UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

ADITIVO NATURAL COMPOSTO POR *Baccharis dracunculifolia*, *Tamarindus indica* L., ÓLEO ESSENCIAL DE CRAVO E LÍQUIDO DA CASTANHA DE CAJU SOBRE O STATUS OXIDATIVO E MODULAÇÃO RUMINAL DE BOVINOS TERMINADOS EM CONFINAMENTO

Autor: Vicente Alfonso Díaz Ávila
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TITULAÇÃO: Doutor em Zootecnia - Área de Concentração Produção Animal

APROVADO em 11 de dezembro de 2020.

Profª Drª Magali Soares dos Santos Pozza

Prof. Dr. Hilário Cuquetto Mantovani

Porf. Dr. Roman David Castañeda Serrano

Dr. Luiz Fernando Costa e Silva

“A hero can be anyone. Even a man doing something as simple and reassuring as putting a coat around a young boy's shoulders to let him know that the world hadn't ended”.

(Bruce Wayne)

“Speak only when your words are more beautiful than the silence”.

(Arabic proverb)

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A minha irmã María Paula e minha sobrinha Alicia,

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A minha parceira da vida Zaira,

A toda a minha família (primos, tios e tias)

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BIOGRAFIA

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Em dezembro de 2000, concluiu o ensino médio na instituição Colegio San Simón, na cidade de Ibagué na Colômbia.

Em setembro de 2008, concluiu o curso de Medicina Veterinária e Zootecnia na Universidad del Tolima em Ibagué – Tolima (COL).

Em fevereiro de 2011, matriculou-se no Programa de Pós-Graduação em Ciências Pecuárias, em nível de Mestrado, área de concentração Produção de Ruminantes, na Universidad del Tolima em Ibagué – Tolima (COL).

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No dia 13 de dezembro de 2019, foi aprovado no Exame Geral de Qualificação.

No dia 11 de dezembro de 2020, submeteu-se à Defesa de Tese junto ao Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá.

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RESUMO

Foram realizados dois experimentos para analisar o efeito dos compostos presentes no extrato vegetal de folhas e caules de baccharis (*Baccharis dracunculifolia),* sementes de tamarindo *(Tamarindus indica L.),* óleo essencial da folha de cravo (*Syzygium aromaticum*), líquido da castanha do caju (LCC) (*Anacardium occidentale*) sobre o estado oxidativo e modulação ruminal de bovinos mestiços (½ Angus *vs.* ½ Nelore) terminados em confinamento. No primeiro experimento, avaliou-se a atividade dos extratos vegetais de folhas e caules de baccharis, sementes de tamarindo, óleo essencial da folha de cravo e LCC sobre o status oxidativo de bovinos terminados com dietas de alto teor de grãos em confinamento. Os bovinos foram distribuídos em quatro tratamentos; CONT – dieta basal; MIX2 – dieta basal mais 2 g/animal/d do extrato vegetal; MIX4 –dieta basal mais 4 g/animal/d do extrato vegetal; MIX6 – dieta basal mais 6 g/animal/d do extrato vegetal. Foram coletadas amostras de sangue em todos os animais. Os níveis dos grupos das proteínas carbonila foram baixos no plasma dos bois suplementados com o MIX. Apresentou aumento na habilidade da redução do ferro no plasma (FRAP) dos bovinos suplementados com o MIX6. Da mesma forma, os níveis das substâncias reativas ao ácido tiobarbitúrico (TBARS) no plasma foram elevados nos tratamentos MIX4 e MIX6. Enquanto para o status hepático, as dietas MIX2 e MIX4 apresentaram melhor perfil que o tratamento CONT. Os resultados mostraram que a inclusão do extrato vegetal melhora o status oxidativo e também a saúde hepática dos bovinos alimentados com dietas de alto teor de grãos em confinamento. No segundo experimento foi testado o efeito do extrato vegetal (baccharis, tamarindo, cravo e LCC), tanto *in vitro* como *in vivo* sobre a população de bactérias ruminais. No experimento *in vitro*, nove bactérias ruminais foram submetidas a quatro concentrações diferentes do extrato vegetal sendo: 0.1; 0.2; 0.5 e 1.0 mg mL^{-1}. No experimento *in vivo*, foram utilizados 32 bovinos machos não castrados (½ Angus *vs.* ½

Nelore), com peso corporal médio de 418 ± 4,51 kg e idade média de 24 ± 2,0 meses, distribuídos em um delineamento inteiramente ao acaso, com quatro tratamentos (0, 2, 4 e 6 g de mix vegetal animal dia^{-1}) e oito repetições por tratamento. A dieta continha 30% de silagem de milho e 70% de concentrado (milho grão, glúten de milho, levedura, calcário, sal mineral e ureia). Foi coletado o liquido ruminal de todos os animais para fazer identificação da população bacteriana. Existiu baixa densidade ótica das bactérias analisadas *in vitro*, principalmente nas concentrações 0.5 e 1.0 mg mL^{-1}. Apenas a *S bovis* não foi afetada pelo extrato vegetal. A adição do extrato vegetal apresentou decréscimo da população de bactérias do gênero Methanobrevibacter, principalmente no tratamento com 6 g/anim/dia. Da mesma forma, houve aumento importante da bactéria *Bifidobacterium ruminantium* na dieta com 4 g/anim/dia. Em conclusão, o extrato vegetal pode influenciar na população bacteriana ruminal e modular o ambiente do rúmen.

Palavras-chave: Atividade antimicrobiana, bactérias ruminais, extratos naturais, estresse oxidativo

ABSTRACT

Two experiments were carried out to analyze the effect of the compounds present in the vegetable extract of leaves and stems of baccharis (*Baccharis dracunculifolia*), tamarind seeds (*Tamarindus indica* L.), essential oil of clove leaf (*Syzygium aromaticum*), liquid from Brazil nut cashew (LCC) (*Anacardium occidentale*) on the oxidative status and ruminal modulation of crossbred cattle (½ Angus *vs.* ½ Nellore) bulls finished in feedlot. In the first experiment, the activity of the plant extracts of baccharis leaves and stems, tamarind seeds, essential oil of clove leaf and LCC was evaluated on oxidative status of bulls finished in feedlot and fed diets with high grain content. The bulls were distributed in four treatments; CONT - basal diet; MIX2 - basal diet plus 2 g/animal/d of plant extract; MIX4 - basal diet plus 4 g/animal/d of plant extract; MIX6 - basal diet plus 6 g/animal/d of plant extract. Blood samples were collected from all bulls. The levels of protein carbonyl groups were low in the plasma of bulls supplemented with MIX diets. There was an increase in the ability to reduce plasma iron (FRAP) in bulls supplemented with MIX6. Likewise, the levels of substances reactive to thiobarbituric acid (TBARS) were high in plasma of bulls fed MIX4 and MIX6 diets. Concerning liver status, MIX2 and MIX4 diets showed a better profile than CONT diet. The results showed that the inclusion of the plant extract improves the oxidative status and also the liver health of bulls fed with diets of high grain content in feedlot. In the second experiment, the effect of the plant extract (baccharis, tamarind, cloves and LCC) was tested, both *in vitro* and *in vivo* on population of ruminal bacteria. In the *in vitro* experiment, nine ruminal bacteria were subjected to four different concentrations of the plant extract: 0.1; 0.2; 0.5 and 1.0 mg mL^{-1}.For *In vivo* experiment 32 bulls (½ Angus *vs.* ½ Nellore) were used, with an average body weight of 418 ± 4.51 kg and an average age of 24 ± 2.0 months, distributed in a completely randomized design, with four treatments (0, 2, 4 and 6 g of animal vegetable

mix day-1) and eight replications per treatment. The diet had 30% corn silage and 70% concentrate (corn grain, corn gluten, yeast, limestone, mineral salt and urea). Ruminal fluid was collected from all bulls to identify the bacterial population. There was a decrease in the optical density of the bacteria analyzed *in vitro*, mainly at concentrations 0.5 and 1.0 mg mL^{-1}. Only *S bovis* was not affected by the plant extract. The addition of the plant extract showed a decrease in the bacteria population of genus Methanobrevibacter, mainly in the treatment with 6 g/anim/day. Likewise, there was an important increase in the bacterium *Bifidobacterium ruminantium* in the treatment with 4 g/anim/day. In conclusion, the plant extract can influence the rumen bacterial population and modulate the rumen environment.

Key words: Antimicrobial activity, ruminal bacteria, natural extracts, essential oils, oxidative stress.

I - INTRODUÇÃO

1. Revisão bibliográfica

Atualmente, na produção de bovinos de corte existe a preocupação não só de produzir em quantidade, mas também, produzir alimentos inócuos e de qualidade. Um dos gargalos na produção de bovinos de corte tem a ver com o uso de aditivos sintéticos que, além de modificar o ambiente ruminal e aumentar os índices produtivos, também pode atuar como agentes que promovem menor conservação da carne devido a promoção da liberação de radicais livres e a lipoperoxidação (Jiang and Xiong, 2016a). Além do uso de aditivos sintéticos na fase de terminação, é comum fazer o confinamento do boi e modificar sua alimentação tendo como base alimentos ricos em carboidratos não fibrosos. O confinamento pode alterar o comportamento do animal e estimular a liberação de hormônios corticosteroides que facilitam o processo de oxidação, tendo como possível efeito um animal propenso a doenças e queda de produção (Kenny et al., 2018). Estes fatores somados com as novas exigências do mercado, em relação aos produtos saudáveis e inócuos, sugerem novos desafios na produção de bovinos de corte.

Igualmente, a área da nutrição de ruminantes, nos últimos anos, vem evoluindo para estudar as implicações do microambiente ruminal na eficiência do alimentar animal. Existe uma estreita relação entre a abundância de alguns microrganismos no rumem, principalmente do gênero *Prevotella*, *Ruminococcus* e *Butyrivibrio* e a melhora na eficiência alimentar (Myer et al., 2015). A variação da população microbiana no rúmen está envolvida no aproveitamento da fibra e diretamente relacionada com a formação dos ácidos graxos voláteis (AGV), podendo haver ou não melhoria da mesma (Li and Guan, 2017). A principal ferramenta que existe para a modificação da população ruminal é pela alimentação. Uma dieta com proporção maior de carboidratos fibrosos aumenta as

populações de bactérias metanogênicas e a proporção de metano produzido no rúmen, pelo contrário, dietas com proporção de grãos e alimentos fibrosos de qualidade apresentam aumento das bactérias Gram– e está relacionada com o aumento na proporção de propionato produzido no rúmen (Witzig et al., 2018). Outra das estratégias para a modificação ruminal é o uso dos aditivos naturais, que vêm sendo usados como substitutos dos aditivos sintéticos como descrito no parágrafo anterior.

Óleos essenciais e metabólitos secundários das plantas estão sendo usados como modificadores do ambiente ruminal, uma vez que tem efeito sobre as bactérias ruminais. Os compostos bioativos são encontrados, na maioria das vezes, como metabólitos secundários das plantas e sua função principal é a de defesa e conservação dos componentes vegetais contra aos agentes externos que possam prejudicar a supervivência da mesma (Marriott, 2000). Devido ao fator de defesa, estes metabólitos secundários possuem efeitos sobre os microrganismos ruminais, uma vez que o animal consome a planta ou produtos derivados como os óleos vegetais e essenciais. Artepellin C, apigenin, naringenin, além de outros compostos fenólicos, taninos e terpenos, têm sido identificados nos últimos anos como compostos ativos com função antimicrobiana, além de também ter propriedades antioxidantes (AGUIAR et al., 2013; SOLÓRZANO-SANTOS; MIRANDA-NOVALES, 2012). Todavia, ainda existe pouco conhecimento tanto do modo de ação como da efetividade destes compostos ao ser usado como aditivos na dieta de animais.

Este trabalho foi realizado para avaliar os efeitos de aditivo natural formulado com extrato vegetal de alecrim do campo (*Baccharis dracunculifolia*) e tamarindo (*Tamarindus indica*), misturados com óleos vegetais de caju (*Anacardium occidentale*) e cravo (*Syzigium aromaticum*), na capacidade antioxidante de bovinos mestiços confinados em fase de terminação e na atividade antimicrobiana nas bactérias ruminais.

1.1 Capacidade antioxidante e sua importância na produção de bovinos de corte

O bem-estar animal é uma área de estudo cujo objetivo está relacionado a gerar condições necessárias para que o animal em produção apresente as mínimas alterações fisiológicas que possam derivar do sistema produtivo e possam causar doenças ou morte. O comportamento normal do animal corresponde às respostas naturais, expressados nos mecanismos fisiológicos que ajudam o animal obter os nutrientes suficientes para sobreviver e se reproduzir para tentar perpetuar sua espécie (Grandin, 2015). Quando o

sistema de produção modifica o ambiente natural do bovino, tendo em conta também as mudanças no sistema de alimentação, o animal precisa adaptar-se aos novos desafios que podem alterar seu estado fisiológico, podendo considerar como gerador de estresse no animal. O estresse está definido como as múltiplas respostas que podem apresentar o animal para modificar seu organismo, quando é submetido à situações que comprometam sua adaptação e sobrevivência (Moreno-Rius, 2019). Uma vez exposto o animal à situação geradora de estresse a produção de radicais livres aumenta e também o gasto energético, aumentando o oxigênio das células gerando distúrbios no estado oxidativo do animal. Quando o animal entra em processo de estresse que não pode ser controlado pela resposta dos hormônios, há acúmulo de fatores pró-inflamatórios que comprometem à saúde animal (McEwen et al., 2015). Quando acontece o confinamento de bovinos de corte, além da modificação ambiental pela diminuição da área para sua mobilização, a alta concentração de grão na dieta pode apresentar como desafio especial para o animal entrar num processo de estresse e alteração do seu estado oxidativo.

No confinamento de bovinos de corte é comum ter dietas com proporção de 80 – 90% de carboidratos não fibrosos, aumentando o risco de modificação no ambiente ruminal e da assimilação de nutrientes. Quando existe aumento de carboidratos não fibrosos, o pH ruminal sofre queda e, consequentemente, ocorre acúmulo de AGV que prejudica a parede ruminal e gera complicações de saúde e queda na produção (Liu et al., 2013). Ao ocasionar esse tipo de mudança nos ruminantes, ocorre também um fenômeno inflamatório pelo aumento de radicais livres em alguns tecidos e alteração no estado oxidativo do animal. Em estados de estresse são ativadas proteínas de fase aguda, além de outros sinais inflamatórias, que estão diretamente relacionadas ao aumento na atividade hepática e maior consumo de oxigênio demandado por este órgão (Xu et al., 2017). Quando acontece este fenômeno, o órgão gera resposta celular que ativa a expressão gênica e a produção de enzimas protetoras, mas também produz gasto energético a mais para o animal, comprometendo a eficiência produtiva. O estresse afeta a eficiência do animal pela oxidação de lipídeos e proteínas pelas células usando o sistema da ubiquitine e gerando produtos como malonaldeído e proteínas carboniladas no processo (Casal et al., 2019). O sistema da ubiquitine é muito dependente de ATP mitocondrial porque o gasto energético celular aumenta para sequestrar os produtos resultantes do processo oxidativo (Russell et al., 2016). O organismo possui mecanismos naturais para controlar o processo de oxidação, já que é um processo fisiológico normal dado principalmente pelos processos metabólicos aeróbicos.

Existem no organismo, complexos enzimáticos que controlam a oxidação e as espécies reativas do oxigênio (ROS). De acordo com alguns autores, existem diferentes níveis de defesa do corpo contra o processo oxidativo, destacando as enzimas antioxidantes superóxido desmutase, catalase e a glutationa peroxidase (Senft et al., 2000; Surai F, 2016). Estas enzimas atuam diretamente na produção de radicais livres, impedindo o desenvolvimento das ROS por meio da decomposição dos peróxidos de hidrogênio, principal forma reativa e geradora dos radicais livres (Ighodaro and Akinloye, 2018). Quando o organismo entra em processo quando a capacidade antioxidante é menor que a produção de radicais livres, então produz o estresse oxidativo no organismo. Todavia, é possível compensar esse desequilíbrio oxidativo gerado pelo estresse com uso de aditivos ou produtos que desencadeie uma resposta antioxidante natural do animal. É possível melhorar a resposta das enzimas antioxidantes, tais como a catalase e a glutationa peroxidase, mediante o fornecimento de suplementos que diminuam o processo oxidativo celular e as espécies reativas do oxigênio (Ran et al., 2019).

