UNIVERSIDADE FEDERAL DE VIÇOSA

FECAL BACTERIOME OF NELLORE STEERS WITH HIGH AND LOW FEED EFFICIENCY PHENOTYPES

Letícia Elisa Rossi
Magister Scientiae

VIÇOSA - MINAS GERAIS
2020

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Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de Magister Scientiae.

Orientador: Hilário Cuquetto Mantovani

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Assentimento:

Letícia Elisa Rossi
Autora

Hilário Cuquetto Mantovani
Orientador

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BIOGRAFIA

LETÍCIA ELISA ROSSI, filha de Maria de Fátima Mapa Rossi e Edson Hugo Rossi, nasceu em 22 de julho de 1991 e é natural de Timóteo – Minas Gerais.

Iniciou o curso de Licenciatura em Ciências Biológicas em março de 2014 na Universidade Federal de Viçosa, concluindo o mesmo em julho de 2018.

Ingressou no mestrado no Programa de Pós-graduação em Microbiologia Agrícola da Universidade Federal de Viçosa em agosto de 2018.

ABSTRACT

ROSSI, Letícia Elisa, M.Sc., Universidade Federal de Viçosa, February, 2020. **Fecal bacteriome of Nellore steers with high and low feed efficiency phenotypes.** Adviser: Hilário Cuquetto Mantovani.

To improve the production performance of beef and dairy cattle herds and reduce the environmental impact related to methane emissions, there is a growing interest of producers and researchers in the study of the factors that influence the phenotype of feed efficiency in cattle and develop methods that to identify more feed-efficient animals in commercial herds. Previous studies suggest that cattle of high and low feed efficiency have differences in the composition of the rumen microbiome, however, the association of these phenotypes with fecal microbiota of ruminants, particularly Nellore cattle, has not yet been established. Thus, in this work, the objective was to evaluate whether the fecal microbiota of Nellore cattle could be used as an indicator of feed efficiency for beef cattle. Fifty-nine Nellore steers, comprising 29 animals showing negative (high feed-efficiency, n-RFI) and 30 showing positive (low feed-efficiency, p-RFI) residual feed intake (RFI), were selected for this study. Fecal samples were collected, genomic DNA was extracted and the V4 region of the 16S rRNA gene was amplified and paired-end reads were generated via Illumina MiSeq sequencing. Metataxonomic analyses indicated significant differences in species richness (Chao-1) and diversity (Shannon) between the fecal microbiota of n-RFI and p-RFI steers (Mann-Whitney test, $P<0.05$). Firmicutes and Bacteroidetes were the most abundant phyla in the fecal-associated microbiota of Nellore steers and members of the genera Ruminococcaceae_UCG-014, dgA-11_gut_group, Treponema_2 e Slackia showed significant differences in fecal samples from the n-RFI and p-RFI groups. Otu00008 (Roseburia spp.) showed the highest abundance among OTUs with significant differences between the two efficiency groups, being more abundant in the n-RFI group. The results suggest that these bacterial groups can be used as potential biomarkers of the feed efficiency phenotype in Nellore cattle. However, further studies will be necessary to validate the results obtained, including functional analysis of fecal microorganisms and using larger cohorts of animals on a commercial scale.

Keywords: Nellore cattle. Feed efficiency. RFI. Fecal microbiota. 16S rRNA gene.

RESUMO

ROSSI, Letícia Elisa, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Bacterioma fecal de novilhos Nelore com fenótipos de alta e baixa eficiência alimentar.** Orientador: Hilário Cuquetto Mantovani.

Com o objetivo de melhorar o desempenho da produção dos rebanhos de bovinos de corte e leiteiros e reduzir o impacto ambiental relacionado com a emissão de metano, tem aumentado o interesse de produtores e pesquisadores pelo estudo dos fatores de influenciam o fenótipo de eficiência alimentar em bovinos e o desenvolvimento de métodos que possibilitem identificar animais mais eficientes em rebanhos comerciais. Estudos anteriores sugerem que bovinos de alta e baixa eficiência alimentar apresentam diferenças quanto à composição do microbioma ruminal, porém a associação desses fenótipos com a microbiota fecal de ruminantes, particularmente os bovinos Nelore, ainda não foi estabelecida. Desse modo, nesse trabalho o objetivo foi avaliar se a microbiota fecal de bovinos Nelore poderia ser utilizada como indicador de eficiência alimentar para bovinos de corte. Cinquenta e nove novilhos Nelore, compreendendo 29 animais que apresentaram consumo alimentar residual (CAR) negativo (alta eficiência alimentar, n-RFI) e 30 animais que apresentaram consumo alimentar residual positivo (baixa eficiência alimentar, p-RFI), foram selecionados para este estudo. As amostras fecais foram coletadas, o DNA genômico extraído e a região V4 do gene que codifica o rRNA 16S foi amplificada. As análises metataxonômicas indicaram diferenças significativas na riqueza de espécies (Chao-1) e na diversidade (Shannon) entre a microbiota fecal de novilhos n-RFI e p-RFI (teste de Mann-Whitney, $P<0,05$). Os filos Firmicutes e Bacteroidetes foram mais abundantes na microbiota associada a fezes de novilhos Nellore e membros dos gêneros Ruminococcaceae_UCG-014, dgA-11_gut_group, Treponema_2 e Slackia, apresentaram diferenças significativas na abundância relativa entre os animais dos grupos n-RFI e p-RFI (White's non-parametric t-test, $P<0,05$). A Otu00008 (Roseburia spp.) apresentou a maior abundância entre as OTUs com diferenças significativas entre os dois grupos de eficiência, sendo mais prevalente no grupo n-RFI. Os resultados sugerem que esses grupos bacterianos podem ser utilizados como potenciais biomarcadores do fenótipo de eficiência alimentar em bovinos Nellore. No entanto, estudos posteriores serão necessários para validar os resultados obtidos, incluindo análise funcional dos microrganismos fecais e utilizando coortes maiores de animais em escala comercial.

Palavras-chave: Bovino Nellore. Eficiência alimentar. CAR. Microbiota fecal. Gene 16S rRNA.

FIGURES LIST

CHAPTER 2

TABLE LIST

Supplementary material list

SUMMARY

GENERAL INTRODUCTION

Brazil has the second-largest cattle herd in the world and beef exports closed the year of 2019 with a record volume and revenue, reaching 1.847 million tons shipped (ABIEC, 2020). The Nellore breed accounts for approximately 80% of Brazilian beef cattle herds, mostly due to the capacity of the zebu cattle to adapt to tropical environments (Neto, 2018). The predominant feeding system for beef cattle in Brazil is extensive, in which animals are kept grazing tropical forages on large shares of land area. Selecting animals that are more efficient in terms of feed conversion can help improve productivity, reduce the reproductive cycle of the ruminant, and decrease production costs as the weight gain for slaughter tends to be faster. In addition, since the daily consumption of dry matter is closely related to the daily production of methane (Hales and Cole, 2017), selecting animals that show a higher conversion of feeds into energy can also reduce the environmental impact of livestock production.

It is estimated that about 80% of the greenhouse gas emissions in the livestock sector comes from the production of ruminants, with 90% of the methane produced resulting from the methanogenic activity of archaea that colonize the rumen (Tapio et al., 2017). Enteric methane accounts for the loss of 2 to 12% of the digestible energy of ruminants, which reduces the efficiency of milk production and weight gain of the animal (Hill et al., 2016). Studies on feed efficiency in beef and dairy cattle have been mainly associated with the microbial metabolism in the rumen since the energy gain in these animals depends primarily on the fermentation of feedstuffs by the ruminal microbiota and the efficiency of the host in transforming volatile fatty acids and microbial protein into meat or milk.

The studies of ruminal physiology and metabolism are usually carried out using fistulated animals (Lam et al., 2018; Artegoitia et al., 2017), allowing the sampling of ruminal digesta directly from the rumen. However, fistulation is an invasive surgical procedure, which limits its application to commercial cattle herds. In addition, ethical issues regarding the welfare of animals used in scientific research further restrict the numbers of experimental animals.

Nonetheless, the characterization of the microbiota in high and low feed efficiency cattle may provide clues about microbial taxa potentially associated with the feed efficiency phenotype (biomarkers), enabling the development of strategies for manipulating the rumen microbiome and improving feed efficiency of the host. For example, the study by (Brooke et al., 2019) suggested

that Prevotella copri could be a microbial biomarker for identifying beef cattle with high feed efficiency at the beginning of their life span and during the production cycle. Myer et al. (2015) found members of the genera Succiniclasticum, Lactobacillus, Ruminococcus, and Prevotella with significant differences in abundance between high and low feed efficiency groups. Recently, it has been found that different operational taxonomical units (OTUs) are shared between the feces and the different compartments of the gastrointestinal tract of Nellore cattle (Lopes et al., 2019). These latter results suggest that the characterization of the fecal microbiota could be a more practical and scalable approach to evaluate differences in the microbiome of high and low feed efficiency steers.

However, there is a lack of studies investigating the association between the compositions of the fecal microbiome of Nellore steers differing in feed efficiency phenotypes kept in tropical grass pastures. Considering the relevance of this subject and aiming to facilitate the metataxonomic study analysis of feed efficiency phenotype, we hypothesized that p-RFI and n-RFI Nellore steers (low and high feed efficiency, respectively) kept on pasture have specific differences in the composition of their fecal microbiota that could be useful for assigning their phenotypes. Therefore, the objective of this work was to characterize the composition of the fecal microbiome of high and low feed efficiency Nellore cattle by targeting the V4 region of 16S rRNA gene.

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CHAPTER 1

BIBLIOGRAPHIC REVIEW

Ruminants have a digestive system characterized by the pregastric fermentation of feedstuffs by symbiotic microorganisms, which gives them an advantage over other herbivores by being able to have better access to the energy of fibrous feeds (Van Soest, 2019). Bacteria, protozoa, and fungi colonizing the rumen are primarily responsible for synthesizing hydrolytic enzymes essential to the process of converting the digestible energy in the feeds into products that can be used as energy and nitrogen sources by the host. Volatile fatty acids, which are produced as a result of ruminal digestion, provide about 80% of the energy required by the ruminant (Krehbiel, 2014). In addition, microbial fermentation provides B-complex vitamins and amino acids that are used in several physiological processes.

Therefore, ruminant nutritionists have been using dietary interventions as an important strategy for manipulating the ruminant gastrointestinal tract microbiota, enabling the improvement of beneficial metabolic processes and the reduction of inefficient or undesirable metabolic processes (Malmuthuge and Guan, 2017). Some dietary interventions are useful to increase volatile fatty acid production and microbial protein synthesis, help maintain the stability of rumen pH, or contribute to reducing methane and ammonia production (Loor et al., 2016). Volatile fatty acids (VFAs) are products of the microbial fermentation process and the main energy substrate of ruminants, the most predominant being acetic, propionic, and butyric acid (Doreau et al., 2016). Bergman (1990) demonstrated that concentrations of organic acids in the rumen vary according to time after feeding and diet composition. For example, starch-rich diets favor propionate production in the rumen (Spore et al., 2018), and propionate contributes significantly as an energy source (glucose synthesis) for the ruminant. Diets rich in fiber, such as tropical forages, tend to promote acetic fermentation and hydrogen production, favoring the growth of methanogenic archaea, and resulting in higher methane production per unit of dry matter consumed by the animal (Yáñez-Ruiz et al., 2008).

