UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

EFEITOS DE EXTRATOS DE PLANTAS E MONENSINA NO CRESCIMENTO DE BACTÉRIAS RUMINAIS E TRATAMENTO AFEX NO VALOR NUTRITIVO DA PALHA DE ARROZ PARA CORDEIROS

Autor: Rodrigo Augusto Cortêz Passetti
Orientador: Prof. Dr. Ivanor Nunes do Prado
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MARINGÁ
Estado do Paraná
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TITULAÇÃO: Doutor em Zootecnia - Área de Concentração Produção Animal

APROVADO em 21 de fevereiro de 2020.

Profª Drª Magali Soares dos Santos Pozza

Prof. Dr. Hilário Cuquetto Mantovani

Prof. Dr. Everson Zotti

Prof. Dr. João Luiz Pratti Daniel

Prof. Dr. Ivanor Nunes do Prado
Orientador

A
Deus...
pela força.

Aos
meus pais, Paulo Cesar Passetti e Maria Célia Cortêz Passetti,
pelo amor e apoio eterno.

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meu avô, Paulo Cortêz,
pelo carinho e admiração.

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esposa Ludmila, Couto Gomes Passetti
pelo incentivo e carinho.

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amigos e familiares,
pelos momentos de grande felicidade.

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BIOGRAFIA

RODRIGO AUGUSTO CORTEZ PASSETTI, filho de Paulo Cesar Passetti e Maria Célia Cortez Passetti, nasceu em Maringá, Paraná, no dia 24 de julho de 1991.

Em dezembro de 2013, concluiu o curso de Zootecnia pela Universidade Estadual de Maringá (UEM - PR), com período sanduíche de setembro de 2012 a agosto de 2013, pelo programa Ciências sem Fronteiras, do Conselho Nacional de Desenvolvimento Científico e Tecnológico, na Van Hall Larenstein University of Applied Sciences, Holanda.

Em 2016, recebeu o título de Mestre área de concentração Produção Animal do Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá. Entre novembro de 2015 a fevereiro de 2016, realizou estágio de três meses na Universidad de Zaragoza, trabalhando em diversos experimentos de análises instrumentais e sensoriais da carne.

Em março de 2016, ingressou no programa de Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá no nível de Doutorado na área de concentração Produção Animal. Entre junho de 2018 a 2019 realizou doutorado sanduíche no Research Center of Lethbridge pertencente a Agriculture and Agri-Food Canada, sobre supervisão do Dr. Tim A. McAllister.

Em junho de 2019, foi aprovado na banca examinadora de qualificação e em fevereiro de 2020, submeteu-se para defesa da presente Tese.

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RESUMO

No primeiro capítulo da tese, foi avaliado o potencial uso de aditivos naturais (óleo essencial de orégano, óleo vegetal de mamona e extrato de *Baccharis dracunculifolia*) e a monensina contra um grupo de bactérias ruminais. Três bactérias gram-negativas (*Prevotella albensis, Prevotella bryantii* e *Treponema saccharophilum*) e três bactérias gram-positivas (*Ruminococcus albus, Ruminococcus flavefaciens* e *Streptococcus bovis)* foram utilizadas. As bactérias foram cultivadas em meio de cultura (Hobson M2) em condições de anaeróbiose em tubos hungate de 10 mL a 39ºC. Para avaliar a capacidade inibitória, as bactérias foram incubadas por 24 horas nas concentrações finais de 10, 20, 50 e 100 mg/L de cada aditivo e a densidade óptica (600nm) foi mensurada às 8, 12 e 24 horas. A monensina inibiu o crescimento das bactérias gram-positivas ($P < 0,05$). Em dosagens mais elevadas (> 20mg/L) foi também capaz de inibir o crescimento das bactéria gram-negativas, possívelmente pelo efeito bactericida. O óleo essencial de orégano inibiu o crescimento ($P < 0,05$) das bactérias gram-negativas e gram-positivas; enquanto os outros compostos tiveram efeito marginal apenas em bactérias gram-positivas (óleo de mamona) ou nenhum efeito (*Baccharis dracunculifolia*). No segundo capítulo da tese avaliou-se o uso da tecnologia *Ammonia Fiber Expansion* (AFEX) que apresenta o potencial para aumentar a digestibilidade da fibra de alimentos volumosos, mas que pode deixar resíduo, a acetamina, na carne. Primeiramente para se avaliar o potencial desta tecnologia, foram incubados alfafa, palha de arroz e palha de arroz tratada em três novilhas fistuladas por até 120 horas, para se determinar a cinética do desaparecimento da MS e FDN e caracterizar o perfil de colonização dos microrganismos. Posteriormente, um estudo com 40 fêmeas ovinas (37,1 ± 3,5 kg) recebendo uma das quatros dietas foram

utilizadas para avaliar a performace e o resíduos de acetamina no sangue e músculo: 1) ALF = 250 g / kg de alfafa; 2) RS = 250 g / kg de palha de arroz; 3) ARS = 250 g / kg de AFEX palha de arroz e 4) ARSW = retirada da dieta ARS sete dias antes do abate. A palha de arroz tratada com AFEX apresentou maiores frações B e A+B e também maior degradabilidade ruminal efetiva ($P < 0.05$). O perfil de colonização da palha de arroz AFEX foi mais similar a alfafa do que para a palha de arroz não tratada, por maior número de *Bacteroidetes.* Ovinos alimentados com dieta RS apresentaram desempenho similar aos ALF. Por outro lado, embora a ingestão de matéria seca dos ARS fora similar aos ALF o seu desempenho e eficiência alimentar foram reduzidos ($P < 0.05$). Uma correlação foi observada entre acetamida no sangue e no músculo dos animais alimentados com ARS. A retirada por até três dias da dieta ARS, foi suficiente para reduzir os níveis de acetamida no sangue ($P < 0.05$). Entretanto, o nível de acetamida no músculo foi similar até o sétimo dia de retirada. Embora o AFEX tenha aumentado a digestibilidade da palha de arroz, a inclusão de 25% deste alimento não melhorou o desempenho dos animais, entretanto um período de retirada superior a sete dias parece ser necessário para retornar os níveis basais de acetamida no músculo.

Palavras chave: acetamida, AFEX, extratos de plantas, ionóforos, óleos essências.

ABSTRACT

The first chapter of this article evaluates the potential use of natural additives (oregano essential oil, castor oil and extract of *Baccharis dracunculifolia*) and monensin against a group of ruminal bacteria. Three gram negative bacteria (*Prevotella albensis*, *Prevotella bryantii* and *Treponema saccharophilum*) and three gram positive bacteria (*Ruminococcus albus*, *Ruminococcus flavefaciens* and *Streptococcus bovis*) were used. The bacteria were grown in culture medium (Hobson M2) under anaerobic conditions in 10 mL suspended tubes at 39ºC. To assess inhibitory capacity, bacteria were incubated for 24 hours in the concentrations of 10, 20, 50 and 100 mg / L of each additive and optical density (600nm) was measured at 8, 12 and 24 hours. Monensin inhibited the gram-positive bacteria growth ($P < 0.05$). Also at higher doses (> 20mg / L) inhibited the gram-negative bacteria growth, possibly due to a bactericidal effect. The oregano essential oil inhibited ($P < 0.05$) the gram-negative and gram-positive bacteria growth; while the other compounds had a marginal effect only on gram-positive bacteria (castor oil) or no effect (*Baccharis dracunculifolia*). In the second experiment it was evaluated the use of Ammonia Fiber Expansion (AFEX) technology, which has the potential to increase fiber digestibility of bulky foods, but can leave residues, like acetamide in the meat. First to assess the potential of this technology, alfalfa, rice straw and treated rice straw were incubated in three fistulated heifers for up to 120 hours to determine the DM and NDF kinetics disappearance and to characterize the microorganisms colonization profile. Subsequently, a study with 40 female sheep (37.1 ± 3.5 kg) receiving one of the four diets was carried out to assess performance and acetamide residues in blood and muscle: 1) ALF = 250 g / kg of alfalfa; 2) RS = 250 g / kg of rice straw; 3) ARS = 250 g / kg of AFEX rice straw and 4) ARSW = withdrawn of ARS diet seven days before the slaughter. AFEX-treated rice straw had higher B and A + B fractions and also greater

effective ruminal degradability (P <0.05). The colonization profile of AFEX rice straw was more like alfalfa than untreated rice straw, due to the greater number of *Bacteroidetes*. Sheep fed with RS diet showed similar performance to ALF. On the other hand, although the ARS dry matter intake was similar to ALF its performance and feed efficiency was reduced (P <0.05). A correlation was observed between acetamide in the blood and muscle of animals fed with ARS. The withdrawal for up to three days from the ARS diet was sufficient to reduce the acetamide levels in the blood (P <0.05). However, the acetamide level in muscle was similar until the seventh day of withdrawal. Although AFEX has increased the rice straw digestibility, the inclusion of 25% of this food did not improve the animals' performance, whereas a withdrawal period longer than seven days seems to be necessary to return the basic acetamide levels in muscle.

Keywords: acetamide, AFEX, plants extracts, ionophores, essential oils

I – REVISÃO DE LITERATURA

1. Introdução a microbiología e fermentação ruminal

Os ruminantes são herbívoros que utilizam principalmente forragens e outros coprodutos pouco adequados na dieta humana como fontes de energia, proteínas e outros nutrientes. Apesar da dependência de forragens na sua alimentação primária, os ruminantes não produzem nenhuma das enzimas necessárias para degradar as paredes das células vegetais. Em vez disso, eles dependem de uma relação simbiótica com microrganismos (bactérias, fungos, archaeas e protozoários) que degradam as paredes das células vegetais até os produtos finais que podem ser usados pelo hospedeiro para produzir leite e carne (HUWS; CREEVEY; OYAMA; MIZRAHI *et al.*, 2018).

Para realizar esta tarefa, os ruminantes têm uma estrutura biologicamente única dentro de seu sistema digestivo conhecido como o retículo-rúmen, que é um ambiente anaeróbio ideal para o crescimento desses microrganismos fermentativos (HOBSON, 2012). O rúmen é essencialmente um fermentador de fluxo contínuo em que o substrato na forma de ração é regularmente fornecido e o pH e a osmolaridade são controlados pela entrada da saliva, absorção de ácidos fermentativos e passagem de proteínas microbianas e a digesta para o trato digestivo posterior (MILLEN; ARRIGONI; PACHECO, 2016). Entretanto, o rúmen também é um sistema dinâmico em que microrganismos têm de ser capazes de se adaptar às mudanças na composição, estrutura física, bem como a quantidade e frequência de disponibilidade de alimentos. Consequentemente, mudanças na razão entre forragem-concentrado, métodos de processamento da dieta e a frequência e quantidade de dietas fornecidas ao hospedeiro podem ter impacto significativo na população microbiana. (BELANCHE; DOREAU; EDWARDS; MOORBY *et al.*, 2012;

FERNANDO; PURVIS; NAJAR; SUKHARNIKOV *et al.*, 2010; HENDERSON; COX; GANESH; JONKER *et al.*, 2015). Neste sentido pode-se afirmar que a fermentação ruminal é dependente do tipo de substrato e o tipo de micro-organismos que habitam o rúmen.

Desde o início das pesquisas em nutrição animal, o objetivo de muitos nutricionistas fora sempre o de poder modular a fermentação ruminal de uma forma mais eficiente. Na presente tese foram avaliadas duas formas de se alterar a fermentação ruminal, a primeira através de uso de aditivos, como ionóforos, óleos essenciais e vegetais que modificam diretamente a população microbiana (ALTERMANN; SCHOFIELD; RONIMUS; BEATTY *et al.*, 2018; DA SILVA; TORRECILHAS; PASSETTI; ORNAGHI *et al.*, 2014; ORNAGHI; PASSETTI; TORRECILHAS; MOTTIN *et al.*, 2017; VALERO; PRADO; ZAWADZKI; EIRAS *et al.*, 2014; ZAWADZKI; PRADO; MARQUES; ZEOULA *et al.*, 2011) e a segunda forma sendo através do uso de tecnologias, como a amonização que melhoram a utilização do substrato, no caso a forragem, pelos micro-organismos ruminais, (BALS; TEYMOURI; HADDAD; JULIAN *et al.*, 2019; BEAUCHEMIN;; RIBEIRO;; RAN;; MILANI; *et al.*, 2019; BLÜMMEL; TEYMOURI; MOORE; NIELSON *et al.*, 2018). Mas antes de aprofundar nestes métodos, deve-se primeiro conhecer melhor a população que habita o rúmen que são divididas em: bactérias, archaeas, protozoários, fungos e bacteriófagos.

1.1. Bactérias

As bactérias do rúmen são o grupo mais abundante e diversificado de microrganismos no ecossistema ruminal. O conteúdo ruminal pode conter 10^{10} a 10^{11} bactérias/mL, que são responsáveis pela maioria da atividade fermentativa no rúmen (MILLEN; ARRIGONI; PACHECO, 2016). Como um todo, eles possuem uma infinidade de atividades enzimáticas, incluindo, amilases, celulases, hemicelulases, proteases e lipases que degradam amido, paredes de células vegetais, proteínas e lipídios, respectivamente (HUWS; CREEVEY; OYAMA; MIZRAHI *et al.*, 2018). A maioria das bactérias (70-80%) está contida na fração sólida do conteúdo ruminal, organizadas em estrutura chamada de biofilme e são as principais responsáveis pela digestão do alimento. No biofilme, os produtos da fermentação (amônia, AGV, CH_4 e H_2) são difundidos no meio ruminal para serem usados por outras colônias, contribuindo assim para o processo de digestão (LENG, 2014).

Outra parcela de microrganismos está presente na fração líquida (20-30%) que utiliza substratos solúveis ou estão em transição de uma partícula de dieta para a próxima. Existe ainda pequena fração de bactérias (< 1%) que estão ligadas ao epitélio ruminal. Estas bactérias utilizam o oxigênio que difunde do sangue e é tóxico para a maioria dos outros microrganismos do rúmen (MILLEN; ARRIGONI; PACHECO, 2016). Estes também hidrolisam a ureia que difunde do sangue para o rúmen, produzindo a amônia que os microrganismos podem combinar com esqueletos de carbono para sintetizar aminoácidos. Embora este pequeno grupo de bactérias não contribua significativamente na digestão, eles ainda contribuem de forma significativa para o microbioma (CHENG; MCCOWAN; COSTERTON, 1979).

A dieta é o principal fator que afeta a composição e a diversidade da população bacteriana ruminal. Quando os bovinos são alimentados com dietas ricas em volumosos, as bactérias celulolíticas proliferam (por exemplo, *Ruminococcus flavefasciens, Ruminococcus albus*, *Bacteroides succinogenes* e *Butyrivibrio fibrisolvens*) e aumenta a diversidade dos microrganismos no rúmen (COUVREUR; HURTAUD; LOPEZ; DELABY *et al.*, 2006). Em contrapartida, se os bovinos são alimentados com dietas ricas em grãos, bactérias amilolíticas (por exemplo, *Ruminobacter amylophilus, Prevotella ruminocola, Streptococus bovis*,) que digerem amido e aquelas que utilizam os produtos finais da digestão do amido (por exemplo, *Megasphaera elsdeni* e *Selenomonas ruminantium)* como, o ácido láctico proliferam**.** Esta mudança reduz a diversidade dos microrganismos e conduz para a diminuição no acetato e aumento na concentração do propionato no rúmen (BELANCHE; DOREAU; EDWARDS; MOORBY *et al.*, 2012; FERNANDO; PURVIS; NAJAR; SUKHARNIKOV *et al.*, 2010). A produção de mais produtos finais da fermentação determina declínio no pH podendo atingir 5,0 ou menos, e irá aumentar a concentração de lactato no rúmen reduzindo ainda mais n pH. (NAGARAJA; TITGEMEYER, 2007). Em pH abaixo de 5,8, muitas das bactérias celulolíticas são inibidas e em consequência a digestão ruminal da fibra também diminui.

1.2. Archaeas

As metanogênicas são membros de um grupo único de microrganismos conhecidos como archaeas e são responsáveis pela produção de metano no rúmen. As metanogênicas não são membros expressivos do microbioma (10^6 células / mL), mas sua capacidade de reduzir as compostos como metilaminas, o formato e o dióxido de carbono ao metano tornam-nas em indivíduos fundamentais da fermentação ruminal (LENG, 2014). A

eructação (não a flatulência) é responsável pela maioria do metano produzido no rúmen. A emissão de metano ruminal não é desejável tanto para o hospedeiro quanto para o ambiente, uma vez que o gás representa perda energética significativa e tem 25 vezes o potencial de aquecimento global do dióxido de carbono (IPCC, 2007)

Por ser considerado uma fonte de perda, consideráveis pesquisas são realizadas sobre os métodos de mitigação de metano. A estratégia de mitigação mais eficaz é a redução da quantidade de unidades de alimento necessária para produzir uma unidade de produto (eficiência animal). Outras estratégias consistem no uso de aditivos alimentares como antibióticos, ionóforos, probióticos ou extratos naturais, (BENCHAAR; CHAVES; FRASER; BEAUCHEMIN *et al.*, 2007; BENCHAAR; GREATHEAD, 2011; COBELLIS; TRABALZA-MARINUCCI; MARCOTULLIO; YU, 2016) que são tóxicos para as archaeas metanogênicas ou atuam no redirecionamento das vias metabólicas do H_2 (LENG, 2014). Mais recentemente, também se tem estudado as enzimas produzidas pelos bacteriófagos, vírus que predam as bactérias (ALTERMANN; SCHOFIELD; RONIMUS; BEATTY *et al.*, 2018). No entanto, pela grande variedade de espécies e a capacidade de adaptação das metanogênicas, várias destas tecnologias desenvolvidas resultaram apenas em redução a curto prazo das emissões de metano e algumas tiveram efeitos secundários indesejáveis na digestibilidade do alimento e na produtividade animal (COBELLIS; TRABALZA-MARINUCCI; MARCOTULLIO; YU, 2016; LENG, 2014).

1.3. Protozoários

Os protozoários estão em menor presença no líquido ruminal (10^3 a 10^6 células / mL); porém, são responsáveis por até 50% da biomassa microbiana ruminal total por causa do tamanho maior (HUWS; CREEVEY; OYAMA; MIZRAHI *et al.*, 2018; NEWBOLD; DE LA FUENTE; BELANCHE; RAMOS-MORALES *et al.*, 2015). O tipo de protozoário mais comumente encontrado no rúmen são os ciliados sendo o gênero *Entodinium* representante de até 90% da população total (DEHORITY; TIRABASSO, 1989). A população de protozoários dificilmente muda ao longo da vida do animal. Entretanto, sua contribuição na fermentação ruminal ainda é controvérsia.

Os protozoários podem contribuir entre 1/4 a 1/3 da digestão da fibra no rúmen, por exemplo, espécies de *Epidinium* estão fortemente associada à parede celular por sua capacidade de utilizar os cloroplastos como fonte de proteína e lipídeos (HUWS; KIM; KINGSTON-SMITH; LEE *et al.*, 2009). Embora os protozoários sejam associados

frequentemente à fração líquida do rúmen, os mesmos podem igualmente se unir à superfície de partículas de alimento ou ao epitélio do rúmen. Protozoários são predadores de bactérias do rúmen, assim, o número de protozoários no rúmen oscila inversamente ao número de bactérias. Em consequência da predação bacteriana, os protozoários são igualmente responsáveis pelo retorno de grande parcela de nitrogênio da proteína microbiana dentro do rúmen (JOUANY, 1996). Nesse sentido, a redução na população de protozoários, poderia melhorar a utilização de nitrogênio pelo animal pelo maior aporte de proteína microbiana no intestino (HRISTOV; IVAN; NEILL; MCALLISTER, 2003).

Os ruminantes podem sobreviver sem quaisquer protozoários no rúmen, mas isso ainda é uma questão de debate entre pesquisadores. Protozoários e archaeas têm relação simbiótica estreita. A remoção de protozoários é muitas vezes acompanhada por declínio transitório nas emissões de metano e podendo aumentar a eficiência dos ruminantes (NEWBOLD; DE LA FUENTE; BELANCHE; RAMOS-MORALES *et al.*, 2015). Embora a redução em emissões do metano e na quebra da proteína ruminal possa ser vantajosa, ela é frequentemente as custas da diminuição na digestibilidade da matéria orgânica e da fibra (NEWBOLD; DE LA FUENTE; BELANCHE; RAMOS-MORALES *et al.*, 2015). Além disso, com o aumento de concentrado na dieta, embora a diversidade de protozoários no rúmen possa diminuir, os protozoários que permanecem são capazes de engolir grânulos de amido, modulando a digestão do amido e reduzindo o risco de acidose ruminal (JOUANY; USHIDA, 1999)

1.4. Fungos

Os fungos são os microrganismos encontrados em menor quantidade no rúmen(10^3 to10^6 zoosporos / mL) representando menos 20% da biomassa microbiana (ELEKWACHI; WANG; WU; RABEE *et al.*, 2017; REZAEIAN; BEAKES; PARKER, 2004). No entanto, os fungos ruminais estão entre os microrganismos mais importantes envolvidos na digestão de forragens de baixa qualidade, como palha de cereais (GRUNINGER; PUNIYA; CALLAGHAN; EDWARDS *et al.*, 2014). Isso se deve à produção de um vasto leque de enzimas degradantes de carboidratos e fibras. Além disso, os fungos produzem estruturas filamentosas conhecidas como rizoides, que têm a capacidade de exercer força física e penetrar paredes de células de plantas altamente lignificadas (GRUNINGER; PUNIYA; CALLAGHAN; EDWARDS *et al.*, 2014). As

enzimas então ficam concentradas na ponta dessas estruturas e nas aberturas que são criadas na parede celular vegetal fornecendo às bactérias acesso ao interior da célula vegetal (KRAUSE; DENMAN; MACKIE; MORRISON *et al.*, 2003). Em culturas puras, os fungos anaeróbios são capazes de produzir diversos produtos como acetato, formato, lactato, CO_2, H_2 etc. No entanto, no rúmen ocorre mudança para uma via metabólica, mais favorável para utilização de H_2 para produção de metano, devido uma associação entre fungos e bactérias metanogênicas. Essa associação é benéfica para ambos os microrganismos possibilitando uma maior produção de enzimas e degradação da fibra (CHENG; EDWARDS; ALLISON; ZHU *et al.*, 2009).

1.5. Bacteriofágo

Os bacteriófagos são "vírus bacterianos" que residem no rúmen em concentrações de 10^{10} partículas/mL de fluido ruminal (LETAROV; KULIKOV, 2009). Essas partículas virais precisam de um hospedeiro (bactérias) para a replicação que pode culminar na ruptura da célula bacteriana (ciclo lítico) ou na integração do genoma do bacteriófago (ciclo lisogênico) no genoma do hospedeiro (DE PAEPE; LECLERC; TINSLEY; PETIT, 2014). A população de bacteriófagos é altamente variável até mesmo entre indivíduos da mesma espécie. Os tipos mais abundantes de bacteriófagos representam fração inferior a 10% da comunidade total. Entretanto, estudos ainda são escassos e muitos bacteriófagos ainda não foram identificados e caracterizados (LETAROV; KULIKOV, 2009). Os bacteriófagos são geralmente hospedeiros específicos, muitas vezes sendo capazes de infectar apenas algumas cepas de uma determinada espécie bacteriana. Alguns bacteriófagos capazes de infectar *Bacteroides*, *Ruminococcus* e *Streptococcus* já foram isolados e tiveram seu genoma mapeado (GILBERT; KLIEVE, 2015). Os bacteriófagos ganham cada vez mais atenção dos cientistas. Apesar de, atualmente não serem aplicáveis além de um nível experimental, os pesquisadores acreditam que futuramente a terapia de bacteriófagos poderia ser administrada para alvejar espécies bacterianas indesejáveis (e. g. *Streptococcus bovis*) como meio de prevenção de algumas doenças digestivas.

2. Aditivos moduladores da fermentação ruminal

2.1. Ionóforos

Grandes avanços na nutrição animal surgiram na década de 1970 com a descoberta de compostos como o poliéster carboxílico (antibióticos ionóforos), capazes de formarem complexos lipossolúveis com certos cátions facilitando o transporte pelas membranas (PRESSMAN, 1976). Em condições normais, as bactérias mantêm o ambiente intracelular com pH neutro e com altas concentrações de potássio (K+) e baixas de sódio (Na+) enquanto no ambiente extracelular ocorre ao contrário, e o pH se encontra mais acidificado pelo acúmulo de ácidos graxos voláteis. que ocorre ao contrário no meio extracelular (AZZAZ; MURAD; MORSY, 2015). Neste sentido as bactérias ruminais são dependentes da troca destes íons para absorver nutrientes. Uma vez estabelecidos na membrana celular os ionóforos irão causar um efluxo de K^+ intracelular da célula e influxo de prótons extracelulares (Na^+ e H^+), aumentando a acidez da célula e inibindo síntese de proteínas (Figura 1). Para reverter a perda de K^+ as bombas de ATPase são desencadeadas para ejetar os prótons, esgotando as reservas de energia para o crescimento da bactéria (AZZAZ; MURAD; MORSY, 2015).

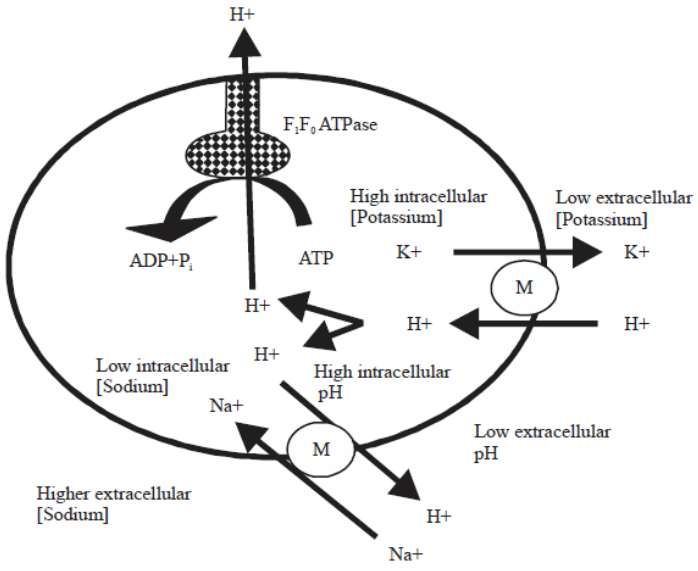

Figura 1. Mecanismo de ação da monensina na membrana celular. Fonte: AZZAZ; MURAD e MORSY (2015).

Os ionóforos são produzidos pelas estirpes de *Streptomyces*, como a monensina, a qual é a mais utilizada na nutrição de ruminantes. A capacidade de modular a fermentação ruminal dos ionóforos se deve às diferenças inerentes entre os organismos Gram - e Gram +. A membrana externa das bactérias Gram-negativas é impermeável a muitas macromoléculas. O movimento do soluto é mediado por porinas. As porinas formam

canais hidrofílicos na membrana externa hidrofóbica com limite de exclusão de aproximadamente 600 daltons (NIKAIDO; NAKAE, 1980). Como os ionóforos são extremamente hidrofóbicos e têm tamanhos moleculares superiores a 500 daltons, a membrana externa das bactérias Gram-negativas atua como barreira protetora. Entretanto, vale ressaltar que algumas espécies de bactérias Gram-negativas não são resistentes a alta concentração de ionóforos, enquanto algumas bactérias Gram-positivas podem desenvolver resistência a eles (DAWSON; BOLING, 1987; NAGARAJA; TAYLOR, 1987).

Apesar do seu efeito positivo, o uso rotineiro de antibióticos como promotores de crescimento na alimentação animal tem preocupado a saúde pública, impondo restrições para a utilização na alimentação animal (1831/2003/CEE; European Commision, 2003) com sentido de prevenção possível surgimento de bactérias resistentes aos antibióticos. Um outro aspecto que tem sido levantado é o da presença destes resíduos nos alimentos. De acordo com a Comissão Diretiva 2009/8/EC o limite máximo de resíduos em leite, ovos e carne é de 8 μg / kg para a monensina e de 150 a 20 μg / kg para a lasolacida (CLARKE; FODEY; CROOKS; MOLONEY *et al.*, 2014). Embora não existam casos em humanos de toxidez aguda, muito se tem questionado sobre possíveis problemas causados por exposição prolongada em baixa dosagem. Por esse motivo, muitos cientistas têm procurado por alternativas naturais como a própolis, óleos essenciais, algas e os extratos de plantas (ORNAGHI; PASSETTI; TORRECILHAS; MOTTIN *et al.*, 2017; VALERO; PRADO; ZAWADZKI; EIRAS *et al.*, 2014; ZAWADZKI; PRADO; MARQUES; ZEOULA *et al.*, 2011).

2.2 Óleos essenciais

As plantas produzem uma extensa variedade de metabólitos secundários, que não estão diretamente ligados com uma função de crescimento ou desenvolvimento da planta, mas são responsáveis pela cor, odores e possuem função ecológica como mensageiros químicos entre planta e ambiente (BALANDRIN; KLOCKE; WURTELE; BOLLINGER, 1985). Os metabólicos secundários são de difícil caracterização por seu complexo metabolismo de síntese, mas podem ser classificados em três grandes grupos: saponinas, taninos e óleos essenciais. Enquanto os efeitos das saponinas e taninos na fermentação ruminal já foram amplamente pesquisados, existe uma gama muito grande

de óleos essenciais que apenas recentemente estão sendo estudados (CALSAMIGLIA; BUSQUET; CARDOZO; CASTILLEJOS *et al.*, 2007).

Os óleos essenciais (OE) são obtidos a partir da destilação a vapor de várias partes de uma planta (raíz, caule, folha e flor). O termo "essencial" deriva da palavra "essência", o que significa cheiro ou gosto e se relaciona com sua propriedade de fornecer sabores e odores. Os óleos essenciais possuem diversas estruturas, mas são classificados em dois grupos: terpenoides (monoterpenoides e sesquiterpenoides) e fenilpropanoides. Os terpenoides, os mais comuns e abundantes nas plantas, geralmente derivam de uma estrutura básica com cinco carbonos (C_5H_8), chamada de isopreno e sendo classificado de acordo com o número destas unidades em seu esqueleto. Os fenilpropanoides são mais raros sendo originados de uma cadeia de três carbonos ligados a um anel aromático de seis carbonos (CALSAMIGLIA; BUSQUET; CARDOZO; CASTILLEJOS *et al.*, 2007).

Diversos OE, como tomilho, orégano, cravo, canela, alecrim e eucalipto estão sendo estudados. Dentre eles, o OE orégano (*Origanum vulgarium*) que é composto principalmente de monoterpenos, como timol e carvacrol, possui ampla atividade antimicrobiana pela presença de um grupo hidroxila em sua estrutura (CALSAMIGLIA; BUSQUET; CARDOZO; CASTILLEJOS *et al.*, 2007). Estudos pioneiros realizados *in vitro* do efeito do timol sobre modulação ruminal foram realizados por BORCHERS (1965) e BRODERICK e BALTHROP JR (1979), os quais reportaram acúmulo de aminoácidos e redução de amônia, sugerindo que a deaminação foi inibida. De acordo com CASTILLEJOS; CALSAMIGLIA e FERRET (2006), doses baixas de timol (50 mg/L) não afetaram a fermentação ruminal *in vitro*, mas em doses mais altas (500 mg/L) as concentrações totais de AGV e amônia diminuíram e aumentou a razão de acetato:propionato. BUSQUET; CALSAMIGLIA; FERRET e KAMEL (2005) relataram que *in vitro*, o carvacrol (2,2 mg/L) diminuiu grandes concentrações de peptídeos e aumentou as concentrações de N de amônia duas horas após a alimentação, sugerindo que o carvacrol inibiu a proteólise ou estimulou a lise de peptídeos. Doses mais altas (300 mg/L) aumentaram a proporção de pH e butirato e diminuiu a razão de acetato e propionato e a concentração total de AGV.

Timol e carvacrol atuam principalmente em bactérias Gram-positivas, e acessam diretamente a membrana celular desestruturando sua integridade e causando lise celular (DUTRA; CASTRO; MENEZES; RAMOS *et al.*, 2019). Por outro lado, outros estudos mostraram que o carvacrol e timol manifestam seus efeitos antimicrobianos também em bactérias Gram-negativas desintegrando sua membrana externa, liberando

lipopolissacarídeos e aumentando a permeabilidade da membrana citoplasmática ao ATP (LAMBERT; SKANDAMIS; COOTE; NYCHAS, 2001). Assim, como a monensina, a maioria dos compostos dos óleos essenciais é lipofílica e não consegue penetrar na membrana externa de bactérias Gram-negativas. Entretanto, esta membrana não é completamente impermeável a substâncias hidrofóbicas e moléculas com baixo peso molecular como timol e carvacrol podem interagir com água (pelas pontes de hidrogênio), atravessar a parede celular por difusão e interagir com a bicamada lipídica interna da célula(DORMAN; DEANS, 2000). Dessa forma, apesar do grande poder antimicrobiano do óleo essencial de orégano, ele possui menor seletividade contra populações específicas tornando a modulação da fermentação ruminal mais difícil.

2.3. Óleos vegetais

Óleos funcionais são extraídos de plantas por prensa ou maceração e apresentam propriedades biológicas que vão além do simples fator nutricional. Diferente dos óleos essenciais, a suas propriedades funcionais não derivam de compostos secundários de essências e especiarias (MURAKAMI; EYNG; TORRENT, 2014). Dentre esses óleos, um que apresenta grande potencial econômico é o óleo de mamona (*Ricinus communis* L) pela sua grande aplicação na indústria, especialmente do biodiesel. O óleo de mamona é obtido facilmente por prensagem da semente de mamona e apresenta grande estabilidade oxidativa que lhe fornece tempo de prateleira maior do que comparado com outros óleos vegetais. De acordo com (BINDER; APPLEWHITE; KOHLER; GOLDBLATT, 1962), essa característica se deve a sua composição que constitui de aproximadamente 90% do ácido graxo insaturado ricinoleico. Além disso, o ácido ricinoleico possui atividade antimicrobiana e vem sendo teorizado como modulador da fermentação ruminal. Entretanto, estudos sobre sua aplicabilidade ainda são escassos (CRUZ; VALERO; ZAWADZKI; RIVAROLI; PRADO *et al.*, 2014).

Alguns autores não encontraram diferença ao suplementarem com óleo de mamona vacas de leite (DE JESUS; DEL VALLE; CALOMENI; SILVA *et al.*, 2016; GANDRA; NUNES GIL; GANDRA; DEL VALE *et al.*, 2014), tourinhos (CRUZ; VALERO; ZAWADZKI; RIVAROLI; DO PRADO *et al.*, 2014; SILVA; TORRECILHAS; ORNAGHI; EIRAS *et al.*, 2014), novilhos (GANDRA; GIL; CÔNSOLO; GANDRA *et al.*, 2012) ovinos e caprinos(MAIA; SUSIN; FERREIRA; NOLLI *et al.*, 2012; MAIA; SUSIN; PIRES; GENTIL *et al.*, 2012), na ingestão e digestibilidade dos alimentos e no

desempenho dos animais. Por outro lado, MORALES; MATA ESPINOSA; MCKAIN e WALLACE (2012) observaram a redução no acetato e aumento no propionato na fermentação ruminal de ovelhas suplementadas com ácido ricinoleico.

