weaning, it can lead to an accumulation of SCFA in the rumen (Aschenbach et al., 2011) – especially when rumen development has been delayed pre-weaning. Such accumulation of SCFA can lead to decreased ruminal pH, which is unfavorable because sustained low rumen pH (5.5) can lead to decreased OM fermentation (Cerrato-Sanchez et al., 2007). Low pH can also influence the cell layers within the stratum corneum (Baldwin and Jesse, 1991), one of the four cell layers of the rumen, and the tight-cell junction protein complexes within the granulosum (Kiddle et al., 1951). The granulosum is controlling the permeability of the epithelium (Gaebel et al., 1989; Baldwin, 1998) and low ruminal pH may lead to compromised ruminal health (Kleen et al., 2004). Moreover, low ruminal pH can trigger a systemic inflammatory response due to the translocation of lipopolysaccharides (**LPS**) into the peripheral circulation in mature animals (Khafipour et al., 2009a).

Acute phase proteins such as serum amyloid A (**SAA**) and LPS binding protein (**LBP**) are commonly used as indicators of inflammation from sub-acute ruminal acidosis (**SARA**), indicating either GIT mucosa damage occurred or immunogenic molecules, such as LPS, entered the circulatory system (Khafipour et al., 2009b). Despite the knowledge of ruminal pH during

weaning (Laarman and Oba, 2011; Laarman et al., 2012; Kim et al., 2016a), there are limited studies monitoring pH adaptations post-weaning in calves. In addition, no studies have investigated the effect of ruminal pH during weaning on indicators of inflammation, i.e. acute phase proteins, in calves.

We hypothesize that in calves (1) there will be a delay in rumen structural development up to 2 wk after weaning; (2) ruminal pH will be depressed up to 2 wk after weaning due to the aforementioned point; and (3) an acute inflammatory response will occur during weaning and up to 2 wk after weaning due to the depressed ruminal pH, causing barrier dysfunction. The objective of this study was to characterize ruminal structural development, fermentation characteristics and acute phase proteins from pre- to post-weaning.

5.2. Materials and methods

5.2.1. Animals and housing

In this study, calves were cared for and handled in accordance with the Canadian Council on Animal Care (CCAC, 2009) regulations and the institutional Animal Care and Use committee (University of Alberta, AB; AUP00002010). A total of 6 Holstein bull calves (45 ± 1.5 kg birth weight; mean ± SE) were obtained from a commercial dairy farm (Millet, Alberta, Canada) 1 wk after birth. It was estimated that 4 animals per timepoint per treatment would be sufficient to detect differences in ruminal pH response variables based on a power calculation, with 80 % power and α of 5 %, based on estimates of variance drawn from previous literature (Wood et al., 2015; Eckert et al., 2015). The calves were housed in individual pens (3.05 × 3.66) with rubber mats (2.42 × 2.42 m) and deep bedding with wood shavings at the Metabolic Unit, University of Alberta, Edmonton, Canada. At wk 2, calves were fitted with rubber ruminal cannulas (2.8 cm diameter; Lesmeister and Heinrichs, 2004) which were placed in the center of the left paralumbar fossa. Before arrival, calves received 3 L of reconstituted powdered colostrum, containing 200 g of IgG (HeadStart, Saskatchewan Colostrum Company, Saskatoon, SK, Canada) within one hour of birth followed by a second feeding (3 L with 200 g IgG) at 12 h after birth. Thereafter, calves were fed 3 L of milk replacer solution (MR; 150 g/L, 26 % CP, 20 % fat, 38 % lactose as fed; Grober Nutrition, Cambridge, Ontario) containing: dried whey protein concentrate, dried skim milk powder, animal and vegetable fat, dried whey powder, Grober Rearing Premix, soy lecithin, calcium chloride, DL-methionine, calcium carbonate L-lysine, yeast autolysate, viable microbial

product (982931), PEG 400 mono and di-oleates, and selenium yeast twice daily (AM and PM) for the first wk of life. From the second wk onwards, MR solution was fed according to 15 % of bodyweight (**BW**) per d in two equal volumes via nipple bucket twice daily (0700 h and 1900 h). Calves began the weaning step-down at wk 6, where the MR solution was restricted to 50 % of the previous wk allocation. Calves were completely weaned by wk 7. Calves were given ad libitum access to texturized calf starter (Trouw Nutrition Canada Inc., Strathmore, AB, Canada; Table 5.7-1), chopped straw (Skyline Harvest, Blumenort MB, Canada, 1-inch chop length) and water from wk 1 onwards.

5.2.2. Data and sample collection

Intake and Growth Performance. Individual MR, CS, straw and water intakes were recorded daily. A sample of CS and straw were collected weekly. The CS and straw were composited by type and stored until chemical analysis. Individual BW were measured at the same time weekly.

Ruminal fluid and pH. Ruminal fluid was sampled through the cannula using a plastic tube and a syringe 3 hr after the AM feeding from the end of wk 5 to 12. Ruminal contents were squeezed through 4 layers of cheesecloth and 5 mL of ruminal fluid was frozen and stored at -20˚C for SCFA analysis. An additional 2 mL of rumen fluid was collected and stored at -20˚C for total bacteria and protozoa density quantification. Ruminal pH was measured continuously every 5 min throughout the experiment from wk 5 to 12, using a T9 pH bolus (Dascor, Escondido, California, US) according to Penner et al. (2006). The pH bolus was removed weekly to download data and for recalibration.

Rumen tissue. Ruminal tissue biopsies were collected at the end of wk 5, 6, 7, 8 and 12 as described by Van Niekerk et al. (2018; Chapter 4). Briefly, the calves were not sedated, but were restrained in a calf chute during sampling. For ruminal tissue sampling, an Allis clamp (15 cm, Jorvet, Loveland CO, USA) was used to retrieve the dorsal coronary pillar through the cannula opening. This exposed the caudodorsal blind sac for sampling, which was done using surgical scissors. To ensure that whole papillae were obtained the cut was made below the base of the papillae. After the samples were obtained, the tissue was washed with sterile phosphate buffered saline (PBS; pH 7.4). For histological analysis, two ruminal tissue samples at each time point were submerged in 10 % formalin solution and stored at room temperature (RT).

Serum acute phase proteins. Blood samples were collected weekly from wk 1 to 12 an hour after the morning feeding via jugular venipuncture. Blood was collected into a silica-coated clot-activating vacutainer tube (Becton Dickinson, Franklin Lakes, NJ), inverted for 30 seconds and stored until clotted (± 30 min) at RT, then centrifuged at 1,500 x g, RT for 15 min. Serum was transferred into microcentrifuge tubes and stored at −20°C for analysis of SAA and LBP.

5.2.3. Sample analyses

Feed Composition. At the end of the trial, CS and straw composited samples were sent for analysis to Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada). The CS and hay samples were analyzed for moisture (AOAC 930.15; analysed, AOAC 922.02; received, NIR), CP (AOAC, method 990.03), crude fat (AOCS Am 5-04), ADF (ANKOM 08-16-06), NDF (ANKOM 08-16-06), starch (enzymatic; UV-method) and minerals (modification of AOAC, 2000 method 968.08, 935.13A, 985.01).

Ruminal SCFA and pH, and total bacteria and protozoa density. The ruminal fluid was analyzed for SCFA and lactate by the Metabolomics Innovation Centre (University of Alberta, Canada) using nuclear magnetic resonance (**NMR**) spectrum analysis (Saleem et al., 2013). Briefly, 250 µL of thawed rumen fluid was mixed with 46.5 µL of a standard buffer solution (54 % D_2O; 46 % 1.75 mM KH_2PO_4 pH 7.0 v/v containing 5.84 mM 2,2-dimethyl-2-silcepentane-5-sulphonate). Then, 250 µL was transferred to an NMR tube (3mm SampleJet, Bruker) and the ^{1}H-NMR spectra were collected by an Avance III spectrometer (700 MHz, Bruker) equipped with a cryoprobe (5 mm, HCN Z-gradient pulsed-field gradient). The Chenomx NMR Suite Professional software package (ver 8.1, Chenomx Inc., Edmonton, AB) was used to quantify the ^{1}H-NMR spectra.

The ruminal pH was averaged by d and then by wk. The minimum, mean and maximum pH were then reported from the summarized data. Ruminal acidosis time span per d was determined from the pH data using three pH thresholds (5.8, 5.5 and 5.2; Penner et al., 2007) and the duration span (min/d) for each threshold was determined.

The repeated bead beating and column method was used to extract DNA from ruminal fluid (Yu and Morrison, 2004). Firstly, the samples were treated with a cell lysing buffer that contained 4 % SDS and were physically disrupted (Zirconium beads, diameter 0.1mm) using a Beads Beater (2,160 x g for 3 min using Biospec Mini Beads Beater 16, BioSpec, Bartlesville,

OK). Thereafter, DNA was isolated, precipitated and purified (QIAmp fast DNA stool mini kit, Qiagen Inc., Germantown, MD) and the quantity and purity were evaluated (NanoDrop 1000, Nanodrop Technologies, Wilmington, DE). The DNA was stored at −20 °C for further analysis.

Real-Time PCR was used to estimate 16S rRNA and 18S rRNA gene copy numbers of total bacteria and protozoa, respectively, with SYBR Green chemistry (Fast SYBR Green Master Mix, Applied Biosystems, Foster City, CA) using a Viia 7 Real-Time PCR System (Thermo-Fisher Scientific). The specific primers used for total bacteria were ACTCCTACGGGAGGCAG (forward) and GACTACCAGGGTATCTAATCC (reverse) with a melting temperature of 60 °C and product size of 467 (Stevenson and Weimer, 2007). The specific primers used for protozoa were GCTTTCGWTGGTAGTGTATT (forward) and CTTGCCCTCYAATCGTWCT with a melting temperature of 60°C and product size of 223 (Sylvester et al., 2004).

Rumen histology measurements. Rumen papillae were retrieved from the formalin (fixative) and submerged in PBS. Individual papillae or a row of papillae (depending on the size) were dissected. A stereo microscope (Olympus Stereo Microscope (SZ2-ILST, Tokyo, Japan) and ScopePhoto were used to obtain images from 10 papillae per calf per sampling timepoint. Papillae were positioned to ensure the entire papillae fit into the image. After imaging, the papillae were placed back in the fixative. The lengths of the 10 papillae were measured from the highest point perpendicular to the base of the papillae using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The papillae width was measured at the midpoint of the papillae. The one-sided surface area of the papillae was measured by outlining the papillae from base to base and multiplied by two to obtain the two-sided surface area.

Rumen papillae cell layer thickness was obtained as described by Van Niekerk et al. (2018; Chapter 3). Ruminal tissue samples were dehydrated overnight and submerged in paraffin wax. Then, the tissue was sectioned in 4 µm-thick slices, and stained with hematoxylin and eosin (Steele et al., 2011). A minimum of 5 papillae per calf per sampling timepoint were imaged using a light microscope (Zeiss Axio Scope.A1, Oberkochen, Germany) and optronics digital camera (PictureFrame Ver, 2.3; Figure 5.7-1C) at x 10 and x 20 magnification. The images obtained from these samples were used to measure the epithelium, corneum, granulosum, spinosum, and basal layer thickness and to count the number of cells per layer using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The thickness of the cell layers was measured 25 % inwards from the base and from the tip of the papillae by drawing a

perpendicular line (to the base and tip) at these two spots and cell numbers were also counted at these two spots. This resulted in 20 measurements (4 measurements per papillae) per calf per timepoint. Whole papillae were scored for sloughing according to (Steele et al., 2015). A score of 1 to 5 was assigned to each papilla, with 1 indicating minor desquamation and 5 indicating severe desquamation. All measurements were performed by two individuals and the averages were reported.

Blood acute phase proteins. Serum amyloid A and LBP concentrations were determined using ELISA kits (TP-802, Tri-Delta Diagnostics Inc., Morris Plains, NJ; HK503, HyCult Biotechnology, Uden, Netherlands, respectively). The serum was diluted 500X for SAA and 100X for LBP measurements initially and the absorbance values were read at 450 nm (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA) for each duplicate sample. For SAA and LBP, the intra-assay coefficient of variation (CV) values were 5.0 and 5.2 % respectively.

5.2.4. Statistical analyses

Data were analyzed as repeated measures using the GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). The model included wk as a fixed effect and calf as a random effect. Data collected daily were summarized by wk. Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$. Calf starter, straw and water intake, total SCFA, lactate, minimum, mean and maximum ruminal pH, and pH thresholds, papillae surface area, length and width, total bacteria and protozoa and SAA and LBP were used to estimate linear relationships through Pearson correlation co-efficient using R software (3.6.1).

5.3. Results

Intake and Growth Performance. Milk replacer intake increased over time due to the milk feeding protocol (Figure 5.7-1A). The maximum average reached 1.44 ± 0.039 kg of DM/d during wk 5, just before step-down weaning. Water intake remained below 2 L/d during the pre-weaning period (Figure 5.7-1B). Thereafter water intake increased, reaching up to 8.1 ± 0.51 L/d during wk 12. For the first 3 wk, calves consumed less than 0.1 kg/d of CS and during wk 4 and 5 consumed approximately 0.13 ± 0.024 kg/d (Figure 5.7-1C). During the step-down wk, CS intake was 3-fold higher than the previous wk. Post-weaning, CS intake steadily increased to 2.8

± 0.07 kg/d during wk 12. Calves consumed minimal straw (1 to 10 g/d) during the pre-weaning period; thereafter, straw intake was approximately 0.03 to 0.04 kg/d (Figure 5.7-1D). During the pre-weaning and step-down period, MR intake contributed to the majority of the ME intake (Figure 5.7-1E) while during the post-weaning period, CS contributed to the majority of the ME intake. During wk 7, calves consumed less ($P < 0.05$) ME than wk 1, as it took calves 6 wk post-weaning to consume the same amount of ME from CS than the amount of ME they consumed from MR just before weaning. Body weight increased ($P < 0.001$) over time reaching 109.3 ± 3.0 kg at wk 12; however, BW were not significantly different between wk 5, 6 7 and 8 (Figure 5.7-2A). During wk 6, calves only gained 0.22 ± 0.052 kg/d and during wk 7, calves lost 0.32 ± 0.0256 kg/d (Figure 5.7-2A). During wk 12, calves started to gain weight at a similar rate (approximately 1.2 kg/d) to wk 4 and 5.

Ruminal fermentation characteristics and total bacteria and protozoa. Overall, total SCFA concentration was the greatest ($P = 0.001$) at wk 7, 10, 11 and 12 (Table 5.7-2). Butyrate, propionate and isobutyrate were unaffected by wk. Acetate proportion was higher ($P < 0.001$) in wk 5 than wk 9, 10, 11 and 12, while valerate had lower ($P = 0.003$) proportions during wk 5 than wk 9, 10, 11 and 12. Isovalerate was 2-fold lower ($P = 0.013$) at wk 7 compared with wk 9. There was no significant difference in lactate over time, however lactate was numerically higher during wk 5. Maximum ruminal pH had a wk effect ($P = 0.003$), reaching the highest pH during wk 12 and the lowest during wk 7 and 8 (Figure 5.7-3A). In regard to duration time/d below a threshold, pH was below < 5.8 for 1203.9 ± 227.65 min/d at wk 8, which was 3-fold higher than wk 12 at 388.1 ± 189.82 min/d (Figure 5.7-3B).

Total bacteria density in rumen fluid were 11.1 ± 0.1, 11.2 ± 0.16, 10.9 ± 0.11, 10.9 ± 0.10, 10.9 ± 0.11, 10.8 ± 0.16, 10.9 ± 0.0.8 and 10.7 ± 0.23 log 10/ml in wk 5 to 12 respectively. Ruminal fluid protozoa density was 3.5 ± 0.22, 3.4 ± 0.18, 3.6 ± 0.25, 3.6 ± 0.26, 3.4 ± 0.15, 3.6 ± 0.18, 3.6 ± 0.26 and 3.7 ± 0.10 log 10/ml in wk 5 to 12 respectively. No difference in total bacteria and protozoa density in ruminal fluid was detected among wk 5 to 12.

Rumen histology measurements. Papillae surface area and length was the greatest ($P < 0.001$) during wk 12 (Table 5.7-3). Papillae width increased ($P < 0.001$) by wk, with wk 5 being the smallest, wk 7 and 8 intermediate, and wk 12 the largest. Supplemental figure S5.7-1 illustrates the papillae growth at different time points. Corneum thickness increased by wk ($P =$

0.003; Table 5.7-4). Spinosum/basale thickness increased (P = 0.009) during wk 8 compared with wk 5. No difference was observed in papillae sloughing score over time.

Serum acute phase proteins. There was a wk effect on LBP (P = 0.004) and SAA (P = 0.002) concentrations (Figure 5.7-3C). The concentration of LBP was the highest during wk 1 (4.58 ± 0.92 μg/ml) which was approximately 2-fold higher than wk 4, 10, 11 and 12, which ranged from 1.79 to 2.04 μg/ml. Concentration of SAA was also the highest during wk 1 (394.84 ± 175.26 μg/ml) and the lowest during wk 4, 6 8, 9, 10, 11 and 12 ranging from 16.52 to 102.96 μg/ml.

Relationship between intake, ruminal structural development, fermentation characteristics and acute phase proteins. The correlation analysis showed that papillae length (r = 0.80, P < 0.001), width (r = 0.90, P < 0.001) and water intake (r = 0.92, P < 0.001) displayed a strong positive correlation with CS intake and that papillae width (r = 0.86, P < 0.001) had a strong correlation with water intake. Total SCFA (r = 0.62, P < 0.001) and papillae surface area (r = 0.75, P < 0.001) had a strong positive correlation with CS intake and papillae surface area (r = 0.67, P < 0.001) and length (r = 0.70, P < 0.001) had a strong positive correlation with water intake. A moderately positive correlation was observed between mean ruminal pH and CS intake (r = 0.41, P = 0.03), straw intake and papillae width (r = 0.41, P = 0.02), papillae width and total SCFA (r = 0.58, P < 0.001). There was also a moderately positive correlation between water intake and total SCFA (r = 0.53, P = 0.003) and total protozoa and pH threshold 5.5 (r = 0.41, P = 0.03). Calf starter intake (r = -0.44, P = 0.01) and water intake (r = -0.43, P = 0.02) had a moderate negative correlation with SAA. Straw (r = -0.48, P = 0.01), water intake (r = -0.42, P = 0.0) and papillae width (r = -0.46, P = 0.01) had a moderate negative correlation with total bacteria and total protozoa and maximum pH (r = -0.40, P = 0.03) also had a moderate negative correlation.