1.2. Uso de aditivos naturais na dieta de bovinos de corte.

A bovinocultura de corte vem evoluindo com a finalidade de se tornar não só competitivo, mas também para atingir um mercado com consumidores preocupados por produtos inócuos e de qualidade. A carne é um produto que sofre deterioração por causa da oxidação lipídica, mesmo sendo um processo natural, pode-se aumentar a vida útil de este produto quando a carne possui um nível de antioxidante que diminua a velocidade do deterioração por este processo (JIANG; XIONG, 2016). Para melhorar o nível de antioxidantes na carne, existe a possibilidade de programar estratégias na produção primária para conseguir um produto final com mais qualidade. A suplementação de animais em confinamento é uma estratégia muito usada com a finalidade de aumentar os rendimentos produtivos, além de tornar mais eficiente o uso dos recursos, principalmente melhorando a eficiência alimentar dos ruminantes (SALAMI et al., 2019). O uso de fontes de antioxidantes naturais na dieta estimula a produção enzimática de antioxidantes e está diretamente relacionada ao decréscimo nos processos inflamatórios e doenças (KRISHNAIAH; SARBATLY; NITHYANANDAM, 2011). Embora, a utilização de aditivos seja uma das estratégias mais usadas para aumentar os parâmetros produtivos, no Brasil o uso de aditivos sintéticos ainda é um problema, principalmente pela proibição em países com potencial de mercado para os produtos da carne. Os aditivos sintéticos têm

efetividade sobre os parâmetros produtivos, mas há comprovação que possuem risco para o consumidor final porque estão sendo associados com aumento na resistência bacteriana e possíveis carcinogênicos (LLONCH et al., 2017). Na atualidade, existe na área de pesquisa diversidade de trabalhos com aditivos de origem natural que estão tendo a mesma efetividade dos sintéticos, além de potencializar a produção de antioxidantes tanto no animal como no produto final.

Os aditivos naturais se destacam por ter como princípios ativos matérias-primas provenientes de plantas, na grande maioria. As plantas possuem dentro de seus compostos, substâncias químicas que são usadas por ela principalmente como sistema de defesa e conservação frente às condições ambientais. Estas substâncias não contêm propriedades nutricionais, mas têm mecanismo de interação no rúmen que permite obter modificação nutricional dos compostos da dieta pelo efeito que têm entre a assimilação dos nutrientes por parte dos microrganismos ruminais (MCSWEENEY et al., 2001). Quando o animal consome este tipo de aditivos, ocorre ação de proteção de alguns compostos nutricionais obtendo melhora na digestibilidade de alguns nutrientes, além da interação que tem estes compostos com microrganismos e sua atividade normal dentro da atividade ruminal. Os compostos fenólicos são metabólitos secundários encontrados comumente nas plantas e estão considerados como modificadores do ambiente ruminal pela interferência no desdobramento das pontes de hidrogênio e na degradação das fibras (JAYANEGARA; LEIBER; KREUZER, 2012). Por outro lado, estes compostos também interagem com a fisiologia de algumas bactérias, inibindo a produção de algumas enzimas essenciais para o crescimento dos microrganismos (WITZIG; ZEDER; RODEHUTSCORD, 2018). Ao obter essas modificações no rúmen, existe alta probabilidade de obter resultados positivos tanto na produção como na qualidade do produto. A suplementação com aditivos tais como óleos essenciais ou metabólitos secundários de plantas, ajudam a melhorar o ganho médio diário de peso e a eficiência alimentar (ELLISON et al., 2017; MIN et al., 2015; RIVERA-MÉNDEZ et al., 2017). Conforme o exposto anteriormente, existe um potencial zootécnico no uso de aditivos naturais na alimentação de bovinos de corte, mas ainda as interações e ações destes compostos no rúmen não são determinadas de forma clara.

Ao manipular o ambiente ruminal mediante o uso de aditivos, existe a possibilidade de obter alteração nas populações microbianas e mudanças na produção de AGV. Tem sido comprovado que ao usar óleos essenciais na dieta de bovinos confinados existe redução na produção de metano ruminal e mudança na razão de acetato:propionato

(ALMEIDA et al., 2019; CHEN et al., 2015). Estas mudanças na produção de AGV estão relacionadas com as alterações nas populações bacterianas no rúmen e com possível ação antibacteriana destes aditivos de origem natural. A variação dos padrões na digestibilidade dos nutrientes dentro do rúmen está relacionada com fatores associados com a diminuição nas populações de protozoários, arquebactérias ou de algumas bactérias produtoras de hidrogênios (PATRA, 2010). Embora a atividade dos aditivos naturais não tenha um alvo definido para agir em um microrganismo especifico, é claro que existe efeito modificador destes compostos nos perfiles de produção de AGV. Grande parte desta ampla ação dos aditivos naturais se deve ao efeito que tem a estrutura molecular dos metabólitos secundários e seu aporte de moléculas de oxigênio e enxofre ao meio ruminal (BENCHAAR; GREATHEAD, 2011). É importante, então para futuras pesquisas, analisar em conjunto dos efeitos que ocasionam cada um dos metabólitos secundários dentro do microambiente ruminal para conseguir aplicar estratégias dirigidas ao tipo de substrato desejado no rúmen.

1.3 Efeito dos metabólitos secundários das plantas sobre o ambiente ruminal

Como foi mencionado anteriormente, as plantas têm metabólitos secundários dentro de seus compostos que são usados como fator de proteção por elas. Os metabólitos secundários, anteriormente, têm sido classificados como fatores antinutricionais porque afetavam o aproveitamento dos nutrientes no rúmen, mas não eram claras as alterações positivas que tinham na produção de AGV nem na saúde ruminal (FEKADU GEMEDE, HABTAMU; RATTA, 2014). Dentro dos metabólitos secundários, os mais importantes para a nutrição de ruminantes, encontram-se os compostos fenólicos, terpenos, taninos e saponinas.

1.3.1 Compostos fenólicos.

Os compostos fenólicos são caraterizados por suas estruturas de anéis aromáticos que permite interagir com moléculas biológicas de forma mais fácil e eficaz. Estão presentes nas plantas e fungos como mecanismo de defesa contra predadores e raios UV, porque grande parte das plantas tropicais possui um conteúdo importante deste composto (GROSS, 1985). Os compostos fenólicos são metabólitos capazes de gerar mudança no ambiente ruminal pelas interações que podem ter a estrutura destes compostos com alguns metabólitos gerados pelos microrganismos. A ação dos compostos fenólicos é similar à

encontrada aos ionóforos, atuando diretamente na alteração de ions dentro das bactérias Gram-positivas (AGUIAR et al., 2014). Dentro dos compostos fenólicos mais caraterizados, encontram-se a *p*-coumarina, ácido cafeico e naringenin. Por outro lado, a interferência no crescimento da população de protozoários por parte dos compostos fenólicos também resulta em menor quantidade de nitrogênio amoniacal e maior disponibilidade de nitrogênio proteico no lúmen do intestino delgado, e aumentaria a eficiência no aproveitamento da proteína da dieta (DE PAULA et al., 2016). Enquanto a produção de AGV no rúmen, os compostos fenólicos promovem maior síntese de acetato e propionato sugerindo que as bactérias se tornam mais eficientes no uso do hidrogênio disposto no meio ruminal (MORSY et al., 2015). No Brasil existem espécies vegetais com quantidades importantes de compostos fenólicos que poderiam ser de importância na área da ruminantes para usar como aditivos, entre elas a *Baccharis dracunculifolia* e seus derivados como, por exemplo, a própolis (VALERO et al., 2014).

1.3.2 Taninos.

Outro dos metabólitos secundários com maior área de estudo dentro da nutrição de ruminantes são os taninos. Os taninos são da classe de flavonoides caracterizados e fazem parte da maioria de sementes e frutas nas plantas tropicais. Os taninos condensados possuem maior importância por sua estrutura química que está composta por três anéis com ligações duplas de carbono que permitem interação mais simples com produtos das reações metabólicas dos microrganismos (BIANCHI et al., 2015). O principal efeito dentro do rúmen está relacionado ao aproveitamento das proteínas. Uma vez que o tanino entra em contato com a saliva do animal, reage junto com a prolina acontecendo uma precipitação do complexo tanino-proteína para limitar sua degradação (ADAMCZYK et al., 2017). Devido a este fator, os microrganismos do rúmen limitam sua atividade quando há presença de compostos que contêm taninos condensados, favorecendo a passagem de proteína para o intestino para obter melhor aproveitamento (ARCHIMÈDE et al., 2016). O anterior tem influência direta no crescimento das bactérias proteolíticas e diminui a proteína microbiana, com consequente redução na digestibilidade das fibras o que promove queda na formação de CH4 e aumento do propionato (HATEW et al., 2016). Devido a que os taninos condensados são compostos encontrados, principalmente nas sementes das árvores produtoras de fruto com semente grande e são as principais fontes de matéria-prima como, por exemplo, o *Tamarindus indica* (AENGWANICH; SUTTAJIT, 2010).

1.3.3 Saponinas.

As saponinas são compostos derivados de esteroides e não solúveis na água, uma vez que sua consistência é parecida com aos sabões. Quimicamente, dentro de sua estrutura, as saponinas contêm ligações dos açúcares junto com triterpenos pela qual sua solubilidade é limitada, assim como o aceso das bactérias para sua degradação (PATRA & SAXENA, 2009b). Basicamente, o efeito que exerce as saponinas dentro do metabolismo ruminal é similar ao sofrido com os taninos condensados. Seu efeito de proteção à proteína contida nos alimentos ingeridos pelo animal vai impactar as populações de protozoários, já que a estrutura esteroidal interage com a membrana do protozoário causando rompimento da mesma (RAMOS-MORALES et al., 2017). Dependendo da concentração de saponinas na dieta animal, o efeito nas bactérias do rúmen pode ser variável. Havendo um efeito antiprotozoário, as bactérias e fungos no rúmen tendem a aumentar suas populações como mecanismo de defesa para evitar efeito na produção de proteína microbiana (PATRA; STIVERSON & YU, 2012), além de algumas adaptações que sofrem as bactérias proteolíticas para aumentar sua eficiência e sobrevivência (WANG et al., 2019). A susceptibilidade dos protozoários frente às saponinas ocasiona também mudanças na produção de AGV. O acetato e butirato são os principais produtos da fermentação entérica dos protozoários. Quando há queda na formação destes AGV, há aumento na produção de propionato com modificação da razão acetato:propionato (BELANCHE et al., 2016). As saponinas podem ser encontradas nas sementes, além dos produtos obtidos delas como óleos vegetais e farinhas. Um dos óleos vegetais com maior uso na alimentação de bovinos no Brasil é o óleo de caju, que contém quantidades importantes de saponinas e terpenos dentro de seus compostos secundários (ORNAGHI et al., 2017; PRADO et al., 2016).

1.3.4 Terpenos.

Os terpenos são moléculas hidrocarbonadas compostos de oxigênio que fazem parte da coloração natural de alguns vegetais e tem papel muito importante na produção de vitaminas e antioxidantes. Estes metabólitos secundários estão associados ao mecanismo muito eficiente de defesa contra predadores e doenças nas plantas, além de estar envolvidas em mecanismo de marcadores hormonal dentro na célula vegetal (SINGH; SHARMA, 2015). Os óleos essenciais, na grande maioria, são produtos

secundários das plantas com conteúdo importante de classes de terpenos. O eugenol, encontrado no óleo essencial de cravo (*Syzygium aromaticum*), é um dos sesquiterpenos com maior estudo na área dos aditivos naturais para bovinos e com importância na modulação do rúmen (MONTESCHIO et al., 2017; ORNAGHI et al., 2017; SOUZA et al., 2019). Embora os efeitos dos terpenos não estejam muito definidos, existe associação no uso de óleos essenciais como aditivos na dieta de ruminantes e decréscimo na comunidade de archeas dentro do rúmen (COBELLIS; TRABALZA-MARINUCCI; YU, 2016b). Igualmente, estes compostos afetam a quebra das proteínas e interferem na deaminação dos aminoácidos por parte das bactérias hiperprodutoras de amônia, aumentando o fluxo de proteína by-pass e diminuindo a excreção de proteína pelo animal (COBELLIS; TRABALZA-MARINUCCI; YU, 2016a). Mesmo tendo efeito na degradação das fibras, ainda não tem demostrado efeitos significativos sobre a produção de AGV no rúmen, mas sim queda na produção de CH4, produto da fermentação ruminal (BENCHAAR; HASSANAT; PETIT, 2015; OH; HARPER; HRISTOV, 2019).

1.4. Modulação ruminal e seus efeitos na produção

Há muitos anos vem sendo pesquisado como os ruminantes poderiam ter redução na emissão de metano, gás relacionado ao efeito estufa. Todavia, a emissão de metano está muito relacionado à saúde do ambiente ruminal, uma vez que no rúmen existe a produção de hidrogênio (H_2) e CO_2 os quais servem, não só para manter o ambiente livre de oxigênio no rúmen, mas também como substrato para as bactérias metanogênicas (CUNHA et al., 2019; RAMÍREZ-RESTREPO et al., 2016). No entanto, existe a possibilidade de modificar as populações microbianas do rúmen com a finalidade de maximizar a eficiência na utilização dos H_2 para produzir maior quantidade de AGV e aumentar a eficiência alimentar dos ruminantes. Os H_2 produzidos pela degradabilidade das fibras têm a possiblidade de ser usados em múltiplas rotas para a formação de AGV e também ser usadas pelos microrganismos metanogênicos para a produção de CH_4. A disponibilidade de H_2 no rúmen depende muito da qualidade da dieta do animal. Quando há mais produção de H_2, há maior possibilidade de produzir maior quantidade de CH_4 e diminuir a eficiência alimentar (BOWEN et al., 2020). Por exemplo, quanto maior a quantidade de fibra na dieta do ruminante, ocorre incremento na produção do acetato o que resulta em maior produção de H_2 porque a rota metabólica do acetato vai aumentar H_2 que não pode ser usado para a produção de mais AGV´s (CUNHA et al., 2019). Essa

influência da dieta na formação de produtos no rúmen está relacionada, evidentemente, à dieta, mas também com a modificação que a mesma provoca na população dos microrganismos ruminais, responsáveis pela produção dos coprodutos no rúmen. Uma dieta rica em amido, pode mudar o perfis e a proporção entre o acetato e o propionato, aumentando a eficiência alimentar (TOMKINS et al., 2015). Essa troca na produção dos AGV poderia ser pela seleção na população das bactérias ruminais. Quando há aumento na proporção de grãos na dieta, existe queda no pH ruminal ocasionando algumas bactérias, especialmente as bactérias hiperprodutoras de amônia no rúmen, sofram queda na população e aumento nas bactérias ácido-lácticas (KHATERI; AZIZI; JAHANI-AZIZABADI, 2017). Devido a essa queda pode ocorrer mudança no só na população das bactérias, mas também nos coprodutos ruminais e na eficiência alimentar.

