Physiological Effects of Feeding High vs Low Levels of Milk Replacer and Starch to Holstein Dairy Calves During the Weaning Transition

by

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Abstract

Current calf nutrition programs revolve around two central themes: liquid feed, to provide early nutritional needs for the calf, and calf starter, to provide the stimuli for rumen development to prepare the calf for weaning. It is currently unclear how milk provision and calf starter starch content affect gastrointestinal physiology and development in the young calf. The aim of this study was to assess how milk provision and dietary starch concentration impact the morphological and physiological development of the gastrointestinal tract. Forty-eight male Holstein calves were blocked by body weight and assigned to one of four treatments with a 2 x 2 factorial arrangement of milk replacer and calf starter starch content: low milk replacer (600 g/d; 4L) and low starch (12% starch, DM; **LL**), low MR and high starch (35.6% starch, DM; **LH**), high MR (1,200 g/d; 8L) and low starch (**HL**), and high MR and high starch (**HH**). Calves were weaned starting on day 42 and ending on day 49, and calves were harvested one week later. Gut permeability was measured on days 28 and 56. Gastrointestinal tissue samples were analyzed for tissue histology, gene expression of metabolic enzymes and nutrient transporter protein abundance. High milk diets resulted in increased intestinal permeability and jejunal sodium-glucose cotransporter 1 abundance. High starch diets resulted in increased monocarboxylate transporter 1 and tended to result in increased anion exchanger 1 abundance as well as decreased papillae sloughing in the rumen. In the lower gut, higher starch diets resulted in increased jejunum villous blunting, and ileal epithelial separation. Higher milk provision likely increased glucose absorption in the jejunum. Increased starch conferred a protective effect on the rumen through increased SCFA absorption capacity and decreased sloughing, but low rumen pH may negatively impact the lower gut.

Preface

The research conducted for this thesis forms part of an international research collaboration between Provimi North America (T.S. Dennis), University of Kentucky (J. Costa), North Dakota State University (K. Swanson), University of Guelph (T.T. Yohe, M.A. Steele), and University of Alberta (M. Bennett, A. Laarman). All animal work was completed following the guidelines by Agriculture Animals in Research and Teaching (FASS, 2010) and the Canadian Council on Animal Care (2009) and were approved by the University of Guelph Animal Care Committee (protocol 4272). Analysis of harvested samples completed at University of Alberta was deemed category A invasiveness by the University of Alberta Research Ethics Office (2022.015 Laarman).

I was responsible for data collection, laboratory analysis, and statistical analysis of chapter 2 of this thesis. I was not responsible for animal husbandry or sample collection. Dennis was responsible for funding, experimental design, and execution. Yohe was responsible for animal husbandry and live animal data collection. In addition, writing and editing two companion manuscripts. Swanson was responsible for enzyme activity analysis. Costa, Steele, and Laarman were responsible for training, experimental design, and editing of manuscripts.

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Table of Contents

List of Tables

List of Figures

Abbreviations

ACAT – Acetoacetyl-CoA thiolase

ADG – Average daily gain

AE2 – Anion exchanger, isoform 2

ATP – Adenosine 5'-triphosphate

B-ACT – ß-Actin

BCS – Body condition score

BHB – ß-hydroxy butyrate

BHBD1 – ß-hydroxy butyrate dehydrogenase, isoform 1

BHBD2 - ß-hydroxy butyrate dehydrogenase, isoform 2

BW – Body weight

CP – Crude protein

DAPI – 4',6-diamidino-2-phenylindole

DM – Dry matter

DMI – Dry matter intake

DRA – Downregulated in adenoma

Gal – Galactose

GI – Gastrointestinal

GIT – Gastrointestinal tract

Glc – Glucose

GLUT2 – Glucose transporter, isoform 2

h – Hour

H&E – Hemotoxylin and eosin

HMGCL – 3-hydroxy-3-methylglutaryl-CoA lyase

IgA – Immunoglobulin A

IM – Isomaltase

KHK – Keto-hexokinase

LAC – Lactase

LPS – Lipopolysaccharide

LSM – Least squares means

MCT1 – Monocarboxylate transporter, isoform 1

MGAM – Maltase glucoamylase

MLC – Myosin light chain

MLCK – Myosin light chain kinase

MR – Milk replacer

mRNA – Messenger ribonucleic acid

NDF – Neutral detergent fiber

NHE1 – Sodium/hydrogen exchanger, isoform 1

NHE2 – Sodium/hydrogen exchanger, isoform 2

NHE3 – Sodium/hydrogen exchanger, isoform 3

PAT1 – Putative anion exchanger, isoform 1

qRT-PCR – Quantitative real time PCR

RPLP0 – Ribosomal protein lateral stalk subunit P0

SAA – Serum amyloid A

SARA – Subacute ruminal acidosis

SCFA – Short chain fatty acid

SEM – Standard error of the mean

SGLT1 – Sodium-dependent glucose transporter, isoform 1

STP – Serum total protein

ZO-1 – Zonula occludin, isoform 1

1.0 Literature Review

1.1 Calf Importance

Raising a heifer is major investment and the animal does not begin to generate revenue for the dairy producer until the first lactation, around 2 years of age. On average, 46% of the cost to raise a heifer from birth to calving comes from feed, and 33% from labor (Hawkins et al., 2019). Despite the cost, raising a heifer is typically more affordable than purchasing a springer heifer (McGuirk, 2008). Health in pre-weaned dairy calves is an on-going challenge. Current morbidity and mortality rates in the US are 33.8% and 5.0%, respectively (Urie et al., 2018a). These factors are also highly correlated, indicating that management of calves to decrease health issues will also decrease mortality rates (Urie et al., 2018a). One way to decrease morbidity and mortality rates is to optimize calf nutrition programs so calves can effectively grow and fight off disease.

In raising a replacement heifer, nutrition (both liquid and solid) and health of the calves during pre-weaning affect a heifer's first lactation performance (Kertz and Loften, 2013; Gelsinger et al., 2016). Calves that are group-housed and fed ad libitum amounts of milk reach age at puberty, age at first service, and age at conception earlier than calves fed restricted amounts of milk and housed individually (Curtis et al., 2018). Providing calves with ad libitum milk up to 56 days of age can increase their first lactation milk yield by 450-1,300 kg compared to restricted milk-fed calves (Soberon et al., 2012). For every one kilogram increase of average daily gain (**ADG**) for calves during the pre-weaning period, those heifers produce 850 kg more milk in their first lactation and sustain an increase in milk yield for the second and third lactation compared to calves with less pre-weaning ADG (Soberon et al., 2012; Chester-Jones et al., 2017). While magnitude of milk yield increase may vary from farm to farm, the relationship between pre-weaning ADG

and post-calving milk yield remains positive (Soberon et al., 2012; Soberon and Van Amburgh, 2013; Chester-Jones et al., 2017). There is a positive relationship between first lactation and combined milk replacer (**MR**) and starter metabolizable energy, where every increase of 1 kg in metabolizable energy intake there is an increase of 1.80 kg of milk production (Rauba et al., 2019). Protein and metabolizable energy intakes from both MR and calf starter increase first lactation performance, but high variation between animals suggests other factors impact both early and later animal performance (Rauba et al., 2019). As previously discussed, some of the factors may include protein content, digestibility, and quality of MR, as well as total energy intake, solid feed nutrition, and post-weaning practices, each contribute to increasing future milk yield (Soberon et al., 2012; Gelsinger et al., 2016). Therefore, optimizing calf nutrition is critical, as they have long-term impacts on dairy herd productivity.

1.1.1 Current Industry Practices

Current industry practices for calf management vary widely. Calves are most commonly fed manually and are housed individually (Costa et al., 2016; Medrano-Galarza et al., 2017; Urie et al., 2018b). Calves are typically offered 4.7 L/d or less of milk (56%; USDA, 2016), and are separated from their dam within 12 hours (73.2%; Vasseur et al., 2010) or 24 hours after birth (97%; Medrano-Galarza et al., 2017) in order to facilitate commercial milk shipment. Once separated from the dam, calves are commonly housed individually to allow for separate management and to reduce adverse health events (Cantor et al., 2019). Conversely, group housing decreases labor costs and increases social development, reducing stress, promoting earlier starter intake, and increasing weight gains (Costa et al., 2015, 2016, 2019). Regardless of housing management, calves are separated from their dam, requiring a robust liquid feeding program.

In the dairy industry, liquid feeding programs show great diversity. Milk is the most expensive component of raising a calf to weaning, driving producers to feed different milk types, primarily to control costs. For manual feeding systems in Canada, calves are fed with MR (40%), saleable milk (36%), waste milk (20%), or a combination of saleable and waste milk (4%; Medrano-Galarza et al., 2017). In the United States, 40.1% of operations feed whole or waste milk, 34.8% feed MR, and 25.1% feed a combination of the milk and MR (Urie et al., 2018b). These liquid feeding practices have varied impacts on calf growth and development, which is why no single milk-feeding type has become predominant.

Beyond milk type, milk volume also varies greatly. Calves are typically fed milk at 6-8 L/d, twice a day on manual feeding systems to reduce labor requirements (Medrano-Galarza et al., 2017; Urie et al., 2018b). However, larger volumes of milk (>8 L/d) fed more frequently (>3 times per day) has been shown to increase calf welfare through decreased abnormal behaviors and signs of hunger (Cantor et al., 2019; Costa et al., 2019). The diversity in calf management programs gives no clear answer for what is optimal, requiring on-going investigation into calf nutrition programs to continuously improve calf development and health.

1.2 Calf Nutrition

1.2.1 Liquid Feed Provision

Due to the limited functionality of the rumen in the first few weeks of life, liquid feed (i.e., milk or MR) is used as the main source of nutrients for the calf (Diao et al., 2019), highlighting the importance of liquid feed management (e.g., milk type, feeding frequency, volume). Feeding milk or MR in high amounts is expensive for both product and labor costs, which motivates producers to wean calves earlier (Dennis et al., 2018). Producers often encourage early intake of

calf starter by feeding lower amounts of milk (10% of body weight; Jasper and Weary, 2002), but this may ultimately compromise lifetime performance.

The type of milk provided to calves also affects both physical calf growth and gastrointestinal development. Calves fed whole milk have 10% higher ADG and higher body weights (BW) both pre- and post-weaning (Lee et al., 2009; Moallem et al., 2010). Additionally, they also have a higher milk yield (~3 kg/d) in the first lactation (Moallem et al., 2010). Developmentally, calves fed whole milk have higher empty jejunum and ileum weights as well as increased crypt depth compared to calves fed MR or MR with supplemental butyrate (Gorka et al., 2011b), indicating the increased weight and growth in response to whole milk is coming from the tissue and not digesta. For rumen development, whole milk fed calves have greater papillae length and width, and increased colonization of cellulolytic bacteria both of which may positively impact their later production compared to MR calves (Gorka et al., 2011b; Ceylan et al., 2021). Furthermore, calves fed whole milk have a better feed to gain ratio and a lower average cost per day ($4.47 vs $6.06, respectively; Lee et al., 2009; Sharpe and Heins, 2021). Overall, calves fed whole milk perform better than calves fed MR.

Whole milk and MR differ in several key aspects that may explain the difference in animal performance. First, bioactive factors, such as antibodies (other than IgG) and growth factors, that are only found in whole milk may be beneficial to the calf's development. Second, macronutrient content in whole milk often differs from MR. In whole milk, on a dry matter (**DM**) basis, crude protein (**CP**) averages 26%, fat ranges 28-32%, and lactose from 38-40% (Moallem et al., 2010; Ceylan et al., 2021; Sharpe and Heins, 2021). In contrast, MR CP ranges from 20-25%, fat ranges from 13-28%, and lactose ranges from 40-48% (Lee et al., 2009; Moallem et al., 2010; Ceylan et al., 2021), meaning most milk replacers have lower CP and fat contents compared to whole milk.

The lower values in MR are to achieve a higher CP to fat ratio which allows for greater intake and growth. For MR to be an equivalent substitute for whole milk in terms of calf performance, more research is needed to determine if the bioactive factors in whole milk play a role in improving performance or if the differences in performance are primarily the result of differing macronutrient composition.

In addition to liquid feed type, liquid feed volume provided also impacts growth and performance. Higher amounts of MR (>0.66 kg of dry matter) in the first month leads to higher gains in calves because they are being provided more lactose which can be more efficiently utilized as an energy source (Hill et al., 2010a). Pre-weaning, higher milk allowance improves disease resistance, ADG, BW, body condition score (**BCS**), and dry matter digestibility, but calves can lose this performance advantage post-weaning (Dennis et al., 2018; Shivley et al., 2018). One study found that MR fed in high amounts led to increased pre-weaning ADG from 0.48 kg/d for conventionally-fed calves (milk provision at 10% of BW) to 0.78 kg/d for ad libitum fed calves, but decreased post-weaning ADG as calves transitioned from liquid to solid feed (Jasper and Weary, 2002); other studies are in agreement that ad libitum-fed calves can have advantages pre-weaning but lose those advantages post-weaning (Terre et al., 2007; Dennis et al., 2018; Hammon et al., 2020). In contrast, some studies have found that feeding higher or ad libitum amounts of milk or MR leads to increased pre-weaning weight gain that can persist through weaning (Appleby et al., 2001; Kiezebrink et al., 2015; Curtis et al., 2018). Therefore, increasing liquid feed allowance improves pre-weaning performance, but shows mixed effects post-weaning demonstrating that further research remains to be done.

As daily milk provision increases, individual meal size becomes more important. Historically, calves were fed no more than 2 L per feeding due to concerns that milk would flow

into the rumen to be fermented, producing lactic acid and decreasing rumen pH (Sjaastad et al., 2010). However, abomasal capacity was recently found to be able to take in a meal size of 6.8 L without this occurring (Ellingsen et al., 2016). On the other hand, larger meals can still lead to abomasal inflammation (Burgstaller et al., 2017; Bus et al., 2019), which can cause abomasal distension, decreased intake, dehydration, diarrhea, and death (Burgstaller et al., 2017; Guarnieri et al., 2020). Therefore, to feed high daily milk allowance without causing abomasal inflammation, calves should be fed smaller, more frequent meals throughout the day until they are ready to wean.

1.2.2 Solid Feed Provision

Although milk is the predominant source of nutrients in early life, preparing calves for weaning requires intake of solid feed. Solid feed consumption is the principal requirement to prepare calves for weaning, as consumption of solid feed stimulates rumination and rumen development (Quigley et al., 1992; Khan et al., 2016). Solid feed for the calf is comprised primarily of concentrate and often, forage. Pre-weaning diets high in forage lead to more physical rumen growth, such as increased rumen volume (Khan et al., 2011a), while diets high in concentrates stimulate rumen epithelial development via the fermentation and production of short chain fatty acids (**SCFA**; Bugaut, 1987; Khan et al., 2016). Therefore, management of solid feed programs must balance rumen papillae development with physical development.

1.2.2.1 Starter

Calf starter intake is the key driver of weaning readiness in dairy calves. Calf starter, primarily via its starch content, positively impacts rumen papillae differentiation (Khan et al., 2016). The fermentation of starch produces SCFA, of which butyrate is one of the major three; butyrate is also the main promoter of rumen papillae development (Gorka et al., 2011a, 2018).

Additionally, increasing starch concentrations in the diet also increases ADG, feed efficiency, and starter intake for calves, without negatively impacting fecal scores (Hill et al., 2016; Hu et al., 2018). One study showed that by increasing starch from 23% to 43% of the total DM, ADG increased by 5.8% in calves 0-8 weeks old, and 9.6% in calves 8-16 weeks old (Hu et al., 2018). Despite evidence that starch is beneficial for rumen development, optimum inclusion rates are not yet established.

Increasing the percentage of starch content in calf starter is typically achieved by lowering neutral detergent fiber (**NDF**) content. The impact of NDF concentrations on calf performance and development are mixed. Increasing NDF content to 25-31% has no impact on starter intake, rumen pH, papillae length, or time spent ruminating pre-weaning (Porter et al., 2007; Poczynek et al., 2020; Vagnoni et al., 2021), although it decreases BW, ADG, and feed efficiency post-weaning (Vagnoni et al., 2021). Conversely, utilizing lower NDF content in the calf starter (~20%) can increase digestibility of DM and CP as well as digestible energy and metabolizable energy (Porter et al., 2007). Variations in impact of starter NDF content on calf performance could be due to a multitude of factors, such as inclusion levels in the diet, sources of NDF (soybean, canola, etc.), and delivery of NDF (starter or forage). Much research remains to be done to identify ideal NDF content in calf starter.

Aside from energy, protein content is also important in promoting rumen development. Calf protein requirements are between 18-25% in starter (NASEM, 2021), with CP concentrations of 16-26% showing no differences in starter intake, BW, ADG, body frame measurements, SCFA concentrations, blood metabolites (glucose, BHB, urea nitrogen), gastrointestinal tract (**GIT**) weight, rumen weight, and feed efficiency (Boorboor et al., 2020; Makizadeh et al., 2020; Stamey Lanier et al., 2021). In terms of calf growth, increased CP has no effect on weight gain when

combined with low energy treatments (Hill et al., 2016; Stamey Lanier et al., 2021), suggesting that energy may be the limiting dietary factor in a calf's physical growth. Whether calves fed high energy diets are similarly unaffected by CP content is unclear. Since absorption and metabolism of CP is understudied, it is unknown what CP concentration in the calf starter optimizes both physical and physiological growth.

Beyond nutrient profile, calf starter texture can impact rumen development. Calf starter texture is affected by particle size, uniformity, and processing. Studies comparing low and high starch diets with different feed textures (pelleted versus texturized) have shown that texturized, high starch diets resulted in higher digestibility, ADG, and BCS, and greater hip width (Hill et al., 2012; Dennis et al., 2017; Quigley et al., 2018). In addition to starch content, the difference in texture could also be a confounding factor for the differences in performance observed. Another study demonstrated that texturized starter, when paired with corn silage, increased BW, ADG, and starter intake compared to calves fed a mashed starter or provided no corn silage (Mirzaei et al., 2016). However, while texturized calf starter appears to most positively impact physical development of the rumen, starter texture is often confounded with starch content (Hill et al., 2012; Dennis et al., 2017; Quigley et al., 2018). More research needs to be done on the effects of texture in starter with the same nutrient contents. If texture is limited in the calf starter (by feeding pelleted or ground starter), it needs to be provided elsewhere to stimulate rumen development physically, such as forage.

1.2.2.2 Forage Provision

Forage provision in a calf's diet is controversial, as there are both benefits and drawbacks. As calves age, forage becomes a progressively more important component of the diet (Suarez-Mena et al., 2016) as forages buffer the rumen (Suarez-Mena et al., 2016) by increasing rumination

time and rumen pH, leading to an overall more stable and functional rumen environment (Khan et al., 2011a; Laarman et al., 2012b; EbnAli et al., 2016). Forages provide mechanical stimuli to the rumen which increase rumen musculature, capacity, and volume (Heinrichs, 2005; Castells et al., 2012; EbnAli et al., 2016), without compromising nutrient digestibility or utilization (Khan et al., 2011a). Feeding forages also decrease abnormal oral behaviors, such as licking surfaces, rolling tongues, and consuming wood shavings (Babu et al., 2004; Castells et al., 2012; Terre et al., 2013). Forage has also been shown to increase starter intake in pre-weaned calves (Castells et al., 2012; EbnAli et al., 2016). Overall, forage provision to calves has several benefits, primarily in the areas of increasing rumen physical development and decreasing abnormal behaviors.

Despite the benefits, forages can also decrease calf performance if forage consumption leads to gut fill. When feeding forages in addition to calf starter, dry matter intake (DMI) of both the forage and starter increases and is accompanied by an increased body weight (Castells et al., 2013). However, this could be due to gut fill and the higher DMI rather than actual BW gain (Jahani-Moghadam et al., 2015; Dennis et al., 2018). Increasing gut fill can also cause decreased starter intake, ADG, and feed efficiency (Hill et al., 2008, 2010b; Imani et al., 2017). Other variables involved in feed forage are the forage type and particle size. Alfalfa has been found to increase total SCFA concentrations in the rumen and increase both forage and starter intake compared to other types of forage (Imani et al., 2017; Omidi-Mirzaei et al., 2018). Generally, forages (regardless of type) have increased DMI and ADG without decreases in nutrient digestibility compared to calves provided with no forage (Castells et al., 2012). However, forage type may also have no impacts on ADG, feed efficiency, rumen pH, body measurements (hip height, with height, body length, heart girth) or final BW (Imani et al., 2017; Omidi-Mirzaei et al.,

2018). Results on forage type are inconsistent and require more research to discern their impacts on calf development.

Particle sizes also have variable results. Medium particle length (2 mm) has been found to increase total SCFA concentrations with no differences in ADG, final BW, or rumen pH compared to long (4 mm) particle length (Omidi-Mirzaei et al., 2018). However a particle length of 3 mm has been shown to increase overall DMI, final BW, overall ADG, and rumen pH with no difference in total SCFA concentrations compared to a fine particle length (1 mm; Nemati et al., 2015). Longer particle sizes (>4 mm) may decrease intake due to an increase in gut fill (Mirzaei et al., 2016). Ultimately, the impact of forage provision to calves is determined by numerous factors including type and processing of starter, forage type, forage particle size, diet type, and the amount of milk provided to calves pre-weaning (Jahani-Moghadam et al., 2015; Suarez-Mena et al., 2016; Vagnoni et al., 2021). Therefore, it is still unclear if there is a single optimal forage feeding program for calves and management goals for separate herds or various stages of development will influence forage feeding.

