UNIVERSITY OF COPENHAGEN

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The effect of adding supplemental fat to the milk replacer on the rumen microbiome in beef calves

Pernille Phennapa Kleebkomut Petersen, LWX374

Animal Science Master thesis

Supervisor: André Luis Alves Neves, Institution for Veterinary- and Animal Science, University of Copenhagen

Supervisor: Einar Vargas Belle Perez, Department of Animal Sciences, School of Agriculture, Policy and Development, University of Reading, UK

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Abstract

There is a large production of beef cattle in Denmark with the purpose to be slaughtered and sold both locally and exported to other countries. To ensure a high-quality product, it is important to keep good health as well as an efficient growth performance. The rumen microbiome plays a crucial role in the digestion of feedstuffs and the health of the host animal, stressing the importance of keeping the structure of the rumen microbial composition undisturbed by external factors. The objective of the study was to determine how supplementation of saturated and unsaturated fats affects the rumen microbiome of pre-weaned beef calves, when the supplement is added to the milk replacer. Studies have shown that the microbiome can be affected by changes in the diet and the age of the animals. Today, the study of the microbiome in ruminants has been widely studied and is a major subject in animal agricultural research.

A total of 18 Holstein-Belgian Blue crossed bull calves weighing (± SD) 69.8 ± 11.1 kg were blocked by weight and randomly assigned to one of three treatments, with two groups (Bovi85 and BoviLM) receiving a total of 21% fat in their diets. Control was fed 811g dry matter of milk replacer containing 16% fat, Bovi85 was fed 753 g dry matter of milk replacer + 58.2 g dry matter BOVI 85 fat supplement (unsaturated fat), and BoviLM was fed 753 g dry matter of milk replacer + 50.6 g dry matter BOVI LM fat supplement (saturated fat). Besides the milk replacer, all groups had *ad libitum* access to water, meadow grass haylage, and starter feed. Fecal samples were taken from the rectum of each animal on days 0, 7, 14, and 21, and analyzed for microbiome composition.

There was no significant effect of the fat sources on the microbiome at any of the taxonomic levels. However, a significant effect of the period was found on the relative abundance of rumen microorganisms at the phylum, family, and genus levels. Firmicutes, Lactobacillaceae, and Lactobacilla decreased from day 14 to day 21 for all groups, while Bacteroidetes, Prevotellaceea, Oscillospiraceae, Alloprevotella, and UCG-005 increased from day 14 to 21. Overall, rumen bacteria populations were not affected possibly because the amount of supplemental fat or the relative short-term of supplementation were insufficient to drive the microbial composition changes.

Preface

This thesis is part of a project named SmartCalf from Kvægafgiftsfonden, built to study the addition of two different types of lipid supplements in the milk replacer and investigate how the fat supplementations would affect the performance and health of beef calves. However, this thesis focused on investigating the effects of the supplemental fats on the rumen microbiome. The SmartCalf project is the first of its nature in Denmark, but similar studies have been made in the US and in other parts of the world.

The thesis aims to contribute to the understanding of how adding saturated or unsaturated fats to the milk replacer diet could affect the microbiome of pre-weaned calves.
As an Animal Science student, I personally have a high interest in the study of animals and chose to join this project as it sounded interesting to get a better understanding of how diets can affect animal production. As the overall project was an investigation of a lot of parameters it was hard to choose just one subject to take on, but due to time, it was chosen to dig deeper into whether there had been a change in the rumen microbiome, as a result of the fat supplementation.

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Signature: Pernille Phennapa Kleebkomut Petersen

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1. Introduction

1.1. Background

In Denmark, beef cattle are raised with the purpose of slaughter and thereafter sold internally or exported to other countries. Some of the animals used for beef production come from dairy farms, but it is also common to find farms that produce beef cattle as their main economic goal (Landbrug og Fødevarer, n.d.).

All around the world a variety of breeds have been used in beef production, and in Denmark alone, there are 18 breeds [e.g., Belgian Blue, Hereford, Danish Red, and Simmental] registered in the database of breed associations. Besides the pure breeds, crossbreeds in the production system can also be found (Landbrug og Fødevarer, n.d.). SEGES makes yearly statistics showing the amount of cattle bred and slaughtered in Denmark. When looking at SEGES (2021), there seems to be an increasing trend in the number of breeding crossbreds compared to the purebreds.

In beef cattle herds, calves and steers are raised with the purpose of slaughtering, but when an old animal (e.g., a cow) is culled from the production system it is also sent to slaughter. The calves in the beef industry are taken from the cow at six months of age and typically slaughtered when they are around one year of age. (Landbrug og Fødevarer, n.d.).

There are three different categories of beef cattle in Denmark which are veal, steers, and beef. In cattle farming there are, however, more categories of animals used in the production of the three types of meat [e.g., old dairy cows, steers, veal, stud, bulls] (Landbrug og Fødevarer, n.d.). Besides the animal of beef production, there are different production systems in beef farm operations: conventional, organic, and free-range (Foedevarestyrelsen, n.d.a.).

It is of great importance to keep a good health and growth performance of weaned beef calves, and although using whole milk would be the natural choice to feed them, milk replacers (MR) are generally used as the feed source due to the high cost of the whole milk (Huuskonen *et* al., 2005). An important factor in the health of young calves is the symbiotic relationship between the gastrointestinal tract (GIT) and the residing microbiome (Kim *et* al., 2011). The rumen microbiota, for example, provides 70% of the daily requirement of cattle through the fermentation of indigestible dietary substrates (Malmuhuge *et* al., 2015). Also, the GIT uses 20% of the oxygen in ruminants and accounts for 30% of the metabolic and protein synthesis

activities. These functions explain why the GIT can affect many biological functions in eukaryotic organisms like animals and plants (Meal *et* al., 2017).

In the rumen, bacteria play an important role in the metabolism of fat-containing substances (Nurzhanov *et* al., 2020), and the rumen microbiome in general plays a major role in the fatty acid metabolism of dietary fats (Cancino-Padilla *et* al., 2021). Some of the natural metabolic conversions of fatty acids (FA) in the rumen occur during an anaerobic process called biohydrogenation (BH) (Yongjae Lee, 2013); the BH of dietary FA, is, however, often incomplete, and intermediate metabolites can reach the duodenum, being absorbed and incorporated into edible products such as milk and meat (Cancino-Padilla *et* al., 2021).

Studies have shown that it is possible to modify the microbiome of the GIT by changing the diet (Gercino *et* al., 2020), and that some fats fed to ruminants can cause an inhibitory effect on the rumen microbiome (Nurzhanow *et* al., 2019). It is also known that the microbiome changes from birth to weaning (Malmuthuge and Guan, 2017). Gercino *et* al. (2020) suggested that the differences in the microbiome due to age can show the best time possible to manipulate the intestinal microbiome to improve health and performance of cattle. Taken all together. The current study aims at unraveling the effects of saturated and unsaturated fats on the microbiome of pre-weaned beef calves that were never tested in previous research. Therefore, the current study is novel and fills the knowledge gap in the field of fat supplementation and its impact on the rumen microbiota of beef calves.

Aim and hypothesis

The aim of this study was to supplement saturated and unsaturated fats in the milk replacer and evaluate how the supplementation affects the rumen microbiome of pre-weaned beef calves.

The hypothesis of the study is that the degree of fatty acid saturation affects the composition of the rumen bacterial populations of pre-weaned beef calves.

2. Literature review

2.1. Production systems

Back in the day, it was common for dairy farms to fatten bull calves for slaughter, but today it is more common for dairy farms to sell the animals to specialized calf producers when the calves are around 2-4 weeks of age. By doing so, good foundation for a more rational production with a focus on a more uniform product has been established in the country (Holdgaard, Justesen and Rasmussen, 2014).

There are three types of animal categories in Danish beef production: veal, steers, and beef. There are, however, different production systems (Landbrug og Fødevarer, n.d.). Calves raised for beef production are typically sent to a calf producer around the age of 2 to 4 weeks, and this production system includes veal, steers, studs, and bulls. Another production system in Denmark includes cows and heifers, which are typically cattle taken out of the dairy industry. This system comprises most of the beef produced in Denmark (Holdgaard, Justesen and Rasmusssen, 2014).

Furthermore, other production systems can be found in Denmark, these are conventional, organic, and free-range. Each production system has specific rules and guidelines that need to be followed. For example, conventional calves do not need to be raised on grasslands, whereas organic calves need to be raised in pasturelands from 15. April to 1. November when they reach 6 months of age (Foedevarestyrelse, n.d.b.). Free ranged cattle are controlled and approved by “Dyrenes Beskyttelse”, this production type focuses on welfare, and calves stay together with the cow during the first five months of their life (Danish Crown, n.d.).

Year statistics from SEGES (2020) showed that in October 2020 there were between 6 and 3,294 registered herds of different beef breeds. Also, there were between 1 and 215 active breeding herds that bred both pure- and crossbreeds. If we only look at the purebreds, the year statistics from 2020 showed that there were between 1 and 201 herds. In October 2020 there were a total of 23,500 cattle in all purebred herds, and a total of 62,343 cattle in all herds with crossbreds. These data shows that there are more breeding crossbreds than purebreds in Denmark at the moment, highlighting the importance of these beef farm operations for the economy of the country.

2.2. Development of the gastrointestinal tract

When a calf is born, the gastrointestinal secretions play a fundamental role in transforming feeds into nutrients for the subsequent absorption in the intestine. Calves are not born as functional ruminants since the rumen function develops as the calf ages and starts to consume solid feed. Therefore, the calves have an 'esophageal groove'. The esophageal groove delivers milk directly to the abomasum where it is digested more efficiently (Guilloteau *et* al., 2009; Strudsholm and Sejrsen, 2003). The esophageal groove is of great importance for the calf because if this groove did not close, the milk could spill into the reticulorumen and be fermented to lactic acid by rumen bacteria. If this were to happen, it would decrease the pH of the rumen, causing rumen acidosis and inhibiting the normal microbial development in the reticulorumen, thereby affecting the calf health negatively (Victoria Aspinall and Melanie Cappello, 2015).

From the time the calf is born and until 16 weeks of age, it can consume and digest both milk and MR; hereafter, the calf will significantly increase the consumption of roughage. If, however, calves are fed concentrates or hay together with the MR, it is possible to offer roughage earlier – from 8 weeks of age (Guilloteau *et* al., 2009; Holdgaard *et* al., 2014).

There are three important digestive enzymes in the GIT of young calves fed MR based on skim milk powder: chymosin, elastase II and lactase. These enzymes are produced by the abomasum, pancreas, and small intestine, and are found in the gastrointestinal tissues after birth. These enzymes increase in activity during the first two days of life, and then gradually decrease their activity with age. Chymosin coagulates milk proteins in the abomasum, as clotting is crucial to reduce the abomasal emptying and to increase the efficiency of the digestive processes in the small intestine of neonatal calves (Guilloteau *et* al., 2009).

2.2.1. Beneficial microflora for the health

The early gut microbiome plays a vital role in the long-term health of the host (Malmuthuge and Leo Guan, 2017). The primary function of the gastrointestinal tract is the digestion and absorption of nutrients, as well as the protection of the host from infections, toxins, and chemicals in the lumen. Furthermore, it prevents unregulated translocation of these into portal circulation (Meale *et* al., 2017).

The intestinal bacteria influence the host health. This is evident, when comparing conventional and germ-free animals. It has been revealed that the gastro-intestinal microflora is important for the normal development of gut morphology and function (REFS). However, some intestinal bacteria are beneficial to health while others can be harmful. For example,

pathogenic bacteria can have a harmful effect on health either through mucosal invasion, production of toxins or both. On the other hand, it is thought that potentially health-promoting bacteria include mainly lactic-acid-producing bacteria such as members of the *Lactobacillus* genera.

Some bacteria of special interest are segmented filamentous bacteria, because these unculturable microbes adhere to the epithelial cells of the ileum and Peyer's patches, stimulating the intestinal immune system and preventing adhesion of pathogenic bacteria (Blok *et* al., 2002). While it is necessary the presence of gut microbiota for the development of the intestinal epithelium, mucosal layer, and lymphoid structures, the gut microbiota is also key for the differentiation of the immune cell repertoire (Malmuthuge and Leo Guan, 2017).

2.3. The rumen microbiome in calves

The rumen microbiome comprises the diversity and function of the entire community of microorganisms that inhabits the rumen (Cancino-Padilla *et* al., 2021). In cattle, the rumen microbiome ferments plant materials and consists of a wide variety of bacteria, archaeal, protozoan, and fungal species, with bacteria being the dominant population. Rumen bacteria contributes mostly to the digestion and conversion of feedstuffs into short-chain fatty acids and microbial protein (Kim *et* al., 2011). Interestingly the microbial-made protein is of high quality regardless of the quality of the nutrient source, meaning that protein in lower quality feeds is improved by the microbial metabolism (Victoria Aspinall and Melanie Cappello, 2015). The ruminal archaeal population is mostly comprised of methanogens that utilize carbon dioxide (CO_2) and hydrogen (H_2) produced by bacterial fermentation to produce methane (CH_4) (Kim *et* al., 2011). To promote the fermentation process in the rumen, it is important for the ruminant to maintain a proper compositional and structural balance of bacteria, protozoa, and fungi. This delicate balance is largely controlled by the diet and the amount of feed that is consumed. An example of this is if the diet has an increase in the grain content, it could lead to an imbalance in the microbial composition in the rumen reticulum compartment. For instance, microbes that break down starch will flourish in a grain-based diet, and microbes that break down complex carbohydrates will diminish considerably (Victoria Aspinall and Melanie Cappello, 2015).

