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USO DE ÓLEOS ESSENCIAIS VIA DIETA LÍQUIDA DE BEZERROS LACTENTES: SEUS EFEITOS NO DESEMPENHO, SAÚDE, NÍVEIS ANTIOXIDANTES E MICROBIOTA INTESTINAL

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Dissertação apresentada como requisito parcial para obtenção do título de mestre em Zootecnia pelo Programa de Pós-Graduação em Zootecnia da Universidade do Estado de Santa Catarina – UDESC. Orientador: Prof. Dr. Aleksandro Schafer da Silva.

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RESUMO

A criação de bezerras é um dos principais desafios da bovinocultura de leite devido a fragilidade imunológica que possuem nas primeiras semanas de vida. É de extrema importância adotar métodos que proporcionem melhores condições imunes, já que se encontra imunologicamente incompetente, e a ocorrência de doenças será maior e seu desenvolvimento será diretamente afetado. Com isso, objetivou-se avaliar se uso de óleos essenciais de orégano, canela e eucalipto fornecidos como um *blend* a bezerros lactentes proporcionam melhorias de desempenho e saúde, bem como a redução da incidência de diarreia e alterações benéficas na composição da microbiota intestinal. Foram conduzidos dois estudos. No primeiro estudo foram utilizados 16 bezerros holandeses (dez dias de idade) aleitados por 60 dias. Foram divididos em dois grupos (n=8 cada), sendo controle (CONTROL) que recebeu sucedâneo comercial, e tratamento (PHYTO), que recebeu sucedâneo reformulado contendo o *blend*. Os animais foram aleitados duas vezes ao dia com 0,25 kg de sucedâneo diluído em dois litros de água a cada aleitamento. O sucedâneo do grupo tratamento continha 5g/kg de aditivo. Os bezerros também receberam concentrado peletizado desde o início do experimento e feno picado a partir dos 28 dias (ambos *ad libitum*). O grupo tratamento apresentou maior ganho de peso e eficiência alimentar, contagem menor de linfócitos, maiores níveis de proteínas totais, e aumento das globulinas. A imunoglobulina A sérica e as imunoglobulinas de cadeia pesada foram maiores no grupo tratamento, e também foram constatados menores níveis de peroxidação lipídica e maiores antioxidantes totais no soro de bezerros do grupo tratamento. No segundo estudo foram utilizados 24 bezerros da raça Holandês, os animais foram aleitados por 60 dias, e depois acompanhados por mais 15 (total de 75 dias). Foram divididos em grupo controle (n=12) e tratamento (n=12), ambos receberam sucedâneo comercial e concentrado peletizado de forma *ad libitum*. O grupo tratamento teve o *blend* adicionado ao sucedâneo duas vezes ao dia nas dosagens de 5 mL/aleitamento nos primeiros 15 dias e 10 mL/aleitamento até o dia 60. O grupo tratamento apresentou melhor conversão e eficiência alimentar, menor contagem de leucócitos e linfócitos e maior concentração de colesterol. Foi observado que os níveis de imunoglobulina A, ceruloplasmina e transferina também diferiram, beneficiando o grupo que recebeu o *blend*, assim como a enzima antioxidante glutationa S-transferase. Nesse segundo estudo foi avaliada a composição da microbiota intestinal e observou-se que não apresentou diferença entre os grupos, mas constatamos que gêneros *Acinetobacter*, *Pseudomonas* e *Psychrobacter* foram os três mais abundantes. E o *blend* de óleos não diminui a população de *E. coli* e *Clostridium spp*. Concluímos que o fornecimento conjunto desses óleos essenciais melhorou as respostas imunes humorais, minimizaram o estresse oxidativo fisiológico e, consequentemente, melhoraram o crescimento e o desempenho. No entanto, essas alterações não estão relacionadas a mudança da microbiota intestinal.

Palavras-chave: 1-8 Cineol; Antimicrobiano; Carvacrol; Cinamaldeído; Sistema imunológico.

ABSTRACT

Raising calf is one of the main challenges of dairy farming due to the immunological fragility they present in the first weeks of life. It is extremely important to adopt methods that provide better immunological conditions, as they are immunologically incompetent, and the occurrence of diseases will be greater and their development will be directly affected. With this, the objective was to evaluate whether the use of essential oils of oregano, cinnamon and eucalyptus supplied in blend form for suckling calves provide improvements in performance and health, as well as a reduction in the incidence of diarrhea and beneficial changes in the composition of the intestinal microbiota. Two studies were conducted. In the first study, 16 Holstein calves (ten days old) suckled for 60 days were used. They were divided into two groups (n=8 each), the control (CONTROL) receiving a commercial substitute and the treatment (PHYTO) receiving a reformulated substitute containing the mixture. The animals were fed twice a day with 0.25 kg of milk replacer diluted in two liters of water at each feeding. The treatment group surrogate contained 5 g/kg of additive. The calves also received pelleted concentrate from the beginning of the experiment and chopped hay from 28 days onwards (both ad libitum). The treatment group showed greater weight gain and feed efficiency, lower lymphocyte counts, higher levels of total protein and increased globulins. Serum immunoglobulin A and heavy chain immunoglobulins were higher in the treatment group, and lower levels of lipid peroxidation and higher total antioxidants were also found in the serum of calves in the treatment group. In the second study, 24 Holstein calves were used, the animals were breastfed for 60 days, and then monitored for another 15 days (total of 75 days). They were divided into control (n=12) and treatment (n=12) groups, both receiving commercial substitute and pelletized concentrate ad libitum. The treatment group had the mixture added to the substitute twice a day at dosages of 5 mL/feed in the first 15 days and 10 mL/feed until day 60. The treatment group showed better feed conversion and efficiency, lower leukocyte and lymphocyte counts and higher concentration of cholesterol. It was observed that the levels of immunoglobulin A, ceruloplasmin and transferin also differed, benefiting the group that received the mixture, as well as the antioxidant enzyme glutathione S-transferase. In this second study, the composition of the intestinal microbiota was evaluated and it was observed that there was no difference between the groups, but we found that the genera *Acinetobacter*, *Pseudomonas* and *Psychrobacter* were the three most abundant. And the blend of oils does not decrease the population of *E. coli* and *Clostridium spp*. We conclude that the joint supply of these essential oils improved humoral immune responses, minimized physiological oxidative stress and, consequently, improved growth and performance. However, these changes are not related to changes in the intestinal microbiota.

Key-words: 1-8 Cineol; Antimicrobial; Carvacrol; Cinnamaldehyde; Immune system.

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1. INTRODUÇÃO

A bovinocultura de leite é um setor produtivo que está em constante evolução e crescimento, e o Brasil é o 4º maior produtor mundial de leite, com destaque na região Sul e do estado de Minas Gerais (FAO, 2023; IBGE, 2023). Destaca-se que a produção está em constante crescimento mesmo com a redução no número de vacas ordenhadas (IBGE, 2017), mostrando a evolução do país em termo de produtividade. Dentro da pecuária nacional e mundial, o leite é um dos produtos de grande relevância (RIBEIRO JUNIOR *et al*., 2020), pois além do alto consumo, é um setor produtivo responsável por empregar diversas famílias.

Dentro da bovinocultura de leite tem-se várias fases produtivas, que vão desde a criação das bezerras até sua fase reprodutiva e lactação. Porém, vale ressaltar que a criação das bezerras é uma em que o animal apresenta grande sensibilidade e assim precisa de muita atenção. São animais pré-ruminantes ao nascerem e também possuem o sistema imunológico virgem, o qual desenvolverá ao longo dos dias, a fim de que e mortalidade seja inferior a 5% (LEITE *et al*., 2017).

A primeira linha de defesa é adquirida de forma passiva através da ingestão do colostro (CHASE *et al*., 2008), que garantirá a sobrevivência do neonato nas primeiras horas de vida e ao longo das semanas até que seu organismo seja capaz de desenvolver sua própria imunidade de forma eficiente. Ao longo das primeiras semanas de vida as bezerras ficam susceptíveis a diversas doenças, e dentre as principais temos as infecções causadoras de diarreia, que o principal problema são as consequências dessa enfermidade, que provoca desidratação e perda de peso, em decorrência da perda de apetite e baixa ingestão de líquido (CHO & YON, 2014), não sendo capaz de repor o que é perdido pelas fezes.

A incidência de diarreia afeta diretamente a microbiota intestinal dos bezerros, e por consequência todo o sistema imunológico, o qual auxilia na proteção contra patógenos no organismo, principalmente contra bactérias causadoras de diarreia (MAYNAR *et al*., 2012). Essa microbiota começa a se desenvolver logo ao nascer, aumentando drasticamente a carga bacteriana até as primeiras 24 horas de vida (ALIPOUR *et al*., 2018), e após o período de aleitamento a microbiota começa a se estabelecer (MAELE *et al*., 2016). O estabelecimento da microbiota intestinal é impactado principalmente pela dieta e fatores externos (KODITHUWAKKU *et al*., 2021; DU *et al*., 2023), inclusive pelo contato com outros bezerros, principalmente os que estejam com a saúde debilitada.

O uso de óleos essenciais vem sendo fortemente estudado por desempenharem um papel de antimicrobianos, mas também de melhoradores de saúde animal. Têm sido utilizados principalmente pela diminuição do uso de antibióticos devido aos riscos à saúde animal e humana (CHAPMAN *et al*., 2016). Os óleos de orégano e canela, possuem como os princípios bioativos carvacrol e cinamaldeído respectivamente, desempenham funções de antimicrobianos, antioxidantes, anti-inflamatórios e melhoradores do sistema imunológico (CALSAMIGLIA *et al*., 2007; CHAPMAN *et al*., 2016), e o óleo de eucalipto, com o 1,8-cineol como principal princípio bioativo também age como antimicrobiano e antioxidante, mas também possui função gastroprotetora (CALDAS *et al*., 2015). O óleo essencial de orégano é um dos mais estudados, demonstrando eficiência na nutrição de ruminantes, modulando a microbiota ruminal e consequentemente melhorando o desempenho desses animais (WU *et al*., 2020).

Atualmente não há conhecimento dos efeitos causados pelos óleos essenciais de orégano, canela e eucalipto utilizados de forma conjunta. Assim, o objetivo desse estudo foi avaliar se um *blend* desses óleos essenciais fornecidos a bezerros em fase de aleitamento proporciona melhoras no desempenho animal, no sistema imunológico e perfil antioxidante, reduz a incidência de diarreia e altera a microbiota intestinal.

2. REVISÃO BIBLIOGRÁFICA

2.1 BOVINOCULTURA DE LEITE

A produção de leite no Brasil vem ganhando bastante destaque, isso porque além da nossa produção estar em constante crescimento, esse setor agropecuário é responsável pela geração de empregos e renda de diversas famílias. No ano de 2021, o Brasil ocupava o 4º lugar em produção mundial, passando de 36 bilhões de litros de leite produzidos (FAO, 2023), com destaque na região Sul por ser a maior produtora, com os estados do Paraná, Rio Grande do Sul e Santa Catarina em 2º, 3º e 4º lugar, respectivamente, e no estado de Minas Gerais que é o maior produtor nacional de leite (IBGE, 2023).

Para entendermos a evolução do nosso país na pecuária leiteira basta olharmos para o cenário brasileiro nas ultimas décadas, no qual o último Censo Agropecuário realizado, sendo no ano de 2017 nos mostra que em 1970 a produção nacional era de pouco mais 6 milhões de litros, já em 2017 a produção foi para 30 bilhões anuais e com uma diminuição de 62% no número de vacas ordenhadas, comprovando o aumento considerável no desempenho em produtividade do nosso país. O aumento da produtividade pode ser justificada pela maior demanda do produto, pela melhoria genética dos animais, nutrição e pelo avanço em termos de tecnologia em cima desse setor como um todo.

O leite é o produto da pecuária de maior relevância dentro do comercio mundial (RIBEIRO JÚINIOR *et al*., 2020), já que é fonte de renda de boa parte da população. Além disso, o leite tem grande importância nutricional, pois é um alimento que contém proteína, vitaminas, minerais e diversos outros nutrientes de extrema importância para o desenvolvimento humano. Devido a essa importância econômica e nutricional, o mercado consumidor está cada vez mais exigente, requerendo produtos mais sustentáveis, funcionais, nutritivos e que visem o bem-estar dos animais e do próprio consumidor (SIQUEIRA, 2019).

Visto o cenário atual da produção leiteira e da sua importância, precisamos olhar com atenção para todas as fases de produção, e pensar sempre em melhorar o desenvolvimento, desempenho, saúde e bem-estar desses animais. Assim, a criação das bezerras se torna um pilar de extrema importância, visto que são as futuras produtoras da propriedade e são animais de grande fragilidade que necessitam de muita atenção para que se desenvolvam de forma adequada, minimizando a chance de possíveis morbidades e reduzindo o percentual de mortalidade.

2.2 CRIAÇÃO DE BEZERRAS LACTENTES

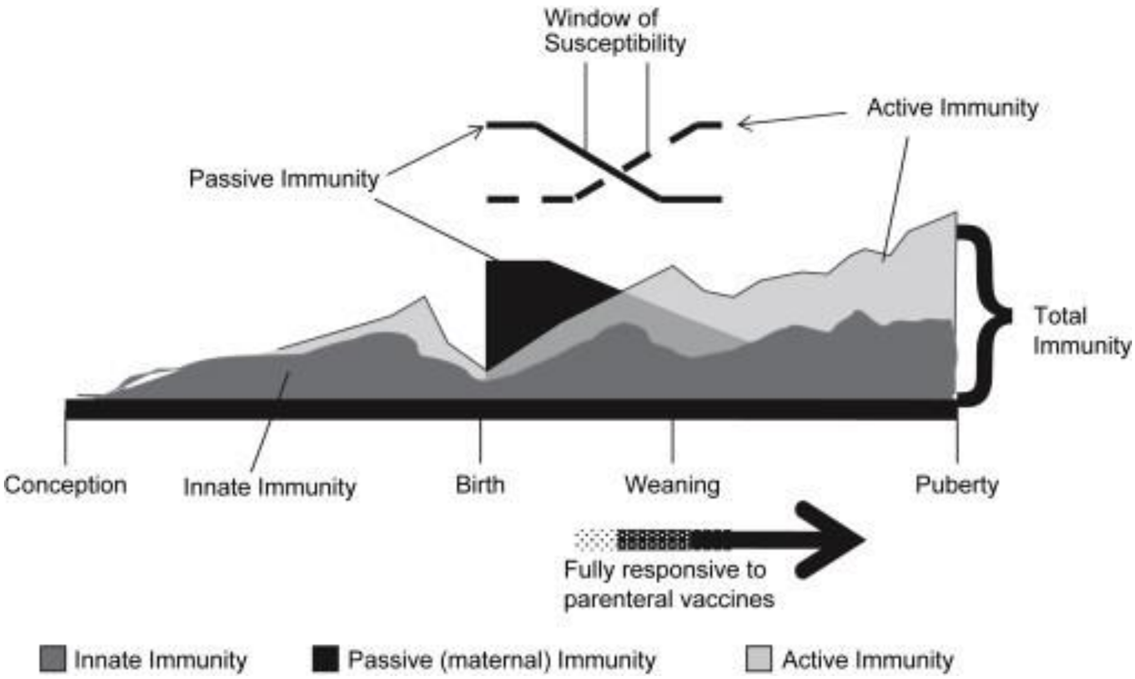
Bezerras são animais consideradas pré-ruminantes, que além disso, são imcompetentes imunologicamente. Esses dois fatores primordiais precisam ser desenvolvidos ao longo das primeiras semanas de vida, pois a estimular o sistema imunológico é importante a fim de evitar quadros infecciosos, reduzir os índices de morbidade e mortalidade de animais neonatos nas propriedades, que deve ser inferior a 5% (LEITE *et al.*, 2017).

Essa fragilidade imunológica acontece principalmente devido ao tipo de placenta (sindesmocorial) que os ruminantes possuem, pois a mesma possui seis camadas, impedindo que proteínas como imunoglobulinas e alguns outros nutrientes sejam passados da mãe para o feto (CHASE *et al.*, 2008; SANTOS *et al.*, 2002). O que precisa ser levado em consideração nessa fase é o fato de que a forma com que os animais se desenvolvem no início da vida implica diretamente no seu desempenho futuro; o que define a qualidade dos animais que farão parte do rebanho produtivo da propriedade (AZEVEDO *et al.*, 2016).

