USE OF CANOLA MEAL AS A PROTEIN SOURCE IN PELLETED STARTER MIXTURES FOR DAIRY CALVES

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ABSTRACT

The broad hypothesis for the series of studies conducted within my Ph.D. research was that canola meal (**CM**), when supplied in pelleted starter mixture, will be an adequate protein source for dairy calves supporting body weight gain and gastrointestinal tract (**GIT**) development, possibly further enhancing those processes when included with other dietary supplements or through processing of CM. Five studies were conducted with a global objective to determine whether the use of CM as protein source in calf starters, when included in combination with other supplements or processing, could help substantiate satisfactory calf growth and GIT development. The first study assessed the different temperatures of heat-treating CM on ruminal and intestinal nutrient digestibility, utilizing in situ and *in vitro* approach in mature Hereford cattle. Heating to 110°C for 10 min resulted in decreased ruminal degradability of dry matter and crude protein relative to a non-heated control, and resulted in greater intestinal crude protein digestibility than when heated to 120°C. In the second study, 28 Holstein bull calves were used in 2 × 2 factorial design, to assess the effect of CM heat-treatment and glycerol supplementation on calf performance and on GIT development at weaning. The interactions between the main effects were minimal. Canola meal heat-treatment negatively affected calf performance, by decreased starter intake, average daily gain (**ADG**), final body weight, and GIT development with lighter ruminal and jejunal tissue weights. Heat-treatment did not impact gene expression of short-chain fatty acids (**SCFA**), peptide and amino acid transporters that were investigated in GIT, nor the activity of brush border enzymes in the small intestine. Glycerol inclusion in the starters increased starter intake and ADG. Additionally, when calves were fed starters with glycerol, the concentration of ruminal SCFA increased and jejunal tissue weight was greater, although no changes were observed for indicators of ruminal development. Glycerol downregulated the expression of *MCT1* in ruminal epithelium and upregulated it in proximal jejunal epithelium. Glycerol also increased activity of dipeptidylpeptidase IV in middle jejunum. Third and fourth study assessed the effect of different protein sources, either CM or soybean meal (**SBM**), as well as microencapsulated sodium butyrate (**MSB**) inclusion in 2 × 2 factorial design. Third study utilized bull calves and fourth study utilized heifer calves to assess starter intake and growth. Additionally, bull calves were also used for the assessment of GIT development. Few interactions between the treatments were present. Canola meal as protein source did not have the same effect across both studies in terms of performance. For bull calves, CM resulted in decreased pre-weaning starter intake, and ADG and feed efficiency

during the weaning phase. Additionally, dry matter digestibility was decreased when CM was used. For heifer calves, CM inclusion increased starter intake without differences in ADG and feed efficiency. Across both studies, CM inclusion resulted in decreased fecal score, indicating lesser severity of diarrhea. Supplementation of MSB increased pre-weaning starter intake; however, it did not affect growth of calves. Canola meal inclusion did not impact the molar proportions of major SCFA in ruminal fluid, but increased jejunal weight and length. Canola meal use in the starters tended to increase abomasal weight and upregulated *MCT4* and *GPR43* expression in the abomasum. While CM did not impact activity of brush border enzymes, CM downregulated expression of *PEPT2* in proximal jejunum and *ATB0+* in ileum. Microencapsulated sodium butyrate decreased the length of ruminal papillae, as well as ruminal epithelial surface area. Additionally, omasal epithelial layers were smaller when MSB was supplemented. When MSB was included in starters, no changes were detected for GIT organ weights. Expression of *MCT1* was upregulated in the ruminal epithelium with MSB supplementation. Microencapsulated sodium butyrate also increased activity of aminopeptidase enzymes in duodenum and ileum. In the final study, the multiple levels (0, 15, 30, 45 and 60% CP) of partial replacement of SBM with CM were investigated using Holstein heifer calves in order to assess starter intake, growth, ruminal fermentation, and digestibility. Linearly increasing CM inclusion in the starters decreased starter intake and crude protein digestibility, but did not affect calf growth. The molar proportion of ruminal propionate increased with increasing CM inclusion, whereas acetate decreased. In conclusion, the partial replacement of SBM with CM can help mitigate the decrease in ADG observed when full replacement of SBM is used, although decreased starter intake may still occur. The use of heat-treated CM, when compared with non-heated CM, decreased starter intake and growth of the calves, and compromised the development of the GIT. To improve calf performance and GIT development, glycerol can be included in the starter at 5% DM. Supplementation of MSB in starters may negatively affect the development of the rumen and omasum. In general, partial replacement of SBM with CM can be reasonably used to ensure adequate protein supply from solid feed in dairy calves.

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LIST OF ABBREVIATIONS

AA = amino acid

AAB = α-aminobutyric acid

ACTB = beta-actin

ADF = acid detergent fiber

ADG = average daily gain

AQP3 = aquaporin 3

ATB0+ = *SLC6A14* = amino acid transporter B0+

BHB = β-hydroxybutyrate

BW = body weight

CM = canola meal

CM0 = inclusion of canola meal at 0% of the CP originally supplied by soybean meal

CM15 = inclusion of canola meal at 15% of the CP originally supplied by soybean meal

CM30 = inclusion of canola meal at 30% of the CP originally supplied by soybean meal

CM45 = inclusion of canola meal at 45% of the CP originally supplied by soybean meal

CM60 = inclusion of canola meal at 60% of the CP originally supplied by soybean meal

CP = crude protein

CRA = cranial sac of rumen

C_T = threshold cycle

d = day

D = potentially degradable fraction

DIST = distal jejunum

DM = dry matter

DMI = dry matter intake

DUO = duodenum

EAAC1 = *SLC1A1* = excitatory amino acid carrier 1

ED = effective degradability

EDCP = effective degradable crude protein

EDDM = effective degradable dry matter

EE = ether extract

F = forward (primer)

G:F = gain to feed ratio

GAPDH = glyceraldehyde-3-phosphate dehydrogenase

GIT = gastrointestinal tract

GLY = effect of glycerol inclusion

GPR41 = *FFAR3* = G protein-coupled receptor 41

GPR43 = *FFAR2* = G-protein-coupled receptor 43

H×G = canola meal heat-treatment by glycerol supplementation interaction

H-G = heat-treated canola meal with glycerol supplementation

H-NG = heat-treated canola meal without glycerol

HEAT = effect of canola meal heat-treatment

HPRT1 = hypoxanthine phosphoribosyltransferase 1

Hyp = hydroxyproline

IgG = immunoglobins G

IGF = insulin-like growth factor

ILE = ileum

K_d = degradation rate of the degradable fraction

K_p = passage rate

MCT1 = *SLC16A1* = monocarboxylic acid transporter 1

MCT4 = *SLC16A3* = monocarboxylic acid transporter 4

ME = metabolizable energy

MF = period of full allocation of milk replacer

MID = middle jejunum

MR = milk replacer

MSB = microencapsulated sodium butyrate

NDF = neutral detergent fiber

NEFA = non-esterified fatty acid

NFC = non-fibrous carbohydrates

NH-G = non-heated canola meal with glycerol supplementation

NH-NG = non-heated canola meal without glycerol

NSC = non-structural carbohydrates

OA = overall period of the whole study

OM = organic matter

PEPT1 = *SLC15A1* = peptide transporter 1

PEPT2 = *SLC15A2* = peptide transporter 2

PROX = proximal jejunum

PS = main effect of protein source in starter mixture (soybean mean vs. canola meal)

PS × MSB = interaction between protein source in starter mixture (soybean mean vs. canola meal) and microencapsulated sodium butyrate

PW = period of post-weaning

R = reverse (primer)

R(t) = percentage of residue during set incubation timepoint (t)

RDP = ruminal degradable protein

RPLP0 = 60S acidic ribosomal protein P0

RUP = ruminal undegradable protein

S = soluble fraction

SBM = soybean meal

SCFA = short chain fatty acid

SD = standard deviation

SEM = standard error of the mean

t = timepoint

T_0 = lag time

Tau = taurine

TCM = treatment of canola meal without microencapsulated sodium butyrate

TCM-MSB = treatment of canola meal with microencapsulated sodium butyrate

TSBM = treatment of soybean meal without microencapsulated sodium butyrate

TSBM-MSB = treatment of soybean meal with microencapsulated sodium butyrate

UT-B = urea transporter B

wk = week

WN = period when milk replacer is gradually reduced to promote weaning

1 GENERAL INTRODUCTION

The global population is projected to increase to 9.3 billion people by 2050 (Lee, 2011) from the current 7.6 billion (United States Census Bureau, 2020), emphasizing the importance of ensuring a safe, affordable, and adequate supply of food for this growing population. Livestock production plays an important role in meeting human food supply requirements. Keeping that goal in mind, the usage of feeds that are not edible by humans has been increasing steadily and currently accounts for 86% of the feed utilized by livestock, which includes forages, straw, off-grade cereal grains, and by-product feeds (Mottet et al., 2017). In addition to providing valuable nutrients for livestock, utilizing agricultural and food processing by-products in animal feeds allows for the elimination of their disposal within landfills.

A by-product arising from canola crushing and oil extraction is canola meal (**CM**). Canola meal is a non-human edible feed that has a high crude protein (**CP**) concentration and is often used in diets for dairy cows, beef cows, small ruminants, poultry, pigs, and fish (Canola Council of Canada, 2015). When utilized for dairy cattle, CM generally increases milk production when compared with other protein sources (Martineau et al., 2013). However, there have been concerns over the use of CM in diets for calves as it may still contain antinutritional factors (Bell, 1993), is not palatable (Miller-Cushon et al., 2014b), may reduce dietary digestibility (Khorasani et al., 1990), and calves may not grow as rapidly. However, recent research has shown that although CM is less palatable to calves than other protein sources (Miller-Cushon et al., 2014b), using CM as partial replacement of soybean meal (**SBM**) may allow for similar quantities of starter intake (Hadam et al., 2016; Terré et al., 2016) and rates of average daily gain (**ADG**) (Terré et al., 2016) relative to SBM.

The palatability issue of CM could potentially be overcome by introducing another feed component that is flavorsome for calves. Often, molasses is used to add flavour, as source of energy and as a binding agent during pelleting to limit the production of fine particles. However, inclusion of molasses in starter mixtures at 10% or above can result in decreased starter intake (Lesmeister and Heinrichs, 2005) and ADG (Hill et al., 2008). Other than molasses, glycerol could be a palatability enhancer due to its sweet taste (Quispe et al., 2013). Glycerol is also a by-product of biodiesel production, that mostly utilizes vegetable oils as substrates (Gerpen, 2005). Additionally, glycerol can be a fermentation substrate for the ruminal microflora, stimulating the

production of propionate and butyrate, and limiting acetate production (Rémond et al., 1993). Butyrate is beneficial for the development of ruminal papillae in calves (Mentschel et al., 2001) and for the development of the whole gastrointestinal tract in general (Górka et al., 2014). Provision of butyrate in the diet, whether it be in milk replacer or solid feed, has been shown to increase body weight gain, starter intake and development of ruminal papillae in calves (Górka et al., 2009; Guilloteau et al., 2009b; Rice et al., 2019). Thus, although CM may appear to be less palatable, there may be strategies to incorporate CM with other dietary components to overcome the negative impacts on intake and growth of calves.

2 LITERATURE REVIEW

2.1 Artificial Rearing of Holstein Calves

2.1.1 Significance in the Industry

In Canada there are an estimated 466,800 calves and an additional 438,500 replacement heifers that allow for steady replacement of the mature dairy cattle population which has been estimated to be 981,300 cows in 2020 (Statistics Canada, 2020). Calf nutrition and management, together with continually improved genetic merit, are important to achieve functioning and high-producing dairy cows. As such, calf health and development are important in the short- and long-term as early life management can impact long-term productivity (Soberon et al., 2012).

Interestingly, while feed efficiency decreases until weaning, since solid feeds are less digestible than milk or milk replacer (**MR**), the costs of feeding calves also decrease (Bach and Ahedo, 2008). Economically, it is important to introduce solid feed to the calves early on. However, care should be taken to avoid poor quality feed and abrupt weaning strategies that can potentially compromise solid feed intake and as a consequence growth of calves. This is especially important when considering potential long-term effects that early-life performance of calves can have on their future productivity as milking cows. Soberon et al. (2012) observed that greater pre-weaning average daily gain (**ADG**) positively impacts first lactation milk yield, with 850 kg more milk being produced for every kilogram of pre-weaning ADG, emphasizing the importance of early-life nutrition. Additionally, although increased MR protein intake does not impact first lactation milk and milk component production, future production can be positively influenced by starter protein intake (Rauba et al., 2019). A similar observation was made by Heinrichs and Heinrichs (2011) where solid feed intake at weaning was positively correlated with first-lactation milk production (which increased 286.7 kg for every 1 kg of solid feed intake). Gelsinger et al. (2016) also showed that although liquid feed and starter intakes combined can positively impact first lactation performance, only ADG above 0.5 kg/d had a positive impact on milk yield during the first lactation. Chester-Jones et al. (2017) observed that both starter intake and ADG at 8 week (**wk**) of age can positively impact first-lactation milk production. When fed *ad libitum*, calves fed whole milk, as opposed to MR, had greater weaning body weight (**BW**), and greater first lactation

milk production (Moallem et al., 2010). This further emphasizes that quality of the feed, feed intake, and rate of calf growth can all have an impact future milk production.

In short-term, calves need to combat several common health issues that can result in their exclusion from the herd early on in life (see Chapter 2.1.5 for more details). Limiting the morbidity and mortality, eliminates lost revenue for their care and related health expenses. Although diarrhea specifically has not been shown to impact future first lactation production, calves that received antibiotic treatments were reported to have lesser milk production by 493 kg during first lactation than untreated calves (Soberon et al., 2012). However, Heinrichs and Heinrichs (2011) observed that number of days with diarrhea and coughing during first 4 months of life was negatively correlated with first-lactation milk production. Heinrichs and Heinrichs (2011) Additionally, diarrhea can decrease BW gain of calves during the first 6 months of life (Donovan et al., 1998), and through that effect, diarrhea can decrease calves' potential future production as lactating cows (Gelsinger et al., 2016). Similarly to diarrhea, pneumonia, and septicemia can also have negative impact on calves' growth (Donovan et al., 1998). Overall, calves play an important role in establishing future dairy herds and their growth pre-weaning can have consequences for the subsequent milk production.

2.1.2 Conventional Practices

Post-natal nutrition of calves begins with colostrum. Colostrum is the first mammary gland secretion secreted after parturition. Colostrum is rich in energy, originating mostly from greater fat content than normal milk (Godden, 2008). However, colostrum is mostly regarded for its high concentration of immunoglobins, especially immunoglobins G (**IgG**), although IgA and IgM are also present in the colostrum (Godden, 2008). Immunoglobins can pass through the gastrointestinal tract (**GIT**) of calf during the early hours of life and provide calf with its first immunological defence (Stott et al., 1979). Monitoring the quality of colostrum through measurement of IgG concentration, pasteurization of fresh colostrum to eliminate potential pathogens, rapid feeding of colostrum relative to birth, and assessing rates for inadequate IgG provision or transfer are all important steps to ensure the survivability (Robison et al., 1988) and later productivity of dairy calves (DeNise et al., 1989). Besides immunoglobins, colostrum is rich in other bioactive components including growth factors, like insulin-like growth factors (**IGF**):

IGF-I and IGF-II which respectively stimulate the proliferation and differentiation of cells (Gauthier et al., 2006), although their effect on intestinal growth is not clear (Blum and Hammon, 2000). Additionally, oligosaccharides are present in colostrum and have prebiotic activity increasing *Bifidobacterium* presence in the feces while limiting the growth of *Escherichia coli,* as well as inhibiting attachment of pathogens to the intestine (Gopal and Gill, 2000). Although some of the bioactive compounds are highlighted in this review, it is not an exhaustive list as multiple others and their effects are investigated by the scientific community.

Following colostrum, dairy calves most often receive either milk (sale milk or waste milk) or MR. In the USA, MR was reported to be fed on 38.5% of farms, while either whole or waste milk on 43.3%, and 38.5% used a combination of both options (Urie et al., 2018). According to Vasseur et al. (2010), waste milk was fed to calves on 47% of farms in eastern Canada, saleable milk was fed to calves on 89% of the farms, and MR was used by 50% of the farms. While not specifically evaluated, the percentages of milk and MR use do not equate to 100% suggesting that individual farms may employ multiple strategies. Importantly, none of the farms surveyed by Vasseur et al. (2010) used pasteurization to treat the whole milk that was being fed to the calves. The use of milk, especially non-saleable milk and when milk is unpasteurized, can result in calves potentially ingesting pathogens (Drackley, 2008a). The use of antimicrobials in MR, although still prevalent in 56% of US farms (USDA, 2007b), is not recommended as a prophylactic measure as it can lead to the development of antibiotic resistant bacteria (Smith, 2015).

Interestingly, the first attempts at development of milk substitute for calves started in late 1910's, with major developments occurring in 1940's (Kertz et al., 2017). Calves fed MR, as opposed to milk, can grow less, require more medical treatments, have a greater risk of mortality, and are economically less profitable (Godden et al., 2005). A similar observation was made by Górka et al. (2011b) where calves fed MR had decreased dry matter intake (**DMI**), BW gain, small intestinal weight, mitotic index in the intestine, and ruminal papillae length and width. However, opposite results have been documented by (Hill et al., 2009), with MR increasing pre-weaning ADG, starter intake and gain to feed (**G:F**) ratio compared with full milk. Feeding MR does not require a pasteurizer, MR is consistent in its composition and the amount fed can be easily adjusted to meet energy requirements of the calf. The quality of MR is also important as for example, soy protein inclusion in MR can negatively affect the development of intestinal villi and decrease brush border enzyme activity (Montagne et al., 1999). Soy protein is the most common non-milk protein

used in MR, however, soy protein is not recommended for calves younger than 3 wk of age (Drackley, 2008a), since calves' digestive enzymatic activities are not yet fully developed (Rey et al., 2012).

Calves are usually offered solid feed early in life, on average at 7 d of age (Vasseur et al., 2010). In terms of solid feed, there are usually two different types, a starter mixture (also referred to as starter) provided in a pelleted or texturized form, and forage sources. Details related to the digestion process of the solid feeds are presented in Chapter 2.2.2. Starter mixtures are usually composed of cereal grains and other by product feeds and are nutrient and energy dense. Calves fed pelleted starter mixtures may consume less starter than those fed texturized starter mixtures; however, BW was not different between the two, implying that feed efficiency may be improved for calves fed pelleted starter mixtures (Bach et al., 2007). When using texturized starter mixtures, there is also potential for sorting of components within that starter (Miller-Cushon and DeVries, 2015).

The two main considerations for designing calf starter mixtures are energy sources and protein sources. Calf energy requirements depend on their age, ADG and environmental temperature and are usually partitioned into net energy requirement for maintenance and for growth (NRC, 2001). The suggested range of metabolizable energy (**ME**), which as opposed to net energy does not account for heat loss within the animal, is between 2.5 and 3.4 Mcal/kg of starter (NRC, 2001). Energy in starters is mostly provided from starch sources such as barley, corn, oat, sorghum, wheat, and their by-products. Previous research has suggested that wheat and sorghum are the most palatable starch sources when provided to post-weaned calves (Miller-Cushon et al., 2014a). However, others have found that when fed within pelleted starters, calves consumed more starter when it contained corn, than wheat, barley and oats containing diets (Khan et al., 2007b). Calves fed corn had greater G:F ratio and BW gain (Khan et al., 2007b), while both corn and wheat resulted in greater ruminal short chain fatty acid (**SCFA**) concentration, ruminal ammonia concentration, greater ruminal weight, and ruminal papillae dimensions (Khan et al., 2008).

When calves consume solid feeds, protein is first digested by the ruminal microbial proteolytic activity and then, either in the form of microbial protein or ruminal undegradable protein (**RUP**), is mostly digested in the intestine of the calves and provide amino acids (**AA**) for metabolism or growth (more detailed description in Chapter 2.2.2). The recommendation for CP

content in starters is around 20% dry matter (**DM**) (NRC, 2001). In the past, Akayezu et al. (1994) assessed starters containing CP at either 14, 16.5, 19 or 22.5% DM, suggesting that above 20% CP does not allow for greater gains than CP below that level. Stamey et al. (2020) evaluated CP concentrations of 19.6 and 25.5% DM in the starter with reported results that calves consumed a greater amount of starter with greater CP concentration, but increasing CP concentration did not result in greater growth. However, greater intake of protein from the starter can have a positive impact on milk and milk component production during first lactation (Rauba et al., 2019). Many high protein feedstuffs have been previously evaluated as potential feed sources for calf starters including SBM, dry distiller grains, sunflower meal, pea meal, CM, and corn gluten meal (Miller-Cushon et al., 2014a; Miller-Cushon et al., 2014b). However, SBM and dry distiller grains appear to be the most preferred by calves (Miller-Cushon et al., 2014a). More details on CM and its use as a protein source are presented in Chapter 2.3.

Fibre is inevitably present in the starter diets as part of its components. Chopped hay or straw can be added separately to the calves' diet, which allows the forage to be present as larger particles and act as source of effective fibre. Terré et al. (2013) reported that increasing the concentration of neutral detergent fibre (**NDF**) in the starter mixture does not change starter intake of calves, and can actually decrease ADG; whereas supplying a separate forage source in the diet stimulates intake of starter, ADG, ruminating time, increases ruminal pH and decreases ruminal SCFA concentration of calves. When hay was added to a barley and a SBM-based starter mixture, the most visible effects occurred at 6 and 8 wk of age, when pH, molar proportion of acetate, and ammonia concentration increased for hay supplemented calves, while molar proportion of propionate decreased (Žitňan et al., 1998). Additionally, only at 9 wk of age were changes in the development of the papillae observed with calves fed both hay and starter having a greater number and longer papillae; however, papillae surface was less when compared with calves fed only starter (Žitňan et al., 1998). Based on review by Khan et al. (2016), calves that consume starter mixtures have greater papillae growth, a more abundant amylolytic bacteria population, greater concentrations of ruminal SCFA, and greater proportions of butyrate and lactate than calves fed forages. On the other hand, calves that consume forage have greater ruminal weight and volume, ruminal motility, cellulolytic and protozoa populations, ruminal pH, and rumination activity (Khan et al., 2016). Therefore, both starter mixtures and forage inclusion play important roles in balanced growth and GIT development of calves during weaning transition.

Water, aside from being supplied in milk or MR, is also essential for proper functioning of the GIT of calves. While this may seem trivial and obvious, artificial rearing systems used for dairy calves may require manual labour to provide water and hence consideration for when water should be provided and whether it should be in excess becomes a labour issue. When calves are deprived of water, starter intake and BW gain decrease (Kertz et al., 1984). On majority of dairies reported in eastern Canadian survey, calves were allowed water access within the first week of life; however, there were 9.6% of farms that did not provide calves with water until after weaning (Vasseur et al., 2010). Across all US calf rearing operations, water was most commonly offered around 15 day (**d**) of age (USDA, 2007a). Wickramasinghe et al. (2019) compared the impacts of providing water access to calves from birth and at 17 d of age. They reported that calves that had water access from birth drank on average 0.75 kg of water during first two weeks of life, as well as had greater BW pre- and post-weaning, greater NDF and ADF digestibility, and greater G:F ratio post-weaning (Wickramasinghe et al., 2019).

2.1.3 Accelerated Feeding Systems

Based on the results of survey conducted by Vasseur et al. (2010), the average amount of milk or MR fed to a calf was 5.5 L per d fed in 2 feedings, with the amount being on average smaller during first week of life (4 L) and last week before weaning (3 L). Strategies for feeding smaller amounts of MR to calves have originated as a way of promoting solid feed intake (Khan et al., 2011a) which in turn can promote ruminal fermentation and development (Khan et al., 2016). However, as a way to promote greater BW gain pre-weaning which can be further beneficial for lactation productivity (Soberon et al., 2012), recent studies, described below, evaluated accelerated feeding strategies with either a greater amount of liquid feed provided to the calves or where MR has a greater concentration of CP or fat.

Calves that are allowed to consume MR *ad libitum* and are weaned at a static age, consume less than 0.5 kg DM/d of solid feed prior to weaning, as opposed to over 1 kg DM/d of solid feed consumed by calves limited in the quantity of MR they consume (Miller-Cushon et al., 2013). As such at weaning, calves fed MR *ad libitum* are not receiving adequate energy supply from solid feed and they experience growth check, when their BW does not grow within the week post-weaning, while normal, continuous growth rate was observed in limit-fed calves (Miller-

Cushon et al., 2013). Similar observations were made by Frieten et al. (2018), who reported that although calves fed MR *ad libitum* grew more prior to the step-down weaning process, it was primarily due to increased intake of MR, since calves actually consumed less starter. At weaning, calves that were fed a restrictive MR quantity had greater ADG, although they did not catch up to the calves fed *ad libitum* MR for BW (Frieten et al., 2018). However, the use of a step-down weaning approach can help improve starter intake despite being fed greater amounts of MR and allows for calves to increase their BW during the weaning transition (Khan et al., 2007a).

As discussed above, calves fed a high milk allowance consume less starter than calves fed a conventional amount of MR, which during weaning transition leads to less metabolizable energy (**ME**) intake, even though post-weaning BW was still greater for calves fed a high milk allowance (MacPherson et al., 2016). A similar observation was made by Kristensen et al. (2007), where providing greater MR supply decreased starter intake, but increased ADG. In that study, they did not observe any effects of increasing milk allowance from 3.10 to 8.34 kg/d on ruminal fermentation, while plasma β-hydroxybutyrate (**BHB**) decreased with greater milk provision. However, some studies have observed that increasing whole milk feeding, increased pre-weaning starter intake of calves as well (Omidi-Mirzaei et al., 2015). Additionally, greater provision of milk resulted in a lighter rumen and omasum, and their respective digesta weights, which most likely relates to smaller intake of starter by those calves (Kristensen et al., 2007). Interestingly, increased amount of MR fed to calves does not impact small intestine epithelial development, nor the expression of genes related to receptors for growth hormone and IGF (Velayudhan et al., 2008). Although pre-weaning growth is very promising for the calves, the trade-off related to decreased starter intake and ruminal development have to be carefully considered when deciding between milk feeding and weaning methods.

2.1.4 Weaning Management

In natural setting, weaning would occur gradually over the course of months, when calves slowly begin to mimic the behaviour of mature animals, repeatedly graze with their herd at 4 to 6 months of age and are weaned at around 10 months of age (Khan et al., 2011a). However, the milk or MR feeding stage of a calf's life is the most expensive in terms of feed cost (Bach and Ahedo, 2008) and as such unsustainable economically for producers for prolonged periods of time, even

though it has greatest G:F conversion. Modern dairy operations try to limit the amount of time the calf consumes liquid feed and begin the weaning transition considerably sooner than it would otherwise occur when calf would be weaned by the dam. The average age of weaning for calves in eastern Canada is around 7 wk of age with almost 90% of farms using gradual weaning approach (Vasseur et al., 2010). In contrast, dairy producers in the United States wean calves around 9 wk of age (Urie et al., 2018). As a result of the expedited weaning process, weaning can be stressful for a calf (Weary et al., 2008) with negative implications on GIT development and post-weaning growth.

There are further few different approaches used to wean calves from liquid feed, most notably ones based on age and ones based on starter intake. In eastern Canadian survey, 66.7% of dairy farms based their weaning practices on calf age, while weaning based on starter intake was conducted on 88.9% of the farms (Vasseur et al., 2010). In the assessment conducted in the United States, calves weaned based on age accounted for 98.1% of farms, while those weaned based on starter intake account for only 49.5% (Urie et al., 2018). The NRC (2001) suggests weaning when starter intake is at least 0.68 kg for 3 consecutive days. Roth et al. (2009) compared conventional weaning (at 12 wk of age using a step-down weaning approach initiated at 8 wk of age) to a concentrate-based weaning approach (step-down weaning approach was initiated when calves consumed 700 g of starter and weaning occurred when starter intake reached 2,000 g for 4 consecutive days). Although Roth et al. (2009) did not observe major differences in ruminal development, BW gain, and number of veterinary treatments, their experimental design weaned calves considerably later than is recommended or typical practice in North America (NRC, 2001; Vasseur et al., 2010), highlighting the importance of weaning age. While the recommended approach, starter intake-based weaning can be hard to implement on commercial farms, and as such generalized weaning programs based on age are common (Urie et al., 2018).

Age-based weaning approaches include abrupt weaning and gradual (also called step-down; Khan et al., 2007a) weaning. Abruptly weaning calves fed a high allowance of MR can have negative consequences on starter intake and post-weaning ADG as these calves have difficulty adapting to the change in feed source and consequently have lower starter and ME intake (Steele et al., 2017). Rather than abrupt weaning, gradual weaning can increase forestomach weight, omasal weight, and small intestine weight, which supports the need for greater starter intake to promote GIT development (Steele et al., 2017). A study by Welboren et al. (2019) showed through

the use of automatic calf feeders, that by spreading the step-down over days instead of weeks, energy intake and growth over weaning transition can be maintained. Age at which the calves are weaned is also important, as calves weaned at 6 wk of age, as opposed to 8 wk of age, had lesser BW, especially when a high plane of nutrition was used early in life, with the decrease continuing for few weeks after weaning as well (Eckert et al., 2015). Smaller BW when calves are weaned earlier in life is mostly the result of insufficient energy intake from solid feed, which cannot fully replace the energy previously provided by MR.

Weaning, with the shift in diet and development of ruminal fermentation impacts digestion processes. For example, protein flowing to the ileum for calves offered milk is mostly comprised of milk protein (50%) while bacterial protein is only estimated to provide 20% (Lallès et al., 1990). In contrast, calves fed a starter mixture as the dominant feed source rely on microbial protein to supply approximately 75% of protein, with bypass protein providing approximately 15% of the ileal protein supply. In both cases endogenous protein is assumed to account for the remainder of the protein present in the ileal digesta. More in-depth information on changes in digestion process is presented in Chapter 2.2.2.

2.1.5 Health Challenges

The most challenging period for calves in terms of health is pre-weaning period. Between 5.7 to 10.8% of heifer calves do not survive until weaning, with diarrhea being responsible for over half of those deaths, and respiratory problems causing a quarter (USDA, 2007b; 2018). Weaned heifers are not as susceptible to disease with mortality rate of 1.8 to 2.8%, attributed mostly to respiratory disease (around 50% of cases), and diarrhea being responsible for only between 6.8 to 10% of cases (USDA, 2007b; 2018). Other causes of calf death include, but are not limited to: complications at calving; navel or joint disease; lameness, injury; uncoordinated movement; lethargic appearance; and poisoning (USDA, 2007b; 2018). While GIT permeability generally decreases with age, the weaning process can lead to increased GIT permeability, especially in the rumen, duodenum, and jejunum (Wood et al., 2015). It is also worth noting that calves spend on average 4 h/d with a ruminal pH below 5.8, which can be considered acidotic in mature animals (Laarman and Oba, 2011). Before and especially during weaning, calves can be

challenged with conditions in their GIT (like decreased pH and disrupted gut permeability) that might increase the risk of pathogen introduction, which could increase the mortality of the calves.

The etiology of infectious diarrhea in calves between 5 and 14 d of age mostly points to rotaviruses, coronaviruses, *Salmonella spp*, and *Cryptosporidium*, while in calves beyond that age, *Escherichia coli*, *Salmonella spp.*, *Eimeria spp.*, and *Giardia spp.* are mostly responsible for induction of diarrhea (McGuirk, 2008). The difference in the presentation and severity of diarrhea is important, as each case requires proper handling with potential veterinary care for further successful growth of the calf. The common effect of calf diarrhea is dehydration, which can often lead to the death of the calf (NRC, 2001). Calves experiencing diarrhea might require an oral electrolyte solution, or an intravenous treatment with electrolyte solution, depending on the level of dehydration (McGuirk, 2008). The incidence of diarrhea in calves (Heinrichs and Heinrichs, 2011), as well as indirect effects of diarrhea, like decreased starter intake and BW (Donovan et al., 1998) can decrease first-lactation milk-production.

After diarrhea, respiratory disease is the most prevalent in calves and responsible for a quarter of calf deaths before weaning (USDA, 2007b; 2018). Pneumonia can result in 10.6 kg less BW gain in the first 6 months of life (Donovan et al., 1998). Diagnosis of pneumonia, especially in early stages can be challenging, since signs, such as a drop in appetite, coughing, or a depressed appearance might not be present or could be mistaken for other diseases (McGuirk, 2008). Health issues of calves can have negative effect on survivability of calves and their growth and therefore, it is important to recognize their impact.

2.2 Gastrointestinal Tract Physiology

2.2.1 Development of the Gastrointestinal Tract in Calves

The development of the rumen and stratification of the ruminal epithelium begin during embryonic development (Warner, 1958). Calves upon birth are not functional ruminants. The size of the GIT organs in neonatal calves is not only smaller than their mature counterparts, but proportionally different. At birth and during early life of the calves, abomasum makes up 57% of the forestomach volume, while rumen only accounts for 42%, and omasum 1% (Warner et al., 1956). This makes anatomical sense as milk is meant to by-pass the rumen through the esophageal

groove for digestion in the abomasum (Ørskov et al., 1970). The function of the esophageal groove is not influenced by bucket feeding or with the use of artificial nipples (Abe et al., 1979). As calves start consuming solid feed, the volumetric proportions of the forestomach compartments change, with the rumen becoming more dominant accounting for an estimated 85% of the forestomach volume, followed by the abomasum (11%) and omasum (3%; Warner et al. (1956)). Although rumen grows considerably in both size and volume, the same does not apply to abomasum, which maintains its size or actually becomes smaller post-weaning than during the neonatal period (Warner et al., 1956).

Formation of ruminal papillae begins during fetal development at between 5 to 6 months of gestation (Arias et al., 1978). After birth, the calves maintained solely on milk, with no hay or starter, have limited ruminal development in terms of weight and volume, as well as ruminal papillae size (Tamate et al., 1962). As previously mentioned, starter mixtures have a positive impact on development of the ruminal epithelium promoting papillae density and absorptive surface area, while consumption of forages results in greater rumen capacity and musculature (Khan et al., 2016). Ruminal papillae are functionally important as they increase the absorptive surface of the rumen. Additionally, rumen, reticulum, and omasum have a unique type of epithelium that consists of 4 layers (or strata): stratum corneum (outermost layer), stratum granulosum, stratum spinosum and stratum basale (Steele et al., 2016). Such a complex structure protects the deeper more metabolically active strata from mechanical damage by feed particles, as well as bacterial colonization (Steele et al., 2016).

The small intestine is important for digestion during both the liquid and solid feeding stages. Within the first week of life the small intestinal weight in calves can actually decrease when compared with birth weight of the small intestine (Guilloteau et al., 2009a). Overall, growth of the intestine does not seem to depend on the form of feed supplied to the calf, as no differences were observed when either MR and starter mixtures were fed to lambs (Baldwin, 1999). However, small intestine growth is greater when more energy is supplied to lambs, especially when a diet high in forage is used, although the effect is still present in high concentrate diets (McLeod and Baldwin, 2000). Additionally, some of the enzymatic activity present in small intestine changes with the shift of diet at weaning, namely lactase activity decreases and maltase and aminopeptidase N activity increase (Le Huerou et al., 1992). Although intestinal tissue is known for increasing its absorptive surface due to the presence of villi, intestinal villi structure is considerably different

from that of the ruminal papillae. As opposed to stratified epithelium in the forestomach, the intestine only has one layer of columnar epithelial cells lining its lumen, which are protected by the mucus layer (Steele et al., 2016). Firstly, after birth enterocytes present in the small intestine are noticeably filled with the vacuoles, which are replaced by enterocytes without vacuoles within 5 to 7 days of age (Guilloteau et al., 2009a). The simple columnar epithelium of the small intestine is comprised of different types of specialized cells. Most abundantly present are the absorptive enterocytes, aptly named for their critical role in the absorption of nutrients in the small intestine (Peterson and Artis, 2014). Small intestine epithelium also includes secretory cells: enteroendocrine cells that are responsible for secretion of hormones; goblet cells which secrete mucin glycoproteins that create the mucus layers; and paneth cells which secrete antimicrobial proteins like defensins, cathelicidins, and lysozymes (Kim and Ho, 2010; Peterson and Artis, 2014). Last but not least, pluripotent stem cells are present in the crypts of the villi and are responsible for renewal of the intestinal epithelium, through proliferation and differentiation (Kim and Ho, 2010; Peterson and Artis, 2014). As calves age, weight of the small intestinal tissue increases (Guilloteau et al., 2009a), however that growth can be inhibited by weaning (Guilloteau et al., 2009a), likely due to greater permeability of duodenum and jejunum observed at weaning (Wood et al., 2015). Gastrointestinal tract of calves changes dynamically both with age and as the feeds that are consumed change as well.

2.2.2 Regional Characterization of Essential Physiological Processes

As mentioned previously, newborn calves do not possess a fully developed GIT capable of digestion to the extent of mature ruminants. Digestive differences stem from both anatomical reasons, discussed in Chapter 2.2.1, as well as from functional processes occurring in GIT.

During the first few weeks of life, calves rely almost exclusively on liquid feed for nutrient supply either via milk or MR. As mentioned previously, liquid feed mostly by-passes the rumen through the esophageal groove and is therefore digested in the abomasum and small intestine. Abomasum functions physiologically very similarly to the monogastric stomach. Casein, which forms about 80% of the protein fraction of liquid feed (Gellrich et al., 2014) is denatured and forms a curd within the abomasum due to the low pH (approximately a pH of 2.0) originating from hydrochloric acid secretion and the presence of rennin (chymosin) (Drackley, 2008a). Curd

formation allows for longer retention and therefore slower digestion of milk protein and fat. Within abomasum, pepsin begins digestion of protein, which is further continued in the small intestine by trypsin, chymotrypsin, elastase and carboxypeptidase secreted by the pancreas, and by peptidases present on the intestinal brush border (Drackley, 2008a). Either di-, tripeptides or AA can be absorbed by intestinal enterocytes, using specialized transporters (Bröer, 2008; Gilbert et al., 2008).

Fat present in milk is partially digested in the abomasum by pregastric lipase originating from saliva, while the remainder of digestion occurs in the small intestine by pancreatic lipase (Drackley, 2008a). Fatty acids and 2-monoacylglycerols, the products of fat digestion in the small intestine, are emulsified and absorbed into the enterocytes, where they are used to form triacylglycerols and consequently chylomicrons, which are used to transport triacylglycerols through lymph vessels into muscles, heart, and adipose tissue (Drackley, 2008a).

As lactose is the only sugar naturally present in milk, calves have capability to digest it using lactase activity within the intestinal brush border (Drackley, 2008a). The activity of other sugar-digesting enzymes, like maltase is negligible during the milk-feeding phase (Le Huerou et al., 1992). As calves age, the activity of lactase diminishes (Le Huerou et al., 1992) as the reliance on milk as a main source of energy also declines.

Shortly after the introduction of starter mixture to the calves, signs of ruminal fermentation can be observed. Rey et al. (2012) observed that calves begin to consume hay at around d 4 of age and starter at around d 9 of age, and the ruminal SCFA concentration noticeably increases after d 10 of age reaching semi-consistent levels at around d 15 of age. Additionally, fibrolytic, amylolytic, ureolytic, and proteolytic enzymatic activities within rumen peaked between d 7 and d 22 of age, stabilising between 1 to 2 month of age (Rey et al., 2012). It should be noted that for the duration of the study, calves were consuming both MR and solid feed and between d 4 to 51 of age consumed on average 424 g/d of starter (Rey et al., 2012). Similar to the increase in ruminal fermentation, ketogenic capability of the ruminal epithelium can be established as early as 4 d of age, however it is only after weaning that it reaches full potential (Baldwin et al., 2004).

Carbohydrates, whether fibrous like cellulose or hemicellulose; or non-fibrous like starch, pectins, dextrans, di-saccharides can be broken down to monosaccharides by microbial enzymes present in the ruminal fluid (Bergman, 1990). Monosacchaarides can in turn be used by the microflora in glycolysis to produce pyruvate (Bergman, 1990). Pyruvate is the precursor for SCFA

production in rumen, with the most important SCFA being acetate, propionate, and butyrate. On average, a greater proportion of acetate is observed when fed high forage diets (approximately 75 mol/100 mol), than when fed high concentrate diets (10 mol/100 mol). With the increased acetate on high forage diets, the molar proportions of propionate (15 vs. 40 mol/100 mol) and butyrate (10 vs. 20 mol/100 mol) are lower than when fed high concentrate diets (Bergman, 1990).

Both propionate and butyrate are metabolised by the ruminal epithelium (Sehested et al., 1999). However, butyrate is the most preferred substrate for ruminal epithelial metabolism, followed by valerate and propionate (Weigand et al., 1975). Portal vein recovery of propionate can be as high as 91%, while for butyrate it is as low as 18% (Kristensen and Harmon, 2004b). Propionate is the major substrate for gluconeogenesis in the liver (Kristensen and Harmon, 2006). Enzymatic changes within liver can be observed when shifting from reliance on glucose and other monosaccharides supplied from milk and glycolysis and pentose phosphate pathway activities in the liver, to gluconeogenesis and ketone metabolism when solid feed starts to dominate the diet (Baldwin et al., 2004).

Protein digestion in weaned calves and mature ruminants is also reliant on the ruminal microflora. The proteolytic activity of ruminal bacteria increases until 15 d of age for calves consuming solid feed and stabilizes thereafter (Rey et al., 2012). Proteolytic digestion occurs extracellularly, and the resulting peptides and AA can be transported into the bacterial cell, and peptides can be further broken down by intestinal peptidases (Bach et al., 2005). Amino acids can either be incorporated into microbial protein or are deaminated and fermented to yield SCFA, ammonia, carbon dioxide, and methane (Tamminga, 1979). The fate of AA are linked to the availability of energy for the ruminal microflora. When energy is in abundance, a greater proportion of AA will be used for microbial protein production, and when energy is lacking, deamination leading to the production of SCFA is more likely to occur (Bach et al., 2005). Additionally, amylolytic bacteria utilize AA and ammonia as their nitrogen source for protein production, whereas cellulolytic bacteria utilize only ammonia (Russell et al., 1992). Additionally, it is important to recognize that feed protein is not fully digested in rumen. Ruminal degradable protein (**RDP**), as the name implies, are the potentially degradable and solubilized protein fraction, consisting mostly of non-protein nitrogen (NRC, 2001). Ruminal degradable protein, through the mechanisms described above, contribute to the creation of microbial protein. Whereas RUP is assumed to not be digested in rumen and in general consists of proteins linked to lignin and tannins,

and heat-damaged proteins (NRC, 2001) and although not digested in the rumen, RUP can potentially be digested in the small intestine along with microbial protein. Overall, the digestion processes occurring in the GIT of calves reflect the changes in the diet, shifting from milk digestion to solid feed digestion.

2.2.3 Strategies to Accelerate Development of the Gastrointestinal Tract

The overall goal of stimulating GIT development as soon as possible in calves is to ensure that during the weaning transition calves will be able to adequately digest solid feed. Digestion of solid feed requires digestive organs of adequate size (mainly rumen and small intestine) and with adequate absorptive surface area (ruminal papillae and intestinal villi and crypt development), as well as established enzymatic activities. When calves are fed a milk-only diet, their forestomach grows only to about 24% the size compared when calves consume solid feed (Warner et al., 1956).

Calves should increase starter mixture consumption as they approach weaning. Provision of good quality, palatable starter is the most basic strategy for stimulating development of GIT, especially rumen, and consequently solid feed intake. Not only does greater starter intake allow for a smoother weaning transition, but it also stimulates the development of the GIT, especially the rumen. Starter mixtures containing starch sources for energy promote establishment of fermentation within the rumen of calves to a greater extent than forage sources, mostly by increasing the production of ruminal butyrate and propionate (Khan et al., 2016). Butyrate especially is implied as the main stimulator for the papillae growth (Sander et al., 1959). Butyrate is metabolized by the epithelial cells in the rumen (Pennington, 1952) and is the preferred substrate for those cells (Weigand et al., 1975).

It is worth noting that although majority of the research focus pertaining to GIT development is on starter mixture feeds, providing calves forage sources can also be beneficial. For mature cattle, forage serves as physical fill for the rumen, helps establish a ruminal mat (Grant, 1997) and helps avoid acidotic conditions (Yang and Beauchemin, 2007). High quality forage provision can result in greater starter intake and ADG post-weaning in calves while also increasing ruminal pH and decreasing SCFA concentration (Terré et al., 2013; Pazoki et al., 2017). Additionally, forage provision helps promote the volumetric growth of rumen (Khan et al., 2016), as well as musculature of the rumen (Hamada et al., 1976; Pazoki et al., 2017). Forage included in

the diet can also help promote greater time spent ruminating by calves (Pazoki et al., 2017), leading to greater saliva flow to the rumen, which can help decrease ruminal SCFA concentration and increase ruminal pH (Castells et al., 2013). Calves fed only concentrates are prone to developing plaque between ruminal papillae which causes the papillae to adhere in clumps and is mostly composed of feed, hair and desquamated epithelial and microbial cells, effectively limiting the absorptive surface area (Suárez et al., 2007). Such plaque buildup can be prevented by the inclusion of a forage source in the diet (Suárez et al., 2007). However, the provision of forage results in a shift in ruminal fermentation towards production of acetate, which can lead to smaller ruminal papillae growth (Castells et al., 2013).

