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PRISCILA FREGULIA LOMAR

The influence of the rumen microbiome on feed efficiency and methane yield - a taxonomic and functional approach

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Priscila Fregulia Lomar

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Orientador: Prof. Dr. Roberto Júnio Pedroso Dias

Co-orientadora: Profa. Dra. Mariana Fonseca Rossi

Colaboradora: Dra. Mariana Magalhães Campos

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I dedicate this work to my mother,
my example of strength.

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What we anticipate seldom occurs; what we least expected generally happens.

(Benjamin Disraeli)

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SUMMARY

ABSTRACT

The rumen microbiome plays a fundamental role in the digestion of plant biomass, and differences in the taxonomic and functional composition of these microorganisms have been demonstrated to influence feed efficiency and methane production. However, the results are not consistent across the studies, and the microbial taxa and the metabolic pathways related to the feed efficiency phenotype and the methane production are not fully elucidated. Additionally, all the studies that explored the relationship between the rumen microbiome and these traits were developed in cattle raised in temperate areas. Section 1 is a review that aimed to discuss how the rumen parameters – and the rumen as a whole – are related to feed efficiency. The rumen is a complex environment and the rumen parameters influence each other, however, most studies do not consider these interactions and discuss the rumen parameters separately. Section 2 compared all the studies published about the relationship between rumen microbiome and residual feed intake (RFI) in order to investigate the influence of the variables of the study and the methodology used (e.g., animal breed, diet, sequencing platform and hypervariable region used on the sequencing) on the microbial data registered. On Section 3 and Section 4 was used Illumina sequencing of the 16S and 18S rRNA in order to explore the taxonomic and functional composition of the rumen microbiome related to feed efficiency and methane yield. These are the first studies to explore the relationship between the rumen microorganisms and these traits in cattle raised under tropical conditions. Advances in omics technologies in the last years have allowed a better exploration of complex microbial communities, such as the rumen. This thesis demonstrates the importance of the meta-omics technologies in order to better understand the rumen microbiome and its relationship with feed efficiency and methane production.

Keywords: Rumen. Rumen microbiology. Feed efficiency. Enteric methane.

RESUMO

Os microrganismos do rúmen são responsáveis pela digestão dos componentes vegetais, e diferenças na composição taxonômica e funcional desses microorganismos tem sido discutidas como relacionadas à eficiência alimentar e produção de metano em bovinos. No entanto, os resultados nãosão consistentes entre os estudos, e os táxons microbianos e vias metabólicas relacionados ao fenótipo de eficiência alimentar e à produção de metano não estão totalmente elucidados. Adicionalmente, todos os estudos que exploraram a relação entre microbiota ruminal e esses traços foram desenvolvidos em bovinos criados em regiões temperadas. A Seção 1 é uma revisão que objetivou discutir como os parâmetros ruminais – e o rúmen como um todo – são relacionados à eficiência alimentar. O rúmen é um ambiente complexo e os parâmetros ruminais influenciam uns nos outros, no entanto, a maioria dos estudos não considera essas interações e discute os parâmetros ruminais separadamente. A Seção 2 comparou todos os estudos publicados sobre a relação entre a microbiota ruminal e consumo alimentar residual (CAR), com o objetivo de investigar a influência das variáveis e metodologias usadas nos estudos (ex.:raça do animal, dieta, plataforma de sequenciamento e região hipervariável) sobre os dados microbianos registrados. Na Seção 3 e Seção 4 foi usado sequenciamento Illumina do 16S e 18S rRNA com o objetivo de explorar a composição taxonômica e funcional da microbiota ruminal relacionada à eficiência alimentar e produção de metano. Esses são os primeiros estudos a explorar a relação entre os microorganismos ruminais e esses traços em bovinos criados sob condições tropicais. Os avanços nas tecnologias ômicas nos últimos anos têm permitido a exploração de comunidades microbianas complexas, como o rúmen. Essa tese demonstra a importância das meta-ômicas a fim de melhor compreender a microbiota ruminal e sua relação com a eficiência alimentar e a produção de metano.

Palavras-chave: Rumen. Microbiologia do rumen. Eficiência Alimentar. Metano entérico.

RESUMO PARA DIVULGAÇÃO CIENTÍFICA

O estômago dos bovinos é dividido em quatro partes: rúmen, retículo, omaso e abomaso. O rúmen funciona como uma câmara de fermentação e é habitado por uma variedade de microrganismos (bactérias, archaea, protozoários e fungos) em uma relação denominada simbiose, que é vantajosa tanto para os microrganismos quanto para os bovinos. Os microrganismos fermentam as fibras que os bovinos ingerem, transformando-as em compostos químicos que podem ser absorvidos pelos animais.

Diferentes grupos de microrganismos podem produzir diferentes compostos para serem absorvidos pelo animal, e a eficiência desse processo pode influenciar no crescimento do animal e na produção de leite. No entanto, a atividade de um grupo específico de archaeas no rúmen, as metanogênicas, gera metano durante o processo de fermentação. Esse processo é essencial para manter o rúmen como uma câmara de fermentação, porém, o metano produzido é liberado na atmosfera por meio da eructação e representa uma perda de energia por parte do animal. O metano contribui significativamente para o aquecimento global e estratégias para reduzir a emissão desse gás são urgentemente necessárias.

Apesar da importância dos microrganismos do rúmen na produção de leite, crescimento do animal e produção de metano, o entendimento de como os diferentes grupos de microrganismos influenciam esses fatores ainda não está claro. Por isso, este estudo investigou como diferentes grupos de microrganismos estão relacionados a bovinos que digerem alimentos de forma mais eficiente e a bovinos que produzem menos metano. A curto prazo, esses dados fornecem a identificação dos microrganismos que são benéficos para o animal e a identificação das funções desses microrganismos na fermentação ruminal. A longo prazo, o entendimento dessa relação entre os microrganismos do rúmen e a produção de leite, carne emetano em bovinos pode levar ao desenvolvimento de probióticos, que são produtos alimentícios que contêm esses microrganismos e podem ser oferecidos aos animais para melhorar sua digestão.

PREFACE

The present thesis contributes to the knowledge of the rumen microbiome related to feed efficiency and methane production. This is the first study that addresses the rumen microbiome related to these phenotype traits under tropical conditions.

The thesis has a small introduction and four sections. Section 1 is a general review of the rumen activity as whole influencing feed efficiency. Section 2 is a meta-analysis of the influence of the methodologies on the microbiome data. For Section 3 and Section 4,the 16S and 18S rRNA were sequenced in order to access the prokaryotic and eukaryotic composition of the rumen microbiome. In Section 3 the taxonomic and predicted functional compositionof the rumen microbiome are discussed as related to feed efficiency, with the animals ranked according to residual feed intake (RFI). In Section 4, the taxonomic and predicted functional composition are discussed as related to methane production, using methane yield as the measurement to rankthe animals.

Section 1 of the thesis has been published (Fregulia et al., 2021; DOI: 10.1016/j.livsci.2021.104761); and Sections 2, 3, and 4 are in the finalization process for submission.

GENERAL INTRODUCTION

Is expected an increase in the global human population, with 9 billion people by 2050. It will demand a 73% increase in the demand for food (ALEXANDRATOS; BRUINSMA, 2012). Thus, it is needed to increase food productivity taking into account sustainability (FREGULIA et al., 2021; OPIO et al., 2013).

Rumen fermentation occurs due to the activity of microorganisms. The rumen microbiome is composed of bacteria, archaea, protozoa, and fungi, which play a role in the fermentation of the plant biomass, transforming cellulose and lignin, primarily indigestible by the host, into digestible components as volatile fatty acids (VFA) (SILVA DE OLIVEIRA; DE MOURA ZANINE; SANTOS, 2007). This process supplies approximately 70% of the energy requirements of the animal (MIZRAHI, 2012), but is also related to methane production. The methane produced on rumen is not metabolized by the animal and is released into the atmosphere, which represents between 2% to 12% of loss of the gross energy ingested (JOHNSON; JOHNSON, 1995). Additionally, methane is a greenhouse gas (GHG) that contributes to global warming 28 times more than CO_2 (GROSSI et al., 2019; BEAUCHEMIN et al., 2020).

Researchers have been selecting bovines with increased feed efficiency. More efficient animals consume less feed and produce more milk and meat, and studies have been discussing that more efficient animals emit less methane (ALEMU et al., 2017; MORAÏS; MIZRAHI, 2019). In this way, the selection of more efficient animals can increase food production and reduce the emission of pollutants.

The taxonomic composition of the rumen microbiome can be related to feed efficiency and methane production (AUFFRET et al., 2020b; WALLACE et al., 2015). However, these results are not consistent across studies, and authors have suggested that particular taxa and their metabolism may be the key to feed efficiency (BOWEN et al., 2020a; BROOKE et al., 2019). On the other hand, authors have suggested that not particular taxa are responsible for increased methane production, but the inter-domain microbial interactions – and consequently, the availability of substrate for methanogenesis (PITTA et al., 2021).

In this way, the present thesis contributes to expanding the knowledge about the rumen microbiome related to feed efficiency and methane production. All the studies relating the rumen microbiome to these phenotypic traits were developed in temperate climates, being this the first study developed in a tropical area and using a breed that is traditionally raised under these conditions.

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SECTION 1

(Section published in the journal *Livestock Science*, A2, IF: 1.943;
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A review of rumen parameters in bovines with divergent feed efficiencies: what do these parameters tell us about improving animal productivity and sustainability?

Abstract

Increased food production is urgently needed to meet the high demand for agricultural products required by the world's growing population. Feed efficiency consists of achieving maximum output using minimum input on animal production. Improving feed efficiency is a valid strategy to increase food production without exhausting the natural resources of the planet. However, the biological mechanisms related to feed efficiency phenotype are not well understood yet. Ruminal activity is the major anaerobic biodigester that supplies energy for the animal, but only recently researchers have focused their attention on correlating rumen parameters to feed efficiency. Among these parameters, rumen microbial composition, gene expression of the rumen epithelium, rumen pH, metabolites, volatile fatty acid, methane production, feed digestibility, and microbial enzymes have been directly associated with feed efficiency. The rumen works as a stable and balanced ecosystem that influences the host, and modulating its microbial activity can be a useful strategy to mitigate emissions of pollutants into the environment (e.g., methane). This review provides an overview of all rumen parameters linked to feed efficiency in bovines and discusses how they can be better understood to improve animal productivity and sustainability.

Key-words: beef cattle, dairy cattle, feed efficiency, microbiome, rumen.

1. Introduction

The most significant challenges that humanity faces today are to produce food for 9 billion people by 2050 and mitigate greenhouse gas emissions (GHGs) to reduce climate

change. The estimated population growth will require a 73% increase in the current milk and meat production to meet the global demand for food (ALEXANDRATOS; BRUINSMA, 2012; FAO, 2019). Researchers have selected livestock animals with improved feed efficiency in order to increase productivity and sustainability. Efforts to improve feed efficiency have been made to achieve maximum output using minimum input (AHOLA; HILL, 2012). Efficient animals are desired because they consume less feed, emit less GHGs (e.g., enteric methane), and produce more milk and meat than the inefficient ones (ALEMU et al., 2017; MORAÏS; MIZRAHI, 2019).

Ruminants can convert plant biomass into nutrient-rich food because of the unique digestive tract (SHABAT et al., 2016). These animals have a four-compartment stomach that combines physical and microbial activities that allow efficient digestion of plant biomass. The pre-gastric fermentation that occurs mainly in the rumen is also an important source of methane that is released into the atmosphere through eructation. Moreover, the fermentation taking place in the rumen provides up to 70% of the energy supply for the animal (VALADARES FILHO; PINA, 2006; WU; PAPAS, 1997).

Dairy and beef cattle have differences in energy utilization (MIZRAHI, 2011; PFUHL et al., 2007). Beef breeds utilize the energy and nutrients to gain more meat, and dairy breeds accumulate more fat as energy to promote homeostasis in lactation (PFUHL et al., 2007). Dairy breeds tend to gain less body weight (BW) with the same amount of nutrients than beef breeds because body fat contains more than twice the energy content of meat protein (PFUHL et al., 2007). The feed energy that is not used by the animal tissues and is not retained in the body is generally lost as heat, urine, feces, and gases, such as methane (MIZRAHI, 2011).

Many parameters may directly or indirectly influence feed efficiency. The main parameters influencing feed efficiency are gene expression, protein turnover, nutrient metabolism, genetic-environmental interactions, behavior, breed, sex, fertility, diet, body composition, and physical activity (HASKELL et al., 2018; HERNANDEZ-SANABRIA et al., 2010; RICHARDSON; HERD, 2004). Feed efficiency can be estimated through different measurements, with residual feed intake (RFI) being the most widely used metric (HERD; ARTHUR, 2009; KOCH et al., 1963).

Herd et al. (2004) estimated that rumen function contribute 23% of feed efficiency variation in beef cattle. Despite the importance of rumen function to animal nutrition, the rumen parameters related to feed efficiency have only been investigated in the last 15 years. Most studies have discussed rumen parameters separately, without considering that the rumen works as a complex microbial environment with a symbiotic relationship with the host. This review

aims to discuss how the rumen parameters cited above are linked to feed efficiency and how a better understanding of their interactions with the host can leverage animal productivity and sustainability.

2. Intake/digestibility and feed efficiency

2.1 Measuring feed efficiency

Different measurements have been used to estimate the influence of biological variables on feed efficiency. The most common feed efficiency measures are average daily gain (ADG) (GREEN et al., 2013), feed conversion ratio (FCR) (VAN DER WESTHUIZEN et al. 2004), feed to gain ratio (F:G) (NKRUMAH et al. 2007), gross feed efficiency (GFE) (SPURLOCK et al., 2012), Kleiber ratio (KR) (KLEIBER, 1961), and dry matter intake (DMI) (RICHARDSON et al., 2020). Each of these measurements has a different mathematical basis that may result in divergent efficiency rankings (FREETLY et al., 2020). Some measures can be used to characterize only dairy or beef breeds, separately, because they can take into account production traits (e.g., body weight – BW and growth rate). As a result, it is difficult to compare the data from studies that use distinct methodologies to study feed efficiency in other livestock breeds (FREETLY et al., 2020; JEWELL et al., 2015a).

Residual feed intake (RFI) was proposed by KOCH et al. (1963) and is the most used feed efficiency measurement for beef and dairy cattle (HILL; AHOLA, 2012; NOEL et al., 2019). It is widely accepted due to its independence from production traits, reflecting only the differences in metabolic processes (ARTHUR et al., 2001; CONNOR et al., 2013; NKRUMAH et al., 2006). RFI is calculated as the difference between the actual feed intake and the predicted intake (ARCHER et al., 1999; BASARAB et al., 2003). The more efficient the animal, the more negative the RFI value is. In this way, efficient animals consume less feed, produce more milk and meet but the body size is usually unaffected (NKRUMAH et al., 2006; HERD; ARTHUR, 2009; RIUS et al., 2012). The selection for improved feed efficiency through RFI is possible because it is a moderately heritable trait 0.33 ± 0.01 (range of 0.07 to 0.62) (BERRY; CROWLEY, 2013).

Even though the results across studies have been inconsistent, variations in RFI among different cattle breeds have been found. The biological factors that affect these variations have been discussed as multifactorial (e.g., digestion, heat increment of feeding, body composition,

activity, protein turnover, tissue metabolism and stress) (HERD; ODDY; RICHARDSON, 2004).

The few studies that used RFI measurements in dairy cattle breeds observed that the predicted intake is greater in the second lactation period. These studies recommended the inclusion of parity order in the energy intake prediction model in RFI calculations for dairy cattle (CONNOR et al., 2013). In addition to fluctuating over the course of the lactation period, RFI measurements can vary across diets, leading to a reranking of animals inside RFI groups (DURUNNA et al., 2011; HERNANDEZ-SANABRIA et al., 2012; OLIVEIRA et al., 2016; ZHOU et al., 2010). Due to these individual variations, the data obtained from studies using beef or dairy cattle need to be analyzed separately.

2.2 Feeding behavior

Feed input represents the largest operational cost in cattle production systems. The selection for animals that better utilize feed nutrients can reduce costs and improve profitability. According to Richardson and Herd (2004), feeding behavior is responsible for about 2% of RFI variations and can be a parameter to estimate feed efficiency in specific situations.

The patterns of feed intake are directly related to the speed of passage rate in the rumen. A slower passage rate increases retention time in the rumen and leads to microbial growth and increased feedstuff colonization, resulting in a greater feed digestion (COLUCCI et al., 1990; MERTENS; LOFTEN, 1980).

Several studies have reported feeding behavior traits correlated with feed efficiency (BINGHAM et al., 2009; NKRUMAH et al., 2007a). Efficient animals consume less dry matter (DM) per unit product (kg meat or milk) compared to inefficient ones (ELOLIMY et al., 2018a; GUIMARÃES et al., 2017; NKRUMAH et al., 2004; LAM et al., 2017). In addition to RFI, dry matter intake (DMI) is also affected by body size, body composition, physiology, age, sex, temperature of the environment, and diet (NRC, 2000). The fact that inefficient animals consume more DM might be partly explained by the lower metabolism during feed digestion to achieve the energy intake levels required to maintain the increased body size (NKRUMAH et al., 2006).

Additionally, feeding behaviors are attributed to different feed efficiency groups. The most efficient animals tend to visit feeding boxes less frequently, consume less feed per visit, and spend more time in head-down feeding events (CHEN et al., 2014; KELLY et al., 2010; NKRUMAH et al., 2007a). A meta-analysis by Kenny et al. (2018) showed that inefficient

animals spend more time eating than efficient ones. Pereira et al. (2016) found that efficient animals spend significantly less time ruminating than the inefficient counterparts.

Nevertheless, not all traits observed by Kenny et al. (2018) showed the same results across studies (e.g., feeding time) (GREEN et al., 2013; LANCASTER et al., 2009; NKRUMAH et al., 2006; RICHARDSON et al., 2003; SILVA et al., 2020). The discrepancy in these findings might be related to the different dietary conditions across studies (MONTANHOLI et al., 2009) or the methodology used, indicating that feeding behavior cannot accurately predict feed efficiency in different experimental designs (FITZSIMONS et al., 2014b; GREEN et al., 2013; KENNY et al., 2018).

Therefore, the standardization of experimental designs is necessary to remove biases coming from differences in feeding regimens in order to enhance our understanding of the correlations between feeding behavior and feed efficiency.

2.3. Digestibility

Ruminants have a stomach divided into four chambers - rumen, reticulum, omasum, and abomasum, and each of these chambers plays a different role in feed digestion. The rumen is the largest chamber and plays a role in digestion and food storage, allowing the ruminant animals to survive long periods without food. Moreover, along with the reticulum, the rumen plays an essential role as a site of anaerobic fermentation (DEPETERS; GEORGE, 2014; WASS, 1971).

Richardson and Herd (2004) estimated that digestibility contributes to 10-14% of the variation in RFI. The factors that influence feed digestibility are digestion kinetics, passage rate of feed particles out of the rumen, enzyme production, diet composition, breed, and environmental conditions (NRC, 1987; KERLEY, 2012; KRUEGER et al., 2009a; RICHARDSON et al., 1996). Improvements in feed digestibility can reduce fecal energy losses and increase energy availability for the animal, thereby enhancing efficiency (KERLEY, 2012).

The relationship between feed efficiency and digestibility is directly affected by the ingredients of the diet. Efficient animals have a greater ability to digest starch, crude protein, DM, organic matter, and neutral detergent fiber (MCDONNELL et al., 2016; NKRUMAH et al., 2006; RIUS et al., 2012). Precision-fed diets result in greater feed efficiency than *ad-libitum* rations, as the latter leads to an increased DMI (PINO et al., 2018).

3. The physical characteristics of the rumen and feed efficiency

3.1 Rumen structure

In adult cattle, the rumen has a volume of approximately 100 liters, occupying a large part of the abdominal cavity (HOBSON; STEWART, 1997). It works as a pre-gastric anaerobic fermentation chamber, with an average temperature of 39 ºC and an average pH of 6.8 (LANA, 2005).

The rumen is covered by a stratified keratinized epithelium without glands, and all digestive processes in the rumen result exclusively from fermentative digestion (MILLEN et al., 2016). The rumen is compartmentalized by thick muscular bands that surround the organ and divide the ruminal space into ventral sac, dorsal sac, blind ventral sac, and blind dorsal sac (MILLEN et al., 2016).

At the cellular level, the rumen epithelium is complex and is covered by leaflike papillae that play a role in short-chain fatty acids (SCFA) absorption (STEVEN; MARSHALL, 1970). Usually, papillae are less numerous and prominent in the ventral sac, denser, and bigger in blind sacs, and less developed in the center of the rumen roof (Millen et al., 2016). About 75% of the SCFA produced in the rumen are absorbed through the epithelial tissue of the rumen and reticulum, and less than 10% of total SCFA reaches the small intestine (HARFOOT, 1978).

Studies have found that inefficient animals have a reticulo-rumen with increased weight (BASARAB et al., 2003; BONILHA et al., 2009; FITZSIMONS et al., 2014a). The greater development of the rumen muscle to mix rumen contents when the fill is greater can explain the increased rumen weight in inefficient animals (FITZSIMONS et al., 2014a; ORTIGUES; DOREAU, 1995). This suggests that the weight of the empty reticulo-rumen could be a parameter for variation in feed efficiency (FITZSIMONS; KENNY; MCGEE, 2014).

3.2 Rumen epithelium

The rumen epithelium exhibits intensive metabolic activity and is responsible for the absorption and metabolism of end-products from microbial fermentation (KERN et al., 2016a; KHIAOSA-ARD; ZEBELI, 2014). It has both a high oxygen demand and mitochondrial concentration. The cellular processes in this tissue (e.g., volatile fatty acids (VFA) uptake, thermogenesis, and protein turnover) contribute to supply the energy required by the animal (DEL BIANCO BENEDETI et al., 2018; HERD; ODDY; RICHARDSON, 2004). In the rumen epithelium, cellular energy is generated through oxidative phosphorylation with the formation

of ATP from VFA metabolism, and the local cellular processes use the ATP produced within the tissue. Thus, a greater oxidative phosphorylation activity in the rumen epithelium can indicate an increased energy expenditure (DEL BIANCO et al., 2018; NELSON; COX, 2009).