5.4. Discussion

The weaning transition is a phase in a calf's life where the rumen experience drastic morphological and metabolic adaptations (Baldwin VI et al., 2004). Calves are fed CS in an effort to initiate rapid rumen development during this phase. However, when SCFA absorption is inadequate due to a delay in rumen development, accumulation of SCFA and reduced ruminal pH may occur, which may lead to ruminal acidosis (Aschenbach et al., 2011). Despite research

of ruminal pH during weaning (Laarman and Oba, 2011; Laarman et al., 2012; Kim et al., 2016a), there are limited studies that monitored ruminal pH adaptations post-weaning in calves. In addition, to our knowledge there are no studies that have investigated the effect of ruminal pH during weaning on acute phase proteins, in calves. Thus, the objective of this study was to evaluate ruminal structural development, fermentation characteristics and acute phase protein levels that occur pre- to post-weaning in calves fed a high MR feeding rate pre-weaning.

Calves consumed minimal CS during the first five wk of life, as a reduction of MR supply is necessary for CS intake to increase to greater amounts (3-fold increase during step-down; Hill et al., 2012; Eckert et al., 2015). One-wk post-weaning (wk 7), calves were not able to consume enough CS to achieve the equal ME intake as the first wk of life. Since calves were unable to consume enough ME from CS, calf performance suffered and no differences were observed in calf BW between wk 5, 6, 7 and 8. Growth depression during weaning has been reported by others in calves fed a high MR feeding rate pre-weaning (Cowles et al., 2006; Van Niekerk et al., 2020; Chapter 2). The time of weaning and weaning method also plays a key role in CS intake and performance (Meale et al., 2015; Steele et al., 2017; Welboren et al., 2019). In the current study, it is likely that calves performed poorly because of early weaning and a one-step (decrease by 50 %) weaning protocol, which is considered an abrupt weaning strategy.

Total SCFA concentration was high during wk 7, which should have stimulated papillae development (Tamate et al., 1962; Stobo et al., 1966). However, during this time it is possible that ME intake was so limited that it was dedicated solely to maintenance and there was not enough ME for rumen development. Based on NRC (2001), a 76.7 kg weaned calf requires 2.97 Mcal ME for maintenance, of which the current calves (at wk 7) only consumed 2.33 Mcal ME. Inasmuch, no changes in ruminal papillae length and surface area during weaning and up to 2 wk post-weaning (wk 8) were observed. The positive correlations between CS intake, SCFA, and papillae size suggest that CS intake is important for rumen development.

Surprisingly, calves in the current study were ruminally acidotic (pH 5.8 for 936.3 min/d) pre-weaning (wk 5), even though CS intake was only approximately 0.130 kg/d at this time. Li et al. (2019) reported that pre-weaning (wk 6) mean ruminal pH was below 5.8, however calves consumed CS up to 1.2 kg/d during this time, which could have resulted in an accumulation of SCFA that lead to low pH. Papillae development was minimal in our study during wk 5, which

could be attributed to the accumulation of SCFA that could not be absorbed because of reduced surface area. At wk 11 and 12 papillae surface area was 6.4-fold higher 6 wk post-weaning (wk 12), which could have facilitated SCFA absorption leading to reduced time that ruminal pH was below 5.8. Numerically acetate and propionate absorption per hr were 14.4 and 6.3 % higher, respectively (Yohe et al., 2019a) and 2-dimensional papillae area increased (Yohe et al., 2019b), in calves fed MR and CS compared with calves fed only MR at 6 wk. This may indicate that an increase in absorption may contribute to increase SCFA absorption. However, this was not reflective during wk 11 and 12 because total SCFA concentrations were the highest during those two wk. Sampling time difference between ruminal pH (every 5 min every d) and ruminal fluid for SCFA (1 timepoint at the end of each wk) may have attributed to the high SCFA concentrations observed because the extend of fermentation that occurred throughout the d might have not been captured. It is interesting that there is no correlation between the duration of pH below the thresholds and papillae size. This lack of correlation may indicate that increasing ruminal pH in calves at this age may rely on other factors, such as rumen buffering from saliva and from the epithelium instead of increased absorption of SCFA from increased surface area of papillae.

In the present study, at 3 wk post-weaning ruminal pH was below pH 5.8 for 902.26 min/d, which is similar to calves fed CS (Kim et al., 2016b). However, calves in the current study also had access to chopped straw, which may have decreased the time below pH 5.8 in the current study due to buffer capacity through increased rumination. In the Kim et al. (2016b) study ruminal pH was below pH 5.8 for 97.6 min/d in the calves fed CS and hay during wk 11 (3 wk post-weaning). Although straw was offered ad libitum in the current study calves only consumed 0.4 kg/d 3 wk post-weaning. The difference observed between the two studies might be due differences in calf age or CS intake, with the current study reporting 1.85 kg/d and Kim et al. (2016b) reporting 1.6 kg/d. Interestingly, Li et al. (2019) demonstrated that calves fed high starch (42.7 %, pelleted) content in CS had poor performance, reduced DMI and BW (from wk 5 onwards), compared with a CS low in starch (35.3 %, whole grains) due to the depression in ruminal pH (measured at −8, −4, 0, 2, 4, 8 and 12 hr relative to CS feeding once every other wk). Based on this data, performance in the current study may not have been influenced by the depression in ruminal pH, as CS starch content was only 32.9 %. In addition, the diet form may also played a role on DMI in Li et al. (2019). Other studies (Kristensen et al., 2007; Laarman and

Oba, 2011) did not measure pH for a long enough duration to identify if ruminal pH had an effect on intake and performance in calves during pre-weaning, weaning transition and post-weaning. It is well known that depressed ruminal pH impacts performance in lactating dairy cows, which is generally attributed to decreased DMI or fibre digestibility (Aschenbach et al., 2019).

Rumen papillae are comprised of four cell layers, namely the stratum corneum, granulosum, spinosum and basal layer. In the current study, corneum thickness was highest during wk 12, which agrees with another study (Gäbel et al., 1987) in mature animals that reported corneum layer thickness increases with high grain diets. However, studies also reported that high grain diets caused corneum thickness to remain similar, but corneum layer sloughing increased (Steele et al., 2011, 2015). It has been reported that in calves, ruminal lesions were observed in both groups fed a 42.7 or 35.3 % starch diet at 17 wk of age, however, lesions were less severe in calves fed the lower starch diet (Gelsinger et al., 2019). The number of cell layers in the corneum increased numerically over time, which may have resulted in increased corneum thickness. In the current study, when calves' experienced ruminal acidosis for multiple hrs/d (wk 7 and 8) there was no difference in corneum thickness, which may indicate that depressed pH does not influence parakeratosis during this time. The granulosum thickness was unchanged in the current study, which contradicts what was reported before (Steele et al., 2011; Liu et al., 2013). A high grain diet fed to mature animals, which is similar to calves undergoing weaning, caused the granulosum layer to be reduced and compromised (Steele et al., 2011; Liu et al., 2013). This negatively impacts barrier function as this layer contains tight-cell junction protein complexes (Kiddle et al., 1951) that prevent unwanted molecules translocating into the blood circulation (Owens et al., 1998; Aschenbach and Gabel, 2000; Nagaraja et al., 2005). The sum of the spinosum and basale layer increased from wk 5 to wk 8; however, previous results contradict this finding, in which cows switched from a high-grain to a high-forage diet displayed no differences in the sum of the spinosum and basale layer thickness (Steele et al., 2011). In our study higher CS intake may have resulted in greater proliferation and differentiation in the spinosum and basale layer which is similar than what Yohe et al. (2019a) reported when calves were fed CS compared with just MR.

Ruminal pH is closely associated with rumen epithelium barrier integrity in mature ruminant experimental models (Penner et al., 2010) but is unknown in dairy calves. However,

ruminal pH is not the only factor impacting ruminal barrier integrity. Low ruminal pH in conjunction with high SCFA concentration can have a significant effect on barrier function and epithelial integrity (Meissner et al., 2017). The translocation of molecules into the blood stream will activate an inflammatory response (Khafipour et al., 2009a). Although SAA increased numerically during wk 7, it appears that the ruminal acidosis the calves experienced during wk 5 to 8 did not influence LBP or SAA. This finding may indicate that barrier function during this time was not influenced because the corneum and the granulosum were not affected during these wk. There was a moderate negative correlation between CS intake and SAA, suggesting that increased CS intake did not evoke an inflammatory response in the current study. This is in contrast to increased blood concentrations of SAA and LBP in cows fed high-grain diets (Khafipour et al., 2009a), indicating inflammatory response activation. A peak in acute phase proteins (SAA and haptoglobin) was observed around 1 wk of life in calves and has been reported before (Orro, 2008; Tóthová et al., 2015). However, thereafter SAA decreased to 80-200 μg/mL, which is considered baseline for a healthy calf (Seppä-Lassila et al., 2013). A similar pattern was observed in the current study. It has been suggested that the peak at approximately the first wk of life is observed because of possible acute phase proteins that are transferred through colostrum intake, which enter calf circulation (McDonald et al., 2001). Another possibility may be that pro-inflammatory cytokines, such as interleukin-6, is ingested via colostrum and stimulates acute phase protein production (Hagiwara et al., 2000). The numerical increase of SAA during wk 7 may be an indication of weaning stress experienced by calves, as Kim et al. (2011) attributed elevated SAA concentrations 3 and 5 d post-weaning to weaning stress. Further research investigating if depressed ruminal pH causes local inflammation is warranted in calves.

5.5. Conclusion

To our knowledge, this is the first study investigating ruminal pH over a long duration (6 wk post-weaning). Metabolizable energy supply from CS may have an effect on rumen development as it took several wk for rumen papillae surface area to increase. Ruminal pH is depressed even prior to step-down weaning in calves with low starter intake, which might be due reduced buffer capacity. It took several wk for the rumen to adapt so as to not be in a severe state of ruminal acidosis post-weaning. The prolonged decreased pH may be caused by a combination

of a delay in rumen development and the inability to buffer the rumen post-weaning. Barrier integrity was likely not affected by the prolonged pH depression and increased SCFA since papillae strata and sloughing score were largely unaffected. Furthermore, acute phase proteins (SAA and LBP) were not influenced during this time, indicating that no inflammatory response occurred. Further investigation is warranted to determine if ruminal acidosis is harmful during the pre- and post-weaning periods in calves as it is in adult animals.

5.6. Literature cited

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5.7. Tables and figures

Table 5.7- 1. Chemical composition of starter and hay fed to dairy calves

Item	Starter	Straw
Dry matter, % as fed	89.4	92.5
DM basis, %		
CP	23.4	4.1
Fat	4.5	1.0
ADF	9.3	53.9
NDF	15.1	75.7
Starch	32.9	1.7
NFC	50.8	9.4
ME[1], Mcal/kg	2.8	1.5

[1]Calculated using NRC (2001).

Table 5.7- 2. Short chain fatty acid characteristics and lactate throughout week 5 to 12 in dairy calves (n = 6) fed) fed up to 1.4 kg/d of milk replacer that were weaned during wk 6.

	Week[1]									*P* value
Item	5	6	7	8	9	10	11	12	SEM	Week
Total SCFA, mM	57.2^{a}	92.0^{ab}	113.6^{b}	103.1^{ab}	105.7^{ab}	118.7^{b}	123.4^{b}	132.8^{b}	12.8	0.003
Acetate, mol/100mol	58.8^{a}	50.4^{ab}	49.5^{ab}	49.8^{ab}	47.4^{b}	47.0^{b}	46.7^{b}	43.2^{b}	2.31	0.006
Butyrate, mol/100mol	12.05	15.75	16.62	17.62	19.27	18.55	17.69	22.75	2.426	0.15
Propionate, mol/100mol	26.44	30.89	30.56	28.76	28.14	29.38	30.51	29.43	1.802	0.69
Isobutyrate, mol/100mol	0.73	0.57	0.57	0.57	0.81	0.67	0.57	0.55	0.094	0.39
Isovalerate, mol/100mol	0.50^{ab}	0.27^{ab}	0.26^{a}	0.33^{ab}	0.54^{b}	0.45^{ab}	0.34^{ab}	0.30^{ab}	0.064	0.013
Valerate, mol/100mol	1.44^{a}	2.09^{ab}	2.44^{ab}	2.90^{ab}	3.81^{b}	3.80^{b}	4.17^{b}	3.80^{b}	0.480	0.003
Lactate, mM	2.95	0.64	0.43	0.30	0.27	0.55	1.44	1.49	0.910	0.45

[1]Values represent means. Different letters represent a significant difference ($P < 0.05$) between week

Table 5.7- 3. Morphometric ruminal papillae parameters and cell density of rumen epithelial strata throughout week 5, 6, 7, 8 and 12 in dairy calves (n = 6) fed) fed up to 1.4 kg/d of milk replacer that were weaned during wk 6.

	Week[1]						*P* value
Item	5	6	7	8	12	SEM	Week
Papilla Surface Area (mm)²	526.7^{a}	615.9^{a}	1056.3^{a}	1195.0^{a}	7682.4^{b}	852.41	<.0001
Papilla Length (µm)	694.9^{a}	723.2^{a}	879.5^{a}	947.8^{a}	3226.50^{b}	247.87	<.0001
Papilla Width (µm)	417.7^{a}	456.4^{ab}	675.5^{b}	686.5^{b}	1391.9^{c}	59.35	<.0001
Corneum thickness (µm)	11.9^{a}	12.0^{a}	13.7^{ab}	15.9^{ab}	17.2^{b}	1.29	0.003
# of cells in corneum	2.05	2.13	2.35	2.67	2.64	0.187	0.036
Granulosum thickness (µm)	17.4	18.5	20.6	19.5	19.3	1.01	0.17
# of cells in granulosum	2.44	2.24	2.50	2.27	2.35	0.096	0.25
Spinosum/basale thickness (µm)	46.8^{a}	48.8^{ab}	67.0^{ab}	70.1^{b}	63.9^{ab}	4.73	0.009
# of cells in spinosum/basale	4.71	4.65	6.24	6.42	5.72	0.455	0.049
Epithelium thickness (µm)	63.4^{a}	66.6^{ab}	87.2^{bc}	90.6^{c}	85.2^{abc}	5.28	0.006
Papillae sloughing score	1.6	2.2	2.4	2.6	2.5	0.27	0.14

[1]Values represent means. Different letters represent a significant difference ($P < 0.05$) between weeks.

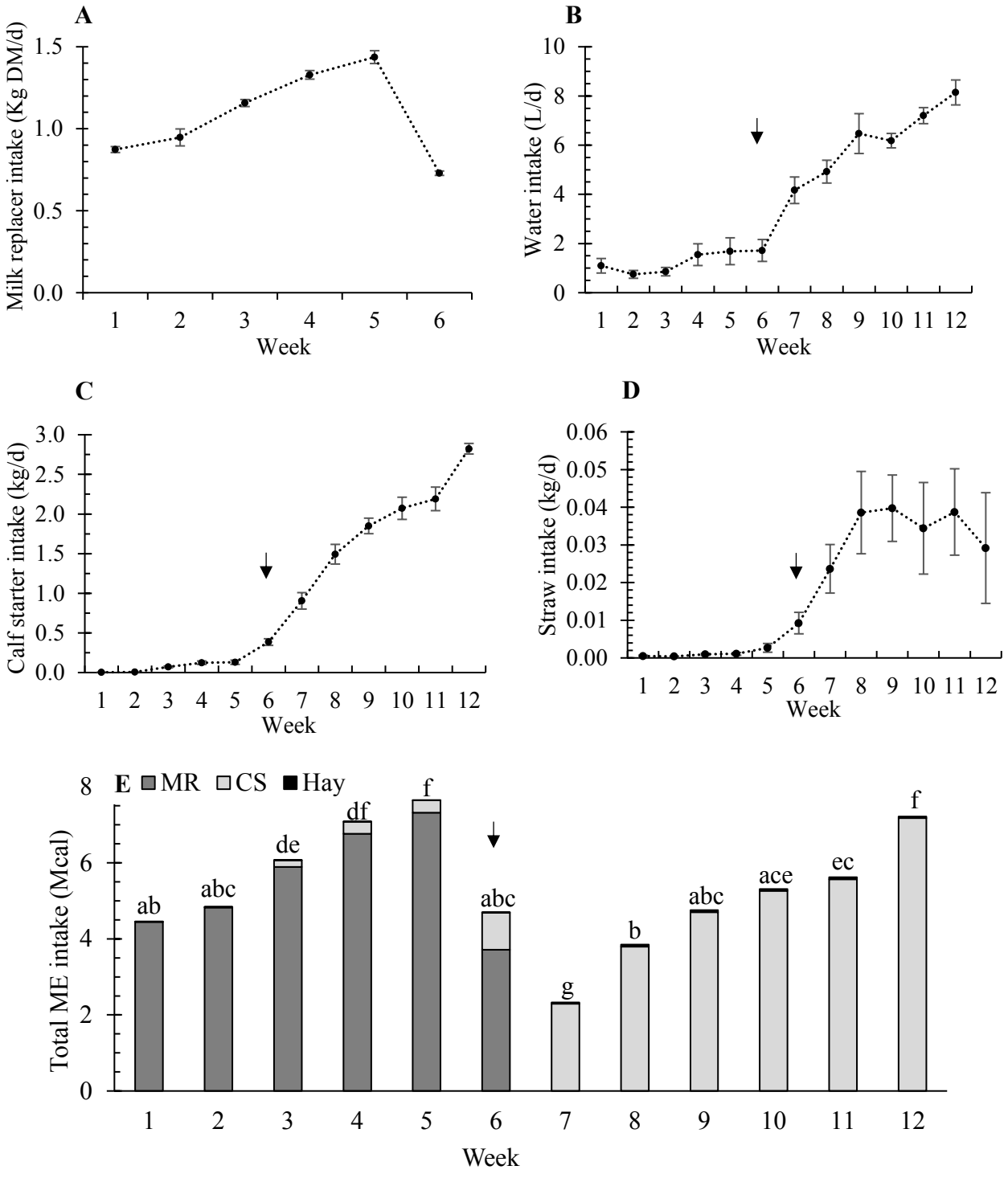


Figure 5.7- 1. Mean weekly (A) MR intake, (B) Water intake, (C) CS intake, (D) Hay intake and (E) Total ME intake in dairy calves (n = 6) fed) fed up to 1.4 kg/d of milk replacer that were weaned during wk 6. Error bars represent SEM and arrows indicate step-down weaning. Different letters represent a significant difference ($P < 0.05$) between weeks.

