Effects of Ruminal Short-chain Fatty Acids and pH on Gastrointestinal Development of Dairy Calves

by

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Abstract

While the importance of pH and short-chain fatty acids (SCFA) on rumen development in calves is well-known, their impact on the small and large intestines are unclear. This study investigated the effects of ruminal SCFA concentrations ([SCFA]) and pH on performance and hindgut fermentation and development of dairy calves. Holstein bull calves (n = 32) were individually housed and fed 900 g/day of milk replacer twice daily and *ad libitum* calf starter and water. At day 10 ± 3 of life, the rumens were fistulated and cannulated. At day 14 of life, calves were grouped by body weight and assigned in a 2 × 2 factorial arrangement of treatments into high or low [SCFA] (285 vs. 10 mM) and high or low pH (6.2 vs. 5.2), creating four treatment groups: high [SCFA], high pH (HS-HP); high [SCFA], low pH (HS-LP); low [SCFA], high pH (LS-HP); and low [SCFA], low pH (LS-LP). Body weight was measured weekly. On days 21, 35, and 49, feces were sampled to calculate apparent total tract digestibility, determinate short-chain fatty acid concentrations and pH. Then, the rumen was evacuated and washed for 4 h with one of four treatment buffers. Buffer samples were taken hourly to calculate ruminal SCFA disappearance rates. On day 49, following the rumen wash, calves were harvested, and the tissue weight and length, and digesta of the rumen, cecum, colon, and rectum were collected to measure organic acid concentrations and pH, followed tissue sampling for histomorphometric and gene expression analysis. The digesta pH of the duodenum, jejunum, and ileum were also recorded. Data were analyzed with main factors (SCFA, pH, and SCFA × pH) as fixed effects and repeated measures for weekly measurements (e.g., body weight, digestibility, and SCFA disappearance rates, as well as fecal and ruminal organic acid concentration and pH). Treatment and day did not affect performance parameters such as apparent total tract digestibility and gut measurements. In the duodenum ($P = 0.05$), jejunum ($P = 0.04$), and ileum ($P < 0.01$), HS-HP had a greater digesta pH

than LS-HP, while the hindgut digesta pH was only affected by the [SCFA] ($P < 0.01$). High [SCFA] increased the concentration of colonic isovaleric acid ($P = 0.05$) and fecal branched-chain fatty acids ($P < 0.01$), while only colonic acetic acid ($P = 0.05$) and fecal lactic acid concentrations ($P < 0.01$) were lower in the HS-LP group. Cecum mucosal thickness tended to be greater in calves in the low pH groups ($P = 0.07$) while decreasing the colonic crypt depth ($P = 0.02$) and tending to decrease relative cyclin A2 expression ($P = 0.09$). The high [SCFA] groups had a better cecal crypt development score ($P = 0.03$), an increase in colonic cyclin A2 ($P < 0.01$) and NBC1 expressions ($P < 0.01$), and a tendency to increase ruminal IGF-1R expression ($P = 0.08$), and the total ruminal SCFA disappearance rate ($P = 0.08$). The HS-LP group had increased propionate ($P = 0.05$) and butyrate disappearance rates ($P = 0.05$). Therefore, 4 h of buffer infusion in the rumen does not change calf performance but does affect hindgut fermentation and epithelium development, in which calves ruminal infused with physiological buffer containing a high short-chain fatty acid concentration and low pH may represent a decreased risk of hindgut acids. However, further investigations are required to understand if calves can experience hindgut acidosis in nutritional trials.

Preface

This thesis is an original work by Matheus Henrique Paez Martins Narciso. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Animal Care and Use Committee – Livestock, Project Name "Interaction of SCFA and pH in promoting cellular differentiation in the rumen of young dairy calves" No. AUP00003823, June 7, 2022. Matheus was responsible for conducting the experiment, sample and data collection, laboratory and statistical analyses, data interpretation and dissemination, and manuscript and thesis writing. Dr. Laarman closely supervised all components of the research and graduate program.

Data from the experiment is presented in Chapters 2 and 3, which were co-authored by Alison R. Wolfe and Richard R. E. Uwiera, who contributed to data interpretation and manuscript edits. In addition, Alison R. Wolfe also assisted in conducting the experiment, sample and data collection, and laboratory analysis. Richard R. E. Uwiera also performed the ruminal cannulation surgery as described in AUP0000382. Chapter 2 is under review by the Journal of Dairy Science by Narciso et al. (unpublished).

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In memoriam, Jack Tequila (2015 – 26/03/2024) – the friendliest cat that ever existed.

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Abbreviations

ADG – Average daily gain

AE2 – Anion exchanger

ATTD – Apparent total tract digestibility

BCFA – Branched-chain fatty acids

DMI – Dry matter intake

DRA – Downregulated-in-adenoma

GIT – Gastrointestinal tract

GLP – Glucagon-like peptide

GLP2R – Glucagon-like peptide 2 receptor

H^+ – hydrogen ion

H-SCFA – Protonated short-chain fatty acids

IGF-1 – Insulin like growth factor 1

IGF-1R – Insulin like growth factor 1 receptor

MCT1 – Monocarboxylate transporter, isoform 1

MCT2 – Monocarboxylate transporter, isoform 2

MCT4 – Monocarboxylate transporter, isoform 4

NBC1 – Na^+/bicabornate cotransporter 1

NHE – Na^+/H^+ exchanger

NHE3 - Na^+/H^+ exchanger, isoform 3

NDF – Neutral detergent fiber

PAT1 – Putative anion exchanger

pH_i – Intracellular pH

SCFA – Short-chain fatty acids

SCFA$^-$ – Ionized short-chain fatty acids

H–SCFA – Protonated SCFA fatty acids

[SCFA] – Short-chain fatty acid concentration

SARA – Subacute ruminal acidosis

1. Literature review

1.1. Introduction

Calves are an essential product of the cattle industry by producing either milk and meat in their adult life. Despite their importance, dairy calves' pre-weaning morbidity and mortality rates may achieve 33.9% and 5%, respectively (Urie et al., 2018a), the first 2 weeks of life being the most critical period (Wells et al., 1997; Urie et al., 2018a). Of the causes of mortality, the incidence of digestive disorders may cause 56.4% of the deaths (United States Department of Agriculture, 2018), representing a major challenge for the industry.

Young calves are nonfunctional ruminants (Khan et al., 2016; Meale et al., 2017; Diao et al., 2019), representing a unique morphophysiological gastrointestinal condition that differs from those of mature cattle. From a morphological perspective, the ruminal papillae of young calves are underdeveloped, which may decrease short-chain fatty acid (SCFA) absorption (Baldwin et al., 2004; Suárez et al., 2006). Simultaneously, lower rumen pH conditions that are commonly associated with subacute ruminal acidosis (SARA) – a major concern for adult cattle (Plaizier et al., 2018; Hossain, 2020), seem to affect positively the performance of dairy calves (Laarman et al., 2012; McCurdy et al., 2019), suggesting a functionality of the calf's gastrointestinal tract (GIT) that is unique from adult cows.

Despite their differences, the current calf nutrition management has targeted chiefly the development of the rumen to reach a stage similar to mature cattle physiology. The calf starter provision to pre-weaning calves, for example, is a common practice in the industry to promote rumen development (Diao et al., 2019), but its effect throughout the GIT, especially large intestine, are poorly understood. Therefore, there is vast room to improve our understanding of the calves'

gastrointestinal physiology further, and this knowledge might be crucial to maximizing the calf's health and performance.

1.2. Dairy industry: Panorama of a historic worldwide activity

The relationship between cattle and humans is dated over 10,000 years ago, in the early Holocene, when evidence suggests that bovine species started being domesticated in India, south-eastern Europe, Africa, and China (Zhang et al., 2020). Likely, the domestication process was primarily triggered by food needs, and later, it was a source of hide, transportation, and labor power (Felius et al., 2014; Zhang et al., 2020). Like other domesticated species such as sheep and goats, cattle presented the right conditions to allow captive management, such as an herbivorous diet, fast growth, the possibility of captive breeding, and social behavior (Felius et al., 2014). Although still debatable, it is traditionally proposed that the usage of cattle for milk production took place later (i.e., Late Neolithic) over the domestication process, as milking requires the development of technical skills (Vigne and Helmer, 2007).

Despite that, only at the beginning of the 20th century, with the development of the pasteurization process, did dairy products start to be widely commercialized, increasing the milk yield demand (Medeiros et al., 2022). It has been estimated a dairy cow population of 265 million heads worldwide in 2020 (FAO, 2021) that contributed to achieving a global milk yield of 906 million tonnes in 2020, in which Asia was the biggest producer, mainly India and China (379 million tonnes), representing 33% of the global milk production, followed by Europe (236 million tonnes) and North America (111 million tonnes). In North America, the United States is the biggest producer, providing 101 million tonnes of milk, followed by Canada with approximately 9.6 million tonnes.

In 2022, the Canadian dairy sector generated a total net farm cash receipt of $8.23 billion and 27,424 jobs associated with the dairy manufacturing sector (Agriculture and Agri-Food Canada, 2022), indicating an essential participation in the Canadian economy. In total, Canada has 9.739 dairy farms responsible for milk shipment, in which Quebec and Ontario are the largest producers, presenting 4,284 and 3,233 dairy farms in 2023, respectively, followed by Alberta (477 farms) and British Columbia (437 farms; Agriculture and Agri-Food Canada, 2023).

1.3. Nutritional management of pre-weaned dairy calves

Considering morbidity and mortality rates of 33.9 and 5%, respectively (Urie et al., 2018a), of the 427,300 dairy calves born annually in Canada (Statistics Canada, 2023), 144,850 calves get sick in their first year of life every year and 21,300 die mainly in the first 2 weeks of life (Wells et al., 1997; Urie et al., 2018a), making the dairy calf operation a major challenge for the dairy industry. Despite the efforts to improve the calf's performance and health, diseases such as diarrhea (scours) and bovine respiratory disease are still the biggest challenges in calf operation, responsible for most of the morbidity and mortality cases in the pre-weaning period (Machado and Ballou, 2022). The incidence of dullness and dehydration associated with diarrhea can reach 17.2% of the calves, while respiratory-related diseases can reach 9.5% (Urie et al., 2018a). In addition, the consequences of bovine respiratory disease cases during the pre-weaning period have negative long-term impacts, such as body weight gain reduction until 12.9 months of life (Hurst et al., 2021) as well as increased culling rate before the first lactation and detrimental reproductive effects (Teixeira et al., 2017). Therefore, meeting the calf's nutritional requirement and developing nutritional management practices are encouraged to overcome the industry challenges.

1.3.1. Colostrum

Among the nutritional management practices, colostrum feeding is the most crucial management practice associated with calf health and survival (Godden et al., 2019; Machado and Ballou, 2022). As cows have a syndesmochorial placenta that prevents immunoglobulin transmission to the fetus, calves are born agammaglobulinemic, consequently depending on colostrum intake for immunoglobulin absorption within 24h after birth (Godden et al., 2019). The intake of colostrum may assist in protecting calves against diseases until their immune system becomes functional, reducing pre-weaning morbidity and mortality rates (Godden et al., 2019).

Conventionally, it is expected that less than 10% of calves have a passive immunity transfer failure (McGuirk and Collins, 2004); however, recently, a new categorization of passive immunity is proposed based on serum IgG concentration: Excellent (≥ 25.0 g/L of IgG), good (18.0 to 24.9 g/L of IgG), fair (10.0 to 17.9 g/L of IgG), and poor (≤ 10.0 g/L of IgG), where 40% of calves should be in the excellent and 10% is expected to be in the poor category (Godden et al., 2019; Lombard et al., 2020). When calves are fed poor quality (≤ 10.0 g/L of IgG) colostrum or even good colostrum after 24h postpartum, in which immunoglobulin absorption is limited, failure of passive immunity transfer is observed, increasing mortality risks (Godden et al., 2019). In addition, colostrum provides immunomodulatory peptides and modulates the neonatal microbiome, suggesting an essential role in the maturation of the immune system beyond that that came from the immunoglobulin provision (Hammon et al., 2020).

As most of the operations separate the dam and calf immediately after birth, colostrum is mostly fed in bottles or esophageal tubes (Urie et al., 2018b); only around 4% of the operations allow the dams to feed colostrum to their calves (Roche et al., 2023). This scenario allows us to monitor the colostrum quality and track the individual colostrum intake. It is recommended to feed

150 to 200 g of IgG by providing 10-12% of body weight (3-4 L for a Holstein calf) of a good quality colostrum (> 50 g/L of IgG) within 4h after birth (Godden et al., 2019; Machado and Ballou, 2022), but an additional feeding 12h after birth, aiming at 6 L of total colostrum intake, has demonstrated to improve the immune system (Roche et al., 2023). This extra step, however, is performed only by 34.1% of the American operations, and in Canada, 33% of the producers still feed less than 4 L (Roche et al., 2023).

1.3.2. Liquid feed

In the dairy industry, milk can be fed as milk replacer, whole, or nonsaleable/waste milk (Machado and Ballou, 2022). In the United States, whole or waste milk is fed to 40.1% of calves, milk replacer is fed to 34.8% of calves, and a combination of both is provided to 25.1% of calves (Urie et al., 2018b). While practices may differ, liquid feeding management consists of three components: milk allowance, quality, and water access.

Milk allowance is essential to pre-weaning nutrition, as pre-weaned calves primarily depend on liquid feed. In the United States, calves are fed 2.6 L per feeding and 2.6 times per day, resulting in a total of 5.6 L of liquid diet fed per day on average (Urie et al., 2018b). In Canada, however, there is a trend in increasing milk allowance, as the maximum milk volume offered is 8.2 L per day, and only 33% of the producers feed a maximum milk volume of $\leq$ 6 L per day (Winder et al., 2018). In addition, younger producers (< 30 years old) are feeding up to 0.8 L more than older producers, and grouped calves are drinking 1.2 L more than individually housed calves (Winder et al., 2018). The current Canadian requirement is to feed at least 2 times per day or free choice, with the total daily intake equal to 20% of the calves' body weight for the first month (about 8L per day for a Holstein calf; proAction - Reference Manual, 2023), which aligns with more recent studies that recommend a higher milk allowance, either by feeding *ad libitum* or offering 20% of

body weight (Fischer et al., 2019; Roche et al., 2023; Welk et al., 2023). Thus, although the feeding frequency requirement has been met, some operations still feed suboptimal milk volume in North America, mainly in the United States.

This milk restriction practice may be partially attributed to increased solid feed intake traditionally reported in the past decades, which is known to promote rumen development before weaning (Khan et al., 2011). However, recently has been demonstrated that a greater milk allowance increases protein and energy supply compared to restricted milk allowance protocols, improving calf growth, organ development, metabolic and endocrine changes, feeding behavior, immune response, and promoting better welfare conditions (Hammon et al., 2020; Roche et al., 2023). In addition, unrestricted milk-fed calves can reach greater body weight and organ development if the weaning transition is performed accordingly by performing later gradual weaning (e.g. step down at 8 weeks of age) or increasing the length of the weaning transition (e.g., > 2 weeks; Hammon et al., 2020; Machado and Ballou, 2022; Welk et al., 2023). Therefore, greater milk allowance demonstrates several benefits in relation to the restricted milk allowance protocols, which should be less commonly practiced as time goes by.

Another vital aspect of liquid feed is the osmolality (Azevedo et al., 2023). Milk replacers are richer in minerals and lactose than whole milk (42 to 45% vs. 35% DM), increasing the percentage of solids per liter of solution, which can range from 12.5 to 20% in commercial milk replacers (Wilms et al., 2019). This percentage difference results in commercial products ranging from slightly hypertonic (just above 300 mOsm/kg; similar to whole milk) to highly hypertonic (>450 mOsm/kg). In addition, mixing errors and the addition of electrolyte powder can increase the osmolality even more (Wilms et al., 2019). The osmolality monitoring is essential for unrestricted milk allowance protocols (Azevedo et al., 2023), as increased osmolality from 439 to

611 mOsm/kg can increase gut permeability, compromise the gut barrier function and, consequently, negatively affect calf health (Wilms et al., 2019). Although more studies are necessary, it is recommended that milk replacers should have an osmolality below 500 mOsm/kg (Azevedo et al., 2023).

Although milk source is the primary liquid feed and provides most of the necessary nutrients, water intake is equally important. In pre-weaning calves, water intake varies according to age, milk allowance, solid feed intake, and environmental and water temperature; however, clean and fresh water must always be available *ad libitum* despite the intake variations (Jensen and Vestergaard, 2021). The consequences of water restriction can be observed at the performance level, in which restricted milk-fed calves with no water access until 17 days of life decreased milk intake by 6% (0.285 kg/d less milk) and tended to have lower body weight and heart girth (Wickramasinghe et al., 2019). Thus, providing water access throughout 24 h since birth is imperative to maximize calf performance and welfare.

1.3.3. Solid feed: Calf starter

Despite the young age, solid feed has been broadly introduced to pre-weaned dairy calves. Calf starter has been offered in 100% of American operations, mostly starting at 4.2 days of life, while a forage source is provided in 43.3% of operations only, mostly at 19.5 days of life (Urie et al., 2018b). Common calf starters contain between 18% and 22% of crude protein, 15% to 20% of NDF, 50% to 55% of non-fiber carbohydrate, and 35% to 40% of starch, being usually presented as a complete pellet or texturized form (Machado and Ballou, 2022), but also as a meal (ingredient blending and mixing without further processing; Quigley, 2019). Several factors can influence calf starter intake, such as palatability, water intake, housing, and social interaction (Costa et al., 2015;

Knauer et al., 2021; Machado and Ballou, 2022), but mainly milk or milk replacer allowance (Machado and Ballou, 2022).

The physical and chemical forms of calf starters affect solid feed intake, rumen development, and growth. However, there is limited information on the characteristics of optimal calf starters (Nikkhah and Alimirzaei, 2023). The calf starter processing type, which is divided into cold methods (e.g. grinding and rolling) and hot methods (pelleting, steam-rolling, steam-flaking, roasting, and extruding), defines the physical and chemical forms of calf starter. While the cold method changes the physical form, the hot methods also change its chemical form (Nikkhah and Alimirzaei, 2023).

In general, more processed calf starter forms (e.g. texturized, pelleted, ground) have increased apparent total tract digestibility (ATTD) than when calf starter is offered as a meal (Quigley, 2019; Nikkhah and Alimirzaei, 2023) Texturized and pelleted-fed calves presented similar ADG and hip width gain, while meal-fed calves had lower ADG and DMI (Hill et al., 2012), which may be partially attributed to effects of processing that increase the surface area, facilitating microbial attachment, and increasing grain digestibility (Nikkhah and Alimirzaei, 2023). However, determining the ideal calf starter form is inconclusive and often controversial. This difficulty might be associated with the apparent relatively low impact of calf starter form on calf's performance compared to different milk feeding regimes. For example, different texturized calf starters had no impact on intakes and ATTD (> 95%), but different milk allowances had a greater effect on fiber digestibility, and ruminal and fecal bacterial communities (van Niekerk et al., 2020). Other studies also did not find differences between texturized and pelleted calf starter on ATTD and body measurements (Ghassemi Nejad et al., 2012), as well as between calf starters with different texturized forms (fine-ground or steam-flaked grains) on body measurements, DMI,

ADG, and ruminal fluid composition, while greater milk allowance showed more significant impacts on calf's performance (Jafari et al., 2020).

On the other hand, other studies have shown performance effects in calves fed different calf starter forms, in which texturized calf starter increased DMI and improved feed efficiency compared to mashed calf starter (Omidi-Mirzaei et al., 2018), and texturized calf starter with steam-flaked corn increased ADG, feed efficiency, and total ruminal SCFA over texturized starter with ground corn (Makizadeh et al., 2020). Additionally, in a meta-analysis by Ghaffari and Kertz (2021), texturized calf starter presents a greater intake (107 g/d) than pelleted calf starters. However, the authors indicated that the significant variations in calf studies do not allow them to recommend the ideal physical form of calf starter.

Furthermore, several grains are currently used in calf starter formulation, such as corn, barley, oats, sorghum, and wheat (Nikkhah and Alimirzaei, 2023). Such variation also alters the chemical composition of calf starter, which ultimately can change its digestibility properties (Quigley, 2019) and, consequently, calves' performance. Calves fed barley-based calf starter had improved feed efficiency and increased ADG and ruminal total SCFA over calves fed corn-based calf starter (Kazemi-Bonchenari et al., 2020). However, this response may change if the grain source is more processed. In a study comparing 18 or 22% protein of steam-flaked barley and steam-flaked corn, pre-weaned calves fed the corn-based calf starter had greater starter intake (616 and 720 g/d vs. 533 and 601 g/d for 18 and 22% of corn- and barley-based starter, respectively), feed efficiency, better fecal score, and higher hip height compared to calves fed steam-flaked barley; thus, steam-flaked corn in texturized calf starter is recommended over steam-flaked barley for young calves, improving ruminal microbial activity and immune function (Sahib et al., 2023). Overall, processed calf starter can increase calf performance compared to meal-based calf starter

or mashed calf starter. In addition, a texturized calf starter seems to increase DMI and feed efficiency compared to other physical forms, but the grain processing utilized might reduce its benefits depending on the grain source. Therefore, more research is needed to evaluate the ideal combination of grain source, physical form, and feeding strategies.

1.3.4. Solid feed: Forages

The rapid fermentation of calf starter promotes rumen development, but also reduces ruminal pH (Diao et al., 2019), which can be assuaged by including forage in the diet (Suarez-Mena et al., 2016b). The offering of forage during the pre-weaning period, however, is debatable. In natural grazing systems, young calves start grazing around the second week of life (Tedeschi and Fox, 2009); however, in the dairy sector, only 43.3% of calf operations offer a forage source, mostly at 19.5 days of life (Urie et al., 2018b). Usually, forage is not added to the calf starter, and when included, it commonly represents less than 5% of the dry matter and is finely chopped (Machado and Ballou, 2022).

