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Desempenho, desenvolvimento do TGI e o perfil de microrganismos ruminais e intestinais de bezerros leiteiros suplementados ou não com extrato de orégano

PORTO ALEGRE
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Tese apresentada como requisito para obtenção do Grau de Doutor em Zootecnia, na Faculdade de Agronomia, da Universidade Federal do Rio Grande do Sul.

Orientador: Dra Vivian Fischer

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Luciano Antônio Ritt
Mestre em Zootecnia

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TÍTULO DA TESE[1] **Desempenho, desenvolvimento do TGI e o perfil de microrganismos ruminais e intestinais de bezerros leiteiros suplementados ou não com extrato de orégano**

Autor: Luciano Antonio Ritt
Orientador: Drª Vivian Fischer

Resumo:
Os óleos essenciais são potenciais substitutos aos antibióticos promotores de crescimento, mas seu modo de ação sobre as populações microbianas e de desenvolvimento do trato gastrintestinal de animais permanece amplamente desconhecido. Para obter mais informações, este estudo investigou a população bacteriana em segmentos do trato gastrointestinal, parâmetros de desenvolvimento do trato gastrintestinal e o consumo e desempenho de bezerros leiteiros pré-desaleitados suplementados com extrato de orégano comercial contendo óleos essenciais. Dez bezerros da raça Holandesa foram alimentados com 6 L / dia de sucedâneo do leite e tiveram livre acesso a água e ração iniciadora desde o primeiro dia, e ao feno a partir da terceira semana do início do período de estudo. Os bezerros foram distribuídos aleatoriamente em dois tratamentos: CON - sem aditivo; e OR - 60 mg / kg de peso corporal por dia com extrato de orégano. O desaleitamento ocorreu no 53º dia do período experimental e no 54º dia, todos os bezerros foram eutanasiados e o conteúdo de rúmen, jejuno, ceco e cólon foram amostrados para determinar a população bacteriana. Foi realizada pesagem do trato cheio e vazio e também retiradas amostras de tecido do rúmen, jejuno, ceco e cólon para análise histológica. A suplementação com OR aumentou o consumo de concentrado inicial, mas não

[1]Tese de Doutorado em Zootecnia - Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (115 p.) Dezembro, 2021.

afetou a conversão alimentar, o ganho médio diário (GMD), o ácido graxo volátil (AGV) total e as concentrações ruminais de acetato (C2), butirato (C4) e propionato (C3), bem como a relação C2:C3. A digestibilidade aparente aumentou no OR para matéria seca (MS), proteína bruta (PB) e nutrientes digestíveis totais (NDT), e apresentou tendência de aumento para matéria orgânica (MO) e carboidratos não-fibrosos corrigidos para cinzas e proteína (NFCap). Em bezerros suplementados com orégano, se observou maior diversidade da população bacteriana do rúmen e do jejuno (índice de Shannon). O extrato de orégano afetou abundância de bactérias gram-positivas e gram-negativas no trato gastrointestinal. Além disso, o OR diminuiu gêneros potencialmente patogênicos, como *Streptococcus*, *Escherichia* e *Clostridium*, embora também tenha diminuído *Bifidubacterium*, reconhecido como potencialmente benéfico no jejuno. Bezerros suplementados tenderam a apresentar maior valores de peso total do abomaso e apresentaram maior peso vazio do abomaso comparado ao controle, sem alterar os pesos dos demais segmentos. No rúmen, bezerros suplementados com orégano apresentaram menor número de ramificações nas papilas, altura das ramificações e número de papilas / ramificações do que CON. No jejuno, bezerros no grupo COM tenderam a apresentar maior espessura da camada muscular comparados ao OR. A concentração de AGVs bem como a proporção C2:C3 no rúmen não foi alterada pela suplementação. A suplementação com OR não alterou expressivamente o consumo, o desempenho, o peso e a morfologia dos segmentos do trato gastrintestinal. A suplementação com OR afetou negativamente aspectos morfológicos das papilas ruminais e não exerceu efeito sobre os demais segmentos do TGI.

Palavras-chave: Bovinocultura de leite, microbiota, histologia, extrato de orégano, óleos essenciais.

THESIS TITLE[2] Performance, GIT development and profile of ruminal and intestinal microorganisms of dairy calves supplemented or not with oregano extract

Author: Luciano Antonio Ritt

Adviser: Dr[a] Vivian Fischer

Abstract:

Essential oils are potential replacements for growth-promoting antibiotics, but their mode of action on microbial populations and development in the gastrointestinal tract of animals remains largely unknown. For more information, this study investigated the bacterial population in gastrointestinal tract segments, gastrointestinal tract development parameters and the performance of pre-weaned dairy calves supplemented with commercial oregano extract containing essential oils. Ten Holstein calves were fed 6 L/day of milk replacer and had free access to water and starter from the first day, and to hay from the third week of the beginning of the study period. The calves were randomly assigned to two treatments: CON - no additive; and OR - 60 mg/kg of body weight per day with oregano extract. Weaning occurred on the 53rd day of the experimental period and on the 54th day, all calves were euthanized and the contents of the rumen, jejunum, cecum and colon were sampled to determine the bacterial population. Weighing of the full and empty tract was also performed, and tissue samples from the rumen, jejunum, cecum and colon were also taken for histological analysis. Supplementation with OR increased initial concentrate intake, but did not affect feed conversion, mean daily gain (GMD), total volatile fatty acids

[2]Doctoral thesis in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (115 p.) December, 2021.

(VFA)and ruminal concentrations of acetate (C2), butyrate (C4) and propionate (C3), as well as the C2:C3 ratio. The apparent digestibility increased in OR for dry matter (DM), crude protein (CP) and total digestible nutrients (TDN), and showed a tendency to increase for organic matter (OM) and non-fifer carbohydrates corrected to ash and protein (NFCap). The alpha diversity (Shannon index) indicated a greater diversity of the bacterial population of the rumen and jejunum in calves supplemented with OR. Oregano extract affected the abundance of gram-positive and gram-negative bacteria in the gastrointestinal tract. Furthermore, OR decreased potentially pathogenic genera, such as *Streptococcus*, *Escherichia* and *Clostridium*, although it also decreased potentially beneficial *Bifidubacterium* in the jejunum. Calves in OR showed a tendency for the total weight of the abomasum to be greater and the empty weight of the abomasum to be greater than the CON, without changing the weights of the other segments. In the rumen, OR had lower branching, number of branches, branch height and number of papillae per branches in the rumen than CON. In the jejunum, CON showed a tendency for muscle layer thickness to be greater than OR. The concentration of VFAs as well as the C2:C3 ratio in the rumen was not altered by supplementation. Suckling calves supplemented with OR showed greater diversity of ruminal and intestinal bacteria, with a reduction in pathogenic bacteria. Supplementation with OR did not significantly alter intake, performance, weight and morphology of the gastrointestinal tract segments. Supplementation with oregano extract negatively affected morphological aspects of the ruminal papillae and had no effect on the other segments of the gastrointestinal tract (GIT).

Keywords: Dairy cattle, microbiota, histology, oregano extract, essential oils.

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LISTA DE ABREVIATURAS E SÍMBOLOS

OR – extrato de orégano (60mg/kg PV)

CON – controle

PV – peso vivo

MR – milk replacer

BW – body weight

CP – crude protein

EE – ether extract

DM – dry matter

GIT – gastrointestinal tract

VFA – volatile fatty acids

C2 – acetate

C3 – propionato

C4 – butirato

OM – organic matter

NDFap – neutral detergent fiber ash and protein corrected

NFCap – non fiber carbohydrates ash and protein corrected

TDN – total digestible nutrients

ADG – average daily gain

CH_4 – methane

TMR – total mixed ration

OE – óleos essenciais

DMI – dry matter intake

H_2S – hydrogen sulfide

CAPITULO I

1. INTRODUÇÃO GERAL

A crescente preocupação com a presença de traços de contaminantes nos produtos de origem animal, somada a resistência bacteriana aos antibióticos convencionais, tem estimulado a busca por alternativas aos agentes químicos, lançando mão do uso de produtos naturais, mais seguros, que poderiam ser utilizados em sistemas de produção. Neste sentido, plantas ou extratos vegetais que contêm flavonóides, taninos, saponinas, óleos essenciais (OE) e uma série de outros compostos secundários vegetais são alternativas para modular a fermentação ruminal (Calsamiglia et al., 2007; Macheboeuf et al., 2008), controlar microrganismos presentes no intestino (Bampidis et al., 2006) e melhorar o desempenho geral dos animais (Hill et al., 2007; Chester-Jones et al., 2010). Estes compostos são geralmente reconhecidos como “seguros” de acordo com a Food and Drug Administration, em virtude de não impactarem negativamente a saúde humana.

Os OEs podem alterar o desenvolvimento ruminal devido seu efeito nas populações de microrganismos e subsequente mudança nos perfis de fermentação ruminal, elevando a produção de butirato, o qual tem papel fundamental no desenvolvimento das papilas ruminais (Lane, Baldwin e Jesse, 2002), sendo o substrato da cetogênese nas papilas (Daniels e Yohe, 2015). Entretanto não há relatos da expressão gênica de enzimas cetogênicas nas papilas ruminais de bezerros leiteiros. Adicionalmente, as mudanças no perfil de ácidos graxos voláteis (AGV) são variáveis, dependendo da dose e da substância ativa do OE avaliado (Busquet et al., 2006; Castillejos et al., 2007). Por outro lado, a concentração de nitrogênio amoniacal ($N\text{-}NH_3$) diminuiu em resposta à suplementação de OE *in vitro* (Busquet et al., 2006; Macheboeuf et al., 2008) ou *in vivo* (Benchaar et al., 2007; Giannenas et al., 2011).

Os estudos que avaliaram o efeito dos OE sobre a produção de AGV e $N\text{-}NH_3$ são na sua maioria ensaios *in vitro*, ou em ruminantes adultos. Alguns estudos recentes (Vakili et al., 2013; Santos et al., 2015; Froehlich et al., 2017; Kazemi-Bonchenari et al., 2018), que utilizaram bezerros, produziram resultados contrastantes. Além disso, as alterações nos AGV e no $N\text{-}NH_3$ ruminal são consequências da modulação das populações de microrganismos no rúmen. Há estudos que relatam mudanças na microbiota ruminal e fecal desde o nascimento até o desaleitamento (Uyeno, Sekiguchi e Kamagata, 2010; Oikonomou et al., 2013; Jami et al., 2013), entretanto, não há estudos sobre os efeitos dos OE sobre a microbiota

ruminal e intestinal (jejuno, ceco e cólon) durante o desenvolvimento dos bezerros de pré-ruminantes a ruminantes funcionais.

Adicionalmente, os OE podem reduzir os microrganismos patogênicos intestinais, como foi demonstrado em estudos anteriores (Zeng et al., 2015; Wei et al., 2016) atuando de forma a reduzir o problema sanitário mais recorrente nas fases iniciais de criação dos mamíferos, a diarreia (Manzanilla et al., 2004; Zeng et al., 2015). Entretanto, os resultados destes três estudos citados, foram obtidos de experimentos com leitões. A diarreia responde por cerca de 80% dos casos de morbidade e mortalidade na criação de bezerros leiteiros (Fruscalso, Olmos e Hötzel, 2020). Mas, com exceção do estudo de Bi et al. (2019), que testou o extrato de amoreira (*Morus alba*) na microbiota intestinal de bezerros desafiados com *E. coli* K99, não existem estudos que tenham avaliado o efeito dos fitoquímicos na redução de microrganismos patogênicos intestinais em bezerros no período pré-desaleitamento.

Com base no supracitado, a presente tese apresentará resultados de desempenho, desenvolvimento e perfil de microrganismos do trato gastrintestinal de bezerros leiteiros durante o período de aleitamento pela suplementação da dieta com extrato de orégano (*Origanum vulgare*).

2. REVISÃO BIBLIOGRÁFICA

2.1. Microrganismos do trato gastrintestinal de ruminantes

Os microrganismos que habitam o trato gastrintestinal (TGI) apresentam importante papel no desempenho e saúde do hospedeiro (Dill-McFarland, Breaker e Suen, 2017), bem como às alterações metabólicas e fisiológicas no TGI de bezerros durante o período crítico da transição de pré-ruminantes para ruminantes funcionais (Davis e Drackley, 1998). A microbiota ruminal consiste em *archaeas* metanogênicas, protozoários, fungos e bactérias (Malmuthuge, Griebel e Guan, 2014), as quais dominam a microbiota ruminal e contribuem na produção de ácidos graxos voláteis e proteína microbiana (Kim, Morrison e Yu, 2011). Populações microbianas distintas foram identificadas como colonizadoras de três diferentes *habitats*: ligadas ao tecido ruminal, ligadas a fluidos e ligadas ao substrato (Cho et al., 2006), sendo que a composição da microbiota também pode variar em função da espécie de ruminante, dieta, idade do hospedeiro, estação do ano e região geográfica (Tajima et al, 2001). No entanto, existem muitas lacunas no conhecimento relacionado à composição da microbiota do TGI de bezerros leiteiros e as possíveis alterações exercidas pela suplementação de fitoquímicos, bem como a sua correlação com respostas biológicas de importância econômica (ou seja, desempenho e sanidade) durante o período de pré-desaleitamento.

2.1.1. Colonização ruminal e intestinal de bezerros pré-desaleitados

Ao nascimento, o trato gastrointestinal de ruminantes jovens é, geralmente, considerado estéril. Durante as primeiras horas de vida, o rúmen torna-se rapidamente colonizado por uma abundante população de bactérias. Entretanto, pesquisas recentes revelam que a colonização por microrganismos já começa durante o desenvolvimento fetal (Alipour et al., 2018).

Em estudo de Bi et al. (2019), a carga inicial de microrganismos intestinais de cordeiros amamentados era derivada principalmente dos tetos da mãe (43%) e do ar ambiente (28%), enquanto que a microbiota de cordeiros alimentados com mamadeira era formada majoritariamente por bactérias da vagina da mãe (46%), ar ambiente (31%), e o piso do curral (12%). A microbiota fecal de bezerros foi altamente variável durante as primeiras 48 horas após o nascimento, mais semelhante à da vagina da

vaca, sugerindo que a microbiota intestinal de bezerros pode ser derivada do canal vaginal durante o parto. No entanto, Alipour et al. (2018) indicaram que a microbiota retal de bezerros recém-nascidos era composta por Firmicutes, Proteobacteria, Actinobacteria e Bacteroidetes, similar à microbiota oral da mãe em vez de à microbiota fecal ou vaginal, mas incluía táxons intestinais típicos.

Anaeróbios facultativos como *Streptococcus* e *Enterococcus* são os primeiros colonizadores do rúmen, os quaisconvertem o rúmen em um ambiente totalmente anaeróbio para promover o rápido estabelecimento de bactérias estritamente anaeróbias (Jami et al., 2013). Aos dois dias de vida, a microflora ruminal de cordeiros atinge 109 células/ml, composta por bactérias estritamente anaeróbias (Fonty et al., 1987). Este mesmo estudo evidenciou que as bactérias aeróbias e anaeróbias facultativas foram 10 a 100 vezes menores do que a contagem bacteriana estritamente anaeróbia observada durante a primeira semana, que continuou a diminuir depois com o avançar da idade (Fonty et al., 1987).

As principais espécies bacterianas proteolíticas e celulolíticas, responsáveis pela degradação dos alimentos, estão presentes no rúmen de bezerros logo após o nascimento (Guzman et al., 2015) e, as atividades fermentativas e enzimáticas microbianas foram rapidamente estabelecidas no rúmen dos bezerros a partir do segundo dia de vida (Rey, Enjalbert e Monteils, 2012). Em um estudo paralelo, a expressão gênica de várias famílias de proteínas relacionadas à degradação de carboidratos foi identificada no rúmen de bezerras aos 14 dias de idade (Li et al., 2012), sugerindo que a microbiota ruminal dos bezerros possui potencial metabólico suficiente e não deve ser considerada rudimentar (Li et al., 2012).

Em comparação com animais mais velhos, a abundância do filo Bacteroidetes foi significativamente menor em bezerros de um dia de idade e foi composta principalmente pelo gênero *Bacteroides*, enquanto os animais mais velhos eram colonizados principalmente por *Prevotella* (Jami et al., 2013). A presença de bactérias celulolíticas e *archaeas* metanogênicas foi observada em cordeiros com 3 a 4 dias de idade, e a população dessas bactérias atingiu um nível semelhante ao observado em ovelhas adultas com sete dias de idade (Fonty et al., 1987). Assim, o estabelecimento de bactérias ruminais ocorre muito antes dos ruminantes jovens terem acesso à dieta sólida.

Dill-McFarland, Breaker e Suen. (2017) indicaram que em bezerros amostrados poucos dias após o desaleitamento, a comunidade bacteriana ruminal foi mais diversa

em comparação com bezerros amostrados durante o aleitamento. Várias unidades taxonômicas operacionais (OTUs) de fungos observadas em bezerros desmamados também estão presentes em adultos. Como os fungos colonizam principalmente sólidos fibrosos, isso pode sugerir que a introdução de forragem permite que fungos anteriormente pouco abundantes ou transitórios persistam e se multipliquem.

Adicionalmente, observou-se que a comunidade bacteriana ruminal muda à medida que os bezerros se desenvolvem (Meale et al., 2016; Dill-McFarland, Breaker e Suen, 2017). No entanto, estudar o impacto da idade do hospedeiro por si só, é difícil em ruminantes, especialmente nos pré-ruminantes, devido ao efeito colateral da dieta. Os pré-ruminantes, especialmente os bezerros leiteiros, passam de uma dieta à base de leite a uma dieta sólida dentro de um curto período de tempo para facilitar o desenvolvimento ruminal; e os estudos acima citados, sobre o impacto da idade dos bezerros na microbiota ruminal, utilizaram animais submetidos a esses regimes alimentares. Portanto, essas mudanças na composição microbiana que seriam devidas a idade e desenvolvimento são, na verdade, devidas em parte, às mudanças na dieta dos bezerros.Além disso, a janela de tempo mais eficaz para intervenções nutricionais sobre os microrganismos permanece desconhecida, necessitando mais estudos para determiná-la (Yáñez-Ruiz, Abecia e Newbold, 2015). Por fim, observou-se que a comunidade bacteriana varia acentuadamente entre as regiões do TGI (rúmen x cólon) e de acordo com o tipo de amostra utilizada para determinação (mucosa x ingesta) dentro de cada região do TGI (Guzman et al., 2016).

A composição da comunidade bacteriana ruminal variou significativamente entre bezerros individuais, sugerindo uma forte especificidade hospedeiro-microbiota (Jami et al., 2013). Da mesma forma, as comunidades de *archaeas* e fungos no rúmen variaram consideravelmente entre os indivíduos (Dill-McFarland, Breaker e Suen, 2017), indicando que a composição da comunidade microbiana ruminal está associada à condição fisiológica do hospedeiro (Jami et al., 2013).

Quando a composição bacteriana ao longo do TGI de bezerros é explorada, o rúmen e o intestino grosso são habitados principalmente pelos filos Bacteroidetes e Firmicutes, sendo os Bacteroidetes em maior quantidade. Estes dois filos guardam certo equilíbrio, enquanto no intestino delgado, mais de 95% das bactérias são Firmicutes (Malmuthuge, Griebel e Guan, 2014). A comunidade bacteriana associada à mucosa do intestino delgado foi composta, principalmente, de Bacteroidetes, Firmicutes e Proteobacteria, representadas por 17 gêneros que são exclusivos desta

região do TGI (Malmuthuge, Griebel e Guan, 2014). Devido à presença de bactérias que unicamente habitam o intestino delgado, sugere-se que os estudos baseados em amostras fecais não revelam o verdadeiro microbioma do TGI. Um estudo comparando as populações bacterianas associadas ao conteúdo ruminal e ao tecido ruminal em bezerros de três semanas de idade revelou que filotipos bacterianos pertencentes a Bacteroidetes seguido de β-Proteobactérias dominava a comunidade aderida ao tecido, já a comunidade aderida ao conteúdo era dominado por filotipos Bacterioidetes seguido de Firmicutes (Malmuthuge, Griebel e Guan, 2014). Desta forma, amostras de líquido ruminal não descrevem adequadamente a diversidade de comunidades bacterianas associadas à mucosa.