Existe, além da dieta, a possibilidade de fazer modificação do ambiente ruminal a partir de compostos sintéticos que exercem efeito antibiótico em algumas das populações no rúmen. Os ionóforos, aditivos usados na alimentação dos ruminantes ocasiona efeito bactericida principalmente nas bactérias Gram-positivas no rúmen (WITZIG; ZEDER; RODEHUTSCORD, 2018). O principal ionóforo usado nas produções bovinas é a monensina. A monensina vem sendo usada há muito tempo tendo efeitos positivos na eficiência alimentar e no ganho de peso diário de bovinos de corte (AKBARIAN-TEFAGHI; GHASEMI; KHORVASH, 2018; BUTAYE; DEVRIESE; HAESEBROUCK, 2003; SALLES et al., 2008). O efeito desta melhora ocorre pelo controle das populações bacterianas no rúmen, principalmente bactérias do gênero *Ruminococcus*, envolvidas na degradação das fibras e na produção de acetato no rúmen. A queda na população deste gênero se encontra relacionada ao aumento nas taxas de produção do propionato e ao maior aproveitamento da energia no rúmen, evitando que a maior produção de H_2 exista maior probabilidade de produzir CH_4, isto indica aumento na digestibilidade da matéria seca e melhora na eficiência alimentar do animal (WITZIG; ZEDER; RODEHUTSCORD, 2018).

Nas últimas décadas, muitas pesquisas estão relacionando com alguns metabólitos secundários das plantas com possível modificação do ambiente ruminal. Os metabólitos secundários das plantas são substâncias químicas naturais que têm ação de proteção contra predadores que poderiam ocasionar dano em sua estrutura ou extinção da espécie por consumo excessivo (WALLACE, 2004). No capítulo anterior foi mencionado alguns dos efeitos que têm os metabólitos secundários dentro da modulação ruminal. É bom ressaltar que os compostos fenólicos são moléculas que têm sido objeto de pesquisa por

suas propriedades antibacterianas. Alguns estudos na área da nutrição de ruminantes relacionam aos compostos fenólicos com a possível redução de bactérias do gênero *Prevotella* e *Ruminococcus* relacionadas com a hiperprodução de amônia no rúmen, este efeito promove maior aproveitamento da matéria seca aumentando a eficiência alimentar (COBELLIS et al., 2016). Os efeitos destes compostos poderiam ser positivos para a produção de ruminantes porque via modificação das populações bacterianas existe também a mudança nos perfis da produção de AGV que ajudaria no aumento dos parâmetros da produção.

2.0. Conclusões e perspectivas

As condições da produção animal na atualidade estão orientadas a oferecer produtos não só com melhor qualidade, mas também com inocuidade que garanta não levar prejuízos à saúde humana. Devido a isso, a preocupação do consumidor está orientada ao consumo de produtos que não tenham resíduos que são usados em algumas ocasiões, nas produções animais, podendo estar associados com problemas como resistência bacteriana e doenças graves. É, por isso, que pela identificação destes produtos de origem natural poderia apresentar um novo tópico que ajudaria a melhorar o produto destinado ao consumidor e ainda melhorar os parâmetros na produção.

A importância de gerar pesquisas com tópicos orientados para a preservação de materiais naturais e a diminuição do impacto gerado pela produção de ruminantes, também poderia ser mais um tópico que se deve ter em consideração na hora de procurar por fontes de alimentação ou suplementação alternativa. Por isso, é importante que desde a zootecnia seja falado a importância de usar produtos (rever a fase), subprodutos e coprodutos originados desde a indústria agrícola para minimizar os custos da produção e o impacto na explotação exagerada dos recursos naturais.

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II - OBJETIVOS GERAIS

Identificar os compostos fenólicos presentes na mistura do extrato vegetal de folhas e caules de baccharis (*Baccharis dracunculifolia*), sementes de tamarindo (*Tamarindus indica* L.), óleo essencial da folha de cravo (*Syzygium aromaticum*) e líquido da castanha do caju (LCC) (*Anacardium occidentale*) para avaliar a atividade antioxidante em bovinos terminados em confinamento, assim como o efeito antibacteriano *in vitro* e *in vivo* nas populações bacterianas do rúmen.

III - Mix of natural extracts to improve the oxidative state and liver activity in bulls finished feedlot

Journal: Livestock Science

Abstract

This study evaluated the effects of a mixture of baccharis (*Baccharis dracunculifolia*) leaves and stems, tamarind (*Tamarindus indica* L.) seed, cashew (*Anacardium occidentale*) nut shell liquid, and clove (*Syzygium aromaticum*) essential oil on the oxidative state of bulls finished in feedlot and fed high-grain diets. A total of 32 bulls (½ Angus *vs.* ½ Nellore) with a mean age of 24 ± 2.0 months and a mean body weight of 418 ± 4.51 kg were distributed in a completely randomized design with four diets and eight replications per diet. The four experimental diets were as follows: CONT – basal diet; MIX2 – basal diet and 2 g/animal/d of extracts from baccharis, tamarind, cashew nut shell liquid and clove; MIX4 – basal diet and 4 g/animal/d of extracts from baccharis, tamarind, cashew nut shell liquid and clove; MIX6 – basal diet and 6 g/animal/d of extracts from baccharis, tamarind, cashew nut liquid shell liquid and clove. The blood samples were collected of the all animals. The levels of protein carbonyl groups were lower ($P < 0.05$) in plasma when bulls were supplemented in any level of MIX. There was an increase in Ferric reducing ability of plasma (FRAP) when MIX6 was used, compared with baseline. Same form, levels of thiobarbituric acid reactive substance (TBARS) in the liver were higher in treatment MIX6 and MIX4, compared with begin of experiment. In relation with liver status, MIX2 and MIX4 showing a better profile that CONTROL. The results show that the inclusion of the mixture of baccharis, tamarind,

cashew nut shell liquid and clove oil improve the oxidative status and could be healthy for liver to bulls that have finished feedlot with high-grain diets.

Keywords: lipid peroxidation, oxidative stress, plant extracts, reactive oxygen species, secondary metabolites

1. Introduction

Equilibrated antioxidant status is necessary to maintain health and improve animal performance. Beef cattle under oxidative stress conditions may be associated with higher production of free radicals, increased tissue inflammation, increased prevalence of diseases and lower parameters of production (Lashkari et al., 2018). Essential oils and plant extracts are nowadays used in diets of animals to replace antibiotics and ionophores, with the intention of improving animal performance, feed efficiency and meat quality (Monteschio et al., 2017; Ornaghi et al., 2017). The increased antioxidant capacity of these natural compounds has been reported to improve animal performance (Akbarian-Tefaghi et al., 2018). Some previous studies have shown a positive relationship between animal antioxidant status, antioxidants offered in the diet and antioxidant activity in beef meat (Biondo et al., 2017; Jiang and Xiong, 2016). Furthermore, the increase in meat antioxidant activity can increase consumer acceptability (Guerrero et al., 2018; Kempinski et al., 2017).

Many tropical trees and co-products of the agroindustry present high contents of flavonoids, phenolic molecules and other secondary metabolites, which present with a known antioxidant activity. *Baccharis dracunculifolia* is a native plant from Brazil. Its foliage contains aliphatic hydrocarbons, terpenes (such as baccharin), flavonoids (such as isosakuranetin) and phenolic acids (such as artepelin C and p-coumaric) (Bonin et al., 2020). Artepelin C has been described as bacchari's principal compound and classified

as a biological antimicrobial-antioxidant agent (Veiga et al., 2017). Tamarind (*Tamarindus indica* L.) is a fruit tree, member of the fabaceae family, with potential additive able to modify ruminal parameters. Seeds and pulp of tamarind could decrease the loss of energy due to fermentation, increasing feed intake, nutrient digestibility and improving growth performance of lambs (Geron et al., 2015). Cashew (*Anacardium occidentale*) is a tree native to the north and northeast regions of Brazil. The cashew nut shell liquid (CNSL) are widely used in the food, chemical and pharmaceutical industries because of their great secondary metabolites content (Prado et al., 2016; Valero et al., 2016). Clove (*Syzygium aromaticum*) essential oil has received a lot of attention due to its high and diverse content of phenolic compounds, its antimicrobial-antioxidant properties and its potential use in animal production and meat industry (Cobellis et al., 2016; Monteschio et al., 2019). The association of all these products listed above could help to improve the meat production system.

Plant extracts and essential oils, besides, have been combined to enhance the biological activity of diet and improve animal performance, due to their synergistic effect (Fugita et al., 2018; Pateiro et al., 2018). In fact, it could be expected that a greater antioxidant effect that will be obtained if several essential compounds are used together, rather than isolated (Hyldgaard et al., 2012). Such use can explain the productive effects in the animal, but its possible effects on cattle health parameters and oxidative status is currently unknown. Cattle confinement can cause stress episodes, such as a heat wave induced by a high-energy diet and contact with humans (Sullivan and Mader, 2018), the use of a mixture of natural compounds perhaps increase the animal performance and protect the animal health from stress disorders (Monteschio et al., 2017). Research concerning the effects of a mixture of plant extracts, vegetable oils and essential oils on antioxidant activity and enzyme stress in bulls is still limited.

The hypothesis of this work was that a mixture of natural plant extracts added in the diets of cattle finished in feedlot could have been improved the oxidative status and liver health, knowing that bulls in feedlot suffer more stress factors. Thus, this study was realized to evaluated the effects of a mixture of baccharis (*Baccharis dracunculifolia*) leaves and stems, tamarind (*Tamarindus indica* L.) seed, cashew (*Anacardium occidentale*) nut shell liquid, and clove (*Syzygium aromaticum*) essential oil on the oxidative state of bulls finished in feedlot and fed high-grain diets.

2. Materials and methods

All animal care and experimental procedures were conducted under the surveillance of the Animal Care and Use Committee of the Universidade Estadual de Maringá, Brazil (protocol number 6680060219) and met the guidelines of the National Council for the Control of Ani-mal Experimentation (CONCEA).

2.1. Location, animals and diets

Study was leaded in Rosa & Pedro Sector, property of State University of Maringá, (Iguatemi-Paraná, Brazil). This region has a humid temperate climate with annual average temperature of 18 ºC and annual average rainfall of 1,114 mm.

A total of 32 (½ Angus vs. ½ Nellore) bulls with mean age of 24 ± 2.0 months and mean body weight of 418.0 ± 4.51 kg were distributed in a completely randomised design, with four diets and eight replications per diet. These bulls are very used in Brazil for meat production. Bulls were weighed at the beginning of the experiment and assigned to 10 m^2 individual pens, partially covered and concrete floors. The bulls were weighed on a 16-hour fast every 28 days using a trunk balance (Beckehauser Cia., Paranavaí, Paraná, Brazil).

The basal diet comprised 700 g/kg concentrate and 300 g/kg corn silage, and was offered ad libitum for 74 days, after adaptation period. Feed intake was recorded daily. The basal diet was the same for all animals, formulated to have the same amount of nitrogen and energy (Table 1) according to the NRC (2016). The four experimental diets were as follows: CONT – basal diet; MIX2 – basal diet and 2 g/animal/d of extracts from baccharis, tamarind, cashew nut shell liquid (CNSL) and clove; MIX4 – basal diet and 4 g/animal/d of extracts from baccharis, tamarind, CNSL and clove; MIX6 – basal diet and 6 g/animal/d of extracts from baccharis, tamarind, CNSL and clove. These concentrations represent typical amounts of compounds from plant extracts and EO supplied to ruminants' diets (Ornaghi et al., 2020, 2017; Rivaroli et al., 2020; Souza et al., 2019). The mixture (MIX) contained 400 g/kg of baccharis (*Baccharis dracunculifolia*) leaves and stems, 400 g/kg of tamarind *(Tamarindus indica L.)* seeds, 100 g/kg of CNSL (*Anacardium occidentale* L.), and 100 g/kg of clove leaf (*Syzygium aromaticum*) essential oil per animal/day. Concentrate with the mixture was prepared every 15 days, adjusting the inclusion according to the intake of dry matter/day per animal, to maintain a constant dosage per animal/day and preserve antioxidant activities.

Leaves and stems of baccharis were collected in Maringá (Paraná-Brazil) (23º27'S 51º59'W) on a latossolo Roxo-Distrófico (Brazilian classification, Embrapa, 2006), during summer in the region. Tamarind seeds were collected from Nova Redenção (Bahia-Brazil) (12º49'S 41º03'W) on a Cambissolo eutrófico (Brazilian classification, Embrapa, 2006), during winter in the region. Both source materials were dried (stove, 55 °C, 72 h), milled (sieve of one millimetre) and then refrigerated at 4 °C. The cashew nut shell liquid (CNSL) was purchased from Safeeds® (Cascavel city, Paraná state, Brazil south) and clove oil was obtained from FERQUIMA® (Vargem Grande Paulista, São Paulo, Brazil) and stored at 4 ºC.

2.2 Chemical analyses

Dry matter (DM) content of diet was determined through oven-drying at 65 °C for 24 h and then drying at 135 °C for 5 h. Organic matter (OM) content was calculated as the difference between the DM and ash contents, with ash determined by combustion at 550 °C for 5 h. Nitrogen content of the samples was determined by the Kjeldahl method (Mertens, 2005). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were determined using the methods described by Van Soest et al. (1991), with heat stable α-amylase and Na_2SO_4 used in NDF procedure and expressed inclusive of residual ash. Metabolizable energy content of feedstuffs was estimated according to NRC (2000) recommendations. The content of non-fiber carbohydrates (NFC) was obtained using the equation:

NFC = 100 – Ash – EE – NDFap – (CP – CPu + U) (Detmann and Valadares Filho, 2010);

where CP is the crude protein; CPu is the crude protein from urea; EE is the ether extract; NDFap, is the neutral detergent fiber corrected for ash and protein; and U is urea. All terms are expressed as % of DM.

The values of total digestible nutrients observed were estimated using the equation:

TDN = CPd + (EEd × 2.25) + NDFd + NFCd (Sniffen et al., 1992);

where: CPd is the digestible crude protein; EEd is the digestible ether extract; NDFd is the digestible neutral detergent fiber; and NFCd is the digestible non-fiber carbohydrates.

2.3. Phytochemical profile

A part of crude extract of mix (27.9 g) was suspended in methanol/water (1:1, 50 mL, v/v), and successively partitioned with n-hexane and ethyl acetate. Then, ethyl acetate fraction was submitted to Nuclear Magnetic Resonance (NMR) analyses. ^{13}C NMR spectrum (Figure 1) of were recorded on a Bruker Avance III HD spectrometer (Bruker®, USA) operating at 75.5 MHz, using DMSO-d6 (Sigma-Aldrich) as solvent. The major compounds were identified based on chemical shift (ppm) data and comparison with data reported in the literature, as eugenol (A) (Fujisawa et al., 1988), cardol (B) and cardanol (C) (Yuliana et al., 2014) (Figure 1).

Antioxidant activity was calculated with the following assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing ability power (FRAP), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and polyphenols (Table 2).

2.4 Blood collection, preparation of plasma and analyses

Blood samples were collected on experimental days 0 and 74 into 10 mL vacutainer tubes (BD - Vacutainer System®) with anticoagulant, via puncture to the jugular vein, before animals were fed. Day 0 was taken as the baseline for each animal. After blood collection, all samples were transferred to centrifuge tubes and centrifuged at 1000×g for 10 minutes (Centribio, Cienlab, Brazil). Plasma samples were immediately separated and transported to the laboratory. Oxidative status in plasma was calculated through measuring protein carbonyl group content, reduced protein thiol group content and the ferric reducing ability of plasma (FRAP). Also, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured in the plasma to evaluate liver damage.

