GUSTAVO FELIPE CORREIA SALES

METAGENOMIC AND METABOLIC ANALYSIS OF THE RUMEN IN CATTLE SUBMITTED TO HEAT STRESS CONDITIONS

LAVRAS - MG

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Dissertação apresentado a Universidade Federal de Lavras, como parte das exigências do programa de pós-graduação em Microbiologia Agrícola, para a obtenção do título de Mestre.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Sales, Gustavo Felipe Correia.
Análise metagenômica e metabólica do rúmen de bovinos submetidos a condições de estresse térmico / Gustavo Felipe Correia Sales. - 2017.
57 p. : il.

Orientador(a): Carla Luiza da Silva Ávila.
Coorientador(a): Mateus Pies Gionbelli, Beatriz Ferreira Carvalho.
Dissertação (mestrado acadêmico) - Universidade Federal de Lavras, 2017.
Bibliografia.

1. Análise Metagenômica. 2. Rúmen. 3. Estresse por calor. I. Ávila, Carla Luiza da Silva. II. Gionbelli, Mateus Pies. III. Carvalho, Beatriz Ferreira. IV. Título.

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Aprovada em 06 de setembro de 2017.

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Dedico a toda minha família, em especial minha mãe Rosemeire, meu pai José Armando e Romeu que me apoiaram e foram fundamentais nesta conquista.

AGRADECIMENTO

Agradeço aos meus pais José Armando e Rosemeire, pelo amor, educação e apoio que me deram em toda minha vida.

Aos meus irmãos Thiago e Marcus pelo companheirismo e incentivos.

Agradeço também ao Romeu, meu segundo pai, por esses anos de conselhos e ensinamentos.

Aos meus tios Rosana e Menoti que foram essenciais para que hoje eu esteja concluindo o curso de mestrado em uma universidade federal.

Agradeço de forma especial a Fernanda por ser minha amiga, companheira e namorada, pelo carinho, apoio e compreensão todo tempo.

À Universidade Federal de Lavras, pela oportunidade.

À CAPES, pela concessão da bolsa de mestrado. À FAPEMIG e ao CNPq.

À professora Carla Luiza da Silva Ávila pela oportunidade, pela convivência, apoio e orientação. Ao professor Mateus Pies Gionbelli e à doutora Beatriz Ferreira Carvalho pelo suporte e orientação.

Aos professores e funcionários do programa de Pós-Graduação em Microbiologia Agrícola pelos ensinamentos e auxílio.

Aos amigos da Universidade Federal de Lavras que me ajudaram a concluir este trabalho.

A todos vocês, minha sincera gratidão.

RESUMO

O estresse térmico é uma condição fisiológica resultante da incapacidade do animal de dissipar calor de forma suficiente para manter a sua homeotermia. Alterações no metabolismo dos bovinos podem induzir alterações na microbiota ruminal e no processo fermentativo do rúmen, e afetar a absorção de nutrientes pelo ruminante, afetando a sua produtividade. O objetivo deste trabalho foi estudar o metagenoma de procariotos e o perfil de metabólitos produzidos no rúmen de bovinos de corte submetidos a condições de conforto e de estresse térmico. Seis novilhas zebuínas puras, canuladas no rúmen, foram utilizados no experimento. Os animais foram confinados em câmaras bioclimáticas dotadas de ventiladores, exaustores, condicionadores de ar, lâmpadas infravermelhas e controladores de umidade. Foram testados 6 tratamentos, em um delineamento em quadrado latino 6 × 6, com um arranjo fatorial 2 × 2 + 2, contendo 2 fatores (temperatura e concentração energética da dieta) e dois tratamentos adicionais, de forma a obterem-se meios de comparação dos efeitos da temperatura sobre as variáveis propostas sem interferência do nível de consumo. As duas temperaturas propostas referem-se a termo neutralidade e estresse por calor (34º C de 6 às 18 horas e 24ºC de 18 às 6 horas). Foi realizada extração de DNA total das amostras coletadas, utilizando protocolo de fenol-clorofórmio. As amostras de DNA total foram sequenciadas pela plataforma de sequenciamento de nova geração MiSeq Sequencing System (Illumina). A partir das sequencias de DNA obtidas foram determinados os índices de diversidade de cada amostra. A classificação taxonômica das sequencias foi determinada utilizando o software de bioinformática Geneious 10.2.3. Os ácidos acético, propiônico, butírico, valérico, isobutírico e isovalérico, foram quantificados por cromatografia líquida de alta eficiência. A fim de caracterizar o perfil de compostos presente no ambiente ruminal foi utilizada a cromatografia gasosa associada a um espectrômetro de massas. As sequencias foram classificadas em 80 diferentes gêneros, pertencentes a 10 filos. Em todos os tratamentos, os filos Firmicutes e Bacteroidetes representam mais de 70% do total de procariotos identificados. Os filos Verrucomicrobia, Spirochaetes e os gêneros *Flavonifractor*, *Treponema* e *Ruminococcus*, apresentam redução (P<0,03) na sua representatividade quando os bovinos foram submetidos a elevadas temperaturas. O gênero *Saccharibacteria genera* (*incertae sedis*), pertencente ao filo de bactérias não cultivadas Candidatus Saccharibacteria, apresentou maior (P=0,05) representatividade de indivíduos nos animais que estavam sob condições de estresse calórico. O aumento de indivíduos pertencentes a este gênero, pode estar associada a diminuição do pH ruminal. A concentração dos ácidos acético e isovalérico foi maior (P=0,03) no rúmen de bovinos em condições de termo neutralidade. Foram identificados 45 diferentes compostos nas amostras do conteúdo ruminal analisadas. Não houve um perfil de compostos padrão de resposta ao estresse por calor. A amina Dihidro-5-pentil-2(3H)-Furanona foi identificada em 100% das amostras referentes aos tratamentos de estresse calórico e pode ser um indicador da condição de estresse térmico em bovinos. O estresse por calor causou alterações na composição da microbiota ruminal e também no processo fermentativo do rúmen e estas alterações podem representar padrões específicos em resposta a alteração fisiológica do hospedeiro em decorrência das condições de estresse por calor.

Palavras-chave: Conforto térmico, Microextração em fase sólida (SPME), Ácidos graxos voláteis (AGV).

ABSTRACT

Heat stress is a physiological condition resulting from an animal's inability to dissipate heat to maintain its homeothermy. Changes in bovine metabolism may induce alterations in the ruminal microbiota and fermentative process, affecting its productivity. The objective of this project was to study prokaryote metagenomes and the metabolic profile in the rumen of beef cattle subjected to comfort and heat stress conditions. Six pure Zebu cattle, cannulated in the rumen, were used in the experiment. The animals were confined in bioclimatic chambers equipped with ventilators, exhausters, air conditioners, infrared lamps and humidity controllers. Six treatments were tested in a latin square design 6 × 6 with a 2 × 2 + 2 factorial arrangement, containing two factors (temperature and diet energy concentration) and two additional treatments, in order to obtain means of comparison of the temperature effect on the proposed variables without consumption level interference. The two proposed temperatures represent thermoneutral and heat stress conditions (34º C from 6 am to 6 pm and 24ºC from 6 am to 6 pm). Total DNA was extracted from each collected sample using a phenol-chloroform protocol. The total DNA was sequenced by the next-generation sequencing platform MiSeq Sequencing System (Illumina), and the diversity index of each sample was determined. The sequences taxonomic classification was determined using Geneious 10.2.3 bioinformatics software. Acetic, propionic, butyric, valeric, isobutyric and isovaleric acids were quantified by high performance liquid chromatography. Gas chromatography-mass spectrometry was used to characterise the compound profile present in the ruminal environment. Sequences were classified into 80 different genera, belonging to 10 phyla. In all treatments, Firmicutes and Bacteroidetes represented more than 70% of all prokaryotes identified. The phyla Verrucomicrobia and Spirochaetes and the genera Flavonifractor, Treponema and Ruminococcus presented a reduction ($P<0.03$) in their population when the cattle were submitted to high temperatures. The genus *Saccharibacteria* (*incertae sedis*), belonging to a phylum of uncultivated bacteria (Candidatus Saccharibacteria), presented higher ($P=0.05$) population in animals under thermal stress conditions. The concentration of isobutyric acid was lower ($P<0.01$) in the rumen of bovine under heat stress conditions. A total of 45 different compounds were identified in the ruminal samples. There was no standard compound profile related to heat stress. The amine dihydro-5-pentyl-2 (3H)-furanone was identified in 100% of the samples following heat stress treatment. The heat stress caused alterations in the ruminal microbiota composition and also in the rumen fermentative process.

Keywords: Thermal comfort, Solid-phase micro-extraction (SPME), Volatile fatty acids (VFA).

Sumário

PRIMEIRA PARTE

1- INTRODUÇÃO

A criação de bovinos no Brasil se destaca no cenário mundial, sendo que a maioria destes animais é criada em ambientes abertos, expostos a fatores ambientais, como temperatura, radiação solar, circulação de ar e precipitação. Dois terços do território brasileiro está situado na região tropical, onde há predominância de temperaturas elevadas, consequentes da alta incidência de radiação solar. A elevada temperatura juntamente com altos índices de umidade pode afetar conforto térmico bovino, submetendo os animais às condições de estresse térmico.

O estresse térmico é uma condição fisiológica resultante de um saldo negativo entre a quantidade líquida de energia que flui do animal para o ambiente externo e a quantidade de energia térmica produzida pelo animal. Este desequilíbrio é induzido por alterações em uma combinação dos fatores ambientais, características do animal e os mecanismos de termo regulação, tais como a condução, radiação, convecção e evaporação (ST-PIERRE et al., 2003). O estresse por calor ambiental resulta em respostas fisiológicas e comportamentais na tentativa de manter a homeostasia, e estes ajustes afetam; o consumo de energia e sua partição dentro do animal, a quantidade de energia disponível para a produção, o nível de produtividade e a eficiência de utilização do alimento pelos animais (NRC, 1981).

Alterações fisiológicas resultantes do estresse por calor afetam diretamente a composição e fisiologia do rúmen, podendo afetar sua comunidade de microrganismos. A simbiose entre os ruminantes e os microrganismos é uma relação extremamente evoluída, onde os microrganismos são beneficiados com abrigo e nutrientes, e o metabolismo desses microrganismos é fundamental para a nutrição do hospedeiro (HOBSON, 1988). Mudanças no metabolismo do hospedeiro podem induzir alterações na microbiota ruminal e afetar a absorção de nutrientes pelo ruminante, gerando menor ganho de peso e também menor produtividade.

O conhecimento da diversidade da microbiota ruminal e dos metabólitos produzidos por estes microrganismos é importante para aumento da eficiência de uso dos nutrientes da dieta e da eficiência do sistema de produção de carne e leite, melhorando as condições de saúde dos animais, no entanto, esse tipo de estudo apresenta limitações. A principal limitação para o estudo dos microrganismos presentes no rúmen é que a maioria desses organismos são considerados não cultiváveis. O fato desses organismos serem anaeróbios e ocorrer forte associação entre os diferentes microrganismos, dificultam o cultivo em laboratório, sendo

fundamental a utilização de técnicas e equipamentos que possibilitem a evolução no estudo desses microrganismos.