1.2.3 Weaning

Weaning, defined as the transition from a liquid to an all-solid feed diet, requires the calf to shift principal energy sources and rely on the still-developing rumen for their energy requirements. Increasing solid feed intake ultimately prepares the calf for this transition. The beginning of the weaning process is determined either by age or by starter intake, and can be accomplished either abruptly or gradually. Most US farms (98.1%) wean calves between 7-8 weeks of age, regardless of starter intake (Medrano-Galarza et al., 2017; Urie et al., 2018b), which may be more practical, but leaves calves with low starter intake at a performance disadvantage post-weaning. On the other hand, weaning at earlier ages, prior to 8 weeks of age, may increase

the calf's starter intake, but also decreases weight gains, especially during weaning, and increases abnormal behaviors (Eckert et al., 2015; de Passille and Rushen, 2016). Pushing weaning ages past 8 weeks may allow calves to transition to eating more starter without declines in weight gains or increases in abnormal behaviors.

Pace of weaning goes along with weaning based on age. Even when calves are weaned at 42 days of age, pace of weaning greatly influences starter intake and growth (Sweeney et al., 2010), suggesting weaning readiness at any given age varies widely from calf to calf. In addition, calves fed higher volumes of milk (>10% of BW) may need to delay weaning to allow them to adjust and increase their starter intake (Bittar et al., 2020). Calves weaned with no more than 50% milk reduction at a time have improved performance by consuming less MR, having greater starter intake, improved rumen function, and no difference in ADG compared to the more abruptly weaned calves (Steele et al., 2017; Klopp et al., 2019; Welboren et al., 2019). Ideally, weaning calves gradually should involve no more than 50% milk reduction at a time, and be completed over a period of at least two weeks to achieve the best performance (Costa, 2017; Klopp et al., 2019; Bittar et al., 2020).

To reduce animal-to-animal variation in solid feed intake at a given weaning age, weaning can be initiated based on calf starter intake rather than age. Weaning based on starter intake is frequently done in several steps to mimic a gradual weaning program (de Passille and Rushen, 2012, 2016; Benetton et al., 2019). For instance, the NASEM (2021) recommends calves consume between 1.25-1.5 kg/d of calf starter before beginning weaning, and other research recommends a threshold of 200-400 g/d for starting weaning and continuing until calves consume 800-1,400 g/d (de Passille and Rushen, 2012, 2016; Benetton et al., 2019). When calves are weaned based on starter intake, their weaning periods are highly variable, lasting 44-84 days, but calves show greater

starter intakes and greater body weight maintenance through weaning (de Passille and Rushen, 2016; Benetton et al., 2019; Neave et al., 2019). Weaning calves gradually, with no more than 50% milk reduction at a time and over a period of at least two weeks, results in the best performance in calves (Costa, 2017; Klopp et al., 2019; Bittar et al., 2020).

Stress can be an early initiator of disease for calves. Weaning is one of the events in the calf's life that can cause stress, as their GIT must rapidly adapt from a post-ruminally digested liquid diet to a ruminally digested solid diet. Stress is often measured by vocalizations as well as feed intake and weight loss, but can also be measured by gut permeability (Wood et al., 2015). Age-dependent and weaning effects on GIT permeability in ruminants has seldom been studied (Wood et al., 2015); however, permeability does decrease with age and weaning has been found to disrupt the decline in permeability status (Wood et al., 2015). The same pattern in permeability has also been shown in pigs, along with early weaning playing a role in increased permeability (Moeser et al., 2007a; Hu et al., 2013; Moeser et al., 2017). It is presumed that decreasing permeability is the natural state, and the increase in permeability around weaning is a negative physiological response to stress; however, the effects of changes in permeability are still unclear. Weaning stress can be reduced if it is the only stressor occurring, as diet change alone has a lesser effect on behavioral stress (Weary et al., 2008). This means that weaning should not be paired with other stressful events (Weary et al., 2008), such as moving to a new pen, in order to reduce stress-induced increases in permeability, which may predispose the animal to increased risk of infection.

In summary, there are many different possible weaning strategies that set calves up for success and prepare them for a solid-feed-only diet. In general, the weaning transition should be on or after 8 weeks of age (Eckert et al., 2015) and reduce milk no more than 50% at a time to

improve the performance of calves (Welboren et al., 2019). Ultimately, weaning success will rely heavily on the extent of development and function of the gastrointestinal tract.

1.3 Gastrointestinal Anatomy and Development

1.3.1 Gastrointestinal Anatomy

The ruminant's foregut is made up of the rumen, reticulum, omasum, and abomasum. The adult rumen capacity ranges from 75-230 liters, accounts for 70% of the GIT mass and 40% of whole body ATP usage, and provides up to 80% of total energy requirements (Bugaut, 1987; Gabel and Sehested, 1997; Millen et al., 2016). The rumen is responsible for nutrient digestion, fermentation, transport, SCFA metabolism, and protection (Graham and Simmons, 2005; Baldwin and Connor, 2017).

Anterior to the rumen, partly separated by a rumen wall, is the reticulum. The reticulum is made up of a rough stratified cutaneous epithelium, which gives the reticulum a honeycomb-like texture; digesta mixes freely from the reticulum to rumen (Millen et al., 2016). Because of this, the two compartments are often referred to in combination as the reticulorumen. The omasum comes after the reticulorumen and is responsible for absorbing water from the digesta (Millen et al., 2016). It makes up approximately 13% of the total stomach weight and contains many laminar folds that increase its surface area to 10% more than the rumen, even with a volumetric capacity of 14-15 L (Millen et al., 2016; Diao et al., 2017). That leads to the abomasum, which is similar to a monogastric stomach, makes up approximately 25% of the total stomach weight, and produces acid (Millen et al., 2016; Diao et al., 2017). The abomasum wall is covered in a protective mucus to prevent the acid from breaking down the stomach lining. This differs from the rumen, which has a different cell lining structure (further discussed in the next section).

The lower gut, consisting of the small intestine and large intestine, begins at the pyloric sphincter at the distal end of the abomasum. The first section of the small intestine is the duodenum, which, due to its proximity to the acidic abomasum, experiences a lower pH (~ pH 5.73) than the distal sections (jejunum, ~ pH 6.51; ileum, ~ pH 7.42; Abuhelwa et al., 2016; Collins et al., 2021). The duodenum is the shortest section of the small intestine (Hewes et al., 2020; Collins et al., 2021) and has connections to the pancreas, liver, and stomach, which provide digestive enzymes. There is no distinct anatomical differentiation between the end of the duodenum and the start of the jejunum, but the mid-jejunum has many folds and also has villi to increase surface area for absorption (Hewes et al., 2020; Collins et al., 2021). The final section of the small intestine, the ileum, also absorbs nutrients; notably, vitamin B12 absorption is high in the ileum (Collins et al., 2021). The ileocecal junction physically marks the end of the ileum and the start of the large intestine, which contains the cecum, colon (ascending, transverse, and descending), and rectum (Standring, 2019; Kahai et al., 2021). Overall, the large intestine is shorter than the small intestine, but has a larger lumen (Kahai et al., 2021). The cecum is a blind pouch that allows for fermentation (Standring, 2019), similar to the rumen. However, the cecum is not the key hindgut fermenter as it is for monogastric animals.

The small intestine is structured into villi, which absorb nutrients, and crypts, which are more secretory than absorptive (Bass and Wershil, 2020; Hewes et al., 2020). There are many different cell types throughout the small intestine that contribute to protection of the GIT via mucous production (goblet cells), antigen sampling and transport to the immune system (microfold cells, tuft cells, dendritic cells), pathogen destruction (macrophages, Paneth cells, dendritic cells), and hormonal signaling (enteroendocrine cells; Steele et al., 2016; Hewes et al., 2020). The ileum has the most immune cells, while the duodenum has the most mucus-producing cells (Steele et al.,

2016), likely to protect against the lower digesta pH coming from the abomasum. The main cell type for nutrient absorption is the enterocyte, which is also the most common cell type along the length of the small intestine. Barrier function via tight junction proteins also plays an important role in luminal defense in the small intestine (Shen et al., 2008; Odenwald and Turner, 2013, 2017) – the tight junctions prevent free passage of pathogens from the lumen of the gut into the bloodstream or lymphatic systems.

Following the cecum, the colon epithelium contains cells similar to the small intestine. Epithelial crypts house enterocytes, goblet cells, and enteroendocrine cells that perform the same function as in the small intestine (Bass and Wershil, 2020). Goblet cells are the second most abundant cell type in the colon and aid in the passage of digesta (Bass and Wershil, 2020). The base of the crypts also house stem cells that proliferate and migrate to become absorptive cells (Bass and Wershil, 2020). In the ruminant, the developed large intestine retains digesta for 10-29 hours and accounts for approximately 12% of cellulose digestion (Hoover, 1978; Stevens et al., 1980). The SCFA profile and pH in the large intestine are similar to the rumen, and account for 8-16% of the total SCFA production in the animal (Hoover, 1978; Stevens et al., 1980).

1.3.2 Rumen Development

The rumen has limited functionality in the newborn calf, requiring it to be developed before weaning so calves can successfully transition to an all-solid feed diet. Early rumen development is primarily driven transcriptional changes that occur before shifts in the microbiome and before the introduction of solid feed (Pan et al., 2021). These transcriptional changes indicates a programmed development response in the rumen occurs in early life regardless of microbes or diet. The rumen exhibits innate immune response before nutrient metabolism is underway, and calves younger than 28 days have development genes comprised of pH homeostasis, nutrient transport,

and antimicrobial categories (Kato et al., 2016; Pan et al., 2021). After weaning, genes involved in ketogenesis and intraruminal pH regulation are up-regulated, while genes involved in inhibiting cell growth are down-regulated (Kato et al., 2016). These gene regulation changes suggest an increase in epithelial proliferation and nutrient metabolism post-weaning. Diet may also impact expression of genes, as ruminants fed starter have increases in gene expression for lipid and carbohydrate metabolism compared to ruminants fed milk only (Wang et al., 2016). Along with genomic changes, sole reliance on all-solid feed diet also requires physical, metabolic, and microbiological development.

Physical development and microbial establishment can be stimulated by solid feed intake, specifically forage for physical development (Khan et al., 2011a; Malmuthuge et al., 2019b), whereas the majority of metabolic development is stimulated by the fermentation of starches and fiber in the feed, specifically from calf starter and the production of SCFA (Baldwin and Connor, 2017; Gorka et al., 2018). Diet is a major contributing factor to rumen development, and proper nutrition (i.e., providing both fermentable starches and forage) can help the calf transition more smoothly into a functioning ruminant. Some studies indicate that age is also a contributing factor to rumen development (Lane et al., 2000; Eckert et al., 2015; Baldwin and Connor, 2017). Therefore, both age and dietary composition need to be balanced to allow the calf to develop their rumen most effectively.

Physical development of the rumen is characterized as an increase in mass, increase in size, increase in wall thickness, and the growth of papillae (Baldwin et al., 2004; Baldwin and Connor, 2017). In the newborn calf, the rumen makes up only 30% of the GIT (Warner et al., 1956; Baldwin et al., 2004; Moran, 2012a; Millen et al., 2016), while the adult rumen makes up 70% of the GIT (Millen et al., 2016). Forage provision is one or the major contributors to the physical growth of

volume and weight in the rumen. Calves fed forage have greater empty rumen weight and musculature than those not provided forage, along with increased rumination time (Khan et al., 2011a; Terre et al., 2013; EbnAli et al., 2016). Increased musculature and rumination time act as markers of rumen function while providing stimuli to continue developing the rumen. Calves fed forage show no differences in starter intake, papillae development (concentration/density, length, and width), blood glucose, and blood ß-hydroxy butyrate (**BHB**), which suggests that metabolic development occurs via different mechanisms than physical development (Khan et al., 2011a).

In addition to physical development, morphological development of papillae is needed to increase the surface area in the rumen for optimal absorptive capacity. Papillae require SCFA, specifically butyrate, for maximum development to occur (Sander et al., 1959; Millen et al., 2016; Baldwin and Connor, 2017). When rapidly fermentable starches are fed to calves, such as those present in calf starter, the starch is fermented into SCFA in the rumen. Of these SCFA, butyrate is produced in greater quantities in high starch diets compared to high forage diets (Gorka et al., 2018). Butyrate is mostly (85-90%) metabolized in the rumen epithelial mitochondria into ketone bodies, BHB, and acetoacetate, to be used as energy sources for extrahepatic tissues (Beck et al., 1984; Baldwin, 1998; Baldwin and Connor, 2017), and is beneficial to the developing rumen because it accelerates rumen epithelium cell proliferation, epithelial blood flow, rumen motility, and increases papillae length (Sakata and Tamate, 1978; Guilloteau et al., 2010; Gorka et al., 2018). Therefore, promoting butyrate abundance in the rumen via feeding rapidly fermentable starches is key to papillae development.

Metabolic development of the rumen can be classified as the rates of microbial SCFA production, ketogenic enzyme activity, and nutrient transport and fermentation in the rumen. The most recent literature shows that production of SCFA and increased rumen enzyme activity occur

in dairy calves as young as 12-14 days old, which have been found to have SCFA concentrations of 49.6-84.4 m*M* (Rey et al., 2012; Suarez-Mena et al., 2015). It is important to note, however that even though natural metabolic development occurs in young calves, that does not mean that they are capable of handling a completely solid feed diet at this age. Calves weaned at 28 days of age have a ketogenic rate ($\mu g \times 2hr^{-1} \times g^{-1}$ of tissue) at 30 days of age that is 40% that of an adult cow, which is insufficient to maintain both basal bodily functions (heartbeat, respiration, etc.) and growth (Bush, 1988). Additionally, calves fed greater volumes of milk have decreased BHB and ketone production rates (μ*M*) that do not reach those of an adult cow until 60 days of age (Eckert et al., 2015). This means that even though these calves are weaned and are no longer consuming milk, the act of removing liquid feed is not sufficient to promote the necessary full metabolic capacity of the rumen.

Regulation of rumen pH also falls under the category of metabolic development. Homeostasis of rumen pH allows for the regulation of SCFA absorption and the microbiome (Connor, 2010). There are many regulatory transporters involved in maintaining rumen pH and countering H^+ increases including bicarbonate and SCFA transporters (anion exchanger 2 [**AE2**], putative anion exchanger 1 [**PAT1**], and downregulated in adenoma [**DRA**]), Na^+/H^+ exchangers (**NHE**; **NHE1**, **NHE2**, **NHE3**) and Na^+/K^+ ATPase pumps (Muller et al., 2000; Graham et al., 2007; Connor et al., 2010). Under physiological conditions, bicarbonate exchangers and Na^+/H^+ exchangers are the most dominant forms of pH regulation as over 50% of acids in the rumen are absorbed or neutralized by the epithelium (Muller et al., 2000; Schlau et al., 2012). Aside from diet-induced metabolic development, there is some metabolic development that occurs over time, regardless of initial diet (Lane et al., 2000, 2002; Baldwin and Connor, 2017). Rumen pH regulation changes from pre-weaning to the weaning transition (Liu et al., 2016; Hiltz et al., 2021),

which suggests it may be influenced more by age than by diet in the young calf. However, nutrient transporter and pH regulation gene and protein expression is highly variable and, depending on the study, has been found to change with diet, change with age and not by diet, or not change with age (Laarman et al., 2012a; Connor et al., 2013; Hiltz et al., 2021).

Microbial establishment is an important factor for rumen development as the microbes aid in the development of intestinal epithelium, mucosal layer, and lymphoid structures, as well as microbial protein metabolism (Malmuthuge and Guan, 2017; Malmuthuge et al., 2019a). The rumen is considered sterile at birth, but microbial colonization begins within the first 20 minutes of life (Arshad et al., 2021) and becomes more diverse and complex with both age and diet (Jiao et al., 2015; Malmuthuge and Guan, 2017). The early rumen microbiome has high diversity variation between calves, which is mainly responsible for triggering adaptive immune responses to new colonization (Abecia et al., 2017; Malmuthuge and Guan, 2017; Malmuthuge et al., 2019a). These immune responses help build early resistance to foreign pathogens. Both the individual variation and immune responses diminish with age, as the microbiome becomes more established (Abecia et al., 2017; Malmuthuge and Guan, 2017; Malmuthuge et al., 2019a). Therefore, early microbial colonization helps establish the microbiome for later life. The colonization of the microbiome can be categorized in three stages. The first occurs within the first 2-3 days of age with the initial colonization of the pioneer species and proteolytic bacteria (Malmuthuge et al., 2019b; Arshad et al., 2021; Pan et al., 2021). The second stage occurs during the shift from colostrum to milk and the microbiome changes from aerobic to anaerobic bacteria (Abecia et al., 2017; Malmuthuge et al., 2019b; Arshad et al., 2021). The final stage occurs during the introduction of solid feed and around the weaning transition where amylolytic, fibrolytic, and methanogenic microorganisms become more prominent and there are less distinct changes in the

microbiome with slower shifts to the adult microbiome (Abecia et al., 2017; Malmuthuge et al., 2019b; Arshad et al., 2021). These stages may be prone to manipulation that provoke change that persists in later life. Additionally, the stages help establish a more homogeneous microbiome, as by 70 days of age, calves have bacteria present for SCFA metabolism, starch degradation, fibrolytic enzyme secretion, and anatomic rumen development (Jiao et al., 2015). While age is an important factor for microbial development, diet is also a major contributor (Malmuthuge et al., 2019a; Arshad et al., 2021). Ruminants fed a milk-only diet have a more diverse microbiome compared to ruminants fed milk and starter, but this may not be ideal as most of the species present are not fermentative in function and there is no set establishment (Wang et al., 2016). Instead, providing starter helps establish a more stable microbiome that can utilize the substrates in the feed (Jiao et al., 2015; Wang et al., 2016; Malmuthuge et al., 2019b).

Though the positive effects of forage and starter provision on physical and metabolic rumen development and microbial establishment have been confirmed by many different studies (Khan et al., 2008, 2016; Suarez-Mena et al., 2016), more research is needed to completely understand how diet and age interact to promote development of the calf forestomach into a fully functional ruminant system.

1.3.3 Lower Gut Development

Along with rumen development, the small and large intestines also go through physical and metabolic growth and development early in the calf's life. The small intestine, which accounts for 2-2.5 % of live weight (Diao et al., 2017), is covered by a single epithelial layer (Pavic et al., 2020). Intestinal mucosa weight and protein content (g/kg BW) peak at two days of age and then decrease (Le Huerou et al., 1992), indicating that small intestine development occurs much earlier than rumen development (small intestine begins changing as early as 20 days of age) and that,

while other organs increase in size, the small intestine is not keeping up. In early life, small intestine length also increases from 11-13 m pre-weaning (Gorka et al., 2011b; Kosiorowska et al., 2011) to 20-25 m post-weaning (Gorka et al., 2017; Steele et al., 2017; Zhang et al., 2018), another sign of physical development. Small intestine and rumen development patterns are impacted by nutrient availability in the diet, which allows the calf to utilize nutrient-dense diets (milk) in early life and transition energy sources from glucose to SCFA.

Beyond physical development, carbohydrate digestive enzymatic activity also changes early in life. Key carbohydrate enzymes in the lower gut include lactase, maltase-glucoamylase (**MGAM**), isomaltase, and keto-hexokinase (**KHK**; Trotta and Swanson, 2021). Genes that encode the enzymes lactase, MGAM, isomaltase, and KHK have the highest expression in the proximal jejunum and lower levels in the duodenum (Le Huerou et al., 1992; Koch et al., 2019; Trotta et al., 2020). Maltase activity increases at weaning and are overall lower than lactase (Huber et al., 1961; Liu et al., 2018), which increases by 1.2-1.6 fold from birth to two days of age and then decreases until 10 weeks of age (Shirazi-Beechey et al., 1991b; Le Huerou et al., 1992). Milk fed to calves either ad libitum or in restricted amounts has no effect on the expression of lactase, isomaltase, or MGAM genes (Koch et al., 2019), indicating that age has a greater effect on genes encoding these enzymes than diet. Calves that were weaned at 56 days had 1.7 times lower lactase levels at 56 days of age compared to the non-weaned calves (Le Huerou et al., 1992). Enzyme activity for the breakdown of milk naturally decreases with time and with the decrease of milk, and calves are not equipped to break down other types of carbohydrates (such as starch) at a young age (Huber et al., 1961; Shirazi-Beechey et al., 1991a). The decrease in lactase is part of the transition to a solid feed diet, as calves begin to utilize starch and SCFA as an energy source instead of glucose. However,

there is still a lot of research to be done on how diet and time influence carbohydrase activity in young ruminants.

Compared to the small intestine, development of the large intestine is not widely studied in the ruminant. Diet seems to have little effect on the growth of the large intestine, as calves fed milk, milk and starter, or additive levels of lactose, have no differences in large intestine weight or its proportion of BW (Inabu et al., 2017; Zhang et al., 2018). Additionally, pre-natal development of the large intestine continues to grow as gestation progresses and developmental progression is unchanged by the dam's diet (Duarte et al., 2013). More research needs to be done on the large intestine of the ruminant to determine if and how diet effects its growth and function.

