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UTILIZAÇÃO DE *BLEND* DE ÓLEOS ESSENCIAIS NA DIETA LÍQUIDA DE BEZERROS

Belo Horizonte

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Utilização de *blend* de óleos essenciais na dieta líquida de bezerros

Versão Final

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Área de concentração: Produção Animal/ Ruminantes

Orientador: Prof. Sandra Gesteira Coelho

Coorientador: Mariana Magalhães Campos

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FOLHA DE APROVAÇÃO

Utilização de *blend* de óleos essenciais na dieta líquida de bezerros

Joana Palhares Campolina

Tese de Doutorado defendida e aprovada, no dia treze de dezembro de dois mil e vinte e um, pela Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Zootecnia da Universidade Federal de Minas Gerais, constituída pelos seguintes membros:

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Para Guilherme, meu porto seguro durante essa jornada.

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“Não haverá borboletas se a vida não passar por longas e silenciosas metamorfoses.”

“Ensinar é um exercício de imortalidade.
De alguma forma continuamos a viver
naqueles cujos olhos aprenderam a ver
o mundo pela magia da nossa palavra.
O professor assim, não morre jamais.”

“O corpo é o lugar fantástico onde mora, adormecido, um universo inteiro…
Tudo adormecido. O que vai acordar é aquilo que a Palavra vai chamar…
As palavras são entidades mágicas, potências feiticeiras, poderes bruxos que despertam os mundos que jazem dentro dos nossos corpos, num estado de hibernação, como sonhos…
A este processo mágico pelo qual a Palavra desperta mundos adormecidos se dá o nome de educação.”

Rubem Alves

RESUMO

O objetivo deste estudo foi avaliar o impacto de uma mistura comercial de óleos essenciais na dieta líquida na: **1)** ingestão alimentar, desempenho, desenvolvimento corporal, células sanguíneas e metabólitos, fator de crescimento semelhante à insulina-1 (IGF-1), fermentação rumenal, escores fecais e respiratórios; e **2)** digestibilidade, peso e histologia dos órgãos, expressão genética e proliferação de células de baço de bezerros leiteiros. As variáveis foram avaliadas durante a fase de aleitamento (4 – 60 d de idade) para machos e fêmeas, bem como efeitos residuais no pós-desaleitamento (61 – 90 d de idade) nas fêmeas. Foram utilizados 45 bezerros recém-nascidos Holandês e Girolando, com peso corporal (PC) ao nascimento de 32,6 ± 4,9 kg. As unidades experimentais foram atribuídas a um tratamento controle (CON, n = 15 fêmeas, e n = 8 machos) ou ao tratamento de suplementação de uma mistura de óleos essenciais (MEO, n = 14 fêmeas, e n = 8 machos). O MEO foi suplementado no sucedâneo com 1 g/d/bezerro (Apex Calf, Adisseo, China). Durante o aleitamento, todos os bezerros receberam 5 L de sucedâneo/d reconstituídos a 15% de sólidos totais, água e concentrado à vontade. Após o desaleitamento, as fêmeas receberam 3 kg de concentrado/d, e silagem de milho à vontade. A ingestão de alimentos, os escores fecais e respiratórios foram avaliados diariamente. O PC foi mensurado a cada três dias, e o desenvolvimento corporal semanalmente. Foram coletadas amostras de sangue nos dias 0, 30 e 60 dias para a contagem total de células sanguíneas, semanalmente para determinar ß-hidroxibutirato, ureia e glicose, e quinzenalmente para IGF-1. Os parâmetros ruminais (pH, ácidos graxos voláteis, amônia-N e proporção acetato:propionato - C2:C3) foram medidos nos dias 14, 28, 42, 60, 74 e 90. A digestibilidade aparente total de nutrientes foi determinada entre 55 e 60 dias de idade. No dia 60 ± 1 os machos foram eutanasiados para avaliação do peso de órgãos, histologia, proliferação de células de baço e análise de expressão genética intestinal. Os dados foram analisados por meio de modelos lineares mistos utilizando o método REML no pacote nlme em R para variáveis contínuas, e testes não paramétrico para variáveis categóricas. Não houve diferenças na ingestão de alimentos, desempenho, desenvolvimento corporal e metabólitos sanguíneos para as fêmeas e machos. No entanto, a proporção de C2:C3 do grupo BEO foi maior em todas as fases ($P = 0,05$). Durante o aleitamento, as fêmeas BEO apresentaram menores contagens de basófilo ($P \leq 0,001$), plaquetas (P = 0,04), efeito cumulativo para linfócitos ($P \leq 0,001$) e menores escores fecais ($P = 0,04$). O ensaio de digestibilidade, expressão gênica intestinal e ensaio de proliferação de células