As técnicas de biologia molecular, como a Reação em Cadeia da Polimerase (PCR) e o Sequenciamento de DNA de Nova Geração (NGS), são importantes ferramentas para auxiliar o estudo da diversidade da microbiota ruminal. Estas técnicas são independentes de cultivo, e permitem a análise da composição da comunidade de microrganismos e de possíveis alterações que possam ocorrer entre as populações em decorrência de alterações fisiológicas. Neste contexto, é de fundamental importância estudar e conhecer mais sobre a biodiversidade da microbiota ruminal, e quais os efeitos do estresse por calor sobre esses microrganismos, já que os dados disponíveis na literatura ainda são muito escassos.

2- REFERENCIAL TEÓRICO

2.1- Bovinocultura e Estresse Térmico

De acordo com o último censo agropecuário realizado pelo Instituto Brasileiro de Geografia e Estatística (IBGE), que foi realizado no ano de 2015, o efetivo de bovinos no Brasil foi de 215,20 milhões de cabeças, representando um aumento de 1,3% em relação a 2014. O estado de Minas Gerais registrou o segundo maior efetivo bovino brasileiro com 11,0% do total nacional. De acordo com o Departamento de Agricultura dos Estados Unidos (United States Department of Agriculture - USDA), o Brasil deteve o segundo maior efetivo de bovinos, sendo responsável por 22,5% do rebanho mundial, atrás apenas da Índia. Em relação à exportação de carne bovina, o Brasil ocupou a terceira posição do ranking internacional em 2015, sendo Índia e Austrália, respectivamente, os maiores exportadores (IBGE, 2015).

Estima-se que até o ano de 2050 a população mundial seja maior do que 7,8 bilhões de pessoas, sendo que o "tempo de dobramento", que é a taxa em que a população dobra o seu tamanho é de apenas 24 anos na África e 35 anos na Ásia e América Latina, em comparação com 98 anos na América do Norte e 1025 na Europa (ROUSH, 1994). Com base nessa estimativa, pode-se concluir que o maior crescimento populacional ocorrerá na região tropical, e consequentemente, é possível estimar também que haverá maior consumo e produção bovina neste local. Animais criados na região intertropical, estão mais expostos ao

estresse por calor, já que a temperatura ambiente média é maior do que nos demais territórios do planeta.

Além do crescimento populacional e da demanda por produtos animais, os efeitos nocivos do estresse térmico ambiental sobre o bem-estar e de produção animal, torna-se um problema maior no futuro se o clima da Terra continuar a aquecer como prevê alguns autores (CUBASCH e MEEHL, 2001; IPCC, 2008). Em um estudo realizado nos Estados Unidos em 2003, o pesquisador ST-Pierre et al. (2003), concluiu que o estresse térmico resulta entre 1,69 e 2,36 bilhões de dólares de perdas econômicas anuais para as indústrias de gado daquele país.

Um dos métodos utilizado para o conforto bovino em relação ao estresse térmico é o Índice de Temperatura e Umidade (ITU), que pode ser utilizado para avaliar o impacto do ambiente sobre os animais. Este índice foi inicialmente descrito para avaliar o conforto térmico humano e posteriormente adotado para avaliação do conforto térmico de animais. Segundo Du Preez et al. (1990), valores de ITU menores ou iguais a 70 são indicadores de um ambiente não estressante; entre 71 e 78 são de alerta; de 79 a 82 a situação é de perigo; e acima de 82, situação de emergência. O Brasil possui um alto ITU, podendo chegar à 80 em determinadas regiões, valor este que é considerado como situação de perigo para o conforto térmico dos animais (INMET, 2017). De acordo com o Instituto Nacional de Meteorologia o ITU pode ser estimado pela equação 1 a seguir, onde, Tbs é a temperatura de bulbo seco (ºC) e Tpo é a temperatura do ponto de orvalho (ºC):

$$\mathbf{ITU = Tbs + 0{,}36 \times Tpo + 41{,}2} \quad (1)$$

O ambiente térmico tem uma forte influência sobre os animais de fazendas, com o efeito da temperatura, vento, chuva, umidade e radiação. Existe uma condição ideal de temperatura para o desenvolvimento do animal, e quando há qualquer mudança nessa condição ideal, os indivíduos respondem alterando a ingestão de alimentos, o metabolismo e a dissipação de calor, que por sua vez altera a partição da energia da dieta pelo animal (NRC, 1981). Quando a temperatura está abaixo ou acima dos valores ideais, a rentabilidade está comprometida, porque os nutrientes são desviados dos fins produtivos, para manter uma temperatura corporal segura. O estresse térmico afeta negativamente uma variedade de

parâmetros produtivos, incluindo a produção de leite, o crescimento, reprodução e características de carcaça (BAUMGARD e RHOADS, 2012).

A ingestão de alimentos e ganho nutricional são comprometidos pelo estresse por calor, já que esta condição causa a redução do consumo de matéria seca, diminui a motilidade e contração ruminal, muda o padrão de fermentação e produção de ácidos graxos voláteis, afetando a digestibilidade e utilização dos nutrientes e, portanto, prejudicando a produtividade (YADAV et al., 2013). O consumo de ração de vacas em lactação começa a declinar em temperatura ambiente de 25 ° C e reduz mais rapidamente acima de 30 ° C (RHOADS et al., 2013). O aumento da carga de calor diminui a absorção de nutrientes em quase todas as espécies e, no caso do gado, a absorção de nutrientes diminui cerca de 30% do consumo de matéria seca (WHEELOCK et al., 2010). Nonaka et al. (2008), concluiu em sua pesquisa que a 33 ° C o consumo de matéria seca foi cerca de 9% inferior em comparação com o consumo de matéria seca, a 20 ° C.

Também tem sido estudado por diversos autores, o efeito do estresse causado por altas temperaturas sobre a digestibilidade dos ruminantes. Christopherson e Kennedy (1983) observaram que há efeitos positivos de alta temperatura ambiente sobre a digestibilidade dos alimentos, sendo que esse aumento foi atribuído à redução da taxa de passagem da digesta, as mudanças na composição da alimentação ou a redução do consumo da matéria seca. Outros autores (CHENG et al., 2014; HAVLIN e ROBINSON, 2015 e YAZDI et al. 2016) concluíram que não houve efeito significativo do estresse por calor sobre a digestibilidade. O mais recente desses estudos, realizado por Yazdi et al. (2016), demostrou que houve redução do consumo de matéria seca induzida pelo estresse calórico. No entanto, o autor considerou que o período em que os animais foram submetidos ao calor foi curto, e que por esse motivo não houve nenhum efeito sobre a digestibilidade da dieta e a morfologia ruminal.

A ruminação é outro sistema afetado quando o animal permanece em ambientes com elevadas temperaturas. Soriani et al. (2013), observaram que com o Índice de Temperatura e Umidade (ITU) acima de 76, há um efeito negativo no tempo de ruminação. Estes resultados, segundo os autores, podem estar relacionados ao efeito do estresse térmico sobre balanço energético, o consumo de matéria seca e o padrão de alimentação diário.

Existe uma necessidade urgente de uma melhor compreensão de como o estresse por calor altera a fisiologia dos bovinos e consequentemente o seu desempenho e produção. Definir a biologia e os mecanismos de como o estresse de calor prejudica o desempenho

animal é fundamental no desenvolvimento de abordagens para solucionar problemas de produção atuais e futuras (BAUMGARD e RHOADS, 2012).

2.2- Microbiota Ruminal e a Metagenômica

Os ruminantes são animais herbívoros que não têm a capacidade de degradar a celulose e outros complexos polissacarídeos de plantas. Para a obtenção de energia a partir desses compostos foi desenvolvido uma relação de simbiose entre os ruminantes e microrganismos, na maioria anaeróbios. Podem ser destacadas três importantes vantagens nutricionais da presença e atividade dos microrganismos no rúmen: [1] as enzimas constitutivas dos ruminantes não hidrolisam a celulose e outros polissacarídeos oriundos da planta, mas os microrganismos são capazes de metabolizar essas substâncias, tornando-as fontes disponíveis de energia, e assim diminuindo a quantidade de matéria seca que passará pela porção pós rúmen trato digestório; [2] a microbiota ruminal pode utilizar o nitrogênio não proteico para o seu crescimento, convertendo-o em proteína microbiana, a qual se torna disponível para a dieta de aminoácidos do animal; [3] a síntese de vitamina pela população microbiana faz com que o animal se torne praticamente independente de outras fontes de vitaminas oriundas da dieta, exceto as vitaminas A e D (DEHORITY, 2003).

A população microbiana no rúmen inclui membros que pertencem aos três Domínios; Eubactérias, Archaea (Metanogênicas) e Eukarya (Protozoários e Fungos). As bactérias do rúmen são adaptadas para viver em acidez entre pH 5,5 e 7,0, na ausência de oxigênio, a uma temperatura entre 39-40°C. O fornecimento constante de alimentos pelo hospedeiro e a remoção contínua dos produtos de fermentação e resíduos alimentares, mantém condições relativamente constantes nas quais uma população extremamente densa se desenvolve (HUNGATE, 1966). Nagaraja (2016) reforça que o rúmen tem uma densa população de bactérias, com números variando de 10^8-10^{11} por grama do conteúdo ruminal. A maioria das bactérias são anaeróbias obrigatórias, embora existam também bactérias anaeróbias facultativas. Além disso, as bactérias são predominantemente gram-negativas, representando 80-90% da população. Já as Archaeas constituem cerca de 2-4% da população de procariotos no rúmen, sendo que, baseado em um método independente de cultivo (sequenciamento do gene 16S rRNA), a maioria das arqueias ruminais pertencem a três grupos: *Methanobrevibacter, Methanomicrobium* e um grupo de metanogênicas ainda não cultivadas, sendo a espécie *Methanobrevibacter ruminantium* a mais prevalente e melhor caracterizada.

Uma grande parcela dos microrganismos ruminais são considerados não cultiváveis, já que as técnicas e equipamentos atuais ainda não fornecem as condições ideais necessárias para o cultivo desses microrganismos em laboratório. Neste cenário, as técnicas de biologia molecular têm se estabelecido com uma ferramenta para auxiliar no estudo de microrganismos, já que essas técnicas não dependem de cultivo e permitem a identificação e análise da biodiversidade dos microrganismos presentes em diferentes amostras. Em um estudo sobre a diversidade de bactérias ruminais de cabras antes e após o desmame, analisando o DNA genômico extraído dos microrganismos do rúmen, o autor Han et al. (2015) observou em sua análise que 72,14% do total de bactérias eram não cultiváveis. Esse resultado evidência a grande quantidade de bactérias que estão presente no rúmen, e ainda não foram totalmente caracterizadas.

