UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS

COMPREENDENDO O MICROBIOMA RUMINAL E OS IMPACTOS NA QUALIDADE DA CARNE DE BOVINOS RECEBENDO ADITIVOS NATURAIS NA DIETA

Autora: Mariana Garcia Ornaghi
Orientador: Prof. Dr. Ivanor Nunes Do Prado
Coorientadora: Prof. Drª Sharon Ann Huws

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Tese apresentada, como parte das exigências para obtenção do título de Doutora em Zootecnia, no Programa de Pós Graduação em Zootecnia da Universidade Estadual de Maringá – Área de concentração: Produção animal.

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TITULAÇÃO: Doutora em Zootecnia - Área de Concentração Produção Animal

APROVADA em 21 de fevereiro de 2020.

Profª Drª Ana Guerrero

Prof. Dr. Hilário Cuquetto Mantovani

Prof. Dr. Everson Zotti

Prof. Dr. João Luiz Pratti Daniel

Prof. Dr. Ivanor Nunes do Prado
Orientador

A mais bela coisa que podemos vivenciar é o mistério. Ele é fonte de qualquer arte verdadeira e qualquer ciência. Aquele que desconhece esta emoção, aquele que não para mais para pensar e não se fascina, está como morto: seus olhos estão fechados.

Albert Einstein

A Deus, por guiar meus passos e me dar forças para seguir a diante.

À minha filha, Ana Clara Ornaghi Bombarda.

Aos meus pais, Emireno Ornaghi Junior (*in memoriam) e* Marieti Garcia Ornaghi *(in memoriam).*

À minha avó, Maria Fernanda Garcia (*in memoriam)*.

Ao meu irmão Angelo Heitor Ornaghi.

DEDICO

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BIOGRAFIA

MARIANA GARCIA ORNAGHI, filha de Marieti Garcia Ornaghi e Emireno Ornaghi Junior, nasceu em 18 de outubro de 1991 em Maringá, Estado do Paraná - Brasil.

Em março de 2009, ingressou no curso de graduação em Zootecnia Bacharelado, na Universidade Estadual de Maringá, em dezembro do mesmo ano iniciou projeto de iniciação científica na área de bovinocultura de corte dando continuidade a esta em 2010 como bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), obtendo o título de Zootecnista em 2013.

Em 2014, ingressou no curso de Pós-graduação em Zootecnia, em nível de mestrado, na área de concentração Nutrição e Produção Animal, pela Universidade Estadual de Maringá, realizando estudos na subárea de Grandes Ruminantes e em fevereiro de 2016 obteve o título de Mestre

Em março de 2016 iniciou no curso de doutorado no programa de Pós-graduação em Zootecnia da Universidade Estadual de Maringá, na área de Nutrição e Produção Animal, desenvolvendo estudos na subárea de Grandes Ruminantes e em maio de 2018 submeteu-se ao exame de qualificação.

Em agosto de 2018 foi contemplada pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) a participar do doutorado sanduíche na Queen's University Belfast, em Belfast, Irlanda do Norte, Reino Unido, até agosto de 2019.

No dia 21 de fevereiro de 2020, submeteu-se a banca de defesa de tese, requerimento para obtenção do título de Doutora em Produção Animal pelo Programa de Pós-Graduação em Zootecnia da Universidade Estadual de Maringá.

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RESUMO

A atual busca por produtos naturais e saudáveis está em expansão. O uso de compostos sintéticos está sendo limitado ou até banido por órgãos da saúde, tanto na indústria alimentícia como no uso de promotores de crescimento na produção animal. No sistema de produção de bovinos em confinamento, principalmente quando são utilizadas dietas com alto teor de concentrado, é necessária a adição de compostos para auxiliar na modulação do rúmen. Neste contexto, é necessário o desenvolvimento de substâncias alternativas não invasivas na alimentação animal. Assim sendo, as substâncias naturais se tornaram promissoras substitutas para os sintéticos, por apresentar ação similar e algumas vezes mais efetiva na produção de ruminantes. Entretanto, para sua adição na alimentação animal é necessário caracterizar os vários produtos de plantas, bem como conhecer o modo de ação destas substâncias. Os óleos essenciais, funcionais e seus compostos apresentam ação antimicrobiana, antioxidante, antiviral, entre outras. Essas propriedades provêm principalmente do efeito sinérgico dos seus constituintes que potencializam os efeitos benéficos. O objetivo de desenvolver este estudo foi avaliar o desempenho e eficiência alimentar, microbioma ruminal, comportamento ingestivo, característica de carcaça, qualidade da carne e aceitabilidade sensorial de 40 novilhos mestiços (½Angus - ½Nelore) com 16 ± 2,2 meses de idade, peso corporal inicial médio de 385,8 ± 20,7 kg sem adição ou com diferentes níveis (1,5; 3,0; 4,5 ou 6,0 g/dia/animal) de um *blend* contendo aditivos naturais, sendo esses, óleo essencial de cravo, óleos funcionais de caju e mamona e compostos microencapsulados (eugenol, timol e vanilina). O período de confinamento foi de 62 dias. O comportamento ingestivo (tempo de ingestão de água, ruminação, alimentação e ócio) foi semelhante entre as dietas (P>0,05). O desempenho animal (ganho médio diário e eficiência alimentar) apresentou aumento linear com inclusão de aditivos naturais (P<0,05). O consumo de matéria seca não apresentou efeitos

(P>0,05) de qualquer nível de dosagem, assim como, as características de carcaça (peso de carcaça quente e rendimento de carcaça). Os produtos da fermentação ruminal (ácidos graxos voláteis e amônia ruminal) apresentaram decréscimo significativo (P<0,05). Além disso, o microbioma ruminal apresentou mudanças significativas com a inclusão dos aditivos naturais (P<0,05). Entretanto, o pH ruminal não diferiu entre os tratamentos (P>0,05). Ainda, os parâmetros de qualidade de carne avaliados pH, textura, oxidação lipídica e coloração foram significativamente diferentes entre os tratamentos (P<0,05), entretanto a capacidade de retenção de água não foi influenciada. Para as avaliações de características organolépticas como odor e *flavour* não foram observadas diferenças significativas, porém, os textura e aceitabilidade geral apresentaram aceitação superior dos animais que receberam adição do *blend* de acordo com os consumidores avaliados (P<0,05). Os resultados indicam que o *blend* de aditivos naturais pode melhorar o desempenho animal a partir da manipulação da fermentação ruminal atuar no produto final melhorando características de qualidade de carne de bovinos terminados em confinamento submetidos à dieta alto grão.

Palavras chave: extratos de plantas, óleo essencial, óleo funcional, microrganismos ruminais.

ABSTRACT

The search for natural products is rising. The use of synthetic compounds is being limited or even banned by health agencies in both food and feed industry. The addition of compounds to improve the rumen fermentation are necessary in high-grain diets fed to feedlot cattle diet. Thus, the substances development in animal feed is necessary and natural substances have become promising substitutes for synthetics since they have a similar or even further effectiveness on ruminant production. However, it is necessary to understand the plant products diversity and its mode of action, as many of them remain unknown. The essential and functional oils, and their compounds present antimicrobial, antioxidant, antiviral, and others actions. These properties are mainly from the synergistic effect of the constituents that potentiate their beneficial effects. The aim with this study was to evaluate the animal performance and feed efficiency, rumen microbiome, intake behavior, carcass characteristics, meat quality and beef sensory acceptability from 40 crossbred steers (½Angus - ½Nelore) with 16 ± 2.2 months old, average initial body weight of 385.8 ± 20.7 kg. Diets had no additive, or different levels (1.5, 3.0, 4.5 or 6.0 g / day/animal) of a blend containing natural additives such as oil clove essential oil, cashew and castor oil functional oils and commercial microencapsulated compounds (eugenol, thymol and vanillin). The feedlot period lasted 62 days. Intake behavior (water intake, rumination, feed intake and idle time) was similar between diets ($P>0.05$). Animal performance (average daily gain and feed efficiency) showed a linear increase with the inclusion of natural additives ($P<0.05$). Dry matter intake had no effects ($P>0.05$) of any dosage used, as well as carcass characteristics (hot carcass weight and hot carcass dressing). Volatile fatty acids and ruminal ammonia showed a decrease ($P<0.05$). Also, the ruminal microbiome showed significant changes with the natural additives inclusion ($P<0.05$). However, ruminal pH did not differ between treatments ($P>0.05$). Furthermore,

the meat quality (pH, shear force, lipid oxidation and meat color) was influenced by diets ($P<0.05$), while the water losses were not influenced by the natural additives blend addition. The sensory evaluation as odor and flavor were similar between treatments ($P>0.05$). Tenderness and overall acceptability had higher scores with natural compounds addition ($P<0.05$). The natural additives blend can improve animal performance through rumen microbiome manipulation, impacting the final product and improving meat quality on cattle finished in feedlot.

Keywords: essential oils, functional oils, microorganisms, plant extracts, rumen.

I. INTRODUÇÃO

Embora o Brasil esteja entre os maiores produtores e exportadores de carne bovina do mundo, ainda apresenta baixa produtividade e baixa qualidade da carne, sobretudo, dos animais terminados em pastagens (Moreira et al., 2003, Rotta et al., 2009a, Rotta et al., 2009b). Assim, observa-se a necessidade de investimentos em tecnologias que promovam a produção de carne com eficiência, qualidade e de forma econômica, com a finalidade de incrementar a margem de lucro do produtor para manter e conquistar novos mercados consumidores. Isto pode ser alcançado com a intensificação do sistema de produção com o uso de ferramentas e práticas de manejo.

No entanto, os sistemas de produção intensiva de carne bovina, como animais terminados em semiconfinamento ou confinamento apresentam maior custo de produção em função da necessidade de aumentar a densidade energética e proteica da ração (Silva et al., 2010).

Os ionóforos são antibióticos que aumentam a eficiência de utilização de alimentos pelos ruminantes (Goodrich et al., 1984, Russell & Strobel, 1989). Russell & Strobel (1989) e Chen & Russell (1991) afirmam que a monensina reduz a produção ruminal de amônia pela inibição da população de bactérias gram-positivas, fermentadoras obrigatórias de aminoácidos e com alta capacidade de produção de amônia, como, por exemplo, as espécies *Peptostreptococcus anaerobius* C, *Clostridium sticklandii* SR e *Clostridium aminophilum*, possibilitando melhor aproveitamento da dieta pelo animal.

O uso rotineiro de antibióticos e promotores de crescimento na alimentação animal tem preocupado a saúde pública (Benchaar et al., 2006, Khorrami et al., 2015). As restrições impostas à utilização de antibióticos na alimentação animal têm como base preocupações ao desenvolvimento de microrganismos resistentes pelo uso inadequado de

ionóforos comprometendo a ação terapêutica dos antibióticos em humanos. Neste contexto, é importante ressaltar o impacto dos ruminantes, os quais podem ser considerados “reservoir” para desenvolvimento e propagação de resistência microbiana pela sua complexidade e abundância microbiológica (Auffret et al., 2017).

Os extratos naturais são metabólitos secundários podendo ser extraídos de várias partes de uma planta, incluindo folhas, flores, sementes, raízes e cascas (Benchaar et al., 2008). Os compostos secundários presentes nesses extratos possuem propriedades antioxidantes, antimicrobianas, analgésica, descongestionantes, anestésica, fungicida entre outras (Burt, 2004). Sua propriedade antimicrobiana é a partir da ação que exercem sobre os microrganismos, principalmente bactérias gram-positivas. De acordo com Bergen and Bates (1984) os óleos essenciais melhoram a eficiência energética, por causa da manipulação da flora bacteriana e pela maior produção de propionato, melhora a utilização de compostos nitrogenados, diminuindo as bactérias proteolíticas e reduzem também a incidência de desordens ruminais, pois podem diminuir a produção de ácido lático.

Extratos naturais de plantas contêm uma ampla variedade de compostos com diferentes funções e mecanismos de ação. Esses atuam de forma específica de acordo com sua estrutura química, ligando-se a sítios específicos na célula bacteriana, acarretando na desintegração da membrana citoplasmática, alterando o fluxo de elétrons e coagulação do conteúdo celular (Burt, 2004). Contudo, esses extratos são promissores como substituto dos ionóforos atualmente utilizados, tornando-se necessário estudar adequadamente aspectos relacionados à composição química, especialmente quanto aos seus princípios ativos, à sua atividade biológica, ao modo de ação, à eficiência no sistema de produção e ao facilitando a sua adoção pelas cadeias produtivas.

II. REVISÃO DE LITERATURA

Aditivos na dieta de ruminantes

Os ionóforos são substâncias que aumentam a eficiência de utilização de alimentos pelos ruminantes (Goodrich et al., 1984, Russell & Strobel, 1989), pois, atuam na microbiota ruminal manipulando os produtos da fermentação a favor do ruminante.

Em uma revisão de pesquisas com grande número de animais, Goodrich et al. (1984) verificaram que a monensina melhora a eficiência alimentar de bovinos em confinamento em 7,5% e o ganho de peso de bovinos em pastagens em 13,5%. Esta melhora na eficiência é decorrente do aumento da eficiência de utilização dos alimentos, provocado, em parte, pela diminuição na produção de amônia ruminal e gás metano (Vyas et al., 2018).

Russell & Strobel (1989) e Chen & Russell (1991) acreditam que a monensina reduz a produção ruminal de amônia pela inibição da população de bactérias gram-positivas, fermentadoras obrigatórias de aminoácidos e com alta capacidade de produção de amônia, como, por exemplo, as espécies *Peptostreptococcu sanaerobius* C, *Clostridium sticklandii* SR e *Clostridium aminophilum.*

Extratos naturais na nutrição de ruminantes

As restrições impostas à utilização de antibióticos na alimentação animal tem como base preocupações com o desenvolvimento de microrganismos resistentes, pelo uso inadequado de ionóforos que prejudicam a atividade terapêutica dos antibióticos em humanos (Russell & Houlihan, 2003, Dewulf et al., 2007, Ray et al., 2007). Em ruminantes a inclusão de ionóforos na dieta tem como objetivo manipular a fermentação ruminal para melhorar os processos benéficos e minimizar processos ineficientes (produção de gás metano – CH_4 e gás carbônico – CO_2). De modo geral, a ação dos ionóforos nas bactérias, principalmente gram-positivas modifica o fluxo de íons na membrana celular (Bergen & Bates, 1984, Russell & Strobel, 1989).

Extratos naturais de plantas contêm ampla variedade de compostos com diferentes funções e mecanismos de ação. Os compostos naturais atuam de forma específica de acordo com sua estrutura química ligando aos sítios específicos na célula bacteriana, acarretando na desintegração da membrana citoplasmática, alterando o fluxo de elétrons e coagulação do conteúdo celular (Kamra & Singh et al., 2019).

Dentre os compostos que apresentam características de ação antimicrobiana presentes nas plantas, encontra-se a classe dos compostos fenólicos (fenóis simples – cetocol, ácidos fenólicos – ácido anacárdico, cinâmico, cafeico e ricininoleico, quinonas – hipericina, flavonóis – totarol, taninos – elagitanina, cumarinas – warfarin); óleos essenciais e terpenoides (capsaicina, thimol, mentol, carvacrol, cânfora, eugenol); alcaloides (berberina, piperina,teofilina); polipetídeos e lectinas (Manose-aglutinina, fabatina, thionina); e poliacetilenos (Heptadeca-dieno-diol), cada um com seu respectivo mecanismo de ação (Kubo et al., 1992, King & Tempesta, 1994, Perrett et al., 1995, Cichewicz & Thorpe, 1996, Fernández et al., 1996, Freiburghaus et al., 1996, Stern et al., 1996, (Peres et al., 1997, , Zhang & Lewis, 1997).

Compostos fenólicos determinam sua capacidade de atuar em função do grau de metoxilação e o número de hidroxilas para atuarem como agentes redutores contra o estresse oxidativo (Verçosa, 2012). O termo ácido fenólico é utilizado a fenóis associados ao ácido carboxílico funcional.

Óleo funcional de caju

O cajueiro é uma planta nativa da Amazônia e nordeste do Brasil, denominada cientificamente de *Anacardium occidentale L*. Além do consumo do fruto e do suco são usados na indústria outros derivados do caju. No processo industrial para obtenção da amêndoa, origina-se o líquido da castanha de caju (LCC). Utilizado para diversas aplicações na indústria (Calo et al., 2015), o LLC possui altas concentrações de lipídeos fenólicos que o torna a maior fonte de origem natural dos ácidos anacárdico, cardol e cardonol. As concentrações dos ácidos variam em função do processo de obtenção da amêndoa (Mazzetto et al., 2009). De acordo com Mazzetto et al. (2009) a concentração dos ácidos graxos no LLC varia de 71,70 a 82,00% para o ácido anacárdico, de 13,80 a 20,10% para o ácido cardol e 1,60 a 9,20% para o ácido cardonol. De modo geral, o LLC é obtido com temperaturas elevadas alterando a estrutura química dos ácidos graxos pela reação de descarboxilação originando maiores teores do ácido cardanol.

Óleo funcional de mamona

A planta mamona denominada de *Ricinus communis* L. está disseminada principalmente na região nordeste pelas características de adaptação ao clima seco com elevadas temperaturas. De acordo com Costa et al. (2004), o óleo extraído da semente da mamona varia de 35 a 55% apresentando altos teores do ácido ricinoleico (cis-12-hydroxyoctadeca-ácido-9-enoico). A concentração do ácido ricinoleico no óleo da semente de *Ricinus communis* L. corresponde de 85 a 90% (Vaisman et al., 2008), seguido de outros ácidos graxos em menor proporção como o ácido linoleico (4,2%), ácido oleico (3,0%), esteárico (1,0%), palmítico (1,0%), ácido hidroxi esteárico (0,7%), ácido linolênico (0,3%) e ácido eicosanoico (0,3%) (Ogunniyi, 2006). De acordo com Ogunniyi (2006), o processo de extração do óleo de mamona pode ser obtido por prensagem mecânica e utilização de solventes. Segundo Costa et al. (2004), a presença de hidroxila (cis-12-hydroxyoctadeca-9-enoic acid) em sua estrutura química aumenta sua densidade e viscosidade em comparação a outros óleos, além de desempenhar ação antimicrobiana semelhante ao ionóforo e ação anti-inflamatória. A versatilidade do ácido ricinoleico permite a utilização do óleo na indústria farmacêutica e cosmética para fabricação de impermeabilizantes, lubrificantes, tintas, sabões, aditivos para polímeros e na produção do biodiesel.

Óleo essencial de cravo

O cravo é uma planta arbórea oriundo das ilhas molucas (conjunto de ilhas da Indonésia) de nome científico *Syzygium aromaticum*. O cravo da índia é uma especiaria muito apreciada, utilizado desde a antiguidade como condimentos e fabricação de remédios. Planta aromática com cheiro característico e pode ser extraído o óleo essencial que possui propriedades singulares como: antisséptico, antimicrobiano, anti-inflamatório, antioxidante, entre outras. Essas características são devidas aos compostos presentes na planta, eugenol, acetato de eugenol, beta-cariofileno entre outros. Dentre desses compostos, o mais abundante é o eugenol, a quantidade desses compostos irá variar de acordo com a parte da planta a ser extraído o óleo, além disso, o potencial de ação apresentado também pode variar de acordo com maturidade da planta, época de colheita para extração e localização geográfica. A ação antimicrobiana já foi relatada por muitos

autores e pode ser observada grande potência desse produto (Abdullah et al., 2015; Calo et al., 2015; Doninelli et al., 2010; Farag et al., 1989).

Potencial antimicrobiano dos extratos naturais

Os extratos naturais têm ampla variedade de efeitos sobre a saúde, incluindo efeitos positivos sobre as doenças cardiovasculares, alguns tumores, processos inflamatórios, e em geral, doenças nas quais ocorre a proliferação descontrolada de radicais livres (Harborne, 1999, Reddy et al., 2003, Trouillas et al., 2003). Estas propriedades dependem de sua capacidade de neutralizar radicais livres, inibir a peroxidação dos lipídeos nas membranas, quelatar os metais e estimular a atividade antioxidante das enzimas (Gutiérrez et al., 2003, Lee et al., 2003). Contudo, as atividades mais importantes destes compostos são como antissépticas e antimicrobianas. As propriedades antissépticas de muitas plantas são conhecidas desde a antiguidade. Os chineses, por exemplo, começaram a usar plantas medicinais em terapias 5.000 anos atrás (3.000 a.C.), os egípcios usavam plantas para a conservação de alimentos e em cerimônias de mumificação (Davidson & Naidu, 2000). No entanto, a primeira prova científica descrevendo suas propriedades antimicrobianas apareceu no início do século 20 (Hoffmann & Evans, 1911).

Desde então, muitos compostos dos óleos essenciais com fortes atividades antimicrobianas foram estudados (Burt, 2004). Terpenoides e fenilpropanoides desenvolvem suas ações contra bactérias interagindo com as membranas celulares (Griffin et al., 1999, Davidson & Naidu, 2000, Dorman & Deans, 2000). Parte desta atividade é pela natureza hidrofóbica dos hidrocarbonetos, que lhes permite interagir com a membrana das células e se acumular na bicamada lipídica das bactérias, ocupando um espaço entre as cadeias dos ácidos graxos (Ultee et al., 1999). Esta interação provoca alterações na conformação nas estruturas das membranas, celulares resultando em sua permeabilização e expansão (Griffin et al., 1999). A desestruturação da membrana altera a estabilidade das trocas de íons pela membrana da célula provocando redução no gradiente de troca iônico na membrana (Figura 1).

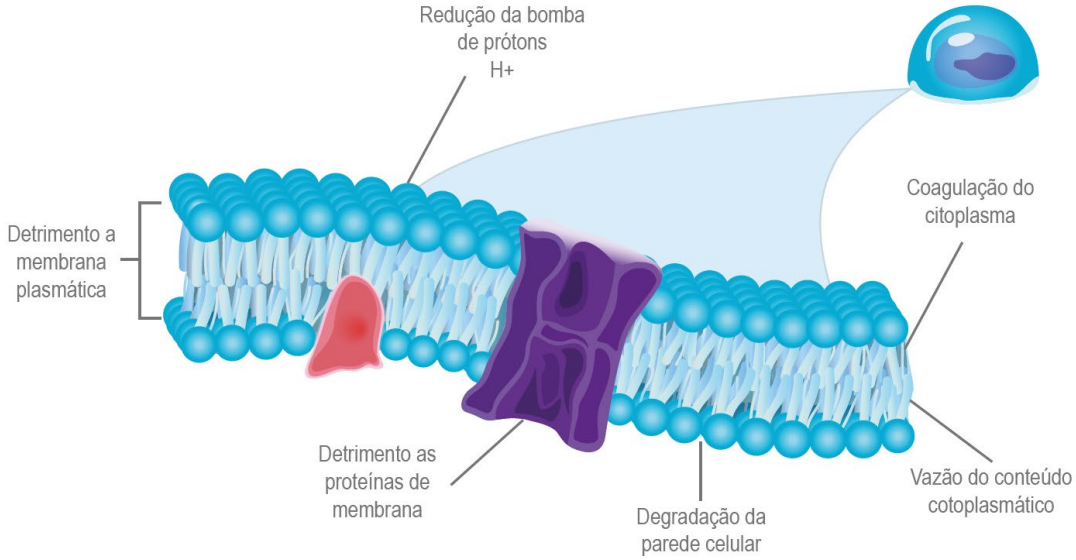

Figura 1. Mecanismo de ação antimicrobiana dos óleos essenciais na célula bacteriana (Burt, 2004).

Na maioria dos casos, as bactérias podem ser tolerantes a estes efeitos por meio de bombas iônicas e não ocorre a morte celular, mas grandes quantidades de energia são desviadas para esta função e o crescimento bacteriano é retardado (Griffin et al., 1999, Ultee et al., 1999, Cox et al., 2001). Alterações nas taxas de crescimento resulta em mudanças na proporção da população bacteriana no rúmen, resultando em mudanças no perfil de fermentação. Em geral, a atividade antimicrobiana mais elevada é em hidrocarbonetos cíclicos, e particularmente em estruturas fenólicas tais como timol e carvacrol, em que o grupo hidroxila e os elétrons deslocados permitem a interação com água pelas pontes de hidrogênio como o principal sítio ativo, tornando-as particularmente ativo contra microrganismos (Griffin et al., 1999, Davidson & Naidu, 2000, Dorman & Deans, 2000, Cox et al., 2001). Ultee et al., (2002) propuseram uma alternativa em que o grupo hidroxila do fenol atua como um transportador de cátions monovalentes e prótons pelas membranas, tais como os antibióticos e ionóforos. Ultee et al., (2002) também observaram que essa hipótese era verdadeira apenas para os grupos hidroxilas dos compostos aromáticos, pelos efeitos observados em compostos como mentol (exatamente igual ao carvacrol, mas não aromático) o qual não apresentou resultados inibitórios significativos. Isto é, provavelmente pela presença de um sistema de elétrons deslocado e a elevada acidez dos fenóis e, por conseguinte, a capacidade do grupo hidroxila liberar seu próton.

Estes mecanismos de ação são mais eficazes contra as bactérias gram-positivas, em que a membrana da célula pode interagir diretamente com a matriz hidrofóbica dos OEs (óleos essenciais) (Smith-Palmer et al., 1998, Chao et al., 2000, Cimanga et al., 2002). Em contraste, a parede celular externa em torno da membrana celular de bactérias gram-negativas é hidrofóbica e não permite a entrada de substâncias lipofílicas. Entretanto, a membrana externa das bactérias gram-negativas não é completamente impermeável e as moléculas de baixo peso molecular pode interagir pelas pontes de hidrogênio, e atravessar a parede celular lentamente por difusão através da camada de lipopolissacarídeos ou pelas proteínas de membrana e interagir com a bicamada lipídica das células (Griffin et al., 1999, Dorman & Deans, 2000). Este é o caso para alguns compostos aromáticos como carvacrol.

Além desses mecanismos de ação da atividade antimicrobiana existe a inibição da síntese de RNA, DNA e proteínas da célula (Feldberg et al., 1988), como por exemplo, os compostos presentes no óleo de alho, óleo de caju, composto timol, entre outros. De fato, muitos estudos relataram que a atividade antimicrobiana dos compostos de enxofre presentes no óleo essencial de alho, como disulfureto de alilo (C6H10S2), favorece a capacidade antimicrobiana do óleo de alho tornando mais poderosa do que a atividade de seu principal compostos individualmente, sugerindo que o efeito está no resultado de uma sinergia entre os diferentes compostos (Reuter et al., 1996, Busquet et al., 2005).

Aditivos naturais sobre a ingestão de alimentos e desempenho de bovinos

Os resultados observados sobre ingestão de alimentos e desempenho de bovinos alimentados com extratos naturais como aditivos são variáveis, isso dependendo dos compostos e doses utilizadas (Patra, 2011). Fornecendo 250 mg/dia de óleo de orégano para cordeiros (Wang et al., 2009), 2 g/dia de óleo de pimenta (35% de α-pineno) para vacas (Yang et al., 2007), 0,75 ou 2 g/dia de um mix de óleos essenciais para vacas leiteiras (Benchaar et al., 2007, Benchaar et al., 2006) e 0,043 ou 0,43 kg/dia para cabras leiteiras (Malecky et al., 2009) não foram observados efeitos sobre a ingestão de alimentos. No entanto, um mix de compostos secundários cinamaldeído (180 mg/dia) e eugenol (90 mg/dia) para bovinos de corte (Cardozo et al., 2006) e doses de cinamaldeído (500 mg/dia) para vacas de leite (Calsamiglia et al., 2007) reduziu de forma significativa a ingestão de alimentos. A redução na ingestão de alimentos poderia estar relacionada

com a palatabilidade dos óleos essenciais, sugerindo, assim, que estes produtos poderiam ser encapsulados para evitar tais problemas (Patra, 2011). Por outro lado, a adição de óleo de pimenta (1 g/dia de extrato de capsicum, contendo 15% de capsaicin) em dieta com alto concentrado para bovinos de corte estimulou a ingestão de alimentos e a fermentação ruminal (Cardozo et al., 2006). Ornaghi et al. (2017) utilizando óleos essenciais de cravo e canela em duas doses (3,5 e 7,0 g/animal/dia) na dieta constataram aumento na ingestão de matéria seca em bovinos terminados em confinamento com alto concentrado.