de baço para os machos não apresentou diferença. Os machos BEO apresentaram pH ruminal mais baixo, trato respiratório mais leve, pâncreas maior, intestinos mais pesados, vilos do íleo maiores, maiores concentrações de butirato cecal e uma maior contagem de eosinófilos (P< 0,05). Esses resultados demonstram que a suplementação com óleos essenciais para bezerros em aleitamento pode contribuir para a manipulação ruminal, desenvolvimento intestinal e função imunológica.

Palavras-chave: Aleitamento, aditivo, bezerro leiteiro, imunidade intestinal, saúde intestinal.

ABSTRACT

The aim of this study was to evaluate if a commercial blend of essential oils supplemented in the liquid diet would affect **1)** feed intake, performance, body development, blood cells and metabolites, insulin-like growth factor-1 (IGF-1), rumen fermentation, fecal scores, and respiratory scores; and **2)** digestibility, internal organs weight and histology, gene expression, and a spleen cell proliferation of pre-weaned calves. All outcomes were evaluated during pre-weaning (4 – 60 d of age) for bull and heifer calves, and carry-over effects during post-weaning (61 – 90 d of age) for heifer calves. It was enrolled 45 newborn Holstein and Hostein × Gyr crossbred calves, with body weight (BW) at birth of 32.6 ± 4.9 kg. Experimental units were assigned to either a control (CON, n = 15 heifers, and n = 8 bull calves) or a blend of essential oil supplementation treatment (BEO, n = 14 heifers, and n = 8 bull calves). The BEO was supplemented in the milk replacer (MR) with 1 g/d/calf (Apex Calf, Adisseo, China). During the pre-weaning phase, all calves were fed daily 5 L of MR/d reconstituted to 15% with water and starter provision *ad libitum*. During the post-weaning, heifers received 3 kg of starter/d, and *ad libitum* corn silage. Feed intake, fecal and respiratory scores were evaluated daily. The BW was measured every three days, while body development was recorded weekly. Blood samples were collected on 0, 30, and 60 days of age for total blood cell count, weekly to determinate ß-hydroxybutyrate, urea and glucose, and biweekly for IGF-1. Ruminal parameters (pH, volatile fatty acids, ammonia-N, and acetate:propionate proportion - C2:C3) were measured on days 14, 28, 42, 60, 74 and 90. Apparent total nutrient digestibility was determined from d 55 to 60 of age, and on d 60 ± 1 animals were euthanized for organ weight, histology, spleen cell proliferation, and intestinal gene expression analysis. Data were analyzed independently using linear mixed models using the REML method in nlme package in R for continuous outcomes, and a non-parametric test was used for ordered categorical outcomes. There were no differences on feed intake, performance, body development, and blood metabolites during both pre-weaning and post-weaning periods for bulls and heifer calves. However, heifer's proportion of C2:C3 during pre- and post-weaning ($P = 0.05$) were affected, as well as basophil ($P \leq 0.001$), and platelet ($P = 0.04$) counts during pre-weaning, and a cumulative effect for lymphocytes ($P \leq 0.001$). Heifers' fecal scores were significant ($P = 0.04$) during pre-weaning, with lower values for BEO. There were no differences for digestibility, intestinal gene expression, and spleen cell proliferation assay for the bull calves. The BEO bull

calves presented a lower ruminal pH, bigger pancreas, heavier intestines, bigger ileum villi, and higher cecum butyrate levels ($P < 0.05$). The bulls from the CON group had a heavier respiratory tract and a higher eosinophil count ($P< 0.05$). These results demonstrate that supplementing essential oils to dairy calves could contribute to ruminal manipulation, gut development, and immune function.

