# DELIVERY OF BUTYRATE PRECURSORS IN THE GASTROINTESTINAL TRACT OF RUMINANTS AS A STRATEGY TO SUPPORT GASTROINTESTINAL FUNCTION AND DEVELOPMENT

A thesis submitted to the College of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the degree of the Doctor of Philosophy in the Department of Animal and Poultry Science

University of Saskatchewan

Saskatoon

By

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**ABSTRACT**

The general hypothesis for the four experiments reported in this thesis was that increasing the intestinal supply of butyrate will stimulate gastrointestinal tract development and functionality, leading to greater growth rates and feed efficiency in ruminants. The first study evaluated the dose response of hydrogenated fat-embedded calcium gluconate (**HFCG**), a butyrate precursor. Growing lambs were assigned to dietary treatments containing HFCG: 0.0% (**CON**), 0.075% (**LOW**), 0.3% (**MED**), or 0.6% (**HIGH**) of the diet (DM basis). Increasing dose of HFCG linearly decreased dry matter intake (**DMI**), average daily gain (**ADG**), and final body weight (**BW**). In the cecum, increased dose of HFCG linearly increased the molar proportion of acetate and linearly decreased the molar proportion of propionate, while the total short-chain fatty acid (**SCFA**) concentration was cubically affected, where LOW had the greatest concentration. Increased butyrate molar proportion was not observed in any of the of the intestinal regions analyzed. The second study aimed to compare the post-ruminal provision of calcium gluconate (**CaG**) and calcium butyrate (**CaB**) to explore the mode of action of CaG relative to butyrate. Four treatments were used, aiming to provide the same amount of butyrate post-ruminally (excluding CON) for beef heifers: 1) negative control (CON; ruminal infusion of double-distilled water); 2) abomasal infusion of CaB (**AB**; 0.029% of BW); 3) abomasal infusion of CaG (**AG**; 0.0077% of BW); and 4) ruminal infusion of HFCG (**RG**; 0.0192% of BW). Relative to CON, both AB and CaG treatments (AG + RG) reduced the molar proportion of acetate and increased the molar proportion of propionate in the colon. The ruminal disappearance of total SCFA, acetate, propionate, and butyrate were increased for AB but not for CaG, relative to CON. Barrier function was not affected by treatments when measured in vivo using urinary excretion of Cr-EDTA; however, when measured using ex vivo conditions, AB tended to decrease mannitol flux across ruminal tissue relative to CON. Neither AB nor CaG increased the intestinal molar proportion of butyrate. In the third experiment, HFCG was fed to finishing heifers at 0.0% (CON), 0.09% (**CG09**), or 0.18% (**CG18**) of the diet (DM basis). Increasing the dose of HFCG did not affect feed intake or growth; however, feed efficiency and ruminal stratum corneum thickness tended to be quadratically affected by HFCG dose. Quadratic effects were observed for jejunal pH, molar proportion of acetate, valerate, and isovalerate in the colon, and meat cook loss; however, changes in the molar proportion of butyrate were not observed in the intestinal regions analyzed. Treatments did not affect carcass characteristics and descriptive sensory analysis of the meat. Finally, in the fourth

experiments, tributyrin (**TB**) was used as a butyrate source and was provided in two forms: unprotected (**UTB**) and rumen protected (**RTB**). Eighty-four lambs were divided in two groups that were fed with different diets containing moderate (MF) or low forage (LF) inclusion, and within each group lambs were assigned to 1 of 6 treatments including a negative control (CON; no dietary inclusion of TB), dietary inclusion of RTB at 0.1% (**RTB1**), 0.2% (**RTB2**), and 0.3% (**RTB3**), or dietary inclusion of UTB at 0.1% (**UTB1**) and 0.3% (**UTB3**). Relative to RTB3, lambs fed UTB3 tended to finish heavier and tended to have an overall higher ADG when fed the MF diet. When lambs were fed the LF diet, RTB tended to increase DMI, and decrease feed efficiency and blood BHBA concentration relative to dietary inclusion of UTB. The use of CaG as a butyrate precursor did not increase the butyrate concentration in the intestine. When compared to the abomasal supply of CaB, post-ruminal supply of CaG did not yield the same results on ruminal SCFA disappearance, leading to the speculation that the site of exposure of butyrate in the gastrointestinal tract could yield different outcomes. The first three studies indicate that post-ruminal supply of CaG when provided at different doses, in differing diets, and ruminant species did not yield positive responses in growth and feed efficiency as the literature reports on the butyrate supply. As well, TB delivery both ruminally and post-ruminally did not increase carcass characteristics in lambs fed either with a MF or a LF diet.

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## LIST OF ABBREVIATIONS

AB = calcium butyrate infused into the abomasum at 0.0029% the body weight

ADF = acid detergent fiber

ADG = average daily gain

AG = calcium gluconate infused into the abomasum at 0.0077% of the body weight

AMP = antimicrobial peptides

$aNDF_{OM}$ = ash-free neutral detergent fiber on organic matter basis

BHBA = β-hydroxybutyrate

BW = body weight

CaB = calcium butyrate

CaG = calcium gluconate

CG09 = dietary inclusion of 0.09% of hydrogenated fat matrix-embedded calcium gluconate

CG18 = dietary inclusion of 0.18% of hydrogenated fat matrix-embedded calcium gluconate

CON = control

CP = crude protein

CV = coefficient of variation

DM = dry matter

DMI = dry matter intake

FFAR – free fatty acid receptor

FGF19 = fibroblast growth factor 19

FXR = Farnesoid X receptor

G:F = gain to feed

GH = Growth hormone

GIT = gastrointestinal tract

GLP-1 = glucagon-like peptide-1

GLP-2 = glucagon-like peptide-2

GLP-2R = glucagon-like peptide-2 receptor

*Gt* = tissue conductance

HCW = hot carcass weight

HFCG = hydrogenated fat matrix-embedded calcium gluconate

HIGH = dietary inclusion of 0.6% of hydrogenated fat matrix-embedded calcium gluconate

IGF-1 = insulin-like growth factor

*Isc* = short-circuit current

LF = low forage

LOW = dietary inclusion of 0.075% of hydrogenated fat matrix-embedded calcium gluconate

LPS = lipopolysaccharide

MCT = monocarboxylate transporters

MED = dietary inclusion of 0.3% of hydrogenated fat matrix-embedded calcium gluconate

MF = moderate forage

MUC-2 = mucin 2

ND = not detectable

NDF = neutral detergent fiber

NEFA = non-esterified fatty acid

NF-κB = nuclear factor kappa B

OM = organic matter

PBS = phosphate-buffered saline

RG = hydrogenated fat matrix-embedded calcium gluconate infused into the rumen at 0.0192% of the body weight

RTB = rumen-protected tributyrin

RTB1 = dietary inclusion of 0.1% of rumen-protected tributyrin

RTB2 = dietary inclusion of 0.2% of rumen-protected tributyrin

RTB3 = dietary inclusion of 0.3% of rumen-protected tributyrin

SCFA = short-chain fatty acid

SED = standard error of the difference

TB = tributyrin

TER = transepithelial resistance

TMR = total mixed ration

$uNDF_{OM}$ = undigested neutral detergent fiber on an organic matter basis

USDA = United States Department of Agriculture

UTB = unprotected tributyrin

UTB1 = dietary inclusion of 0.1% of unprotected tributyrin

UTB3 = dietary inclusion of 0.3% of unprotected tributyrin

ZO-1 = zonula occludens-1

ZO-2 = zonula occludens-2

# 1. GENERAL INTRODUCTION

Butyrate is a short-chain fatty acid (**SCFA**) produced by ruminal fermentation that gained attention due its effects on the development and maintenance of the gastrointestinal tract (**GIT**; Mentschel et al., 2001; Guilloteau et al., 2010). Identified as a signalling molecule (Li and Elsasser, 2005), butyrate increases the ruminal papillae surface area (Sakata and Tamate, 1978; Mentschel et al., 2001) by upregulating growth and proliferation (Li and Elsasser, 2005) and downregulating apoptosis (Mentschel et al., 2001) in ruminal epithelial cells. Thus, ruminal provision of an external source of butyrate in sheep, led to an increase of the ruminal absorptive surface area (Sakata and Tamate, 1976, 1978). Moreover, the cell proliferation promoted by an increase in butyrate concentration has also been observed in the small intestine of calves fed milk replacer containing sodium butyrate (Górka et al., 2014), and in colonic cells on in vitro systems treated with an external source of butyrate (Cook and Sellin, 1998; Furusawa et al., 2013). The increase in brush border enzyme activity (Basson and Hong, 1998), and in forestomach absorptive capacity as a result of increased of blood flow (Storm et al., 2011), and the promotion of a more stable intestinal ecosystem by the inhibition of the growth of pathogenic bacteria (Manzanilla et al., 2006), are additional effects of the provision of butyrate in the GIT. These responses can result in an enhanced growth rate and feed efficiency in livestock animals (Guilloteau et al., 2009).

The health of the host is also enhanced by increased concentration of butyrate in the GIT, as it has been reported to increase the gastrointestinal barrier function (Guilloteau et al., 2010) though the support of cellular tight junctions (Zhang et al., 2006) as a result of increased tight cell junction gene and protein expression (Peng et al., 2009; Wang et al., 2012). In vitro, the presence of butyrate increased transepithelial resistance (**TER**; Peng et al., 2009), and accelerated the assembly of tight junction proteins in colonic cells (Plöger et al., 2012; Wang et al., 2012). Butyrate has also been reported as an anti-inflammatory agent, controlling the local inflammation on the gastrointestinal epithelia (Dianzani et al., 2006) caused by inflammatory bowel disease (Serpe et al., 2010), ulcerative colitis (Vieira et al., 2012), or by repairing damaged intestinal cells (Piva et al., 2002; Mazzoni et al., 2008; Le Gall et al., 2009).

However, the dietary provision of free forms of butyrate has limited use due its strong odour and its potential to reduce diet palatability (Lallès et al., 2009; Guilloteau et al., 2010). Thus, the use of less odorous forms as butyrate salts (Guilloteau et al., 2010), butyrins (Edelman et al., 2003),

and butyrate precursors (Tsukahara et al., 2002) may facilitate its use as a feed additive. In an in vitro system containing porcine cecal digesta, Tsukahara et al. (2002) investigated glucose, sorbitol, and gluconic acid as potential butyrate precursors. Among them, gluconic acid resulted in high butyrate production and slowed fermentation, decreasing the production of lactic acid, compared to the other two substrates. Found in rice, honey, wine, and vinegar (Asano et al., 2005), gluconic acid is an organic acid derived from glucose and it can form gluconate salts with sodium, calcium, potassium, and zinc (Asano et al., 1994). In porcine cecal digesta, the increase in butyrate through sodium gluconate fermentation has been reported to stimulate the growth of *Lactobacillus* spp. and *Megasphaera elsdenii*; and it has been identified as a potential alternative to antibiotics, as it promoted growth in piglets (Biagi et al., 2006). In addition, sodium gluconate also increased jejunum villus height and crypt depth in piglets (Poeikhampha and Bunchasak, 2011). In ruminants, inclusion of calcium gluconate (CaG) in the total mixed ration (**TMR**) of lactating dairy cows resulted in contradictory results. Emery et al. (1960) reported an increase in fat-corrected milk yield, while McKnight et al. (2019) reported a reduction of milk protein and milk lactose yields. However, post-ruminal infusion of CaG in lactating dairy cows increased feed efficiency by decreasing dry matter intake (**DMI**; McKnight et al., 2019), increasing milk fat yield (Doelman et al., 2019a; McKnight et al., 2019), and energy-corrected milk yield (Doelman et al., 2019a). In addition, dietary provision of a rumen-protected form of CaG (hydrogenated fat-embedded CaG; **HFCG**) increased milk fat yield (Seymour et al., 2021), DMI, and milk protein yield (Seymour et al., 2022).

On the other hand, supplementation with tributyrin (**TB**, a butyrin) increased the DMI (Gu et al., 2017), final body weight (**BW**), average daily gain (**AGD**), and feed efficiency (Sotira et al., 2020) in piglets, probably as a result of the support of morphological development in the GIT (Sakdee et al., 2016). In pre-weaned calves, the addition of TB in milk replacer increased the plasma concentration of glucagon-like peptide-2 (**GLP-2**), but did not affect feed intake, growth, and feed efficiency (Araujo et al., 2016). Additionally, dietary provision of TB to weaned lambs tended to decrease DMI, increase neutral detergent fiber (**NDF**) digestibility, and increase the richness and diversity of the ruminal microbial community (Li et al., 2022). Dietary provision of TB also reduced the DMI of adult ewes (Ren et al., 2018) and reduced the inflammation response of lactating dairy cows subjected to heat stress (Guo et al., 2021). A reduction for the inflammatory response was also observed in mice induced to colonic inflammation (Leonel et al., 2013). It

appears that the provision of both butyrins and butyrate precursors seem to yield the same growth performance and morphological effects found with the provision of free forms of butyrate.

## 2. LITERATURE REVIEW

### 2.1. Introduction

Short-chain fatty acids (**SCFA**) are end products of microbial fermentation (Cummings and Macfarlane, 1991) that can be absorbed and metabolized by the gastrointestinal tract (**GIT**) and supply part of the energy requirements of cattle (Augenlicht et al., 1999). The proportion of energetic contribution from SCFA depends on the type of the diet consumed, where for ruminants which possess a developed fermentation chamber as the reticulorumen, can meet up to 80% of the energy requirements (Rechkemmer et al., 1988). The main SCFA produced during ruminal fermentation are: acetate, propionate, and butyrate (Pennington, 1952), where their amount and molar proportions are altered by the type of diet consumed (Sutton et al., 2003). Lactating dairy cows consuming a 40:60 forage-to-concentrate diet had ruminal fluid with a total SCFA production of 86.6 m*M* and a molar proportion of 67.0, 18.9, and 12.4 mol/100 mol of acetate, propionate, and butyrate, respectively. On the other hand, when consuming a 10:90 forage-to-concentrate diet, the ruminal fluid of lactating dairy cows presented a total SCFA production of 92.8 m*M* and a molar proportion of 50.1, 37.0, and 9.1 mol/100 mol of acetate, propionate, and butyrate, respectively (Sutton et al., 2003). It shows, that among the main SCFA produced in the ruminal fluid, butyrate is produced in the smallest proportion (Bergman, 1990). However, given the potency of butyrate to support maintenance and stimulate development of the GIT epithelium, butyrate supplementation has been vastly studied (Velazquez et al., 1996; Pender et al., 2000; Inagaki and Sakata, 2005).

### 2.2. General effects of butyrate in the gastrointestinal tract

Butyrate is an important direct energy source for the epithelium of the GIT, and the majority of the butyrate produced in the rumen (90%) is metabolized by the gut tissues (Bergman, 1990; Britton and Krehbiel, 1993). In the large intestine, Roediger (1980) reported butyrate as the main energy source of colonocytes with approximately 75% of the oxygen consumed by these cells being related to butyrate metabolism. Butyrate metabolism is initiated by its activation to butyryl-CoA (Britton and Krehbiel, 1993), which allows its oxidation via the β-oxidation pathway (Pennington, 1954) to acetyl CoA (Bergman, 1990). The acetyl CoA produced will be either converted to ketone bodies (as acetoacetate, acetone, and β-hydroxybutyrate [**BHBA**]) by

ketogenesis or enter the citric acid cycle where it is oxidazed to produce ATP (Emmanuel, 1980; Kolb et al., 2021).

In addition to energy supply, increasing ruminal butyrate has been reported to increase the mitotic index, leading to increased cell proliferation and consequently to increased absorptive surface area (Sakata and Tamate, 1976). In addition, in in vitro systems containing isolated carcinogenic cells, butyrate was able to induce differentiation (Chen and Breitman, 1994) and to suppress cell proliferation by inducing apoptosis and regulating the expression of oncogenes (Scheppach et al., 1995), showing the importance of butyrate to gastrointestinal epithelia development and maintenance.

Low (2m*M*) but not high (8 m*M*) concentration of butyrate in Caco-2 monolayer cells enhanced barrier function (Peng et al., 2007). Low concentrations of butyrate are able to positively regulate the expression of the major tight junction proteins (occludin, claudin-1, claudin-4, and zonula occludens-1) and to activate the AMP-activated protein kinase in Caco-2 cells; as well as it accelerates the relocation of the occludin and zonula occludens-1 to the periphery of the cell, facilitating tight junction assembly and, consequently increasing barrier function (Peng et al., 2009). In addition, butyrate indirectly controls inflammation (Ducastel et al., 2020) by signalling the activation of the Farnesoid X Receptor (**FXR**) through the free fatty acid receptor (**FFAR**) as FFAR2 and FFAR3 (G-protein coupled receptors 41 and 43, respectively). The FXR receptor plays an important role in the control of homeostasis, and its activation is important to control inflammation (Gege et al., 2019). Moreover, colonic defense is promoted by butyrate by inhibiting the growth of intestinal undesirable bacteria (Cook and Sellin, 1998), and increasing nutrient absorption through more blood flow to the ruminal epithelium (Storm et al., 2011). Thus, dietary supply of butyrate or diet manipulation to increase butyrate concentration at certain sites of the GIT could be a strong substitute for antibiotic growth promoters in production animals (Piva et al., 2002).

### 2.3. Effects of butyrate supplementation in the rumen

Ruminal supplementation of butyrate has been reported to increase ruminal papillae development in calves (Mentschel et al., 2001; Kato et al., 2011; C. Wang et al., 2017) and sheep (Sakata and Tamate, 1976; Sakata and Tamate, 1978) resulting in greater length and width of papillae and consequently larger absorptive surface area. The increased ruminal papillae

development is the consequence of an increased mitotic index (Sakata and Tamate, 1976; Sakata and Tamate, 1978; Mentschel et al., 2001) and reduced apoptosis (Mentschel et al., 2001). However, as reviewed by Penner et al. (2011), the direct effect of butyrate added to in vitro cell cultures containing ruminal epithelial cells are the opposite to the effects observed in vivo, as the addition of butyrate into these systems inhibited the epithelial proliferation (Gálfi et al., 1981; Neogrády et al., 1989). The contradictory effects show that the stimulatory effects of butyrate on the proliferation of the rumen epithelium are likely indirectly stimulated through the release of hormones and growth factors (Penner et al., 2011) such as insulin-like growth factor 1 (**IGF-1**; Wang et al., 2017; Xu et al., 2018), growth hormone (**GH**; Kato et al., 2011; Wang et al., 2017), insulin (Kato et al., 2011; Herrick et al., 2017), glucagon (Hatew et al., 2019; Inabu et al., 2019), and glucagon-like peptide-2 (**GLP-2;** Elsabagh et al., 2017; Inabu et al., 2019). Additionally, ruminal infusion of butyrate in lactating dairy cows led to an increase of blood flow to the reticulorumen epithelia, resulting in increased absorption of propionate (Storm et al., 2011). Thus, butyrate supplementation into the reticulorumen, likely increases SCFA absorption through a combination of increased absorptive surface area as a long-term effect and increased blood flow as an acute mechanism.

Zhang et al. (2018) evaluated the effects of butyrate to mitigate ruminal epithelial damage caused by a high-concentrate diet. In that study, sodium butyrate was added to the diet of goats fed a high-grain diet and the authors observed that butyrate enhanced the expression of the tight-junction proteins claudin-1, claudin-4, occludin, and zonula occludens-1 in the ruminal epithelium. These authors also observed that the addition of sodium butyrate (1% of the diet; DM basis) to high-concentrate diets inhibited protein kinase C (**PKC**) and mitogen-activated protein kinases (**MAPK**) signaling pathways and worked as a protective factor in the rumen epithelium as these kinases downregulated expression of tight junction proteins, leading to disruption of barrier function. Thus the addition of butyrate in high-concentrate diets, protects the ruminal epithelium against episodes of subacute acidosis.

As forementioned, increasing ruminal supply of exogenous butyrate affect hormones (IGF-1, GH, insulin, and glucagon) and metabolites (ketone bodies as β-hydroxybutyrate) concentration in the blood (Huhtanen et al., 1993; DeFrain et al., 2006; Herrick et al., 2017). In addition, GLP-2 is also reported to be increased by ruminal butyrate supply (Elsabagh et al., 2017; Inabu et al.,

2019; Fukumori et al., 2020); a response which will be discussed in future sections. The metabolic alterations caused by ruminal supply of exogenous butyrate may also affect feed intake and milk composition, as butyrate may increase dry matter intake (**DMI**), reduce milk urea nitrogen (DeFrain et al., 2004), increase milk fat and protein concentration (Huhtanen et al., 1993), and increase dry matter and organic matter digestibility (Fukumori et al., 2020) in lactating dairy cows. In fact, there is evidence for mitigation of DMI depression in pre-partum cows (DeFrain et al., 2006). On the other hand, negative effects have been observed including a decrease in feed efficiency in lactating dairy cows (Huhtanen et al., 1993), lighter calves at weaning due to reduced feed efficiency (Araujo et al., 2016), and disruption of the selective permeability of the isolated ruminal epithelium when fed high doses of butyrate into the rumen (1.25 and 2.50% of the diet; Wilson et al., 2012). Peng et al. (2009) reported that a low dose of butyrate (2 m*M*) promoted increased barrier function in Caco-2 cells, while a high dose (8 m*M*) decreased the barrier function, increasing intestinal permeability, indicating that dose-dependent responses to butyrate may behave in a quadratic manner.

### 2.4. Effects of butyrate supplementation in the small intestine

Although there is limited information available in the literature about the effects of butyrate in the small intestine, it is reported that in nonruminants, butyrate increased the ileal villus height (Sakdee et al., 2016) and decreased *E. coli* counts in the small intestine (Gu et al., 2017) of piglets supplemented with tributyrin. In addition, supplementation modulated permeability of the small intestine decreasing diarrhea incidence in piglets supplemented with sodium butyrate (Huang et al., 2015). The diminished responses might be due to the reduced amount of not only butyrate, but total SCFA naturally produced in the small intestine. Even with the increased production of SCFA in forestomach of the GIT of ruminants, the amount of SCFA observed in the small intestine coming from ruminal fermentation is very low. In steers fitted with cannula in the rumen, omasoabomasal orifice, abomasum, and duodenum, the concentration of SCFA found in the rumen versus duodenum decreased from 84 - 109 m*M* to 7 – 14 m*M*, respectively; reflecting considerable SCFA disappearance in the abomasum (Rupp et al., 1994). Pederzolli et al. (2018) analyzed the gastrointestinal contents of Holstein steers and showed a total concentration of total SCFA of 170.8 m*M* in the rumen, 46.2 m*M* in the cecum, and 45.6 m*M* in the colon; as compared to 6.4 and 9.7 m*M* in the duodenum and jejunum, respectively.

In ruminants, provision of sodium butyrate has been reported to function as a growth promoter in calves as compared to flavomycin. In that experiment, Guilloteau et al. (2009) reported that compared to flavomycin, sodium butyrate increased crypt depth and mitotic index in both the proximal and middle jejunum, and crypt depth in the distal jejunum. Moreover, duodenal brush-border enzyme activity (dipeptidase and maltase) was enhanced when sodium butyrate was added to milk replacer. Additionally, Górka et al. (2011) reported that the substitution of whole milk with milk replacer retarded the maturation and the development of the small intestine, but the addition of sodium butyrate to milk replacer helped to alleviate these negative effects. Górka et al. (2014) compared the effects of the supplementation of sodium butyrate in the starter mixture (ruminal supply) vs milk replacer (post-ruminal supply) and found that the site of delivery yielded different outcomes for small intestinal development. When added to the starter mixture, sodium butyrate tended to increase the weight of the small intestine, the crypt depth in the proximal jejunum, and villus height in the distal jejunum. However, when added to milk replacer, sodium butyrate decreased villus height in the proximal jejunum as well as villus height, crypt depth, and tunica mucosa thickness in the middle jejunum. Moreover, sodium butyrate added to the milk replacer enhanced brush-border enzyme activity in the jejunum and ileum. As reported in the rumen, calves supplemented with sodium butyrate showed increased cell proliferation and decreased apoptosis in the middle jejunum mucosa. Although both sites of release had positive effects on small intestine development, the release post-ruminally (milk replacer) showed a more pronounced effect (Górka et al., 2014). In addition, when infused into the abomasum of lambs, butyrate tended to increase oxygen uptake and increased the glucose, glutamine, and glutamate uptake by the portal-drained viscera (Foote and Freetly, 2016). A subsequent study verified that when lambs received an infusion of butyrate in the abomasum, there was an upregulation of genes related to glycolysis and gene clusters related to mitochondrial functions, changing the metabolic flux in the duodenum (Foote et al., 2017).

### 2.5. Effects of butyrate supplementation in the large intestine

The administration of butyrate to rodents (Vieira et al., 2012; Ducastel et al., 2020) and humans (Scheppach et al., 1992; Vernia et al., 1995; Lührs et al., 2002) has shown potential to control inflammation in the large intestine through the modulation of several pathways. For example, goblet cell expression increases leading to elevated mucus production (Rhodes, 1997).

In piglets fed tributyrin, butyrate protected them against a lethal lipopolysaccharide (**LPS**) challenge, that led to a reduction in cecal goblet cells expression, paired with a down regulation of inflammatory cytokine production (Gu et al., 2017). On the other hand, in colonic cell culture, butyrate stimulated mucus production mediated by an increase in prostaglandin production and mucin 2 (**MUC-2**) expression (Willemsen et al., 2003). Additionally, Inan et al. (2000) reported that butyrate reduced the activation of nuclear factor-κB (**NF-κB**) in a human colonocyte cell line. The NF-κB plays a central role in the regulation of immune and inflammatory responses, which consequently regulates the expression of several cytokines (Baeuerle and Henkel, 1994). As such, butyrate acts to downregulate cytokine-activated gene expression thereby suppressing colonic mucosal inflammation (Gibson and Rosella, 1995). From a human health perspective, butyrate has been consistently reported to act against ulcerative colitis (Chapman et al., 1994; Vieira et al., 2012) and inflammatory bowel disease (Serpe et al., 2010).

In cell culture, butyrate has been reported to increase barrier function of large intestine epithelial cells. In a Caco-2 cell monolayer model, low concentration (2 m*M*) of butyrate resulted in an increase in the barrier function verified by increased transepithelial electrical resistance (**TER**) and decreased permeability of inulin, indicating a reduction in permeability (Peng et al., 2007). Moreover, using the same cell culture model and the same dose of sodium butyrate, Peng et al. (2009) reported that the increase in barrier function was mediated by an increase in AMP-activated protein kinase activity. The AMP-activated protein kinase is reported to accelerate tight junction protein assembly and reorganization (Zheng and Cantley, 2007). Similar results for enhanced barrier function were reported by Wang et al. (2012) in cdx2-IEC monolayer cell cultures with reported outcomes including increased expression of tight junction proteins (claudin-1), redistribution of zonula occludens-1 and occludin to the cellular membrane, and greater TER. However, it is important to note that butyrate yielded the opposite result (decreased barrier function) when cells were treated with high doses of sodium butyrate (8 m*M*) as described by (Peng et al., 2007) which agrees with the results described in the rumen section (Wilson et al., 2012).

In the large intestine, adequate concentrations of butyrate have been reported as being essential to maintain immune homeostasis (Tan et al., 2014), as well as responsible for the proliferation, differentiation, and maturation of the cecal and colonic epithelial cells (Kosaka et

al., 1991; McNabney and Henagan, 2017). It should be noted that stimulatory effects are not observed with other SCFA (Kurata et al., 2019). Thus, treatment with butyrate has been reported to be a promising treatment against colorectal cancer as butyrate is capable of modulating the expression, growth, and apoptosis of carcinogenic cells (Berenson et al., 1995; Kurata et al., 2019).

Finally, the delivery of butyrate (in the form of sodium butyrate) post-ruminally in calves (added to the milk replacer) has been reported to affect colonic SCFA composition, with an increase in total SCFA concentration, and the molar proportions of acetate and propionate without an increase in butyrate. Butyrate supply also has altered the cecal microbiota with a decrease in the abundance of *Butyrivibrio* and *Shuttleworthia* and an increase in the abundance of *Phascolarctobacterium*, a propionate producer (O'Hara et al., 2018). In addition, these authors also observed a decrease in the abundance of *Mogibacterium*, a bacteria related to impaired gut health which agreed with previous reports on the effects of butyrate on this intestinal pathogen (Steinmann et al., 2009). Changes in SCFA and microbial profile in the large intestine tended to increase the growth rate in the pre-weaning phase, leading to heavier calves at weaning. However, provision of sodium butyrate did not affect DMI and only tended to improve feed efficiency of calves relative to the control (O'Hara et al., 2018).

### 2.6. Effects of glucagon-like peptide-2 on the gastrointestinal tract development

Glucagon-like peptide-2 is a gut peptide secreted by the L-cells located in the gastrointestinal tract (D. Burrin et al., 2003), and its production and secretion have been linked to nutritional, hormonal, and neural stimulation (Ørskov and Holst, 1987; Brubaker et al., 1997; Hartmann et al., 2000). In livestock, an increase in plasma GLP-2 concentration has been reported in dairy calves ruminally infused with sodium butyrate (0.3, 0.6, 0.9, and 1.2 g per kg of BW; Hatew et al., 2019), in calves fed a starter mixture supplemented with sodium butyrate (0.6% as fed; Górka et al., 2011a), in dairy cows provided with sodium butyrate in the diet (1.1% of the dietary DM; Fukumori et al., 2020), and for adult sheep ruminally infused with sodium butyrate (up to reach 10% of the daily energy requirements; Elsabagh et al., 2017). As such, it is postulated that some of the responses to butyrate are mediated by GLP-2.

The effects described by the increase of plasma GLP-2 concentration are associated with intestinal growth (Gleeson et al., 1971), development (Burrin et al., 2003), and repair (Drucker, 2001; Drucker, 2002; Campbell and Drucker, 2013). In rats with surgically induced small intestine

resection, increased concentration of plasma GLP-2 promoted a faster morphological and functional adaptation of the intestine, rapidly enhancing the size of the intestinal regions (Ljungmann et al., 2001). In restricted fed dairy cows, provision of exogenous GLP-2 largely mitigated the negative effects of feed restriction on intestinal mucosa villus height, villus width, and crypt depth (Kvidera et al., 2017b). Drucker et al. (1996) showed that mice subcutaneously injected with GLP-2 had increased mucosal mass, villus height, and crypt cell proliferation along the small intestine. In a study with pig fetuses and neonates, exogenous GLP-2 increased the expression and activity of aminopeptidase A in fetuses, and maltase in neonates (Petersen et al., 2001). In rats, exogenous GLP-2 increased the expression of ileal sucrase-isomaltase, and tended to increase its expression in the jejunum (Kitchen et al., 2000). These results show that GLP-2 not only supports the initial development of the GIT but also acts to reestablish the morphology and function of the GIT after a stress episode.

Additionally, beyond the stimulus on intestinal epithelium development and increased activity of brush-border enzymes, increasing concentration of plasma GLP-2 also stimulates intestinal nutrient transport and increases intestinal blood flow and consequently nutrient utilization (D. Burrin et al., 2003). The increase in blood flow stimulated by GLP-2 has also been reported in calves (Taylor-Edwards et al., 2011; Walker et al., 2015; Connor et al., 2016). Moreover, subcutaneous infusion of GLP-2 has been reported to increase GIT barrier function in calves (Taylor-Edwards et al., 2011; Walker et al., 2015) and in mice (Benjamin et al., 2000; Hadjiyanni et al., 2009). It also increases the expression of tight junction proteins (ZO-1; Walker et al., 2015) and reduces GIT permeability. Finally, GLP-2 controls intestinal inflammation based on a reduction of circulating concentrations of haptoglobin and serum amyloid A in feed-restricted dairy cows (Kvidera et al., 2017b), and inhibition of NF-κB activation in mice exposed to LPS-induced inflammation (Xie et al., 2014). In conclusion, effects reported for GLP-2 are similar to the ones reported for butyrate and it is possible that butyrate functions indirectly through GLP-2. That said, few studies have evaluated the mechanisms between butyrate and increases in plasma GLP-2, thus it can only be speculated whether butyrate has an indirect effect or is directly responsible for increases in plasma GLP-2 concentration.

### 2.7. Effects of tributyrin supplementation as a butyrate source

Tributyrin (**TB**) is a triacylglycerol found in butter (Leonel et al., 2013) and is composed of three butyrate molecules esterified to a glycerol (Edelman et al., 2003). Compared to free butyric acid, TB is not volatile and has less odour, which decreases adverse properties associated with manufacturing and its inclusion in diets (Guilloteau et al., 2010; Inabu et al., 2019). In humans, the odour of butyrate impairs its use in cancer treatment and use of TB could be more readily orally administered with low toxicity, improving patient well-being and pain control (Edelman et al., 2003). In nonruminants, the release of butyrate from the TB molecule in the small intestine by intestinal lipase, altered in the site of absorption of free butyrate from the stomach to the small intestine (Li et al., 2015).

The use of TB in nonruminants diets has been mostly reported in pre-weaned and weaned piglets, with results being inconsistent. For suckling piglets with intrauterine growth restriction, TB increased body weight (**BW**) relative to the non-supplemented group, but it was insufficient to overcome the growth restriction (Dong et al., 2016). The increase in growth was associated with the development of the immune organs (spleen) and small intestine morphology, digestive enzyme activity, and improved barrier function. For weaned pigs, Sakdee et al. (2016) did not observe any differences in growth, gastrointestinal pH, intestinal microbial populations, or SCFA composition in the large intestine. However, Piva et al. (2002) observed that feeding TB (combined with lactitol) to weaned piglets led to a reduction in mortality and weight loss related to weaning, followed by increased BW and average daily gain (**ADG**), relative to control. Moreover, Gu et al. (2017) reported that feeding TB prevented growth retardation in weaned piglets by stimulating appetite, increasing ileal epithelium morphological development, modulating the fibroblast growth factor 19 (**FGF19**) ileal expression, and decreasing *E. coli* counts in the intestine. These authors also reported TB modulated inflammatory cytokine concentration, protecting the animals from a lethal LPS challenge. Finally, Sotira et al. (2020) observed that compared to the control group, TB increased ADG in the second half of the supplementation period and improved the feed efficiency of weaned piglets, while increasing serum glucose and insulin.

In ruminants, the release of butyrate from TB should occur in the rumen through microbial hydrolysis of triacylglycerols (Noble, 1978). When added to milk replacer for Holstein calves, TB did not affect growth, fecal score, or most of the measured blood metabolites (glucose, BHBA and IGF-1; Inabu et al., 2019). However, an increase in blood GLP-2 was detected (Inabu et al., 2019).

On the other hand, a different outcome was observed by Araujo et al. (2016), where the supplementation of TB in milk replacer of Holstein calves negatively impacted growth, and tended to decrease feed efficiency. Likewise, TB did not glucose, insulin, BHBA, and glucagon-like peptide-1 (**GLP-1**) in blood. For weaned lambs, dietary provision of TB (combined with glycerol monolaurate) increased feed efficiency and the apparent digestibility of neutral detergent fiber (**NDF**), and increased of Chao1 and Shannon indices of the ruminal microbiome (Li et al., 2022).

In lactating goats, intraruminal infusion of high doses of TB (168 g/d) resulted in lethargy two hours after infusion, an increase in blood BHBA and insulin concentrations, and a decrease in blood glucose concentration (Drackley et al., 1989). In an in vitro study using ruminal fluid from ewes fed a diet containing a 60:40 forage-to-concentrate ratio, increased doses of TB (0, 2, 4, 6, and 8 g/kg; DM basis) linearly increased apparent degradation of DM, crude protein (**CP**), NDF, and acid detergent fiber (**ADF**), followed by a linearly decrease in $CO_2$ production and a linear increase in methane production (Ren et al., 2018). In addition, in the same study TB also increased the activity of fibrolytic enzymes. In an in vivo study using ewes, increased TB (0, 2, 4, 6, and 8 g/kg; DM basis) linearly decreased DMI and ruminal pH, followed by a linear increase in total SCFA, acetate, propionate, butyrate, and branched-chain fatty acid, and a linear increase in the molar proportion of butyrate in ruminal fluid (Ren et al., 2018). In dairy cows, a rumen protected form of TB was tested under heat stress conditions and the authors observed that provision of TB increased milk fat, protein, and energy-corrected milk yield while it reduced milk urea nitrogen and somatic cell counts. Additionally, TB reduced the expression of inflammatory cytokines (tumor necrosis factor α, interleukin 1β, and interleukin 6), indicating an overall reduction in the inflammatory response to heat stress in dairy cows (Guo et al., 2021).

### 2.8. Effects of gluconic acid supplementation as a butyrate precursor

Gluconic acid has been identified as a butyrate precursor and unlike butyrate, gluconate salts do not affect the taste of foods (Asano et al., 2005). The fermentation profile of gluconic acid was compared to glucose and sorbitol (a slowly fermented sugar) and after 24 h of incubation in an in vitro system containing porcine cecal digesta, it presented a slower fermentation rate and increased production of butyrate (Tsukahara et al., 2002). Moreover, gluconic acid fermentation resulted in lesser production of lactate compared to glucose fermentation (Tsukahara et al., 2002). These authors suggested that the main gluconic acid fermenters were lactic acid bacteria (*Lactobacillus*

*reuteri* and *L. mucosae*) and acid-utilizing bacteria (*Megasphaera esldenii* and *Mitsuokella miltiacida*), where initially, lactic acid bacteria ferment gluconic acid to acetate and lactate and acid-utilizing bacteria use these products to form butyrate (Figure 2.1). Additionally, Tsukahara et al. (2006), showed that porcine cecal digesta incubated in vitro with sodium gluconate and inoculated with *Lactobacillus acidophilus* and *Megasphaera elsdenii* increased the production of butyrate by 60% and valerate by 50% relative to the uninoculated culture. On the other hand, Asano et al. (1994) identified that supplementation with gluconic acid in humans, increased the count of fecal *Bifidobacterium*.

Katagiri and Imai (1955) investigated the fermentation of gluconate, glucose, and xylose using in vitro systems inoculated with lactic acid bacteria and reported that the fermentation of 2 moles of gluconate in a static system generated 2 moles of lactic acid, 1 mol of acetic acid, 1 mol of ethanol, 2 moles of carbon dioxide, and 1 mol of water. The resulting stoichiometry is reported below.

$$2\ C_6H_{12}O_7 \rightarrow 2\ C_3H_6O_3 + C_2H_4O_2 + C_2H_5OH + 2\ CO_2 + H_2O$$ (Katagiri and Imai, 1955).