1.4 Gastrointestinal Physiology

1.4.1 Foregut Physiology

At birth, the rumen is non-functional, requiring considerable development into a mature rumen with functional epithelium. The mature rumen epithelium is stratified and squamous, consisting of four strata that vary in thickness depending on diet, developmental stage, and feeding pattern (Baldwin and Connor, 2017). The stratum basale is the basal-most layer and is the most important layer for energy metabolism (Baldwin and Jesse, 1991; Baldwin, 1998; Graham and Simmons, 2005). The stratum spinosum and stratum granulosum are the middle strata, and they lack a distinct division between them (Tamate et al., 1974; Graham and Simmons, 2005). The strata spinosum and granulosum contain the tight junction barrier, semi-selective to prevent invasion of microbes that circumvent the outer defenses (Baldwin and Connor, 2017; Meissner et al., 2017; Aschenbach et al., 2019). The stratum corneum is the outermost layer that continuously

renews and sloughs cells and varies in thickness to act as a defense against mechanical damages (Aschenbach et al., 2019).

The majority of SCFA fermented in the rumen are transported across the epithelium and into the bloodstream via passive diffusion or facilitated transport. Passive diffusion can only transport SCFA in the associated form. Associated SCFA make up only 1% of SCFA present in the rumen due to the pK_a of SCFA (~4.8) and physiological rumen pH (~6.5; Aschenbach et al., 2009; Aluwong et al., 2010). If passive diffusion were the predominant or only form of SCFA absorption, there is an expectation that as rumen pH decreases, the SCFA absorption rate would increase linearly. However, this linear increase is absent and shows the need for facilitated transport via SCFA transporters (Aschenbach et al., 2009).

Several SCFA transport mechanisms exist on the apical and basolateral membranes. The most widespread SCFA transporter is monocarboxylate transporter 1 (**MCT1**), which is found in abundance in most tissues (Halestrap, 2012). The MCT1 transporter is located on the basolateral membrane and moves one H^+ ion and one $SCFA^-$ ion from the cell, into the bloodstream (Figure 1.1; Graham et al., 2007; Connor et al., 2010; Laarman et al., 2013). It is also capable of transporting SCFA-metabolites such as lactate, BHB, and acetoacetate, and is thought to be the bottleneck for SCFA transport (Dengler et al., 2015; Baaske et al., 2020). Other transporters include PAT1, DRA, and AE2 (Benedeti et al., 2018). These transporters are embedded in the luminal membrane and move one bicarbonate ion out of the cell and one SCFA into the epithelium (Omer, 2016).

Changes in SCFA transporters in response to age or diet vary widely. Expression of MCT1 and DRA genes increase as calves age (Liu et al., 2016; Nakamura et al., 2018), although MCT1 protein abundance does not (Hiltz et al., 2021). For example, lambs provided starter at 7 days of

age compared to 42 days of age had lower expression of DRA gene and increased expression of MCT1 gene (Liu et al., 2016). Additionally, calves fed MR and starter tended to have higher expression of MCT1 gene, but no difference in transporter abundance (Yohe et al., 2019; Hiltz et al., 2021). In contrast, lambs nursing off the ewe, compared to lambs fed MR, in addition to eating starter tend to decrease expression of MCT1 and PAT1 genes, and have no differences in expression of DRA gene (Sun et al., 2018a). The differences seen in SCFA transporter abundance are likely multi-factorial, and potentially include species, feeding strategy, starter composition, and feeding management. It is still unclear how SCFA transporters respond to diet and age.

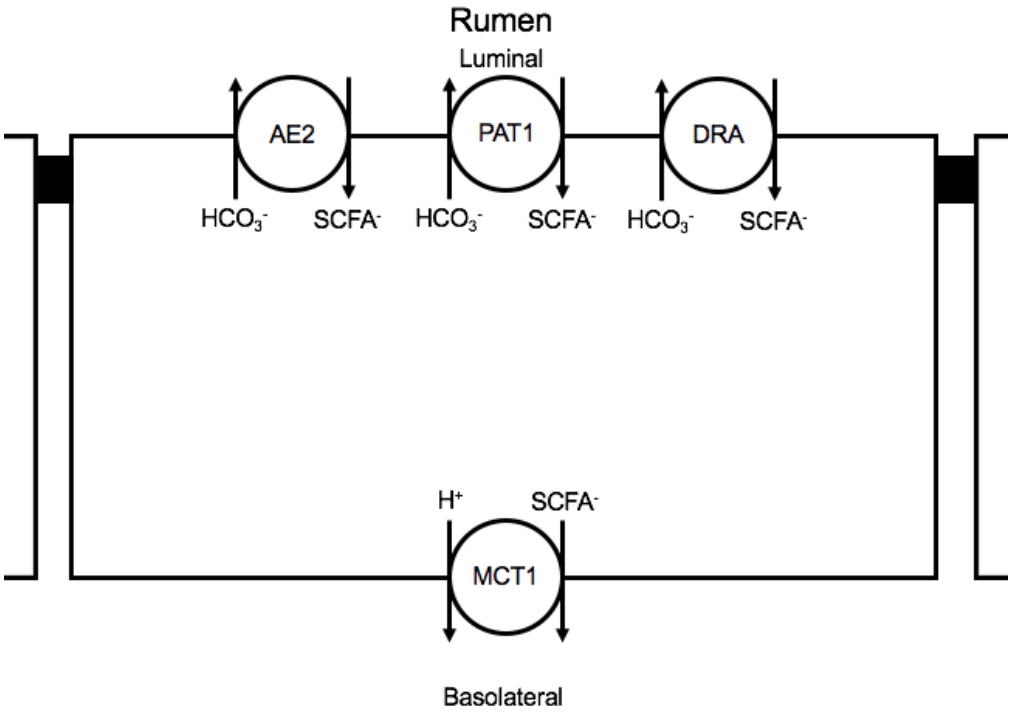

Figure 1.1 Common SCFA transporters in the rumen epithelium. The luminal side transporters are anion exchanger 2 (AE2), putative anion transporter 1 (PAT1) and downregulated in adenoma (DRA). The luminal transporters move one bicarbonate (HCO_3^-) out of the cell and into the rumen lumen, and one dissociated SCFA ($SCFA^-$) into the cell. The basolateral transporter,

monocarboxylate transporter 1 (MCT1), moves one $SCFA^-$ and one hydrogen ion (H^+) out of the cell and into the bloodstream.

1.4.2 Foregut Barrier Function

Since the rumen houses a diverse microbial population as well as rough feeds that can cause mechanical damage, the rumen epithelium must form a layer of protection in addition to the other roles it plays in nutrient transport. Beneath the stratum corneum, other epithelial layers house the tight junction protein complexes that act as a dynamic barrier to prevent pathogens from freely flowing into the bloodstream (Shen et al., 2008; Stumpff et al., 2011; Meissner et al., 2017). Tight junction complexes are made up of a variety of protein structures that ensure their integrity, including occludin, zonula occludin-1 (ZO1), and claudins. Occludin is expressed most abundantly in the apical-most layers of barrier-forming epithelium cells to maintain and regulate barrier function with ZO1, which is present in the stratum granulosum and stratum spinosum (Graham and Simmons, 2005; Shen et al., 2011; Stumpff et al., 2011). The claudins are predominantly barrier-forming and help prevent water loss from cells and regulate paracellular ion conductance (Graham and Simmons, 2005; Gunzel and Yu, 2013; Zihni et al., 2016). There are many different claudin types, which dictate the properties of the tight junction; the rumen epithelium expresses claudins 1, 3, 4, 7, 12, and 23 (Shen et al., 2011; Stumpff et al., 2011; Baldwin et al., 2012). Expression of claudin genes is up-regulated by butyrate, which also helps maintain barrier function (Baldwin et al., 2012). Tight junctions are a major component of barrier function within the rumen that allow for protection against pathogens and other harmful components.

Increased butyrate production may increase tight junction integrity; however, increased SCFA production can lead to decreases in rumen pH and metabolic disorders such as subacute

ruminal acidosis (**SARA**). Subacute ruminal acidosis is a nutrition-based issue for both the dairy and beef industry often triggered by an increase in rapidly fermentable carbohydrates, such as starch, and impacts the functionality of the rumen epithelium. Subacute ruminal acidosis leads to decreases in milk yield and feed intake, and to additional gastrointestinal disorders (Abdela, 2016). Additionally, SARA can cause epithelial barrier dysfunction and increases in permeability (Emmanuel et al., 2007; Meissner et al., 2017). One of the benchmarks for SARA is a rumen pH at or below 5.8 for more than 3 hours/day (Beauchemin et al., 2003; Schwartzkopf-Genswein et al., 2003; Villot et al., 2018). However, it is important to note that SARA is more than just a decrease in rumen pH; it is also marked by an increase in osmolarity and SCFA and toxin concentrations (Penner et al., 2010). Ruminants with only a decrease in rumen pH have no increases in permeability or changes in barrier function (Emmanuel et al., 2007; Penner et al., 2010). To induce barrier dysfunction, additional insults (such as increased SCFA concentrations or repeated episodes of SARA) must be present. When high starch diets are rapidly introduced, the total rumen SCFA concentration increases and damage to the rumen papillae can be seen by decreases in stratum thickness and lesions forming on papillae (Steele et al., 2011; Liu et al., 2013; Sun et al., 2018b). Increases in passive ion leak and permeability marker flux are also observed (Meissner et al., 2017; Sun et al., 2018b), indicating that barrier function has been impaired. Additionally, decreases in both mRNA and protein expression for claudins, occludin, and ZO-1 are seen in ruminants with induced SARA (Liu et al., 2013; Meissner et al., 2017; Sun et al., 2018b). The effects of SARA may also spread beyond the rumen through immune responses and increases in lipopolysaccharide (LPS) flux (Emmanuel et al., 2007; Steele et al., 2009), which can decrease production and compromise immune function.

1.4.3 Lower Gut Physiology

In neonatal ruminants, lactose from milk is the principal carbohydrate energy source and must be digested into glucose and galactose, then absorbed. Ruminants' energy utilization when under three weeks of age are limited by how much lactose can be hydrolyzed, rather than how much glucose and galactose can be absorbed (White et al., 1971; Gerrits, 2019). Lactose not broken down enzymatically can be fermented by lactic acid bacteria in the small intestine (Liu et al., 2019; Malmuthuge et al., 2019a). By six weeks of age, however, decreased intestinal lactase activity reduces glucose absorption by half (Gerrits, 2019; MacPherson et al., 2019) and decreases glucose oxidation, especially after weaning (Baldwin and Jesse, 1992; Baba et al., 2005). This means that, prior to weaning, the small intestine's rate of glucose absorption exceeds lactose digestion, indicating that glucose absorption is more efficient in the young ruminant.

Glucose absorption occurs primarily via facilitated diffusion/active transport, involving two key transporters. First, an apically-located sodium-dependent glucose cotransporter-1 (SGLT1), which transports glucose from the small intestine lumen into the epithelial cell. Sodium-dependent glucose cotransporter 1 has a high affinity for glucose, but a low capacity for transport (Figure 1.2; Zheng et al., 2012; Pavic et al., 2020). This means that most glucose traveling through the GIT, during non-meal periods (<10 mM; Zheng et al., 2012), are bound and transported by SGLT1. Protein abundance for SGLT1 can vary based on amount of glucose in the lumen, duration of fasting, and diurnal rhythmicity (day/night cycle; Roder et al., 2014). The decline in glucose supply and absorption in older ruminants corresponds with the decrease in SGLT1 activity post-weaning (Harmon and McLeod, 2001). In SGLT1 knockout mice, glucose absorption decreases by 80% and plasma glucose levels are 73% lower (Roder et al., 2014), highlighting its central, but not sole, role in glucose transport in the small intestine.

The second key glucose transporter is the basolateral-located facilitated glucose transporter 2 (GLUT2), which transports glucose into the bloodstream (Wright, 1993; Navale and Paranjape, 2016; Pavic et al., 2020). Glucose transporter 2 has a low affinity for glucose, but a high transport capacity (Zheng et al., 2012; Pavic et al., 2020), meaning that during periods of increased glucose concentrations, GLUT2 can transport glucose quickly into the bloodstream. Abundance for GLUT2 is highest in the duodenum and jejunum, and is regulated by how much glucose is in the small intestine, with lower abundance occurring right before a meal (Kellett and Helliwell, 2000; Lohrenz et al., 2011; Trotta et al., 2020). The impact of diet on GLUT2 mRNA abundance is unclear. Different volumes and types of milk have no difference on GLUT2 abundance (Steinhoff-Wagner et al., 2014; Brito et al., 2018), while increasing concentrates can increase abundance (Li et al., 2016). However, GLUT2 abundance does decrease with age (Li et al., 2016).

Nutrient transport is required to be able to utilize the digested nutrients; however, barrier function and protection from intruding pathogens is also a critical role for GIT epithelium.

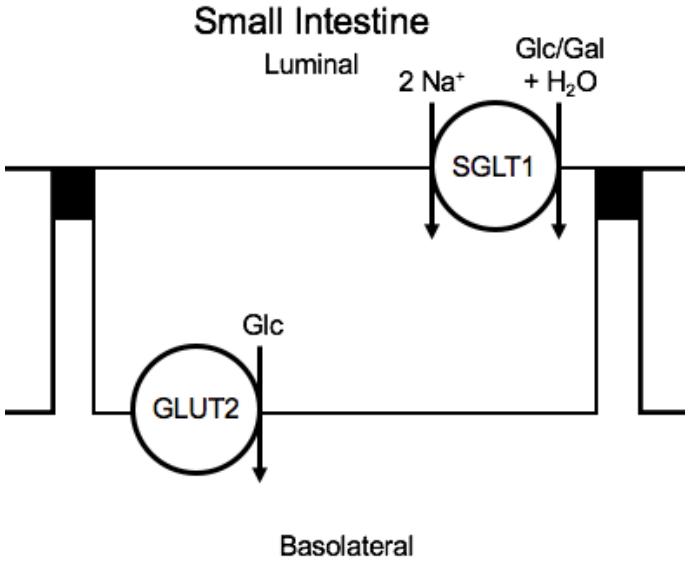

Figure 1.2 The most abundant luminal side glucose transporter is the sodium-dependent glucose transporter 1 (SGLT1), which moves two sodium ions (Na^+) and one glucose/galactose (Glc/Gal)

and one water molecule (H_2O) from the small intestine lumen into the cell. The basolateral transporter, glucose transporter 2 (GLUT2), moves one glucose (Glc) out of the cell and into the bloodstream.

1.4.4 Lower Gut Barrier Function

Transepithelial transport is controlled by establishing a barrier between the luminal and serosal sides of the epithelium. As with the rumen, the small intestine epithelium also contains tight junctions that determine paracellular permeability (Shen et al., 2008; Odenwald and Turner, 2013, 2017). Other parameters for barrier function in the small intestine include mucins produced by goblet cells that prevent bacteria from adhering to the intestinal wall, commensal bacteria and Paneth cells secreting antimicrobial substances, and secretory IgA from enterocytes (Turner, 2009; Camilleri et al., 2012; Moeser et al., 2017).

Loss of tight junction barrier is often referred to as “leaky gut”, regardless of location or mechanism of permeability (Van Uytzel et al., 2021). Loss of tight junction barrier function can trigger immune activation and disease in susceptible animals, though tight junction barrier loss does not directly cause disease (Odenwald and Turner, 2013). Bacteria that cause tight junction permeability also typically cause apoptosis, which leads to loss of epithelial integrity and diarrhea (Krug and Fromm, 2020). Loss of epithelial integrity is only correlated, not causative, to disease,and no human or animal model has established a direct link between loss of barrier function and disease (Odenwald and Turner, 2013; Herrmann and Turner, 2016). Further studies are required to determine whether loss of barrier function is directly, or only indirectly, associated with disease and poor-doing.

1.5 Knowledge Gap

Current calf nutrition programs revolve around two central feed groups: 1) liquid feed, to provide early nutritional needs for the calf; and 2) calf starter, to provide the stimuli for rumen development to prepare the calf for weaning. Current nutritional strategies vary widely, including factors such as the amount of milk fed to calves (from 10% of BW to ad libitum), amount of concentrate included in the diet (starter fed a restricted amount, ad libitum, or in a total mixed ration), and the nutrient composition of the concentrates (starch ranging from 10-53% of diet composition). While calves can survive on a solid feed diet at weaning, it is unknown how the digestion, absorption, and metabolic capacity are best developed to prepare calves for weaning. Increasing milk allowance has the potential to increase weight gains and lower gut development by taking advantage of the early nutrient utilization efficiency of milk. Increasing calf starter starch content has the potential to promote rumen development through the production and absorption of SCFA. The overall objective of my M.Sc. research is to improve our understanding of how milk allowance and calf starter starch content impact gut physiology and development in the young calf. We hypothesize that calves fed high volumes of milk or a starter with high starch content would have increased gastrointestinal nutrient absorption capacity, increased digestion and metabolism enzyme expression, decreased permeability, and healthy tissue morphology.

2.0 Physiological effects of feeding high vs low levels of milk replacer and starch to Holstein dairy calves during the weaning transition

2.1 Introduction

Calves are born with a non-functioning rumen that comprises 30% of the gastrointestinal tract (**GIT**) mass (Warner et al., 1956; Baldwin et al., 2004; Moran, 2012b). In contrast, the adult rumen makes up 70% of the GIT mass and provides up to 80% of their energy requirements in the form of short chain fatty acids (**SCFA**; Warner et al., 1956; Bugaut, 1987; Gabel and Sehested, 1997). To wean successfully, calves must transition energy reliance from lactose in liquid feed to starch in solid feed, requiring physiological development of both the rumen and the microbiome that can effectively use starch as an energy source (Radostits and Bell, 1970; Moran, 2012b; Gomez et al., 2016). Calves are fed milk typically at 10-20% of their body weight (**BW**; Jasper and Weary, 2002; NFACC, 2009; Medrano-Galarza et al., 2017). Increased milk provision can lead to increased pre-weaning average daily gain (**ADG**), health, and future lactation performance (Jasper and Weary, 2002; Hill et al., 2010a), but may also delay rumen development or decrease post-weaning ADG (Khan et al., 2011b). Increasing milk provision, while beneficial to calf growth and welfare, delays solid feed intake.

Eating and fermenting solid feed stimulates rumination in the calf (Quigley et al., 1992; Khan et al., 2016). Starch in starter is a potent stimulator of rumen epithelial development through fermentation and production of SCFA, especially butyrate (Bugaut, 1987; Khan et al., 2016). While the importance of starch in rumen development is well-established, optimal inclusion rates are unclear. High starch diets (~40%) increase nutrient digestibility and ADG (Hill et al., 2012;

Dennis et al., 2017; Quigley et al., 2018) but starch contents in calf starter can range down to 8-10% (Dennis et al., 2017; Quigley et al., 2018; Aragona et al., 2020). Overall, the goal of a calf nutrition program is to promote high growth and development when feeding milk, while increasing starter intake to promote rumen development and prepare the calf for weaning.

Rumen development in early life involves important physiological adaptations. Intestinal permeability and rumen pH are two areas that fluctuate with the developmental changes occurring through diet and time. While gastrointestinal (**GI**) permeability decreases after birth, weaning can increase permeability (Wood et al., 2015). When intestinal dysfunction (generally through increased permeability) is induced, it causes inflammation, altered metabolism, and decreased feed intake (Kvidera et al., 2017). Most research done on increased permeability focuses on disease and the negative impacts of permeability (Odenwald and Turner, 2017; Vanuytsel et al., 2021). However, some research suggests tight junction proteins can be regulated by other physiological mechanisms to increase permeability which increases nutrient flux (Turner et al., 1997; Turner, 2000, 2009). It is currently unclear if increases in permeability are beneficial to nutrient absorption in the animal, as barrier loss alone does not cause disease (Odenwald and Turner, 2013; Herrmann and Turner, 2016).

The effects of rumen pH on the growing calf are also currently unclear. In adult cows, periods of low rumen pH (below 5.8) for more than 3 hours/day is termed the benchmark for subacute ruminal acidosis (**SARA**; Beauchemin et al., 2003; Schwartzkopf-Genswein et al., 2003; Villot et al., 2018), and can cause decreases in weight gain and feed intake, as well as decreased papillae length and width, and increased keratinization (Abdela, 2016). However, studies have shown calves to have rumen pH ranges between 5.5-5.9 for long periods of time (7-18 hours/day) without the negative impacts of acidosis in cows (Laarman et al., 2012b; Wood et al., 2015; van

Niekerk et al., 2021). Evidence continues to accumulate suggesting the impacts of low rumen pH on calves is unclear and may not have the same effects as seen in adult cows.

Previous studies investigating high and low planes of macro-nutrition focused primarily on physical growth parameters. Since calves are transitioning energy sources from glucose to short chain fatty acids, considerable physiological development may be overlooked. Calf physical performance, nutrient digestibility, and inflammation were explored in companion papers (Yohe et al., 2022b; a) but they did not explain the physiological development of the GIT. The objective of this study was to investigate how milk provision and calf starter starch content impact the morphological and physiological development of the gastrointestinal tract, specifically digestion, absorption, and metabolic capacity. The hypothesis was that calves fed high volumes of milk or a starter with high starch content would have increased gastrointestinal nutrient absorption capacity, increased digestion and metabolism enzyme expression, decreased permeability, and healthy tissue morphology.

2.2 Materials and Methods

2.2.1 Animals and Treatments

Animal work was published in companion papers (Yohe et al., 2022a; b). Briefly, all animal work was completed following the guidelines by Agriculture Animals in Research and Teaching (FASS, 2010) and the Canadian Council on Animal Care (2009) and were approved by the University of Guelph Animal Care Committee (protocol 4272). Analysis of harvested samples for this study was deemed category A invasiveness by the University of Alberta Research Ethics Office (2022.015 Laarman). At 4 days of age, 48 male, Holstein calves were transported to the Nurture Research Center in Brookville, Ohio. Upon arrival, BW was taken for

each calf. Each calf was housed individually with straw bedding covered by garden mats to prevent calves from consuming bedding. All calves were provided unlimited access to water.