Keywords: Additive, dairy calf, gut immunity, intestinal health, pre weaning.

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CAPÍTULO III – ARTIGO I

ACRONYMS AND ABBREVIATION LIST

A

ADF – Acid Detergent Fiber

ADG – Average Daily Gain

AGP – Antimicrobial Growth Promoter

B

BEO – Blend of essential oils

BHB – Beta-hydroxybutirate

BrECC – Control of *Escherichia Coli* Extratc

BW – Body weight

C

C2 – Acetic Acid

C3 – Propionic Acid

C4 - Butyric Acid

CFU – Colony Forming Unit

CI – Confidence Interval

CO_2 – Carbon Dioxide

CON – Control Group

CP – Crude Protein

D

D – Day

DEI – Digestible Energy Intake

DM – Dry Matter

DMI – Day Matter Intake

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

E

ECE – *Escherichia Coli* Extratc

EDTA – Ethylenediamine Tetraacetic Acid

ELISA – Enzyme-Linked Immunoassay

EO – Essential Oil

G

GAPDH – Glyceraldehyde 3-Phosphate Dehydrogenase

GE – Gross Energy

GEF – Fecal Gross Energy

GEI – Gross Energy Intake

GEMR – Milk Replacer Gross Energy

GER – Refusals Gross Energy

GES – Starter Grosse Energy

GEU – Urinary Gross Energy

GIT – Gastrointestinal Tract

H

HB – Hemoglobin

HG – Heart Girth

I

IGF-1 – Insulin Growth Factor Type I

IL-6 – Interleukin 6

IL-10 – Interleukin 10

L

LPS – *E. coli* Lipopolysaccharide

M

MCHC - Mean Corpuscular Hemoglobin Concentration

MCV – Mean Corpuscular Value

MEI – Metabolizable Energy Intake

MI – Mitotic Index

MIC – Minimum Inhibitory Concentration

MR – Milk replacer

MTT -

MW – Metabolic Weight

N

N – Nitrogen

NDF – Neutral Detergent Fiber

NF – Fecal Nitrogen

NFR – Nutrient Feces Recovery

NI – Nitrogen Intake

NLR – Neutrophils Lymphocytes Ratio

N-NH_3 – Ammonia Nitrogen

NU – Urinary Nitrogen

NUI – Nutrient Intake

P

PC – Peso corporal

PCV – Packed Cell Volume

PH – Potential of Hydrogen

PLR – Platelet to Lymphocytes Ratio

PKC – Protein Kinase C

PMA – Phorbol 12-Myristate 13-Acetate

R

RBC – Red Blood Cell

RH – Rump Height

RNA – Ribonucleic acid

RT-qPCR – Real Time Polymerase Chain Reaction

RW – Rump Width

S

SD – Standard Deviation

SEM – Standard Error of the Mean

T

T – Treatment

TNF – Tumor Necrosis Factor

V

VFA – Volatile Fatty Acids

W

W – Week

WH – Withers Height

SUMÁRIO

1. CHAPTER I – GENERAL INTRODUCTION

Pre weaning is one of the most important phase of calves' development and performance (Bach and Ahedo 2008). Therefore, the use of nutritional additives for neonatal animals has been under evaluation to help achieve more efficiency in the rearing phase, improving body development, accelerating the conversion of pre-ruminants to ruminants, boosting immunity, and helping to deal with the health challenges of the neonatal phase (Benchaar et al. 2008; Drackley 2008).

For many years dairy industry routinely used large amounts of antimicrobial growth promoters in animal nutrition as the main additive (Gonzalez Ronquillo and Angeles Hernandez 2017). However, public concern about the probable emergence of antimicrobial resistance, fear over human health, and pressure to eliminate non-plant xenobiotic agents from animal diets caused changes in the use of these additives (Greathead 2003). In 2006 European Union banned antimicrobial use as an animal growth promoter. Therefore, the research for alternatives increased substantially (Jouany and Morgavi 2007). Furthermore, a growing concern of cattle impact over greenhouse gases emission and increased nitrogen excretion on soil made the race to find a viable and good alternative even more urgent.