Collectively, these data indicate that initially or at least the first step of the fermentation process is likely conducted by lactic acid bacteria and that butyrate is not immediately produced. The production of butyrate could be explained by an in vitro experiment incubating human fecal microflora conducted by Bourriaud et al. (2005), where the authors showed that two of the three microflora tested fermented lactate to butyrate. According to the C-labelled technique used by the authors, they verified that the butyrate was produced through the acetyl CoA pathway indicating that lactate was fermented to pyruvate, pyruvate was fermented to acetate, and then acetate was converted to acetyl CoA and then to butyrate (the detailed pathway is shown of Figure 2.1, the pathway adapted from Bourriaud et al., 2005). The fermentation pathway from lactate to butyrate using an external source of acetate has also confirmed in human colonic cultures (Muñoz-Tamayo et al., 2011) and in dark fermentation in bioreactors (Detman et al., 2019; Detman et al., 2021).

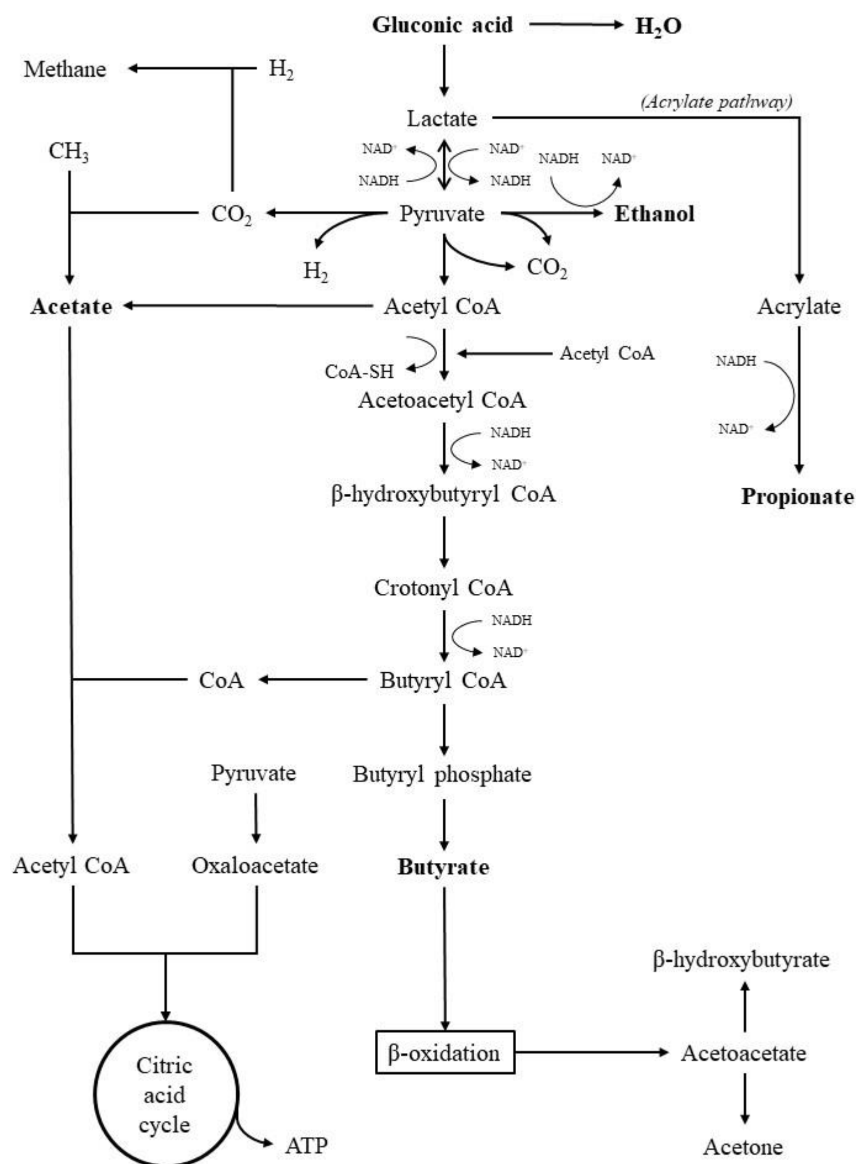


**Figure 2.1.** Schematic representation of the metabolic pathways of the static fermentation of gluconic acid resulting in butyrate, propionate, acetate, ethanol, carbon dioxide, and water as end-products (Katagiri and Imai, 1955). The oxidation of gluconic acid to pyruvate could result in the production of ethanol (Eram and Ma, 2013), propionate (Den Besten et al., 2013), acetate, or butyrate (Bourriaud et al., 2005), and the acetate could be used as external source of carbons to produce butyrate (Detman et al., 2019). Two metabolic pathways are available for acetate production, where it can be formed directly from acetyl CoA (resulted from pyruvate oxidation) or by the Wood-Ljungdahl pathway, where a carbon dioxide and a methyl radical are used for acetate production; while the acrylate pathway is available to propionate production (Den Besten et al., 2013).

Unlike butyrate, gluconic acid is poorly absorbed in the small intestine (19.9 and 11.6% in the upper and in the lower portion of the small intestine of rats, respectively; Asano et al., 1994) and when gluconic salts were incubated in situ in the loops of small intestine of rats, 100% of the incubated salts remained unaltered (Asano et al., 1997), most likely due the low bacterial density in the proximal small intestine ($10^3$ and $10^4$ bacteria/gram of digesta in the duodenum and jejunum, respectively; compared to $10^{12}$ bacteria/gram of digesta in the colon; Dieterich et al., 2018). In addition, Asano et al. (1997) tested the digestion of gluconic acid and gluconate salts in different digestive juices (human saliva, artificial gastric juice, porcine pancreatin, and rat intestinal mucosa) and concluded that gluconate salts were stable in various media. Thus, feeding gluconate to nonruminants provides a mechanism to deliver a potential butyrate precursor in the distal GIT, as microbial conversion from gluconate to butyrate is required to release butyrate. The hypothesis for distal delivery of butyrate using Na-gluconate was tested in an in vivo study with rats fed a carcinogenic compound to induce colon cancer. In that study, the dietary supply of sodium gluconate increased cecal butyrate concentration and decreased the incidence of cancerous tumors in the large intestine. The authors attributed the antitumorigenic effects of sodium gluconate to butyrate and its stimulation of apoptosis in cancerous cells (Kameue et al., 2004).

Additionally, similar effects among butyrate supplementation and sodium gluconate supplementation have been observed in piglets, where Poeikhampha and Bunchasak (2011) observed a decrease in cecal ammonia concentration and increase in cecal lactic acid, along with a tendency for an increase in total SCFA and butyrate concentrations in cecal digesta. Jejunal crypt depth was positively affected by supplementation with sodium gluconate, leading to an increase in ADG and final BW. Moreover, Biagi et al. (2006) added gluconic acid to the diet of piglets at 0, 3,000, 6,000, and 12,000 ppm, and observed a quadratic response with piglets fed 6,000 ppm of gluconic acid having greater ADG and final BW.

In dairy cows, the provision of 57 g/d calcium gluconate (**CaG**) in a total mixed ration (**TMR**) fed to dairy cows was initially reported to increase milk fat percentage and fat-corrected milk yield, but DMI was not reported (Emery et al., 1960). When administrated post-ruminally to target an increase in butyrate production in the GIT, CaG increased milk fat concentration and tended to increase milk fat yield and energy-corrected milk yield (Doelman et al., 2019a). In that study, relative to control, an increase in fecal isobutyrate and blood BHBA, and a decrease of

glucose and non-esterified fatty acids (**NEFA**) were observed in cows supplemented with CaG. Moreover, McKnight et al. (2019) reported that provision of ruminally available CaG and post-ruminal supply yield outcomes differed in dairy cows. When added to the diet, CaG did not affect DMI but decreased both milk protein and milk lactose yields. Feeding CaG increased the proportion of ruminal acetate, decreased the proportion of butyrate, and decrease the concentration of propionate, without affecting blood metabolites (glucose, NEFA, and BHBA). On the other hand, post-ruminal infusion of CaG decreased DMI and increase milk fat yield, increasing the feed efficiency of lactating dairy cows. Although the results of ruminal provision are not consistent between Emery et al. (1960) and McKnight et al. (2019), post-ruminal supply of CaG seems to have more consistent benefits on milk fat yield. Seymour et al. (2021) fed dairy cows a rumen-protected form of CaG (hydrogenated fat-embedded CaG; **HFCG**), where in the first experiment, the authors observed a reduction of fecal isobutyrate and an increase on circulating NEFA and milk urea. Additionally, feeding HFCG also tended to increase fat- and energy-corrected milk yields, milk fat and lactose yields, and lactose concentration, but tended to decrease the protein content relative to control. Seymour et al. (2022) fed HFCG to primiparous and multiparous dairy cows, and while primiparous cows did not differ in milk yield or milk composition; feeding HFCG increased DMI and milk protein yield and tended to increase milk fat yield and fat- and energy-corrected milk yields in the first 8 weeks of lactation of multiparous cows. Although these studies did not compare the effects of CaG versus the effect of butyrate provided post-ruminally, it seems that CaG has potential to increase milk performance.

### 2.9. Short-chain fatty acid absorption

Microbial populations in the GIT of mammals produce SCFA that have great importance for the metabolism of the host as they can meet part of their energy requirements. As reviewed by Rechkemmer et al. (1988), the energy requirements met by SCFA metabolism depends on the species, and it is highly dependent on the feeding type (i.e., carnivores, herbivores, omnivores, insectivores), on the size and development of the hindgut, diet and on the presence or absence of a forestomach. The contribution to the energy requirements varies from less than 10% for rats, dogs, and humans; to 30 to 40% for ponies and rabbits; 30 to 76% for pigs (Rechkemmer et al., 1988); and up to 80% for ruminants (Bergman, 1990).

Successful utilization of SCFA as an energy source depends on absorption and both primary and secondary metabolism. Based on the measurement of the unabsorbed SCFA bypassing the rumen, the absorption of ruminal SCFA ranges from 50 to 85% with the wide range in absorption being attributed to different absorptive capacities, different digesta passage rates among species, and differing diets fed (Aschenbach et al., 2011). Despite long-chain fatty acids and SCFA belonging to the same chemical family (i.e., fatty acids) the absorptive mechanisms are quite different. The absorption of long-chain fatty acids are driven by hydrolyzation of triacylglycerols to chylomicrons with absorption initially reaching lymphatic vessels before entering systemic circulation (Iber, 1998). However, SCFA absorption differs as they have a hydrophilic character, resulting in a different absorption mechanism (Bézard and Bugaut, 2018).

The capacity of the epithelium to absorb SCFA varies throughout the regions of the GIT. As SCFA are products of microbial fermentation (Roy et al., 2006), the forestomach (reticulorumen) and hindgut (large intestine, cecum and colon) are regions with greater SCFA production and absorption. Environmental conditions in the small intestine (fast passage rate combined with the presence of bile and digestive enzymes) limit growth and maintenance of microbial communities (Kastl et al., 2020). Consequently, the duodenum and jejunum present a lower microbial count ($10^3$ and $10^4$ to $10^5$ CFU/mL of digesta, respectively), compared to the colon ($10^{12}$ CFU/mL of digesta; Hayashi et al., 2005).

Ruminal pH is closely linked to the rate of SCFA production and its absorption, thus ruminal pH is dependent on the ingredients in the diet (rate of fermentation and amount of physically effective fiber, Allen, 1997). In normal conditions, ruminal pH ranges from 5.5 to 7.0 (Grünberg and Constable, 2009) and given the dissociation constant (pKa) of SCFA (4.8; Cistola et al., 1982), most of the SCFA in the ruminal fluid are found in the dissociated form ($SCFA^-$) rather than in the undissociated form (HSCFA; Aschenbach et al., 2011). The production of SCFA and the consequent accumulation of protons ($H^+$) in the ruminal fluid has the potential to reduce pH, resulting in sub-acute ruminal acidosis that can negatively affect growth and productivity of cattle (Nocek, 1997). Proton removal is mediated by buffers present in saliva and other buffering substances; however, the main mechanism of proton removal is through absorption, accounting for more than half of the total proton removal (Allen, 1997). Short-chain fatty acid absorption pathways differ dependent whether SCFA are in the dissociated or undissociated form. The

absorption of the undissociated SCFA occurs through lipophilic diffusion (Walter and Gutknecht, 1986; Gäbel et al., 2002). On the other hand, the absorption of dissociate SCFA, which are practically impermeable to the membrane, occurs primarily through a bicarbonate-dependent transporter (Aschenbach et al., 2011). Other pathways of absorption for undissociated SCFA include that facilitated through the monocarboxylate transporters (**MCT**) and maxi-anion channels (Stumpff et al., 2018).

While predominant absorption pathways for SCFA are known, regulation of absorption is complex (Aschenbach et al., 2011). Acetate, propionate, and butyrate, the main SCFA produced by microbial fermentation, have similar pKa ($pKa_{acetic\ acid}$ = 4.75, $pKa_{propionic\ acid}$ = 4.87, and $pKa_{butyric\ acid}$ = 4.82; Cistola et al., 1982) values, but their production rates are widely different. In grass-fed sheep provided a diet meeting maintenance requirements, the rate of SCFA production was 3.7, 1.0, and 0.7 mol/day for acetate, propionate, and butyrate, respectively (Bergman, 1990). Due to the chain length, the permeability of butyrate is greater than propionate, and propionate is greater than acetate and as a result the permeation coefficient of butyrate is14 times greater than for acetate (Walter and Gutknecht, 1986). The rates of intracellular metabolism (which results in faster clearance, creating a gradient that increases permeability and absorption) follows the same order as the permeability rates (Kristensen and Harmon, 2004), as reviewed by Aschenbach et al. (2011). As stated by Walter and Gutknecht (1986), individual SCFA production rates are inversely related to the lipophilic capacity to permeate through the epithelial membrane. Gäbel et al. (2001) demonstrate that blocking ATP delivery into ruminal epithelial tissue causes a reduction in butyrate metabolism and a consequent intracellular accumulation, decreasing butyrate flux. Moreover, branched-chain fatty acids have been reported to have a slower rate of absorption as compared to straight-chain fatty acids (Bugaut, 1987); a finding supported in vivo as the absorption of butyrate has been reported to be greater than iso-butyrate (Gäbel et al., 2001).

The bicarbonate-dependent transport of SCFA, on the other hand, depends on a transport protein which is bicarbonate dependent. The $SCFA^-/HCO_3^-$ exchanger located in the apical membrane of the cells transports dissociated SCFA into the cell exchanging it with bicarbonate ($HCO_3^-$) which is released into the rumen (Kramer et al., 1996; Aschenbach et al., 2009). Opposite to absorption by diffusion, in which the SCFA carries a proton while it crosses the membrane

(HSCFA), this electroneutral transport mechanism neutralizes a proton in the rumen through the carbonic anhydrase reaction ($HCO_3^- + H^+ \rightarrow H_2O + CO_2$).

The capacity of ruminants to be resilient to acidosis or to be more resistant to high-grain diets could be related to the capacity of proton removal or SCFA absorption by the ruminal epithelium. In an experiment conducted by Penner et al. (2009), sheep were divided into three treatments described as the control (which did not receive any acidosis challenge), a non-responsive group (which received a acidosis challenge – glucose drench into the rumen – but did not responded to it), and responsive group (which received the acidosis challenge and respond in terms of developing acidosis). Ruminal tissue was mounted in Ussing chambers to evaluate acetate and butyrate uptake with the non-responsive group having greater uptake of both acetate and butyrate as compared to the responsive and control groups, indicating a greater capacity of SCFA absorption by the ruminal epithelia. In addition, the higher plasma BHBA concentration found in vivo in the non-responsive group, compared to responsive and control group, likely reflected higher butyrate absorption in vivo. This experiment provided evidence for the importance to balance SCFA production and proton removal from the ruminal environment, and the individual response that each animal can have to an acidotic challenge (Penner et al., 2009a).

With regard to SCFA absorption in the large intestine, data shows that the majority of the SCFA produced in the large intestine are absorbed (more than 95%; Rechkemmer et al., 1988). In the human rectum, the absorption rate of butyrate and propionate are higher than the absorption rate of acetate; however, no differences were observed between the absorption rates of propionate and butyrate (Saunders, 1991). These responses are closely linked to chain length and the metabolization capacity of the SCFA in the ruminal epithelium (Rönnau et al., 1989). In addition, as reported in the ruminal epithelium (Rübsamen and von Engelhardt, 1981), SCFA absorption is closely related to the colonic transport of electrolytes and water (Roediger and Moore, 1981). Although the $Na^+$ absorption mechanism is not related to each individual SCFA, it is closely related to SCFA absorption. Unlike in the rumen, these authors also observed that a pH reduction (from 6.4 to 5.0) results in a reduction (70%) in $Na^+$ absorption, suggesting that $Na^+$ absorption might be strongly related to the luminal concentration of $H^+$ (Rousseau and Sladen, 1971).

Finally, it is important to mention that, despite lactate not being classified as a SCFA, the rate of its absorption across the ruminal epithelium is 10 times lower than the rate of SCFA

absorption (Williams and Mackenzie, 1965). The slow rate of absorption is the main reason for accumulation of lactate in the ruminal environment and lactate has been implicated as a driver for ruminal acidosis (Nagaraja and Titgemeyer, 2007). The identified lactate transporter on the ruminal epithelial tissue is a MCT that seems to vary between different ruminant species, and it could be one of the factor that determines the resiliency and resistance to ruminal acute acidosis (Nagaraja and Titgemeyer, 2007).

## 2.10. Barrier function and tight junction proteins

The gastrointestinal epithelium is one of the largest surfaces areas in contact with the outer world of the mammalian body, and structures present on the epithelium, like crypts, are responsible for increasing the surface area estimated at approximately 400 $m^2$ (Van Der Flier and Clevers, 2009). The epithelium is formed by continuous cells lining the GIT that regulate the selective permeability of substances, allowing the absorption of nutrients and preventing the paracellular movement of non-desired molecules. This line of cells also has the function of sensing (nutrients and microorganisms), regulating the luminal microbiota, and initiating an immune response if necessary (Jutfelt, 2011).

The gastrointestinal barrier function is controlled by the specialized intestinal epithelial cells as the enteroendocrine, goblet, and Paneth cells. The enteroendocrine cells maintain communication between the central and enteric neuroendocrine system by secreting hormones that regulate digestive function; the goblet cells the physically protect cells through the secretion of mucus; and Paneth cells produce antimicrobial proteins (**AMP**; Gallo and Hooper, 2012) which are essential to minimize the risk of inflammation due the high microbial colonization in the GIT (Peterson and Artis, 2014). Opposite to the intestinal epithelium (monolayer), the rumen epithelium is a stratified epithelium containing four strata: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale (Graham and Simmons, 2005). The outer stratum, stratum corneum, is a keratinized cell layer which works as the first physical barrier of the epithelium to the constant exposure to the rumen microbiota and it is similar to the mucus layer formed in the intestinal epithelia (Meyer et al., 2014; Baaske et al., 2020). The stratum corneum contains papillae which increases the absorptive surface area of the ruminal epithelium. The second barrier of the ruminal epithelium is located in the stratum granulosum, which expresses tight junction proteins,

such as claudin-1 and zonula occludens-1 (Graham and Simmons, 2005) which promote the selective permeability of the epithelium to nutrients (Aschenbach et al., 2019).

Junctional complexes are composed by the tight junctions, adhered junctions, gap junctions, and desmosomes (Farquhar and Palade, 1963) and they are located near the apical surface of the epithelium (Schneeberger and Lynch, 1992; Tsukita et al., 2001). The tight junctions are responsible for the seal between adjacent cells with the point of sealing called “kissing points”; moreover they also act like a fence separating the apical from the basolateral membrane of the cells (Claude and Goodenough, 1973; Schneeberger and Lynch, 1992). As a result, tight junctions determine the paracellular permeability to solutes (Tsukita et al., 2001). Tight junctions are composed of tight junction proteins, which are responsible for maintaining adherence between adjacent intestinal cells. Tight junctions, also are responsible for regulating selective permeability using the cytoplasmatic actin and myosin networks (Blair et al., 2006). Gastrointestinal cells have a rapid and constant rate of renewal, thus the control of the expression of the tight junction proteins are important in maintaining epithelial integrity (Eisenhoffer et al., 2012). Dysregulated turnover of intestinal epithelial cells could result in disruption of barrier function, increasing intestinal permeability (Peterson and Artis, 2014).

Additionally, the second element of the junctional complex, the adhered junctions, are located immediately behind the tight junctions (Farquhar and Palade, 1963). In a single layer epithelia, as found in the intestine, adhered junctions are responsible for the mechanical attachment between the adjacent cells (Schneeberger and Lynch, 1992) and for intercellular communication, but they are not responsible for the paracellular permeability (Oda and Takeichi, 2011). Desmosomes are the third element of the junctional complex (Farquhar and Palade, 1963) and acts along the adhered junctions (Oda and Takeichi, 2011), and the gap junctions which are specialized structures that facilitates cell to cell communication (Schneeberger and Lynch, 1992).

As presented by Nejdfors et al. (2000) and Penner et al. (2010), different regions of the GIT present different barrier function, which means that the tightness of the tight junctions varies in a tissue-dependent manner, resulting in differential permeability (Schneeberger and Lynch, 1992). Thus, mucosal permeability is adaptable and may be regulated by extracellular stimuli such as, nutrients, cytokines, and bacteria. In addition, the relationship between the luminal stimuli and the mucosa can shape pro-inflammatory and immune-regulatory responses, aiming to keep

homeostasis and consequently integrity (Turner, 2009). On the other hand, disease like blind loop syndrome could over stimulate the tight junction assembly resulting in a progressive loss of the junctional structure and, leading to a decrease in nutrient absorption and ion permeation, of the epithelium (Schulzke et al., 1990).

It is important to note that disruption of barrier function has being speculated to be one of the major causes of intestinal bowel disease (Mankertz and Schulzke, 2007), resulting in a systematic immune activation (Sandler et al., 2011). The expression of tight junction proteins and, consequently the transepithelial integrity, is highly regulated by signals from the commensal bacteria, including activation of toll-like receptor-2 (Cario et al., 2004). Toll-like receptors are responsible for signal recognition of microbial ligands or endogenous signals associated with pathogens (Abreu, 2010). Consequently, the ability to sense commensal bacteria in the GIT is essential to keep the integrity of the barrier function (Peterson and Artis, 2014). Other ligands identified to recognize microbial associated molecular patterns are the nucleotide-binding oligomerization domain-like receptors and the retinoic acid-inducible gene-like receptors as they both are responsible to protect the epithelium against injuries and tumour development (Lamkanfi and Dixit, 2012; Peterson and Artis, 2014).

Gastrointestinal permeability is not a constant throughout the GIT as in nonruminants, permeability decreases from the small intestine to the colon, but increases at the distal part of the GIT, which is indirectly proportional to the endotoxic capacity of sites (Nejdfors et al., 2000). In ruminants, permeability has been reported to be affected by region, species, and molecule size of the nutrient. Analyzing the gastrointestinal permeability in Holstein calves, Penner et al. (2014) observed that the flux of mannitol was greater in the jejunum compared to rumen and omasum and numerically higher than ileum, cecum, and colon. Mannitol is often used in permeability studies and its flux indicates the paracellular movement through the tissue (Penner et al., 2010). This data supports the speculation that the decreased permeability and consequently a more organized barrier function are located at the GIT sites that are subject to a higher endotoxic challenge (presence of higher counts of microorganisms).

Acute ruminal acidosis has been proposed as a cause of a rapid reduction in the epithelial barrier function in the rumen, increasing the potential of toxins and antigens to cross the epithelial barrier and reach the systemic circulation of the host (Aschenbach and Gäbel, 2000). In addition,

Zhang et al. (2013) restricted heifers to 75, 50, and 25% of ad libitum intake and demonstrated that only severe feed restriction (intake restricted to 25% of ad libitum intake) compromised the barrier function and reduce the fractional rate of total SCFA, acetate, and propionate. Moreover, beef heifers consuming either a high- or a moderate-forage diet, also showed a reduction in ruminal absorption of total SCFA and acetate as a result of feed restriction (feed intake of approximately 25% of the ad libitum intake; Albornoz et al., 2013).

## 2.11. Summary of research rationale

For livestock, the development and maintenance of the GIT are essential to maximize growth and feed efficiency, and to ensure welfare in animal production. As explored in the literature review, the GIT faces several challenges due to the large area of exposure to the outer world and to the high density of microbial inhabitants. Thus, the capacity of selective permeability of the gastrointestinal epithelium plays an important role to maintain the undesirable agents outside of the systemic circulation of the animal and to maximize nutrient absorption. The effects of butyrate in the GIT reported in the literature have shown some inconsistencies, mainly due to the differences in dosage and site of exposure. However, in summary butyrate has shown positive effects on the development of the GIT of pre-weaned animals (piglets and calves) enhancing their health status and efficiency. Due to the limitation of butyrate usage in its free form, related to strong and undesirable odour and volatile characteristic, the use of precursors and other forms of butyrate (CaG and TB, respectively) seems to be logical and promising are of exploration. Nonetheless, the reports in the literature about the use of these strategies focus mainly on lactating dairy cows. Butyrate is a production of microbial fermentation and consequently a dietary change can lead to a change in butyrate product in the GIT, exploring the mode of action of these molecules and their interaction with diets containing different forage-to-concentrate ratio seems to be logical to verify their potential use as feed additives.

### 2.12. Overall hypothesis

The increase of intestinal supply of butyrate by using butyrate precursors will increase the morphological developments and the intestinal functionality, by increasing the nutrient absorption, the brush border enzyme activity, and the barrier function, leading to an increase in growth and feed efficiency in ruminants.

### 2.13. Overall objective

To determine the effects of provision of either CaG and TB on the development and functionality of the GIT of ruminants fed with diets containing different forage-to-concentrate ratio.

# 3. EFFECT OF FEEDING CALCIUM GLUCONATE EMBEDDED IN A HYDROGENATED FAT MATRIX ON FEED INTAKE, GASTROINTESTINAL FERMENTATION AND MORPHOLOGY, INTESTINAL BRUSH BOARDER ENZYME ACTIVITY AND BLOOD METABOLITES IN GROWING LAMBS[1]


## 3.1. Abstract

Gluconate salts have been identified as a butyrate precursor when fed to non-ruminant species and may increase the butyrate concentration in the large intestine supporting gastrointestinal health and development. The objective of this study was to evaluate the dose response of hydrogenated fat-embedded calcium gluconate (**HFCG**) on performance and gastrointestinal tract (**GIT**) development in growing lambs. Thirty-two wether lambs were used in a randomized complete block design and assigned to 1 of 4 treatments differing in the inclusion of HFCG: 0.0% (**CON**), 0.075% (**LOW**), 0.30% (**MED**), and 0.60% of the diet (**HIGH**). Lambs were allocated into individual pens and fed ad libitum with feed delivered twice daily. Feed intake was recorded daily, and body weight (**BW**) was assessed at the beginning and the end of the 29-d period. Blood was sampled on d 21, prior to feeding and 6 h post-feeding to evaluate changes in β-hydroxybutyrate, glucose, and insulin concentrations. Total fecal collection was conducted during d 25 to 28 to assess apparent total tract digestibility. On d 29, lambs were slaughtered, and the entire GIT was separated by region to enable sampling of tissue and digesta. Data were analyzed to assess linear, quadratic, and cubic effects of HFCG dose. Final BW, average daily gain, and dry matter intake decreased linearly ($P \leq 0.02$) with increasing HFCG. Increasing inclusion of HFCG linearly decreased ($P = 0.01$) the thickness of the stratum corneum in ruminal papillae but did not affect other strata ($P \geq 0.34$). Omasal digesta weight linearly decreased ($P = 0.01$) as the concentration of HFCG increased and abomasal digesta weight was cubically affected ($P = 0.03$) the increasing dose of HFCG. Short-chain fatty acid concentration in the cecum was cubically affected ($P < 0.01$) with increasing dose of HFCG where low dose had the greatest concentration.


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[1]A version of this chapter has been published in the Journal of Animal Science. Watanabe, D. H. M., J. Doelman, M. A. Steele, L. L. Guan, D. J. Seymour, J. A. Metcalf, and G. B. Penner. 2022. Effect of feeding calcium gluconate embedded in a hydrogenated fat matrix on feed intake, gastrointestinal fermentation and morphology, intestinal brush border enzyme activity and blood metabolites in growing lambs. J. Anim. Sci. 100:1–12. doi:10.1093/JAS/SKAC205. **Author contribution:** The author contributed to this publication with conceptualization, funding acquisition, experimental planning and implementation, caring of the animals, sample collection, majority of the laboratory analysis, statistical analysis, preparation of the original draft, and implementation of co-author reviews.

Moreover, increasing the dietary supply of HFCG linearly increased the proportion of acetate ($P = 0.04$) in the cecum and linearly decreased the proportion of propionate in the digesta of both the cecum ($P < 0.01$) and colon ($P = 0.01$). Colon crypt depth was quadratically ($P = 0.03$) affected with the increasing dose of HFCG, where lambs fed MED had greatest crypt depth. We conclude that feeding HFCG to growing lambs did not increase butyrate concentration in the large intestine and consequently does not increase the absorptive surface area of the whole tract, the size of the GIT, or the functionality of the intestine.

## 3.2. Introduction

Gluconic acid is an organic acid derivate of glucose that can be found in rice, honey and royal jelly, wine, and vinegar (Asano et al., 2005). Gluconic acid forms gluconate salts by complexing with sodium, calcium, potassium, or zinc, and these forms are used in the food industry as acids, coagulants, and as a mineral supplement (Asano et al., 1994). Identified as a butyrate precursor in the non-ruminant intestine (Biagi et al., 2006), sodium gluconate increases butyrate synthesis by stimulating the growth of *Lactobacillus* spp. and *Megasphaera elsdenii* when studied in vitro using porcine cecal digesta as an inoculant (Tsukahara et al., 2002, 2006). Biagi et al. (2006) reported a linear increase in gas production, a quadratic reduction in ammonia concentration, and a linear increase in total short-chain fatty acid (**SCFA**) concentrations when measured in vitro using cecal inoculum from pigs. In that study, the increase in SCFA concentration was partially mediated by increased butyrate concentration with increasing gluconic acid. These data suggest that gluconic acid and gluconate salts might serve as a novel strategy to supply butyrate to the gastrointestinal tract in vivo.

Butyrate has been studied for its ability to increase the absorptive surface area (Mentschel et al., 2001; Li and Elsasser, 2005), brush-border enzyme activity (Basson and Hong, 1998), and tight cell junction gene and protein expression (Peng et al., 2009; Wang et al., 2012) throughout the gastrointestinal tract. In non-ruminant species, gluconic acid has been investigated as a potential alternative to antibiotics in addition to promoting growth as inclusion of gluconic acid in the diets of piglets quadratically increased ADG (Biagi et al., 2006). In another study, the inclusion of sodium gluconate in the diet of piglets increased final BW and average daily gain, reduced ammonia concentration in the cecum digesta, and increased jejunum villus height and crypt depth (Poeikhampha and Bunchasak, 2011). For dairy cows, providing a ruminally-available source of calcium gluconate (**CaG**) increased fat corrected milk yield (Emery et al., 1960). A more recent study provided contrasting results where inclusion of 0.2% CaG in the total mixed ration (**TMR**) reduced yields of milk, protein, and lactose; however, postruminal infusion of CaG decreased dry matter intake (**DMI**) and increased milk fat yield (McKnight et al., 2019). Doelman et al. (2019) fed dairy cows CaG embedded in a hydrogenated fat matrix to reduce ruminal availability, and although no changes were detected for fecal SCFA concentration, these authors observed a quadratic response where milk fat and milk protein yields increased and then decreased with

increasing the dose of CaG. Seymour et al. (2021) reported tendencies for increased milk fat yield primarily driven by increased incorporation of long chain fatty acids of endogenous origin when supplementing dairy cattle with approximately 16 g/d of a hydrogenated fat-embedded CaG (**HFCG**) supplement. Considering that most CaG should not be absorbed across the small intestine and would have limited caloric value (Asano et al., 1997), it is possible that CaG alters the intestinal milieu thereby promoting increased absorptive surface area, activity of intestinal epithelia, and microbial activity. Strategies that stimulate intestinal development may be critical given the linkage between inflammation and production outcomes (Kvidera et al., 2017b) and the hypothesized importance of the post-ruminal regions on intestinal permeability (Penner et al., 2014; Fischer et al., 2018) in dairy (Horst et al., 2019) and beef cattle (Briggs et al., 2020).

We hypothesized that feeding HFCG would increase butyrate concentration in the large intestine and indirectly increase absorptive surface area in the intestine, size of the gastrointestinal tract, and functionality of the intestine in a dose-dependent manner. The objective of this study was to evaluate the dose response of HFCG on feed intake and digestibility, SCFA concentrations throughout the gastrointestinal tract, gastrointestinal morphology, and brush-border enzyme activity for growing lambs.

## 3.3. Material and methods

Use of lambs and cattle in this experiment and the procedures implemented were approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20100021, Saskatoon, SK, Canada) and followed the guidelines of the Canadian Council on Animal Care (Ottawa, ON, Canada).

### 3.3.1. Experimental design, dietary treatments, and dry matter intake

Thirty-two Canadian Arcott wether lambs with an initial BW of 35.0 ± 1.82 kg (mean ± SD) were used in a randomized complete block design. To remove potential BW effects on gastrointestinal tract (**GIT**) size and weight, lambs were blocked by BW into 1 of 8 blocks. Within blocks, lambs were randomly assigned to 1 of 4 treatments differing in the inclusion of HFCG (50% hydrogenated palm fat, 40% calcium gluconate, 10% calcium carbonate; Selko Lactibute, Trouw Nutrition, Amersfoort, the Netherlands). The HFCG was provided at 0.0% (**CON**), 0.075% (**LOW**), 0.30% (**MED**), and 0.60% of the diet (**HIGH**). The dose of 0.075% was similar to that in

Doelman et al. (2019) and Seymour et al. (2021), and the dose range used in the present study extends beyond that previously reported by McKnight et al. (2019).

The basal diet was common among treatments (Table 3.1) with oat flour (Keyleaf, Saskatoon, SK) used as a carrier for the HFCG to ensure a homogeneous distribution. In addition, calcium carbonate and palmitic acid (Jefo Dairy Fat, Jefo, Saint-Hyacinthe, QC) were used as replacements for HFCG to balance for Ca and ether extract. All lambs were provided their respective treatment for 28 d prior to slaughter with 1 block being handled on an individual day. As such, blocks started the experiment in a staggered manner. Prior to the start of the study, lambs were group-housed in an outside pen with continuous access to water and *ad libitum* provision of grass hay. Two days before of the start of the experimental period (d -1), all lambs within a block were moved into the barn and lambs were allocated into individual pens (3 $m^2$) with wood shavings as bedding. During these two days, lambs were fed grass hay.

Throughout the study, lambs received a total mixed ration twice daily at 0900 h (60% of the diet) and 1600 h (40% of the diet). The quantity of feed offered was adjusted based on the weight of the refusals to ensure refusal weights ranged between 5.0 and 15.0% of the total amount of feed offered. Samples of forages were collected twice weekly, while concentrates were collected once weekly for DM determination. These DM values were used, if necessary, to adjust the diet twice a week to ensure the delivery of the diet based on the DM formulation.

Body weight was assessed at the beginning and the end of the experimental period on two consecutive days (d 0 and 1 and d 28 and 29, respectively), and the average of the two measurements were used to calculate initial and final body weight (**BW**), and average daily gain (**ADG**). In addition, samples of feed ingredients and the feed refused were collected from d 25 through d 28 for each lamb to enable calculation of nutrient intake. The ingredient and refusal samples were dried in a forced-air oven at 55ºC until a constant weight was achieved. Dry matter intake (**DMI**) was expressed in kg/d and as a percentage of BW based on the assumption of linear growth.

### 3.3.2. Apparent total tract digestibility

On d 22, lambs were fitted with fecal collection harnesses to allow for adaptation to the harness and a secure fit. Total fecal collection occurred from d 25 to 28. Bags attached to the fecal

**Table 3.1.** Feed ingredients of experimental diets containing increasing dose of hydrogenated fat-embedded Ca-gluconate (HFCG).

| | Treatment[1] | | | |
|---|---|---|---|---|
| Ingredient, % of DM | CON | LOW | MED | HIGH |
| Barley silage | 35.00 | 35.00 | 35.00 | 35.00 |
| Alfalfa hay | 15.00 | 15.00 | 15.00 | 15.00 |
| Steam flaked corn | 20.00 | 20.00 | 20.00 | 20.00 |
| Steam flaked barley | 13.67 | 13.67 | 13.67 | 13.67 |
| Pelleted beet pulp | 3.09 | 3.09 | 3.09 | 3.09 |
| Vitamin and mineral pellet[2] | | | | |
| Ammonium chloride | 2.336 | 2.336 | 2.336 | 2.336 |
| Limestone | 1.025 | 1.025 | 1.025 | 1.025 |
| Salt | 0.285 | 0.285 | 0.285 | 0.285 |
| Lasalocid[3] | 0.079 | 0.079 | 0.079 | 0.079 |
| Premix[4] | 0.222 | 0.222 | 0.222 | 0.222 |
| Barley grain, ground | 3.300 | 3.300 | 3.300 | 3.300 |
| Calcium phosphate mono | 2.393 | 2.393 | 2.393 | 2.393 |
| Treatment carrier | | | | |
| HFCG | 0.000 | 0.075 | 0.300 | 0.600 |
| Oat flour | 3.000 | 3.000 | 3.000 | 3.000 |
| Palmitic acid | 0.300 | 0.263 | 0.150 | 0.000 |
| Ca-carbonate | 0.300 | 0.263 | 0.150 | 0.000 |
| Chemical composition, % of DM ± SD[5] | | | | |
| Dry matter | 57.48 ± 0.17 | 57.48 ± 0.17 | 57.48 ± 0.17 | 57.48 ± 0.17 |
| Organic matter | 92.69 ± 0.32 | 92.69 ± 0.32 | 92.69 ± 0.32 | 92.69 ± 0.32 |
| Crude protein | 12.65 ± 0.30 | 12.65 ± 0.30 | 12.65 ± 0.30 | 12.65 ± 0.30 |
| Acid detergent fiber | 16.61 ± 3.59 | 16.61 ± 3.59 | 16.61 ± 3.59 | 16.61 ± 3.59 |
| Neutral detergent fiber | 31.69 ± 5.88 | 31.69 ± 5.88 | 31.69 ± 5.88 | 31.69 ± 5.88 |
| Ether extract | 2.99 ± 0.15 | 2.99 ± 0.15 | 2.99 ± 0.15 | 2.99 ± 0.15 |
| Starch | 31.82 ± 4.30 | 31.82 ± 4.30 | 31.82 ± 4.30 | 31.82 ± 4.30 |
| Ca | 0.77 ± 0.22 | 0.77 ± 0.22 | 0.77 ± 0.22 | 0.77 ± 0.22 |
| P | 0.37 ± 0.05 | 0.37 ± 0.05 | 0.37 ± 0.05 | 0.37 ± 0.05 |

[1]Sheep were assigned with CON (*n* = 8; no inclusion of HFCG in the diet), LOW (*n* = 8; inclusion of 0.075% of HFCG in the diet, % DM), MED (*n* = 8; inclusion of 0.3% of HFCG in the diet, % DM), and HIGH (*n* = 8; inclusion of 0.6% of HFCG in the diet, % DM).
[2]Contained (% DM) 77.3 g/kg of Ca; 56 g/kg of P; 4.3 g/kg of Mg; 2.1 g/kg of K; 12.4 g/kg of Na; 185.9 g/kg of Cl; 3.6 g/kg of S; 722 mg/kg of Mn; 3.6 mg/kg of Cu; 2,994 mg/kg of Fe; 799 mg/kg of Zn; 31 mg/kg of I, 21 mg/kg of Co, 5.2 mg/kg of Se; 170,947.5 IU/kg of vitamin A; 18,106.9 IU/kg of vitamin D; and 1,163.94 IU/kg of vitamin E.
[3]Bovatec (Zoetis Canada Inc., Kirkland, QC), 20% of lasalocid sodium.
[4]Gowan's Feed Consulting lamb premix (MasterFeeds, Lethbridge, AB) which contained (on DM basis): 7,980,172 IU/kg of vitamin A; 842,090 IU/kg of vitamin D; 84,131 IU/kg of vitamin E, 8,500 mg/kg of biotin; 28,985 mg/kg of Mn; 834 mg/kg of Co; 1,429 mg/kg of I; 238.2 mg/kg of Se; 75,000 mg/kg of calcium carbonate; 10,000 mg/kg of mineral oil; and 174,275 mg/kg of wheat midds.
[5]Average ± SD of 8 samples representing block 1 to 8.

harnesses were emptied daily before feeding (0800 h) and the wet weight was recorded. At the end of the 4-d collection, a single 1 kg composite sample was prepared proportionally to the daily output. Fecal samples were dried at 55°C until a constant weight was achieved.