Since concentrate diets can lead to plaque formation on the surface of ruminal papillae (Suárez et al., 2007) and ruminal pH considered to be indicative of ruminal acidosis for prolonged period of time, inclusion of additional starch sources in the starters might not be beneficial to GIT development and function. Targeted supplementation of butyrate has been used to stimulate both ruminal and intestinal development in calves (Górka et al., 2009; Guilloteau et al., 2009b; Górka et al., 2014). Butyrate is produced during ruminal fermentation and is preferentially metabolized by the ruminal epithelium (Weigand et al., 1975); however, it is also present in milk fat as part of the triacylglycerides at concentration of 7.5 to 13.0 mol/100 mol (Parodi, 1997). Butyrate, when present in liquid feed, can bypass the rumen due to the esophageal groove. As such, butyrate can be a source of energy to the enterocytes by entering β-oxidation pathway and being converted to acetoacetyl-coenzyme A, although butyrate in excess of energy requirements can be used for production of ketone bodies (Astbury and Corfe, 2012). Although cell culture studies have suggested that butyrate might decrease cell proliferation and increase differentiation and apoptosis by inhibiting histone deacetylation (Candido et al., 1978; Davie, 2003), there is a disparity in literature regarding the effects of butyrate when comparing *in vitro* and *in vivo* studies (Mentschel et al., 2001; Górka et al., 2011b; Górka et al., 2014).

Butyrate can be supplemented as a salt, with sodium butyrate being the most common salt, and calcium, magnesium and potassium butyrate being less common (Górka et al., 2018a). Butyrate can be either in the unprotected (crystallized) or protected form (encased in a triglyceride matrix also referred to as microencapsulation), or as glycerol ester tributyrin (Górka et al., 2018a). The form in which butyrate is supplemented, as well as mode of supplementation, either in liquid feed or solid feed, changes the site to which the butyrate is delivered. When supplemented in liquid

feed, or when supplemented in solid feed in a microencapsulated form, butyrate will mostly be supplied to the abomasum and small intestine; whereas when supplemented in an unprotected form in solid feed, butyrate would mostly be delivered to the rumen (Górka et al., 2018a). Since butyrate is naturally present in milk (Parodi, 1997), supplementation of butyrate in MR could help mitigate some of the issues that are associated with MR feeding when compared with whole milk.

For neonatal calves, butyrate inclusion in colostrum decreased serum IgG concentrations (Hiltz and Laarman, 2019). When butyrate was supplemented in MR for calves, it increased BW (Górka et al., 2009; Guilloteau et al., 2009b; Górka et al., 2011a), ADG (Górka et al., 2011a) and decreased feed to gain ratio, and therefore improved feed efficiency (Guilloteau et al., 2009b). More importantly, butyrate supplementation in MR can increase papillae dimensions (Górka et al., 2009; Górka et al., 2011a; Kato et al., 2011), ruminal weight (Górka et al., 2009; Górka et al., 2011b), length of small intestine (Górka et al., 2014), height of intestinal villi (Guilloteau et al., 2009b; Górka et al., 2014), depth of intestinal crypts (Guilloteau et al., 2009b; Górka et al., 2014), intestinal mitotic index (Górka et al., 2011b; Górka et al., 2014) and decrease intestinal apoptotic index (Górka et al., 2011b; Górka et al., 2014). Additionally, brush border enzymatic activities are increased by butyrate supplementation in MR, including increased lactase (Górka et al., 2014), maltase (Górka et al., 2014), aminopeptidase N (Guilloteau et al., 2009b), and dipeptidylpeptidase IV (Guilloteau et al., 2009b).

When included in starter mixtures for calves, the most often used form of supplemental butyrate supplementation is microencapsulated sodium butyrate (**MSB**). Starter supplementation with butyrate results in increased starter mixture intake, ADG (Ślusarczyk et al., 2010; McCurdy et al., 2019), papillae dimensions (Górka et al., 2011a), weight of the small intestine (Górka et al., 2014), intestinal mitotic index (Górka et al., 2014), intestinal villi length (Górka et al., 2014) and dipeptidylpeptidase IV and aminopeptidase N activities (Górka et al., 2014). In addition, inclusion of butyrate in diets for post-weaning heifers decreased coccidia count when used at 0.8%DM (Rice et al., 2019), which could be one of the ways butyrate can decrease the number of days with diarrhea (Górka et al., 2011a). Additionally, the level of butyrate supplementation can be important, as with increasing butyrate supplementation, ADG and starter intake can decrease, while fecal fluidity increases (Wanat et al., 2015).

Another form of butyrate supplementation is as tributyrin, which is an ester of butyrate and glycerol. Although tributyrin had positive effects in piglets, with increased ADG and villus

height, improved intestinal barrier function and decreased fecal scores (Wang et al., 2019a; Wang et al., 2019b), same was not observed in calves. When butyrate is supplemented as tributyrin in MR, it does not impact starter intake, nor final BW of calves and may decrease ADG and the G:F ratio (Araujo et al., 2016). Overall, supplemental butyrate, in either crystalline or MSB form, within both MR and solid feed positively affects both performance of the calves and the GIT development, however timing and level of inclusion may need to be considered carefully.

Another potential additive that could benefit GIT development of calves is glycerol. As a by-product of biodiesel production, crude glycerol (85% purity) is created following removal of methanol after transesterification process, which can be further refined to 99.5-99.7% purity (Gerpen, 2005). Considering varying purity levels, the quality of glycerol that is added to the feed should be monitored to avoid inclusion of contaminants into the diet. Currently, glycerol is only approved as a feed additive for growing bull calves in Canada (Minister of Justice, 1983). Glycerol is however allowed as a general feed material for animals in European Union, although its purity needs to be declared below 99% (European Union Commission, 2013). The net energy for lactation originating from glycerol has been estimated to be similar or greater than for corn (Drackley, 2008b). Glycerol has been investigated in the diets in mature dairy cattle (DeFrain et al., 2004; Chung et al., 2007; Paiva et al., 2016); however, its effects vary, likely due to different glycerol purity, glycerol dose, different forage to concentrate ratio of the diets and the physiological state of the cows. For example, a reduction of DMI in response to glycerol inclusion was observed in transition cows fed 0.43 or 0.86 kg of glycerol per day (DeFrain et al., 2004), late-lactation cows fed 0.14 or 0.21 kg glycerol/ kg DM (Paiva et al., 2016), and beef calves fed glycerol at 10 or 20% of diet (Ramos and Kerley, 2012); while, increased DMI and milk yield were observed for mid-lactation cows fed 15.3% DM of glycerol in the diet (Bajramaj et al., 2017). Additionally, no effect of glycerol on DMI was observed in post-partum cows fed 0.25 kg glycerol/d (Chung et al., 2007) nor in Holstein bulls weighing over 300 kg and fed between 0 and 12% DM of glycerol (Mach et al., 2009). The levels of inclusion of glycerol, as well as experimental models, varied considerably between the studies, which could contribute to the differences in the observed results.

Glycerol is fermented by ruminal microflora (Hobson and Mann, 1961), with butyrate (Rémond et al., 1993; Paiva et al., 2016) and propionate (Ramos and Kerley, 2012; Paiva et al., 2016) as main end products. It has also been suggested that only 25% of glycerol is fermented in rumen, around 45% of glycerol may be absorbed through the ruminal epithelium, while the

remaining 30% is washed out to the omasum (Werner Omazic et al., 2015). Although the absorption of glycerol accounts for a considerable amount of glycerol, that glycerol can be utilized in the liver as a substrate for gluconeogenesis (Werner Omazic et al., 2015). Research with glycerol in pre-weaning calves is scarce and has concentrated more on energy replacement. For example, the ability to utilize glycerol as a partial replacement for lactose in MR (Pantophlet et al., 2016) or glucose in oral rehydration solutions (Werner Omazic et al., 2013). As shown by an *in vitro* study, even 2 g of glycerol within 1-L fermentation container can increase the molar proportion of butyrate (Rémond et al., 1993). Supplementation of glycerol in pre-weaning calves that do not consume large amounts of starter mixture may increase ruminal butyrate concentrations and may be beneficial in terms of ruminal development. Overall results in mature dairy cattle varied in regards to DMI (DeFrain et al., 2004; Chung et al., 2007; Paiva et al., 2016; Bajramaj et al., 2017). Since calves consume greater amounts of sweetened concentrate (Montoro et al., 2012), the sweet taste of glycerol (Quispe et al., 2013) could potentially improve the starter intake of calves.

2.3 Canola Meal

2.3.1 Canola and Canola Meal

Canola, also known as double-zero or double-low rapeseed, is a cultivar of rapeseed that was produced from the *Brassica napus* Bronkowski variety in Saskatoon (SK, Canada) in the 1960's and 1970's (Bell, 1982). Canola is now produced around the world in mostly moderate climates, with European Union being the greatest producer in 2018/2019, followed by China, Canada, and India (USDA, 2020). Even though both European Union and China produce the most CM, those two regions also import the most CM; whereas Canada is the greatest world exporter of CM (USDA, 2020). World production of CM was 39.08 million mT in 2018/2019, which is still considerably smaller than SBM production which is estimated at 233.83 million mT (USDA, 2020). However, considering the average price per mT, which in 2018/2019 was $329 US/mT for SBM and $247 US/mT for CM (USDA, 2020) and the protein concentration of both protein sources, 51.8 and 41.6% DM respectively (Broderick et al., 2016), the price per mT of protein could be calculated, as $635 US and $594 US for SBM and CM, respectively. When compared

with SBM, CM can be an economically suitable protein source, even if not the most commonly used one.

Canola meal is produced from the crushing of whole canola seed as a means of extracting canola oil for human consumption. The leftover CM cannot be utilized for human use and is therefore repurposed as animal feed. There are two main processing methods for canola resulting in different CM: mechanical and solvent extraction. The more commonly used processing method is pre-pressed solvent extraction due to more efficient of oil removal (Table 2.1). The main steps of the canola oil extraction process are outlined in Figure 2.1 and include: seed cleaning, preconditioning, flaking, cooking, solvent extraction, desolventizing, toasting, cooling and drying (Canola Council of Canada, 2015).

From nutritional perspective, the main changes that occur during oil extraction are the decrease in ether extract content between whole canola seed (40.5%, Table 2.1) and CM (3.6-5.4%), while both NDF and ADF increase. Additionally, although CM is a high protein source, with yearly variation in CP content (on average between 40.6 to 42.4%; (Broderick et al., 2016), CP concentration of CM is less than that in SBM (53.6%, Table 2.1).

Mechanical extraction, also known as double pressing, is not used as often as solvent extraction due to its lower efficiency of oil extraction (Figure 2.2). The basic difference between the two processes is that during mechanical extraction, canola is pressed twice through the expeller, and omits the solvent extraction and any further steps tied to the solvent use. As such, the meal produced from the mechanical extraction has a greater concentration of ether extract ranging from 8 to 11% which can increase the energy concentration of CM. Mechanically pressed CM is not exposed to additional heat that is applied to the meal during desolventizing and toasting (Canola Council of Canada, 2015).

As opposed to rapeseed cultivars, canola was designed to contain lesser amounts of antinutritional factors than rapeseed, namely less than 30 μmol glucosinolates/g DM and less than 2% of erucic acid (13-docosenoic acid) within the oil (Bell, 1993). Glucosinolates are group of over 100 known plant compounds, of which around 30 are important for the quality of CM (Sørensen, 1990). The main negative effect of glucosinolates pertains to their goitrogenic effect and it can be worsened by the presence of myrosinase enzyme, which can hydrolyse glucosinolates into more toxic products: oxazolidinethione and isothiocyanates (Bell, 1993).

Table 2.1. Chemical composition of canola seed, mechanically extracted canola meal (CM), solvent extracted canola meal and soybean meal (SBM)

Variable	Canola seed[1]	Mechanically extracted CM[1]	Solvent extracted CM[2]	SBM[2]
DM, %	89.9	90.3	90.7	88.6
CP, %DM	20.5	37.8	40.7	51.5
NDF, % DM	17.8	29.8	28.8	7.5
ADF, %DM	11.6	20.5	18.5	4.1
Ether extract, %DM	40.5	5.4	4.2	1.3
Ash, %DM	4.6	7.4	8.2	6.8
RUP[3], %CP	17.2[4]	29.3[4]	24.3	31.2

[1]NRC (2001).
[2]Paz et al. (2014).
[3]RUP = rumen undegradable protein.
[4]Based on equation described by Schwab et al. (2003) and digestion kinetics values from NRC (2001).

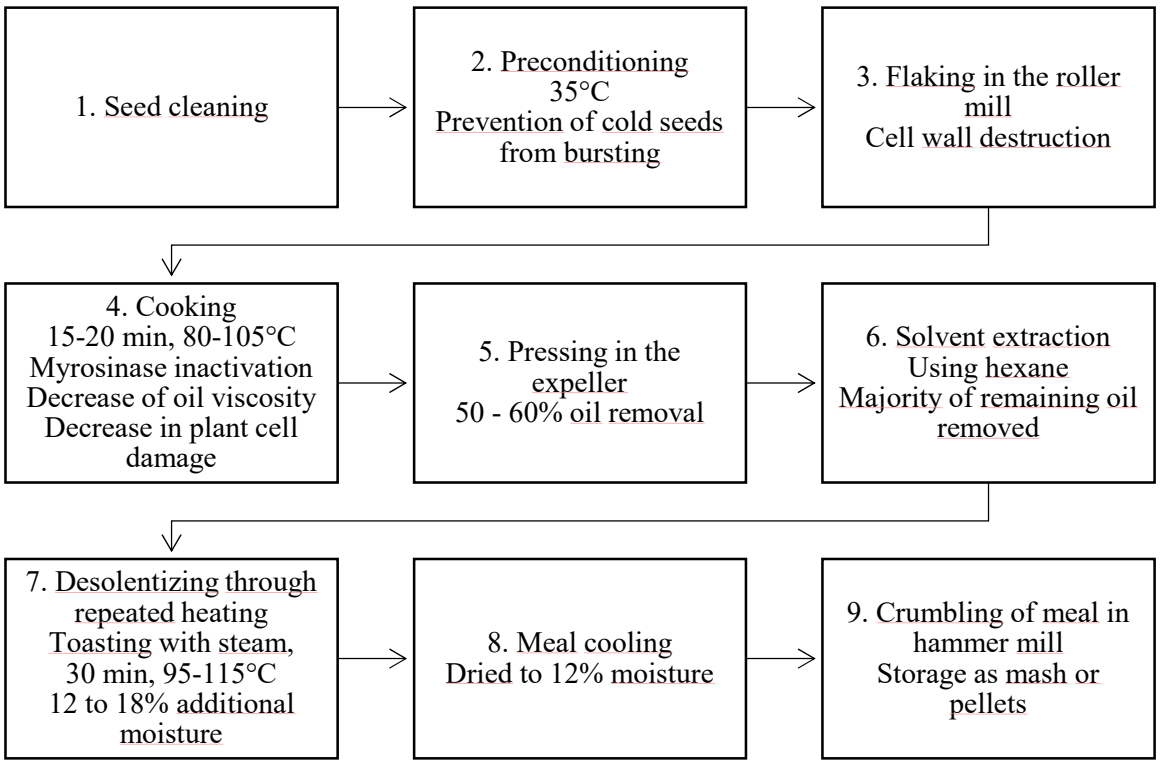

Figure 2.1. The main steps, conditions, and objectives of canola oil extraction using solvent. Based on Canola Council of Canada (2015).

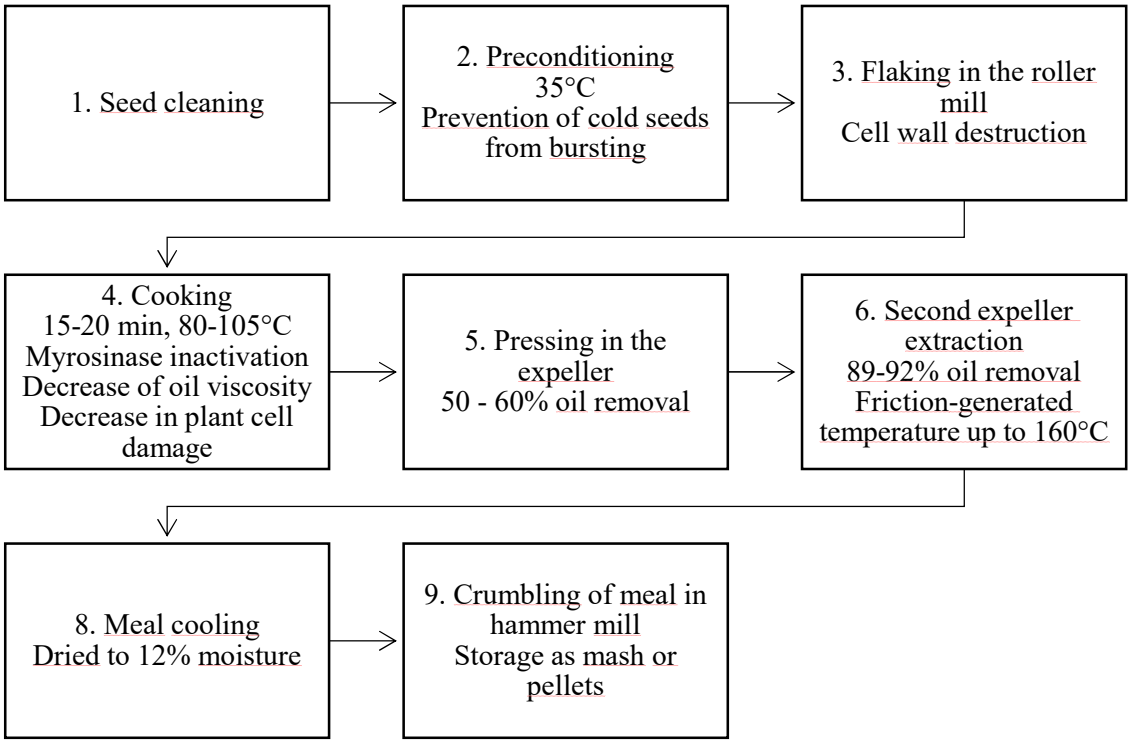

Figure 2.2. The main steps, conditions, and objectives of canola oil extraction through mechanical extraction (double-pressing). Based on Canola Council of Canada (2015).

Glucosionalates can also have a negative effect on the taste of CM (Sørensen, 1990). Current levels of glucosinolates in CM are considerably below the previously established threshold of 30 µmol/g DM, with yearly variation between 4.00 to 5.20 µmol/g DM within Canadian processing plants (Adewole et al., 2016). While yearly variation of glucosinolate concentration was noted, Adewole et al. (2016) only determined concentrations of 5 individual glucosinolates, which does not account for the presence of other components of the same group. Erucic acid can have negative effects on growth and fat deposition in heart and skeletal muscles, mostly in rodents (Dupont et al., 1989). However, these potential negative effects were sufficient to warrant the development of low erucic acid canola varieties (Bell, 1982).

While the previously described glucosinolates and erucic acid are the main antinutritional factors considered within CM, they are not the only ones. Other antinutritional factors that can be present in CM include (Bell, 1993):

- sinapine at 0.6 to 1.8%, which can contribute to the bitter flavor of CM, although it is likely overshadowed by glucosinolates;
- tannins at 1.5 to 3.0%, which can interfere with digestion processes, especially relating to protein digestion;
- phytic acid at 3.0 to 6.0%, which can negatively impact mineral absorption.

2.3.2 Use of Canola Meal in Diets for Dairy Cattle

Canola meal can be used in diets for many different types of animals, including dairy, beef, small ruminant, poultry, swine and fish operations (Bell, 1993; Canola Council of Canada, 2015). In the 1970's CM utilization by dairy cattle accounted for 20% of total livestock CM use in Canada (Bell, 1982). According to Brennan et al. (1999), the Australian dairy industry accounted for 26% of livestock CM usage and was the single biggest source of livestock CM use.

When CM is included in diets for mature dairy cattle, on an isonitrogenous basis, and compared to other protein sources (e.g. corn gluten meal, cottonseed meal, distiller grains), DMI, milk yield, and fat- and energy-corrected milk yields, as well as milk protein yield can be increased (Martineau et al., 2013). However, this effect is not as visible when CM replaces SBM with the only detectable effect remaining being increased milk protein yield (Martineau et al., 2013). In the meta-analysis by Martineau et al. (2013) which included 27 different experiments, the average

inclusion rate of CM was 11.7% DM, with a maximum inclusion of 17.2% DM (Martineau et al., 2013). Huhtanen et al. (2011) compared the use of CM and SBM in diets for dairy cows comparing responses from 122 different studies. They found that CM may increase DMI, milk production, and milk protein yield in dairy cows fed diets based around grass silage with average CM inclusion of 11.7% DM. Overall, CM is often used in dairy cow diets due to its many production benefits.

Summary of major effects of CM is in Table 2.2. A more recent study replacing SBM with CM observed decreased ruminal DM and CP digestibility, as well as total tract DM digestibility (Paz et al., 2014). A similar observation was made when CM was included in diets for feedlot cattle, where total tract CP digestibility was $_{\text{decreased}}$ (Zinn, 1993). Additionally, an in situ experiment observed that SBM results in greater degradability of DM and CP than CM (Maxin et al., 2013). However, when Paula et al. (2018) compared CM and SBM in terms of performance and digestion, they observed that when CM was fed to dairy cows, it resulted in lesser milk urea nitrogen concentration, greater milk lactose, greater CP, OM, DM, and NDF digestibility, greater CP intake, greater ruminal ammonia concentration, and a tendency for greater RDP supply. Thus, despite the decrease in ruminal digestibility, use of CM still can result in increased milk production.

2.3.3 Use of Canola Meal in Diets for Holstein Calves

Even though the use of CM in mature dairy cows is common and can result in production benefits as outlined in Chapter 2.3.2, the same is not clear when it comes to CM use in diets for dairy calves. The research when pertaining to use of CM in calf rations does not provide a definitive pattern of effect on calf performance (Table 2.2).

Some early work on rapeseed use in calf diets showed no differences in terms of growth and digestibility (Ingalls and Seale, 1971; Fisher, 1980) but reductions in starter intake when rapeseed was included (Stake et al., 1973). However, results were not consistent as the inclusion of low glucosinolate rapeseed meal (included at 20% DM) within calf diets did not affect ADG nor starter intake, while high glucosinolate variety of CM decreased starter intake (Fiems et al., 1985). Khorasani et al. (1990) observed that replacing SBM with CM in diets for calves decreased DM and CP apparent total tract digestibility.

Table 2.2. Major observations of canola meal (CM) inclusion research in mature cattle and calves, as compared with soybean meal (SBM), unless otherwise stated

CM inclusion	CM effect			Reference
	Increased	Decreased	Not affected	
Adult cattle				
20% DM[1]		Ruminal and total tract CP[2] digestibility		Zinn (1993)
Average 11.7% DM, metanalysis	DMI[3], milk production, milk protein yield			Huhtanen et al. (2011)
Average 16.4% DM, metanalysis	Other protein: DMI, milk yield, milk protein yield SBM: milk protein yield			Martineau et al. (2013)
In situ analysis		DM and CP digestibility		Maxin et al. (2013)
In situ analysis	Ruminal degradation of Met and Glu	Ruminal DM and CP, and DM total tract digestibility		Paz et al. (2014)
11.2% DM	Milk lactose %, CP, OM[4], DM and NDF[5] digestibility, ruminal ammonia concentration, RDP[6] supply	Milk urea nitrogen concentration	Milk yield, milk fat and protein %	Paula et al. (2018)
Calves				
6.8 or 13.7% rapeseed meal			Starter intake, ADG[7], feed efficiency	Ingalls and Seale (1971)
26.20%		Starter intake	BW gain, DM and CP digestion	Stake et al. (1973)
31.6% rapeseed meal	Apparent lignin and fat digestibility		Starter intake, ADG, digestibility	Fisher (1980)
17.60%		Post-weaning ADG	Starter intake, pre-weaning ADG	Claypool et al. (1985)
0, 10, 20%			ADG, starter intake	Fiems et al. (1985)
43% DM		DM and CP apparent total tract digestibility		Khorasani et al. (1990)
30%		Starter intake, less preferred/palatable than soybean meal		Miller-Cushon et al. (2014b)
35.0% as fed or 16.5% as fed	At 35%: fecal fluidity and d with diarrhea between 1-35 d of age	At 35%: ADG, G:F[8] ratio, fecal fluidity d36-56; At 16.5%: starter intake d 1-35, fecal fluidity d36-56		Hadam et al. (2016)
15% DM		Pre-weaning G:F ratio	Starter intake, straw intake, ADG	Terré et al. (2016)

[1]DM = dry matter.
[2]CP = crude protein.
[3]DMI = dry matter intake.
[4]OM = organic matter.
[5]NDF = neutral detergent fibre.
[6]RDP = rumen degradable protein.
[7]ADG = average daily gain.
[8]G:F = gain to feed.

Considering CM decreases CP digestibility in mature ruminants (Zinn, 1993; Paz et al., 2014), it is not surprising to observe similar effect in calves (Khorasani et al., 1990). It is likely that the digestibility issues with CM digestibility could stem from the relatively high fibre fraction of the meal, which could impede digestibility.

Additionally, Huhtanen et al. (2011) also observed across the different dairy nutrition studies that heat-treatment of CM decreases CP digestibility, which can lead to greater supply of RUP. Heat-treatment of CM can be carried out at different temperatures, times and methods; however, excessive temperature or duration of heat can result in the Maillard reaction which can decrease protein digestibility (van Soest and Mason, 1991). This was observed by McKinnon et al. (1995) who reported that heating CM to 145°C resulted in decreased DM and CP disappearance in both the rumen and intestine, whereas heating CM to 125°C only resulted in decreased DM and CP disappearance in the rumen. Heat-treatment of CM can also cause changes to the AA composition of CM, by increasing concentration of Met and Gln (Newkirk et al., 2003). Additionally, after heat-treatment AA disappearance in the rumen decreases, while the disappearance in the intestine increases (Moshtaghi Nia and Ingalls, 1995).

Canola meal was shown to be less palatable to calves than SBM (Miller-Cushon et al., 2014a; Miller-Cushon et al., 2014b). When Terré et al. (2016) compared pelleted CM and SBM (in combination with wheat and barley straw), no differences in DMI or growth were observed; however, SBM tended to result in greater G:F ratio. Hadam et al. (2016) compared both full replacement of SBM with CM, and when 50% of CP supply was replaced by CM. When fully replacing SBM, CM resulted in decreased ADG, while no changes in BW and intake were observed, and an increased number of days with diarrhea, while fecal fluidity was increased only pre-weaning, and was decreased around weaning (Hadam et al., 2016). However, when CM replaced only 50% of CP from SBM, the only negative effect observed was a tendency for a smaller starter intake pre-weaning (Hadam et al., 2016). As such, although notions about CM in calf starters are generally negative, some research suggests comparable performance to that of SBM. Additionally, the inclusion rate of CM in calf diets can range from 15% DM (Terré et al., 2016), to 35% as fed (Hadam et al., 2016) and even up to 43% on a DM basis (Khorasani et al., 1990), which can be considerably greater than in diets for mature dairy cows averaging 11.7% DM (Martineau et al., 2013). The variation in the inclusion rate could also partially be responsible for differing responses observed within the literature.

2.4 Summary of Research Rationale

Calves play an important role in maintaining the continuity of dairy herds. Based on the conducted literature review, early life nutrition of calves and their consequent growth help ensure the calf becomes a highly producing dairy cow. As such, good quality starter mixture needs to be provided to calves early in life, as starter intake helps with establishing ruminal fermentation producing SCFA with butyrate is regarded as the main stimulator of GIT development. Considering that regional production of canola and CM is substantial in Canada, it would be desirable to include CM into starter mixtures for calves. The use of CM for calf starters has been investigated in the past with quite variable results. In fact, some studies reported comparable performance when contrasted with SBM, or decreased performance, especially due to palatability and digestibility issues. However, by modifying CM with heat-treatment processing, the protein fractions digested in the rumen and small intestine can be altered. Also, the potential negative effects of CM could be mitigated by supplementation with glycerol, which can improve palatability of the starter and potentially stimulate ruminal development) and MSB which is known for enhancing ruminal and small intestine development. Overall, this project can help clarify conflicting research regarding CM use in calf starters, both by itself and in settings designed to mitigate the known negative effects of CM.

2.5 Overall Hypothesis

Canola meal, when supplied in pelleted starter mixture, will be an adequate protein source for dairy calves supporting both their growth and gastrointestinal tract development, possibly further enhancing those processes by additional supplementation or processing.

2.6 Overall Objective

To determine the effects of canola meal on growth and intake performance characteristics and on ruminal and small intestinal development of Holstein calves, while also evaluating the effects of additional supplementation with other compounds or different processing characteristics of canola meal.

3 EFFECT OF HEAT-TREATED CANOLA MEAL AND GLYCEROL INCLUSION ON PERFORMANCE AND GASTROINTESTINAL DEVELOPMENT OF HOLSTEIN CALVES[1]

3.1 Abstract

The objectives of this study were to assess the effect of using heat-treated canola meal (**CM**) and glycerol inclusion in starter mixtures on starter intake, growth, and gastrointestinal tract (**GIT**) development in Holstein bull calves. In the first study, a protocol for the heat-treatment of CM was evaluated by comparing commercial CM that was exposed to 0, 100, 110, or 120°C of heat-treatment for 10 min. Following heat-treatment, in situ crude protein (**CP**) ruminal degradability and estimated intestinal CP digestibility were assessed. It was observed that the degradable fractions of dry matter and CP in CM decreased linearly with increasing temperature of heat-treatment. The estimated intestinal CP digestibility was greatest when CM was heated to 110°C. In the second study, 28 bull calves were used in a randomized complete block design. Calves were fed pelleted starters containing CM or CM that was heat-treated to 110°C for 10 min. Diets also contained 0 or 5% glycerol on a dry matter basis. The study lasted 51 d, ending on the first day of weaning. Starter intake, average daily gain (**ADG**), ruminal short-chain fatty acid concentrations, morphology of the rumen and small intestine, gene expression at mRNA level (*MCT1*, *GPR41*, *GPR43*, *UT-B*, *AQP3*, *PEPT1*, *PEPT2*, *ATB0+*, and *EAAC1*) in the ruminal,

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Author contribution

My contribution to this publication includes: changes to the original experimental design; practical implementation of the experimental design; caring for animals used during the studies; conducting data collection pertaining the animals; collection of samples; coordination of sample collection during dissections when help of other people was required; conducting majority of laboratory work; conducting statistical analysis; preparation of original manuscript; and implementation of co-authors reviews.

jejunal, and ileal epithelium, and brush border enzyme activities in the duodenum, jejunum, and ileum were investigated. Few interactions between heat-treated CM and glycerol inclusion were observed. Feeding heat-treated CM did not affect starter intake. However, feeding heat-treated CM to calves tended to reduce ADG and decreased the weight of ruminal and jejunal tissue. Heat-treatment did not affect gene expression or brush border enzyme activities in the small intestine. Glycerol inclusion tended to increase cumulative starter intake and increased body weight gain during whole study period. Use of glycerol reduced ruminal pH and increased the concentration of ruminal short-chain fatty acids. Additionally, glycerol inclusion increased abomasal, duodenal, jejunal, and cecal digesta weights and tended to increase the weight of the jejunal tissue. Glycerol supplementation tended to downregulate the expression of *MCT1* in the ruminal epithelium, and upregulated the expression of *MCT1* in the epithelium of proximal jejunum. In conclusion, heat-treatment of CM may negatively affect calf growth and GIT development. Glycerol inclusion may increase starter intake, ADG, ruminal fermentation, and intestinal development in calves when CM is used as a main source of protein in pelleted starter mixture.

3.2 Introduction

The weaning transition represents a critical phase for calves (Weary et al., 2008), with marked nutritional changes occurring as the provision of milk is reduced and calves start to rely on solid feed intake. The shift from milk to solid feed both stimulates and relies on gastrointestinal tract (**GIT**) development (Roth et al., 2009; Khan et al., 2016). Though considerable focus has been placed on stimulating ruminal digestion around weaning as a strategy to promote ruminal development, Górka et al. (2011b) reported that development of the small intestine may also influence ruminal development. As such, dietary strategies that provide nutrients to promote ruminal fermentation while ensuring substrate availability in the small intestine may provide benefit for calves at weaning.

Canola meal (**CM**) is a common protein source used in diets for lactating cows and has been reported to increase milk yield when used as a replacement for soybean meal (**SBM**) or wheat dried distiller grains (Huhtanen et al., 2011; Martineau et al., 2013). However, CM is not commonly used in diets for calves around weaning due to concerns over palatability (Miller-Cushon et al., 2014a; Miller-Cushon et al., 2014b) and digestibility (Fiems et al., 1985; Khorasani et al., 1990). In contrast, some studies have suggested that CM inclusion has limited effects on starter intake and growth, at least when inclusion is limited to a portion of the SBM (Claypool et al., 1985; Hadam et al., 2016). However, mildly heat-treated CM might be beneficial because it may increase the RUP fraction (McKinnon et al., 1991) and deliver a greater supply of AA to calves, and thus potentially hasten small intestinal development, starter intake, and consequently, whole GIT development in calves before weaning. Nevertheless, the effect of heat-treated sources of protein, likely to increase rumen bypass protein content in starter mixture, for calves at weaning has not been extensively evaluated.

Glycerol is a by-product of bio-diesel production (Gerpen, 2005) that is rapidly fermented in the rumen (Rémond et al., 1993) and shifts fermentation to increase the proportion of butyrate and propionate in ruminal fluid (Rémond et al., 1993; Paiva et al., 2016). Butyrate is known to stimulate development of the ruminal epithelium (Sander et al., 1959; Mentschel et al., 2001), and thus glycerol may be a valuable feed ingredient in diets for calves during the weaning transition. Additionally, due to its sweet taste (Quispe et al., 2013), glycerol may improve palatability of feed,

which may be an important contributing factor when CM is used as a source of protein in calf starters.

I hypothesized that the use of heat-treated CM in a starter mixture will promote the development of the small intestine, likely through greater supply of AA to the small intestine, which will have a positive effect on starter intake and intestinal and ruminal development. Also, I hypothesized that glycerol inclusion in a starter mixture containing CM would increase the proportion of butyrate in ruminal fluid, and thus further stimulate ruminal development and starter intake. The objectives were to evaluate the optimal heat-treatment temperature of CM in regard to ruminal and estimated intestinal digestibility of nutrients, and to determine the effect of CM heat-treatment, glycerol supplementation, and their interaction on starter intake, calf growth, and the development of the GIT of dairy calves at weaning.

3.3 Materials and Methods

Experimental procedures were pre-approved by the University of Saskatchewan Animal Research Ethics Board (protocol no. 20100021) in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, ON, Canada).

3.3.1 Study 1: Optimal Heat-treatment Temperature of CM

3.3.1.1 Heat-treatment of CM

Four subsamples of commercial solvent-extracted CM (100 kg each) from a single source were used to evaluate the effect of heat-treated CM on in situ ruminal and estimated intestinal CP digestibility. For each treatment, a 25-kg portion from each subsample was subjected to 1 of 4 treatments: (1) no additional heat (**CON**), or (2) additional heat-treatment to achieve a temperature of 100°C (**H-100**), (3) 110°C (**H-110**), or (4) 120°C (**H-120**). All treatment temperatures were held at the designated temperature for 10 min (McKinnon et al., 1991, 1995). Heat-treatment was conducted in a tumble dryer (POS Bioscience, Saskatoon, SK, Canada) and the temperature was steadily increased from ambient temperature to the treatment temperature. A total of (mean ± SEM) 26 ± 2.1 min for H-100, 43 ± 4.4 min for H-110, and 67 ± 6.7 min for H-120 were required

to achieve target temperatures. Following 10 min of heat exposure at the specific temperature, the CM was cooled to 50°C. The durations required for cooling were 21 ± 2.3 min for H-100, 24 ± 4.1 min for H-110, and 30 ± 0.3 min for H-120. All temperature changes occurred linearly.

3.3.1.2 In Situ Ruminal Degradability

Four ruminally cannulated (internal diameter of 10 cm, Bar Diamond Inc., Parma, ID) Hereford-cross heifers (616 ± 26.0 kg, initial BW ± SD) were used for in situ incubations. Heifers were housed in individual pens (3 × 3 m) at the Livestock Research Building (University of Saskatchewan, Saskatoon, SK, Canada) and were fed a TMR twice a day at 0900 and 1700 h. The diet consisted of barley silage (25% DM), rolled barley grain (47% DM), grass hay (20% DM), and a mineral and vitamin pellet (8% DM) with the amount of TMR provided at 2.5% of BW. Feed refusals were weighed and collected daily at 0830 h. Barley grain and mineral and vitamin pellet samples were collected once per wk and forage samples were collected twice per wk. Feed and refusal samples were analyzed for DM content by drying at 55°C in a forced-air oven until achieving a constant weight. The DM content of the ingredients was used to adjust their proportions in the diet on an as-fed basis, as necessary. Feed samples were ground to pass through a 1-mm sieve (Christy and Norris, Christy Turner Ltd., Chelmsford, UK) and were pooled by sampling date and analyzed for chemical composition as described below.

Empty polyester bags (53 ± 10 μm pore size; #BG510, Bar Diamond Inc.) were dried at 55°C for 1 h, placed in a desiccator for 15 min, and weighed. Approximately 7 g of CM was weighed into each bag and then bags were sealed. The sequential-in all-out procedure, as recommended by the NRC (2001), was used for in situ incubations. Incubation times for the nylon bags were 0, 2, 4, 8, 12, 16, 24, and 48 h. Incubations were completed in 2 separate, consecutive, randomized runs with treatments and times balanced among heifers. Nylon bag insertion into the rumen was initiated at 0900 h on d 1 and all bags were removed at 0900 h on d 3. Bags for the 0 h of incubation were soaked in warm (37 to 39°C) distilled water for 30 min. As the in situ incubation approach used a sequential-in all-out method, the number of nylon bags inserted into a single heifer did not exceed 44 bags. In addition, 4 bags for each treatment replicate were incubated for 12 h to allow for a 3-step *in vitro* procedure to measure estimated intestinal digestibility (Calsamiglia and Stern, 1995).

Upon removal from the rumen, bags were immediately placed in cold water (4°C) and washed 5 times. For each wash, 60 bags were placed in 15 L of cold water and manually agitated for 1 min. After washing, bags were placed on a flat pan and frozen at −20°C for 24 h. Following this step, bags were removed from the freezer, placed in cold water, and rinsed 1 additional time to reduce microbial contamination (Kamel et al., 1995). The bags were then dried at 55°C until achieving a constant weight, placed in desiccators for 15 min, and weighed. The residual feed from each time point within a treatment replicate were pooled for chemical analysis.

3.3.1.3 Estimated Intestinal Digestibility

The 3-step estimated intestinal digestibility procedure was conducted as described by Calsamiglia and Stern (1995). The residues remaining after 12 h of ruminal incubation were pooled for each treatment and subjected to digestion in pepsin (1 g/L; No. P-7012, Sigma, St. Louis, MO) and HCl (0.1 N) for 1 h at 38°C in a shaking water bath. Samples were then neutralized using 1 N sodium hydroxide and subsequently incubated in the presence of pancreatin (3 g/L; No. P-7545, Sigma; in 0.5 M KH2PO4 buffer) for 24 h. During incubation, samples were placed in a shaking water bath (38°C). At the end of incubation, enzymatic activity was stopped by addition of trichloroacetic acid (100% wt/vol, T6399, Sigma). Samples were then stored at 4°C until further analysis of CP content.

3.3.1.4 Chemical Analysis of Feed and In Situ Samples

Analytical DM content was determined by drying samples at 135°C for 2 h. Crude protein concentration was measured using the Kjeldahl method (984.13, AOAC, 1994). (984.13, AOAC International, 1994). Ash was determined according to method 942.05 (AOAC, 1994) (AOAC International, 1994) and was used to calculate OM concentration. The NDF and ADF concentrations were analyzed independently using Ankom F57 filter bags in the Ankom 200 Fiber Analyzer (ANKOM Technology Corp., Fairport, NY) according to the method of van Soest et al. (1991); NDF samples were treated with α-amylase and sodium sulfite. Ether extract analysis was carried out in Goldfisch Fat Extraction Apparatus (Model 35001, Goldfisch, ExpoTechUSA, Houston, TX) according to method 920.39 (AOAC, 1994). (AOAC International, 1994). Amino

acid concentrations of the CM samples were analyzed by Evonic Nutrition and Care GmbH (Essen, Germany).

3.3.2 Study 2: Effect of Heat-Treated CM and Glycerol on Calves

3.3.2.1 Animals, Housing, and Feeding Regimen

Twenty-eight newborn Holstein bull calves were sourced from a single commercial herd near Saskatoon (SK, Canada) for this study. All calves were separated from their dam immediately after birth and provided a commercial colostrum replacer (Headstart bovine dried colostrum; The Saskatoon Colostrum Co. Ltd., Saskatoon, SK, Canada) to target total supply of 180 g of IgG within first 12 h of life. Two bags of colostrum replacer (120 g of IgG) were fed within 6 h of birth, and a third bag (60 g of IgG) was fed within 12 h of birth. Colostrum replacer was fed from a bottle with a nipple. Following colostrum, all calves received a commercial milk replacer (**MR**, Grober Nutrition, Cambridge, ON, Canada, Table 3.1) containing dried skim milk powder (Grober Energy Supplement 983681), dried whey powder, vegetable oil, Grober rearing premix (950261), autolyzed yeast, soy lecithin, calcium chloride, dl-methionine, calcium carbonate l-lysine, flavor (983030; Grober Nutrition), viable microbial product (982931; Grober Nutrition), polyethylene glycol, mono and di-oleates, and selenium enriched yeast (982026; Grober Nutrition) with a mixing rate of 150 g (DM basis) of MR powder in 1 L of water. Until 7 d of age, all calves were fed 4 L/d of MR in 2 equal feedings. Within 1 wk of birth (3.6 ± 2.1 d of age), calves were transported to the Livestock Research Building (University of Saskatchewan, Saskatoon, SK, Canada). Upon arrival, calves were placed in individual pens (1.2 × 2.4 m) with wood shavings as bedding. Bedding was kept at a minimum and replaced as needed.

At 8 d of age calves were weighed (mean 43.2 ± 3.9 kg) and BW was recorded weekly thereafter. The amount of MR provided was adjusted to deliver 10% BW on d 8 and 9, 11.5% BW on d 10 and 11, 13% BW on d 12 and 13, and 15% BW from d 14 until d 28 of age with the MR fed in 3 equal feedings at 0800, 1200, and 1600h. Calves were exposed to a gradual weaning protocol by reducing the amount of MR offered to 10% of BW starting on d 29, 5% BW starting on d 36 (both fed in 2 equal feedings at 0800 and 1600 h), and 2.5% BW from d 43 (fed in 1 feeding at 0800 h) with no further MR offered after d 50. The amount of MR offered and refused

Table 3.1. Ingredient and nutrient composition of pelleted starter mixtures containing canola meal either nonheated (NH) or heated to 110°C for 10 min (H), without (NG) or with (G) inclusion of 5% DM of glycerol, and nutrient composition of milk replacer

Variable	Treatment				Milk replacer[1]
	NH-NG	NH-G	H-NG	H-G	
Ingredient composition, %DM					
Non-heated canola meal	34.00	34.00	-	-	
Heated canola meal	-	-	34.00	34.00	
Barley grain	32.00	26.82	32.00	26.82	
Corn grain	29.00	29.00	29.00	29.00	
Whey-permeate (deproteinized)	2.00	2.00	2.00	2.00	
Mineral supplement[2]	3.00	3.00	3.00	3.00	
Urea	-	0.18	-	0.18	
Glycerol	-	5.00	-	5.00	
Chemical composition[3], %DM					
DM, %	91.5 ± 0.6	91.5 ± 0.8	92.6 ± 0.2	92.7 ± 0.8	93.2 ± 0.9
CP	20.4 ± 0.5	20.6 ± 0.5	21.2 ± 0.7	21.2 ± 0.4	27.3 ± 0.7
ADF	10.2 ± 0.2	10.1 ± 0.4	10.3 ± 0.2	10.7 ± 0.3	n/a[4]
NDF	16.1 ± 0.2	17.1 ± 0.8	17.0 ± 0.4	17.0 ± 0.2	n/a
Starch	39.2 ± 0.2	35.9 ± 0.9	38.1 ± 0.8	33.9 ± 0.8	n/a
NSC	41.1 ± 0.1	44.7 ± 1.4	42.5 ± 1.0	46.0 ± 0.8	n/a
Ether extract	2.99 ± 0.2	3.00 ± 0.1	3.23 ± 0.2	3.00 ± 0.0	17.7 ± 0.1
OM	91.6 ± 0.1	91.9 ± 0.3	91.9 ± 0.2	91.5 ± 0.3	92.4 ± 0.1
Ca	1.31 ± 0.0	1.19 ± 0.0	1.24 ± 0.0	1.32 ± 0.0	1.1 ± 0.0
P	0.96 ± 0.0	0.94 ± 0.0	0.97 ± 0.0	1.01 ± 0.0	0.9 ± 0.0
ME[5], Mcal/kg	3.09 ± 0.01	3.10 ± 0.02	3.12 ± 0.01	3.09 ± 0.01	4.71 ± 0.01

[1]Grober Nutrition (Cambridge, ON, Canada). The milk replacer contained dried skim milk powder (Grober Energy Supplement 983681), dried whey powder, vegetable oil, Grober Rearing Premix (950261), autolyzed yeast, soy lecithin, calcium chloride, DL-methionine, calcium carbonate L-lysine, flavour (983030), viable microbial product (982931), polyethylene glycol, mono and di-oleates, and selenium yeast (982026).