Enquanto ainda feto, a resposta predominante no organismo do bovino é a inata, mas ao passar das semanas de gestação essa resposta imune vai diminuindo devido ao aumento de hormônios como o cortisol no final da gestação e durante o parto (CHASE *et al.*, 2008; GOMES *et al.*, 2014). A primeira imunidade do neonato vai ser adquirida somente após a ingestão de colostro, que possui os nutrientes necessários para a fase inicial, imunoglobulinas, leucócitos maternos, hormônios, e outros componentes que garantirão a sobrevivência do bezerro nos primeiros dias (CHASE *et al.*, 2008; GODDEN, 2008). O colostro para ter a eficiência esperada deve ser fornecido no máximo nas primeiras seis horas de vida, mas o ideal é que seja imediatamente após o nascimento, pois conforme passa o tempo pós-parto, a capacidade de absorção da bezerra diminui consideravelmente (RUFINO, 2014).

Os animais em questão devem receber de 10 a 15% do peso vivo em colostro, quando fornecido colostro dentro da qualidade ideal (LASKOSKI *et al.*, 2020), assim como de 24 a 48 horas após a colostragem os índices séricos de IgG e proteína total devem ser de no mínimo 10mg/mL e 5,5 g/dL respectivamente, de acordo com o método utilizado para avaliação (GOMES, 2018). Assim podemos dizer que a colostragem foi bem realizada e houve uma boa transferência de imunidade passiva. Os anticorpos obtidos pela transferência de imunidade passiva se mantém até a 4ª a 6ª semana de vida, e após esse período, diminuem o animal entra num período crítico chamado "janela imunológica" (Figura 1), pois os anticorpos endógenos ainda não são suficientes (BITTAR, *et al.*, 2018; CHASE *et al.*, 2008), tendo entre 6 a 8 semanas de vida como o período de maior estresse e desafio para os bezerros (UYENO *et al*., 2010), incluindo o período de desmame, que sabemos que causam grande estresse a eles também.

Figura 1 – Desenvolvimento da resposta imunológica de bezerros



Fonte: CHASE *et al*. (2008).

Além da colostragem existem outros fatores que precisam ser considerados quando falamos em desenvolvimento imunológico de bezerras, como a cura do umbigo, fornecimento de abrigo adequado, água limpa e concentrado para estimular seu desenvolvimento ruminal e desempenho. A cura do umbigo deve ser realizada porque o mesmo serve de porta de entrada para vários microrganismos (TORQUATO, 2018). Devemos ter um olhar geral sobre estes animais, ficar atentos a qualquer alteração de comportamento e sinais clínicos, pois eles podem adquirir infecções e desenvolver doenças com rápida evolução para quadros agudos. Outro fator de extrema importância para o desenvolvimento desses pré-rumiantes é a parte nutricional, que além da dieta líquida normalmente a base de leite ou de seus substitutos como o sucedâneo, é preciso ofertar aos animais uma dieta sólida a base de concentrado que irá promover o desenvolvimento ruminal de forma adequada (HEINRICHS e JONES, 2003).

Os bezerros devem ser alojados de forma adequada em instalações que proporcionem condições ideais para seu desenvolvimento, pois neonatos não são capazes de regular sua temperatura corporal quando desafiados por condições climáticas adversas, induzindo condições de estresse térmico, o que irá debilitar ainda mais seu sistema imunológico (CHO e

YON, 2014). Davis e Drackley (1998) mostraram que a termoneutralidade de bezerros jovens varia de 15 a 25ºC, variando de acordo com peso, idade, condições ambientais e alguns outros fatores estressores.

As infecções mais frequentes em bezerras lactentes são as intestinais que causam diarreia e as que causam pneumonia (SLANZON, 2018), e são casos que normalmente precisam de intervenção com medicamentos, principalmente antimicrobianos, que quando em excesso se tornam um grande problema devido ao desenvolvimento de cepas bacterianas resistentes aos antibióticos em questão.

A diarreia como já citado é um dos principais fatores prejudiciais ao animal, e acaba sendo um sinal clínico ocasionado pela infecção de algum microrganismo patogênico na microbiota intestinal, e junto com a diarreia normalmente se tem outros sinais clínicos, como desidratação, perda de apetite e perda de peso (CHO e YON, 2014). Além da diarreia infecciosa, a não infecciosa também afeta os animais, e ela ocorre devido a problemas na digestão dos nutrientes da dieta líquida, o que se da principalmente por problemas de manejo, como o fornecimento do mesmo na temperatura fora do adequado, má higienização dos utensílios, mudanças bruscas na dieta e uso de substitutos do leite de baixa qualidade (BITTAR et al., 2018). A evolução do quadro de diarreia pode ser rápida, e por consequência trazer outros distúrbios que levem o animal ao óbito. Schinwald *et al.* (2022) relataram que durante seu estudo de mapeamento que a mortalidade por diarreia chegou 33,3%. Urie *et al*. (2018) evidenciaram que em fazendas dos Estados Unidos da América, 50,9% dos bezerros doentes estavam acometidos com doenças digestivas, e de todos que morreram, 32% morreram por conta dessas doenças digestivas, em ambos os casos tendo o número de animais com doenças respiratórias como a segunda doença mais importante.

2.2.1 Microbiota intestinal de bezerros

A microbiota do trato intestinal tem um papel de extrema importância no estabelecimento do sistema imunológico e na proteção do hospedeiro contra patógenos no organismo (MAYNARD *et al*., 2012). Além disso, o trato gastrointestinal tem como função primária fazer a digestão e absorção dos nutrientes (MAELE *et al*., 2017). O mesmo é considerado estéril ao nascer, mas começa a ser colonizados logo após o nascimento de forma rápida (UYENO *et al*., 2010; KLEIN-JOBSTL *et al*., 2014; ALIPOUR *et al*., 2018), e esse desenvolvimento é impactado pela interação do hospedeiro com os microrganismos, pela microbiota materna, momento do parto, e principalmente pela dieta e fatores externos

(KODITHUWAKKU *et al*., 2021; DU *et al*., 2023), do nascimento até as primeiras 24 horas de vida a carga bacteriana intestinal dos bovinos pode aumentar em 7000 vezes (ALIPOUR *et al*., 2018)

Sabemos que durante a gestação a mãe não consegue passar ao bezerro proteínas como as imunoglobulinas, destacando aqui a IgA que é um componente importante do sistema imunológico que está presente na mucosa intestinal (DU *et al*., 2023). A IgA, especificamente a secretora (SIgA), possui capacidade de detectar bactérias que são prejudiciais ao hospedeiro e elimina-las do organismo ao opzoniza-las, isso porque essas bactérias liberam toxinas que se aderem às células do epitélio facilitando o processo de detecção, fazendo com que as IgA selecionem a microbiota benéfica que irá colonizar o intestino, mas isso claro, se o colostro fornecido for de alta qualidade (DU *et al*., 2023).

A diversidade da microbiota intestinal dos bezerros recém-nascidos se apresenta maior que em bezerros mais velhos (ALIPOUR *et al*. 2018), ou seja, essa diversidade vai diminuindo conforme a microbiota vai se adaptando as condições que o animal está, pois de acordo com os pesquisadores a microbiota dos bezerros aos 7 dias de vida se assemelha muito a microbiota de bovinos adultos. Uyeno *et al*. (2010) sugerem que bezerros tenham grandes mudanças na microbiota intestinal nos primeiros três meses de vida, estando ligado ao desenvolvimento fisiológico do trato gastrointestinal. Meale *et al*. (2016) também mostrou que no pós-desmame a microbiota fecal de bezerras é mais uniforme do que durante a fase de aleitamento, sugerindo uma certa estabilidade da população microbiana.

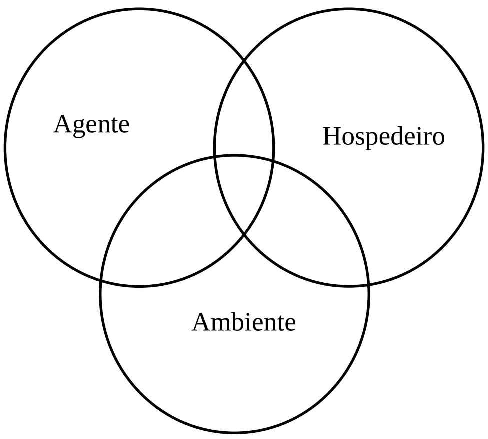
Como um dos fatores que afetam a microbiota intestinal do bezerro, temos a microbiota da mãe. Klein-Jöbstl *et al*. (2019) sugerem que a microbiota fecal do bezerro é afetada grandemente pelas bactérias presentes no canal vaginal da mãe no momento do parto, pois em seu estudo é possível ver essa semelhança de microbiotas logo ao nascer, e que inclusive é distinta da microbiota do colostro.

Quando falamos que a dieta influencia na microbiota intestinal temos que levar em consideração a dieta líquida e sólida, assim como o fornecimento de água. A dieta líquida consiste basicamente no fornecimento de leite e/ou seus substitutos, principalmente o leite em pó, o sucedâneo e leite não comercializável. O leite integral é a fonte de dieta líquida mais recomendada devido à sua composição. O sucedâneo é um substituto do leite feito de forma industrial, que tem por característica a fácil conservação e a ampla gama de ingredientes na sua produção, sendo considerado estéril microbiologicamente mas muitas vezes formulados com alta inclusão de produtos vegetais para aumentar as concentrações de proteína e carboidratos de forma mais econômica, o que afeta diretamente a sua digestibilidade podendo causar um desbalanço no organismo do bezerro provocando diarreia não infecciosa, eliminando microrganismos benéficos pelas fezes e deixando o animal ainda mais frágil e susceptível a infecção de microrganismos patogênicos causadores de diarreia (BITTAR *et al*., 2018).

Outro problema de extrema importância é o uso de leite não comercializável, que são basicamente leites oriundos de vacas com mastite ou que estão em tratamento por antimicrobianos e por isso não podem ser comercializados. O leite de vacas com mastite apresenta composição nutricional variável e alta carga bacteriana, aumentando a incidência de diarreia; e no caso de vacas em tratamento, há uma alta probabilidade de haver resíduos dos antibióticos no leite podendo desenvolver resistência das bactérias aos fármacos (BITTAR *et al*., 2018). Já dieta sólida desses animais é composta basicamente por concentrado, ao passar das semanas o consumo desse alimento vai aumentando a fim de suprir a demanda do animal para um bom desempenho, e durante o consumo desse alimento vários microrganismos que estão aderidos as partículas colonizam o trato gastrintestinal, além de que a ingestão de alimento sólido favorece o desenvolvimento e o início da fermentação ruminal, alterando por consequência a microbiota intestinal de forma indireta (DU *et al*., 2023).

Além desses fatores, o ambiente em que o bezerro está alojado também pode afetar o desenvolvimento da microbiota intestinal. O sistema de criação pode estimular o crescimento de microrganismos patogênicos no ambiene expondo o animal a doenças e afetando seu sistema imunológico, já que o ambiente faz parte da tríade epidemiológica das doenças apresentada na Figura 2 (MOORE *et al*., 2007).

Figura 2 - Tríade epidemiológica



Fonte: adaptado de Moore *et al*. (2007).

Existem diferentes tipos de alojamentos para esses animais, mas a característica principal deles é ser individual ou coletivo, e cada um traz seus prós e contras. O que precisamos ressaltar é que quando falamos de microbiota intestinal falamos em agentes causadores de diarreia (que podem ser patogênicos ou não), e que são contagiosos entre os animais, e o ideal é que pelo menos nas primeiras 4 semanas de vida os animais fiquem em abrigos individuais para tentar minimizar o contagio de microrganismos causadores de infecções intestinais e outras doenças (MOORE *et al*., 2007). Um ponto importante em relação ao ambiente é a limpeza e desinfecção do local, principalmente quando já se tem algum animal doente que possa ter deixado alguma secreção, pois as bactérias ficam no ambiente por um determinado tempo, e na Tabela 1 conseguimos observar as principais bacterias causadoras de diarreia e o tempo que as mesmas resistem no ambiente.

Tabela 1 - Características das duas principais bactérias causadoras de diarreia em bezerros

Bactérias	**Transmissão**	**Tempo de Excreção**	**Persistência no Ambiente**
Salmonella spp.	Oral/fecal e ambiente	30 a 50 dias	Até 2 anos
E. coli	Oral/fecal e ambiente	2 dias	Até 3 meses

Fonte: adaptado de Bittar *et al*. (2018) e Moore *et al*. (2007).

A microbiota do trato gastrointestinal como um todo pode ser alterada e modulada de diversas maneiras, por situações do ambiente ou propositalmente; e a principal forma de alteração/modulação é com o uso de aditivos via dieta, como pro e prébioticos, leveduras, extratos vegetais de diferentes formas e com óleos essenciais (JOUANY e MORGAVI, 2007). Essa modulação se torna mais eficaz quando feita no início da vida do animal, antes que os microrganismos presentes se tornem estáveis e, consequentemente mais difíceis de se alterarem (DILL-McFARLAND *et al*., 2018). No Quadro 1 podemos observar experimentos que avaliaram a composição da microbiota intestinal de bezerros em fase de aleitamento.

Quadro 1 - Trabalhos que avaliaram a microbiota intestinal de bezerros em fase de aleitamento

Autores	Tratamento	Resultado
Luo *et al*., 2022	Suplementação de β-glucana	Aumentou a abundância da microbiota intestinal.
Hang *et al*., 2021	Se a microbiota intestinal do bezerro está relaciona a microbiota do colostro	Não houve associação entre as microbiotas.
Hummel *et al*., 2021	Nutrição da mãe: - Controle com e sem suplementação; - Restrição alimentar com e sem suplementação.	Suplementação mineral da mãe aumentou a diversidade da microbiota intestinal fetal.
Liu *et al*., 2023	Rastreamento da dinâmica da microbiota fecal	A alfa-diversidade aumentou com a idade e o desenvolvimento dos bezerros.
Liu *et al*., 2022	Suplementação com tibutirina	Tibutirina estimulou a colonização de bactérias produtoras de AGCC.
Lu *et al*., 2022	Fornecimento de *Saccharomyces cerevisiae*	Aumentou a abundância de *Lactobacillus* **e** *Blautia, e diminuiu de Fusobacterium.*
Kodithuwakku *et al*., 2021	Fornecimento de fibra durantes os primeiros 21 dias de vida.	Melhorou a colonização de bactérias benéficas e reduziu a abundância das patogênicas.
Zhang *et al*., 2022	Aleitamento com sucedâneo e leite de descarte em diferentes proporções.	Aleitamento com 100% de leite de descarte aumentou a abundância de *Prevotellaceae* e diminuiu de *Bacteroides*.
Badman *et al*., 2019	Fornecimento de dois diferentes sucedâneos.	A composição dos sucedâneos pode afetar o estabelecimento da microbiota intestinal.

Fonte: adaptado pelo autor.

