Influence of the liquid diet on gastrointestinal development in dairy calves

by

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ABSTRACT

INFLUENCE OF THE LIQUID DIET ON GASTROINTESTINAL DEVELOPMENT

IN DAIRY CALVES

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Due to the prevalence of gastrointestinal diseases and disorders in dairy calves, optimizing nutritional strategies to enhance gastrointestinal (**GIT**) development is at the forefront of industry concerns. This thesis investigates the effects of whole milk powder (**WP**) and milk replacer (**MR**) of similar macronutrient composition on GIT development, function, and composition in Holstein calves. Calves fed the WP treatment had greater gut mass, specifically the mass of the rumen and small intestine. When evaluating histomorphological measures, the surface area index of the distal jejunum of WP calves was greater. Additionally, the transcellular permeability of WP calves was increased. The treatments in this thesis also altered the free fatty acid and phospholipid profile of small intestine tissue. These findings suggest that the fatty acid profiles of WP and MR affect GIT physiology from gross morphology to phospholipid composition, which may cause changes to membrane fluidity and cell function.

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LIST OF ABBREVIATIONS

ADG: Average daily gain
ALA: α – linolenic acid
AI: Artificial insemination
BW: Body weight
CP: Crude protein
CP:ME: Crude protein to metabolizable energy ratio
d: day
DM: Dry matter
DMI: Dry matter intake
DHA: docosahexaenoic acid
EPA: Eicosapentaenoic acid
FPT: Failure passive transfer
g: Grams
GIT: Gastrointestinal tract
h: Hour
Ig: Immunoglobulin
IgG: Immunoglobulin-G
LA: Linoleic acid
LSMEAN: Least square mean
ME: Metabolizable energy
MFG: Milk fat globule
MFGM: Milk fat globule membrane
MR: Milk replacer
MUFA: monounsaturated fatty acids
PUFA: poly unsaturated fatty acids
SCFA: Short chain fatty acids
SD: Standard deviation
SEM: Standard error of the mean
SPT: Successful passive transfer
WM: Whole milk
WP: Whole milk powder
WPNI: whey protein nitrogen index

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1 Literature Review

1.1 Introduction

Heifers in the dairy industry represent the future of the milking herd, as well as a significant financial investment to producers. As a result, producers aim to optimize the health, welfare, and efficient growth of heifer calves while keeping costs low. In an effort to optimize milk production, dairy calves are not raised with the dam, partially contributing to the many unique challenges and stressors that lead to very high rates of morbidity and mortality in the pre-weaning phase (33.9% and 5%, respectively; NAHMS, 2014). Veal calves in particular face greater stressors as they are commonly not raised on farm of origin and must endure transport, social stressors and increased disease challenges contributing to even higher rates of mortality (7 to 8%; Winder et al., 2016). Diseases and disorders of the gastrointestinal tract (**GIT)** account for more than 50% of calf morbidity and approximately 33% of calf mortality (NAHMS, 2014; Urie et al., 2018a,b). Consequently, strategies to prevent or reduce GIT disease are important to producers, industry members and researchers alike and modifying traditional nutritional management strategies is key to achieving optimal calf health, welfare, and productivity.

Numerous studies have shown that colostrum is critical to achieving passive immunity and ensuring calf health by influencing GIT development, digestion, and nutrient uptake (Blum and Hammon, 2000; van Keulen et al., 2021). While colostrum is extremely important to the young calf, the largest portion of the pre-weaning diet is comprised of either whole milk (**WM**) or calf milk replacer (**MR**). Calves are born with an esophageal groove that allows WM or MR to bypass the underdeveloped rumen and

enter the small intestine, which has a high capacity for digesting lactose, the major carbohydrate in milk (Huber et al., 1964). In North America, WM and MR are the predominant liquid diets for dairy calves – approximately 50% of dairy calves are fed MR and the remainder are fed some form of WM (USDA, 2014). Producers choose WM or MR for their calves based on several factors: MR is clean, convenient, and nutritionally consistent, while WM is available on farm and is hypothetically, evolutionarily designed to promote calf development and health. Alternatively, WM is variable and influenced by many factors including season (Pacheco-Pappenheim et al., 2021), parity (Yang et al., 2013) and breed (Armstrong, 1959). Additionally, when management is poor or the health status of the cow is compromised, WM can have an increased pathogen load (Vasavada, 1988) compared with MR.

Recently, studies investigating the relationship between pre-weaning growth and future production in commercial herds have found that each additional 1 kg of pre-weaning average daily gain (**ADG**) results in an additional 1 113 kg of milk in the first lactation, although it does not affect future lactations (Soberon et al., 2012). However, it is important to consider that nutrient quality and type (WM or MR) during this period is critically important to first lactation milk yield (Moallem et al., 2010). Heifer calves allowed to suckle dams three times daily during the pre-weaning period had greater BW and tended to produce more milk in the first lactation versus heifers fed ad libitum MR (Bar-Peled et al., 1997). Increasing future production is just one of the many goals of calf nutrition, along with reducing the cost of heifer raising, optimizing growth of the calf to maximize future production, and minimizing incidence of disease (Khan et al., 2011).

This section will review the importance of the liquid diet, including colostrum, WM and MR, on calf heath and development, as well as the developmental adaptations that occur as a result of the composition of the liquid diet during the pre-weaning period.

1.2 Nutritional management of the pre-weaning calf

1.2.1 Colostrum feeding

Due to the structure of the bovine placenta, neonatal calves are born without immunoglobulins derived from maternal serum, and as a result, have naïve immune systems at birth (Arthur et al., 1996; Weaver et al., 2000). Bovine colostrum contains very high amounts of immunoglobulins compared to WM (94.1 g/L IgG vs. 1.0 g/L IgG), which must cross the intestinal barrier to generate humoral immunity until calves develop adaptive immunity (Weaver et al., 2000). Therefore, it is critically important to provide high-quality colostrum containing > 50 g IgG/L to prevent morbidity and mortality (Robison et al., 1988; Wells et al., 1996). Previously, when calves had serum IgG < 10 g/L between 1 to 7 d of age, it was considered failure of passive transfer (**FPT**; Tyler et al., 1996). In Ontario, 24% of calves on 109 farms were considered to have FPT (Renaud et al., 2020). It is estimated that FPT occurs in approximately 19% of calves and 31% of early life mortality is due to FPT (Beam et al., 2009). Due to the correlation between FPT and morbidity, FPT also represents a substantial economic loss to farmers (approximately $87.52 CAD per calf; Raboisson et al., 2016). Recently, a four-category classification system has been recommended to further reduce calf morbidity (Lombard et al., 2020). The new categories use four serum IgG categories: excellent (≥25.0), good (24.9-18.0), fair (17.9-10.0) and poor (≤10 g/L) passive transfer of

immunity. Unfortunately, colostrum quality varies widely, ranging from 7.1 to 159.1 g/L of IgG (Quigley et al., 2013), and thus, rates of passive transfer also vary accordingly, leading to development of colostrum replacement products that consistently supply adequate amounts of immunoglobulins when maternal colostrum quality is low.

Overall, there are four primary factors that result in successful colostrum management programs: quantity, cleanliness, quality, and quickness (Fischer-Tlustos et al., 2021). Quantity has been emphasized by Dunn et al. (2017), who demonstrated that feeding colostrum at 10% BW compared to 5% BW increased serum IgG concentrations in the first 3 d of life. Cleanliness refers to the importance of feeding bacteria- and pathogen-free colostrum which can occur when feeding maternal colostrum through mammary contamination or during the collection or handling on farm (Stewart et al., 2005). Finally, the neonatal calf intestine is very permeable to large macromolecules such as IgG, but gradually becomes less permeable over the first 24 h of life (Lecce and Morgan, 1962; Stott et al., 1979). Therefore, high quality colostrum (> 50 g IgG/L, Wells et al., 1996) must be provided to neonates in a timely manner – for, as Fischer et al. (2018) found, delaying the first colostrum feeding up to 6 or 12 hours after birth negatively impacts IgG absorption.

Despite the historic focus of colostrum research on IgG, colostrum contains a diverse array of bioactive compounds present at higher concentrations than in WM. The bioactive compounds include, but are not limited to, oligosaccharides, growth factors and hormones (Odie et al., 1996; Blum and Hammon, 2000; Blum, 2006). While the immune components of colostrum have been extensively studied (Nissen et al., 2017l

Honan et al., 2020), colostrum also contains high concentrations of essential lipid molecules, such as n-3 polyunsaturated fatty acids (**PUFA**), palmitic acid, phospholipids, and cholesterol (Nobel et al., 1978; Contarini et al., 2014; O'Callaghan et al., 2020). Moreover, studies have suggested that milkings 2 to 6, classified as transition milk, are distinct from colostrum and WM (Blum and Hammon, 2000). Transition milk contains greater amounts of IgG, protein, and solids than WM (Foley and Otterby, 1978). Numerous studies have shown that additional colostrum and transition milk feedings stimulate GIT development and digestive efficiency due to the elevated levels of growth stimulating hormones, IgG and protein (Bühler et al., 1998; Blättler et al., 2001; Berge, et al., 2009). By increasing intestinal surface area, colostrum and transition milk intake in the first days of life can enhance nutrient absorption (Pyo et al., 2020). Additionally, Carter et al. (2021) put forward a growing body of evidence for the use of colostrum to treat diarrhea. Despite the benefits of proper colostrum management strategies and feeding transition milk, many producers do not assess colostrum quality and discard transition milk, or mix it with whole milk for calves, resulting in an inconsistent source of nutrients for calves.

1.2.2 Liquid diet feeding strategies

Due the underdeveloped rumen and presence of the esophageal groove, the digestion and metabolism during pre-weaning period is essentially considered "pseudo-monogastric", as opposed to ruminant, and thus requires a high-quality liquid diet (Radostits and Bell, 1970). Additionally, dairy heifers represent financial investments for producers until first calving at approximately two years of age, unless sold, and

subsequently, represent a financial investment for producers (Geiger, 2019). Cost of feed represents over 50% of the total cost in raising heifers (Karszes et al., 2008), however, an alternative option is to feed waste milk, which is non-saleable whole milk. Using a partial budget model, Godden et al. (2005) found that feeding pasteurized waste milk resulted in cost savings of $0.69USD per calf per day by improving growth and reducing morbidity and mortality rates. Waste milk, however, may contain antibiotic residues, varies in its nutritional composition and quality (similar to WM), and may contain an increased pathogen load (Ruzante et al., 2008; Moore et al., 2009). Although concerns with pathogen load may be mitigated by pasteurization (Godden et al., 2005), waste milk may result in increased antimicrobial resistance (Langford et al., 2003).

When comparing macronutrient composition (**Figure 1-1**), MR contains higher levels of lactose (35-50% vs. 33-38% DM; Pantophlet et al., 2016), lower levels of fat (16-22% vs. 30-40% DM; Berends et al., 2020), comparable proteins levels (20-28% vs. 25-27% DM, respectively; Morrison et al., 2017; Drackley, 2020; Chapman et al., 2020), and higher amounts of ash (8-12% vs. 7-9% DM; Gaucheron, 2005) compared to WM. Traditional MR formulations contain 20% protein and 20% fat and are offered at a restricted (low plane of nutrition) feeding rate but formulations can also be enhanced (25% protein, 20% fat) and fed at a higher plane of nutrition (Raeth-Knight et al., 2009). For the purpose of this thesis, restricted MR programs will refer to feeding rates of 10% BW, intensified MR programs refer to higher feeding rates (15% or 20% BW), and conventional MR programs refer to 20% protein, 20% fat composition. Since the fat content of MR is significantly lower regardless of the feeding rate, WM (325 g) provides

an average of 15% greater energy density at ~5.4 vs. ~4.7 Mcal of ME/kg of DM (MR: 325 g), Drackley, 2008). Ergo, the energy content of MR is lower than WM, meaning calves consuming MR intake less energy per L than calves consuming WM as well as MR has a lower ME:CP ratio. The traditional high lactose-low fat formulation of MR stems from traditional objectives for lean growth in calves and the historic availability of whey for MR formulations.

In white veal production, calves obtain the majority, if not all, of their nutrient requirements from high volumes of milk between two and six months of life. Conversely, in the dairy industry, calves are traditionally fed restricted amounts of milk and are weaned onto starter feeds at approximately 2 months of age. Over half of U.S. dairy farms provide heifers with 4.0 – 5.0 L of milk/d based on the conventional recommendation of feeding milk at 10% of BW (NAHMS, 2014). This level of feeding is considered a low plane of nutrition (Rosenberger et al., 2017) and is associated with reduced growth (Jasper and Weary, 2001; Khan et al., 2011; Kiezebrink et al., 2015). In addition to reducing feed costs, this strategy also encourages calves to consume starter, promote rumen development, and allow for an easier weaning transition (Savage and McCay, 1942; Kertz et al., 1979; Baldwin et al., 2004).

Feeding calves at an intensified feeding rate (i.e. 20% of birth BW) is currently recommended because increased preweaning ADG from increased milk intake is associated with increased first lactation and lifetime productivity (Soberon et al., 2012; Soberon and Van Amburgh, 2013). Increased pre-pubertal growth and bodyweight is highly correlated with onset of puberty (Serjse, 1994) and translates to earlier puberty,

thereby reducing time before first artificial insemination (**AI**), calving and rearing costs. While cost/kg of BW does not always differ between WM and MR (Bhatti et al., 2012b), multiple studies have stated that overall, WM has a greater economic benefit due to increased returns over rearing costs (Bhatti et al., 2012a; Bhatti et al., 2013; Korst, et al., 2017). Moallem et al. (2010) and Shamay et al. (2005) found that calves fed WM reached puberty sooner, and Moallem et al. (2010) found that WM fed calves had reduced age at first AI compared to calves fed MR (ad libitum 24% protein, 13% fat and ad libitum 23% protein and 15% fat, respectively). Conversely, in a study comparing calves fed a conventional MR (20% protein, 20% fat) vs. an enhanced MR program (28% protein, 18% fat), conventional MR calves had higher growth rates, reduced age at first calving and no effect on milk yield (Raeth-Knight et al., 2009). Bach and Ahedo (2008), Bach (2012) and Sobeorn et al. (2012) found increased nutrient intakes from increase milk volume during early life resulted in increased first lactation yields. Davis Rincker et al. (2011) and Terre et al. (2009) found no differences between enhanced MR diets compared with conventional MR formulations on first lactation milk production. The suggested mechanism behind this limitied first lactation production is that an increase in pre-pubertal weight gain combined with increased mammary fat pad might interfere with the development of parenchymal tissue (Hovey et al., 1999; Geiger, 2019). Consequently, the literature currently does not agree on the benefits of enhanced feeding programs on future heifer production and concerns remain regarding intensive feeding programs and their effects on the early development of the mammary gland (Lammers et al., 1999; Silva et al., 2002).

Another important factor of MR feeding is its source of protein and fats. Skim milk powder and whey have greater digestibility than vegetable proteins, at ~94% (Guilloteau et al., 1986; Raeth et al., 2016). Skim milk contains similar nutrients to WM, but whey protein has a superior amino acid profile with sufficient levels of essential amino acids (McDonaugh et al., 1976). Lammers et al. (1998) found that feeding MR sourced from 67% of 100% whey yielded better growth than 100% skim milk-based MR when fed ad libitum without starter. Whey, a milk protein and by-product of cheese manufacturing, was a common historical ingredient in MR formulations due to its low market value. However, the increasing demand for whey in functional foods and infant formulas has increased whey prices and, therefore, it is becoming less available for use in calf MR formulations. Milk proteins have greater digestibility due than vegetable proteins, which are a cheaper alternative protein source for use in MR (Guilloteau et al., 1986). Wheat and soy are common vegetable proteins used in MR formulations, although they have sub-optimal amino acid profiles with low content of methionine and contain anti-nutritional factors that reduce growth, GIT morphology and enzyme activity in the small intestine (Branco-Pardal et al., 1995; Montagne, et al., 1999; Miqueo et al., 2017). Recently, the amino acid profile of MR has been investigated for its impacts on calf growth and development (van Niekerk et al., 2021). Increasing MR allowance in calves from 10% to 20% of birth BW (26% protein, 26% fat) and supplementing arginine or glutamine improved intestinal surface area by increasing cell proliferation or decreasing cell atrophy (Van Keulen et al., 2020). Additionally, Ahangarani et al. (2020) demonstrated that supplementing glutamic acid at 0.3% DM to a MR (~24% CP, 18%

fat DM) sourced from skim and whey did not affect growth or intestinal permeability but increased amino acid levels in plasma. Hydrolyzed wheat protein may be a good alternative to expensive milk proteins when fed at 4.5% DM and supplemented with methionine and lysine, producing similar growth rates to milk-protein based MR (Castro et al., 2016). Whey protein concentrate can also be substituted by plasma protein or plant peptide powder without negatively affecting growth (Raeth et al., 2016; Morrison et al., 2017).