[2]Composition: Ca 1.2%; P 0.6%; Mg 0.4%; K 1.2%; S 0.3%; Na 0.4%; Cl 0.7%; Se 0.5%; Fe 230 ppm; Mn 120 ppm; Zn 220 ppm; Cu 50 ppm; Vit. A 10,000 IU; Vit. D 1,600 IU; Vit. E 50 mg.

[3]Mean ± SD.

[4]n/a = not analyzed.

[5]ME = metabolizable energy, calculated based on NRC (2001) for the starters as: (1.01 × DE – 0.45) + 0.0046 × (EE% – 3), where DE = digestible energy, calculated as (0.057 CP% + 0.094 EE% + 0.0415 (100 – CP% – EE% – ash%)) × 0.82; and for the milk replacer as: 0.96 × (0.97 × (0.057 CP% + 0.092 EE% + 0.0395 × (100 – CP% – EE% – ash%))).

were recorded at each feeding and summarized to reflect each feeding level and the cumulative amount fed over the course of the study. Representative samples of MR were collected from each 25-kg bag and used for DM analysis to monitor and ensure the mixing rate was static at 150 g of DM/L. Samples of MR were also composited monthly and used for chemical analysis (described below).

Calves were blocked by birth date and within block randomly assigned to 1 of 4 treatments, using BW at 8 d of age as a secondary blocking factor. The treatments differed in composition of offered pelleted starter mixtures that contained: (1) CM without glycerol (NHNG), (2) CM with glycerol (NH-G), (3) heat-treated CM without glycerol (H-NG), or (4) heat-treated CM with glycerol (H-G) in a 2 × 2 factorial arrangement (Table 3.1). Starters were formulated to be isonitrogenous and isoenergetic. To balance the nitrogen content between glycerol supplemented and non-supplemented diets, urea was included in the glycerol starters. Based on results of study 1 (see Results section), the heat-treated CM was heated in a tumble dryer (POS, Saskatoon) at 110°C for 10 min before mixing with other starter ingredients and pelleting. The crude glycerol made from soybean (minimum of 80% purity and maximum of 2.30% sodium) was sourced from Canadian Feed Research Centre (North Battleford, SK, Canada) and was included at 5% of the pellet DM. Glycerol was included in the starters in exchange for barley grain. Starter mixtures were pelleted at the Canadian Feed Research Centre. First, the starter mash was prepared for pelleting with UAS Muyang pellet conditioner (model MUTZ350-J Triple layered twin; Muyang, Yangzhou, China) by addition of 2.5% moisture at atmospheric pressure. Next, the mash was pelleted using a 3.5-mm die at 65°C for 20 to 25 s in the UAS-Muyang pellet mill (model MUZL350II). The compression ratio of the die was 14.0 as calculated by dividing the effective length of the die hole (50 mm) by the diameter of the die hole (3.57 mm). Pellets were then cooled to 5°C above ambient temperature in a UAS-Muyang counter-flow cooler (model SLNF14X14A). Pelleting added 2.5% moisture, which was removed by the cooling process.

Starter was offered from d 8 of age at a rate of 400 g/d and the amount was adjusted daily to ensure *ad libitum* intake by increasing the amount offered by 500 g when refusals dropped below 250 g initially, and when refusals were less than 500 g for the rest of the study. Refusals of the starter were removed and recorded daily before the morning MR feeding, and fresh starter offered after provision of MR. Furthermore, refusals from each calf were collected and pooled on a weekly basis and analyzed for DM as previously described. In addition, samples of starters were collected

weekly for DM determination and chemical analysis. Samples were then composited to yield a monthly sample for each treatment and were then ground to pass through a 1-mm sieve (ZM 200 Retsch, Haan, Germany). Data on starter intake were summarized to indicate starter intake from d 8 to 28, 29 to 35, 36 to 42, and 43 to 49 to represent periods of time where MR was provided at 15% of BW, 10% of BW, 5% of BW, and 2.5% of BW, respectively. In addition, cumulative starter intake over the duration of the study was calculated.

Samples of the starter pellets and MR were sent to Cumberland Valley Analytical Services (Hagerstown, MD) for analysis of DM, CP, ether extract, OM, ADF, NDF, starch, ethanol soluble carbohydrates, Ca, and P. Dry matter was analyzed using method 930.15 by drying the sample at 135°C (AOAC, 2000). Crude protein was analyzed using method 990.03 (AOAC, 2000) using Leco FP-528 Nitrogen Combustion Analyzer (Leco, St. Joseph, MI). Acid detergent fiber was analyzed using method 973.18 (AOAC, 2000) with modifications; I used Whatman 934-AH glass microfiber filters with 1.5-µm particle retention. Neutral detergent fiber was analyzed as described by van Soest et al. (1991) using sodium sulfate and α-amylase with the same microfiber filters as used for ADF. Starch was analyzed according to Hall (2009). Ethanol soluble carbohydrates were analyzed according to DuBois et al. (1956). Ether extract was analyzed using method 2003.05 (AOAC, 2006) using Tecator Soxtec System HT 1043 Extraction unit (Tecator, Foss, Eden Prairie, MN). To calculate OM, ash was analyzed with method 942.05 (AOAC, 2000) using a 1.5-g sample with a 4-h ashing duration. Mineral analysis was conducted using method 985.01 (AOAC, 2000) and Perkin Elmer 5300 DV ICP (Perkin Elmer, Shelton, CT).

3.3.2.2 Blood Sample Collection and Analysis

Blood samples were collected on d 22 (15% BW of MR provision), d 43 (5% BW of MR provision) and on d 51 (1 d after weaning) from the jugular vein at 1000 h (2 h post-feeding). Two samples (10 mL each) were collected at each time point with blood placed in a tube containing Na-heparin (plasma separation; 158 IU, Becton Dickinson, Franklin Lakes, NJ) and a tube without anticoagulant (serum separation; Becton Dickinson). Blood collected into the tube with Na-heparin was immediately placed on ice until centrifugation for 15 min at 2,600 × g at 4°C (Sorvall ST 16R, Thermo Scientific, Waltham, MA), whereas blood collected into the tube without an anticoagulant was allowed to clot for 1 h before centrifugation at the same conditions as for plasma separation.

The supernatant from both samples was then transferred into separate vials and frozen at −20°C until analysis of glucose, insulin, β-hydroxybutyrate (**BHB**), non-esterified fatty acids (**NEFA**), and urea.

Plasma glucose concentration was analyzed by enzymatic reaction of glucose with glucose oxidase/peroxidase (No. P7119 Sigma) and dianisidine dihydrochloride (No. D3252 Sigma) in a 96-well plate and the absorbance was measured at 450 nm using a plate reader (Epoch 2, BioTek Instruments Inc., Winooski, VT). Plasma insulin concentration was analyzed using a bovine-specific ELISA kit (no. 10–120–01, Mercodia, Uppsala, Sweden). Concentration of BHB in serum was measured based on the method described by Williamson et al. (1962). Plasma NEFA concentration was analyzed using a commercial kit (HR Series NEFAHR(2); Wako Diagnostics, Mountain View, CA) using 2 solvents (no. 995–34791 and 993–35191) and 2 color reagents (no. 999–34691 and 991–34891). Plasma urea-N concentration was analyzed using the method described by Fawcett and Scott (1960).

3.3.2.3 Post-slaughter Data and Sample Collection

One day after weaning (d 51), all calves were killed via captive bolt stunning followed by exsanguination at 1400 h, or at 1330 h and 1430 h if 2 calves were killed on the same day. The whole GIT was dissected to determine tissue and digesta weights and to obtain tissue and digesta samples. The weights, both with and without digesta, were recorded for the rumen, omasum, abomasum, duodenum, jejunum, ileum, cecum, and colon. The rumen was cut open along the dorsal sac and digesta was transferred into a clean container. Ruminal tissue samples were collected from the cranial ventral sac of the rumen. For intestinal tissues, length measurements were conducted for each region. Then, tissue samples were taken from the mid-point of the duodenum (the distal portion of the duodenum was determined at the ligament of Treitz), 3 regions of jejunum including the proximal (25% of the total length starting at the ligament of Treitz), middle (50% of the length), and distal regions (75% of the length) with the region adjacent to the ileocecal fold used to denote the end of the jejunum and start of the ileum. The mid-point of the ileum was also collected using the ileocecal fold and ileocecal junction to define the ileum. All tissue samples were gently washed in ice-cold sterile phosphate buffered saline (Sigma) to remove digesta before any further processing.

3.3.2.4 Ruminal Fermentation Characteristics

A representative sample of ruminal digesta was strained through 2 layers of cheesecloth and pH was measured using a portable pH meter (Accumet AP110, Fischer Scientific, Ottawa, ON, Canada). Ten milliliters of strained ruminal fluid were mixed with 2 mL of metaphosphoric acid (25% wt/vol). The sample was stored at −20°C until analysis of short-chain fatty acid (SCFA) concentration using gas chromatography (Agilent Technologies Inc., Santa Clara, CA) as described by Khorasani et al. (1996). A second sample of strained ruminal fluid (10 mL) was collected and mixed with 2 mL of 1% sulfuric acid and frozen at −20°C until analysis of ammonia concentration based on the method described by Fawcett and Scott (1960).

3.3.2.5 Tissue Collection for Gene Expression

Representative tissues from the cranial sac of the rumen (**CRA**), proximal jejunum (**PROX**), and ileum (**ILE**) were used to obtain epithelia and to measure the mRNA expression of selected target genes Table 3.2. The genes of interest were monocarboxylate transporter 1 (***MCT1***) analyzed in CRA, PROX, and ILE; G-protein-coupled receptors (***GPR41***, ***GPR43***) analyzed in CRA, PROX, and ILE; urea transporters (***UT-B*** and ***AQP3***) analyzed in CRA; peptide transporters (***PEPT1*** and ***PEPT2***) and AA transporters (***EAAC1*** and ***ATB0+)*** analyzed in PROX and ILE; as well as reference genes (***GAPDH***, ***RPLP0***, and ***HPRT1***) analyzed in CRA, PROX, and ILE. The ruminal epithelium was manually peeled from the underlying muscle layer and cut into small pieces, the samples of the intestinal tissues were cut open through the mesenteric line, and the mucosa was scraped off using a glass slide. All tissues were collected using sterile equipment, rinsed in sterile ice-cold phosphate buffered saline, and transferred to 2-mL test tubes with 1.8 mL of RNAlater solution (Applied Biosystems, Foster City, CA). Tubes were stored for 24 h at 4°C and then frozen at −20°C.

Table 3.2. Target gene name, accession number, primer sequences, and function of the genes of interest.

Target gene abbreviation[1]	Accession number	Forward (F) and reverse (R) primers (3 – 5)	Efficiency, %	Amplicon length, bp
Reference genes				
GAPDH[2]	NM_001034034	F: TCTGGCAAAGTGGACATCGT R: ATGACGAGCTTCCCGTTCTC	101.3	134
RPLP0[2]	NM_001012682	F: TTGTGGGAGCAGACAACGTG R: GCCGGGTTGTTTTCCAGATG	102.2	136
HPRT1[3]	NM_001034035	F: AGGTTGCGAGCTTGCTGAT R: AGGGCATATCCCACAACAAA	103.3	103
Short chain fatty acid receptors and transporters				
MCT1[3] *(SLC16A1)*	NM_001037319	F: CTGATGGACCTTGTGGGACC R: CGGTAATTGATGCCCATGCC	98.5	206
GPR41[3] *(FFAR3)*	FJ562214.1	F: CCACCATCTATCTCACGTCCC R: GGGAGGAGTTCCCCGAGAAT	99.6	192
GPR43[4] *(FFAR2)*	FJ562212.1	F: TCATGGGTTTCGGCTTCTAC R: GGGGAAGAAGAAGAGGAGGA	107.0	323
Urea transporters				
UT-B[5] *(SLC14A1)*	NM_001008666	F: TATGTCCATGACGTGTCCAGTCT R: GCAGGTCCCATTTGCTCAGC	104.9	65
AQP3[5]	NM_001079794	F: CGCGAGCCCTGGATCA R: CCCAGATCGCATCGTAATACAA	103.3	103
Peptide and amino acid transporters				
PEPT1[6] *(SLC15A1)*	NM_001099378	F: AGCAAAGGCTTTCGAAGACA R: ACAGCCATCCTGCTTGAAGT	107.1	170
PEPT2[3] *(SLC15A2)*	NM_001079582	F: TGCTGACTCATGGTTGGGAA R: TTGGAAGCAAGAGGCTAGAAGA	107.7	110
ATB0+[7] *(SLC6A14)*	NM_001098461	F: AGTCGGAGCAGCATTATTTAAAGGAA R: ACCAGGATGAGTAGGACAACATAGG	103.3	93
EAAC1[3] *(SLC1A1)*	NM_174599	F: TGTGCTACATGCCGATTGGT R: GATTGCAAGCCCACTCAGGA	110.4	122

[1]*GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *RPLP0* = 60S acidic ribosomal protein P0; *HPRT1* = hypoxanthine phosphoribosyltransferase 1; *MCT1* = monocarboxylic acid transporter 1; *GPR41* = G protein-coupled receptor 41; *GPR43* = G protein-coupled receptor 43; *UT-B* = urea transporter B; *AQP3* = aquaporin 3; *PEPT1* = peptide transporter 1; *PEPT2* = peptide transporter 2; ATB0+ = amino acid transporter B0+; *EAAC1* = excitatory amino acid carrier 1.
[2]Verdugo (2016).
[3]Designed using NCBI Primer-BLAST (NCBI, 2018).
[4]Wang et al. (2009).
[5]Røjen et al. (2011).
[6]Connor et al. (2010a).
[7]Liao et al. (2009).

3.3.2.6 Quantitative Real-Time PCR

Samples of epithelia were thawed, removed from RNAlater, and ground with a mortar and pestle while purged with liquid nitrogen to keep the tissue frozen. Approximately 100 mg of tissue was transferred into a 2-mL sterile microcentrifuge tube. I extracted RNA through the phenol-chloroform extraction process based on the Chomczynski and Sacchi (1987) and Trizol (Thermo Fisher Scientific) protocols, with an additional isopropanol precipitation step.

The nucleic acid concentration and the 260:280 nm absorbance ratio were measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific). Samples were then evaluated for RNA integrity on a 1.2% agarose denaturing gel. The RNA was considered good quality if there was distinct separation of the 18S and 28S ribosomal RNA bands. Four samples were suspected to contain genomic DNA contamination. As such, they were DNase-treated using the TURBO DNA-free Kit (Thermo Fisher Scientific) and re-evaluated on the agarose gel to ensure lack of contamination.

For each sample, 2 µg of RNA was reverse transcribed to produce cDNA (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). Primer efficiency (Table 3.2) was determined (Ramakers et al., 2003) using a composite sample of cDNA from all calves in the study. The melting curve of the primers was analyzed to ensure their specificity. The primer efficiency values ranged from 98 to 110%, with an average of 103.6%. Quantitative real-time PCR was carried out using the CFX96 Real-Time PCR system and SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). I ran the reaction in duplicate with different gastrointestinal regions for the same gene analyzed on consecutive 96-well plates (Bio-Rad).

The expression (C_T, threshold cycle) of reference genes (*GAPDH*, *RPLP0*, *HPRT1*) in all analyzed regions of GIT were used to determine whether there were differences between the treatments. All 3 reference genes were deemed to have stable C_T as treatment effects were not present ($P \geq 0.15$, data not shown). The expression of target genes was analyzed by calculating the difference between the C_T for the gene of interest and the geometric mean of C_T for the reference genes (ΔC_T). With gene expression calculated in this manner, greater ΔC_T values indicate lesser mRNA expression, and lesser ΔC_T values indicate greater expression.

3.3.2.7 Brush Border Enzyme Activity Assays

The epithelium from the duodenum, the proximal, middle, and distal jejunum, and the ileum were scraped using sterile glass slides on a clean surface on ice. Epithelia samples were placed into 2-mL cryo-vials, snap frozen in liquid nitrogen, and then stored at −80°C until analysis of lactase (EC 3.2.1.23), maltase (EC 3.2.1.20), dipeptidylpeptidase IV (EC 3.4.14.5), aminopeptidase A (EC 3.4.11.7), and N (EC 3.4.11.2) enzyme activity (Maroux et al., 1973; Nagatsu et al., 1976; Dahlqvist, 1984). Briefly, small intestine mucosa samples were thawed on ice and 1 g of tissue was weighed and transferred into a 10-mL vial. Five milliliters of reverse osmosis water was added per 1 g of tissue and samples were then homogenized for 30 s, and following a 10 s break, for another 30 s, while kept on ice at all times. The homogenate was centrifuged (SORVALL ST 16R, Thermo Scientific) at 1,000 × g, 4°C for 5 min and the supernatant was transferred into multiple 1.5-mL tubes and frozen at −80°C.

Protein content was determined for each supernatant of tissue homogenate using the bicinchoninic acid method described by Smith et al. (1985). Lactase and maltase were analyzed according to Dahlqvist (1984) with minor modifications. The released glucose was measured according to the same procedure as plasma glucose described above. The L-glutamyl-p-nitroanilide and L-leucyl-p-nitroanilide (Bachem, Bubendorf, Switzerland) were, respectively, used as substrates for analysis of aminopeptidase A and N activities (Maroux et al., 1973) and L-glycyl-p-nitroanilide (Bachem) was the substrate for dipeptidylpeptidase IV activity (Nagatsu et al., 1976). The reaction was carried out in glass cuvettes and absorbance was measured using spectrophotometer at 410 nm for 10 min (Beckman Coulter, Brea, CA) fitted with a connection to a circulating water bath (1130–1S, VWR International, Mississauga, ON) to ensure stable reaction temperature. Brush border enzyme activities were calculated based on the amount of released product over the reaction period, either glucose for lactase and maltase or p-nitroanilidine for aminopeptidase A and N, and for dipeptidase IV. Enzyme activity was normalized per unit of protein in the tissue homogenate and 1 min of reaction.

3.3.3 Statistical Analysis

3.3.3.1 Optimal Heat-Treatment Temperature

Degradation rates were initially analyzed using the PROC NLIN of SAS (ver. 9.4, SAS Institute, Cary, NC) following Ørscov's model (Ørskov and McDonald, 1979) with the equation

$$R(t) = U + D \times e^{-K_d \times (t - T_0)} \quad \textbf{Equation 3.1}$$

where R(t) is the percentage of residue at a given incubation time point (t), U is the undegradable fraction (%), D is the potentially degradable fraction (%), e is Euler's number, K_d is the degradation rate of D (%/h), and T_0 is lag time (h). Effective degradability was calculated following the equation

$$ED = S + \frac{(D \times K_d)}{K_d + K_p} \quad \textbf{Equation 3.2}$$

where ED is the effective degradability, S is the soluble fraction, which was washed out from 0-h incubation bags, and K_p is the rate of passage (assumed K_p = 5%/h).

The effect of CM heat-treatment on degradation rates for DM and CP, the degradable fractions, the effective degradability, and the estimated intestinal digestibility were analyzed using the mixed model in SAS version 9.4, considering heat-treatment as the fixed effect with polynomial contrasts used to determine the linear or quadratic effect of heat-treatment. Significance was declared when $P \leq 0.05$ and tendencies when $0.05 < P \leq 0.10$.

3.3.3.2 Effect of Heat-Treated CM and Glycerol on Calves

Data were analyzed as a randomized complete block design with a 2 × 2 factorial design using the mixed model of SAS. The model included the fixed effects of CM heat-treatment (untreated and heated), glycerol inclusion (0 and 5%), and the 2-way interaction. Block was considered as a random effect. Data were tested to verify whether the data and residuals were normally distributed and homoscedastic. Data that were collected over time (BW, ADG, starter intake, and MR intake) were also analyzed using repeated measures with time (wk or d) as a fixed effect and the relevant 2- and 3-way interactions. Covariance error structures were tested and the error structure for each variable with the least Akaike's and Bayesian Information Criterion was chosen. In case of a significant interaction between fixed effects, the Bonferroni mean separation

was used to determine if treatment means differed. Differences were declared significant when $P \leq 0.05$, and tendencies were declared when $P \leq 0.10$. As there were no interactions (2- or 3-way) of treatment with time, only the main treatment effects and the interaction among the heat-treatment and glycerol inclusion are reported.

3.4 Results

3.4.1 Study 1: Optimal Heat-Treatment Temperature for CM

Increasing the temperature for the heat-treatment of CM increased DM concentration at a decreasing rate (quadratic, $P = 0.001$; Table 3.3), decreased OM concentration at a decreasing rate (quadratic, $P = 0.002$), increased CP concentration at a decreasing rate (quadratic, $P = 0.002$), increased NDF and ADF concentrations at an increasing rate (quadratic, $P < 0.001$), and increased ether extract concentration linearly ($P = 0.006$). The increases in CP, NDF, ADF, and ether extract were offset by a quadratic reduction in NFC concentration with the rate of reduction increasing as the temperature of heat-treatment for CM increased ($P < 0.001$).

The rate of DM disappearance from the rumen decreased at a decreasing rate with the increase of heat-treatment temperature for CM (quadratic, $P = 0.017$; Table 3.3). The soluble fraction of DM decreased at a decreasing rate (quadratic, $P = 0.024$). The degradable fraction of DM decreased at an increasing rate with the increase of temperature (quadratic, $P < 0.001$), with values of 60.0% for CON and 34.1% for H-120. The undegraded fraction of DM increased at an increasing rate (quadratic, $P < 0.001$) with values of 14.1% for CON and 47.0% for H-120. The effectively degradable DM, assuming a constant passage rate of 5%/h, decreased at an increasing rate with increasing temperature of treatment (quadratic, $P < 0.001$) from 54.2 for CON to 31.7% for H-120. The rate of CP disappearance from the rumen decreased from CON (4.89%/h) to H-110 (3.54%/h) and increased for H-120 (7.77%/h; quadratic, $P = 0.005$). The soluble fraction of CP was not affected by heat-treatment ($P \geq 0.14$). The degradable fraction of CP decreased at an increasing rate when exposed to greater treatment temperatures (quadratic, $P < 0.001$) with values ranging from 79.0 and 22.3% for CON and H-120 treatments, respectively, and the CP undegraded fraction increased at an increasing rate (quadratic, $P < 0.001$).

Table 3.3. Chemical composition and digestion characteristics of the nonheated (CON) and heat-treated canola meal when heated to achieve temperatures of 100 (H-100), 110 (H-110), or 120°C (H-120) for 10 min (n = 3)

Variable	Treatment				SEM	*P*-value	
	CON	H-100	H-110	H-120		Linear	Quadratic
Chemical composition							
DM, %	89.7	92.7	94.4	95.6	0.3	<0.001	0.001
OM, % DM	93.6	93.3	93.1	93.0	0.0	<0.001	0.002
CP, % DM	32.7	34.7	35.5	36.3	0.2	<0.001	0.002
NDF, % DM	25.5	30.9	36.8	44.2	0.7	<0.001	<0.001
ADF, % DM	19.3	22.4	25.2	33.4	0.4	<0.001	<0.001
NFC[1], % DM	31.9	23.9	17.0	8.8	0.8	<0.001	<0.001
Ether extract, % DM	3.53	3.65	3.87	3.93	0.08	0.006	0.073
In situ DM degradation							
K_d[2], %/h	4.50	4.20	3.14	3.06	0.21	0.002	0.017
Soluble, %	25.8	21.9	19.6	18.8	0.5	<0.001	0.024
Degradable, %	60.0	57.1	52.7	34.2	1.9	<0.001	<0.001
Undegradable, %	14.1	21.0	27.7	47.0	2.1	<0.001	<0.001
EDDM[3], %	54.2	46.8	39.1	31.7	0.8	<0.001	<0.001
In situ CP degradation							
K_d, %/h	4.89	4.59	3.54	7.77	0.59	0.28	0.005
Soluble, %	16.4	11.8	8.6	12.1	2.9	0.14	0.73
Degradable, %	79.0	70.0	59.0	22.3	3.5	<0.001	<0.001
Undegradable, %	4.6	18.2	32.3	65.7	3.9	<0.001	<0.001
EDCP[3], %	55.5	45.3	32.7	25.4	2.7	<0.001	0.003
Intestinal CP digestibility[4], %	45.9	46.1	51.0	37.2	2.6	0.48	0.034

[1]NFC calculated based on NRC (2001): 100 – (NDF, % + CP, % + ether extract, % + ash, %).
[2]K_d = the degradation rate of degradable fraction.
[3]Effectively degradable dry matter or crude protein assuming a 5%/h passage rate.
[4]Estimated CP intestinal digestibility was based on a three-step procedure described by Calsamiglia and Stern (1995).

The effectively degradable CP decreased at an increasing rate after exposure to increasing heat-treatment temperatures (quadratic, $P = 0.003$) from 55.5% for CON to 25.4% for H-120. The estimated intestinal digestibility responded quadratically to the increase of heat-treatment temperature ($P = 0.034$), with digestibility increasing from CON (45.9%) to H-110 (51.0%) and decreasing for H-120 (37.2%).

With increased heat-treatment temperature of CM, linear decreases in the concentration of Arg ($P = 0.002$; Table 3.4), His ($P = 0.003$), Met ($P = 0.048$), Asp ($P = 0.026$), Cys ($P = 0.001$), Glu ($P = 0.035$), Pro ($P = 0.043$), and Ser ($P = 0.049$) were detected. In addition, Lys ($P = 0.059$), Phe ($P = 0.058$), Thr ($P = 0.099$), and Val ($P = 0.088$) concentrations tended to decrease linearly. Isoleucine, Leu, Ala, and Gly concentrations were not affected by the heat-treatment ($P \geq 0.101$).

3.4.2 Study 2: Effect of Heat-Treated CM and Glycerol on Calves

Even though our treatment diets were designed to be isonitrogenous and could not be statistically evaluated, I observed numerically greater CP content in the heat-treated treatment diets (Table 3.1). Treatments that contained glycerol had numerically less starch and greater NSC concentration. There were no 2- or 3-way interactions of treatments with time for BW, ADG, starter intake, and MR intake ($P \geq 0.21$, data not shown). The aforementioned variables increased with time ($P \leq 0.001$, data not shown); however, these observations were expected for growing calves.

Initial BW of calves ($P = 0.66$; Table 3.5) was not affected by CM heat-treatment. Heat-treatment of CM decreased final BW ($P = 0.041$) and tended to reduce ADG ($P = 0.079$) and total BW gain ($P = 0.097$). When comparing starters without glycerol to those with glycerol, glycerol inclusion tended to increase ADG ($P = 0.088$) and increased total BW gain ($P = 0.003$). Heat-treatment of CM in the starter did not affect starter intake, with the exception of a tendency for reduced starter intake ($P = 0.090$) from d 43 to 49 - a time when MR provision was limited to 2.5% of BW. Inclusion of glycerol tended to increase starter intake from d 29 to 35 ($P = 0.056$), d 36 to 42 ($P = 0.087$), d 43 to 49 ($P = 0.087$), and the cumulative starter intake throughout the study ($P = 0.069$). Milk replacer intake did not differ among treatments ($P \geq 0.14$).

Table 3.4. Amino acid composition of nonheated (CON) and heat-treated canola meal when heated to achieve temperatures of 100 (H-100), 110 (H-110), or 120°C (H-120) for 10 min (n = 3)

Variable	Treatment				SEM	*P*-value	
	CON	H-100	H-110	H-120		Linear	Quadratic
Essential amino acids, % CP							
Arg	6.19	5.89	5.74	5.58	0.09	0.002	0.11
His	2.72	2.62	2.52	2.52	0.04	0.003	0.27
Ile	4.17	4.07	3.63	3.97	0.14	0.11	0.88
Leu	7.30	7.12	6.95	6.78	0.17	0.101	0.72
Lys	5.44	4.95	4.97	4.36	0.30	0.059	0.28
Met	2.06	2.00	1.92	1.94	0.04	0.048	0.58
Phe	4.25	4.11	3.94	4.02	0.09	0.058	0.73
Thr	4.56	4.46	4.33	4.24	0.11	0.099	0.63
Val	5.36	5.18	5.00	5.09	0.12	0.088	0.83
Non-essential amino acids, % CP							
Ala	4.66	4.55	4.34	4.43	0.11	0.104	0.69
Asp	7.31	7.10	6.78	6.83	0.14	0.026	0.37
Cys	2.45	2.32	2.25	2.25	0.03	0.001	0.40
Gly	5.30	5.17	4.94	5.05	0.13	0.12	0.73
Glu	18.06	17.50	16.73	17.03	0.35	0.035	0.61
Pro	6.57	6.33	6.10	6.14	0.14	0.043	0.56
Ser	4.41	4.28	4.08	4.15	0.09	0.049	0.57

Table 3.5. Body weight, BW gain, feed intake for Holstein bull calves (n = 7/treatment) between 8 and 51 d of age, and ruminal fermentation characteristics on 51 d of age (1 d post-weaning) when fed pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal with (G) or without (NG) glycerol supplementation (5% DM)

Variable	Treatments				SEM	*P*-value[1]		
	NH-NG	NH-G	H-NG	H-G		HEAT	GLY	H×G
Initial BW, kg	43.9	43.1	42.5	43.1	1.6	0.66	0.95	0.69
Final BW, kg	65.4	69.1	61.3	64.1	2.4	0.041	0.12	0.82
ADG, g/d	511.3	615.1	443.3	508.6	54.4	0.079	0.088	0.69
BW gain[2], kg	21.5	26.0	18.7	23.9	1.6	0.097	0.003	0.82
Starter intake[3], kg DM/d	0.363	0.355	0.359	0.355	0.027	0.63	0.23	0.69
d 8 to 28, kg DM/d	0.026	0.049	0.020	0.030	0.008	0.16	0.056	0.54
d 29 to 35, kg DM/d	0.116	0.174	0.113	0.103	0.045	0.39	0.58	0.43
d 36 to 42, kg DM/d	0.329	0.508	0.305	0.416	0.093	0.48	0.087	0.68
d 43 to 49, kg DM/d	0.753	0.959	0.602	0.766	0.117	0.090	0.069	0.83
Cumulative, d 8 to 50, kg DM	9.98	13.87	8.51	10.56	1.93	0.14	0.075	0.57
Milk replacer intake, kg DM/d	0.700	0.715	0.754	0.746	0.028	0.14	0.90	0.69
d 8 to 28, kg DM/d	0.921	0.952	0.909	0.929	0.045	0.65	0.51	0.92
d 29 to 35, kg DM/d	0.856	0.866	0.824	0.859	0.036	0.53	0.46	0.68
d 36 to 42, kg DM/d	0.456	0.463	0.430	0.449	0.015	0.19	0.37	0.70
d 43 to 49, kg DM/d	0.233	0.243	0.223	0.232	0.007	0.17	0.18	0.96
Cumulative, d 8 to 50, kg DM	32.2	33.2	31.5	32.8	1.2	0.59	0.37	0.87
Ruminal fermentation								
pH	5.15	5.12	5.46	4.98	0.13	0.47	0.040	0.070
SCFA[4] concentration, mM	130.1	149.7	117.0	140.4	9.7	0.11	0.005	0.78
Acetate, mol/100 mol	59.7	48.5	49.7	53.2	4.3	0.50	0.34	0.075
Propionate, mol/100 mol	23.5	35.9	28.2	26.3	4.2	0.51	0.18	0.069
Iso-butyrate, mol/100 mol	0.280	0.159	0.346	0.210	0.076	0.43	0.092	0.92
Butyrate, mol/100 mol	11.4	11.1	17.9	15.2	3.1	0.100	0.62	0.70
Iso-valerate, mol/100 mol	0.283	0.144	0.321	0.283	0.077	0.45	0.076	0.85
Valerate, mol/100 mol	3.69	3.56	2.86	4.44	0.40	0.96	0.083	0.041[5]
Caproate, mol/100 mol	1.11	0.61	0.67	0.57	0.30	0.36	0.25	0.45
Ammonia, mg/dL	15.2	14.8	11.9	14.4	2.3	0.30	0.54	0.41

[1]HEAT = effect of canola meal heat-treatment; GLY = effect of glycerol inclusion; H×G = canola meal heat-treatment by glycerol supplementation interaction.
[2]BW gain = final BW – initial BW.
[3]d8 = provision of starter to the calves begun; d28 = provision of milk replacer was gradually decreased over time to facilitate weaning; d50 = first d with no milk provision.
[4]SCFA = short-chain fatty acid.
[5]No mean separation was detected with the Bonferroni post-hoc analysis.

I observed a tendency for a heat-treatment × glycerol interaction for ruminal pH (P = 0.070; Table 3.5) with the numerically greatest pH for the H-NG treatment, intermediate for the NH-NG and NH-G treatments, and the least for the H-G treatment. Heat-treatment of CM did not affect ruminal SCFA concentration (P = 0.11), but glycerol inclusion increased SCFA concentration (P = 0.005). There was a tendency for an interaction between heat-treatment of CM and glycerol inclusion for the molar proportions of acetate and propionate (P = 0.075 and P = 0.069, respectively). In general, the greatest molar proportion of acetate was observed for the NH-NG treatment, intermediate for the H-G treatment, and the least for the H-NG and NH-G treatments. The molar proportion of propionate was the greatest for the NH-G treatment, intermediate for the H-NG treatment, and H-G treatment, and the least for the NH-NG treatment. Glycerol inclusion tended to decrease the molar proportions of iso-butyrate (P = 0.092); however, the molar proportion of iso-butyrate was not affected by heat-treatment (P = 0.43). The molar proportion of butyrate was not affected by the glycerol supplementation (P = 0.62) but tended to be greater when heat-treated canola was included in the starters (P = 0.100). The molar proportion of isovalerate tended to be decreased by the glycerol supplementation (P = 0.076). A heat-treatment × glycerol interaction was observed for valerate (P = 0.041), but means that differed could not be detected with the Bonferroni post-hoc analysis. The molar proportion of caproate and the ammonia concentration were not affected by the experimental treatments ($P \geq 0.25$).

The weight of the ruminal tissue and ruminal digesta were reduced ($P \leq 0.011$; Table 3.6) for calves fed heat-treated CM compared with those fed CM without additional heat-treatment. Glycerol inclusion in the starter did not affect the weight of the ruminal tissue or digesta ($P \geq 0.18$). Omasal tissue and digesta, and abomasal tissue weights were not affected by the experimental treatments ($P \geq 0.14$). Abomasal digesta weight tended to be greater with glycerol supplementation (P = 0.080). Duodenal tissue weight and length were not affected by experimental treatments ($P \geq$ 0.15), and digesta weight tended to be greater with glycerol supplementation (P = 0.087). Jejunal tissue weight was less with heat-treatment (P = 0.024) and tended to be greater with glycerol supplementation (P = 0.079). Glycerol supplementation also increased jejunal digesta weight (P = 0.001). Heat-treatment of CM tended to decrease the jejunal length (P = 0.075). Ileal tissue weight and length were not affected by the experimental treatments ($P \geq 0.23$). Heat-treatment of CM decreased ileal digesta weight (P = 0.004).

Table 3.6. Gastrointestinal tract morphometry of Holstein bull calves (n = 7/treatment) at 51 d of age (1 d post-weaning) fed pelleted starter mixtures containing either nonheated (**NH**) or heat-treated (**H**) canola meal and with (**G**) or without (**NG**) glycerol supplementation (5% DM)

Variable		Treatment				SEM	*P*-value[1]		
		NH-NG	NH-G	H-NG	H-G		HEAT	GLY	H×G
Rumen	Tissue, kg	1.16	1.26	0.884	1.03	0.11	0.011	0.18	0.78
	Digesta, kg	4.37	3.82	2.68	2.97	0.29	<0.001	0.61	0.12
Omasum	Tissue, kg	0.179	0.233	0.205	0.221	0.025	0.77	0.14	0.42
	Digesta, g	96.7	120.6	135.6	127.4	34.4	0.40	0.77	0.56
Abomasum	Tissue, kg	0.343	0.361	0.343	0.355	0.019	0.86	0.41	0.87
	Digesta, kg	0.534	0.550	0.327	0.632	0.089	0.48	0.080	0.12
Duodenum	Tissue, g	84.3	90.0	74.5	84.9	6.2	0.23	0.20	0.71
	Digesta, g	18.8	19.7	13.8	21.5	2.4	0.50	0.087	0.17
	Length, m	0.650	0.657	0.590	0.633	0.028	0.15	0.38	0.52
Jejunum	Tissue, kg	1.33	1.55	1.14	1.28	0.10	0.024	0.079	0.68
	Digesta, kg	0.776	1.278	0.768	0.992	0.103	0.14	0.001	0.17
	Length, m	18.2	18.1	15.8	17.9	0.8	0.075	0.18	0.16
Ileum	Tissue, kg	0.166	0.187	0.135	0.167	0.022	0.25	0.24	0.79
	Digesta, g	69.7	63.9	19.6	27.1	13.8	0.004	0.95	0.63
	Length, m	1.029	1.000	0.831	0.943	0.120	0.23	0.69	0.50
Cecum	Tissue, g	80.1	73.9	66.7	76.6	7.0	0.38	0.85	0.26
	Digesta, kg	0.288	0.228	0.236	0.156	0.030	0.018	0.009	0.69
	Length, m	0.300	0.279	0.274	0.284	0.018	0.42	0.95	0.26
Colon	Tissue, kg	0.355	0.359	0.301	0.374	0.023	0.33	0.072	0.099
	Digesta, kg	0.330	0.426	0.267	0.327	0.055	0.13	0.14	0.73
	Length, m	2.79[a]	2.59[ab]	2.39[b]	2.54[ab]	0.10	0.01	0.78	0.038

[a,b] Means with uncommon superscripts differ ($P < 0.05$).

[1]HEAT = effect of canola meal heat-treatment; GLY = effect of glycerol inclusion; H×G = canola meal heat-treatment by glycerol supplementation interaction.

Cecal tissue weight and length were not affected by the experimental treatments ($P \geq 0.26$). The CM heat-treatment decreased cecal digesta weight ($P = 0.018$) and glycerol supplementation increased it ($P = 0.009$). A treatment interaction was observed for the colonic length ($P = 0.038$) and tended to be observed for colonic tissue weight ($P = 0.099$). Colonic length was the greatest for NH-NG treatment, intermediate for NH-G and H-G, and shortest for H-NG treatment. Colonic tissue weight was numerically the greatest for the H-G treatment, intermediate for NH-G and NH-NG treatments, and the least for H-NG treatment. Colonic tissue weight also tended to be increased by the glycerol supplementation ($P = 0.072$), and colonic length was decreased by CM heat-treatment ($P = 0.010$). Colonic digesta was not affected by the experimental treatments ($P \geq 0.13$).

Heat-treatment did not affect the gene expression of any of the analyzed genes of interest ($P \geq 0.22$; Table 3.7). A heat-treatment × glycerol interaction was observed for *AQP3* ($P = 0.021$) and tended to be observed for the expression of *MCT1* ($P = 0.076$) in ruminal epithelium from the cranial sac. For the expression of *AQP3*, the Bonferroni mean separation test did not identify means that differed, but numerically the greatest expression of *AQP3* was for the H-NG treatment, followed by NH-G and NH-NG treatments, and the least for the H-G treatment. The expression of *MCT1* was numerically greatest for the H-NG treatment, intermediate for the NH-NG and NH-G treatments, and the least for the H-G treatment. Expression of *UT-B*, *GPR41*, and *GPR43* in the ruminal epithelium was not affected by the glycerol inclusion ($P \geq 0.26$). Glycerol inclusion resulted in greater expression of *MCT1* ($P = 0.017$) in the epithelium of the PROX. A tendency for a heat-treatment × glycerol supplementation interaction was observed for the expression of *GPR41* in the PROX ($P = 0.089$) with the least values for NH-G and H-NG and the greatest for NH-NG and H-G. Glycerol did not affect any of the genes of interest in the ileal epithelium ($P \geq 0.46$).

I did not detect any treatment interactions nor effect of heat-treatment of CM on brush border enzyme activities ($P \geq 0.16$; Table 3.8). Inclusion of glycerol did not affect activity of aminopeptidase A, aminopeptidase N, lactase, or maltase ($P \geq 0.19$), but tended to increase dipeptidylpeptidase IV activity in the middle jejunum ($P = 0.059$).

Heat-treatment of CM did not affect plasma glucose concentration ($P \geq 0.18$, Table 3.9). Glycerol supplementation tended to decrease plasma glucose concentration on d 43 of age ($P = 0.076$), but had no effect on glucose concentration on d 22 and 51 of age ($P \geq 0.45$). Plasma insulin concentration did not differ between the heat-treated and nonheated CM ($P \geq 0.27$).

Table 3.7. Gene expression in the cranial sac of the rumen (CRA), proximal jejunum (PROX), and ileum (ILE) of Holstein bull calves (n = 7/ treatment) at 51 d of age (1 d post-weaning) as influenced by feeding pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal and with (G) or without (NG) glycerol supplementation (5% DM)

Gene of interest[1]		Treatment				SEM	*P*-value[2]		
		NH-NG	NH-G	H-NG	H-G		HEAT	GLY	H×G
CRA	*MCT1*	1.79[3]	1.84	1.70	2.18	0.13	0.30	0.034	0.076
	UT-B	5.18	5.25	5.34	5.29	0.17	0.44	0.94	0.63
	AQP3	0.492	0.303	0.237	0.753	0.164	0.49	0.26	0.021[4]
	GPR41	15.2	15.3	15.0	14.5	0.4	0.29	0.64	0.50
	GPR43	14.1	14.5	14.0	13.7	0.4	0.22	0.95	0.33
PROX	*MCT1*	3.57	3.15	3.83	3.33	0.18	0.22	0.017	0.83
	PEPT1	4.75	5.31	5.15	5.01	0.24	0.83	0.38	0.16
	PEPT2	13.2	13.0	12.6	13.1	0.3	0.41	0.57	0.23
	EAAC1	7.73	8.39	8.33	7.92	0.38	0.85	0.73	0.15
	ATB0+	4.15	4.03	3.72	4.18	0.24	0.48	0.39	0.16
	GPR41	9.33	9.55	9.52	9.29	0.12	0.81	0.95	0.089
	GPR43	9.30	9.18	9.00	8.98	0.23	0.28	0.76	0.83
ILE	*MCT1*	6.66	6.57	6.57	6.77	0.21	0.63	0.63	0.22
	PEPT1	6.02	5.96	5.83	6.11	0.32	0.93	0.73	0.59
	PEPT2	10.5	10.4	10.5	10.7	0.2	0.36	0.92	0.30
	EAAC1	6.12	6.24	5.96	5.65	0.48	0.41	0.83	0.63
	ATB0+	5.94	5.76	5.40	6.06	0.38	0.74	0.54	0.27
	GPR41	7.59	7.43	7.66	7.53	0.21	0.66	0.46	0.93
	GPR43	10.2	10.1	10.3	10.7	0.4	0.41	0.71	0.51

[1]*MCT1* = monocarboxylic acid transporter 1; *GPR41* = G protein-coupled receptor 41; *GPR43* = G-protein-coupled receptor 43; *UT-B* = urea transporter B; *AQP3* = aquaporin 3; *PEPT1* = peptide transporter 1; *PEPT2* = peptide transporter 2; *ATB0+* = amino acid transporter B0+; *EAAC1* = excitatory amino acid carrier 1.

[2]HEAT = effect of canola meal heat-treatment; GLY = effect of glycerol inclusion; H×G = canola meal heat-treatment by glycerol supplementation interaction.

[3]Values of gene expression are presented as ΔC_T = threshold cycle (C_T) for the gene of interest – C_T for the reference genes; greater ΔC_T values represent lesser mRNA expression.

[4]No mean separation was detected with the Bonferroni post-hoc analysis.