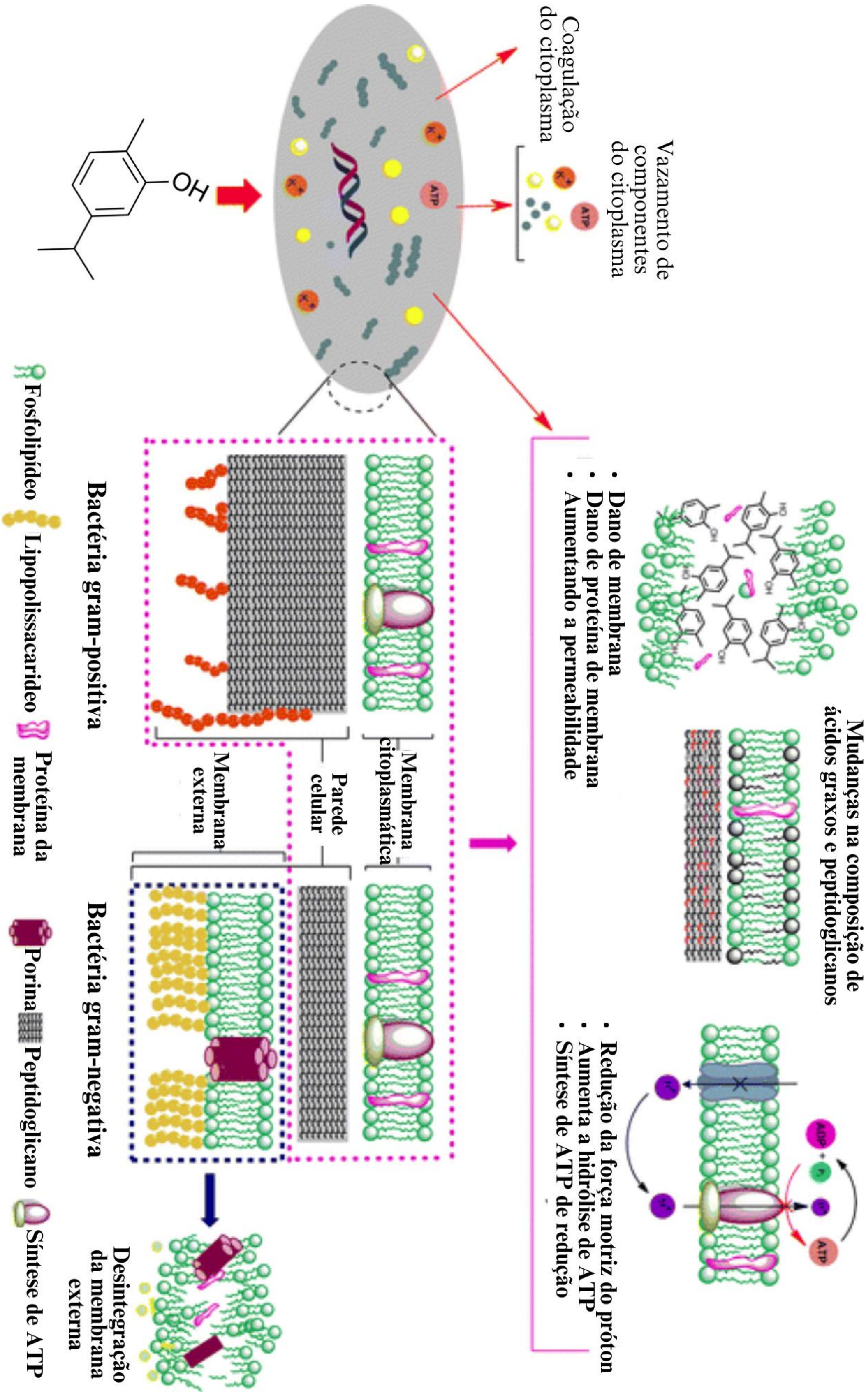
E analisando todos esses fatores relacionados as bezerras, é necessário encontrar alternativas para minimizar esses impactos negativos e melhorar os fatores de saúde e desempenho. Nesse sentido, os óleos essenciais apresentam resultados positivos na melhoria de saúde e desempenho de bovinos como um todo, mas também de bezerros, principalmente relacionado a colonização de microrganismos e a saúde do animal.

2.3 ÓLEOS ESSENCIAIS

São aditivos líquidos, aromático e voláteis que são extraídos de fontes vegetais como sementes, folhas, galhos, cascas de árvores e outras fontes (BURT, *et al* 2004; CUI *et al*., 2019). Os óleos essenciais estão ganhando cada vez mais espaço no meio científico e consequentemente no meio comercial, pois os mesmos ocupam espaço de melhoradores de desempenho, antioxidantes, antimicrobianos e potencializadores de sistema imunológico. Têm sido utilizados principalmente pela diminuição do uso de antibióticos na nutrição animal, devido aos riscos à saúde animal e humana (CHAPMAN *et al*., 2016; VAKILI *et al*., 2013). É de extrema importância entender a composição de cada óleo essencial, pois irá influenciar a forma de ação do produto e as suas funções, bem como determinam seu odor, viscosidade, densidade, textura, forma de penetração celular e fixação, e também onde cada óleo irá atuar dentro do organismo (BAPTISTA-SILVA *et al*., 2020). Os óleos essenciais utilizados nesse estudo são os de canela, orégano e eucalipto, motivo pelo qual serão os principais relacionados em nossa revisão de literatura.

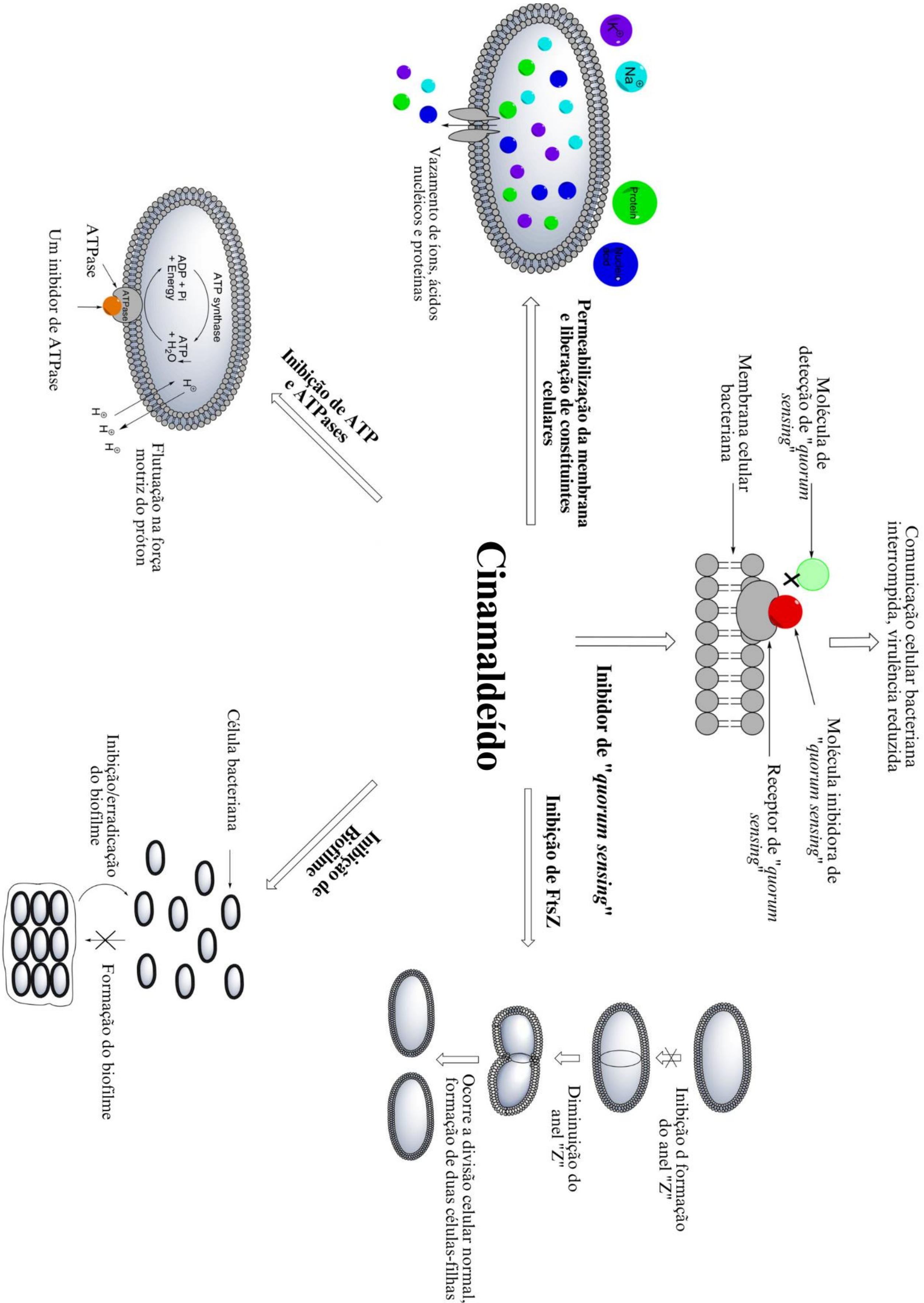
As espécies mais comuns de canela são *Cinnamomum cassia* (canela chinesa) e *Cinnamomum verum* (canela verdadeira), sendo que o óleo essencial de canela é extraído da *C. verum* devido a melhor qualidade da casca (SHREAZ *et al*., 2016). O cinamaldeído é o principal componente bioativo da canela, e o carvacrol é o composto bioativo presente no orégano e no tomilho, e ambos os componentes possuem grande atividade antimicrobiana (CALSAMIGLIA *et al*., 2007; CHAPMAN *et al*., 2016). O óleo essencial de orégano além disso possui potencial de melhorador de desempenho em ruminantes, pois o mesmo pode melhorar a conversão alimentar e a saúde dos mesmos ao atuar na modulação da fermentação ruminal (WU *et al*., 2020). Nas Figuras 3 e 4 estão descritos os mecanismos pelos quais o cinamaldeído e o carvacrol atuam como antimicrobiano.

Figura 3 - Mecanismos de ação antibacteriano do carvacrol



Fonte: adaptado de Marinelli *et al*. (2018)

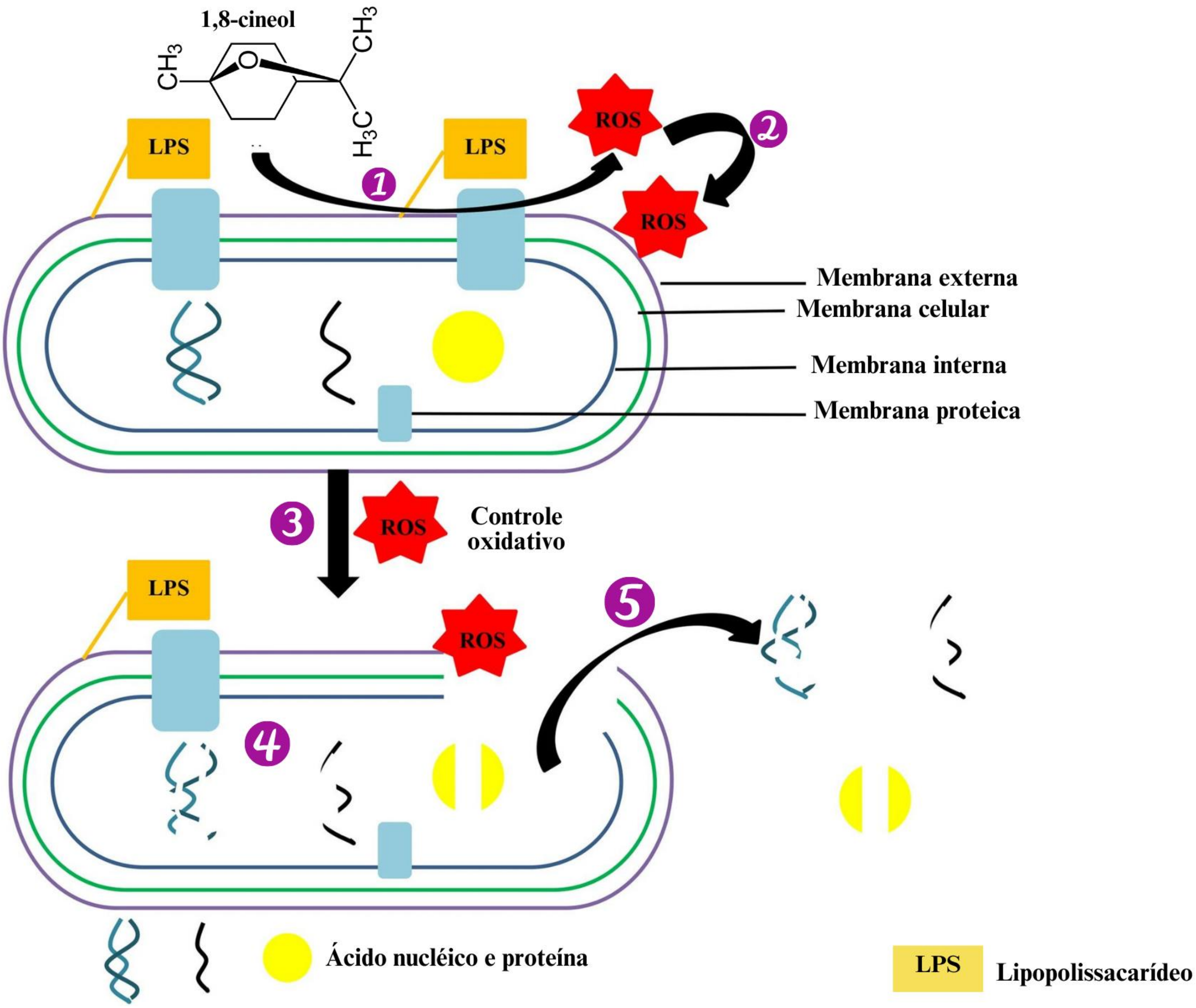
Figura 4 - Mecanismos antibacterianos do cinamaldeído



Fonte: adaptado de John e Stephens (2019).

De acordo com Kumar *et al*. (2012), o óleo essencial extraído das folhas de eucalipto é composto majoritariamente por 1,8-cineol, α-pineno e D-limoneno. O 1,8-cineol se torna o principal componente do óleo de eucalipto (BAPTISTA-SILVA *et al*., 2020) devido a sua alta concentração, que pode chegar a 97,32% da composição de acordo com o tipo de eucalipto de onde o óleo for extraído (ALDOGHAIM *et al*., 2018). O 1,8-cineol também age como antimicrobiano, o seu mecanismo de ação está demonstrado na Figura 5 (MOO *et al*., 2021), e possui atividade gastroprotetora e antioxidante, no sistema gastrointestinal o composto possui capacidade de regenerar as células gástricas e aumentar muco gástrico (CALDAS *et al*., 2015).

Figura 5 - Ação antimicrobiana do 1,8-cineol



Fonte: adaptado de Moore *et al*. (2021).

A capacidade antioxidante é atribuída a capacidade que o composto tem de fazer a ligação com o sistema keap1/nrf2, que resulta no aumento de enzimas como a superóxido dismutase e a catalase, que desempenham papel antioxidante (CAI *et al*., 2020). Conforme demostrado no trabalho dos mesmos autores, a propriedade antimicrobiana do 1,8-cineol não é bem definida, porém é possível observar seu papel adjuvante a outras substâncias com capacidade de combate às bactérias.

No Quadro 2 estão descritos alguns resultados de artigos que utilizaram óleos essenciais e/ou os princípios ativos destacado ao longo desse trabalho com bezerros em fase de aleitamento.

Quadro 2 - Artigos publicados utilizando os óleos essenciais e princípios ativos abordados nesse trabalho com bezerros em fase de aleitamento

Autores	Tratamento	Resultado
Ritt *et al*., 2021	Óleo de orégano	Maior diversidade da população bacteriana no rúmen e jejuno.
Volpato *et al*., 2019	*Blend* de óleos com carvacrol, cinamaldeído, aroma de eucalipto e resina de óleo de páprica	Minimizou as infecções bacterianas, melhorou a saúde geral e aumentou o ganho de peso.
Katsoulos *et al*., 2017	Óleo de orégano por apenas 10 dias	Diminui a gravidade da diarreia.
Tapki *et al*., 2020	Óleo de orégano	Melhorou eficiência alimentar, saúde e desempenho.
Seirafy e Sobhanirad, 2017	Óleo de orégano e tomilho de forma isolada e conjunta	O óleo de orégano aumentou o nível de linfócitos; e o uso conjunto melhorou os parâmetros de ingestão de concentrado.
Selvi e Tapki, 2019	Óleo de orégano	Diminuiu a idade de início do consumo de concentrado.
Soltan, 2009	*Blend* com óleo de eucalipto, cristal de mentol e óleo de menta.	Reduziu a incidência de diarreia e melhorou o escore geral de saúde.
Santos *et al*., 2015	Carvacrol, cineol, cinamaldeído e resina de óleo de pimenta	Maior proporção de microrganismos benéficos na microbiota intestinal.

Fonte: adaptado pelo autor.

Visto as propriedades dos óleos essenciais, bem como suas principais funções, entende-se que podem proporcionar efeito antimicrobiano em bactérias entéricas patogênicas causadoras de distúrbios como diarreia em bezerros em fase de aleitamento, além de proporcionar a modulação do perfil fermentativo por parte das bactérias nos mesmos animais, afetando então o aproveitamento dos alimentos, bem como, o desempenho dos animais.

3. ARTIGO 1: Inclusion of essential oils in a calf milk replacer and their effects on growth performance and the immune and oxidative systems

Os resultados desta dissertação são apresentados na forma de artigo, com a seções de acordo com as orientações da Revista Ciência Rural.

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Highlights

- Added of essential oils in the milk replacer improved calf performance.

- The milk replacer provides a source of humoral immune elements and antioxidants, minimizing the physiological oxidative stress of calves.

- The inclusion of essential oils in the milk replacer has a strong odor of essences; and caused 25% of the animals to reject the milk.