To fill this gap, a large number of different kinds of additives have been tested and used. The most common alternatives used are probiotics, prebiotics, dicarboxylic acids, enzymes, polyunsaturated fatty acids, and plant components (Ballou, Davis, and Kasl 2019), being this last one the latest researched choice. Plants components are organic chemical compounds produced to help the plant cells. The essential oils (EO) are one of these components that have antimicrobial, antioxidant, and antiseptic effects and help animal immunity and performance (Benchaar et al. 2008), being a promising alternative to be used as an additive for calves.

Currently, it has been used for EO supplementation in dairy farms through a liquid or solid diet. Commercial starters and milk replacers have been using EO as one of the ingredients of their product composition. Impact in development and health have been varying according to dose, through, and type o EO. However, it is important to mention that negative impacts have not been found yet.

Thus, the aim of this study was to evaluate EO supplementation in the liquid diet to pre-weaned dairy heifer and bull calves. We evaluated 45 calves' performance, nutrient digestibility, ruminal and metabolic parameters, immunity, health scores, organ growth and inflammatory gene expression

outcomes during pre-weaning, and carryover effects on post-weaning. Our hypothesis was that supplementation a commercial blend of essential oils in the liquid diet would improve performance and immunity and decrease neonatal disease impact.

2. CHAPTER II – LITERATURE REVIEW

Additives have been widely used in animal production with the main objective to help animal efficiency (Bacanlı and Başaran, 2019). However, with the demand for more sustainable production that incorporates animal, human and environmental perspectives, the use of some additives has been questioned lately, especially due to the rise of zoonotic multidrug-resistant microbes (Millet and Maertens, 2011). Thus, natural plant-based or microbial additives have received attention as a possible alternative to improve animal performance and efficiency (Salazar et al., 2019).

Nutraceuticals are compounds used to improve immune responses and performance. This term is derived from the word nutrition added to pharmaceutical (Ballou et al., 2019). They are normally divided into probiotics, prebiotics, polyunsaturated fatty acids, and phytonutrients (essential oils). Briefly, probiotics are directed fed microbials used to improve the gastrointestinal tract (GIT) community, thus regulating this organ integrity and regulating inflammation. Prebiotics are indigestible carbohydrates that improve animal health, serving as an energy source for commensal or probiotic bacteria. Polyunsaturated fatty acids are fats that manipulate the degree of inflammation. Furthermore, phytonutrients are a group of plant secondary compounds isolated from plants with potential therapeutic application (Gaggìa et al., 2010).

2.1. What are essential oils?

The EO are delivered from plants, and to survive environmental stressors such as pathogen attack, plant competition, or herbivore ingestion, those plants develop defense mechanisms. These mechanisms can be mechanical – thorn, prickle, spines – or chemical – plant secondary compounds. The plant secondary compounds are not essential for plant survival or growth (Wina, 2012). Their main purpose is stopping animals from eating the vegetable, causing aversion, bitter taste, and interfering with the animal digestive process. Because of that, they are considered "anti-nutritive" agents and affect animal organs' functionality as well as animal behavior (Durmic and Blache, 2012). They are also responsible for the odor and color of plants and spices, having an important function as plant chemical messenger, attracting insects for pollination, and dispersing seeds.

There are innumerable chemical secondary compounds divided into many classes, such as flavonoids, tannins, saponins, alkaloids, non-protein amino acids, cyanogenic glycosides, glycosinolates, and terpenes (Hart et al., 2008). These classes are structured into three big groups: saponins, tannins, and essential oils (EO) (Calsamiglia et al., 2007). Saponins and tannins effects are well known for ruminants. However, besides long-time use in human medicine, EO information and use for ruminants, especially calves, is scarce.

The OE are blends of these secondary chemical complexes, characterized as volatile aromatic compounds, with 20 to 60 different chemical substances (Benchaar et al., 2008). Terpenoids and phenylpropanoids are the main active compounds and the most important (Jouany and Morgavi, 2007). The name “essential oil” comes from “essence”, related to the property that these substances of providing flavor and odors, and “oil” since they are mostly arranged with low-density lipid composts, but they are not true oils (Benchaar et al., 2007; Calsamiglia et al., 2007).

