FACULTY OF BIOSCIENCE ENGINEERING

# METATAXONOMIC ANALYSIS OF THE MICROBIAL COMMUNITY IN THE RUMEN AND FAECES OF DAIRY COWS DURING THE PERIOD AROUND CALVING IN RELATION TO HAEMATOLOGICAL BIOMARKERS

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# List of Abbreviations

| | |
|---|---|
| A:G | Albumin/globulin |
| ANOVA | Analysis of Variance |
| APR+ | Acute phase response plus diseased cows |
| ASV | amplicon sequence variants |
| BHB | Beta-hydroxybutyrate |
| DMI | Dry matter intake |
| GH | Growth hormone |
| GHR | Growth hormone receptor |
| GIT | Gastrointestinal tract |
| GPx | Glutathione peroxidase |
| GSH | Reduced glutathione |
| GSSH | Oxidized glutathione |
| Hp | Haptoglobin |
| IGF-I | Insulin-like growth factor |
| LPS | Lipopolysaccharides |
| MDA | Malondialdehyde |
| NEB | Negative energy balance |
| NEFA | Non esterified fatty acids |
| Non-APR | No acute phase response |
| ORAC | Oxygen radical absorbance capacity |
| PCoA | Principal coordinate analysis |
| PERMANOVA | Permutational multivariate analysis of variance |
| PG | Propylene glycol |
| ROS | Reactive oxygen species |
| SAA | Serum amyloid A |
| SARA | Subacute ruminal acidosis |
| SCFA | Short chain fatty acids |
| SOD | Superoxide dismutase |
| TAC | Total antioxidant capacity |
| VFA | Volatile fatty acids |

## Summary

The transition period, three weeks before and three weeks after calving, is a stressful time for dairy cows because their bodies undergo physiological and metabiological changes and need to provide energy for the onset of milk synthesis. Some cows go through this period less smoothly than others and consequently may experience imbalances and metabolic disorders such as mastitis, SARA, ketosis, etc. Therefore, there is likely an inter-animal difference in the adaptation to the metabolic changes among dairy cows during the transition period. There are, of course, various factors that contribute to each animal's uniqueness including genetics, management conditions, etc. However, this research focused on the microbial community (bacteria, archaea, protozoa and fungi) in the rumen and hindgut, using faecal samples as a proxy for the latter. This study tried to see if there is a link between the microbial composition of the cow's gastrointestinal tract, specifically the microbial composition of the rumen and hindgut, and the health status of the cow specifically metabolic and inflammatory status during the transition period.

The inflammation cluster was divided into two groups: acute phase response plus diseased animals (APR+) and no acute phase response (non-APR). Additionally, the metabolic cluster was divided into a LoBHB and SloBHB group. The LoBHB group included cows that navigated the transition period with minimal metabolic imbalances, while the SloBHB group exhibited slightly greater imbalances accompanied by an adaptive response. In the inflammation cluster, a few rumen bacteria were increased in the APR+ group, while some faecal archaea (day -7 and +21) were more abundant in the non-APR group. In the metabolic cluster, two faecal bacterial species (day +21) were more abundant in the LoBHB group, whereas one faecal archaeal species (day +21) and several faecal fungal taxa (day -7 and +21) were increased in the SloBHB group. There are some possible explanations for the presence of these microorganisms. They may benefit from the changing state of the body during the transition period. Some might help maintain balance, while others could potentially contribute to an imbalance.

In this thesis no differences were observed in alpha diversity between the metabolic and inflammatory clusters for all the samples except for the fungal community in the faeces samples (day -7) between the inflammation clusters. Additionally, the beta diversity indices of rumen bacteria, archaea, and protozoa differed or tended to differ between the inflammation clusters, while differences in rumen and faecal (day +21) fungi were observed between the metabolic clusters. In the archaeal community, there was also a tendency for a difference in faeces samples (day -7) between the inflammation clusters. Differences in rumen and faecal (day +21) fungal composition were observed between the metabolic clusters. In the archaeal community, there was also a tendency for a difference in faeces samples (day -7) between the inflammation clusters. Only, a limited number of indicator genera, phyla, or ASVs could be identified within the rumen bacterial and faecal archaeal communities, differentiating between the immunological clusters, while no microbial indicator genus or ASV could be identified in the rumen to distinguish between metabolic clusters. In the faecal samples 21 days post-partum, indicator bacterial, archaeal, and fungal ASVs or genera were identified as differing between metabolic clusters.

But, overall diversity differences, as well as the identification of indicators to differentiate between the various health clusters, were limited. Several factors might have contributed to this, such as the use of samples from two different farms. Additionally, in this study, clusters were identified that exhibited statistically significant differences in immunological (inflammatory clusters) and metabolic parameters (metabolic clusters). However, literature suggests that the differences between the inflammation clusters may be relatively modest. In the metabolic cluster, cows in the SloBHB group may not be metabolically impaired but are instead adapting to higher body fat mobilization.

## Samenvatting

De transitieperiode, drie weken voor en drie weken na het kalven, is een stressvolle periode voor melkkoeien omdat hun lichamen fysiologische en metabole veranderingen ondergaan en energie moeten leveren voor de start van de melkproductie. Sommige koeien doorlopen deze periode minder vlot dan andere en kunnen daardoor onevenwichtigheden en metabole aandoeningen zoals mastitis, SARA, ketose, enz. ervaren. Daarom is er waarschijnlijk een verschil tussen dieren in de aanpassing aan de metabole veranderingen tijdens de transitieperiode. Er zijn uiteraard verschillende factoren die bijdragen aan de uniciteit van elk dier, zoals genetica, management, enz. Dit onderzoek richtte zich echter op de microbiële gemeenschap (bacteriën, archaea, protozoa en fungi) in de pens en de endeldarm, waarbij fecale monsters werden gebruikt als een proxy voor de laatste. Deze studie probeerde te onderzoeken of er een verband bestaat tussen de microbiële samenstelling van het maagdarmkanaal van de koe, specifiek de microbiële samenstelling van de pens en de endeldarm, en de gezondheidstoestand van de koe, met name de metabole en inflammatoire status tijdens de transitieperiode.

Het inflammatiecluster werd verdeeld in twee groepen: acute fase respons plus zieke dieren (APR+) en geen acute fase respons (non-APR). Daarnaast werd het metabole cluster onderverdeeld in een LoBHB- en een SloBHB-groep. De LoBHB-groep bestond uit koeien die de transitieperiode doorliepen met minimale metabole onevenwichtigheden, terwijl de SloBHB-groep iets grotere onevenwichtigheden vertoonde, vergezeld van een adaptieve respons. In het inflammatiecluster waren enkele pensbacteriën verhoogd in de APR+ groep, terwijl enkele fecale archaea (dag -7 en +21) meer voorkwamen in de non-APR-groep. In het metabole cluster waren twee fecale bacteriesoorten (dag +21) meer aanwezig in de LoBHB-groep, terwijl een fecale archaeale soort (dag +21) en verschillende fecale fungi (dag -7 en +21) verhoogd waren in de SloBHB-groep. Er zijn enkele mogelijke verklaringen voor de aanwezigheid van deze micro-organismen. Ze kunnen profiteren van de veranderende toestand van het lichaam tijdens de transitieperiode. Sommigen kunnen helpen de balans te behouden, terwijl anderen mogelijk bijdragen aan een onevenwicht.

In deze thesis werden geen verschillen in alpha-diversiteit waargenomen tussen de metabole en inflammatoire clusters voor alle monsters, behalve voor de fungi in de fecale monsters (dag -7) tussen de inflammatieclusters. Daarnaast verschilden of neigden de beta-diversiteitsindexen van pensbacteriën, archaea en protozoa tussen de inflammatieclusters, terwijl verschillen in pens- en fecale (dag +21) fungi werden waargenomen tussen de metabole clusters. In de archaeale gemeenschap was er ook een neiging tot een verschil in fecale monsters (dag -7) tussen de inflammatieclusters. Verschillen in pens- en fecale (dag +21) schimmel-samenstelling werden waargenomen tussen de metabole clusters. In de archaeale gemeenschap was er ook een neiging tot een verschil in fecale monsters (dag -7) tussen de inflammatieclusters. Slechts een beperkt aantal indicatorgenera, -phyla of -ASV's konden worden geïdentificeerd binnen de pensbacteriële en fecale archaeale gemeenschappen, die het verschil maakten tussen de immunologische clusters, terwijl geen microbiële indicatorgener of ASV in de pens kon worden geïdentificeerd om de metabole clusters te onderscheiden. In de fecale monsters 21 dagen na de bevalling werden indicatorbacteriële, archaeale en fungale-ASV's of -generra geïdentificeerd die verschilden tussen de metabole clusters.

Maar de algemene diversiteitsverschillen, evenals de identificatie van indicatoren om te differentiëren tussen de verschillende gezondheidclusters, waren beperkt. Verschillende factoren kunnen hieraan hebben bijgedragen, zoals het gebruik van monsters van twee verschillende boerderijen. Verder, werden in deze studie clusters geïdentificeerd die statistisch significante verschillen vertoonden in immunologische (inflammatoire clusters) en metabole parameters (metabole clusters). De literatuur suggereert echter dat de verschillen tussen de inflammatieclusters relatief bescheiden kunnen zijn. In het metabole cluster lijken de koeien in de SloBHB-groep niet metabool aangetast, maar passen zij zich eerder aan aan een verhoogde mobilisatie van lipiden.

# INTRODUCTION

The rising global demand for dairy products has necessitated a simultaneous increase in milk production. Historically, this increase was primarily due to genetic selection and improved nutrition. It is well known that the period around calving is a huge metabolic challenge for dairy cows. High-yielding dairy cows, due to selection pressures, face additional strain in adapting to early lactation. During lactation, several factors influence milk production, including feed intake, blood glucose levels, cell activity, protein, and fat synthesis, as well as metabolic and regulatory pathways for essential nutrients.

In early lactation, dairy cows typically experience a period of negative energy balance (NEB). Indeed, the very high energy demands for milk production during this period often exceed the energy intake from the cow's diet. As a result, the cow needs to mobilize its body reserves to make up for the energy deficit. Lipolysis is a natural, hormone-driven state, commonly observed in mammals at the onset of lactation. However, the NEB in modern high-genetic-merit dairy cows with high milk yields is considered the result of 'stretching' this natural phenomenon during the genetic selection process. While some cows can navigate this period with minimal issues, others face major imbalances and encounter conditions like for example ketosis, subacute ruminal acidosis, and uterine infections. Consequently, they commence their lactation in an unfavourable state. This may cause a reduction in profits for producers as well as veterinary and culling expenses. This period is marked by significant physiological changes and metabolic challenges which can lead to oxidative stress, metabolic imbalance, and inflammation.

The health status of the cow can be determined using some key oxidative, inflammation and metabolic parameters in the blood. Oxidative stress can occur when free radicals or other reactive oxygen species, generated during metabolic processes or infections are not adequately neutralized and cleared away. Metabolic stress occurs when metabolic homeorhesis is disrupted. During the transition period, dairy cows are facing tremendous changes in physiological status and failed adaptation may result in metabolic stress. The combination of prioritizing energy and nutrients for milk production, along with oxidative and metabolic stress, can result in insufficient resources to adequately support the immune system. This can lead to disrupted immune function and increased inflammation.

It is well-known that host-microbiome interaction plays an important role in an individual's metabolism and overall health status (Sanz et al., 2010; Shreiner et al., 2015). A balanced microbiome is crucial for ruminants as healthy rumen microbes contribute to and are essential for optimal rumen function, digestion, and consequently, good metabolism. Imbalances in the microbiome during the transition period, often result from dietary changes, metabolic stress, or disease. In addition, gastrointestinal microbial composition can also influence an animal's ability to handle metabolic stress during the transition period. However, the mutual relationship between the dairy cow's health status in early lactation and the microbial population in the gastrointestinal tract is largely unknown.

This study investigates whether a link can be established between the microbial composition of the cow's gastrointestinal tract, specifically the microbial composition of the rumen and hindgut, and the health status of the cow specifically metabolic and inflammatory status. Additionally, specific microbes serving as indicators were identified for the above-mentioned health status. Two types of samples were used in this study: rumen samples collected at 21 days after calving and faecal samples collected 7 days before and 21 days after calving. Since obtaining rumen samples are often invasive, this study explored whether faecal samples could serve as an alternative in studying the health status of the dairy cows during the transition period. Data obtained from two different dairy farms, where animals were monitored during the period around calving, were used in this study. This includes metabolic, and inflammatory parameters in the blood that can determine the health status of the animals. In this study, we investigated the relationship between the microbial communities in the rumen and faeces and the health status of the animals, as determined by blood parameters. The microbial communities were analysed using metataxonomic analysis from DNA extracted from rumen and faecal samples.

# 2 LITERATURE STUDY

## 2.1 Microbial community in the ruminant's gastrointestinal tract

(Flint & Bayer, 2008).The cow's diet is predominantly plant-based. Lignocellulose-rich plant material is a major component of the diet of herbivorous mammals. Lignocellulose is composed of two types of polymers: cellulose, and hemicellulose. This material is largely undegradable by mammalian. Thus, its breakdown within the gut is accomplished by communities of resident microorganisms that live in symbiotic or mutualistic association with the host. The rumen microbiota mainly consists of bacteria ($10^{10}$-$10^{11}$ cells/ml), protozoa ($10^{4}$-$10^{6}$ cells/ml), archaea ($10^{6}$-$10^{8}$ cells/ml), and fungi ($10^{3}$-$10^{6}$ zoospores/ml) (Matthews et al., 2019). They can produce volatile fatty acids (VFA), microbial proteins and vitamins for the host. VFA are the main energy source for the host. The digestive capability of the dairy cow is directly proportional to the existing rumen microflora activity (Flint & Bayer, 2008).

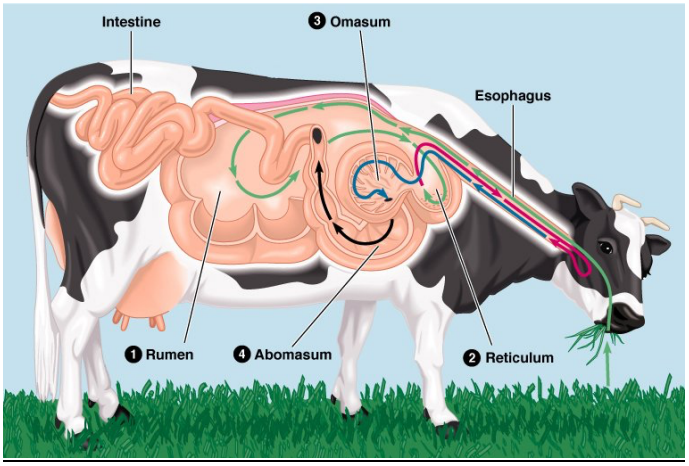


***Figure 1.*** *The ruminant digestive tract (Addison Wesley Longman Inc, 1999).*

(L. Liu et al., 2023; Stevens & Hume, 1998).The gastrointestinal tract (GIT) of ruminants can be divided into four compartments between oesophagus and duodenum: rumen, reticulum, omasum, and abomasum. The cow has three forestomaches consisting of: rumen, reticulum, omasum. These comprise nonglandular epithelium. They function as chambers for microbial digestion and fluid absorption. As seen in figure 1 plant material is first ingested into the rumen (1), where it is processed mechanically and exposed to rumen microbes for foregut fermentation. The reticulum (2) allows the animal to further regurgitation before passing more finely processed cud to the omasum (3). The abomasum (4) is the "true stomach", this one contains glandular mucosa for production and secretion of digestive juices. The abomasum contains the host-derived digestive enzyme that can break down the bacteria to release nutrients (Niehaus, 2009). After passing through the abomasum, the food proceeds into the small intestine. This organ is divided into three main compartments: the duodenum, jejunum, and ileum. The presence of villi creates a large surface area in the intestinal wall, thereby increasing the absorption capacity of nutrients. Subsequently, the food passes by the cecum before entering the colon. Any remaining food residues progress to the hindgut, where additional microbial fermentation occurs. The result of this digestive process, known as faeces, is ultimately excreted through the rectum. Around 30% of the fibrous material, including xylose and hemicellulose, undergoes fermentation and decomposition in the hindgut (L. Liu et al., 2023; Stevens & Hume, 1998).

### 2.1.1 Bacteria

(Hua et al., 2022; Jami & Mizrahi, 2012).Bacteria are the most abundant and diverse group of rumen microorganisms (~95% of total microbiota). Carbohydrates, such as starch and cellulose, constitute the primary energy source in the diets of dairy cows. Consequently, cellulolytic and amylolytic bacteria play a crucial role in supporting the carbohydrate breakdown. Cellulolytic bacteria (e.g. *Ruminococcus* and *Fibrobacter* spp.) are pivotal contributors to the breakdown of cellulose, an otherwise indigestible component for the host. On the other hand, amylolytic bacteria (e.g. *Succinivibrio* and *Selenomonas* spp.) are responsible for the degradation of starch (Hua et al., 2022; Jami & Mizrahi, 2012). Besides the amylolytic and cellulolytic bacteria, there is another subgroup present in the GIT called the proteolytic bacteria (e.g. *Prevotella* and *Bacteroides*). They are responsible for the breakdown of proteins. In a study by Pitta et al (2016), it was concluded that the rumen microbiome was dominated by the following phyla: Bacteroidetes (70%), Firmicutes (15 – 20%) and Proteobacteria (7%). Considering that this is an estimation, it's important to note that the bacterial composition of the host varies based on diet composition, genetics, and age (Pitta et al., 2016).

