UNIVERSIDADE FEDERAL DO PAMPA

CAMPUS SÃO GABRIEL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

ANDERSON SANTOS DE FREITAS

RELAÇÃO ENTRE A MICROBIOTA DO RÚMEN E O AUMENTO NO GANHO DE PESO EM BOVINOS DE CORTE

São Gabriel

2020

ANDERSON SANTOS DE FREITAS

RELAÇÃO ENTRE A MICROBIOTA DO RÚMEN E O AUMENTO NO GANHO DE PESO EM BOVINOS DE CORTE

Dissertação apresentada ao programa de Pós-Graduação *stricto sensu* em Ciências Biológicas da Universidade Federal do Pampa como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Prof. Dr. Luiz Fernando Würdig Roesch

São Gabriel

2020

FICHA CATALOGRÁFICA

Ficha catalográfica elaborada automaticamente com os dados fornecidos
pelo(a) autor(a) através do Módulo de Biblioteca do
Sistema GURI (Gestão Unificada de Recursos Institucionais) .

F866r Freitas, Anderson Santos de
Relação entre a microbiota do rúmen e o aumento no ganho de peso em bovinos de corte / Anderson Santos de Freitas.
48 p.

Dissertação(Mestrado)-- Universidade Federal do Pampa, MESTRADO EM CIÊNCIAS BIOLÓGICAS, 2020.
"Orientação: Luiz Fernando Würdig Roesch".

1. Cadeia Produtiva. 2. Criação de gado. 3. Microbiomas. 4. Microbiota Runinal. I. Título.

ANDERSON SANTOS DE FREITAS

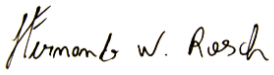
RELAÇÃO ENTRE A MICROBIOTA DO RÚMEN E O AUMENTO NO GANHO DE PESO EM BOVINOS DE CORTE

Dissertação apresentada ao programa de Pós-Graduação stricto sensu em Ciências Biológicas da Universidade Federal do Pampa como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Áreas de concentração: Biologia Molecular; Microbiologia.

Dissertação defendida e aprovada em 21/12/2020.

Banca Examinadora:



Prof. Dr. Luiz Fernando Würdig Roesch
Orientador
UNIPAMPA

Prof[a]. Dra. Tsai Siu Mui

Universidade de São Paulo – USP

Prof. Dr. Victor Satler Pylro

Universidade Federal de Lavras -UFLA

AGRADECIMENTOS

Agradeço primeira e principalmente a meus pais, Nildo e Valdirene que me deram a vida e nunca permitiram que nada faltasse, além de me darem amor e apoio incondicional para todas as etapas da minha vida.

Ao meu orientador, professor Dr. Luiz Roesch, por ser orientador, amigo, conselheiro de carreira e tantas outras incumbências que o tornaram alguém que eu sempre pude contar independente do tópico.

Ao grupo de pesquisa, em especial a Priscila, Susi, Flavia e Bia pelas trocas de conhecimento, parceria e pelo socorro mútuo sempre que o R de alguém desistia de funcionar.

A meus amigos de grandes grupos históricos: Reunião dos Templários, S I N C R O N I A, Melhores 2.0, Ações & Injeções, A Confraria e Clube do Sargento Pimenta. A vida é muito mais fácil e divertida com os amigos e vocês são a prova disso.

Aos amigos e colegas do Biotec em Pauta, o podcast que me permitiu conhecer e trabalhar com pessoas incríveis e viver excelentes momentos nesse ano tão complicado.

A Sérgio, Teresinha e Mateus, a família que salvou a minha vida quando eu precisei de internet às pressas no final deste trabalho e fiquei sem a quem recorrer.

À Universidade Federal do Pampa e seu corpo de docentes, técnicos e funcionários terceirizados por proporcionar a estrutura física e humana para que este trabalho fosse possível.

“O mato é grosso, mas a gente é fina”

Estagiário do Globo Rural

RESUMO

Bovinos (*Bos* taurus) são animais de grande importância econômica, sobretudo na produção de carne e leite, movimentando bilhões de dólares todos os anos. São bastante difundidos no Brasil, em especial no Pampa, desde o período das Grandes Navegações quando foram trazidos por colonizadores europeus. Tratam-se de animais classificados como ruminantes por terem um estômago multicavitário onde a primeira porção é chamada de rúmen. Nesse ambiente, microrganismos como bactérias e fungos atuam na degradação dos alimentos ingeridos e na produção de compostos absorvíveis pelo animal ao longo de todo o trato gastrointestinal. O presente trabalho objetivou analisar a microbiota do rúmen de bovinos e sua relação com a nutrição dos animais. Foram coletadas amostras de líquido ruminal de novilhas cedidas pelo Departamento de Diagnóstico e Pesquisa Agropecuária (DDPA) na cidade de São Gabriel, RS. Estas novilhas foram acompanhadas por 60 dias para análise de aspectos nutricionais (como por exemplo o ganho médio de peso) e, posteriormente, agrupadas em grupos relativos a esse acompanhamento para a realização de análises comparativas. O DNA microbiano (região ITS2 para fungos e região V4 do rRNA 16S para bactérias) foi amplificado por PCR e sequenciado na plataforma Ion PGM. As sequências foram processadas de acordo com as recomendações do Brazilian Microbiome Project e analisadas no ambiente R. Com isso, identificou-se um padrão de microrganismos ligados a animais com maior ganho de peso, sendo estes organismos conhecidos por produzirem enzimas que degradam com maior eficiência a matéria orgânica vegetal. Os dados apontam para uma combinação de fungos e bactérias que estão associados a um maior ganho de peso, incluindo os gêneros bacterianos *RFN20*, *Prevotella* e *Anaeroplasma* bem como os gêneros fúngicos *Aureobasidium*, *Cryptococcus* e *Sarocladium*. Em táxons mais abrangentes, microrganismos anotados como pertencentes à família *RF16* e às ordens *Tremellales* e *Pleosporales* também se mostraram relacionadas a um ganho de peso elevado. Entre as archaeas, foi encontrado apenas o filo *Euryarchaeota* (0,2% das sequências), que não mostrou diferença entre os grupos. Estes reportes sugerem que é possível a utilização destes microrganismos em estudos posteriores tanto como marcadores de eficiência quanto em terapias microbianas para aumento do ganho de peso.

Palavras-chave: Cadeia Produtiva, Criação de gado, Microbiomas, Microbiota Ruminal.

ABSTRACT

Bovines (*Bos taurus*) are animals with great economic importance, especially in the production of meat and milk, moving billions of dollars every year. They are very widespread in Brazil, especially in the Pampa, since the Great Navigations period when they were brought by European colonists. These animals are classified as ruminants due they have a multi-cavity stomach where the first portion is called rumen. In this environment, microorganisms such as bacteria and fungi act in the degradation of ingested food and in the production of absorbable compounds by the animal throughout the gastrointestinal tract. The present work aimed to analyze the bovine rumen microbiota and its relationship with animal nutrition. Rumen samples of ruminal liquid from heifers provided by the Department of Diagnosis and Agricultural Research (DDPA) were collected in the city of São Gabriel, RS. These heifers were monitored for 60 days for analysis of nutritional aspects (such as average weight gain) and, subsequently, grouped into groups related to this monitoring for comparative analyzes. The microbial DNA (ITS2 region for fungi and V4 region of 16S rRNA for bacteria) was amplified by PCR and sequenced on the Ion PGM platform. The sequences were processed according to the recommendations of the Brazilian Microbiome Project and analyzed in the R environment. With this, a pattern of microorganisms linked to animals with greater weight gain was identified, being these organisms known to produce enzymes that degrade more efficiently vegetable organic matter. The data point to a combination of fungi and bacteria that are associated with greater weight gain, including the bacterial genera *RFN20*, *Prevotella* and *Anaeroplasma* as well as the fungal genera *Aureobasidium*, *Cryptococcus* and *Sarocladium*. In higher taxa, microorganisms assigned as belonging to the family *RF16* and to the orders *Tremellales* and *Pleosporales* also showed relationship with a higher gain of weight. Among archaeas, it was found only the phylum *Euryarchaeota* (0.2% of sequences), which did not show difference between the groups. These reports suggest that is possible the use of these microorganisms in later studies both as efficiency markers and in microbial therapies to increase weight gain.

Keywords: Livestock, Microbiomas, Productive Chain, Ruminal Microbiota.

SUMÁRIO

1 INTRODUÇÃO

1.1 O gado bovino e sua importância econômica

Gado bovino é um termo utilizado para designar animais herbívoros de grande porte, pertencentes à espécie *Bos taurus*, compreendendo bois, vacas e touros. Sua chegada ao Brasil remonta à época das Grandes Navegações, sendo trazidos por navegadores portugueses, espanhóis e holandeses principalmente nos séculos XVI e XVII (SILVA; BOAVENTURA; FIORAVANTI, 2012). Tratam-se de animais de grande importância histórica e econômica, sobretudo na produção de carne, couro e leite, sendo bastante difundidos na região sul do Brasil, principalmente no Pampa gaúcho (ALMEIDA, DE; MICHELS, 2012; MINISTÉRIO DO MEIO AMBIENTE, 2019).

O rebanho bovino brasileiro hoje soma mais de 215,5 milhões de cabeças de gado (IBGE, 2019) espalhados por mais de 172 milhões de hectares de áreas de pastejo, sendo considerado o maior rebanho comercial do mundo (MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO, 2018; SCHLESINGER, 2010). Dados da Embrapa pontuam que a carne bovina sozinha representa 6% do PIB total do Brasil e 30% do PIB do agronegócio nacional. São abatidos em torno de 40 milhões de animais todo ano, movimentando apenas com vendas de carne um valor superior a 400 bilhões de reais (EMBRAPA, 2020).

No tocante à exportação, segundo a Associação Brasileira de Frigoríficos (ABRAFRIGO, 2020), foram exportados mais de 1,8 milhões de toneladas de carne bovina e derivados no último ano, gerando mais de 7,5 bilhões de dólares, dado que coloca a carne bovina em sétimo lugar entre os principais produtos exportados pelo Brasil. Os principais países importadores estão no mercado asiático, primariamente China, Hong Kong, Arábia Saudita e Rússia (FARMNEWS, 2020). E as previsões para o futuro da bovinocultura brasileira são extremamente positivas: as estimativas apontam para um aumento de 20% na produção de carne até 2027 onde se espera produzir 11,5 milhões de toneladas de carne somente em território nacional (MALZONI, 2018).

Dado este contexto, é necessário buscar alternativas para ampliar e otimizar a produção de carne, a fim de propor um crescimento efetivo e ao mesmo tempo sustentável da cadeia produtiva, uma vez que a criação de gado gera críticas quanto à produção de gases causadores do efeito estufa, por exemplo, e também pela perda de espaço para a criação de gado em favor

de culturas agrícolas como soja e milho. É justamente nesse ponto que o presente trabalho se propõe a contribuir.

1.2 A nutrição dos bovinos e a importância dos micro-organismos

Para propor melhorias na criação de gado é necessário compreender como funciona o processo nutricional destes animais, um processo que, como será visto adiante, é altamente dependente e conduzido por microrganismos de todos os domínios da vida.

A principal característica morfológica dos bovinos quanto à nutrição consiste no seu estômago multicavitário, composto por rúmen, retículo, omaso e abomaso (LINN *et al.*, 2018). Esta configuração estomacal permite que o processo de alimentação compreenda um ciclo de pastejo bem definido. Em geral, os bovinos passam um terço do seu tempo pastando a maior quantidade de alimento possível e armazenando-o na primeira dessas cavidades: o rúmen. O restante do tempo compreende o terço dedicado a regurgitar o alimento do rúmen que é mascado na boca para depois ser engolido novamente, num processo que é denominado ruminação o qual auxilia na quebra física e enzimática de substâncias como amido e lignina, e o terço dedicado ao ócio pelo animal (PARISH; RIVERA, 2017).