While calves can tolerate high quantities of lactose and can digest it well, the high lactose content of MR can pose challenges to health and GIT development (Hof, 1980; Wilms et al., 2019). When lactose exceeds the GIT's ability for absorption, a greater amount of carbohydrates will enter the lower gut for fermentation by intestinal microbes. The increased amount of carbohydrates and fermented products may alter the osmolality of the lumen contents and impair nutrient and water absorption, and may ultimately result in osmotic diarrhea (Hof, 1980; Jodal and Lundgren, 1986). The osmolality of WM is approximately 300 mOsm/kg while MR can be ~450 mOsm/kg due to the high lactose and ash content in conjunction with a high percentage of solids (12.5 – 20%; Wilms et al., 2019). Feeding calves a hypertonic MR (>400 mOsm/kg) has been demonstrated to impair absorptive surface area and barrier function (Glosson et al., 2015; Wilms et al., 2019). It is established that in bovines that GIT barrier function is impaired during diarrhea (Klein et al., 2008). Therefore, while adequate lactose consumption is important for meeting nutrient requirements, a MR formulated at 45% lactose DM and 15% solids for a 45 kg calf should not exceed approximately 6.7 L of

MR per day (Hof, 1980, van Niekerk et al., 2021). Interestingly, Welboren et al. (2021) found that calves fed a MR partially replacing lactose with fat resulted in heavier, yet more permeable, GIT; thus, the balancing of the energy sources in MR warrants further investigation.

Overall, there is little consensus over whether WM or MR feeding produces calves with lower rates of morbidity, greater intestinal integrity or growth. Previous studies comparing WM and MR differ widely in design, including use of differing sources of macronutrients, digestibility of the diets, differing energy and macronutrient contents, and DM feeding rates. As such, these differences must be carefully considered when interpreting results and comparing amongst different studies. Numerous studies have shown that WM fed calves have lower morbidity and mortality compared to MR fed calves (Jamaluddin et al., 1996; Godden et al., 2005; Lee et al., 2009); in contrast, some studies have found the reverse to be true regarding morbidity (Gleeson et al., 2012) and WM fed calves tended to have increased days with scours (Gorka et al., 2011a).

1.2.2.1 Whole milk vs. milk replacer feeding

Efficient growth is a major goal of dairy rearing programs, and as such, numerous studies have attempted to quantify which diet promotes more efficient growth. Some researchers have seen greater growth rates (Niwinska, 2005; Flaga et al. 2015; Zhang et al., 2019) and increased skeletal growth in WM calves (Shamay et al., 2005, Lee et al., 2009). Recently, Qadeer et al. (2021) found that the type of liquid diet influences the growth rate of calves, but amount fed (10% vs. 15%) has no effect on

growth. The authors recommended feeding WM at 10% of bodyweight compared to feeding a conventional MR (20% CP, 20% fat DM) and attributed the differences in growth to superior nutrient intake in the WM group. Lee et al. (2009) fed either WM or MR in 1.8 L meals 4 times daily, with both diets having similar gross compositions (~24% CP, 25.6% fat, ~38% lactose, DM basis). It was found that growth was greater for WM fed calves, however the MR in this experiment included soy protein concentrate, which has a sub-optimal amino acid profile with low levels of methionine that may have been responsible for the observed differences in growth. However, it has been suggested that the nutritional consistency provided by MR can support greater BWs than WM (Hill et al., 2009).

While significantly less quantified, the different diets can even affect GIT development (Niwinska, 2005; Moallem et al., 2010). Zhang et al. (2019) emphasized the importance of the type and composition of the liquid diet on GIT development. It is difficult to assess existing data on GIT development in WM vs MR fed calves due to the numerous gaps and variables in the research, including level of feeding intensity, nutrient source, and nutrient content. Zhang et al. (2019) found greater forestomach and rumen mass but smaller abomasum mass in MR than WM fed calves fed the same feeding intensity (12% BW twice daily), while Moallem et al. (2010) found contradictory results in WM and MR fed calves (ad libitum twice daily). Both MR formulations provided significantly less fat than WM (16% and 13% DM, respectively). Moallem et al. (2010) found that the omasum, abomasum, small intestine, omentum and total digestive tract weight were greater in WM calves. Therefore, liquid diets influence both gross

morphology and histomorphology of the GIT (Niwinska, 2005). These results demonstrated that feeding an MR (34% CP, 24% fat) does not support duodenum weight, villus height and submucosa thickness to the same extent as WM, although this study also fed a soy protein concentrate. Ultimately, more research is required to understand how the different feeding strategies of the liquid diet influence GIT development in dairy calves.

1.2.3 Whole milk and milk replacer fat

1.2.3.1 Differences in composition

While it can be argued that historical WM composition is an optimal composition for calf development, MR provides a more consistent macronutrient supply (Hill et al., 2009). While MR's can be formulated from plant or animal sources, digestibility of the ingredients is crucial to ensure optimal nutrient uptake. In Europe, the fat sources used in MR are primarily mixtures of vegetable oils such as coconut, palm, or rapeseed (Huuskonen et al., 2005), while in North American, MRs are sourced from animal fats such as lard and tallow (Essleburn et al., 2013). One of the primary goals of calf rearing is lean growth, however studies by Tikofsky et al. (2001), Hill et al. (2008), and Bartlett et al. (2006) highlighted the propensity of high fat MR diets to increase fat deposition, possibly interfering with optimal mammary development (Serjsen and Purup, 1997). However, a longitudinal study by Urie et al. (2018a) found that the mortality risk was three times greater (9%) for calves receiving <150 g/d of fat from the liquid diet than calves fed >150 g/d (3%), underscoring the important role of dietary fat as an energy source for the immune system as well as precursors to pro- and anti-inflammatory

eicosanoids. Berends et al. (2020) demonstrated that calves fed high fat MR (23% DM) ad libitum required fewer therapeutic interventions in early life. Furthermore, calves fed restricted diets (6.0 to 7.0 L/d) of a high-fat MR had lower preweaning fecal scores compared to calves fed a traditional high-lactose MR (>40% lactose DM; Amado et al., 2019). These differences may be due to the effect of the diet on altering adipose tissue which has potent metabolic and endocrine functions (Frayn et al., 2003).

Dietary fat from WM provides ~50% of dietary energy in addition to essential fatty acids and bioactives which are important to the metabolic function of young animals (Deplanque et al., 2015; Grote et al., 2016; van Niekerk et al., 2021). Recent studies have investigated the use of traditional MR formulations (<2% fat) in comparison to WM (Amado et al., 2019, Welboren et al., 2021) as well as MR containing a fat content similar to WM (>23%; Amado et al., 2019; Berends et al., 2020). However, based on the ingredients in MR (i.e. milk-based, animal or vegetable), it may be lacking in important dietary elements such as fatty acids (Hill et al., 2008, 2011; Kato et al., 2011; Essleburn et al., 2013).

1.2.3.2 Chemical and physical structure of fat in MR and WM

The differences between WM and MR extend beyond macronutrient composition, the two diets also have different milk fat globule structures (Lopez et al., 2015), triglyceride structures (Lien, 1994), and fatty acid profiles (Hanus et al., 2018). In milk, fatty acids are secreted almost exclusively in the milk fat globules (**MFG**), which consists of a triglyceride core covered with the milk fat globule membrane (**MFGM**). The MFGM is comprised of three layers of phospholipids and proteins from the cellular

membrane of the mammary gland (Argov-Argaman, 2019). In contrast, the lipid droplets in MR do not contain MFGM and are coated with milk proteins and casein which are still readily absorbed but do not confer important effects on the GIT (Lopez et al., 2015). For example, sphingomyelin is one of the major constituents of the MGFM and when orally supplemented to young rodents improves intestinal function such as decreasing lactase activity (Motouri et al., 2003). Additionally, supplementation of bovine MFGM in formula has been demonstrated in rats to improve health and gut development by influencing the gut microbiome, intestinal growth, and tight junction protein structure (Bhinder et al., 2017).

Triglycerides make up the vast majority of milk fat (~98%;Taylor and MacGibbon, 2002) and are comprised of a glycerol backbone and three fatty acids with important spatial positioning (sn1, sn2, sn3; Lammi-Keefe and Jensen, 1984). For example, in WM, approximately 40% of C16:0 is in the s*n*2 position (Freeman et al., 1965), compared with 5 to 20% in vegetable oils (Mattson and Lutton, 1958). This has implications on digestion because when C16:0 is hydrolyzed from vegetable oils it often binds with calcium and forms insoluble calcium soaps that may slow passage rate and decrease fat and calcium absorption (Straarup et al., 2006). The fatty acid profile of WM compared with a MR sourced from coconut and palm oil can be seen in **Figure 1-2** and **Figure 1-3**. A lower concentration of medium chain fatty acids and higher concentration of long chain fatty acids are seen in MR than WM which can alter absorption in the small intestine (Hocquette and Bauchart, 1999). Additionally, Bascom et al. (2007) compared WM and MR (20% protein, 20% fat, 27% protein, 33% fat, 29% protein,16%

fat) and hypothesized that the superior growth and feed efficiency in the WM group was due to higher levels of medium chain FA, whereas MR contains more long chain fatty acids. When MR is enriched with coconut oil the amount of medium chain fatty acids increased and resulted in comparable growth rates to WM fed Jersey calves which may be more beneficial due to the higher medium chain fatty acid content of Jersey milk compared to Holstein milk (Bowen Yoho et al., 2013).

It is important to consider that some fatty acids are essential since they cannot be synthesized de novo by the animal. Garcia et al. (2015) previously demonstrated that increasing the amount of essential fatty acids such as linoleic acid (**LA**) and α-linolenic acid (**ALA**) between 0.0321 and 0.036 g/kg $BW^{0.75}$ improved ADG, wither and hip heights. Further to this point, Hill et al. (2009) fed increased amounts of LA using higher content of flax oil and found alkaline phosphatase, an indicator of bone formation, was elevated (Watkins et al., 2001). There is a great saturated fatty acid (**SFA**) content in WM and a smaller n-6:n-3 ratio than MR. Therefore, calves fed a MR with an equivalent fat composition as WM will be provided with a larger amount of precursors for arachidonic acid (**ARA**), a pro-inflammatory eicosanoid, than eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**), which are precursors to anti-inflammatory eicosanoids (Noble et al., 1978; Sardesai, 1992; Calder, 2010). Generally, n-3 fatty acids are studied for their anti-inflammatory function, but also play a role in bone turnover, and therefore, growth (Kajarabille et al., 2013). It is important to consider that when overfeeding fatty acids, some metabolic functions may be altered, thereby reducing the synthesis of other derivatives (Hill et al., 2011). While LA is a precursor for

DHA, the synthesis of DHA can be decreased when LA is added in extreme quantities to diets (Buccioni et al., 2012).

1.2.3.3 Effects of fatty acid profile on GIT physiology

Since the importance of fat in the diet of young animals has become more prominent, studies have sought to quantify the effect of these diets on the physiology of the GIT. While Wilms et al. (2019) demonstrated the risk of hypertonic high lactose diets to increasing GIT permeability in calves, conversely, high fat diets in rats have also been demonstrated to increase paracellular intestinal permeability as measured by Cr-EDTA recovery, (Suzuki and Hara, 2010). This change was attributed to a reduction in the expression of tight junction proteins also observed in the study. In calves, the effects of higher fat diets on GIT physiology are inconsistent. High fat MR have previously been associated with an increase in paracellular permeability but positively affected fecal scores (Amado et al., 2019). Following these results, it was determined that when MR composition is designed to partially replace lactose with fat, calves had heavier but more permeable GITs than conventional formulations (Welboren et al., 2021). It is possible that altering the fatty acid composition of fat in MR may further support optimal gut development.

Numerous studies have demonstrated that supplementing MR with fatty acids to better mimic the profile of WM can influence health outcomes and GIT development (Gorka et al., 2011a, b; Hill et al., 2011). Supplementing n-3 and n-6 PUFA in MR or WM has been shown to decrease severity of diarrhea and improve performance of calves (Garcia et al., 2015). This may be because long chain PUFA are used to

synthesize inflammatory molecules including prostaglandins, leukotriens and resolvins and may modulate an inflammatory response (Schmitz and Ecker, 2008). Butyric acid is the most extensively supplemented fatty acid in calf nutrition and has been found to improve performance and reduce diarrhea incidence when supplemented in a cocktail of fatty acids, including myristic acid, lauric acid and ALA (Essleburn et al., 2013; Garcia et al., 2015; Hill et al., 2016), By promoting development of the intestinal barrier, butyric acid supplementation in MR can improve rumen and intestinal growth (Gorka et al., 2009; Gorka et al., 2011a, b; Guiolloteau et al., 2009). When sodium-butyrate was supplemented at 0.3%, as fed in a 22% protein, 17% fat MR, it indirectly increased reticulorumen weight and papillae length (Gorka et al., 2011a). Interestingly, when sodium-butyrate was supplemented at 3 g/kg DM when calves received up to 2 300g of DM daily, jejunum crypt depth was increased by 14% and 25% in the proximal and distal regions, and greater mitotic index in the proximal and mid jejunum, indicating that sodium-butyrate supplementation can influence both forestomach and intestinal development (Guiolloteau et al., 2009).

When supplemented with sodium-butyrate, the GIT of calves undergoes proliferative changes and these changes are hypothesized to occur at GIT level due to the lack of butyric acid found in blood (Guilloteau et al., 2009; Gorka et al., 2011a). Frieten et al. (2017) compared feeding ad libitum or restricted MR (21.7% CP, 18.6% fat DM) and found that butyrate supplementation had no effect on calf performance when calves were fed a high plane of nutrition. While the mechanisms of butyric acid's influence on the GIT are undefined, Luo et al. (2019) demonstrated that butyrate is

proliferative in small concentrations and potentially inhibitory in large concentrations. An experiment using Holstein steers determined that when butyrate exceeds the metabolic capacity of the rumen, it causes apoptosis (Kristensen and Harmon, 2004). Several studies have compared how different fatty acid supplementations affect growth, performance, and health, whereas little is known on how the entire fatty acid profiles of WM and MR influence GIT development.

1.3 GIT growth and development in early life

1.3.1 Importance of GIT development in calves

Liquid feed is the primary source of nutrients for young calves until they regularly consume solid feed, and the presence of the esophageal groove bypasses the majority of the foregut. Therefore, the main site of digestion for young calves is the small intestine. Since small intestine development affects overall digestion and nutrient absorption, it is also critical to overall health and growth in young calves. Small intestine development may also affect rumen development thereby influencing future solid feed intake (Gorka et al., 2011a). Thus, since the rumen is the main digestive tissue of the lactating dairy cow, the pre-weaning diet and its influence on gut development may be significant to growth and future lactation.

Development of the GIT is measured numerous ways including gross morphology (weight, length; Gorka et al., 2011a), histomorphology, (Welboren et al., 2021), and immunohistochemistry, such as Ki-67 antigen detection to investigate cell proliferation (Pyo et al., 2020). There is a well-established positive relationship between increased maintenance energy costs of splanchnic tissues and plane of nutrition (Ferrell

et al., 1986, Ferrell, 1988; Reynolds, 2002). While increased gross morphology is generally considered an improvement in GIT development, it is important to note that a larger mass will require more energy for maintenance (Reynolds, 2002).

1.3.2 GIT permeability

A primary role of the epithelium of the GIT is to prevent pathogens and toxins from entering the bloodstream of the animal. Permeability of the GIT is maintained through two primary mechanisms: transcellularly through a cell and paracellularly between cells. The most studied mechanism of GIT permeability is the function of tight junctions that connect adjacent enterocytes and regulate paracellular permeability, which are mainly responsible for large molecules (Zihni et al., 2016). Tight junctions are made up of tight junction proteins including occludin, claudin and zonula occluden (Zihni et al., 2016). The proper function of tight junctions can be influenced by numerous factors including inflammation. Elevated levels of inflammatory cytokines such as tumor necrosis factor alpha (**TNFα**) occur during pathogen invasion and are correlated with loss of intestinal barrier function due decreasing gene expression and altering distribution of tight junction proteins (Cao et al., 2013; Liu et al., 2013; Wang et al., 2018).