The available substrate present for the microbiota in the rumen is different from that in the hindgut. This can result in a distinct bacterial composition in the rumen compared to that in the hindgut. In a study by Liu et al (2016), the investigation aimed to discern potential differences in the bacteria between the rumen and the hindgut. The following results were observed, a higher abundance of Firmicutes and Verrucomicrobia at the phylum level in faeces compared to ruminal content. In contrast, ruminal content showed a greater abundance of Bacteroidetes, Chloroflexi, Lentisphaerae, Tenericutes, and unclassified bacteria. At the genus level, the abundance of *Clostridium*, *Turicibacter*, *Cellulosilyticum*, *Akkermansia*, *Treponema*, and unclassified Peptostreptococcaceae was greater in faeces compared to ruminal content. Meanwhile, lower percentages were observed for the following genera in faeces: *Ruminococcus*, *Acetitomaculum*, *Mogibacterium*, *Prevotella, Saccharofermentans*, *Succiniclasticum*, unclassified Christensenellaceae, unclassified *Bacteroidales*, unclassified *Rikenellaceae*, unclassified *Lachnospiraceae*, unclassified *Prevotellaceae*, unclassified *bacteria*, and unclassified *Mollicutes* (Liu et al., 2016).

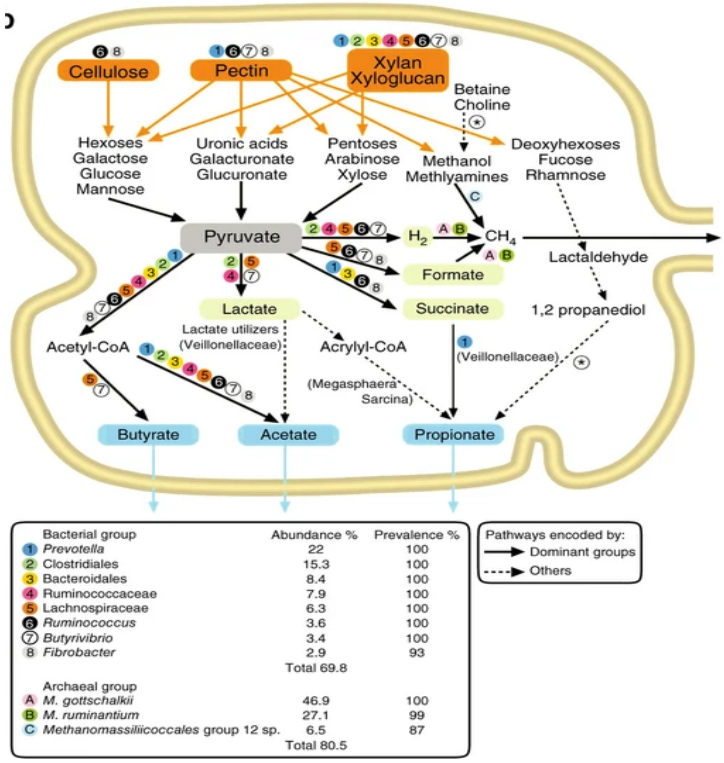


***Figure 2.*** *The degradation and metabolism of carbohydrates by the dominant bacterial and archaeal groups in the rumen* (Seshadri et al., 2018)

#### 1.1.1.1 Phylum Bacteroidetes

*Prevotella* is the dominant genus within the phylum Bacteroidetes in the rumen microbiome. Characterized as non-spore-forming gram-negative species, *Prevotella* exhibits a primarily saccharolytic nature. This microbe showcases an impressive capacity to process an extensive array of proteins and polysaccharides. Notably, one of its notable fermentation byproducts is propionate. The significance of propionate lies in its unique quality of sequestering hydrogen, consequently reducing the availability of this element for methane production. This property can be interesting to mitigate methane emissions (Betancur-Murillo et al., 2023). Furthermore, its glucogenic nature is of major importance for gluconeogenesis in the liver, which is the main glucose source for ruminants. Another group of rumen bacteria, belonging to the same phylum and regarded as one of the core rumen bacteria, is the unclassified Bacteroidales (Henderson et al., 2015).

#### 1.1.1.2 Phylum Firmicutes

(Hungate & Bryant, 1964; Willems & Collins, 2015).The phylum Firmicutes includes Gram-positive bacteria. This phylum includes many rumen cellulolytic bacteria. Genera *Butyrivibrio*, and *pseudobutyrivibrio* belonging to this phylum can break down proteins and, furthermore, exhibit the ability to utilize various hemicelluloses and xylans-types of plant fibers (Hungate & Bryant, 1964; Willems & Collins, 2015). The genus *Ruminococcus* falls within the same phylum and is another important rumen bacteria. *Ruminococcus* species can degrade cellulose and cellobiose but cannot utilize glucose or other sugars. The primary end-products of this degradation include acetate, ethanol, formate, lactate, hydrogen, and $CO_2$, with various combinations and proportions as the major outcomes. *Ruminococcus albus* is generally more prevalent than *Ruminococcus flavefaciens*. Remarkably, *Ruminococcus albus* produces ethanol, whereas *Ruminococcus flavefaciens* produces succinate (Hua et al., 2022).

#### 1.1.1.3 Phylum Proteobacteria

(Bryant & Small, 1956; Strobel, 1992)The genus *Succinivibrio* belongs to this phylim are gram-negative bacteriaa. In a study of Henderson et al. (2015), it was observed that animals receiving concentrate-rich diets showed a higher abundance of *Succinivibrio*. Based on previous research, it is likely that these organisms are major producers of propionate and its precursor, succinate. (Bryant & Small, 1956; Strobel, 1992). Consequently, they contribute to the elevated levels of propionate generated from concentrate-rich diets. (Henderson et al., 2015). This phylum also includes a wide variety of pathogenic genera such as *Escherichia coli*. In studies by Mu and Xue, a higher abundance of Proteobacteria in the rumen was observed in high-producing dairy cows compared to low-producing cows (Mu et al., 2019; Xue et al., 2019).

#### 1.1.1.4 Phylum Fibrobacteres

Fibrobacteres is not a dominant phylum in the rumen. However, an important fiber-degrading bacterium *Fibrobacter* belongs to this phylum. In a study by Henderson et al. (2015), *Fibrobacter* abundances were compared across various ruminant species, revealing significantly higher levels in bovines than in deer, sheep, or camelids. This observation suggests that *Fibrobacter* is favored in the bovine rumen and likely plays a crucial role in the degradation of plant fiber in cattle (Henderson et al., 2015).

### 2.1.2 Archaea

(Janssen & Kirs, 2008)Methanogens are an important subgroup of archaea present in the rumen and are obligate anaerobic. Methanogens are organisms that produce methane as a metabolic by-product (also called methanogenesis). Two groups of methanogens are present in the rumen: hydrogenotrophic methanogens and methylotrophic methanogens. The fermentation end-products, including $H_2$, $CO_2$, methanol, and methylamines, are not used by the host animal, but provide substrates for rumen hydrogenotrophic and methylotrophic methanogens leading to atmospheric $CH_4$ emissions. Methanogens that utilize acetate as substrate are called acetotrophic (Boone et al., 1993). It has been indicated that they grow too slowly on this substrate to persist in the rumen. Therefore, acetotrophic methanogens are suggested to be rare in the rumen (Janssen & Kirs, 2008). In a study of Henderson et al. (2015) it was reported that the two largest groups of methanogens in the rumen were members of the *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* clades. Alongside a *Methanosphaera* sp. and two Methanomassiliicoccaceae - affiliated groups, these five dominant methanogen groups comprised 89.2% of the archaeal communities (Henderson et al., 2015). It is remarkable that archaea are less diverse than bacteria in the rumen. This can be explained by the fact that archaea use a smaller range of substrates than bacteria.

Recent studies have unveiled a positive correlation between *Methanosphaera*, and *Methanomassiliicoccales* lineages with methane emissions. These less prevalent methanogens may play a more significant role in overall $CH_4$ production compared to more abundant $CO_2$-reducing methanogens (Pitta et al., 2022). In contrast (Shi et al., 2014) reported that the abundance of *Methanosphaera* was higher in low-$CH_4$ emitting animals compared to the high-$CH_4$ emitting animals.

### 2.1.3 Protozoa

(Mizrahi & Jami, 2018).Rumen protozoa have been characterized as degraders of plant fibers as well as non-structural polysaccharides such as starch (Williams et al., 2020). Between 20–45% of the amylolytic activities in the rumen have been attributed to protozoa. They depend on the host for food supply, but they also transform an array of plant and bacterium constituents into cell components and metabolites that are used by the host (Coleman, 2006; Hua et al., 2022). In comparison to other single-cell eukaryotic microorganisms, protozoa are relatively large (10 to >100 µm). Despite protozoa being somewhat less diverse than bacteria, they still occupy a considerable portion of microbial biomass in the rumen, approximately 10-50% of the total microbial biomass (Mizrahi & Jami, 2018). Protozoa also known for their predatory activity on prokaryotes in the rumen. It has been estimated that 0.1% of rumen prokaryotes are digested by the rumen protozoal population every minute (Coleman, 2006).

Two major groups of protozoa were observed in the mammalian gut, namely the flagellates and ciliates. Since the flagellates are often present only in low numbers, resulting in a relatively small contribution to digestion, the focus is mainly on ciliates (Dehority, 1986). Holotrichs (order: Vestibuliferida) and entodiniomorphs (order: Entodiniomorphida) are dominant among the ciliates that are often observed in the rumen of the cow (Andersen et al., 2023; Williams, 1986). *Entodinium* and *Epidinium* are the most abundant genera of protozoa observed in the rumen. They belong to the order Entodiniomorphida (Coleman, 2006).
Holotrich species belonging to the order Vestibuliferida, such as *Dasytricha* and *Isotricha*, are typically observed in lower abundances compared to entodiniomorphs. However, they are well-known for their ability to ferment soluble carbohydrates and generate volatile fatty acids (VFAs) and hydrogen (Williams, 1986).

### 2.1.4 Fungi

Fungi in the rumen were only discovered in 1975 by Orpin, before that they were mistakenly identified as flagellate protozoa (Orpin, 1975). They account for a small proportion (8-10%) of the rumen microbial biomass where they are involved in degrading structural carbohydrates by producing a wide range of enzymes (Hua et al., 2022). For example, they can produce following enzymes that are necessary for plant biomass decomposition: cellulases, xylanases, mannases, esterases, glucosidases and glucanases. These enzymes, also known as hydrolases, can degrade the plant cell wall. This allows them to release fermentable sugars that were previously inaccessible to surface-acting bacteria, colonize the robust plant structures, and ultimately, break down and weaken plant tissues, thereby reducing the size of plant particles (Fliegerova et al., 2015).

New gene datasets and metagenomic techniques have enabled the classification of fungi present in the gastrointestinal tract, belonging to the phylum *Neocallimastigomycota*, including one class, *Neocallimastigomycetes*, one order, *Neocallimastigales*, and a single family, *Neocallimastigaceae*. Within the family there are currently six genera: *Neocallimastix*, *Piromyces*, *Caecomyces*, *Orpinomyces*, *Anaeromyces* and *Cyllamyces* (Gruninger et al., 2014)

Generally, fungi tend to be more abundant in cattle that are fed a high-fiber diet. Drawing from various studies, the following overall conclusions can be made: the genera *Caecomyces* and *Cyllamyces* were abundant in feacal samples. In rumen samples, the genera *Orpinomyces*, *Anaeromyces* and *Neocallimastix* exhibited higher levels of abundance. Genera *Piromyces* was observed in both faecal and rumen samples (Griffith et al., 2009; Kittelmann et al., 2013; Liggenstoffer et al., 2010).

## 2.2 Metabolism around calving

### 2.2.1 Lactation

(Capuco & Akers, 2009).The primary aim of milk production is to provide essential nutrients crucial for the growth and development of the offspring. Additionally, it helps in enhancing the immunological and endocrine competence of the calf (Capuco & Akers, 2009). During early lactation, there is a rapid initial increase in milk production. After 6 weeks, the milk yield reaches its peak. Subsequently, it gradually decreases until the end of the lactation period (Figure 3).

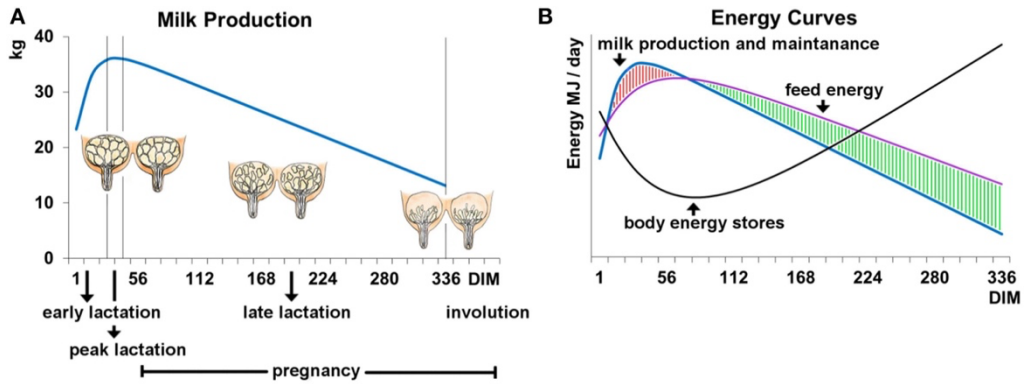


***Figure 3A.*** *Milk production as a function of time after calving; 3B: Energy curves of dairy cow around parturition* (Strucken et al., 2015).

The transition period, spanning approximately three weeks before and after calving, poses significant challenges for high-producing Holstein cows. This critical phase involves physiological stress as the cow readies herself for parturition and subsequently recovers from it. During this six-week timeframe, the cow undergoes a notable metabolic shift to provide essential nutrients for milk synthesis to the mammary gland. At the onset of the lactation, the cow experiences less appetite, leading to a decrease in dry matter intake (Allen et al., 2009; Kuhla, 2020). This metabolic alteration results in a negative energy balance and immunosuppression. A negative energy balance occurs when the net energy intake falls short of the net energy requirements. Immunosuppression refers to a temporary or permanent impairment of the immune response, increasing susceptibility to diseases due to insults to the immune system (Strucken et al., 2015).

### 2.2.2 Triangular relation between metabolic stress, oxidative stress, and inflammation

(Sordillo & Raphael, 2013)As previously mentioned, cows experience a NEB during the transition period. Low glucose levels in the bloodstream will be accompanied by a reduction in insulin levels. Consequently, this stimulates the release of non-esterified fatty acids (NEFA) from adipose tissue. But an excessive release of lipids can cause accumulation of free fatty acids in the blood. Elevated levels of NEFA are associated with an increase of reactive oxygen species (ROS), causing oxidative stress. Subsequently, the prioritization of energy and nutrients towards lactogenesis, coupled with metabolic and oxidative stress, can result in insufficient resources allocated to the immune system. Disrupted immune function can cause inflammation (Sordillo & Raphael, 2013). Furthermore, oxidative stress elevates inflammation mainly by activating proinflammatory pathways. For example, ROS can initiate NF-kB signaling pathways, leading to increased production of cytokines that coordinate inflammatory responses. Next to ROS, beta-hydroxybutyrate (BHB) and NEFA can also activate the NF-kB pathway (De Heredia et al., 2012; X. Shi et al., 2014). These interrelationships are nicely illustrated in Figure 4 (Abuelo et al., 2019).

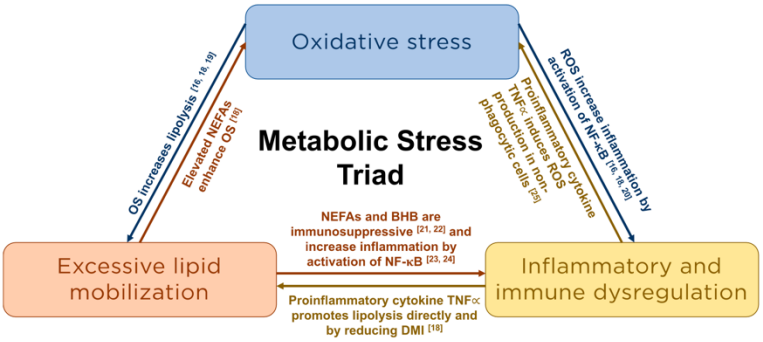


***Figure 4.*** *Schematic representation of the intrinsic relationships among the components of the metabolic stress triad. NEFA = non-esterified fatty acid; OS = Oxidative Stress; ROS = Reactive oxygen species; NF-kb = nuclear factor kappa-b: BHB = beta-hydroxybutyrate; TNFα = Tumor necrosis factor alpha; DMI = Dry matter intake* (Abuelo et al., 2019).