Outro óleo vegetal que apresenta capacidade moduladora (VAN NEVEL; DEMEYER; HENDERICKX, 1971) é o óleo de caju (*Anacardium accidentale*) e vem sendo utilizado junto com o óleo de mamona. O blend dos óleos de caju e mamona foi primeiramente testado como anticocidiostático em poedeiras (MURAKAMI; EYNG; TORRENT, 2014) e posteriormente em ruminantes (CRUZ; VALERO; ZAWADZKI; RIVAROLI; DO PRADO *et al.*, 2014; DE JESUS; DEL VALLE; CALOMENI; SILVA *et al.*, 2016; PRADO; CRUZ; VALERO; ZAWADZKI *et al.*, 2016; VALERO; PRADO; ZAWADZKI; EIRAS *et al.*, 2014). O blend de óleos funcionais melhorou a digestibilidade da matéria seca (CRUZ; VALERO; ZAWADZKI; RIVAROLI; DO PRADO *et al.*, 2014) a eficiência alimentar (VALERO; PRADO; ZAWADZKI; EIRAS *et al.*, 2014) e a qualidade de carne (PRADO; CRUZ; VALERO; ZAWADZKI *et al.*, 2016) de novilhos em confinamento e também alterou a fermentação ruminal aumentando a concentração de proprionato em vacas de leite(DE JESUS; DEL VALLE; CALOMENI; SILVA *et al.*, 2016)

2.4. Própolis

O uso da própolis na alimentação de ruminantes também tem apresentado resultados positivos. PRADO; ZEOULA; MOURA; FRANCO *et al.* (2010) observaram aumento na digestibilidade da matéria seca e proteína bruta e redução no número de bactérias metanogênicas. Além disso, bovinos terminados em confinamento alimentados com adição de própolis na dieta apresentaram melhor eficiência alimentar e desempenho produtivo (VALERO; PRADO; ZAWADZKI; EIRAS *et al.*, 2014; ZAWADZKI; PRADO; MARQUES; ZEOULA *et al.*, 2011). Embora a própolis apresente resultados zootécnicos interessantes, sua produção em escala é difícil por se tratar de um produto produzidos pelas abelhas e apresentar grande variação em sua composição.

De maneira geral, a própolis apresenta 50% de resina, em que são encontrados os flavonoides e ácidos fenólicos, 30% de cera, 10% de óleos essenciais, 5% de pólen e 5% de outras substâncias orgânicas (GÓMEZ-CARAVACA; GÓMEZ-ROMERO; ARRÁEZ-ROMÁN; SEGURA-CARRETERO *et al.*, 2006). Entretanto, a sua composição química é complexa sendo extremamente dependente das plantas disponíveis

na região. Em seu trabalho, YONGKUN; IKEGAKI; DE ALENCAR e DE MOURA (2000) classificaram a própolis brasileira em 12 grupos distintos.

Uma das formas de se descobrir a origem botânica da própolis é a análise comparativa entre a sua composição química e a sua provável fonte vegetal (ALENCAR; AGUIAR; PAREDES-GUZMÁN; PARK, 2005). Os mesmos autores identificaram por cromatografia líquida de alta precisão e cromatografia gasosa com espectrometria de massas, que a planta, *Baccharis*. *Dracunculifolia*, popularmente conhecida como alecrim-do-campo é a principal fonte de resina para a elaboração das própolis produzidas nos estados de São Paulo e Minas Gerais. MARÓSTICA JUNIOR; DAUGSCH; MORAES; QUEIROGA *et al.* (2008) ao compararem extratos metanólicos e óleos essenciais de "própolis verde" e de *Baccharis dracuncolifolia* observaram um perfil semelhante entre as amostras. Foram observados 13 flavonoides nos extratos metanólicos e 17 compostos voláteis nos óleos essenciais em ambas as amostras.

2.5. Baccharis dracuncolifolia

Baccharis dracuncolifolia, apresenta potencial de exploração pela indústria por seu forte aroma exótico em medicamentos, cosméticos e defensivos agrícolas(DE SOUSA; JORGE; LEITE; FURTADO *et al.*, 2009). Além disso, ela pode ser uma alternativa para produzir em maior escala os princípios ativos benéficos que são encontrados na "própolis verde".

FERRONATTO; MARCHESAN; PEZENTI; BEDNARSKI *et al.* (2007) observaram que o óleo essencial de *Baccharis dracuncolifolia*, obtido pelo processo de hidrodestilação, possuí ação antimicrobiana contra bactérias Gram-negativas *Escherichia coli e Pseudomonas aeruginosa*o, e bactérias Gram-positivas como *Staphylococcus aureus*. PARREIRA; MAGALHÃES; MORAIS; CAIXETA *et al.* (2010) observaram a presença de e mono/sesquiterpenos, dos quais germacreno D (2,18%), b-cariofileno (2,28%), biciclogermacreno (3,42%), d-cadineno (3,66%), a-muurolol (4,66%), espatulenol (16,24%) e nerolidol (33,51%) os quais representavam 66% da composição do óleo essencial de *Baccharis dracuncolifolia* obtido pela hidrodestilação. Esses autores reportaram que o óleo essencial exibiu elevada ação vermífuga, uma vez que todos os pares de *Schistosoma mansoni* de vermes adultos foram mortos, após incubação com o óleo essencial (10, 50, e 100 mg/mL). Entretanto, o óleo essencial de *Baccharis dracunculifolia* não apresentou ação ativa nos ensaios antimicrobianos (*C. albicans*, *C.*

glabrata, C. krusei, C. neoformans). Os mesmos autores testaram a ação sozinha do principal composto observado no óleo essencial de *Baccharis dracuncolifolia* (nerolidol), entretanto esse não apresentou nenhuma atividade esquistossomosicida.

JOHANN; OLIVEIRA; SIQUEIRA; CISALPINO *et al.* (2012) extraíram a fração hexana da parte aérea de *Baccharis dracuncolifolia* e identificaram três compostos (ácido ursólico, metil linolenato, óxido de cariophileno e trans-nerolidol) com atividade antifúngica contra quatro espécies isoladas de *Paracoccidioides brasiliensis*, os quais são os principais fungos causadores de micose sistêmica na América Latina. Os mesmos autores concluíram que o meio de ação óxido de cariophileno pode estar relacionado com o ergosterol, um tipo de colesterol presente na membrana das células vegetais e fúngicas.

3. Concentração inibitória mínima

Para avaliar o potencial de um determinado aditivo para modular a fermentação ruminal, um dos métodos mais usados é a determinação de sua concentração inibitória mínima (CIM ou MIC), que pode ser definida como a menor concentração de um composto capaz de inibir o crescimento de um organismo desafiador. No caso de microrganismos aeróbicos pode-se utilizar de discos de difusão, em que o agente é aplicado a um poço ou disco de papel no centro de uma placa de ágar semeada com o microrganismo de teste sendo então mensurado posteriormente o raio (mm ou cm) de seu crescimento (MANN; MARKHAM, 1998). O método mais utilizado ou clássico é o teste de diluição em caldo é que envolve a preparação de diluições duplas do aditivo a ser testado em meio de crescimento líquido dispensado em tubos de ensaio. Os tubos contendo os aditivos são inoculados com uma suspensão bacteriana padronizada. Após incubação durante a noite a 35°C, os tubos são examinados quanto ao crescimento bacteriano visível, sendo evidenciado pela turbidez (DWIVEDI; PANDEY; PANDEY; RAMTEKE *et al.*, 2017).

Entretanto, no caso dos micro-organismos ruminais a manipulação se torna mais complexa por se tratar de indivíduos estritamente anaeróbios que não toleram oxigênio. Os trabalhos de Robert Hungate, o pai da microbiologia ruminal, trouxe muitas das tecnologias de cultura para bactérias anaeróbicas que ainda são amplamente utilizadas no mundo (HUNGATE, 1966). A técnica consiste em cultivar os micro-organismos em tubos contendo meio de cultura em um ambiente anaeróbicos. Muitos gases comerciais apresentam 99.998% de pureza, o que pode ser tóxico para os micro-organismos, sendo

necessário purificar os gases passando-os em tubos de cobre. O gás então purificado e aspergido no meio de cultura que é fervido e resfriado para criar um ambiente livre de O_2. Agentes redutores como a cisteína são adicionados ao meio para criar um ambiente altamente reduzido que é necessário para o crescimento dos micro-organismos. Por fim, os tubos são selados com tampas feitas de borracha butílica, que é mais resistente a oxidação e à penetração de oxigênio (HUNGATE, 1966; RUSSELL, 2002).

A partir deste meio de cultivo, diversos autores avaliaram o potencial de ionóforos e óleos essenciais contra culturas puras de bactérias pela mensuração de turbidez (650 nm). NEWBOLD; WALLACE; WATT e RICHARDSON (1988) avaliaram dois ionóforos (tetronasina e monensina) contra diversas bactérias ruminais. Eles incubaram em tubos hungate culturas puras de bactérias por 48 horas nos tubos com menores concentrações de ionóforos e repetiram o processo sucessivamente até que as bactérias fossem inibidas em 50% (MIC50). As bactérias mais succeptíveis à monensina e tetranosina foram a Gram-positivas como a *Ruminococcus flavefasciens* e *Ruminococcus albus*, entrentanto a *Ruminococcus flavefasciens* apresentou maior suscetibilidade para se adaptar à tetranosina.

MCINTOSH; WILLIAMS; LOSA; WALLACE *et al.* (2003) avaliaram o efeito de um blend de OE contendo timol, eugenol, vanilina e limoneno sobre o crescimento de culturas puras de bactérias. Eles também determinaram a MIC50 após 48 horas de incubação e observaram que os óleos essenciais foram capazes de inibir a maioria dos microrganismos em concentrações acima de 100 mg/L. *Streptococcus bovis* foi a bactéria mais resistente enquanto a *Prevotella ruminocola* foi uma da mais susceptíveis. Os mesmos autores ainda reportaram que algumas espécies, incluindo *Prevotella ruminicola* e *Prevotella bryantii* se adaptaram e foram capazes de crescer em maiores concentrações de OE, enquanto outras, incluindo *Clostridium sticklandii* e *Peptostreptococcus anaerobius*, que são bactérias hiper produtoras de amônia, permaneceram sensíveis.

Mais recentemente, DE AGUIAR; ZEOULA; FRANCO; PERES *et al.* (2013) avaliaram a atividade antimicrobiana de três extratos brasileiros de própolis contra cultura puras de bactérias ruminais. Os extratos de própolis inibiram o crescimento da *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, *Ruminococcus albus* 7, *Butyrivibrio fibrisolvens* D1, *Prevotella albensis* M384, *Peptostreptococcus sp*. D1, *Clostridium aminophilum F* e *Streptococcus bovis* Pearl11, enquanto *Ruminococcus albus* 20, *Prevotella bryantii* B14 e *Ruminobacter amylophilus H18* foram resistentes a todos os extratos. Além disso, as cepas que apresestaram maiores sensibilidades foram as

das bactérias hiper produtoras de amônia como a *Costridium aminophilum* F e *Peptostreptococcus sp*. Os autores também identificaram os principais compostos encontrados no própolis (naringenina, crisina, ácido cafeico, ácido p-cumarico e artepilina C). Entretanto, somente a narigenina apresentou efeito contra todas as cepas testadas.

No primeiro estudo da presente tese foi utilizada esta técnica para avaliar o potencial destes aditivos em afetar o crescimento de três bactérias Gram-posítivas (*Prevotella albensis, Prevotella bryantti,* e *Treponema sacharophilum*) e três Gram-negativas (*Ruminococcus albus*, *Ruminococcus flavefasciens* e *Streptococcus bovis*) que são de interessse na fermentação ruminal.

Bactérias do gênero *Prevotella* são uma das bactérias mais predominantes no rúmen e desempenham papel importante na degradação de peptídeos (CAMMACK; AUSTIN; LAMBERSON; CONANT *et al.*, 2018). Essas bactérias também são conhecidas por sua alta atividade da enzima dipeptil peptidase, que remove dipeptídeos das proteínas (WALLACE; MCKAIN, 1991). Uma redução no número de bactérias proteolíticas pode levar ao aumento de aminoácidos que escapam do rúmen, e pode beneficiar os animais pela maior eficiência na utilização de nitrogênio(DE AGUIAR; ZEOULA; FRANCO; PERES *et al.*, 2013).

Existe grande variedade de microrganismos capazes de fermentar pectina, no entanto, o crescimento da *Treponema saccharophilum* depende frequentemente da abundância de pectina como substrato(LIU; WANG; ZHU; PU *et al.*, 2014). A pectina é um carboidrato estrutural, mas não fibroso, que normalmente é encontrado nos alimentos para animais. A pectina é rapidamente degradada no rúmen, mas diferentes do amido, acetato e propionato são os principais produtos formados. Assim, favorecer o crescimento de bactérias utilizadoras de pectina pode ser benéfico para o animal, reduzindo o risco de acidose e outros distúrbios metabólicos causados por dietas ricas em amido (HATFIELD; WEIMER, 1995).

Ruminococcus. flavefaciens e *R. albus* são importantes bactérias celulolíticas encontradas no rúmen (CAMMACK; AUSTIN; LAMBERSON; CONANT *et al.*, 2018). Este grupo de bactérias é capaz de hidrolisar a celulose usando a enzima celulase (PELL; SCHOFIELD, 1993) e formando uma vasta gama de produtos finais como acetato, formato de succinato de butirato, CO2, etanol e CO2 (HUNGATE, 1966).

A acidose do rúmen geralmente ocorre com o acúmulo de lactato quando o gado é alimentado com dietas de alto concentrado. *Streptococcus. bovis* é uma bactéria

amilolítica frequentemente associada a esse distúrbio metabólico (CHEN; LIU; WANG; WANG *et al.*, 2016). No entanto, *S. bovis* é dependente do pH, produzindo lactato quando o pH é menor que 5,5, mas fermentação de formato, acetato e etanol quando o pH é maior que 6,0 (RUSSELL; HINO, 1985).

Um outro aspecto importante na avaliação da MIC, que também foi explorada em nosso estudo, é a relação do crescimento dos micro-organismos ao longo do tempo. Dentre os principais fatores que irão afetar a velocidade o crescimento dos micro-organismos se destacam a temperatura, o pH, o potencial redox, e a quantidade de substrato disponível no meio (ROBAZZA; TELEKEN; GOMES, 2010). Em um sistema fechado, sabe-se que o crescimento dos micro-organismos pode ser dividido em 4 fases (Figura 2).

Figura 2. Curva de crescimento típica de micro-organismos em ambientes finitos. Fonte: (ROBAZZA; TELEKEN; GOMES, 2010)

A primeira é chamada de fase "*lag",* na qual os micro-organismos ainda estão se adaptando ao meio, a fase exponencial, na qual os micro-organismos já adaptados e irão destinar a maioria de seu metabolismo para a reprodução, a fase estacionária, que pela escassez de nutrientes o surgimento e morte de micro-organismos são iguais e por fim, a fase de declínio que pela escassez de nutrientes o número de mortes supera a de novos micro-organismos (ROBAZZA; TELEKEN; GOMES, 2010). No caso de bactérias ruminais alguns autores avaliaram o crescimento de cultura pura de bactérias por até 48 horas (DE AGUIAR; ZEOULA; FRANCO; PERES *et al.*, 2013; MCINTOSH; WILLIAMS; LOSA; WALLACE *et al.*, 2003). Já, no primeiro estudo, baseado em ensaios preliminares de laboratório, observou-se que o tempo de 24 horas correspondia

ao final da fase estacionaria, portanto, trabalhando com apenas três pontos de observação 8, 12 e 24 horas.

4. Tratamentos de alimentos volumosos

4.1 Importância dos alimentos volumosos

Existe uma demanda crescente por um sistema de produção mais eficiente e por consequência mais sustentável. Uma das formas de se melhorar a eficiência do animal, é fornecendo forrageiras de melhor qualidade. A alfafa (*Medicado sativa*) também conhecida como a "Rainha das Forrageiras" é amplamente utilizada na produção de ruminantes pela sua alta produção, qualidade nutricional e palatabilidade (RADOVIĆ; SOKOLOVIĆ; MARKOVIĆ, 2009). No entanto, para se manter competitivo, os produtores sempre buscam por produtos alternativos, para diminuir seus custos com a alimentação, que podem representar até 80% dos custos totais dos animais em confinamento (ABRAHÃO; PRADO; PEROTTO; MOLETTA, 2005; CARVALHO; DE ZEN, 2017; SILVA; PRADO; CARVALHO; SILVA *et al.*, 2010).

Por exemplo, a palha de arroz é uma fonte de biomassa que se encontra em grande disponibilidade no mundo. Em 2018, a produção global de arroz foi de aproximadamente 770 milhões de toneladas, sendo a China, a Índia e o Brasil os principais produtores (FAOSTAT, 2018). Para cada kg de arroz colhido, mais de 1,5 kg de palha é produzido LAL (2005). Apesar de sua abundância, a palha de arroz é pouca utilizada na nutrição de ruminantes, pois apresenta baixa digestibilidade por seu alto conteúdo de sílica e estruturas recalcitrantes da parede celular (KOPP, 2003; MAKKAR; SÁNCHEZ; SPEEDY, 2007). Para dispor deste resíduo, a palha de arroz é frequentemente queimada no solo, contribuindo assim com as emissões de gases na atmosfera e redução da qualidade do ar (CHEN; LI; RISTOVSKI; MILIC *et al.*, 2017). Neste sentido, para melhorar a digestibilidade dos volumosos de baixa qualidade os pesquisadores desenvolveram diversos métodos de tratamentos que serão discutidos a seguir.

4.2 Tipos de tratamentos

Os tratamentos dos alimentos volumosos podem ser de origem, biológica, física ou química. Os tratamentos biológicos são aqueles que utilizam da inoculação de micro-organismos, como fungos por exemplo, que são capazes de degradar a lignina, por exemplo reduzindo de 11,7 para 5,7% a lignina na palhada de trigo e melhorando sua

digestibilidade de 29,7 para 58,1% sem prejudicar a quantidade de celulose e hemicelusose (MOYSON; VERACHTERT, 1991). Os tratamentos biológicos apresentam a vantagem sobre os físicos e químicos pois não poluem o meio-ambiente, entretanto necessitam de longos períodos para o desenvolvimento dos fungos podendo ocasionar perdas ou contaminação (CASTRO; PAIVA; DIAS; SANTOS, 2004).

Dentro os tratamentos físicos, a moagem apesar de não melhorar a digestibilidade do volumoso permite maior consumo do animal por causa da redução do tamanho de partícula (DE MORAIS; DE DEUS NEPOMUCENO; DE CARVALHO ALMEIDA, 2017). Outros tratamentos físicos utilizam da pressão e do vapor durante determinado tempo. A alta pressão permite a solubilização da hemicelulose, rompendo as ligações com a lignina enquanto a rápida descompressão junto com vapor de água afrouxa a estrutura da fibra celular (DE MORAIS; DE DEUS NEPOMUCENO; DE CARVALHO ALMEIDA, 2017). Já os tratamentos químicos mais utilizados se caracterizam pela utilização de diversos produtos alcalinos como a cal hidratada (Ca(OH)2), o hidróxido de sódio (NaOH) e a ureia ou amônia (amonização).

A amonização é um tratamento químico, que permite que a parede celular de forrageiras de baixa qualidade se tornem mais acessíveis para os microrganismos do rúmen e também melhorem a quantidade de nitrogênio não proteico, resultando em maior digestibilidade (BALS; TEYMOURI; HADDAD; JULIAN *et al.*, 2019; BEAUCHEMIN;; RIBEIRO;; RAN;; MILANI; *et al.*, 2019; BLÜMMEL; TEYMOURI; MOORE; NIELSON *et al.*, 2018). No método tradicional de amonização, utiliza-se de ureia ou amônia durante um período que pode variar de 7 a 30 dias dependendo da temperatura (inverno e verão), concentração de amônia ou ureia (2-4% da MS total) e a quantidade de água na forragem(PAIVA; GARCIA; QUEIROZ; REGAZZI, 1995).

4.3 *Ammonia Fiber Expansion*

Ammonia Fiber Expansion (AFEX) é uma tecnologia emergente que vem sendo explorada pela indústria do bicombustível, mas tem demonstrado potencial para ser utilizada na produção de ruminantes (BEAUCHEMIN;; RIBEIRO;; RAN;; MILANI; *et al.*, 2019; GRIFFITH; RIBEIRO JR; OBA; MCALLISTER *et al.*, 2016; MOR; BALS; TYAGI; TEYMOURI *et al.*, 2018). O tratamento por AFEX envolve expor a biomassa a altos níveis de amônia à elevada temperatura e pressão (100°C e 2 MPa) por pelo menos uma hora, com a vantagem de parte da amônia ser recuperada e reciclada no processo

(CAMPBELL; TEYMOURI; BALS; GLASSBROOK *et al.*, 2013; MOR; BALS; TYAGI; TEYMOURI *et al.*, 2018).

Recentes estudos *in vitro* e *in vivo* têm demonstrado o potencial desta tecnologia para melhorar o valor das forragens de baixa qualidade. BLÜMMEL; TEYMOURI; MOORE; NIELSON *et al.* (2018) testaram 10 tipos de palhas de cereais tratados com AFEX e observaram aumento no conteúdo de proteína bruta, produção de gás e digestibilidade aparente e verdadeira. Utilizando de um rúmen artificial (RUSITEC) GRIFFITH; RIBEIRO JR; OBA; MCALLISTER *et al.* (2016) observaram aumento no desaparecimento da matéria seca, matéria orgânica e fibra em detergente neutro de palhas de cevada tratada com AFEX. Outros autores observaram que ao trocar a palha de trigo por pellets de palha de trigo tratados com AFEX, aumentou a digestibilidade e a energia disponível de búfalos e vacas lactantes (MOR; BALS; TYAGI; TEYMOURI *et al.*, 2018). Em outro estudo, MOR; BALS; KUMAR; TYAGI *et al.* (2019) observaram redução na ingestão de matéria seca, e no crescimento, de cabritos alimentando com pellets de palha de trigo tratadas com AFEX em substituição ao concentrado. Os mesmos autores ainda observaram nenhuma diferença em relação aos parâmetros de fermentação ruminal e metabólitos no sangue, com exceção do aumento da atividade da acetamidade no rúmen. A acetamida (CH_3CONH_2) é produzida e incorporada na forragem pela reação entre amônia e acetato que ocorre durante o tratamento de AFEX. A quantidade de acetamida encontrada em diferentes palhadas por BALS; TEYMOURI; HADDAD; JULIAN *et al.* (2019) foi de 6,6 mg/g para o milho, 5,6 mg/g para o trigo, 4,3 mg/g para a cevada e 4,4 mg/g para o arroz. A acetamida pode ser utilizada como fonte de nitrogênio pelos microrganismos ruminais (DRAPER, 1967; NAGAYAMA; KONNO; OKA, 1961). Devido a sua simples estrutura química, tem-se sugerido que a acetamida é formada por diversos processos da digestão, podendo ser encontrada naturalmente em alimentos como leite e carne nos valores de 0.27 a 0.77 mg/kg.(VISMEH; HADDAD; MOORE; NIELSON *et al.*, 2017).

Todavia, recentes estudos demonstraram aumento nas concentrações de acetamida no leite de vacas (16−23 vezes) e búfalas (19−28 vezes) após três semanas de alimentação contendo palhas tratadas com AFEX (BALS; TEYMOURI; HADDAD; JULIAN *et al.*, 2019). Embora não exista atualmente nenhuma regulação para a quantidade de acetamida a ser consumida, o aumento deste composto nos alimentos levanta questionamentos sobre saúde dos consumidores uma vez que a acetamida é classificada como grupo 2B cancerígeno por sua capacidade de induzir tumores em ratos (IARC, 1999). Estima-se

que a exposição diária de acetamida na população dos Estados Unidos seja de 1.5 mg (BERCU; GALLOWAY; PARRIS; TEASDALE *et al.*, 2018). Esse valor está substancialmente abaixo aos associados ao músculo de ratos (7.000 mg/kg) que receberam doses letais de acetamida (KEGLEY; HILL; ORME; CHOI, 2014). Embora o impacto à saúde dos consumidores seja incerta, atualmente a acetamida é considerada um contaminante, podendo ser um ponto crítico na implementação da tecnologia AFEX.

5. Literatura citada

ABRAHÃO, J. J. S.; PRADO, I. N.; PEROTTO, D.; MOLETTA, J. L. Características de carcaças e da carne de tourinhos submetidos a dietas com diferentes níveis de substituição do milho por resíduo úmido da extração da fécula de mandioca. **Revista Brasileira de Zootecnia**, 34, n. 5, p. 1640-1650, 2005.

ALENCAR, S. M. d.; AGUIAR, C. L. d.; PAREDES-GUZMÁN, J.; PARK, Y. K. Composição química de Baccharis dracunculifolia, fonte botânica das própolis dos estados de São Paulo e Minas Gerais. **Ciência Rural**, 35, n. 4, 2005.

ALTERMANN, E.; SCHOFIELD, L. R.; RONIMUS, R. S.; BEATTY, A. K. *et al.* Inhibition of rumen methanogens by a novel archaeal lytic enzyme displayed on tailored bionanoparticles. **Frontiers in microbiology**, 9, p. 2378, 2018.

AZZAZ, H. H.; MURAD, H. A.; MORSY, T. A. Utility of ionophores for ruminant animals: a review. **Asian Journal of Animal Sciences**, 9, n. 6, p. 254-265, 2015.

BALANDRIN, M. F.; KLOCKE, J. A.; WURTELE, E. S.; BOLLINGER, W. H. Natural plant chemicals: sources of industrial and medicinal materials. **Science**, 228, n. 4704, p. 1154-1160, 1985.

BALS, B.; TEYMOURI, F.; HADDAD, D.; JULIAN, W. A. *et al.* Presence of Acetamide in Milk and Beef from Cattle Consuming AFEX-Treated Crop Residues. **Journal of agricultural and food chemistry**, 67, n. 38, p. 10756-10763, 2019.

BEAUCHEMIN;, K. A.; RIBEIRO;, G. O.; RAN;, T.; MILANI;, M. R. M. *et al.* Recombinant fibrolytic feed enzymes and ammonia fibre expansion (AFEX) pretreatment of crop residues to improve fibre degradability in cattle. **Animal Feed Science and Technology**, 256, p. 114260, 2019.

BELANCHE, A.; DOREAU, M.; EDWARDS, J. E.; MOORBY, J. M. *et al.* Shifts in the Rumen Microbiota Due to the Type of Carbohydrate and Level of Protein Ingested by Dairy Cattle Are Associated with Changes in Rumen Fermentation–3. **The Journal of nutrition**, 142, n. 9, p. 1684-1692, 2012.

BENCHAAR, C.; CHAVES, A.; FRASER, G.; BEAUCHEMIN, K. *et al.* Effects of essential oils and their components on in vitro rumen microbial fermentation. **Canadian journal of animal science**, 87, n. 3, p. 413-419, 2007.

BENCHAAR, C.; GREATHEAD, H. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. **Animal Feed Science and Technology**, 166, p. 338-355, 2011.

BERCU, J.; GALLOWAY, S.; PARRIS, P.; TEASDALE, A. *et al.* Potential impurities in drug substances: Compound-specific toxicology limits for 20 synthetic reagents and by-products, and a class-specific toxicology limit for alkyl bromides. **Regulatory Toxicology and Pharmacology**, 94, p. 172-182, 2018.

BINDER, R.; APPLEWHITE, T.; KOHLER, G.; GOLDBLATT, L. Chromatographie analysis of seed oils. Fatty acid composition of castor oil. **Journal of the American Oil Chemists' Society**, 39, n. 12, p. 513-517, 1962.

BLÜMMEL, M.; TEYMOURI, F.; MOORE, J.; NIELSON, C. *et al.* Ammonia Fiber Expansion (AFEX) as spin off technology from 2nd generation biofuel for upgrading cereal straws and stovers for livestock feed. **Animal Feed Science and Technology**, 236, p. 178-186, 2018/02/01/ 2018.

BORCHERS, R. Proteolytic activity of rumen fluid in vitro. **Journal of Animal Science**, 24, n. 4, p. 1033-1038, 1965.

BRODERICK, G.; BALTHROP JR, J. Chemical inhibition of amino acid deamination by ruminal microbes in vitro. **Journal of Animal Science**, 49, n. 4, p. 1101-1111, 1979.

BUSQUET, M.; CALSAMIGLIA, S.; FERRET, A.; KAMEL, C. Screening for the effects of natural plant extracts and secondary plant metabolites on rumen microbial fermentation in continuous culture. **Anim. Feed Sci. Technol**, 123, n. 124, p. 597-613, 2005.

CALSAMIGLIA, S.; BUSQUET, M.; CARDOZO, P. W.; CASTILLEJOS, L. *et al.* Invited Review: Essential Oils as Modifiers of Rumen Microbial Fermentation. **Journal of Dairy Science**, 90, n. 6, p. 2580-2595, 2007/06/01/ 2007.

CAMMACK, K. M.; AUSTIN, K. J.; LAMBERSON, W. R.; CONANT, G. C. *et al.* RUMINANT NUTRITION SYMPOSIUM: Tiny but mighty: the role of the rumen microbes in livestock production. **Journal of animal science**, 96, n. 2, p. 752-770, 2018.

CAMPBELL, T. J.; TEYMOURI, F.; BALS, B.; GLASSBROOK, J. *et al.* A packed bed ammonia fiber expansion reactor system for pretreatment of agricultural residues at regional depots. **Biofuels**, 4, n. 1, p. 23-34, 2013.

CARVALHO, T. B.; DE ZEN, S. A cadeia de Pecuária de Corte no Brasil: evolução e tendências. **Revista iPecege**, 3, n. 1, p. 85-99, 2017.

CASTILLEJOS, L.; CALSAMIGLIA, S.; FERRET, A. Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in in vitro systems. **Journal of dairy science**, 89, n. 7, p. 2649-2658, 2006.

CASTRO, A. L. A. d.; PAIVA, P. C. d. A.; DIAS, E. S.; SANTOS, J. d. Avaliação das alterações bromatológicas e de degradabilidade do resíduo de lixadeira do algodão após tratamento biológico com Pleurotus sajor-caju. **Ciência e agrotecnologia**, 28, n. 3, p. 608-613, 2004.

CHEN, J.; LI, C.; RISTOVSKI, Z.; MILIC, A. *et al.* A review of biomass burning: Emissions and impacts on air quality, health and climate in China. **Science of The Total Environment**, 579, p. 1000-1034, 2017/02/01/ 2017.

CHEN, L.; LIU, S.; WANG, H.; WANG, M. *et al.* Relative significances of pH and substrate starch level to roles of Streptococcus bovis S1 in rumen acidosis. **AMB Express**, 6, n. 1, p. 80, 2016/09/22 2016.

CHENG, K.; MCCOWAN, R.; COSTERTON, J. Adherent epithelial bacteria in ruminants and their roles in digestive tract function. **The American journal of clinical nutrition**, 32, n. 1, p. 139-148, 1979.

CHENG, Y. F.; EDWARDS, J. E.; ALLISON, G. G.; ZHU, W.-Y. *et al.* Diversity and activity of enriched ruminal cultures of anaerobic fungi and methanogens grown together on lignocellulose in consecutive batch culture. **Bioresource technology**, 100, n. 20, p. 4821-4828, 2009.

CLARKE, L.; FODEY, T. L.; CROOKS, S. R. H.; MOLONEY, M. *et al.* A review of coccidiostats and the analysis of their residues in meat and other food. **Meat Science**, 97, n. 3, p. 358-374, 2014/07/01/ 2014.

COBELLIS, G.; TRABALZA-MARINUCCI, M.; MARCOTULLIO, M. C.; YU, Z. Evaluation of different essential oils in modulating methane and ammonia production, rumen fermentation, and rumen bacteria *in vitro*. **Animal Feed Science and Technology**, 215, p. 25-36, 2016.

COUVREUR, S.; HURTAUD, C.; LOPEZ, C.; DELABY, L. *et al.* The linear relationship between the proportion of fresh grass in the cow diet, milk fatty acid composition, and butter properties. **Journal of Dairy Science**, 89, n. 6, p. 1956-1969, 2006.

CRUZ, O. T. B.; VALERO, M. V.; ZAWADZKI, F.; RIVAROLI, D. C. *et al.* Effect of glycerine and essential oils (Anacardium occidentale and Ricinus communis) on animal performance, feed efficiency and carcass characteristics of crossbred bulls finished in a feedlot system. **Italian Journal of Animal Science**, 13, n. 4, p. 3492, 2014.

CRUZ, O. T. B.; VALERO, M. V.; ZAWADZKI, F.; RIVAROLI, D. C. *et al.* Effect of glycerine and essential oils (*Anacardium occidentale* and *Ricinus communis*) on animal performance, feed efficiency and carcass characteristics of crossbred bulls finished in a feedlot system. **Italian Journal of Animal Science**, 13, n. 4, p. 790-797, 2014.

DA SILVA, L. G.; TORRECILHAS, J. A.; PASSETTI, R. A. C.; ORNAGHI, M. G. *et al.* Glycerin and cashew and castor oils in the diets for bulls in finished in feed lot: Ingestive behavior. **Semina: Ciências Agrárias**, 35, n. 5, p. 2723-2737, 2014.

DAWSON, K. A.; BOLING, J. A. Effects of potassium ion concentrations on the antimicrobial activities of ionophores against ruminal anaerobes. **Applied and Environmental Microbiology.**, 53, n. 10, p. 2363-2367, 1987.

DE AGUIAR, S. C.; ZEOULA, L. M.; FRANCO, S. L.; PERES, L. P. *et al.* Antimicrobial activity of Brazilian propolis extracts against rumen bacteria in vitro. **World Journal of Microbiology and Biotechnology**, 29, n. 10, p. 1951-1959, 2013.

DE JESUS, E. F.; DEL VALLE, T. A.; CALOMENI, G. D.; SILVA, T. H. d. *et al.* Influence of a blend of functional oils or monensin on nutrient intake and digestibility, ruminal fermentation and milk production of dairy cows. **Animal Feed Science and Technology**, 219, p. 59-67, 2016.

DE MORAIS, L. F.; DE DEUS NEPOMUCENO, D.; DE CARVALHO ALMEIDA, J. C. Tratamentos de volumosos de baixo valor nutritivo para ruminantes-uma revisão. **Acta tecnológica**, 11, n. 1, p. 67-81, 2017.

DE PAEPE, M.; LECLERC, M.; TINSLEY, C. R.; PETIT, M.-A. Bacteriophages: an underestimated role in human and animal health? **Frontiers in cellular and infection microbiology**, 4, p. 39, 2014.

DE SOUSA, J. P. B.; JORGE, R. F.; LEITE, M. F.; FURTADO, N. A. *et al.* Seasonal variation of the (E)-nerolidol and other volatile compounds within ten different cultivated populations of Baccharis dracunculifolia DC (Asteraceae). **Journal of Essential Oil Research**, 21, n. 4, p. 308-314, 2009.

DEHORITY, B. A.; TIRABASSO, P. A. Factors affecting the migration and sequestration of rumen protozoa in the family Isotrichidae. **Microbiology**, 135, n. 3, p. 539-548, 1989.

DORMAN, H.; DEANS, S. G. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. **Journal of applied microbiology**, 88, n. 2, p. 308-316, 2000.