3.1 ABSTRACT

This study determined whether a reformulated milk replacer containing a blend of essential oils composed of cinnamon, oregano, and eucalyptus improves suckling calves' immune and oxidative systems, consequently improving productive performance. Sixteen Holstein calves (ten days old) suckled for 60 days. The animals were divided into a control group (n = 8), which received the commercial milk replacer, and a treatment group (n = 8), which received a milk replacer containing oils. The animals were fed twice daily with 0.25 kg of milk replacer diluted in two liters of water at each feeding. The milk replacer in the treatment group contained 5 g of additive per kg. The milk replacer containing essential oils had a strong aroma and odor and consequently was rejected by two calves in the treatment group. These animals were withdrawn from the experiment after five days of attempting to adapt them. The calves also received pelleted concentrate from the beginning of the experiment and chopped hay from 28 days onwards (both ad libitum). The treatment group showed more weight gain and feed efficiency than control animals. Lower lymphocyte counts were observed in calves fed milk replacers containing essential oils. Total serum protein was higher due to increased globulins in calves in the treatment group than in the control group. Serum immunoglobulin A and heavy-chain immunoglobulins were higher in the treatment group. Lower levels of lipid peroxidation and higher total antioxidants were observed in the serum of calves in the treatment group. We conclude that some animals may not adapt to the milk replacer containing essential oils due to the odor; however, the calves that consumed the milk containing the essential oils showed stimulated humoral immune responses that minimized the physiological oxidative stress of the rearing phase and consequently favored growth and weight gain.

Keywords: carvacrol; thymol; 1,8-cyneol; cinnamaldehyde; antimicrobial.

3.2 RESUMO

Este estudo avaliou se um sucedâneo do leite contendo uma mistura de óleos essenciais compostos de canela, orégano e eucalipto melhora os sistemas imunológico e oxidativo de bezerros lactentes, consequentemente melhorando o desempenho produtivo. Dezesseis bezerros holandeses (dez dias de idade) amamentaram por 60 dias. Os animais foram divididos em grupo controle (n = 8), que recebeu sucedâneo comercial do leite, e grupo tratamento (n = 8), que recebeu sucedâneo contendo óleos. Os animais foram alimentados duas vezes ao dia com 0,25 kg de sucedâneo do leite diluído em dois litros de água a cada aleitamento. O sucedâneo no grupo de tratamento continha 5 g de aditivo por kg. O sucedâneo contendo óleos essenciais tinha forte aroma e odor e, conseqüentemente, foi rejeitado por dois bezerros do grupo de tratamento. Esses animais foram retirados do experimento após cinco dias de tentativas de adaptação. Os bezerros também receberam concentrado peletizado desde o início do experimento e feno picado a partir dos 28 dias (ambos ad libitum). O grupo tratamento apresentou maior ganho de peso e eficiência alimentar do que os animais controle. Contagem menor de linfócitos foram observadas em bezerros alimentados com sucedâneos do leite contendo óleos essenciais. A proteína sérica total foi maior devido ao aumento de globulinas em bezerros no grupo de tratamento do que no grupo controle. A imunoglobulina A sérica e as imunoglobulinas de cadeia pesada foram maiores no grupo de tratamento. Menores níveis de peroxidação lipídica e maiores antioxidantes totais foram observados no soro de bezerros do grupo tratamento. Concluímos que alguns animais podem não se adaptar ao sucedâneo do leite contendo óleos essenciais devido ao odor; no entanto, os bezerros que consumiram o leite contendo os óleos essenciais apresentaram respostas imunes humorais estimuladas que minimizaram o estresse oxidativo fisiológico da fase de recria e, conseqüentemente, favoreceram o crescimento e o ganho de peso.

Palavras-chave: carvacrol; timol; 1,8-cineol; cinamaldeído; antimicrobiano.

3.3 INTRODUCTION

Calf development is a significant challenge in dairy production due to the high fragility of these animals, which directly affects productivity. Their development is compromised when their health is challenged, primarily because the immune system begins to develop only after birth. Ruminant placentas, which include a protective layer, do not allow immunoglobulins to pass from the mother to the fetus (CHASE et al., 2008), making their immune system dependent on passive immunity, arising from the colostrum produced by the mother, which must be consumed in the first hours after birth. Colostrum is rich in immunoglobulins and other nutrients, providing the necessary components for the animal to face the microbiological challenges of the environment in the first days of life (HILL, 2010) and develop active immunity.

Many infectious agents cause disease in calves. *Enterobacteria* and *Escherichia coli* are the most common; these organisms are responsible for lesions in the intestinal epithelium (CHO & YOON, 2014) and cause diarrhea, which leads to dehydration and loss of appetite, affecting development. The high incidence of bacterial diarrhea in the first weeks of life makes antimicrobial therapy necessary; however, these treatments lead to antibiotic resistance. For these reasons, replacing antibiotics with alternative antimicrobials, which act as growth promoters, is imperative. Such replacements include commercially-available plant extracts. Many plant components inhibit pathogenic gram-negative (e.g., Escherichia coli, Campylobacter jejuni) and gram-positive (e.g., Bacillus subtilis, Clostridium) bacteria. Typically, gram-negative organisms are less sensitive to essential oils due to their external lipopolysaccharide membrane that restricts the diffusion of phenolic compounds (CHRISTAKI et al., 2020).

The potential of essential oils in animal nutrition is well understood, especially regarding the role of growth promoters, immunostimulants, and antioxidants (CHRISTAKI et al., 2020). Antioxidant capacity is directly related to health, and under normal conditions, there is a balance between the generation and elimination of free radicals. Imbalance results in oxidative stress in the animal and reduces performance; however, compounds in essential oils can prevent and even inhibit this stress by eliminating free radicals (PAN et al., 2023).

Essential oils are aromatic and volatile products extracted from plant sources such as branches, seeds, bark, leaves, and flowers (BURT, 2004). These compounds are characterized by strong odors that can interfere with feed ingestion. This concern is frequent among researchers, like when cinnamon, oregano, and eucalyptus essential oils were added to the replacement. These oils are compelling for researchers and industry because they contain cinnamaldehyde (cinnamon oil), carvacrol, thymol (oregano oil), 1,8-cineol, and D-limonene (eucalyptus oil), which display antimicrobial activity (CALSAMIGLIA et al., 2007; Chapman et al., 2016; KUMAr et al., 2012; CIMANGA et al., 2002; BARANSKA et al., 2005). Oregano's essential oil reduces feed conversion and positively modulates ruminal fermentation (WU et al., 2020). 1,8-cineol increases gastric mucus production inhibits lesions, and protects the mucosa, suggesting that these compounds have a gastroprotective function (CALDAS et al., 2015). The antioxidant capacity of essential oils, especially eucalyptus, is attributed to the ability of the compound to associate with the keap1/nrf2 system, which results in increased antioxidant enzymes such as superoxide dismutase and catalase (CAI et al., 2020).

Based on all that is known about essential oils in animal diets, we hypothesized that consuming them by calves would improve their health and favor their growth performance. However, there is some concern due to the pungent odor of the essential oils transferred to the powdered milk, there would be interference with consumption. Therefore, the present study determined whether a milk replacer containing a blend of essential oils (oregano, cinnamon, and eucalyptus) would improve the immune system, antioxidants, and growth performance in suckling calves.

3.4 MATERIALS AND METHODS

The experiment was performed at the Experimental Farm of the Centro de Educação Superior do Oeste in Guatambu, Santa Catarina. We used 16 male Holstein calves (ten days old) divided into control and treatment groups, with eight animals each. The animals were housed in pairs in collective pens, with four pens in the control group and four in the treatment group. The experiment lasted 60 days, which was the total period of breastfeeding. Before the experiment, the transition from cow's milk to milk replacer was carried out gradually. At the beginning of the experiment, the supply of the milk replacer with the additive was started for the animals in the treatment group; however, some calves rejected it, likely due to the strong odor of essential oils, of which cinnamon was predominant. However, six of the eight animals adapted to the phytoobiotic-containing milk replacer in two or three days, unlike the other two animals that did not consume the milk even when forced. After seven days after the beginning of the treatment, we excluded these calves from the experiment because they had become dehydrated (when milk without phytobiotics was provided, both animals consumed it in total).

The additive used was liquid Enterosan® (Tecphy, Canoinhas, SC, Brazil), based on essential oils of cinnamon, oregano, and eucalyptus, and it has a purity of 10%. This liquid product was lyophilized and used in powdered form in the milk replacer during manufacture at 5 g of additive/kg of a milk replacer. The replacement was stored in sealed plastic bags to avoid odor loss or volatilization of the product. The animals in both groups received 0.25 kg of milk replacer diluted in two liters of water per feeding, with two feedings per day, at 8:00 am and 4:30 pm (a total of 0.5 kg of milk replacer/calf/day). In addition to the liquid diet, the animals received a solid diet based on pelletized concentrate. From the 28th day of the experiment onwards, the animals received chopped hay ad libitum. The composition of the feeds is described in Table 2.

The animals were weighed weekly using a digital scale. Blood samples were collected on days 1, 15, 30, 45, and 60 to analyze hematologic, biochemical, and immunological variables. Blood collections were performed through the jugular vein using needles and vacuolated tubes. We used tubes with EDTA for hematologic analysis and others with a clot activator to obtain serum for biochemical, immunological, and antioxidant analyses.

Hematologic analyses were performed using the Equipe Vet 3000® automatic equipment. To separate the serum, the tubes were centrifuged without anticoagulant (3500 RPM for 10 min), and the serum was transferred to identified microtubes and stored at –20 °C until analysis. In the biochemical analyses, serum levels of total proteins, glucose, albumin, cholesterol, and urea were measured using the semi-automatic analyzer Bio-2000 (BioPlus®) and following the instructions of the Analisa® commercial kits for each variable. Globulins were determined using a mathematical calculation (total proteins – albumin).

According to TOMASI et al. (2018), using mini-gels (10 x 10 cm), proteins were separated using sodium dodecyl-polyacrylamide gel. The gels were stained with Coomassie Blue and photographed to identify and quantify the protein fractions using Labimage1D software (Loccus Biotechnology). A standard containing fractions with molecular weight between 10 and 250 kD (Kaleidoscope - BIO-RAD) was used as a reference for the identification of protein fractions.

Lipid peroxidation was determined as thiobarbituric acid reactive substances (TBARS) levels using the methods described by JENTZSCH et al. (1996) for sera samples. Serum TBARS levels were expressed as nM malondialdehyde/mL. Antioxidant levels were measured through the ferric-reducing ability of plasma (FRAP) in serum. According to BENZIE & STRAIN (1996), FRAP was measured and expressed as mol/L.

All data were analyzed using the SAS MIXED procedure (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed effects test. Weight gain and average daily gain were tested for fixed treatment effects using a pen (treatment) and animal (pen) as random variables. All other variables (body weight, biochemistry, blood count, protein count, oxidants) were analyzed as repeated measures and tested for treatment fixed effects and treatment × day. All results on d1 for each variable were included as covariants; however, the command for covariates was removed from the model when $P > 0.05$. Averages were distinguished using PDIFF, and all results were expressed as LSMEANS with standard errors. Significance was defined when differences had $P \leq 0.05$, and trend which $P > 0.05$ and ≤ 0.10.

3.5 RESULTS

Body weights in the treatment group were higher than controls from day 28 ($P \leq 0.05$). Weight gain during the experiment was 8.7 kg higher ($P \leq 0.05$) in the treatment group than in the control group. Feed consumption did not differ between the groups; however, the treatment group had a greater feeding efficiency from 1 to day 60 (Table 3). There was no treatment effect or treatment x day interaction for red blood cell counts (erythrocytes, hematocrit, and hemoglobin). The number of white cells differed, with fewer leukocytes due to the reduction of lymphocytes in the treatment group ($P \leq 0.05$, Table 4).

There were differences in cholesterol, total protein, and globulin between groups. Calves in the treatment group had lower cholesterol levels in the treatment x day interaction ($P \leq 0.05$) and a tendency to be lower when we evaluated only the treatment ($P \geq 0.05$ and ≤ 0.10). The total protein level differed on day 45; treatment group animals were superior to the control group animals ($P \leq 0.05$). However, when the protein was fractionated, higher globulin was observed in the treatment group on days 45 and 60 ($P \leq 0.05$; Table 4). There was no effect of treatment and treatment x day interaction for glucose, albumin, or urea levels (Table 4).

The results of the proteinogram are presented in Table 5. The effect of treatment and interaction treatment x day was verified for IgA and IG-heavy chain; calves that consumed the milk replacer with essential oils had higher concentrations of these variables. Haptoglobin levels, by contrast, showed only the effect of the treatment, with higher concentrations in the treatment group. There was no treatment effect or treatment x day interaction for ceruloplasmin and transferrin levels ($P > 0.05$).

The results for lipid peroxidation and total antioxidants are shown in Figure 6. On days 45 and 60, lower levels of TBARS and higher levels of FRAP were observed in the treatment group. The treatment x day interaction showed that the primary differences occurred on days 45 and 60 of the experiment. These findings suggest that animals in the treatment group had lower lipid peroxidation and more significant antioxidant action from day 45 onwards.

3.6 DISCUSSION

There are several studies of essential oils in ruminant nutrition, and they have demonstrated that they improve health and performance in several ways. In summary, we found that calves that consumed the phytogenic in replacement milk showed stimulation of humoral and antioxidant immune responses, which was probably reflected in better health and increased weight gain. Other researchers included a mixture of essential oils in milk replacer and found that essential oils contributed to ruminal manipulation in pre-weaning and carry-over effects in post-weaning, improved immunity, and decreased neonatal diarrhea in the pre-weaning phase (PALHARES CAMPOLINA et al., 2021).

Similar to our study, there have been reported positive effects on zootechnical performance when phytogenics were added to the animal diet. AKBARIAN-TEFAGHI et al. (2018) provided plant extracts to suckling calves containing eucalyptus and found a higher average daily gain and better feed efficiency in the post-weaning period. LIU et al. (2020) added a blend of essential oils containing carvacrol, thymol, and cineol to the pelleted concentrate and recorded more significant weight gain, reaching 11% higher in these calves; in addition, the authors reported that these animals had higher dry matter intake and lower feed conversion. FROEHLICH et al. (2017) reported similar results, using essential oils containing carvacrol, thymol, and cineol at various doses in suckling calves. The authors found a higher average daily gain in the suckling phase. When researchers provided essential oils rich in thymol, eugenol, vanillin, limonene, and guaiacol formulated with different levels of protein in the diet of suckling calves, they found that regardless of protein levels in the diet, average daily gain, weaning weight, and efficiency feed were higher in animals that received the essential oils (KAZEMI-BONCHENARI et al. 2018).

The primary hypothesis for these effects is the robust antimicrobial effect, suggesting that this additive is a performance enhancer (MCINTOSH et al., 2003). Essential oils and active components at high doses inhibited ruminal microbial fermentation; moderate doses could modify ruminal fermentation by altering the production of volatile fatty acids, protein metabolism, or both (CALSAMIGLIA et al., 2007), favoring nutrition and, consequently, weight gain.

Essential oil components influence health, as verified in this study, by stimulating the immune and antioxidant response. Similar to those found in the present study, LIU et al. (2020) provided a blend of essential oils to calves and found higher IgM, IgG, and IgA values, also suggesting an improvement in the immune response. Using cinnamaldehyde-based essential oil in suckling calves via a liquid diet resulted in a lower use of antimicrobials until the animals' sixth week of life and a lower risk of navel inflammation than the control (PEMPEK et al. 2018), a substantial problem in calf rearing. AL-SUWAIEGH et al. (2020) provided a blend of essential oils (clove, oregano, and juniper) to cows during early lactation and found positive effects on milk production; there were also higher total protein and globulin levels. These studies demonstrated a typical result: essential oils increased serum immunoglobulins, which positively affects calves. There was a small increase in haptoglobin, which is an acute phase protein not normally found in serum levels in bovine organisms; its presence is associated with inflammatory conditions and oxidative stress, which can be seen as an indicator of health and an indicator of an inflammatory condition (MURATA et al., 2004; PETERSEN et al., 2004)

The precise mechanism by which essential oils induce a humoral immune response is poorly understood; however, researchers found that the essential oil of *Pituranthos tortuosus* when consumed by mice, boosted lipopolysaccharide-induced splenocyte proliferation, suggesting improved B cell and humoral immune response activation (KRIFA et al., 2015). One possibility for an increase in immunoglobulins would be an increase in lymphocytes, as described in studies of essential oils (PALHARES CAMPOLINA et al., 2021); by contrast, in the present study, we reported a lower count of this immune cell.

In a study of pigs supplemented with carvacrol and thymol, the researchers concluded that the compounds reduced the post-weaning intestinal oxidative stress of the animals and decreased the presence of *Enterococcus* and *E. coli* (WEI et al., 2017). The calves that consumed the blend of essential oils, likewise, had an effect of minimizing the serum and physiological oxidative stress; similarly, we found less lipid peroxidation and an increase in the total antioxidant capacity.