### 2.2.3 Metabolic stress

(Bauman & Bruce Currie, 1980).Before delving deeper into the metabolic stress that a cow may experience around calving, the concepts of homeostasis and homeorhesis are introduced. Homeostatic control is responsible for preserving the stability of physiological balance and ensuring the consistency of environmental conditions within the organism. On the other hand, homeorhesis refers to the synchronized and well-coordinated regulation of metabolic processes in body tissues, essential for sustaining a specific physiological state. Metabolic stress occurs when homeorhesis is disrupted (Bauman & Bruce Currie, 1980).

#### 2.2.3.1 Biomarkers of metabolic responses

When the cow is in its transition period, body reserves will be utilized to counteract the energy deficit of the Negative Energy Balance (NEB). To release energy stored in these reserves, stored lipids must be mobilized. Subsequently, lipolysis will take place, leading to the release of non-esterified fatty acids (NEFA) from adipose tissues into the bloodstream (Leblanc, 2010). In the liver NEFA undergo ß-oxidation, converting them into acetyl-CoA. Subsequently, acetyl-CoA can be further oxidized in the Krebs cycle to provide energy. However, in cases of NEFA overproduction, there aren't enough intermediaries to participate in the Krebs cycle, leading to the oxidation of NEFA into ketones with Beta-Hydroxybutyrate (BHB) being the most common.

(De Koster & Opsomer, 2013)At the onset of lactation, all available glucose in the body is directed towards the mammary glands for lactose synthesis. This also means that muscles and adipose tissue uptake minimal amounts of glucose. The mammary gland operates independently of insulin for glucose uptake because glucose transporter type 4 is not expressed. Meanwhile, reduced circulating levels of insulin and glucose can initiate lipolysis, leading to the liberation of non-esterified fatty acids (NEFA) from adipose tissues into the bloodstream (De Koster & Opsomer, 2013)

Furthermore, metabolism is controlled by hormones like growth hormone (GH) and insulin-like growth factor (IGF-I), collectively included in the somatotropic axis. The binding of GH to a growth hormone receptor (GHR) in the liver stimulates the secretion of IGF-I. Thanks to negative feedback, the IGF-I concentrations will regulate the secretion of GH (Renaville et al., 2002). In the transition period, the reduced expression levels of hepatic GHR leads to a lower production of IGF-I (Witte et al., 2019). Accordingly, the negative feedback of IGF-I on GH secretion is suppressed. As a result, there will be a continuous synthesis of GH, which further promotes lipolysis to NEFA.

#### 2.2.3.2 Metabolic stress vs metabolic adaptation

An example of metabolic imbalance is ketosis. It can occur during a Negative Energy Balance (NEB). During lipid mobilization, an excess of Non-Esterified Fatty Acids (NEFA) enters the bloodstream. However, there are insufficient intermediates available to facilitate their oxidation via the Krebs cycle. As a result, NEFA is oxidized into ketones. Acetone, acetoacetate, and beta-hydroxybutyric acid (BHB) are known as ketone bodies. There are two types of ketosis: subclinical and clinical ketosis. Subclinical ketosis is characterized by the presence of ketone bodies in blood, urine, and milk without clinical signs. Subclinical ketosis can progress to clinical ketosis. Clinical ketosis is caused by a high concentration of ketone bodies in body fluids. This condition is accompanied by symptoms such as loss of appetite, decreased milk production, rapid weight loss, and dry faeces. The BHB concentration in the blood is an accepted method for diagnosing ketosis. Concentrations of 1,2-1,4 mmol/L (Suthar et al., 2013) are classified as subclinical, and concentrations higher than 3 mmol/L are classified as clinical (Oetzel et al., 2007).

### 2.2.4 Oxidative stress

(Storz & Imlay, 1999; Tufarelli et al., 2023)An increased demand for oxygen enhances the production of oxygen-derived substances, collectively known as reactive oxygen species (ROS). Mitochondria are the main sources of ROS, which are produced as byproducts of the respiration process. Notable ROS include superoxide ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$), hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$) radicals. These reactive oxygen intermediates can cause damage to proteins, lipids, and DNA. An elevated release of ROS can result in the inadequate elimination or neutralization of these free radicals, leading to oxidative stress (Storz & Imlay, 1999; Tufarelli et al., 2023).

Antioxidants play a crucial role in the prevention or elimination of oxidative damage. Two types can be distinguished: enzymatic and non-enzymatic. Among the enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, while the non-enzymatic antioxidants include vitamins (e.g. C and E), minerals (e.g. selenium and zinc) and metabolites (e.g. uric acid and

melatonine) (Kharrazi et al., 2008). SOD catalyze the conversion of $O_2$•– to $H_2O_2$ (Younus, 2018). Next, hydrogen peroxide can be converted to water by glutathione peroxidase (GPx). The activity of the GPx enzyme depends on the selenium concentration available in the body.

Malondialdehyde (MDA) is a product of lipid peroxidation. Polyunsaturated fatty acids are susceptible to oxidation by reactive oxygen species (ROS) and free radicals. When exposed, they readily interact with hydroxyl radicals, leading to the formation of lipid peroxyl radicals. These radicals then react with other polyunsaturated fatty acids, resulting in the creation of lipid hydroperoxide and additional lipid peroxyl radicals. Additionally, lipid peroxyl radicals can react with intramolecular double bonds, producing cyclic endoperoxide, which subsequently breaks down to generate MDA. Therefore, MDA serves as a marker indicating the occurrence of lipid peroxidation, which is often associated with oxidative stress and cellular damage (Castillo et al., 2006).

Glutathione is a non-enzymatic antioxidant and comes in two forms: reduced glutathione (GSH) and oxidized (GSSH). Enzymes such as glutathione peroxidase (GPx) can use GSH as a substrate. GSH is important to maintain the intracellular redox status by neutralizing peroxidic radicals and peroxides. An excess of ROS can also lead to a toxic buildup of GSSH. The determination of GSH/GSSH is useful tool to determine oxidative stress (Nuhu et al., 2020)

Due to the complexity of individually measuring each antioxidant component and their interactions within plasma, methods have been developed to assess total antioxidant capacity (TAC). These methods consider the cumulative action of all antioxidants present in plasma and body fluids. They can therefore represent a comprehensive status rather than a simple sum of measurable antioxidants. Oxygen Radical Absorbance Capacity (ORAC) is an example of TAC (Cao et al., 1993; Celi, 2011).

### 2.2.5 Inflammation

Almost all cows experience a certain degree of inflammation during the transition period. The inflammatory status is identified by increased blood levels of pro-inflammatory cytokines and positive acute-phase proteins. In reaction to stimuli linked to infection and tissue injury, elements of both innate and adaptive immunity launch coordinated responses, thereby initiating inflammation (Bradford et al., 2015).

Albumin and globulin are proteins present in the blood and are valuable indicators of the animal's inflammatory status. They are primarily synthesized by the liver. A decrease in albumin concentration may result from reduced synthesis by the liver due to increased inflammation. Albumin is classified as a negative acute phase protein. Conversely, an increase in globulins can be observed as an immune response by the body. Globulin is classified as a positive acute phase protein. The albumin-to-globulin ratio represents a synthetic index for the blood protein profile and is widely utilized in clinical biochemistry (Cattaneo et al., 2021).

Other acute proteins that are indicators of the health status of the cow include serum amyloid A (SAA) and haptoglobin (Hp). These are both considered positive acute phase proteins. An increase in SAA is observed in the blood associated with various disorders, making it a frequent choice for assessing the inflammatory status of the animal. Hp is synthesized in the liver, and during inflammatory stress, an elevation of Hp can be detected in the blood. Hp can eliminate released metabolites originating from cellular degradation and helping in preventing reactive oxygen intermediates from causing cell damage (Alsemgeest et al., 1994; Moisá et al., 2019; Trela et al., 2022).

## 2.3 Diet around calving and its influence microbial community in the ruminant's gastrointestinal tract

### 2.3.1 Diet around calving and its influence on the microbial community

As previously mentioned, the transition period is a critical phase for the cow. The body prepares for parturition and then recovers from it. This period is associated with Negative Energy Balance (NEB). Dairies with high-producing cows aim to mitigate negative energy balance by transitioning from a prepartum high-fibre, low-energy diet to a postpartum low-fibre, high-energy diet. The postpartum diet, rich in highly fermentable carbohydrates, will result in an increased production of volatile fatty acids and lactic acid within the rumen. Consequently, these acids accumulate as the rumen mucosa's capacity to absorb them is limited. This accumulation lowers the pH and increases the osmolality of the digesta in the rumen (Mao et al., 2012; Plaizier et al., 2008, 2012). Cows fed a high-grain diet spend less time chewing compared to those on a forage-based diet. Chewing prompts saliva production, serving as a buffer for the rumen digesta. Therefore, reducing the forage content in the diet also reduces the rumen pH (Plaizier et al., 2008). Consequently, feeding high-grain diets may result in subacute ruminal acidosis (SARA), a metabolic disorder. Furthermore, excessive grain feeding can benefit some microorganisms by providing more substrate and specific niches. But it can be detrimental to microorganisms that rely on fibre and are sensitive to a low pH of digesta or any of the gut metabolites and compounds whose concentrations are increased by grain feeding. In conclusion, this dietary shift induces changes in both physiological conditions and microbial composition in the rumen and the hindgut (Khafipour et al., 2016).

Excessive grain levels in the diet can lead to a reduction in cellulolytic bacteria and, additionally, an increase in gram-positive bacteria. In a study by Lima, it was discovered that certain bacteria, specifically *Fibrobacter*, *Ruminobacter*, *Selenomonas*, *Butyrivibrio*, and *Succinivibrio*, played a crucial role in differentiating the microbes between the pre- and postpartum stages. Further, the prepartum microbes were characterized by an increased prevalence of fungi, which subsequently transitioned to a postpartum pattern marked by an elevated presence of protozoa associated with starch digestion (Lima et al., 2015). Whereas in a study Khafipour feeding high-grain diets showed a decrease in the populations of ciliate protozoa (Khafipour et al., 2009). Studies have shown that when cattle are fed high amounts of grains, the relative abundances of the phyla *Bacteroidetes* and *Fibrobacter* decline. This decline facilitates the proliferation of *Firmicutes* and other opportunistic phyla, including gram-negative *Proteobacteria* (Khafipour et al., 2009; Mao et al., 2013).

### 2.3.2 Diseased cows around calving and association with changes in the rumen microbial community

#### 2.3.2.1 LPS - associated changes in the rumen microbial community

Numerous studies have demonstrated that elevated grain feeding leads to increased concentrations of various metabolites in rumen digesta. While many of these metabolites serve as substrates for beneficial microorganisms, some are toxic and inflammatory. A toxic metabolite linked to high-grain feeding is lipopolysaccharides (LPS). It is a bacterial endotoxin, released from the cell walls of Gram-negative bacteria present in the rumen and hindgut. Within this context, members of the Phylum Proteobacteria are one of the major contributors to the pool of free LPS endotoxins in both compartments. But these endotoxins in their free form can cause an immune response (Plaizier et al., 2008, 2012). Furthermore, the changes in the abundance of the microbes and microbial composition can compromise host immunity, making it easier for pathogenic species to colonize. As a result, colonization by pathogenic species may become more prevalent.

Mastitis in cattle is an inflammatory reaction of the udder tissue within the mammary gland, resulting from either physical injury or infections by microorganisms. According to Hu's study, disturbances in rumen microbiota were linked to mastitis development in cows. It is possible that this occurs because LPS from the rumen could migrate to the mammary gland through the bloodstream, initiating inflammation. Furthermore, an elevated presence of Stenotrophomonas in the rumen could contribute to mastitis development (Hu et al., 2022).

While certain microorganisms thrive in shifting environments during transition period, others play a pivotal role in restoring equilibrium to microbial communities. For instance, a study by Chen observed that Megasphaera elsdenii and Butyrivibiro fibrosolvens can divert the production of short-chain fatty acids (SCFA) from lactate to butyrate. This redirection enhances ruminal pH, thereby mitigating the risk of subacute rumen acidosis. (Chen et al., 2019). Furthermore, it should be noted that a minimal growth rate was observed for these bacteria was at a pH of 5.5, thus indicating that a pH that is too low is detrimental to the growth of Megasphaera elsdenii and Butyrivibiro fibrosolvens.

#### 2.3.2.2 ketosis associated changes in the rumen microbial community

In a study by Wang et al. (2023), metataxonomic analysis was conducted on rumen samples. 16S rRNA sequencing was used for the metataxonomic analysis. The study concluded that Christensenellaceae, Ruminococcaceae, Lachnospiraceae, and Prevotellaceae were enriched in ketotic cows (Wang et al., 2023).

# 3 MATERIALS AND METHODS

## 3.1 Problem and Objective

The transition period is challenging for dairy cows. This period is marked by significant physiological changes and metabolic challenges which can lead to oxidative stress, metabolic imbalance, and inflammation. While some cows can navigate this period with minimal issues, others face major imbalances. This occurs despite them being fed the same feed and being under the same management conditions. Therefore, it is assumed that there is an inter-animal differences in dealing this stressful period. One of the reasons for this inter-animal differences may be the gastro-intestinal microbial composition. This study investigates whether a correlation can be established between the microbial composition of the cow's gastrointestinal tract, specifically the microbial composition of the rumen and hindgut, and the health status of the cow specifically metabolic, and inflammatory status. Additionally, we aimed to identify indicator microbes that could potentially be linked to the metabolic or inflammatory status of the dairy cows.

## 3.2 Animals diet and management

Sixty multiparous Holstein Friesian dairy cows from two research farms were used in this study. Forty cows were from the ILVO research farm (Flanders Research Institute for Agriculture, Fisheries, and Food, Melle, Belgium). These animals were part of a group of 99 dairy cows (117 lactations) monitored during their transition period from October 2018 to October 2020. The experiment and sample collection were approved by the Ethical Committee of ILVO (2018/329). The remaining 20 cows were from a group of 73 animals (86 lactations) monitored at Hooibeekhoeve (Geel, Belgium) during their transition period from January 2021 to December 2022. Ethical approval for this experiment was granted by the institutional animal care and use committee of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering at Ghent University (2020-078).

### 3.2.1 Feed

From three weeks before calving, the cows were fed a partial mixed ration (PMR), supplemented with dry cow mineral premix (Prolacta, AVEVE, Merksem, Belgium) with on average 1 kg balanced (in terms of net energy for lactation and intestinally digestible protein) concentrate per cow, per day. This ration was fed until 2 days after calving. The PMR consisted of maize silage, pressed beet pulp, soybean meal, pre-wilted grass silage and balanced compound feed. This PMR is designed to fulfil the nutritional requirements for cows with an average body weight of 650 kg and producing 26 kg fat-protein corrected milk a day. The supply of the balanced concentrate changed according to the animal's lactation stage (see Table 1 and 2). Cows were offered the PMR as two equal meals at roughly 07:30 h and 16:30 h ad libitum and had free access to water.

***Table 1.*** *Ingredients and chemical composition (g/kg of DM) of the diet offered from 3 weeks before calving (Close-up) up to the first 2 days of lactation, as well as the diets given on day 3 (Lac3) and day 20 (Lac20) of lactation. The build-up of balanced compound feed A and B, Covasoy, and Soybean meal, created a gradual and linear shift from Lac3 to Lac20.*

| Item | Close-up | Lac 3 | Lac 20 |
|---|---|---|---|
| **Maize silage** | 344 | 321 | 248 |
| **Grass silage** | 344 | 321 | 248 |
| **Beet pulp** | 84,2 | 78,4 | 60,7 |
| **Urea** | 1,4 | 1,3 | 1,01 |
| **Straw** | 9,57 | 8,92 | 6,9 |
| **Barley** | 11 | 10,2 | 7,89 |
| **Maize** | 41,6 | 38,8 | 30 |
| **Soybean meal** | 79,8 | 111 | 73,7 |
| **Covasoy**[1] | - | 24,5 | 54 |
| **Balanced compound feed A**[2] | - | 24,5 | 108 |
| **Balanced compound feed B**[2] | 67,2 | 61,2 | 162 |
| **Prolacta**[3] | 16,8 | - | - |
| **Chemical composition, g/kg of DM (unless noted otherwise)** | | | |
| **DM (g/kg)** | 392 | 440 | 543 |
| **VEM**[4] | 998 | 1027 | 1053 |
| **CP** | 138 | 160 | 168 |
| **FOM**[5] | 592 | 591 | 587 |
| **Starch** | 168 | 164 | 180 |
| **$NE_L$ (MJ/kg of DM)**[6] | 6,89 | 7,09 | 7,27 |

***Table 2.*** *The linear build-up between day 3 and 23 of lactation of the supplemental part of the diet. This was individually supplied to the cows during milking via the concentrate dispenser available in the barn (kg/d).*

| Item | Amount at day 3 | Build-up duration (d) | Amount at day 23 |
|---|---|---|---|
| **Balanced compound feed A** | 0,2 | 20 | 2 |
| **Balanced compound feed B** | 1,5 | 10 | 4 |
| **Covasoy** | 0,2 | 7 | 1 |
| **Soybean meal** | 0,3 | 20 | 3 |

### 3.2.2 Sample collection

Rumen samples were collected 21 days after parturition using a stomach tube. The tube was inserted through the mouth and the esophagus to access the rumen. The tube was inserted into the ventral ruminal sac according to the method from (Geishauser et al., 2012) and rumen sample was collected. Sample collection was performed 2 h after feeding. To avoid contamination with saliva, a flora scoop was used. The collected rumen sample was transferred to a cryovial and snap-frozen in liquid nitrogen for microbial analysis. These vials were stored at -80 °C until DNA extraction (Yang, Heirbaut, Jeyanathan, et al., 2022).