A maioria dos estudos baseados em sequenciamento tem se concentrado nas comunidades bacterianas (Meale et al., 2016), enquanto a diversidade de *archaea* metanogênicas, fungos anaeróbios e protozoários no TGI de bezerros permanece pouco caracterizada. As informações atuais relacionadas à comunidade de *archaeas* e fungos no TGI de bezerros, permanecem limitadas a poucos trabalhos (Dill-McFarland, Breaker e Suen, 2017), sendo que nenhum destes estudos objetivou promover alterações nestas classes de microrganismos por meio de suplementação dietética com fitoquímicos.

Fonty et al. (1987) relataram que o estabelecimento ruminal de outras classes de microrganismos somente ocorre depois que as bactérias estejam estabelecidas, sendo que, os fungos e *archaeas* metanogênicas iniciam a colonização entre 8 a 10 dias após o parto, enquanto os protozoários surgem somente 15 dias após o parto. Contudo, um estudo recente identificou espécies de *archaea* (isto é, *Methanobrevibacter mobile* e *Methanobrevibacter votae*) no rúmen de bezerros apenas vinte minutos após o nascimento, sugerindo que a colonização de *archaeas* metanogênicas ocorre antes do parto e levanta questões sobre a afirmação de que o TGI dos bezerros é estéril no momento do nascimento (Guzman et al., 2015).

A metanogênese é um processo inerente ao metabolismo energético de espécies *archaeas*, que por sua vez, contribui para a manutenção de uma baixa pressão parcial de hidrogênio (H_2) no rúmen, o que é indispensável para o funcionamento de enzimas microbianas (Janssen e Kirs, 2008). Por outro lado, a metanogênese afeta negativamente o meio ambiente e a eficiência de utilização de energia da dieta. Assim, a análise abrangente de comunidades que utilizam H_2, como as *archaeas* metanogênicas e as bactérias acetogênicas no TGI dos bezerros, pode

facilitar os esforços na busca de estratégias para diminuir as emissões de metano entérico (Dias, 2017).

Em relação aos fungos anaeróbios, existem apenas dois estudos que caracterizaram a comunidade fúngica em ruminantes jovens. Fonty et al. (1987) identificaram *Neocallimastix frontalis* e *Caecomyces communis* no rúmen de cordeiros aos oito dias de idade, mas essas espécies desapareceram em quase 100% dos cordeiros após o oferecimento de concentrado e feno. Dill-McFarland, Breaker e Suen. (2017) acompanharam as mudanças na microbiota ruminal e fecal de bezerros leiteiros (de 2 semanas até 2 anos de idade) e relataram que os fungos anaeróbios estavam em quantidades abaixo da detecção até o desaleitamento.

Assim como os fungos anaeróbios, os protozoários ciliados têm uma íntima associação com as comunidades de bactérias e *archaeas* do rúmen e podem afetar a digestibilidade dos nutrientes, a fermentação e a metanogênese (Newbold et al., 2015). No entanto, ao contrário de outros grupos microbianos, o estabelecimento de protozoários ciliados no rúmen de animais jovens é dependente do contato direto ou indireto com a saliva de animais adultos (Coleman, 1979).

Em geral, mais pesquisas são necessárias para caracterizar a microbiota e identificar os fatores nutricionais que afetam o estabelecimento, a distribuição e a sobrevivência de comunidades de *archaeas*, bactérias, fungos e protozoários no TGI de bezerros leiteiros. Há necessidade de pesquisas que busquem evidenciar os benefícios da utilização de produtos naturais alternativos aos antibióticos comerciais no controle de problemas sanitários que acometem os bezerros leiteiros no período pré-desaleitamento, como a diarreia, a qual, juntamente com as doenças respiratórias, responde por aproximadamente 80% da morbimortalidade de bezerros leiteiros nesta fase (Fruscalso, Olmos e Hötzel, 2020).

Adicionalmente, outra questão importante a considerar é que as técnicas baseadas em cultivo utilizadas até recentemente limitavam o conhecimento obtido nas análises microbiológicas, sendo recuperada apenas uma pequena fração da diversidade microbiana presente no TGI (Coballo e Sanz, 2007). Com o advento das técnicas moleculares, independentes de cultivo, têm impulsionado os estudos de caracterização de comunidades microbianas como um todo (Uyeno, Sekiguchi e Kamagata, 2010). O sequenciamento do gene que codifica o rRNA 16S (bacteriano), permite que microrganismos presentes nas amostras sejam classificados e que sejam realizadas análises de riqueza das comunidades e determinados outros índices de

diversidade. Com base neste conhecimento mais aprofundado, é possível estabelecer pontos de distinção entre as comunidades de animais frente a diferentes tratamentos e analisar correlações entre grupos específicos de microrganismos e variáveis como ganho de peso, consumo voluntário, redução na produção de metano, eficiência alimentar alterações no sistema imune e no status antioxidante, etc. Por fim, a análise abrangente do microbioma do TGI de ruminantes pode orientar a elaboração de estratégias para promover a colonização de grupos microbianos ligados à saúde e desempenho de bezerros leiteiros, especialmente criados em sistemas desafiadores.

2.2. Desenvolvimento ruminal

O desenvolvimento da microflora, morfológico e metabólico do rúmen são os três processos vitais que devem ocorrer para que um animal pré-ruminantes se torne um ruminante funcional. Até o momento, pouco se sabe sobre como cada um destes processos ocorre e como podem, de forma sinérgica, afetar o crescimento dos bezerros bem como a utilização de nutrientes, dois importantes processos que acarretam a vida produtiva futura.

A transição de pré-ruminante para ruminante funcional está alicerçada na capacidade do rúmen em suportar a fermentação, capacidade esta que é dependente de cinco elementos-chave: estabelecimento da microflora, disponibilidade de substrato, presença de líquido, habilidade absortiva do epitélio ruminal e, taxa de passagem ruminal (Daniels e Yohe, 2015). Entretanto, apesar de haver conhecimento da necessidade desses processos para o desenvolvimento ruminal, ainda não é completamente compreendido como o rúmen sofre as mudanças metabólicas ao nível molecular para suportar a fermentação; como, quando e sob que circunstâncias as classes de microrganismos povoam o rúmen; ou como as populações microbianas do rúmen modificam-se em resposta à dieta.

A superfície luminal do rúmen funcional é revestida por numerosas papilas, as quais consistem em estruturas epiteliais compostas de múltiplas camadas celulares. As principais funções das papilas são aumentar a área de superfície de absorção do rúmen e absorver AGV. As papilas ruminais absorvem os AGV por difusão passiva e facilitada (Aschenbach et al., 2011), assim entram na corrente sanguínea do animal. O acetato e o propionato entram, principalmente, na circulação portal, enquanto cerca

de 85 a 90% do butirato é oxidado formando cetonas antes de entrar na circulação portal. O butirato é principalmente oxidado em β-hidroxibutirato (BHB) e, em menor grau, em acetoacetato. Devido àessa mudança na forma do butirato, o mesmo é comumente visto como um substrato energético para as células epiteliais do rúmen e também está implicado no crescimento das papilas ruminais.

O desenvolvimento morfológico do rúmen refere-se principalmente às características das papilas, à espessura do músculo e ao tamanho do órgão (Van Soest, 1994). O comprimento, a largura e a área das papilas aumentam com a idade e respondem à dieta e ao butirato, como mencionado anteriormente. O fornecimento de dietas altamente fermentáveis (Bull et al., 1965) ou dietas peletizadas (Hinders e Owen, 1965), ou a infusão de butirato (Tamate et al., 1962), ou ainda a modificação do metabolismo ruminal objetivando aumentar a proporção de butirato podem causar alterações morfológicas nas papilas ruminais. O butirato estimula o crescimento das papilas do rúmen através de meios desconhecidos, existindo muitas teorias a esse respeito, entretanto, nenhum mecanismo foi elucidado (Daniels e Yohe, 2015).Por outro lado, uma das teorias que tentam explicar o mecanismo do butirato diz respeito à insulina e o glucagon como prováveis mediadores do crescimento epitelial no rúmen. Quando insulina mais glicose foram injetados na veia jugular externa de ovinos, houve maior proliferação de células epiteliais do rúmen comparado à infusão isolada de glicose. Observou-se estímulo no crescimento das células epiteliais ruminais *in vitro* quando essas foram incubadas em meio contendo $1,6x10^{-9}$M de insulina, independentemente da ação inibitória de ácido butírico presente no meio de cultura. Entretanto, quando as células foram incubadas em meio contendo $3,0x10^{-12}$ M de glucagon, observou-se proliferação celular apenas na ausência de butirato (Gálfi et al., 1991). Mediante esses resultados, infere-se que a insulina seja o mediador da estimulação mitótica causada pelos AGV *in vivo*.

Propionato e butirato parecem ser mais estimuladores do crescimento papilar em bezerros do que acetato. Considerando-se que o mecanismo de ação se dê por efeito indireto sobre a secreção de insulina (Gálfi et al., 1991), butirato seria o maior estimulador do crescimento papilar, já que em ovinos, a infusão sanguínea de menor quantidade de butirato foi requerida para desencadear a mesma resposta em insulina plasmática que propionato (Sano et al., 1995).

O desenvolvimento metabólico do rúmen concentra-se na capacidade de células epiteliais do rúmen produzirem cetonas a partir de produtos finais da

fermentação absorvidos (AGVs). Dados existentes sugerem que a entrada de AGVs nas células epiteliais ruminais pode ocorrer via transporte facilitado e difusão passiva (Aschenbach et al., 2011) e provavelmente depende da camada celular dentro do epitélio do rúmen. A entrada via transporte facilitado requer transportadores de membrana. Sendo que o mais importante transportador de AGV encontrado é o MCT1 (Minuti et al., 2015).

O tecido ruminal dos neonatos é incapaz de oxidar o butirato (o principal substrato cetogênico para células epiteliais ruminais) nas cetonas, BHB ou acetoacetato, logo após o nascimento (Daniels e Yohe, 2015). Como resultado, as concentrações sanguíneas desses metabólitos são baixas. As duas enzimas relacionadas com a cetogênese nas papilas ruminais são a HMG-CoA sintase e AcAc-CoA tiolase (Leighton, Nicholas e Pogson, 1983). Lane, Baldwin e Jesse. (2002) demostraram que a abundância de mRNA de 3-hidroxi-3-metilglutaril CoA sintase (HMG-CoA sintase) aumentou em paralelo com a cetogênese ruminal antes de 49 dias de idade em cordeiros e sugeriu que a HMG-CoA sintase é a enzima limitante da taxa de cetogênese ruminal. Duas isoformas da HMG-CoA sintase são conhecidas: HMGCS1, que é citoplasmática, e HMGCS2, que é mitocondrial. Naeem et al. (2012) sugeriram que a cetogênese intramitocondrial é a principal via para a geração de BHB nas células epiteliais do rúmen de bezerros jovens. Na ausência da cetogênese ruminal, a teoria que está sendo desenvolvida é que o butirato formado no rúmen, mesmo em pequenas quantidades, parece estimular a transcrição de gênica das enzimas relacionadas ao desenvolvimento das papilas (isto é, as isoformas da HMC-CoA sintase), que em última análise, regulam a maturação metabólica do rúmen.

Com relação ao papel do butirato no desenvolvimento intestinal, se tem conhecimento que, de forma similar aos animais monogástricos, o butirato é produzido na parte distal do intestino delgado e no intestino grosso em ruminantes (Bergman, 1990). Sua concentração na digesta do intestino grosso pode ser semelhante à encontrada no líquido ruminal, em ruminantes adultos (Li et al., 2012). Embora a importância do butirato para as funções do intestino grosso em ruminantes não tenha sido extensivamente investigada, acredita-se que seja similar àquela conhecida para os monogástricos (Górka et al., 2018), como por exemplo, efeito positivo no desempenho do crescimento, desenvolvimento do epitélio intestinal e efeitos antimicrobianos em aves (Leeson et al., 2005). Pesquisas têm demonstrado que o butirato também pode servir como fonte de energia para os enterócitos (Mahdavi e

Torki, 2009), o que pode levar ao aumento no crescimento das vilosidades e a área de superfície geral de absorção no intestino (Guilloteau et al., 2010).

2.3. Potencial do uso de extrato de orégano (*Origanum vulgare*) na alimentação de bezerros

2.3.1. Óleos essenciais

Os óleos essenciais (OE) são um grupo diversificado de metabólitos secundários vegetais que contêm componentes voláteis responsáveis pelo olfato e o sabor das plantas (Calsamiglia, et al. 2007). Na natureza, desempenham papel importante na comunicação alelopática entre plantas, na atração de insetos polinizadores, redução na herbivoria e, atuam como antibacterianos, antivirais, antifúngicos, inseticidas ou herbicidas (Miguel, 2010). Os OE são classificados em dois grupos químicos específicos, os terpenóides (mais comuns) e os fenilpropanóides, derivados de diferentes precursores metabólicos, contribuindo com mais de 15.000 componentes únicos (Miguel, 2010).

Recentes descobertas têm demonstrado que os OE podem ser uma alternativa aos antibióticos convencionais, uma vez que apresentam potencial de melhoria da eficiência alimentar, na utilização de nutrientes e na saúde animal. No entanto, neste momento ainda há uma necessidade de mais pesquisas para fornecer informações para recomendações práticas de sua utilização dietética. Adicionalmente, os resultados dos estudos mostram que a eficácia destes compostos varia dependendo da parte da planta utilizada, da época do ano em que foi cultivada e da estrutura química do OE (Calsamiglia, et al. 2007). Os resultados de pesquisas também nos levam a creditar às diferentes dietas, dosagens e manejo animal pelas inconsistências dos resultados de estudos com óleos essenciais.

2.3.2. Modos de ação dos óleos essenciais

A natureza hidrofóbica dos óleos essenciais contribui para suas propriedades antimicrobianas, as quais permitem a alteração do metabolismo e crescimento bacteriano, causados não por um modo específico, mas por várias áreas-alvo na célula bacteriana (Benchaar et al., 2008). Os mecanismos de ação antimicrobianos variam dependendo do tipo de OE e da cepa do microrganismo (Chouhan, Sharma e

Guleria, 2017). Os OE têm alta afinidade pelos componentes lipídicos nas membranas celulares bacterianas afetando e até interrompendo os processos da membrana celular,isto é, transporte de elétrons, gradientes iônicos, translocação de proteínas, fosforilação e outras reações dependentes de enzimas (Dorman e Deans, 2000). Estudos demonstraram que a ação dos OE sobre a integridade da membrana celular leva à alteração na permeabilidade da membrana, acarretando na perda de conteúdos intracelulares vitais, como proteínas, açúcares redutores, ATP e DNA, enquanto que inibe a geração de energia (ATP) e enzimas relacionadas à destruição de células e vazamento de eletrólitos (Lakehal et al., 2016). A atividade antimicrobiana dos OE é, portanto, atribuída a uma cascata de reações envolvendo toda a célula bacteriana (Macwan et al., 2016).

Uma das reações envolve alterações conformacionais na estrutura da membrana celular bacteriana, causando perda da estabilidade da membrana. Hipoteticamente, isso ocorre quando os OE se acumulam na camada lipídica da membrana, resultando em fluidificação e expansão da membrana, fazendo com que a membrana vaze e, desta forma, diminuindo o gradiente iônico transmembrana (Griffen et al., 1999). A perda de estabilidade da membrana afeta uma ampla variedade de microrganismos, incluindo bactérias gram-positivas e gram-negativas, e é bem documentado que, em comparação com bactérias gram-negativas, as gram-positivas são mais susceptíveis aos OE (Huang et al., 2014; Azhdarzadeh e Hajjati, 2016). Isso se deve muito provavelmente porque a parede celular externa é hidrofílica, impedindo assim que os OE lipofílicos atravessem esta barreira (Benchaar et al., 2008). Ou então pode ser atribuído ao fato de as bactérias gram-negativas possuírem uma membrana externa complexa, rica em lipopolissacarídeos e mais complexa, limitando a difusão de compostos hidrofóbicos através dela, enquanto que a membrana das gram-positivas é menos complexa, sendo circundada por uma camada de peptidoglicano não suficientemente densa para resistir às pequenas moléculas antimicrobianas, facilitando o acesso à parede celular (Zinaviadou, Koutsomanis e Biliaderis, 2009). Essa interação dos OE resulta na perda de estabilidade da membrana, entretanto, tipicamente não resultam em morte celular bacteriana, mas acarreta o retardo do crescimento bacteriano, o que também causa impacto no perfil de fermentação ruminal (Griffen, et al. 1999). Entretanto, relatos têm demonstrado que os compostos bioativos do OE podem se ligar à superfície da célula e, posteriormente, penetrar a bicamada fosfolipídica da membrana celular (Chouhan, Sharma e Guleria,

2017), e isso leva à perturbação da integridade estrutural da membrana, devido ao acúmulo dos compostos bioativos, influenciando no metabolismo celular, causando a morte da célula (Bajpai, Sharma e Baek, 2013). Em última análise, os óleos essenciais podem apresentar efeitos bactericidas e bacteriostáticos.

2.3.3. Orégano

O orégano (*Origanum vulgare*) é uma fonte de OE bem documentada, tendo já demonstrado ser capaz de romper o gradiente iônico da membrana celular bacteriana, inibindo tanto bactérias gram-positivas quanto gram-negativas (Dorman e Deans 2000). Os compostos ativos majoritários do óleo de orégano são o carvacrol e o timol, mas também são encontrados o *p*-cimeno e Y-terpineno, sendo que os dois últimos são classificados como terpenos. Várias unidades de isopreno (C_5H_8), após serem combinadas, resultam na produção de hidrocarbonetos chamados terpenos (Chouhan, Sharma e Guleria, 2017). Por meio de modificações bioquímicas dos terpenos através de enzimas que adicionam moléculas de oxigênio e movem ou removem grupos metila, resulta na formação dos terpenóides (Caballero, 2003). Tem-se sugerindo que compostos fenólicos, tais como carvacrol e o timol, apresentam atividade antimicrobiana efetiva, devido aos grupos hidroxila na estrutura fenólica (Benchaar et al., 2008). Helander et al., 1998) demonstraram o rompimento da força próton-motriz e dissipação dos gradientes iônicos dos íons H^+ e K^+, afetando os processos intracelulares ATP-dependentes. Outro mecanismo proposto por estes autores é semelhante ao dos antibióticos ionóforos, envolvendo o grupo hidroxila, atuando como um portador de prótons transmembrana (Benchaar et al., 2011).