Despite the low provision rate in the industry, in pre-weaning dairy calves, forages can promote rumen development by improving ruminal muscularity, volume, and motility; however, some studies have shown a decreased growth rate due to its lower digestibility, which may increase gut fill, reducing solid feed intake (Xiao et al., 2020; Nikkhah and Alimirzaei, 2022). In a study, calves without hay supplementation had lower total DMI compared to hay-supplemented calves starting at 2 or 6 weeks of life (0.64, 0.77, 0.99 kg/d, respectively), ruminal pH (5.39, 5.69, 5.99, respectively), reduced rumination time, and increased non-nutritive oral behaviour (Lin et al., 2018). In addition, hay-supplemented calves had a greater forestomach weight and ruminal volume (46.3% and 37.0% increase for calves hay-supplemented at 2 and 6 weeks of age, respectively) than calves without hay supplementation (Lin et al., 2018). On the other hand, in another study,

the authors concluded that calves fed hay earlier in life tended to have negative effects on growth, as well as decreased nutrient digestibility, while having similar ruminal fermentation and rumination time compared to calves fed hay later (Xiao et al., 2023). These controversial results may be associated with different nutritional strategies (i.e., restricted vs. non-restricted milk allowance), forage sources and allowance, forage particle size, feeding methods, and physical forms of calf starters (Diao et al., 2019; Nikkhah and Alimirzaei, 2022), suggesting the forage inclusion in pre-weaning dairy calves is a complex subject that needs to be further investigated.

The balance between forage allowance and source is essential to calf performance. Comparing weaned calves fed free-choice calf starter along with either 5% chopped hay or ad libitum long grass hay, calves fed ad libitum long grass hay showed a reduction of 17% in total DMI, 20% in ADG, and 23% in hip width compared to calves fed 5% chopped hay (Aragona et al., 2020). In another study, compared to non and 15% inclusion of chopped wheat straw in diets of pre-weaning calves, 7.5 % inclusion tended to improve total solid feed intake (659, 685, and 826 g/d, respectively) and ADG (519, 553, and 620 g/d, respectively; Hosseini et al., 2019). Collectively, those studies and others indicate benefits in feeding pre-weaning calves with lower levels of forage, limited to 10% of intake (Xiao et al., 2020).

The positive response to lower inclusion of forage is inconsistent in the literature, potentially attributed to forage quality differences (Xiao et al., 2020). Calves seem to prefer high-quality forage, such as grass or alfalfa, over low-quality forage, such as straw (Muruz and Aksu, 2024). For example, calves fed free-choice alfalfa hay had the highest forage intake (14% of solid DMI) compared to calves fed lower forage sources, resulting in inferior ADG and total DMI (Castells et al., 2012). These results reflect the preference for high-quality and palatable forages, which ultimately decreased calf starter intake (e.g., gut fill), limiting calf performance. In addition, given

that even straw, at a low intake of approximately 5%, can improve total DMI compared to calves fed 10% high-quality forage (Muruz and Aksu, 2024). Including 7.5% of low-quality forage (e.g. wheat straw) also seems to improve ruminal fermentation and growth performance, as it increases calf starter intake (Jalayerinejad et al., 2024). Also, in another study comparing different hay supplementation rates, calves fed 7.5% bromegrass hay tended to have higher ADG (1.22 vs. 1.02 kg) and greater feed efficiency (0.66 vs. 0.58 body weight gain/DMI) than calves fed 15% grass hay after weaning (Coverdale et al., 2004). Therefore, a balance between forage allowance and forage quality, 5 to 10% of high-quality forage (e.g., alfalfa), seems to be beneficial for pre-weaning dairy calves.

Furthermore, the forage particle size is another factor that can affect calf performance due to its potential effects in increasing chewing activity, which increases saliva production that, ultimately, buffers rumen fermentation (Xiao et al., 2020; Muruz and Aksu, 2024). Despite controversial results in the literature, overall, increased particle size (from 2 mm long up to 5 mm) of high-quality forage can increase calf starter intake, prevent stereotypical behaviors, improve nutrient digestibility, and promote better rumen development (Xiao et al., 2020; Muruz and Aksu, 2024); therefore, particle size is also a crucial aspect to be considered when feeding forage to young calves. Other aspects can also affect calves' response to forage provision, such as the time of forage introduction and the calf starter physical form. Studies have shown that providing alfalfa hay at the 2nd week of life improved DMI, growth performance, and rumen development compared to calves fed forage at 4th and 6th week of life (Xiao et al., 2020). In addition, forage provision separated from calf starter can be beneficial, especially for calves fed pelleted and fine ground calf starter (Xiao et al., 2020; Muruz and Aksu, 2024). In summary, providing forage to young calves can be beneficial if considering the forage source and allowance, particle size, method (e.g. mixed

or separated from calf starter) and introduction time. Along with limited forage provision, feeding calves in the first weeks of life with long forage particle size (e.g., 3 to 5 mm long) likely improves feed intake, growth rate, chewing activity, feeding sorting behaviors and maintains a healthy rumen environment, especially when they are fed a non-texturized calf starter (Xiao et al., 2020; Nikkhah and Alimirzaei, 2022; Muruz and Aksu, 2024).

1.3.5. Weaning transition management

Weaning transition is a considerable stressful experience for young calves (Enríquez et al., 2011; Carulla et al., 2023). In the natural environment, calves are weaned at about 10 months of age (Reinhardt and Reinhardt, 1981), while in the industry, calves are typically weaned at about 6 to 8 weeks of life (Fischer et al., 2019). Given the economic impacts associated with the pre-weaning period as well as the benefits of early ruminal development, as discussed previously, an early weaning transition, about 4 to 6 weeks of life, has been targeted for decades (Fischer et al., 2019). As such, milk allowance is restricted to 10% of BW, promoting greater calf starter intake and, consequently, rumen development, however, more recently, later weaning protocols have been studied (Fischer et al., 2019). Compared to calves weaned at 6 weeks, calves weaned at 8 weeks of life had greater ADG for the week pre-weaning (0.34 vs. 0.79 kg/d, respectively) and post-weaning (Eckert et al., 2015). In fact, in Canada, calves must be at least 8 weeks old before weaning is achieved (Dairy Code of Practice 2023 - National Farm Animal Care Council, 2023), and, in the United States, the mean age at weaning is 65.7 d of life (Urie et al., 2018b).

How the weaning transition is performed is also an essential aspect. In Canada, for example, it is also required to gradually wean the calves for at least 5 days (Dairy Code of Practice 2023 - National Farm Animal Care Council, 2023). While different protocols have been tested, a gradual decrease in milk allowance over a certain period is the concept behind the gradual transition

strategies (Fischer et al., 2019; Whalin et al., 2021), differently from the abrupt weaning protocols, in which there is a minimal transition period or, mostly, no transition period between pre- and post-weaning (Whalin et al., 2021). In the last decade, abrupt weaning was performed in only 16.5% of the Canadian herds (Vasseur et al., 2010), while gradual weaning protocols have been taken place (Fischer et al., 2019) given the increased growth rates, especially post-weaning, observed in calves gradually weaned (Steele et al., 2017). In addition, the duration of weaning transition is another important factor, in which calves over longer periods of weaning transition seem to have an increased calf starter intake (Welk et al., 2024). Therefore, weaning after 8 weeks of life, along with a gradual milk allowance reduction over 2 weeks of duration has been recommend to improve calf performance and health and meet the Canadian requirement.

Although age has been commonly a criterion for weaning transition, alternative methods, such as a minimum calf starter intake, have been proposed (Welk et al., 2024), in which calves should consume at least 1 kg/d of calf starter (Carulla et al., 2023). In fact, in a study comparing different calf starter allowances, calves fed 0.8 kg/d of calf starter for 3 consecutive days had the greatest DMI at weaning compared to calves fed lower amounts of calf starter, but the authors concluded that calves should consume at least 0.5 kg/d for 3 consecutive days in order to improve performance, nutrient digestibility and general health conditions (Ghassemi Nejad et al., 2013), suggesting that increased calf starter intake over the pre-weaning period is also important to minimize performance losses.

1.3.6. Calf nutrition summary

In summary, calf nutrition is crucial to maximizing performance throughout the lifetime. A high-quality colostrum provision is encouraged as early as 4 h after birth to potentialize the immune system development. If increased milk allowance, up to 8 L/d, is observed, then

osmolality should be less than 500 mOsm/kg. Furthermore, despite the broad use of calf starter, there has yet to be a consensus on its ideal characteristics. More processed forms, such as texturized calf starter, seem to affect calf performance better. Providing a small amount (5 to 10%) of high-quality forage, such as alfalfa hay, with long particle size – 3 to 5 mm long – at least at 2nd week of life has potential positive effects in calf performance and rumen development maximization. Regarding weaning strategies, it is recommended the implementation of step-down protocols over 2 weeks aiming for a calf fully weaned after 8 weeks of life. Despite the focus of calf nutrition management on calf performance and rumen development, more is needed to know about the effects of such strategies on the lower gut.

1.4. Anatomy & Physiology

1.4.1. Foregut

The GIT of ruminants is a massive metabolically active tissue responsible for digestion, nutrient absorption, and protection against pathogen entrance (Membrive, 2016). The GIT of ruminants has three forestomachs (rumen, reticulum, and omasum), abomasum, and lower gut (small intestine and large intestine; Hofmann, 1986; Clauss and Hofmann, 2014; Membrive, 2016; Meale et al., 2017).

In mature cattle, the rumen is the largest component of the forestomachs, representing approximately 64% of the total size of the forestomachs (Membrive, 2016; adapted from Dárce, 1977). The rumen is responsible for feed fermentation, maintaining a symbiotic relationship with microorganisms, avoiding pathogens' entrance into the bloodstream, and absorbing 50 to 85% of SCFA. The rumen epithelium has "finger-like" structures named papillae that are formed by the differentiation of ruminal epithelial cells, which increase the absorptive surface area (Arias et al., 1978; Baldwin et al., 2004; Steele et al., 2014). The rumen papillae, in turn, comprise a multilayer

stratified squamous epithelium that allows nutrient absorption and promotes protection against pathogens (Steele et al., 2016) and feed abrasiveness (Membrive, 2016). Attached to the rumen, the reticulum is mainly responsible for sorting the particles through the reticular-omasal orifice, potentially setting off the rumination process (Hofmann, 1986; Membrive, 2016). The omasum, however, is mainly responsible for nutrient and metabolite absorption and forwards the digesta into the abomasum that starts the protein digestion by enzymatic action, which is completed in the lower gut (Hofmann, 1986; Clauss and Hofmann, 2014; Membrive, 2016).

Contrary to mature cattle, young calves are nonfunctional ruminants whose abomasum is the largest and most functional compartment of the forestomachs; however, as calves get older, the rumen becomes the largest compartment (Khan et al., 2016; Meale et al., 2017; Diao et al., 2019). Beyond size, young calves' ruminal musculature, epithelium, and digestive mechanism are also underdeveloped as they supposedly consume milk in their first months of age only (Baldwin et al., 2004). As such, they present a structure named esophageal groove that forwards milk to the abomasum (Kaba et al., 2018; Diao et al., 2019). However, when solid feed is consumed, that structure is not “activated”, forwarding solid feed into the rumen instead of the abomasum (Jones and Heinrichs, 2017; Diao et al., 2019), which promotes rumen development (Diao et al., 2019).

1.4.2. Lower gut

Although the rumen has been the focus of many studies, little is known about the lower gut of ruminants (Plaizier et al., 2018; Sanz-Fernandez et al., 2020). Typically, the digestive mechanism of the lower gut of mature cattle has been considered similar to monogastric (Harmon, 2009; Brake and Swanson, 2018); however, the interaction between the rumen and the lower gut is unique. The small intestine (i.e., duodenum, jejunum, and ileum) has a columnar epithelium covered by crypts and villi constituted by a cell monolayer, where multiple specialized cells take

place, such as nutrient absorptive cells, mucus-producing cells (i.e., goblet cells), immune cells, and enteroendocrine cells (Peterson and Artis, 2014; Steele et al., 2016). Compared to the rumen, the small intestine is energetically more efficient (Huntington, 1995; Sanz-Fernandez et al., 2020), although the efficiency of the digestion mechanism in the small intestine of mature cattle is still debatable due to a potential enzymatic limitation (Huntington, 1995; Plaizier et al., 2018; Campos Rocha et al., 2022).

On the other hand, in pre-weaning calves, milk is the primary energy source, and it bypasses the rumen, reaching the abomasum straightly. It implies that, in calves, the small intestine is the leading site responsible for digestion and absorption, suggesting that the lower gut has a fundamental role in the physiology of young calves, a function potentially more critical than that of mature cows. In the small intestine, lactose, the most important component of milk, is enzymatically degraded into glucose and galactose to be absorbed (Meale et al., 2017), a digestive process assumed to be similar to monogastric animals (Diao et al., 2019). Also, the intestines of neonatal calves are able to absorb large macromolecules (e.g., IgG) from colostrum for the first 24 h of life (Meale et al., 2017), suggesting a vital role of those GIT sections in the calf's immune system.

In the cattle's small intestine, feed particles may not be completely digested, potentially reaching the large intestine (i.e., cecum, colon, and rectum), also known as the hindgut (Gressley et al., 2011; Sanz-Fernandez et al., 2020; Campos Rocha et al., 2022). In mature cattle, the hindgut has a bacterial population responsible for a second fermentation, which may correspond to 14% of the fermentation capacity of the rumen (Gressley et al., 2011). Although there is limited information, it has been suggested that there is an association between the microbial population of the large intestine and cattle health and performance (O'hara et al., 2020), where the microbiota

influences the mucosal immune response development, prevents intestinal infections (Malmuthuge et al., 2015), and produces SCFA that, along with branched-chain fatty acids (BCFA) such as isovalerate and isobutyrate (Gressley et al., 2011; Song et al., 2018; Xin et al., 2021) may contribute from 5 to 10% of the cattle dietary energy (Gressley et al., 2011). Moreover, the cattle hindgut microbiome seems to be very dynamic, being composed of digesta- and mucosa-associated bacterial populations which are influenced by several factors such as diet, age, management, and GIT section (Malmuthuge et al., 2012, 2015; Song et al., 2018), revealing to be a complex knowledge field that must be explored to improve the cattle's health and productivity. In pre-weaning calves, however, little is known about the hindgut physiological role. Although some studies have suggested, through fecal analyses, that young calves are active hindgut fermenters (Song et al., 2018; Kumar et al., 2021; Xin et al., 2021), whether hindgut fermentation plays a major role in calf digestion is still unknown.

1.5. Gut development

1.5.1. Foregut

Considering that calves, in their natural environment, are weaned approximately at 10 months of age (Reinhardt and Reinhardt, 1981) and that, in the dairy industry, they often are weaned approximately at 6-8 weeks of age, the calf rumen is typically underdeveloped at weaning transition (Fischer et al., 2019). In order to optimize rumen development, calf starter has been broadly added to the diet of calves (Meale et al., 2017; Palczynski et al., 2020), as it is a well-known source of rapidly fermentable carbohydrates that increases SCFA production, triggering rumen development (Aschenbach et al., 2011; Membrive, 2016; Diao et al., 2019). To evaluate rumen development, histomorphometric analyses have been performed (Lesmeister et al., 2004; Steele et al., 2014; Diao et al., 2019), as the ruminal papillae have been associated with increased

SCFA absorptive capacity (Dieho et al., 2016). Although rapidly fermented carbohydrates are beneficial for rumen development in young calves, it is known that the excess of grains in diets of high-producing dairy cows may induce an imbalance between H^+ removal and production caused by the accumulation of SCFA (Aschenbach et al., 2019). Such a scenario may jeopardize ruminal barrier function through ruminal acidification that damages the epithelial cells, leading to increased permeability of the epithelium (Aschenbach et al., 2019; Baaske et al., 2020); however, whether those adverse effects matter for young calves, it is not entirely known.

1.5.2. Lower gut

The lower gut development also can be affected by the increased grain intake. In SARA conditions, inflammatory responses may happen potentially damaging the ruminal epithelium, hence decreasing ruminal digestibility (Gressley et al., 2011; Plaizier et al., 2018). That condition, along with the usage of small particle size (i.e., concentrate) that increases the passage rate, may contribute to increasing starch and SCFA flow to intestines, potentially leading to hindgut acidosis (Gressley et al., 2011; Plaizier et al., 2018; Sanz-Fernandez et al., 2020), a condition where the accumulation of organic acids (e.g., SCFA, lactic acid, BCFA) originated from the excess of fermentation reduces digesta pH, leading to the shift of the hindgut microbiota and damages the intestinal epithelium (Gressley et al., 2011; Köhler, 2020; Sanz-Fernandez et al., 2020), however, there is no defined pH threshold described in the current literature that suggests hindgut acidosis (Köhler, 2020).

Although the relationship between ruminal and hindgut acidosis is not clear, in fact, some symptoms associated with ruminal acidosis, such as diarrhea and the presence of blood and mucin casts in the feces, actually arise from intestinal epithelium damage (Gressley et al., 2011; Sanz-Fernandez et al., 2020), suggesting a potential association between ruminal and hindgut acidosis.

Suarez-Mena et al. (2015) found that as calves aged, their fecal starch concentration increases as a response to increased starter intake, causing an increase in passage rate. They also hypothesized that lower rumen pH could affect starch digestion in the small intestine due to the potential effect of rumen pH on the digesta, which may become more acid than the small intestine is able to buffer, decreasing its enzymatic activity, which may decrease small intestine digestibility and, therefore, increasing the starch concentration in the large intestine, explaining such fecal starch increasing.

However, despite the efforts, to the best of our knowledge, the controlling and monitoring of hindgut fermentation patterns through nutritional strategies has yet to be described in the literature for dairy calves. On the other hand, in mature cows, the modulation of gut functionality has been achieved by changing the dietary composition. For instance, the proportion of dietary corn and barley grains has been associated with changes in the site where feed is mostly fermented (Oba and Kammes-Main, 2023), in which increased corn proportions increase the lower gut fermentation, as corn has a lower ruminal digestibility. In comparison, barley grain has greater ruminal digestibility and is primarily fermented in the rumen (Oba and Kammes-Main, 2023). Therefore, if corn grain intake provokes increased lower gut fermentation in mature cows, corn-based calf starter might benefit younger calves as they are more intestinal dependent. While some studies have shown that a barley-grain-based diet increased ruminal fermentation and feed efficiency compared to a corn-based diet in dairy calves (Kazemi-Bonchenari et al., 2020), whether and how such dietary changes affect the hindgut of calves during the pre-weaning period is unclear.

Along with the lack of information on how to nutritionally modulate hindgut fermentation, the potential impacts of hindgut acidosis on health and performance in pre-weaned calves still need to be determined. Some recent studies have been investigating molecular expression associated with gut development and cecal digesta/feces analyses, such as pH, starch, lactic acid, BCFA, and

SCFA concentrations to understand further hindgut fermentation (Kumar et al., 2021; Campos Rocha et al., 2022; Poier et al., 2022). In terms of molecular expression, some studies have proposed analysis of microRNAs (miRNAs) associated with gut development, GLP-1 and 2 blood concentration (Steele et al., 2016; Meale et al., 2017) and GLP-2 receptor (GLP2R) mRNA expression (Taylor-Edwards et al., 2010; Connor et al., 2015; Sun et al., 2018, 2019) and protein expression (Connor et al., 2015) as potential gut development indicators. Together, the findings above suggest a potential role of molecular expression as an indicator of intestinal epithelium development in dairy cattle, which is affected by nutritional management, especially in pre-weaned calves, which are most intestinal-dependent. In addition, given the fermentative process that occurs in the calves' GIT that results in SCFA production and pH reduction, the gene expression of SCFA transporters and epithelium cell homeostasis-related genes have been evaluated (Connor et al., 2010b; Naeem et al., 2012), which may reveal essential information on impacts of SCFA and pH in calves' gut epithelium physiology.

1.5.3. Nutrient metabolism: SCFA absorption & pH regulation

One of the most markable features of ruminants is their capacity to degrade complex carbohydrates, making them highly efficient animals. Given the rumen environment characteristics such as pH ranging from 5.5 to 7, minimum oxygen concentration, and the constant presence of substrate, the rumen is rich in a variety of microorganisms such as bacteria, archaebacteria, protozoa and fungi (Membrive, 2016). Among the ruminal microbial population, bacteria have received significant attention due to their role in feed fermentation, mainly carbohydrates. As end-products of this fermentation, SCFA, such as acetate, propionate, and butyrate, are produced, playing a pivotal role in supporting the energy demand of ruminants (Russell et al., 1992; Suárez et al., 2006). In a healthy GIT, nutrients are mostly transcellularly absorbed while cell junctions

seal the paracellular route (i.e., the space between adjacent cells), a process known as barrier function (Turner, 2009; Aschenbach et al., 2019). Both ruminal and intestinal epithelium have junction structures composed of desmosomes, adherence junction, and tight junction proteins (Turner, 2009; Steele et al., 2015, 2016), acting avoiding pathogenic microbes and their toxins entering the bloodstream that are known to lead to local and systemic inflammatory responses (Aschenbach et al., 2019; Sanz-Fernandez et al., 2020).