### 3.3.3. Chemical analysis

All feed, refusals, and fecal samples were ground using a Christy Turner Miracle Mill (Christy Turner Ltd., Suffolk, United Kingdom) with a 1-mm screen. Dried and ground feed ingredient samples were composited by experimental block prior to analysis and composites of feed refusals and feces for each lamb were prepared. Subsequently, the composited feed ingredient, feed refusals, and fecal samples were submitted to SGS Crop Science Canada (Guelph, ON, Canada) for analysis of starch, crude protein (**CP**), neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**) and ether extract. To determine organic matter (**OM**) concentration, samples were ashed for 2 h at 600ºC and the residue was subtracted from 100% (method 942.05; AOAC, 2000). Starch was determined based the methodology described by Hall (2009), including digestion with amylase and measurement of the resulting glucose (method 996.11; AOAC, 2007). Nitrogen was analyzed using a Leco FP-528 Nitrogen Combustion Analyzer (LECO Corp., St. Joseph, MI) and CP was calculated by multiplying the nitrogen by 6.25 (AOAC, 2007). Neutral detergent fiber was determined as described by Van Soest et al. (1991) with, minor modifications, such that samples were filtered using Whatman 934-AH glass micro-fiber filters with 1.5 µm particle retention (GE Healthcare Life Sciences, Piscataway, NJ). The NDF assay included the use of α-amylase and sodium sulfite. The ash-free NDF (**aNDF$_{OM}$**) was determined using the residue of the NDF analysis followed by ashing at 535ºC for 2 h. The ADF concentration was determined as described by AOAC (2000) with the same filtration modifications as for NDF.

### 3.3.4. Blood collection and analysis

An intravenous catheter (13.3 cm 16-gauge intravenous catheter; BD Angiocath, Becton Dickinson, North Ryde, New South Wales, Australia) was inserted into a jugular vein of each lamb on d 20. Blood sampling was initiated on d 21 at 0900 h (immediately prior to feeding) and at 1500 h (6 h post-feeding) to evaluate changes in β-hydroxybutyrate (**BHBA**), glucose, and insulin concentrations. For plasma, blood was collected into a 7-mL vacutainer containing lithium heparin (144 IU; Fisher Scientific, Nepean, ON, Canada), mixed by inversion and immediately placed on ice until centrifugation at 2,500 × *g* for 15 min at 4°C. For serum, blood was collected into a 7-mL

vacutainer tube containing silica gel (Fisher Scientific) and allowed to clot for 30 min prior being centrifuged. Catheters were removed following the final blood sample.

Plasma glucose concentration was measured using a glucose oxidase/peroxidase enzyme (PGO Enzyme Preparation, Sigma-Aldrich, St. Louis, MO) and *o*-dianisidine dihydrochloride (Sigma-Aldrich). Absorbance was determined at 450 nm with a plate reader (Microplate 96 wells flat-bottom clear, Greiner Bio-One North America Inc., Monroe, NC). Plasma insulin concentration was measured using a commercial kit (Mercodia Ovine Insulin ELISA; Mercodia AB, Sylveniusgatan 8A, Uppsala, Sweden) and absorbance was measured using a plate reader at 450 nm with a minimum sensitivity of 0.05 μg/L. Serum samples were used to determine the BHBA concentration using the enzyme 3-hydroxybutyrate dehydrogenase (C756W35; Roche Diagnostics, Laval, QC, Canada) to catalyze the oxidation of BHBA to acetoacetate as described by Williamson et al. (1962). All samples were analyzed in triplicate and samples with CV higher than 5% were reanalyzed until a CV ≤ 5% was achieved.

### 3.3.5. Gastrointestinal tract dissection

On d 29, lambs were slaughtered using captive bolt stunning, pithing, and exsanguination at 1300 h (4 h after feeding). The abdominal cavity was opened immediately after confirmation of death, and the distal portions of the esophagus and rectum were tied off to avoid loss of digesta. The entire GIT was removed and placed on a clean table for identification of each region as described by Penner et al. (2014). To separate the regions of the GIT, two zip ties were placed at each junction to avoid the loss of digesta from both segments. Initially, the forestomach compartments were separated from the intestine at the pyloric sphincter. The reticulorumen, omasum, and abomasum were then isolated. For the small intestine, the duodenum was identified between the pyloric sphincter and ligament of Treitz, the jejunum between the ligament of Treitz and ileocecal fold, and the ileum between the ileocecal fold and ileocecal valve. Finally, the large intestine was divided into cecum and colon by sectioning it at the ceco-colic junction. The entire dissection process was completed within approximately 20 min for each lamb. The slaughter sequence was randomized within blocks by treatment to minimize slaughter order effect in the samples collected.

The reticulo-rumen, omasum, and abomasum were weighed with the digesta. The digesta was then removed, tissues were washed with tap water and drip-dried, and weighed again to

determine the tissue and digesta weights, respectively. The regions of the small and large intestine were also emptied. During the removal of digesta, tissue was carefully handled to avoid damage. The length of the duodenum, jejunum, ileum, cecum, and colon were measured and recorded, being careful not to apply force and stretch the tissue. The liver, kidneys, and spleen were also collected and weighed. Digesta from the jejunum, ileum, cecum, and colon were diluted with double-distilled water at a 1:1 (v:v) ratio and mixed thoroughly. Duodenal digesta was not collected due to low volume and risk of digesta movement from the abomasum. Ruminal digesta and diluted digesta from the jejunum, ileum, cecum, and colon were strained through 4 layers of cheese cloth and used for measurement of pH using a portable pH meter (Accumet AP110; Fisher Scientific). Following pH measurement, 10 mL of the digesta from each region was mixed with 2 mL of metaphosphoric acid (25% wt/v), placed in a 15-mL tube, and stored at -20ºC until measurement of SCFA concentration.

Short-chain fatty acids were separated and quantified by gas chromatography (Agilent 6890 Series GC System, Santa Clara, CA, USA) as described by Khorasani et al. (1996). Briefly, digesta from the intestinal regions centrifuged to clarify digesta, filtered through a 25-μm syringe filter (Fisher Scientific), and the ruminal and intestinal filtrate were centrifuged again. Isocaproic acid was used as an internal standard and the relative response factors for acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids were calculated with reference to standards. For each sample, 1 μL was injected using a 17:1 split ratio and an injector temperature of 260ºC. The column (7HM-G009-11 Zebron, 30 m × 0.32 mm × 0.25 μm, Phenomenex, Torrance, CA) was incubated in an oven initially at 90ºC and then raised to 170ºC at 10ºC/min. A flame ionization detector set at 250ºC was used.

An 8 × 8 cm sample of the ventral sac of the rumen was collected and stored at -20ºC until DM determination of the epithelial and submucosal layers, as described by Schurmann et al. (2014). Briefly, the sample was thawed and trimmed to result in a 5 × 5 cm section. After trimming, the epithelial layer was peeled from the submucosal layer by hand and the wet weight of both parts were recorded. The layers were placed in a forced-air over for 8 h at 105ºC and the dry weight was recorded. Calculation of epithelial and sub-mucosal tissues were determined and reported as mg/cm$^2$.

Ruminal (caudal blind sac), jejunal (proximal, near the ligament of Treitz), and colon (end of the centripetal turn) tissue samples were collected for histological evaluation. Prior to collection, tissues were gently rinsed with phosphate buffered saline until the total removal of the digesta (**PBS**, Sigma-Aldrich; pH =7.2) to remove any remaining digesta. For the rumen, papillae were cut at the basal connection under a dissecting microscope (10× magnification) prior to placing papillae in cassettes (Simport, Beloeil, QC, Canada). For the jejunum and colon, tissues were cut longitudinally and a 1-$cm^2$ section of tissue was included. Samples were then placed in biopsy cassettes (Simport) and fixed in 10% formalin (10% formaldehyde, v/v, in neutral phosphate buffer; Ricca Chemical Company, Arlington, TX). Samples were submitted to Prairie Diagnostic Services (Saskatoon, SK, Canada) where the tissues were dehydrated in increasing concentrations of ethanol (2 washes with 90% ethanol for 1.5 min and 3 washes with 100% ethanol for 15 sec each) and washed with xylene at the end (two washes with a duration of 1 and 2 min). The dehydrated tissues were then embedded in paraffin wax, cut into 4-µm sections and then mounted onto glass slides, as described by Kent-Dennis et al. (2019).

Histomorphometric analyses were made by a technician that was blinded to treatments. For ruminal tissue, five papillae were randomly selected, and each stratum were measured in two different parts of the papillae, totaling 10 measurements per experimental unit. Measurements were taken at 10× objective lens, and strata were defined as described by Steele et al. (2011), where the stratum corneum was defined as the layer of cells located at the outermost tip of the ruminal papillae, heavily stained and absence of nuclei. Subsequently, the stratum granulosum was defined as the layer of cells with lighter staining, long axes, and presence of nuclei. Due to the lack of discernable difference between the stratum spinosum and basale cells, they were measured as a single group, defined as the nested cells between the lamina propria and the stratum granulosum. For colon samples, measurements were taken using a lens with 5× magnification and crypt depth was determined based on the protocol adapted by Steele et al. (2011). Tissue from jejunum was collected to be analyzed; however, tissues were too damaged for evaluation.

Tissue from jejunum (proximal, near to the ligament of Treitz) was washed with sterile PBS (Sigma-Aldrich) and placed on a cold sterile surface, opened transversely, scraped using a sterile glass slide to remove the mucosal surface, snap frozen in liquid nitrogen, and stored at -80ºC for evaluation of activity of brush border enzymes as described by Górka et al. (2011). Briefly, 1 g of

mucosa was homogenized with 5 mL of double distilled water and centrifuged for 5 min at 1,000 × *g* at 4ºC. The supernatant of the homogenized mucosa was used to determine the total mucosal protein concentration as described by Smith et al. (1985), and the activity of brush border maltase and sucrase as described by Dahlqvist (1984).

### 3.3.6. In situ ruminal DM disappearance of HFCG

This experiment was conducted to evaluate the DM disappearance and the effectiveness of the ruminal protection of the HFCG. Six ruminally-cannulated Angus heifers were fed a diet to that used for the main study (54.0% of barley silage, 11% of alfalfa hay, 14.0% of steam flaked corn, 10.0% of dry-rolled barley, 2,0% of pelleted beet pulp, and 1.0% of mineral/vitamin supplement; on the DM basis) for 8 d (7 d of adaptation + 1 d of ruminal incubation). The HFCG was weighed (7 g) into polyester bags (R510; Ankom Technology, Macedon, NY ; 5 × 10 cm, pore size = 50 μm) and incubated in the rumen for 24 h. Bags were incubated in triplicate and a blank bag was added to account for weight changes during incubation. After 24 h of incubation, bags were removed from the rumen, washed for 5 times in cold tap water (1 min/wash), and dried at 55ºC until achieving constant weight. The DM disappearance was calculated using the initial and final weight of the bags using the blank correction. Bags were opened, emptied, and the content was sent to away to be analyzed for CaG concentration (MasterLab, Boxmeer, The Netherlands). Using hot water (<93 °C) the gluconic acid was extracted and diluted for LC-MS/MS analysis. Using a Vanquish HPLC system (Fisher Scientific, Landsmeer, The Netherlands) 2 μl of sample was separated on an ion exchange column. After the separation the triple quadrupole Quantis LC-MS (Fisher Scientific) analyzed the gluconic acid concentration using a heated electrospray source.

### 3.3.7. Statistical analysis

Data were analyzed using PROC MIXED of SAS v9.4 (SAS Institute Inc., Cary, NC) including the dietary treatment as a fixed effect with block and lamb within block as random effects. The Kenward-Roger method was used to calculate the adjusted denominator degrees of freedom. Polynomial orthogonal contrasts were used to assess linear, quadratic, and cubic effects of HFCG dose, once HFCG doses were not equally spaced, the PROC IML was used to calculate the correct contrasts values to input on the SAS code. For outcomes measured at multiple time points (i.e., concentrations of glucose, insulin and BHBA), the fixed effects of time and the treatment × time interaction were included in the model. Time was considered as a repeated

measure and the variation within animal was modelled using either a compound symmetric or heterogenous compound symmetric covariance structure, which was selected based on the lowest Akaike information criterion.

The compound symmetry covariance structure was selected for glucose, while the heterogeneous compound symmetric structure was selected for insulin and BHBA. Original data and their residuals were tested for normality based on visual assessment and the Shapiro-Wilk test. Residuals for blood insulin and cecal length were not normally distributed when initially analyzed; as such, the data were transformed using the equations $x^{1/3}$ and $x^{-2}$, respectively, where *x* is the original data. For the transformed data, standard errors of the differences (**SED**) and *P*-values were reported based on the analysis of transformed data, while means presented are back-transformed. In all cases, significance was declared when $P < 0.05$ and tendencies were declared when $0.05 \leq P < 0.10$.

## 3.4. Results

### 3.4.1. Body weight, dry matter intake, and growth performance

Initial BW did not differ among treatment groups ($P = 0.51$); however, final BW, ADG, G:F ratio, and DMI (in kg and % BW) linearly decreased ($P \leq 0.02$) as the concentration of HFCG increased (Table 3.2). There were no differences detected between CON and LOW for BW, DMI, ADG, or G:F ratio ($P \geq 0.31$).

### 3.4.2. Rumen, omasum, and abomasum parameters

A tendency for a cubic response was observed for ruminal digesta weight ($P = 0.09$) with greatest weight for the LOW dose, while no difference was observed ($P \geq 0.13$) for ruminal tissue weight with the increasing dose of HFCG (Table 3.3). Moreover, both ruminal epithelia weights and submucosal layer weights did not differ ($P \geq 0.11$) among treatments. No differences ($P \geq 0.21$) were observed for digesta pH, total SCFA, and the molar proportions of SCFA. Increasing the dose of HFCG linearly decreased ($P = 0.01$) the thickness of the stratum corneum in the ruminal epithelium, while it did not affect the thickness of strata granulosum ($P \geq 0.34$), spinosum ($P \geq 0.43$), or basale ($P \geq 0.51$; Table 3.3). Omasal digesta weight linearly decreased ($P = 0.01$; Table 3.3) as the concentration of HFCG increased without changing omasal weight ($P \geq 0.18$). While abomasal digesta was affected cubically where increasing dose of HFCG initially decreased, then

increased, and decreased digesta weight ($P = 0.03$). Abomasal tissue weight was not affected by linear, quadratic, or cubic responses ($P > 0.11$).

### 3.4.3. Small intestine parameters

Duodenal length was quadratically ($P = 0.01$; Table 3.4) affected by increasing HFCG dose where lambs fed MED had a shorter duodenum compared to lambs on the other treatments. There were no differences ($P \geq 0.48$) in the digesta pH in this region. Likewise, no differences ($P \geq 0.15$) were observed for length and digesta pH for the jejunum and ileum. However, total SCFA concentration in the jejunum tended to be affected quadratically ($P = 0.06$), where lambs fed MED had lower SCFA concentration compared to the lambs on the other treatments. No differences ($P \geq 0.59$) in total ileal SCFA concentrations were observed, and for both the jejunum and ileum, acetate was the only SCFA able to be detected. The protein content of the jejunal mucosal and the maltase activity were not affected by the increasing HFCG dose. Sucrase activity was not detected.

### 3.4.4. Large intestine parameters and organs weight

Length of the cecum and colon, along with the pH of the digesta from those regions, were not affected by increasing dose of HFCG ($P \geq 0.22$; Table 3.5). Cecal SCFA was affected cubically ($P = 0.01$) with the increasing dose of HFCG where dose was greatest for LOW. Increasing dietary supply of HFCG linearly increased the proportion of acetate ($P = 0.04$) and linearly decreased ($P < 0.01$) the proportion of propionate in cecal digesta. There were no other differences for the molar proportion of individual SCFA in cecal digesta. Increasing the dose of HFCG did not affect total SCFA concentration in the colon; however, there was a linear reduction ($P = 0.01$) in the molar proportion of propionate. There were no differences in the concentrations of acetate, isobutyrate, butyrate, isovalerate, and valerate among treatments in colonic digesta ($P \geq 0.14$). Colon crypt depth was quadratically ($P = 0.03$) affected by increasing the dose of HFCG, where lambs fed MED had the greatest crypt depth. There were no changes in the width of the crypts ($P \geq 0.48$; Table 3.5). Kidney, liver, and spleen weights were not affected ($P \geq 0.10$) by increasing addition of HFCG (Table 3.6).

**Table 3.2.** Body weight, dry matter intake, and growth of lambs (n = 32) fed increasing doses of hydrogenated fat-embedded Ca-gluconate (HFCG).

| Item | Treatment[1] | | | | SED[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | LOW | MED | HIGH | | Linear | Quadratic | Cubic |
| Initial BW, kg | 35.0 | 34.8 | 35.1 | 35.1 | 0.47 | 0.55 | 0.92 | 0.51 |
| Final BW, kg | 36.9 | 37.9 | 35.8 | 34.6 | 1.29 | 0.02 | 0.87 | 0.28 |
| ADG[3], g/day | 67 | 114 | 25 | -20 | 45.2 | 0.01 | 0.90 | 0.18 |
| G:F[4] ratio, kg/kg | 0.055 | 0.110 | 0.020 | -0.063 | 0.0533 | 0.01 | 0.554 | 0.24 |
| DMI, kg/d | 1.1 | 1.0 | 1.0 | 0.8 | 0.10 | 0.02 | 0.27 | 0.34 |
| DMI, % of BW | 2.96 | 2.73 | 2.90 | 2.29 | 0.254 | 0.02 | 0.25 | 0.26 |

[1]Sheep were assigned with CON (*n* = 8; no inclusion of HFCG in the diet), LOW (*n* = 8; inclusion of 0.075% of HFCG in the diet, % DM), MED (*n* = 8; inclusion of 0.3% of HFCG in the diet, % DM), and HIGH (*n* = 8; inclusion of 0.6% of HFCG in the diet, % DM).
[2]SED: Standard error of the difference.
[3]ADG: average daily gain.
[4]G:F: gain-to-feed.

**Table 3.3.** Rumen, omasum, and abomasum parameters of lambs (n = 32) fed increasing doses of hydrogenated fat-embedded Ca-gluconate (HFCG) for 28 days.

| Item | Treatment[1] | | | | SED[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | LOW | MED | HIGH | | Linear | Quadratic | Cubic |
| *Rumen* | | | | | | | | |
| Digesta weight, kg | 3.48 | 4.36 | 3.43 | 3.63 | 0.561 | 0.59 | 0.91 | 0.09 |
| Tissue weight, g | 826 | 865 | 865 | 197 | 63.4 | 0.48 | 0.33 | 0.74 |
| Muscle wet weight, mg/cm² | 87.72 | 100.24 | 91.24 | 107.68 | 11.490 | 0.19 | 0.58 | 0.23 |
| Muscle dry weight, mg/cm² | 15.54 | 16.71 | 16.15 | 19.53 | 2.277 | 0.11 | 0.52 | 0.54 |
| Muscle dry matter, % | 17.67 | 16.74 | 17.84 | 17.22 | 0.657 | 0.91 | 0.71 | 0.15 |
| Epithelia wet weight, mg/cm² | 97.18 | 106.48 | 104.84 | 107.20 | 13.440 | 0.61 | 0.79 | 0.57 |
| Epithelia dry weight, mg/cm² | 17.77 | 19.36 | 19.68 | 18.68 | 2.765 | 0.86 | 0.53 | 0.73 |
| Epithelia dry matter, % | 18.42 | 18.30 | 18.82 | 18.97 | 0.485 | 0.16 | 0.79 | 0.56 |
| Digesta pH | 6.18 | 5.95 | 6.03 | 6.12 | 0.180 | 0.88 | 0.39 | 0.29 |
| Total SCFA, m*M* | 99.2 | 108.6 | 108.5 | 107.8 | 11.77 | 0.62 | 0.60 | 0.55 |
| Acetate, mol/100 mol | 61.67 | 62.89 | 60.97 | 62.15 | 1.697 | 0.89 | 0.58 | 0.32 |
| Propionate, mol/100 mol | 21.00 | 19.88 | 22.25 | 20.18 | 2.320 | 0.97 | 0.45 | 0.42 |
| Isobutyrate, mol/100 mol | 0.66 | 0.55 | 0.49 | 0.58 | 0.146 | 0.46 | 0.79 | 0.38 |
| Butyrate, mol/100 mol | 14.41 | 14.96 | 14.59 | 15.05 | 1.037 | 0.68 | 0.91 | 0.57 |
| Isovalerate, mol/100 mol | 0.43 | 0.45 | 0.41 | 0.49 | 0.135 | 0.86 | 0.85 | 0.55 |
| Valerate, mol/100 mol | 1.05 | 0.99 | 1.06 | 1.11 | 0.113 | 0.43 | 0.84 | 0.60 |
| Histology | | | | | | | | |
| Stratum corneum, µm | 48.25 | 46.73 | 41.92 | 36.44 | 3.240 | 0.01 | 0.92 | 0.99 |
| Stratum granulosum, µm | 15.25 | 15.94 | 16.66 | 15.79 | 1.270 | 0.74 | 0.34 | 0.90 |
| Stratum spinosum, µm | 48.47 | 48.30 | 45.14 | 45.12 | 4.072 | 0.43 | 0.72 | 0.85 |
| Stratum basale, µm | 62.92 | 60.87 | 63.45 | 65.64 | 5.535 | 0.51 | 0.85 | 0.73 |

**Table 3.3 continued.** Rumen, omasum, and abomasum parameters of lambs (n = 32) fed increasing doses of hydrogenated fat-embedded Ca-gluconate (HFCG) for 28 days.

| Item | Treatment[1] | | | | SED[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | LOW | MED | HIGH | | Linear | Quadratic | Cubic |
| *Omasum* | | | | | | | | |
| Digesta weight, g | 82 | 93 | 69 | 57 | 12.3 | 0.01 | 0.98 | 0.22 |
| Tissue weight, g | 119 | 105 | 108 | 101 | 10.3 | 0.18 | 0.76 | 0.23 |
| *Abomasum* | | | | | | | | |
| Digesta weight, g | 460 | 298 | 387 | 363 | 68.1 | 0.64 | 0.55 | 0.03 |
| Tissue weight, g | 223 | 214 | 180 | 205 | 23.8 | 0.38 | 0.11 | 0.75 |

[1]Sheep were assigned with CON (*n* = 8; no inclusion of HFCG in the diet), LOW (*n* = 8; inclusion of 0.075% of HFCG in the diet, % DM), MED (*n* = 8; inclusion of 0.3% of HFCG in the diet, % DM), and HIGH (*n* = 8; inclusion of 0.6% of HFCG in the diet, % DM).
[2]SED: Standard error of the difference.

**Table 3.4.** Small intestine parameters of lambs (n = 32) fed with three different doses of hydrogenated fat matrix-embedded Ca-gluconate (HFCG) for 28 days.

| Item | Treatment[1] | | | | SED[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | LOW | MED | HIGH | | Linear | Quadratic | Cubic |
| *Duodenum* | | | | | | | | |
| Length, cm | 55.38 | 54.13 | 47.25 | 53.38 | 2.663 | 0.28 | 0.01 | 0.43 |
| Digesta pH | 4.42 | 4.84 | 4.59 | 4.21 | 0.60 | 0.49 | 0.58 | 0.54 |
| *Jejunum*[4] | | | | | | | | |
| Length, m | 22.85 | 21.5 | 20.88 | 21.26 | 0.953 | 0.17 | 0.15 | 0.44 |
| Digesta pH | 7.28 | 7.26 | 7.27 | 7.25 | 0.116 | 0.82 | 0.99 | 0.88 |
| Total SCFA, m*M* | 3.9 | 4.7 | 3.7 | 5.7 | 1.35 | 0.59 | 0.06 | 0.59 |
| Acetate, mol/100 mol | 100.00 | 100.00 | 99.63 | 99.26 | 0.585 | 0.15 | 0.99 | 0.87 |
| Butyrate, mol/100 mol | ND[3] | ND | 0.37 | ND | - | - | - | - |
| Protein, mg/g of mucosa | 46.4 | 48.8 | 45.8 | 44.5 | 5.20 | 0.53 | 0.92 | 0.60 |
| Maltase, U/mg of protein | 16.0 | 19.3 | 15.7 | 17.6 | 3.59 | 1.00 | 0.80 | 0.28 |
| *Ileum*[5] | | | | | | | | |
| Length, cm | 81.13 | 74.5 | 81.38 | 83.00 | 12.246 | 0.66 | 0.91 | 0.56 |
| Digesta pH | 7.86 | 7.74 | 7.83 | 7.89 | 0.148 | 0.50 | 0.74 | 0.41 |
| Total SCFA, m*M* | 6.5 | 9.5 | 10.7 | 8.3 | 2.53 | 0.58 | 0.16 | 0.45 |
| Acetate, mol/100 mol | 100.00 | 100.00 | 98.87 | 99.47 | 0.677 | 0.12 | 0.17 | 0.36 |

[1]Sheep were assigned with CON (*n* = 8; no inclusion of HFCG in the diet), LOW (*n* = 8; inclusion of 0.075% of HFCG in the diet, % DM), MED (*n* = 8; inclusion of 0.3% of HFCG in the diet, % DM), and HIGH (*n* = 8; inclusion of 0.6% of HFCG in the diet, % DM).
[2]SED: Standard error of the difference.
[3]ND: not detectable.
[4]Jejununal concentration of propionate, isobutyrate, isovalerate, valerate, and sucrase activity were not detectable.
[5]Ileal concentration of propionate, isobutyrate, butyrate, isovalerate, and valerate were not detectable.

**Table 3.5.** Large intestine parameters of lambs (n = 32) fed with three different doses of hydrogenated fat matrix-embedded Ca-gluconate (HFCG) for 28 days.

| Item | Treatment[1] | | | | SED[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | LOW | MED | HIGH | | Linear | Quadratic | Cubic |
| *Cecum* | | | | | | | | |
| Length, cm | 21.75 | 22.38 | 22.00 | 22.75 | 2.130 | 0.58 | 0.81 | 0.70 |
| Digesta pH | 7.09 | 7.07 | 7.02 | 7.04 | 0.072 | 0.46 | 0.47 | 1.00 |
| Total SCFA, m*M* | 60.2 | 80.8 | 65.3 | 71.1 | 7.84 | 0.78 | 1.00 | 0.01 |
| Acetate, mol/100 mol | 73.94 | 74.95 | 75.50 | 75.87 | 0.835 | 0.04 | 0.39 | 0.49 |
| Propionate, mol/100 mol | 17.60 | 17.13 | 16.54 | 15.86 | 0.546 | <0.01 | 0.67 | 0.71 |
| Isobutyrate, mol/100 mol | 1.66 | 1.27 | 1.37 | 1.56 | 0.267 | 0.87 | 0.27 | 0.27 |
| Butyrate, mol/100 mol | 4.00 | 4.13 | 3.91 | 3.92 | 0.309 | 0.59 | 0.89 | 0.59 |
| Isovalerate, mol/100 mol | 1.43 | 1.11 | 1.22 | 1.36 | 0.283 | 0.86 | 0.45 | 0.37 |
| Valerate, mol/100 mol | 1.36 | 1.38 | 1.29 | 1.43 | 0.100 | 0.53 | 0.28 | 0.60 |
| *Colon* | | | | | | | | |
| Length, cm | 4.67 | 4.89 | 4.39 | 4.58 | 0.272 | 0.35 | 0.37 | 0.19 |
| Digesta pH | 7.18 | 7.08 | 7.09 | 7.21 | 0.102 | 0.51 | 0.22 | 0.47 |
| Total SCFA, m*M* | 68.0 | 73.3 | 69.7 | 62.5 | 5.66 | 0.14 | 0.32 | 0.43 |
| Acetate, mol/100 mol | 74.42 | 75.09 | 75.41 | 75.86 | 0.976 | 0.16 | 0.70 | 0.66 |
| Propionate, mol/100 mol | 17.14 | 17.24 | 16.46 | 15.77 | 0.586 | 0.01 | 0.95 | 0.63 |
| Isobutyrate, mol/100 mol | 1.54 | 1.25 | 1.45 | 1.65 | 0.262 | 0.31 | 0.45 | 0.31 |
| Butyrate, mol/100 mol | 4.18 | 4.05 | 3.99 | 3.90 | 0.290 | 0.33 | 0.82 | 0.75 |
| Isovalerate, mol/100 mol | 1.32 | 1.04 | 1.25 | 1.41 | 0.316 | 0.46 | 0.61 | 0.41 |
| Valerate, mol/100 mol | 1.37 | 1.36 | 1.41 | 1.42 | 0.108 | 0.55 | 0.88 | 0.90 |
| Histology | | | | | | | | |
| Crypt depth, µm | 569.58 | 607.52 | 617.33 | 552.05 | 29.355 | 0.29 | 0.03 | 0.53 |
| Crypt width, µm | 39.51 | 39.35 | 39.94 | 37.83 | 2.350 | 0.48 | 0.54 | 0.82 |

[1]Sheep were assigned with CON (*n* = 8; no inclusion of HFCG in the diet), LOW (*n* = 8; inclusion of 0.075% of HFCG in the diet, % DM), MED (*n* = 8; inclusion of 0.3% of HFCG in the diet, % DM), and HIGH (*n* = 8; inclusion of 0.6% of HFCG in the diet, % DM).
[2]SED: Standard error of the difference.

### 3.4.5. Apparent total tract digestibility and blood parameters

Increasing dose of HFCG did not affect fecal DM concentration, nor did it affect digestibility coefficients for DM ($P \geq 0.10$), NDF ($P \geq 0.33$), ADF ($P \geq 0.58$), starch ($P \geq 0.38$), and ether extract ($P \geq 0.21$; Table 3.7). Crude protein digestibility tended to be cubically affected ($P = 0.09$) by the increasing dose of HFCG with the numerically greatest digestibility for those fed LOW. However, neither linear ($P = 0.76$) nor quadratic ($P = 0.76$) effects were observed for CP digestibility (Table 3.7). Treatment did not affect ($P \geq 0.10$; Table 3.8) glucose, insulin, or BHBA concentrations; however, insulin ($P < 0.01$) and BHBA ($P < 0.01$) were affected by time, where the concentrations were greater 6 h after feeding compared to immediately prior to feeding.

### 3.4.6. In situ ruminal DM disappearance of HFCG and ruminal protection of CaG

After 24 h of incubation HFCG presented 13.4 ± 2.89% (mean ± SD) of DM disappearance (data not shown). The final concentration of CaG was 38.7 ± 1.36% which resulted in a 16.1 ± 4.73% of CaG disappearance.

## 3.5. Discussion

Although there is no published data regarding the rumen-protection efficacy of the HFCG, based on in situ degradation we confirmed the efficacy of the rumen protection with 86.6% of the HFCG recovered after 24 h of ruminal incubation. Wu et al. (2012), used a similar coating technique as in the current study to protect lysine; however, oleic acid was used (2 and 4% of the product) in this study and they reported that the ruminal escape of the DM ranged from 86.5 to 94.2%. Based on the concentration of CaG within the HFCG after the incubation, there was similar degradation when comparing the total HFCG and CaG components. As such, my data support that the HFCG had reasonable ruminal protection and the extent of protection is similar to that of Wu et al. (2012).

Given that limited ruminal release of CaG was expected, small or no changes in the SCFA profile in the rumen occurred. While it could be expected that some of the CaG should be available in the small intestinal regions, we did not observe changes in the SCFA profile throughout the small intestine. My results are supported by previous in situ where Asano et al. (1997) incubated gluconic acid in the small intestine of rats and reported recovery of 86% of the amount infused 30 min later suggesting that gluconic acid is stable in the small intestine and poorly absorbed. Changes

**Table 3.6.** Organ weight of lambs (n = 32) fed with three different doses of hydrogenated fat matrix-embedded Ca-gluconate (HFCG) for 28 days.

| Item | Treatment[1] | | | | SED[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | LOW | MED | HIGH | | Linear | Quadratic | Cubic |
| Kidney, kg | 99 | 91 | 93 | 89 | 4.8 | 0.10 | 0.73 | 0.16 |
| Liver, kg | 652 | 643 | 624 | 612 | 34.6 | 0.22 | 0.80 | 0.99 |
| Spleen, kg | 85 | 141 | 115 | 191 | 75.6 | 0.24 | 0.78 | 0.45 |

[1]Sheep were assigned with CON (*n* = 8; no inclusion of HFCG in the diet), LOW (*n* = 8; inclusion of 0.075% of HFCG in the diet, % DM), MED (*n* = 8; inclusion of 0.3% of HFCG in the diet, % DM), and HIGH (*n* = 8; inclusion of 0.6% of HFCG in the diet, % DM).
[2]SED: Standard error of the difference.

**Table 3.7.** Fecal excretion (DM) and DM digestibility of lambs (n = 32) fed with three different doses of hydrogenated fat matrix-embedded Ca-gluconate (HFCG) for 28 days.

| Item | Treatment[1] | | | | SED[2] | *P*-value | | |
|---|---|---|---|---|---|---|---|---|
| | CON | LOW | MED | HIGH | | Linear | Quadratic | Cubic |
| Fecal DM output, g | 323 | 333 | 262 | 259 | 52.8 | 0.12 | 0.56 | 0.55 |
| Digestibility, % | | | | | | | | |
| DM | 74.65 | 74.37 | 74.56 | 71.66 | 1.789 | 0.10 | 0.39 | 0.76 |
| CP | 59.34 | 67.39 | 62.35 | 64.00 | 3.533 | 0.76 | 0.76 | 0.09 |
| NDF | 47.22 | 52.75 | 47.79 | 44.02 | 6.376 | 0.33 | 0.65 | 0.46 |
| ADF | 39.77 | 43.65 | 40.36 | 37.12 | 7.317 | 0.58 | 0.76 | 0.91 |
| Starch | 99.42 | 99.53 | 99.43 | 99.49 | 0.094 | 0.91 | 0.88 | 0.94 |
| Ether extract | 62.06 | 67.77 | 67.18 | 70.04 | 4.773 | 0.21 | 0.74 | 0.18 |

[1]Sheep were assigned with CON (*n* = 8; no inclusion of HFCG in the diet), LOW (*n* = 8; inclusion of 0.075% of HFCG in the diet, % DM), MED (*n* = 8; inclusion of 0.3% of HFCG in the diet, % DM), and HIGH (*n* = 8; inclusion of 0.6% of HFCG in the diet, % DM).
[2]SED: Standard error of the difference.

**Table 3.8.** Blood parameters from lambs (n = 32) fed with three different doses of hydrogenated fat matrix-embedded Ca-gluconate (HFCG) for 28 days in two different timepoints (before the morning feeding [0 h] and 6 h after the morning feeding [6 h]).

| | Treatment[1] | | | | Time | | SED[2] | *P*-values | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CON | LOW | MED | HIGH | 0 h | 6 h | | Time | Linear | Quadratic | Cubic |
| Glucose, mmol/L | 2.58 | 2.66 | 2.98 | 2.81 | 2.70 | 2.81 | 0.323 | 0.592 | 0.323 | 0.243 | 0.840 |
| Insulin, pmol/L | 17.7 | 24.7 | 19.4 | 27.2 | 17.6 | 26.9 | 5.31 | 0.003 | 0.066 | 0.164 | 0.253 |
| BHBA, mmol | 1.515 | 1.622 | 1.504 | 1.412 | 1.330 | 1.702 | 0.0801 | <0.01 | 0.331 | 0.59 | 0.415 |

[1]Sheep were assigned with CON (*n* = 8; no inclusion of HFCG in the diet), LOW (*n* = 8; inclusion of 0.075% of HFCG in the diet, % DM), MED (*n* = 8; inclusion of 0.3% of HFCG in the diet, % DM), and HIGH (*n* = 8; inclusion of 0.6% of HFCG in the diet, % DM).
[2]SED: Standard error of mean.

in the large intestinal digesta composition were expected based on studies conducted by Tsukahara et al. (2002, 2006) and Biagi et al. (2006). In those studies, porcine cecal digesta was used as an inoculum for in vitro fermentation and the addition of gluconate increased n-butyrate, acetate, propionate, and total production of SCFA after 24 h of incubation. While we observed a cubic change for total SCFA in the cecum and a linear increase in acetate concentration in the cecum, no other changes in large intestinal fermentation were detected. It should be noted that other in vivo studies have reported only increases in fecal isobutyrate in response to HFCG supplementation on lactating dairy cows (Doelman et al., 2019; Seymour et al., 2021) suggesting that the effective dose may be too small to elicit changes or that changes do not persist across the large intestine due to rapid absorption of SCFA. Likewise, Biagi et al. (2006) fed weaned piglets gluconic acid and did not observe changes in SCFA concentrations in the cecum compared to piglets not supplemented with gluconate.