DRAPER, P. The aliphatic acylamide amidohydrolase of Mycobacterium smegmatis: its inducible nature and relation to acyl-transfer to hydroxylamine. **Microbiology**, 46, n. 1, p. 111-123, 1967.

DUTRA, T. V.; CASTRO, J. C.; MENEZES, J. L.; RAMOS, T. R. *et al.* Bioactivity of oregano (Origanum vulgare) essential oil against Alicyclobacillus spp. **Industrial crops and products**, 129, p. 345-349, 2019.

DWIVEDI, C.; PANDEY, I.; PANDEY, H.; RAMTEKE, P. W. *et al.* Chapter 9 - Electrospun Nanofibrous Scaffold as a Potential Carrier of Antimicrobial Therapeutics for Diabetic Wound Healing and Tissue Regeneration. *In*: GRUMEZESCU, A. M. (Ed.). **Nano- and Microscale Drug Delivery Systems**: Elsevier, 2017. p. 147-164.

ELEKWACHI, C. O.; WANG, Z.; WU, X.; RABEE, A. *et al.* Total rRNA-seq analysis gives insight into bacterial, fungal, protozoal and archaeal communities in the rumen using an optimized RNA isolation method. **Frontiers in microbiology**, 8, p. 1814, 2017.

FAOSTAT. Statistics division. Food and agriculture organization of the United States. 2018.

FERNANDO, S. C.; PURVIS, H.; NAJAR, F.; SUKHARNIKOV, L. *et al.* Rumen microbial population dynamics during adaptation to a high-grain diet. **Applied and Environmental Microbiology**, 76, n. 22, p. 7482-7490, 2010.

FERRONATTO, R.; MARCHESAN, E. D.; PEZENTI, E.; BEDNARSKI, F. *et al.* Atividade antimicrobiana de óleos essenciais produzidos por Baccharis dracunculifolia DC e Baccharis uncinella DC (Asteraceae). **Revista Brasileira farmacognosia**, 17, p. 224-230, 2007.

GANDRA, J.; NUNES GIL, P.; GANDRA, E.; DEL VALE, T. *et al.* Productive performance of simmental dairy cows supplemented with ricinoleic acid from castor oil. **Archivos de Zootecnia**, 63, n. 244, p. 575-585, 2014.

GANDRA, J. R.; GIL, P. N.; CÔNSOLO, N. R. B.; GANDRA, E. *et al.* Addition of increasing doses of ricinoleic acid from castor oil (Ricinus communis L.) in diets of Nellore steers in feedlots. **Journal of Animal and Feed Science** 21, p. 566-576, 2012.

GILBERT, R. A.; KLIEVE, A. V. Ruminal viruses (bacteriophages, archaeaphages). *In*: **Rumen microbiology: From evolution to revolution**: Springer, 2015. p. 121-141.

GÓMEZ-CARAVACA, A.; GÓMEZ-ROMERO, M.; ARRÁEZ-ROMÁN, D.; SEGURA-CARRETERO, A. *et al.* Advances in the analysis of phenolic compounds in products derived from bees. **Journal of Pharmaceutical and Biomedical Analysis**, 41, n. 4, p. 1220-1234, 2006.

GRIFFITH, C. L.; RIBEIRO JR, G. O.; OBA, M.; MCALLISTER, T. A. *et al.* Fermentation of ammonia fiber expansion treated and untreated barley straw in a rumen simulation technique using rumen inoculum from cattle with slow versus fast rate of fiber disappearance. **Frontiers in microbiology**, 7, p. 1839, 2016.

GRUNINGER, R. J.; PUNIYA, A. K.; CALLAGHAN, T. M.; EDWARDS, J. E. *et al.* Anaerobic fungi (phylum Neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. **FEMS microbiology ecology**, 90, n. 1, p. 1-17, 2014.

HATFIELD, R. D.; WEIMER, P. J. Degradation characteristics of isolated and in situ cell wall lucerne pectic polysaccharides by mixed ruminal microbes. **Journal of the Science of Food and Agriculture**, 69, n. 2, p. 185-196, 1995.

HENDERSON, G.; COX, F.; GANESH, S.; JONKER, A. *et al.* Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. **Scientific Reports**, 5, p. 14567, 10/09/online 2015. Article.

HRISTOV, A. N.; IVAN, M.; NEILL, L.; MCALLISTER, T. Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. **Animal Feed Science and Technology**, 105, n. 1-4, p. 163-184, 2003.

HUNGATE, R. E. **The Rumen and it Microbes**. New York: Academic Press, 1966.

HUWS, S. A.; CREEVEY, C. J.; OYAMA, L. B.; MIZRAHI, I. *et al.* Addressing global ruminant agricultural challenges through understanding the rumen microbiome: Past, present, and future. **Frontiers in microbiology**, 9, 2018.

HUWS, S. A.; KIM, E. J.; KINGSTON-SMITH, A. H.; LEE, M. R. *et al.* Rumen protozoa are rich in polyunsaturated fatty acids due to the ingestion of chloroplasts. **FEMS microbiology ecology**, 69, n. 3, p. 461-471, 2009.

IARC. **Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide**. IARC, 1999. 9283212711.

IPCC. **Climate change: The physical science basis. In: Contribution of Working**

Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, 2007. 0521705967.

JOHANN, S.; OLIVEIRA, F. B.; SIQUEIRA, E. P.; CISALPINO, P. S. *et al.* Activity of compounds isolated from Baccharis dracunculifolia DC (Asteraceae) against Paracoccidioides brasiliensis. **Medical mycology**, 50, n. 8, p. 843-851, 2012.

JOUANY, J.-P. Effect of rumen protozoa on nitrogen utilization by ruminants. **The Journal of nutrition**, 126, n. suppl_4, p. 1335S-1346S, 1996.

JOUANY, J.; USHIDA, K. The role of protozoa in feed digestion-Review. **Asian-Australasian Journal of Animal Sciences**, 12, n. 1, p. 113-128, 1999.

KEGLEY, S.; HILL, B.; ORME, S.; CHOI, A. PAN pesticide database, pesticide action network, North America (Oakland, CA). 2014.

KOPP, J. Using Straw in Cattle Rations-Frequently Asked Questions. 2003.

KRAUSE, D. O.; DENMAN, S. E.; MACKIE, R. I.; MORRISON, M. *et al.* Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. **FEMS microbiology reviews**, 27, n. 5, p. 663-693, 2003.

LAL, R. World crop residues production and implications of its use as a biofuel. **Environment International**, 31, n. 4, p. 575-584, 2005/05/01/ 2005.

LAMBERT, R.; SKANDAMIS, P. N.; COOTE, P. J.; NYCHAS, G. J. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. **Journal of applied microbiology**, 91, n. 3, p. 453-462, 2001.

LENG, R. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. **Animal Production Science**, 54, n. 5, p. 519-543, 2014.

LETAROV, A.; KULIKOV, E. The bacteriophages in human-and animal body-associated microbial communities. **Journal of applied microbiology**, 107, n. 1, p. 1-13, 2009.

LIU, J.; WANG, J.-K.; ZHU, W.; PU, Y.-Y. *et al.* Monitoring the rumen pectinolytic bacteria Treponema saccharophilum using real-time PCR. **FEMS Microbiology Ecology**, 87, n. 3, p. 576-585, 2014.

MAIA, M. d. O.; SUSIN, I.; FERREIRA, E. M.; NOLLI, C. P. *et al.* Intake, nutrient apparent digestibility and ruminal constituents of sheep fed diets with canola, sunflower or castor oils. **Revista Brasileira de Zootecnia**, 41, n. 11, p. 2350-2356, 2012.

MAIA, M. d. O.; SUSIN, I.; PIRES, A. V.; GENTIL, R. S. *et al.* Growth, carcass characteristics, chemical composition and fatty acid profile of the longissimus dorsi muscle in goat kids fed diets with castor oil. **Revista Brasileira de Zootecnia**, 41, n. 11, p. 2343-2349, 2012.

MAKKAR, H. P. S.; SÁNCHEZ, M.; SPEEDY, A. W. **Feed supplementation blocks. Urea-molasses multi-nutrient blocks: simple and effective feed supplement technology for ruminant agriculture**. Rome, Italy: FAO, 2007. 978-92-5-105438-3

MANN, C.; MARKHAM, J. A new method for determining the minimum inhibitory concentration of essential oils. **Journal of applied microbiology**, 84, n. 4, p. 538-544, 1998.

MARÓSTICA JUNIOR, M. R.; DAUGSCH, A.; MORAES, C. S.; QUEIROGA, C. L. *et al.* Comparison of volatile and polyphenolic compounds in Brazilian green propolis and its botanical origin Baccharis dracunculifolia. **Food Science and Technology (Campinas)**, 28, n. 1, p. 178-181, 2008.

MCINTOSH, F.; WILLIAMS, P.; LOSA, R.; WALLACE, R. *et al.* Effects of essential oils on ruminal microorganisms and their protein metabolism. **Appl. Environ. Microbiol.**, 69, n. 8, p. 5011-5014, 2003.

MILLEN, D. D.; ARRIGONI, M. D. B.; PACHECO, R. D. L. **Rumenology**. Springer, 2016. 3319305336.

MOR, P.; BALS, B.; KUMAR, S.; TYAGI, N. *et al.* Influence of replacing concentrate mixture with AFEX pellets on rumen fermentation, blood profile and acetamide content in the rumen of crossbred (Alpine × Beetle) female goats. **Small Ruminant Research**, 170, p. 109-115, 2019/01/01/ 2019.

MOR, P.; BALS, B.; TYAGI, A. K.; TEYMOURI, F. *et al.* Effect of ammonia fiber expansion on the available energy content of wheat straw fed to lactating cattle and buffalo in India. **Journal of Dairy Science**, 101, n. 9, p. 7990-8003, 2018/09/01/ 2018.

MORALES, E. R.; MATA ESPINOSA, M. A.; MCKAIN, N.; WALLACE, R. J. Ricinoleic acid inhibits methanogenesis and fatty acid biohydrogenation in ruminal digesta from sheep and in bacterial cultures. **Journal of Animal Science**, 90, n. 13, p. 4943-4950, 2012.

MOYSON, E.; VERACHTERT, H. Growth of higher fungi on wheat straw and their impact on the digestibility of the substrate. **Applied Microbiology and Biotechnology**, 36, n. 3, p. 421-424, 1991.

MURAKAMI, A.; EYNG, C.; TORRENT, J. Effects of functional oils on coccidiosis and apparent metabolizable energy in broiler chickens. **Asian-Australasian journal of animal sciences**, 27, n. 7, p. 981, 2014.

NAGARAJA, T.; TAYLOR, M. Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives. **Applied Environmental Microbiology**, 53, n. 7, p. 1620-1625, 1987.

NAGARAJA, T.; TITGEMEYER, E. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. **Journal of Dairy Science**, 90, p. E17-E38, 2007.

NAGAYAMA, H.; KONNO, K.; OKA, S. Formamidase in mycobacteria and its use in differentiating saprophytic mycobacteria from other mycobacteria. **Nature**, 190, n. 4782, p. 1219, 1961.

NEWBOLD, C. J.; DE LA FUENTE, G.; BELANCHE, A.; RAMOS-MORALES, E. *et al.* The role of ciliate protozoa in the rumen. **Frontiers in microbiology**, 6, p. 1313, 2015.

NEWBOLD, C. J.; WALLACE, R. J.; WATT, N.; RICHARDSON, A. J. Effect of the novel ionophore tetronasin (ICI 139603) on ruminal microorganisms. **Applied Environmental Microbiology**, 54, n. 2, p. 544-547, 1988.

NIKAIDO, H.; NAKAE, T. The outer membrane of Gram-negative bacteria. *In*: **Advances in microbial physiology**: Elsevier, 1980. v. 20, p. 163-250.

ORNAGHI, M. G.; PASSETTI, R. A. C.; TORRECILHAS, J. A.; MOTTIN, C. *et al.* Essential oils in the diet of young bulls: Effect on animal performance, digestibility, temperament, feeding behaviour and carcass characteristics. **Animal Feed Science and Technology**, 234, p. 274-283, 2017/12/01/ 2017.

PAIVA, J.; GARCIA, R.; QUEIROZ, A. d.; REGAZZI, A. Efeitos dos níveis de amônia anidra e períodos de amonização sobre os teores dos constituintes da parede celular na palhada de milho (Zea mays L.). **Revista Brasileira de Zootecnia**, 24, n. 5, p. 683-692, 1995.

PARREIRA, N. A.; MAGALHÃES, L. G.; MORAIS, D. R.; CAIXETA, S. C. *et al.* Antiprotozoal, schistosomicidal, and antimicrobial activities of the essential oil from the leaves of Baccharis dracunculifolia. **Chemistry & biodiversity**, 7, n. 4, p. 993-1001, 2010.

PELL, A. N.; SCHOFIELD, P. Microbial adhesion and degradation of plant cell walls. **Forage cell wall structure and digestibility**, p. 397-423, 1993.

PRADO, I.; CRUZ, O.; VALERO, M.; ZAWADZKI, F. *et al.* Effects of glycerin and essential oils (Anacardium occidentale and Ricinus communis) on the meat quality of crossbred bulls finished in a feedlot. **Animal Production Science**, 56, n. 12, p. 2105-2114, 2016.

PRADO, O. P. P.; ZEOULA, L. M.; MOURA, L. P. P.; FRANCO, S. L. *et al.* Isolation and expeditious morphological, biochemical and kinetic characterization of propolis-tolerant ruminal bacteria. **Revista Brasileira de Zootecnia**, 39, n. 9, p. 2048-2054, 2010.

PRESSMAN, B. C. Biological applications of ionophores. **Annual review of biochemistry**, 45, n. 1, p. 501-530, 1976.

RADOVIĆ, J.; SOKOLOVIĆ, D.; MARKOVIĆ, J. Alfalfa-most important perennial forage legume in animal husbandry. **Biotechnology in Animal Husbandry**, 25, n. 5-6-1, p. 465-475, 2009.

REZAEIAN, M.; BEAKES, G. W.; PARKER, D. S. Distribution and estimation of anaerobic zoosporic fungi along the digestive tracts of sheep. **Mycological research**, 108, n. 10, p. 1227-1233, 2004.

ROBAZZA, W. S.; TELEKEN, J. T.; GOMES, G. A. Modelagem Matemática do Crescimento de Microrganismos em Alimentos. **2010**, 11, n. 1, p. 10, 2010-06-01 2010.

RUSSELL, J. B. **Rumen microbiology an its role in ruminant nutrition**. New York: Cornell University, 2002.

RUSSELL, J. B.; HINO, T. Regulation of lactate production in Streptococcus bovis: a spiraling effect that contributes to rumen acidosis. **Journal of Dairy Science**, 68, n. 7, p. 1712-1721, 1985.

SILVA, L. G. d.; TORRECILHAS, J. A.; ORNAGHI, M. G.; EIRAS, C. E. *et al.* Glycerin and essential oils in the diet of Nellore bulls finished in feedlot: animal performance and apparent digestibility. **Acta Scientiarum. Animal Sciences**, 36, n. 2, p. 177-184, 2014.

SILVA, R. R.; PRADO, I. N.; CARVALHO, G. G. P.; SILVA, F. F. *et al.* Níveis de suplementação na terminação de novilhos Nelore em pastagens: aspectos econômicos. **Revista Brasileira de Zootecnia**, 39, n. 9, p. 2091-2097, 2010.

VALERO, M. V.; PRADO, R. M. d.; ZAWADZKI, F.; EIRAS, C. E. *et al.* Propolis and essential oils additives in the diets improved animal performance and feed efficiency of bulls finished in feedlot. **Acta Scientiarum. Animal Sciences**, 36, n. 4, p. 419-426, 2014.

VAN NEVEL, C.; DEMEYER, D.; HENDERICKX, H. Effect of fatty acid derivatives on rumen methane and propionate in vitro. **Applied Environmental Microbiology**, 21, n. 2, p. 365-366, 1971.

VISMEH, R.; HADDAD n, D.; MOORE, J.; NIELSON, C. *et al.* Exposure Assessment of Acetamide in Milk, Beef, and Coffee Using Xanthydrol Derivatization and Gas Chromatography/Mass Spectrometry. **Journal of agricultural and food chemistry**, 66,. 1, p. 298-305, 2017.

WALLACE, R. J.; MCKAIN, N. A survey of peptidase activity in rumen bacteria. **Microbiology**, 137, n. 9, p. 2259-2264, 1991.

YONGKUN, P.; IKEGAKI, M.; DE ALENCAR, S.; DE MOURA, F. Evaluation of Brazilian propolis by both physicochemical methods and biological activity. **Honeybee Science**, 21, n. 2, p. 85-90, 2000.

ZAWADZKI, F.; PRADO, I. N.; MARQUES, J. A.; ZEOULA, L. M. *et al.* Sodium monensin or propolis extract in the diets of feedlot-finished bulls: effects on animal performance and carcass characteristics. **Journal of Animal and Feed Sciences**, 20, n. 1, p. 16-25, 2011.

II - OBJETIVOS

O objetivo deste estudo foi de avaliar o potencial uso de aditivos naturais e o tratamento da palha de arroz por AFEX na produção de ruminantes.

Mais especificamente, objetivou-se então avaliar a capacidade de inibição da monensina, óleo essencial de orégano, óleo vegetal de mamona, extrato de *Baccharis dracunculifolia* em três bactérias gram-negativas e três bactérias gram-positivas encontradas no rúmen. Também se objetivou avaliar o potencial da tecnologia AFEX no desempenho e quantificar os resíduos de acetamida na carne de cordeiros.

III - RUMENSIN, OREGANO ESSENTIAL OIL, CASTOR OIL, AND BACCHARIS HYDROETHANOLIC EXTRACT ON GROWTH INHIBITION OF RUMEN GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

(Animal Feed Science and Technology)

ABSTRACT. Natural additives are a promising tool to modulate ruminal fermentation due to the recently concern against antimicrobial resistance to antibiotics. We evaluate the antimicrobial capacity of different levels (10, 20, 50 and 100 mg / L) of monensin, oregano essential oil (*Origanum vulgare L)*, castor oil (*Ricinus communis*), and hydroetanolic extract of *Baccharis* (*Baccharis dracuncuculifolia)* against Gram-negative (*Prevotella albensis, Prevotella bryantii* and *Treponema saccharophilum*), and Gram-positive bacteria (*Ruminococcus albus, Ruminococcus flavefaciens* and *Streptococcus bovis*). Optical density (600 nm) was measured at 8, 12 and 24 h. Monensin inhibited Gram-positive bacteria growth ($P < 0.05$), However, higher dosages was also effective against Gram-negative bacteria, possible due to a toxicological effect. Oregano essential oil inhibited ($P < 0.05$) both Gram-negative and Gram-positive bacteria. Castor oil had marginal or no effect on Gram-negative but inhibited the cellulolytic bacteria (*R. albus*) growth at 12 h. Concentrations of *Baccharis dracunculifolia* used in this study (up to 100 mg / L) had no inhibitory effect on ruminal bacteria. Natural additives are a promising tool to modulate ruminal fermentation, which highlights the importance of further studies to evaluate the potential of new products.

1. Introduction

According to ANUALPEC (2019), Brazil cattle herd has approximately 215 million heads, being the majority, raised on pasture and low input production systems. Extensive production systems increases the slaughter age, which promotes high methane (CH_4) emissions per kg of product (Cardoso et al., 2016). However, the number of cattle finished in feedlot in Brazil have doubled in the last 10 years (ANUALPEC, 2019), and it have been estimated that intensification practices could reduce up to 48% the greenhouse gases emissions per kg carcass and to 7 fold the total land area used per kg carcass (Cardoso et al., 2016).

In more intensive production systems, antibiotics or ionophores, like monensin, are frequently added into ruminants diets as a strategy to modulate rumen fermentation and improve animal efficiency (Zawadzki et al., 2011). In contrast, the routine antibiotics usage as growth-promoters in feed has generated public health concerns due to the emergence of antibiotic resistant-bacteria that could represent risks to human health (Schäberle and Hack, 2014). Consequently, considerable efforts have been employed towards the development of natural alternatives to antibiotics and ionophores (Ornaghi et al., 2017; Souza et al., 2019; Ornaghi et al., 2020). Among these substances, plants extracts and essential oils have attracted the most attention (Monteschio et al., 2017; Rivaroli et al., 2017; Fugita et al., 2018; Ornaghi et al., 2020).

The oregano essential oil have monoterpenes like carvacrol and thymol, that are capable to affect the cellular membrane permeability of Gram-negative bacteria (Lambert et al., 2001). Castor oil, a co-product of castor seed is composed by 90% of ricinoleic acid which is commonly known due to its antimicrobial properties (Gandra et al., 2014). More recently, others studies have been focusing in a plant commonly found in South America, *Baccharis dracunculifolia*, which is the raw material used by bees (*Apis melifera*) to produce the green propolis (Maróstica Junior et al., 2008), which could be used to modulate ruminal fermentation due to its richness on secondary metabolites, like flavonoids, that have antioxidants and antimicrobials properties (Ferronatto et al., 2007; Bonin et al., 2020).

Considerable scientific information has been generated to show that these alternatives additives can alter rumen fermentation, feed digestion and bacterial and archaeal communities (Cobellis et al., 2016b). However, due to large plants variety as well as the extraction and processing methods, results in literature are still divergent. This highlight the importance but also the potential of news studies. However, animal studies are costly and time consuming, but the use of *in vitro* techniques testing additives against the pure culture bacteria growth could be used for screening the selection of new products (McIntosh et al., 2003; Aguiar et al., 2013) to be further tested on animals.

This study was realized to evaluate alternative natural additives (oregano essential oil, castor oil, and *B. dracunculifolia* extracts) in comparison to monesin in the pure culture bacteria growth.

2. Material and methods

2.1. Monensin and natural additives

Rumensin™ 200 (20% of sodium monensin - Elanco®) was purchased in feeding mill supply shop (AB Araújo – Maringá, Paraná Brazil). Oregano essential oil was obtained from FERQUIMA® (Vargem Grande Paulista, São Paulo, Brazil). Castor plant oil was obtained from SAFEEDS® (Cascavel, Paraná, Brazil) and stored ± 4º C.

Oregano essential oil have been previously characterized and showed a great amount of gama-terpinene and ortho-cymene (8.0% and 9.4%, respectively), while carvacrol was found as the major compound (68.3%) (Biondo et al., 2017). *Baccharis dracunculifolia* samples were collected at Maringá city, Paraná, Brazil south, with geographic coordinates 23°27'S and longitude 51°59'W. Climatic conditions of region have be annual average temperature of 18° C and annual average rainfall of 1,114 mm. Whole plants collected were weighed and dried in a forced air circulation oven (TECNAL - TE-394/2 – Piracicaba, São Paulo, Brazil) at a temperature of 40° C. The dry material was then, milled using 1 mm sieve and a knife mill (WILLEY). Then, 10 g of the material was mixed with hydroethanolic solution (70:30, v / v) and placed in agitation (20 min) with rest (15 min) for 2 h. The extract was kept in a water bath (35° C) for 24 h. The extract was then filtered and concentrated using a rotary evaporator (FISATOM – São Paulo, Brazil) at ambient temperature, until the solvent was completely evaporated. The remaining crude extract was lyophilized and stored at 4° C.

Lyophilized *B. dracunculifolia* samples were diluted in acetonitrile in a 1:1 ratio and analyzed by UHPLC-HRMS using a Nexera X2 ultra-high performance liquid chromatography system as described by Bonin et al. (2020). MS and MS / MS spectra were visualized using Software Data Analysis 4.3, and then compared to the existing literature and analyzed using a free-access mass spectrometry database such as Human Metabolome Database (HMDB) (Wishart et al., 2012). Twelve compounds were identified: germacrene B, spathulenol, naringenin, kaempferol, artepillin C, alpha-pinene, hydroxycinnmic acid, apigenin, kaempferide, limonene, phenylethanol, β-caryophyllene (Figure 1) as described by Bonin et al. (2020). The same procedure was used for castor oil; in which ricinoleic acid was identified as the main compost (Figure 2).

2.2. Bacteria strain and culture conditions

Three Gram-negative bacteria: *Prevotella albensis* (DSMZ 11370), *Prevotella bryantii* (DSMZ 11371) and *Treponema saccharophilum* (DSMZ 2985), and three Gram-positive bacteria: *Ruminococcus albus* (DSMZ 20455,) *Ruminococcus flavefaciens* (DSMZ 25089) *Streptococcus bovis* (DSMZ 20480) were purchased (DSMZ – Brunswick, Germany). Lyophilized bacteria were activated according manufactures recommendation, and then replicates were frozen in Hungate tubes with Hobson M2 media (Hobson and Stewart, 2012) plus glycerol (20%) and stored at -80ºC. The medium consisted of 2 g glucose, 2 g maltose, 4 g sodium bicarbonate, 10 g bacto-casitone, 2.5 g yeast, 2 g cellobiose, 150 mL mineral solution I, 150 mL of mineral solution II, 200 mL of clarified rumen fluid, 10 mL of 60% (w / v) sodium lactate solution, 1 mL of 0.1% resazurin solution, in 1 L of distilled water. The mineral solution I consists of 3 g of dipotassium phosphate in 1 L of distilled water. Mineral solution II consists of 3 g of monopotassium phosphate, 6 g of ammonium sulfate, 6 g of sodium chloride, 6 g of sulfate of magnesium, 0.6 g of calcium chloride in 1 L of distilled water. The medium was prepared under anaerobic conditions by boiling, addition of reducing agent and continued flushing of O_2 free CO_2 into the flask and tubes, using the Hungate (1966) technique. After the culture medium reduction, the tubes were sealed with butyl stoppers and autoclaved, before inoculation.

2.3. Effect of additives on growth of pure culture bacteria

Hungate tubes of each bacteria were thawed overnight and subcultures (3 stepwise repetitions) were growth in Hobson M2 media at 39ºC for 24 h to washout glycerol before the start of the assay. The assay was carried out in duplicates tubes containing 9 mL of the culture medium and 0.5 mL of cultured medium containing bacteria and 0.5 mL of each additive working solution. For the working solution, additives (Rumensin, oregano essential oil, castor oil, and extract of *B. dracunculifolia*) were solubilized in Tween 5%, at the concentrations of 200, 400 1.000 and 2.000 mg / L. The tubes were inoculated under anaerobic conditions (stream of O_2-free CO_2 while the tube is open) and incubated at 39ºC. Preliminary data indicated that 12 h of growth corresponded to early stationary phase. Then, bacterial growth was assessed at 0, 8, 12 and 24 h at 39ºC by using optical density (OD) at 600 nm. The incubation time 0 was used with the only purpose of a baseline as at this time there is no action of the compounds over the bacteria growth. The absorbance of culture medium tubes containing the additives but not inoculated was

measured and subtracted from the absorbance of the assay tube. The antimicrobial activity was assessed in the Hungate tubes using the final concentration of 10, 20, 50 and 100 mg / L for plants additives and Rumensin (20% of sodium monensin). Tubes containing only culture medium were also inoculated and used as controls (0 mg / L).

2.4. Statistical analyses

Optical density was interpreted by analysis of variance using the GLM of *IBM Statistical Package for the Social Sciences (SPSS version 22*), with the effect of incubation time analyzed as a repeated measurement. Differences among means were then identified using the Bonferroni procedure with significance declared at $P \leq 0.05$. Regression was performed to analyze concentrations effect (0, 10, 20, 50 and 100 mg / L) and the equation used to estimate the additive (mg / L) amount necessary to inhibit 50% of bacterial growth (MIC_e).

3. Results

Considering the three Gram-negative bacteria: *Prevotella albensis* (DSMZ 11370, proteolytic and amylolytic), *Prevotella bryantii* (DSMZ 11371 proteolytic and amylolytic) and *Treponema saccharophilum* (DSMZ 2985 pectinolytic), and three Gram-positive bacteria: *Ruminococcus albus* (DSMZ 20455, fibrolytic) *Ruminococcus flavefaciens* (DSMZ 25089 fibrolytic) *Streptococcus bovis* (DSMZ 20480 proteolytic and amylolytic), when no additive was added (0 mg / L) the growth of most bacteria used in our study reached stationary phase between 8 and 12 h, with exception of *Ruminococcus albus* that continued growing until 24 h.

3.1. Rumensin

The *P. albensis* growth plateaued at 8 h incubation, with OD values ranging from 1.45 to 1.50, and no differences ($P > 0.05$) over time was observed (Table 1). In contrast, Rumensin lightly reduced ($P < 0.05$) the *P. albensis* growth at 8 h ($P < 0.05$). At high concentrations (20, 50 and 100 mg) there was still bacteria grow over time, with the highest OD values at 12 h, which was reduced at 24 h. This could be caused by the

bactericidal effect due to the long exposure to sodium monensin. However, this bacterium presented a high tolerance to Rumensin (MIC_e > 200 mg / L).

Prevotella bryantii continued to grow until 12 h but a slightly reduction in the OD was observed at 24 h when no additive was added (Table 1). A quadratic effect showed that Rumensin reduced growth of this species, but concentrations higher than 50 mg / L had no detrimental effects in the OD values. This species was less resistant to Rumensin than *P. albensis* as this inhibitory effect continued at 24 h, with MIC_e values around 45 mg / L.

Treponema saccharophilum growth plateaued at 8 h of incubation and no differences ($P > 0.05$) over time was observed (Table 1). In contrast, when Rumensin was added these bacteria presented a reduction in the OD values that continued over time. Like *P. albensis*, a quadratic effect of Rumensin concentration was also observed. However, *T. sacharophilum* was more resistant as MIC_e values were around 65 mg / L.

Rumensin successfully inhibited the Gram-positive bacteria growth, like *R. albus* (Table 2). However, the normal growth of these bacteria was slower, as OD values started at 0.51 and continued to increase between 12 and 24 h ($P < 0.05$). Similarly, when these bacteria were incubated with Rumensin, it also presented a slightly OD increase ($P < 0.05$) over time with exception to the higher concentrations which OD values remained around 0.10. *Ruminococcus albus* was the most sensitive bacteria to Rumensin in our study, with MIC_e value of 2 mg / L between 8 and 12 h of incubation.

The *R. flavefaciens* growth plateaued at 8 h with an OD of 1.33 and no differences over time was observed (Table 2). Rumensin concentration showed both linear and quadratic effects ($P < 0.05$) resulting on a reduction in the OD. Optical density increased at 12 h but returned to decrease at 24 h of incubation. Surprisingly, this species was much more resistant than *R. albus* with MIC_e values around 45 mg / L.

Streptococcus bovis growth plateaued between 8 and 12 h, but a slightly reduction in the OD was observed at 24 h (Table 2). Similar effects were observed over time when Rumensin was added. Rumensin significantly reduced ($P < 0.05$) the OD of *S. bovis* but a quadratic effect showed that concentrations higher than 50 mg / L had no improvements on the inhibitory capacity. In addition, the MIC_e observed at *S.bovis* were also around 45 mg / L.

3.2. Oregano essential oil

Oregano essential oil concentration reduced ($P < 0.05$) the *P. albensis* growth at 8 h, however these values remained around 0.50 independent of the concentration tested (Table 3). However, these were temporary effects as bacterial returned to normal levels at 12 and 24 h of incubation. Oregano essential oil was more effective to inhibit the initial *P. albensis* growth in comparison to monensin, as its MIC_e was lower than 50 mg / L at 8 h. Similarly, the growth of the other two Gram-negative bacteria, *P bryantii* and *T. saccharophilum*, (Table 3) were also inhibited at 8 h by oregano essential oil (MIC_e of 37 and 48 mg / L respectively) but returned to normal level at 12 and 24 h ($P < 0.05$).

The *R. albus* growth was inhibited ($P < 0.05$) at 8 and 12 h, but these bacteria returned to grow at 24 h (Table 4). Interestingly, the MIC_e were 48 and 69 mg / L at 8 and 12 h of incubation, showing that concentrations higher than 50 mg / L of oregano essential oil are necessary to extend the inhibitory effect on these bacteria. Oregano essential oil reduced the *R. flavefacien*s growth at 8 h ($P < 0.05$), but bacteria returned to grow at 12 and 24 h. A quadratic effect was observed at 12 h, possible indicating a slightly OD reduction and a weak inhibitory capacity at this time point, however when compared at 8 h it was more susceptible (MIC_e of 28 mg / L) at 8 h than *R. albus*. *Streptococcus bovis* (Table 4) was the only bacteria that was not affected by oregano essential oil.

3.3. Castor oil

Castor oil had marginal effects over Gram-negative bacteria (Table 5). The *P. albensis* growth was slightly reduced at 8 h of incubation, while *P. bryantii* was not affected neither by concentration nor incubation time. The OD of *T. saccharophilum* was linearly reduced at 8 h, but this vegetal oil presented an MIC_e of 660 mg / L for these bacteria.

Castor oil showed a tendency ($P < 0.10$) to reduce the OD of *R. albus* at 8 h but bacteria returned to grow after 12 h (Table 6). However, castor oil continued to inhibit the growth at 12 h ($P < 0.05$), of *R. albus* with an MIC_e value of 88 mg / L. In contrast, castor oil showed only a tendency to slightly inhibit the *R. flavefaciens* growth at 12 h. *Stretococcus bovis* growth was not affected by castor oil concentration, however a tendency to reduce the OD over time was observed.

3.4. Baccharis dracunculifolia extract

Baccharis dracunculifolia concentration did not inhibit the Gram-negative and Gram-positive bacteria growth (Table 7). Surprisingly, *R. albus*, when incubated with *B. dracunculifolia* extract, presented a higher OD than control.

4. Discussion

4.1. Effect on Gram-negative bacteria

Modulating rumen fermentation using additives, for a more favorable volatile fatty acids ratio and reduced deamination and methane production have always been the objective of ruminant nutritionists (Zawadzki et al., 2011; de Aguiar et al., 2013; Valero et al., 2014). There are two major groups of bacteria found in the rumen responsible for ammonia production. The first one that is presented in low numbers but with very high specific activity (hiper-ammonia producing bacteria or HAP) and those in high number but with low specific activity like *Prevotella* sp (Krause and Russell, 1996). The HAP are generally Gram-positive and represent less than 1% of total rumen microbiota, thus considering that monensin inhibit around 30% of ammonia forming activity in the rumen, it is theorized that others monensin-insensitive bacteria plays a bigger role (Wallace et al., 2002).

Bacteria from the genus *Prevotella* are one of the most predominant bacteria in the rumen and plays an important role in the peptides degradation (Cammack et al., 2018). These bacteria are also known due to their high activity of the enzyme dipeptidyl peptidase that breaks dipeptides from proteins (Wallace and McKain, 1991). Thus, a reduction in the total number of proteolytic bacteria could lead to an increase of aminoacids escaping the rumen which could benefit animal by a higher efficiency of nitrogen utilization (de Aguiar et al., 2013).