Gessner et al. (2017) report the potential of plant polyphenols to combat oxidative stress and inflammatory processes in farm animals, emphasizing the productivity benefits. Polyphenols in essential oils block NF-κB activation by inhibiting phosphorylation and proteasomal degradation of IκB, an effect that is due in part to the antioxidant properties of polyphenols (VENDRAME & KLIMIS-ZACAS, 2015).

Another possible mechanism is that polyphenols directly eliminate reactive oxygen species and induce Nrf2 activation, which, in turn, leads to the activation of several antioxidant enzymes (NA & SURH, 2008). In the present study, the direct elimination of reactive oxygen species and the activation of Nrf2 help to minimize the development of physiological oxidative stress in calves; the literature suggests that these events trigger pro-inflammatory pathways activating NF-κB, mitogen-activated protein kinases, and activator protein 1 (CHUANG & MCINTOSH, 2011).

3.7 CONCLUSION

We conclude that including essential oils in the milk replacer improved calf performance, manifesting as more significant weight gain and better feed efficiency. This milk replacer provides a source of humoral immune elements and antioxidants, minimizing the physiological oxidative stress of calves in the rearing phase when health and nutritional challenges are substantial. However, we also concluded that not all animals voluntarily consume the essential oil replacer, probably due to the strong odor of this feed additive.

Ethics approval

The project was approved by the ethics committee on the use of animals in research of university (number protocol nº 2322230522).

3.8 ATTACHMENTS

Table 2: Chemical composition of feed fed to calves.

Itens		Quantity (DM) d1–28	Quantity (DM) d28–60
Concentrate[1]		*Ad libitum*	*Ad libitum*
Milk replacer		0.5 kg/day	0.5 kg/day
Hay		0.0	*Ad libitum*
Chemical composition (%)			
	Concentrate	Replacer	Hay
Dry matter	89.8	96.7	83.6
Crude protein	24.3	15.1	8.09
Ethereal extract	4.95	15.9	3.67
Mineral matter	7.12	9.80	6.21
Neutral detergent fiber	29.3	4.57	70.6
Acid detergent fiber	15.1	2.43	33.8

[1.] The commercial concentrate (Nutrialfa Bezerra®) was formulated based on micronized soybean meal, ground corn, wheat bran, minerals, vitamins, and sodium monensin.

Table 3. Performance of growth and consumption of calves fed with milk replacer with and without the phytobiotic

	Control	Phyto	SEM	P – Treat	P – Treat x day
Body weight (kg)				**0.05**	**0.02**
d1	40.6	44.5	0.57		
d7	43.2	46.6	0.55		
d14	46.5	50.5	0.55		
d21	50.1	55.2	0.54		
d28	54.8[b]	61.8[a]	0.55		
d35	60.1[b]	65.3[a]	0.55		
d42	63.0[b]	70.6[a]	0.56		
d49	65.7[b]	75.6[a]	0.56		
d56	69.4[b]	82.3[a]	0.55		
d60	70.0[b]	82.6[a]	0.55		
Weight gain (kg)					
d1–60	29.4[b]	38.1[a]	0.45	**0.03**	**–**
d1–28	14.1	17.2	0.44	**0.07**	**–**
d28–60	15.2[b]	20.8[a]	0.44	**0.05**	**–**
Average daily weight gain (kg DM)					
d1–60	0.490[b]	0.635[a]	0.04	**0.03**	**–**
d1–28	0.504	0.614	0.04	**0.07**	**–**
d28–60	0.475[b]	0.650[a]	0.05	**0.05**	**–**
Daily concentrate consumption (kg DM)					
d1–60	0.707	0.826	0.13	0.61	–
d1–28	0.484	0.522	0.11	0.56	–
d28–60	0.907	1.141	0.16	0.17	–
Daily hay consumption (kg DM)					
d28–60	0.219	0.254	0.09	0.29	–
Daily milk consumption (kg DM)					
d1–60	0.490	0.490	0.00	–	–
Daily feed consumption (kg DM)					
d1–28	0.97	1.01	0.18	0.49	–
d28–60	1.61	1.88	0.24	0.51	–
Total feed consumed (kg/DM)					
d1–60	78.9[b]	88.6[a]	1.06	**0.08**	**–**
Feed efficiency					
d1–60	0.372[b]	0.430[a]	0.07	**0.05**	**–**
d1–28	0.517[b]	0.607[a]	0.07	**0.04**	**–**
d28–60	0.294	0.345	0.05	**0.09**	**–**

Phyto: animals receiving the phytobiotic.
1. Demonstrates treatment effect where different letters in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.
2. Demonstrates the interaction between treatment × day where different letters on the same line show a significant difference ($P \leq 0.05$) and trend ($P > 0.05$ and ≤ 0.1) between groups.

Table 4: Blood count and serum biochemistry of calves fed milk replacer with and without phytobiotic.

Variables	Control	Phyto	SEM	P - Treat	P - Treat x day
Leukocytes (x10^3 cel/µL)				**0.01**	**0.01**
d1	21.08	19.23	1.69		
d15	16.40^a	9.78^b	1.65		
d30	18.30^a	10.40^b	1.65		
d45	6.89	6.60	1.41		
d60	8.11^a	6.80^b	1.41		
Lymphocytes (x10^3 cel/µL)				**0.05**	**0.01**
d1	14.33	12.14	1.25		
d15	11.89^a	7.31^b	1.24		
d30	13.85^a	7.47^b	1.06		
d45	3.87	4.72	1.06		
d60	5.32	4.69	1.05		
Granulocytess/monocytes (x10^3 cel/µL)				**0.08**	**0.01**
d1	6.75	7.08	0.74		
d15	4.38^a	2.47^b	0.74		
d30	4.53^a	2.97^b	0.74		
d45	3.01^a	1.88^b	0.7		
d60	2.80	2.14	0.71		
Erythrocytes (x10^6 cel/µL)				0.89	0.86
	8.06	7.94	0.05		
Hemoglobin (mg/dL)				0.93	0.88
	10.14	10.36	0.29		
Hematocrit (%)				0.91	0.76
	28.90	29.20	1.5		
Glucose (mg/dL)				0.86	0.82
	78.2	76.9	2.81		
Cholesterol (mg/dL)				**0.03**	**0.01**
d1	67.1	70.3	3.75		
d15	109	95.1	3.71		
d30	118	126	3.70		
d45	153^a	131^b	3.72		
d60	120	110	3.71		
Total protein (g/dL)				0.15	**0.02**
d1	6.58	6.96	0.07		
d15	5.85	5.65	0.06		
d30	5.68	5.75	0.06		
d45	8.16^b	8.95^a	0.06		
d60	7.12	7.75	0.05		
Albumin (g/dL)				0.35	0.13
	3.01	2.87	0.02		
Urea (mg/dL)				0.79	0.42
	31.7	28.8	3.71		
Globulin (g/dL)				**0.05**	**0.01**
d1	4.00	3.55	0.05		
d15	3.25	3.23	0.06		
d30	2.96	3.23	0.05		
d45	4.28^b	5.10^a	0.05		
d60	4.27^b	5.01^a	0.05		

Phyto: animals receiving the phytobiotic.
1. Demonstrates treatment effect where different letters in the same row show significant differences (P ≤ 0.05) and trends (P > 0.05 and ≤ 0.1) between groups.
2. Demonstrates the interaction between treatment × day where different letters on the same line show a significant difference (P ≤ 0.05) and trend (P > 0.05 and ≤ 0.1) between groups.

Table 5: Proteinogram of calves fed milk replacer with and without phytobiotic.

	Control	**Phyto**	**SEM**	**P - Treat**	**P - Treat x day**
IgA (g/dL)				**0.04**	**0.01**
d1	0.84	0.81	0.04		
d15	0.87	0.86	0.04		
d30	0.76[b]	0.87[a]	0.04		
d45	0.77[b]	0.95[a]	0.05		
d60	0.72[b]	0.92[a]	0.05		
heavy chain Ig (g/dL)				**0.01**	**0.01**
d1	0.99	1.04	0.05		
d15	1.08	1.10	0.05		
d30	1.01	1.34	0.07		
d45	0.97	1.47	0.07		
d60	0.99	1.29	0.06		
Ceruloplasmin (g/dL)				0.94	0.77
	0.55	0.53	0.02		
Haptoglobin (g/dL)				**0.05**	0.13
	0.31[b]	0.34[a]	0.02		
Transferrin (mg/dL)				0.86	0.90
	0.22	0.23	0.03		

Phyto: animals receiving the phytobiotic.
1. Demonstrates treatment effect where different letters in the same row show significant differences (P ≤ 0.05) and trends (P > 0.05 and ≤ 0.1) between groups.
2. Demonstrates the interaction between treatment × day where different letters on the same line show a significant difference (P ≤ 0.05) and trend (P > 0.05 and ≤ 0.1) between groups.

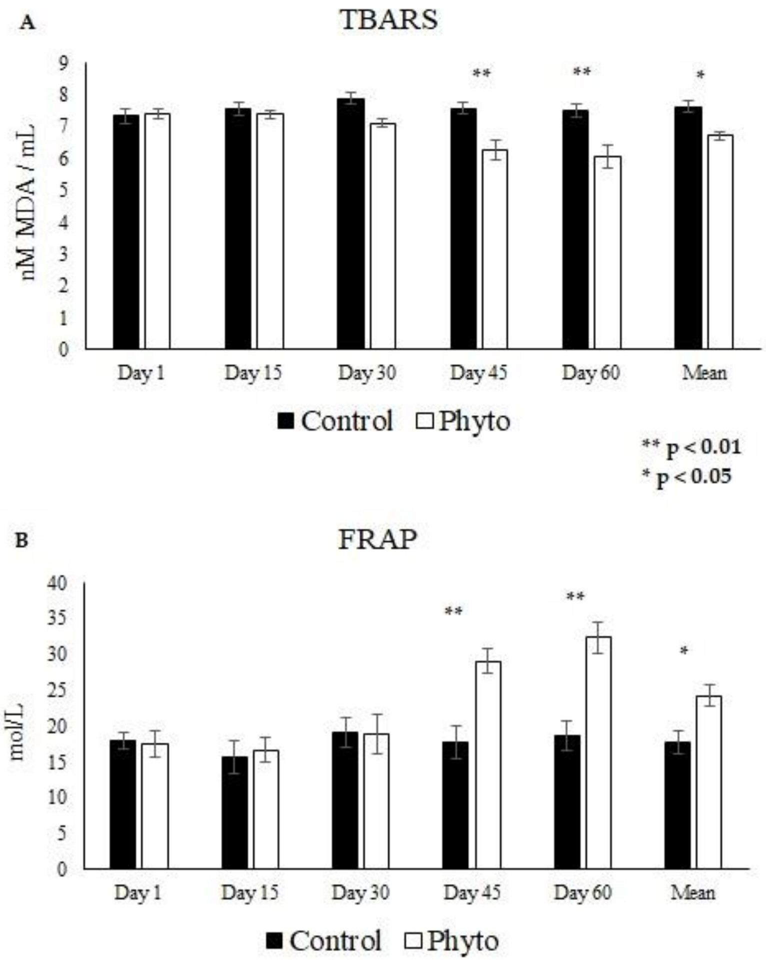

Figure 6: Antioxidant marker levels of calves fed milk replacer with and without phytobiotic. Phyto: animals receiving the phytobiotic. Note: the asterisk over the bars shows the difference between groups (* $P < 0.05$; ** $P < 0.01$).

4. ARTIGO 1: Effects of a blend of essential oils in the milk of suckling calves on performance, immune and antioxidant systems, and intestinal microbiota

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4.1 ABSTRACT

Neonatal diarrhea is a significant problem in calves; this clinical sign can be modified by several factors, especially infection by pathogenic bacteria in the intestine or environment. There is also the fragile health of these animals due to their immature immune system, which increasingly potentiates diseases at this stage. The objective of this study was to determine whether the addition of a blend of essential oils with cinnamon, oregano, and eucalyptus stimulates the immune system, minimizes the oxidative response, and alters the intestinal microbiota of suckling calves, enhancing their growth. Twenty-four male Holstein calves (approximately five days old) suckled for 60 days, underwent a weaning process, and were followed up until day 75 of the experiment. The calves were divided into control (n = 12) and phytobiotic (n = 12) groups, receiving commercial substitutes and pelleted concentrate ad libitum. The phytobiotic group added the blend to the liquid diet twice daily at 5 mL/feeding in the first 15 days and 10 mL/feeding until day 60. We detected no differences in weight gain, but animals in the phytobiotic group tended to consume less food. The phytobiotic group showed better conversion and feed efficiency than the animals in the control group. He also had a lower leukocyte and lymphocyte count and a higher cholesterol concentration. Immunoglobulin A (IgA), ceruloplasmin, and transferrin also differed between groups, with higher IgA and lower levels of acute phase proteins (ceruloplasmin and transferrin) in calves that consumed the phytobiotic. Higher glutathione S-transferase (GST) activity was found in the serum of calves in the treatment group. The intestinal microbiota did not differ between groups; however, the genera *Acinetobacter*, *Pseudomonas*, and *Psychrobacter* were the most abundant regardless of treatment. The ingestion of the oil blend did not reduce *Escherichia coli* and *Clostridium* spp., the most common causative agents of gastroenteritis in calves. We concluded that the blend of oils based on cinnamon, oregano, and eucalyptus improved the immune and antioxidant systems, improving feed efficiency without affecting the intestinal microbiota.

Keywords: cinnamaldehyde, 1,8-cineole, carvacrol, thymol, intestinal microorganisms.

4.2 INTRODUCTION

Dairy production is improving internationally and in Brazil, one of the largest milk producers worldwide (FAO, 2023). These advances have been taking place to minimize problems and enhance production. In dairy production, several phases of animal husbandry, including calf production, are considered the most critical because they have an extremely immature and fragile immune system (Chase et al., 2008). Calves face several problems during the first weeks of life; however, the primary problem affecting this animal class is diarrhea, a clinical sign associated with infections caused by some pathogenic microorganisms. The diarrhea problem can be greater when accompanied by loss of appetite and dehydration, further weakening the animal (Cho & Yon, 2014). They can die quickly, depending on the degree of infection and lack of response from the animal.

Low ambient temperature, a high number of calves housed in the same facility, lack of heating, inadequate ventilation, and an inadequate method of supplying a liquid diet (milk and its substitutes) increase the incidence of diarrhea and promote a higher mortality rate (Zhao et al., 2021). Understanding the intestinal microbiota is essential for understanding diarrhea; however, this microbiota is also responsible for the digestion, absorption of some nutrients, and helping to establish the animal's immune system regarding protection against pathogenic microorganisms (Maynard et al., 2012; Maele et al., 2017).

The intestinal microbiota of calves begins to be established from birth quickly and is affected by several factors, including the microbiota of the mother's vaginal canal at the time of delivery, the environment in which the animal is housed, the origin of the food provided to the animal, whether it is consumable or discarded milk, and the ingestion of solid food (Alipour et al., 2018; Kodithuwakku et al., 2021). Several non-pathogenic bacteria form this microbiota and can often act as barriers to other pathogenic bacteria (Bischoff, 2011). Some bacteria in the gastrointestinal tract are harmless while the animal is healthy; however, they can cause illness when the calf is immunosuppressed. The gastrointestinal microbiota can be modulated to establish specific microorganisms, and this modulation can be accomplished in several ways, including using essential oils (Bento et al., 2013); they are more effective when performed at the beginning of the animal's life. Calves that at this stage have a fluctuating microbiota that depends on environmental conditions (Dill-Mcfarland et al., 2018).

Essential oils are products extracted from several plant sources (Cui et al., 2019), which because of their antimicrobial action (Calsamiglia et al., 2007; Chapman et al., 2016) have been used as an alternative to reduce the use of antibiotics in animal nutrition (Chapman et al., 2016; Vakili et al., 2013). Cinnamon, eucalyptus, and (mainly) oregano oils have been frequently studied due to their biological properties, which allow effects such as antimicrobial, antioxidant, and anti-inflammatory (Haddi et al., 2017; Leyva-López et al., 2017; Dhakad et al., 2017), which are used for additives in animal feed. Cinnamon essential oil is extracted from *Cinnamomum* spp. (Shreaz et al., 2016) as a source of cinnamaldehyde, the primary bioactive component (Calsamiglia et al., 2007). Another component extracted from essential oil is carvacrol, which derives primarily from oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) (Chapman et al., 2016). Oregano oil also acts as a performance enhancer in ruminants, as it improves feed conversion and health by modulating ruminal fermentation (Wu et al., 2020). The essential oil of eucalyptus is extracted from the leaves, and 1,8-cineole is the primary component of interest (Kumar et al., 2012; Baptista-Silva et al., 2020) due to its gastroprotective and antioxidant activities; it regenerates gastric cells and increases gastric mucus (Caldas et al., 2015).