While a healthy ruminal epithelium provides a protection against pathogens, it also responsible for absorption of nutrients and metabolites such as SCFA. From a general perspective, the SCFA absorption in the rumen consists of two steps: 1) crossing from the lumen to epithelium through the mucosal (apical) side; 2) crossing from the epithelium to the bloodstream through the serosal (basolateral) side (Penner, 2019; Baaske et al., 2020). There are several pathways through which SCFA can cross the epithelium on both sides, depending on the SCFA molecular structure. Briefly, SCFA are presented in the ionized form ($SCFA^-$) and protonated form (H–SCFA), associated with H^+ (Aschenbach et al., 2011; Millen et al., 2016). The latter is absorbed from the lumen through lipophilicity in a passive diffusion process, while $SCFA^-$ cross the epithelium by several bicarbonate-dependent and independent protein-transporters such as anion exchanger 2 (AE2), downregulated-in-adenoma (DRA), putative anion exchanger 1 (PAT1), and monocarboxylate transporter 1 (MCT1), 2 (MCT2) and 4 (MCT4; Petri et al., 2019; Baaske et al., 2020).

Moreover, the proportion of these different forms of SCFA is luminal-pH-dependent (Millen et al., 2016). SCFA are weak acids ($pK_a = 4.8$), which means that, at pH 4.8, 50% of SCFA are in H–SCFA form (Millen et al., 2016; Penner, 2019). In this sense, in the rumen of healthy cows, $SCFA^-$ should be the predominant form, as ruminal pH does not commonly reach such a

threshold (Bergman, 1990; Millen et al., 2016). Interestingly, the optimal ruminal pH has been shown to be different between mature cows and calves. The ruminal pH of calves commonly achieves a pH of 5.8 or lower (Diao et al., 2017; van Niekerk et al., 2021b; Parsons et al., 2022), a threshold associated with SARA in mature cows, a condition of low ruminal pH whose thresholds are commonly assumed to remain from 5.5 to 5.8, although this definition is still debatable (Humer et al., 2018; Plaizier et al., 2018; Hossain, 2020). Beyond that, possibly lower ruminal pH in young calves even positively affects their performance, proving a faster rate of increased calf starter intake (Laarman et al., 2012) and increasing average daily gain (McCurdy et al., 2019). These physiological differences between mature cows and calves may mean that cows whose ruminal pH used to be higher may have a greater percentage of $SCFA^-$, which means predominancy of protein-transporter absorption pathways, while passive diffusion may represent greater importance for pre-weaned calves, as their ruminal pH seems to be lower, a condition associated with a higher percentage of H–SCFA. In addition, such mechanisms could also be seen in the lower gut. The intestinal pH, especially in the distal sections, is higher than the calf's ruminal pH, possibly representing transporter-dependence for SCFA absorption in lower gut sections. In fact, many studies have found SCFA transporters in the lower gut (Kirat and Kato, 2006; Kirat et al., 2006, 2007; Connor et al., 2010b; Guilloteau et al., 2010; Stumpff, 2018; Zhan et al., 2018), however whether different ruminal pH and SCFA concentrations may affect the SCFA transporters in the lower gut is still unknown, particularly in pre-weaned calves.

In addition, when SCFA are produced, hydrogen ions (H^+) are released within the lumen, contributing to decreased luminal pH (Aschenbach et al., 2011; Millen et al., 2016; Penner, 2019). One of the strategies to avoid that is H^+ absorption through epithelial cells, which reduces the concentration of free H^+ in the lumen (Aschenbach et al., 2011; Millen et al., 2016). However,

within the epithelium cells, H^+ accumulation may decrease the intracellular pH (pH_i), and then acid-base transporters are responsible for H^+ uptake from the lumen as well as H^+ exportation to the bloodstream or returning them to the lumen (Aschenbach et al., 2011; Stumpff, 2018). For example, beyond some SCFA absorptive transporters (AE2 and bicarbonate-dependent protein transporters), Na^+/H^+ exchanger isoform (NHE) 1, 2, and 3 (Laarman et al., 2012) and Na^+/Bicarbonate cotransporter (NBC) have been associated with intracellular pH regulation (Stumpff, 2018; Petri et al., 2019; Baaske et al., 2020), indicating a complex cell homeostatic mechanism to preserve cell integrity.

In summary, while increased feed fermentation may lead to SARA and hindgut acidosis in mature cows, little is known about the impacts of increased fermentation on the GIT of pre-weaning calves, especially in the lower gut. Whether calves may experience such disorders while still in a rumen-underdeveloped stage is unclear. Therefore, understanding the fermentation profile of feedstuffs and gut physiology is fundamental to maximizing ruminant performance and health.

1.6. Knowledge gap

Even when investigating dairy calves' physiology, the focus and objectives are primarily associated with making calves, as early and as much as possible, similar to mature cows through promoting rumen development. Calves, however, are not miniature cows. Significant physiological differences, such as calves' lack of rumen function and reliance on the small intestine for nutrient digestion and absorption, mean conclusions from cows may be inappropriately extrapolated to calves. In calves, the interaction between the rumen and the lower gut, especially the hindgut, is unclear. Building our understanding of how ruminal SCFA and pH may impact the hindgut in calves throughout the pre-weaning period may improve nutritional and management strategies that meet the singular physiological requirements of pre-weaned calves. The general

hypothesis of this study is that ruminal environmental changes can be reflected in the lower gut in pre-weaning dairy calves, leading to hindgut acidosis. Therefore, the first general objective of this study is to investigate how ruminal environmental changes affect the hindgut physiology via ruminal infusion of different SCFA concentrations and pH. In addition, the second general objective is to evaluate the risk and impact of such infusions on the incidence of hindgut acidosis in pre-weaning dairy calves.

2. Effects of single-dose ruminal infusions of high or low short-chain fatty acid concentrations and high or low pH on apparent total tract digestibility and hindgut fermentation of pre-weaned dairy calves

2.1. Abstract

While the importance of pH and short-chain fatty acids (SCFA) on rumen development are well-known, their impact on the small and large intestine are unclear. This study investigated how single-dose ruminal infusions with high or low short-chain fatty acid concentrations and high or low pH affect dairy calves' productivity as well as physiological parameters associated with hindgut acidosis at three-time points in 49 days. Holstein bull calves (n=32) were individually housed and fed milk replacer (900 g/d) twice daily and *ad libitum* calf starter and water. At d 10 ± 3 of life, the rumens were fistulated and cannulated. At d 14 of life, calves were grouped by body weight and assigned in randomized complete block design with a 2 × 2 factorial arrangement of treatments: high or low [SCFA] (285 vs. 10 mM) and high or low pH (6.2 vs. 5.2), creating four treatment groups: high [SCFA], high pH (HS-HP); high [SCFA], low pH (HS-LP); low [SCFA], high pH (LS-HP); and low [SCFA], low pH (LS-LP). On d 21, 35, 49, feces were sampled to calculate apparent total tract digestibility, determinate organic acid concentrations (i.e., SCFA, BCFA and lactic acid), and pH. Afterward, the rumen was evacuated and underwent a single-dose infusion for 4 h with one of four treatment buffers. After completion of rumen infusion on d 49, calves were harvested, and the tissue weight and length, and digesta pH of the rumen, cecum, colon, and rectum were recorded along with the digesta pH of duodenum, jejunum, and ileum only at d 49 after dissection. Data were analyzed with main factors as fixed effects and repeated measures for weekly measurements. Treatments did not affect performance parameters such as feed intake, average daily gain, apparent total tract digestibility and gut measurements. In the duodenum, jejunum, and ileum, HS-HP had a greater digesta pH than LS-HP, while the hindgut

digesta pH was only affected by the [SCFA]. High [SCFA] increased the concentration of colonic isovaleric acid and fecal branched-chain fatty acids (BCFA), while only colonic acetic acid and fecal lactic acid concentrations were lower in the HS-LP group. Fecal SCFA and BCFA concentrations increased mainly on d 35. In conclusion, 4 h of physiological buffer infusion in the rumen does not change apparent total tract digestibility and gut measurements but does affect hindgut fermentation parameters (i.e., organic acid concentrations and digesta pH). In addition, calves can experience increased risks of hindgut acidosis around 35 days of life; therefore, understanding the effects of calves' ruminal development on hindgut physiology is encouraged.

2.2. Introduction

Calves are an important product of the cattle industry as they will produce milk and meat in their adult life. Despite that, dairy calves' morbidity and mortality rates may achieve 33.9% and 5%, respectively (Urie et al., 2018a), and their first 2 weeks of life are the most critical period (Wells et al., 1997; Urie et al., 2018a). Of the causes of morbidity, the incidence of digestive disorders is a major challenge (Wells et al., 1997; United States Department of Agriculture, 2018; Urie et al., 2018a). Currently, calf nutritional management has been focused mainly on rumen development, as calves are weaned approximately at 6-8 weeks of age, when their rumen is typically underdeveloped (Fischer et al., 2019). As such, calf starter has been broadly used (Meale et al., 2017; Palczynski et al., 2020), as it is a well-known source of rapidly fermentable carbohydrates that increases short-chain fatty acid (SCFA) production (Aschenbach et al., 2011; Membrive, 2016; Diao et al., 2019). Despite promoting rumen development, the effects of calf starter intake on the lower gut are not completely understood. In a study comparing calves fed calf starter with high or low starch concentration (35.6% vs. 12% DM, respectively), cecal pH was higher in calves fed high starch concentration, and no differences were found in inflammation

markers, despite the proposed increase in hindgut fermentation (Yohe et al., 2022). However, while in high-producing dairy cows, the excess intake of grains may lead to disorders that negatively affect their health and performance due to changes in the rumen and hindgut environment, in pre-weaned calves such effects, especially in the hindgut, are unclear.

Within those disorders associated with grain intake, subacute ruminal acidosis and hindgut acidosis are well-documented in mature cows. Briefly, carbohydrate fermentation releases protons that decrease luminal pH (Aschenbach et al., 2011), causing feed intake reduction, laminitis, and liver abscesses (Plaizier et al., 2018; Hossain, 2020; Pinedo and Melendez, 2022). This process can also decrease ruminal digestibility due to damage in the epithelium (Gressley et al., 2011; Plaizier et al., 2018). The decreased ruminal digestibility, along with the usage of small feed particle size (i.e., concentrate), increases the passage rate, which contributes to an increase in the starch and SCFA flow to intestines, potentially leading to hindgut acidosis (Gressley et al., 2011; Plaizier et al., 2018; Sanz-Fernandez et al., 2020). This disorder is characterized by the accumulation of organic acids such as SCFA, branched-chain fatty acid (BCFA, such as isobutyric and isovaleric acids), and lactic acid, which originate from the excess of fermentation, causing digesta pH reduction, hindgut microbiota shift, and intestinal epithelium damage (Gressley et al., 2011; Sanz-Fernandez et al., 2020). Despite the efforts, in the literature, there is no organic acid concentration or pH value that characterizes hindgut acidosis (Köhler, 2020; Sanz-Fernandez et al., 2024).

Although the relationship between ruminal and hindgut acidosis is unclear, some symptoms associated with ruminal acidosis, such as diarrhea and the presence of blood and mucin casts in the feces, are from intestinal epithelium damage (Gressley et al., 2011; Sanz-Fernandez et al., 2020), suggesting a potential association between ruminal and hindgut acidosis. Pederzolli et

al. (2018) found that steers with ruminal acidosis had lower digesta pH in the rumen but also in the cecum and proximal colon. Additionally, Suarez-Mena et al. (2015) found that lower rumen digesta pH could affect starch digestibility in the small intestine, which may become more acidic than the small intestine is able to buffer, causing a reduction in the small intestine enzymatic activity, thus decreasing small intestine digestibility. They suggested that this physiological change could increase the starch concentration in the large intestine, explaining the observed increased fecal starch concentrations in their study.

To further understand hindgut fermentation, some recent studies have been investigating hindgut digesta and fecal parameters such as pH, starch, and organic acid concentrations (i.e., lactic acid, BCFA and SCFA; Kumar et al., 2021; van Gastelen et al., 2021; Poier et al., 2022). It has been proposed that pre-weaned calves are active hindgut fermenters, presenting increased BCFA and SCFA concentrations and decreased fecal pH, ultimately characterizing greater hindgut fermentation. While the effects of known SCFA concentrations have been tested in the calves' rumen through ruminal physiological buffer infusions (Yohe et al., 2019), to the best of our knowledge, the impacts of the ruminal infusions on the hindgut functionality of pre-weaning calves were not tested, which might give an insight of the effects of rumen environmental changes on the lower gut functionally and calves' performance. Physiological responses in the rumen, especially pH, varies wildly in calves given the same dietary treatment (Laarman et al., 2012; McCurdy et al., 2019), so a dietary approach may result in wide variability in rumen environments.

This study aims to investigate how single-dose ruminal infusions with high or low short-chain fatty acid concentrations ([SCFA]) and high or low pH affect physiological hindgut fermentation parameters and performance parameters, such as apparent total tract digestibility, feed intake and average daily gain (ADG) of pre-weaned calves. Due to the close association

between SCFA and pH regarding their physiological effects on ruminants, we hypothesize that the interaction of high ruminal [SCFA] and low pH (HS-LP) will increase the risk of hindgut acidosis in pre-weaning calves, leading to decreased digesta and fecal pH, increased digesta and fecal organic acid concentrations (i.e., SCFA, BCFA, and lactic acid), and decreased apparent total tract digestibility.

2.3. Materials and Methods

2.3.1. Animal & Housing

This study was approved by the Institutional Animal Care and Use Committee at the University of Alberta (AUP#00003823). Holstein bull calves (n = 32; body weight = 42.17 ± 4.49 kg) from a commercial farm were fed 4 L of colostrum: the first feeding (2 L) was within the first hour after birth, followed by 2 more feedings (1 L each) every 12 h. Then, calves were transported in the first week of life to the Metabolic Unit Research Station at the University of Alberta, Edmonton, Canada, in a temperature-humidity monitored barn and they were housed in individual pens (4 m × 3 m) with rubber mat flooring covered with shavings. When calves completed 14 days of life, the shavings were removed, and extra rubber mats were added to provide extra cushioning. In the first seven days of life, they were fed milk replacer (Table 2.1) twice daily up to 600 g (150 g/L) and 900 g throughout the remaining experimental period (i.e., no weaning occurred), along with free access to calf starter and water. Feed intake and body weight were recorded daily and weekly, respectively. Calf health was monitored daily based on the recommendations of the attending veterinarians, calf morbidities were either treated, or calves were removed from the study.

2.3.2. Experimental Design

The rumens of Holstein bull calves (n = 32) were fistulated and cannulated at d 10 ± 3 of life (Castillo and Hernández, 2021). At d 17 of life, they were blocked by body weight and randomly assigned via Excel randomizer in a 2×2 factorial arrangement of treatments (n = 8): high [SCFA] (285mM; Yohe et al., 2019) vs. low [SFCA] (10 mM; Schurmann et al., 2014), and high pH (6.2) vs. low pH (5.2), creating four treatment groups: high [SCFA], high pH (HS-HP); high [SCFA], low pH (HS-LP); low [SCFA], high pH (LS-HP); and low [SCFA], low pH (LS-LP). The SCFA mixture was a 50:35:15 mixture of acetate:propionate:butyrate (Yohe et al., 2019), and pH and osmolality were adjusted with sodium gluconate and sodium hydroxide.

On days 21, 35, and 49 of life, after the morning feeding (about 10:00h), calves were submitted to the reticulorumen-washing technique as described by Yohe et al. (2018; 2019). Briefly, a vacuum device was built to remove the rumen digesta content; at the same time, approximately 50 ml of digesta were sampled and stored at -80°C. After which, 3 L of a wash buffer (Table 2.2) was poured through the cannula with a tube connected to a funnel followed by aspiration with the vacuum device. That procedure was performed three times (1 L of wash buffer per time) to maximize the digesta removal. Subsequently, a known amount (from 1 to up to 3 L) of physiological buffer containing one of four SCFA buffer treatments was infused in a single dose for 4 h, as rumen nadir pH following AM feeding is reached between 3-4 h post-prandial (Laarman et al., 2012; Suarez-Mena et al., 2016a). Although all infusions were performed in a single dose at their respective time points (e.g., d 21, 35 and 49), the volume of the physiological buffer was dependent on the rumen volume capacity of each calf, which was determined based on visual observation through the cannula. Thus, as calves grew and their rumen developed and increased

in volume capacity, the volume of the single-dose infusion increased up to 3 L. After the 4 h infusion, calves had free access to water and calf starter.

Fecal samples were taken by rectal palpation 24 h before (pre-treatment) and 48 h after (post-treatment) infusion as well as every 2 h over the infusion period (at 0 h, 2 h, and 4 h). Immediately after collection, a fecal subsample was mixed with distilled water in a 1:1 ratio (Poier et al., 2022) to measure fecal pH (Mettler Toledo FiveGo F2 pH/mV Meters, Catalog No. 01-912-358, Fisher Scientific). The remaining samples from pre- and post-treatment were divided into 2 subsamples: one was frozen at -80 °C for determination of organic acid concentrations (i.e., SCFA, BCFA, lactic acid), and the other one was pre-dried at 55 °C for apparent total tract digestibility estimation.

Calves were not weaned prior to euthanasia on d 49. Within 1h of completion of the pulse infusion, calves were harvested by captive bolt and exsanguination, followed by gastrointestinal tract removal from the body cavity. The intestinal sections were identified and cable-tied to mitigate digesta translocation, after which the digesta collection points were identified and ziplocked (defined as: duodenum = 0 - 100 cm caudal to the pyloric sphincter; jejunum = 100 cm caudal from the darker-pink, twist-shaped segment that differs from duodenum morphological characteristics; ileum = 100 cm cranial to the proximal ileocecal junction; cecum = ligature at the base of the main intestinal segment; colon = 100 cm caudal from the cecal-colonic junction; rectum = 10 cm cranial from the anus; (adapted from Cangiano et al., 2023). Next, the hindgut sections were separated, and cecum, colon, and rectum weights and lengths and rumen weight were recorded. Digesta samples were collected from each intestine section accordingly with the respective digesta collection point. The digesta sample's pH was measured immediately after collection, and the samples were stored in 50 ml conic plastic tubes and frozen at -80°C.

2.3.3. Laboratory Analysis

Fecal samples from the pre-treatment period, ruminal digesta, and intestinal section digesta samples collected at the time of harvest were analyzed by gas chromatography (Varian 430 GC with FID detector, and Stabilwax-DA column) for determination of organic acid concentrations such as SCFA (acetic, propionic, and butyric acids), BCFA (isobutyric and isovaleric acids) and lactic acid, as hindgut fermentation indicators, along with the hindgut digesta and fecal pH (Castro et al., 2016; Kumar et al., 2021; Virgínio Júnior and Bittar, 2021). The dry matter (DM; calculated by the sample weight differences before and after overnight drying at 110 °C), organic matter (OM; calculated as 100 - ash content), protein (Organic elemental analyzer, Flash 2000, Thermo Fisher Scientific, Mississauga, Ontario, Canada), fat (hexane fat extraction), and starch (AOAC Official Method 996.11) content of feed samples and pre-dried fecal samples from pre-treatment and post-treatment of d 21 and 35 were assessed to evaluate the apparent total tract digestibility (van Niekerk et al., 2020; Barnett et al., 2022; Xu et al., 2022) using acid-insoluble ash as internal marker (van Niekerk et al., 2020; Du et al., 2021; Xu et al., 2022).

2.3.4. Statistical Analysis

The experimental unit was the individual calf. By assigning 8 animals per treatment, a priori power analysis estimated that the data analysis can detect a 10% difference between means of treatments with a coefficient of variation of 10%, with 80% power (Berndtson, 1991). To test the model fit, normality was verified in the SAS Software (SAS Institute Inc., Cary, NC, USA.) with PROC UNIVARIATE along with lognormal and gamma distributions. The best-fit distribution was used in PROC GLIMMIX with SCFA and pH as main fixed effects and block (body weight) and day as random effects. Tukey adjustments were made for multiple comparisons. Significance was declared at < 0.05.

The statistical model is provided below:

$$Y = \mu + S_i + P_j + S \times P_{ij} + B_k + D_l + D \times S \times P_{lij} + D \times S_{li} + D \times P_{lj} + \varepsilon_{ijklm}$$

S_i is the effect of SCFA, P_j is the effect of pH, B_k is the random block effect, D_l is the effect of day, and ε_{ijklm} is the error term.

For weekly measurements (e.g., feed intake, ADG, digestibility, and fecal and rumen digesta pH), repeated measures were performed by adding time effect (D_l) and its interactions to the model as a fixed effect. As such, along with the distribution test, the model fit was tested by the Akaike information criterion testing four covariant structures. The one with the smallest AIC was used. Time × treatment interactions were reported. The LSM and SEM were reported for normal data (e.g. ADG, tissue weight and length, rumen, intestinal digesta and fecal pH, ruminal isobutyric, isovaleric, and lactic acid concentrations, and fecal acetic, propionic, isobutyric, lactic acid concentrations, cecal organic acid concentrations, and colonic acetic, propionic, butyric, isobutyric acid concentrations), and the geometric means and CI were reported for non-normal data for legibility (lognormally distributed = milk replacer and calf starter intake, apparent total tract digestibility, ruminal acetic, propionic, butyric acid concentrations, fecal butyric and isovaleric acid concentrations, and colonic isovaleric and lactic acid concentrations). Outliers were not identified.

Our treatment group's initial body weight averages were HS-HP: 44.76 ± 4.02 kg, HS-LP: 46.20 ± 4.7 kg, LS-HP: 44.74 ± 4.63 kg, and LS-LP: 44.73 ± 6.14 kg. Body weight was not different among treatments.