Estudos têm demonstrado que os óleos essenciais são altamente eficazes contra microrganismos específicos (Dorman e Deans, 2000; Benchaar et al., 2008). Helander et al. (1998) relataram que o orégano inibiu bactérias gram-negativas, tais como a *Escherichia coli* e a *Salmonella typhimurium*. Marino, Bersani e Comi. (2001), corroboram os achados do estudo anterior e, adicionalmente, relataram que o orégano não apenas inibiu as bactérias gram-negativas (*Escherichi coli* e *Salmonella typhimurium*), mas também as bactérias *Proteus mirabilis*, *Proteus vulgaris*, *Serratia marcescens*, *Yersinia enterocolitica*, *Pseudomonas fluorescens* e *Pseudomonas putida*. Foram observados efeitos inibitórios contra bactérias gram-positivas, tais como *Micrococcus sp*., *Sarcina flava*, *Streptococcus aureus*, *Bacillus licheniformis*, *Bacillus thuringiensis* e *Listeria innocua*. Em um estudo *in vivo*, Bampidis et al. (2006),

utilizando bezerros desafiados com *Escherichia coli*, relataram que a suplementação com folhas de orégano secas (10 mg/kg de peso vivo - PV) diluídas em uma solução líquida, foi eficaz na redução das estirpes de *E. coli*.

Os efeitos do OE são dependentes da dose. Em estudo *in vitro*, Busquet et al. (2006) testaram a inclusão de diferentes doses de óleo de orégano (padronizado em 69% de carvacrol), isto é, 3,0 mg, 30 mg, 300 mg e 3 g/L de meio de incubação, relataram que a alta dosagem (3,0 g/L) resultou na diminuição da concentração total de AGV, da concentração de N-NH_3 e uma elevação no pH ruminal. Entretanto, doses menores (300 mg) podem ser administradas com segurança sem inibir a fermentação ruminal (Busquet, et al. 2006). Esses resultados são reforçados por Castillejos et al. (2007), onde indicaram que uma alta dose (500 mg/L) de OE levou à redução das concentrações de AGV, de N-NH_3, elevando o pH ruminal e as relações acetato:propionato. Já, dosagens menores (5,0 e 50 mg/L) não apresentaram efeito sobre o N-NH_3, o pH ruminal nem sobre relação acetato:propionato, mas levou ao aumento na concentração de AGV (Castillejos et al., 2007).

Quando testados, os componentes puros do orégano, como o timol, também produzem resultados semelhantes em termos de fermentação ruminal (Calsamiglia et al., 2007). Em doses mais altas, o timol pode afetar a digestão total de nutrientes, diminuir a produção de ácidos graxos voláteis, diminuir as concentrações de N-NH_3 e inibir o metabolismo microbiano ruminal. Tem sido sugerido que uma dose ideal de timol varia aproximadamente entre 50 e 500 mg/L (Calsamiglia et al., 2007).

O carvacrol tem sido sugerido como inibidor da proteólise. Um estudo *in vitro* usando doses mais altas de carvacrol (300 mg/L) diminuiu as proporções de acetato para propionato, a produção de AGV e elevou o pH e o butirato (Calsamiglia et al., 2007). A dosagem ideal pode depender do tipo de dieta alimentada ao animal, mas o efeito antimicrobiano do timol dependeu pH, ou seja, quanto menor o pH (6,5 x 5,5), mais eficaz foi o efeito. As estruturas químicas do timol e do carvacrol são diferentes e essas variações nas estruturas químicas podem afetar os resultados (Calsamiglia et al., 2007).

Fenóis terpenóides, como o carvacrol, provaram efeitos inibitórios sobre a produção de toxina diarreica por *Bacillus cereus* (Chouhan, Sharma e Guleria, 2017). O precursor do carvacrol é o *p*-cimeno, que é um monoterpeno com um anel de benzeno, sem quaisquer grupos funcionais nas suas cadeias laterais. Quando usado isoladamente, o *p*-cimeno não apresenta função antimicrobiana eficiente

(Bagamboula, Uyttendaele e Debevere, 2004), mas a atividade de compostos como o carvacrol é potencializada pelo *p*-cimeno (Rattanachaikunsopon e Phumkhachorn, 2010). Além disso, *p*-cimeno mostrou efeito negativo sobre a síntese de proteínas em células de *E. coli* (Chouhan, Sharma e Guleria, 2017). Esses resultados mostram que o extrato de orégano tem potencial de beneficiar a saúde intestinal de bezerros pré-desaleitados.

2.3.4. O uso de óleos essenciais na alimentação de bezerros

Os resultados publicados são variáveis, porém demonstram muitos benefícios promissores. No entanto, mais pesquisas precisam ser feitas sobre a dosagem ideal e as formas de suplementação de óleos essenciais. Hill et al. (2007) relataram a melhoria nos ganhos médios diários, no consumo de ração *starter* e na eficiência alimentar de bezerros pré-desaleitados, alimentados com uma mistura comercial de OE. Mas os resultados são controversos, por exemplo, Santos et al. (2015), em um estudo onde foi suplementada uma mistura comercial de OE (carvacrol, cineol, cinamaldeído e resina de óleo de pimenta) na dose de 400 mg/kg de MS (misturado ao sucedâneo lácteo ou metade ao sucedâneo e metade na ração *starter*) não relataram aumento no desempenho nem nos parâmetros imunológicos testados.

No estudo de Katsoulos et al. (2017), foi testado o efeito da suplementação de OE de orégano (12,5 mg/kg de PV) sobre o escore fecal e incidência de diarreia em bezerros até os 10 dias de vida. Os escores fecais médios ao longo do experimento, a incidência de diarreia, a duração e a severidade dos episódios de diarreia foram significativamente menores nos bezerros suplementados com OE em comparação aos bezerros controle. Os autores concluíram que a suplementação com OE de orégano possui um efeito preventivo contra a síndrome diarreica neonatal.

Froehlich et al. (2017), testando diferentes doses (1,25, 2,5 e 3,75 g de OE por alimentação – duas alimentações diárias com 270 g de ração cada) de uma mistura comercial de OE (isto é, carvacrol, cariofileno, *p*-cimeno, cineole, terpimeno e timol) em bezerros, observaram que os bezerros que consumiram a dose de 1,25 g apresentaram maior ganho de peso diário e, consequentemente, maior peso corporal, quando comparados às demais doses de OE e ao controle negativo. Neste estudo, os autores também observaram que a dose de 1,25 g elevou as quantidades de IgA e IgG, o que corresponde ao melhor desempenho destes bezerros e, que os escores fecais foram melhorados com a suplementação de OE. Entretanto, as concentrações

de AGV no sangue não foram modificadas em nenhuma das doses, sugerindo que os OE não inibiram nem melhoraram o desenvolvimento ruminal (Froehlich et al., 2017).

Em um estudo Vaikili et al. (2013), utilizando bezerros Holandês após desmame, consumindo uma dieta contento 15% de feno de alfafa e 85% de concentrado, suplementada com óleos essenciais de tomilho (5 g de OE/dia/bezerro) ou canela (5 g de OE/dia/bezerro). Semelhantemente ao estudo de Santos et al. (2015), Vakili et al. (2013) não relataram nenhum efeito no desempenho dos bezerros, nem em relação aos metabólitos sanguíneos (glicose, N-ureico, triglicerídeos, colesterol total, BHB, alanina-aminotransferase e aspartato-aminotransferase). A fermentação ruminal também foi similar entre os tratamentos para pH ruminal, N-NH_3 e AGV. Entretanto, houve uma diminuição nas proporções molares de acetato para propionato e nos níveis de acetato nos bezerros suplementados. A proporção de butirato foi significativamente maior nos bezerros suplementados com OE de canela em comparação com os bezerros controle (Vakili et al., 2013).

Mais recentemente, Kazemi-Bonchenari et al. (2018) suplementaram bezerros Holandês com uma mistura de OE (timol, eugenol, vanilina, lemoneno e guaiacol) na dose de 1 g/kg MS de ração *starter*. Os autores observaram que a suplementação com a mistura de OE melhorou a eficiência alimentar e o ganho de peso diário. Também foram observados aumentos na concentração de AGV, bem como a concentração de butirato e propionato e uma redução no acetato (Kazemi-Bonchenari et al., 2018).

A suplementação da ração *starter* (300 mg/kg de ração) de bezerros Holandês com uma mistura de OE (*Rosmarinus officinalis* L., *Zataria multiflora* Boiss e *Mentha pulegium* L.) elevaram o consumo de ração, o ganho médio diário e apresentaram peso mais elevado ao momento do desmame se comparados aos controle-negativos (Jeshari et al., 2016).

Como se pode observar há certa inconsistência em relação aos resultados dos estudos quando a dieta de bezerros é suplementada com OE. Observa-se também que as doses utilizadas não obedecem a um padrão entre os estudos e também a forma de suplementar varia entre os estudos (misturado ao sucedâneo ou à ração *starter*, ou a ambos) e também uma grande variedade de extratos vegetais são usados como fontes de OE nestes estudos. Isto tudo pode ser responsável pela inconsistência observada.

3. HIPÓTESES E OBJETIVOS

3.1. Hipóteses

- O extrato de orégano altera a microbiota ruminal e intestinal em bezerros leiteiros no período pré-desaleitamento, com efeito, principalmente sobre as bactérias gram-positivas;
- A suplementação de extrato de orégano aos bezerros leiteiros no período pré-desaleitamento, reduz cepas bacterianas patogênicas intestinais, isto é, *E. coli*, *Salmonella* e *Clostridium*, melhorando assim a saúde intestinal;
- A suplementação com extrato de orégano modifica a morfologia e a histologia, e melhora o desenvolvimento (elevação do número, comprimento e largura)

das papilas ruminais e vilosidades intestinais de bezerros leiteiros no período pré-desaleitamento;

- O extrato de orégano melhora o desempenho (eficiência alimentar, ganho médio diário e digestibilidade aparente) dos bezerros no período pré-desaleitamento.

3.2. Objetivos

- Caracterizar as comunidades de bactérias no rúmen e nos segmentos do TGI (isto é, jejuno, ceco e cólon) de bezerros leiteiros, suplementados ou não, com extrato de orégano durante o período pré-desaleitamento;
- Avaliar cepas bacterianas patológicas relacionadas à diarreia, no intestino de bezerros leiteiros no período pré-desaleitamento, suplementados ou não com extrato de orégano;
- Avaliar modificações morfométricas e histológicas das papilas ruminais e vilosidades intestinais de bezerros leiteiros no período pré-desaleitamento, consumindo dieta suplementada ou não com extrato de orégano.
- Medir indicadores de desempenho dos bezerros suplementados ou não com extrato de orégano.

CAPÍTULO II

Article 1: Oregano extract fed to pre-weaned dairy calves alters rumen and intestinal bacteria microbiota, starter intake and apparent digestibility

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Oregano extract fed to pre-weaned dairy calves alters rumen and intestinal bacteria microbiota, and apparent digestibility

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Running title: Oregano extract affects rumen and intestine microbiota in dairy calves

Abstract

Essential oils have become attractive candidates for use in the livestock industry, yet their mode of action over microbial populations of animals remains largely unknown. To gain further insight, this study investigated the bacterial population in

segments of gastrointestinal tract and performance of pre-weaned dairy calves supplemented with a commercial Oregano extract containing essential oils. Ten Holstein calves were fed 6 L/day of milk replacer, and had free access to water and calf starter from the first day, and to hay from the third week of the start of the study period. The calves were randomly assigned to two treatments: CON - without additive; and OR - 60 mg/kg body weight per day with oregano extract. Weaning occurred on the 53rd day of the experimental period and on the 54th day, all calves were euthanized, and their rumen, jejunum, cecum and colon contents were sampled to determinate bacterial population. The OR supplementation increased starter concentrate intake, but did not affect feed conversion, average daily gain (ADG), total VFA acetate, butyrate, and propionate ruminal concentrations, as well as C2 to C3 ratio. Apparent digestibility increased in OR for DM and CP, and showed a trend of increasing for OM e NFCcp. The alpha diversity analysis (Shannon index) indicated a higher diversity of rumen and jejunum bacterial population in OR calves. Oregano extract affected positively and negative the abundance of both positive and gram-negative bacteria in the gastrointestinal tract. Moreover, OR decreased potentially pathogenic genera such *Streptococcus*, *Escherichia* and *Clostridium*, albeit it also decreased the population of the potentially beneficial *Bifidubacterium* in the jejunum.OR supplemented to dairy calves also improved concentrate intake and apparent digestibility, but it did not change feed efficiency or ADG. This study indicates that oregano extract supplemented to pre-weaned dairy calves modulate bacteria microbiota and may contribute to reduce pathogenic bacteria in the gastrointestinal tract with slight positive effects on their performance.

KEYWORDS: dairy calf, essential oils, growth performance, intestine bacteria, oregano, rumen bacteria

INTRODUCTION

Antibiotics are used in dairy farms to improve immunity, reduce stress and susceptibility to pathogens, improving rumen development and animal performance, while decreasing calf mortality risk due to neonatal diarrhea incidence (Poudel *et al*., 2019). However, with the limitations in the prophylactic use of antimicrobials, apart from the increasing concern over the presence of contaminants in animal products, combined with bacterial resistance to conventional antibiotics, animal production is being directed towards the prohibition of ionophores and antibiotics in livestock (Tang *et al*., 2017).

A viable alternative to chemicals is needed to promote animal welfare and optimize livestock production, while ensuring no risks to human health and the environment (Cheng *et al*., 2014). Plants or plant extracts containing flavonoids, tannins, saponins, essential oils and many other secondary plant compounds were explored as possible alternatives capable of bringing the above-mentioned benefits for livestock (Patra & Saxena, 2010). Plant extracts are among the most promising alternatives to antibiotics due to their extensive biological effects, and can be used in calf feed to prevent diarrhea. Moreover, effects of plant extracts on the colonization of microbial populations remains to be determined in calves (Diao et al., 2019).

Essential oils vary in chemical structure and biological effects. Terpenoids and phenylpropanoids represent the most common types of essential oils (Patra and Saxena, 2010). Studies conducted in ruminants show its potential to modulate ruminal fermentation (Calsamiglia et al., 2007), control pathogenic microorganisms (Bampidis et al., 2006; Ozkaya et al., 2018) and improve animal performance (Hill et al., 2007). Other studies have reported decreased methane production in dairy cows (Kolling et al., 2018), increased average daily gain (ADG - Hill et al., 2007; Froehlich et al., 2017;

Kazemi-Bonchenari et al., 2018), increased feed intake (FI) and improved feed efficiency (Hill et al., 2007; Kazemi-Bonchenari et al., 2018).

Oregano extracts or leaves contain essential oils, such as carvacrol and thymol, and oregano extracts supplementation has been studied with dairy cows (Kolling et al., 2018; Stivanin et al., 2019; Benchaar, 2020; Vizzotto et al., 2020) and dairy calves (de Paris et al., 2020; Heisler et al.,2020), showing positive effects on feeding behavior (Stivanin et al., 2019; Heisler et al., 2020), reducing methane emission (Kolling et al., 2018) and improving redox status of calves (de Paris et al., 2020) and cows (Vizzoto et al., 2020). However, there are controversial results for body weight gain, feed efficiency and rumen short-chain volatile fatty acids. For instance, some studies also showed that oregano or its essential oils did not improve weight gain (Kolling et al., 2016; de Paris et al., 2020) and milk production and composition (Kolling et al., 2018; Vizzoto et al., 2020, Benchaar, 2020). On the contrary, a blend of essential oils containing thymol and carvacrol increased average daily gain (Froehlich *et al*., 2017; Kazemi-Bonchenari *et al*., 2018), feed intake and improved feed efficiency (Kazemi-Bonchenari *et al*., 2018).

These responses may result from changes in the composition of microbiota at the gastrointestinal tract caused by the antimicrobial activities of essential oils. Bacterial microbiota abundance in the rumen of dairy calves present marked differences from birth to maturity, e.g. 24 months of age (Jami et al., 2013) and might change in response to diet manipulation during the early stages of growth (Poudel et al., 2019). Thus, the early modulation of the microbiota may provide long-term benefits for adult health and performance (Malmuthuge & Guan, 2017).Jonova et al. (2021) and Arne and Ilgaza (2021)found that the supplementation of the prebiotic and probiotic to Holstein calves positively impacted the development of almost all morphological

structures of the dorsal saccus and saccus ventralis of the rumen and intestine. However, the impact of essential oils supplementation on young ruminant's microbiota remainspoorly known. The objectives of this study were to evaluate the use of a commercial product composed of oregano extract (Oreganol®) on gastrointestinal tract bacterial population and on performance of pre-weaned dairy calves.

MATERIALS AND METHODS

Experimental design, animals, and treatment diets

Ten male Holstein calves aged approximately four days were distributed in a completely randomized design with 2 treatments and 5 replicates (calves) per treatment. The calves were sourced from dairy farms located within 15 km of the site of the experiment. The average weight of the calves was 37.1 kg (± 3.6 kg standard deviation). They received colostrum (10% of birth weight - BW) in the 1st day and transition milk 2nd and 3rd days of life. The calves were randomly assigned to two treatments: control without feed additive (CON); and with addition of 60 mg/kg body weight of oregano extract (OR; Oreganol®, Asteri Veterinary Medicines Industry Ltd., São Paulo, Brazil), composed mainly by carvacrol 80% and thymol 2%. The oregano extract was top-dressed on the concentrate starter fed every morning. The milk replacer (MR - Bovimix Vitamilk® 19.0% CP, 15.0% EE of dry matter; Vitamix Nutrição Animal Ltd, Nova Itaberaba, Santa Catarina, Brazil – 125 g/L) was divided into two daily meals fed at 08:00 and 18:00 h totaling 6 L/day, except during the last 5 days before weaning, when calves received 2 L/day, aiming to increase the solid feed intake. Calves had free access to drinking water, starter concentrate (ground corn grain – 40.5%, soybean meal – 33.3%, wheat meal – 26.0% and Premix vitamin+mineral –

0.2%) and coast-cross grass hay (*Cynodon* dactylon (L.) Pers). Animals were kept in individual pens of 1.50 m × 1.00 m) with wood shaving bedding. The feed composition (*Supplementary Table S1*) was formulated according to the National Research Council (2001) guidelines.

Measurements and sample collection

Body weights (BW) were recorded on days 1, 15, 30, 45 and 53 of the experiment, before the morning feeding, so adjustments of dietary supplements were made accordingly. Every morning (07:00 h), leftovers were weighed to obtain the daily intake of starter and hay. During the last five days of the experiment, fecal samples were collected directly from the rectal ampoule, weighed and identified to determine the apparent digestibility of dry matter (DM) and nutrients. Fecal production was estimated using titanium dioxide (TiO_2 – 0.5 g/day) during the last nine days of the study at the moment of morning feeding, as an external fecal marker (Ohmori *et al*., 2013). On day 53 of the experiment calves were weaned and on day 54 they were euthanized with acepromazine (0.013 mg/kg BW), thiopental (0.125 mg/kg BW) and potassium chloride (80-120 mL). The abdominal cavity was opened, and the rumen was isolated from the other parts of the gastrointestinal tract (GIT) with zip lock to prevent reflux of digesta. Samples of approximately 100 mL of rumen content (50 mL for microbial DNA analysis and 50 mL VFA analysis), were then taken and stored at -20°C until DNA extraction and VFA analysis.

Laboratory measurements

Chemical analyzes were conducted on each diet and fecal sample in duplicate and values are presented as dry matter (DM) basis. DM, crude protein (CP), ash, and ether extract (EE) were analyzed according to methods described by AOAC(2005). For further details, please refer to *Supplementary material*.

The concentration of VFA (e.g. acetic, propionic and butyric acids), were determined in the ruminal liquid samples by gas chromatography (GC-FID; Varian Star 3400CX, Chrompack, Middelburg) and an auto sampler system was used for VFA determinations. The analytical calibration parameters are shown in *Supplementary Table S2*.The results were expressed in mmol of each VFA per 100 mL of rumen fluid.