The EO are extracted from various aromatic plants that are normally found in warm countries and include tea tree oil, lemon oil, clove oil, garlic oil, cinnamon oil, thyme oil, mustard oil, oregano oil, lavender oil, eucalyptus oil, peppermint oil (Bhavaniramya et al., 2019). There are more than 3000 known EO, with 300 of these with economic importance. This is because EO has a variable composition and depends on several aspects such as plant species, life stage, part of the plant – flower, bud, seed, leaves, twigs, bark, herbs, wood, fruits, and roots – and the environment where it grows – type o soil, humidity, solar exposure, temperature, etc. To produce it, there are different methods, such as hydrodistillation, steam distillation, or dry distillation, being the steam distillation the most common (Burt, 2004; Nazzaro et al., 2013).

2.2. Biological properties and mechanisms of action

The EO properties are well known, but their exact mechanism is still unclear. They have several effects on human and animal health, including their antimicrobial, antioxidant, antiseptic, and immunomodulatory effect (Calsamiglia et al., 2007; Ballou et al., 2019)

The antiseptic properties of EO have been known for many years; however, the first scientific evidence was only described in the XX century (Greathead, 2003). The EO molecule has and

hydrophobic nature due to cyclic hydrocarbons. This aspect allows interaction with lipids in the cell membranes or mitochondria, causing disturbance and changes in membrane structure and cell morphology, leading to increase permeability. Thus, leakage of cells' ions and contents can happen, leading to spending large amounts of energy on cell death (Dorman and Deans, 2000; Calsamiglia et al., 2007). Entering the cell, they can also coagulate some cell constituents through protein denaturation (McGrath et al., 2018) and reduce intracellular ATP pool (Nazzaro et al., 2013), causing inhibition of bacteria growth and functionality. Therefore, they have dose-dependent antimicrobial – bactericidal and bacteriostatic – effects on several microorganisms (bacteria, fungi, viruses, and protozoa) (Greathead, 2003). Due to differences on its membrane, Gram-negative bacteria have a less penetrable membrane and are more resistant to the effect of the EO (Jouany and Morgavi, 2007). However, with small molecular weight, some EO can disintegrate the external membrane of these gram-negative bacteria and interrupt cellular energy metabolism, bacterial replication, and functionality (Benchaar et al., 2008; Ballou et al., 2019).

2.3. Use and impact on calves' performance

The use of EO to young or older animals has already been shown to modulate ruminal and gut microbiota and ecosystem, impaction on performance, nutrient use, and health (Arshad et al., 2021). Its supplementation impacted intestinal track development and influenced ruminal microbiological activity (Cobellis et al., 2016) by suppressing some microbiota species (McIntosh et al., 2003). Consequently, they optimize ruminal function mitigating ammonia and decreasing methane production through deamination and methanogenesis (McIntosh et al., 2003; Patra and Yu, 2012), increasing propionate to improve energy available to the animal, reducing feed protein degradation leading to an increase of amino acids available in the small intestine, reducing degradation of rapidly fermented carbohydrates controlling the lactic acid concentration and improving fiber digestion (Jouany and Morgavi, 2007). The EO use for young calves has already been shown to be efficient in positively modulating ruminal fermentation parameters (Vakili et al., 2013). They have been cited as the natural replacement for ionophores on rumen fermentation and animal performance (McGrath et al., 2018) since they can increase flavoring, palatability, and digestibility of feed, reflection on calf's

zootechnical performance increasing calf's starter intake, feed efficiency, body weight gain and nutrient digestibility (Santos et al., 2015; Froehlich et al., 2017; Zhou et al., 2020).

The EO can modulate ruminal VFA gut microbiota and improve animal performance. They increase ingestion of dry matter and growth performance due to antimicrobial activity and ruminal manipulation (Ornaghi et al., 2017). Thus, manipulation of gastrointestinal tract metabolism will reflect animal performance parameters and increase daily weight gain, animal growth, and feed efficiency (Spanghero et al., 2009). However, no effect over body frame development was found for supplemented animals. Kazemi-Bonchenari et al. (2018) suggested that EO supplementation could be only effective in structural growth when associated with higher protein concentration on the starter.