A correta escolha e dose de adição de compostos naturais é um fator importante pois podem estimular a ingestão ou provocar efeito inverso reduzindo a ingestão de alimentos pelos animais (Patra, 2011). Yang et al. (2010) observaram que cinamaldeído apresentava melhor efeito em baixas doses (0,4 g/dia), enquanto as doses mais elevadas (1,6 g/dia) não tiveram efeito sobre a ingestão de alimentos em bovinos.

Por outro lado, a literatura é limitada sobre o efeito dos óleos essenciais e seus compostos sobre o desempenho de ruminantes. Bampidis et al. (2005) não observaram efeito sobre o ganho médio diário e eficiência alimentar quando cordeiros em crescimento foram alimentados com dietas suplementadas com folhas de orégano, fornecendo 144 ou 288 mg/kg de concentrado de folha de orégano (85% de carvacrol). Da mesma forma, Benchaar et al. (2006) não observaram efeito sobre o ganho médio diário em bovinos de corte alimentados com dieta à base de silagem e suplementados com 2 ou 4 g/dia/animal de um mix de óleos essenciais à base de timol, eugenol, vanilina e limoneno. No entanto, o mix de óleos essenciais teve efeito quadrático sobre a eficiência alimentar, sendo que a dose 2 g/dia melhorou e eficiência quando comparado com a dose de 4 g/dia. Chaves et al. (2008) também observaram que o carvacrol ou cinamaldeído (0,2 g/dia) não tiveram efeito sobre o desempenho de ovinos alimentados com deitas à base de milho ou cevada durante 11 semanas, embora o ganho tenha sido numericamente maior para os animais alimentados com dieta à base de cevada quando comparado com os animais alimentados com a dieta controle (288 vs. 310 g/dia). No entanto, maior ganho médio diário (250 ou 254 vs. 217 g/dia) foi observado quando óleos essenciais (cinamaldeído ou pimenta) foram adicionados às dietas à base de cevada. Desta forma, a ação dos óleos essenciais sobre o desempenho animal poderia ser dose-dependente (Patra, 2011).

Aditivos naturais sobre a qualidade da carne

A ação antioxidante está ligada a capacidade de se ligar a radicais livres e inibir processos de estresse oxidativo que potencializam a oxidação dos lipídeos presentes na carne, os quais ocasionam o *off-flavor* (cheiro e sabor indesejável) oriundo da rancidez do produto (Gutierrez et al., 2018).

Geralmente, a proteção celular contra o estresse oxidativo é mediada por dois mecanismos de capacidade antioxidantes (Figura 2), e geralmente apresentam baixo peso molecular, assim como os compostos secundários presentes nas plantas. Primeiramente, tem-se os compostos que exercem sua função como antioxidantes diretos, são redox ativo e inibem a ação de espécies reativas ao oxigênio (ROS), enquanto no segundo tipo os antioxidantes atuam de forma indireta como indutores de antioxidantes e outras enzimas citoprotetoras (Dinkova-Kostova & Talalay, 2008). Muitos óleos essenciais, cujos principais componentes são monoterpenos e sesquiterpenos, possuem propriedades antioxidantes (Amorati et al., 2013).

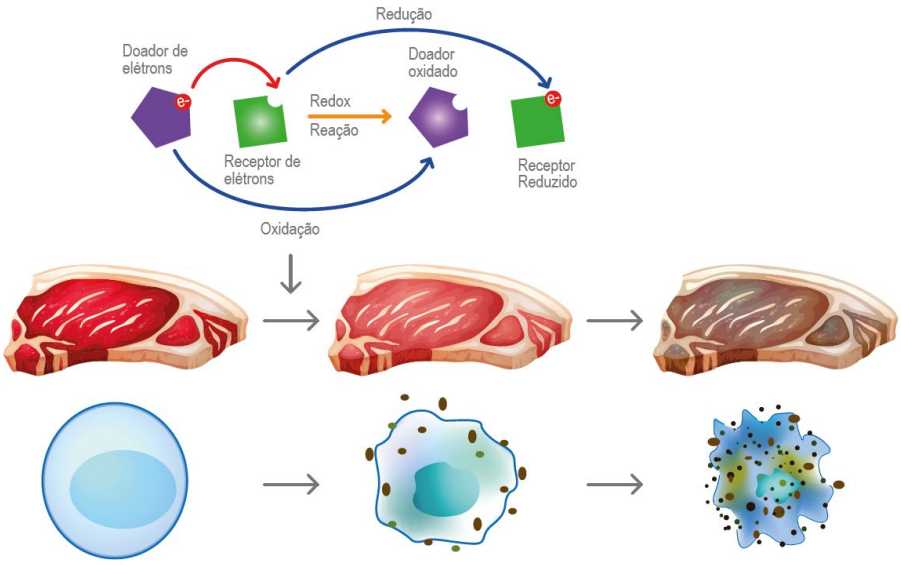

Figura 2. Processo de Oxidação e ação antioxidante

O primeiro contato do consumidor é com a coloração da carne, esse aspecto é altamente relacionado com fator de qualidade sendo considerado como o ponto mais importante na percepção e momento de decisão da compra, isso porque é associado ao frescor da carne. Alterações na cor da carne são pela oxidação da oximioglobina a metamioglobina, conferindo à carne a cor marrom pouco atraente (Nerín et al., 2006).

Entretanto, poucos relatos sobre o uso dos óleos essenciais na dieta de bovinos são elucidados na literatura, assim como também existem divergências nos seus resultados sobre o efeito na qualidade de carne. Esses compostos podem sofrer alguma metabolização microbiana ou serem absorvidos quando adicionados a dieta de forma livre, a microencapsulação pode proteger e potencializar os efeitos benéficos no produto final (carne). Rivaroli et al. (2016), utilizando adição de um *blend* de óleos essenciais (orégano, alho, limão, alecrim, tomilho, eucalipto e laranja doce) na dieta de bovinos terminados em confinamento em duas doses 3,5 e 7,0 g resultou em menor oxidação lipídica na carne de animais alimentados com 3,5 g /animal dia. Em um estudo utilizando óleos essenciais de orégano, alecrim, alho e gengibre a 0,05 % da dieta de suínos Janz et al. (2007), observaram uma tendência a menor oxidação lipídica na carne dos animais recebendo na dieta óleo de orégano, mas sem apresentar efeitos significativos nos outros parâmetros de qualidade avaliados (textura, coloração e perdas de água). Da mesma forma, Simitzis et al. (2007) adicionando óleo essencial de orégano na dieta de cordeiros (1ml/kg) observou redução significativa na oxidação lipídica da carne mesmo após período longo de armazenamento (quatro meses).

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III. OBJETIVOS GERAIS

Objetivou-se avaliar o desempenho, eficiência alimentar, fermentação e microbioma ruminal, comportamento ingestivo e qualidade da carne de bovinos meio sangue recebendo dieta alto grão em confinamento com adição de um *blend* de aditivos naturais (óleo essencial de cravo, óleos funcionais de mamona e caju e compostos microencapsulados) em diferentes níveis.

CAPÍTULO IV

(Animal Microbiome)

Natural plant-based additives can improve ruminant performance by influencing the rumen microbiome

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Abstract

Backgroud

The use of synthetic compounds as growth promoters in animal production, is now limited or even banned by health agencies globally due to human safety concerns. In feedlot cattle, when using high grain diets, it is necessary to supplement the diet with compounds capable of modulating the rumen in order to reduce the incidence of acidosis and improve growth. In this context, natural substances have become promising substitutes. The objective of this study was to evaluate the effects of a natural additive blend (NA) on animal performance, the rumen microbiome and ingestive behavior in 40 young bulls.

Results

The initial and final average body weight was similar ($P > 0.05$) for all diets, although average daily gain increased linearly ($P < 0.01$) when NA was fed. However, feed efficiency improved linearly ($P < 0.05$) by including NA in the diet. Principal volatile fatty acid: acetic, butyric, isovaleric and valeric decreased linearly ($P < 0.02$) following NA addition. Similarly, NA addition linearly decreased ($P < 0.02$) the acetate/propionate ratio. The propionate and isobutyric acid concentrations showed a positive quadratic effect ($P < 0.05$). Furthermore, NA addition reduced ammonia concentrations ($P < 0.001$) and ruminal pH was not affected ($P > 0.05$) by the diets. The rumen microbiome was significantly different between beef cattle fed the different treatments ($P < 0.05$), with a reduction in the archaea, and within the Clostridium, Robinsoniella, Acidaminococcus, Acetitomaculum, Succinimonas and Weissella ($P < 0.05$) seen when NA was fed. The functional capacity of the rumen microbiome was affected following NA supplementation. Overall, we observed Aldehyde oxidase/xanthine

dehydrogenase, molybdopterin binding; RecG, N-terminal antiparallel four helix bundle; Transposase, ISC1217; Restriction endonuclease, type II, XamI; Acyl-protein synthetase, LuxE; ABC-2 transporter; which could be related to the natural additives mechanism of action.

Conclusions

Animal performance was improved in a dose-dependent manner by natural additive addition to the diet of bulls. These beneficial effects are correlated to changes in the rumen microbiome. Our findings suggest that the natural additive blend used in this study could be used as an alternative natural substitute to synthetic antibiotics for animal production.

Keywords: essential oils; feedlot; microbiome; microbiota; rumen.

Background

Ruminants obtain their energy for maintenance and production largely through the feed and the fermentative capacity of the rumen microbiome, resulting in the production of short-chain fatty acids, especially acetate, propionate and butyrate. However, the fermentation process also produces secondary gases, like methane, which can represent losses of up to12% of the total energy intake, thus affecting feed efficiency [1, 2]. Additionally, the accumulation of short-chain fatty acids in the rumen for long periods can result in ruminal abnormal function, and additives are often used to prevent this occurrence. Of those, antibiotics are additives largely used to prevent metabolic disorders and to improve animal efficiency in many non-EU countries [3]. However, there is increasing public concern regarding antibiotic resistance [3]. Thus, some

67 countries are limiting (FDA, 2015) or even banning (EU; OJEU, 2003) the use of
68 antibiotics in animal feed as precautionary measures against antimicrobial resistance.
69 This is pivotal given that there is evidence that the rumen is likely a reservoir of
70 antibiotic resistance genes [4].

71 There is potential to use natural products as substitutes to antibiotics in ruminant
72 nutrition, such as natural additives (NA) from plant extracts, and essential and
73 functional oils [5, 6, 7, 8, 9]. Essential and functional oils have active secondary
74 metabolites produced by plants. These secondary metabolites are reported as having
75 antibacterial, antifungal and antioxidant activity [10, 11]. Secondary metabolites having
76 antimicrobial effects can act by inhibiting RNA, DNA and protein synthesis, and even
77 damaging cell membrane [12]. Therefore, these metabolites may manipulate rumen
78 fermentation resulting in improved feed efficiency. Furthermore, there is evidence that
79 the volatile and odorant compounds in secondary metabolites improve palatability of
80 the diet [13].

81 Active compounds in plants are dependent on biotic (i.e. species, portion, etc.) and
82 abiotic (i.e. temperature, humidity, etc.) factors. Clove oil (Syzygium aromaticum) is
83 enriched in eugenol, which was reported as having antimicrobial properties [14].
84 Vanilla (Vanilla planifolia) and thyme (Thymus vulgaris) are enriched in vanillin and
85 thymol, respectively, which were reported as having antimicrobial [15] and antioxidant
86 activity [16]. Cashew nut oil (Anacardium occidentale) and castor oil (Ricinus
87 communis), which are enriched in cardanol, cardol and anacardic acid, were also
88 reported as having antimicrobial properties [17]. These active compounds have
89 potential to affect Gram-positive and Gram-negative bacteria [18] and synergetic
90 effects of using plants extracts have been reported [19].

The authors have recently reported improved performance of beef cattle supplemented with either 3.5 or 7.0 g/day per animal of essential oils from clove or cinnamon [20]. However, mechanistic effects of NA on the rumen microbiome remains poorly explored, but it is assumed that the rumen function is likely different. Thus, in this study we fed beef cattle with increasing levels of NA (essential oil from clove leaf, castor and cashew functional oils, and a commercial blend composed of vanillin, eugenol and thymol) and evaluated animal performance and rumen parameters. Furthermore, we used shotgun metagenomics to explore underlying changes in the rumen microbiome. In summary, this study provides a comprehensive understanding of the effects of a commercially available natural plant-based additive blend on ruminant performance alongside a comprehensive understanding of the mechanism of action within the rumen.

Results

Animals diet

Bulls were fed a basal diet comprised of 70% concentrate containing corn grain offered ad libitum and protein supplement (soybean meal; premix composed of: urea, vitamins and minerals; limestone; yeast and salt) and 30% corn silage for 62 days (Table 1).

Feeding behavior activities

There were no effects of NA blend addition to bull diets on rumination, feed intake, water intake and idle time ($P > 0.05$; Table 2).

Animal performance

The initial body weight and final body weight (FBW) were similar for all diets ($P > 0.05$), nonetheless average daily gain (ADG) of bulls increased linearly ($P < 0.01$) when the NAs blend was added in diets (Table 3). The NA addition in diets had no effect ($P > 0.05$) on Dry Matter Intake (DMI) (kg/day – 9.9 or kg/100 kg body weight – 2.3%). However, feed efficiency improved linearly ($P < 0.04$) with the NA addition to diets (Table 3). In addition, the HCW (Hot Carcass Weight) and HCD (Hot Carcass Dressing) did not differ between cattle fed with NA blend ($P > 0.05$; Table 3).

Ruminal ammonia and volatile fatty acid (VFA).

The NA blend addition affected rumen fermentative characteristics and resultant VFAs produced (Table 4). The major VFAs: acetate, butyrate, isovalerate, and valerate were reduced linearly when animals were fed NAs ($P < 0.05$). Similarly, NA addition in diets linearly reduced ($P < 0.02$) the acetate/propionate ratio. NA supplementation resulted in a quadratic effect on propionate and isobutyric acid concentrations ($P < 0.05$). Furthermore, animals supplied with NA had linear reductions in rumen methane concentration ($P < 0.001$). Ammonia concentration had a quadratic effect following NA blend supplementation of bull diets ($P < 0.001$). The ruminal pH was not affected ($P > 0.05$) by NA inclusion in diets (Table 4).

Rumen bacterial diversity and abundance

In our study, the major phyla present in the rumen were Bacteroidetes (47%) and Firmicutes (36%; Figure 1). Bacteroidetes ($P < 0.05$) were reduced when NA was included in the diet. A quadratic response was seen for Candidatus Saccharibacteria, Chytridiomycota, Elusimicrobia, Eukaryota Unassigned, Fibrobacteres, Firmicutes,

Spirochaetes, Synergistetes and Tenericutes ($P < 0.05$). Source data are included in supplementary material (Table S1).

The families Prevotellaceae (43%) and Ruminococcaceae (20%) were observed as the most abundant across treatments (Figure 2). Significant changes were observed in the families causing quadratic responses in Cardiobacteriaceae, Clostridiales_Family_XIII_Incertae_Sedis, Prevotellaceae, Ruminococcaceae. Our data also showed a decrease in Acidaminococcaceae, Coriobacteriaceae, Defluviitaleaceae, Desulfovibrionaceae, Neisseriaceae, Paenibacillaceae, Peptococcaceae, Porphyromonadaceae and an increase in Christensenellaceae, Bacillaceae, Lactobacillaceae, Ophryoscolecidae, Rikenellaceae, Trichomonadidae ($P < 0.05$) post NA supplementation of bull diets. Source data are included in supplementary material (Table S2).

The most common rumen bacterial genera across the treatments were Succinivibrio, Succiniclasticum, Marvinbryantia and Prevotella (12%, 11%, 9% and 6%, respectively; Figure 3). A quadratic effect was observed when NA was supplemented into the bull diet with respect to the genera Alistipes, Asteroleplasma, Dorea, Elusimicrobium, Entodinium, Faecalibacterium, Haemophilus, Holdemanella, Paraprevotella, Pseudoscardovia, Pyramidobacter, Roseburia, Ruminobacter, Sphaerochaeta, Subdoligranulum, Syntrophococcus. A decrease in Acetitomaculum, Acidaminococcus, Akkermansia, Alloprevotella, Candidatus_Saccharimonas, Citreitalea, Clostridium, Fretibacterium, Mailhella, Moryella, Phascolarctobacterium, Prevotella, Robinsoniella, Succinimonas, Suttonella, Tetratrichomonas and Weissella and an increase in Anaerostipes, Atopobium, Bacillus, Bavariicoccus, Fibrobacter, Hydrogenoanaerobacterium, Paenibacillus and Sporobacter ($P < 0.05$) was noted post

NA dietary supplementation. Source data are included in supplementary material (Table S3).

Methanogen diversity and abundance

Archaeal abundance was reduced on the whole with the inclusion of NA in the bull diets ($P < 0.05$; Table 5). The families Methanobacteriaceae and Methanomicrobiaceae ($P < 0.05$); orders Methanomicrobiales, Methanobacteriales and Methanomassiliicoccales ($P < 0.05$) and the genera Methanobrevibacter and Methanosphaera, showed a significant decrease with NA supplementation, whilst the genus Methanomicrobium showed a tendency to be present at lower abundance ($P = 0.051$). Furthermore, on a species level, a decrease in Methanobrevibacter ruminantium, Methanobrevibacter sp D5 and Methanobrevibacter sp G16 was seen following NA supplementation of bull diets ($P < 0.05$).

Gene Network correlations

We observed close to 13,000 functionally annotated genes in total across the experimental samples using shotgun metagenomics and 28 were significantly differentially abundant when the bull diet contained NA (Fig. 4; Table S4). Functional annotation data showed significantly biological responses due to the NA addition whereas mostly related to protection against foreign attack to DNA and DNA maintenance, replication and repair (Restriction endonuclease, type II, XamI; Restriction endonuclease, type II, EcoRV; Host-nuclease inhibitor protein Gam; RecG, N-terminal antiparallel four helix bundle; Type IV secretion system protein TraG/VirD4; Type IV secretion system, VirB10 / TraB / TrbI and Transposase, ISC1217). There were also functional process associated with membrane protection and maintenance

(ABC-2 transporter; Conjugal transfer, TrbG/VirB9/CagX and Capsule biosynthesis protein CapC), metabolic role (Lyase, catalytic; Acyl-protein synthetase, LuxE; Phenolic acid decarboxylase, bacterial; Peptidase G2, IMC autoproteolytic cleavage domain; Glycyl radical enzyme, HI0521, predicted; Transposase, ISC1217 and Tetrahydrodipicolinate-N-succinyltransferase, chain A, domain 1), oxidative stress response (Thiol peroxidase conserved site and Aldehyde oxidase/xanthine dehydrogenase, molybdopterin binding), attack protection and resistance (Bacterial virulence protein VirB8; KorB, C-terminal and Siphovirus Gp157), plasmid replication (KorB, C-terminal), and unknown biologic process (Protein of unknown function DUF4244; Protein of unknown function DUF4054; Protein of unknown function DUF4912; Protein of unknown function DUF4294; Protein of unknown function DUF4416; Protein of unknown function DUF3853).

Specifically, the functional annotations Restriction endonuclease, type II, XamI; Lyase, catalytic; Acyl-protein synthetase, LuxE; Host-nuclease inhibitor protein Gam; ABC-2 transporter; Transposase, ISC1217; RecG, N-terminal antiparallel four helix bundle and Protein of unknown function DUF4294 were decreased with NA inclusion in the diet. Furthermore, the annotations that showed an increase post NA inclusion in the diet were: Glycyl radical enzyme, HI0521, predicted; Aldehyde oxidase/xanthine dehydrogenase, molybdopterin binding, Peptidase G2, IMC autoproteolytic cleavage domain; Siphovirus Gp157; Type IV secretion system protein TraG/VirD4; Type IV secretion system, VirB10 / TraB / TrbI; Conjugal transfer, TrbG/VirB9/CagX; KorB, C-terminal and Protein of unknown function DUF4416. Nevertheless, a quadratic response was also noted for: Bacterial virulence protein VirB8; Capsule biosynthesis protein CapC; Phenolic acid decarboxylase, bacterial; Restriction endonuclease, type II, EcoRV; Thiol peroxidase conserved site; Tetrahydrodipicolinate-N-

succinyltransferase, chain A, domain 1; Protein of unknown function DUF3853 and Protein of unknown function DUF4912.

The family Succinivibrionaceae had a strong positive correlation (average r = > 0.9) with Tetrahydrodipicolinate-N-succinyltransferase, chain A, domain 1; Type IV secretion system, VirB10 / TraB / TrbI; Phenolic acid decarboxylase, bacterial; Thiol peroxidase conserved site; Type IV secretion system, VirB10 / TraB / TrbI; Bacterial virulence protein VirB8; Conjugal transfer, TrbG/VirB9/CagX; KorB, C-terminal gene abundances. The Paenibacillaceae bacterial family (Phylum Firmicutes) had a positive correlation (r = > 0.9) with Peptidase G2 and Glycyl radical enzyme, HI0521, predicted gene abundance. The Victivallaceae interacted with Protein Function DUF4416 and Capsule Biosynthesis Protein CapC (r = > 0.9). The Glycyl radical enzyme, HI0521, predicted, showed a major correlation with Bacillaceae (r = > 0.9). Prevotellaceae had a negative correlation (r = − 0.8) with Ruminococcaceae, and Methanobacteriaceae also had a negative correlation (r = > − 0.7) with Protein Function DUF4294 and ABC-2 transporter gene abundances. Source data are included in supplementary material (Table S4, Fig. 6).

Discussion

In this study we evaluated the mechanism of action of a commercially available blend of essential oil, at increasing concentrations, on the rumen microbiome and host phenotype. Feeding behavior of ruminants is dependent on diet and the environment [21], and as expected, no differences were observed between treatments in this study. On average, animals spent 336 minutes at the feeder, 236 minutes ruminating, 35 minutes drinking water and the remaining at rest. Beef cattle tend to spend an average of 400 minutes eating and 300 ruminating when finished in feedlot [21]. Fiber content

238 is a known factor influencing time spent ruminating and consequently in water ingestion
239 due to the stimulus on the salivary glands [22]. The observed values in this study
240 provide evidence of a healthy rumen, which is supported by the pH values, which are
241 higher than 6.90 for all treatments. Ornaghi et al. [20], also observed similar feeding
242 behavior when young bulls were fed diets with essential oils and 70:30 concentrate to
243 roughage ratio. Moreover, Zotti et al. [23], fed monensin (included at 30 mg/kg or 40
244 mg/kg) and functional oils (blend of castor oil and cashew nut shell liquid included at
245 400 mg/kg) to a high concentrate diet (92.25% concentrate) with 12 steers and
246 observed no effects on feeding behavior parameters.

247 Essential oils are volatile and odorant compounds which can impact the palatability
248 of the diet, positively or negatively [13], nonetheless we found no effects on DMI in this
249 study. Our results are in agreement with those from Valero et al. [8], whereby bulls fed
250 with 3 g/animal/day of ricinoleic acid (extracted from castor oil seed), anacardic acid,
251 cardanol and cardol (extracted from the cashew nut shell liquid) during finishing had
252 similar DMI (kg/day). On other hand, Yang et al. [24] reported an increase in DMI when
253 cinnamaldehyde (0.4, 0.8 and 1.6 g/day per animal) was fed to feedlot cattle during 28
254 days of observation. These variations might be related to the differing effects of the
255 essential oils in isolation as opposed to presence in a mixture.

256 Secondary metabolites extracted from plants often have antimicrobial properties
257 [25, 26]. In our study, the main compounds present in the blend were: eugenol, vanillin,
258 thymol, cardol, cardanol, ricinoleic acid, which can modulate the rumen fermentation
259 and reduce methanogens abundance [27]. These compounds may improve the animal
260 performance by modulating rumen fermentation [8, 10, 20]. Indeed, the ADG and feed
261 efficiency increase linearly when NA were added to the diets. Furthermore, acetate,
262 butyrate, isovaleric, valeric, and ammonia concentration were reduced when NA were

added to the diets. Ornaghi et al. [20], also reported a significant increase in ADG using NA (clove essential oil and cinnamon essential oil in two different doses 3.5 and 7.0 g/animal/day) in the diet of young bulls finished in feedlot. However, most studies using NA are in vitro, and in vivo experiments are still scarce in literature. VFAs provide energy for the ruminant maintenance and to produce milk and meat. Nearly 252 kcal are necessary to produce 1 mol of acetate, compared to 62 kcal net gain to produce propionate [28], which also release free hydrogens used to produce methane by archaea (methanogens). We observed a reduction of Acetitomaculum, an important acetogenic bacterial genus, which utilizes monosaccharides to produce acetate, and is often found when cattle are fed high grain diets [29]. We also observed a reduction of the Acidaminococcus genus, which have acetate as major end-product [30]. Reducing the production of acetate can be positive to reduce environmental impact of beef cattle production as more energy is available to the animal as opposed to being lost in the form of methane.

Methanogens are commonly found in association with protozoa [31], which use hydrogenosomes to produce methane. In this study, the use of NA linearly reduced acetate and the archaeal population, that likely reduced methane production suggested by the reduction in archaea abundance. This decrease in the archaeal population post NA supplementation of diets could be due to hydrophobicity of phenolic compounds present in the NA, allowing permeation of the phospholipidic membrane resulting on cell lysis [32; 33]. Khorrami et al. [34] supplemented thyme and cinnamon essential oils (500 mg/kg DM) into ruminant diets and evaluated rumen fermentation and observed decreased protozoal and methanogens abundance, thus corroborating our data. Macheboeuf et al. [35], studied the production of methane in vitro following the inclusion of essential oils from five plants: Thymus vulgaris, Origanum vulgare,

288 thymol chemo-type of O. vulgare, Cinnamomum verum, and Anethum graveolens);
289 and three pure compounds: thymol, carvacrol, and cinnamaldehyde, and observed a
290 decrease of methanogenesis up to 76% with the highest doses. Patra and Yu [6], also
291 provided evidence for the inhibition of methanogenesis and decreases in protozoal
292 density following addition of five essential oils from clove, eucalyptus, garlic, origanum
293 and peppermint oils and using three different doses in vitro (0.25, 0.50, and 1.0 g/L).
294 The effects of the NA blend on propionate production was quadratic and showed
295 the maximum concentration at 4.5 level of natural mix addition. Propionate is the
296 principal precursor of liquid glucose and is related to gluconeogenesis. In addition,
297 production of propionate causes a net gain of around 62 kcal of energy, therefore
298 propionate is beneficial for ruminant production. There was a linear decrease of
299 butyrate following the supplementation of NA to the diet of bull diets. Butyrate can
300 inhibit propionate absorption, therefore is not as beneficial as an energy source for the
301 ruminant [36]. Watanabe et al. [37], observed reduction of butyrate, acetate and
302 methane production when raw cashew nut shell liquid was added to in vitro cultures. It
303 is therefore important to highlight the dose-type dependent effect of the natural
304 additives, which are enhanced when administered as a blend.
305 NA had a quadratic effect on ruminal ammonia concentration and was higher in bulls
306 fed the control diet compared with those fed NA (21.82 vs 4.78 mg/dL). This lower
307 production may be related to the reduction in hyper-ammonia bacterial abundances,
308 for example the Clostridium genus abundance was significant lower compared to the
309 control diet. The Clostridium genus is one of the major ammonia producers and is
310 highly affected by NA [39]. Furthermore, the genus Acidaminococcus and
311 Robinsoniella were linearly reduced. The genus Acidaminococcus produces ammonia
312 as the major end product through glutamate fermentation [30]. The genus

313 Robinsoniella is correlated with high ruminal ammonia concentration and with
314 methanogens, which is due to a reflection of metabolic interaction among microbial
315 consortium [40]. Thus, abundance decreases for both genera could impact the
316 microbial consortium leading to lower methane production. Furthermore, the potential
317 antimicrobial power of NA can be potentiated when the ruminal pH is low as in the
318 grain diets such as in this study [39]. Furthermore, this decrease likely increases
319 absorption of amino acids that are not broken to ammonia, which will be available for
320 absorption in the gut [35]. In contrast, Jesus et al. [41], observed no significant effect
321 on ruminal ammonia but an increase in propionate and lower blood urea concentration,
322 suggesting a potential rumen fermentation shift, when a commercial blend (cashew nut
323 shell liquid and castor oil) was fed to dairy cattle, these responses might be related to
324 the animal basal diet. Recently, Cobellis et al. [17], reported that some essential oils
325 can affect VFA production in the rumen but that it is dose and compound dependent,
326 thus, they have specific effects on the rumen microbiome. As the rumen microbiome
327 present a higher variability, some biological role can interact with the results of this
328 study such as animal effect.