Total DNA extraction

Samples of rumen content (solid and liquid phase), jejunum, cecum and colon were collected in 50 ml falcon tubes and immediately frozen at -20°C, then sent to the laboratory. The samples were thawed and homogenized and ~200 mg of each sample was used for DNA extraction from the microbial genome using the E.Z.N.A.®Stool DNA Kit (Omega), according to the manufacturer's instructions. DNA concentrations were measured by Fluorometer Qubit® 3.0. From the extraction phase to the DNA purification and quantification phases were performed for each of the 5 animals in the treatment. For the analysis of the bacterial community of the rumen and the 3 sections of the intestine (jejunum, colon and cecum), equal amounts of DNA were combined and 3 samples grouped per treatment (5 animals per group) of isolated DNA were created before DNA amplification.

PCR-amplification and sequencing

After amplification of the DNA strands, for each pool of DNA from the 3 pools of each section (rumen, jejunum, cecum and colon), the V4 region of the bacterial 16S rRNA gene was amplified using F515 (5´CGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGA3´) and R806-(5´GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCA3´) primers to characterize the rumen bacterial composition, both modified to contain an Illumina adapter region as described by Caporaso *et al*.(2010). The amplification was performed in a 25 µL mixture consisting of 12.5 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 µM of each primer, 200 µM dNTP, 2 U Platinum *Taq* High Fidelity DNA polymerase (Life Technologies), and 1 × reaction buffer. An automated thermal cycler (BioRad, Hemel Hempstead, UK) was used for PCR amplification which was programmed for an initial denaturation of 94°C for 2 min, followed by 25 cycles of denaturation (94°C for 45 s), annealing (55°C for 45 s) and extension (72°C for 1 min) and a final extension of (72°C for 6 min) as per manufacturer's instructions. Five microliters of each PCR product was used to verify amplification by gel electrophoresis on a 1% agarose gel.

Amplicons were purified using Agencourt AMPure XP beads following manufacturer instructions. Purified products were again quantified using Qubit® Fluorometric Quantitation Indexes were added to DNA libraries following the manufacturer instructions (Illumina Inc., San Diego, California, USA). Sequencing was conducted on platform Illumina Mi Seq with a v2 500 kit, which generates paired end reads of 250 bp.

Microbial communities' analysis

The algorithm utilized in this analysis was a high-performance implementation of the Ribosomal Database Project (RDP) described in Wang *et al*. (2007), which is utilized in the Base Space™ platform from Illumina. The data base utilized for taxonomic assignment was according to Ali Alishum (2019). DADA2 formatted 16S rRNA gene sequences for both bacteria and *archaea* (Version 2). Diversity analysis was calculated in R studio (R Core Team, 2017), according to the formula below:

$$H = -\sum_{j=1}^{S} pi\ 1n\ pi$$

Where, H = Shannon's diversity index; S = total number of species in the community (richness); p_i = proportion of *S* made up of the *i*th species; and E_H = equitability (evenness).

Statistical analysis

Data of VFA, and bacterial diversity (genus) of rumen and intestine microbiota were analyzed by ANOVA using the General Linear Model (GLM) procedure of SAS (Statistical Analysis System, software package 9.0, SAS Institute Inc., Cary, NC, USA), the level of significance of 5% (*P*<0,05) was considered, and 0,05 >*P*> 0,10 were considered as tendency, using the following statistical model:

$$Yij = \mu + Ti + eij$$

Where Y_i= observation; μ = overall mean of each parameter; T_i= effect of supplement; and e_{ii} = random error used to test supplement.

Data analysis of starter, hay and total DM intakes, BW, feed efficiency and ADG were performed using SAS (Statistical Analysis System, software package 9.0, SAS

Institute Inc., Cary, NC, USA). All data were checked for normality and outliers by using the UNIVARIATE procedure before any statistical analyzes were conducted. All data were subjected to least squares ANOVA for a completely random design with 2 treatments via the MIXED procedure with period (two weeks each) as a repeated measurements. The statistical model used was:

$$Yij = \mu + Ti + Pi + (TP)ij + eij$$

Where Y_{ij} = observation, μ = overall mean of each parameter, T_i =effect of supplement, P_i = effect of period, $(TP)_{ij}$= treatment by period, and e_{ij} = random error. Birth weight was tested as a covariate but did not improve statistical significance (P=0,283) and therefore was removed from the model. Treatment, period, and treatment × period interactions were considered the fixed effects, whereas period (P_j) was considered a repeated measurement. The calves and error were considered as random effect. Initial analyses tested nine covariance structures (first-order autoregressive, ar(1); compound symmetry, CS; compound variance, VC; Heterogeneous compound symmetry, CSH; first-order autoregressive moving-average, ARMA (1,1); no diagonal factor analytic, TOEP; first-order autoregressive, arH(1); Banded main diagonal unstructured, UN(1); and ante-dependence, ANTE(1)) and the model with smallest AICC value was selected for final analysis. Level of significance of 5% (P<0.05) was considered, and 0.05 >P>0.10 were considered as trends.

RESULTS

Comparative analysis of feed intake, digestibility, rumen fermentation and animal growth performance between Control and OR supplemented diets

There was significant interaction between treatments and periods for starter concentrate intake and total DMI (Table 1). In the period from of 1 to 14 d, calves in the control group consumed more the starter (P<0.05) than the OR calves, while in the

last period (43-53 d), we verified the opposite. During the period 1 – 14 d, total DMI was higher in CON compared with OR, while in the other periods total DMI was similar between treatments. Oregano extract did not affect hay intake, however, calves consumed a greater (P<0.05) amount in the period 29 - 42 d compared with the other periods.

There were significant effects of treatment and periods on BW (Table 2), and significant effects of period in Feed conversion, but ADG did not vary between treatments and periods. Considering the whole trial, BW of OR calves were greater (P<0.05) in comparison to the control calves. Feed conversion was reduced as calves aged.

The dietary treatment did not affect the concentration of VFA or the acetate-to-propionate ratio (P>0.05; Table 3). The feed addition of OR improved total tract apparent digestibility of DM, CP and TDN (P<0.05; Table 3) and tended (0.05<P<0.10) to increase that of the OM and NCFap.

Effects of OR on the taxonomic composition of ruminal and intestinal bacteria in pre-weaned calves

A total of 3,089,759 high quality DNA sequences were obtained from the 12 pools of rumen, jejunum, cecum and colon digesta samples, with a mean of 153.63 ± 57.48 sequences/sample retained after filtration for quality and chimera removal for each sample. The average length of sequences used for analysis was 253 base pairs. Taxonomic analysis identified 47 phyla, and Firmicutes was the most numerically abundant for all GIT segments of CON and OR calves. However, the inspection of the abundance in each GIT segment and diet group reveals differences for the secondary most abundant phyla. In the rumen, the second numerically most abundant phyla was

Bacteroidetes, in the jejunum of OR was Cyanobacteria and in CON was Actinobacteria. In the cecum, the second most abundance phyla in OR was Proteobacteria and Bacteroidetes in CON calves, and in the colon, in OR was Bacteroidetes and Cyanobacteria in CON calves. The relative abundances of the top 19 phyla found in this study are shown in Figure 1.

In the rumen digesta, phyla Bacteroidetes, Proteobacteria, Spirochaetas and Euryachaeota were relatively more abundant in OR calves than in control. In the jejunum digesta, plyla protebacteria and Euryachaeota were relatively more abundant in OR calves than in CON, while Actinobacteria phylum was much more abundant in CON than in OR calves.

In the cecum digesta, phyla Proteobacteria and Actinobacteria were relatively more in OR calves than in control, while Bacteroidetes phylum was much more abundant in control than in OR calves. Finally in colon digesta, phylum Bacteroidetes was relatively more abundant in OR calves than in CON, while phyla Proteobacteria and Actinobacteria were relatively more abundant in control than in OR calves.

In the rumen digesta, 11 genera showed high abundance, *Ruminococcus*, *Acetobacterium*, *Turicibacter*, *Prevotella*, *Methanobrevibacter*, *Butyrivibrio*, *Desulfovibrio*, *Streptococcus*, *Escherichia*, *Clostridium* and *Clostridium_XI* (Figure 2). The abundance of the genera *Turicibacter*, *Streptococcus*, *Escherichia*, *Clostridium* and *Clostridium_XI* were higher ($P \leq 0{,}05$) in CON calves, while the abundance of *Ruminococcus*, *Acetobacterium*, *Prevotella*, *Methanobrevibacter*, *Butyrivibrio* and *Desulfovibrio* were higher ($P \leq 0.05$) in OR calves.

In the jejunum digesta, 11 genera showed high abundance specially Bulleidia, *Escherichia*, *Prevotella*, *Roseburia*, *Clostridium*, *Bifidobacterium*, *Streptococcus*, *Methanobrevibacter*, *Ruminococcus*, *Turicibacter* and Romboutsia (Figure 3). The

abundance of genum *Bifidobacterium* was higher in CON calves, while the abundance of genera *Prevotella* and *Methanobrevibacter* were higher in OR calves ($P \leq 0.05$).

On the other side, a smaller number of genera showed expressive abundance in the digesta present in the cecum, Bulleidia, *Roseburia*, *Bifidobacterium*, Ruminococccus, *Streptococcus* and *Clostridium* (Figure 4). Only the genera *Roseburia*and *Streptococcus* differed statistically between treatments ($P \leq 0.05$), with their abundance being reduced in OR calves.

In the colon, 10 genera stood out in abundance: *Lactobacillus*, *Methanobrevibacter*, *Streptococcus*, Campylobacter, *Prevotella*, *Bifidobacterium*, *Ruminococcus*, *Escherichia*, *Clostridium* and *Roseburia*. *Prevotella*, *Roseburia*, *Streptococcus* and *Escherichia* had their abundances reduced ($P \leq 0.05$) in OR animals.

Moreover, oregano extract reduced the abundance of the main bacterial genera causing diarrhea in calves. In the rumen, the genera *Streptococcus*, *Escherichia*, *Clostridium* and *Clostridium_IX* were reduced ($P < 0.05$) in OR calves. In the cecum, the genus *Streptococcus* was reduced, and in the colon, the genera *Streptococcus* and *Escherichia* were reduced ($P < 0.05$) in OR calves.

Microbiota in the rumen and jejunum of the OR calves were more diverse ($P < 0.001$) compared to the CON, but in the other TGI segments (colon and cecum) there were no differences between CON and OR calves (Figure 6).

DISCUSSION

Dietary changes have been reported as an important factor influencing the dynamics of rumen microbial populations and the resulting metabolic changes, leading to significant changes in ruminant production (Ornaghi et al., 2020). Recent questions about residues in products and antibiotic resistance have raised the interest in natural dietary additives with the potential to modulate the performance of ruminants. Many

encouraging results have been observed about the application of different food additives, including organic acids, probiotics, enzymes and phytochemicals (Hassan et al., 2020). One of the main phytochemicals with potential in the nutrition of ruminants are the essential oils, which can affect ruminal development due to their action on populations of microorganisms, changing the ruminal fermentation profile (Santos et al., 2015).

The main contribution of this study was to highlight the positive impact of OR on bacteria microbiota in the gastrointestinal tract, increasing diversity and some potential benefic genera such as *Ruminococcus*, *Butyrivibrio*, *Desulfovibrio*, *Prevotella* (cellulolytic capability) and also reducing potential pathogenic genera such *Streptococcus*, *Clostridium* and *Escherichia*.

The greater diversity (Shannon index) in the bacterial community found in the rumen and jejunum digesta in OR calves (Figure 6) is in agreement with the greater richness and diversity of bacterial communities observed in dairy heifers supplemented with carboxylic acids or polyphenols (De Nardi et al., 2016), or copper and grape pomace (Biscarini et al., 2018). This response can be attributed to the fact that essential oils are not only bactericidal substances, but also bacteriostatic (Griffin et al., 1999). As bacterial growth of a certain taxa is reduced (Griffin et al., 1999), other uninhibited taxa may increase, increasing overall bacterial diversity. These results are corroborated by Patra & Yu (2012), as these authors observed an increase in the Shannon diversity index for bacterial populations using essential oils (clove oil, eucalyptus oil, garlic oil, oregano oil and peppermint oil. pepper) in an *in vitro* study.

At the phylum level, the high abundance of Firmicutes followed by Bacteroidetes in the rumen microbiota in newborn calves and aged of 2, 6 and 24 months was reported by Jami et al. (2013). These authors noticed a lower abundance of Firmicutes

in 2-months calves, with highest prevalence of Bacteroidetes and Proteobacteria, and only further with 24-months calves Firmicutes together with Bacteroidetes were the main phyla. In the present study, Firmicutes phylum presented the highest abundance in the rumen for both OR and CON calves, but apparently OR calves enhanced the abundance of Bacteroidetes and Protebacteria, anticipating changes that would occur later in life (Jami et al., 2013).

At the genus level, in the rumen microbiota, seven of the eleven genera that differed in their abundance between OR and CON calves are within the gram-positive group, such as *Ruminococcus*, *Acetobacterium*, *Turicibacter*, *Methanobrevibacter*, *Streptococcus*, *Clostridium*, and *Clostridium*_IX. Three of these genera were more abundant in OR calves (*Ruminococcus*, *Acetobacterium* and *Methanobrevibacter*), while the others within the gram-negative group, namely *Prevotella*, *Buthyrivibrio*, *Desulfovibrio* and *Escherichia*, were more abundant in OR calves. The greater abundance of these gram-negative genera in calves supplemented with OR may be explained by the greater susceptibility of gram-positive bacteria to essential oils, because they do not have a protective outer membrane – a hydrophilic bilayer (Dorman&Deans, 2000). As a result, lower abundance of some gram-positive genera was observed in OR group, as was also found by Patra and Yu (2015). The fact that not all gram-positive bacteria were affected by OR may be due to the less activity of the major essential oils in OR (carvacrol and thymol) against this type of bacteria (Zengin and Baysal, 2014).

Additionally, low pH of the medium improves the action of essential oils by causing greater permeability of the bacterial membrane (Soltan et al., 2018). The greater feed intake, as it contains greater amounts of quickly fermentable carbohydrates, can lead to a greater production and concentration of VFA, acidifying

the medium. In the present study, no difference was found between the CON and OR groups in the concentration of VFAs in the rumen, however, the supplemented group consumed a greater amount of concentrate (Table 1) in the last experimental period, which in turn, may have led to a better action of oregano essential oils. It is worth to notice that we measured diversity and bacterial abundance in euthanized calves, so probably the effects of higher concentrate intake in the last days of life were observed.

Prevotella was one of the most abundant genus found in the present study (Figure 2), which is in agreement with other studies with young ruminants (Mohammadzadeh et al., 2014; Poudel et al., 2019). The abundance of *Prevotella* was higher in OR animals, in the rumen and in the jejunum, corroborating with Lei et al. (2019), supplementing goats fed with essential oils, (52 mg/animal/day). Zhou et al. (2020) also carried out an *in vitro* study with increasing doses of essential oils of oregano. They noticed augment of the abundance of the *Prevotella* genus as the doses of essential oils were increased. The increase in the abundance observed for *Prevotella* might be associated with better utilization of fibrous and non-fibrous carbohydrates by calves (Wu et al., 2012). These results demonstrate the potential of essential oils as those in OR in modulate fermentation in the rumen and reduce methane emissions (Kolling et al., 2018).

In this study, the genus *Ruminococcus* was one of the most abundant genus (Figure 2), and it showed increased abundance in OR compared with CON calves. This genus is composed of two species highly specialized in fiber degradation (*Ruminococcus*albus and *Ruminococcus*flavefaciens), which can produce large amounts of cellulases and hemicellulases (Doerner and White, 1990), and might be related to the trend (P= 0.076) of improved total tract NDF digestibility, and significant improved total tract DM digestibility (Table 3).

Supplementation of oregano extract increased the population of *Desulfovibrio* in the rumen. The genus *Desulfovibrio* is responsible for most of the sulfate-reducing dissimilatory activity in the rumen and can convert sulfate to sulfide. The end product, hydrogen sulfide (H_2S) is recognized as the third molecular gaseous signal (Wallace, 2010), which plays important roles in mucosal defense in the digestive system (Wang et al., 2002; Kimura et al., 2005). H_2S had a protective effect on gastric ischemia-reperfusion injury by decreasing oxidative stress and anti-inflammatory effect (Liu et al., 2010, Ju et al., 2013). The same was observed in the study by Zhang et al. (2019), supplementing resveratrol for calves in the pre-weaning period.

The absence of significant effects on VFA production and ratio observed in thes study has been already reported in several studies (Kolling et al., 2018; Santos et al., 2015; Benchaar et al., 2007). The amount of oregano extract tested in the present study, 60 mg of OE/kg of BW (3,7 g/kg BW of essential oils), was not able to modulate rumen fermentation concerning production of VFA, possibly due to the low concentration of essential oils (6.5%). It is worth to notice that the amount of OR supplied to calves in this study is higher than those recommended for commercial operations. Benchaar (2020) supplemented cows with oregano oil (containing 70% carvacrol) or carvacrol (98% purity) at 50 mg/kg of total mixed ration (3.8 g/kg BW of essential oils), and they did not find any effect on rumen fermentation parameters (i.e., pH, ammonia, VFA).

Distinct phytochemicals present in essential oils have different properties; thymol and carvacrol act mainly as potent antimicrobials against pathogens such as *Escherichia* coli, *Salmonella* typhimurium, *Staphylococcus* aureus, *Staphylococcus* epidermidis and *Lysteria* monocytogenes (Benchaar et al., 2008). The results found in the present study corroborate the aforementioned data, as *Streptococcus*, *Escherichia*,

Clostridium and *Clostridium_IX* were reduced in the rumen with OR supplementation. A similar effect was shown in the cecum, with a reduction in the genus *Streptococcus*, as well in the colon, where the genera *Streptococcus* and *Escherichia* were reduced in OR calves.

The abundance of the genus *Methanobrevibacter* was increased in the rumen and jejunum of OR calves compared with the CON. Although it seems controversial as oregano extract decreased methane emissions in dairy cows (Kolling et al., 2018), it might not be so as essential oils, such as carvacrol and thymol in oregano may reduce CH_4 emissions by not only by direct negative effects on methanogens, but by indirect effect on some bacteria and protozoa that are critical for methanogen metabolism or by reducing feed fermentation (Ohene-Adjei et al., 2008; Cobellis et al., 2016).

The reduced abundance of the genera *Turicibacter* in the rumen and *Roseburia* in both cecum and colon of OR calves was probably due the inhibitory action of oregano essential oils as *Roseburia* is a gram-positive anaerobic bacteria (Patra and Yu, 2015).

Surprisingly the abundance of genus *Bifidubacterium* was reduced in the jejunum in OR calves compared with CON. On the contrary, He et al. (2017) added oregano essential oils to hens increasing the number of intestinal *Bifidubacterium* while *Salmonella* was significantly ($P< 0.01$) decreased.

Supplementation with ORhad small effects on feed intake. The lower starter intake observed in the beginning of the trial in OR calves compared with control might had been due to strong smell and possibly characteristic taste, as oregano extract was top-dressed onto the concentrate. However, during the last period of the trial (43 - 53 d), calves adapted to the supplement's smell and flavor, and they consumed higher amounts of the starter compared with control calves. Kolling et al. (2016) noticed some

changes in the behavior of heifers supplemented with oregano extract such as increased time spent eating the concentrate, increasing the occurrence of post-ingestive licking the feed bunk with abundant saliva production and the occurrence of sneeze events. Volatile compounds present in the oregano extract (Liu et al., 2020) might stimulate orexis and flavor of plants (Calsamiglia et al., 2007). Results reported by Jeshari et al. (2016), Froehlich et al. (2017) and Kazemi-Bonchenari et al. (2018) support the findings of the present study, as these authors observed that a commercial essential oils blend improved both the starter intake and feed efficiency.