The microbes in ruminants are of great importance for the host animal as they provide up to 70% of their daily energy requirement through the fermentation of indigestible dietary substrates. The GIT can affect several biological functions, highlighting the importance of maintaining a healthy and functional GIT to benefit animal energy harvest (Meal et al., 2017).

Studies of the ruminant gut microbiota have mainly been focused on the microbiome's impact on meat and milk production (Malmuthuge *et* al., 2015). Also, the GIT uses 20% of the oxygen in ruminants and accounts for 30% of the metabolic and protein synthesis activities. The environment in the reticulorumen needs to be of low oxygen concentrations to allow the growth of facultative anaerobic bacteria and protozoa; as mentioned before, these facultative anerobic microbes are crucial for the fermentation process of feed particles and the survival of strictly anerobic microbes (Victoria Aspinall and Melanie Cappello, 2015).

It is well-known that ruminants are not born with the microbial ecosystem established and that the microorganisms that will be crucial for their survival must be acquired from the environment (Victoria Aspinall and Melanie Cappello, 2015). The rumen microbiota has a significant impact on pre-ruminant management, and especially in the weaning process, because it depends on the development of the rumen and the ability of the microbiome to ferment complex carbohydrates. For example, the presence of volatile fatty acids (acetate, propionate, and butyrate) produced by rumen microbes need to be absorbed, requiring the development of rumen papillae (Malmuthuge *et* al., 2015).

Early gut colonization is critically important to the immunological development of the gastrointestinal tract, development of a functional fermentative environment, and neonatal resistance to pathogenic challenges (Yeoman and White, 2014). A pathogenic challenge could be *Escherichia coli*, which is related to neonatal diarrhea. This condition could be improved by administering *Bifidobacterium* and *Lactobacillus* to the diets of newborn calves during their first week of life to help increase weight gain and feed conversion ratio while decreasing diarrhea incidences (Malmuthuge *et* al., 2015).

Though it is well-known that the intestinal microbial colonization in ruminants occurs from birth to weaning, several microbial representatives have been found in the feces of calves less than 20 minutes after birth. The mode of microbial acquisition from the environment is still debatable, but the microbes may start to colonize the GIT during the delivery or even before birth (Meale *et* al., 2017). The early microbiome consists of bacterial species from *Propionobacterium*, *Clostridium*, *Peptostreptococcus*, and *Bifidobacterium* genera, and the cellulolytic bacterial population *Ruminococcus* (Malmuthuge and Guan, 2017). Through meconium samples it has been found that the first colonizers in the GIT are *Citrobactor*, *Lactococcus*, *Leuconostoc*, and *Lactobacillus*, as the meconium microbial composition is very similar to the fecal microbiota at 6 and 12 h after birth. This similarity does, however, decrease

after 24 h of life due to the increased diversity, suggesting that the establishment of a complex microbiome may occur very early in life in ruminants (Malmuthuge and Guan, 2017).

2.3.1. Fecal microbiome

Studies using fecal samples to investigate the microbiota of ruminants, have reported a simple, less diverse bacterial community at birth which increases in complexity and diversity with the age and dietary changes. In majority of studies using fecal samples on pre-weaned calves, a higher abundance of *Bacteroides* at the genus level has been reported (Malmuthuge and Guan, 2017).

For the fecal microbiome, studies have consistently found *Prevotella*, *Bacteroides, Ruminoccus, Feacalbacterium*, *Rosbria*, and *Clostridium* in fecal samples from ruminants. Therefore, these are believed to be a part of the core microbiota of the fecal microbiome (Huws *et* al., 2018).

Noel *et* al. (2019) found that Rumococcaceae, Lachnospirachae, Bacteroidales, Clostridaceae, and Rikenellaceae were the seven most dominant species at the family level, when using fecal samples to study the gut microbiome. Furthermore, it was observed that when comparing rumen and fecal samples, the differences in the microbiota caused by breed or diets, where less pronounced in fecal samples. It was stated that for the fecal samples, these differences may only differ in the less dominant taxa of the microbiome.

Rumen samples may show a greater picture of differences due to breed or diet, in the gut microbiome. However, Shanks *et* al (2011) stated that a complete characterization of the fecal bacterial community composition, could help to address research gaps such as odor emissions and the shedding of fecal indicators used for recreational water testing.

2.3.2. Effect of lipids on the rumen microbiome

It is possible to modify the microbiome by changing the diet. A dietary change could potentially initiate a “bloom” of specific microbial populations, or it could even enhance the abundance of stress-response genes within the microbial community (Gercino *et* al., 2020; Auffret *et* al., 2017). Native fats fed to ruminants cause an inhibitory effect on the rumen microflora (Nurzhanow *et* al., 2019). An unbalanced microbiome is called “dysbiosis” and will generally be observed after dietary changes and alterations in the ruminal volatile fatty acid concentration. Dysbiosis can potentially be associated with a lower ruminal pH, the use of

antibiotics, the presence of heavy metals or toxic substances, or even infection with pathogenic bacteria (Auffret *et* al., 2017).

Rumen bacteria play an important role in the metabolism of fat-containing substances as a significant proportion of lipids absorbed in the intestine come from from microbial fermentation, whose role is crucial to the hydrogenation of unsaturated fatty acids, hydrolysis of lipids, and their synthesis from non-lipid components. With a low rate of lipolysis, the intensity of hydrogenation is decreased (Nurzhanov *et* al., 2020).

According to Gercino *et* al., (2020) the differences in the microbiome due to age can show the best time possible to manipulate the intestinal microbiome and improve the health and performance of cattle. Enjalbart *et* al. (2017) states that fat addition to the diet shapes the rumen microbial community, hence modulating the rumen function and creating opportunities to improve growth and performance.

2.3.3. Unsaturated fatty acids

Unsaturated fatty acids are a dominant component in the commonly used fat sources for ruminants (Enjalbart *et* al., 2017). To break down unsaturated fatty acids, the microbial community in the rumen promotes biohydrogenation, where fatty acids are both saturated and desaturated to provide energy for the host animals. This energy is in the form of volatile fatty acids (Abbas *et* al., 2018). The unsaturated fatty acids increase the fermentation pH of the rumen, where the production of acetate, butyrate, total volatile fatty acids, total gas, and methane is decreased; simultaneously, there is an increase in the unsaturation of C18 fatty acids (Li *et* al., 2012). Enjalbart *et* al. (2017) state that unsaturated fatty acids have a negative effect on microbial growth, especially protozoa and fibrolytic bacteria, but the effect on the different components of the microbiome depends on the fat source. For example, *Prevotella ruminicola* and some strains of *Butyrivibrio fibrisolvens* can be negatively affected by palmitic and stearic acids. Oleic acids were far more inhibitory on the growth of most fibrolytic bacteria, but they stimulated the growth of *Selenomonas ruminantium* and *P. ruminicola*. Furthermore, Fiorentini *et* al. (2013) found that soybean oil seems to be more efficient regarding nutrient intake, as they reduce the numbers of fungi and protozoa, consequently improving the efficiency of microbial protein synthesis.

Bacteria are the main microbial agents responsible for biohydrogenation. It was thought that protozoa were not actively involved in the biohydrogenation, but it has now been proved that protozoa affect the composition of the bacterial population in the rumen and may therefore have a role in biohydrogenation indirectly. In addition to this, it has been shown that protozoa

directly incorporate unsaturated fatty acids, protecting them in the rumen from biohydrogenation, allowing a direct transfer into the milk and meat production (Newbold and Ramos-Morales, 2020).

2.3.4. Saturated fatty acids

Saturated fatty acids suppress ruminal methanogenesis. Some of the most effective saturated fatty acids to suppress CH_4 production are nonesterified lauric acid (C_{12}) followed by myristic acid (C_{14}). In contrast to these, long-chained saturated fatty acids such as palmitic acid (C_{16}) and stearic acid (C_{18}) are less effective in suppressing ruminal methanogenesis. (Zhou *et* al., 2013)

Findings from Cancino *et* al. (2021) shows that feeding saturated fatty acids to ruminants increased the abundance of *Firmicutes* and decreased the abundance of *Bacteroidetes*. At the genus level *Prevotella* was dominant amongst the *Bacteroidetes*, while *Succiniclasticum* was the most dominant amongst the *Firmicutes*. Furthermore Cancino *et* al. (2021) observed that the abundance of *Prevotella* was reduced on day 63 in the saturated fatty acid diet.

Henderson (2009) did a study on how fatty acids affect the pure cultures of rumen bacteria. It was found that species such as *Bacteroides ruminicola* and *Selenomonas ruminantium* were unaffected by oleic acid. Growth of *Butyrivibrio* was stimulated by a low concentration of oleic acid when given at a low concentration, but if given at a high concentration, oleic acid seemed to have an inhibitory effect on *Butyrivibrio*. Furthermore, Henderson (2009) found that palmitic and stearic acids were inhibitory for these species.

2.4. Rumen microbiome and performance

In recent years, research has confirmed the link between the ruminant microbiome and productivity of the host animals and the environment in which they are raised. This piece of evidence explains why studies on the rumen microbiome are at the forefront of animal agriculture research (Mizrahi and Jai, 2018). The production of CH_4 and ammonia (NH_3) in the rumen is increased when feed is fermented and digested. Both CH_4 and NH_3 contribute to increase enteric greenhouse gas (GHG) emissions from livestock agriculture. Studies have been conducted to reduce GHG emissions by tackling enteric production of CH_4 and NH_3. There has been found strategies which redirect rumen carbon and nitrogen metabolism away from these

products can provide opportunities for a significant improvement in productivity, not only by reducing GHG emissions but also by improving nutrient retention (Bath *et* al., 2013).

Studies have shown that an increased dietary energy content increase the ruminal propionate concentration and reduce the ammonia concentration, but it can also affect microorganisms in the microbiome that are positively or negatively related to the intramuscular saturated fatty acid content of the meat (Hu *et* al., 2020). Furthermore, fat supplements may modulate the rumen activity of FA profiles of meat in beef cattle and milk in dairy cattle (Hu *et* al., 2020), and that the ruminal microbiota plays a pivotal role in defining the FA composition in milk which, in turn, can be affected by the diet (Cremonesi *et* al., 2018).

Studies have been made to determine how the rumen microbiome plays a role in the marbling of meat in beef cattle (Abbas *et* al., 2018). It has been proven that the microbial protein produced by microorganisms in the rumen provides a highly digestible source of amino acids used for muscle growth and milk protein. This suggests that the microorganisms play an essential role in optimizing nutrient utilization from the feed (Loor *et* al., 2016).

When diving into the link between the microbiome and ruminants' performance, Hu *et* al. (2020) observed that an increased dietary energy increases the ratio of *Firmicutes* to *Bacteroidetes*, causing an increase in ruminal amylolytic and propionate-producing bacteria populations. These findings agreed with Wang *et* al. (2019) who stated that the dietary energy level can affect the ruminal microbiota, and furthermore affect rumen fermentation and fatty acid synthesis. Both studies found the dominant microbial phyla to be *Bacteroidetes* and *Firmicutes*. Hu *et* al. (2019) found that *Prevotella* was positively related to intramuscular polyunsaturated fatty acid contents and negatively related to intramuscular saturated fatty acid content. For further investigation on the intramuscular fat content in beef cattle, and more specifically the marbling of the meat Abbas *et* al. (2018), found that the microbial species in Wagyu cattle are correlated to the highest marbling grades. These microbial taxa belonged predominantly to the families *Mogibacteriaceae*, *Lachnospiraceae*, and *Clostridiaceae*. These findings indicate that the microbial community might have an influence on the marbling grade of the meat, and that manipulating the microbiome could increase the amount of marbling (Abbas *et* al., 2018).

A way of manipulating the microbiome is through dietary interventions. For example, fat supplements can influence the rumen microbiome in such a way that the rumen activity of FA profile of both meat and milk are altered (Hu *et* al., 2020). Studies have shown that the microbes digested in the abomasum serve as the major protein sources for the host (Mizrahi

and Jami, 2018). These microbial proteins are highly digestible and play an essential role in optimizing nutrient utilization by the host (Loor *et* al., 2016). Mizrahi and Jami (2018) stated that there is a correlation between the ratio of *Firmicutes* to *Bacteroidetes* and the daily fat production in milk.