Vários autores já utilizaram técnicas moleculares para estudar os microrganismos ruminais (BENTO et al., 2015; FOUTS et al., 2012; GRUNINGER et al., 2014; INDUGU et al., 2016; JIAO et al., 2015; PETRI et al., 2012). Entre estas técnicas, o Sequenciamento de DNA de Nova Geração (NGS) tem se destacado, pois é uma técnica independente de cultivo e que apresenta uma elevada precisão. Em princípio, o conceito por trás de algumas tecnologias de NGS, como por exemplo a plataforma de sequenciamento MiSeq Sequencing System da Illumina (Illumina Ltd., Cambridge, UK), é semelhante ao sequenciamento por Eletroforese Capilar (Sequenciamento de Primeira Geração), onde a DNA polimerase catalisa a incorporação de desoxirribonucleótidos trifosfatos (dNTPs) marcados com fluorescência em uma fita molde de DNA, durante ciclos sequenciais de síntese de DNA. Durante cada ciclo, no ponto de incorporação, os nucleotídeos são identificados através da excitação do fluoróforo. A principal diferença é que, ao invés de sequenciar um único fragmento de DNA, o NGS estende este processo para milhões de fragmentos de DNA de forma paralela, permitindo assim sequenciar um grande número de fragmentos ao mesmo tempo, possibilitando, entre outros estudos, a análise da composição, abundância e relações filogenéticas de uma comunidade microbiana (MARDIS, 2008).

2.3- Efeitos do Estresse Térmico sobre a Microbiota Ruminal

Estudos têm mostrado que o estresse térmico pode causar mudanças fisiológicas em ruminantes. Essas mudanças afetam diretamente a produtividade animal, e possivelmente deve afetar a comunidade microbiana (Yadav et al., 2013).

Tajima et al. (2007), analisou a diversidade microbiana do rúmen de vacas leiteiras, sob três tratamentos diferentes: [1] bovinos com 250 kg, sob três diferentes temperaturas (20, 28 e 33ºC) com 60% de umidade relativa do ar; [2] animais com o mesmo peso, sob as mesmas temperaturas (20, 28 e 33º C) mas com 80% de umidade relativa do ar; [3] bovinos com 430 kg, sob as mesmas três diferentes temperaturas e com a 60% de umidade relativa do ar. No total, nove bibliotecas de Unidades Taxonômicas Operacionais (OTUs) da região 16S do rRNA bacteriano foram construídas, o que representou três intervalos de temperatura nos três tratamentos. O programa LIBSHUFF, que compara duas diferentes bibliotecas de sequências de DNA, foi utilizado a fim de determinar se há diferença significativa entre as amostras. O autor constatou que ocorreu alterações na diversidade de bactérias, nos dois 2 e, em decorrência do aumento da temperatura de 20 para 33ºC. Não foram definidas quais são as alterações que ocorreram na microbiota, apenas que existe diferença significativa entre as bibliotecas. Em todos os tratamentos, as sequências pertencentes aos filos Bacteroidetes e Firmicutes foram dominantes com uma proporção maior que 80% da diversidade bacteriana ruminal total. Utilizando a técnica de Reação em Cadeia da Polimerase em Tempo Real (qPCR), os autores quantificaram a presença de três espécies de bactérias, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* e *Prevotella ruminicola*, além de um grupo de bactérias não cultivadas. Apenas a quantidade de bactérias do grupo ainda não cultivadas, apresentaram um declínio com o aumento da temperatura e umidade.

O estudo realizado por Uyeno et al. (2010), utilizou as amostras dos tratamentos 2 e 3 do trabalho anteriormente citado (Tajima et al., 2007) para analisar de forma específica os diferentes grupos de microrganismos que haviam sido identificados no rúmen. Neste trabalho, foram desenhadas sondas específicas para diferentes grupos de microrganismo, inclusive para o grupo de bactérias não cultivadas (que o autor dividiu em três grupos e os nomeou de grupo desconhecido A, B e C). Os autores concluíram, que em ambos os tratamentos, as espécies *Clostridium coccoides*, *Eubacterium rectale*, espécies do gênero *Streptococcus* e também as bactérias do grupo desconhecido B aumentaram significativamente com o aumento da temperatura ambiente. Além disto, com o aumento da temperatura ambiental, a quantidades de bactérias do gênero *Fibrobacter* (uma das principais bactérias fibrolíticas) diminuíram mais de 50% em ambos os tratamentos. Já as bactérias pertencentes ao gênero *Streptococcus* (uma das principais bactérias sacarolíticas) aumentaram 2,5 vezes (no tratamento 2) e 10 vezes (no tratamento 3). Embora as populações relativas destes gêneros sejam relativamente pequenos na comunidade bacteriana no rúmen, as alterações na população dos referidos

gêneros poderão ser consideradas importantes na resposta adaptativa ruminal. Uyeno et al. (2010) concluíram também, que essas alterações pareceram ser resultado indireto do estresse por calor através da alteração da ingestão alimentar.

2.4- Metabólitos Produzidos pela Fermentação no Rúmen

Os carboidratos da alimentação dos bovinos incluem polissacarídeos, que podem ser estruturais (celulose, hemiceluloses e pectina) ou não estrutural (amido) e açúcares. Bactérias, protozoários e fungos produzem uma variedade de enzimas beta-glicosidases que quebram as ligações glicosídicas para produzir, primeiro oligossacarídeo, e depois os di- e monossacarídeos. Várias espécies de bactérias ruminais possuem atividades celulolíticas, e também podem digerir hemiceluloses e pectina, além disso, algumas bactérias não celulolíticas, como por exemplo, *Prevotella* (*P. albensis, P. brevis, P. bryanti* e *P. rumincola*), *Butyrivibrio, Fibrisolvens, Pseudobutyrivibrio xylanivorans*, também podem digerir hemiceluloses. Embora a pectina seja um polissacarídeo estrutural, ele é completamente digerido no rúmen, e as principais bactérias com atividade pectinolítica incluem *Prevotella sp.*, *Lachnospira multiparus*, *Streptococcus bovis* e *Trepnema* (*T. bryantii e T. saccharophilum*). O amido é rapidamente digerido no rúmen, sendo que a digestão depende do tipo e do grau de processamento do grão. As principais bactérias amilolíticas no rúmen incluem *Ruminobacter amylophilus*, *Selenomonas ruminantium*, *Streptococcus bovis* e espécies de *Lactobacillus* e *Bifidobacterium*, e as principais enzimas envolvidas são a alfa-amilase e pullulanase. O rúmen também possui bactérias que fermentam açúcar, pertencentes aos gêneros *Streptococcus, Bifidobacterium, Lactobacillus* e *Treponema* (NAGARAJA, 2016).

Durante a fermentação microbiana de carboidratos e substratos endógenos, no trato gastrointestinal dos ruminantes, são produzidos ácidos graxos voláteis (AGVs) de cadeia curta, sendo que considerável energia é obtida a partir desses ácidos orgânicos em espécies herbívoras. As estimativas são que estes ácidos contribuem com aproximadamente 70% das necessidades calóricas dos ruminantes. Além disso, a produção e a absorção de AGV tem um efeito muito significativo sobre o crescimento das células epiteliais, o fluxo de sangue e funções normais de secreção e absorção pelo intestino grosso, ceco e rúmen (BERGMAN, 1990).

Os principais AGVs produzidos no rúmen são o acetato, propionato, e butirato, numa proporção variando desde 75:15:10 a 40:40:20, dependendo da composição da dieta, além dos

ácidos isobutíricos, valéricos, isovaléricos, que estão presentes em quantidade relativamente menores. A absorção dos ácidos ocorre no local de produção de forma rápida, sendo que grandes quantidades são metabolizadas pelo epitélio intestinal ruminal antes de chegarem a corrente sanguínea (BERGMAN, 1990). Os ácidos acético, propiónico e butírico podem ser usados para gerar ATP no metabolismo intermediário, no entanto, ao contrário do ácido acético e butírico, o ácido propiónico pode ser usado também como precursor para a síntese de glicose (DIJKSTRA, 1993). A maior parte do ácido butírico é convertido em corpos cetônicos e CO_2 pelas células epiteliais, e quase toda a restante é removida pelo fígado. Já o ácido acético é utilizado principalmente pelos tecidos periféricos, em especial as gorduras e os músculos (BERGMAN, 1990).

3- CONSIDERAÇÕES FINAIS

As condições ambientais de elevada temperatura e umidade podem levar os bovinos a uma condição fisiológica de estresse por calor, que gera mudanças no metabolismo do animal e pode gerar alterações na microbiota presente no rúmen e afetar o processo fermentativo que ocorre neste ambiente. Os microrganismos que vivem em simbiose com os animais ruminantes são essenciais para o processo digestivo do animal, sendo responsáveis por gerar grande parte da energia calórica requerida pelo hospedeiro, além de ser fonte de aminoácidos e vitaminas.

Apesar da importância dos microrganismos para a nutrição animal, são escassos os estudos sobre as possíveis alterações na microbiota ruminal e sobre o processo fermentativo que ocorre no rúmen, em resposta as mudanças fisiológicas causadas pelo estresse calórico no hospedeiro. As técnicas de biologia molecular são ferramentas fundamentais para o melhorar o conhecimento sobre a microbiota presente no rúmen de bovinos sob condições de estresse térmico, pois são técnicas independentes de cultivo e com alta sensibilidade e precisão.

O conhecimento sobre alterações causadas pelo estresse por calor sobre os bovinos e consequentemente sobre a microbiota e o processo fermentativo do rúmen é um pré-requisito para a geração de novas estratégias, que possam melhorar o bem-estar animal, o seu desempenho e produtividade.

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SEGUNDA PARTE

METAGENOMIC AND METABOLIC ANALYSIS OF THE RUMEN IN CATTLE SUBMITTED TO HEAT STRESS CONDITIONS

Article formatted in accordance with the standards of the Anaerobe journal

Abstract

Heat stress is a physiological condition resulting from an animal's inability to dissipate heat to maintain its homeothermy. Changes in bovine metabolism may induce alterations in the ruminal microbiota and fermentative process, affecting its productivity. The objective of this project was to study prokaryote metagenomes and the metabolic profile in the rumen of beef cattle subjected to comfort and heat stress conditions. Six pure Zebu cattle, cannulated in the rumen, were used in the experiment. The animals were confined in bioclimatic chambers equipped with ventilators, exhausters, air conditioners, infrared lamps and humidity controllers. Six treatments were tested in a latin square design 6 × 6 with a 2 × 2 + 2 factorial arrangement, containing two factors (temperature and diet energy concentration) and two additional treatments, in order to obtain means of comparison of the temperature effect on the proposed variables without consumption level interference. The two proposed temperatures represent thermoneutral and heat stress conditions (34º C from 6 am to 6 pm and 24ºC from 6 am to 6 pm). Total DNA was extracted from each collected sample using a phenol-chloroform protocol. The total DNA was sequenced by the next-generation sequencing platform MiSeq Sequencing System (Illumina), and the diversity index of each sample was determined. The sequences taxonomic classification was determined using Geneious 10.2.3 bioinformatics software. Acetic, propionic, butyric, valeric, isobutyric and isovaleric acids were quantified by high performance liquid chromatography. Gas chromatography-mass spectrometry was used to characterise the compound profile present in the ruminal environment. Sequences were classified into 80 different genera, belonging to 10 phyla. In all treatments, Firmicutes and Bacteroidetes represented more than 70% of all prokaryotes identified. The phyla Verrucomicrobia and Spirochaetes and the genera Flavonifractor, Treponema and Ruminococcus presented a reduction ($P<0.03$) in their population when the cattle were submitted to high temperatures. The genus *Saccharibacteria* (*incertae sedis*), belonging to a phylum of uncultivated bacteria (Candidatus Saccharibacteria), presented higher ($P=0.05$) population in animals under thermal stress conditions. The concentration of isobutyric acid was lower ($P<0.01$) in the rumen of bovine under heat stress conditions. A total of 45 different compounds were identified in the ruminal samples. There was no standard compound profile related to heat stress. The amine dihydro-5-pentyl-2 (3H)-furanone was identified in 100% of the samples following heat stress treatment. The heat stress caused alterations in the ruminal microbiota composition and also in the rumen fermentative process.