The absence of effect on hay intake was expected and probably due to the fact the oregano extract was top-dressed onto the concentrate, so there was no influence on the sensorial characteristics of hay. The variation in hay intake during the 4° period was probably due to the substitution effect, as MR supply was reduced while preparing calves for the weaning and the starter consumption was increased.

The variation in total DMI followed the variation of starter concentrate intake, mainly during the first period. On the last period, it followed the variation in MR and starter concentrate intakes. As MR is an important source of DM, with its reduction in the last period, total DMI was also reduced, despite the increase in starter intake. Starter intake is usually negatively related to the liquid diet volume (milk or MR) fed (Davis & Drackley, 1998). Additionally, it is important to notice that the last period was eight days shorter than the other periods, and this fact impacted the absolute values of the intake per period.Tapki et al. (2020) tested the supplementation of two doses of OE oregano (Low dose 100 and High dose 150 mg/l milk) and observed that low dose calves started the consumption of hay and starter feed earlier than high dose and control. This resulted from increased saliva secretion, increased appetite, and promotion of rumen development (Taki et al., 2020). These authors report that the odor

and/or high dose of EO can have deleterious effects on the microbiota and ruminal fermentation, since a threshold profile, characterized by a virtual halt of fermentation when the doses are higher than the level threshold, occurs for the essential oil based on carvacrol and thymol (Tapki et al., 2020).

The greater BW in the OR-fed group was probably due to the greater BW at the beginning of the trial as ADG and feed conversion were similar between treatments. It is worth to notice the greater apparent digestibility coefficients for OR calves, but this fact was not enough to improve ADG and feed conversion. Other studies also did not verify differences in total feed intake in dairy calves (Paris et al., 2020) and heifers (Kolling et al., 2016).

On the other hand, Froehlich et al. (2017) reported that calves supplemented with blend of essential oils (carvacrol, caryophyllene, *p*-cymene, cineole, terpineneand thymol) had enhanced growth rates compared with the control group. Liu et al. (2020) observed that calves fed blend of essential oils (carvacrol,caryophyllene, *p*-cymene, cineole, terpinene,and thymol) had greater BW than calves fed a control treatment.

CONCLUSIONS

Supply of oregano extract to pre-weaned dairy calves increased the diversity of bacterial population in the rumen and jejunum. Supplementation of oregano extract to dairy calves affected both gram-positive and negative bacteria and decreased the abundance of potential pathogenic bacteria, enhancing apparent digestibility. Albeit oregano extract does not improve feed intake and body weight gain, its actions on bacteria microbiota at the gastrointestinal tract might be a useful tool to help dairy calves to overcome the challenges faced during the pre-weaning period.

ANIMAL WELFARE STATEMENT

Animal experiments were carried out according to the Animal Care and Use Committee of the Federal University of Rio Grande do Sul, Rio Grande do Sul, Brazil. The authors confirm that journal's ethical policies, as noted on the journal author's guidelines page, have been respected.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the Use of Animals from the Federal University of Rio Grande do Sul, Rio Grande do Sul (CEUA/UFRGS), protocol number 35666.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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TABLE 1 Average starter, hay and total DM intake of calves fed-control or OR-Supplemented

Period	Starter (kg DM)		Average, per period
	CON	OR	
d 1-14	0.92^{Ba}	0.31^{Bb}	0.62 (0.09)
d 15-28	2.00^{Ba}	1.52^{Ba}	1.76 (0.09)
d 29-42	5.86^{Aa}	6.21^{Aa}	6.03 (0.09)
d 43-53	7.25^{Ab}	8.72^{Aa}	7.99 (0.09)
Average, d 1-53	4.01 (0.28)	4.19 (0.28)	
	Hay (kg DM)		
d 1-14	-	-	-

d 15-28	0.25	0.39	0.32 (0.12)^{C}
d 29-42	1.38	1.71	1.55 (0.12)^{A}
d 43-53	1.04	1.25	1.15 (0.12)^{B}
Average, d 1-53	0.67 (0.10)	0.84 (0.10)	
	Total (kg DM)		
d 1-14	11.51^{BCa}	10.89^{Cb}	11.20 (0.10)
d 15-28	12.83^{Ba}	12.50^{Ba}	12.66 (0.10)
d 29-42	17.35^{Aa}	18.03^{Aa}	17.69 (0.10)
d 43-53	10.41^{Ca}	12.09^{BCa}	11.25 (0.10)
Average, d 1-53	13.02 (0.31)	13.38 (0.31)	

CON = control; OR (oregano extract) = 60 mg kg/body weight; Starter = starter fed intake in kg DM; Hay = Hay fed intake in kg DM; Total = starter + hay + milk replace intake in kg DM.

Capital letters = statistical difference within the columns; small letters = statistical differences in the same row; numbers in parentheses = standard error mean.

TABLE 2 Average body weight, feed efficiency and average daily gain of calves fed-control or OR-Supplemented

Period	BW (kg)		Average, per period
	CON	OR	
d 1-14	37.20	42.90	40.05 (3.68)^{C}
d 15-28	42.90	49.10	46.00 (3.68)^{BC}
d 29-42	48.70	55.50	52.10 (3.68)^{AB}
d 43-53	52.90	60.40	56.65 (3.68)^{A}
Average, d 1-53	45.42 (3.22)^{b}	51.97 (3.22)^{a}	
	Feed conversion		

d 1-14	0.48	0.57	0.53 (0.06)^{C}
d 15-28	0.87	0.99	0.93 (0.06)^{B}
d 29-42	0.97	1.05	1.01 (0.06)^{B}
d 43-53	2.02	1.96	1.99 (0.06)^{A}
Average, d 1-53	1.09 (0.06)	1.14 (0.06)	
	ADG (kg/d)		
d 1-14	0.40	0.44	0.42 (0.03)
d 15-28	0.41	0.44	0.42(0.03)
d 29-42	0.41	0.46	0.44(0.03)
d 43-53	0.36	0.45	0.40(0.03)
Average, d 1-53	0.39 (0.03)	0.45(0.03)	

CON = control; OR (oregano extract) = 60 mg kg/body weight; BW = body weight in kg; Feed conversion (dry matter intakedivided by BWG); ADG = average daily gain in kg/d^{-1}.

Capital letters = statistical difference within the columns; small letters = statistical differences in the same row; numbers in parentheses = standard error mean.

TABLE 3 Apparent digestibility of feed fractions and volatile fatty acids (VFA) in calves fed-control or OR-Supplemented

Itens	Treatments		s.e.m	*P*-value
	COM	OR		
Digestibility coefficients				
DM	0.72^{a}	0.76^{b}	0.01	0.050
Organic Matter	0.74^{c}	0.77^{d}	0.01	0.080
NDFap	0.55	0.61	0.03	0.330
CP	0.74^{a}	0.78^{b}	0.01	0.050

EE	0.79	0.76	0.02	0.449
NFCap	0.67[c]	0.73[d]	0.02	0.076
VFA (mmol/100mL)				
Acetic acid(C2)	53.11	66.84	4.89	0.173
Propionic acid(C3)	39.56	48.45	5.06	0.412
Butyric acid (C4)	15.31	18.83	3.64	0.658
Total	108.00	134.10	12.40	0.321
C2 : C3	1.44	1.47	0.12	0.910

CON = control; OR (oregano extract) = 60 mg kg/body weight; DM = dry matter; NDFap =ash protein-corrected neutral detergent insoluble fiber; CP = crude protein; EE = ether extract; NFCap = ash protein-corrected non fiber carbohydrates; s.e.m = standard error mean; means followed by a and b represents statistical significance ($P<0.05$); c and d represents trends ($0.05<P<0.10$).

FIGURE 1. Phylum-level taxonomic composition of bacterial populations in the rumen digesta of pre-weaned calves fed a control diet and supplemented with OR.

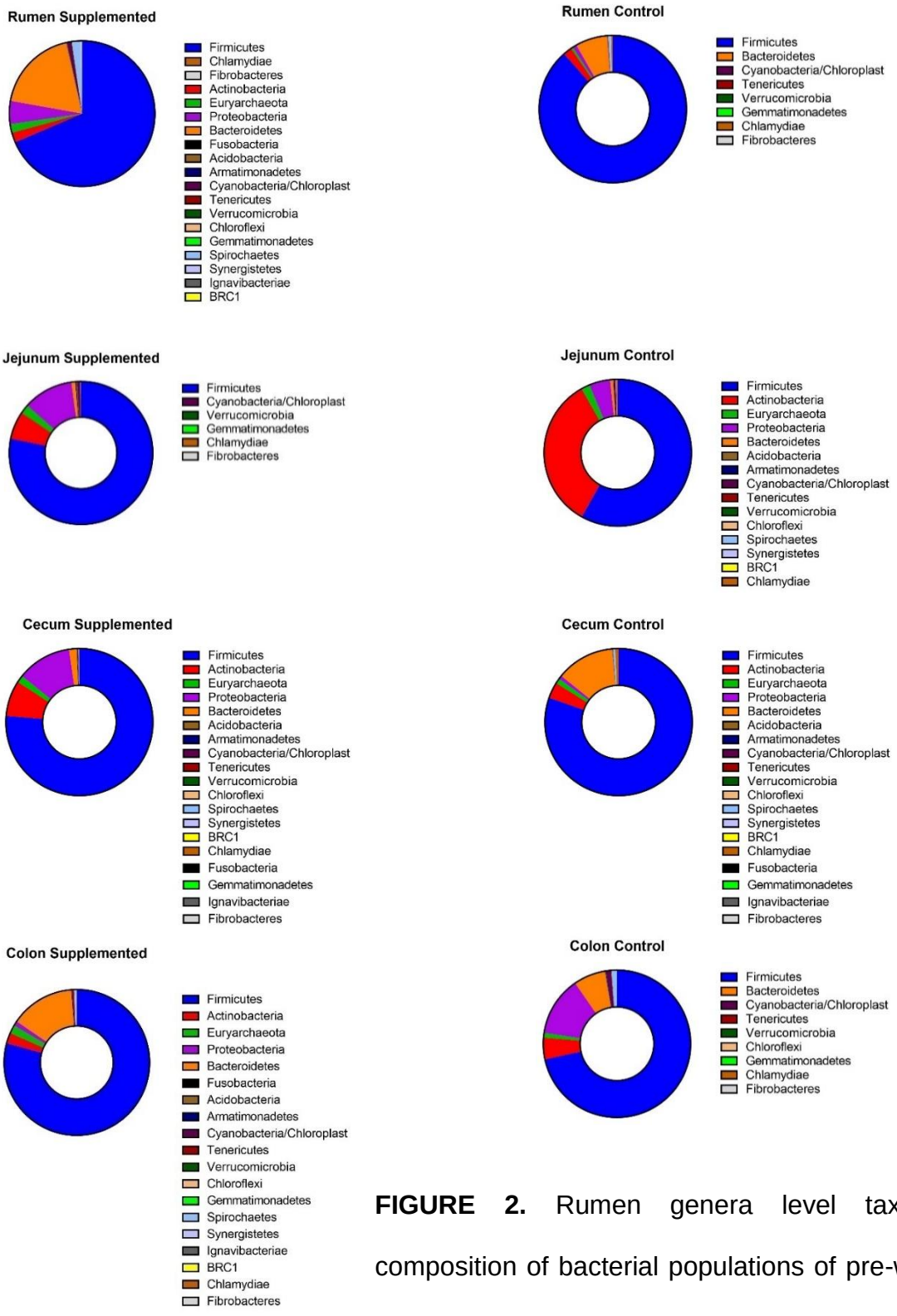


FIGURE 2. Rumen genera level taxonomic composition of bacterial populations of pre-weaned

calves fed a control diet and OR-Supplemented. Statistical differences (P<0.05) between genera are represented by “*”.

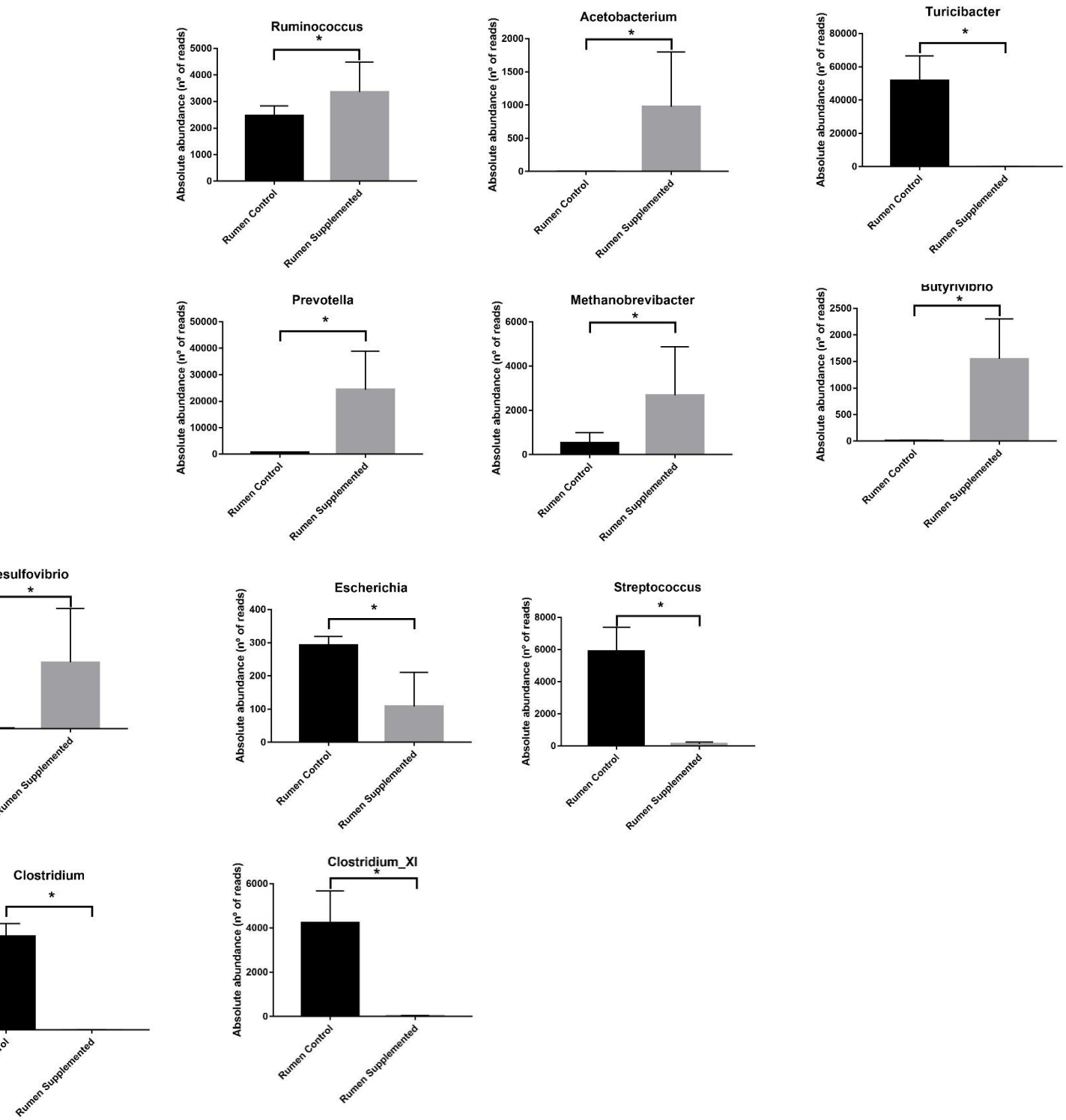


FIGURE 3. Jejunum genus level taxonomic composition of bacterial populations of pre-weaned calves fed a control diet and OR-Supplemented. Statistical differences (*P*<0.05) between genera are represented by "*".

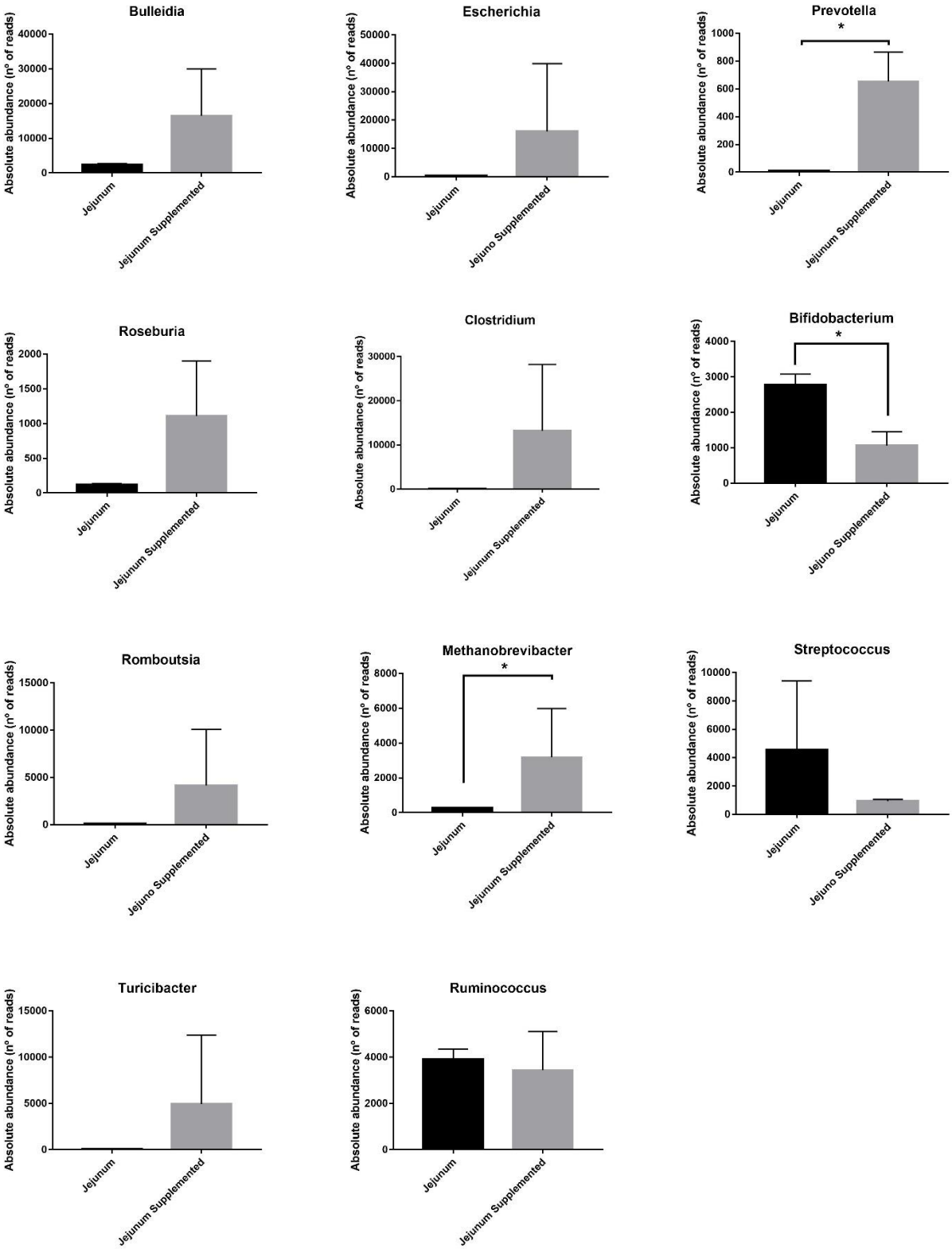


FIGURE 4. Cecum genera level taxonomic composition of bacterial populations of pre-weaned calves fed a control diet and OR-Supplemented. Statistical differences (*P*<0.05) between genera are represented by "*".