2.4. Results

2.4.1. Feed Intake & Average Daily Gain (ADG)

Total milk replacer intake and calf starter intake were unaffected by treatment (*P = 0.55* and *P = 0.85,* respectively; Table 2.3). Calf starter intake and ADG were unaffected by the interaction of time × treatment (*P* = 0.45 and *P* = 0.11*,* respectively) but they increased over time (calf starter: d 21 = 0.015 kg/d (CI = 0.012 to 0.017), d 35 = 0.09 kg/d (CI = 0.073 to 0.113), d 49 = 0.23 kg/d (CI = 0.178 to 0.274); *P* < 0.01); ADG: d 21 = 0.23 kg (CI = 0.191 to 0.269), d 35 = 0.60 kg (CI = 0.561 to 0.639), and d 49 = 0.77 kg (CI = 0.731 to 0.809); SEM = 0.1; *P* < 0.01*;* Figure 2.1).

2.4.2. Gut Measurements & Apparent Total Tract Digestibility

No treatment differences were found in rumen weight (*P* = 0.65) and cecum weight (*P* = 0.42) and length (*P* = 0.20), colon weight (*P* = 0.14) and length (*P* = 0.65), and rectum (*P* = 0.14) and length (*P* = 0.43; Table 2.4). The apparent total tract digestibility was unaffected by SCFA × pH × time interaction (DM – *P* = 0.55; OM – *P* = 0.72; protein – *P* = 0.54; fat – *P* = 0.82; starch – *P* = 0.97; Table 2.5).

There was no difference in apparent total tract digestibility between pre- and post-treatment (long-term effects) on d 21 (digestibility pre- and post-treatment, respectively: DM = 91.71 and 97.49, SEM = 2.39, *P* = 0.45; OM = 79.12 and 85.81, SEM = 11.72, *P* = 0.66; protein = 83.56 and 91.37, SEM = 2.77, *P* = 0.23; fat = 82.61 and 91.17, SEM = 4.81, *P* = 0.32; starch = 97.79 and 98.79, SEM = 1.12, *P* = 0.64) and d 35 (digestibility pre- and post-treatment, respectively: DM = 94.41 and 98.31, SEM = 0.36, *P* = 0.20; OM = 85.11 and 89.65, SEM = 1.58, *P* = 0.14; protein = 70.08 and 82.90, SEM = 2.45*, P* = 0.11; fat = 97.10 and 96.28, SEM = 0.54, *P* = 0.37; starch = 97.64 and 98.91, SEM = 0.27, *P* = 0.22). In addition, no day differences were observed on d 21, d

35, and d 49 (digestibility on d 21, d 35, d 49, respectively: DM = 92.38, 94.05, and 97.10, SEM = 7.50, *P* = 0.54; OM = 80.35, 85.11, and 84.27, SEM = 12.32, *P* = 0.90; protein = 87.64, 74.69, and 79.66, SEM = 9.97, *P* = 0.36; fat = 91.64, 97.23, and 95.49, SEM = 4.68, *P* = 0.27; starch: 98.07, 97.61, and 98.78, SEM = 1.54; *P* = 0.44).

2.4.3. Fermentation Profile

No SCFA × pH × time effect was found in rumen digesta and fecal pH (*P* = 0.55 and *P* = 0.60*,* respectively*)*. Similarly, there was no SCFA × pH effect of those variables (*P* = 0.42 and *P* = 0.14, respectively; Table 2.6), but fecal pH was higher in calves in the high [SCFA] groups (High [SCFA] = 7.27 (CI = 7.22 to 7.32), Low [SCFA] = 6.75 (CI = 6.70 to 6.80); SEM = 0.13; *P* < 0.01). Rumen digesta pH was higher on d 21 (CI = 6.50 to 6.66) than on d 49 (CI = 6.04 to 6.2; SEM = 0.20; *P* < 0.01), while fecal pH was lower on d 21 (CI = 6.95 to 7.07) than on d 49 (CI = 7.33 to 7.45; SEM = 0.16; *P* = 0.03). Ruminal organic acid concentrations (SCFA, BCFA, lactic acid) were not affected by SCFA × pH × time interaction. Regarding age effects, ruminal lactic acid concentration was lower on d 49 (CI = 2.16 to 2.4; SEM = 0.30; *P* < 0.01), while SCFA (acetic - CI = 8.56 to 12.4; SEM = 4.89; propionic - CI = 2.3 to 4.74; SEM = 3.10; and butyric acids - CI = 0.102 to 1.72l; SEM = 2.06) were lower on d 21 (*P* < 0.01) and ruminal BCFA (isobutyric - CI = 0.306 to 0.354; SEM = 0.06l; *P* = 0.05; and isovaleric - CI = 0.286 to 0.334; SEM = 0.06; *P* = 0.04) concentrations were higher on d 35 and lower in calves submitted to low buffer pH (mean = 0.34; CI = 0.32 to 0.35; 0.04; *P = 0.04,* and mean = 0.32; CI = 0.304 to 0.336; SEM = 0.04; *P* = 0.03, respectively).

The digesta pH of the small intestine sections was different among the treatments: the group HS-HP had a greater duodenum (mean = 6.03; CI = 5.91 to 6.15; SEM = 0.31; *P* = 0.05), jejunum (mean = 6.22; CI = 6.05 to 6.39; SEM = 0.44; *P* = 0.04), and ileum digesta pH (mean = 7.38; CI

= 7.34 to 7.42; SEM = 0.11; $P < 0.01$), compared to the group LS-HP (mean = 6.97; CI = 6.93 to 7.01; SEM = 0.11; $P < 0.01$), which also had lower ileum digesta pH compared to the group LS-LP (Figure 2.2). The digesta pH of the hindgut sections (cecum, colon, and rectum) was not affected by treatments, but calves submitted to high [SCFA] had increased digesta pH in the colon (mean = 6.98; CI = 6.91 to 7.05; SEM = 0.17; $P < 0.01$) and rectum (mean = 7.00; CI = 6.92 to 7.09; SEM = 0.22; $P < 0.01$), while cecum digesta pH tended to increase (mean = 6.49; CI = 6.42 to 6.55; SEM = 0.17; $P = 0.06$).

Cecal and colonic digesta organic acid concentrations were not affected by treatment, except for colonic acetic acid, in which the HS-HP (mean = 53.2; CI = 50.1 to 56.2) group had a greater acetic acid concentration compared to the HS-LP group (mean = 36.8; CI = 33.7 to 39.8; SEM = 7.86; $P = 0.05$; Table 2.7). Calves in the high [SCFA] group had greater colonic isovaleric acid concentration (mean = 0.51; CI = 0.463 to 0.557; SEM = 0.12; $P = 0.05$).

Fecal pH decreased in the short-term (over the 4 h infusion) on d 35 and d 49 ($P < 0.01$ and $P = 0.04$, respectively; Figure 2.3); however, it was not affected in the long-term (pre- and post-treatment) on d 21 (fecal pH pre- and post-treatment, respectively: 7.00 and 7.26, SEM = 0.15, $P = 0.09$) and d 35 (fecal pH pre- and post-treatment, respectively: 7.32 and 7.32, SEM = 0.13, $P = 0.98$). Fecal SCFA and BCFA were not affected by treatment × time, but the lactic acid concentration of HS-LP was lower (mean = 3.71; CI = 3.56 to 3.86) than LS-LP (mean = 4.75; CI = 4.60 to 4.90; SEM = 0.39; $P < 0.01$), and fecal BCFA concentrations (isobutyric – mean = 1.01; CI = 0.973 to 1.05; SEM = 0.10; $P < 0.01$; and isovaleric – mean = 0.58; CI = 0.54 to 0.617; SEM = 0.10; $P < 0.01$) were greater in calves submitted to high [SCFA]. Fecal organic acid concentrations were affected by age: fecal lactic acid concentration decreased over time ($P < 0.01$); on d 35, calves presented higher acetic (mean = 17.78; CI = 17.20 to 18.40; SEM = 1.59; $P < 0.01$*)*

and butyric acid (mean = 2.58; CI = 2.42 to 2.74; SEM = 0.41; $P < 0.01$) concentration; and, on d 21, BCFA concentrations (isobutyric - mean = 0.60; CI = 0.553 to 0.647; SEM = 0.12: $P < 0.01$; and isovaleric - 0.21; CI =0.143 to 0.277; SEM = 0.17; $P < 0.01$) were lower when compared to d 35.

2.5. Discussion

Despite the hindgut fermentation-related physiological changes (i.e., modification in the intestinal digesta pH and organic acid concentrations) after the ruminal infusion with SCFA buffers, indicators of performance such as calf starter intake, ADG, apparent total tract digestibility (i.e., DM, OM, protein, fat, and starch digestibility), and gut measurements (i.e., rumen weight, and cecum, colon, and rectum weight and length) of pre-weaned calves were not affected by the treatments. While the digesta pH of the small intestine sections was affected by the treatments, in which HS-HP had a greater digesta pH than LS-HP, the hindgut digesta pH was only affected by the [SCFA]. Similarly, high [SCFA] increased the concentration of colonic isovaleric acid and fecal BCFA (i.e., isobutyric and isovaleric acids), while only colonic acetic acid concentration and fecal lactic acid were affected by treatment, in which the group HS-LP had the lowest concentrations.

2.5.1. Impact of Rumen SCFA and pH on Performance and Fermentation Profile

The digestibility results are consistent with previous studies (van Niekerk et al., 2020; Quigley et al., 2021; Xu et al., 2022), indicating that, although their young age is associated with rumen underdevelopment (Diao et al., 2019), pre-weaned calves have high feed efficiency, and digested nearly 90% of the nutrients consumed, and more than 80%, 70%, 90%, and 95% of OM, protein, fat, and starch, respectively. In addition, the lack of changes in the apparent total tract digestibility after infusions (i.e., pre- vs. post-treatment) and the lack of effect of treatment on feed

intake and ADG suggest that calves can temporarily adapt to rumen-environmental changes without compromising their productivity despite the changes in digesta pH and organic acid concentration. Similarly to our findings, Yohe et al. (2019) found that a 6h SCFA infusion did not show SCFA absorption capacity differences in calves fed different diets. However, some performance parameters, such as nutrient digestibility, can change after ruminal [SCFA] modulations due to longer-term ruminal microbiome changes (Hendawy et al., 2022), suggesting that time might have been a limiting factor. Such a time limitation could also explain why there were no treatment differences in rumen weight and hindgut section weights and lengths (i.e., cecum, colon, and rectum). In addition, changes in ruminal epithelium might have been more noticeable than rumen weight, as the rumen epithelium of 60-days-old calves supplemented with yeast cell wall were positively affected despite the lack of changes in rumen weight (Ma et al., 2020).

Regarding the physiological fermentation parameters, the ruminal digesta pH and organic acid concentrations (i.e., SCFA, BCFA, and lactic acid) were not affected by treatments, which could be explained by the fact that the rumen digesta samples were taken prior to the infusions as part of rumen preparation for the SCFA buffer administration. On the other hand, treatment effects were observed on digesta pH in the lower gut, indicating that it can be physiologically affected by the rumen environment in a short period (i.e., within 4 h), despite the lack of treatment effects on the productivity parameters. Specifically, while the small intestine was affected by the interaction of ruminal [SCFA] and pH, the hindgut sections were affected by [SCFA] only, which may suggest two potential mechanisms: a) the small intestine may be more susceptible to changes in the pH and SCFA of the foregut, and b) the hindgut seems to be better able to manage the lower digesta

pH, explaining why no treatment differences were observed either on the cecum, colon, and rectum digesta pH or on fecal pH.

In fact, many studies have shown a close relationship between the rumen and the small intestine in calves (Diao et al., 2019). Górka et al. (2011) found that a whole milk diet can increase the empty jejunum and ileum weight and crypt depth, as well as increase rumen papillae length and width. They also found significant positive correlations between the small intestine and reticulorumen weights. Moreover, increased dietary starch decreases rumen and small intestine starch digestibility but does not affect hindgut starch digestibility (Sanz-Fernandez et al., 2020), suggesting that the small intestine, compared to the hindgut, is more likely to be affected morphologically, physiologically, and functionally, maybe due to its closer location to the rumen compared to the hindgut. Alternatively, it takes an infusion for longer than 4 h to compromise the hindgut because of the microclimate in that environment.

Regarding the proposed hindgut pH regulation mechanism, it has been suggested that the hindgut is more susceptible to digesta pH changes compared to the rumen, as it does not have saliva and protozoa populations, which assist the ruminal digesta pH regulation (Gressley et al., 2011). However, in humans and rodents, some studies have shown that the colon and rectum mucus can create a microclimate that preserves mucosal pH despite the luminal pH variations (McNeil et al., 1987; Sanz-Fernandez et al., 2020), which might explain why, in our study, the buffer pH did not affect hindgut digesta pH. Whether there is a pH regulation mechanism in the hindgut, it seems to be affected by buffer [SCFA], as we found that groups submitted to high [SCFA] had the greatest hindgut digesta pH.

Interestingly, the group HS-LP had the greatest pH variation over the whole intestine (i.e., 5.50 in duodenum up to 7.06 in the rectum). The greatest pH recovery might also be associated

with the release of sodium bicarbonate into the lumen, suggesting that the HS-LP had the greatest SCFA absorption capacity. Although SCFA absorptive capacity and pH regulation mechanisms were not investigated in this study, this hypothesis aligns with the lowest colonic acetic and lactic acid concentration of the group HS-LP, which might also be partially associated with greater SCFA and lactic acid absorption capacity (Aschenbach et al., 2009; Baaske et al., 2020). Therefore, the lowest colonic acetic acid and fecal lactic acid concentrations and higher digesta pH suggest that, opposite to our hypothesis, the HS-LP may have a reduced risk of hindgut acidosis, as digesta and fecal SCFA and lactic acid concentrations have been used as hindgut fermentation parameters (Castro et al., 2016; Köhler, 2020). In fact, the HS-LP group represents calf starter-fed calves, as grain fermentation increases SCFA ruminal production along with ruminal pH reduction, conditions associated with increased rumen development in pre-weaning calves (Aschenbach et al., 2011; Diao et al., 2019). Therefore, our findings suggest that calf starter intake may be beneficial not only for rumen development but also for hindgut functionality. Nevertheless, more studies on dietary strategies, such as calf starter composition, physical form, and allowance, are needed to understand the impacts of calf starter intake throughout the gastrointestinal tract.

2.5.2. Impact of Age on Susceptibility to Rumen Environment

In calves, apparent total tract digestibility did not change either over time (d 21, d 35, and d 49) or between pre- and post-treatment (i.e., long-term) on d 21 and d 35. As the digestibility depends on the development of ruminal fermentation and intestinal digestion in calves (Quigley, 2019), the 4 h buffer infusion may not be enough time to trigger a difference in the apparent total tract digestibility when evaluating pre- and post-treatment effects. Many studies have shown apparent total tract digestibility differs in calves fed different diets (Dong et al., 2019; Quigley, 2019; van Niekerk et al., 2021a), which could be a result of the longer ruminal exposure to the

imposed condition that may trigger the gastrointestinal tract development more substantially than our pulse dose infusions, explaining why we did not find such differences between pre- and post-treatment.

Additionally, the lack of differences in apparent total tract digestibility over time (i.e., between d 21, d 35, and d 49) could be associated with the overall low calf starter intake, as pre-weaned calves do not consume much calf starter (Dong et al., 2019), especially before d 35 when starter intake averaged 0.10 kg/d among all treatment groups. Despite the limited milk replacer allowance (900 g/d) and higher crude protein and lower fat profile of offered calf starter, the average calf starter intake of this study was lower than previous studies on calves at a similar age (Hu et al., 2019; Quigley et al., 2019), potentially associated with recovery effects from cannulation surgery, suggesting a limitation of this study. Interestingly, some studies have shown that the effect of age could be greater on the site of digestibility, in which the hindgut digestibility decreases over time while rumen digestibility increases as a response to rumen development (Quigley, 2019). Therefore, although the total tract digestibility did not differ from d 21 to d 49 in our study, the site of the digestibility might have changed as the calf starter intake increased over time, which is known for promoting rumen development even in pre-weaned calves (Diao et al., 2019).

Regarding the physiological mechanisms, the over-time reduction of ruminal digesta pH could be associated with the increased calf starter intake, a physiological response well described in the literature (Diao et al., 2019; Nikkhah and Alimirzaei, 2023). Interestingly, despite the over-time ruminal digesta pH reduction, the fecal pH increased. This inverse relationship might be partially explained by a hindgut pH regulation capacity, especially when considering the lack of buffer pH effects on the hindgut digesta pH and the re-establishment of pre-treatment fecal pH

levels within 48 h post-treatment after their short-term reduction over the infusion time (i.e., 0, 2, 4 h), suggesting that pre-weaned calves can physiologically compensate for lower ruminal digesta pH. In addition, different from our study, calves in the industry may have access to potential buffers such as forage sources or bedding. In fact, calves fed a mixture of calf starter with 10% chopped hay sorted for long particles, which might be associated with the prevention of health-related issues related to low rumen pH (Engelking et al., 2020). Therefore, calves may be able to modulate their ruminal pH along with the physiological compensation suggested in our study, though rumen pH in calves does not appear to affect calf starter intakes or growth prior to, or during, weaning (McCurdy et al., 2019).

On the other hand, Poier et al. (2022) proposed that changes in rumen development and fermentation capacity make the hindgut fermentation proportionally smaller in older pre-weaned calves, potentially explaining such inverse relationship between ruminal digesta and fecal pH found in our study. The greater over-time rumen fermentation capacity may have been achieved in this study as ruminal [SCFA] increased over time while lactic acid concentration and ruminal pH decreased (Aschenbach et al., 2011; Penner, 2019; Baaske et al., 2020). Independently, if such an inverse relationship is due to gut site fermentation change or due to some hindgut pH regulation mechanisms, the apparent total tract digestibility did not change, and feed intake and ADG increased over time. Therefore, those findings suggest that pre-weaned calves are resilient and able to thrive despite the intestinal physiological changes that were necessary to increase fecal pH despite the decreased ruminal pH.

2.6. Conclusion

Overall, 4 h of a single-dose infusion with physiological buffers in the rumen does not change performance parameters such as feed intake, ADG, rumen and hindgut weight and length,

and apparent total tract digestibility. Rumen environmental changes such as [SCFA] and pH can affect the small intestine digesta pH and physiological hindgut fermentation parameters. High [SCFA] and pH increased intestinal digesta pH in the small intestine, but only high [SCFA] increased the digesta pH in the hindgut. The HS-LP group had lower colonic acetic acid and fecal lactic acid concentrations. Therefore, contrary to our hypothesis, nutritional practices aimed at combining high ruminal SCFA concentration and low pH may present a lower hindgut acidosis risk. Further investigations are necessary to understand if the SCFA absorption and pH regulation mechanisms are associated with those physiological changes.

Table 2.1. Milk replacer and calf starter composition

	Milk Replacer	**Calf Starter**
Composition, % of DM		
Dry matter, %	95.14	87.88
Organic matter	93.35	93.17
Crude protein	27.05	21.63
Crude fat	18.39	4.47
NDF	-	19.28
Starch	2.51	31.16
Ash	6.65	6.83

Table 2.2. Chemical composition of wash buffer and SCFA buffer

Components (mmol/L)	Wash buffer	SCFA buffer[1]	SCFA buffer[2]
NaCl	106.01	20.01	20.01
$NaHCO_3$	24.00	24.00	24.00
NH_4Cl	-	2.50	2.50
NaOH	6.50	212.03	212.03
KOH	19.99	19.99	19.99
K_2HPO_4	2.00	2.00	2.00
$CaCl_2$	1.51	1.50	1.50
$MgCl_2$	1.50	1.50	1.50
Acetic Acid	-	143.02	6.00
Propionic Acid	-	100.01	3.00
Butyric Acid	-	40.58	1.00
HCl	49.72	-	-
pH[3]	7.4	5.2 or 6.2	5.2 or 6.2

[1]High SCFA concentration (Yohe et al., 2019)
[2]Low SCFA concentration (Schurmann et al., 2014)
[3]Buffers had the same initial NaOH concentration (212.03 mmol/L) until pH adjustment (5.2 or 6.2) with NaOH and gluconic acid

Table 2.3. Effects of ruminal SCFA concentration and pH on total feed intake of pre-weaned dairy calves infused with a physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively)

Variables	SCFA × pH						*P*-value		
	High SCFA		Low SCFA		Variability		SCFA × pH	SCFA	pH
	HP	LP	HP	LP					
Total milk replacer intake[1], L	263	264	257	251	5.49[3]	5.60	0.55	0.12	0.71
Total calf starter intake[2], kg	10.1	10.9	6.8	8.5	2.74		0.85	0.27	0.64

[1]Data lognormally distributed; values were back-transformed and reported in geometric means. Variability was reported in a 95% coefficient interval (CI). The largest CI range among treatment comparisons is reported.
[2]Data normally distributed; values and variability are reported in LSM and SEM, respectively. The highest SEM among treatment comparisons is reported.
[3]Lower and upper CI values, respectively

Table 2.4. Effects of ruminal SCFA concentration and pH on gastrointestinal tract weight and length of pre-weaned dairy calves infused with a physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively)

Variables	SCFA × pH						*P*-value		
	High SCFA		Low SCFA		CI[1]		SCFA × pH	SCFA	pH
	HP	LP	HP	LP					
Tissue weight[2], g									
Rumen, × 10^3	1.3	1.3	1.2	1.1	897	1687	0.65	0.28	0.73
Cecum	164	200	177	168	93	236	0.42	0.73	0.65
Colon	635	467	540	537	500	770	0.14	0.82	0.14
Rectum	94.2	91.3	71.8	99.6	63.9	125	0.14	0.48	0.22
Tissue length[2], cm									
Cecum	22.3	29.5	25.2	25.8	16.6	28.0	0.19	0.89	0.13
Colon	306	316	299	298	275	342	0.65	0.32	0.73
Rectum	17.5	17.7	14.5	17.1	13.4	20.9	0.43	0.26	0.39

[1]Lower and upper confidence Interval (CI), respectively. The largest range is reported.
[2]Data normally distributed; values are reported in LSM.