This performance impact could be explained by changes in blood metabolites concentrations and present carry-over effects after EO supplementation withdrawal (Akbarian-Tefaghi et al., 2018). Extracts of EO from cinnamon improve insulin receptor function, increasing insulin sensitivity and liver function (Jeshari et al., 2016). Coriander extract can stimulate insulin secretion and gluconeogenesis by increasing glucose metabolism and uptake by the muscle (Greathead, 2003), leading to increased blood triglycerides (Asghari et al., 2021). Experiments with EO have already shown differences in blood glucose, with lower values for supplemented animals. The serum glucose is a valuable indicator of nutritional status and provides a rapid assessment of animal stress (Lakhani et al., 2019). Additionally, it has been recently discovered that EO affects mineral metabolism, influencing ruminal calcium transportation and impaction over calcium metabolism (Braun et al., 2019).

2.4. Impact on health and immune status

The immune system plays an important role in dairy calves' health and can be classified into two distinct types: innate and adaptive immune responses. The innate system is the most important for young calves since the animal is born with it. It uses external mechanisms such as physical barriers and internal mechanisms such as phagocytic cells and signaling proteins (Chase and Kaushik, 2019). The innate system is the first line of defense and acts in a non-specific way. On the other hand, the adaptive immune response is a specific system that recognizes, remembers, and acts against specific

pathogens (Chase et al., 2008). It can be divided into passive immunity, developed through received antibodies, or active immunity, developed against antigen or pathogen using humoral and cell-mediated approaches (Jain, 1998). However, the calf will only be fully immunologically developed around puberty (Mark et al., 2014). For that reason, improving heifer immunity as fast and as soon as is possible is extremely necessary.

Some EO have been reported as an alternative to increasing ruminant resilience with its immunostimulant properties (Franz et al., 2010). They can enhance immune functions through anti-inflammatory effects, increase mucosal blood flow, increase humoral and cellular immunity, and modulation of immune pathway through specific receptors, enzymes, or molecules such as cytokines and neuropeptides (Ballou et al., 2019). An increase in platelet, white blood cell counts, hemoglobin, packed cell volume, and mean corpuscular volume (Seirafy and Sobhanirad, 2017) and IgA titers have been reported (Durmic and Blache, 2012; Lakhani et al., 2019). Higher globulin levels for EO supplemented animals also indicate a higher humoral immune response (Lakhani et al., 2019). Pérez-Rosés et al. (2015), studying 15 different OE, observed changes in leucocyte phagocytic activity and inhibition of complement system, proving that EO affects the immune system and inflammatory response.

The effect on inflammatory responses reflect on cytokines, acute proteins, and blood immune cells release (Oh et al., 2017) since they have the property of modulating anti-inflammatory responses by inhibition of leukotriene synthesis and lipoxygenase effect, as well as the inhibition of pro-inflammatory cytokines such as TNF-α and IL-6 (Miguel, 2010). Thus, EO and its blends have been used to treat chronic inflammation conditions in humans (de Lavor et al., 2018), acting as an anti-eosinophilic agent (Rogerio et al., 2010). They help this modulation lowering oxidative process due to its antioxidant activity (Bhavaniramya et al., 2019). This antioxidant activity happens by scavenging free radicals such as reactive oxygen and nitrogen species, produced during inflammatory processes or diseases, inhibiting membrane lipids peroxidation, chelating metals, and stimulating antioxidant activities (Burt, 2004; Oh et al., 2017). This prevents the accumulation of reactive oxygen intermediates and helps intraluminal elimination of free radicals.

The EO can also act over the calf's microbiota, increasing beneficial microorganisms in the intestinal track (Santos et al., 2015). This could help disease morbidity decrease in young animals, reduce disease severity, or be an alternative for veterinary treatment since they have already been

shown to decrease *E. Coli* shedding in young calves (Asghari et al., 2021). Pempek et al. (2018) found that EO supplementation in young calves decreased omphalophlebitis incidence and Bampidis et al. (2006) compared neomycin and EO treatment against colibacillosis found the same efficacy from both treatments.