329 In terms of gene network interactions and function of the rumen microbiome, we
330 found that Glycyl Radical and Peptidase function, were positively correlated to each
331 other. The Ruminococcaceae family undergo changes with the inclusion of NA and
332 had a positivel correlation with the abundance of protein Glycyl Radical genes, which
333 are found to contribute to environmental resilience, and are also potentially related with
334 VFA production [42]. The abundance of Prevotellaceae was negatively correlated with
335 Ruminococcaceae; the two major bacterial families found in our study. Both families
336 are known to compete for the same niche in the rumen [43] perhaps explaining their
337 negative correlations to each other. Blautia tended to increase linearly, even in a low

concentration. This taxon can improve polysaccharides utilisation, improving the rumen fermentation [44]. Some Blautia species can consume H2 increasing the acetogenesis, which can lead to competition with the methanogens [45]. Nonetheless, the Peptococcaceae family was reduced using the blend of NA. This family is a producer of H2 from amino acids or carbohydrates fermentation. The impact on Blautia genus and Peptococcaceae family might be a secondary cause of the methanogens reduction as the competition for substrates and H2 lower production can reduce the archaea abundance [46].

There is no doubt that the rumen is a complex environment [47]. Understanding the abundance of the microbes is and their function is nonetheless crucial when investigating the mechanisms of action of a novel additive and to ensure no detrimental effects are encountered. In this study, we show that the essential oil blend used affected the rumen microbiome, potentially through disruption of bacterial cell membranes and breakdown in DNA replication [17, 18, 26, 38]. Important bacterial defense mechanisms used by microbes were observed in our study, such as DNA replication and protection against attack from outsider metabolites, being mostly from membrane sites in response to encountering the blend of essential oils. Furthermore, one of the major protein annotations in our study was the ABC transporter group, the key role of this protein is translocating molecules across the membrane to the maintenance of the cell, followed by multidrug or antimicrobial efflux pumps [30]. This protein was affected and decreased by NA addition. [30]. We also noted some DNA restrictions modification mechanisms used for protection of bacterial and archaea against invading foreign DNA were reduced by NA addition, both Restriction endonuclease, type II XamI and EcoRV, to date the difference between them are in the mode of recognition process and cleavage [48].

Conclusions

In our study, the blend of natural additives improved animal performance by beneficial modulating the rumen microbiome. Furthermore, our data suggest that methane emissions may be decreased with NA levels from 3 g/animal/day addition in this study, suggested by the archaeal reduction. Ammonia concentrations were also reduced which is also of major benefit for the environment. Also, we can conclude that the level 4.5 g/animal/day in this study had improved animal performance, thus, may replace the use of antibiotics in beef cattle finished in a feedlot with high grain diets. These positive results are mainly a consequence of the ability of the NA blend to beneficially modulate the rumen microbiome.

Materials and Methods

Animals and diets

A total of 40 (½ Angus vs ½ Nellore) young bulls of 16 ± 2.2 months of age and with a body weight of 385.8 ± 20.7 kg were used in this study. A 14-d adaptation period before starting the experiment was used, during which the concentrate was gradually increased for animals. The bulls were weighed every 28 days at a trunk balance (Beckehauser Cia. Paranavaí city, Paraná, South Brazil).

Bulls were fed with a basal diet comprised of 70% concentrate and 30% corn silage offered *ad libitum* for 62 days (Table 1), and the feed intake was recorded individually every day for 5% leftovers. Feed samples were collected every day, and stored at -20°C prior to analysis. Bulls were randomized on five treatments: control (CON), without the naturals additives addition; NA15, with the addition of 153.07 mg per kg of DM of a naturals additives blend (1.5 g/day/animal); NA30, 305.2 mg per kg of DM of

388 a naturals additives blend (3.0 g/day/animal); NA45, 444.66 mg per kg of DM of a
389 naturals additives blend (4.5 g/day/animal): NA60, addition of 594.65 mg per kg of DM
390 of a naturals additives blend (6.0 g/day/animal). The natural additives blend contained
391 clove leaf essential oil (Ferquima®, Vargem Grande Paulista, São Paulo, Brazil), castor
392 and cashew functional oils (Safeeds®, Cascavel, Paraná, Brazil) and a commercial
393 blend composed of vanillin, eugenol and thymol (Safeeds®, Cascavel, Paraná, Brazil).
394 Each treatment contained 37.5% of clove essential oil, 37.5% of the commercial blend
395 containing vanillin, eugenol and thymol, 12.5% of castor oil and 12.5% of cashew oil.
396 Following day 62 in the feedlot, the animals were weighed after 16 hours of fasting
397 (482 ± 31.9 kg) and transported to a commercial slaughterhouse (Campo Mourão city,
398 Paraná, South Brazil). The truck stocking density was 0.8 ± 0.2 bulls/m^2, and the
399 transport distance was less than 90 km. The bulls were slaughtered following the usual
400 practices of the Brazilian beef industry. The animals were stunned using a captive-bolt
401 pistol. Then, they were bled through exsanguinations by cutting the neck vessels, and
402 the head hide, viscera, tail, legs, diaphragm and excess internal fat were removed.
403 Afterwards, the carcasses were divided medially from the sternum and spine, resulting
404 in two similar halves, which were weighed to calculate the hot carcass weight (HCW).
405 Then, the half-carcasses were washed, weighed, identified and stored in a chilling
406 chamber at 4°C, where they remained for a 24 h period and drip loss measured by the
407 difference between the hot carcass weight and the carcass weight observed 24 hours
408 later after chilling. The hot carcass dressing (HCD) percentage was defined as the hot
409 carcass weight divided by the FBW 16 hours before slaughter and calculated by using
410 the equation: HCD = (HCW/FBW) x 100.
411
412 **Diet chemical analyses**

The dry matter (DM) content of the ingredients was determined by oven-drying at 65°C for 24 h and then drying at 135°C for 3 h (Method 930.15) [49]. The 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 [49]. The N content in the samples was determined by the Kjeldahl method (Method 976.05) [49]. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using the methods described by Van Soest et al. [21], using heat stable α-amylase and sodium sulfite for the NDF procedure, and residual ash. The factor of 0.82 was used to convert metabolizable energy requirement to digestible energy requirements, and the factor 4.1868 was used to convert total digestible nutrients requirement to megajoules (NRC, 2000).

Feeding behavior

In order to evaluate feeding behavior, the young bulls were subjected to two periods of 24h of observation using five-minute intervals and three trained evaluators. A total of 288 observations were performed for each animal. Animals were adapted to feeding behavior evaluation for five days prior to the start of evaluations. Water and feed intake, and rumination and idle periods were obtained by the sum of 288 observations (minute/day). Observations were performed without interfering with the animal's routine. The water intake was considered when animals were at the individual water reservoir, and feed intake was considered when animals were at the feeder. Rumination was considered when animals were chewing a bolus. Idle was considered when animals were not performing any of the activities described previously [50].

437 **Rumen sampling**

438 Fresh rumen content was collected at the end of the experimental period (5 days before
439 the slaughter) 4h before animals feeding, from 25 animals chosen at random (5 on
440 each treatment). Rumen contents were sampled by a trained veterinarian using an
441 esophageal probe and vacuum pump. Rumen liquor (50 mL) were sampled from the
442 ventral region of the rumen and was then strained through two layers of muslin. The
443 pH was recorded immediately using a pH meter (Hanna instruments model HI99163,
444 Romaria – Brazil); the electrode was previously calibrated and then inserted into the
445 rumen fluid. Sub-samples used to evaluate volatile fatty acids (VFA) and ammonia
446 concentrations were preserved by the addition of trichloroacetic acid (25%; v/v)
447 following storage in ultra-freezer (- 80°C). Sub-samples used to evaluate protozoal
448 count were preserved using formaldehyde (4%; v/v/).

449

450 **Ruminal ammonia and VFA measurements**

451 Ruminal ammonia-N concentration was determined using the distillation method
452 (Kjeltec Auto 1030 Analyzer Tecator, Hoganas, weden). Ruminal fluid samples were
453 analyzed for VFA by gas chromatography (Shimadzu, Model GC-2014, automatic
454 injection model AOC – 20i) equipped with a 30-m (0.32 mm ID) silica-fused column
455 (HP INNOwax – 19091N - Capillary Column, Varian, Palo Alto, CA, USA). Helium and
456 crotonic acid (trans-2-butenoic acid) were used as carrier gas and internal standard,
457 respectively. Oven initial and final temperatures were 55 and 195°C, respectively, and
458 detector and injector temperatures were adjusted at 250°C.

459

DNA extraction, Metagenomic Library Preparation and Sequencing

DNA was extracted from the rumen liquid after thawing samples at 4°C using a FastDNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA) according to manufacturer's guidelines. The DNA integrity was verified using agarose gel electrophoresis. DNA was quantified using Pico 100 (Picodrop, Ltd., Hinxton, UK). Extracted genomic DNA were normalized to 10 ng/μL with PCR grade water (Roche Diagnostics Limited, Mannheim, Germany) and 50 ng were used to prepare metagenomic libraries using the Nextera® DNA kit (Illumina, San Diego, United States) following standard instructions. Nextera® DNA libraries were quantified. Sample libraries were pooled in equimolar concentrations following Illumina guidelines and sequenced at 2 x 151 bp using an Illumina HiSeq 2500 rapid run, with samples duplicated over two lanes, and following standard manufacturer's instructions. Sequence data quality control and analyses were performed using the QIIME pipeline, version 1.7.0 [51]. Illumina adapters and primers were removed, and the forward and reverse reads were paired.

Rumen microbiome diversity, function and gene network correlations

Taxonomic and functional analysis data were assessed with MGnify (http://www.ebi.ac.uk/metagenomics) following the pipeline version 5.0. Differential abundances of gene functional categories were assessed between dietary treatments using DESeq2 [52]. The input for correlation analysis was performed with the normalized counts taken over all samples from the internal normalization calculated by DESeq2. We applied a P-value cut-off of 0.01 to the resulting domain predictions and counted the number of gene functional which were assigned domains using volcano plots to the differences between control diet and the treatments. Correlations between

485 datasets (biological taxonomy and functional annotation) were calculated using
486 Pearson's rank correlation using R software and visualized with ggplot package. The
487 differences were considered significant at Bonferroni corrected p-value < 0.05. After
488 the correlation procedure and p adjusted values the results were used to develop the
489 functional annotation of proteins and biological taxonomy network using standard
490 procedures of the software Cytoscape.
491
492 **Statistical analyses**
493 In the current study, only microbial taxa with a relative abundance higher than 10
494 reads were considered and used for the analysis. Bacterial abundance profiles were
495 summarized at phyla, family and genus levels, and archaeal communities were
496 summarized to species level. Relative abundances of microbial taxa were normalized
497 to the lowest reads number for bacteria, and then compared among diet using analysis
498 of variance (ANOVA) and the MIXED procedure to determine the linear and quadratic
499 effects and assess the effects of the treatment control versus blend of NA. All
500 performance data were tested for normality and showed a normal distribution. The data
501 were analyzed using ANOVA and by use of regression equations using the MIXED
502 procedure. In all statistical analyses, the diet was considered a fixed effect, and the
503 animals considered a random effect. Treatment means were computed with the
504 LSMEANS option.
505 $Yij = \beta 0 + \beta_1 X_i + \beta_2 X_i^2 + \varepsilon ij;$
506 where:
507 Yij observation of the repetition j on treatment i;
508 β0 general coefficient;
509 β1 linear regression coefficient of the variable observed depending on the levels;

510 β2 quadratic regression coefficient of the variable observed depending on the
511 levels;
512 Xi independent variables (blend of NA levels);
513 Eij residual error.
514 The statistical analyzes were performed using SAS (2004) (Institute Inc., Cary, NC)
515 for Windows and R package.
516

517 **Availability of data and materials**

518 The raw FASTA files of the sequence data were submitted to European Bioinformatics
519 Institute (EMBL-EBI) Sequence Read Archive database with accession number
520 ERP112000 (https://www.ebi.ac.uk/metagenomics).
521

522 **Abbreviations**

523 **ADF:** acid detergent fiber
524 **ANOVA:** Analysis of variance
525 **bp**: base pairs
526 **CON:** Control
527 **DM:** Dry matter
528 **DNA:** Deoxyribonucleic acid
529 **FBW:** Final body weight
530 **HCD:** Hot carcass dressing
531 **HCW:** Hot carcass weight
532 **NA:** Natural additives
533 **NDF:** neutral detergent fiber
534 **OM:** Organic matter
535 **pH:** Potential hydrogenation

VFA: Volatile fatty acid

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Author information

Contributions

Designed the experiment: MO, IP; Field work conduction: MO, TR; Rumen fluid collection: MO, CM, TR, FC; Laboratory procedures: MO, RP; Generation and analysis of the microbiome data: MO, SH, CC. Wrote the manuscript: MO, RP, SH, CC, IP. All authors read and approved the final manuscript.

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Ethics declaration

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 (approval Nº 8583060318) and met the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

Declaration of interest

The authors declare no conflicts of interest.

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

Ingredients	Diet
Corn silage	275.9
Corn grain	613.2
Soybean meal	51.0
Premix[1]	50.5
Mineral salt	4.5
Limestone	4.5
Yeast	0.4
Chemical composition	
Dry matter	577
Crude protein	132
Organic matter	968
Ash	31.4
Ether extract	40.1
Neutral detergent fiber	288
Acid detergent fiber	117
Total digestible nutrients	790
Metabolizable energy (MJ/kg DM)	11.9
Calcium	6.82
Phosphorus	3.56

713 [1]Premix: magnesium (57 g/kg), sodium (81 g/kg), sulphur (3.75 g/kg), cobalt (20 mg/kg), copper (500
714 mg/kg), iodine (25 mg/kg), manganese (1 500 mg/kg), selenium (10 mg/kg), zinc (2 000 mg/kg), vitamin
715 A (400 000 UI/kg), vitamin D3 (50 000 UI/kg), vitamin E (750 UI/kg), ether extract (168 g/kg) and urea
716 (200 g/kg).

Table 2 Feeding behavior from young bulls finished in feedlot with and without natural additive addition to diet

Activities, min/day	Experimental diets					SEM[6]	*P* – value
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		
Rumination	245.0	219.5	209.5	262.0	245.0	9.911	0.550
Feed intake	343.5	349.5	344.5	305.5	337.5	9.182	0.394
Water Ingestion	35.0	34.5	38.0	32.0	37.0	2.451	0.932
Idle	816.5	836.5	848.0	840.5	820.5	11.392	0.883

[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 –6.0 g/animal/day of natural additives addition. Naturals additives contained clove leaf essential oil (Ferquima®), castor and cashew functional oils (Safeeds®) and a commercial blend composed of vanillin, eugenol and thymol (Safeeds®); [6]Standard error of means; [7]Linear effect; [8]Quadratic effect.

Table 3 Animal performance and feed efficiency of young bulls finished in the feedlot with and without natural additive addition to diet

	Experimental diets						*P* – value		
Item	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	*SEM*[6]	L[7]	Q[8]	0% *vs* blend
Initial body weight, kg	382.8	388.0	385.6	385.4	387.3	2.941	0.762	0.641	0.623
Final body weight, kg	473.0	478.7	481.4	486.9	490.0	3.942	0.131	0.322	0.267
Average daily gain, kg	1.43	1.44	1.52	1.61	1.63	0.031	0.013	0.047	0.145
Dry matter intake, kg/d	9.85	9.80	9.83	10.12	10.09	0.144	0.300	0.521	0.706
Dry matter intake, %/BW	2.30	2.26	2.27	2.32	2.33	0.024	0.542	0.670	0.909
Feed efficiency, kg	0.145	0.147	0.155	0.160	0.160	0.014	0.043	0.134	0.216
Hot carcass weight, kg	248.1	252.0	246.6	253.9	246.1	2.521	0.900	0.879	0.816
Hot carcass dressing, %	52.37	52.62	51.25	52.18	51.51	0.302	0.178	0.195	0.357

[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 – 6.0 g/animal/day of natural additives addition. Naturals additives contained clove leaf essential oil (Ferquima®), castor and cashew functional oils (Safeeds®) and a commercial blend composed of vanillin, eugenol and thymol (Safeeds®); [6]Standard error of means; [7]Linear effect; [8]Quadratic effect.

Table 4 Ruminal volatile fatty acids and ruminal ammonia concentration from rumen fluid of young bulls finished in feedlot with and without natural additive addition to diet

	Experimental diets						*P* – value		
Item	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	SEM[6]	L[7]	Q[8]	0% *vs blend*
Acetate (mol/100 mol)	56.15	56.16	43.64	43.98	43.74	1.31	<.0001	<.0001	<.0001
Propionate (mol/100 mol)	17.45	17.00	14.44	16.37	13.69	0.73	0.350	0.054	0.682
Isobutyric (mol/100 mol)	0.91	1.18	0.85	0.79	0.93	0.03	<.0001	0.038	0.623
Butyrate (mol/100 mol)	10.87	13.89	8.67	6.33	7.30	0.67	<.0001	0.221	0.262
Isovaleric (mol/100 mol)	3.07	3.75	2.08	1.85	2.39	0.18	0.002	0.055	0.144
Valeric (mol/100 mol)	1.23	1.33	0.94	0.92	1.07	0.06	0.018	0.210	0.226
A/P* ratio	3.22	3.37	3.02	2.73	3.24	0.12	0.023	0.945	0.434
Ammonia (mg/dL)	21.82	5.95	5.94	3.02	4.20	1.72	0.006	<.0001	<.0001
pH	6.91	6.95	7.05	6.95	7.07	0.06	0.270	0.968	0.326

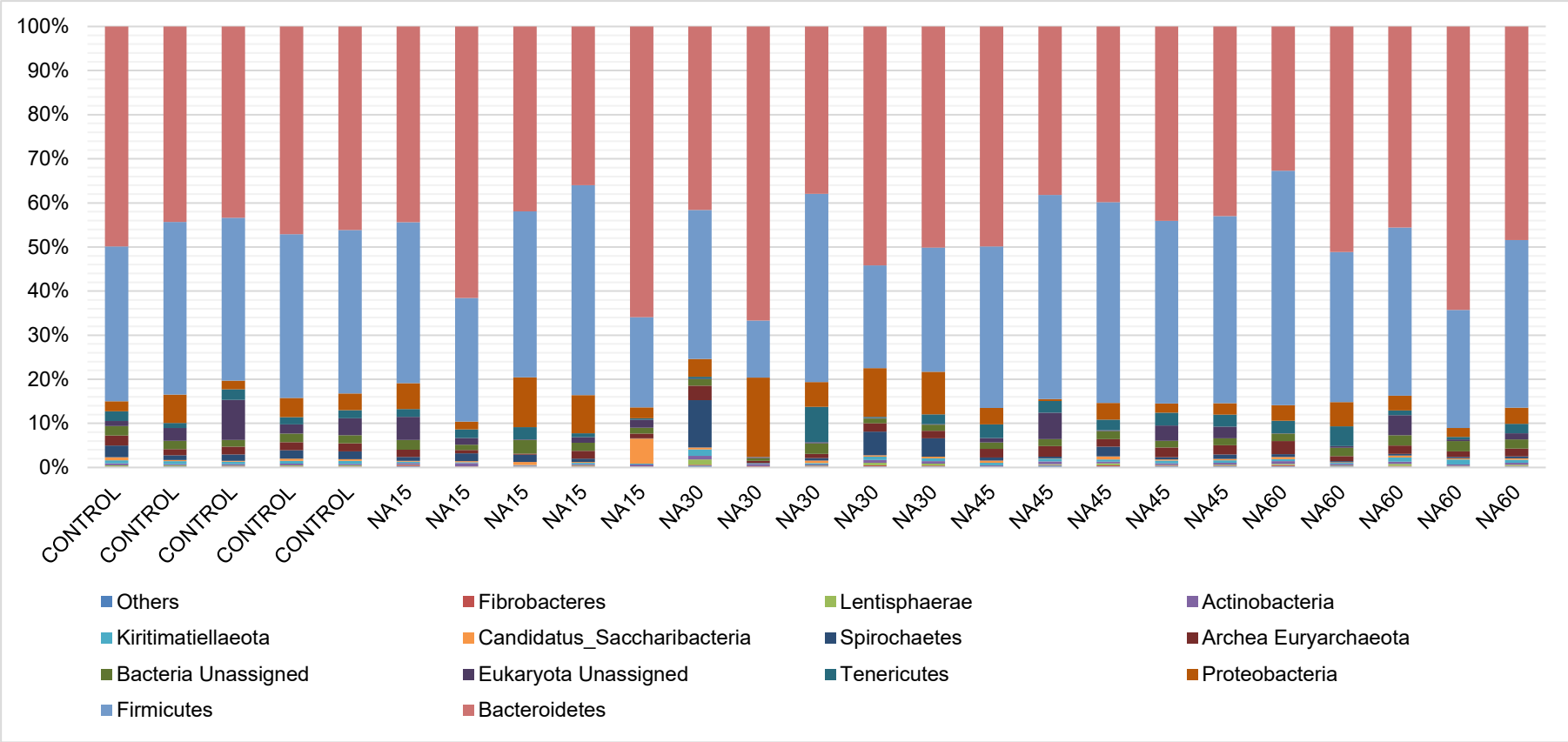
[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 – 6.0 g/animal/day of natural additives addition. Naturals additives contained clove leaf essential oil (Ferquima®), castor and cashew functional oils (Safeeds®) and a commercial blend composed of vanillin, eugenol and thymol (Safeeds®); [6]Standard error of means; [7]Linear effect; [8]Quadratic effect. *A/P = acetate/propionate ratio.

Table 5 Archaea diversity and abundances from young bulls finished in feedlot with and without natural additive and without natural additive addition to diet

	Experimental diets						*P* – value		
Archaea taxonomy	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	*SEM*[6]	L[7]	Q[8]	0% *vs* blend
Archaea Euryarchaeota	2.00	2.22	2.08	1.74	1.93	0.422	0.434	0.847	0.977
f_Methanobacteriaceae	88.53	17.29	19.61	18.40	14.56	13.800	0.956	0.918	<.0001
f_Methanomicrobiaceae	0.27	0.02	0.03	0.00	0.06	0.060	0.826	0.739	0.002
o_Methanomicrobiales	21.89	2.74	5.77	4.09	3.94	4.794	0.844	0.692	0.005
o_Methanobacteriales	19.84	2.90	3.34	3.87	2.48	3.442	0.845	0.991	<.0001
o_Methanomassiliicoccales	1.66	0.13	0.27	0.02	0.17	0.207	0.728	0.440	<.0001
g_Methanobrevibacter	211.22	42.48	36.31	40.08	43.89	14.733	0.909	0.786	<.0001
g_Methanomicrobium	0.74	0.04	0.23	0.05	0.18	0.264	0.981	0.557	0.051
g_Methanosphaera	7.56	2.12	1.97	1.83	2.63	1.255	0.869	0.995	<.0001
s_Methanobrevibacter ruminantium	0.72	0.04	0.43	0.20	0.21	0.118	0.336	0.044	0.001
s_Methanobrevibacter sp D5	0.98	0.40	0.24	0.12	0.26	0.139	0.162	0.936	<.0001
s_Methanobrevibacter sp G16	0.74	0.05	0.13	0.04	0.11	0.262	0.983	0.791	0.039

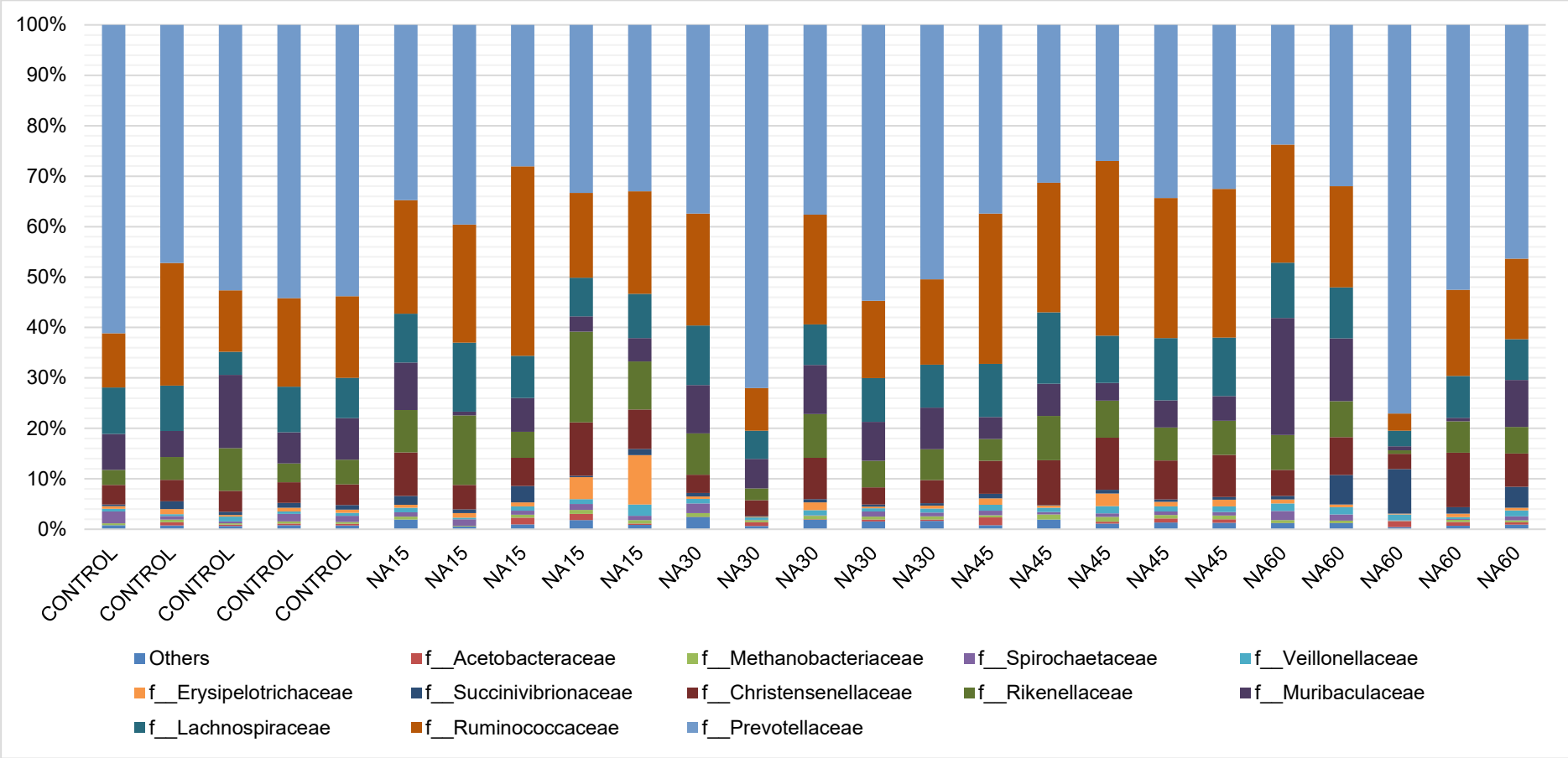
[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 – 6.0 g/animal/day of natural additives addition. Naturals additives contained clove leaf essential oil (Ferquima®), castor and cashew functional oils (Safeeds®) and a commercial blend composed of vanillin, eugenol and thymol (Safeeds®); [6]Standard error of means; [7]Linear effect; [8]Quadratic effect; f_ = family taxonomy, g_ genus taxonomy; o_ = order taxonomy; s_= species taxonomy.