2.5. Studies on milk replacers and performance of calves

Around the world, studies have been made to investigate the effects of milk replacers on both beef and dairy cattle and their performance. It is important to know the nutritional requirements of calves, because if they are not fed appropriately to meet their nutritional needs, there will not be enough energy to support optimal growth and development (Palczynski *et* al., 2020).

Several studies have been conducted on the determination of the ideal content of fat percentages in milk replacers and their effects on the average daily gain in beef calves. Bascom *et* al. (2007) compared three milk replacer diets with different levels of protein and fat contents, and a diet consisting of whole milk. All groups received 180 g/day of crude protein, which supports an average daily gain of 650 g of body weight according to the NRC 2001. In the study, it was observed that the group receiving a diet with 27.3% protein and 33.4% fat and the group receiving whole milk had the greatest percentage of body fat gained. Additionally, these groups gained more grams of fat than the groups fed a diet of 28.5% protein and 16.4% fat, and 20.6% protein and 20.6% fat. It was, however, noted that all groups had a lower average daily gain than that estimated by the NRC 2001.

To further investigate the relationship between the fat concentration and a high protein concentration in milk replacers, Hill *et* al. (2009a) constructed a study where Holstein calves were fed a diet of 0.6 kg dry matter of milk replacer per calf daily. The fat percentages of the MR ranged from 14 to 23%, and the crude protein content ranged from 51.6 to 56.7 g crude protein per Mcal of ME. Here they saw that the pre-weaning starter intake responded quadratically to fat supplementation when the fat supplementation was at 14% and at 23%. There was a reduction in the digestibility of the diet and starter intake, which contributed to a decreased average daily gain at the higher fat concentrations in the milk replacer. Hill *et* al. (2009a) concluded that there was a maximized pre-weaning average daily gain at diets consisting of 27% crude protein, 17% fat milk replacer with a total of 55 g crude protein/Mcal of ME. This outcome was observed when the fat concentration varied to obtain various crude

protein to ME ratios. Also, calves fed 27% crude protein, 20% fat milk replacer with 53g crude protein/Mcal supported the overall average daily gain.

It has been reported that different sources of energy in the milk replacer can alter the body composition of calves (Bascom *et* al., 2007). Through research, it has been seen that the dietary crude protein requirement of pre-ruminant calves is dependent on energy intake and can minimize fat deposition in the growth phase. This has the potential to benefit long-term productivity, especially in dairy cattle. This is because an excessive body fat deposition is negatively correlated with mammary development, dry matter intake (DMI), and future milk yield. But when the goal is to expedite the rate of gain, an enhanced calorie intake from adding extra fat can be beneficial to growth (Tikofsky *et* al., 2001).

This increased weight gain from supplementing fat to the milk replacer is supported by Esselburn *et* al. (2013), who saw that when the beef calves were fed a milk replacer diet consistent of animal fat and supplemented with a commercial product, (consisting of butyrate, medium-chain fatty acids, and linolenic acid) there was an improved growth and feed efficiency.

That the dietary crude protein is dependent on the energy intake is supported by Hill *et* al. (2009b). In a study, they investigated the optimal crude protein to energy ratio at two different amounts of milk replacer. They had a total of eight groups, where the calves were fed different levels of crude protein at both high and low feeding rate. Hill *et* al. (2009b) noted that the pre-weaning average daily gain was greater at a high milk replacer feeding rate, but at the same time the starter intake was lower. For both pre- and post-weaning calves, there was a quadratic increase in the average daily gain as the crude protein increased. In the study, it was concluded that feeding of 51.5 g crude protein/Mcal would be optimal at a low feeding rate to maximize the average daily gain. In contrast, for the high feeding rate, the optimal value would be to feed 55 g crude protein/Mcal for the average daily gain to be maximized.

2.6. Milk replacers

In Denmark, there are companies that produce and sell milk replacers. All milk replacers mentioned here can be seen in Table 1.

The company ‘Himmerlands Grovvare’ mentions three types of milk replacers that can be beneficial to calves after they have been fed colostrum. Skimmed milk-based with a minimum of 60% skimmed milk replacer coagulates in the calf’s esophageal groove and gives

the best growth in the first 2-3 weeks of the calf's life. They state that their products with around 20-30% skimmed milk do not coagulate in the calf's stomach compared to the product with 60% of skimmed milk. The last product offered by Himmerlands Grovvare is whey-based, and because it does not coagulate in the calf's esophageal groove it is not recommended before the calf is 3-4 weeks. In this product there is a digestibility of protein that is almost as great as that of skimmed milk-based MR (Himmerlands, n.d).

DLG announced that the amount of milk and dry matter concentration in their milk replacers can be adapted to target the growth rate of the calves. They have two series of products called 'Friska' and 'MilkFoss'. For both brands, it is possible to choose a milk replacer with either 60%, 40%, or 20% of skimmed milk powder. For the MilkFoss it is also possible to buy one product called 'MilkFoss Lac'. DLG recommends MilkFoss Lac for the slightly bigger calves, because it contains a high amount of vegetable proteins that are only digestible for calves when they have reached a certain age. DLG announces that their Friska 60 has the highest amount of energy in the form of protein and fat, and the fat content should be the greatest available in the market. Both Friska 60 and MilkFoss 60 have a content of milk proteins which makes them both ideal to feed the calves after the colostrum. Friska 40 and MilkFoss 40 have a content of 40% skimmed milk powder and are thought to be used as a mixture in the whole milk. In both products there is a high amount of E-vitamin, and both are suited to supplement cow milk. In the Friska 20 and MilkFoss 20, there is a content of 20% skimmed milk powder, DLG announced that Friska 20 and MilkFoss 20 are ideal for bigger calves and have a high content of E-vitamin. The 40% skimmed milk powder products are suitable for supplementing cow milk (DLG, n.d.).

Viking also produces MRs. Their brand is called 'Kip' and can be found with different amounts of milk percentages. 'Kip Excellent' is made of whey powder, vegetable oils and fatty acids such as palm, coconut, rapeseed, and soy oils. Besides 'Kip Excellent' they have 'Kip 40' and 'Kip 60' which are made of 40% and 60% skimmed milk powder respectively. They also contain whey power, vegetable oils and fatty acids like the Kip Excellent (Viking Danmark, n.d).

Another company is 'Mosegården', announces that their milk replacer is based on milk. According to the manufacture's information Mosegården's MR gives the calves a high drinking lust due to taste. Their MRs contain a high amount of E-vitamin and organic selenium (Hatting, n.d.).

E-lac states that their milk replacer is based on milk, that it is very tasteful and gives the calves a greater drinking lust. Their milk replacers are divided into different color groups, where

they have three types of "red", one type of "blue", one type of "green" and one type of "yellow" milk replacer.

Company	Milk Replacer	Usage	Price (DKK)
DLG	Friska (20, 40, 60%)	Both great to use for calves after colostrum	523.75-468.75
	MilkFoss (20, 40, 60%)		510-615
	MilkFoss Skimm	Unit mix throughout milk feeding period	577.50
	MilkFoss Lac	Recommended for larger calves as final mixture	368.75
	Eurolac Bio (Organic)	Organic milk replacer for caves, pigs, lambs, and kid	1,250
Viking	Kip Excellent	Whey based for calves over 4 weeks of age	570.31
	Kip 60 Opti	For newborn calves	726.56
	Kip 40 (40%)	Skimmed milk based for calves over 4 weeks of age	640.63
	Kip 60 (60%)	For newborn calves	695.31
Mosegården	Maternor (60%)	Supposed to increase drink lust in calves	19.82*
E-lac**	E-lac rød 60%	60% skimmed milk powder Full feed mixture for calves	-
	E-lac rød 60% sur	Same as above	-
	E-lac rød 50%	50% skimmed milk powder Full feed mixture for calves	-
	E-lac blå	30.5% skimmed milk powder Full feed mixture for calves	-
	E-lac grøn	Full feed mixture for calves	-
	E-lac gul	Full feed mixture for calves	-

Table 1 *- Types of milk replacers sol in Denmark *The price when buying 1,250 kg (50 sacks of 25 kg/sack) **Price is not listed on E-lac homepage*

2.7. Fat supplements

Bergafat F100 consists of palmitic acid, stearic acid, oleic acid, and linoleic acid. There are in total 91.9% saturated fatty acids in it and 8.1% unsaturated fatty acids. When used at a low to moderate dosage, during the early lactation, it can increase the energy density of a diet and provide energy and fat for efficient milk production. When used in a moderate to high dosage, during mid-lactation, providing energy and fat to the cow it can give an efficient milk production; it can also be used for shifting energy towards milk production. (Tobias Gorniak, 2019)

Lipitec Bovi 85 is a granulated product that consists of calcium saponified palm fatty acids. It has been developed with the intention of increasing the production of milk and milk fat in high-yielding dairy cows. This fat supplement can be fed in its raw form or be used in a feed mixture. Lipitec claims that it is both an economic and efficient energy source.

Lipitec Bovi HF is a sprayed dry fat, developed with the intention of increasing the production of milk and milkfat in high-yielding dairy cows and is based on saturated fatty acids palmitic acid and stearic acid. Typically, it increases the fat content in milk by 0.3% units. It can be fed in the raw form or used in a feed mixture.

Lipitec Bovi LM is a sprayed dry fat that was developed with the intention of increasing the production of milk and milkfat in high-yielding dairy cows. The fat supplement is based on saturated fatty acids, which are added to the diet together with mono- and diglycerides to improve the emulsifying ability and digestibility. Typically, it increases the fat content of milk by 0.3% units and gives more milk than regular saturated fats. It can be fed in its raw form or used in a feed mixture.

Lipitec Glycofat is granulated calcium saponified palm fatty acids and glycerol. It is claimed to have a better smell and taste than traditionally saponified fat and can be used unmixed or as a supplement in a feed mixture. It should be both an economic and efficient energy source.

Fats mentioned in this section can be found in Table 2, with an explanation of the content of fat types.

Fat supplement	Types of fat
Bergafat F100	Palmitic acid (85.5%) Stearic acid (3.7%) Oleic acid (6.9%) Linoleic acid (1.2%)
Lipitec Bovi85	Myristic acid (1%) Palmitic acid (45%) Stearic acid (5%) Oleic acid (40%) Linoleic acid (9%)
Lipitec BoviHF	Myristic acid (1%) Palmitic acid (50%) Stearic acid (43%) Oleic acid (3%) Other (2%)
Lipitec BoviLM	Myristic acid (max. 1.5%) Palmitic acid (40-55%) Stearic acid (40-55%) Oleic acid (max 8%) Other (max. 3%)
Lipitec Glycofat	Myristic acid (1%) Palmitic acid (45%) Stearic acid (5%) Oleic acid (40%) Linoleic acid (9%)

***Table 2**. Types of fat supplements sold in Denmark, and the types of lipids they contain*

3. Methodology

3.1. Experimental design and diets

The experimental site was at a beef calf farm in Eskilstrup, Denmark (54°52'24.5"N, 11°56'23.8"E). Eighteen Holstein-Belgian Blue crossbred bull calves weighing (± SD) 69.8 ± 11.1 kg on day 0 (day of arrival at the beef farm), were blocked by weight (70.3 ± 11.9 kg, 69.0 ± 11.5 kg, 70.2 ± 11.8 kg) and assigned randomly to receive one of the three treatments. The control group (70.3 ± 11.9 kg body weight) was fed 811 g DM of MR containing 16% fat (Table 3), without the fat supplement. Group 2 (69.0 ± 11.5 kg body weight) was fed 753 g DM of MR + 58.2 g DM BOVI 85 fat supplement (Lipitec, NLM Vantinge, Ringe, Denmark) (Table 3), and group 3 (70.2 ± 11.8 kg body weight) was fed 753 g DM of MR + 50.6 g DM BOVI LM

fat supplement (Lipitec, NLM Vantinge A/S, Ringe, Denmark). Bovi85 and BoviLM groups were fed the corresponding diets to achieve 21% fat in the diets. A commercial milk replacer (Table 3) served as the basal liquid feed for all groups. The fat supplements were fed over a 21-days period in November 2021.

The amount of fat supplement given to Bovi85 and group BoviLM was calculated using the method of 'two equations with two unknowns', such that all treatment groups were fed the same amount of DM as well as the liquid feed. In the MR there was 96.59% DM which was used as the baseline for the calculations. Bovi85 and BoviLM had respectively, 96.50% DM and 99.28% DM. From the calculations it was concluded that group Bovi85 should be given 30 g of Bovi 85 supplement and 390 g MR twice daily. The group BoviLM should be given 25.8 g of Bovi LM fat supplement and 394.2 g MR twice daily. For the full calculations, please see Appendix 1 for more details.

3.2. Animals and Housing

All groups had *ad libitum* access to water, meadow grass haylage and starter feed (Table 3.). Haylage was fed in hayracks placed in the wall of the hutches. The starter feed was fed from a group feeder, with a capacity of 31 kg, placed in each group. Intakes of haylage and starter feed were monitored daily to ensure that feed was always available. All groups were housed in outdoor hutches measuring 3.7 m × 3.6 m (13.32 m^2) and bedded with fresh straw supplied daily. The front of the hutches was equipped with headlock panels where the calves were fetched during feeding time. All calves were fed a fixed rate of three liters of milk replacer. Milk replacer was fed at around 40 °C in buckets twice daily at around 0800 h and 1700 h. After feeding, all calves were offered three liters of water with sodium chloride and an E vitamin supplement.