Petri et al. (2012) have previously shown that changing dietary concentrate and forage proportions can help alter dry matter intake, rumen pH, as well as ruminal microbial community composition. The removal of forage in high concentrate diets increased bacterial diversity, despite the reduction in ruminal pH does not favor the growth of fibrolytic bacterial species. Zhang et al.

(2017) aimed to evaluate the effects of different ratios of forage to concentrate (80:20, 60:40, 40:60 and 20:80) with a diet composed of corn silage as the only forage, steam-flaked corn, soybean meal, and mineral mix. The authors showed that propionate and butyrate proportions increased linearly when concentrate was increased in the diet. These organic acids have been associated with increased levels of gene expression of MCT4 and CD147, which are proton-linked monocarboxylate transporters that mediate the transport of short-chain fatty acids across the plasma membrane (Nakamura et al., 2018). In addition, there was a decrease in the relative abundance of bacteria (e.g. Fibrobacter, Succinimonas) and some cellulolytic ciliates (e.g. Polyplastron and Ostracodinium), as well as an increase in Entodinium, a non-fibrous carbohydrate degrader, in the samples of cows fed higher proportions of concentrate (steam-flaked corn and soybean meal). These findings are useful to develop strategies aiming to improve the efficiency of ruminal fermentation and ruminant productivity.

In ruminants, improving the efficiency of ruminal fermentation can have a positive impact on feed conversion efficiency, allowing better conversion of feeds into energy, better utilization of available nutrients, and ultimately, greater production of meat and milk. The potential to increase the utilization of nutrients available in the feeds can improve profitability due to productivity increases, reduction of costs associated with less feed consumed, and decreased losses of dietary energy related to methane production (the lower the feed intake, the lower the emission of gases such as methane) (McGovern et al., 2018). Steers that are more feed-efficient appear to show significantly higher concentrations of butyrate and valerate and a higher concentration of total VFA in the rumen (Guan et al., 2008). These results suggest that more efficient animals also harbor microbial communities in the rumen that lead to more efficient fermentation of dietary substrates, resulting in differences in energy metabolism between animals with higher and lower feed efficiency.

The Residual Feed Intake (RFI) is a parameter frequently used to estimate the feed efficiency phenotype of beef cattle and select animals that are more efficient in feed conversion. The RFI is calculated based on the difference between observed dry matter intake and the expected amount of feed consumption by each animal (Koch et al., 1963). Expected intake is based on a linear regression equation of feed intake measured as a function of animal development measures such as average daily weight gain (kg/day). Therefore, animals with negative RFI (n-RFI) show observed consumption that is lower than the expected consumption, being classified as high feed

efficiency. Animals with positive RFI (p-RFI) present observed feed consumption higher than the expected and are classified as low feed efficiency (Archer et al., 1997).

Ruminant feed efficiency is directly linked to economic profitability in cattle production since feeding costs represent approximately 60 to 70% of the total production costs (Karisa et al., 2014). Thus, animals with a lower feed conversion ratio would generate a high cost for the producer. Daily methane production is another important factor to consider since animals that require less dry matter intake to achieve a desired average daily gain are more efficient and have reduced methane emissions (Velazco et al., 2017). Methane accounts for 16% of total anthropogenic greenhouse gas emissions, and about 30% of these emissions are estimated to come from ruminants (Wallace et al., 2015). Thus, strategies to reduce the emission of CH_4 prioritizing the improvement of feed and productive efficiency of ruminants have been the focus of several studies (Nkrumah et al., 2006; Basarab et al., 2013; Waghorn and Hegarty, 2011).

Previous studies have used different strategies to identify genetic markers involved in important biological mechanisms associated with feed efficiency (Sherman et al., 2010; Abo-Ismail et al., 2014; Saatchi et al., 2014). Abo-Ismail et al. (2014) performed bovine genomic analysis of tissue and blood samples from different breeds of cattle (Angus, Simmental, Piedmontese, Gelbvieh, Charolais, and Limousin) and identified new SNPs (single nucleotide polymorphisms) potentially associated with feed efficiency traits for use in marker-assisted animal selection. Significant SNPs ($P < 0.05$) explained 26% of the genetic variance for RFI. In that study, the authors identified several positional and functional candidate genes involved in important biological mechanisms associated with feed efficiency and performance. Some studies have indicated that the heritability of RFI in cattle is moderate to low. Grion et al. (2014) reported estimates of 0.33 ± 0.10, while Santana et al. (2014) estimated that the heritability for dry matter intake, RFI, and body weight gain at 0.40, 0.38, and 0.54, respectively, suggesting that it is necessary to use high feed efficiency selection indices that combine more than one trait of efficiency, such as microbiome composition, reproductive efficiency and growth characteristics, to develop more effective strategies for the improvement of the Nellore cattle.

Therefore, analysis of the microbiota composition in different portions of the ruminant gastrointestinal tract (mainly the bovine rumen), has been performed aiming to identify potential biomarkers associated with more efficient animals (Jami et al., 2014; Hernandez-Sanabria et al., 2012; Guan et al., 2008). For example, Jewell et al. (2015) used the gross feed efficiency (GFE)

parameters, calculated dividing the energy-corrected milk by dry matter intake for each period of lactation cycle and residual feed intake (RFI) to evaluate the production efficiency of dairy cows. Some genera, such as Prevotella, had several operational taxonomic units (OTUs) strongly associated with high and low-efficiency cows. Myer et al. (2015) evaluated the rumen microbial composition of beef cattle and also found significant differences between efficiency groups, including the genera Succiniclasticum, Lactobacillus, Ruminococcus, and Prevotella. The most dominant phyla were Firmicutes (23-33%) and Bacteroidetes (53-63%), in which the highest abundance of Firmicutes was associated with animals with higher average daily body weight gain and lower average dry matter intake. In addition, Prevotella has been reported as the most abundant rumen genus. These results suggest that it is necessary to perform taxonomic analyzes at the species level to associate relative abundances with the feed efficiency phenotype and identify potential microbial biomarkers.

Lopes et al. (2019), to characterize the composition of the bacterial and fungal microbiota colonizing the gastrointestinal tract (GIT) of Nellore steers, identified significant differences in beta diversity between the evaluated segments (solid and ruminal fluid, small intestine, cecum, and feces). Despite this, twenty-eight bacterial OTUs and six fungal OTUs were found to be shared in all segments of the TGI in at least 50% of the steers. Thus, the hypothesis that steers with high and low feed efficiency have differences in their fecal microbiota was also evaluated and differences were found between OTUs belonging to the families Lachnospiraceae, Prevotelaceae, Coriobacteriaceae, and Ruminococcaceae between the groups.

As shown earlier, the use of fecal samples to analyze microbiota differences in high and low feed efficiency animals is a more comprehensive method without the need for a fistula or probe, being a more practical and less invasive approach. Fecal samples associated with the feed efficiency phenotype have also been used in studies with pigs to find efficiency biomarkers. Yang et al. (2017) identified 31 OTUs that were potentially linked to pig feed efficiency, with the majority belonging to the Clostridiales group, while McCormack et al. (2017) observed that the Christensenellaceae, Oscillibacter, and Cellulosilyticum groups were enriched in pigs with low RFI. In addition, Brooke et al. (2019) evaluated the composition of the fecal microbiota of feedlot cattle to identify microbial markers linked to efficiency traits, which could provide a fast and practical method to classify ruminants according to their phenotypes, and could bring economic benefits to cattle producers. In their study, Brooke et al. (2019) suggested that Prevotella copri

could be a microbial marker to identify high feed efficiency beef cattle early in their life and during the production cycle. The authors also suggested that the development of more refined quantification techniques could allow the correlation of P. copri with the feed efficiency phenotype, promoting the application by ranchers.

Given the aforementioned, these previous studies provide evidence that animals with high and low feed efficiency have differences in the abundance of ruminal microorganisms that are relevant to the degradation of dietary components. However, it is necessary to expand these studies to evaluate not only the composition of the ruminal microbiota but also the interactions and functionality of these microbial groups and potential biomarkers (Li and Guan, 2017). Additionally, it is imperative to develop studies targeting the use of less invasive methods (e.g. fecal sampling) to evaluate differences in the microbiome of steers that differ in their feed efficiency phenotypes. This can help expand the comparisons of the microbiota composition in different efficiency groups under different management and feeding conditions, validating potential biomarkers in larger cohorts of animals and at commercial scale.

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CHAPTER 2

Title: Characterization of the fecal bacteriome of Nellore steers with high and low feed efficiency phenotypes

Abstract

Anaerobic microorganisms that colonize the digestive tract of ruminants are largely responsible for the degradation of complex substrates from plant biomass, allowing ruminants to obtain nutrients and energy available in the ingested feedstuffs. The feed efficiency of cattle has been increasingly investigated to reduce the cost of feeds, reduce the emission of methane gas, in addition to improving the reproductive performance and weight gain of the animals. In this study, we aimed to characterize the fecal microbiota of grazing Nellore steers allocated into distinct feed efficiency phenotypes. Fifty-nine Nellore steers comprising 29 animals with negative residual feed intake (n-RFI, high feed efficiency) and 30 with positive residual feed intake (p-RFI, low feed efficiency) grazing tropical forages were selected for sample collection. The fecal microbiome was assessed by targeting the V4 region of the 16S rRNA gene. The results indicated significant differences in species richness (Chao-1) and diversity (Shannon) between the fecal microbiota of n-RFI and p-RFI steers (Mann-Whitney test, $P < 0.05$). Bacteroidetes and Firmicutes were the dominant phyla in both groups, but without significant differences in abundance. However, the abundances of genera Ruminococcaceae_UCG-014, dgA-11_gut_group, Treponema_2, and Slackia were significantly different in fecal samples of the n-RFI and p-RFI steers. Among the 14 OTUs showing differences in abundance between both efficiency groups, Otu00008 (Roseburia spp.) was more abundant in the n-RFI group. These bacterial taxa appear to be associated with the feed efficiency phenotype of Nellore steers suggesting that analysis of the fecal microbiome could be a useful non-invasive approach to characterize the feed efficiency phenotype in beef cattle.

Keywords: Nellore cattle; Pasture; Feed efficiency; RFI; Fecal microbiota; 16S rRNA gene.

Introduction

Cattle production has great social and economic relevance worldwide, providing high-quality proteins and highly bioavailable essential minerals to human diets through the production of meat and milk. Brazil's beef exports hit record numbers in 2019, with the volume of meat exported reaching 1.847 million tons generating a total revenue of US $ 7.59 billion (ABIEC, 2020). The Nellore breed comprises a large part of the Brazilian beef cattle herd due to its adaptability to tropical climate conditions, sexual longevity, and resistance to ecto and endoparasites (Neto N., 2018).