Fig. 1



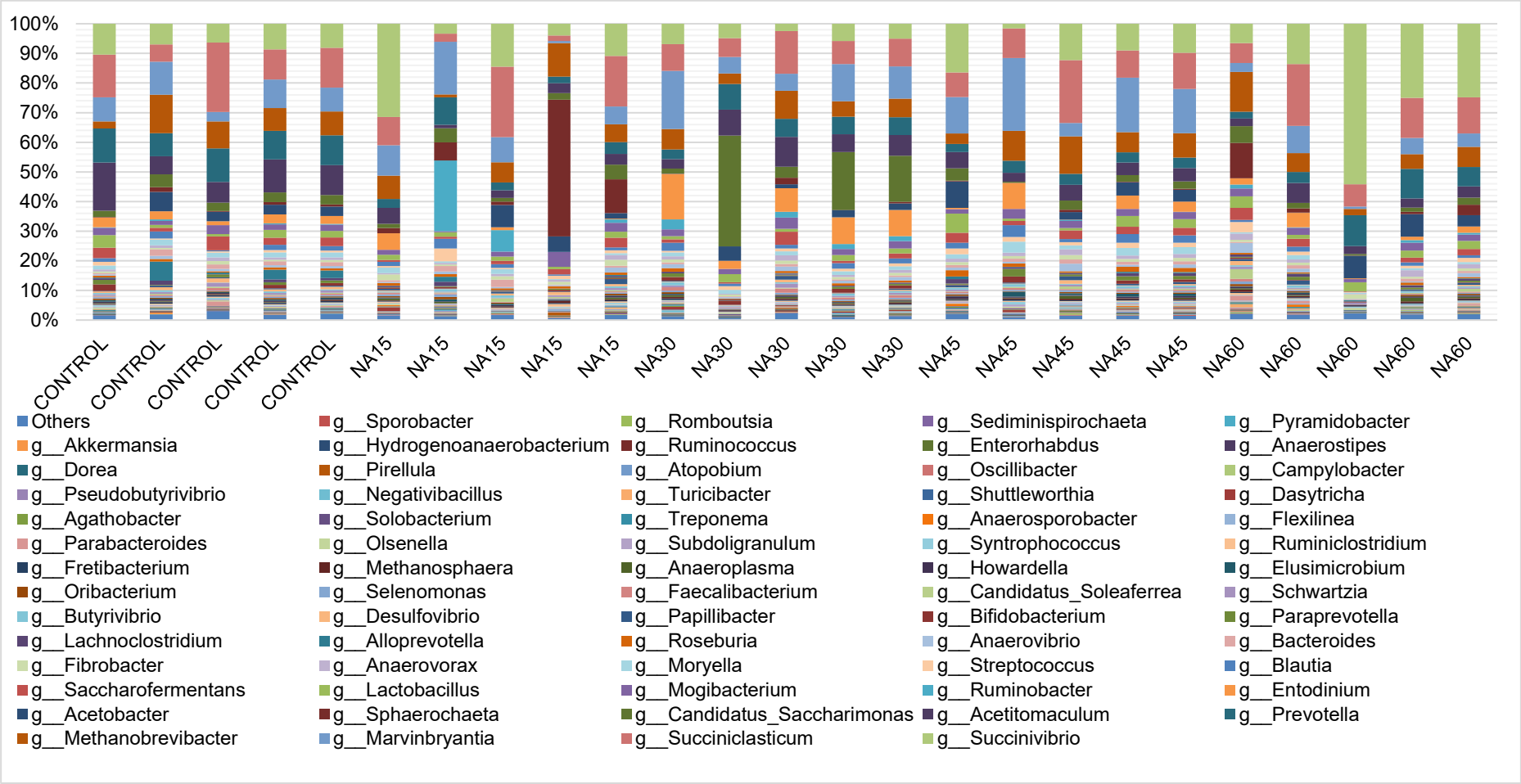
Relative abundance of rumen microbiota based on phyla level and taken from young bulls finished in a feedlot and fed with and without natural additives. Sequences that represented < 10% in a sample were combine in others (blue) to aid the visualization.

Fig. 2



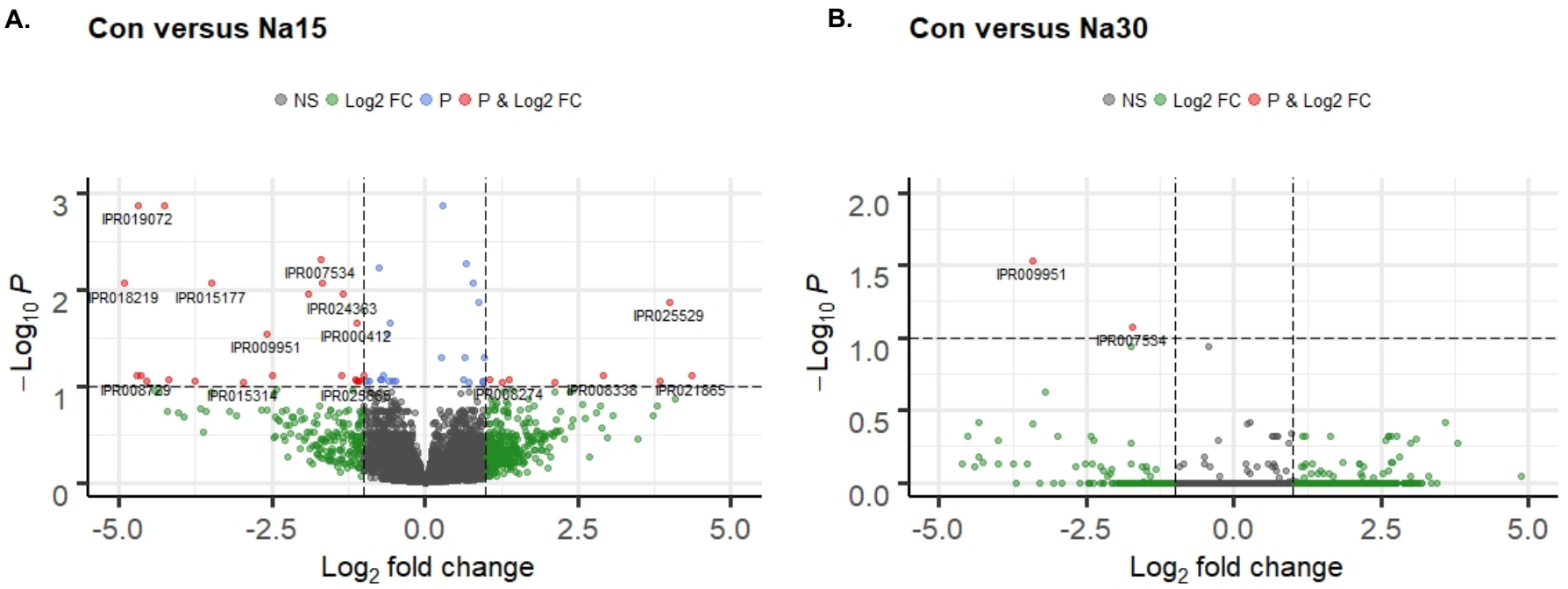
Relative abundance of rumen microbiota on family level of young bulls finished in feedlot and fed natural additives. Sequences that represented < 10% in a sample were combine in others (blue) to aid the visualization.

Fig. 3



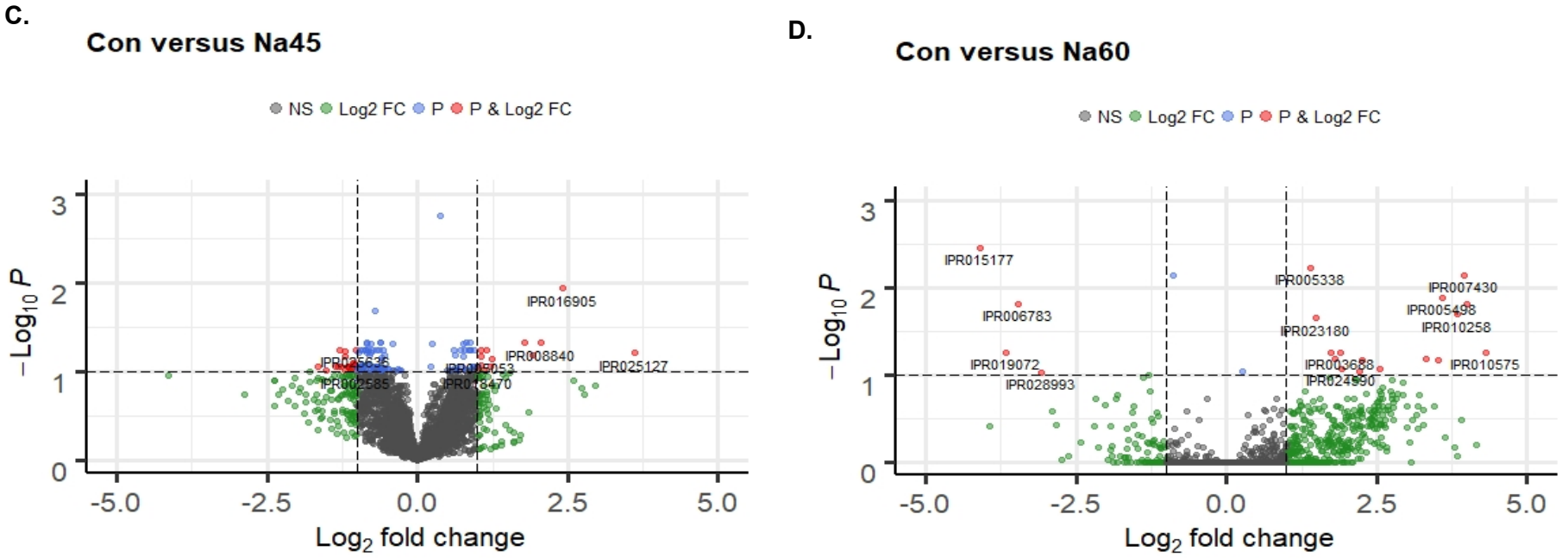
Relative abundance of rumen microbiota on a genera level and taken from young bulls finished in feedlot and fed with and without natural additives. Sequences that represented < 10% in a sample were combine in others (blue) to aid the visualization.

Fig. 4



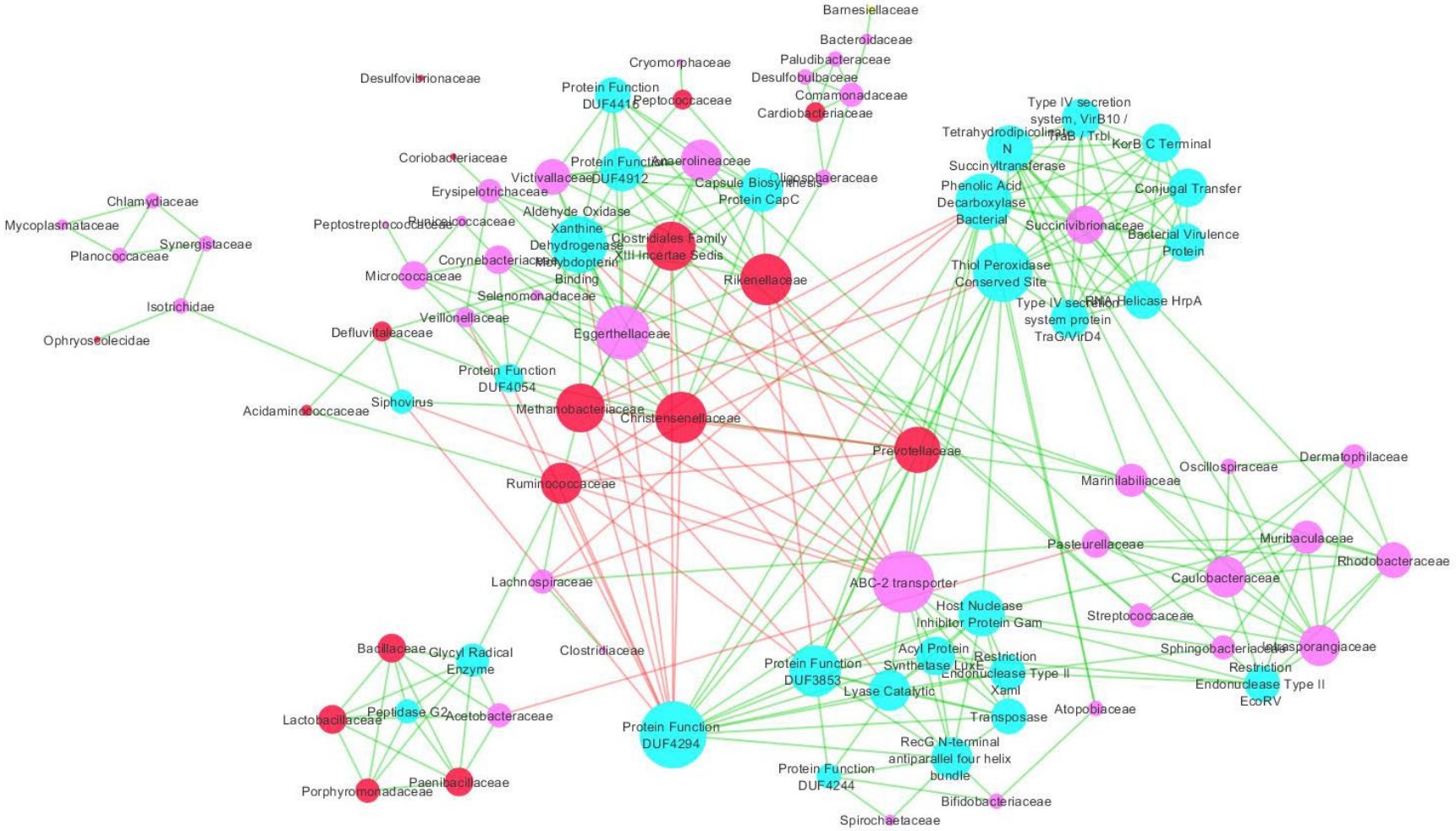
To be continued

Fig. 4 Continuation



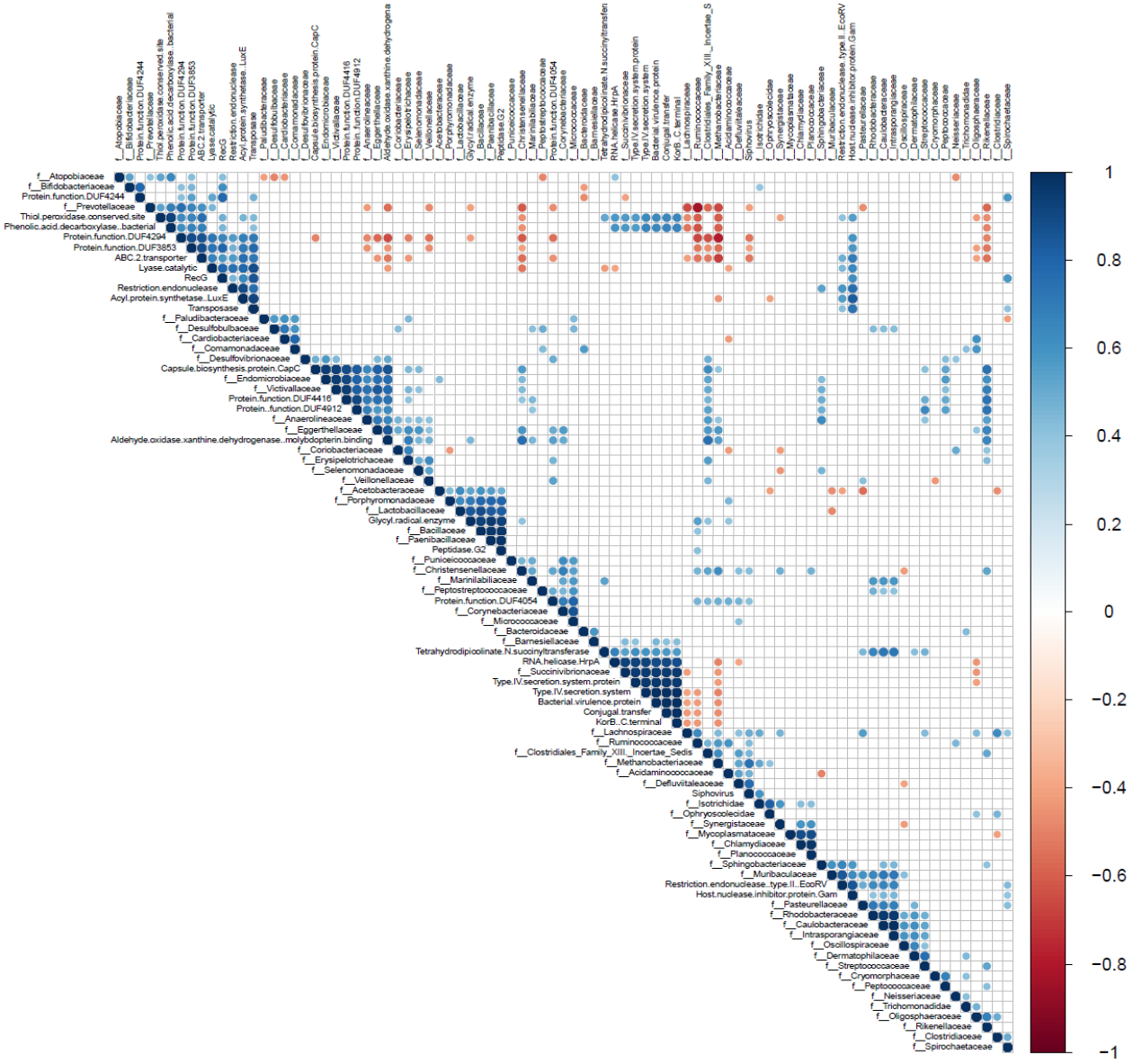
Volcano plot of rumen microbial genes following shotgun metagenomic sequencing of samples obtained from young bulls finished in the feedlot and fed with and without natural additives. Black dots represent non-significantly differentially expressed proteins, green dots represent proteins significantly differentially expressed at pFDR < 0.05 while red dots represent the most significantly differentially expressed proteins; A - Control diet versus Na15 (1.5 g/animal/day of natural additives addition), B - Control diet versus Na30 (3.0 g/animal/day of natural additives addition), C - Control diet versus Na45 (4.5 g/animal/day of natural additives addition), D – Control diet versus Na60 (6.0 g/animal/day of natural additives addition).

Fig. 5



Gene network correlation between rumen diversity and gene functional annotation P < 0.05; light blue nodes) and biological taxonomy family abundance (pink nodes) of young bulls finished in feedlot and fed natural additives. The nodes size is related to the number of directed edges. Green lines are positive correlation (r^2 = > 0.5) and red lines negative correlation (r^2 = < - 0.5). Family taxonomy abundance with significant effect between treatments (P < 0.05; red nodes).

Fig. 6



Correlogram between functional annotation of genes and biological taxonomy on a family levels from samples taken from young bulls finished in feedlot and fed with and without natural additives ($P < 0.05$).

Supplementary Data

Table S1 Comparison of rumen microbiota abundance and diversity on a phyla level and taken from young bulls finished in a feedlot with and without natural additive addition to diet

	Experimental diets						P - value		
Item	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	SEM[6]	L[7]	Q[8]	0% *VS* NA
Acidobacteria	0.38	0.18	0.00	0.18	0.69	0.090	0.9956	0.4442	0.2394
Actinobacteria	111.06	113.71	119.29	137.37	110.30	8.811	0.4348	0.8104	0.6147
Armatimonadetes	0.62	3.15	0.59	1.52	2.03	0.366	0.1466	0.0749	0.2128
Ascomycota	2.98	5.58	3.49	6.20	3.38	0.916	0.8407	0.3714	0.4054
Bacteria Unassigned	433.76	575.71	456.95	533.21	453.11	27.140	0.6257	0.2041	0.2224
Bacteroidetes	16160.51	11326.14	14614.40	10228.94	14757.65	558.359	0.3308	0.0007	0.0002
Candidatus_Melainabacteria	0.57	1.40	0.16	0.64	1.79	0.294	0.4207	0.2982	0.8348
Candidatus_Saccharibacteria	96.63	126.12	650.91	94.89	82.16	68.575	0.8633	0.0023	0.1993
Chlamydiae	0.34	6.19	2.35	1.47	2.50	1.063	0.1804	0.6209	0.2935
Chytridiomycota	0.00	0.00	1.71	0.50	0.15	0.168	0.1796	0.0002	0.0214
Elusimicrobia	16.18	29.03	6.30	36.54	10.10	3.459	0.4058	0.0025	0.2943
Eukaryota Unassigned	292.52	385.65	1465.95	657.81	321.10	133.601	0.4362	0.0047	0.0660
Euryarchaeota	373.89	484.60	424.23	559.15	368.18	38.519	0.5523	0.3715	0.2652
Fibrobacteres	20.71	57.45	31.85	43.45	34.86	3.680	0.1495	0.0323	0.0058
Firmicutes	8638.23	11173.60	8667.37	12985.44	8435.01	501.631	0.1391	0.0032	0.0263
Fusobacteria	0.53	1.69	2.40	1.69	0.87	0.441	0.997	0.5776	0.2519

To be continued

Table S1 continuation

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Ignavibacteriae	0.00	0.52	0.75	0.53	0.40	0.118	0.9688	0.4988	0.0604
Kiritimatiellaeota	159.89	143.67	147.88	134.34	116.10	17.630	0.8783	0.8664	0.7191
Lentisphaerae	18.77	98.15	49.19	46.69	35.70	12.932	0.2206	0.5172	0.1823
Proteobacteria	718.33	1869.80	694.15	882.44	2246.77	208.117	0.0913	0.1725	0.3549
Spirochaetes	284.19	900.12	182.81	147.33	283.95	114.013	0.0404	0.2653	0.6583
Streptophyta	1.58	1.18	1.28	4.28	0.29	0.511	0.0509	0.2747	0.5917
Synergistetes	19.25	15.21	15.49	23.87	16.59	1.257	0.0301	0.222	0.7313
Tenericutes	564.86	461.80	360.21	1305.04	549.34	99.138	0.0028	0.0242	0.4842
Verrucomicrobia	8.29	10.03	4.36	9.09	11.20	1.234	0.8131	0.1436	0.8859

[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 – 6.0 g/animal/day of natural additives addition. Naturals additives contained clove leaf essential oil (Ferquima®), castor and cashew functional oils (Safeeds®) and a commercial blend composed of vanillin, eugenol and thymol (Safeeds®); [6]Standard error of means; [7]Linear effect; [8]Quadratic effect.

Table S2 Comparison of rumen microbiota abundance and diversity on a family level and taken from young bulls finished in a feedlot with and without natural additive addition to diet

	Experimental diets						P - value		
Item	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	SEM[6]	L[7]	Q[8]	0% *VS* NA
Acetobacteraceae	54.331	100.215	55.982	118.683	85.867	16.422	0.7362	0.2670	0.4082
Acidaminococcaceae	1.115	1.449	0.683	2.749	0.924	0.206	0.0163	0.0036	0.2208
Anaerolineaceae	0.123	0.681	0.469	0.441	0.457	0.099	0.4580	0.7404	0.1317
Atopobiaceae	15.260	13.590	9.853	16.583	12.948	1.421	0.5271	0.2084	0.6188
Bacillaceae	0.460	0.772	0.290	5.661	0.855	0.650	0.0105	0.0650	0.2222
Bacteroidaceae	3.861	4.013	2.841	3.547	3.905	0.394	0.7300	0.4245	0.7208
Barnesiellaceae	1.822	0.565	2.007	0.000	4.124	0.652	0.7825	0.3365	0.5654
Bifidobacteriaceae	6.396	3.336	4.619	4.230	3.485	0.721	0.7107	0.6887	0.2429
Cardiobacteriaceae	0.616	0.512	2.875	0.000	0.620	0.334	0.5816	0.0035	0.4996
Caulobacteraceae	0.000	0.000	0.000	0.000	4.744	0.775	1.0000	1.0000	1.0000
Chlamydiaceae	0.123	2.178	0.724	0.407	1.357	0.403	0.1868	0.6177	0.3653
Christensenellaceae	733.769	1344.960	820.652	1501.560	1190.320	89.216	0.4982	0.0061	0.0158
Clostridiaceae	1.257	1.118	2.276	1.980	1.139	0.258	0.3087	0.3213	0.4372
Clostridiales_Family_XIII._Incertae_Sedis	0.535	1.660	0.179	1.850	0.327	0.187	0.6551	0.0003	0.0552
Comamonadaceae	0.123	0.279	0.869	0.328	0.293	0.153	0.9221	0.2057	0.376
Coriobacteriaceae	1.098	1.592	0.931	0.402	0.834	0.157	0.0197	0.8724	0.752
Corynebacteriaceae	1.338	2.942	2.461	5.660	5.019	1.090	0.4588	0.5616	0.4333
Cryomorphaceae	1.323	0.882	0.497	0.000	0.615	0.185	0.1335	0.9101	0.0752
Defluviitaleaceae	18.501	8.874	5.495	33.789	7.671	2.610	0.0001	0.0022	0.5707
Dermatophilaceae	0.000	0.844	0.359	0.164	0.654	0.151	0.1688	0.7276	0.2552

To be continued

Table S2 continuation

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Desulfobulbaceae	0.123	0.281	0.683	0.402	0.648	0.090	0.6642	0.1651	0.1526
Desulfovibrionaceae	2.339	4.519	0.145	2.043	1.685	0.548	0.1416	0.0367	0.9382
Eggerthellaceae	12.227	29.950	20.147	22.128	14.544	3.400	0.4822	0.5404	0.199
Endomicrobiaceae	2.150	11.168	0.179	3.672	2.439	1.736	0.1792	0.1362	0.5233
Erysipelotrichaceae	116.685	591.156	111.579	229.610	98.629	70.791	0.0954	0.1105	0.2634
Intrasporangiaceae	0.000	0.000	0.000	0.000	2.127	0.347	1.0000	1.0000	1.0000
Isotrichidae	0.343	1.854	3.455	3.800	0.327	0.609	0.3013	0.6969	0.0872
Lachnospiraceae	1427.700	1728.430	1526.870	2081.600	1454.130	90.932	0.1960	0.1138	0.1189
Lactobacillaceae	0.508	0.878	0.862	2.766	1.092	0.248	0.0087	0.1031	0.0755
Marinilabiliaceae	0.123	1.336	0.000	0.730	2.698	0.325	0.5015	0.1929	0.4432
Methanobacteriaceae	69.378	102.964	107.117	139.585	71.720	8.165	0.1051	0.4574	0.0144
Micrococcaceae	0.123	0.163	0.145	0.657	0.591	0.126	0.2339	0.4558	0.5530
Muribaculaceae	1479.060	876.607	1476.590	881.527	1668.200	171.398	0.9929	0.2204	0.3788
Mycoplasmataceae	0.589	1.060	0.434	0.000	0.377	0.163	0.0504	0.8304	0.8283
Neisseriaceae	4.637	8.160	3.303	1.307	3.782	1.020	0.0422	0.6066	0.8843
Oligosphaeraceae	1.497	2.514	3.117	2.161	0.586	0.306	0.6871	0.3108	0.1353
Ophryoscolecidae	33.795	60.325	198.408	82.155	44.667	18.433	0.6542	0.0062	0.0552
Oscillospiraceae	0.000	0.423	0.786	0.000	0.491	0.113	0.2125	0.0567	0.1486
Paenibacillaceae	0.123	0.200	0.000	2.403	0.000	0.273	0.0039	0.0380	0.1927
Paludibacteraceae	0.370	0.419	0.434	0.402	0.343	0.106	0.9633	0.9401	0.8727
Pasteurellaceae	0.172	0.379	0.248	0.475	0.750	0.098	0.7602	0.5110	0.4459
Peptococcaceae	2.150	2.756	0.179	1.058	0.879	0.329	0.0837	0.0450	0.2953

To be continued

Table S2 continuation

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Peptostreptococcaceae	1.942	0.824	1.441	1.517	2.367	0.316	0.5089	0.7648	0.4274
Planococcaceae	0.123	6.688	2.999	2.098	4.246	1.024	0.1658	0.6195	0.1597
Porphyromonadaceae	0.833	0.000	0.000	1.696	0.214	0.177	0.0004	0.0248	0.4266
Prevotellaceae	9655.940	6053.800	9052.390	5831.610	8313.770	498.941	0.8689	0.0137	0.0228
Puniceicoccaceae	0.172	0.479	0.786	1.228	2.960	0.353	0.4653	0.9391	0.4316
Rhodobacteraceae	0.165	0.000	0.000	0.803	1.636	0.269	0.3374	0.5767	0.8796
Rikenellaceae	886.272	1971.880	1099.030	1211.420	940.601	127.199	0.0380	0.1122	0.0671
Ruminococcaceae	2904.550	4332.780	3040.840	5290.690	2870.990	283.216	0.1881	0.0087	0.0327
Selenomonadaceae	11.204	10.309	10.308	12.683	8.390	0.988	0.4774	0.6802	0.9695
Sphingobacteriaceae	0.616	0.518	0.393	0.204	0.620	0.137	0.5023	0.9367	0.5231
Spirochaetaceae	224.925	176.088	141.433	123.213	146.101	20.803	0.4428	0.8896	0.1724
Streptococcaceae	1.269	7.247	2.220	5.067	5.520	1.016	0.4978	0.1653	0.1806
Succinivibrionaceae	155.679	258.673	85.399	100.275	743.708	72.568	0.3946	0.5574	0.9599
Synergistaceae	1.789	2.388	1.538	3.082	2.060	0.344	0.5451	0.2374	0.5574
Trichomonadidae	0.165	1.129	0.538	0.000	0.130	0.147	0.0141	0.9417	0.2682
Veillonellaceae	103.460	187.525	135.379	201.587	202.634	15.800	0.7676	0.1610	0.0774
Victivallaceae	0.743	19.671	2.565	2.319	3.189	3.235	0.0994	0.3439	0.3748

[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 – 6.0 g/animal/day of natural additives addition. Naturals additives contained clove leaf essential oil (Ferquima®). castor and cashew functional oils (Safeeds®) and a commercial blend composed of vanillin. eugenol and thymol (Safeeds®); [6]Standard error of means; [7]Linear effect; [8]Quadratic effect.