Protein carbonyl group contents were measured spectrophotometrically in plasma and liver homogenates using 2,4-dinitrophenylhydrazine (DNPH) (ε370 = 22×103 M^{-1} cm^{-1}), and values were expressed as nmol (mg protein)$^{-1}$ (Levine et al., 1990). Reduced protein thiol groups in plasma were determined using the compound 5,5′-dithiobis 2-nitrobenzoic acid (DTNB), (Sedlak and Lindsay, 1968). Levels of carbonylated proteins were measured through by spectrophotometer on the liver supernatant with 2,4-dinitrophenylhydrazine, in the same form as described above for the plasma. Lipoperoxide content was measured by means of thiobarbituric acid reactive substance (TBARS) assay. TBARS levels were measured from the standard curve prepared with 1,1′,3,3′-tetraethoxypropane. Total ROS content was quantified via the 2′-7′-dichlorofluorescein diacetate (DCFH-DA) assay (Rodrigues Siqueira et al., 2005), which quantifies the oxidation of DCFH-DA to the fluorescent 2′, 7′-dichlorofluorescein (DCF) in the presence of ROS. Formation of DCF was measured using a spectrofluorometer RF-5301 (Shimadzu, Kyoto-Japan; 504 nm for excitation and 529 nm for emission). Rate mitochondrial ROS production (real-time ROS production) was calculated by linear fluorescence increase due to DCF formation. The results were expressed as both nmol·min^{-1}·(mg protein)$^{-1}$ and the effective concentration of methyl jasmonate (MeJA) that stimulates a half-maximal ROS production (EC_{50}). EC_{50} was calculated by numerical interpolation using Stineman interpolation formula. Reduced (GSH) and oxidised glutathione (GSSG) were gauged spectrofluorimetrically by means of o-phthalaldehyde (OPT) assay. Activities of catalase, superoxide dismutase (SOD) and myeloperoxidase (MPO) were measured by spectrophotometry on liver homogenate supernatant. Catalase activity was estimated at 240 nm using H_2O_2 as the substrate. Activity SOD was estimated according to the pyrogallol autoxidation method. MPO activity was measured as described above for the plasma (Nakanishi et al., 2018). FRAP was measured by

spectrophotometry (595 nm) using tripyridyltriazine (TPTZ) and ferric chloride ($FeCl_3$), as described by literature, but with a few modifications (Benzie and Strain, 1996). Quickly, 900 µL of freshly prepared FRAP reagent was blended with 90 µL distilled water more 50 µL of plasma, for obtained as appropriate control reagent. Final dilution of test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of a 10 mM 2,4,6-tripyridyl-S-triazine solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3 \bullet 6H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6. The temperature was maintained at 37 °C and the reaction was monitored for up to 30 min at 595 nm. Trolox been used to make a standard curve and results were shown in µmol L^{-1}. Activities of AST and ALT were measured using commercial kits (Gold Analisa®, Minas Gerais, Brazil).

2.5. Preparation of liver homogenates and analyses

At last day of the feedlot experiment (74 day), bulls were transported to a commercial slaughterhouse localized at a distance of less than 40 km from the farm (Colorado, Paraná, Brazil). Animals were slaughtered following usual practices of Brazilian beef industry. The bulls were stunned using a captive-bolt pistol, and subsequently hoisted and bled, through by cutting the neck vessels. After exposing the digestive compartments, liver was immediately removed, freeze-clamped and stored in liquid nitrogen for transportation to the laboratory for analyses. The liver was then homogenized in a van Potter homogeniser with 10 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and aliquots were separated for use as total homogenates. The remaining homogenates were centrifuged at 11,000 g for 15 min and the supernatants were separated as the soluble fractions of the homogenates. For determination of hepatic and oxidative status, we conducted the following analyses: thiobarbituric acid reactive substances (TBARS), reactive oxygen species (ROS), reduced glutathione (GSH),

oxidised glutathione (GSSG), catalase activity (CAT), superoxide dismutase activity (SOD) and measured protein carbonyl group content in the liver.

TBARS was carried out to determine lipid peroxidation in liver (Buege and Aust, 1978). Amount lipoperoxides (mg protein)$^{-1}$ was measured with a standard curve prepared with 1,1′,3,3′-tetraethoxypropane. ROS content was quantified via the 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) assay (Rodrigues Siqueira et al., 2005), and the acetate groups of DCFH-DA allow it to enter the organelles. These groups are removed by esterases producing the reduced DCFH within the organelle, which can be oxidized by peroxides to the fluorescent oxidized dichlorofluorescein (DCF). Formation of DCF was measured immediately after stopping the reaction on ice with a spectrofluorometer RF-5301 (Shimadzu, Kyoto-Japan) in which the excitation and emission wavelengths were set at 504 and 529 nm, respectively. A standard curve with oxidized dichlorofluorescein (DCF) has been used to express results on how nmol (mg of protein)$^{-1}$. Glutathione (GSH) and oxidized glutathione (GSSG) were measured in the liver total homogenate. GSH and GSSG contents were measured spectrofluorimetrically (excitation 350 nm and emission 420 nm) by the o-phthalaldehyde assay (Senft et al., 2000). For testing GSSG, sample has been incubated with 10 mM N-ethylmaleimide (NEM) and then with a mix containing 1 M NaOH and 0.4 µM phthalaldehyde to find out the fluorescence. The results were calculated using a standard curve prepared with GSH or GSSG and the values were expressed as nmol (mg protein)$^{-1}$. Antioxidant enzymatic activities were assessed in the liver homogenate supernatant. CAT activity was estimated by measuring changes in absorbance at 240 nm using H_2O_2 as the substrate and expressed as µmol min^{-1} (mg protein)$^{-1}$ (Slocombe and Cote, 1977). The SOD activity was estimated by its capacity to inhibit the pyrogallol autoxidation in an alkaline medium, with the latter being measured at 420 nm. One SOD unity has been considered to be

quantity of enzyme that able to promote 50% inhibition. Protein carbonyl group contents were measured in the same way as they were analyzed in plasma.

2.6. Statistical analysis

Plasma concentrations of all enzymes and liver were analyzed using the MIXED procedure of SAS (Statistical Analysis System, version 9.0) with KR ddfm approximation to determine the denominator degrees of freedom for the test of fixed effects. Differences between diets were made through the Tukey-Kramer test with a 95% reliability range.

3. Results

3.1. Oxidative status and enzyme activities in the plasma

Plasma oxidative status was evaluated before (day 0) and after supplementation with baccharis, tamarind extracts, CNSL, and clove oil mix (day 74). The levels of protein carbonyl groups, and oxidative injury parameter, were different between treatments in the plasma of bulls had been supply with the MIX compared to CONT and day 0. Moreover, the level of thiol groups and FRAP (antioxidant parameters) were modified in the plasma ($P > 0.05$) by MIX4 and MIX6 supplementation but there not difference between CONT. Comparison with DAY0, these groups could be better antioxidant performance considered the possible stress conditions under feedlot state to the animals. Albumin levels also to showing that MIX4 supplementation has been a better performance compared with the other groups, that demonstrating a liver status healthy. Likewise of the antioxidant parameters (FRAP and THIOLS), levels of Day 0 are better compared with the other groups because of animals not yet under feedlot conditions.

The plant mix supplementation modifies the activities of AST enzyme but not ALT enzyme in plasma. The AST values in the study showing that MIX2 and MIX4 have

been a better performance compared with MIX6 and CONT (Table 3). This result could be suggesting that supplementation with these two quantities provide more benefit to liver health.

3.2. Oxidative status and enzyme activities in the liver

Hepatic oxidative injury direct was evaluated by measuring the levels of TBARS and protein carbonyl groups in the liver homogenates. Levels of TBARS were different in MIX6 compared with the other groups, showing an improvement in the oxidative status of the animals in this group. As to enzyme activity to the liver, catalase levels had to show that animals of MIX4 showing an increase in its levels and is different than the other groups (Table 4). This increase in value is related with an improvement of the oxidative status to the animal and major protection against pathogen agents.

4. Discussion

Feedlot systems and high contents of non-structural carbohydrates in cattle could be associated with increases in health problems, principally by animal stressing. The stress process affects directly to immune cells because they have membranes with high concentrations of polyunsaturated fatty acids, which are highly susceptible to lipid peroxidation (Spears and Weiss, 2008). A mixture of secondary metabolites found in plants, such as phenolic compounds, terpenes, and flavonoids, possess antioxidants activities and, therefore, could be used as natural antioxidants in the animal production system (Kempinski et al., 2017; Monteschio et al., 2019; Valero et al., 2016). Indeed, several tropical plants, such as baccharis and tamarind, could have valuable applications in animal nutrition (Archimède et al., 2016). Use of mixtures of these sources would potentiate the synergistic effect on animal health, improve the production and shelf-life

of meat. Some combinations of essential oils and plant extracts have been enhancing performance and improve antioxidant activity in the animal, obtained quality meat products (Ornaghi et al., 2017; Sharifi et al., 2018).

Protein carbonyl levels are a biomarker of protein oxidation associated with metabolic disorders and, more frequently, with age (Hopps et al., 2010). Any process that induces oxidative stress in an animal can produce elevated protein carbonyl levels. Secondary metabolites found in the plant extracts can improve the antioxidant activity of the animals and could be reduced the lipid peroxidation process in tissues (Aengwanich and Suttajit, 2010; de Aguiar et al., 2014). In agreement with this, natural mix of this study showed a better performance in oxidative status decreasing of protein carbonyl levels in all treatments compared with the beginning of experiment and control group. FRAP and THIOLS is a measure of antioxidant power and provides information above the equilibrium about the level of oxidants and antioxidant agents (Benzie and Strain, 1996). Mitochondrial activities are a great cellular oxidative and can modulate oxidation of THIOL groups, when occurring antioxidant activity this modulates could be stopping and avoid formation to ROS (Santofimia-Castaño et al., 2016). The antioxidant activity of eugenol is powerful versus other antioxidant compounds and when it is included in animal diet, it improves the synthetic capacity of metabolic organs and plasma concentrations of FRAP and THIOLS (Konvičná et al., 2015). Agreement with previously, all secondary metabolites present in the mixture of this study can be reduced the oxidative stress and, consequently, improve animal health. Levels of albumin and AST in plasma have been a relationship with hepatic function, metabolic capacity, and health to the animal. When occurring an increase of AST levels and decrease of plasma albumin values has been a hepatic dysfunction, rate increase generalized infections, and inflammatory processes (Kostadinova et al., 2018; Wang et al., 2017). Its important

highlight, that in this study levels of AST and albumin showing could be existed a better functional hepatic in animals of MIX2 and MIX4, compared with the experimental animals.

As to enzymatic hepatic functionally and oxidative status in the liver, levels of TBARS and catalase enzyme levels in the liver indicate a reflex of the animal immunological system. When diets contain new plant sources, like those used in this study, they could be toxic to the animal causing chronic liver damage (Chen et al., 2019; Garg et al., 2018; Knupp et al., 2016). Thus, the treatment with MIX6 led to a better liver condition as compared with the CONT treatment. Immune responses can be modulated by the coupling of some secondary compounds presents in plants, for example, cardanol. Cellular receptors responsible for generating an anti-inflammatory response and the stimulation of defensive cells could modify their response thought stimulating the linking of hydrogen peroxide with phenolic compounds, avoiding hydrogen peroxide effects on the cellular membrane (Dosoky et al., 2016; Oh et al., 2013). Despite the results observed in this study, references about the effects of secondary metabolites on oxidative status in ruminant production are limited. However, it has been observed that secondary metabolites act as a synergetic agent, exhibit a protective hepatic effect, and likewise inhibited the hepatotoxicity and liver damage caused by oxidative stress (Abdel-Kader et al., 2019; Oh et al., 2017).

5. Conclusion

The use of a mixture of baccharis, tamarind, CNSL, and clove oil in bulls finished in a feedlot with high-grain diets led to a reduction in lipoperoxidation and had a protective action on the liver, improving the oxidative status of the bulls. This was happened especially case in animals supplied with 2 and 4 g/anim./d.. Also, the use of

these natural compounds in the mix could be generated a synergistic action on ruminant production, since it improves oxidative status and health to the animal.

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Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Table 1. Ingredients and chemical composition of basal diet (g/kg DM)

Ingredients	**Diet**
Corn silage	308.4
Corn grain	636.1
Refinazil	42.4
Limestone	3.5
Mineral salt	3.6
Urea	5.7
Yeast	0.4
Chemical composition	
Dry matter	710.3
Crude protein	129.0
Organic matter	933.0
Ash	68.7
Ether extract	33.6
Neutral detergent fibre	255.0
Acid detergent fibre	74.4
Total digestible nutrients	791.0
Metabolisable energy (Mcal/kg DM)	2.9
Calcium	5.2
Phosphorus	3.6

Table 2. Antioxidant activity of the crude extract of mix.

Antioxidant activity	
Polyphenols (mg/g)	456.13
FRAP (mg/g)	10.43
ABTS (%)	7.47
DPPH (%)	27.21

FRAP: ferric reduction antioxidant power; ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DPPH: 2,2-diphenyl-1-picrylhydrazyl.

Table 3. Plasma concentrations of antioxidant enzymes of crossbred bulls in feedlot with natural additive supplementation. *P < 0.05 difference exist.

ITEM	TREATMENT					SEM*	P-value
	Day 0	CONT	MIX2	MIX4	MIX6		Treatment
Antioxidant status							
Protein carbonyls (nmol-mg protein^{-1})	7,93^{a}	7,19^{a}	5,61^{b}	5,24^{b}	5,36^{b}	0,362	<0.0001
FRAP (nmol-mL^{-1})	0,25^{a}	0,13^{b}	0,11^{c}	0,11^{c}	0,13^{b}	0,006	<0.0001
THIOLS (nmol-mL^{-1})	363,23^{b}	378,83^{ab}	366,79^{b}	389,25^{a}	401,45^{a}	8,902	0.0297
Albumina (g/dL)	3,45^{a}	1,90^{c}	2,24^{bc}	2,43^{b}	2,00^{c}	0,174	<0.0001
Enzyme activity							
AST (U-L^{-1})	37,31^{bc}	40,23^{ab}	33,42^{c}	35,44^{bc}	44,90^{a}	1,929	0.0025
ALT (U-L^{-1})	16,10	16,13	17,92	17,50	20,47	1,237	0.1034

Day 0: before starting the study. CONT: control group. MIX2: 2 g/anim./d. MIX4: 4 g/anim./d. MIX6: 6 g/anim./d. ^{ab}*Values with different letters in the same row statistically different by Tukey's test (P=0,05).*

**SEM, Standard error of means.*

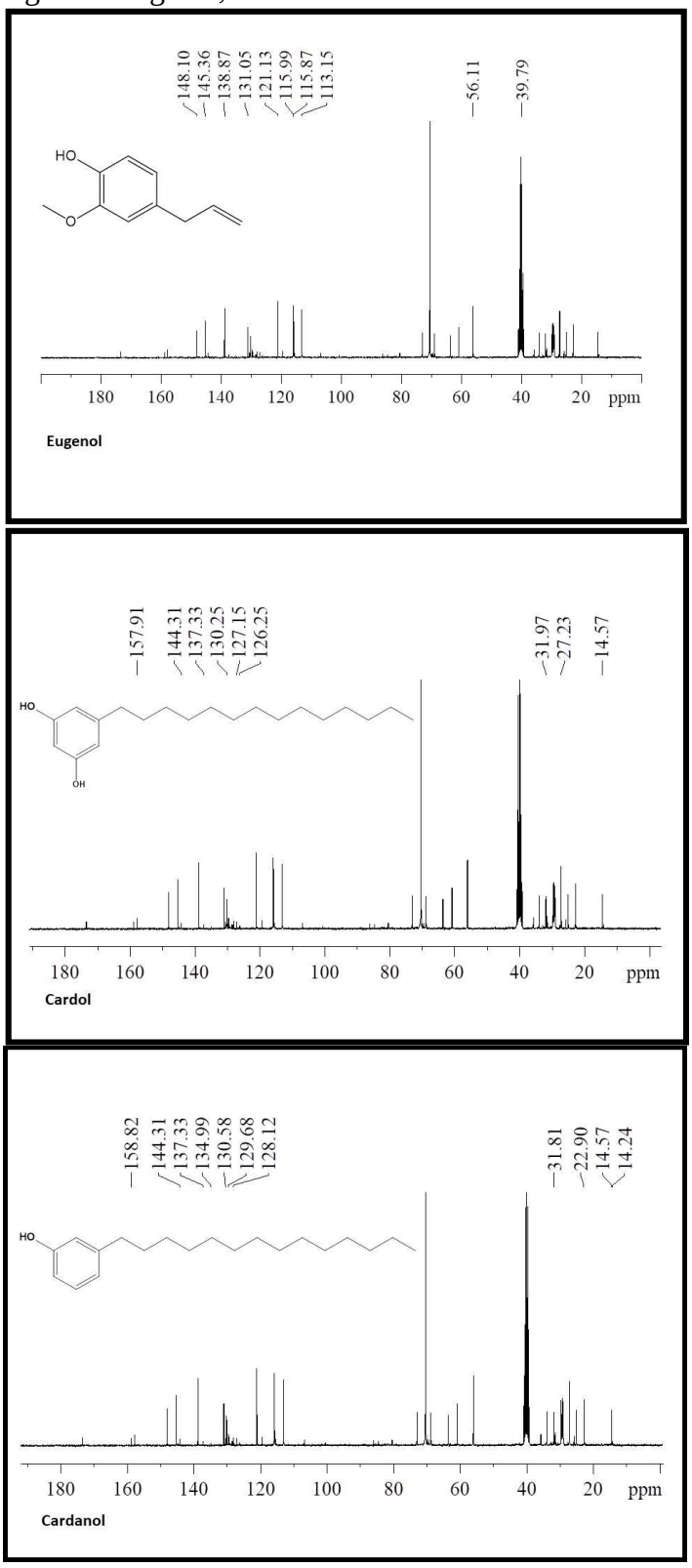
Table 4. Concentrations of antioxidant enzymes to liver of crossbred bulls in feedlot with natural additive supplementation. *P < 0.05 difference exist.