Surprisingly, in our study, the Gram-negative *P. bryantii* and *T. saccharophilum* bacteria were susceptible to the action of Rumensin in high dosages. The potential of monesin in modulate rumen fermentation is related to the ability to selectively inhibit Gram-positive over Gram-negative bacteria promoting a shift in the acetate to propionate ratio toward more propionate. (McGuffey et al., 2001; Appuhamy et al., 2013). As Rumensin had 20% of sodium monensin the corresponding concentrations used in our study were of 0, 0.5, 1.0, 2.5 and 5 mg / L of monensin. Newbold et al. (1988) showed that some species of Gram-negative bacteria were more sensitive (*Bacteroides*

succinogenes) to monensin (0.023 mg / L) than others (*Bacteroides ruminicol*a, 2.702 mg / L). In this sense, the reduced OD observed for dosages greater than 20 mg / L of Rumensin may be explained by a toxicity concentration of monensin (>2.5 mg / L of monensin), instead of its ability to interact with the outside membrane.

In our study, oregano essential oil reduced the OD of Gram-negative bacteria. Similar results were observed by McIntosh et al. (2003) which evaluated a blend of essential oils (thymol, eugenol, vanillin, and limonene) against a wide range of rumen bacteria. More recently, Cobellis et al. (2016a) using the quantitative PCR technique observed that oregano essential oil (1.125 mL / L) decreased the total bacteria abundance, especially on *Prevotella spp* and archaeas. Generally, Gram-positive bacteria are more sensitive to essential oils than Gram-negative bacteria, but small compounds, such as carvacrol and thymol are able to interact with cell membrane of Gram-negative bacteria, leading to cell content loss and cell lysis (Benchaar and Greathead, 2011). Previous *in vitro* studies demonstrated that these compounds affect ruminal fermentation. As example, carvacrol in low dosages (2.2 mg / L) inhibited proteolysis or stimulated peptide lyses of bacteria (Busquet et al., 2005). In contrast, in greater dosages (300 mg / L), it increased pH and butyrate, and decrease of acetate and propionate, and total VFA concentration (Busquet et al., 2005). Low thymol dosages (50 mg / L) had no effect on ruminal fermentation, but at greater dosages (500 mg / L) it reduced total VFA (Castillejos et al., 2006).

The toxic effect of high monensin dosages, or the lower selectivity of oregano essential oil is a concern on modulating rumen fermentation, as other bacteria of interest may be compromised, like *T. saccharophilum*. There is a large variety of microorganisms capable to ferment pectin, however the growth of *T. saccharophilum* is often dependent of the pectin abundance as substrate (Liu et al., 2014). Pectin is a structural but nonfibrous carbohydrate, that is normally found in plant feedstuffs. Pectin is rapidly degraded in the rumen, but different from starch, acetate is the major product. Thus favoring the growth of pectin users bacteria could be beneficial for animal, by reducing the risk of acidosis and other metabolic disorders caused by diets rich in starch (Hatfield and Weimer, 1995).

4.2. Effect on Gram-positive bacteria

Ruminococcus. flavefaciens, and *R. albus* are important Gram-positive cellulolytic bacteria found in the rumen (Cammack et al., 2018). This group of bacteria are capable of hydrolyzing cellulose using the enzyme cellulase (Pell and Schofield, 1993) and

303 forming a vast range of end products like acetate, butyrate succinate and formate, CO_2,
304 H_2 ethanol and lactate(Hungate, 1966). As expected, Rumensin successfully inhibit the
305 Gram-positive bacteria growth . Interestingly, *R. flavefaciens* showed lower susceptibility
306 to Rumensin than *R. albus*. Similar results were observed by Newbold et al. (1988) in
307 which, after a series of stepwise adaptation to monensin, *R. flavefaciens* presented an
308 increase on the MIC_e (0.388 to 0.588 mg / L) while *R. albus* remained the same (0.064
309 mg / L).
310 Rumen acidosis generally occurs with VFA accumulation when cattle are fed high
311 concentrate diets. *Streptococcus bovis* is an important proteolytic and amylolytic bacteria
312 often associated with this metabolic disorder (Chen et al., 2016). In normal conditions, *S.*
313 *bovis* fermentation end products are formate, acetate and ethanol, however when pH is
314 lower than 5.5 it change to lactate which enhance even more the pH decline (Russell and
315 Hino, 1985). Thus, controlling the *S. bovis* growth has been of interest of research due to
316 their role in ruminal acidosis (Fernando et al., 2010; Belanche et al., 2012)**.** In our
317 study, Rumensin inhibited *S. bovis*, especially after 24 h of incubation. However, our
318 lowest concentration (10 mg / L) were higher (<1.0 mg / L) to those observed by other
319 authors (Newbold et al., 1988; Newbold et al., 2013).
320 Oregano essential oil affected cellulolytic bacteria (*R. albus* and *R. flavefasciens*). This
321 was expected, since oregano essential oil is rich in carvacrol and thymol, which are
322 monoterpenes with a strong antimicrobial activity against a wide range of Gram-positive
323 bacteria (Calsamiglia et al., 2007). However, Cobellis et al. (2016a) only observed
324 changes in the relative abundance of *R. albus* when oregano essential oil was mixed with
325 cinnamon and rosemary essential oil which is explained by the synergistic effect of them.
326 More recently, Castañeda-Correa et al. (2019) observed that thymol was more efficient
327 to reduce methane production than carvacrol, possible due to an indirect effect of EO on
328 microorganisms that produce substrates such as hydrogen or formate which are used by
329 the methanogens bacteria. The *S. bovis* growth were not affected by oregano essential oil,
330 which corroborates with the findings of Evans and Martin (2000), that tested different
331 thymol concentrations, in which only those > 100 mg / L reduced the OD.
332 Castor oil (*Ricinus communis*) is one of the most important crops in developing
333 countries because of its potential to be used in biodiesel industry. It can be obtained by
334 pressing the castor seeds which are rich in ricinoleic acid (Gandra et al., 2014). Past
335 studies showed the antimicrobial potential against a wide range of anaerobic bacteria
336 (Novak et al., 1961; Ferreira et al., 2002). Because of that, castor oil has been theorized

to modulate ruminal fermentation and improve animal performance. In fact, some *in vivo* trials reported improvements on animal performance of young bulls fed with ricinoleic acid (2 g/animal day) (Gandra et al., 2012), or a mix of cashew and castor oil (3 g/animal day) (Valero et al., 2014).

In our study, high castor oil concentrations affected the *R. albus* growth which is an important bacteria responsible by fiber degradation (Cammack et al., 2018). It is well known that the inclusion of plant oils in diets can reduce DMI and NDF digestibility(Weld and Armentano, 2017). More recently, Ibrahim et al. (2018) observed that the inclusion of oils (palm, olive and sunflower) in diet of goats at 6% of DM base significantly reduced the population of *R. albus*, but no differences were observed on other cellulolytic bacteria like *Fibrobacter succinogenes* and *R. flavefaciens*.

Baccharis dracunculifolia, popularly known as "Alecrim-do-campo", is the main raw material used by bees (*A. mellifera*) to produce green propolis, whose benefits to human health have been widely studied (Maróstica Junior et al., 2008). More recently, studies revealed that propolis had antimicrobial activity against ruminal Gram-positive and Gram-negative bacteria (Aguiar et al., 2013) and that feeding propolis (3 g/animal day) improved the performance of young bulls (Valero et al., 2014). The propolis antimicrobial properties could be explain due to its composition which has 50% resin, where is found flavonoids and phenolic acids, 30% wax, 10% essential oils, 5% pollen and 5% other organic (Gómez-Caravaca et al., 2006).

To the best to our knowledge, no studies with ruminal micro-organisms have been conducted with *B. dracunculifolia* yet. However, due to the similar chromatography profile to propolis (Maróstica Junior et al., 2008) it have been theorized that this plant could have the same benefits as propolis, with the advantage of reduced costs. Our research group evaluated the addition of *B. dracunculifolia* leaves in diet (5, 10 and 15 mg/day) of Nellore steers but no differences on blood parameters, final body weight, average daily gain, dry matter intake, or feed efficiency were observed (Souza, 2020) (data not published yet).

Baccharis dracunculifolia did not affect the Gram-negative and Gram-positive bacteria growth. In fact, it increased the OD in some cases (*R. albus*). This bias may be caused by the slower growth rate of this bacteria. On the other hand, when tested against aerobic microorganisms, the *B. dracunculifolia* essential oil (up to 10µL/ disc) presented antimicrobial activity against Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) *and* Gram-positive *(Staphylococcus aureus)* (Ferronatto et al. 2007). More

recently, Bonin et al. (2020) evaluated the antimicrobial of *B. dracunculifolia* extracts and observed better antimicrobial action against Gram-positive bacteria, in which the MIC for both bacteria *Staphylococcus aureus* and *Bacillus subtilis* were 125 mg / L and *Bacillus cereus* was 250 mg / L. The same plant extracts used by Bonin et al. (2020) were used in our study. However, the final concentrations tested were lower than Bonin et al. (2020), which could explain this marginal response. Furthermore, different methods of extraction, (hydroethanolic extract vs essential oil) may affect composition and consequently the concentration necessary to inhibit the growth of rumen microorganisms.

5. Conclusions

As expected Rumensin showed a great antimicrobial capacity against Gram-positive bacteria, however high dosage (≥ 20 mg / L) was also toxic for Gram-negative bacteria. Oregano essential oil showed great antimicrobial activity against both Gram-positive and Gram-negative bacteria, thus despite of its antimicrobial potential its inability to select Gram-positive over Gram-negative organism limits its usage for modulating ruminal fermentation. Castor oil showed more selectivity against Gram-positive bacteria; however, it reduced the *R. albus* growth, which is an important fiber degrader instead of *S. bovis* which is a bacteria often associated with rumen acidosis. The *B. dracunculifolia* extract concentration, (up to 100mg / L) used in this trial were ineffective against all ruminal bacteria tested. Future studies with higher concentrations and different extraction methods may be necessary to fully understand the potential of *B. dracunculifolia*.

6. Future implications

This was the first study conducted in our research group with rumen anaerobic microorganism. The technique was satisfactory to antimicrobial capacity of natural additives against rumen bacteria of research interest, especially those having proteolytic, amylolytic, cellulolytic and pectinolytic activity. Other microorganisms of interest, that were not evaluated in this study, are the hyper ammonia-producing bacteria and archaea methanogens, that could be addressed in future researches. In addition, complimentary techniques like batch culture, in vitro rumen fermentation, or RUSITEC could also be used to further support the findings.

South America is rich on biodiversity, castor oil and *B. dracunculifolia* are just two additives from a vast number of plants with potential to be explored. Furthermore, natural additives are dependent of the part of the plants (root, stem, leaf and flower) harvest season (summer, autumn, winter, or spring) and extraction methods (ethanolic, methanolic etc.). This highlights the importance and also the potential of future studies. Thus, this technique could be applied as the first step for screening potential additives, targeting a more accurate range of concentrations, with a greater spectrum of action, and reducing cost and time spent on future animal trials.

7. Conflict of interests

The authors declare no conflict of interests.

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9. References

Aguiar, A.C.R., Oliveira, C.R., Caldeira, L.A., Junior, V.R.R., Oliveira, S.J., Soares, C., Silva, D.A., Menezes, J.C., Borges, L.D.A., 2013. Consumo, produção e composição do leite e do queijo de vacas alimentadas com níveis crescentes de ureia. Revista Brasileira de Ciência Veterinária 20, 37-42. http://dx.doi.org/10.4322/rbcv.2014.048.

ANUALPEC, 2019. Anuário da Pecuária Brasileira. Instituto FNP, São Paulo, São Paulo, Brasil.

Appuhamy, R.N.J.A.D., Strathe, A.B., Jayasundara, S., Wagner-Riddle, C., Dijkstra, J., France, J., Kebreab, E., 2013. Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. Journal of Dairy Science 96, 5161-5173. http://dx.doi.org/10.3168/jds.2012-5923.

441 Belanche, A., Doreau, M., Edwards, J.E., Moorby, J.M., Pinloche, E., Newbold, C.J.,
442 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein
443 ingested by dairy cattle are associated with changes in rumen fermentation. The Journal
444 of Nutrition 142, 1684-1692. https://doi.org/10.3945/jn.112.159574.
445 Benchaar, C., Greathead, H., 2011. Essential oils and opportunities to mitigate enteric
446 methane emissions from ruminants. Animal Feed Science and Technology 166, 338-355.
447 https://doi.org/10.1016/j.anifeedsci.2011.04.024.
448 Biondo, P.B.F., Carbonera, F., Zawadzki, F., Chiavellia, L.U.R., Pilau, E.J.P., Prado, I.N.,
449 Visentainer, J.V., 2017. Antioxidant capacity and identification of bioactive compounds
450 by GC-MS of essential oils commercialized in Brazil. Current Bioactive Compounds 13,
451 137-143. http://dx.doi.org/10.2174/1573407212666161061408084.
452 Bonin, E., Carvalho, V.M., Avila, V.D., Santos, N.C.A., Zanqueta, É.B., Lanchero,
453 C.A.C., Previdelli, I.T.S., Nakamura, T.U., Abreu Filho, B.A., Prado, I.N., 2020.
454 Baccharis dracunculifolia: Chemical constituents, cytotoxicity and antimicrobial activity.
455 LWT - Food Science and Technology 120, 1-10.
456 https://doi.org/10.1016/j.lwt.2019.108920.
457 Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C., 2005. Screening for effects of plant
458 extracts and active compounds of plants on dairy cattle rumen microbial fermentation in
459 a continuous culture system. Animal Feed Science and Technology 123–124, 597-613.
460 http://dx.doi.org/10.1016/j.anifeedsci.2005.03.008.
461 Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L., Ferret, A., 2007. Invited
462 Review: Essential Oils as Modifiers of Rumen Microbial Fermentation. Journal of Dairy
463 Science 90, 2580-2595. https://doi.org/10.3168/jds.2006-644.
464 Cammack, K.M., Austin, K.J., Lamberson, W.R., Conant, G.C., Cunningham, H.C.,
465 2018. RUMINANT NUTRITION SYMPOSIUM: Tiny but mighty: the role of the rumen
466 microbes in livestock production. Journal of animal science 96, 752-770.
467 10.1093/jas/skx053.
468 Cardoso, A.S., Berndt, A., Leytem, A., Alves, B.J., Carvalho, I.N.O., Soares, L.H.B.,
469 Urquiaga, S., Boddey, R.M., 2016. Impact of the intensification of beef production in
470 Brazil on greenhouse gas emissions and land use. Agricultural Systems 143, 86-96.
471 http://dx.doi.org/10.1016/j.agsy.2015.12.007.
472 Castañeda-Correa, A., Corral-Luna, A., Hume, M.E., Anderson, R.C., Ruiz-Barrera, O.,
473 Castillo-Castillo, Y., Rodriguez-Almeida, F., Salinas-Chavira, J., Arzola-Alvarez, C.,
474 2019. Effects of thymol and carvacrol, alone or in combination, on fermentation and
475 microbial diversity during in vitro culture of bovine rumen microbes. Journal of
476 Environmental Science and Health, Part B 54, 170-175.
477 https://doi.org/10.1080/03601234.2018.1536580.
478 Castillejos, L., Calsamiglia, S., Ferret, A., 2006. Effect of essential oil active compounds
479 on rumen microbial fermentation and nutrient flow *in vitro* systems. Journal of Dairy
480 Science 89, 2649-2658. http://dx.doi.org/10.3168/jds.S0022-0302(06)72341-4.
481 Chen, L., Liu, S., Wang, H., Wang, M., Yu, L., 2016. Relative significances of pH and
482 substrate starch level to roles of Streptococcus bovis S1 in rumen acidosis. AMB Express
483 6, 80. 10.1186/s13568-016-0248-2.
484 Cobellis, G., Trabalza-Marinucci, M., Marcotullio, M.C., Yu, Z., 2016a. Evaluation of
485 different essential oils in modulating methane and ammonia production, rumen
486 fermentation, and rumen bacteria *in vitro*. Animal Feed Science and Technology 215, 25-
487 36. http://dx.doi.org/10.1016/j.anifeedsci.2016.02.008.
488 Cobellis, G., Trabalza-Marinucci, M., Yu, Z., 2016b. Critical evaluation of essential oils
489 as rumen modifiers in ruminant nutrition: A review. Science of the Total Environment
490 545, 556-568. http://dx.doi.org/10.1016/j.scitotenv.2015.12.103.

491 de Aguiar, S.C., Zeoula, L.M., Franco, S.L., Peres, L.P., Arcuri, P.B., Forano, E., 2013.
492 Antimicrobial activity of Brazilian propolis extracts against rumen bacteria in vitro.
493 World Journal of Microbiology and Biotechnology 29, 1951-1959.
494 http://dx.doi.org/10.1007/s11274-013-1361-x.
495 Evans, J.D., Martin, S.A., 2000. Effects of thymol on ruminal microorganisms. Current
496 Microbiology 41, 336-340.
497 Fernando, S.C., Purvis, H.T., Najar, F.Z., Sukharnikov, L.O., Krehbiel, C.R., Nagaraja,
498 T.G., Roe, B.A., DeSilva, U., 2010. Rumen microbial population dynamics during
499 adaptation to a high-grain diet. Applied and Environmental Microbiology 76, 7482-7490.
500 Ferreira, C.M., Rosa, O.P.S., Torres, S.A., Ferreira, F.B.d.A., Bernardinelli, N., 2002.
501 Activity of endodontic antibacterial agents against selected anaerobic bacteria. Brazilian
502 Dental Journal 13, 118-122. http://dx.doi.org/10.1590/S0103-64402002000200008
503 Ferronatto, R., Marchesan, E.D., Pezenti, E., Bednarski, F., Onofre, S.B., 2007. Atividade
504 antimicrobiana de óleos essenciais produzidos por Baccharis dracunculifolia DC e
505 Baccharis uncinella DC (Asteraceae). Revista Brasileira de farmacognosia 17, 224-230.
506 http://dx.doi.org/10.1590/S0102-695X2007000200016
507 Fugita, C.A., Prado, R.M., Valero, M.V., Bonafé, E.G., Carvalho, C.B., Guerrero, A.,
508 Sañundo, C., Prado, I.N., 2018. Effect of the inclusion of natural additives on animal
509 performance and meat quality of crossbred bulls (Angus vs. Nellore) finished in feedlot.
510 Animal Production Science 58, 2076-2083. https://dx.doi.org/10.1071/AN16242.
511 Gandra, J.R., Gil, P.C.N., Cônsolo, N.R.B., Gandra, E.R.S., Gobesso, A.A.O., 2012.
512 Addition of increasing doses of ricinoleic acid from castor oil (*Ricinus communis* L.) in
513 diets of Nellore steers in feedlots. Journal of Animal and Feed Sciences 21, 566-576.
514 https://doi.org/10.22358/jafs/66131/2012.
515 Gandra, J.R., Nunes Gil, P.C., Gandra, E.R.S., Del Vale, T.A., Barletta, R.V., Zanferari,
516 F., Jesus, E.F., Takiya, C.S., Mingoti, R.D., Almeida, G.F., 2014. Productive performance
517 of simmental dairy cows supplemented with ricinoleic acid from castor oil. Archivos de
518 zootecnia 63, 575-585. http://dx.doi.org/10.4321/S0004-05922014000400002
519 Gómez-Caravaca, A.M., Gómez-Romero, M., Arráez-Román, D., Segura-Carretero, A.,
520 Fernández-Gutiérrez, A., 2006. Advances in the analysis of phenolic compounds in
521 products derived from bees. Journal of Pharmaceutical and Biomedical Analysis 41,
522 1220-1234. https://doi.org/10.1016/j.jpba.2006.03.002.
523 Hatfield, R.D., Weimer, P.J., 1995. Degradation characteristics of isolated and in situ cell
524 wall lucerne pectic polysaccharides by mixed ruminal microbes. Journal of the Science
525 of Food and Agriculture 69, 185-196. 10.1002/jsfa.2740690208.
526 Hobson, P.N., Stewart, C.S., 2012. Rumen microbial ecosystem. Blackie Academic &
527 Professional, Londo, UK.
528 Hungate, R.E., 1966. The rumen and its microbes Academic Press New York and
529 London. Academic Press, New York, Estados Unidos.
530 Ibrahim, N.A., Alimon, A., Yaakub, H., Abdullah, N., Samsudin, A., 2018. Effects of
531 Dietary Oil Supplementation with Different Fatty Acid Profiles on Rumen Fibre
532 Degrading Bacteria Population in Goats.
533 Krause, D.O., Russell, J.B., 1996. An rRNA approach for assessing the role of obligate
534 amino acid-fermenting bacteria in ruminal amino acid deamination. Applied and
535 Environmental Microbiology 62, 815-821.
536 Lambert, R.J.W., Skandamis, P.N., Coote, P.J., Nychas, G.J.E., 2001. A study of the
537 minimum inhibitory concentration and mode of action of oregano essential oil, thymol
538 and carvacrol. Journal of Applied Microbiology 91, 453-462.
539 https://doi.org/10.1046/j.1365-2672.2001.01428.x.

Liu, J., Wang, J.-K., Zhu, W., Pu, Y.-Y., Guan, L.-L., Liu, J.-X., 2014. Monitoring the rumen pectinolytic bacteria Treponema saccharophilum using real-time PCR. FEMS Microbiology Ecology 87, 576-585. 10.1111/1574-6941.12246.

Maróstica Junior, M.R., Daugsch, A., Moraes, C.S., Queiroga, C.L., Pastore, G.M., Parki, Y.K., 2008. Comparison of volatile and polyphenolic compounds in Brazilian green propolis and its botanical origin *Baccharis dracunculifolia*. Food Science and Technology 28, 178-181. http://dx.doi.org/10.1590/S0101-20612008000100026

McGuffey, R.K., Richardson, L.F., Wilkinson, J.I.D., 2001. Ionophores for dairy cattle: current status and future outlook. Journal of Dairy Science 84, E194-E203. https://doi.org/10.3168/jds.S0022-0302(01)70218-4.

McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., Beever, D.A., Newbold, C.J., 2003. Effects of essential oils on ruminal microorganisms and their protein metabolism. Applied and Environmental Microbiology 69, 5011-5014. 10.1128/aem.69.8.5011-5014.2003.

Monteschio, J.O., Souza, K.A., Vital, A.C.P., Guerrero, A., Valero, M.V., Kempinski, E.M.B.C., Barcelos, V.C., Nascimento, K.F., Prado, I.N., 2017. Clove and rosemary essential oils and encapsuled active principles (eugenol, thymol and vanillin blend) on meat quality of feedlot-finished heifers. Meat Science 130, 50-57. http://dx.doi.org/10.1016/j.meatsci.2017.04.002.

Newbold, C.J., Wallace, R.J., Walker-Bax, N.D., 2013. Potentiation by metal ions of the efficacy of the ionophores, monensin and tetronasin, towards four species of ruminal bacteria. FEMS Microbiology Letters 338, 161-167. http://dx.doi.org/10.1111/1574-6968.12044.

Newbold, C.J., Wallace, R.J., Watt, N.D., Richardson, A.J., 1988. Effect of the novel ionophore tetronasin (ICI 139603) on ruminal microorganisms. Applied and Environmental Microbiology 54, 544-547.

Novak, A., Clark, G., Dupuy, H., 1961. Antimicrobial activity of some ricinoleic acid oleic acid derivatives. Journal of the American Oil Chemists' Society 38, 321-324. http://dx.doi.org/10.1007/BF02638439.

Ornaghi, M.G., Guerrero, A., Vital, A.C.P., Souza, K.A., Passetti, R.A.C., Mottin, C., Castilho, R.A., Sañudo, C., Prado, I.N., 2020. Improvements in the quality of meat from beef cattle fed natural additives. Meat Science in press. https://doi.org/10.1016/j.meatsci.2020.108059.

Ornaghi, M.G., Passetti, R.A.C., Torrecilhas, J.A., Mottin, C., Vital, A.C.P., Gurerrero, A., Sañudo, C., Campo, M.M., Prado, I.N., 2017. Essential oils in the diet of young bulls: Effect on animal performance, digestibility, temperament, feeding behaviour and carcass characteristics. Animal Feed Science and Technology 234, 274-283. http://dx.doi.org/10.1016/j.anifeedsci.2017.10.008.

Pell, A.N., Schofield, P., 1993. Microbial adhesion and degradation of plant cell walls. Forage cell wall structure and digestibility, 397-423.

Rivaroli, D.C., Ornaghi, M.G., Mottin, C., Prado, R.M., Ramos, T.R., Guerrero, A., Jorge, A.M., Prado, I.N., 2017. Essential oils in the diet of crossbred (½ Angus vs. ½ Nellore) bulls finished in feedlot on animal performance, feed efficiency and carcass characteristics. Journal of Agricultural Science 9, 205-212. http://dx.doi.org/10.5539/jas.v9n10p205-212.

Russell, J.B., Hino, T., 1985. Regulation of lactate production in Streptococcus bovis: a spiraling effect that contributes to rumen acidosis. Journal of Dairy Science 68, 1712-1721.

588 Schäberle, T.F., Hack, I.M., 2014. Overcoming the current deadlock in antibiotic
589 research. Trends in Microbiology 22, 165-167.
590 http://dx.doi.org/10.1016/j.tim.2013.12.007.
591 Souza, K.A., 2020. Leaves of Baccharis dracunculifolia added in the diets of steers
592 finished in feedlot, effect on performance and immune response.
593 Souza, K.A., Monteschio, J.O., Mottin, C., Ramos, T.R., Pinto, L.A.M., Eiras, C.E.,
594 Guerrero, A., Prado, I.N., 2019. Effects of diet supplementation with clove and rosemary
595 essential oils and protected oils (eugenol, thymol and vanillin) on animal performance,
596 carcas characteristics, digestibility, and behavior activities for Nellore heifers finished in
597 feedlot. Livestock Science 220, 190-195. http://dx.doi.org/10.1016/j.livsci.2018.12.026.
598 Valero, M.V., Prado, R.M., Zawadzki, F., Eiras, C.E., Madrona, G.S., Prado, I.N., 2014.
599 Propolis and essential oils additives in the diets improved animal performance and feed
600 efficiency of bulls finished in feedlot. Acta Scientiarum. Animal Sciences 36, 419-426.
601 http://dx.doi.org/10.4025/actascianimsci.v36i4.23856.
602 Wallace, R.J., McEwan, N.R., McIntosh, F.M., Teferedegne, B., Newbold, C.J., 2002.
603 Natural Products as Manipulators of Rumen Fermentation. Asian-Australas J Anim Sci
604 15, 1458-1468. 10.5713/ajas.2002.1458.
605 Wallace, R.J., McKain, N., 1991. A survey of peptidase activity in rumen bacteria.
606 Microbiology 137, 2259-2264. https://doi.org/10.1099/00221287-137-9-2259.
607 Weld, K., Armentano, L., 2017. The effects of adding fat to diets of lactating dairy cows
608 on total-tract neutral detergent fiber digestibility: A meta-analysis. Journal of dairy
609 science 100, 1766-1779.
610 Wishart, D.S., Jewison, T., Guo, A.C., Wilson, M., Knox, C., Liu, Y., Djoumbou, Y.,
611 Mandal, R., Aziat, F., Dong, E., 2012. HMDB 3.0—the human metabolome database in
612 2013. Nucleic Acids Research 41, D801-D807. http://dx.doi.org/10.1093/nar/gks1065.
613 Zawadzki, F., Prado, I.N., Marques, J.A., Zeoula, L.M., Rotta, P.P., Sestari, B.B., Valero,
614 M.V., Rivaroli, D.C., 2011. Sodium monensin or propolis extract in the diets of feedlot-
615 finished bulls: effects on animal performance and carcass characteristics. Journal of
616 Animal and Feed Sciences 20, 16-25. https://doi.org/10.22358/jafs/66153/2011.

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618

619 **Table 1.** Influence of Rumensin in the anaerobic Gram-negative bacteria growth

Rumensin[1] concentration, mg / L	Time (h)			SEM	Repeated measures
	08	12	24		*P < Value*
Prevotella albensis					
0	1.45	1.50	1.48	0.023	0.886
10	1.55	1.55	1.42	0.042	0.244
20	1.43AB	1.55A	1.38B	0.038	0.023
50	1.20	1.44	1.24	0.038	0.128
100	1.23B	1.49A	1.25B	0.046	0.003
SEM	0.040	0.027	0.025		
L	0.003	0.480	<0.001		
Q	0.006	0.719	<0.001		
MIC_e (mg / L)	251	-	347		
Prevotella bryantii					
0	1.30B	1.39A	1.32B	0.027	0.038
10	0.98A	0.92B	0.71C	0.036	0.004
20	0.80B	0.85A	0.60B	0.032	<0.001
50	0.32B	0.35A	0.28B	0.010	0.023
100	0.31B	0.38A	0.33B	0.011	<0.004
SEM	0.088	0.089	0.086		
L	<0.001	<0.001	<0.001		
Q	<0.001	<0.001	<0.001		
MIC_e (mg / L)	47	45	33		
Treponema saccharophilum					
0	1.35	1.37	1.37	0.022	0.898
10	1.06A	1.04B	0.75C	0.042	<0.001
20	0.92A	0.94B	0.71C	0.032	0.004
50	0.55A	0.53A	0.43B	0.016	<0.001
100	0.54B	0.61A	0.50C	0.014	<0.001
SEM	0.071	0.070	0.077		
L	<0.001	<0.001	0.030		
Q	<0.001	<0.001	<0.001		
MIC_e (mg / L)	67	65	49		

620 [1] Rumensin = 20% of sodium monensin; A B = Different uppercase letters means difference in the same
621 line at Bonferroni ($P < 0.05$). L = linear effect; Q = quadratic effect; MIC_e = estimated amount of additive
622 concentration (mg / L) necessary to reduce 50% of optical density

623 **Table 2.** Influence of Rumensin in the anaerobic Gram-positive bacteria growth

Rumensin[1] concentration mg / L	Time (h)			SEM	Repeated measures
	08	12	24		*P < Value*
Ruminococcus albus					
0	0.51C	0.84B	0.98A	0.085	0.052
10	0.09C	0.13B	0.34A	0.035	<0.001
20	0.11C	0.13B	0.26A	0.022	<0.001
50	0.07	0.11	0.09	0.011	0.164
100	0.05C	0.08B	0.13A	0.012	0.098
SEM	0.047	0.071	0.074		
L	0.030	0.020	0.002		
Q	0.008	0.002	<0.001		
MIC_e (mg / L)	2	2	18		
Ruminococcus flavefaciens					
0	1.33	1.41	1.38	0.043	0.762
10	1.03A	0.97B	0.92C	0.014	0.016
20	0.82A	0.84A	0.67B	0.022	0.005
50	0.38B	0.40A	0.40A	0.007	0.019
100	0.30B	0.40A	0.34B	0.012	<0.001
SEM	0.091	0.088	0.088		
L	<0.001	<0.001	<0.001		
Q	<0.001	<0.001	<0.001		
MIC_e (mg / L)	45	46	41		
Streptococcus bovis					
0	1.34A	1.29A	1.25B	0.021	0.019
10	0.94A	0.94A	0.79B	0.019	<0.001
20	0.79A	080A	0.63B	0.023	<0.001
50	0.31A	0.33A	0.26B	0.009	0.004
100	0.29B	0.34A	0.28B	0.007	0.005
SEM	0.091	0.085	0.083		
L	<0.001	<0.001	<0.001		
Q	<0.001	<0.001	<0.001		
MIC_e (mg / L)	41	45	45		

624 [1] Rumensin = 20% of sodium monensin A B = Different uppercase letters means difference in the same
625 line at Bonferroni ($P < 0.05$); L = linear effect; Q = Quadratic effect; MIC_e = estimated amount of additive
626 concentration (mg / L) necessary to reduce 50% of optical density.

627 **Table 3.** Influence of oregano essential oil in the anaerobic Gram-negative bacteria growth

Oregano essential oil concentration, mg / L	Time (h)				Repeated Measures
	08	12	24	SEM	*P < Value*
Prevotella albensis					
0	1.45	1.50	1.48	0.023	0.886
10	NE	NE	NE	NE	NE
20	0.51B	1.33A	1.55A	0.137	0.010
50	0.45C	1.27B	1.62A	0.150	0.001
100	0.53B	1.44A	1.50A	0.135	<0.001
SEM	0.108	0.027	0.032		
L	0.010	0.829	0.919		
Q	<0.001	<0.001*	0.233		
MIC_e (mg / L)	43	-	-		
Prevotella bryantii					
0	1.30B	1.39A	1.32B	0.027	0.038
10	NE	NE	NE	NE	NE
20	0.40B	1.17A	1.57A	0.148	0.006
50	0.39C	1.14B	1.59A	0.152	0.009
100	0.42B	1.22A	1.61A	0.152	0.008
SEM	0.100	0.029	0.048		
L	0.009	0.130	0.066		
Q	<0.001	0.001*	0.059		
MIC_e (mg / L)	37	-	-		
Treponema saccharophilum					
0	1.35	1.37	1.37	0.022	0.898
10	NE	NE	NE	NE	NE
20	0.49C	1.38B	1.60A	0.145	0.004
50	0.47B	1.34A	1.57A	0.146	0.004
100	0.51B	1.37A	1.46A	0.132	0.009
SEM	0.095	0.020	0.038		
L	0.008	0.942	0.764		
Q	<0.001	0.946	0.103		
MIC_e (mg / L)	48	-	-		

628 A B = Different uppercase letters means difference in the same line at Bonferroni ($P < 0.05$). L = linear
629 effect; Q = quadratic effect MIC_e = estimated amount of additive concentration (mg / L) necessary to reduce
630 50% of optical density; * = equation to calculate MIC_e does not have real square roots; NE = not evaluated.