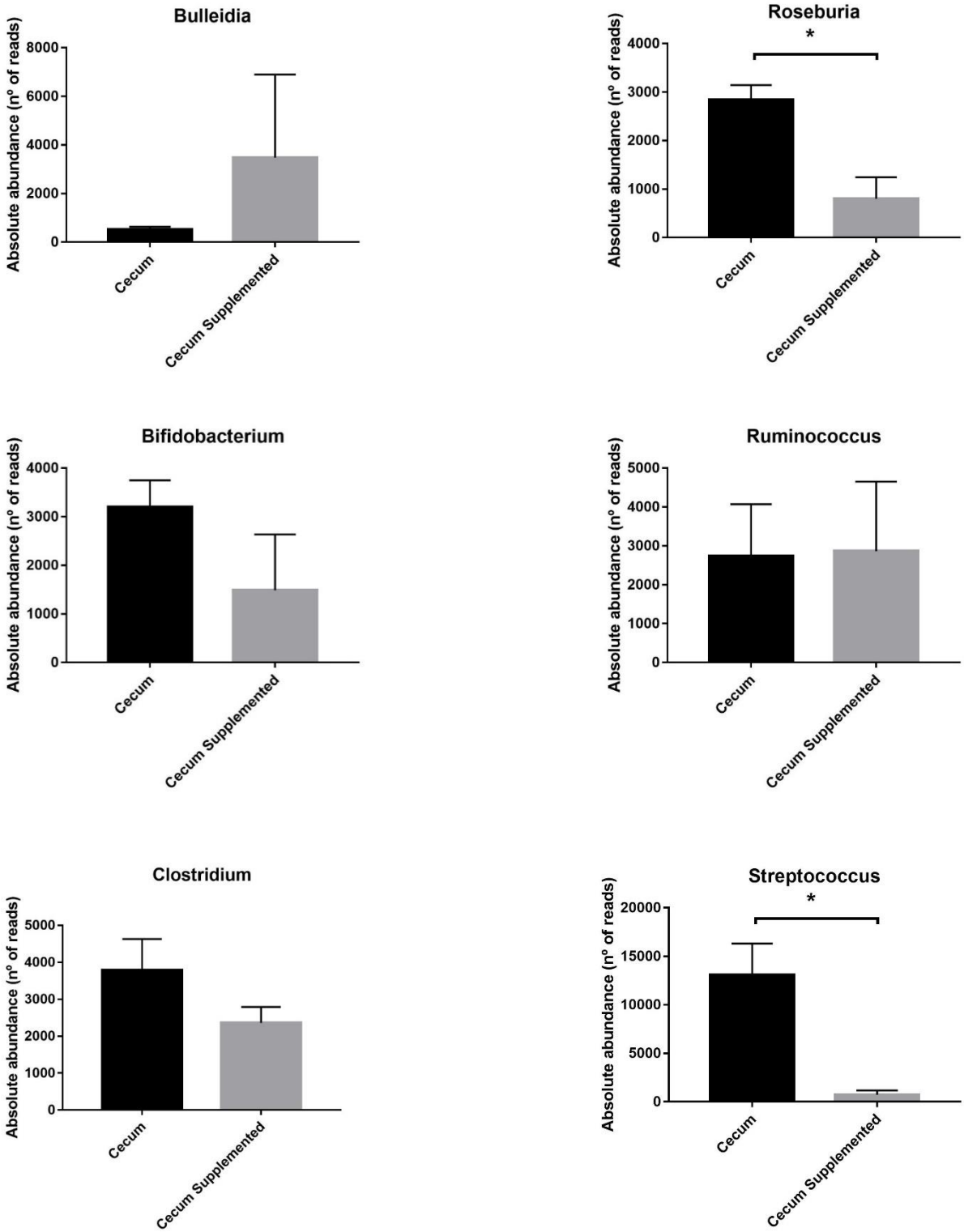


FIGURE 5. Colon genera level taxonomic composition of bacterial populations of pre-weaned calves fed a control diet and OR-Supplemented. Statistical differences (*P*<0.05) between genera are represented by "*".

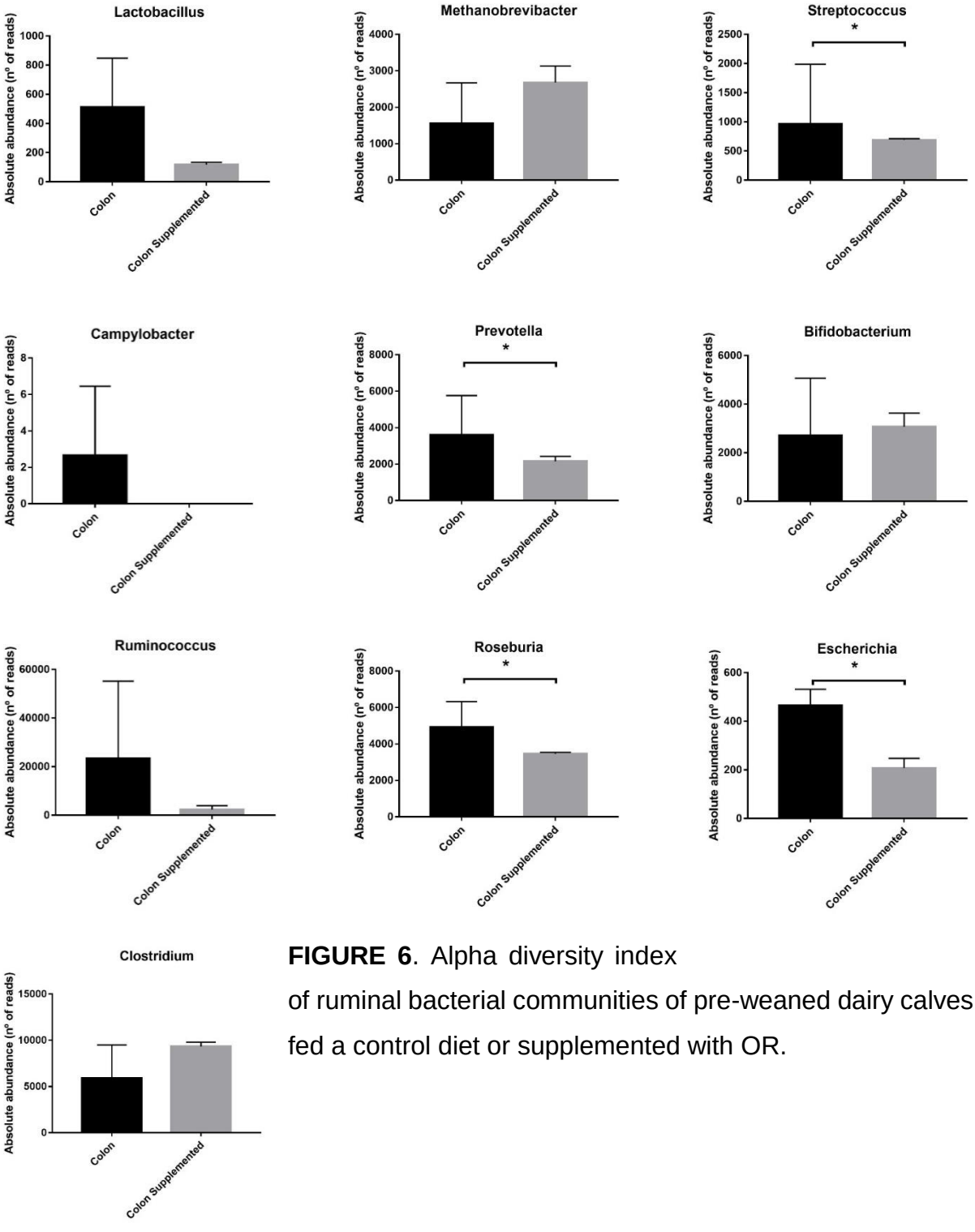
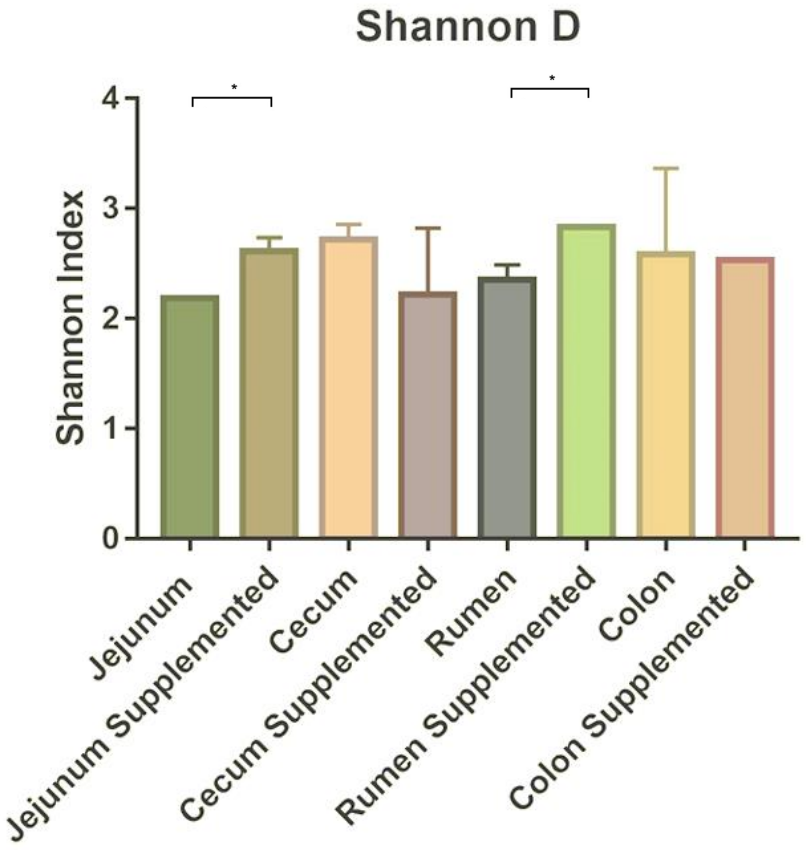


FIGURE 6. Alpha diversity index of ruminal bacterial communities of pre-weaned dairy calves fed a control diet or supplemented with OR.


Shannon D
Shannon Index
0
1
2
3
4
*
*
Jejunum
Jejunum Supplemented
Cecum
Cecum Supplemented
Rumen
Rumen Supplemented
Colon
Colon Supplemented

CAPÍTULO III

Article 2: Effect of supplemental oregano extract on ruminal and intestinal morphology of pre-weaned calves

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Effect of supplemental oregano extract on ruminal and intestinal morphology of pre-weaned calves

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Abstract

The aim of this study was to evaluate the effects of oregano extract (*Origanum vulgare*) supplementation on the histological and morphological variables of suckling dairy calves in a 53-day experiment. Ten male Holstein calves from commercial dairy farms were used, distributed in two treatments: 1) a basal diet without additive (CON); 2) the same diet, but containing 60mg/kg bodyweight/day of oregano extract (OR). Weaning occurred on the 53rd(56th day of live) day of the experimental period and on the 54th day, all calves were euthanized and the full and empty tract were weighed and tissue samples from the rumen, jejunum, cecum and colon were also taken for histological analysis. The variables observed were: full and empty weight of the

gastrointestinal tract; papilla height, tunica of the submucosa, number of papillae, number of branches, branch height and number of papillae/branches in the rumen; number of glands, villi length, mucosal thickness, submucosal thickness, muscle thickness in the jejunum, cecum and colon. Oregano extract tended to increase the full and empty weight of the abomasum without changing the weight of the other segments. In the rumen, OR-supplemented calves showed lower branching, number of branches, branch height and number of papillae/branches. In the jejunum, calves in CONtended to have thicker muscle layer than calves in OR. Most parameters evaluated were not affected by OR supplementation. Essential oils are promising replacements for antibiotics. However, the dose, administration routes and supply time deserve further studies, allowing for better performance and health of the animal to be achieved.

Keywords: oregano extract, calves, morphology, rumen, intestine

Introduction

The role of essential oils in the intestinal morphology of monogastric animals is well documented. Several *in vivo* studies have reported the role of essential oils as growth promoters in the intestine, e.g. in broilers (Amad et al., 2011). Essential oils have been reported to reduce the production of toxic compounds and damage to broiler intestinal epithelial cells (Samadian et al., 2013, Heydarian et al., 2020). Bacterial toxins are known to have negative effects on intestinal morphology (Samadian et al., 2013).

However, the impact of essential oil supplementation on the gastrointestinal tract development of young ruminants remains largely unexplored. The aim of this

study was to evaluate the use of a commercial product composed of oregano extract (Oreganol®) in the development and histology of GIT in pre-weaned dairy calves.

Material and Methods

Experimental design, animals, and treatment diets

Ten male Holstein calves aged approximately four days were distributed in a completely randomized design with 2 treatments and 5 replicates (calves) per treatment. The calves were sourced from dairy farms located within 15 km of the site of the experiment. The average weight of the calves was 37.1 kg (± 3.6 kg standard deviation). They received colostrum (10% of birth BW) on the first day of live, and transition milk until the 3rd day of life. The calves were randomly assigned to two treatments: 1) a basal diet without additive (CON); 2) the same diet, but containing 60mg/kg bodyweight/day of oregano extract (OR - Oreganol®, Asteri Veterinary Medicines Industry Ltd., São Paulo, Brazil - carvacrol 80% and thymol 2%). The oregano extract was top-dressed on the concentrate starter fed every morning. The milk replacer (MR - Bovimix Vitamilk® 19.0% CP, 15.0% EE of dry matter; Vitamix Nutrição Animal Ltd, Nova Itaberaba, Santa Catarina, Brazil – 125 g/L) was divided into two daily meals fed at 08:00 and 18:00 h totaling 6 L/day, except during the last 5 days before weaning, when calves received 2 L/day, aiming to increase the solid feed intake. Calves had free access to drinking water, starter concentrate (ground corn grain – 40.5%, soybean meal – 33.3%, wheat meal – 26.0% and Premix vitamin+mineral – 0.2%) and coast-cross grass hay (*Cynodon dactylon* (L.) Pers). Animals were kept in individual pens of 1.50 m × 1.00 m) with wood shaving bedding. The feed composition

(Supplementary Table S1) was formulated according to the National Research Council (2001) guidelines.

Measurements and sample collection

Body weight (BW) was recorded on days 1, 15, 30, 45 and 53 of the experiment, before the morning feeding, so adjustments of dietary supplements were made accordingly. Every morning (07:00 h), leftovers were weighed to obtain the daily intake of starter and hay. During the last five days of the experiment, fecal samples were collected directly from the rectal ampoule, weighed to determine the apparent digestibility of dry matter (DM) and nutrients of each animal. Fecal production was estimated using titanium dioxide (TiO_2 – 0.5 g/day) during the last nine days of the study at the moment of morning feeding, as an external fecal marker (Ohmori et al., 2013). On day 53 of the experiment calves were weaned and on day 54 they were euthanized with acepromazine (0.013 mg/kg BW), thiopental (0.125 mg/kg BW) and potassium chloride (80-120 mL). The abdominal cavity was opened, and the rumen was isolated from the other parts of the gastrointestinal tract (GIT) with zip lock to prevent reflux of digesta.

The esophagus, rumen, reticulum, omasum, abomasum, small intestine and large intestine of the five calves of each treatment were first weighed with digesta and then emptied and samples were collected for histological analysis, then washed under running water and weighed empty. Then, two samples of $2cm^2$ of the rumen tissue, and one sample of the jejunum, cecum and colon of each animal were taken. Rumen sampling was performed close to the central pillar, while sampling of intestinal sections was collected from the medial portions of each section. For fixation, the samples were washed with saline solution to remove any digesta residues that could deteriorate the

sample. Subsequently, the samples were placed in Falcon 50mL tubes (one sample per tube), where a 40 mL aliquot of paraformaldehyde was added, remaining for 24 h at room temperature. After 24 h, the paraformaldehyde was discarded in a waste drum and 40 mL of ethyl alcohol was added to the Falcon tube for conservation until the samples were processed in the laboratory.

Mounting of histological slides

The processing of histological slides of the rumen, jejunum, cecum and colon was performed at the Institute of Basic Health Sciences (ICBS) of UFRGS using a Thermo SCIENTIFIC MICROM STP 120 tissue processor that automatically performed the processes of dehydration, clarification, impregnation and inclusion of materials. The dehydration process, which consists of removing water from the tissues, took place over a period of 8 hours with ethyl alcohol. Paraffin does not mix homogeneously with alcohol, so it is necessary to carry out the clarification process. In this experiment, this step lasted 8 hours, using xylol to replace the alcohol inside the tissue. The impregnation process took 5 hours and consists of infiltration of paraffin into the tissues and as a means of tissue inclusion. The embedding was done placing the tissue infiltrated with paraffin in a mold with liquid paraffin to be sectioned in the microtome with the surface downwards, after cooling the embedded material is obtained in the paraffin blocks. Histological sections were made with a Leica model RM2155 microtome, 5 micrometers thick, in transverse or longitudinal orientation, with hematoxylin eosin staining.

Image capture process

The histological images of the tissue slides were performed equally at ICBS with the aid of a Zeiss Axioimager M2 electron microscope coupled to a computer with the Zeiss 2012 program, using 40X objective in all images and saving them in a TIF file format. The measurements were performed using the ImageJ 1.53f/java 1.8.0_261 program. The same 40x objective calibration was performed on all images so that the number of pixels could be associated with real measurements, in micrometers. The scale bar was inserted into all images and it was determined to 3.9 micrometers/pixels.

The following measurements were taken from the rumentissue: 1) papilla count per area of 15,000 µm; 2) thickness of the tunica submucosa measured from the end of the tunica mucosa to the beginning of the tunica muscularis; 3) height of the ruminal papillae, measured from the base of the papilla in the tunica submucosa to the tip of the papilla. Five repetitions per slide of each measure were analyzedand the results were expressed in micrometers. It was considered whether or not the rumen tissue had ramifications. When there were branches, they were counted, performing three repetitions per slide.The height of the branch and the number of papillae per branch was also measured.

In the jejunum, cecum and colon samples, the number of glands in an area of 1,000 µm was measured, as well as the thickness of the mucosal, submucosal and muscular tunics, with five repetitions per slide for each measurement. In the jejunum, the measurement of the length of the villi was also performed, going from the end of the gland to the tip of the villi, with five repetitions per slide.

Statistical analysis

A completely randomized design was used, followed by analysis of variance and T- test procedure of SAS (Statistical Analysis System, software package 9.0, SAS Institute Inc., Cary, NC, USA), the level of significance of 5% ($P<0.05$) was considered, and $0.05 > P > 0.10$ were considered as tendency to compare the means between treatments with and without supplementation with oregano extract.

Results

Weights of GIT segments

The full and empty weights of the esophagus, rumen, reticulum, omasum, abomasum, small intestine and large intestine of the calves are shown in table 2. There was no statistical difference ($P \leq 0.05$) for full and empty weights of the GIT sections between CON and OR calves, except for the abomasum. Calves in OR had higher abomasal empty weight ($P = 0.013$) and tended to have higher abomasal full weight ($P = 0.062$) compared to CON.

Histological variables of the rumen, jejunum, cecum and colon

The histological variables of the rumen, presence of branches, number of branches, height of branches and number of branches/papilla were significantly higher ($P = 0.019$; $P = 0.018$; $P< 0.001$; and $P = 0.007$, respectively) for control calves, when compared to OR-Supplemented animals (Table 3).

There was no statistical difference between CON and OR animals for the variables studied in the other tissues of the GIT, except for the thickness of the muscle layer of the jejunum, which tended ($P = 0.063$) to be thicker in CON animals compared

with OR (Table 3). Images of histological analysis are presented in the supplementary material (Appendix 2).