Keywords: Thermal comfort, Solid-phase micro-extraction (SPME), Volatile fatty acids (VFA).

Introduction

Ruminant animals reared in grazing systems are constantly exposed to natural climatic conditions. The tropical and subtropical regions are characterised by high average temperatures and low seasonal variation. Heat stress is a physiological condition resulting from a negative balance between the net quantity of energy that flows from an animal to the external environment and the amount of thermal energy produced by the animal [1]. Animal productivity is maximised within specific environmental conditions. When the temperature is above or below the ideal values, profitability is compromised [2]. Diet is considered the main variable affecting rumen microbial fermentation [3]. However, changes in the host metabolism caused by heat can induce changes in ruminal microbiota and affect the absorption of nutrients [4, 5, 6 and 7]. Heat stress can reduce productivity and reproductive efficiency due to reduction in dry matter intake, reduction in ruminal motility and contraction, change in the fermentation pattern and volatility in fatty acid production, affecting the dynamic characteristics of digestion and nutrient utilisation [2, 8, 9, 10 and 11]. Some studies have been carried out with the purpose of analysing the effects of heat stress on the physiology of bovine animals [5, 10, 11, 12 and 13]. However, there are few studies that have analysed the possible effects of heat stress on the diversity of ruminal microorganisms and the production of metabolites during feed fermentation. To the best of our knowledge, only two studies describe the effect of temperature on cattle rumina; these studies evaluated Holstein heifers [6 and 7]. We did not find studies evaluating the effect of heat stress on beef cattle microbiota. Tajima et al. [6] and Uyeno et al. [7] concluded that heat stress caused a change in the composition of ruminal microbiota, and also the number of metabolites produced by fermentation. These changes may have been caused by changes in food intake resulting from heat stress [7].

The main limitation to the study of microorganisms present in the rumen is that a large proportion of these microorganisms are considered uncultivable. The fact that these organisms are anaerobic and there are strong associations between different groups of microorganisms hinders their cultivation in the laboratory. The technique of Next-Generation DNA Sequencing (NGS) has been highlighted in the analysis of these microorganisms because it is a technique independent of cultivation and has a high level of accuracy [14-16]. The objective of this work was to study the metagenome of prokaryotes and the profile of metabolites produced in the rumen of cattle subjected to

conditions of comfort and heat stress, in free or restricted consumption of high- or low-energy diets, in order to isolate the effect of the consumption level.

Materials and Methods

The trial was conducted at the Federal University of Lavras, in Brazil. All the experimental procedures involving animals followed the ethical precepts for studies involving animals at this University (protocol 022/2015). Six pure-breed zebu heifers, with initial average weight of 280 kg, a mean age of 12 months, and fitted with cannulas were used in the study. The experiment was carried out in an environmentally controlled laboratory (bioclimatic chamber). Each one of the bioclimatic chambers was equipped with exhaust fans for forced circulation and constant renewal of air, air conditioners, infrared heating lamps and humidity controllers. Six treatments were tested in a 6 × 6 Latin Square with a 2 × 2 + 2factorial arrangement, containing two factors (dietary temperature and energy concentration) and two additional treatments, one for each diet, in order to obtain the means of comparison of the temperature effects on the proposed variables without interference of the consumption level. Two temperatures were evaluated: term neutrality (24ºC permanently) and heat stress (34º C from 6:00 AM to 6:00 PM, and 24ºC from 6:00 PM to 6:00 AM). In two treatments (HHL and HLL), the animals were under heat stress conditions (H), on a diet of high (H) or low (L) energy values, feeding without restriction (ad libitum) (L). In treatments three and four (CHL and CLL), the animals were in thermal comfort conditions (C), consuming, respectively, diets of high (H) and low (L) energy values, with no restriction on the amount of food (L). In the two additional treatments (CHR and CLR), as in the two previous treatments, the animals were in thermally comfortable conditions, consuming diets with high and low energy levels, but in these treatments the animals had their feeding restricted (R), the same amount as when they were in heat stress conditions. The proposed diets were isonitrogenated, with variations in energy concentration, consisting of corn silage and corn, soybean meal, and a urea and mineral mixture for composition of the concentrate. The diets represented, respectively, a diet used in the majority of Brazilian feedlots with high concentrate content, called here a high energy level diet [20]; and a diet with a level of total digestible nutrients (TDN), similar to grazing systems, with low/medium levels of supplementation, called a low energy diet [21]. The proportions of the ingredients and energy levels of protein and fiber for both diets are presented in Table 1.

Corn silage used in the diet was obtained from a single silo, for which there was no effect of different silage on ruminal microbiota. The animals were fed twice a day, at 6:00 AM and 06:00 PM.

Table 1- Ingredients and chemical composition of the high and low energy level diets used in the experiment

Item	**Diet**	
	High energy level	Low energy level
Ingredients Proportion (%)		
Corn Silage	35.0	85.0
Corn Grain	49.3	4.3
Wheat bran	10.0	4.5
Soy Bran	4.0	4.0
Urea + Ammonium Sulphate	0.6	1.1
Mineral Solution	1.1	1.1
Dry Matter	69.4	74.8
Nutritional Composition (%)		
OM	91.1	93.2
CP	12	12
FDNp	20.9	37
NFC	50.5	37
NDFi	14.6	15.5
EE	4.44	3.19

OM= Organic matter; CP= Crude protein; NDFp= Neutral Detergent Fiber corrected for protein NFC= Non-fibrous carbohydrates; NDFi= Indigestible Neutral Detergent Fiber; EE= Ethereal Extract.

Experiment comprised six periods of 17 days. In each period there were 16 days of adaptation to the diets and climate conditions. On the seventeenth day of each period one sample was collected with the animal in fasting and one sample two hours after feeding. Collection of the samples was performed using the ruminal emptying method. For this experiment, these samples (before and after) were mixed, one composite sample being taken for each animal in each period. The sample was divided into two parts, one being used for DNA extraction and the other for chromatographic analysis and to determine the rumen pH level. The samples were immediately frozen in liquid nitrogen to stop fermentation and stored at -20ºC.

Metagenomic analysis

Rumen fluid samples (40 mL) were centrifuged (20 min, 480 g) and the pellet used for metagenomic DNA extraction. DNA was extracted according to the protocol for phenol/chloroform extraction as described by Bashir et al. [22]. After the extraction, the DNA integrity was conferred by agarose gel electrophoresis and the concentration and purity of DNA was determined using QIAxpert (Qiagen, USA).

DNA samples were submitted to amplification reactions to amplify the ribosomal 16S DNA region, following the protocol described by Klindworth et al. [14]. The primer pairs used were: S-D-Bact-0341-B-S-17, 5'-CCTACGGGNGGCWGCAG-3', and S-D-Bact-0785-a-A-21, 5'-GACTACHVGGGT ATCTAATCC-3'. The reaction was carried out in 50 ml volumes containing 0.3 mg/ml Bovine Serum Albumin (BSA), 250µM dTNPs, 0.5 µM of each primer, 0.02 U DNA Polymerase enzyme and 5x buffer containing 1.5mM $MgCl_2$. The following PCR conditions were used: initial denaturation at 95ºC for 5 minutes, followed by 25 cycles consisting of denaturation (95ºC for 40 s), annealing (55ºC for 2 minutes) and extension (72ºC for 1 minute) and a final extension step at 72ºC for 7 minutes. The amplicons were sequenced by the latest generation sequencing platform, MiSeqSequencing System (IlluminaLtd., Cambridge, UK).

DNA sequences were analyzed using Geneious 10.2.3 software [23]. Initially the primer pairs were removed from the forward and reverse sequences using the TrimEnds tool. The forward and reverse sequences were aligned using the Merge Paired reads tool to form a single sequence consensus. Sequences were analyzed in order to remove the low quality ones and the Workflows tool was used to exclude the sequences with less than 200 pb. After filtering the sequences, the CHIMERAS were removed, through the UCHIME algorithm, where the sequences were purchased with the SILVA database [23]. Then, the 16S Biodiversity tool was used for taxonomic analysis of the microorganisms in the samples. This tool assigned taxonomy to each sequence, comparing them with the Ribosomal Database Project (RDP) Classifier [25].

The Menhinick (Dmn) index was calculated to evaluate the richness (abundance) of species in each treatment. To identify the diversity of microorganisms in each sample the Shannon (H') index was analyzed, which takes into account both the uniformity (evenness) and the species richness, recommended for situations in which the entire community cannot be inventoried. The higher the value of H', the greater the diversity of species in the sampled environment. Species dominance was calculated by

the Simpson (D) index, which is based on the probability of two individuals randomly selected in a community belonging to the same species. The Simpson index varies from 0 to 1, and values closer to 1 indicate domination of one or more species in a given environment [26].

Characterization of Compounds by High Performance Liquid Chromatography (HPLC)

Organic acids (acetic, propionic, butyric, valeric, isovaleric and isobutyric) were determined by HPLC (Shimadzu, model LC-10Ai, Shimadzu Corp., Kyoto, Japan) equipped with a detection system of Ultraviolet-Visible (UV–Vis) detector (SPD 10Ai). HPLC was operated at 50 C for acids and detected via UV absorbance (210 nm). Column used for separation was a Shimadzu ion exclusion column SCR-101H (Shim-pack, 7.9 mm × 30 cm, Shimadzu, Kyoto, Japan) with a mobile phase of Perchloric acid (100 mM) at a flow rate of 0.6 mL/min. Chemical compounds used as standards (purity 99.8%) were purchased from Merck (Darmstadt, Germany).