Milk Replacer (MR)

	Control	Bovi 85	Bovi LM
g DM/day	811	753	753
Composition, % in DM			
DM, %	96.6	96.6	96.6
Crude Protein, %	24.0	24.0	24.0
Ether Extract, %	15.7	21	21
Ash, %	6.0	6.0	6.0
Calcium, %	0.8	0.8	0.8
Phosphorus, %	0.78	0.78	0.78
Sodium, %	0.52	0.52	0.53
Cellulose, %	0.1	0.1	0.1
Iron, mg/kg	100	199	100
Zink, mg/kg	64	64	64
Selenium, mg/kg	0.3	0.3	0.3
Cupper, mg/kg	4	4	4
Manganese, mg/kg	64	64	64
Iodine, g/kg	0.16	0.16	0.16

Ingredients, % of inclusion	**Starter feed**	**Haylage**
Pelleted Concentrate	40	-
Wheat	30	-
Nearly	24	-
Pea	6	-
Grass	-	100
Chemical composition, % of DM		
Dry matter	84.2	69.9
Crude Protein	21.0	11.9
Ether extract	3.4	-
Ash	8.0	5.9
NDF	20.7	70.4

Chemical composition, % in DM	**Lipid supplements**	
	Bovi 85	Bovi LM
g DM/day	58.2	50.6
Dry Matter	96.6	99.3
Ash	20.5	0
Fat	89.9	100
Fatty acid profile, %		
C14:0	1	< 1.5
C16:0	45	40-55
C18:0	5	40-55
C18:1	40	< 8
C18:2	9	< 3

Table 3. Chemical compositions of the MR, including the lipid supplement Bovi 85 and Bovi LM, starter feed and haylage.

3.3. Sampling and monitoring

Fecal samples for analysis of microbiome and metabolomics were taken from the rectum of each calf, using clean gloves for each animal, placed in individual containers, and transported back to the University of Copenhagen to be stored in a freezer (-80 °C) until later analysis.

Samples were taken on days 0, 7, 14, and 21, which in this thesis will be referred to as P_0, P_1, P_2, and P_4 indicating the periods at which samples were collected.

3.4. Feces analysis

Frozen fecal samples were sent to Clinical Microbiomics for 16S rDNA sequencing-based microbiome analysis.

Firstly, the samples went through a DNA extraction protocol using the NucleoSpin® 96 stool (Macherey-Nagel) kit, where bead beating was done in a Vortex-Genie 2 horizontally at 2700 rpm for 5 minutes. In all laboratory procedures one positive control (ZymoBIOMICS™ Microbial Community Standard, Zymo Research) and two negative controls were included.

After DNA extraction, a PCR test was done with the forward *S-D-Bact-0341-b-S-17* and reverse primers *S-D-Bact-0785-a-A-21* attached to Illumina adapters.

Products from the nested PCR were pooled based on band intensity and the resulting library cleaned with magnetic beads. DNA concentration of pooled libraries was measured fluorometrically, and sequencing was done on an Illumina MiSeq desktop sequencer using the MiSeq Reagent Kit V3 (Illumina) for 2x 300 bp paired-end-sequencing. (Clinical Microbiomics, 2022)

3.4.1. Bioinformatics analysis

The bioinformatic analysis was done by Clinical Microbiomics. Here an adjusted dada2 pipeline was used for bioinformatic processing of the sequence data into an amplicon sequence variant (ASV) abundance table (Clinical Microbiomics, 2022).

In the first step the primer sequence was removed from raw reads using cutadapt. Reads without a perfect match or with ambiguous bases were filtered out, this was also true for reads shorter or longer than expected. There was an additional filtering and trimming step, where reads were first trimmed at the 3 prime ends based on sample-specific quality scores. The trimmed reads based on quality scores of nucleotides that were expected to contain more than

one error were removed, and the remaining reads were dereplicated into unique sequences and then denoised separately for forward and reverse reads for each of the samples. Denoised forward and reverse reads were merged thereby discarding read pairs without sufficient overlap or with any mismatch in the overlap region (Clinical Microbiomics, 2022).

Suspected chimeras were removed from the generated abundance table by internal abundance and sequence comparison. The taxonomic assignment of the detected ASVs was done using a naïve Bayesian classifier algorithm (implemented in *qiime2*) comparing the ASV sequences to the SILVA reference database (version 138). Alpha and beta diversity measures were calculated from the rarefied data (Clinical Microbiomics, 2022).

3.5. Statistical analysis

The rumen microbiome data were analyzed using a model that included diet, time, and diet × time as fixed effects and calves withing treatment as random effect. Data was analyzed using R i386 4.1.3 with a two-way ANOVA, and any significant statistical difference was determined at the probability of $p < 0.05$. To analyze alpha-diversity metrics, evenness and Shannon's diversity and Faith's phylogenetic diversity (PD) were calculated. To analyze beta-diversity metrics, Jaccard index, weighted UniFrac distance and Bray–Curtis dissimilarity index were calculated. The dissimilarity matrices and the distances between the rumen microbiota and categorical groups were tested using unweighted UniFrac distance matrices using permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. Plots were generated using the visualizer of the *q2-diversity* plugin implemented in *qiime2*.

The *mixMC* (mixOmics microbial community) R package was used to perform the multivariate analysis to identify associations between microbial composition and the explanatory variables (fat supplements). In the analysis, we considered only microbial taxa and microbial functions with a relative abundance > 0.01% and prevalent in at least 50% of the samples (36 out 71).

4. Results

In total, 20M raw sequencing reads were generated and processed using the *qiime2* pipeline. For the 71 biological samples, it was obtained sufficient sequencing data, with an average sequencing depth of 73,234 read pairs per sample after quality filtering. The minimum high-quality sequence of a sample was set at 36,096 reads.

4.1. Taxonomic overview

Taxonomic classification was assigned to all amplicon sequence variants (ASVs) based on the SILVA reference database. Overall, 99.9% of the 2,532 detected ASVs could be assigned to the phylum level, 87% could be assigned to the family level, and 64% could be assigned to the genus level. The relative abundance profiles aggregated to the order, phylum, family, and genus levels are shown in Figures 1, 3, 5, and 7, respectively, with the intention to provide a general overview of the fecal microbiome composition.

Figures 2, 4, 6, and 8 show the changes in the relative abundance of the microbes according to the order, phylum, family and genus levels.

4.1.2. Phylum

Phyla	**Treatment**				***p*-value**		
	Control	**Bovi LM**	**Bovi 85**	**SEM**	**Treatment (T)**	**Period (P)**	**T × P**
Firmicutes	69.9	67.1	61.9	2.01	0.86	0.009	0.22
Bacteroidota	26.2	26.6	31.7	2.22	0.9	0.01	0.26
Actinobacteriota	2.3	2.7	2.9	0.52	0.99	0.36	0.92
Proteobacteria	1.2	3.01	1.2	0.48	0.85	0.8	0.91
Fusobacteriota	0.23	0.06	0.05	0.06	0.60	0.73	0.94
Patescibacteria	0.13	0.16	0.04	0.04	0.74	0.16	0.52
Cyanobacteria	0.11	0.02	0.11	0.02	0.945	0.0485	0.179
Desulfobacterota	0.08	0.03	0.05	0.02	0.29	0.328	0.587
Euryarchaeota	0.02	0.05	0.02	0.12	0.39	0.5666	0.759

Table 4 – Microorganisms are represented in their relative abundance percentage. Effect of saturated (Bovi LM) and unsaturated (Bovi 85) lipids on the microbiome, at phylum level. Control, no fat supplement; BoviLM, supplemented with 50.6g DM saturated fat; Bovi85, supplemented with 58.2g DM unsaturated fat; SEM, standard error of the mean; results were declared significant at $p < 0.05$.

In the fecal samples 17 phyla was identified irrespective of the diet, with Firmicutes (66.9%) being the dominant taxa, followed by Bacteroidota (27.6%), Actinobacteriota (2.7%),

and Proteobacteria (1.9%). Together, these four phyla accounted for 97.2% of the fecal microbiome. Less abundant phyla with an average of relative abundance of 0.1% or less was grouped as ‘Others’. Unidentified phyla were grouped as ‘Unclassified’ and corresponded to 0.003% of the phylum abundance. Table 4 compares the relative abundance (%) at different time points of the most abundant phylum between Control, BoviLM and Bovi85. There were no significant effects of the treatments on the general phyla classification.

A two-way ANOVA test showed that Firmicutes significantly decreased in relative abundance over the period ($p = 0.009 < 0.05$), while Bacteroidota significantly increased in relative abundance over the period ($p = 0.01 < 0.05$). For the Control group there was an increase in Firmicutes in P_1 from P_0, and in both P_2 and P_3 the relative abundance decreased. For the BoviLM group, there was a decrease in the relative abundance of Firmicutes in P_1, an increase in P_2 and another decrease in P_3. Bovi85 had an increase in the relative abundance of Firmicutes from P_0 until P_2, followed by a large decrease in P_3.

For the relative abundance of Bacteroidota there was a decrease from P_0 to P_2 for both the control group and Bovi85, where there was an increase in the relative abundance at P_3. BoviLM had an increase in the relative abundance of Bacteroidota throughout all sampling days.

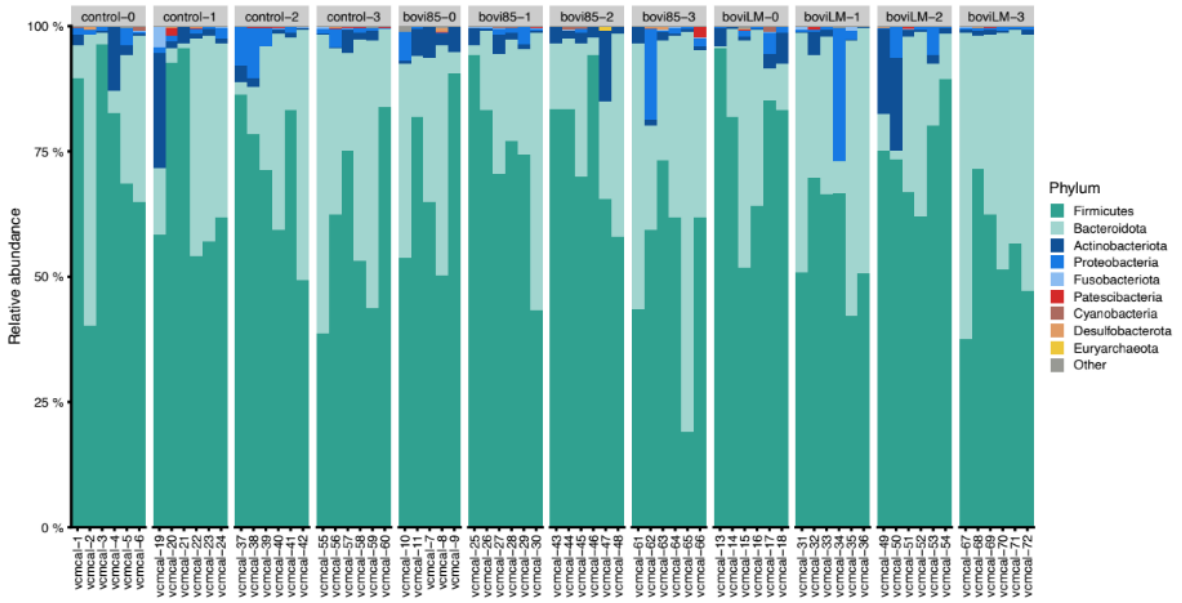

Figure 1. Taxonomic profiles at the phylum level based on relative abundances, grouped by a combination of the treatment and period variables. The top ten abundant phyla across all samples are shown. Less abundant phyla are summarized as “Other".