Symbiotic anaerobic microorganisms that colonize the digestive tract of ruminants are primarily responsible for the degradation of complex sources of fibers, allowing the host to harvest the essential nutrients and energy from ingested feeds (Krehbiel, 2014). Due to its essential role in the nutrition of the ruminant, the rumen microbiome has been the main target for studies trying to link the feed efficiency phenotype with the microbiota of the cattle gastrointestinal (GI) tract (Myer et al., 2015; Jami et al., 2014; Guan et al., 2008). A parameter often used to estimate the feed efficiency phenotype of beef cattle is Residual Feed Intake (RFI), which consists of the difference between the actual dry matter consumption and the expected amount of feed consumed based on some animal development metric (Koch et al., 1963). Expected consumption is determined from linear regression of measured feed intake versus development measures such as average daily weight gain (kg/day). However, previous studies have suggested that the feed efficiency phenotype is also affected by non-genetic factors due to low to moderate RFI heritability (Grion et al., 2014; Santana et al., 2014).

Therefore, linking microbial markers with efficiency traits could allow the selection of animals showing greater productivity of meat and milk, reduce costs with feeding and contribute to decreasing methane emission and the overall environmental impact of cattle production systems. Previous work has focused mainly on the analyzes of the ruminal microbiota aiming to identify taxa correlated with the feed efficiency phenotype (Li and Guan, 2017; Jewell et al., 2015). Nonetheless, rumen sampling requires invasive techniques such as the use of fistula, oral probes, or even slaughter of the animals. Recent studies have suggested the use of fecal samples as a more practical, more comprehensive, and less invasive method to associate microbiota composition with phenotypic traits of the host (Lopes et al., 2019; Brooke et al., 2019). In this study, we examined

the hypothesis that grazing Nellore steers could be differentiated according to their feed efficiency group based on differences in the composition of the fecal microbiota. The aim was to evaluate whether the fecal microbiota of Nellore cattle could be used as an indicator of feed efficiency for beef cattle. For that, we sampled fecal contents from 59 Nellore steers (29 with negative RFI and 30 with positive RFI) and characterized the bacterial community by targeting the V4 region of the 16S rRNA gene.

Material and Methods

Animal selection, diets, and sample collection

A contemporary herd of 124 Nellore bulls from the Centro Avançado de Pesquisa Tecnológica dos Agronegócios de bovinos de corte (São Paulo State, Brazil) were submitted to feeding performance tests for 98 days (28 days of adaptation, followed by 70 days of data collection). A growing diet was formulated according to the weight gain requirements of 1 kg/day, consisting of 615 g/kg corn silage, 33 g/kg Brachiaria hay, 167 g/kg dry ground corn, 163 g/kg soybean meal, 3.6 g/kg urea, 0.4 g/kg ammonium sulfate and 18 g/kg mineral mixture (dry matter basis). The steers were fed ad libitum using the GrowSafe® automated feeding system (GrowSafe Systems Ltd, Airdrie, Canada). Subsequently, the residual feed intake (RFI) was calculated as previously described (Fidelis et al., 2017). After the RFI analysis period, the steers were kept on pasture for 124 days with a diet composed of Brachiaria brizantha cv. Marandu ad libitum and mineral salt. Fecal samples were collected from a group of 59 steers classified as low and high RFI, with a mean live weight of 380.41±16.27 kg and an age of 507±21.70 days.

The experimental procedures were approved by the Ethics Committee on Production Animal Use of the Universidade Federal de Viçosa (CEUAP-UFV, Protocol 026). Fecal samples were collected and stored in plastic bags at -20ºC.

DNA extraction and sequencing

Total genomic DNA from fecal samples was extracted using a phenol/chloroform extraction protocol and mechanic disruption (bead-beating) of microbial cells, as described by

Stevenson and Weimer (2009). Extracted DNA was quantified on a spectrophotometer, lyophilized, and sent for sequencing to the Department of Bacteriology/University of Wisconsin-Madison, United States. The V4 hypervariable region of the 16S rRNA bacterial gene was amplified as previously described (Lopes et al., 2019) using primers designed by Kozich et al. (2013). The amplicons were subjected to paired-end sequencing (v2 kit, 2 x 250 bp) on an Illumina MiSeq platform (Illumina, Inc., San Diego, California, USA). Raw sequence reads from all samples were submitted to the NCBI's Sequence Read Archive (SRA) under BioProject accession number PRJNA605191.

Sequence analysis

Bacterial sequences were processed using Mothur v.1.43.0 (Schloss et al., 2009) following the MiSeq SOP (https://www.mothur.org/) using paired-end reads. Initially, the paired-end reads were combined into continuous sequences using the **make.contigs** command and reads (R1 and R2) without sequence overlap were eliminated. Then, sequences containing ambiguous base calls or longer than 300 bp bases were removed with the **screen.seqs** command to reduce PCR and sequencing errors. Identical sequences were then merged using the **unique.seqs** command and treated together as unique sequences in the downstream steps.

The unique sequences were aligned to the V4 region of the Silva Database release 132 (Quast et al., 2013) using the **align.seqs** command and alignments containing columns without information were filtered using the **filter.seqs.** Similar sequences within the same sample were grouped using the **pre.cluster** command, allowing a maximum difference of 2 bp between sequences. Subsequently, chimeric and undesirable sequences (such as Archaea, 18S rRNA, 16S rRNA gene fragments, chloroplasts and mitochondria sequences) were identified and removed using the **chimera.uchime** and **remove.seqs** commands, respectively.

Sequences were grouped into operational taxonomic units (OTUs) using uncorrected pairwise distances clustered with the furthest neighbor method, based on 97% sequence identity and classified using the Silva database as reference (Quast et al., 2013). The commands used were **classify.seqs** and later **classify.otu**. To confirm the taxonomic classification of OTUs, the sequences were extracted and used for database alignment Ribosomal Database Project (RDP) by the Naive Bayesian Classifier method (Wang et al., 2007). To determine the alpha diversity

indexes (Chao1, Shannon and Simpson) and calculate the relative abundance of OTUs, it was first necessary to normalize the OTU tables using the **normalize.shared** command due to different sequencing depths. Normalization was established by the sample with the lowest number of sequences and thus a count of equal sequences was established for all samples.

Statistical analysis

Grouping of the samples according to the feed efficiency phenotype (negative and positive RFI, respectively) was represented by Non-metric Multidimensional Scaling (NMDS) analysis performed using the Bray-Curtis dissimilarity metric in the PAST software (Hammer et al., 2001). Non-parametric analyses of similarities (ANOSIM, with 10,000 permutations) was carried out to evaluate the composition of OTUs with the feed efficiency phenotypes using the PAST software.

Normality of the data was evaluated using the Kolmogorov-Smirnov test and analysis of correlation between feed efficiency phenotypes and alpha diversity indices were performed on the SPSS version 22 software for Windows (SPSS Inc. Chicago, IL, USA) (Norusis, 1993). For data with non-normal distribution, the Mann-Whitney non-parametric test for independent samples was used, with $P<0.05$ considered as significant. Relative abundances at the bacterial taxa at the level of OTU, phylum, family, and genus were calculated using the STAMP v2.1.3 software (Parks et al., 2014) using the two-sided White's non-parametric t-test, with correction of false discovery rate (FDR) using the Benjamini-Hochberg method ($P<0.05$ for significant values).

Analysis of organic acids

Fecal samples were centrifuged (12,000 × g, 10 min) and the cell-free supernatants were treated as previously described (Lopes et al., 2019). Organic acid analysis was performed on a Dionex Ultimate 3000 dual detector HPLC apparatus (Dionex Corporation, Sunnyvale, CA, USA). Analytes were separated on a Phenomenex Rezex ROA (300 × 7.8 mm) ion exclusion column (Phenomenex Inc. Torrance, CA, USA) that was maintained at 45 °C and detected on a Shodex RI-101 refractive index (RI) detector. Standards were prepared from stock solutions of the following organic acids: acetic (20 $mmol.l^{-1}$), succinic (10 $mmol.l^{-1}$), propionic (10 $mmol.l^{-1}$), valeric (10 $mmol.l^{-1}$), isovaleric (5 $mmol.l^{-1}$), isobutyric (10 $mmol.l^{-1}$) and butyric (10 $mmol.l^{-1}$).

The calibration curve for each organic acid was constructed by plotting the concentrations of each acid (following serial 2-fold dilutions of the stock solution) and the corresponding peak area.

Results

Animal selection

The 59 Nellore steers were subjected to the RFI calculation to be classified into feed efficiency groups. Thus, 29 n-RFI and 30 p-RFI steers were classified (Table 1). The groups n-RFI (-0.336 ± 0.348) and p-RFI (0.392 ± 0.412) showed a significant difference between them (t-test, $P < 0.05$ for significant values).

Table 1. Residual feed intake (RFI) of the 59 Nellore steers evaluated in this study.

Animal	n-RFI	Animal	p-RFI
3656	-0.274	3619	0.757
3658	-0.490	3651	0.116
3790	-0.203	3834	0.250
3791	-0.010	3638	0.418
3696	-0.173	3784	0.399
3684	-0.653	3712	1.464
3605	-0.070	3682	0.204
3842	-0.048	3652	1.938
3672	-0.107	3800	0.117
3641	-0.783	3608	0.175
3753	-0.065	3703	0.057
3890	-0.087	3835	0.104
3685	-0.461	3596	0.315
3647	-0.079	3831	0.647
3743	-0.016	3632	0.053
3673	-0.345	3829	0.060
3679	-0.004	3604	0.315
3665	-0.345	3804	0.303
3893	-1.352	3639	0.111
3873	-0.150	3824	0.598
3847	-0.269	3666	0.802
3614	-1.187	3870	0.019
3848	-0.019	3721	0.444
3745	-0.263	3706	0.131
3657	-0.357	3623	0.194
3628	-0.071	3675	0.168
3773	-0.388	3823	0.600
3621	-0.947	3748	0.454
3781	-0.540	3814	0.233
		3603	0.323

Sequencing

Amplicon sequencing of the 16S rRNA gene from 59 fecal samples of Nellore steers with high and low feed efficiency phenotypes generated 8,051,365 crude sequences, with a maximum size of 500 bp, an average size of 253 bp, and a minimum length of 242 bp. After filtering and cleaning, with the removal of chimeras and homopolymers, 2,271,199 good quality sequences were obtained, of which 1,051,408 were from high feed efficiency steers and 1,219,791 belonged to the low feed efficiency steers. The average Good's coverage obtained in the samples was > 98%, indicating that most of the diversity (OTUs) was probably covered during sequencing. The mean values as well as the standard deviation of the sequencing data are shown in Table 2.

Table 2. Summary of sequencing data from fecal samples of low feed efficiency (p-RFI) and high feed efficiency (n-RFI) Nellore steers.