631 **Table 4.** Influence of oregano essential oil in the anaerobic Gram-positive bacteria growth

Oregano essential oil concentration, mg / L	Time (h)			SEM	Repeated measures
	08	12	24		*P* < *Value*
Ruminococcus albus					
0	0.51C	0.84B	0.98A	0.085	0.052
10	NE	NE	NE	NE	NE
20	0.22C	0.50B	1.38A	0.151	0.003
50	0.23C	0.40B	0.58A	0.045	0.005
100	0.12C	0.36B	1.16A	0.135	0.001
SEM	0.050	0.057	0.079		
L	0.009	0.003	0.913		
Q	0.016	0.001	0.291		
MIC_e (mg / L)	48	69	-		
Ruminococcus flavefaciens					
0	1.33	1.41	1.38	0.043	0.762
10	NE	NE	NE	NE	NE
20	0.25C	0.98B	1.52A	0.160	0.003
50	0.30C	1.12B	1.52A	0.155	0.003
100	0.34C	1.23B	1.51A	0.152	<0.001
SEM	0.119	0.044	0.036		
L	0.018	0.657	0.357		
Q	<0.001	0.014*	0.375		
MIC_e (mg / L)	28	-	-		
Streptococcus bovis					
0	1.34A	1.29A	1.25B	0.021	0.019
10	NE	NE	NE	NE	NE
20	1.34	1.35	1.38	0.014	0.584
50	1.31	1.43	1.33	0.031	0.294
100	1.32	1.36	1.33	0.023	0.747
SEM	0.024	0.020	0.016		
L	0.746	0.289	0.428		
Q	0.909	0.052	0.195		
MIC_e (mg / L)	-	-	-		

632 A B = Different uppercase letters means difference in the same line at Bonferroni ($P < 0.05$). L = linear
633 effect; Q = quadratic effect; MIC_e = estimated amount of additive concentration (mg / L) necessary to
634 reduce 50% of optical density, * = equation to calculate MIC_e does not have real square roots; NE = not
635 evaluated

636 **Table 5.** Influence of castor oil in the anaerobic Gram-negative bacteria growth

Castor oil concentration, mg / L	Time (h)				Repeated measures
	08	12	24	SEM	*P* < *Value*
Prevotella albensis					
0	1.45	1.50	1.48	0.023	0.886
10	1.33B	1.45A	1.46A	0.030	0.032
20	1.24	1.53	1.54	0.050	0.087
50	1.25	1.49	1.30	0.039	0.277
100	1.30B	1.48A	1.45A	0.033	0.030
SEM	0.023	0.020	0.026		
L	0.189	0.702	0.308		
Q	0.011*	0.875	0.132		
MIC_e (mg / L)	-	-	-		
Prevotella bryantii					
0	1.30B	1.39A	1.32B	0.027	0.038
10	1.46	1.48	1.39	0.020	0.089
20	1.39	1.38	1.37	0.029	0.929
50	1.49	1.52	1.42	0.035	0.458
100	1.45	1.38	1.40	0.038	0.320
SEM	0.024	0.030	0.019		
L	0.113	0.889	0.260		
Q	0.071	0.494	0.261		
MIC_e (mg / L)	-	-	-		
Treponema saccharophilum					
0	1.35	1.37	1.37	0.022	0.898
10	1.40	1.47	1.44	0.022	0.536
20	1.27	1.37	1.36	0.021	0.239
50	1.22B	1.43A	1.44A	0.039	0.015
100	1.26	1.41	1.35	0.028	0.117
SEM	0.021	0.018	0.019		
L	0.044	0.751	0.604		
Q	0.022	0.805	0.461		
MIC_e (mg / L)	660	-	-		

637 A B = Different uppercase letters means difference in the same line at Bonferroni ($P < 0.05$). L = linear
638 effect ($P < 0.05$); Q = Quadratic effect; MIC_e = estimated amount of additive concentration (mg / L)
639 necessary to reduce 50% of optical density; * = equation to calculate MIC_e does not have real square roots
640

641 **Table 6.** Influence of castor oil in the anaerobic Gram-positive bacteria growth

Castor oil concentration mg / L	Time (h)			SEM	Repeated measures
	08	12	24		*P < Value*
Ruminococcus albus					
0	0.51C	0.84B	0.98A	0.085	0.052
10	0.17C	1.07B	1.31A	0158	0.001
20	0.16C	0.29B	0.93A	0.106	0.001
50	0.20B	0.51A	0.42A	0.041	0.006
100	0.16	0.48B	0.99A	0.110	0.001
SEM	0.040	0.069	0.069		
L	0.101	0.041	0.289		
Q	0.064	0.024	0.005*		
MIC_e (mg / L)	-	88	-		
Ruminococcus flavefaciens					
0	1.33	1.41	1.38	0.043	0.762
10	1.28	1.46	1.32	0.028	0.061
20	1.28	1.39	1.32	0.020	0.291
50	1.22	1.41	1.35	0.035	0.161
100	1.19B	1.36A	1.35A	0.037	0.038
SEM	0.025	0.025	0.017		
L	0.060	0.367	0.942		
Q	0.147	0.670	0.826		
MIC_e (mg / L)	-	-	-		
Streptococcus bovis					
0	1.34A	1.29A	1.25B	0.021	0.019
10	1.37	1.29	1.21	0.025	0.164
20	1.37A	1.30A	1.20B	0.026	0.007
50	1.39	1.29	1.22	0.029	0.114
100	1.33	1.33	1.26	0.018	0.165
SEM	0.015	0.014	0.013		
L	0.599	0.396	0.316		
Q	0.287	0.648	0.332		
MIC_e (mg / L)	-	-	-		

642 A B = Different uppercase letters means difference in the same line at Bonferroni ($P < 0.05$). L = linear
643 effect; Q = quadratic effect; MIC_e = estimated amount of additive concentration (mg / L) necessary to
644 reduce 50% of optical density; * = equation to calculate MIC_e does not have real square roots.

Table 7. Influence of *Baccharis dracunculifolia* hydroethanolic extract in the anaerobic Gram-negative bacteria growth

Baccharis dracunculifolia extract concentration, mg / L	Time (h)				Repeated Measures
	08	12	24	SEM	*P < Value*
Prevotella albensis					
0	1.45	1.50	1.48	0.023	0.886
10	1.45	1.52	1.54	0.038	0.176
20	1.46	1.49	1.42	0.029	0.454
50	1.48	1.44	1.49	0.040	0.723
100	1.54	1.65	1.44	0.043	0.057
SEM	0.029	0.032	0.019		
L	0.261	0.132	0.441		
Q	0.528	0.093	0.750		
MIC_e (mg / L)	-	-	-		
Prevotella bryantii					
0	1.30B	1.39A	1.32B	0.027	0.038
10	1.54	1.53	1.43	0.049	0.433
20	1.55	1.55	1.35	0.048	0.081
50	1.51A	1.51A	1.37B	0.047	0.030
100	1.46	1.46	1.39	0.040	0.509
SEM	0.043	0.028	0.025		
L	0.497	0.944	0.634		
Q	0.767	0.440	0.889		
MIC_e (mg / L)	-	-	-		
Treponema saccharophilum					
0	1.35	1.37	1.37	0.022	0.898
10	1.49	1.49	1.40	0.034	0.349
20	1.38	1.38	1.37	0.028	0.985
50	1.33	1.33	1.41	0.030	0.175
100	1.40	1.40	1.37	0.018	0.887
SEM	0.021	0.026	0.014		
L	0.844	0.681	0.883		
Q	0.911	0.710	0.707		
R^2	0.002*	0.010	0.001*		
MIC_e (mg / L)	-	-	-		

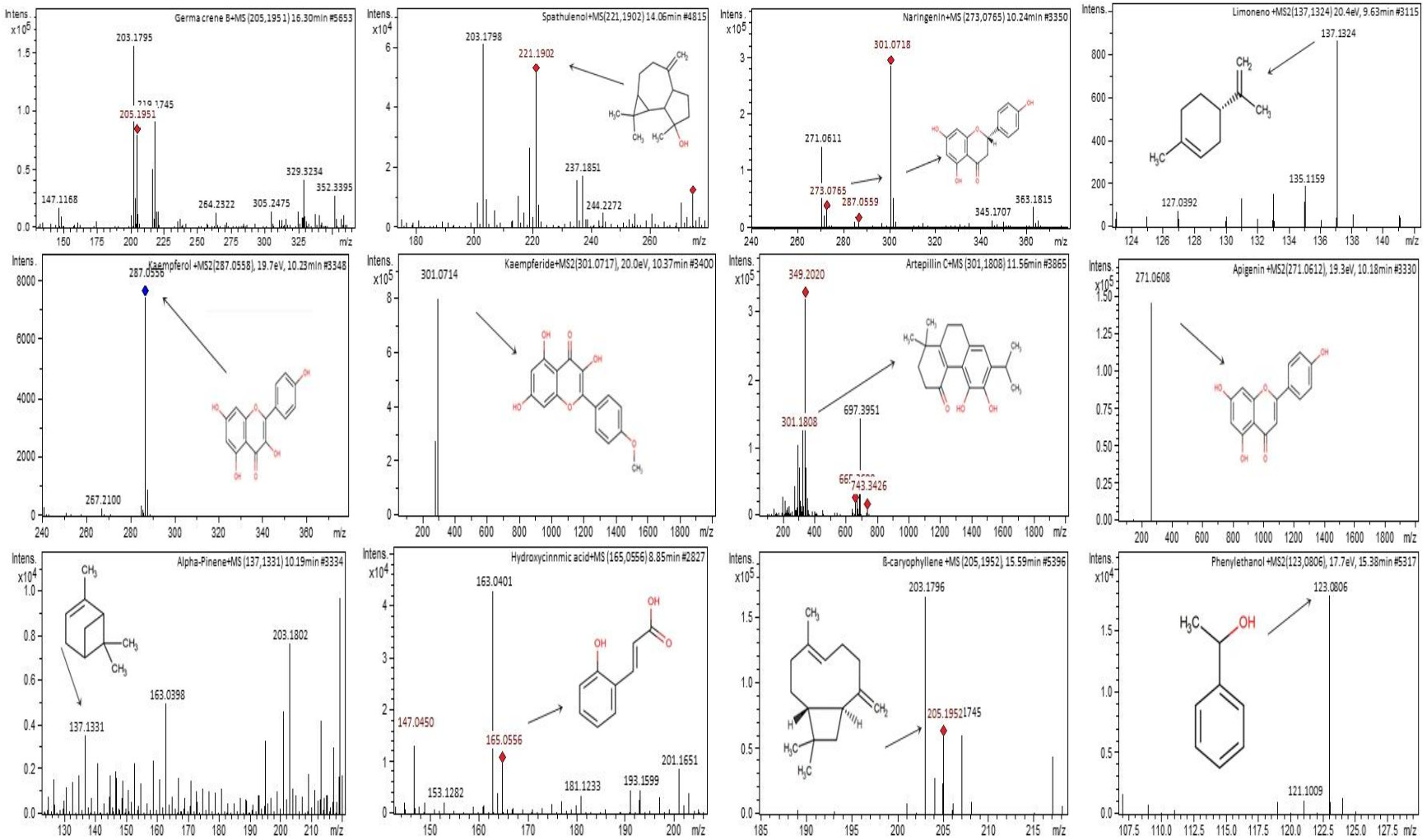
A B = Different uppercase letters means difference in the same line at Bonferroni ($P < 0.05$). L = linear effect; Q = quadratic effect; MIC_e = estimated amount of additive concentration (mg / L) necessary to reduce 50% of optical density; * = equation to calculate MIC_e does not have real square roots

650 **Table 8.** Influence of *Baccharis dracunculifolia* hydroethanolic extract in the anaerobic Gram-
651 positive bacteria growth

Baccharis dracunculifolia extract concentration, mg / L	Time (h)			SEM	Repeated measures *P* < *Value*
	08	12	24		
Ruminococcus albus					
0	0.51C	0.84B	0.98A	0.085	0.052
10	0.73B	1.15A	0.97AB	0.059	0.008
20	0.73B	1.19A	0.92AB	0.059	0.005
50	0.82B	1.14A	1.12AB	0.046	0.007
100	0.90	1.16	1.14	0.039	0.063
SEM	0.040	0.039	0.029		
L	0.003	0.118	0.005		
Q	0.004*	0.047	0.019		
MIC_e (mg / L)	-	-	-		
Ruminococcus flavefaciens					
0	1.33	1.41	1.38	0.043	0.762
10	1.40	1.36	1.33	0.022	0.590
20	1.40	1.43	1.27	0.033	0.279
50	1.39	1.49	1.35	0.027	0.118
100	1.40	1.40	1.38	0.025	0.070
SEM	0.026	0.021	0.020		
L	0.742	0.720	0.495		
Q	0.864	0.348	0.497		
MIC_e (mg / L)	-	-	-		
Streptococcus bovis					
0	1.34A	1.29A	1.25B	0.021	0.019
10	1.41A	1.30B	1.19B	0.030	0.035
20	1.36	1.33	1.29	0.026	0.344
50	1.31	1.40	1.34	0.0333	0.572
100	1.38	1.44	1.33	0.018	0.213
SEM	0.010	0.025	0.021		
L	0.927	0.024	0.052		
Q	0.291	0.071	0.088		
MIC_e (mg / L)	-	-	-		

652 A B = Different uppercase letters means difference in the same line at Bonferroni ($P < 0.05$). L = linear
653 effect; Q = quadratic effect; MIC_e = estimated amount of additive concentration (mg / L) necessary to
654 reduce 50% of optical density * = equation to calculate MICe does not have real square roots

1



2 **Figure 1.** Chromatographic extract profile of Baccharis draunculifolia extract.

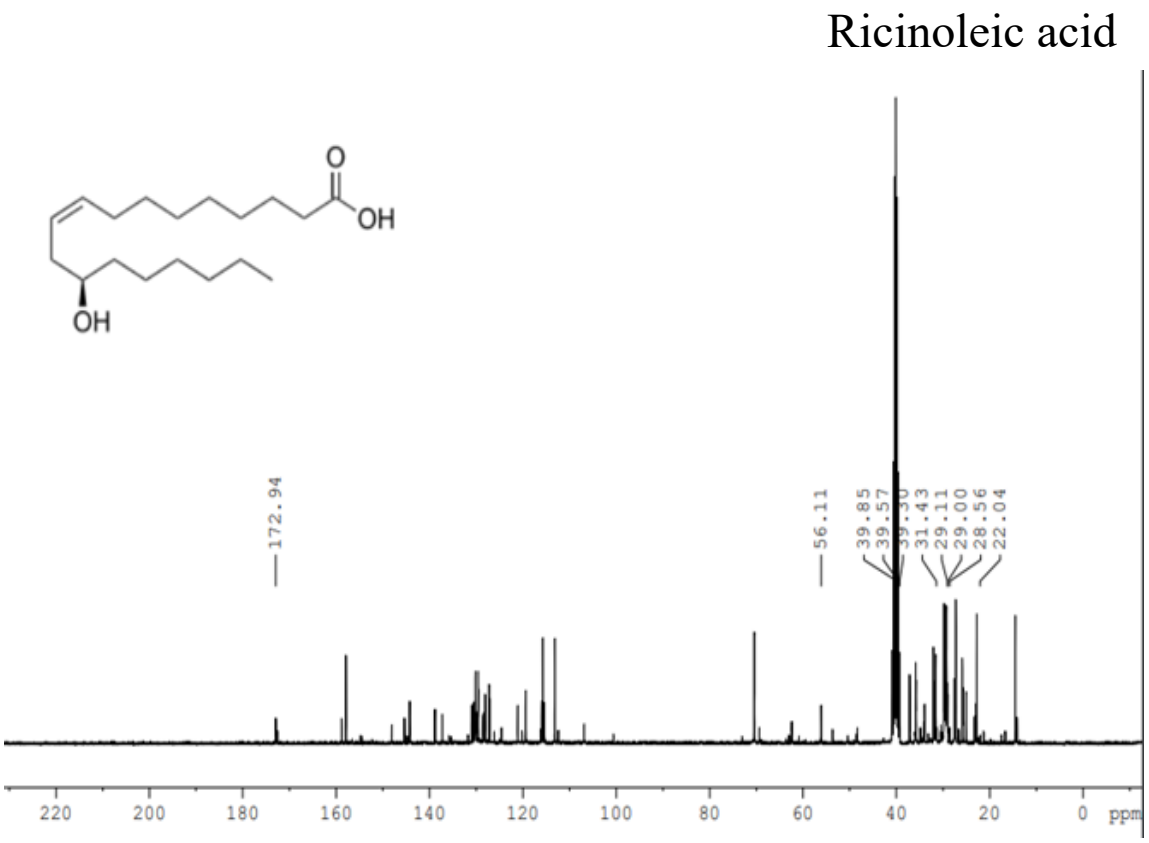


Figure 2. Chromatographic profile of castor oil.

IV - EFFECT OF AMMONIA FIBRE EXPANSION (AFEX) TREATMENT OF RICE STRAW ON IN SITU DIGESTIBILITY, MICROBIAL COLONIZATION, ACETAMIDE LEVELS AND GROWTH PERFORMANCE OF LAMBS

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ABSTRACT. The objective of this study was to evaluate the effect of AFEX treatment (ARS) of rice straw (RS) on the *in situ* degradability, microbial colonization, growth performance and acetamide levels in ewe lambs. Alfalfa, rice straw and AFEX-treated rice straw were incubated in nylon bags in the rumen for 0, 1, 3, 6, 12, 24, 36, 48, 72 and 120 h to determine DM and NDF disappearance kinetics. Sequencing of 16S rRNA was used to characterize colonizing bacterial and archaeal profiles. Lambs (N =40; 37.1 ± 3.5 kg) were fed pelleted diets that contained: 1) ALF = 250 g/kg of alfalfa; 2) RS = 250 g/kg of rice straw; 3) ARS = 250 g/kg of AFEX rice straw; 4) ARSW = ARS withdrawn from the diet 7 d before slaughter. Blood samples were collected biweekly and after ARSW on d 1, 3, 5 and 7 and at slaughter, the diaphragm muscle was used for measurement of acetamide. Alfalfa had greater Kd and A fraction ($P < 0.05$), whereas ARS had higher ($P < 0.05$) B and A+B fractions. Alfalfa DM and NDF degradability was greater at 12 h, but lower than ARS thereafter. Effective ruminal degradability (Ed) at 0.02, 0.04 and 0.06/h was greater ($P< 0.05$) for ARS than other forages. Digestion of ALF and ARS plateaued after 48 h, while RS continued to be degraded. Compared to other forages, alpha and beta microbial diversity of ARS was reduced ($P< 0.05$). The phylogenetic profile of initial colonizers of ARS was more similar to ALF than RS and was dominated by *Bacteroidetes*. Lambs fed RS exhibited similar growth to those fed ALF, while the DMI of ARS lambs was similar, but ADG and feed efficiency were reduced ($P < 0.05$). ALF exhibited greater ($P < 0.05$) DM, OM, CP, NDF, ADF and starch digestibility than ARS. ARS exhibited lower CP, but higher NDF and ADF digestibility than RS.

A strong correlation ($R^2 = 0.81$) was observed between blood and muscle acetamide levels in lambs fed ARS. Withdrawal of ARSW reduced ($P < 0.05$) blood acetamide levels after 3 d, but levels in the diaphragm remained similar to ARS lambs at slaughter. Although AFEX improved the NDF and DM digestibility of RS and altered the phylogenetic profile of primary colonizers, it did not improve the growth of ewe lambs, likely as a result of reduced intake.

1. Introduction

In 2018, global rice production was approximately 770 million tonnes with China, India and Brazil being the primary producers (FAOSTAT, 2018). For each kg of rice grain harvested an additional 1.5 kg of rice straw is residue (Lal (2005). Although rice straw is an abundant resource of biomass, its digestibility and feed value are low due to its high silica content and recalcitrant plant cell walls restricting it usage in ruminant diets (Sarnklong et al., 2010). To dispose of the residue, rice straw is often burned in the field, contributing to emissions and a substantial reduction in air quality (Chen et al., 2017). Physical and chemical pre-treatments can improve the nutritional quality of rice straw and increase its use as feed. Ammoniation,makes the cell wall of low quality forages more accessible to rumen microbiota and increases its non-protein nitrogen content, resulting in overall higher digestibility (Blümmel et al., 2018; Bals et al., 2019; Beauchemin; et al., 2019). One promising technology is Ammonia Fibre Expansion (AFEX) which involves exposing biomass to high levels of ammonia at elevated temperature and pressure (approximately 100 °C and 2 MPa) for less than 1 h, with the added advantage of the ammonia being recovered and recycled (Campbell et al., 2013; Mor et al., 2018).

Recent *in vitro* and *in vivo* studies have demonstrated the potential of AFEX to improve the feed value of low quality forages. Blümmel et al. (2018) tested 10 AFEX-treated cereal straws and observed an increase in crude protein content, *in vitro* gas production, and *in vitro* apparent and true digestibility. Using an artificial rumen simulation technique (Rusitec), Griffith et al. (2016) observed an increase in disappearance of dry matter, organic matter, and neutral detergent fiber as a result of AFEX treatment of barley straw. Others have observed that replacing wheat straw with pelleted AFEX wheat straw increased the digestibility and energy available to lactating buffalo and cattle (Mor et al., 2018). In a second study, Mor et al. (2019) observed a decrease in dry matter intake, growth and rumen fermentation of goats that were fed pelleted AFEX wheat straw as a

replacement for concentrate. They found that AFEX did not alter rumen fermentation or blood metabolites, but it did increase acetamidase activity in rumen. Acetamide is produced and incorporated into the forage as a result of the reaction of ammonia with acetate during treatment, with concentrations differing among forage sources (corn stover 6.6 mg/g; wheat straw 5.6 mg/g; barley straw 4.3 mg/g and rice straw 4.4 mg/g; Bals et al; 2019) . Acetamide can serve as a non-protein nitrogen source for rumen microflora (Mor et al., 2019) and is naturally found in milk and meat at concentrations from 0.27 to 0.67 mg/kg (Vismeh et al., 2017). Levels in milk have been shown to exceed this range in cattle and buffalo fed AFEX-treated straw (Bals et al., 2019). Acetamide has been classified as a Group 2B carcinogen since the 80's based on its ability to induce liver tumours in rats (IARC, 1999). However, only recently has it been considered a food contaminant and to date no regulatory agencies have defined those standard levels that would be considered safe for human consumption (Bals et al., 2019).

Dietary interventions can impact the rumen microbiome, especially changes in the forage : concentrate ratio (Fernando et al., 2010a; Belanche et al., 2012b). AFEX straws have been assessed for their ability to replace low-quality forages and concentrates in ruminant diets (Mor et al., 2018; Mor et al., 2019). To date, the impact of AFEX treatment on the sequential colonization of crop residues such as rice straw has not been examined. With fresh ryegrass, rumen colonization is a bi-phasic process with primary colonizers establishing after 1-2 h and secondary colonizers, after 4-8 h in the rumen (Huws et al., 2015). We theorized that AFEX treatment would alter the rumen bacterial colonization of rice straw in a manner that is more reflective of higher quality forages. Thus, the objective of this study was to evaluate the *in situ* digestibility and the adherent bacterial profile of AFEX treated rice straw and its effect on growth and acetamide levels in lambs.

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2. Material and Methods

2.1 Ethics committee

This experiment was conducted at Agriculture and Agri-Food Canada in Lethbridge, Alberta. Lambs used in this experiment were cared for in accordance with Canadian Council of Animal Care(CCAC, 2009). All procedures and protocols used in this study were reviewed and approved by the Animal Care Committee at the Lethbridge Research and Development Centre (number ACC1812).

2.1 In situ measurements and biofilm formation

Three ruminally cannulated Angus × Hereford heifers were housed in tie stalls and adapted to a diet consisting of 592 g/kg of DM of barley silage, 300 g/kg of DM of barley straw, 82 g/kg of DM of dry roll barley and 26 g/kg of DM of a trace-mineralized supplement for 21 d. To prevent sorting, barley straw was fed at 1000 h, 30 min after the provision of concentrate. Barley straw was chopped to a length of 6 to 10 cm and provided *ad libitum* to ensure 10% to 20% orts. Heifers were bedded with wood shavings on top of rubber mats and exercised daily for 2 h.

Samples of AFEX rice straw [g/kg of DM; CP (129), NDF (575), ADF (442) and reducing sugars (0.60)], rice straw [g/kg of DM; CP (69), NDF (753), ADF(519) and reducing sugars (0.0.45)] and alfalfa [g/kg of DM; CP (157), NDF (556), ADF(459) and reducing sugars (1.25)], were ground to pass through a 4 mm screen. Polyester Bags (10 × 20 cm; R1020, ANKOM Technology, Macedon, NY; 50-μm porosity) were loaded with 6.0 ± 0.05 g of forage and sealed with zip ties 1 cm from the top. Bags were incubated in the rumen for 0, 1, 3, 6, 12, 24, 36, 48, 72, and 120 h. Triplicate bags for each time point were placed inside larger mesh bags (30 × 30 cm)

and incubated in each heifer. There were a total of 9 polyester bags per mesh bag and 1 mesh bag per time point. Input and collection of bags were scheduled so that there was no more than 5 mesh bags (45 polyester bags) inside the rumen at any point in time.

Prior to introduction into the rumen, bags were placed in water at 39°C for 10 min to promote hydration. Upon removal from the rumen, bags were immediately submerged in ice water, removed from the mesh bags and briefly rinsed to remove rumen contents and placed into mesh laundry bags. Laundry bags were washed twice in cold water in a large top-loading washing machine set to a gentle cycle (≈ 5 min, no spin cycle).

Bags were removed from the mesh bags, briefly rinsed under cold running water until the water ran clear and gently squeezed to remove excess water. Bags were then placed in foil trays in a forced-air oven at 55°C for 72 h, cooled in desiccators, and hot-weighed. Weights from individual bags were used to calculate DM disappearance. The 0-h bags were placed in a 2 L beaker of water on a hotplate at 39°C for 30 min to estimate the washout fraction. Bags were stirred every 10 min, and after 30 min they were washed and dried as described above. Triplicate samples were composited, ground to pass through a 1-mm screen, and analyzed sequentially for aNDF (Mertens, 2002) with modifications to each procedure for use in a fibre analyzer [F57 Fibre Filter Bags, 200 Fibre Analyzer, ANKOM Technology; (Vogel et al., 1999)], with heat-stable α amylase (Termamyl 120, Sigma- Aldrich, St. Louis, MO) and sodium sulfite included (S430-3sodium sulfite anhydrous, Fisher Scientific, Pittsburgh, PA).

To examine biofilm formation, rice straw, AFEX rice straw or alfalfa were placed in polyester bags and incubated in the rumen of heifers for 2 , 4, 8 and 48 h. After removal from the rumen, nylon bags were gently rinsed twice with 150 mL of phosphate buffered saline. The rinsate was transferred to a labelled falcon tube and flash frozen in liquid nitrogen. Rinsate samples were

then freeze dried and ground with a coffee grinder. The DNA was extracted from ~0.1 g of the freeze dried, ground residue using the Zymobiomics DNA extraction kit (Zymo Research, Irvine CA) and assessed for quality using gel electrophoresis and quantified using a flurospectrophotometerat$A_{260/280}$ (ND-3300 Nanodrop, Wilmington, DE, U.S.A).A PCR reaction was conducted to amplify the full length 16s rRNA gene using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1398R (5'- TACGGYTACCTTGTTACGACTT-3') to confirm the absence of PCR inhibitors in the sample.

Sequencing was performed at McGill University and Genome Quebec Innovation Center, Montreal, Canada using the Illumina MiSeq Reagent Kit v2 (500 cycle) following the manufacturer's guidelines. The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) targeting the V4 region of the 16S rRNA gene were used to examine both bacterial and archaeal diversity. A 33 cycle PCR using 1 μL of a 1 in 10 dilution of genomic DNA and the Fast Start High Fidelity PCR System (Roche, Montreal, PQ) was conducted with the following conditions: 94 °C for 2 min, followed by 33 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final elongation step at 72 °C for 7 min. Fluidigm Corporation (San Francisco, CA) barcodes were incorporated in a second PCR reaction using the FastStart High Fidelity PCR System under the following conditions: 95 °C for 10 min, followed by 15 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min, followed by a final elongation step at 72 °C for 3 min. After amplification, PCR products were assessed in a 2% agarose gel to confirm successful amplification. All samples were quantified using the Quant-iTPicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and were pooled in equal proportions. Pooled samples were then purified using calibrated AmpureXP beads (Beckman Coulter, Mississauga, ON). The pooled samples (library) were quantified using the Quant-iTPicoGreen

dsDNA Assay Kit (Life Technologies, Carlsbad, CA) and the Kapa Illumina GA with Revised Primers-SYBR Fast Universal kit (Kapa Biosystems, Wilmington, MA). Average fragment size was determined using a LabChip GX (PerkinElmer, Waltham, MA, USA) instrument.

Raw fastq files were imported into qiime2 for sequence analysis. Primer and adapter sequences were removed from sequence files with the plugin cutadapt (Martin, 2011). Following removal of primer and adapter sequences, the program dada2 (Callahan et al., 2016) was used for quality control, filtering of any phiX reads in the sequence data and removal of chimeric sequences. Following Dada2, the mafft program was used for alignment and phylogenetic trees were generated using FastTree (Price et al., 2010). Taxonomy was assigned to sequences using a Naïve-Bayes classifier trained with the Silva 128 reference database and the feature-classifier plugin (Bokulich et al., 2018). Samples were rarefied to the lowest number of sequences in all samples to ensure that α- and β-diversity analysis used the same number of sequences per sample. The diversity plugin core-diversity-metrics was used to asses microbial diversity within (α-diversity) and among samples (β-diversity). α-Diversity measures for richness (Shannon's diversity index), phylogenetic diversity (Faith's phylogenetic diversity), number of observed OTU, evenness (Pielou's Evenness) and taxonomic abundance were evaluated. β-Diversity analysis was carried out using weighted and unweighted UniFrac (Lozupone et al., 2011). The significance of differences in taxon abundance, and alpha/beta diversity metrics was tested using the GLM procedure in SPSS. Sequences have been deposited to the Small Reads Archive (NCBI) with accession number PRJNA557266.

2.2 Lambs performance and acetamine analyses

171 To evaluate the inclusion of AFEX rice straw as forage source, Canadian Arcot x Suffolk
172 ewe lambs (n= 40; 37.1 ± 3.5 kg of BW) were randomLy distributed to one of the four treatments:
173 1) ALF = 25% alfalfa; 2) RS = 25% of rice straw; 3) ARS = 25% AFEX-treated rice straw (N =
174 10) and 4) ARSW which was the same diet as "3", but with AFEX rice straw removed from the
175 diet 7 d prior to slaughter. Diets were pelleted and contained the same concentrate ingredients
176 (canola meal, canola oil, barley, beet pulp and corn DDGS), with urea added to ensure that diets
177 were isonitrogenous (Table 1). Lambs were housed in individual pens and weighed on two
178 consecutive days at the start and end of the experiment. Experimental diets were offered *ad libitum*
179 at 0930 h each day for the duration of the trial with water freely available. Feed delivery was
180 recorded daily, with orts collected and weighed weekly for determination of weekly DMI. Dry
181 matter content of feed and orts were determined weekly by oven-drying samples at 55°C for 72 h.
182 Individual BW was recorded weekly to determine ADG, and G:F was calculated as the ratio of
183 ADG to DMI until the withdrawal period (42 d on feed). Lambs were slaughtered after 48 d at a
184 commercial packing plant and hot carcass weight (HCW), dressing percent, grade and muscle
185 score at the shoulder, loin, and hind leg muscle were recorded (Sunterra Meats Ltd, Innisfail, AB).
186 To measure apparent digestibility, feed and fecal samples were collected daily in the morning prior
187 to feeding between d 21 and 28. Acid insoluble ash (AIA) was used as a digestibility marker.
188 Faeces were collected weekly just before feeding from the rectum of all lambs. Samples were
189 stored at −20°C until analysed. Feed and fecal samples were oven dried at 55 °C and ground
190 through a 1-mm screen (Standard model 4 Wiley mill; Arthur H. Thomas, Philadelphia, PA).
191 Apparent total tract digestion of DM, OM, CP, NDF, ADF, and starch was calculated as 100 − 100
192 × [(AIA concentration in feed/AIA concentration in feces) × (nutrient concentration in
193 feces/nutrient concentration in feed)].

194 To measure the impact of removal of AFEX rice straw from the diet on acetamide levels in
195 blood and the diaphragm, 10 lambs from the AFEX group were randomLy selected and switched
196 to the alfalfa diet, 7 d before slaughter (ARSW). Blood samples were collected via the jugular vein
197 before feeding from all lambs biweekly and after withdrawal after 1, 3, 5, and 7 d. Blood was
198 collected in a 6 mL vacuum tube, containing sodium heparin (BD Vacutainer® REF 367878,
199 Franklin Lakes, NJ, USA). Plasma was obtained by centrifugation (2,000 × g for 20 min at 4°C)
200 and stored in a 7 mL screw-cap tube at −20°C until analyzed. After slaughter diaphragm (~10 g
201 each) was collected from each lamb. Samples were placed in a Thermos filled with ice, transported
202 to the lab and stored at −20°C.
203
204 *2.3Chemical analyses*
205
206 *In situ* residues, diets and faeces were analyzed for analytical DM, (AOAC 2005; method
207 930.15), OM (method 942.05), ash (method 942.05), aNDF, and ADF. Ash content was
208 determined by combustion of samples in a muffle furnace at 550 °C for 5 h. Acid insoluble ash
209 (AIA) was used as internal digestibility marker (Van Keulen and Young, 1977). Samples of feed
210 and faeces (5 g) were placed in pre-weighed crucibles, dried, weighed, and ashed overnight at
211 450℃ . To estimate AIA, ash was transferred to a 600 mL beaker and boiled in 2N HCl for 5 min
212 and then filtered through Whatman 41 filter paper. The filter was placed back in a crucible, ashed
213 overnight at 450℃, cooled and weighed.
214 To measure reducing sugars, 1 g of forage ground through a 4 mm screen was boiled with
215 20 mL of distilled water for 1 h, cooled and centrifuged at 5000 x g for 20 min. The extract (9 mL)
216 was then mixed with 1 mL of 0.15 N HCL and boiled for 10 min. An additional 20 mL of distilled
217 water was added to the mixture and reducing sugars were determined using the Nelson-Somogyi

method (Nelson, 1944; Somogyi, 1952). For standards, a 15 µmol glucose/mL water stock solution (270.2 mg glucose/100 mL water) was prepared. Extracts were measured in triplicate and OD was read at 630 ηm using a Dynatech MRX Plate Reader and reducing sugars expressed as g/kg.

Acetamide was quantified in plasma and diaphragm samples as described by Vismeh et al. (2017). Plasma (100uL) was transferred to a 2-mL Eppendorf tube, and 15 µL of an internal standard (5 ug/mL propionamide in methanol) was added to achieve 0.5 mg of propionamide/L. The reaction volume was brought up to 150 uL with distilled water. To precipitate proteins, 300 uL of 0.5 M HCl in MeOH was added to each tube. Tubes were vortexed, and placed in -80˚C freezer for 1 h to promote protein precipitation. Tubes were centrifuged at 20,800 x g for 10 min and 250 uL of plasma was mixed with 200 uL of xanthydrol solution (5%) and incubated in the dark at 40ºC for 2 h.

Diaphragm samples were ground and extracted with methanol. Propionamide was used as an internal standard at a ratio of 0.50 µg/g of meat. The meat extract was derivatized with 9-xanthydrol at 40°C for 2.5 h. Potassium hydroxide was used to neutralize the solution and ethyl acetate to separate the xanthydrol-derivatized acetamide. After centrifugation, the collected ethyl acetate portion was evaporated to dryness, and the precipitate re-suspended in ethyl acetate, centrifuged and the supernatant transferred to vials for GC/MS analysis

The GC/MS analyses was performed at the Michigan Biotechnology Institute (Lansing, Michigan USA) using an Agilent 7890N GC/MS system equipped with Agilent 7683 auto-sampler and a 5973C single quadrupole mass spectrometer (Agilent Technologies, USA). A VF-5ms column (Agilent CP9013, 0.25mm I.D., 30m length, 0.25µm film thickness) was used for analytical separation and helium was the carrier gas.

2.4 Calculations

Disappearance of DM and aNDF at each time was calculated for each substrate within heifer and period. The duplicate values at each time were averaged and then fitted using a nonlinear procedure (Package 'minpack.lm') in R (Team, 2018), available at: (https://cran.r-project.org/web/packages/minpack.lm/index.htmL), to the model:

$$Y = A + B(1 - e^{-c(t-L)}) \text{ for } t > L ;$$

Where A is the soluble fraction (%), B the slowly degradable fraction (%), C the fractional rate of disappearance (%/h), L the lag time (h), and t is the incubation time (h). Effective ruminal disappearance (Ed) was also estimated using the model described by (Baah et al., 2005).