Table 2.5. Effects of ruminal SCFA concentration and pH on apparent total tract digestibility of pre-weaned calves infused with a physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively)

Variables	SCFA × pH				*P*-value				
	High SCFA		Low SCFA		CI[2]		Time	SCFA × pH	SCFA × pH × Time
	HP	LP	HP	LP					
Apparent total tract digestibility[1], %									
Dry matter	97.0	98.1	98.3	96.4	4.54	4.64	0.31	0.43	0.55
Organic matter	90.9	89.2	90.1	78.7	4.24	4.75	0.50	0.57	0.72
Protein	83.4	91.7	89.4	87.1	4.39	4.60	< 0.01	0.14	0.54
Fat	91.9	95.3	101	96.7	3.99	5.18	0.72	0.88	0.82
Starch	98.0	99.2	98.5	98.6	4.57	4.62	0.32	0.49	0.97

[1]Data lognormally distributed; values were back-transformed and reported in geometric means. Variability was reported in a 95% coefficient interval (CI).
[2]Lower and upper confidence Interval (CI), respectively. Lower and upper CI, respectively.

Table 2.6. Effects of age and ruminal infusion of physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively) on the ruminal digesta and fecal organic acid concentrations and pH of pre-weaned dairy calves

Variables	SCFA × pH					*P*-value								*P*-value
	High SCFA		Low SCFA		Variability	SCFA	pH	SCFA × pH	SCFA × pH × Time	d 21	d 35	d 49	Variability	
	HP	LP	HP	LP										
Rumen digesta pH[1]	6.20	6.13	6.47	6.14	0.23	0.21	0.36	0.42	0.55	6.58[a]	6.01[b]	6.12[b]	0.20	< 0.01
Ruminal organic acids, mM														
Acetic acid[2]	18.2	21.6	14.8	17.6	2.19[3] 3.15	0.36	0.45	0.99	0.40	10.5[b]	23.7[a]	22.2[a]	2.63[3] 3.53	< 0.01
Propionic acid[2]	8.29	10.2	6.54	8.01	1.28[3] 2.40	0.35	0.43	0.99	0.22	3.52[b]	12.5[a]	11.8[a]	1.90[3] 2.98	< 0.01
Butyric acid[2]	3.43	5.14	2.33	2.77	0.16[3] 1.66	0.21	0.47	0.77	0.30	0.91[b]	6.28[a]	5.43[a]	1.11[3] 2.47	< 0.01
Isobutyric acid[1]	0.43	0.30	0.49	0.37	0.08	0.26	0.04	0.96	0.98	0.33[b]	0.47[a]	0.39[ab]	0.06	0.05
Isovaleric acid[1]	0.40	0.29	0.50	0.35	0.08	0.19	0.03	0.72	0.96	0.31[b]	0.48[a]	0.37[ab]	0.06	0.04
Lactic acid[1]	2.83	2.80	2.74	2.80	0.23	0.77	0.90	0.79	0.91	3.19[a]	2.91[a]	2.28[b]	0.30	< 0.01
Fecal pH[1]	7.19	7.36	6.86	6.63	0.47	< 0.01	0.81	0.14	0.60	7.01[b]	7.34[ab]	7.39[a]	0.16	0.03
Fecal organic acids, mM														
Acetic acid[1]	14.8	16.5	16.2	16.2	1.89	0.67	0.55	0.50	0.10	12.6[b]	17.8[a]	17.3[a]	1.59	< 0.01
Propionic acid[1]	5.85	6.65	6.11	5.36	0.84	0.38	0.97	0.19	0.14	4.98	7.04	5.95	1.03	0.16
Butyric acid[2]	1.56	2.55	1.81	1.74	0.53[3] 1.31	0.56	0.26	0.19	0.17	1.36[ab]	2.58[a]	1.88[b]	0.78[3] 1.11	< 0.01

Isobutyric acid[1]	0.90	1.13	0.66	0.77	0.14	< 0.01	0.09	0.55	0.60	0.60[b]	1.12[a]	0.87[ab]	0.17	< 0.01
Isovaleric acid[2]	0.48	0.72	0.27	0.29	-0.79[3] 0.08	< 0.01	0.27	0.41	0.45	0.21[b]	0.70[a]	0.45[a]	-0.72[3] -0.02	< 0.01
Lactic acid[1]	4.56[ab]	3.71[b]	3.93[ab]	4.75[a]	0.39	0.45	0.95	< 0.01	0.21	5.62[a]	4.39[b]	2.70[c]	0.49	< 0.01

[1]Data normally distributed; values and variability are reported in LSM and SEM, respectively. The highest SEM among treatment comparisons is reported.

[2]Data lognormally distributed; values were back-transformed and reported in geometric means. Variability was reported in a 95% coefficient interval (CI). The largest CI range among treatment comparisons is reported.

[3]Lower and upper CI values, respectively. [a-c]Values in the same row with different superscripts differ ($P < 0.05$).

Table 2.7. Effects of ruminal SCFA concentration and pH on hindgut organic acid concentrations of pre-weaned dairy calves infused with a physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively)

Variables	SCFA × pH				Variability		*P*-value		
	High SCFA		Low SCFA				SCFA × pH	SCFA	pH
	HP	LP	HP	LP					
Cecal organic acid concentrations, mM									
Acetic acid[1]	70.6	60.9	63.0	64.4	7.42		0.29	0.70	0.45
Propionic acid[1]	24.3	21.2	20.2	20.2	2.70		0.21	0.39	0.76
Butyric acid[1]	9.01	7.75	7.83	8.29	1.19		0.31	0.71	0.64
Isobutyric acid[1]	1.09	1.25	1.61	1.61	0.36		0.94	0.06	0.58
Isovaleric acid[1]	0.63	0.37	0.46	0.45	0.12		0.17	0.55	0.15
Lactic acid[1]	0.68	0.88	0.78	0.81	0.09		0.12	0.74	0.07
Colonic organic acid concentrations, mM									
Acetic acid[1]	53.7[a]	36.8[b]	36.9[b]	43.3[ab]	7.86		0.05	0.39	0.38
Propionic acid[1]	20.0	14.5	14.5	15.5	3.58		0.21	0.38	0.38
Butyric acid[1]	6.52	4.58	4.78	5.53	1.26		0.14	0.66	0.52
Isobutyric acid[1]	1.61	1.28	1.19	1.30	0.41		0.44	0.50	0.71
Isovaleric acid[2]	0.75	0.38	0.22	0.29	-1.58[3]	0.72	0.21	0.05	0.60
Lactic acid[2]	0.64	0.50	0.63	0.54	-0.82[3]	-0.10	0.76	0.82	0.24

[1]Data normally distributed; values and variability are reported in LSM and SEM, respectively. The highest SEM among treatment comparisons is reported.
[2]Data lognormally distributed; values were back-transformed and reported in geometric means. Variability was reported in a 95% coefficient interval (CI). The largest CI range among treatment comparisons is reported.
[3]Lower and upper CI values, respectively. [a-b]Values in the same row with different superscripts differ ($P < 0.05$).

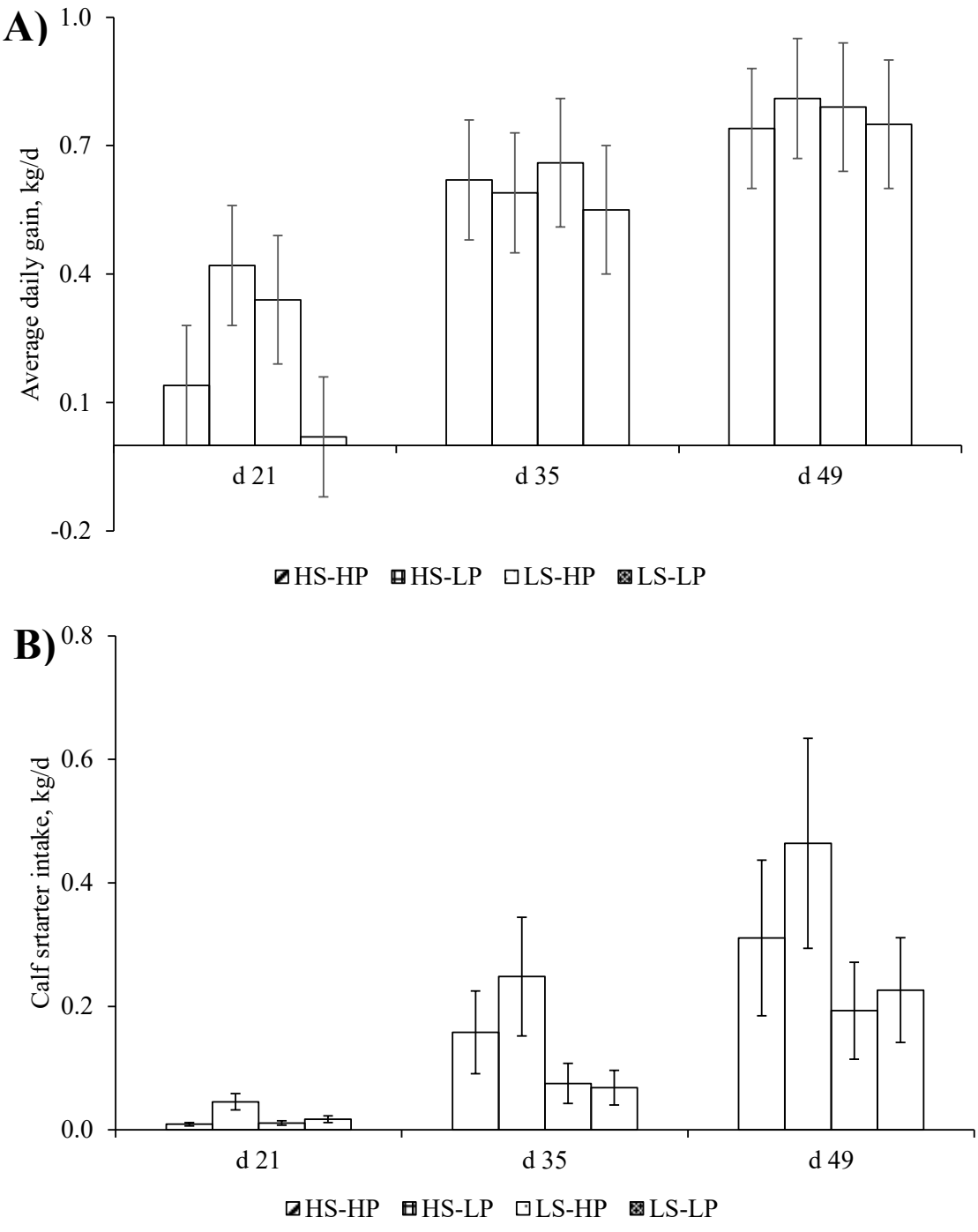


Figure 2.1. Interaction effect of age and treatments on A) ADG (LSM and SEM) and B) calf starter intake (geometric means and CI) of pre-weaned calves infused with a physiological buffer containing high (280 mM) or low (10 mM) SCFA concentration (HS or LS, respectively). Statistical difference is defined as $P < 0.05$.

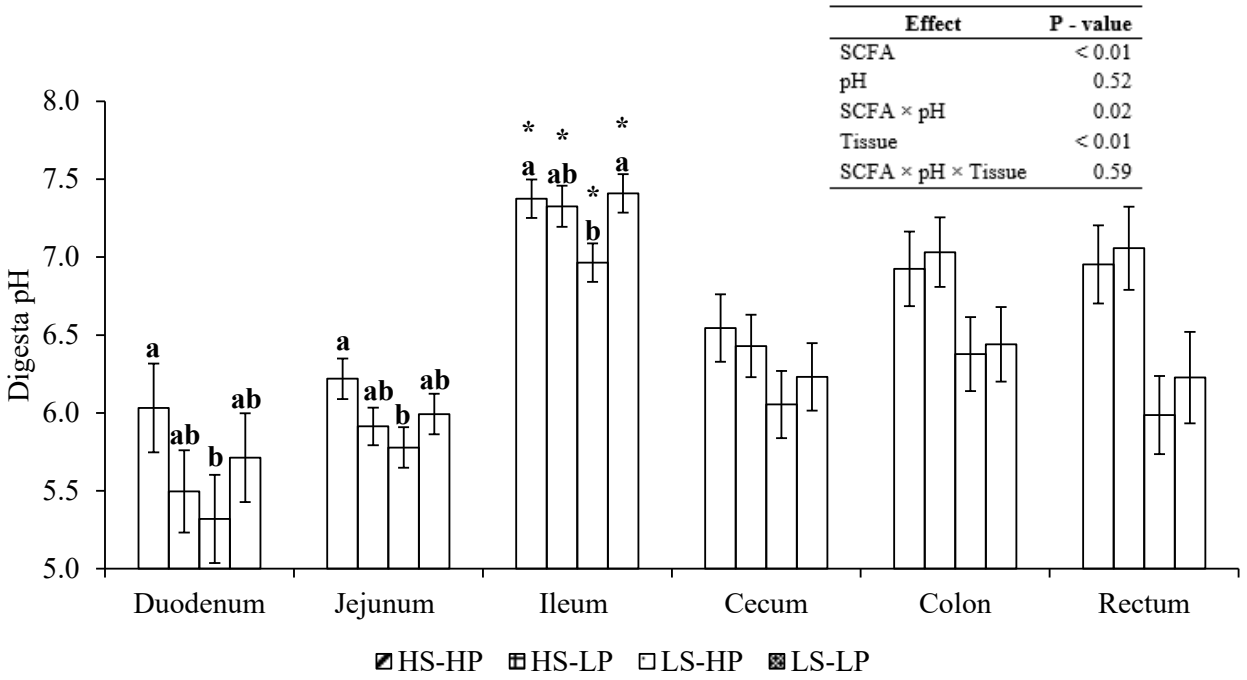


Figure 2.2. LSM and SEM of the interaction effect of SCFA and pH on digesta pH of the duodenum, jejunum, ileum, cecum, colon, and rectum collected at harvesting of pre-weaned calves pulse-dosed with a physiological buffer containing high (280 mM) or low (10 mM) SCFA concentration (HS or LS, respectively) and high (6.2) or low (5.2) pH (HP or LP, respectively). Different letters mean statistical differences within each gastrointestinal tract section ($P < 0.05$). * $P < 0.01$.

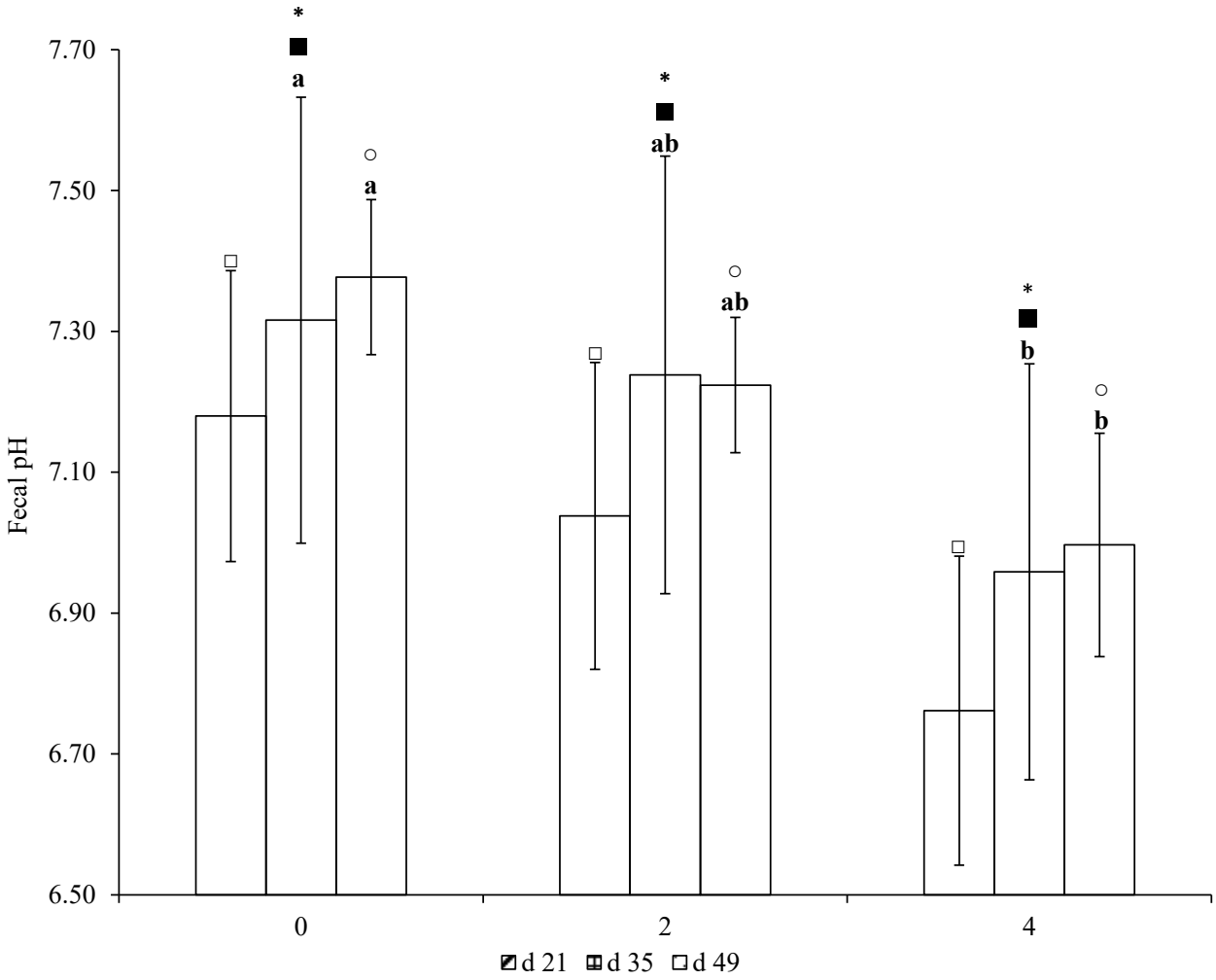


Figure 2.3. LSM and SEM of fecal pH at 0, 2, 4 h of infusion (short-term effects) on days 21, 35, and 49. Same symbols indicate statistical comparison. Different letters mean statistical difference between hours for each infusion day ($P < 0.05$). ***** $P < 0.01$.

3. Effects of ruminal infusion of short-chain fatty acid concentrations and pH on rumen and hindgut epithelium morphology and physiology of pre-weaned dairy calves

3.1. Abstract

While the impacts of high short-chain fatty concentrations ([SCFA]) and low pH on rumen development are well-known, their impact on hindgut development is unclear. The objective of this study is to evaluate how [SCFA] and pH impact rumen and hindgut epithelium development of pre-weaning dairy calves. Holstein bull calves (n = 32) were individually housed and fed milk replacer (900 g/d) twice daily, and *ad libitum* calf starter and water. At d 10 ± 3 of life, the rumens were fistulated and cannulated. At d 14 of life, calves were grouped by body weight and assigned in a 2 × 2 factorial arrangement of treatments: high or low [SCFA] (285 vs. 10 mM) and high or low pH (6.2 vs. 5.2), creating four treatment groups: high [SCFA], high pH (HS-HP); high [SCFA], low pH (HS-LP); low [SCFA], high pH (LS-HP); and low [SCFA], low pH (LS-LP). On d 21, 35, and 49, the rumen was evacuated and underwent a pulse-dose 4 h infusion with one of four treatment buffers, which was sampled hourly to calculate SCFA disappearance rates. After completion of the infusion on d 49, calves were harvested, and tissue samples were taken from the rumen, cecum, and colon for histomorphometric analysis, tissue scoring, and determination of expression of genes involved in SCFA absorption and epithelium cell homeostasis, proliferation, and apoptosis. Data were analyzed with main factors (SCFA and pH) and their interaction as fixed effects and repeated measures for weekly measurements. Cecum mucosal thickness tended to be greater in calves in the low pH groups ($P = 0.07$) while decreasing the colonic crypt depth ($P = 0.02$) and tending to decrease relative cyclin A2 expression ($P = 0.09$). The high [SCFA] groups had a better cecal crypt development score ($P = 0.03$), an increase in colonic cyclin A2 ($P < 0.01$) and NBC1 expressions ($P < 0.01$), and a tendency to increase ruminal IGF-1R expression ($P = 0.08$), as well as the total SCFA disappearance rate ($P =$

0.08). The HS-LP group had increased propionate ($P = 0.05$) and butyrate disappearance rates ($P = 0.05$). In conclusion, high [SCFA] improved gut epithelium development and a HS-LP condition is shown to benefit ruminal SCFA absorption capacity, potentially reducing risks of hindgut acidosis, and, at least, maintaining gut epithelium development.

3.2. Introduction

Despite dairy calves' importance in ensuring milk and meat production in adult life, their morbidity and mortality rates may reach 33.9% and 5% (Urie et al., 2018a), respectively, with digestive disorders being the major cause (United States Department of Agriculture, 2018). While pre-weaning calf nutrition has often prioritized rumen development by providing calf starter (Diao et al., 2019), little is known about the effects of such practice on hindgut development. Calf starter is a known source of rapidly fermentable carbohydrates that produces short-chain fatty acids (SCFA) and protons, thereby reducing ruminal pH (Aschenbach et al., 2011; Diao et al., 2019). A high SCFA/low pH scenario is beneficial for rumen epithelium development (Meale et al., 2017; Diao et al., 2019). However, there is limited information on SCFA absorption and pH regulation mechanisms in pre-weaning calves, which might be different from mature cows, as the calves' ruminal pH commonly reaches a threshold lower than mature individuals without negatively affecting their performance (Laarman et al., 2012; McCurdy et al., 2019). How the ruminal fermentation environment affects the hindgut, however, is unclear.