Feed efficiency group	Steers (n)	Good's coverage	After filtering and clean-up		After normalization		After cut-off*	
			Reads	OTUs	Reads	OTUs	Reads	OTUs
p-RFI	30	0.99 ± 0.004	40660 ± 14482	1736 ± 224	18720 ± 191	1484 ± 163	17383 ± 1426	1071 ± 83
n-RFI	29	0.99 ± 0.004	36255 ± 15541	1759 ± 236	18831 ± 178	1627 ± 181	17719 ± 817	1122 ± 103

Values represent the mean ± standard deviation. *Reads and OTUs that were detected in at least half of the steers in each feed efficiency group.

Specific fecal bacteria OTUs are associated with the p-RFI and n-RFI phenotype in Nellore steers

Sample correlation analysis according to the feed efficiency phenotype (n-RFI and p-RFI) was performed and graphically represented by Non-metric Multidimensional Scaling (NMDS) plots using Bray-Curtis dissimilarity as distance metric (Figure 1). Ordination of the data and non-parametric analysis of similarities (ANOSIM) did not show significant differences (P=0.075) in distance metrics, indicating similar species composition between the fecal microbiota on n-RFI and p-RFI steers.

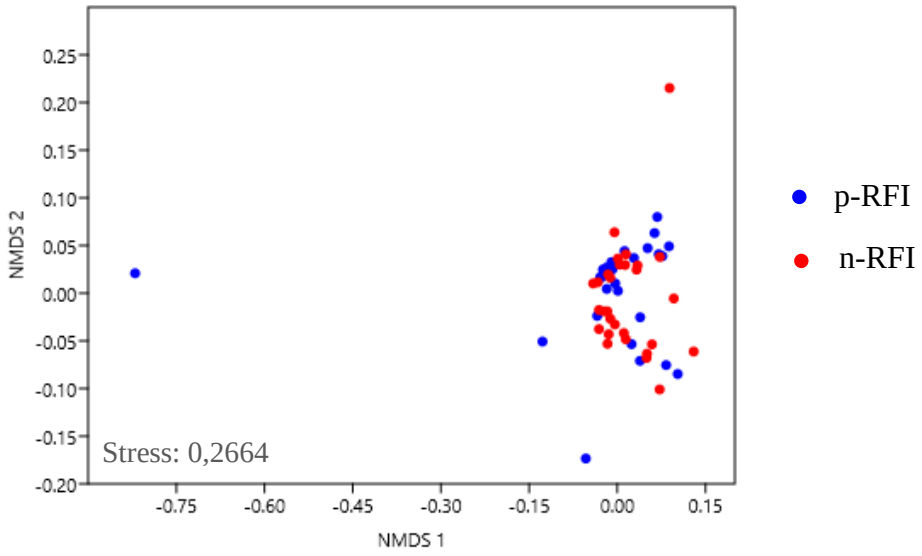


Figure 1. Non-metric Multidimensional Scaling (NMDS) plot using Bray-Curtis dissimilarity metric for bacterial communities for feed efficiency.

Beta diversity analyses were also performed selecting the animals with extreme RFI values for Non-metric Multidimensional Scaling (NMDS) plots using Bray-Curtis dissimilarity as the distance metric. OTUs data from low-efficiency animals with RFI greater than 0.5 and high-efficiency animals with values less than -0.5 were selected (Figure 2). Ordination of the data and the non-parametric analysis of similarities (ANOSIM) also did not show significant differences

(P=0.412) in the distance metrics, confirming that even animals with extreme RFI values present similar composition of the fecal microbiota species.

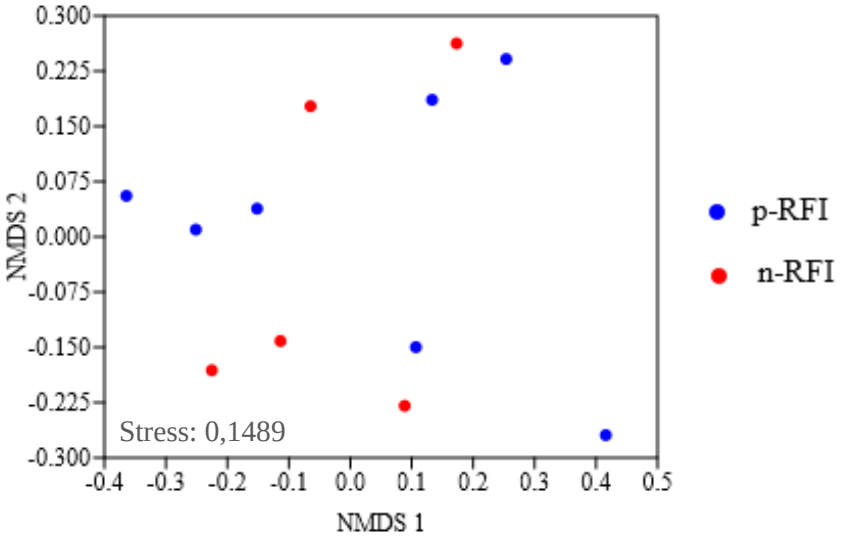
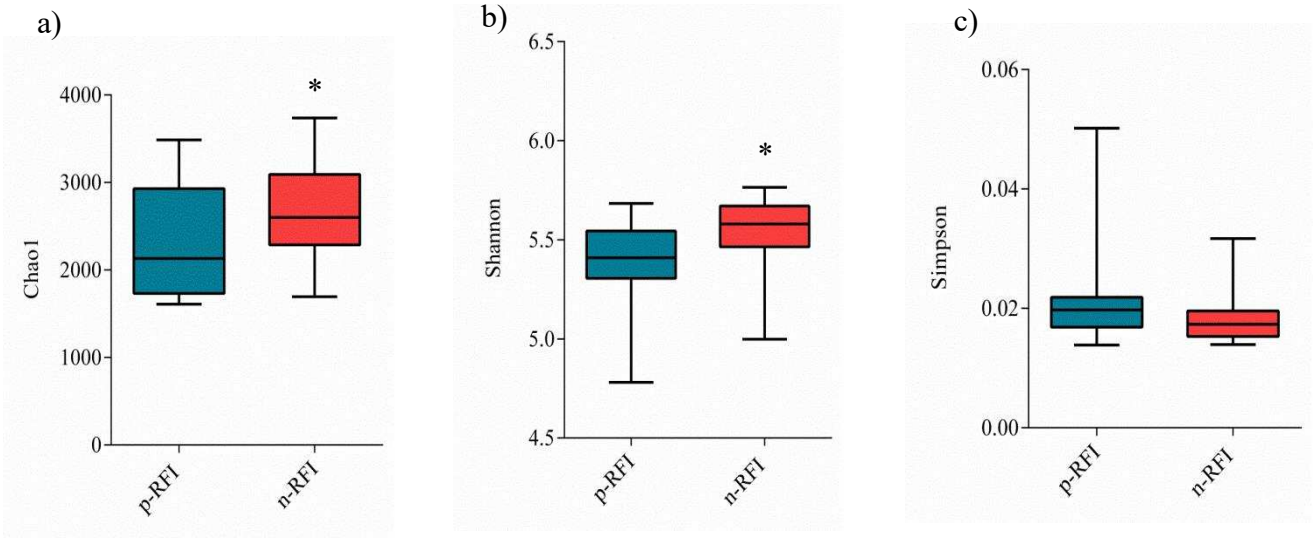


Figure 2. Non-metric Multidimensional Scaling (NMDS) plot using Bray-Curtis dissimilarity metric for bacterial communities from fecal samples from animals with extreme RFI values (greater than 0.5 for p-RFI and less than -0.5 for n-RFI).

However, analyses of diversity within samples of n-RFI and p-RFI Nellore steers (alpha diversity) indicated significant differences in Chao1 richness (non-parametric Mann-Whitney test, P=0.01) and Shannon-Wiener diversity function (non-parametric Mann-Whitney test, P=0.007) between fecal samples of n-RFI and p-RFI Nellore steers (Figure 3). Even though Simpson diversity did not indicate a significant difference between bacterial communities in fecal samples of steers within each feed efficiency phenotype, this metric showed a trend (P=0.05, non-parametric Mann-Whitney test) for distinct similarities between the fecal microbiota of n-RFI and p-RFI steers.



* Significant at 0.05% probability by the non-parametric Mann-Whitney test.

Figure 3. Alpha diversity of the fecal bacterial community from high and low feed efficiency Nellore steers. Values of alpha diversity indexes represent the mean ± standard deviation.

Taxonomic analyzes of the bacterial communities in the fecal samples of Nellore steers, revealed 21 phyla, 175 families, and 401 genera. Analysis of relative abundances for each phylum demonstrated a predominance of Firmicutes in n-RFI (68.60%±1.74) and p-RFI (68.55%±2.05) steers, while the abundance of Bacteroidetes varied from 9.67%± 0.91 in the fecal samples of n-RFI steers to 10.06%±1.14 in the p-RFI steers. Actinobacteria and Tenericutes were also predominant in these samples, with abundances greater than 5.0% for both feed efficiency phenotypes. Nonetheless, no significant differences ($P>0.05$) were observed between feed efficiency phenotypes for the abundance of microbial taxa at the phylum level (Figure 4 and Supplementary Figure S1). Unclassified phyla represented 1.75%±0.31 and 1.58%±0.43 of the OTUs in n-RFI and p-RFI steers, respectively.

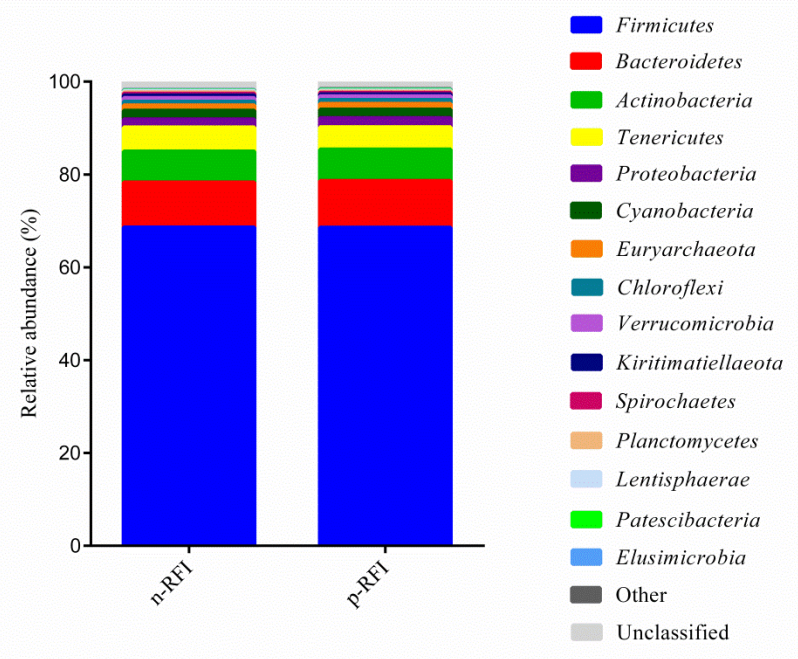


Figure 4. Bacterial community composition at the phylum level in fecal samples of n-RFI and p-RFI Nellore steers. Each bar represents the mean relative abundances (%) of the major phyla found in fecal samples of each animal group. "Other" represents the phyla with relative abundances <0.1%.