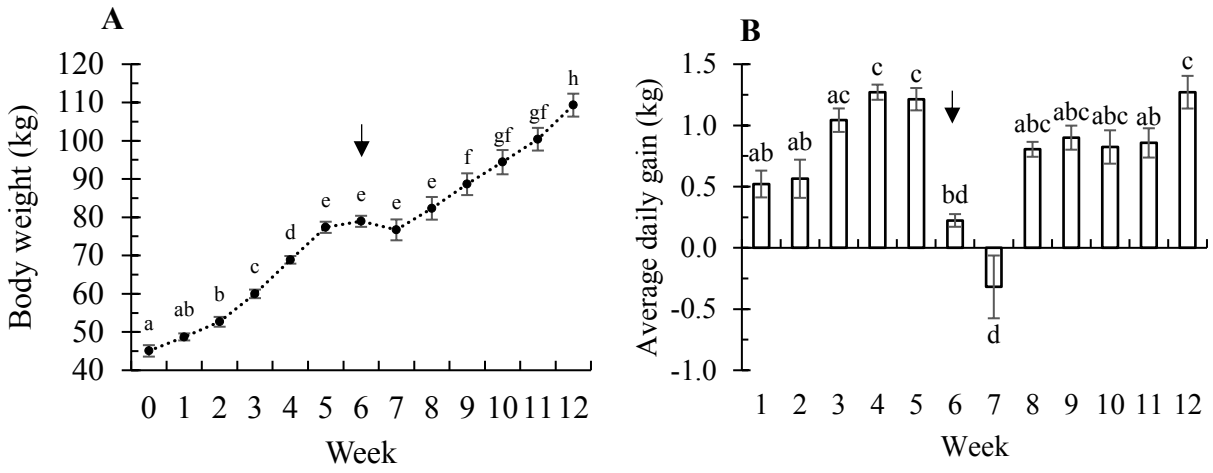


Figure 5.7- 2. Mean weekly (A) BW and (B) average daily gain in dairy calves (n = 6) fed) fed up to 1.4 kg/d of milk replacer that were weaned during wk 6. Error bars represent SEM and arrows indicate step-down weaning. Different letters represent a significant difference ($P < 0.05$) between weeks.

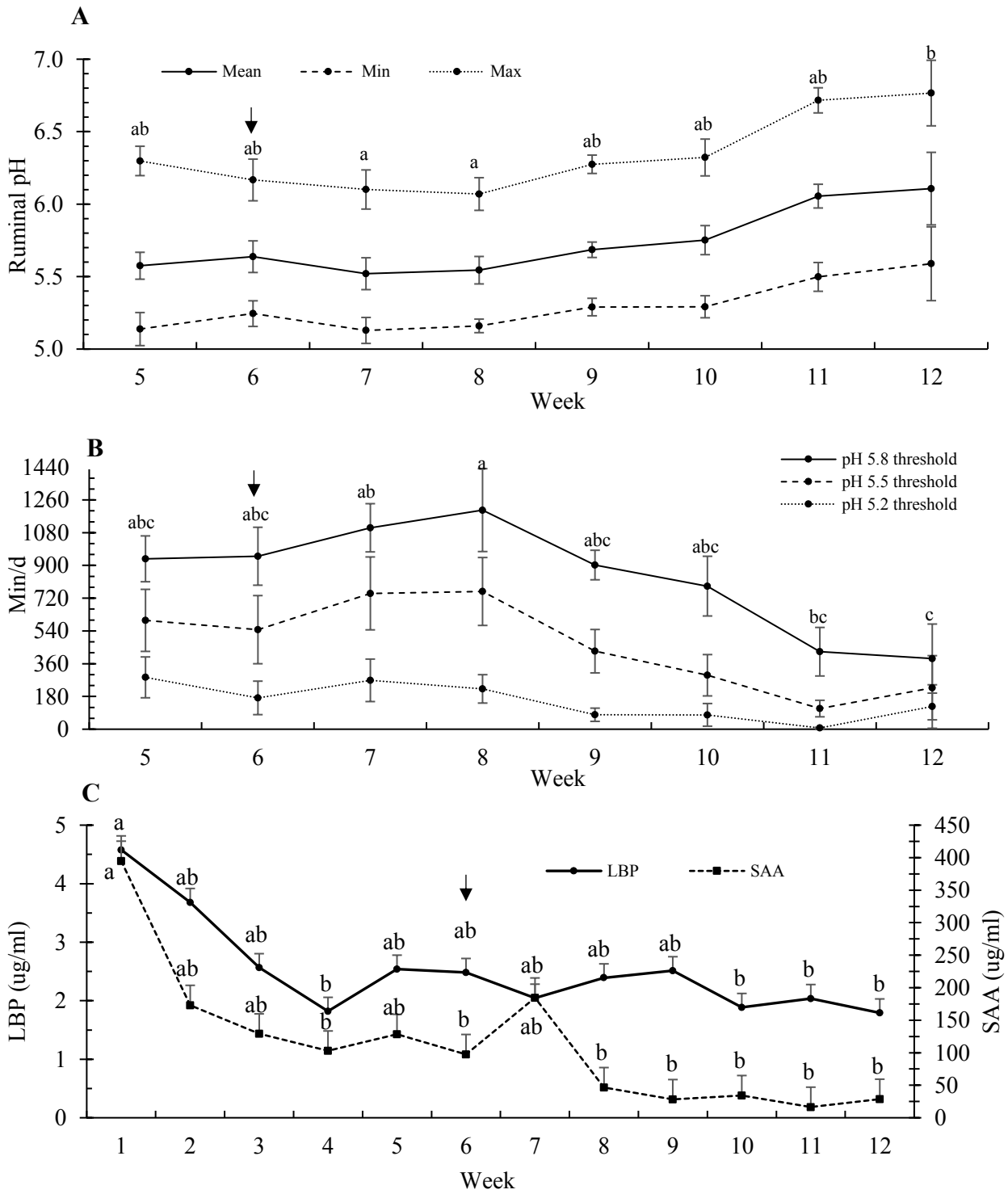


Figure 5.7- 3. (A) Daily ruminal pH summarized by week: maximum, mean and minimum ruminal pH from dairy calves (n = 6) fed up to 1.4 kg/d of milk replacer that were weaned during wk 6. (B) The duration(min/d) the pH was below pH threshold 5.8, 5.5 and 5.2. (C) Average lipopolysaccharide binding protein and serum amyloid A. Error bars represent SEM and arrows indicate step-down weaning. Different letters represent a significant difference ($P < 0.05$) between weeks.

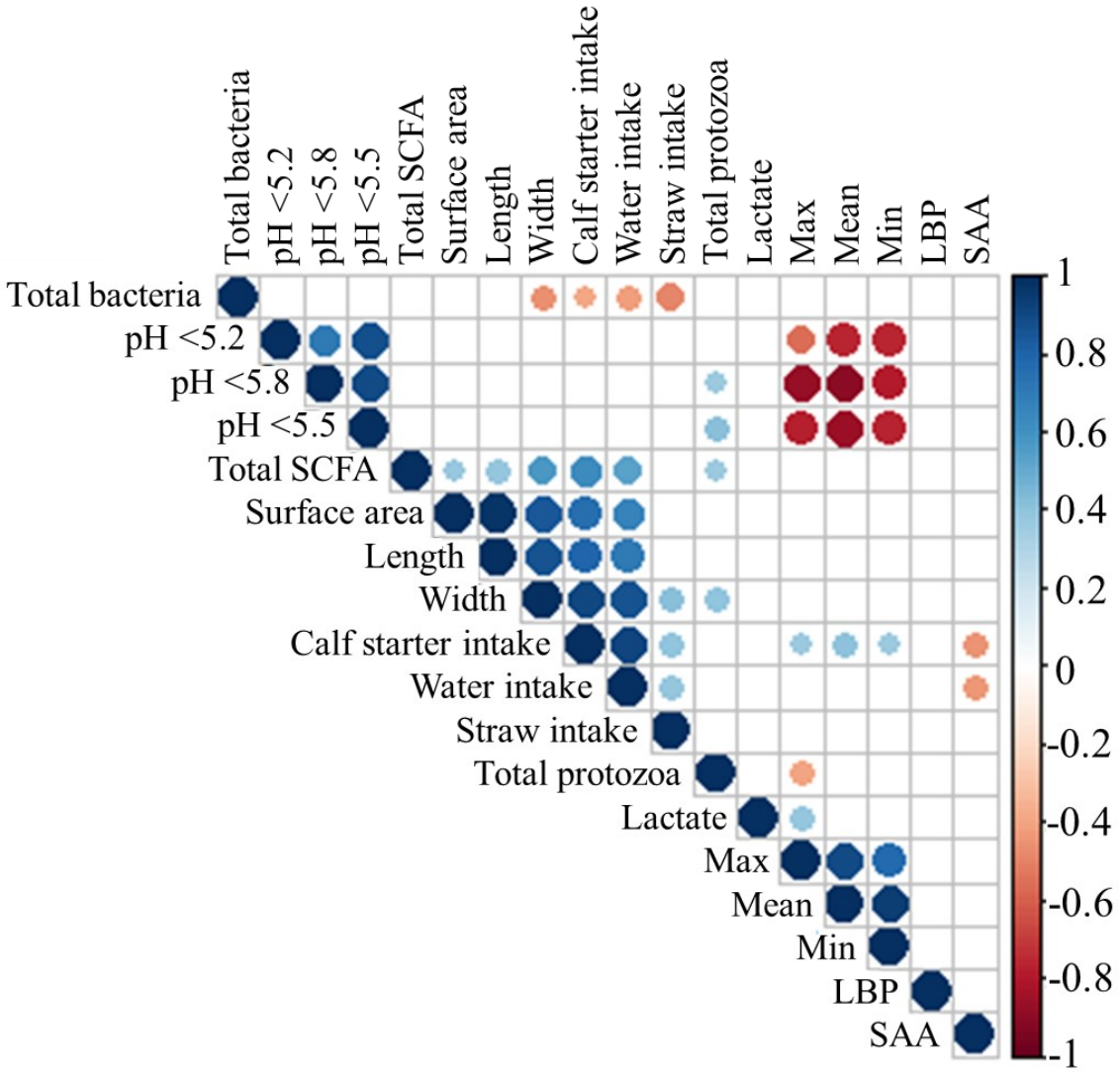
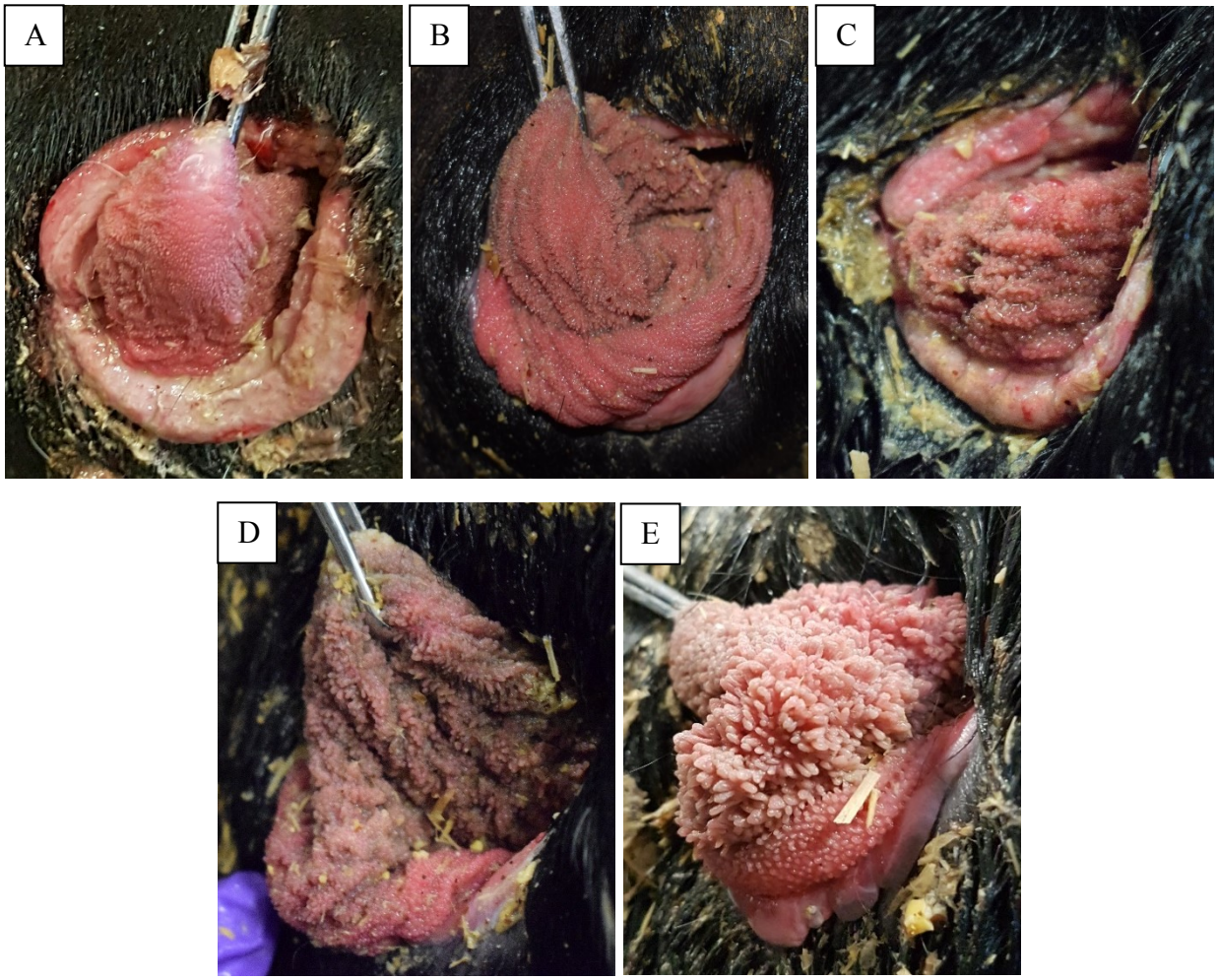


Figure 5.7- 4. A heatmap illustrating Pearson's correlation coefficients between total bacteria, pH thresholds (5.2, 5.8 and 5.5), total short chain fatty acids (SCFA), papillae surface area, length and width, calf starter, water and straw intake, total protozoa, lactate, maximum, mean and minimum ruminal pH, lipopolysaccharide binding protein (LBP) and serum amyloid A (SAA). Circle size indicates significant correlations $P \leq 0.05$, the bigger the circle the higher the significance.



Supplemental figure S5.7- 1. Representative images of the dorsal coronary pillar retrieved through the cannula hole using Allis clamps illustrating different stages of papillae development. (A) week 5, (B) week 6, (C) week 7, (D) week 8 and (E) week 12.

6. Assessment of the colon transcriptome changes from pre- to post-weaning in calves fed high milk replacer feeding rate.

6.1. Introduction

In the hindgut, undigested feed undergoes microbial fermentation, producing short chain fatty acids (**SCFA**), which may contribute up to 9 % of metabolizable energy requirements in cattle (Bergman, 1990). It is known that colon fermentation plays an important role in feed digestion and production of SCFA. It has been reported that the colon is colonized with diverse microbiota that produces SCFA in dairy calves from birth to 6 wk of age (Song et al., 2018). When calves consume calf starter the expression of genes related to immune response were up-regulated in the colon of calves (*TLR2*; Malmuthuge et al., 2013) and down-regulated in colon of lambs (*TLR4, TNFα* and *IFNγ*; Liu et al., 2017) compared to animals fed milk only. Despite our knowledge of gastrointestinal tract (**GIT**) changes that occur, the hindgut is highly understudied in ruminants, and only received attention recently due to its role in fermentation and immunity. With the advancement of molecular approaches to characterize gene expression it has been useful to describe rumen adaptations during weaning. However, there has been a only few studies that used this approach to characterize colonic targeted gene changes in transitioning cows (Bach et al., 2018) and goats (Tao et al., 2014a,b) and transcriptomic changes in dairy calves (He et al., 2018) and steers (Wang et al., 2017) due to the challenges in obtaining biological samples without sacrificing the animals.

During weaning, calves may experience increased fermentation in the hindgut. Excessive fermentation in the hindgut can result in reduced pH and may lead to hindgut acidosis (Gressley et al., 2011). It has been reported that during weaning and post-weaning, fecal starch increased (Eckert et al., 2015; Steele et al., 2017), which is an indication that higher amounts of starch reach the hindgut, possibly increasing the likelihood of hindgut acidosis incidences in calves. When hindgut acidosis occurs, cecum and colon tissue becomes damaged (Tao et al., 2014a), leading to inflammation and possibly systemic inflammatory responses indicated by elevated serum amyloid A (**SAA**) and lipopolysaccharide binding protein (**LBP**) concentrations in the blood (Khafipour et al., 2009a, b). However, knowledge regarding the influence of increased starch reaching the hindgut on colon function and the relationship between colon function and SAA and LBP of calves are lacking.

We hypothesized that in calves, gene expression in the colon tissue will respond to increased starch reaching the hindgut from pre- to post-weaning, and that these changes will be associated with a rise in systemic inflammatory response indicators. The objectives of this study were to investigate the functional changes in the colon of calves that occur at molecular level using RNA-seq based transcriptomics and to link such changes to calf phenotypic changes, including colon mucosa thickness, fecal starch and serum inflammatory marker concentrations pre- and post-weaning and during the weaning transition.

6.2. Materials and methods

6.2.1. Animals and housing

Animals, housing and feeding were described in Chapter 4. In this study, 6 Holstein bull calves were cared for and handled in accordance with the Canadian Council on Animal Care (CCAC, 2009) regulations and all experimental procedure were reviewed and approved by the institutional Animal Care and Use committee (University of Alberta, AB; AUP00002010).

Briefly, calves were individually housed in a barn with pens (3.05 × 3.66 m) with rubber mats (2.42 × 2.42 m) and wood shavings. Calves were fed 6 L of milk replacer (MR; 150 g/L, 26 % CP, 20 % fat, 38 % lactose as fed; Grober Nutrition, Cambridge, Ontario) solution per d for 7 d, thereafter MR solution was fed at 15 % of bodyweight (BW; adjusted every 7 d based on new BW) per d. During wk 6 calves received 50 % of previous wk MR allocation and were fully weaned at the end of wk 6. Milk replacer was fed twice daily and calves had ad libitum access to texturized calf starter (CS; 23.4 CP %, 32.9 % starch; Trouw Nutrition Canada Inc., Strathmore, AB, Canada), straw (4.1 % CP, 1.7 % starch; Skyline Harvest, Blumenort MB, Canada, 1-inch chop length) and water.

6.2.2. Data and sample collection

Fecal samples. Fecal samples were collected from each calf three hours after the morning feeding weekly, at the end of wk 4 to 12. All samples were stored at -20 °C for fecal DM and starch analysis. The fecal samples were later analyzed (AOAC International, 2000) for DM (oven method 930.15) and starch (α-amylase method; Hall, 2009).