Table S3 Comparison of rumen microbiota abundance and diversity on a genus level and taken from young bulls finished in a feedlot with and without natural additive addition to diet

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Acetanaerobacterium	0.50	0.31	0.55	0.18	0.35	0.07	0.5935	0.1471	0.4425
Acetitomaculum	423.40	133.21	293.38	188.53	158.05	28.64	0.3987	0.0271	0.0005
Acetobacter	135.95	121.93	91.33	167.65	158.81	23.42	0.5684	0.4428	0.8905
Acidaminococcus	2.14	0.11	0.00	0.00	1.38	0.34	0.9116	0.9489	0.0203
Agathobacter	6.34	6.08	7.93	8.04	6.91	0.74	0.4403	0.5691	0.6244
Akkermansia	9.57	3.74	1.83	0.90	3.89	1.05	0.3481	0.8516	0.0061
Alistipes	2.74	1.80	1.70	4.18	0.96	0.35	0.0187	0.1259	0.8206
Alloprevotella	110.06	18.54	2.81	16.24	7.78	12.17	0.9424	0.5971	0.0011
Anaerobiospirillum	0.74	1.58	0.77	0.55	7.75	1.19	0.7801	0.9528	0.9402
Anaerofustis	0.46	1.02	1.49	1.10	1.43	0.30	0.9393	0.6305	0.3809
Anaeroplasma	7.59	12.73	14.54	14.82	21.52	2.49	0.7972	0.9135	0.3371
Anaerosporobacter	4.57	5.65	4.09	12.51	10.40	1.81	0.2498	0.3311	0.5528
Anaerostipes	3.72	3.53	0.43	6.62	6.94	0.62	0.0293	0.0006	0.8544
Anaerotruncus	0.26	0.40	0.68	0.62	0.33	0.11	0.5332	0.5724	0.2980
Anaerovibrio	29.98	30.62	45.57	48.65	55.44	6.14	0.3787	0.7357	0.4850
Anaerovorax	36.67	34.45	39.86	56.49	57.69	4.51	0.1292	0.6469	0.5493
Asteroleplasma	1.73	0.28	1.53	1.70	0.17	0.21	0.0168	0.2624	0.2264
Atopobium	2.93	3.34	2.52	9.38	4.79	0.69	0.0012	0.0119	0.1161
Bacillus	0.50	2.12	0.21	8.18	1.89	0.92	0.0216	0.0295	0.1459
Bacteroides	58.74	48.27	22.72	43.80	27.55	5.14	0.7701	0.0899	0.1125

To be continued

Table S3 continuation

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Bavariicoccus	0.00	0.00	0.00	1.74	0.25	0.21	0.0054	0.0868	0.2174
Bifidobacterium	36.94	15.14	42.88	35.31	8.55	5.30	0.2142	0.2093	0.6547
Bilophila	0.72	1.67	0.43	0.00	1.22	0.26	0.0503	0.5624	0.9749
Blautia	59.92	60.49	70.51	104.59	37.81	7.98	0.0690	0.5518	0.3325
Brevibacterium	0.00	0.00	0.00	0.00	1.92	0.22	0.9990	0.9990	1.0000
Brevundimonas	0.00	0.00	0.00	0.00	4.92	0.80	1.0000	1.0000	1.0000
Butyricicoccus	2.32	2.69	1.66	2.58	3.28	0.37	0.9330	0.3725	0.9937
Butyrivibrio	17.45	22.25	25.49	28.65	18.80	2.33	0.4061	0.9946	0.2074
Campylobacter	2.00	6.81	1.75	4.77	7.54	0.83	0.3878	0.0573	0.2101
Candidatus_Saccharimonas	130.78	123.83	651.40	101.98	98.45	66.68	0.9011	0.0019	0.2677
Candidatus_Soleaferrea	13.22	21.64	6.01	18.42	40.35	5.49	0.8532	0.3576	0.8807
Candidatus_Symbiothrix	4.57	0.45	0.64	0.55	0.17	0.43	0.9122	0.8626	<.0001
Caproiciproducens	1.54	2.05	0.98	0.95	2.75	0.39	0.3899	0.6365	0.8394
Catenibacterium	1.48	1.11	0.43	0.90	0.89	0.17	0.7062	0.2450	0.1547
Catenisphaera	2.63	0.00	0.21	1.66	0.00	0.42	0.2017	0.5774	0.0650
Cellulosilyticum	0.00	0.98	0.00	0.00	0.25	0.20	0.3660	0.3812	0.5338
Citreitalea	1.04	1.24	0.00	0.00	0.00	0.20	0.0429	0.2263	0.1934
Clostridium	6.48	0.20	0.00	0.18	1.06	1.05	0.9968	0.9459	0.0240
Collinsella	0.68	0.77	0.90	1.11	0.36	0.13	0.4301	0.9007	0.4946
Comamonas	1.18	0.29	0.85	1.32	6.50	0.72	0.5999	0.9777	0.8230
Corynebacterium	0.74	1.34	2.26	2.93	3.14	0.60	0.4294	0.9423	0.3841
Dasytricha	0.23	9.82	16.93	12.17	0.36	2.70	0.7740	0.4067	0.0678

To be continued

Table S3 continuation

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Desulfobulbus	2.12	1.23	1.53	2.54	4.76	0.43	0.2818	0.7368	0.7158
Desulfotomaculum	0.23	0.74	0.34	0.00	0.70	0.14	0.1051	0.9388	0.7138
Desulfovibrio	37.11	22.26	15.43	17.82	19.22	3.57	0.6934	0.6365	0.0534
Dialister	4.02	0.44	0.00	0.93	6.98	1.04	0.8731	0.7987	0.1687
Dorea	4.71	2.60	9.12	5.38	2.65	0.61	0.0393	0.0001	0.3469
Eisenbergiella	2.52	1.34	0.43	2.41	2.04	0.30	0.2314	0.0681	0.1284
Elusimicrobium	17.35	16.20	7.55	35.45	7.68	2.99	0.0178	0.0103	0.6997
Enterorhabdus	3.30	3.85	4.22	5.80	3.92	0.54	0.2893	0.7017	0.3783
Entodinium	108.29	57.41	367.78	144.36	89.00	31.65	0.2703	0.0007	0.2075
Faecalibacterium	20.93	11.42	35.08	20.64	15.49	2.73	0.2371	0.0087	0.8163
Fibrobacter	28.40	54.55	37.00	46.44	38.10	3.41	0.4272	0.1350	0.0436
Flavonifractor	2.48	3.12	1.19	1.80	2.07	0.38	0.3023	0.2528	0.6671
Flexilinea	2.20	10.45	7.25	9.59	8.37	1.43	0.8496	0.4867	0.0765
Fretibacterium	19.35	9.70	14.45	15.65	12.99	1.00	0.0350	0.4450	0.0103
Fusicatenibacter	0.52	0.44	0.34	0.22	1.07	0.13	0.5852	0.9677	0.5652
Fusobacterium	0.46	0.62	1.70	1.10	0.88	0.31	0.6495	0.3600	0.4314
Haemophilus	0.23	0.14	1.71	0.00	0.52	0.21	0.8203	0.0051	0.4400
Holdemanella	0.26	0.00	1.11	1.27	1.11	0.17	0.0115	0.2413	0.1678
Howardella	15.04	13.97	12.23	20.11	16.55	1.16	0.1005	0.1350	0.8928
Hydrogenispora	0.26	0.46	0.64	0.40	1.19	0.15	0.9095	0.6127	0.5439
Hydrogenoanaerobacterium	2.44	4.46	5.54	5.58	1.83	0.48	0.3716	0.6286	0.0125
Intestinimonas	3.60	2.28	3.84	2.24	1.89	0.34	0.9716	0.0897	0.3426

To be continued

Table S3 continuation

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Kocuria	0.24	0.48	0.43	1.28	1.33	0.29	0.4122	0.5879	0.5370
Lachnobacterium	3.66	1.83	2.60	1.48	1.23	0.43	0.7992	0.4284	0.1411
Lachnoclostridium	31.21	23.52	15.26	38.72	26.80	3.44	0.1659	0.0987	0.5404
Lachnospira	1.77	0.85	3.28	2.40	1.35	0.36	0.1663	0.0926	0.6486
Lactobacillus	90.81	62.80	71.27	121.98	113.57	11.38	0.1151	0.5049	0.8542
Mailhella	1.78	1.85	0.21	0.40	0.25	0.23	0.0258	0.0950	0.0643
Marvinbryantia	336.21	360.84	453.81	624.69	191.88	50.59	0.0803	0.7567	0.2337
Megasphaera	2.52	3.55	0.55	1.54	1.37	0.58	0.2975	0.2346	0.6812
Mogibacterium	91.03	98.45	102.94	99.18	91.64	7.72	0.9784	0.8592	0.6768
Moraxella	0.68	0.75	0.00	0.00	0.53	0.14	0.0995	0.3306	0.2341
Moryella	68.52	40.67	53.79	95.57	31.65	6.41	0.0020	0.2984	0.6866
Mycoplasma	2.12	3.24	0.85	0.83	1.44	0.43	0.0919	0.3280	0.6681
Negativibacillus	4.53	6.88	11.47	6.21	1.73	1.39	0.8771	0.1953	0.3037
Olsenella	14.98	8.42	9.76	8.72	10.67	1.40	0.9473	0.7645	0.1198
Oribacterium	17.16	18.24	12.62	15.84	17.39	1.47	0.6287	0.3079	0.6935
Oscillibacter	4.41	5.87	4.01	4.21	4.09	0.55	0.3772	0.5227	0.8512
Oscillospira	0.00	1.67	0.68	1.31	1.88	0.33	0.7315	0.3794	0.1645
Paenibacillus	0.72	1.28	1.41	3.53	1.22	0.36	0.0410	0.2769	0.1239
Papillibacter	15.39	26.61	18.67	30.07	27.07	3.63	0.7753	0.3615	0.3309
Parabacteroides	15.90	0.92	1.53	1.86	25.04	3.72	0.3917	0.9878	0.1189
Paraprevotella	36.44	9.80	24.60	50.42	21.44	4.82	0.0071	0.6432	0.4686
Phascolarctobacterium	2.10	1.27	0.64	1.17	0.48	0.21	0.8666	0.2682	0.0380

To be continued

Table S3 continuation

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Pichia	0.48	0.57	0.21	0.58	0.50	0.12	0.9736	0.3250	0.9441
Pirellula	1.00	9.40	5.37	2.75	1.46	1.68	0.2314	0.8817	0.2846
Polyplastron	1.53	2.32	2.22	0.22	0.35	0.46	0.1681	0.4639	0.9628
Porphyromonas	0.00	0.34	1.07	0.22	0.90	0.20	0.8496	0.1716	0.3127
Prevotella	420.17	176.86	249.22	146.86	276.41	26.51	0.6351	0.1209	0.0002
Pseudobutyrivibrio	4.38	6.00	3.41	5.93	6.54	0.64	0.9760	0.1703	0.6709
Pseudoflavonifractor	0.72	1.16	0.43	0.93	0.35	0.14	0.6099	0.1208	0.7371
Pseudoscardovia	0.00	0.00	3.41	0.00	0.00	0.41	1.0000	0.0014	0.2065
Pyramidobacter	4.24	2.46	2.52	6.70	3.17	0.56	0.0150	0.1499	0.7905
Raoultibacter	0.46	0.43	0.64	0.40	1.14	0.11	0.9355	0.4617	0.9057
Robinsoniella	5.25	0.00	1.92	0.28	0.00	0.64	0.8717	0.2383	0.0038
Romboutsia	0.00	1.38	1.32	6.12	7.29	1.08	0.1435	0.3784	0.2608
Roseburia	29.29	32.81	30.61	57.97	18.61	3.78	0.0136	0.0812	0.1566
Ruminiclostridium	11.31	18.61	7.84	10.77	10.53	1.55	0.1133	0.1108	0.7786
Ruminobacter	10.43	270.77	72.99	3.66	24.20	40.59	0.0398	0.5484	0.3007
Ruminococcus	5.36	3.83	5.20	2.47	4.17	0.68	0.5493	0.3046	0.4154
Saccharofermentans	124.26	62.24	70.38	106.81	87.99	10.72	0.1974	0.6304	0.1191
Schwartzia	32.34	15.73	29.71	15.58	19.08	2.72	0.9848	0.0553	0.0801
Sediminispirochaeta	1.18	5.32	1.83	3.01	4.58	0.58	0.1875	0.1270	0.1262
Selenomonas	16.04	18.47	20.16	18.75	14.17	1.71	0.9618	0.7570	0.5153
Sharpea	3.80	0.00	2.13	0.83	1.51	0.57	0.6425	0.2736	0.0640
Shuttleworthia	9.60	3.90	5.50	6.71	6.77	0.82	0.2732	0.9291	0.0511

To be continued

Table S3 continuation

Item	Experimental diets					SEM[6]	P - value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *VS* NA
Solobacterium	5.86	7.75	8.48	7.96	2.94	0.80	0.9279	0.7646	0.2704
Sphaerochaeta	30.41	555.78	23.49	14.66	149.18	78.72	0.0292	0.2043	0.3835
Sphingobacterium	0.00	0.11	0.85	0.22	0.70	0.15	0.8292	0.1092	0.3182
Sporobacter	1.94	2.53	2.05	7.55	2.81	0.50	<.0001	0.0008	0.0085
Streptococcus	29.40	63.03	38.15	64.75	58.43	7.80	0.9459	0.2493	0.2198
Subdoligranulum	11.93	8.46	8.57	20.83	7.24	1.22	<.0001	0.0078	0.7266
Succiniclasticum	563.59	460.78	393.19	510.09	508.53	49.67	0.7712	0.5314	0.4345
Succinimonas	3.64	0.74	0.00	0.00	1.17	0.46	0.5625	0.7374	0.0037
Succinivibrio	338.21	536.55	209.73	410.38	1039.20	95.89	0.6370	0.2610	0.8279
Suttonella	0.96	0.10	1.19	0.00	0.36	0.21	0.8778	0.0486	0.3151
Syntrophococcus	15.00	8.76	6.91	18.53	11.94	1.53	0.0371	0.0906	0.3254
Tetratrichomonas	1.28	4.50	1.32	0.00	0.72	0.50	0.0031	0.4330	0.5524
Treponema	9.06	6.32	9.81	2.73	8.71	1.25	0.3743	0.1380	0.3998
Turicibacter	1.64	5.51	3.50	5.64	14.52	1.66	0.9785	0.6224	0.4185
Weissella	1.76	0.31	0.43	0.87	0.88	0.19	0.3073	0.7206	0.0107

[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 –6.0 g/animal/day of natural additives addition. Naturals additives contained clove leaf essential oil (Ferquima®). castor and cashew functional oils (Safeeds®) and a commercial blend composed of vanillin, eugenol and thymol (Safeeds®); [6]Standard error of means; [7]Linear effect; [8]Quadratic effect.

Table S4 Functional gene annotation using InterPro results with significance level (P < 0.05) from DESeq (-Log$_{10}$P)

Item	Experimental diets					*SEM*[6]	*P* – value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[7]	Q[8]	0% *vs* blend
IPR000412	271.20	84.40	114.00	103.00	167.00	2.13	0.2859	0.0941	<.0001
IPR003688	1479.00	1770.40	1158.20	2219.20	4790.80	73.03	0.0037	0.007	0.7416
IPR005498	185.80	327.40	133.20	182.20	1872.80	39.41	0.0169	0.004	0.9442
IPR006783	520.00	80.80	46.20	126.00	47.60	8.58	0.0013	<.0001	<.0001
IPR007430	127.20	182.00	60.00	84.20	1649.60	35.65	0.0159	0.0027	0.9593
IPR007534	530.00	139.40	138.20	238.80	214.80	7.03	<.0001	0.0003	<.0001
IPR008274	2521.20	5073.20	2469.80	4450.20	3035.20	62.79	0.8597	0.4546	0.0328
IPR008338	19.80	110.40	14.80	71.00	26.80	2.72	0.3272	0.0370	0.1699
IPR008729	470.00	16.00	25.00	27.80	412.60	15.11	0.8518	0.0221	0.0205
IPR008840	244.80	295.40	506.80	889.20	317.00	12.07	0.0001	0.4401	0.0054
IPR009951	354.00	50.40	28.80	79.20	191.60	6.37	0.1948	0.0001	<.0001
IPR010258	173.80	192.20	90.00	120.80	2040.00	44.99	0.0178	0.0034	0.9327
IPR010575	29.80	25.00	9.20	15.00	487.60	11.14	0.0180	0.0034	0.9081
IPR015177	269.20	20.40	122.80	40.20	16.80	4.38	0.0006	0.0005	<.0001
IPR015314	367.20	39.40	137.80	145.20	296.60	7.89	0.9022	0.0237	0.0080
IPR016905	134.00	274.80	181.20	708.80	203.00	10.92	0.1419	0.1105	0.0157
IPR018219	151.00	4.40	6.80	13.20	166.80	4.20	0.7923	0.0005	0.0025
IPR019072	378.80	13.40	31.00	25.20	30.60	7.88	0.0107	0.0014	0.0001
IPR021865	3.00	46.40	9.00	355.80	31.20	8.19	0.0082	0.0481	0.1348
IPR023180	100.20	138.60	86.00	122.80	266.00	3.09	0.0019	<.0001	0.5090
IPR024363	995.00	332.40	446.00	446.80	645.00	11.09	0.1385	<.0001	<.0001
IPR024590	156.20	285.40	130.80	189.00	514.00	7.80	0.0213	0.0084	0.5525
IPR025127	90.80	491.20	153.80	927.00	354.00	21.75	0.2178	0.3121	0.1047

To be continued

Table S4 continuation

Item	Experimental diets						*P* – value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	*SEM*[6]	L[7]	Q[8]	0% *vs* blend
IPR025338	5.20	1.20	3.80	3.20	2.00	0.14	0.3734	0.6242	0.1754
IPR025529	11.20	134.60	188.00	13.80	47.00	3.37	0.0207	0.1985	0.2703
IPR025636	2394.20	1246.40	1584.20	1128.20	1775.60	21.88	0.0792	0.0003	<.0001
IPR028993	54.20	10.40	8.00	13.20	6.80	1.01	0.0066	0.0017	0.0002
IPR032585	7.00	20.20	4.00	8.800	11.60	0.40	0.0573	0.0444	0.3963

[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 – 6.0 g/animal/day of natural additives addition. Naturals additives contained clove leaf essential oil (Ferquima®), castor and cashew functional oils (Safeeds®) and a commercial blend composed of vanillin, eugenol and thymol (Safeeds®); [6]Standard error of means; [7]Linear effect; [8]Quadratic effect. IPR019072 = Restriction endonuclease, type II, XamI; IPR018219 = Thiol peroxidase conserved site; IPR015177 = Lyase, catalytic; IPR007534 = Acyl-protein synthetase, LuxE; IPR024363 = Protein of unknown function DUF3853; IPR008729 = Phenolic acid decarboxylase, bacterial; IPR015314 = Restriction endonuclease, type II, EcoRV; IPR009951 = Host-nuclease inhibitor protein Gam; IPR000412 = ABC-2 transporter; IPR025529 = Protein of unknown function DUF4416; IPR008274 = Aldehyde oxidase/xanthine dehydrogenase, molybdopterin binding; IPR008338 = Capsule biosynthesis protein CapC; IPR021865 = Peptidase G2, IMC autoproteolytic cleavage domain; IPR025636 = Protein of unknown function DUF4294; IPR032585 = Protein of unknown function DUF4912; IPR016905 = Glycyl radical enzyme, HI0521, predicted; IPR008840 = Siphovirus Gp157; IPR025127 = Protein of unknown function DUF4054; IPR006783 = Transposase, ISC1217; IPR028993 = RecG, N-terminal antiparallel four helix bundle; IPR025338 = Protein of unknown function DUF4244; IPR023180 = Tetrahydrodipicolinate-N-succinyltransferase, chain A, domain 1; IPR003688 = Type IV secretion system protein TraG/VirD4; IPR024590 = RNA helicase HrpA, C-terminal; IPR007430 = Bacterial virulence protein VirB8; IPR005498 = Type IV secretion system, VirB10 / TraB / TrbI; IPR010258 = Conjugal transfer, TrbG/VirB9/CagX; IPR010575 = KorB, C-terminal.

CAPÍTULO V

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Improvements in the quality of meat from beef cattle fed natural additives

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Abstract

Forty young bulls were fed with five different treatments (n = 8, 62 days): control, without the addition of natural additives (CON); NA15, a mixture of natural additives (1.5 g/animal/day); NA30, a mixture of natural additives (3.0 g/animal/day); NA45, a mixture of natural additives (4.5 g/animal/day); and NA60, a mixture of natural additives (6.0 g/animal/day). The hot carcass weight and dressing percentage, fat thickness, *Longissimus* muscle area, marbling, pH, and carcass tissue composition were measured. In addition, the instrumental meat quality (colour, water holding capacity, texture and lipid oxidation) and consumer acceptability attributes, across display were evaluated. Diet had no effect ($P > 0.05$) on the carcass characteristics evaluated (except pH). The diets significantly influenced the pH, shear force, tenderness, lipid oxidation and overall acceptability evaluated by consumers ($P < 0.05$). Globally, natural additives have some potential use in animal feed to improve meat quality.

Keywords: clove leaf essential oils, castor oil, cashew oil, encapsulate compounds, consumer acceptability

1. Introduction

Nowadays, global meat consumption is rising, along with concerns about food quality (Sans & Combris, 2015). In general, animal production and welfare, environmental issues, nutrition, feeding characteristics, and growth promoting additives are some of the factors of interest. In order to meet the meat demand, growth promoters such as antibiotics have been used to improve animal efficiency. However, due to concerns about the development of bacterial resistance, the use of these antibiotics is limited or banned in

many countries. Thus, natural additives have shown potential to replace antibiotics in promoting higher animal performance, without changing or even improving meat quality (Fugita et al., 2018; Monteschio et al., 2017; Rivaroli et al., 2016). These natural additives contain many compounds with high antimicrobial and antioxidant activities (Nikmaram et al., 2018).

Among these compounds, it is possible to find: the essential oil of clove leaf (*Eugenia caryophyllus*), which contains an average of 83 % to 90 % eugenol (Biondo et al., 2017) and has been widely used due to its high bactericidal, fungicidal, and antioxidant potential (Ornaghi et al., 2017; Souza et al., 2019); cashew oil (*Anacardium occidentale*), which has antimicrobial action attributed to the active anacardic and cardolic acids that act as monovalent ionophores (Valero et al., 2016), and anti-inflammatory and antioxidant activities attributed to the compound cardanol (Amorati et al., 2001; Trevisan et al., 2006); and castor oil (*Ricinus communis* L.), which contains predominantly ricinoleic acid which, together with other unsaturated fatty acids, corresponds to 97 % of the oil mass (Cruz et al., 2014; Valero et al., 2016). These fatty acids reduce the acetate:propionate ratio, inhibit methane production, alter bacterial resistance, increase microbial synthesis, and reduce ruminal ammonia concentrations (Ramírez-Restrepo et al., 2016).

Vanillin, eugenol, and thymol are known as performance enhancers in animal production (Hausmann et al., 2018; Souza et al., 2019). Understanding the benefits of adding microencapsulated forms of these compounds to animal feed might have a positive impact on meat quality, since the desired action on the metabolism is placed at the intestinal level (Vinceković et al., 2017). In addition, due to the possibility of absorption in the gut without the compounds being degraded in the rumen and losing their main

properties, these compounds might be absorbed and their properties, such as antioxidant activity, transferred to the animal's meat (Monteschio et al., 2017).

The synergism between compounds can enhance their antioxidant and antimicrobial effects when they are blended, and in addition, each compound can perform specific functions. Therefore, it is of great interest to search for products that improve animal performance and also bring benefits or do not change the quality of the final product (meat) (Rivaroli et al., 2016, 2017). In this regard, the development of products that have potential in animal production and maintain or improve the quality of meat is a challenge.

Based on previous studies by our research group (Fugita et al., 2018; Monteschio et al., 2017; Ornaghi et al., 2017; Passetti et al., 2017, Rivaroli et al., 2016, 2017; Souza et al., 2019; Valero et al., 2014, 2016) oils were selected to be blended and tested at different levels for potential synergism to improve animal performance and meat quality.

The aim of this study was to investigate the effects of a blend containing natural additives (clove essential oil, castor and cashew oil, and a commercial microencapsulated blend composed of vanillin, eugenol, and thymol) on the instrumental and sensorial attributes (consumer acceptability) of beef.

2. Material and Methods

2.1. Location, animals, diets, slaughter procedure, and muscle sampling

The experiment was approved by the Department of Animal Production and Research Ethic Committee at the State University of Maringá, Brazil, and followed the guiding principles of biomedical research with animals, number 081/2014. The experiment was

carried out at the Rosa & Pedro Sector of the Iguatemi Experimental Farm of State University of Maringá, Maringá, Paraná, South Brazil.

A total of 40 cross-bred (Angus × Nellore) young bulls of 16 ± 2.2 months of age and with a body weight of 385.82 ± 20.67 kg were used. The bulls were fed a basal diet comprised of 70 % concentrate and 30 % corn silage offered *ad libitum* for 62 days in individual pen (10m^2, partially covered, with concrete floors and automatic waterers). The animals were randomised across five treatments: control, without the addition of natural additives (CON); NA15, with the addition of 153.07 mg per kg of DM of a mixture of natural additives (1.5 g/day); NA30, with the addition of 305.2 mg per kg of DM of a mixture of natural additives (3.0 g/day); NA45, with the addition of 444.66 mg per kg of DM of a mixture of natural additives (4.5 g/day); and NA60, with the addition of 594.65 mg per kg of DM of a mixture of natural additives (6.0 g/day). The natural additives contained 37.5 % essential oils from clove leaf (*Eugenia aromatica*) (Ferquima®), 12.5 % functional oil of castor (*Ricinus communis*), 12.5 % functional oil of cashew (*Anacardium occidentale*) (Safeeds®), and 37.5 % a commercial blend composed of active compounds (vanillin, eugenol, and thymol) (Safeeds®).

The animals were transported to a commercial slaughterhouse (Campo Mourão city, Paraná, south Brazil) and slaughtered at 18 ± 2.2 months of age with an average final body weight of 482 ± 31.9 kg. The truck stocking density was 0.8 ± 0.2 bulls/m^2 and the transport distance was less than 90 km. The young bulls were slaughtered following the usual practices of the Brazilian beef industry. The animals were stunned using a captive-bolt pistol. Then, they were bled by exsanguination by cutting the neck vessels, and the head, hide, viscera, tail, legs, diaphragm, and excess internal fat were removed. Afterwards, the carcasses were divided medially from the sternum and spine, resulting in two similar halves, which were weighed to calculate the hot carcass weight. Then, the

half-carcasses were washed, identified, and stored in a chilling chamber at 4 °C, where they remained for a 24 h period.

2.2. Carcass measurements and meat sampling

The hot carcass dressing (HCD) percentage was calculated according to the following equation: HCD = (HCW/FBW) × 100, where HCW = hot carcass weight, and FBW = final body weight, 16 hours before slaughter.

After 24 hours *post mortem,* the *Longissimus* (*thoracis*) *muscle* (LM) was excised from the right half carcass from 5^{th} to the 13^{th} vertebra. Steaks were cut between the 6^{th} and 13^{th} ribs, vacuum packaged individually, and assigned to 1, 7, or 14 day ageing periods before being frozen and stored at -18 °C until analysis (< 1 month of storage).