Gut permeability in calves is known to be high in the first 24 h after birth, prior to gut closure, and is also elevated in calves with diarrhea compared to non-diarrhetic calves (Klein et al., 2008; Arajuo et al., 2015). Gut permeability can also be altered by diet. High fat diets in calves have been shown to increase paracellular small intestine permeability. Amado et al. (2019) found that MR with 23% fat increased transcellular

permeability in calves but improved fecal scores. In piglet studies, when oleic acid was perfused into the intestine, permeability increased (Velasquez et al., 1993). EPA and DHA have been demonstrated to increase tight junction function (Hossain and Hirata, 2008) while Usami et al. (2001) showed similar results in vitro with EPA and ALA improving tight junction permeability. However, little is known about how differing fatty acid contents influence gut permeability in pre-weaning calves.

Transcellular permeability is related to diet and membrane fluidity (Jedlovszky and Mezei, 2003; Ibarguren et al., 2014). Oleic acid, ARA and DHA increase membrane fluidity once incorporated into the phospholipid membrane (Ibarguren et al., 2014). Cholesterol is one of the main components of the cellular membrane and may increase membrane fluidity because it interacts with sphingomyelin, which is predominantly saturated (Cullis and Hope, 1991). Therefore, since SFA are an important precursor for cholesterol, synthesis should theoretically decrease cholesterol in the membrane and decrease epithelial permeability (Pizzo et al., 2002). Additionally, some integral membrane proteins are preferentially located between sphingomyelin and cholesterol (Samsonov et al., 2001). The interaction between phospholipids and cholesterol can also change the confirmation of the hydrocarbon chain, thereby increasing membrane thickness (Garcia-Saez et al., 2007). Both Ibarguren et al. (2014) and Jedlovszky and Mezei (2003) have confirmed that greater cholesterol inclusion in diets decreases cellular membrane permeability. Whether cholesterol differs between bovine WM and MR is not well classified and therefore, the influence of cholesterol incorporation into enterocyte membranes of calves is unknown.

The GIT has regional differences in permeability and these variations are hypothesized to be determined by nutrient absorption (Zhang et al., 2013), endoocytoxic potential (Nejdfors et al., 2000) and microbial fermentation (Penner et al., 2014). Penner et al. (2014) determined that the jejunum of ruminants boasts the highest transcellular permeability of the intestinal region, as indicated by mannitol transport, the omasum is the region of greatest large molecule permeability, as measured by inulin transport, and the duodenum is the region of the GIT with greatest transcellular permiability. After the omasum, the rumen is the second most prominent site of paracellular permeability, followed by the jejunum. Thus, in calves who are functionally monogastric, it can be hypothesized that the jejunum is the major site of paracellular permeability.

1.3.2.1 Measuring GIT permeability

Gut permeability in vivo can be measured non-invasively using non-nutritional markers to assess epithelial integrity. Lactulose and Cr-EDTA are two large molecules that are transported paracellularly through the tight junctions while mannitol is a marker that can be used to assess transcellular transport through the epithelial cells via active or passive transport (Wijtten et al., 2011). Permeability to the markers can vary in response to physiological, pharmaceutical, and nutritional factors (Branco-Pardal et al., 1995; Klein et al., 2007). For example, Amado et al. (2019) reported that the interaction of markers with milk fat may increase marker absorption, suggesting that pulse dosing may be more suitable. It should also be considered that researchers may analyze marker permeability in blood (serum or plasma) or urine, or both, and the accuracy,

sensitivity and difference between these values is not well quantified (Amado et al., 2019; Welboren et al., 2021).

Gut permeability can also be measured ex vivo, with the use of Ussing chambers to distinguish between different segments of the GIT. Tissues must be incubated in buffers (glucose-containing or short-chain fatty acid-containing) depending on the type of tissue and serosal or mucosal side of the tissue, to provide similar luminal energy to in vivo conditions. In Ussing chambers, a section of intestinal mucosa is mounted between two chambers and marker probes are placed in solution in the chamber on the mucosal side. The passage and appearance of these marker probes in the opposite chamber represents the permeability of the tissue (Wijtten et al., 2011). Ussing chambers can be used to gather three different permeability measurements including paracellular and active ion transport, which gives insights into the movement of water through the epithelia (Boudry, 2005). While Ussing chambers may be tissue-specific, they require animals to be killed, while non-nutritional markers may be used to assess permeability repeatedly, therefore, there are advantages to both techniques (Wijten et al., 2011).

1.3.2.2 Gut tissue composition

All mammalian cell membranes consist of a lipid bilayer and the composition of the cell membrane is reflective of diet composition (Spector and Yorek, 1985). This is true in dairy calves, as demonstrated by Jenkins and Kramer (1990). Calves were fed tallow and coconut-based MR (20% fat, 25% protein DM) as the control, with one group fed half of the tallow and coconut as corn oil, and the remaining two groups fed a 2:1 or

1:2 mixture of corn oil and fish oil. High LA intake increased LA and decreased oleic, ARA, and ALA in tissue phospholipids, while supplementing fish oil, which is high in EPA and DHA, increased phospholipid concentrations of EPA and DHA. The tissues measured in this experiment were liver platelets, muscle, and heart, as well as plasma and changes were observed in all tissues. Therefore, changes in MR fat composition may have important ramifications on the structure and function of cell membranes and integration of pro- or anti-inflammatory phospholipids.

Enterocyte membrane fluidity is an important parameter in regulating numerous functional processes including ion permeability and nutrient absorption (Lee, 1975). Like GIT permeability, the membrane fluidity of enterocytes varies within intestinal regions, decreasing as the intestine becomes more distal, which has functional impacts on nutrient absorption (Putkey et al., 1982; Brasitus and Schachter, 1984). Since enterocyte fluidity is mainly determined by cholesterol content and saturation level, it is not surprising that Garriga et al. (2002) found that the distal regions of the small intestine in chickens have decreased cholesterol content and saturation compared with the proximal regions. Fatty acids are rapidly integrated into cell membranes and interact with the phospholipids in the bilayer. The incorporation of long chain saturated fatty acids decreased fluidity and permeability compared with MUFA or PUFA (Ibarguren et al, 2014). Piglets fed diets deficient in PUFA had decreased fluidity due to the incorporation of the fatty acids present into the membrane phospholipids, as seen by the reduction in membrane and total phospholipid contents of LA, EPA and DHA (Daveloose et al., 2002). Lipid composition may also change a membrane's biophysical

properties, by altering the optimal conformation for catalytic activity of proteins and enzymes in the membrane (Lenaz, 1987).

Fatty acids are readily incorporated into the phospholipids of membranes, thus due to differences in concentration of saturated long chain fatty acids it can be expected that MR and WM will alter phospholipid composition differently. Unlike most calf studies, human studies have investigated increasing membrane fluidity as a strategy to increase drug absorption, which subsequently leads to increased intestinal permeability (Khajuria et al., 2002). Therefore, to some extent, increased transcellular permeability may increase nutrient absorption and allow for superior growth (Khajuria et al., 2002). Inasmuch, MR formulations may be able to optimize this strategy in the future by altering the fatty acid profile.

1.4 Hypothesis and objective

The hypothesis of this thesis is that the fatty acid profile of the fat fraction of WP would alter tissue composition, promote GIT growth, development in gross morphology, villous development, proliferation markers, and decrease GIT permeability. Therefore, the objective of this thesis was to compare a high-fat MR to a WP, having similar macronutrient inclusion in terms of fat, protein, and lactose, on GIT morphology, permeability, and composition. This knowledge may be used to optimize MR formulations and to optimize pre-weaning GIT development in order to minimize morbidity and maximize growth.

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1.6 Tables and Figures

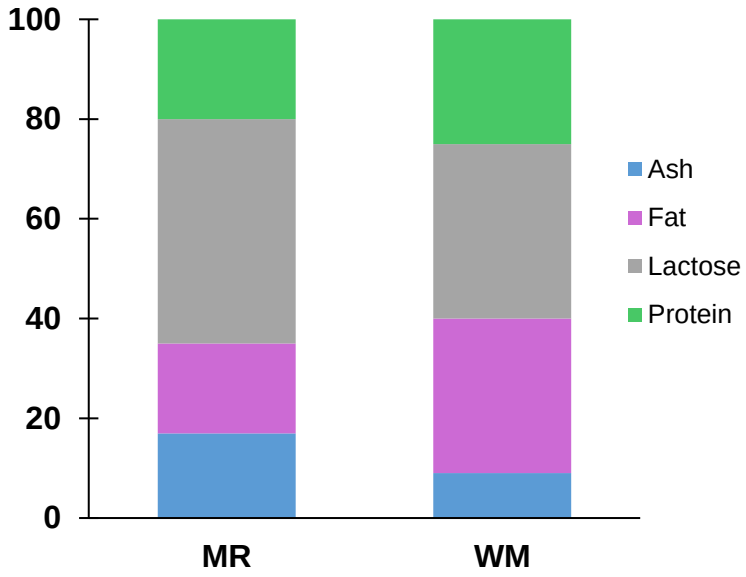

Figure 1-1. Macronutrient composition of milk replacer for calves and whole bovine milk. Milk replacer formulations currently contain higher levels of lactose (35-50% vs. 33-38% DM in WM; Pantophlet et al., 2016), lower levels of fat (16-22% vs. 30-40% DM; Berends et al., 2020), comparable proteins levels (20-26% vs. 25-27% DM, respectively; Morrison et al., 2017; Chapman et al., 2020), and higher amounts of ash (8-12% vs. 7-9% DM)

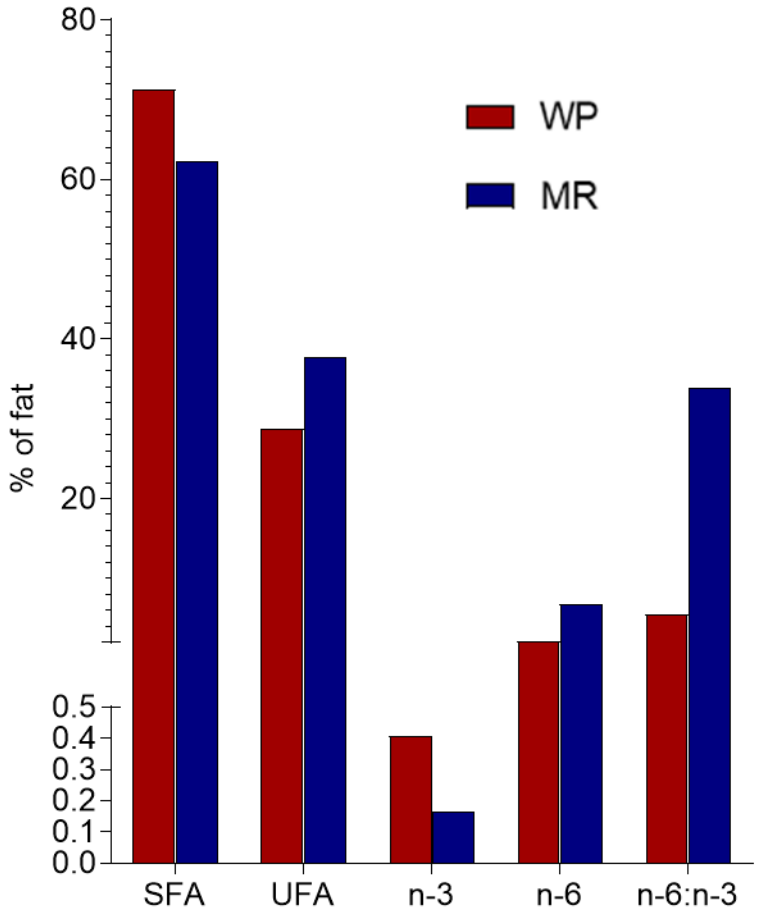

Figure 1-2. Composition of the fat fraction of WM and a MR based on coconut (35%) and palm (65%; Trouw Nutrition).

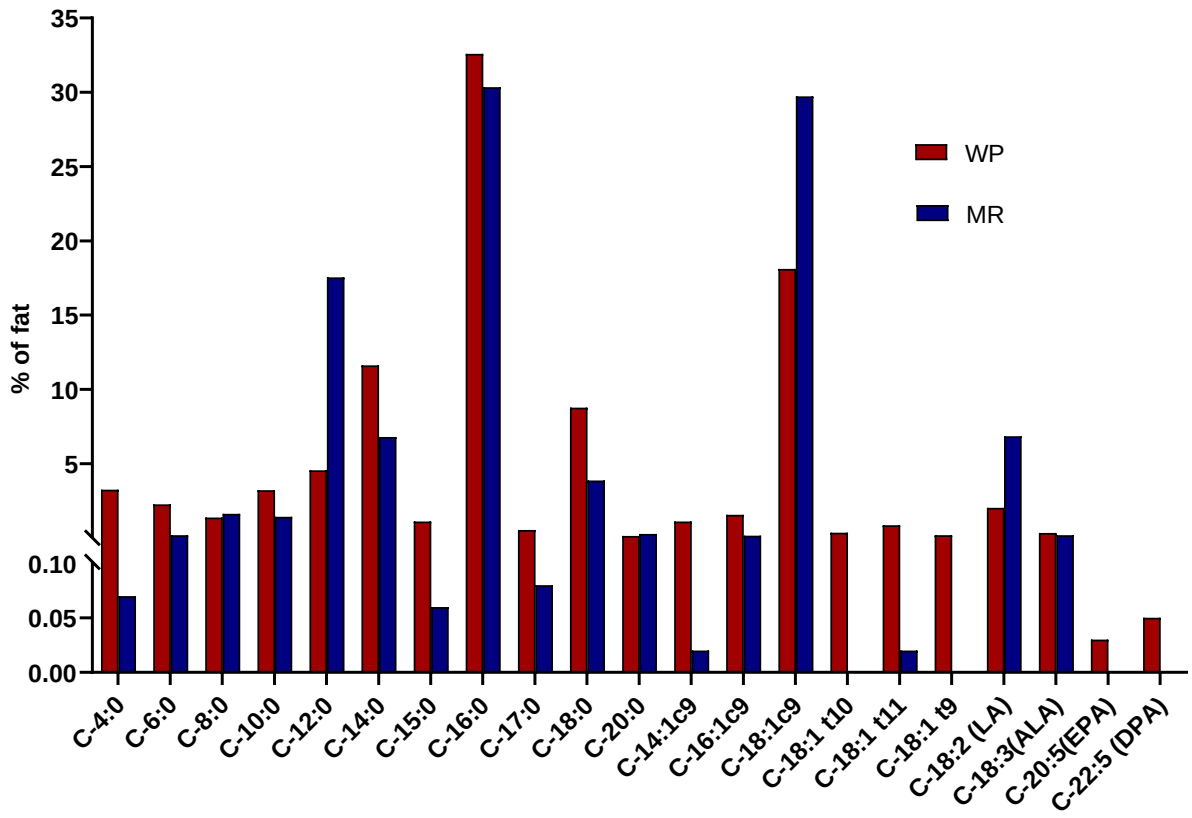

Figure 1-3. Fatty acid profile of WM and a MR based on coconut (65%) and palm (35%; Trouw Nutrition).

2 Gastrointestinal structure, function and composition of pre-weaned dairy calves fed whole milk powder or a high fat milk replacer

2.1 Abstract

The objective of the current study was to compare GIT morphology, permeability, and composition in response to feeding either a whole milk powder (**WP**) or a milk replacer (**MR**) formulated to have similar fat, protein, and lactose content as whole milk. Eighteen male Holstein calves (46.6 ± 1.21 kg; 56.4 h ± 1.30 h of age at arrival) were individually housed for four weeks. Upon arrival, calves were randomly assigned to three times daily of 3 L (135 g/L) of either WP (WP, 25% fat, 23.5% protein, 36.5% lactose DM basis, n = 9) or a high-fat MR (MR, 24% fat, 21.8% protein, 37.4% lactose DM basis, n = 9). Calves had no access to solid feed, but water was available ad libitum. Bodyweight was measured weekly and feed intakes were recorded daily. On d 21, intestinal permeability was assessed using Cr-EDTA, D-lactulose and mannitol. Calves were euthanized on d 29 of life to obtain organ weights and intestinal samples to assess gene expression, GIT structure via histological analysis and tissue composition. Overall, no differences were found in milk intakes (8.5 L/d WP vs 8.7 L/d MR ± 0.22 L) or BW prior to dissection (68.9 ± 1.4 kg). Dissection results showed whole forestomach weight without contents was 22% greater in WP calves. Specifically, empty rumen, reticulum, and omasum weights were 23%, 31% and 36% greater in the WP calves, respectively. Furthermore, weights of the duodenum and ileum were similar between treatment groups, but distal jejunum weight and whole small intestine weight were 19% and 15% greater, respectively, in WP. Surface area of the duodenum and ileum did not

differ between treatment groups; however, surface area of distal jejunum was 33% greater in WP. Urinary Cr-EDTA recovery tended to be greater in WP fed calves at both 6 and 24 h post marker administration. Gene expression of tight junction proteins did not differ in the proximal jejunum or ileum between treatments. Jejunum free fatty acid and phospholipid composition were different between treatments and generally reflected the fatty acid profile of the respective diets. Feeding WP or MR alters GIT structure, function and composition, future studies should investigate the implications of these changes on long term health and productivity.