[1]Covasoy: formaldehyde treated soybean meal to bypass rumen degradation.
[2]Balanced compound feed A and B see Table 2.
[3]Contains: 2,2 g Ca, 39,5 g P, 0 g K, 81,6 g Mg, 30,1 g Na, 2000 mg choline chloride, 20 mg calcium iodate, 15 mg cobalt sulphate, 1000 mg copper sulphate, 1,250 mg manganese oxide, 2,500 mg zinc sulphate, 40 mg sodium selenite, 1000.000 IU vitamin A, 200.000 IU vitamin D3, 4440 of vitamin E.
[4]VEM: feed unit lactation (reference).
[5] FOM: fermented organic matter (reference).
[6]NEL: Net Energy Lactation calculated based on the Belgian-Dutch net energy evaluation system: 1000 VEM = 6,9 MJ NEL (reference).

Faecal samples were collected 7 days before parturition and 21 days after parturition. Two hours after the morning feeding, faeces were sampled by grab sampling (± 200g) directly from rectum from the cow. After homogenizing, faecal samples were immediately transferred to cryovials and snap-frozen with liquid nitrogen and stored at -80 °C until DNA-extraction (Yang, Heirbaut, Jing, et al., 2022).

Between approximately 9:15 and 9:45 am, serum and plasma samples of blood were harvested at -7, 3, 6 and 9 days relative to the calving from coccygeal vessels and at 21 days after calving, it was harvested from the jugular vein. Plasma was collected in NaF tubes (4 mL) for blood and for serum samples, serum blood tubes (10 mL) were used (Heirbaut et al., 2023).

### 3.2.3 Analysis of blood for metabolic, oxidative and inflammatory parameters

At all sampling days, the serum tubes were kept at room temperature for 30 minutes before centrifuging at 1500 *g* for 15 minutes. The NaF tubes on the other hand were kept in an ice box until centrifuged at 1000 g for 10 minutes at room temperature. After the centrifugation step, the upper layer of both tubes was divided and transferred to Eppendorf tubes. Serum samples to determine the concentrations of BHBA, NEFA, insulin, Hp, SAA, albumin: globulin and plasma samples from the NaF tubes for glucose analysis were stored at -20 °C, while serum samples for IGF-I were stored at -80 °C until analysis. Blood samples which were obtained from the jugular vein using lithium heparin tubes at 21 d after parturition was used for the analysis of oxidative parameters (GSSH, GPx, MDA, ORAC and SOD).

Blood parameters glucose, BHBA, NEFA, IGF-1 and insulin were used as the metabolic markers. Glucose, BHBA, and NEFA were analyzed using GalleryTM Discrete Analyzer (ThermoFisher Scientific, MA, USA) and Randox kits (Randox Laboratories Ltd, Ibach, Switzerland) by the lab DGZ (Belgium). Serum IGF-I was analyzed by a radioimmunoassay method using the Non- Non-Extraction IGF-I IRMA DSL-2800 (LifeSpan Biosciences, Seattle, USA) by Poznań University of Life Sciences. The concentration of this hormone was determined using the isotope I125 and an automatic gamma radiation reader (Wizard2 2-Detector Gamma Counter, Perkin Elmer). Lastly, insulin was measured using the Insulin ELISA kit (Bio-connect Diagnostics, Huissen, The Netherlands) (Girma et al., 2024; Heirbaut et al., 2023).

Blood parameters SAA, Hp and albumin:globulin were used as the inflammatory markers. SAA and Hp were analyzed using the SAA assay-multispecies ELISA kit and Haptoglobin assay kit respectively (BioRepair, Sinsheim, Germany). Albumin and globulin were analyzed using MINICAP PROTEIN(E) 6 (Sebia, Lisses, France) by the lab DGZ (Belgium) (Zhang et al., 2024).

Oxidative parameters such as GPx activity, SOD, MDA, ORAC and GSSG (%) were also analyzed in the blood. The GPx activity assay follows the method described by Hernández et al. (2004) by measuring the consumption of NADPH at 340 nm, with adjusted volumes of reagents to fit the 96-well plate. The SOD assay kit 19160–1KT-F (Sigma Aldrich) was used to determine the SOD activity by an indirect spectrophotometric method. The concentration of MDA was assessed based on the modified method of Grotto et al. (2007). The ORAC assay was performed according to the method of Nimalaratne et al. (2011). The GSSG (%) analysis was performed according to the method of Degroote et al. (2012) by HPLC [Agilent 1200 series, with autosampler, quaternary pump, column oven, and DAD detector (Agilent Technologies)] (Zhang et al., 2023).

### 3.2.4 Clustering of cows based on inflammatory status

The inflammation clusters previously made by a PhD student (Zhang et al., 2024) were used in this study. The clustering of the inflammation cluster is based on the entire dataset, from which 60 random cows were chosen for this thesis research. Parameters related to inflammation (Hp, SAA and A:G) were used to sort the cows in different clusters. The k-medoids clustering algorithm available in the R package cluster (Maechler et al., 2023) was used for this purpose. Before clustering, the data were standardized (z-scores) and the number of clusters was determined by k-means clustering using R package NbClust (Charrad et al., 2014). Then, the clusterboot function in the R package fpc (Hennig, 2023) was used to ensure the cluster stability. The Jaccard index was calculated for each replication and values above 0,8 were considered as acceptable clustering results. In this case, the two clusters obtained a score higher than 0,8 Jaccard index. As such, the two clusters were referred to as the "acute phase response + diseased cows (APR+)" and "non-APR cows (Non-APR)" based on the inflammatory parameters.

### 3.2.5 Clustering of cows based on metabolic status

The metabolic clusters made by a PhD student were used in this study (Girma et al., 2024). The clustering of the metabolic cluster is based on the entire dataset, from which 60 random cows were chosen for this thesis research. The metabolic clusters were created using longitudinal data of blood BHB levels (Girma et al., 2024). These clusters were based on the trajectories of this metabolite (Figure 5). The BHB levels in the blood were measured on the following days: 3, 6, 9, and 21. Accordingly, there were three clusters: quickly increasing BHB (QuiBHB), slowly increasing BHB (SloBHB), and low BHB (LoBHB). However, due to the low number of cows in the QuiBHB cluster (4 cows) in our subset of animals with faecal and rumen samples, it was omitted from this study. Therefore, comparisons in this study were made between the two remaining clusters: SloBHB and LoBHB.

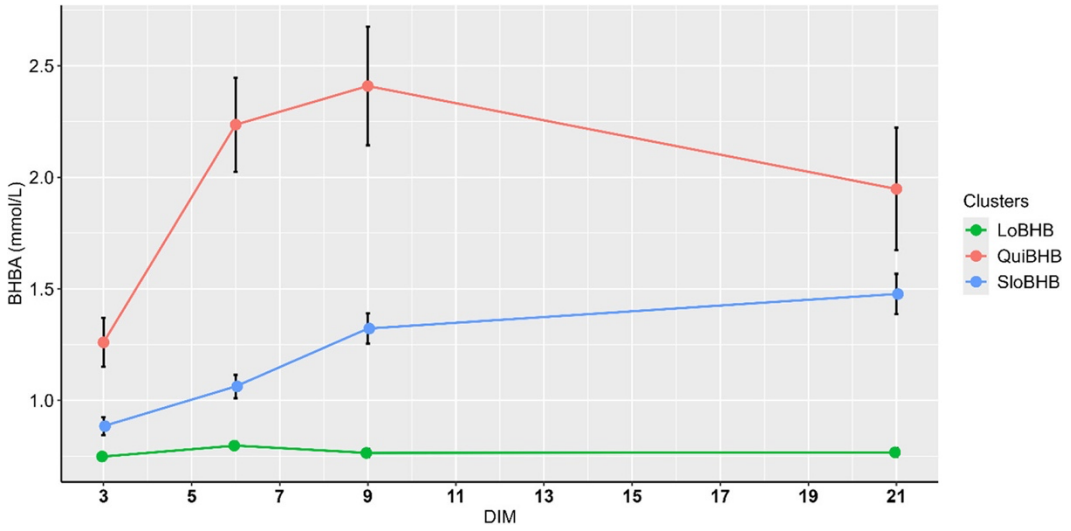


***Figure 5****.The BHB trajectories of the three metabolic clusters: quickly increasing BHB (QuiBHB), slowly increasing BHB (SloBHB) and low BHB (LoBHB)* (Girma et al., 2024)

## 3.3 Microbial analysis

### 3.3.1 DNA extraction from rumen faecal samples

Yu & Morrison, 2004The DNA extraction was performed following the protocol set up by Yu & Morrison, 2004. For the rumen fluid, 1 mL of sample was centrifuged at 4 °C for 15 min at 16,000 × g to collect the pellet, which was then used in the DNA extraction procedure. For the faecal samples 50 mg of freeze-dried faeces were homogenized and weighed before performing the DNA extraction. The quality and concentration of the DNA was tested using the Nanodrop spectrophotometer (VWR International BVBA, Leuven, Belgium) before being stored at -20°C.

### 3.3.2 Metataxonomic analysis

The extracted DNA was sent to Edinburgh Genetics (Midlothian, United Kingdom)) for the amplicon sequencing. Amplicon sequencing was performed for all four microbial groups: bacteria, archaea, protozoa and fungi. The targeted genes and primers used for this amplicon sequencing are given in Table 3. The sequencing was carried out by using the Illumina MiSeq V3-technology (2 × 250bp). The sequence was demultiplexed and the barcodes were cut off by the company. This data was then analyzed by utilizing the QIIME2 software (version 2024.2), using the DADA2 pipeline (Bolyen et al., 2019). This pipeline is needed because the process of amplicon sequencing brings errors in the sequencing data. The DADA2 algorithm models the errors to construct amplicon sequence variants (ASV). The DADA2 pipeline was thus used to detect and correct Illumina amplicon sequences, remove the primers and chimeric reads and assemble the data into ASV. A filtering step was performed to remove low abundance sequences (frequencies below 0,01% or present in less than 2 samples). Based on the number of reads/sample normalization of reads were performed using QIIME2 software. Taxonomy of bacteria was assigned using a naïve Bayes classifier trained on the Greengenes2 database (release 2022,10) at 99% similarity followed by the removal of the features of archaea and unassigned taxa. Taxonomy of archaea was assigned using the full-length Greengenes2 database (release 2022,10) at 99% similarity followed by the removal of the features of bacteria and unassigned taxa. For the taxonomy classification of the protozoa database created by Kittelmann et al., (2015) was used and the fungal taxonomy was assigned using the LSU_database v 2.0 (Hanafy et al., 2020).

***Table 3.*** *Regions used for gene amplicon sequencing and corresponding primers per species.*

| Microbial group | Gene amplicon | Primer | Reference |
|---|---|---|---|
| Bacteria | 16S rRNA | 515F-806R | Caporaso et al., (2011) |
| Archaea | 16S rRNA | Arch349F-806R | Takai & Horikoshi, (2000) |
| Protozoa | 18S rRNA | P-ssu-316F-GIC758R | Ishaq & Wright, (2014) |
| Fungi | D1-D2 region (large subunit 28S) | AGF-LSU-EnVs (D1-D2) | Young et al., (2022) |

### 3.3.3 Diversity

Alpha diversity indices analyze the richness of species or the quantity of species within an ecosystem, whereas beta diversity measures the diversity among ecosystems by evaluating alterations in species composition. Essentially, alpha diversity relates to diversity within an ecosystem, whereas beta diversity focuses on diversity between ecosystems (Whittaker, 1972).

There are different ways to measure both α- and β-diversity and these diversities were calculated using the Phyloseq software in R. A nonparametric Kruskal-Wallis test was used to evaluate the differences of α-diversity metrics between clusters. Additionally, distance-based (Bray-Curtis distances) permutational multivariate analysis of variance (PERMANOVA) was carried out to check whether bacterial composition varies between clusters (β-diversity). Based on the obtained P-value, there can be a significant difference ($P<0,05$) or a tendency ($0,05<P<0,10$). A brief description of diversity indices calculated in this study is given below.

*Alpha diversity:*

The Shannon index was computed for each sample by summing the product of the proportion of each species and the natural logarithm of that proportion. This calculation provides a measure of diversity that accounts for both the number of species present (richness) and how evenly distributed the individuals are among these species (evenness) (Lynch, 2024).

The Simpson index operates on a concept similar to the Shannon Index. It relies on the likelihood that two entities (such as microbes or reads) randomly selected from the sample belong to different types (e.g., species). Because this is a probability-based calculation, resulting scores fall within the range of 0 to 1 (Lynch, 2024).

Observed features is a simple metric for quantifying microbiome diversity. This metric involves counting the features present in a given sample. This qualitative diversity metric treats each feature as either observed or not, without considering the frequency of observation (G. Caporaso, 2021).

*Beta diversity:*

(Bray & Curtis, 1957)The Bray-Curtis dissimilarity examines the abundance of microbes that are shared between two samples and takes the total number of microbes into account. The method gives a score from 0 to 1. If the sample has the same number of microbes, in the same abundance, a score of 0 is given. If they share no microbes, a score of 1 will be given as they have maximum dissimilarity. Important to note is that because this is a bounded scale, amplified or contrasted differences might occur because of the total amount of microbes present in the sample (Bray & Curtis, 1957).

Another approach to compute beta diversity involves utilizing weighted or unweighted UniFrac. This technique considers the phylogenetic relationship among the microbes detected in two samples, presuming that closely related microorganisms likely share similar functions. Consequently, a sample containing various species from the same genus is considered more similar than a sample containing different species from distinct genera. The distinction with weighted UniFrac lies in its consideration of the abundance of microorganisms as well (Lozupone et al., 2006).

Differential sequence analysis was performed using the DeSeq2 method within the R environment (R Core Team, 2020) at both the ASV and genus levels. This approach aimed to identify bacterial groups with varying abundance across clusters of cows. DeSeq2 is a widely used tool for comparative analysis of sequenced data. While it is primarily designed for analyzing RNA sequencing data to detect differentially expressed genes among groups, it is also applicable in metataxonomic analysis. DeSeq2 utilizes negative binomial generalized linear models, employing dispersion estimates and logarithmic fold changes for quantitative analysis.

## 3.4 Statistical analysis

Differences between the clusters on production parameters (DMI, Milk production, and Milk intensity), metabolic parameters, inflammatory parameters and oxidative parameters were analyzed by R (version 2023.03.0+386). The model used was mixed model ANOVA, with the farm and the clusters as the fixed factors and the cows as the random factor. A nonparametric Kruskal–Wallis test was used to evaluate the differences of α diversity metrics between clusters in R software. In addition, distance-based (Bray–Curtis distance) permutational multivariate analysis of variance (PERMANOVA) was carried out to check whether microbial composition varies between clusters in R. If there were more than 2 groups, pairwise comparisons were made between groups to identify which groups differ with each other. Beta diversity based on the Bray-Curtis and weighted unifrac distance matrices was visualized on principal coordinate analysis (PCoA) plot.

## 3.5 Generative Artificial Intelligence (GAI)

ChatGPT is in some cases used to generate a better written formal grammatical text.

# 4 RESULTS

## 4.1 Overview of microbial communities in the rumen and faeces

Three different types of samples were collected: rumen fluid (days +21), faeces (days -7), and (days +21). The mentioned days are related to the calf's birth.

### 4.1.1 Bacteria

Figure 6 shows the variation in bacterial composition at the phylum level across different samples. In the rumen the predominant phyla are Bacteroidetes (53,5 %) and Firmicutes (36,5 %). The predominant genus in rumen is *Prevotella* (41,5%) which belongs to Phylum Bacteroidetes followed by the genus *Succiniclasticum* (15,7%) from Phylum Firmicutes. In the faecal samples, Bacteroidetes and Firmicutes are once again the most abundant phyla present. But now it is noticeable that Firmicutes (days -7): 50,3%; (days +21): 61,6%) is the most dominant compared to Bacteroidetes. The predominant genus in the faecal sample is *uncultured Ruminococcaceae*: 23,5% and 21,6% in faecal sample collected before and after parturition respectively. Additionally, there is also a noticeable difference in abundance of bacteria between the two faecal samples (days -7) and (days +21).