Colon tissue collection. Colon tissue biopsies were taken at the end of wk 5, 6, 7, 8 and 12 using and endoscope as described by Van Niekerk et al. (2018; Chapter 4). Briefly, to obtain

colon samples with an endoscope, the calves were restrained in a calf chute and the lubricated distal tip of the endoscope (100 cm length, 9.8 mm diameter; GIF-Q140, Olympus, Tokyo, Japan) connected to a light source and processor (CLV-U40 and CV-140, Olympus) was gently inserted 40 cm into the calf's anus. From the distal colon, tissue samples were obtained at 30 to 40 cm from the calf's anus with endoscopic biopsy forceps (Captura hot biopsy forceps, HDBF-2.4-230-S, Cook Medical). The tissue samples were washed with phosphate buffered saline (PBS; pH 7.4). Two colon tissue samples were obtained at wk 5, 6, 7, 8 and 12 and submerged in 10 % formalin solution and stored at room temperature at each timepoint for histological analysis. An additional 4 colon tissue samples were obtained at the end of wk 5, 7 and 12 and placed in RNA stabilization fluid (RNALater, ThermoFisher Scientific, Burlington, ON, Canada) for RNA analysis and the samples were stored at room temperature for 24 h and then frozen at −20 °C.

6.2.3. Sample and statistical analyses

Colon mucosa thickness measurements. Colon tissue samples were retrieved from the fixative, dehydrated overnight, submerged in paraffin wax, sectioned in 4 µm thick slices, and stained with hematoxylin and eosin (Steele et al., 2011). A minimum of 4 micrographs were taken per sample per calf per sampling timepoint using a light microscope (Zeiss Axio Scope.A1, Oberkochen, Germany) and optronics digital camera (PictureFrame Ver, 2.3; Figure 6.7-1C) at x 20 magnification. The images obtained from these samples were used for the measurements of mucosa thickness by placing a grid on the image and measuring the mucosa thickness at 5 different equally spaced regions using ImageJ (National Institutes of Health, Bethesda, MD, USA). All measurements were performed by two separate individuals and differences between both were tested by using SAS and there were no differences, therefore the averages are reported.

Statistical Analysis of fecal starch and mucosa thickness. Fecal DM and starch and mucosa thickness data were analyzed using GLIMMIX procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC). The model included wk as a fixed effect and calf as a random effect and were as follow:

$$Y_{ij} = \mu + wk_i + \varepsilon_{ij},$$

where, Y_{ij} = the j^{th} observation in the i^{th} wk; μ = the overall mean; wk_i = the fixed effect of the i^{th} wk; and ε_{ij} = the random error in the j^{th} observation in the i^{th} wk. Significance were declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RNA Extraction and RNA Sequencing. A RNeasy mini kit (QIAGEN Inc, Germantown, MD, USA) was used to isolate total RNA from each biopsy sample (average total RNA concentration was 9,961 ± 662 ng/sample) as per manufacturer's instructions. Total RNA was used to construct the libraries using TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Thereafter, cDNA libraries were pooled and then sequenced at Génome Québec (Montréal, Quebec, Canada) using the Illumina HiSeq 4000 system (Illumina) to obtain 100bp paired-end reads.

mRNA Mapping and Normalization. Fastq-mcf set at quality score ≥ 20 and length ≥ 75 were used to trim and filter adapters and low-quality bases from the obtained sequences (Aronesty, 2013). Then, the clean sequencing reads were aligned to the reference bovine genome (UMD 3.1; Ensembl v.83.31) and assembled (Zimin et al., 2009) using software packages TopHat2 (v2.0.9) (Kim, D. et al., 2013) and Bowtie2 (v2.3.3.1) (Langmead and Salzberg, 2012). The BAM files generated by TopHat2 were then converted into SAM files using Samtools (v1.1) (Li et al., 2009). Thereafter the number of mapped reads per gene in the SAM file were quantified using HTSeq-count (v0.6.1) (Anders et al., 2015). The mapped reads were normalized using counts per million (**CPM**), which are as follow: (number of reads mapped to a gene in a sample) ÷ (total number of reads mapped to all annotated genes in a sample) × 10^6. Genes were considered expressed when CPM >1 and in at least 4 animals per age group.

Identification of differentially expressed and uniquely expressed genes. Differentially expressed (**DE**) genes were identified based on pairwise comparison assigned as short term (wk 7 vs. 5), long term (wk 12 vs. 5) and post-wean (wk 12 vs. 7) using Bioconductor edgeR (v3.4.1l) package in R (Kim et al., 2013). Raw p-values were adjusted by false discovery rate (**FDR**) based on the Benjamini and Hochberg method. Genes with fold change >1.5 or < −1.5 and FDR ≤ 0.05 were considered DE. The genes that were only expressed at one time point were defined as uniquely expressed genes.

Weighted Gene Co-expression Network Analysis (WGCNA) of expressed genes and trait relationships. To examine the relationship between expressed genes and phenotypic

measures, the WGCNA R software package (v1.41.1) (Langfelder and Horvath, 2008) was used to identify the co-expressed genes that were correlated with the traits. The co-expressed genes were grouped into modules (distinguished by different color name) by using the automatic one-step function, blockwiseModules. The modules were then used to identify module–trait relationships (calculated by Pearson correlation) with calf starter intake, fecal starch, colon mucosa thickness and acute phase proteins (lipopolysaccharide binding protein and serum amyloid A; **LBP** and **SAA**; obtained from Chapter 4). The modules that were significantly correlated ($-0.5 \geq r \geq 0.5$; $P \leq 0.05$) with more than one phenotypic trait were chosen for further functional analysis (darkturquoise, green, royalblue and grey60).

Function analysis of core and uniquely expressed and DE genes and trait-associated genes. The functions of core transcriptome, DE genes and uniquely expressed genes obtained were then identified using the Protein Annotation Through Evolutionary Relationship (PANTHER; Mi et al., 2019) gene tool. The results were summarized at molecular function, biological process, and cellular component levels. Ingenuity Pathway Analysis (**IPA**) was used for enriched molecular and cellular functions of DE genes and trait associated genes and significance was declared at $P < 0.05$.

6.3. Results

Fecal starch and colon mucosa thickness. Fecal starch had a wk effect ($P = 0.0002$), with wk 9 higher ($P \leq 0.01$) than wk 4, 5 and 6 (Figure 6.7-1A). Overall, fecal DM tended to be different ($P = 0.08$) over wk, reaching the lowest ($P = 0.04$) fecal DM at wk 10 compared with wk 7. There was a wk effect on colon mucosa thicknesses ($P = 0.0016$) and it was lower during wk 7 and 8 compared with wk 5 (Figure 6.7-1B).

Colon epithelial transcriptome. A total of 14, 311 genes were expressed (CPM >1 and in more than 4 out of 6 animals per age group) in the colon tissue of calves. A total of 13,466, 13,855 and 13,442 genes were expressed in wk 5, 7 and 12, respectively with 12,886 expressed in all age groups which was defined as core transcriptome in this study (Fig 2). In addition, 192, 400 and 153 genes were identified as uniquely expressed genes for wk 5, wk 7 and wk 12, respectively. Functional analysis of core transcriptome using PANTHER revealed that the top molecular functions were ‘catalytic activity’ (37 %) and ‘binding’ (37 %; Figure 6.7-3). The top cellular components were ‘cell’ (43 %) and ‘organelle’ (32 %) whereas the top biological

processes were ‘cellular’ (32 %) and ‘metabolic processes’ (23 %). For the unique genes, ‘cellular process’ and ‘cell’ were the top ones in the biological processes and cellular components categories, respectively at wk 5, 7 and 12. (Figure 6.7-4A and B). At wk 5 ‘catalytic activity’ was the top molecular function and at wk 7 and 12 ‘binding’ was the top molecular function (Figure 6.7-4C).

With regarding the DE genes, a total of 65 DE genes were identified in the colon between wk 12 and wk 5 (defined as long term), and 10 DE and 18 DE genes for comparisons between wk 7 vs 5 (defined as short term), and wk 12 vs 7 (defined as post-wean; Figure 6.7-1B), respectively. Of these DE genes, 3, 38 and 11 were up-regulated and 7, 37 and 7 were down-regulated for short term, long term and post-wean comparisons, respectively (Supplemental table S6.7-4).

Co-expressed genes using WGCNA. WGCNA identified co-expression gene modules that were significantly correlated with fecal starch, colon mucosa thickness, LBP, SAA and CS intake respectively. In total, 24 gene modules were identified and 10 of the modules were significantly correlated with phenotypic traits (Figure 6.7-5). The darkturquoise module (183 genes) had a negative correlation with CS intake (r = -0.57, $P = 0.02$). The darkturquoise module also had a positive correlation with SAA (r = 0.72, $P = 0.002$). The green module (517 genes) had a negative correlation with fecal starch (r = -0.60, $P = 0.01$) and CS intake (r = -0.76, $P < 0.001$) and a positive correlation with colon mucosa thickness (r = 0.57, $P = 0.02$). The grey60 module (241 genes) had a negative correlation with colon mucosa thickness (r = -0.05, $P = 0.05$) and a positive correlation with CS intake (r = 0.67, $P = 0.005$). Similarly, the royalblue module (226 genes) had a negative correlation with colon mucosa thickness (r = -0.53, $P = 0.04$) and a positive correlation with CS intake (r = 0.79, $P < 0.001$).The yellow (1111 genes; r = 0.53, $P = 0.04$,), greenyellow (331 genes; r = 0.61, $P = 0.01$) and lightyellow (236 genes; r = 0.71, $P = 0.002$) modules were all positively correlated with CS intake. A negative correlation (r = -0.55, $P = 0.03$) was observed between midnightblue module (266 genes) with fecal starch and cyan (298 genes; r = -0.70, $P = 0.003$) module and CS intake. A positive correlation (263 genes; r = 0.66, $P = 0.005$) was observed with lightcyan module with SAA.

Functional analysis of co-expressed genes related to the traits. The functional analysis was further analyzed for co-expressed genes in darkturquoise, green and royalblue and grey60

modules because they were associated with more than one phenotypic trait. Functional analysis using IPA revealed that that the top five enriched molecular and cellular functions of co-expressed genes in the darkturquoise module were cell death and survival, cell morphology, cell-to-cell signaling and interaction and cell cycle (Supplemental table S6.7-1). Co-expressed genes in the green module had cellular function and maintenance, cellular movement, molecular transport, cellular development and amino acid metabolism as the top five functions. Top five functions of co-expressed genes in the royalblue module also included cellular function and maintenance, cellular movement, molecular transport, cellular development and also had small molecular biochemistry. Cell cycle, cell morphology, drug metabolism, lipid metabolism and small molecular biochemistry were the top five molecular and cellular functions that were enriched in from the co-expressed genes in the grey60 module. The top five molecular functions, cellular components, and biological processes analysis with Panther for genes in darkturquoise, green, royalblue and grey60 module are presented in Supplemental table S6.7-2. Overall, the top molecular functions were binding, catalytic activity, molecular function regulator, molecular transducer activity, transcription regulator activity and transporter activity. The top five cellular components cell, organelle, membrane, protein-containing complex, extracellular region; and the biological processes were cellular process, metabolic process, biological regulation, localization, and response to stimulus for the co-expressed genes in four modules.

Top five pathway of co-expressed genes identified by PANTHER are presented in Supplemental table S6.7-3. The pathway with the most genes were inflammation mediated by chemokine and cytokine signaling pathway (8 genes) from the co-expressed genes in the darkturquoise module, integrin signaling pathway (9 genes) from the co-expressed genes in the green module, CCKR signaling map (7 genes) from the co-expressed genes in the grey60 module and inflammation mediated by chemokine and cytokine signaling pathway (5 genes) from the co-expressed genes in the royalblue module.

Functional analysis of short term, long term, and post-wean DE genes. Functional analysis using IPA revealed that that the top five molecular and cellular functions of short-term DE genes were cell morphology, cellular function and maintenance, molecular transport, protein synthesis and amino acid metabolism (Supplemental table S6.7-5). For the long-term DE genes, cellular function and maintenance, molecular transport, small molecular biochemistry, cell death and survival and cellular movement were the top five functions. Cell-to-cell signaling and

interaction, cellular development, cellular growth and proliferation, drug metabolism and molecular transport were the top five molecular and cellular functions for post-wean DE genes.

Genes identified as co-expressed and DE. The meprin A subunit beta (*MEP1B*) gene was a long-term DE gene (1.5 FC down regulated) that was also a co-expressed gene (darkturquoise module) negatively correlated with CS intake and fecal starch and positively correlated with SAA and colon mucus thickness (Table 6.7-1). From the co-expressed genes in the green module, 13 genes were also DE genes from which 3, 12 and 1 were in the short term, long term and post-wean groups respectively. In the short-term comparison, the expression of aquaporin 8 (*AQP8*), solute carrier family 13 member 2 (*SLC13A2*) and solute carrier family 7 member 8 (*SLC7A8, LAT-2*) were down regulated by 1.76, 1.64 and 1.55 FC, respectively. Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2 (*ACE2*; FC =-2.04), cubilin (*CUBN*; FC =-2.52), endothelial PAS domain protein 1 (*EPAS1*; FC =-2.05), heme oxygenase 1 (*HMOX1*; FC =-1.79), potassium voltage-gated channel subfamily J member 15 (*KCNJ15*; FC =-1.96), nephronectin (*NPNT*; FC =-1.65), S100 calcium binding protein A12 (*S100A12*; FC =-1.58), solute carrier family 13 member 2 (*SLC13A2*; FC = -3.74), solute carrier family 40 member 1 (*SLC40A1*; FC =-2.21), solute carrier family 6 member 14 (*SLC6A14, ATB0*,+; FC =-1.95), solute carrier family 7 member 8 (*SLC7A8*, *LAT-2*; FC =-2.44) and solute carrier family 9 member A3 (*SLC9A3, NHE3*; FC =-1.98) were DE genes in the long term comparison. Post-wean DE gene solute carrier family 13 member 2 (*SLC13A2*; FC=-2.12) was also one of the co-expressed genes in the green module. Short term DE gene flavin containing monooxygenase 2 (*FMO2*; FC=2.14) was found to be one of co-expressed genes in the grey60 module that was positively correlated with CS intake and negatively correlated with colon mucosa thickness. The long term (FC= 2.03) and post-wean group (FC=1.58) DE gene collagen type XVII alpha 1 chain (*COL17A1*) were the co-expressed genes from the royalblue module that was negatively correlated with colon mucosa thickness and positively correlated with CS intake.

6.4. Discussion

During weaning, the GIT undergoes significant changes when calves are transitioned from a pre-dominantly milk-based diet to a solid feed diet to facilitate calf maintenance and growth (Baldwin VI et al., 2004; Guilloteau et al., 2009). During weaning, CS intake increases rapidly which may lead to increased fecal starch due to poor GIT adaptations at this time (Steele

et al., 2017) as demonstrated in the current study by higher fecal starch shortly after weaning. Fecal starch concentration may be influenced by CS type and weaning method, feeding texturized CS (Quigley et al., 2019), weaning calves early (Eckert et al., 2015) and abruptly (Steele et al., 2017) increase fecal starch. Such increase in fecal starch may lead to increased hindgut fermentation (Gressley et al., 2011), increasing the likelihood of hindgut acidosis.

Hindgut acidosis has been identified and has been reported to damage cecum and colon tissues in cattle (Gressley et al., 2011) and goats (Tao et al., 2014a,b); however, it is unclear whether this could also happen in the hindgut of calves during the weaning transition. Histological analyses revealed that mucosa of the cecum and colon can be damaged by feeding high grain diets to goats (Tao et al., 2014a). It is suggested that endotoxins such as LPS may be translocated into the blood stream when barrier function is impaired, which may cause an inflammatory response (Khafipour et al., 2009a). In the current study we did not observe colon mucosa damage in all of the samples collecte and the normal levels of serum inflammatory markers that have been observed in Chapter 4 indicate that even though a reduction in mucosa thickness were observed, it may not lead to an inflammatory response in the colon of these calves.

Very few studies have studied colon function of ruminants at molecular level. In dairy calves 51 h of age, different colostrum feeding times does not influence colon gene expression (He et al., 2018). However, a total of 122 colon genes involved in immune function were positively or negatively correlated with *Lactobacillus* and *Faecalibacterium prausnitzii* abundance. Beef steers that shed more *Escherichia coli* O157 have higher expression levels of descending colon genes involved in immune function (T-cell migration and proliferation) and cholesterol absorption (Wang et al., 2017). According to both these studies colon genes involved in immune function play an important role in the health of young calves and beef steers. In the current study, 32 % and 23 % of the core genes of the colon mucosa were involved in cellular and metabolic processes, respectively. These two processes are also reported to be the top processes in neonatal ruminants (He et al., 2018) and beef steers (Wang et al., 2017). The colon does play a role in SCFA absorption and metabolism and the SCFA absorbed may account for up to 9 % of metabolizable energy requirements in adult cattle (Bergman, 1990) thus, the colon may also play an important role in cellular and metabolic processes such as SCFA absorption to support calf growth.

Among the 16 genes that overlapped between DE genes and co-expressed genes, to our knowledge none of them have been reported in the colon tissue of calves. Six out of the 16 genes (*AQP8, SLC7A8, SLC13A2, SLC9A3, SLC6A14 and SLC40A1*) were involved in nutrient or mineral transport and one gene that is an upstream regulator for inflammatory responses or tissue growth pathways (*S100A12*). A summary is presented in Figure 6.7-6 of the functional chances (tissue growth, inflammatory response, and nutrient absorption) that the aforementioned seven genes are involved in and these gene relationships with the changes that occur in the phenotypic traits which will be further discussed below.

The colon is responsible for water, amino acid, and mineral absorption (Sherwood et al., 2013). The expression of genes involved in water or urea (*AQP8*), amino acid (*SLC7A8* and *SLC6A14*) and mineral absorption (*SLC9A3, SLC40A1*) decreased post-weaning. The expression of these genes was also negatively correlated with CS intake and fecal starch. The first DE and co-expressed gene to be discussed is *AQP8,* which are part of the aquaporin (AQP) family that transport water and may also have the ability to transport urea (Ma and Verkman, 1999). From this family, *AQP3, AQP7, AQP9*, and *AQP10* have been reported to be able to transport urea, however the ability of *AQP8* to transport urea is still under investigation (Echevarria and Ilundain, 1998; Rojek et al., 2008; Litman et al., 2009). The *AQP8* is expressed in human and rat colon tissue (LaPointe et al., 2008) and was reported to be expressed in ruminal epithelium of Nellore bulls on a finishing diet (Benedeti et al., 2018), however *AQP8* expression in the ruminant colon is unknown. It has been reported that the protein abundance (using immunoblots) of *AQP8* is higher in the rumen epithelium when dairy cows received a higher protein diet (Røjen et al., 2011), which is the opposite than what was expected because with high protein intake urea recycling decreases. Our results are also similar to the Røjen study as the calves received 26 % CP in the MR and only 23 % in the CS and during wk 7 (post-weaning) CS intake was still low (< 1.0 kg; Chapter 4), which means that calves were not consuming a lot of protein. This may indicate that *AQP8* expression is independent of protein intake and that other factors, such as water, may regulate *AQP8*. However, further investigation into the regulation of this AQP in the colon is warranted.