On day one, the subcutaneous fat was measured at the level of the 12^{th} rib after a cross-section in the LM, using a digital calliper with a reading accuracy of 150 mm/6 " 0.01 mm (King tools, São Paulo, Brazil). The LM area was measured on a transverse cut between the 12^{th} and 13^{th} ribs using a compensating planimeter. Marbling was measured on the LM from the 12^{th} rib using the Brazilian scoring system (18 to 16: abundant, 15 to 13: moderate, 12 to 10: mean, 9 to 7: small, 6 to 4: light, and 3 to 1: traces).

The pH was determined using a pH metre (Hanna instruments model HI99163, Romaria, Brazil); the electrode was calibrated and inserted into the muscle between the 12^{th} and 13^{th} ribs at the time of slaughter and 24 hours post slaughtering.

The carcass tissue composition was estimated by the physical separation of the components (muscle, fat, bone, and other tissues) from the 6^{th} rib, and the percentage of each was calculated (Robelin & Geay, 1975).

148 *2.3. Meat ageing*

149

150 Samples from day 1 were analysed immediately. The samples aged for 7 and 14 days
151 were vacuum packed in 25 × 15 × 0.18 cm transparent polyamide/polyethylene pouches
152 120 μm; with 1 cm^3/m^2/24 h O_2 permeability and 3 cm^3/m^2/24 h CO2 permeability at 4
153 °C and 75 % relative humidity; with a 3 g/m^2/24 h water vapour transmission rate at 38
154 °C and 100 % relative humidity; a 97 °C Vicat softening temperature; and 1.3 g dart drop
155 strength, and sealed using Sulpack SVC 620 equipment (VAC). The samples stored for 7
156 and 14 days were exposed in a chilling chamber (4 ± 1º C) simulating typical Brazilian
157 market conditions with artificial light from a 50/50 siliconised Light Emitting Diode
158 (LED), 4.8 W, for 12 hours/day.

159

160 *2.4. Instrumental meat colour*

161

162 The colour was evaluated after 30 min of blooming at 1, 7, and 14 days of ageing using
163 the CIE L*a*b* system with a Minolta CR-400 Chroma metre (Japan) (with a 10° view
164 angle, D65 illuminant, and 8 mm aperture with a closed cone). Six measurements at
165 randomly selected points were recorded per sample, obtaining values for lightness (L*),
166 redness (a*), and yellowness (b*).

167

168 *2.5. Thawing, drip, and cooking losses*

169

170 The steaks were thawed at 4 °C for 24 h. They were then weighed, and the thawing
171 losses were calculated as the percentage difference between the fresh and thawed weights.

Drip loss was measured using the method described by Honikel (1998). One steak of each animal was taken 24 h *post mortem*, placed in a plastic bag, and kept at 4 °C. After 24 h, the sample was removed from the bag, dried on absorbent paper, and reweighed. The amount of drip at 48 h *post mortem* was expressed as a percentage.

$$\%\ drip\ loss = \frac{initial\ weight - final\ weight}{initial\ weight} * 100$$

For cooking losses, the raw steaks were weighed and wrapped in aluminium foil at each individual ageing time. Each sample was cooked in a pre-heated grill (Grill Philco Jumbo Inox, Philco SA, Brazil) at 200 °C until an internal temperature of 72 °C was reached, which was monitored using an internal thermocouple (Incoterm, 145 mm, Incoterm LTDA, Brazil). The sample was then removed from the heat and left at ambient temperature to cool. Once the steaks reached 25 °C, they were weighed and the cooking losses calculated as the percentage difference in weight before and after cooking.

2.6. Texture measurement

The texture of the previously cooked steaks was analysed using a Stable Micro Systems TA.XTplus texture analyser fitted with a 490.33 N load cell (Texture Technologies Corp., Serial Number 41288, Godalming, Surrey, UK) with a Warner-Bratzler blade, crosshead speed 19.98 cm/min, distance 3 cm, calibration weight 49.03 N, following to the protocol described by Honikel (1998). The meat was cut into rectangular pieces of 1 cm^2 cross-section (eight pieces per animal), which were cut perpendicular to the direction of the muscle fibres.

197 *2.7. Lipid oxidation*

198

199 The lipid oxidation was accessed as malonaldehyde (MDA) content in meat. It was
200 quantified using the thiobarbituric acid reactive substances (TBARS) assay according to
201 Souza et al. (2011). The meat sample (5 g) was mixed with TCA solution (7.5% TCA,
202 0.1% EDTA and 0.1% gallic acid) (10 mL), homogenized using an Ultra Turrax, then
203 centrifuged at 4°C for 15 min and 4.000 rpm. The supernatant was filtered and mixed
204 with TBARS reagent (1% thiobarbituric acid, 562.5 μM, HCl, 15% TCA) (1:1 v/v). The
205 mixture was boiled (100°C) for 15 min, cooled, then the absorbance measured at 540 nm
206 against an MDA standard. Results were expressed as mg MDA kg-1 of meat. Lipid
207 oxidation assays were performed at 1, 7 and 14 days of ageing.

208

209 *2.8. Consumer test*

210

211 Sensory evaluation analyses were approved by the State University of Maringá,
212 Maringá, Pr, Brazil (CAAE: 56154816.2.0000.0104). Four steaks per animal (2.5 cm-
213 thick) were cut between the 11^{th} and 13^{th} ribs, vacuum packaged individually, and
214 assigned to 1 and 7 days ageing periods. Those steaks with one day of ageing were frozen
215 immediately. The rest of the samples were kept at 4 °C until reaching 7 days before being
216 frozen and stored at -18 °C for the consumers' analysis.
217 The test involved a total of 120 consumers. They were selected based on the Brazilian
218 demographic characteristics regarding gender (48.7 % males, 51.3 % females) and age
219 (25.5 % of the individuals was < 24 years old, 39.6 % was between 25 and 44 years old,
220 21.5 % was between 45 and 64 years old, and 13.4 % was > 65 years old).

The frozen samples, previously aged for 1 or 7 days, were thawed for 24 h at 4 ± 1 °C before the analyses. Afterwards, they were cooked at 200 °C on a pre-heated, double-grill hotplate (Philco Grill Jumbo Inox, Philco S.A., Brazil) until the internal temperature reached 75 °C, which was monitored using a penetration thermocouple (Incoterm, 145 mm, Incoterm LTDA). Subsequently, 10 homogeneous cubes (2 × 2 × 2 cm) per steak were obtained, wrapped individually in aluminium foil, marked with a three-digit code, and kept warm at 50 °C for less than 10 min until they were served. Consumers were given instructions before the test and were supervised to ensure that the proper procedures were followed. Each consumer evaluated ten samples, one from each treatment group (five diets and two ageing times), which were tasted individually in a random order to avoid the effect of sample order presentation, first-order, or carry-over effects (Macfie, Bratchell, Greehoff, & Vallis, 1989).

To standardise the condition of the mouth before each sample, consumers were instructed to eat a small piece of bread and drink some mineral water at the beginning of the sensory evaluation and between samples. Consumers evaluated the odour, flavour, tenderness, and overall acceptability using a 9-point structured hedonic scale (1 = dislike extremely and 9 = like extremely), without a neutral central point (Font-i-Furnols et al., 2009).

2.9. Statistical analyses

All study data were tested for normality (Shapiro-Wilk test) and showed a normal distribution. The data were analysed by analysis of variance using the R statistical software, with the animal identity as a random effect.

The experimental diet effect evaluated from an orthogonal contrast was used to assess the effects of the control treatment *versus* natural additives, linear and quadratic response ($P \leq 0.05$). The effect of ageing (1, 7, or 14 days) on instrumental meat colour; Warner Bratzler shear force; and thawing, drip cooking losses and lipid oxidation was analysed. Differences between the means for different ageing times and diets were assessed using the Tukey Test ($P \leq 0.05$).

The consumer test results were assessed by an analysis of variance using the General Lineal Model (GLM) procedure in SPSS v15.0 for Windows (IBM SPSS Statistics, SPSS Inc., Chicago. USA). Diet and ageing were considered as fixed effects and consumers as random effect in the sensory test. The mean and standard error of the mean (SEM) were calculated for each variable. Statistical differences between the diets and ageing periods were assessed using a Duncan's Test ($P \leq 0.05$).

Ward's method was used to develop hierarchical cluster analysis and determine the different segments of consumers according to the overall acceptability. XLSTAT (v.19.01) was used to analyze. The number of clusters was selected by a dendrogram that divide by groups finding a compromise between homogeneity within clusters and heterogeneity between clusters.

A Principal Component Analyses was used to identify the relationships between treatments and meat attributes. The results are presented graphically in a biplot including the attributes and the treatment.

In all statistical analyses, the diet was considered a fixed effect, and the animals considered a random effect. The diets means were computed using the LSMEANS option.

$Yij = \beta 0 + \beta_1 X_i + \beta_2 X_i^2 + \varepsilon ij;$

where:

Yij = observation of the repetition j on diet i;

270 β0 = general coefficient;

271 β1 = linear regression coefficient of the variable observed depending on the level;

272 β2 = quadratic regression coefficient of the variable observed depending on the level;

273 Xi = independent variables (blend of NA levels);

274 Eij = residual error.

275

276 **3. Results**

277

278 *3.1. Carcass characteristics and pH*

279

280 The carcass weight and hot carcass dressing percentage (Table 1) did not differ
281 between treatments ($P > 0.05$). In addition, significant differences in the fat thickness,
282 area of the *Longissimus* muscle, or marbling were not observed ($P > 0.05$). The tissue
283 composition also did not differ ($P > 0.05$) among treatments. All diets presented a similar
284 percentage of muscle, fat, bone, and other tissues (Table 1).

285 When the pH was analysed, a significant effect was observed ($P < 0.05$), showing a
286 linear and quadratic behaviour (Table 1).

287

288 *3.2. Instrumental meat colour*

289

290 The diet did not affect the parameters L*, a*, or b* (lightness, redness, and yellowness,
291 respectively) used to measure the meat colour ($P > 0.05$; Table 2). The values observed
292 for L* on the first day were approximately 38, for a* were approximately 14, and for b*
293 were approximately 13. However, the L* and b* values increased when the effect of

294 increasing ageing time was evaluated on the diets with 3.0 and 4.5 g/animal/day of natural
295 additives ($P < 0.05$). There was no interaction between diet and ageing time ($P > 0.05$).
296
297 *3.3. Thawing, drip, and cooking losses, Warner Bratzler shear force and lipid oxidation*
298
299 No changes in water losses by any of the procedures used in the current study ($P >$
300 0.05) were observed when natural additives were included in the diet (Table 3).
301 However, ageing influenced the thawing losses, with significantly higher values
302 (approximately a 38 % loss) after one week of ageing than one day ($P < 0.05$) for all
303 treatments. In relation to cooking losses, ageing influenced only the treatments with 1.5
304 (9.9 % loss) and 4.5 g/animal/day of the natural additives (11.48 % loss) ($P < 0.05$).
305 The blend inclusion influenced the shear force (Table 4) on day one and showed a
306 tendency ($P = 0.054$) to decrease from 78.65 to 64.82 N, and on day seven, the tenderness
307 decreased linearly ($P = 0.030$) from 50.60 to 41.68 N. In addition, the ageing time also
308 decreased the Warner Bratzler shear force values of meat by 50 % in all treatments ($P <$
309 0.05). An interaction of diet and ageing time was not observed ($P > 0.05$).
310 Lipid oxidation showed a linear reduction of 0.052 and 0.130 mg malonaldehyde/kg
311 (Table 5) when the blend was added to the diet of young bulls ($P < 0.05$) on day seven
312 and fourteen of storage, respectively. Moreover, the lipid oxidation increased
313 significantly with ageing time ($P < 0.05$) without present an interaction between diet and
314 ageing ($P > 0.05$).
315

316 *3.4. Consumer acceptability*

317

318 In this study, the overall acceptability was correlated mostly with flavour (R = 0.943),
319 followed by tenderness (R = 0.941) (Figure 1). The acceptability of treatments NA15
320 (ageing day seven, A7) and NA30 (A7) was the most related to odour, and the
321 acceptability of CON (A7), NA45 (A7), and NA60 (A7) was associated with flavour,
322 tenderness, and overall acceptability, which are all attributes strongly correlated at an
323 ageing period of seven days; they are located on the right side of the biplot, inversely
324 related to day one.

325 The acceptability for odour and flavour were similar between diets ($P > 0.05$, Table
326 6). However, the tenderness and overall acceptability were affected by diet ($P < 0.05$),
327 with higher scores for tenderness for NA30 compared to CON and NA15 in the overall
328 acceptability NA30 and NA45 values were higher compared to NA15.

329 Regarding ageing time, a significant difference was observed ($P < 0.05$). Although
330 both ageing times were well accepted, the consumers scored meat aged for 7 days higher
331 than that aged for 1 day. In addition, the principal component analyses showed a
332 correlation between aged beef and the sensory attributes (Figure 1). The first two principal
333 component axes explained 97.63 % of the total variance. Attributes related to odour,
334 flavour, tenderness, and overall acceptability were placed on the right side of F1, close to
335 the treatments with seven days of ageing. Meats with a short ageing period (one day) were
336 located on the left side of F1, inversely related to the acceptability attributes. The samples
337 from the NA30, NA45, and NA60 groups were more closely related to acceptability than
338 those from the control or NA15 groups.

339

4. Discussion

4.1 Carcass characteristics

In the current study, the values of fat thickness and marbling (3.8 mm and 1.5 points, respectively) can be considered as representative of a low fatness grade, which might be due to the presence of Nellore genes that can result in a low fat deposition. Although the values found were low, they might still be adequate, since fat thickness must be between 3 and 6 mm to effectively protect the carcass during cooling (Rotta et al., 2009).

The *Longissimus* muscle area of the bulls was on average of 83 cm^2, demonstrating an adequate muscle deposition in the animals, which is similar to some studies using *Bos taurus* × *Bos indicus* (Maggioni et al., 2010; Ornaghi et al., 2017; Prado et al., 2009). Similarly, Monteschio et al. (2017), when using clove and rosemary essential oils and encapsulated active principle ingredients (eugenol, thymol, and vanillin blend) in the diet of heifers, did not find significant differences in fat thickness, marbling points, or the *Longissimus* muscle area.

The mean of tissue composition (muscle 64 %, fat 16 %, bone 13 %, and other tissues 7 %) was similar in all diets. Corroborating our data, Yang, Ametaj, Benchaar, He, and Beauchemin (2010) evaluated cinnamaldehyde levels (400, 800, 1600 mg/bulls per day) in the diet of steers in a feedlot and did not observe significant differences in carcass characteristics. Rivaroli et al. (2017) fed 27 crossbred bulls (Angus × Nellore) a mix of essential oils (oregano, garlic, lemon, rosemary, thymus, eucalyptus, and sweet orange) at two inclusion levels (500 and 1000 mg/kg of DM/animal/day) and also did not find differences in carcass characteristics. These results demonstrate that the addition of many natural additives in a blend to the animals' diet does not affect the carcass characteristics.

365 *4.2. Instrumental meat colour*

366

367 The mean lightness (L*) value observed on day 1 was approximately 38.4 points. This
368 value suggests an attractive lightness to the consumer. It is likely that the *Bos taurus*
369 influence could affect the results by increasing the L* values, since *Bos taurus* presents
370 lower calpastatin activities, which are highly correlated with lower L* values (Page, Wulf,
371 & Schwotzer, 2001).

372 The value of L* increased with ageing for only NA45 ($P < 0.05$). The meat colour can
373 be influenced by several factors, such as age, breed, diet, and sex (Guerrero et al., 2018).
374 The animals were a crossbreed of *Bos indicus* and *Bos taurus; Bos taurus* animals
375 generally present higher values for lightness than *Bos indicus*, which can be explained by
376 their lower temperament scores (they are less excitable animals), which are highly
377 correlated with the 24 h calpastatin activity and pH values, and, therefore, muscle colour
378 (in this case L*) (Page et al., 2001; Wulf, O'Connor, Tatum, & Smith, 1997). The
379 lightness makes the meat more attractive to the consumers; the brightness of red meat is
380 associated with a fresh product.

381 The redness values (a*) were unchanged with the ageing time and showed means of
382 approximately 14 points, which demonstrated a maintenance of the red colour ($P > 0.05$).
383 This might be explained by the values of pH (5.7) and the storage mode (vacuum
384 packaging).

385 The yellowness values (b*) increased during ageing for NA30 and NA45 only ($P <$
386 0.05). The variation in b* values might be related specifically to the degree of
387 oxygenation of Mb to MbO_2, which is also supported by the fact that the yellowness
388 increases during blooming (Lindahl, Lundström, & Tornberg, 2001; Rosenvold &
389 Andersen, 2003) even when vacuum packing slows down the oxygenation process.

390 *4.3. Thawing, drip, and cooking losses, Warner Bratzler shear force and lipid oxidation*

391

392 The thawing, drip, and cooking losses were not affected by the diets ($P > 0.05$).
393 However, the thawing and cooking losses were affected by ageing; the thawing loss was
394 influenced by all treatments and the cooking loss only by the treatments NA15 and NA45
395 ($P < 0.05$), increasing with the ageing time. Lipid and protein oxidation decreases the
396 quality of meat, including water losses (Pearce, Rosenvold, Andersen, & Hopkins, 2011).
397 In addition, some variations in the pH and muscular structure modifications could have
398 affected our results, making the losses higher after one day of ageing.

399 Regarding the cooking losses, differences were only observed for the treatments NA15
400 and NA45 during display ($P < 0.05$), increasing on day 7. Although, the marbling value
401 was not significantly different between treatments, animals fed with NA15 and NA45 had
402 numerically less marbling than the others, which could be associated with the higher
403 cooking loss. During cooking, intramuscular fat serves as a barrier against juice losses,
404 increasing the meat water retention and juiciness (Pearce et al., 2011).

405 Related to shear force, on day one, the meat from all the treatments could not be
406 considered as tender meat (values > 48.05 N) (Shackelford, Morgan, Cross, & Savell,
407 1991). However, as expected, at the end of the ageing period, all treatments presented
408 lower values (below 43.84 N).

409 On day seven and fourteen, a linear effect between diets was observed ($P < 0.05$; Table
410 5), which might be explained by the oxidation process with values for TBARS ranging
411 among 0.563, 0.530, 0.473, 0.482, 0.511 and 0.781, 0.748, 0.726, 0.725, 0.651 mg
412 malonaldehyde/kg on days seven and fourteen to CON, NA15, NA30, NA45 and NA60,
413 respectively, and also the decline in pH, as shown in Table 1. Oxidation can lead to the
414 production of free radicals that can initiate further lipid and protein oxidations. In a recent

415 review article, Falowo, Fayemi, & Muchenje (2014) noted that the free radical chains of
416 protein oxidation and lipid oxidation in animal muscle are similar. The peroxyl radicals
417 formed during lipid oxidation are absorbed by hydrogen atoms in proteins to form protein
418 radicals which might adversely affect calpain activity by modifying the highly susceptible
419 cysteine residues in the active site (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004).
420 The incorporation of antioxidants into the meat through the addition of natural additives
421 to the animals' diet might decelerated the oxidative process and have delayed oxidation,
422 resulting in improving proteolysis and meat tenderness.

423 It has also been demonstrated that oxidative stress affects meat tenderness. Oxidative
424 stress in tissues results in functional and/or structural damage to muscle (Lykkesfeldt &
425 Svendsen, 2007). It has been found that the myofibril protein is affected during meat
426 ageing and storage (Martinaud et al., 1997), and that a high production of free radicals
427 and reactive oxygen species (ROS) results in degenerative damage to the cellular structure
428 and affects meat quality (Piccione et al., 2013). Nonetheless, ROS production is related
429 to collagen synthesis and solubility and can, therefore, increase meat toughness, since the
430 delay in oxidation and consequent decrease in ROS production can benefit meat
431 tenderness (Falowo et al., 2014).

432

433 *4.4. Consumer acceptability*

434

435 The results showed that the addition of natural additives at 3.0 g/animal/day (NA30)
436 improved tenderness acceptability compared to CON group, however no statistical
437 differences were observed between the CON and the other treatments with natural
438 additives. The higher tenderness scores (compared to CON and the highest and lower
439 levels (NA15 and NA60)) given to NA30 and NA45, might be associated with the lower
440 pH, since pH is highly correlated with the meat toughness through the calpain/calpastatin
441 proteolytic system (Wulf et al., 1997). The sensory values for tenderness were higher with
442 increasing ageing time due to the enzymatic activity, which is related to the observed
443 shear force measurements.

444 The ageing time was a determining factor for meat acceptance; the meat aged for seven
445 days was the best accepted. This preference for ageing meat had already been observed
446 in other studies (Eiras et al., 2017; Guerrero et al., 2016). Ageing time leads to the
447 development of flavour precursors and to a tender meat, which improve the acceptability
448 (Mónson, Sañudo, & Sierra, 2005). However, with several ageing days, off-flavours can
449 develop resulting in rejection by consumers (Legako et al., 2015).

450 In this study, none of the treatments presented lower scores than the control (tenderness
451 and overall acceptability) or had the same appreciation (flavour and odour), which
452 indicates that the addition of the blend did not negatively affect meat acceptance;
453 contrariwise, it improved the acceptance in some aspects. Corroborating our findings,
454 Guerrero et al. (2017), in a study using a commercial blend of essential oils (oregano,
455 garlic, lemon, rosemary, thyme, eucalyptus, and sweet orange) at two different inclusion
456 levels of 3.5 and 7.0 g/animal/day, observed that the blend improved the overall
457 acceptability the most at the 3.5 g/animal/day concentration.

458 *4.5. Cluster analysis*

459

460 Resulting from other consumer studies (Guerrero et al., 2018; Vital et al., 2018),
461 showed as preferences in beef acceptability are not homogenous among consumer groups
462 (clusters). There are different groups of consumers, with differentiated perceptions and
463 overall acceptability of the product, which establish significant beef market segments.
464 Cluster 1 was composed by the 26.66% of consumers (78.1% of the cluster had less
465 than 40 years old and 50% of the group were women). In this group of participants, diet
466 and ageing were significant factors ($P < 0.010$ and $P < 0.001$, respectively), with a
467 significant interaction between them ($P < 0.050$). Beef from those diet with 3.0 or 4.5 g
468 per animal and day of natural additives were preferred respect to higher dosages (NA60).
469 Also, there were almost 2 points of differences between ageing, presenting significant
470 higher scores 7 days (6.56 points) respect beef from 1 day of ageing (4.93 points).
471 The largest group of consumers (cluster 2), compiled the 70.0% of the participants on
472 the study. That cluster included a similar number of men (47.6%) with a low percentage
473 of men with more than 55 years (2.5%) and between women (52.4 % of the sample) there
474 were presence in each of the four age ranges analyzed. In this cluster, diet and ageing also
475 were significant factors ($P < 0.010$ and $P < 0.001$, respectively) being 7 days also
476 preferred respect 1 day of ageing but without a significant interaction between diet and
477 ageing ($P < 0.050$). This group evaluated all treatments with high scores, over 7.21 on a
478 9-point scale. Although, there were significant differences between diets, being the
479 highest (NA60) and medium dosages (NA 30) scored significantly higher than lows
480 addition treatments (NA30).
481 Hierarchical cluster analysis showed a small cluster (3) composed only by 3.33% of
482 participants (75% women in the group) with specific and different characteristics. For this

group, no studied effect was significant, and they rejected samples from all treatments and ageing.

5. Conclusions

The addition of natural additives to the diet of young bulls did not affect the carcass characteristics, meat colour, water holding capacity, or acceptability of the odour and flavour by consumers. The diets with natural additives improved the pH, shear force, oxidative stability and tenderness acceptability evaluated by consumers. In general, ageing time influenced the quality parameters, with the meat aged for 7 days receiving higher scores by consumers than meat that was not aged. Thus, it is possible to observe that the addition of natural additives to the cattle's diet did not worsen the quality of the meat and improved some parameters, such as the sensory attributes (tenderness), making them a promising natural alternative in animal feed. In our study, the recommended level of inclusion to achieve the benefits of natural additives is 3 g/animal/day.

Conflict of interest

The authors declare no conflict of interest.

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681 **Table 1.** Effect of the inclusion of natural additives on carcass characteristics

Item	Diets					SEM[7]	P-value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]		L[8]	Q[9]	0% *vs* NA[6]
Hot carcass weight, kg	248.1	252.0	246.6	253.9	246.1	2.521	0.900	0.879	0.816
Hot carcass dressing, %	52.37	52.62	51.25	52.18	51.51	0.302	0.178	0.195	0.357
Fat thickness, mm	3.69	3.49	4.82	3.30	3.92	0.204	0.846	0.720	0.683
Muscle area, cm^2	83.5	81.7	81.1	87.6	81.0	1.492	0.935	0.980	0.863
Marbling, points	1.50	1.44	1.73	1.25	1.38	0.094	0.480	0.670	0.817
Muscle, %	63.03	63.57	63.06	65.13	63.71	0.711	0.562	0.827	0.648
Fat, %	15.73	16.72	16.52	15.63	16.91	0.571	0.756	0.953	0.632
Bone, %	13.82	12.75	14.31	13.88	12.43	0.412	0.568	0.619	0.642
Others, %	7.42	6.97	6.10	5.35	6.95	0.344	0.302	0.202	0.217
pH	5.74a	5.77a	5.65b	5.72ab	5.57b	0.027	0.003	0.007	0.131

682 [1]CON = control (without natural additives); [2]NA15 = addition of 1.5 g/animal/day of natural additives; [3]NA30 = addition of 3.0 g/animal/ day of
683 natural additives; [4]NA45 = addition of 4.5 g/animal/day of natural additives; [5]NA60 = addition of 6.0 g/animal/day of natural additives; [6]NA =
684 natural additives; [7]standard error of means; [8]linear effect; [9]quadratic effect; a, b: indicate statistical differences in the same row ($P \leq 0.05$).

685

686 **Table 2.** Effect of the inclusion of natural additives in the diet and ageing period on meat colour

	Diets						P-value		
Day	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	SEM[7]	L[8]	Q[9]	0% *vs* NA[6]
			L*						
1	38.18	38.07	38.76	38.27c	38.89	0.397	0.562	0.844	0.757
7	40.16	39.57	39.87	40.44b	41.86	0.372	0.101	0.096	0.767
14	40.89	40.54	41.73	42.56a	42.11	0.376	0.085	0.228	0.361
SEM	0.551	0.468	0.540	0.471	0.652		P D×A*		
P <	0.112	0.084	0.065	0.001	0.098		0.934		
			a*						
1	14.44	14.11	14.04	14.17	14.62	0.197	0.763	0.533	0.681
7	14.90	14.50	15.09	13.93	14.26	0.228	0.232	0.488	0.408
14	14.72	14.24	14.91	13.85	14.12	0.189	0.222	0.472	0.340
SEM	0.240	0.259	0.248	0.180	0.298		P D×A*		
P <	0.730	0.834	0.155	0.775	0.812		0.836		
			b*						
1	13.11	12.99	13.25b	13.29b	13.62	0.175	0.263	0.480	0.677
7	13.99	13.68	14.26ab	13.89ab	14.28	0.164	0.478	0.736	0.926
14	14.18	13.96	14.65a	14.55a	14.35	0.134	0.333	0.501	0.558
SEM	0.221	0.202	0.221	0.188	0.323		P D×A*		
P <	0.117	0.140	0.018	0.002	0.458		0.989		

687 [1]CON = control (without natural additives); [2]NA15 = addition of 1.5 g/animal/day of natural additives; [3]NA30 = addition of 3.0 g/animal/ day of
688 natural additives; [4]NA45 = addition of 4.5 g/animal/day of natural additives; [5]NA60 = addition of 6.0 g/animal/day of natural additives; [6]NA =
689 natural additives; [7]standard error of means; [8]linear effect; [9]quadratic effect; *interaction between diet and ageing time; a, b: indicate statistical
690 differences in the same column ($P \leq 0.05$).