	TREATMENT					P-value
ITEM	**Control**	**MIX2**	**MIX4**	**MIX6**	**SEM***	**Treatment**
Antioxidant status						
Protein carbonyls (nmol-mg protein^{-1})	6,74	5,94	6,23	5,67	0,304	0.1162
TBARS (nmol-g^{-1})	56,54^{a}	53,69^{a}	52,56^{a}	31,92^{b}	3,651	0.0009
ROS (nmol-g^{-1})	17,47	25,54	20,79	23,69	3,291	0.1065
GSH (nmol-g^{-1})	3,75	3,78	3,46	3,66	0,122	0.5058
GSSG (nmol-g^{-1})	0,106	0,103	0,101	0,095	0,005	0.5189
Enzyme activity						
Catalase (μmol/min)	675,90^{b}	670,53^{b}	761,51^{a}	666,27^{b}	19,141	0.0003
SOD (U mL^{-1})	1,32	1,58	1,48	1,61	0,198	0.7859

Day 0: before starting the study. CONT: control group. MIX2: 2 g/anim./d. MIX4: 4 g/anim./d. MIX6: 6 g/anim./d. ^{ab}*Values with different letters in the same row statistically different by Tukey's test (P=0,05).*

**SEM, Standard error of means.*

Figure 1. 13C-NMR spectrum (DMSO-d6, 75.5 MHz) of crude extract of mix: marked signs for eugenol, cardol and cardanol.



IV - Effects of natural mix of Brazilian Agricola products and co-products on in vitro and in vivo ruminal bacterial population

Journal: The International Journal of Animal Bioscience

Abstract

Natural additives are management with more frequency in livestock production due to the restriction of beef products raised with synthetics products, as ionophores. These natural products have been probed antimicrobial properties that can be reduced some ruminal bacterial population and improve feed efficiency. This study was carried out to evaluated the effects of a mixture of baccharis (*Baccharis dracunculifolia*) leaves and stems, tamarind (*Tamarindus indica* L.) seed, cashew (*Anacardium occidentale*) nut shell liquid, and clove (*Syzygium aromaticum*) essential oil on ruminal bacterial populations. For the *in vitro* test, nine bacteria were used, of which they were grown in an anaerobic medium containing 0.1, 0.2, 0.5, and 1.0 mg mL^{-1} of the extracts or oils. Growth was evaluated by monitoring the optical density ($OD_{600\ nm}$) at intervals of 0, 8, 12, and 24 hours of incubation at 39 °C. For the *in vivo test*, we used 32 bulls, of which were distributed in four diets: CONT – basal diet; NM2 – basal diet and 2 g/animal/d of the natural mix; NM4 – basal diet and 4 g/animal/d of the natural mix; NM6 – basal diet and 6 g/animal/d of the natural mix. Except *S. bovis*, the addition of 0.5 and 1.0 mg mL^{-1} concentrations of the natural mix extract resulted in a greater impact on growth dynamics, with a reduction in optical density in all intervals of

observations. PCR analysis of ruminal content of animals showing that the addition of natural mix as an additive can be modified the ruminal bacterial population. Natural mix addition showing a decreased in operational taxonomic units of Methanobrevibacter genus, especially in NM6. In addition, NM4 presented an important increase in *Bifidobacterium ruminantium*, compared with the other treatments, that could be indicated a better use of hydrogens for volatile fatty acids production on the rumen. In conclusion, natural mix of baccharis, tamarind seed, cashew nut shell liquid, and clove essential oil can modulate the ruminal bacterial population.

Keywords: Firmicutes, Methanobrevibacter, rumen environmental, ruminal modulation, secondary metabolites

Implications

Tropical plants are rich in secondary metabolites, such as phenolic and flavonoids compounds. These compounds are potential rumen fermentation modulators, and without causing a risk to human and animal health. The hypothesis of this study was that the inclusion of extracts of leaves and stems of Baccharis, tamarind seeds, cashew and cloves oils could be modified ruminal bacterial population in beef cattle. The study contributes to new information about the efficacy of the extracts plant as antimicrobial agent on ruminant production.

Introduction

Rumen microbiome has been an area of livestock production with great advanced regard to feeding efficiency and animal performance. Modifications on ruminal bacterial

populations through additives supplied in cattle production have been reported many times showing a better animal performance due to methane production decreased and higher volatile fatty acids (VFA) (McCann et al., 2014; Piñeiro-Vázquez et al., 2018; Witzig et al., 2018). Rumen functionally depends on microbial interactions and every bacterial population fulfils a function either in the degradation of products as ruminal environment maintenance (Clemmons et al., 2019). Therefore, it is necessary to determine rumen bacterial composition and its interaction with different subtracts to determine an equilibrating between production and ruminal healthy in cattle. Dietary content plays a principal role in microbial ruminal profile causing more significant changes in bacterial populations. Forage and concentrate are the main source that affecting the ruminal microbiota profile and their co-products production since the feeding proportioned could be caused changing in pH, temperature, and density of ruminal content (Lengowski et al., 2016; Jin et al., 2018). For example, high forage fibrous content in diet could be promoting more acetate production and methane as fermentable ruminal co-products (Ellison et al., 2017). Indeed, feed efficiency in ruminant production has a close relation with less or more methane ruminal production. The production of ruminal co-products, so, involving interactions between diet and ruminal population (bacterial, fungus, archaea, protozoans).

In livestock, exist synthetic products that cause a ruminal modification acting as antibiotics and countering, principally, Gram-positive bacterial population which is the principal fiber degrade population in the rumen. Ionophores are the principal additives used in livestock due to their principal role is to decrease energy ruminal losses, improve feed efficiency and increase daily gain weight (Benchaar et al., 2006; Tomkins et al., 2015). Monensin is the more useful and common ionophore until now in beef production from America. Monensin can be caused changes in a ruminal microbiota, affecting fungi

populations and bacterial communities, principally bacteria of taxa Firmicutes in charge of H_2 production and fiber degradation (Shen et al., 2017). This alteration could be decreased methane production and enhanced feed efficiency in cattle. However, these ionophore products have been prohibited in some countries because it's associated with antimicrobial-resistant and cancer risk in humans (Landers et al., 2012). Alternative methods in last years have been investigating plant resources, especially tropical vegetation, with great contents of secondary metabolites, which could be have the same modification effect in ruminal environment and enhancing cattle production. For example, phenols compounds of some tropical plants, as cashew (*Anacardium occidentale* L.) or baccharis (*Baccharis dracunculifolia*), can promote improvements in total nutrient digestibility and decreased protozoal ruminal population, generating enhance in animal performance (Valero et al., 2016; Fugita et al., 2018). Indeed, these natural additives have been great potential in ruminal bacterial population modification. Some effects of natural additives on bacterial ruminal population are attribute decrease of hyper ammonia bacteria in the rumen, such as *P. ruminicola* and *B. fibrisolvens*, resulting in a reduction starch and protein degradation in the rumen that could be beneficially in a total nutrient digestibility in cattle (Patra and Saxena, 2009a; Patra, 2010). It’s an important finding to continue researches in natural additives by livestock production, especially beef production. Inclusive, it is would be necessary to include mixes of various plant extracts for finding synergy and enhance their beneficial effect.

Therefore, the hypothesis of this work was a mixture of natural plant extracts could be modified ruminal bacterial population in beef cattle. Thus, this study was realized to evaluate the *in vitro* and *in vivo* effects of a mixture of baccharis (*Baccharis dracunculifolia*) leaves and stems, tamarind (*Tamarindus indica* L.) seed, cashew (*Anacardium occidentale*)

nut shell liquid, and clove (*Syzygium aromaticum*) essential oil in ruminal bacterial population of beef cattle.

Materials and methods

Natural extract obtention and phytochemical profile

The natural extract was made with baccharis (*Baccharis dracunculifolia*) leaves and stems, tamarind (*Tamarindus indica* L.) seed, cashew (*Anacardium occidentale*) nut shell liquid, and clove (*Syzygium aromaticum*) essential oil. The natural materials were collected in the Northeast and South Brazilian regions during the summer. CNSL and clove essential oil were purchased from FERQUIMA® (Vargem Grande Paulista city, São Paulo state, Brazil, southeast).

Natural mix was analyzed by Bruker Avance III HD spectrometer (Bruker®, USA) for identified the major chemical compounds composition.

In vitro antibacterial evaluation of natural mix

In vitro analysis was made in the microbiology laboratory of Animal Science Department of State University of Maringá (Parana-Brazil). Natural material (leaves, stems, and seeds) was collecting and grounding with a blade mill with a 1 mm sieve (Wiley TE-650/1). Once ground, natural materials, and oils were mixed in a proportion at 40% of baccharis, 40% of tamarind, 10% of CNLS and 10% of clove oil proportions and diluted in a Tween® 80 solution (5%). The extracts were made in different concentrations of 0.1; 0.2; 0.5 and 1.0 mg mL^{-1}. These concentrations represent typical amounts of compounds from plant extracts and EO supplied to ruminants diets in many researches (Tomkins et al., 2015; Prado et al., 2016; Ornaghi et al., 2017).

Every natural compound and oil were putting in an Erlenmeyer flask, then, they were mixing in 50 mL of Tween solution. Erlenmeyer flasks were shaking through a magnetic stirrer (Tecnal TE-0851) for 15 minutes three times, with 20 minutes of repose between stir, for homogenization of all materials. After shaking, each solution was filtered using filter papers (Whatman No1, 90 mm) and stored refrigeration (4 ºC). For the medium bacterial growing preparation, it was taken into account proposal Hobson's methodology (Hobson, 1969). The Hobson's M2 medium contained (per L): bacto-casitone (10.0 g); sodium hydrogen carbonate (4.0 g); yeast extract (2.5 g); cellobiose (2.0 g); maltose (2.0 g); glucose (2.0 g); cysteine-HCL (1.0 g); mineral solution I (150 mL); mineral solution II (150 mL); clarified rumen Fluid (200 mL); sodium lactate solution (10 mL); resazurin Solution (1 mL); and distilled water until complete 1000 mL. The medium bacterial growing culture was prepared under anaerobic conditions and a pH of approximated 6.8. After being ready, medium culture was distributing in Hungate tubes (9.0 mL), under the continuous flux of CO_2 and sealed with rubber septa and plastic caps, then, tubes were putting in the autoclave for 30 minutes and stored light free (Hungate, 1967).

Once the tubes with the medium are ready, the bacterial were cultured. Nine bacterial were using for the in vitro assay (Table 1). These nine bacterial are the most representative and significant in the ruminal functionality (Henderson et al., 2015; Roehe et al., 2016; Loor et al., 2016). For growing bacterial evaluated was calculated the optical density (OD) with a spectrophotometer (Thermo scientific, Genesys 10UV Scanning) having use a wave longitude at 600 nm. All bacteria, previously activation, were cultivated in a Hungate tube with 9 mL of medium bacterial growing. Six replicates of each concentration and one control by every bacterium were used in the assay, for a total of 36 tubes per bacteria. Treatment distribution into the tube were randomized and putting as follows: CONTROL (0.5 mL of

bacteria + 0.5 mL of Hobson's M2 medium); 0.1 mg mL^{-1} (0.5 mL of bacteria + 0.5 mL of natural extract at 0.1 mg mL^{-1} of concentration); 0.2 mg mL^{-1} (0.5 mL of the test bacteria + 0.5 mL of natural extract at 0.2 mg mL^{-1} of concentration); 0.5 mg mL^{-1} (0.5 mL of the test bacteria + 0.5 mL of natural extract at 0.5 mg mL^{-1} of concentration); 1.0 mg mL^{-1} (0.5 mL of the test bacteria + 0.5 mL of natural extract at 1.0 mg mL^{-1} of concentration). Incubation was making on a stove at 39 ºC for 24 hours. OD was measured at 0, 8, 12, and 24 hours while passed incubation.

In vivo antibacterial evaluation of natural mix

A total of 32 (½ Angus vs. ½ Nellore) bulls were used for in vivo assay. Bulls had a mean age of 24 ± 2.0 months and body weight of 418.0 ± 4.51 kg were distributed in a completely randomized design, with four diets and eight replications per diet. Bulls were weighed at the beginning of the study and, after, every 28 days using a trunk balance (Beckehauser Cia., Paranavaí, Paraná, Brazil). Every single bull was assigned to a 10 m^2 individual pen, with a feeder and drinker in each one. The research was made in Iguatemi experimental farm, a property of the State University of Maringá, (Iguatemi-Paraná, Brazil). This region has a humid temperate climate with an annual average temperature of 18 ºC and an annual average rainfall of 1,114 mm.

Diets were the same for all animals, formulated to have the same amount energy and protein contents (Table 2) according to the NRC (2016) (NRC, 2016). The basal diet was composed of 700 g/kg dry matter (DM) concentrate and 300 g/kg DM corn silage fed *ad libitum* for 74 days (Table 2). Four treatments were given to the animals with different concentration supplies of the natural mix as an additive, with the same concentration handled in the in vitro assay: CONT – basal diet; NM2 – basal diet and 2 g/animal/d of natural mix

compound of baccharis, tamarind, cashew nut shell liquid (CNSL) and oil clove; NM4 – basal diet and 4 g/animal/d of natural mix; NM6 – basal diet and 6 g/animal/d of natural mix. The mix contained 400 g/kg of baccharis (*Baccharis dracunculifolia*) leaves and stems, 400 g/kg of tamarind (*Tamarindus indica* L.) seeds, 100 g/kg of CNSL (*Anacardium occidentale* L.), and 100 g/kg of clove (*Syzygium aromaticum*) oil. The natural mix was prepared every 15 days to maintain a constant dosage per animal/day with a preserve antioxidant activities y of the secondary chemical compounds of the same.

Collect and processing of the ruminal liquid samples

Ruminal fluid was sampled 70 days after starting the experimental period, before the morning feeding. A flexible PVC tube (6 mm of diameter), previously sterilized, was inserted to a depth of 120-150 cm, approximately, via the esophagus. 100 mL of ruminal content sample were obtained using an electric vacuum pump and putting into an Erlenmeyer flask, then, carried to microbiology Laboratory in the State University of Maringá for centrifugation and filtration with the purpose of separate liquid and solid phase. Posteriorly, the liquid phase was frozen at -80 °C until processing. It was made a pooled of ruminal fluid for each treatment for the DNA extraction and sequencing, posteriorly.

DNA extraction, amplification, and sequencing

The extraction of the genetic material was carried out with a protocol of magnetic beads standardized in the laboratory. Bacterial identification was carried out by high throughput sequencing of the V3 / V4 regions of the 16S rRNA gene. Sequencing was performed on the MiSeq kit (Illumina Inc., USA), using the 300-cycle single-ended V2 kit, without standardization of the libraries. The sequences for each sample were processed using the

QIIME v1.5.1 pipeline at Laboratories Neoprospecta Microbiome Technologies (Florianopolis-Santa Catarina, Brazil), considering a maximum cumulative error of 1% in sequencing. To identify the species of microorganisms, present in the samples, the DNA sequences obtained were aligned with the Greengenes reference database (https://greengenes.secondgenome.com/).