The rumen epithelium is stratified, and its papillae have high metabolic activity. The leaflike papillae increase the absorptive surface area and allow increased microbial attachment to the rumen wall. They also promote the exchange of metabolites between the rumen and bloodstream, and support immune protection for the host (GALFI et al., 1991; GRAHAM; SIMONS, 2005; STEVEN; MARSHALL, 1970). Lam et al. (2017) observed that the rumen epithelium thickness was greater in efficient cattle than in their inefficient counterparts. An increased epithelial thickness might be related to a greater metabolic and functional activity in the epithelium papillae (TAMATE; FELL, 1977).

Dietary modifications can cause variations in papillary number and size, sloughing of surface epithelial cells, and variations in gene expression (HEISENBERG; BELLAÏCHE, 2013; KONG et al., 2016). Diet composition leads to the production of different SCFAs, promoting an adaptive response in rumen papillae to the diet (GOODLAD, 1981; GRAHAM; SIMMONS, 2005; KHIAOSA-ARD; ZEBELI, 2014; MENTSCHEL et al., 2001; STEELE et al., 2015). Butyrate is the most potent stimulator of epithelial proliferation among SCFAs (short-chain fatty acids) (MENTSCHEL et al., 2001). However, studies have found that most of the morphological characteristics of the papillae (number, length, width, weight, absorptive surface area, density) were not associated with feed efficiency (KERN et al., 2016a; PEREIRA et al., 2016).

Despite the small number of studies conducted in this area of research, the results mentioned above suggest that metabolic pathways in the rumen epithelium may play a role in feed efficiency.

4. Rumen genetics and chemistry

4.1 Epithelium gene expression

The rumen epithelial transcriptome has an increased variation in gene expression profiles including those related to tissue morphogenesis, energy pathways, VFA absorption and metabolism, and immune functions. According to Kong et al. (2016), who used Weighted Gene Co-expression Network Analysis (WGCNA), 47.5% of the core genes in the rumen epithelial tissue are involved in metabolic processes. They observed differential expression in 122 genes

in the rumen tissue of animals with divergent feed efficiencies. They found that the entire transcriptome profiles of the inefficient animals did not cluster as in the efficient animals, indicating a clear separation between the gene expression profiles of the two groups.

Genes involved in tissue morphogenesis are among those with increased expression in the rumen epithelial tissue of efficient cattle. These upregulated genes can change the number, shape, and size of cells and increase paracellular permeability for nutrient absorption (HEISENBERG; BELLAÏCHE, 2013; KONG et al., 2016). Moreover, efficient animals have a greater expression of genes involved in energy-generating pathways (e.g., oxidative phosphorylation, glycolysis, and tricarboxylic acid cycle). The greater genetic expression of these genes leads to increments in energy inputs required by the increased tissue morphogenesis in these animals (DEL BIANCO et al., 2018; KONG et al., 2016). These insights into rumen epithelium gene expression were observed using different types of methodologies, such as WGCNA by Kong et al. (2016) and Quantitative real-time PCR (qRT-PCR), by Del Bianco et al. (2018).

Genes related to VFA absorption and metabolism are also differentially expressed between feed efficiency phenotypes. Guan et al. (2008) observed that efficient animals exhibited an increased butyrate concentration in the rumen that may result in an increased absorptive capacity in these animals. An increased butyrate concentration can also lead to the transcription of genes related to VFA uptake, as observed by Elolimy et al. (2018a) using qRT-PCR and by Kong et al. (2016) using WGCNA.

Kern et al. (2016b) reported several genes that appear to have downregulated immune functions in inefficient animals as observed in a RNA-seq data analysis. The authors suggested that the lower weight gain in inefficient animals could be related to the energy expenditure of the immune response against pathogenic microorganisms rather than for growth and performance. It is known that efficient cattle have a greater abundance of rumen pathogenic microorganisms than inefficient cattle (AUFFRET et al., 2020). Therefore, the expression of immune-related genes in efficient animals may not necessarily be related to feed efficiency phenotype, but a casual response of the immune system to the increased abundance of these pathogens.

Differential expression in the rumen epithelium between feed efficiency phenotypes has been observed for genes involved in cytoskeletal organization, modulation of intercellular adhesion through adherent junctions, collagen metabolism, protein turnover, ketogenesis, pyruvate metabolism, cellular oxidative stress, transport in the epithelium, and cell migration signaling pathways (ELOLIMY et al., 2018a; KERN et al., 2016b, 2017; KONG et al., 2016).

In addition to gene expression, other authors have observed differences in the rate of transcription in the rumen epithelium. Kong et al. (2016) demonstrated that the relative mitochondrial genome copy number per epithelium cell is positively correlated with RFI. These authors noted a greater expression of mitochondrial genes and lower copy numbers of mitochondrial genomes in efficient cattle, suggesting a greater rate of transcription in the rumen epithelium. Furthermore, Del Bianco et al. (2018) reported that efficient animals may have a lower mitochondrial activity that could decrease the production and energy expenditure in the epithelium tissue.

4.2.Rumen pH

The rumen pH normally ranges from 5.5-7.0 in cattle fed high-quality forages, with total mixed rations containing up to 50% concentrates (CUNNINGHAM et al., 2018; LANA, 2005; SMITH, 2009). When the rumen pH remains below 5.5 for an extended period, the animal can develop subacute ruminal acidosis. This disease constitutes a common and serious health problem in bovines worldwide (DUFFIELD et al., 2004; NORDLUND, 2001).

Ruminal fluid pH fluctuations are affected by meal patterns, fluid passage rate through the rumen, and organic matter degradation (ALLEN et al., 1997). Changes in rumen pH can influence several ruminal parameters, mainly the population of microorganisms (CARBERRY et al., 2012), synthesis and absorption of VFA (LÓPEZ et al., 2003), and inhibition of methanogens activity (PITT et al., 1996; VAN KESSEL; RUSSELL, 1996).

Lam et al. (2017) using a method to record pH every 5 min along the day found that efficient animals spend more time between acidotic and optimal rumen pH than the inefficient ones. They observed that the ruminal pH was not associated with feed intake but with the feed efficiency phenotypes. Other studies, however, failed to find differences in the rumen pH between feed efficiency groups when rumen samples were collected orally at specific times of the day (FITZSIMONS et al., 2013; KRUEGER et al., 2009a, 2009b; LAWRENCE et al., 2011a, 2013; MCDONNELL et al., 2016). All studies mentioned above used oral techniques to collect rumen fluids, except for Lam et al. (2017), who used data loggers inserted via the esophagus into the ventral sac of the rumen. Rumen pH recorded through data loggers may be advantageous over the other methodologies since McDonnel et al. (2016) recognized the possibility of saliva contamination of the orally sampled rumen digesta.

5. Rumen microbiome and its metabolites

5.1 Rumen metabolites

The rumen fluid is composed of many chemical compounds involved in various chemical reactions. These compounds are amino acids, dicarboxylic acids, fatty acids (e.g., linoleic and alpha-linolenic), volatile fatty acids, glycerides, carbohydrates, cholesterol esters, phospholipids, inorganic ions, and gases; and many of them are microbial fermentation end-products or intermediate metabolites (CLEMMONS et al., 2020; SALEEM et al., 2013). The rumen metabolites can have both endogenous or xenobiotic origins, and the latter is derived from microbes or plants. Therefore, these compounds are associated with different compositions of the microbiota (FONTANESI, 2016; LI et al., 2020).

Differences in the production of rumen metabolites, mainly those involved in intermediary metabolism, contribute to the efficient use of nutrients (CLEMMONS et al., 2020; HUNTINGTON, 1990; OKINE; MATHISON, 1991). However, it can be difficult to quantify the actual rumen metabolite production, as these chemicals are constantly absorbed into the bloodstream (DIJKSTRA et al., 1993).

Despite these limitations, studies have demonstrated differences in rumen metabolites between animals with divergent feed efficiencies (ARTEGOITIA et al., 2017; CLEMMONS et al., 2020; LI et al., 2020). These compounds include phospholipids, organic acids and derivatives, amino acids, fatty acids, glycerides, cholesterol esters, nucleosides, organooxygen compounds, organoheterocyclic compounds, and biogenic amines (LI et al., 2020).

Linoleic and alpha-linolenic metabolic pathways are the most common functional pathways associated with feed efficiency (ARTEGOITIA et al., 2017; LI et al., 2020). Biogenic amines, which might disturb the appetite of the animals, were also identified, such as tyramine and histamine (LI et al., 2020). Metabolites involved in genetic material recycling and protein metabolism, and those involved in carbohydrate and lipid metabolism are functions differentially abundant between feed efficiency phenotypes (CLEMMONS et al., 2020). Despite the few studies, the data are promising and suggest that signatures in the rumen metabolome may be useful to identify variations in feed intake and feed efficiency (LI et al., 2020).

5.2 Ruminal volatile fatty acids

Short-chain fatty acids (SCFAs) are volatile fatty acids (VFAs) that are end-products of ruminal fermentation. SCFAs supply approximately 70% of the net energy requirements of the animal (MIZRAHI et al., 2011; SEYMOUR et al., 2005). Acetic, propionic and butyric acids are the main VFAs in the ruminal fluid (CARTER; GROVUM, 1990). The diet type and the ruminal microorganisms that ferment the different substrates available in the diets determine the production and proportion of SCFAs in the rumen (CARBERRY et al., 20114a, 2014b; DE LA TORRE et al., 2019).

SCFAs are absorbed in the rumen epithelium through protein-mediated transport and simple diffusion (LÓPEZ et al., 2003). Epithelium absorption can be influenced by moderate feed restriction, feeding time, and physiological responses to stress conditions, like animal transportation (ASCHENBACH et al., 2009; BOURGON et al., 2017; WARNER, 1966). The SCFA concentration in the rumen represents the balance between microbial production and epithelial absorption (HERNANDEZ-SANABRIA et al., 2011).

The production and absorption of SCFA regulate the luminal pH in the rumen. The decrease in pH associated with high SCFA concentration increases rumen absorption (KHIAOSA-ARD; ZEBELI, 2014; LÓPEZ et al., 2003) and causes adaptative responses, such as epithelial proliferation (PENNER et al., 2011). An increased SCFA accumulation leads to metabolic disorders that may affect the health and productivity of the animals (BARKER et al., 1995). The total SCFA concentration is positively associated with the time after feeding, with its highest level occurring approximately nine hours after the morning feeding. These concentrations usually do not differ if other sampling times are recorded throughout the day (LI et al., 2009).

The total concentration of SCFA and the concentrations of specific VFAs have been associated with DMI (CARBERRY et al., 2012; HERD et al., 2019; HERNANDEZ-SANABRIA et al., 2011, 2012). Since DMI is a fundamental parameter in RFI measurement, a better understanding of the differences in VFA metabolism in divergent feed efficiency phenotypes is necessary (HERNANDEZ-SANABRIA et al., 2010, 2012). However, fewstudies have reported the relationship between rumen metabolites and feed efficiency (GUANet al., 2008). Most metabolome studies have investigated serum metabolites (CLEMMONS et al., 2017).

Some authors found that efficient animals may possess a higher concentration of total SCFA that might increase microbial fermentation (GUAN et al., 2008). On the other hand, other

authors have suggested that an increased SCFA concentration hinders animal performance, as it is commonly associated with lower rumen pH (KLIEVE et al., 2003; KOIKE; KOBAYASHI, 2001). Thus, no clear patterns have been observed across studies regarding the associations of total VFA concentrations with feed efficiency (**Table 1**).

Since butyrate was associated with efficient animals by Guan et al. (2008), the correlation of this SCFA with feed efficiency has been widely referenced in various studies. Butyrate is a particularly important energy source for most tissues, and its increase in the rumen of efficient animals could be due to a shift in bacterial groups capable of metabolizing products with greater energy contents (GUAN et al., 2008). Nonetheless, several other studies failed to find a clear relationship between feed efficiency and butyrate concentrations in the rumen (**Table 1**), suggesting that the various butyrate metabolic pathways may contribute differently to feed efficiency phenotyping.

The metabolic pathways encoded in the genomes of rumen microbes utilize different substrates and generate various end-products during SCFAs production. Propionate and butyrate syntheses compete for the same substrate as methanogenesis – the rumen hydrogen (MOSS et al., 2000; UNGERFELD, 2015). It is widely accepted that acetate formationpromotes CH_4 production (CARBERRY et al., 2014b) while the increase in the propionate-to-acetate ratio is associated with a decline in CH_4 production (RUSSELL, 1998). Thus, it is expected that feed efficient animals exhibit a greater concentration of propionate, butyrate, propionate-to-acetate ratio, and a lower concentration of acetate in the rumen. However, the results are highly variable and inconsistent across studies, as observed in **Table 1**.

5.3 Microbial enzymes

Studies have investigated the (meta)genome and (meta)transcriptome of rumen microorganisms to understand the role of microbial enzymes in the degradation of recalcitrant fibers of plant cell walls (WANG et al., 2019). Rumen microbes produce carbohydrate-active enzymes (CAZymes), which act in the deconstruction of plant polysaccharides and breakdown of glycoside bonds of complex sugars into simple sugars (BOHRA et al., 2019; BRULC et al., 2009). In addition to the role in feed digestion of ruminants, rumen CAZymes have been considered potential candidates for biotechnological applications (NOEL et al., 2019; SESHADRI et al., 2018). However, identifying CAZymes in the rumen is difficult due to the fact that most rumen microbes are currently unculturable (PATEL et al., 2014).

Recently, a few studies have described CAZyme profiles related to feed efficiency. CAZymes are clustered into families through amino acid sequence similarities, although a single family member may perform vastly different functions and catalyze many different chemical reactions (LEVIN et al., 2017, LOMBARD et al., 2014). Li and Guan (2017) identified several CAZymes families with differential abundance between divergent feed efficiency groups. However, Neves (2019) did not find differences in CAZymes between the two groups of feed efficiency phenotyping. Neves (2019) discussed that the main limitation of the current methods applied to discover rumen CAZymes (e.g., metagenomic screening) arises from the impossibility to identify individual members of CAZyme families. Therefore, considering that Li and Guan (2017) observed different abundances in CAZymes families related to feed efficiency phenotypes, more studies are necessary to identify the relationship between the individual members of CAZyme families and feed efficiency.

5.4 Methane production

Hydrogen (H_2) is one of the major fermentation products in the rumen (DEMEYER, 1991). Methanogenic archaea utilize H_2 and carbon substrates, mainly CO_2, acetate, or methanol, to produce methane, reducing the hydrogen pressure in the rumen in a process known as methanogenesis (CARBERRY et al., 2014a; HEDDERICH; WHITMAN, 2006). This fermentative process causes a significant energy loss (2-15%) relative to the dietary gross energy intake (JOHNSON et al., 1990; VAN NEVEL; DEMEYER, 1996). This energy could be saved and redirected for the performance and growth of the animal instead of being released to the atmosphere in the form of CH_4 (CARBERRY et al., 2014b). Livestock animals produce enteric methane that is emitted into the atmosphere, resulting in a considerable impact on the global greenhouse gas emissions budget. Therefore, the reduction of enteric methane emissions would have both economic and environmental benefits (CARBERRY et al., 2014b).

Methane production depends on DMI (ESCOBAR-BAHAMONDES et al., 2017; KENNEDY; CHARMLEY, 2012), rumen feed retention time, and the rumen microbiome composition (BASARAB et al., 2013; HERD; ARTHUR, 2009). Diet also influences methane production, as concentrate-fed animals emit less CH_4 per kg DMI than forage-fed animals (LLONCH et al., 2018; ROEHE et al., 2016). The decreased methane production by concentrate-fed animals results from a greater propionate production observed during starch fermentation. In this scenario, hydrogen availability for methanogenesis is lowered and less CH_4 is produced by rumen archaea (COTTLE et al., 2011; JANSSEN, 2010).

Nkrumah et al. (2006) suggested that efficient cattle produce 24-28% less methane (L/kg of BW) compared to inefficient ones (HEGARTY et al., 2007; JONES et al., 2011; NKRUMAH et al., 2006). Since then, the selection for efficient bovines has been widely used as an alternative method to reduce CH_4 emissions without affecting performance (BASARAB et al., 2013; DINI et al., 2018; FITZSIMONS et al., 2013; HEGARTY et al., 2007; JONES et al., 2011; MCDONNELL et al., 2016; PICKERING et al., 2015; SHARMA et al., 2014; SILVA et al., 2020). The selection for animals that produces less methane is possible because methane production has a low-to-moderate heritability (h^2 = 0.13 to 0.38) (CROWLEY et al., 2010; DE HAAS et al., 2011).

The differences in methane production between feed efficiency groups have been explained through three hypotheses: 1) Efficient animals emit less methane because they lose less energy during methanogenesis (FREETLY et al., 2020); 2) Efficient animals consume less feed, and methane production is expected to be proportionally decreased relative to the amount of feed consumed (FITZSIMONS et al., 2013; HEGARTY et al., 2007); 3) Differences in the composition of methanogenic archaea (DELGADO et al., 2019; LI et al., 2016, 2017).

Efficient animals that produce less methane are not consistently found across all studies, as some researchers have shown that inefficient animals can yield less methane (MCDONNELL et al., 2016; OLIJHOEK et al., 2018). Moreover, others studies have failed to detect differences between feed efficiency phenotypes and CH_4 production (ALEMU et al., 2017; FLAY et al., 2019; FREETLY; BROWN-BRAND, 2013; MERCADANTE et al., 2015; RENAND et al., 2019). Authors have suggested that efficient animals might produce more methane due to a greater digestibility of neutral detergent fiber (OLIJHOEK et al., 2018). This efficient fiber digestion results in a greater nutrient availability in the rumen, and stimulates acetate production that may lead to hydrogen formation – the substrate used by methanogenic archaea to produce methane (FREETLY et al., 2020).

Considering the contradictory results in the literature, the relationship between methane production and feed efficiency is subtle and may depend on the microbial population composition and diet (RENAND et al., 2019). Thus, it remains unclear whether the differences in methane production in the bovine rumen are due to the inherent variation in digestive efficiency related to feed efficiency phenotypes or are merely a result of the reduction in DMI associated with efficient animals (KELLY et al., 2010; LAWRENCE et al., 2011a). Therefore, more studies with larger population sizes are needed to obtain a more realistic picture of the variations in CH_4 production between feed efficiency groups (RENAND et al., 2019). Moreover, CH_4 data need to be integrated with other characteristics of the animals, such as the

rumen microbiota, which can be used as an additional variable to improve the interpretation of CH_4 data generated by feed efficiency phenotypes (DINI et al., 2018).

6. Structure and composition of the rumen microbiome

Ruminants consume plant materials that are usually composed of indigestible polysaccharides for the animal (FLINT et al., 2008). However, the rumen microorganisms have specialized enzymes to degrade these plant components (DEHORITY, 1991; MACKIE, 2002), and this degradation generates end-products such as SCFA, carbon dioxide, methane, and ammonia (DILL-MCFARLAND et al., 2018; FLINT et al., 2008; HERNANDEZ-SANABRIA et al., 2010; LI et al., 2019).

The overall richness of the bovine rumen microbiota comprises two groups of prokaryotes (2-5% archaea and 95% bacteria) and two groups of eukaryotes (0.1% fungi and 1% protists) (MIZRAHI, 2013; WEIMER, 2015). Due to its microbial diversity, the rumen plays a key role not only in feed digestion but also in the immune responses and overall health of the host (KHIAOSA-ARD; ZEBELI, 2014).

Bacteria are ubiquitous inhabitants of the rumen. They are essential for the survival of the animal due to their role in the degradation of plant fiber and are classified according to the substrate they ferment (ARCURI et al., 2006; MORAÏS; MIZRAHI, 2019; PITTA et al., 2010).

Methanogens constitute the main group of archaea in the rumen, accounting for approximately 90% of the total rumen archaea composition (BAN et al., 2021). These methanogens are essential to maintain a low H+ concentration in the rumen, by reducing CO_2 to CH_4 (BODAS et al., 2012). The efficient H_2 removal favors VFA formation and increases feed fermentation rates (MCALLISTER; NEWBOLD, 2008; WOLIN, 1979).

The rumen protozoans are divided into flagellates and ciliates groups. Although there is little knowledge about the role of flagellate protozoans in the rumen, it is suggested that they play a role in fiber digestion (ZHANG et al., 2020).

Ciliate protozoans constitute about 50% of the rumen biomass (Newbold et al., 2015). They are also involved in bacterial predation and have a role in methane production due to their association with methanogenic archaea (GUYADER et al., 2014; MORGAVI et al., 2010). However, a quantitative meta-analysis by Eugène et al. (2004) found that ciliate-free animals still exhibit an efficient use of nutrients, especially when they are given poor diets.

Anaerobic fungi are found in the rumen in two biological life stages: zoospores and sporangia (found attached to solid particulate material) and free-living zoospores (found in the

rumen fluid) (BAUCHOP, 1979). These microorganisms play a role in fiber degradation since they can penetrate both the cuticle and the cell wall of lignified material (HOBSON; STEWART, 1997). However, rumen fungi are not detected in all ruminant individuals raised under different management conditions (MORAÏS; MIZRAHI, 2019).

The composition of the rumen microbiota is directly affected by the diet, feeding frequency, DMI, rumen size, passage rate, pH, antibiotic use, age, sex, breed, the health of the host animal, and geographical location (CARBERRY et al., 2012; LI et al., 2019; DILL-MCFARLAND et al., 2018; STEWART et al., 1997). Among these factors, diet is one of the main components influencing the composition of the rumen microbiota (CARBERRY et al., 2012).