My findings contrast previous in vitro findings (Tsukahara et al., 2002, 2006; Biagi et al., 2006) as we observed a cubic response to increasing HFCG dose where only the LOW dose increased total SCFA concentration in cecal digesta. The lack of increase in SCFA in cecal digesta for MED and HIGH could be expected given the linear reduction in DMI with increasing HFCG dose. Moreover, we observed a linear increase in acetate and linear decrease in propionate in cecal digesta, without any change in butyrate, with increasing dose of HFCG which may also be related to the decreased DMI observed. Supporting this suggestion, changes in the molar proportions of isovalerate and valerate and for acetate, propionate, and butyrate have been reported in the cecum and colon, respectively of calves when exposed to feed restriction relative ad libitum fed calves (Pederzolli et al., 2018). In addition to the change in cecal SCFA molar proportions, the proportion of propionate linearly decreased in the colon with increasing CaG. Unlike the in vitro studies, we could only measure SCFA concentration at a single time point, 4 h after feeding, and used a rumen-protected form of CaG. It is possible that shifts in SCFA concentrations are time-dependent or that, when considering meal sizes and passage rate, the dose applied was insufficient for a measurable change in butyrate. Additionally, it is possible that differences in SCFA concentration in vivo are not detectable once SCFA are absorbed, whereas, with in vitro systems, SCFA production can be assessed due its accumulation into the system (den Besten et al., 2013). The confounding effect for differential absorption of individual SCFA may be further supported as compared to acetate, the rates of butyrate transport are greater for butyrate than acetate and the intra-epithelial

metabolism of butyrate is more extensive (Bugaut, 1987; Gäbel et al., 2001; Aschenbach et al., 2009). Considering that lambs did not sort their diets, lambs ingested 0, 0.75, 3.0, and 4.8 g of HFCG for CON, LOW, MED, and HIGH respectively; showing that the reduced DMI impact at the most the lambs submitted to the HIGH dose.

We also hypothesized that use of CaG would lead to morphological modifications in the large intestine and in the entire GIT, mainly by increasing in butyrate production. As described by Guilloteau et al. (2010), butyrate has both direct and indirect effects on the GIT, stimulating energy supply and proliferation of the epithelium. Indirect effects of butyrate on the gastrointestinal tract, even beyond the region of exposure, are mediated by the release of IGF-1, which stimulates ruminal epithelial cell proliferation (Baldwin et al., 2004), or GLP-2, which is a well-known stimulator and regulator of GIT development and function (Burrin et al., 2003). In the current study, the linear thinning of the stratum corneum in the ruminal papillae might be related to the linear DMI which led to a reduced amount of substrate in the rumen and, consequently a lesser extent of fermentation or possibly via paracrine responses. However, the latter is unlikely given we did not observe an increase in butyrate throughout the GIT. That said, there was a quadratic deepening of colon crypt depth with the increasing dose of HFCG, although the impact was not sufficient to affect performance. This is in contrast to the findings of Poeikhampha and Bunchasak (2011) who supplemented piglets with sodium gluconate and observed an increase in jejunum villous height and crypt depth, leading to increased final BW and ADG compared to piglets that did not receive sodium gluconate supplementation.

The linear reduction in final BW and the reduction in ADG with the increasing HFCG dose was unexpected and can likely be explained by the linear reduction for DMI. Others have reported a reduction in DMI in response to post-ruminal infusion of CaG (McKnight et al., 2019); however, increased feed efficiency was observed in the same study while we observed a reduction. Doelman et al. (2019) also infused CaG into the abomasum of dairy cows at 0, 44, 93, 140, and 187 g/cow/d and reported a quadratic response in DMI, where they observed the lowest DMI in cows infused with a dose of 140 g/d CaG, partially supporting the findings of the current study.

While the cause for the linear decrease in DMI with increasing dose of HFCG is not fully understood, it may be partially explained by the linear increase in the molar concentration of acetate in the cecum. While propionate is largely considered as hypophagic in ruminants (Allen et

al., 2009), previous research in rats has demonstrated that perfusion of the colon with acetate, but not propionate, stimulated GLP-1 release, which was not mediated by free fatty acid receptors 2 and 3, suggesting that the satiety effect may be related to improved luminal nutrient supply (Christiansen et al., 2018). Previous research has implicated acetate as an anorexic agent in mice (Frost et al., 2014) and speculated that the acetate-driven release of GLP-1 may mediate feed intake by signaling the hypothalamus. In the present study, there was a linear increase in the molar proportion of acetate without corresponding changes in the total SCFA concentration, suggesting greater exposure of the epithelia to acetate may have stimulated GLP-1 and the subsequent reduction in DMI (Honda, 2016). In addition, D'Alessio et al. (1994) and Drucker (2002) reported that greater secretion of GLP-1 leads to a stimulation of insulin secretion, fitting well with the observed tendency for a linear increase for insulin in the present study. Additionally, Faulkner and Pollock (1991) reported that intravenous administration of GLP-1 led to an increase in insulin secretion in starved sheep in hyperglycemia situations. A subsequent experiment conducted by Martin and Faulkner (1993) studied the intravenous administration of glucose combined with GLP-1 and reported that GLP-1 increased the insulin concentration in ruminants and regulated glucose homeostasis similarly to that described in non-ruminants species. These authors also stated that the increase in insulin concentration is dose-dependent with GLP-1 and this effect is less pronounced in fed than starved sheep. Thus, increasing dose of HFCG may have exerted a greater effect arising from GLP-1 as DMI decreased. The same authors suggested that the effects of GLP-1 may not be glucose dependent as glucose supply in the intestine are expected to be lower in ruminants than non-ruminant species and that other nutrients influence the GLP-1 and insulin release mechanisms. However, Relling et al. (2014) verified that intravenous GLP-1 infusions in sheep did not affect the DMI, thus future studies are needed to measure GLP-1 concentrations to confirm this speculated mode of action.

### 3.6. Conclusion

In the present study, the use of HFCG was designed to deliver CaG to the intestine. My in situ results suggested that over 86% of the HFCG was protected from ruminal degradation which were further supported by a lack of effects on ruminal digesta. In addition, changes in the molar proportion of acetate and propionate in the cecum and propionate in the colon, and the decrease for feed intake and ADG are interpreted to confirm at least partial release of CaG in the large

intestine. Future research is needed to evaluate the rate and site of release for HFCG as well as its mode of action in ruminants. In addition, we conclude that feeding HFCG to growing lambs does not increase butyrate concentration in the intestine and consequently does not increase the absorptive surface area of the intestine, the size of the GIT, or the functionality of the intestine in a dose-dependent manner.

# 4. A COMPARISON OF POST-RUMINAL PROVISION OF CA-GLUCONATE AND CA-BUTYRATE ON GROWTH PERFORMANCE, GASTROINTESTINAL BARRIER FUNCTION, SHORT-CHAIN FATTY ACID ABSORPTION, INTESTINAL HISTOLOGY, AND BRUSH-BORDER ENZYME ACTIVITY IN BEEF HEIFERS[2]

The experiment described on Chapter 3 was developed to identify an optimal dose of CaG to be used in the subsequent studies and to identify its mode of action. Once a linear decrease in DMI, growth and feed efficiency were identified, an optimal dose of HFCG could not be identified; thus, a different approach was necessary for the subsequent experiment. On the experiment described in this Chapter, an optimal dose of post-ruminal provision of CaG found by Doelman et al. (2019) and McKnight et al. (2019) on lactating dairy cows were used to allow the investigation of the effects of post-ruminal provision of CaG and CaB.

## 4.1. Abstract

The objective of this study was to compare the effects of post-ruminal provision of Ca-butyrate (**CaB**) when delivered in a ruminally protected form or via abomasal dosing and Ca-gluconate (**CaG**) on SCFA concentration throughout the GIT, nutrient digestibility, GIT barrier function, ruminal SCFA absorption, ruminal morphometrics, intestinal brush border enzyme activity, and blood parameters of beef heifers. Thirty-two beef heifers fitted with ruminal cannulas were used in a randomized complete block design and assigned to 1 of 4 treatments: 1) negative control (ruminal infusion of double-distilled water; **CON**); 2) abomasal infusion of CaB (**AB**; 0.0029% of BW); 3) abomasal infusion of CaG (**AG**; 0.0077% of BW); and 4) ruminal infusion of a hydrogenated fat-embedded CaG (**RG**; 0.0192% of BW). Excluding CON, treatments were designed to deliver the same amount of butyrate in the small intestine. Heifers were allocated in individual pens and DMI was limited to 95% of voluntary intake to minimize a potential confounding effect. Total GIT barrier function was assessed on d 17 and SCFA disappearance was

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[2]A version of this chapter has been published in the Journal of Animal Science. Watanabe, D. H. M., J. Doelman, M. A. Steele, L. L. Guan, D. J. Seymour, and G. B. Penner. 2023. A comparison of post-ruminal provision of Ca-gluconate and Ca-butyrate on growth performance, gastrointestinal barrier function, short-chain fatty acid absorption, intestinal histology, and brush-border enzyme activity in beef heifers. J. Anim. Sci. 101:1–15. doi:10.1093/jas/skad050.
**Author contribution:** The author contributed to this publication with conceptualization, funding acquisition, experimental planning, and implementation, caring of the animals, sample collection, majority of the laboratory analysis, statistical analysis, preparation of the original draft, and implementation of co-author reviews.

conducted on d 21 using the temporarily isolated and washed reticulo-rumen technique. On d 28, heifers were slaughtered, and ruminal and colonic digesta were collected to assess SCFA concentration, while ruminal, jejunal, and colonic tissues were collected to assess SCFA fluxes and regional barrier function ex vivo using the Ussing chamber technique. For colonic digesta, both AB and CaG treatments reduced the proportion of acetate ($P = 0.05$) and increased the proportion of propionate ($P = 0.05$) compared to CON. Relative to CON, AB but not CaG treatments increased in vivo ruminal disappearance of total SCFA ($P = 0.01$), acetate ($P = 0.03$), propionate ($P = 0.01$), and butyrate ($P < 0.01$). Treatments did not affect ($P \geq 0.10$) acetate and butyrate fluxes in the ruminal and colonic tissues when measured ex vivo; however, compared to CON, AB tended to decrease ($P = 0.09$) mannitol flux across ruminal tissue. In addition, mannitol flux was affected ($P < 0.01$) by region, with greater mannitol flux across the jejunum than rumen and colon. It can be concluded that while both abomasal infusion of CaB and CaG affect the molar proportion of acetate and propionate in the colon, only abomasal CaB stimulated ruminal SCFA absorption for growing beef heifers.

## 4.2. Introduction

Among the short-chain fatty acids (**SCFA**) produced by ruminal fermentation, butyrate has received considerable attention as low concentrations stimulate development and maintenance of gastrointestinal tract (**GIT**) barrier function (Sakata and Tamate, 1978; Mentschel et al., 2001; Guilloteau et al., 2010). Within the rumen, butyrate has been reported to increase the ruminal papillae surface area, and consequently the absorptive surface (Sakata and Tamate, 1976) through a reduction in apoptosis (Mentschel et al., 2001). Butyrate also indirectly increases the forestomach absorptive capacity by increasing blood flow (Storm et al., 2011). Additionally, butyrate is a key energy source for the ruminal epithelium (Bergman, 1990) and primary ketogenesis by the ruminal epithelium provides energetic substrates to peripheral tissues. Butyrate is also the preferred energy source of colonocytes (Roediger, 1980) and the effects of the increased supply of butyrate to the intestine of non-ruminants include a reduction of colitis-related inflammation (Dianzani et al., 2006; Serpe et al., 2010; Vieira et al., 2012), repair of intestinal damage in piglets (Piva et al., 2002), and increased intestinal cell proliferation (Cook and Sellin, 1998) leading to increased growth performance in pigs (Mazzoni et al., 2008; Le Gall et al., 2009). In calves, the addition of sodium butyrate to milk replacer led to an increase in growth which may be partially mediated by increased ruminal papillae length and width (Gorka et al., 2009, 2011a). Butyrate also acts as a signalling molecule that helps to support maintenance of cellular tight junctions (Zhang et al., 2006). Peng et al. (2009) incubated colonic cells with and without butyrate and reported that the presence of butyrate increased the transepithelial electrical resistance (**TER**) by accelerating the relocation of the tight junction proteins, zonula occludens-1 (**ZO-1**) and occludins to the cell membrane. Others have reported that cells incubated with butyrate can increase the expression and redistribution of tight junction proteins such as claudin-1 (Wang et al., 2012), claudin-2, occludin, cingulin, ZO-1, and ZO-2 (Plöger et al., 2012).

Due the strong odor and volatility of butyric acid, its use as a dietary supplement has been limited. However, more stable and less odorous forms of butyric acid, such as butyrate salts and encapsulated products (Guilloteau et al., 2010) may facilitate greater use. Another approach to increase butyrate supply is through the use of butyrate precursors. Tsukahara et al. (2002) incubated glucose, sorbitol, or gluconic acid in porcine cecal digesta and concluded that in comparison with glucose and sorbitol, gluconic acid is a more efficient butyrate precursor, as there

was greater butyrate production and lesser lactate production. There is debate in the literature as to whether gluconic acid or Ca-gluconate (**CaG**) alters butyrate concentrations in intestinal digesta and presumably butyrate supply. McKnight et al. (2019) fed CaG in a total mixed ration to dairy cattle and reported a greater proportion of butyrate and acetate, and a reduction in the propionate concentration in ruminal fluid relative to those not provided CaG. However, a study providing CaG through abomasal infusion did not show an increase in fecal butyrate (Doelman et al., 2019a) and when provided as a rumen protected product (Watanabe et al., 2022) no increases in the molar proportion of butyrate in digesta from the rumen, small intestinal, or large intestinal digesta was observed. While those studies have not reported greater butyrate concentrations with post-ruminal CaG provision, authors have suggested that preferential absorption of butyrate and relatively low doses of CaG may preclude such changes (Watanabe et al., 2022). As such, a lack of response to intestinal butyrate concentration does limit the potential for greater butyrate supply with CaG provision.

While not directly compared within a study, outcomes arising from butyrate and gluconate supplementation may be similar. As reported by Manzanilla et al. (2006), early weaned pigs provided sodium butyrate had greater feed efficiency and increased crypt depth in the jejunum. In comparison, supplementation with sodium gluconate to piglets by Poeikhampha and Bunchasak (2011) resulted in increased intestinal development, demonstrated by the increased jejunum villus height and crypt depth leading to a greater average daily gain (**ADG**) and final body weight (**BW**) compared to piglets without supplementation. In addition, Biagi et al. (2006) also observed increased ADG and a tendency to increase the total SCFA in the jejunum of piglets supplemented with gluconic acid. When combined with potential for gluconate fermentation to butyrate (Tsukahara et al., 2002), it is plausible that the Ca-gluconate may be a viable strategy to increase intestinal butyrate supply.

It was hypothesized that relative to control (**CON**), post-ruminal provision of Ca-butyrate (**CaB**) and CaG, whether provided using a ruminally protected source or via abomasal infusion, would increase butyrate concentration in the large intestine, leading to an increase in nutrient and SCFA absorption, and GIT barrier function and functionality. It was also hypothesized that the effects of post-ruminal provision of CaB and CaG would not differ. The objective of this study was to compare the effects of post-ruminal provision of CaB and CaG on SCFA concentration

throughout the GIT, nutrient digestibility, barrier function, SCFA absorption, ruminal morphometrics, intestinal brush border enzyme activity, and blood parameters for beef heifers.

## 4.3. Material and methods

Use of heifers for this experiment was approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20100021, Saskatoon, SK, Canada). Heifers and their use followed the guidelines of the Canadian Council on Animal Care (Ottawa, ON, Canada).

### 4.3.1. Experimental design and treatments

Twenty-eight Angus cross and four Speckle Park ruminally-cannulated yearling beef heifers (BW 388 ± 31 kg, mean ± SD) were used to evaluate the mode of action of HFCG on GIT function. BW was recorded and used to block the heifers into 1 of 8 blocks. Blocks were used to minimize the effect of BW and breed, and, within block, heifers were randomly assigned to 1 of 4 dietary treatments. Heifers were cannulated 6 mo before the start of the experiments, at which time they were fitted with a 7.62 cm diameter cannula (model 4C 3"; Bar Diamond Inc., Parma, ID). Body temperature was measured daily for each heifer and the surgery site was cleaned and disinfected. After two weeks of recovery, sutures were removed, and the cannula was replaced with a 9 cm cannula (model 9C; Bar Diamond Inc.). Prior to treatment exposure, heifers were housed indoor in individual pens (3 m × 3 m) equipped with rubber mats on the floor, with a suspended ball for environmental enrichment, and a water bowl. Heifers were allowed 5 d of environmental adaptation during which they were fed a common diet containing (dry matter [**DM**] basis) grass hay (66.3%), dry-rolled barley (16.9%), and a vitamin and mineral mash (16.9%) offered *ad libitum*. Pens were scraped and washed once daily before feeding (0800 h). Following adaptation, all heifers were fed a common diet composed of (DM basis) barley silage (40.0%), grass hay (10.0%), dry-rolled barley (33.2%), a barley-grain based vitamin and mineral mash (16.4%), and limestone (0.34%). The diet contained (mean ± SD) 50.8 ± 0.02% of DM, 33.2 ± 1.11% of starch, 15.5 ± 0.57% of crude protein (**CP**), 31.2 ± 0.77% of ash-free neutral detergent fiber (**aNDFom**), 16.5 ± 0.62% of acid detergent fiber (**ADF**), 2.89 ± 0.22% of ether extract, 1.55 ± 0.16% of Ca, and 0.27 ± 0.02 of P. For the first 7 days of the experimental period, the diet was fed to achieve *ad libitum* intake to determine voluntary intake. From d 8 onwards, dry matter intake (**DMI**) was limited to 95% of average voluntary intake during the first 7 d to minimize its confounding effect on treatment responses. Heifers were fed once daily at 0900 h.

The treatments in this study included: a negative control (**CON**; ruminal infusion of double-distilled water, 1 L); a positive control which consisted of CaB infused into the abomasum at 0.0029% of BW (**AB**; Bonding Chemical, Katy, TX); CaG infused into the abomasum at 0.0077% BW (**AG**; Global calcium PVT. LTD., Tamil Nadu, India); and a hydrogenated fat matrix-embedded CaG infused into the rumen at 0.0192% BW (**RG**; 50% hydrogenated palm fat, 40% calcium gluconate, 10% calcium carbonate; Selko Lactibute, Trouw Nutrition, Amersfoort, the Netherlands). Excluding the negative control, treatments were designed to deliver the same amount of butyrate to the small intestine based on previous studies (Gorka et al., 2009, 2011a,b, 2014) where calves supplemented with Na-butyrate at 0.003% of BW (via milk replacer) presented greater BW (Gorka et al., 2009, 2011a) increased reticulo-rumen weight, ruminal papillae weight, and width (Gorka et al., 2009, 2011a,b), increased proliferation and reduced apoptosis in the mid-jejunal mucosa (Górka et al., 2011b, 2014), and enhanced brush border enzyme activity in the small intestine (Górka et al., 2014). Given that, the dose of CaB was established at 0.003% of BW. For CaG treatments, the conversion of gluconate to butyrate was based on results of an in vitro study (Tsukahara et al., 2002) where 1 mol of Na gluconate was converted to 0.305 mol of butyrate. Thus, to achieve a similar butyrate supply, it was estimated that a CaG dose of 0.0077% of BW was required. Finally, as the HFCG contained 40% CaG, the dose of the rumen protected CaG was designed to provide the same quantity of CaG as the AG treatment (0.0192% of BW) with the assumption that 100% of the rumen protected CaG would flow to the small intestine. On average, AB heifers were infused with 11.56 g/d of CaB, AG heifers were infused with 30.69 g/d of CaG, and RG heifers were infused with 76.52 g/d of HFCG which represented 0.15, 0.40, and 1.03% of the total DMI. All infusions were provided daily immediately after feeding (0930 h). The CaB and CaG were suspended in 250 mL of reverse osmosis water and an additional 750 mL of water was used to rinse the infusion line to ensure complete infusion into the abomasum. The total amount of HFCG infused in the rumen was divided into 5 equal parts using filter paper (Whatman 1441-150 41 Ashless Quantitative Filter Paper, Whatman International Ltd., Maidstone, England) packages that were placed in different regions of the rumen. To ensure the same rate of water addition for all treatments, the HFCG and control treatments received 1 L of water in the rumen. Weekly BW measurements were taken prior to feeding to adjust the dose of each treatment on a percent of BW basis.

The daily amount of feed offered and refused were recorded for each heifer to determine as fed feed intake. The DM content of the feed ingredients were determined weekly to ensure the diet delivered reflected the formulated composition. From d 20 to 26, data for DMI were used for analysis. During this time, ingredient and refusal samples were collected daily and the daily samples were composited proportionally (as fed basis) across the 7-d collection period to determine DM content. The DM was determined by placing samples in a forced-air oven at 55ºC for 72 h or until a constant weight was achieved. In addition to the weekly BW measurements, BW was measured on 2 consecutive d at the beginning (d 0 and 1) and end (d 21 and 22) of the experimental period.

### 4.3.2. Apparent total tract digestibility

During the digestibility evaluation period, samples of feed ingredients, refusals, and feces were collected for 4 consecutive days. Total tract digestibility was determined using the ash-corrected undigested neutral detergent fiber (**uNDFom**) as an internal marker. Spot samples of feces (200 g/sample) were collected directly from the rectum every 12 h on d 17 (0600 and 1800 h), d 18 (0900 and 2100 h), d 19 (1200 and 2400 h), d 20 (1500 h), and d 21 (0300 h). Immediately following collection, the sample was thoroughly mixed, after which a 125 g subsample was removed and added to a separate container to create a composite for each heifer. Feces were stored at -20°C during collections and until determination of DM.

All samples were dried at 55ºC until they achieved a constant weight, and ground using a Christy Turner Miracle Mill (Christy Turner Ltd., Suffolk, England) with a 1-mm screen. The dried and ground samples were submitted to SGS Crop Science Canada (Guelph, ON, Canada) for analysis of organic matter (**OM**), starch, CP, neutral detergent fiber (**NDF**), ADF, ether extract, and uNDFom. The OM concentration was determined by ashing the samples for 2 h at 600ºC and subtracting the residue from 100% (method 942.05; AOAC, 2000). The starch concentration was determined by digesting it with amylase and measuring the resulting glucose (Hall, 2009; method 996.11; AOAC, 2007). The CP concentration was calculated by analyzing the nitrogen content of the samples using a Leco FP-528 Nitrogen Combustion Analyzer (LECO Corp., St. Joseph, MI) and the value was multiplied by 6.25 (method 992.03; AOAC, 2007). The NDF concentration was determined by the methodology proposed by Van Soest et al. (1991) with the modification that Whatman 934-AH glass micro-fiber filters with 1.5 µm particle retention (GE Healthcare Life

Sciences, Piscataway, NJ) and included the use of α-amylase and sodium sulfite. The residue of the NDF analysis was ashed at 535ºC for 2 h and used to calculate the ash-free neutral detergent fiber (**aNDFom**). The ADF determination was undertaken using the same modifications proposed for the NDF methodology, based on the protocol established by AOAC (2000).

#### 4.3.3. Total gastrointestinal tract barrier function

On d 16, heifers were fit with a urinary catheter (24 Fr Foley catheter, Bardex Lubricath, Franklin Lakes, NJ) to allow for total urine collection. On d 17, Cr-EDTA was infused into the rumen of each heifer to evaluate the paracellular permeability of the gastrointestinal tract, as described by Zhang et al. (2013). Briefly, a 1 L solution of a 180 m*M* Cr-EDTA solution was infused into the rumen via the cannula. Total urine output was collected every 24 h over 48-h (d 18 and 19, at 1100 h). Urine output was recorded, and a representative sample was collected, stored at -20ºC, and submitted to Prairie Diagnostic Services (Saskatoon, SK) to determine Cr concentration using inductively coupled plasma emission coupled with mass spectroscopy, as described by Connor et al. (2017).

#### 4.3.4. Short-chain fatty acid absorption in vivo

Ruminal SCFA absorption was measured using the temporarily isolated and washed reticulo-rumen technique as described by (Zhang et al., 2013) and (Care et al., 1984). Briefly, the reticulo-ruminal digesta was evacuated, after which the reticulo-rumen was washed with pre-heated water (39ºC) twice. Subsequently, a washing buffer solution (105 m*M* NaCl, 10 m*M* Na acetate, 20 m*M* Na propionate, 25 m*M* $NaHCO_3$; pH 6.2, 297 ± 3.3 mOsmol/kg) was pre-heated to 39ºC and used for an additional 5 to 6 washes (5 L/wash) to allow for removal of residual feed particles while suppling essential nutrients. Once the reticulo-rumen was clean, an esophageal occluding device was placed in the distal portion of the esophagus to prevent saliva inflow into the rumen. Saliva was removed from the esophagus using a 2-stage vacuum pump (model N86KT45P; KNF Neuberger Inc., Trenton, NJ, USA). An occluding device (24 Fr Foley catheter 7cc ribbed balloon, Bardex Lubricath, Franklin Lakes, NJ) was inserted into the omasal orifice to prevent liquid passage out of the rumen. The reticulo-rumen was washed again, and all residual buffer was removed. Next, 15 L of buffer solution (2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 5 m*M* NaCl, 5 m*M* KCl, 30 m*M* Na acetate, 35 m*M* K acetate, 35 m*M* Na propionate, 8 m*M* Na butyrate, 7 m*M* butyric acid, 5 m*M* L-lactic acid, 25 m*M* $NaHCO_3$, 2 m*M* Cr-EDTA; osmolality 275 ± 3.9 mOsmol/kg, pH 6.2)

was infused into the rumen. Samples of the incubation buffer were collected before infusion (0 minutes), and at 5 minutes and 45 minutes after infusion. The buffer samples collected were used to measure the ruminal concentration of Cr and SCFA enabling calculation of liquid volume and SCFA disappearance rates according to Care et al. (1984). Disappearance rates were assumed to be equal to absorption rates.

### 4.3.5. Blood sampling and analysis

Jugular catheters (8.3 cm 14-gauge intravenous catheter, BD Angiocath, Becton Dickinson, North Ryde, New South Wales, Australia) were inserted into the jugular vein on d 14, with blood collection initiated at 0 (0900 h) and 6 h following the morning feeding to evaluate changes in β-hydroxybutyrate (**BHBA**), glucose, and insulin. For plasma, blood was collected into a 10-mL vacutainer tube containing lithium heparin (158 IU, BD, Franklin Lake, NJ), centrifuged at 3,000 × *g* for 15 min at 4°C to harvest plasma, and stored at -20°C. For serum, blood was collected into a 10-mL vacutainer tube containing a clot activator (Serum blood collection tubes, BD Vacutainer, Franklin Lakes, NJ). Serum was chilled on ice for 30 min prior to being centrifuged following the same conditions as for plasma. Plasma glucose concentration was measured using a glucose oxidase/peroxidase enzyme (P7119, Sigma-Aldrich, St. Louis, MO) and dianisidine dihydrochloride (F5803, Sigma-Aldrich). Absorbance was determined at 450 nm with a plate reader (Epoch 2, BioTek Instruments Inc., Winooski, VT). Plasma insulin concentration was measured using a commercial kit (Mercodia Ovine Insulin ELISA, Uppsala, Sweden) and absorbance was measured using a plate reader (Epoch 2) at 450 nm. Serum BHBA concentration was measured using the enzyme 3-hydroxybutyrate dehydrogenase (H6501, Roche, Mississauga, Ontario Canada) to catalyze the oxidation of BHBA to acetoacetate, as described by Williamson et al. (1962). The reduction of NAD to NADH was measured using a plate reader at the wavelength of 340 nm and the increase in absorbance was directly proportional to the amount of BHBA in the sample. Both plasma and serum samples were analyzed in triplicate for glucose and BHBA and samples with CV higher than 5% were repeated until achieving a CV ≤ 5%. For insulin samples, the CV limit was restricted to 7%. Catheters were removed following the final blood sample.

### 4.3.6. Gastrointestinal tract measurements

At 1000 h on d 24, 25, 27, and 28, heifers were slaughtered by captive bolt stunning, followed by pithing and exsanguination. The order of slaughter was balanced across blocks to avoid

potential bias with days of treatment exposure. Reticulo-ruminal digesta samples were collected prior to slaughter via the ruminal cannula. After confirmation of death, the gastrointestinal tract was removed and digesta from jejunum (proximal, near to the ligament of Treitz) and colon (end of the centripetal turn) were collected. Ruminal digesta was strained through 2 layers of cheesecloth and stored in a sterile container. Digesta from jejunum and colon were also stored in sterile containers, and colon digesta was diluted 1:1 (wt/wt) with double-distilled water. Digesta pH was assessed in duplicate using a portable pH meter (Accumet AP110, Fisher Scientific, Nepean, ON, Canada). Subsequently, 10 mL of digesta from each region was transferred into a container containing 2 mL of meta-phosphoric acid (25% w/v) and stored at -20ºC until analyzed for SCFA as described by Khorasani et al. (1996) using gas chromatography (Agilent 6890 Series GC System, Santa Clara, CA, USA) to separate and quantify each SCFA. Digesta samples were thawed, centrifuged (at 12,000 × *g*, 4ºC for 10 min), and the supernatant were filtered through a 25-µm syringe filter (Fisher Scientific), and centrifuged (at 16,000 × *g*, 4ºC for 10 min) one more time. Acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate concentrations were calculated by the relative response of the internal standard (isocaproate). For each sample, 1 µL was injected using a 17:1 split ratio and an injector temperature of 260ºC. The column (7HM-G009-11 Zebron, 30 m × 0.32 mm × 0.25 µm, Phenomenex, Torrance, CA) was incubated in an oven initially at 90ºC and then raised to 170ºC at 10ºC/min. A flame ionization detector set at 250ºC was used.

For ruminal histological evaluation, 10 papillae from the caudal blind sac were cut as close as possible from the basal connection and placed in biopsy cassettes (Simport, Beloeil, QC, Canada). Jejunum tissue samples (proximal, near to the ligament of Treitz) and colon tissue samples (end of the centripetal turn) were taken as previously described by Penner et al. (2014) for histological evaluation. These tissues were cut longitudinally and a 1 $cm^2$ section was placed in a biopsy cassette. Biopsy cassettes from ruminal, jejunal, and colonic samples were fixed in 10% formalin (10% formaldehyde, v/v, in neutral phosphate buffer; Ricca Chemical Company, Arlington, TX, USA) and then submitted to Prairie Diagnostic Services, where tissues were sequentially dehydrated with increasing concentration of ethanol (2 washes with 90% ethanol for 1.5 min and 3 washes with 100% ethanol for 15 sec) followed by washing with xylene (two washes with a duration of 1 and 2 min). The dehydrated tissues were then embedded in paraffin wax, cut into 4 µm sections, and mounted onto glass slides, as described by Kent-Dennis et al. (2019).

Tissues were then stained with hematoxylin and eosin. Jejunum and colon tissue measurements were attempted, but tissue sections were damaged, preventing evaluation.

A technician, blinded to treatments, evaluated five ruminal papillae from each heifer for histomorphometric analysis as described by Steele et al. (2011). This entailed measurement of each stratum at two representative spots of the papillae, totaling 10 measurements per experimental unit. Measurements were taken at 10× magnification and measured using the ImageJ software v.1.53e (National Institutes of Health, Bethesda, MD). Stratum were defined as corneum (the layer of cells located at the apical border, heavily stained and absent of nuclei), granulosum (the layer of cells with lighter staining, long axes, and with sporadic presence of nuclei and intracellular organelles), spinosum (the layer of cuboidal cells with nuclei), and basale (the layer of columnar cells adjacent to the lamina propria, abundant with of nuclei and intracellular organelles). Similar analyses were conducted for colon tissue, where 15 randomly selected crypts per heifer were evaluated at 5× magnification.

A 20-cm section from the jejunum (proximal to the ligament of Treitz) was collected, opened longitudinally using sterile forceps and scissors (conducted on a clean surface on top of an ice layer), and the mucosal layer was scraped using a glass slide. The mucosal scrapings were transferred into an empty 1.8mL cryotube vial (Fisher) and frozen at -80ºC until analysis of total protein content and brush border enzyme activity as described by Górka et al. (2011b). Sample preparation consisted of homogenizing a 1 g sample of tissue with 5 mL of distilled water followed by centrifugation for 5 min at 1,000 × *g* at 4ºC. The total protein content was determined from the supernatant according to Hartree (1972), using bovine serum albumin as a standard. Maltase and sucrase activity were determined, as described by Dahlqvist (1984).

### 4.3.7. Evaluation of SCFA flux and barrier function ex vivo

From each heifer, a sample of the rumen (caudal ventral sac), jejunum (mid-point of the jejunum), and colon (proximal region, at the end of centripetal turns) were collected as described by Penner et al. (2014) and Pederzolli et al. (2018). Tissues were washed immediately upon collection in a heated (38.5°C) and oxygenated (95% $O_2$:5% $CO_2$) buffer solution (Table 4.1). The ruminal epithelium was separated from the underlying musculature by hand stripping. Intestinal tissues were split along the mesenteric connection, washed, and transported to the laboratory in the same buffer solution as the ruminal epithelia. In the lab, the intestinal tissues were gently hand-

stripped to separate the mucosa from the submucosal tissues. Ruminal tissues were mounted in Ussing chambers with an exposed surface area of 3.14 $cm^2$ while tissues from the jejunum and ileum had an exposed surface area of 1 $cm^2$ as previously described (Penner et al., 2014).

Four Ussing chambers for each region were used to incubate the tissues to evaluate barrier function. The buffers (Table 4.1) used to incubate ruminal and colonic tissue on the mucosal side (15 mL, pH 6.2) contained SCFA, while the buffer at the serosal side (15 mL, pH 7.4) contained glucose as an energy source. For jejunal tissue, both mucosal and serosal sides were incubated with a buffer solution (15 mL, pH 7.4) containing glucose. As described by Pederzolli et al. (2018), this approach was used to mimic available energy sources on each side of each region. All epithelia were incubated under short-circuit conditions (Aschenbach and Gäbel, 2000). Argenthal reference electrodes (Mettler Toledo, Urdorf, Switzerland) were used to measure the potential difference across the epithelia via agar bridges (3% agar in 3 M KCl) with voltage measured using a computer-controlled voltage clamp device (Syscon Ingenierbüro GmbH, Kusterdingen, Germany). Based on the voltage measured, current was passed in the opposite direction such that the transepithelial potential difference was equal to 0 mV. The inverse value of the clamp current represented the short-circuit current ($I_{sc}$), which is indicative of the transcellular charge transfer across the epithelium. The tissue conductance ($G_t$) was determined every 6 sec according to Ohm's law by measuring the impulse-induced change in the transepithelial potential difference following the application of short bipolar current impulses. Tissues were provided with 20 min for stabilization of electrophysiology before being assigned to ex vivo treatments.

#### 4.3.8. Measurements of barrier function

After stabilization of electrophysiology, D-1-$^{14}$C-mannitol (74 kBq/15 mL; Perkin Elmer, Waltham, MA, USA) was added to the mucosal side to achieve a concentration of 2.5 m*M* and the mucosal-to-serosal flux rate of mannitol was used as an indicator for paracellular permeability (Penner et al., 2010, Wilkens et al., 2011). For barrier function, quadruplicate chambers for each region were used as technical replicates and the average value was used for statistical analysis. Following the addition of the isotope, 45 min was provided to allow for isotope equilibration prior

**Table 4.1.** Chemical composition of the buffers used to transport the tissue and incubate the tissues in the Ussing chambers.

| | Buffer | | | | | |
|---|---|---|---|---|---|---|
| | Transport | Barrier Function[2] | Bicarbonate Serosal[2] | Bicarbonate Mucosal[2] | Bicarbonate free, serosal[2] | Bicarbonate free, mucosal[2] |
| Chemical (m*M*) | | | | | | |
| Acetazolamide | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.1 |
| Acetic acid | 0.0 | 5.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Calcium chloride | 1.8 | 1.8 | 0.0 | 0.0 | 0.0 | 0.0 |
| Calcium gluconate | 0.0 | 0.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| D-glucose | 10.0 | 5.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| Disodium hydrogen phosphate | 0.0 | 0.0 | 2.4 | 2.4 | 2.4 | 2.4 |
| Gluconic acid | 5.0 | 5.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| HEPES - free acid | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| L-glutamine | 0.0 | 0.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Magnesium chloride | 1.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Mannitol | 0.0 | 0.0 | 106.5 | 106.5 | 127.0 | 127.0 |
| Magnesium gluconate | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 |
| Potassium chloride | 1.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Potassium gluconate | 0.0 | 0.0 | 5.5 | 5.5 | 5.5 | 5.5 |
| Potassium phosphate dibasic | 2.0 | 2.0 | 0.0 | 0.0 | 0.0 | 0.0 |

**Table 4.1 continued.** Chemical composition of the buffers used to transport the tissue and incubate the tissues in the Ussing chambers.

| | Buffer | | | | | |
|---|---|---|---|---|---|---|
| | Transport | Barrier Function[2] | Bicarbonate Serosal[2] | Bicarbonate Mucosal[2] | Bicarbonate free, serosal[2] | Bicarbonate free, mucosal[2] |
| Chemical (m*M*) | | | | | | |
| Sodium acetate | 0.0 | 19.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Sodium bicarbonate | 25.0 | 25.0 | 25.0 | 25.0 | 0.0 | 0.0 |
| Sodium butyrate | 10.0 | 4.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Sodium chloride | 92.0 | 92.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Sodium gluconate | 15.0 | 1.0 | 60.0 | 60.0 | 69.5 | 29.5 |
| Sodium hydroxide | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 5.0 |
| Sodium nitrate | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 40.0 |
| Sodium phosphate monobasic | 0.5 | 0.5 | 0.6 | 0.6 | 0.6 | 0.6 |
| Sodium propionate | 0.0 | 12.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| pH | 7.4 | 7.4 | 7.4 | 6.2 | 7.4 | 6.2 |
| Osmolality (mOsmol/kg)[1] | 292 ± 6.8 | 291 ± 7.4 | 291 ± 0.8 | 291 ± 1.9 | 291 ± 2.6 | 291 ± 0.4 |
| Temperature (ºC) | 38.5 | 38.5 | 38.5 | 38.5 | 38.5 | 38.5 |

[1] Osmolality values are expressed as means ± SE.