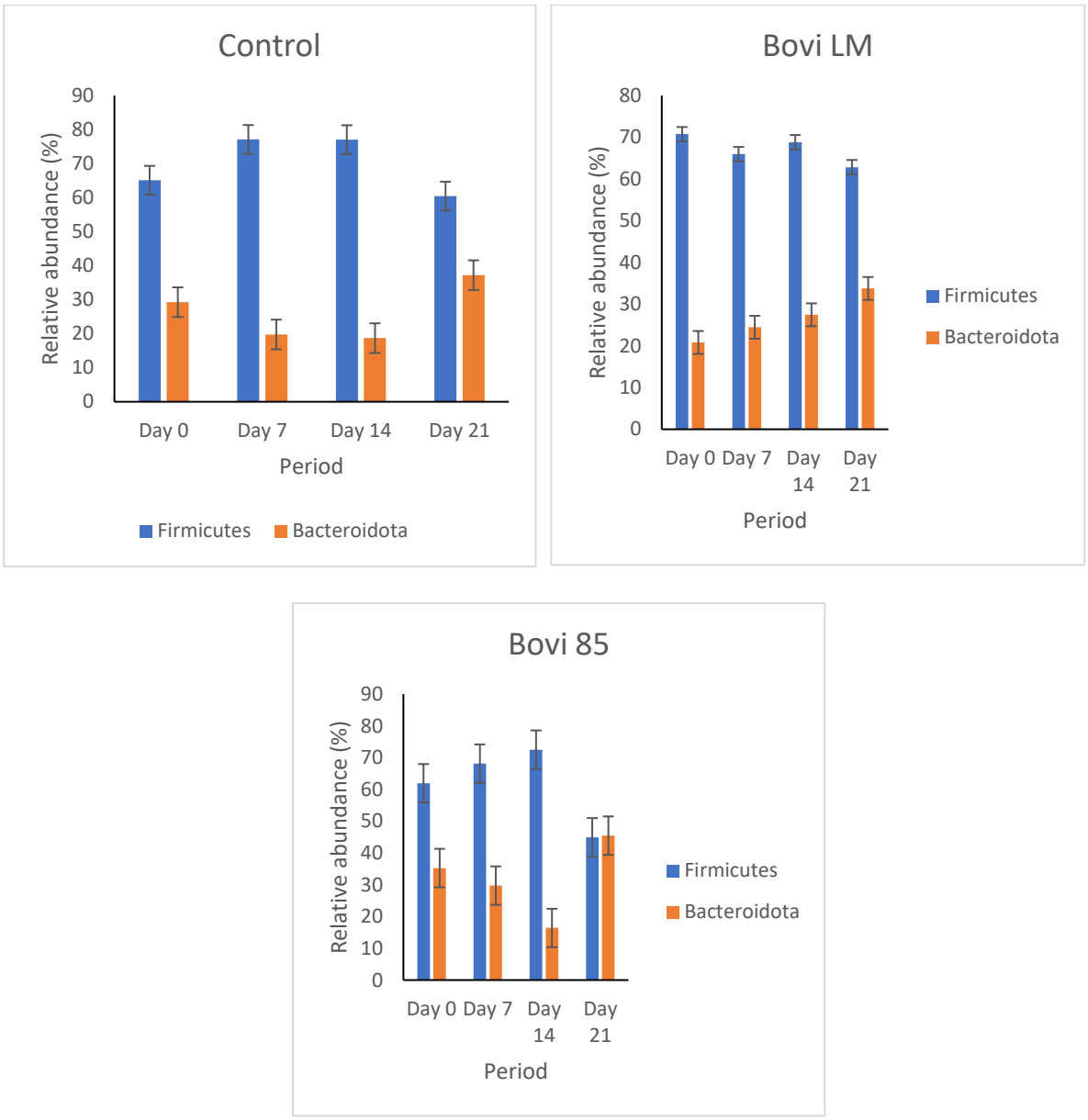

Figure 2. The changes of relative abundance over the period at the phylum level, for Firmicutes and Bacteroidota in each treatment group.

4.1.1. Order

Order	Treatment				*p*-value		
	Control	**Bovi LM**	**Bovi 85**	**SEM**	**Treatment (T)**	**Period (P)**	**T × P**
Bacteroidales	26.33	28.77	27.46	2.22	0.90	0.01	0.02
Oscillospirales	22.04	27.94	24.27	1.52	0.32	0.75	0.58
Lachnospirales	27.07	18.69	22.72	1.76	0.15	0.17	0.22
Lactobacillales	8.66	6.92	6.15	1.18	0.65	0.009	0.11
Clostridia UCG-014	4.94	5.98	5.98	0.70	0.83	0.24	0.53
Coriobacteriales	2.35	2.46	2.50	0.50	0.99	0.59	0.98
Erysipelotrichales	2.18	1.85	2.55	0.24	0.46	0.86	0.89
Enterobacterales	1.38	1.97	1.18	0.46	0.77	0.996	0.94
Veillonellales-Selenomonadales	0.31	1.19	1.83	0.34	0.16	0.74	0.55

Table 5 – Microorganisms are represented in their relative abundance percentage. Effect of saturated (Bovi LM) and unsaturated (Bovi 85) lipids on the microbiome, at the order level. Control, no fat supplement; BoviLM, supplemented with 50.6g DM saturated fat; Bovi85, supplemented with 58.2g DM unsaturated fat; SEM, standard error of the mean; results were declared significant at $p < 0.05$.

From the fecal samples, 44 species were identified at the order level, with Bacteroidales accounting for 27.52%. These microbes were the dominant taxa in the fecal microbiome, followed by Oscillospirales (24.87%), Lachnospiraes (22.83%), and Lactobacillales (7.24%) and 0.05% were unclassified. Table 5 compares the relative abundance (%) at different time points of the most abundant orders. There was found no significant effect of the treatments.

Two-way ANOVA showed a significant increase in the relative abundance of Bacteroidales ($p = 0.01 < 0.05$) for the period, and a significant decrease in the relative abundance of Lactobacillales ($p = 0.009 < 0.05$) with the period. For the Control group there was a decrease in the relative abundance of Bacteroidales from P_0 to P_3. BoviLM had an increase in Bacteroidales from P0 to P1, followed by a decrease from P_1 to P_2, and then an increase from P_2 to P_3. For Bovi85 there was a decrease in the relative abundance of

Bacteroidales from P_0 to P_2, followed by an increase at P_3, where a higher percentage of relative abundance was reached compared to P_0 (P_0 = 25.96%, P_3 = 41.17%).

Lactobacillales decreased in its relative abundance in the control and BoviLM from P_0 to P_3. For BoviLM, there was a small increase between P_1 and P_2 (P_1 = 5.26%, P_2 = 5.95%). Bovi85 had a decrease in the relative abundance of Lactobacillales from P_0 to P_1, followed by an increase at P_2, and thereafter it decreased at P_3.

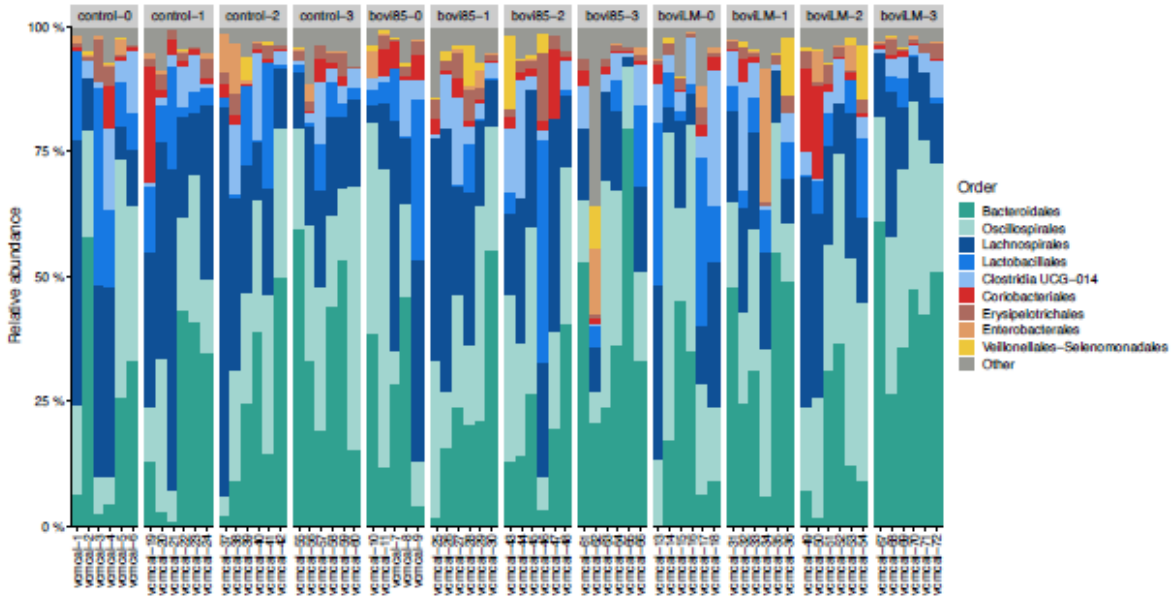

Figure 3 Taxonomic profiles at order level based on relative abundances, grouped by a combination of the treatment and period vaiable. The top ten abundant orders across all samples are shown. Less abundant phyla are summarized as "Other".

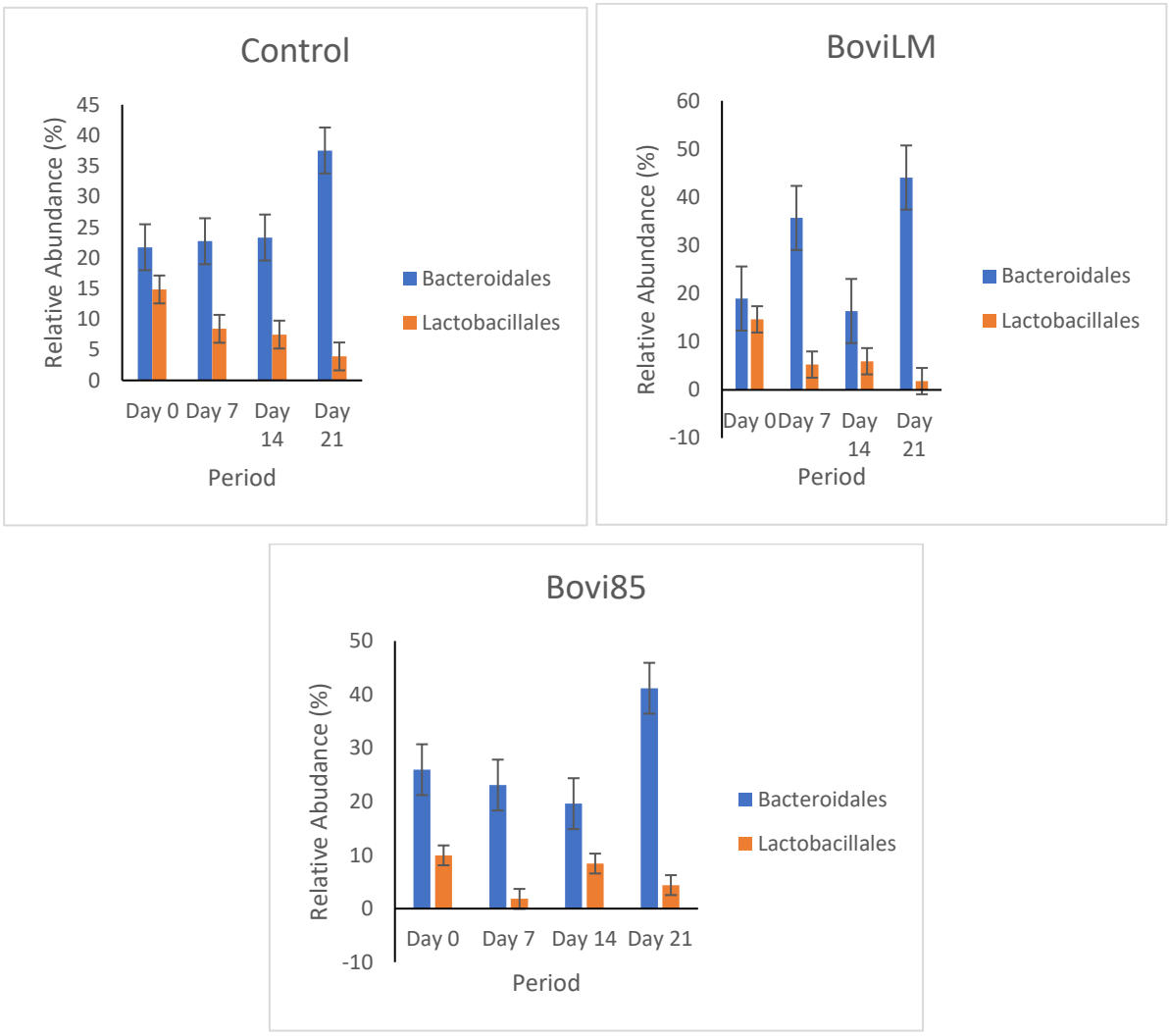

Figure 4. The changes of relative abundance over the periods at the order level, for Bacteroidales and Lactobacillales in each treatment group.