2.2 Introduction

Approximately half of all dairy calves in North America are fed MR (USDA, 2014) during the preweaning period, which differs in composition from whole milk (**WM**). Typically, MR contains 16 to 20% fat (%DM), whereas WM contains approximately 30% (Park, 2009). Differing fat content and source between MR and WM can influence the digestibility and the physiological impact on the calf. Previous studies comparing WM and MR have examined the influence of different milk allowances and MR using different fat sources on feed intake, digestibility, and calf growth (Lynch et al., 1978; Huuskonen et al., 2005). When the composition of MR was formulated similar to WM (25.6% fat, vegetable and animal source), WM fed calves gained 36% more BW than MR fed calves in the pre-weaning period (Lee et al., 2008). These differences can largely be attributed to differences in digestibility and nutrient availability. Recently, high fat MRs have been investigated to better replicate WM composition and authors report that calves fed high fat MR (≥23%) had reduced number of therapeutic interventions,

lower fecal scores and improved growth and gastrointestinal tract (**GIT)** development than calves fed a low-fat MR (≥17%; Amado et al., 2019; Berends et al., 2020; Welboren et al., 2021b). Therefore, fat is of great importance to the pre-weaning diet of dairy calves.

Lipids are an efficient source for energy deposition, as well as essential fatty acids and bioactives, conferring important structural and metabolic functions for newborn animals (Koletzko et al., 2011; Delplanque et al., 2015; Grote et al., 2016). In contrast, MR has substantially different fatty acid and amino acid profiles, depending on the raw materials used (e.g., dairy, vegetable, or animal products; Hill et al., 2008, 2011; Kato et al., 2011; Esselburn et al., 2013). Within the fat fractions of WM and MR, fatty acid profile, lipid profile and triglyceride structure also differ, which can negatively affect fat absorption and digestion by generating calcium soaps (Lien, 1994; Agostoni et al., 1995). Several studies have shown that supplementing different fatty acids in MR to better mimic WM can influence health outcomes and GIT development (Gorka et al., 2011a, b; Hill et al., 2011). For example, supplementing MR with butyric acid, medium chain fatty acids and α-linolenic acid (**ALA**) resulted in increased growth and feed efficiency and decreased the number of days with diarrhea (Hill et al., 2007,2011; Garcia et al., 2014). Butyric acid is generally absent from MR and supplementation in MR has been shown to increase rumen and intestinal growth, and specifically, to promote development of the intestinal barrier (Gorka et al., 2009; Gorka et al., 2011a,b; Kato et al., 2011). Meanwhile, very little is known about how feeding WM vs. MR influences the development of the GIT.

The liquid diet composition has been demonstrated to alter numerous aspects of GIT development. Zhang et al. (2019) observed that rumen development is closely associated with the type and composition of the liquid feeding diet while MR composition affects intestinal permeability (Amado et al., 2019); however, no data exists comparing these factors between WM and MR. In addition, changes in intestinal permeability may be reflective of changes in mRNA of tight junction genes (Suzuki and Hara, 2011) in intestinal tissue. Fatty acid profile of the diet is also involved in modifications of intestinal permeability and tissue composition (Spector and Yorek, 1985; Jenkins and Kramer, 1990; Ibarguren et al., 2014). Omega 3 and 6 fatty acids have been suggested to alter intestinal permeability based on their roles as anti- and pro-inflammatory precursors, respectively. Usami et al. (2011) demonstrated that eicosapentaenoic acid (**EPA**) and ALA (both omega 3) decrease tight junction permeability in vitro. It is plausible that physiochemical differences between WM and MR mediate changes in permeability by facilitating changes in gene expression and tissue composition during GIT growth and development, which in turn alters intestinal permeability.

Based on this data, we hypothesized that fatty acid profile of WP would alter GIT composition and promote GIT growth, development, and decrease permeability. Therefore, the objective of this study was to compare a high-fat MR to a WP – both having similar macronutrient inclusion in terms of fat, protein, and lactose – on GIT structure, function, and composition in pre-weaned calves.

2.3 Materials and methods

This study was conducted between March and June 2019 at the Calf Research Facility of Trouw Nutrition Research & Development (Sint Anthonis, the Netherlands). All procedures described in this article comply with the Dutch Law on Experimental Animals and Directive 2010/63/EC and were accordingly approved by the Animal Care and Use Committee of Utrecht University (DEC no. AVD2040020173425).

2.3.1 Animals and experimental design

A total of 18 male Holstein-Friesian calves (46.6 ± 1.21 kg BW; 56.4 ± 1.30 h after birth) were collected from 7 dairy farms within 14 km of the research farm. Histological data from previous calf research was considered to evaluate the sample size. Based on the outcome of Pyo et al. (2020), investigating villous growth in calves, a standard deviation of 20.85 µm was assumed for villus length. The minimal meaningful difference was considered to be 90 µm. Therefore, for a power (1-β) of 80% (Kempthorne, 1973), and a significance level of 0.05, the smallest meaningful sample size to detect relevant differences was 8 calves per treatment group. It was then estimated that 9 calves per treatment would be sufficient to detect differences in the current study. This study is part of a larger experiment that compared the effect of WP feeding against three MR formulations on health and growth (Wilms et al., in review).

At the farm of origin, a standardized protocol for colostrum management was used in the first 24 h after birth. A first meal of 3 L was offered within the first 3 h after birth, followed by two feedings of 2 L within 24 h of birth. Colostrum was tested with a portable Multi-Test Analyzer (DVM Rapid Test II, Vetlab, Palmetto, Florida, USA) and

required a reading of 22% Brix or greater, indicating an immunoglobin content of 50 mg/L or greater (NAHMS, 2007). Successful completion of this protocol was evaluated by assessing blood IgG upon arrival at the research farm within 48 to 72 h after birth using a portable Multi-Test Analyzer (DVM Rapid Test™ II). Following completion of the colostrum protocol, calves were provided 2.5 L of a commercial MR at a concentration of 135 g/L (Sprayfo Excellent, Trouw Nutrition, Deventer, the Netherlands) twice daily until arrival at the research farm. Upon arrival, calves were randomly assigned to 1 of 2 treatments of either dehydrated WM (WP; 25% fat DM basis; n = 9; Table 2-1) or high fat MR (MR; 24% fat DM basis; n = 9; Table 2-1) which were offered three times daily (0600, 1200 and 1800 h) in 3 L meals (135 g/L of WP or MR) through teat buckets. No solid feed was provided to control nutrient intake. Throughout the experiment, water was available ad libitum through additional buckets.

2.3.2 Housing

Calves were housed indoors in individual pens (1.22 X 2.13 m), separated by galvanized bar fences, and equipped with rubber-slatted floors in the front (50% of total pen area) and a laying area in the back, including a mattress covered with flax straw. During total urine collection, calves were tethered to the front of the pen and an elevated plateau covered with rubber was added to the front of the pen to elevate the animals to ease urine collection. The temperature and humidity in the calf facility was maintained at 15°C or greater and below 80%, respectively. Calves were exposed to daylight and/or artificial light from 0600 to 2200 h.

Health was visually monitored daily by caretakers and a standard veterinary protocol was followed in the case of disease. A veterinarian was consulted if clinical symptoms were not described in the standard protocol. Fecal scoring and intakes of liquid feed (WP or MR) and water were recorded daily throughout the study period, as described in Amado et al. (2019). Bodyweight was measured on the day of arrival and once weekly.

2.3.3 Chemical Analysis

Feed and urine were analyzed at MasterLab (Boxmeer, the Netherlands). Milk replacer and WP samples were analyzed for DM, crude ash, crude fat, CP, macro-minerals, and carbohydrates (lactose, glucose and starch). Dry matter content was determined by drying at 103°C for 4 h (EC 152/2009; EC, 2009). Crude ash was analyzed by incineration in a muffle furnace for 4 h at 550°C (EC 152/2009; EC, 2009). Crude fat was determined by hydrochloric acid hydrolysis followed by extraction with petroleum (EC 152/2009; EC, 2009). Crude protein content was analyzed by combustion, according to the Dumas method (Etheridge et al., 1998; ISO 16634-1:2008). Macro-minerals were analyzed using inductively coupled plasma mass spectrometry (PerkinElmer ICP-MS 300D) according to NEN-EN 2017. Chloride was analyzed as described in Wilms et al. (2019). Carbohydrates in MR and treatments were determined by titrimetric method according to 1971 71/250/EEG for lactose and EC 152/2009 for glucose.

To prepare the chromium measurements, 0.5 mL of nitric acid was added to 1 mL of urine sample. Following a 4 h incubation at 95°C, MilliQ water (Millipore, Billerica,

MA) was added to the solution to achieve a final volume of 15 mL. Samples were analyzed using ICP-MS (PerkinElmer, ICP-MS 300D). A calibration curve of chromium (0, 0.005, 0.02, 0.1 and 0.5 mg/L) was used to quantify the samples and the results were corrected using an internal standard (74Ge). For extraction of D-mannitol and lactulose, urine samples were diluted using D-Mannitol and labelled Lactulose (13-c) as internal standards. The extracts of the solution were then analyzed using a Phenomenex Luna 3 µm SUGAR 100A 150 mm x 4.6mm column (Phenomenex, Utrecht, the Netherlands) on a Thermo TSQ Quantis liquid chromatography tandem mass spectrometer (heated electrospray ionization source) with an Vanquish pump, oven and autosampler (ThermoFisher Scientific, Waltham, MA). The elution buffer used was a blend of 80% acetonitrile and 20% water containing 1 mM of formate.

Quantification of peroxide value, lipid oxidation and total fat and fatty acids was performed by Merieux Nutrisciences (Chicago, USA; Tables 2-1, 2-2). Peroxide value was determined by weighing 5 g of either WP or the high-fat MR into a 250 mL Erlenmeyer flask and dissolving 50 mL of acetic acid-isoactone and 0.5 mL of KI solution. The flask was then left to stand with occasional shaking for 1 min, at which point 30 mL of water was added to the solution. Iodine was liberated by titrating with standardized thiosulfate solution and peroxide value was calculate and reported in mEq/kg. To determine lipid oxidation, a thiobarbituric acid (**TBA**) test was performed by first homogenizing and weighing samples into a blender jar and blending with deionized water. Sulfanilamide solution was added to cure samples, after which they were transferred to a distillation flask. Hydrochloric acid was added, after which the solution

was distilled and distillate was collected over 10 min. TBA reagent was combined with distillate in a test tube, boiled for 35 min and then left to cool to room temperature. Absorbance was measured against a blank, as described in Kirk and Sawyer (1991). Total fat and fatty acids were determined via gas chromatography. Samples were weighed into a Mojonnier flask and pyrogallol, internal standard and hydrochloric acid and/or ammonium hydroxide were added. The flask was placed into a water bath for 45 to 90 min. Fat was extracted 4 x with petroleum ether and ethyl ether and then dried with sodium sulfate. Using a Rotovap, samples were evaporated at 45 ± 5°C and then methylated with BF3. The solvent layer was then transferred to a gas chromatography autosampler vial and injected. Fatty acids and total fat were calculated using an internal standard method.

Whey protein nitrogen index (**WPNI**) is a measure of protein quality and was determined by mixing 5 g of MR or WP in a jar with 50 mL of distilled water. The jar was then placed in a water bath at 37°C for 30 min. During the 30 min, the solution was shaken 5-to-6 times. The jar was removed from the water bath and 20 g of sodium chloride was added before placing the jar back into the water bath for another 30 min. During the first 15 min, the solution was shaken 6 times. The solution was set to rest for 15 min and filtered using Whatman No. 3 filter paper to determine the casein-nitrogen content (CNC). The first liquid through the filter was discarded and approximately 15 mL of the second liquid was collected and 0.24 mL of 10% HCl was added to 8 mL of the filtrate and mixed. The solution was rested for 30 min and filtered over a S&S blueband filter to determine the NPN fraction. Flow through was discarded and both the CNC and

NPN fractions were diluted 1x using a phosphate buffer. Absorption was determined at 272 nm using a standard curve. The difference between CP, CNC and NPN equates to WPNI.

The amino acid profile of WP and MR (Table 1-2) were analyzed using ultra-performance liquid chromatography (UPLC). A total of 0.1 g of WP and MR were measured in duplicates into small digestion tubes and 5 mL of 6 M HCl-phenol solution were added to each tube. Each tube was then flushed with nitrogen gas for 10 s. Digestion tubes were sealed and samples were hydrolyzed in a heating block for 24 h at 110°C. Following digestion, samples were removed from the heating block and cooled to room temperature. A volumetric pipette was used to add1 mL of norvaline standard solution (5 mM) to each tube and vortexed. Samples of 1 mL were then aliquoted into microcentrifuge tubes and stored at -20°C until the day of UPLC analysis. On the day of UPLC analysis, samples were vortexed and centrifuged at 5,000 × g for 5 min. Using a microcentrifuge tube, 120 µL of acidic sample was transferred into another microcentrifuge tube with 100 µL of 6 M NaOH and 400 µL of milliQ water and vortexed to homogenize. Microcentrifuge tubes were centrifuged again at 5,000 × g for 5 min. Derivation and UPLC amino acid analysis were performed according to Camara et al. (2020).

2.3.4 Intestinal Permeability Assessment

Permeability of the GIT was assessed using indigestible markers. The volume of each marker solution was adjusted for each calf to provide an individual dose of Cr-EDTA (0.1 g/kg BW; Masterlab, Boxmeer, the Netherlands), lactulose (0.4 g/kg of BW;

Sigma-Aldrich, Zwijndrecht, the Netherlands) and D-mannitol (0.12 g/kg of BW; Sigma-Aldrich). The marker solutions were orally pulse-dosed using 100 mL syringes (BD Plastipak, Merkala, Alkmaar, the Netherlands) to calves at 0600 h on d 21 instead of the morning MR meal. This method was used to avoid potential interferences in marker absorption due to different treatment composition (Amado et al., 2019). After marker administration, total quantitative 6- and 24-h urine collection was done using urine collection bags attached with medical glue, as described in Wilms et al. (2020b), to evaluate marker recovery in urine. Additionally, a spot urine sample was taken using plastic bags on d 20 (one day prior to quantitative urine collection) as a background measurement. Samples were transported in boxes with cooling aids and stored at -18ºC. Urine samples were processed at the University of Nottingham (Nottingham, United Kingdom). Urine Cr-EDTA concentrations were determined using ICP-MS (Thermo Fisher XSeriesII, Thermo Fisher Scientific, Waltham, MA).

2.3.5 Post-mortem Analysis

Calves were euthanized 3 h after the morning meal (33.0 ± 0.44 d of age) by injecting 6 mL of Tetracaine (T61; MSD Animal Health Nederland, Boxmeer, Netherlands) into the jugular vein. After the calves achieved a surgical plane of sedation, exsanguination was performed. The esophagus and rectum were tied using zip-ties to prevent loss of contents when the GIT was removed and placed on a surgical table. Each segment of the GIT was weighed and measured for length, as described in Pyo et al. (2020). Samples of the small intestine were taken as follows: samples of duodenum were taken 7.5 cm distal from the pyloric sphincter, proximal jejunum

samples were taken 100 cm distal to the duodenum sampling site, and distal jejunum samples were taken 30 cm proximal to the collateral branch of the cranial mesentery artery. Ileum samples were collected 30 cm proximal to the ileo-cecal junction and colon samples were collected 30 cm distal to the ileo-cecal junction. Tissue samples were cut longitudinally, washed with phosphate buffered saline and cross sections were fixed in 10% buffered formalin solution (4% formaldehyde; Fisher Scientific, Hampton, NH, USA), and placed in RNALater (Invitrogen), after which the samples were stored at -80°C until analysis.