In mature cows, both the rumen and hindgut can be affected by ruminal SCFA concentration and pH, as is shown with subacute ruminal acidosis. Subacute ruminal acidosis, a digestive disorder observed in cows fed high-concentrate diets, is associated with high SCFA concentration and low pH, leading to a variety of negative health outcomes (Plaizier et al., 2018; Hossain, 2020; Pinedo and Melendez, 2022). During subacute ruminal acidosis, the increased concentrate intake increases digesta

passage rate, increasing starch flow to the hindgut. In the hindgut, fermentation of the starch increases organic acid production (e.g., SCFA, branched-chain fatty, and lactic acids) and decreases pH, leading to hindgut acidosis, a secondary disorder equivocally interpreted as ruminal acidosis (Gressley et al., 2011; Sanz-Fernandez et al., 2020). Although hindgut acidosis has not been studied as deeply as ruminal acidosis, hindgut acidosis can cause diarrhea, bloody feces, intestinal epithelium damage, and local inflammation (Gressley et al., 2011; Sanz-Fernandez et al., 2020).

The incidence of ruminal acidosis and hindgut acidosis is well-recognized in cows but not in pre-weaning dairy calves. For example, the rumen nutrient digestibility in younger calves is limited (Quigley, 2019), which could increase hindgut fermentation as more feed particles escape towards the lower gut (Gressley et al., 2011). However, as calves start consuming more solid feed, their rumen becomes more developed, increasing ruminal digestibility (Quigley, 2019), which decreases the starch concentration in the lower gut, reducing the risk of hindgut acidosis (Sanz-Fernandez et al., 2020).

Nevertheless, rumen and hindgut epithelium morphology and physiology in calves may differ from that of cows. Feeding weaned calves 70% concentrate with 40% starch decreased ruminal n-butyrate proportion, but its proportion doubled in the colon, suggesting either the presence of different microbial communities among gut segments (Hartinger et al., 2024) or different uptake physiology. In addition, the colonic expression of immune response-related genes was greater than in the rumen in cows and in the cecum in calves, suggesting that the colon has a greater role in the gastrointestinal tract immune system (Malmuthuge et al., 2013; Bach et al., 2018). Yohe et al. (2022) proposed that, given the higher fecal starch concentration, calves fed calf starter with a high starch concentration had an increased hindgut fermentation despite presenting a higher cecal digesta pH. In addition, they found that those calves tended to have a lower colonic digesta pH, suggesting that the cecum and colon may have different responses to high starch concentration. However, how the rumen, cecum, and colon

epithelium development are affected by known [SCFA] and pH is still a knowledge gap that should be investigated, as it may provide important information on the physiological requirements of each gut site.

Given the importance of SCFA and pH on gut epithelium development, the objective of this study is to evaluate how high or low [SCFA] and high or low pH impact rumen and hindgut epithelium morphology and physiology of pre-weaning dairy calves. We hypothesize that high [SCFA] and low pH (HS-LP) conditions will increase ruminal SCFA disappearance, associated with an increase in SCFA absorption, while damaging the ruminal epithelium (e.g. given the supraphysiological condition). On the other hand, calves under HS-LP condition may present a greater hindgut epithelium development due to lower SCFA concentrations and increased pH in the hindgut (e.g. as a response to the increased ruminal SCFA absorption), decreasing the risk of hindgut acidosis.

3.3. Materials and Methods

3.3.1. Animal & Housing

This study was approved by the Institutional Animal Care and Use Committee at the University of Alberta (AUP#00003823). The complete description of the material and methods is described by Narciso et al. (unpublished). Briefly, Holstein bull calves (n = 32; body weight = 42.17 ± 4.49 kg) from a commercial farm were fed 4 L of colostrum and then transported to the Metabolic Unit Research Station at the University of Alberta, Edmonton, Canada. In the first seven days of life, calves were fed milk replacer twice daily up to 600 g (150 g/L) and 900 g throughout the remaining experiment time, along with free access to calf starter and water.

3.3.2. Experimental Design

The rumens of Holstein bull calves (n = 32) were fistulated and cannulated at d 10 ± 3 of life (Castillo and Hernández, 2021). At d 17 of life, they were blocked by body weight and randomly

assigned in randomized complete block design with a 2 × 2 factorial arrangement of treatments: high [SCFA] (285 mM; Yohe et al., 2019) vs. low [SFCA] (10 mM; Schurmann et al., 2014), and high pH (6.2) vs. low pH (5.2), creating four treatment groups: high [SCFA], high pH (HS-HP); high [SCFA], low pH (HS-LP); low [SCFA], high pH (LS-HP); and low [SCFA], low pH (LS-LP). The SCFA mixture was a 50:35:15 mixture of acetate:propionate:butyrate (Yohe et al., 2019) and pH and osmolality were adjusted with gluconic acid and sodium hydroxide. On days 21, 35, and 49 of life, calves were submitted to a reticulorumen-washing technique, in which a known amount of physiological buffer containing one of four treatments (SCFA buffer) was infused for 4 h. During the pulse-dose infusion, SCFA buffer was sampled hourly, as described by Yohe et al. (2018), and frozen at -80 °C. On d 49, within 1 h after third infusion, calves were euthanized by captive bolt and exsanguination, followed by gastrointestinal tract removal from the body cavity. The rumen and hindgut segments (e.g. cecum and colon) were identified, and tissue samples were taken from the rumen (dorsal sac immediately above the longitudinal pillar), cecum (sac ending – the furthest point from the main ileocecal junction), and colon (100 cm caudal from the ileocecal junction). Tissue samples were divided into two subsamples: one for gene expression analysis, which was snap-frozen and stored at -80 °C; and one for histomorphometric analysis, which was immediately washed in PBS, transferred to 10% buffered formalin for a minimum of 24 h at room temperature to preserve their structure, then suspended in 70% ethanol for dehydration (Yarpuzlu et al., 2014).

3.3.3. Laboratory Analysis

3.3.3.1. Histomorphometric Analysis & Tissue Scoring

Tissue samples were prepared by taking 3 mm-thick tissue blocks, obtained from each tissue using a surgical blade and placed into embedding cassettes (Fisherbrand, 15-200-403H). Cassettes were then placed in a tissue processor and were embedded in paraffin wax. Samples from each specimen were

sectioned at 7–10 μm in thickness using a microtome (Leica M72S). The sections were then stained with Hematoxylene (Hematoxylene solution modified to Gill III, Merck) and Eosin (Eosin Y solution 0.5% alcoholic, Merck Inc.). All sections were examined under a light microscope (Axio Imager, Carl Zeiss Inc.) with images captured from three different magnifications (2.5 ×, 10 ×, and 40 ×).

For histomorphometrics analysis, the tissue images were analyzed with the software ImageJ (Schneider et al., 2012). For rumen samples, 2.5 × objective images were used to evaluate papillae length, width, perimeter, and area, and 40 × objective images were used to assess the thickness of keratin and non-keratin layers and total papillae epithelium thickness (adapted from da Silva et al., 2020; Terler et al., 2023). As such, 5 well-defined, -oriented, and representative papillae were measured. For papillae length, a line in the center of the papillae was drawn from its base to the apex. To minimize potential measurement errors, this line was measured 3 times for each papilla, and a variance of up to 5% between measurements was allowed to determine the average of 3 measures. Once the average of each papilla was determined, a second average of those 5 papillae was calculated to obtain the final average value. The same approach was performed for the papillae area and perimeter measurements. For papillae width, 3 measurements were performed at different points (base, middle, and top) and lines were drawn perpendicular to the papillae orientation. Similarly, to obtain the thickness of epithelium layers, the average of 3 measurements was determined. For hindgut tissue samples, 10 × objective images were evaluated to measure crypt depth and width, as well as mucosal thickness (Fleige et al., 2007; Montanholi et al., 2013; Nishihara et al., 2023). Ten well-defined, oriented, and representative crypts were measured. The crypt depth and width were estimated with the

same approach described for papillae length and width, respectively. Mucosal thickness was obtained as an average of 5 different points perpendicular to the mucosal base.

For histological scoring, hindgut tissue samples were scored using 2.5 × objective images for crypt development scoring and 10 × objective images for Goblet cell loss scoring. The scoring procedure was performed according to Bennett (2022). Using a 1 to 5 scale, 3 treatment-blinded, trained, and calibrated scorers subjectively measured cecal and colonic crypt development and goblet cell loss. For each variable, the average of the scores was obtained to calculate a final score. The scale for crypt development evaluation was: score 1 - indicates all or almost all of the crypts present are elongated and uniform in shape; score 2 - indicates most of the crypts present are elongated, with more variation than a score of 1; score 3 - indicates some of the crypts present are elongated, with more variation than a score of 2; score 4 - indicates few of the crypts present are elongated, with more variation than a score of 3; and score 5 - indicates none or very few of the crypts present are elongated, with most of the crypts being short and round. The scale for Goblet cell loss evaluation was: score of 1 - indicates all or almost all of the crypts present contain goblet cells in great number; score 2 - indicates most of the crypts present contain many goblet cells, with fewer goblet cells present than a score of 1; score 3 - indicates some of the crypts present contain goblet cells, with fewer goblet cells present than a score of 2; score 4 - indicates few of the crypts present contain few goblet cells, with fewer goblet cells present than a score of 3; and score 5 - indicates none or very few of the crypts present very few goblet cells, with fewer goblet cells present than a score of 4.

3.3.3.2. RNA Extraction, cDNA Synthesis & Reverse Transcription-quantitative Polymerase Chain Reaction

RNA was isolated using TRIzol, chloroform, and isopropanol with ethanol washing steps. RNA concentration was determined using a NanoDrop 200c spectrophotometer (NanoDrop Technologies,

Rockland, DE); cDNA synthesis was performed from the obtained RNA by TC-3123 thermocycler (Techne) using Superscript II, which was inactivated by heat to stop cDNA synthesis.

Gene expression was analyzed using the primers listed in Table 3.1, which were obtained either from the literature or designed with the NCBI Primer-Blast (National Center for Biotechnology Information, 1998). Target genes were selected based on function association, including: 1) Gut epithelium cell proliferation-associated genes – cyclin A2, cyclin D1, insulin like growth factor 1 receptor (IGF-1R), and pro-glucagon (GCG; Steele et al., 2016; Liu et al., 2019); 2) Cell apoptosis – caspase 3 (Bach et al., 2018); 3) SCFA absorption, monocarboxylic acid transporter 1 (MCT1 = SLC16A1; Bach et al., 2018); and 4) Intracellular pH regulation – Na^+/H^+ exchanger (NHE3 = SLC9A3), carbonic anhydrase 2 (CA2), and sodium bicarbonate cotransporter 1 (NBC1 = SCL4A4; (Supuran, 2016; Baaske et al., 2020). Internal control genes used were ATP synthase subunit beta (ATP5B), beta-actin (ACTB), peptidylprolyl isomerase (PPIA), ribosomal protein S15A (RPS15A), and ribosomal protein S9 (RPS9). All primers were optimized to check stability, melt curve, and efficiency [calculated as $\mathbf{10}^{(-1\,/\,Slope)}$]. Reverse transcription-quantitative polymerase chain reactions were performed in triplicates using Fast SYBR Green Master Mix (Applied Biosystems. Reactions were carried out using a QuantStudio 3 Real-Time PCR instrument (Applied Biosystems). The resulting C_t values were used to calculate the relative expression of target genes by relative quantification using the geometric means of the internal control genes following the method described by Pfaffl (2004).

3.3.3.3. Ruminal SCFA Disappearance Rates

The acetate, propionate, and butyrate concentrations in SCFA buffer samples were determined by gas chromatography (Varian 430 GC with FID detector and Stabilwax-DA column) using the following conditions: GC Column – Stabilwax-DA 30 meter, 0.53 mm ID, 0.5 um df (RestekCorp.);

Head Pressure – 7.5 psi.; Split vent flow – 20 ml/minute or adjusted as required; Injector Temperature – 170ºC; Column Temperature – 90ºC held for 0.1 min, increased to 170ºC at 10ºC/min and held for 2 min.; Run time – 10 min.; Detector Temperature – 190ºC; Internal standard – 25% phosphoric acid, approximately 20 ml water and 300 µl of isocaproic acid (4-methyl-valeric acid MW 116.20 g/mol) with a total volume brought up to 100 ml with water.

Absolute and fractional disappearance rates were calculated hourly for total SCFA, as well as acetate, propionate, and butyrate individually (Chibisa et al., 2020); then, the average of the 4 samples was determined per infusion to obtain the final rates. As a limitation of this study, the passage rate was not determined; thus, absolute and fractional rates are referred to as disappearance rates, representing both absorption across the rumen epithelium and passage through the digestive tract.

3.3.4. Statistical Analysis

Data was analyzed in SAS Software (SAS Institute Inc., Cary, NC, USA). For non-parametric data (e.g. hindgut tissue scoring), PROC GLIMMIX was used with Poisson distribution and a Linklog function to fit count data along with SCFA and pH, and SCFA × pH as main effects. For the remaining variables, data distributions were characterized with PROC UNIVARIATE testing of three different distributions (normal, lognormal and gamma). The best-fit distribution was used in PROC GLIMMIX with SCFA, pH, and SCFA × pH as fixed effects. Repeated measures were performed for weekly measurements (e.g., ruminal SCFA disappearance rates). As such, the time (D_l) effect and its interactions were added to the model as a fixed effect, and four covariant structures were tested. The variance-covariance structure with the lowest AIC was used. α was established as < 0.05, and tendency was defined as $P < 0.10$. The estimates and SEM of non-normal data were backtransformed for ease

of reading. Tukey adjustments were made for multiple comparisons. The statistical model is provided below:

$$Y = \mu + S_i + P_j + S \times P_{ij} + B_k + D_l + D \times S \times P_{lij} + D \times S_{li} + D \times P_{lj} + \varepsilon_{ijklm}$$

S_i is the effect of SCFA, P_j is the effect of pH, B_k is the random block effect, D_l is the effect of day, and ε_{ijklm} is the error term.

3.4. Results

3.4.1. Ruminal & Hindgut Epithelium Morphology

Papillae length, width, perimeter, and area, as well as epithelium layer thickness and crypt length and width, were unaffected by the SCFA × pH interaction (Table 3.2). Similarly, crypt development and Goblet cell loss scoring were unaffected by the main factor interaction in the cecum and colon. Cecum mucosal thickness tended to be thicker in calves in the low pH groups (high pH = 435.76; low pH = 488.47; SEM = 26.68; $P = 0.07$), and the colonic crypt depth was higher in calves in the high pH groups (high pH = 325; low pH = 276; SEM = 19; $P = 0.02$). High [SCFA] had a lower cecal crypt development score (high SCFA = 2.87; low SCFA = 3.59; SEM = 0.23; $P = 0.03$).

3.4.2. Gene Expression

Gene expression data are listed in Table 3.3. The SCFA × pH interaction did not affect the expression of any target gene in any tissue. In the rumen, calves in the high [SCFA] groups tended to have increased relative IGF-1R expression (high SCFA = 1.33; low SCFA = 0.50; SEM = 0.44; $P = 0.08$), but no SCFA effect was found in other cell proliferation-associated genes: cyclin A2 (SEM = 0.38; $P = 0.74$) and cyclin D1 (SEM = 0.75; $P = 0.21$). In the colon, calves in the high [SCFA] groups had increased relative cyclin A2 (high SCFA = 1.40; low SCFA = 0.71; SEM = 0.23; $P < 0.01$) and NBC1 expression (high SCFA = 1.50; low SCFA = 0.83; SEM = 0.22; $P < 0.01$), but no SCFA effect was found in the other cell proliferation-associated genes (cyclin D1, SEM = 0.57, $P = 0.11$; IGF-1R,

SEM = 0.18, P = 0.18; and GCG, SEM = 0.29, P = 0.22) and cell homeostasis-associated genes (NHE3, SEM = 0.36, P = 0.42; and CA2, SEM = 2.02, P = 0.31), respectively. In addition, calves in the high pH groups tended to have decreased relative cyclin A2 expression (high pH = 0.85; low pH = 1.26; SEM = 0.23; P = 0.09), but no pH effect was found in the other cell proliferation-associated genes (cyclin D1, SEM = 0.60, P = 0.72; IGF-1R, SEM = 0.20, P = 0.27; and GCG, SEM = 0.29; P = 0.92).

3.4.3. Ruminal SCFA Disappearance Rates

Absolute and fractional disappearance rates are shown in Figure 3.1. The absolute total SCFA disappearance rate tended to be higher in calves in the HS-HP and HS-LP groups than calves in LS-HP and LS-LP groups at d 21, d 35, and d 49 (P = 0.08). While SCFA × pH × time effect was not found in the absolute acetate disappearance rate (P = 0.11), the absolute propionate disappearance rate tended to be affected by SCFA × pH × time interaction (P = 0.09) and was greater in the HS-LP group no matter the calves age (P = 0.05). In addition, at d 21, calves in the HS-LP group had the greatest absolute butyrate disappearance rate and, along with the HS-HP group, had a greater absolute butyrate disappearance rate than LS-HP and LS-LP groups at d 35 and 49 (P = 0.05). Fractional disappearance rates were not affected by SCFA × pH × time interaction for total SCFA (P = 0.74), acetate (P = 0.43), propionate (P = 0.67) and butyrate (P = 0.16). However, the fractional total SCFA and acetate disappearance rates increased over time (P = 0.05 and P = 0.01, respectively), and calves in the high [SCFA] groups had a greater total SCFA (P < 0.01), acetate (P = 0.02), propionate (P < 0.01) and butyrate (P < 0.01) fractional disappearance rates.

3.5. Discussion

In summary, while ruminal epithelium morphology was unaffected by [SCFA] and pH exposure, hindgut epithelium was affected: calves in the high [SCFA] groups had a better cecal crypt

development score, and while calves in the low pH groups had increased mucosal thickness in the cecum, they had a decreased colonic crypt depth. In addition, while ruminal and cecal epithelial gene expression was less affected by treatments, showing only a tendency for increased ruminal relative IGF-1R expression in the high [SFCA] groups, the colonic epithelium showed an increased relative cyclin A2 expression in the low pH groups and an increased cyclin A2 and NBC1 expression in high [SCFA] groups. Furthermore, fractional SCFA disappearance rates were unaffected by treatments; however, absolute SCFA disappearance rates were higher in calves in the high [SCFA] groups, mainly in the HS-LP treatment as hypothesized, which had the greatest absolute propionate disappearance rate regardless of the calves' age, and greatest absolute butyrate disappearance rate at d 21.

3.5.1. Differences Between Rumen and Hindgut Epithelium

Considering the lack of ruminal histomorphologic changes and the limited gene expression alterations compared to the hindgut epithelium, the rumen of pre-weaning calves seems to be less vulnerable to dietary changes compared to the hindgut in agreement with other studies in mature cows (Gressley et al., 2011). In mature cows, the corneal layer of the epithelium plays a key role in protecting the epithelium against feed abrasiveness, fermentation side effects, and the pathogen's entrance (Membrive, 2016). The physical protective mechanism of the corneum might have contributed to the lack of changes in the ruminal epithelium in our study despite the typical ruminal epithelium underdevelopment observed in pre-weaning calves (Steele et al., 2016; Diao et al., 2019). In addition to the physical protective mechanism, the rumen epithelium has chemical mechanisms, such as saliva buffering, whose production is triggered by rumination (Paudyal, 2021; Srivastava et al., 2021). Although pre-weaning calves have limited rumination activity (Lopreiato et al., 2018; Costa et al., 2021), rumination is not necessarily absent at this age, which was visually conformed in some

calves between d 35 and 49. Therefore, the lack of treatment effects on rumen epithelium in our study could be partially attributed to physical and chemical ruminal epithelium protective mechanisms.

In contrast to the rumen epithelium, the hindgut epithelium protective mechanisms rely almost exclusively on mucus and antimicrobial peptide production and cell junctions (Gressley et al., 2011; Steele et al., 2016; Sanz-Fernandez et al., 2020). Given its known effects on pH regulation in the intestine (McNeil et al., 1987; Sanz-Fernandez et al., 2020), mucus production might be the principal mechanism protecting the hindgut epithelium against our treatments. However, given the treatment differences in gene expression and histology in the hindgut epithelium, the mucus production might not have successfully protected the epithelium against our treatments, contrary to the rumen's protective mechanisms.

Interestingly, the cecum and colon seem to have different physiological requirements, as calves in the low pH groups showed an increased mucosal thickness in the cecum but a decreased crypt depth in the colon. In a study evaluating different feeding regimes, calves fed calf starter with high starch concentrations had a greater cecal pH and a tendency for a lower colonic pH (Yohe et al., 2022), suggesting potential physiological differences associated with pH between the cecum and colon, potentially leading to some differences found in the cecal and colonic microbiomes (Virgínio Júnior and Bittar, 2021). In addition, while treatment effects did not affect gene expression in the cecum at all, our treatment did affect gene expression in the colon. Thus, collectively, such differences between hindgut segments might suggest that compared to the cecum, the colon is more susceptible to dietary stimuli.

In fact, the colon epithelium was the most histologically and molecularly affected tissue in our study. While low pH groups had decreased colonic crypt depth, their colonic cyclin A2 expression increased, which is associated with epithelium cell proliferation acting in the S-phase and mitosis

cycle (Bendris et al., 2011; Loukil et al., 2015; Liu et al., 2019). Considering that the same pH condition (e.g. low pH) decreased colonic crypt depth, such an increased cyclin A2 expression might be related to a cellular turnover response as an epithelium recovery mechanism (Seidelin, 2004), suggesting that we may have induced at least mild colonic acidosis in low pH groups, as hindgut acidosis is associated with decreased pH and damage to the intestinal epithelium (Gressley et al., 2011; Sanz-Fernandez et al., 2020). Therefore, our study suggests that a lower pH condition may be beneficial for the cecum but not for the colon and that the hindgut epithelium is more susceptible to dietary challenges than the ruminal epithelium.