At arrival, calves were blocked by BW and assigned one of four treatments in a 2 × 2 factorial of milk provision and calf starter starch content. The milk provision treatments were low milk replacer (**MR**; 600 g/d; 4 L) and high MR (1,200 g/d; 8 L); the starch treatments were low starch (12% starch, DM) and high starch (35.6% starch, DM). The four treatments were low MR and low starch (**LL**), low MR and high starch (**LH**), high MR and low starch (**HL**), and high MR and high starch (**HH**). All treatments used the same MR (24.5% CP, 19.8% fat DM) that was fed twice a day (0700h and 1700h) beginning on day 1 (day after arrival). Weaning started on day 42 with calves receiving half their treatment allotment of MR (LL & LH: 2 L/d; HL & HH: 4 L/d) and ended on day 49, when milk allotment was removed.

Whole gut permeability was measured on days 22-23 and 50-51 after the morning MR meal by dosing a 200 mL total volume of Cr-EDTA (179 m*M* Cr-EDTA solution), lactulose (0.4 g/kg of BW; Sigma-Aldrich, St. Louis, MO, USA), D-mannitol (0.12 g/kw of BW; Sigma-Aldrich, St. Louis, MO, USA) via an esophageal tube feeder. Blood samples were collected at 0, 2, 4, 6, 8, and 10 h relative to marker dosing via jugular catheter into a 10 mL serum vacuum tube (BD Vacutainer Serum Blood Collection Tubes, Franklin Lakes, NJ, USA). The blood was allowed to clot at room temperature before centrifuging at 3,000 × *g* at 4ºC for 20 min (Marathon 21000R; Fisher Scientific, Pittsburgh, PA, USA) and separating the supernatant, which was stored at -20ºC until analysis.

Calves were harvested on days 57-60 by captive bolt gun and exsanguination, at a rate of 12 calves per day (n = 3 per treatment, per day). The harvest schedule was staggered based on the calves' age at arrival, so calf age at harvest was closely clustered. Tissue samples were taken

from the rumen, duodenum, proximal jejunum, distal jejunum, ileum, and colon. Each sample was cut and rinsed with PBS to prevent contamination with digesta or blood. Tissue samples for gene expression analysis were cut into 2-3 cm sections, placed in cryovials containing RNAlater (Invitrogen, Waltham, MA, USA) and stored at room temperature until being moved to a -20 °C freezer the following day and then at -80 °C for long-term storage. Tissue samples for histology analysis were cut into 5 cm sections and stored in vials containing formalin. Histology samples were then moved to a 70% ethanol solution two days later before being processed and mounted in paraffin wax. Paraffin wax cassettes were stored at room temperature.

2.2.2 Gut Permeability

Three permeability markers (CrEDTA, lactulose, and mannitol) were used to strengthen the output of the results along with other measurements such as histology (Galipeau and Verdu, 2016). Both lactulose and mannitol are known to be metabolized by colon microbes, so are used to assess intestinal permeability (Galipeau and Verdu, 2016; Gonzalez-Gonzalez et al., 2019). Whereas CrEDTA is not metabolized by colon microbes, so it can be used to assess whole tract permeability (Galipeau and Verdu, 2016). There are only two published studies on the fermentability of lactulose and mannitol in the rumen to assess their suitability as permeability markers in ruminants, with conflicting results (Ahmed et al., 2013; Ellett et al., 2022). One study found that lactulose and mannitol may largely escape rumen fermentation (Ahmed et al., 2013) and the other found that lactulose and mannitol can be fermented in the rumen (Ellett et al., 2022). It has also been noted that pre-ruminant calves may not ferment lactulose and mannitol in the rumen (Klein et al., 2007), and other studies have used these markers to assess calf intestinal permeability (Araujo et al., 2015; Amado et al., 2019).

Gut permeability was performed as described previously (Welboren et al., 2021). Briefly, for Cr-EDTA detection, all serum samples were diluted 10 times with a basic diluent. The diluent consisted of 4% (w/v) 1-butanol, 0.01% (w/v) EDTA, 0.01% (w/v) Triton X-100, and 1% (w/v) tetramethylammonium hydroxide made up with high purity water (>18 MΩ). Samples were then analyzed for total Cr at Trent University Water Quality Center (Peterborough, ON Canada) by inductively coupled mass spectrometry with an Agilent 8800 ICP-QQQ-MS (Santa Clara, CA, USA) using H2 reaction gas in MS/MS mode.

Serum lactulose and D-mannitol were analyzed in duplicate where internal standard solutions of lactulose ($^{13}C_{12}$ lactulose, Omicron Biochemicals Inc) and D-mannitol ($^{13}C_6$ mannitol, Cambridge Isotope Laboratories, Inc), as well as ammonium formate, were added to each unknown sample. Samples were vortexed and centrifuged before the supernatant was collected into vials to be analyzed via liquid chromatography-mass spectrometry (LC-MS). All samples were analyzed by a Thermo Vanquish™ Duo, tandem UHPLC system coupled to a TSQ Altis, triple quadruple mass spectrometer (ThermoX Fisher Scientific, Waltham, MA, USA). Lactulose, D-mannitol, and their corresponding internal standards ($^{13}C_{12}$ lactulose and $^{13}C_6$ mannitol, respectively) were monitored and quantified. Quantification was performed in Thermo TraceFinder 5.0 software (ThermoFisher Scientific, Waltham, MA, USA) with 1/x weighting, with method detection limits for both analytes at 150 ng mL^{-1}. After concentration was analyzed, the area under the curve was calculated and summed for each calf for each week to get the permeability index used for statistical analysis.

2.2.3 Gut Histology

Rumen, duodenum, jejunum, ileum, and colon tissues were processed and embedded in paraffin wax (Animal Health Laboratory, University of Guelph, Guelph, ON, Canada), and were

then sliced into 5 μm sections to be mounted on charged microscope slides (Globe Scientific, Mahwah, NJ, USA) and analyzed as described previously (Laarman et al., 2013). Briefly, all slides were blinded prior to analysis. Then, slides were deparaffinized and rehydrated using three one-minute washes of xylene, one of 100% isopropanol, and one of 70% isopropanol. Slides were then stained using a hematoxylin and eosin dye (H&E) and imaged using a Zeiss Primo Star compound light microscope (Carl Zeiss Microscopy, White Plains, NY, USA). Three images were taken per slide and randomized before quantification. Before each scoring category, three evaluators were calibrated using a random selection of 10 images. For scoring, each image was blinded and then rated by three evaluators on a scale of one to five, with one being ideal for each gut health parameter, three being moderate, and five being most severe; scales were adapted from previous research (Erben et al., 2014; Steele et al., 2015). Rumen tissue was scored based on papillae development, and papillae cell sloughing. A score of 1 for rumen papillae development was all or almost all papillae in the image were single, uniform, and finger-like in shape (Figure 2.1). A score of 2 was most papillae are uniform but may show minor irregularities in shape. A score of 3 was some of the papillae are uniform but are more irregular in shape than a 2 score. A score of 4 was few papillae are uniform and most papillae in the image are severely irregular in shape compared to a score of 3. A score of 5 was very few or almost no uniform shapes were present and all or almost all papillae in the image were very branched and irregular. A score of 1 for rumen papillae sloughing was all or almost all of the corneal layer was intact with no separation from the papillae (Figure 2.2). A score of 2 was most of the corneal layer was intact with minimal separation from the papillae. A score of 3 was some of the corneal layer was intact, with more separation than a score of 2. A score of 4 was little of the corneal layer was intact, with more separation than a score of 3. A score of 5 was none or very little of the corneal

layer remained intact with the papillae, with severe separation. Duodenum, jejunum, and ileum tissue were scored based on goblet cell loss, villous blunting, and separation of epithelial cells (Erben et al., 2014). A score of 1 for small intestine goblet cell loss was all or almost all of the villi present contained goblet cells in great number (Figure 2.3). A score of 2 was most of the villi present contained many goblet cells, with less goblet cells present than a score of 1. A score of 3 was some of the villi present contained goblet cells, with less goblet cells present than a score of 2. A score of 4 was few of the villi present contained few goblet cells, with less goblet cells present than a score of 3. A score of 5 was none or very few of the villi present contained very few goblet cells, with less goblet cells present than a score of 4. A score of 1 for small intestine villous blunting was all or almost all of the villi present were elongated and uniform in shape (Figure 2.4). A score of 2 was most of the villi present were elongated, with more variation than a score of 1. A score of 3 was some of the villi present were elongated, with more variation than a score of 2. A score of 4 was few of the villi present were elongated, with more variation than a score of 3. A score of 5 was none or very few of the villi present were elongated, with most of the villi being short and round. A score of 1 for small intestine epithelial separation was no or very little separation from the epithelium is visible throughout the image (Figure 2.5). A score of 2 was little separation from the epithelium was visible, and more than a score of 1. A score of 3 was some separation from the epithelium was visible, and more than a score of 2. A score of 4 was much separation from the epithelium was visible, and more than a score of 3. A score of 5 was severe separation from the epithelium was greatly visible. Colon tissue was scored based on crypt development, and goblet cell loss. A score of 1 for colon crypt development was all or almost all of the crypts present were elongated and uniform in shape (Figure 2.6). A score of 2 was most of the crypts present were elongated, with more variation than a score of 1. A

score of 3 was some of the crypts present were elongated, with more variation than a score of 2. A score of 4 was few of the crypts present were elongated, with more variation than a score of 3. A score of 5 was none or very few of the crypts present were elongated, with most of the crypts being short and round. A score of 1 for colon goblet cell loss was all or almost all of the crypts present contained goblet cells in great number (Figure 2.7). A score of 2 was most of the crypts present contained many goblet cells, with less goblet cells present than a score of 1. A score of 3 was some of the crypts present contained goblet cells, with less goblet cells present than a score of 2. A score of 4 was few of the crypts present contained few goblet cells, with less goblet cells present than a score of 3. A score of 5 was none or very few of the crypts present contained very few goblet cells, with less goblet cells present than a score of 4.

2.2.4 Expression of Genes (Quantitative Real-Time PCR)

Total RNA was extracted from rumen and jejunum tissue using a Trizol (Invitrogen, Waltham, MA, USA) and chloroform method (Chomczynski and Sacchi, 2006). Rumen and jejunum tissue was used as genes of interest were in highest abundance in those two tissues. Concentration and purity were measured at 260 nm and 280 nm using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). All sample absorbance ratios (A260/A280) were above 1.80 and determined to be a high enough quality. Then, the same amount of total RNA for each sample was reverse-transcribed with Superscript II and Oligo-d(T) (Invitrogen, Waltham, MA, USA). Primers (Invitrogen, Waltham, MA, USA) for quantitative real-time PCR (**qRT-PCR**) were designed and verified using NCBI Primer BLAST (Table 2.1). Primers were also verified for accuracy by evaluating the melt curve for one distinct peak using the thermocycler (StepOnePlus Real-Time PCR System, Applied Biosystems, Carlsbad, CA, USA) set to one cycle of 95°C for 15s, 60°C for 60s, and 95°C for 15s. Genes targeted were:

acetoacetyl-CoA thiolase (***ACAT***), *ß-hydroxybutyrate dehydrogenase 1* (***BHBD1***), *ß-hydroxybutyrate dehydrogenase 2* (***BHBD2***), *3-hydroxy-3-methylglutaryl-CoA lyase* (***HMGCL***), *isomaltase* (***IM***), *keto-hexokinase* (***KHK***), *lactase* (***LAC***), *maltase-glucoamylase* (***MGAM***), and *pancreatic amylase* (***PA***). *Pancreatic amylase* can be measured in jejunum tissue (Gardner et al., 1970; Russell et al., 1981). Two genes were used as reference genes: *β-actin* (***B-ACT***) and *Ribosomal Protein Lateral Stalk Subunit P0* (***RPLP0***). Reference genes were selected based on stability and independence from changes in diet (Brym et al., 2013; Trotta et al., 2020).

Quantitative real-time PCR was performed using a Power SYBR Green PCR Master Mix (ThermoFisher Scientific, Waltham, MA, USA). The thermocycler (StepOnePlus Real-Time PCR System, Applied Biosystems, Carlsbad, CA, USA) was set to 40 cycles of 95°C for 15s, and 60°C for 60s. All samples were run in triplicate. All genes were analyzed on a standard curve to determine the amplification efficiency. The mRNA abundance of the target genes was calculated using the amplification efficiency and Ct values, correcting for the geometric mean of the reference genes (Pfaffl, 2001; Vandesompele et al., 2002; Gao and Oba, 2016).
Equation used for fold change calculation:

$$\frac{E_{target}^{(Min\ Ct_{target} - Ct_{target})}}{Geo\ Avg\ (E_{HKG(n)}^{(Min\ Ct_{HKG} - Ct_{HKG})})}$$

Where E is the amplification efficiency (calculated as: $10^{(-1/slope)}$), Ct is the cycle threshold value for each sample, Min Ct is the lowest Ct value out of all the samples for one gene, target is the single gene of interest, HKG is the housekeeping gene or reference gene, n is the number of housekeeping genes, and Geo Avg is the geometric average of the housekeeping genes for each sample.

2.2.5 Immunofluorescence

Rumen and jejunum tissues were processed using the same methods as the gut histology described above, up to the deparaffinization step. Samples were then incubated in a 10 m*M* sodium citrate solution at 95°C for 15 min for antigen retrieval and afterwards cooled to room temperature. After cooling, samples were washed with PBS (pH 7.4) and blocked using a buffer containing 10% goat serum (ThermoFisher Scientific, Waltham, MA, USA) and 0.3% Triton-X100 (Fisher Scientific, Ottawa, ON, Canada) in PBS for 30 min. A primary rabbit polyclonal antibody (Bioss, Woburn, MA, USA; or Signalway Antibody, College Park, MD, USA), dissolved in blocking buffer, was added to each sample and incubated for 90 min at room temperature. Samples were triple-rinsed again in PBS, before adding a fluorescent secondary anti-rabbit goat antibody (Alexa Fluor 488, ThermoFisher Scientific, Waltham, MA, USA) for 40 min and mounted using ProLong Antifade with 4',6-Diamidino-2-Phenylindole (DAPI) nuclear stain (ThermoFisher Scientific, Waltham, MA, USA). Primary antibody and secondary antibody dilutions varied depending on the optimal signal for each antibody (Table 2.2). A negative control without primary antibody was used for each primary antibody, as well as a control containing no antibodies. After staining was complete, slides were stored at -20ºC until they were analyzed. Antibody targets in the rumen were anion exchanger 2 (AE2), monocarboxylate transporter 1 (MCT1), downregulated in adenoma (DRA), and putative anion transporter 1 (PAT1). Antibody targets in the jejunum were sodium-dependent glucose co-transporter 1 (SGLT1) and glucose transporter 2 (GLUT2).

Slides were visualized using an Olympus FV3000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan). Microscope settings were optimized for each antibody and kept the same across samples within each antibody. For each slide, three different areas of

papillae or villi were imaged (Laarman et al., 2013). For each image analysis, quantification of transporter abundance was done in the CellProfiler software version 3.1.9 using a pipeline for each tissue (McQuin et al., 2018). For the rumen, the corneal layer was identified and removed to prevent background noise. Nuclei were then identified to determine the approximate number of cells per image. Each papilla was isolated from the background and intensity of fluorescence was measured. For the jejunum, a villus was isolated from the background to determine the intensity of fluorescence and nuclei were identified to determine the approximate number of cells per image. For both tissues, the overall intensity was divided by the number of cells to achieve the average intensity per cell. Three images were used for each calf and the intensities were averaged to get the average intensity per calf for each protein. Values are reported as relative protein abundance in arbitrary units (AU).

2.2.6 Statistical Analysis

Statistical analysis was performed using R version 3.6.1 (R Core Team, 2019) with calf as the experimental unit. Least square means (LSM) was calculated using the emmeans package, and standard error of the mean (SEM) was calculated using the plotrix package. An ANOVA with a 2 × 2 factorial was used for all analysis except for gut permeability which used repeated measures in the rstatix package. Data was analyzed for normality using a Shapiro test, homogeneity of variances was tested using a Levene test, and homogeneity of covariances was tested using a Box's M-test. Unless otherwise noted, data was normally distributed, there was homogeneity of variances, and homogeneity of covariances. Significance was determined at a P value of < 0.05 and tendencies declared when P value was between 0.05-0.10. Unless noted otherwise, all values represented are LSM ± SEM. Correlation analysis was done using a Kendall correlation coefficient as data was not normally distributed, as determined by a Shapiro test. The

data for SCFA and rumen pH were obtained from Yohe et al. (2022b) and used for correlation analysis. The rumen pH parameters used were minimum pH, average pH, maximum pH, minutes per day under a pH of 6, minutes per day under a pH of 5.6, and pH magnitude per day. The correlation coefficients (r) were defined as 0.00 to 0.30 or 0.00 to -0.30 = negligible; 0.30 to 0.50 or -0.30 to -0.50 = low; 0.50 to 0.70 or -0.50 to -0.70 = moderate; 0.70 to 0.90 or -0.70 to -0.90 = high; and 0.90 to 1.00 or -0.90 to -1.00 = very high.

Statistical model used for ANOVA was:

$$Y_{ij} = \mu + M_i + S_j + (M \times S)_{ij} + \varepsilon_{ij}$$

Where Y_{ij} is the independent variable, μ is the overall mean, M_i is the effect of milk provision level, S_j is the effect of starch provision level, $(M \times S)_{ij}$ is the interaction between milk provision level and starch provision level, and ε_{ij} is the residual error.

Statistical model used for repeated measures was:

$$Y_{ijk} = \mu + M_i + S_j + W_k + (M \times S)_{ij} + (M \times S \times W)_{ijk} + \varepsilon_{ijk}$$

Where Y_{ij} is the independent variable, μ is the overall mean, M_i is the effect of milk provision level, S_j is the effect of starch provision level, W_k is the effect of week, $(M \times S)_{ij}$ is the interaction between milk provision level and starch provision level, $(M \times S \times W)_{ijk}$ is the interaction between milk, starch and week, and ε_{ij} is the residual error.

2.3 Results

2.3.1 Gut Permeability

All three permeability markers (Cr-EDTA, Lactulose, and Mannitol) increased the permeability index in calves provided with more milk (P = 0.04, P < 0.01, and P < 0.01, respectively; Figure 2.9). Postweaning calves (week 8) had a significantly lower permeability

index compared to pre-weaning (week 4) for Cr-EDTA and lactulose markers ($P = 0.03$ and $P < 0.01$, respectively), but not mannitol ($P = 0.11$). None of the permeability markers were affected by starch concentration in the diet ($P = 0.42$, 0.87, 0.46, respectively), the milk × starch interaction ($P = 0.81$, 0.16, 0.22, respectively), or the milk × starch × week interaction ($P = 0.46$, 0.14, 0.89, respectively).

2.3.2 Gut Histology

Subjective papillae development, was not different between treatments for milk ($P = 0.12$; Table 2.3), starch ($P = 0.79$) or milk × starch interaction ($P = 0.64$). For epithelial sloughing in the rumen, there was a significant decrease in the amount of sloughing for higher starch diets ($P < 0.01$). However, there was no difference in treatments for milk ($P = 0.52$) or milk × starch interaction ($P = 0.34$).

In the duodenum, there was no effect of milk, starch, or milk × starch interaction on goblet cell loss ($P = 0.61$, 0.76, 0.29), villous blunting ($P = 0.61$, 0.33, 0.21), or epithelial cell separation ($P = 0.40$, 0.60, 0.57). In the jejunum, there was no effect of milk or starch treatments on goblet cell loss ($P = 0.98$, 0.24) or epithelial separation ($P = 0.51$, 0.19). Jejunum goblet cell loss was affected by milk × starch interaction ($P = 0.02$), but villus blunting ($P = 0.29$) and epithelial separation were not ($P = 0.60$). Jejunum villous blunting tended to increase with higher concentrations of starch ($P = 0.07$) but was not significant for milk treatments ($P = 0.31$). In the ileum, milk, starch, and milk × starch treatments did not affect goblet cell loss ($P = 0.37$, 0.20, 0.64) or villous blunting ($P = 0.66$, 0.51, 0.41). Ileal epithelial separation was not affected by milk treatments or milk × starch interactions ($P = 0.80$, 0.25), but tended to increase with high starch treatments ($P = 0.08$). In the colon, milk, starch, and milk × starch treatments did not affect crypt development ($P = 0.12$, 0.41, 0.46) or goblet cell loss ($P = 0.94$, 0.29, 0.64).

2.3.3 qPCR

For the genes analyzed in the rumen, expression of *ACAT* was higher in calves provided with more milk ($P = 0.02$; Table 2.4) but there was no difference due to starch treatment ($P = 0.80$) or the milk × starch interaction ($P = 0.69$). The expression for the remaining three genes (*BHBD1*, *BHBD2*, and *HMGCL*) showed no difference for either milk treatment ($P = 0.98$, 0.72, 0.37, respectively), starch treatment ($P = 0.98$, 0.49, 0.73, respectively), or milk × starch interaction ($P = 0.80$, 0.24, 0.77, respectively).