The microbiota is different in solid and liquid fractions in the rumen, and the diversity of bacteria is greater in the liquid fraction than in the solid. Bacteria present in the solid fraction form a group of microbes that are specialized in degrading plant cell walls. Once established, this group tends to decrease into a minimally functional consortium. On the other hand, bacteria present in ruminal liquid are composed of low-abundance taxa that are sensitive to dietary shifts and provide enhanced metabolic flexibility (JEWELL et al., 2015).

Ruminants are born with a non-functional rumen, and newborns acquire the microorganisms as the rumen develops with the age of the animal (DILL-MCFARLAND et al., 2018; REY et al., 2014). These microorganisms are transferred to the newborn from contact with other animals, humans, feed, or the environment (ARCURI et al., 2006; DILL-MCFARLAND et al., 2017). The factors that affect microbial acquisition and the long-term establishment of microbes in the rumen, as well the assembly of the microbial community, are not well understood (DILL-MCFARLAND et al., 2018). However, all individuals have a “core” bacterial structure in the rumen that is commonly found in most animals despite changes in the diet and geographical locations (HENDERSON et al., 2015). This core microbiome constitutes most of the microbial taxa present in the rumen, although less prevalent operational taxonomic units (OTUs) may represent species that are present only under specific management conditions of the host animal (ZHOU et al., 2010). Based on their 16S nucleotide sequences, the prokaryote OTUs that constitute the core microbiome are phylogenetically related, and it is suggested that they present greater heritability than the non-core members (SASSON et al., 2017).

Several studies have shown a relationship between rumen microbiota and production traits, such as weight gain and milk yield (CARBERRY et al., 2012; JAMI et al., 2014), and methane emissions (WALLACE et al., 2015). Recently, associations between the rumen microbiome and cattle feed efficiency have been identified (AUFFRET et al., 2020; SHABAT

et al., 2016). Investigating this relationship is essential because dysbiosis – the imbalance of the gut microbiota - can cause diseases and reduce the performance of the animals (ROSS et al., 2013).

The first studies discussing the relationship between rumen bacteria and feed efficiency were carried out using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). They found a clear separation of the microbial profiles generated from feed efficiency phenotypes (CARBERRY et al., 2012; GUAN et al., 2008; HERNANDEZ-SANABRIA et al., 2012; ZHOU et al., 2010). Efficient animals have lower diversity and richness indices of rumen microorganisms when compared to their inefficient counterparts (BOWEN et al., 2020; CLEMMONS et al., 2019; SHABAT et al., 2016; ZHOU et al., 2009). Moreover, there is a higher dominance of certain species and genera in efficient animals (SHABAT et al., 2016).

However, many studies have not found significant differences in the total microbiota composition between divergent feed efficiency groups (AUFFRET et al., 2020; BOWEN et al., 2020; CARBERRY et al., 2012, 2014a; HERNANDEZ-SANABRIA et al., 2010; ZHOU et al., 2009, 2010). Nonetheless, other studies have shown a differential abundance in specific OTUs related to each feed efficiency group (DELGADO et al., 2019; HERNANDEZ-SANABRIA et al., 2010; LI et al., 2019; ZHOU et al., 2009, 2010) (**Figure 1**). The abundance of some specific taxa is significantly correlated with feed efficiency phenotype (CARBERRY et al., 2014a; DELGADO et al., 2019; ELOLIMY et al., 2018), or directly correlated to the intensity of the phenotype (SHABAT et al., 2016). These findings suggest that particular taxa and their metabolism may be a key to feed efficiency (AUFFRET et al., 2020; BOWEN et al., 2020; ELOLIMY et al., 2020; HERNANDEZ-SANABRIA et al., 2010; JEWEL et al., 2015; SASSON et al., 2017).

Zhou et al. (2010) changed diets during an experiment from low-energy to high-energy diet, which led some animals to switch from one efficiency group to another. The PCR-DGGE analysis showed that some bands (gradient gel representing microbial taxa) disappeared, while new ones appeared. Carberry et al. (2012) and Hernandez-Sanabria et al. (2012) also changed the diet from high-forage to high-energy and observed that the effect of RFI on bacterial profiles was influenced by the diet type. Under high-forage and high-energy feeding, PCR-DGGE bands clustered according to RFI phenotype, while under low forage and low-energy diets the bands grouped separately irrespective of RFI ranking. These findings suggest that the effect of low-forage and low-energy diets is greater on feed efficiency than that observed on inter-animal variation (CARBERRY et al., 2012). Therefore, Carberry et al. (2012) suggested that, although

the rumen microbiota may play a role in host feed efficiency, this effect is likely modulated by the type of diet offered.

Comparisons of results across studies reveal a trend towards a greater abundance of Proteobacteria, Actinobacteria, and Ascomycota in efficient animals (**Figure 1**). Despiteseveral of these studies have not found differences at the phylum level, variations in the abundance of genus and species levels have been reported. Thus, when analyzing the result of all studies together, the differences at the phylum level might be the result of the small differences observed at the genus and species levels. However, a meta-analysis with more robust statistics is necessary to verify whether this trend is significant or not.

Interestingly, Auffret et al. (2020) found that genera known to be potential pathogens such as *Staphylococcus* (Firmicutes), *Eimeria* (Alveolata), *Sphaerochaeta*, *Treponema* (Spirochaetes), *Vibrio* and *Lawsonia* (Proteobacteria) were all significantly more abundant in efficient animals.

Researchers have been looking for taxonomic markers that could be used to predict feed efficiency in bovines (BROOKE et al., 2019). *Prevotella* has many OTUs strongly associated with feed efficiency groups (BROOKE et al., 2019; CARBERRY et al., 2012; JEWELL et al., 2015; MCCANN et al., 2014). Considering that particular OTUs may have different functional roles, studies have suggested that *Prevotella* could be a candidate for further investigation at the species level. Future results could provide insights into the importance of specific taxa in the rumen microbiota and feed efficiency (CARBERRY et al., 2012; JEWELL et al., 2015; MCCANN et al., 2014).

Although many studies have focused on the taxonomic composition of the microbial community in the rumen, Li et al. (2019) argued that microorganisms belonging to different taxonomic groups may utilize similar substrates and produce similar products. Thus, investigating the functional profiles could add an extra layer of information to understand the biology behind feed efficiency than taxonomic classification alone.

Considering the differences in the microbiome between the two groups of feed efficiency and changes in functional profiles, Shabat et al. (2016) suggested that the flow through metabolic pathways is different between these groups. Shabat et al. (2016) found that there is a significantly lower number of KEGG-enriched pathways in the microbiomes of efficient cows compared to their inefficient counterparts. Similarly, studies using PCR-DGGE also observed that the profiles from efficient animals were grouped more closely than those from inefficient ones. Taken all together, rumen bacteria from efficient animals have more similar metabolic pathways (GUAN et al., 2008; ZHOU et al., 2010).

Thus, Shabat et al. (2016) suggested that inefficient animals have a more diverse use of resource compounds, which results in a more diverse array of metabolites produced that may negatively affect energy harvest by the animal. The use of a limited number of metabolic pathways by efficient animals may favor a better utilization of the compounds. Additionally, the rumen microbiome of inefficient animals is less dominated by specific taxa, which suggests that the microbiome of efficient animals is less complex and more specialized to support the energy requirements of the host.

It is worth noting that the studies discussed in this review have used different methodologies in order to understand the relationship between rumen microbiota and feed efficiency. Inconsistencies observed across these studies can be attributed not only to the diet and inter-animal variations but also to the methodologies used. The sampling method can directly influence the detection of microbial diversity, considering that the solid and liquid phases of the rumen present a difference in the microbiota composition (JEWELL et al., 2015; NOEL et al., 2019). Regarding the molecular analyses, the primers utilized, methods for DNA preparation, genetic material employed (DNA *vs*. RNA), and the targeted microbial populations (selected microbial taxa *vs*. entire active microbiome) may influence the results (BROOKE et al., 2020; ZHANG et al., 2020). The size of the reference library also affects the performance of predictions from rumen microbiome profiles (ROSS et al., 2013). Several studies have found many unclassified sequences (CARBERRY et al., 2014a; GUAN et al., 2008; HERNANDEZ-SANABRIA et al. 2010, 2012; LI et al., 2017; MYER et al., 2015; ZHANG et al., 2020; ZHOU et al., 2009), which highlights the importance of international collaborations to build larger reference databases, such as the Hungate 1000 project (http://www.rmgnetwork.org/hungate1000.html). In addition, standardizing experimental, laboratorial, and computational protocols across studies could improve the accuracy and interpretation of the results, generating a better understanding of which differences in the microbiota are related to the phenotype and which are a result of the methodology used.

Conclusion

The current paper provides an overview of studies that were generally contrasting. This review suggests that comprehending how the environmental factors (e.g., the chemical composition of the diet) affect host physiology is essential to understand feed efficiency phenotyping. Moreover, it was stressed that there is an urgent need for standardization of the

methodologies to measure feed efficiency in order to provide data that can be comparable across studies.

To understand the effect of feeding patterns and digestibility on feed efficiency, more studies are needed to investigate different breeds. This is especially important for beef and dairy cattle operations. These two groups of animals utilize feed energy and are fed and managed differently, and as a result, variations in feed efficiency are expected to be observed. Therefore, well-designed experiments and appropriate statistical analyses, such as meta-analysis and advanced statistical models, are required to understand the biological parameters associated with beef cattle *versus* dairy cattle feed efficiency.

Ruminal volatile fatty acids, metabolism, pH, and production of both methane and enzymes vary during the day at different times after feeding. This highlights the importance of longitudinal studies in order to obtain samples at different times of the day and periods of animal life. Histological studies with samples from multiple locations in the rumen are also warranted to provide a complete understanding of tissue metabolism as well as intercellular spaces, cell migration, and nutrient absorption.

Moreover, it is necessary to determine to what degree the rumen microbiome influences the host and vice-versa. To achieve this goal, meta-omics technology could generate usable data on transcripts, proteins, and metabolites to depict the functional activity of the rumen community and complement information provided by DNA sequencing. This approach can offer a more complete understanding of how the rumen microbiome profile influences feed efficiency. We also suggest that future studies correlate feed efficiency to as many different rumen variables as possible to remove inconsistencies of results across studies and provide in-depth information concerning the complex host-microbe interactions.

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Table 1. Short-chain fatty acids (SCFA) in the rumen fluid found in studies that investigated feed efficiency in bovines. (X) represents where SCFAs were more abundant.

Short-chain fatty acids (SCFAs)	**L-RFI**	**H-RFI**	**No difference**	**Diet/Author**
Total SCFA	X			Low forage [1, 2]
		X		Low forage [3]
			X	Low forage [4, 7]
			X	High forage [5, 6]
Butyrate	X			Low forage [1, 2]
	X			Milk and starter grain mix [8]
		X		Low forage [9]
			X	Low forage [4, 7, 13]
			X	High forage [5, 6, 10]
Acetate	X			Low forage [1,14]
			X	Low forage [4, 7, 13]
			X	High forage [5, 6, 10]
			X	Milk and starter grain mix [8]
Propionate	X			Low forage [2]
		X		Low forage [14]
	X			Milk and starter grain mix [8]
	X			High forage [12]
		X		Low forage [4]

		X		High forage [5]
			X	Low forage [1, 14]
			X	High forage [6, 10]
			X	Milk and starter grain mix [8]
Valerate	X			Low forage [1, 2]
		X		High forage [6]
			X	Low forage [4]
			X	Milk and starter grain mix [8]
			X	High forage [10]
Isobutyrate	X			Low forage [3]
			X	Low forage [4]
Isovalerate	X			Low forage [2]
	X			High forage [11]
		X		Low forage [9]
			X	Low forage [4]
Acetate: Propianate	X			Low forage [4]
	X			High forage [5]
		X		High forage [11]
			X	Low forage [2]

[1] Guan et al. (2008); [2] Shabat et al. (2016); [3] Hernandez-Sanabria et al. (2012); [4] McGovern et al. (2018); [5] Krueger (2009); [6] De la Torre et al. (2019); [7] Lam et al. (2017); [8] Elolimy et al. (2020); [9] Hernandez-Sanabria et al. (2012); [10] Rius et al. (2012); [11] Carberry et al. (2012); [12] Lawrence et al. (2011); [13] Artegoitia et al. (2017), [14] Lages et al. (2020).

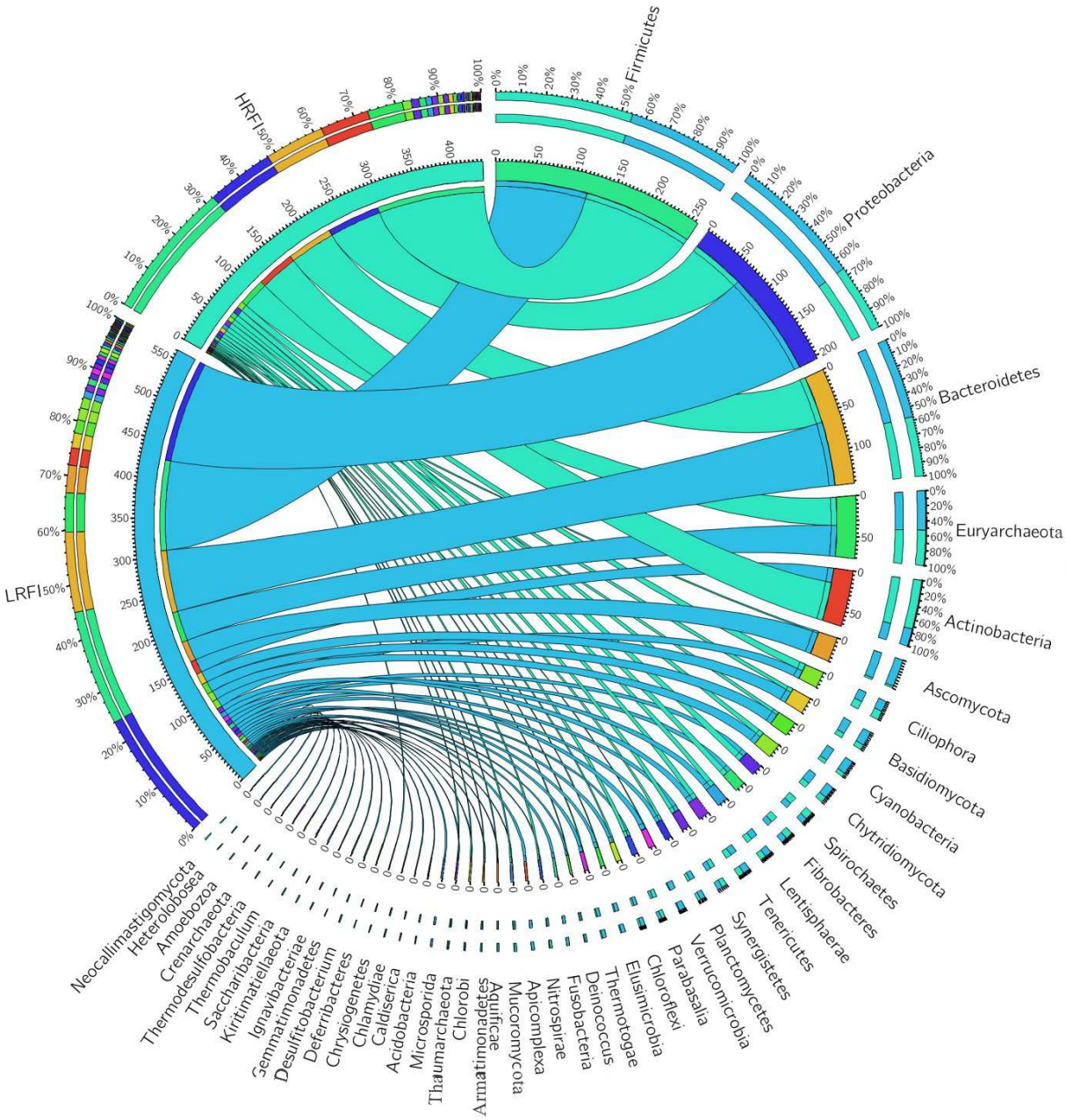

Figure 1. The frequency in which each phylum was more abundant on each RFI group. More efficient animals are identified as L-RFI – low RFI, and less efficient are identified as H-RFI – high RFI. The figure represents studies where the rumen microbiota was related to bovine RFI. (Zhou et al., 2009, 2010; Hernandez-Sanabria et al., 2012; Rius et al., 2012; Jami et al., 2014; McCan et al., 2014; Jewel et al., 2015; Li et al., 2016, 2017, 2019a, b, 2020; Carberry et al., 2012, 2014; Shabat et al., 2016; Cunningham et al., 2018; Elolimy et al., 2018a, b; McGovern et al., 2018; Clemmons et al., 2019; Delgado et al., 2019; Auffret et al., 2020; Bowen et al., 2020; Zhang et al., 2020). The width of the tapes represents the number of studies where each phylum was more abundant in each group of feed efficiency.

Section 2

(Section to be submitted to the journal *Animal Production Science*,
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Does the methodology influence the relationship between feed efficiency and residual feed intake? A meta-analysis

Abstract

Rumen microorganisms provide about 70% of the energy to the host through fermentation, and studies have shown that the microorganisms taxa can be differentially correlated to the feed efficiency. Besides the growing efforts in this research field in the last years, provided specially by the advent of next-generation sequencing (NGS), results obtained from studies are still contradictory, which can be related to the variables across the studies. These studies show differences in the breed, diet, DNA sequencing platform and hypervariable region of DNA sequenced. So, the objective of this study was to compare the composition of the rumen microbiome, sequenced by NGS and correlated to residual feed intake (RFI), in order to understand how these variables affect the abundance of taxa found. Besides that, this study presents a review of all studies that approach the relationship between feed efficiency and rumen microbiome.

Key-words: Feed efficiency, residual feed intake, RFI, rumen microbiome.

1. Introduction

The rumen harbors a dense diversity of microorganisms, which consists of bacteria, archaea, protozoa and fungi. These microorganisms are responsible for providing about 70% of the energy to the host through the fermentation of feed particles and degradation of plant fibers into digestible compounds for the ruminants, having volatile fatty acids (VFAs), carbon dioxide, methane, and ammonia as final products (BERGMAN, 1990; HERNANDEZ-SANABRIA et al., 2010). Recently, Wallace et al. (2019) used a large number of animals in order to prove the existence of a core rumen microbiome, phylogenetically linked and present in all animals. Despite this part of the rumen microbiota, there is another part highly responsive to changes in diet, age, sex, breed, geographical location, antibiotic use and the health of the host animal (CUNNINGHAM; AUSTIN; CAMMACK, 2018; PAZ et al., 2018). Considering

the fundamental role of microorganisms in ruminal fermentation and the great influence of animal physiology on the composition of the rumen community, the pioneer study by Guan et al. (2008) highlighted that microorganism taxa could be differentially correlated to the feed efficiency. Since then, omics have been revolutionizing the study of microbial diversity, making possible to compare the structure of microorganism communities, especially those that are not cultivable, such as the rumen microbiota. Furthermore, several studies have used these methodologies in order to better understand how the microbial taxonomic composition and microbial genes responsible for specific functions can be related to feed efficiency.

Despite the growing effort in this field, the data generated from each study is individually affected by diet and animal physiology, as well as by the methodology of the experiment, and may end up expressing not the variations related to feed efficiency, but the variations related with methodology used on each study. Moreover, considering differences in physiology across the different breeds, it is important to understand whether the information obtained from a breed can be applied to others. In this way, the objective of this study was to compare the composition of the rumen microbiome and its relationship with feed efficiency in order to understand how the variables across the studies affect the abundance of taxa found. For this, we performed a meta-analysis in order to access the differences through the total abundance of ruminal microbiota from animals with divergent phenotypes for residual feed efficiency (RFI). Besides that, this study is a review of this field of research.

2. Material and Methods

2.1 Literature Search

We performed an extensive electronic literature search that accessed the influence of rumen microbial in RFI, considering the more abundant taxa in each group of efficiency. Studies were searched in Scopus, Web of Science, PubMed and Google Scholar databases using the codes in English ("feed efficiency" OR "residual feed intake" OR RFI) AND rumen, published beforeJanuary 2021. In addition, we also searched references cited by the articles selected in order towiden our search related to this topic. There was no restriction to peer-reviewed journals and only papers published in journals were included, excluding abstracts, conference proceedings and thesis. The abstracts of the articles found in the databases were read in order to identify those that met the search criteria.

2.2 Review and state of the art

All the studies that correlated rumen microbiome and feed efficiency were included in the review, including studies that used all methodologies of measurement of feed efficiency and molecular sequencing methodologies, in order to perform the review of this research field.

2.3 Meta-analysis approach

In order to provide a robust comparison, only those studies which met the following selection criteria were included in the meta-analysis: 1) Studies that used samples from rumen; 2) studies that used RFI as a measurement of feed efficiency; 3) studies that directly reported the relation between rumen microbiota related to both more efficient animals (low residual feed intake, L-RFI) and less efficient animals (high residual feed intake, H-RFI), listing the taxa that occurred in each group. The exclusion criteria were: 1) studies that used samples from other gastrointestinal tract segments; 2) studies that did not discuss exactly the relation between rumen microbiome and feed efficiency (i.e. studies that discuss the influence of the diet on efficiency and, separately, discuss microbiome).

Studies that used more than one diet (low forage – LF and high forage - HF) and more than one breed, and did not specify in which of these variables each taxon was found, were excluded from the meta-analysis of these topics. Studies that did not specify the hypervariable region used also were not included in the meta-analysis about this issue.