691 **Table 3.** Effect of the natural additives to the diet and the ageing period on water losses of beef

	Diets						P-value		
Day	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	SEM[7]	L[8]	Q[9]	0% *vs* NA[6]
				Drip losses					
1	3.92	5.60	3.03	3.57	4.38	0.401	0.696	0.854	0.823
				Thawing losses					
1	7.92b	8.09b	6.31b	7.88b	6.84b	0.332	0.313	0.573	0.436
7	12.87a	15.06a	11.36a	13.02a	12.52a	0.496	0.439	0.744	0.921
14	11.80a	12.04a	10.63a	11.40a	10.66a	0.264	0.116	0.294	0.348
SEM	0.630	0.744	0.702	0.611	0.650		P D×A*		
P <	0.001	0.001	0.003	0.001	0.001		0.924		
				Cooking losses					
1	33.97	32.90b	31.49	33.01b	32.54	0.503	0.446	0.468	0.254
7	36.83	37.17a	35.21	36.64a	36.41	0.507	0.701	0.808	0.713
14	34.68	34.55ab	34.51	33.58ab	33.30	0.515	0.303	0.570	0.599
SEM	0.591	0.750	0.711	0.610	0.861		P D×A*		
P <	0.117	0.049	0.078	0.044	0.158		0.969		

692 [1]CON = control (without natural additives); [2]NA15 = addition of 1.5 g/animal/day of natural additives; [3]NA30 = addition of 3.0 g/animal/ day of
693 natural additives; [4]NA45 = addition of 4.5 g/animal/day of natural additives; [5]NA60 = addition of 6.0 g/animal/day of natural additives; [6]NA =
694 natural additives; [7]standard error of means; [8]linear effect; [9]quadratic effect; *interaction between diet and ageing time; a, b: indicate statistical
695 differences in the same column ($P \leq 0.05$).

Table 4. Effect of the inclusion of natural additives in the diet and the ageing period on the Warner Bratzler shear force (N)

	Diets						P - value		
Day	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	SEM[7]	L[8]	Q[9]	0% *vs* NA[6]
			WBSF						
1	76.65a	79.34a	61.39a	69.82a	64.82a	2.745	0.054	0.131	0.147
7	50.60ABb	54.82Ab	40.70Bb	42.56Bb	41.68Bb	2.027	0.030	0.093	0.236
14	43.84b	43.34c	35.79b	36.08b	38.54b	1.745	0.108	0.157	0.168
SEM	0.461	0.360	0.348	0.340	0.410		P D×A*		
P <	0.002	0.001	0.003	0.001	0.010		0.976		

[1]CON = control (without natural additives); [2]NA15 = addition of 1.5 g/animal/day of natural additives; [3]NA30 = addition of 3.0 g/animal/ day of natural additives; [4]NA45 = addition of 4.5 g/animal/day of natural additives; [5]NA60 = addition of 6.0 g/animal/day of natural additives; [6]NA = natural additives; [7]standard error of means; [8]linear effect; [9]quadratic effect; *interaction between diet and ageing time; a, b: indicate statistical differences in the same column ($P \leq 0.05$); A, B: indicate statistical differences in the same row ($P \leq 0.05$).

701 **Table 5.** Effect of the inclusion of natural additives in the diet and the ageing period on the lipid oxidation

	Diets						P - value		
Day	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	SEM[7]	L[8]	Q[9]	0% *vs* NA[6]
1	0.398b	0.402b	0.330b	0.387b	0.376c	0.022	0.154	0.360	0.569
7	0.563Aa	0.530ABa	0.473Ba	0.482Bab	0.511Bb	0.030	0.001	0.211	0.349
14	0.781Aa	0.748ABa	0.726ABa	0.725ABa	0.651Ba	0.012	0.006	0.020	0.369
SEM	0.023	0.032	0.025	0.021	0.024		P D×A*		
P <	0.018	0.005	0.001	0.003	0.001		0.983		

702 [1]CON = control (without natural additives); [2]NA15 = addition of 1.5 g/animal/day of natural additives; [3]NA30 = addition of 3.0 g/animal/ day of
703 natural additives; [4]NA45 = addition of 4.5 g/animal/day of natural additives; [5]NA60 = addition of 6.0 g/animal/day of natural additives; [6]NA =
704 natural additives; [7]standard error of means; [8]linear effect; [9]quadratic effect; *interaction between diet and ageing time; a, b: indicate statistical
705 differences in the same column ($P \leq 0.05$); A, B: indicate statistical differences in the same row ($P \leq 0.05$).

706 **Table 6**. Effect of the inclusion of natural additives on consumer acceptability of attributes of grilled *Longissimus* aged for 1 and 7 days (n = 120
707 consumers) §

Acceptability	Diets					Ageing time		SEM[6]	P-value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	1 d	7 d		Diet (D)	Ageing (A)	D × A
Odour	6.80	6.78	6.90	6.71	6.80	6.72	6.91	0.048	0.242	0.001	0.795
Flavour	6.92	6.87	7.03	7.00	6.99	6.67	7.26	0.049	0.336	0.003	0.965
Tenderness	6.57b	6.61b	7.02a	6.81ab	6.92ab	6.20	7.23	0.059	0.049	0.008	0.615
Overall	6.81ab	6.68b	7.09a	7.00a	6.88ab	6.45	7.26	0.050	0.047	0.019	0.718

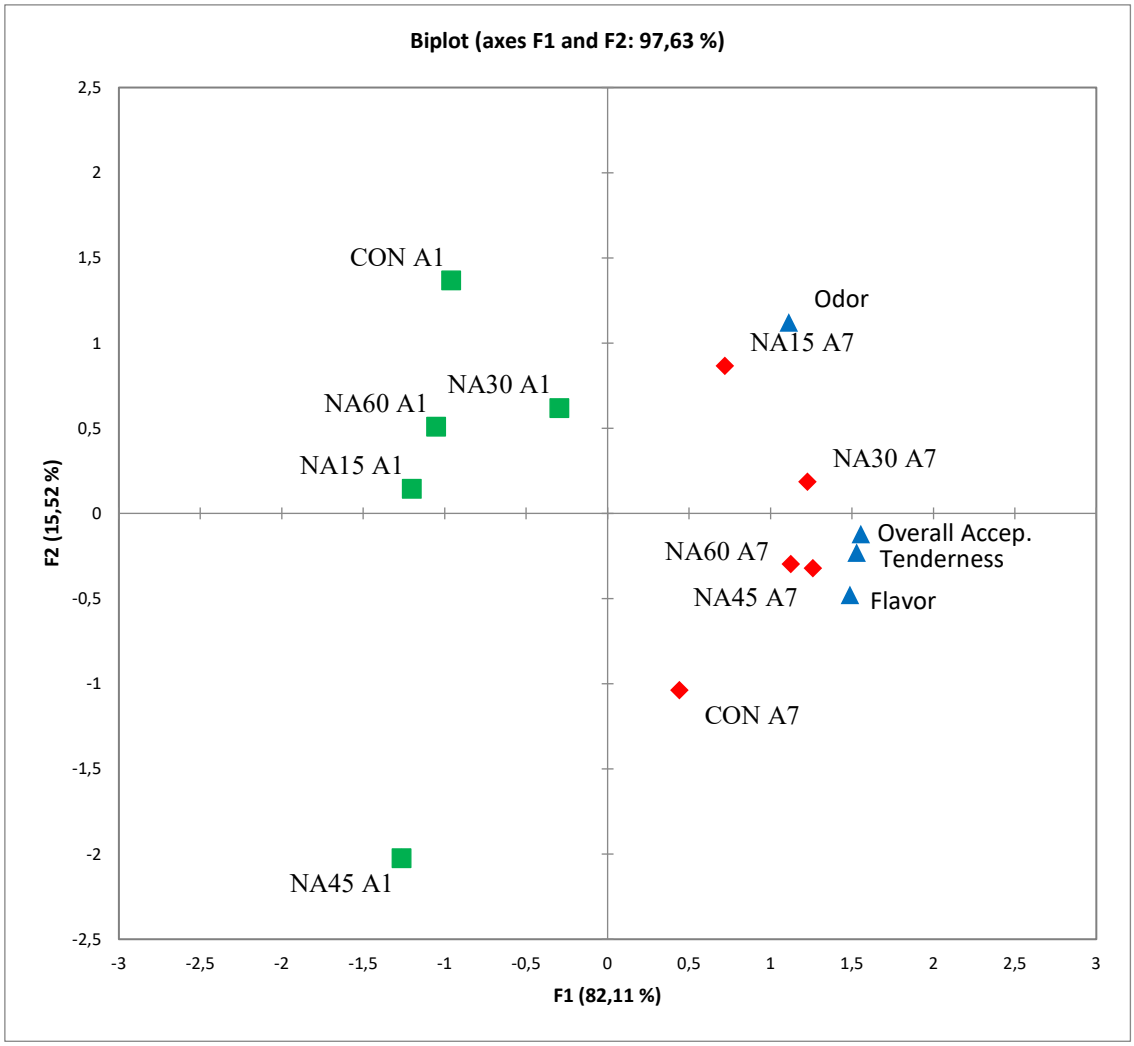
708 [1]CON = control (without natural additives); [2]NA15 = addition of 1.5 g/animal/day of natural additives; [3]NA30 = addition of 3.0 g/animal/ day of
709 natural additives; [4]NA45 = addition of 4.5 g/animal/day of natural additives; [5]NA60 = addition of 6.0 g/animal/day of natural additives; [6]standard
710 error of means; a, b: indicate statistical differences in the same row ($P \leq 0.05$).
711 §Based on a 9-point scale (1: dislike extremely; 9: like extremely).

712 **Table 7.** Effect of the inclusion of natural additives on overall acceptability of attributes of grilled *Longissimus* aged for 1 and 7 days by segmented
713 by clusters of consumers (*n* = 120 consumers) §

Acceptability	Diets					Ageing time		SEM[6]	P-value		
	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	1 d	7 d		Diet (D)	Ageing (A)	D x A
Cluster 1 (n=32)	5.59 ab	5.69 ab	6.14 a	6.11 a	5.17 b	4.93	6.56	0.108	0.007	<0.001	0.015
Cluster 2 (n=84)	7.38 ab	7.21 b	7.59 a	7.39 ab	7.63 a	7.21	7.67	0.045	0.003	<0.001	0.553
Cluster 3 (n=4)	2.13	2.13	3.88	2.50	2.25	2.10	3.05	0.297	0.352	0.068	0.289

714 [1]CON = control (without natural additives); [2]NA15 = addition of 1.5 g/animal/day of natural additives; [3]NA30 = addition of 3.0 g/animal/ day of
715 natural additives; [4]NA45 = addition of 4.5 g/animal/day of natural additives; [5]NA60 = addition of 6.0 g/animal/day of natural additives; [6]standard
716 error of means; a, b: indicate statistical differences in the same row ($P \leq 0.05$).
717 §Based on a 9-point scale (1: dislike extremely; 9: like extremely).
718

719 **Figure 1.** Principal component analysis of the scores for tenderness, flavour, and overall
720 acceptability of beef from young bulls fed with natural additives and aged for either 1 or
721 7 days.



722
723 CON = control (without natural additives); NA15 = addition of 1.5 g/animal/day of
724 natural additives; NA30 = addition of 3.0 g/animal/ day of natural additives; NA45 =
725 addition of 4.5 g/animal/day of natural additives; NA60 = addition of 6.0 g/animal/day of
726 natural additives; A1 = ageing day 1, green squares; A7 = ageing day 7, red rhombs.
727

CAPÍTULO VI

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Natural additives in diets of young bulls as an antioxidant source to improve meat quality

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747 **Abstract**

748 Forty (½ Angus *vs.* ½Nellore) young bulls of 16 ± 2.2 months of age, with a body weight
749 of 385.82 ± 20.67 kg were fed (62 days) with different diets: without or with different
750 inclusion levels of a naturals additives blend (from 1,500 to 6,000 mg/animal/day). The
751 blend was composed by a mix of essential oil from clove´s leaf, functional oils from castor
752 and cashew and a blend of natural compounds (vanillin, eugenol and thymol). Colour,
753 antioxidant activity (DPPH, ABTS and FRAP assays), lipid oxidation and visual
754 acceptability were evaluated through aging (14 days). Both factors (diet and storage) had
755 effect in all parameters evaluated ($P < 0.05$). The diets with natural additives reduced lipid
756 oxidation, due to the increase of antioxidant potential which improved shelf-life ($P <$
757 0.05). The studied natural additives can be used in animal feed to improve meat quality
758 during shelf-life.

759

760 **Keywords:** essential oils, eugenol, functional oils, lipid oxidation, thymol, vanillin.

1. Introduction

Consumer awareness about the impacts of food on human health has being increasing in recent decades, especially on the consumption of animal origin products (Clonan et al., 2015). Thus, there is increasing interest to control several aspects of the livestock production chain to produce safe, healthy and affordable products (Bosona & Gebresenbet, 2013). As there is also increasing evidence of pathogen antibiotic resistance caused due to the antibiotics use in the livestock as part of production practices, studies on natural alternatives are encouraged (Ronquillo & Hernandez, 2017). Natural additives (NAs) can be used to improve the animal performance and meat quality (Hayajneh, 2019; Pateiro et al., 2018; Jiang & Xiong, 2017).

One of the biggest economic challenges in the meat industry is to improve the products shelf-life. The main causes of meat deterioration are microbiological and non-microbiological (Fletcher et al., 2018). The first one decreases the product quality due to microbial spoilage (i.e breakdown of the meat components due to bacterial; fungal growth). The second cause is related to the lipids and proteins oxidation of the meat during the storage which affects the major variables of product´s quality like colour, odour, flavour and texture. Meat has a great concentration of saturated and unsaturated fatty acids, being the latter prone to oxidation due to the instability provided by the larger surface for reaction by the double bond contained (Xiao, Zhang, Lee, & Ahn, 2013).

The susceptibility of meat components to oxidation can be influenced by animal species, breed, fibre type, anatomical location, diet and stress (Min, Nam, Cordray, & Ahn, 2008). Animal exposed to stress are at risk of oxidative stress, which will accelerate meat oxidation.

785 The cellular system is responsible for the oxidative stress and production of free
786 radicals, which are products of reaction of metabolic processes. Free radical's
787 accumulation cause functional and structural damage to muscle organelles, cells and
788 tissues (Sies, Berndt & Jones, 2017). For example, myofibril protein is affected by a high
789 free radicals and ROS (reactive oxygen species) production during the meat storage
790 (Martinaud, Mercier, Marinova, & Tassy, 1997; Piccione et al., 2013), leading to
791 degenerative damage of cellular structure, ageing of tissue and then affecting the meat
792 quality.

793 Furthermore, the diet consumed by animals during their productive phase has a great
794 influence on the meat susceptibility to oxidation *post-mortem* (Wood & Enser, 2017), and
795 additives can be used to mitigate such effects. Substances, such as natural products, can
796 be used to delay oxidation. There is evidence that plant extracts have strong free radical
797 scavenging activity, and may protect the cells integrity (Kleinberg et al., 2019; Scipioni
798 et al., 2018; Al-Zubiri et al., 2017). However, improving meat quality and increasing
799 storage time (shelf-life) of red meat is challenging due to the rumen nature, a fermentation
800 chamber that host bacteria, fungi, protozoa and bacteriophages that degrades and modify
801 dietary components (Richardson et al., 2019).

802 Meat colour if one of the most important factors that influence the preference of
803 consumers, and cherry red colour will be correlated to freshness, which is desirable
804 (Passetti et al., 2017). However, meat oxymyoglobin exposed to air will rapidly be
805 oxidized to metmyoglobin, thus providing a brown colour which is rejected by consumers
806 (Suman & Joseph, 2013). NAs not only can improve animal performance, but can also
807 improve the meat antioxidant capacity, improving colour stability and resulting in
808 extended shelf-life (Falowo et al., 2014; Velasco & Williams, 2011).

Consequently, there is a need to explore suitable alternatives from natural sources, such as plant-derived antioxidants, to combat the challenges of oxidative instability of lipids and protein in meat. Furthermore, while the interest in oxidative stress and antioxidant activities continues to expand, many questions still remain unanswered as to how the reactions chain prior to the conversion of muscle to meat can reduce oxidative stress in meat. Thus, the aim of the current study was to investigate the effect of the NAs (blend: clove essential oil, cashew and castor oil, thyme, vanillin and eugenol protected compounds) addition on the finishing diet of young bulls and its effect on meat lipid oxidation, antioxidant activity and shelf-life.

2. Material and Methods

2.1 Local, animals, diets and experimental design

This experiment was approved by the Department of Animal Production and Research Ethic Committee at the State University of Maringá, and it followed the guiding principles of biomedical research with animals n° 081/2014 (approval Nº 8583060318). The study was conducted at the Rosa & Pedro Sector, State University of Maringá, Experimental Farm Station at Iguatemi city, Paraná, southern Brazil. A total of 40 (½ Angus *vs.* ½Nellore) young bulls of 16 ± 2.2 months of age, with a body weight (BW) of 385.82 ± 20.67 kg were used in a completely randomized design. The bulls were weighed at the beginning of the experiment and assigned to 10 m^2 individual pens, partially covered and with concrete floors.

The bulls were distributed into five diets according to initial BW. The adaptation period before starting the experiment lasted two weeks, when the concentrate was

834 supplied gradually. The bulls were weighed every 28 days in a trunk balance
835 (Beckehauser Cia. Paranavaí city, Paraná, south Brazil).

836 The basal diet comprised of 70% concentrate and 30% corn silage, and it was offered
837 *ad libitum* for 62 days. The feed intake was recorded daily. The basal diet was similar for
838 all animals, formulated to be isonitrogenous and isoenergetic (Table 1), according to NRC
839 (2000). The animals were randomized in five diets (n=8 animals per treatment) without
840 or with different inclusion levels of a naturals additives blend (from 1,500 to 6,000
841 mg/animal/day) which was composed by essential oil from clove´s leaf (Ferquima®),
842 functional oils from castor and cashew (Safeeds®) and a commercial blend composed by
843 a mix of natural compounds (vanillin, eugenol and thymol; Safeeds®). The diets were:
844 control (CON): without addition; AN15: 153.07 mg/animal/kg of dry matter (DM) in a
845 total of 1,500 mg/day; AN30: 305.2 mg/animal/kg of DM in a total 3,000 mg/day; AN45:
846 444.66 mg/animal/kg of DM in a total 4,500 mg/day; and AN60: 594.65 mg/animal/kg
847 of DM in a total 6,000 mg/day. The oils use was defined based on previous findings of
848 our research group (Valero et al., 2014; Valero et al., 2016; Ornaghi et al., 2017; Passetti
849 et al., 2017) where it provided evidence of potential synergism between compounds, thus
850 improving animal performance and meat quality.

851

852 *2.2 Sample preparation*

853

854 At day 62 in the feedlot, the bulls were weighed after 16 hours of fasting (482 ± 31.9
855 kg) and transported to a commercial slaughterhouse (Campo Mourão city, Paraná, South
856 Brazil). The truck stocking density was 0.8 ± 0.2 bulls/m^2, and the transport distance was
857 less than 90 km. The young bulls were slaughtered following the usual practices of the
858 Brazilian beef industry. The bulls were stunned using a captive-bolt pistol. Then, they

were bled through exsanguinations by cutting the neck vessels, and the head hide, viscera, tail, legs, diaphragm and excess internal fat were removed. Afterwards, the carcasses were divided medially from the sternum and spine, resulting in two similar halves, which were weighed to calculate the hot carcass weight. Then, the half-carcasses were washed, identified and stored in a chilling chamber at 4 °C, where they remained for a 24 h period. Then, the *Longissimus* muscle (LM) was excised from the left half of the carcass from the seventh to the last lumbar vertebra. The LM was transported to the Laboratory of Animal Science, State University of Maringá. Homogenous steaks of 2.5 (colour, antioxidant activity, lipid oxidation) and 2.0 cm (visual analysis) thick were then obtained. The steaks were distributed randomly for experimental meat instrumental analysis in two different package methods (vacuum and film packages, see technical specifications below) during different times of display, to antioxidant and lipid oxidation: 1, 3, 7 and 14 days; colour: 1, 7 and 14 and visual acceptability displayed until 14 days. The assays were assessed on meat displayed in film packages with the aim to observe the major impact of oxygen contact and NA protective effect.

2.3 Meat display

Samples from day 1 were analysed immediately as a reference point. To the film storage the steaks were packaged individually in polystyrene trays (Darnel Embalagens LTDA, Curitiba, Paraná, Brazil, 14 × 21 cm) wrapped with a retractile film (Goodyear®, Americana, São Paulo, Brazil), with oxygen permeability of 8,200 $cm^3/m^2/d$, rates of 262 $cm^3/m^2/d$. To the vacuum storage, samples were vacuum packed in 25 × 15 × 0.18 cm transparent polyamide/polyethylene pouches 120 μm; 1 $cm^3/m^2/24$ h O^2 permeability; 3 $cm^3/m^2/24$ h CO^2 permeability at 4 °C; in 75 % relative humidity; 3 $g/m^2/24$ h water

884 vapour transmission rate at 38 °C; 100 % relative humidity; 97 °C Vicat softening
885 temperature; and 1.3 g dart drop strength; and sealed using a Sulpack SVC 620 equipment
886 (VAC). The samples were stored for 3, 7 and 14 days and were exposed in a chilling
887 chamber (4 ± 1 °C) simulating typical Brazilian market conditions with artificial light
888 from a 50/50 siliconized Light Emitting Diode, 4.8 W, for 12 hours/day.
889
890 *2.4 Instrumental meat colour*
891
892 The colour was evaluated using the CIELab system with a Minolta CR- 400 Chroma
893 meter (Japan) with a 10 ° view angle, D65 illuminant and 8 mm of aperture with a close
894 cone. Six measurements at randomly selected points were recorded per sample, obtaining
895 lightness (L*), redness (a*) and yellowness (b*). Vacuum packed samples were allowed
896 to bloom for 30 min before colour evaluation.
897
898 *2.5 Antioxidant activity*
899
900 Antioxidant activity was assessed on meat samples on permeable to oxygen film at 1,
901 7 and 14 days of display (1:1 w/v with methanol), after extraction. Extracts were obtained
902 by homogenization (in ultra turrax for meat), centrifugation (15 min, 4,000 rpm) and
903 filtration (filter paper). Antioxidant activity was assessed using the ferric reducing
904 antioxidant power (FRAP), ABTS and DPPH radical scavenging assays.
905
906
907

908 *2.5.1 FRAP assay*

909

910 The FRAP method was performed according to Zhu et al. (2002). Samples were mixed
911 with methanol and an aliquot (250 μL) was mixed with 50 mM sodium phosphate buffer
912 pH 7 (1.25 mL) and 1% potassium ferricyanide (1.25 mL), and incubated at 50 °C for 20
913 min. Then, trichloroacetic acid (TCA) (10%) (1.25 mL) was added and the mixture was
914 centrifuged at 3000 rpm for 10 min. The upper layer (2.5 mL) was mixed with 0.1% ferric
915 chloride (500 μL) and the absorbance was measured at 700 nm. Results were expressed
916 as mg of gallic acid equivalent (GAE) g^{-1} oil, mg of GAE g^{-1} coating and mg of GAE per
917 100 g^{-1} of meat. The standard curve of gallic acid ranged from 0−300 mg per l^{-1}.

918

919 *2.5.2 ABTS assay*

920

921 The ABTS assay was conducted according to Re et al. (1999), with modifications.
922 $ABTS^{\cdot+}$ was generated through the interaction of 7 mM ABTS (5 mL) with 140 mM
923 potassium persulfate (88 μL). The mixture was incubated in the dark at 25 °C for 16 h.
924 The ABTS-activated radical was diluted with ethanol to an absorbance of 0.70 ± 0.02.
925 The radical scavenging activity (%) was measured at 734 nm. Samples (40 μL) were
926 mixed with $ABTS^{\cdot+}$ solution (1960 μL) and absorbance was recorded at 6 min. The
927 radical scavenging activity (%) was calculated as:

928

929 $$ABTS \text{ radical scavenging activity } (\%) = (1 - (A_{sample\ t=0} / A_{sample\ t}) * 100$$

930

931 where: $A_{sample\ t=0}$: sample absorbance at time zero; $A_{sample\ t}$: sample absorbance at 6 min.

2.5.3 DPPH assay

DPPH scavenging activity was measured according to Li et al. (2009), with modifications. Samples (150 µL) were mixed with 2850 µL of a methanolic solution containing DPPH (60 µM) and reacted for 30 min. The absorbance at 515 nm was measured against a blank of pure methanol. Antioxidant activity was calculated as:

$$\text{DPPH scavenging activity (\%)} = (1 - (A_{sample\ t=0} / A_{sample\ t}) * 100$$

where: $A_{sample\ t=0}$: sample absorbance at time zero; $A_{sample\ t}$: sample absorbance at 30 min.

2.6 Lipid oxidation

The meat malonaldehyde (MDA) content was quantified using the thiobarbituric acid reactive substances (TBARS) assay according to Souza et al. (2011). The sample (5 g) was mixed with TCA solution (7.5% TCA, 0.1% EDTA and 0.1% gallic acid) (10 mL), homogenized using an Ultra Turrax, then centrifuged at 4°C for 15 min and 4,000 rpm. The supernatant was filtered and mixed with TBARS reagent (1% thiobarbituric acid, 562.5 µM, HCl, 15% TCA) (1:1 v/v). The mixture was boiled (100 °C) for 15 min, cooled, then the absorbance measured at 540 nm against an MDA standard. Results were expressed as mg MDA kg/1 of meat. Lipid oxidation assays were performed at 1, 7 and 14 days of display.

957

958 *2.7 Visual acceptability*

959

960 Standardised conditions for photography were prepared according to a previous study
961 (Chan, Moss, Farmer, Gordon, & Cuskelly, 2013). Steaks were photographed every 2
962 days until they reach 14 days of display, using a NIKON D3100 digital camera mounted
963 on a photographic stand and containing two D65 fluorescent light tubes as standard
964 illuminate. An additional grey-colour cardboard was used to cover the cabinet entrance
965 to provide lighting evenly distributed across the sample and to avoid exposure to external
966 light.

967 The camera was fixed perpendicularly 45 cm to the surface of the meat sample. In
968 accordance with other experiments (Passetti et al., 2017; 2019), the following camera
969 parameters were chosen: manual mode; shutter speed, 1/20; aperture size, F5.3; ISO,
970 1600; focal distance 40 mm. Images were exported as JPEG files. A Gretag Macbeth mini
971 Colour-Checker (Colour-confidence, Birmingham, UK), which contains 24 coloured
972 patches, was photographed with each meat sample to check the colour reproduction
973 capability.

974 Consumer-based sensory panels were conducted with semi-trained evaluators (n = 61
975 evaluators) to evaluate the meat colour acceptability. Photos were presented in random
976 order (Passetti et al., 2017). Consumers evaluated the meat using a 9-point structured
977 hedonic scale (1= dislike extremely to 9= like extremely) to assess the visual meat
978 acceptability. The shelf-life was limited by the number of days at which the samples were
979 assigned with scores equal or higher than 4.5. Each consumer evaluated correspondent
980 photographs of the samples, which were presented in random order (Passetti et al., 2017;

Passetti et al., 2019), using a 9-point structured hedonic scale (1= dislike extremely to 1= like extremely) to assess the meat colour visual acceptability.

2.8 Statistical analyses

The experimental design was completely randomized with five diets and eight replications. Data were tested for normality (Shapiro-Wilk test). Those that showed a normal distribution were analysed by analysis of variance using the R statistical software (R Development Core Team, 2014). The experimental diet effect was evaluated using orthogonal contrast, which was used to assess the effects of control diet versus diets with NAs, linear and quadratic response ($P \leq 0.05$). The effect of display on meat quality variables (colour, antioxidant, lipid oxidation) and the instrumental meat colour variables were evaluated and differences between display time means were assessed by using the Tukey Test ($P \leq 0.05$). Further, once the fitted regression equations were determined, the response surface plots were drawn using the R statistical software (R Development Core Team, 2014).

Data of visual acceptability were imported into an Excel matrix after checking for missing data and outliers. Visual acceptability scores were analysed in the IBM Statistical Package for the Social Sciences (SPSS version 20), using a General Linear Model (GLM) with days of display and experimental diet considered as fixed effects. To analyse the scores evolution among the display period, a simple regression for the effect of days was performed.

In all statistical analyses, the experimental diet was considered as fixed effect and the animal was considered a random effect. Diets means were computed with the LSMEANS option.

$Yij = \beta 0 + \beta_1 X_i + \beta_2 X_i^2 + \varepsilon ij;$

where:

Yij observation of the repetition j on diet i;

β0 general coefficient;

β1 linear regression coefficient of the variable observed depending on the levels;

β2 quadratic regression coefficient of the variable observed depending on the levels;

Xi independent variables (experimental diet);

Eij residual error.