Dentro da cavidade ruminal, por sua vez, existe uma espécie de câmara de fermentação altamente vascularizada e com projeções em suas paredes adaptada para o melhor e mais rápido aproveitamento do alimento (LINN *et al.*, 2018). Fungos, bactérias, archaeas e protistas lá presentes produzem enzimas que degradam a matéria vegetal ingerida, produzindo vitaminas, aminoácidos e ácidos graxos voláteis de cadeia curta (como os ácidos propanoico e butílico), que são a fonte primária de energia para os bovinos (WIRTH *et al.*, 2018; WLODARSKI *et al.*, 2017). São estes micro-organismos ruminais que fazem a maior parte do processo digestivo, sendo responsáveis por 50 a 65% do amido consumido pelo animal, por exemplo (PARISH; RIVERA, 2017).

Os principais microrganismos envolvidos neste processo digestório pertencem aos filos bacterianos *Firmicutes* e *Bacteroidetes*, a maioria inclusa nos gêneros *Prevotella*, *Butyrivibrio* e *Ruminococcus* (STEWART *et al.*, 2019), e aos filos fúngicos *Ascomycota* e *Neocallimastigomyota* (SONG; JEONG; KIM, 2017), bem como archaeas metanogênicas, especialmente da classe *Methanobacteria* (WIRTH *et al.*, 2018). Estes micro-organismos desempenham funções diversas dentro do processo digestivo e são igualmente importantes para

o melhor desempenho nutricional possível do animal. Prova disso é que essa configuração microbiana ruminal é comum a animais de diversas partes do mundo, numa espécie de microbiota consenso do rúmen (STEWART *et al.*, 2019).

1.3 Sequenciamento de Nova Geração: uma nova forma de entender a microbiologia na nutrição animal

Compreender os microrganismos do rúmen de bovinos é, portanto, chave para encontrar mecanismos que melhorem a nutrição destes animais e torne-os ainda mais eficientes na cadeia produtiva.

Indo de encontro à microbiologia clássica, muito ligada à cultura de microrganismos, as novas tecnologias em biologia molecular desenvolvidas nas últimas décadas permitiram um crescimento exponencial no conhecimento sobre diversas áreas da vida, inclusive sobre a microbiota ruminal (FOUTS *et al.*, 2012). O principal avanço nesse sentido se deve ao Sequenciamento de Nova Geração (*NGS*, na sigla em inglês), uma técnica que permite a identificação de organismos de um ambiente sem a necessidade prévia de cultura em meio de cultivo, obtendo resultados diretamente do DNA extraído da amostra (SCHUSTER, 2007), ROESCH et al. 2007).

Através do *NGS*, os cientistas se libertaram da necessidade de mimetizar o ambiente ruminal em laboratório, tarefa que é extremamente difícil (COCOLIN; DOLCI; RANTSIOU, 2011), e puderam focar em entender o funcionamento microbiano do rúmen diretamente da natureza. Alguns artigos já postulam interessantes descobertas, como a alta na abundância dos gêneros *Entodinium* e *Prevotella* em animais submetidos a dietas com baixo teor de forragem (CARBERRY *et al.*, 2012) e o aumento de fungos raros em animais sob acidose ruminal induzida (ISHAQ *et al.*, 2017). No entanto, ainda há um vasto campo de descobertas a serem desvendadas sobre como os microrganismos do rúmen influenciam no processo digestório e como esse conhecimento pode ser aplicado na cadeia produtiva a fim de melhorar e ampliar a produção da área.

2 OBJETIVOS

Com base nesse contexto, o objetivo da presente dissertação foi correlacionar a composição microbiana do rúmen ao ganho de peso em bovinos de corte e procurar por táxons microbianos como marcadores de alto ou baixo ganho de peso, bem como propor o uso destes táxons em possíveis terapias microbianas a fim de melhorar a cadeia produtiva.

3 APRESENTAÇÃO DO MANUSCRITO

O presente trabalho é apresentado na forma do manuscrito do artigo publicado na revista Antonie van Leeuwenhoek (eISSN: 1572-9699, ISSN: 0003-6072) e formatado conforme suas normas. O manuscrito está dividido em Resumo, Introdução, Material e Métodos, Resultados, Discussão, Conclusões e Referências.

4 MANUSCRITO

Microbial Patterns in Rumen Are Associated with Gain of Weight in Beef Cattle

Anderson Santos de Freitas [1,+], Diego Bitencourt de David [2,+], Beatriz Midori Takagaki [1] and Luiz Fernando Würdig Roesch [1,*]

Affiliations:

[1] Centro Interdisciplinar de Pesquisas em Biotecnologia – CIP-Biotec, Campus São Gabriel, Universidade Federal do Pampa, São Gabriel, Rio Grande do Sul, Brasil.

[2] Departamento de Diagnóstico e Pesquisa Agropecuária – DDPA, Secretaria Estadual da Agricultura, Pecuária e Desenvolvimento Rural – SEADPR/RS, São Gabriel, Rio Grande do Sul, Brasil.

[+] These authors have contributed equally to this work.

ORCID ID:

AS Freitas: https://orcid.org/0000-0002-7726-0180

DB de David: https://orcid.org/0000-0002-2403-0429

BM Takagaki: https://orcid.org/0000-0002-4320-1617

LFW Roesch: https://orcid.org/0000-0003-1450-8828

***Corresponding author:**

Luiz Fernando Wurdig Roesch

Centro Interdisciplinar de Pesquisas em Biotecnologia – CIP-Biotec, Campus São Gabriel, Universidade Federal do Pampa, São Gabriel, Rio Grande do Sul, Brasil.

Email: luizroesch@unipampa.edu.br

Abstract

Ruminal microorganisms play a pivotal role in cattle nutrition. The discovery of the main microbes or of a microbial community responsible for enhancing the gain of weight in beef cattle might be used in therapeutic approaches to increase animal performance and cause less environmental damages. Here, we examined the differences in bacterial and fungal composition of rumen samples of Braford heifers raised in natural grassland of the Pampa Biome in Brazil. We aimed to detect microbial patterns in the rumen that could be correlated with the gain of weight. We hypothesized that microorganisms important to digestion process are increased in animals with a higher gain of weight. The gain of weight of seventeen healthy animals was monitored for 60 days. Ruminal samples were obtained and the 16S gene and ITS1 region were amplified and sequenced to identify the closest microbial relatives within the microbial communities. A predictive model based on microbes responsible for the gain of weight was build and further tested using the entire dataset., The main differential abundant microbes between groups included the bacterial taxa *RFN20*, *Prevotella, Anaeroplasma* and *RF16* and the fungal taxa *Aureobasidium, Cryptococcus, Sarocladium, Pleosporales* and *Tremellales*. The predictive model detected some of these taxa associated with animals with the high gain of weight group, most of them being organisms that have been correlated to the production of substances that improve the ruminal digestion process. These findings provide new insights about cattle nutrition and suggest the use of these microbes to improve beef cattle breeding.

Keywords: bacteria, fungi, livestock, microbiome, next generation sequencing.

Introduction

Microorganisms from all domains of life are associated with cattle nutrition. Bacteria, Archaea and micro Eukaryotes are present across the entire gastrointestinal tract (GIT) of these animals. They digest complex molecules, such as cellulose and lignin, and help the absorption of water and nutrients (Malmuthuge et al. 2014; Wirth et al. 2018). Microbes from the rumen, in particular, are crucial for this process, once the rumen acts as a fermentation chamber hosting microbial fermentation of the food ingested by the cattle. The rumen environment is responsible for about 50 to 65 percent of starch and soluble sugars utilized by an animal and produces the main food and energy sources for bovines (i.e. proteins, volatile fatty acids, and synthesis of B and K vitamins) (Parish and Rivera 2017; Linn et al. 2018).

Since mimicking the rumen conditions in laboratories to isolate those microbes is a challenging task, the rising of culture-free techniques, mainly Next Generation Sequencing (NGS), has facilitated the study of these microbes. Over the achievements accumulated by classic culture-based approaches, NGS has provided a wide range of knowledge about rumen microbes and its behavior in the last decades (Mccann et al. 2014; Myer et al. 2016). Several studies, using different techniques and bovine races, have uncovered the structure of the rumen environment and pointing to a consensus ruminal core microbiome across a large geographical range (Jami and Mizrahi 2012; Henderson et al. 2015). This core is dominated by the bacterial phylum *Bacteroidetes*, mainly composed by *Prevotella* species, either in Brazilian Nelore bovines (Jesus et al. 2015) or in Holstein bull calves from Canada (Malmuthuge et al. 2014), for example. Indeed, this predominance of *Bacteroidetes* and its *Prevotella* species is extensively described by reports that denote these taxa as important microorganisms for digestion of organic matter in the rumen, being considered as biomarkers for good ruminal microbiota (Nathani et al. 2015). With respect to fungi, the ruminal microbial core includes a range of anaerobic genera, mainly from *Neocallimastigomycota*, *Basidiomycota* and *Ascomycota* phyla (Fliegerova et al. 2015; Song et al. 2017).

Although geographical standards of ruminal microbiota are known, previous works also support external aspects like diet may influence the rumen microbial composition. Animals submitted to a low-forage diet, for example, showed a higher abundance of *Entodinium* and *Prevotella* species (Carberry et al. 2012) whereas animals with induced sub-acute ruminal acidosis (SARA) presented an increase of rare fungal taxa (Ishaq et al. 2017). Similarly, different microbial composition affect production chains, such as cows with high milk yield, which presented higher levels of *Prevotella* (Indugu et al. 2017) and the correlation of milk-fat yield to a higher *Firmicutes/Bacteroidetes* ratio in rumen (Jami et al. 2014).

All this knowledge comes to meet the growing need for food supply and the concern about climate changes in the world. These areas may be benefitted by new insights about how to optimize cattle breeding with more production and less environmental damages (Stewart et al. 2019). Alongside, the ruminal microbiology has historically failed in optimize animal performance by microbial information and therapies. Much of this is due to the lack of knowledge about how microorganisms act biochemically through the digestion processes, a situation that is being reveled by novel high throughput techniques (Mccann et al. 2014).

In this context, and considering the economic importance of cattle breeding as well as the constant search for improvements in production, we purpose ourselves to find microbial patterns in rumen that could be correlated with gain of weight, the key topic in beef cattle breeding. Here, we tested the differences of microbial communities in a set of heifers raised in natural grasslands from the Pampa Biome. Heifers with a high daily gain of weight were compared with the ones with a low daily gain. Our hypothesis was that animals with a higher gain of weight might have microbial patterns that can explain a better digestion process and, consequently, an enhanced gain of weight.

Materials and Methods

Cattle management and experimental design

In this work, we attempted to build a predictive model for microbial biomarkers of enhanced gain of weight using a subset of rumen samples and further test the model using the entire dataset. For this, we selected animals in the same natural grasslands from the Pampa Biome and calculated the average daily weight gain (ADG) for all of them. Based on ADGs, we selected the upper and lower quartile groups of weight gain and compared them for composition of both bacteria and fungi, targeting microorganisms that could be increased in one of the groups.

The animals were raised by the Departamento de Diagnóstico e Pesquisa Agropecuária (DDPA, SEAPI-RS), located in São Gabriel, RS, Brazil. All animal management and research procedures were approved by the Animal Welfare Committee of the State Foundation of Agricultural Research (FEPAGRO) registered under the number 15/14. We sampled seventeen Braford heifers (36 months, 343±30 kg) randomly distributed in paddocks with natural grasslands from the Pampa Biome for more than two years. These animals were submitted to a continuous grazing stock method, with a forage allowance of 12 kg of dry matter/100 kg of live

weight adjusted by a put and take animals method. The pasture structure during the sampling period is described in Table 1. The native pastures include mainly the species *Eryngium horridum, Vernonia nudiflora, Erianthus angustifolius, Eupatorium bunifolium, Paspalum notatum, Eragrostis plana, Axonopus affinis, Paspalum umbrosum, Desmodium incanum* and *Paspalum plicatulum.*

We monitored the weight gain for 60 days, with the sampling occurring in the middle of this period (30 days) in the autumn, aiming to minimize effects of extreme climate conditions as well as microbial seasonal variations (Noel et al. 2017). Based on this monitoring, we estimate the average daily weight gain (ADG) for each animal and proceed to downstream analysis.