Characterization of Volatile Compounds by Headspace-Solid Phase Micro-extraction Gas Chromatography-Mass Spectrometry

Gas Chromatography associated with Mass Spectrometry was used to identify the volatile compounds from rumen samples relating to the HHL, HLL, CHR and CLR treatments, to identify compounds in treatments with different temperatures, isolating the effect of the amount of feed consumed by the animals in these different treatments. Extraction of the compounds was performed using the Solid Phase Micro-extraction (SPME) technique, according to Spinhirne et al. [27]. Initially, 2 mL of each sample were added to a vial (15 mL) and hermetically sealed. A divinylbenzene / carboxen / polydimethylsiloxane (DVB/CAR/PDMS) 50/30 mm SPME fiber (Supelco Co., Bellefonte, PA, USA.) was used to extract volatile constituents from the headspace. The fiber was equilibrated for 5 minutes at 39°C and then exposed to the samples for 15 minutes at the same temperature. Injections were performed by fiber exposition for 3 minutes. The column oven initial temperature was 60ºC, followed by a first ramp of 60ºC/min to 110ºC, a second ramp of 10ºC/min to 210ºC, and finally a third ramp of 60ºC/min to 250ºC with a final hold time of 3 minutes, and the total running time was 35 minutes. Helium gas (99.9% purity) was used in the mobile phase at a constant flow of 1 mL/min along the range of compounds. Analytes were separated in a Carbowax

20M (30 m x 0.25 mm x 1.5 µm) capillary column. A quadruple mass analyzer was used, with an electron impact ionization system, operated at 70 eV and 260°C. Volatile compounds were identified by comparing the mass spectra compounds in the samples with the National Institute of Standards and Technology (NIST library, Gaithersburg, MD, USA) data base and the retention time with literature data using the n-Alkane index.

Statistical analysis

Data obtained from the metagenomic analysis (diversity index and taxonomy) and High-Performance Liquid Chromatography were analyzed by variance analysis, using SAS software, version 9.4 (SAS Inst. Inc., Cary, NC), adopting the treatment as fixed and animal and period as random effects. Means were compared by means of contrasts considering two types of 2×2 factorial arrangement, containing two temperatures (thermal comfort and heat stress) and containing two diets (high or low energy concentration). The additional treatments in the first evaluation were not considered, therefore the comparisons were in 'Free consumption.' In the second evaluation, called 'Restricted consumption,' data from the two additional treatments were used in place of the treatments in thermal comfort and free consumption. Thus, for metagenomic and liquid chromatography analysis there is the effect of temperature, diet and interaction between the two in a scenario of possible interference on consumption, or not. Gas Chromatography-Mass Spectrometry identified volatile compounds in repetition, aiming only to identify the compounds without doing statistical analysis.

Results

Diversity of Prokaryotes - Metagenomic Analysis

Estimates of diversity, based on the number of Operational Taxonomic Units (OTUs) present in each sample, are shown in Table 2. When the diet was restricted, the animals present in the thermal comfort treatments presented higher (P=0.04) species richness than the animals that were under heat stress. There was no significant change in the values calculated for the Shannon diversity index (H') and the Simpson dominance index (D). There was no difference in the number of OTUs among the treatments.

Table 2 - Estimates of diversity based on metagenomic analysis

	Treatment						Contrasts					
Number	1	2	3	4	5	6	Free Consumption 1 and 2 vs. 3 and 4 1 and 3 vs. 2 and 4			Restricted Consumption 1 and 2 vs. 5 and 6 1 and 5 vs. 2 and 6		
Temperature	Heat	Heat	Confort	Confort	Confort	Conforto						
Diet* Consumption	High Free	Low Free	High Free	Low Free	High Restricted	Low Restricted	T	D	TxD	T	D	TxD
	Index (% de OTUs)						P-valor					
Menhinick (Dmn)	5.27±0.55	6.35±0.55	7.02±0.61	6.44±0.55	6.76±0.61	7.24±0.61	0.10	0.63	0.13	0.04	0.17	0.58
Shannon (H')	4.42±0.25	4.49±0.25	4.95±0.27	4.49±0.25	4.72±0.27	4.88±0.27	0.26	0.40	0.25	0.16	0.63	0.86
Simpson (D)	0.032±0.021	0.063±0.021	0.017±0.023	0.058±0.021	0.034±0.023	0.027±0.023	0.63	0.10	0.82	0.44	0.57	0.39
OTUs	250±34.9	278±34.9	274±38.3	270±34.9	264±38.3	322±38.3	0.78	0.67	0.59	0.34	0.17	0.63

** High and low energy level diets. T= temperature. D= diet. TxD= interaction between temperature and diet. ND = Not detectable*

Using Geneious 10.2.3 software and the RDP classifier database, the sequences (operational taxonomic unit) were classified from phyla to genera. The treatments showed different taxonomic compositions (Figure 1, Tables 3 and 4). Ten different phyla were identified (Figure 1), among which Firmicutes and Bacteroidetes were the most abundant, representing together more than 70% of the total number of microorganisms identified. Among the phyla identified, Euryarchaeota (Archaea) and Proteobacteria accounted for on average 4% and 3%, respectively (Table 3). Candidatus Saccharibacteria, Actinobacteria, Spirochaetes, Lentisphaerae, Planctomycetes and Verrucomicrobia were the other phyla identified in all samples. On average, 3% of the phyla present in the samples were not classified.

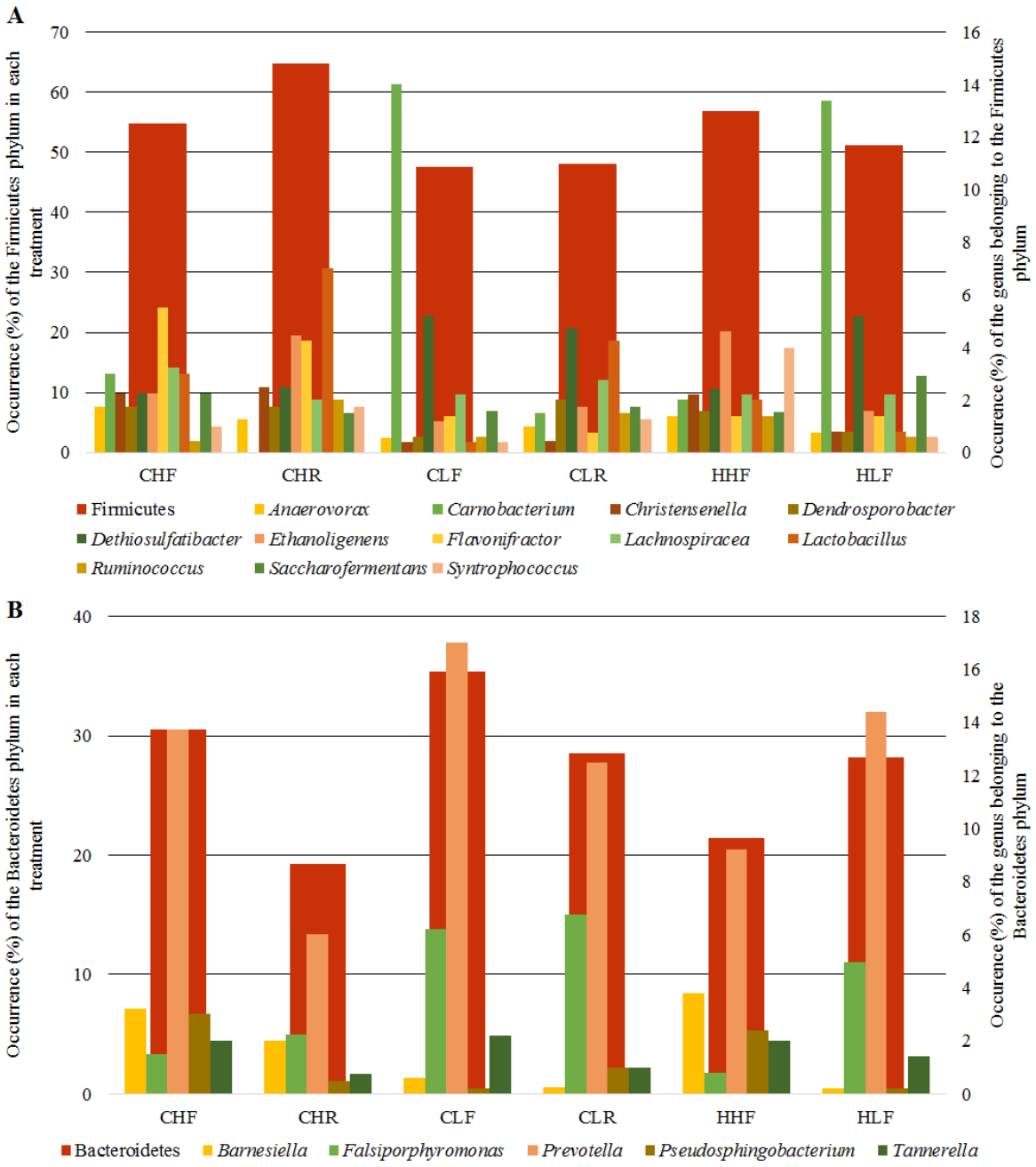

Figura 1: Porcentagem de ocorrência dos filos Firmicutes e Bacteroidetes e dos gêneros pertencentes à esses filos em cada tratamento. Treatments: **CHF** - termalc onfort, high energy, free consumption; **CHR** - termal confort, high energy, restricted consumption; **CLF** - termal confort, low energy, free consumption; **CLR** - termal confort, low energy, restricted consumption; **HHF** - heat stress, high energy, free consumption; **HLF** - heat stress, low energy, free consumption.

Changes in ambient temperature, diet and level of consumption changed the percentages of some phyla and genera. The percentage of individuals belonging to the phylum Firmicutes increased ($P=0.05$) in the treatments with high-energy diets, while the phyla Planctomycetes and Lentisphaerae showed reductions ($P<0.05$) in these treatments (Table 3). Heat stress reduced ($P=0.03$) the percentages of the phyla Verrucomicrobia and Spirochaetes. The effect of heat stress on the Spirochaetes was lower in high-energy diets. There was a tendency ($P=0.06$) towards an increase in the percentage of bacteria belonging to the phylum Candidatus Saccharibacteria in treatments with conditions of heat stress.