Analysis of the relative abundances at the family level revealed a predominance of Ruminococcaceae in n-RFI (30.26%±2.30) and p-RFI (29.64%±2.14) steers. Lachnospiraceae was the second most abundant family, corresponding to 16.90%±2.16 of the sequences in fecal samples of n-RFI steers and 17.20%±1.69 in the p-RFI steers. Cristensenellaceae was also predominant, being the third most abundant family, with relative abundances of 8.41%±0.74 and 8.42%±0.84 in n-RFI and p-RFI steers, respectively. Although the Veillonellaceae family showed a low relative abundance in n-RFI (0.07%±0.06) and p-RFI (0.11%±0.09) steers, a significant difference in abundance was observed (P=0.03) for this family among feed efficiency phenotypes (Figure 5 and Supplementary Figure S2). Unclassified families corresponded to approximately 6.00% of all OTUs in both groups.

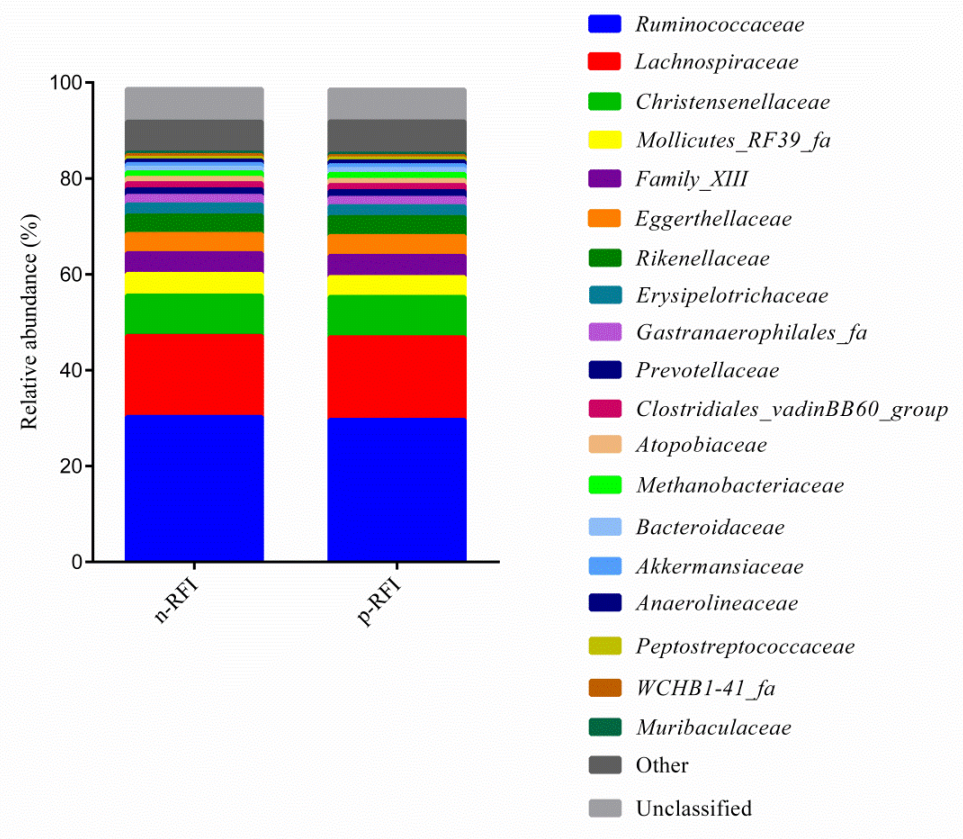


Figure 5. Bacterial community composition at the family level in fecal samples of n-RFI and p-RFI Nellore steers. Each bar represents the mean relative abundances (%) of the major families found in fecal samples of each animal group. "Other" represents the families with relative abundances <0.5%.

From the analysis of relative abundances of the genera present in the bacterial communities of fecal samples of Nellore steers, Ruminococcaceae_UCG-010 was the most abundant genus for both efficiency groups, varying from 8.87%±1.36 in the fecal samples of n-RFI steers to 8.50%±1.60 in p-RFI steers. Christensenellaceae_R-7_group was the second most abundant genus with relative abundances of 8.58%±1.10 and 8.30%±1.30 for n-RFI and p-RFI steers, respectively. Unclassified genus represented 27.11%±5.86 (n-RFI) and 29.33%±7.50 (p-RFI) of OTUs for feed efficiency groups (Figure 6 and Supplementary Figure S3). Of the 401 genera revealed by the taxonomic analysis, Ruminococcaceae_UCG-014, dgA-11_gut_group, Treponema_2, and Slackia showed significant differences ($P<0.05$) between feed efficiency groups with an abundance greater than 0.1% (Table 3).

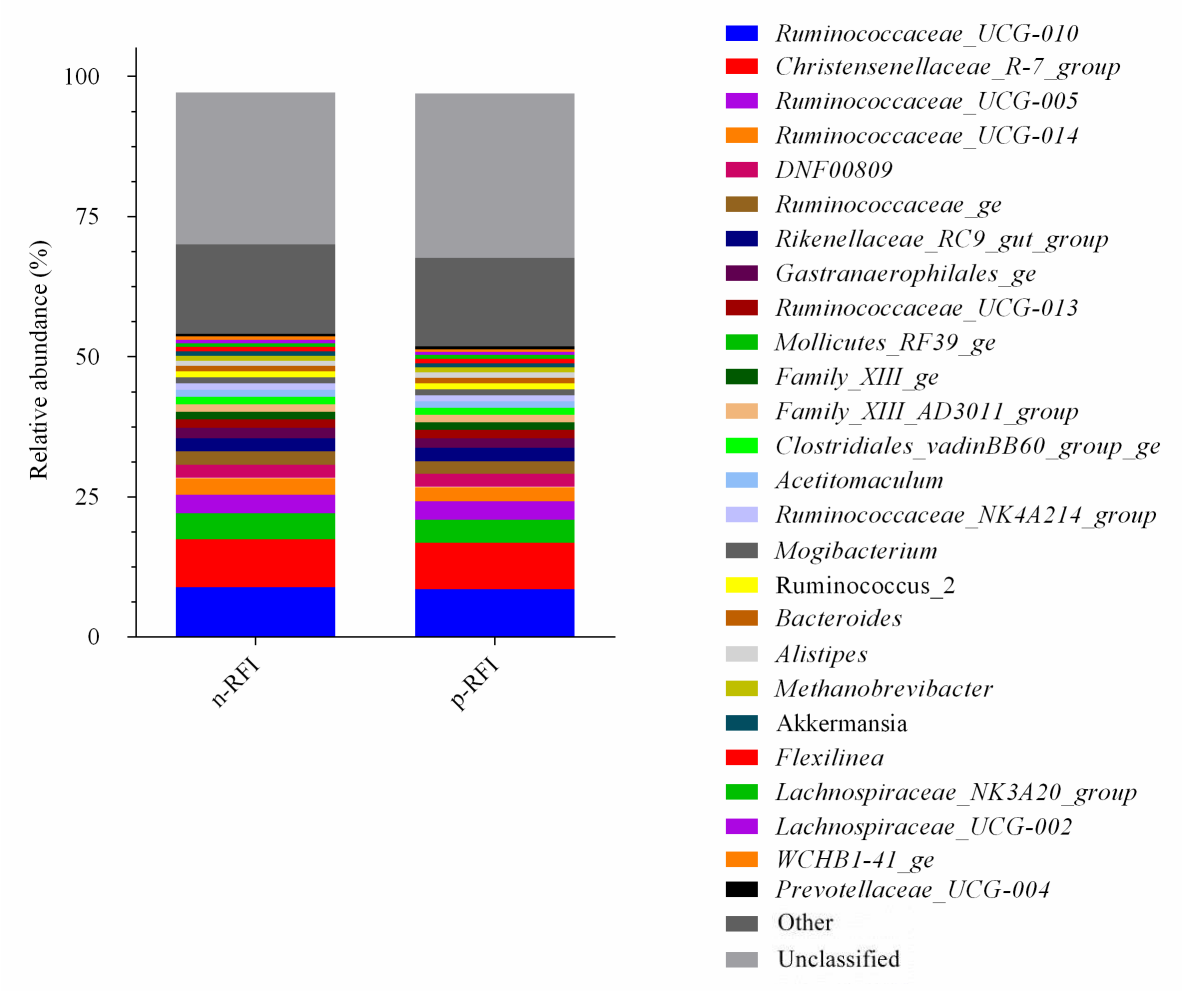


Figure 6. Bacterial community composition at the genus level in fecal samples of n-RFI and p-RFI Nellore steers. Each bar represents the mean relative abundances (%) of the major genera found in fecal samples of each group animal group. "Other" represents the genera with relative abundances <0.5%.

Table 3. Bacterial genera present in fecal samples of Nellore steers with relative abundance greater than 0.1% and which showed a significant difference between the n-RFI and p-RFI feed efficiency groups. The gray column represents the feed efficiency group that shows the highest abundance for each taxon.

Taxon	n-RFI	p-RFI	p-value
Ruminococcaceae_UCG-014	2,92 ± 0,64	2,53 ± 0,55	0,02
dgA-11_gut_group	0,48 ± 0,09	0,43 ± 0,09	0,02
Treponema_2	0,36 ± 0,13	0,29 ± 0,10	0,03
Slackia	0,13 ± 0,05	0,10 ± 0,04	0,02

Values represent mean ± standard deviation.

A total of 5,715 OTUs were obtained after sequence normalization. Among these, 904 OTUs were unique to n-RFI steers, corresponding to 0.39% of the total relative abundance, while the p-RFI steers had 745 unique OTUs representing 0.44% of total relative abundance (Figure 7). In addition, a total of 4,066 OTUs were shared between the feed efficiency groups, and those with an abundance greater than 0.1% did not show significant differences between feed efficiency groups (P>0.05) (Table 4). Of the shared OTUs, the Ruminococcaceae family showed greater abundance for both groups, with the genus Ruminococcaceae_UCG-005 being the most abundant, representing a total of 15.82%±0.56 for Nellore n-RFI steers and 16.11%±0,71 for the p-RFI steers. The second most abundant family was Peptostreptococcaceae, which revealed a predominance of the genus Romboutsia in n-RFI (3.39%±2.78) and p-RFI (3.50%±2.02) steers. Lachnospiraceae was also predominant, with the Agathobacter genus being the most abundant, with relative abundances of 3.18%±1.50 and 2.49%±0.95 in n-RFI and p-RFI steers, respectively.

Among the exclusive OTUs, the most abundant belonged to the families Prevotellaceae (0.05%±0.23) and Lachnospiraceae (0.01%±0.03) in animals with high feed efficiency (n-RFI) and Chryseomicrobium (0.07%±0.32), Lachnospiraceae (0.06%±0.30), and Ruminococcaceae (0.04%±0.23) in low-feed efficiency steers (p-RFI) (Supplementary Table S1).