Table 3.8. Brush border enzyme activities in duodenum (DUO), proximal (PROX), middle (MID) and distal (DIST) jejunum, and ileum (ILE) of Holstein bull calves (n = 7/ treatment) at 51 d of age (1 d post-weaning) as influenced by feeding pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal and with (G) or without (NG) glycerol supplementation (5% DM)

Brush border enzyme activity		Treatment				SEM	*P*-value[1]		
		NH-NG	NH-G	H-NG	H-G		HEAT	GLY	H×G
Aminopeptidase A	DUO	2.94	2.37	2.31	2.52	0.29	0.42	0.54	0.19
	MID	35.3	36.2	24.6	30.5	6.9	0.25	0.63	0.73
	ILE	37.9	24.1	22.4	22.9	6.0	0.16	0.26	0.23
Aminopeptidase N	DUO	8.41	10.91	9.52	8.67	2.29	0.80	0.72	0.47
	MID	16.6	14.4	23.5	17.7	4.7	0.28	0.39	0.70
	ILE	32.3	20.4	23.7	23.0	5.0	0.55	0.22	0.27
Dipeptidylpeptidase IV	MID	3.37	5.31	3.57	4.16	0.64	0.47	0.059	0.30
	ILE	5.65	4.54	4.66	4.37	0.75	0.41	0.33	0.56
Lactase	DUO	105.6	122.9	113.8	97.2	15.6	0.52	0.98	0.22
	PROX	144.5	128.0	119.5	112.7	20.9	0.32	0.56	0.81
	MID	59.7	68.2	66.3	74.3	11.4	0.54	0.43	0.98
	DIST	20.6	21.6	19.9	20.2	1.6	0.47	0.65	0.80
	ILE	19.2	18.2	19.9	19.3	1.1	0.35	0.45	0.85
Maltase	DUO	19.8	23.2	20.4	20.2	1.8	0.35	0.21	0.18
	PROX	32.0	27.8	33.9	26.5	4.8	0.94	0.19	0.71
	MID	27.9	32.8	27.0	27.8	3.6	0.43	0.29	0.61
	DIST	36.7	34.3	34.6	41.2	5.6	0.61	0.66	0.34
	ILE	20.7	18.6	18.3	20.5	2.8	0.92	0.98	0.37

Data are presented as enzymatic units per milligram of protein, which describes the amount of micromoles of glucose or *p*-nitroanilide released per minute of reaction at 37°C.

[1]HEAT = effect of canola meal heat-treatment; GLY = effect of glycerol inclusion; H×G = canola meal heat-treatment by glycerol supplementation interaction.

Table 3.9. Concentration of plasma glucose, insulin, non-esterified fatty acid (NEFA), and urea, and serum BHB of Holstein bull calves (n = 7/treatment) 2 h after solid feed presentation as influenced by feeding of pelleted starter mixtures containing either nonheated (NH) or heat-treated (H) canola meal and with (G) or without (NG) glycerol supplementation (5% DM)

Variable	d of age[1]	Treatment				SEM	*P*-value[2]		
		NH-NG	NH-G	H-NG	H-G		HEAT	GLY	H×G
Glucose, mg/dL	22	94.7	92.2	91.3	96.8	5.4	0.34	0.45	0.83
	43	95.5	84.4	91.7	88.2	4.7	0.99	0.076	0.35
	51	66.5	69.9	64.6	62.1	3.6	0.18	0.91	0.41
Insulin, µg/L	22	0.671	0.463	0.704	0.271	0.121	0.51	0.016	0.36
	43	0.426	0.206	0.534	0.259	0.102	0.27	0.003	0.70
	51	0.110	0.126	0.126	0.107	0.010	0.89	0.89	0.099
BHB, mmol/L	22	0.454	0.447	0.325	0.402	0.054	0.096	0.33	0.26
	43	0.365	0.400	0.465	0.474	0.036	0.022	0.53	0.72
	51	0.492	0.463	0.611	0.456	0.049	0.26	0.061	0.18
NEFA, mEq/L	22	147.1	144.9	141.7	133.7	8.7	0.35	0.56	0.74
	43	199.3	146.6	144.5	142.7	17.8	0.072	0.092	0.11
	51	196.6	155.1	151.9	161.6	20.6	0.36	0.45	0.23
Urea-N, mg/dL	22	7.90	8.29	9.06	8.13	0.61	0.42	0.65	0.28
	43	9.36	7.93	8.44	9.78	0.86	0.45	0.95	0.035[3]
	51	9.01	9.61	10.22	10.42	0.68	0.13	0.53	0.75

[1]D of age: 22 = during 15% BW milk replacer provision; 43 = at 5% BW milk replacer provision; 51 = a day after weaning.
[2]HEAT = effect of canola meal heat-treatment; GLY = effect of glycerol inclusion; H×G = canola meal heat-treatment by glycerol supplementation interaction.
[3]No mean separation was detected with the Bonferroni post-hoc analysis.

On d 22 and 43 of age, plasma insulin was decreased by the glycerol supplementation ($P = 0.016$ and $P = 0.003$, respectively). I observed a tendency for a heat-treatment by glycerol supplementation interaction for plasma insulin concentration on d 51 of age ($P = 0.099$), with the numerically greatest concentration for the both NH-G and H-NG treatments relative to NHNG and H-G. Serum BHB concentration on d 22 of age tended to be reduced with heat-treatment ($P = 0.096$), yet on d 43, BHB concentration was increased by heat-treatment ($P = 0.022$). On d 51 of age, serum BHB tended to be decreased by glycerol supplementation ($P = 0.061$). Plasma NEFA concentration was not affected by the experimental treatments on d 22 and 51 of age ($P \geq 0.23$). On d 43, plasma NEFA concentration tended to be less for calves fed heat-treated CM ($P = 0.072$) and tended to be less for glycerol inclusion ($P = 0.092$). A heat-treatment × glycerol supplementation interaction was observed for plasma urea-N at 43 d of age ($P = 0.035$). Mean separation for this variable was not achieved; however, the greatest plasma urea-N concentration was observed for the NH-NG and H-G treatments, intermediate for H-NG and the least for the NH-G treatment. The main effects of heat-treatment and glycerol inclusion did not affect plasma urea-N concentration on any of the 3 d of sample collection ($P \geq 0.13$).

3.5 Discussion

3.5.1 Optimal Temperature for Heat-Treatment of CM

As CM is a by-product from the canola crushing industry, processing conditions imposed within and among processing plants affect composition, especially for RDP and RUP proportions (Bell and Keith, 1991). The CON CM in the present study had similar proportions of the undegradable protein fraction as those reported by Huang et al. (2015) and Maxin et al. (2013). Moreover, I observed that heat-treating CM increased the proportion of RUP, an observation reported previously (McKinnon et al., 1991; Dakowski et al., 1996). Though the intent of the heat-treatment in the present study was to increase the rumen undegradable fraction, the heat-treating process also increased the NDF and ADF content, as observed in previous research (McKinnon et al., 1995). The increase in NDF and RUP, and subsequent decrease in NFC, were likely partially due to the Maillard reaction (van Soest and Mason, 1991), which occurs when soluble carbohydrate components react with AA to form indigestible compounds that are insoluble in

neutral detergent solution. The latter statement is supported by the general reduction in AA concentrations observed with increasing heat-treatment. Similar observations for decreased essential AA concentrations due to the Maillard reaction have been made by Ajandouz and Puigserver (1999); although, their treatments occurred in aqueous conditions. Past studies have also reported no effect of heat-treatment on the CP content of CM (McKinnon et al., 1991; McKinnon et al., 1995; Dakowski et al., 1996). Conversely, I observed that CP concentration increased at a decreasing rate with increasing heat-treatment temperature. It is unclear why CP concentration was altered with heat-treatment.

As expected, heat-treatment decreased the proportion of potentially degradable CP in the rumen, reduced the degradation rate of the degradable fraction (K_d) relative to untreated CM, and quadratically decreased the estimated intestinal digestibility of CP. Heat-treatment also decreased the concentration of Glu, a response that was opposite to that previously reported (Newkirk et al., 2003). It is unclear why results in the present study and that of Newkirk et al. (2003) differ, but may be related to the heat-treatment protocol and the inherent composition of the CM. The increase in the undegradable fraction and stimulatory effect of heat-treatment on intestinal CP digestibility indicate that I achieved an increase in the RUP without having marked reductions in the intestinal digestibility using heat by up to 110°C. Although I measured in situ degradation using heifers, rather than calves, Vazquez-Anon et al. (1993) suggested that measurements made with cows may reasonably approximate in situ digestion in calves within 4 wk of weaning. Other studies have also suggested that ruminal fermentation at the time of weaning is not different from that occurring several weeks after weaning (Quigley et al., 1985; Lalles and Poncet, 1990).

3.5.2 Interactions Between CM Heat-Treatment and Glycerol Supplementation

When designing the current study, I anticipated interactions between heat-treated CM and glycerol inclusion in starters because glycerol inclusion could increase the concentration of ruminal butyrate (Rémond et al., 1993; Paiva et al., 2016) and potentially stimulate ruminal development (Sander et al., 1959; Mentschel et al., 2001), whereas heat-treated CM could increase the supply of AA to the intestine, potentially promoting intestinal growth and function. Altogether, feed intake and calf performance could be enhanced. However, only a few interactions were observed, and the detected interactions were primarily only tendencies. It is unclear why the

interactions did not occur, but a possible explanation is that heat-treatment of CM may have reduced apparent total-tract CP digestibility in calves, thereby limiting one of the potential synergistic effects. This previous explanation is partially supported by reduced BW gain with heat-treated CM; however, I did not measure apparent total-tract digestibility and cannot confirm the digestibility response. Others have also reported that heat-treatment of protein sources (SBM) for calves did not alter the intestinal supply of protein from microbial protein or RUP, despite the reduction of effective ruminal degradability measured using in situ technique in a steer from heat-treatment (Obitsu et al., 1995). Additionally, glycerol did not increase molar proportion of ruminal butyrate in the present study, and hence the secondary proposed mode of action for beneficial effects of heat-treated CM and glycerol inclusion was not observed. As such, the main effects of heat-treated CM and glycerol will be discussed separately and only the most important interactions will be mentioned and discussed.

3.5.3 Effect of Heat-Treating CM on Calf Performance and Development of the GIT

Studies have evaluated the use of CM in starter mixtures for calves, suggesting that use of CM may decrease starter intake (Stake et al., 1973; Miller-Cushon et al., 2014b), CP digestibility (Schingoethe et al., 1974; Fiems et al., 1985; Khorasani et al., 1990), and ADG (Miller-Cushon et al., 2014b; Hadam et al., 2016) relative to SBM; however, in some studies starter intake (Ingalls and Seale, 1971; Fisher, 1980), CP digestibility (Stake et al., 1973; Fisher, 1980), and ADG (Stake et al., 1973; Fisher, 1980; Fiems et al., 1985) were not affected by protein source. In the present study, I did not compare different protein sources, but compared untreated and heat-treated CM to evaluate the effect of altering the RUP supply.

In the current study, I observed decreased ADG and, as a result, a lighter final BW when calves were fed starters containing heat-treated CM. Calves fed heat-treated CM tended to have less cumulative starter intake from d 8 to 50; of which the most notable response was from d 43 to 49. In the 2 studies reported by Obitsu et al. (1995), feeding calves heat-treated SBM did not affect DMI when compared with SBM that was not heated, but calves in those studies received 30 or 40% of their diet as hay, which may have masked treatment effects. In the present study, calves that ate diets containing heat-treated CM also had lighter ruminal and jejunal weights. Given the importance of the rumen for fermentation of feed and SCFA absorption, and the importance of the

jejunum to facilitate protein digestion and absorption, the reduction in tissue weights could indicate decreased digestive capacity. Additionally, ruminal, ileal, and cecal digesta weights were lighter for the heat-treated CM treatment, likely reflecting a smaller GIT size and the tendency for lesser DMI. However, the effect appears to be influenced primarily by the changes in tissue weight as gene expression and brush border enzyme activities were, in general, not affected by heat-treatment.

A critical assumption in this study was that a large portion of the RUP from heat-treated CM could be digested in the small intestine, as indicated by the *in vitro* measurement approach employed. However, the lack of a positive effect of heat-treatment may be indicative of reduced CP digestibility with heat-treatment, thereby eliminating potential increases for the total metabolizable protein supply, or that provision of RUP may not provide added benefit for calves around weaning. I did not confirm digestibility of the starters *in vivo*, and therefore cannot determine the cause for the lack of effect. The increase of RUP due to heat-treatment of CM may not be effective in calves, part of this response can be attributed to the reduction in starter intake. Using simple predictions from the in situ degradability of CM in study 1 combined with starter intake and the composition of the starter in study 2, the RUP supply from CM can be estimated. As expected, until 35 d of age, CM provision in the starter was estimated to supply up to 5% of the total RUP. The low contribution of the CM in the starter toward the RUP supply were mostly due to the relatively small quantities of starter intake, large quantities of MR intake, and the assumption that the CP in MR was 100% RUP (data not shown). Comparatively, during the step-down weaning phase, the estimated contribution of CM to the supply of RUP ranged from 42.90 to 50.11%, resulting in the predicted supply of 48 to 64 g/d. However, between 43 and 49 d of age, heat-treatment of CM may have only marginally increased the supply of RUP from 54 g/d for CM that was not heated to 57 g/d for CM that was heated, again mostly due to the negative effect of heated CM on starter intake. Importantly, the predicted RUP contribution from CM in the starter increased the metabolizable protein supply from MR by 75 to 100%, showing that during the step-down phase of the weaning process, solid feed contributes substantially to the potential RUP supply. Even though these calculations are essential to approximate the contribution of the treatments to the RUP supply, they do not account for differences in the digestibility of the RUP fraction and rely on the use of in situ degradation measurements conducted in heifers to predict the response. Moreover, these calculations do not account for potential differences in the RUP

supply with pelleting of the treatments (Huang et al., 2015) or other ingredients within the pellet, and rely on the assumption that capacity for ruminal digestion of CP in CM was similar to that for mature cattle. The latter suggestion is supported by previous research where ruminal SCFA concentrations and the contribution of bacterial N to the omasal N supply were not different during the week of weaning relative to that measured post-weaning (Quigley et al., 1985). Additionally, the lining of the intestinal tissue can be renewed every 2 to 3.5 d, depending on species (Darwich et al., 2014), which suggests that even short exposure to differing ration RUP fraction might have potential to influence the development of the intestine. With that said, there is a paucity of research evaluating the digestive capability of calves around weaning and how RUP affects metabolizable protein supply (Hill et al., 2013), especially when originating from heat-treatment of a protein source (Vazquez-Anon et al., 1993).

Given the relatively high AA requirement for growing calves (NRC 2001), the dietary changes occurring during weaning, and that ruminal fermentation may not provide sufficient quantities of microbial protein to meet metabolizable protein requirements, I hypothesized that providing additional RUP may enhance the growth of Holstein calves. However, use of plant-based proteins in MR reduced digestibility (Guilloteau et al., 2011) and altered the intestinal epithelium (Górka et al., 2011b); furthermore, cooking soy protein at 50°C overnight further reduced digestibility (Dawson et al., 1988). As such, I speculated that despite the limited effects of heat-treatment on the estimated intestinal digestibility determined *in vitro*, heat-treatment of CM may have decreased total-tract CP digestibility, thereby limiting AA supply to promote growth of the GIT and BW growth. The potential reduction in digestibility is further supported by Montagne et al. (2003), as they suggested that the digestion of plant-based protein might be limited at oligopeptide digestion and AA absorption steps in calves. On the contrary, Obitsu et al. (1995) reported that there were no differences in bacterial N flow to the intestine when calves were fed SBM or heated SBM, suggesting that rather than a reduction in digestibility of CP, heat-treatment may not have increased the flow of RUP. This latter suggestion is supported by the empirical calculations previously described. Given that I did not observe any changes in proteolytic brush border enzyme activity or gene expression for AA transporters, I can postulate that intestinal digestibility was not improved or that intestinal supply of microbial N and the flow of RUP were not altered. Nevertheless, it is clear that heat-treatment of CM does not stimulate growth or intestinal development in calves around weaning. Alternatively, it cannot be excluded that heat-

treatment of CM had a suppressive effect on rumen development, particularly ruminal fermentation development; however, ruminal pH and SCFA concentrations in calves were not affected by feeding them heat-treated CM, challenging such a hypothesis. Moreover, heat-treated CM did not affect ruminal ammonia-N, suggesting that the RDP supply was adequate.

Heat-treatment of CM had only small effects on the blood metabolites evaluated by decreasing BHB on d 22 and increasing BHB in d 43, as well as NEFA concentration on d 43. The calves consuming heat-treated CM overall consumed less starter between d 8 and 28, which could result in the lesser BHB concentration on d 22. On the other hand, heat-treatment of CM tended to result in increased ruminal butyrate proportion post-weaning, which could result in the increased BHB concentration in the serum. The reason why heat-treatment of CM increased ruminal butyrate is not clear.

3.5.4 Effect of Glycerol on Calf Performance and Development of the GIT

Calves that consumed starters supplemented with glycerol cumulatively consumed more starter during the whole study and especially during the step-down period. A possible reason for the increase in starter intake is that glycerol has been suggested to have a sweet taste (Quispe et al., 2013) and, therefore, may increase palatability of starter mixture. However, a recent review has suggested that glycerol has relatively inconsistent effects on DMI, and the response is likely due to purity of the glycerol source and dose (Kholif, 2019). The glycerol used in the present study had greater than 80% purity and was included at a relatively low rate, supporting that glycerol may enhance DMI at low inclusions (Ariko et al., 2015).

As a result of greater starter intake, both ADG and total BW gain were greater in glycerol supplemented calves. In a study conducted on beef calves (309.3 ± 14.0 kg), ADG increased when glycerol was included at 10% of the DM and decreased when included at 20% (Ramos and Kerley, 2012); however, a following study within the same publication did not yield any changes in ADG. Pantophlet et al. (2016) also reported no effect of glycerol on growth of calves. Although calves used for the previous studies were older than the calves in the present study, there is evidence that, even with a low inclusion rate, glycerol might have positive effects on growth of young ruminants.

As expected, with rapid glycerol fermentation in the rumen (Rémond et al., 1993), ruminal SCFA concentration increased in calves fed glycerol supplemented starters, and as a

consequence, a decrease in ruminal pH was noted. Similar effects have been previously observed in dairy cows (DeFrain et al., 2004; Mach et al., 2009); however, others have reported no changes for ruminal SCFA concentrations in calves (Ramos and Kerley, 2012) or cows (Paiva et al., 2016) when glycerol was included in the diet. According to Hall et al. (2015), SCFA concentration might not be the most reliable measure of fermentation, as it does not account for the ruminal volume, ruminal dilution, or absorption rates. The ruminal dilution rate may be particularly important in young calves (Yohe et al., 2018) considering that I observed increased digesta weight in the abomasum, duodenum, jejunum, and cecum, and increased tissue mass of the jejunum and colon when calves were supplemented with glycerol without differences in ruminal tissue weight. I suspect that glycerol might promote faster digesta passage through the GIT because glycerol increased starter intake, did not affect ruminal digesta weight, and increased digesta weight in regions of the GIT distal to the rumen. As a consequence, more nutrients were likely digested post-ruminally, as indicated by greater dipeptidase IV activity in jejunum, especially when glycerol was combined in the starter with heat-treated CM. Similar observations of increased dipeptidase IV activity in the distal jejunum were made when supplying microencapsulated sodium butyrate, another energy substrate, to calves (Górka et al., 2014). Similarly, Werner Omazic et al. (2015) estimated that 25% of glycerol is fermented, 45% is absorbed, and 30% flows into the omasum of dairy cows; however, such data are not available for calves. Given the potential for ruminal fermentation in the present study and outflow from the rumen, it is likely that some of the glycerol was fermented in the intestine, possibly promoting intestinal growth. Further support for this suggestion includes that the expression of *MCT1* was upregulated in the jejunum, implying greater potential for SCFA absorption from the intestine for calves supplemented with glycerol. Unfortunately, in the present study, I did not measure SCFA concentration in the small or large intestines, and thus cannot determine whether SCFA concentrations were altered. Nevertheless, SCFA released post-ruminally or delivered with digesta to lower regions of the GIT may have an important effect on GIT development in calves (Górka et al., 2018a).

Werner Omazic et al. (2015) noted, as mentioned previously, that in addition to being fermented in the rumen and passing to the omasum, glycerol can be transported through the epithelium. Apparent transport of glycerol into portal circulation was observed both in mature dairy cows (Werner Omazic et al., 2015) and in calves (Werner Omazic et al., 2013), following a large pulse-dose of glycerol into the rumen. In the current study, I did not observe differences in

the expression of *AQP3* in the CRA due to glycerol, although I did observe a heat-treatment by glycerol inclusion interaction. Aquaporin 3 is a water channel within the ruminal epithelium and is within a subgroup classified as aquaglyceroporins, as they are able to transport small particles such as urea (Rojek et al., 2008; Walpole et al., 2015) or glycerol (Wu and Beitz, 2007). It is possible that the dose of glycerol in the present study was not sufficient to stimulate a response for *AQP3* expression or that the transport was mediated by other aquaglyceroporins such as *AQP7*, *AQP9*, or *AQP10* (Rojek et al., 2008) that were not analyzed in the present study.

Glycerol can modulate ruminal fermentation and has been reported to increase the molar proportion of propionate (Rémond et al., 1993; DeFrain et al., 2004; Ramos and Kerley, 2012) and butyrate (Rémond et al., 1993; DeFrain et al., 2004; Paiva et al., 2016) and decrease acetate (Rémond et al., 1993; Ramos and Kerley, 2012; Paiva et al., 2016). In the present study, I did not observe changes in the molar proportions of acetate, propionate, or butyrate with glycerol inclusion. Reasons for the discrepancy between the present study and past studies may be due to the single spot-sampling approach used in the present study compared with more frequent measurements in the previous studies, and that calves in the present study were transitioning to solid feed. Differences could also arise from a lower dose of glycerol in the present study (5% DM). For example, Paiva et al. (2016) used glycerol inclusions ranging from 7 to 21% of DM, Ramos and Kerley (2012) used 0 to 20% DM, and Rémond et al. (1993) dosed ruminally cannulated cows at 20 or 40% of DM. However, DeFrain et al. (2004) used glycerol doses within a similar range as the current study with only very minor changes in SCFA concentration. Thus, in the current study, the positive effect of glycerol inclusion in starter mixture was likely not a result of its effect on ruminal butyrate, and thus stimulation of rumen epithelium development, which was originally hypothesized.

Glycerol decreased both plasma glucose and insulin concentrations on d 43—a time point corresponding to when calves were exposed to the step-down weaning process. The reduction in glucose and insulin might be due to the decrease in the starch supply in the glycerol treatments due to glycerol introduction into the feed. Similar observations for reduced glucose have been reported in mature ruminants when corn was replaced by glycerol (Carvalho et al., 2011). Surprisingly, glycerol also resulted in decreased NEFA concentration on d 43, further suggesting that the reduction in glucose and insulin were not driven by reduced nutrient intake. However, I observed lesser BHB concentration on d 51 for calves fed glycerol, suggesting that the potentially added

nutrient supply was likely not mediated through butyrate as was observed by Carvalho et al. (2011). That said, glycerol increased both the total SCFA concentration in the rumen and starter intake during the step-down period and would be expected to increase, rather than decrease BHB (Quigley et al., 1991). There was also no effect of glycerol on tissue mass in the proximal parts of GIT, and therefore the decreased of BHB concentration is difficult to explain.

In the present study, calves fed starters with heat-treated CM with glycerol had the greatest gene expression of *MCT1* in the CRA and the least when fed heat-treated CM without glycerol. The gene *MCT1* is a monocarboxylate transporter responsible for the removal of SCFA and ketones out of the rumen (Halestrap and Price, 1999). Nakamura et al. (2018) showed that *MCT1* was upregulated in post-weaned calves, although *MCT1* was not affected by changes in SCFA profiles. The increased *MCT1* observed in the present study may be related to greater starter intake between d 29 and 50 of age, as calves supplemented with glycerol consumed more starter than calves not supplemented with glycerol. However, heat-treatment increased ruminal butyrate, and butyrate is known to increase *MCT1* expression in ruminal epithelium (Dengler et al., 2015).

3.6 Conclusions

Increasing heat-treatment temperature decreased the ruminally degradable DM and CP fractions in CM with minimal effects on estimated intestinal digestibility in cannulated heifers when heated at 110°C for 10 min. Heat-treated CM and glycerol inclusion do not interact to promote development of the GIT in calves around weaning. Feeding heat-treated CM to calves before and during weaning may reduce ADG and GIT development. Inclusion of glycerol at 5% DM may positively affect ADG in pre-weaned calves. Furthermore, glycerol may increase ruminal fermentation and *MCT1* expression in the small intestine, suggesting stimulation of GIT development.

4 CANOLA MEAL OR SOYBEAN MEAL AS PROTEIN SOURCE AND THE EFFECT OF MICROENCAPSULATED SODIUM BUTYRATE SUPPLEMENTATION IN PELLETED STARTER MIXTURES FOR DAIRY CALVES. I. GROWTH PERFORMANCE, FEED EFFICIENCY, NUTRIENT DIGESTIBILITY AND SELECTED BLOOD PARAMETERS

During the in situ study described in Chapter 3, heat-treated CM resulted in decreased ruminally degradable DM and CP fractions, while the intestinal digestibility was decreased at temperatures greater than 110°C. Overall, use of heat-treated CM in starters had negative impact on ADG and GIT development in calves. Therefore, for subsequent studies, heat-treated CM was not used. Additionally, as shown in Chapter 3, addition of glycerol to the starters can improve starter intake and growth of calves, and therefore glycerol was included as part of the all treatment diets in the present studies. Following the insight into effects of additional CM processing and glycerol supplementation, the subsequent studies compare CM with SBM as protein sources within starter mixtures for calves, as well as evaluate further supplementation with microencapsulated sodium butyrate.

4.1 Abstract

Two studies were conducted to assess the effect of protein source and microencapsulated sodium butyrate (**MSB**) inclusion in pelleted starter mixtures on growth performance, gain to feed (**G:F**) ratio, nutrient digestibility and selected blood metabolites in calves. In Study 1, 28 Holstein bull calves (8.7 ± 0.8 d of age and 43.0 ± 4.4 kg; mean ± SD) were allocated to 1 of 4 treatments in a 2 × 2 factorial arrangement and fed a pelleted starter mixture containing canola meal (**CM**, 34.8% DM) or soybean meal (**SBM**, 23.7% DM) as the main source of protein, with or without supplemental MSB (0.33% DM). Starter mixtures were formulated to be similar for CP, Lys, and Met and were fed *ad libitum*. Calves were weaned after 42 d of milk replacer feeding (51.7 ± 0.8 d of age) and observed for another 21 d. Furthermore, selected blood parameters were measured on 21, 42, and 63 d of the study and nutrient digestibility was measured post-weaning. In Study 2, 60 Holstein heifer calves (9.1 ± 0.8 d of age and 43.2 ± 4.2 kg) were assigned to the same

treatments as in Study 1. The calves were weaned after 49 d of milk replacer feeding (59.1 ± 0.8 d of age) and observed for an additional 14 d. Milk replacer and starter mixture intake, and fecal score were recorded daily, whereas BW was recorded weekly. In Study 1, calves fed starter mixtures containing CM had or tended to have lesser pre-weaning starter intake, weaning ADG, weaning and overall G:F ratio, and post-weaning total tract DM digestibility, as opposed to those fed starter mixtures with SBM. However, these differences did not affect overall starter intake and ADG as well as final BW. Feeding starter mixtures with SBM resulted in greater fecal score at weaning, post-weaning, and in the whole study period, compared to feeding starter mixtures with CM, indicating greater fecal fluidity for calves fed starter mixture containing SBM. Supplementation with MSB only tended to increase pre-weaning starter mixture intake. In Study 2, heifer calves that were fed starter mixtures with CM had greater cumulative post-weaning starter intake, but the protein source in starter mixture had no effect on ADG, BW, or G:F ratio. Similar to Study 1, fecal score post-weaning and in the whole study period was greater for calves fed starter mixtures containing SBM. Inclusion of MSB in starter mixtures for calves tended to decrease post-weaning starter mixture intake. In conclusion, use of CM or SBM as a main source of protein in starter mixture resulted in similar growth performance of bull and heifer calves; however, CM use in starter mixtures reduced starter intake, ADG, and G:F ratio at least at some points of rearing. Canola meal use in starter mixtures might contribute to decreased fecal score of calves, suggesting firmer feces. Under the conditions of the current study, supplementation of MSB had minor impact on the growth performance of the calves.

4.2 Introduction

Canola meal (**CM**) is a common protein source used in diets for dairy cows (Huhtanen et al., 2011; Martineau et al., 2013). However, its use in starter mixtures for calves is often discouraged. This is a result of concerns with palatability (Fiems et al., 1985) and digestibility (Fiems et al., 1985; Khorasani et al., 1990), causing lesser feed efficiency (Hadam et al., 2016; Terré et al., 2016) and ADG (Hadam et al., 2016) for calves fed starter mixtures containing CM. Considering the above mentioned concerns, CM is not commonly used in starter mixtures for calves with soybean meal (**SBM**) remaining the preferred source of protein. However, some studies did not show differences in starter intake (Fiems et al., 1985; Hadam et al., 2016; Terré et al., 2016) and ADG (Fiems et al., 1985; Terré et al., 2016) when CM was used in starter mixtures instead of SBM, suggesting that CM has the potential to be an effective source of protein in starter mixtures for calves. It should be also noted that some past studies have evaluated rapeseed meal and meal originating from 'old' varieties of canola and rapeseed [e.g., Fiems et al. (1985)], which could have a greater concentration of antinutritional factors that are known to reduce feed intake and growth of calves (Montagne et al., 1999).

Besides the presence of various antinutritional factors (e.g., glucosinolates, erucic acid, tannins, phytic acid), lesser CP, and greater fiber content, CM contains less Lys and more Met compared to SBM (NRC, 2001; Paz et al., 2014). Both Lys and Met are considered limiting AA for growth of newborn calves (Abe et al., 1998). Depending on ingredient composition of the diet, as well as nutrient composition of CM and SBM used for feed formulation, contents of Lys and Met may differ between diets utilizing CM and SBM (Khorasani et al., 1990; Zinn, 1993), resulting in another confounding factor when comparing CM and SBM. Therefore, equalizing Lys and Met in starter mixtures containing CM or SBM, may allow for a more robust comparison between CM and SBM effects when used in calf starter mixtures.

Efficiency of starter use, especially CM, for calves could be increased by dietary butyrate supplementation. Butyrate is naturally produced in gastrointestinal tract (**GIT**) and is present in milk (Guilloteau et al., 2010a). Its addition into milk replacer (**MR**) or starter mixtures was reported to have a positive impact on GIT development and growth performance of calves in numerous studies (Górka et al., 2018a). In pre-weaning calves, butyrate is the main stimulator of ruminal epithelium development (Sander et al., 1959; Mentschel et al., 2001). Thus,

supplementation of butyrate in solid feed can be used to accelerate ruminal epithelial cell proliferation and differentiation in calves (Górka et al., 2011a), particularly when provided in a rumen-unprotected form (Górka et al., 2018a). When supplemented in a protected form, for example microencapsulated, butyrate is only partially released in the forestomach and has been reported to stimulate the development and function of lower regions of GIT (Piva et al., 2007; Górka et al., 2018a). Taking into account that CM contains various antinutritional factors, which have a negative impact on GIT of mammals, particularly the small intestine (Bell, 1993), it may be possible that protected butyrate supplementation in starter mixtures may have a positive impact on the performance of calves when CM is used as a main source of protein in starter mixture. Further evidence is provided by reports of reduced negative impact of antinutritional factors such as plant-based proteins on the development of the small intestine, as well as improved pancreatic function in calves receiving butyrate supplementation (Guilloteau et al., 2009b; Guilloteau et al., 2010b; Górka et al., 2011b). Furthermore, in calves, microencapsulated sodium butyrate (**MSB**) inclusion in pelleted starter mixture can positively impact ruminal development (Górka et al., 2011a; Górka et al., 2014).

Considering the above, my hypothesis was that feeding a starter mixture containing CM and MSB to calves will result in at least similar or improved growth performance to that observed in calves fed a starter mixture containing SBM. This hypothesis was tested while equalizing Lys and Met concentration among treatments. The objective of the study was to compare the use of CM and SBM in starter mixtures on growth characteristics of both bull and heifer calves and to determine whether MSB supplementation will additionally influence those responses when Lys and Met content is similar between diets. Moreover, the impact of CM and SBM as the main protein source and MSB supplementation in starter mixture on nutrient digestibility and some blood parameters in calves were investigated.

4.3 Materials and Methods

Two studies were conducted to on a commercial dairy farm (Top Farms Głubczyce, Głubczyce, Poland). Study 1 was conducted from January until April 2016, while Study 2 from July until October 2016. The experimental protocol was approved by the Local Institutional Animal Care and Use Committee (Kraków, Poland; protocol No. 211/2015).

4.3.1 Study 1

4.3.1.1 Animals, Housing and Feeding Regimen

Twenty-eight Holstein bull calves (8.7 ± 0.8 d of age and 43.0 ± 4.4 kg; mean ± SD) were allocated to 4 treatments in a 2 × 2 factorial treatment arrangement (7 calves/treatment) with the main treatments being the source of protein and inclusion of MSB. As such, calves were fed a pelleted starter mixture containing SBM as a main source of protein without (**TSBM**) or with MSB (**TSBM-MSB**), or were fed pelleted starter mixture containing either CM as a main source of protein without (**TCM**) or with MSB (**TCM-MSB**). Starters were formulated to be isonitrogenous and similar for Lys and Met concentration (Table 4.1 and Table 4.2). In order to ensure similar Lys and Met content in starters, the most abundant ingredients to be used in the starters were analyzed for macronutrients and AA concentration prior to pelleting. Based on this initial analysis (data not shown), only addition of Met (L-Methionine, production no. GDAF150607, distributed by "Blattin Polska" Sp. z o.o., Izbicko, Poland) to starters containing SBM was required. For all starters, feeds from the same batch were used. Microencapsulated sodium butyrate (BIOLEK Sp. z o.o., Macierzysz, Ożarów Mazowiecki, Poland) which contained 30% sodium butyrate and 70% of a triglyceride matrix was included in starters at 0.33% DM by replacing barley grain. This inclusion rate was based on past studies with calves (Górka et al., 2011a; Górka et al., 2014) and that greater inclusion rates induced negative responses (Wanat et al., 2015). Chromium oxide (Cr_2O_3; Sigma-Aldrich, Saint Louis, MO) was included at 0.22% DM of starters and used as digestibility marker. Based on results from Chapter 3, glycerol was added at 5.07% DM into all starters.

Calves were sourced from two commercial dairy farms belonging to one operator (Top Farms Głubczyce, Głubczyce, Poland), both located near (<15 km) the calf rearing facility where calves were housed during the study period. Prior to the study, calves underwent standardized procedures for newborn calves. Briefly, calves were separated from dams immediately after birth, housed in individual hutches, and provided with 4 L of colostrum within 2 h of life, and then with an additional 2 L of colostrum by 12 h of life. On d 2 and 3 of life, calves were fed 2 L of transition milk 2 times per day, which on consecutive days was followed by being

Table 4.1. Ingredient composition of pelleted starter mixtures used for Study 1 and Study 2 to test the effect of using canola meal or soybean meal as a protein source with or without microencapsulated sodium butyrate

Component (% DM)	Study 1				Study 2			
	TSM[1]	TSM-MSB	TCM	TCM-MSB	TSM	TSM-MSB	TCM	TCM-MSB
Canola meal	-	-	34.95	34.94	-	-	34.74	34.73
Soybean meal	23.71	23.70	-	-	23.65	23.65	-	-
Barley	28.94	28.63	18.91	18.60	29.03	28.72	19.09	18.78
Corn grain	29.38	29.37	29.35	29.34	29.72	29.71	29.75	29.73
Wheat bran	5.02	5.02	5.01	5.01	4.92	4.92	4.93	4.92
Whey protein (dry)	2.71	2.71	2.70	2.70	2.69	2.69	2.69	2.69
Mineral-vitamin supplement[2]	1.06	1.06	1.06	1.06	1.05	1.05	1.05	1.05
Glycerol	5.07	5.07	5.07	5.07	5.04	5.04	5.05	5.04
MSB[3]	-	0.33	-	0.33	-	0.33	-	0.33
Methionine	0.06	0.06	-	-	0.09	0.09	-	-
Monocalcium phosphate	1.10	1.10	-	-	1.10	1.10	-	-
Limestone	2.21	2.21	2.21	2.21	2.20	2.20	2.20	2.20
Sodium chloride	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52
Chromium oxide	0.22	0.22	0.22	0.22	-	-	-	-

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).

[2]Blattin Super Premium (Blattin Sp. z o.o., Izbicko, Polska): 18.3% Ca; 3% P; 8.5% Na; 5.5% Mg; 11,500 mg Zn; 5,000 mg Mn; 1,500 mg Cu; 27 mg Co; 83 mg I; 87 mg Se; 1,000,000 IU vitamin A; 160,000 IU Vit. D; 5,000 mg vitamin E; 20 mg vitamin B1; 30 mg vitamin B2; 2156 mg vitamin B3; 67 mg vitamin B5; 100,000 µg vitamin B7; 4 mg vitamin B9; 25 mg vitamin B6; 250 mg vitamin B12.

[3]MSB = microencapsulated sodium butyrate (BIOLEK Sp. z o.o., Macierzysz, Ożarów Mazowiecki, Poland).

Table 4.2. Nutrient composition of pelleted starter mixtures used for Study 1

Variable	Treatment/Feed[1]				
	TSBM	TSBM-MSB	TCM	TCM-MSB	MR
DM, %	90.1 ± 0.3[2]	90.9 ± 0.2	90.2 ± 0.7	90.4 ± 0.3	95.0 ± 0.2
Ash, % DM	9.1 ± 0.2	9.1 ± 0.3	8.4 ± 0.2	8.3 ± 0.3	10.8 ± 0.4
OM, % DM	90.9 ± 0.2	90.9 ± 0.3	91.6 ± 0.2	91.7 ± 0.3	89.2 ± 0.4
CP, % DM	21.9 ± 0.4	21.7 ± 0.1	20.7 ± 0.2	20.3 ± 0.3	21.7 ± 0.2
EE[3], % DM	1.8 ± 0.0	2.0 ± 0.2	2.9 ± 0.3	3.4 ± 0.1	18.1 ± 0.3
NDF, % DM	13.4 ± 1.2	15.0 ± 0.6	18.7 ± 1.3	18.6 ± 1.2	-
ADF, % DM	6.5 ± 0.4	7.3 ± 0.3	10.8 ± 1.0	11.5 ± 0.5	-
NFC[4], %DM	53.9 ± 1.0	52.3 ± 0.9	49.2 ± 1.4	49.4 ± 0.9	
Sugar, % DM	7.8 ± 0.2	8.5 ± 0.2	8.7 ± 0.2	8.4 ± 0.2	-
Cr, g/kg DM	1.2 ± 0.2	1.2 ± 0.2	1.4 ± 0.1	1.3 ± 0.0	-
ME[5], Mcal/kg	3.02 ± 0.00	3.04 ± 0.02	3.09 ± 0.01	3.11 ± 0.01	4.52 ± 0.03
Amino acid, g/kg DM					
Ala	8.26 ± 0.16	8.78 ± 0.33	8.33 ± 0.20	7.99 ± 0.29	-
Arg	14.28 ± 0.28	14.98 ± 0.78	12.93 ± 0.28	12.26 ± 0.46	-
Asp	17.82 ± 0.26	18.72 ± 0.35	14.13 ± 0.09	13.21 ± 0.19	-
Cys	3.36 ± 0.14	3.54 ± 0.03	4.25 ± 0.15	4.43 ± 0.02	-
Glu+Gln	37.87 ± 1.08	40.24 ± 1.15	35.25 ± 0.82	32.56 ± 1.38	-
Gly	8.01 ± 0.23	8.58 ± 0.32	8.88 ± 0.28	8.54 ± 0.24	-
His	5.51 ± 0.12	5.89 ± 0.39	5.74 ± 0.21	5.54 ± 0.21	-
Ile	7.54 ± 0.09	7.50 ± 0.25	6.69 ± 0.41	6.30 ± 0.19	-
Leu	15.01 ± 0.03	15.73 ± 0.42	14.24 ± 0.42	13.74 ± 0.33	-
Lys	9.00 ± 0.11	9.44 ± 0.38	8.70 ± 0.26	8.50 ± 0.08	-
Met	3.87 ± 0.11	3.78 ± 0.07	3.66 ± 0.08	3.76 ± 0.05	-
Phe	8.98 ± 0.24	9.49 ± 0.24	7.84 ± 0.16	7.50 ± 0.21	-
Pro	10.40 ± 0.22	11.27 ± 0.82	11.48 ± 0.64	11.65 ± 1.52	-
Ser	8.17 ± 0.04	9.00 ± 0.75	7.91 ± 0.09	7.58 ± 0.42	-
Thr	6.46 ± 0.09	6.91 ± 0.26	7.31 ± 0.06	6.84 ± 0.14	-
Tyr	5.92 ± 0.05	5.91 ± 0.06	5.89 ± 0.03	5.80 ± 0.30	-
Val	9.54 ± 0.03	9.64 ± 0.58	9.81 ± 0.73	9.09 ± 0.63	-

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM); MR = milk replacer.

[2]mean ± SD.

[3]EE = ether extract.

[4]NFC calculated according to NRC (2001), as 100 – (NDF, % + CP, % + EE, % + ash, %).

[5]ME = metabolizable energy, calculated based on NRC (2001) for the starters as: (1.01 × DE – 0.45) + 0.0046 × (EE% – 3), where DE = digestible energy, calculated as (0.057 CP% + 0.094 EE% + 0.0415 (100 – CP% – EE% – ash%)) × 0.82; and for the milk replacer as: 0.96 × (0.97 × (0.057 CP% + 0.092 EE% + 0.0395 × (100 – CP% – EE% – ash%))).

fed 2 L of whole milk 2 times per day, until the calves were transported to the calf barn. During this time calves did not have access to a starter mixture.

Between 8 and 10 d of age (8.7 ± 0.8 d), calves were transported from the farm of birth to a commercial naturally ventilated calf barn (Top Farms, Głubczyce). Calves were transported twice a week (Mondays and Thursdays), always at around 1000 h and 4 or 8 calves/wk were sourced for the study, resulting in 7 blocks of calves. Within each block, calves were allocated to 1 of 4 treatments based on their BW and farm of birth, ensuring that treatments were similar for initial BW and balanced for number of calves from each farm. Only healthy calves were allocated to the study. Calves were housed in 1.5 × 1.2 m indoor individual pens with wood shavings as bedding material, and had permanent access to an additional 3.0 × 1.2 m individual outdoor concrete paddock. Upon arrival, calves were weighted, prophylactically treated with a broad-spectrum antibiotic (Zactran, Merial, Lyon, France) to prevent pulmonary diseases, and fed 2 L of electrolytes (Rehyvet, Univit, Olomouc, Czech Republic or Rehydrat, Biowet, Puławy, Poland; depending on the block of calves). Additionally, calves were given an oral coccidiostat (Baycox 5%, Bayer, Leverkusen, Germany) on d 15 of the study as part of routine treatment protocols on the farm.

After transport to calf barn, calves were fed a commercial MR (Polmass Milk Red Full Instant, Polmass S.A., Bydgoszcz, Poland; Table 4.2) with the exception that the original formula of MR was altered to remove calcium butyrate. Milk replacer was mixed at a rate of 150 g of MR powder (as fed) per 1 L of water. To ease the transition from whole milk to MR, calves received 2 L of MR at the 1700 h feeding on the d of transport (d 1 of the study). On d 2 of the study, calves received 2.5 L of MR per feeding fed twice daily at 0730 and 1700 h, which was increased to 3 L per feeding on d 3 fed twice daily as well. Calves received 3 L of MR per feeding until 1 wk before weaning. Starting on d 36 of the study, calves were limited to one 3-L feeding daily at 0730 h. Weaning took place on d 43 of the study, which was the first day with no MR provision (51.7 ± 0.8 d of age). Refusals of MR were measured and recorded after each feeding. After weaning, the study was continued for 3 wk.

The pelleted starter was offered *ad libitum* from d 1 of the study. Daily, after the morning MR feeding (0800 h), refusals were recorded, and fresh starter was provided. Initially, 500 g/d of starter was offered. When calves consumed more than half of the allocation, the amount of starter offered was increased by another 500 g. Subsequently, whenever calves refused less than 500 g,

the amount of starter fed was increased by an additional 500 g. Samples of starter mixtures were collected twice a week, while samples of MR were collected upon opening of a new bag. All samples were composited by month of the study.

4.3.1.2 Measurements and Observations

Body weight and withers height of calves were measured weekly at 1000 h. All disease events and medical treatments were recorded. Fecal scores (fluidity) of the calves were recorded daily, using a 4-point scale [1 = normal, 2 = semi-formed, 3 = loose feces but staying together, 4 = diarrhea, watery feces; Larson et al. (1977)]. Calves displaying a fecal score ≥ 3 were offered at least 2 L of electrolyte solution between MR feedings (Rehyvet, Univit, Olomouc, Czech Republic or Rehydrat, Biowet, Puławy, Poland; depending on the block of calves) and monitored for further clinical signs. Additionally, in cases of a persistent fecal score ≥ 3, severe diarrhea (fecal score 4), or dehydration, calves were treated on an individual basis according to a veterinarian's recommendation.