We used the total abundance of ruminal microbiota in order to compare the composition of the rumen microbiome and its relationship with feed efficiency. The standardized sensitivity was calculated using the z–score approach according to the following equation:

$$z = \frac{x - \mu}{\sigma}$$

where x is the total abundance data described measuring the efficiency per different kind of methodologies (i.e., hypervariable region, sampling method, sequencing platform, breed and diet), μ is the overall mean of all data per approach of efficiency, and σ is the standard deviation of all data for all approach of efficiency included in this study.

The z–score is a meta-analytical approach commonly used to assess differences and overall effects between heterogeneity in different outcomes (VILAS–BOAS et al., 2020). All calculations were performed in MS Excel by firstly calculating the standardized and partial z–score values, standard deviation, confidence interval, overall effect size, and later calculating the sum of the squared deviations being the figures performed in SigmaPlot (version 12.0).

3. Results

3.1 Data availability

Our article search yielded a total of 21,448 studies: 462 from Scopus, 719 from PubMed, 869 from Web of Science, 19,400 from Google Scholar and 5 additional ones taken from references cited in these studies.

3.2 Literature review

After screening all studies, 34 studies correlating rumen microbiome and feed efficiency (**Table S1**) were found. The majority of the studies (82%) sequenced only the 16S rRNA, and most of them were focused on bacteria (**Figure 1A**), and the RFI was the most common feed efficiency index across studies, being used in 85% of them (**Figure 1B**). Most of the breeds used in these studies are from Europe (**Figure C**). Concerning methodologies of DNA sequencing used in these studies, there has been a change over time with the introduction of New Generation Sequencing (NGS) (**Figure 1D**), and despite most of the studies have used 16S rRNA, the hypervariable region sequenced was highly different across studies (**Figure 1E**); on the other hand, most studies that used 18S rDNA did not specify which hypervariable region was used.

3.3 Meta-analysis approach

After screening all studies, only 10 studies were selected for the meta-analysis (**Figure 2, Table 1**). The studies were based on purebreds, hybrids and composite bovines, and there is a large difference between the number of animals sampled across studies (31.5 ≠ 18.59). The majority of studies (6) collected samples from slaughter, and there are some differences across the type of probe used in the other 4 studies. Illumina was the most common sequencing method used, being part of the methodology of 7 studies, and the 16S rRNA was the most accessed molecular marker to identify the rumen microorganisms (**Table 1**), being the microorganism groups differentially sampled between the studies.

The selected studies provided 878 abundance values for 47 phyla of microorganisms and 515 genera, and bacteria was the most sampled group.

4. Discussion

4.1 Literature review

4.1.1 Groups differentially sampled

The bovine rumen microbiota is composed mostly of bacteria (95%) (MIZRAHI, 2013), and this high abundance is related to its capacity to decompose feed into short-chain fatty acids (C1 to C5), amino acids, H_2, and CO_2, which is essential to supply the energetic requirements of the host. These organisms are widely investigated (**Figure 1A**), and studies have shown that different taxa of bacteria are affected differentially by feed efficiency phenotype. Authors have pointed out that some taxa could be used as markers to feed efficiency, as species from *Prevotella* genus, that are highly responsive to this phenotype independently of the diet (CARBERRY et al., 2014; MCCANN et al., 2014; PAZ et al., 2018).

The second most sampled group is Archaea, which constitutes 2–5% of the rumen composition. Since authors have suggested that more efficient animals produce 28% less methane than the less efficient ones (HEGARTY et al., 2007; JONES et al., 2011; NKRUMAH et al., 2006), in the early studies on this field some authors wondered whether the community composition or abundance of methanogens could be strongly related to feed efficiency (ZHOU; HERNANDEZ-SANABRIA; LE, 2009; ZHOU; HERNANDEZ-SANABRIA; LUO GUAN, 2010a). However, despite these studies finding differences in methanogen composition between the feed efficiency phenotypes, these data could not completely explain the difference that exists in both feed efficiency groups and methane production. Afterward, studies have shown that less efficient animals could present less methane yield (MCDONNELL et al., 2016; OLIJHOEK et al., 2018), and methane production may not be the key that links rumen microbiome and feed efficiency.

The eukaryote ruminal community is composed of protozoa and fungi (0.1–1%), and despite their low abundance, protozoan ciliates contribute to about one-third of fermentative digestion and also control bacterial populations through predation, but studies about the importance of these microorganisms in the rumen have had contradictory results (GUYADER et al., 2014; MORGAVI et al., 2010b; SANTRA et al., 1998). Additionally, rumen fungi play a role in fiber degradation since they can penetrate both the cuticle and the cell wall of lignified material (CARBERRY et al., 2012). Despite eukaryotic rumen microorganisms being highly specialized in fiber degradation, limited attention was given to them until recently, when Zhang et al. (2020) deeply investigated the role of these microorganisms on feed efficiency and found that the less efficient animals have a higher richness of eukaryotic microorganisms in the rumen. Also, interestingly Auffret et al. (2020) found that some pathogenic eukaryotic are more abundant in more efficient animals.

Only three studies sampled the whole microbiome (AUFFRET et al., 2020a; LAM et al., 2018; RIUS et al., 2012), and besides most of these studies have shown that few taxa are correlated to the feed efficiency, the knowledge about how specific microbial groups affect the whole microbial profile are poor yet (HERNANDEZ-SANABRIA et al., 2012), and there is a considerable number of sequences attributed to *unknown* taxa.

4.1.2 Feed efficiency indexes

Several indexes have been used to address the animals into feed efficiency phenotypes, and each of them has a distinct mathematic basis, which may result in divergent efficiency rankings (FREETLY et al., 2020; JEWELL et al., 2015a). For this reason, the multiple definitions of feed efficiency make it difficult to describe the relationship between the rumen microbiota and feed efficiency (Freetly et al., 2020).

The RFI, proposed by Koch et al. (1963) and expanded recently (HILL, 2012), is the most used feed efficiency index. This measurement is highly accepted due to its independence from production traits, body weight (BW), and growth rate, so the inter-animal differences are mostly related to variations in metabolic processes (ARTHUR; RENAND; KRAUSS, 2001; NKRUMAH et al., 2006). However, studies have shown that the ranking of animals selected based on RFI phenotypic may change when the animals receive a different diet (low- to high energy and vice versa) (DURUNNA et al., 2011; ZHOU et al. 2010), which can hamper comparison of data from studies with animals under different diets.

4.1.3 Breed sampling

Despite North America being the main continent producing research in this field (58% of the articles included in this review), most of the breed used in these studies is from Europe (**Figure 1C**). It happens due to the massive imports of domestic animals during the late seventeenth and early eighteenth centuries in North America, and a subsequent good adaptation of these breeds to a new environment (COSSETTE; HORARD-HERBIN, 2003). However, considering that the studies in this field are mostly from Europe and North America, there is great repeatability in the breeds used, which can influence the results found. Several breed-associated biological factors play a role in the rumen microbiome, such as eating frequency, dry matter intake (DMI), and rumen size (LI et al., 2019a). The small variation on the breeds used in the studies brings advantages in order to provide comparisons across the data obtained on them, on the other hand, it is not advantageous considering that the influence of breed in

rumen microbiome is not well understood and more studies are needed in order to understand the variation correlated to the feed efficiency and that correlated to the breed.

4.1.4 Sequencing platforms

The advent of next-generation sequencing (NGS) technology allowed the study of uncultivable rumen microorganisms, making it possible to analyze deeper the role of the microbial community function and its influence on the host (MYER et al., 2015a).

Despite the high resolution of NGS sequencing, the different NGS platforms might recover different diversities. A comparison between the two most frequently used platforms, Illumina and Roche 454, showed that despite both platforms providing a comparable view of the community sampled, there are differences between them, and Illumina showed more explicitly both sequence coverage and limit of detection than Roche 454 (LI et al., 2014; LUO et al., 2012). This difference is shown in the results of the studies, which had lower throughput, not allowing the identification of most of the taxa at the genus level. These differences led to an increase in the use of the Illumina platform, which occurred simultaneously with a growing interest in this research field, in a way that most of the studies correlating the rumen microbiome and feed efficiency are based on Illumina sequencing (**Figure 1D**).

4.1.5 Hypervariable region

Over time, the hypervariable regions of DNA amplified in the studies were changed. This occurred mainly due to the changing of the sequencing platform, since regions V6-V8 were chosen for their length is within the sequencing metrics of the 454 platform (JEWELL et al., 2015a) and subsequently the regions V3-V4 have shown an increased resolution on data from microbiome profiles and improved reproducibility, although they still have existing limitations (SOERGEL et al., 2012; TREMBLAY et al., 2015) (**Figure 1E**).

4.2 Meta-analysis approach

4.2.1 Breed

The data found in our study shows that purebreds have lower variation in the abundance of the rumen microbiome than those from the hybrid Holstein-Friesian (**Figure 3A**). These data support the data previously discussed about the influence of the breed on the rumen microbiome.

The core microbiome has a higher heritability than the non-core rumen microorganisms, and these taxa with high heritability are more correlated to physiologic characteristics from the

host, such as feed intake and metabolism (LI et al., 2019b; SASSON et al., 2017). Moreover, Guan et al. (2008) suggested that purebreds generate different microbial profiles from those from hybrid animals, and it was also observed that some breeds have a higher influence on the rumen microbiome than the feed efficiency phenotype, which does not occur in hybrid animals (GUAN et al. 2008).

In this way, our study reinforces that there is an influence of the breed, and consequently of the host physiology on the rumen microbiome. Our study also shows that data about the rumen microbiome obtained from a host breed may not be considered for all breeds, and the taxa of microorganisms might influence differently the feed efficiency across the breeds. In this way, studies that have been searching for a taxa key for feed efficiency should take into account whether it is possible to apply that for all the breeds.

4.2.2 Diet

Our data show that animals fed with a low forage (LF) diet have higher variation in the abundance of the rumen microbiome than those under high forage (HF) (**Figure 3B**). It is widely known that the diet modulates the microbial profile, however, it was observed that the role of the rumen microbiome on feed efficiency also may be modulated by the diet, differing across the diets offered (CARBERRY et al., 2012). Moreover, Li et al. (2019) observed that taxa with a lower heritability are more affected by the diet than the phenotype. In this way, Li et al. (2019) suggest that the diet needs to be the same across all animals in order to understand the link between both breed and rumen microbiome on feed efficiency (LI et al. 2019).

4.2.3 Sequencing platforms

Our analysis support that there is a higher sequencing throughput obtained from Illumina sequencing (**Figure 3C**), showing that Illumina can identify taxa occurring in a relatively low frequency in the sample. This advantage is also demonstrated by the change of methodology used in this study field over time, with the adoption of Roche 454 becoming unusual while Illumina 454 had considerable growth in its use (**Figure 1D**).

On the other hand, despite the increasing quantity and quality of data generated from NGS sequencing, a significant number of sequences is assigned to unclassified microorganisms yet, which hampers a more accurate analysis of the whole microbiome(LI et al., 2019b; ZHANG et al., 2020a). In this way, more efforts are necessary in order to comprehend and

characterize rumen microorganisms, especially expanding the coverage of rumen microbial genomes in databases (LI et al., 2019b).

4.2.4 Hypervariable region

The meta-analysis made here shows that the V6-V8 regions can assign microorganisms in a large range of abundance (**Figure 3D**), allowing that microorganisms in a low abundance on the sample might be sequenced. However, the V3-V4 regions, most used nowadays, show a lower variation between the data obtained, allowing a more appropriate comparison between studies.

In this way, it is important to be careful when comparing data considering that they can be biased based on the hypervariable region selected (MYER et al., 2015a).

6. Conclusion

This study demonstrates that is urgently needed to standardize the methodology used to explore the rumen microbiome, since the methodology used (eg., hypervariable region and sequencing platform) can influence the taxonomic composition of the rumen microbiome identified.

Despite the recent advance in this research field, the NGS allowed the obtention of a large quantity of data in a short period of time. However, it is yet necessary to improve the computational methodology to allow the identification of a greater percentage of the taxa and to enable a general view of the microbial profile.

Moreover, it is fundamental to better understand which part of the microbiome profile is correlated to feed efficiency phenotype and which part is correlated to the variables of the studies.

It is imperative to make comparisons across the different breeds in order to better understand the influence of the host physiology on the rumen microbiome, and also to utilize different diets in order to understand the influence of the diet on the microbiome in each breed.

Thereon, this study shows that it is crucial to standardize the methodologies used in the studies, such as sequencing methodology and hypervariable sequences, in order to generate data that allow comparison between studies allowing to find patterns in rumen microbiome between the RFI groups. Furthermore, it is important to highlight that studies that search for a key taxon to improve the RFI need to take into account that this taxon may not be the same for all bovines, considering that the role of each taxon on the host is influenced by its physiology and diet.

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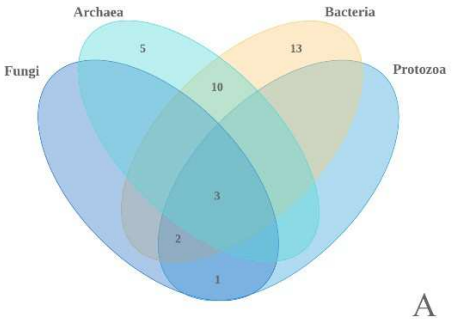
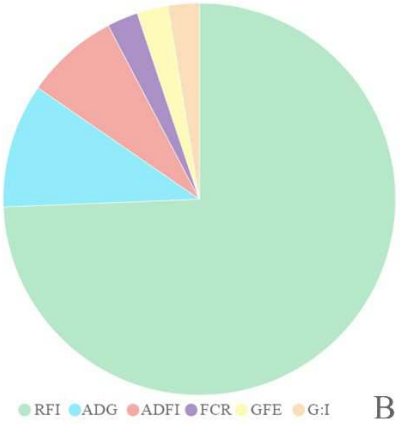
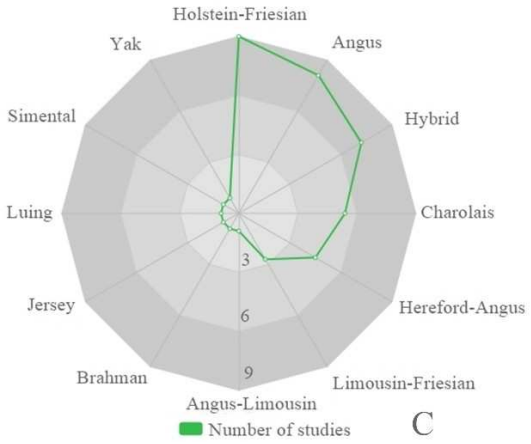

Archaea
Bacteria
Fungi
Protozoa
5
13
10
3
2
1

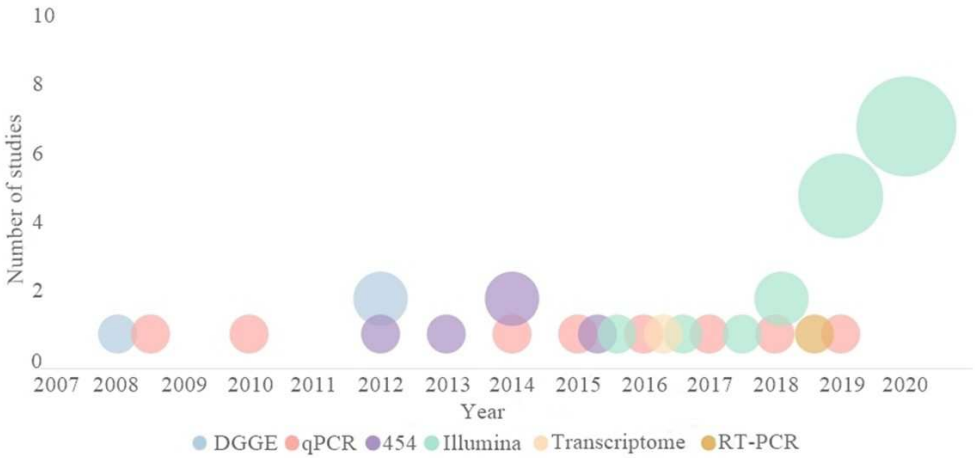
A


RFI
ADG
ADFI
FCR
GFE
G:I

B


Holstein-Friesian
Angus
Hybrid
Charolais
Hereford-Angus
Limousin-Friesian
Angus-Limousin
Brahman
Jersey
Luing
Simental
Yak
3
6
9
Number of studies

C


Number of studies
Year
DGGE
qPCR
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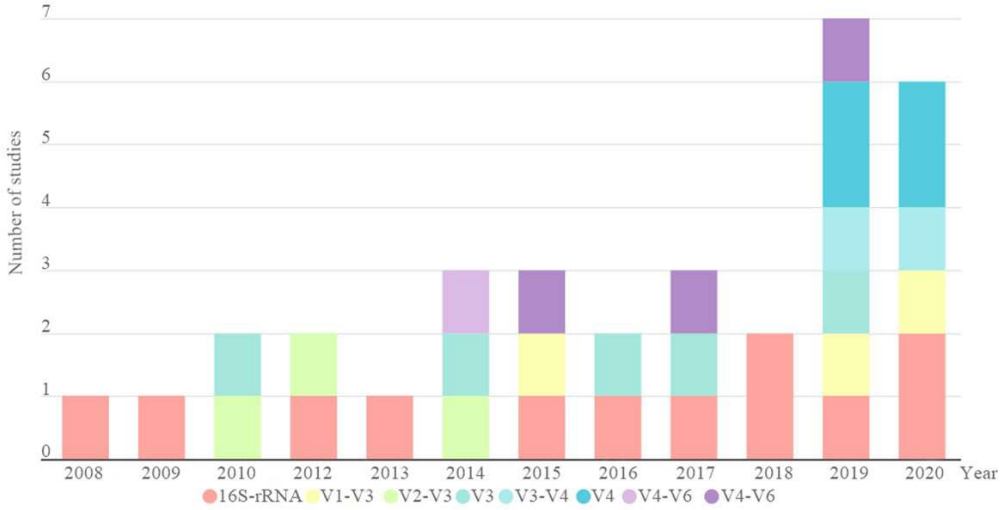
Illumina
Transcriptome
RT-PCR

D


Number of studies
Year
16S-rRNA
V1-V3
V2-V3
V3
V3-V4
V4
V4-V6
V4-V6

E

Figure 1. Studies included in the review. **A.** Venn diagram of the groups sampled. Each circle represents the number of studies in which each microbial group was sampled; **B.** Percentageof studies in which the different feed efficiency indexes were used; **C.** Breeds used in the studies. Each circle represents three studies; **D.** Methodologies used for microbial taxa identification over time; **E.** Number of studies in which each hypervariable region was used.

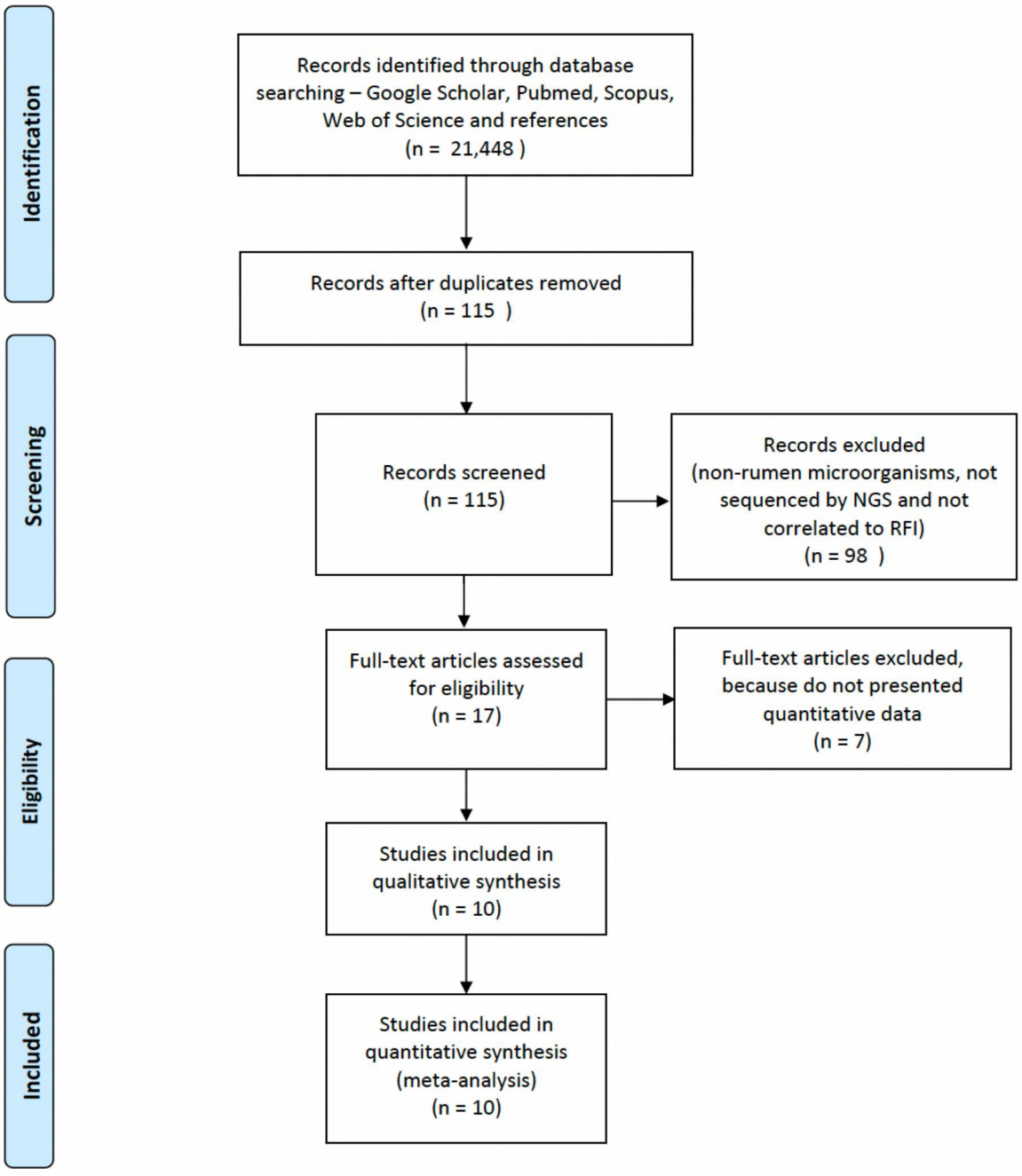


Fig. 2. Flow diagram of the literature searched in Google Scholar, Pubmed, Scopus, Web of Science and references within articles found and included in our study. Figure adapted from PRISMA (MOHER et al., 2009); RFI: Residual Feed Intake.