2.3.6 Total Lipid Analysis of Small Intestine Tissue

Frozen samples of jejunum tissue were ground under liquid nitrogen using mortar and pestle and 0.05 g were weighed for analysis. Samples were placed into 1 mL of 0.1 M potassium chloride in a glass tube. A homogenizer was used to grind the sample matrix which was then added to another glass tube with 50 µL of 17:0 Free fatty acid standard (1 mg/mL). A 2 mL 2:1 chloroform-methanol mixture was added into the original tube and then transferred to the second tube. This process was repeated twice to ensure complete transfer of sample between tubes. Tubes were vortexed for 5 to 10 s or until mixed and then tubes were flushed with nitrogen gas for 5 to 10 s. Samples were left overnight at 4°C. Samples were centrifuged at 1460 rpm for 10 min at 21°C. A 22.9 cm Pasteur pipette was used to transfer the lower the chloroform layer into a leak-proof 15 ML tube that was washed with acid. The tube was completely dried under a gentle stream of nitrogen. The drying apparatus was cleaned with hexane before and after use. In each tube 2 mL of 0.5 *M* potassium hydroxide in methanol was added. Both

tubes were saponified at 100°C for 1 h in the oven. Tubes were checked every 10 min to ensure no solvent evaporated and the level of solvent remained equal across all tubes. Tubes were cooled for 10 min at room temperature in a fume hood. To stop methylation, 2 mL of double-distilled water was added to each tube then vortexed. Tubes were then centrifuged at 1460 rpm for 10 min at 21°C to separate phases. A 14.6 cm Pasteur pipet was used to extract the top hexane layer into a clean gas chromatography vial and dried with nitrogen. For gas chromatography analysis, samples were reconstituted in 250 µL of hexane and placed in a glass insert. The insert was returned to the gas chromatography vial, capped tightly, and run with 30 µL of MA6 as the standard.

2.3.7 Phospholipid Analysis of Small Intestine Tissue

Lipids including phospholipids (**PL**) were extracted from samples according to the method of Bligh and Dyer (1959) in the presence of the internal C17:0-phosphatidyl choline (PC) standard. Processing of the samples was performed as described previously (Bligh and Dyer, 1959; Holub et al., 2011). Sample extracts were dried under nitrogen after the addition of butylated hydroxytoluene. Extracts were resolubilized in 200 µL of dichloromethane. Separation of extracts was performed by solid-phase extraction by spotting 50 µL of lipid extracts containing internal standards on silica gel 60 thin-layer chromatography plate (Merck 5721-7). A standard solution was spotted 50 µL in the adjacent sample lanes for PL band identification. TLC plates were developed in mobile phase heptane:isopropyl ether:acetic acid 60:40:3 v/v/v (Holub et al., 2011). After the solvent had moved 80% up the plate, the plate was removed from the TLC

tank and allowed to dry for 5 min in the fume hood. The plate was sprayed with 8-anilino-1-napthalene sulfonic acid in methanol in order to identify bands of interest based on RF comparisons to appropriate standards by using long wave UV. A single edged razor was used to scrape off bands of interest into screw top 16 mm x 125 mm glass test tubes. Fatty acid methyl esters were prepared using boron trichloride in methanol and the methylation tubes were heated by hot plate for 50 mins at 95°C. The fatty and methyl esters were then analyzed on an Agilent 7890B gas-liquid chromatograph with a 60-m DB-23 capillary column (0.32 internal diameter: Morrison and Smith, 1964).

2.3.8 Small Intestine Histomorphology

Samples of duodenum, distal jejunum and ileum were dehydrated overnight by incubation in ethanol. Once the samples were successfully dehydrated, they were embedded in paraffin wax, sectioned (5 µm) using a rotary microtome and stained with hematoxylin and eosin (Gezondheidsdienst voor Dieren, Deventer, the Netherlands). Slides were viewed using a Leica ICC50W microscope at 40× magnification connected to the Leica Airlab app (Leica Microsystems, Wetzlar, Germany) for imaging. ImageJ software (ImageJ, National Institute of Health, Bethesda, MD, USA) was used to determine villus height and width, crypt depth and width, and muscularis layer thickness. Villus height was measured from the fully attached epithelial cell to the villus-crypt junction and villus widths were measured perpendicularly at mid-villus height. Crypt depth was measured from the villus-crypt junction to the bottom of the crypt and crypt width was measured at the opening between villi at the villus-crypt interface (Wongdee et al., 2016).

Muscularis layer thickness was measured from the apical side below the crypt to the serosal side. Two to three measurements were taken from 3 to 5 images per slide (10 measurements/small intestinal region). All measurements were averaged by sample and analyzed as one observation per dependent variable. Villi height and crypt depth were used to calculate villi:crypt ratio. Mucosal surface area index (**SA**) was calculated (shown in Eq. 1) from the mucosal-to-serosal amplification ratio as seen in Kisielinksi et al. (2002),

Equation 1.

$$M = \frac{\left[(a \times b) + \left(\frac{a}{2} + \frac{c}{2}\right)^2 - \left(\frac{a}{2}\right)^2\right]}{\left(\frac{b}{2} + \frac{c}{2}\right)^2}$$

where: M = Mucosal SA index; a = Villi width (µm); b = Villi height (µm); and c = Crypt width (µm).

2.3.9 Ki-67 Immunohistochemistry of Small Intestine Tissue

Duodenum, proximal jejunum and ileum tissue embedded in paraffin were also evaluated for expression of the Ki-67 antigen using immunohistochemistry (Histotechnology lab; University of Guelph, Guelph, Canada). Ki-67 is a marker for proliferation (Hall et al., 1990). An automated Leica microtome was used to section the tissue. Slides were then deparaffinized, rehydrated and treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. Slides were then placed into the Dako PT Link (Dako Auostainer, Agilent Technologies, Missisauga, Canada) for heat-induced epitope-retrival at high pH, cooled and placed on the staining instrument. A

universal non-serum blocker was applied for 10 min. Sections were then incubated with mouse anti-human Ki-67 monoclonal antibody (1:50 dilution; clone MIB-1, Agilent Technologies), followed by anti-mouse/anti-rabbit polymer (EnVision Flex HRP, Agilent Technologies) detection and Nova Red chromogen (Vector Laboratories, Burlington, Canada). Duplicate sections of one calf per treatment group (2 total) were subjected to the same immunohistochemistry procedure as negative reagent controls with substitution of antibody diluent alone for the primary antibody. Images were captured using a Leica ICC50W microscope at 100x magnification connected to the Leica Airlab app (Leica Microsystems). Five images were taken per tissue section per calf. The fraction of cells stained positive for Ki-67 antigen out of the total cells was quantified using bioanalysis software QuPath (Bankhead et al., 2017). The mucosal area of the crypts of each image were selected and cells stained positive for the Ki-67 antigen within the selected area were detected using positive cell detection. A subset of 2 animals and all three tissues were used to optimize settings for background radius (16 px), sigma (1.8 px) and threshold (1.35) to enable accurate detection of the positive and negative cells at the right colour intensity. For duodenum, proximal jejunum, and ileum % CV was 8.9, 7.1 and 14.8, respectively.

2.3.10 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Frozen tissues from the proximal jejunum and ileum were ground with liquid nitrogen using a mortar and pestle. Extracted RNA from tissues stored in RNAlater using Pure Link RNA Mini Kit (Invitrogen), according to manufacturer's protocol. Quantity and integrity number of RNA were measured using ultraviolet-visible spectroscopy (Nanodrop

One Microvolume, Thermo Fisher Scientific, Maltham, MA, USA) and electrophoresis (TapeStation, Agilent Technologies, Santa Clara, CA, USA). The average RNA integrity number was 6.92 and 7.11 for the proximal jejunum and ileum samples, respectively, and the minimum threshold was 5.6. Then, 1 µg RNA was used to generate cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA) which was analyzed for the expression of genes encoding several tight junction proteins and cytokines by performing qRT-PRC. The primer pair sequences used are shown in Table 2-4. The geometric mean of all three housekeeping genes (*β-actin, RPL19, GAPDH*) were used as reference in both the proximal jejunum and ileum. Primer efficiencies were determined using a standard curve and ranged between 92% and 110% (Table 2-9). The qRT-PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) by the Genomics Facility at the University of Guelph (Guelph, Canada). In each well, 5 µl of cDNA, 10 µl of DNA polymerase-containing supermix (SsoAdvanced Universal Inhibitor-Tolerant SYBR Green, Bio-Rad, Hercules, CA, USA), 0.8 µl of 5 µM forward and reverse primer mix and 4.2 µl of nuclease-free H2O was added. The activation step was 3 min at 98°C polymerase activation step, followed by 40 cycles of a two-step qPCR (10 s at 98°C for denaturation and 30 s at 60°C of combined annealing/extension). Then, a melting curve was generated at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s to confirm specificity of the PCR amplicon. The expression of the target gene relative to the target gene was calculated according to Pfaffl (2001) using the average of the MR group as the control.

2.3.11 Calculations and Statistical Analysis

Data were analyzed in a mixed model using the GLIMMIX procedure in SAS software (version 9.4, SAS Institute Inc., Cary, USA) and fecal score was analyzed using the Chi square test in PROC FREQ. Differences were considered when the Chi square $p \leq 0.05$. Pearson correlation values were analyzed using PROC CORR. We adjusted the response distribution in the model statement to lognormal when it improved the fit of the model indicated by the distribution of studentized residuals and the Shapiro-Wilk statistic. To present the results data was back transformed. In all analyses, the experimental unit was the calf. Body weight entered the model as a covariate for all mass-related measurements but was not included in the model for gene expression analysis. The model included the fixed effect of treatment, time, and interaction of treatment-by-time in the case of repeated measures. The statistical model for repeated measure analyses was

$$y_{ijkl} = \mu + \text{treatment}_i + week_j + (Trt \times W)_{ij} + e_{ijk}$$

where y_{ij} is the lth observation ith treatment at the jth time, μ is the intercept, treatment_i is the fixed effect of the ith treatment, week_j is the fixed effect of the jth week, $(Trt \times W)_{ij}$ is the fixed effect of the interaction of the ith treatment-at the jth week. BW_k is the observed bodyweight upon arrival, and e_{ij} is the random error associated with the lth observation in the ith treatment at the jth time. The covariance structure's heterogenous autoregressive, autoregressive, and compound symmetry were tested for each variable. It was found that autoregressive was the best fit based on the lowest Aikaike's and Bayesian information criteria. The model for free fatty acid and phospholipid tissue composition included the fixed effect of treatment, tissue, and interaction of treatment by time; but for the purpose

of this thesis only the tissue effects are presented. The Tukey post-hoc adjustment was used to separate means. All values reported are presented as least square means with significance declared at $P < 0.05$ and tendencies are considered at $0.05 \leq P < 0.10$.

2.4 Results

2.4.1 General Health, Growth, Intakes, and Intestinal Permeability

Serum IgG measured at arrival did not differ between treatments ($P = 0.66$; Table 2-5). The dietary treatments did not influence ADG nor daily treatment intakes ($P = 0.82$, $P = 0.44$, respectively; Table 2-5). Consequently, ME intake and feed efficiency did not differ between treatments (Table 2-5). Proportion of calves that experienced diarrhea between WP and MR were similar: 71% vs 68%, ($P = 0.60$), respectively. Results for urinary Cr-EDTA, mannitol and lactulose recovery are presented in Figure 2-1. Recovery of Cr-EDTA between 0 and 6 h was 60% greater ($P = 0.007$) in WP fed calves and tended to be 26% greater ($P = 0.082$) between 0 and 24 h in calves fed WP. Recovery of lactulose in urine between 0 and 6 h was 47% greater ($P = 0.047$) in WP fed calves. There were no differences in recovery of lactulose between 0 and 24 h or recovery of D-mannitol.

2.4.2 Gastrointestinal Tract Weights and Lengths

Weights and lengths of the GIT are presented in Table 2-6. Omasum, reticulum, and total forestomach weight were 36%, 31% and 22% greater, respectively, in WP fed calves ($P \leq 0.048$). Rumen weight tended to be 23% greater ($P = 0.053$) in the WP fed calves. In addition, whole small intestine weight and jejunum weight were 15 and 19% greater ($P = 0.031$ and $P = 0.013$, respectively) in WP fed calves. No other differences

were observed in GI segment weights or lengths. No differences were observed in the weights of the pancreas, liver or kidney however, spleen weight was 18% greater in MR fed calves (P = 0.004).

2.4.3 Intestinal Histomorphology, Immunohistochemistry and Gene Expression

Liquid feed treatments had no effect on intestinal development apart from SA index, which was greater in the distal jejunum of calves fed WP compared with those fed MR (P = 0.031; Table 2-7). The villus height:crypt depth ratio of the ileum was lower (P = 0.037) in calves fed WP (Table 2-7). No differences were observed in detection of Ki-67 antigen (Table 2-7). No differences were observed in either the tight junction genes or nutrient transporters in either the proximal jejunum or ileum (Figure 2-2).

2.4.4 Total Lipid and Phospholipid of Small Intestinal Tissue

The free fatty acid results of jejunal tissue are presented in Table 2-8. Calves fed WP had 2.64-fold greater concentration of n-3 FA in jejunal tissue (P < 0.001) while n-6 concentrations were unaffected (P = 0.361) and accordingly, the n-6:n-3 ratio was 3.2-fold greater in MR calves (P < 0.001). Total FA, SFA, UFA, MUFA and PUFA were unaffected by treatments (P = 0.729). However, the concentration of C12:0 tended to be 4.8-fold greater in MR calves (P = 0.074) and the concentration of C15:0 free FA tended to be 5.5-fold greater in WP calves (P = 0.051). The concentration of C17:1 cis-10 tended to be greater in WP calves (P = 0.06) and the concentration of linoleic acid (**LA**) tended to be greater in MR calves (P = 0.095). Calves fed WP had 3.7-fold higher concentrations of ALA in jejunal tissue (P < 0.001) and tended to have greater concentrations of C18:4 n-3 (P = 0.084). Concentrations of C20:4 n-6, C20:3 n-3 and

C20:5 n-3 were 51%, 100% and 2.1-fold greater in WP calf jejunum tissue ($P = 0.049$, $P = 0.032$, and $P < 0.001$, respectively). Lastly, the concentration of C22:5 n-3 as a free fatty acid was 2.5-fold greater in WP calves ($P < 0.001$). The free fatty acid profile of ileum tissue is presented in Appendix 1.

The phospholipid fatty acid results of jejunal tissue are presented in Table 2-9. Calves fed MR had 6.0% greater total saturated fatty acids, ($P < 0.001$), 53% greater C15:0 ($P = 0.006$), 16% greater C16:0 ($P < 0.001$) and 13% greater concentration of C24:0 ($P = 0.039$). Unsaturated fatty acids and MUFA were 7.9% and 20% greater in WP calves ($P < 0.001$ and $P < 0.001$) while PUFA content was unaffected ($P = 0.451$). Total content of n-3 FA was 2.4 times greater in WP calves ($P < 0.001$) while n-6 content was 18% greater in MR calves ($P = 0.002$). The ratio of n-6:n-3 FA was 2.9 fold greater in MR calves ($P < 0.001$). Of the MUFA, C16:1 and C18:1 was 3.4-fold and 25% greater in WP calves ($P < 0.001$, $P < 0.001$, respectively) and C24:1 was 55% greater in MR calves ($P < 0.001$). Concentration of C18:3 n-6 and C18:3 n-3 were 55% and 5-fold greater in WP ($P = 0.070$, $P < 0.001$, respectively), while C18:2 n-6 was 41% greater in MR ($P < 0.001$). All three n-6 with 20 carbons (C20:2, C20:3, C20:4) were greater (2-fold, 31% and 25%, respectively) in WP calves ($P < 0.001$, $P = 0.010$ and $P = 0.063$ respectively). Both C20:4 n-3 and C20:5 n-3 were greater (2.6-fold and 3.6-fold, respectively) in WP calves ($P < 0.001$). While C22:4 n-6 was 47% greater in MR jejunum tissue ($P = 0.013$), C22:5 n-3 and C22:6 n-6 were 2.3-fold and 49% greater in WP calf jejunum tissue ($P \leq 0.001$). The phospholipid profile of ileum tissue is presented in Appendix 2.