3. Results and discussion

3.1 Instrumental meat colour, antioxidant power and lipid oxidation

In this study a greater antioxidant activity was observed in the meat from the animals that received NAs in diet and it was higher in the higher addition levels ($P < 0.05$; Fig. 1; Table 2). Greater antioxidant capacity was observed for all the three methods used to evaluated the antioxidant power (ABTS, DPPH and FRAP). There was also effect of diet and storage time (Fig. 1), which revealed an influence of the NAs levels on the days of meat exposition in the vacuum and film packages.

The NAs used in the diet can pass through the rumen and may be deposited in tissues (e.g. meat) resulting in a higher antioxidant power (Falowo, Fayemi, & Muchenje, 2014). Compounds present in the NA blend, such as phenolic compounds, can attract electrons and delay the oxidation, and likely protected meat oxidation (Fig. 2). Furthermore, such compounds can activate antioxidants enzymes (e.g. catalase or superoxide dismutase) in the circulatory system (Frankič, Voljč, Salobir, and Rezar, 2009).

The beef lipid oxidation, expressed by the MDA production, was affected by diets ($P < 0.05$) and an increase with aging time (1, 3, 7 and 14 days) was expected. The antioxidant power delayed the oxidation and consequently increased the shelf-life through the maintenance of the meat colour (Table 3 and 4). Oxidative stress can also be delayed by the use of natural antioxidants that improve the balance between production of ROS (reactive oxygen species) and the body's defence mechanisms, which prevents a future oxidation on tissue after conversion of the muscle to meat (Falowo, Fayemi, & Muchenje, 2014; Mc Cord, 2000; Rock, Jacob & Bowen, 2009). Peroxy radicals can react with unsaturated fatty acids in meat, resulting in rancid odour and off flavours from the volatile compounds formed in this reaction, thus interfering on meat quality and consumer acceptability. According to Min & Ahn (2005), aldehydes influence ROS formation, triggers the deterioration of meat colour and flavour, protein stability and functionality. Besides the balance between ROS and antioxidants oxidation can be affected by different factors, such as pH, diet, fatty acids, iron content on meat, and others (Gatellier et al., 2007).

In contrast of our results Rivaroli et al., (2016), using an essential oils blends in two doses (3.5 and 7.0 g/animal/day) in beef cattle diet, observed an increase in lipid oxidation on the highest blend inclusion level. Higher NAs quantities can act as pro-oxidant (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). On the other hand, corroborating with our data, Monteschio et al., (2017) found a positive effect in lipid oxidation delay with the essential oils blend addition (clove and rosemary essential oils and encapsulate active principles (eugenol, thymol and vanillin blend) in different doses 2, 4 or 1.33 g/animal/day, respectively) in diet of beef heifers. Thus, the dose and the compounds added to the diets of ruminants need to be considered.

Evolution of colour variables along display is compiled on Table 3. There was not a significant interaction between the two effects studied (diet and display). The L* did not show a significant effect at 1 and 14 days of storage ($P > 0.05$) when NAs were added to diet. However, at day 7 of storage a tendency ($P = 0.061$) of an increase in the L* value can be observed. This parameter is correlated with the meat freshness and consequently with higher consumers acceptability, since, the colour is the first attribute that the consumers take into consideration on the purchase moment (Resconi et al., 2012).

The NAs addition had no effect on a* values ($P > 0.05$). Nevertheless, a superior stability during storage was observed with the NAs when analysed within each day (NA30, NA45 and NA60) compared to control diet, or with the lowest blend addition concentration level (NA15). This may be associated to the protection caused by the antioxidant incorporation in cells membranes, which delay the myoglobin oxidation. These compounds act in the capture of free radicals which are formed during lipid oxidation, delaying the conversion of the cherry red pigment (oxymyoglobin [oxyMb (Fe^{2+})]) to the brown pigment (metmyoglobin [MetMb (Fe^{3+})]; Hayes et al., 2009). High ROS levels in meat could reduce meat sensory quality, cause loss of protein functionality, degradation of polyunsaturated fatty acid and also the conversion of oxymyoglobin to metmyoglobin pigment resulting generation of free radicals which could result deterioration of meat protein (Suman & Joseph, 2013).

The diets influenced ($P < 0.05$) the yellowness (b*), where the values presented an increase until day 7, the values ranged from 12.42 to 15.47. On other hand, Rivaroli et al. (2016) did not observed significant effect on meat b* value with essential oils blend addition (oregano, garlic, lemon, rosemary, thyme, eucalyptus and sweet orange) in two different levels (3.5 and 7.0 g/animal/day).

3.3 Visual acceptability

There was a significant interaction between diets and display on visual acceptability ($P < 0.001$; Table 4; Fig 3). The display consumer acceptability was decreased, possibly due to oxidation and discoloration of meat surface. A gradual decline in visual appraisal was expected because oxidative processes are a natural cause of meat deterioration, producing a less attractive appearance of meat for consumers, as is usually reported (Ornaghi et al., 2020; Eiras et al., 2017; Passetti et al., 2017; Prado et al., 2015; Vitale et al., 2014).

Visual acceptability scores of consumers ranged from 6.23 to 6.78 in the first day, but no clear effect was observed when the NAs were added. At the third day of evaluation, an increase on the meat acceptability with NAs compared to the diet was observed ($P < 0.05$). This is likely due to a change from purple-reddish for a cherry red colour of meat. Consumers have preference scale first for cherry-red (oxymyoglobin state), than for purple-reddish (deoxymyoglobin state), and the less desirable was the brown colour (metmyoglobin state; Hayes et al., 2009). The mechanism of myoglobin states changes during display due to several factors (oxidation, spoilage, etc) and even the used methodology affects the maximum scores. In sequential designs (display on trays or in photos in sequential order) consumers give higher scores in the first days, because they knew that the meat is fresh. Eiras et al. (2017) and Prado et al. (2015) reported a lower shelf-life for beef steaks, between 5 and 7 days, where visual analyses were done in person directly observing trays. Thus, when this additional information is not provided consumers scores relies only on their visual perception of meat colour, as we can observe in this study. Passetti et al. (2017) used random photos and reported shelf life between 7.19 and 7.66 following the addition of 5,000 mg/animal/day clove or cinnamon essential oil in the diet of beef cattle/day. Contrary to our results, the essential oils addition resulted

in lower meat visual scores (Passetti et al., 2017). The complexity of these compounds and the NAs mixture used in the current study seem to have a synergistic positive effect, reducing meat oxidation and discoloration.

According to Passeti et al. (2017) findings, the increase of acceptability values on the first 3 days of display is due to the own methodology used. Presenting the photographs in a random way, as it was used on the current analyses, force consumers to associate meat freshness and its acceptability only/exclusively with color aspects. Deleting the influence of others inevitable information's as sample real days of display, which is knew with a sequential presentation of photos or which a daily presence visual analysis, where unconscious has influence on final scores assignation, being those punctuations higher in the first days and lower in the last with a progressive decrease. Being a tool more accurately to evaluate the direct impact in meat discoloration.

From the 7^{th} to the 13^{th} day of display visual acceptability scores remained higher than 5.0. Scores lower than 5.0 reflects rejection by the consumers, which only occurred after the 14th day of display. Past studies observed that read meat could be displayed for up to 6 or 7 days, and the score at 14 days observed in our study was unexpected. A linear effect was observed when the blend on days 7^{th}, 11^{th} and 13^{th}. The higher scores could be explained to the antioxidant activity present on NAs which reduced the change of the red cherry colour (oxymyoglobin) to the brown colour (metmyoglobin).

There was a significant linear effect for the NAs addition in the cattle diet when meat was evaluated at 14^{t} days ($P < 0.050$). The highest dosages NA45 and NA60 presented higher acceptability scores compared to CON, which is likely due to a less oxidative status of those treatments, as previously commented. Nevertheless, a superior stability during storage was observed with the NAs (NA30, NA45 and NA60) compared to control diet or with the lower blend addition concentration (NA15). The antioxidant power delayed

the oxidation and consequently increase the shelf- life through the meat colour maintenance (Table 4 and 5).

To determinate the display shelf-life of the meat a regression analyses was performed (Table 5). Control group presented meat with lower shelf-life 8.53 days. The essential oil inclusion improved the visual shelf-life being NA 60 group the one that presented the highest shelf-life: 9.58 days. This reflects the beneficial effect of the synergism between the natural compounds on blend content on the myoglobin oxydation.

The regression analyses of our study presented low R^2 values, and this could be explained due to the scores that remained higher than 5.0 after 13 days of evaluation. According to Passetti et al. (2019), the amount of days of display to be evaluated in visual analyses could be reduced, but it will depend on the inflection point (the day which scores are below 5.0). However, our results show high acceptability results until the last day of evaluation, which suggests that meat in this experiment would still be accepted even after 14 days of display. Shelf-life defined by regression equations, which compile the number of days that consumers evaluated meat with scores equal or higher than 5.0 was higher for blend addition with respect to control treatment (Table 5). The addition of the lowest and highest dosage of NAs blend added an extra day of shelf-life of the product.

4. Conclusion

The natural additives addition in the diet of young bulls, specially the higher doses (NA60), reduced the lipid oxidation and colour losses in relation to control diet, improving the antioxidant potential and acceptably by consumers. Natural additives can be used in animal feed to improve meat quality during shelf-life, however the type of additive and the concentration must be considered.

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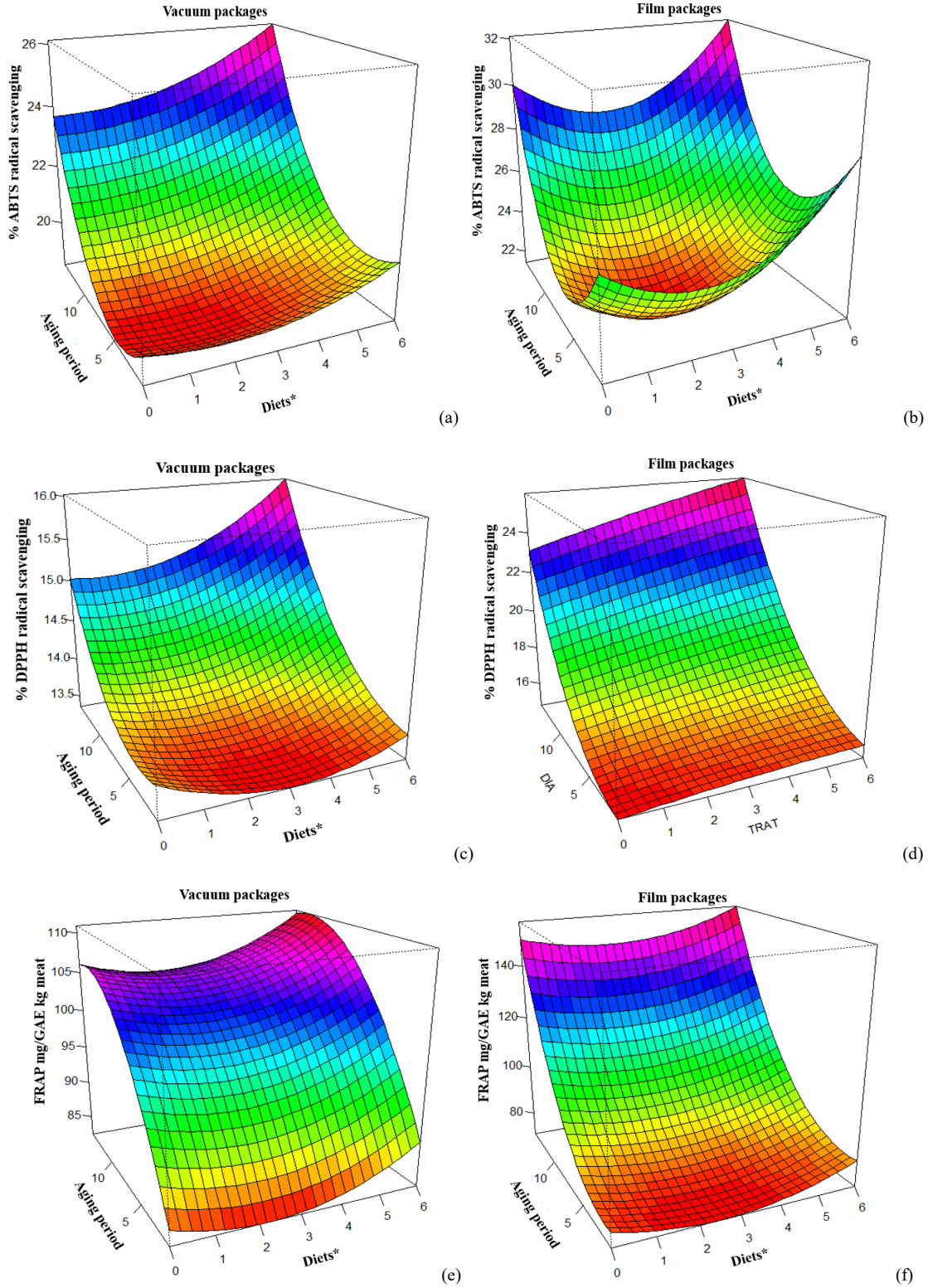
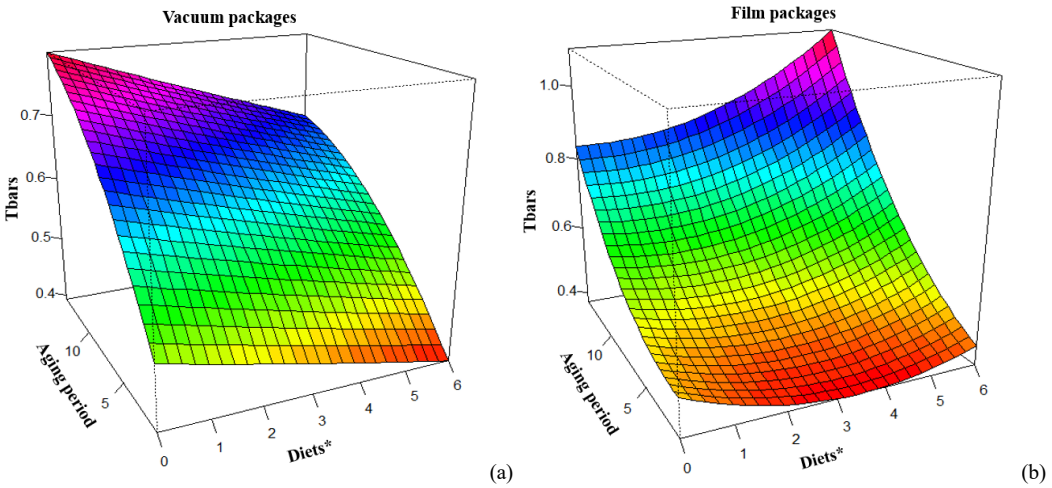
Figure 1. Response surface of the antioxidants activity on meat of young bulls finished in feedlot with natural additives: (a) ABTS radical scavenging (%) on meat storage in vacuum packages, (b) ABTS radical scavenging (%) on meat storage in film packages, (c) DPPH radical scavenging (%) on meat storage in vacuum packages (d) DPPH radical scavenging (%) on meat storage in film packages, (e) Ferric reducing power (FRAP mg/EAG kg meat) on meat storage in vacuum packages, (f) Ferric reducing power (FRAP mg/EAG kg meat) on meat storage in film packages.



*Diets (experimental diets: 0 = without blend; 1 – 6 = blend addition levels 1.5; 3.0; 4.5; 6.0)

Figure 2. Lipid oxidation on meat of young bulls finished in feedlot with natural additives: (a) in vacuum packages and (b) film packages; (Tbars) expressed as mg malonaldehyde/kg of meat during storage time.



(a) (b)

*Diets (experimental diets: 0 = without blend; 1 – 6 = blend addition level 1.5; 3.0; 4.5; 6.0)

Table 1

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

Ingredients	Diet
Corn silage	275.9
Corn grain	613.2
Soybean meal	51.0
Premix[1]	50.5
Mineral salt	4.5
Limestone	4.5
Yeast	0.4
Chemical composition	
Dry matter	577
Crude protein	132
Organic matter	968
Ash	31.4
Ether extract	40.1
Neutral detergent fiber	288
Acid detergent fiber	117
Total digestible nutrients	790
Metabolizable energy (MJ/kg DM)	11.9
Calcium	6.82
Phosphorus	3.56

[1]Premix: magnesium (57 g/kg), sodium (81 g/kg), sulphur (3.75 g/kg), cobalt (20 mg/kg), copper (500 mg/kg), iodine (25 mg/kg), manganese (1 500 mg/kg), selenium (10 mg/kg), zinc (2 000 mg/kg), vitamin A (400 000 UI/kg), vitamin D3 (50 000 UI/kg), vitamin E (750 UI/kg), ether extract (168 g/kg) and urea (200 g/kg).

Table 2

Regression coefficients of the proposed model for the variables of response surface: Tbars (vaccum package), Tbars (Film package), Abts (vaccum package), Abts (Film package), Dpph (vaccum package), Dpph (Film package), Frap (vaccum package) and Frap (Film package).

Item	Tbars (Vaccum package)	Tbars (Film package)	Abts (Vaccum package)	Abts (Film package)	Dpph (Vaccum package)	Dpph (Film package)	Frap (Vaccum package)	Frap (Film package)
Constant	0.4949	0.4782	19.7859	27.0112	13.8184	14.8393	80.2965	77.8940
Diet	-0.0170	-0.0625	-0.2762	-1.2984	-0.2509	0.1569	-18.0010	-4.2361
Day	0.0397	-0.0068	-0.5310	-1.3427	-0.0924	-0.1519	4.2261	-1.7967
Diet x Day	-0.0005	0.0040	0.0178	-0.0071	0.0139	0.0276	0.0098	0.0262
R^2	0.4244	0.3843	0.3498	0.2116	0.0339	0.8680	0.4440	0.9020
Lack of fit	0.1062	1.2250	281.3700	204.4000	24.1420	33.8600	3211.1000	2810.0000
P-Value Diet	0.0101	0.0478	0.001703	0.0005	0.1334	0.0664	0.1023	0.0941
P-Value Day	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Table 3

Colour variables during beef display from young bulls finished in feedlot with natural additives.

	Diets (Dt)						P - value		
Display (Dp)	CON[1]	NA15[2]	NA30[3]	NA45[4]	NA60[5]	SEM[6]	L[7]	Q[8]	0% *vs blend*
			L*						
1	38.93a	38.39	38.66	40.64	39.77	0.254	0.099	0.262	0.254
7	41.29b	40.11	41.36	42.36	42.21	0.313	0.061	0.136	0.769
14	40.19b	40.10	40.25	41.35	41.60	0.395	0.138	0.297	0.520
SEM	0.36	0.36	0.52	0.36	0.50		P (Dt x Dp)[9]		
P <	0.017	0.720	0.088	0.140	0.111		0.931		
			a*						
1	13.30a	13.58a	13.68a	13.74a	13.48a	0.142	0.626	0.588	0.406
7	15.39b	15.57b	15.68b	15.72b	15.70b	0.189	0.552	0.803	0.562
14	14.46b	14.59b	14.36ab	14.56ab	14.49ab	0.197	0.971	0.999	0.933
SEM	0.289	0.302	0.247	0.268	0.300		P (Dt x Dp)[9]		
P <	0.006	0.015	0.001	0.004	0.004		1.000		
			b*						
1	12.45a	12.81a	12.62a	13.51a	12.99a	0.156	0.032	0.080	0.583
7	14.79b	14.45b	15.16b	15.41b	15.47b	0.164	0.037	0.114	0.400
14	14.14b	13.92b	14.06b	14.56b	14.42b	0.168	0.299	0.549	0.806
SEM	0.261	0.209	0.320	0.220	0.290		P (Dt x Dp)[9]		
P <	0.001	0.002	0.001	0.001	0.001		0.885		

[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 – 6.0 g/animal/day of natural additives addition; [6]Standard error of means; [7]Linear effect; [8]Quadratic effect. [9]P (Dt x Dp): P value interaction Diet x Display.

a,b: Different lowercase letters in the same column are significantly different (P < 0.05).

Table 4

Visual acceptability (n=61) of meat of young bulls finished in feedlot with natural additives and display time §.

	Diets						P - value		
Day	CON[1]	AN15[2]	AN30[3]	AN45[4]	AN60[5]	SEM[6]	L[7]	Q[8]	0% *vs blend*
1	6.58b	6.23bc	6.38bc	6.78b	6.36b	0.031	<0.001	0.1543	0.143
3	7.00a	7.40a	7.19a	7.27a	7.15a	0.047	0.381	0.240	0.017
7	5.87c	5.68d	5.86de	6.30c	6.30c	0.032	<0.001	0.128	0.382
11	5.59d	5.31e	5.58e	5.67e	5.95c	0.040	0.005	0.395	0.534
13	5.34e	5.29e	5.24f	5.93d	6.10c	0.036	<0.001	<0.001	0.106
14	4.61f	4.71f	4.58g	4.82f	4.96d	0.050	0.522	0.182	0.481
SEM	0.026	0.035	0.036	0.025	0.025		[9]P (Dt x Dp)		
P <	0.0001	0.0001	0.0001	0.0001	0.0001		0.0001		

[1]CON = control (without natural additives); [2]NA15 – 1.5 g/animal/day of natural additives addition; [3]NA30 – 3.0 g/animal/ day of natural additives addition; [4]NA45 – 4.5 g/animal/day of natural additives addition; [5]NA60 – 6.0 g/animal/day of natural additives addition; [6]Standard error of means; [7]Linear effect; [8]Quadratic effect. [9]P (Dt x Dp): P value interaction Diet x Display.

a,b: Different lowercase letters in the same column are significantly different ($P < 0.05$).

§Based on a hedonic 9 points scale (1 = dislike extremely; 9 = like extremely).

Table 5

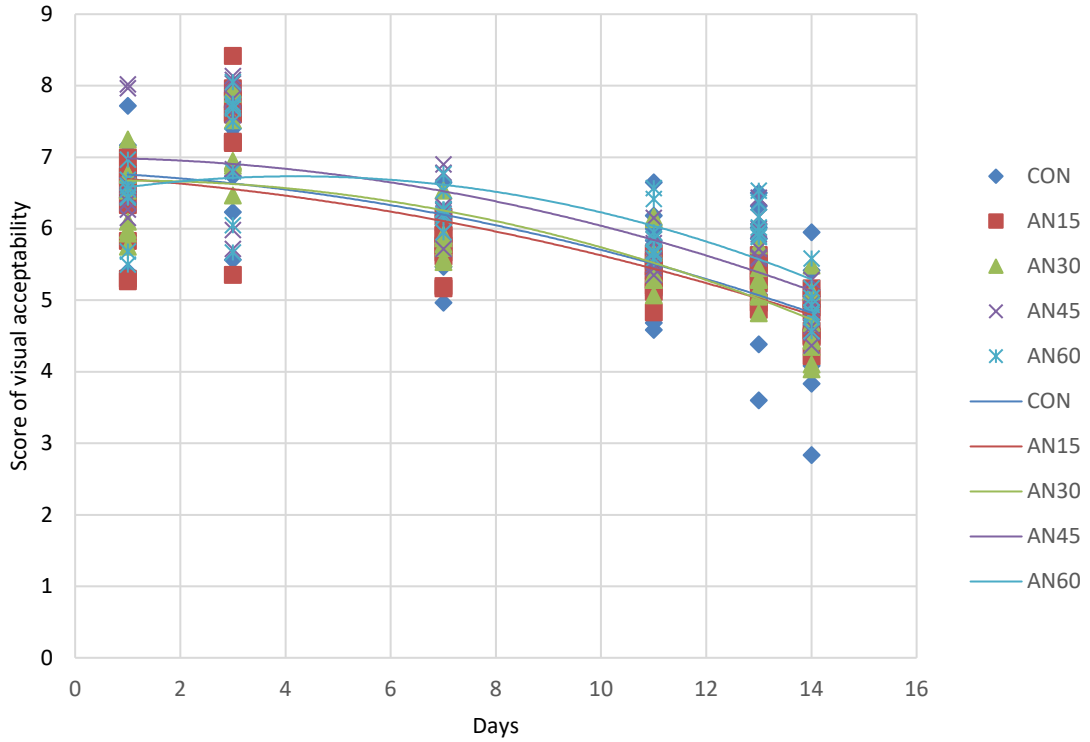
Regression analysis of visual acceptability of meat from bulls finished in feedlots fed with or without natural additives addition.

Diets[a]	Days[b]	Equation	R^2	F	P - Value
CON[1]	8.53	$Y = 12.937 – 0.477x – 0.053x^2$	0.161	275.05	<0.001
AN15[2]	9.36	$Y = 13.618 – 1.014x – 0.010x^2$	0.128	211.28	<0.001
AN30[3]	8.72	$Y = 14.884 - 1.264x + 0.015x^2$	0.174	303.85	<0.001
AN45[4]	8.64	$Y = 11.816 + 0.042x – 0.096x^2$	0.157	268.87	<0.001
AN60[5]	9.58	$Y = 11.916 – 0.357x – 0.038x^2$	0.081	126.92	<0.001

[a]Diets: [1]CON – without essential oil; [2]AN15 – 1.5 g/animal/day of natural additives addition; [3]AN30 – 3.0 g/animal/ day of natural additives addition; [4]AN45 – 4.5 g/animal/day of natural additives addition; [5]AN60 – 6.0 g/animal/day of natural additives addition.

[b]Days: Number of days which consumers evaluated meat with scores equal or higher than 5.0.

Fig. 3. Visual acceptability (1 = dislike extremely; 9 = like extremely) of meat from bulls finished in feedlots fed with or without natural additives addition.



VII CONCLUSÕES GERAIS

No geral, a inclusão do *blend* proporcionou maior ganho médio diário e melhor eficiência alimentar dos animais, apresentando aumento linear com a inclusão das doses, sem haver alterações sobre o consumo de matéria seca. Com a adição dos aditivos naturais observou-se diminuição na produção de acetato e a redução drástica na produção de nitrogênio amoniacal. Quando se observou a microbiota ruminal através de análise de sequenciamento (metagenômica) foi possível observar a redução de bactérias relacionadas com a produção de acetato e amônia bem como a redução linear de *archaeas* (microrganismos produtores de metano), sugerindo a redução de metano. Assim, pode-se confirmar uma modulação no ambiente ruminal que proporcionou melhor desempenho animal. Nas medidas de característica de carcaça a inclusão do aditivo natural não resultou em diferenças entre os tratamentos, exceto no pH. O pH da carne reduziu em relação ao tratamento controle. Essa medida é diretamente ligada a maciez da carne. Em consequência disso, observa-se a redução linear da textura (aumento da maciez) na carne dos animais recebendo aditivos naturais. Isso pode estar relacionado com o aumento no potencial antioxidante consequente menor oxidação lipídica observado no estudo, que pode afetar o sistema calpaína/calpastatína aumentando a maciez da carne. No teste sensorial, em que 120 consumidores (divididos de acordo com o censo do IBGE, 2010, levando em consideração gênero e idade) provaram pedaços de carne de todos os tratamentos e avaliaram de acordo com sua preferência atribuindo notas de 1 (desgosto

extremamente) a 9 (gosto extremamente) para as características organolépticas: *flavour*, textura e aceitabilidade geral do produto. Neste teste, observou-se aumento da preferência em relação a textura e aceitabilidade geral, ou seja, o produto apresentou maior maciez e maior aceitabilidade de acordo com os consumidores. No teste de aceitabilidade visual, foi levado em conta a aceitabilidade do consumidor em relação a coloração da carne exposta por 14 dias em uma gôndola simulando as reais condições do mercado brasileiro (iluminação por LED, temperatura ± 4°C e forma de apresentação do produto com bandejas recobertas por papel filme). Neste caso, 60 consumidores receberam fotografias dos bifes (bifes porcionados da 6° vertebra de todos animais de todos os tratamentos), de forma aleatória, (as fotografias foram realizadas de forma padrão para todas as amostras) para avaliarem de acordo a sua preferência em uma escala de 1 a 9 como no teste sensorial. Ao final, pode-se observar a preferência do consumidor pela carne dos animais recebendo o *blend* na dieta. Com o aumento da inclusão do *blend* as notas para aceitabilidade aumentaram linearmente e ao final concluiu-se que é possível aumentar em um dia a *shelf life* do produto final, podendo ocasionar em impactos positivos na indústria. Esse aumento na vida de prateleira (*shelf life*) é resultado do aumento no potencial antioxidante observado no estudo. Os aditivos naturais apresentam completo potencial de ação, pois, abrange efeitos desde o desempenho animal através da modulação do microbioma ruminal até o produto final melhorando processos oxidativos que vão refletir em melhor qualidade da carne.