4.3.1.3 Blood Sample Collection and Analysis

Blood samples were collected on d 21 (pre-weaning period), 42 (weaning period), and 63 (post-weaning period) of the study at 1000 h. Blood samples were collected from the jugular vein of the calves into tubes containing lithium heparin (plasma collection; 16 IU lithium heparin/mL blood, Sarstedt, Nümbrecht, Germany) and a tube containing a clot activator (serum collection; Sarstedt, Nümbrecht, Germany). Blood for serum collection was allowed to clot for 1 h, while blood for plasma collection was placed on ice and allowed to cool down for at least 15 min. Both tubes with heparin and the clot activator were centrifuged for 15 min at 2,300 × *g* and the resulting plasma and serum were then transferred into 2-mL vials and stored at -20°C until analysis for plasma glucose, urea, AA concentration, and serum insulin, BHB, and IGF-1 concentration.

Plasma glucose and urea, as well as serum BHB were determined by a commercial laboratory (WDL, Gietrzwałd, Poland) on an automatic chemical analyzer (Hitachi 902, Hitachi, Japan) as previously described by Górka et al. (2011b). For glucose and urea determination,

commercial BioSystems (Barcelona, Spain) kits were utilized. A different commercial kit, Diagnostic System Laboratories Inc. (Sinsheim, Germany), was used to determine BHB concentration. Serum insulin and IGF-1 concentrations were analyzed using radioimmunoassay and a commercial set of reagents (INS-IRMA and IGF-1-RIA-CT kits for insulin and IGF-1 determination, respectively; DIAsource, Louvain-La-Neuve, Belgium). Plasma AA concentration (only in samples collected on d 63 of the study) was analyzed using Pico-Tag AA analysis system (Waters, Milford, MA) at the Jagiellonian University (Kraków, Poland).

4.3.1.4 Digestibility Measurement and Chemical Analysis of Feeds and Feces

On either 51 (10 calves) or 54 (18 calves) d of the study (52.9 ± 1.5 d or 61.6 ± 1.8 d of age), the digestibility measurement was initiated. Fecal sampling was conducted over three days, always on Friday, Saturday, and Sunday. Days of sampling (and thus days of study when sampling was initiated for each calf) were dictated by staff accessibility for sampling in the calf barn and allowed for streamlining the sampling procedure and increasing sampling accuracy. The sampling day was assessed as a covariate and found to be not significant ($P = 0.35$, data not shown) and not included in the actual statistical analysis of digestibility variables. Eight samples of feces were collected every 9 hours from each calf at 0700 and 1600 h on d 1 of sampling, 0100, 1000, 1900 h on d 2, and 0400, 1300, 2200 h on d 3. At each sampling timepoint, 100 g of fresh feces was collected through manual stimulation of the calf's rectum and then frozen at -20°C in a composited sample for each calf until the end of sampling. Subsequently, fecal samples were thawed. Both fecal and starter samples were dried at 55°C in a forced air oven until achieving a constant weight. The samples were ground to pass through 1-mm screen using Fritsch mill (Type EFB3H/4D90SA-2; Fritsch GmbH, Idar-Oberstein, Germany) and analyzed for nutrient composition, as described below.

Analysis of DM, ash, CP, NDF, and ADF in feeds and feces were performed as previously described by Górka et al. (2017b). Water soluble sugars in feeds were analyzed using a method described by DuBois et al. (1956) with glucose as a standard. Ether extract in MR was analyzed using procedure 920.39 (AOAC, 2005) following hydrolysis with 4*M* HCl. Chromium concentration in feeds and feces were analyzed using flame atomic absorption spectrophotometry

after ashing the sample in nitric and perchloric acid (PN-EN 1233:2000P, Poland). Amino acid composition in feed was determined using an AA analyzer AAA-400 (INGOS, Czech Republic) by first hydrolyzing the protein in 6*M* HCl at 110°C for 24 h. Sulfur-containing AA were determined after performing acid oxidation in mixture of 18N formic acid and perchlorate (9:1 vol/vol) at 110°C for 24 h. Apparent nutrient digestibility was calculated using Cr as a marker.

4.3.2 Study 2

4.3.2.1 Animals, Housing, and Feeding Regimen

Sixty Holstein heifer calves (9.1 ± 0.8 d of age and 43.2 ± 4.2 kg; mean ± SD) were allocated to this study. The experimental design and feed ingredients (Table 4.1) of starters were similar to those used in the Study 1. Briefly, the experiment was conducted as a 2 × 2 factorial treatment arrangement with calves assigned to 1 of 4 treatments: TSBM, TSBM-MSB, TCM or TCM-MSB (15 calves/treatment). The calves were sourced from two commercial dairy farms belonging to one operator (Top Farms Głubczyce). Prior to being assigned to the study, heifers were treated in the same manner as bulls in Study 1. Transport to the calf barn (Top Farms Głubczyce) occurred twice a week (Tuesdays and Fridays; between 1000 and 1100h) between 8 to 10 (9.1 ± 0.8) d of age. In the Study 2, heifer calves were also blocked by the week of transport to the calf barn. All calves were collected over 6 wk, resulting in 6 blocks of 4, 12, 16, 12, 8, and 8 calves each. Within block, calves were allocated to treatments based on their BW and farm of birth ensuring that treatments were similar for initial BW and balanced for number of calves from each farm.

The composition of starters used in Study 2 differed only slightly from those used in Study 1. This was mostly a result of differences in the chemical composition of the feed ingredients used between studies. Starters used for Study 2 did not contain chromium oxide (Table 4.1).

The heifer calves in Study 2 were cared for in the same manner as the bull calves in Study 1, with minor modifications. Specifically, pens were bedded with straw instead of wood shavings, which resembled more standard housing conditions of calves used on the farm. Furthermore, the MR feeding protocol was similar. Milk replacer was provided by Trouw Nutrition Poland (Grodzisk Mazowiecki, Poland) and its original formula was altered to remove sources of butyrate.

In order to ease the transition from whole milk to MR, the amount of MR fed to the calves was gradually increased, in the same way as described for Study 1. Feeding 3 L/feeding twice daily at 0730 and 1700 h was continued until 2 week before weaning. On d 36 of the study, calves were limited to one feeding per d at 0730 h. Weaning took place on d 50 of the study (59.1 ± 0.8 d of age), which was the first d with no MR provision and the study lasted for two wk post-weaning. Refusals of MR were measured and recorded after each feeding.

The pelleted starter mixture was offered *ad libitum* from d 1 of the study. Daily, after morning MR feeding (0800 h), refusals were recorded, and fresh pellet was provided. The amount of starter offered was adjusted daily when necessary to ensure *ad libitum* intake as described in Study 1.

4.3.2.2 Sampling, Measurements and Observations

Samples of the starter mixtures and MR powder were collected weekly, composited by month of the study. Samples were dried, ground (with exception to MR powder), and analyzed for chemical components as described for Study 1. Additionally, starters were analyzed for starch as described by Faisant et al. (1995). Body weight and withers height of calves were measured every week. Fecal scores and disease incidence were monitored daily, similar to Study 1. Calves were treated as described for Study 1 in terms of health management.

4.3.3 Statistical Analysis

The power analysis was conducted using one-way analysis of variance (SAS, version 9.4, SAS Institute, Cary, NC) based on the observed means and SD of DMI and ADG (Chapter 3, Hadam et al., 2016), resulting in estimated statistical power of minimum 0.88 at n = 7 and 0.99 for n = 15.

In Study 1, one bull calf died shortly before weaning due to bloat, and another two calves that experienced severe bloat issues [treated according to veterinarian's advice, including rumenostomy and treating with Biovetalgin (BIOWET DRWALEW S.A., Drwalew, Poland)] were eliminated from the dataset. This resulted in 6, 6, 6 and 7 bull calves that were used for statistical analysis for treatment TSBM, TSBM-MSB, TCM and TCM-MSB, respectively.

In Study 2, one heifer calf died due to pneumonia 2 wk after allocation to the study after treatment according to the veterinarian's recommendation, and another one had to be removed from the dataset due to constipation issues. This resulted in 15, 14, 15, 14 heifer calves for TSBM, TSBM-MSB, TCM and TCM-MSB treatments, respectively.

For both studies, data were analyzed as a 2 × 2 factorial design using SAS software (version 9.4, SAS Institute, Cary, NC). Normally distributed variables were analyzed using PROC MIXED. Protein source, MSB inclusion, and the protein source × MSB inclusion interactions were considered in the statistical model as fixed effects, whereas block of calves was considered as a random term. Variables analyzed over time on each calf were analyzed as repeated measurements by including the effect of time (day for milk replacer and starter intake or wk for ADG and withers height) and the REPEATED statement in the model. The effect of time was significant for majority of analyzed variables ($P \leq 0.032$; both in Study 1 and 2) with the exception of post-weaning ADG in Study 1 ($P = 0.57$). However, as the time effect was not the focus of the current research, it will not be discussed. Also, two and three-way interactions with time effects were considered in the statistical model. In Study 1 the two- and three-way interactions were not significant for all analyzed variables ($P \geq 0.19$) except there was a MSB × day effect ($P = 0.046$) for fecal score during the weaning phase. Thus, for Study 1, the 2 and 3-way interactions were removed from the model. In Study 2, significant treatment × time interactions or tendencies for their interactions were observed for some variables and thus two- and three-way interactions were left in the model. However, these interactions did not further advanced interpretation of the data or testing of the hypothesis and as such, will not be discussed. For each variable analyzed as repeated measure, the most suitable covariance structure was selected based on the least values of the Akaike's and Bayesian Information Criterions. For ADG, starter intake, and gain to feed (**G:F**) ratio, the initial BW was included in the model as a covariate. Parameters measured in the blood of calves were not analyzed as repeated measures because their initial concentrations (prior to dietary treatments allocation) were not considered in analysis. Additionally, for analysis of blood parameters on d 21 of the study, data from calves that were excluded due to bloat were included in these statistical analyses, as those health events only affected calves in the later part of the study. Non-normally distributed variables (fecal score and days with diarrhea) were analyzed using PROC GLIMMIX. Fecal score data were analyzed using Poisson distribution with a random statement for _residual_ and calf specified as subject of analysis to allow for accounting repeated measure on each animal,

and autoregressive 1 as the covariance structure, with the exception of the pre-weaning period for Study 1 in which covariance error structure was used and the weaning period during Study 1 when compound symmetry covariance error structure was used. Days with diarrhea were analyzed using lognormal distribution. Unless otherwise stated, data are presented as least square means and corresponding standard errors. When a significant effect of protein source × MSB inclusion was found, means were separated using Bonferroni adjustment. Significance was declared when $P < 0.05$, while a tendency was declared when $0.05 \leq P \leq 0.10$.

4.4 Results

4.4.1 Study 1

Crude protein content was numerically greater (21.8% vs. 20.5%; Table 4.2) for starter mixtures containing SBM then for those containing CM, whereas NDF and ADF content was less for SBM treatments than for CM treatments (14.5% vs. 18.7% for NDF; 6.9 vs. 11.2% for ADF). Although starter mixtures were formulated to be similar for Lys and Met, content of Lys was slightly lesser (by 6.7%) in starter mixtures containing CM than in those containing SBM. Moreover, starter mixtures containing CM had lesser concentrations of Arg (by 13.9%), Asp (by 25.2%), Glu with Gln (by 15.2%), Ile (by 15.7%), Phe (by 20.4%), and Ser (by 10.9%), and greater concentration of Cys (by 25.8%) compared to those containing SBM.

Both daily and cumulative MR intake did not differ among treatments ($P \geq 0.42$; Table 4.3). During the pre-weaning period, daily starter intake was less for calves fed the starter mixtures containing CM rather than SBM ($P = 0.013$), and tended to be greater for calves fed starter mixtures containing MSB ($P = 0.065$) independently of the protein source that was used in the starter. Daily starter intake during the weaning and post-weaning period was not affected by treatments ($P \geq 0.21$), but overall starter intake tended to be affected by the protein source × MSB interaction ($P = 0.081$). This was a result of greater daily starter intake for TCM-MSB and TSBM, intermediate for TSBM-MSB, and the least for TCM. However, when considering starter intake cumulatively, no differences between treatments were detected for any of the periods of the study ($P \geq 0.28$). Initial and final BW did not differ between experimental treatments ($P \geq 0.44$). Pre-weaning, post-weaning, and overall ADG of calves did not differ among treatments ($P \geq 0.20$).

Table 4.3. Milk replacer and starter intake, BW, ADG, feed efficiency, fecal score, and DM digestibility of calves in Study 1

Variable	Treatment[1]				SEM	*P*-value[2]		
	TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS × MSB
Milk replacer intake, g DM/d[3]	753.7	758.6	755.0	757.7	5.4	0.96	0.42	0.80
Cumulative milk replacer intake, kg	31.7	31.8	31.7	31.8	0.2	0.94	0.42	0.81
Starter intake, g DM/d								
Pre-weaning[3,4]	246.7	270.2	219.7	239.5	20.2	0.013	0.065	0.87
Weaning[3]	1221.6	1393.3	1253.0	1309.1	122.0	0.77	0.21	0.52
Post-weaning[3]	1942.7	1905.4	2003.2	2142.2	168.8	0.36	0.75	0.58
Overall[3]	913.9	874.5	820.3	919.8	46.9	0.54	0.46	0.081
Cumulative starter intake, kg DM								
Pre-weaning	9.17	9.79	5.96	9.14	1.76	0.28	0.29	0.48
Weaning	19.25	18.51	17.05	19.03	1.81	0.65	0.74	0.46
Post-weaning	28.70	26.77	27.34	29.23	2.13	0.79	0.99	0.37
Overall	57.14	55.25	50.33	57.40	5.24	0.67	0.63	0.40
Initial BW, kg	42.7	43.5	43.0	42.6	1.9	0.87	0.93	0.75
Final BW, kg	84.9	86.8	83.6	81.8	4.5	0.44	0.65	0.98
ADG, kg/d								
Pre-weaning[3]	0.652	0.646	0.594	0.614	0.044	0.30	0.87	0.76
Weaning[3]	0.643	0.647	0.445	0.505	0.098	0.100	0.75	0.77
Post-weaning[3]	0.942	0.842	0.897	0.900	0.092	0.94	0.59	0.55
Overall[3]	0.715	0.692	0.627	0.654	0.047	0.20	0.97	0.61
Withers height[3], m	0.837	0.828	0.832	0.833	0.010	1.00	0.72	0.60
Gain to feed ratio, kg gain/kg DM								
Pre-weaning	0.621	0.598	0.585	0.589	0.036	0.51	0.76	0.64
Weaning	0.396	0.362	0.257	0.257	0.04	0.018	0.63	0.60
Post-weaning	0.456	0.459	0.470	0.429	0.057	0.88	0.77	0.71
Overall	0.496	0.499	0.482	0.460	0.015	0.092	0.54	0.42
Fecal score[5]								
Pre-weaning[3]	1.83	1.91	1.83	1.73	0.10	0.097	0.89	0.097
Weaning	1.42	1.27	1.11	1.17	0.08	0.024	0.62	0.23
Post-weaning	1.45	1.21	1.08	1.15	0.07	0.008	0.35	0.067
Overall	1.61	1.58	1.48	1.45	0.05	0.011	0.59	0.98
Overall d with diarrhea[6]	6.9	6.7	5.9	4.6	1.5	0.33	0.60	0.67
DM digestibility, %	65.2	61.9	60.0	57.2	3.8	0.083	0.28	0.93

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).

[2]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.

[3]Significant effect of time ($P \leq 0.002$).

[4]Pre-weaning = d 1 until d 35 of the study; Weaning = d 36 until d 49 of the study; Post-weaning = d 50 until d 63 of the study; Overall = d 1 until d 63 of the study.

[5]Four point scale, where 1 = normal and 4 = diarrhea.

[6]Fecal score ≥ 3.

However, during the weaning period, calves fed starter mixtures containing CM tended to have smaller ADG than those fed starter mixtures containing SBM (P = 0.100). The MSB supplementation had no effect on weaning ADG (P = 0.75). Experimental treatments did not impact the pre-weaning and post-weaning G:F ratio ($P \geq 0.51$), but G:F was less during the weaning period (P = 0.018) and tended to be less ($P \leq 0.092$) in the overall study period when calves were fed starters containing CM. Gain to feed ratio was not affected by MSB supplementation ($P \geq 0.54$). Withers height did not differ between treatments during the study ($P \geq 0.60$). Fecal score was less or tended to be less during all periods of the study for calves fed starters containing CM ($P \leq 0.097$), implying firmer feces, compared to calves fed starter mixtures containing SBM. During the pre-weaning and post-weaning period, there was a tendency for a protein source × MSB interaction for fecal score ($P \leq 0.097$), with the greatest fecal score observed for TSBM, intermediate for TSBM-MSB and TCM-MSB, and the least for TCM. Supplementation of MSB did not impact fecal score during the pre-weaning, weaning, post-weaning periods, as well as during the whole study ($P \geq 0.35$). Number of days with diarrhea did not differ among treatments ($P \geq 0.33$).

Calves fed starters containing CM tended to have lesser post-weaning apparent total tract DM digestibility ($P = 0.083$; Table 4.3). However, these results should be interpreted with caution. For NDF and ADF, the calculated digestibility values were negative for some animals (data not shown). Negative NDF and ADF could indicate that calves consumed part of the wood shavings used as a bedding, potentially confounding this analysis. Thus, only results for DM digestibility are presented.

No protein source × MSB supplementation interaction effects was observed on any of the analyzed blood parameters ($P \geq 0.22$; Table 4.4). Protein source did not impact plasma glucose concentration ($P \geq 0.13$), serum IGF-1 concentration ($P \geq 0.31$), nor serum BHB concentration ($P \geq 0.41$). Serum insulin concentration on d 21 (pre-weaning) and d 42 (weaning) of the study were also not affected by the protein source, but a tendency (P = 0.090) for lesser insulin concentration was observed on d 63 (post-weaning) of the study for calves fed starters containing CM, compared with those fed starters with SBM. Plasma urea concentration tended to be greater for calves fed CM on d 21 (P = 0.087) and less on d 63 (P = 0.059) of the study, but was not affected by protein source on d 42 of the study (P = 0.80). Both plasma glucose and serum insulin concentrations were not affected by MSB supplementation ($P \geq 0.36$). Serum IGF-1 tended to be less on d 21 of the

Table 4.4. Concentration of selected parameters in the blood of calves in Study 1

Variable	Day of study	Treatment[1]				SEM	*P*-value[2]		
		TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS × MSB
Glucose, mg/dL	21	136.1	139.0	130.8	134.1	5.7	0.39	0.60	0.97
	42	131.3	142.6	129.5	125.3	6.3	0.13	0.58	0.22
	63	115.2	119.5	112.5	115.9	4.1	0.45	0.36	0.91
Insulin, µIU/mL	21	39.4	54.8	56.1	54.3	12.1	0.47	0.54	0.44
	42	33.3	40.3	53.4	41.1	9.2	0.27	0.78	0.31
	63	23.7	27.6	18.1	19.2	3.9	0.090	0.53	0.74
IGF[3]-1, ng/mL	21	41.3	31.4	42	35.8	4.6	0.59	0.099	0.69
	42	54.1	44.6	39.3	40.4	9.2	0.31	0.65	0.57
	63	102.5	97.2	88.8	88.4	21.4	0.52	0.87	0.89
BHB[4], mmol/L	21	0.046	0.069	0.041	0.057	0.010	0.41	0.059	0.73
	42	0.184	0.143	0.161	0.151	0.022	0.74	0.25	0.46
	63	0.408	0.377	0.423	0.380	0.052	0.85	0.47	0.91
Urea, mg/dL	21	14.9	14.1	16.8	15.7	1.0	0.087	0.36	0.84
	42	22.3	18.0	21.6	17.9	1.7	0.80	0.019	0.86
	63	22.8	18.7	18.8	15.6	2.0	0.059	0.061	0.81

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).

[2]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.

[3]IGF = insulin-like growth factor.

[4]BHB = β-hydroxybutyrate.

study when calves were supplemented with MSB ($P = 0.099$), but was not affected on d 42 nor d 63 of the study ($P \geq 0.65$). On the other hand, MSB supplementation tended to increase serum BHB concentration on d 21 of the study ($P = 0.059$) but had no effect on BHB concentration on d 42 and d 63 of the study ($P \geq 0.25$). Supplementation of MSB resulted in lesser plasma urea concentration on d 42 of the study ($P = 0.019$) and tended to have the same effect on d 63 of the study ($P = 0.061$).

For essential AA, plasma Met concentration tended to be greater for calves fed starter mixtures containing CM ($P = 0.068$; Table 4.5), whereas plasma Phe concentration was less ($P = 0.027$). A protein source × MSB interaction was observed for plasma Trp concentration ($P = 0.002$) with the greatest concentration observed for TSBM-MSB, while the other three treatments had significantly lesser plasma Trp concentration but were not different from each other. Starter mixtures containing MSB did not affect any essential AA concentrations in plasma ($P \geq 0.33$). Plasma concentration of Lys, Arg, His, Ile, Leu, Thr, Val, and total essential AA were not affected by the experimental treatments ($P \geq 0.12$). For non-essential AA, a protein source × MSB interaction was observed for plasma concentrations of hydroxyproline (**Hyp**), Gln, taurine (**Tau**), Pro, and α-aminobutyric acid (**AAB**) ($P \leq 0.041$), and tended to be significant for the total non-essential AA concentration ($P = 0.069$). Proline and AAB concentrations were the greatest for SBM-MSB, intermediate for TSBM and TCM, and the least for CM-MSB. For Gln, Hyp, and Tau, the Bonferroni post-hoc mean separation did not detect differences among means. However, similar to Pro and AAB, the numerically the greatest concentrations of Gln and Tau were observed for TSB-MSB and the least for TCM-MSB. Plasma Hyp concentration was the greatest for TSBM-MSB and TCM and the least for TSBM and TCM-MSB. Since only a tendency was observed for the total non-essential AA concentration, no mean separation was observed using Bonferroni analysis. However, similar to the individual AA described above, numerically the greatest values were observed for the TSBM-MSB treatment, followed by TCM, then TSBM and the least TCM-MSB. Feeding calves starters containing CM tended to result in greater Glu ($P = 0.069$) and Gly ($P = 0.066$) concentrations in plasma when compared with feeding starter mixtures containing SBM. Canola meal use in starter mixture also resulted in lesser concentration of plasma Asn, Gln, and Tau ($P \leq 0.028$), compared to SBM. Inclusion of MSB in starters tended to decrease plasma concentration of Ala and Tau ($P \leq 0.088$). Plasma concentrations of Asp, Cit, Hyp, Orn, Ser and Tyr did not differ between treatments ($P \geq 0.12$).

Table 4.5. Amino acids concentration in the blood of calves in Study 1[1]

Concentration, μmol/L	Treatments[2]				SEM	*P*-value[3]		
	TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS × MSB
Essential amino acid								
Met	15.4	15.6	20.5	18.9	2.2	0.068	0.75	0.68
Lys	81.2	99.8	84.9	75.6	8.6	0.25	0.59	0.12
Arg	148.8	155.9	142.9	121.6	14.7	0.19	0.64	0.35
His	49.0	55.0	57.4	49.7	5.0	0.76	0.87	0.18
Ile	93.8	94.7	87.1	82.7	8.1	0.26	0.83	0.75
Leu	124.0	125.6	111.4	106.1	10.2	0.13	0.86	0.74
Phe	61.6	62.7	54.9	48.4	4.4	0.027	0.55	0.40
Thr	78.6	86.6	88.9	84.0	10.2	0.71	0.88	0.54
Trp	30.8[b]	43.9[a]	28.3[b]	21.0[b]	2.9	0.001	0.33	0.002
Val	205.3	237.5	207.4	192.3	16.1	0.19	0.60	0.16
Total	895.8	972.8	882.4	805.9	70.2	0.21	0.99	0.29
Non-essential amino acid								
AAB[4]	6.44[ab]	10.45[a]	6.94[ab]	6.37[b]	0.98	0.070	0.086	0.026
Ala	164.0	158.9	177.0	145.1	10.5	0.97	0.088	0.20
Asn	66.9	77.9	60.9	46.4	7.9	0.028	0.82	0.12
Asp	11.4	7.2	11.6	13.1	1.9	0.12	0.48	0.14
Cit	68.3	64.4	61.6	66.1	5.2	0.64	0.96	0.44
Gln	265.6	381.8	278.7	231.5	23.4	0.008	0.16	0.002
Glu	71.4	71.4	88.8	82.1	7.3	0.069	0.65	0.65
Gly	389.6	432.7	481.8	450.8	30.9	0.066	0.83	0.20
Hyp[5]	38.5	45.2	45.8	36.0	2.8	0.70	0.57	0.006
Orn	78.6	88.8	76.2	70.1	7.6	0.18	0.80	0.30
Pro	70.2[ab]	78.9[a]	75.1[ab]	59.5[b]	4.8	0.12	0.46	0.016
Ser	111.7	74.2	110.3	117.6	15.4	0.19	0.34	0.16
Tau[6]	28.8	39.4	16.2	16.1	2.6	0.001	0.053	0.041
Tyr	51.1	55.2	51.9	48.0	5.0	0.53	0.98	0.43
Total	1431.4	1582.9	1550.4	1388.6	81.1	0.65	0.95	0.069

[1]In blood samples collected on d 63 of the study (post-weaning period).
[2]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).
[3]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.
[4]AAB = α-aminobutyric acid.
[5]Hyp = hydroxyproline.
[6]Tau = taurine.
[a,b]Means with uncommon superscripts differ ($P < 0.05$).

4.4.2 Study 2

The starters used were balanced for CP, as well as Lys and Met content (Table 4.6). Contents of NDF and ADF were numerically greater for the CM treatments than for SBM; whereas, starch was less for CM treatments, compared to SBM. Starters containing CM, when compared with starters containing SBM, had lesser contents of Arg (by 12.0%), Asp (by 35.8%), Glu with Gln (by 12.3%), Ile (by 13.41%), Phe (by 18.8%) and Ser (by 11.6%), and greater content of Cys (by 33.3%).

Protein source did not affect daily and cumulative MR intake, BW, ADG, G:F ratio, or withers height ($P \geq 0.23$; Table 4.7). A protein source × time interaction was observed for daily milk replacer intake ($P = 0.023$), which was a result of greater milk replacer intake by calves fed starters with CM during first days of the study when milk replacer volume was gradually increased to adapt calves to its intake. Daily starter intake ($P = 0.010$) during the overall study period and cumulative starter intake ($P = 0.031$) were greater for calves fed starters with CM compared to calves fed starters with SBM; however, starter intake was not affected by experimental treatments during the pre-weaning, weaning, and post-weaning periods ($P \geq 0.22$). Supplementation of MSB did not affect daily and cumulative MR intake, daily and cumulative starter intake, BW, ADG, and G:F ratio of calves ($P \geq 0.23$) but tended to decrease withers height ($P = 0.095$). However, protein source × MSB × time interaction was observed for ADG post-weaning (P = 0.047), due to TCM-MSB treatment having the least ADG in the first week post-weaning (0.808 kg/d vs. 1.199, 0.937 and 1.000 kg/d for TSBM, TSBM-MSB and TCM, respectively) and the greatest ADG in the second week post-weaning (1.347 kg/d vs. 0.932, 0.932 and 1.185 kg/d for TSBM, TSBM-MSB and TCM, respectively). Fecal score tended to be greater during the pre-weaning ($P = 0.077$), and was greater post-weaning ($P = 0.035$) and overall in the study ($P = 0.077$) for calves fed starters with SBM compared to calves fed starters with CM, but did not differ between those treatments during weaning period ($P = 0.11$). A tendency for protein source × MSB supplementation interaction was observed for days with diarrhea ($P = 0.069$). Numerically, the greatest number of days with diarrhea was observed for the TSBM-MSB treatment, intermediate for TCM and TSBM, and the least for TCM-MSB.

Table 4.6. Nutrient composition of pelleted starter mixtures used for Study 2

Variable	Treatment/Feed[1]				
	TSBM	TSBM-MSB	TCM	TCM-MSB	MR
DM, %	90.8 ± 0.2[2]	90.6 ± 0.1	90.8 ± 0.2	90.5 ± 0.1	95.3 ± 0.4
Ash, % DM	7.5 ± 0.1	8.0 ± 0.1	7.7 ± 0.1	7.8 ± 0.2	6.7 ± 0.1
OM, % DM	92.5 ± 0.1	92.1 ± 0.1	92.3 ± 0.1	92.2 ± 0.2	93.3 ± 0.1
CP, % DM	19.7 ± 0.6	20.5 ± 0.4	20.4 ± 0.1	20.3 ± 0.1	23.8 ± 0.1
EE[3], % DM	2.9 ± 0.4	3.1 ± 0.5	3.0 ± 0.0	3.2 ± 0.1	17.9 ± 0.2
NDF, % DM	13.3 ± 1.7	11.6 ± 0.6	17.8 ± 0.5	17.5 ± 0.2	-
ADF, % DM	4.9 ± 0.1	4.8 ± 0.4	9.6 ± 0.8	11.3 ± 0.4	-
Starch, % DM	44.9 ± 2.6	42.2 ± 0.9	37.7 ± 1.6	35.9 ± 0.7	-
Sugar, % DM	8.1 ± 0.3	8.4 ± 0.2	9.0 ± 0.6	8.1 ± 0.2	-
ME[4], Mcal/kg	3.11 ± 0.01	3.11 ± 0.02	3.11 ± 0.00	3.12 ± 0.01	4.66 ± 0.01
Amino acid, g/kg DM					
Ala	8.10 ± 0.22	8.17 ± 0.09	7.94 ± 0.33	7.80 ± 0.20	-
Arg	13.05 ± 0.75	13.19 ± 0.14	11.82 ± 0.64	11.60 ± 0.91	-
Asp	17.22 ± 0.85	18.44 ± 0.10	13.47 ± 0.06	12.79 ± 0.04	-
Cys	3.39 ± 0.14	3.40 ± 0.02	4.33 ± 0.01	4.72 ± 0.11	-
Glu+Gln	36.77 ± 0.36	37.31 ± 0.30	33.39 ± 0.45	32.59 ± 1.40	-
Gly	7.61 ± 0.24	7.52 ± 0.13	8.28 ± 0.26	8.23 ± 0.01	-
His	5.10 ± 0.36	5.04 ± 0.14	5.27 ± 0.17	5.21 ± 0.01	-
Ile	7.09 ± 0.02	7.20 ± 0.13	6.44 ± 0.06	6.16 ± 0.08	-
Leu	14.92 ± 0.48	14.78 ± 0.18	13.79 ± 0.58	13.59 ± 0.09	-
Lys	8.54 ± 0.39	8.63 ± 0.12	8.21 ± 0.26	8.13 ± 0.11	-
Met	3.98 ± 0.05	4.11 ± 0.11	4.22 ± 0.08	3.81 ± 0.10	-
Phe	9.04 ± 0.41	8.91 ± 0.01	8.01 ± 0.06	7.22 ± 0.05	-
Pro	11.59 ± 0.26	12.75 ± 0.79	12.03 ± 0.21	12.14 ± 0.05	-
Ser	7.87 ± 0.61	8.56 ± 0.14	7.35 ± 0.26	7.37 ± 0.29	-
Thr	6.00 ± 0.16	6.47 ± 0.11	6.44 ± 0.07	6.88 ± 0.24	-
Tyr	5.98 ± 0.41	5.95 ± 0.02	5.73 ± 0.39	5.53 ± 0.21	-
Val	9.24 ± 0.19	9.01 ± 0.16	8.90 ± 0.12	8.82 ± 0.21	-

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM); MR = milk replacer.

[2]mean ± SD.

[3]EE = ether extract.

[4]ME = metabolizable energy, calculated based on (NRC, 2001) for the starters as: (1.01 × DE -0.45) + 0.0046(EE% – 3), where DE = digestible energy calculated as (0.057 CP% + 0.094 EE% + 0.0415 (100-CP% - EE% - ash%)) × 0.82; and for the milk replacer as: 0.96 × (0.97 × (0.057 CP% + 0.092 EE% + 0.0395 (100 – CP% - EE% - ash%))).

Table 4.7. Milk replacer and starter intake, BW, ADG, feed efficiency and fecal fluidity of calves in Study 2

Variable	Treatment[1]				SEM	*P*-value[2]		
	TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS×MSB
Milk replacer intake, g DM/d[3,4]	692.4	685.7	694.1	695.8	5.1	0.23	0.62	0.40
Cumulative milk replacer intake, kg	34.2	33.7	34.1	34.2	0.3	0.32	0.36	0.13
Starter intake, g DM/ day								
Pre-weaning[3,5]	97.8	99.0	105.4	124.8	23.4	0.22	0.45	0.50
Weaning[3]	880.4	793.3	898.3	852.2	90.7	0.56	0.36	0.73
Post-weaning[3]	2105.7	2162.2	2257.5	2142.0	173.2	0.71	0.87	0.62
Overall[3]	715.3	664.7	757.2	745.1	30.6	0.010	0.18	0.41
Cumulative starter intake, kg DM								
Pre-weaning	3.52	3.15	3.3	3.64	0.90	0.76	0.98	0.44
Weaning	11.91	10.5	12.16	11.38	1.07	0.60	0.31	0.77
Post-weaning	29.48	27.54	31.6	30.74	1.20	0.031	0.25	0.66
Overall	44.61	41.35	47.22	46.15	2.78	0.17	0.42	0.68
Initial BW, kg	43.6	43.2	43.2	43.2	1.3	0.87	0.83	0.85
Final BW, kg	86.3	82.5	85.7	85.3	2.3	0.61	0.34	0.43
Average daily gain, kg/d								
Pre-weaning[3]	0.581	0.527	0.544	0.566	0.035	0.98	0.60	0.21
Weaning[3]	0.600	0.560	0.620	0.640	0.077	0.46	0.93	0.61
Post-weaning[3,6]	1.003	0.926	1.072	0.999	0.071	0.32	0.30	0.98
Overall[3]	0.678	0.622	0.676	0.677	0.031	0.37	0.36	0.34
Withers height[3], m	0.822	0.811	0.82	0.813	0.005	0.97	0.095	0.71
Feed efficiency, kg gain/kg DM								
Pre-weaning	0.643	0.576	0.600	0.590	0.036	0.62	0.23	0.47
Weaning	0.482	0.428	0.481	0.560	0.074	0.16	0.80	0.27
Post-weaning	0.477	0.491	0.468	0.439	0.045	0.43	0.85	0.66
Overall	0.540	0.515	0.524	0.52	0.017	0.72	0.34	0.57
Fecal score[7]								
Pre-weaning[3]	1.35	1.45	1.33	1.31	0.05	0.077	0.42	0.17
Weaning	1.04	1.08	1.01	1.02	0.03	0.11	0.34	0.46
Post-weaning	1.09	1.09	1.03	1.02	0.03	0.035	0.86	0.92
Overall[3]	1.21	1.28	1.19	1.17	0.03	0.013	0.40	0.13
Days with diarrhea[8]	3.3	4.2	3.4	2.1	0.7	0.098	0.57	0.069

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).

[2]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.

[3]Significant effect of time ($P \leq 0.032$).

[4]Significant PS × day interaction ($P = 0.023$).

[5]Pre-weaning = d 1 of the study (9.1 ± 0.8 d of age) to d 35 of study; Weaning = d 36 of the study to d 49; Post-weaning = d 50 of study to d 63; Overall = d 1 of study to d 63.

[6]Significant PS × MSB × day interaction ($P = 0.047$).

[7]Four point scale, where 1 = normal and 4 = diarrhea.

[8]Fecal score ≤ 3.

4.5 Discussion

With few interactions observed between protein source and MSB effects, the hypothesis was only partially confirmed as no major improvements were achieved for calf performance when TCM-MSB treatment was used. In Study 1, pre-weaning starter intake and weaning ADG of calves, and feed efficiency during the overall study period were negatively affected by CM use in the starter, and incorporation of MSB did not fully reverse those negative effects. Such negative consequences of CM use were not observed in Study 2, but MSB supplementation also did not improve the performance of calves. Nevertheless, in both studies, overall starter intake and ADG of calves were comparable regardless of protein source used in starter mixture. Thus, CM use in starter mixtures for calves as the main source of protein may result in similar but not improved performance of calves relative to SBM, even when MSB is supplemented in the feed. However, decreased G:F ratio may be expected when CM fully replaces SBM.

Prior to more in depth discussion, specificity and limitations of the current experimental model have to be addressed. Starter mixtures were formulated to be similar for CP, Lys, and Met content, but not for NDF, ADF, and starch content. The aim of equalizing Lys and Met contents between starters was to limit the number of confounding variables in the current studies, as well as to increase probability of similar performance of calves fed starter mixtures containing CM or SBM. Starter mixtures containing CM were expected to contain less Lys, due to lesser Lys concentration in CM than in SBM (NRC, 2001; Paz et al., 2014), which could be an important factor limiting efficiency of CM use in calf starter mixtures, particularly pre-weaning. In fact, during the pre-weaning period most of the protein consumed with solid feed flows to the small intestine (Lallès et al., 1990). Thus, AA composition of solid feed may affect growth when the forestomach is not yet developed. However, based on preliminary AA analysis in components of starters, Lys content in starter mixtures was comparable between treatments (data not shown). Therefore, only unprotected Met was added to starter mixtures containing SBM, which could actually increase efficiency of SBM use in starter mixtures. However, even though Met was supplemented in starters containing SBM and Met intake post-weaning was greater for SBM treatments (due to numerically greater starter intake), plasma Met tended to be greater for treatments receiving CM in starter mixtures. Although this may indicate that CM provided Met in excess or delivery of other AA (e.g., Lys) that limited Met use by calves, supplemental unprotected

Met is rapidly degraded in the rumen (Mangan, 1972; Chalupa, 1975). Moreover, Met flow into intestine has the most apparent impact on its plasma concentration (Papas et al., 1974; Chalupa, 1975). As such, the addition of Met to starters containing SBM rather did not impact the calf's AA supply directly, but was most likely used by the ruminal microbes, which in turn have a considerably stable AA composition (Quigley et al., 1985). Furthermore, additional Lys supplementation in starter mixtures containing CM as a main source of protein had no impact on performance of calves (Krupa, 2018), allowing us to conclude that some differences in Lys content between treatments were also of minor importance. Therefore, elimination of differences in Lys and Met concentration between diets was rather a precaution and had only minor, if any, impact on the results of the study. To further support the latter discussion, energy intake has more profound impact on ADG than protein intake (Hill et al., 2016) and when starch and energy content is equalized between starters, CM inclusion in order to replace up to 60% of SBM protein had no substantial impact on performance of calves (Chapter 6). Within the multiple experiments comparing CM and SBM use as protein sources in calf starters (Fiems et al., 1985; Khorasani et al., 1990; Hadam et al., 2016), including the present studies, CM partially replaced a starch source within the starter mixture, to maintain the isonitrogenous state of the diet. As such, the possible reduction in the starch content when CM is used as source of protein in starter can be also an important factor contributing to the observed differences between treatments.

4.5.1 Effect of Protein Source

When CM was previously compared with SBM as a protein source in starter mixtures for calves, frequently the results varied. Some studies found that when calves were fed starter mixture containing CM instead of SBM, feed efficiency (Hadam et al., 2016; Terré et al., 2016) and ADG (Hadam et al., 2016) were reduced. These responses are not consistent within the literature, as Fiems et al. (1985) and Terré et al. (2016) did not report differences in starter intake and ADG when CM was used in starter mixtures instead of SBM. As mentioned previously, these differing responses can possibly be an effect of the inevitable change in nutrient composition of the starters as CM inclusion replaces the starch source to a greater extent than SBM. The studies mentioned above varied in level of CM inclusion, 10 and 20% in Fiems et al. (1985), 15% in Terré et al. (2016), and 16.5 and 35% in Hadam et al. (2016), whereas in the current study CM was present at

on average 34.8% DM. These considerable differences in the inclusion rates could have contributed to disparity of observed results among different publications. An older publication by Schingoethe et al. (1974), utilizing CM-predecessor, rapeseed meal, reported a minor decrease in starter intake of calves pre-weaning, with no effect on feed efficiency when diets were balanced for fiber content. Similarly, Fisher (1980) did not report any negative impact of CM use in starter diet for calves when a low fiber variety of CM was used as a source of protein, compared to SBM. These two studies provide examples of how the concentrations of other nutrients in starter, like starch or fiber, can impact on the response of calves. Furthermore, the remainder of starter composition is also highly variable between studies, with major starch sources alternating between barley grain, corn grain and oat, which also may likely contribute to different results between studies.

Additionally, results of the two studies differed. Slight differences between the experimental design of Study 1 and 2 might have contributed to these differences. Firstly, Study 1 was conducted on bull calves, whereas Study 2 on heifer calves. Bull calves can gain BW at a faster rate than heifer calves (Koch et al., 1973; Ware et al., 2015). Additionally, greater frame size can be observed for bull calves when compared with heifers (Hopkins, 1997); however, these effects are not repeatable among various experiments (Tahmasbi et al., 2014; Ware et al., 2015). As such, potential differences originating from using both bulls and heifers in two consecutive experiments need to be acknowledged. Secondly, the weaning phase was 1 wk longer in Study 2 compared with Study 1. Thirdly, between the two studies different MR were used that additionally numerically differed in energy content. Finally, Study 1 was conducted over winter, while Study 2 over summer. The difference in seasons likely resulted in over two times greater pre-weaning starter intake by bull calves in Study 1, compared to the intake by heifer calves in Study 2. Although I do not possess precise data about the temperatures that calves were exposed to, it can be speculated that bull calves from Study 1 might have more readily consumed starter earlier in life to meet their energy demands, since temperatures below 15°C already can result in increased demand for energy in calves (NRC, 2001). However, increased requirement for energy during winter and associated increased starter intake pre-weaning could have led to increased consumption of antinutritional factors present in CM during a period of life when calf's GIT is the most susceptible to their influence. This could be a reason for lesser pre-weaning starter mixture intake when CM was used as a source of protein in Study 1. Hadam et al. (2016) observed that full

replacement of SBM by CM in starter mixture for calves has a negative impact on ADG and feed efficiency mostly in the first 4 to 5 wk of age. On the other hand, with the longer weaning period in Study 2, there was a greater chance for the GIT to adapt to the efficient digestion of potentially less digestible feed. To support that, the weaning period in Study 1 was shorter than in Study 2 and a tendency for decreased DM digestibility after weaning was observed when CM was used as a protein source in starter mixture, consistent with results of other studies (Khorasani et al., 1990). Lesser efficiency of nutrient digestion likely also contributed to the tendency toward decreased ADG in bull calves fed starter mixtures with CM during the weaning period, compared to those fed starter mixtures with SBM. It can be speculated that the delay of intake for starters containing CM [e.g. by feeding more milk or MR; (Khan et al., 2011b)], and extending transition period from milk or MR to solid feed, may improve efficiency of CM use in starter mixture for calves. Thus, in a more traditional rearing systems (low to moderate amounts of milk, early weaning) some apparent negative consequences can be expected when CM is used to fully replace SBM, as reported by others (Hadam et al., 2016; Terré et al., 2016).

As indicated by results of Study 1, full replacement of SBM by CM may have a negative impact on the starter intake of calves. It should be noted that this decrease in starter intake happened even though glycerol was present in the starters, which can enhance the palatability of the CM-containing starters (Chapter 3). However, cumulative starter intake during the post-weaning period in Study 2 was actually greater when calves were fed starters containing CM than SBM. However, no consistent trend was observed across the two studies that would clarify the difference between the CM and SBM, likely due to differences between studies. Additionally, changes in starter intake did not translate into better G:F ratio nor increased growth characteristics of calves. In general, greater fecal scores were observed in calves fed starters containing SBM, in both studies, which indicated that those calves were more prone to experiencing more liquid feces, particularly at and after weaning. When considering the overall period for Study 2, there was a tendency for a protein source × day interaction ($P = 0.094$, data not showed), with calves fed SBM showing more liquid feces during early days of the experiment than calves fed CM. Hadam et al. (2016) observed that post-weaning fecal fluidity was decreased when calves were fed starter mixture containing CM instead of SBM, although they observed an opposite effect in the pre-weaning period. Since CM was added into the starters at a greater rate than SBM to achieve the same CP content, inclusion of barley and thus content of starch were greater in starters containing

SBM. Greater intake of starch by calves fed starters with SBM likely resulted in more starch escaping digestion within the rumen and small intestine and entering the hindgut (Gressley et al., 2011). Increased inflow of starch into the hindgut can lead to hindgut acidosis, with diarrhea being a common symptom (Gressley et al., 2011). Furthermore, more bloat was observed in calves that were consuming starters with SBM. Greater starch content in starter mixture in combination with glycerol (5.07% DM of starter in the current study), which is highly fermentable in the rumen (Rémond et al., 1993), could have contributed to the bloat issues. This may indicate that SBM use in starter mixtures for calves may result in some disadvantages, and CM could be used to at least partially prevent them, e.g. by partial replacement of SBM by CM (Hadam et al., 2016, Chapter 6).

It has been shown that replacement of SBM with CM in the diet leads to decreased digestibility of nutrients in calves (Khorasani et al., 1990), lambs (Matras et al., 1990), and feedlot cattle (Zinn, 1993), but not necessarily in dairy cows (Paula et al., 2018). The results of nutrient digestibility are consistent with results of Khorasani et al. (1990). Specifically, I also observed a tendency for decreased digestibility of DM after weaning when calves consumed starter mixtures with CM. The decrease in digestibility might be due to greater concentration of fiber in starters containing CM, which is less digestible, but also the negative impact of antinutritional factors other than glucosinolates or erucic acid [which are present in small concentrations in CM (Adewole et al., 2016)], such as tannins or phytic acid, on GIT development and function in calves.