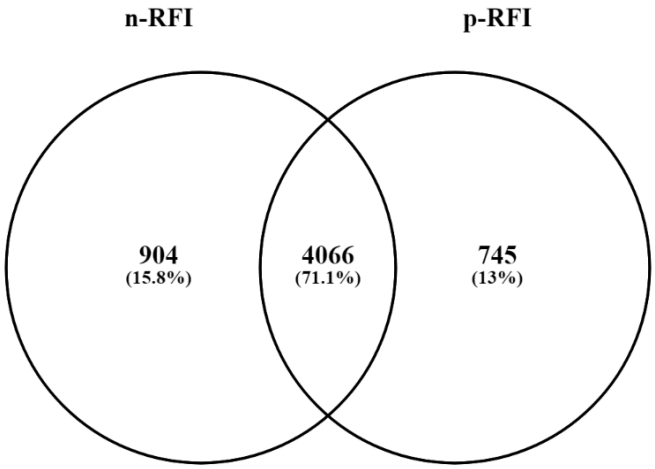


Figure 7. Venn diagram showing the number of bacterial OTUs unique and shared in the fecal samples of p-RFI and n-RFI Nellore steers. The percentage corresponds to the total value of 5,715 OTUs.

Table 4. Shared operational taxonomic units (OTUs) between feed efficiency groups that showed greater relative abundance in n-RFI and p-RFI Nellore steers. Values represent mean and standard deviation (SD).

						n-RFI		p-RFI		
Domain	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Mean**	**SD**	**Mean**	**SD**	**P -value**
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcaceae_UCG-005	7,035	1,294	7,026	1,508	1,241
						3,339	0,545	3,547	1,137	1,077
						2,279	0,488	2,348	0,496	1,027
						1,172	0,358	1,045	0,425	1,004
						1,085	0,313	1,112	0,290	1,068
						0,908	0,353	1,036	0,411	1,019
					Ruminococcaceae_NK4A214_group	0,666	0,250	0,727	0,310	1,086
						0,514	0,223	0,505	0,161	1,132
					Ruminococcaceae_unclassified	0,441	0,159	0,405	0,147	1,062
					Saccharofermentans	0,460	0,179	0,372	0,147	0,566
				Peptostreptococcaceae	Romboutsia	3,390	2,776	3,505	2,017	1,147
					Peptostreptococcaceae_unclassified	0,730	0,686	0,846	0,603	0,931
					Paeniclostridium	0,562	0,855	0,548	0,518	1,200
				Lachnospiraceae	Agathobacter	3,18	1,50	2,49	0,95	0,677
					Dorea	0,671	0,205	0,702	0,195	0,971
				Family_XIII	Mogibacterium	2,672	1,077	2,150	0,953	0,681
					Family_XIII_AD3011_group	0,509	0,267	0,441	0,173	0,983
				Christensenellaceae	Christensenellaceae_R-7_group	1,116	0,247	1,020	0,218	0,842
	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotellaceae_UCG-004	1,484	1,139	1,822	1,041	1,024
				Bacteroidaceae	Bacteroides	0,495	0,441	0,407	0,382	1,134
				Rikenellaceae	Alistipes	0,414	0,239	0,388	0,199	1,024
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	Akkermansia	0,740	0,697	0,858	1,262	1,058
	Actinobacteria	Coriobacteriia	Coriobacteriales	Eggerthellaceae	DNF00809	0,561	0,314	0,534	0,303	1,076
					Others	0,014	0,015	0,014	0,017	0,967

Heatmap

0

8

The volatile fatty acids analyzed in the fecal samples of Nellore steers showed no correlation (t-test, $P<0.05$ for significant values) with the feed efficiency phenotype (Table 5), even though there was a trend ($P<0.10$) for increased concentration of acetic and butyric acid in fecal samples of n-RFI steers.

Table 5. Composition of volatile fatty acids present in the fecal samples of p-RFI and n-RFI Nellore steers.

	n-RFI		p-RFI		
Parameter	**Mean**	**SEM**	**Mean**	**SEM**	**p-value**
Acetic acid (%)	50.89	3.57	49.67	5.09	0.07
Propionic acid (%)	26.00	4.31	26.60	5.62	0.35
Isobutyric acid (%)	9.86	3.04	10.14	4.35	0.30
Butyric acid (%)	8.84	3.93	8.74	3.69	0.06
Valeric acid (%)	4.42	2.86	4.84	4.21	0.88
Total VFA (mmol/L)	11.77	0.11	10.75	0.16	0.88

Values represent mean and standard error of the mean (SEM).

Discussion

The domestication of ruminants holds enormous significance for humanity due to the capacity of these animals to make the energy stored in plant biomass accessible to humans. This is possible because anaerobic microorganisms that colonize the gastrointestinal tract (GIT) of ruminants synthesize an enzymatic arsenal that allows the host to convert lignocellulosic substrates from their diets into products that are highly valuable for human nutrition (e.g. meat and milk) (Kruger Ben Shabat et al., 2016). However, raising cattle herds in extensive production regimes has some disadvantages such as the high cost for feeding, large use of arable lands, consumption of hydric resources, and higher environmental impact due to methane emissions (Paz et al., 2018). Thus, several studies have sought for strategies that could help improve the sustainability of food-animal production systems. Recent approaches have emphasized the role of the cattle GIT microbiota in the feed conversion efficiency of the animals as a potential tool for improving the productivity of production animals (Li and Guan, 2017).

Therefore, the selection of animals that are more feed-efficient can help farmers to reduce costs with feeding and supplementation of cattle. This is particularly relevant in tropical countries such as Brazil, where beef cattle are kept grazing during a great portion of their life cycle and tropical forages may have their nutritional composition affected by seasonality, such as in the dry period (Detmann et al., 2014). In addition, for cattle raised mostly on grasses, RFI evaluations pose a heavy burden on the producers because the animals need to be confined for long periods (up to 80 days) to monitor their feed consumption and weight gain. Given that previous studies indicated that the microbiota colonizing the ruminant gastrointestinal tract (GIT) play an important role in converting feeds into usable energy to the host and could potentially affect the feed efficiency phenotype of cattle, there is a growing interest in evaluating the GIT microbiome of production animals to develop predictive tools for feed efficiency.

The rumen ecosystem has been a natural target for studies investigating the association between animal microbiome and feed efficiency. However, access to rumen is very limited, especially in commercial herds, where fistulation and use of oral probe is not an option or not practical. Therefore, understanding the composition of the fecal microbiota of cattle and identifying biomarkers associated with the host's feed efficiency phenotype has been suggested as a more practical and non-invasive alternative to characterize certain phenotypic traits (e.g. feed efficiency) in cattle (Lopes et al., 2019; Brooke et al., 2019).

In this study, we characterized the fecal microbiota of 59 Nellore steers on pasture categorized into two groups according to their feed efficiency phenotype (RFI). Non-metric Multidimensional Scaling (NMDS) analysis indicated that the fecal microbiota of n-RFI and p-RFI steers were similar (Figure 1 and Figure 2). These results are probably because the animals were fed the same diet during the finishing period. Studies have shown that diet has an important role in the microbial composition of the gastrointestinal tract of ruminants, being used as a mechanism for manipulating the microbiota (Petri et al., 2012; Zhang et al., 2017). Kim et al. (2014) investigated the individual animal variation of bovine fecal microbiota by pyrosequencing technology. A group of 426 steers fed three different diets varying in the composition of dry rolled corn, corn silage, alfalfa, and supplement were analyzed. The objective was to investigate the variation of the fecal microbiota according to the forage and grain composition of the diet. The study showed that diet was the greatest factor altering fecal the composition of the bacterial communities, while gender and age only had minor effects on the fecal microbiota. The impact of

the diet on fecal communities was also investigated by Shanks et al. (2011). The fecal microbial communities of beef cattle from three different feeding operations and residing in four different geographic locations were profiled using massively parallel pyrosequencing of a hypervariable region of the 16S rRNA coding region. These operations consisted of three management groups, the forage group, the processed grain group, and the unprocessed grain group. The study demonstrated that bovine fecal bacterial communities are strongly influenced by animal feeding operations. The results also indicated that feeding operations were more important and determinant for the cattle microbiome than the geographic location of the feedlot.

Diversity and richness are important ecological metrics since an alteration on these indices are usually related to changes in the ecosystem functionality (Reese and Dunn, 2018). When analyzing the alpha diversity indices in the current study, results indicated differences among the feed efficiency groups in terms of richness and diversity of the operational taxonomic units (OTUs), with the values of both indices being higher in n-RFI steers (Figure 3). These results suggest that the fecal microbiome composition might be related to the capacity of the host to extract energy from their diets, similar to what has been demonstrated for the ruminal ecosystem (Kruger Ben Shabat et al., 2016). However, contrary to what was observed in the present study, previous observations indicated a lower species richness in the rumen microbiome of steers with high feed efficiency, suggesting a more "specialized" ruminal microbiota in these animals.

The population of bacteria colonizing the lower intestinal tract of cattle is dominated by strict anaerobes (Dowd et al., 2008). In the present study, Firmicutes and Bacteroidetes were the most abundant groups in fecal samples for both efficiency groups, followed by Actinobacteria, Tenericutes, and Proteobacteria (Figure 4). These results agree with the findings of Durso et al. (2010), who also found Firmicutes (62.8% of the OTUs) Bacteroidetes (29.5% of the OTUs) as the most abundant groups of bacteria in fecal samples of beef cattle. Additionally, Proteobacteria (4.4% of the OTUs), Actinobacteria (0.79% of the OTUs) and Tenericutes (0.63%) were also reported. The study characterized fecal bacteria from six feedlot cattle by full-length capillary sequence analysis of 16S rRNA gene. Kim and Wells (2016) conducted a meta-analysis on 16S rRNA gene sequences of bovine fecal origin publicly available in the Ribosomal Database Project (RDP). Firmicutes and Bacteroidetes were the most abundant phyla and accounted for 49% and 42% of all 13,663 sequences, respectively. Proteobacteria was the third-largest phylum and accounted for 6% of the total sequences.

Studies have also demonstrated that the bacterial community of the ruminant gastrointestinal tract plays an important role in defining the physiological traits of the host (Jami et al., 2014; Koliada et al., 2017). Myer et al. (2015) found that increases in Firmicutes as well as many bacterial genera belonging to this phylum were associated with animals showing higher average daily body weight gain. They suggested that a greater abundance of Firmicutes could have an impact on feed efficiency in cattle. In addition, previous work indicated that a higher Firmicutes/Bacteroidetes ratio is associated with an increase in body mass index in humans (Koliada et al., 2017; Armougom and Raoult, 2008) and with the production of milk fat in dairy cows (Jami et al., 2014).