Essential oils, natural antimicrobials in calf production, are increasingly promising, especially in blend form. The powerful aroma of essential oils can be a problem in feeding calves because, when added to milk, they can lead to a lack of interest in consumption; in concentrate, these oils can reduce consumption. The benefits of isolated oregano (Wu et al., 2020), cinnamon (Vakili et al., 2013), and eucalyptus (Abo-Donia et al., 2021) oil for young ruminants are known; however, the effect of these three essential oils when combined and given to calves during the suckling phase is not known. Thus, the objective of the present study was to determine whether the addition of a blend based on essential oils of cinnamon, oregano, and eucalyptus added to the liquid diet of calves would stimulate the immune system combined with anti-inflammatory action, minimize oxidative responses, and alter the intestinal microbiota, consequently enhancing animal growth.

4.3 MATERIALS AND METHODS

The study was carried out at the Experimental Farm of the Centro de Educação Superior do Oeste – FECEO of the State University of Santa Catarina – UDESC, in the municipality of Guatambu – SC. It lasted 75 days: 60 days of breastfeeding, six days of gradual weaning, and nine days post-weaning. The Committee on Ethics in the Use of Animals of the State University of Santa Catarina approved all experiments (protocol number 2322230522).

4.3.1 Experimental Design and Feeding

Twenty-four male Holstein calves with a maximum of five days of life were used and acquired through donations from regional producers. The calves were housed in individual suspended cages and were divided into two groups: Control and phytobiotic, with 12 calves in each group, where each animal was considered an experimental unit. The animals received a substitute as a liquid diet at a dosage of 0.25 kg/day (in natural matter) diluted in two liters of water twice a day, at 08:00 and 16:30. For the animals in the phytobiotic group, a commercial blend of essential oils (cinnamon, oregano and eucalyptus) was added, in the purity of 6% of essential oils, that is, 60 mL/L in an emulsifying vehicle.

In the first 15 days of the experiment, a dose of 5 mL/feeding was given to each animal, and after that, the dose was increased to 10 mL/feeding. The lowest initial dose was to adapt the animal's taste to the product since it has a strong cinnamon smell. Both groups of animals received pelleted concentrate and water ad libitum. At the beginning of weaning (day 61), the amount of milk was proportionally reduced for six days, and the calves began to receive hay ad libitum in addition to the concentrate limited to 2.5 kg/day to avoid metabolic disturbances. The composition of the food provided is displayed in Table 1.

4.3.2 Efficiency, Feed Conversion, and Stool Score

The feed provided and the leftovers the following day were weighed to obtain average daily consumption values (ADC), in dry matter. Weekly, the animals were weighed using a digital scale to monitor the daily weight gain (ADG). Daily the stool score was evaluated using a visual analysis methodology proposed by Bednar et al. (2000). Information on weight gain, feed conversion (ADC/GMD – kg/kg), and feed efficiency (GMD/ADC – kg/kg) were obtained using mathematical calculations based on the weight of the animals and their daily consumption.

4.3.3 Blood Tests

Blood samples were collected for hematologic, biochemical, and immunological analyses on days 1, 15, 30, 45, 60, and 75. Collections were made through the jugular vein with needles and vacuolated tubes. Tubes with EDTA were used to prevent clotting and enable hematologic analysis, and another contained a clot activator to obtain serum. For serum separation, the tubes without anticoagulant were centrifuged at 3500 RPM for ten min, and the serum was transferred to identified microtubes and stored at –20 °C until further analyses were performed.

Hematologic analyses were performed on Equipe Vet 3000® automatic equipment using fresh blood. The equipment made it possible to quantify the total number of leukocytes and the differential (lymphocytes, granulocytes, and monocytes) total erythrocyte count, hemoglobin concentration, and hematocrit.

In the biochemical analyses, total protein, glucose, albumin, cholesterol, and urea serum levels were measured on a semi-automatic analyzer Bio-2000 BioPlus® and commercial kits Analisa®. The levels of globulins were obtained using a mathematical calculation (total proteins – albumin).

For protein fractionation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed, according to Fagliari et al. (1998), adapted by Tomasi et al. (2018) using a mini gel (10 x 10 cm). The gel was stained with Coomassie Blue and photographed to identify and quantify the protein fractions using Labimage1D software (Loccus Biotechnology). A standard containing fractions with molecular weight between 10 and 250 KD (Kaleidoscope - BIORAD) was used as a reference.

Lipid peroxidation was determined as TBARS levels using the methods described by Jentzsch *et al*. (1996) for sera samples. Serum TBARS were expressed as nM malondialdehyde (MDA)/mL. Antioxidant levels were measured as the ferric-reducing ability of plasma (FRAP) in serum. According to Benzie and Strain (1996), FRAP was measured, and the results were expressed as mol/L.

4.3.4 Intestinal Microbiota

On days 1, 35, and 60, feces were collected directly from the rectal ampulla and stored in 3M™ Quick Swabs for qualitative and quantitative detection of microorganisms using metagenomics by sequencing the 16S rRNA gene, which was performed by the laboratory BPI - Biotechnology Research and Innovation®.

Total DNA was extracted from 200 mg (wet weight) of samples with the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, USA). Primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) were selected to amplify the V3–V4 region of bacterial 16S rRNA gene by polymerase chain reaction (PCR) (Hjelmsø et al. 2014).

Libraries were quantified by q-PCR using the Kapa Library Quantification Kit (Illumina, San Diego, CA, US) following the manufacturer's recommendations. Samples were normalized to a final concentration of 2 nM. and sequenced with an Illumina MiSeq for 250 cycles from each end.

4.3.5 Statistical Analysis

All data were analyzed using the SAS MIXED procedure (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed effects test. Weight gain, average daily gain, feed conversion, and feed efficiency were tested for fixed treatment effects and using the animal as random variables. All other variables (body weight, biochemistry, blood count, protein count, and oxidants) were analyzed as repeated measures and tested for fixed effects of treatment and treatment × day. All results obtained on d1 for each variable were also included as covariants; however, the command for covariates was removed from the model when $P > 0.05$. Averages were determined using PDIFF, and all results were reported as LSMEANS followed by the standard error. The chi-square test was used for stool score analysis. Significant differences were defined when $P \leq 0.05$, and trends when $P>0.05$ and >0.10.

The sequence data was processed with Mothur v.1.39.5 (Schloss et al. 2009), in line with the mothur MiSeq SOP (Kozich et al. 2013). Taxonomy was then assigned by querying the representative sequence of each oligotype against the SILVA database (release 132) (Quast et al. 2013).

Closed-reference clustered OTU data was exported for analysis with Phyloseq v1.41 (McMurdie and Holmes, 2013) in R 4.3.1 (R Core Team, 2023). Alpha diversity values for bacterial communities were obtained using inverse Simpson diversity index. The inverse Simpson calculator is preferred to other measures of alpha diversity since it indicates the richness of a community with uniform evenness that has the same level of diversity and thus has some biological interpretation (Morris et al., 2014). The tax_glom function of Phyloseq was used to aggregate abundance data by genera to generate relative abundance plots of the 10 most abundant genera across all samples, which were plotted by averaging abundances by study and by animal. Relative abundance of well know potentially pathogenic *Clostridium* and *Escherichia* (Songer and Anderson, 2006) was performed. All figures were generated with the ggplot2 package v3.2.1 (Wickham, 2016).

4.4 RESULTS

4.4.1 Consumption, Weight Gain, Conversion, and Feed Efficiency

Dry matter intake tended to be lower in the phytobiotic group ($P > 0.05$ and ≤ 0.1) than in the control group when we evaluated the total dry matter consumed throughout the experiment. The weight gain did not differ between groups in any of the measurements; however, animals in the phytobiotic group had better feed conversion and efficiency ($P \leq 0.05$). The data are expressed in Table 2.

4.4.2 Stool Score

There was no effect of treatment on the stool score ($P > 0.05$). During the experiment, the score was 1.80 in the control group and 1.87 in the phytobiotic group. According to the methodology, scores of 1 and 2 were considered normal; however, it is worth noting that these values are the average of the entire experimental period, so we cannot say that the animals did not have diarrhea at any time.

4.4.3 Blood Tests

In the hematologic analysis, the animals in the phytobiotic group had lower leukocyte and lympgocyte counts on days 45 and 60 ($P < 0.05$). On day 30, the granulocyte count was also lower in the phytobiotic group ($P < 0.05$). Regarding the other hematologic parameters, no difference was observed at any time during the experiment. The results are displayed in Table 8.

Glucose, total protein, albumin, urea, and globulin showed no difference throughout the experimental period. However, on days 30, 45, and 60, cholesterol levels were higher in the phytobiotic group (Table 9).

In Table 10, we can see the results of the oxidative stress biomarkers. GST showed an effect of treatment and interaction treatment x day on days 15, 30, 45, and 60, higher in the serum of animals in the phytobiotic group. There was no treatment effect or treatment x day interaction for TBARS or total thiols.

The results of the proteinogram are displayed in Table 11, where we can observe an effect of treatment and interaction treatment x day for levels of IgA, ceruloplasmin, and transferrin. Serum IgA was higher in the phytobiotic group on day 60, ceruloplasmin was lower on days 30 and 60, and transferrin was lower on day 45. As an effect of treatment, it led to higher IgA and lower ceruloplasmin and serum transferrin in calves that consumed the blend of essential oils. There was no effect of treatment and interaction treatment x day for the concentration of heavy chain immunoglobulins, haptoglobin, C-reactive protein, and ferritin.

4.4.4 Intestinal Microbiota

In calf feces, nine genera of the most abundant microorganisms were detected, shown in Figures 1 and 2, detailed by treatment and experimental period, respectively. The genera *Acinetobacter*, *Bacteroides*, *Pseudomonas*, and *Psychrobacter* were the most numerous, with numerical differences between groups but without statistical differences between treatments ($P > 0.05$). The designation “other” refers to genera that appeared in small amounts, and “unclassified” refers to genera that could not be identified against the SILVA database.

A greater number of taxa was observed in the samples of animals from the phytobiotic group, demonstrating a greater alpha diversity than the control. Observing this alpha diversity, it is also possible to observe that the greatest diversity occurs later at the end of the experiment but without significant differences ($P > 0.05$), as shown in Figures 3 and 4.

The abundance of the primary bacteria that cause diarrhea in suckling calves was also determined (*Clostridium*, *Escherichia*, and *Salmonella*), the first two appearing in greater abundance, unlike the rarely identified *Salmonella*. In both cases, comparing the treatments, there was no significant difference regarding the abundance of *Clostridium* and *Escherichia* (Figures 11 and 12).

4.5 DISCUSSION

Intake of the blend of essential oils provided better efficiency and feed conversion for the calves because feed intake was lower, as Tapiki et al. (2020) had described when providing oregano oil to calves. Likewise, Silva et al. (2020) obtained greater feed efficiency in lactating cows when supplied with a commercial additive composed of capsaicin, carvacrol, cinnamaldehyde, and eugenol, with carvacrol and cinnamaldehyde being two of the components present as bioactives in the product used in the experiment in question. Furthermore, Farshid et al. (2021) found greater feed efficiency for calves supplemented with cinnamon essential oil than oil from other plants and a probiotic. The greater weight gain with lower dry matter intake can be explained by a small modulation of ruminal microorganisms allowing greater efficiency in using available nutrients (Cardozo et al., 2005). Therefore, our results corroborate the literature, showing that this tested essential oil blend is a way to save on raising calves without losing productivity.

The components of the essential oils in this work act as antimicrobials and intestinal flora modulators, and one of the possible responses to this antimicrobial action is a decrease in the incidence of diarrhea. Tapiki et al. (2020) and Katsoulos et al. (2017) gave oregano oil to calves and obtained a lower incidence of diarrhea; there was less use of antibiotics in a group of animals that were consuming essential oil (Katsoulos et al., 2017). Ritt et al. (2023) concluded that an oregano extract with 80% carvacrol positively affected the intestinal and ruminal bacterial population, decreasing the abundance of the pathogenic bacteria *Streptococcus*, *Clostridium*, and *Escherichia*, unlike the present study, where no difference was obtained for *Clostridium* and *Escherichia* between treatments. The difference between the supply doses can explain this since, in the present study, it was low concerning that used in the literature.

Cinnamon, oregano, and eucalyptus oils also improve the immune and antioxidant systems. Like our study, Liu et al. (2020) obtained a better immune response, with higher values of IgM, IgG, and IgA, when feeding calves with an essential oil also containing probiotics (carvacrol, caryophyllene, p-cymide, cineol, terpinene, and thymol). Al-Suwaiegh et al. (2020) achieved higher levels of total protein and globulin, in addition to positive results in cows' milk production with the supply of a mixture of clove, oregano, and juniper oils. IgA is an antibody predominantly produced on mucosal surfaces and is an indicator of intestinal immunity (Pabst,

2012) because they coat pathogenic microorganisms protecting the host against infection (Palm et al., 2014). However, the function of IgA is to act on the integrity of the intestinal barrier, enhancing pro-inflammatory immune responses and effectively contributing to intestinal homeostasis (Pabst, 2012). This fact demonstrates that the animals in the phytobiotic group had greater intestinal protection, probably due to the blend of oils, avoiding the incidence of diarrhea and diseases caused by pathogens and enhancing the immune responses.

Acute phase proteins such as ceruloplasmin, haptoglobin and transferrin are produced by the liver (Rocha et al., 2016; Bozukluhan et al., 2018); they were detected in lower concentrations in the phytobiotic group at some times during the experimental period. The response of these proteins is a nonspecific reaction that occurs shortly after the damage caused by the pathogen; in addition, the concentrations of acute phase proteins, in general, indicate the intensity of the inflammatory reaction in the body (Bozukluhan et al., 2018); this may have occurred in the present study due to the known anti-inflammatory effect of essential oils. Erkiliç et al. (2019) showed that neonatal calves that show clinical signs of diarrhea, absence of sucking reflex, dehydration, cold extremities, lateral recumbency, and inability to stand have higher serum levels of ceruloplasmin, showing that high levels indicate that the animal is in good health. Saleh et al. (2022) showed that calves with diarrhea had increased levels of ceruloplasmin (which is expected) as it is an inflammatory response. In the present study, we believe in an anti-inflammatory effect caused by the blend of essential oils since transferrin and ceruloplasmin are markers of inflammatory intensity.

Lower levels of leukocytes and lymphocytes were detected in lambs supplemented with essential oil containing carvacrol, thymol, and cinnamaldehyde (Favaretto et al., 2020), as in the present experiment. This finding can be explained by the antimicrobial action of oils (Favaretto et al., 2020) because oils change the permeability of the bacteria membrane (Dorman and Deans, 2000). The action of oils minimizes energy expenditure during inflammatory responses and directs this energy toward growth (Molosse et al., 2022), associated with better feed efficiency. The blend of essential oils provided the animals with antioxidant mechanisms since the lipid peroxidation of the phytobiotic group was lower than that of the control group. This finding was due to a higher activity of GST, which inhibits oxidative reactions (Brunetto et al., 2023). With higher levels of IgA, lower levels of leukocytes, and acute phase proteins of the phytobiotic group, we found that the calves improved the immune system and overall health.

The nine bacterial genera detected in the feces of calves in our experiment, both control and treatment, were *Acinetobacter, Alloprevotella*, *Bacteroides*, *Collinsella*, *Fusobacterium*, *Megamonas*, *Prevotella*, *Pseudomonas*, and *Psychrobacter*; among them, *Acinetobacter*, *Pseudomonas*, and *Psychrobacter* were the most abundant. *Acinetobacter* has animals as one of its natural habitats, as it has high resistance to antimicrobials and the environment, with the species of *A. baumannii* as the most significant of its kind in animals (Doughari et al., 2011). This bacterium can cause infections of different types, including in soft tissues such as the intestine, also causing symptoms of pneumonia (Hou et al., 2022), a common disease in calves (Bittar et al., 2018). In the distribution of genders by experimental period, *Acinetobacter* increased significantly on days 35 and 60, which can be explained by the fact that it belongs to the normal intestinal microbiota of calves, and its growth occurs as the microbiota is established.

Pseudomonas, by contrast, live in different environments using animal tissues as a substrate, also having substantial resistance to antimicrobials (Rojo, 2010; Winsor et al., 2016; Mcvey et al., 2016; Zarei, 2023;). It is a bacterium associated with meat deterioration and rarely associated with animal health (Mcvey et al., 2016; Zarei, 2023). *P. aeruginosa* is the most clinically important in animals because it is not a constituent of the normal intestinal microbiota and can be found in the feces of healthy animals, where it is considered an opportunistic pathogen (Mcvey et al., 2016; Winsor et al., 2016). This genus showed a lower abundance over the days; since it is not part of the normal microbiota, we can have this decrease as beneficial to the health of our calves.