For the genes analyzed in the jejunum, expression of *MGAM* tended to be higher in the high starch concentration treatments ($P = 0.07$) but there was no difference due to milk treatment ($P = 0.76$) or the milk × starch interaction ($P = 0.33$). The expression for the remaining four genes (*IM*, *KHK*, *LAC*, and *PA*) showed no difference for either milk treatment ($P = 0.99$, 0.44, 0.66, 0.15, respectively), starch treatment ($P = 0.72$, 0.21, 0.23, 0.59, respectively), or milk × starch interaction ($P = 0.38$, 0.49, 0.19, 0.80, respectively).

2.3.4 Immunofluorescence

For the proteins analyzed in the rumen, AE2 tended to increase in calves fed higher concentrations of starch ($P = 0.09$; Figure 2.10) and MCT1 showed a significant increase in calves fed higher concentrations of starch ($P = 0.02$). For both AE2 and MCT1, there was no effect of the milk treatment ($P = 0.73$, 0.86, respectively) or the milk × starch interaction ($P = 0.28$, 0.79, respectively). The other two proteins (DRA and PAT1) showed no difference in abundance for either milk treatment ($P = 0.96$, 0.14, respectively), starch treatment ($P = 0.88$, 0.24, respectively), or milk × starch interaction ($P = 0.67$, 0.55, respectively).

For the proteins analyzed in the jejunum, SGLT1 had a significant increase in abundance for calves provided with more milk ($P < 0.01$; Figure 2.11) but there was no difference due to

starch treatment ($P = 0.86$) or the milk × starch interaction ($P = 0.34$). For GLUT2, there was no difference in abundance for either milk treatment ($P = 0.28$), starch treatment ($P = 0.23$), or milk × starch interaction ($P = 0.46$).

2.3.5 Correlation

In the rumen, there was a positive correlation between ruminal butyrate and PAT1 abundance (Table 2.5) but a negative correlation between ruminal butyrate and AE2 abundance. An increase in rumen pH had a positive correlation with MCT1 and PAT1 abundance. There were also negative correlations between *ACAT* and *HMGCL* mRNA abundance and total ruminal SCFA, ruminal acetate, and ruminal butyrate (Table 2.6).

2.4 Discussion

The overall objective of this study was to look at the physiological development of the rumen and other organs in the digestive tract to assess the digestion, absorption, and metabolism capacity of the energy source found in calves fed diets varying in milk provision and starch levels. Additionally, we wanted to evaluate epithelial cell structure and function as a measure of nutrient absorption capacity and development in calves fed different levels of milk and starch. We hypothesized that calves with higher milk provision and higher starch content in the starter would have the greatest nutrient absorption capacity, ketogenic and amylolytic enzyme expression as they were fed the highest concentration of nutrients. Increased milk feeding increased rumen papillae size (Yohe et al., 2022a), but not sloughing, papillae development, or SCFA transporter abundance. Increased milk feeding also increased jejunal glucose transporter abundance as well as gut permeability. Increased starch concentrations increased SCFA transporter abundance and

decreasing papillae sloughing (indicative of damage); but, in the small intestine, high starch groups tended to show increased villi blunting and epithelial separation (indicative of damage).

2.4.1 Ruminal Response to Milk Provision and Starter Starch Content

It is possible that a temporary increase in milk provision could benefit the calves by increasing pre-weaning performance (ADG and BW) without sacrificing post-weaning rumen development and weight gains. Our study showed that none of the transporters (MCT1, AE2, DRA, and PAT1) were impacted by milk volume. One study found that MCT1 protein and mRNA abundance increased in calves fed whole milk compared to MR (Flaga et al., 2015). This increase could indicate that changes in MCT1 abundance are impacted by whole milk rather than milk volume, as our study showed no difference in protein abundance between milk treatments. Overall, our study found no differences in postweaning physiological rumen development (histology, nutrient transporter abundance, and enzyme expression) regardless of amount of milk provided to calves. Additionally, a companion study found that calves fed higher volumes of milk had tendencies towards increased final BW and ADG, without any differences in small intestine measurements (Yohe et al., 2022a). This lack of differences suggests that calves can be fed higher amounts of milk to benefit their early pre-weaning performance without sacrificing rumen or intestinal development, which is in agreement with other studies (Schaff et al., 2018; Koch et al., 2019; Tummler et al., 2020).

Instead of increased starch leading to low rumen pH, which causes harm to the rumen, there may be a protective effect of increased fermentation. In the companion study, average rumen pH ranged from 5.7-5.9 and spent between 6-10 hours per day under a pH of 5.6 post-weaning across treatments (Yohe et al., 2022b). Rumen pH was positively correlated to MCT1 and PAT1 abundance; further, MCT1 increased and AE2 tended to increase in high starch treatments. The

increase in both MCT1 and AE2 may be helping to buffer the rumen, as MCT1 transports H^+ out of the epithelium and AE2 transports bicarbonate into the rumen lumen. Additionally, in high starch calves there were decreases in papillae sloughing, indicating less damage, which is the opposite effect seen in adult cows (Steele et al., 2011; Liu et al., 2013; Sun et al., 2018b). In the companion study, there were no differences seen in rumen papillae measurements between the starch treatments (Yohe et al., 2022a). Due to the wide range of morphological development across the GIT (Figure 2.8), scoring images better captured the variation among samples than epithelial measurements. In the rumen, papillae width and length vary tremendously during the weaning transition, which can lead to discrepancies when using measurements (Steele et al., 2014). Additionally, scoring assesses the degree of various factors that may otherwise be missed by measurement analysis methods such as inflammation, architectural distortion (i.e. villous blunting), and epithelial integrity (Brown et al., 2005; Steele et al., 2015; Mosli et al., 2017).

Our results agree with other work showing that there is no correlation between low rumen pH and starter intake, papillae size, corneum thickness, or acute phase proteins (Kristensen et al., 2007; van Niekerk et al., 2021). While there were no differences in total SCFA concentrations or rumen pH (Yohe et al., 2022b), it is unclear if the lack of difference is due to similar fermentation between the diets or if more SCFA are being transported out in the high starch diets. We suspect that more SCFA are being transported out, as there were increases in MCT1 protein abundance and tendencies for increases in AE2 protein abundance. However, this was a potential for increased transport as nutrient flux was not measured in this study. If SCFA transport is able to increase enough to remove SCFA and buffer rumen pH, high starch calf starters may confer a protective effect on the rumen epithelium. Other studies agree, as calves with a lower pH have increased intake, ADG, and the ability to regulate pH (McCurdy et al., 2019; Hiltz et al., 2021) and increased

SCFA transport increases resistance to SARA (Penner et al., 2009). Together, this means that high starch calf starters are able to provide morphological and SCFA transport capacity benefits without causing morphological damage to the rumen epithelium in calves.

In regards to starch content and nutrient transport, MCT1 and AE2 protein abundance are increased by higher starch concentrations in the diet, but we saw no change in protein abundance for DRA or PAT1. Few papers have looked at the impacts of starter starch content and nutrient transporter abundance in calves. The closest comparisons are calves (Laarman et al., 2012a; Yohe et al., 2019; Hiltz et al., 2021) or lambs (Sun et al., 2018a) fed either milk only or milk and starter. In lambs fed only milk MCT1 and PAT1 mRNA abundance tended to be higher compared to lambs fed milk and starter (Sun et al., 2018a). In contrast, calves fed milk and starter had increases in MCT1 mRNA compared to calves fed only milk (Laarman et al., 2012a; Yohe et al., 2019); however, there was no difference in MCT1 protein abundance (Yohe et al., 2019; Hiltz et al., 2021). Our study disagrees with those above, as we saw increases in MCT1 protein abundance in calves fed higher amounts of starch. These differences could be due to changes in species, diet composition, or SCFA concentrations, as ruminants in the above studies saw increases in total SCFA concentrations for animals fed milk and starter (Laarman et al., 2012a; Sun et al., 2018a; Hiltz et al., 2021). As neither DRA nor PAT1 were impacted by either starch or milk treatment, this indicates DRA and PAT1 may not be influenced by diet. Other studies agree, as ruminants fed either milk or milk and starter had no differences in DRA or PAT1 mRNA abundance (Laarman et al., 2012a; Sun et al., 2018a).

2.4.2 Small Intestinal Response to Milk Provision and Starter Starch Content

2.4.2.1 Gut Permeability in Growing Calves

High milk provision was linked to increased gut permeability, in agreement with other studies have shown increases in permeability (Moeser et al., 2007b; Wilms et al., 2019; Welboren et al., 2021). Although the exact linkage between milk provision and gut permeability is unclear, the phenomenon could be caused by impact of stress, luminal osmolality, physiological dilation of barrier function, or some combination of the above.

The first possibility of increased gut permeability by high milk provision is the result of a stress response. Weaning can cause stress responses such as increased vocalizations, abnormal oral behaviors, increased cortisol, and increased permeability (Moeser et al., 2007b; Jasper et al., 2008; Loberg et al., 2008). The high milk calves were provided with more milk, and therefore their weaning transition represents a greater decrease in milk consumption than calves on a low milk provision. An increase in stress response between week 7 and week 8 was reflected in overall increases in haptoglobin (Yohe et al., 2022b). More milk consumption and subsequent lactose digestion would increase glucose concentrations in the lumen, and, in turn, increase glucose transport (Zheng et al., 2012; Pavic et al., 2020). Simultaneously, haptoglobin was either highly variable (Yohe et al., 2022b) or stable (van Niekerk et al., 2021) over weeks of time, and serum amyloid A (SAA) was no different with increased milk provision (Yohe et al., 2022b). While SAA increases the week after weaning (van Niekerk et al., 2021; Yohe et al., 2022b), permeability in our study decreased. If the increased permeability were a stress response, it should have increased with the inflammatory markers. Together, the increased gut permeability in high milk-fed calves

without concomitant inflammatory response in the markers analyzed or increase in permeability after weaning suggests a stress response is unlikely to be the sole factor leading to gut permeability.

Aside from stress response, another possible explanation for increased gut permeability is the impact of increased luminal osmolality on epithelial cell function. When replacing lactose with glucose (to increase osmolality), gut permeability increased with higher levels of glucose (Wilms et al., 2019). When MR lactose was partially replaced with fat, Welboren et al. (2021) found that digesta osmolality did not change, but permeability increased. Our calves were fed the same MR in different volumes with the same nutrient concentrations. Since recent studies failed to find a direct link between osmolality and gut permeability, and our study used the same MR formulation, it appears unlikely osmolality would contribute to the differences in gut permeability seen in this study.

The third possibility for the increase in gut permeability is a dilation of barrier tight junctions to acutely increase intestinal glucose uptake beyond SGLT1 transporter capabilities. Increased gut permeability through SGLT1 activation occurs when feeding liquid feed but not dry feed (Turner and Madara, 1995). Post-prandial glucose concentrations typically exceed 25 m*M*, the highest capacity of SGLT1 (Zheng et al., 2012; Pavic et al., 2020). When stimulated, SGLT1 activates myosin light chain kinase (MLCK; Turner and Madara, 1995; Turner et al., 1997; Camilleri, 2019), dissociating tight junction proteins from the cytoskeleton. As a result, tight junctions dilate and increase paracellular permeability (Harhaj and Antonetti, 2004; Chelakkot et al., 2018; Vanuytsel et al., 2021). This tight junction dilation increases net clearance of small radius molecules such as mannitol (radius = 3.6 Å), but not inulin (radius = 11.5 Å) or lactulose (radius = 9.5 Å) from the small intestine lumen (Liang and Weber, 2014; Hollander and Kaunitz, 2020). Since a glucose molecule has a radius of 5 Å (Maroudas, 1970), it would be able to pass through

such a dilated tight junction. As permeability is not transporter mediated, it should be clear that SGLT1 is not permeability. Instead, SGLT1 may be a component of a mechanism that effects the regulation of permeability. If dilation were occurring, we would expect no changes in tight junction protein abundance as no damage is being done. When dilated, tight junctions are not being broken down, as they are when increased permeability is associated with disease (Gunzel and Yu, 2013; Hu et al., 2013). Permeability decreased after weaning, lending support to increasing permeability to increase nutrient flux during the milk feeding period. However, measurement of tight junction protein abundance and glucose kinetics would be required to differentiate between the causes of increased permeability. Since pre-weaned calves obtain glucose from their liquid feed supply, tight junction dilation may explain why calves fed a higher amount of milk increased SGLT1 transporter abundance and gut permeability.

While gut permeability is often worrisome, it is not a direct cause of disease (Odenwald and Turner, 2013; Camilleri, 2019). Although restoration of barrier function will improve gastrointestinal disease recovery, barrier function itself is not directly linked to disease (Camilleri, 2019). Consequently, tight junction dilation may allow a pre-weaned calf to increase post-prandial glucose uptake. Indeed, a pre-weaned calf may prioritize increased glucose uptake at the expense of increased maintenance cost (Welboren et al., 2021). As a result, increased gut permeability in calves may not be a pathological result that needs to be managed but is instead an adaptive response to increase energy uptake in growing calves with a high milk provision.

2.4.2.2 Starch Content and Lower Gut Health

Higher starch diets were beneficial in the rumen but were detrimental in the small intestine. When dietary starch increases, it can lead to an increase in rumen passage rate (Moharrery et al., 2014; Liu et al., 2019), which means rumen digestibility decreases and starch flow to the small

intestine increases. Our study agreed as increased starch in the diet increased *MGAM* mRNA abundance, which could indicate more starch is entering the small intestine. Small intestine morphometric measurements and luminal pH were unaffected by milk provision and starch content (Yohe et al., 2022a), in partial agreement with other studies showing tendencies to decrease ileal villus width and crypt depth or showing no differences (Wang et al., 2009; Zhang et al., 2018). Contrary to morphometrics, histological scoring showed high starch starters tended to increase small intestine villous blunting and epithelial separation, both signs of damage or disease in both humans (Serra and Jani, 2006; Kamboj and Oxentenko, 2017), and cows and calves (Baines et al., 2008; Foster and Smith, 2009). Damage to the small intestine may not be coming from the starch entering or being broken down in the small intestine, but instead from the increased rumen fermentation.

The effects of SCFA concentration and rumen pH on the small intestine and colon are conflicting. Some studies have found that when high starch diets lower the rumen pH, the colon pH will lower as well, but the small intestine pH will remain unaffected (Wang et al., 2009; Pederzolli et al., 2018; Liu et al., 2019). It has also been found that when the rumen pH decreases, the small intestine pH will also decrease (Asadollahi et al., 2018). Differences between studies may be due to age of the animals, stage of production, or diet composition. Our study agrees with others (Asadollahi et al., 2018; Pederzolli et al., 2018) that high starch may cause damage to the small intestine, but it does not cause an increase in permeability, indicating that lower gut morphology is linked to rumen pH, not gut permeability. In other studies on calves and goats, increased starch content decreased rumen pH to 5.5-6.0 (Wang et al., 2009; Zhang et al., 2018), which may not be low enough to induce small intestine morphological changes. Potentially,

acidotic levels of rumen pH could lead to downstream morphological damage in the small intestine and colon, despite decreased papillae sloughing increased SCFA transport capacity in the rumen.

A potential concern with increasing starch content is hindgut acidosis. Hindgut acidosis is triggered by a low pH and presence of LPS, usually following SARA (Gressley et al., 2011; Sanz-Fernandez et al., 2020). As dietary starch increases, rumen passage rate and intestinal starch flow increase (Gressley et al., 2011; Sanz-Fernandez et al., 2020), which can hinder intestinal digestion and lead to hindgut acidosis. The colon is more sensitive to pH changes compared to the rumen because of the lack of saliva and having a single-layered epithelium, even though the colon has a protective mucus layer that the rumen lacks (Gressley et al., 2011). Common symptoms associated with hindgut acidosis are an immune response, mucosal damage (seen in the feces) and increased permeability (Sanz-Fernandez et al., 2020). As we saw no increases in gut permeability and no morphological damage to the colon with high starch diets, it is unlikely that hindgut acidosis occurred in our study. A combination of microbiota changes, increase in LPS, increase in SCFA, and decrease in pH may be required to damage the small intestine epithelium and trigger an immune response (Liu et al., 2019). However, inconsistent results from this and other studies indicate that the impact of rumen pH on small intestine morphology remains unclear.

2.5 Conclusion

Calves fed high amounts of milk have increased gut permeability and increased small intestine SGLT1 abundance, without impairing rumen epithelial development, sloughing, and transport capacity. Calves fed high concentrations of starch have increased ruminal SCFA transporter abundance and increased rumen papillae development. However, decreased rumen pH, independent of diet, is associated with morphological damage in the small intestine.

Increasing the pre-weaning plane of nutrition and starch content for calves can lead to increased GIT development and nutrient transport for calves postweaning.

2.6 Tables

Table 2.1 List of primers for the targeted genes in this study.

Gene Symbol	Gene Name	Accession Number	Tissue	Primer Sequence	Amplification Efficiency (%)	Amplicon Length	Tm
ACAT	*Acetoacetyl-CoA thiolase*	NM_001046075.1	Rumen	Forward: CATATGCTGTTCCTAAGGTTCT Reverse: ACAGCTCCTCCGTTGATATT	121.98	154	60
BHBD1	*B-hydroxybutyrate dehydrogenase 1*	NM_001034600.2	Rumen	Forward: CCAGTTTGTTTTGGGAAC Reverse: AGCGAGCCTCTTGTC	91.32	151	56.8
BHBD2	*B-hydroxybutyrate dehydrogenase 2*	NM_001034488.2	Rumen	Forward: GCTGCTCTAAAATGTGACG Reverse: GATGATCAAATGAGGCTGT	103.01	132	58.7
HMGCL	*3-hydroxy-3-methylglutaryl-CoA Lyase*	NM_001075132.1	Rumen	Forward: GAAAATTGTGGAAGTTGGTC Reverse: ATGGTCAGCCATCTGTG	102.33	156	58.8
IM	*Isomaltase*	NM_001114189.2	Jejunum	Forward: ACATCAACAAATGCTGGAGT Reverse: CTGACTTTGAGTTGTGAGGA	100.03	102	59.3
KHK	*Keto-hexokinase*	NM_001076351	Jejunum	Forward: TGATGGACAAATACCCAGA Reverse: TCGTCCAGGACAAAATCAG	101.47	169	60.6
LAC	*Lactase*	XM_592166	Jejunum	Forward: AGTTCCAATCTACCTAGCTG Reverse: GAGTCTTCTTTGATAGCCTTG	104.32	129	56.8
MGAM	*Maltase-glucoamylase*	XM_024991197.1	Jejunum	Forward: GATGGGGAAACAAAAGATAC Reverse: ATGCTAAATTATTGGGGTCT	108.94	124	57.6
PA	*Pancreatic amylase*	NM_001035016.1	Jejunum	Forward: AATGATGACTGGGCGTTAT Reverse: TTACCATCACAAGAAACATTG	122.44	131	59
B-ACT	*B-Actin*[1]	NM_173979.3	Both	Forward: CTCTTCCAGCCTTCCTTCCT Reverse: TAGAGGTCCTTGCGGATGTC	102.61 (Rumen), 100.48 (Jejunum)	101	64.4
RPLP0	*Ribosomal Protein Lateral Stalk Subunit P0*[1]	NM_001012682.1	Both	Forward: CAACCCTGAAGTGCTTGACAT Reverse: AGGCAGATGGATCAGCCA	122.01 (Rumen), 118.76 (Jejunum)	227	64.8

[1]Used as a reference gene in this manuscript

Table 2.2 Antibody information for proteins used in immunofluorescence.

Protein	Tissue	Primary Antibody Dilution	Secondary Antibody Dilution	Company
Anion exchanger 2 (AE2)	Rumen	50	100	Bioss[1]
Downregulated in adenoma (DRA)	Rumen	100	50	Signalway Antibody[2]
Facilitated glucose transporter 2 (GLUT2)	Jejunum	50	100	Bioss
Monocarboxylate co-transporter 1 (MCT1)	Rumen	100	100	Bioss
Putative anion transporter 1 (PAT1)	Rumen	100	50	Bioss
Sodium-dependent glucose transporter 1 (SGLT1)	Jejunum	50	100	Bioss

[1]Bioss: Woburn, MA, USA

[2]Signalway Antibody: Greenbelt, MD, USA

Table 2.3 Tissue scoring based on H&E staining between treatments. Calves fed low or high amounts of milk (600 g/d or 1,200 g/d, respectively) and ad libitum starter with low or high amounts of starch (12% starch, DM or 35.6% starch, DM, respectively). Treatments were low milk and low starch (LL), low milk and high starch (LH), high milk and low starch (HL), and high milk and high starch (HH).