Discussion

Weights of GIT segments

The main contribution of the present study was to highlight the still limited effects of supplementation with oregano extract on the development and histology of the GIT segments. At the dose used (60 mg/kg BW) of oregano extract, the supplemented animals showed an increase in the weight of the abomasum. Consumption of solid foods, especially concentrates, promotes rumen colonization, organ size increase and papillary development (Diao et al., 2019). On the other hand, according to Huber(1969), factors that stimulate ruminal development, such as grain intake and VFA production, also induce greater growth of the abomasum glands. In the present study, the higher full weight of the abomasum in OR animals were not expected, since, only in the last experimental period, this group had a higher starter consumption compared with control, with no differences for the total starter consumption, liquid or hay diets (*Supplementary Table* S2). It is important to note that this supposedly greater digestive capacity of the abomasum occurred without prejudice to the development of pre-stomach weight, especially the rumen, since there was no difference in empty and full weight between the other pre-stomach compartments. It is worth to notice the absence of studies evaluating the influence of essential oil supplementation on the development of this organ.

Histological variables of the rumen, jejunum, cecum and colon

The animals in the control group showed greater values for the indicators of rumen papillary development (presence of branches, number of branches, height of branches and number of branches/papilla), without significant effects on the histological aspects of the other segments (i.e., jejunum, cecum and colon). In the present study, no difference was found between the CON and OR groups in the concentration of VFAs in the rumen, possibly because the total consumption of starter and hay was similar between the groups. The higher consumption of starter observed at the end of the experiment probably was not enough to promote an increase in VFAs. Volatile fatty acids, fermentation end products, are highly correlated with starter intake (Santos et al., 2015). This result corroborates with Santos et al. (2015), when they supplemented calves with 400mg/kg of a commercial blend of essential oils, not finding differences in the concentrations of VFAs in the rumen fluid between the groups. These authors suggested that apparently, EO at the doses tested in the study were not able to modulate the rumen fermentation. Changes in the profiles of VFAs in rumen fluid would also alter the acetate:propionate (C2:C3) ratios. As butyrate and propionate are important for the development of the ruminal papilla, and especially propionate is used in the gluconeogenesis pathway (Drackley, 2008), while butyrate is used as an energy source in the rumen epithelium, there is potential for change in the development of length, width and area of ruminal papillae (Tamate et al., 1962). In this way, a smaller proportion of the C2:C3 ratio is desired. In this experiment, OR supplementation did not change the VFA values, as well as the C2:C3 ratio (*Supplementary Table* S4).

Due to our results regarding the similarity in the concentration of VFA in the rumen fluid, one could expect similar histological results in the rumen, which did not occur. Results with essential oils show variable effect on VFA production. Santos et al.

(2015) did not show changes in VFA concentration in ruminal fluid. Palhares Campolina et al. (2021) supplemented 1g/calf/day of a blend of essential oils and also found no differences in VFA concentrations in the rumen fluid, but reported changes in the C2:C3 ratio. In the present study, there was no statistical difference in the concentration of volatile fatty acids between treatments

The occurrence of deformed or branched papillae may be due to the effect of excess ruminal butyrate and propionate on cell proliferation. McGavin and Morrill (1976) proposed that the branching of the ruminal papillae occurs as a way to compensate for the loss of absorption capacity in response to the thickening of the keratin layer. This hypothesis is also based on the principle proposed by Greenwood et al. (1997), who observed that the length of the papillae increases with the increase in the percentage of keratin in the epithelium. A thickening of the keratin layer in the rumen epithelium due to reduced particle size of the starter feed was observed by Strusinska et al. (2009) in 90-day-old calves. Thus, the keratinization and deformation of papillae, i.e., the process called parakeratosis, implies a lower capacity for absorption of ruminal fermentation products. However, this phenomenon was only reported when finely processed diets were offered to animals. In the present work, the starter ration consisted of ground corn, soybean bran, wheat bran and vitamin and mineral premix, with no difference in particle size between OR and CON group.

Essential oils can be supplemented to calves through milk or milk replacer (Santos et al., 2015; Hassan et al., 2020) or mixed with starter feed (Jeshari et al., 2016; Salazar et al., 2019). The administration of medications and food additives via milk or milk replacer is considered better due to its ability to bypass the rumen. However, essential oils are mainly hydrophobic or lipophilic compounds and therefore are not soluble in milk, which can result in uneven mixing, poor stability and poor

bioavailability. Based on the above, we chose to supplement the oregano extract would be via starter feed. Our hypothesis was that the oregano extract, in addition to modulating the development of the rumen, would also pass to the lower gastrointestinal tract and modulate the development of this organ.

The lack of statistical differences in the evaluated histological variables in the intestine, may be due to a low rate of passage of the oregano extract to the intestine. There was a tendency for the thickness of the jejunal muscle tissue to be greater in CON calves. It is accepted that the increase in the thickness of the intestinal mucosa is correlated with an increase in the digestive and absorptive function of the small intestine due to the increase in the absorptive surface area (Aslan et al., 2021)

Due to the scarcity of literature on the subject, we hypothesize that as the calves were slaughtered after 53 days of the experiment, it could be that there was not enough time for the extract to have shown an effect on the morphology of the gastrointestinal tract. This speculation if time was sufficient to highlight morphological changes in the GIT of pre-weaned calves was raised based on recent studies testing other additives. Jonova et al. (2021) studied the supplementation of the prebiotic inulin and its combination with *Saccharomyces cerevisiae* in Holstein calves lasting eight weeks, starting from the 32nd day of life. The authors report that the combination of inulin and yeast positively impacted the development of almost all morphological structures of the saccus dorsalis and saccus ventralis of the rumen and intestine. In another study Arne and Ilgaza (2021) tested the supplementation of the prebiotic inulin in combination with the probiotic *Enterococcus faecium* in calves aged 23 to 90 days. The authors concluded that the addition of 12 g of inulin to milk improves the development of the rumen papillae (length and width), especially in the saccus ventralis region. Combining this dose of inulin with 0.25 g of *E. faecium*, a significant increase in the length and

width of the papillae was observed. Based on the above, we hypothesized that the increase in the age of the animals, could lead to the achievement of the expected results.

Another hypothesis is that the number of animals was insufficient, since there was a high variation in the means of the measured parameters. The dose and product studied were used in previous studies with calves (Heisler et al., 2020; De Paris et al., 2020). Using the same product (Oreganol®) and dose (60mg/kg BW), Heisler et al. (2020) observed that the oregano extract anticipated the occurrence of the first rumination and intake of roughage by approximately seven days compared to the control group, but did not modify body weight gain, incidence of diarrhea, health conditions and other behavioral variables. De Paris (2019), supplementing 70mg/BW of oregano extract, observed an improvement in the redox status in supplemented animals compared to the control group.

During the study, no occurrence of severe diarrhea was observed, regardless of the group. Importantly, the animals were raised under good conditions. In one study, Katsoulos et al. (2017) administered 12.5 mg/day of oregano essential oil to calves in the first 10 days of life and the mean fecal score throughout the experiment, diarrhea incidence, duration and severity of diarrhea episodes were significantly lower in the group supplemented with essential oils of oregano compared to controls, showing that essential oils have a role at the intestinal level.

Conclusions

In the present study, OR showed limited effects on the weight of the GTI segments, improving weight of the abomasum. Moreover, OR affected negatively branching, number of branches, branch height and number of papillae/branches in the rumen. In the jejunum, CON showed a tendency for muscle layer thickness to be greater than OR. Most indicators of development and morphology, i.e. organs weight and histological analysis of GIT were not affected by OR supplementation, or positively affected compared with control, by reducing branched papillae. However, the dose, administration routes and supply time deserve further studies, allowing for better performance and health of the animal to be achieved.

Author Statement

The idea for the paper was conceived by **L.A. Ritt and V. Fischer**. The experiment was designed by **L.A. Ritt and V. Fischer**. The experiment was conducted by **L.A. Ritt**. The data were organized and analyzed by **V. Fischer and E.C. Modesto**. The paper was written by **L.A. Ritt**. The manuscript was reviewed and formatted by **V. Fisher, E.C. Modesto and G. Heisler.**

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Ethical considerations

This study was approved by the Ethics Committee of the Use of Animals from the Federal University of Rio Grande do Sul, Rio Grande do Sul (CEUA/UFRGS), protocol number 35666.

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Nutrição Animal Ltd for the vitamin and mineral mix used in calf starter.

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Table 1. Chemical composition of starter concentrate, hay and milk replacer fed to pre-weaned dairy calves

	Feeds		
	Starter	Hay	Milk replacer[1]
DM (g/kg)	884.4	876.6	940.7
Ash (g/kg DM)	36.9	100.7	68.5
CP (g/kg DM)	220.6	96.4	209.5
Ether Extract (g/kg DM)	26.6	16	141
NDFa (g/kg DM)	388.5	769.4	-
ADFa (g/kg DM)	65.8	390	-
Total Digestible Nutrients (g/kg DM)	744.7	535	-

MS = dry matter; MN = natural matter; PB = crude protein; NDFa = ash-corrected insoluble neutral detergent insoluble fiber; ADFa = ash-corrected acid detergent insoluble fiber.
[1]Bovimix Vitamilk® Vitamix Animal Nutrition – Nova Itaberaba, Santa Catarina, Brazil.

Table 2. Full and empty weight (kg) of the esophagus, rumen, reticulum, omasum, abomasum, small intestine and large intestine of suckling calves supplemented or not with OR.

Organ Weight	Treatment		CV	*P*-value
	CON	OR		
FULL				
Esophagus	0.095	0.103	28.340	0.672
Rumen	2.917	3.876	35.053	0.238
Reticulum	0.232	0.260	47.828	0.711
Omasum	0.321	0.324	52.339	0.981
Abomasum	0.668	1.038	31.603	0.062
Small intestine	2.895	3.407	24.962	0.333
Large intestine	1.332	1.457	17.489	0.440
EMPTY				
Esophagus	0.073	0.075	21.134	0.815
Rumen	0.481	0.639	28.292	0.153
Reticulum	0.114	0.135	24.327	0.313
Omasum	0.188	0.220	30.166	0.433
Abomasum	0.265[b]	0.364[a]	15.724	0.013
Small intestine	1.512	1.915	55.161	0.519
Large intestine	0.786	0.898	15.514	0.213

N=10; CON = without oregano extract supplementation; OR = supplemented with oregano extract; CV = coefficient of variation; *P*-value = 0.05.

Table 3. Histology of the rumen, jejunum, cecum and colon of pre-wasted calves supplemented or not with oregano extract

Variáveis	Treatment		CV	*P*-value
	CON	OR		
RUMEN				
Papilla height	36.093	35.943	25.608	0.980
Submucosal tunic	15.723	8.961	57.113	0.167
Number of papillae	7.700	6.478	36.358	0.474
Branch	1.000[a]	0.778[b]	31.792	0.019
Number of branches	6.000[a]	3.444[b]	67.432	0.018
Branch height	84.370[a]	37.240[b]	54.039	0.001
Number of papillae/branches	5.591[a]	2.765[b]	54.443	0.007
JEJUNUM				
Number of glands	5.861	6.044	14.174	0.777
Villi length	12.326	10.138	35.934	0.476
Mucosal thickness	46.643	42.914	38.848	0.757
Submucosal thickness	5.389	4.243	34.928	0.373
Muscle thickness	22.007	16.911	16.251	0.063
CECUM				
Number of glands	4.507	4.917	31.445	0.691
Mucosal thickness	30.313	37.300	33.207	0.379
Submucosal thickness	0.686	0.542	26.869	0.239
Muscle thickness	10.453	8.862	69.546	0.736
COLON				
Number of glands	3.900	4.427	43.081	0.677

Mucosal thickness	33.734	44.714	39.644	0.334
Submucosal thickness	4.289	5.685	26.595	0.213
Muscle thickness	51.454	62.578	37.425	0.467

N=10; CON = without oregano extract supplementation; OR = supplemented with oregano extract; CV = coefficient of variation; *P*-value = 0.05.

Supplementary material

SupplementaryTable S1. Average starter, hay and total DM intake of calves fed-control or OR-Supplemented

Period	Starter (kg DM)		Average, per period
	Com	OR	
d 1-14	0.92^{Ba}	0.31^{Bb}	0.62 (0.09)
d 15-28	2.00^{Ba}	1.52^{Ba}	1.76 (0.09)
d 29-42	5.86^{Aa}	6.21^{Aa}	6.03 (0.09)
d 43-53	7.25^{Ab}	8.72^{Aa}	7.99 (0.09)
Average, d 1-53	4.01 (0.28)	4.19 (0.28)	
	Hay (kg DM)		
d 1-14	-	-	-
d 15-28	0.25	0.39	0.32 (0.12)^{C}
d 29-42	1.38	1.71	1.55 (0.12)^{A}
d 43-53	1.04	1.25	1.15 (0.12)^{B}
Average, d 1-53	0.67 (0.10)	0.84 (0.10)	
	Total (kg DM)		
d 1-14	11.51^{BCa}	10.89^{Cb}	11.20 (0.10)
d 15-28	12.83^{Ba}	12.50^{Ba}	12.66 (0.10)
d 29-42	17.35^{Aa}	18.03^{Aa}	17.69 (0.10)
d 43-53	10.41^{Ca}	12.09^{BCa}	11.25 (0.10)
Average, d 1-53	13.02 (0.31)	13.38 (0.31)	

CON = control; OR = 60 mg kg/body weight; Starter = starter fed intake in kg DM; Hay = Hay fed intake in kg DM; Total = starter + hay + milk replace intake in kg DM.
Capital letters = statistical difference within the columns; small letters = statistical differences in the same row; numbers in parentheses = standard error mean.

Supplementary Table S2. Average body weight, feed efficiency and average daily gain of calves fed-control or OR-Supplemented

Period	BW (kg)		Average, per period
	Com	OR	
d 1-14	37.20	42.90	40.05 (3.68)^{C}
d 15-28	42.90	49.10	46.00 (3.68)^{BC}
d 29-42	48.70	55.50	52.10 (3.68)^{AB}
d 43-53	52.90	60.40	56.65 (3.68)^{A}
Average, d 1-53	45.42 (3.22)^{b}	51.97 (3.22)^{a}	
	Feed Conversion		
d 1-14	0.48	0.57	0.53 (0.06)^{C}
d 15-28	0.87	0.99	0.93 (0.06)^{B}
d 29-42	0.97	1.05	1.01 (0.06)^{B}
d 43-53	2.02	1.96	1.99 (0.06)^{A}
Average, d 1-53	1.09 (0.06)	1.14 (0.06)	
	ADG (kg/d)		
d 1-14	0.40	0.44	0.42 (0.03)
d 15-28	0.41	0.44	0.42(0.03)
d 29-42	0.41	0.46	0.44(0.03)
d 43-53	0.36	0.45	0.40(0.03)
Average, d 1-53	0.39 (0.03)	0.45(0.03)	

CON = control; OR = 60 mg kg/body weight; BW = body weight in kg; Feed conversion = (ADG divided by dry matter intake); ADG = average daily gain in g/d^{-1}.
Capital letters = statistical difference within the columns; small letters = statistical differences in the same row; numbers in parentheses = standard error mean.

Supplementary Table S3. Apparent digestibility of calves fed-control or OR-Supplemented

Itens	Treatments		s.e.m	*P*-value
	CON	OR		
DM	72.11[a]	75.84[b]	1.00	0.050
Organic Matter	73.82[c]	77.39[d]	1.03	0.080
NDFap	54.71	60.52	2.84	0.330
CP	73.85[a]	78.39[b]	1.18	0.050
EE	78.81	76.31	1.54	0.449
NFCap	66.55[c]	73.40[d]	1.96	0.076
TDN	71.74[a]	76.10[b]	1.08	0.034

CON = control; OR = 60 mg kg/body weight; DM = dry matter; NDFap =ash protein-corrected neutral detergent insoluble fiber; CP = crude protein; EE = ether extract; NFCap = ash protein-corrected non fiber carbohydrates; TDN = total digestible nutrients, s.e.m = standard error mean; means followed by a and b represents statistical significance ($P<0.05$); c and d represents trends ($0.05<P<0.10$).

Supplementary Table S4. Mean values of volatile fatty acids (VFA) of calves fed-control or OR-Supplemented

VFA (mmol/100mL)	Treatments		s.e.m.	*P*-value
	CON	OR		
Acetic acid(C2)	53.11	66.84	4.89	0.173
Propionic acid(C3)	39.56	48.45	5.06	0.412
Butyric acid (C4)	15.31	18.83	3.64	0.658
Total	108.00	134.10	12.40	0.321
C2 : C3	1.44	1.47	0.12	0.910

CON = control; OR = 60 mg kg of body weight $^{-1}$; s.e.m = standard error mean.

4. CONSIDERAÇÕES FINAIS

No presente estudo, fornecimento de extrato de orégano a bezerros leiteiros em fase de pré-desaleitamento aumentou a diversidade da população bacteriana no rúmen e jejuno e diminuiu a abundância de bactérias patogênicas potenciais, aumentando a digestibilidade aparente. Por outro lado, a suplementação do extrato de orégano não melhorou a ingestão de ração, eficiência alimentar e o ganho de peso corporal. A suplementação de extrato de orégano também apresentou tendência de o peso total do abomaso ser maior e o peso vazio do abomaso maior, sem alterar os pesos dos demais segmentos, sem também melhorar as variáveis histológicas do rúmen, ceco e cólon. Entretanto, no rúmen, reduziu a ocorrência de papilas ramificadas. No jejuno, o extrato de orégano mostrou uma tendência para redução daespessura da camada muscular. As doses, as vias de administração e o tempo de suplementação merecem mais estudos, permitindo que se alcance melhor desempenho e saúde do animal. O fato de os resultados de estudos disponíveis na literatura serem bastante variáveis, pode-se inferir que essas diferenças derivam das diferentes espécies e idades utilizadas e/ou forma do óleo.Entretanto, suas ações sobre a microbiota de bactérias no trato gastrointestinal podem ser uma ferramenta útil para ajudar bezerros leiteiros a superar os desafios enfrentados durante o período pré-desaleitamento.