2.5 Statistical analyses

Data were analyzed by the MIXED procedure of SPSS version IBM Statistical Package for the Social Sciences (SPSS version 22). For the *in situ* study, the model included the random effect of heifer and period, and the fixed effect of forage. For the relative abundance of bacteria and archaea at each incubation time point, substrate was considered as a fixed and heifer as a random effect. For the lamb study, DM intake, average daily gain and feed efficiency were analyzed as a completely randomized design with diet as a fixed effect, lambs nested within diet and week as a repeated measure. Initial BW, final BW, hot carcass weight, hot carcass dressing %, grade rule, nutrient digestibility, and acetamide concentrations in plasma and muscle were analyzed using the same procedure without repeated measures. The withdrawal period for AFEX diets was analyzed separately using the MIXED procedure with day as a repeated measure. Differences among diets were identified using the LSD mean procedure with significance declared at $P \leq 0.05$.

3. Results

3.1 In situ degradability

Alfalfa had a greater ($P < 0.05$) A fraction for both DM and NDF degradation than rice straw and AFEX rice straw (Table 2). In contrast, AFEX rice straw exhibited greater ($P < 0.05$) B and A+B fractions than alfalfa and rice straw. Kinetic parameters for rice straw were all lower ($P < 0.05$) as compared to alfalfa and AFEX rice straw. Despite having a greater DM A+B fraction, AFEX rice straw exhibited a lower ($P<0.05$) Kd than alfalfa, but a higher Kd than rice straw. The Kd of AFEX rice straw for NDF degradation was lower ($P < 0.05$) than alfalfa, but similar to rice straw. The Ed of DM at a Kp of 0.02, 0.04 or 0.06, were greater for AFEX rice straw ($P < 0.05$), than alfalfa and rice straw. Similar results were observed for the Ed of NDF, with the exception of Ed calculated at 0.06 where alfalfa and AFEX rice straw did not differ (Table 2). The lag time calculated for DM and NDF did not differ ($P > 0.05$) among treatments. Up to 12 h of incubation, alfalfa exhibited greater DM and NDF disappearance than either AFEX or untreated rice straw (Figure 1). However, after 24 h of incubation AFEX rice straw DM and NDF disappearance were greater than alfalfa. The DM and NDF disappearance of AFEX rice straw (90 and 70%, respectively) and alfalfa (60 and 50%, respectively) plateaued after 48 h, with the DM disappearance of rice straw increasing until 120 h and NDF disappearance plateauing at 96 h.

3.2. Temporal diversity of adherent biofilms

Incubation time in rumen did not affect ($P > 0.05$) species richness, Faith's phylogenetic diversity, Pielou'sevenenss or the Shannon index (data not shown). Observed OTUs of AFEX rice straw

(1035 ± 99) were lower than alfalfa (1150 ± 111; P = 0.02), and rice straw (1233 ± 136; P = 0.002). Faith's PD also differed among substrates with AFEX rice straw having the lowest and rice straw the highest diversity. Shannon Diversity was also lower for AFEX rice straw (8.2 ± 0.4) than rice straw (8.6 ± 0.4; $P < 0.05$), but did not differ ($P > 0.05$) from alfalfa. These data show that the alpha diversity of the microbial community attached to rice straw was reduced as a result of AFEX and that this treatment lowered the richness of the colonizing community.

Principle component plots of weighted and unweighted UniFrac distances was used to investigate the similarity/dissimilarity of the overall microbiome composition of the colonizing microbiome in all three forages over time (Figure 2).The strongest feature impacting the composition of the microbiome was the individual animal (Figure 2a). Plots of both unweighted and weighted UniFrac distances showed that all samples from each animal clustered ($P < 0.05$). Permanova analysis identified an effect of forage type in both the PcoA plots of unweighted and weighted unifrac ($P < 0.05$) with rice straw and AFEX rice straw exhibiting differences ($P < 0.05$) in colonizing microbial communities. However, the samples did not distinctly cluster (Figure. 2b). There was a trend ($P < 0.10$) for differences in the microbial communities colonizing alfalfa as compared to rice straw and AFEX rice straw. Weighted unifrac did not show obvious clustering of samples based on ruminal incubation time, with the exception that populations at 48 h differed ($P < 0.002$) from other incubation times (Figure. 2c). None of the samples differed ($P < 0.05$) based on the unweighted unifrac for incubation time, however populations associated with 1 and 48 h tended to differ ($P < 0.10$).

Firmicutes, Bacteroidetes, Euryarchaeota, Spirochaetae, Proteobacteria and Fibrobacteres were the 5 most abundant phyla, with ≈75% of sequences attributable to *Bacteroidetes* and *Firmicutes*. No interactions among treatments were noted at the genera level (supplemental file 1).

313 At the phylum level, the early colonization of AFEX rice straw was more similar to alfalfa than to
314 rice straw due to a higher percentage ($P < 0.05$) of *Bacteroidetes* and lower percentages ($P < 0.05$)
315 of *Euryarchaeota*, *Proteobacteria* and *Actinobacteria* (Table 3). Between 4 and 8 h of incubation,
316 AFEX rice straw had higher percentage of *Bacteroidetes* than rice straw and alfalfa, while rice
317 straw had higher ($P < 0.05$) percentage of *Firmicute*s than alfalfa and AFEX rice straw. AFEX rice
318 straw had a lower percentage of *Proteobacteria* until 4 h and lower percentages of *Euryarchaeota*
319 *and Actionobacteria* until 8 h than rice straw ($P < 0.05$). *Spirochaetae* percentage was higher ($P <$
320 0.05) in AFEX after 8 h of incubation. At 48 h of incubation, AFEX rice straw showed a colonizing
321 bacterial profile similar to other forages with the exception of having a lower ($P <0.05$), percentage
322 of *Actinobacteria*. A difference in *Fibrobacteres* percentage was observed only between alfalfa
323 and rice straw at 48 h of incubation.
324
325 *3.3 Performance trial and acetamide contents*
326
327 Lambs fed RS or ARS exhibited similar ($P > 0.05$) final BW, HCW and HC dressing and
328 grade rule to lambs fed ALF (Table 4). Lambs fed RS also had ADG, DMI (kg and % of BW) and
329 feed efficiencies that were similar ($P < 0.05$) to those fed ALF. However, the DMI and ADG of
330 lambs fed ARS- was lower ($P< 0.05$) than those fed RS. Feed efficiency of lambs fed ARS was
331 lower ($P < 0.05$) than those fed RS or ALF (Table 4). Ruminal pH measured before feeding
332 averaged above 6.0 in all lambs, but the rumen pH in lambs fed ALF tended to be lower ($P < 0.06$)
333 than in those fed RS. Nutrient digestibility was greater ($P < 0.05$) in lambs fed ALF than in those
334 fed either ARS or RS. Both diets with rice straw (ARS and RS) presented similar DM and OM
335 digestibility. However, CP digestibility was greater ($P<0.05$) for RS, while NDF and ADF were
336 greater for ARS. A strong correlation existed between acetamide in plasma and the diaphragm for

lambs fed AFEX rice straw (Figure. 3). Acetamide was greater ($P<0.05$) in both plasma and the diaphragm (18.93 and 2.66 ppm, respectively) of lambs fed ARS as compared to ALF (0.94 and 0.83, respectively) (Table 5). Withdrawal of AFEX rice straw from the diet (ARSW) reduced acetamide concentrations in plasma, but diaphragm concentrations remained similar to those in lambs continuously fed ARS. After the 5 days of withdraw, acetamide concentrations in plasma had nearly returned to pre-treatment levels.

4. Discussion

4.1 In situ degradability

Ruminants are able to digest structural carbohydrates within plant cell walls that are unsuitable as an energy source for mongastrics, including humans. Alfalfa (*Medicago sativa*), is a high quality forage due to its high yield, nutritional value and palatability resulting in its extensive use in the beef, dairy and sheep industries (Radović et al., 2009). However, to be competitive, producers have to also look for local by-products to reduce feed costs. Rice straw is an abundant source of low quality biomass which could be better used by ruminants when processed (Beauchemin; et al., 2019; Mor et al., 2019; Ribeiro et al., 2019) . Ammonia Fibre Expansion is a process that uses liquid ammonia at moderate temperature and high pressure to deconstruct the plant cell wall and increase the access of rumen microorganisms to cell wall carbohydrates (Hahn-Hägerdal et al., 2006). A recent study showed that AFEX treatment increased the digestibility of wheat straw for lactating buffalo and cattle compared to untreated wheat straw (Mor et al., 2018). In addition, Mor et al. (2019) replaced concentrate with AFEX wheat straw and observed similar growth in doe goats in the later part of their experiment.

In our study, the rapidly digestion fraction (A), of AFEX rice straw was higher than rice straw but lower than alfalfa. Alfalfa (0.1.25 g/kg) had more than twice the reducing sugar content of AFEX treated rice straw (0.60 g/kg), which was expected since alfalfa is known to be a highly digestible forage (Wang et al., 2012). AFEX rice straw presented both higher potential degradable and total potential degradable fractions (B and A+B fractions) than alfalfa and rice straw. AFEX doubled the DM (45 to 90%) and NDF (35 to 70%) degradability of rice straw after 48 h of incubation. AFEX treatment promotes de-crystallization of cellulose, partial de-polymerization of hemicellulose, de-acetylation of acetyl groups, and cleavage of lignin - carbohydrate complexes (O'Connor, 1971; Chundawat et al., 2010). Lignin is a highly complex aromatic heterogeneous polymer that is covalently cross-linked with polysaccharides by covalent bonds that limit the hydrolysis of plant cell walls (Sarkanen and Ludwig, 1971).

The AFEX treatment improved Ed of DM and NDF of rice straw at all three passage rates. Logically, the Ed of the three forages was reduced with increased passage rate. When passage rate is slow, a faster degradation rate increases the effective ruminal degradability of forage (Beauchemin; et al., 2019). Our findings are in accordance with those of Beauchemin; et al. (2019) who observed a higher Ed for AFEX treated straws from barley, corn, rice and wheat.

4.2.3.2. Temporal diversity of adherent biofilms

Temporal changes in the colonizing microbiota of AFEX rice straw were consistent with the formation of a primary and a secondary colonizing communities in a manner similar to that observed when ryegrass was incubated in the rumen (Huws et al., 2015). The composition of microbiota on the surface of AFEX treated rice straw was more similar to alfalfa hay than rice straw during the early stages of colonization. As the incubation time increased, the composition of

colonizing microbiota became similar in all forages as mature microbial communities became established. This would suggest that AFEX treatment altered the initial stage of digestion in the rumen through changes in the structure and chemistry of structural carbohydrates, but that populations eventually evolved to similar climax populations after prolonged ruminal incubation. Consequently, it is likely that the structural and chemical nature of the indigestible components of the plant cell walls were similar across all forage types.

Ammonia Fibre Expansion is an efficient method for increasing the yield of fermentable sugars from lignocellulosic biomass (Dale et al., 1996). The increase in reducing sugars (from 0.45 to 0.60 g/kg) could explain why AFEX altered the microbial profile involved in the early stages of rice straw digestion to a profile that was more similar to alfalfa. Differences in the microbial profile of AFEX rice straw and alfalfa between 4 and 8 h could be attributed to the faster rate of degradation (Kd) and the increase in reducing sugars (1.25 g/kg), increasing *Bacteroidetes*. It has been shown that members of the phylum *Bacteroidetes* are more abundant with grain (45%) than forage-based diets (25%), due to the availability of more soluble carbohydrates (Fernando et al., 2010a). However, it is not only the concentration, but the rate of release of soluble carbohydrates that limits the growth of these microorganism (Kingston-Smith et al., 2003). Huws et al. (2015) observed that the shift in secondary colonization (4-8 h) was characterized by an increase of *Firmicutes* and a numerical reduction in *Bacteroidetes* associated with fresh ryegrass. The similarity among forages in the secondary phase of colonization is likely linked to a decrease in soluble sugars and colonizing *Bacteroidetes* as climax bacterial populations associate with the indigestible fraction of forage.

The *Euryarchaeota* in biofilms colonizing AFEX treated rice straw were lower than in other forages. *Methanobrevibacter* represent the majority of the *Euryarchaeota* group (Henderson et al.,

2015). These members of the archaea utilize H_2 as substrate to reduce CO_2 to CH_4, through a series of reactions that are coupled to ATP synthesis (Leahy et al., 2010). The most successful method to mitigate CH_4, emissions is by improving feed efficiency (Leng, 2014). Thus, the improvements in cellulose digestion (less complicate pathway for micro-organism) may explain the reduction in the number of archaea. *Proteobacteria* can represent around 14% of the of the core microbiome in the rumen and are more associated with ruminants fed a high grain diet (Petri et al., 2013). In our study, *Proteobacteria* were lower in AFEX rice straw than other forages, with values below 3%. Ribeiro et al. (2019) observed that *Proteobacteria* represented around 1% of total microbial population associated with solid feed in lambs fed a 50:50 concentrate forage diet containing AFEX- wheat straw, confirming the lower abundance of this phylum with diets that contain more forage.

Differences in *Fibrobacteres* were observed between alfalfa and rice straw after 48 h of incubation. Similar results were also reported by (Liu et al., 2016) who found differences in the abundance of *Fibrobacteres* between rice straw and alfalfa after16 and 48 h of ruminal incubation. *Fibrobacteres.* are one of the principal cellulolytic bacterial species in the rumen, with some reports that they are more effective at degrading plant cell walls than *Ruminococcus* species (Ralph and Helm, 1993; Ransom-Jones et al., 2012). The greater abundance of *Fibrobacteres* in rice straw could account for the continued degradation of this substrate beyond 48 h of incubation in the rumen (Liu et al., 2016). There is a lack of information on the role of *Actinobacteria* in the rumen, but a recent study with composted rice straw demonstrated that *Actinobacteria* may play a role in the degradation of complex lignocellulose (Wang et al., 2016). Interestingly, *Actinobacteria* were lower in AFEX rice straw than in other forages, possible because AFEX disrupted lignin-carbohydrate complexes (O'Connor, 1971; Chundawat et al., 2010).

Liu et al. (2016) observed a shift in adherent microbial populations in alfalfa and rice straw after 6 h of incubation in the rumen, which was characterized by an increase in *Spirochaetae* with both forages, but a reduction in this phylum after 48 h in alfalfa. In our study, *Spirochaetae* were higher in AFEX rice straw than in either alfalfa or rice straw, and increased over time. Bacteria specialized in fibre digestion, like *Fibrobacter* and *Treponema* may play a more important role in secondary colonization (Liu et al., 2016). *Treponema*, is a member of the *Spirochaetae*, and is a highly motile bacterium that acts in symbiosis with non-motile cellulolytic bacteria by using their fermentation end-products (Leng, 2014). Thus, the higher amount of *Spirochaetae* with AFEX rice straw may be associated with a higher availability of end products arising from the enhanced DM and NDF digestion observed with this alkali treatment.

4.3Lambs Performance and acetamide

The DM digestibility of ARS was lower than ALF but similar to RS, which was not expected based on the *in situ* data. This could be related to a higher passage rate of AFEX treated rice straw due to an increase in its specific gravity as a result of smaller particle size (Welch, 1986). In the *in situ* study, AFEX rice straw remained in the rumen, inside bags, while in the lambs study, a portion of the AFEX rice straw could have been washed out of the rumen and not digested. Lambs fed ARS still had higher NDF and ADF digestibility than RS, which is in agreement with our *in situ* results. Ammonia fibre expansion disrupts plant cell walls, and as ammonia evaporates it deposits end products of the hydrolysis process including amides, arabinoxylan oligomers and lignin-based phenolics. This process forms nanopores within the plant cell wall that can enhance the accessibility of enzymes to structural carbohydrates (Chundawat et al., 2011; Campbell et al.,

2013). However, this improvement in NDF digestibility was not reflected in an improvement in the growth of lambs fed the pelleted AFEX rice straw diet.

Surprisingly, rice straw did not limit intake as a result of gut fill and lambs fed RS had a higher intake than ARS. Straw is often included in the the diet of finishing ruminants as a source of effective NDF so as to mitigate the metabolic disorders associated with high concentrate diets (Bodas et al., 2010). However, in this case straw is typically limited to no more than 15% of the diet DM. Haddad and Ata (2009) observed that inclusion of wheat straw (10-15% DM) improved average daily gain and feed efficiency by 29 and 19% respectively, as compared to concentrate diets that lacked or contained only 5% wheat straw. In ruminants, few large feed particles (2 to 16 mm) are excreted (<2%) as they are retained in rumen and further subject to breakdown as result of digestion and rumination (Hummel et al., 2018). In our study, rice straw comprised a substantial portion of the diet (25%), but it was ground (4 mm) and pelleted, reducing the particle size and its residence time in the rumen. This may have reduced the negative feed back loop on intake as a result of rumen fill, increasing the DMI and ADG of lambs. In addition, pelleting a total mixed ration (TMR) can reduce sorting of ingredients and optimize rumen fermentation, leading to improved growth in finishing lambs (Zhong et al., 2018). According to Blanco et al. (2014) the amount of ground barley straw that can be fed can be higher in pelleted diets as compared to if it is fed as full length forage, with no negative effect on the growth lambs when it was fed as a TMR pellet at 25% of DM.

The DMI can also be regulated by energy requirements and the concentration of volatile fatty acids in the rumen (Allen, 2000). Lambs fed ALF had similar intake to ARS, but had the highest DM digestibility, likely accounting for the lower ruminal pH with this diet. The accumulation of organic acids in the rumen decreases pH, which can lead to subclinical acidosis

and economic losses. However, the pH values observed in our study were well within a normal range (5.6 – 6.5) (Nagaraja and Titgemeyer, 2007), making it unlikely that differences in fibre digestibility were related to low ruminal pH. Thus, other factors like lower palatability may have limited the intake of lambs fed ARS. Sheep and goats are very sensitive to concentrate palatability. Lower palatability generally results from post-ingestive signals due to accumulation of fermentation end products (Baumont et al., 2000). Some amides (nicotinamide, propionamide), are rapidly degraded into ammonia in rumen, however acetamide is known to be slowly metabolized by microorganisms (Arner, 1964). Amines have been reported to slightly increase rumen osmolality and to reduce palatability in sheep due to a reduction at the initial eating rate at the beginning of the meal.(Van Os et al., 1995). Amides could increase osmolality as well, however we could not find a study that reported negative effects on palatability as a result of the accumulation of acetamide in the rumen.

Lambs fed ARS had lower CP digestibility than RS. Urea was added to RS to achieve isonitrogenous diets, while a large portion of the CP in ARS diet originated from the acetamide (4.4 mg/g) present in AFEX pellets (Bals et al; 2019).. Mor et al. (2019) associated the improvements in the ADG of goats to a reduction in acetamide in the rumen as rumen microbiota adapted to utilize it as a source of non-protein N. In addition, a recent metabolic study conducted by our group observed a 30% decline in N retention in wethers fed a diet containing AFEX wheat straw as compared to alfalfa (Ribeiro et al., 2019). Thus, the lower digestibility of CP may have promoted the lower feed intake and feed efficiency of the lambs fed AFEX rice straw. Previous growth trials evaluating the partial substation of forage or concentrate by AFEX treated forages have not reported similar decreases in feed intake in ruminants (Mor et al., 2018; Mor et al., 2019; Ribeiro et al., 2019).

Acetamide (CH3CONH2) can be used by rumen microorganisms as a source of nitrogen (Nagayama et al., 1961; Draper, 1967). Due to its simplicity, this molecule is suggested to be formed naturally or as by-product of other processes and naturally occurs in milk and meat at concentrations of up to 0.4 mg/kg (Vismeh et al., 2017). However, acetamide has been classified as a Group 2B human carcinogen, due to its capacity to induce cancer in rats (Williams, 1980; IARC, 1999). Bals et al. (2019) observed that acetamide levels in milk increased 16−23 times in cattle and 19-28 times in buffalo after 3 weeks of feeding AFEX pellets. However, the amount of acetamide excreted in milk only represented 0.2% of that which was ingested. Acetamide is extensively metabolized by microorganisms in the rumen and utilized as a N source to synthesize amino acids for microbial protein synthesis (Bergner, 1984). Mycobacteria have also been shown to be able to metabolize acetamide (Nagayama et al., 1961; Draper, 1967) and more recent studies have shown that rumen microbiota have an enhanced ability to metabolize acetamide arising from AFEX after adaptation (Mor et al., 2019).

In contrast to milk, no difference in acetamide content was observed between beef purchased from a supermarket and that arising from cattle fed AFEX pellets (Bals et al., 2019). However, for lambs it was observed that acetamide in the diaphragm was greater in lambs fed AFEX wheat straw (8.27%) than in those fed a diet with alfalfa (1.83%; Ribeiro et al. 2019). Differences in the microbiomes and metabolism between cattle and sheep may account for these observations, especially if the shorter time to finish lambs does not allow for sufficient time for microbial adaptation. We hypothesized that a 7 d withdrawal of AFEX straw from the diet would be sufficient to return acetamide to basal levels. Acetamide contents in plasma was higher than in muscle, and declined substantially3 d after AFEX rice straw was removed from the diet According to Putcha et al. (1984), around 70% of oral or intravenous acetamide was metabolised by rats

within 72 h after administration. The removal of AFEX straw from the diet resulted in a slight decline in acetamide in the diaphragm (2.14mg/kg), but it was still higher than the recommended daily acetamide exposure (1.5 mg/) in the United States (Bercu et al., 2018). However, these concentrations are substantially lower than that associated in the muscle tissue (7,000 mg/kg) of rats that received a lethal doses of acetamide (Kegley et al., 2014). Thus, more studies are still necessary to determine if acetamide levels encountered in food from livestock fed AFEX treated forages pose a health risk.

5. Conclusion

AFEX transformed rice straw into a highly digestible forage source and altered the microbial profile of early formed biofilms (higher *Bacteroidetes* and lower *Firmicutes*) so that they more closely resembled those of alfalfa than untreated rice straw. However, improvements in digestibility did not result in improved weight gain in lambs fed a pelleted diet that contained 25% AFEX rice straw as compared to untreated rice straw due to reductions in intake and feed efficiency. The AFEX process shows considerable potential to improve the feed value of crop residues, but different feeding strategies still need to be defined so as to ensure that increases in fibre digestibility translate into improved growth performance and efficiency in ruminants. Acetamide was increased (blood and diaphragm) but quickly declined in blood after withdrawal of AFEX from the diet for 3 d, but a longer withdrawal period would be needed to return acetamide to basal levels in muscle tissue. Further studies are necessary to determinate if this increase of acetamide in muscle caused by AFEX presents a health risk to humans as compared to the natural acetamide levels that are found in milk and meat.

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8. Declarations of interest

None

9. References

Abrahão, J.J.S., Prado, I.N., Perotto, D., Moletta, J.L., 2005. Características de carcaças e da carne de tourinhos submetidos a dietas com diferentes níveis de substituição do milho por resíduo úmido da extração da fécula de mandioca. Revista Brasileira de Zootecnia 34, 1640-1650.

Aguiar, A.C.R., Oliveira, C.R., Caldeira, L.A., Junior, V.R.R., Oliveira, S.J., Soares, C., Silva, D.A., Menezes, J.C., Borges, L.D.A., 2013. Consumo, produção e composição do leite e do queijo de vacas alimentadas com níveis crescentes de ureia. Revista Brasileira de Ciência Veterinária 20, 37-42. http://dx.doi.org/10.4322/rbcv.2014.048.

Alencar, S.M.d., Aguiar, C.L.d., Paredes-Guzmán, J., Park, Y.K., 2005. Composição química de Baccharis dracunculifolia, fonte botânica das própolis dos estados de São Paulo e Minas Gerais. Ciência Rural 35.

Allen, M.S., 2000. Effects of Diet on Short-Term Regulation of Feed Intake by Lactating Dairy Cattle. J. Dairy Sci. 83, 1598-1624. https://doi.org/10.3168/jds.S0022-0302(00)75030-2.

Altermann, E., Schofield, L.R., Ronimus, R.S., Beatty, A.K., Reilly, K., 2018. Inhibition of rumen methanogens by a novel archaeal lytic enzyme displayed on tailored bionanoparticles. Front. Microbiol. 9, 2378.

ANUALPEC, 2019. Anuário da Pecuária Brasileira. Instituto FNP, São Paulo, São Paulo, Brasil.

AOAC, 2005. Official methods of analysis.
Appuhamy, R.N.J.A.D., Strathe, A.B., Jayasundara, S., Wagner-Riddle, C., Dijkstra, J., France, J., Kebreab, E., 2013. Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. Journal of Dairy Science 96, 5161-5173. http://dx.doi.org/10.3168/jds.2012-5923.
Arner, A., 1964. The breakdown of asparagine, glutamine, and other amides by microorganisms from the sheep's rumen. Aust. J. Biol. Sci. 17, 170-182. https://doi.org/10.1071/BI9640170.
Azzaz, H.H., Murad, H.A., Morsy, T.A., 2015. Utility of ionophores for ruminant animals: a review. Asian Journal of Animal Sciences 9, 254-265.
Baah, J., Shelford, J., Hristov, A., McAllister, T., Cheng, K., 2005. Effects of Tween 80 and fibrolytic enzymes on ruminal fermentation and digestibility of feeds in Holstein cows. Asian-Aust. J. Anim. Sci 18, 816-824. https://doi.org/10.5713/ajas.2005.816.
Balandrin, M.F., Klocke, J.A., Wurtele, E.S., Bollinger, W.H., 1985. Natural plant chemicals: sources of industrial and medicinal materials. Science 228, 1154-1160.
Bals, B., Teymouri, F., Haddad, D., Julian, W.A., Vismeh, R., Jones, A.D., Mor, P., Van Soest, B., Tyagi, A., VandeHaar, M., 2019. Presence of Acetamide in Milk and Beef from Cattle Consuming AFEX-Treated Crop Residues. J. Agr. Food Chem. 67, 10756-10763. https://doi.org/10.1021/acs.jafc.9b04030.
Baumont, R., Prache, S., Meuret, M., Morand-Fehr, P., 2000. How forage characteristics influence behaviour and intake in small ruminants: a review. Livest. Prod. Sci. 64, 15-28. https://doi.org/10.1016/S0301-6226(00)00172-X.
Beauchemin;, K.A., Ribeiro;, G.O., Ran;, T., Milani;, M.R.M., Yang;, W.Z., Khanaki;, H., Gruninger;, R., Tsang;, A., McAllister, T.A., 2019. Recombinant fibrolytic feed enzymes and ammonia fibre expansion (AFEX) pretreatment of crop residues to improve fibre degradability in cattle. Anim. Feed Sci. Technol. 256, 114260. https://doi.org/10.1016/j.anifeedsci.2019.114260.
Belanche, A., Doreau, M., Edwards, J.E., Moorby, J.M., Pinloche, E., Newbold, C.J., 2012a. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. The Journal of Nutrition 142, 1684-1692. https://doi.org/10.3945/jn.112.159574.
Belanche, A., Doreau, M., Edwards, J.E., Moorby, J.M., Pinloche, E., Newbold, C.J., 2012b. Shifts in the Rumen Microbiota Due to the Type of Carbohydrate and Level of Protein Ingested by Dairy Cattle Are Associated with Changes in Rumen Fermentation–3. The Journal of nutrition 142, 1684-1692. https://doi.org/10.3945/jn.112.159574.
Benchaar, C., Chaves, A., Fraser, G., Beauchemin, K., McAllister, T., 2007. Effects of essential oils and their components on in vitro rumen microbial fermentation. Can. J. Anim. Sci. 87, 413-419.
Benchaar, C., Greathead, H., 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Animal Feed Science and Technology 166, 338-355. https://doi.org/10.1016/j.anifeedsci.2011.04.024.
Bercu, J., Galloway, S., Parris, P., Teasdale, A., Masuda-Herrera, M., Dobo, K., Heard, P., Kenyon, M., Nicolette, J., Vock, E., 2018. Potential impurities in drug substances: Compound-specific toxicology limits for 20 synthetic reagents and by-products, and a class-specific toxicology limit for alkyl bromides. Regul. Toxicol. Pharm. 94, 172-182. https://doi.org/10.1016/j.yrtph.2018.02.001.
Bergner, H., 1984. Metabolism of 14C-and 15N-labelled acetamide and acetylurea as NPN sources. Can. J. Anim. Sci. 64, 37-38. https://doi.org/10.4141/cjas84-145.

625 Binder, R., Applewhite, T., Kohler, G., Goldblatt, L., 1962. Chromatographie analysis of seed oils.
626 Fatty acid composition of castor oil. Journal of the American Oil Chemists' Society 39, 513-517.
627 Biondo, P.B.F., Carbonera, F., Zawadzki, F., Chiavellia, L.U.R., Pilau, E.J.P., Prado, I.N.,
628 Visentainer, J.V., 2017. Antioxidant capacity and identification of bioactive compounds by GC-
629 MS of essential oils commercialized in Brazil. Current Bioactive Compounds 13, 137-143.
630 http://dx.doi.org/10.2174/1573407212666161061408084.
631 Blanco, C., Bodas, R., Prieto, N., Andrés, S., López, S., Giráldez, F.J., 2014. Concentrate plus
632 ground barley straw pellets can replace conventional feeding systems for light fattening lambs.
633 Small Rumin. Res. 116, 137-143. https://doi.org/10.1016/j.smallrumres.2013.11.008.
634 Blümmel, M., Teymouri, F., Moore, J., Nielson, C., Videto, J., Kodukula, P., Pothu, S.,
635 Devulapalli, R., Varijakshapanicker, P., 2018. Ammonia Fiber Expansion (AFEX) as spin off
636 technology from 2nd generation biofuel for upgrading cereal straws and stovers for livestock feed.
637 Anim. Feed Sci. Technol. 236, 178-186. https://doi.org/10.1016/j.anifeedsci.2017.12.016.
638 Bodas, R., López, S., Rodríguez, A.B., Andrés, S., Mantecon, A., Giráldez, F.J., 2010. Feed intake,
639 digestibility, and carcass characteristics of lambs fed a diet supplemented with soluble fibre. Anim.
640 Prod. Sci. 50, 45-51. https://doi.org/10.1071/AN09094.
641 Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A.,
642 Caporaso, J.G., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences
643 with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 90. https://doi.org/10.1186/s40168-
644 018-0470-z.
645 Bonin, E., Carvalho, V.M., Avila, V.D., Santos, N.C.A., Zanqueta, É.B., Lanchero, C.A.C.,
646 Previdelli, I.T.S., Nakamura, T.U., Abreu Filho, B.A., Prado, I.N., 2020. Baccharis
647 dracunculifolia: Chemical constituents, cytotoxicity and antimicrobial activity. LWT - Food
648 Science and Technology 120, 1-10. https://doi.org/10.1016/j.lwt.2019.108920.
649 Borchers, R., 1965. Proteolytic activity of rumen fluid in vitro. J. Anim. Sci. 24, 1033-1038.
650 Broderick, G., Balthrop Jr, J., 1979. Chemical inhibition of amino acid deamination by ruminal
651 microbes in vitro. J. Anim. Sci. 49, 1101-1111.
652 Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C., 2005a. Screening for effects of plant extracts
653 and active compounds of plants on dairy cattle rumen microbial fermentation in a continuous
654 culture system. Animal Feed Science and Technology 123–124, 597-613.
655 http://dx.doi.org/10.1016/j.anifeedsci.2005.03.008.
656 Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C., 2005b. Screening for the effects of natural
657 plant extracts and secondary plant metabolites on rumen microbial fermentation in continuous
658 culture. Anim. Feed Sci. Technol 123, 597-613.
659 Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016.
660 DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581.
661 https://doi.org/10.1038/nmeth.3869.
662 Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L., Ferret, A., 2007. Invited Review:
663 Essential Oils as Modifiers of Rumen Microbial Fermentation. Journal of Dairy Science 90, 2580-
664 2595. https://doi.org/10.3168/jds.2006-644.
665 Cammack, K.M., Austin, K.J., Lamberson, W.R., Conant, G.C., Cunningham, H.C., 2018.
666 RUMINANT NUTRITION SYMPOSIUM: Tiny but mighty: the role of the rumen microbes in
667 livestock production. Journal of animal science 96, 752-770. 10.1093/jas/skx053.
668 Campbell, T.J., Teymouri, F., Bals, B., Glassbrook, J., Nielson, C.D., Videto, J., 2013. A packed
669 bed ammonia fiber expansion reactor system for pretreatment of agricultural residues at regional
670 depots. Biofuels 4, 23-34. https://doi.org/10.4155/bfs.12.71.

671 Cardoso, A.S., Berndt, A., Leytem, A., Alves, B.J., Carvalho, I.N.O., Soares, L.H.B., Urquiaga,
672 S., Boddey, R.M., 2016. Impact of the intensification of beef production in Brazil on greenhouse
673 gas emissions and land use. Agricultural Systems 143, 86-96.
674 http://dx.doi.org/10.1016/j.agsy.2015.12.007.
675 Carvalho, T.B., De Zen, S., 2017. A cadeia de Pecuária de Corte no Brasil: evolução e tendências.
676 Revista iPecege 3, 85-99. http://dx.doi.org/10.22167/r.ipecege.2017.1.85.
677 Castañeda-Correa, A., Corral-Luna, A., Hume, M.E., Anderson, R.C., Ruiz-Barrera, O., Castillo-
678 Castillo, Y., Rodriguez-Almeida, F., Salinas-Chavira, J., Arzola-Alvarez, C., 2019. Effects of
679 thymol and carvacrol, alone or in combination, on fermentation and microbial diversity during in
680 vitro culture of bovine rumen microbes. Journal of Environmental Science and Health, Part B 54,
681 170-175. https://doi.org/10.1080/03601234.2018.1536580.
682 Castillejos, L., Calsamiglia, S., Ferret, A., 2006a. Effect of essential oil active compounds on
683 rumen microbial fermentation and nutrient flow in in vitro systems. J. Dairy Sci. 89, 2649-2658.
684 Castillejos, L., Calsamiglia, S., Ferret, A., 2006b. Effect of essential oil active compounds on
685 rumen microbial fermentation and nutrient flow *in vitro* systems. Journal of Dairy Science 89,
686 2649-2658. http://dx.doi.org/10.3168/jds.S0022-0302(06)72341-4.
687 CCAC, 2009. CCAC guidelines on: The care and use of farm animals in research, teaching and
688 testing, Canada Ottawa, ON.
689 Chen, J., Li, C., Ristovski, Z., Milic, A., Gu, Y., Islam, M.S., Wang, S., Hao, J., Zhang, H., He,
690 C., Guo, H., Fu, H., Miljevic, B., Morawska, L., Thai, P., Lam, Y.F., Pereira, G., Ding, A., Huang,
691 X., Dumka, U.C., 2017. A review of biomass burning: Emissions and impacts on air quality, health
692 and climate in China. Sci. Total Environ. 579, 1000-1034.
693 https://doi.org/10.1016/j.scitotenv.2016.11.025.
694 Chen, L., Liu, S., Wang, H., Wang, M., Yu, L., 2016. Relative significances of pH and substrate
695 starch level to roles of Streptococcus bovis S1 in rumen acidosis. AMB Express 6, 80.
696 10.1186/s13568-016-0248-2.
697 Cheng, K., McCowan, R., Costerton, J., 1979. Adherent epithelial bacteria in ruminants and their
698 roles in digestive tract function. The American journal of clinical nutrition 32, 139-148.
699 Cheng, Y.F., Edwards, J.E., Allison, G.G., Zhu, W.-Y., Theodorou, M.K., 2009. Diversity and
700 activity of enriched ruminal cultures of anaerobic fungi and methanogens grown together on
701 lignocellulose in consecutive batch culture. Bioresour. Technol. 100, 4821-4828.
702 Chundawat, S.P., Donohoe, B.S., da Costa Sousa, L., Elder, T., Agarwal, U.P., Lu, F., Ralph, J.,
703 Himmel, M.E., Balan, V., Dale, B.E., 2011. Multi-scale visualization and characterization of
704 lignocellulosic plant cell wall deconstruction during thermochemical pretreatment. Enen. Environ.
705 Sci. 4, 973-984. https://doi.org/10.1039/C0EE00574F.
706 Chundawat, S.P., Vismeh, R., Sharma, L.N., Humpula, J.F., da Costa, S.L., Chambliss, C.K.,
707 Jones, A.D., Balan, V., Dale, B.E., 2010. Multifaceted characterization of cell wall decomposition
708 products formed during ammonia fibre expansion (AFEX) and dilute acid based pretreatments.
709 Bioresour. Technol. 101. https://doi.org/10.1016/j.biortech.2010.06.027.
710 Clarke, L., Fodey, T.L., Crooks, S.R.H., Moloney, M., O'Mahony, J., Delahaut, P., O'Kennedy,
711 R., Danaher, M., 2014. A review of coccidiostats and the analysis of their residues in meat and
712 other food. Meat Science 97, 358-374. https://doi.org/10.1016/j.meatsci.2014.01.004.
713 Cobellis, G., Trabalza-Marinucci, M., Marcotullio, M.C., Yu, Z., 2016a. Evaluation of different
714 essential oils in modulating methane and ammonia production, rumen fermentation, and rumen
715 bacteria *in vitro*. Animal Feed Science and Technology 215, 25-36.
716 http://dx.doi.org/10.1016/j.anifeedsci.2016.02.008.