Our analyses indicated that the Ruminococcaceae was the most abundant family in the fecal bacterial community of both n-RFI and p-RFI steers, followed by members of the family Lachnospiraceae (Figure 5). These families of bacteria comprise several species with cellulolytic activity, which are capable of decomposing substrates that are not digestible by the ruminant and also produce short-chain fatty acids that serve as a source of energy to the host (Biddle et al., 2013). The fecal community of beef cattle characterized in the current study shared the main groups described for dairy cattle although the abundance may vary between these hosts. Dowd et al. (2008) analyzed fecal samples from 20 lactating dairy cows fed a diet composed predominantly of concentrate using bacterial tag-encoded FLX 16S rDNA amplicon pyrosequencing. The study showed that Clostridium spp. (Clostridiaceae family) and Bacteroides spp. (Bacteroidaceae family) were highly prevalent in the fecal samples and distributed across all of the animals, followed by Porphyromonas ssp., Ruminococcus ssp., Alistipes ssp., Lachnospira ssp., and Prevotella spp. However, for all these studies, the influence of the diet on the composition of microbial communities must be considered. For example, Kim et al. (2014) shown that both Prevotella and Bacteroides were rarely detected in cattle fed a diet composed of silage/forage (70% corn silage and 30% alfalfa haylage).

Results in the present study revealed a significant difference in the relative abundance of members of the Veillonellaceae family between the n-RFI and p-RFI steers, being higher in the p-RFI steers. Some genera of the Veillonellaceae family, including the genus Megasphaera, can utilize lactic acid and convert this substrate into other organic acids, such as acetate and propionate, as the main fermentation end-products (Uchiyama et al., 2020). Although the increase in abundance of bacteria from this phylogenetic group in the rumen environment can improve the

fermentation of easily degradable carbohydrates (Ushakova et al., 2013), the role of these microorganisms in the fecal microbiome is not well known. In addition, since in the current study their abundances in the fecal microbiota were low (around 0.09%), it is not possible to speculate about the relevance and functionality of this group for the efficiency phenotype in Nellore steers.

The genera with mean relative abundance greater than 0.1% showing significant differences between the n-RFI and p-RFI groups were Ruminococcaceae_UCG-014, dgA-11_gut_group, Treponema_2, and Slackia, the first being the most abundant (2,92 ± 0.64) (Figure 6, Table 3). All these genera showed greater abundance in the n-RFI group (more efficient animals), demonstrating a possible positive relationship with high feed efficiency. Zhang and Wang (2018) demonstrated that members of the Ruminococcaceae_UCG-014 group are essential for fiber degradation in the rumen by positively associating their abundances with neutral detergent fiber (NDF) degradability in the goat diet. These results emphasize the important role played by this bacterial group in the metabolism of digestible energy for ruminants.

Our results revealed OTUs associated with Nellore steers showing high and low feed efficiency, but there were no significant differences between groups (Table 4). Among the OTUs shared between the n-RFI and p-RFI groups, the genus Ruminococcaceae_UCG-005 was the most representative. Ruminococcaceae_UCG-005 belongs to the family Ruminococcaceae, which plays an important role in the digestion of fibers in ruminants and animals fed with forage, as in the case of the present study, tend to present a greater abundance in this group (Zhang et al., 2018). Qiu et al. (2019) evaluated variations in the fecal bacterial composition of Holstein steers fed with different energy densities and identified a decrease in the genus Ruminococcaceae_UCG-005 by decreasing the fiber content of the diets. The genus Romboutsia, belonging to the Peptostreptococcaceae family, also showed a representative abundance in both groups of feed efficiency. Members of Romboutsia are anaerobes associated with the intestinal environment of mammals and their main end products of metabolism are acetate and format (Gerritsen et al., 2014). Although their specific roles are not well established, Gerritsen et al. (2019) demonstrated that Romboutsia has a wide fermentation capacity of unique amino acids and carbohydrate utilization metabolisms.

When analyzing the unique OTUs of each feed efficiency group (supplementary Table S1), the Prevotellaceae family stood out as the most abundant in n-RFI steers although its abundance was low (0.049%). This family is reported in many studies associated with the rumen microbiome

of animals predominantly fed concentrate diets, and Prevotella has been reported as the most abundant bacterial genus in the rumen of cattle (Myer et al., 2015; Stevenson and Weimer, 2009). Thus, the low abundance of this family in the present study can be explained by the grazing condition of the animals. As demonstrated by Shanks et al. (2011), the relative abundances of members of the Prevotellaceae family in fecal samples from cattle fed unprocessed grain rations is more than 10 times higher than that in animals fed exclusively forage rations. Then Prevotella genus has been described as an important taxon associated with feed efficiency in cattle (Paz et al., 2018; Li and Guan, 2017; Jewell et al., 2015). Carberry et al. (2012) showed that Prevotella abundance was higher ($P<0.0001$) in inefficient animals, while Jewell et al. (2015) identified OTUs of the Prevotella genus associated with both high and low-RFI animals. They concluded that although bacterial enumeration is difficult to estimate from sequencing data, it is worth considering that proportional changes in microbial taxa belonging to the core species may be important and needs further investigation. Furthermore, a study evaluating fecal samples of cattle fed a diet consisting of 69.8% corn grain, described Prevotella copri as a potential microbial biomarker to identify beef cattle with high feed efficiency at the beginning of their life and during the production cycle. Prevotella copri has been associated with the utilization of several carbohydrates as substrates and described as a key player in the establishment of the intestinal function and in the health of the host (Brooke et al., 2019).

Studies about the impact of ruminal diversity and composition on the feed efficiency of animals are closely associated with their ruminal metabolism. In the present study, the concentration of short-chain fatty acids did not vary in the feces of Nellore steers with high and low feed efficiency (Table 5). However, in a study with lactating cows, Kruger Ben Shabat et al. (2016) showed significant differences in rumen short-chain fatty acids composition between animals of high and low efficiency. Propionate, butyrate, valerate, and isovalerate were found at higher concentrations in the rumen of the more efficient cows. In addition, the total concentration of short-chain fatty acids was also higher in the more efficient animals. This may be explained by functional changes in the rumen microbiota leading to differences in metabolic pathways for substrate utilization between high and low feed efficiency animals. Therefore, while evaluation of the rumen ecosystem suggests that lower microbial diversity and higher production of specific short-chain fatty acids seems to be related to higher efficiency in feed conversion, analysis of the

fecal microbiota indicates that differences in diversity and abundance of bacterial taxa are relevant for the characterization of the efficiency phenotype of Nellore steers.

The results presented here showed that the fecal samples harbor considerable bacterial diversity and suggest bacterial groups from these samples associated with the feed efficiency phenotype. These results are promising since few studies have associated the fecal microbiota of Nellore cattle to the efficiency phenotype (Lopes et al., 2019). Fecal samples have already been widely used in different studies, such as with humans, to assess the relationship between the composition of the microbiota and the health status of the host, being associated with several diseases including depression, autism, inflammatory bowel disease, and obesity (Jiang et al., 2015; Kang et al., 2018; Sokol et al., 2006; Duncan et al., 2008). The collection of fecal samples, therefore, is a non-invasive, more practical method, which allows the analysis of a larger number of individuals. In ruminants, the result of this study is promising, since the analysis of fecal samples to evaluate the efficiency phenotype could be performed without requiring fistulation and slaughter of the animals, reducing ethical issues inherent to animal experimentation. However, large-scale studies will be needed to validate these findings using larger cohorts of animals on a commercial scale, which may expand our current knowledge about the relationship between animal traits and the microbial community of the Nellore gastrointestinal tract.

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Supplementary material

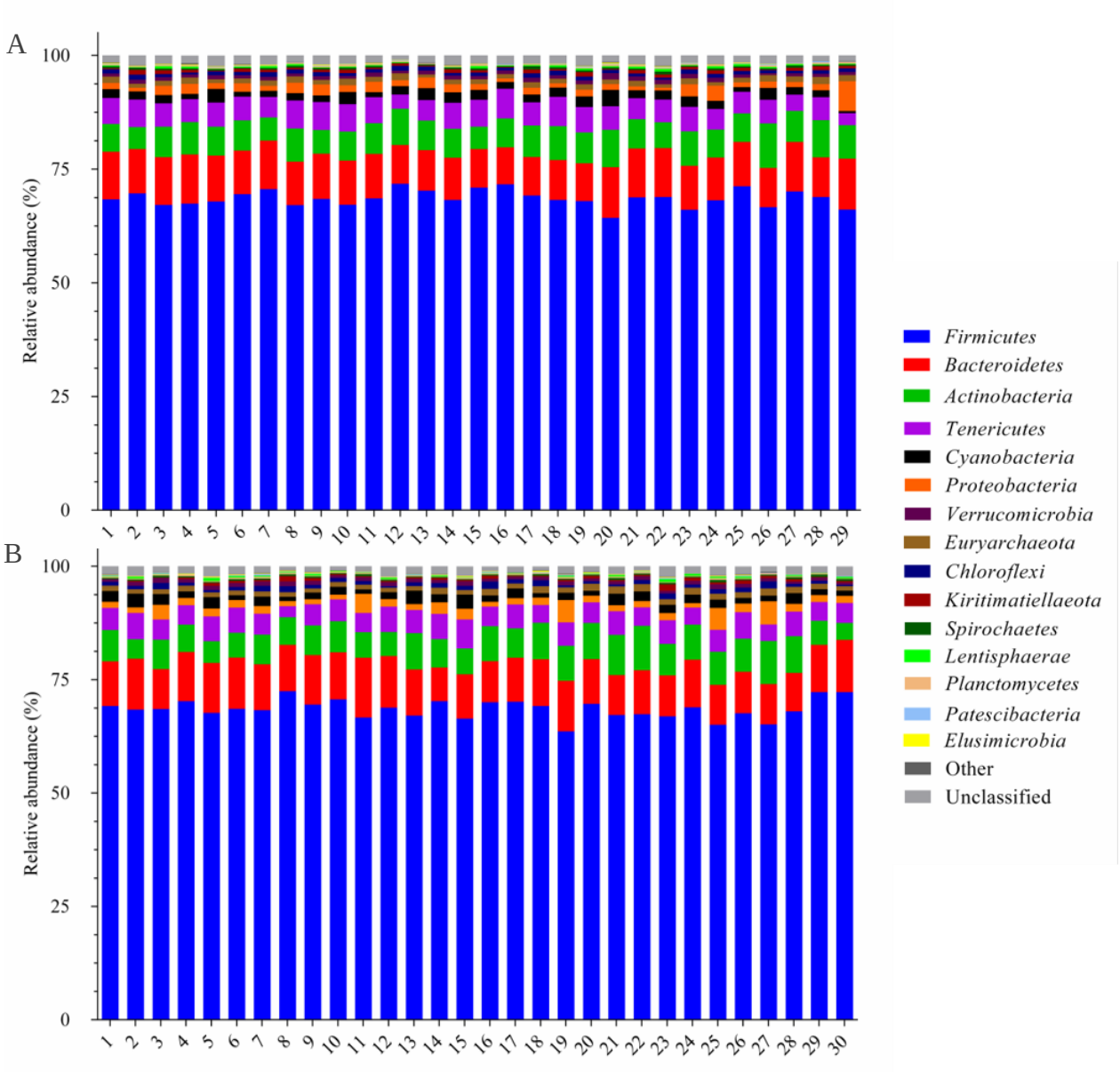

Figure S1. Bacterial community composition at the phylum level in fecal samples of n-RFI (A) and p-RFI (B) Nellore steers. Each bar represents a different steer with its average relative abundance (%) of the major phyla found in fecal samples of each animal group. "Other" represents the phyla with relative abundances <0.1%.