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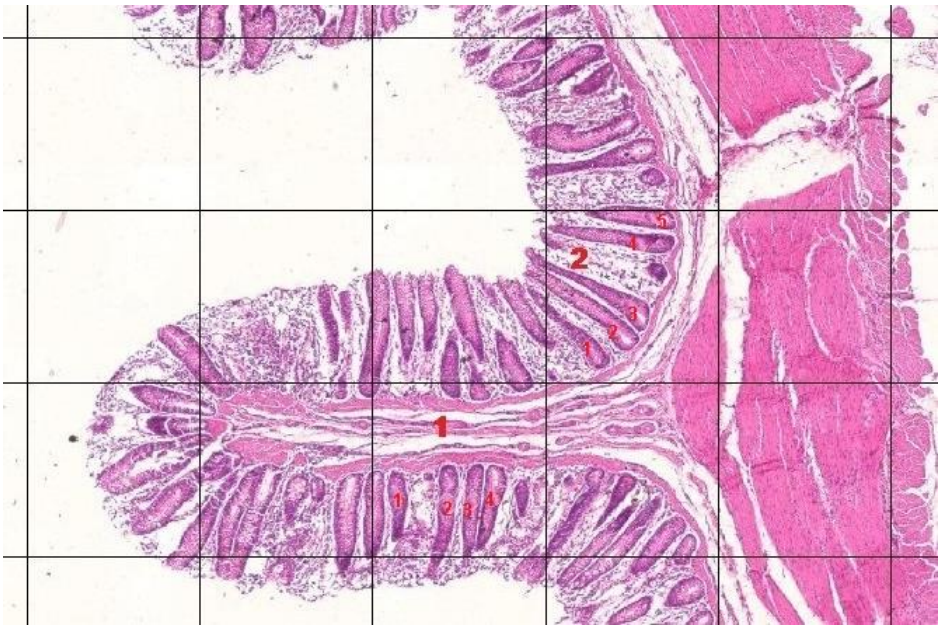
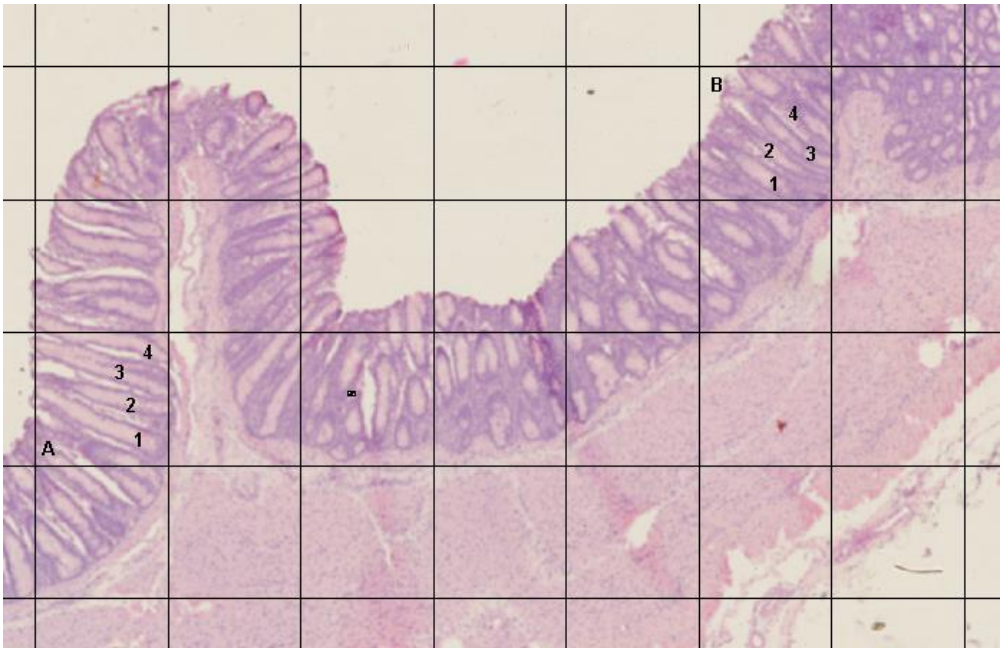
APÊNDICE 1

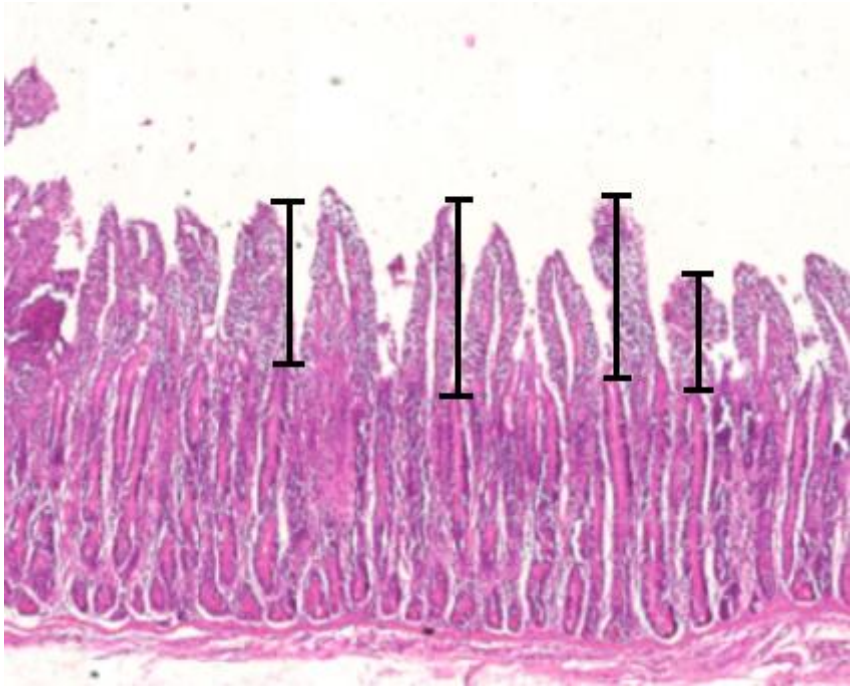
Histology supplementary images



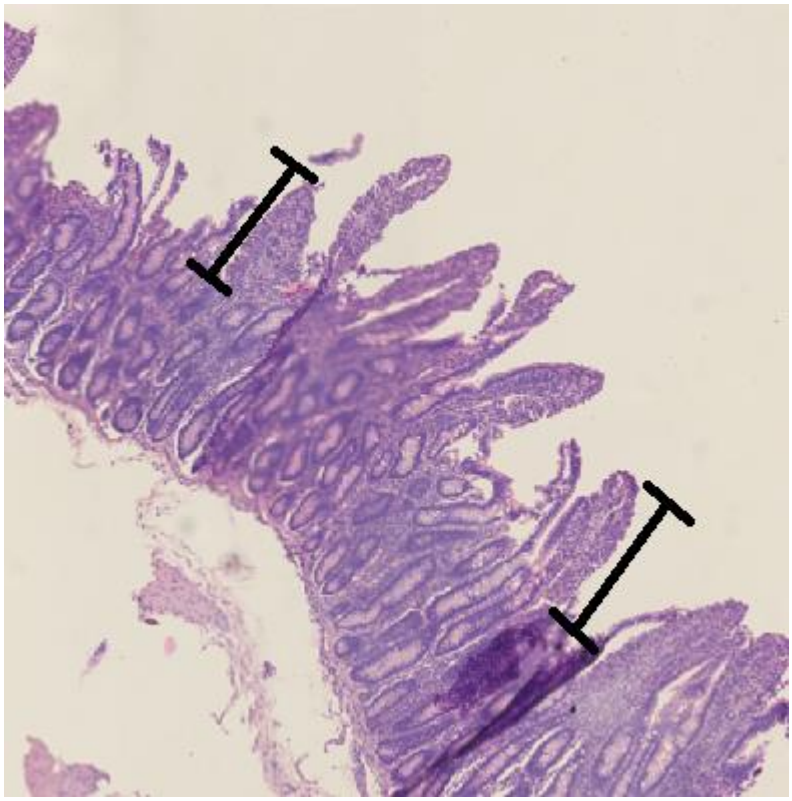
Supplementary figure S1: histological image of the calf colon from the CON group. Observe the number of glands per 1000µ area, with area 1 having 4 glands and area 2 having 5 glands.



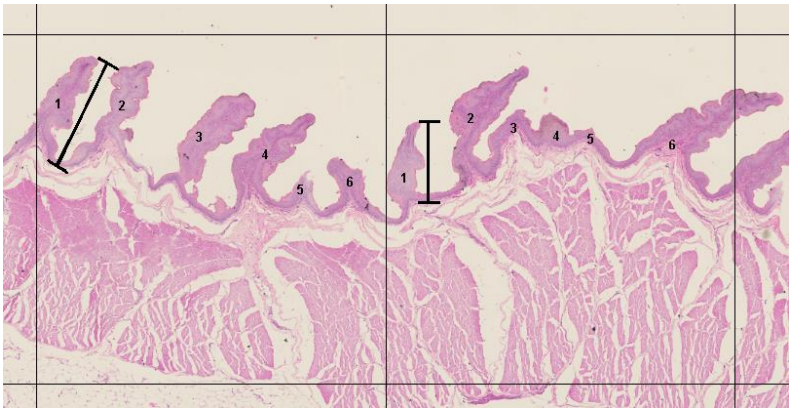
Supplementary figure S2: histological image of the calf colon from the OR group. Observe the number of glands per area of 1000µ, being area A with 4 glands and area B with 4 glands.



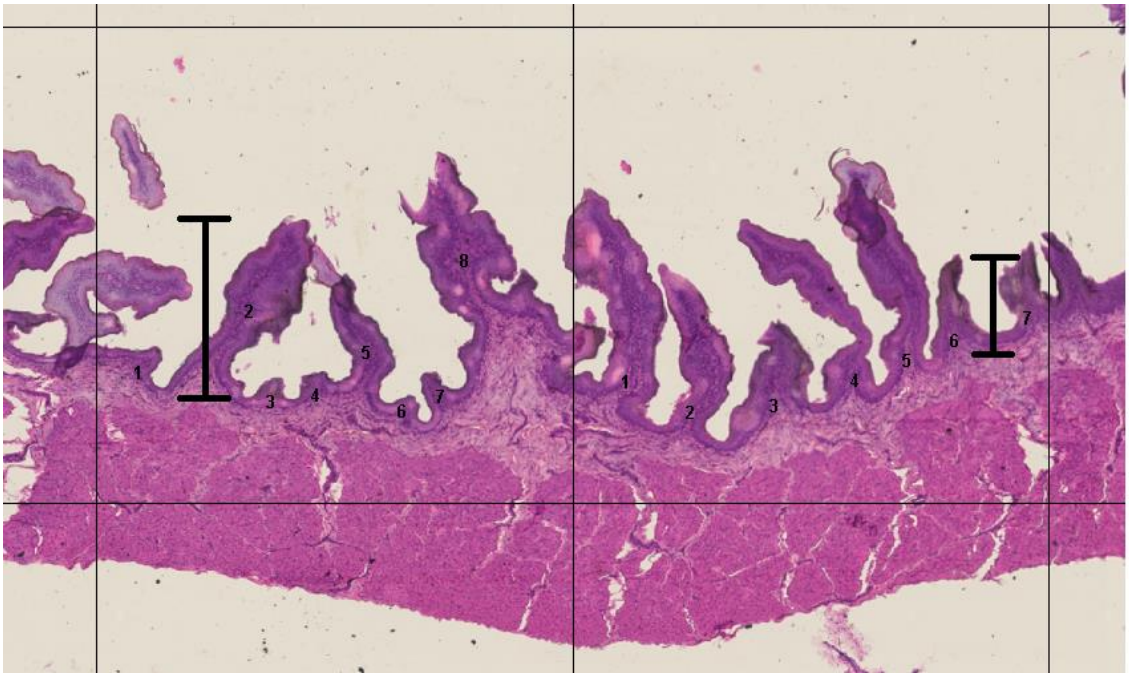
Supplementary figure S3: histological image of the jejunum of a calf from the CON group. Observe the tissue villi with the defined height from the end of the gland to the tip of the villi.



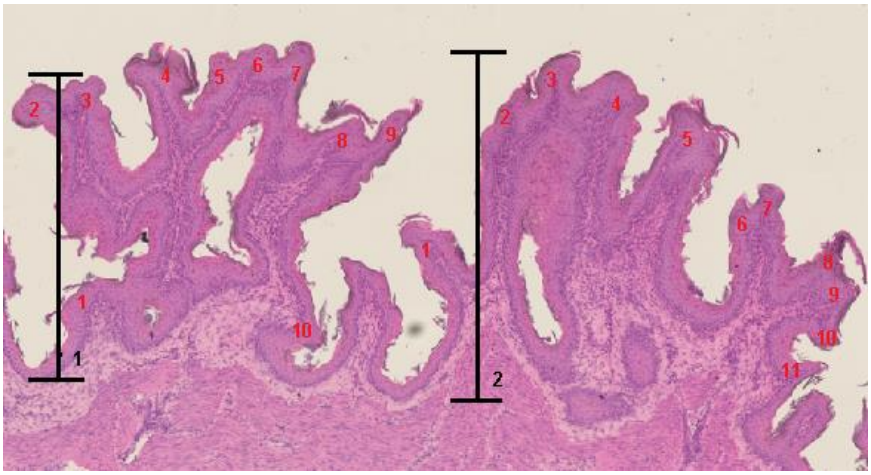
Supplementary figure S4: histological image of the jejunum of a calf from the OR group. Observe the tissue villi with the defined height from the end of the gland to the tip of the villi.



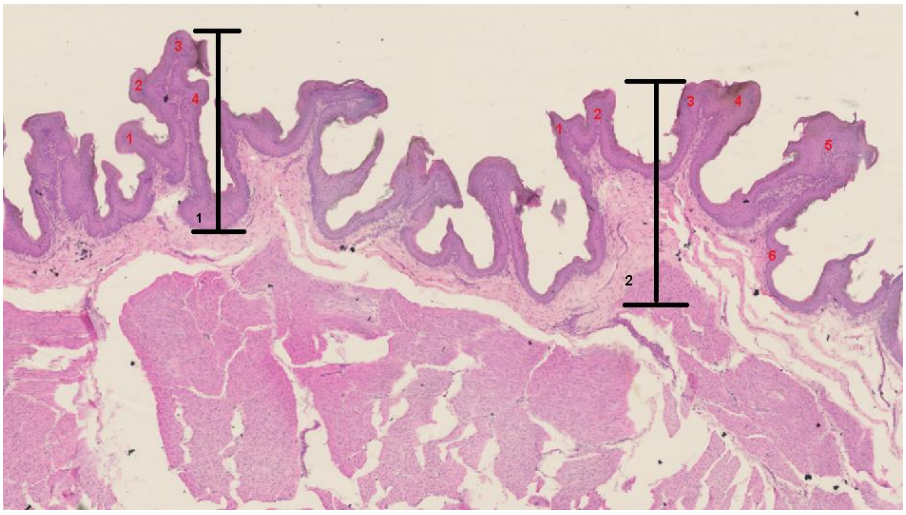
Supplementary figure S5: histological image of the calf rumen in the CON group. Observe the height of the ruminal papillae and the amount of ruminal papillae per area of 15000µ, with area A with 6 glands and area B with 6 glands



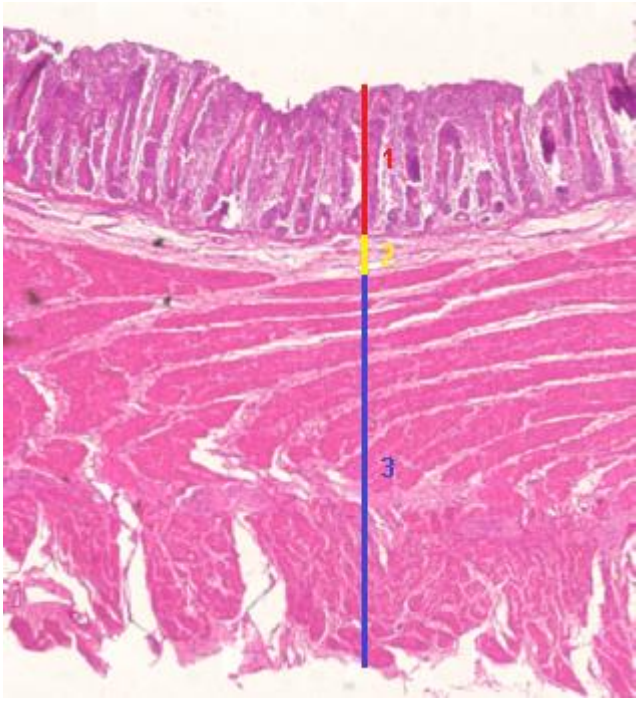
Supplementary figure S6: histological image of the rumen of the OR group calf. Observe the height of the ruminal papillae and the amount of ruminal papillae per area of 15000µ, with area A having 8 glands and area B having 7 glands.



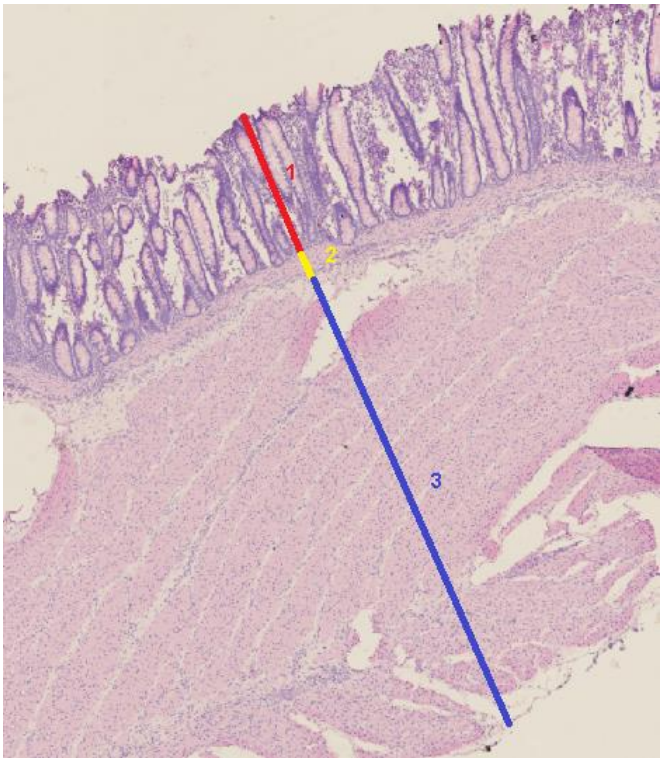
Supplementary figure S7: histological image of the calf rumen in COM group. Note the height of the branches and the number of ruminal papillae per branch, with branch 1 having 10 papillae and branch 2 having 11 papillae.



Supplemental figure S8: histological image of the rumen of the OR group calf. Note the height of the branches and the number of ruminal papillae per branch, with branch 1 having 4 papillae and branch 2 having 6 papillae



Supplementary figure S9: histological image of the calves cecum in the CON group. Note the thickness of the tunics: 1- mucosal tunic, 2- submucosal tunic and 3- muscular tunic.



Supplementary figure S10: histological image of the calves cecum of the OR group. Note the thickness of the tunics: 1- mucosal tunic, 2- submucosal tunic and 3- muscular tunic.

APÊNDICE 2

Carta de aprovação da CEUA

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 35666
Título: EXPRESSAO GENICA DE ENZIMAS E O PERFIL DE MICRORGANISMOS RUMINAIS E INTESTINAIS COM REFLEXOS SOBRE A SAUDE DE BEZERROS LEITEIROS SUPLEMENTADOS OU NAO COM EXTRATO DE OREGANO

Vigência: 01/11/2018 à 31/07/2020

Pesquisadores:

Equipe UFRGS:

VIVIAN FISCHER - coordenador desde 01/11/2018
André Gustavo Cabrera Dalto - pesquisador desde 01/11/2018
ELISA CRISTINA MODESTO - pesquisador desde 01/11/2018
GUILHERME HEISLER - Aluno de Mestrado desde 01/11/2018
LISIANE DA SILVEIRA GARCIA - Aluno de Especialização desde 01/11/2018
CINDY ANNE KLAUSBERGER XIMENES - Aluno de Especialização desde 01/11/2018
Angelica Tarouco Machado - Aluno de Mestrado desde 01/11/2018

Equipe Externa:

Camila Soares Souza - pesquisador desde 01/11/2018

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 03/12/2018 - Sala 323 - Anexo I do prédio da Reitoria - Campus Centro da UFRGS- Bairro Farroupilha - Porto Alegre, em seus aspectos éticos e metodológicos, para a utilização de 16 bezerros da raça Holandês, macho de três até sete dias de vida, oriundos de fazenda leiteira localizada na região da Serra Gaúcha; de acordo com os preceitos das Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008, o Decreto 6899 de 15 de julho de 2009, e as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), que disciplinam a produção, manutenção e/ou utilização de animais do filo Chordata, subfilo Vertebrata (exceto o homem) em atividade de ensino ou pesquisa.

Porto Alegre, Sexta-Feira, 14 de Dezembro de 2018

MARCELO MELLER ALIEVI
Coordenador da comissão de ética