In addition, the expression of genes involved in amino acid transport (LAT-2 and ATB0,+) differed between pre- and post-weaning and are positively correlated with colon mucosa thickness and negatively correlated with fecal starch and CS intake. Amino acid transporter

solute carrier family 7 member 8 (*LAT-2*) has medium affinity for neutral amino acids and high transport capacity, whereas amino acid transporter solute carrier family 6 member 14 (*ATB0,+*) transports cationic and neutral amino acids (Broer, 2008). The *LAT-2* gene is expressed in the equine colon (Woorward et al., 2014). Since equine are hindgut fermenters the importance of this gene in the colon in young ruminants might not be the same because the small intestine is the main site where amino acids are absorbed in ruminants. The *ATB0,+* gene has been reported to be expressed in the ruminant duodenum (Waters et al., 2013) and its role in the colon most likely is not of the same importance as in the duodenum because the small intestine is the main site of amino acids absorption. In our study these two amino acid transporters are more expressed pre-weaning than post-weaning, which may indicate that when calves drink milk these transporters in the colon might contribute to both cationic and neutral amino acid transport. It may be possible that during the milk fed period there are amino acids that escape absorption in the small intestine and are absorbed in the colon; therefore, the amino acid transporters in the colon are more abundant pre-weaning. Furthermore, during the post-weaning period the calf relies on microbial protein produced in the rumen for metabolizable protein (50-80 %; NRC, 2001) and it may be possible that less amino acids escape small intestinal absorption, therefore less amino acid transporters are required in the colon.

There are genes expressed in colon tissue that can regulate the pH of the digesta. The expression of sodium/hydrogen exchanger 3 (*NHE3*) differed between pre- and post-weaning and are positively correlated with colon mucosa thickness and negatively correlated with fecal starch and CS intake It has been reported that low ruminal pH may decrease *NHE3* expression in the rumen (Laarman et al., 2012). With the increase in CS intake in our study more starch is reaching the hindgut (shown by increased fecal starch), suggesting the increased fermentation occurring in the hindgut. Less protons are pumped back into the colon lumen with decreased expression of *NHE3*, which will reduce the risk of digesta pH decreasing further (Stumpff, 2018). The altered expression of *NHE3* may suggest altered digesta pH, however, pH was not measured in this study and future work is needed to investigate the change in pH since it plays an important role in minimizing hindgut acidosis.

The expression of *S100A12*, a gene involved in immune function, were reduced post-weaning compared with pre-weaning and was positively correlated with colon mucosa thickness and negatively correlated with fecal starch and CS intake. The S100 proteins are a family of

genes, calcium-binding cytosolic proteins, that have intracellular and extracellular functions involved in immunity, inflammation, cell proliferation, apoptosis, differentiation, migration, protein phosphorylation, energy metabolism and calcium balance (Marenholz et al., 2004; Donato et al., 2013; Gross et al., 2014). In humans, the *S100A12* gene is used as a gene marker of inflammation (Funk et al., 2015) because inflammatory diseases, such as allergies and inflammatory bowel disease increase *S100A12* expression (Hofmann Bowman et al., 2011; Däbritz et al., 2013). In a recent study, it was reported that *S100A12* expression measured in somatic cell mRNA from dairy cow milk may be used as a gene marker for subclinical mastitis (Zhong et al., 2018). The decrease in *S100A12* expression in the current study may indicate that pre-weaning calves experienced inflammation in the colon. More so, this gene is negatively correlated with CS intake and fecal starch but positively correlated with mucosa thickness. This may indicate that MR intake may have a bigger influence on this gene than starch reaching the hindgut, which was unexpected. During wk 5, calves received up to 0.7 kg (DM) of MR per meal which is a large quantity, that may have caused increased passage rate and thereby undigested MR could have reached the colon influencing the *S100A12* gene. Further research on the impact of MR on *S100A12* is warranted to elucidate the reason why the *S100A12* had higher expression pre-weaning than post-weaning. Since *S100A12* is involved in cell proliferation and apoptosis and inflammation, it is possible that this gene can be used as a gene marker of colon mucosa thickness and inflammation in calves. More investigation on *S100A12* gene as a marker for inflammation and colon mucosa thickness is warranted in young ruminants.

In addition, the gene marker *S100A12* is an upstream regulator of the p53 signaling, MAPK cascade and NF-kB pathways (Nazari et al., 2017) that are involved in proliferation, differentiation, survival, apoptosis, motility and stress response (Pimienta and Pascual, 2007; Raman et al., 2007; Shaul and Seger, 2007). Three genes including mitogen-activated protein kinase 1 (*MAPK1*), tumor protein p53 (*TP53*), mitogen-activated protein kinase 6 (*MAPK6*) involved in these three pathways were co-expressed genes that were negatively correlated with CS intake and fecal starch, and positively correlated with colon mucosa thickness are all part of the MAPK cascade. More genes (*MAP2K4* and *MAPK13*) were found as part of the MAPK cascade pathway that were co-expressed and negatively correlated with CS intake and fecal starch concentration and positively correlated with colon mucosa thickness, SAA and LBP concentrations. This suggests that as CS intake and fecal starch increased the expression of these

co-expressed genes decreased. The regulation of cell proliferation and apoptosis of intestinal epithelial cells is a very important process as the intestinal epithelium experiences rapid turnover (every 3–8 d; Cheng and Leblond, 1974), and a balance needs to be maintained between cell proliferation and apoptosis. The p53 pathway plays an important role in the coordination of proliferation and apoptotic signals (Purvis et al., 2012) and the MAPK genes are involved in cascade pathways that are important in cellular processes such as proliferation, differentiation, survival, apoptosis motility and stress response (Pimienta and Pascual, 2007; Raman et al., 2007; Shaul and Seger, 2007). The p53 and MAPK pathway together may indicate that during pre-weaning when calves consume MR the colon mucosa cells experience stress. The targeted proteasome-mediated degradation of the *TP53* protein may be influence during this period and thereby cell proliferation and apoptosis are influence and that is why there is an increase in colon mucosa thickness when the calves consume MR.

Inhibitor of nuclear factor kappa B kinase subunit beta (*IKBKB***)** gene was also a co-expressed gene negatively correlated with CS intake and fecal starch concentration and positively correlated with colon mucosa thickness, SAA and LBP concentrations, which means that as CS intake and fecal starch increased the expression of this co-expressed genes decreased. The *IKBKB* gene have been reported to be expressed in ruminant duodenum (Li et al., 2019) and ileum (Do et al., 2018) but not in the ruminant colon to our knowledge. Normally, nuclear transcription factor kappa-B (*NF-kB*) is bound to an inhibitory protein (*IkB*) keeping it inactive (Oeckinghaus and Ghosh, 2009). Stimuli such as DNA damages, cellular stresses, inflammatory cytokines, bacterial or viral products increases TNFα levels, thereafter *IKBKB* phosphorylates *IkB* releasing *NF-kB*, which enters the cell nuclease (Peterson et al., 2011). *NF-kB* then activates various genes involved in functions such as pro-inflammatory proteins, cell adhesion molecules, acute phase proteins, stress response genes, growth factors, and factors regulating cell proliferation and apoptosis (Barnes and Karin, 1997; Sica et al., 1997). Thus, *IKBKB* play a major role in the NF-kB pathway which may have cause an increase in inflammatory response pre-weaning in calves.

6.5. Conclusion

When calves were fed MR, increased gene expression in the colon was observed for genes involved in tissue growth, nutrient absorption and immune function compared to when

calves were weaned and fed CS. This is the first study to provide evidence that *AQP8, SLC7A8, SLC13A2, SLC9A3, SLC6A14, SLC40A1,* and *S100A12* are expressed in the colon of young ruminants. Furthermore, the observed changes in the response genes suggests that these genes play an important role in young ruminants and may be used as future gene markers to evaluate nutrient absorption and immune function.

6.6. Literature cited

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6.7. Tables and figures

Table 6.7- 1. Genes that are deferentially expressed (DE) and associated with a co-expression gene module.

Module	Pairwise comparison	Gene name and symbol	Fold change	Function
Darkturquoise	Long	Meprin A subunit beta (*MEP1B*)	-1.50	Mediates protein-protein interactions
Green	Short	Aquaporin 8 (*AQP8*)	-1.76	Transport water and urea
		Solute carrier family 13 member 2 (*SLC13A2*)	-1.64	Cotransport of sodium ions and dicarboxylates such as succinate and citrate
		Solute carrier family 7 member 8 (*SLC7A8, LAT-2*)	-1.55	Amino acid transporter, L-type
	Long	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 2 (*ACE2*)	-2.04	Angiotensin-converting enzyme
		Cubilin (*CUBN*)	-2.52	Calcium-binding EGF-like domain
		Endothelial PAS domain protein 1 (*EPAS1*)	-2.05	Act as transcription factors
		Heme oxygenase 1 (*HMOX1*)	-1.79	Metal ion binding and apoptotic process
		Potassium voltage-gated channel subfamily J member 15 (*KCNJ15*)	-1.96	Inward rectifier potassium channel
		Nephronectin (*NPNT*)	-1.65	Calcium-binding EGF domain and mediates protein-protein interactions
		S100 calcium binding protein A12 (*S100A12*)	-1.58	Inflammatory response
		Solute carrier family 13 member 2 (*SLC13A2*)	-3.74	Cotransport of sodium ions and dicarboxylates such as succinate and citrate
		Solute carrier family 40 member 1 (*SLC40A1*)	-2.21	Iron-regulated transporter
		Solute carrier family 6 member 14 (*SLC6A14, ATB0,+)*	-1.95	Cationic amino acid transporter
		Solute carrier family 7 member 8 (*SLC7A8, LAT-2*)	-2.44	Amino acid transporter, L-type
		Solute carrier family 9 member A3 (*SLC9A3, NHE3*)	-1.89	Sodium/hydrogen exchanger 3
	Post-wean	Solute carrier family 13 member 2 (*SLC13A2*)	-2.12	Cotransport of sodium ions and dicarboxylates such as succinate and citrate
Grey60	Short	Flavin containing monooxygenase 2 (*FMO2*)	2.14	Xenobiotic-metabolizing enzymes
Royalblue	Long	Collagen type XVII alpha 1 chain (*COL17A1*)	2.03	Hemidesmosome assembly
	Post-wean	Collagen type XVII alpha 1 chain (*COL17A1*)	1.58	Hemidesmosome assembly

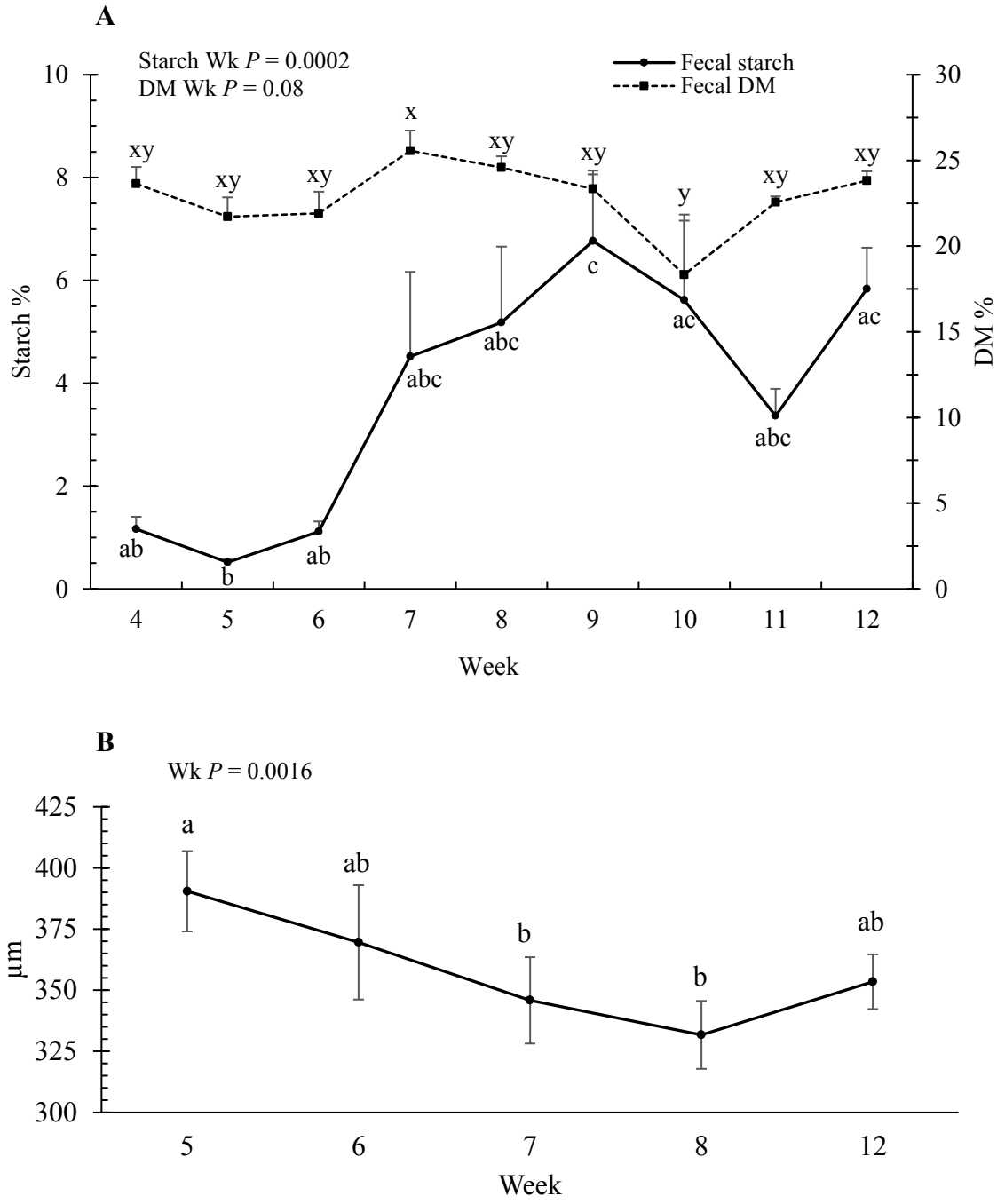

Figure 6.7- 1. (A) Weekly fecal starch, fecal DM and (B) colon mucosa thickness in dairy calves (n=6) fed fed up to 1.4 kg/d of milk replacer that were weaned during wk 6. Different letters represent a significant difference (abc, $P<0.05$; xy, $0.05 \leq P < 0.1$) and between weeks.

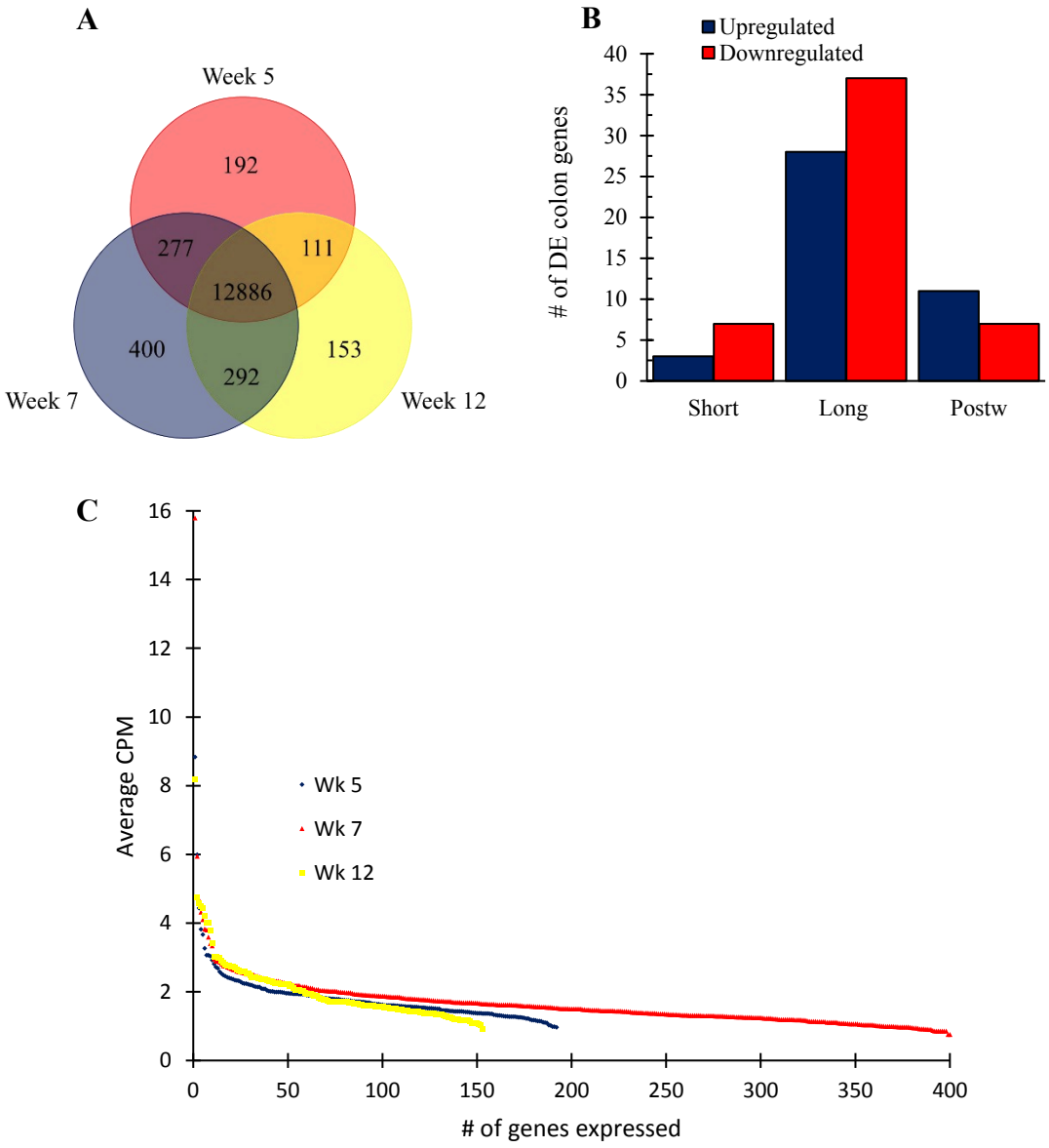

Figure 6.7- 2. (A) Expressed genes in Wk 5, 7 and 12 in Venn diagram. (B) The number of deferentially expressed genes that are up- or downregulated between pairwise comparisons (short, long and post-wean). (C) Genes that are uniquely expressed in wk 5, 7 and 12.