Parameter[1]	Treatment[2]				*P*-Value		
	LL	LH	HL	HH	Milk[3]	Starch[4]	Milk×Starch[5]
Rumen							
Papillae Development	2.89 ± 0.25	2.73 ± 0.23	2.52 ± 0.17	2.56 ± 0.19	0.21	0.79	0.64
Epithelial Sloughing	3.61 ± 0.23	2.91 ± 0.18	3.31 ± 0.18	2.96 ± 0.14	0.52	< 0.01	0.34
Duodenum							
Goblet Cell Loss	2.97 ± 0.24	2.78 ± 0.28	2.75 ± 0.17	2.92 ± 0.30	0.61	0.76	0.29
Villous Blunting	3.52 ± 0.19	3.12 ± 0.17	3.38 ± 0.15	3.44 ± 0.19	0.61	0.33	0.21
Epithelial Separation	2.15 ± 0.11	2.14 ± 0.18	1.91 ± 0.21	2.09 ± 0.17	0.40	0.60	0.57
Jejunum							
Goblet Cell Loss	3.73 ± 0.25	3.42 ± 0.22	3.16 ± 0.29	4.00 ± 0.10	0.98	0.24	0.02
Villous Blunting	3.10 ± 0.21	3.26 ± 0.22	3.09 ± 0.21	3.70 ± 0.19	0.31	0.07	0.29
Epithelial Separation	2.04 ± 0.25	2.21 ± 0.19	1.77 ± 0.13	2.18 ± 0.29	0.51	0.19	0.60
Ileum							
Goblet Cell Loss	3.97 ± 0.19	3.79 ± 0.27	3.88 ± 0.15	3.48 ± 0.27	0.37	0.20	0.64
Villous Blunting	3.40 ± 0.20	3.37 ± 0.15	3.19 ± 0.14	3.44 ± 0.16	0.66	0.51	0.41
Epithelial Separation	2.33 ± 0.23	2.45 ± 0.22	2.02 ± 0.23	2.65 ± 0.17	0.80	0.08	0.25
Colon							
Crypt Development	3.08 ± 0.14	3.37 ± 0.18	2.92 ± 0.23	2.93 ± 0.19	0.12	0.41	0.46
Goblet Cell Loss	2.48 ± 0.32	2.31 ± 0.28	2.59 ± 0.28	2.15 ± 0.27	0.94	0.29	0.64

[1] Parameters are scored on a scale of 1-5, with 1 being ideal and 5 being furthest from ideal for each category.

[2] Values shown are least square means (LSM) ± standard error of the means (SEM).
[3] Effects of milk (low or high) on tissue scoring.
[4] Effects of starch (low or high) on tissue scoring.
[5] Effects of milk and starch interaction on tissue scoring.

Table 2.4 Fold change in mRNA abundance in calves fed low or high amounts of milk (600 g/d or 1,200 g/d, respectively) and ad libitum starter with low or high amounts of starch (12% starch, DM or 35.6% starch, DM, respectively). Treatments were low milk and low starch (LL), low milk and high starch (LH), high milk and low starch (HL), and high milk and high starch (HH).

Gene[1]	Treatment[2]				*P*-Value		
	LL	LH	HL	HH	Milk[3]	Starch[4]	Milk×Starch[5]
Rumen							
ACAT	2.93 ± 1.04	2.08 ± 0.67	5.41 ± 1.71	5.56 ± 1.25	0.02	0.80	0.69
BHBD1	0.65 ± 0.13	0.70 ± 0.18	0.70 ± 0.12	0.66 ± 0.17	0.98	0.98	0.80
BHBD2	4.97 ± 1.85	10.90 ± 4.59	9.95 ± 2.30	8.73 ± 2.10	0.72	0.49	0.24
HMGCL	1.50 ± 0.63	1.97 ± 0.71	2.34 ± 0.67	2.40 ± 0.76	0.37	0.73	0.77
Jejunum							
IM	10.52 ± 5.88	8.08 ± 4.69	6.55 ± 2.89	12.34 ± 4.36	0.99	0.72	0.38
KHK	1.49 ± 0.64	0.61 ± 0.12	0.93 ± 0.19	0.60 ± 0.11	0.44	0.21	0.49
LAC	2.62 ± 1.00	9.73 ± 4.73	4.78 ± 2.93	4.48 ± 1.61	0.66	0.23	0.19
MGAM	1.95 ± 0.79	12.25 ± 5.30	6.12 ± 2.79	9.42 ± 4.48	0.76	0.07	0.33
PA	11.80 ± 6.76	14.00 ± 3.38	21.80 ± 9.53	28.30 ± 10.20	0.15	0.59	0.80

[1] *ACAT = acetyl-CoA thiolase*; *BHBD1 = ß-hydroxybutyrate dehydrogenase 1*; *BHBD2 = ß-hydroxybutyrate dehydrogenase 2*; *HMGCL = 3-hydroxy-3-methylglutaryl-CoA lyase*; *IM = isomaltase*; *KHK = keto-hexokinase*; *LAC = lactase*; *MGAM = maltase-glucoamylase*; *PA = pancreatic amylase*

[2] Values shown are least square means (LSM) ± standard error of the means (SEM).

[3] Effects of milk (low or high) on tissue scoring.

[4] Effects of starch (low or high) on tissue scoring.

[5] Effects of milk and starch interaction on tissue scoring.

Table 2.5 Kendall correlation coefficients[1] between total rumen bolus pH measurements from week 8 and protein abundance determined by immunofluorescence.

Variables[2]	AE2	DRA	MCT1	PAT1
Rumen				
Total SCFA	.	.	.	.
Acetate	.	.	.	.
Propionate	.	.	.	.
Butyrate	-0.25	.	.	0.40
Minimum pH	.	.	0.31	.
Average pH	.	.	0.33	.
Maximum pH	.	.	0.32	0.25
Mins <6[3]	.	.	-0.33	.
Mins <5.6[4]	.	.	.	.
Magnitude[5]	.	.	.	0.36

[1] Cell color and symbol denotes significance. *Green cells are significant ($P \leq 0.05$) and †yellow cells are a tendency ($P = 0.10$-0.05). A negative sign (-) indicates a negative correlation, whereas no sign indicates a positive correlation.

[2] Column abbreviations: "AE2" = Anion exchanger 2 protein abundance, "DRA" = Downregulated in adenoma protein abundance, "MCT1" = Monocarboxylate co-transporter 1 protein abundance, "PAT1" = Putative anion transporter 1 protein abundance, "GLUT2" = Glucose transporter 2 protein abundance, "SGLT1" = Sodium-dependent glucose transporter 1 protein abundance.

[3] "Mins <6" = Minutes per day under a pH of 6

[4] "Mins <5.6" = Minutes per day under a pH of 5.6

[5] "Magnitude" = pH magnitude per day

Table 2.6 Kendall correlation coefficients[1] between total rumen bolus pH measurements from week 8 and rumen mRNA abundance and tissue scoring parameters.

					Rumen[2]	
Variables[3]	***ACAT***	***BHBD1***	***BHBD2***	***HMGCL***	**Papillae**	**Sloughing**
Rumen						
Total SCFA	-0.44	.	.	-0.41	.	.
Acetate	-0.54	.	.	-0.48	.	.
Propionate	.	.	.	.	.	.
Butyrate	-0.34	.	.	-0.29	.	.
Minimum pH	.	.	.	.	.	.
Average pH	.	.	.	.	.	.
Maximum pH	.	.	.	.	.	.
Mins <6[4]	.	.	.	.	.	.
Mins <5.6[5]	.	.	.	.	.	.
Magnitude[6]	.	.	.	.	.	.

[1] Cell color and symbol denotes significance. *Green cells are significant ($P \leq 0.05$) and †yellow cells are a tendency ($P = 0.10$-0.05). A negative sign (-) indicates a negative correlation, whereas no sign indicates a positive correlation.

[2] Rumen abbreviations: "Papillae" = Papillae development, "Sloughing" = Epithelial sloughing

[3] Column abbreviations: "*ACAT*" = *Acetoacetyl-CoA thiolase* mRNA abundance, "*BHBD1*" = *ß-hydroxybutyrate dehydrogenase 1* mRNA abundance, "*BHBD2*" = *ß-hydroxybutyrate dehydrogenase 2* mRNA abundance, "*HMGCL*" = *3-hydroxy-3-methylglutaryl-CoA Lyase* mRNA abundance, "*IM*" = *Isomaltase* mRNA abundance, "*LAC*" = *Lactase* mRNA abundance, "*MGAM*" = *Maltase-glucoamylase* mRNA abundance, "*PA*" = *Pancreatic abundance*.

[4] "Mins <6" = Minutes per day under a pH of 6

[5] "Mins <5.6" = Minutes per day under a pH of 5.6

[6] "Magnitude" = pH magnitude per day

2.7 Figures

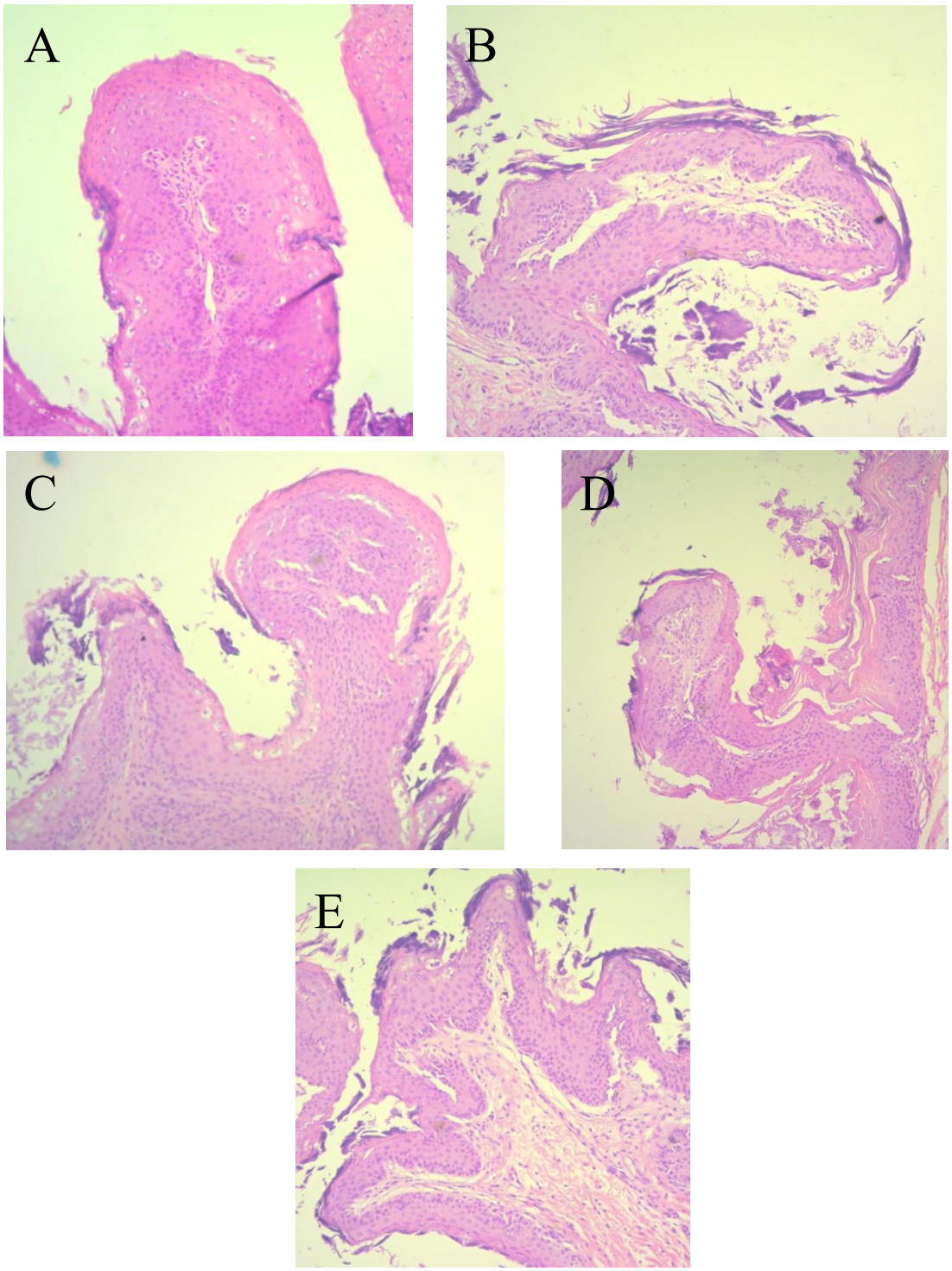

Figure 2.1 Tissue histology for the rumen papillae development scoring range. A score of 1 (A) indicates all or almost all papillae in the image were single, uniform, and finger-like in shape. A score of 2 (B) indicates most papillae are uniform but may show minor irregularities in shape. A

score of 3 (C) indicates some of the papillae are uniform but are more irregular in shape than a 2 score. A score of 4 (D) indicates few papillae are uniform and most papillae in the image are severely irregular in shape compared to a score of 3. A score of 5 (E) indicates very few or almost no uniform shapes were present and all or almost all papillae in the image were very branched and irregular.

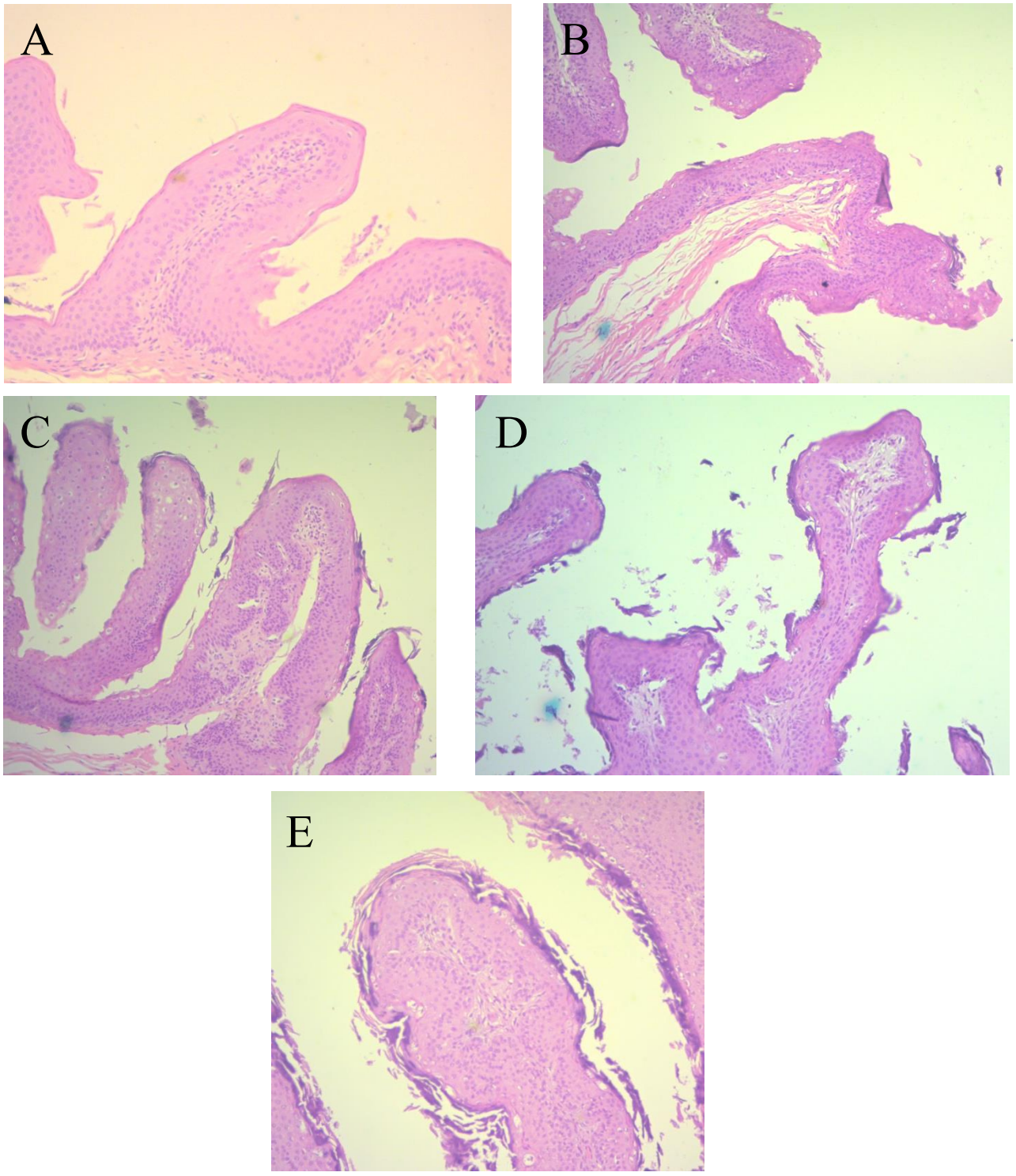

Figure 2.2 Tissue histology for the rumen papillae sloughing scoring range. A score of 1 (A) indicates the all or almost all of the corneal layer is intact with no separation from the papillae. A score of 2 (B) indicates that most of the corneal layer is intact with minimal separation from the

papillae. A score of 3 (C) indicates some of the corneal layer is intact, with more separation than a score of 2. A score of 4 (D) indicates little of the corneal layer is intact, with more separation than a score of 3. A score of 5 (E) indicates none or very little of the corneal layer remains intact with the papillae, with severe separation.

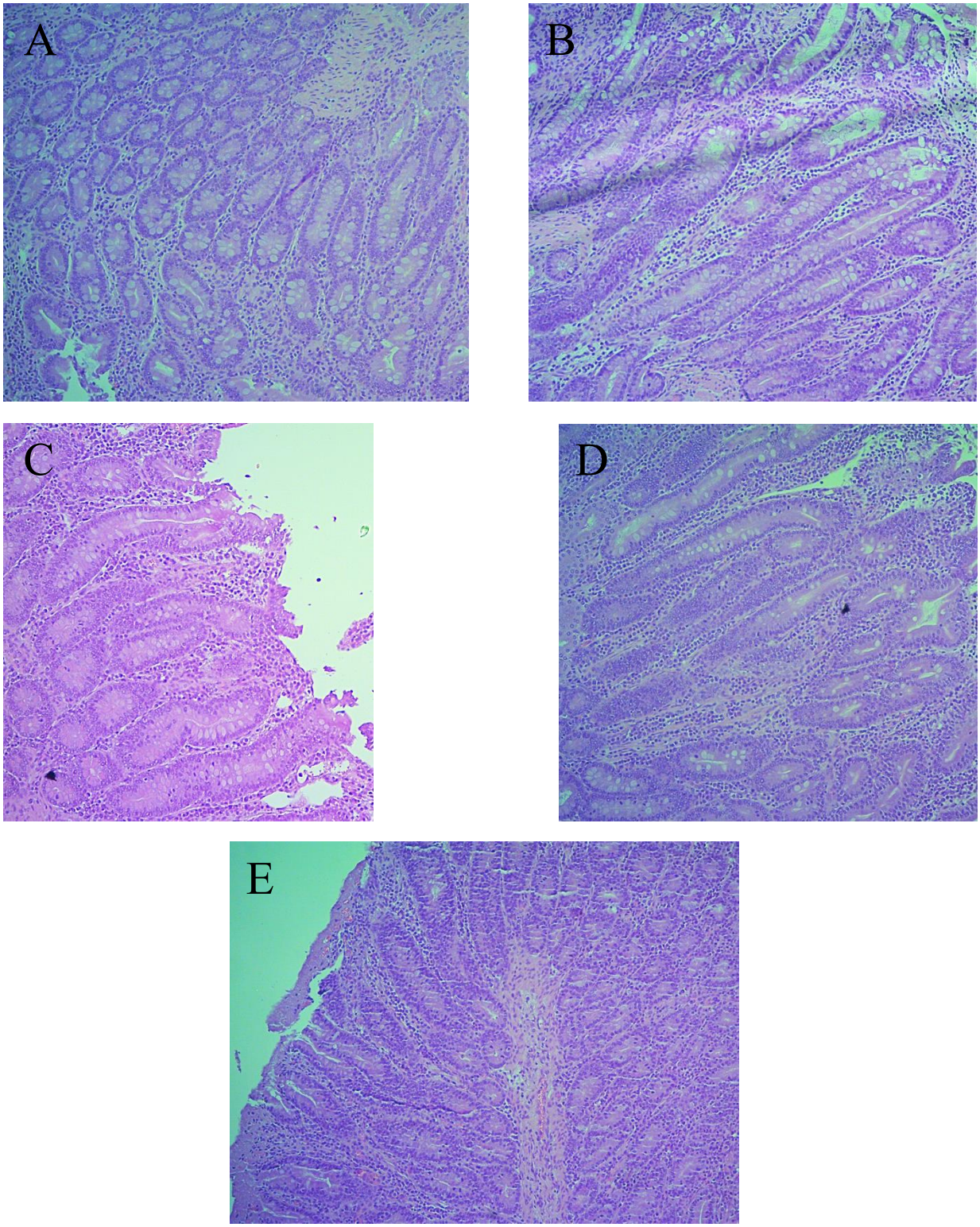

Figure 2.3 Tissue histology for the small intestine (duodenum, jejunum, and ileum) goblet cell loss scoring range. A score of 1 (A) indicates all or almost all of the villi present contain goblet cells in great number. A score of 2 (B) indicates most of the villi present contain many goblet

cells, with less goblet cells present than a score of 1. A score of 3 (C) indicates some of the villi present contain goblet cells, with less goblet cells present than a score of 2. A score of 4 (D) indicates few of the villi present contain few goblet cells, with less goblet cells present than a score of 3. A score of 5 (E) indicates none or very few of the villi present contain very few goblet cells, with less goblet cells present than a score of 4.