Statistically analysis

For to the determinant in vitro effects of natural extract over each bacterium were used the gamma model with log connection function of the mixed generalized linear models. R software make use of the geeglm package was used to determinate the statistically difference between treatments and wherein all models were adjusted with the "AR1" correlation structure (R Core Team, 2017).

Adjusted model is as follows:

$Y = \beta o + \beta 1 \text{ time} + \beta 2 \text{ concentration} + \beta 3 \text{ time*} \text{ concentration} + \text{Ɛ(error)}$

The composition of each group at the taxonomic level was determined and plotted into histograms. Sequences were classified into operational taxonomic units (OTUs) based on a similarity threshold of 97%. The bioinformatics and statistical analyses were performed on OTUs. The QIIME software was used for representative sequences of each bacterial OTUs and was compared with databases (Nguyen et al., 2016).

Results and discussion

Phytochemical profile of Natural Mix Extract (NME)

NME phytochemical profile is showing in Figure 1. Within identified compounds more relevant it was determined that the cardanol, cardol, and eugenol presenting the most outstanding peaks in the ^{13}C-NMR spectrum of the NME.

Tropical plants contain a great quantity of secondary metabolites. Many antimicrobial compounds have been describing in tropical plants with antimicrobial effects (Gupta et al., 2016; Biondo et al., 2017). The major metabolites proportion of these plants are part of the phenolic compounds. Eugenol is the major compound finding in clove oil and it has great antimicrobial activity. The antimicrobial activity of this clove oil depending on the quantity of eugenol inside which can vary between 9.4 at 14.7 g by 100 g of dry matter of clove (*S. aromaticum*) (Shan et al., 2005). Likewise, cardol and cardanol, the other identify compounds finding in the natural mix, are secondary metabolites recognized in CNSL present a high antimicrobial activity. The chemical structure of cardol and cardanol having a similar composition as for the synthetic antibiotic, with an aliphatic and aromatic ring that has a potential against bacterial activity (Yuliana et al., 2014; Huang et al., 2019). Natural extracts are gaining importance as new antibiotics because synthetics molecules used in pharmaceutical industries for antibiotic fabrication have been creating bacterial resistance. Exist some reports that relate to the presence of tetracyclic and aminoglycoside on food with gastrointestinal diseases associated with Gram-positive bacterial resistance (Landers et al., 2012; Chen et al., 2019). With the comprehensive study of metabolites secondary in plants has been determined to could be existed an antimicrobial function in this kind of compounds and don’t be collateral effects in food products or human health (Patra and Saxena, 2009a; b; Wanapat et al., 2012; Olagaray and Bradford, 2019).

In vitro effect of Natural Mix Extract (NME) on ruminal bacterial

The effects of natural mix extract over Bacteroidetes showing in figure 2. There is an effect on optical density that indicates a decrease in growing pattern bacterial, mainly in 0.5 and 1 mg/mL^{-1} concentrations. *P. ruminicola* has been the most affected bacteria showing a less optical density significative in all times on the 0.5 and 1 mg/mL^{-1} concentrations, compared with the CONTROL group (0 mg/mL^{-1}). As for the *P. bryantii*, all concentrations have been decreased effect over optical density, compared with the CONTROL group, but only until 8 hours. After this time, existed an adverse effect of natural mix extract, increasing the optical density especially at 12 hours. *P. albensis* was the least affected bacterial by the natural mix extract, showing a decrease over optical density from 12 hours in the 0.5 and 1.0 mg/mL^{-1} concentrations.

Bacterial belonging to the *Bacteroides* phylum has been Gram-negative bacterial. Ionophores are the antibiotics more using for ruminal modification in livestock but reporting a low effect in Bacteroides phylum, specially *Prevotella* genus, in some cases ionophore cause an increased percentage of phyla *Bacteroides* (Shen et al., 2017). For the other hand, the metabolites associated with oil essentials have been a potential reduction in *Bacteroides* bacterial, especially in those bacterial related with ammonia production. *Prevotella* is a genus involved in 'hyper-NH_3-producing' (HAP) and eugenol has a good antimicrobial action against this genus because could be inhibited the breakdown of amino acids to NH_3 contributing with less substrate for the sustainability of this kind of bacterial (Wallace, 2004). However, *P. albensis* don't showing an important descend regarding other *Prevotellas* this may be due to this bacteria have to action in peptides degradation, which not presenting any affectation with to eugenol (Hartinger et al., 2018).

The effect of natural mix extract on *T. saccharophilum* and *S. dextrinosolvens* was notorious (Figure 3). It can be observed that all concentrations at the natural mix extract have been a decreasing effect over optical density, compared with CONTROL. This effect is more outstanding in 0.5 and 1.0 mg. mL^{-1} concentrations on *T. saccharophilum* and in the 0.5 mg. mL^{-1} concentration on *S. dextrinosolvens*.

Spirochaetes and *Proteobacteria* phylum are bacterial involved in the fermenting process, principally polysaccharide fermentation. These two bacterial having an enzyme complex of β-galactosidase and β-glucosidase, by which the role in ruminal degradation is over monosaccharides and simple sugars (Newbrook et al., 2017). Although they can act over non-fibrous carbohydrates also they are more sensitive to pH changes, principally acid ruminal environment caused by high-grain diets (Guo et al., 2020). Due to high sensitivity could have obtained the response of *T. saccharophilum* and *S. dextrinosolvens* in this *in vitro* assay. It has been reported that exists a slight decline of ruminal pH when supply natural plant extracts in the diet for ruminants (Patra and Saxena, 2011; Ornaghi et al., 2017).

Firmicutes phylum presents a variable performance in the optical density when it is submitted to the natural mix extract (Figure 4). *S. bovis* has been not showing any effect in some concentrations within the application of the natural mix extract. For other hand, Ruminococcus bacterial were affected highly. *R. albus* was decreased optical density in the minimum concentrations (0.1 and 0.2 $mg.mL^{-1}$) of the natural mix extract, while *R. flavefaciens* had less optical density in higher concentrations (0.5 and 1.0 $mg.mL^{-1}$) during all hours. As for the *L. multiparus*, 0.5 and 1.0 $mg.mL^{-1}$ concentrations were showing a decreasing effect over optical density only at the 12 and 24 hours, compared with CONTROL.

Firmicutes phylum has the principal groups of bacterial that attacking the fiber and implication in structural carbohydrates degradation is important. The bacterial of Firmicutes phylum are Gram-positive bacterial for what they are more sensitive to antibiotics or ionophore action (Gaskins et al., 2002; Tomkins et al., 2015). Phenolic compounds, as eugenol or cardol, have a similar response on ruminal bacterial to ionophore products. Phenolic compounds could form hydrogen complexes that interacting with the cell walls of the bacteria and inhibiting bacteria growth (Witzig et al., 2018). *R. albus* and *R. flavefaciens* are the more susceptible bacterial of *Firmicutes* phylum. Natural plant extracts can be properties that inhibited the dynamic growth of Gram-positive bacterial due to reduction of peptidolytic activity and therefore a decrease of nutrients for bacteria (Patra and Saxena, 2009a). The resistance of the genus Streptococcus is recognized in the literature because Streptococcus bacteria have R genes that increase survival against any external attack (Pompilio et al., 2019).

Characterization of rumen bacterial population

In the current study, it was identified bacterial populations and classified by phylum, genus, and species for each treatment. Within the phylum that has been found into the treatments were show that *Euryarchaeota* phylum has more proportion at the CONTROL (31%), while *Firmicutes* phylum had a major proportion (65.1%) in NM2 treatment and *Actinobacteria* phylum was the most proportion (56.8%) in NM4 treatment (Figure 5). Besides, NM6 has present the Bacteroidetes phylum as a major representative (3.6%) but the proportion is very low compared with the other phylum identified.

The rumen ecosystem has a variability as for bacterial, protozoal, and fungi population, but bacterial population has been the most important microorganism into the rumen

(Henderson et al., 2015). The phylum has presented an important variation between treatments but it is important to clarify that some effects on the phylums could be caused by the high content of grains in the diet. For example, phylum *Bacteroidetes* is finding in high proportions when the animals have a high forage diet, but when diets have high grain proportion this phylum decreasing notorious (Petri et al., 2014), as in this study. Firmicutes phylum was affected negatively at increases natural extract into the diets. Inclusive, in NM2 Firmicutes value has been an increasing percentage compared with CONTROL. This effect could be due to a bacterial selection beginning for natural additive. The chemical structure of secondary metabolites finding in the natural mix can affect the interactions to the substrate and bacteria action, modulating the ruminal microbiome and digestion, principally, of fiber (Cobellis et al., 2016; Du et al., 2019).

According to the variability of the genus between the treatments (Figure 6), we can observe that CONTROL has more genus of bacterial (3076) compared with the other treatments, and NM6 presents a lower value as for ruminal bacterial population variability (1686). Among the values that can be highlighted, *Methanobrevibacter* genus has been more present in CONTROL than in the other treatments (955 vs. 747 (NM2) vs. 729 (NM4) vs. 259(NM6). Also, the *Butyrivibrio* genus has been shown more important value, principally in NM2 (814) than in the other treatments (9 (CONTROL) vs. 7 (NM4) vs. 30 (NM6). *Bifidobacterium* genus is the most relevant genus into NM4 treatment represented 1422 OTUs into this treatment, while in CONTROL only 365, NM2 51 and NM6 143 OTUs of this genus. Into NM6 treatment, the most representative genus is *Olsenella* having 622 OTUs, compared with the other treatments that have less OTUs (303 (CONTROL) vs. 62 (NM2) vs. 249 (NM4).

It is important to show the variability of bacterial species that exist between treatments (Figure 7). Exist a great difference between CONTROL and the other treatments that is the higher number of OTUs of *Methanobrevibacter olleyae* (435) and *Methanobrevibacter thaueri* (408). While, *Butyrivibrio fibrisolvens* (795) is the specie more representative in treatment NM2, *Bifidobacterium ruminantium* (799) had more OTUs in NM4 treatment, and *Olsenella umbonata* (622) the bacteria with high value in NM6. Also, it should have a special focus on decreasing the methanogenic bacteria in NM6.

Referring to the bacterial modulation in genus and species between the treatments exist difference important in OTUs values that could be affecting co-products ruminal production. *Methanobrevibacter* genus decreased with natural mix supply what can indicate that natural mix has an effect on the methanogenic ruminal population. CNSL and clove oil are implicating in hydrogen-producing bacteria decreased and ammonia formation (Kobayashi et al., 2016; Díaz et al., 2018). When the formation of ammonia and hydrogens is affected by the decrease of formate in the rumen and, at the same time, cause growing inhibition of Methanobrevibacter species, especially *Methanobrevibacter thaueri* (de Paula et al., 2016; Kenny et al., 2018). For other hand, it is important to highlight the increase in OTUs of the *Butyrivibrio* genus in NM2. *Butyrivibrio* genus is implicated in hemicellulose degradation in the rumen, especially in xylans fermentation (Emerson and Weimer, 2017). Increment xylans degradation could be caused better butyric acid production in the rumen and improve intramuscular fat deposition and meat quality in cattle (Miguel et al., 2019). Indeed, the *Olsenella* genus, the main genus to NM6, is also associated with butyrate production and fermentation of non-structural carbohydrates (Watanabe et al., 2019; Fan et al., 2020). Even if, Olsenella species can be involved in methylamine production, a CH_4 precursor, *Olsenella*

umbonata don't produce methyl group by the CH_4 for that methane production is low or null and used pectin degradation, principally, for butyrate production (Kelly et al., 2019).

Conclusions

The use of natural plant extracts affects ruminal bacterial development. This effect could be indicated a change of ruminal population and improve the feed efficiency. *In vitro* effect was different in some bacterial species, compared with in vivo assay, it's could be due to a possible synergism of the different compounds into materials used for natural mix and product dry presentation. The answer to the natural extract against nine evaluated bacterial was higher in 0.5 and 1.0 $mg.mL^{-1}$ concentrations, in almost all bacteria, except *S. bovis*. As for the natural mix, the additive has been a marked response decreasing the *Methanobrevibacter* bacterial in NM6, what could suppose an improvement in feed efficiency decreasing methane production and increasing the AGV's. Another response related to the addition to natural mix in animal diets was the great proportion of *Actinobacteria* phylum in NM4, principally. Bacteria of *Actinobacteria* phylum could be related to an increase of non-structural carbohydrates degradation and occurring a decrease in acetate: propionate proportion, which also could be improved the feed efficiency of the animal. Comprehension of bacterial population changes causing natural additives could be determining a new way in organic production and improve, principally, feed efficiency.

Ethics approval

All experimental procedures were conducted under the surveillance of the Animal Care and Use Committee of the Universidade Estadual de Maringá, Brazil (protocol nº.

1103290719), and met the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

Conflict of interest

The authors declare that they have no conflicts of interest.

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Table 1. Ruminal bacterial using in the experiment

Phylum	Bacteria
Firmicutes	*Ruminococcus albus* (DSM 20455)
	Ruminococcus flavefaciens (DSM 25089)
	Streptococcus equinus (DSM 20558)
	Lachnospira multiparus (DSM 3073)
Bacteroidetes	*Prevotella albensis* (DSM 11370)
	Prevotella bryantii (DSM 11371)
	Prevotella ruminicola (ATCC® 19189™)
Spirochaetes	*Treponema saccharophilum* (DSM 2985)
Proteobacteria	*Succinivibrio dextrinosolvens* (DSM 3072)

Table 2. Ingredients and chemical composition of basal diet (g/kg of DM)

Ingredients (g/kg of DM)	Diet
Corn silage	308.4
Corn grain	636.1
Corn gluten	42.4
Limestone	3.5
Mineral and vitamin supplement[1]	3.5
Urea	5.7
Yeast (*Saccharomyces cerevisiae*)	0.4
Chemical composition (g/kg of DM)	
Dry matter	710.3
Crude protein	122.8
Organic matter	930.9
Ash	69.1
Ether extract	33.6
Non-fiber carbohydrates.	610.4
Neutral detergent fiber	255.4
Acid detergent fiber	74.4
Total digestible nutrients	791.4
Metabolizable energy[2] (Mcal/kg DM)	2.9
Calcium	5.2
Phosphorus	3.6

[1] Mineral salt composition (kg): calcium, 50 g; magnesium, 57 g; sodium, 81 g; sulphur, 3.75 g; cobalt, 20 mg; copper, 500 mg; iodine, 25 mg; manganese, 1.500 mg; selenium, 10 mg; zinc, 2.000 mg; vitamin A, 400.000 UI; vitamin D3, 50.000 UI; vitamin E, 750 UI; ether extract, 168 g; urea, 200 g.

[2] Estimated based on NRC (2000).

Figure 1. 13C-NMR spectrum (DMSO-d6, 75.5 MHz) of natural mix extract (NME).