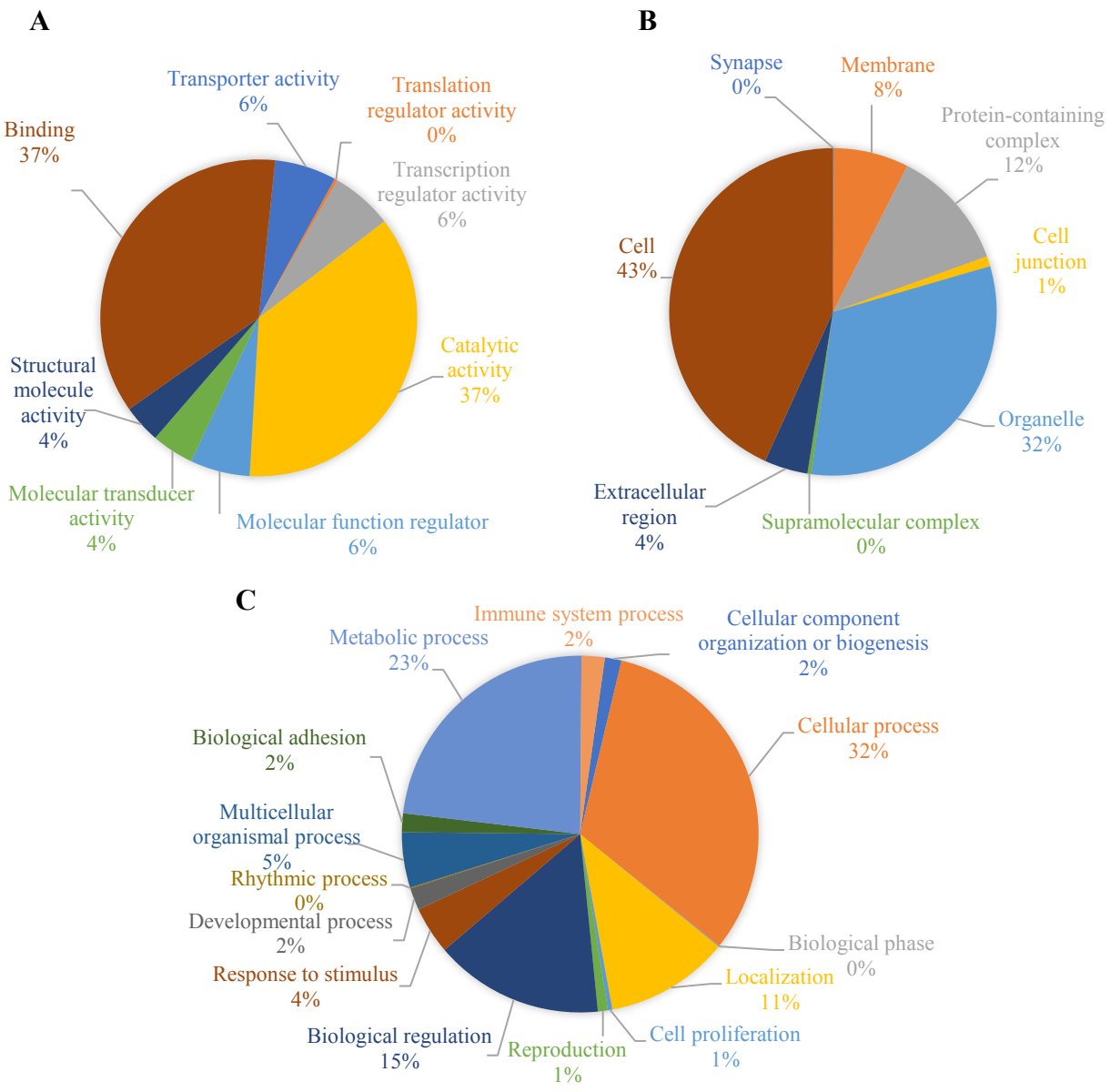

Figure 6.7- 3. (A) Molecular functions, (B) cellular components and (C) biological processes of the core colon mucosa genes identified by PANTHER.

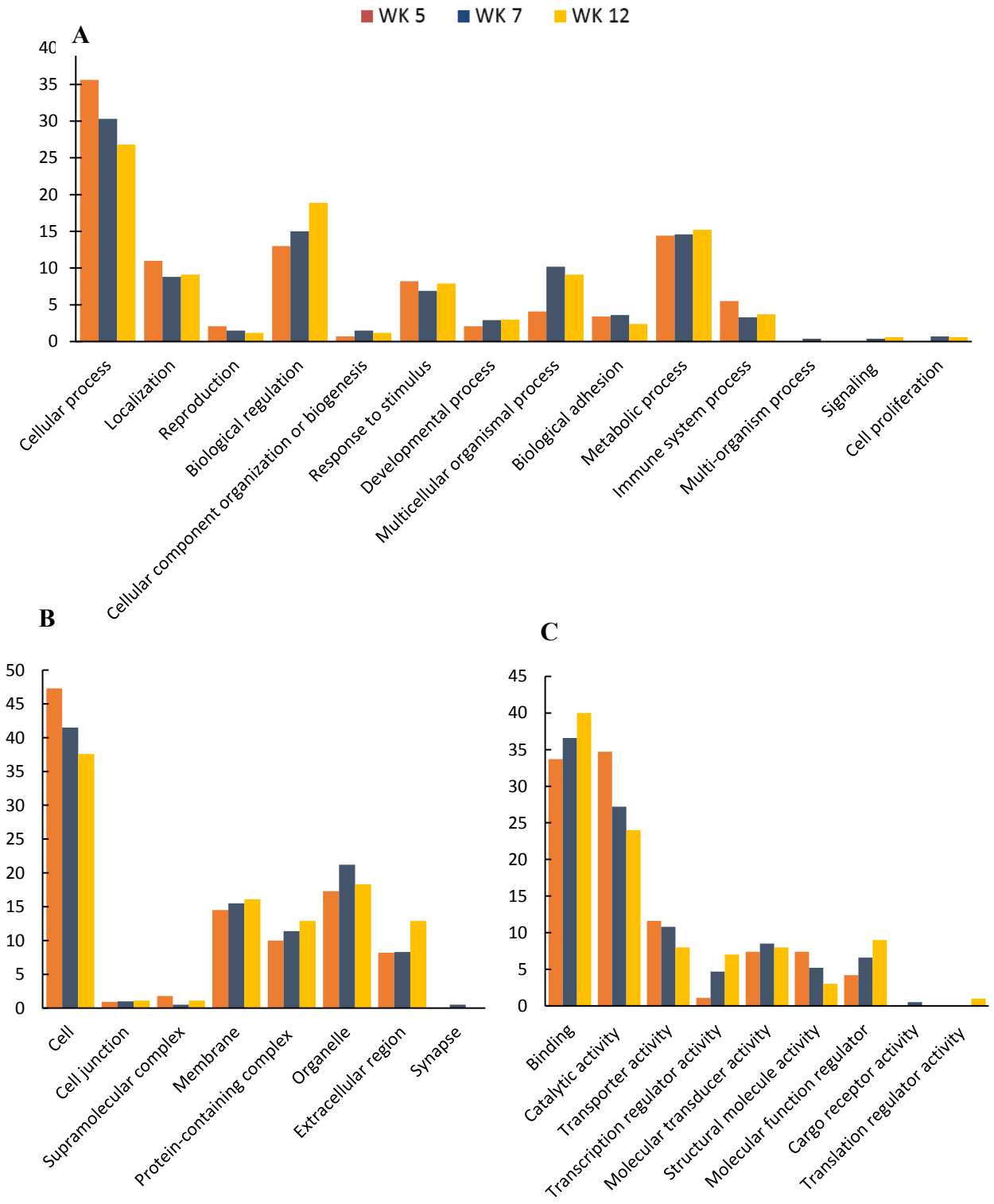

Figure 6.7- 4. (A) Biological processes, (B) cellular components and (C) molecular functions of the unique colon mucosa genes identified by PANTHER.

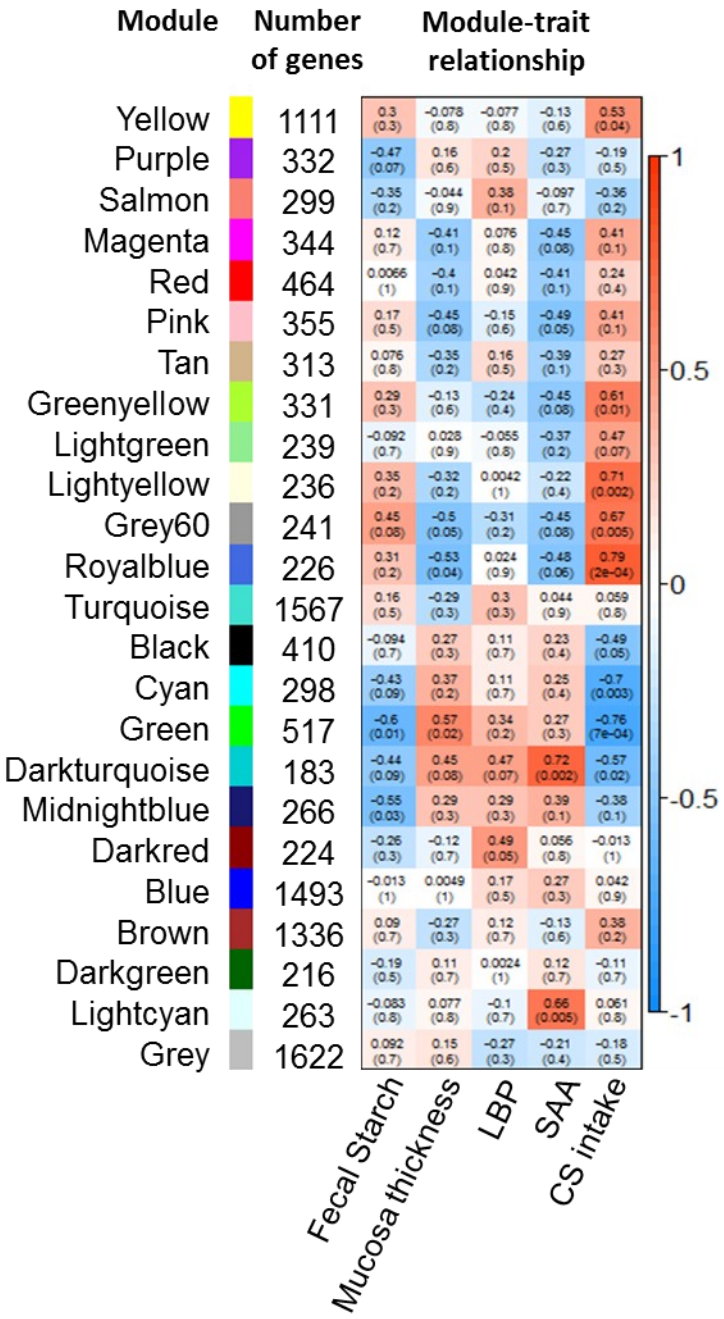

Figure 6.7- 5. WGCNA identification of colon mucosa gene modules correlated with fecal starch, colon mucosa thickness, lipopolysaccharide binding protein (LBP), serum amyloid A (SAA) and calf starter intake. The top number indicate the correlation coefficient and the bottom number indicate the p-value. A positive correlation is indicated by red and a negative correlation is indicated by blue. The strength of the correlation is indicated by the intensity of the color (red or blue).

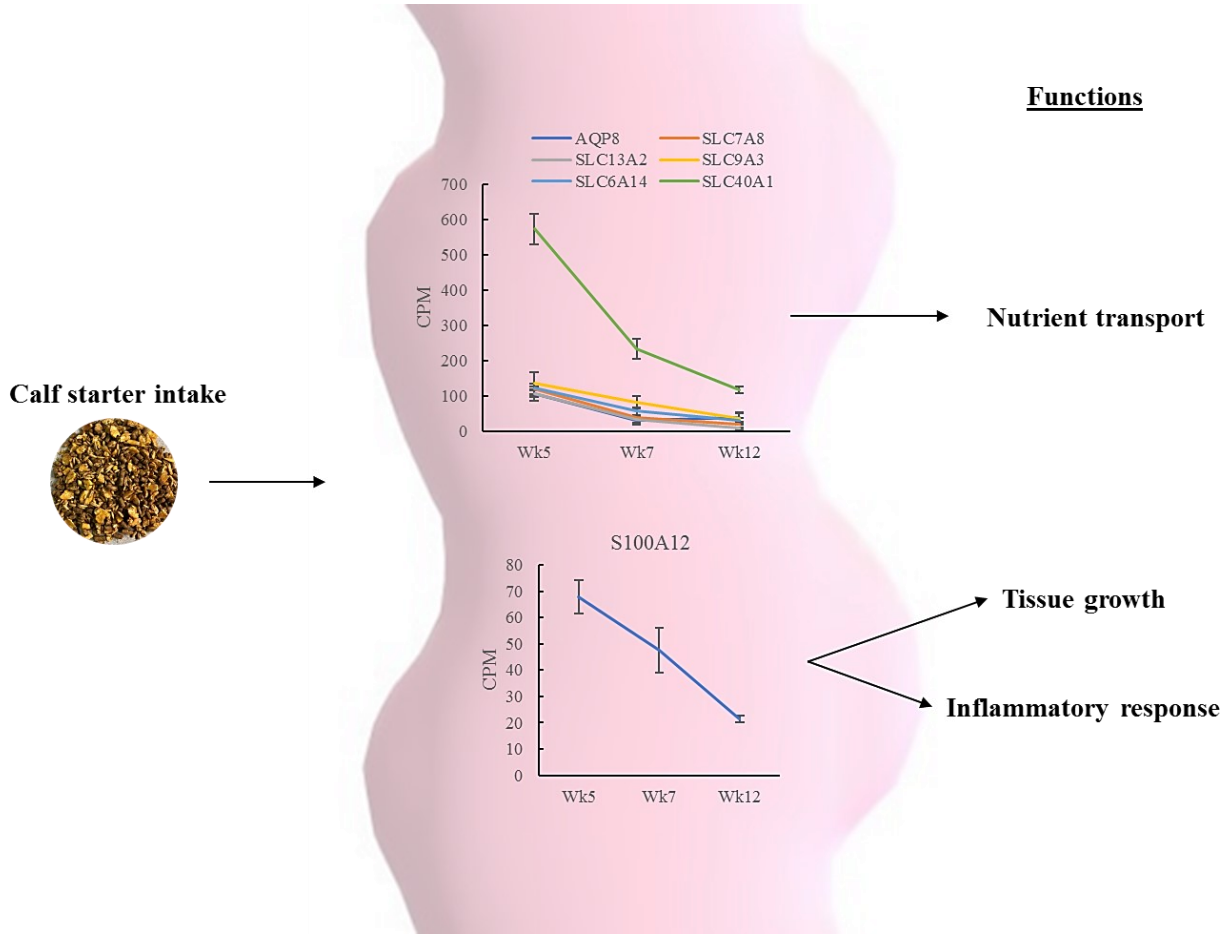

Figure 6.7- 6. Summary of 3 functional chances (nutrient absorption, inflammatory response and tissue growth) in the colon that occur at molecular level by using the transcriptome and to link changes including calf starter intake, fecal starch, colon mucosa thickness and serum inflammatory marker concentrations in dairy calves.

Supplemental table S6.7- 1. Top 5 molecular and cellular functions enriched in the darkturquoise, green, royalblue and grey60 module with IPA

Module	Molecular and cellular functions	Number of genes	*P*-Value
Darkturquoise	Cell death and survival	52	0.031
	Cell morphology	32	0.031
	Cell-to-cell signaling and interaction	23	0.031
	Cell cycle	32	0.031
	Free radical scavenging	3	0.002
Green	Cellular function and maintenance	129	0.027
	Cellular movement	108	0.029
	Molecular transport	102	0.029
	Cellular development	47	0.027
	Amino acid metabolism	21	0.029
Royalblue	Molecular transport	56	0.011
	Cellular development	55	0.011
	Cellular movement	18	0.009
	Cellular function and maintenance	45	0.009
	Small molecular Biochemistry	46	0.011
Grey60	Cell cycle	26	0.014
	Cell morphology	40	0.014
	Drug metabolism	8	0.014
	Lipid metabolism	29	0.014
	Small molecular Biochemistry	34	0.014

Supplemental table S6.7- 2. Top 5 molecular functions, cellular components and biological processes analysis with Panther for genes in darkturquoise, green, royalblue and grey60 module

Module	Molecular functions	Number of genes	Cellular components	Number of genes	Biological processes	Number of genes
Darkturquoise	binding (GO:0005488)	60	cell (GO:0005623)	61	cellular process (GO:0009987)	56
	catalytic activity (GO:0003824)	50	organelle (GO:0043226)	33	metabolic process (GO:0008152)	39
	molecular function regulator (GO:0098772)	9	membrane (GO:0016020)	12	biological regulation (GO:0065007)	31
	molecular transducer activity (GO:0060089)	9	protein-containing complex (GO:0032991)	7	localization (GO:0051179)	27
	transcription regulator activity (GO:0140110)	9	extracellular region (GO:0005576)	7	response to stimulus (GO:0050896)	8
Green	catalytic activity (GO:0003824)	131	cell (GO:0005623)	156	cellular process (GO:0009987)	174
	binding (GO:0005488)	126	organelle (GO:0043226)	87	metabolic process (GO:0008152)	113
	transporter activity (GO:0005215)	34	protein-containing complex (GO:0032991)	30	biological regulation (GO:0065007)	73
	molecular function regulator (GO:0098772)	23	membrane (GO:0016020)	28	localization (GO:0051179)	59
	transcription regulator activity (GO:0140110)	22	extracellular region (GO:0005576)	21	response to stimulus (GO:0050896)	27
Grey60	binding (GO:0005488)	71	cell (GO:0005623)	73	cellular process (GO:0009987)	85
	catalytic activity (GO:0003824)	52	organelle (GO:0043226)	67	metabolic process (GO:0008152)	54
	transporter activity (GO:0005215)	14	protein-containing complex (GO:0032991)	15	biological regulation (GO:0065007)	37
	transcription regulator activity (GO:0140110)	11	membrane (GO:0016020)	10	localization (GO:0051179)	27
	molecular transducer activity (GO:0060089)	7	extracellular region (GO:0005576)	6	response to stimulus (GO:0050896)	15
Royalblue	catalytic activity (GO:0003824)	66	cell (GO:0005623)	68	cellular process (GO:0009987)	66
	binding (GO:0005488)	55	organelle (GO:0043226)	52	metabolic process (GO:0008152)	51
	transporter activity (GO:0005215)	11	protein-containing complex (GO:0032991)	17	biological regulation (GO:0065007)	30
	transcription regulator activity (GO:0140110)	10	membrane (GO:0016020)	11	localization (GO:0051179)	28
	molecular function regulator (GO:0098772)	10	extracellular region (GO:0005576)	11	response to stimulus (GO:0050896)	12

Supplemental table S6.7- 3. Top 5 pathway analysis with Panther for genes in darkturquoise, green, royalblue and grey60 module