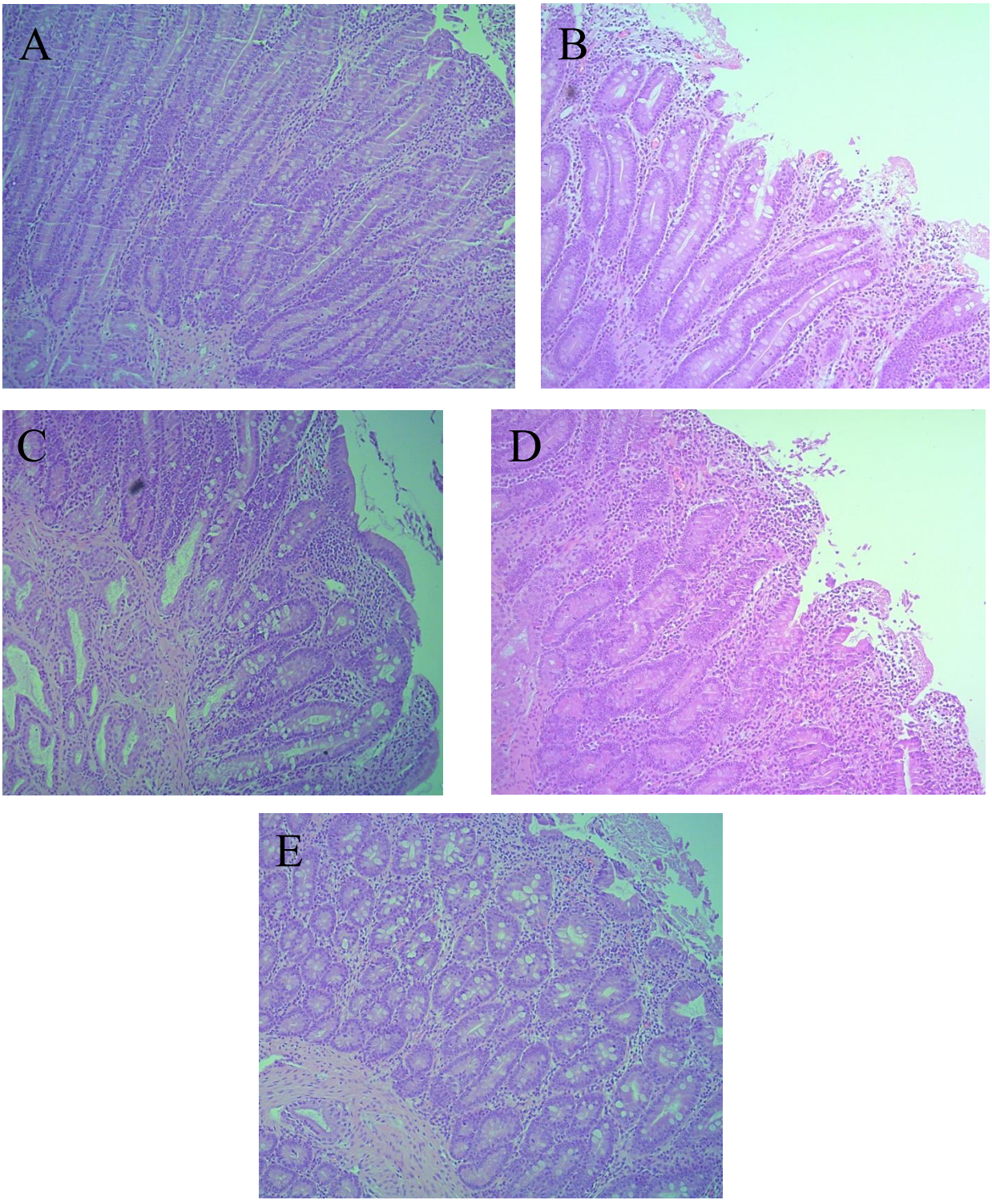

Figure 2.4 Tissue histology for the small intestine (duodenum, jejunum, and ileum) villous blunting scoring range. A score of 1 (A) indicates all or almost all of the villi present are elongated and uniform in shape. A score of 2 (B) indicates most of the villi present are

elongated, with more variation than a score of 1. A score of 3 (C) indicates some of the villi present are elongated, with more variation than a score of 2. A score of 4 (D) indicates few of the villi present are elongated, with more variation than a score of 3. A score of 5 (E) indicates none or very few of the villi present are elongated, with most of the villi being short and round.

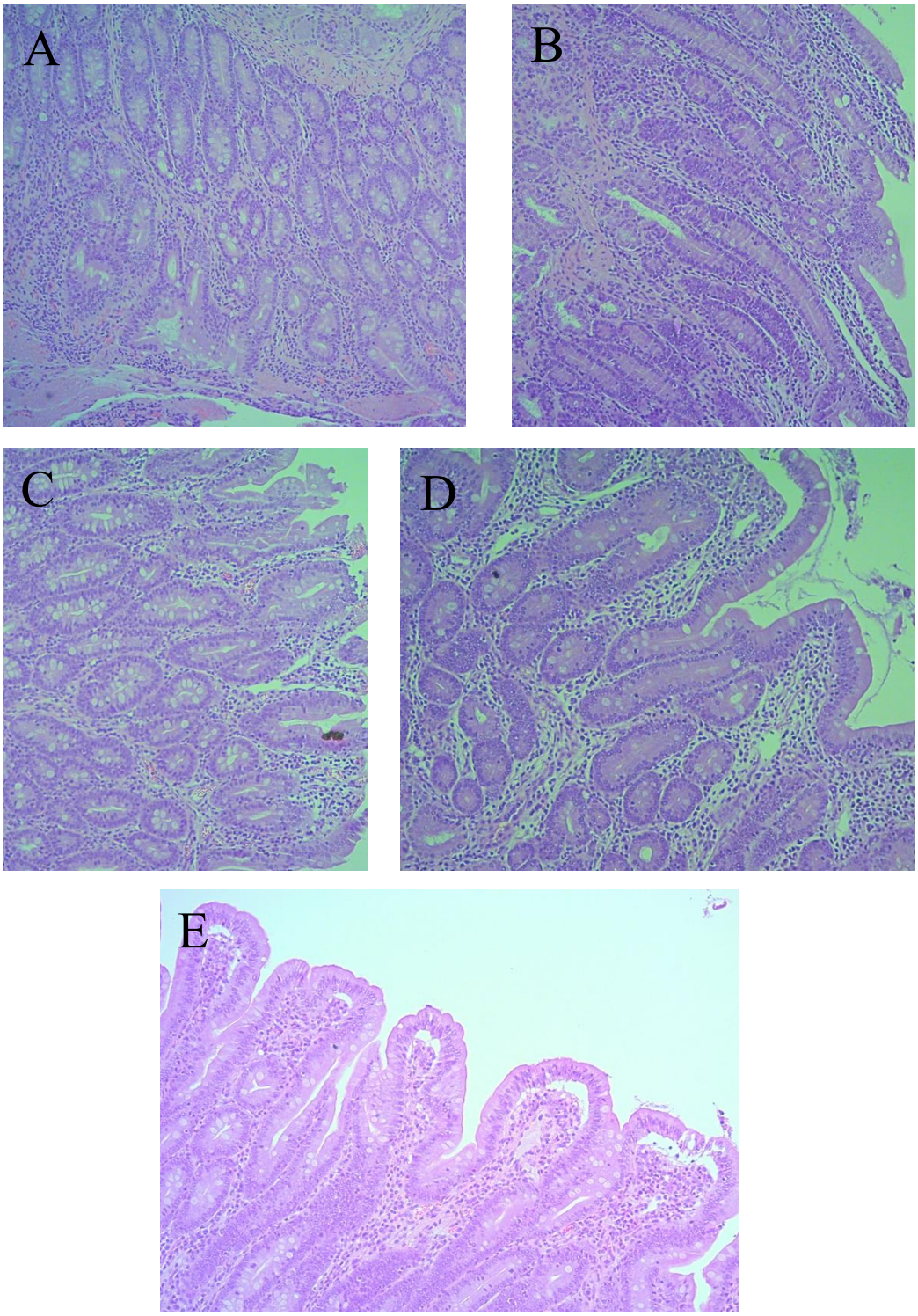

Figure 2.5 Tissue histology for the small intestine (duodenum, jejunum, and ileum) epithelial separation scoring range. A score of 1 (A) indicates no or very little separation from the epithelium is visible throughout the image. A score of 2 (B) indicates little separation from the

epithelium is visible, and more than a score of 1. A score of 3 (C) indicates some separation from the epithelium is visible, and more than a score of 2. A score of 4 (D) indicates much separation from the epithelium is visible, and more than a score of 3. A score of 5 (E) indicates severe separation from the epithelium is greatly visible.

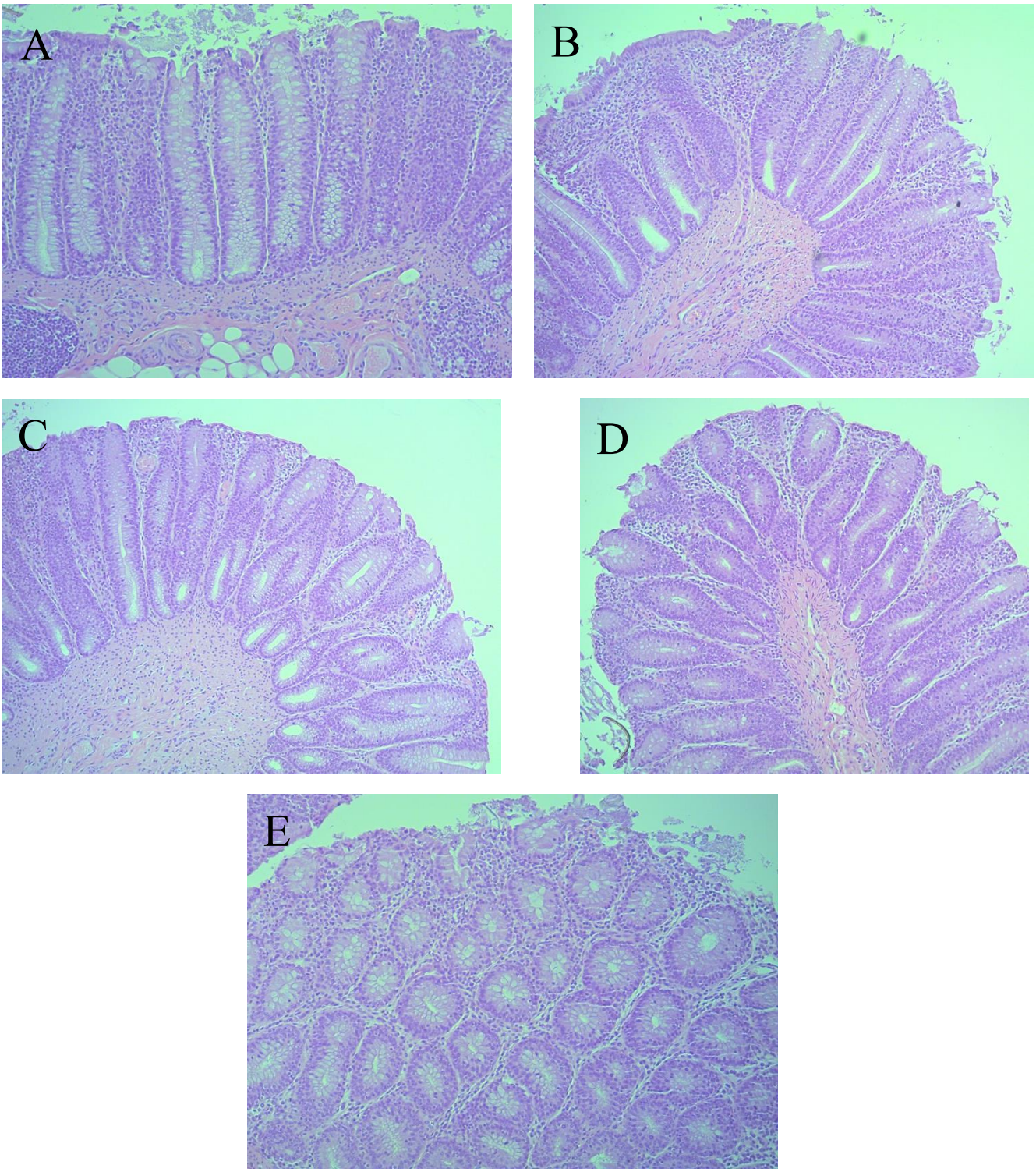

Figure 2.6 Tissue histology for the colon crypt development scoring range. A score of 1 (A) indicates all or almost all of the crypts present are elongated and uniform in shape. A score of 2 (B) indicates most of the crypts present are elongated, with more variation than a score of 1. A

score of 3 (C) indicates some of the crypts present are elongated, with more variation than a score of 2. A score of 4 (D) indicates few of the crypts present are elongated, with more variation than a score of 3. A score of 5 (E) indicates none or very few of the crypts present are elongated, with most of the crypts being short and round.

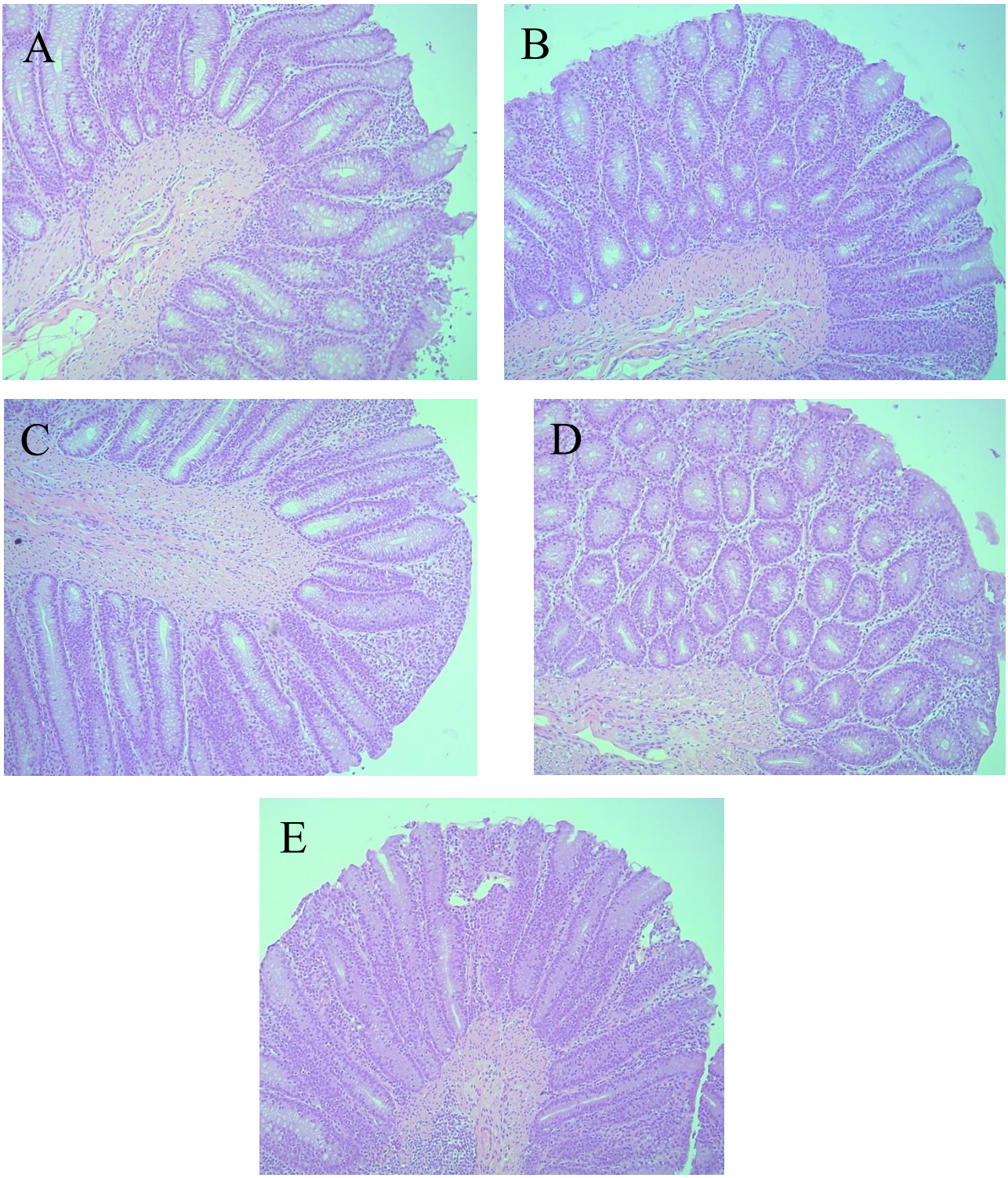

Figure 2.7 Tissue histology for the colon goblet cell loss scoring range. A score of 1 (A) indicates all or almost all of the crypts present contain goblet cells in great number. A score of 2 (B) indicates most of the crypts present contain many goblet cells, with less goblet cells present

than a score of 1. A score of 3 (C) indicates some of the crypts present contain goblet cells, with less goblet cells present than a score of 2. A score of 4 (D) indicates few of the crypts present contain few goblet cells, with less goblet cells present than a score of 3. A score of 5 (E) indicates none or very few of the crypts present contain very few goblet cells, with less goblet cells present than a score of 4.

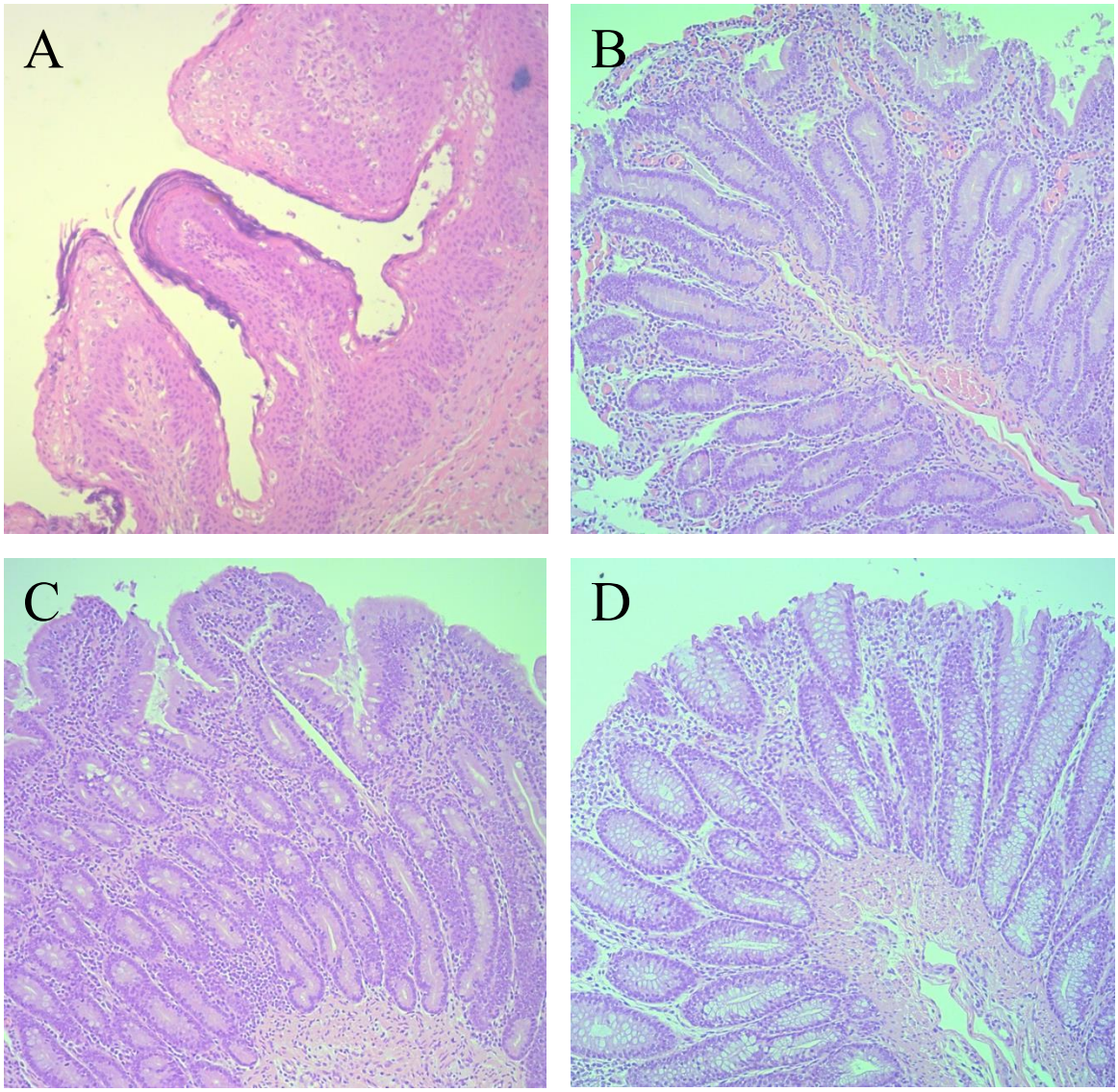

Figure 2.8 Gut histology variation throughout the different tissues: rumen (A), jejunum (B), ileum (C), and colon (D). Displaying variation in papillae and villi lengths and widths within the same image.