[2] Buffers contained 60.0 mg/L of pennicillin G, 50.0 mg/L of flurocytosine and 100 mg/L of kanamycin sulfate flurocytosine.

to measurement of mannitol flux (Sehested et al., 1999). Hot samples (100 µL; mucosal side) were collected at the beginning (0 min) and at the end (120 min) of the incubation. In addition, cold samples (500 µL; serosal side) were collected at the beginning (0 min), middle (60 min), and at the end (120 min) of the incubation. Fresh buffer was added to the chambers following the collection of the cold samples to avoid changes in volume and hydrostatic pressure. The hot and cold samples were placed in 7-mL scintillation vials (Perkin Elmer) and 400 µL of fresh buffer solution were added to the hot samples to equalize the volume among hot and cold samples. Five milliliters of scintillation cocktail (Ultima Gold, Perkin Elmer) were added to enable measurement of radioactivity using a scintillation counter (Tricarb 2910, Perkin Elmer).

### 4.3.9. Short-chain fatty acid absorption across the ruminal and colonic epithelia

For ruminal and colonic tissues, 8 chambers were used (4 chambers/tissue region) and two treatments were assigned (n = 2/treatment) within tissue type balancing for tissue $G_t$. Half of the chambers received a buffer solution (Table 4.1) that was free of SCFA but contained $HCO_3$ to determine the non-inhibited SCFA flux. The second treatment received a buffer without $HCO_3^-$ and with $NO_3^-$, to maximally inhibit SCFA transport (Penner et al., 2009a). Subsequently, a solution containing 1 *M* N-acetate that was spiked with 2-$^3$H-acetate (120 kBq $^3$H-acetate added to each chamber) and a solution containing 1 *M* Na-butyrate spiked with 1-$^{14}$C-butyrate (74 kBq $^{14}$C-butyrate added to each chamber) was added to the mucosal column to achieve final concentrations of 15 m*M* each of acetate and butyrate. Tissues were provided with 45 min for isotope equilibration and two 1-h flux periods commenced with hot (100 µL) and cold (500 µL) buffer samples collected at the start and end of each flux period, as described above. The mean flux over the 2 flux periods and duplicate tissues were used to determine the average flux rate. From these data, total SCFA flux (that measured in buffer containing $HCO_3$) and maximal inhibitable flux were calculated. By difference, protein-mediated flux was determined as described by Schurmann et al. (2014).

### 4.3.10. Statistical analysis

Data were analyzed using the MIXED procedure of SAS v9.4 (SAS Institute Inc., Cary, NC) considering the effect of treatment as fixed and the effects of block and heifer within block as

random. The Kenward-Roger correction was used to adjust the denominator degrees of freedom. Pre-planned contrasts were used to evaluate 1) the effect of AB vs. CON; 2) the effect of AB relative to CaG (i.e., AB vs. AG + RG); and 3) the pooled response to CaG (i.e., AG + RG vs. CON). For blood parameter outcomes (concentration of glucose, insulin and BHBA) that were measured at multiple time points, the fixed effects of time and the treatment × time interaction were included in the model. The variation within animal was modelled with heterogeneous compound symmetric covariance structure selected based on achieving the lowest Akaike information criterion. For fluxes of mannitol, acetate, and butyrate by region, the fixed of region and treatment × region were included in the model, and the contrasts were tested as mentioned before.

Original data and their residuals were tested for normality using both Shapiro-Wilk testing and visual assessment of the data distribution. Only rumen acetate proportion residuals were not normally distributed, and the dataset was transformed using $x^3$ to achieve a normal distribution. The standard error of the difference (**SED**) and *P*-values were reported based on the analysis of the transformed data and means reported are based on the original data. In all cases, significance was declared when $P < 0.05$ and tendencies were declared when $0.05 \leq P < 0.10$.

## 4.4. Results

Infusion with either of AB or CaG (AG and RG) did not affect ($P \geq 0.87$) initial BW, final BW ($P \geq 0.21$), or DMI ($P \geq 0.49$); expressed both in kg/day and % of BW; Table 4.2) compared to CON. Ruminal digesta pH and total SCFA concentration were not affected by either AB or CaG infusion ($P \geq 0.12$; Table 4.3) compared to CON. On the other hand, the molar proportion of isobutyrate was less when heifers were infused with CaG compared to CON (1.60 *vs.* 1.34%; $P =$ 0.04). Jejunum digesta pH was not affected by any treatment ($P \geq 0.45$). For colon digesta, the infusion with CaG tended ($P = 0.05$) to increase the digesta pH compared to AG. In addition, infusion with CaG tended to decrease the SCFA concentration compared to CON (53.6 *vs*. 45.2 m*M*; $P = 0.09$). The infusion of both AB ($P = 0.04$) and CaG (76.17 *vs.* 73.98%; $P < 0.05$) decreased the proportion of acetate in the colon digesta compared to CON. Infusion with both AB ($P > 0.05$) and CaG (16.57 *vs.* 17.55%; $P = 0.02$) increased the proportion of propionate in the colon digesta. Moreover, infusion with AB increased the colonic valerate proportion relative to CON ($P = 0.04$).

The apparent total tract digestibility was not affected by neither AB nor CaG infusion ($P \geq 0.14$; Table 4.4). Treatments did not affect the urinary chromium excretion ($P \geq 0.59$; Table 4.5). Plasma glucose concentration tended to be less for heifers infused with AB compared to CON ($P = 0.08$) and was less for CaG (58.0 *vs*. 44.7 mg/dL; $P > 0.01$) when compared to CON (Table 4.5). Plasma insulin concentration was only affected by time ($P > 0.01$), where concentrations measured 6 h after treatment administration (time 6) were greater than at time 0 (before the treatment administration). In addition, plasma BHBA for heifers infused with CaG were less when compared to heifers infused with AB (1.197 *vs*. 1.063 mmol; $P = 0.04$) and tended to be lesser when compared to CON (1.172 *vs*. 1.063 mmol; $P = 0.09$).

Abomasal infusion with AB increased ruminal disappearance of total SCFA ($P = 0.01$; Table 4.6), acetate ($P = 0.03$), propionate ($P = 0.01$), and butyrate ($P > 0.01$) when reported as mmol/h, compared to CON. The AB also increased total SCFA ($P = 0.03$), propionate ($P = 0.02$), and butyrate ($P = 0.01$) disappearance in %/h, and tended to increase the acetate disappearance in %/h ($P = 0.05$) when compared to CON. In addition, heifers infused with AB had greater total SCFA (677.7 *vs*. 548.8 mmol/h; $P = 0.04$), propionate (226.8 *vs*. 180.3 mmol/h; $P = 0.04$), and butyrate (101.0 *vs*. 82.7 mmol/h; $P = 0.04$) disappearance rates and tended to have greater acetate disappearance (349.9 *vs*. 272.3 mmol/h; $P = 0.06$) compared to heifers infused with CaG. Total SCFA (37.9 *vs*. 29.9%/h; $P = 0.03$), acetate (34.5 *vs*. 26.7%/h; $P = 0.04$), propionate (41.3 *vs*. 33.1%/h; $P = 0.02$), and butyrate (44.7 *vs*. 37.0%/h; $P = 0.04$) disappearance (%/h) were also greater for heifers infused with AB compared to CaG. The SCFA disappearance rates were not affected by the infusion of CaG when compared to CON ($P \geq 0.18$). There was no effect of CaG relative to CON for SCFA disappearance rates. The rumen strata histological measurements were not affected by treatment ($P \geq 0.45$; Table 4.7), nor were jejunal epithelial protein concentration and maltase brush-border enzyme activity ($P \geq 0.32$). There was no sucrase brush-border enzyme activity detected.

The ex vivo acetate and butyrate fluxes across ruminal and colonic epithelia were not affected by treatment ($P > 0.10$) regardless of whether incubated with bicarbonate-containing or buffers excluding bicarbonate (Table 4.8). Both acetate and butyrate fluxes (without inhibition and $HCO_3$-independent) were affected by region ($P < 0.01$), with greater flux rates across the ruminal epithelia than the colonic epithelia (data not shown). Mannitol flux across the isolated ruminal epithelia tended to be lower ($P = 0.08$) for AB infusion when compared to CON, and jejunal $I_{sc}$

**Table 4.2.** Body weight and dry matter intake for heifers (n = 32) receiving negative control (CON), abomasal Ca butyrate (AB; 0.0029% of BW), abomasal Ca gluconate (AG; 0.0077% of BW), or ruminal hydrogenated fat-embedded Ca gluconate (RG; 0.0192% of BW) designed to deliver equal post-ruminal supply of butyrate.

| | Treatment | | | | | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| Item | CON | AB | AG | RG | SED[2] | CON *vs*. AB | CON *vs*. CaG | AB *vs*. CaG |
| Initial body weight, kg | 388.8 | 387.8 | 387.0 | 389.6 | 6.45 | 0.87 | 0.93 | 0.93 |
| Final body weight, kg | 415.3 | 410.5 | 406.4 | 402.8 | 13.69 | 0.62 | 0.21 | 0.49 |
| Dry matter intake, kg/d | 7.82 | 7.84 | 7.70 | 7.32 | 0.559 | 0.96 | 0.54 | 0.50 |
| Dry matter intake, %BW | 1.92 | 1.95 | 1.91 | 1.82 | 0.128 | 0.80 | 0.68 | 0.49 |

[1]CON *vs*. AB: contrast between control and CaB; AB *vs*. CaG: contrast between CaB and CaG (AG + RG); CON *vs*. CaG: contrast between control and CaG (AG + RG).
[2]SED: Standard error of the difference.

**Table 4.3.** Rumen and colon short-chain fatty acids (SCFA) and jejunum digesta pH for heifers (n = 32) receiving negative control (CON), abomasal Ca butyrate (AB; 0.0029% of BW), abomasal Ca gluconate (AG; 0.0077% of BW), or ruminal hydrogenated fat-embedded Ca gluconate (RG; 0.0192% of BW) designed to deliver equal post-ruminal supply of butyrate.

| | Treatment | | | | SED[2] | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| Item | CON | AB | AG | RG | | CON *vs*. AB | CON *vs*. CaG | AB *vs*. CaG |
| *Rumen* | | | | | | | | |
| Digesta pH | 7.41 | 7.26 | 7.35 | 7.27 | 0.089 | 0.12 | 0.22 | 0.55 |
| Total SCFA, m*M* | 47.1 | 52.4 | 46.5 | 49.7 | 5.92 | 0.38 | 0.84 | 0.42 |
| Acetate, % | 59.46 | 61.51 | 64.33 | 61.52 | 2.805 | 0.43 | 0.16 | 0.61 |
| Propionate, % | 24.06 | 23.96 | 20.34 | 24.99 | 3.016 | 0.97 | 0.60 | 0.63 |
| Isobutyrate, % | 1.60 | 1.45 | 1.45 | 1.22 | 0.141 | 0.28 | 0.04 | 0.36 |
| Butyrate, % | 10.52 | 8.73 | 9.87 | 8.64 | 1.180 | 0.14 | 0.23 | 0.61 |
| Isovalerate, % | 2.30 | 2.50 | 2.04 | 2.55 | 0.497 | 0.66 | 1.00 | 0.63 |
| Valerate, % | 1.53 | 1.46 | 1.29 | 1.38 | 0.279 | 0.80 | 0.42 | 0.60 |
| *Jejunum* | | | | | | | | |
| Digesta pH | 7.46 | 7.50 | 7.53 | 7.57 | 0.143 | 0.77 | 0.45 | 0.70 |
| *Colon* | | | | | | | | |
| Digesta pH | 6.96 | 6.80 | 7.01 | 6.97 | 0.102 | 0.14 | 0.75 | 0.05 |
| Total SCFA, m*M* | 53.6 | 53.0 | 44.9 | 45.4 | 5.70 | 0.92 | 0.09 | 0.12 |
| Acetate, % | 76.17 | 73.54 | 72.96 | 75.00 | 1.127 | 0.04 | 0.05 | 0.65 |
| Propionate, % | 16.57 | 17.79 | 18.16 | 17.55 | 0.571 | 0.05 | 0.02 | 0.90 |
| Isobutyrate, % | 0.95 | 1.06 | 1.13 | 1.14 | 0.170 | 0.52 | 0.24 | 0.62 |
| Butyrate, % | 5.04 | 5.47 | 5.21 | 4.85 | 0.376 | 0.27 | 0.96 | 0.18 |
| Isovalerate, % | 0.82 | 0.94 | 1.11 | 0.73 | 0.237 | 0.62 | 0.63 | 0.93 |
| Valerate, % | 1.03 | 1.20 | 1.19 | 1.07 | 0.080 | 0.04 | 0.15 | 0.28 |

[1]CON *vs*. AB: contrast between control and CaB; AB *vs*. CaG: contrast between CaB and CaG (AG + RG); CON *vs*. CaG: contrast between control and CaG (AG + RG).
[2]SED: Standard error of the difference.

**Table 4.4.** Total tract digestibility coefficient for heifers (n = 28) receiving negative control (CON), abomasal Ca butyrate (AB; 0.0029% of BW), abomasal Ca gluconate (AG; 0.0077% of BW), or ruminal hydrogenated fat-embedded Ca gluconate (RG; 0.0192% of BW) designed to deliver equal post-ruminal supply of butyrate.

| | Treatment | | | | SED[2] | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| Item | CON | AB | AG | RG | | CON *vs*. AB | CON *vs*. CaG | AB *vs*. CaG |
| *Digestibility, %*[3] | | | | | | | | |
| DM | 70.64 | 69.47 | 71.05 | 70.33 | 1.630 | 0.48 | 0.97 | 0.40 |
| CP | 72.10 | 70.48 | 70.92 | 70.96 | 2.161 | 0.46 | 0.54 | 0.80 |
| NDF | 44.32 | 41.88 | 45.37 | 46.73 | 3.136 | 0.45 | 0.53 | 0.14 |
| ADF | 38.00 | 35.41 | 37.13 | 40.72 | 3.727 | 0.50 | 0.78 | 0.29 |
| Starch | 98.80 | 98.63 | 98.89 | 98.44 | 0.384 | 0.65 | 0.69 | 0.91 |
| Ether extract | 58.35 | 57.14 | 63.59 | 44.16 | 6.689 | 0.86 | 0.45 | 0.58 |

[1]CON *vs*. AB: contrast between control and CaB; AB *vs*. CaG: contrast between CaB and CaG (AG + RG); CON *vs*. CaG: contrast between control and CaG (AG + RG).
[2]SED: Standard error of the difference.
[3]DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber.

**Table 4.5.** Urinary chromium-EDTA excretion as a measurement of in vivo total tract barrier function, and plasma glucose and insulin, and serum BHBA concentrations for heifers (n = 32) receiving negative control (CON), abomasal Ca butyrate (AB; 0.0029% of BW), abomasal Ca gluconate (AG; 0.0077% of BW), or ruminal hydrogenated fat-embedded Ca gluconate (RG; 0.0192% of BW) designed to deliver equal post-ruminal supply of butyrate.

| | Treatment | | | | Time[1] | | SED[3] | *P*-values[2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CON | AB | AG | RG | time 0 | time 6 | | Time | CON *vs.* AB | CON *vs.* CaG | AB *vs.* CaG |
| Urinary Cr, mg/48 h | 216.92 | 203.51 | 216.09 | 212.56 | - | - | 24.403 | - | 0.59 | 0.90 | 0.61 |
| *Blood parameters* | | | | | | | | | | | |
| Glucose, mg/dL | 58.0 | 49.2 | 49.0 | 40.3 | 49.0 | 49.3 | 4.82 | 0.925 | 0.08 | <0.01 | 0.29 |
| Insulin, μg/L | 0.625 | 0.665 | 0.495 | 0.547 | 0.415 | 0.751 | 0.1893 | 0.003 | 0.83 | 0.53 | 0.39 |
| BHBA, mmol | 1.172 | 1.197 | 1.121 | 1.005 | 1.093 | 1.155 | 0.0710 | 0.185 | 0.73 | 0.09 | 0.04 |

[1]Time 0: before feeding; Time 6: 6 hours after feeding.
[2]CON *vs*. AB: contrast between control and CaB; AB *vs.* CaG: contrast between CaB and CaG (AG + RG); CON *vs*. CaG: contrast between control and CaG (AG + RG).
[3]SED: Standard error of the difference.

tended to differ (0.51 *vs.* -0.33 μEq/[cm$^2$ × h]; $P = 0.09$) between AB and CaG infusions (Table 4.9). Treatments did not affect the mannitol flux $G_t$, or $I_{sc}$ for colon tissues used to evaluate barrier function ($P \geq 0.25$). Additionally, it was observed that mannitol flux ($P < 0.01$), $G_t$, ($P < 0.01$) and $I_{sc}$ ($P > 0.01$) were affected by region. Mannitol flux were greater across the jejunum (63.18 nmol/[cm$^2$ × h]) relative to the rumen (22.72 nmol/[cm$^2$ × h]) and colon (34.83 nmol/[cm$^2$ × h]), which did not differ from each other. $G_t$ did not differ between jejunum (24.08 mS/cm$^2$) and colon (19.84 mS/cm$^2$) but were greater relative to rumen (10.04 mS/cm$^2$). Finally, colon had greater $I_{sc}$ (1.04 μEq/[ cm$^2$ × h]) relative to rumen (0.31 μEq/[ cm$^2$ × h]) and jejunum (-0.04 μEq/[ cm$^2$ × h]), which did not differ from each other.

## 4.5. Discussion

Increasing the intestinal supply of butyrate for ruminant species has been reported to elicit responses across the entire gastrointestinal tract. For example, provision of sodium butyrate in milk replacer for dairy calves increased BW, ADG (Guilloteau et al., 2009; Górka et al., 2011b), and feed efficiency (Guilloteau et al., 2009), enhanced absorptive surface area in the rumen (papillae length; Mentschel et al., 2001; Górka et al., 2011b), stimulated small intestine development (increased mitotic index in the jejunum; Mentschel et al., 2001; Guilloteau et al., 2009; Górka et al., 2014), and increased total SCFA, acetate, and propionate concentrations in the colon (O'Hara et al., 2018). While provision of butyrate is possible, I evaluated the use of CaG as a prebiotic in an effort to promote postbiotic supply of butyrate to the intestine. The use of CaG as a butyrate precursor was based on the conversion of gluconic acid to butyrate in a closed in vitro fermentation system conducted by Tsukahara et al. (2002). While this assumption may be valid for the AG, it was assumed that 100% of the HFCG would bypass the rumen. More recent data obtained using in situ incubations has suggested that 13.4 ± 2.89% of the HFCG may be degraded in the rumen, implying that the supply of CaG to the small intestine for the HFCG treatment may have been overestimated. Nevertheless, it was hypothesized that provision of both CaB and CaG, whether infused into the abomasum (AG) or into the rumen with a ruminally protected form (RG), would elicit changes in colonic SCFA profile, nutrient absorption, intestinal barrier function, blood metabolites, and GIT function and development in a manner not different to CaB.

**Table 4.6.** Disappearance rates of short-chain fatty acids (SCFA) determined using the temporarily isolated and washed reticulo-rumen technique for heifers (n = 32) receiving negative control (CON), abomasal Ca butyrate (AB; 0.0029% of BW), abomasal Ca gluconate (AG; 0.0077% of BW), or ruminal hydrogenated fat-embedded Ca gluconate (RG; 0.0192% of BW) designed to deliver equal post-ruminal supply of butyrate.

| | Treatment | | | | SED[2] | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| Item | CON | AB | AG | RG | | CON *vs*. AB | CON *vs*. CaG | AB *vs*. CaG |
| *SCFA disappearance, mmol/h* | | | | | | | | |
| Total | 486.0 | 677.7 | 514.0 | 556.6 | 78.24 | 0.01 | 0.44 | 0.04 |
| Acetate | 250.0 | 349.9 | 252.4 | 292.3 | 46.71 | 0.03 | 0.56 | 0.06 |
| Propionate | 164.1 | 226.8 | 178.3 | 182.2 | 24.44 | 0.01 | 0.42 | 0.04 |
| Butyrate | 71.8 | 101.0 | 83.3 | 82.2 | 9.89 | <0.01 | 0.18 | 0.04 |
| *SCFA disappearance, %/h* | | | | | | | | |
| Total | 29.2 | 37.9 | 29.2 | 30.6 | 4.01 | 0.03 | 0.83 | 0.03 |
| Acetate | 26.4 | 34.5 | 25.2 | 28.1 | 4.07 | 0.05 | 0.95 | 0.04 |
| Propionate | 32.2 | 41.3 | 33.2 | 32.9 | 4.09 | 0.02 | 0.80 | 0.02 |
| Butyrate | 34.3 | 44.7 | 37.7 | 36.3 | 4.08 | 0.01 | 0.43 | 0.04 |

[1]CON *vs*. AB: contrast between control and CaB; AB *vs*. CaG: contrast between CaB and CaG (AG + RG); CON *vs*. CaG: contrast between control and CaG (AG + RG).

[2]SED: Standard error of the difference.

**Table 4.7.** Rumen strata histological measurements and jejunum brush-border enzyme activity for heifers (n = 32) receiving negative control (CON), abomasal Ca butyrate (AB; 0.0029% of BW), abomasal Ca gluconate (AG; 0.0077% of BW), or ruminal hydrogenated fat-embedded Ca gluconate (RG; 0.0192% of BW) designed to deliver equal post-ruminal supply of butyrate.

| Item | Treatment | | | | SED[2] | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|
| | CON | AB | AG | RG | | CON *vs*. AB | CON *vs*. CaG | AB *vs*. CaG |
| *Rumen Strata, µm* | | | | | | | | |
| Corneum | 27.29 | 26.47 | 27.88 | 24.98 | 3.081 | 0.79 | 0.75 | 0.99 |
| Granulosum | 11.73 | 11.27 | 11.32 | 11.35 | 0.901 | 0.61 | 0.61 | 0.94 |
| Spinosum | 39.17 | 37.96 | 34.46 | 38.98 | 3.975 | 0.76 | 0.48 | 0.72 |
| Basale | 70.30 | 66.13 | 63.00 | 69.73 | 5.858 | 0.48 | 0.45 | 0.96 |
| *Jejunum brush-border enzyme activity* | | | | | | | | |
| Protein, mg/g of mucosa | 48.3 | 48.8 | 43.2 | 40.6 | 7.86 | 0.94 | 0.36 | 0.32 |
| Maltase, U/mg of protein | 32.5 | 39.9 | 32.5 | 39.0 | 9.59 | 0.42 | 0.69 | 0.60 |
| Sucrase, U/mg of protein | ND[3] | ND | ND | ND | - | - | - | - |

[1]CON *vs*. AB: contrast between control and CaB; AB *vs*. CaG: contrast between CaB and CaG (AG + RG); CON *vs*. CaG: contrast between control and CaG (AG + RG).
[2]SED: Standard error of the difference.
[3]ND: Not detectable.

**Table 4.8.** Mucosal-to-serosal flux of acetate and butyrate across the isolated ruminal and colonic epithelia in Ussing chambers for heifers (n = 32) receiving negative control (CON), abomasal Ca butyrate (AB; 0.0029% of BW), abomasal Ca gluconate (AG; 0.0077% of BW), or ruminal hydrogenated fat-embedded Ca gluconate (RG; 0.0192% of BW) designed to deliver equal post-ruminal supply of butyrate.

| Item | Treatment | | | | SED[2] | *P*-value[1] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CON | AB | AG | RG | | CON *vs.* AB | CON *vs.* CaG | AB *vs.* CaG | Region |
| *Total flux* | | | | | | | | | |
| Acetate flux, μmol/(cm$^2$ × h) | | | | | | | | | |
| Rumen | 1.17 | 1.48 | 1.24 | 1.38 | 0.197 | 0.10 | 0.38 | 0.32 | <0.01 |
| Colon | 0.35 | 0.33 | 0.39 | 0.39 | 0.088 | 0.88 | 0.58 | 0.47 | |
| Butyrate flux, μmol/(cm$^2$ × h) | | | | | | | | | |
| Rumen | 1.09 | 1.10 | 1.08 | 1.09 | 0.204 | 0.96 | 0.97 | 0.92 | <0.01 |
| Colon | 0.58 | 0.56 | 0.62 | 0.57 | 0.121 | 0.90 | 0.85 | 0.74 | |
| *Flux with maximal inhibition* | | | | | | | | | |
| Acetate flux, μmol/(cm$^2$ × h) | | | | | | | | | |
| Rumen | 0.65 | 0.78 | 0.67 | 0.92 | 0.158 | 0.44 | 0.32 | 0.92 | <0.01 |
| Colon | 0.28 | 0.25 | 0.19 | 0.29 | 0.067 | 0.72 | 0.57 | 0.88 | |
| Butyrate flux, μmol/(cm$^2$ × h) | | | | | | | | | |
| Rumen | 0.93 | 0.93 | 0.81 | 0.98 | 0.153 | 1.00 | 0.80 | 0.80 | <0.01 |
| Colon | 0.58 | 0.48 | 0.47 | 0.55 | 0.112 | 0.36 | 0.47 | 0.74 | |

[1]CON *vs*. AB: contrast between control and CaB; AB *vs*. CaG: contrast between CaB and CaG (AG + RG); CON *vs*. CaG: contrast between control and CaG (AG + RG); Region: contrast among regions.
[2]SED: Standard error of the difference.

**Table 4.9.** Ex vivo mannitol flux used as an indicator of barrier function, and tissue conductance ($G_t$), and short-circuit current ($I_{sc}$) across ruminal, jejunal, and colonic epithelia using the Ussing chamber technique for heifers (n = 32) receiving negative control (CON), abomasal Ca butyrate (AB; 0.0029% of BW), abomasal Ca gluconate (AG; 0.0077% of BW), or ruminal hydrogenated fat-embedded Ca gluconate (RG; 0.0192% of BW) designed to deliver equal post-ruminal supply of butyrate.

| | Treatment | | | | SED[2] | *P*-value[1] | | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | CON | AB | AG | RG | | CON *vs.* AB | CON *vs.* CaG | AB *vs.* CaG | Region |
| *Mannitol flux, nmol/($cm^2$ × h)* | | | | | | | | | |
| Rumen | 30.25[a] | 18.40 | 19.76 | 22.48 | 6.522 | 0.08 | 0.12 | 0.63 | |
| Jejunum | 74.31[b] | 61.89 | 53.61 | 62.91 | 14.327 | 0.40 | 0.21 | 0.77 | <0.01 |
| Colon | 33.11[a] | 28.91 | 44.45 | 32.86 | 9.538 | 0.66 | 0.51 | 0.25 | |
| *$G_t$[3], mS/$cm^2$* | | | | | | | | | |
| Rumen | 10.83[b] | 8.45 | 10.85 | 10.03 | 2.294 | 0.31 | 0.85 | 0.33 | |
| Jejunum | 19.11[a] | 24.64 | 26.01 | 26.16 | 6.097 | 0.36 | 0.17 | 0.79 | <0.01 |
| Colon | 17.93[a] | 18.99 | 19.31 | 23.12 | 4.247 | 0.79 | 0.39 | 0.52 | |
| *$I_{sc}$[4], μEq/($cm^2$ × h)* | | | | | | | | | |
| Rumen | 0.29[b] | 0.37 | 0.29 | 0.28 | 0.125 | 0.57 | 0.94 | 0.47 | |
| Jejunum | -0.15[b] | 0.51 | -0.17 | -0.48 | 0.532 | 0.21 | 0.69 | 0.09 | 0.02 |
| Colon | 0.54[a] | 1.23 | 1.29 | 1.17 | 0.828 | 0.37 | 0.33 | 0.99 | |

[1]SED: Standard error of the difference.
[2]CON vs. AbCaB: contrast between control and CaB; AbCaB vs. CaG: contrast between CaB and CaG (AbCaG + RHFCaG); CON vs. CaG: contrast between control and CaG (AbCaG + RHFCaG); Region: contrast among regions.
[3]$G_t$: tissue conductance.
[4]$I_{sc}$: short-circuit current.
[a,b]Within the parameters analyzed (mannitol flux, $G_t$, and $I_{sc}$), regions with different superscripts in a column differ ($P < 0.05$). Letters are only shown for the CON treatment to represent the overall region effect.

Although no treatments affected ruminal papillae strata dimensions, provision of AB stimulated in vivo ruminal SCFA disappearance with rates that were nearly 30%/h greater than CON and more than 20% greater than CaG. Moreover, AB tended to decrease circulating glucose concentrations relative to CON. However, increases in SCFA flux rates were not detected under ex vivo conditions. Differences in the response between in vivo absorption and *ex vivo* flux may be related to the effect of butyrate on stimulation of blood flow (Storm et al., 2011), as blood flow and removal of metabolites are not replicated ex vivo in Ussing chambers. While not measured in the present study, SCFA have been reported to be a driving factor increasing vasodilation to the gastrointestinal tract. In cattle, butyrate is the most effective SCFA (within physiological concentrations) to stimulate increased blood flow to the ruminal epithelia (Bergman, 1990). Nevertheless, greater rates of SCFA absorption observed in vivo in response to AB may help to stabilize ruminal pH and deliver more energy substrates for maintenance and production outcomes (Aschenbach et al., 2011; Penner et al., 2011). That said, differences in ruminal pH, nor major changes in ruminal SCFA concentrations were detected in the present study; however, measurements for ruminal pH were based on a single spot sample collected prior to feeding. Hence, future research is required to evaluate the role of abomasal butyrate supply on ruminal pH.

Paired with greater rates of SCFA absorption in vivo, the effect of butyrate is thought to be mediated through luminal sensing mechanisms (Baldassano and Amato, 2014) resulting in the secretion of glucagon-like peptide-2 (**GLP-2**) in ruminants (Górka et al., 2011a; Elsabagh et al., 2017; Hatew et al., 2019) and non-ruminants (Tappenden and McBurney, 1998; Yadav et al., 2013). In this regard, increasing the post-ruminal supply of butyrate through AB could be expected to stimulate glucagon-immunoreactive cells in the duodenum (Alumets et al., 1983) and the secretion of GLP-2 by the L-cells (Ørskov and Holst, 1987; Brubaker et al., 1997; Hartmann et al., 2000). Reported increases in barrier function have been observed in mice (Benjamin et al., 2000), lactating cows (Kvidera et al., 2017a), and dairy calves (Taylor-Edwards et al., 2011; Walker et al., 2015) administered GLP-2. Butyrate has been reported to stimulate improved barrier function of intestinal epithelia when measured in vitro (Peng et al., 2009; Zheng et al., 2017) and similar responses are expected for ruminants (Plöger et al., 2012; Zhang et al., 2018). In the present study, it was observed that AB tended to decrease mannitol flux across the ruminal epithelium, which is indicative of improved barrier function. However, the lack of response in other regions of the GIT,

lack of effect for epithelial tissue conductance, and lack of differences in Cr-EDTA excretion in urine, suggest that the effect is small at best under the conditions of the present study.

Although it was hypothesized that AB and CaG would yield similar responses, there is limited data to support this hypothesis. Both AB and CaG altered the colonic acetate and propionate concentrations and plasma glucose concentration relative to CON, but only AB increased in vivo ruminal SCFA absorption and tended to increase the ex vivo ruminal barrier function. Moreover, there was a greater effect to increase in vivo SCFA absorption for AB relative to CaG. As mentioned previously, my hypothesis was based on the conversion of CaG to butyrate in an in vitro study conducted by Tsukahara et al. (2002) using porcine cecal digesta. However, these authors observed not only an increase in butyrate concentration, but also acetate and total SCFA. Although such effects can be detected in vitro, in vivo studies using treatments that deliver CaG post-ruminally have reported inconsistent responses for indicators of small and large intestinal fermentation. Doelman et al. (2019) reported an increase in fecal isobutyrate in dairy cows with post-ruminal infusion of CaG, while Seymour et al. (2021) reported a decrease in fecal isobutyrate from dairy cows supplemented with HFCG in the TMR. Watanabe et al. (2022) reported a linear increase in the molar proportion of acetate and a linear decrease in propionate in cecal digesta of growing lambs supplemented with increased doses of HFCG in the TMR; however, none of the previously mentioned experiments have reported increased butyrate molar proportion. Thus, future in vitro studies with colon and cecal digesta from cattle should be conducted to test whether CaG yields greater production of butyrate.

Differences in the results from AB and CaG could be related to the site of exposure for butyrate on the GIT and the instantaneous supply. Very little SCFA pass out of the rumen with the liquid outflow to reach the duodenum, indicating a high capacity for SCFA absorption by the omasal, and potentially abomasal, epithelia (Masson and Phillipson, 1952). While absorption of SCFA across the abomasum has not been investigated in the ruminant stomach, there is evidence of butyrate absorption in the human stomach (Saunders, 1991). The available evidence suggests that the low pH of abomasal digesta would result in SCFA that are in an undissociated state, thereby increasing the ability of butyrate and other SCFA to passively diffuse across the epithelia (Gäbel et al., 2002). As such, there may be site of exposure differences among my treatments. Abomasal and proximal small intestine release and absorption of AB resembles that expected with supplementation of sodium butyrate in milk replacer for dairy calves (Guilloteau et al., 2009;

Górka et al., 2014). In contrast, CaG must be fermented to butyrate, leading to a high probability for more distal fermentation and absorption given limited microbial density in the proximal regions of the small intestine (Plaizier et al., 2020). For HFCG, intestinal digestion of the hydrogenated fat matrix may further shift the CaG supply distally. With all treatments, a single pulse dose was provided daily; however, provision of HFCG in the rumen could be expected to lead to a more sustained flow of CaG out of the rumen and hence less potential for pulsatile provision of butyrate to the intestine.

In the present study, the SCFA absorption was measured in vivo and ex vivo; while treatment responses differed between the two methodologies, the SCFA disappearance and flux data agrees with numerous past reports. Firstly, the fractional rates of SCFA absorption increased with increasing chain length when incubated in vivo using molar proportions of acetate, propionate, and butyrate reflective of that observed under typical conditions and when incubated ex vivo using equimolar proportions of SCFA (Danielli et al., 1945; Gray, 1947; Aschenbach et al., 2011). Secondly, the ex vivo flux data confirm that a greater proportion of the acetate flux, relative to butyrate flux, occurs via protein-mediated flux given the lesser inhibitable portion of the flux (Aschenbach et al., 2009; Penner et al., 2009b; Schurmann et al., 2014). These data support that acetate flux is mediated largely by SCFA/bicarbonate anion exchange with greater potential for butyrate flux via passive diffusion, or at least mechanisms that were not inhibited in the present study.

There is very limited data available on SCFA flux across intestinal tissues in ruminants and there are no studies comparing ruminal and colonic SCFA absorption. That said, studies published more than 30 years ago have reported a positive relationship between SCFA absorption and colonic pH in sheep (Rübsamen and von Engelhardt, 1981; Bugaut, 1987). My data shows that ruminal fluxes of both acetate and butyrate were greater across the ruminal epithelia compared to colonic epithelia. Relative to the single layer epithelium present in the colon, the ruminal epithelium appears to be specialized for SCFA absorption, particularly when considering that the stratified squamous epithelial structure increases the diffusional distance between the mucosal and serosal sides. While flux differed, it appears that broad categorical pathways of transport across the ruminal and colonic epithelia are similar with greater inhibitory potential for acetate relative to butyrate.

Similarly, regional effects for mannitol flux support previous studies conducted by Penner et al. (2014) and Pederzolli et al. (2018). The greater flux of mannitol across the jejunum, when compared to the fluxes observed for ruminal and colonic tissue, helps to support the hypothesis that the small intestinal regions have greater permeability, and hence lesser barrier function, than ruminal and large intestinal regions. However, Ussing chamber approaches can only assess direct effects of tissues as they exclude microbial and other protective effects such as mucus on barrier function. As such, future studies are needed to test regional permeability responses in vivo. That said, the mannitol flux data from the present study support the general trend where regions with greater microbial density have improved barrier function (Plaizier et al., 2020).

### 4.6. Conclusion

The gastrointestinal tracts of growing beef heifers displayed differential responses to the infusions of CaB and CaG. However, both AB and CaG altered the molar proportion of SCFA in the colon. Furthermore, AB stimulated SCFA absorption across the reticulo-rumen in vivo, highlighting that the effects of butyrate can extend beyond the tissue of exposure. Future studies are needed to confirm the conversion rate of CaG to butyrate, the site of release of HFCG, and the effect of provision of butyrate in different sites of the intestine (proximal *vs.* distal).

# 5. EFFECT OF FEEDING CALCIUM GLUCONATE EMBEDDED IN A HYDROGENATED FAT MATRIX ON PERFORMANCE, GASTROINTESTINAL FERMENTATION, RUMINAL MORPHOLOGY, CARCASS MERIT AND MEAT QUALITY OF BEEF HEIFERS

The experiment described on Chapter 4 attempted to compare if post-ruminal provision of CaG (both by abomasal infusion of by the feeding of a rumen protected form; HFCG) would yield similar results to the post-ruminal provision of butyrate. It seems that only partial responses in the large intestine were similar, with the most important finding being an increase of SCFA disappearance in the rumen as a result of post-ruminal infusion of CaB. This response was not observed for the post-ruminal provision of CaG. However, in the experiments described in both Chapter 3 and 4, the post-ruminal provision of CaG was associated with ruminants fed with diets containing moderate amounts of forage. Changes in the forage-to-concentrate ratio of the diet alters the total production and the molar concentration of butyrate in the GIT, and as a result it is reasonable to verify the effects of supplying HFCG in low forage diets.