In dairy cows, feeding diets with CM results, compared to other protein sources, in changes in plasma AA concentration, likely as a result of changes in the composition of AA flowing out of the rumen, post-ruminal protein digestion, and AA absorption or metabolism. Specifically, based on meta-analysis of available data (Martineau et al., 2014), it was concluded that feeding CM to cows results in increased total plasma AA, mostly due to increased essential plasma AA concentration, whereas concentrations of plasma urea and milk urea were actually decreased, likely due to more efficient whole-body N utilization (Martineau et al., 2014). In calves, around 20% of the protein flowing into the small intestine is of bacterial origin when calves are fed milk, and it increases to about 60% post-weaning (Lallès et al., 1990). Furthermore, the microbial protein flowing out of the rumen in calves was shown to have relatively stable AA composition, independently on the source of protein in the diet (Quigley et al., 1985). During present study, differences were observed in plasma AA composition between treatments, even

though protein from solid feed account only 10% of intestinal protein flow after weaning (Lallès et al., 1990). However, results of the current study did not show the same increase in essential AA when feeding CM in starter mixtures, as did Martineau et al. (2014) when CM was fed in diets for dairy cows. Most of observed differences between protein sources occurred in the non-essential plasma AA concentrations (specifically plasma Phe, Trp, AAB, Asn, Gln and Tau), which were generally greater for calves fed starters with SBM. Only plasma Met, Glu, and Gly were increased when CM was fed in the starter mixture. When highly concentrated diets containing CM are fed to ruminants, including calves, intestinal digestibility of CP is reduced, although CP ruminal outflow may be increased (Khorasani et al., 1990; Matras et al., 1990; Zinn, 1993). This reduced intestinal digestion and absorption of AA, as also shown through reduced gene expression of peptide transporter *PEPT2* and AA transporter *ATB0* in the intestinal epithelium (Chapter 5), likely contributed to decrease of some plasma AA in calves fed starters with CM. Such an impact does not apply to Met (Khorasani et al., 1990), which corresponds to its greater plasma concentration in calves fed starter mixture with CM in the current study. However, despite likely decreased efficiency of post-ruminal CP digestion in calves fed starters with CM, calves' growth performance was not negatively affected, indicating that calves could compensate for this lesser nutrient digestion by increasing efficiency of AA use. On 63 d of the study, CM use in starter mixtures tended to result in reduced plasma urea concentration, which is a similar observation to that of Martineau et al. (2014) in dairy cows. This decreased plasma urea concentration could be a result of lesser ruminal ammonia in calves fed starters with CM (Chapter 5) but also can be interpreted as more efficient AA use for metabolic processes (Martineau et al., 2014).

Feeding starter mixtures with SBM resulted in greater plasma Gln concentration in calves, while feeding starter mixtures with CM resulted in greater plasma Glu concentration, although at roughly three-fold decreased levels than Gln. Some absorbed AA can be used by enteric tissues, with Gln and Glu having a negative flux across the GIT tissue (Koeln et al., 1993). Glutamate is a precursor for production of Gln and both can be used as energy substrates by enterocytes (Reeds and Burrin, 2001), with Gln being a preferred source (Bertolo and Burrin, 2008). Starter mixtures containing CM as a main source of protein contained less Glu and Gln. Since it is estimated that in humans over 80% of luminal Glu will be utilized by enteric tissue and minimal quantities are transported back from blood (Bertolo and Burrin, 2008), increased Glu concentration in the blood is likely due to further metabolic processes, including being the indirect product of the

tricarboxylic acid cycle (Boisen et al., 2000). Considering increased tissue size of the small intestine in calves fed CM starters (Chapter 5), the greater tissue mass might have contributed to greater utilization of Gln and therefore lesser concentration in the blood.

On d 63 of the study, a tendency was observed for decreased plasma insulin concentration in calves fed CM, while at the same time no difference was observed for plasma glucose concentration. Lesser insulin concentration in calves fed starter with CM was likely a result of decreased intake of starch and as a result decreased supply of glucose. Use of CM as an alternative protein source to SBM in starter mixtures and associated other differences in starter mixture composition likely had substantial impacts on metabolic processes in calves, especially after weaning. To sum up, protein source used in starter mixture had substantial impact on metabolic process in calves, especially protein and AA metabolism.

4.5.2 Effect of MSB Supplementation

Based on the previous research, I was expecting that MSB supplementation would have more profound effect on performance of calves than I observed, and that this effect would be apparent when CM was used in the starter mixture. The effect of butyrate previously observed includes increased starter intake (Górka et al., 2009; Ślusarczyk et al., 2010; McCurdy et al., 2019) and ADG of calves (Górka et al., 2009; Ślusarczyk et al., 2010; McCurdy et al., 2019; Rice et al., 2019), and less incidence of diarrhea (Górka et al., 2011a). Across the two studies I observed a tendency for greater pre-weaning starter intake, lesser withers height, greater pre-weaning plasma BHB concentration, and lesser weaning and post-weaning plasma urea concentration when MSB was supplemented. Greater plasma BHB was likely a result of greater metabolic capabilities of the ruminal tissue of calves consuming starters supplemented with MSB and not a direct effect of MSB supplementation, given the low dose (e.g., conversion of supplemental butyrate to BHB by ruminal epithelium). Rice et al. (2019) observed greater blood BHB when butyrate was supplemented in solid feed for calves, although such differences were not observed by McCurdy et al. (2019). A positive impact of MSB supplementation on starter mixture intake pre-weaning and on ruminal epithelium development (Chapter 5) did not translate into greater starter intake in subsequent periods of the present study. The fact that supplementation of MSB decreased plasma

urea concentration at and after weaning was surprising. The present results differ from those of Rice et al. (2019), as they did not observe an effect of butyrate supplementation in solid feed on plasma urea nitrogen concentration. However, Rice et al. (2019) utilized an unprotected source of butyrate, that would be almost entirely released and absorbed in the rumen. In the current studies, a protected form of butyrate was used, which can impact not only the rumen, but also regions of the intestine.

Previous research showed that calves fed starter mixtures with MSB had less incidence of diarrhea, more solid fecal consistency, and fewer electrolyte treatments pre-weaning (Górka et al., 2011a), none of which were observed within the present studies. The mechanism behind the MSB effect on fecal fluidity improvement was suspected to involve an increased mitotic index and decreased apoptosis of GIT epithelium due to butyrate action, and thus enhanced GIT epithelium functions (Mentschel et al., 2001; Górka et al., 2014). Butyrate can also affect the barrier function of the intestinal epithelium, by up-regulation of genes responsible for the tight junctions (Wang et al., 2012). However, a recent publication by Rice et al. (2019) observed another possible mode of action. In that study, supplementing calves with sodium butyrate decreased the coccidia count in feces. In both of the current studies, calves received an oral coccidiostat on d 15 of the study as a preventative measure, which could have diminished or eliminated the MSB response on fecal score and number of days with diarrhea.

4.5.3 Interactions Between Protein Sources and MSB Supplementation

Considerably more interactions were expected between the experimental factors than were observed during the studies. Of the few observed, the plasma AA concentrations were the most often affected by protein source × MSB interactions. This included plasma Trp, AAB, Gln, Hyp, Pro, Tau, and total non-essential AA. For all these AA, the greatest concentrations were observed for TSBM-MSB, and the lowest for TCM-MSB. Microencapsulated sodium butyrate supplementation increased activity of aminopeptidase A and N in distal sections of small intestine, independently of the protein source that was used in the starter mixture (Chapter 5). Butyrate supplementation was also shown to increase pancreatic enzyme secretion (Guilloteau et al., 2010b). Although no data on protein digestibility is available from current studies, it was likely less in calves fed starter mixtures containing CM, compared to those fed starter mixtures

containing SBM [based on greater total tract DM digestibility for those fed SBM, as well as the results of the study of Khorasani et al. (1990)]. It can be speculated that MSB supplementation, enhanced protein digestion, especially when it was combined with SBM in the starter mixture, which resulted in increased plasma concentrations of AA. This may indicate that the increased brush border enzyme activity observed when MSB was used in the starter was not sufficient to offset the potentially less digestible protein in CM (Chapter 5). Greater expression of peptide transporter *PEPT2* and AA transporter *ATB0* in the intestinal epithelium was observed when CM was used in the starter mixture (Chapter 5). This may explain the rather minor impact of MSB supplementation on growth performance of calves fed starter mixture with CM and lack of interaction between those factors; however, the impact on growth of calves was also minor when SBM was used in starter mixture and limited mostly to the pre-weaning (greater starter mixture intake) and weaning period (tendency for greater ADG). In Study 1, a tendency for a treatment interaction was observed for the overall daily starter intake with TCM-MSB resulting in the greatest intakes, followed by TSBM, TSBM-MSB and TCM resulting in the least. The tendency for the interaction might suggest that starter mixture containing CM was less willingly consumed than SBM, and MSB supplementation might offset that protein source effect.

Additionally, post-weaning fecal score during Study 1 was affected by an interaction of experimental treatments, with TSBM resulting in the greatest values and TCM resulting in the least values, indicating CM might be beneficial for the fecal fluidity of the calves. A similar tendency was observed in Study 2 for number of days with diarrhea, where TSBM resulted in the greatest values, and TCM-MSB resulting in the least, further suggesting that combination of CM with MSB might positively influence the fecal fluidity of calves and reduce number of incidence of diarrhea. Canola meal can in some aspects positively influence the development of the small intestine (Chapter 5) and possibly in combination with MSB's ability to stimulate mitosis and decrease apoptosis (Górka et al., 2014), it can help stabilize the permeability of the gut to help minimize the amount of scouring.

4.6 Conclusions

The combination of CM and MSB in starter mixtures did not result in the hypothesized improved growth performance of calves, compared to feeding starter mixtures with SBM, only partially confirming the hypothesis. Furthermore, results suggest that the use of CM in starter mixture as a main source of protein might negatively affect pre-weaning starter intake and weaning ADG, and overall G:F ratio, while SBM use might negatively affect fecal score. Although the negative CM impact was not universally observed across the two studies, with CM increasing starter intake and reducing the number of days with diarrhea in one of the studies. Complete replacement of SBM with CM, though likely to result in comparable performance, should carry further considerations of possible impacts on feed efficiency and nitrogen metabolism. Use of supplemental MSB in calf starters had little effect on performance, most notably increasing pre-weaning starter intake.

5 CANOLA MEAL OR SOYBEAN MEAL AS PROTEIN SOURCE, AND THE EFFECT OF MICROENCAPSULATED SODIUM BUTYRATE SUPPLEMENTATION IN PELLETED STARTER MIXTURE FOR DAIRY CALVES. II. THE DEVELOPMENT OF THE GASTROINTESTINAL TRACT

To allow a more in-depth insight into the differences between the CM and SBM as protein sources for calves, evaluation of GIT development for bull calves from Study 1 in Chapter 4 was conducted. Considering that processing of CM can have an impact on GIT development, as shown in Chapter 3 with heat-treatment of CM, the next step was to assess the differences in GIT development of calves fed different protein sources, either CM or SBM. Microencapsulated sodium butyrate was tested as an additional factor to evaluate its effect on GIT development.

5.1 Abstract

The aim of this study was to assess the effect of protein source, either soybean meal (**SBM**) or canola meal (**CM**), and microencapsulated sodium butyrate (**MSB**) supplementation in a pelleted starter mixture on the development of the gastrointestinal tract (**GIT**) in dairy calves. Twenty-eight bull calves (8.7 ± 0.8 d of age and 43.0 ± 4.4 kg; mean ± SD) were assigned to one of four treatments in a 2 × 2 factorial arrangement: SBM as a main source of protein without or with MSB or CM without or with MSB. Calves were fed starters *ad libitum* and exposed to a gradual weaning program with weaning taking place on 51.7 ± 0.8 d of age. Calves were observed for additional 3 wk after weaning and killed on d 72.1 ± 0.9 of age after which the GIT was dissected. Morphometric measurements were recorded and samples for ruminal fermentation, histology, gene expression, and brush border enzyme activities were collected. Canola meal use in the starter mixture increased abomasal tissue weight, jejunal tissue weight and length and the mRNA expression of *MCT4* and *GPR43* in the ruminal epithelium, and decreased ruminal ammonia and mRNA expression of *PEPT2* and *ATB0+* in the proximal small intestine and ileum, respectively. On the other hand, MSB inclusion in the starter mixture decreased ruminal papillae length, ruminal epithelial surface area and ruminal epithelium dry weight, while increasing mRNA expression of *MCT1* in ruminal epithelia. Reduced ruminal surface area due to MSB

supplementation was the most apparent when MSB was combined with CM in the starter mixture. Additionally, MSB supplementation decreased thickness of omasal epithelium, omasal epithelium living strata and stratum corneum, and increased duodenal and ileal aminopeptidase A enzymatic activity and ileal aminopeptidase N enzymatic activity. Overall, CM might increase growth of the GIT of calves, particularly the small intestine, but may negatively affect intestinal epithelium function and peptide and AA absorption. Supplementation of MSB did not impact the GIT development and might have negative effect on ruminal and omasal epithelium development, particularly when combined in a starter mixture with CM.

5.2 Introduction

Weaning is a challenging time in the life of calves, as reliance on liquid feeds for energy, proteins, vitamins and minerals is diminished, and digestion of solid feeds has to meet nutritional requirements. In order to ensure efficient digestion of solid feed after weaning, the gastrointestinal tract (**GIT**) must undergo major developmental changes. Important changes must occur during rumen development, including increase of ruminal volume, development of ruminal papillae and establishing of ruminal fermentation (Tamate et al., 1962; Connor et al., 2013). Most of those changes are driven by solid feed intake, as calves only consuming milk or milk replacer (**MR**) do not develop papillae or increase the size of the rumen to the same extent as calves consuming solid feed (Tamate et al., 1962; Connor et al., 2013). However, other regions of GIT develop prior to and after weaning (Baldwin et al., 2004). This includes substantial developmental changes of the small intestine, an important site of feed digestion and absorption in calves, especially prior to weaning (Steele et al., 2016).

Soybean meal (**SBM**) is a commonly used protein source in starter mixtures for dairy calves. While canola meal (**CM**) is a popular protein source for mature dairy cows (Huhtanen et al., 2011), it is not often considered for use in dairy calf rations. This is due to variable results of studies comparing CM with SBM use (Fiems et al., 1985; Khorasani et al., 1990; Miller-Cushon et al., 2014b; Hadam et al., 2016). Besides relatively high fiber content, which may negatively affect total tract nutrient digestibility and thus calves' growth performance, CM contains various antinutritional factors (e.g. tannins, phytic acid, sinapines), that are known to challenge the GIT, particularly the small intestine (Bell, 1993). However, the effect of using CM in calf diets has mostly been analyzed from a performance perspective, by assessing feed intake, growth, feed efficiency, feeding behaviour, or health standpoints (Fiems et al., 1985; Miller-Cushon et al., 2014b; Hadam et al., 2016). There is currently limited research characterizing the impact of CM use in calf starters on development of the GIT.

From the products arising during ruminal fermentation, butyrate has the greatest impact on the development of the ruminal papillae (Sander et al., 1959; Mentschel et al., 2001). Butyrate supplementation in MR or starter mixtures for dairy calves has been shown to increase ruminal papillae length and width, as well as ruminal weight (Górka et al., 2011a; Górka et al., 2014). However, it is not only rumen development that is stimulated from supplementary butyrate. When

supplemented in a microencapsulated form (i.e. embedded in triglyceride matrix), which prevents active substance from being fully released in the stomach (rumen, omasum and abomasum), butyrate can be delivered to the intestine (Górka et al., 2018a). Supplementation with microencapsulated sodium butyrate (**MSB**) in a starter mixture has been shown to increase villi height and weight of the intestine, as well as to increase the mitotic index and decrease the apoptotic index of the jejunal epithelium of calves (Górka et al., 2014). As such, supplementation of MSB can stimulate the development of the entire GIT in dairy calves.

The hypothesis of this study was that feeding a starter mixture containing CM and MSB will prevent potential negative impact of CM on calf's GIT development, and thus will result in at least similar or improved development of the GIT to that observed in calves fed starter mixture containing SBM. This hypothesis was tested while Lys and Met concentration in starter mixtures were equalized among treatments. The objective of the study was to compare the effects of feeding calves starter mixtures containing SBM or CM as the main protein source for calves on rumen and small intestine development, as well as establishing whether MSB supplementation will enhance GIT development.

5.3 Materials and Methods

The study was conducted on a commercial dairy farm (Top Farms Głubczyce, Głubczyce, Poland), according to the animal care protocol approved by the Local Institutional Animal Care and Use Committee (protocol No. 211/2015), and it took place from January until April 2016.

5.3.1 Animals, Housing and Feeding Regimen

A detailed description of experimental design, housing and feeding of calves is presented elsewhere (Chapter 4, Study 1). Briefly, 28 bull Holstein calves (8.7 ± 0.8 d of age and 43.0 ± 4.4 kg; mean ± SD) were allocated to four treatments in a 2 × 2 factorial treatment arrangement (7 calves/treatment) and fed a pelleted starter mixture containing SBM as a main source of protein without (**TSBM**) or with MSB (**TSBM-MSB**), or CM as a main source of protein without (**TCM**) or with MSB (**TCM-MSB**). Starters were formulated to be isonitrogenous and similar for Lys and Met content, the most limiting AA for growth of pre-weaned calves (Abe et al., 1998), but differed

in starch, fiber, and energy content. Lysine and Met concentration were similar among treatments to limit the number of confounding factors. Microencapsulated sodium butyrate (BIOLEK Sp. z o.o., Macierzysz, Ożarów Mazowiecki, Polska) contained 30% of sodium butyrate and 70% of triglyceride matrix and was included in starter mixtures at 0.33% DM in exchange for barley. Calves were sourced from two commercial dairy farms belonging to one operator (Top Farms Głubczyce, Głubczyce, Poland) and were transferred from the place of birth to the calf barn between 8 to 10 (8.7 ± 0.8) d of age. The transfer occurred twice a week (Monday and Thursday) and each week 4 calves were allocated to the study (one calf per treatment), resulting in 7 blocks of calves. During the study period, calves were fed 3 L of commercial MR (Polmass Milk Red Full Instant, Polmass S.A., Bydgoszcz, Poland) twice daily (0730 h and 1700 h) until d 36 of the study when number of feedings was limited to one per day (0730 h). Weaning occurred on d 43 of the study (51.7 ± 0.8 d of age) and calves remained in the study for additional 3 wk. Starter mixtures were fed free of choice throughout whole study period.

5.3.2 Gastrointestinal Tract Dissection and Sample Collection

Three weeks after weaning (72.1 ± 0.9 d of age), two times per week (Monday and Wednesday), calves were transported at 0830 h to a nearby slaughterhouse (approximately 8 km), where they were killed via captive bolt stunning, followed by exsanguination. The first calf each day was killed at 0900 h and as soon as the dissection of the GIT was completed, the next calf was killed. Within each day, the sequence of killing was randomized among treatments.

Right after killing, the whole GIT was removed and dissected. The reticulorumen was weighed, cut open through the dorsal sac, and digesta was deposited into a designated container. Ruminal tissue samples were collected from the cranial ventral sac. Using sterile equipment, four small pieces (2 × 2 cm) of whole ruminal tissue were taken and rinsed five times with ice-cold sterile saline. The ruminal epithelium was then manually separated from the muscle layer. Small pieces of the epithelium were transferred into 2-mL test tubes with 1.8 mL of RNAlater (Applied Biosystems, Waltham, MA), stored for 24 h at 4°C, and then were frozen at -20°C. Additionally, two whole-thickness tissue samples were taken from the ventral sac. One 4 × 4 cm and one 2 × 2 cm tissue samples were rinsed thoroughly with tap water, gently blotted using paper towel and

preserved in 50 mL of 10% neutral buffered formalin solution (Sigma-Aldrich, St. Louis, MO). After 24-h storage in formalin, samples were transferred into 50 mL of 70% ethanol and stored until further processing for histological measurements. The remaining ruminal tissue was rinsed repeatedly with tap water, pressed by hand, and weighed. Then, two 5 × 5 cm whole tissue samples from the cranial sac of the rumen were taken, stored in plastic bag and frozen on dry ice for subsequent dry tissue content measurements. It should be noted that the tissue sample weights collected for histology and gene expression are not included in the empty weight measurements for rumen and following GIT regions, as time sensitivity of gene expression analysis and integrity of the samples for histology were prioritized during the sampling procedure.

The omasum was weighed with the digesta and subsequently cut open. Tissue samples were collected from five large-order omasal laminae (from the tip to the bottom, 2 cm thick), and treated as the ruminal samples for histology, described above.

The abomasum was weighed with digesta, cut open, and the digesta were removed and discarded. Abomasal epithelium was clipped using sterile scissors and forceps from the pyloric region of the abomasum (10 cm from the pylorus), and four small pieces were rinsed in ice-cold sterile saline, transferred into sterile tubes with RNAlater, stored for 24 h at 4°C, and then frozen at -20°C. A whole thickness 4 × 4 cm abomasum tissue sample was collected from the same region, and processed in the same manner as ruminal histology samples. The remaining tissue was rinsed repeatably with tap water, pressed by hand and weighed.

The small intestine was divided into the following regions: duodenum (the end marked by the ligament of Treitz), proximal (at 25% of total length), middle (at 50% of total length), and distal (at 75% of total length) jejunum (with the end of jejunum determined by the ileocecal fold), and the ileum (with the end denoted by the ileocecal junction). From each region, tissue samples (approximately 20 cm length) were collected, the inside was gently washed with ice-cold sterile saline from a squeeze-bottle in order to remove digesta, and tissues were placed on sterile paper towel located on top of glass cooled by ice. Intestinal tissues were opened along the mesenteric line. From the proximal jejunum and ileum, the mucosal layer was scrapped off, using sterile glass slides, and deposited into 2-mL sterile test tubes with 1.8 mL of RNAlater, were stored for 24 h at 4°C, and then frozen at -20°C. From all 5 intestinal regions, additional mucosal scrapings were collected into cryogenic tubes (approximately 3 g/region), that were frozen in liquid nitrogen, and

subsequently stored at -80°C until brush border enzyme activity analyses. After sampling, remaining digesta were manually squeezed out of the intestine, and the tissue was weighed.

5.3.3 Ruminal Fermentation Characteristics

Ruminal digesta were deposited in a container and mixed thoroughly. The pH was measured in duplicate using hand-held pH meter (N517, Meratronic, Warszawa, Poland). A representative sample of ruminal digesta was strained through 2 layers of cheesecloth and 10 mL of supernatant was mixed with 2 mL of meta-phosphoric acid (25% wt/v). The sample was stored at -20°C until analysis for short-chain fatty acid (**SCFA**) concentration. In addition, 4 mL of supernatant was collected, mixed with 0.2 mL saturated $HgCl_2$, and frozen at -20°C until analysis for ammonia concentration.

Short-chain fatty acids in the ruminal digesta were determined by gas chromatography (3400 CX, Varian Star, Palo Alto, CA) equipped with a flame ionization detector as described previously by Górka et al. (2017a) using DB-FFAP column (30 m × 0.5 mm, J&W Scientific, Folsom, CA) and argon as a carrier. Ammonia concentration in the ruminal digesta was analyzed as described by Conway (1962).

5.3.4 Dry Weight of the Ruminal Tissue

Frozen duplicate 5 × 5 cm whole-tissue fragments from the cranial ventral sac of the rumen were thawed and their actual dimensions were measured. The epithelium was manually peeled from the muscle layer, and both were placed on separate aluminum trays and their weight was recorded. The samples were dried in a forced flow oven at 105°C for 5 h and placed in a desiccator for 2 h. The dry tissue weight, for both epithelium and muscle layers, was recorded again and calculated per cm^2 of the tissue.

5.3.5 Histometric Measurements

Ruminal, omasal, and abomasal tissue samples preserved in formalin and stored in ethanol were used for histometric measurements. One cm^2 of ruminal tissue was used to determine ruminal papillae length, width and density, and ruminal muscle thickness, as described previously by Górka et al. (2011a) and Górka et al. (2018c) with some modifications. Briefly, all papillae were cut off at the base using forceps and scissors under a microscope (SteREO Discovery V12 ZEISS microscope, Oberkochen, Germany) with a PlanApo S 0.63x FWD 81mm ZEISS lens (Oberkochen, Germany). Papillae were counted and the length and width (middle point) of each papillae was measured using AxioVision 40 (V 4.8.2.0, ZEISS, Oberkochen, Germany) software. The epithelial surface area (mm^2/cm^2) was determined as the length × width × density × 2 (Malhi et al., 2013). The remaining portion of the tissue (approximately 2 cm^2) was cut into four 0.5- to 1-cm thick pieces, positioned on one edge, and muscle layer thickness was also measured in five locations on each piece of the tissue resulting in 20 measurements for each sample. Abomasal tissues samples were also divided into four 0.5- to 1-cm thick pieces. Using the same microscope that was used for rumen papillae and rumen muscle measurements, five measurements of epithelial thickness and muscle layer thickness in the abomasum were performed for each piece of tissue, resulting in 20 measurements for each parameter and sample. Furthermore, omasal tissue samples and also individual ruminal papillae were used for histometric measurements, as previously described by Górka et al. (2018b) with some modifications. Briefly, each omasal tissue sample (n = 5/calf) was divided into 5 smaller 0.5 cm thick pieces and embedded in 5 separate paraffin blocks (P3683, Sigma-Aldrich, St. Louis, MO). Six section cuts, at least 200 µm apart, were completed on each omasal paraffin block. For each section cut, 6 measurements of the epithelial layer (stratum granulosum, stratum spinosum, stratum basale) and 12 measurements of the keratinized layer (stratum corneum) were conducted using a light microscope with a 10× objective lens and 16× ocular (Nikon E600, Tokyo, Japan) with a JVC TK-C1380E color digital camera (Victor Company of Japan Ltd., Yokohama, Japan) and MultiScanBase v. 14.02 software (Computer Scanning System Ltd., Warsaw, Poland). Similarly, five ruminal papillae/calf were embedded in separate paraffin blocks. For easier embedding, the largest papillae were chosen from each calf. Four section cuts of each ruminal papillae were analyzed with 6 measurements of the epithelial layer and 12 measurements of the keratinized layer performed using the same microscope as describe

above. Epithelium thickness and stratum corneum thickness were used to mathematically calculate living strata thickness. Additionally, integrity of the embedded and sectioned tissue (damage index) was assessed using a 3-point scale as described by Steele et al. (2015). Data for histometric measurements (e.g., epithelium and muscle thickness) were pooled by animal and region of GIT to obtain a mean value for each animal.

5.3.6 Quantitative Real-Time PCR

Total RNA from ruminal (cranial ventral sac), abomasal, proximal jejunal and ileal tissues stored in RNAlater was isolated using method of Chomczynski and Sacchi (1987). The concentration and integrity of RNA was estimated spectrophotometrically using a photospectrometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA) and verified using electrophoresis with an agarose gel. Samples of total RNA of good quality (OD_{260nm}/OD_{280nm} between 1.8 and 2.0) and without signs of degradation on an agarose gel were subjected to the reverse transcription reaction. Reverse transcription was conducted using 2 µg of total RNA and High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instruction, using a thermocycler (Eppendorf, Hamburg, Germany). The obtained cDNA was stored at –20°C until further use for gene expression analyses of G protein-coupled receptor 41 (***GPR41***;Table 5.1), G-protein-coupled receptor 43 ***(GPR43),*** monocarboxylic acid transporter 1 (***MCT1),*** monocarboxylic acid transporter 4 (***MCT4***), peptide transporter 1 (***PEPT1***), peptide transporter 2 (***PEPT2),*** aquaporin 3 (***AQP3***), urea transporter B (***UT-B***), amino acid transporter B0+ (***ATB0+***), excitatory amino acid carrier 1 (***EAAC1),*** and 3 reference genes: glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***), 60S acidic ribosomal protein P0 (***RPLP0***), and beta-actin (***ACTB***). Target genes were chosen strategically to address different aspects of GIT function: *GPR41, GPR43, MCT1,* and *MCT4* were used to assess SCFA sensing and transport within the GIT; *AQP3* and *UT-B* were used to assess ruminal urea transport; *PEPT1*, *PEPT2* were used to assess transport of peptides in the intestine regions; and *ATB0+* and *EAAC1* were used as indicators for the transport of select AA in the intestinal regions. Target and reference gene mRNA level was analyzed in duplicate using StepOnePlus™ Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA).

Table 5.1. Target gene names, accession numbers, primer sequences and function of the genes of interest for the performance study utilizing bull calves to evaluate either canola meal or soybean meal as protein source, and microencapsulated sodium butyrate supplementation in the starter mixtures

Target gene name abbreviation[1]	Accession number	Forward and reverse primers (3 – 5)[7]	Region[8]	Gene function
GAPDH[2]	NM_001034034	F: TCTGGCAAAGTGGACATCGT R: ATGACGAGCTTCCCGTTCTC	CRA, PROX, ILE	Endogenous control
RPLP0[3]	NM_001012682	F: GGAAACTCTGCATTCCCGCT R: AAGGCCTTGACCTTTTCAGC	CRA, PROX, ILE	Endogenous control
ACTB[2]	AY141970	F: GCGGCATTCACGAAACTACC R: GCCAGGGCAGTGATCTCTTT	CRA, PROX, ILE	Endogenous control
MCT1 (SLC16A1)[4]	NM_001037319	F: CTTCTGTAACACTGTGCAGGAACT R: CGTAGATCATAAAGAAAGCCTGGT	CRA, PROX, ILE	SCFA[9] transporter
MCT4 (SLC16A4)[5]	NM_001109980	F: CATGGTGTCTGCGTCCTTCTGTGG R: AGCGGTTGAGCATGATGAGTGAGG	CRA, PROX, ILE	SCFA transporter
GPR41 (FFAR3)[3]	FJ562214.1	F: GGCTAGAGATGGCAGTGGTC R: TAGCTCCCCTCCCGAGTATG	CRA, PROX, ILE	SCFA receptor
GPR43 (FFAR2)[3]	FJ562212.1	F: ATGCTCTGTTCCTCTTTCCCA R: GGCATGGCTGTCCTTGTCTT	CRA, PROX, ILE	SCFA receptor
UT-B (SLC14A1)[3]	NM_001008666	F: CAGGACAGGTCCGCCATTAC R: AGACAGGCAGGTCCCATTTG	CRA	Urea transporter
AQP3[3]	NM_001079794	F: TCCTCGTGATGTTTGGCTGT R: TGATGGTGAGGAAACCACCG	CRA	Water transporter (urea salvaging)
PEPT1 (SLC15A1)[3]	NM_001099378	F: GTTTGATCAGCAGGGCTCCA R: GATGACGATCAGGATGGCGT	PROX, ILE	Oligopeptide transporter
PEPT2 (SLC15A2)[3]	NM_001079582	F: TGCTGACTCATGGTTGGGAA R: TTGGAAGCAAGAGGCTAGAAGA	PROX, ILE	Oligopeptide transporter
ATB0+ (SLC6A14)[6]	NM_001098461	F: AGTCGGAGCAGCATTATTTAAAAGGAA R: ACCAGGATGAGTAGGACAACATAGG	PROX, ILE	Glutamate transporter
EAAC1 (SLC1A1)[3]	NM_174599	F: TGTGCTACATGCCGATTGGT R: GATTGCAAGCCCACTCAGGA	PROX, ILE	Cationic and neutral amino acid transporter

[1]*GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *RPLP0* = 60S acidic ribosomal protein P0; *ACTB* = beta-actin; *MCT1* = monocarboxylic acid transporter 1; *MCT4* = monocarboxylic acid transporter 4; *GPR41* = G protein-coupled receptor 41; *GPR43* = G protein-coupled receptor 43; *UT-B* = urea transporter B; *AQP3* = aquaporin 3; *PEPT1* = peptide transporter 1; *PEPT2* = peptide transporter 2; *ATB0+* = amino acid transporter B0+; *EAAC1* = excitatory amino acid carrier 1.
[2]Verdugo (2016).
[3]Designed using NCBI Primer-BLAST (NCBI, 2018).
[4]Kuzinski and Röntgen (2011).
[5]Benesch et al. (2014).
[6]Liao et al. (2009).
[7]F = forward primer, R = reverse primer.
[8]CRA = cranial sac of rumen; PROX = proximal jejunum (at 25% length); ILE = ileum.
[9]SCFA = short chain fatty acid.

Each reaction was performed with 5 µL of PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, Waltham, MA), 900 n*M* of each primer (forward or reverse), 1 µL of cDNA sample, and filled up to 10 µL with nuclease-free water (Thermo Fisher Scientific, Waltham, MA). Each run consisted of holding stage (95°C/20 s) and 40 cycling stages (95°C for 3 s and 60°C for 30 s). To ensure correct product of analysis, melting curve analysis was performed for each sample. Additionally, products of the reaction for each primer were run in an agarose gel and sequenced to confirm the homology with target sequence. The efficiency of primers ranged from 96.0% to 105.1%, with the average being 100.3% ± 2.8%. The inter- and intra-assay CV were 0.234% ± 0.044% and 0.602 ± 0.699%, respectively. Relative expression of the investigated genes is presented as ΔC_T, where:

$$\Delta C_T = \text{treshold cycle } (C_T) target - C_T reference \qquad \textbf{Equation 5.1}$$

Therefore, a higher ΔC_T value represented lower mRNA expression and vice versa. The three reference genes (*GAPDH*, *RPLP0* and *ACTB*) were analyzed using NormFinder (0.953, Molecular Diagnostics Laboratory, Aarhus, Denmark), according to Andersen et al. (2004), in order to determine if there were differences between experimental groups and regions of GIT. *GAPDH* expression was the most stable across GIT regions and within regions, and did not differ significantly between the treatments ($P \geq 0.16$) and thus was used for ΔC_T calculations.

5.3.7 Brush-border Enzyme Activity

The scraped epithelia from duodenum, proximal, middle, and distal jejunum, and the ileum were used for the analysis of protein content and brush border enzymes activities. Enzymes evaluated were: lactase (EC 3.2.1.23), maltase (EC 3.2.1.20), dipeptidylpeptidase IV (EC3.4.14.5), aminopeptidase A (EC 3.4.11.7), and aminopeptidase N (EC 3.4.11.2); and their activity was determined as described previously (Górka et al., 2017). Briefly, frozen samples of the epithelium were kept on ice and homogenized with distilled water (1 g of mucosa:5 mL of water). The homogenate was centrifuged for 5 min at 1,000 × *g* at 4°C and the supernatant was collected. The protein content of the supernatant was analyzed according to Hartree (1972). Lactase and maltase activities were analyzed according to Dahlqvist (1984), with minor modifications: increase in

analyze tissue homogenate volume to 100 µL and glucose detection through enzymatic reaction of glucose with glucose oxidase/peroxidase (No. P7119 Sigma-Aldrich, St. Louis, MO) and dianisidine dihydrochloride (No. D3252 Sigma-Aldrich, St. Louis, MO) with the reaction analyzed at 450 nm wavelength using plate reader SpectraMax Plus UV/Vis spectrometer (Molecular Devices, San Jose, CA). L-glutamyl-p-nitroanilide and L-leucyl-p-nitroanilide (Bachem, Budendorf, Switzerland) were used as substrates for analysis of aminopeptidase A and N activity, respectively (Maroux et al., 1973), while L-glycyl-p-nitroanilide (Bachem) was the substrate for analysis of dipeptidylpeptidase IV activity (Nagatsu et al., 1976). Enzymatic activities are reported as enzymatic units (U) per mg of protein and correspond to the released glucose or p-nitroanilide (µmol/min of reaction) at 37°C.

5.3.8 Statistical Analysis

One bull calf died shortly before weaning due to bloat, and another two calves that experienced severe bloat issues (treated according to veterinarian's advice) were eliminated from the dataset (Chapter 4). This resulted in 6, 6, 6 and 7 calves that were used for statistical analysis for TSBM, TSBM-MSB, TCM and TCM-MSB treatments, respectively.

Data in this study were analyzed as a 2 × 2 factorial design using PROC MIXED of SAS (version 9.4, SAS Institute, Cary, NC). Protein source, MSB inclusion, and protein source × MSB interaction were considered in the statistical model as fixed effects, whereas block was included as a random term. For analysis of GIT compartments, tissue and digesta weight, and length, final BW of calves was included in the statistical model, as a covariate. When a significant effect of protein source × MSB inclusion interaction was observed, means were separated using the Bonferroni adjustment in SAS. Unless otherwise stated, data are presented as least square means and corresponding standard errors. Significance was declared when $P \leq 0.05$, while a tendency was declared when $0.05 < P \leq 0.10$.

5.4 Results

Data on feed intake, growth performance, feed efficiency and health (i.e. fecal score, number of days with diarrhea) can be found in Chapter 4, Study 1. Briefly, calves fed starters containing CM had decreased pre-weaning starter intake, weaning ADG, weaning and overall G:F ratio, and post-weaning total-tract digestibility. However, overall starter intake and ADG, and final BW did not differ among treatments. Supplementation of MSB positively affected pre-weaning starter intake. Fecal score was increased, indicating more liquid feces, when calves were consuming starters with SBM compared to starters with CM.

There were no interactions between different protein sources and MSB supplementation for ruminal fermentation characteristics ($P \geq 0.22$; Table 5.2). MSB supplementation did not affect ruminal fermentation ($P \geq 0.13$). Ruminal pH, SCFA concentration, and the molar proportion of individual SCFA were not affected by protein source ($P \geq 0.27$), except for valerate, which was less for calves fed CM as compared with SBM ($P = 0.021$). Ruminal ammonia concentration tended to be less for CM treatments as compared to SBM treatments ($P = 0.084$).

Only one interaction was observed for the calf GIT morphometry for the omasal digesta weight *(*$P = 0.005$, Table 5.3), which was the greatest for TSBM-MSB, intermediate for TCM and TCM-MSB and the least for TSBM. No other interactions were observed for the calf morphometry ($P \geq 0.12$). Ruminal and omasal tissue weights did not differ between treatments, and neither did the ruminal digesta weight. Inclusion of CM in the starters tended to result in heavier abomasal tissue weight ($P = 0.063$) and a longer jejunum ($P = 0.065$), and greater jejunal tissue weight ($P = 0.046$), when compared with inclusion of SBM. Omasal digesta was greater for calves supplemented with MSB ($P = 0.008$). Small intestinal digesta weights were not affected by the protein source, nor MSB supplementation ($P \geq 0.23$). Throughout the GIT, MSB did not affect the weight or length of the GIT tissue ($P \geq 0.22$).

Protein source × MSB interactions were observed for ruminal epithelial surface area ($P = 0.049$; Table 5.4), papillae density ($P = 0.035$), and thickness of abomasal epithelium ($P = 0.038$). For all mentioned interactions, the Bonferroni post-hoc mean separation did not detect differences among treatments. However, ruminal epithelial surface, papillae density, and abomasal epithelium thickness were numerically the greatest for TCM, intermediate for TSBM and TSBM-MSB, and the least for TCM-MSB.

Table 5.2. Rumen fermentation characteristics

Variable	Treatment[1]				SEM	*P*-value[2]		
	TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS×MSB
Rumen pH	5.19	5.55	5.49	5.41	0.18	0.67	0.44	0.23
SCFA[3] concentration, mMol/L	182.7	186.6	180.2	177.1	22.5	0.78	0.98	0.87
Acetate, mMol/100 mMol	44.4	43.7	42.1	42.3	2.9	0.49	0.93	0.86
Propionate, mMol/100 mMol	30.1	32.2	35.4	32.9	2.7	0.27	0.95	0.40
Iso-butyrate, mMol/100 mMol	0.302	0.088	0.270	0.244	0.074	0.42	0.13	0.22
Butyrate, mMol/100 mMol	19.1	17.3	18.3	20.1	2.6	0.62	0.98	0.40
Iso-valerate, mMol/100 mMol	0.500	0.442	0.406	0.407	0.088	0.48	0.75	0.74
Valerate, mMol/100 mMol	5.49	6.36	3.29	4.01	0.91	0.021	0.39	0.93
Ammonia, mg/dL	18.8	19.4	13.6	14.2	2.8	0.084	0.83	0.99

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).

[2]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.

[3]SCFA = short-chain fatty acid.

Table 5.3. Gastrointestinal tract morphometry

Variable		Treatment[1]				SEM	*P*-value[2]		
		TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS×MSB
Rumen	Tissue, kg	1.95	1.91	2.02	1.99	0.08	0.35	0.68	0.95
	Digesta, kg	4.24	4.03	3.53	3.99	0.39	0.36	0.75	0.40
Omasum	Tissue, kg	0.349	0.376	0.380	0.396	0.021	0.21	0.29	0.70
	Digesta, kg	0.232[b]	0.394[a]	0.297[ab]	0.327[ab]	0.032	0.97	0.008	0.005
Abomasum	Tissue, kg	0.371	0.382	0.418	0.419	0.021	0.063	0.79	0.81
	Digesta, kg	0.406	0.328	0.468	0.418	0.084	0.35	0.43	0.85
Duodenum	Tissue, kg	0.099	0.096	0.105	0.100	0.009	0.59	0.67	0.93
	Digesta, kg	0.010	0.016	0.009	0.012	0.004	0.61	0.29	0.78
	Length, m	0.45	0.48	0.49	0.43	0.04	0.85	0.74	0.30
Jejunum	Tissue, kg	2.23	2.03	2.5	2.35	0.14	0.046	0.22	0.82
	Digesta, kg	1.16	1.29	1.18	1.53	0.19	0.52	0.23	0.55
	Length, m	20.7	20.6	23.3	21.7	0.9	0.065	0.38	0.45
Ileum	Tissue, kg	0.256	0.204	0.221	0.281	0.035	0.55	0.91	0.12
	Digesta, kg	0.033	0.076	0.076	0.092	0.030	0.35	0.34	0.66
	Length, m	1.32	1.15	1.25	1.29	0.13	0.78	0.57	0.36

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).

[2]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.

[a,b] Means with uncommon superscripts differ ($P < 0.05$).

Table 5.4. Ruminal epithelium development in the ventral sac of the rumen, omasum and abomasum

Variable	Treatment[1]				SEM	*P*-value[2]		
	TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS×MSB
Rumen								
Epithelial surface, mm^2/cm^2	1136.1	1088.0	1249.6	820.6	102.3	0.40	0.019	0.049
Papillae density, n/cm^2	140.4	158.4	181.1	129.0	15.7	0.71	0.28	0.035
Papillae length, mm	2.41	2.02	2.22	2.15	0.12	0.83	0.080	0.21
Papillae width, mm	1.61	1.63	1.56	1.49	0.07	0.14	0.71	0.47
Epithelium thickness, µm	158.3	156.8	160.6	156.8	7.0	0.68	0.88	0.95
Stratum corneum thickness, µm	20.6	21.0	21.3	22.6	1.1	0.30	0.43	0.68
Living strata thickness, µm	136.8	136.1	138.4	131.9	6.8	0.82	0.53	0.61
Muscle layer thickness, mm	2.44	2.64	2.63	2.61	0.17	0.60	0.54	0.44
Damage index[3]	2.6	2.5	2.3	2.1	0.1	0.019	0.25	0.57
Dry epithelium mass, mg/cm^2	117.5	79.8	109.2	88.1	7.2	0.99	<0.001	0.15
Dry muscle mass, mg/cm^2	33.0	29.9	30.8	32.0	3.7	0.99	0.80	0.58
Omasum								
Epithelium thickness, µm	140.4	127.6	137.4	127.3	8.5	0.81	0.064	0.85
Stratum corneum thickness, µm	16.5	14.8	16.0	14.8	0.8	0.72	0.029	0.66
Living strata thickness, µm	123.8	112.8	121.8	112.4	7.7	0.83	0.087	0.89
Damage index	1.78	1.85	1.97	1.93	0.10	0.20	0.89	0.60
Abomasum								
Epithelium thickness, mm	1.01	1.10	1.13	0.99	0.05	0.94	0.58	0.038
Muscle layer thickness, mm	2.26	2.06	2.29	2.16	0.15	0.69	0.31	0.81

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).

[2]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.

[3]Described as a 3-point scale: 1 = not damaged, whole tissue; 2 = minor sloughing and tissue separation; 3 = major sloughing and tissue separation (Steele et al., 2015).

Different protein sources did not influence any of the papillae dimensions, epithelial measurements, nor dry tissue weight in the cranial ventral sac of the rumen ($P \geq 0.14$), except for ruminal damage index ($P = 0.019$), which was greater in calves consuming starters with SBM rather than CM. Supplementation of MSB in starters resulted in decreased dry epithelium mass ($P < 0.001$) and tended to decrease papillae length ($P = 0.080$). Papillae density and width, dry muscle mass, and the epithelial measurements within the rumen were not affected by the MSB supplementation ($P \geq 0.25$). Omasal histological characteristics did not differ between protein sources ($P \geq 0.20$). Meanwhile, MSB supplementation decreased the thickness of omasal stratum corneum ($P = 0.029$) and tended to decrease the thickness of the entire epithelium ($P = 0.064$) and that living strata ($P = 0.087$), but did not affect damage index of the omasal epithelium ($P = 0.89$). Abomasal epithelium and muscle layer thickness did not differ among treatments ($P \geq 0.31$).