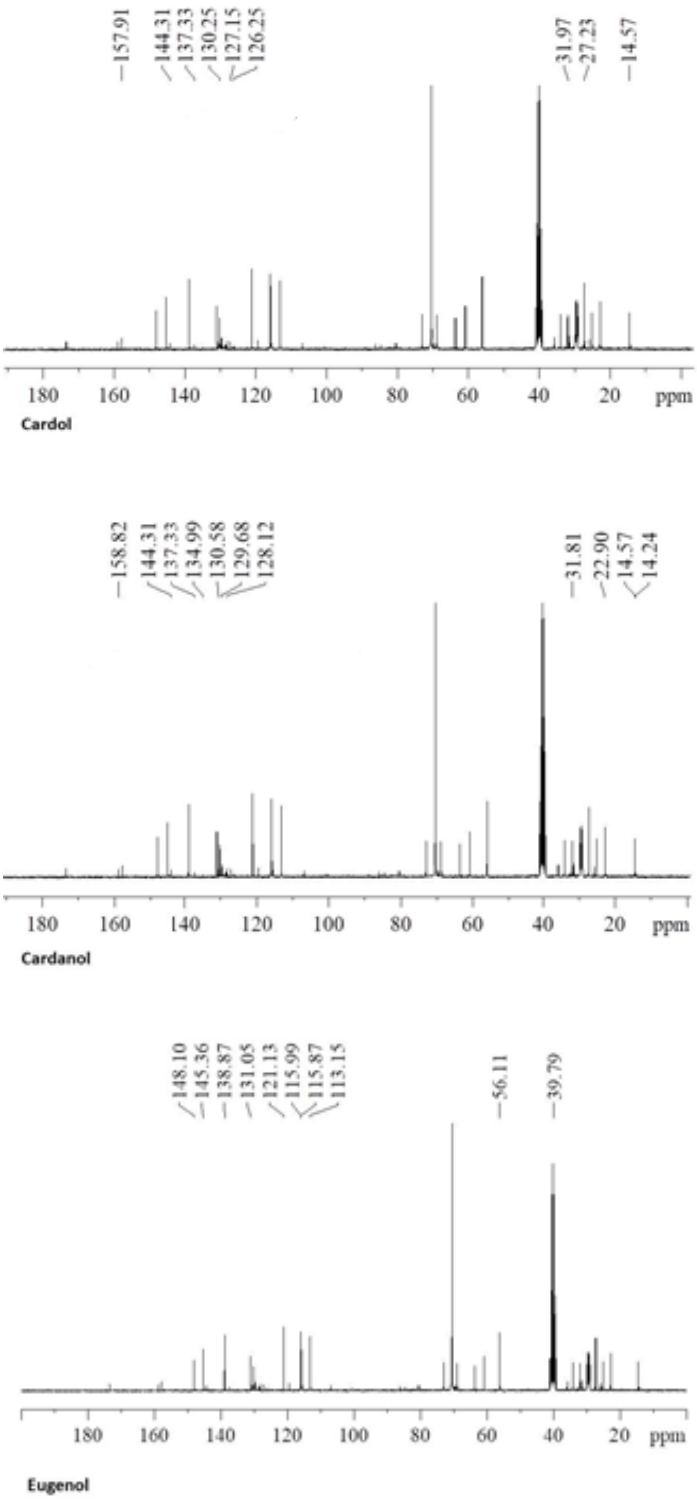


Figure 2. In vitro effect of natural mix extract against *Bacteroidetes*

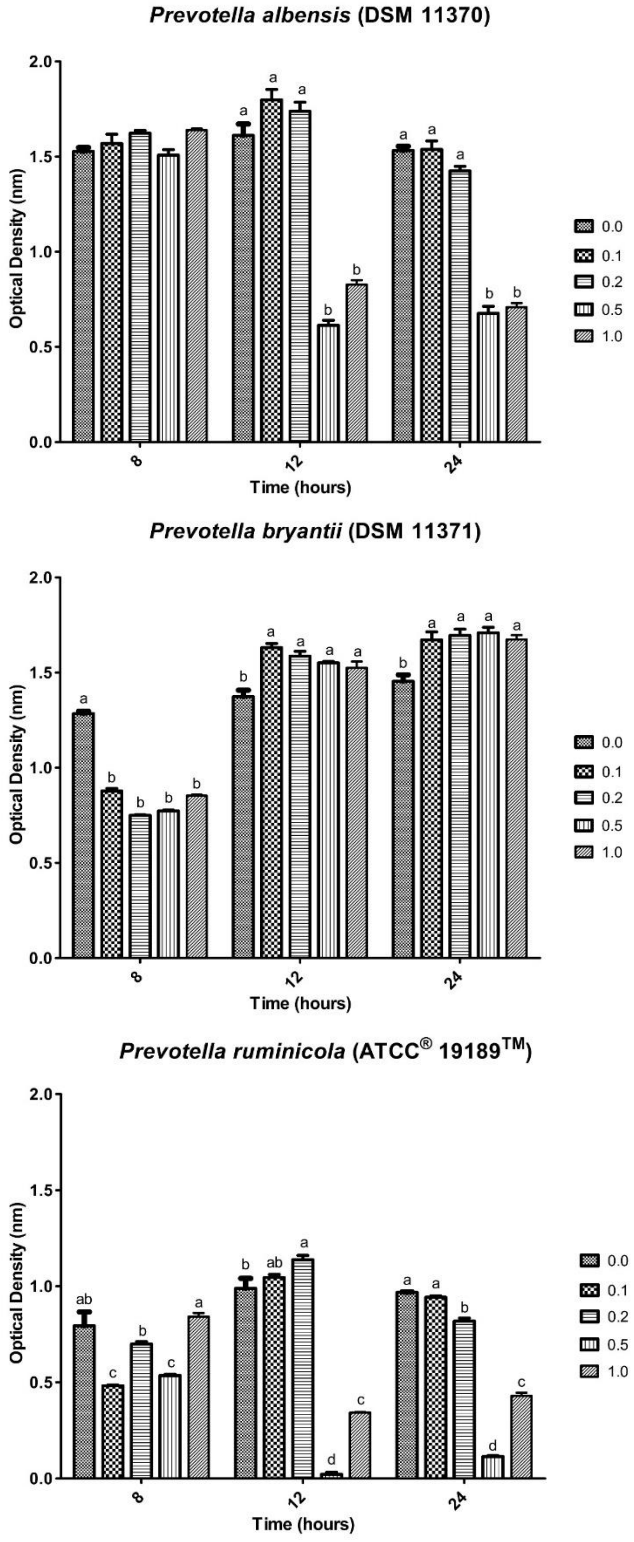


Figure 3. In vitro effect of natural mix extract against *Spirochaetes* and *Proteobacteria*

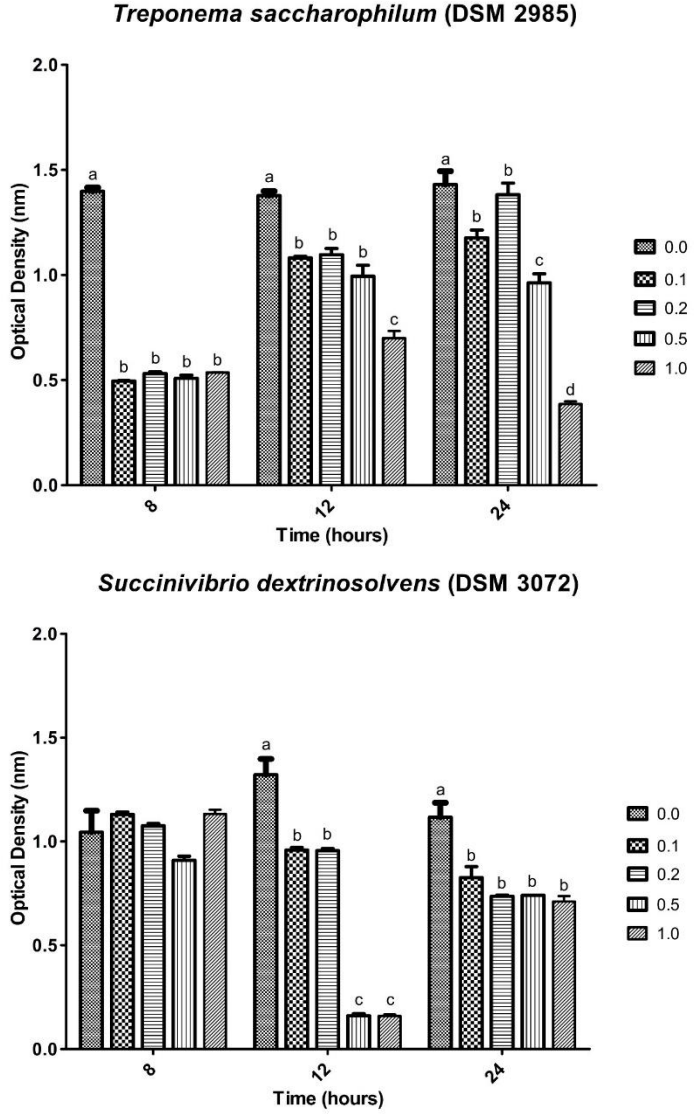


Figure 4. In vitro effect of natural mix extract against *Firmicutes*

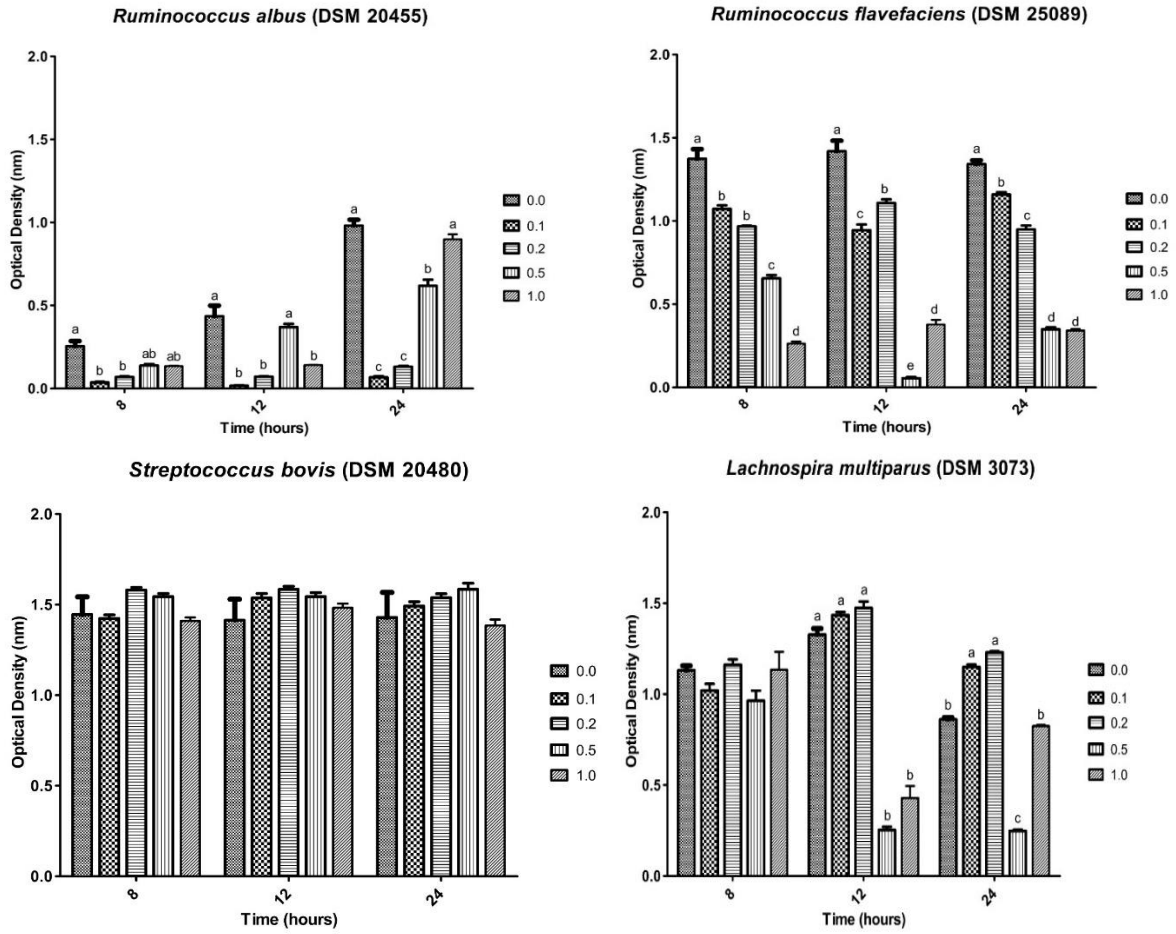


Figure 5. Effects of natural mix additive on rumen bacterial population (% phylum) in bulls feeding with high grain content.

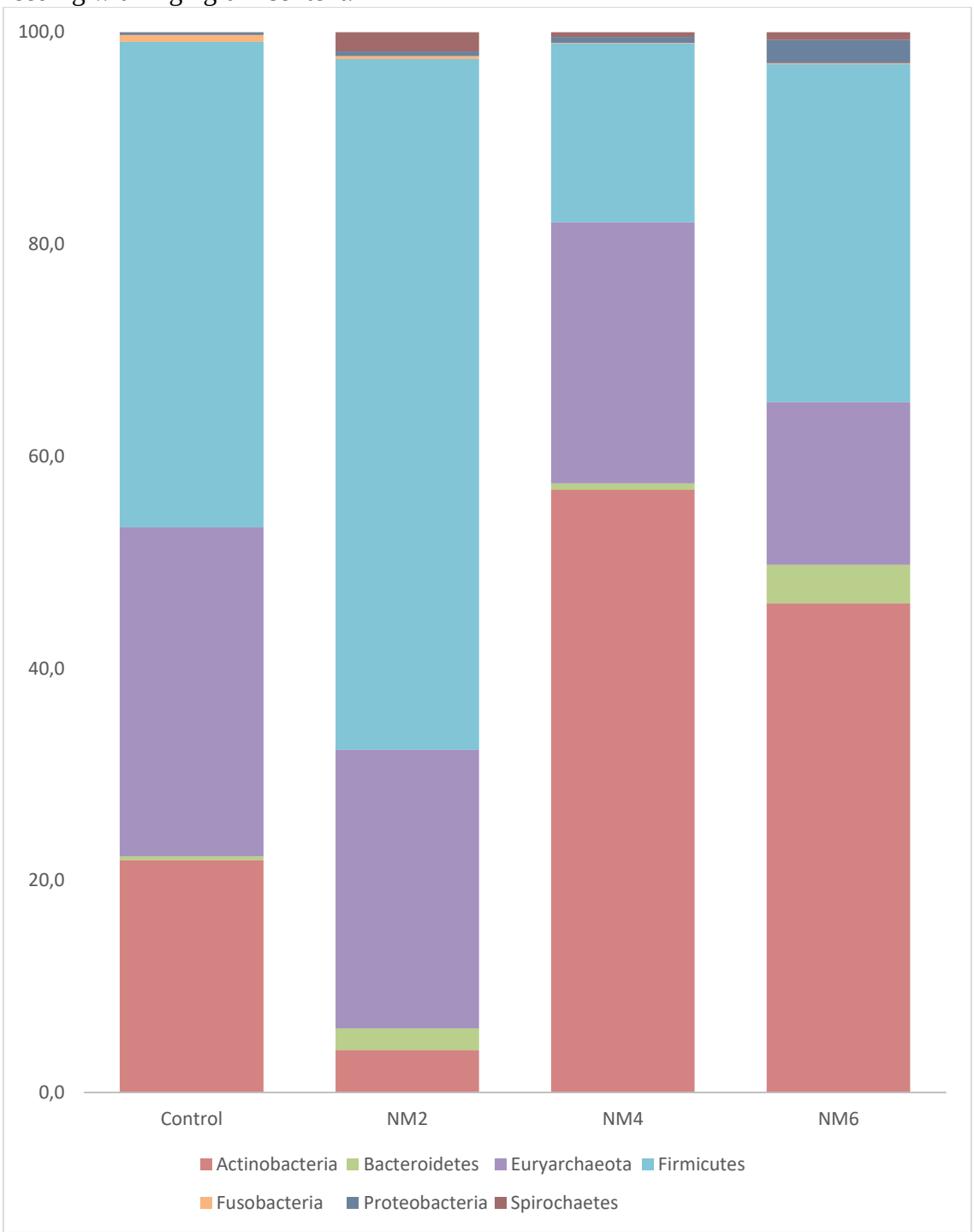


Figure 6. Effects of natural mix additive on rumen bacterial genus in bulls feeding with high grain content.