## 5.1. Abstract

Twenty-one beef heifers were used in a randomized complete block design for 206 ± 8 d (mean ± SD) to evaluate the dose response of hydrogenated fat-embedded calcium gluconate (**HFCG)** when included at 0.0% (**CON**), 0.09% (**CG09**), and 0.18% (**CG18**) of diet DM on feed intake, gastrointestinal fermentation, carcass merit, and meat quality characteristics of finishing beef heifers. Heifers were fed individually with weekly feed intake recorded. Fecal samples were collected on d 171 and 192, and gastrointestinal tissue, digesta, carcass merit, and meat quality and sensory characteristics were assessed after slaughter. Data were analyzed to determine the treatment and linear effect of HFCG dose. Feed intake, ADG, G:F and carcass characteristics were not affected ($P \geq 0.15$) by HFCG. Gastrointestinal fermentation was minimally affected where CG09 decreased ($P = 0.03$) jejunal digesta pH and increased ($P = 0.02$) the proportion of isovalerate in the colon relative to CG18. Carcass ($P \geq 0.46$), meat quality ($P \geq 0.15$), and sensory characteristics ($P \geq 0.15$) were not affected by increasing doses of HFCG. Based on these results, inclusion of HFCGin high-grain diet fed to beef heifers did not affect either feed intake, growth, gastrointestinal fermentation parameters, carcass merit, meat quality or meat palatability.

## 5.2. Introduction

Butyrate has been identified as a signaling molecule that regulates cell growth and proliferation of the gastrointestinal tract (Li and Elsasser, 2005). Dietary supplementation of butyrate has been reported to increase the absorptive surface area of the ruminal epithelia in sheep (Sakata and Tamate, 1976; Sakata and Tamate, 1978) and calves (Mentschel et al., 2001) by stimulating the mitotic index and decreasing apoptosis. Increased epithelial proliferation has also been observed in the small intestine of dairy calves fed milk replacer or starter mixture containing added butyrate (Górka et al., 2014). Butyrate affects the assembly of tight junction proteins (Plöger et al., 2012) increasing the gastrointestinal barrier function when measured in vivo (in goats; Zhang et al., 2018) and in vitro (Peng et al., 2009; Wang et al., 2012). In line with its effects on barrier function, butyrate reduces inflammation of the gastrointestinal epithelia exhibiting inflammatory bowel disease (Serpe et al., 2010) or ulcerative colitis (Vieira et al., 2012).

Despite potential benefits of dietary butyrate supplementation in animal production, including growing cattle, its volatile and odorous characteristics have promoted investigation into butyrate salts and encapsulated forms (Guilloteau et al., 2010). Alternatively, providing butyrate fermentation precursors as prebiotics, such as gluconic acid, may provide another strategy to increase butyrate in the intestine, while minimizing the production of lactate (Tsukahara et al., 2002). Supplementation of unprotected sodium gluconate improved average daily gain (**ADG**) and increased the concentration of short-chain fatty acids (**SCFA**), including butyrate, in the intestine of pigs (Biagi et al., 2006; Poeikhampha and Bunchasak, 2011), suggesting it may be an effective precursor for butyrate.

Post-ruminal infusions of Ca-gluconate (**CaG**) in lactating dairy cows has been reported to increase milk fat yield (Doelman et al., 2019a; McKnight et al., 2019) but unprotected dietary CaG led to a reduction in milk yield as well as milk protein and lactose (McKnight et al., 2019). Given positive responses to post-ruminal but not ruminal CaG, several authors have evaluated the supplementation of rumen-protected CaG (CaG embedded in a hydrogenated fat matrix; **HFCG**) for dairy cows (Seymour et al., 2021; Seymour et al., 2022; Seymour et al., 2023) and sheep (Watanabe et al., 2022). Provision of HFCG increased milk fat yield (Seymour et al. 2023) and decreased concentration of isobutyrate in feces (Seymour et al., 2021), while increasing DMI and milk protein yield (Seymour et al., 2022). However, increasing HFCG inclusion in diets containing

50% forage for sheep led to reductions in DMI, ADG, and final body weight (**BW**; Watanabe et al. 2022). Moreover, HFCG did not increase butyrate concentration in the large intestine but did increase the molar proportion of acetate and decrease the molar proportion of propionate (Watanabe et al. 2022). Conversely, post-ruminal provision of CaG as compared to post-ruminal butyrate yielded similar responses in terms of increased propionate and decreased acetate in the colon of beef heifers fed 50% forage diet (Watanabe et al., 2023). Feeding HFCG to growing steers increased acetate, propionate, and butyrate concentrations in the hindgut (Rowland et al., 2022) and impacted microbial populations important in gut homeostasis and health (Koyun et al., 2022). These data collectively support that provision of post-ruminal CaG may alter fermentation profiles with potential increases in butyrate supply.

Feeding high-grain diets could lead to luminal acidosis due to the accumulation of organic acids in the rumen (Bevans et al., 2005) and intestine (Gressley et al., 2011; Pederzolli et al., 2018) as a consequence of high levels of fermentable carbohydrates in the diet (Owens et al., 1998). The ruminal pH reduction has been reported to increase ruminal epithelial permeability (Aschenbach and Gäbel, 2000) and lead to a local inflammatory response (Humer et al., 2018). In addition, high-grain diets are known to increase digesta passage rate and consequently post-ruminal digestion (NASEM, 2016), increasing fermentation in the hindgut. The large intestine has limited buffering capacity as compared to the rumen due the lack saliva inflow and a lesser abundance of protozoa which may help to modulate starch digestion and hence pH decline (Gressley et al., 2011). As a result, ruminal acidosis can also reduce large intestinal pH and modulate permeability (Pederzolli et al., 2018). Thus, it might be justified to include a protected form of butyrate or its precursors in diets of finishing cattle to ensure butyrate delivery not only to the small intestine but also to the large intestine. However, it is unclear whether increasing the supply of butyrate precursors improves butyrate supply and promotes gastrointestinal tract function in finishing cattle.

Thus, based on the effects of butyrate on the gastrointestinal tract (**GIT**), it is hypothesized that supplementing HFCG would increase intestinal butyrate concentration, and increase growth and carcass and meat quality characteristics of beef heifers in a dose-dependent manner. The objective of this study was to evaluate feed intake, indicators of gastrointestinal fermentation, and carcass and meat quality characteristics in finishing beef heifers receiving increasing levels of HFCG.

## 5.3. Material and methods

Use of cattle in this experiment and the procedures implemented were approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20190138, Saskatoon, SK, Canada) and followed the guidelines of the Canadian Council on Animal Care (Ottawa, ON, Canada).

### 5.3.1. Experimental design, treatments, and receiving protocol

Twenty-one 10-mo Simmental × Limousin cross heifers were used in a randomized complete block design. Heifers were blocked (7 blocks) by initial BW (323 ± 35.3 kg; mean ± SD) to remove potential effects on feed intake, growth, and GIT development, and to allow for staggered slaughter dates at the end of the study. Within blocks, heifers were randomly assigned to 1 of 3 treatments that differed in the inclusion level of HFCG (50% hydrogenated palm fat, 40% calcium gluconate, 10% calcium carbonate; Selko Lactibute, Trouw Nutrition, Amersfoort, the Netherlands). The inclusion rates of HFCG (dry matter [**DM**] basis) were 0.0% (**CON**), 0.09% (**CG09**), and 0.18% (**CG18**) with their being substituted for barley grain in the diet. Tested doses of HGCG were based on Seymour et al. (2021) with dietary inclusion of HFCG of 0.07% (DM basis) offering 16 g/d of HFCG for dairy cows consuming 22.31 kg/d. The CG18 dose was designed to provide a similar quantity (16 g/d) for beef heifers with a predicted average DMI of 8.9 kg/d. The CON finishing diet consisted of (DM basis; Table 5.1) barley silage (8.72%), dry-rolled barley grain (87.44%), and a mineral and vitamin premix (3.84%).

At arrival, heifers were kept in a common pen with a covered shelter and fed a diet containing (DM basis) 50.0% barley silage, 46.2% dry-rolled barley grain, and 3.8% of a mineral and vitamin premix for 14 d. At the end of this period, heifers were dewormed (Solmectin Pour-on, Solvet, Calgary, AB, Canada), vaccinated (Bovi-shield Gold/One Shot, Zoetis, Canada, Kirkland, QC; Ultrabac 7/Somubac, Zoetis, Canada, Kirkland, QC), and implanted with 200 mg of trenbolone acetate and 40 mg of estradiol (Revalor-XH, Merck Animal Health, Roseland, NJ). Heifers were weighed on two consecutive days to assess initial BW and facilitate blocking. After blocking, heifers were allocated to individual pens (3 × 6 m), where half of the pen area was a covered shelter with straw bedding and a heated water trough. Body weight was assessed every 28 d, with all animals weighed on a single day. The experiment was conducted between January and September

of 2016, for the first 18 weeks, heifers were not weighed due to weather conditions. Body weights were used to calculate average daily gain (**ADG**) by regression using the observed BW and days on feed.

### 5.3.2. Adaptation, feeding protocol, and feed analysis

Heifers were adapted to the high-grain diet using a 5-step transition protocol. As described in Table 5.1, diets from steps 1 and 2 were offered for 4 d each, while steps 3, 4, and 5 were fed for 6 d each. An extended transition was used relative to standard practices (Samuelson et al., 2016) due to variable feed intake and evidence of loose manure. The final finishing diets were fed from d 27 to the end of the study (206 ± 7.8 d; mean ± SD). Heifers were fed a total mixed ration once daily (0930 h) targeting refusals equating to 3% of the feed offered (as fed basis) to allow for *ad libitum* intake. Feed bunks were checked and assessed by visual score every morning before feeding (0700 h) and the amount of feed offered was based on the daily score, to avoid DMI fluctuations. Feed bunks were emptied weekly, and refusals were collected, weighed, and sampled.

Feed ingredients were collected twice a week, and the feed and refusals samples were dried in a forced-air oven at 55ºC until a constant weight was achieved. Ingredient DM coefficients were updated twice a week to ensure the correct inclusion of ingredients on an as fed basis. Ingredient and refusal DM coefficients and weights were used to calculate weekly DMI. Samples were composited by collection time where the total experimental period was divided into three equal durations and three samples of each ingredient were used for chemical analysis. At the end of the experimental period, feed samples were ground using a Christy Turner Miracle Mill (Christy Turner LTD., Suffolk, UK) with a 1-mm screen and sent to Cumberland Valley Analytical Services (Waynesboro, PA) for analysis of chemical composition. Dry matter was determined by drying the samples at 135ºC (method 930.15; AOAC, 2000), ash was determined by ashing 1.5 g of sample for 4 h at 600ºC (adapted method 942.05; AOAC, 2000), and the organic matter (**OM**) content was determined by subtracting the ash content from 100%. Crude protein was determined using a LECO FP-528 Nitrogen Combustion Analyzed (LECO, St. Joseph, MI) by the method 990.03 of AOAC (2000). The NDF and ADF content were analyzed as proposed by Van Soest et al. (1991) and method 973.18 of AOAC (2000), respectively. Both methods were adapted to use Whatman 934-AH (GE, Healthcare Life Sciences, Chicago, IL) glass 1.5-µm microfiber filters. In addition, α-amylase and sodium sulfite were used in the NDF analysis. Free glucose was used to determine

starch concentration as described by Hall (2009). Ether extract was determined according to AOAC (2000), method 2003.05 using the Tecator Soxtec System HT 1043 Extraction unit (Tecator, FOSS, Eden Prairie, MN). The Ca and P content were determined using the method 958.01 from AOAC (2000) with the modification of ashing a 0.35 g sample for 1 h at 535ºC, followed by digestion in open crucibles for 25 min in 15% nitric acid on a hotplate. Samples were then diluted to 50 mL and analyzed on axial view using a Perkin Elmer 5300 DV ICP (Perkin Elmer, Shelton, CT). Dietary composition was determined mathematically using the nutrient concentration of individual feeds and their DM inclusion rate in the diet.

### 5.3.3. Fecal collections

Feces were collected directly from the rectum of the heifers on d 171 and 192 with collection occurring 6 h after feeding. Feces were then diluted with double-distilled water at a 2:1 (v/v) ratio and strained through 4 layers of cheesecloth. Subsequently, fecal pH was measured in duplicate, and 10 mL was transferred into a vial containing 2-mL of meta-phosphoric acid (25% w/v) for SCFA analysis. Short-chain fatty acids were separated and quantified by gas chromatography (Agilent 6890 Series GC System, Santa Clara, CA, USA) using isocaproic acid as an internal standard and the relative response factors for acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids were calculate with reference to the standard (Khorasani et al., 1996).

### 5.3.4. Gastrointestinal tract sampling and carcass characteristics

Upon reaching a mean group BW of 600 kg, heifers were transported to a provincially inspected abattoir (Martensville, SK, Canada) by block prior to feeding. This resulted in 197 d on feed (**DOF**) for blocks 6 and 7, 204 DOF for blocks 4 and 5, 211 DOF for blocks 2 and 3, and 218 DOF for block 1. Final BW was assessed before feeding on both the day before and the day of slaughter. The slaughter order at the plant was random and recorded to allow for carcass identification and gastrointestinal tract collections. Heifers were stunned and exsanguinated starting at 0900 h. After removal of the feet, hide, and the head, the abdominal cavity was opened and the whole GIT was placed on a table in a separate room to allow for identification of the rumen (caudal blind sac), jejunum (proximal, near to the ligament of Treitz), and colon (end of the centripetal turn) as described by Penner et al. (2014) and Watanabe et al. (2023).

**Table 5.1.** Adaptation protocol and diet composition of experimental diets containing increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**).

| | Experimental diets | | | | | |
|---|---|---|---|---|---|---|
| | Step 1 | Step 2 | Step 3 | Step 4 | Step 5 | Finishing |
| Days on feed | 4 | 4 | 4 | 6 | 6 | 206 ± 8 |
| *Ingredient, %DM* | | | | | | |
| Barley silage | 40.00 | 30.00 | 20.00 | 15.00 | 11.00 | 8.72 |
| Barley grain[1] | 56.16 | 66.16 | 76.16 | 81.16 | 85.16 | 87.44 |
| Mineral/vitamin premix[2] | 3.84 | 3.84 | 3.84 | 3.84 | 3.84 | 3.84 |
| *Chemical composition, % DM ± SD* | | | | | | |
| Dry matter | 56.22 ± 0.03 | 92.38 ± 0.01 | 70.46 ± 0.01 | 75.02 ± 0.01 | 78.82 ± 0.02 | 79.90 ± 0.02 |
| Organic matter | 92.52 ± 0.17 | 93.20 ± 0.16 | 93.87 ± 0.20 | 94.21 ± 0.22 | 94.48 ± 0.25 | 94.64 ± 0.26 |
| Crude protein | 14.85 ± 0.16 | 14.61 ± 0.19 | 14.37 ± 0.21 | 14.26 ± 0.22 | 14.16 ± 0.23 | 14.11 ± 0.23 |
| Acid detergent fiber | 17.44 ± 0.79 | 14.71 ± 0.56 | 11.98 ± 0.35 | 10.62 ± 0.26 | 9.52 ± 0.23 | 8.90 ± 0.22 |
| Neutral detergent fiber | 32.72 ± 1.81 | 29.16 ± 1.53 | 25.61 ± 1.25 | 23.83 ± 1.11 | 22.41 ± 1.01 | 21.60 ± 0.95 |
| Starch | 44.07 ± 0.91 | 49.68 ± 0.98 | 55.28 ± 1.10 | 58.09 ± 1.17 | 60.33 ± 1.24 | 61.61 ± 1.28 |
| Ca | 0.81 ± 0.01 | 0.78 ± 0.01 | 0.74 ± 0.01 | 0.73 ± 0.01 | 0.71 ± 0.01 | 0.071 ± 0.01 |
| P | 0.40 ± 0.01 | 0.040 ± 0.01 | 0.41 ± 0.01 | 0.041 ± 0.01 | 0.041 ± 0.01 | 0.041 ± 0.01 |

[1]Hydrogenated fat matrix-embedded Ca-gluconate was added through substitution of barley grain resulting in a barley inclusion of 56.07 and 55.98% for step 1, 66.07 and 65.98% for step 2, 76.07 and 75.98 for step 3, 81.07 and 90.98 for step 4, 85.07 and 84.98 for step 5, and 87.36 and 87.26 for finishing on the CG09 and CG18 respectively.

[2]Mineral/vitamin premix contained: 40.9% of canola meal, 15.9% of urea, 34.8% of limestone, 5.0% of NaCl, 1.9% of potassium magnesium sulfate, 0.23% of selenium, 0.11% of copper sulfate, 0.02% of manganese sulfate, 0.02% of zinc sulfate, 0.01% of vitamin A, 0.002% of vitamin D3, 0.32% of vitamin E, 0.38 mg/d of melengesterol acetate (MGA), and 38 mg/kg of sodium monensin (Elanco Animal Health, Greenfield, IN).

Ruminal digesta were mixed and a subsample was collected. For the intestinal regions, the sections were identified, tied to avoid digesta loss, after which samples of digesta were collected. Samples from all regions were strained through 4 layers of cheesecloth and colon digesta was diluted with double-distilled water at 1:1 (v/v) ratio and mixed thoroughly. After straining, digesta pH was measured in duplicate using a portable pH meter (Accumet AP110; Fisher Scientific, Nepean, ON, Canada). Following pH measurements, 10 mL of strained digesta from each region were placed in two 15 mL tubes containing either 2 mL of metaphosphoric acid (25%, w/v) or 2 mL of sulfuric acid (1%, v/v) for SCFA and ammonia-N concentration determination, respectively. Short-chain fatty acid concentration was determined as described above. Ammonia-N concentration was determined by the colorimetric phenol hypochlorite method described by Fawcett and Scott (1960).

A 5 × 5 cm area of the caudal blind sac of the rumen and a sectional length of approximately 15 cm of both proximal jejunum and colon were collected and gently rinsed with cold phosphate buffered saline (**PBS**, Millipore Sigma, St. Louis, MO; pH = 7.2) until clean. Approximately 10 rumen papillae were cut from the rumen tissue at the basal connection and placed in biopsy cassettes (Simport, Beloeil, QC, Canada). Jejunum and colon tissues were cut longitudinally and 1 $cm^2$ epithelial samples were also placed in biopsy cassettes. Cassettes were immediately placed in 10% formalin (10% formaldehyde, v/v, in neutral phosphate buffer; Ricca Chemical Company, Arlington, TX, USA) until submitted to Prairie Diagnostic Services (Saskatoon, SK, Canada) where they were dehydrated. The dehydration was accomplished by washing cassettes in solutions containing increasing concentrations of ethanol followed by a wash with xylene at the end. Dehydrated tissues were embedded in paraffin wax, and sectioned into 4-μm sections and finally mounted onto glass slides to be stained as described by Kent-Dennis et al. (2019). Histomorphometric measurements were recorded by a technician blinded to treatments, where for ruminal tissue, 5 papillae were randomly selected, and each stratum were measured at two distinct locations totaling 10 measurements per experimental unit. Measurements were taken at 10× magnification and strata were defined as follows: stratum corneum, the layer of cells located at the outermost tip of the ruminal papillae, heavily stained and absence of nuclei; stratum granulosum, the layer of cells with lighter staining, long axes, and presence of nuclei; stratum spinosum, the layer of cuboidal shaped cells with nuclei; and stratum basale, the layer of columnar cells closer to the lamina propria with presence of nuclei (adapted from Graham and Simmons, 2005 and Steele

et al., 2011). Tissues from the jejunum and colon were not suitable for evaluation of histology as the villi and crypts were not intact.

### 5.3.5. Carcass collection, grading, meat quality, composition, and sensory panel

Hot carcass weight (**HCW**) was recorded and used to calculate the dressing percentage relative to the shrunk final BW (final BW × 0.96). Carcasses were then refrigerated for 13 d and a 4-rib cut-out from the left ribeye (including the $11^{th}$ to the $14^{th}$ ribs) was shipped under refrigeration conditions to the Lacombe Research and Development Centre, Agriculture and Agri-Food Canada (Lacombe, AB, Canada) to be evaluated by a certified grader according to standards of the Canadian Beef Grading Agency (Calgary, AB, Canada). At the grade site (between the $12^{th}$ and the $13^{th}$ ribs) fat thickness (at the three-quarter position of the spinous process) and rib-eye area (the *M. longissimus thoracis* area in $cm^2$) were taken according to Lopez-Campos et al. (2018).

As described by Jones et al. (1991), grade fat was measured in a scale from 1 to 15, on the fourth quadrant from the spinous process; the measurement was taken at the minimum point of thickness and each point of the scale increased in 2 mm of increments, starting at 2 to 3 mm (fat class 1). Muscle score was determined with a 1 to 4 scale using the width and length of the *M. longissimus thoracis* (Jones et al., 1991). As described by Segura et al. (2021), retail cut yield was calculated using the developed matrix of the percentage of the estimated retail cut yield using muscle score and fat class. Total lean yield was calculated based on the muscle score and grade fat as total lean yield (%) = 63.5 + 1.05 × (muscle score) – 0.76 × (grade fat) described by the CBGA (2020). The United States Department of Agriculture (**USDA**) beef marbling scoring system was used to assess the marbling scores (USDA, 1989).

Subsequently, four 1-inch steaks were fabricated. Starting from the caudal side, the first steak was ground using a Blixir (Model BX3) (Robot-Coupe USA Inc., Ridgeland, MS, USA) and one 50-g sub-sample was analyzed for moisture and fat content according to the AOAC (2008) Method 2008.06 using a Smart Turbo Moisture Analyzer (Model 907,990) and a Smart Trac Fat Analyzer (Model 907,955; CEM Corporation, Matthews, NC, USA). Another 50-g sub-sample was analyzed for protein according to the AOAC (2011) Method 2011.04 using a Rapid N Cube (Elementar Analyses System GmbH, Hanau, Germany). The second steak was weighed prior to cooking and grilled on a preheated (210 °C) Garland grill (Model ED30B, Condon Barr Food Equipment Ltd.,

Edmonton, AB) to an internal temperature of 35.5 °C, turned, and cooked to a final temperature of 71 °C. Cooking time was then recorded and, upon removal from the grill, the steak was sealed in polyethylene bags and submerged in an ice-water bath to avoid further cooking. The cooked steak was subsequently stored for 1 d at 2 °C. After chilling, the steak weight was measured to determine cooking losses and six cores (1.90 cm in diameter) were cut parallel to the longitudinal orientation of the muscle fibers. In order to determine sample shear force perpendicular to the fiber grain, a TA-XT Plus Texture Analyzer equipped with a Warner-Bratzler shear head (at a 200 mm/min crosshead speed) and a 30-kg load cell were used together with Texture Exponent 32 Software (Texture Technologies Corp., Hamilton, MA). Shear force (N) for each steak was calculated as the average of the six cores. The third and fourth steaks were used for descriptive sensory and flavor profile analyses, respectively, performed by a ten-member expert meat evaluation panel. Treatments were balanced to each session and samples were randomly assigned to sessions and panelists. Steaks were cooked as mentioned above and sub-samples were cubed (1.3 × 1.3 × 1.3 cm) before being handed to the panelists. For descriptive attribute sensory analyses, samples were evaluated for initial and overall tenderness, initial and sustained juiciness, beef flavor and off-flavor intensity, and the amount of perceptible connective tissue, using nine-point descriptive scales. For flavor profile analysis, samples were evaluated using a 15-cm line scale with standard reference points for detected aromas, tastes and flavors (0 = none, 15 = extremely intense), according to the flavor lexicon described by AMSA (2016).

#### 5.3.6. Statistical analysis

Data were analyzed using PROC MIXED of SAS v9.4 (SAS Institute Inc., Cary, NC) where the dietary treatment was considered the fixed effect, and block and heifer were considered as random effects. For the descriptive sensory analyses and flavor profile data, the session and the panelist within session were added as random effects. The adjusted denominator of degrees of freedom were calculated using the Kenward-Roger method. Polynomial contrasts were used to assess the linear and quadratic effects of HFCG dose. Original data and the residuals were tested for normality based on visual assessment and the Shapiro-Wilk test, and it was confirmed that data were normally distributed.

Fecal variables and DMI (both in kilograms and as a % BW) were analyzed as a repeated measure where the timepoints for DMI were defined based on the weighing intervals. For these

variables, the fixed effects of time and treatment × time interaction were added in the model with time included as a repeated measure. The variation within animal was modeled using the covariance structure which presented the lowest Akaike information criteria. First-order autoregressive structure was selected for DMI expressed in both kilograms and as a percentage of the BW and compound symmetric structure was selected for fecal pH, total SCFA, and isobutyrate, butyrate, and valerate proportions, while the heterogeneous compound symmetric structure was selected for fecal acetate and propionate proportions.

To evaluate the differences attributed for each variable evaluated on the descriptive sensory and flavor profile panels the counts of each score were calculated using the FREQ procedure of SAS v9.4. The attributes identified on the descriptive sensory and flavor profile panels were categorized relative to their scores. For the descriptive sensory data, scores averaging less than 5 were classified as "negative", equal to 5 as "neutral", and greater than 5 as "positive". On the other hand, the profile panel data were classified as "present", when the score was greater than 1 and "absent" when it was less or equal to 1 (adapted from Barragán-Hernández et al. 2021). The scores were calculated using the average response of each panelist within each session per block. Using the GLIMMIX procedure of SAS, scores obtained for the descriptive sensory data were analyzed by conditional logistic regression using maximum likelihood estimation with Laplace approximation obtaining the odds ratio (OR) and the Chi-square value based on the individual exposure of each dose of HFCG on a multinomial distribution. Scores were considered as a weighting variable and were modelled using a cumulative logit function assuming the data followed a multinomial distribution. For the flavor profile data, the GLIMMIX was also used but with binary distribution to assess the probability of detection of each aroma, taste, or flavor descriptor. For both descriptive sensory attributes and flavor profile data, three contrasts were assessed to evaluate the OR of CG09 having a worse score than CON, the OR of CG18 having a worse flavor than CON, and the OR of CG18 having a worse score than CG09. In all cases, significance was declared when $P < 0.05$, and tendencies were declared when $0.05 \leq P < 0.10$.

## 5.4. Results

### 5.4.1. Body weight, dry matter intake, and growth performance

Increasing doses of HFCG did not affect ($P \geq 0.36$; Table 5.2) initial or final BW, DMI, or ADG; however, G:F ratio tended to be quadratically ($P = 0.08$) affected where heifers on the CG09 treatment tended to have higher G:F ratio. In addition, DMI expressed either as kg/d or % BW was affected by time ($P < 0.01$); as it increased until the fifth month and remained constant thereafter. The DMI reported as a % BW was the greatest at 4 and 5 mo and decreased thereafter. Treatment × time interactions were not observed for DMI ($P \geq 0.87$; data not shown).

### 5.4.2. Fecal parameters

Fecal pH, total SCFA concentration, and the molar proportion of SCFA in feces were not affected by the increasing dose of HFCG ($P \geq 0.18$). Day of sampling did not affect fecal pH ($P = 0.40$) or the molar proportions of SCFA ($P \geq 0.21$); however, it affected total SCFA concentration ($P < 0.01$) where they were higher on d 192 relative to d 171 (Table 5.3). The treatment × time interaction was only significant for the molar proportion of fecal valerate ($P < 0.01$), where heifers fed HFCG independent of dosage had reduced fecal molar proportions on d 192 relative to CON animals, (data not shown).

### 5.4.3. Ruminal fermentation and histology

Post-mortem ruminal digesta variables (pH, SCFA concentration, and ammonia nitrogen concentration) were not affected ($P \geq 0.11$) by dosage of HFCG (Table 5.4). Stratum corneum thickness tended to be quadratically ($P = 0.05$) affected by HFCG dose where CG09 tended to have a thinner stratum. However, dose of HFCG did not affect ($P \geq 0.32$) the thickness of the other strata.

### 5.4.4. Intestinal fermentation

Jejunal digesta pH was quadratically ($P = 0.03$) affected by increasing dose of HFCG, where heifers fed with CG09 had lower jejunal pH (Table 5.5). Provision of HFCG did not affect jejunal SCFA ($P \geq 0.20$) or ammonia nitrogen concentration ($P \geq 0.11$), with acetate being the only SCFA detected. Colonic digesta pH, total SCFA concentration, and ammonia nitrogen concentration were not affected ($P \geq 0.15$) by dose of HFCG. However, molar proportion of isovalerate was quadratically ($P = 0.01$) affected by HFCG dose, where CG09 had a greater molar proportion. In addition, acetate ($P = 0.05$) and valerate ($P = 0.08$) molar proportions tended to be quadratically

affected by the HFCG dose, where CG09 tended to have lesser acetate and greater valerate molar proportions.

### 5.4.5. Carcass characteristics, meat quality, and sensory analysis

Hot carcass weight ($P \geq 0.36$; Table 5.6), dressing percentage ($P \geq 0.70$), rib eye area ($P \geq 0.70$), marbling score ($P \geq 0.75$), fat thickness ($P \geq 0.30$), retail cut yield ($P \geq 0.48$), and lean meat yield ($P \geq 0.57$) were not affected by increasing dose of HFCG. Additionally, meat quality variables (Table 5.7) including moisture ($P \geq 0.20$), fat ($P \geq 0.39$), protein ($P \geq 0.49$), shear force ($P \geq 0.13$), and cook time ($P \geq 0.5$) were not affected by treatment. On the other hand, cook loss tended to be quadratically ($P = 0.06$) affected by HFCG dose, where CG09 meat had a greater loss.

Dietary treatment with HFCG did not affect initial tenderness ($P = 0.28$), juiciness ($P = 0.36$), beef flavor intensity ($P = 0.97$), off-flavor intensity ($P = 0.21$), amount of perceived connective tissue ($P = 0.35$), overall tenderness ($P = 0.44$), and sustainable juiciness ($P = 0.88$) indicating that CG09 and CG18 do not affect meat eating quality evaluated by trained panelists (Table 8; the frequency obtained for each score of each variable is presented on Supplementary Table 5.1). In addition, treatments did not affect the intensity of aromas ($P \geq 0.22$), tastes ($P \geq 0.25$), or flavor descriptors (Supplementary Table 5.2).

## 5.5. Discussion

In an attempt to increase the provision of butyrate to the intestine of beef heifers fed a high-grain diet, a hydrogenated fat embedded form of CaG was fed. Ruminal protection of HFCG has been reported in Chapter 3 using a 24-h ruminal incubation resulting in a recovery rate of 86.6% providing proof of the efficacy of the ruminal protection. Ruminal protection likely helps explain the lack of differences within ruminal digesta in response to increasing dose of HFCG and its consequent bypass to the intestine. Due to the low microbial concentration in the proximal parts of the small intestine (J. Wang et al., 2017) and the proven stability of gluconic acid in the small intestine of rats (Asano et al., 1997), the reduction of jejunal pH may not be a direct effect of CaG fermentation; moreover, total concentration of SCFA were not affected by treatment as a result of the pH reduction does not appear to be linked to SCFA concentration. In addition, Watanabe et al. (2022) did not find changes in the fermentation in the ileum of lambs fed increasing HFCG, leading to speculation that fermentation of CaG is most likely to occur in the large intestine.

**Table 5.2.** Body weight (**BW**), dry matter intake (**DMI**), and growth parameters of beef heifers (n = 21) fed a finishing diet with increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| | Treatment[1] | | | SED[2] | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|
| Item | CON | CG09 | CG18 | | Linear | Quadratic | Time[4] |
| Initial BW, kg | 323.4 | 322.8 | 323.4 | 3.95 | 0.99 | 0.86 | - |
| Final BW, kg | 628.9 | 638.0 | 625.7 | 16.02 | 0.85 | 0.45 | - |
| DMI, kg/d | 8.57 | 8.46 | 8.38 | 0.498 | 0.65 | 0.97 | <0.01 |
| DMI, % BW | 1.78 | 1.76 | 1.76 | 0.087 | 0.78 | 0.85 | <0.01 |
| ADG[5], kg/d | 1.48 | 1.53 | 1.47 | 0.069 | 0.86 | 0.36 | - |
| G:F[6], kg/kg | 0.177 | 0.184 | 0.180 | 0.0036 | 0.45 | 0.08 | - |

[1]Heifers were assigned with CON (*n* = 7; no inclusion of HFCG in the diet), CG09 (*n* = 7; inclusion of 0.09% of HFCG in the diet, % DM), or CG18 (*n* = 7, inclusion of 0.18% of HFCG in the diet, % DM).
[2]SED, standard error of the difference.
[3]Treatment × time interaction: DMI, kg/d ($P = 0.87$) and DMI, % BW ($P = 0.87$).
[4]Time effect of DMI calculated using a four-week interval throughout the experimental period.
[5]ADG, Average daily gain.
[6]G:F, gain-to-feed.

**Table 5.3.** Fecal pH and SCFA concentration for beef heifers (n = 21) fed a finishing diet with increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| | Treatment[1] | | | Time | | SED[2] | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | CON | CG09 | CG18 | d 171 | d 192 | | Linear | Quadratic | Time |
| Fecal pH | 7.23 | 7.15 | 7.17 | 7.22 | 7.15 | 0.113 | 0.56 | 0.57 | 0.40 |
| Total SCFA[4,5], m*M* | 31.89 | 33.99 | 29.55 | 22.93 | 40.69 | 3.638 | 0.51 | 0.32 | <0.01 |
| Acetate, mol/100 mol | 73.59 | 74.43 | 76.14 | 75.27 | 74.18 | 1.913 | 0.18 | 0.80 | 0.21 |
| Propionate, mol/100 mol | 17.06 | 15.77 | 15.04 | 15.64 | 16.27 | 2.020 | 0.19 | 0.83 | 0.34 |
| Butyrate, mol/100 mol | 8.66 | 9.86 | 8.78 | 9.18 | 9.03 | 1.342 | 0.93 | 0.35 | 0.86 |
| Valerate, mol/100 mol | 0.47 | 0.14 | <0.01 | <0.01 | 0.41 | 0.090 | <0.01 | 0.24 | <0.01 |

[1]Heifers were assigned with CON ($n$ = 7; no inclusion of HFCG in the diet), CG09 ($n$ = 7; inclusion of 0.09% of HFCG in the diet, % DM), or CG18 ($n$ = 7, inclusion of 0.18% of HFCG in the diet, % DM).

[2]SED, standard error of the difference.

[3]Treatment × time interaction: fecal pH ($P = 0.35$), total SCFA ($P = 0.95$), acetate ($P = 0.73$), propionate ($P = 0.41$), butyrate ($P = 0.34$), and valerate ($P < 0.01$).

[4]SCFA, short-chain fatty acids.

[5]Fecal isobutyrate and isovalerate were not detectable.

**Table 5.4.** Ruminal digesta and ruminal epithelial histology of beef heifers (n = 21) fed a finishing diet with increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| Item | Treatment[1] | | | SED[2] | *P*-value | |
|---|---|---|---|---|---|---|
| | CON | CG09 | CG18 | | Linear | Quadratic |
| Digesta pH | 6.84 | 6.47 | 6.27 | 0.325 | 0.11 | 0.77 |
| Total SCFA[3], m*M* | 102.25 | 100.80 | 117.73 | 18.845 | 0.42 | 0.58 |
| Acetate, mol/100 mol | 49.52 | 52.41 | 51.08 | 2.393 | 0.53 | 0.33 |
| Propionate, mol/100 mol | 38.47 | 31.84 | 34.63 | 4.454 | 0.41 | 0.25 |
| Isobutyrate, mol/100 mol | 1.23 | 1.18 | 1.26 | 0.250 | 0.91 | 0.78 |
| Butyrate, mol/100 mol | 6.87 | 10.73 | 8.81 | 2.164 | 0.38 | 0.14 |
| Isovalerate, mol/100 mol | 1.79 | 2.00 | 2.22 | 0.499 | 0.41 | 0.99 |
| Valerate, mol/100 mol | 2.12 | 1.83 | 2.01 | 0.358 | 0.77 | 0.46 |
| $NH_3$-N, mg/dL | 11.91 | 12.32 | 15.36 | 3.659 | 0.36 | 0.69 |
| Histology | | | | | | |
| Stratum corneum, µm | 27.94 | 20.28 | 23.79 | 2.931 | 0.18 | 0.05 |
| Stratum granulosum, µm | 8.72 | 8.04 | 8.66 | 0.514 | 0.91 | 0.17 |
| Stratum spinosum, µm | 35.67 | 34.02 | 36.13 | 4.543 | 0.92 | 0.64 |
| Stratum basale, µm | 48.83 | 47.25 | 56.25 | 7.298 | 0.32 | 0.41 |

[1]Heifers were assigned with CON (*n* = 7; no inclusion of HFCG in the diet), CG09 (*n* = 7; inclusion of 0.09% of HFCG in the diet, % DM), and CG18 (*n* = 7, inclusion of 0.18% of HFCG in the diet, % DM).
[2]SED, standard error of the difference.
[3]SCFA, short-chain fatty acids.

**Table 5.5.** Intestinal digesta parameters of beef heifers (n = 21) fed a finishing diet with increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| Item | Treatment[1] | | | SED[2] | *P*-value | |
|---|---|---|---|---|---|---|
| | CON | CG09 | CG18 | | Linear | Quadratic |
| *Jejunum* | | | | | | |
| Digesta pH | 7.26[ab] | 6.88[b] | 7.73[a] | 0.296 | 0.13 | 0.03 |
| Total SCFA[3,4], m*M* | 1.81 | 0.93 | 3.96 | 1.647 | 0.20 | 0.20 |
| $NH_3$-N, mg/dL | 21.14 | 16.42 | 18.69 | 2.373 | 0.32 | 0.11 |
| *Colon* | | | | | | |
| Digesta pH | 7.16 | 7.10 | 6.99 | 0.107 | 0.15 | 0.76 |
| Total SCFA[5], m*M* | 57.38 | 63.46 | 67.22 | 8.758 | 0.29 | 0.88 |
| Acetate, mol/100 mol | 77.31 | 74.74 | 76.29 | 1.092 | 0.37 | 0.05 |
| Propionate, mol/100 mol | 15.08 | 16.80 | 15.79 | 1.149 | 0.55 | 0.21 |
| Butyrate, mol/100 mol | 6.57 | 6.49 | 7.05 | 0.776 | 0.55 | 0.65 |
| Isovalerate, mol/100 mol | 0.18[ab] | 0.50[a] | 0.01[b] | 0.112 | 0.27 | 0.01 |
| Valerate, mol/100 mol | 0.69 | 1.15 | 0.85 | 0.207 | 0.47 | 0.08 |
| $NH_3$-N, mg/dL | 13.41 | 11.18 | 10.97 | 2.196 | 0.30 | 0.61 |

[1]Heifers were assigned with CON (*n* = 7; no inclusion of HFCG in the diet), CG09 (*n* = 7; inclusion of 0.09% of HFCG in the diet, % DM), and CG18 (*n* = 7, inclusion of 0.18% of HFCG in the diet, % DM).
[2]SED, standard error of the difference.
[3]SCFA, short-chain fatty acids.
[4]Only acetate was detectable.
[5]Colonic isobutyrate was not detectable.
[a,b]Means within a row with uncommon superscript letter differ ($P < 0.05$) among the different doses of HFCG.