Rumen sampling strategy and diet quality measurement

We collected ruminal fluid from 17 heifers shortly after the morning grazing cycle (approximately between 9:00 and 12:00 h). All animals were kept in a closed corral without food assessment during this procedure. Samples of the ruminal fluid (100 ml) were collected through an esophageal probe equipped with a vacuum system. For each animal, a sterilized set of hoses and kitassato flasks were used in order to avoid cross-contamination. Ruminal liquid samples were immediately stored in 50 mL tubes properly identified and stored in ice-cold box for transportation to the laboratory. Approximately two hours after the collection, the tubes were frozen at -80°C. Before sampling the ruminal fluid, a fecal sample was collected via rectum using sterile gloves. This fraction was designated for analysis of diet quality as described below.

Analysis of relationship between fecal protein concentration and diet digestibility provided dietary quality estimation by methods previously described by Lancaster (Lancaster 1949) and Rosa and colleagues (Rosa et al., n.d.). Briefly, fecal samples were oven dried with forced air circulation at 55ºC for 72 hours. After partially dried and grounded, feces were subjected to dry matter determination after drying at 105°C for 12 hours (Easley et al., 1965). The organic matter was determined by flaring at 550°C and crude protein (CP) was determined by the Kjeldahl method (Kirk, 1950). The concentration of fecal CP in the organic matter (CPf:g/kg.OM) was used in the equations (1), (2) and (3) described below to estimate organic matter digestibility (OMD), protein concentration in the diet (PC) and daily protein consumption (CP Intake). The equations were constructed specifically for native grasslands of Pampa Biome by using the Rosa's et al. methods (Rosa et al., n.d.):

$$OMD=0.942-38{,}619/fecalCP \quad (1)$$

$$PC=1.346*(fecalCP)-47.63\ (2) \quad (2)$$

$$CPintake=8.0728*(fecalCP)-347.38 \quad (3)$$

DNA extraction and PCR amplification

For DNA extraction, we used 1 ml of homogenized ruminal fluid samples from each animal. DNA extraction was performed with the PowerSoil®DNA kit (MoBio, USA) according to the manufacturer's instructions. Concentrations and purity of the DNA were determined using the NanoVue™ spectrophotometer (GE Healthcare, USA). All DNA samples were stored at -20°C until use for the PCR reactions.

The amplification, sequencing and processing of the data were performed according to the recommendations of the Brazilian Microbiome Project - available at http://www.brmicrobiome.org - (Pylro et al. 2014b). For each sample (n=17), the v4 region from the 16S gene was amplified by using the 515F/806R primers (Caporaso et al. 2011) for amplification of microorganisms of the Bacteria and Archaea domain simultaneously. The primer was synthesized together with the A-Key (5'CCATCTCATCCCTGCGTGTCTCCGACTCAG'3) and P1-Key (5'CCTCTCTATGGGCAGTCGGTGAT'3) adapters to obtain a sequence composed for A-barcode-806R and P1-515F adapter and primers. A similar approach was used to the internal transcribed spacer region (ITS1) which was amplified by means of the ITS1F/ITS2R primers (White et al. 1990; Gardes and Bruns 1993; Walters et al. 2016) for the amplification of microorganisms from the Fungi kingdom in each sample (n=12). Twelve known bases (barcodes) were added to the 5' region of the primers, which were used to identify the origin of each sequence, according to the methodology proposed by Hamady and collaborators (Hamady et al. 2008) either for bacteria and fungi.

PCR reactions were performed for each DNA isolated from the samples with a different barcode per reaction. For bacteria, each of the 25 mL of PCR mixture consisted of 2U of Platinum Taq DNA High Fidelity Polymerase (Invitrogen, Carlsbad, CA, United States), 4 uL 10X High Fidelity PCR Buffer, 2 mM MgSO4, 0.2 mM dNTPs, 0.1 mM of both the 806R barcoded primer and the 515F primer, 25 ug of Ultrapure BSA (Invitrogen, Carlsbad, CA, United States) and approximately 50 ng of DNA template. The conditions used in these reactions were: 94° C for 2 minutes, 30 cycles at 94° C for 45s for denaturation, 55 °C for 45s for annealing, and 72° C for 1 minute for extension; followed by 72 °C for 6 minutes of final

extension. For fungi, each of the 50 µl of PCR mixture consisted of 2U of Platinum Taq DNA High Fidelity Polymerase (Invitrogen, Carlsbad, CA, United States), 5 uL 10X High Fidelity PCR Buffer, 2 mM MgSO4, 0.2 mM dNTPs, 0.1 mM of both the ITS2R barcoded primer and the ITS1F primer, 25 ug of Ultrapure BSA (Invitrogen, Carlsbad, CA, United States) and approximately 100 ng of DNA template. The conditions used in the reactions for fungi were: 94° C for 2 minutes, 35 cycles at 94° C for 45s for denaturation, 55° C for 60s for annealing the primer oligonucleotides, and 72° C for 1.5 minute for extension; followed by 72° C for 10 minutes of final extension.

The resulting PCR products were purified with the Agencourt® AMPure® XP Reagent kit (Beckman Coulter, USA). The final concentration of the PCR product DNA was quantified using the Qubit Fluorometer kit (Invitrogen) following the manufacturer's recommendations. Finally, the PCR products combined into equimolar concentrations were used to library preparation win Ion One-Touch 2 System using Ion PGM Template OT2 400 Kit (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing was performed in Ion PGM Sequencing 400 on the Ion PGM System using 318 Chip v2. The Raw sequences were deposited in the Sequence Read Archive (SRA), BioProject accession PRJNA599105.

Statistical analysis

After building the Operational Taxonomic Unity (OTU) table following the Brazilian Microbiome pipeline (Pylro et al. 2014a), the OTU tables were imported into the R environment and transformed by centered log-ratio to reflect the compositional nature of these type of data (Gloor and Reid 2016). All analysis were performed independently for bacteria and fungi, but keeping the same parameters. Possible confounding variables were tested by a permutational analysis of variance (PERMANOVA) based on Euclidean distance with the vegan package (Dixon 2003). Initial insights were provided by analysis of microbial richness. After, we tested the main differences between the communities from higher ADG and lower ADG groups by differential abundance of both phyla and OTUs using the ALDEx2 package (Gloor). We considered as differentially abundant all phyla or OTUs with effect size higher than 1 and a p-value calculated by the Welch's test lower than 0.1 (Gloor et al. 2016). Finally, we tested all OTUs matched as differentially abundant between groups as models of regression including all 17 initial samples aiming to validate our findings in the whole dataset.

Results

Subjects Characteristics

Ten subjects were selected from the initial set of 17, by using the descriptive statistics summarized in Figure 1. These samples comprised the extreme quartiles of daily gain of weight in the dataset. We included samples with marginal values (matching with extreme quartile's borderlines, Figure 1) once this information kept the groups with widely different gains of weight and increased the power for downstream analyses (Table 2). Our goal was to build a model with maximum contrast between ADG groups keeping similar diet parameters among the tested animals. Indeed, both animal groups presented very similar rates of organic matter digestion, crude protein concentration in feces, estimated crude protein intake and live weight at the beginning of the experiment, representing a very homogeneous sample set (Table 2, Supplementary Figure S1). On the other hand, the average dairy gain of weight was significantly different between groups, overcoming, in mean, differences of 400 grams of weight gain per day (Table 2).

Sequencing Report and Control for Confounding Variables

A total of 289,137 high quality sequences were obtained from all 17 samples, with a mean of 12,072 bacterial sequences and 6,993 fungal sequences per sample. In average, the Good's coverage of the bacterial samples was higher than 95% and the coverage of the fungal samples was higher than 99% in all samples, what means our sequencing depth was sufficient for the investigation of the ruminal microbiota in each sample properly (Good 1953; Lemos et al. 2011).

To avoid possible false positives/negatives in downstream analysis, the presence of confounding variables (Pourhoseingholi et al. 2012) were tested by stratification. For doing this, a PERMANOVA analysis using all measured variables was performed and the results are shown in Table 3. No significant bias related to any possible confounding variable tested such as *Eragorstis plana* cover, percentage of crude protein in feces and the paddock effect was detected ($p > 0.05$ in all analyses, Table 3). In addition, analysis indicated that the bacterial communities in different ADG classes were strongly different ($R^2 = 0.14$; $p = 0.03$). On the other hand, no differences within the fungal community was observed between ADG classes.

Overall description of bacterial and fungal communities

We found 24 microbial phyla within all samples: 19 from Bacteria, 1 from Archaea (Supplementary Figure S2) and 4 from Fungi (Supplementary Figure S3). Bacterial communities were dominated by species from *Bacteroidetes* and *Firmicutes*, the only ones which presented values of centered log-ratio transformed abundance higher than 4.

Euryarchaeota was the only archaeal phylum found in the analysis and presented marginal abundance (< 0.2%) in all samples. In fungal samples, we saw a huge domain of *Ascomycota* OTUs, followed by *Basidiomycota*. Differential abundance analyses showed that phyla distribution between the ADG groups was very similar either in bacteria/archaea or in fungi (effect size < 1 and p-value > 0.1 for all tested phyla).

Both bacterial and fungal communities also presented similar number of richness Operational Taxonomic Unities (OTUs) of when comparing extreme ADG classes (Figure 2). Although no differences were detected within this alpha diversity measurement, PERMANOVA outputs (Table 3) showed significant differences at least in bacterial composition between the two ADG classes, what led us to believe that may be some differences in specific taxonomic levels. We proceed to downstream analyses targeting to find these differences.

Differential abundance analysis revealed microbial patterns associated with enhanced gain of weight

Differential abundance analysis at the OTU level (using the highest possible taxonomy rank) reveled many microorganisms differentially abundant between extreme ADG classes (Table 4). Three OTUs belonging to the genus *RFN20* presented higher abundance in the higher ADG group, as well as OTUs belonging to the genus *Anaeroplasma* and the family *RF16*. Two OTUs assigned as *Prevotella* genus also presented higher abundance in higher ADG group, but another OTU also assigned as *Prevotella* presented opposite trend, with higher abundance in the lower ADG group. Among fungal OTUs, the most important taxa with differential abundance were the genera *Aureobasidium*, *Cryptococcus* and *Sarocladium*, all of them increased in the higher ADG group. In addition, OTUs closest related to the orders *Tremellales* and *Pleosporales* were also more abundant in the higher ADG group. The only OTU significantly more abundant in lower ADG group was assigned to the genus *Ceratobasidium*.

Testing the biomarkers with the entire dataset

Our approach defined a group of microbial taxa differentially abundant in the Higher and Lower ADG groups. To confirm these findings, we correlated the average daily gain of weight with the relative abundance (normalized by centered log ratios) of each taxa from the Table 4 using all the initial 17 samples. Our goal was to test whether was a real trend in microbial abundance of these taxa correlated with gain of weight in whole dataset. The results

indicate that many OTUs found here were associated with gain of weight in beef cattle (Figures 3 and 4).

OTUs closest related to *RFN20* (1), *Prevotella* (1) and *Anaeroplasma* presented the highest and most confident positive correlations ($R > 0.6$ and $p < 0.01$) with average daily gain of weight. These results indicate the abundance of each one of these OTUs can individually explain more than 60% of the ADG from tested animals with a confidence higher than 99%.

Correlations between differential abundant fungal taxa and average dairy gain of weight were also confirmed using the entire dataset (Figure 4). *Ceratobasidium* provided the main (and only) negative correlation between fungal taxa and ADG ($R = -0.65$), with high confidence ($p=0.023$). On the other hand, the non-identified OTU closest related to the order *Tremellales*, provided the highest positive correlation with ADG ($R = 0.67$ and $p = 0.018$). The genus *Cryptococcus* was also positively correlated with ADG ($R = 0.58$ and $p = 0.049$). All other fungi previously identified as increased by the ALDEx analysis presented weaker correlations.

Discussion

This study identified differences in microbial communities from rumen of heifers with increased weight gain. To our knowledge, it's the first work that attempted to measure these differences in heifers in natural grasslands from the Pampa Biome, a biome historically linked to cattle breeding (Rouse 1977; Roesch et al. 2009). Here, we presented multiple evidences supporting previous works about general structure of rumen microbiota and added knowledge about the association of microbes with weight gain, a key factor for beef production.