Module	Pathway	Number of genes
Darkturquoise	Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	8
	Huntington disease (P00029)	6
	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	6
	Apoptosis signaling pathway (P00006)	5
	Integrin signalling pathway (P00034)	5
	Wnt signaling pathway (P00057)	5
	EGF receptor signaling pathway (P00018)	5
	B cell activation (P00010)	5
Green	Integrin signalling pathway (P00034)	9
	Apoptosis signaling pathway (P00006)	6
	Angiogenesis (P00005)	6
	Wnt signaling pathway (P00057)	5
	Cadherin signaling pathway (P00012)	5
	Gonadotropin-releasing hormone receptor pathway (P06664)	5
Grey60	CCKR signaling map (P06959)	7
	Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	5
	Wnt signaling pathway (P00057)	5
	Gonadotropin-releasing hormone receptor pathway (P06664)	5
	Huntington disease (P00029)	4
	p53 pathway (P00059)	4
	PDGF signaling pathway (P00047)	4
Royalblue	Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	5
	Thyrotropin-releasing hormone receptor signaling pathway (P04394)	3
	Metabotropic glutamate receptor group III pathway (P00039)	2
	Apoptosis signaling pathway (P00006)	2
	Angiogenesis (P00005)	2
	5HT2 type receptor mediated signaling pathway (P04374)	2
	Integrin signalling pathway (P00034)	2
	GABA-B receptor II signaling (P05731)	2
	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	2
	Wnt signaling pathway (P00057)	2
	Oxytocin receptor mediated signaling pathway (P04391)	2
	Parkinson disease (P00049)	2
	Histamine H1 receptor mediated signaling pathway (P04385)	2
	Metabotropic glutamate receptor group II pathway (P00040)	2
	Gonadotropin-releasing hormone receptor pathway (P06664)	2

Supplemental table S6.7- 4. Colon mucosa DE genes (fold change >1.5 or < −1.5 and FDR ≤ 0.05) between pairwise comparisons are presented

Short term (Week 7 vs. 5)				
ID	**Gene name (Gene symbol)**	**logFC**	**PValue**	**FDR**
ENSBTAG00000002974	flavin containing monooxygenase 2(FMO2)	2.14	3.26E-10	8.64E-07
ENSBTAG00000004414	solute carrier family 30 member 10(SLC30A10)	-4.37	2.84E-09	4.66E-06
ENSBTAG00000007415	solute carrier family 7 member 8(SLC7A8)	-1.55	4.74E-12	3.11E-08
ENSBTAG00000008788	solute carrier family 13 member 2(SLC13A2)	-1.64	2.69E-09	4.66E-06
ENSBTAG00000010163	sodium channel epithelial 1 gamma subunit(SCNN1G)	2.24	1.09E-06	8.37E-04
ENSBTAG00000012290	sodium channel epithelial 1 beta subunit(SCNN1B)	2.42	3.30E-10	8.64E-07
ENSBTAG00000017020	S100 calcium binding protein G(S100G)	-3.23	2.31E-09	4.66E-06
ENSBTAG00000020184	aquaporin 8(AQP8)	-1.76	2.58E-05	1.25E-02
ENSBTAG00000025035	prolactin receptor(PRLR)	-1.75	1.90E-05	9.59E-03
ENSBTAG00000031497	fibroblast growth factor binding protein 1(FGFBP1)	-1.78	1.07E-07	1.08E-04
Long term (week 12 vs. 5)				
ID	**Gene name (Gene symbol)**	**logFC**	**PValue**	**FDR**
ENSBTAG00000000185	solute carrier family 6 member 14(SLC6A14)	-1.95	4.56E-26	1.58E-22
ENSBTAG00000000973	potassium voltage-gated channel subfamily J member 15(KCNJ15)	-1.96	1.18E-05	2.17E-03
ENSBTAG00000001021	cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1(CYP1A1)	3.08	2.09E-06	5.26E-04
ENSBTAG00000001244	plasminogen activator, tissue type(PLAT)	-1.79	1.08E-09	6.23E-07
ENSBTAG00000001595	metallothionein 1E(MT1E)	-3.40	1.69E-05	2.92E-03
ENSBTAG00000001936	phosphoenolpyruvate carboxykinase 1(PCK1)	1.75	2.67E-13	3.36E-10
ENSBTAG00000002181	KH RNA binding domain containing, signal transduction associated 3(KHDRBS3)	2.33	2.72E-04	2.45E-02
ENSBTAG00000002430	collagen type XVII alpha 1 chain(COL17A1)	2.03	8.19E-18	1.42E-14
ENSBTAG00000002974	flavin containing monooxygenase 2(FMO2)	3.63	1.04E-25	2.87E-22
ENSBTAG00000003669		2.12	2.26E-04	2.18E-02
ENSBTAG00000003711	endothelial PAS domain protein 1(EPAS1)	-2.05	6.72E-35	3.10E-31
ENSBTAG00000004013	fibroblast growth factor 7(FGF7)	1.57	6.63E-05	8.86E-03
ENSBTAG00000004272	TLH29 protein precursor-like(ISG12(B))	3.01	2.08E-08	9.00E-06
ENSBTAG00000004414	solute carrier family 30 member 10(SLC30A10)	-3.99	2.42E-08	1.02E-05

ENSBTAG00000004629	solute carrier family 9 member A3(SLC9A3)	-1.89	2.35E-06	5.71E-04
ENSBTAG00000005072	cubilin(CUBN)	-2.52	4.42E-07	1.53E-04
ENSBTAG00000005353	desmin(DES)	1.87	7.91E-04	5.04E-02
ENSBTAG00000006686	nephronectin(NPNT)	-1.65	3.06E-20	6.06E-17
ENSBTAG00000006975	sucrase-isomaltase(SI)	1.57	4.44E-05	6.39E-03
ENSBTAG00000007415	solute carrier family 7 member 8(SLC7A8)	-2.44	9.32E-25	2.15E-21
ENSBTAG00000007554	interferon alpha inducible protein 6(IFI6)	2.13	7.38E-04	4.87E-02
ENSBTAG00000008788	solute carrier family 13 member 2(SLC13A2)	-3.74	5.87E-54	8.14E-50
ENSBTAG00000009596	chromosome 8 open reading frame, human C9orf43(C8H9orf43)	-1.90	7.02E-07	2.16E-04
ENSBTAG00000010498	solute carrier family 40 member 1(SLC40A1)	-2.21	6.04E-42	4.19E-38
ENSBTAG00000010820	Wnt family member 11(WNT11)	-1.52	7.15E-04	4.74E-02
ENSBTAG00000011036	carcinoembryonic antigen related cell adhesion molecule 20(CEACAM20)	-3.43	3.34E-08	1.32E-05
ENSBTAG00000011207	calponin 1(CNN1)	1.76	2.77E-04	2.45E-02
ENSBTAG00000011424	tropomyosin 2 (beta)(TPM2)	1.61	4.75E-04	3.57E-02
ENSBTAG00000011473	myosin light chain 9(MYL9)	1.52	2.74E-04	2.45E-02
ENSBTAG00000011534	receptor activity modifying protein 1(RAMP1)	1.50	5.91E-04	4.07E-02
ENSBTAG00000011585	macrophage stimulating 1(MST1)	-1.61	2.52E-06	5.88E-04
ENSBTAG00000011752	synemin(SYNM)	2.33	8.93E-05	1.10E-02
ENSBTAG00000012638	S100 calcium binding protein A12(S100A12)	-1.58	5.81E-13	6.70E-10
ENSBTAG00000012884	putative mucosal pentraxin homolog(MPTX)	-1.93	1.26E-05	2.26E-03
ENSBTAG00000014600	adenylate cyclase 8(ADCY8)	-2.43	2.43E-04	2.25E-02
ENSBTAG00000015441	actin, gamma 2, smooth muscle, enteric(ACTG2)	2.09	1.53E-04	1.64E-02
ENSBTAG00000015582	heme oxygenase 1(HMOX1)	-1.79	3.57E-09	1.83E-06
ENSBTAG00000015988	myosin heavy chain 11(MYH11)	1.50	4.26E-04	3.41E-02
ENSBTAG00000016276	histidine ammonia-lyase(HAL)	-2.20	2.47E-06	5.88E-04
ENSBTAG00000016327	solute carrier family 28 member 2(SLC28A2)	-2.32	2.99E-06	6.79E-04
ENSBTAG00000017020	S100 calcium binding protein G(S100G)	-4.19	5.56E-12	5.51E-09
ENSBTAG00000017280	complement C3(C3)	-1.67	8.48E-07	2.50E-04
ENSBTAG00000017357	chloride channel accessory 4(CLCA4)	-2.51	1.06E-04	1.23E-02
ENSBTAG00000018125	kinesin family member 5C(KIF5C)	2.28	4.90E-04	3.63E-02
ENSBTAG00000020391	meprin A subunit beta(MEP1B)	-1.50	3.42E-05	5.39E-03
ENSBTAG00000020921	protein kinase C beta(PRKCB)	1.57	7.60E-06	1.55E-03

ENSBTAG00000021220	transmembrane protein 72(TMEM72)	1.63	3.14E-04	2.72E-02
ENSBTAG00000021501	calcium-activated chloride channel(LOC784768)	-2.10	4.47E-05	6.39E-03
ENSBTAG00000022715		-2.45	1.49E-08	6.88E-06
ENSBTAG00000022779	olfactomedin 4(OLFM4)	-1.50	9.43E-16	1.45E-12
ENSBTAG00000025071	teneurin transmembrane protein 2(TENM2)	2.15	9.05E-06	1.77E-03
ENSBTAG00000025242	chromosome 19 open reading frame, human C17orf78(C19H17orf78)	-3.48	1.16E-10	8.96E-08
ENSBTAG00000027225	lingual antimicrobial peptide(LAP)	-2.57	7.93E-04	5.04E-02
ENSBTAG00000029317		-1.99	3.19E-04	2.73E-02
ENSBTAG00000031160	immunoglobulin lambda-like polypeptide 1(IGLL1)	1.80	1.56E-05	2.78E-03
ENSBTAG00000031497	fibroblast growth factor binding protein 1(FGFBP1)	-1.55	9.18E-06	1.77E-03
ENSBTAG00000032774	adipogenesis regulatory factor(ADIRF)	1.63	5.30E-04	3.81E-02
ENSBTAG00000034402	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2(ACE2)	-2.04	1.13E-09	6.28E-07
ENSBTAG00000045494		6.38	1.58E-04	1.67E-02
ENSBTAG00000046386	serine rich and transmembrane domain containing 1(SERTM1)	-2.16	3.96E-04	3.30E-02
ENSBTAG00000047046		-4.95	1.58E-04	1.67E-02
ENSBTAG00000047600	ROS proto-oncogene 1, receptor tyrosine kinase(ROS1)	-2.48	2.12E-07	7.72E-05
ENSBTAG00000047700		2.11	1.53E-06	4.16E-04
ENSBTAG00000047986		1.77	2.67E-04	2.43E-02
ENSBTAG00000048135		2.08	2.05E-06	5.26E-04

Post-wean (Week 12 vs. 7)

ID	Gene name (Gene symbol)	logFC	PValue	FDR
ENSBTAG00000002430	collagen type XVII alpha 1 chain(COL17A1)	1.58	8.00E-13	1.12E-08
ENSBTAG00000003668	chromosome X open reading frame, human CXorf57(CXHXorf57)	2.68	4.29E-05	1.82E-02
ENSBTAG00000004272	TLH29 protein precursor-like(ISG12(B))	2.69	2.90E-07	4.51E-04
ENSBTAG00000006234	natriuretic peptide receptor 1(NPR1)	1.69	1.43E-04	3.65E-02
ENSBTAG00000008788	solute carrier family 13 member 2(SLC13A2)	-2.12	2.71E-08	1.26E-04
ENSBTAG00000012290	sodium channel epithelial 1 beta subunit(SCNN1B)	-1.69	7.74E-07	9.04E-04
ENSBTAG00000016276	histidine ammonia-lyase(HAL)	-1.92	4.45E-05	1.83E-02
ENSBTAG00000018570	short chain dehydrogenase/reductase family 16C, member 5(SDR16C5)	1.54	3.75E-05	1.65E-02
ENSBTAG00000021501	calcium-activated chloride channel(LOC784768)	-2.16	8.97E-07	9.67E-04
ENSBTAG00000025035	prolactin receptor(PRLR)	1.68	1.95E-04	4.18E-02
ENSBTAG00000025242	chromosome 19 open reading frame, human C17orf78(C19H17orf78)	-2.55	1.58E-04	3.82E-02

ENSBTAG00000025952	odorant-binding protein(OBP)	4.57	1.77E-06	1.55E-03
ENSBTAG00000039861	2',5'-oligoadenylate synthetase 1, 40/46kDa(OAS1Y)	1.71	1.07E-05	6.51E-03
ENSBTAG00000040034	uncharacterized LOC507550(LOC507550)	-2.50	7.42E-07	9.04E-04
ENSBTAG00000045494		9.45	2.20E-06	1.71E-03
ENSBTAG00000047600	ROS proto-oncogene 1, receptor tyrosine kinase(ROS1)	-3.11	2.77E-06	2.05E-03
ENSBTAG00000047700		1.66	4.70E-05	1.88E-02
ENSBTAG00000047986		1.61	1.26E-04	3.53E-02

Supplemental table S6.7- 5. Top 5 molecular and cellular functions enriched in the DE genes with IPA

Module	Molecular and cellular functions	Number of genes	*P*-Value
Short term	Cell morphology	6	0.041
	Cellular function and maintenance	2	0.020
	Molecular transport	7	0.049
	Protein synthesis	3	0.009
	Amino acid metabolism	3	0.049
Long term	Cellular function and maintenance	12	0.010
	Molecular transport	21	0.010
	Small molecular biochemistry	15	0.010
	Cell death and survival	8	0.008
	Cellular movement	18	0.010
Post-wean	Cell-to-cell signaling and interaction	2	0.040
	Cellular development	2	0.040
	Cellular growth and proliferation	3	0.040
	Drug metabolism	3	0.013
	Molecular transport	6	0.042

7. General discussion

7.1. Importance of the research

Dairy calf nutrition is important for GIT development and function, calf performance and health, and profitability. One challenge that can negatively influence growth and compromise calf health is the weaning transition because the GIT undergoes dramatic structural and metabolic changes and adaptations (Baldwin VI et al., 2004; Khan et al., 2011b) during this time. Many studies have researched different feeding strategies to optimize GIT development and function and calf performance and health. Yet, little is known about the combination of feeding rate of MR and starter composition on performance, digestibility and rumen and hindgut fermentation. Additionally, ruminal fermentation characteristics and the effect of the aforementioned parameters on inflammatory marker changes in dairy calves have not been quantified in depth from pre- to post-weaning. Furthermore, little is known about changes that may occur in the colon from pre- to post-weaning in calves.

Chapters 2 and 3 evaluated how the amount of MR fed and the processing of corn in starter impacts growth performance, nutrient digestibility, rumen and hindgut fermentation, rumen and fecal fibrolytic bacteria, and inflammatory biomarkers of dairy calves. To my knowledge, the findings presented in Chapters 2 and 3 are the first to explore feeding high MR feeding rate in combination with whole corn or steam flaked corn in calf starter (CS). Overall, the MR feeding rate had a larger impact on calf performance and fibre digestibility (pre-weaning) and rumen and fecal fibrolytic bacterial populations. Furthermore, CS intake influenced by MR feeding rate or calf age had the biggest impact on rumen and hindgut fermentation, while the processing of the corn did not impact these parameters. Feeding dairy calves more milk during the pre-weaning phase recently gained attention because it has been reported to improve weight gain (Jasper and Weary, 2002; Khan et al., 2011a; Rosenberger et al., 2017) and feed conversion (feed to gain; Khan et al., 2007; Moallem et al., 2010; Davis-Rincker et al., 2011). However, calves that received higher amounts of milk pre-weaning struggle during the weaning transition and part of the increased pre-weaning growth (Cowles et al., 2006) and feed efficiency advantage is compromised due to inadequate nutrient consumption (Hill et al., 2006) and reduced nutrient digestibility (Hill et al., 2016; Dennis et al., 2018), as reported in Chapter 2. It is possible that the reduced CS intake during pre-weaning in the calves fed the

higher MR feeding rate caused inadequate rumen microbial fermentation and thereby calves experienced reduced fibre digestibility, as reported previously (Hill et al., 2016; Dennis et al., 2018). Chapter 2 is the first study that investigated selected fibrolytic bacterial species in relation to fibre digestion in calves fed different MR feeding rates. Calves consuming less CS, due to higher MR feeding rates, had a decreased *Butyrivibrio fibrisolvens* abundance and *Butyrivibrio fibrisolvens* and *Clostridium cluster IV* proportions in the rumen, and a decreased proportion of *Clostridium cluster IV* and *Faecalibacterium sp.* in fecal matter during wk 5 (pre-weaning). These results suggest that reduced rumen and hindgut fibre digestion may occur pre-weaning in calves fed a high MR feeding rate compared with calved fed a low MR feeding rate.

Hindgut fermentation is a new area of interest and has not been investigated in calves. Fermentation in the rumen and hindgut of calves is dependent on CS intake, as reported in Chapter 3. The calves fed the low MR feeding rate had more total SCFA in both the rumen and fecal samples. Furthermore, fecal starch increased and fecal pH decreased as CS intake increased overtime. With increased fermentation, SCFA accumulate and pH decreases, which may cause lysis of gram-negative bacteria (Mao et al., 2013). This may release endotoxins, such as LPS, resulting in endotoxin accumulation in the digesta and translocation into the peripheral circulation (Khafipour et al., 2009), possibly causing a systemic inflammatory response. According to the acute phase proteins measured (SAA and LBP) in Chapter 3, their concentrations were within the healthy range for calves, albeit free fecal LPS concentrations were high during the 4 wk measuring period – presumably causing an inflammatory response. This study is the first to investigate the effect of ruminal and hindgut pH on select acute phase proteins (SAA and LBP) in dairy calves, which have only been reported in mature ruminants before. The combination of whole corn and high MR feeding rate resulted in decreased fecal pH compared to the other treatments at wk 8; yet, there were no changes in inflammatory biomarkers at this time. Based on the results in Chapters 2 and 3, low and high feeding rate of MR can be combined with either flaked corn or whole corn in the CS. The price between the whole or flaked corn in texturized CS might be different because processing grain is generally costlier. Based on our results, there are minimal differences between flaked or whole corn, and therefore it is the producer's choice which CS they prefer to feed to calves.