717 Cobellis, G., Trabalza-Marinucci, M., Yu, Z., 2016b. Critical evaluation of essential oils as rumen
718 modifiers in ruminant nutrition: A review. Science of the Total Environment 545, 556-568.
719 http://dx.doi.org/10.1016/j.scitotenv.2015.12.103.
720 Couvreur, S., Hurtaud, C., Lopez, C., Delaby, L., Peyraud, J.-L., 2006. The linear relationship
721 between the proportion of fresh grass in the cow diet, milk fatty acid composition, and butter
722 properties. J. Dairy Sci. 89, 1956-1969.
723 Cruz, O.T.B., Valero, M.V., Zawadzki, F., Rivaroli, D.C., do Prado, R.M., Lima, B.S., do Prado,
724 I.N., 2014a. Effect of glycerine and essential oils (Anacardium occidentale and Ricinus communis)
725 on animal performance, feed efficiency and carcass characteristics of crossbred bulls finished in a
726 feedlot system. Italian Journal of Animal Science 13, 3492.
727 Cruz, O.T.B., Valero, M.V., Zawadzki, F., Rivaroli, D.C., Prado, R.M., Lima, B.S., Prado, I.N.,
728 2014b. Effect of glycerine and essential oils (*Anacardium occidentale* and *Ricinus communis*) on
729 animal performance, feed efficiency and carcass characteristics of crossbred bulls finished in a
730 feedlot system. Italian Journal of Animal Science 13, 790-797.
731 http://dx.doi.org/10.4081/ijas.2014.3492.
732 da Silva, L.G., Torrecilhas, J.A., Passetti, R.A.C., Ornaghi, M.G., Eiras, C.E., Rivaroli, D.C.,
733 Valero, M.V., do Prado, I.N., 2014. Glycerin and cashew and castor oils in the diets for bulls in
734 finished in feed lot: Ingestive behavior. Semina: Ciências Agrárias 35, 2723-2737.
735 Dale, B.E., Leong, C., Pham, T., Esquivel, V., Rios, I., Latimer, V., 1996. Hydrolysis of
736 lignocellulosics at low enzyme levels: application of the AFEX process. Bioresour. Technol. 56,
737 111-116. https://doi.org/10.1016/0960-8524(95)00183-2.
738 Dawson, K.A., Boling, J.A., 1987. Effects of potassium ion concentrations on the antimicrobial
739 activities of ionophores against ruminal anaerobes. Appl. Environ. Microbiol. 53, 2363-2367.
740 de Aguiar, S.C., Zeoula, L.M., Franco, S.L., Peres, L.P., Arcuri, P.B., Forano, E., 2013.
741 Antimicrobial activity of Brazilian propolis extracts against rumen bacteria in vitro. World Journal
742 of Microbiology and Biotechnology 29, 1951-1959. http://dx.doi.org/10.1007/s11274-013-1361-
743 x.
744 de Jesus, E.F., Del Valle, T.A., Calomeni, G.D., Silva, T.H.d., Takiya, C.S., Vendramini, T.H.A.,
745 Paiva, P.G.d., Silva, G., Netto, A., Rennó, F.P., 2016. Influence of a blend of functional oils or
746 monensin on nutrient intake and digestibility, ruminal fermentation and milk production of dairy
747 cows. Anim. Feed Sci. Technol. 219, 59-67.
748 De Paepe, M., Leclerc, M., Tinsley, C.R., Petit, M.-A., 2014. Bacteriophages: an underestimated
749 role in human and animal health? Frontiers in cellular and infection microbiology 4, 39.
750 de Sousa, J.P.B., Jorge, R.F., Leite, M.F., Furtado, N.A., Bastos, J.K., da Silva Filho, A.A.,
751 Queiroga, C.L., de Magalhaes, P.M., Soares, A.E., 2009. Seasonal variation of the (E)-nerolidol
752 and other volatile compounds within ten different cultivated populations of Baccharis
753 dracunculifolia DC (Asteraceae). Journal of Essential Oil Research 21, 308-314.
754 Dehority, B.A., Tirabasso, P.A., 1989. Factors affecting the migration and sequestration of rumen
755 protozoa in the family Isotrichidae. Microbiology 135, 539-548.
756 Dorman, H., Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant
757 volatile oils. Journal of applied microbiology 88, 308-316.
758 Draper, P., 1967. The aliphatic acylamide amidohydrolase of Mycobacterium smegmatis: its
759 inducible nature and relation to acyl-transfer to hydroxylamine. Microbiology 46, 111-123.
760 https://doi.org/10.1099/00221287-46-1-111.

761 Dutra, T.V., Castro, J.C., Menezes, J.L., Ramos, T.R., do Prado, I.N., Junior, M.M., Mikcha,
762 J.M.G., de Abreu Filho, B.A., 2019. Bioactivity of oregano (Origanum vulgare) essential oil
763 against Alicyclobacillus spp. Industrial crops and products 129, 345-349.
764 Dwivedi, C., Pandey, I., Pandey, H., Ramteke, P.W., Pandey, A.C., Mishra, S.B., Patil, S., 2017.
765 Chapter 9 - Electrospun Nanofibrous Scaffold as a Potential Carrier of Antimicrobial Therapeutics
766 for Diabetic Wound Healing and Tissue Regeneration, In: Grumezescu, A.M. (Ed.), Nano- and
767 Microscale Drug Delivery Systems, Elsevier, pp. 147-164.
768 Elekwachi, C.O., Wang, Z., Wu, X., Rabee, A., Forster, R.J., 2017. Total rRNA-seq analysis gives
769 insight into bacterial, fungal, protozoal and archaeal communities in the rumen using an optimized
770 RNA isolation method. Front. Microbiol. 8, 1814.
771 Evans, J.D., Martin, S.A., 2000. Effects of thymol on ruminal microorganisms. Current
772 Microbiology 41, 336-340.
773 FAOSTAT, 2018. Statistics division. Food and agriculture organization of the United States.
774 Fernando, S.C., Purvis, H., Najar, F., Sukharnikov, L., Krehbiel, C., Nagaraja, T., Roe, B.,
775 DeSilva, U., 2010a. Rumen microbial population dynamics during adaptation to a high-grain diet.
776 Applied and Environmental Microbiology 76, 7482-7490. https://doi.org/10.1128/AEM.00388-
777 10.
778 Fernando, S.C., Purvis, H.T., Najar, F.Z., Sukharnikov, L.O., Krehbiel, C.R., Nagaraja, T.G., Roe,
779 B.A., DeSilva, U., 2010b. Rumen microbial population dynamics during adaptation to a high-grain
780 diet. Applied and Environmental Microbiology 76, 7482-7490.
781 Ferreira, C.M., Rosa, O.P.S., Torres, S.A., Ferreira, F.B.d.A., Bernardinelli, N., 2002. Activity of
782 endodontic antibacterial agents against selected anaerobic bacteria. Brazilian Dental Journal 13,
783 118-122. http://dx.doi.org/10.1590/S0103-64402002000200008
784 Ferronatto, R., Marchesan, E.D., Pezenti, E., Bednarski, F., Onofre, S.B., 2007a. Atividade
785 antimicrobiana de óleos essenciais produzidos por Baccharis dracunculifolia DC e Baccharis
786 uncinella DC (Asteraceae). Rev bras farmacogn 17, 224-230.
787 Ferronatto, R., Marchesan, E.D., Pezenti, E., Bednarski, F., Onofre, S.B., 2007b. Atividade
788 antimicrobiana de óleos essenciais produzidos por Baccharis dracunculifolia DC e Baccharis
789 uncinella DC (Asteraceae). Revista Brasileira de farmacognosia 17, 224-230.
790 http://dx.doi.org/10.1590/S0102-695X2007000200016
791 Fugita, C.A., Prado, R.M., Valero, M.V., Bonafé, E.G., Carvalho, C.B., Guerrero, A., Sañundo,
792 C., Prado, I.N., 2018. Effect of the inclusion of natural additives on animal performance and meat
793 quality of crossbred bulls (Angus vs. Nellore) finished in feedlot. Animal Production Science 58,
794 2076-2083. https://dx.doi.org/10.1071/AN16242.
795 Gandra, J., Nunes Gil, P., Gandra, E., Del Vale, T., Barletta, R., Zanferari, F., Ferreira de Jesus,
796 E., Takiya, C., Mingoti, R., Almeida, G., 2014a. Productive performance of simmental dairy cows
797 supplemented with ricinoleic acid from castor oil. Archivos de zootecnia 63, 575-585.
798 Gandra, J.R., Gil, P.C.N., Cônsolo, N.R.B., Gandra, E.R.S., Gobesso, A.A.O., 2012a. Addition of
799 increasing doses of ricinoleic acid from castor oil (*Ricinus communis* L.) in diets of Nellore steers
800 in feedlots. Journal of Animal and Feed Sciences 21, 566-576.
801 https://doi.org/10.22358/jafs/66131/2012.
802 Gandra, J.R., Gil, P.N., Cônsolo, N.R.B., Gandra, E., Gobesso, A.A.d.O., 2012b. Addition of
803 increasing doses of ricinoleic acid from castor oil (Ricinus communis L.) in diets of Nellore steers
804 in feedlots. J Anim Feed Sci 21, 566-576.
805 Gandra, J.R., Nunes Gil, P.C., Gandra, E.R.S., Del Vale, T.A., Barletta, R.V., Zanferari, F., Jesus,
806 E.F., Takiya, C.S., Mingoti, R.D., Almeida, G.F., 2014b. Productive performance of simmental

807 dairy cows supplemented with ricinoleic acid from castor oil. Archivos de zootecnia 63, 575-585.
808 http://dx.doi.org/10.4321/S0004-05922014000400002
809 Gilbert, R.A., Klieve, A.V., 2015. Ruminal viruses (bacteriophages, archaeaphages), Rumen
810 microbiology: From evolution to revolution, Springer, pp. 121-141.
811 Gómez-Caravaca, A., Gómez-Romero, M., Arráez-Román, D., Segura-Carretero, A., Fernández-
812 Gutiérrez, A., 2006a. Advances in the analysis of phenolic compounds in products derived from
813 bees. Journal of Pharmaceutical and Biomedical Analysis 41, 1220-1234.
814 Gómez-Caravaca, A.M., Gómez-Romero, M., Arráez-Román, D., Segura-Carretero, A.,
815 Fernández-Gutiérrez, A., 2006b. Advances in the analysis of phenolic compounds in products
816 derived from bees. Journal of Pharmaceutical and Biomedical Analysis 41, 1220-1234.
817 https://doi.org/10.1016/j.jpba.2006.03.002.
818 Griffith, C.L., Ribeiro Jr, G.O., Oba, M., McAllister, T.A., Beauchemin, K.A., 2016. Fermentation
819 of ammonia fiber expansion treated and untreated barley straw in a rumen simulation technique
820 using rumen inoculum from cattle with slow versus fast rate of fiber disappearance. Front.
821 Microbiol. 7, 1839. https://doi.org/10.3389/fmicb.2016.01839.
822 Gruninger, R.J., Puniya, A.K., Callaghan, T.M., Edwards, J.E., Youssef, N., Dagar, S.S.,
823 Fliegerova, K., Griffith, G.W., Forster, R., Tsang, A., 2014. Anaerobic fungi (phylum
824 Neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and
825 biotechnological potential. FEMS Microbiol. Ecol. 90, 1-17.
826 Haddad, S.G., Ata, M.A., 2009. Growth performance of lambs fed on diets varying in concentrate
827 and wheat straw. Small Rumin. Res. 81, 96-99.
828 https://doi.org/10.1016/j.smallrumres.2008.11.015.
829 Hahn-Hägerdal, B., Galbe, M., Gorwa-Grauslund, M.F., Lidén, G., Zacchi, G., 2006. Bio-ethanol–
830 the fuel of tomorrow from the residues of today. Trends Biotechnol. 24, 549-556.
831 https://doi.org/10.1016/j.tibtech.2006.10.004.
832 Hatfield, R.D., Weimer, P.J., 1995. Degradation characteristics of isolated and in situ cell wall
833 lucerne pectic polysaccharides by mixed ruminal microbes. Journal of the Science of Food and
834 Agriculture 69, 185-196. 10.1002/jsfa.2740690208.
835 Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census, C., Abecia,
836 L., Angarita, E., Aravena, P., Nora Arenas, G., Ariza, C., Attwood, G.T., Mauricio Avila, J., Avila-
837 Stagno, J., Bannink, A., Barahona, R., Batistotti, M., Bertelsen, M.F., Brown-Kav, A., Carvajal,
838 A.M., Cersosimo, L., Vieira Chaves, A., Church, J., Clipson, N., Cobos-Peralta, M.A., Cookson,
839 A.L., Cravero, S., Cristobal Carballo, O., Crosley, K., Cruz, G., Cerón Cucchi, M., de la Barra, R.,
840 De Menezes, A.B., Detmann, E., Dieho, K., Dijkstra, J., dos Reis, W.L.S., Dugan, M.E.R., Hadi
841 Ebrahimi, S., Eythórsdóttir, E., Nde Fon, F., Fraga, M., Franco, F., Friedeman, C., Fukuma, N.,
842 Gagić, D., Gangnat, I., Javier Grilli, D., Guan, L.L., Heidarian Miri, V., Hernandez-Sanabria, E.,
843 Gomez, A.X.I., Isah, O.A., Ishaq, S., Jami, E., Jelincic, J., Kantanen, J., Kelly, W.J., Kim, S.-H.,
844 Klieve, A., Kobayashi, Y., Koike, S., Kopecny, J., Nygaard Kristensen, T., Julie Krizsan, S.,
845 LaChance, H., Lachman, M., Lamberson, W.R., Lambie, S., Lassen, J., Leahy, S.C., Lee, S.-S.,
846 Leiber, F., Lewis, E., Lin, B., Lira, R., Lund, P., Macipe, E., Mamuad, L.L., Cuquetto Mantovani,
847 H., Marcoppido, G.A., Márquez, C., Martin, C., Martinez, G., Eugenia Martinez, M., Lucía
848 Mayorga, O., McAllister, T.A., McSweeney, C., Mestre, L., Minnee, E., Mitsumori, M., Mizrahi,
849 I., Molina, I., Muenger, A., Muñoz, C., Murovec, B., Newbold, J., Nsereko, V., O'Donovan, M.,
850 Okunade, S., O'Neill, B., Ospina, S., Ouwerkerk, D., Parra, D., Pereira, L.G.R., Pinares-Patiño,
851 C., Pope, P.B., Poulsen, M., Rodehutscord, M., Rodriguez, T., Saito, K., Sales, F., Sauer, C.,
852 Shingfield, K., Shoji, N., Simunek, J., Stojanović-Radić, Z., Stres, B., Sun, X., Swartz, J., Liang

Tan, Z., Tapio, I., Taxis, T.M., Tomkins, N., Ungerfeld, E., Valizadeh, R., van Adrichem, P., Van Hamme, J., Van Hoven, W., Waghorn, G., John Wallace, R., Wang, M., Waters, S.M., Keogh, K., Witzig, M., Wright, A.-D.G., Yamano, H., Yan, T., Yáñez-Ruiz, D.R., Yeoman, C.J., Zambrano, R., Zeitz, J., Zhou, M., Wei Zhou, H., Xia Zou, C., Zunino, P., Janssen, P.H., 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci. Rep-UK. 5, 14567. https://doi.org/10.1038/srep14567.

Hobson, P.N., Stewart, C.S., 2012. Rumen microbial ecosystem. Blackie Academic & Professional, Londo, UK.

Hristov, A.N., Ivan, M., Neill, L., McAllister, T., 2003. Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. Anim. Feed Sci. Technol. 105, 163-184.

Hummel, J., Scheurich, F., Ortmann, S., Crompton, L.A., Gerken, M., Clauss, M., 2018. Comparative selective retention of particle size classes in the gastrointestinal tract of ponies and goats. J. Anim. Physiol. An. N. 102, 429-439. https://doi.org/10.1111/jpn.12763.

Hungate, R.E., 1966a. The Rumen and it Microbes. Academic Press, New York.

Hungate, R.E., 1966b. The rumen and its microbes Academic Press New York and London. Academic Press, New York, Estados Unidos.

Huws, S.A., Creevey, C.J., Oyama, L.B., Mizrahi, I., Denman, S.E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S.M., Hess, M., 2018. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: Past, present, and future. Front. Microbiol. 9.

Huws, S.A., Edwards, J.E., Creevey, C.J., Rees Stevens, P., Lin, W., Girdwood, S.E., Pachebat, J.A., Kingston-Smith, A.H., 2015. Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. FEMS Microbiol. Ecol. 92, fiv137. https://doi.org/10.1093/femsec/fiv137.

Huws, S.A., Kim, E.J., Kingston-Smith, A.H., Lee, M.R., Muetzel, S.M., Cookson, A.R., Newbold, C.J., Wallace, R.J., Scollan, N.D., 2009. Rumen protozoa are rich in polyunsaturated fatty acids due to the ingestion of chloroplasts. FEMS Microbiol. Ecol. 69, 461-471.

IARC, 1999. Re-evaluation of some organic chemicals, hydrazine and hydrogen peroxide. IARC.

Ibrahim, N.A., Alimon, A., Yaakub, H., Abdullah, N., Samsudin, A., 2018. Effects of Dietary Oil Supplementation with Different Fatty Acid Profiles on Rumen Fibre Degrading Bacteria Population in Goats.

IPCC, 2007. Climate change: The physical science basis. In: Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press.

Johann, S., Oliveira, F.B., Siqueira, E.P., Cisalpino, P.S., Rosa, C.A., Alves, T.M., Zani, C.L., Cota, B.B., 2012. Activity of compounds isolated from Baccharis dracunculifolia DC (Asteraceae) against Paracoccidioides brasiliensis. Medical mycology 50, 843-851.

Jouany, J.-P., 1996. Effect of rumen protozoa on nitrogen utilization by ruminants. J. Nutr. 126, 1335S-1346S.

Jouany, J., Ushida, K., 1999. The role of protozoa in feed digestion-Review. Asian-Australas. J. Anim. Sci. 12, 113-128.

Kegley, S., Hill, B., Orme, S., Choi, A., 2014. PAN pesticide database, pesticide action network, North America (Oakland, CA).

Kingston-Smith, A.H., Bollard, A.L., Thomas, B.J., Brooks, A.E., Theodorou, M.K., 2003. Nutrient availability during the early stages of colonization of fresh forage by rumen micro-organisms. New Phytol. 158, 119-130. https://doi.org/10.1046/j.1469-8137.2003.00709.x.

Kopp, J., 2003. Using Straw in Cattle Rations-Frequently Asked Questions.

Krause, D.O., Denman, S.E., Mackie, R.I., Morrison, M., Rae, A.L., Attwood, G.T., McSweeney, C.S., 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. FEMS microbiology reviews 27, 663-693.

Krause, D.O., Russell, J.B., 1996. An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. Applied and Environmental Microbiology 62, 815-821.

Lal, R., 2005. World crop residues production and implications of its use as a biofuel. Environ. Int. 31, 575-584. https://doi.org/10.1016/j.envint.2004.09.005.

Lambert, R., Skandamis, P.N., Coote, P.J., Nychas, G.J., 2001a. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of applied microbiology 91, 453-462.

Lambert, R.J.W., Skandamis, P.N., Coote, P.J., Nychas, G.J.E., 2001b. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology 91, 453-462. https://doi.org/10.1046/j.1365-2672.2001.01428.x.

Leahy, S.C., Kelly, W.J., Altermann, E., Ronimus, R.S., Yeoman, C.J., Pacheco, D.M., Li, D., Kong, Z., McTavish, S., Sang, C., 2010. The genome sequence of the rumen methanogen Methanobrevibacter ruminantium reveals new possibilities for controlling ruminant methane emissions. PloS one 5, e8926. https://doi.org/10.1371/journal.pone.0008926.

Leng, R., 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim. Prod. Sci. 54, 519-543. https://doi.org/10.1071/AN13381.

Letarov, A., Kulikov, E., 2009. The bacteriophages in human-and animal body-associated microbial communities. Journal of applied microbiology 107, 1-13.

Liu, J., Wang, J.-K., Zhu, W., Pu, Y.-Y., Guan, L.-L., Liu, J.-X., 2014. Monitoring the rumen pectinolytic bacteria Treponema saccharophilum using real-time PCR. FEMS Microbiology Ecology 87, 576-585. 10.1111/1574-6941.12246.

Liu, J., Zhang, M., Xue, C., Zhu, W., Mao, S., 2016. Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants. J. Dairy Sci. 99, 9668-9681. https://doi.org/10.3168/jds.2016-11398.

Lozupone, C., Lladser, M.E., Knights, D., Stombaugh, J., Knight, R., 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J. 5, 169. https://doi.org/10.1038/ismej.2010.133.

Maia, M.d.O., Susin, I., Ferreira, E.M., Nolli, C.P., Gentil, R.S., Pires, A.V., Mourão, G.B., 2012a. Intake, nutrient apparent digestibility and ruminal constituents of sheep fed diets with canola, sunflower or castor oils. Revista Brasileira de Zootecnia 41, 2350-2356.

Maia, M.d.O., Susin, I., Pires, A.V., Gentil, R.S., Ferreira, E.M., Mendes, C.Q., Alencar, S.M.d., 2012b. Growth, carcass characteristics, chemical composition and fatty acid profile of the longissimus dorsi muscle in goat kids fed diets with castor oil. Revista Brasileira de Zootecnia 41, 2343-2349.

Makkar, H.P.S., Sánchez, M., Speedy, A.W., 2007. Feed supplementation blocks. Urea-molasses multi-nutrient blocks: simple and effective feed supplement technology for ruminant agriculture. FAO, Rome, Italy.

Mann, C., Markham, J., 1998. A new method for determining the minimum inhibitory concentration of essential oils. Journal of applied microbiology 84, 538-544.

Maróstica Junior, M.R., Daugsch, A., Moraes, C.S., Queiroga, C.L., Pastore, G.M., Parki, Y.K., 2008a. Comparison of volatile and polyphenolic compounds in Brazilian green propolis and its

945 botanical origin *Baccharis dracunculifolia*. Food Science and Technology 28, 178-181.
946 http://dx.doi.org/10.1590/S0101-20612008000100026
947 Maróstica Junior, M.R., Daugsch, A., Moraes, C.S., Queiroga, C.L., Pastore, G.M., Parki, Y.K.,
948 2008b. Comparison of volatile and polyphenolic compounds in Brazilian green propolis and its
949 botanical origin Baccharis dracunculifolia. Food Science and Technology (Campinas) 28, 178-
950 181.
951 Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads.
952 EMBnet. J. 17, 10-12. https://doi.org/10.14806/ej.17.1.200.
953 McGuffey, R.K., Richardson, L.F., Wilkinson, J.I.D., 2001. Ionophores for dairy cattle: current
954 status and future outlook. Journal of Dairy Science 84, E194-E203.
955 https://doi.org/10.3168/jds.S0022-0302(01)70218-4.
956 McIntosh, F., Williams, P., Losa, R., Wallace, R., Beever, D., Newbold, C., 2003a. Effects of
957 essential oils on ruminal microorganisms and their protein metabolism. Appl. Environ. Microbiol.
958 69, 5011-5014.
959 McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., Beever, D.A., Newbold, C.J., 2003b. Effects
960 of essential oils on ruminal microorganisms and their protein metabolism. Applied and
961 Environmental Microbiology 69, 5011-5014. 10.1128/aem.69.8.5011-5014.2003.
962 Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds
963 with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85, 1217-1240.
964 Millen, D.D., Arrigoni, M.D.B., Pacheco, R.D.L., 2016. Rumenology. Springer.
965 Monteschio, J.O., Souza, K.A., Vital, A.C.P., Guerrero, A., Valero, M.V., Kempinski, E.M.B.C.,
966 Barcelos, V.C., Nascimento, K.F., Prado, I.N., 2017. Clove and rosemary essential oils and
967 encapsuled active principles (eugenol, thymol and vanillin blend) on meat quality of feedlot-
968 finished heifers. Meat Science 130, 50-57. http://dx.doi.org/10.1016/j.meatsci.2017.04.002.
969 Mor, P., Bals, B., Kumar, S., Tyagi, N., Reen, J.K., Tyagi, B., Choudhury, P.K., Tyagi, A.K., 2019.
970 Influence of replacing concentrate mixture with AFEX pellets on rumen fermentation, blood
971 profile and acetamide content in the rumen of crossbred (Alpine × Beetle) female goats. Small
972 Rumin. Res. 170, 109-115. https://doi.org/10.1016/j.smallrumres.2018.10.016.
973 Mor, P., Bals, B., Tyagi, A.K., Teymouri, F., Tyagi, N., Kumar, S., Bringi, V., VandeHaar, M.,
974 2018. Effect of ammonia fiber expansion on the available energy content of wheat straw fed to
975 lactating cattle and buffalo in India. J. Dairy Sci. 101, 7990-8003.
976 https://doi.org/10.3168/jds.2018-14584.
977 Morales, E.R., Mata Espinosa, M.A., McKain, N., Wallace, R.J., 2012. Ricinoleic acid inhibits
978 methanogenesis and fatty acid biohydrogenation in ruminal digesta from sheep and in bacterial
979 cultures. Journal of Animal Science 90, 4943-4950. http://dx.doi.org/10.2527/jas2011-4670.
980 Murakami, A., Eyng, C., Torrent, J., 2014. Effects of functional oils on coccidiosis and apparent
981 metabolizable energy in broiler chickens. Asian-Australas. J. Anim. Sci. 27, 981.
982 Nagaraja, T., Taylor, M., 1987. Susceptibility and resistance of ruminal bacteria to antimicrobial
983 feed additives. Appl. Environ. Microbiol. 53, 1620-1625.
984 Nagaraja, T., Titgemeyer, E., 2007. Ruminal acidosis in beef cattle: the current microbiological
985 and nutritional outlook. J. Dairy Sci. 90, E17-E38. https://doi.org/10.3168/jds.2006-478.
986 Nagayama, H., Konno, K., Oka, S., 1961. Formamidase in mycobacteria and its use in
987 differentiating saprophytic mycobacteria from other mycobacteria. Nature 190, 1219.
988 https://doi.org/10.1038/19012.
989 Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of
990 glucose. J. biol. Chem 153, 375-380.

991 Newbold, C.J., de la Fuente, G., Belanche, A., Ramos-Morales, E., McEwan, N.R., 2015. The role
992 of ciliate protozoa in the rumen. Front. Microbiol. 6, 1313.
993 Newbold, C.J., Wallace, R.J., Walker-Bax, N.D., 2013. Potentiation by metal ions of the efficacy
994 of the ionophores, monensin and tetronasin, towards four species of ruminal bacteria. FEMS
995 Microbiology Letters 338, 161-167. http://dx.doi.org/10.1111/1574-6968.12044.
996 Newbold, C.J., Wallace, R.J., Watt, N., Richardson, A.J., 1988a. Effect of the novel ionophore
997 tetronasin (ICI 139603) on ruminal microorganisms. Appl. Environ. Microbiol. 54, 544-547.
998 Newbold, C.J., Wallace, R.J., Watt, N.D., Richardson, A.J., 1988b. Effect of the novel ionophore
999 tetronasin (ICI 139603) on ruminal microorganisms. Applied and Environmental Microbiology
1000 54, 544-547.
1001 Nikaido, H., Nakae, T., 1980. The outer membrane of Gram-negative bacteria, Advances in
1002 microbial physiology, Elsevier, pp. 163-250.
1003 Novak, A., Clark, G., Dupuy, H., 1961. Antimicrobial activity of some ricinoleic acid oleic acid
1004 derivatives. Journal of the American Oil Chemists' Society 38, 321-324.
1005 http://dx.doi.org/10.1007/BF02638439.
1006 O'Connor, J., 1971. Ammonia explosion pulping: new approach to fiber separation. Paper trade
1007 journal.
1008 Ornaghi, M.G., Guerrero, A., Vital, A.C.P., Souza, K.A., Passetti, R.A.C., Mottin, C., Castilho,
1009 R.A., Sañudo, C., Prado, I.N., 2020. Improvements in the quality of meat from beef cattle fed
1010 natural additives. Meat Science in press. https://doi.org/10.1016/j.meatsci.2020.108059.
1011 Ornaghi, M.G., Passetti, R.A.C., Torrecilhas, J.A., Mottin, C., Vital, A.C.P., Guerrero, A., Sañudo,
1012 C., del Mar Campo, M., Prado, I.N., 2017a. Essential oils in the diet of young bulls: Effect on
1013 animal performance, digestibility, temperament, feeding behaviour and carcass characteristics.
1014 Anim. Feed Sci. Technol. 234, 274-283. https://doi.org/10.1016/j.anifeedsci.2017.10.008.
1015 Ornaghi, M.G., Passetti, R.A.C., Torrecilhas, J.A., Mottin, C., Vital, A.C.P., Gurerrero, A.,
1016 Sañudo, C., Campo, M.M., Prado, I.N., 2017b. Essential oils in the diet of young bulls: Effect on
1017 animal performance, digestibility, temperament, feeding behaviour and carcass characteristics.
1018 Animal Feed Science and Technology 234, 274-283.
1019 http://dx.doi.org/10.1016/j.anifeedsci.2017.10.008.
1020 Paiva, J., Garcia, R., Queiroz, A.d., Regazzi, A., 1995. Efeitos dos níveis de amônia anidra e
1021 períodos de amonização sobre os teores dos constituintes da parede celular na palhada de milho
1022 (Zea mays L.). R. Soc. Bras. Zootec 24, 683-692.
1023 Parreira, N.A., Magalhães, L.G., Morais, D.R., Caixeta, S.C., de Sousa, J.P., Bastos, J.K., Cunha,
1024 W.R., Silva, M.L., Nanayakkara, N., Rodrigues, V., 2010. Antiprotozoal, schistosomicidal, and
1025 antimicrobial activities of the essential oil from the leaves of Baccharis dracunculifolia. Chemistry
1026 & biodiversity 7, 993-1001.
1027 Pell, A.N., Schofield, P., 1993. Microbial adhesion and degradation of plant cell walls. Forage cell
1028 wall structure and digestibility, 397-423.
1029 Petri, R.M., Schwaiger, T., Penner, G.B., Beauchemin, K.A., Forster, R.J., McKinnon, J.J.,
1030 McAllister, T.A., 2013. Characterization of the core rumen microbiome in cattle during transition
1031 from forage to concentrate as well as during and after an acidotic challenge. PloS one 8, e83424.
1032 https://doi.org/10.1371/journal.pone.0083424.
1033 Prado, I., Cruz, O., Valero, M., Zawadzki, F., Eiras, C., Rivaroli, D., Prado, R., Visentainer, J.,
1034 2016. Effects of glycerin and essential oils (Anacardium occidentale and Ricinus communis) on
1035 the meat quality of crossbred bulls finished in a feedlot. Anim. Prod. Sci. 56, 2105-2114.

1036 Prado, O.P.P., Zeoula, L.M., Moura, L.P.P., Franco, S.L., Paiva, S.B., Arcuri, P.B., 2010. Isolation
1037 and expeditious morphological, biochemical and kinetic characterization of propolis-tolerant
1038 ruminal bacteria. Revista Brasileira de Zootecnia 39, 2048-2054.
1039 Pressman, B.C., 1976. Biological applications of ionophores. Annual review of biochemistry 45,
1040 501-530.
1041 Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2–approximately maximum-likelihood trees
1042 for large alignments. PloS one 5, e9490. https://doi.org/10.1371/journal.pone.0009490.
1043 Putcha, L., Griffith, D.P., Feldman, S., 1984. Disposition of 14C-acetohydroxamic acid and 14C-
1044 acetamide in the rat. Drug Metab. Dispos. 12, 438-443.
1045 Radović, J., Sokolović, D., Marković, J., 2009. Alfalfa-most important perennial forage legume in
1046 animal husbandry. Biotech. Anim. Husb. 25, 465-475. https://doi.org/10.2298/BAH0906465R.
1047 Ralph, J., Helm, R., 1993. Forage Cell Wall Structure and Digestibility, Madison.
1048 Ransom-Jones, E., Jones, D.L., McCarthy, A.J., McDonald, J.E., 2012. The Fibrobacteres: an
1049 Important Phylum of Cellulose-Degrading Bacteria. Microbial Ecol. 63, 267-281.
1050 https://doi.org/10.1007/s00248-011-9998-1.
1051 Rezaeian, M., Beakes, G.W., Parker, D.S., 2004. Distribution and estimation of anaerobic
1052 zoosporic fungi along the digestive tracts of sheep. Mycological research 108, 1227-1233.
1053 Ribeiro, G.O., Gruninger, R.J., Jones, D.R., Beauchemin, K.A., Yang, W., Wang, Y., Abbott,
1054 D.W., Tsang, A., McAllister, T.A., 2019. Effect of ammonia fiber expansion (AFEX) treated
1055 wheat straw and a recombinant fibrolytic enzyme on rumen microbiota and fermentation
1056 parameters, total tract digestibility, and performance of lambs. EAAP. Public 138, 169 - 170.
1057 https://doi.org/10.3920/978-90-8686-891-9_24.
1058 Rivaroli, D.C., Ornaghi, M.G., Mottin, C., Prado, R.M., Ramos, T.R., Guerrero, A., Jorge, A.M.,
1059 Prado, I.N., 2017. Essential oils in the diet of crossbred (½ Angus vs. ½ Nellore) bulls finished in
1060 feedlot on animal performance, feed efficiency and carcass characteristics. Journal of Agricultural
1061 Science 9, 205-212. http://dx.doi.org/10.5539/jas.v9n10p205-212.
1062 Robazza, W.S., Teleken, J.T., Gomes, G.A., 2010. Modelagem Matemática do Crescimento de
1063 Microrganismos em Alimentos. 2010 11, 10. 10.5540/tema.2010.011.01.0101.
1064 Russell, J.B., 2002. Rumen microbiology an its role in ruminant nutrition. Cornell University, New
1065 York.
1066 Russell, J.B., Hino, T., 1985. Regulation of lactate production in Streptococcus bovis: a spiraling
1067 effect that contributes to rumen acidosis. Journal of Dairy Science 68, 1712-1721.
1068 Sarkanen, K.V., Ludwig, C.H., 1971. Liguins. Occurrence, formation, structure, and reactions.
1069 Sarnklong, C., Cone, J.W., Pellikaan, W., Hendriks, W.H., 2010. Utilization of Rice Straw and
1070 Different Treatments to Improve Its Feed Value for Ruminants: A Review. Asian-Australas. J.
1071 Anim. Sci. 23, 680-692. https://doi.org/10.5713/ajas.2010.80619.
1072 Schäberle, T.F., Hack, I.M., 2014. Overcoming the current deadlock in antibiotic research. Trends
1073 in Microbiology 22, 165-167. http://dx.doi.org/10.1016/j.tim.2013.12.007.
1074 Silva, L.G.d., Torrecilhas, J.A., Ornaghi, M.G., Eiras, C.E., Prado, R.M.d., Prado, I.N.d., 2014.
1075 Glycerin and essential oils in the diet of Nellore bulls finished in feedlot: animal performance and
1076 apparent digestibility. Acta Scientiarum. Animal Sciences 36, 177-184.
1077 Silva, R.R., Prado, I.N., Carvalho, G.G.P., Silva, F.F., Almeida, V.V.S., Santana Júnior, H.A.,
1078 Paixão, M.L., Abreu Filho, G., 2010. Níveis de suplementação na terminação de novilhos Nelore
1079 em pastagens: aspectos econômicos. Revista Brasileira de Zootecnia 39, 2091-2097.
1080 Somogyi, M., 1952. Notes on sugar determination. J. Biol. Chem. 195, 19-23.