4.1.3. Family

Family	Treatment				*p*-value		
	Control	Bovi LM	Bovi 85	SEM	Treatment (T)	Period (P)	T × P
Lachnospiraceae	27.07	18.69	22.25	1.76	0.149	0.165	0.22
Ruminococcaceae	14.10	18.84	15.96	1.43	0.388	0.09	0.43
Prevotellaceae	11.52	10.59	12.13	1.28	0.877	0.007	0.12
Lactobacillaceae	8.50	6.89	5.45	1.18	0.559	0.01	0.099
Bacteroidaceae	6.21	9.49	3.99	0.95	0.70	0.76	0.17
Oscillospiraceae	6.26	5.46	6.79	0.73	0.84	0.0004	0.012
Muribaculaceae	5.77	5.61	4.72	0.85	0.28	0.11	0.36
Rikenellaceae	2.11	2.21	3.31	0.47	0.52	0.47	0.55
[Eubacterium] coprostanoligenes group	2.53	1.999	2.17	0.23	0.99	0.18	0.568

Table 6 - Microorganisms are represented in their relative abundance percentage. Effect of saturated (Bovi LM) and unsaturated (Bovi 85) lipids on the microbiome, at family level. Control, no fat supplement; BoviLM, supplemented with 50.6g DM saturated fat; Bovi85, supplemented with 58.2g DM unsaturated fat; SEM, standard error of the mean; results were declared significant at $p < 0.05$

At the family level, 198 family groups were identified and 6.2% were grouped as 'Unclassified'. The main family groups were Lachnospiraceae (22.7%), Ruminococcaceae (16.3%), Prevotellaceae (11.4%), and Lactobacillacea (6.9%), which accounted for 57.33% of the fecal microbiome. Table 6 compares the relative abundance (%) at different time points of the most abundant family groups between control, BoviLM and Bovi85. There were no significant effects between treatments.

Among all groups, Ruminoccoccaceae significantly decreased in relative abundance ($p = 0.007 < 0.05$) at P_1 and P_3, but with an increase in P_2. Prevotellaceae increased in relative abundance with the trial period in all treatments ($p = 0.01 < 0.05$), however, there was a decrease from P_1 to P_2. For BoviLM and Bovi85 there was an increase in the relative abundance of Prevotellaceae at P_3. The control group had a significant decrease in the relative abundance of

Lactobacillaceae ($p = 0.01 < 0.05$) during the trial period. For both BoviLM and Bovi85 there was a decrease in the relative abundance of Lactobacillaceae from P_0 to P_1, followed by an increase in P_2 and decrease in P_3, ending in a lower relative abundance for both groups in P_3 than at P_0. Oscillospiraceae showed a significant effect on the Treatment × Period ($p = 0.012$). For both control and BoviLM the relative abundance increased during the trial period. For Bovi85 there was an increase at P_1, followed by a decrease in P_2 and an increase in P_3.

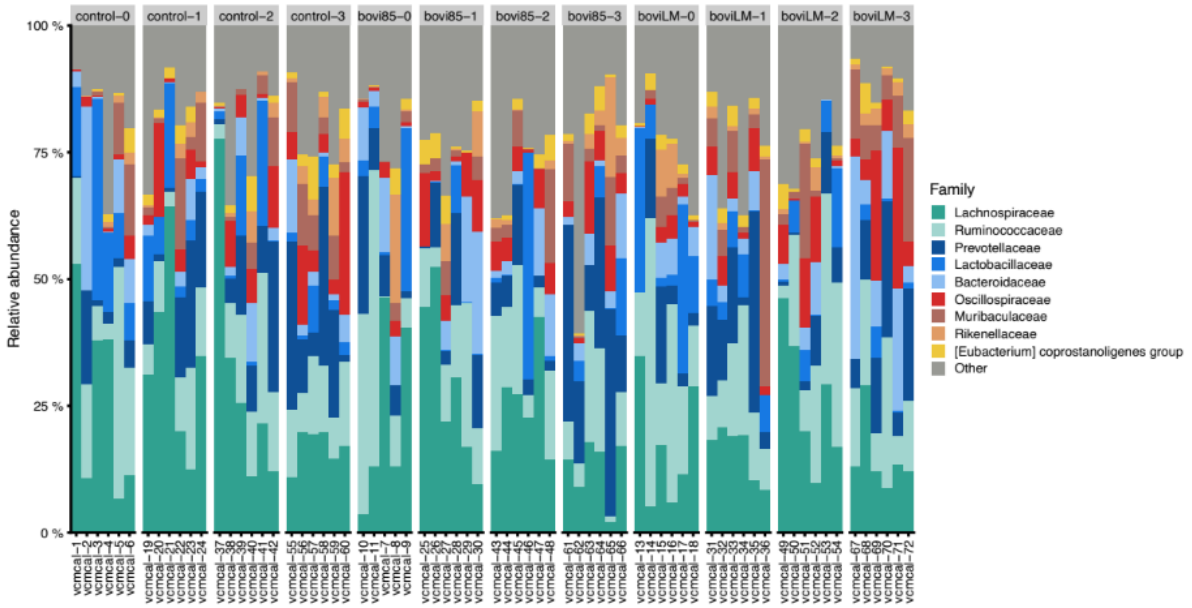

Figure 5. Taxonomic profiles at family level based on relative abundances, grouped by a combination of the treatment and period variable. The top ten abundant families across all samples are shown. Less abundant families are summarized as “Other".

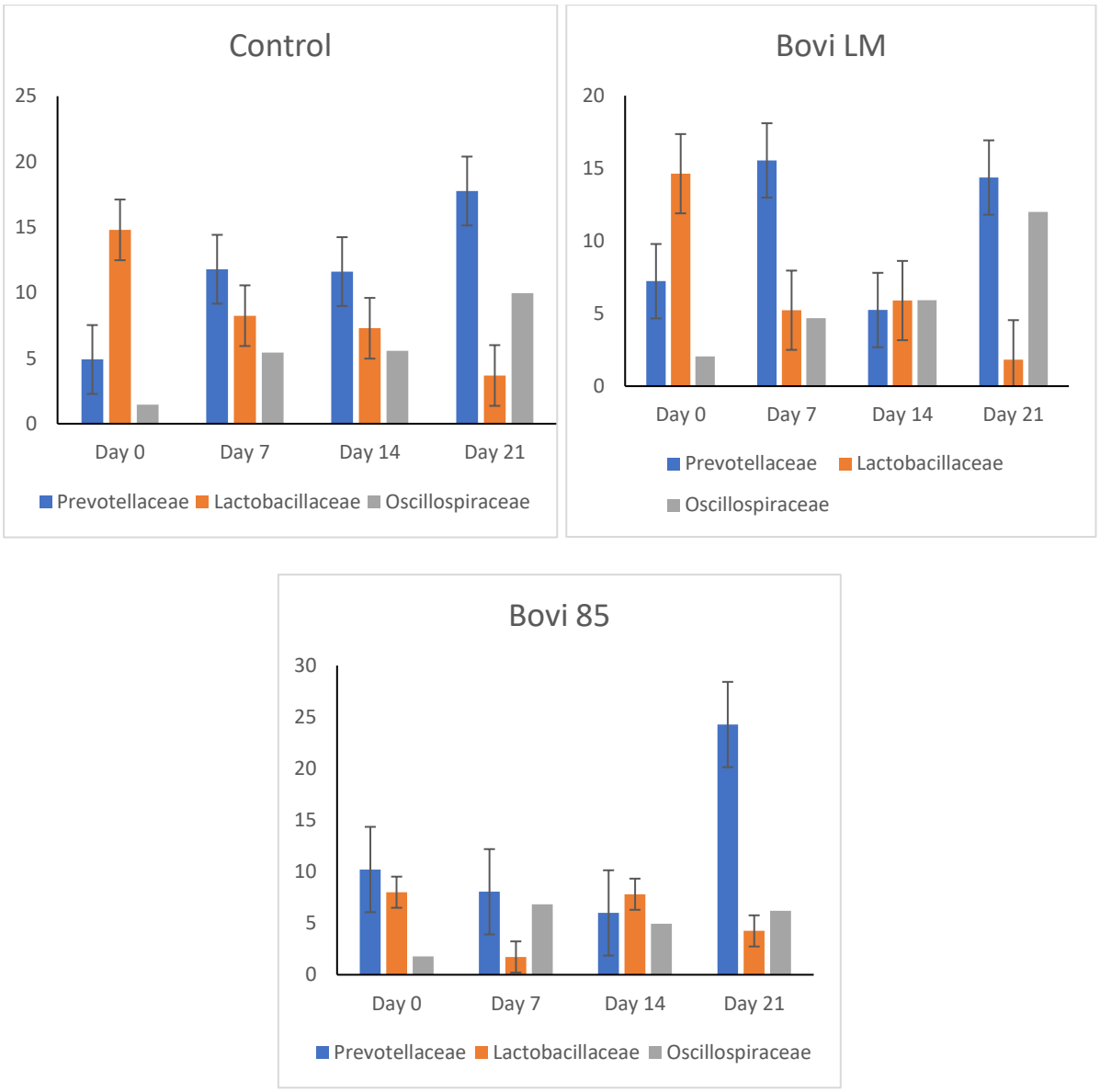

Figure 6. The changes of relative abundance over the period at the family level, for Prevotellaceae, Lactobacillaceae, and Oscillospiraceae in each treatment group.

4.1.4. Genus

Genus	Treatment			*p*-value			
	Control	Bovi LM	Bovi 85	SEM	Treatment (T)	Period (P)	T × P
Blautia	15.49	8.03	10.38	1.54	0.03	0.06	0.08
Faecalibacterium	8.78	14.78	9.03	1.35	0.12	0.38	0.40
Alloprevotella	7.70	7.97	8.37	1.16	0.96	0.001	0.047
Bacteroides	5.23	7.18	6.59	0.95	0.70	0.76	0.17
Lactobacillus	4.80	4.16	3.11	0.74	0.63	0.03	0.16
Subdoligranulum	3.75	3.95	3.35	0.82	0.488	0.08	0.019
UCG-005	1.81	3.47	4.87	0.67	0.72	0.0007	0.017
[Ruminococcus] torques group	2.30	1.56	2.99	0.36	0.045	0.5489	0.239
HT002	3.84	1.47	2.29	0.50	0.75	0.02	0.23

Table 7 - Microorganisms are represented in their relative abundance percentage. Effect of saturated (Bovi LM) and unsaturated (Bovi 85) lipids on the microbiome, at genus level. Control, no fat supplement; BoviLM, supplemented with 50.6g DM saturated fat; Bovi85, supplemented with 58.2g DM unsaturated fat; SEM, standard error of the mean; results were declared significant at $p < 0.05$

At the genus level, 20.1% of the microorganisms were unsigned to the corresponding genera. The main genera groups that were correctly assigned in this study were *Blautia* (11.3%), *Faecalibacterium* (10.9%), *Alloprevotella* (8%), *Bacteroides* (6.3%), and *Lactobacillus* (4%), accounting for 40.5% of the overall abundance. Table 7 compares the relative abundance (%) at different time points of the most abundant genus between control, BoviLM and Bov85.

Blautia showed a significant effect of the Treatment × Period interaction ($p = 0.08 < 0.05$). There was an increase in relative abundance for the control group, in P_1, followed by a decrease for the rest of the trial. In BoviLM, there was a decrease from P_0 to P_1, in P_2 there where observed an increase, and in P_3 a decrease was observed. Bovi85 had a decrease in the relative abundance of *Blautia* throughout the trial period. The relative abundance of *Alloprovetella* ($p = 0.001 < 0.05$) increased throughout the trial period for the control group.

For BoviLM there was a decrease in the relative abundance in P_2 which was followed by an increase at P_3. However, for Bovi 85, *Alloprevotella* decreased in its relative abundance from P_0 to P_2, followed by an increase at P_3. The relative abundance of *Lactobacillus* ($p = 0.03 < 0.05$) decreased in P_1 and P_3 but had an increase at P_2 for both control and Bovi85. For BoviLM there was a decrease in the relative abundance of *Lactobacillus* throughout the trial period. *Subdoligranulum* showed a significant effect of Treatment × Period ($p = 0.019 < 0.05$). For both control and BoviLM there was a decrease in its relative abundance throughout the trial period. For Bovi85 there was a decrease from P_0 to P_1, an increase at P_2 and another decrease at P_3. The relative abundance of UCG-005 ($p = 0.0007 < 0.05$) increased from P_0 to P_1, followed by a decrease at P_2 and an increase at P_3 for both control and BoviLM. For Bovi85 there was a decrease in its relative abundance from P_0 to P_2, followed by an increase at P_3. UCG-005 also showed a significant effect of Treatment × Period ($p = 0.017 < 0.05$). HT002 ($p = 0.02 < 0.05$) decreased during the trial period for control. For BoviLM there was a decrease in its relative abundance from P_0 to P_2, followed by a slight increase in P_3. For Bovi85 there was a decrease from P_0 to P_1, an increase at P_2, followed by a decrease in P_3.