Studying the GIT tissue when different feeding regimes may help us understand what changes occur at a histological or molecular level. However, these techniques normally require

euthanization of the animal to obtain GIT tissue. For instance, when researchers aim to investigate GIT changes during weaning, serial slaughtering is commonly used (Warner et al., 1956; Lane et al., 2002). However, variation might be introduced between temporal samples because the GIT tissue is not from the same animal and there are large individual variations within the same group. If a method can be developed to collect GIT tissue over time from the same animal, it will enable researchers to study GIT changes that may occur in the animal due to age, weaning or diet changes. Therefore, Chapter 4 evaluated methods to biopsy the rumen and colon of calves to confirm suitable quality for histological and molecular evaluation. Based on the results, it is best to obtain rumen epithelium tissue by retracting the rumen epithelium through the rumen cannula and to use surgical scissors for taking samples. To collect colon mucosa samples, endoscopic biopsying can be used in calves. Both the rumen and colon tissue samples collected by the respective sampling techniques did yield optimal samples for use in histological or molecular analysis. By using cannulated animals or endoscopic biopsying as a tool to collect rumen and colon tissue in the same animal throughout time, fewer animals are required per research trial. This method will also allow the dairy research community to efficiently investigate the impact of weaning regimens, feed rations and age on tissue structure.

Many studies reported that during weaning the calf GIT experience dramatic changes, structurally and metabolically, in order to adapt to solid feed as it becomes the main nutrient source post- weaning (Baldwin VI et al., 2004; Khan et al., 2011b). Therefore, Chapters 5 and 6 focused on studying the changes that may occur from pre- to post weaning by using the method developed in Chapter 4 for collecting tissue samples from the rumen and colon.

It is important to note that this is the first study (Chapter 5) to characterize the rumen development and environment up to 6 wk post-weaning. In general, rumen papillae observed did not develop until later during the post-weaning period. Metabolizable energy intake supplied by CS may have influenced the rumen development because ME intake were below calf maintenance requirements post-weaning. Interestingly, the rumen environment during the pre-weaning period was acidotic despite low CS intake at this time. This may indicate that there is a lack of buffer capacity during the pre-weaning period. A study reported that calves fed a low amount of milk with a CS intake of 1.2 kg/d pre-weaning had ruminal pH below 5.8 (Li et al., 2019), which is expected when the rumen is undeveloped and fermentation is high. Furthermore, in our study the rumen environment was acidotic several wk post-weaning, which may have been

a consequence of CS intake increasing rapidly during this time. This rapid increase in CS intake results in depressed pH, suggesting that there may be an imbalance between the rate of fermentation, buffer capacity and rumen development. The serum acute phase proteins (SAA and LBP) indicate that no systemic inflammatory response occurred despite the rumen being in an acidotic state pre-weaning and several weeks post-weaning. The ruminal pH levels reported in Chapter 5 are equivalent to adult ruminant SARA or acute ruminal acidosis during some weeks (Penner et al., 2007; Aschenbach et al., 2011). However, calves in Chapter 5 did not display an inflammatory response, rumen papillae did not exhibit sloughing, and the cell layers (basal, spinosum, and granulosum) stayed intact, unlike in adult ruminants where rapid sloughing of the stratum corneum (Steele et al., 2009), decreased thickness of cell layers (Steele et al., 2011) and fragmentation of cell layers in the stratum granulosum (Liu et al., 2013) were observed when animals experienced SARA. Taking into consideration all the data presented in Chapter 5, ruminal acidosis may be part of rumen development and not a problem in dairy calves during weaning. Future research is needed to validate this speculation by investigating calf rumen function under reduced ruminal pH conditions.

It is unclear why such large differences in ruminal pH were observed between calves in Chapter 3 and Chapter 5 when the calves received the same amount of MR (used only HI calves from Chapter 3) and were weaned using the same method at the same age. The calves in Chapter 3 received a texturized CS containing a high amount of starch (41.5 %) compared with the starch the calves in Chapter 5 received (32.9 %). However, ruminal pH was not as expected, with only the calves in chapter 5 experiencing ruminal acidosis even pre-weaning despite the lower starch content in the diet. The different sampling techniques may have had the biggest influence, as a Geishauser probe was used to obtain spot ruminal fluid samples, and possible saliva contamination could have influence the SCFA concentrations and pH values observed in Chapter 3 (Duffield et al., 2004). This is contrast to Chapter 5, in which rumen fluid was obtained directly from the rumen through a cannula. The calves in Chapter 5 also had access to chopped straw, which in theory should have increased the buffer capacity of the rumen; however, the calves only consumed a small amount. The calves in Chapter 3 were not fed forage but were bedded on long particle wheat straw. The bedding consumption is unmeasurable, but it may have played a role in the buffering capacity of the rumen. In conclusion, sampling method and

bedding type can influence ruminal pH in calves, and therefore it is hard to compare among studies and draw conclusions.

The above-mentioned research (Chapter 5) led to more insight on the changes that occur in the rumen from pre- to post-weaning. By characterizing ruminal pH changes, we identified that depressed ruminal pH does not cause a systemic inflammatory response based on the two acute phase proteins that were used as markers. The rumen histology samples analysis also gave us further insight into how quickly structural changes occur in the rumen from pre- to post weaning. This study is the first to investigate the development of the rumen and to characterize the changes that occur in the ruminal fermentation environment within the same calf over time. This is very important because all the decisions about solid feed diet formulations for calves are made to mitigate or prevent ruminal acidosis. More so, due to these decisions, calf growth may be limited because higher starch diets are associated with ruminal acidosis. As such, feeding lower starch diets may prevent ruminal acidosis. However, starch content in the diet is positively associated with weight gain of the calf (Hu et al., 2018). Therefore, this study provides the scientific knowledge needed for the development of better calf management strategies.

In addition to rumen fermentation, colon fermentation also plays an important role in providing energy to ruminants. Bergman, (1990) reported that up to 9% of metabolizable energy requirements in cattle come from SCFA production in the hindgut. Furthermore, research has shown that the hindgut is more efficient than the rumen in digesting starch when calculated as the proportion of starch entering the fermentation site (Haimoud et al., 1995; Callison et al., 2001). Since the colon may play an important role in energy provision for the calf, Chapter 6 examined the functional changes of the colon mucosa at molecular level in calves (the same calves in Chapter 5) from pre- to post-weaning. Additionally, these molecular changes were linked to calf phenotypic changes (colon mucosa thickness, fecal starch, and serum inflammatory marker concentrations).

In general, fecal starch increased as CS intake increased up to wk 9 (Chapter 6), which is similar to that reported in Chapter 3. Interestingly, 2 wk after weaning fecal starch values reported in Chapter 6 were approximately 5%, which is half of that reported in Chapter 3 (10.9 %). This difference between studies might be due to the amount of starch in CS fed to the calves, with calves in Chapter 6 fed 0.25 g/d less starch during wk 8. This data suggests that consuming

CS containing higher amounts of starch leads to more starch reaching the hindgut, increasing the possibility of hindgut fermentation in dairy calves.

Seven colon tissue genes involved in nutrient absorption, tissue growth and immune function were identified as possible gene markers that may be used in future studies to evaluate changes that may occur in the colon. The expression of six genes involved in water or urea (*AQP8*), amino acid (*SLC7A8*, *SLC6A14*) and mineral absorption (*SLC9A3, SLC40A, SLC9A3*) decreased post-weaning and were negatively correlated with CS intake and fecal starch. The expression of an upstream regulator gene (*S100A12*), which plays a role in inflammatory responses and tissue growth pathways, also decreased post-weaning. Additionally, the expression of *S100A12* was negatively correlated with fecal starch and CS intake and positively correlated with the colon mucosa thickness. It is important to note that this study is the first to characterize functional changes of the colon mucosa occurring from pre- to post-weaning at the molecular level in calves. By studying the relationship between mucosa thickness and gene expression from colon tissue samples, it provides us with a greater insight into the molecular mechanisms of structural functional adaptation of the colon from pre- to post-weaning in calves.

The colon plays an important role in providing the calf with energy as it is responsible for nutrient, mineral and water absorption (Sherwood et al., 2013). It may be possible that the colon does not experience functional changes, such as those in metabolic processes, to the same extent as the rumen when calves are weaned, or the diet is changed; however, this is only speculated based on four published studies that have investigated the effect of diet change on the colon. It has been reported that the expression of only a few genes in the colon were influenced in cows transitioned from a dry to a lactating diet (Bach et al., 2018), and from, goats, fed a high concentrate diet (Tao et al., 2014a,b). Previous work has shown that delaying feeding colostrum after birth did not influence colon gene expression in neonatal calves (He et al., 2018). However, it may be possible that the bacteria present in the colon play a bigger role in gene expression changes in the colon. The abundance of *Lactobacillus* and *Faecalibacterium prausnitzii* were positively or negatively correlated with expression of 122 colon immune function genes in neonatal calves (He et al., 2018) and immune function genes have higher expression in beef steers that shed more *Escherichia coli* O157 (Wang et al., 2017). Thus, based on the above-mentioned studies and the study in Chapter 6, the colon only undergoes a few changes at molecular level during the transition period or when different diets are fed. This may indicate

that the colon transcriptome is more resilient to changes in diet and/or weaning transitioning periods.

7.2. Limitation and future directions

Based on the results obtained from the two studies conducted, a few knowledge gaps still exist and few new knowledge gaps have been uncovered. The effect of early life nutrition and weaning is of significant economic importance to dairy producers as it not only directly impacts mortality rates, disease susceptibility and growth, but also function of the gut and milk production later in life. The utilization of high plane of milk nutrition feeding regimens to take advantage of the improved growth and the potential increased milk production later in life has become more popular around the world (Kertz and Loften, 2013; Kertz et al., 2017; Winder et al., 2018). It is well known that calves struggle to consume enough CS at a young age, and as seen in Chapter 5, calves were below maintenance requirements for metabolizable energy (**ME**) during weaning and the wk after. Therefore, future research needs to focus on the combination of MR feeding and CS composition because both play a large role in providing enough nutrients for maintenance and growth of the calf – both forms of energy that complement one another.

The CS intake of calves fed the low MR feeding rate ranged from 16.0 – 46.4 kg, while CS intake of calves fed the high MR feeding rate ranged from 7.4 – 37.1 kg during the 8 wk period in Chapters 2 and 3. These data show that CS intake is highly variable in calves, even between calves fed MR at the same rate. These differences between calves may have also resulted in the minimal differences in ruminal and hindgut fermentation parameters, as well as the inflammatory biomarkers reported. Based on a power calculation, the number of animals used per treatment should be at least 18 to detect a significant difference over the 8 wk. In addition, calves might have the ability to chew the whole corn in the CS, and therefore, they may be able to more effectively utilize the feed compared to adult ruminants (Owens, 2005). This may provide reasoning as to why we did not observe differences between the whole corn and flaked corn treatments in Chapter 2 and 3. Future research should investigate if calves have the ability to chew whole corn, and if so, up to what age or DMI. Moreover, they should use a higher number of animals per treatment. This knowledge would be valuable to the dairy industry, because if calves are able to chew whole corn, this would result in whole corn being a cost-efficient feeding strategy compared to processed corn.

There are multiple grain types that differ in starch content that can be used in CS as the starch source (Huntington, 1997). Starch content of wheat are 77%, 70 to 73% for corn sorghum, and rice (Huntington, 1997), and 57 to 58% barley and oats (Poore et al., 1993). The cereal grains, wheat, barley, and oats have more readily available fermentable starch than corn and sorghum (Swan et al., 2006). It has been reported that CS that contain corn increase CS, hay, NDF, CP and starch intake compared with barley, oats, or wheat (Khan et al., 2007b), subsequently increasing calf body weight (BW) and body measurements. Replacing oats with corn in a diet that contained both had no influence on intake and BW (Hill et al., 2008). Both studies in the thesis fed texturized CS that contained corn and oats as starch sources, and given the lack of systemic inflammation (Chapters 3 and 5) and disruption to ruminal epithelium structure (Chapter 5) due to the reduced ruminal pH (Chapter 5) or the high CS starch content (Chapters 2 and 3), corn and oats may be the optimum grain type in CS. These grains can be used simultaneously to reach the starch content needed. The optimum starch content of CS is unknown however, increasing levels of starch in the CS is associated with increasing CS intake and BW (Hu et al., 2018). Further research is warranted to investigate the optimum starch level for calves.

The ruminal fluid for pH measurements in Chapter 3 was only collected at one time point per day, thus diurnal changes that may occur were not captured in this study. This may have also resulted in the higher ruminal pH reported when compared to Chapter 5, despite starch content being higher in Chapter 3. Continuous ruminal pH measurements are more comprehensive, and according to our knowledge there are only four studies (Laarman and Oba, 2011; Laarman et al., 2012; Kim et al., 2016a,b) that elucidate ruminal pH changes using continuous measurements in calves during the weaning period. Two of these studies measured ruminal pH continuously for three (Laarman and Oba, 2011) and four (Laarman et al., 2012a) days, thus knowledge on long term ruminal pH changes is scarce. Furthermore, previous work has shown that CS intake and performance increased when starch content in the diet increased (Hu et al., 2018). Thus, a study investigating CS differing in starch content on ruminal pH using continuous ruminal pH measurements during weaning would give us a better understanding if a relationship between CS starch content and the extent of depressed ruminal pH exists.

In Chapter 2, targeted fibrolytic bacteria were investigated to understand whether they played a role in the differences observed in fibre digestibility between the HI and LO fed calves.

However, only a small part of the ruminal microbiota has been quantified by using qPCR and primers from the literature, providing limited information about the overall microbiota in the GIT. For example, fungi may have contributed to fibre digestibility in the GIT, as they have greater cellulase-complexes, making them more efficient at breaking down fibre compared to bacteria (Stewart, 1994). Therefore, it is important to note that this method is only for proof of concept. More insight into the microbiome and its role in fibre digestion can be obtained by using more advanced methods, like amplicon sequencing.

The changes that were observed in Chapters 5 and 6 in the rumen and colon, respectively, were measured within the same animals from pre- to post-weaning. However, these changes do not consider the additional changes that occur as the calves age. A future study is needed to truly identify the specific changes that occur due to weaning and those that occur due to age. For example, one group of calves fed just milk and not weaned compared with another group of calves that are weaned. This will help identify age dependent negative effects that cannot be mitigated through manipulation of the diet or weaning strategy.

Most of the current knowledge regarding ruminal pH and its effect on rumen and animal health pertains to adult animals and most studies assume that this knowledge can be transferred to the calf; however, according to Chapter 5, this might not be true. The rumen environment was acidotic pre-weaning and for several weeks post-weaning, yet, papillae strata, sloughing score, and acute phase proteins (SAA and LBP) were unaffected. Therefore, further research regarding the effect of depressed ruminal pH in calves during weaning on local inflammation and tissue integrity in the rumen is warranted. Tissue inflammation or integrity can be used to define GIT tissue health parameters. Histological quantification and gene expression are methods that can be used to identify tissue inflammation or to evaluate tissue integrity, as numerous genes and proteins are identified in the literature for their involvement in barrier function (claudins, occludens, zonula occludens, and gap junctions; Graham and Simmons, 2005; Stumpff et al., 2011), inflammation (*TLR2, TLR4, TLR9, TNFA, ,PTGS1, PTGS2, TGFB, ALOX5, ALOX5AP, LTA4H*, and *LTC4S*; Kent-Dennis et al., 2019; *MyD88* and *NF-kB*; Baldwin, 1996), cell-cycle-regulation (Cyclin D1, Cyclin A2, Cyclin E1, CDK-2, CDK-4 and CDK-6; Xu et al., 2018), proliferation (*IGFBP 2-6*; Steele et al., 2011; Xu et al., 2018; Nishihara et al., 2018, 2019) and apoptosis (Caspase 3, Caspase 8, Bcl-2 and Bad; Xu et al., 2018). The assays proliferating cell nuclear antigen (PCNA; Hall et al., 1990) and antigen Ki67 (Mentschel et al., 2001) both stain

nuclei brown during active phases of the cell cycle, and the assay terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) can be used for identifying positive apoptotic cells (brown-stained nuclei indicates the presence of DNA fragmentation due to apoptosis; Tao et al., 2014b). Using some of the above-mentioned methods to study tissue integrity and health will provide a better understanding of how ow ruminal pH affects ruminal tissue in calves. This is important because, as speculated earlier, it may be possible that ruminal acidosis is necessary for calves to transition to functional ruminants and may not actually be a concern.

We used fecal pH, SCFA and bacterial population densities as measurements for hindgut fermentation to determine if calves experienced hindgut acidosis. However, this is not colon digesta and it is possible that the fecal sample collected was altered between the colon and rectum. Consequently, a clear relationship between colon samples and fecal samples needs to be identified in order for fecal samples to be used as a proxy for fermentation parameters of the colon. Furthermore, unlike ruminal acidosis, there are no hindgut pH or fecal pH thresholds set to identify hindgut acidosis, thus future research should quantify the possible thresholds for hindgut acidosis in ruminants.

The acute phase proteins used as inflammatory markers of the GIT in this thesis were SAA and LBP (Chapters 3, 5 and 6). In addition to the aforementioned acute phase proteins, haptoglobin (Hp) and fibrinogen (Fb) are among the commonly used markers for GIT inflammation in ruminants (Paulina and Tadeusz, 2011). One of the biggest limitation with blood inflammatory markers is that stressors or any kind of inflammation in the body can influence their concentration. This includes, but is not limited to, disease, poor nutrition, transition period (weaning), transportation, heat, dehorning, and castration. It has been reported that weaning increases SAA (Kim et al., 2011), Hp (Qiu et al., 2007; Kim et al., 2011) and Fb (Qiu et al., 2007) and that diarrhea increases SAA (Paulina and Tadeusz, 2011). In both our studies (Chapters 3 and 5), we did not observe an increase in SAA during or after weaning. Therefore, because calves may experience multiple forms of inflammation or stress at the same time it is difficult to distinguish the specific process that activated the acute phase proteins. In future research, it may be important to investigate which acute phase proteins are the most accurate or useful for measuring inflammatory responses induced by the diet. Furthermore, it would be helpful to determine the optimal time to collect measurements after diet changes occur.

Overall, the findings of this thesis provide new knowledge on the combination of MR feeding rate and the processing of corn in CS fed to calves. There was no interaction between the amount of MR feeding rate and the processing of corn in CS on calf growth, digestibility, rumen and hindgut fermentation and calf health, therefore different amounts of MR in combination with CS containing flaked or whole corn can be fed to dairy calves. Calves do not only experience ruminal acidosis during the weaning period, and based on our findings, it may be from pre-weaning and last up to 6 wk post-weaning. However, blood inflammatory markers and papillae structure integrity remained unchanged, which suggest that reduced ruminal pH does not influence calf health. A developing rumen may be more resistant to reduced ruminal pH than a mature rumen. Furthermore, weaning calves only influenced a few changes at a molecular level in the colon, which may indicate that the colon transcriptome in calves is not influenced by diet and/or weaning transitioning periods. All of these findings are important to the dairy industry given the key role nutrition plays in calf development, health, and ultimately the profitability of dairy operations.

7.3.Literature cited

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