A tendency for a protein source × MSB supplementation interaction for the expression of *GPR43* in ruminal epithelium was observed ($P = 0.091$; Table 5.5). Since only a tendency was observed, no mean separation was achieved. However, numerically, the greatest expression was observed for TSBM-MSB, intermediate for TCM and TSBM, and the least for TCM-MSB. Protein source did not affect expression of any analyzed genes ($P \geq 0.21$) in the cranial ventral sac of the rumen, while MSB supplementation resulted in greater expression of *MCT1* ($P = 0.018$), but did not affect any other genes ($P \geq 0.39$). Starters containing CM resulted in greater expression of *GPR43* ($P = 0.005$) and tended to result in greater expression of *MCT4* ($P = 0.065$) in the abomasal epithelium. In the proximal jejunal epithelium, the only gene affected by protein source was *PEPT2* ($P = 0.044$), which showed decreased expression when CM, rather than SBM, was included in the starters. Also, a tendency for decreased expression for *ATB0+* ($P = 0.085$) was observed in the ileal epithelium when CM was present in the starters. Inclusion of MSB in the starters did not affect gene expression in the abomasum, proximal jejunum, or ileum ($P \geq 0.104$).

Protein source × MSB interactions were observed for lactase activity in the proximal jejunum ($P = 0.012$; Table 5.6) and maltase activity in the ileum ($P = 0.028$); however, the Bonferroni post-hoc mean separation did not detect differences among treatment means. Although the mean separation was not achieved, for lactase activity in the proximal jejunum, the greatest activity was observed for TSB-MSB, which was followed by TCM, TCM-MSB, and TSB. For the ileal maltase activity, the greatest activity was observed for TCM-MSB, then TSBM, TSB-MSB, and the least for TCM.

Table 5.5. Relative gene expression in the cranial sac of the rumen (CRA), abomasum (ABOM), proximal jejunum (PROX), and ileum (ILE)

Gene of interest[1]		Treatment[2]				SEM	*P*-value[3]		
		TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS×MSB
CRA	*MCT1*	0.771[4]	0.138	0.525	0.002	0.228	0.37	0.018	0.79
	MCT4	11.08	11.23	10.90	11.18	0.24	0.64	0.39	0.80
	UT-B	0.952	1.090	0.357	0.627	0.376	0.18	0.60	0.86
	AQP3	1.79	1.66	1.55	1.38	0.20	0.21	0.48	0.92
	GPR41	13.49	13.41	13.27	13.14	0.55	0.65	0.85	0.96
	GPR43	16.30	15.87	16.01	17.26	0.48	0.25	0.40	0.091
ABOM	*MCT1*	6.75	6.57	6.81	6.72	0.29	0.69	0.63	0.85
	MCT4	10.46	10.21	10.05	10.01	0.19	0.065	0.36	0.50
	GPR41	9.25	9.30	9.41	8.92	0.25	0.65	0.39	0.29
	GPR43	15.47	15.77	15.19	15.00	0.20	0.005	0.76	0.15
PROX	*MCT1*	4.92	4.88	4.80	4.58	0.22	0.34	0.57	0.68
	MCT4	8.38	8.61	8.06	8.54	0.32	0.37	0.14	0.57
	PEPT1	4.06	4.69	4.05	4.63	0.36	0.93	0.104	0.95
	PEPT2	11.45	11.79	12.37	12.30	0.33	0.044	0.68	0.55
	EAAC1	7.61	7.75	7.46	7.74	0.43	0.85	0.63	0.88
	ATB0+	0.046	0.173	0.043	-0.287	0.297	0.45	0.74	0.45
	GPR41	10.09	10.15	10.32	10.20	0.21	0.46	0.88	0.63
	GPR43	12.15	12.57	12.21	12.68	0.28	0.76	0.13	0.93
ILE	*MCT1*	5.27	5.47	5.19	5.06	0.19	0.23	0.86	0.39
	MCT4	8.99	8.92	8.94	8.97	0.16	0.98	0.90	0.74
	PEPT1	3.41	3.83	3.74	3.76	0.43	0.76	0.61	0.66
	PEPT2	10.81	10.53	10.37	10.09	0.28	0.11	0.29	0.99
	EAAC1	7.71	6.58	7.48	6.68	0.80	0.93	0.24	0.84
	ATB0+	4.38	4.87	5.41	5.12	0.37	0.085	0.76	0.28
	GPR41	7.83	7.32	7.30	7.36	0.24	0.21	0.26	0.15
	GPR43	12.46	12.52	12.87	13.13	0.36	0.17	0.67	0.78

[1]*MCT1* = monocarboxylic acid transporter 1; *MCT4* = monocarboxylic acid transporter 4; *GPR41* = G protein-coupled receptor 41; *GPR43* = G protein-coupled receptor 43; *UT-B* = urea transporter B; *AQP3* = aquaporin 3; *PEPT1* = peptide transporter 1; *PEPT2* = peptide transporter 2; *ATB0+* = neutral amino acid transporter; *EAAC1* = excitatory amino acid carrier 1.

[2]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).

[3]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.

[4]Values of gene expression are presented as ΔC_T = threshold cycle (C_T) for the gene of interest – C_T for the reference genes; greater ΔC_T values represent lesser mRNA expression.

Table 5.6. Brush border enzymes activity (U/mg of protein) in the small intestine of bull calves

Variable		Treatment[1]				SEM	*P*-value[2]		
		TSBM	TSBM-MSB	TCM	TCM-MSB		PS	MSB	PS×MSB
Aminopeptidase A	DUO[3]	1.35	2.88	2.01	2.76	0.66	0.64	0.066	0.50
	PROX	2.74	2.32	2.97	2.90	0.60	0.51	0.69	0.78
	MID	18.94	13.52	22.75	20.78	4.81	0.23	0.42	0.70
	DIST	32.09	36.80	27.34	37.39	7.23	0.78	0.32	0.72
	ILE	8.45	12.20	9.32	14.40	1.66	0.37	0.015	0.69
Aminopeptidase N	DUO	18.29	19.56	18.85	16.72	2.32	0.61	0.85	0.44
	PROX	19.87	19.83	19.46	19.83	1.77	0.91	0.93	0.91
	MID	46.37	47.86	40.41	43.37	7.67	0.50	0.78	0.92
	DIST	39.31	40.61	34.81	38.73	5.15	0.54	0.62	0.80
	ILE	33.49	34.83	29.5	42.07	3.88	0.65	0.073	0.13
Dipeptidylpeptidase IV	DUO	nd[4]	nd	nd	nd	-	-	-	-
	PROX	nd	nd	nd	nd	-	-	-	-
	MID	12.35	13.18	13.54	12.5	2.87	0.93	0.97	0.75
	DIST	11.18	13.84	13.72	13.9	2.66	0.63	0.60	0.64
	ILE	9.36	9.07	8.74	12.85	1.44	0.29	0.20	0.14
Lactase	DUO	36.3	30.33	35.93	28.36	7.09	0.86	0.31	0.90
	PROX	55.89	150.8	101.42	71.03	22.64	0.46	0.17	0.012
	MID	38.73	40.54	39.58	34.66	5.96	0.64	0.78	0.54
	DIST	2.82	2.50	2.38	2.85	0.30	0.87	0.79	0.16
	ILE	1.55	1.48	1.41	1.53	0.12	0.71	0.83	0.44
Maltase	DUO	4.23	4.98	4.75	5.14	0.32	0.42	0.19	0.67
	PROX	8.90	11.11	9.63	8.62	1.33	0.52	0.66	0.24
	MID	10.45	9.58	8.45	8.43	1.54	0.32	0.77	0.79
	DIST	8.16	8.39	7.04	7.60	0.90	0.30	0.66	0.85
	ILE	4.13	3.86	3.59	4.66	0.30	0.64	0.17	0.028

[1]TSBM = soybean meal used as the main source of protein in the starter mixture; TSBM-MSB = TSBM with microencapsulated sodium butyrate (0.33% DM); TCM = canola meal used as the main source of protein in the starter mixture; TCM-MSB = TCM with microencapsulated sodium butyrate (0.33% DM).
[2]PS = main effect of protein source in starter mixture (soybean mean vs. canola meal); MSB = main effect of microencapsulated sodium butyrate inclusion in starter mixture (0 vs. 0.33% DM); PS × MSB = interaction between main effects.
[3]DUO = duodenum; PROX = proximal jejunum (at 25% length); MID = middle jejunum (at 50% length); DIST = distal jejunum (at 75% length); ILE = ileum.
[4]not detected.

Protein source did not affect brush border enzyme activity in the small intestine ($P \geq 0.23$). Aminopeptidase A activity tended to be greater in calves fed starters supplemented with MSB in the duodenum ($P = 0.066$) and was greater in the ileum ($P = 0.015$). Similarly, aminopeptidase N activity in the ileum tended ($P = 0.073$) to be greater for MSB supplemented calves. Dipeptidylpeptidase IV, lactase and maltase activities were not affected by MSB supplementation ($P \geq 0.17$).

5.5 Discussion

The study did not confirm the hypothesis that a combination of CM as protein source in starter mixtures containing MSB would prevent a potential negative impact of CM on development of GIT in calves. Nevertheless, prior to discussion of results, specificity of the experimental model needs to be mentioned. In order to limit the number of confounding factors between protein sources, Lys and Met were equalized among treatments (Chapter 4), both considered the most limiting AA for growth in calves (Abe et al., 1998). This was achieved by Met supplementation in starters containing CM, whereas no AA (Lys) were supplemented to in starters containing CM (Chapter 4). The addition of Met to SBM starters may have favoured SBM treatments in terms of growth performance as well as GIT development. However, as the additional Met is likely to be utilized by ruminal microflora first (Chalupa, 1975), the increased Met flow to the small intestine of the calves was not anticipated, although that was not evaluated in this study. To support that, while SBM treatments contained added Met to balance treatments, CM treatments had greater plasma Met concentration (Chapter 4). In general, Met supplementation in starter mixtures containing SBM was a precaution and was assumed to have only minor impact on results of the current study (Chapter 4).

5.5.1 Effect of Protein Source

The starters containing CM as a protein source contained numerically less CP than those with SBM (20.5 vs. 21.8% DM, respectively) and greater NDF (18.7 vs. 14.2% DM) and ADF (11.2 vs. 6.9% DM) than starters containing SBM (Chapter 4). This resulted in less non-fibrous carbohydrate content in starters containing CM than SBM (49.3 vs. 53.1% DM), mostly due to

differences of starch content. Although slightly less CP intake could partially contribute to the decreased ruminal ammonia in calves fed starters with CM treatments and a decreased plasma urea concentration for calves fed CM treatments (Chapter 4), it is unlikely to fully explain observed differences between protein sources in the current study. Compared to SBM, it was shown that CM contains more RUP than SBM (Broderick et al., 2016), which could result in a lesser amount of AA available for bacteria to convert into ammonia in the rumen. Furthermore, protein of CM was shown to be less digestible post-ruminally (Khorasani et al., 1990), which could result in less AA available for tissue growth and metabolic processes. Both could lead to the observed decreased ruminal ammonia and plasma urea concentration in calves fed starters with CM when compared to SBM. Nevertheless, it is worth mentioning that pre-weaning plasma urea was greater in calves fed starters with CM, compared to calves fed starters with SBM, but this reversed after weaning (Chapter 4). Such changes in the plasma urea concentration could indicate that when calves were fed starters containing CM, digestion and metabolism of protein and AA may have changed substantially with age, increasing BW, and starter intake. However, the changes in the ruminal ammonia in the current study were only recorded at one sampling point and might not fully reflect changes that could be occurring, especially considering the differing waiting time of calves at the abattoir. In mature dairy cows, the ruminal ammonia concentration changes during the day and is the greatest post-feeding (Robinson and McQueen, 1994). In calves, greatest ruminal ammonia increase can be observed about 8 h post-feeding (Quigley et al., 1992). More studies are needed to fully explain impact of CM use in starter mixtures on protein and AA metabolism in calves.

Feeding different protein sources did not impact ruminal SCFA concentrations, nor development of ruminal tissue. These results were not surprising as ruminal protein digestion is not meant to directly influence major SCFA concentrations, nor epithelial development. The only exception was the molar proportion of valerate, which was decreased for the calves fed starters with CM. A similar response for reduced valerate has also been reported by Getachew et al. (2004) after 24-h *in vitro* incubation of CM samples, when compared with SBM, in ruminal fluid of mature Holstein cows that were fed an oat hay diet. Ruminal valerate can stem from either carbohydrate or from fermentation of certain AA, namely Pro, Arg, Lys and Met (Andries et al., 1987). Although the rate at which the two processes occur is not clear, nor how much the individual AA are utilized, it is interesting to note that when considering Pro, Arg, Lys and Met together, their total concentration was lesser in CM starters by about 2 g/kg DM (Chapter 4). Based on post-

weaning starter intake, the timepoint for which SCFA concentrations were measured, starter intakes were approximately 2 kg DM, leading to estimated differences of approximately 4 g/d in those AA. As such, I could speculate that the difference in the AA concentrations between the two protein sources used in the present study was responsible for the change in the ruminal valerate concentration. Getachew et al. (2004) also observed a correlation between ruminal fluid valerate concentration and CP content of feed. Lesser concentration of valerate in ruminal fluid for calves fed starters with CM could also be attributed to lesser CP content in CM starters, as well as the lesser concentrations of individual AA from which valerate can originate. It cannot be excluded that lesser ruminal valerate in ruminal fluid of calves fed starters with CM was due to lesser ruminal CM protein degradation.

Additionally, I have observed that ruminal tissue from calves fed CM was less damaged than in those fed SBM. High grain diets are known to negatively affect the tight junctions in the ruminal tissue (Liu et al., 2013) and starters containing SBM had greater concentration of NFC, mostly due to greater content of rapidly fermentable starch derived from barley (Chapter 4). Although no differences among treatments were observed for ruminal SCFA and ruminal pH, ruminal pH had low values confirming results of others (Laarman et al., 2012) and potentially posing a challenge for the ruminal epithelium. As already mentioned, lack of differences for ruminal SCFA concentration and especially ruminal pH was likely a result of variation in time calves were without feed before euthenization, which ranged between 0.37 to 4.42 h after feeding time, although they had constant access to feed before. Thus, potential differences between treatments in terms of ruminal SCFA and ruminal pH could have been missed. More frequent measurements should have been collected; however, it was not possible within the current experimental setting.

Interestingly, in the present study, I observed an impact of protein source on abomasal development with an increase in tissue weight, as well as increased expression of *MCT4* and *GPR43* in the abomasal epithelium for calves fed starters with CM. As indicated previously, ruminal fermentation did not differ among treatments (with the exception of valerate); however, the influx of SCFA into the abomasum was not evaluated, which has been estimated to be 10% of that in ruminal fluid (Johnston et al., 1961). Within the abomasum, which is considerably more acidic than the rumen, most SCFA present would be undissociated and it is expected that absorption would be mediated through passive diffusion (Aschenbach et al., 2011). Within the

abomasal tissue, *MCT4* is located on the basolateral membrane of epithelial cells, as opposed to the apical side in ruminal cells, and as such *MCT4* may be important to facilitate basolateral release of SCFA or their metabolites into portal circulation (Kirat et al., 2007). The abundance of *MCT4* in abomasal epithelium has been reported to be similar to that in small intestinal epithelium in cattle (Kirat et al., 2007). Given that both *GPR43*, which is a SCFA receptor (Wang et al., 2009), and *MCT4* were upregulated in the abomasum in the CM treatments, I speculate that abomasal SCFA supply may have been increased. A small scale infusion study in sheep conducted by Williams et al. (1968) suggested that rates of SCFA absorption within the rumen and abomasum can be comparable. As shown by Pennington (1952), abomasal tissue is capable of utilizing SCFA, especially butyrate and acetate, and producing ketone bodies, although not as efficiently as ruminal tissue. As such, it might be likely that, at least partially, the SCFA in the abomasal tissue could have served as an energy source for the tissue, which could explain the increase in the tissue weight that was observed. However, I only observed a general increase in size of the abomasal tissue, rather than in epithelium thickness. Since I did not detect major differences in ruminal fermentation or digesta weight between protein sources, I can only speculate about the origins of abomasal changes. Nevertheless, it should be also considered that CM resulted in decreased DM digestibility (Chapter 4). A reduction in digestibility could alter the rate at which the digesta flows out of the rumen. Specifically, more digesta could flow out of the rumen necessitating increased tissue size for digesta mixing and passage to lower regions of the GIT, as also supported by greater intestine tissue weight (see below).

I observed increased jejunal tissue weight and length for calves fed CM compared to those fed SBM. However, there were no differences in brush border enzyme activity, and CM treatments downregulated *PEPT2* in proximal jejunum epithelium and *ATB0+* in the ileal epithelium. *ATB0+* is an AA transporter for anionic and neutral AA, such as Gln (Bode, 2001). Decreased expression of one of the Gln transporters could be at least partially a result of lesser Gln content in starters containing CM, and explains to some extent lesser plasma Gln concentration in calves fed starters with CM (Chapter 4). As a result, absorption of peptides and AA from the small intestine was likely limited in calves fed starter mixtures with CM, although this could be at least partially compensated by a longer small intestine. Reduced peptide and AA uptake could be also a result of a negative impact of antinutritional factors, namely phytic acid, present in CM, on the investigated peptides and AA transporters. Phytic acid can negatively affect small intestinal epithelium function

in piglets by impeding digestive enzymes, limiting nutrient presence, and slowing down enterocyte proliferation (Woyengo et al., 2011). It should be noted that during weaning, calves fed starters containing CM, tended to have decreased ADG and G:F ratio, and feeding starters with CM resulted in lesser DM digestibility (Chapter 4). The decrease in digestibility could partially explain greater intestinal tissue mass, which would be needed to support adequate feed digestion. This in turn, in combination with reduced expression of peptide and AA transporters, could explain the decrease in feed efficiency and ADG of calves without a change in BW, as more energy would be needed to maintain greater GIT tissue while simultaneously less AA could be used for muscle growth (due to reduced efficiency of AA absorption). However, since even post-weaning calves are still growing and increasing their intakes, a more developed intestinal tract might provide an advantage for feed digestion in latter stages of rearing and indicates adaptation of GIT to consumed feed.

5.5.2 Effect of MSB Supplementation

Microencapsulated butyrate supplementation in the starter did not have as profound effect on the GIT as previous research would have suggested (Górka et al., 2011a; Górka et al., 2014). Ruminal fermentation was not affected, which could be expected as the sodium butyrate was protected by microencapsulation, and would only minorly be released in the rumen. Additionally, rate of MSB inclusion in the current study was relatively low at 0.33%DM. Previous research suggests that greater inclusion rates might negatively affect ADG and starter intake of calves (Wanat et al., 2015). Also, no effect on GIT development in terms of size was observed when MSB was included in the diet. Previously, MSB supplementation in a starter mixture increased ruminal tissue weight (Górka et al., 2011a) and jejunal tissue weight in calves (Górka et al., 2014). However, in those studies, calves were slaughtered at 26 d of age, when starter intake was still rather low, but positively affected by MSB supplementation. Taking into account that in the present study post-weaning starter intake did not differ between treatments, which inevitably has an impact on GIT tissue weight, a lack of MSB impact on ruminal and intestinal tissue weights, as well as omasal and abomasal tissue weights seems to be logical.

Within ruminal epithelial tissue, MSB supplementation resulted in decreased ruminal epithelial surface area. Papillae length tended to be decreased by MSB supplementation in the calf

starter. Microencapsulated sodium butyrate inclusion also reduced dry epithelium weight in ventral sac of rumen, suggesting that both papillae and overall epithelium development in the rumen might be negatively affected. This is in direct opposition to what was observed by Górka et al. (2011a) where an increase in ruminal papillae length and width, as well as reticuloruminal tissue weight when calves were supplemented with MSB. Butyrate is commonly associated with promoting ruminal epithelium development (Sander et al., 1959; Bergman, 1990). However, when MSB was supplemented in a starter mixture, I observed increased expression of *MCT1* in ruminal epithelium. No differences were observed in ruminal fermentation, and specifically molar proportions of SCFA present in ruminal fluid, including butyrate. Although it might be possible that MSB was partially released in the rumen of the calves, with the small dose of MSB used, its direct effect on ruminal *MCT1* expression is questionable. *MCT1* is mostly expressed on the basolateral side of the ruminal epithelium (Kirat et al., 2005) and besides SCFA, *MCT1* plays a role in the transport of other cell metabolites such as BHB or lactate (Connor et al., 2010b). Likely the mentioned metabolites are responsible for increased *MCT1* expression; however, more detailed insight into the ruminal epithelium metabolism might be necessary to fully explain the described effect. Although surface area for SCFA absorption was reduced, functions of ruminal epithelium (e.g. SCFA absorption) could be enhanced by MSB supplementation, which could allow for compensation of decreased epithelial surface area.

In line with reduced papillae surface area in the rumen, the epithelium thickness, living strata, and keratinized strata thickness in the omasum were all decreased by MSB inclusion in starters. Those differences were accompanied by greater digesta mass in the omasum of calves supplemented with MSB. In general, concentration of SCFA present in the omasum is estimated to be about half that in ruminal fluid, and decreases further within the omasum before digesta move to the abomasum (Johnston et al., 1961). Greater digesta mass in the omasum might suggest a longer retention time in this region of the GIT. Additionally, exogenous butyrate was shown to negatively impact fibre digestibility in sheep (Górka et al., 2017b; Górka et al., 2018b), which could lead to a greater supply of fibre getting entrapped in the omasum, helping to explain the greater omasal digesta mass. Additionally, butyrate can stimulate ruminal muscle contractions (Kendall and McLeay, 1996), which could encourage more outflow of digesta from rumen to the omasum. However, while considering the greater omasal digesta weight, it is worth noting that omasal tissue weight did not differ among treatments, which could suggest that omasum likely

increased in size to accommodate additional digesta for MSB treatment, which could in turn lead to thinner lamina.

Similarly to results of Górka et al. (2014), MSB supplementation in starter mixture increased brush enzyme activity in the small intestine of calves. However, while Górka et al., (2014) observed increased dipeptydylpeptidase activity, in the current study activity of aminopeptidase A in duodenum and ileum and aminopeptidase N in ileum was increased. Even though MSB should not directly influence digestion of protein, it is possible that through stimulation of enterocyte mitosis and a decrease in apoptosis (Górka et al., 2014), butyrate may support epithelial function and brush border enzymatic activity. Although I have observed increased brush border enzymatic activity in calves supplemented with MSB, it did not have positive impact on growth performance (Chapter 4), likely due to negative impacts on ruminal absorptive surface area and a lack of effect on peptide and AA transporter expression in the small intestine. In humans, butyrate supplementation was shown to stimulate expression of the peptide transporter *PEPT1* in colonic enterocytes (Dalmasso et al., 2008), which was not observed in the current study. Therefore, results of this study indicate that MSB is unlikely to prevent potential negative consequences of feeding CM to calves on GIT functions.

5.5.3 Interactions Between the Protein Source and MSB Supplementation

Not as many interactions were observed in the present study as were anticipated. For ruminal epithelial surface, ruminal papillae density and abomasal epithelial layer thickness, I observed that TCM resulted in the greatest values, while TCM-MSB resulted the least values. A similar treatment interaction pattern was also observed for the *GPR43* expression in the ruminal epithelium, although TSBM-MSB resulted in the greatest expression. Expression of *GPR43* is stimulated by SCFA, mostly by acetate, to lesser extent by propionate and butyrate (Brown et al., 2003), which could imply that there were some changes in ruminal fermentation between treatments that the current experimental model could not have detected. The treatment TCM-MSB resulted in the least number of ruminal papillae and epithelial surface area, which was the opposite result to what was hypothesized. The reason why TCM-MSB combination in the starter mixture had a negative impact on ruminal epithelium development is not clear; however, it could be related to lesser digestibility of CM, lesser content of NFC in starter (Chapter 4), and the impact of

butyrate on rumen motility. With butyrate being able to promote ruminal contractions (Kendall and McLeay, 1996), it could lead to greater movement of digesta within the rumen, possibly leading to desquamation of the epithelial cells by feed particles and deterioration of the epithelial layer, eventually limiting the absorptive capabilities. Alternatively, MSB could increase digesta flow out of the rumen, as indicated by increased omasal digesta mass and maltase activity in the last sections of the small intestine (see below). When combined with CM in the starter, such a mode of MSB action would lead to a reduction of rapidly fermentable carbohydrates available in the rumen, already at a lesser amount in starters containing CM, which reduced SCFA production in the rumen and ruminal epithelium growth. Therefore, the utilization of MSB when combined with CM might require evaluation of optimal MSB dose rate.

Additionally, few main effect interactions were observed in intestinal tissue variables, which pertain to the proximal jejunum and ileum of calves. In summary, TSBM-MSB treatment had the greatest lactase activity in proximal small intestine, whereas TCM-MSB had the greatest maltase activity in distal small intestine. Górka et al. (2014) did not observe any changes in lactase and maltase activities when supplementing MSB in the starter mixture; however, when fed in unprotected form in MR, butyrate increased middle and distal jejunal lactase activity and distal jejunal maltase activity. Considering post-weaned calves were used in the current study, the lactase activity should be slowly diminishing as they are no longer reliant on milk (Le Huerou et al., 1992). Maltase activity steadily increases with age after weaning (Le Huërou et al., 1990) and is an important part of the digestion process. The greatest maltase activity in the ileum was observed for TCM-MSB treatment, which also resulted in the smallest ruminal epithelial surface area and abomasal epithelial thickness. Interestingly, calves consuming TCM-MSB starter tended to consume the most starter overall, followed by TSBM (Chapter 4). This could have allowed for more feed to pass through the GIT and stimulate greater maltase activity in the ileum.

5.6 Conclusions

Calves fed CM as a protein source showed increased tissue weight and length of the small intestine, suggesting adaptation to less digestible feed. However, greater GIT tissue mass could contribute to less efficient feed use for calves fed starter mixtures with CM (Chapter 4). Furthermore, feeding starter mixture with CM to calves resulted in lesser expression of peptide

and AA transporters (*PEPT2* and *ATB0+*) in the small intestinal epithelium, suggesting a negative impact of CM on small intestinal epithelium function. Canola meal use in starter mixture may have reduced starter intake pre-weaning and G:F ratio during weaning and whole study periods; however, its negative impact on ADG was only visible during weaning (Chapter 4). However, similar negative impacts were not observed in the second study conducted within a project, in which even a greater starter mixture intake was noted when it contained CM (Chapter 4). As such, CM might be a potential replacement for SM as a major protein source in starter mixtures.

Microencapsulated sodium butyrate did not have as profound effect on performance (Chapter 4) nor GIT development as anticipated. Particularly, although MSB supplementation increased brush border aminopeptidase A and N activity, it did not improve performance of calves, especially in calves fed starter mixtures with CM. Furthermore, MSB supplementation resulted in decreased ruminal papillae length and papillae surface area in ventral sac of rumen, especially when MSB was combined with CM in starter mixture. Overall, MSB supplementation through whole rearing period (from birth trough weaning) might not benefit GIT development as previously anticipated.

6 EFFECTS OF CANOLA MEAL INCLUSION RATE IN STARTER MIXTURES FOR HOLSTEIN HEIFER CALVES ON DRY MATTER INTAKE, AVERAGE DAILY GAIN, RUMINAL FERMENTATION, PLASMA METABOLITES, AND TOTAL TRACT DIGESTIBILITY

Canola meal when compared with SBM, as a protein source in calf starters can decrease performance of calves as shown by lesser pre-weaning starter intake, weaning ADG, feed efficiency, and DM digestibility (Chapter 4, Study 1), while also increasing indicators of GIT development (Chapter 5), especially that of the abomasum and jejunum. However, that effect was not consistent among the two studies conducted in Chapter 4, where in Study 2, CM increased overall starter intake. Additionally, a recent publication by Hadam et al. (2016) showed that although full replacement of SBM with CM might decrease ADG and feed efficiency, only minor reductions in pre-weaning starter intake were observed when CM replaced 50% of the SBM crude protein. Considering the above, this study was designed to assess how CM inclusion rates, between 0% to 60% of CP supply, impact starter intake, growth, ruminal fermentation, blood metabolites, and apparent total tract digestibility for Holstein heifer calves.

6.1 Abstract

The objective of this study was to determine the effects of canola meal (**CM**) inclusion rate in pelleted starter mixtures for Holstein heifer calves on dry matter intake, average daily gain, ruminal fermentation, plasma metabolites, and total tract digestibility. At birth, 50 heifer calves were blocked by birth date and BW and within block, randomly assigned to 1 of 5 treatments containing pelleted starters with 0, 15, 30, 45, or 60% of crude protein supplied by CM instead of soybean meal (**SBM**). Pellets were formulated to have similar crude protein and starch content, and were presented to calves starting on d 8.0 ± 0.0 (mean ± SD) of age with starter intake measured daily. From 8.0 ± 0.0 d of age through d 35.3 ± 2.4 of calves were fed milk replacer at 15% of BW offered in 3 equal feedings at 0600, 1500, and 2100 h. Beginning on d 36.3 ± 2.4 a gradual 21-day step-down weaning process was imposed where no further milk replacer was provided starting on d 57.0 ± 0.0. Data for starter intake were compared prior to weaning, during

weaning transition, and post-weaning. On d 62.2 ± 0.8 of age, blood was collected every 4 h for a 24-h profile and analyzed for glucose, β-hydroxybutyrate, insulin, and urea concentrations. From d 66.2 ± 0.8 of age and extending for three days, fecal samples were collected to measure fecal nutrient output and to determine total tract digestibility. Additionally, ruminal fluid (d 70.2 ± 0.8 of age) was sampled at 1300 h through an esophageal tube connected to a vacuum pump. The pH of the ruminal fluid was measured and analyzed to determine short-chain fatty acid and ammonia concentrations. Starter intake tended to linearly decrease with increasing CM inclusion, especially during weaning and post-weaning stages of the study; however, average daily gain, body weight and G:F ratio did not differ among treatments. The molar proportion of acetate tended to linearly decrease, while propionate tended to linearly increase with greater CM inclusion in the starters. Crude protein digestibility linearly decreased, ruminal ammonia concentration was not affected by treatments, and plasma urea concentration tended to linearly decrease with increasing CM inclusion in the starter. In conclusion, CM can be used as a partial replacement for SBM by up to 60% of CP but increasing the inclusion rate can negatively affect starter intake and protein digestion.

6.2 Introduction

Canola meal (**CM**) is a common protein source used in diets for lactating dairy cattle and has been reported to increase milk yield when used as a replacement for soybean meal (**SBM**; (Huhtanen et al., 2011; Martineau et al., 2013). However, CM is not commonly used in diets for calves due to concerns with palatability (Miller-Cushon et al., 2014b). Full replacement of SBM with CM decreased starter intake (Chapter 4), ADG (Miller-Cushon et al., 2014b; Hadam et al., 2016), feed efficiency, and increased fecal fluidity (Hadam et al., 2016). Such negative responses are often related back to potential antinutritional factors such as glucosinolates, tannins, and phytic acid present in CM (Bell, 1993). However, CM currently used in animal nutrition has very low levels of glucosinolates and erucic acid (Bell, 1993), suggesting other factors, such as increased NDF, may influence the negative response. Relative to SBM, CM contains less CP and greater NDF concentrations (NRC, 2001; Paz et al., 2014). Thus, the use of CM as a replacement for SBM requires greater inclusion rates of CM to achieve the same dietary CP concentration, but also results in less starch and greater NDF concentrations. Neutral detergent fibre content of CM can be variable among years of production (Broderick et al., 2016) providing an additional cause for variability in responses among studies.

While negative effects on feed intake and growth of calves have been reported when CM completely replaces SBM, replacing 50% of the CP from SBM with CM did not negatively affect DMI or ADG (Hadam et al., 2016). Amino acid profile of CM also differs from SBM, with a greater concentration of Met (Paz et al., 2014); one of the limiting amino acids (**AA**) for growth of calves (Abe et al., 1998). Thus, CM use in calf starter mixtures could also yield positive results. Additionally, in Chapter 4, CM when compared with SBM, resulted in improved fecal score (implying more solid feces), further indicating that there might be potential benefits for its use. However, there are a limited number of studies evaluating graded CM inclusion rates, rather than full replacement of SBM (Hadam et al., 2016).

The hypothesis of this study was that including CM in a pelleted starter mixture by replacing up to 60% of the CP supplied by SBM would not affect negatively calf performance before and after weaning. This hypothesis was tested while starch and fiber contents were similar in experimental starters. The objective of this study was to determine the effect of CM inclusion as a partial replacement for SBM on DMI, ADG and feed efficiency before weaning along with

DMI, ADG, feed efficiency ruminal fermentation, nutrient digestibility, and blood metabolites in Holstein heifers after weaning.

6.3 Materials and Methods

This study was conducted between December 2016 and January 2018. Animal handling and sampling procedures were pre-approved by the University of Saskatchewan Animal Research Ethics Board (protocol no. 20100021) in accordance with the guidelines from the Canadian Council on Animal Care (Ottawa, ON).

6.3.1 Heifers, Housing, and Feeding Regimen

A total of 50 new-born Holstein heifers, with a birth weight of 39.8 ± 3.4 kg (mean ± SD), from the University of Saskatchewan Rayner Dairy Research and Teaching Facility (Saskatoon, SK, Canada) were enrolled in the study. Heifers were included in the study unless they were from a twin pregnancy. Calves were separated from their dam immediately after birth. Within 2 h of birth, calves were fed colostrum replacer (120 g of IgG, Headstart Bovine Dried Colostrum, The Saskatoon Colostrum Co. Ltd., Saskatoon, SK, Canada), and within 12 h of birth a second feeding of colostrum replacer was provided (60 g of IgG, Headstart Bovine Dried Colostrum). Within the first 24 h of life, calves were moved to the calf barn and their weight was recorded. Heifers were blocked by birth date and birth weight and within block were randomly assigned to 1 of 5 pelleted starter treatments differing in the CM inclusion rate where CM replaced 0 (**CM0**), 15 (**CM15**), 30 (**CM30**), 45 (**CM45**), or 60% (**CM60**) of crude protein (**CP**) supplied by SBM (Table 6.1). Animal care technicians were blinded to treatments using color-coded worksheets and containers. Starter CP and starch concentrations were balanced among treatments by adjusting the inclusion rates of corn grain, barley grain, corn gluten meal, and wheat bran. Starters were pelleted at the Canadian Feed and Research Centre (North Battleford, SK, Canada) by conditioning the starter mash in a Buhler ACHA System (Bühler Group, Uzwil, Switzerland) to add 2.5% moisture at atmospheric pressure. Secondly, using a UAS-Muyang pellet mill (model MUZL350II, Muyang, Yangzhou, China) the mash was pelleted using a 4.0 mm die, at 68°C for 25 s.

Table 6.1. Ingredient composition of the milk replacer and pelleted calf starters differing in canola meal inclusion rate: 0 (CM0), 15 (CM15), 30 (CM30), 45 (CM45) and 60% (CM60) of the CP originally supplied by soybean meal

Variable (%DM)	Treatment					Milk replacer[1]
	CM0	CM15	CM30	CM45	CM60	
Soybean meal	28.4	24.1	19.8	15.7	11.4	n/a
Canola meal	0.0	5.2	10.4	15.7	20.7	n/a
Barley	15.8	17.7	18.0	18.7	20.7	n/a
Corn	21.8	21.4	22.3	22.8	22.5	n/a
Corn gluten meal	1.0	1.5	2.0	2.5	3.0	n/a
Wheat bran	21.1	18.1	15.7	12.7	9.8	n/a
Salt	0.5	0.5	0.5	0.5	0.5	n/a
Limestone	2.2	2.2	2.2	2.2	2.2	n/a
Molasses	2.2	2.2	2.2	2.2	2.2	n/a
Mineral supplement[2]	1.1	1.1	1.1	1.1	1.1	n/a
Whey protein	2.6	2.6	2.6	2.6	2.6	n/a
Glycerol	2.5	2.5	2.5	2.5	2.5	n/a
Monocalcium phosphate	0.6	0.6	0.6	0.6	0.6	n/a
Titanium oxide	0.2	0.2	0.2	0.2	0.2	n/a

[1]Excel Pro-Gro, Grober Nutrition, Cambridge, ON, Canada.

[2]Composition: 17.1% Ca; 2.7% P; 5.0% Mg; 0.1% K; 7.0% Na; 10.8% Cl; 723.5 ppm Cu; 2,584.2 ppm Fe; 5,694.4 ppm Zn; 41.9 ppm I; 17.2 ppm Co; 43.0 ppm Se; 927,500 IU Vit. A; 158,230 IU Vit. D; 5,250 IU Vit. E.

Finally, the pellet was cooled in UAS-Muyang Counter Flow Cooler (model SLNF14X14A, Muyang, Yangzhou, China) to 5°C above room temperature. Cooling also removed the moisture that was incorporated during conditioning.

Calves were housed individually in pens (1.5 × 2 m) with wood shavings as bedding. Calves were weighed weekly at 1000 h with BW used to assess ADG and to adjust the amount of milk replacer (**MR**) provided. Milk replacer (Excel Pro-Gro, Grober Nutrition, Cambridge, ON, Canada) was mixed using 150 g (DM basis) of MR powder/L water. Milk replacer amounts offered and refused were recorded daily. During the first week, MR feeding rate was gradually increased until achieving 15% of BW offered in 3 equal feedings at 0600, 1500, and 2100h. Starting on d 35.3 ± 2.4 d of age, based on the day of the weekly BW measurement, calves were exposed to a 3-wk step-down weaning process. Calves received 10% of their BW in MR from d 36.3 ± 2.4 to 42.3 ± 2.4, 7% of BW from d 43.3 ± 2.4 to 49.3 ± 2.4 offered in two equal feedings at 0600 and 2100h, and 3% of BW from d 50.3 ± 2.4 to 56.0 ± 0.0 offered in a single meal at 0600 h. No further MR was provided starting on d 57.0 ± 0.0.

Calves received their respective pelleted starter from 8.0 ± 0.0 d of age with the amount of starter offered and refused recorded daily. Calves were provided sufficient starter to ensure that voluntary starter intake was achieved with fresh starter provided daily at 0700 h immediately after measuring pellet refusals. Corresponding to provision of starter, fresh water was available *ad libitum*. Samples of the starter mixtures and MR powder were collected weekly and dried at 55°C for 72 h. Samples were subsequently composited to yield monthly samples and ground to pass through a 1-mm sieve (Christy and Norris, Christy Turner Ltd., Chelmsford, United Kingdom). Samples were sent to Cumberland Valley Analytical Services (Hagerstown, MD) for chemical analysis of DM, organic matter, CP, NDF, ADF, starch, water soluble carbohydrates, ether extract, calcium, and phosphorus. Dry matter was analyzed using method 930.15 (AOAC, 2000) by drying the sample at 135°C. Crude protein was analyzed using method 990.03 (AOAC, 2000) with a Leco FP-528 Nitrogen Combustion Analyzer (Leco, St. Joseph, MI). Acid detergent fibre was analyzed using method 973.18 (AOAC, 2000) modified to incorporate the use of Whatman 934-AH glass microfiber filters with 1.5 μm particle retention. Neutral detergent fiber was analyzed as described by van Soest et al. (1991) using sodium sulfite and α-amylase and the same modifications as for the ADF method. Starch was analyzed according to Hall (2009). Ash, which served to calculate organic matter content, was analyzed with method 942.05 (AOAC, 2000), with 1.5 g sample

weight, 4-h ash time and hot weight being used. Fat was analyzed using method 2003.05 (AOAC, 2006) using Tecator Soxtec System HT 1043 Extraction unit (Tecator, Foss, Eden Prairie, MN). Water soluble carbohydrates were analyzed according to the method of DuBois et al. (1956). Mineral analysis was conducted using method 985.01 (AOAC, 2000) using Perkin Elmer 5300 DV ICP (Perkin Elmer, Shelton, CT).

On d 62.2 ± 0.8 (mean ± SD), jugular catheters (1.7 × 83 mm, 16 GA, BD Angiocath, Franklin Lakes, NJ) were inserted and blood samples were collected the following day at 0600, 1000, 1400, 1800, 2200, 0200 h to harvest plasma. At each sampling timepoint, 10 mL of blood was collected into a tube containing Na-heparin (plasma separation; 158 IU, Becton Dickinson, Franklin Lakes, NJ). Heparinized saline (2000 IU/L) was used to maintain catheter patency by flushing catheters following blood collection. Immediately after sampling, blood sample was placed on ice, and was centrifuged for 15 min at 1,600 × *g* at 4°C (Sorvall ST 16R, Thermo Scientific, Waltham, MA), following which the supernatant was collected and stored at -20°C. Plasma glucose concentration was analysed using an enzymatic reaction with glucose oxidase and peroxidase (No. P7119 Sigma, St. Louis, MO), dianisidine dihydrochloride (No. D3252 Sigma, St. Louis, MO) in a 96-well plate, and absorbance was measured at 450 nm using a spectrophotometer (Epoch 2, BioTek Instruments, Inc., Winooski, VT). Plasma insulin concentration was analyzed using the Mercodia Bovine Insulin ELISA kit (Mercodia, Uppsala, Sweden). B-hydroxybutyrate concentration in plasma was measured after deproteinization (Daykin et al., 2002) based on the method described by Williamson et al. (1962). Plasma urea concentration was analyzed using method by Fawcett and Scott (1960).

On d 66.2 ± 0.8 of age, fecal sampling was initiated to determine apparent total-tract digestibility. To facilitate this process, the starters fed during the study contained 0.22% DM titanium oxide (Table 6.1). Sampling was initiated on d 66.2 ± 0.8 and continued for 4 d resulting in 8 samples that represented every 3 h of a 24-h cycle. Fecal samples (100 g) were collected every 12 h directly from the rectum through manual stimulation, with a 3-h offset in collection times among subsequent days. Samples were composited by calf and frozen until being analyzed for DM and chemical composition as previously described for feed samples. Additionally, titanium concentration in feed, refusal, and fecal samples was measured according to the method of Myers et al. (2004). Determination of titanium included a pre-digestion of samples (Tecator Digestor Auto 1011 3844, FOSS) with 98% sulfuric acid in the presence of a catalyzer (FisherTab™ CT-

37 Kjeldahl Tablets K3011000, Fisher Scientific) for 2 h, addition of 30% hydrogen peroxide, filtration of samples through Whatman 541 filter paper (Maidstone, United Kingdom), and measurement of absorbance at 410 nm.

6.3.2 Ruminal Fermentation Characteristics

On d 70.2 ± 0.8 of age (13.2 ± 0.8 d after weaning), ruminal fluid was collected 6 h post feeding (1300 h) to evaluate ruminal short-chain fatty acid (**SCFA**) and ammonia concentrations. Ruminal fluid was sampled through an esophageal tube (Geishauser, 1993) connected to a vacuum pump (DOA-P704-AA, Gast Manufacturing, Inc., Benton Harbor, MI) with a digital timer (H5CX, OMRON, Tokyo, Japan) that allowed for momentary pressure release after every 3 s. The esophageal tube consisted of two components: a stainless steel end piece with a rounded tip (64.3 mm in length with a 15.8-mm outside diameter) that had 16 holes measuring 5.8 mm in diameter located 9.9 mm apart in 4 rows; and a 1.5-m plastic tube with an inner diameter of 9.0 mm and outer diameter of 15.0 mm. The first ruminal fluid collected (approximately 50 mL) was discarded to avoid saliva contamination. Afterwards, using a fresh container, ruminal fluid was collected, strained through two layers of cheese cloth, and pH was measured using portable pH meter (Accumet AP110, Fischer Scientific, Ottawa, ON, Canada). Two 10-mL ruminal fluid samples were collected and were added to 2 mL of meta-phosphoric acid (25% wt/vol) or 2 mL of 1% sulfuric acid. In some instances, the volume collected did not allow for two full 10-mL samples to be obtained. In such cases, the amount of ruminal fluid placed in each vial was recorded to correct for dilution as the amount of meta-phosphoric acid and sulfuric acid were held constant. Ruminal fluid could not be obtained from 6 calves (2 from CM15, 1 from CM30, 2 from CM45 and 1 from CM60) due to clogging of the sampling tube. Samples were frozen at -20°C until being analyzed for SCFA concentration using gas-chromatography (Agilent Technologies Inc., Santa Clara, CA) as described by Khorasani et al. (1996), and ammonia concentration was analyzed using the method detailed by Fawcett and Scott (1960).