Once we established groups of animals with highly contrasting weight gain (Figure 1), kept for more than two years in the same conditions (Table 1), we showed the overall community of heifers was similar in diversity of phyla and in richness of microbial taxa (Figure 2), irrespective of the weight gain. Indeed, several studies postulated the rumen microbiome presents a stable community (Tapio et al. 2016; Indugu et al. 2017; Malmuthuge and Guan 2017; Wirth et al. 2018; Sun et al. 2019; Stewart et al. 2019). These reports indicated a high dominance of *Bacteroidetes* species inhabiting the ruminal fluid within various environments and conditions, followed by a smaller, but consistent, presence of *Firmicutes*. Our results are aligned with those findings, as well as with findings about fungal samples which were not correlated to any dominant phyla in rumen neither in the literature nor in our work (Akin and Borneman 1990; Malmuthuge and Guan 2017; Wirth et al. 2018; Stewart et al. 2019).

Differences between ADG classes were tested at the OTU level. Results from PERMANOVA analyses (Table 3), indicated reliable differences between higher and lower

ADG groups. In fact, analyzing samples at the OTU level, we showed a strong increment of various taxa associated to digestion processes in animals with higher ADG (Table 4). The unclassified *RFN20*, for example is a rumen-specific bacterium member of the subclass *Erysipelotrichia*, which was previously correlated to mice under high fat diets (Turnbaugh et al. 2009; Greiner and Bäckhed 2011). Additionally, *Anaeroplasma* is a genus characterized by its anaerobic fermentation which products fatty acids as propionate (Brown et al. 2015) whereas *Prevotella* produces majorly succinic and acetic acids (Shah et al. 2015). All of them are short chain fatty acids important to the glucose biosynthesis in cattle, being considering a primary energy source for these animals (Linn et al. 2018; Wirth et al. 2018). Lastly, the family *BF16* is also a rumen-native taxon with higher abundance in mature calves and also correlated to methane production, a clear signal of higher digestion rate (Smith et al. 2018; Cunha et al. 2019). All of these taxa were previously reported as members of the regular and efficient microbiota from rumen and their increased abundance may indicate an improved ability to digestion or, at least, a need for more specialized fermentation in rumen due to, for example, more food intake.

Nonetheless, the case of *Prevotella* OTUs deserves additional attention. Despite two OTUs assigned as *Prevotella* presented higher abundance in higher ADG samples, another OTU also related to the genus *Prevotella* was increased in the lower ADG group (Table 4, Figure 4). *Prevotella* is a very complex genus and its species can adapt to the most different environments (Gupta et al. 2015). In addition, this dubious role of ruminal *Prevotella* species in states of good or bad production were previously reported in milk production. Chiquette and colleagues, for example, showed that a strain of *Prevotella bryantii* acts as probiotic increasing the fat content in milk (Chiquette et al. 2008) whereas Jami and collaborators found an OTUs assigned as *Prevotella* negatively correlated with milk-fat yield (Jami et al. 2014). Our approach is not suitable to identify species precisely so, for this reason, we believe that we found different *Prevotella* species that may be related to higher and lower weight gain.

Within the fungal communities, the three genera assigned as increased in higher AGD heifers also present characteristics that may increase the animal's digestibility (Table 4). *Aureobasidium* is a genus of yeast-like fungi with a great genomic plasticity that allows it to adapt to the most different environments (Slepecky and Starmer 2009; Gostinčar et al. 2011). This genus produces a high number of Carbohydrate Active Enzymes (CAZy), which synthesize or break down saccharides and are very important for the ruminal digestion of organic matter (Gostinčar et al. 2014). *Cryptococcus* is a genus of soil-resident fungi correlated to degradation of wood and associated with bird excreta (Kobayashi et al. 2005; Refai et al.

2017). This curious association may be explained by the production of phenol oxidases, enzymes that increase the absorption of carbon and nitrogen besides acting in lignin degradation, carbon mineralization and dissolved organic carbon exportation (Sinsabaugh 2010). *Sarocladium*, is a known phytopathogen specialized in secretion of enzymes that degrade the cell wall and other hard plant cellular components (Giraldo et al. 2015; Guimarães et al. 2017; Hou et al. 2019). Additionally, *Sarocladium* is an endophytic of *Poaceae* species, the main family in the native Pampa grasslands (Liu et al. 2017).

Alongside the identified genera, we identified two OTUs also correlated to the higher weight gain only classified at the order level (Table 4). *Tremelalles* and *Pleosporales* represents a wide range of fungi from yeasts to filamentous fungi. Most of them are cosmopolitan saprophytes, but others are described living in vascular plants (Shearer et al. 2009; Suetrong et al. 2009; Zhang et al. 2009; Findley et al. 2009). Because of this, we are not able to suggest biochemical mechanisms that explain the relationship among these families and a higher weight gain. Nonetheless, the aforementioned genus *Cryptococcus* is included in the order *Tremellales* (Findley et al. 2009), which may support the presence of it and/or other close related taxa to an increased weight gain.

On the other hand, we found an OTU closest related to the genus *Ceratobasidium* as increased in lower ADG group. This genus comprises a set of cosmopolitan fungi, most of them saprophytes in the soil (McMahon and Purwantara 2016). Some strains of this genus, however, have been successfully tested as a tool for biocontrol of the plant pathogen *Rhizoctonia solani* (Mosquera-Espinosa et al. 2013). These findings may suggest an action of *Ceratobasidium* as a strong competitive microbe in the rumen environment of these animals and may lead to a low abundance of other microorganisms with phytopathogenic activities that may improve the digestion process, what is supported by its direct correlation with lower gain of weight (Figure 4).

The relationships between the rumen microbiota in beef cattle has mainly focused on feedlot production systems, but the information on grazing cattle is very limited due to the complexity of the production system. As our study was conducted with cattle raised in natural grasslands, we were not able to measure precisely the animal food intake. The lack of such measurement might represent a limitation of our study. However, our goal was to find differences in microbial composition without a controlled breeding. The methodologies accepted for measuring food intake based on dose markers change the grazing patterns and stress the animals interfering with the weight gain and masking the microbial relationships.

Besides, the protocols for measuring the weight gain present high variability making it necessary to follow the animals for long and continuous time.

In addition, although the low number of samples limits our statistical power, our data were suitable to point a tendency to a core of microbes correlated to an increased weight gain in cattle. Either in bacteria or in fungi we found taxa associated directly or indirectly with higher fermentation and digestion rates increased in higher ADG group. Most of them are known to be specialists in producing substances important for plant organic matter degradation, what increases the digestion (and maybe the food intake) and consequently, the absorption of nutrients and gain of weight either. In practice, we provide a list of microbes here discussed that could be hereafter tested to be included in cattle nutrition as enhancers of gain of weight.

The use of microorganisms in cattle nutrition is not novel. However, the currently available products are focused in a narrow range of species, mainly yeasts from the genera *Saccharomyces* and *Kluyveromyces* (Shurson 2018). Our results have found no significant correlation of these genera with weight gain. On the other hand, we found a yeast that is naturally present in rumen environment correlated with a higher weight gain: *Aureobasidium*. Its aforementioned production of CAZymes may be linked to a higher efficiency in degradation of plant carbohydrates and supports future studies to evaluate possible benefits that this genera can induce in beef cattle breeding. The same perspective is expected for some strains of the bacterial genus *Prevotella*. Some *Prevotella* species have shown good results in production chain with some lineages (Chiquette et al. 2008). Besides, *Anaeroplasma* and *RFN20*, both of them highly correlated with high weight gain (Figures 3 and 4), may be important for cattle nutrition either individually or in a consortium. The study of *Ceratobasidium*, on the other hand, as a suppressor of weight gain may also be necessary, once this genus was pointed as strongly correlated with lower weight gain (Figure 4). All these taxa need to be tested by further works, but the target to find differences and suggest ADG-linked microbes has been achieved.

5. Conclusions

Here, we showed microbial patterns associated with increased weight gain in beef cattle from natural grasslands of the Brazilian Pampa. The results suggest a relationship of specific microorganisms with improved ability to produce specific compounds related to a better digestion process and animals with a higher gain of weight. Those microorganism are not currently available in commercial products focused in improving cattle nutrition and might be focus of studies for the development of new and more efficient probiotic products. This report

may also be important for future research for biomarkers of productivity and for microbial therapies to improve productivity in beef cattle causing less environmental damages.

Author Contributions: conceptualization, A.S.F, D.B.D, and L.F.W.R; methodology, A.S.F., D.B.D. and B.M.T.; validation, D.B.D; formal analysis, A.S.F and L.F.W.R; investigation, A.S.F., D.B.D. and B.M.T.; resources, D.B.D. and L.F.W.R; data curation, A.S.F and L.F.W.R.; writing—original draft preparation, A.S.F and D.B.D.; writing—review and editing, A.S.F, D.B.D and L.F.W.R; visualization, B.M.T; supervision, L.F.W.R; project administration, D.B.D and L.F.W.R.; funding acquisition, D.B.D and L.F.W.R".

Ethical Statement: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. All animal management and research procedures were approved by the Animal Welfare Committee of the State Foundation of Agricultural Research (FEPAGRO) registered under the number 15/14.

References

Akin DE, Borneman WS (1990) Role of Rumen Fungi in Fiber Degradation. Journal of Dairy Science 73:3023–3032. https://doi.org/10.3168/jds.S0022-0302(90)78989-8

Brown DR, Bradbury JM, Johansson K-E (2015) *Anaeroplasma*. In: Whitman WB, Rainey F, Kämpfer P, et al. (eds) Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons, Ltd, Chichester, UK, pp 1–5

Caporaso JG, Lauber CL, Walters WA, et al (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences 108:4516–4522. https://doi.org/10.1073/pnas.1000080107

Carberry CA, Kenny DA, Han S, et al (2012) Effect of Phenotypic Residual Feed Intake and Dietary Forage Content on the Rumen Microbial Community of Beef Cattle. Applied and Environmental Microbiology 78:4949–4958. https://doi.org/10.1128/AEM.07759-11

Chiquette J, Allison MJ, Rasmussen MA (2008) *Prevotella bryantii* 25A Used as a Probiotic in Early-Lactation Dairy Cows: Effect on Ruminal Fermentation Characteristics, Milk Production, and Milk Composition. Journal of Dairy Science 91:3536–3543. https://doi.org/10.3168/jds.2007-0849

Cunha CS, Marcondes MI, Veloso CM, et al (2019) Compositional and structural dynamics of the ruminal microbiota in dairy heifers and its relationship to methane production. Journal of the Science of Food and Agriculture 99:210–218. https://doi.org/10.1002/jsfa.9162

Dixon P (2003) VEGAN, a package of R functions for community ecology. Journal of Vegetation Science 14:927–930

Findley K, Rodriguez-Carres M, Metin B, et al (2009) Phylogeny and Phenotypic Characterization of Pathogenic *Cryptococcus* Species and Closely Related Saprobic Taxa in the Tremellales. Eukaryotic Cell 8:353–361. https://doi.org/10.1128/EC.00373-08

Fliegerova K, Kaerger K, Kirk P, Voigt K (2015) Rumen Fungi. In: Puniya AK, Singh R, Kamra DN (eds) Rumen Microbiology: From Evolution to Revolution. Springer India, New Delhi, pp 97–112

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2:113–118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Giraldo A, Gené J, Sutton DA, et al (2015) Phylogeny of *Sarocladium* (*Hypocreales*). Persoonia - Molecular Phylogeny and Evolution of Fungi 34:10–24. https://doi.org/10.3767/003158515X685364

Gloor G ANOVA-Like Differential Expression tool for high throughput sequencing data. 19

Gloor GB, Macklaim JM, Fernandes AD (2016) Displaying Variation in Large Datasets: Plotting a Visual Summary of Effect Sizes. Journal of Computational and Graphical Statistics 25:971–979. https://doi.org/10.1080/10618600.2015.1131161