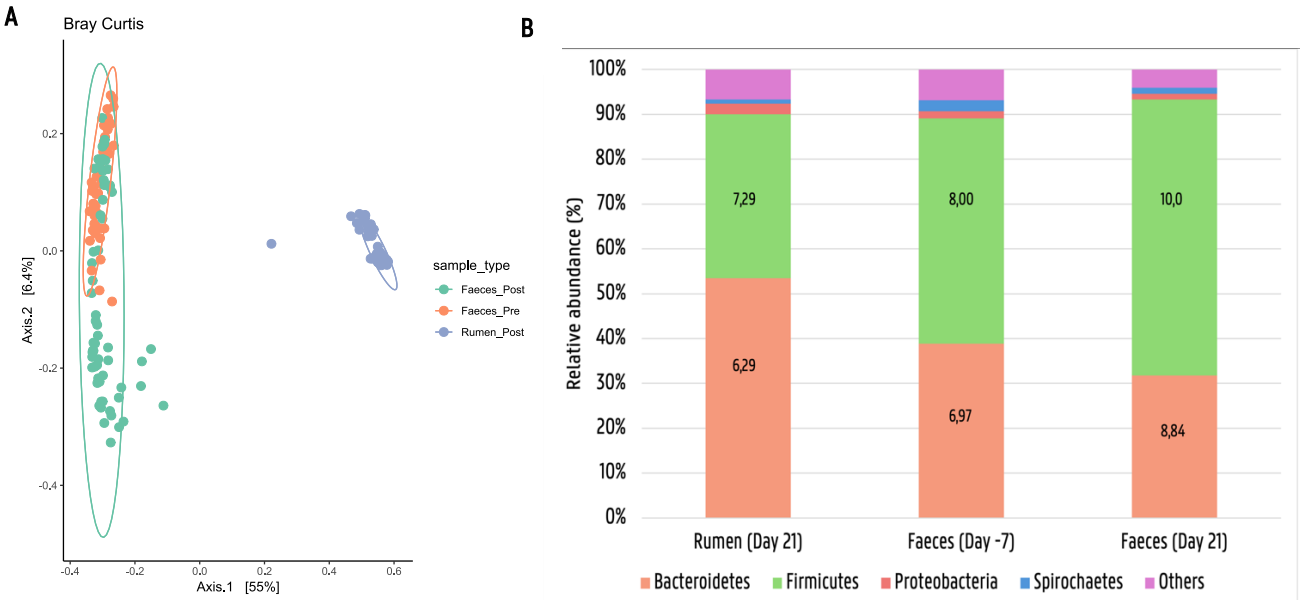


***Figure 6A.*** *A principal coordinate analysis (PCoA) plot of bacterial 16S rRNA gene distribution at ASV level in the rumen and faeces samples collected 7 days before parturition (faeces pre, n=45) and 21 days after parturition (faeces post n=60 and rumen_post n=58). The PCoA plot is based on Bray-Curtis distances.* ***B****: Relative abundances (%) in major phyla of bacteria in the rumen and faecal samples with standard deviation inside the corresponding block..*

### 4.1.2 Archaea

Figure 7A is a principal coordinate analysis (PCoA) plot, based on Bray-Curtis distances at archaeal 16S rRNA ASV level of the collected samples. The rumen and faecal samples cluster away from each other along axis 1, which explains 18,5% of the variation. The PERMANOVA analysis performed with phyloseq revealed that the rumen bacterial composition at the ASV level is different (P<0.05) from that of faecal samples. Additionally, the faecal bacterial composition varies(P<0.05) between samples collected before (-7 days) and after (+21 days) parturition. Alpha diversity indices such as Shannon index, Simpson index and the observed AVS did not differ between the faecal samples collected before and after parturition. However, all these alpha diversity indices were higher in rumen samples compared to both faecal samples. Figure 7B shows the variation in Archaea composition at the family level across different samples. In all the sample the predominant family is *Methanobacteriaceae.* In rumen *Methanobacteriaceae* (90,3%) and *Methanomethylophilaceae* (9,44%) are the most abundant families. In the faecal samples ((day -7) and (day +21)), it is noticeable that *Methanomethylophilaceae* is remarkably less present (almost absent) compared to the rumen samples. The dominant genera in the rumen and faecal samples are Methanobrevibacter A (rumen-post: 59,3%, Faeces-Post: 64,3% and Faeces-pre: 71,1%), *Methanobrevibacter* D 1148 (rumen-post: 15,3%, faeces-post: 14,2% and faeces-pre: 15,8%) and *Methanosphaeara* (rumen-post: 14,3%, faeces-post: 21,3% and faeces-pre: 12,1%).

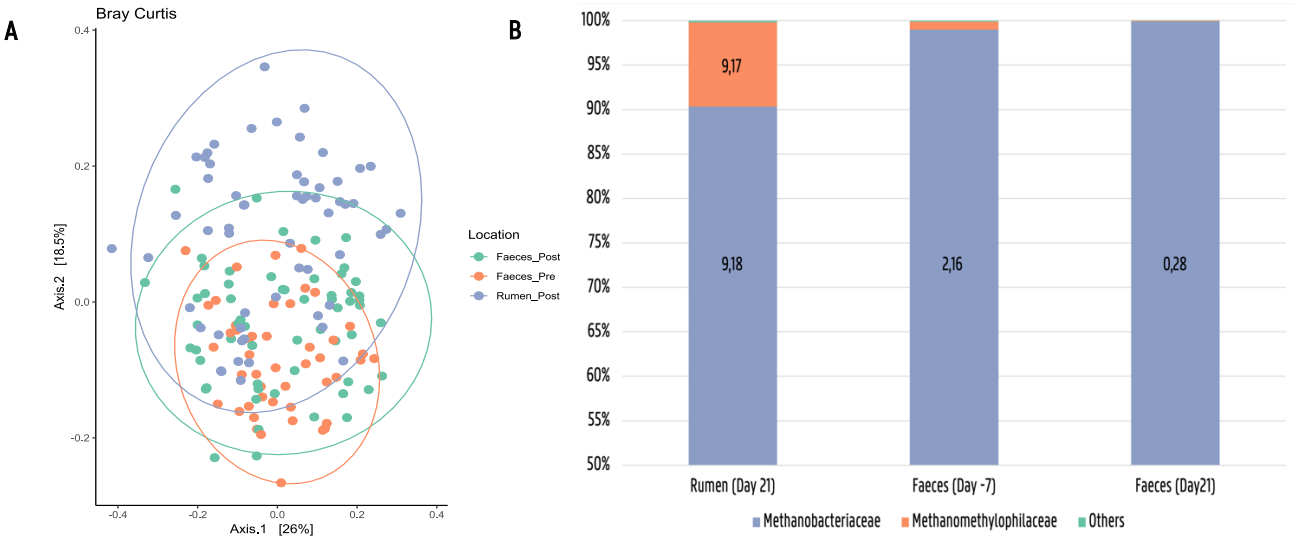


***Figure 7A.*** *A principal coordinate analysis (PCoA) plot of archaeal 16S rRNA gene distribution at ASV level in the rumen and faeces samples collected 7 days before parturition (faeces pre) and 21 days after parturition (faeces post and rumen). The PCoA plot is based on Bray-Curtis distances.* ***B****: Relative abundances (%) in major families of archaea in the rumen and faecal samples with standard deviation inside the corresponding block.*

### 4.1.3 Protozoa

The protozoa are absent in Faecal samples. Figure 8 shows the most abundant genera of protozoa present in the rumen. All rumen protozoa belong to phylum *Ciliophora* and genera *Epidinium* (33,1%), *Entodinium* (28,0%) and *Isotricha* (11,1%) are the most abundant ones.

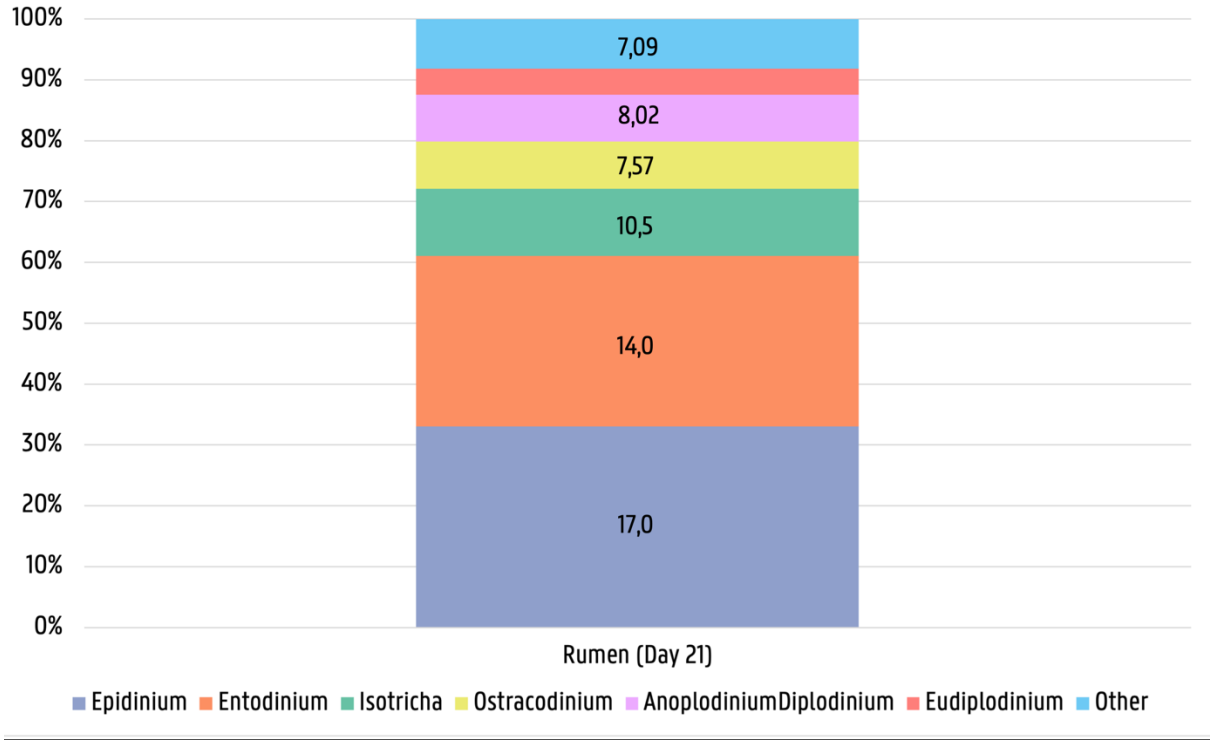


***Figure 8**. Relative abundances in major genera of protozoa in rumen with standard deviation inside the corresponding block*

### 4.1.4 Fungi

Figure 9A is a principal coordinate analysis (PCoA) plot, based on Bray-Curtis distances at fungal D1-D2 region (large subunit 28S) ASV level of the collected samples. The post parturition and pre parturition samples cluster away from each other along axis 1, which explains 24,7% of the variation. The PERMANOVA analysis performed with phyloseq revealed that the rumen fungal composition at the ASV level is different ($P < 0,05$) from that of faecal samples. Additionally, the faecal fungal composition varies ($P < 0,05$) between samples collected before (day -7) and after (day +21). Shannon index did not differ among 3 sample types. However, the Simpson index was higher in the rumen samples compared to both faecal samples. All the fungi belong to the phylum Neocallimastigomycota, class Neocallimastigomycetes and the order Neocallimastigales. In the samples taken after the delivery, *Neocallimastigaceae* is the most abundant family (rumen: 43,0% and faeces (D (+21)): 32,8%). In the other faecal samples (D (-7)) are *Genera incertae sedis* (27,0%) and other (25,5%) families the most abundant. The dominant rumen fungi belong to genus *Neocallimastix* (42,2%) followed by *NY05* (21%). Faeces samples collected before parturition were dominated by uncultured *Neocallimastigales* (25,2%) and genus *Neocallimastix* (19,8%), while faeces samples collected after parturition dominated by genus *Neocallimastix* (32,3%) and genus *Piromyces* (28,4%).

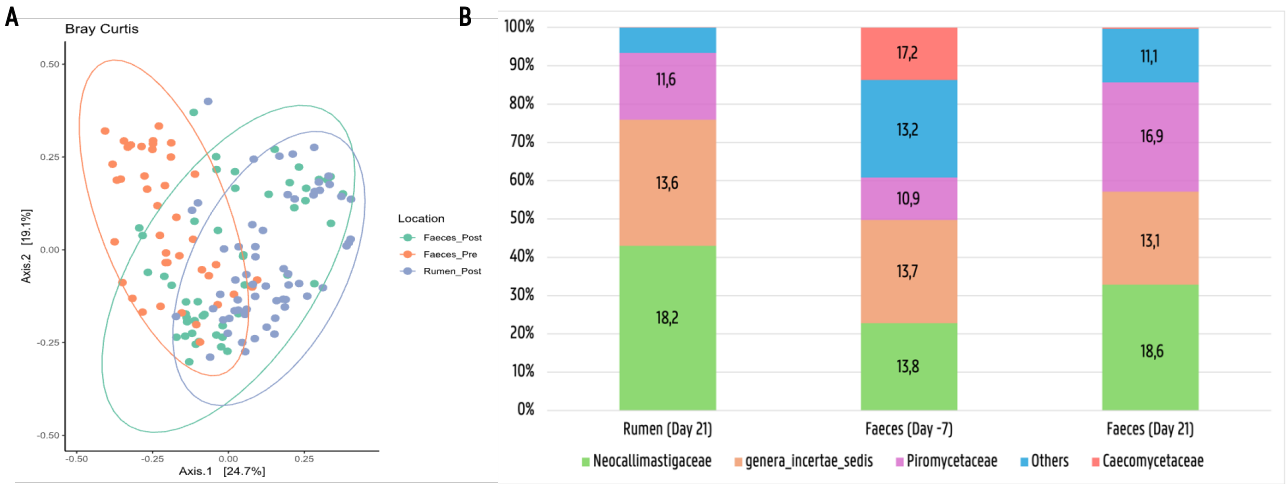


***Figure 9A.*** *A principal coordinate analysis (PCoA) plot of Fungal D1-D2 region (large subunit 28S) distribution at ASV level in the rumen and faeces samples collected 7 days before parturition (faeces pre) and 21 days after parturition (faeces post and rumen). The PCoA plot is based on Bray-Curtis distances.* ***B:*** *Relative abundances in major families of fungi in rumen and faecal samples with standard deviation in the corresponding block.*

## 4.2 Clustering based on the inflammatory and metabolic biomarkers

In this study, the dairy cows were divided into two clusters based on inflammation or metabolic biomarkers. This information can be found in Table 4. The detailed information about the biomarkers used for this clustering was previously described in the Materials and Methods section.

***Table 4.*** *Distribution of cows among the metabolic and inflammation clusters within different sample types per farm. Inflammation clusters are categorized as non-APR or APR+ cows, where APR denotes acute phase response, and '+' signifies inclusion of clinically diseased cows in this cluster. Cows in the metabolic cluster are categorized as either SloBHB or LoBHB.*

| Sample-type | Farm | Inflammation-based clusters | | Metabolic-based clusters | |
|---|---|---|---|---|---|
| | | Acute phase response+ (APR+) | Non-acute phase response (non-APR) | SloBHB | LoBHB |
| Rumen D+21 | ILVO | 15 | 25 | 9 | 28 |
| | Hooibeekhoeve | 5 | 11 | 5 | 13 |
| Faeces D+21 | ILVO | 16 | 25 | 9 | 29 |
| | Hooibeekhoeve | 5 | 11 | 5 | 13 |
| Faeces D-7 | ILVO | 13 | 19 | 8 | 23 |
| | Hooibeekhoeve | 4 | 11 | 4 | 10 |

## 4.3 Differences in production parameters and inflammation, metabolic and oxidative biomarkers between APR+ cows and non-APR cows

In Table 5, a significant difference is observed in the production parameters within the inflammation cluster and farm for DMI ($p < 0.05$). APR+ cows have a lower DMI compared to non-APR cows. However, there is no significant difference in milk production. As a result, the Milk production/DMI tends to be higher ($p = 0,056$) in APR+ cows than in non-APR cows.

As blood inflammation parameters were used for the clustering, all 3 inflammation markers differed ($p < 0,05$, Hp and albumin:Globulin) or tended to differ ($p = 0,06$, SAA) between inflammation clusters.

Strong farm effects were observed on several metabolic and oxidative parameters (Table 5). Among the metabolic parameters, BHB (day -7) and NEFA (day 21) significantly differed between the two inflammation clusters. Additionally, average NEFA, insulin (day -7), and average insulin showed a tendency to differ between clusters. However, none of the oxidative parameters differed between the two inflammation clusters.