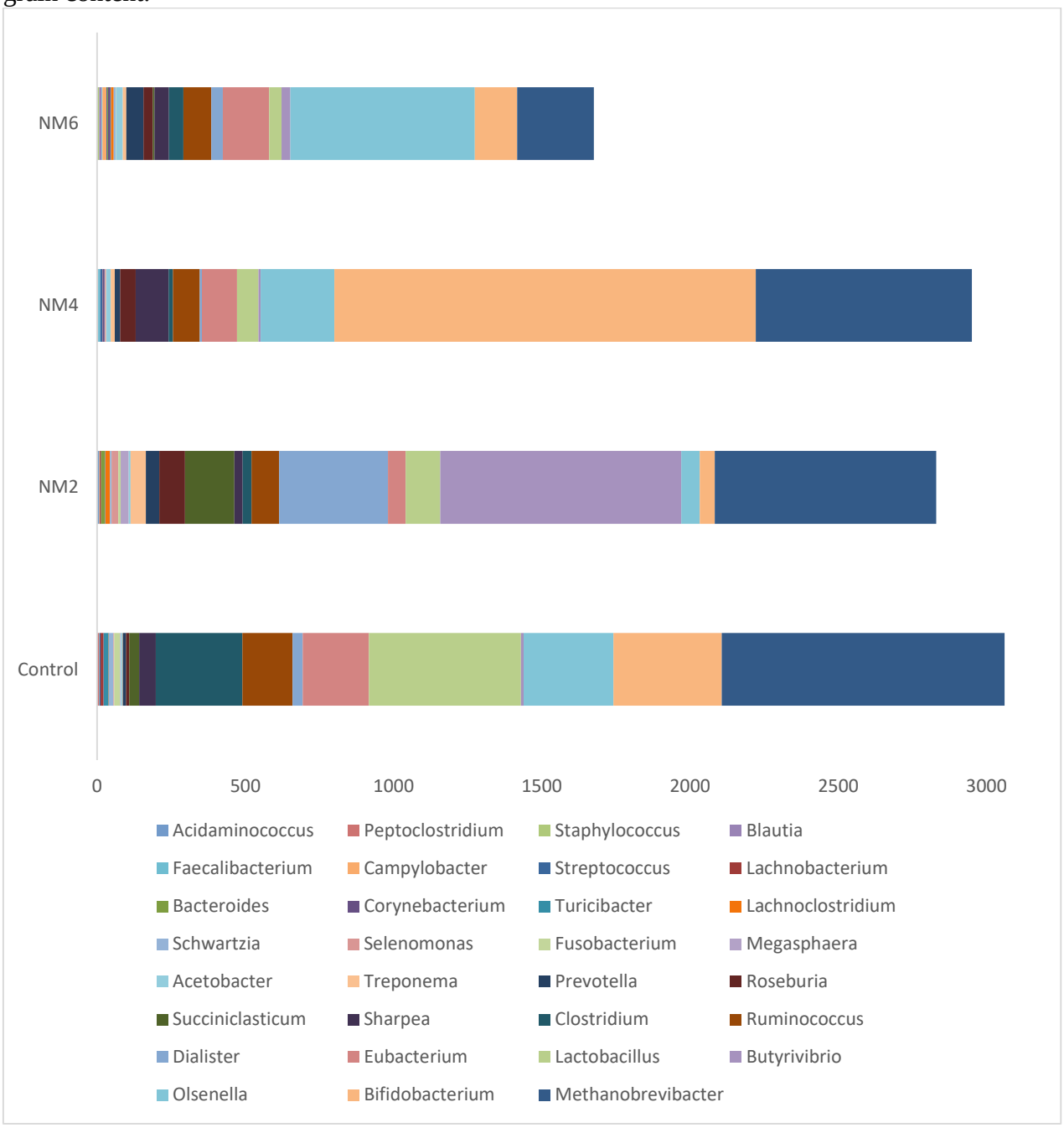
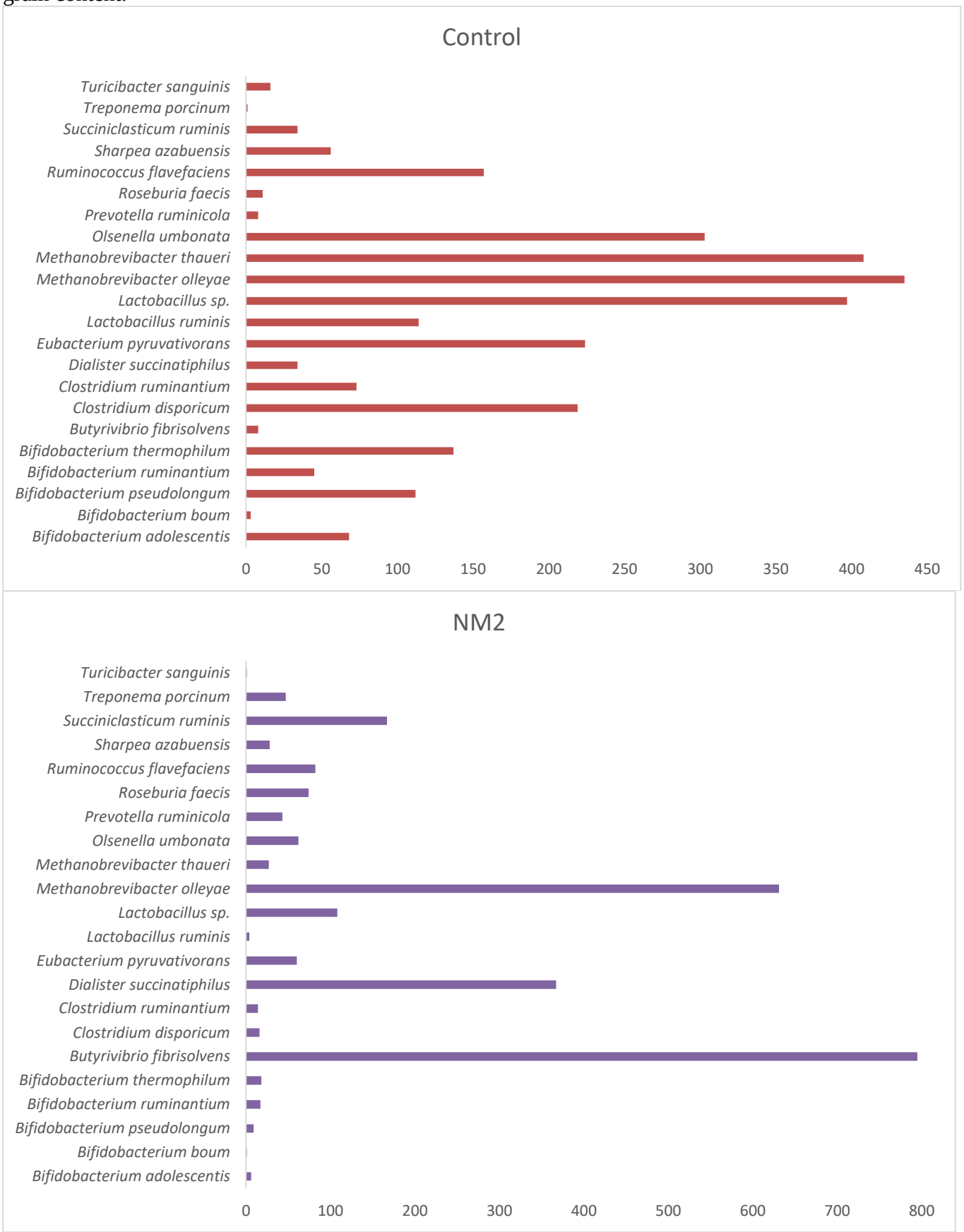
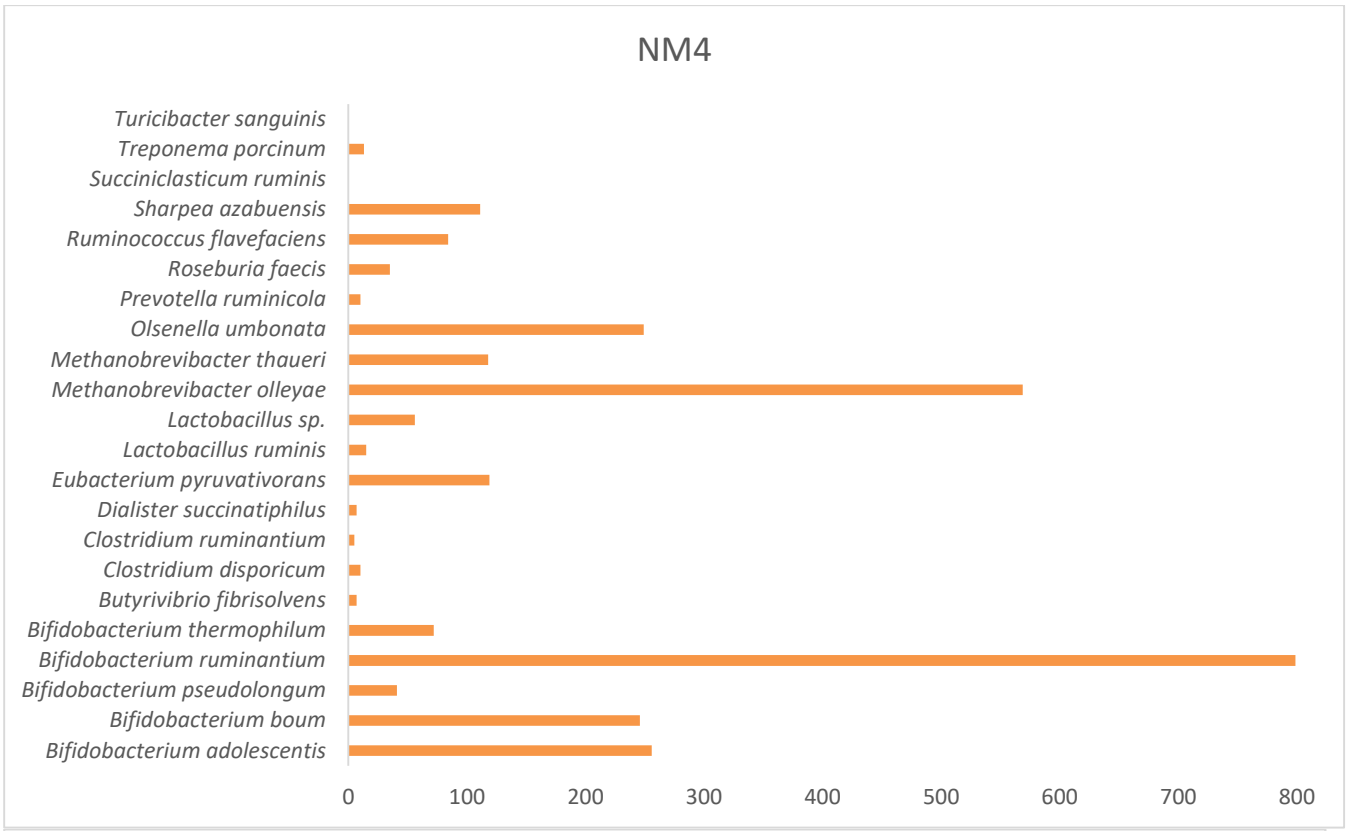
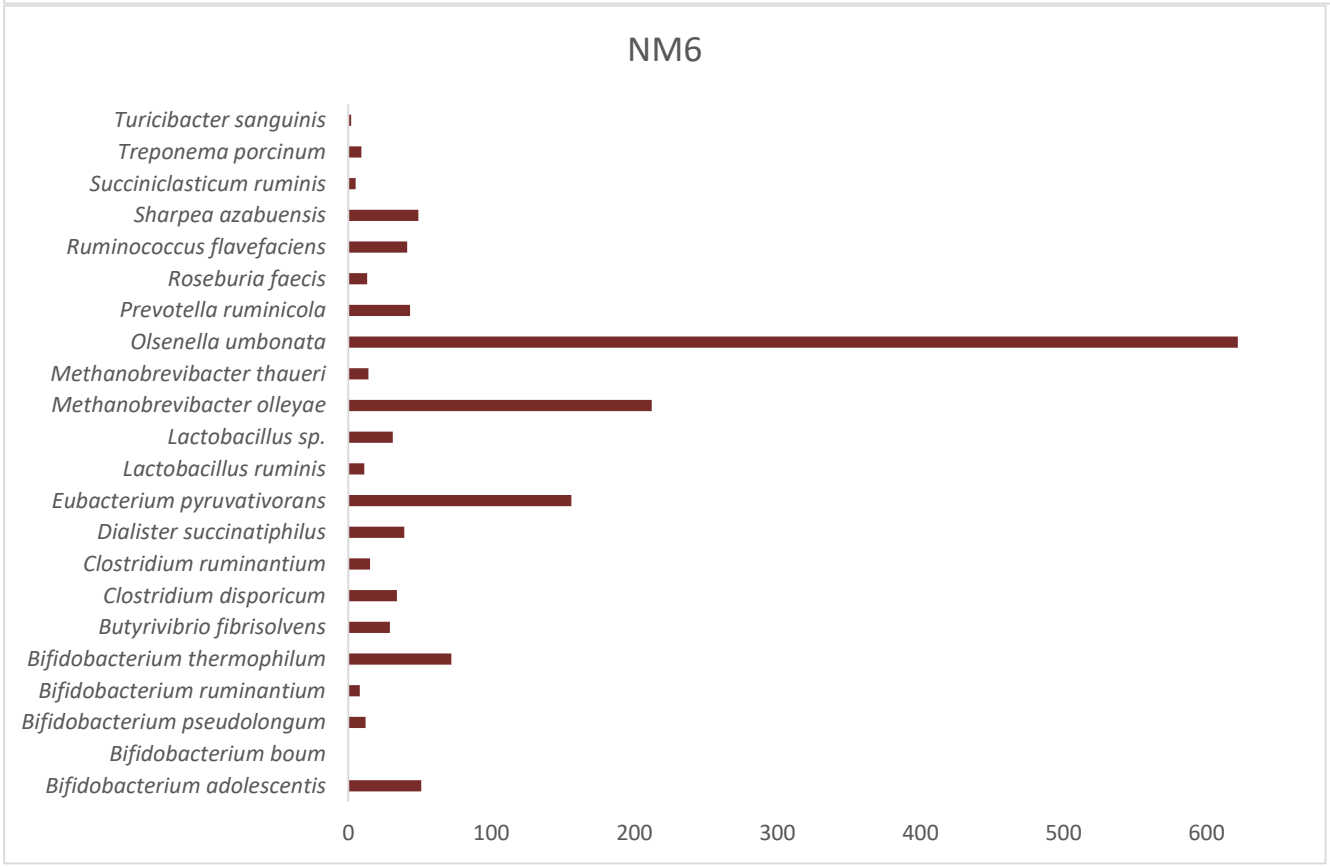


Figure 7. Effects of natural mix additive on rumen bacterial species in bulls feeding with high grain content.




NM4
Turicibacter sanguinis
Treponema porcinum
Succiniclasticum ruminis
Sharpea azabuensis
Ruminococcus flavefaciens
Roseburia faecis
Prevotella ruminicola
Olsenella umbonata
Methanobrevibacter thaueri
Methanobrevibacter olleyae
Lactobacillus sp.
Lactobacillus ruminis
Eubacterium pyruvativorans
Dialister succinatiphilus
Clostridium ruminantium
Clostridium disporicum
Butyrivibrio fibrisolvens
Bifidobacterium thermophilum
Bifidobacterium ruminantium
Bifidobacterium pseudolongum
Bifidobacterium boum
Bifidobacterium adolescentis
0
100
200
300
400
500
600
700
800


NM6
Turicibacter sanguinis
Treponema porcinum
Succiniclasticum ruminis
Sharpea azabuensis
Ruminococcus flavefaciens
Roseburia faecis
Prevotella ruminicola
Olsenella umbonata
Methanobrevibacter thaueri
Methanobrevibacter olleyae
Lactobacillus sp.
Lactobacillus ruminis
Eubacterium pyruvativorans
Dialister succinatiphilus
Clostridium ruminantium
Clostridium disporicum
Butyrivibrio fibrisolvens
Bifidobacterium thermophilum
Bifidobacterium ruminantium
Bifidobacterium pseudolongum
Bifidobacterium boum
Bifidobacterium adolescentis
0
100
200
300
400
500
600