**Table 5.6.** Carcass characteristics of beef heifers (n = 21) fed a finishing diet with increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| Item | Treatment[1] | | | SED[2] | *P*-value | |
|---|---|---|---|---|---|---|
| | CON | CG09 | CG18 | | Linear | Quadratic |
| Hot carcass weight, kg | 371.9 | 379.7 | 370.7 | 10.19 | 0.91 | 0.36 |
| Dressing percentage, % | 61.62 | 61.98 | 61.71 | 0.922 | 0.92 | 0.70 |
| Rib eye area, $cm^2$ | 102.7 | 103.6 | 105.3 | 6.55 | 0.70 | 0.94 |
| Marbling score[3] | 469 | 484 | 474 | 31.8 | 0.90 | 0.75 |
| Fat thickness[4], mm | 15.71 | 12.86 | 14.14 | 2.244 | 0.49 | 0.30 |
| Retail cut yield[5], % | 49.9 | 50.6 | 50.4 | 0.74 | 0.48 | 0.51 |
| Lean meat yield[6], % | 57.25 | 57.14 | 58.08 | 1.429 | 0.57 | 0.66 |

[1]Heifers were assigned with CON (*n* = 7; no inclusion of HFCG in the diet), CG09 (*n* = 7; inclusion of 0.09% of HFCG in the diet, % DM), and CG18 (*n* = 7, inclusion of 0.18% of HFCG in the diet, % DM).
[2]SED, standard error of the difference.
[3]Marbling scores assessed as described the United States Department of Agriculture standards for grades of beef carcasses, where: 0 = devoid, 100 = practically devoid, 200 = traces, 300 = slight, 400 = small, 500 = modest, 600 = moderate, 700 = slightly abundant, 800 = moderately abundant, and 900 = abundant.
[4]Fat thickness measured between the 12th and the 13th ribs at the three-quarter position of the spinous process.
[5]Treatment average according to the developed matrix using muscle score and fat class proposed by the Canadian Beef Grading Agency (CBGA).
[6]Treatment average calculated based on the muscle score and grade fat as follows: total lean yield (%) = 63.5 + 1.05 × (muscle score) – 0.76 × (grade fat) described by the CBGA.

**Table 5.7.** Meat quality of beef heifers (n = 21) fed a finishing diet with increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| | Treatment[1] | | | SED[2] | *P*-value | |
|---|---|---|---|---|---|---|
| Item | CON | CG09 | CG18 | | Linear | Quadratic |
| Moisture, % WMB[3] | 71.62 | 72.09 | 71.51 | 0.450 | 0.81 | 0.20 |
| Fat, % WMB | 4.02 | 3.55 | 3.96 | 0.568 | 0.91 | 0.39 |
| Protein, % WMB | 23.32 | 23.27 | 23.58 | 0.356 | 0.49 | 0.58 |
| Shear force, kg | 5.30 | 5.78 | 5.17 | 0.323 | 0.76 | 0.13 |
| Cook loss, mg/g | 209.9 | 241.2 | 199.5 | 20.66 | 0.63 | 0.06 |
| Cook time, sec/g | 4.17 | 4.45 | 4.13 | 0.561 | 0.93 | 0.55 |

[1]Heifers were assigned with CON (*n* = 7; no inclusion of HFCG in the diet), CG09 (*n* = 7; inclusion of 0.09% of HFCG in the diet, % DM), and CG18 (*n* = 7, inclusion of 0.18% of HFCG in the diet, % DM).
[2]SED, standard error of the difference.
[3]WMB, wet matter basis.

**Table 5.8.** Odds ratios relative to the meat descriptive sensory attributes of beef heifers (n = 21) fed a finishing diet with increasing doses[1] of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| | CG09 *vs*. CON | | CG18 *vs*. CON | | CG18 *vs*. CG09 | | |
|---|---|---|---|---|---|---|---|
| Attributes[1] | Odds ratio | Confidence limits (95%) | Odds ratio | Confidence limits (95%) | Odds ratio | Confidence limits (95%) | *P*-value |
| Initial tenderness | 0.250 | 0.041 - 1.525 | 0.391 | 0.063 - 2.416 | 1.563 | 0.265 - 9.229 | 0.28 |
| Initial juiciness | 0.312 | 0.037 - 2.625 | 0.262 | 0.032 - 2.154 | 0.840 | 0.104 - 6.774 | 0.36 |
| Beef flavor intensity | 0.875 | 0.168 - 4.557 | 0.844 | 0.162 - 4.401 | 0.965 | 0.187 - 4.967 | 0.97 |
| Off flavor intensity | 0.374 | 0.095 - 1.477 | 1.032 | 0.244 - 4.362 | 2.758 | 0.688 - 11.058 | 0.21 |
| Amount of connective tissue | 0.364 | 0.089 - 1.484 | 0.530 | 0.125 - 2.242 | 1.455 | 0.389 - 5.440 | 0.35 |
| Overall tenderness | 0.337 | 0.058 - 1.962 | 0.624 | 0.101 - 3.871 | 1.850 | 0.338 - 10.133 | 0.44 |
| Sustainable juiciness | 0.837 | 0.137 - 5.094 | 0.661 | 0.110 - 3.969 | 0.790 | 0.133 - 4.689 | 0.88 |

[1]Heifers were assigned with CON (n = 7; no inclusion of HFCG in the diet), CG09 (n = 7; inclusion of 0.09% of HFCG in the diet, % DM), and CG18 (n = 7, inclusion of 0.18% of HFCG in the diet, % DM). [2]Nine-point descriptive scales: 9 = extremely tender, extremely juicy, extremely intense beef flavour, extremely bland off-flavour, and no perceptible connective tissue; 1 = extremely tough, extremely dry, extremely bland beef flavour, extremely intense off-flavour, and extremely abundant perceptible connective tissue.

**Supplementary table 5.1.** Frequency (score count) for meat sensory attributes of beef heifers (n = 21) fed a finishing diet with increasing doses[1] of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| | Score count frequency[2], *n* | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| Attributes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| Initial tenderness | | | | | | | | | | |
| CON[1] | 0 | 0 | 0 | 3 | 3 | 8 | 20 | 19 | 14 | 67 |
| CG09 | 1 | 1 | 1 | 7 | 1 | 12 | 14 | 19 | 10 | 66 |
| CG18 | 2 | 0 | 0 | 5 | 5 | 8 | 17 | 21 | 9 | 67 |
| Initial juiciness | | | | | | | | | | |
| CON | 0 | 1 | 1 | 2 | 5 | 18 | 23 | 16 | 1 | 67 |
| CG09 | 0 | 0 | 4 | 5 | 7 | 12 | 30 | 7 | 1 | 66 |
| CG18 | 0 | 1 | 1 | 8 | 9 | 18 | 21 | 8 | 0 | 66 |
| Beef flavor desirability | | | | | | | | | | |
| CON | 0 | 0 | 0 | 2 | 3 | 10 | 16 | 33 | 3 | 67 |
| CG09 | 0 | 0 | 1 | 2 | 1 | 12 | 24 | 25 | 1 | 66 |
| CG18 | 0 | 0 | 0 | 1 | 2 | 9 | 23 | 28 | 3 | 66 |
| Beef flavor intensity | | | | | | | | | | |
| CON | 0 | 0 | 0 | 0 | 4 | 8 | 27 | 26 | 2 | 67 |
| CG09 | 0 | 0 | 0 | 0 | 4 | 13 | 25 | 22 | 2 | 66 |
| CG18 | 0 | 0 | 0 | 1 | 4 | 10 | 25 | 24 | 2 | 66 |
| Off flavor intensity | | | | | | | | | | |
| CON | 0 | 0 | 0 | 4 | 2 | 1 | 5 | 8 | 47 | 67 |
| CG09 | 0 | 0 | 1 | 8 | 0 | 3 | 6 | 9 | 39 | 66 |
| CG18 | 0 | 0 | 0 | 4 | 2 | 1 | 4 | 12 | 43 | 66 |

**Supplementary table 5.1 continued.** Frequency (score count) for meat sensory attributes of beef heifers (n = 21) fed a finishing diet with increasing doses[1] of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| | Score count frequency[2], *n* | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|
| Attributes | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| Amount of connective tissue | | | | | | | | | | |
| CON | 0 | 0 | 0 | 1 | 1 | 4 | 8 | 29 | 24 | 67 |
| CG09 | 0 | 1 | 0 | 1 | 4 | 6 | 10 | 23 | 21 | 66 |
| CG18 | 0 | 0 | 0 | 1 | 3 | 6 | 7 | 27 | 22 | 66 |
| Overall tenderness | | | | | | | | | | |
| CON | 0 | 0 | 0 | 0 | 1 | 7 | 14 | 34 | 11 | 67 |
| CG09 | 0 | 0 | 1 | 2 | 1 | 10 | 18 | 25 | 9 | 66 |
| CG18 | 0 | 0 | 0 | 1 | 1 | 8 | 17 | 32 | 7 | 66 |
| Sustainable juiciness | | | | | | | | | | |
| CON | 0 | 0 | 0 | 3 | 8 | 16 | 32 | 8 | 0 | 67 |
| CG09 | 0 | 0 | 0 | 5 | 5 | 16 | 37 | 3 | 0 | 66 |
| CG18 | 0 | 0 | 0 | 1 | 12 | 15 | 33 | 5 | 0 | 66 |
| Overall palatability | | | | | | | | | | |
| CON | 0 | 0 | 0 | 2 | 4 | 7 | 29 | 20 | 5 | 67 |
| CG09 | 0 | 0 | 2 | 2 | 4 | 10 | 31 | 17 | 0 | 66 |
| CG18 | 0 | 3 | 1 | 1 | 7 | 37 | 17 | 1 | 0 | 67 |

[1]Heifers were assigned with CON (n = 7; no inclusion of HFCG in the diet), CG09 (n = 7; inclusion of 0.09% of HFCG in the diet, % DM), and CG18 (n = 7, inclusion of 0.18% of HFCG in the diet, % DM). [2]Nine-point descriptive scales: 9 = extremely tender, extremely juicy, extremely intense beef flavour, extremely bland off-flavour, and no perceptible connective tissue; 1 = extremely tough, extremely dry, extremely bland beef flavour, extremely intense off-flavour, and extremely abundant perceptible connective tissue.

**Supplementary table 5.2.** Flavor profile based on a sensory panel of beef heifers (n = 21) fed a finishing diet with increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| | Probability of detection | | | | CON vs. CG09 | | CON vs. CG18 | | CG09 vs. CG18 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CON[1] | CG09 | CG18 | SEM[2] | Odds ratio | Confidence Limit | Odds ratio | Confidence Limit | Odds ratio | Confidence Limit | *P*-value |
| *Aromas detected* | | | | | | | | | | | |
| Beef | - | - | - | - | - | - | - | - | - | - | - |
| Barnyard | 0.180 | 0.177 | 0.230 | 0.054 | 0.980 | 0.365 - 2.635 | 1.354 | 0.525 - 3.494 | 0.724 | 0.281 - 1.867 | 0.72 |
| Bloody/serumy | 0.066 | 0.161 | 0.148 | 0.0470 | 2.740 | 0.742 - 10.122 | 2.466 | 0.655 - 9.281 | 1.111 | 0.389 - 3.174 | 0.27 |
| Brown roasted | 0.984 | 0.952 | 0.967 | 0.023 | 0.328 | 0.028 - 3.823 | 0.492 | 0.036 - 6.631 | 0.667 | 0.094 - 4.716 | 0.64 |
| Burnt | 0.062 | 0.031 | 0.030 | 0.037 | 0.481 | 0.057 - 4.061 | 0.458 | 0.053 - 3.953 | 1.050 | 0.096 - 11.509 | 0.22 |
| Buttery | 0.410 | 0.403 | 0.443 | 0.064 | 0.973 | 0.450 - 2.104 | 1.144 | 0.530 - 2.469 | 0.851 | 0.395 - 1.833 | 0.90 |
| Corn | 0.066 | 0.065 | 0.066 | 0.032 | 0.983 | 0.211 - 4.568 | 1.000 | 0.215 - 4.650 | 0.983 | 0.211 - 4.568 | 1.00 |
| Cruciferous | 0.127 | 0.080 | 0.089 | 0.051 | 0.597 | 0.132 - 2.693 | 0.674 | 0.154 - 2.955 | 0.885 | 0.182 - 4.298 | 0.75 |
| Fatty | 0.035 | 0.048 | 0.035 | 0.032 | 0.569 | 0.117 - 2.775 | 1.00 | 0.250 - 4.002 | 0.569 | 0.117 - 2.775 | 0.71 |
| Grainy | 0.066 | 0.032 | 0.028 | 0.032 | 0.475 | 0.074 - 3.053 | 0.737 | 0.141 - 3.845 | 0.644 | 0.091 - 4.559 | 0.71 |
| Green hay | 0.148 | 0.177 | 0.098 | 0.049 | 1.246 | 0.444 - 3.949 | 0.630 | 0.194 - 2.050 | 1.977 | 0.631 - 6.193 | 0.47 |
| Livery | 0.016 | 0.016 | 0.016 | 0.0020 | 0.984 | 0.049 - 19.898 | 1.000 | 0.049 - 20.232 | 0.984 | 0.049 - 19.898 | 1.00 |
| Metallic | - | - | - | - | - | - | - | - | - | - | - |
| Rancid | - | - | - | - | - | - | - | - | - | - | - |
| Sour dairy | 0.226 | 0.196 | 0.131 | 0.056 | 1.937 | 0.669 - 5.607 | 1.625 | 0.548 - 4.817 | 1.192 | 0.449 - 3.163 | 0.43 |
| Stale/cardboard | - | - | - | - | - | - | - | - | - | - | - |
| Other | - | - | - | - | - | - | - | - | - | - | - |
| Unidentified | - | - | - | - | - | - | - | - | - | - | - |
| *Tastes detected* | | | | | | | | | | | |
| Salty | 0.803 | 0.839 | 0.771 | 0.054 | 1.273 | 0.472 - 3.434 | 0.822 | 0.324 - 2.086 | 1.549 | 0.589 - 4.075 | 0.64 |
| Sour | 0.590 | 0.661 | 0.656 | 0.0630 | 1.356 | 0.618 - 2.974 | 1.323 | 0.602 - 2.906 | 1.025 | 0.461 - 2.279 | 0.67 |
| Sweet | 0.246 | 0.145 | 0.148 | 0.055 | 0.521 | 0.195 - 1.390 | 0.531 | 0.199 - 1.418 | 0.981 | 0.336 - 2.866 | 0.29 |
| Bitter | 0.246 | 0.242 | 0.295 | 0.058 | 0.979 | 0.405 - 2.365 | 1.284 | 0.544 - 3.031 | 0.762 | 0.323 - 1.798 | 0.76 |
| Umami | 0.787 | 0.823 | 0.836 | 0.052 | 1.187 | 0.481 - 3.276 | 1.381 | 0.519 - 3.678 | 0.909 | 0.332 - 2.491 | 0.77 |

**Supplementary table 5.2 continued.** Flavor profile based on a sensory panel of beef heifers (n = 21) fed a finishing diet with increasing doses of hydrogenated fat-embedded Ca-gluconate (**HFCG**) for 206 ± 8 days.

| | Probability of detection | | | SEM[2] | CON vs. CG09 | | CON vs. CG18 | | CG09 vs. CG18 | | *P*-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CON[1] | CG09 | CG18 | | Odds ratio | Confidence Limit | Odds ratio | Confidence Limit | Odds ratio | Confidence Limit | |
| *Flavors detected* | | | | | | | | | | | |
| Beef | - | - | - | - | - | - | - | - | - | - | - |
| Barnyard | - | - | - | - | - | - | - | - | - | - | - |
| Bloody/serumy | 0.230 | 0.258 | 0.328 | 0.06 | 1.168 | 0.482 - 2.826 | 1.638 | 0.694 - 3.865 | 0.713 | 0.309 - 1.646 | 0.47 |
| Brown roasted | 0.962 | 0.936 | 0.934 | 0.032 | 1.150 | 0.076 - 3.159 | 0.483 | 0.075 - 3.106 | 1.018 | 0.219 - 4.729 | 0.68 |
| Burnt | 0.049 | 0.032 | 0.049 | 0.028 | 0.644 | 0.091 - 4.559 | 1.000 | 0.172 - 5.808 | 0.644 | 0.091 - 4.559 | 0.87 |
| Buttery | 0.279 | 0.323 | 0.377 | 0.062 | 1.232 | 0.538 - 2.821 | 1.567 | 0.692 - 3.547 | 0.787 | 0.355 - 1.744 | 0.52 |
| Corn | - | - | - | - | - | - | - | - | - | - | - |
| Cruciferous | 0.047 | 0.054 | 0.044 | 0.034 | 1.178 | 0.154 - 9.013 | 0.942 | 0.114 - 7.768 | 1.250 | 0.160 - 9.737 | 0.97 |
| Fatty | 0.033 | 0.048 | 0.016 | 0.023 | 1.500 | 0.212 - 10.612 | 0.492 | 0.036 - 6.631 | 3.051 | 0.262 - 35.579 | 0.64 |
| Grainy | 0.015 | 0.031 | 0.028 | 0.025 | 2.005 | 0.114 - 35.343 | 1.831 | 0.101 - 33.296 | 1.095 | 0.091 - 13.148 | 0.87 |
| Green hay | 0.033 | 0.032 | 0.066 | 0.032 | 0.983 | 0.116 - 8.324 | 2.070 | 0.322 - 13.309 | 0.475 | 0.074 - 3.053 | 0.61 |
| Livery | 0.016 | 0.032 | 0.082 | 0.035 | 2.000 | 0.148 - 26.969 | 5.357 | 0.519 - 55.299 | 0.373 | 0.062 - 2.260 | 0.25 |
| Metallic | 0.131 | 0.082 | 0.097 | 0.043 | 0.710 | 0.213 - 2.366 | 0.592 | 0.167 - 2.093 | 1.200 | 0.317 - 4.549 | 0.67 |
| Rancid | 0.033 | 0.016 | 0.016 | 0.023 | 0.484 | 0.036 - 6.521 | 0.492 | 0.036 - 6.631 | 0.984 | 0.049 - 19.670 | 0.78 |
| Sour dairy | 0.328 | 0.242 | 0.262 | 0.06 | 0.654 | 0.281 - 1.525 | 0.729 | 0.315 - 1.685 | 0.898 | 0.375 - 2.149 | 0.55 |
| Stale/cardboard | - | - | - | - | - | - | - | - | - | - | - |
| Other | - | - | - | - | - | - | - | - | - | - | - |
| Unidentified | 0.033 | 0.016 | 0.016 | 0.023 | 0.484 | 0.036 - 6.521 | 0.492 | 0.036 - 6.631 | 0.984 | 0.049 - 19.670 | 0.78 |

[1]Heifers were assigned with CON (*n* = 7; no inclusion of HFCG in the diet), CG09 (*n* = 7; inclusion of 0.09% of HFCG in the diet, % DM, and CG18 (*n* = 7, inclusion of 0.18% of HFCG in the diet, % DM).
[2]SEM, standard error of the mean.

The quadratic responses observed in the molar proportions of colonic acetate, isovalerate, and valerate are an indication of altered fermentation caused by CaG; however, it did not follow the linear responses observed in growing lambs (in vivo) fed with increasing dose of HFCG (Watanabe et al., 2022), or on the in vitro incubation of gluconic acid in large intestinal digesta, which was reported to increase butyrate concentration (Tsukahara et al., 2002, 2006; Biagi et al., 2006). Although the doses used in vitro are substantially greater than that provided in the present study, in vitro systems are unable to mimic the removal of end-products, leading to its accumulation.

The provision of butyrate for feedlot cattle have been reported previously by Moreira et al. (2016) where the dietary provision of 10 g/bull/d of encapsulated calcium butyrate showed that relative to control (no supplementation) increased duodenal villi height. In addition, the dietary provision of encapsulated butyric acid and zinc at 1, 2, and 3 g/steer/d (Francis et al., 2022) increased the carcass dressing percentage relative to controls. However, the diets in both experiments had relatively high forage inclusion for finishing diets (30%) compared to industry standards (Samuelson et al., 2016) and neither study reported the effective dose of calcium butyrate and butyric acid of the supplements used. Based on the average DMI throughout the experimental period, heifers fed with CG09 and CG18 ingested 7.6 and 15.1 g/d of HFCG, respectively. Considering that HFCG contains 40% of CaG and the conversion rate of CaG to butyrate has been estimated at 30.5% (Watanabe et al., 2023), the theoretical butyrate production was approximately 0.9 and 1.8 g/heifer/d for CG09 and CG18, respectively. These intakes are comparable to the intermediate doses (1 and 2 g/steer/d) reported by Francis et al. (2022); however, increased dressing percentage was not observed in the current study. Moreover, the quadratic response for cook loss shows that supplementation with CG09 could negatively affect the meat juiciness, however it was not identified on the descriptive sensory attributes of the meat, as there was no difference on the overall juiciness among the treatments.

While providing greater quantities of HFCG may seem logical, recent studies have provided evidence suggesting a linear reduction in DMI and growth without increases in the molar proportion of butyrate in any section of the gastrointestinal tract for lambs (Watanabe et al., 2022). Additionally, inclusion of large proportions of unprotected sodium butyrate in diets for lambs (1.25 and 2.50% of the diet; DM basis) resulted in large increases in the molar concentration of ruminal

butyrate and reduced selective permeability of the isolated ruminal epithelia due the reduced butyrate absorption (Wilson et al., 2012). On the other hand, in studies by Górka et al. (2017, 2018), large amounts of unprotected butyrate infused into the rumen or unprotected sodium butyrate supplementation in the diet, resulted in increased butyrate flow to the small intestine, affecting its length and selected functions. Also, protected butyrate supplementation, although by-passing the rumen, was shown to affect rumen papillae dimension (Kowalski et al., 2015). Considering that less than 15% of butyrate produced in the rumen flows to the intestine (Peters et al., 1990; López et al., 2003), the previously mentioned studies help provide support for a low effective dose of butyrate and its delivery to the intestine to stimulate gastrointestinal tract function in ruminants. As reviewed by Guilloteau et al. (2010), low doses of butyrate provision are capable of increasing the mitotic index in the small intestinal epithelium, inducing cytoprotective effects on the GIT epithelium, affecting the gastrointestinal microbiota composition, increasing nutrient digestibility and growth, and reducing oxidative stress and cytokine synthesis. In support of the present findings, the vast majority of previous studies evaluating the effect of CaG have observed little or inconsistent changes in fecal SCFA concentrations. While Doelman et al. (2019) observed an increase for fecal isobutyrate in dairy cows post-ruminally infused with CaG (at 44, 93, 140, and 187 g/cow/d), Seymour et al. (2021) observed a decrease of fecal isobutyrate in dairy cows fed with HFCG, targeting a lower post-ruminal delivery CaG (6.4 g/cow/d); with only Rowland et al. (2022) reporting greater acetate, propionate, and butyrate concentrations in the hindgut. As such, questions remain for whether HFCG supply may lead to greater butyrate production in the intestine of ruminants and if the single time-point collection is a limitation to assess the conversion of CaG to butyrate due its rapidly absorption on the hindgut epithelia.

There are very few studies evaluating CaG for beef cattle fed with high-grain diets (Rowland et al., 2022), as previously mentioned, a more intensive intestinal fermentation was expected due to changes in passage rate related to the diet. However, the average SCFA concentration in the large intestine of heifers in the current study (62.7 m*M* on the average) are similar to the average SCFA concentration reported in studies with ruminants fed with a 50:50 forage:concentrate ratio diet (69.4 and 68.4 m*M*) in the cecum and colon of sheep (Watanabe et al., 2022), 49.2 m*M* in the colon of beef heifers (Watanabe et al., 2023), and 41.7 and 62.4 m*M* in the cecum and colon of Holstein steers (Pederzolli et al., 2018). The subtle differences measured in the current experiment are mainly related to change in the large intestine fermentation. The quadratic effects detected in

the variables measured showed that CG09 tended to have a thinner stratum corneum, a decreased jejunal pH, and a tended to have decreased molar proportion of acetate, and a greater molar proportion of valerate in the colon digesta, and an increased molar proportion of colonic isovalerate. The decrease in jejunum digesta pH is puzzling as CG09 heifers had numerically the lowest SCFA concentrations. Coincidentally, CG09 heifers numerically outperformed the other 2 treatments and tended to have greater feed efficiency (G:F ratio); however, producing a meat which tended to be more susceptible to loss during cooking. Nevertheless, the lack of a clear phenotypic response to the treatments makes it difficult to interpret these results.

## 5.6. Conclusion

It is concluded that despite HFCG alter the colonic fermentation, it did not increase butyrate concentration in a dose-dependent manner in the GIT and only induced slightly changes on rumen morphometrics. However, it seems that a lower dose of supplementation of HFCG, CG09, can lead to beneficial effects on feed efficiency of beef heifers fed with a high-grain diet.

# 6. EFFECT OF FEEDING UNPROTECTED OR RUMEN PROTECTED TRIBUTYRIN ON FEED INTAKE, GROWTH, CARCASS CHARACTERISTICS, AND BLOOD METABOLITES OF GROWING RAM LAMBS FED DIETS CONTAINING MODERATE OR LOW FORAGE INCLUSION

The experiments described in Chapters 3 and 5 used the approach of feeding a rumen-protected form of CaG (HFCG) to lambs fed a moderate forage diet and to beef heifers fed a low amount of forage, respectively. Based on the results observed in both experiments, the delivery of a butyrate precursor into the large intestine did not lead to an increase in growth and feed efficiency as observed in the provision of post-ruminal butyrate in both ruminants and nonruminants. Thus, the experiment presented in the Chapter 6 changed the approach and fed a different form of butyrate, tributyrin, where the butyrate molecules need to be released from glycerol, but there is no need for conversion through fermentation as CaG. Moreover, the experiments described in this chapter used lambs fed diets containing two different levels of forage with tributyrin in an unprotected and rumen-protected form.

## 6.1. Abstract

Tributyrin (**TB**) is a triglyceride with three butyrate molecules that has been reported to increase growth in nonruminants. The objective of this study was to evaluate the effect of unprotected TB (**UTB**) and rumen-protected TB (**RTB**) on growth, feed efficiency, carcass characteristics, and serum β-hydroxybutyrate (**BHBA**) in growing lambs fed diets containing either moderate forage (**MF**; Study 1) or low levels of forage (**LF**; Study 2). Eighty-four lambs were assigned into 1 of 2 studies (MF or LF) of 42 lambs and, within each study they were assigned to 1 of 6 treatments including a negative control (**CON**; no dietary inclusion), dietary inclusion of RTB at 0.1% (**RTB1**), 0.2% (**RTB2**), or 0.3% (**RTB3**), or dietary inclusion of UTB at 0.1% (**UTB1**) or 0.3% (**UTB3**). Body weight (BW) was assessed at the start, every 2 wks and at the end of each study. Blood samples were collected at 0 and 6 h after feeding on d 93. After 97 d, lambs were slaughtered and carcass characteristics were recorded. Within each study, data were analyzed as a 2 × 2 + 2 factorial, to evaluate the effect of protection, dose, the protection × dose interaction, and linear and quadratic responses to increasing dose of RTB. In Study 1, final BW (*P* = 0.06) and average daily gain (*P* = 0.06) tended to be greater for UTB3 lambs relative to RT3 lambs and serum BHBA concentration increased after 6 h of feeding (*P* = 0.01). In Study 2, RTB tended to increase

the dry matter intake ($P = 0.07$) and tended to reduce both feed efficiency ($P = 0.10$) and serum BHBA concentration ($P = 0.10$) relative to UTB. Although tendencies were observed in both studies, TB did not positively affect the carcass characteristics, or the growth of ram lambs fed either a MF or LF diet.

## 6.2. Introduction

Butyrate is an end product of anaerobic fermentation providing an important source of energy for gastrointestinal tract (**GIT**) tissues (Bergman, 1990) and acts as a signalling molecule (Guilloteau et al., 2010). Numerous studies have evaluated responses of the GIT to butyrate and outcomes include reduced local inflammation (Dianzani et al., 2006; Serpe et al., 2010; Vieira et al., 2012), modulation of the innate immune system (Weber and Kerr, 2006), accelerated regeneration of damaged intestinal epithelia (Mazzoni et al., 2008; Le Gall et al., 2009; Serpe et al., 2010), increased proliferation and differentiation of intestinal epithelial cells (Cook and Sellin, 1998; Furusawa et al., 2013), and inhibited growth of selected pathogenic bacteria (Cook and Sellin, 1998; Manzanilla et al., 2006). In calves, dietary provision of butyrate has been reported to increase ruminal papillae length (Mentschel et al., 2001; Górka et al., 2011a; Kato et al., 2011) by reducing the apoptosis index (Mentschel et al., 2001), and to increase surface area of the small intestine when it is added to milk replacer. In addition, butyrate has been reported to enhance the production of digestive enzymes resulting in a greater growth rate and feed efficiency (Guilloteau et al., 2009).

As a free acid, butyrate has a strong odour and has been postulated to reduce dietary palatability (Lallès et al., 2009; Guilloteau et al., 2010). Butyrate precursors (Tsukahara et al., 2002) and butyrins (i.e. tributyrin; **TB**; Edelman et al., 2003) have been evaluated as potential replacements for butyrate given their lower volatility. However, butyrate precursors and TB may have differing sites of release in the gastrointestinal tract (Guilloteau et al., 2010). Dietary supply of TB to piglets has been reported to increase BW, ADG, feed efficiency (Sotira et al., 2020), and DMI (Gu et al., 2017). As a butyrate source, TB has also been reported to stimulate the development of the intestinal epithelium of nursery piglets (Sakdee et al., 2016). That said, when added to milk replacer fed to calves, Araujo et al. (2016) reported no effects of TB on DMI, growth, or feed efficiency and Inabu et al. (2019) reported a reduction in DMI followed by an increase in glucagon-like peptide-2 (**GLP-2**). In weaned lambs, dietary TB tended to decrease DMI but increased NDF digestibility, and increase the richness and diversity of the ruminal microbial community (Li et al., 2022). A reduction in DMI with dietary TB was also observed in adult ewes (Ren et al., 2018).

Based on the information available, it appears that TB generates inconsistent responses and there are few studies that have evaluated use of TB when lambs are fed moderate and high concentrate diets. Variability in responses may be related to site of butyrate release when TB is fed along with the inherent microbial production of butyrate as high doses of butyrate may induce negative effects (Wilson et al., 2012). When provided in the diet, as a triglyceride, ruminal microbes may hydrolyze TB to yield butyrate and glycerol (Jenkins, 1993) thereby increasing ruminal butyrate. Additionally, dietary characteristics such as the forage-to-concentrate ratio and fermentability of the diet also alter SCFA production, the molar concentration of the individual SCFA (Bergman, 1990; Sutton et al., 2003), and the passage rate of digesta throughout the GIT (NASEM 2016). These responses could change the site of digestion and potentially the composition of produced SCFA. There are suggestions that post-ruminal exposure to butyrate may be more impactful than ruminal exposure (Guilloteau et al., 2010) and hence, ruminal protection of TB may facilitate post-ruminal supply of butyrate. However, there are no studies comparing ruminal and post-ruminal TB supply.

It is hypothesized that provision of ruminally protected TB (**RTB**), but not unprotected TB (**UTB**), to lambs fed a moderate forage (**MF**; Study 1) or a low forage diet (**LF**; Study 2) would improve growth, feed efficiency, serum BHBA, and carcass yield in a dose-dependent manner, without affecting DMI. The objective of this study was to evaluate the effect of dose of UTB and RTB on DMI, growth, feed efficiency, serum BHBA, and carcass characteristics of lambs fed diets containing moderate forage (**MF**) or low levels of forage (**LF**) inclusion.

## 6.3. Material and methods

Use of lambs in this experiment was approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20190138, Saskatoon, SK, Canada) and followed the guidelines of the Canadian Council on Animal Care (Ottawa, ON, Canada).

### 6.3.1. Experimental design, dietary treatments, and growth performance

Eighty-four ram lambs were stratified by initial BW and randomly assigned into 1 of 2 studies (42 lambs/study) that evaluated use of TB in diets containing 40% (MF; 27.9 ± 2.44 kg; mean ± SD) or 10% (LF; 27.9 ± 2.12 kg) forage as described in Tables 6.1 and 6.2. Within each study, lambs were stratified by BW and within strata assigned to 1 of 6 treatments (*n* = 7 rams/treatment)

including a negative control (**CON**; no dietary inclusion), dietary inclusion of RTB (containing 22.5% of TB) at 0.1% (**RTB1**), 0.2% (**RTB2**), and 0.3% (**RTB3**), or dietary inclusion of UTB (containing 90% of TB) at 0.1% (**UTB1**) and 0.3% (**UTB3**).

At receiving, lambs were group-housed in an outside pen with continuous access to water and fed grass hay (ad libitum). Lambs were housed indoor in individual pens ($3m^2$) bedded with wood shavings and fed once daily (1000 h) for 97 d. At the start of each study, lambs were adapted either to a MF or a LF diet. The MF diet transition consisted of a 5-d single-step adaptation protocol with lambs initially fed a diet containing 65% forage until they were transitioned to their final diet containing 40% forage (MF) on d 6. In Study 2, the transition to the LF diets consisted of a 20-d adaptation protocol utilizing intermediary 4 diets. The initial and intermediary diets contained 65, 40, 30, and 20% forage with each diet fed for 5 d. Lambs were then fed their final diet containing 10% forage (LF) with feeding of this diet initiated on d 21.

To ensure ad libitum intake, refusals were collected and weighed prior to feeding, and the amount of feed offered was adjusted to target refusals that weighed 3 to 5% of the feed offered (as is basis). Refusals were composited for each lamb on a weekly basis and a representative sample was collected for determination of DM concentration. Corn silage was collected twice weekly and dry ingredients (barley grain, canola meal, and the mineral and vitamin supplement) were collected weekly. Feed ingredients and refusals were dried in a forced-air over at 55ºC until a constant weight was achieved. Dry matter coefficients were used to calculate weekly DMI and for feed samples, to ensure that the mixed diet represented the DM formulation**.**

Body weights were measured at the start and end of the study, and every two weeks during the study. Initial and final BW were assessed using the average BW measured on two consecutive days, and additional BW measurements were collected every two weeks on a single day. The ADG was calculated by regressing BW and days on feed with the slope equal to ADG. The ADG and average DMI were used to calculate gain-to-feed (**G:F**) ratio.

### 6.3.2. Blood collection and analysis

On day 93, blood samples were collected from each lamb prior to feeding (1000 h) and 6 h later to evaluate changes in BHBA. Samples were collected from the jugular vein into a 10-mL vacutainer vial containing a clot activator (BD Vacutainer, Franklin Lakes, NJ). Blood samples

**Table 6.1.** Feed ingredients of the experimental diets fed to growing ram lambs on the moderate forage (MF) group (n = 42) containing increasing doses of either rumen-protected tributyrin (RTB) or unprotected tributyrin (UTB).

| | Diet | |
|---|---|---|
| | Step 1 | MF |
| Days on feed | 5 | 92 |
| Ingredient, % of DM | | |
| Corn silage | 65.00 | 40.00 |
| Barley grain | 18.34 | 43.34 |
| Canola meal | 11.67 | 11.67 |
| Vitamin and mineral supplement[1] | | |
| Ammonium chloride | 0.833 | 0.833 |
| Limestone | 0.366 | 0.366 |
| Salt | 0.102 | 0.102 |
| Lasalocid[2] | 0.058 | 0.058 |
| Premix[3] | 0.079 | 0.079 |
| Barley grain, ground | 1.177 | 1.177 |
| Calcium phosphate mono | 0.854 | 0.854 |
| Treatment carrier[4] | 1.52 | 1.52 |
| Chemical composition, % DM ± SD[5] | | |
| Dry matter | 43.57 ± 0.09 | 53.35 ± 2.14 |
| Organic matter | 93.66 ± 0.59 | 94.16 ± 0.40 |
| Crude protein | 14.89 ± 0.40 | 15.55 ± 0.26 |
| Acid detergent fiber | 19.67 ± 1.94 | 15.36 ± 0.93 |
| Neutral detergent fiber | 34.33 ± 2.99 | 28.34 ± 1.53 |
| Starch | 29.38 ± 2.99 | 38.49 ± 2.17 |
| Ca | 0.54 ± 0.02 | 0.49 ± 0.02 |
| P | 0.53 ± 0.01 | 0.56 ± 0.01 |

[1]Mineral and vitamin premix contained (% DM) 77.3 g/kg of Ca; 56 g/kg of Mg; 4.3 g/kg of Mg; 2.1 g/kg of K; 12.4 g/kg of Na; 185.9 g/kg of Cl; 3.6 g/kg of S; 722 mg/kg of Mn; 3.6 mg/kg of Cu; 2,994 mg/kg of Fe; 799 mg/kg of Zn; 31 mg/kg of I, 21 mg/kg of Co, 5.2 mg/kg of Se; 170,947.5 IU/kg of vitamin A; 18,106.9 IU/kg of vitamin D; and 1,163.94 IU/kg of vitamin E.
[2]Bovatec (Zoetic, Canada Inc., Kirkland, QC), 20% of lasalocid sodium.
[3]Gowan's Feed Consulting lamb premix (MasterFeeds, Lethbridge, AB) which contained (on DM basis): 7,980,172 IU/kg of vitamin A; 842,090 IU/kg of vitamin D; 84,131 IU/kg of vitamin E, 8,500 mg/kg of biotin; 28,985 mg/kg of Mn; 834 mg/kg of Co; 1,429 mg/kg of I; 238.2 mg/kg of Se; 75,000 mg/kg of calcium carbonate; 10,000 mg/kg of mineral oil; and 174,275 mg/kg of wheat midds.
[4]Treatment carrier was a mixture of palmitic acid (Jefo Dairy Fat, Jefo, Saint-Hyacinthe, QC) and either rumen-protected tributyrin (RTB) or unprotected tributyrin (UTB) as follows: CON: 1.52% of carrier; RTB1: 1.01% of carrier and 0.51% of RTB; RTB2: 0.51% of carrier and 1.01% of RTB; RTB3: 1.52% of rumen-protected tributyrin; UTB1: 1.405% of carrier and 0.117% of UTB; and UTB3: 1.173% of carrier and 0.348% of UTB.
[5]Average ± SD of 3 samples representing beginning, middle, and final of the experimental period.