Table 1. Summary of the studies included in the meta-analysis.

Study	Diet	Breed	Sample method	Sequencing method	Hypervariable region
Auffret et al. (2020)	HF and LF	Charolais and Luing	Slaughter	Illumina	-[1,2]
Zhang et al. (2020)	LF	Angus, Charolais and Kinsella Hybrid	Slaughter	Illumina	-[2]
Welch et al. (2020)	LF	Angus	Slaughter	Illumina	-[1]
Li et al. (2019)	LF	Charolais and Kinsella Hybrid	Slaughter	Illumina	V1-V3[3] and V6-V8[4]
Noel et al. (2019)	HF and LF	Holstein-Friesian and Jersey	Transesophageal device	Illumina	V3-V4[3,4]
McGovern et al. (2018)	LF	Simmental	Slaughter	Illumina	V4[3,4]
Li et al. (2017)	LF	Hybrid	Slaughter	Illumina	V1-V3[3] and V6-V8[4]
Jewell et al. (2015)	HF	Holstein-Friesian	Rumen cannula	454	V6-V8[3]
McCan et al. (2014)	HF and LF	Limousin-Friesian	Oral-rumen tube	454	V4-V6[3]
Rius et al. (2012)	HF	Holstein-Friesian	Rumen cannula	454	-[1,2]

[1]16S rRNA, unspecified region; [2]18S rRNA, unspecified region; [3]Bacteria; [4]Archaea

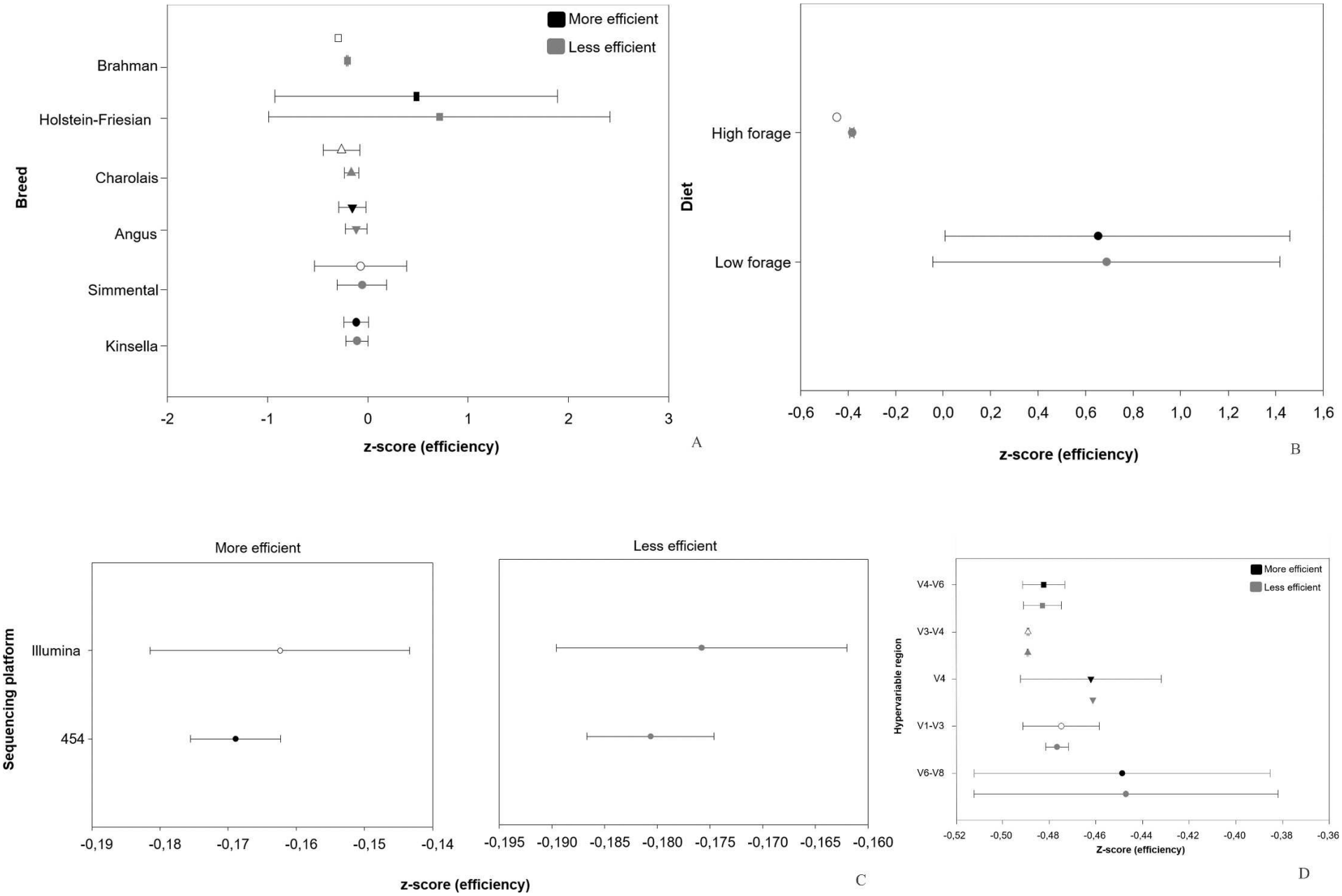

More efficient
Less efficient
Brahman
Holstein-Friesian
Charolais
Angus
Simmental
Kinsella
Breed
-2
-1
0
1
2
3
z-score (efficiency)
A
High forage
Low forage
Diet
-0,6
-0,4
-0,2
0,0
0,2
0,4
0,6
0,8
1,0
1,2
1,4
1,6
z-score (efficiency)
B
More efficient
Less efficient
Illumina
454
Sequencing platform
-0,19
-0,18
-0,17
-0,16
-0,15
-0,14
-0,195
-0,190
-0,185
-0,180
-0,175
-0,170
-0,165
-0,160
z-score (efficiency)
C
More efficient
Less efficient
V4-V6
V3-V4
V4
V1-V3
V6-V8
Hypervariable region
-0,52
-0,50
-0,48
-0,46
-0,44
-0,42
-0,40
-0,38
-0,36
Z-score (efficiency)
D

Figure 3. The meta-analysis of rumen microbiome abundance and its correlation with RFI. **A.** Abundance of rumen microorganisms correlated to the breed of the host; **B.** Abundance of rumen microorganisms according to the diet received by the host; **C.** Abundance of rumen microorganisms correlated to molecular sequencing method, divided between RFI groups; **D.** Abundance of rumen microorganisms correlated to the hypervariable region of 16S rDNA or 18S rDNA sequencing.

Section 3

(Section to be submitted to the journal *Frontiers in Microbiology*, Qualis A1, IF: 5.640)

Taxonomic and predicted functional signatures reveal linkages between the rumen microbiota and feed efficiency in dairy cattle raised in tropical areas

Abstract

Ruminants can digest plant biomass due to the symbiosis with a complex microbiota residing in the rumen environment. However, the relationship between the rumen taxonomic and functional microbial composition and feed efficiency (FE) remains unclear, especially in hybrid dairy cattle (Holstein x Gyr) raised under tropical conditions. In this study, twenty-two F1 Holstein x Gyr heifers were selected and divided into two groups according to their residual feed intake (RFI) ranking: 1) high efficiency (HE) (n = 11) and 2) low efficiency (LE) (n = 11). Rumen contents were collected using a stomach tube apparatus and analyzed using amplicon sequencing targeting the 16S and 18S rRNA genes. The diversity indexes revealed no differences in the rumen microbiome between the HE and LE groups. The multivariate analysis (sPLS-DA) showed a separation in bacterial taxonomic profiles, but no differences in archaeal and protozoal profiles were detected. sPLS-DA showed a clear separation in the predicted functional profile for bacteria, archaea, and protozoa between the HE and LE groups. Specific microbial taxa (e.g., *Howardella*, *Shuttleworthia*, *Eremoplastron,* and *Polyplastron*) and functions (e.g., K03395, K05882, and K13643) associated with each FE group were observed. This study demonstrates that the differences in the rumen microbiome relative to FE ranking are not directly observed through diversity indexes but by identifying specific taxa and microbial functions that characterize each FE group. This identification may allow the discovery of biomarkers that could improve feed efficiency through manipulation of the rumen microbiota and fermentation and illuminate how the ruminal taxonomic and functional profiles work in hybrid dairy cattle raised in tropical conditions.

Key-words: archaea, bacteria, protozoa, microbiome, RFI, SSUrRNA.

1. Introduction

Feed fermentation in the rumen is powered by the activity of a vast array of anaerobic microbes that live in perfect symbiosis with the host animal (OLIVEIRA et al., 2007). These microbes comprise representative taxa from prokaryotic (bacteria and archaea) and eukaryotic (fungi and protozoa) organisms. Bacteria are the most abundant rumen microorganisms, playing an essential role in the degradation of plant fiber and starch (MORAÏS; MIZRAHI, 2019). Archaea, mainly constituted of methanogens, reduce CO_2 to CH_4 to maintain a low hydrogen pressure in the rumen (BODAS et al., 2012). Fungi are related to fiber digestion, penetrating both the cuticle and the cell wall of lignified materials (MORAÏS; MIZRAHI, 2019). Rumen protozoa predate bacteria and enhance methanogenesis (MORGAVI et al., 2010a). Despite the importance of protists for rumen fermentation and interspecies association with methanogens, few studies have attempted to understand the relationship between rumen protozoa and feed efficiency (FE) (CLEMMONS et al., 2021; ZHANG et al., 2020b).

The rumen microbes are believed to affect the host FE, and this effect has been observed mainly when residual feed intake (RFI) is used as the FE measurement (GUAN et al., 2008; AUFFRET et al., 2020). Most studies correlating the rumen microbiome with RFI have predominantly been developed in cattle raised in temperate climates (AUFFRET et al., 2020; WELCH et al., 2021). However, little is known about the linkage between the rumen microbiome and RFI in breeds raised in tropical regions. In addition to the diet, the rumen microbiome composition is affected by the breed (LI et al., 2019c) and the environmental conditions (e.g., temperature) where the animal is raised (DEHORITY; ORPIN, 1997). The rumen functioning and microbial fermentation are affected when temperate climate breeds are exposed to the high atmospheric temperatures in tropical areas (BAUMGARD et al., 2007; PASSINI et al., 2009), indicating that the association of the microbiome and FE within the breed are altered in animals raised under heat stress.

Brazil is one of the largest milk producers in the world (IBGE, 2018), relying on the cross-bred Holstein x Gyr to support its milk production. These hybrid cattle are the most common dairy breeds in the country and are fully adapted to tropical areas. They combine the high milk yield from Holstein with the Gyr adaptation and tolerance to high temperatures, ecto- and endoparasites (COSTA et al., 2010; FERREIRA et al., 2010). About 64% of the world's cattle are raised in the tropics (AZEVEDO et al., 2005), but these animals are usually less productive than those reared in

temperate areas. Hybrid cattle are necessary to increase productivity because pure breeds from temperate climates perform poorly in tropical areas owing to heat stress (BACCARI Jr, 1990; MARCHEZAN, 2013). Taken together, these studies suggest that improving ruminant productivity in tropical areas requires, among other things, a full understanding of the contribution of the rumen microbiome composition and functions to FE.

Yet, the relationship between the rumen microbiome and RFI has not reached a general consensus across studies (AUFFRET et al., 2020b; BOWEN et al., 2020b; FREGULIA et al., 2021). Some authors say that the relationship between the rumen microbiome and RFI phenotype may not be explained at the community level because of the redundant role played by the microbial taxa in the rumen function (FREGULIA et al., 2021). Other authors report that the functional profile of the rumen microbiota is more related to FE than the taxonomic profile itself (CLEMMONS et al., 2019; LI et al., 2019). Shabat et al. (2016) found that efficient cattle had a lower number of biochemical-enriched pathways than their inefficient counterparts, suggesting that the rumen microbiome of efficient animals has more restricted metabolic pathways and maintains only those functions that are relevant to the host animal. The current study investigated the effect of the RFI phenotypes on the rumen microbial taxa (bacteria, archaea, and protozoa) and their functions in hybrid dairy cattle (Holstein x Gyr) raised under tropical conditions.

2. Materials and methods

All experimental procedures involving animals in this study were approved by the Ethics Committee of Embrapa Dairy Cattle (number: 05/2015). The experiment was conducted at the Embrapa Dairy Cattle Experimental Farm - Coronel Pacheco, Minas Gerais, Brazil.

Animal experiments and sample collection

This experiment was conducted as part of a larger study designed to examine the biological parameters in F1 Holstein x Gyr related to feed efficiency (CABRAL DA SILVA et al., 2020; FONSECA et al., 2020; LEÃO et al., 2018; MARÇAL-PEDROZA et al., 2020; ORNELAS et al., 2019). A detailed description of diet composition, performance data, calculation of FE indexes, and group classifications are provided in Cabral et al. (2020).

Briefly, twenty-two F1 Holstein x Gyr heifers were used, averaging 258 ± 20 d (mean ± SD) of age and 293 ± 21.5 kg body weight (BW) at the beginning of the metabolism study. Heifers

were housed in individual tie stalls (2.5 x 1.2 m) with bedding made of rubber mats (WingFlex, Kraiburg TPE GmbH & Co., Waldkraiburg, Germany). Diet had dry matter (DM) and crude protein (CP) concentrations of 437 g/kg and 178 g/kg DM, respectively, and included (DM basis) 75% corn silage and 25% concentrate (96% soybean meal and 4% mineral premix, DM basis). The animals were evaluated according to the RFI index and classified into two groups: 1) high efficiency (HE) and 2) low efficiency (LE), with 11 animals per group.

Rumen contents were collected using a stomach tube with a rumen vacuum sampler, snap-frozen using liquid nitrogen, and stored under − 80 °C for further analysis.

DNA extraction, library preparation, and sequencing

Total DNA was extracted from 2mL of each rumen fluid sample using bead-beating and phenol-chloroform extraction methods (adapted from Oliveira et al., 2007). Briefly, 2 mL of rumen fluid sample were transferred to a new tube and washed with 1 mL of lysis buffer (500 mM NaCl; 50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 4% SDS). Subsequently, 2 µl RNase were added to the samples and the tubes were incubated at 37° C for 15 minutes. Twenty microliters of proteinase K were added to the tubes and the cells were lysed by physical disruption using bead beating with a BioSpec Mini Bead-Beater (BioSpec, Bartlesville, OK, USA) at 4,800 rpm for 4 minutes. The supernatant was obtained from each sample and transferred to a new tube for the subsequent phenol-chloroform-isoamyl alcohol extraction. The extracted DNA was precipitated with ammonium acetate 10M and cold 100% isopropanol. After 30 minutes in the freezer, the tubes were centrifuged at 16,000 x g for 10 minutes and the supernatant was removed. Cold 70% ethanol was added to the samples and the tubes were centrifuged at 16,000 x g for 2 minutes. The supernatant was removed and the content was resuspended in 200 µl of buffer TE (10 mM Tris; 1 mM EDTA). All samples were analyzed using both the NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and Qubit Quantification Platform (Invitrogen Ltd., Paisley, UK) to accurately assess DNA quantity and quality.

Amplicon library preparation (n = 22) was performed by PCR amplification of the V4 region of the 16S rRNA gene of bacteria and archaea, using the primers 515F (5′-Adaptor/ GTGCCAGCMGCCGCGGTAA) and 806R (5′-Adaptor/GGACTACHVGGGTWTCTAAT) (CAPORASO et al., 2011); and by the amplification of the V3-V4 region of the 18S rRNA gene of protozoa, using the customized primers 316F (5′-Adaptor/GCTTTCGWTGGTAGTGTATT)

and 539R (5′- Adaptor/CTTGCCCTCYAATCGTWCT) (SYLVESTER et al., 2004). Illumina TruSeq libraries were prepared and sequenced on the Illumina HiSeq 2500 sequencing platform (Illumina, Inc., SanDiego, CA, USA).

Amplicon sequencing data analysis

Bioinformatics analysis followed the same procedure as previously described by Liu (2020). In detail, sequencing data were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2) 2020.8 version (BOLYEN et al., 2019). The data were demultiplexed, the sequence reads were quality-filtered, denoised, and merged. After quality control, the Divisive Amplicon Denoising Algorithm (DADA2) plugin implemented in QIIME2 was used to remove chimeric sequences, and the amplicon sequencing variants (ASVs) table was generated (CALLAHAN et al., 2016). Representative sequences were aligned to the SILVA 132 Small Subunit rRNA Database for bacteria and the classifier was pre-trained on the Silva 18S rRNA database (release 132) for protozoa and on the Rumen and Intestinal Methanogens Database (RIM-DB) for archaea (QUAST et al., 2012; SEEDORF et al., 2014a), using the *fit-classifier-naive-bayes* method from the *q2-feature classifier* plugin.

Predicting functional profile

Microbial functions were predicted by reconstruction of the unobserved states for 16S and 18S rRNA sequences. The tool Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) in QIIME2 (DOUGLAS et al., 2020) is based on the Integrated Microbial Genomes (IMG) database (MARKOWITZ et al., 2012) and was used to predict MetaCyc pathways for bacterial, archaeal, and protozoal ASVs (CASPI et al., 2014).

Statistical analysis

To analyze the microbial diversity among samples, the sequence count of all samples was standardized by rarefying them to the same number of sequences (the smallest sampling size) using the *q2-feature-table* plugin. The plugin *q2-diversity* used the rarefied feature table and the phylogenetic tree to calculate the diversity metrics. To investigate the Alpha-diversity metrics, Faith's Phylogenetic Diversity (PD), Evenness, and Shannon's diversity were calculated. And to investigate Beta-diversity metrics, weighted UniFrac distance, Jaccard index, and Bray–Curtis

dissimilarity index were calculated. The dissimilarity and distance among rumen microbiota and categorical RFI groups were tested on unweighted UniFrac distance matrices Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations. Based on this analysis, plots were generated using the visualizer of the *q2-diversity* plugin.

Finally, the mixMC multivariate method implemented in the *mixOmics* R package was used to identify associations between microbial profiles and functions (microbial and functional signatures) and the RFI groups. For this analysis, only microbial taxa and microbial functions with a relative abundance > 0.01% and prevalence in at least 50% of the samples were considered (11 out 22). Then, sparse partial least square discriminant analysis (sPLS-DA) (LÊ CAO et al., 2016) was applied to identify microbial signatures related to RFI.

3. Results

Sequencing information

Ruminal samples were collected from dairy cattle (n = 22) in order to determine the relationship between taxonomic and predicted functional profiles of rumen microbiome and RFI. A total of 2,132,659 million of 16S rRNA reads and 7,084,856 million of 18S rRNA reads were generated from the rumen samples collected from the 22 animals. After quality control, combining paired-end reads, and filtering chimeras, on average 91% of the sequences passed the filters, with 1,661,299 sequences separated as 16S rRNA and 6,111,590 as 18S rRNA. An average of 75,513 (±12,226) and 277,799 (±58,359) quality-filtered sequences were generated per animal for 16S rRNA and 18S rRNA, respectively. Good's coverages for both 16S and 18S rRNA were higher than 98%, suggesting that the sequencing depth had sufficient coverage for the microbial communities.

Microbial community structure

The bacterial, archaeal, and protozoal communities were examined using Faith's Phylogenetic Diversity (PD), Evenness, and Shannon's diversity analysis, and none of the alpha-diversity metrics were different between HE and LE animals. Additionally, beta-diversity metrics such as unweighted UniFrac, did not show a significant difference between the two groups (**Supplementary Table S1**). The weighted UniFrac, Jaccard index, and Bray–Curtis dissimilarity

matrix demonstrated that there was no clustering between the divergent RFI groups (data not presented).

Taxonomic profile

Taxonomic profiling revealed a total of 22 procaryotic taxa at the phylum level identified from 16S rRNA and 8 eukaryotic taxa at the phylum level from 18S rRNA. From 16S rRNA, 74% of the ASVs belonged to the Bacteria kingdom, 24% to Archaea, and 2% were unclassified. The dominant prokaryotic phylum was *Firmicutes* (52%), followed by *Euryarchaeota* (24%) and *Bacteroidota* (18%). At the genus level, the predominant taxa were *Methanobrevibacter* (23%), *Christensenellaceae*_R-7_group (10%), and *Prevotella* (8%). From 18S rRNA, 95% of the ASVs were categorized as Protozoa, <0,001% were classified as Fungi, and 5% were unclassified. The dominant phylum was Ciliophora (95%) followed by an unassigned group (5%). At the genus level, the predominant taxa were *Entodinium* (53%), *Diplodinium* (22%), and the unassigned group (15%). Notably, 14% and 20% of the reads could not be assigned to a known genus of 16S and 18S rRNA, respectively (**Figure 1**).

In order to better represent the taxonomic and predict functional profile of the rumen microbiota related to RFI, all detected taxa, including the unclassified taxa, were included in the analysis. Fungi taxa sequenced by 18S rRNA primer were removed from the analysis, since this molecular marker is not suitable for fungi due to a low-quality classification.

Predicted functional profile

To improve the accuracy of the analysis, Using the PICRUSt2 package in QIIME2, a total of 6,774 and 7,636 MetaCyc pathways were predicted based on 16S rRNA and 18S rRNA, respectively.