3.5.2. Rumen Environmental Changes Affect the Hindgut Physiology in Pre-weaned Calves

While luminal pH affects gut segments differently, as discussed previously, high [SCFA] improved the overall gut epithelium (e.g. rumen, cecum and colon) in pre-weaning calves. In the rumen, calves in the high [SCFA] groups tended to have a greater relative expression of IGF-1R, a membrane receptor that binds IGF-1, a mitogenic peptide hormone that stimulates DNA synthesis in gut epithelium cells (Forsyth, 1996; Steele et al., 2016; Liu et al., 2019). Similarly to our findings, lambs orally infused with sodium butyrate for 39 d at 0.36 g/kg body weight had greater plasmatic concentration of IGF-1 and IGF-1R mRNA expression (Liu et al., 2019), indicating that the high [SCFA] may have benefited ruminal epithelium development.

In addition, although dietary changes have shown no effects on ruminal SCFA absorption (Yohe et al., 2019), calves exposed to high [SCFA] had the greatest absolute propionate and butyrate disappearance rates and tended to have greater total ruminal SCFA disappearance rate. Despite not determining the liquid passage rate, given the different treatment effects among the SCFA, it suggests that ruminal SCFA absorption might have been the main responsible for the treatment differences in SCFA disappearance rates, as the liquid passage rate should be a constant for all SCFA. In addition,

the liquid passage rate seems to be relatively more resilient to treatment effects, ranging between 11 to 13% (Gelsinger et al., 2020) with no significant differences in other studies (Yohe et al., 2019; Gelsinger et al., 2020). Therefore, if calves in the high [SCFA] showed an increase in absolute propionate and butyrate disappearance rates, it is more likely to be an effect of SCFA absorption than the liquid passage rate differences. The potential increase in SCFA absorption in the high [SCFA] groups, however, does not seem to be associated with MCT1, which is a SCFA basolateral membrane transporter that exports intracellular SCFA by either H^+ symport or sodium bicarbonate exchange (Baaske et al., 2020), given the lack of treatment effects on its gene expression.

Regarding the hindgut epithelium, although the HS-LP group did not show increased hindgut epithelium development as hypothesized, calves in the high [SCFA] groups had a better cecal crypt development score and increased colonic expression of cyclin A2. Collectively, these findings might suggest that high [SCFA] triggered hindgut epithelium development, as cyclin A2 is associated with cell proliferation (Loukil et al., 2015).

In addition, the high [SCFA] groups had an increase in colonic NBC1 expression, which might be indirectly associated with increased SCFA absorption. A higher luminal SCFA gradient may promote SCFA absorption in exchange with sodium bicarbonate by apical-sided transport proteins (Aschenbach et al., 2009; Baaske et al., 2020). If this mechanism was relevant for SCFA absorption, pH_i regulation mechanisms associated with sodium bicarbonate exchange might have been necessary to maintain cell homeostasis while promoting SCFA absorption. Among the cell homeostasis-associated genes evaluated in this study, NBC1 was the only one that operates by importing sodium bicarbonate from the bloodstream (Soleimani and Burnham, 2001), potentially providing fuel for luminal SCFA absorption. This theory might explain the lack of treatment effects in other pH regulation mechanisms, such as NHE3, which exports H^+ from the cell to the lumen while importing

Na^+ (Baaske et al., 2020; Dominguez Rieg and Rieg, 2024), and CA2, which reversely converts CO_2 and H_2O to H_2CO_3 in the cytosol that is ultimately dissociated in H^+ and $HCO3^-$ to regulate pH_i (Supuran, 2016). Therefore, despite our limitation on not determining SCFA absorption via sodium bicarbonate exchange, our results collectively suggest that it is possible that the colon epithelium pH_i regulation of pre-weaning calves rely mostly on sodium bicarbonate exchange mechanisms, potentially as a response to SCFA absorption, which may explain the increased hindgut digesta pH in calves in the high [SCFA] groups (Narciso et al., unpublished).

Moreover, the group HS-LP had the highest absolute propionate disappearance rate regardless of calves' age and the highest absolute butyrate disappearance rate at d 21. In addition, this group tended to have a greater absolute total SCFA disappearance rate than the LS-HP and LS-LP groups, which collectively suggests that the HS-LP group potentially had a greater SCFA absorption capacity, in agreement with our hypothesis. Despite that, the HS-LP group did not show a significant superior hindgut epithelium development, contrary to our hypothesis. Collectively, our findings indicate that the HS-LP group had a lower risk of experiencing hindgut acidosis, which agrees with our hypothesis, despite not improving hindgut epithelium development.

3.6. Conclusion

Overall, rumen environmental changes such as [SCFA] and pH seem less impactful in rumen epithelium than hindgut epithelium. In addition, although more research is needed to verify it, cecal and colon epithelium have different physiological requirements, as low pH can be more detrimental to the colonic epithelium while increasing mucosal thickness in the cecum. Despite this difference, high [SCFA] showed to benefit the rumen, cecum and colon epithelium, by increasing ruminal propionate and butyrate disappearance rates, cell proliferation-associated gene expression in the colon, and crypt development in the cecum. Moreover, the HS-LP group showed

greater propionate and butyrate disappearance rates as hypothesized, however, contrary to the hypothesis, no effects were found in the rumen and hindgut epithelium, suggesting that high [SCFA] and low pH conditions may be beneficial for ruminal SCFA absorption capacity, potentially reducing risks of hindgut acidosis, and at least, maintaining a gut epithelium development.

Table 3.1. Primer pair sequences of genes analyzed in rumen, cecum and colon tissue collected at harvest on d 49 of pre-weaned dairy calves infused with a physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively).

Genes[1]	Accession Number	Primer Sequence 5' – 3'	Efficiency, %	Amplicon Size (bp)	References
Rumen					
Cyclin A2	NM_001075123.1	For: TGGATGGTAGTTTTGAGTCTCC Rev: ACGTGTGAATGTCCTCATGGT	88.0	111	Current study
Cyclin D1	NM_001046273.2	For: GGTCCTGGTGAACAAACTC Rev: TTGCGGATGATCTGCTT	101	114	Malhi et al., 2013; Zhang et al., 2018
Caspase-3	NM_001077840.1	For: CAGCGTCGTAGCTGAACGTA Rev: TGCTTCCATGTAAGATCTTTGTCT	92.8	107	Current study
MCT1	NM_001037319.1	For: AACACTGTGCAGGAACTTTACTTTTC Rev: TGCCAGCGGTCGTCTCTTAT	86.8	90	Dieho et al., 2017
NHE3	NM_001192154.2	For: CCCGGCAGGAGTACAAACAT Rev: TTGGCCGACTTGAAGGACTC	95.7	93	Dieho et al., 2017
NBC1	XM_001788699.1	For: AGACGGCGAGGTGGATTAAGTT Rev: TCAAATAAGCTGTGAAGGGACAAG	114	100	Naeem et al., 2012
CA2	NM_178572.2	For: AAGGTTCTGAGCATACTGTGG Rev: CTGTTCCAAAGTCCCCGTAC	112	104	Gao and Oba, 2016
IGF-1R	NM_001244612.1	For: CACGAGTGGAGAAATCTGCG Rev: ATGTGGAGGTAGCCCTCGAT	109	102	Current study
ATP5B	NM_175796	For: CCCTCAAGGAGACCATCAAA Rev: GGACACCATGGAGGATGAGT	99.6	184	Connor et al., 2010
ACTB	NM_173979.3	For: GAGCTACGAGCTTCCTGACGGGC Rev: AATGCCGCAGGATTCCATGCCCAG	99.5	109	Kent-Dennis et al., 2020
Cecum					
Cyclin D1	NM_001046273.2	For: GGTCCTGGTGAACAAACTC Rev: TTGCGGATGATCTGCTT	108	114	Malhi et al., 2013; Zhang et al., 2018

Caspase-3	NM_001077840.1	For: GCGTCGTAGCTGAACGTAAAT Rev: TCCAAGGATATTCCAGAGTCCA	111	133	Current study
MCT1	NM_001037319	For: TTAATGCCACCACCAGTGAA Rev: AAGCCACTGCCTGACAAGAT	105	148	Nakamura et al., 2018
NHE3	NM_001192154.2	For: CCCGGCAGGAGTACAAACAT Rev: TTGGCCGACTTGAAGGACTC	111	93	Dieho et al., 2017
NBC1	NM_174605.1	For: TCTGACTGGGCTGTCAGTCT Rev: ACGATCCATGAACTGCACAC	111	121	Current study
CA2	NM_178572.2	For: TCGCGGAGAATGGTCAACAA Rev: GTGAACCAGGTGTAGCTCGG	104	201	Tan et al., 2023
PPIA	XM_010804358.2	For: TCTGAGCACTGGAGAGAAAGGATTTG Rev: GAAGTCACCACCCTGGCACATA	93.6	88	Rosa et al., 2018, 2021
RPS15A	XM_585783	For: GCAGCTTATGAGCAAGGTCGT Rev: GCTCATCAGCAGATAGCGCTT	90.7	151	Bionaz and Loor, 2007
Colon					
Cyclin A2	NM_001075123.1	For: TGGATGGTAGTTTTGAGTCTCC Rev: ACGTGTGAATGTCCTCATGGT	88.2	111	Current study
Cyclin D1	NM_001046273.2	For: GGTCCTGGTGAACAAACTC Rev: TTGCGGATGATCTGCTT	95.1	114	Malhi et al., 2013; Zhang et al., 2018
Caspase-3	NM_001077840.1	For: GCGTCGTAGCTGAACGTAAAT Rev: TCCAAGGATATTCCAGAGTCCA	90.9	133	Current study
MCT1	NM_001037319	For: TTAATGCCACCACCAGTGAA Rev: AAGCCACTGCCTGACAAGAT	112	148	Nakamura et al., 2018
NHE3	NM_001192154.2	For: AGCTACGTGGCCGAGGG Rev: AGACAGAGGCCTCCACGGT	92.6	121	Etschmann et al., 2006; Yan et al., 2014
NBC1	NM_174605.1	For: TCTGACTGGGCTGTCAGTCT Rev: ACGATCCATGAACTGCACAC	117	121	Current study
CA2	NM_178572.2	For: TCGCGGAGAATGGTCAACAA Rev: GTGAACCAGGTGTAGCTCGG	95.3	201	Tan et al., 2023
IGF-1R	NM_001244612.1	For: ACGAGTGGAGAAATCTGCGG Rev: AGTCCTCGGCCTTGGAAATG	110	127	Current study
GCG	NM_173916.3	For: ATTGCTTGGCTGGTGAAAGG Rev: AATGAATGACACACTTACTTCCTGT	101	200	Current study
ACTB	NM_173979.3	For: ACCGCAACCAGTTCGCCAT Rev: CTTGCTCTGAGCCTCATCCC	91.1	200	Current study

RSP9	NM_001101152.2	For: CTTGCTCTGAGCCTCATCCC Rev: TGCTTGCGGACCCTGATGT	90.8	204	Current study

[1]MCT1 = SLC16A1, monocarboxylic acid transporter 1; NBC1 = SCL4A4, sodium bicarbonate cotransporter; NHE3 = SLC9A3, Na^+/H^+ exchanger; CA2, Carbonic anhydrase 2; IGF-1R, insulin like growth factor 1 receptor; GCG, pro-glucagon; ATP5B, ATP synthase subunit beta; ACTB, beta actin; PPIA, peptidylprolyl isomerase; RPS15A, ribosomal protein S15A; RPS9, ribosomal protein S9.

Table 3.2. Effects of ruminal SCFA concentration and pH on rumen and hindgut epithelium of pre-weaned dairy calves infused with a physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively).

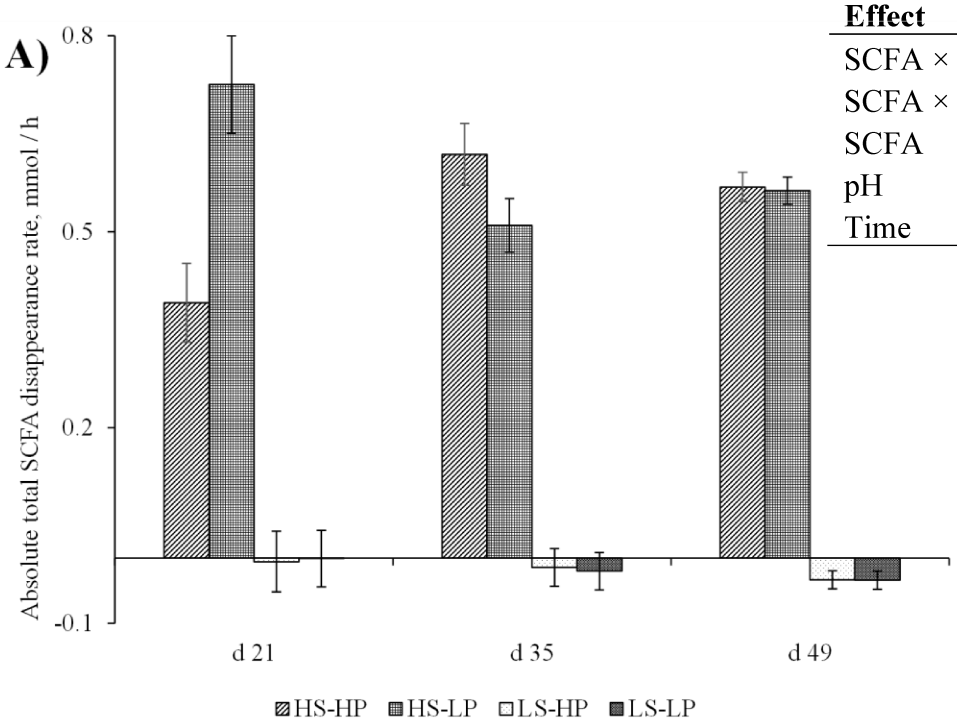
Variables	SCFA × pH					*P*-value		
	HS-HP	HS-LP	LS-HP	LS-LP	SEM*	SCFA × pH	SCFA	pH
Ruminal papillae, µm								
Length	683	733	642	646	169	0.89	0.69	0.87
Width	285	321	257	285	61	0.93	0.45	0.45
Perimeter	2.1×10^3	2.1×10^3	1.8×10^3	1.8×10^3	624	0.97	0.56	0.95
Area, μm^2	2.13×10^5	2.75×10^5	1.68×10^5	2.37×10^5	1.16×10^5	0.94	0.65	0.48
Ruminal papillae epithelium thickness, µm								
Keratin layer	12.0	11.4	9.8	10.2	1.5	0.64	0.13	0.89
Non-keratin layer	62.5	65.4	56.6	60.0	11.5	0.98	0.60	0.77
Total epithelium	74.3	78.0	66.7	70.0	12.2	0.99	0.51	0.77
Cecum epithelium, µm								
Crypt depth	305	335	269	308	37	0.86	0.23	0.19
Crypt width	58.0	51.5	53.8	55.6	4.1	0.18	0.99	0.47
Mucosal thickness	457	495	415	482	38	0.58	0.30	0.07
Cecum scoring								
Crypt development	2.79	2.97	3.57	3.63	0.33	0.82	0.03	0.69
Goblet cell loss	2.35	2.44	2.52	1.80	0.29	0.13	0.32	0.22
Colon epithelium, µm								
Crypt depth	331	294	319	259	26	0.54	0.21	0.02
Crypt width	58.2	54.8	60.8	59.8	4.6	0.75	0.34	0.58
Mucosal thickness	550	541	534	512	64	0.83	0.51	0.66
Colon scoring								
Crypt development	3.08	3.40	2.80	2.63	0.37	0.51	0.16	0.88
Goblet cell loss	1.85	1.87	2.46	1.79	0.27	0.18	0.32	0.22

*Highest SEM among the treatment comparisons. Different letters mean statistical difference between groups ($P < 0.05$).

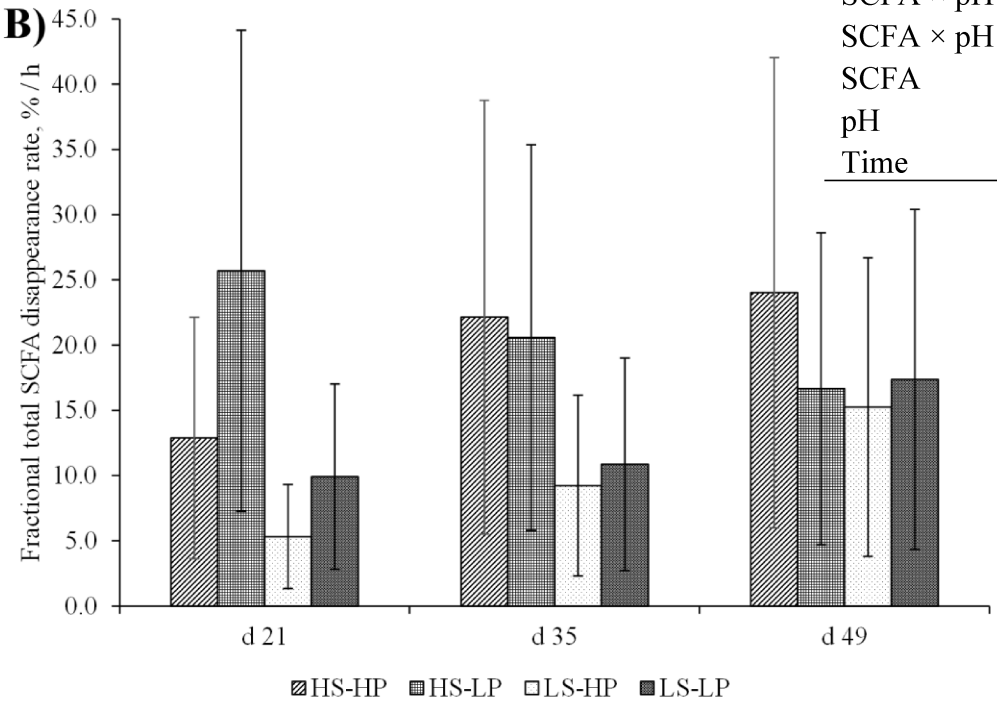
Table 3.3. Least squares means of the normalized Q relative gene expression of pre-weaned dairy calves infused with a physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively).

Genes[1]	SCFA × pH					*P*-value		
	HS-HP	HS-LP	LS-HP	LS-LP	SEM*	SCFA × pH	SCFA	pH
Rumen								
Cyclin A2	1.14	0.62	1.15	0.36	0.57	0.73	0.74	0.11
Cyclin D1	0.74	1.10	1.52	2.27	1.11	0.80	0.21	0.80
Caspase-3	1.32	1.59	0.79	0.50	0.52	0.56	0.11	0.98
MCT1	0.99	1.12	1.30	0.82	0.49	0.36	0.99	0.62
NHE3	1.06	1.09	1.67	0.82	0.54	0.23	0.64	0.29
NBC1	1.98	1.89	1.31	1.30	0.74	0.94	0.22	0.94
CA2	0.88	0.92	1.20	0.86	0.28	0.33	0.49	0.43
IGF-1R	1.43	1.24	0.58	0.42	0.66	0.97	0.08	0.69
Cecum								
Cyclin D1	2.72	0.96	2.82	3.07	2.94	0.62	0.59	0.73
Caspase-3	0.40	0.69	0.41	0.65	0.35	0.91	0.96	0.31
MCT1	0.48	1.06	0.73	0.36	0.48	0.16	0.50	0.75
NHE3	0.88	1.14	1.58	0.72	0.66	0.23	0.76	0.51
NBC1	1.13	1.14	1.41	0.36	0.60	0.21	0.55	0.22
CA2	0.43	0.49	0.33	0.15	0.25	0.49	0.23	0.74
Colon								
Cyclin A2	1.23	1.57	0.47	0.95	0.34	0.75	< 0.01	0.09
Cyclin D1	0.51	0.86	1.64	1.74	0.86	0.84	0.11	0.72
Caspase-3	1.35	0.98	0.94	1.24	0.36	0.19	0.77	0.87
MCT1	0.87	1.15	1.00	0.50	0.65	0.39	0.55	0.81
NHE3	0.47	0.47	0.58	0.95	0.54	0.62	0.42	0.63
NBC1	1.44	1.56	0.61	1.05	0.33	0.48	< 0.01	0.25
CA2	1.24	1.24	5.10	0.68	3.03	0.30	0.31	0.43
IGF-1R	0.68	0.47	0.64	0.39	0.28	0.94	0.76	0.27
GCG	0.67	0.86	0.52	0.26	0.43	0.92	0.22	0.92

[1]MCT1 = SLC16A1, monocarboxylic acid transporter 1; NBC1 = SCL4A4, sodium bicarbonate cotransporter; NHE3 = SLC9A3, Na^+/H^+ exchanger; CA2, Carbonic anhydrase 2; IGF-1R, insulin like growth factor 1 receptor; GCG, pro-glucagon.
*Highest SEM among the treatment comparisons.