2.5 Discussion

In the current study, it was hypothesized that the fatty acid profile of the WP treatment would improve GIT morphology and alter tissue composition in calves fed WP as well as reduce intestinal permeability compared with calves fed a high fat MR. Calves receiving the WP treatment had increased foregut and hindgut mass, as well as increased SA index in the distal jejunum, but had greater intestinal permeability. These results corroborate the findings of Niwinska et al. (2005) where increased small intestinal development was observed in WM fed calves compared to an iso-energetic MR (34% protein, 24% fat, soy and whey based), yet this study did not correct for macronutrient composition. The results of the present study are the first to suggest that for a similar macronutrient inclusion in terms of total fat, protein, and lactose, differences in fatty acid profile may affect GIT growth and function. Previous studies comparing WP and MR differed in feeding rates, composition of the liquid diet or digestibility. In the current study, both treatments were offered at the same feeding rate and contained milk-based products, although protein digestibility was numerically lower and DM digestibility was lower in the WP treatment (Wilms et al, in review). The difference in digestibility is caused by the lower WPNI in the WP treatment which likely occurred due to overheating during the drying process. The largest treatment differences were seen in the composition of the fat fraction, namely the fatty acid profile. Amino acids, such as leucine, have also been shown to promote intestinal development, while others, such as phenylalanine, inhibit intestinal development in milk-fed Holstein calves (Cao et al., 2019), although this topic is less explored. The amino acid profiles in these treatments

were similar and likely not responsible for the observed differences in this study. Several studies have suggested that supplementing butyric acid, medium chain FA, ALA, lauric acid and myristic acid in MR improves performance and reduces diarrhea incidence (Esselburn et al., 2013; Garcia et al., 2014; Hill et al., 2016). Therefore, it is plausible that differences in fatty acid profile between MR and the WP, could explain part of the observed differences in GIT mass in the foregut and hindgut.

With respect to fatty acids, butyrate has been extensively studied as a supplement for MR and is commonly administered in the salt form sodium-butyrate. Butyrate has a dose-dependent effect and is proliferative when administered to calves in either starter or MR within the first week of life at approximately 0.3% DM (Guilloteau et al., 2009; Niwinska et al., 2017). In the present study, butyrate content was 0.84 and 0.02% DM (3.24% and 0.07% of total fat) in the WP treatment and MR treatment, respectively, and may have contributed to increased intestinal tissue mass and SA in the WP group. Histology results from previous studies show proliferative effects on the GIT at low concentrations (≤ 0.3%) and apoptotic effects at high concentrations (Guilloteau et al., 2009). Results of butyrate supplementation are contradictory, showing increases in mitotic index in the jejunum (Guilloteau et al., 2009) and decreases in mitosis:apoptosis index in the epithelium of the small intestine both (Gorka et al., 2011a) further demonstrating it proliferative and apoptotic funcitons. Butyrate supplementation in MR has been found to increase foregut weight, as well as rumen papilla length and width (Gorka et al., 2009; Gorka et al., 2011a). This suggests that in the present study butyrate may have had a proliferative effect in the rumen, however, the detection of the

Ki-67 antigen in the present study does not support this mechanism in intestinal tissue. It is hypothesized that butyrate improves GIT development via increasing GLP-2 mediated intestinal proliferation (Gorka et al., 2011b; Penner et al., 2011), however, GLP-2 receptors in the rumen of mature animals have not been detected (Taylor-Edwards 2010). The WP treatment resulted in increased organ mass in the foregut, potentially increasing rumen capacity and may increase absorption (Kristensen et al., 2007). Conversely, increased organ mass may also increase maintenance requirements (Reynolds, 2002) and therefore, may not benefit calves. Rumen development is critically important to optimize absorption of nutrients to maximize growth in the pre-weaning period (Baldwin et al., 2004). While nutrient absorption was not directly measured, increase in GIT mass in the foregut and small intestine may be attributed to differences in butyric acid intake between the treatments.

During the pre-weaning period, villus development (i.e., improving surface area) is an important factor for enhancing intestinal nutrient absorption, and therefore, is important to overall calf growth (Seegraber and Morrill, 1982; Hammon and Blum, 1997). Greater SA index in the distal jejunum of WP fed calves in this experiment, as well as greater mass in the distal jejunum and whole small intestine, indicates greater potential for nutrient absorption. Villus height to crypt depth ratio was only increased in the ileum of MR-fed calves in this study and is an indicator of increased intestinal proliferation (Jeurissen et al., 2002). In contrast, detection of Ki-67 antigen was not different in any segments of the small intestine indicating that changes in microstructure in the jejunum may not be due to changes in proliferation. Villus height and crypt depth

individually were not different between treatments. Villus height is known to correlate directly with growth performance in calves (Vente-Spreeuwenberg et al., 2003) but no differences were found in the present study (Wilms et al., 2021, in review). The histological measurements in this study indicate that the fatty acid profiles of WM and MR could affect both intestinal mass and epithelial microstructure differently, however the overall effect of these changes on nutrient absorption is unknown and the lower digestibility of the WP treatment may have influenced results (Wilms et al., in review)

Amino acid profile and other protein characteristics may also play a role in the development of the neonatal GIT. The protein source in WP was largely casein (approximately, 82% casein, 18% whey), whereas the MR used in this study was both casein and whey based (approximately, 50:50). However, the differences between whey and casein when milk-based are not known to affect health or digestion in dairy calves (Terosky et al., 1997; Lammers et al., 1998). Results from Castro et al. (2016) found that milk replacer formulated with hydrolyzed wheat protein and provided as 4.5% DM with methionine and lysine supplement, supports growth as well as milk-based diets for calves. In the current experiment, hydrolyzed wheat protein comprised 4.1% DM, and therefore, differences in protein source likely did not contribute to the observed differences. However, the WP treatment had numerically lower protein digestibility and lower DM digestibility (Wilms et al., in review), which may have influenced calf development. Amino acids are known to influence GIT development. In piglets, both glutamine and arginine supplementation improved intestinal morphology such as absorptive area, and in weaned pigs, glutamine reduced the severity of diarrhea (Wu et

al., 1996; Jiang et al., 2009; Wang et al., 2012). Histological measurements have been shown to increase from arginine and glutamine supplementation in calves (Van Keulen et al., 2020) however, arginine and glutamine content in both WP and MR were comparable, thus, it is unlikely that the changes in GIT growth and microstructure were affected by these amino acids. Ahangarani et al. (2020) queried whether glutamic acid supplementation in MR affected intestinal permeability and found that the amino acid supplementation did not alter permeability. While the amino acid levels in both treatments were very similar, the bioavailability of some amino acids in the WP may have been affected when overheated. Overall, when the present results are combined with previous literature, the role of amino acid profile in pre-weaning GIT development is unlikely but cannot be excluded.

Recent studies have found that many aspects of the preweaning diet affect the intestinal permeability of calves. In the current study, WP fed calves had increased Cr-EDTA and lactulose recovery in the first 6 h after marker administration and there was a trend towards greater Cr-EDTA recovery within the 24 h after marker administration. Therefore, due to the greater recovery of lactulose and Cr-EDTA, these results indicate a greater paracellular intestinal permeability in WP fed calves. It should be noted that analyzing permeability markers in urine vs. blood yields different results, as demonstrated by Welboren et al. (2021a), where differences in lactulose recovery were only detected in blood. Increased intestinal permeability has previously been associated with a loss of epithelial barrier integrity of the GIT (Araujo et al., 2015; Thiagarajah et al., 2018), but it is important to note that greater permeability is not necessarily associated

with diarrhea or indicate a damaged epithelium. Araujo et al. (2015) found diarrhetic calves had increased paracellular permeability and observed no differences in transcellular permeability. Unlike metabolic conditions such as sub-acute rumen acidosis, which has a known threshold for sub-clinical and clinical manifestations (AlZahal et al., 2007), no such threshold exists for intestinal permeability. It would be beneficial to develop such a threshold to quantify what different degrees of permeability mean to the animal in terms of health and production.

Studies in piglets demonstrate an inverse relation between butyrate intake and intestinal permeability, and studies in pigs and calves show butyrate-associated decreases in diarrhea incidence (Hill et al., 2007; Gorka et al., 2011a; Huang et al., 2015). Decreases in intestinal permeability have been associated with increases in jejunal and colonic occludin protein expression (Huang et al., 2015), however, differences in tight junction and inflammatory cytokine gene expression were not seen in this study, despite the differences in intestinal permeability. It should be noted that, gene expression results do not necessarily represent these molecules at the level of functional protein. The results of the present study demonstrate that WP and MR feeding alters intestinal permeability without affecting gene expression of tight junction proteins or cytokines.

Differences in fatty acid content of diets have previously been demonstrated to alter intestinal composition and function (Daveloose et al., 2002; Christon et al., 1989). These studies evaluated membrane fluidity and composition, however, results from Khajuria et al. (2002) suggest that intestinal permeability, membrane fluidity and

histomorphology of intestinal tissue are related. In the present study, the phospholipid profile of jejunal tissue and permeability results were correlated, as seen in Appendix 3. Furthermore, n-3 and n-6 have been evaluated for their ability to influence permeability due to their anti and pro-inflammatory functions and incorporation into cellular membranes (Sardesai, 1992; Wesley, 1998; Calder, 2010). Jejunum composition was largely reflective of diet, as demonstrated by the higher levels of EPA, a lower n-6:n-3 ratio and less LA in the diet of the WP group, which was reflected in the free fatty acid and phospholipid profiles of jejunum tissue. While previous in vitro studies found that EPA improved tight junction functionality, and consequently decreases intestinal permeability (Usami et al., 2001; Hossain and Hirata, 2008), this effect was not seen in this study, which may be due to the damaged protein in the WP treatment. Amount, bioavailability, and form of EPA may also be playing a role as Piazzi et al. (2014) found that supplementing EPA as a free fatty acid at 1% of corn oil caused a switch in membrane composition from omega-6 to omega 3, related to a reduction in pro-inflammatory prostaglandin production. In conclusion, the fatty acid profiles of WP and MR alter the free fatty acid and phospholipid composition of small intestinal tissue and may play a role in altering GIT function.

This study was the first to examine the effect of liquid diets with similar macronutrient composition, but different physiochemical characteristics on GIT development. The WP treatment aimed to represent the biological reference: bovine whole milk however, the WP treatment was in the form of a dehydrated powder, which is not necessarily representative of fresh WM. Major differences between dehydrated

and fresh WM can be seen in fat globule structures that largely differ in size, as dehydrated WP was also homogenized. There is also a potential for loss and inactivation of bioactive compounds during the drying process. Additionally, the drying process of WP is not intended to maintain the quality standards for feeding to newborn animals. As a result, the WPNI index was lower in the WP treatment, which resulted in a lower quality protein fraction in that treatment. Due to the nature of the facility, WP was used instead of fresh WM, however, future studies looking at GIT development and permeability could benefit from the inclusion of fresh WM in order to get biological references and establish numerical boundaries for permeability and health. The findings of this study suggest that differences in fat and protein composition affect the GIT morphology, permeability and tissue composition in pre-weaned calves. This should be investigated further to identify which fatty acids are most important for improving milk replacer formulations and optimizing calf health and GIT development.

2.6 Conclusion

The results of the current study demonstrate that the whole milk powder treatment increased gastrointestinal mass of the foregut, as well as mass of the whole small intestine and jejunum. The distal jejunum of whole milk powder fed calves showed greater surface area index and tended to have increased intestinal permeability. Dietary fatty acid profiles were largely reflected in free fatty acid and phospholipid profiles of intestinal tissue. There were no observed differences in gene expression of tight junction proteins between treatments. The effects of nutrient profiles, especially fatty acid profile, on gut development warrant further investigation to understand its influence

on growth and implications for milk replacer formulations. Future studies should evaluate which elements of nutrient profiles and physicochemical characteristics play the most relevant roles in gut development to optimize milk replacer formulations.

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2.8 Tables and Figures

Table 2-1. Nutrient compositions of whole milk powder (WP) and a high fat milk replacer (MR)[1] fed 3.0L three times daily to male Holstein-Friesen calves (n=18)

Item	WP	MR
Ingredient (% DM)		
Whole milk powder	100	-
Skim milk powder	-	29.5
Palm and coconut oil mixture (2:1)	-	22.6
Whey products	-	42.1
Hydrolyzed wheat protein	-	4.1
Premix	-	1.7
Nutrient composition[2]		
DM	960	968
Fat	250	242
Lactose	365	374
Protein	235	218
Glucose	0	4.84
Starch	0	13.7
Na	5.20	4.38
K	13.5	11.1
Cl	11.4	8.02
Ca	13.5	7.74
P	9.36	6.40
Mg	1.25	1.12
ME (Mcal/kg)[3]	4.95	4.93
CP:ME ratio	45.6	42.8
SID (mEq/L)[4]	31.7	42.6
WPNI (mg/g)[5]	1.82	6.68
Osmolality (mOsm/kg)[6]	301	316
Peroxide value (meq/kg)	10.5	11.9
TBA Concentration (mg/kg)[7]	0.883	0.203
Para-Anisidine value	7.00	4.15

[1]Treatments included: whole milk powder (WP; n = 9) and a high-fat milk replacer (MR; n = 9). Treatment concentration was 135.0 g DM/L to reflect the solid percentage of bovine whole milk.

[2]Expressed as g/kg DM unless specified otherwise

[3]Calculated following NRC (2001)

[4]Strong ion difference

[5]SWhey protein nitrogen index

[6]Osmolality (in moles per kg of solvent and expressed in mOsm/kg) was calculated by adding osmolality of carbohydrates and minerals as described in Wilms et al. 2020a

[7]Thiobarbutric acid test for lipid oxidation

Table 2-2. Fatty acid profiles of whole milk powder (WP) and a high fat milk replacer (MR) with similar macronutrient composition fed three times daily to male Holstein bull calves (n=18).

Fatty acid (% of total fat)	Treatment[1]	
	WP	MR
C4:0	3.24	0.07
C6:0	2.25	0.17
C8:0	1.37	1.61
C10:0	3.21	1.42
C12:0	4.55	17.5
C14:0	11.6	6.78
C14:1	1.10	0.02
C15:0	1.09	0.06
C16:0	32.6	30.3
C16:1	0.05	<0.01
C17:0	0.52	0.08
C18:0	8.76	3.85
trans-9 C18:1	0.18	0.00
trans-10 C18:1	0.34	0.00
trans-11 C18:1	0.84	0.002
Total 18:1 *trans*	1.60	0.02
cis-9 C18:1	18.1	29.7
C18:2 n - 6	2.01	6.84
C20:0	0.12	0.26
C18:3 n - 3	0.33	0.17
cis-9, *trans*-11 CLA	0.37	0.06
C20:4 n – 6	0.09	0.00
C20:5 n – 3	0.03	0.00
C22:5 n - 3	0.05	0.00
Total SFA[2]	71.2	62.3
Total UFA[3]	28.8	37.7
Total MUFA[4]	24.8	30.4
Total PUFA[5]	3.1	7.1
Total n-6 FA[6]	2.2	6.8
Total n-3 FA[7]	0.4	0.2
n-6:n-3 ratio	5.5	34

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.
[2]Sum of saturated fatty acids from 14 to 24 carbons in length
[3]Sum of unsaturated fatty acids from 14 to 24 carbons in length
[4]Sum of monounsaturated fatty acids from 14 to 24 carbons in length
[5]Sum of polyunsaturated fatty acids from 18 to 22 carbons in length
[6] Sum of omega 3 polyunsaturated fatty acids from 18 to 22 carbons in

[7]Sum of omega 6 polyunsaturated fatty acids from 18 to 22 carbons in length

Table 2-3. Amino acid profiles of whole milk powder (WP) and a high fat milk replacer (MR) with similar macronutrients fed three times daily to male Holstein bull calves. (n=18).

Amino acid (g/100g)	WP	MR
Alanine	1.09	1.07
Arginine	0.93	0.82
Aspartate	2.39	2.30
Glutamate	6.02	5.38
Glycine	0.88	0.76
Histidine	0.62	0.54
Isoleucine	1.12	1.18
Leucine	2.72	2.61
Lysine	2.08	2.17
Phenylalanine	1.52	1.31
Proline	3.11	2.65
Serine	2.00	1.67
Threonine	1.22	1.27
Tyrosine	1.48	1.24
Valine	1.36	1.36

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.