***Table 5.*** *The differences in the production parameters and the metabolic and inflammatory markers between the inflammation clusters: acute phase response + diseased (APR+) and no acute phase response (non-APR) with farm as a factor. Average metabolic markers mean the average value of respective metabolic markers in the samples collected on days 3, 6, 9 and 21 relative to the birth of the calf.*

| | APR+ | Non-APR | SEM | Farm | Clusters | Farm x Cluster |
|---|---|---|---|---|---|---|
| DMI (kg/d) | 18,4 | 20,8 | 0,379 | 0,026 | 0,001 | 0,295 |
| Milk production (kg/d) | 36,5 | 38,1 | 0,719 | 0,372 | 0,434 | 0,867 |
| Milk kg/kg DMI | 2,01 | 1,86 | 0,0465 | 0,013 | 0,056 | 0,340 |
| | | | | | | |
| Inflammation markers (day 21) | | | | | | |
| HP (mg/L) | 331 | 136 | 30,0 | 0,963 | 0,040 | 0,099 |
| SAA (mg/L) | 67,3 | 18,8 | 7,32 | 0,918 | 0,065 | 0,007 |
| Albumin:Globulin (mol/mol) | 0,620 | 0,816 | 0,0169 | 0,032 | 1,13E-07 | 0,232 |
| | | | | | | |
| Metabolic biomarkers | | | | | | |
| Glucose (day 21) (mmol/L) | 3,09 | 3,11 | 0,0579 | 0,839 | 0,383 | 0,141 |
| BHB (day -7) (mM) | 0,60 | 0,64 | 0,0201 | 0,001 | 0,038 | 0,097 |
| BHB (day 21) (mM) | 1,27 | 1,00 | 0,0766 | 0,959 | 0,174 | 0,783 |
| Average BHB (mM) | 1,15 | 0,91 | 0,170 | 0,546 | 0,149 | 0,346 |
| NEFA (day -7) (mM) | 0,21 | 0,18 | 0,0206 | 0,373 | 0,329 | 0,617 |
| NEFA (day 21) (mM) | 0,72 | 0,52 | 0,140 | 0,701 | 0,027 | 0,594 |
| Average NEFA (mM) | 0,73 | 0,57 | 0,113 | 0,330 | 0,068 | 0,770 |
| IGF-1 (day -7) (µg/L) | 178 | 186 | 5,54 | 0,001 | 0,395 | 0,017 |
| IGF-1 (day 21) (µg/L) | 121 | 167 | 32,0 | < 0,001 | 0,129 | 0,835 |
| Average IGF-1 (µg/L) | 118 | 163 | 32,0 | < 0,001 | 0,113 | 0,925 |
| Insulin (day -7) (µg/L) | 0,77 | 1,07 | 0,212 | 0,021 | 0,084 | 0,654 |
| Insulin (day 21) (µg/L) | 0,34 | 0,36 | 0,0154 | 0,183 | 0,545 | 0,357 |
| Average insulin (µg/L) | 0,24 | 0,36 | 0,848 | 0,180 | 0,052 | 0,447 |
| | | | | | | |
| Oxidative markers (day 21) | | | | | | |
| GSSH (%) | 7,92 | 8,90 | 0,692 | 0,034 | 0,456 | 0,997 |
| GPx (U/mL) | 0,06 | 0,05 | 0,00462 | 0,628 | 0,461 | 0,891 |
| MDA (mmol/L) | 4,80 | 4,67 | 0,0957 | 0,002 | 0,570 | 0,989 |
| ORAC (µM) | 10,6 | 10,2 | 0,311 | < 0,001 | 0,614 | 0,126 |
| SOD (U/mL) | 14,3 | 11,0 | 2,32 | 0,130 | 0,329 | 0,385 |

### 4.3.1 Differences in bacterial composition

None of the three alpha diversity indices (Shannon index, Simpson index and Observed ASVs) and in none of the three types of samples (rumen, faeces -7 and +21) differences in the bacterial communities were observed between the inflammation clusters.

Table 6 shows the results of the beta diversity tests, specifically Bray-Curtis and weighted UniFrac. The farm effects on bacterial composition are very evident, as there is a significant difference in each sample with both tests ($p << 0,05$). Despite the farm effects, there was a tendency for a difference ($p = 0,08$) in rumen bacterial composition between two inflammation clusters for the rumen samples.

***Table 6**. P-values per sample type obtained from beta diversity tests called Bray-Curtis and weighted UniFrac with farm as a factor*

| | Bray-Curtis | | Weighted Unifrac | |
|---|---|---|---|---|
| | Inflammation | Farm | Inflammation | Farm |
| Rumen +21 | 0,055 | 0,001 | 0,128 | 0,001 |
| Faeces +21 | 0,422 | 0,001 | 0,544 | 0,046 |
| Faeces -7 | 0,223 | 0,001 | 0,215 | 0,001 |

Figure 10 shows the corresponding graphics for the Bray Curtis test of Table 6.

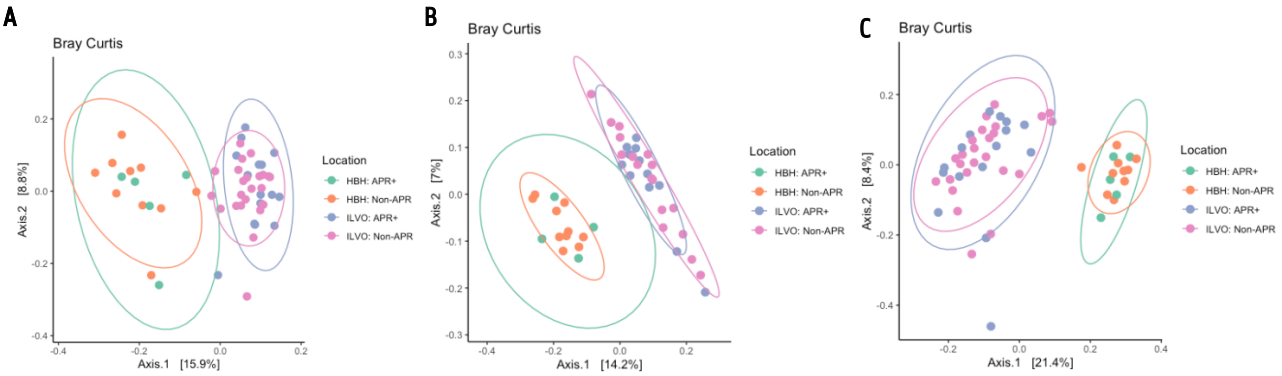


***Figure 10.** Principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity indices at ASV level, of the bacterial community structure of following sample types: A: Rumen, B: Faeces (-7) and C: Faeces (+21). APR: acute phase response + diseased cows (APR+) and non-APR: non-acute phase response cows based on the inflammatory parameters.*

A DESeq2 test was performed in R at the ASV, phylum, and genus levels to determine which bacterial groups showed differential abundance within the inflammation cluster.

When examining the inflammation-based clusters at the phylum level, four bacterial phyla were identified as differentially abundant between APR+ and non-APR cows in rumen samples collected on day 21. Additionally, at the genus level, one bacterial genus was found to differ significantly between the APR+ and non-APR groups. The corresponding Log2FoldChange and p-values are shown in Table 7. For these phyla and genus all the Log2FoldChange values are negative which means that there has been an increase of these bacteria in the APR+ group compared to non-APR cows.

***Table 7.*** *Differential sequence analysis within kingdom Bacteria found in rumen between the non-acute phase and acute phase + diseased animals. A positive value of the Log2FoldChange means that the bacterial abundance was increased in the non-APR group while a negative value indicates an increase in the APR+ group.*

| Genus level | | | | | | |
|---|---|---|---|---|---|---|
| Log2FoldChange | P-value | Phylum | Class | Order | Family | Genus |
| -1,053 | 0,00131 | *Verrucomicrobia* | *Verruco-5* | *WCHB1-41* | *RFP12* | (Unidentified) |
| **Phylum level** | | | | | | |
| -1,025 | 0,0112 | *Cyanobacteria* | | | | |
| -0,726 | 0,0101 | *Verrucomicrobia* | | | | |
| -0,569 | 0,00969 | *Synergistetes* | | | | |
| -2,060 | 0,00623 | *Elusimicrobia* | | | | |

No bacterial taxa showed differences between inflammation clusters in faecal samples (day -7 and +21).

### 4.3.2 Differences in archaeal composition

The alpha diversity indices did not show significant differences in the archaeal communities across all types of samples (rumen, faeces -7 and +21) between inflammation clusters.

In Table 8, a significant difference was observed in the rumen samples with the weighted UniFrac test between the inflammation clusters. Next, a tendency for a difference between inflammation clusters is observed in the Weighted Unifrac test on the faeces (day -7) samples (p = 0,088). A strong farm effect was evident between inflammation clusters on archaeal composition in all three types of samples.

***Table 8****. P-values per sample type obtained from beta diversity tests called Bray-Curtis and weighted UniFrac with farm as a factor*

| | Bray-Curtis | | Weighted Unifrac | |
|---|---|---|---|---|
| | Inflammation | Farm | Inflammation | Farm |
| Rumen +21 | 0,108 | 0,001 | 0,024 | 0,001 |
| Faeces +21 | 0,243 | 0,001 | 0,444 | 0,001 |
| Faeces -7 | 0,629 | 0,001 | 0,088 | 0,001 |

Figure 11 shows the corresponding graphics for the Weighted Unifrac test from table 9.

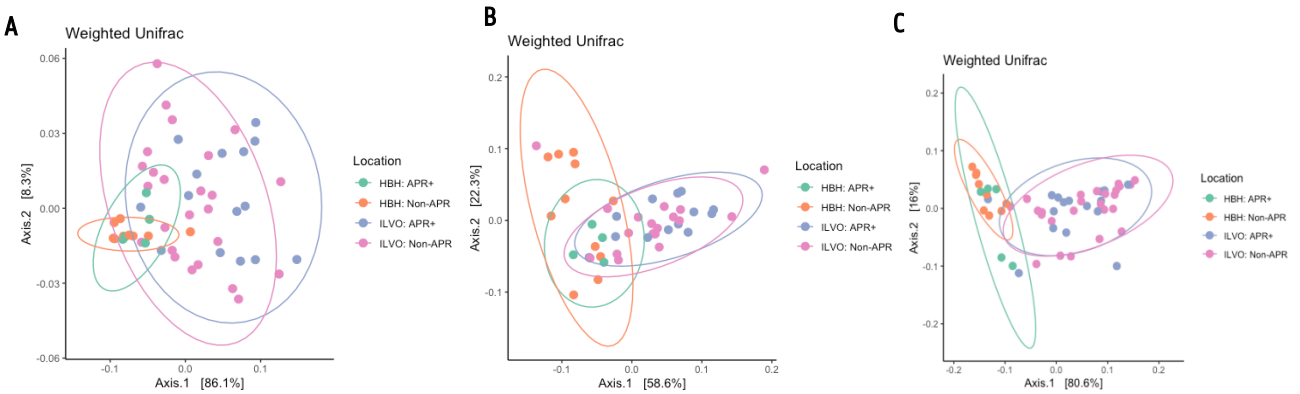


***Figure 11.*** *Principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity indices at ASV level, of the archaeal community structure of following sample types: A: Rumen, B: Faeces (-7) and C: Faeces (+21). APR: acute phase response + diseased cows (APR+) and non-APR: non-acute phase response cows based on the inflammatory parameters.*

A Deseq2 analysis did not show any rumen archaeal taxa differed between the inflammation clusters. In the faeces (day +21) samples, an archaeal phylum was observed to be more prevalent in APR+ group compared to the non-APR group. In the faeces (day -7) samples, two archaea at the ASV level were observed to be more prevalent in the non-APR group than the APR+ group.

***Table 9.*** *Differential sequence analysis within kingdom Archaea found in faeces (+21 and -7) between non-acute phase and acute phase + diseased animals. A positive value of the Log2FoldChange means that the Archaea was increased in the non-APR group, while a negative value indicates an increase in the APR+ group.*

| Log2FoldChange | P-value | Phylum | Order | Family | Genus | Species |
|---|---|---|---|---|---|---|
| Faeces (+21): phylum level | | | | | | |
| -2,951 | 0,030 | *Thermoplasmatota* | | | | |
| Faeces (-7): ASV level | | | | | | |
| 6,485 | < 0,001 | *Thermoplasmatota* | *Methanomassiliicoccales* | *Methanomethylophilaceae* | MX-02 | (Unidentified) |
| 5,280 | < 0,001 | *Thermoplasmatota* | *Methanomassiliicoccales* | *Methanomethylophilaceae* | UBA71 | sp006954425 |

### 4.3.3 Differences in protozoal composition

As protozoa are absent in the faecal samples, the microbial analysis is only performed in rumen samples. The three different alpha diversity indices showed no significant differences in the diversity of protozoa in the rumen samples.

Beta diversity analysis showed a strong farm effect. As shown in Table 10, only the Weighted Unifrac shows a difference ($P < 0,05$) in the inflammation clusters. However, after performing a DESeq2 analysis, no difference in the abundance of protozoa groups at genus and ASV level was observed between APR+ and non-APR cows.

***Table 10*** *P-values per sample type obtained from beta diversity tests called Bray-Curtis and weighted UniFrac with farm as a factor*

| | Bray-Curtis | | Weighted Unifrac | |
|---|---|---|---|---|
| | Inflammation | Farm | Inflammation | Farm |
| Rumen +21 | 0,116 | 0,001 | 0,044 | 0,020 |

Figure 12 shows the corresponding graphics for the Weighted Unifrac test from Table 11.

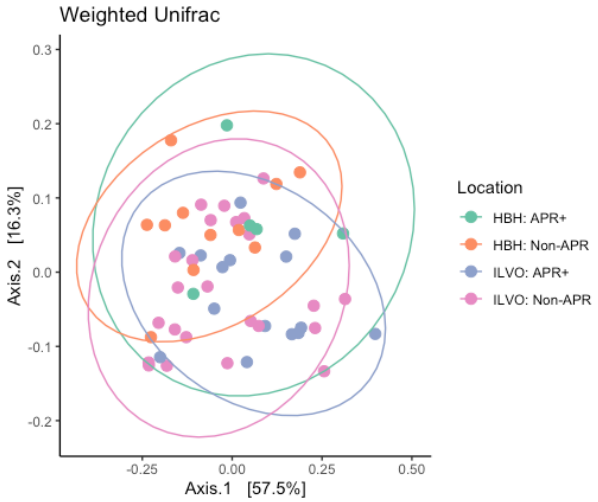


***Figure 12.*** *Principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity indices at ASV level, of the rumen protozoal community structure. APR: acute phase response + diseased cows (APR+) and non-APR: non-acute phase response cows based on the inflammatory parameters.*

### 4.3.4 Differences in fungal composition

The three different alpha diversity indices showed no significant differences in diversity in the fungal communities in the rumen and faeces (day 21). However, for the faeces (day -7) the Shannon and Simpson indices did show a significant difference.

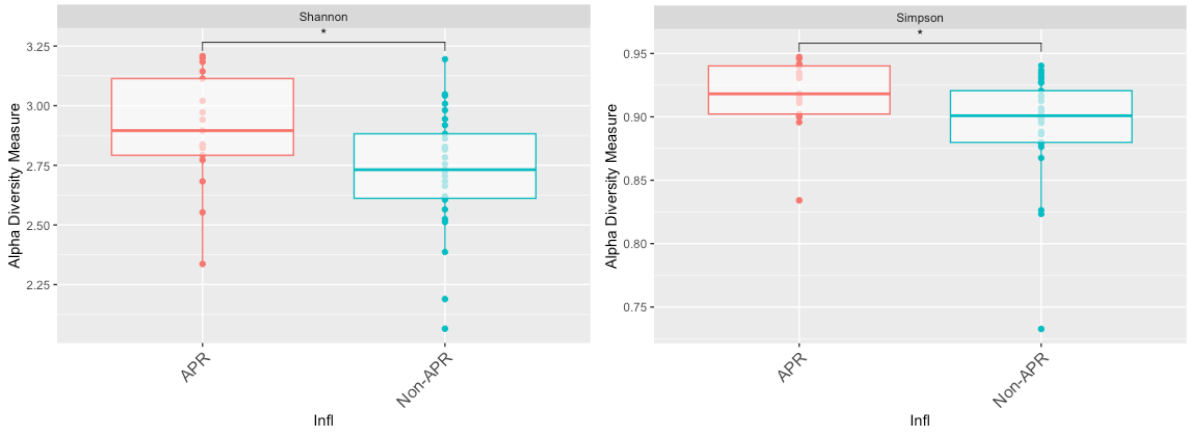


***Figure 13.*** *Alpha diversity tests (left: Shannon and right: Simpson) of the fungal community in the faeces samples (day -7) of the inflammation clusters APR: acute phase response + diseased cows (APR+) vs non-APR: no acute phase response.*

Table 11 shows the results of the Bray-Curtis test. Apart from the farm effect, no significant difference in the inflammation cluster was observed when using Bray-curtis and weighted unifrac distances. Additionally, the farm effect is also very evident.

***Table 11.*** *P-values per sample type obtained from beta diversity tests called Bray-Curtis and weighted UniFrac with farm as a factor*

| | Bray-Curtis | | Weighted Unifrac | |
|---|---|---|---|---|
| | Inflammation | Farm | Inflammation | Farm |
| Rumen +21 | 0,579 | 0,001 | 0,601 | 0,092 |
| Faeces +21 | 0,509 | 0,001 | 0,212 | 0,001 |
| Faeces -7 | 0,285 | 0,001 | 0,207 | 0,001 |

In addition to the Bray-Curtis test, a DESeq2 analysis was also performed in R, but no significant differences in fungi were found between the APR+ and non-APR groups at genus and ASV level.