Psychrobacter is also an opportunistic pathogen (Garcia-López & Maradona, 2014). Rama et al. (2023) identified *Psychrobacter* as one of the most abundant genera in the intestinal microbiota of healthy broilers, whether in conventional systems or systems without antibiotics. As they are opportunistic, the presence of these three genera, as already mentioned, will only cause damage to the animal's health when it is immunosuppressed.

When we analyze the distribution of the nine genera within each experimental period (days 0, 35, and 60), we notice a fluctuation in the bacterial population as the animal ages and the microorganisms become established. In addition to *Acinetobacter*, the genus *Bacteroides* was also practically non-existent on day zero; however, it was evident throughout the experimental period, although without much prominence when only the treatment was evaluated. This genus is also part of the normal intestinal microbiota; however, *B. fragilis* is clinically significant (Mcvey et al., 2016; Cao et al., 2022), as they produce an enterotoxin that causes diarrhea in calves and other young animals (Knoop, 2014), and has pathogenic potential very similar to that of *E. coli* (Sears, 2001) which is one of the main microorganisms that cause diarrhea in calves and is related to a high mortality rate (Cao et al., 2022). However, many *Bacteroides* sp. produce enzymes such as superoxide dismutase, which acts directly on the antioxidant system (Knoop, 2014), beneficial to the animal's body when it is not immunosuppressed.

Another genus found is *Fusobacterium*, which remained regular during the experimental period and without difference between groups. *F. necrophorum* is one of the most prevalent Fusobacteria, being a normal microorganism of the gastrointestinal tract (Nagajara et al., 2005), as well as being an opportunistic pathogen (Ichikawa-Seki et al., 2019). The genus *Megamonas* can be associated with healthy individuals (Suchodolsk et al., 2015) and is related to the production of beneficial substances and the reduction of pathogenic bacteria (Chevrot et al., 2008; Wu et al., 2022). In addition, it is negatively related to intestinal dysfunctions, decreasing the intestinal microbiota of sick individuals (Gungor et a., 2016).

The genus *Alloprevotella* became evident only after 60 days of the experiment, being a group of beneficial bacteria since they can produce short-chain fatty acids (Cao et al., 2023), allowing them to be associated with improved performance. animal (Virginio Junior et al., 2021). The literature suggests that the *Alloprevotella* genus acts as an intestinal barrier stimulator, reducing pathogenic organisms through competition and the need for antibiotics (Fan et al., 2019; Rolinec et al., 2020). Due to the high presence of *Prevotella* in healthy individuals, the genus is considered to belong to the gastrointestinal tract of humans and animals (Larsen, 2017; Whitman et al., 2015), similar to Lourenço et al. (2020), who also identified the genus *Prevotella* in calves as one of the predominant ones. According to the literature, the genera *Prevotella*, *Bacteroides*, and *Fusobacterium* can be found in greater abundance from the 15th day of life in calves, when solid intake is increasing (Enjalbert et al., 2014), which explains the greater presence of these genera in feces collected on days 35 and 60.

No difference was observed in the population abundance of *E. coli* and *Clostridium* spp. between treatments. *E. coli* is the primary bacterium causing calf diarrhea (Foster & Geof, 2009; Gull, 2022), but its presence does not mean that the animal will be sick since its pathogenicity depends on two virulence factors that need to be present (Foster & Geof, 2009); therefore, the bacteria is a constituent of the normal microbiota. *Clostridium* is another bacteria responsible for causing diarrhea in calves, primarily *C. perfringens*; however, it is less common and is found in the microbiota of healthy animals (Gull, 2022). The presence of these two bacteria throughout the experiment did not harm their health, as they were immunologically protected. In addition, the calves' feces score remained within the ideal range most of the time; the average score was 1.80 and 1.87 in the control and phytobiotic groups, respectively. These values are not considered diarrhea, showing that, in general, the animals demonstrated adequate intestinal conditions.

4.6 CONCLUSION

The supply of a blend of essential oils with cinnamon, oregano, and eucalyptus provided the calves with humoral and antioxidant immune system stimulation, minimizing physiological oxidative stress and better efficiency and feed conversion. Furthermore, the blend dosage was insufficient to reduce the incidence of *E. coli* and *Clostridium* spp. The assessment of abundance and biodiversity did not differ between animals that consumed the blend of essential oils and those that did not; however, it allowed identifying the main genera in the feces of calves in the first weeks of age.

4.7 ATTACHMENTS

Table 6: Ingredients and chemical composition of feed fed to calves.

Ingredients		Quantity (DM) d1–60	Quantity (DM) d 61–75
STEP 1			
Concentrate[1]		*Ad libitum*	*Ad libitum*
Milk replacer		484.4 g/day	363.3 g/day (d 61–62), 242.2 g/day (d63–64); and 121.1 g/day (d65–66)
Hay		0	*Ad libitum*
Chemical composition (%)			
	Concentrate	Replacer	Hay
Dry matter	89.31	96.89	85.0
Crude protein	26.10	15.16	7.23
Ethereal extract	3.44	16.51	0.80
Mineral matter	6.44	6.64	7.23
NDF	26.03	4.63	70.21
ADF	9.48	2.93	34.20

[1]The concentrate was based on wheat bran, micronized soy bran, ground corn, and whey powder.

Table 7. Growth performance and consumption of calves supplemented with cinnamon, oregano, and eucalyptus essential oils.

Variable	Control	Phytobiotic	SEM	P – Treat	P – Treat x day
Body weight, kg				0.86	0.87
d1	40.7	41.0	0.34		
d7	40.9	40.9	0.34		
d14	40.2	40.4	0.33		
d21	41.8	40.9	0.34		
d28	44.0	42.8	0.33		
d35	46.6	45.4	0.35		
d42	50.2	49.0	0.35		
d49	53.9	53.3	0.36		
d56	57.5	57.1	0.36		
d60	61.2	62.4	0.34		
d75	78.1	79.6	0.35		
Weight gain, kg					
d1–60	16.6	16.0	0.21	0.84	–
d1–75	34.4	36.0	0.22	0.39	–
d60–75	17.6	20.0	0.20	0.42	–
Average daily weight gain, g					
d1–60	276	266	0.18	0.88	–
d60–75	1240	1333	0.21	0.80	–
Consumo de concentrado, kg DM					
d1–60	31.2	25.0	3.09	0.14	–
d60–75	26.2	22.8	2.41	0.11	–
Daily milk consumption, kg DM					
d1–60	0.484	0.484	0.00	–	–
Total food consumed (kg/DM)					
d1–60	60.2[a]	54.1[b]	2.59	**0.09**	–
Feed efficiency					
d1–60	0.279[b]	0.296[a]	0.03	**0.05**	–
Feed conversion					–
d1–60	3.58[a]	3.37[b]	0.16	**0.05**	–

Phytobiotic: treatment group, which received the blend of essential oils.
1. Shows treatment effect, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.
2. Shows treatment × day interaction, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.

Table 8. Blood count of calves supplemented with a blend of cinnamon, oregano, and eucalyptus essential oils.

Variable	Control	Phytobiotic	SEM	P - Treat	P - Treat x day
Leukocytes (x10^3 cel/µL)				0.25	**0.01**
d1	14.8	16.6	0.69		
d15	16.6	15.8	0.66		
d30	21.2	17.3	0.77		
d45	12.4[a]	9.91[b]	0.65		
d60	8.00[a]	5.17[b]	0.66		
d75	8.24	7.53	0.66		
Lymphocytes (x10^3 cel/µL)				**0.07**	**0.01**
d1	8.22	7.87	054		
d15	10.8	8.58	0.54		
d30	10.9	10.5	0.54		
d45	8.53[a]	5.60[b]	0.52		
d60	6.70[a]	3.74[b]	0.52		
d75	4.62	3.96	0.52		
Monocytes (x10^3 cel/µL)				0.52	0.21
	1.78	1.38	0.61		
Granulocytes (x10^3 cel/µL)				0.75	**0.05**
d1	3.78	4.91	0.35		
d15	3.32	4.37	0.35		
d30	6.57[a]	4.72[b]	0.35		
d45	2.02	2.66	0.34		
d60	2.07	2.35	0.29		
d75	2.38	2.32	0.28		
Erythrocytes (x10^6 cel/µL)				0.95	0.96
	8.05	8.31	0.03		
Hemoglobin (mg/dL)				0.98	0.95
	10.4	10.4	0.18		
Hematocrit (%)				0.96	0.94
	28.4	28.3	0.71		

Phytobiotic: treatment group, which received the blend of essential oils.
1. Shows treatment effect, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.
2. Shows treatment × day interaction, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.

Table 9. Clinical biochemistry of calves supplemented with cinnamon, oregano, and eucalyptus essential oils.

Variables	Control	Phytobiotic	SEM	P - Treat	P - Treat x day
Glucose (mg/dL)					
	83.5	83.1	2.12		
Cholesterol (mg/dL)				**0.05**	**0.01**
d1	59.5	59.8			
d15	75.8	82.4			
d30	144[b]	161[a]			
d45	109[b]	150[a]			
d60	112[b]	147[a]			
d75	120	131			
Total protein (g/dL)				0.56	0.30
	6.52	6.34	0.25		
Albumin (g/dL)				0.95	0.92
	2.64	2.65	0.09		
Urea (mg/dL)				0.91	0.88
	17.2	16.8	0.55		
Globulin (g/dL)				0.81	0.76
	3.87	3.68	0.18		

Phytobiotic: treatment group, which received the blend of essential oils.
1. Shows treatment effect, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.
2. Shows treatment × day interaction, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.

Table 10. Oxidative status of calves supplemented with cinnamon, oregano, and eucalyptus essential oil blend.

Variables	Control	Phytobiotic	SEM	P - Treat	P - Treat x day
TBARS (g/dL)				0.81	0.86
	17.3	16.3	1.02		
GST (g/dL)				**0.01**	**0.01**
d1	432	414	5.84		
d15	420[b]	457[a]	5.85		
d30	411[b]	525[a]	5.74		
d45	410[b]	492[a]	5.79		
d60	420[b]	539[a]	5.78		
Total Thiols (g/dL)				0.76	0.62
	62.2	65.7	1.03		

Phytobiotic: treatment group, which received the blend of essential oils.
1. Shows treatment effect, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.
2. Shows treatment × day interaction, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.

Tabela 11: Proteinograma de bezerros suplementados ou não com *blend* de óleos essenciais de canela, orégano e eucalipto.

Variables	Control	Phytobiotic	SEM	P - Treat	P - Treat x day
IgA (g/dL)				**0.05**	**0.03**
d1	0.69	0.65	0.02		
d15	0.67	0.64	0.02		
d30	0.70	0.71	0.02		
d45	0.65	0.70	0.03		
d60	0.62[b]	0.75[a]	0.02		
Heavy chain Ig (g/dL)				0.38	0.17
	0.96	1.01	0.04		
Ceruloplasmin (g/dL)				**0.05**	**0.02**
d1	0.52	0.54	0.02		
d15	0.53	0.50	0.02		
d30	0.58[a]	0.51[b]	0.01		
d45	0.52	0.48	0.01		
d60	0.50[a]	0.39[b]	0.02		
Haptoglobin (g/dL)				0.89	0.60
	0.29	0.30	0.01		
C-reactive protein (g/dL)				0.45	0.23
	0.18	0.16	0.01		
Ferritin (g/dL)				0.54	0.42
	0.23	0.20	0.02		
Transferrin (mg/dL)				**0.01**	**0.01**
d1	0.20	0.20	0.02		
d15	0.21	0.20	0.02		
d30	0.20	0.17	0.02		
d45	0.21[a]	0.16[b]	0.01		
d60	0.22	0.17	0.02		

Phytobiotic: treatment group, which received the blend of essential oils.
1. Shows treatment effect, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.
2. Shows treatment × day interaction, where different letters on the same line show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and ≤ 0.1) between groups.

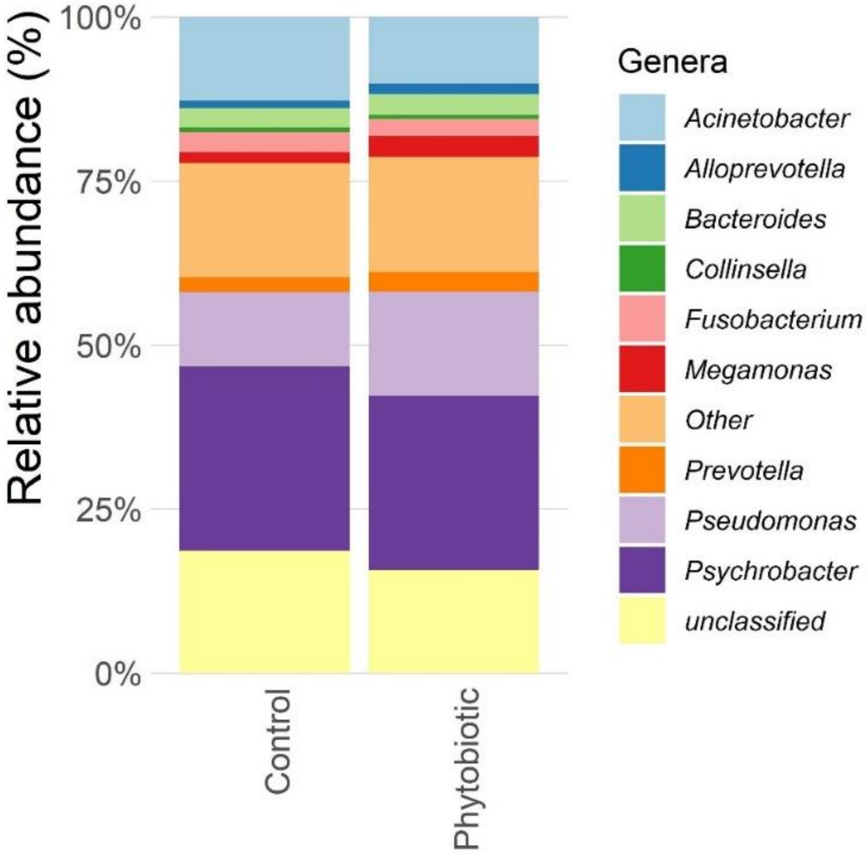

Figure 7. Most abundant bacterial genera found in the feces of calves supplemented with a blend of cinnamon, oregano, and eucalyptus essential oils. NOTE: Control: group of animals that did not receive the blend of essential oils; Phytobiotic: group of animals that received the blend. *Data from the first collection (time 0) were excluded for this analysis.

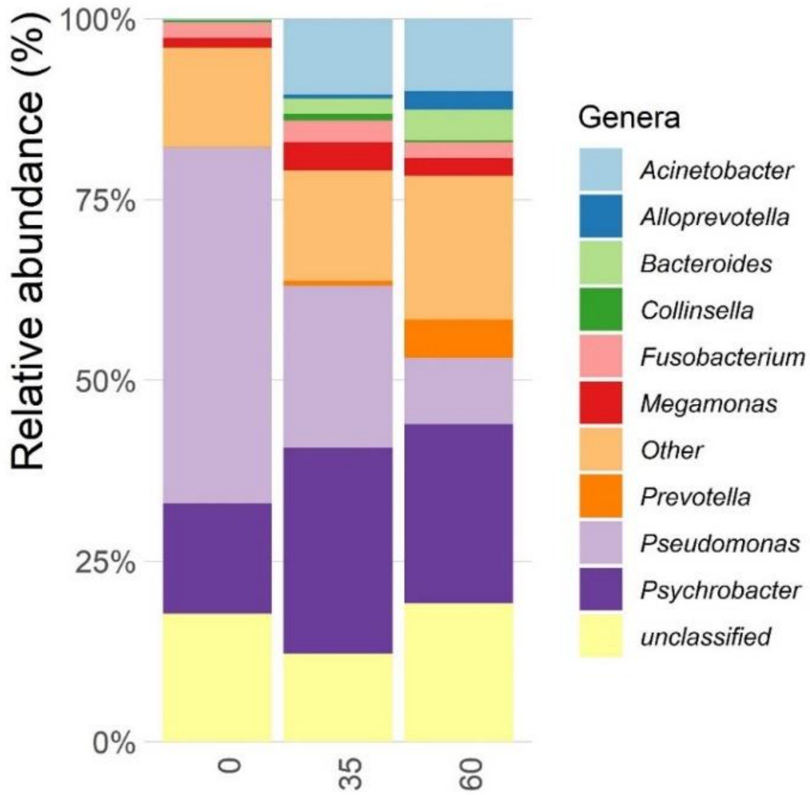

Figure 8. Most abundant bacterial genera found in calf feces on days 0, 35, and 60, dates that represent the days of the experiment.