Table 2-4. Primer pair sequences of genes analyzed in proximal jejunum and ileum tissue collected from male Holstein calves (n=18) fed a whole milk powder (WP) or a high fat milk replacer (MR) of similar macronutrient composition

Gene[1]	Function[2]	Forward primer (5'-3') Reverse primer (5'-3')	Amplicon Size	Efficiency, %	Source
ACTB	Reference gene β-actin	CTAGGCACCAGGGCGTAATG CCACACGGAGCTCGTTGTAG[2]	177	101	Current study
GAPDH	Reference gene Glyceraldehyde 3-phosphate dehydrogenase	CCGTAACTTCTGTGCTGTGCC TTGATGGCGACGATGTCCACT	104	100	Malmathuge et al., 2013
RNA pol	Reference gene RNA polymerase	GGGACAACGGTGATCGGATT ATGTGCCGTTCCACCTTGTA	88	108	Current study
CLDN2	Transmembrane tight junction protein	CCAGGCCATGATGGTGACAT GAAGAAGACTCCGCCCACAA[3]	136	105	Walker et al., 2015
OCLN	Protein regulating passage of macromolecules	CGCAGGAAGTGCCTTTGGTAGC GCAGCCATGGCCAGCAGGAA[2]	124	107	Malmathuge et al., 2013
TJP1	Scaffold protein in tight junction (ZO-1)	AATGCATCCTGACCACCAGG GATGGTGCCGGGTTTGTTTC[3]	162	110	Walker et al., 2013
IL-1β	Pro-inflammatory cytokine	AGCATCCTTTCATTCATCTTTGAAG GGGTGCGTCACACAGAAACTC[4]	78	108	Galvão et al., 2011
TNF-α	Pro-inflammatory cytokine	CCCCAGAGGGAAGAGCAGT GAGGGCATTGGCATACGAGT[4]	168	110	Current study
CDC42	Controlling actin-myosin dynamics	ACGACCGCTGAGTTATCCAC TCTCAGGCACCCACTTTTCTT[5]	101	106	Current study

[1]CLDN2 = claudin-2, OCLN = occludin, TJP1 = tight junction protein 1, IL-1β = interleukin 1 beta, TNF-α = tumour necrosis factor alpha, CDC42 = cell division cycle 42

[2]Krug et al., 2014; Quiros and Nusrat, 2014; Rohr et al., 2019

Table 2-5. Arrival parameters of general measurements of male Holstein bull calves (n=18) fed whole milk powder (WP) and a high fat milk replacer (MR) with similar macronutrient composition three times daily.

	Treatment[1]			*P*-value[2]		
Item	WP	MR	SEM	T	W	T*W
IgG (mg/dL)	1991	1838	239.0	0.66	-	-
ADG	0.69	0.67	0.064	0.82	<0.001	0.16
Bodyweight					-	-
Arrival BW (kg)	47.1	45.6	1.72	0.54	-	-
Mean BW (kg)[3]	57.5	57.5	1.39	0.98	<0.001	0.16
Final BW (kg)[4]	69.9	67.5	1.93	0.40	-	-
Milk intake (L/d)	8.5	8.7	0.22	0.44	<0.001	0.18
ME Intake	5.95	5.83	0.15	0.48	<0.001	0.13
FE (ME/kg gain)	0.61	0.58	0.054	0.65	<0.001	0.23

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.

[2]T = effect of treatment (either WP or MR); W = effect of week (when applicable); T*W = interaction between treatment and week

[3]Least squares mean bodyweight analyzed as a repeated measure between weeks 1-4

[4]Final BW = bodyweight on the day of dissection after the week 4 bodyweight measurement, not analyzed as repeated measure

Table 2-6. The effect of feeding whole milk powder (WP) or milk replacer (MR) with similar macronutrient inclusion on gastrointestinal lengths and weights in male Holstein calves fed three times daily (n=18).

Item	Treatment[1]			*P*-value	
	WP	MR	Pooled SEM	T	BW[2]
Weight (g)					
Whole forestomach[3]	1245.8	1019.0	60.16	0.018	0.012
Abomasum	454.1	406.3	25.65	0.211	0.252
Omasum	201.9	148.2	14.86	0.022	0.265
Rumen	473.5	384.0	29.89	0.053	0.004
Reticulum	97.5	74.7	7.45	0.048	0.082
Whole small intestine[4]	1808.5	1570.9	70.15	0.031	0.013
Duodenum	34.0	27.9	3.40	0.219	0.092
Jejunum	1432.6	1200.6	61.56	0.013	0.171
Ileum	365.6	387.8	40.69	0.449	0.010
Whole large intestine[5]	592.8	577.9	27.19	0.707	0.041
Cecum	79.5	88.9	9.26	0.622	0.926
Colon	446.4	414.6	24.44	0.377	0.031
Rectum	66.1	70.8	5.15	0.533	0.451
Visceral organs					
Liver	1812.2	1823.1	55.73	0.893	0.004
Kidney	364.7	355.4	10.75	0.555	0.026
Spleen	231.0	272.6	8.45	0.004	0.010
Pancreas	49.9	44.2	2.65	0.154	0.246
Length (cm)					
Whole small intestine	2001.8	1902.1	71.61	0.345	0.685
Jejunum	1734.3	1641.4	69.66	0.366	0.925
Ileum	266.5	278.5	21.37	0.698	0.501
Whole large intestine	289.2	271.7	15.19	0.431	0.821
Cecum	24.8	22.1	1.11	0.107	0.506
Colon	264.2	248.9	15.06	0.488	0.835

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.

[2]Bodyweight at the time of dissection used as a covariate

[3]Forestomach = Abomasum + omasum + rumen + reticulum

[4]Small intestine = duodenum + jejunum + ileum

[5]Large intestine = colon + cecum

Table 2-7. Histomorphometric measurements of villi, crypts and muscularis layer thickness within the duodenum, distal jejunum and ileum of male Holstein bull calves (n = 18) fed a whole milk powder (WP) or a high fat milk replacer (MR) with similar macronutrient inclusions three times daily.

Item	Treatment[1]			
	WP	MR	SEM	*P*-value
Duodenum				
Villus length (µm)	247.7	283.8	25.57	0.336
Villus width (µm)	117.7	113.9	11.45	0.820
Crypt depth (µm)	291.1	262.0	33.64	0.553
Crypt width (µm)	123.9	145.1	9.22	0.126
Villus height: crypt depth ratio	0.85	1.09	0.15	0.329
SA index	0.92	0.88	0.082	0.697
Muscularis thickness (µm)	614.8	653.6	43.46	0.541
Ki-67 (% positive cells)	19.62	17.91	1.61	0.472
Distal jejunum				
Villus length (µm)	214.3	265.4	25.39	0.178
Villus width (µm)	105.3	86.5	3.98	0.005
Crypt depth (µm)	166.7	216.9	22.94	0.143
Crypt width (µm)	117.9	107.7	6.41	0.284
Villus height: crypt depth ratio	1.28	1.23	0.19	0.844
SA index	1.16	0.87	0.086	0.031
Muscularis thickness (µm)	485.8	522.0	25.77	0.340
Ki-67 (% positive cells)	21.91	20.59	2.16	0.676
Ileum				
Villus length (µm)	238.4	267.8	20.20	0.323
Villus width (µm)	87.6	102.7	9.26	0.267
Crypt depth (µm)	198.5	141.0	31.54	0.219
Crypt width (µm)	103.7	111.9	8.89	0.523
Villus height: crypt depth ratio	1.20	1.91	0.22	0.037
SA index	0.95	1.01	0.074	0.603
Muscularis thickness (µm)	538.0	619.2	38.06	0.155
Ki-67 (% positive cells)	23.33	21.99	2.20	0.675

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.

Table 2-8. Free fatty acid composition of jejunum tissue from male Holstein calves (n = 18) fed fed a whole milk powder (WP) or a high fat milk replacer (MR) of similar macronutrient inclusions three times daily.

	Treatment[1]			
Fatty acids (ug/100 mg of tissue)	WP	MR	SEM	*P* - value
C12	4.79	22.56	6.923	0.074
C14	43.9	33.4	24.09	0.760
iso-C14	5.06	1.22	2.202	0.223
C15	5.15	0.93	1.497	0.051
C15:1 *cis*-10	1.57	1.70	0.637	0.890
C16	196.8	165.5	93.77	0.814
C16:1 *cis*-9	16.2	14.9	6.94	0.890
C17:1 *cis*-10	4.59	1.36	0.795	0.060
C18	110.0	70.3	31.08	0.370
C18:1 *cis*-9	167.4	168.4	91.01	0.994
C18:1 *cis*-11	14.32	7.04	4.022	0.205
C18:2 n-6	54.5	85.6	12.95	0.095
C18:2 *t*	1.26	0.54	0.706	0.472
C18:3 n-6	0.75	0.34	0.231	0.217
C19	1.13	0.77	0.176	0.145
C19:1 *cis*-7	0.76	0.00	0.224	0.020
C18:3 n-3	6.13	1.65	0.678	<0.001
C18:4 n-3	1.92	0.58	0.539	0.084
C20	1.45	1.12	0.342	0.503
C20:1c5&8	0.85	0.68	0.260	0.646
C20:1 cis-11	1.76	1.74	0.613	0.989
C20:2 n-6	1.76	2.36	0.514	0.407
C20:3 n-9	0.58	0.48	0.064	0.260
C20:3 n-6	3.87	2.54	1.29	0.465
C20:4 n-6	23.15	15.32	2.755	0.049
C20:3 n-3	0.20	0	0.064	0.032
C20:5 n-3	2.77	1.31	0.293	0.001
C22	2.92	2.42	0.267	0.191
C22:1 n-9	0.67	0.63	0.126	0.822
C22:2 n-6	5.07	5.02	3.278	0.991
C23	0.68	0.64	0.390	0.933
C22:4 n-6	3.25	3.61	0.624	0.679
C22:5 n-6	0.31	0.33	0.201	0.936
C22:5 n-3	6.65	2.64	0.663	<0.001
C24	2.16	1.74	0.296	0.322
C22:6 n-3	2.15	1.28	0.547	0.262
C24:1 n-9	1.75	2.22	0.338	0.335
Total	699.4	622.8	273.76	0.844

Total SFA[2]	369.5	299.4	155.76	0.751
Total UFA[3]	329.8	323.5	119.89	0.970
Total MUFA[4]	215.5	199.9	103.93	0.916
Total PUFA[5]	113.7	123.1	19.03	0.729
Total n-3[6]	19.8	7.5	1.94	<0.001
Total n-6[7]	92.6	115.1	17.26	0.361
n-6:n-3	4.69	15.2	0.58	<0.001

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.
[2]Sum of saturated fatty acids from 14 to 24 carbons in length
[3]Sum of unsaturated fatty acids from 14 to 24 carbons in length
[4]Sum of monounsaturated fatty acids from 14 to 24 carbons in length
[5]Sum of polyunsaturated fatty acids from 18 to 22 carbons in length
[6] Sum of omega 3 polyunsaturated fatty acids from 18 to 22 carbons in
[7]Sum of omega 6 polyunsaturated fatty acids from 18 to 22 carbons in length

Table 2-9. Phospholipid composition of jejunum tissue from male Holstein calves (n = 18) fed fed a whole milk powder (WP) or a high fat milk replacer (MR) of similar macronutrient inclusions three times daily.

	Treatment[1]			
Fatty acids (ug/g)	WP	MR	SEM	*P* - value
C14	1.26	1.33	0.083	0.550
C14:1	0.10	0.11	0.056	0.842
C15:0	0.52	0.34	0.044	0.006
C16:0	25.1	29.2	0.52	<0.001
C16:1	0.99	0.29	0.026	<0.001
C18:0	24.0	23.0	0.62	0.277
C18:1	21.2	17.0	0.56	<0.001
C18:2 n-6	9.1	12.8	0.45	<0.001
C18:3 n-6	0.17	0.11	0.020	0.070
C18:3 n-3	0.85	0.17	0.032	<0.001
C18:4 n-3	0.01	0.01	0.013	0.960
C20:0	0.81	0.86	0.080	0.632
C20:1	0.49	0.54	0.025	0.176
C20:2 n-6	0.47	0.23	0.029	<0.001
C20:3 n-6	0.77	0.59	0.046	0.010
C20:4 n-6	4.19	3.36	0.304	0.063
C20:3 n-3	0.04	0.03	0.011	0.487
C20:4 n-3	0.08	0.03	0.010	0.001
C20:5 n-3	0.27	0.07	0.014	<0.001
C22:0	1.59	1.63	0.065	0.619
C22:1	0.81	0.91	0.046	0.133
C22:2 n-6	0.13	0.13	0.035	0.970
C22:4 n-6	1.31	1.93	0.166	0.013
C22:5 n-6	0.26	0.29	0.021	0.283
C22:5 n-3	2.04	0.90	0.082	<0.001
C22:6 n-6	0.67	0.45	0.043	0.001
C24:0	1.53	1.73	0.066	0.039
C24:1	1.30	2.01	0.083	<0.001
Total SFA[2]	54.8	58.1	0.55	<0.001
Total UFA[3]	45.2	41.9	0.55	<0.001
Total MUFA[4]	24.9	20.8	0.64	<0.001
Total PUFA[5]	20.3	21.1	0.69	0.451
Total n-3 PUFA[6]	3.96	1.65	0.112	<0.001
Total n-6 PUFA[7]	16.4	19.4	0.62	0.002
n-6:n-3	4.14	11.8	0.61	<0.001

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.

[2]Sum of saturated fatty acids from 14 to 24 carbons in length

[3]Sum of unsaturated fatty acids from 14 to 24 carbons in length

[4]Sum of monounsaturated fatty acids from 14 to 24 carbons in length
[5]Sum of polyunsaturated fatty acids from 18 to 22 carbons in length
[6] Sum of omega 3 polyunsaturated fatty acids from 18 to 22 carbons in
[7]Sum of omega 6 polyunsaturated fatty acids from 18 to 22 carbons in length

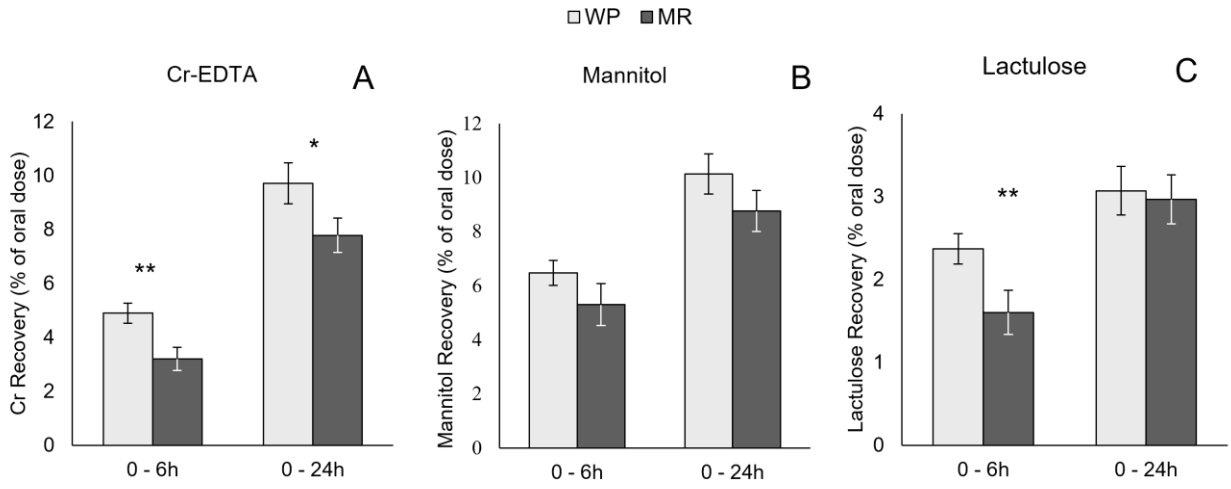

Figure 2-1. Urinary recovery of Cr-EDTA (A), Lactulose (B) and D-Mannitol (C) after an oral dose of the markers in calves fed whole milk powder (26% fat, 21.6 MJ/kg; WP; n = 9), and a milk replacer (MR) with high fat (25% fat; 21.3 MJ/kg; HF; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk. Urine was collected on d 21 using urine bags over 2 collection periods; 0 – 6 h and 0 – 24 h. **P* < 0.1, ***P* < 0.05.