**Table 6.2.** Feed ingredients of the experimental diets fed to growing ram lambs on the low forage (LF) group (n = 42) containing increasing doses of either rumen-protected tributyrin (RTB) or unprotected tributyrin (UTB).

| | Diet | | | | |
|---|---|---|---|---|---|
| | Step 1 | Step 2 | Step 3 | Step 4 | LF |
| Days on feed | 5 | 5 | 5 | 5 | 77 |
| Ingredient, % of DM | | | | | |
| Corn silage | 65.00 | 40.00 | 30.00 | 20.00 | 10.00 |
| Barley grain | 18.34 | 43.34 | 54.54 | 65.81 | 77.01 |
| Canola meal | 11.67 | 11.67 | 10.47 | 9.20 | 8.00 |
| Vitamin and mineral supplement[1] | | | | | |
| Ammonium chloride | 0.833 | 0.833 | 0.833 | 0.833 | 0.833 |
| Limestone | 0.366 | 0.366 | 0.366 | 0.366 | 0.366 |
| Salt | 0.102 | 0.102 | 0.102 | 0.102 | 0.102 |
| Lasalocid[2] | 0.058 | 0.058 | 0.058 | 0.058 | 0.058 |
| Premix[3] | 0.079 | 0.079 | 0.079 | 0.079 | 0.079 |
| Barley grain, ground | 1.177 | 1.177 | 1.177 | 1.177 | 1.177 |
| Calcium phosphate mono | 0.854 | 0.854 | 0.854 | 0.854 | 0.854 |
| Treatment carrier[4] | 1.52 | 1.52 | 1.52 | 1.52 | 1.52 |

**Table 6.2 continued.** Feed ingredients of the experimental diets fed to growing ram lambs on the low forage (LF) group (n = 42) containing increasing doses of either rumen-protected tributyrin (RTB) or unprotected tributyrin (UTB).

| | Diet | | | | |
|---|---|---|---|---|---|
| | Step 1 | Step 2 | Step 3 | Step 4 | LF |
| Chemical composition, % DM ± SD[5] | | | | | |
| Dry matter | 43.57 ± 0.09 | 53.35 ± 2.14 | 60.24 ± 0.14 | 67.89 ± 0.26 | 76.44 ± 1.41 |
| Organic matter | 93.66 ± 0.59 | 94.16 ± 0.40 | 94.41 ± 0.34 | 94.67 ± 0.29 | 94.92 ± 0.27 |
| Crude protein | 14.89 ± 0.40 | 15.55 ± 0.26 | 15.44 ± 0.22 | 15.32 ± 0.19 | 15.21 ± 0.18 |
| Acid detergent fiber | 19.67 ± 1.94 | 15.36 ± 0.93 | 13.48 ± 0.53 | 11.59 ± 0.21 | 9.71 ± 0.40 |
| Neutral detergent fiber | 34.33 ± 2.99 | 28.34 ± 1.53 | 25.71 ± 0.94 | 23.08 ± 0.36 | 20.45 ± 0.23 |
| Starch | 29.38 ± 2.99 | 38.49 ± 2.17 | 42.88 ± 1.86 | 47.32 ± 1.55 | 51.71 ± 1.26 |
| Ca | 0.54 ± 0.02 | 0.49 ± 0.02 | 0.46 ± 0.02 | 0.43 ± 0.02 | 0.40 ± 0.02 |
| P | 0.53 ± 0.01 | 0.56 ± 0.01 | 0.56 ± 0.01 | 0.56 ± 0.01 | 0.56 ± 0.01 |

[1]Mineral and vitamin premix contained (% DM) 77.3 g/kg of Ca; 56 g/kg of Mg; 4.3 g/kg of Mg; 2.1 g/kg of K; 12.4 g/kg of Na; 185.9 g/kg of Cl; 3.6 g/kg of S; 722 mg/kg of Mn; 3.6 mg/kg of Cu; 2,994 mg/kg of Fe; 799 mg/kg of Zn; 31 mg/kg of I, 21 mg/kg of Co, 5.2 mg/kg of Se; 170,947.5 IU/kg of vitamin A; 18,106.9 IU/kg of vitamin D; and 1,163.94 IU/kg of vitamin E.

[2]Bovatec (Zoetic, Canada Inc., Kirkland, QC), 20% of lasalocid sodium.

[3]Gowan's Feed Consulting lamb premix (MasterFeeds, Lethbridge, AB) which contained (on DM basis): 7,980,172 IU/kg of vitamin A; 842,090 IU/kg of vitamin D; 84,131 IU/kg of vitamin E, 8,500 mg/kg of biotin; 28,985 mg/kg of Mn; 834 mg/kg of Co; 1,429 mg/kg of I; 238.2 mg/kg of Se; 75,000 mg/kg of calcium carbonate; 10,000 mg/kg of mineral oil; and 174,275 mg/kg of wheat midds.

[4]Treatment carrier was a mixture of palmitic acid (Jefo Dairy Fat, Jefo, Saint-Hyacinthe, QC) and either rumen-protected tributyrin (RTB) or unprotected tributyrin (UTB) as follows: CON: 1.52% of carrier; RTB1: 1.01% of carrier and 0.51% of RTB; RTB2: 0.51% of carrier and 1.01% of RTB; RTB3: 1.52% of rumen-protected tributyrin; UTB1: 1.405% of carrier and 0.117% of UTB; and UTB3: 1.173% of carrier and 0.348% of UTB.

[5]Average ± SD of 3 samples representing beginning, middle, and final of the experimental period.

were chilled on ice for 30 min and then centrifuged at 1,800 × *g* for 20 min at 4°C. Serum was transferred into a vial and stored at -20°C until analysis. The serum BHBA concentration was determined using 3-hydroxybutyrate dehydrogenase (H6501, Roche, Mississauga, Ontario Canada) to catalyze the oxidation of BHBA to acetoacetate (Williamson et al., 1962). Samples were analyzed in triplicate and samples with an intra-assay CV greater than 5% were repeated until achieving a CV ≤ 5%. The resulting BHBA inter- and intra-assay CV were 3.50% and 4.92, respectively.

### 6.3.3. Slaughter and carcass traits measurements

After 97 d of feeding, lambs were transported to SunGold Specialty Meats Ltd. (Innisfail, AB, Canada) and the hot carcasses weight (**HCW**) and back-fat thickness were assessed. Hot carcass weight was expressed as a percentage of final full BW to calculate dressing percentage.

### 6.3.4. Statistical analysis

Within study (Study 1 and Study 2), data were analyzed using the PROC MIXED of SAS (version 9.4, SAS Institute Inc., Cary, NC) as a 2 × 2 + 2 factorial. The treatments included in the 2×2 factorial included RTB1, RTB3, UTB1, and UTB3. For this analysis the fixed effects of protection (RTB vs. UTB), dose (0.1 vs. 0.3%), and the protection × dose interaction were included along with the random effect of lamb. Data were also analyzed using the fixed effect of dietary treatment, random effect of lamb, and polynomial contrasts were used to determine the linear and quadratic responses for increasing dose of RTB. For BHBA, the fixed effects also included time and treatment × time interactions with time included as a repeated measure. The heterogeneous compound symmetry was selected as it yielded the least Akaike's and Bayesian Information Criterion values for both Study 1 and Study 2. For all variables, significance was declared when $P \leq 0.05$ and tendencies were considered when $0.05 < P \leq 0.10$.

## 6.4. Results

### 6.4.1. Study 1: Effect of TB for lambs fed a diet containing a moderate forage inclusion

Based on study design, initial BW was not affected by treatment ($P \geq 0.96$); however, final BW tended to be affected by a protection × dose interaction ($P = 0.06$), where lambs fed UTB3 tended to finish the experiment heavier than lambs fed RTB3. In addition, ADG tended to be

affected by a protection × dose interaction ($P = 0.06$), where lambs fed with both RTB1 and UTB3 tended to have greater overall ADG than RTB3. Dry matter intake ($P \geq 0.19$), G:F ($P \geq 0.30$), and carcass characteristics ($P \geq 0.11$) were not affected by treatments (Table 6.3). Serum BHBA was not affected by treatments but was affected by time ($P = 0.01$), where the concentration of BHBA increased from pre feeding (0.336 mmol/L; data not shown) to 6 h after feeding (0.389 mmol/L).

### 6.4.2. Study 2: Effect of TB for lambs fed a diet containing a low forage inclusion

For lambs fed with the low forage diet, treatment did not affect initial ($P \geq 0.96$) or final BW ($P \geq 0.61$), ADG ($P \geq 0.47$), or carcass characteristics ($P \geq 0.11$). However, use of RTB tended ($P = 0.10$) to increase DMI (RTB = 1.71 kg/d and UTB = 1.50 kg/d; $P = 0.07$) and decrease G:F relative to UTB. The UTB-fed lambs tended to have greater serum BHBA relative to RTB fed lambs (RPT = 0.341 mmol/L and UTB = 0.405 mmol/L; $P = 0.10$) as shown in Table 6.4. In addition, BHBA was affected by time ($P = 0.03$), where the BHBA concentration increased from pre-feeding (0 h; 0.352 mmol/L; data not shown) to 6 h after feeding (0.395 mmol/L).

## 6.5. Discussion

Provision of butyrate has been reported to enhance gastrointestinal health by acting as an anti-inflammatory agent (Serpe et al. 2010; Dianzani et al. 2006; Vieira et al. 2012), while promoting functionality (Basson and Hong, 1998; Peng et al., 2007), and development of the GIT (Manzanilla et al., 2006; Górka et al., 2011a; Liu et al., 2017). However, effects of butyrate for ruminants have mostly been evaluated in pre-weaned calves (Guilloteau et al., 2009; Górka et al., 2011a; Araujo et al., 2016) and in lactating dairy cows (Hamada et al., 1984; Kowalski et al., 2015; Fukumori et al., 2020). There is minimal information on the effects of ruminal or intestinal provision of butyrate fed in diets with differing forage-to-concentrate ratios. Feeding diets containing different forage-to-concentrate ratios (i.e., high-forage *vs.* high-grain diets) lead to differences in SCFA production and changes the molar proportions of individual SCFA (Bergman 1990; Sutton et al., 2003). The forage-to-concentrate ratio also affects digesta passage rate (NASEM 2016), potentially leading to differences in nutrient digestion and consequently fermentation in the distal GIT.

Although the experiments were not designed to compare MF and LF directly, the data from these two experiments provide new information on how TB impacts growth performance of

lambsfed MF and LF. In Study 1, tendencies for greater BW and G:F were observed when lambs were provided either dose (0.1 and 0.3% DM) of RTB and when provided the high dose (0.3%) of the UTB, relative to the low dose of UTB (0.1%). It is postulated that providing the greater dose of UTB and both doses of RTB may have led to greater post-ruminal supply of butyrate than the low UTB dose. However, the lack of a dose response for RTB challenges the previous suggestion. Recently, Watanabe et al. (2023) reported that abomasal provision of butyrate stimulated absorption of SCFA across the rumen relative to heifers not provided butyrate. Stimulation of SCFA absorption could account for greater performance without changes in DMI. Several other studies have reported that intestinal supply of butyrate stimulates ruminal development (Górka et al., 2009; Górka et al., 2011a) and it has been hypothesized that post-ruminal butyrate supply may stimulate these effects via paracrine or endocrine signalling (Plöger et al., 2012). Future research is needed to confirm whether dose and form of TB affect post-ruminal butyrate flow and its release as a mechanism to alter growth and feed efficiency.

In Study 2, it was observed that RTB tended to increase DMI without changes in ADG, thereby resulting in a tendency for reduced G:F relative to UTB. Moreover, BHBA tended to be greater for lambs fed UTB than those fed RPT, supporting the potential for increased ruminal butyrate supply with UTB as compared ti RTP. Incubation of TB in vitro and provision of TB in vivo have been reported to increase butyrate concentration; however, in vitro there were very limited effects on other SCFA while in vivo TB increased acetate, propionate, and butyrate while decreasing pH (Ren et al., 2018). It is unclear why provision of RPT may have stimulated intake without altering growth, but this response may be related to a larger GIT driven be greater post-ruminal butyrate supply (Górka et al., 2009; Guilloteau et al., 2009). However, the lack of differences in hot carcass weight and dressing percent challenge this interpretation.

**Table 6.3.** Growth, feed intake, carcass characteristics and serum β-hydroxybutyrate (**BHBA**) of growing ram lambs (n = 42) fed for 97 d with a moderate forage diet and supplemented with different levels of tributyrin in a rumen-protected (**RTB**) and unprotected form (**UTB**).

| | Treatments[1] | | | | | | SED[2] | *P*-values[3] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | CON | RTB1 | RTB2 | RTB3 | UTB1 | UTB3 | | P | D | I | L | Q |
| Initial body weight, kg | 27.91 | 27.93 | 27.93 | 28.00 | 27.90 | 27.94 | 1.490 | 1.00 | 0.97 | 1.00 | 0.96 | 0.98 |
| Final body weight, kg | 54.76 | 54.17 | 55.40 | 50.71 | 51.19 | 55.05 | 2.340 | 0.72 | 0.92 | 0.06 | 0.15 | 0.23 |
| Average daily gain, kg/d | 0.277 | 0.281 | 0.278 | 0.242 | 0.251 | 0.279 | 0.0238 | 0.84 | 0.74 | 0.06 | 0.16 | 0.23 |
| Dry matter intake, kg/d | 1.43 | 1.35 | 1.56 | 1.31 | 1.32 | 1.55 | 0.146 | 0.33 | 0.35 | 0.19 | 0.78 | 0.41 |
| Gain-to-feed ratio, kg/kg | 0.196 | 0.199 | 0.183 | 0.182 | 0.182 | 0.181 | 0.0176 | 0.46 | 0.45 | 0.48 | 0.30 | 0.87 |
| How carcass weight, kg | 24.64 | 24.09 | 24.84 | 22.64 | 23.36 | 24.47 | 1.144 | 0.57 | 0.86 | 0.19 | 0.16 | 0.32 |
| Dressing percentage, % | 45.07 | 44.42 | 44.83 | 44.63 | 45.58 | 44.46 | 0.785 | 0.39 | 0.43 | 0.25 | 0.72 | 0.69 |
| Fat thickness, mm | 13.86 | 13.86 | 15.29 | 12.43 | 14.00 | 15.43 | 1.643 | 0.11 | 1.00 | 0.15 | 0.59 | 0.23 |
| BHBA[4], mmol/L | 0.431 | 0.349 | 0.352 | 0.354 | 0.335 | 0.350 | 0.0518 | 0.80 | 0.80 | 0.89 | 0.17 | 0.26 |

[1]CON: no dietary inclusion of TB, 1.52% of carrier; RTB1: 1.01% of carrier and 0.51% of RTB; RTB2: 0.51% of carrier and 1.01% of RTB; RTB3: 1.52% of rumen-protected tributyrin; UTB1: 1.405% of carrier and 0.117% of UTB; and UTB3: 1.173% of carrier and 0.348% of UTB (% of DM).

[2]SED: standard error of the difference.

[3]P: protection main effect between the dietary inclusion of rumen-protected (RTB1 and RTB3) and unprotected (UTB1 and UTB3) tributyrin; D: dose main effect between the dietary inclusion of 0.1 (RTB1 and UTB1) and 0.3% (RTB3 and UTB3) of tributyrin; I: interaction between protection and dose main effects; L: linear contrast among the dietary inclusions of rumen-protected tributyrin (CON, RTB1, RTB2, and RTB3); Q: quadratic contrast among the dietary inclusions of rumen protected tributyrin (CON, RTB1, RTB2, and RTB3).

[4]β-hydroxybutyrate; BHBA was analysed at time 0 (prior to feeding) and time 6 (6 h after feeding); $P_{\text{treatment}} = 0.43$, $P_{\text{time}} = 0.01$, and $P_{\text{treatment} \times \text{time}} = 0.90$; $BHBA_{\text{time 0}}$ = 0.336 mmol/L and $BHBA_{\text{time 6}}$ = 0.389 mmol/L.

**Table 6.4.** Growth, feed intake, carcass characteristics and serum β-hydroxybutyrate (**BHBA**) of growing ram lambs (n = 42) fed for 97 d with a low forage diet and supplemented with different levels of tributyrin in a rumen-protected (**RTB**) and unprotected form (**UTB**).

| | Treatments[1] | | | | | | SED[2] | *P*-values[3] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Item | CON | RTB1 | RTB2 | RTB3 | UTB1 | UTB3 | | P | D | I | L | Q |
| Initial body weight, kg | 27.95 | 27.93 | 27.90 | 27.98 | 27.91 | 27.94 | 1.268 | 0.98 | 0.96 | 0.99 | 0.99 | 0.96 |
| Final body weight, kg | 55.13 | 57.57 | 52.79 | 57.25 | 56.57 | 56.86 | 2.726 | 0.74 | 0.99 | 0.88 | 0.86 | 0.61 |
| Average daily gain, kg/d | 0.295 | 0.324 | 0.268 | 0.313 | 0.315 | 0.302 | 0.0193 | 0.54 | 0.46 | 0.95 | 0.97 | 0.56 |
| Dry matter intake, kg/d | 1.56 | 1.67 | 1.36 | 1.75 | 1.52 | 1.48 | 0.156 | 0.07 | 0.83 | 0.62 | 0.60 | 0.21 |
| Gain-to-feed ratio, kg/kg | 0.182 | 0.188 | 0.192 | 0.175 | 0.199 | 0.202 | 0.0159 | 0.10 | 0.66 | 0.46 | 0.74 | 0.31 |
| How carcass weight, kg | 25.26 | 26.47 | 24.34 | 26.20 | 24.81 | 25.69 | 1.503 | 0.32 | 0.78 | 0.60 | 0.88 | 0.77 |
| Dressing percentage, % | 45.77 | 45.98 | 46.10 | 45.66 | 43.92 | 45.15 | 0.934 | 0.11 | 0.56 | 0.33 | 0.94 | 0.62 |
| Fat thickness, mm | 15.86 | 16.43 | 18.00 | 18.42 | 16.57 | 15.71 | 2.326 | 0.38 | 0.70 | 0.33 | 0.22 | 0.97 |
| BHBA[4], mmol/L | 0.372 | 0.335 | 0.378 | 0.347 | 0.391 | 0.420 | 0.0447 | 0.10 | 0.59 | 0.81 | 0.81 | 0.92 |

[1]CON: no dietary inclusion of TB, 1.52% of carrier; RTB1: 1.01% of carrier and 0.51% of RTB; RTB2: 0.51% of carrier and 1.01% of RTB; RTB3: 1.52% of rumen-protected tributyrin; UTB1: 1.405% of carrier and 0.117% of UTB; and UTB3: 1.173% of carrier and 0.348% of UTB (% of DM).

[2]SED: standard error of the difference.

[3]P: protection main effect between the dietary inclusion of rumen-protected (RTB1 and RTB3) and unprotected (UTB1 and UTB3) tributyrin; D: dose main effect between the dietary inclusion of 0.1 (RTB1 and UTB1) and 0.3% (RTB3 and UTB3) of tributyrin; I: interaction between protection and dose main effects; L: linear contrast among the dietary inclusions of rumen-protected tributyrin (CON, RTB1, RTB2, and RTB3); Q: quadratic contrast among the dietary inclusions of rumen protected tributyrin (CON, RTB1, RTB2, and RTB3).

[4]β-hydroxybutyrate; BHBA was analysed at time 0 (prior to feeding) and time 6 (6 h after feeding); $P_{treatment} = 0.58$, $P_{time} = 0.03$, and $P_{treatment \times time} = 0.84$; $BHBA_{time\ 0} = 0.352$ mmol/L and $BHBA_{time\ 6} = 0.395$ mmol/L.

The differing results observed in Study 1 and Study 2 lead to the suggestion that dietary factors such as the forage-to-concentrate ratio may alter outcomes arising from butyrate supplementation. It has been reported that the forage-to-concentrate ratio affects ruminal pH such that a reduction in forage inclusion from 91.7 to 35.8% (DM basis) resulted in a reduction in mean ruminal pH from 6.48 to 6.03, respectively (Penner et al., 2009b). In beef heifers fed high forage (92% of forage) and moderate forage (60% of forage) diets, the mean ruminal pH only tended to be reduced for heifers fed moderate-forage relative to a high-forage. However, the minimum ruminal pH was reduced from 6.3 to 5.9. Thus, due to limited data collection in Study 1 and Study 2, it is only possible to speculate that due to the different forage-to-concentrate ratio, that ruminal pH was reduced to lambs on Study 2, relative to lambs on Study 1.

Lipolytic enzymes are produced by several microorganisms in the rumen, and their activity is dependent on pH and temperature (Privé et al., 2013; Privé et al., 2015). Thus lipase activity could change based on the fermentability of the diet (Henderson, 1971). As an example, lipase activity was reduced by 38% when the pH was decreased from 6.8 to 5.9 in vitro (Van Nevel and Demeyer, 1996). As lipase is responsible for hydrolyzing glycerol to release free fatty acids (Jenkins, 1993; Privé et al., 2015) alterations in lipase activity may partially explain differential results between Study 1 and Study 2. Thus, it is possible that the butyrate release in the rumen was partially inhibited due to lesser lipase activity. However, as lipase activity was not measured, future research is needed to evaluate flow of tributyrin and its release as affected by dietary formulation.

Despite the inconsistency in data, it seems that the site of butyrate delivery into the GIT is essential to the expression of positive outcomes. When butyrate (sodium butyrate) was provided to dairy calves mixed either in the milk replacer (post-ruminally) or in the starter mixture (ruminally), both stimulated ruminal papillae growth. However, only the addition into milk replacer positively affected growth and tended to reduce the duration on electrolyte therapy within the first week of the experiment (Górka et al., 2011a). On the other hand, small intestine development was stimulated for both dietary forms of provision, but the authors concluded that intestinal development were more pronounced when butyrate was added to milk replacer than into the starter mixture (Górka et al., 2014). In a recent study, abomasal butyrate infusion in beef heifers increased SCFA absorption across the reticulo-rumen relative to the negative control, showing that butyrate delivered post-ruminally stimulated ruminal epithelial functionality (Watanabe et al.,

2023). It may be possible that butyrate supply to the abomasum or proximal duodenum may have a greater stimulatory effect on gastrointestinal function than more distal release as would occur with TB. The previous statement may help explain why independent of diet, provision of TB did not affect HCW or carcass characteristics. More research is needed to confirm the efficacy of protection and the site of release of the rumen-protected TB and to evaluate how site of butyrate exposure affects responses.

## 6.6. Conclusion

In Study 1, where lambs were fed a MF diet, the provision of TB (both rumen-protected and unprotected) did not affect growth, feed efficiency, serum BHBA, or carcass characteristics relative to CON. However, in Study 2, where lambs were fed a LF diet, tendencies for increased feed efficiency and serum BHBA for lambs fed with UTB relative to lambs fed with RTB, indicated the importance of site of release of butyrate within the GIT of ruminants. Overall, unprotected and rumen protected forms of tributyrin do not seem to have marked effects on feed intake, feed efficiency, or carcass characteristics for growing lambs fed medium or low forage diets.

# 7. GENERAL DISCUSSION

## 7.1. Potential modes of action for HFCG

As noted throughout this thesis, provision of low doses of HFCG have been reported to increase milk fat yield for dairy cattle and Trouw Nutrition (Amersfoort, the Netherlands) is currently working on a submission to register a claim with the Canadian Food Inspection Agency indicating increased production of milk, fat, and protein IN dairy cattle. Based on the reported outcomes, the mode of action for rumen protected HFCG was proposed (Doelman et al., 2019a). The most promoted theory was that HFCG could serve as a butyrate precursor as CaG can be fermented to lactate and subsequently butyrate. Support for this theory was driven largely based on in vitro studies (Tsukahara et al., 2002; Biagi et al., 2006). As such, the experiments in this thesis were designed to test effects of CaG as a butyrate precursor and the impact of butyrate or butyrate derivatives (butyrins) on gastrointestinal tract function and growth of cattle.

## 7.2. Calcium gluconate as a butyrate precursor

It was hypothesized that provision of CaG whether provided in a rumen protected form (HFCG; Chapter 3, 4, and 5) or using abomasal infusion of CaG (Chapter 4) would stimulate butyrate production or increase butyrate supply in the intestine. Calcium gluconate has been identified as a butyrate precursor based on results from in vitro studies conducted by Tsukahara et al. (2002, 2006) and a biochemical pathway for metabolism has been presented (Figure 2.1) which results in the conversion of gluconate to lactate and then butyrate. Furthermore, it has been speculated that CaG will have the same affect in ruminants given that post-ruminal infusion of CaG in lactating dairy cows resulted in increased milk fat concentration and tended to increase energy-corrected and milk fat yields (Doelman et al., 2019a), while improving feed efficiency (McKnight et al., 2019). Similar responses were observed when lactating dairy cows were fed a rumen-protected form of CaG (HFCG) as reported by Seymour et al. (2021, 2022). These authors also reported that feeding HFCG tended to increase fat-, energy-corrected milk yield, and milk fat yields. However, due the nature of these studies, none of them were able to confirm an increase of butyrate concentration in the intestine. Results for increased butyrate in the intestine are limited with Rowland et al. (2022) reporting that HFCG increased acetate concentration in the cecum and rectum, propionate concentration in the cecum, colon, and rectum, and tended to increase butyrate concentration in the colon of steers fed a finishing diets. Similar to Doelman et al. (2019) and

Seymour et al. (2021) where changes in the fecal SCFA composition were observed, the studies reported in this thesis reported changes in intestinal and fecal SCFA concentrations, but no change in butyrate.

The possibility of CaG being degraded in the rumen due to inadequate ruminal protection can be challenged as the in situ incubation described in Chapter 3 showed only limited (13.4 ± 2.89% of DM) disappearance after 24 h of incubation. In addition, the lack of detection of increased intestinal butyrate in in vivo studies relative to in vitro studies could be due to the accumulation of end-products in in vitro systems as there is no removal of end-products. Moreover, among the main SCFA, butyrate is reportedly absorbed the fastest due its chain length (Walter and Gutknecht, 1986) and rapid intracellular metabolism rate resulting in a flux that increases its permeability (Kristensen and Harmon, 2004). Thus, a single time-point for collection of the intestine digesta (at slaughter for Chapters 3, 4, and 5) may be insufficient to show whether HFCG is being converted to butyrate in the intestine of ruminants. In addition, rectal sampling (fecal collection on Chapter 5) may not detect butyrate concentration increases due its conversion and absorption in the proximal large intestine so that it does not reach distal regions. As previously mentioned, the first step in the fermentation of gluconic acid is its transformation to lactate (Figure 2.1), as confirmed by the in vitro findings of Tsukahara et al. (2002, 2006). These authors showed that the addition of gluconic acid in an in vitro system containing porcine cecal digesta increased the *Lactobacillaceae*, leading to an initial increase in acetate and lactate concentration. However, the same studies showed that this increase occurs in the presence of *Megasphaera elsdenii* which can convert both acetate and lactate to butyrate. In addition, as reported by Koyun et al. (2022), dietary inclusion of HFCG in feedlot steers increased the abundance *Firmicutes* in selected regions of the large intestine. As *Megasphaera elsdenii* is a member of the phylum *Firmicutes* it is possible to speculate that dietary provision of HFCG in feedlot steers (Koyun et al., 2022) is leading to similar alteration in the large intestine microbiota as suggested by the in vitro studies of Tsukahara et al. (2002, 2006).

While at low doses, increased butyrate was not observed, it is clear that HFCG has the potential to modulate intestinal fermentation (Chapter 4), as some of the changes mirrored that observed with AB in terms of a decrease in the molar proportion of acetate and an increase of molar proportion in the propionate in colonic digesta. However, the increase in SCFA

disappearance in the rumen observed with AB, was not observed for CaG. In Chapter 3, HFCG modulated cecal fermentation as indicated by increasing acetate and decreasing propionate concentration, which was the opposite observed in the colonic digesta in Chapter 4. In addition, in Chapter 5, heifers fed the intermediate dose (CG09) tended to have a decreased molar proportion of acetate in the colon and increased valerate and tended to have greater proportions of isovalerate. Based on in vivo experiments reported in the literature, piglets receiving increased doses of gluconic acid did not have altered SCFA composition in the jejunum, ileum, and colon (Biagi et al., 2006). However, dairy calves supplemented with sodium butyrate had increased concentrations, but not molar proportions, of total SCFA, acetate, and propionate. However, the question for whether HFCG serves as a butyrate precursor in the intestine of ruminants remains unclear and future research is needed to confirm whether intestinal microbiota from cattle fed a variety of diets converts CaG to butyrate.

### 7.3. Dose of HFCG

Doses of HFCG or CaG used in this thesis were based on doses that yielded positive outcomes for dairy cows as described by Doelman et al. (2019), McKnight et al. (2019), and Seymour et al. (2021; Chapters 3 and 5) or doses that were expected to provide an equimolar release of butyrate to the intestine relative to AB (Chapter 4). Abomasal infusion of unprotected CaG in lactating dairy cows at doses of 44, 93, 140, and 187 g/d resulted in an increase in milk fat concentration and a tendency to increased milk fat yield and ECM yield compared to controls (no infusion of CaG; Doelman et al., 2019). In addition, quadratic responses were observed for fecal isobutyrate and plasma BHBA, indicating that the lowest dose (44 g/cow/d) maximized the outcomes of both variables. Lactating dairy cows infused with a daily dose of 44 g of CaG had an average DMI of 25.4 kg/d throughout the experiment, thus the abomasal infusion of 44 g of CaG represented a dietary inclusion of 0.17% (DM basis). Using the optimal dose described by Doelman et al. (2019), McKnight et al. (2019) titrated smaller doses of abomasally infused CaG resulting in the doses 0, 17, 32, and 46 g/cow/d. These authors reported a linear decrease in DMI without any changes in milk yield, resulting in a linear increase in feed efficiency. As responses were linear, the greatest dose infused (46 g/cow/d) yielded the best outcome. Following the same process as above to determine dietary concentration, cows infused with 46 g/d of CaG had an

average DMI of 23.1 kg/d, resulting in a dosage of 0.20% on a DM basis. Thus, addition of 0.20% ruminal bypass of CaG in the diet yielded the most positive results for lactating dairy cows.

To allow for a fair comparison among dairy cows and beef heifers, my approach used a dose that was adjusted for body weight (Chapters 4). The optimal dose, 44 and 46 g/cow/d of CaG infused into the abomasal of dairy cows as per Doelman et al. (2019) and McKnight et al. (2019) represented dosages of 0.006% and 0.007% of BW, respectively. Thus, the abomasal infusion of CaG in beef cows was set at 0.0077% of BW, resulting in the infusion of approximately 30.5 g/d of CaG into the abomasum, which led to a HFCG ruminal infusion of 0.0192% of BW (RG), to achieve the same amount of CaG as administered into the abomasum (HFCG contains 40% of CaG). As described by Seymour et al. (2021), the dietary inclusion of HFCG for dairy cows was 0.07% of the diet (DM basis) to reach a daily intake of 16 g of HFCG and 6.40 g of CaG at a DMI of 22.3 kg. To reach the same level of supplementation to beef heifers fed with a high-grain diet in Chapter 5, the overall DMI was estimated at 8.9 kg/d leading to a dietary inclusion of 0.18% of HFCG. As a percentage of BW, CG09 and CG18 represents 0.002 and 0.004% of BW, respectively (Table 7.1). The intake of HFCG based on percentage of BW can then be compared to the doses administered to growing lambs in Chapter 3, where the lowest dose tested on beef heifers (CG09; Chapter 5) and the lowest dose tested on growing lambs (LOW; Chapter 3) represented an intake of 0.002% of BW. In addition, Chapter 3 explored a wide range of dosages, from 0.002 to 0.014% of BW (Table 7.1), possibly allowing for negative effects of high supplementation of butyrate to be detected as per Wilson et al. (2012).

**Table 7.1.** Doses of HFCG represented as g/d, %DM, and %BW in Chapter 3, 4, and 5.

| Chapter | Animal | Treatment[1] | HFCG doses, g/d[2] | HFCG dose, % DM | HFCG dose, % BW[3] |
|---|---|---|---|---|---|
| 3 | Lambs | CON | 0.00 | 0.000 | 0.000 |
| | | LOW | 0.75 | 0.075 | 0.002 |
| | | MED | 3.00 | 0.300 | 0.008 |
| | | HIGH | 4.80 | 0.600 | 0.014 |
| 4 | Beef heifers | AG[4] | 30.55 | 0.397 | 0.008 |
| | | RG | 76.06 | 1.039 | 0.019 |
| 5 | Beef heifers | CON | 0.00 | 0.000 | 0.000 |
| | | CG09 | 7.61 | 0.090 | 0.002 |
| | | CG18 | 15.08 | 0.180 | 0.004 |

[1]CON: Control, no inclusion of HFCG in the diet; LOW: inclusion of 0.075% of HFCG in the diet; MED: inclusion of 0.3% of HFCG in the diet; HIGH: inclusion of 0.6% of HFCG in the diet; AG: abomasal infusion of CaG at 0.0077% of BW; RG: ruminal infusion of HFCG at 0.0192% BW; CG09: inclusion of 0.09% of HFCG in the diet; and CG18: inclusion of 0.18% of HFCG in the diet.

[2]Daily doses of HFCG intake were calculated based on the average DMI throughout the entire experimental period.

[3]Doses of HFCG based on the percentage of BW were calculated based on the average between the initial and final BW.

[4]AG information is based on the abomasal infusion of CaG and not HFCG.

### 7.4. Effect of butyrate and butyrins on gastrointestinal tract function

Initially, the rationale behind the TB doses focused on the highest dose tested. The higher dose of both RTB and UTB (0.3%, DM basis) used in Chapter 6 with growing ram lambs, was based on the reports of the dosages of sodium butyrate (**NaB**) included in the diet for weaned piglets (Manzanilla et al., 2006) and dairy calves (Górka et al., 2009). Where piglets supplemented with NaB (0.3% DM basis) had deeper jejunal crypts and an increased number of colonic Goblet cells (Manzanilla et al., 2006), the dietary provision of NaB in milk replacer for dairy calves (0.3% as fed basis), reduced the weight loss in the first 15 days of life, resulting in heavier lambs at the end of the experiment. The dietary provision of NaB for calves also resulted in increased ruminal papillae length and width and plasma GLP-2 concentration (Górka et al., 2009).

As proposed, post-ruminal provision of CaG was expected to increase butyrate concentration through microbial fermentation in the large intestine, due its ability to resist degradation in the intestine by mammalian enzymes and its low absorption in the small intestine (Asano et al., 1994, 1997). However, given there were no observed increases in butyrate concentration, studies also utilized butyrate (Chapter 4) and butyrins (Chapter 5) to evaluate effects of post-ruminal butyrate supply. In Chapter 4, CaB was infused into the abomasum and increased ruminal SCFA disappearance and reduced mannitol flux across ruminal tissue. The greater rates of SCFA disappearance may be linked to increased blood flow to the GIT with butyrate exposure (Storm et al., 2011) and reduced ex vivo mannitol flux ex vivo may indicate increased tight junction expression and assembly (Peng et al., 2007). These results support that low doses of post-ruminal butyrate may enhance gastrointestinal tract function supporting the hypothesis in this thesis even without detectable changes in butyrate.

While butyrate effects were clear in Chapter 4, effects of tributyrin in Chapter 6 were largely absent. Post-ruminal provision should have occurred with the ruminally protected tributyrin, with release occuring more distally in the intestine than with CaB. For example, it is expected that CaB would be absorbed in the abomasum or proximal portion of the duodenum, based on the study of the flow of SCFA from the rumen to the duodenum as reported by Rupp et al. (1994). Due to the triglyceride structure of tributyrin and protection in a hydrogenated fat, exposure to lipases would be required to release free butyrate. Few studies have evaluated site of release for butyrate and increasing the intestinal supply is commonly stated as the strategy of rumen protected butyrate

(Guilloteau et al., 2010). However, when provided in milk replacer, it is likely that butyrate would be absorbed in the abomasum. Relating to the data found in the literature for newborn calves, Guilloteau et al. (2009) reported enhanced growth rate, increased crypt depth and mitotic index in the proximal and distal jejunum, and increased brush border enzyme activity with the addition of sodium butyrate to milk replacer. As well, Górka et al. (2011a) reported increased ruminal papillae length and width that consequently increased BW gain, and Górka et al. (2014) reported an effect on small intestine development driven by an increase in cell proliferation. As the addition of butyrate to milk replacer would lead to its delivery and absorption in the abomasum (Rupp et al., 1994) due to the action of the esophageal groove, these studies are highly correlated with the butyrate provision in the AB treatment in Chapter 4.

Although the increase in butyrate concentration in the large intestine has been reported to be beneficial for growth (Biagi et al., 2006), the data observed for heifers provided AB infusions leads to the speculation that not only the dose but also the site of release and absorption may affect the responses to butyrate. Additionally, it is worth mentioning that supplementation of dairy calves with encapsulated sodium butyrate in starter mixtures (Górka et al., 2009) increases the chances of post-ruminal release along with increased plasma GLP-2 (Górka et al., 2009; Górka et al., 2011a). These responses tended to increase reticulorumen weight and ruminal papillae length and width (Górka et al., 2009), crypt depth and tunica mucosa thickness in the duodenum and, brush border enzyme activity in the jejunum (Górka et al., 2014). These responses resulted in a positive effect in feed intake and growth (Górka et al., 2011a). Thus, future studies are necessary to identify not only butyrate precursors but coating techniques that allow the release of butyrate in the abomasum instead of in small or large intestine. In addition, a better understanding of the interaction between the site of release and the outcomes yielded by butyrate provision are necessary to help to understand the disparity of results found in the literature.

# 8. GENERAL CONCLUSION

The fermentation data found in the studies using HFCG did not confirm that CaG may increase butyrate concentration in the intestine of ruminants. Moreover, post-ruminal provision of CaG negatively affected growth and feed efficiency of growing lambs, while it did not negatively affect beef heifers fed with either a moderate forage or low forage diet. The dosing of butyrate into the abomasum yielded the greatest effects throughout all the treatments explored in the four studies presented in this thesis confirming the potential for butyrate to stimulate gastrointestinal tract function. Moreover, these results lead to the speculation that in addition to the dose of butyrate provided, the site of release, and absorption play an important role in physiological responses. The previous statement is further supported as ruminal and post-ruminal provision of TB did not yield differences in the variables of interest, such as carcass characteristics, showing that independently of the diet consumed and the dose provided, TB does not yield beneficial effects for finishing ruminants.

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