Metabolic pathways were predicted for bacteria and archaea separately, but the RIM-DB was used to improve the classification of archaea and generated a new dataset. Even using these two different datasets (bacteria and archaea) as inputs for PICRUSt2, it was possible to observe that the MetaCyc pathways predicted for both datasets were the same, with identical frequency per feature. The ten most abundant pathways reconstructed for each microbial group were considered the major predicted functions of the rumen microbiome (**Supplementary Table S2**).

Taxonomic and functional signatures related to RFI

The sPLS-DA multivariate analysis was used to identify microbial taxa and functions that best characterize each group of RFI. For this analysis, only microbial taxa and functions with a relative abundance > 0.01% and prevalent in at least 50% of the samples (11 out 22) were considered.

Twenty-one phyla and 49 genera of bacteria and archaea were detected from 16S rRNA, and two phyla and seven genera of protozoa were detected from 18S rRNA. After pre-training the classifier in order to improve the archaeal classification, a new dataset was generated from 16S rRNA including only archaea, with one phylum and two genera

Following the centered log-ratio transformation procedures, it was observed a clear separation in bacterial taxonomic profile differentiating the rumen microbiome in HE and LE, but no differences were observed in archaeal and protozoal profiles (**Figure 2**). However, although the taxonomic profile reveals no differences for archaeal and protozoal, a clear separation of the functional profile for bacteria, archaea, and protozoa from divergent groups of RFI was observed (**Figure 2**).

Overall, 55% of the bacterial signature selected in component 1 of the sPLS-DA characterized the rumen microbiome of HE animals, which included members of the taxa *Howardella*, *Shuttleworthia*, *Coprococcus*, *Colidextribacter*, *Solobacterium*, *Carnobacterium*, [*Eubacterium*]_xylanophilum_group, and four unclassified taxa. From the new dataset generated for archaea, 50% of the archaeal signature selected in component 1 characterized the rumen microbiome of HE animals, having also members of one unclassified taxon present. On the other hand, 60% of the protozoal signature selected on this same component of the sPLS-DA characterized the rumen microbiome of LE animals and comprised the taxa *Eremoplastron*, *Polyplastron,* and one unclassified taxon (**Figure 3**).

The most important MetaCyc pathways for component 1 in bacteria and archaea are related to metabolism (50%), signaling and cellular processes (30%), and genetic information processing (20%). For protozoa, the most important MetaCyc pathways on component 1 are related to metabolism (72%), environmental information processing (9%), genetic information processing (9%), and unknown functions (9%) (**Figure 4**).

In terms of functional signature, 70% of the bacterial and archaeal signatures selected in component 1 of the sPLS-DA characterize the rumen MetaCyc pathways of HE animals, including functions related to signaling and cellular processes (eg., K03395, K18833, and K03304),

metabolism (eg., K05882, K03822, K13669, K15781, K18382) and genetic information processing (eg., K13643 and K07445). For protozoa, 100% of the signature selected on this same component of the sPLS-DA characterize the rumen MetaCyc pathways of LE animals, including functions related to metabolism (eg., K16177, K08265, K14082, K16183, K08264, K16180, K16181, and K16182), environmental information processing (eg., K01539), genetic information processing (eg., K11627) and unknown functions (eg., K09706) (**Figure 4**).

4. Discussion

This is the first report that investigated the linkage between the rumen microbial community and its functions and the RFI phenotype of hybrid dairy cattle raised in tropical conditions. First, alpha- and beta-diversity indexes did not differ between the two RFI groups. In agreement with previous studies on temperate climate breeds, these findings suggest that the diversity indexes may not be significant to differentiate feed efficiency phenotypes (CLEMMONS et al., 2019; MYER et al., 2015b). Second, the microbial signatures are useful to detect correspondences between specific taxa and RFI phenotypes in ruminants (SHABAT et al., 2016a; DELGADO et al., 2019). Third, the MetaCyc pathways predicted on PICRUSt2 and analyzed using mixMC were able to separate functional microbial profiles related to RFI for bacteria, archaea, and protozoa, and also specific metabolic pathways associated with each RFI group. These results are in line with Shabat et al. (2016a) and suggest that the functional profile of the rumen microbiota can be more informative about FE than the taxonomic profile of the whole microbial community.

The lack of differences in alpha- and beta-diversity suggest that RFI phenotypes may not be reflected in the diversity of the microbial community, but are the result of dissimilarities at a finer resolution, such as specific microbial taxa (MCGOVERN et al., 2020). Microorganisms belonging to different taxonomic groups may play the same role in the rumen, utilizing similar substrates and producing similar products (CLEMMONS et al., 2019; LI et al., 2019c). This may indicate that detecting specific microbial taxa and their functions is fundamental to understanding the linkage between the RFI phenotype and the taxonomic structure of the rumen microbiome. Studies that used PCR-DGGE to understand the linkage between microbial community structure and FE have reported that the bacterial profiles generated from LE animals were grouped and separated from the profiles obtained from HE animals (GUAN et al., 2008). In this study, the sPLS-DA models showed a clear separation in the bacterial and archaeal profiles differentiating the two

RFI groups, except for the protozoal profiles (Figure 1). Carberry et al. (2012) found that the diet influenced the effect of RFI on the bacterial profile, especially when the animals were fed a higher forage diet, in agreement with our results. Nevertheless, most recent studies have suggested that specific microbial taxa and not the whole rumen microbiome are the main drivers that explain the differences in FE phenotypes regardless of the diet type (CARBERRY et al., 2012; ELOLIMY et al., 2018; BROOKE et al., 2019).

The microbial signatures identified in this article provide a further understanding of the relationships between RFI and the rumen microbiome and its functions. The bacterial signature of HE animals included members of the families *Lachnospiraceae* (*Howardella*, *Shuttleworthia*, *Coprococcus,* and *Eubacterium xylanophilum*), *Oscillospiraceae* (*Colidextribacter*), *Erysipelotrichaceae* (*Solobacterium*), *Carnobacteriaceae* (*Carnobacterium*), and four not identified taxa (**Figure 2**). The genus members of *Lachnospiraceae* found here have been previously related to feed efficiency in cattle (ELOLIMY et al., 2018; JEWELL et al., 2015b; SHABAT et al., 2016) as well as in other animals, such as pigs and chickens (ALIAKBARI et al., 2021; LEE; KIL; SUL, 2017). Among all genera described above, the following three genera are the only ones with known functions in the rumen. *Howardella* plays a role in urea hydrolysis (COOK et al., 2007). Ureolytic bacteria are the most important organisms in the rumen involved in N metabolism and are responsible for the breakdown of urea to NH_3 used for the synthesis of microbial protein for the host (HAILEMARIAM et al., 2021). *Shuttleworthia* participates in lipid and carbohydrate metabolism and regulates the endocrine system via SCFA production, which can potentially increase the host feed efficiency (LIU et al., 2021). *Coprococcus* has been extensively related to high feed efficient cattle (JEWELL et al., 2015b; SHABAT et al., 2016) and plays a role in metabolizing carbohydrates for the host (WHITMAN and UBO, 2015).

The bacterial signature of the LE group included members of the families *Pirellulaceae* (p-1088-a5_gut_group), *Desulfovibrionaceae* (*Desulfovibrio*), *Peptostreptococcaceae* (*Romboutsia*), *Fibrobacteraceae* (*Fibrobacter*), *Clostridia*_UCG-014, WCHB1-41, and three not identified taxa. While *Pirellulaceae* p-1088-a5_gut_group is associated with inefficient cattle, it has been related to more efficient pigs (GARDINER; METZLER-ZEBELI; LAWLOR, 2020) and contributes to calcium digestibility in goats (LIU et al., 2020). *Desulfovibrio* is responsible for removing the toxic hydrogen sulfide gas from the rumen when ruminants consume increased concentrations of sulfate. Hydrogen sulfide can inhibit the production of VFA, especially butyrate, thereby impacting feed

efficiency (ZHANG et al., 2021). *Romboutsia* is related to less severe immune responses, as demonstrated by the decreased concentrations of pro-inflammatory cytokines on plasma levels (LIANG et al., 2016) in inefficient animals exhibiting downregulated immune functions (KERN et al., 2016b).

The archaeal signature of HE animals is composed of unclassified taxa. Myer et al. (2016) also found many unassigned taxa that could be the key to understanding feed efficiency. Projects such as the Hungate 1000 (http://www.rmgnetwork.org/hungate1000.html) are crucial to investigating the rumen microbiome and the relationship between the archaeal taxa and feed efficiency. The archaeal signature of LE is entirely composed of *Methanobrevibacter*, which accounts for the majority of the rumen methanogens in cattle. This genus is more abundant in inefficient cattle and is associated with enteric methane emissions (DELGADO et al., 2019).

The protozoal signature comprehends members of only one family, Ophryoscolecidae. For HE, the signature included members of the genera *Ophryoscolex* and *Metadinium*, and for LE included members of *Eremoplastron*, *Polyplastron,* and one genus not identified. Rumen protozoans play a role in microbial protein synthesis, nitrogen balance, and contribute up to 50% of the bio-mass in the rumen (MORGAVI et al., 2010b; GUYADER et al., 2014; NEWBOLD et al., 2015). Different from our results, previous studies detected a differential abundance of *Diplodinium* and *Entodinium* in divergent FE groups (CLEMMONS et al., 2021; ZHANG et al., 2020b). However, the relationship between rumen protozoa and feed efficiency is still not clear, indicating a need for further study of protozoal functions on the rumen.

For the predicted MetaCyc pathways, even though no statistical differences were found between the two RFI groups, there is a clear separation in the microbial functions for bacteria, archaea, and protozoa related to each group. In bacteria and archaea, 48 and 45%, respectively, of the metabolic pathways in HE animals were associated with various metabolism functions (e.g., carbohydrates and lipids). For protozoa, 36% of the MetaCyc pathways related to LE animals were associated with energy and amino acid metabolism, indicating that, unlike the bacterial metabolic functions, the protozoal metabolic pathways may be detrimental to feed efficiency (**Figure 3**). Our results agree with Shabat et al. (2016) and Li and Guan (2017), who showed that less efficient cattle have more diverse activities of rumen microbiomes than their efficient counterparts. According to Shabat et al. (2016), in more efficient cattle, simpler metabolic pathways networks may result in a

higher concentration of products that are more relevant for the rumen fermentation, supporting a greater energy harvest efficiency for the host.

5. Conclusion

This study has revealed compositional differences in specific taxa and MetaCyc pathways related to RFI phenotypes in dairy cattle raised in tropical conditions. Several taxa were unassigned when we profiled the microbial community at more specific levels (e.g., genus, species). This limitation points to the necessity of using sequencing platforms that utilize longer reads sequencing to improve the resolution of the microbial taxonomic classification (e.g., species and strain levels) to identify novel microbial biomarkers related to FE. It is imperative to build reference databases tailored for the rumen environment to overcome this limitation. Despite the existence of rumen microbiome databases such as the RIM-DB for methanogens (SEEDORF et al., 2014b), the AF-RefSeq for anaerobic fungi (PAUL et al., 2018), and the ureC database for ureolytic bacteria (JIN et al., 2017), these libraries are still limited, with a large number of rumen microorganisms not yet identified and cultured.

This article suggests that discovering biomarkers for FE phenotypes could be accomplished by identifying specific taxa and metabolic pathways that characterize each RFI phenotype. In this way, specific microbes and metabolic pathways could be manipulated in the rumen to improve FE. Additionally, we suggest that meta-omics data (e.g., metagenomics, metatranscriptomics, metabolomics) be incorporated in future studies to facilitate biomarkers discovery and provide a better overview of the rumen microbiome functionality and its association with FE phenotype. To achieve these goals, future research has to harness the power of new technologies (e.g., third-generation sequencing followed by advances in bioinformatic analysis) to reveal the complex interplay between the rumen microbiome (composition and functions) and FE.

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FIGURES

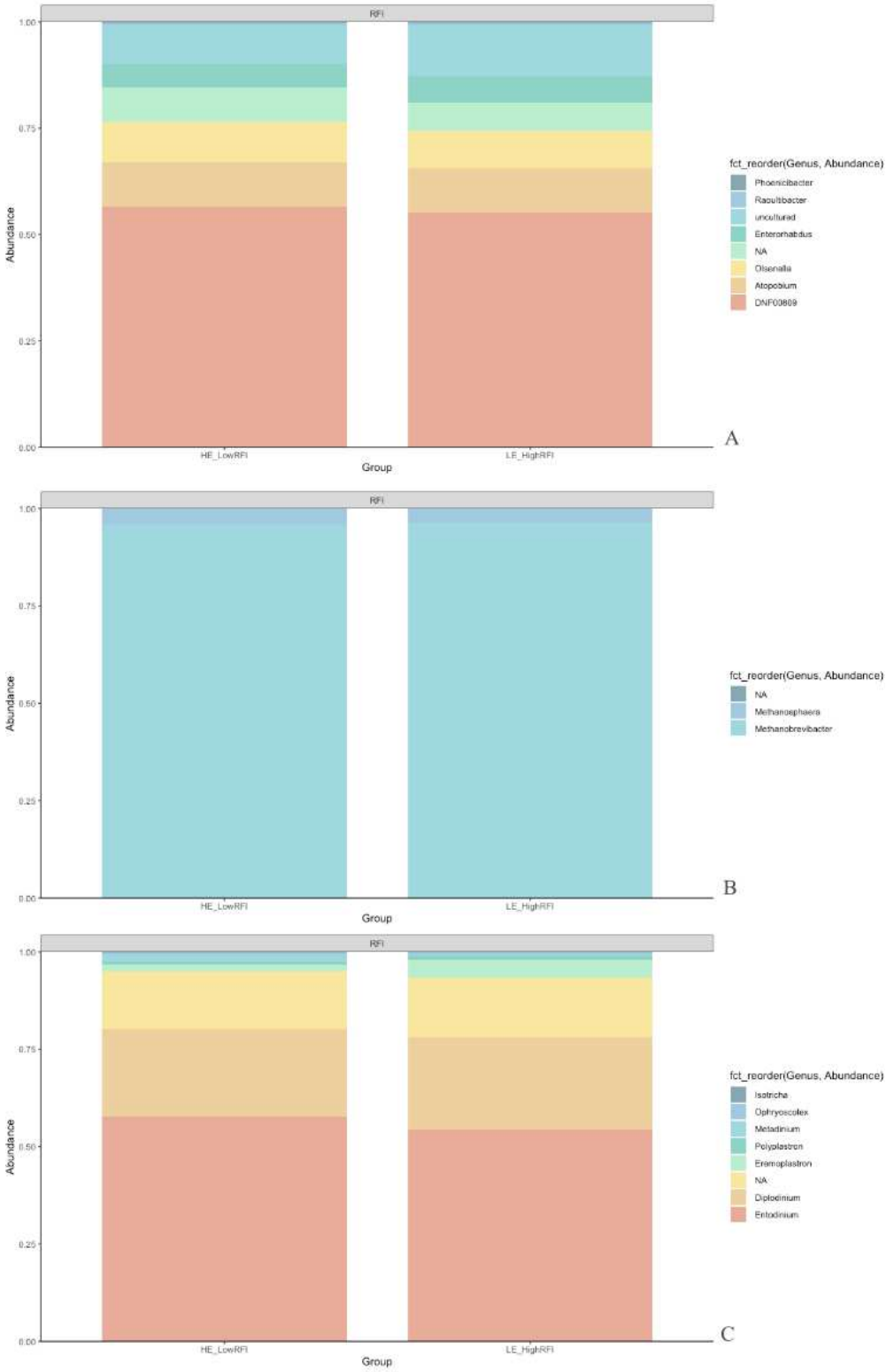

RFI
Abundance
1.00
0.75
0.50
0.25
0.00
HE_LowRFI
LE_HighRFI
Group
fct_reorder(Genus, Abundance)
Phoenicibacter
Raoultibacter
uncultured
Enterorhabdus
NA
Olsenella
Atopobium
DNF00809
A
RFI
Abundance
1.00
0.75
0.50
0.25
0.00
HE_LowRFI
LE_HighRFI
Group
fct_reorder(Genus, Abundance)
NA
Methanosphaera
Methanobrevibacter
B
RFI
Abundance
1.00
0.75
0.50
0.25
0.00
HE_LowRFI
LE_HighRFI
Group
fct_reorder(Genus, Abundance)
Isotricha
Ophryoscolex
Metadinium
Polyplastron
Eremoplastron
NA
Diplodinium
Entodinium
C

Figure 1. Taxa summary plot between the two feed efficiency groups (FE and LE). **A.** Bacteria; **B.** Archaea; **C.** Protozoa.

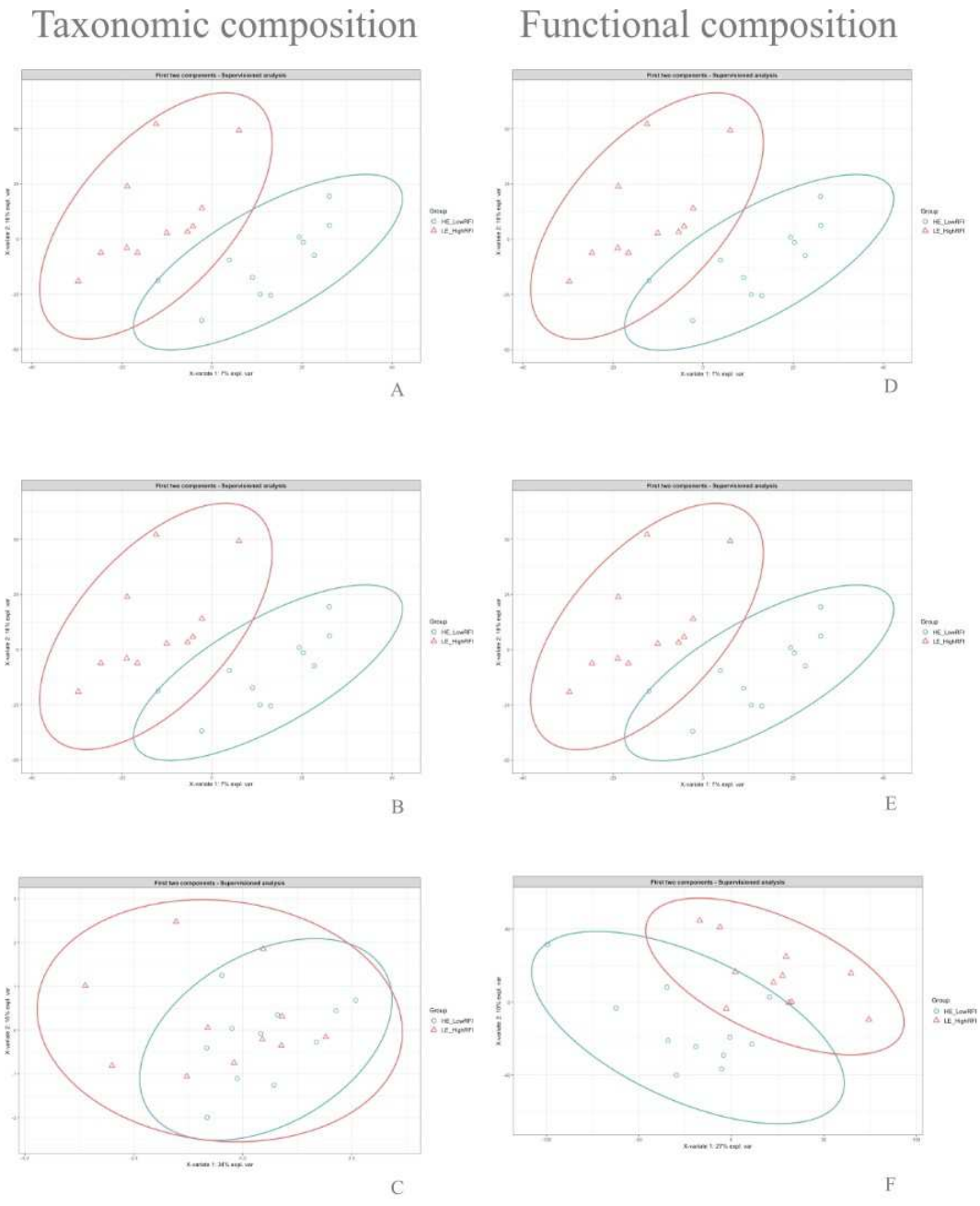


Figure 2. Sparse partial least square discriminant analysis results on rumen microbiome in two FE groups of dairy cattle. Sample plot on the two first sPLS-DA components with 95% confidence level ellipse plots. **A.** bacterial taxonomic composition; **B.** archaeal taxonomic composition; **C.** protozoal taxonomic composition; **D.** bacterial functional composition; **E.** archaeal functional composition; **F.** protozoal functional composition.


Low efficiency (LE)
High efficiency (HE)
NA
WCHB1-41
Clostridia_UCG-014
[Eubacterium]_xylanophilum_group
Carnobacterium
NA
uncultured
Fibrobacter
Solobacterium
Colidextribacter
NA
Romboutsia
Coprococcus
NA
NA
Desulfovibrio
p-1088-a5_gut_group
NA
Shuttleworthia
Howardella
-0.2
0.0
0.2
0.4
A
NA
Methanobrevibacter
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
B
NA
Polyplastron
Metadinium
Ophryoscolecidae
Eremoplastron
-0.6
-0.4
-0.2
0.0
0.2
0.4
C

Figure 3. Contribution of each microbial taxa selected on the first component. The length of the bar represents the importance of the microbial taxa to the component – importance from the bottom to the top. Colors indicate the FE group in which the microbial taxa is more abundant. **A.** Bacteria; **B.** Archaea; **C.** Protozoa.