6.3.3 Statistical Analysis

One calf had to be prematurely withdrawn from the study due to navel infection; however, it was replaced with another calf by the end of the study to maintain 10 calves per treatment. The number of calves per treatment was determined based on observed means and standard deviation for DMI and ADG in Chapter 4 and Hadam et al. (2016). Using this data, a power analysis utilizing a one-way analysis of variance (SAS, version 9.4, SAS Institute, Cary, NC) was conducted, resulting in estimated statistical power above 0.97 for both variables. Milk replacer intake, starter intake, and ADG were determined based on: 1) the full study (d 8 to d 71 of age; **overall**); 2) while calves were fed their full allocation of MR (d 8 to d 35 of age, **MR fed**; 3) the period where MR was gradually reduced to promote weaning (d 36 to d 56 of age; **weaning**); and 4) the post-weaning phase (d 57 to d 71 of age; **post-weaning**). These data were considered as distinct biological periods given the changes in MR and starter feeding. Data collected that yielded a single observation for each calf (initial and final BW, average MR intake, average starter intake, feed efficiency, ADG, ruminal pH, SCFA concentration and molar proportions of individual SCFA, ammonia concentration, apparent total tract digestibility) were analyzed as a randomized complete block design including treatment as a fixed effect and block as a random effect. Polynomial contrasts were used to evaluate the linear and quadratic responses to increasing CM inclusion in the pelleted starter mixtures (SAS, version 9.4, SAS Institute, Cary, NC).

For variables collected over time (plasma glucose, insulin, urea, and BHB) the model also included hour and the treatment × hour interaction as fixed effects with time included as a repeated measure. Additionally, starter and MR intakes were run as repeated measures with day as a measure of time, whereas ADG was analyzed weekly. The covariance error structure that yielded the least Akaike's and Bayesian Information Criterion values were determined and used for each variable independently.

6.4 Results

As designed, starters were similar in CP and starch concentrations (Table 6.2); although, ADF and NDF concentrations numerically increased and the water-soluble carbohydrate concentration numerically decreased with increasing CM inclusion. When analyzed as

Table 6.2. Nutrient composition of the milk replacer and pelleted calf starters differing in canola meal inclusion rate: 0 (CM0), 15 (CM15), 30 (CM30), 45 (CM45) and 60% (CM60) of the CP originally supplied by soybean meal

Variable (%DM)	Treatment					Milk replacer[1]
	CM0	CM15	CM30	CM45	CM60	
n	13	13	13	13	14	13
DM, %	90.8 ± 1.4[5]	90.4 ± 1.5	90.6 ± 1.2	90.5 ± 1.2	90.8 ± 1.7	94.3 ± 0.2
Ash	8.6 ± 0.3	7.6 ± 1.0	7.7 ± 0.9	8.8 ± 0.2	8.7 ± 0.9	6.6 ± 0.2
CP	24.4 ± 0.2	24.6 ± 0.4	24.2 ± 0.4	24.2 ± 0.3	24.1 ± 0.2	27.0 ± 0.4
ADF	7.2 ± 0.6	7.9 ± 0.3	8.3 ± 0.4	9.7 ± 0.4	9.9 ± 0.6	0.8 ± 0.5
NDF	17.4 ± 0.8	16.9 ± 0.6	18.4 ± 1.1	18.1 ± 0.3	18.2 ± 0.5	n/a
Starch	26.8 ± 0.9	26.8 ± 0.4	26.2 ± 0.4	26.6 ± 0.6	26.7 ± 0.4	n/a
WSC[2]	13.6 ± 1.0	12.9 ± 1.0	13.6 ± 1.3	11.8 ± 2.0	12.3 ± 0.6	55.6 ± 6.5
EE[3]	2.8 ± 0.1	2.5 ± 0.1	2.9 ± 0.1	2.7 ± 0.1	2.7 ± 0.2	20.2 ± 0.7
Ca	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	n/a
P	1.0 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	n/a
ME[4], Mcal/kg	3.13 ± 0.01	3.14 ± 0.03	3.16 ± 0.03	3.11 ± 0.01	3.12 ± 0.03	4.86 ± 0.03

[1]Excel Pro-Gro, Grober Nutrition, Cambridge, ON, Canada.
[2]WSC = water soluble carbohydrates.
[3]EE = ether extract.
[4]ME = metabolizable energy, calculated based on NRC (2001) for the starters as: (1.01 × DE – 0.45) + 0.0046 × (EE% – 3), where DE = digestible energy, calculated as (0.057 CP% + 0.094 EE% + 0.0415 (100 – CP% – EE% – ash%)) × 0.82; and for the milk replacer as: 0.96 × (0.97 × (0.057 CP% + 0.092 EE% + 0.0395 × (100 – CP% – EE% – ash%))).
[5]Mean ± SD.

repeated measures, no interactions between CM level treatment and time were discovered for MR intake, starter intake and ADG ($P \geq 0.36$, data not shown), however the three variables differed across time ($P < 0.001$, data not shown). When analyzed as polynomial contrasts, initial and final BW, MR intake, cumulative MR intake, ADG and G:F ratio did not differ among treatments ($P \geq 0.15$, Table 6.3). Starter intake during the MR fed period did not differ among treatments ($P \geq 0.17$); however, when measured overall ($P = 0.055$), or during weaning ($P = 0.063$) and post-weaning ($P = 0.053$) phases, starter intake tended to linearly decrease with increasing CM starter content. Similar observation was made for cumulative starter intake, which did not differ during the MR fed period ($P \geq 0.12$), and decreased linearly during weaning period ($P = 0.042$) and tended to decrease linearly during overall and post-weaning periods ($P = 0.098$).

Ruminal pH and SCFA concentration were not affected by the CM inclusion rate ($P \geq 0.33$;Table 6.4). The molar proportion of acetate tended to decrease linearly with increasing CM inclusion in the starters ($P = 0.069$), whereas the molar proportion of propionate tended to linearly increase with increasing CM concentration ($P = 0.075$). The molar proportions of iso-butyrate, butyrate, and iso-valerate did not differ among treatments ($P \geq 0.17$) while the molar proportion of valerate decreased linearly with increased CM inclusion ($P = 0.034$). Caproate was not affected by treatment ($P \geq 0.14$) and neither was ruminal ammonia concentration ($P \geq 0.18$).

Apparent total tract digestibility of DM and starch were not affected by CM inclusion rate ($P \geq 0.12$,Table 6.5). However, CP digestibility tended to decrease linearly with increasing CM inclusion rate ($P = 0.084$). Ether extract digestibility responded quadratically ($P = 0.011$), decreasing from CM0 to CM15, increasing for CM30 and CM45, and decreasing for CM60. Unfortunately, digestibility could not be calculated for the NDF and ADF portion of the starters, likely due to bedding consumption.

I did not observe treatment by time interactions for any of the analyzed blood metabolites ($P \geq 0.28$; data not shown); however, for glucose ($P < 0.001$), insulin ($P = 0.023$), and BHB ($P < 0.001$) differences were observed among hours of sampling (data not shown). Plasma glucose nor BHB concentrations did not differed among treatments ($P = 0.46$, Table 6.6). Plasma insulin concentration resulted in a quadratic response to increasing CM inclusion ($P = 0.045$), with insulin concentration being numerically greater for CM0, CM15, and CM60 treatments and least for CM30 and CM45. Plasma urea concentration tended to linearly decrease with greater CM inclusion in the starters ($P = 0.056$).

Table 6.3. Daily milk replacer (MR) and starter intake, and performance characteristics of Holstein heifer calves fed different levels of canola meal inclusion: 0 (CM0), 15 (CM15), 30 (CM30), 45 (CM45) and 60% (CM60) of the crude protein originally supplied by soybean meal

Variable	Treatment					SEM	*P*-value	
	CM0	CM15	CM30	CM45	CM60		Linear	Quadratic
n	10	10	10	10	10			
Initial BW, kg	39.6	39.7	40.0	40.0	39.5	1.1	0.97	0.73
Final BW, kg	101.1	103.5	101.2	99.2	99.0	2.4	0.26	0.58
MR intake[1], kg DM/d								
Overall	0.92	0.94	0.94	0.92	0.94	0.02	0.79	0.84
MR fed	1.03	1.07	1.05	1.05	1.06	0.03	0.80	0.59
Weaning	0.80	0.80	0.81	0.77	0.82	0.03	0.70	0.53
Cumulative MR intake, kg								
Overall	51.70	52.89	52.69	51.65	52.82	1.18	0.79	0.84
MR fed	36.60	37.94	37.22	37.19	37.39	0.85	0.75	0.61
Weaning	15.08	14.32	15.45	14.44	15.59	0.50	0.40	0.29
Starter intake[1], kg DM/d								
Overall	1.00	1.00	1.00	0.94	0.84	0.06	0.055	0.30
MR fed	0.12	0.14	0.13	0.11	0.09	0.02	0.17	0.27
Weaning	0.89	0.93	0.90	0.87	0.67	0.09	0.063	0.14
Post-weaning	2.82	2.77	2.68	2.59	2.48	0.14	0.053	0.84
Cumulative starter intake, kg								
Overall	63.46	64.93	62.80	60.03	53.72	4.03	0.054	0.27
MR-fed	3.29	3.89	3.55	2.92	2.33	0.63	0.12	0.22
Weaning	18.90	19.74	19.10	18.33	14.09	1.83	0.042	0.104
Post-weaning	41.30	41.32	40.16	38.80	37.26	1.98	0.098	0.66
Feed efficiency, kg gain/ kg DM								
Overall	0.535	0.543	0.531	0.530	0.559	0.013	0.40	0.29
MR fed	0.712	0.697	0.712	0.685	0.745	0.026	0.52	0.27
Weaning	0.506	0.546	0.529	0.540	0.540	0.026	0.43	0.56
Post-weaning	0.384	0.383	0.353	0.366	0.368	0.026	0.56	0.63
ADG[2], kg/d								
Overall	0.88	0.92	0.89	0.86	0.86	0.031	0.30	0.58
MR fed	0.78	0.83	0.82	0.76	0.81	0.035	0.97	0.65
Weaning	0.84	0.89	0.87	0.84	0.78	0.062	0.37	0.30
Post-weaning	1.10	1.13	1.06	1.13	1.17	0.067	0.77	0.83

[1]Overall = d 8 to 71 of age; MR fed = d 8 to 35 of age; Weaning = d 36 to 56 of age; Post-weaning =d 57 to 71 of age.
[2]ADG = average daily gain.

Table 6.4. Ruminal fermentation characteristics on d 70.2 ± 0.8 of age in Holstein heifer calves fed pelleted starter mixtures differing in canola meal inclusion rate: 0 (CM0), 15 (CM15), 30 (CM30), 45 (CM45) and 60% (CM60) of the crude protein originally supplied by soybean meal

Variable	Treatment					SEM	P-value	
	CM0	CM15	CM30	CM45	CM60		Linear	Quadratic
n	10	8	9	8	9			
Ruminal pH	5.65	5.54	5.70	5.51	5.57	0.15	0.64	0.97
SCFA[1] concentration, m*M*	118.3	133.1	111.3	132.6	128.0	6.7	0.33	0.85
Acetate, mol/100 mol	47.6	47.1	46.5	48.9	43.8	1.1	0.069	0.12
Propionate, mol/100 mol	36.0	38.0	36.0	38.3	40.9	2.5	0.075	0.39
Iso-butyrate, mol/100 mol	0.32	0.22	0.31	0.40	0.31	0.10	0.43	0.90
Butyrate, mol/100 mol	8.54	10.18	8.43	5.81	9.41	1.44	0.48	0.48
Iso-valerate, mol/100 mol	0.322	0.343	0.342	0.434	0.360	0.092	0.24	0.58
Valerate, mol/100 mol	3.73	4.49	3.45	3.50	3.23	0.43	0.034	0.36
Caproate, mol/100 mol	1.30	0.73	1.19	1.15	0.83	0.23	0.35	0.98
Ammonia, mg/dL	4.00	3.00	3.40	5.05	3.42	0.71	0.63	0.99

[1]SCFA = short-chain fatty acid.

Table 6.5. Apparent total tract digestibility post-weaning of pelleted calf starters differing in canola meal inclusion rate: 0 (CM0), 15 (CM15), 30 (CM30), 45 (CM45) and 60% (CM60) of the CP originally supplied by soybean meal

Apparent total tract digestibility, % DM	Treatment					SEM	*P*-value	
	CM0	CM15	CM30	CM45	CM60		Linear	Quadratic
n	10	10	10	10	10			
DM, %	69.9	69.2	68.7	69.9	68.8	0.7	0.45	0.77
CP	73.0	70.7	72.6	72.5	70.0	1.0	0.084	0.44
Starch	98.5	98.1	98.2	99.2	98.8	0.3	0.12	0.48
Ether extract	67.1	65.2	74.4	74.0	65.0	2.4	0.54	0.011

Table 6.6. Blood plasma parameters of calves on 62.2 ± 0.8 d of age as influenced by different canola meal inclusion rates in pelleted starter mixtures: 0 (CM0), 15 (CM15), 30 (CM30), 45 (CM45) and 60% (CM60) of crude protein originally supplied by soybean meal

Variable	Treatment					SEM	*P*-value	
	CM0	CM15	CM30	CM45	CM60		Linear	Quadratic
n	10	10	10	10	10			
Glucose, mg/dL	62.7	61.1	61.8	58.8	61.7	2.0	0.50	0.46
Insulin, µg/L	0.549	0.559	0.433	0.403	0.594	0.066	0.73	0.045
BHB[1], mg/dL	2.40	2.45	2.53	2.07	2.71	0.32	0.81	0.58
Urea, mg/dL	13.1	12.3	11.7	12.1	12.0	0.5	0.056	0.15

[1]BHB = β-hydroxybutyrate.

6.5 Discussion

Although previous studies have reported that incorporation of CM in starter mixtures as a full replacement for SBM reduces starter intake (Chapter 4) and growth (Hadam et al., 2016) for calves around weaning, no studies known to the author have assessed how a series of CM inclusion rates affects calf responses. Fiems et al. (1985) evaluated the use of rapeseed meal with low or high glucosinolate content in calf starters when included at either 10 or 20% of dietary DM. In that study, they reported that starter intake was decreased only when the high glucosinolate variety of CM was fed. In a more recent study, Hadam et al. (2016) reported that partial replacement (50% of CP) of SBM with CM only tended to decrease pre-weaning starter intake, as opposed to decreased ADG, G:F ratio and number of days with diarrhea when full replacement of SBM with CM was fed. Using weaned lambs, Mandiki et al. (1999) suggested that CM inclusion rate in starter of 25 to 30% was optimal based on growth rate and hormonal concentrations. These studies have provided evidence that use of CM can be acceptable with partial but not full replacement of SBM. However, the studies available have used a limited number of inclusion rates (Hadam et al., 2016) and do not consistently consider the comparison of CM and SBM contribution on an equal protein basis. Additionally, most studies do not balance the starch content of the starters, being more concerned about the CP content of the starters. When CM is included as a protein source in calf starters, the starch concentration numerically decreases (Chapter 4), whereas NDF and ADF concentration numerically increases (Chapter 4; Hadam et al., 2016).

In the present study, increasing CM inclusion to account for an increasing proportion of starter CP tended to decrease starter intake, especially in later stages of the study (weaning and post-weaning) when calves were consuming greater quantities of starter. However, the decrease in starter intake did not affect ADG, BW and G:F ratio. Past research has reported that complete substitution of SBM with CM reduces starter intake pre-weaning when fed to bull calves but may increase cumulative starter intake post-weaning when fed to heifers (Chapter 4). Hadam et al. (2016) reported decreased starter intake pre-weaning with the partial replacement (50% of CP) of SBM, but not when measured post-weaning. A possible explanation for the reduced starter intake with increasing CM inclusion in the present study could be low palatability of CM that has previously been reported (Miller-Cushon et al., 2014b). As such, the palatability of CM would have a lesser impact on starter intake with low inclusion rates or lesser consumption rates such as

observed pre-weaning. While I cannot attribute specific factors that caused the reduction in starter intake, antinutritional compounds such as tannins or phytic acid present in CM (Bell, 1993), may be candidates and the concentration of these compounds would increase within increasing CM inclusion. However, the decrease in starter intake was not severe enough to impact ADG nor G:F ratio.

While ADG and G:F ratio were not affected, I observed a linear decrease in the apparent total-tract CP digestibility with increasing CM inclusion in starters. I have expected a reduction in ADG with the decreased CP digestibility, which did not occur. Others have also reported a reduction in CP digestibility when replacing SBM with CM in starter mixtures for calves (Khorasani et al., 1990) and feedlot cattle (Zinn, 1993). Given that CM can have a greater proportion of RUP than SBM (Broderick et al., 2016), shifting the site of digestion towards post-ruminal regions of GIT may limit total tract digestibility in calves. This statement is further supported by the relatively low ruminal ammonia concentrations when compared with previous studies (Rey et al., 2012, Chapter 3). The low ruminal ammonia may suggest that, in general, ruminal protein digestibility was limited; however, relatively high starch concentration in the pellet provided a source of rapidly fermentable carbohydrate could also allow microbes to utilize the available ammonia. As reported in Chapter 5, protein source (CM compared to SBM) does not affect the activity of intestinal proteolytic brush-border enzyme activity in calves nor did it affect pancreatic secretions (Khorasani et al., 1990), further suggesting that increasing RUP supply may decrease total tract CP digestibility. This speculation is further supported by the tendency for a linear decrease in plasma urea concentration in the current study which has been also reported previously when CM fully replaces SBM (Chapter 4). Additionally, in the same study, expression of peptide transporter 2 in proximal jejunum and neutral amino acid transporter in the ileum was decreased when calves were fed CM starters, which could have contributed to decreased absorption and lesser CP digestion in the whole GIT. Unfortunately, I did not assess ruminal nitrogen kinetics or indicators for microbial protein supply and hence, reasons for the reduction in CP digestibility and plasma urea cannot be confirmed.

The total concentration of SCFA tended to differ among treatments without any detectable linear or quadratic responses. Canola meal has been reported to decrease SCFA concentrations when compared with SBM during *in vitro* incubation (Getachew et al., 2004; Paula et al., 2017). Also, as discussed by Paula et al. (2017), the shift in SCFA concentration might stem

from differences in NFC content of the feed, rather than from the protein source itself. Even though carbon chains arising from AA can be fermented to produce SCFA, this effect is promoted when carbohydrate availability is limited (Bach et al., 2005). Considering that the ruminal SCFA concentration was at or slightly above what has been reported for calves of a similar age (Rey et al., 2012) and that cumulative ADG was more than 1 kg/d, there is little evidence to support that carbohydrate availability or energy supply were limited in the current diets. As such, in the present study, the differences in SCFA concentration may be attributed to changes of the starter composition to allow for iso-nitrogenous and iso-starch starters. For example, CM0 contained the least barley (15.8% DM) and the most wheat bran (21.1% DM); whereas, CM60 contained the most barley (20.7% DM) and the least wheat bran (9.8% DM). This statement becomes important in substitution studies as CM and SBM cannot be simply and equally replaced and hence, while I attribute results to the protein source effect, I cannot ignore potential confounding factors such as starch and fiber sources.

The molar proportion of acetate tended to decrease while the molar proportion of propionate tended to increase with increasing CM inclusion rate. Paula et al. (2017) reported no differences for acetate and propionate concentration when comparing CM and SBM *in vitro* using continuous culture, but observed a decrease in the molar proportion of butyrate with CM. In another study, Getachew et al. (2004) observed no change in acetate and butyrate, but a decrease in propionate. Thus, changes in molar proportions of individual SCFA differ based on the experimental model with no clear pattern when CM replaces SBM. Based on the results of this study and when considering previous studies, it is possible that the protein did not directly influence the molar proportions of acetate and propionate, as discussed previously for the total SCFA concentration. In fact, it is possible that changes in ruminal acetate and propionate concentrations could be due to changes in NDF concentration or composition of starch source with increasing CM inclusion. With respect to starch, diets in the present study were balanced for the starch content by increasing predominantly the barley inclusion with increasing CM inclusion rate coupled with a decrease in the inclusion of wheat bran content. These changes were necessary as CM and SBM have differing CP concentrations. While these changes did not affect total tract starch digestibility, I cannot confirm whether rate of ruminal fermentation or the site of digestion was altered.

I also observed a linear decrease for the molar proportion of valerate in ruminal fluid. The molar proportion of valerate observed during the current study was similar to that observed in the other calf studies (Laarman et al., 2012; Terré et al., 2013) and greater than that in mature dairy cows (Beauchemin and Yang, 2005; Paddick et al., 2019). Similar to the observed decrease in the molar proportion of valerate in the present study, a previous study had also observed a reduction in the molar proportion of valerate when SBM was fully replaced with CM and was attributed to the AA composition and fermentation of those AA within the protein sources (Chapter 4 and 5). A reduction in valerate concentration has also been observed during 24-h *in vitro* incubation of CM when compared with SBM (Getachew et al., 2004); however, opposite effects have been observed using continuous culture comparing inclusion of CM with the replacement of SBM (Paula et al., 2017). Although valerate is present in the rumen at a relatively low concentration compared to other SCFA, it is a preferred substrate for the ruminal epithelium, second only to butyrate (Weigand et al., 1975). Hence, increased valerate concentrations may help to stimulate ruminal epithelial development and be an important metabolite for calves in the early post-weaning phase.

In current study, I observed a quadratic response for plasma insulin concentration, and the pattern of response was similar to the change in ruminal propionate concentration. For example, the greatest concentration of both propionate and insulin was observed for the CM60 treatment. A large proportion of the propionate can be recovered in the portal-drained viscera of ruminants (Kristensen and Harmon, 2004a); whereas, in the normally functioning rumen infused with SCFA the recovery is not as great. In general, ruminal epithelial tissue was suggested to convert between 3 to 15% of propionate into lactate (Bergman, 1990). Propionate stimulates the release of insulin in sheep and cattle (Horino et al., 1968) and hence the increase in propionate concentration may explain the insulin response despite differing sampling schedules for blood (measured 6 times over a 24-h period) and ruminal fluid (measured once at 1300 h).

Glucose concentrations did not differ among treatments and were within normal ranges for weaned calves (Quigley et al., 1991). The concentration of BHB did not differ among treatments despite reductions in starter mixture intake with increasing CM inclusion. Although BHB concentration in serum is positively linked to starter intake (Quigley et al., 1991), the lack of effect likely stems from no differences in ruminal butyrate (Kristensen and Harmon, 2006).

6.6 Conclusions

Increasing the CM inclusion rate in the starter mixture reduced starter intake, ruminal molar proportion of valerate, CP digestibility, and plasma urea concentration. However, ADG and BW gain of the calves was not affected. As such, CM could be considered as suitable protein source used to partially replace SBM in starter diets for diary heifers by up to 60% of CP.

7 GENERAL DISCUSSION

7.1 Summary of Findings

The success of calf rearing during the weaning transition heavily depends on adequate intake of a high-quality starter mixture. Soybean meal is a commonly used protein source within calf starters; whereas, CM has been regarded as not suitable for calf consumption, due to issues with palatability, performance, and digestibility (Stake et al., 1973; Khorasani et al., 1990; Miller-Cushon et al., 2014a). However, available research has not consistently reported reductions for indicators of calf performance when CM is used in calf starters (Table 2.2). My hypothesis was that CM can be a comparable protein source to SBM both in terms of calf performance and GIT development, and that the response of calves fed CM could be improved by inclusion of ingredients that can either mask the flavour of CM or stimulate the development of the GIT, as well as by additional processing of CM to increase the RUP fraction of CM. In order to test my hypothesis, I conduced 5 different experiments.

As described in Chapter 3, a calf study was designed to establish if heat-treatment of CM and glycerol supplementation would affect the performance and GIT development of calves at weaning. Firstly, using an in situ approach with mature Hereford-cross heifers, I established that a temperature of 110°C held for 10 min was optimal for achieving the least ruminal protein DM and CP degradation, while maintaining estimated intestinal CP digestibility. Increasing that temperature to 120°C further decreased RDP, but also decreased the estimated intestinal CP digestibility. Consecutively, in a calf study, it was established that only few interactions between CM heat-treatment and glycerol supplementation were present, most with no physiological relevance. Whereas, heat-treatment of CM resulted in reduced growth parameters of calves based on final BW, ADG, and total BW gain. Heat-treated CM decreased starter intake during the week before weaning. Heat-treatment of CM increased the molar proportion of ruminal butyrate without affecting total SCFA concentration. This surprisingly resulted in decreased weight of rumen and jejunum. Inclusion of glycerol resulted in increased starter intake, ADG, and total BW gain. Glycerol increased ruminal SCFA concentration, without affecting molar proportion of butyrate, nor development of the rumen, based on ruminal tissue weight. Glycerol inclusion led to greater weight of jejunum. Glycerol increased digesta weight in abomasum, duodenum, jejunum and

cecum. Glycerol downregulated the expression of SCFA transporter *MCT1* in the rumen and upregulated it in the jejunum.

Chapters 4 and 5 form one cohesive unit analyzing the effect of protein source, either CM or SBM, and MSB supplementation in dairy calf starters on performance (Chapter 4) and GIT development (Chapter 5). In order to analyze the performance of calves, two studies were conducted, with the first one using bull calves (Study 1) and second one using heifer calves (Study 2). There were minor differences between the designs of the two studies. During Study 1 (Chapter 4) the use of CM in the starters in place of SBM, resulted in decreased pre-weaning starter intake, although no changes in starter intake were observed during weaning and post-weaning periods and when evaluated overall. During weaning period, ADG and G:F ratio were decreased when CM was fed. Use of CM in starters decreased fecal score during weaning and post-weaning periods, and when considering whole study period, indicating firmer feces during those times. The effect of MSB during Study 1 included increased pre-weaning starter intake, and decreased blood urea on d 43 and d 63 of the study. During Study 2 (Chapter 4), use of CM as a protein source increased overall starter intake, as well as post-weaning cumulative starter intake, and decreased post-weaning and overall fecal score, as well as number of days with diarrhea. Meanwhile the only effect of butyrate supplementation was decreased withers height.

Concerning GIT development (Chapter 5) when CM was used as protein source in calf starters, CM decreased ruminal ammonia concentration, ruminal molar proportion of valerate, ruminal damage index, and increased abomasal and jejunal tissue mass, jejunal length, upregulated expression for *MCT4* and *GPR43* in the abomasum, and downregulated *PEPT2* in the proximal jejunum and *ATB0+* in the ileum. Meanwhile, MSB supplementation decreased ruminal papillae length and epithelial surface area, upregulated *MCT1* expression in the rumen, increased omasal digesta weight, decreased omasal epithelium layer thickness, and increased duodenal aminopeptidase A, and ileal aminopeptidase A and N activities.

The study described in Chapter 6 evaluated how the level of CM inclusion rate in calf starters affects performance (ADG, DMI intake, feed efficiency) and ruminal fermentation of heifer calves. In this study, increasing CM inclusion rate decreased starter intake when considered overall, and during the step-down and post-weaning periods. However, the reduction in starter intake did not affect BW, ADG, or feed efficiency. Additionally, with increasing CM inclusion, the molar proportion of acetate decreased, while propionate increased in ruminal fluid. Apparent

digestibility of CP decreased linearly, while EE digestibility was affected quadratically. Plasma urea concentration decreased with greater rate of CM in the starters.

7.2 Constraints of the Experimental Designs

In Chapter 3, CM heat-treatment was first tested to establish a heat-treatment protocol used for the calf study. Ruminal digestibility was assessed using in situ experimental incubation in mature Hereford-cross heifers that were ruminally cannulated, while intestinal digestibility was estimated from *in vitro* digestion. Vazquez-Anon et al. (1993) postulated that in situ digestion measured in mature cows can be used to estimate digestion in calves around weaning. Especially considering that ruminal fermentation characteristics in calves can be similar at weaning and for few weeks following (Quigley et al., 1985; Lalles and Poncet, 1990). As such, the in situ approach using mature cattle was chosen to qualitatively establish the effects of CM heat-treatment on ruminal and intestinal digestibility of DM and CP.

One of the likely limitations of the studies evaluating the heat-treatment of CM was the application of heat-treatment post-processing. As part of the current canola processing practices, canola is heated to temperatures between 95 and 115°C for 30 min (Canola Council of Canada, 2015). However, the idea of additional heat application has been evaluated for CM (McKinnon et al., 1991; McKinnon et al., 1995) and SBM (Obitsu et al., 1995) in the past and served as the basis for the current experimental design.

To ensure treatments imposed had a great enough magnitude to measure responses (Chapter 3), the amount of RUP supplied by CM in the starter mixture was calculated. Until, 35 d of age, when MR was the dominant source of energy for the calves, CM in the starter supplied only around 5% of total RUP. This changed during the step-down phase when CM supplied 42.9 to 50.11% of RUP, and the amount did not shift much until weaning. Although these changes only affected that last two weeks before weaning, when considering RUP fraction, it is meant to affect the small intestine of calves, which can replace its intestinal lining within 2 to 3.5 d (Darwich et al., 2014). With these calculations, I confirmed that the amount of RUP supplied by CM during step-down and weaning was substantial when compared with MR RUP supply and could have physiological impact on calves.

The calf studies were conducted using both heifer calves and bull calves. From practical standpoint, the studies that involved dissections (Chapter 3 and 5) were conducted on bull calves, whereas the performance studies were conducted using heifer calves. Both heifer and bull calves serve different purposes on dairy farms: heifers will form the future replacements for the milking dairy herd; while bulls may be kept as breeding stock (very limited number) or raised for veal or beef production. Differences can be observed between bull and heifer calves in terms of BW gain and frame size where bull calves grow bigger than heifers (Koch et al., 1973; Hopkins, 1997; Ware et al., 2015). It is unclear if GIT development can be affected by sex of the calves. Tahmasbi et al. (2014) observed that up to 6 week of age, the size of the stomach (measured in cm) does no differ between heifers and bull calves; however, this observation does not allow for more in depth analysis of epithelial development or changes in the ruminal fermentation or enzymatic activities tied to digestion processes. Considering the changes in growth with no clear response observed for starter intake between calf sexes (Hopkins, 1997), establishing whether those differences are a result of GIT adaptations or post-digestion partitioning of energy could be imperative. This issue could be addressed in future research, utilizing ruminal cannulation (Kristensen et al., 2010) or biopsy techniques (van Niekerk et al., 2018) to ensure that dissection studies conducted in bull calves are representative of those in heifer calves.

Additionally, while calves in Chapter 3 and 6 were protected from the environmental elements, the calves used in Chapters 4 and 5 were housed in semi-open barn that allowed each calf constant and free choice access to an individual outdoor paddock. Although the outdoor paddocks could be considered a more enriched housing style for the calves, the calves in Chapter 4 and 5 were subject to weather conditions which could have altered response to the experimental treatments. Study 1 (Chapter 4 and 5) using bulls was conducted during winter/spring time, whereas Study 2 (Chapter 4) with heifers was conducted in summer. Rauba et al. (2019) observed previously that calves born in winter consume more MR and starter, likely due to greater energy requirements with decreased ambient temperature (NRC, 2001). The time of year when the studies were conducted could introduce additional variability. However, due to space limitations in the barn, the separation of the studies in time was unavoidable.

Another potential source of variability was that calves' pens were bedded with either wood shavings (Chapter 3, Chapter 4 – Study 1, Chapter 5, Chapter 6) or straw (Chapter 4 – Study 2). Calves can potentially consume the bedding material. However, use of bedding material is a

common practice during calf rearing. Keeping calves dry and allowing them a comfortable place to lay down is a requirement according to Canadian Code of Practice for the Care and Handing of Dairy Cattle (NFACC, 2009). Especially calves that were housed in the barn with constant outdoor access in Chapters 4 and 5, were at a greater risk of getting sick with diarrhea (Hänninen et al., 2003). Even some studies that compare different bedding materials for calves do not contain a negative control of no bedding (Hill et al., 2011). The consumption of bedding by calves could have introduced additional NDF into the calves' diet, which I was not able to quantify. Unknown consumption of bedding would result in an underestimation of NDF intake and hence digestibility. This was observed in Chapter 4, Study 1 and Chapter 6, when NDF and ADF digestibility resulted in negative values. Hill et al. (2019) observed that digestibility values did not change when calves were bedded with straw as opposed to rubber mats, implying that consumption of bedding was negligible. Considering benefits of bedding from an animal care perspective, the use of bedding is justified. Although the digestibility measurements might not be quantitatively compared with other experiments, they can serve to distinguish differences between treatments, since calves in those experiments had uniform bedding practices.

The constraints of the present studies listed above could add sources of variability among the studies. It should be recognized that differences in housing, bedding and environment, among others, exist between individual dairy farms. Awareness of such issues is important for adequate interpretation of data and should be further utilized when applying the results to the dairy industry operations.

7.3 Effect of CM on Dairy Calf Performance

Additional heat-treatment of CM at 120°C for 10 min can decrease apparent intestinal digestibility of CM, whereas, increasing heat-treatment temperature increases the ruminal undegradable fraction of both DM and CP. As such, the effect of heat-treatment of CM at 110°C on calf performance has been evaluated. Both final weight and ADG of calves fed heat-treated CM were less than for calves consuming non-heated CM. Decreased growth of the calves was likely due to decreased cumulative intake of calves that consumed heat-treated CM. The reason for lesser starter intake likely stemmed from lower palatability of heat-treated CM, which may have been affected by the Maillard reaction (van Soest and Mason, 1991). Additionally, calves consuming

heat-treated CM had a lighter rumen and jejunum, which could also imply smaller digestive capability of the GIT. Unfortunately, with no digestibility measurements in this study, the reasons for decrease in GIT weight could only be speculated. The effect of heat-treatment could have been greater in calves than in mature ruminants, by affecting either RUP fraction content or RUP intestinal digestibility.

When CM replaced SBM, it numerically increased the content of ADF and NDF (Chapter 4 and 6). As described in Chapter 4, CM inclusion decreased pre-weaning starter intake for bull calves, but increased starter intake during post-weaning phase and during whole experiment for heifers. In Chapter 6, increasing CM inclusion rate in starters linearly decreased starter intake during step-down and post-weaning phases, and when starter intake was considered for the whole experiment. However, the changes in starter intake generally did not affect the growth of the calves, only exception being decreased ADG and G:F ratio in Chapter 4 during weaning period for bull calves. Especially, ADG was not affected when CM was included at up to 60% of CP supply in the starters (Chapter 6). The factors that could have potentially influence the differences in the response were discussed previously (Chapter 7.2). The results, considering their variability, could be partially due to other changes in the starter resulting from different inclusion rates of CM and SBM required to achieve the same CP content. Overall, CM can have a negative effect on starter intake, which could relate to the lack of palatability of CM for calves (Miller-Cushon et al., 2014b), which can be further exacerbated by the heat-treatment application. Although some antinutritional factors can still be present in CM that can affect taste of the starter, like glucosinolates and sinapine, or its digestibility, like tannins (Bell, 1993). Additionally, the decrease in starter intake could also be due to the increase in NDF and ADF fraction of the starter when CM is included. However, considering few observed changes in ADG and G:F ratio, the use of CM could be promising as it would not compromise calf growth, especially at smaller inclusion rates of up to 60% CP.

7.4 Effect of CM on Dairy Calf Gastrointestinal Tract Development

The use of CM as replacement for SBM in dairy calf starters can result in greater abomasal and jejunal tissue weight. Whereas, heat-treated CM resulted in decreased ruminal and jejunal tissue weights. Even though heat-treatment to 110°C increased the estimated intestinal digestibility

in vitro, the digestible fraction of CM still decreased with greater application of heat. Such a decrease in digestibility could have a more severe effect on calves with not yet fully developed GIT. Although the heat-treatment of CM in the present study was used additionally to the already processed CM, this study showcased how important the quality of processing can be, especially if over-processing was to occur. With calves still developing during weaning transition, the quality of CM that is being fed to the calves needs to be assessed to ensure high quality of the meal. In addition to increasing weight of abomasum, CM also increased expression of *MCT4* and *GPR43*, SCFA transporter (Kirat et al., 2007) and receptor (Wang et al., 2009) respectively. This could indicate that use of CM, as opposed to SBM, can modify either post-ruminal SCFA supply or SCFA metabolism in the abomasum (Pennington, 1952). Both length and weight of jejunum increased with CM feeding, however, the expression of *PEPT2* in proximal jejunum and *ATB0+* in ileum were at the same time downregulated. These results could suggest that absorption of peptides and AA was limited in the small intestine when CM was included in the starters. Greater tissue weight could be a result of compensating for limited digestion, especially considering decreased ADG and G:F ratio accompanying CM feeding in those calves. However, brush border enzymes activity remained unchanged between CM and SBM feeding. Heat-treatment did not affect neither gene expression in the GIT of calves, nor activity of brush border enzymes in small intestine, suggesting that the major effects of heat-treated CM were a result of decreased starter intake. Use of CM in starters can have complex effect on development and digestion processes in GIT of calves.

An interesting observation across Chapter 5 and 6 was made, where the molar proportion of ruminal valerate decreased when CM was present in the starter mixture. Although valerate is present at relatively small proportion of the total SCFA (on average 3.91% in the current studies), it is a known source of energy for epithelial cells, preferentially following butyrate (Weigand et al., 1975; Kristensen et al., 2000). Based on the analysis of the AA content in CM and SBM starters (Chapter 4), the likely cause for greater valerate may be due to alterations in the AA concentrations (e.g. Pro, Arg, Lys and Met) which serve as precursors for valerate synthesis by the ruminal microbes (Andries et al., 1987). This decrease in valerate; however, did not translate into altered ruminal tissue weight, and might not be of physiological importance. Overall, the effects of CM on ruminal fermentation were scarce.

7.5 Glycerol and MSB as Supplements in Dairy Calf Starters

Originally, I hypothesized that glycerol included in starter mixture will increase starter intake, ruminal butyrate, and ruminal development in calves. The effects of including CM in starters only partially aligned with the experimental hypothesis. Glycerol increased starter intake of calves (Chapter 3), as was expected due to its sweet taste (Quispe et al., 2013). I expected glycerol to be fermented in the rumen (Hobson and Mann, 1961) and to increase the proportion of ruminal butyrate (Rémond et al., 1993; Paiva et al., 2016), therefore stimulating ruminal development. Although glycerol supplementation did increase the concentration of ruminal SCFA (Chapter 3), there were no changes observed regarding the molar proportions of acetate, propionate, or butyrate. Since there was no change in the butyrate molar proportion, it was not surprising that ruminal weight did not differ among treatments. That said, I observed greater weight of jejunum and colon, upregulation of *MCT1* in the proximal jejunum and greater dipeptydylpaptidase activity in middle jejunum in calves supplemented with glycerol. It has been previously estimated that 30% of glycerol may flow out of the rumen in mature dairy cows (Werner Omazic et al., 2015) and if a similar proportion of glycerol could pass into omasum of calves, it could contribute to an increase in intestinal weights. The likelihood for post-ruminal glycerol action is further supported by greater digesta weights in the abomasum, duodenum, jejunum and cecum in calves were fed starters containing glycerol in 6 h post-feeding. Although not through the expected mechanism, as ruminal butyrate did not differ among treatment, glycerol did stimulate the development of the calves' GIT, especially in the intestine.

MSB supplementation, is known to increase the ruminal development of calves by increasing both weight of the ruminal tissue and the size of the papillae (Mentschel et al., 2001; Górka et al., 2009) as well as increasing the size of intestinal villi (Guilloteau et al., 2009b; Górka et al., 2014). However, in the present study (Chapter 5) the effects of MSB inclusion in the starters were opposite to that described in the literature. In the present study, no changes were observed for GIT tissue weights; however, ruminal epithelial development was affected negatively when MSB was present in the starters. The papillae length, and consequently the ruminal epithelial surface, was decreased with MSB supplementation. Similarly to previous research (Górka et al., 2009), no changes were observed in terms of ruminal fermentation as MSB is a protected form that only partially dissociates in the rumen. MSB supplementation also upregulated expression of

MCT1 in ruminal epithelium. However, since MCT1 is actually located on basolateral side of ruminal epithelium (Kirat et al., 2005), this change was likely due to transport of other cell metabolites out of the cells (Connor et al., 2010b). The negative effects of MSB on GIT development are surprising; however, they might be partially justified by the age of calves. A lot of research evaluating use of MSB has been done on young, mostly still MR fed calves, even though the effect of MSB inclusion in the starters was also evaluated. For example, in study conducted by Górka et al. (2014), calves were killed at 26 d of age, as compared with an average age of 72 d in the present study (Chapter 5). One study (McCurdy et al., 2019) did evaluate the post-weaning response to MSB starter inclusion, finding that it can increase starter intake and ADG, while decreasing the ruminal SCFA concentration and increasing the proportion of propionate. However, McCurdy et al. (2019) did not evaluate the GIT development. Therefore, the negative effect of MSB in post-weaning calves cannot be ruled out. Additionally, MSB supplementation increased aminopeptidase A activity in duodenum and ileum and aminopeptidase N in ileum. Since MSB can stimulate proliferation of enterocytes (Górka et al., 2014), this was likely the mode of action. However, with no effect of the size of GIT and no impact on growth performance, it is likely that the change in brush border enzyme activity is not likely to have further influence on metabolism and growth of calves. MSB supplementation did increase starter intake pre-weaning, and with the numerous past research documenting stimulatory effect of supplemental butyrate on both performance and GIT development in pre-weaned calves, the MSB use pre-weaning should still be considered.

7.6 Overall Considerations for CM Use in Calf Starters

Considering all the experimental approaches utilized to test the overall hypothesis, a general conclusion is that CM can be included in calf starters with some stipulations. Using CM as main protein source for calves has the potential to decrease starter intake; however, inclusion does not appear to consistently affect ADG. As such with ensuring adequate ADG of calves is maintained, CM could be used in calf starters with similar results as SBM, while being a more economically viable feed source for dairy producers due to its lower price (USDA, 2020). My research has further shown that the inclusion of CM to provide up to 60% of CP supply in the calf starter does not decrease calf growth. Although not directly investigated, based on heat-treatment

calf study (Chapter 3), indirect conclusion can be speculated regarding the quality of CM. The over-processed CM should not be used for calves as it can compromise growth and GIT development. The quality of CM should be adequate, primarily avoiding obviously burnt CM based on the organoleptic assessment, or CM with odd nutrient composition, as excessive heat-treatment can increase CP, NDF and ADF content.

Additionally, utilizing glycerol in calf starters at 5% DM can be beneficial in terms of calf growth and GIT development. Whereas, MSB supplementation through the whole rearing period at 0.33% DM might have a negative effect on GIT development in post-weaning calves; however, its use pre-weaning, might be still considered due to increased starter intake, as well as known stimulation of ruminal and small intestinal development.

7.7 Future Research

Rauba et al. (2019) suggested that level of protein originating from both starter mixture and MR fed to the calves can positively affect their first lactation milk production. In that analysis, the level of starter protein increased fat and protein production during first lactation. Considering the small differences in performance of calves (Chapter 4 and 6) and greater size of the GIT in calves fed CM as oppose to SBM, future research could determine if the utilization of different protein sources in calf starters pre- and post-weaning could have an impact on first lactation production. Additionally, the effect of protein source change on the ruminal microbiome of calves could be established, especially in the *in vitro* setting, where other diet components are not confounding the experimental model. Both suggested research concepts could be combined together, as change in the protein source does introduce variability in AA composition of the diet, which could potentially influence the ruminal microbes. Whether the changes in ruminal microbiome would be quantitative (change in the microbial protein amount present for example) or qualitative (affecting the species present) would need to be determined as well.

More in-depth research should be conducted into the level of microbial protein that is being supplied to calves and the balance of microbial protein with the RUP supplied from the feed. Although some work has already been done (Lallès et al., 1990) setting the foundation in this respect, more detailed analysis could help establish the optimal level of RUP, RDP and microbial protein that need to be supplied to the calf, in order to support its growth and metabolism.

Additionally, considering the current weaning practices (Chapter 2.1.4) more research should be conducted to determine the comparative level of GIT development between calves weaned at a conventional age and calves weaned by the dam at around 10 months of age. This research could be conducted both on Holstein calves, that are currently weaned early in life, as well as on beef calves, that are more likely to be weaned later in life. The calf breed comparison could help establish the baseline for both dairy and beef calves and whether the development of GIT in calves when weaned at such an early age compares to what it would need to be otherwise.

8 CONCLUSION

The major hypothesis for this thesis has been partially confirmed in that CM can be successfully used in starter mixtures for calves as a substitute for SBM. Care needs to be taken when using CM in the starters, as decreased starter intake may occur; however, the CM effect on the growth of the calves was inconsistent between different studies. However, CM can increase the size of the abomasum and jejunum, which could be viewed as adaptation to decreased digestibility. Additionally, inclusion of CM to supply up to 60% of the CP can further help mitigate impacts on ADG and feed efficiency. The quality of CM is an important factor, as CM that is over-processed, represented in this study as heat-treatment, can have negative effect on both performance of calves and result in inferior development of the GIT.

Although the glycerol mode of action is different than what was originally anticipated. Glycerol did not increase the molar proportion of ruminal butyrate, while increasing the overall concentration of SCFA. Additionally, glycerol resulted in increased starter intake and ADG, as well as increased jejunal tissue weight. All these factors suggest that use of glycerol in calf starters can have a beneficial effect on calf performance from birth till weaning.

Supplementation of MSB in the starters did not produce the results that were expected considering the available literature. With the potential to decrease ruminal papillae length, ruminal epithelial surface area and omasal epithelial thickness, the use of MSB post-weaning should be avoided.

Overall, partial use of CM in starter mixtures for calves is a viable substitute for SBM as a protein source, whereas glycerol supplementation can be used to improve both calf growth and their GIT development. However, the use of MSB and heat-treatment of CM can compromise the calf GIT maturation.

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