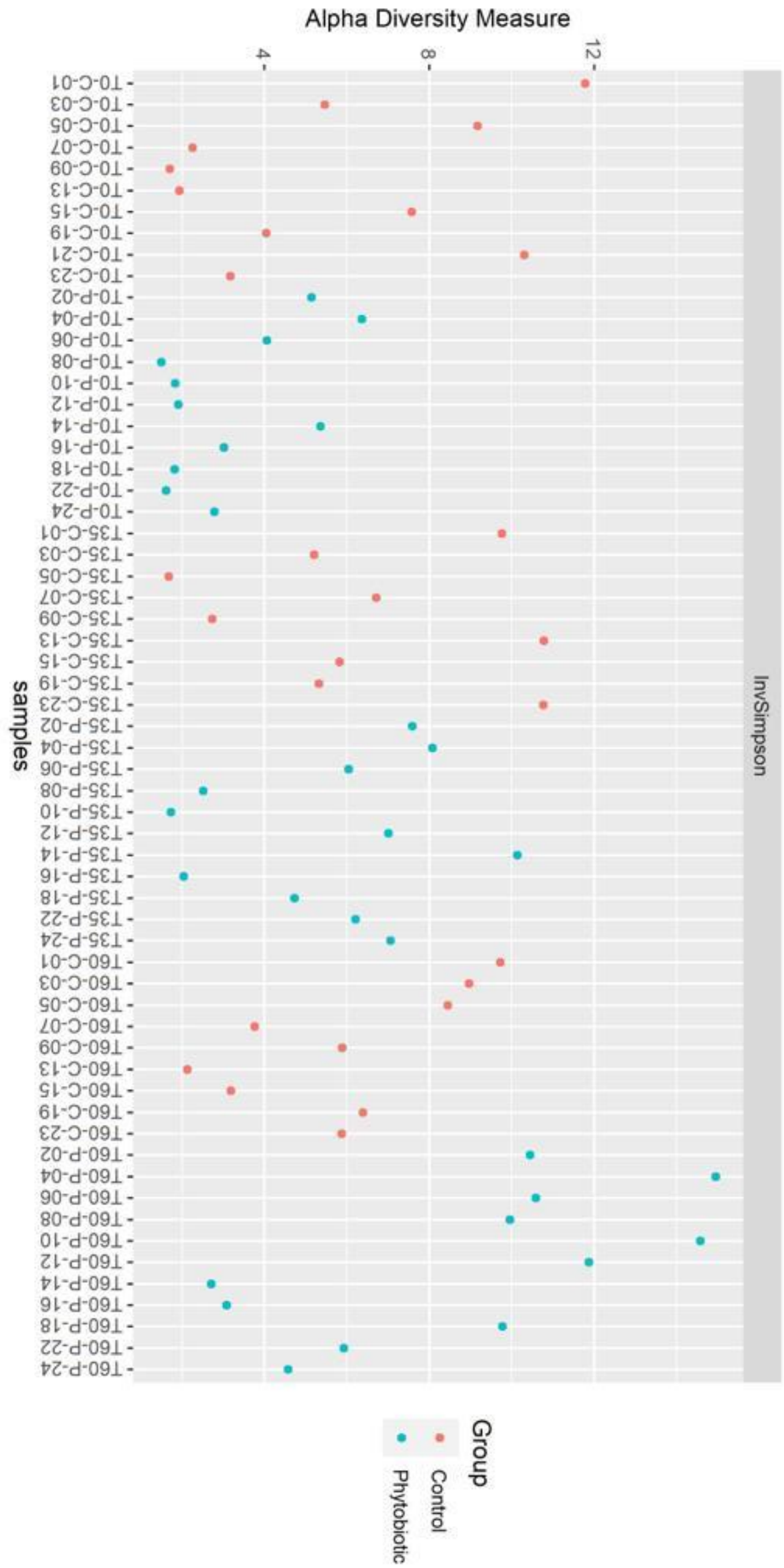

Figure 9. Alpha diversity of each sample. Phytobiotc refers to the group that received the blend of essential oils.

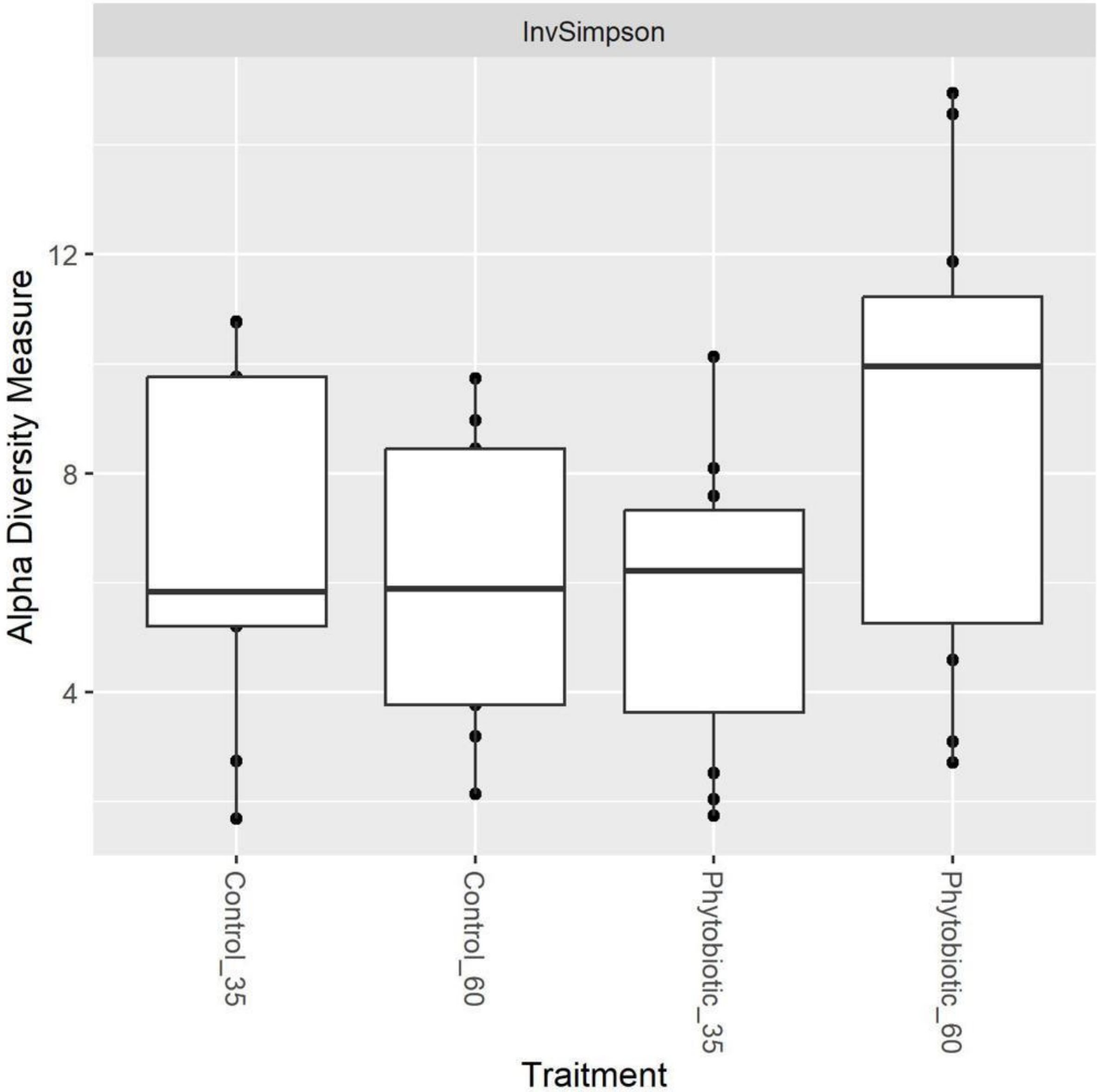

Figure 10. Alpha diversity based on treatment and days 35 and 60 of the experiment.

Phytobiotc refers to the group that received the blend of essential oils.

*Data from the first collection (time 0) were excluded for this analysis.

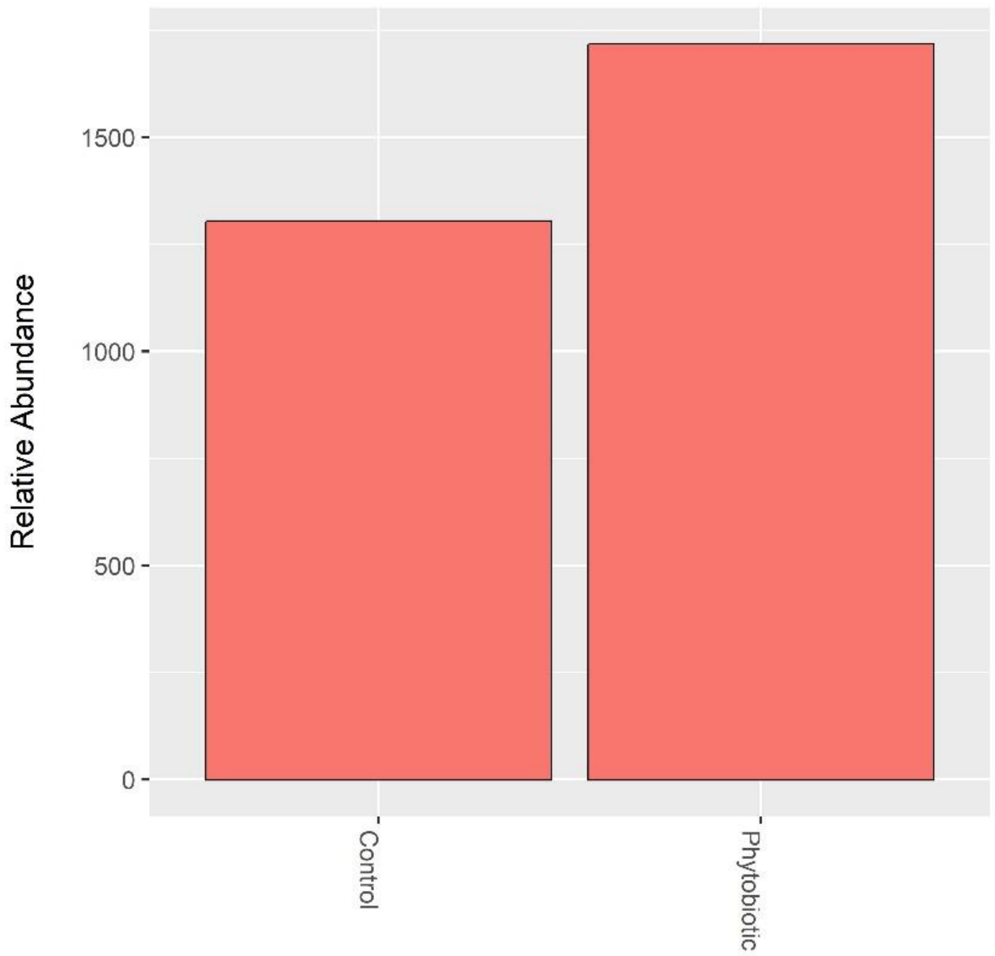

Figure 11. Abundance of *Clostridium* was detected in the feces of calves that did or did not receive the essential oil blend (No difference between treatments – P > 0.05). Phytobiotc refers to the group that received the blend of essential oils.

*Data from the first collection (time 0) were excluded for this analysis.

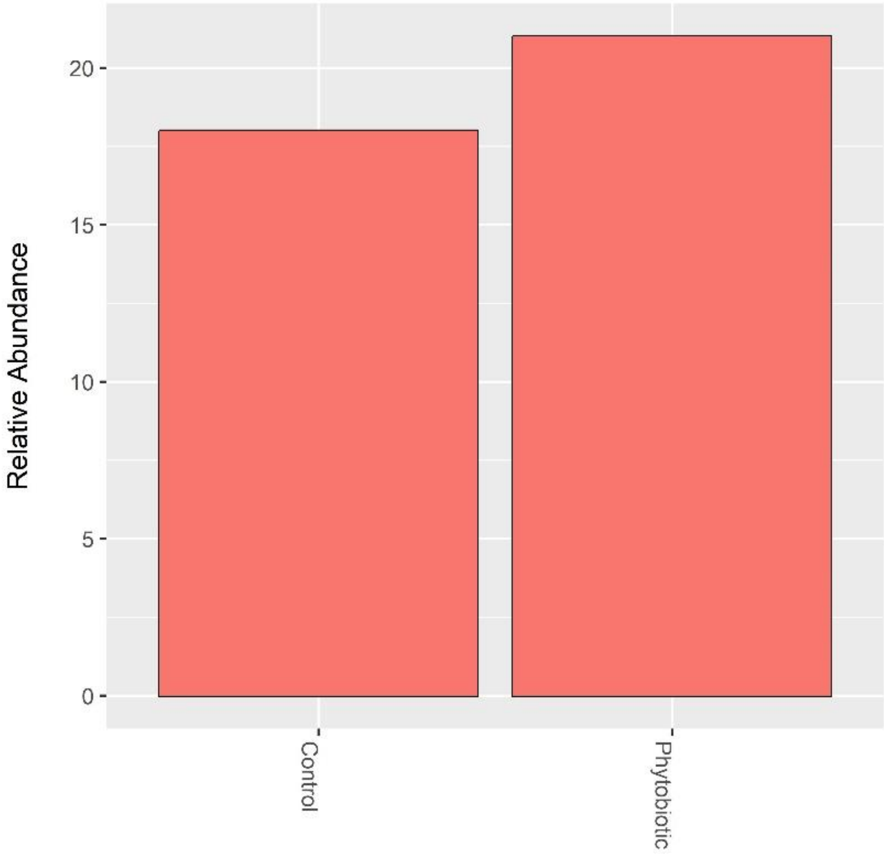

Figure 12. Abundance of *Escherichia* in feces of calves with or without the essential oil blend (No difference between treatments – P > 0.05). Phytobiotc refers to the group that received the blend of essential oils.

*Data from the first collection (time 0) were excluded for this analysis.

5. CONSIDERAÇÕES FINAIS

O uso dos óleos essenciais de orégano, canela e eucalipto de forma conjunta traz benefícios a saúde de bezerros lactentes, melhorando seu sistema imune humoral, aumentando os níveis de antioxidantes e diminuindo a peroxidação lipídica. Contribui pra uma melhor eficiência alimentar, e quando fornecido em uma dose maior, é capaz de proporcionar um melhor ganho de peso desses animais. Porém, as dosagens fornecidas, que são recomendadas pelos fabricantes nesses dois experimentos, não foram suficientes para modificar positivamente a microbiota intestinal, isto é, reduzir as bactérias patogênicas e aumentar as benéficas a saúde do intestino.

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ANEXO A – COMPROVANTE CEUA

UDESC
UNIVERSIDADE DO ESTADO DE SANTA CATARINA | LAGES
CENTRO DE CIÊNCIAS AGROVETERINÁRIAS

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Uso de óleos essências na dieta de bezerros: efeitos sob o desempenho, saúde, microbiologia intestinal e ruminal e sistema antioxidante e imunológico ", protocolada sob o CEUA nº 2322230522 (ID 001574), sob a responsabilidade de **Aleksandro Schafer da Silva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade do Estado de Santa Catarina (CEUA/UDESC) na reunião de 27/05/2022.

We certify that the proposal "Use of essential oils in the diet of calves: effects on performance, health, intestinal and rumen microbiology and antioxidant and immune system", utilizing 48 Bovines (48 males), protocol number CEUA 2322230522 (ID 001574), under the responsibility of **Aleksandro Schafer da Silva** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of Santa Catarina State (CEUA/UDESC) in the meeting of 05/27/2022.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 06/2022 a 05/2023 Área: Zootecnia

Origem:	Animais de proprietários						
Espécie:	Bovinos	sexo:	Machos	idade:	3 a 150 dias	N:	48
Linhagem:	Holandês			Peso:	30 a 150 kg		

Local do experimento: bezerreiro - setor de ruminantes na FECEO - UDESC Oeste

Lages, 31 de maio de 2022

José Cristani
Coordenador da Comissão de Ética no Uso de Animais
Universidade do Estado de Santa Catarina

Pedro Volkmer de Castilhos
Vice-Coordenador da Comissão de Ética no Uso de Animais
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