Souza, K.A., 2020. Leaves of Baccharis dracunculifolia added in the diets of steers finished in feedlot, effect on performance and immune response.
Souza, K.A., Monteschio, J.O., Mottin, C., Ramos, T.R., Pinto, L.A.M., Eiras, C.E., Guerrero, A., Prado, I.N., 2019. Effects of diet supplementation with clove and rosemary essential oils and protected oils (eugenol, thymol and vanillin) on animal performance, carcas characteristics, digestibility, and behavior activities for Nellore heifers finished in feedlot. Livestock Science 220, 190-195. http://dx.doi.org/10.1016/j.livsci.2018.12.026.
Team, R.C., 2018. R: A Language and Environment for Statistical Computing.
Valero, M.V., Prado, R.M., Zawadzki, F., Eiras, C.E., Madrona, G.S., Prado, I.N., 2014a. Propolis and essential oils additives in the diets improved animal performance and feed efficiency of bulls finished in feedlot. Acta Scientiarum. Animal Sciences 36, 419-426. http://dx.doi.org/10.4025/actascianimsci.v36i4.23856.
Valero, M.V., Prado, R.M.d., Zawadzki, F., Eiras, C.E., Madrona, G.S., Prado, I.N.d., 2014b. Propolis and essential oils additives in the diets improved animal performance and feed efficiency of bulls finished in feedlot. Acta Scientiarum. Animal Sciences 36, 419-426.
Van Keulen, J., Young, B., 1977. Evaluation of Acid-Insoluble Ash as a Natural Marker in Ruminant Digestibility Studies 1, 2. J. Anim. Sci. 44, 282-287. https://doi.org/10.2527/jas1977.442282x.
Van Nevel, C., Demeyer, D., Henderickx, H., 1971. Effect of fatty acid derivatives on rumen methane and propionate in vitro. Appl. Environ. Microbiol. 21, 365-366.
Van Os, M., Dulphy, J., Baumont, R., 1995. The effect of protein degradation products in grass silages on feed intake and intake behaviour in sheep. Brit. J. Nutr. 73, 51-64.

https://doi.org/10.1079/BJN19950008.
Vismeh, R., Haddad, D., Moore, J., Nielson, C., Bals, B., Campbell, T., Julian, A., Teymouri, F., Jones, A.D., Bringi, V., 2017. Exposure Assessment of Acetamide in Milk, Beef, and Coffee Using Xanthydrol Derivatization and Gas Chromatography/Mass Spectrometry. J. Agr. Food Chem. 66, 298-305. https://doi.org/10.1021/acs.jafc.7b02229.
Vogel, K.P., Pedersen, J.F., Masterson, S.D., Toy, J.J., 1999. Evaluation of a filter bag system for NDF, ADF, and IVDMD forage analysis. Crop Sci. 39, 276-279. https://doi.org/10.2135/cropsci1999.0011183X003900010042x.
Wallace, R.J., McEwan, N.R., McIntosh, F.M., Teferedegne, B., Newbold, C.J., 2002. Natural Products as Manipulators of Rumen Fermentation. Asian-Australas J Anim Sci 15, 1458-1468. 10.5713/ajas.2002.1458.
Wallace, R.J., McKain, N., 1991. A survey of peptidase activity in rumen bacteria. Microbiology 137, 2259-2264. https://doi.org/10.1099/00221287-137-9-2259.
Wang, C., Dong, D., Wang, H., Müller, K., Qin, Y., Wang, H., Wu, W., 2016. Metagenomic analysis of microbial consortia enriched from compost: new insights into the role of Actinobacteria in lignocellulose decomposition. Biotechnol. Biofuels 9, 22. https://doi.org/10.1186/s13068-016-0440-2.
Wang, Y., Majak, W., McAllister, T.A., 2012. Frothy bloat in ruminants: Cause, occurrence, and mitigation strategies. Anim. Feed Sci. Technol. 172, 103-114. https://doi.org/10.1016/j.anifeedsci.2011.12.012.
Welch, J.G., 1986. Physical Parameters of Fiber Affecting Passage from the Rumen1. J. Dairy Sci. 69, 2750-2754. https://doi.org/10.3168/jds.S0022-0302(86)80723-8.

Weld, K., Armentano, L., 2017. The effects of adding fat to diets of lactating dairy cows on total-tract neutral detergent fiber digestibility: A meta-analysis. Journal of dairy science 100, 1766-1779.

Williams, G.M., 1980. The pathogenesis of rat liver cancer caused by chemical carcinogens. BBA-Rev. Cancer 605, 167-189. https://doi.org/10.1016/0304-419X(80)90003-7.

Wishart, D.S., Jewison, T., Guo, A.C., Wilson, M., Knox, C., Liu, Y., Djoumbou, Y., Mandal, R., Aziat, F., Dong, E., 2012. HMDB 3.0—the human metabolome database in 2013. Nucleic Acids Research 41, D801-D807. http://dx.doi.org/10.1093/nar/gks1065.

YongKun, P., Ikegaki, M., de Alencar, S., de Moura, F., 2000. Evaluation of Brazilian propolis by both physicochemical methods and biological activity. Honeybee Science 21, 85-90.

Zawadzki, F., Prado, I.N., Marques, J.A., Zeoula, L.M., Rotta, P.P., Sestari, B.B., Valero, M.V., Rivaroli, D.C., 2011. Sodium monensin or propolis extract in the diets of feedlot-finished bulls: effects on animal performance and carcass characteristics. Journal of Animal and Feed Sciences 20, 16-25. https://doi.org/10.22358/jafs/66153/2011.

Zhong, R.Z., Fang, Y., Zhou, D.W., Sun, X.Z., Zhou, C.S., He, Y.Q., 2018. Pelleted total mixed ration improves growth performance of fattening lambs. Anim. Feed Sci. Technol. 242, 127-134. https://doi.org/10.1016/j.anifeedsci.2018.06.008.

1144 **Table 1.** Ingredients and chemical composition (g/kg of DM) of diets fed to finishing lambs

	ALF[1]	RS[2]	ARS[3]
Alfafa	250.0	0.0	0.0
Rice straw	0.0	250.0	0.0
Rice straw AFEX[4]	0.0	0.0	250.0
Canola meal	10.0	10.0	10.0
Canola oil	10.0	10.0	10.0
Barley	150.0	150.0	150.0
Beet pulp	424.0	414.0	422.0
DDGS corn	115.0	115.0	115.0
Molasses (sugar beet)	20.0	20.0	20.0
Mineral sheep[5]	10.0	10.0	10.0
Chloride ammonia	5.0	5.0	5.0
Urea	0.0	10.0	3.0
Dical	3.0	3.0	3.0
Calcium carbonate	3.0	3.0	3.0
Vitamin ADE	0.0	0.0	0.0
Bovatec[6]	0.2	0.2	0.2
DM	942 ± 3.1	934 ± 1.0	938 ± 1.7
OM	856 ± 3.7	837 ± 1.3	841 ± 0.4
CP	161 ± 8.8	159 ± 3.6	155 ± 3.3
Starch	77 ± 11.5	70 ± 7.4	97 ± 13.9
aNDF	344 ± 10.7	402 ± 28.2	334 ± 17.2
ADF	227 ± 19.3	253 ± 11.7	245 ± 13.7

1145 [1]ALF = 25% alfalfa; [2]RS = 25% of rice straw; [3]ARS = 25% AFEX-treated rice straw;[4]Ammonia Fiber Expansion [5]White salt,

1146 dynamite, zinc sulfate, manganese sulfate, Ethylenediamine Dihydriodide (80%), selenium premix 1% cobalt carbonate, canola

1147 oil; [6]Lasolacid 20%

Table2. *In situ* DM and NDF degradation (g/kg) of alfalfa, rice straw and ammonia fiber expansion (AFEX) treated rice straw .

	Alfalfa	Rice straw	AFEX rice straw	SEM	P-value
DM					
A	357.7c	176.0a	289.2b	8.67	0.001
B	397.7b	244.8a	654.4c	12.14	0.001
A+B	602.5b	573.7a	943.7c	5.70	0.001
Kd	0.80c	0.20a	0.40b	0.040	0.001
Ed (0.02/h)	551.4b	388.7a	735.2c	8.38	0.001
Ed (0.04/h)	518.0b	321.4a	627.9c	9.52	0.001
Ed (0.06/h)	494.5b	286.5a	562.3c	9.56	0.001
lag time	0.778	0.516	0.589	0.427	0.907
NDF					
A	272.6c	136.7a	216.5b	5.02	0.001
B	239.4a	287.1b	494.7c	9.31	0.001
A+B	512.0b	423.8a	711.2c	5.34	0.001
Kd	0.70b	0.30a	0.40a	0.050	0.001
Ed (0.02/h)	459.2b	301.6a	547.5c	3.24	0.001
Ed (0.04/h)	425.7b	252.5a	465.2c	3.56	0.001
Ed (0.06/h)	402.4b	226.0a	415.6b	3.88	0.001
lag time	0.895	0.445	0.921	0.278	0.372

A = the soluble fraction (%), B = the slowly degradable fraction (%), A+B the potential degradable fraction, Ed = Effective ruminal disappearance (Ed). Different letters means difference at LSD<0.05

1152 Table 3. Microbial profiles of bacteria colonizing alfalfa, rice straw and ammonia fiber expansion (AFEX)
1153 treated rice straw after 1, 4, 8 and 48 h of incubation in the rumen.

		Alfalfa	Rice straw	AFEX rice straw	SEM	P-value
1 h						
	Firmicutes	36.71	42.74	36.56	1.485	0.085
	Bacteroidetes	42.92b	33.41a	45.34b	2.230	0.014
	Euryarchaeota	3.76b	4.82c	2.54a	0.417	0.003
	Spirochaetae	2.55a	3.86b	3.31ab	0.204	0.014
	Proteobacteria	2.56b	2.63b	1.32a	0.274	0.032
	Absconditabacteria	1.17	1.10	1.49	0.490	0.409
	Fibrobacteres	4.15	5.19	4.42	0.699	0.118
	Actinobacteria	1.53b	1.40b	0.52a	0.174	0.002
	Verrucomicrobia	1.44	1.54	1.58	0.053	0.648
	Cyanobacteria	0.66	0.57	0.70	0.166	0.697
	Tenericutes	1.05	0.81	0.96	0.087	0.483
	Planctomycetes	0.69	0.79	0.46	0.107	0.069
	Chloroflexi	0.32	0.48	0.31	0.061	0.182
	Synergistetes	0.16	0.21	0.16	0.018	0.558
	Elusimicrobia	0.13	0.15	0.1	0.018	0.313
	Others	0.20	0.31	0.25	0.050	0.056
4 h						
	Firmicutes	35.44a	41.29b	31.90a	1.470	0.004
	Bacteroidetes	42.80b	34.91a	46.55c	2.058	0.001
	Euryarchaeota	4.11b	4.63b	2.30a	0.510	0.014
	Spirochaetae	2.99a	4.19ab	5.60b	0.417	0.010
	Proteobacteria	1.25a	1.86b	1.23a	0.120	0.025
	Absconditabacteria	1.09	0.96	1.37	0.470	0.373
	Fibrobacteres	5.93	6.44	7.00	1.465	0.331
	Actinobacteria	0.77b	1.00c	0.25a	0.112	<0.001
	Verrucomicrobia	1.13	1.14	1.08	0.026	0.620
	Cyanobacteria	0.59	0.45	0.65	0.165	0.276
	Tenericutes	2.21b	1.32ab	0.73a	0.239	0.020
	Planctomycetes	0.75	0.65	0.56	0.099	0.452
	Chloroflexi	0.46	0.49	0.29	0.102	0.206
	Synergistetes	0.24	0.26	0.21	0.017	0.498
	Elusimicrobia	0.12	0.18	0.10	0.035	0.426
	Others	0.14	0.24	0.17	0.043	0.323
8 h						
	Firmicutes	40.72ab	42.95b	37.04a	1.046	0.037
	Bacteroidetes	34.39a	32.46a	39.00b	1.399	0.004
	Euryarchaeota	4.95b	4.95b	3.30a	0.352	0.021
	Spirochaetae	4.99a	5.23a	7.89b	0.525	0.003

	Proteobacteria	2.49	1.91	2.21	0.336	0.181
	Absconditabacteria	1.00	1.04	1.04	0.385	0.773
	Fibrobacteres	5.25	6.86	6.04	0.996	0.545
	Actinobacteria	0.89b	0.77b	0.38a	0.079	<0.001
	Verrucomicrobia	1.00ab	0.85a	1.45b	0.052	0.043
	Cyanobacteria	0.53	0.48	0.57	0.150	0.585
	Tenericutes	1.80b	1.10a	0.67a	0.170	0.002
	Planctomycetes	0.72b	0.50a	0.59ab	0.096	0.025
	Chloroflexi	0.40	0.37	0.31	0.071	0.380
	Synergistetes	0.29b	0.27b	0.21a	0.015	0.019
	Elusimicrobia	0.09	0.11	0.06	0.017	0.051
	Others	0.11	0.16	0.13	0.028	0.334
48 h						
	Firmicutes	44.01	41.91	36.36	1.775	0.104
	Bacteroidetes	32.11	31.16	30.51	1.416	0.759
	Euryarchaeota	6.68	6.05	4.56	0.770	0.141
	Spirochaetae	3.22	5.68	14.01	2.321	0.139
	Proteobacteria	1.21	1.00	1.42	0.118	0.181
	Absconditabacteria	1.24	0.95	1.34	0.456	0.328
	Fibrobacteres	3.88a	6.74b	6.37ab	0.569	0.032
	Actinobacteria	1.12b	0.86b	0.43a	0.104	0.002
	Verrucomicrobia	1.77	1.40	1.90	0.125	0.308
	Cyanobacteria	0.66	0.50	0.78	0.203	0.572
	Tenericutes	1.07	0.50	0.58	0.111	0.068
	Planctomycetes	1.49	1.23	1.09	0.208	0.339
	Chloroflexi	0.64	0.47	0.42	0.098	0.130
	Synergistetes	0.50b	0.37ab	0.30a	0.063	0.045
	Elusimicrobia	0.17	0.15	0.16	0.033	0.949
	Others	0.22a	0.54b	0.25ab	0.083	0.039

1154 Different letters means difference at LSD<0.05

Table 4. Performance, ruminal pH and nutrient digestibility in lambs fed diets containing alfalfa, rice straw or ammonia fiber expansion (AFEX) treated rice straw diets

	Diets				
	ALF[1]	RS[2]	ARS[3]	SEM	P-value
Initial BW (kg)	36.86	36.75	37.38	0.551	0.881
Final BW (kg)	52.71	52.90	51.12	0.617	0.419
ADG (kg)	0.363b	0.378b	0.301a	0.017	0.044
DMI(kg)	1.718ab	1.824b	1.665a	0.017	<0.001
DMI (%BW)	3.942ab	4.051b	3.767a	0.028	0.020
Feed efficiency (G:F)	0.200b	0.203b	0.179a	0.009	0.001
HCW (kg)	24.41	24.15	23.31	0.343	0.365
HC dressing (%)	46.30	45.58	45.61	0.436	0.534
Grade rule (mm)	17.50	17.88	17.60	0.259	0.188
Rumen pH					
Mean	6.46a	6.95b	6.76ab	0.068	0.032
Min	5.58	6.57	6.02		
Max	7.15	7.33	7.35		
Coefficient of digestibility					
DM	0.821b	0.667a	0.665a	0.012	<0.001
OM	0.838b	0.698a	0.703a	0.011	<0.001
CP	0.782c	0.678b	0.564a	0.016	<0.001
aNDF	0.735c	0.521a	0.578b	0.016	<0.001
ADF	0.726c	0.533a	0.593b	0.015	<0.001
Starch	0.992b	0.983a	0.986b	0.001	0.024

Different letters means difference at LSD P <0.05. [1]ALF = 25% alfalfa; [2]RS = 25% of rice straw; [3]ARS = 25% AFEX-treated rice straw

Table 5. Acetamide content in blood and diaphragm muscle tissue of lambs fed with or without ammonia fiber expansion (AFEX) treated rice straw

	Diets					
Acetamide (ppm)	ALF[1]	RS[2]	ARS[3]	ARSW[4]	SEM	*P-value*
Plasma (day 0)	0.64a	0.77ab	0.85ab	0.90b	0.033	0.004
Plasma (day 49)	0.94a	2.13a	18.93b	1.79a	1.559	<0.001
Diaphragm	0.83a	0.70a	2.66b	2.14ab	0.279	0.022
	Acetamide in blood (ppm) during withdraw period					
AFEX diets	1st d	3rd d	5th d	7th d	SEM	*P-value*
ARSW	46.75c	17.14b	1.83a	1.79a	4.310	0.006
ARS	44.176	NA*	52.17	18.93	7.949	0.152

Different letters means difference at LSD P <0.05. [1]ALF = 25% alfalfa; [2]RS = 25% of rice straw; [3]ARS = 25% AFEX-treated rice straw (N = 10) and [4]ARSW which was the same diet as "3" but with AFEX straw removed from the diet 7 d prior to slaughter, NA = not analyzed.

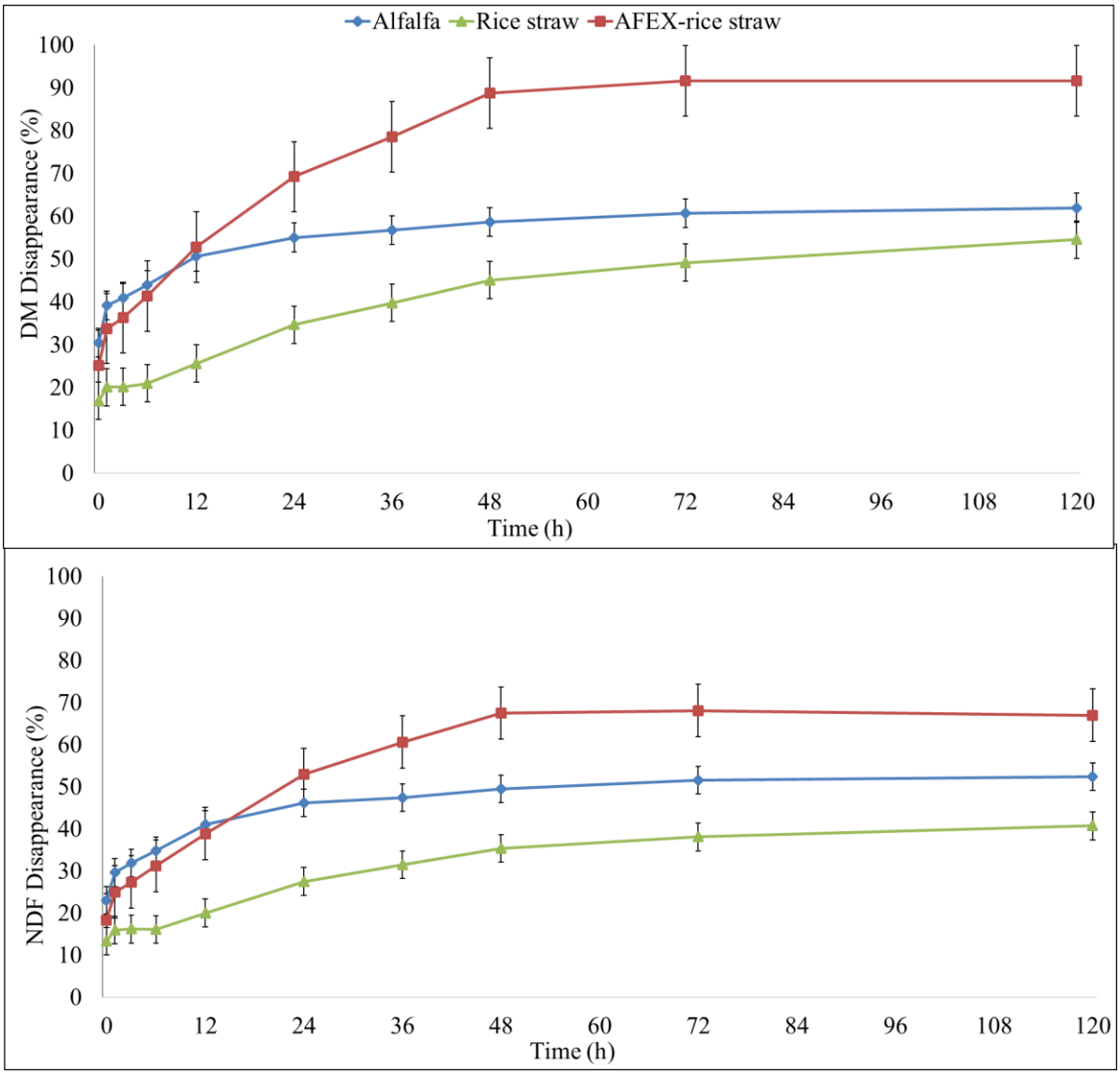

Figure 1. DM and NDF disappearance of substrates until 120 h of in situ incubation.

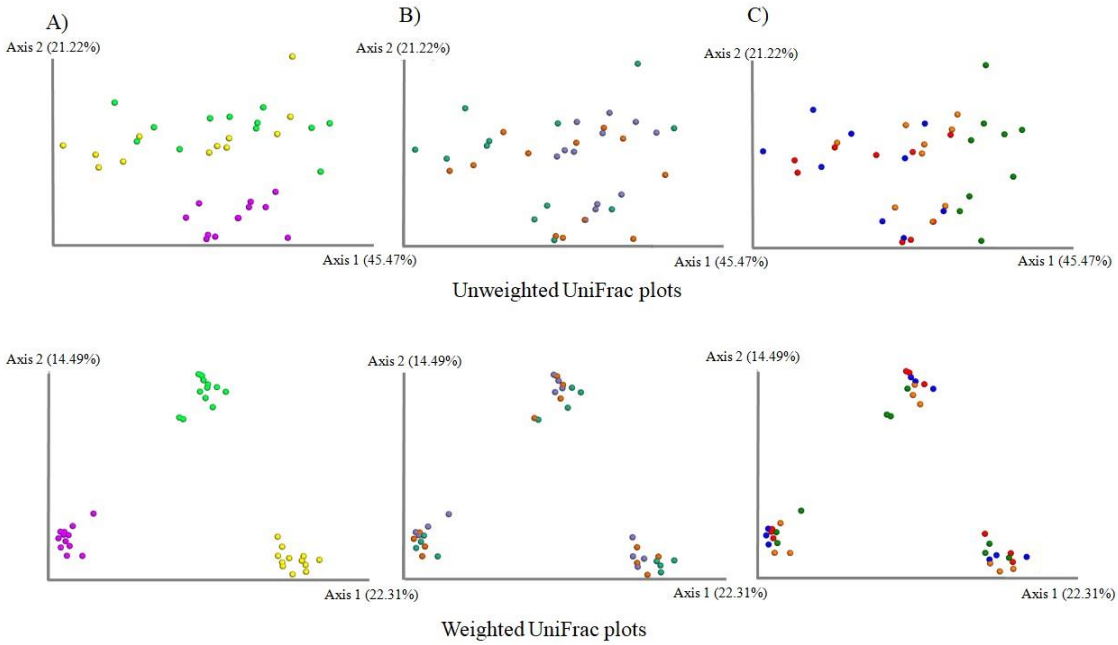

Figure 2. Weighted and unweighted unifrac plots for: a = Heifer (green = #4, magenta = #14, yellow = #18;);b = Forage (rice straw – Purple, Red-Orange alfalfa, Teal – AFEX rice straw) and c = Incubation time (1 h, Blue – 4 h, Orange – 8h, dark green – 48h).

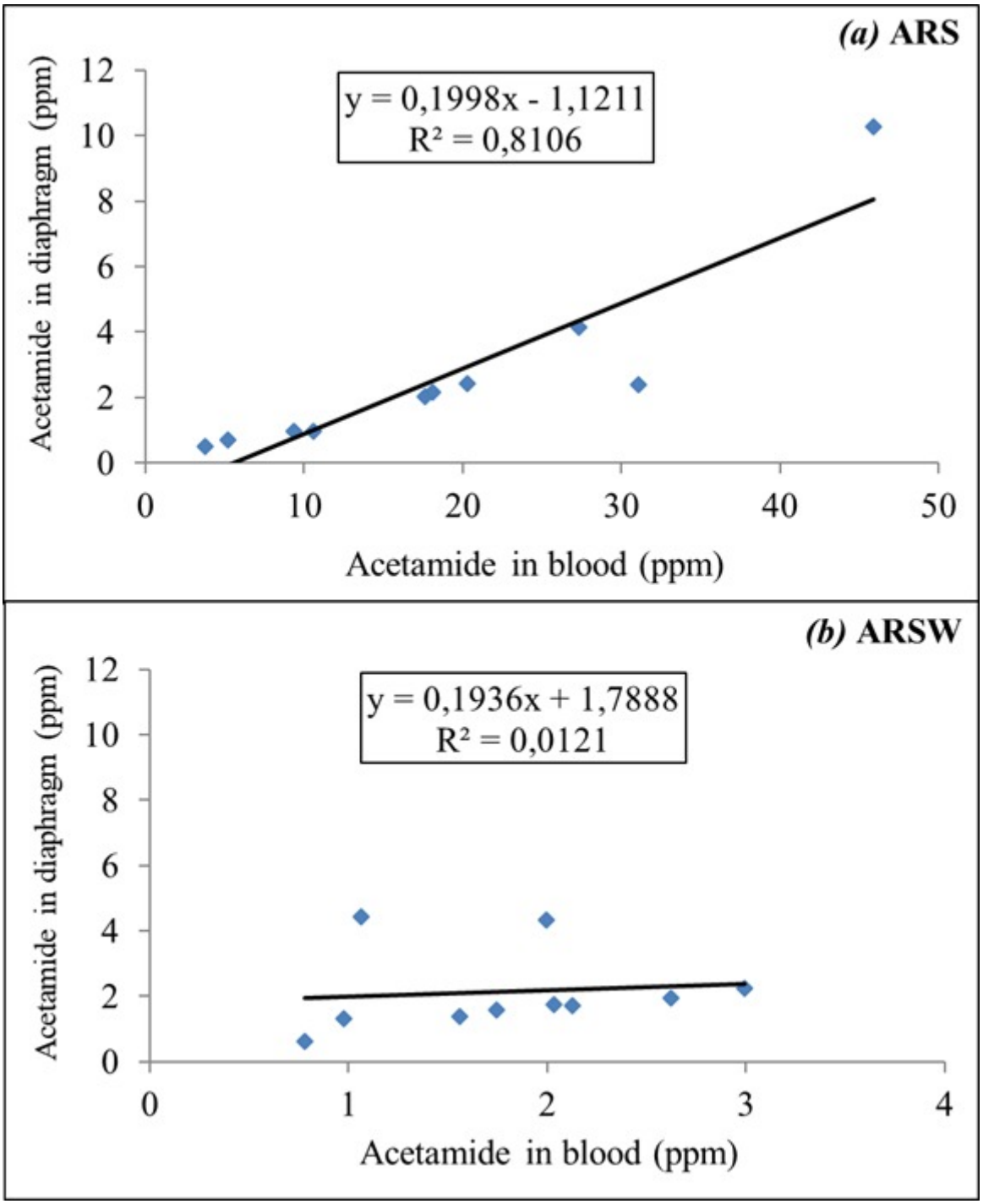

Figure 3. Correlation between acetamide content in diaphragm and blood of lambs after 48 days in feedlot.

V - CONSIDERAÇÕES FINAIS

A monensina é um ionophoro que apresenta potencial para modular fermentação ruminal, devido a sua capacidade de inibir o crescimento de bactérias gram positivas. Entretanto a sua utilização de forma incorreta pode causar prejuízos uma vez que dosagens acima de 20 mg/L passam a ser tóxicas as bactérias. O óleo de orégano apesar de se mostrar eficaz contra bactérias de interesse na modulação da fermentação ruminal como a *Streptococcus bovis*, apresenta uma menor seletividade no espectro de ação dos micro-organismos ruminais. Isso é devido a seus compostos de baixo peso molecular (timol e carvacrol) que são capazes de penetrar e interagir com a membrana de bactérias gram negativas. Outros produtos por outro lado como o óleo de mamona apresentaram potencial de seletividade ao conseguir inibir algumas espécies de bactérias gram positivas, entretanto esta ação teve um efeito marginal, que não se prolongou após 24 horas de incubação. Apesar de estudos anteriores demonstrarem a ação da *Baccharis dracunculifolia* em inibir bactérias aeróbicas, em nosso estudo as concentrações utilizadas não afetaram o crescimento das bactérias ruminais, possivelmente concentrações acima de 200 mg/L são necessários para atuar nestes micro-organismos.

No segundo estudo podemos observar que o AFEX parece ser uma tecnologia bastante promissora, pois aumenta significativamente a digestibilidade da palha de arroz. Isso se deve a uma abertura da parede celular que facilitan o acesso a carboidratos pelos micro-orgnismos. Por outro lado seu uso na alimentação de animais ainda precisa ser melhor elucidado, uma vez que a inclusão deste alimento a 25 % da dieta não refletiu diretamente em melhorias no desempenho e a eficiência alimentar destes animais. Acetamina no sangue rapidamente retornou aos níveis basais após 3 dias de retirada de dietas contendo AFEX, mas períodos mais longos são necessários para se observar este

efeito no músculo. Por outro lado a carne de animais alimentados com palhas tratadas com AFEX não apresenta riscos diretos a saúde humana uma vez que os níveis observados (2,66 ppm) estão muito abaixo dos níveis considerados cancerígenos em ratos (7.000 ppm).

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Effect of ammonia fibre expansion (AFEX) treatment of rice straw on *in situ* digestibility, microbial colonization, acetamide levels and growth performance of lambs

Rodrigo A.C. Passetti[a,*], Ludmila C.G. Passetti[b], Rob J. Gruninger[c], Gabriel O. Ribeiro[d], Mohammed R. Marami Milani[c], Ivanor N. Prado[a], Tim A. McAllister[c,*]

[a] Departamento de Zootecnia, Universidade Estadual de Maringá, Maringá, Paraná, 87020-900, Brazil
[b] Departamento de Zootecnia, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Campus de Unaí, Unaí, Minas Gerais, 38610-000, Brazil
[c] Lethbridge Research and Development Centre, Agriculture and Agri-Food Canada, Lethbridge, Alberta, T1J 4B1, Canada
[d] Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 5A8, Canada

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ABSTRACT

The objective of this study was to evaluate the effect of AFEX treatment (ARS) of rice straw (RS) on the *in situ* degradability, microbial colonization, growth performance and acetamide levels in ewe lambs. Alfalfa, rice straw and AFEX-treated rice straw were incubated in nylon bags in the rumen for 0, 1, 3, 6, 12, 24, 36, 48, 72 and 120 h to determine DM and NDF disappearance kinetics. Sequencing of 16S rRNA was used to characterize colonizing bacterial and archaeal profiles. Lambs (N = 40; 37.1 ± 3.5 kg) were fed pelleted diets that contained: 1) ALF = 250 g/kg of alfalfa; 2) RS = 250 g/kg of rice straw; 3) ARS = 250 g/kg of AFEX rice straw; 4) ARSW = ARS withdrawn from the diet 7 d before slaughter. Blood samples were collected bi-weekly and after ARSW on d 1, 3, 5 and 7 and at slaughter, the diaphragm muscle was used for measurement of acetamide. Alfalfa had greater Kd and A fraction ($P < 0.05$), whereas ARS had higher ($P < 0.05$) B and A + B fractions. Alfalfa DM and NDF degradability was greater at 12 h, but lower than ARS thereafter. Effective ruminal degradability (Ed) at 0.02, 0.04 and 0.06/h was greater ($P < 0.05$) for ARS than other forages. Digestion of ALF and ARS plateaued after 48 h, while RS continued to be degraded. Compared to other forages, alpha and beta microbial diversity of ARS was reduced ($P < 0.05$). The phylogenetic profile of initial colonizers of ARS was more similar to ALF than RS and was dominated by *Bacteroidetes*. Lambs fed RS exhibited similar growth to those fed ALF, while the DMI of ARS lambs was similar, but ADG and feed efficiency were reduced ($P < 0.05$). ALF exhibited greater ($P < 0.05$) DM, OM, CP, NDF, ADF and starch digestibility than ARS. ARS exhibited lower CP, but higher NDF and ADF digestibility than RS. A strong correlation ($R^2 = 0.81$) was observed between blood and muscle acetamide levels in lambs fed ARS. Withdrawal of ARSW reduced ($P < 0.05$) blood acetamide levels after 3 d, but levels in the diaphragm remained similar to ARS lambs at slaughter. Although AFEX improved the NDF

Abbreviations: A, soluble fraction; A + B, total potentially degradable fraction; AFEX, ammonia fibre expansion; ALF, diet containing 25 % alfalfa; aNDF, neutral detergent fibre using heat-stable amylase and sodium sulfite; ARS, diet containing 25 % AFEX treated rice straw; ARSW, same diet as ARS but with AFEX straw removed from the diet 7 d prior to slaughter; B, potentially degradable fraction; ED, effective rumen degradable proportion; Kd, fractional rate of degradation of fraction B; Kp, fractional particle outflow rate from the rumen; RS, diet containing 25 % rice straw

* Corresponding authors.
E-mail addresses: racpassetti@gmail.com (R.A.C. Passetti), tim.mcallister@canada.ca (T.A. McAllister).

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