Table 3 - Differences between the occurrence of major phyla in the different treatments evaluated

	Treatment						Contrasts					
Number	1	2	3	4	5	6	Free Consumption			Restricted Consumption		
Temperature	Heat	Heat	Confort	Confort	Confort	Conforto	1 and 2 vs. 3 and 4 1 and 3 vs. 2 and 4			1 and 2 vs. 5 and 6 1 and 5 vs. 2 and 6		
Diet*	High	Low	High	Low	High	Low	T	D	TxD	T	D	TxD
Consumption	Free	Free	Free	Free	Restricted	Restricted						
	Phyla (%)						P-valor					
Firmicutes	57.0±5.8	51.0±5.8	56.5±6.2	47.7±5.8	63.0±6.2	50.0±6.2	0.67	0.11	0.74	0.57	0.05	0.45
Bacteroidetes	21.4±7.0	28.2±7.0	28.5±7.6	35.4±7.0	21.1±7.6	26.5±7.6	0.21	0.22	0.99	0.86	0.30	0.90
Euryarchaeota	5.8±1.9	3,0±1.9	1.5±2.2	3.0±1.9	6.3±2.2	4.7±2.2	0.30	0.74	0.29	0.59	0.30	0.76
Actinobacteria	2.2±1.3	2.6±1.3	1.4±1.4	0.9±1.3	2.3±1.4	5.0±1.4	0.29	0.96	0.69	0.29	0.21	0.36
Proteobacteria	5.0±2.1	3.0±2.1	2.0±2.3	2.8±2.1	1.2±2.3	3.3±2.3	0.47	0.78	0.52	0.43	0.98	0.37
CandidatusSaccharibacteria	4.4±1.1	3.8±1.1	1.3±1.2	2.4±1.1	2.5±1.2	2.5±1.2	0.06	0.80	0.44	0.18	0.81	0.80
Planctomycetes	0.7±0.4	1,0±0.4	0.4±0.4	1.5±0.4	0.4±0.4	1.7±0.4	0.63	0.05	0.26	0.44	0.03	0.18
Lentisphaerae	0.2±0.7	1.6±0.7	0.6±0.8	2.4±0.7	0.4±0.8	1.1±0.8	0.32	0.01	0.71	0.77	0.09	0.52
Verrucomicrobia	ND	0.4±0.4	0.7±0.4	0.8±0.4	0.9±0.4	1,0±0.4	0.08	0.38	0.65	0.03	0.44	0.60
Spirochaetes	1.2±0.8	0.4±0.8	4.4±0.8	0.4±0.8	0.9±0.8	0.1±0.8	0.02	<0.01	0.02	0.66	0.25	0.98
Others	2.4±1.2	4.6±1.2	2.6±1.4	3.0±1.2	2.2±1.4	3.8±1.4	0.59	0.31	0.48	0.67	0.15	0.83

** High and low energy level diets. T= temperature. D= diet. TxD= interaction between temperature and diet. ND = Not detectable*

Eighty different genera were identified in the samples evaluated (Supplementary Table S1). The twenty genera with greater abundance and prevalence are presented in Table 4. Among the twenty major genera, twelve belong to the phylum Firmicutes, while five belong to the phylum Bacterioidetes (Figure 1). Only one genus each was identified as belonging to the phyla Spirochaetes, Euryarchaeota and Candidatus Saccharibacteria. On average, 26% of the genera were not classified (Supplementary Table S1). The genus *Prevotella* presented the greatest abundance in all treatments, except in the CHR treatment, where the genus *Lactobacillus* was dominant (Table 4). The genus *Methanobrevibacter* was the only genus found from the Archaea domain.

The genera *Anaerovorax*, *Christensenella*, *Pseudosphingobacterium* and *Syntrophococcus* exhibited a higher ($P<0.03$) proportion when the cattle were consuming high-energy diets. While the genera *Carnobacterium*, *Dethiosulfatibacter* and *Falsiporphyromonas* showed a greater ($P<0.03$) percentage of individuals in animals fed low-energy diets. The genera *Flavonifractor* and *Treponema* showed a greater ($P<0.01$) percentage of individuals when the animals were on high-energy diets, and the thermal comfort accentuated this result. Individuals of the genus *Ruminococcus* were of higher ($P=0.03$) occurrence in treatments with thermal comfort and feed restrictions, when compared with the animals under heat stress and free consumption. The genus of bacteria not cultivated, *Saccharibacteria* (*incertae sedis*), presented a higher ($P=0.05$) percentage of individuals in treatments where the heifers were under heat stress conditions. The pH values were lower in high-energy diets ($P<0.03$) and in the treatments under heat stress ($P=0.07$), when compared with treatment under thermal comfort and feed restrictions (Table 5).

Table 4 - Differences between the occurrence of main genera in the different treatments evaluated

	Treatment						Contrasts					
Number	1	2	3	4	5	6	Free Consumption			Restricted Consumption		
Temperature	Heat	Heat	Confort	Confort	Confort	Conforto	1 and 2 vs. 3 and 4 1 and 3 vs. 2 and 4			1 and 2 vs. 5 and 6 1 and 5 vs. 2 and 6		
Diet* Consumption	High Free	Low Free	High Free	Low Free	High Restricted	Low Restricted	T	D	TxD	T	D	TxD
	Genera (%)						P-valor					
Anaerovorax	1.4±0.4	0.7±0.4	1.8±0.4	0.6±0.4	1.3±0.4	1,0±0.4	0.62	0.01	0.42	0.77	0.16	0.66
Barnesiella	3.8±1.8	0.2±1.8	3.3±2,0	0.6±1.8	2.1±2,0	0.3±2,0	0.96	0.09	0.79	0.65	0.15	0.61
Carnobacterium	2.0±5.1	13.4±5.0	3.0±5.6	14.0±5.1	ND	1.5±5.6	0.87	0.03	0.96	0.15	0.18	0.37
Christensenella	2.2±0.5	0.8±0.5	2.3±0.6	0.4±05	2.5±0.6	0.5±0.6	0.74	0.01	0.67	0.96	<0.01	0.55
Dendrosporobacter	1.6±0.7	0.8±0.7	1.7±0.8	0.6±0.7	1.8±0.8	2.0±0.8	0.96	0.16	0.80	0.31	0.67	0.46
Dethiosulfatibacter	2.4±1.1	5.0±1.1	2.4±1.3	5.5±1.1	2.9±1.3	4.4±1.3	0.78	0.01	0.84	0.98	0.07	0.61
Ethanoligenens	4.6±1.5	1.6±1.5	2.3±1.7	1.2±1.5	4.4±1.7	1.8±1.7	0.36	0.17	0.52	0.99	0.07	0.89
Falsiporphyromonas	0.8±1.8	5.8±1.8	0.9±2,0	5.9±1.8	1.5±2,0	6.8±2,0	0.96	<0.01	0.97	0.59	<0.01	0.93
Flavonifractor	1.4±0.7	1.4±0.7	5.6±0.7	1.4±0.7	4.2±0.7	0.8±0.7	<0.01	<0.01	<0.01	0.07	0.01	0.01
Lachnospiracea	2.3±0.6	2.2±0.6	3.2±0.7	2.2±0.6	2.0±0.7	2.8±0.7	0.45	0.32	0.42	0.78	0.60	0.48
Lactobacillus	2.0±2.1	0.8±2.1	3.0±2.4	0.4±2.1	6.8±2.4	4.3±2.4	0.89	0.37	0.74	0.07	0.39	0.76
Methanobrevibacter	5.6±1.9	3,0±1.9	1,0±2.1	2.6±1.9	6.3±2.1	4.5±2.1	0.21	0.80	0.29	0.60	0.29	0.84
Prevotella	9.0±3.4	14.4±3.4	12.5±3.7	17.1±3.4	6.5±3.7	11.2±3.7	0.31	0.11	0.89	0.35	0.11	0.91
Pseudosphingobacterium	2.4±1.0	0.3±1.0	3.0±1.1	0.2±1.0	0.4±1.1	1,0±1.1	0.80	0.03	0.72	0.56	0.47	0.22
Ruminococcus	1.5±0.5	0.4±0.5	0.5±0.5	0.6±0.5	1.9±0.5	1.8±0.5	0.29	0.22	0.13	0.03	0.13	0.27
Saccharibacteriagenera	4.2±1.1	3.8±1.1	1,0±1.3	2.4±1.1	1.7±1.3	2.5±1.3	0.05	0.66	0.43	0.11	0.85	0.60
Saccharofermentans	1.6±0.7	2.9±0.7	2.1±0.8	1.5±0.7	1.3±0.8	2.1±0.8	0.52	0.56	0.16	0.40	0.12	0.70
Syntrophococcus	4.0±1,0	0.4±1,0	1.2±1.1	0.6±1,0	1.8±1.1	1.5±1.1	0.15	0.03	0.10	0.57	0.05	0.09
Tannerella	1.9±1.4	1.4±1.4	2.0±1.5	2.2±1.4	1.7±1.5	0.5±1.5	0.64	0.88	0.75	0.65	0.47	0.79
Treponema	1.0±0.7	0.2±0.7	4.4±0.8	0.2±0.7	0.6±0.8	0.2±0.8	0.01	<0.01	0.01	0.70	0.34	0.74
Others	44.2±3.3	40.8±3.3	41.4±3.7	40.0±3.3	49.3±3.7	47.6±3.7	0.61	0.49	0.76	0.11	0.47	0.81

** High and low energy level diets. T= temperature. D= diet. TxD= interaction between temperature and diet. ND = Not detectable*

Metabolites Profile - High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Spectrometer (GC-MS)

Acetic, propionic and butyric acids presented the greatest concentrations independently of treatment (Table 5). The acetic and butyric acids presented lower ($P<0.05$) concentrations in the treatments in which the animals were consuming high-energy diets, regardless of the level of consumption and without temperature effect. In relation to the total number of VFAs, the percentages of these two acids were also lower ($P<0.01$) in the treatments with high-energy diets.

Although there have been no significant changes in the concentration of propionic acid in different treatments, there was a greater ($P<0.01$) percentage of this acid in relation to the total number of VFAs in treatments where the cattle were consuming high-energy diets (Table 5). The data shows a tendency ($P=0.07$) towards an increase in propionic acid in relation to the total VFAs in treatments under heat stress. The isobutyric acid was the only one that presented alteration in its concentration due to the temperature effect. In the treatments in which animals were under heat stress conditions, there was a greater ($P<0.01$) concentration of isobutyric acid regardless of the diet consumption level. The low-energy diet emphasised this fact by increasing the concentration of this acid.

Table 5– Concentration of organic acids quantified by High Performance Liquid Chromatography (HPLC)

	Tratamento						Contrastes					
Número	1	2	3	4	5	6	Free Consumption			Restricted Consumption		
Temperatura	Heat	Heat	Confort	Confort	Confort	Conforto	1 and 2 vs. 3 and 4 1 and 3 vs. 2 and 4			1 and 2 vs. 5 and 6 1 and 5 vs. 2 and 6		
Dieta* Consumo	High Free	Low Free	High Free	Low Free	High Restricted	Low Restricted	T	D	TxD	T	D	TxD
	Acids (mM)						P-valor					
Acetic	50.47±7.22	71.31±6.51	56.04±7.20	61.66±6.52	56.19±7.21	63.35±7.20	0.74	0.05	0.23	0.86	0.04	0.29
Propionic	16.89±2.31	17.40±2.10	12.08±2.31	15.96±2.10	13.35±2.30	15.16±2.31	0.12	0.27	0.38	0.16	0.56	0.75
Butyric	7.49±1.01	11.21±0.91	8.89±1.01	10.47±0.91	8.03±1.01	9.44±1.01	0.72	0.01	0.26	0.51	0.01	0.23
Isobutyric	2.93±0.67	5.71±0.60	2.86±0.67	2.20±0.60	1.84±0.67	2.50±0.67	0.01	0.10	0.01	<0.01	0.01	0.11
Isovaleric	3.56±1.04	1.14±0.93	2.08±1.04	0.65±0.93	1.51±1.04	0.98±1.04	0.33	0.07	0.62	0.29	0.16	0.36
Valeric	0.87±0.18	0.96±0.16	1.12±0.18	0.84±0.16	0.76±0.18	0.79±0.18	0.67	0.53	0.26	0.39	0.72	0.88
AGV total[2]	70.48±9.10	89.24±8.23	72.55±9.07	77.01±8.25	76.50±9.08	79.58±9.07	0.50	0.14	0.35	0.81	0.18	0.33
% Acetic	71.44±2.33	80.21±2.11	77.02±2.32	79.96±2.12	73.80±2.33	80.41±2.32	0.18	<0.01	0.15	0.51	<0.01	0.59
% Propionic	18.01±2.44	6.87±2.20	10.65±2.43	6.20±2.21	15.78±2.43	7.70±2.43	0.07	<0.01	0.12	0.73	<0.01	0.48
% Butyric	12.11±1.04	10.53±1.04	13.92±0.95	12.17±1.04	10.75±1.04	12.81±0.95	0.16	0.04	0.88	0.62	0.05	0.81
Acetic/Propionic	3.11±0.53	3.62±0.53	4.05±0.62	3.39±0.53	4.80±0.53	3.37±0.62	0.53	0.88	0.31	0.22	0.42	0.10
pH	5.69±0.22	6.30±0.22	5.53±0.24	6.43±0.24	6.21±0.24	6.47±0.24	0.93	<0.01	0.43	0.07	0.03	0.35