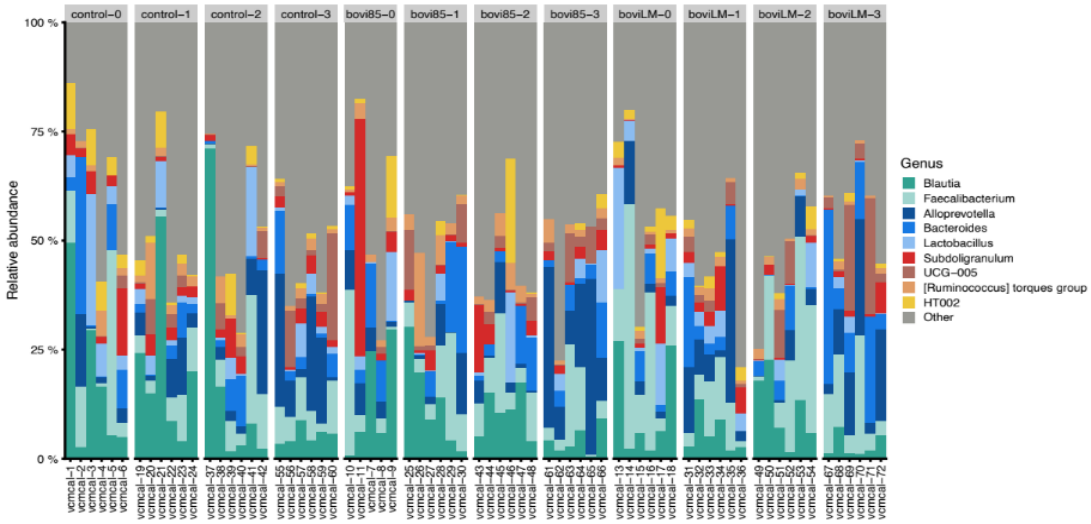

Figure 7. Taxonomic profiles at genus level based on relative abundances, grouped by a combination of the treatment and period variable. The top ten abundant genera across all samples are shown. Less abundant genera are summarized as “Other".

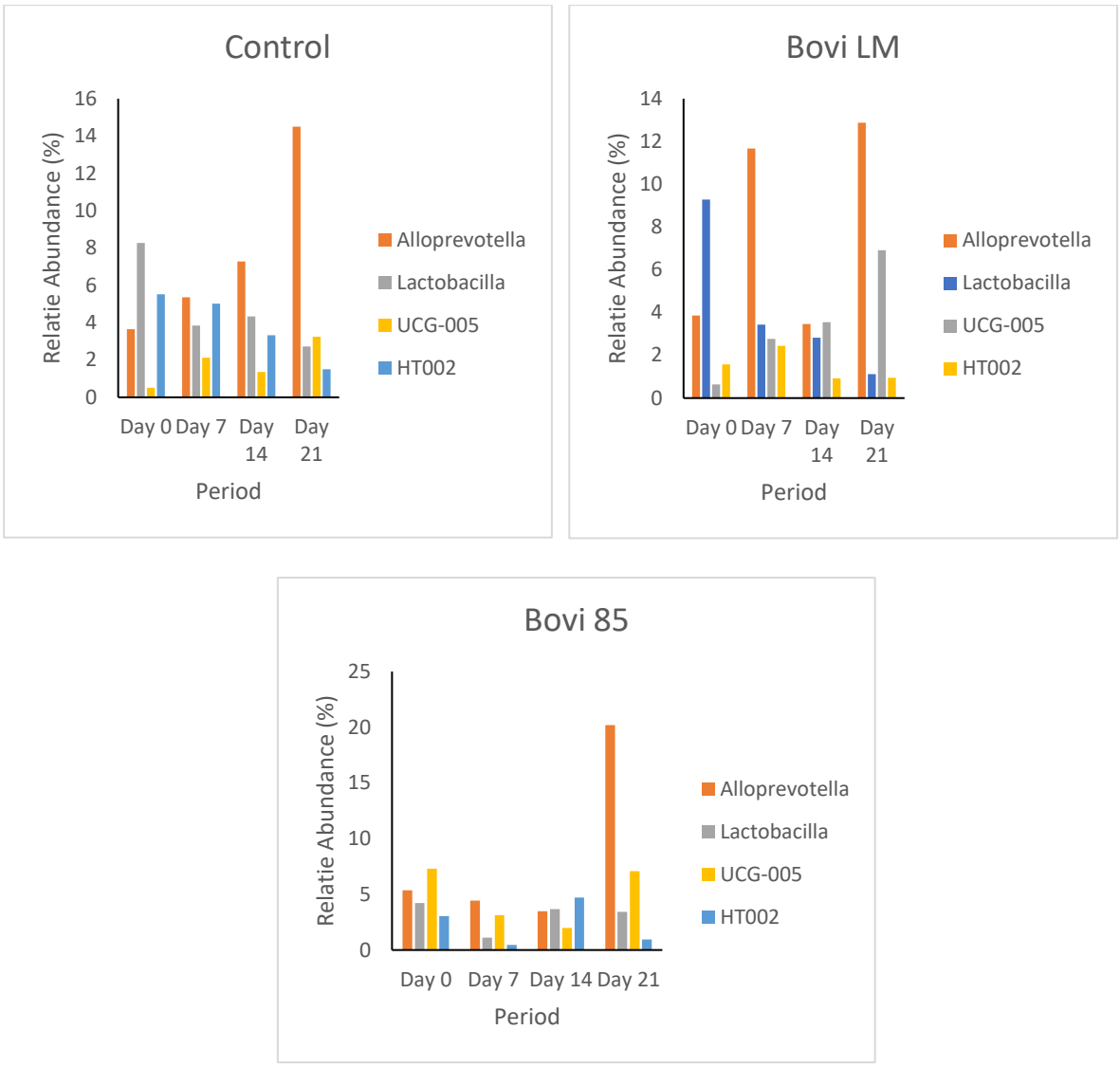

Figure 8. The changes of relative abundance over the period at genus level, for Alloprevotella, Lactobacilla, UCG-005, and HT002 of each treatment group.

4.2. Alpha and Beta diversity

Diversity metric	Bacteria	Archaea
	***P*-value**	***P*-value**
Period		
Faith's Phylogenetic Diversity	0.006	0.003
Simpson's Evenness	0.02	0.02
Good's coverage	> 98%	> 98%
Unweighted UniFrac	0.001	0.001
Treatment		
Faith's Phylogenetic Diversity	0.89	0.77
Simpson's Evenness	0.84	0.86
Good's coverage	> 98%	> 98%
Unweighted UniFrac	0.44	0.32
Period x Treatment		
Faith's Phylogenetic Diversity	0.14	0.09
Simpson's Evenness	0.39	0.38
Good's coverage	> 98%	> 98%
Unweighted UniFrac	0.001	0.002

Table 8 - Alpha-diversity and Beta-diversity statistics of the rumen microbiota related to Period, Treatment and Period x Treatment. Significance determined at $p \leq 0.05$.

No significant Faith's phylogenetic diversity and Simpson's evenness were found for bacteria or archaea across treatments; however, we found a significant difference in the diversity indices for bacteria ($p = 0.006 < 0.05$; $p = 0.02 < 0.05$) and archaea ($p = 0.003 < 0.05$; $p = 0.02 < 0.05$) according to the period. The Good's coverage showed that less than 2% of the ASV's were not covered by the sequencing depth.

Beta diversity between the samples at four different timepoints during the trial was evaluated. In the unweighted UniFrac distances, the Principal Component Analysis (PCoA) showed that it was not possible to separate the microbial profiles across treatment. An ANOVA test showed no significant differences in the microbial community between the treatments, but a significant difference in the microbial community related to period and period × treatment was detected.

5. Discussion

The rumen microbiome performs several symbiotic functions that are essential for the survival of the host, especially those physiological functions that cannot be undertaken by the host itself (Cancino-Padilla *et* al., 2021). One of the central roles of the microbiome is to ensure the productivity and health of ruminants. However, external factors, such as the age of the host, diet, feed, and feed additives can affect the composition and diversity of the microbiota (Kim *et* al., 2011). The current study aimed to investigate, through Illumina Miseq sequencing technology, the effect of dietary supplementation of saturated and unsaturated fats on the rumen microbiome of young bull calves. Malmuthuge *et* al. (2015) reported a possible link between the gut microbiota and host health. Auffret *et* al. (2017) stated that the risk of dysbiosis is caused by an unbalance in the microbiome, due to dietary changes and modifications in the ruminal volatile fatty aid profiles. Therefore, the current study expected to find significant differences in the compositions of the fecal microbiomes driven by the fat supplementation.

Surprisingly, there was no significant effects of the fat supplementation on the composition of the fecal microbiome in any of the treatment groups. The lack of effect of the fat sources on the fecal microbiome composition may stem from the fact that the supplemented fats used in this study were based on by-pass fats. Same results were found by Nurzhanov *et*. al (2019), who used two different fat-containing supplements of by-pass fats based on palm oils with different fat contents. Nurzhanov *et* al. (2019) investigated the taxonomic structure of the rumen microbiome in calves, when feeding a fat supplement. In their study, a selection of 12 bulls aging 12 months were divided into three groups, with one group being the control. All groups were fed the same basic ratio of feedstuffs, which consisted of grass hay, corn silage, crushed barley, fodder syrup, and premix. In contrast, the calves used in this study were fed both MR and *ad libitum* starter feed and haylage. These findings suggest that by-pass fats do not alter the microbiome in significant ways, regardless of whether the fat supplements are offered to pre-weaned or post-weaned calves.

In general, the overall taxonomic classification of the fecal microbiome determined in this study agrees with what has been found in other studies looking into the rumen microbiome of cattle (Neves, 2020; Malmuthuge *et* al., 2015; Kim *et* al., 2011; Cancino-Padilla *et* al., 2021; Nurzhanov *et* al., 2019).

Malmuthuge *et* al. (2015) stated that changes in the relative abundance of bacteria belonging to *Firmicutes, Bacteroidetes,* and *Proteobacteria* have age-dependent variations led by the shifts of bacteria species caused by dietary changes and development of the rumen function. These findings could explain the significant effect of the period on the microbiome. Yet, Hu *et* al. (2020) and Wang *et* al. (2019) found that the relation between *Firmicutes* and *Bacteroidetes* is affected by an increased dietary energy level. BoviLM and Bovi85 had a higher fat content in the diet, but no significant effect of these two treatments on the changes in the microbiome was found. As explained previously, the lack of treatment effect could, be due to the nature of the fat sources (by-pass fats) since these types of supplements are easier for the rumen to ferment.

5.1 Taxonomy of microbiome

The most abundant phyla found in this study were Firmicutes (66.9%), Bacteroidetes (27.6%), Actinobacteriota (2.7%), and Proteobacteria (1.9%). These findings agree with similar studies of the composition of the gut microbiome in ruminants. For instance, Neves et. al (2020) collected the rumen fluid from 6 purebred bulls, which had been fed a forage-based diet containing alfalfa hay, corn silage, limestone, salt, and mineral. The rumen fluid was collected using a Geishauser oral probe at four time points (0, 80, 100 and 180 days) and some of the most abundant microorganisms in the phyla was found to be Bacteroidetes, Firmicutes, Proteobacteria, Spirochaetes, and Actinobacteria respectively. In this study, Spriochaetes was however not found to be one of the most abundant species in the microbiome, which could be due to the feed differences, as the calves in this study had a MR-based diet. Furthermore, Neves et. al found a higher relative abundance of Bacteroidetes (46.3%) than that found in this study (27.6%). A reason for these differences could be the differing ages and diets of the animals used in each study. Another explanation could be the different sequencing methods. Neves *et* al (2020) used metatranscriptomics, whereas this study used 16S amplicon. Kim *et* al. (2011) who investigated differences between sampling methods, found that the results of the composition in the microbiome can differ depending on factors such as the sampling methods as well as the diet. Sampling could also explain the differences in the abundances and compositions of microbiomes between and across studies.

Malmuthuge *et* al. (2015) discussed the potential strategies that may be used to manipulate the early microbiome of calves to improve both production and health of newborn calves. A key information they found in their review is that the abundance of Bacteroidetes increases with the age of the host. They explained that the increase in Bacteroidetes is related

to shifts of the diet from milk to fibrous diet. This dietary change could explain the findings in this study, where the relative abundance of Bacteroidetes increased from P_0 to P_3 in all groups.

The relative abundance of Bacteroidota and Firmicutes decreased or increased in this study, suggesting a possible relationship between these taxa. Cancino-Padilla *et* al. (2021), investigated the long-term effect (63 days) of lipid supplementation on the rumen microbiome of dairy cows. The treatments were as follows; a control basal diet with no lipid supplementation, a basal diet containing hydrogenated vegetable oil as a saturated FA source, and a basal diet containing olive oil as an unsaturated FA source. The authors did not find significant effects of the lipid supplementations on the microbiome, agreeing with this study. But when they investigated the temporal changes between day 21 and day 63, the same relationship found in this study between Bacteroidetes and Firmicutes was detected in their research.

In the order rank, the most abundant species were Bacteroidales (27.52%), Oscillospirales (24.87%), Lachnospirales (22.83%), and Lactobacillales (7.24%), accounting for 82.46% of the whole order level. Only Bacteroidales and Lactabacillales were found to be significant with the period. Malmuthuge *et* al. (2017) noted that the fecal microbiota of calves fed MR had a higher prevalence of Clostriadales and Bacteroidales than calves fed a pasteurized waste milk. In this study, we fed the calves MR and Bacteroidales were the most dominant order found in the fecal microbiome. Mao *et* al. (2015) found that Bacteroidales are more abundant in the large intestine, supporting our results.