## 4.4 Differences in production parameters and metabolic, inflammatory and oxidative biomarkers between SloBHB and LoBHB cows

In Table 12, strong farm effects were shown in DMI and MP/DMI. However, the production parameters did not differ between the two metabolic clusters, SloBHB and LoBHB. We also observed an interaction effect between farm and metabolic clusters.

No significant findings were established for any inflammation marker between the metabolic clusters. Strong farm effects were observed in several of metabolic and oxidative biomarkers. Additionally, a significant difference between the LoBHB and SloBHB groups was observed for glucose, BHB (day 21 and average), NEFA (day 21 and average) and MDA (day 21).

Finally, a significant difference was observed in the interaction between farm and trajectory for the following three metabolic parameters: NEFA (average) and IGF-I (day 21 and average).

***Table 12.*** *The differences in the production parameters and the metabolic and inflammatory markers between the metabolic clusters: LoBHB and SloBHB with farm as a covariate. Average metabolic markers mean the average value of respective metabolic markers in the samples collected on days 3, 6, 9 and 21 relative to the birth of the calf.*

| | LoBHB | SloBHB | SEM | Farm | Trajectory | Farm x Trajectory |
|---|---|---|---|---|---|---|
| DMI (kg/d) | 20,1 | 19,6 | 0,372 | 0,001 | 0,146 | 0,046 |
| Milk production (kg/d) | 37,0 | 38,3 | 0,713 | 0,917 | 0,789 | 0,571 |
| Milk kg/kg DMI | 1,87 | 1,96 | 0,0478 | 0,007 | 0,234 | 0,273 |
| | | | | | | |
| Inflammation markers (day 21) | | | | | | |
| HP (mg/L) | 201 | 118 | 29,4 | 0,387 | 0,157 | 0,663 |
| SAA (µg/mL) | 31,0 | 21,4 | 7,24 | 0,646 | 0,692 | 0,668 |
| Albumin:Globulin | 0,75 | 0,77 | 0,0168 | 0,160 | 0,586 | 0,122 |
| | | | | | | |
| Metabolic biomarkers | | | | | | |
| Glucose (day 21) (mmol/L) | 3,18 | 2,74 | 0,0593 | 0,671 | 1,91E-05 | 0,059 |
| BHB (day -7) (mM) | 0,63 | 0,58 | 0,0198 | 0,001 | 0,243 | 0,744 |
| BHB (day 21) (mM) | 0,83 | 1,60 | 0,0731 | 0,258 | 1,53E-06 | 0,817 |
| Average BHB (mM) | 0,81 | 1,21 | 0,0532 | 0,318 | 2,26E-08 | 0,954 |
| NEFA (day -7) (mM) | 0,18 | 0,17 | 0,0170 | 0,302 | 0,954 | 0,534 |
| NEFA (day 21) (mM) | 0,52 | 0,66 | 0,0414 | 0,870 | 0,049 | 0,254 |
| Average NEFA (mM) | 0,54 | 0,77 | 0,0397 | 0,086 | 0,001 | 0,017 |
| IGF-1 (day -7) (µg/L) | 207 | 196 | 22,9 | < 0,001 | 0,322 | 0,174 |
| IGF-1 (day 21) (µg/L) | 154 | 170 | 16,5 | 0,005 | 0,155 | 0,003 |
| Average IGF-1 (µg/L) | 149 | 149 | 13,5 | 0,010 | 0,108 | 0,012 |
| Insulin (day -7) (µg/L) | 1,03 | 0,78 | 0,0982 | 0,056 | 0,389 | 0,971 |
| Insulin (day 21) (µg/L) | 0,38 | 0,24 | 0,0327 | 0,176 | 0,125 | 0,989 |
| Average insulin (µg/L) | 0,36 | 0,22 | 0,0297 | 0,056 | 0,121 | 0,740 |
| | | | | | | |
| Oxidative markers (day 21) | | | | | | |
| GSSH (%) | 8,90 | 8,93 | 0,494 | 0,070 | 0,249 | 0,059 |
| GPx (U/mL) | 0,06 | 0,05 | 0,00342 | 0,761 | 0,689 | 0,629 |
| MDA (mmol/L) | 4,36 | 5,39 | 0,190 | 0,002 | 0,012 | 0,408 |
| ORAC (µM) | 11,2 | 9,44 | 0,360 | 0,010 | 0,176 | 0,425 |
| SOD (U/mL) | 12,9 | 11,5 | 2,08 | 0,041 | 0,981 | 0,859 |

### 4.4.1 Differences in bacterial composition

The three different alpha diversity indices showed no significant differences in diversity in the bacterial communities across all types of samples (rumen, faeces -7 and +21).

Table 13 shows the results of the Bray-Curtis test. Apart from the farm effect, no significant differences in the metabolic cluster were observed in any of the 3 types of samples collected.

***Table 13.*** *P-values per sample type obtained from beta diversity tests called Bray-Curtis and UniFrac with farm as a factor*

| | Bray-Curtis | | Weighted Unifrac | |
|---|---|---|---|---|
| | Metabolic | Farm | Metabolic | Farm |
| Rumen +21 | 0,387 | 0,001 | 0,356 | 0,001 |
| Faeces +21 | 0,173 | 0,001 | 0,761 | 0,027 |
| Faeces -7 | 0,264 | 0,001 | 0,588 | 0,001 |

When examining the metabolic-based clusters at the ASV level, three bacteria were identified as differentially abundant between SloBHB and LoBHB cows in faeces samples collected on day 21. The corresponding Log2FoldChange and p-values are shown in Table 14. Both bacteria were more abundant in the LoBHB cluster.

***Table 14.*** *Differential sequence analysis at ASV level within kingdom Bacteria found in faeces +21 between SloBHB and LoBHB animals. A positive value of the Log2FoldChange means that the Bacteria was increased in the SloBHB group, while a negative value indicates an increase in the LoBHB group.*

| Log2FoldChange | P-value | Phylum | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|---|---|
| -6,704 | < 0,001 | *Bacteroidetes* | *Bacteroidia* | *Bacteroidales* | (Unidentified) | (Unidentified) | (Unidentified) |
| -5,614 | < 0,001 | *Bacteroidetes* | *Bacteroidia* | *Bacteroidales* | *Bacteroidaceae* | *5-7N15* | (Unidentified) |

### 4.4.2 Differences in archaeal composition

The three different alpha diversity tests showed no significant differences in diversity in the archaeal communities across all types of samples (rumen, faeces -7 and +21).

Table 15 shows the results of the Bray-Curtis test. Apart from the farm effect, no significant difference between the metabolic cluster was observed in any of the 3 sample types.

***Table 15.*** *P-values per sample type obtained from beta diversity tests called Bray-Curtis and UniFrac with farm as a factor*

| | Bray-Curtis | | Weighted Unifrac | |
|---|---|---|---|---|
| | Metabolic | Farm | Metabolic | Farm |
| Rumen +21 | 0,428 | 0,001 | 0,260 | 0,001 |
| Faeces +21 | 0,435 | 0,001 | 0,741 | 0,001 |
| Faeces -7 | 0,787 | 0,001 | 0,688 | 0,001 |

Only in the faecal samples, an abundance difference was observed at ASV level in archaeal groups between the LoBHB and SloBHB groups. In the faeces (day +21) samples, the archaeal genus *Methanobacteriota A1229* was observed to be more prevalent in SloBHB group compared to the LoBHB group (Table 16).

***Table 16.*** *Differential sequence analysis at ASV level within kingdom Archaea found in faeces +21 between SloBHB and LoBHB animals. A positive value of the Log2FoldChange means that the Archaea was increased in the SloBHB group, while a negative value indicates an increase in the LoBHB group.*

| Log2FoldChange | P-value | Phylum | Class | Order | Family | Genus | Species |
|---|---|---|---|---|---|---|---|
| 1,262 | 0,001 | *Methano-bacteriota A 1229* | *Methano-bacteria* | *Methano-bacteriales* | *Methano-bacteriaceae* | *Methano-brevibacter D 1148* | *Methano-brevibacter D 1148 olleyae* |

### 4.4.3 Differences in protozoal composition

The three different alpha diversity indices showed no significant differences in diversity in the protozoal communities across the rumen samples.

The p-values of the beta diversity tests shown in Table 18 indicate no significant differences in protozoa between the LoBHB and SloBHB groups. Only a farm effect was observed. This was confirmed by a DESeq2 test.

***Table 17.*** *P-values per sample type obtained from beta diversity tests called Bray-Curtis and UniFrac with farm as a factor*

| | Bray-Curtis | | Weighted Unifrac | |
|---|---|---|---|---|
| | Metabolic | Farm | Metabolic | Farm |
| Rumen +21 | 0,312 | 0,001 | 0,605 | 0,004 |

### 4.4.4 Differences in fungal composition

The three different alpha diversity indices showed no significant differences in diversity in the fungal communities across all types of samples (rumen, faeces -7 and +21).

In Table 18, a significant difference ($p < 0,05$) was observed with the Bray-Curtis test on the rumen and faeces (-7) samples between the metabolic clusters. Additionally, the Weighted Unifrac test also showed a significant difference for the rumen samples. The farm effect is very evident, as both tests indicated a significant difference in all samples.

***Table 18.*** *P-values per sample type obtained from beta diversity tests called Bray-Curtis and UniFrac with farm as a factor*

| | Bray-Curtis | | Weighted Unifrac | |
|---|---|---|---|---|
| | Metabolic | Farm | Metabolic | Farm |
| Rumen +21 | 0,017 | 0,001 | 0,005 | 0,022 |
| Faeces +21 | 0,037 | 0,001 | 0,055 | 0,001 |
| Faeces -7 | 0,543 | 0,001 | 0,380 | 0,001 |

Figure 14 shows the corresponding graphics for the Bray Curtis test from Table 20.

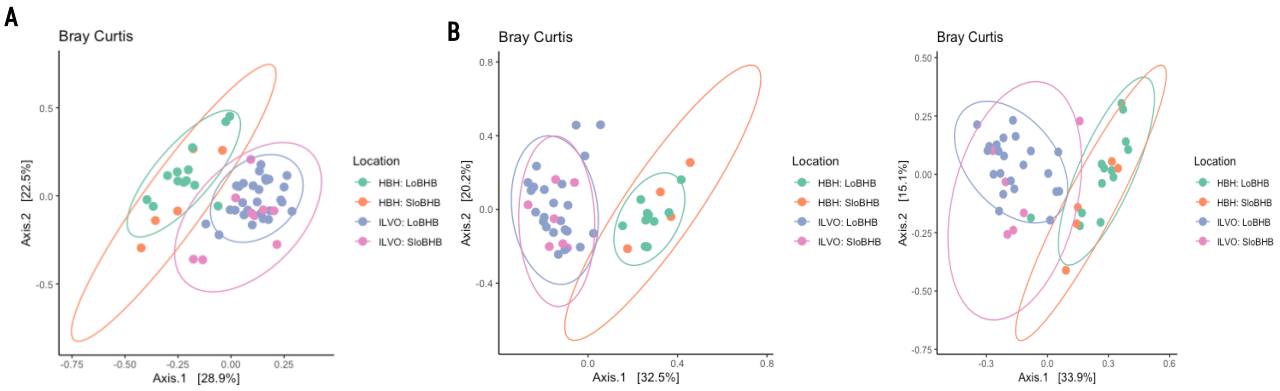


***Figure 14.*** *Principal coordinate analysis (PCoA), based on Bray-Curtis dissimilarity indices at ASV level, of the fungal community structure of following sample types: A: Rumen, B: Faeces (-7) and C: Faeces (+21). SloBHB and LoBHB cows based on BHB levels observed in the blood.*

After performing a DESeq2 analysis in R, one species was identified at the ASV level and three at the genus level, all in faeces samples, to differ between the clusters. The corresponding Log2FoldChange and p-values are shown in Tables 19 and 20. All the Log2FoldChange values are positive, indicating that these fungi are more prevalent in the SloBHB group.

***Table 19.*** *Differential sequence analysis at ASV level within kingdom Fungi found in faeces (-7) between SloBHB and LoBHB animals. A positive value of the Log2FoldChange means that the Bacteria was increased in the SloBHB group, while a negative value indicates an increase in the LoBHB group.*

| Log2FoldChange | P-value | Phylum | Order | Family | Genus | Species |
|---|---|---|---|---|---|---|
| 10,981 | < 0,001 | *Neocallimastigomycota* | *Neocallimastigales* | **genera incertae sedis* | *Buwchfawromyces* | *Buwchfawromyces eastonii* |

***Table 20.*** *Differential sequence analysis at genus level within kingdom Fungi found in faeces (-7 and +21) between SloBHB and LoBHB animals. A positive value of the Log2FoldChange means that the Bacteria was increased in the SloBHB group, while a negative value indicates an increase in the LoBHB group.*

| Faeces (-7) | | | | | |
|---|---|---|---|---|---|
| Log2FoldChange | P-value | Phylum | Order | Family | Genus |
| 10,754 | 0,001 | *Neocallimastigomycota* | *Neocallimastigales* | **genera incertae sedis* | *Buwchfawromyces* |
| Faeces (+21): | | | | | |
| 2,453 | < 0,001 | *Neocallimastigomycota* | *Neocallimastigales* | *Piromycetaceae* | *Piromyces* |
| 6,591 | < 0,001 | *Neocallimastigomycota* | *Neocallimastigales* | *Neocallimastigaceae* | *Pecoramyces* |

# 5 DISCUSSION

The transition period, three weeks before and three weeks after calving, is a stressful time for dairy cows because their bodies undergo physiological and metabiological changes and need to provide energy for the onset of milk synthesis. Some cows go through this period less smoothly than others and consequently may experience imbalances and metabolic disorders such as mastitis, SARA, ketosis, etc. Therefore, there is likely an inter-animal difference in the adaptation to the metabolic changes among dairy cows during the transition period. There are, of course, various factors that contribute to each animal's uniqueness including genetics, management conditions, etc. However, this research focused on the microbial community in the rumen and hindgut, using faecal samples as a proxy for the latter. This study tried to see if there is a link between the microbial composition of the cow's gastrointestinal tract, specifically the microbial composition of the rumen and hindgut, and the health status of the cow specifically metabolic and inflammatory status during the transition period.

Results could be summarised as follows: No differences were observed in alpha diversity between the metabolic and inflammatory clusters for all the samples except for the fungal community in the faeces samples (day -7) between the inflammation clusters. Additionally, the beta diversity indices of rumen bacteria, archaea, and protozoa differed or tended to differ between the inflammation clusters, while differences in rumen and faecal (day +21) fungi were observed between the metabolic clusters. In the archaeal community, there was also a tendency for a difference in faeces samples (day -7) between the inflammation clusters. Differences in rumen and faecal (day +21) fungal composition were observed between the metabolic clusters. In the archaeal community, there was also a tendency for a difference in faeces samples (day -7) between the inflammation clusters. Only, a limited number of indicator genera, phyla, or ASVs could be identified within the rumen bacterial and faecal archaeal communities, differentiating between the immunological clusters, while no microbial indicator genus or ASV could be identified in the rumen to distinguish between metabolic clusters. In the faecal samples 21 days post-partum, indicator bacterial, archaeal, and fungal ASVs or genera were identified as differing between metabolic clusters. Hence, overall diversity differences as well as the identification of indicators to differentiate between the different health clusters were limited.

Several factors might have played a role in this, such as the use of samples from two farms. In section 5.1., the reasons for choosing an experimental setup involving two farms and the associated challenges are discussed. Additionally, in this study, clusters were identified that exhibited statistically significant differences in immunological (inflammatory clusters) and metabolic parameters (metabolic clusters). However, these statistical differences may not necessarily correspond to biologically relevant distinctions. Therefore, the absolute values of immunological and metabolic parameters across the various groups will be compared with existing literature to assess their biological significance. Thirdly, microbial taxonomic data have been used in this study to assess differences between health clusters, while microbial functionality and activity have not been considered. These aspects, along with the indicator species and their potential biological links to the clusters, are discussed below.

## 5.1 Farm effect

In this study, samples from cows originating from two farms were used. This approach potentially makes the differences more robust and valid across farms. By including both cluster and farm in the model, the 'cross-farm' differences between clusters can be identified. A farm effect was observed in the microbial communities of both the rumen and hindgut, indicating a significant difference in the microbial communities between cows from ILVO and those from Hooibeekhoeve, despite similar management conditions at both farms. This farm effect may present challenges in discovering indicator organisms. Various factors may have contributed to this effect, which will be discussed below.

(Ryu & Davenport, 2024)All cows in this study were multiparous Holstein Friesians. Host genetics influence the composition of the microbial community in the GIT (Ryu & Davenport, 2024). However, since all the cows are of the same breed, this minimizes the likelihood that genetic differences are the primary cause of variations between the farms. Further, environmental factors play an important role in shaping the microbial composition of the cows' gut (Cholewińska et al., 2021). For instance, microbes from the immediate environment - such as soil, water, and other animals - can have a considerable influence. Another major factor is the cows' feed, which is known to greatly affect gut microbial composition. While overall, dietary characteristics are comparable across both farms, feeds and dietary composition as well as other farm practices differed, which might have contributed to variations in the gut microbiota. For example, a difference in milking management between the two farms was observed. Hooibeekhoeve employed an automatic milking system, allowing cows to autonomously decide when to be milked, potentially leading to more frequent milking. In contrast, cows at ILVO were milked twice a day at fixed times. Cows milked more frequently may require additional energy, which could influence both their feed intake and feeding patterns, such as the frequency of feeding (Azizi et al., 2009). These differences in milking practices could, in turn, affect the composition of the gut microbial community.