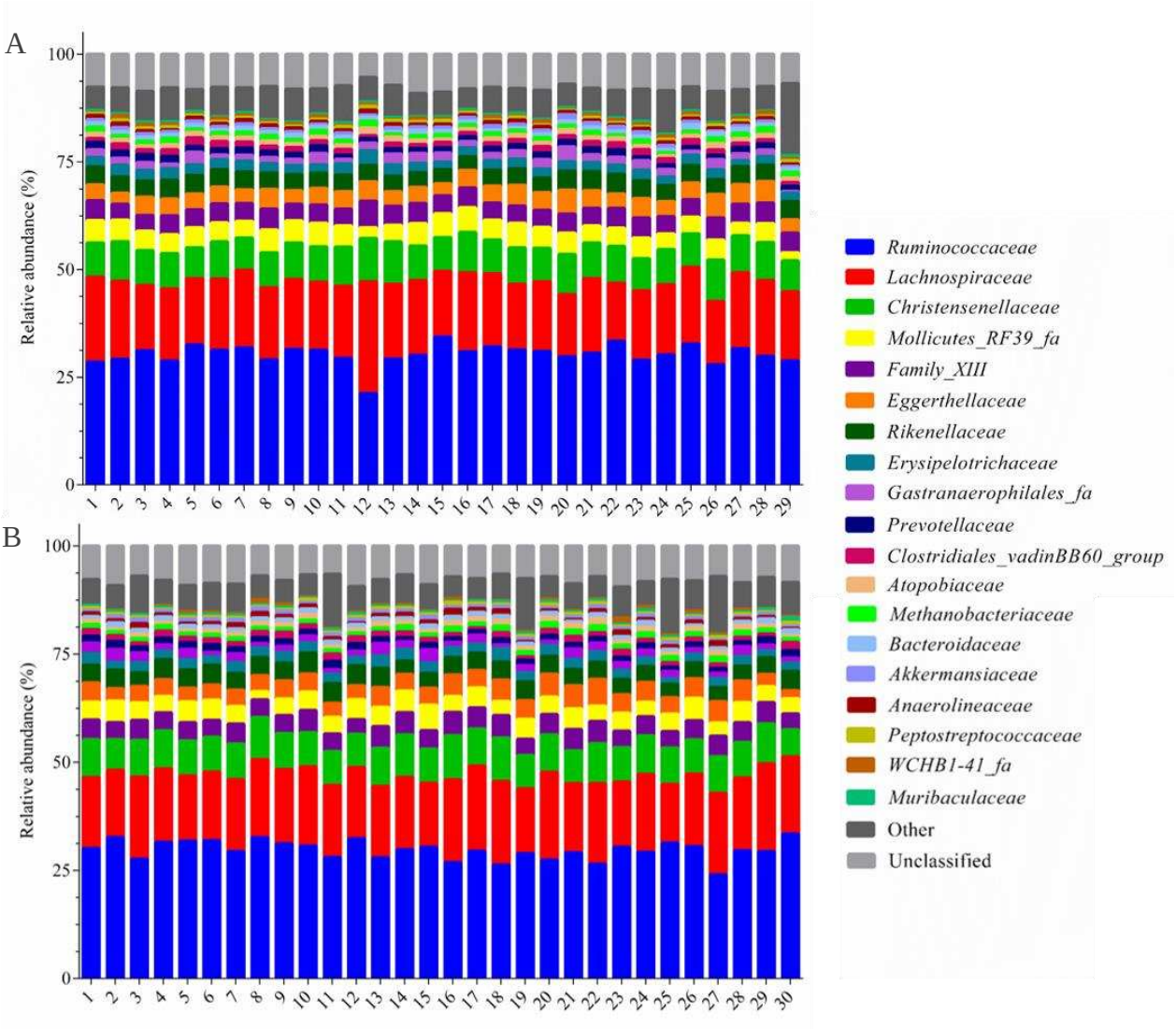

Figure S2. Bacterial community composition at the family level in fecal samples of n-RFI (A) and p-RFI (B) Nellore steers. Each bar represents a different steer with its average relative abundance (%) of the major families found in fecal samples of each animal group. "Other" represents the families with relative abundances <0.5%.

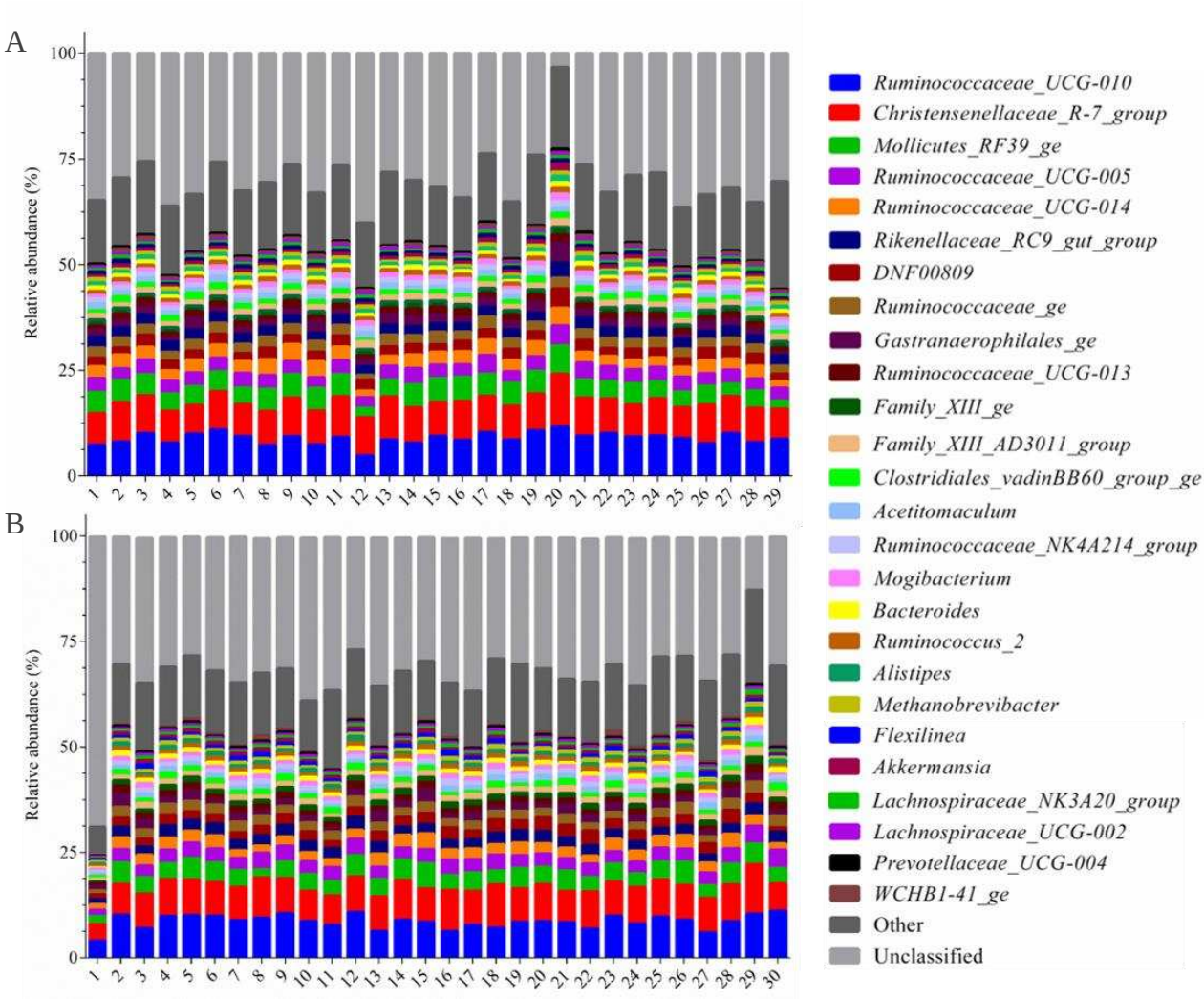

Figure S3. Bacterial community composition at the genus level in fecal samples of n-RFI (A) and p-RFI (B) Nellore steers. Each bar represents a different steer with its average relative abundance (%) of the major genera found in fecal samples of each animal group. "Other" represents the genera with relative abundances <0.5%.

Table S1. Exclusive operational taxonomic units (OTUs) that showed greater relative abundance in n-RFI (A) and p-RFI (B) Nellore steers. Values represent mean and standard deviation (SD).

A

Phylum	Class	Order	Family	Genus	Mean	SD
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae_unclassified	0,009	0,032
			Ruminococcaceae	Ruminococcaceae_UCG-005	0,004	0,020
				Ruminococcaceae_unclassified	0,003	0,011
			Christensenellaceae	Christensenellaceae_R-7_group	0,003	0,012
			Eubacteriaceae	Anaerofustis	0,003	0,008
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotellaceae_UCG-003	0,049	0,226
			M2PB4-65_termite_group	M2PB4-65_termite_group_ge	0,007	0,037
Actinobacteria	Coriobacteriia	Coriobacteriales	Eggerthellaceae	DNF00809	0,006	0,023
Cyanobacteria	Melainabacteria	Gastranaerophilales	Gastranaerophilales_fa	Gastranaerophilales_ge	0,003	0,013
					0,002	0,007
				Other	0,000	0,001

Heatmap **n-RFI**

0

0,05

B

Phylum	Class	Order	Family	Genus	Mean	SD
Firmicutes	Bacilli	Bacillales	Planococcaceae	Chryseomicrobium	0,068	0,318
			Bacillaceae	Oceanobacillus	0,007	0,037
	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae_unclassified	0,058	0,297
					0,004	0,015
			Ruminococcaceae	Ruminococcaceae_UCG-013	0,042	0,229
				Ruminococcaceae_UCG-010	0,014	0,077
			Christensenellaceae	Christensenellaceae_R-7_group	0,004	0,015
			Family_XI	Tissierella	0,004	0,016
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas	0,006	0,028
Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Alloprevotella	0,005	0,029
				Other	0,000	0,001

Heatmap **p-RFI**

0

0,07

GENERAL CONCLUSIONS

Nellore steers classified as high and low feed efficiency phenotypes and grazing tropical forages show specific differences in the taxa composition of their fecal microbiota.

Members of the families Veillonellaceae, Ruminococcaceae (Ruminococcaceae_UCG-014), and Prevotellaceae, which are essential for the fermentation of dietary components in beef cattle, showed significant differences in relative abundances between n-RFI and p-RFI Nellore steers.

There were no significant differences between the VFA concentration in the fecal samples of n-RFI and p-RFI Nellore steers.

Fecal sampling could be a useful, non-invasive approach, to evaluate large cattle herds for feed efficiency on a commercial scale.