Low efficiency (LH)
High efficiency (HE)
K18382
K15781
K13669
K03822
K07445
K03304
K13643
K18833
K05882
K03395
A
K18382
K15781
K13669
K03822
K07445
K03304
K13643
K18833
K05882
K03395
B
K16182
K16181
K16180
K08264
K16183
K14082
K11627
K08265
K01539
K16177
K09706
C

Figure 4. Contribution of each microbial function selected on the first component. The length of the bar represents the importance of the microbial taxa to the component – importance from the bottom to the top. Colors indicate the FE group in which the microbial function is more abundant. **A.** bacterial function; **B.** archaeal function; **C.** protozoal function.

Supplementary Material

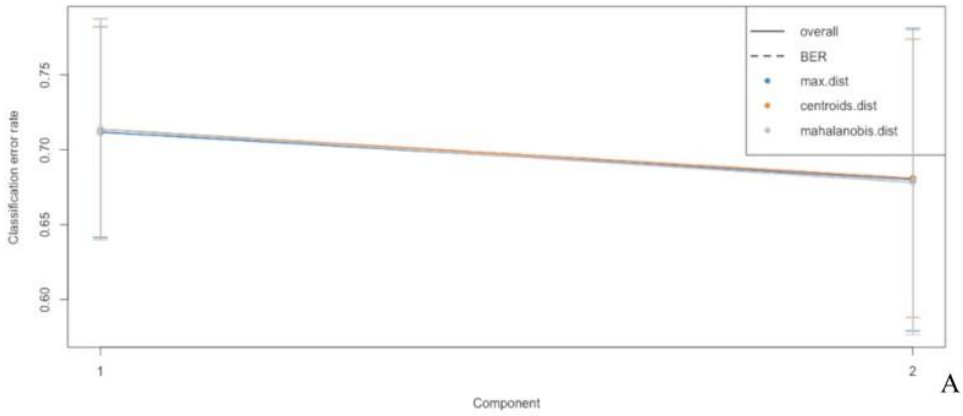
Supplementary Table S1. Alpha-diversity and Beta-diversity statistics of the rumen microbiota in HE and LE dairy cattle. Significance determined at $p \leq 0.05$.

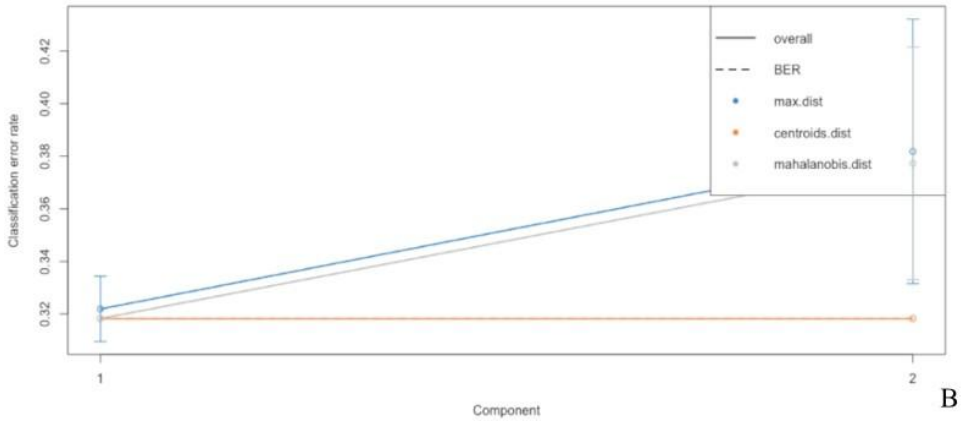
Diversity metric	Bacteria	Archaea	Protozoa
	P-value	*P*-value	*P*-value
Faith's Phylogenetic Diversity	0.12	0.09	0.10
Simpson's Evenness	0.71	0.66	0.92
Good's coverage	98%	98%	99%
Unweighted UniFrac	0.98	0.82	0.63

Supplementary Table S2. The main predicted microbial functions on rumen of dairy cattle divergent to feed efficiency.

Group	KEGG		
	Category	KEGG	Description
Bacteria and Archaea	Signaling and cellular processes	K01990	Transport system ATP-binding protein
		K06147	ATP-binding cassette, subfamily B, bacterial
		K01992	Transport system permease protein
		K02004	Transport system permease protein
		K02003	Transport system ATP-binding protein
	Genetic information processing	K03088	RNA polymerase sigma-70 factor, ECF subfamily
		K02529	LacI family transcriptional regulator
		K03657	DNA helicase II / ATP-dependent DNA helicase PcrA [EC:3.6.4.12]

	Lipid metabolism	K00059	3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]
	Function unknown	K07133	Uncharacterized protein
Protozoa	Signaling and cellular processes	K01990	Transport system ATP-binding protein
		K01992	Transport system permease protein
		K02004	Transport system permease protein
		K02003	Transport system ATP-binding protein
		K02015	Iron complex transport system permease protein
		K06147	ATP-binding cassette, subfamily B, bacterial
		K02016	Iron complex transport system substrate-binding protein
	Genetic information processing	K03088	RNA polymerase sigma-70 factor, ECF subfamily
		K02529	LacI family transcriptional regulator
	Signal transduction	K03406	Methyl-accepting chemotaxis protein





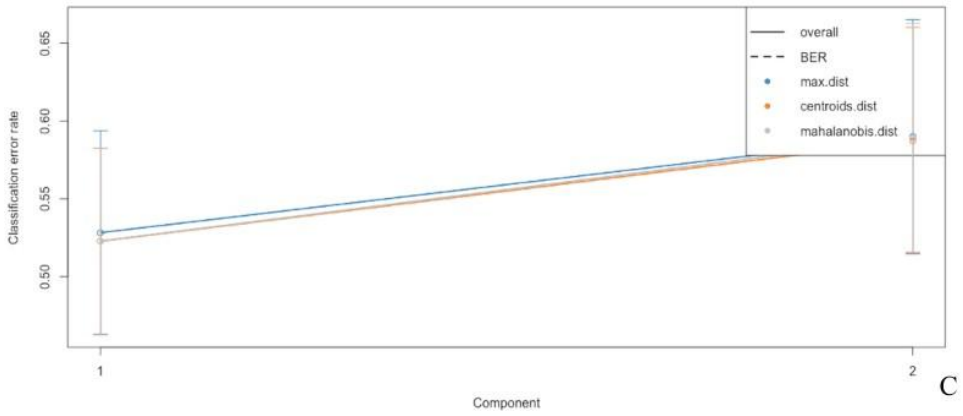


Figure S1. Classification performance per component for two predictions distances using repeated stratified cross-validation (10 × 5-fold Cross Validation). To estimate the classification error rate

for the dataset, the distance metrics used for sPLS-DA (CLR transformed data) was the "centroids.dist". **A.** Bacterial data; **B.** Archaeal data; **C.** Protozoal data.

Section 4

(Section to be submitted to the *Journal of Animal Science*, Qualis A1, IF: 3.159)

Methane yield in dairy cattle and its association with rumen taxonomic and functional composition of the rumen microbiome

Abstract

The rumen fermentation is related to methane production, a potent greenhouse gas (GHG), being the cattle responsible for 11% of the GHGs produced in the world. This methane emission also represents a loss of the gross energy ingested by the animal, and high methane emitters are less efficient in milk and meat production. Considering that the rumen microorganisms are the responsible for the rumen methanogenesis, we used twenty-two F1 Holstein x Gyr heifers divided into two groups according to their methane yield (high CH_4 yield (High_CH_4) and low CH_4 yield (Low_CH_4)), with eleven animals per group, in order to better understand the taxonomic and functional composition of the rumen microbiome related to the methane yield phenotype in animals raised under tropical areas. Rumen contents were collected and analyzed using amplicon sequencing targeting the 16S and 18S rRNA genes. The diversity indexes revealed no differences related to the methane yield phenotype. The multivariate analysis (sPLS-DA) showed only a discrete separation in taxonomic and functional profiles of bacteria, archaea and protozoa. Despite this analysis characterizing microbial taxa and functions that are more related to each methane yield group, based on the knowledge about rumen methanogenesis, we suggest that inter-domain interactions and functional niches can be more explanatory about methane yield than specific taxa or functions.

Key-words: Methane yield, enteric methane, rumen microbiome, 16S rRNA, 18S rRNA.

1. Introduction

The rumen fermentation occurs due to the activity of symbiotic microorganisms that act on components of the diet that are indigestible by the host, making them digestible (Oliveira et al., 2007). This process provides up to 70% of the energy supply for the animal, but is also related to methane production, which is a greenhouse gas (GHG) that contributes to global warming 28 times more than CO_2, being the ruminants responsible for 11% of the GHGs produced in the world (GROSSI et al., 2019; BEAUCHEMIN et al., 2020). In addition, the methane produced during the rumen fermentation is not metabolized by the animal and is eliminated in the atmosphere, mainly by respiration and eructation, which represents between 2% to 12% of loss of the gross energy ingested (JOHNSON; JOHNSON, 1995). In this way, in order to mitigate methane emissions and improve feed efficiency is needed to improve knowledge about enteric methane production.

The methanogen archaea are responsible for the CH_4 production on rumen, using the substrate released from the diet fermentation (MARTÍNEZ-ÁLVARO et al., 2020). The synthesis of methane can occur from three different pathways: methylotrophy, hydrogenotrophy and acetoclastic methanogenesis, and in general occurs due to the activity of methanogenic archaea reducing CO_2, methanol or methylamines to form CH_4 (BEAUCHEMIN et al., 2008; MARTÍNEZ-ÁLVARO et al., 2020; PITTA et al., 2022). Despite the methanogenesis process being well characterized, new methanogen taxa are still being discovered, and the extent to which the rumen microbiome influence CH_4 production on the host is not yet well known (DIFFORD et al., 2018; MARTÍNEZ-ÁLVARO et al., 2020).

All methanogens work toward the same goal of generating energy through methane synthesis, however, methanogenic lineages differ in their metabolic and physiological capabilities, which may differentially affect the CH_4 formation, with lineages related to greater CH_4 emissions (PITTA et al., 2022). Additionally, several authors revealed the importance of the other rumen microbial groups on methanogenesis, mainly through interactions with bacteria and protozoa supplying H_2 that posteriorly will be used on methanogenesis or being involved in other metabolic pathways related to methane production (KAMKE et al., 2016; MARTÍNEZ-ÁLVARO et al., 2020; SA et al., 2016).

Based on the need to clarify the relationship between methane yield in dairy cattle and the rumen microbiome, the objective of this study was to evaluate the relationship between divergent

animals to methane yield phenotype and the taxonomic and functional composition of the rumen microbiome.

2. Materials and methods

The procedures in this study were approved by the Ethics Committee of Embrapa Dairy Cattle (number: 05/2015). The experiment was conducted at the Embrapa Dairy Cattle Experimental Farm, located in Coronel Pacheco, Minas Gerais, Brazil.

This work is part of a larger study designed that aims to understand the biological parameters related to feed efficiency in F1 Holstein x Gyr, including methane measurements (CABRAL DA SILVA et al., 2020; FONSECA et al., 2020; LEÃO et al., 2018; MARÇAL-PEDROZA et al., 2020; ORNELAS et al., 2019). Ornelas et al. (2019) provide a detailed description of differences in methane production, yield and intensity, and also the calculation and group classification of the animals from this study into each methane index.

Briefly, twenty-two F1 Holstein x Gyr heifers were used, averaging 293 ± 21.5 kg body weight (BW) and 258 ± 20 d (mean ± SD) of age at the beginning of the metabolism study. Animals were housed in individual tie stalls (2.5 x 1.2 m) with rubber mats (WingFlex, Kraiburg TPE GmbH & Co., Waldkraiburg, Germany).

Diet was composed of dry matter (DM) and crude protein (CP) contents (437 g/kg and 178 g/kg DM, respectively) and included (DM basis) 75% corn silage and 25% concentrate (96% soybean meal and 4% mineral premix, DM basis).

Rumen contents were collected using a stomach tube with a rumen vacuum sampler, snap-frozen using liquid nitrogen, and stored under − 80 °C until analysis.

The animals were evaluated in gas exchange chambers for the calculation of methane yield. Observations of individual animal gas exchanges were collected using open-circuit respiratory chambers equipped with a data acquisition system (Sable Systems International, Las Vegas, USA). Based on CH_4 data (CH_4 yield (g/kg DMI)), the animals were divided into two groups, with eleven animals per group: high CH_4 yield (High_CH_4) and low CH_4 yield (Low_CH_4), as described by Ornelas et al. (2019). In order to explore the rumen microbiome, total DNA was extracted from 2mL of each rumen fluid sample using bead-beating and phenol-chloroform extraction methods (adapted from Oliveira et al., 2007). Briefly, was transferred 2 mL of rumen fluid sample to a new tube and washed with 1 mL of lysis buffer (500 mM NaCl; 50 mM Tris-HCl, pH 8.0, 50 mM

EDTA, 4% SDS). 2 µl RNase were added and the tubes were incubated at 37° C for 15 minutes. Were added 20 µl of proteinase K to the tubes and the cells were lysed by physical disruption using bead beating with a BioSpec Mini Bead-Beater (BioSpec, Bartlesville, OK, USA) at 4,800 rpm for 4 minutes. The supernatant was transferred to a new tube for phenol-chloroform-isoamyl alcohol extraction. The DNA was precipitated with ammonium acetate 10M and cold 100% isopropanol. The tubes remained for 30 minutes in the freezer and were centrifuged at 16000 xg for 10 minutes. The supernatant was removed and cold 70% ethanol was added. The tubes were centrifuged at 16000 xg for 2 minutes. The supernatant was removed and the content was resuspended in 200 µl of buffer TE. The NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) and Qubit Quantification Platform (Invitrogen Ltd., Paisley, UK) were used to accurately assess DNA quantity and quality.

Amplicon library preparation (n = 22) was implemented by PCR amplification of the V4 region of the 16S rRNA gene of bacteria and archaea, using the primers 515F (5′-Adaptor/ GTGCCAGCMGCCGCGGTAA) and 806R (5′-Adaptor/GGACTACHVGGGTWTCTAAT) (CAPORASO et al., 2011); and by the amplification of the V3-V4 region of the 18S rRNA gene of protozoa, using the primers 316F (5′-Adaptor/GCTTTCGWTGGTAGTGTATT) and 539R (5′-Adaptor/CTTGCCCTCYAATCGTWCT) (SYLVESTER et al., 2004). Were prepared Illumina TruSeq libraries and then sequenced on the IlluminaHiSeq2500 sequencing platform (Illumina, Inc., San Diego, CA, USA).

For the bioinformatic analysis, the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) 2020.8 version (BOLYEN et al., 2019) was used to analyze sequencing data. The data were demultiplexed, the sequence reads were quality-filtered, denoised, and merged. The Divisive Amplicon Denoising Algorithm (DADA2) plugin in QIIME2 was used to remove chimeric sequences, and the amplicon sequencing variants (ASVs) table was generated (CALLAHAN et al., 2016). Representative sequences were aligned to the SILVA 132 Small Subunit rRNA Database for bacteria(QUAST et al., 2012; SEEDORF et al., 2014a), and the classifier was pretrained on the Silva 18S rRNA database (release 132) for protozoa and on the Rumen and Intestinal Methanogens Database (RIM-DB) for archaea, using the fit-classifier-Naive–Bayes method from the q2- feature classifier plugin.

In order to predict MetaCyc metabolic pathways for bacterial, archaeal, and protozoal ASVs (CASPI et al., 2014), was used the package Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) in QIIME2 (DOUGLAS et al., 2020).

For the statistical analysis, to compare the microbial diversity among samples, the sequence count of all samples was standardized by rarefying them to the same number of sequences (the smallest sampling size) using the rarefy command of the q2-feature-table plugin. The plugin q2-diversity plugin used the rarefied feature table and the phylogenetic tree to calculate the diversity metrics. To investigate the Alpha-diversity metrics, were calculated Faith's Phylogenetic Diversity (PD), Evenness and Shannon's diversity. And to investigate Beta-diversity metrics were calculated weighted UniFrac distance, Jaccard index and Bray–Curtis dissimilarity index. The dissimilarity of the samples was tested on unweighted UniFrac distance matrices Permutational Multivariate Analysis of Variance (PERMANOVA) with 999 permutations. Based on this analysis, plots were generated using the visualizer of the q2-diversity plugin.

Furthermore, the multivariate methods in the mixMC (mixOmics microbial community) R package have been used in order to identify specific associations between microbial profiles and functions, and explanatory variables. For this analysis, were considered only microbial taxa and microbial functions with a relative abundance > 0.01% and prevalent in at least 50% of the samples (11 out 22). Then, sparse partial least square discriminant analysis (sPLS-DA) (LÊ CAO et al., 2016) was applied to identify microbial signatures related to methane yield.

3. Results

Twenty-two dairy cattle were used. The objective was to understand the differences in the taxonomic and predicted functional profile of the rumen microbiome related to methane yield. In total, were generated 2,074,658 million of 16S rRNA reads and 7,084,856 million of 18S rRNA reads from the rumen samples. After quality control, combining paired-end reads, and filtering chimeras, on average 91% of the sequences passed the filters, of which 1,622,048 were from 16S rRNA and 6,111,590 from 18S rRNA. Per animal, were generated an average of 73,729 (±14,905) filtered sequences from 16S rRNA and 277,799 (±58,359) from 18S rRNA. Good's coverages for both 16S and 18S rRNA were higher than 98%, which demonstrated a sufficient coverage of the sequencing depth.

Alpha- and Beta-diversity indexes were used in order to understand the relationship between the community structure of bacteria, archaea and protozoa, and the methane yield phenotype. Beta-diversity is a measure used in ecology in order to assess the differences between environments or samples (in this case, the cows). Contrarily, the alpha-diversity takes into account the diversity within cows (DIFFORD et al., 2018). Here, for alpha-diversity analysis were used Faith's Phylogenetic Diversity (PD), Evenness, and Shannon's diversity analysis. For beta-diversity, was used as unweighted UniFrac. None of the diversity metrics used showed a significant difference between the methane yield groups. Additionally, weighted UniFrac, Jaccard index, and Bray–Curtis dissimilarity matrix did not show a clustering between the divergent methane yield groups (data not shown).

For the bioinformatics and statistical analysis all taxa were considered, even unclassified taxa. Fungi taxa were removed from the analysis since the 18S rRNA molecular marker is not suitable for the classification of this microbial group.

The package PICRUSt2 in QIIME2 was used in order to predict the most abundant microbial functions on the rumen of animals divergent for methane yield. Were identified a total of 6,774 MetaCyc pathways from 16S rRNA and 7,639 from 18S rRNA. Since the classifier was pre-trained on RIM-DB in order to improve the accuracy of the analysis for archaea, it generated a new dataset for these microorganisms. In this way, the MetaCyc pathways were predicted separately for bacteria and archaea from the two different datasets. Even analyzing these two datasets separately, the MetaCyc pathways predicted for bacteria and archaea were similar.

To identify microbial taxa and functions that best characterize each group of methane yield was used the sPLS-DA multivariate analysis. For this statistical method, were considered only microbial taxa and functions and prevalent in at least 50% of the samples (11 out 22) and with a relative abundance > 0.01%. After filtering, were detected 21 phyla and 49 genera of bacteria; one phylum and two genera of archaea; and two phyla and seven genera of protozoa. To generate the barplot, only the taxa with the abundance > 5% were considered (**Figure 1**).

After the centered log-ratio transformation (CLR), the taxonomic profile for bacteria, archaea and protozoa was estimated. There is no clear separation for the taxonomic profile of the archaeal and protozoal profile between the two methane yield groups. However, the bacterial profile showed a separation of the two methane yield groups. For the functional profile, was observed a discrete differentiation for bacteria, archaea and protozoa, with part of the profile being

overlapped for the two groups, showing that a part of the community structure is not exclusive to high or low yield group (**Figure 2**).

To deeply understand the taxonomic and functional signatures that characterize the rumen microbiome in divergent animals for methane yield, the sPLS-DA was used to identify specific microbial taxa and functions. At the taxonomic level, the bacterial signature selected in component 1 characterizes 70% of the rumen microbiome of the animals of the group Low CH_4, including members of the taxa [*Eubacterium*]_*ruminantium*_group, *Lachnospiraceae*_UCG-006, SP3-e08, [*Eubacterium*]_*hallii*_group, UCG-001, and two taxa not identified. For archaea, the signature selected in component 1 predominantly characterizes the rumen of High CH_4 animals and is composed by the taxa *Methanobrevibacter*. For protozoa, the rumen of Low CH_4 animals is predominantly characterized by *Entodinium* (**Figure 3**).

At the functional level, for bacteria and archaea, 90% of the signature selected in component 1 characterize the rumen MetaCyc pathways of animals with low CH_4, and comprehends MetaCyc pathways related to signaling and cellular processes (30%), metabolism (20%), genetic information processing (20%), environmental information processing (10%) and unknown functions (20%). For protozoa, 80% of the functional signature selected in component 1 of the sPLS-DA characterize the rumen of Low CH_4 animals, and the functions are related to environmental information processing (42%), metabolism (42%), signaling and cellular processes (7%) and cellular processes (7%) (**Figure 4**).

4. Discussion

There are genetic and phenotypic variations related to methane production in cattle, having a low-moderate heritability (h2 = 0.13 to 0.38), which offers the possibility of breeding animals with lower methane emission (CROWLEY et al., 2010; DE HAAS et al., 2011; HERD et al., 2016).

Studies have reported that animals with higher feed efficiency produce less methane (NKRUMAH et al., 2006). Despite this relationship, most breeding programs aim to improve feed efficiency, and there is still no breeding program selection cattle with low methane emissions (RENAND et al., 2019). If there is a correlation between feed efficiency and methane production, these programs should also be focused on the selection of low emitters cattle.

The rumen is considered one of the most diverse ecosystems on the planet in terms of functional richness and species diversity (MIZRAHI; JAMI, 2018). Zhou et al. (2010) found that the methanogenic communities in efficient animals were more diverse than those in the inefficient counterparts. However, our study found no difference in the diversity of archaea, bacteria or protozoa between the two groups of methane yield, suggesting that these differences can be also driven by the diet.