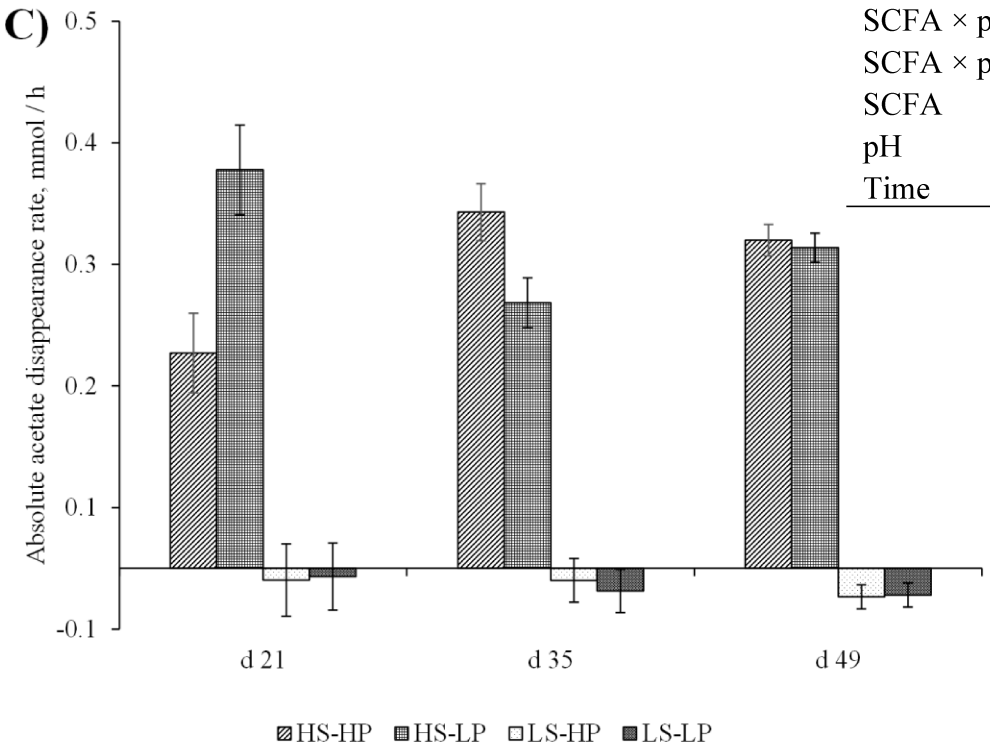


Effect	*P* - value
SCFA × pH × Time	0.08
SCFA × pH	0.12
SCFA	< 0.01
pH	0.13
Time	0.78



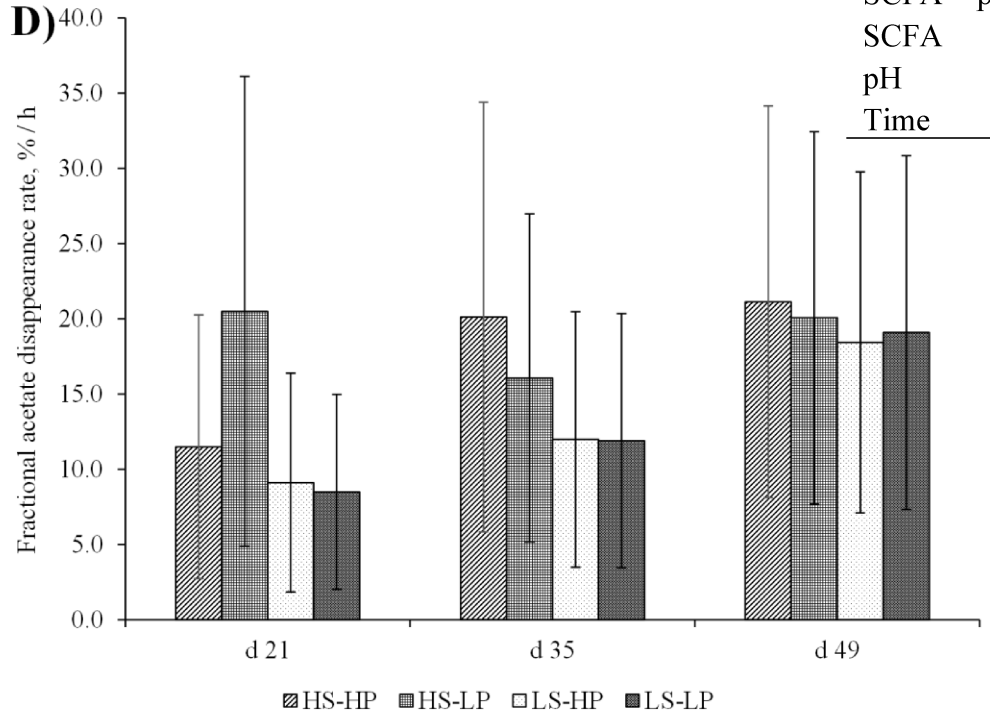
Effect	*P* - value
SCFA × pH × Time	0.74
SCFA × pH	0.46
SCFA	< 0.01
pH	0.18
Time	0.05

C)



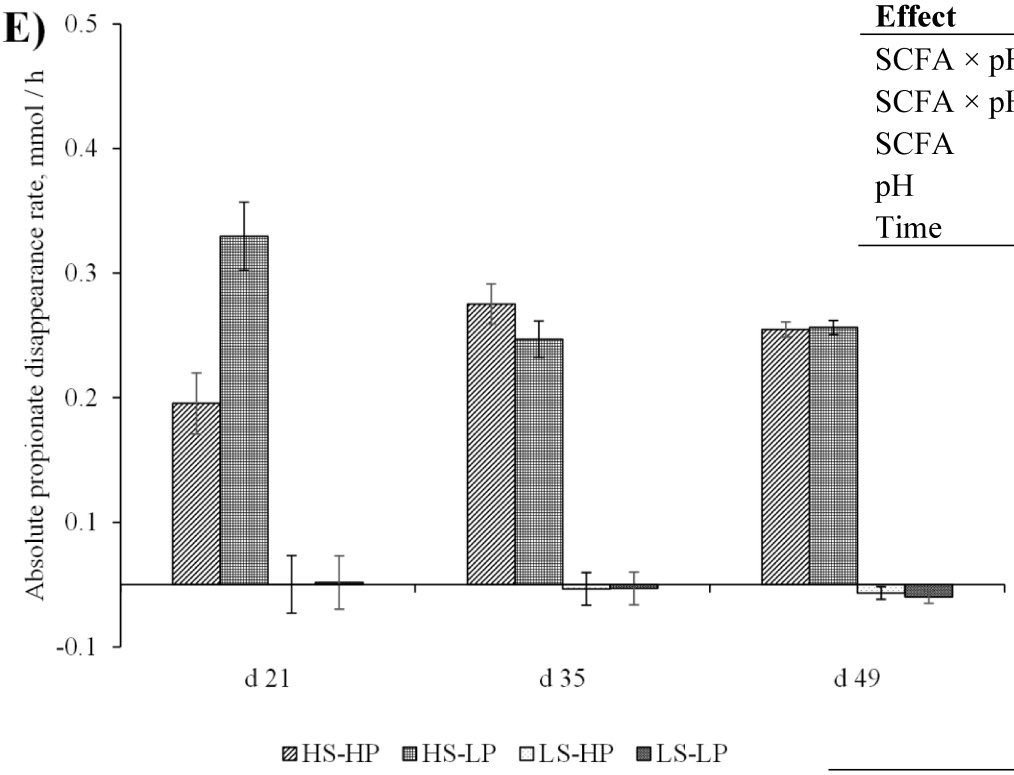
Effect	*P* - value
SCFA × pH × Time	0.11
SCFA × pH	0.31
SCFA	< 0.01
pH	0.39
Time	1.00

D)



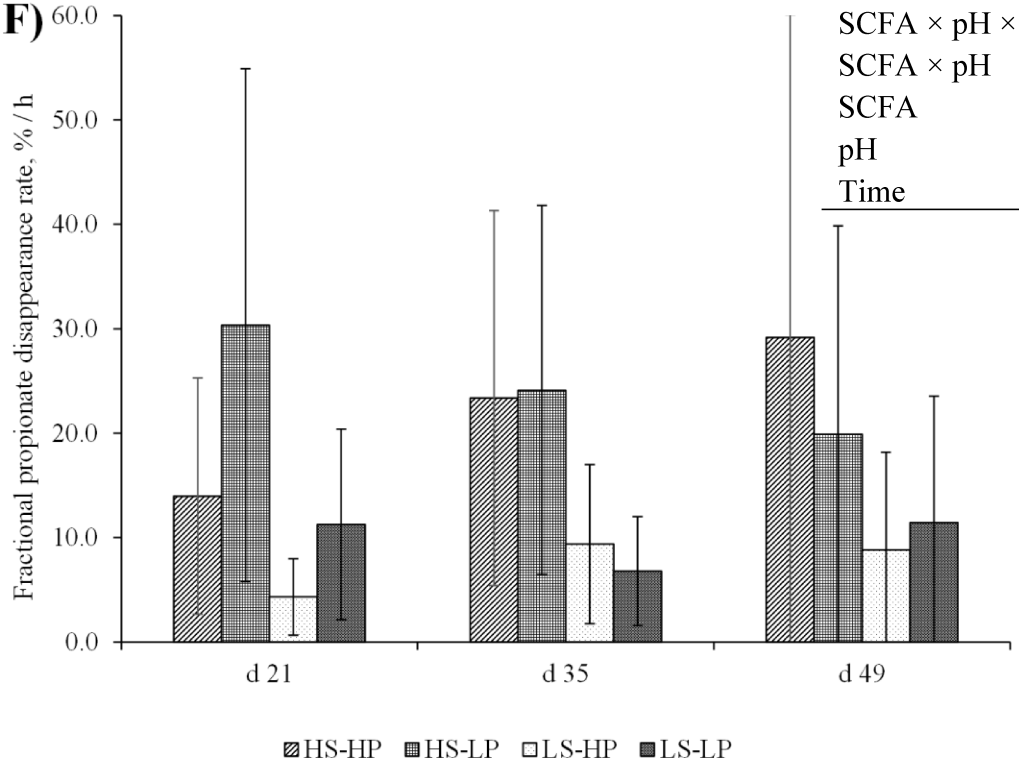
Effect	*P* - value
SCFA × pH × Time	0.43
SCFA × pH	0.70
SCFA	0.02
pH	0.76
Time	0.01

E)

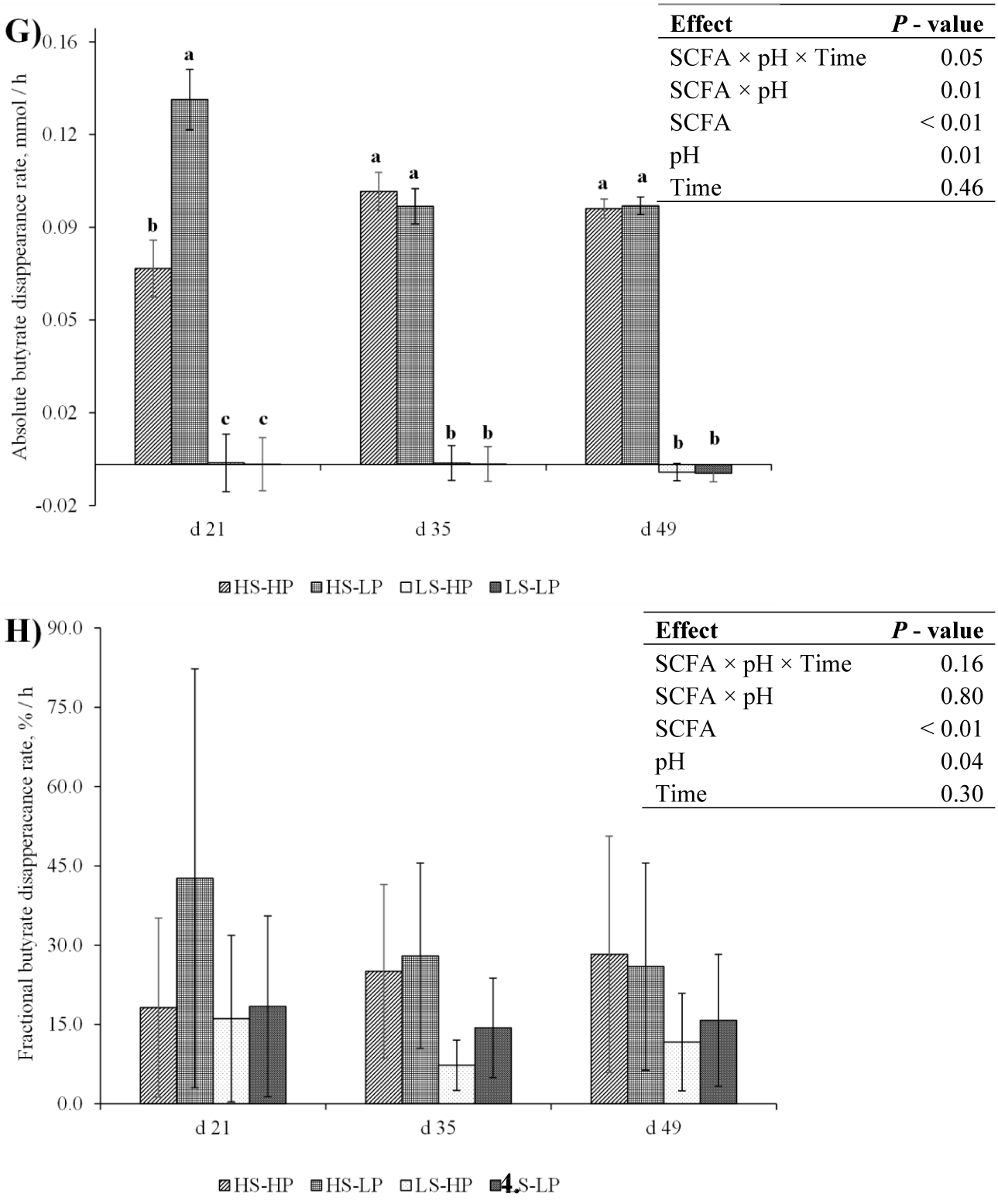


Effect	*P* - value
SCFA × pH × Time	0.09
SCFA × pH	0.05
SCFA	< 0.01
pH	0.07
Time	0.31

F)



Effect	*P* - value
SCFA × pH × Time	0.67
SCFA × pH	0.68
SCFA	< 0.01
pH	0.22
Time	0.74



Effect	*P* - value
SCFA × pH × Time	0.05
SCFA × pH	0.01
SCFA	< 0.01
pH	0.01
Time	0.46

Effect	*P* - value
SCFA × pH × Time	0.16
SCFA × pH	0.80
SCFA	< 0.01
pH	0.04
Time	0.30

Figure 3.1. Geometric means and back-transformed SEM of the effects of ruminal SCFA concentration and pH on the ruminal absolute and fractional disappearance rates of total SCFA (A) and (B), respectively; acetate (C) and (D), respectively; propionate (E) and (F), respectively; and butyrate (G) and (H), respectively, of pre-weaned dairy calves infused with a physiological buffer containing high or low SCFA concentration (HS or LS, respectively) and high or low pH (HP or LP, respectively). Different letters indicate statistical differences ($P < 0.05$)

4. General Discussion

4.1. Overview

This study aimed to investigate how changes in the ruminal environment affect the productivity and lower gut physiology and development, especially the hindgut, of pre-weaning dairy calves. In the second chapter, we evaluated the effects of ruminal SCFA concentration ([SCFA]) and pH on performance parameters, such as calf starter intake, ADG, and apparent total tract digestibility, and physiological hindgut fermentation parameters, such as digesta pH and organic acid concentration. We hypothesized that the interaction of high ruminal [SCFA] and low pH would increase the risk of hindgut acidosis in pre-weaning calves, leading to decreased digesta and fecal pH, increased digesta and fecal organic acid concentrations and decreased apparent total tract digestibility. High [SCFA] and pH increased intestinal digesta pH in the small intestine, but only high [SCFA] increased the digesta pH in the hindgut. The HS-LP group had lower colonic acetic acid and fecal lactic acid concentrations and the greatest pH recovery from the small to large intestines, potentially reducing the risk of hindgut acidosis, contrary to our hypothesis.

In the third chapter, we evaluate how known [SCFA] and pH impact calf' rumen and hindgut epithelium morphology, expression of gut epithelium development-related genes, and ruminal SCFA disappearance rates. We hypothesized that supraphysiological high [SCFA] and low pH (HS-LP) conditions would increase ruminal SCFA disappearance while damaging the ruminal epithelium. In addition, such a condition would lead to greater hindgut epithelium development due to lower SCFA concentrations and increased pH in the hindgut, decreasing the risk of hindgut acidosis. Overall, we found that, regardless of pH, high [SCFA] improved gut epithelium development and a HS-LP condition was shown to benefit ruminal SCFA absorption capacity, potentially reducing risks of hindgut acidosis and, at least maintaining gut epithelium development. In addition, the cecum and colon responded differently to lower pH conditions.

Therefore, the results from both studies collectively suggest that nutritional practices combining high ruminal SCFA concentration and low pH may present a lower risk of hindgut acidosis. However, further investigation is necessary to understand the impacts of such a condition in each gut segment.

4.2. Major Findings

4.2.1. Calves are resilient to endogenous physiological changes in the rumen environment

Despite the increased calf starter intake and consequently decreased rumen digesta pH as calves got older, their fecal pH increased, and ATTD did not change. In addition, ruminal epithelium development-related gene expression did not substantially change even in groups exposed to supraphysiological SCFA conditions, which showed increased SCFA disappearance rates. Collectively, those findings suggest that calves are resilient animals, able to thrive despite physiological changes. In fact, calves underwent unique conditions that highlight important physiological differences between calves and mature cattle that must be addressed. For example, it was proposed that young calves are active hindgut fermenters, given their decreased fecal pH and increased organic acid concentrations (Kumar et al., 2021; van Gastelen et al., 2021; Poier et al., 2022), and that the primary site of digestibility changes from the intestines to the rumen as calves age and start consuming more solid feed, such as calf starter (Quigley, 2019). The digestibility site transition may partly explain the over-time physiological differences (e.g., ruminal and fecal pH), as it changes the fermentation site, which increases H^+ production and decreases luminal pH (Aschenbach et al., 2019).

In addition, we observed increased fecal organic acid concentrations on d 35 when calf starter intake started increasing, suggesting that calves can experience an increased risk of hindgut acidosis depending on their diets. Hindgut acidosis happens when increased organic acid

concentrations and decreased pH increase the osmolarity pressure, which can jeopardize gut permeability, ultimately leading to inflammation and performance reduction (Gressley et al., 2011; Sanz-Fernandez et al., 2020). Considering that, the increased organic acid concentrations do not necessarily indicate the incidence of hindgut acidosis but do suggest an increased risk of presenting this disorder.

4.2.2. Lower gut is affected by the rumen environment in a short time period

Our relative short-term ruminal infusions showed considerable changes in the pH and organic acid concentrations of the gastrointestinal tract digesta and feces, along with alteration in the hindgut epithelium morphology and gene expression. These findings indicate that rumen environmental changes can be reflected in the lower gut in pre-weaning calves, highlighting the importance of knowledge of lower gut physiological requirements.

In addition, contrary to our hypothesis, nutritional practices combining high SCFA concentrations and low pH increased the hindgut digesta pH and decreased organic acid concentrations, potentially associated with an increased ruminal SCFA absorption capacity, that ultimately may have decreased the risk of hindgut acidosis. Interestingly, a HS-LP condition is similar to when calves are fed calf starter, as its fermentation also increases the ruminal SCFA concentration and decreases ruminal pH (Diao et al., 2019). Therefore, feeding calf starter potentially reduces the physiological risk of hindgut acidosis in pre-weaning calves when they are adapted to that. This could be an indirect effect of rumen development, improving ruminal digestibility and SCFA absorption capacity while decreasing hindgut fermentation, as discussed previously. Nevertheless, more studies are needed to investigate how rumen development affects hindgut fermentation and whether calf starter affects rumen and hindgut development differently.

4.3. Industry Applications

We demonstrated that changes in the ruminal environment can affect the entire gastrointestinal tract in a short period and that, although high ruminal SCFA concentrations and low pH conditions may decrease risks of hindgut acidosis, some adaption to this condition should be investigated given the increased fecal organic acid concentrations when calf starter started to be consumed more substantially. In addition, we do have some evidence suggesting that calves experience at least mild colonic acidosis in low pH conditions. Given these findings, the industry should start considering the lower gut requirements and potential impacts of calf nutrition management on the lower gut. Monitoring how the calves' hindgut responds to calf nutrition might be necessary to maximize calf performance and health.

4.4. Limitations and Future Research

A limitation of this study is the short period of ruminal buffer exposure. A more extended period of ruminal buffer exposure might provide a better understanding of how the rumen environment can affect lower gut physiology. In addition, more frequent infusions might be more impactful in evaluating rumen development than an extended infusion period, given that physiological changes associated with feed intake, such as ruminal pH changes, happen within 4 hours post-feeding (Laarman et al., 2012; Suarez-Mena et al., 2016a). However, given the invasiveness of the wash-reticulum technique, it may be challenging to keep calves healthy while investigating the physiological effects. Moreover, further investigation on how acidosis impacts the cecum and colon epithelium development is necessary as it seems that those gut segments may have different physiological requirements, which was observed not only with our results but also when checking the primers for qPCR, which seems to be gut segment-specific.

In addition, this study was based on supraphysiological conditions that might not necessarily reflect industry settings. Therefore, future studies are necessary to examine our findings in nutritional trials. As calf starter fermentation, degradation and digestion also rely on microbe activity (Membrive, 2016), our research questions should also be investigated using dietary modulations (i.e., changes in calf starter and forage composition, adaption protocol to calf starter) on non-cannulated calves. In summary, although this study provided important information on calf physiology and gut development, nutritional trials will be able to translate our findings into nutritional practices more applicable in the industry.

4.5. Conclusions

This study provided important information on the interaction between rumen and hindgut physiology of pre-weaning calves. Overall, 4 h of physiological buffer infusion in the rumen does not change performance; however, rumen environmental changes such as [SCFA] and pH can affect the lower gut in a short period. The HS-LP conditions may represent a decreased risk of hindgut acids, but an adaption might be necessary. Further investigations are required to understand if calves can experience hindgut acidosis in nutritional trials.

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