It is also important to note that while differences in microbial communities between farms were observed, this does not necessarily imply differences in microbial function.

## 5.2 Production parameters and inflammatory, metabolic and oxidative biomarkers

The results for this section can be found in Tables 5 and 12. When investigating differences in metabolic parameters between inflammation clusters, a few parameters (BHB (day -7) and NEFA (day +21)) showed significant differences or tended (average NEFA, insulin (day -7) and average insulin) to differ between APR+ and non-APR cows. This suggests a possible association between inflammation and metabolic stress. However, no differences in inflammation parameters were observed between the metabolic clusters. Therefore, while inflammation seems to be related to metabolic stress, the reverse - that is, differences in metabolic clusters with respect to inflammation parameters - was not observed. This suggests that metabolic disorders can play a role in inflammation, although metabolic imbalance does not result in inflammation.

### 5.2.1 Differences in the inflammation-based clusters

(Sordillo & Raphael, 2013).Since the inflammation clusters were formed based on inflammation markers, significant differences (Hp and Albumin:Globulin ratio) or trends SAA) were observed between the two inflammation clusters for each of the inflammatory markers (Table 5). However, all cows experience an enhanced inflammatory status around calving. This is a natural response to the changes the body undergoes during the transition period. During the calving physical stress can occur, potentially leading to tissue damage. This, in combination with the metabolic and hormonal changes during the transition period, can further exacerbate the inflammatory response in cows. Additionally, it helps the cow in the recovery process (Bradford et al., 2015). However, it is crucial for the inflammation to subside after a few days. If it persists, it can lead to systemic inflammation and other health problems (Sordillo & Raphael, 2013).

To evaluate how much the obtained values of inflammatory markers deviate from expected values or those typically observed when inflammatory problems occur, our findings were compared with those of several studies. In a study by Huzzey et al. (2009), the potential of using haptoglobin (Hp) concentration as an indicator for metritis, a uterine infection, was investigated. The healthy group in this study had average Hp concentrations of 580 ± 120 mg/L on day +3 and 310 ± 80 mg/L on day +6. Cows with concentrations greater than 1 g/L had an increased risk of developing metritis (Huzzey et al., 2009). Although the measurements in the current thesis were obtained on day +21, the Hp concentrations (APR+: 331 mg/L and Non-APR: 136 mg/L) in this thesis are considerably lower than the threshold value considered in Huzzey's study for increased risks of an inflammatory problem. For serum amyloid A (SAA), concentrations in healthy dairy cattle range from 0 to 70 mg/L (Trela et al., 2022). The SAA concentrations in both the APR+ and non-APR groups fall within this range. However, the average concentration in the APR+ group is close to the upper limit, with a value of 67,3 mg/L. Lastly, the albumin:globulin ratio for clinically healthy cows is 0.88 ± 0.43 (Alberghina et al., 2011). The non-APR cluster is closest to this value, and the APR+ cluster also falls within this interval.

In conclusion, while there are statistical differences in inflammatory parameters between the non-APR and APR+ groups, the values for the inflammatory impaired group are not exceptionally high. This suggests that, from a physiological or biological perspective, the differences between the clusters may be relatively modest.

### 5.2.2 Differences in the metabolic-based clusters

Table 12 shows significant differences in several metabolic parameters, namely glucose, BHB (day +21), NEFA (day +21), average BHB, and average NEFA, as expected. These findings align with the results of Girma et al. (2024), and the clustering in this thesis is also based on Girma's work with clustering based on the BHB trajectory. In Girma's study an additional third cluster was identified (QuiBHB), where animals had an initially higher BHB concentration than the other clusters and an average BHB of 2,4 mM. This QuiBHB cluster was associated with lower DMI, and milk yield compared to the SloBHB and LoBHB clusters. Although the clustering of Girma's study was maintained in the current thesis, only a subgroup of animals was included here, as the number of cows with rumen and faecal samplings was limited to 40 at ILVO and 20 at HBH. As a result, only 4 animals of the subset belonged to the more extreme QuiBHB group and hence, we could not include the QuiBHB in this study.

To evaluate how the obtained values of metabolic biomarkers deviate from expected values, the findings of several studies will be examined. First, BHB concentration in the blood is an accepted method for diagnosing ketosis. Concentrations of 1,2-1,4 mmol/L (Suthar et al., 2013) are classified as subclinical, and concentrations higher than 3 mmol/L are classified as clinical (Oetzel et al., 2007). The BHB (day +21) concentrations in Table 12 indicate that the cows in the SloBHB cluster (1,60 mmol/L) falls outside the range of subclinical ketosis, indicating that – according to the classical definition, this group would already be tending towards clinical ketosis. Additionally, the average BHB (day 3, 6, 9 and 21) concentration in the SloBHB cluster (1,21 mmol/L) falls within the

range for diagnosing subclinical ketosis. However, SloBHB cows exhibited higher milk yield , suggesting that the elevated BHB in these cows may reflect an adaptive response, rather than a metabolic disorder (Girma et al., 2024).

Furthermore, normal glucose levels for cows range between 2,22 and 3,33 mmol/L. Cows are considered hypoglycemic if their glucose concentration is below 2,22 mmol/L, and hyperglycemic if it exceeds 3,33 mmol/L (Adewuyi et al., 2005). In Table 12, the concentrations fall within the normal range although the SloBHB cluster has a lower glucose concentration compared to the LoBHB cluster.
During the period leading up to calving, NEFA concentrations typically rise gradually, with levels generally ranging from 0,5 to 1 mM. In the week immediately before calving, NEFA concentrations can vary from less than 0,5 to 2 mM. After calving, NEFA levels drop sharply. If a cow's NEFA concentration exceeds 0,6 mM post-calving, it indicates a severe negative energy balance (NEB) (Adewuyi et al., 2005). In Table 12, the NEFA (day +21) concentration in the SloBHB cluster is on the higher side (0,66 mM), indicating a greater NEB in these cows.
In early lactation, a drop in plasma insulin concentration from 1.36 ± 0.21 to 0.85 ± 0.17 µg/L is observed (Dębski et al., 2017). Notably, the SloBHB (day -7 and +21 and average) cluster exhibit significantly lower insulin concentrations compared to the LoBHB cluster. But the LoBHB group also has a relatively low insulin value after calving compared to the values reported in the literature. After calving, a cow's energy demands increase significantly due to the onset of milk production and the body's need to recover from the birthing process. This energy demand triggers a reduction in insulin, which is a natural response to promote the mobilization of stored fat as an energy source. However, if the insulin drop is too pronounced, it can exacerbate the negative energy balance (NEB), leading to more severe metabolic stress.

In conclusion, although the SloBHB cluster indicates more mobilisation of body reserves, resulting in elevated BHB, this group maintained DMI and milk production. As such, cows in this cluster potentially are not metabolically impaired, but rather metabolically coping with a higher body fat mobilisation.

It should also be noted that both farms added propylene glycol (PG) to the animals' feed during the postpartum period if the animals showed signs of metabolic imbalance. Research suggests that PG helps restoring metabolic balance during early lactation (Zhang et al., 2020). Propylene glycol reduces the molar ratio of acetate to propionate in rumen volatile fatty acids because part of the PG is metabolized into propionate in the rumen. This could result in a decrease in ketone production. The remaining PG is directly absorbed and contributes to gluconeogenesis via pyruvate, which enhances energy supply. Additionally, plasma concentrations of NEFA and BHB generally decrease (Nielsen & Ingvartsen, 2004). Therefore, the use of PG may have potentially reduced the differences between the metabolic clusters.

## 5.3 Metataxonomic analysis

Metataxonomic analysis also has its limitations, which will be further explored below.

### 5.3.1 Metataxonomic databases

The choice of databases for taxonomic classification can significantly impact the results obtained. An outdated or infrequently updated database might overlook recently discovered microorganisms. Conversely, even some up-to-date databases, which are still under development, may not yet include all known microorganisms. For bacteria, an updated and regularly maintained database was used, ensuring comprehensive coverage of known bacterial taxa. For archaea classification, three different databases were available: RDB, Greengenes2, and the Silva database. When our sequences were classified using these databases, each produced different results. In this study, the full-length Greengenes2 database (release 2022.10) was used to assign taxonomy to archaea. Given that this database is up-to-date, we do not anticipate significant issues, though future research should

explore any remaining discrepancies. On the other hand, the database employed for protozoa taxa dates back to 2015, meaning it lacks more recent discoveries. Finally, the database used for fungi is recent but still in the development phase.

### 5.3.2 Alpha diversity

It is remarkable that no difference in alpha diversity indices was observed, except for faecal fungi (day -7) between the metabolic clusters. Previous research indicated that lower richness in the microbiome gene and taxa is associated with higher feed efficiency. Feed efficiency refers to a cow's ability to extract energy from its feed. The microbiomes of inefficient cows exhibit greater diversity in microbial species and metabolic pathways, leading to a broader range of metabolites. In contrast, efficient cows have simpler, more specialized microbiomes that produce higher concentrations of metabolites essential for the host's energy needs. This suggests that, while inefficient microbiomes are more complex, efficient microbiomes are better optimized to meet the host's metabolic demands (Shabat et al., 2016). Every cow experiences a NEB during the transition period, but the intensity can vary from one cow to another. One might expect that cows with a less severe NEB, would be "more efficient". However, this should be approached with caution, as there are also cows with a high NEB that maintain high milk production.

## 5.4 Differences in the microbial community in rumen and faeces

### 5.4.1 Differences in the microbial community in rumen and faeces between inflammation clusters

The observed differences between APR+ and non-APR clusters occur in bacterial or archaeal phyla, families, or genera that exhibit very low abundances. Additionally, the bacterial composition of the rumen is highly redundant. Therefore, caution should be taken to avoid overinterpretation.

Table 7 shows the bacteria identified as differentially abundant between APR+ and non-APR cows in rumen samples collected on day 21. All these bacteria are increased in the APR+ compared to the non-APR cows. Both the abundance of some minor phyla, namely Cyanobacteria, Verrucomicrobia, Synergistetes, and Elusimicrobia (average abundance of 0,55%, 0,33%, 0,17%, and 0,027%) and the family RFP12 (0,31%) belonging to the phylum Verrucomicobia, increased in the APR+ group. The family RFP12 remains poorly characterized, and they are one of the key members from the phylum Verrucomicobia. In our study, the family RFP12 contributes to >67% of the abundance of Verrucomicrobia. A study by Pinnell investigated microbial communities within the rumen of cattle, specifically focusing on how different micro-environments within the rumen harbour distinct microbial populations. They distinguished between three micro-environments in the rumen: fluid, fibrous pack, and mucosa. The Lefse method showed that four microbial groups were more abundant in the fibrous pack, including *RFP12* (*Verruco-5*) (Pinnell et al., 2022). In a study by (Meyer et al., 2021), cows were given a lipopolysaccharide (LPS) injection to induce an acute phase reaction in dairy cows. Immediately after the injection, feed intake was lower, accompanied by reduced rumination activity, so the retention time increased. This was beneficial for the fibrolytic bacteria. In a study by Tröscher-Mußotter et al. (2022), an increase in fibrolytic bacteria was also observed after the injection of LPS. This might possibly explain why RFP12 bacteria were observed in the APR+ cluster.
Furthermore, the presence of cyanobacteria is somewhat unusual, as the rumen is predominantly an anaerobic environment while cyanobacteria are aerobic. This presence may be attributed to the uptake of limited amounts of oxygen and the fermentation of polysaccharides in the rumen, which may not be completely anaerobic. It is possible that the cyanobacteria were taken up by the cows from their immediate environment (McGorum et al., 2015).

Table 9 shows the different archaea identified as differentially abundant between APR+ and non-APR cows in faeces samples (day -7 and +21). The average abundance for the phylum Thermoplasmatota is 0,10% in faeces (day +21). Furthermore, the observed

taxa, MX-02 and UBA71, have the following average proportions: 0,41% and 0,37% in faeces (day -7). Figure 7B also demonstrated that the *Methanomethylophilaceae* family is found in very low quantities in the faeces samples. In a study by Chen et al., fecal samples from parental and offspring pigs were used for metagenomic sequencing. This analysis identified, among other findings, that Thermoplasmatota at the phylum level, and MX-02 and UBA71 at the genus level, were associated with methylotrophic pathways. Additionally, they also observed that the methanogenesis function was positively correlated with fiber decomposition functions and negatively correlated with the starch decomposition function (Chen et al., 2024).

### 5.4.2 Differences in the microbial community in rumen and faeces between the metabolic LoBHB- and SloBHB-clusters

Table 14 shows the different bacteria identified as differentially abundant between SloBHB and LoBHB cows in faeces samples collected on day 21. The observed taxa, Bacteriodales and *5-7N15*, have the following average proportions: 6,08% and 5,93% in SloBHB and LoBHB respectively. These observed taxa were increased in the LoBHB cluster. Both belong to the order Bacteriodales, which can produce acetate (figure 2). Acetate is a VFA that can be used as a source of energy (Seshadri et al., 2018). According to Li et al. (2022), *5-7N15* had a significant positive correlation with the crude fiber digestibility (Li et al., 2022). This available energy source may have helped these cows experience less NEB compared to the SloBHB cluster.

Table 16 shows the different archaea identified as differentially abundant between SloBHB and LoBHB cows in faeces samples collected on day 21. *Methanobrevibacter D 1148 olleyae* was increased in SloBHB cluster. This species has an average abundancy of 7,48% in faeces (day +21). *Methanobrevibacter olleyae* is a hydrogenotrophic methanogens which can utilize $CO_2$, formate and $H_2$ for growth (Rea et al., 2007). Since methane production leads to an energy loss for the animal, this could be a possible explanation for why these archaea were observed in the SloBHB group (Khairunisa et al., 2023). These archaea may have contributed to a greater negative energy balance. A larger negative energy balance results in increased lipid mobilization, which means more NEFA in the blood, subsequently leading to increased BHB production.

In Tables 19 and 20 the different fungi identified as differentially abundant SloBHB and LoBHB in faeces samples (day -7 and +21) are shown. The species *Buwchfawromyces eastonii* and the genus *Buwchfawromyces* showed a higher abundance in the faeces (day -7) in the SloBHB cluster (Tables 19 and 20). Both the species and genus had an average proportion of 0,26% in the faeces (day -7). Additionally, the genera *Piromyces* and *Pecoramyces* had a higher abundance in the SloBHB cluster in the faeces (day +21), with average proportions of 28,5% and 0,39%, respectively. The anaerobic fungi have highly active cellulolytic and semi-cellulolytic enzymes, which might explain why these fungi show a higher abundance in the faecal samples. (Gruninger et al., 2014). By the time substrates reach the hindgut, they have already travelled through the gastrointestinal tract, often leaving behind the more difficult-to-digest fractions towards the end (L. Liu et al., 2023). The SloBHB group has been suggested to better adapt to the transition changes (e.g. by producing BHB in the liver to metabolize the mobilized NEFA). Perhaps, an enhanced fiber digestibility could indicate that the animals not only were more adaptive/efficient at the level of the liver, but also during the digestion.

## 5.5 Recommendations for future research

Several questions remain unanswered, including, for example: Who is the driver? Do the microbes initiate the disease, or does the disease alter the microbial community in the gastrointestinal tract? Or is it just a "coincidental association" which is observed, This study cannot answer these questions, and further research is needed.

Combining metataxonomic approaches with metatranscriptomics and/or metabolomics can provide valuable insights into the transcriptionally and metabolically active microbes. Metatranscriptomics provides more insights about the biochemical pathways that are active into the microbial community. Additionally, PICRUSt2, based on 16S rRNA, offers predictions of metagenome functions, but most of its data is derived from human microbes (Caicedo et al., 2020). In contrast, CowPI, which is still in the development stage, applies the same principles as PICRUSt2 but is tailored specifically for the rumen of cows.

To achieve more reliable results, future studies should include a larger number of animals and potentially involve multiple farms.

# 6 GENERAL CONCLUSION

It can be concluded that there are indeed differences in microbial composition between cows experiencing imbalance around the transition period. This was observed in both the inflammation and metabolic clusters. The use of animals from two different farms made the results more robust and reliable. However, a notable finding in this study was the farm effect. The microbial community in cows from ILVO differed significantly from that in cows from Hooibeekhoeve. Several factors may contribute to this difference, so further research will be needed to determine which factors are most influential.

There were some indicator organisms observed in both rumen and faeces samples between the clusters. There are some possible explanations for the presence of these microorganisms. They may benefit from the changing state of the body during the transition period. Some might help maintain balance, while others could potentially contribute to an imbalance.

Further research is needed to determine the exact influence these microorganisms have on imbalances and balances in cows.

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