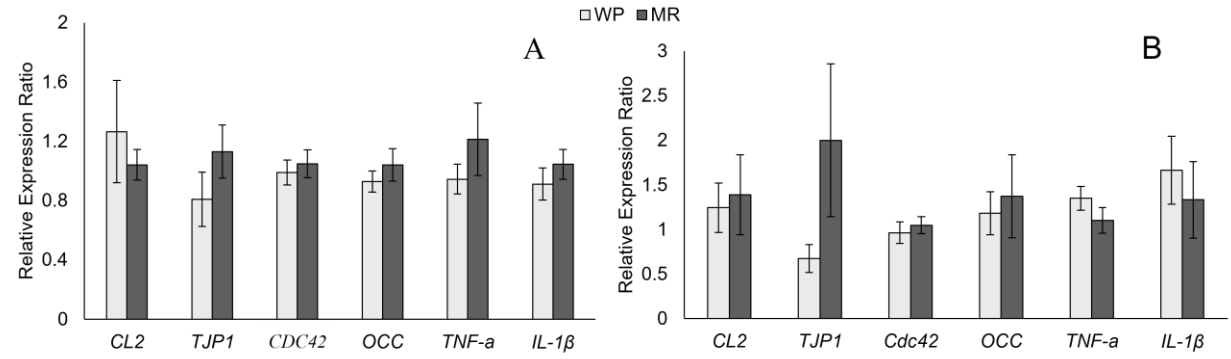

Figure 2-2. Effect of feeding either whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), or a milk replacer (MR) with high fat (25% fat; 21.3 MJ/kg; MR; n = 9) on gene expression of tight junction and inflammatory genes in the proximal jejunum (A,) and ileum (B) of Holstein bull calves at d 28. Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk. Gene expression is normalized to 3 housekeeping gene (*β-actin, GADPH, RNA-pol*) and expressed relative to the average of the WP group. Significance declared at $P \leq 0.05$.

2.9 Appendices

Appendix 1. Free fatty acid composition of ileum tissue from male Holstein calves (n = 18) fed a whole milk powder (WP) or a high fat milk replacer (MR) of similar macronutrient inclusion three times daily.

	Treatment[1]			
Fatty acids (ug/g)	WP	MR	SEM	*P* - value
C12	5.03	32.1	6.923	0.007
C14	53.4	75.9	24.09	0.512
iso-C14	6.15	3.54	2.202	0.404
C15	5.38	1.45	1.497	0.067
C15:1 *cis*-10	1.93	2.35	0.637	0.642
C16	251.9	345.0	93.77	0.485
C16:1 *cis*-9	18.2	20.9	6.94	0.788
C17:1 *cis*-10	4.18	1.95	0.795	0.052
C18	108.3	101.6	31.08	0.881
C18:1 *cis*-9	204.0	369.0	91.01	0.205
C18:1 *cis*-11	17.2	14.5	4.022	0.629
C18:2 n-6	28.8	71.4	12.95	0.024
C18:2 *t*	2.33	0.83	0.706	0.138
C18:3 n-6	0.47	0.70	0.231	0.486
C19	1.17	0.79	0.176	0.130
C19:1 *cis*-7	0.85	0.16	0.224	0.033
C18:3 n-3	3.01	1.98	0.678	0.289
C18:4 n-3	2.24	0.95	0.539	0.096
C20	1.37	1.74	0.342	0.450
C20:1c5&8	0.90	0.73	0.260	0.639
C20:1 cis-11	1.65	2.87	0.613	0.164
C20:2 n-6	2.49	2.25	0.514	0.739
C20:3 n-9	0.31	0.38	0.064	0.444
C20:3 n-6	4.00	3.38	1.29	0.732
C20:4 n-6	21.1	18.8	2.755	0.551
C20:3 n-3	0.10	0.04	0.064	0.546
C20:5 n-3	2.41	0.84	0.293	<0.001
C22	2.41	2.04	0.267	0.327
C22:1 n-9	0.56	0.60	0.126	0.823
C22:2 n-6	1.14	1.23	3.278	0.986
C23	0.55	0.45	0.390	0.849
C22:4 n-6	3.44	5.44	0.624	0.027
C22:5 n-6	0.33	0.36	0.201	0.915
C22:5 n-3	7.92	3.24	0.663	<0.001
C24	1.86	1.51	0.296	0.413
C22:6 n-3	1.92	1.21	0.547	0.363
C24:1 n-9	2.17	2.10	0.338	0.889

Total	772.9	1095.3	273.76	0.408
Total SFA[2]	432.3	563.2	155.76	0.555
Total UFA[3]	340.5	532.1	119.89	0.263
Total MUFA[4]	258.5	419.1	103.93	0.279
Total PUFA[5]	81.8	112.6	19.03	0.256
Total n-3 PUFA[6]	17.6	8.26	1.94	0.001
Total n-6 PUFA[7]	61.8	103.5	17.26	0.093
n-6:n-3	3.47	11.9	0.931	<0.001

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.
[2]Sum of saturated fatty acids from 14 to 24 carbons in length
[3]Sum of unsaturated fatty acids from 14 to 24 carbons in length
[4]Sum of monounsaturated fatty acids from 14 to 24 carbons in length
[5]Sum of polyunsaturated fatty acids from 18 to 22 carbons in length
[6] Sum of omega 3 polyunsaturated fatty acids from 18 to 22 carbons in
[7]Sum of omega 6 polyunsaturated fatty acids from 18 to 22 carbons in length

Appendix 2. Phospholipid composition of jejunum tissue from male Holstein calves (n = 18) fed a whole milk powder (WP) or a high fat milk replacer (MR) of similar macronutrient inclusions three times daily.

	Treatment[1]			
Fatty acids (ug/g)	WP	MR	SEM	*P* - value
C14	1.64	1.72	0.083	0.497
C14:1	0.38	0.22	0.056	0.051
C15:0	0.60	0.47	0.044	0.040
C16:0	27.1	30.0	0.52	<0.001
C16:1	0.76	0.42	0.026	<0.001
C18:0	21.0	20.3	0.62	0.396
C18:1	18.5	17.7	0.56	0.307
C18:2 n-6	4.58	5.97	0.45	0.039
C18:3 n-6	0.23	0.18	0.020	0.144
C18:3 n-3	0.37	0.08	0.032	<0.001
C18:4 n-3	0.01	0.04	0.013	0.081
C20:0	1.08	1.08	0.080	0.967
C20:1	0.65	0.62	0.025	0.338
C20:2 n-6	0.81	0.43	0.029	<0.001
C20:3 n-6	1.00	0.86	0.046	0.034
C20:4 n-6	6.95	6.78	0.304	0.705
C20:3 n-3	0.03	0.03	0.011	0.884
C20:4 n-3	0.04	0.03	0.010	0.572
C20:5 n-3	0.32	0.07	0.014	<0.001
C22:0	2.09	1.82	0.065	0.006
C22:1	0.89	9.89	0.046	0.971
C22:2 n-6	0.41	0.32	0.035	0.055
C22:4 n-6	2.04	3.51	0.166	<0.001
C22:5 n-6	0.36	0.40	0.021	0.233
C22:5 n-3	0.41	0.32	0.082	<0.001
C22:6 n-6	1.02	0.66	0.043	<0.001
C24:0	1.71	1.49	0.066	0.021
C24:1	2.02	2.53	0.083	<0.001
Total SFA[2]	55.3	56.9	0.55	0.051
Total UFA[3]	44.7	43.1	0.55	0.051
Total MUFA[4]	23.2	22.3	0.64	0.346
Total PUFA[5]	21.5	20.8	0.69	0.463
Total n-3 PUFA[6]	5.15	2.36	0.112	<0.001
Total n-6 PUFA[7]	16.4	18.4	0.62	0.025
n-6:n-3	3.18	7.86	0.184	<0.001

[1]Treatments included: whole milk powder (26% fat; 21.6 MJ/kg; WP; n = 9), and a milk replacer with high fat (25% fat; 21.3 MJ/kg; MR; n = 9). Treatment concentration was 135.0 g/L to reflect the solid percentage of bovine whole milk.

[2]Sum of saturated fatty acids from 14 to 24 carbons in length
[3]Sum of unsaturated fatty acids from 14 to 24 carbons in length
[4]Sum of monounsaturated fatty acids from 14 to 24 carbons in length
[5]Sum of polyunsaturated fatty acids from 18 to 22 carbons in length
[6] Sum of omega 3 polyunsaturated fatty acids from 18 to 22 carbons in
[7]Sum of omega 6 polyunsaturated fatty acids from 18 to 22 carbons in length

Appendix 3. Pearson correlation values (r) between phospholipid profile of jejunum tissue and permeability marker recovery in urine of male Holstein calves (n=18) fed whole milk powder or high fat milk replacer with similar macronutrient composition three times daily.

Fatty acid	6h Chromium	6h Lactulose	6h Mannitol	24h Chromium	24h Lactulose	24h Mannitol
C15:0	0.67**	0.67**	0.45	0.56*	0.58*	0.13
C16:1	0.50*	0.59*	0.27	0.45	0.32	0.18
C18:3 n-6	0.47	0.59*	0.48	0.40	0.47*	0.19
C18:3 n-3	0.48*	0.59*	0.26	0.39	0.40	0.14
C20:2 n-6	0.49*	0.54*	0.18	0.44	0.23	0.21
C20:3 n-3	0.10	-0.24	0.15	0.0	0.14	-0.55*
C20:5 n-3	0.48*	0.58*	0.26	0.35	0.40	0.08
C22:4 n-6	-0.66**	-0.77***	-0.53*	-0.40	-0.28	-0.43
C24:1	-0.35	-0.54*	-0.33	-0.32	-0.37	-0.27
n – 3	0.43	0.50*	0.24	0.35	0.35	0.03
n – 6	-0.58*	-0.45	-0.22	-0.50*	-0.14	-0.23

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

3 General Discussion

There are considerable differences between WM and modern MR formulations in the source and composition of their macronutrients. Traditional MR formulations contain high amounts of lactose and low levels of fat, therefore MR formulations provide less energy by ~15% compared with WM when fed in similar amounts. Additionally, current feeding programs for raising calves do not provide the volume of milk that they would consume in a natural setting. The global trend in calf nutrition towards higher planes of nutrition and greater milk volumes has brought the discrepancy between MR formulation

and WM to the forefront (van Niekerk et al., 2021). As MR allowances increase, the level of every ingredient increases in an equivalent manner, raising the question of whether current MR formulations are providing an optimal nutrient supply to calves.

In the present study, a MR with a macronutrient composition similar to WP was compared with WP when fed 9 L daily divided over three meals (135 g/L). The fat micro-compositions of the two diets differed, namely in fatty acid profile, triglyceride structure and presence of milk fat globule membrane. Therefore, this study is the first to compare WM and MR with similar macronutrient inclusions and similar intake of the diets. It is important to note that some of the differences seen between groups may be influenced by the damaged protein fraction in the WP group, thus lowering the DM digestibility of the WP treatment (Wilms et al., in review). The amino acid profile between treatments did not differ substantially however, bioavailability of the WP protein fraction may have been affected during the drying process as demonstrated by the lower WPNI and the lower DM digestibility. It should also be noted that the fatty acid profile of WM varies widely from numerous factors, including season, parity, breed, stage of lactation and maternal nutrition (Palmquist et al., 1993; Jensen, 2002; Kelsey et al., 2003). Consequently, this study may not reflect WM feeding in North America as it is fed in commercial settings. This study still provides valuable insight on the effects of WM and MR on GIT development at the level of histology, tissue mass and GIT permeability.

There is evidence that some of the differences in permeability and GIT development may be explained by the differences in fatty acid profiles between the treatments. However, this study does not entirely reflect WM as the drying process may have

damaged milk fat globules and milk fat globule membranes. Diets enriched with milk fat globule membranes have been shown to have a protective effect against LPS-induced GIT permeability by decreasing the inflammatory response (Snow et al., 2011). The GIT permeability assessment in this study could be further interpreted if analyzed in blood, as results differed between measurements in blood and urine, as demonstrated by Welboren et al. (2021a). It should also be noted that when evaluating permeability and comparing experimental results, age may influence recovery, as more calves experience diarrhea between 7 and 14 d of age and calves in this thesis were over 21 d of age (Araujo et al., 2015). While inflammatory states can alter tight junction protein functioning, neither cytokine nor tight junction protein gene expression were different between treatment groups. However, gene expression results cannot be interpreted to represent these molecules at the level of functional proteins, which should be measured in future studies. Physical structure and distribution of proteins should also be analyzed in future studies, as these parameters are highly correlated with GIT permeability (Hamada et al., 2010; Joly Condette et al., 2014).

Cholesterol content and membrane fluidity are also affected by diet and influence tissue function and GIT permeability (Cullis and Hope, 1991; Pizzo et al., 2002; Jedlovszky and Mezei, 2003). Therefore, while the free fatty acid and phospholipid fatty acid profiles of small intestine tissue are quantified in this thesis, cholesterol content and fluidity would also contribute to understanding the effect of feeding WM and MR on GIT composition and function. While transcellular permeability was not affected by the treatments, altering membrane fluidity through the diet may allow for optimized

absorption of nutrients or drugs (Khajuria et al. 2002) and improved calf growth and development.

This experiment also did not evaluate how the differing fatty acid profiles may have influenced the intestinal microbial population in calves. One strategy to decrease calf diarrhea is to promote a healthy GIT microbial population by dietary interventions (Yu et al., 2013). The present study did not offer starter to calves to better isolate the effect of the liquid diet, however, starter consumption is important to rumen development and weaning, and further influences the microbial population (Flatt et al., 1958; Malmuthuge et al., 2013; Connor et al., 2013). Calves fed all liquid diets have underdeveloped rumens and consumption of solid feed promotes rumen growth and development to support weaning and future digestion (Flatt et al., 1958; Connor et al., 2013). Malmuthuge et al. (2013) found that feeding starter vs just MR (22% CP, 17% fat) tended to alter microbial populations and increased tight junction gene expression in calves during weaning suggesting that when the rumen is fully populated with microbes GIT permeability may be reduced. Future studies should investigate the effect of the differing fatty acid composition on microbial population, tight junction gene expression and the implications of these differences on GIT permeability.

Additionally, past literature hypothesized that improvements in growth from fatty acid supplementation is a function of improved immune responses (Ballou and DePeters, 2008; Essleburn et al., 2013; Garcia et al., 2014). In WM and MR, the ratio between LA and ALA differs greatly, typically 40 for MR and 1-2 in WM. In juvenile fish, optimizing the LA:ALA ratio has been shown to improve growth and activity and

expression of digestive and brush border enzymes (Zeng et al., 2016). There is limited information on the optimal ratio for calves. In calves, supplementing n-3 FA has been shown to improve the acute immune response without affecting growth performance (Ballou and DePeters, 2008; Ballou et al., 2008). More recently, Garcia et al. (2014) determined that LA and ALA promoted growth and immune status of pre-weaned calves when supplemented within the ranges of 3 to 5 g/d and 0.3 to 0.6 g/d. This was likely due to the balance of LA acting as a precursor of proinflammatory mediators and the anti-inflammatory functions of ALA (Calder, 2006; Schmitz and Ecker, 2008). In this thesis, the MR calves had higher intakes of LA, with 20.78 g/d vs. 6.35 g/d for WP, and similar amounts of ALA (1.04 g/d and 0.52 g/d, WP, and MR respectively). Therefore, the fatty acid composition of WP and MR may also alter immune function, requiring further investigation. Future studies should examine how the differences observed in the GIT of calves fed fresh WM or MR affect their performance through weaning, as well as future lactation performance and feed efficiency. Additionally, membrane fluidity in intestinal tissues should be investigated as many studies have demonstrated that it is affected by diet composition, and this may have implications for metabolism and development of calves. Lastly, it should be noted that the calves in this study originated from 7 different farms, and this may influence results compared to a study with calves that originate from the same farm especially when considering the effect of the microbiota.

3.1.1 Overall Conclusions

Overall, this thesis demonstrates that it is not only macronutrient composition, but also fatty acid profile that influences calf GIT development, ranging from gross morphology to microstructure. To that end, this thesis also demonstrates that the fatty acid characteristics likely alter GIT physiology including paracellular permeability. Tissue composition was also affected by the dietary treatments which may have implications on GIT permeability, membrane fluidity and nutrient transport. Therefore, future efforts to optimize MR formulations should aim to adjust the fatty acid profile to better support the growth, development, and health of calves.

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