Gloor GB, Reid G (2016) Compositional analysis: a valid approach to analyze microbiome high-throughput sequencing data. Canadian Journal of Microbiology 62:692–703. https://doi.org/10.1139/cjm-2015-0821

Good IJ (1953) The Population Frequencies of Species and the Estimation of Population Parameters. Biometrika 40:237. https://doi.org/10.2307/2333344

Gostinčar C, Grube M, Gunde-Cimerman N (2011) Evolution of Fungal Pathogens in Domestic Environments? Fungal Biology 115:1008–1018. https://doi.org/10.1016/j.funbio.2011.03.004

Gostinčar C, Ohm RA, Kogej T, et al (2014) Genome sequencing of four *Aureobasidium pullulans* varieties: biotechnological potential, stress tolerance, and description of new species. BMC Genomics 15:549. https://doi.org/10.1186/1471-2164-15-549

Greiner T, Bäckhed F (2011) Effects of the gut microbiota on obesity and glucose homeostasis. Trends in Endocrinology & Metabolism 22:117–123. https://doi.org/10.1016/j.tem.2011.01.002

Guimarães RA, Lobo VL da S, Côrtes MVCB, et al (2017) Characterization of Sarocladium oryzae and its reduction potential of rice leaf blast. Pesquisa Agropecuária Tropical 47:41–52. https://doi.org/10.1590/1983-40632016v4742738

Gupta VK, Chaudhari NM, Iskepalli S, Dutta C (2015) Divergences in gene repertoire among the reference Prevotella genomes derived from distinct body sites of human. BMC Genomics 16:153–153. https://doi.org/10.1186/s12864-015-1350-6

Hamady M, Walker JJ, Harris JK, et al (2008) Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nature Methods 5:235–237. https://doi.org/10.1038/nmeth.1184

Henderson G, Ganesh S, Jonker A, et al (2015) Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Scientific Reports 5:. https://doi.org/10.1038/srep14567

Hou YM, Zhang X, Zhang NN, et al (2019) Genera *Acremonium* and *Sarocladium* Cause Brown Spot on Bagged Apple Fruit in China. Plant Disease 103:1889–1901. https://doi.org/10.1094/PDIS-10-18-1794-RE

Indugu N, Vecchiarelli B, Baker LD, et al (2017) Comparison of rumen bacterial communities in dairy herds of different production. BMC Microbiology 17:. https://doi.org/10.1186/s12866-017-1098-z

Ishaq SL, AlZahal O, Walker N, McBride B (2017) An Investigation into Rumen Fungal and Protozoal Diversity in Three Rumen Fractions, during High-Fiber or Grain-Induced Sub-Acute Ruminal Acidosis Conditions, with or without Active Dry Yeast Supplementation. Frontiers in Microbiology 8:. https://doi.org/10.3389/fmicb.2017.01943

Jami E, Mizrahi I (2012) Composition and Similarity of Bovine Rumen Microbiota across Individual Animals. PLoS ONE 7:e33306. https://doi.org/10.1371/journal.pone.0033306

Jami E, White BA, Mizrahi I (2014) Potential Role of the Bovine Rumen Microbiome in Modulating Milk Composition and Feed Efficiency. PLoS ONE 9:e85423. https://doi.org/10.1371/journal.pone.0085423

Jesus RB de, Omori WP, Lemos EGDM, Souza JAM de (2015) Bacterial diversity in bovine rumen by metagenomic 16S rDNA sequencing and scanning electron microscopy. Acta Scientiarum Animal Sciences 37:251. https://doi.org/10.4025/actascianimsci.v37i3.26535

Kobayashi CCBA, Souza LKH e, Fernandes O de FL, et al (2005) Characterization of *Cryptococcus neoformans* isolated from urban environmental sources in Goiânia, Goiás State, Brazil. Revista do Instituto de Medicina Tropical de São Paulo 47:203–207. https://doi.org/10.1590/S0036-46652005000400005

Lancaster RJ (1949) Estimation of Digestibility of Grazed Pasture from Fæces Nitrogen. Nature 163:330–331. https://doi.org/10.1038/163330b0

Lemos LN, Fulthorpe RR, Triplett EW, Roesch LFW (2011) Rethinking microbial diversity analysis in the high throughput sequencing era. Journal of Microbiological Methods 86:42–51. https://doi.org/10.1016/j.mimet.2011.03.014

Linn J, Hutjens M, Shaver R, et al (2018) The ruminant digestive system. In: University of Minnesota Extension. https://extension.umn.edu/dairy-nutrition/ruminant-digestive-system#large-intestine-1000463. Accessed 23 May 2019

Liu XB, Guo ZK, Huang GX (2017) Sarocladium brachiariae sp. nov., an endophytic fungus isolated from Brachiaria brizantha. Mycosphere 8:827–834. https://doi.org/10.5943/mycosphere/8/7/2

Malmuthuge N, Griebel PJ, Guan LL (2014) Taxonomic Identification of Commensal Bacteria Associated with the Mucosa and Digesta throughout the Gastrointestinal Tracts of Preweaned Calves. Applied and Environmental Microbiology 80:2021–2028. https://doi.org/10.1128/AEM.03864-13

Malmuthuge N, Guan LL (2017) Understanding host-microbial interactions in rumen: searching the best opportunity for microbiota manipulation. Journal of Animal Science and Biotechnology 8:. https://doi.org/10.1186/s40104-016-0135-3

Mccann JC, Wickersham TA, Loor JJ (2014) High-throughput Methods Redefine the Rumen Microbiome and Its Relationship with Nutrition and Metabolism. Bioinformatics and Biology Insights 8:BBI.S15389. https://doi.org/10.4137/BBI.S15389

McMahon P, Purwantara A (2016) Vascular Streak Dieback (*Ceratobasidium theobromae*): History and Biology. In: Bailey BA, Meinhardt LW (eds) Cacao Diseases: A History of Old Enemies and New Encounters. Springer International Publishing, Cham, pp 307–335

Mosquera-Espinosa AT, Bayman P, Prado GA, et al (2013) The double life of *Ceratobasidium* : orchid mycorrhizal fungi and their potential for biocontrol of *Rhizoctonia* solani sheath blight of rice. Mycologia 105:141–150. https://doi.org/10.3852/12-079

Myer PR, Kim M, Freetly HC, Smith TPL (2016) Evaluation of 16S rRNA amplicon sequencing using two next-generation sequencing technologies for phylogenetic analysis of the

rumen bacterial community in steers. Journal of Microbiological Methods 127:132–140. https://doi.org/10.1016/j.mimet.2016.06.004

Nathani NM, Kothari RK, Patel AK, Joshi CG (2015) Functional Characterization Reveals Novel Putative Coding Sequences in ***Prevotella ruminicola*** Genome Extracted from Rumen Metagenomic Studies. Journal of Molecular Microbiology and Biotechnology 25:292–299. https://doi.org/10.1159/000437265

Noel SJ, Attwood GT, Rakonjac J, et al (2017) Seasonal changes in the digesta-adherent rumen bacterial communities of dairy cattle grazing pasture. PLOS ONE 12:e0173819. https://doi.org/10.1371/journal.pone.0173819

Parish J, Rivera D (2017) Understanding the Ruminant Animal Digestive System. Mississippi State University 8

Pourhoseingholi MA, Baghestani AR, Vahedi M (2012) How to control confounding effects by statistical analysis. Gastroenterology and Hepatology From Bed to Bench 5:79–83

Pylro VS, Roesch LFW, Morais DK, et al (2014a) Data analysis for 16S microbial profiling from different benchtop sequencing platforms. Journal of Microbiological Methods 107:30–37. https://doi.org/10.1016/j.mimet.2014.08.018

Pylro VS, Roesch LFW, Ortega JM, et al (2014b) Brazilian Microbiome Project: Revealing the Unexplored Microbial Diversity—Challenges and Prospects. Microbial Ecology 67:237–241. https://doi.org/10.1007/s00248-013-0302-4

Refai MK, El-Hariri M, Alarousy R (2017) *Cryptococcosis* in Animals and Birds: A Review. 22

Roesch LF, Vieira F, Pereira V, et al (2009) The Brazilian Pampa: A Fragile Biome. Diversity 1:182–198. https://doi.org/10.3390/d1020182

Rosa FQ, Pagel R, David DB, et al Measurement of nutritional parameters by fecal markers in cattle fed heterogeneous natural grasslands. In Press

Rouse JE (1977) The criollo: Spanish cattle in the Americas. University of Oklahoma Press.

Shah HN, Chattaway MA, Rajakurana L, Gharbia SE (2015) *Prevotella*. In: Bergey's Manual of Systematics of Archaea and Bacteria. American Cancer Society, pp 1–25

Shearer CA, Raja HA, Miller AN, et al (2009) The molecular phylogeny of freshwater Dothideomycetes. Studies in Mycology 64:145–153. https://doi.org/10.3114/sim.2009.64.08

Shurson GC (2018) Yeast and yeast derivatives in feed additives and ingredients: Sources, characteristics, animal responses, and quantification methods. Animal Feed Science and Technology 235:60–76. https://doi.org/10.1016/j.anifeedsci.2017.11.010

Sinsabaugh RL (2010) Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil Biology and Biochemistry 42:391–404. https://doi.org/10.1016/j.soilbio.2009.10.014

Slepecky RA, Starmer WT (2009) Phenotypic plasticity in fungi: a review with observations on *Aureobasidium pullulans*. Mycologia 101:823–832. https://doi.org/10.3852/08-197

Smith KE, Garza AL, Butterfield KM, et al (2018) Succession of ruminal bacterial species and fermentation characteristics in preweaned Brangus calves1. Translational Animal Science 2:S48–S52. https://doi.org/10.1093/tas/txy043

Song J, Jeong JY, Kim M (2017) Diversity Census of Fungi in the Ruminal Microbiome: A meta-analysis. Journal of the Korea Academia-Industrial Cooperation Society 18:466–472. https://doi.org/10.5762/KAIS.2017.18.12.466

Stewart RD, Auffret MD, Warr A, et al (2019) Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. Nature Biotechnology 37:953–961. https://doi.org/10.1038/s41587-019-0202-3

Suetrong S, Schoch CL, Spatafora JW, et al (2009) Molecular systematics of the marine Dothideomycetes. Studies in Mycology 64:155–173. https://doi.org/10.3114/sim.2009.64.09

Sun H-Z, Xue M, Guan LL, Liu J (2019) A collection of rumen bacteriome data from 334 mid-lactation dairy cows. Scientific Data 6:. https://doi.org/10.1038/sdata.2018.301

Tapio I, Shingfield KJ, McKain N, et al (2016) Oral Samples as Non-Invasive Proxies for Assessing the Composition of the Rumen Microbial Community. PLOS ONE 11:e0151220. https://doi.org/10.1371/journal.pone.0151220

Turnbaugh PJ, Ridaura VK, Faith JJ, et al (2009) The Effect of Diet on the Human Gut Microbiome: A Metagenomic Analysis in Humanized Gnotobiotic Mice. Science Translational Medicine 1:6ra14-6ra14. https://doi.org/10.1126/scitranslmed.3000322

Walters W, Hyde ER, Berg-Lyons D, et al (2016) Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. mSystems 1:. https://doi.org/10.1128/mSystems.00009-15

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: PCR - Protocols and Applications - A Laboratory Manual. Academic Press, pp 315–322

Wirth R, Kádár G, Kakuk B, et al (2018) The Planktonic Core Microbiome and Core Functions in the Cattle Rumen by Next Generation Sequencing. Frontiers in Microbiology 9:. https://doi.org/10.3389/fmicb.2018.02285

Zhang Y, Schoch CL, Fournier J, et al (2009) Multi-locus phylogeny of Pleosporales: a taxonomic, ecological and evolutionary re-evaluation. Studies in Mycology 64:85–102. https://doi.org/10.3114/sim.2009.64.04

Table 1. Description of the pasture structure during the sampling period.

Measurements	Mean	Standard Deviation
Herbage accumulation (kg/day)	17.28	1.18
Pasture height (cm)	9.74	1.44
Herbage mass (kg)	2530	240
Forage allowance ([kg of DM]/[100 kg of LW])	11.43	2.98
**Eragrostis plana* cover (%)	14	6.68

*Exotic gramineous plant considered an aggressive invasive plant with low nutritional quality and high resistance to mechanic traction.