[1]High and low energy level diets. T= temperature. D= diet. TxD= interaction between temperature and diet. [2]AGV total = Acetic + Propionic + Butyric

Volatile compounds present in the samples from the HHL, HLL, CHR and CLR treatments were analysed by gas chromatography associated with a mass spectrometer. Forty-nine compounds were identified in all samples of the ruminal fluid analysed (Table 6). Among the compounds found, 10 are classified as acids, 12 as esters, 9 as alcohols, 4 as phenols, 4 as aldehydes, 8 as alkanes/ketones and 2 as amines. The presence of these compounds occurred differently in the treatments evaluated. Enanthic acid, tetradecanoic acid, stearic acid ethyl ester, and 1-butanol-3-methyl were only present in animals under heat stress conditions. The compounds heptadecanoic acid ethyl ester and 2-pentadecanone were found only in animals in thermal comfort. The other compounds occurred at random in the treatments on stress and thermal comfort.

Acetic, propionic, butyric, valeric, isobutyric and caproic acids, together with tphenol, were found in 100% of the samples analysed, regardless of treatment. Amine dihydro-5-pentil-2(3H)-furanona, was found in higher frequency in animals under conditions of heat stress. This amine was identified in 100% of the samples of treatments with high temperatures, and in less than 50% of the samples collected in the animals that were in conditions of term neutrality. Regardless of environmental conditions, the majority of the esters were identified in the treatments with high-energy diets. Amine 3-methyl-1H-Indole was found with greater frequency in samples of treatments with high-energy diets, and was present in 100% of the samples from the HHL and CHR treatments.

The phenolic compounds and the aldehydes showed no variations related to heat stress. However, these compounds had a higher occurrence in animals that belonged to the treatments with low-energy diets; for example, p-cresol was found in 100% of the samples from the HLL and CLR treatments.

Table 6– Percentage of occurrence of volatile compounds identified by Gas Chromatography - Mass Spectrometer.

Compounds	Heat stress		Comfort	
	High	Low	High	Low
Acids 20,4%				
Acetic acid				
Propanoic acid				
Butanoic acid				
Valeric acid				
Isobutyric acid				
Caproic acid				
Isovaleric acid				
Enanthic acid				
Phosphonic acid				
Butanoic acid, 2-methyl-				
Esters 24,5%				
Benzeneacetic acid, ethyl ester				
Pentadecanoic acid, ethyl ester				
Heptadecanoic acid, ethyl ester				
Docosanoic acid, ethyl ester				
Benzenepropanoic acid, ethyl ester				
Tetradecanoic acid, ethyl ester				
Hexadecanoic acid, ethyl ester				
Stearic acid, ethyl ester				
Lauric acid, ethyl ester				
Myristic acid, ethyl ester				
Palmitic acid, ethyl ester				
Salicylic acid methyl ester				
Alcohols 18,4%				
1-Pentanol				
1-Hexanol				
Phenylethyl Alcohol				
1-Dodecanol				
Benzeneethanol				
Benzenepropanol				
3-Phenylpropanol				
1-Butanol, 3-methyl				
1-Tridecanol				
Fenóis 8,2%				
Phenol				
Phenol, 3-propyl				
Phenol, 4-ethyl-				
p-Cresol				

Compounds	Heat stress		Comfort	
	High	Low	High	Low
Aldehydes8,2%				
Tridecanal				
Tetradecanal				
Pentadecanal				
Hexadecanal				
AlkanesandKetones 16,3%				
Pentadecane				
Hexadecane				
Heptadecane				
Octadecane				
Nonadecane				
Heneicosane				
2-Pentadecanone				
Acetophenone				
Amines 4,1%				
1H-Indole, 3-methyl-				
2(3H)-Furanone, dihydro-5-pentyl-				

Occurrence (% of samples) of each compound found	80-100%	60-75%	40-50%	20-25%	0%

Discussion

Bovines subjected to treatments in thermal comfort with restricted dietary consumption showed greater (P=0.04) richness (abundance) of species when compared with animals in heat stress conditions, but this value does not result in higher (P>0.16) diversity of prokaryotes. The elevated values of the diversity index (H'>4.42) calculated for all treatments, along with the low values of dominance (D<0.063), indicate that no species are dominant, thereby ensuring the uniformity of species in the rumen, maintaining the diversity of prokaryotes. The high values calculated of H' allow us to conclude that conditions of heat stress, even when affecting certain groups of prokaryotes, do not lead to a reduction of diversity. Rumen microbiome composition is influenced by several factors such as diet, environment, age and physiological status [3, 6]. However, this microbiota tends to maintain homeostasis for the perfect functioning of the fermentation process [3]. To the best of our knowledge, studies that have evaluated the effect of heat stress on rumen microbial composition are scarce in the literature. In this study, we observed that heat stress changed the microorganism

population (number of individuals) in the rumen, but did not alter the composition of the microbiota. The same result was observed in Holstein heifers, where no differences were found in the total number of OTUs and in the values of the Shannon index [6].

It is already known that heat stress causes alterations in the physiology of an animal, the decline in food consumption being the most obvious [3.28]. The reduction in consumption may be one of the causes of changes in the microbiota due to heat stress [6]. To isolate this effect, we used treatments where the animals were on thermal comfort and feed restrictions (CHR and CLR). These treatments have reinforced the effect of heat stress on some variables and highlighted changes in the phylum Firmicutes and in the genus *Ruminococcus*.

In all treatments, the phyla Firmicutes and Bacteroidetes represented more than 70% of the total number of prokaryotes observed. These values are in agreement with the results already described, according to which these phyla are the most abundant in the rumen of cattle subjected to different conditions and treatments [5, 15, 29]. Heat stress did not cause significant changes in the percentage of individuals in the two major phyla. The phylum Firmicutes showed a higher percentage of OTUs in heifers fed high-energy diets, but this result was only evident when there were restrictions on the consumption. However, two genera belonging to this phylum did not respond in the same way to a change of diet. Five genera belonging to the phylum Firmicutes *(Anaerovorax*, *Christensenella*, *Flavonifractor*, *Pseudosphingobacterium* and *Syntrophococcus*) had a higher percentage in the treatments with a high-energy diet; however, the genera *Carnobacterium* and *Dethiosulfatibacter* had a higher percentage in treatments where the diet was low-energy. Fernando et al. [30] also observed different behaviours between genera within the same phylum. During the adaptation to a high-energy diet, there was no change in the population of Firmicutes, but the families *Clostridiaceae* and *Acidaminococcaceae*, belonging to this phylum, had a population increase due to the increase of energy available in the diet [30].

The genus *Prevotela*, recognised by pectinolytic, proteolytic and hemicellulolytic activity [31], was the genus that presented greater representativeness in the treatments. Other studies have already demonstrated that this genus is the most abundant in the rumen of cattle and that individuals belonging to this genus play different metabolic functions in the rumen ecosystem [32-34]. The genus *Methanobrevibacter* was the only genus of Archaea found. This genus is one of the

main genera of Archaea described for the ruminal environment [31, 35]. Most species of methanogenic Archaeas can grow using H_2 and formate as energy sources, using the electron derivatives of these compounds to reduce the CO_2 in CH_4. The removal of the H_2 in the ruminal environment ensures a standard of VFA production and decreases the inhibitory effect of H_2 for microbial fermentation [35, 36].

Thermal comfort with feed restrictions increased the population of *Ruminococcus*. This genus represents one of the main groups described of cellulolytic bacteria in the rumen ecosystem [33, 37]. In free consumption there was no effect of temperature on the percentage of individuals of this genus. Tajima et al. [5] analysed the effect of heat stress on bacteria of the species *Ruminococcus flavefaciens* present in the rumen of cattle, and concluded that there was no significant difference in the population of this species as a result of increased ambient temperature. In the study of Tajima et al. [5], there were no restrictions on the dietary consumption of the animals that were on thermal comfort conditions, and these results are consistent with those obtained in this study. The increase in temperature reduced the *Ruminococcus* population ($P=0.03$) only when there was a restriction on the cattle's consumption. The reduction in the percentage of *Ruminococcus* due to heat stress may represent a reduction in cellulose degradation in the rumen.

The largest percentage of individuals related to the phylum Spirochaetes (genus *Treponema*) was found in the treatments with thermal comfort and high-energy diets. A study by Pastert and Canale-Parola [38] of the physiological diversity of bacteria belonging to the phylum Spirochaetes in the rumen of cattle revealed that all the representatives of this phylum that were identified belonged to the genus *Treponema*. According to these authors, the species belonging to this genus are capable of using polymers, such as pectin, xylan and arabinogalactan, as fermentable substrates, but not cellulose, which explains the higher occurrence of this phylum in high-energy diets.

The genus *Flavonifractor* also presented a higher percentage of individuals in the treatment of thermal comfort. This genus was described by Carlier et al. [39]: they are strictly anaerobic bacilli that can ferment glucose, fructose and ribose, and metabolise flavonoids; butyric and acetic acids are the main final products of their metabolism. Flavonoids are a group of metabolites in the phenols chemical class. Phenolic compounds were found with greater frequency in the treatments with low-

energy diets and this fact can be associated with a reduction in the percentage of individuals belonging to the genus *Flavonifractor* precisely in these treatments.