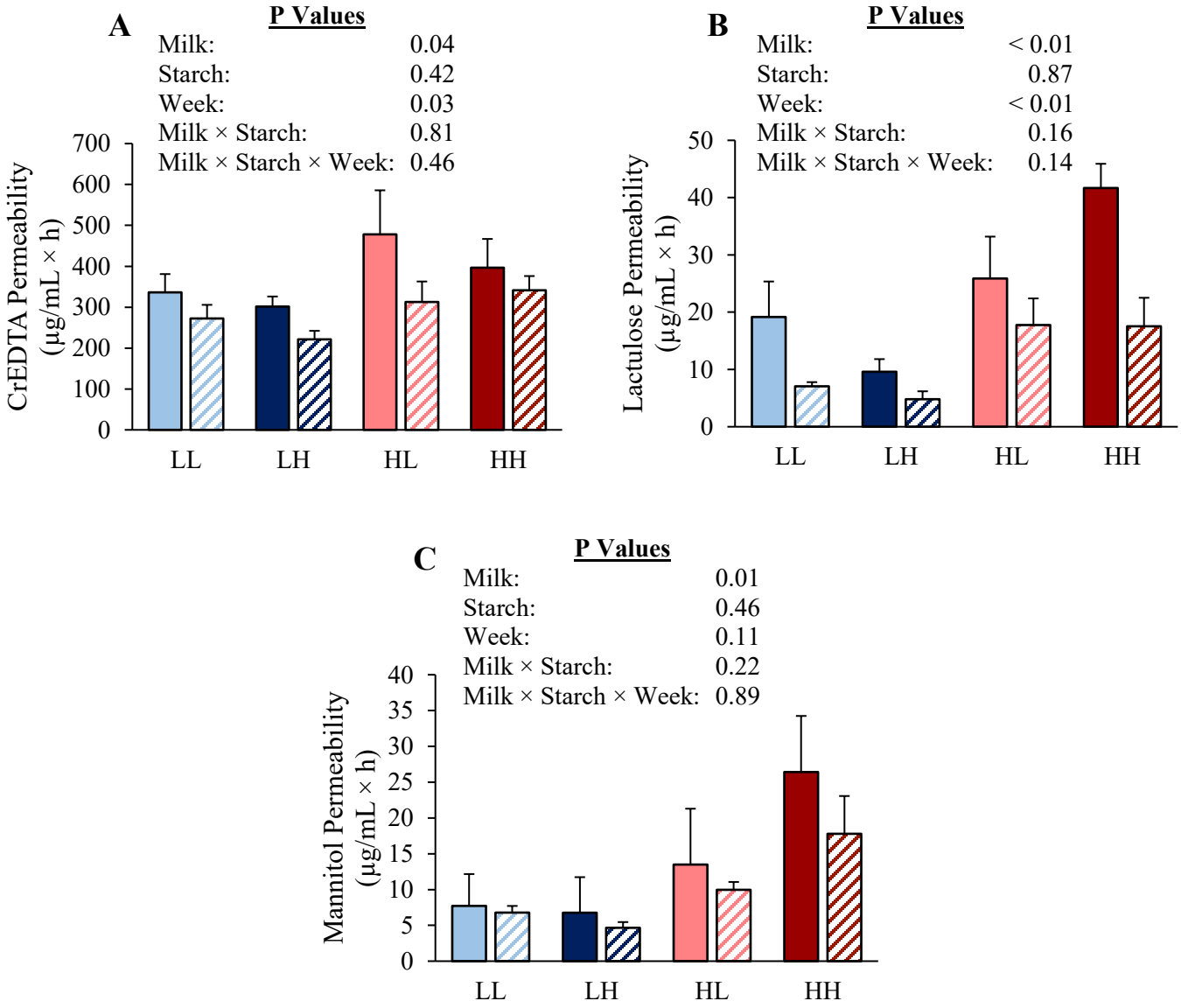

Figure 2.9 Chromium EDTA (A), lactulose (B), and mannitol (C) permeability between treatments dosed and collected at weeks 4 (pre-weaning; solid bars) and 8 (post-weaning; striped bars) of the trial and measured in the blood. Calves fed low or high amounts of milk (600 g/d or 1,200 g/d, respectively) and ad libitum starter with low or high amounts of starch (12% starch, DM or 35.6% starch, DM, respectively). Treatments were low milk and low starch (LL), low milk and high starch (LH), high milk and low starch (HL), and high milk and high starch (HH). Data shown as least square means (LSM) ± standard error of the means (SEM).

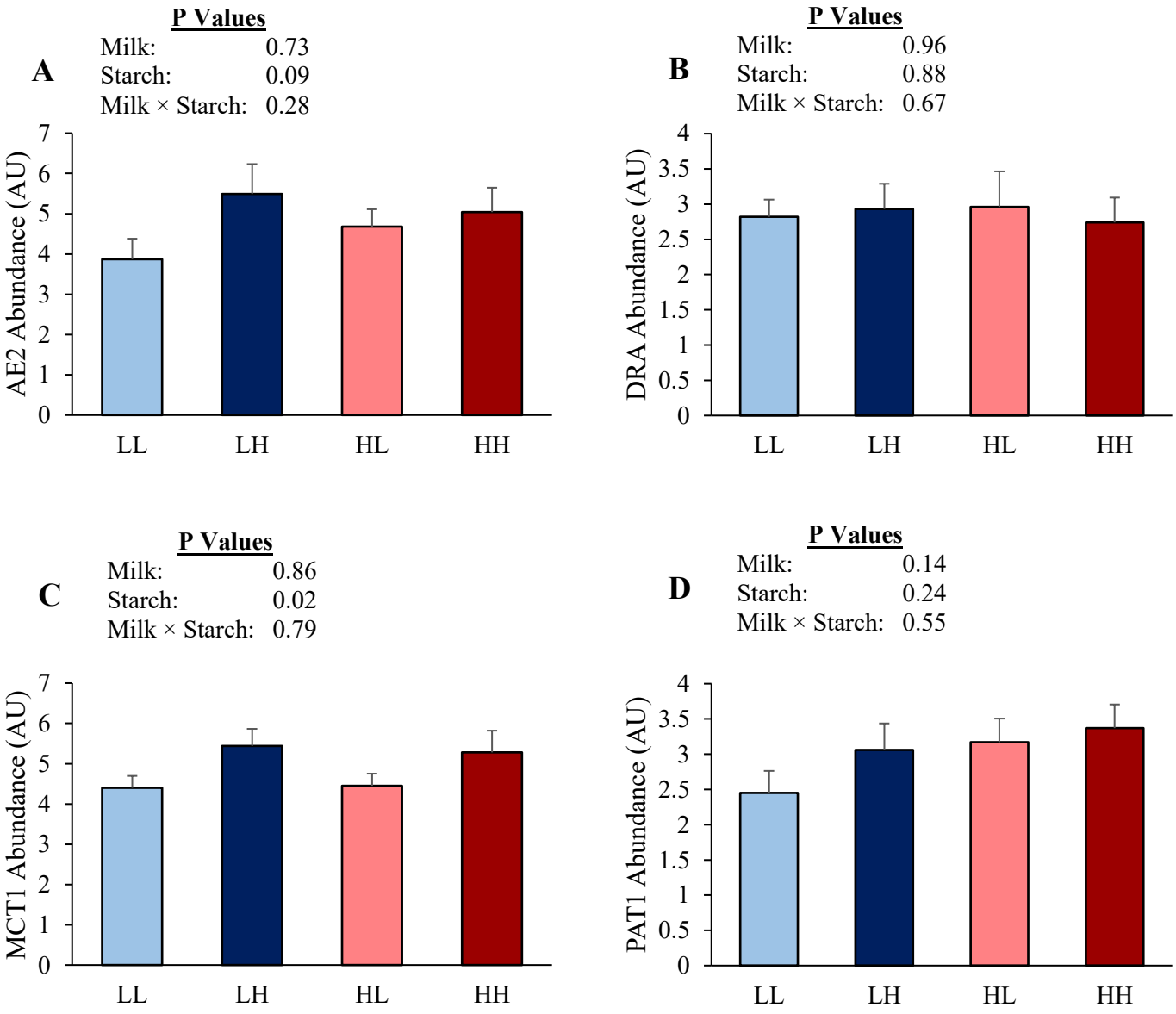

Figure 2.10 Relative protein abundance (in arbitrary units, AU) based on immunofluorescence for rumen proteins: anion exchanger 2 (AE2; A), downregulated in adenoma (DRA; B), monocarboxylate co-transporter 1 (MCT1; C), and putative anion transporter 1 (PAT1; D). Calves fed low or high amounts of milk (600 g/d or 1,200 g/d, respectively) and ad libitum starter with low or high amounts of starch (12% starch, DM or 35.6% starch, DM, respectively). Treatments were low milk and low starch (LL), low milk and high starch (LH), high milk and low starch (HL), and high milk and high starch (HH). Data shown as least square means (LSM) ± standard error of the means (SEM).

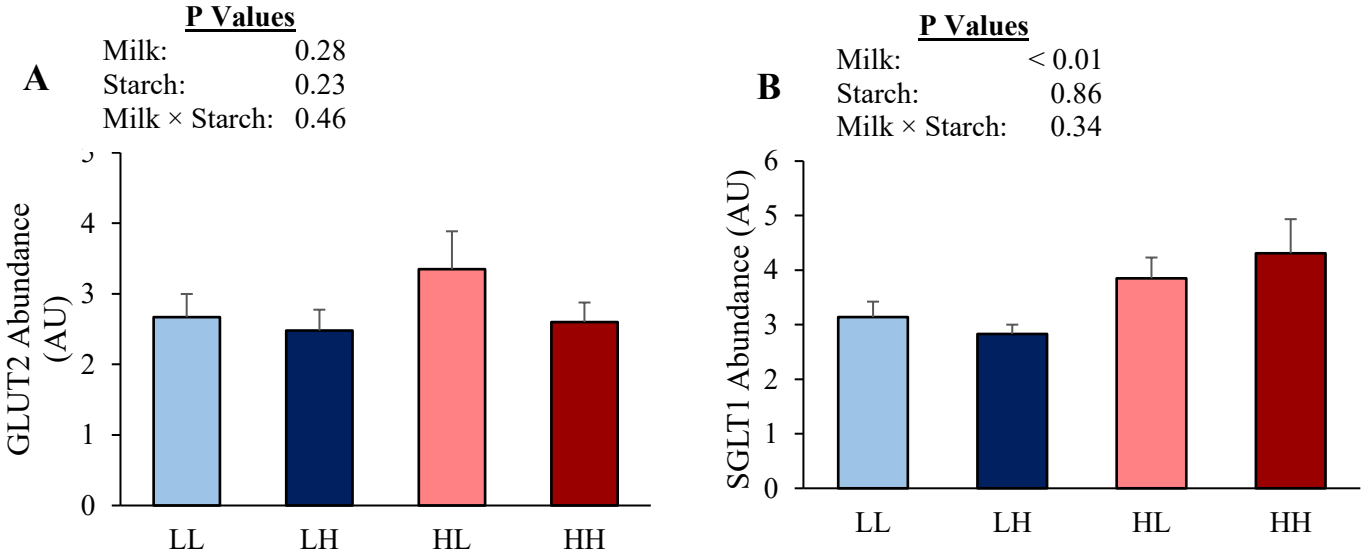

Figure 2.11 Relative protein abundance (in arbitrary units, AU) based on immunofluorescence for proximal jejunum proteins: facilitated glucose transporter 2 (GLUT2; A) and sodium-dependent glucose transporter 1 (SGLT1; B). Calves fed low or high amounts of milk (600 g/d or 1,200 g/d, respectively) and ad libitum starter with low or high amounts of starch (12% starch, DM or 35.6% starch, DM, respectively). Treatments were low milk and low starch (LL), low milk and high starch (LH), high milk and low starch (HL), and high milk and high starch (HH). Data shown as least square means (LSM) ± standard error of the means (SEM).

3.0 General Discussion

The objective of this study was to investigate how the morphological and physiological development of the gastrointestinal tract is impacted by milk provision and starch content of the solid diet. Specifically, this study investigated how gut permeability and histology, as well as digestive, absorptive, and metabolic genes and proteins were impacted by milk provision and starter starch content. I hypothesized that calves fed the high milk provision and high starch starter would have the greatest digestion and metabolism enzyme expression, and SCFA and glucose absorption capacity because they have the highest energy nutrient provision in the diet. Increasing the pre-weaning plane of nutrition and starch content for calves can lead to increased GIT development and nutrient transport for calves postweaning. However, more work needs to be done on calves fed different dietary compositions through the weaning transition while looking at physiological parameters such as nutrient transporters and enzymes (both mRNA and protein), barrier and tight junction proteins, specific organ permeability and digestibility, and histology.

3.1 Major Findings

3.1.1 Impacts of Starch Content on Rumen and Lower Gut

Increased starch concentration in starter increased SCFA transporter abundance, decreased rumen papillae sloughing, and tended to increase jejunum villous blunting and ileal epithelial separation, without changes in rumen pH. Other work agrees that, regardless of rumen pH, rumen papillae surface area, length, width, and stratum thickness increase with age, and there is no correlation between starter intake, rumen pH, and papillae size (Kristensen et al.,

2007; van Niekerk et al., 2021). Instead, our study shows there may be a protective effect on the rumen of increased fermentation, as rumen pH was positively correlated to MCT1 and PAT1 abundance. These correlations, as well as the increase in both luminal and basolateral SCFA transporter abundance in high starch diets, could reveal a protective effect on the rumen if SCFA transporter abundance is able to increase enough to buffer the rumen pH. Other studies agree, as calves with a lower pH have increased intake, ADG, and the ability to regulate pH (Wood et al., 2015; Hiltz et al., 2021; van Niekerk et al., 2021). Altogether, calf productivity is not affected by low rumen pH in the same way as adult cows. Our study advances the knowledge that high starch calf starters are able to provide morphological and SCFA transport capacity benefits without causing morphological damage to the rumen epithelium in calves.

While increased starch intake can increase rumen epithelial morphological development and function, there is the potential for increased starch to lead to damage in the small intestine if the rumen pH drops. While morphometric measurements were unaffected by milk provision and starch content (Yohe et al., 2022a), histological scoring showed high starch starters tended to increase villous blunting and epithelial separation in the small intestine. It is currently unclear what is causing damage to the small intestine morphology. Damage to the small intestine may not be coming from the starch entering or being broken down in the small intestine, but instead from the increased rumen fermentation which leads to increases in SCFA concentration and lower rumen pH. However, the link between rumen pH and SCFA concentration on both the small intestine and colon are unclear. High starch diets that lower the rumen pH may have the potential to lower colon pH with (Asadollahi et al., 2018) or without (Wang et al., 2009; Pederzolli et al., 2018; Liu et al., 2019) lowering small intestine pH.

In agreement with others (Asadollahi et al., 2018; Pederzolli et al., 2018), high starch diets did not have an increase in gut permeability, indicating that lower gut morphology is linked to rumen pH and not gut permeability. Potentially, acidotic levels of rumen pH could lead to downstream morphological damage in the small intestine and colon, even though lower rumen pH decreased ruminal papillae sloughing and increased ruminal SCFA transport capacity. However, inconsistent results from other studies (Wood et al., 2015; Pederzolli et al., 2018; van Niekerk et al., 2021) indicates that more research needs to be done on the impact of rumen pH on small intestine morphology.

3.1.2 Glucose Uptake Via Lower Gut Barrier Dilation

Increased milk provision had the largest impact in the small intestine through increased glucose transporter abundance and intestinal permeability, indicating a possible glucose transport mechanism other than SGLT1. Post-prandial glucose concentrations exceed 25 mM, and, by extension, SGLT1 transport capacity (Zheng et al., 2012; Pavic et al., 2020). In periods of high glucose concentrations, intestinal tight junction may dilate to increase small molecule transport via facilitated diffusion (Herrmann and Turner, 2016; Klinger et al., 2016). The proposed mechanism for SGLT1 to tight junction dilation pathway is SGLT1 activates in the presence of glucose, which activates the brush-border sodium/hydrogen exchanger 3 (NHE3), which increases MLCK activity (Turner et al., 2000; Herrmann and Turner, 2016). Myosin light chain kinase is part of the smooth muscle contraction mechanism and phosphorylates myosin light chain (MLC) which leads to the contraction of the apical actin cytoskeleton within the tight junction protein complex (Turner et al., 1997; Turner, 2000; Herrmann and Turner, 2016). This contraction causes the tight junction to dilate, increasing permeability and decreasing transepithelial resistance (Harhaj and Antonetti, 2004; Chelakkot et al., 2018; Vanuytsel et al.,

2021). Increasing tight junction dilation allows the flow of water and smaller nutrients, such as glucose, to diffuse paracellularly, increasing the net clearance of nutrients (Turner and Madara, 1995; Odenwald and Turner, 2013), but only when both glucose and water are present at high concentrations (Turner et al., 2000). This means that diets high in liquid, or liquid-based diets may have increased permeability as a means of increasing post-prandial glucose transport.

Gut permeability increases through activation of MLCK also occurs via pathogenic stimuli (Turner, 2000; Vanuytsel et al., 2021). Compared to pathogenic stimuli, the SGLT1-induced tight junction dilation is more rapid, size-selective, reversable, and tightly regulated compared to the other disease-type mechanisms (Turner, 2000; Herrmann and Turner, 2016). Intermediate events between a disease state permeability and SGLT1 permeability may be similar, but their initial stimuli and end effects are different (Turner, 2000). Pathogenic stimuli may also cause damage or destruction of the tight junction proteins (Gunzel and Yu, 2013; Hu et al., 2013). While this analysis showed acute tight junction dilation as a possible mechanism, glucose transport kinetics and tight junction protein abundance were not studied. Both analyses would lend merit to the potential mechanism of SGLT1-linked tight junction dilation. Nevertheless, increased milk intake is able to increase small intestine nutrient transporter abundance, without impairing rumen epithelial cell structure and function.

3.2 Industry Application

This study showed increased nutrient transport abundance and enzyme mRNA expression, and improved tissue morphology with increased milk provision and increased levels of starch in the calf diet. Increasing milk provision increases permeability which may be a physiological response rather than a stress response. Industry may not need to be concerned about a temporary increase in gut permeability in pre-weaned calves if that is associated with

increased nutrient uptake rather than pathological breakdown of the epithelium. Increases in permeability during stressful periods, such as weaning, should be measured along with markers of stress (cortisol, serum amyloid A, etc.) to conclude that the increased permeability is occurring from stress rather than increasing nutrient flux (Hu et al., 2013; Wood et al., 2015; Moeser et al., 2017). Increased permeability is an inconsistent marker of stress, as it can occur with or without a stress response.

Industry should be aware of the impacts of starch on the development of the rumen and small intestine. Our study showed that increased starch may have a protective effect on the rumen, as there were increases in SCFA transporter protein abundance and decreases in papillae sloughing (indicative of damage). Increases in starch in the calf starter may be beneficial to both calf growth, as seen in other studies (Hill et al., 2016; Hu et al., 2018), and rumen development, as seen in our study. However, it should be noted that increases in starch may have damaging effects on the small intestine through a decreased rumen pH (Pederzolli et al., 2018). Overall, increased starch has many benefits and calves fed higher amounts of starch should be monitored for signs of acidosis and other health problems.

3.3 Limitations and Future Research

One of the limitations of this study was the lack of chronic inflammatory marker or stress marker measurements, such as cortisol. If the changes in nutrient transport, enzyme expression, or permeability induced by high or low milk or starch are causing physical and physiological differences, it should be evaluated to understand if the changes are being influenced by stress. Another limitation was the length of the trial. Calves were only weaned for a week before harvest, so any post-weaning effects seen were only representing a very short period. Future

research should look at the long-term effects of pre-weaning diet on later performance, ideally through the first lactation.

No microbial factors were evaluated for this study. Microbes play an important role in the rumen through development and the establishment of the microbiome, as well as the small intestine through the fermentation of lactose. The role of microbes in starch and lactose breakdown in calves should be evaluated in future studies.

It should be noted the differences in mRNA abundance compared to protein abundance. The enzymes and nutrient transporters analyzed in this study were only evaluated through one process (mRNA or protein). There are known changes in post-transcriptional abundance in enzymes and transporters (Wood et al., 2000; Iwanaga and Kishimoto, 2015), and differences between mRNA and protein could have varied the results of the study.

Future research should focus on the dietary factors that impact rumen pH in calves. More research needs to be done on the pH regulation methods and their development in calves. Rumen pH regulation in young calves appears to be different than in adult cows, as the rumen pH of calves is sustained at lower values (Laarman et al., 2012b; Wood et al., 2015; van Niekerk et al., 2021). At what age the low rumen pH impacts begins to resemble those of an adult cow remains unclear. Another limitation was the lack of mucus layer analysis. Research in humans and mice has shown that high fiber diets can increase the colon mucus layer (Makki et al., 2018), and similar effects may be seen through high starch diets in the small intestine. However, mucus characteristics have not been described in ruminants (Steele et al., 2016) and paucity of research has been done on starch impacts to the small intestine mucus layer.

Another aspect of this study that warrants future research is the increased gut permeability post-weaning in calves that were fed high milk plane of nutrition. Previous studies

largely focused on gut permeability and how it relates to disease states, rather than a physiological state. This study suggested increased gut permeability may be a post-prandial physiological response to increased luminal glucose abundance, facilitated by a transient dilation of epithelial tight junctions. While SGLT1 is not a mediator of permeability itself, it acts as a sensor to activate the tight junction dilation mechanism that affects permeability. More research needs to be done on the mechanisms causing the increase in permeability, how long the increased permeability status lasts, and if there are long term effects of increased permeability. Because blood glucose is tightly regulated, tight junction dilation may not result in measurable changes in blood glucose. Future studies should include tight junction protein abundance and transepithelial glucose kinetics to assess the tight junction dilation permeability mechanisms and its occurrence in young calves. If glucose kinetics, SGLT1 abundance, and permeability all increase, along with potential changes to tight junction proteins, it may show a link between SGLT1 and permeability. Research also needs to be done on the differences between pathological and physiological increases in gut permeability, as not all increases in permeability may be negative.

3.4 Conclusions

This research shows the benefits of higher milk provision without delaying morphological rumen development. Higher starch concentrations in the starter diet benefit rumen development through increasing SCFA transport capacity and reducing epithelial sloughing; however, increased morphological damage occurs in the lower gut when calf starter starch content is high. While the benefits of increased milk provision are evident, higher starch content has mixed impacts on gastrointestinal development and requires more optimization in calf nutrition programs.

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