Table 2. Description of the diet variables from Higher Average Dairy Gain of Weight group (Higher ADG) versus the Lower Average Dairy Gain of Weight group (Lower ADG).

Parameters	Higher ADG	Lower ADG	p value
Average Daily Gain (g/day)	534.2 ± 42.2	131.4 ± 32.8	*0.01*
Organic Matter Digestion (%)	64.2 ± 1.39	65.3 ± 0.67	0.88
Crude Protein Concentration (g/kg OM)	117.4 ± 8.52	120.4 ± 5.91	1
Crude Protein Intake (g/day)	642.4 ± 51.1	660.3 ± 35.5	1
Initial Live Weight (kg)*	337.5 ± 15.0	337.3 ± 15.6	1

Variables were summarized as average ± SEM and compared using the Mann-Whitney-Wilcoxon test. Significant p-values were highlighted in italic. g = grams; kg = kilograms; OM = organic matter. * Mean of live weight measured in the beginning of the experiment.

Table 3. Detection of possible confounding variables associated with the lower and higher classes of Average Daily Gain of Weight (ADG).

	Variables	**R^2**	**p-value**
Bacteria	ADG Class	0.14	*0.03*
	Eragrostis plana Cover	0.10	0.47
	CP in feces	0.20	0.37
	Paddock	0.35	0.09
Fungi	ADG Class	0.22	0.30
	Eragrostis plana Cover	0.22	0.30
	CP in feces	0.42	0.47
	Paddock	0.57	0.60

R^2 and p-values were obtained by Permutational analysis of variance (PERMANOVA) based on the Euclidean dissimilarities applied to the Centered log-ratio (clr) transformed abundance of 16S rRNA sequences for bacterial/archaeal and fungal communities. Significant value is set in italics. p values are based on 999 permutations. ADG = Average Daily Gain; CP = Crude Protein.

Table 4. List of microbial taxa with significant difference between higher and lower group of Average Daily Gain of Weight (ADG).

	Taxon	rab.win.Lower [a]	rab.win.Higher [b]	effect [c]	overlap [d]	we.ep [e]
Bacteria	*RFN20* (1)	2.47	4.21	1.54	0.04	0.01
	RFN20 (2)	2.68	4.18	1.28	0.10	0.03
	RFN20 (3)	4.62	5.88	1.20	0.12	0.04
	Anaeroplasma	-2.74	1.82	1.54	0.04	0.03
	Prevotella (1)	-1.35	1.99	1.25	0.07	0.04
	Prevotella (2)	1.61	3.01	1.10	0.09	0.05
	Prevotella (3)	2.46	0.76	-1.08	0.10	0.06
	RF16	-0.05	1.88	1.17	0.10	0.07
Fungi	*Aureobasidium*	-1.52	3.59	1.90	0.02	0.05
	Tremellales fam Incertae sedis	-1.14	6.41	1.70	0.03	0.06
	Ceratobasidium	5.04	-3.09	-1.62	0.04	0.07
	Pleosporales	-1.35	4.65	1.71	0.02	0.07
	Cryptococcus	0.31	6.07	1.60	0.00	0.09
	Sarocladium	-1.91	4.99	1.38	0.05	0.09

[a] median centered log-ratio value for the Lower ADG group of samples; [b] median centered log-ratio value for the Higher ADG group of samples; [c] effect size of the difference, median of difference between groups on a log base 2 scale/largest median variation within groups, positive values indicate a higher abundance in the Higher ADG group whereas negative values indicate higher abundance in the lower ADG group; [d] confusion in assigning an observation to Higher

or Lower group; [e] the expected value of the Welch Test P value. Table includes all OTUs with effect > 1 and p-value < 0.1.

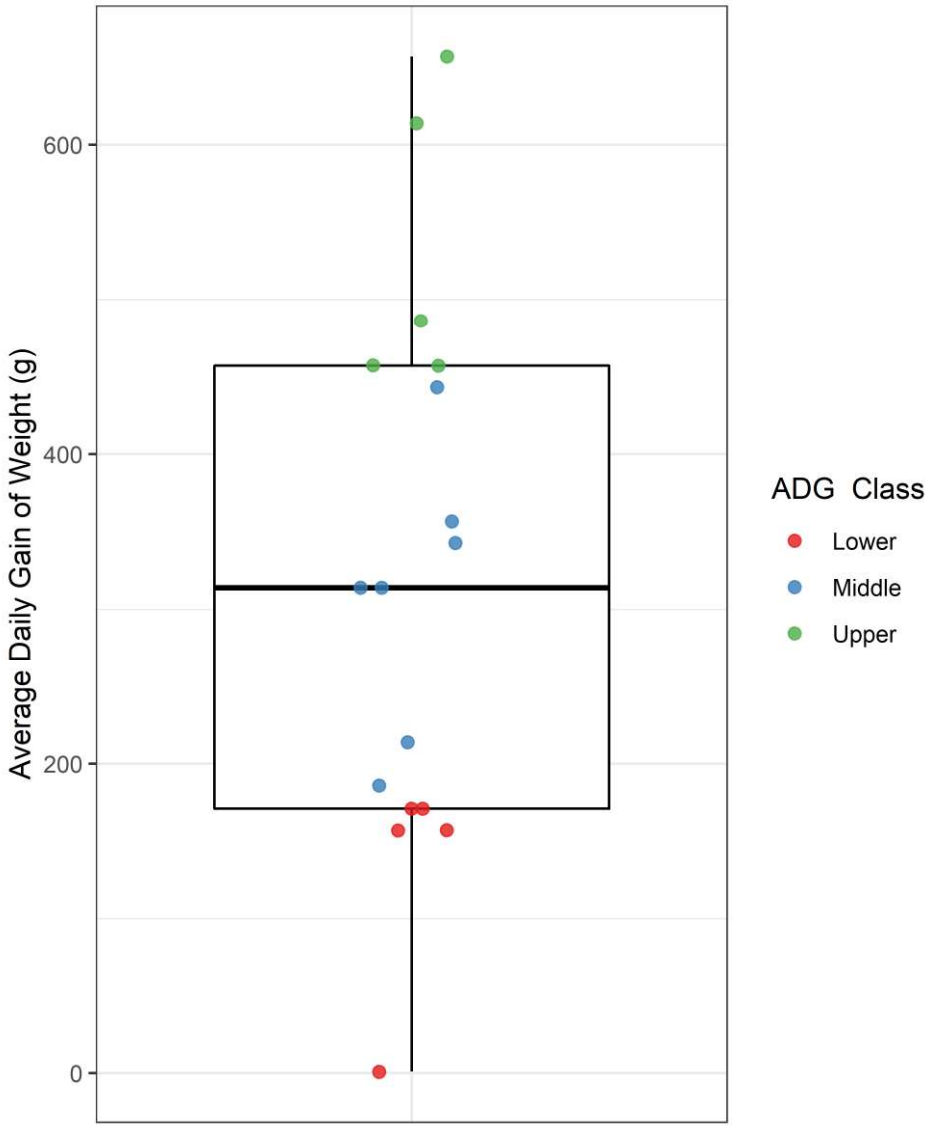


Figure 1. Distribution of average daily gain of weight (ADG) in the bovine samples. The first quartile was assigned to Lower ADG (red) and the fourth quartile to Higher ADG (green). Samples comprised in the second and third quartiles (blue) were classified as Middle ADG and were not included in differential abundance analysis.

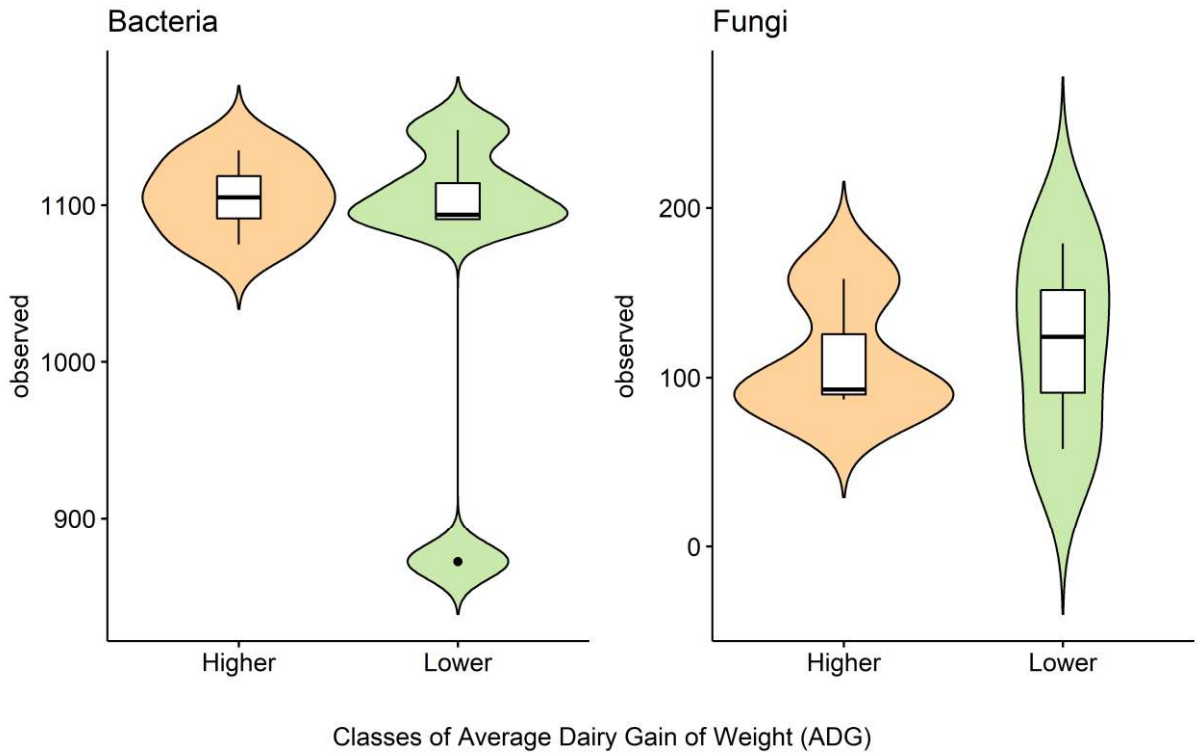


Figure 2. Number of observed OTUs of bacterial (left panel) and fungal (right panel) communities in the Higher ADG and Lower ADG groups. Graphs represent the number of different operational taxonomic units (OTUs) found in each group. Boxes inside the violins span the first to third quartiles; the horizontal line inside the boxes represents the median. Whiskers extending vertically from the boxes indicate variability outside the upper and lower quartiles, and the single black circles indicate outliers.