The phylum Candidatus Saccharibacteria is a phylum of uncultivated bacteria evidenced by Albertsenet et al. [40] after sequencing of the complete genome of several members of the Division Candidate TM7, which had already been described in different ecosystems, as shown in a study by Hugenholtzet et al. [41]. This result was confirmed by an analysis of the genus *Saccharibacteria* (*incertae sedis*), belonging to the phylum Candidatus Saccharibacteria, and showed a greater (P=0.05) percentage of individuals in cattle under heat stress conditions. Albertsenet et al. [40] state in their study (based on genomic analysis) that bacteria belonging to this phylum have an obligatory fermentative metabolism, fermenting glucose and other sugars, and producing lactic acid. Uyeno et al. [6] also observed in their study that a group of uncultivated bacteria had increased their population in the rumen when the cattle were in a high-temperature environment. Some studies show that one of the physiological responses of cattle to heat stress is a decrease in ruminal pH [4, 5, 42]. According to Mishra et al. [42], a decrease in ruminal pH as a result of increased ambient temperature is associated with the accumulation of lactic acid in the rumen. An increase in the percentage of individuals belonging to the genus *Saccharibacteria* (*incertae sedis*) can be associated with a higher production of lactic acid in the rumen and consequently a decline in pH. Although no significant difference (P=0.07) in pH values was observed, we could observe that animals under heat stress conditions presented lower values of pH compared with animals that were on thermal comfort and with feed restrictions.

Acetic, propionic and butyric acids were the acids in greatest abundance in all samples, regardless of treatment. These are the main volatile fatty acids produced by rumen microbial fermentation, and this result is widely described in the literature [3, 5, 6.43, 44]. The authors Nonaka et al. [4], Tajima et al. [5] and Kelley et al. [44] concluded that increased ambient temperature resulted in a decline in the concentration of acetic and butyric acids and of total VFAs produced in the rumen of Holstein cows and heifers. In the studies conducted by these three groups of authors, the food amounts were not restricted, and it was not possible to separate the temperature effect from the consumption level effect. In this study, there was no effect of temperature on these two acids, but there was an effect of the diet. The cattle that were consuming a high-energy diet showed lower concentrations of these acids in the rumen. A tendency (P=0,07) was

observed in this study towards an increase in propionic acid in relation to the total VFAs in treatments under heat stress, similar to Nonaka et al. [4] and Kelly et al. [44].

Isobutyric acid is a branched chain fatty acid, used as a calorie source for cattle and as a growth factor for rumen cellulolytic bacteria [3, 31, 45, 46]. Bacteria of the species *Fibrobacter succinogenes* (cellulolytic) use isobutyric acid for the synthesis of long-chain fatty acids and aldehydes [47]. Isobutyric acid supplementation in Simmental steers resulted in an increase in the concentration of total VFAs in the rumen and also in an increase in the digestibility of organic matter, crude protein and neutral detergent fibre [48]. The higher digestibility of dry matter in animals under heat stress can be explained by the greater accumulation of isobutyric acid in the rumen [4, 5, 9]. In the literature, there are few studies that have evaluated the heat stress effects on ruminal fermentation parameters, and the studies that performed this analysis did not evaluate the effect of high temperature and humidity on isobutyric acid concentration in the rumen.

The cattle consuming high-energy diets presented a reduction in ruminal pH. According to Berman [3], when there is a reduction in ruminal pH, AGVs become more protonated or undissociated, which increases the rate of absorption. In fact, animals consuming a high-energy diet presented a lower pH value in the rumen, and a lower concentration of acetic, butyric and isobutyric acids. This result may be due to the higher absorption, or the lower proportion, of fibre in the diet [49]. Simultaneously with a reduction in the concentration of acetic and butyric acids, there was an increase in the percentage of propionic acid in relation to the total VFAs in the treatments with high-energy diets.

The technique of Solid Phase Micro-Extraction (SPME) is a practical technique, indicated for the extraction and concentration of metabolites present in environmental samples [26 e 50]. After gas chromatography analysis associated with a mass spectrometer, it was possible to identify 49 analytes from seven different classes of chemical compounds. This shows that the SPME extraction technique can be successfully used for analysis of ruminal liquid samples. With the exception of dihydro-5-pentyl-2 (3H) -furanone amine, which was most frequently identified in high ambient temperature treatments, there was no standard compound profile in response to heat stress conditions. 3-methylindole amine (3MI) was identified with higher frequency in high-energy diets. The amines are associated with protein synthesis by ruminal

microorganisms, and subsequently become sources of amino acids for the host [51]. 3MI is one of the main products of degradation of tryptophan in the rumen [52, 53]. Like other compounds of the indole class, 3-methylindole, found in the rumen of animals fed high-energy diets, can cause malabsorption syndrome, and hepatic anaemia in humans [54]. Yokoyama et al. [55] concluded that the presence of 3MI in ruminal fluid and blood plasma, following intra-ruminal administration of tryptophan, and the relationship between 3MI concentration and the severity of clinical signs, indicate that 3MI is the major metabolite resulting from ruminal fermentation related to development of acute pulmonary edema and pulmonary emphysema in cattle.

Conclusion

Under the conditions evaluated, heat stress had an effect on the ruminal microbiota and the concentration of some of the volatile fatty acids of beef cattle. Although there was a reduction in the richness of species in the rumen of cattle under caloric stress, this alteration did not affect the high diversity of prokaryotes present in this environment. The main alterations were related to an increase in microorganisms belonging to the phylum Candidatus saccharibacteria and the concentration of isobutyric acid in animals under heat stress, which may be associated with a reduction in ruminal pH. The diet energy level had a greater effect on the ruminal parameters evaluated than heat stress, which highlights the importance of diet control and the level of consumption in studies to evaluate ruminal fermentation and on its microbiota. The effect of heat stress on the richness index and on the populations of the phylum Firmicutes and the genus *Ruminococcus* were only observed when there was restriction in the dietary consumption level. In view of the results, we can understand the reasons for some changes in the ruminal environment that are related to the performance of beef cattle under heat stress. This can help in the management of these animals and minimise the losses caused by the effects of heat stress in production systems.

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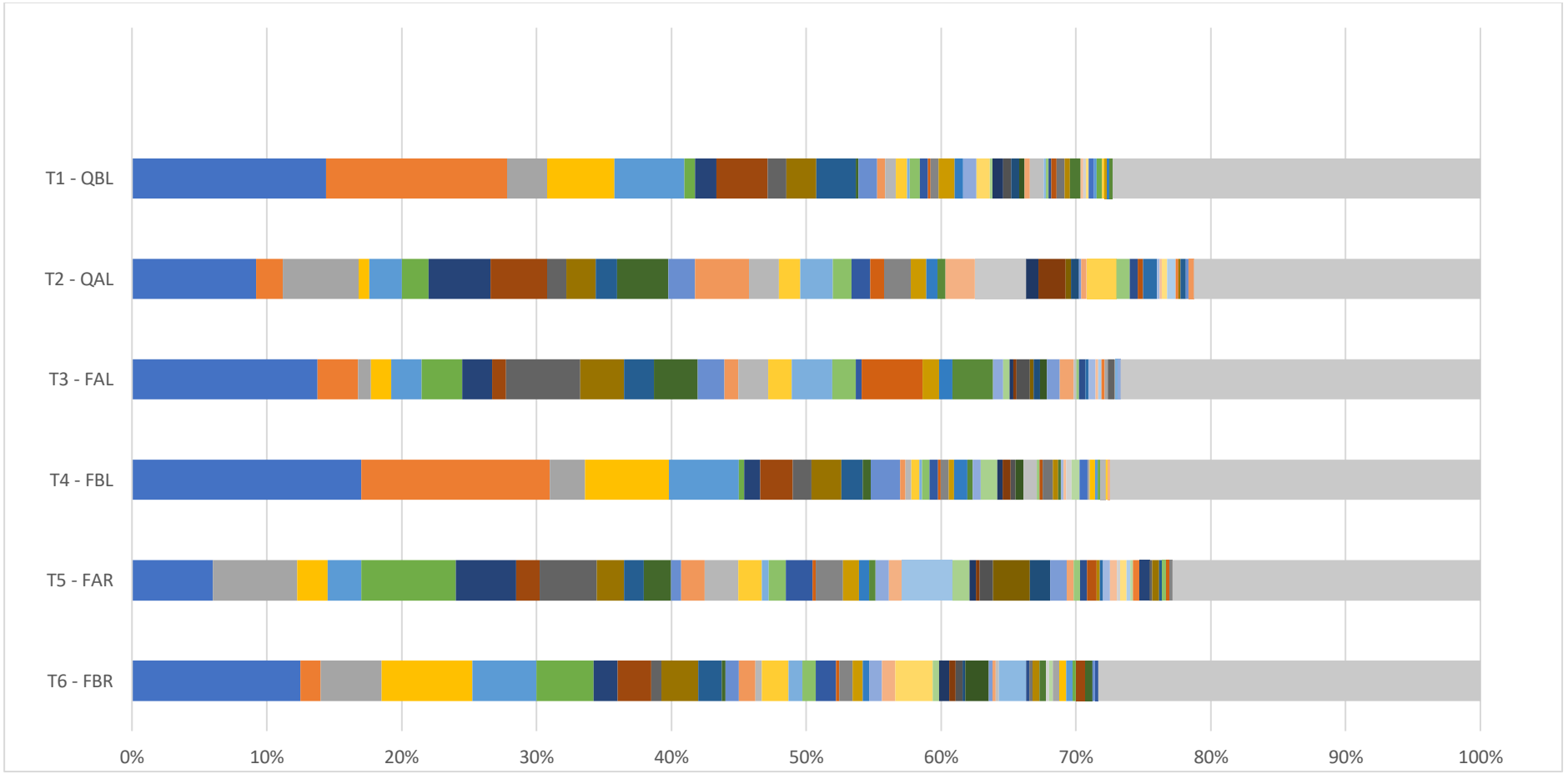
Supplementary Table S1 - Occurrence of 80 genera identified in the different treatments




Prevotella
Dethiosulfatibacter
Flavonifractor
Tannerella
Pseudosphingobacterium
Coriobacterineae
Coprococcus
Euryarchaeota
Anaerophaga
Sporobacter
Desemzia
Gimesia
Coprobacter
Robinsoniella
Schwartzia
Saccharibacteria genera
Cellulosibacter
Lacticigenium
Blastopirellula
Arthrobacter
Outros
Carnobacterium
Lactobacillus
Lachnospiracea incertae sedis
Syntrophococcus
Anaerovorax
Butyrivibrio
Mogibacterium
Ornithobacterium
Stomatobaculum
Clostridium IV
Alkalibacter
Tangfeifania
Intestinimonas
Alkalitalea
Lutaonella
Slackia
Enterococcus
Prolixibacter
Desulfospira
Faecalitalea
Methanobrevibacter
Ethanoligenens
Saccharofermentans
Christensenella
Ruminococcus
Paraprevotella
Pseudomonas
Gracilibacter
Anaerobacterium
Vampirovibrio
Hydrogenibacillus
Oscillibacter
Alloprevotella
Victivallis
Ornatilinea
Acetobacteroides
Acetatifactor
Acidaminobacter
Howardella
Hespellia
Falsiporphyromonas
Saccharibacteria genera incertae sedis
Barnesiella
Dendrosporobacter
Treponema
Pseudoflavonifractor
Bifidobacterium
Succiniclasticum
Psychrobacter
Brochothrix
Eisenbergiella
Oligosphaera
Holdemania
Papilibacter
Pseudobutyrivibrio
Anaerotruncus
Acinetobacter
Bavariicoccus
Anaerosalibacter
Paludibacter