The study of Lactobacillales in the fecal microbiome can monitor the health status of the calves. Hall *et* al. (2017) investigated how feeding weaned beef calves Selenium-enriched alfalfa hay for 8 weeks could alter the nasal microbiota. In their study they used 17 heifers and 28 steer calves, which were sorted into three treatment groups. Samples were taken by nasal swabs and blood were sampled for selenium analyses. For the microbial DNA, Illumina MiSeq were used in the analysis. Hall *et* al. (2017) observed that bacterial orders such as Lactobacillales were increased in healthy control calves. In this study, the relative abundance of Lactobacillales decreased with the period, which could indicate a decreasing health of the animals when considering the findings of Hall *et* al. (2017). It should be noted that Hall *et* al. (2017) investigated the nasal microbiota, while this study investigated the fecal microbiota.

When looking at the family level, the most abundant microorganisms were Lachnospiraceae (22.7%), Ruminococcaceae (16.3%), Prevotellaceae (11.4%), and

Lactobacillaceae (6.9%); these findings agree with the results found by Neves (2020), Malmuthuge *et* al. (2015), and Nurzhanov *et* al. (2019). For Bovi85, it was noted that when the abundance of Lachnospiraceae decreased the abundance of Ruminococcaceae increased, agreeing with the findings of Nurzhanov *et* al. (2019). The same pattern was not found for Control or BoviLM groups likely because the fat content in the Bovi85 treatment (85%) was closer to the fat content (86.9%) of the supplement used by Nurzhanov *et* al. (2019). In contrast, the control group was not fed fat. Furthermore, the fat content in BoviLM was as high as 90%, which might have had an impact on the microorganism's composition, as bacteria are sensitive to the oil supplementations (Zened *et* al., 2011).

At the genus level the most abundant microorganisms were *Blautia* (11.3%), *Faecalbacterium* (10.9%), *Alloprevotella* (8%), *Bacteroides* (6.3%), and *Lactobacillus* (4%). These findings agree with the most abundant genera found in studies done by Neves, 2020 and Malmuthuge *et* al., 2015, although this study did not find a similar relative abundance percentage as those found by Neves (2020). These differences in the relative abundance f the genera could be due to the sampling and sequencing methods (amplicon vs metatranscriptomics) used in each study. Also, *Blautia* was found to be the most dominant microorganism in this study which is not in agreement with Maynou *et* al., 2019 or Neves, 2020.

5.2 The most abundant microorganisms

Mao *et* al. (2015) analyzed the bacterial communities in ten distinct GIT sites in six Holstein dairy cattle aged five years old. The cows used in the study were between 140 to 189 days in lactation. The diets were a total mixed ration given *ad libitum* formulated to either meet or exceed the energy requirements. They found that the digesta-associated microbiota of the forestomach exhibits a greater relative abundance of Firmicutes and Bacteroidetes, which were also the microorganisms with the highest relative abundance in this study. Mao *et.* al. (2015) explained that taxa belonging to Firmicutes can be detected widely in the rumen and play an important role in the degradation of starch and fiber. In this study, Firmicutes was the phyla with the highest relative abundance in all groups because; our experimental animals had *ad libitum* access to both starter feed and haylage. Referring to Mao *et* al (2015) it is of great importance that Firmicutes are present in the GIT, as these microbes are involved in the fermentation of starch and fiber. Mao *et* al (2015) also states that Bacteroidetes have in previous studies been found to be significantly less abundant in the digesta-associated

microbiota of the small and large intestine, but in the rumen, it is prominent and mainly composed of the genus Prevotella. However, in this study, Prevotella was affected by the treatments and was only the 16th most abundant genus. On the other hand, Bacteroidota was found to be the second largest group in the phylum ranking, since we collected the samples directly from the rectum. Mao *et* al. (2015) found that Bacteroidetes were the second most prevalent phyla in regions of the GIT such as the colon and rectum.

On the family level, we observed a significant effect of the period on the relative abundances of Prevotellaceae, Lactobacillaceae and Oscillospiraceae.

Li and Guan (2017) applied a total RNA-based metatranscriptomics to show the linkage between the active rumen microbiome and feed efficiency in beef cattle. In their study, twenty beef steers were used, and sampling was performed from the rumen environment. Li and Guan (2017) explained that Prevotellaceae utilize various substrates such as starch, protein, peptides, hemicellulose, and pectin to generate a wide range of end products, mainly short-chain fatty acids, including acetate, succinate, and propionate. In this study, there was an increase in the relative abundance of Prevotellaceae during the periods of sampling, which can be explained by the statements of Li and Guan (2017), although we did not obtain sufficient information to support their hypothesis – e.g., we did not measure short-chain volatile fatty acids in the rumen fluid.

Li and Guan (2017) discovered that Lactobacillaceae have a higher relative abundance in less efficient animals in feed-related traits such as dry matter intake, average daily gain, and feed conversion ratio in steers and heifers. In this study there was a decrease in the relative abundance of Lactobacillaceae over the periods of collection for the Control and BoviLM groups. This reduction in the relative abundance of Lactobacillaceae agrees with the results seen for the effect of lipids on the average daily gain observed by Pedersen (2022), where Bovi85 seemed to have a lesser efficient growth rate than that of the Control and BoviLM groups. According to Li et. al (2018), Oscillospiraceae are mainly butyrate producers and can use glycans as a source of energy. In this study the relative abundance of Oscillospiraceae increased with the period, suggesting that a greater source of energy could have been used in the form of glycans by the Oscillospiraceae microbes.

On the genus level there was a significant effect of the period on the relative abundance of *Alloprevotella, Lactobacillus*, *UCG-005*, and *HT002*.

Li *et.* al (2018) found that *Alloprevotalla* function in piglets is to produce succinate and acetate to improve the gut barrier against inflammatory agents. Also, the abundance of *Alloprevotella* seemed to decrease in weaned piglets. In this study, the relative abundance of *Alloprevotella* increased with the period. If we assume that *Alloprevotella* function is the same in cattle as in pigs, its increased abundance in the fecal microbiome is possible associated with the increased diarrhea indices observed by Pedersen (2022), who worked with the same group of calves as us.

Malmuthuge *et* al. (2015) has found that *Lactobacillus* can influence calf health, and if given during the first week of the calf life, it will lead to a decreased diarrhea incidence and an improved weight gain. This effect of *Lactobacillus* has been observed mostly in pre-weaned calves than in weaned calves, which indicates that *Lactobacilla* is not as effective to decrease diarrhea when the microbiome is already established. In this study, the relative abundance of *Lactobacilla* decreased with the period, which would agree with the assumption for the increased relative abundance of *Alloprevotella*. However, more researched is warranted to investigate the use of *Alloprevotella* as a biomarker to monitor the health status of beef calves from direct sampling of the fecal microbiome.

6. Conclusion

No significant effect of the different lipid supplements on the rumen microbiome of beef calves was found in this study. However, a significant effect of the period was found on Firmicutes and Bacteroidetes at the phylum level. In a similar fashion, a significant effect of the period was found on Bacteroidales and Lactobaccillales at the order level. As for the families, a significant effect of the period was found on Prevotellaceae and Lactobacillaceae, and at the genus level, there was found a significant effect of the period on *Alloprevotella, Lactobacillus*, *UCG-005*, and *HT002*.

Overall, it was concluded that the supplement of either saturated (BOVI LM) or unsaturated lipids (BOVI 85) did not have a significant effect on the microbiome of young bull calves, showing that these fat sources are safe to use from the microbiome perspective. It should be noted that the lipids used in this study were by-pass fats, and this may be the main reason behind the lack of significant effect of the fat supplements on the fecal microbiome. Further investigations with other fat sources (with different degrees of fatty acid saturation) should be conducted to better understand how the lipid supplementation to milk replacer for young beef calves affect the gut microbiome.

7. Perspectives

To follow up the investigations of this study, it would be interesting to increase the amount of supplemented lipids in the diet. Future research could follow up on previous studies that showed the benefits of a higher energy intake for beef cattle, especially the positive effects of fat supplementations on the growth rate and health.

Furthermore, to achieve a more accurate understanding of the gut microbiome it will be necessary to use direct sampling techniques (e.g., tubes, probes, etc.) to collect rumen fluid, as previous studies have shown that the composition of the microbiota in ruminants differs throughout the GIT.

This study was part of a larger project, and, therefore, this thesis is only a piece of the whole picture. The main goal of the whole project was to replicate the daily management of beef calves and to investigate whether we could improve the production system (e.g., with calf performance) by supplementing either saturated or unsaturated fats to the milk replacer. In this part of the project, the goal was to investigate whether the supplemented lipids would cause any significant changes in the fecal microbiome of beef calves, as the diet influences the gut microbiome composition and diversity. It was, however, found that the supplemented lipids (saturated and unsaturated) used in the study did not affect the fecal microbiome, confirming our expectations that the amount of added lipids would not cause health problems to the animals, though not agreeing with our hypothesis that a significant effect would be found on the microbiome.

The addition of extra lipids to the diet of beef calves could be beneficial to health and be recommended to be added during the winter or the summer. In both periods it may be beneficial for the calves to have extra energy for thermoregulation, as cold weather could cause cold stress, while warm weather could cause heat stress. When lipids are added to the diet in these situations (cold and warm weather), there will be more energy for thermoregulation without compromising energy the available for the growth of the animal.

In the performance experiment described by Pedersen (2022), the saturated fat caused a slight increase in body weight and body measures when compared to the control group. Although there was not a significant effect of the two fat supplements on the performance of the calves, the addition of saturated fat increased (at least numerically) the average daily gain when compared to unsaturated lipids.

Further investigation would be to investigate if the addition of saturated and unsaturated lipids to the diets can have a significant effect on the microbiome when added to the starter feed

as well as the milk replacer. Also, it could be investigated if there are other differences in the gut microbiome when lipids are added after weaning the calves.

It is important to investigate the gut microbiome of ruminants as it could be significantly altered through dietary manipulations. Scientists are currently working to create dietary interventions to decrease enteric CH_4 production and improve the overall health of the ruminants. Finally, the research focus must shift towards the early days of the ruminant life, as this period is essential for the immune system development. If diet manipulations can improve the immune system development of young calves, then the overall production system will be benefited because there will be a decrease in the production costs associated with feeding and health (e.g., reduction in the use of antibiotics).

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Appendix

Appendix 1. Calculation of supplemented lipids in MR

Bovi 85 supplement

Milk Replacer: 140 g L^{-1}, $x + y = 140$, $y = 140 - x$

$$0.966 * 0.157 * x + 0.97 * 0.897 * y = 0.21(0.966x + 0.97y)$$

$$\rightarrow\ 0.152x + 0.870y = 0.203x + 0.204y$$

Replacing $140 - x$ in place of y:

$$0.152x + 0.870(140 - x) = 0.203x + 0.204(140 - x)$$

$$\rightarrow 0.152x + 121.8 - 0.870x = 0.203x + 28.56 - 0.204x$$

$$\rightarrow 93.24 = 0.717x \rightarrow x = 130.0g$$

$$\rightarrow y = 10.0g$$

Checking for fat content:

$$0.966 * 0.157 * 130.0 + 0.97 * 0.0897 * 10 = 19.7 + 8.7$$

$$= 28.4$$

$$DM = 0.966 * 130 + 0.97 * 10 = 135.28 \cong 135.3\ g\ DM$$

$$\rightarrow [fat] = \frac{28.4}{135.3} * 1000 \cong 210 g/kg^{-1}\ DM$$

$$Milk\ Replacer\ (x) = 130 g/L^{-1} \rightarrow 3 * 130 = 390g$$

$$Bovi\ 85\ (y) = 10 g/L^{-1} * 3 = 30g$$

Therefore, for the Bovi 85 group, the calves were each given 30g of Bovi 85 two times a day meaning that each calf was given 60g/day.

Bovi LM supplement

Milk Replacer: 140 g L^{-1}, $x + y = 140$, $y = 140 - x$

$$0.966 * 0.157x + 0.99y = 0.23(0.966x + 0.99y)$$

$$\rightarrow 0.152x + 0.99y = 0.203x + 0.208y$$

Replacing $140 - x$ in place of y:

$$0.152x + 0.99(140 - x) = 0.203x + 0.208(140 - x)$$

$$138.6 - 29.12 = 0.833x$$

$$x = 131.4g \rightarrow y = 140 - 131.4 = 8.6g$$

Checking for fat content:

$$0.966 * 0.157 * 131.4 + 0.99 * 1 * 8.6 = 28.44g\ fat$$

$$DM = 0.966 * 131.4 + 0.99 * 8.6 = 135.45\ g\ DM$$

$$[fat] = \frac{28.44}{135.45} * 1000 = 210g/kg^{-1}\ DM$$

$$Milk\ Replacer = 3 * 131.4g/L^{-1} = 394.2g$$

$$Bovi\ LM = 3 * 8.6g/L^{-1} = 25.8g$$

Therefore, for the Bovi LM group, the calves were each given 25.8g two times a day, meaning that each calve was given 51.6 g/day.