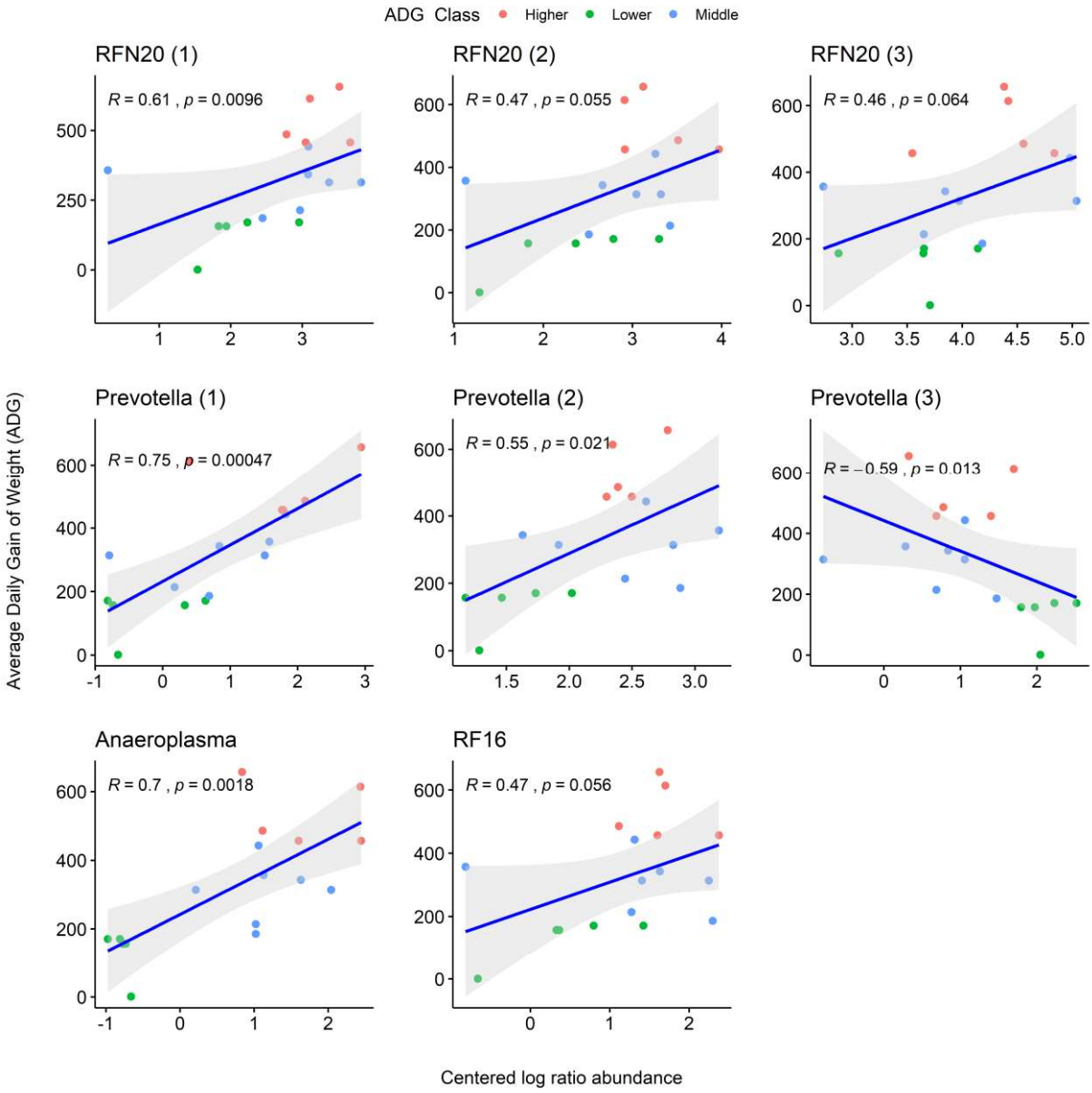


Figure 3. Ratios of OTUs closest related to the bacterial taxa and their correlation with daily gain of weight in beef cattle's rumen.

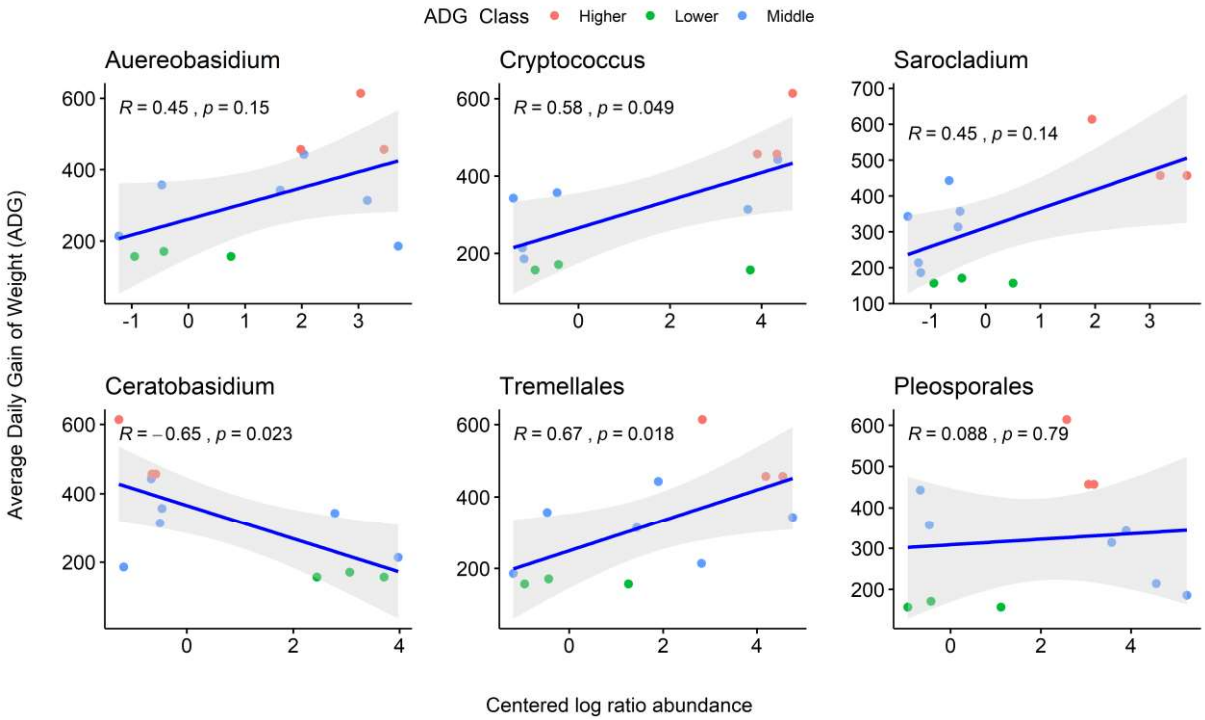
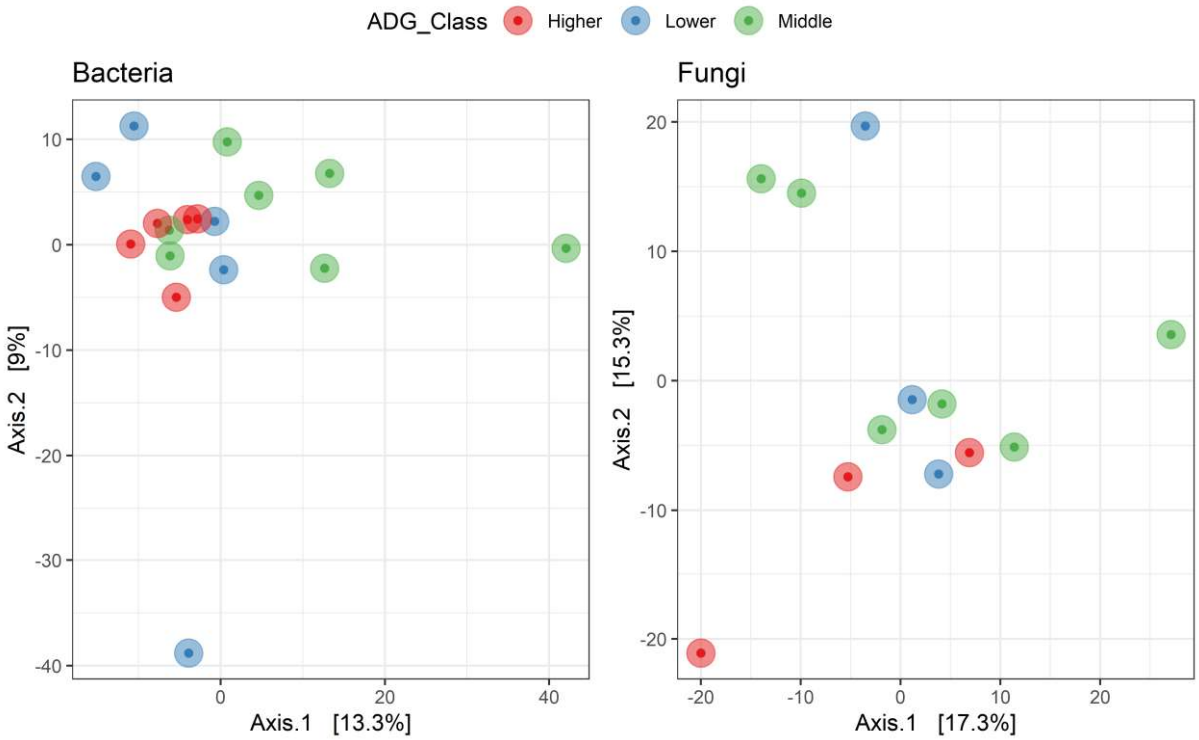
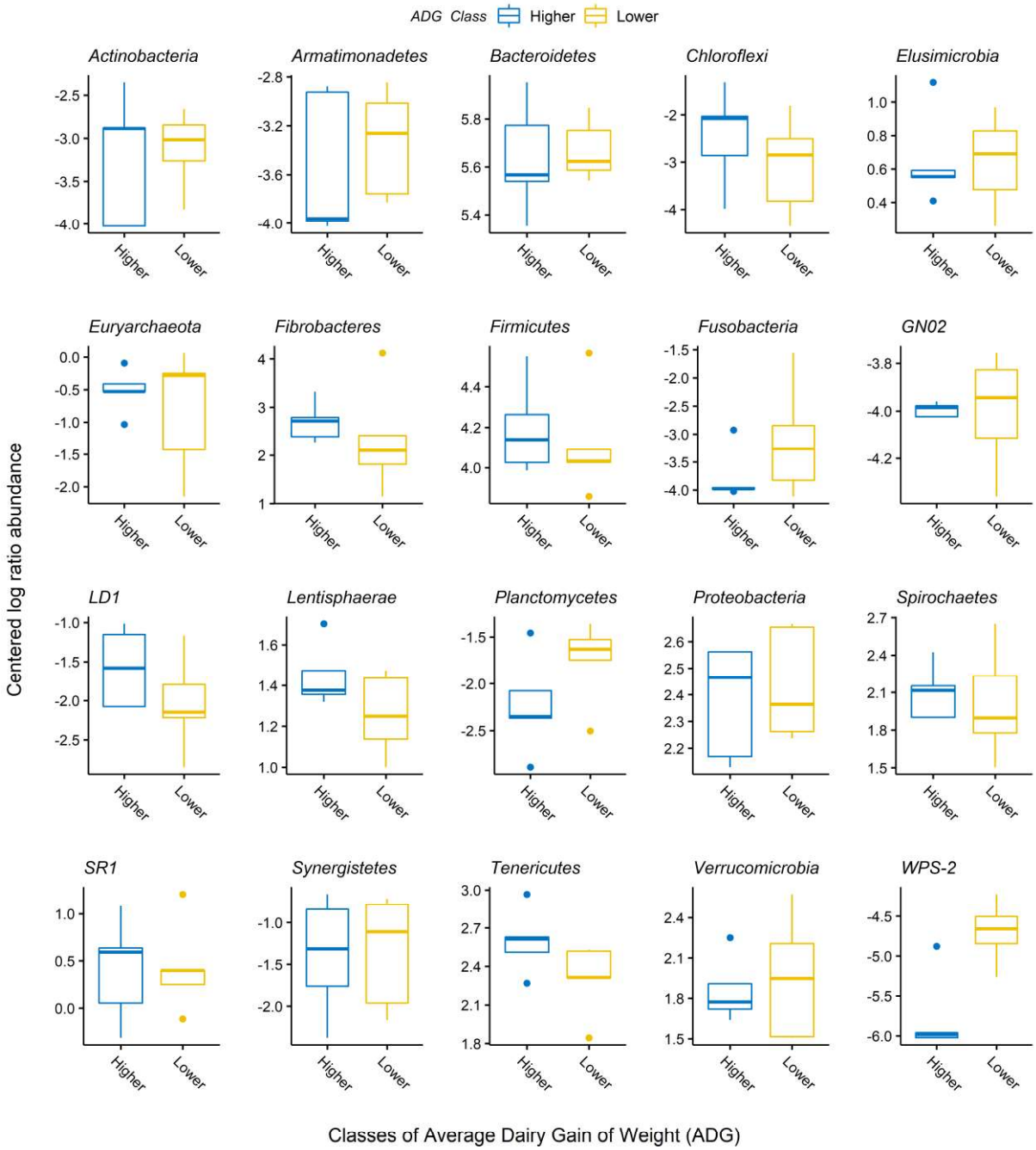