Our study showed no significant differences in the microbial community structure for archaea, bacteria and protozoa between the two groups divergent for methane yield (**Figure 2**). Nevertheless, when observing at specific level, is possible to note microbial taxa and functions that better characterize each methane yield group. Among the most significative results, are the archaea of the genus *Methanobrevibacter* strongly related to the High CH_4 group and the protozoal genus *Entodinium* more related to the Low CH_4 group (**Figure 3**), in agreement with previous studies that pointed out the relevance of *Methanobrevibacter* in animals with high methane emission (WALLACE et al., 2015).

Methane production is strongly related to microbial hydrogen production through fermentation processes (KAMKE et al., 2016), and recent studies have suggested that the production of hydrogen and other microbial substrates drive CH_4 production on rumen (TAPIO et al., 2017). The bacterial fermentation supply substrate for methanogenesis in the rumen, including hydrogen, carbon dioxide (CO_2), acetate and methyl compounds, being the hydrogen and CO_2 the main substrate for methanogenesis in the rumen (HOOK; WRIGHT; MCBRIDE, 2010; HUNGATE et al., 1970; KAMKE et al., 2016). Ciliated protozoa are prominent H_2 producers, keeping a physical association with methanogens archaea, which favors H_2 transfer from one to the other (MORGAVI; JOUANY; MARTIN, 2008). Thus, there is a strong correlation between bacterial and protozoa hydrogen production and methane formation by methanogenic archaea on the rumen. In this way, bacterial and protozoal communities and their activities contribute to the methane yield phenotype of the animal (KAMKE et al., 2016). Because of that, despite the analysis of the sPLS-DA characterizing the MetaCyc Pathways as more related to methane yield (**Figure 4**), we suggest that there are no specific functions responsible for the methane yield phenotype, but a set of functions composing a functional niche.

It is needed to explore not only the microbial taxa related to methane emission, but also the microbial ecology on rumen, including mainly the interactions that can help to explain the

relevance of the different microbial groups on methane production (MARTÍNEZ-ÁLVARO et al., 2020). It has been postulated that archaea abundance should be proportional to CH_4 production (WALLACE et al., 2014), but recent studies demonstrated that not only the abundance but also the diversity of methanogenic archaea and its interactions with other rumen microorganisms are important to the amount of CH_4 formed (PITTA et al., 2021). As have been discussed by other authors, our results also suggest that differences in methane yield are mainly explained by the interactions of the rumen microorganisms and their functions rather than being driven only by methanogens. It demonstrates the importance of studies that address inter-domain microbial interactions, which highlights the necessity of network analysis approaches using patterns of co-occurrence and correlations based on abundance to better understand these connections (LAYEGHIFARD; HWANG; GUTTMAN, 2017).

Since the metabolic pathways can supply substrate to others, as well as account differently for the methane yield, is needed to characterize the functional niches for the different microbial groups in the rumen in order to identify potential mechanisms having an impact on CH_4 emissions.

Despite our study characterizing specific microbial taxa and functions more strongly related to each group of methane yield phenotype, based on the knowledge about methanogenesis in the rumen, it is more likely that the inter-domain associations may better explain the methane yield. It shows the necessity of exploring the rumen microbiome using meta-omics technologies (e.g., metagenomic, metaproteomic and metabolomic) to generate a complete overview of the rumen microbiome and to associate it to network analysis approaches in order to understand the co-occurrence and correlations based in taxonomic and functional abundance. The increased understanding of the rumen microbiome and its effect on methane production may lead to novel strategies toward an improved host phenotype for increased sustainability aligned with increased agricultural productivity.

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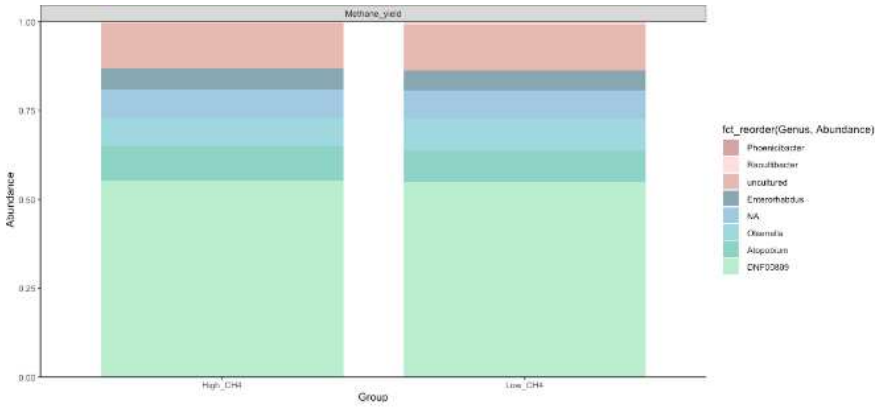
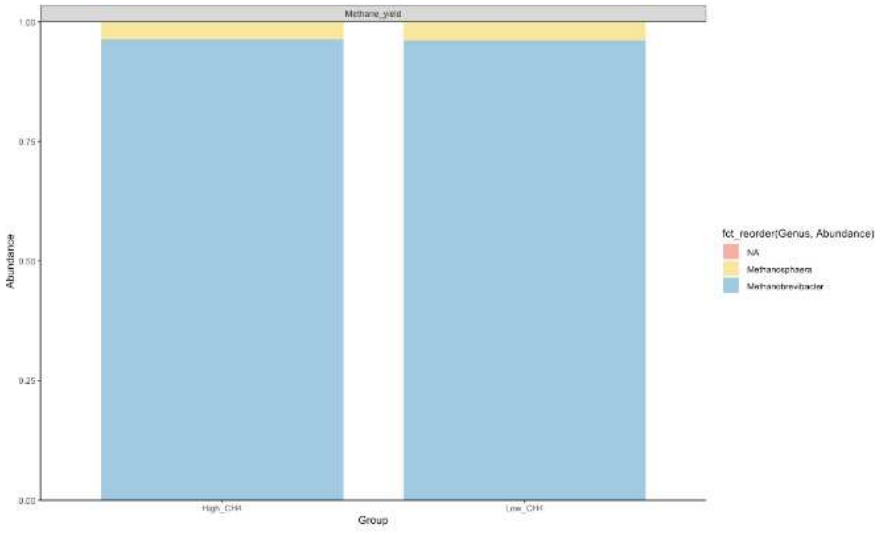

Methane_yield
1.00
0.75
0.50
0.25
0.00
Abundance
High_CH4
Low_CH4
Group
fct_reorder(Genus, Abundance)
Phoenicibacter
Ruminibacter
uncultured
Enterorhabdus
NA
Olsenella
Atopobium
DNF00809


Methane_yield
1.00
0.75
0.50
0.25
0.00
Abundance
High_CH4
Low_CH4
Group
fct_reorder(Genus, Abundance)
NA
Methanosphaera
Methanobrevibacter

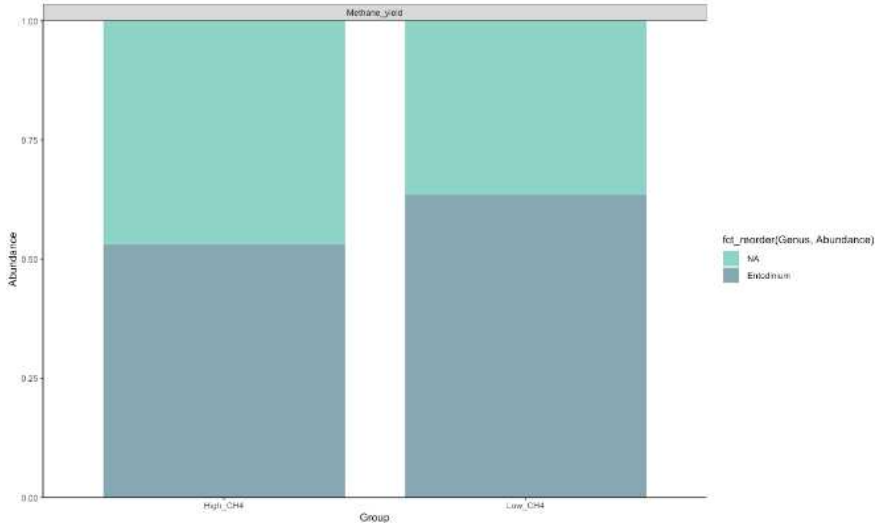

Methane_yield
1.00
0.75
0.50
0.25
0.00
Abundance
High_CH4
Low_CH4
Group
fct_reorder(Genus, Abundance)
NA
Eritrichium

Figure 1. Taxa summary plot between the two methane yield groups (High CH_4 and Low CH_4). Groups with abundance > 5%. **A.** Bacteria; **B.** Archaea; **C.** Protozoa.

Taxonomic composition

Functional composition

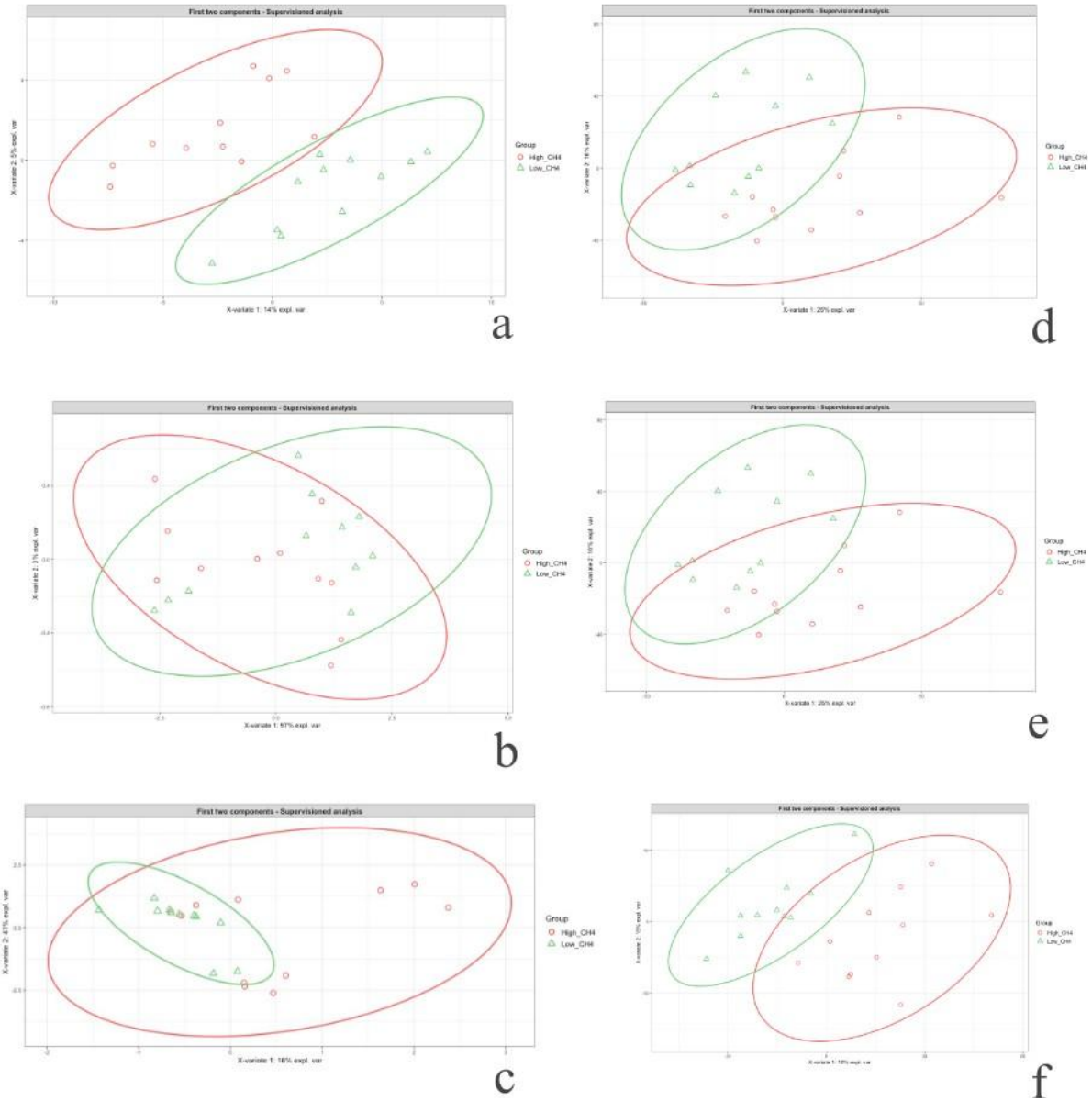
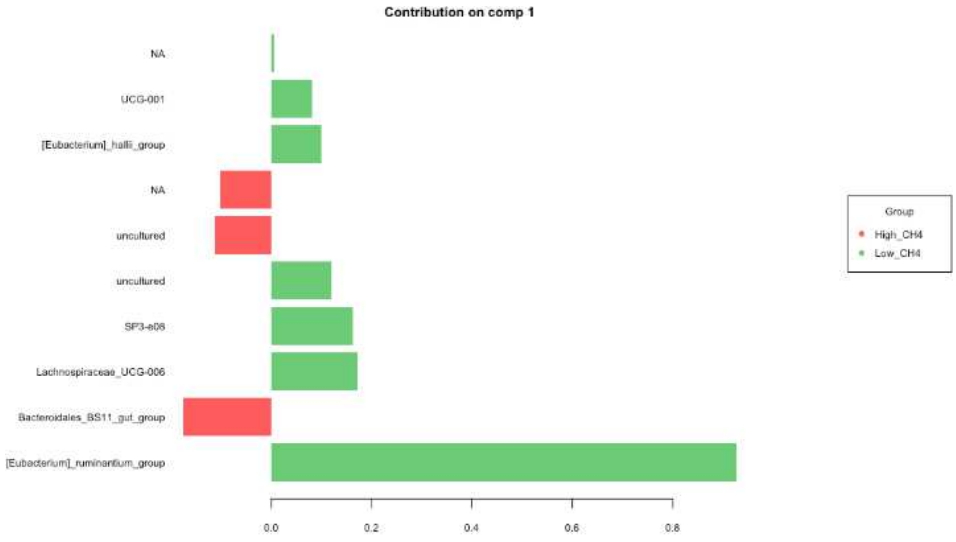
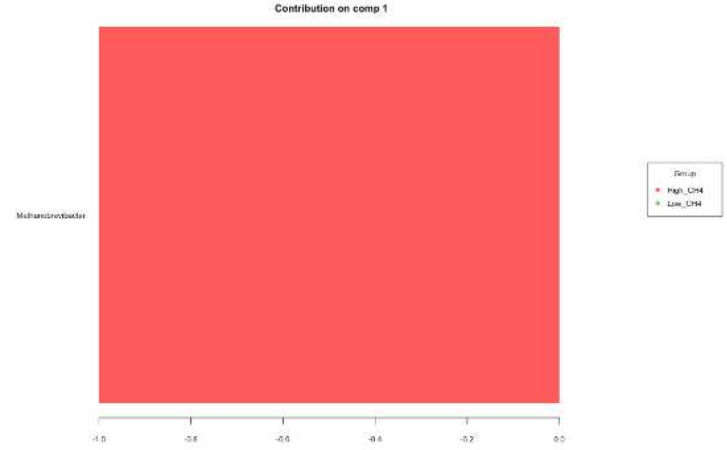


Figure 2. Sparse partial least square discriminant analysis results on rumen microbiome in two methane yield groups. **A.** bacterial taxonomic composition; **B.** archaeal taxonomic composition; **C.** protozoal taxonomic composition; **D.** bacterial functional composition; **E.** archaeal functional composition; **F.** protozoal functional composition.


Contribution on comp 1
NA
UCG-001
[Eubacterium]_hallii_group
NA
uncultured
uncultured
SP3-e08
Lachnospiraceae_UCG-006
Bacteroidales_BS11_gut_group
[Eubacterium]_ruminantium_group
0.0
0.2
0.4
0.6
0.8
Group
High_CH4
Low_CH4


Contribution on comp 1
Group
High_CH4
Low_CH4

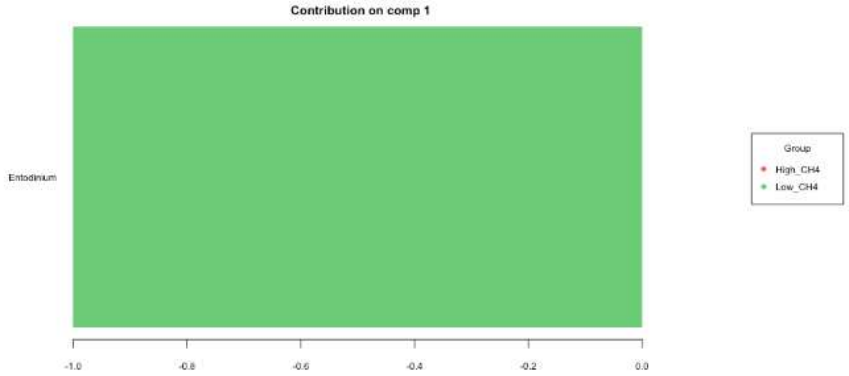
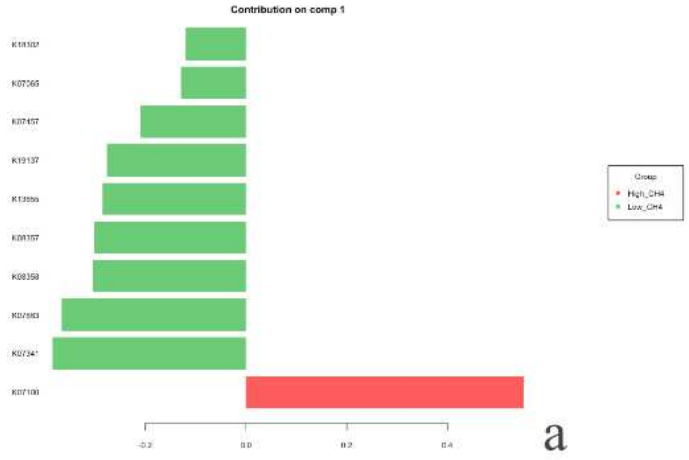
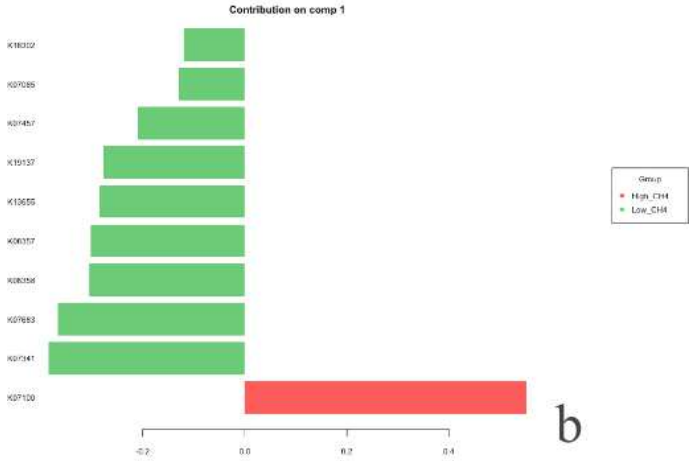

Contribution on comp 1
Entodinium
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
Group
High_CH4
Low_CH4

Figure 3. Contribution of each microbial taxa selected on the first component. The length of the bar represents the importance of the microbial taxa to the component – importance from the bottom to the top. Colors indicate the methane yield group in which the microbial taxa is more abundant. **A.** Bacteria; **B.** Archaea; **C.** Protozoa.


Contribution on comp 1
Group
High_CH4
Low_CH4
a


Contribution on comp 1
Group
High_CH4
Low_CH4
b

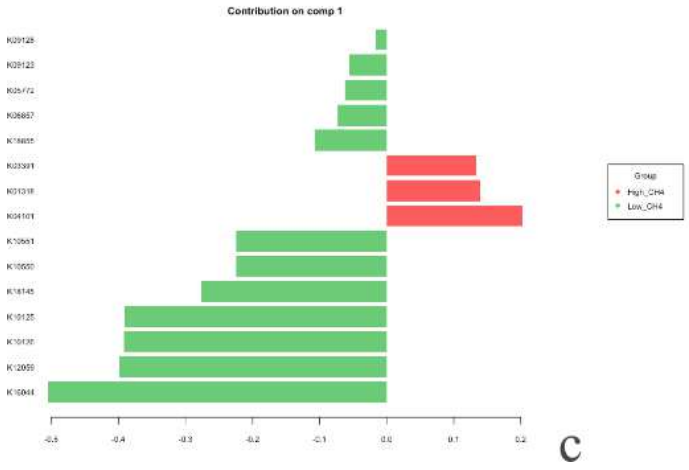

Contribution on comp 1
Group
High_CH4
Low_CH4
c

Figure 4. Contribution of each microbial function selected on the first component. The length of the bar represents the importance of the microbial taxa to the component – importance from the bottom to the top. Colors indicate the methane yield group in which the microbial function is more abundant. **A.** bacterial function; **B.** archaeal function; **C.** protozoal function.

FINAL CONSIDERATIONS

The present study contributes significantly to expanding the knowledge relating rumen microbiome to feed efficiency and methane yield in dairy cattle raised under tropical conditions. All studies that correlated rumen microbiome and these phenotypes are developed in temperate climate, most of them sequencing only the 16S rRNA. The integration of the data from 16S and 18S rRNA offers a better overview of the microbial community on rumen than when analyzing only the prokaryotic community. Additionally, our analysis to predict functional MetaCyc pathways allows the identification of the potential functions that can be more relevant for the phenotypes of interest, supplying a more complete overview of the rumen microbiome activity. The highlights pointed out here suggest that future studies investigate the microbial species and functions to better understand the feed efficiency phenotype; and investigate the microbial interactions and functional niches to better understand the methane yield phenotype.