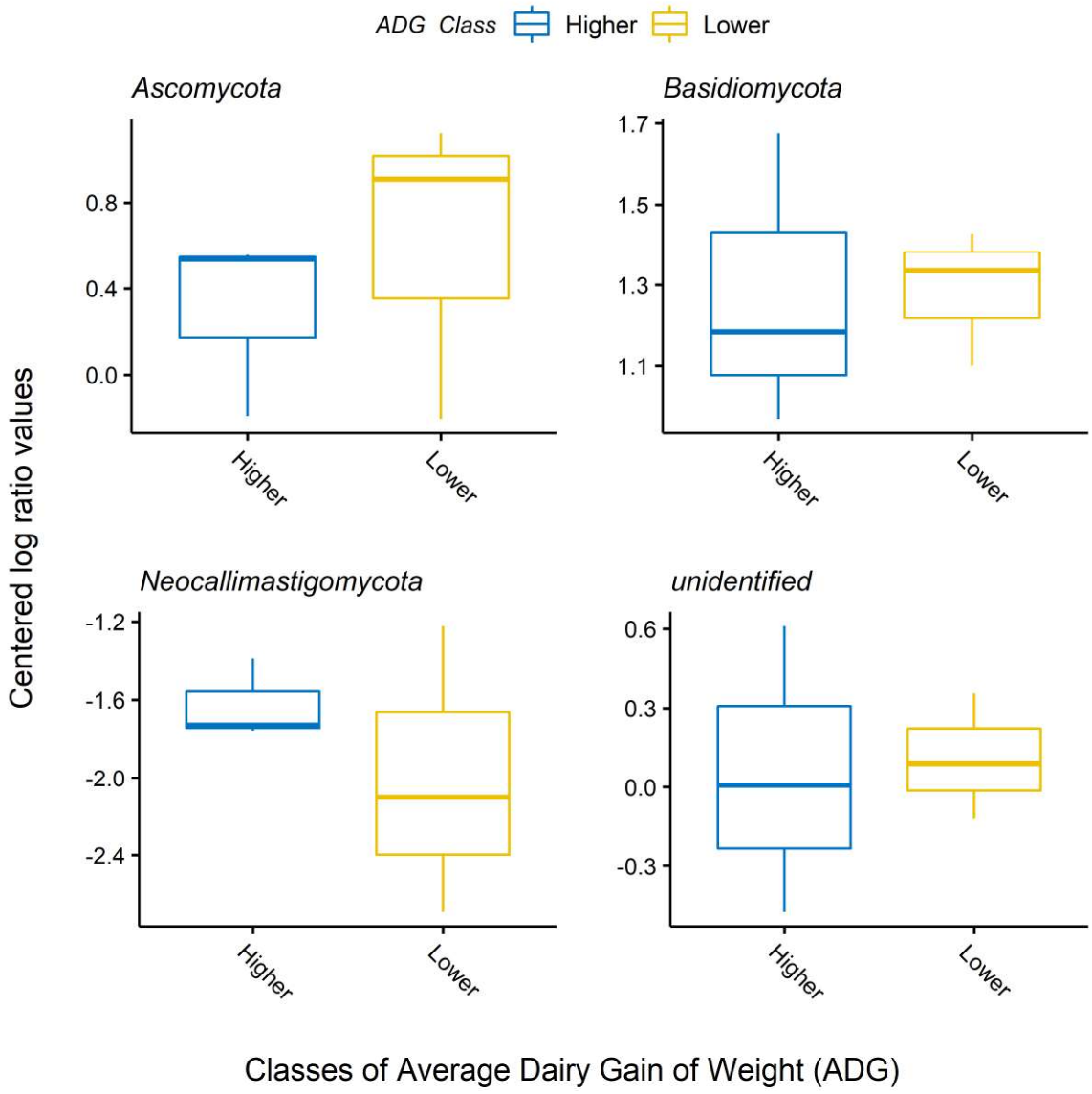
Figure 4. Ratios of OTUs closest related to fungal taxa and their correlation with daily gain of weight in beef cattle's rumen.



Supplementary Figure S1. Overall comparisons of bacterial and fungal communities respectively at OTU level based on principal coordinates analysis (PCoA), depicting clusters of bacterial communities in 17 samples each from the Higher (red), Lower (blue) and Middle (green) gain of weight groups. Each point represents a microbial community in a sample. Points closer to each other represent similar microbial communities, while points farther from each other represent dissimilar microbial communities. The statistical significance of sample groupings was tested with the Adonis function using distance matrices as primary input. R2 values were 0.140 (p = 0.052) for Bacteria and 0.198 (p = 0.195) for Fungi.



Supplementary Figure S2. Centered log ratio transformed abundance of all bacterial phyla present in ruminal samples from bovines with Higher ADG (yellow) and Lower ADG (blue). Differences were tested with the ALDEx package. No significant difference between groups was found (effect size < 1, p-value > 0.1). Note that negative clr values indicate very low abundance.



Supplementary Figure S3. Centered log ratio transformed abundance of all fungal phyla present in ruminal samples from bovines with Higher ADG (yellow) and Lower ADG (blue). Differences were tested with the ALDEx package. No significant difference between groups was found (effect size < 1, p-value > 0.1). Note that negative clr values indicate very low abundance.

5 CONCLUSÕES

A microbiota do rúmen tem papel crucial na digestão de ruminantes, entre eles os bovinos. No presente trabalho, a importância destes microrganismos se evidencia e mostra-se que, de fato, há padrões microbianos que estão associados a um maior ganho de peso em gado de corte do Pampa brasileiro. Alguns gêneros de bactérias e fungos foram encontrados como mais abundantes naqueles animais com um ganho de peso elevado.

Os microrganismos aqui descritos como associados a um alto ganho de peso, no entanto, não estão atualmente disponíveis em produtos comerciais focados em melhorar a nutrição animal de bovinos. Dessa forma, os achados deste trabalho podem ser um passo inicial para a produção de probióticos novos e mais eficientes focados em maior e melhor nutrição de bovinos de corte. Da mesma forma, os dados aqui reportados também surgem como promissoras unidades taxonômicas para futuros estudos com biomarcadores de produtividade em gado de corte e aumento da produtividade com menores danos ambientais.

6 REFERÊNCIAS BIBLIOGRÁFICAS

ABRAFRIGO. Exportação Brasileira de Carnes Bovina e Derivados. **Estatísticas – ABRAFRIGO**, [S.l.], 2020. Disponível em: <http://www.abrafrigo.com.br/index.php/estatisticas/>. Acesso em: 17 dez. 2020.

ALMEIDA, A. K. DE; MICHELS, I. L. O Brasil e a economia-mundo: o caso da carne bovina. 2012. v. 33, n. 1, p. 24.

CARBERRY, C. A. *et al.* Effect of Phenotypic Residual Feed Intake and Dietary Forage Content on the Rumen Microbial Community of Beef Cattle. **Applied and Environmental Microbiology**, 15 jul. 2012. v. 78, n. 14, p. 4949–4958.

COCOLIN, L.; DOLCI, P.; RANTSIOU, K. Biodiversity and dynamics of meat fermentations: The contribution of molecular methods for a better comprehension of a complex ecosystem. **Meat Science**, nov. 2011. v. 89, n. 3, p. 296–302.

EMBRAPA. Carne bovina - Portal Embrapa. **Portal Embrapa**, [S.l.], 2020. Disponível em: <https://www.embrapa.br/qualidade-da-carne/carne-bovina>. Acesso em: 17 dez. 2020.

FARMNEWS, E. Principais importadores de carne bovina do Brasil no 1° semestre de 2020. **Farmnews**, [S.l.], 7 jul. 2020. Disponível em: <http://www.farmnews.com.br/mercado/principais-importadores-de-carne-bovina-3/>. Acesso em: 17 dez. 2020.

FOUTS, D. E. *et al.* Next Generation Sequencing to Define Prokaryotic and Fungal Diversity in the Bovine Rumen. **PLoS ONE**, 7 nov. 2012. v. 7, n. 11, p. e48289.

IBGE. PPM 2019: após dois anos de queda, rebanho bovino cresce 0,4% | Agência de Notícias | IBGE. **Agência IBGE Notícias**, [S.l.], 2019. Disponível em: <https://agenciadenoticias.ibge.gov.br/agencia-sala-de-imprensa/2013-agencia-de-noticias/releases/29163-ppm-2019-apos-dois-anos-de-queda-rebanho-bovino-cresce-0-4>. Acesso em: 17 dez. 2020.

ISHAQ, S. L. *et al.* An Investigation into Rumen Fungal and Protozoal Diversity in Three Rumen Fractions, during High-Fiber or Grain-Induced Sub-Acute Ruminal Acidosis Conditions, with or without Active Dry Yeast Supplementation. **Frontiers in Microbiology**, 10 out. 2017. v. 8. Disponível em: <http://journal.frontiersin.org/article/10.3389/fmicb.2017.01943/full>. Acesso em: 14 nov. 2019.

LINN, J. *et al.* The ruminant digestive system. **University of Minnesota Extension**, [S.l.], 2018. Disponível em: <https://extension.umn.edu/dairy-nutrition/ruminant-digestive-system#large-intestine-1000463>. Acesso em: 23 maio. 2019.

MALZONI, M. Carta Boi - Em dez anos a projeção do crescimento da produção de carne bovina é de 20,5%. Atenção às oportunidades de mercado. **Scot Consultoria**, [S.l.], 2018. Disponível em: <https://www.scotconsultoria.com.br/noticias/cartas/47848/carta-boi---em-dez-anos-a-projecao-do-crescimento-da-producao-de-carne-bovina-e-de-205.-atencao-as-oportunidades-de-mercado.htm>. Acesso em: 17 dez. 2020.

MINISTÉRIO DA AGRICULTURA, PECUÁRIA E ABASTECIMENTO. Dados de rebanho bovino e bubalino no Brasil – 2017. **MAPA**, [S.l.], 2018. Disponível em: <http://www.agricultura.gov.br/assuntos/sanidade-animal-e-vegetal/saude-animal/programas-de-saude-animal/febre-aftosa/documentos-febre-aftosa/DadosderebanhobovinoebubalinodoBrasil_2017.pdf>. Acesso em: 23 maio. 2019.

MINISTÉRIO DO MEIO AMBIENTE. Pampa. **Ministério do Meio Ambiente**, [S.l.], 2019. Disponível em: <http://www.mma.gov.br/biomas/pampa.html>.

PARISH, J.; RIVERA, D. Understanding the Ruminant Animal Digestive System. **Mississippi State University**, 2017. Beef, Goats and Sheep. p. 8.

SCHLESINGER, S. **Onde pastar? o gado bovino no Brasil**. 1. ed ed. Rio de Janeiro: FASE, 2010.

SCHUSTER, S. C. Next-generation sequencing transforms today's biology. **Nature Methods**, 19 dez. 2007. v. 5, p. 16.

SILVA, M. C. Da; BOAVENTURA, V. M.; FIORAVANTI, M. C. S. História do Povoamento Bovino no Brasil Central. **Revista UFG**, 2012. v. 13, p. 34–41.

SONG, J.; JEONG, J. Y.; KIM, M. Diversity Census of Fungi in the Ruminal Microbiome: A meta-analysis. **Journal of the Korea Academia-Industrial Cooperation Society**, 31 dez. 2017. v. 18, n. 12, p. 466–472.

STEWART, R. D. *et al.* Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. **Nature Biotechnology**, ago. 2019. v. 37, n. 8, p. 953–961.

WIRTH, R. *et al.* The Planktonic Core Microbiome and Core Functions in the Cattle Rumen by Next Generation Sequencing. **Frontiers in Microbiology**, 24 set. 2018. v. 9. Disponível em: <https://www.frontiersin.org/article/10.3389/fmicb.2018.02285/full>. Acesso em: 2 abr. 2019.

WLODARSKI, L. *et al.* Microbiota ruminal: diversidade, importância e caracterização. **Revista Electrónica de Veterinaria**, 2017. v. 18, n. 11, p. 20.