The microbiota and immune manipulation caused by this plant secondary compound also can affect the nervous system through impact over physiological and psychological responses. Thus, changing animal behavior decreases fear, depression, and anxiety. It is suggested that the EO interact with neurotransmissior sensitizing agents involved in nociception. However, it is not fully understood how the precise mechanism occurs (Durmic and Blache, 2012). It is known that EO can also decrease cortisol levels (Lakhani et al., 2019). Since cortisol is related to behavior and immune response, this would explain some comportment changes.

2.5. References

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3. CHAPTER III – PAPER I – PUBLISHED ON PLOS ONE

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Effects of a blend of essential oils in milk replacer on performance, rumen fermentation, blood parameters, and health scores of dairy heifers

3.1. Abstract

The aim of this study was to evaluate how the inclusion of a blend of essential oils in milk replacer (MR) affects different outcomes of dairy heifers. The outcomes evaluated: feed intake, performance, body development, blood cells and metabolites, insulin-like growth factor-1 (IGF-1), rumen fermentation, fecal scores, and respiratory scores. All outcomes were evaluated during pre-weaning (4 – 60 d of age), and carry-over effects during post-weaning (61 – 90 d of age) periods. The experimental units utilized were 29 newborn Holstein × Gyr crossbred dairy heifers, with genetic composition of 5/8 or more Holstein and 3/8 or less Gyr and body weight (BW) at birth of 32.2 ± 5.2 kg. Experimental units were assigned to either a control (CON, n = 15) or a blend of essential oil supplementation (BEO, n = 14) treatment, maintaining a balance of genetic composition. The BEO was supplemented in the MR with 1 g/d/calf of a blend of essential oils (Apex Calf, Adisseo, China) composed by plant extracts derived from anise, cinnamon, garlic, rosemary, and thyme. During the pre-weaning phase, all heifers were fed 5 L of MR/d reconstituted to 15% (dry matter basis), divided into two equal meals. Water and starter were provided *ad libitum*. During the post-weaning, animals received a maximum of 3 kg of starter/d, and *ad libitum* corn silage, divided into two meals. Feed intake, fecal and respiratory scores were evaluated daily. The BW was measured every three days, while body development was recorded weekly. Blood samples were collected on 0, 30, and 60 d of age for total blood cell count, weekly and on the weaning day to determinate ß-hydroxybutyrate, urea and glucose, and biweekly for IGF-1. Ruminal parameters (pH, volatile fatty acids, ammonia-N, and acetate:propionate proportion - C2:C3) were measured on days 14, 28, 42, 60, 74 and 90. A randomized complete block design with an interaction between treatment and week was the experimental method of choice to test the hypothesis of the BEO's effect on all outcomes. An ANOVA procedure was used for continuous outcomes, and a non-parametric test was used for the ordered

categorical outcomes, both adopting a CI = 95%. Results indicated that there was not enough evidence to accept the alternative hypothesis of the effect of BEO in MR on feed intake, performance, body development, and blood metabolites during both pre-weaning and post-weaning periods. However, results indicated that the inclusion of BEO in MR significantly affects the proportion of C2:C3 during pre- and post-weaning ($P = 0.05$). Similarly, the effect was significant for basophil ($P \leq 0.001$), and platelet ($P = 0.04$) counts pre-weaning. The interaction between week and treatment was also significant for lymphocytes ($P \leq 0.001$), revealing a cumulative effect. Lastly, fecal scores were also significant ($P = 0.04$) during pre-weaning, with lower values for BEO. The BEO contributed to ruminal manipulation in pre-weaning and carry-over effects in post-weaning, immunity improvement, and decreased morbidity of neonatal diarrhea in the pre-weaning phase.

3.2. Introduction

A good calf-rearing program should embrace aspects that encompass from body development, stress reduction, meet nutritional requirements, and housing management to optimize calf health status. Average daily gain (ADG) and body weight (BW) at weaning are key metrics used to measure the success of the rearing program. It is well known that these parameters are related to the success of the rearing program, as well as the heifer's future milk production. Therefore, a bad life start can negatively impact animal adult performance [1]. Nutritional problems and neonatal diseases, especially diarrhea and respiratory syndrome, are some examples of negative impacts on the calf's young life. They can act as stressors, lowering calf immunity, increasing animal susceptibility to other disorders, and raise mortality rates [2,3].

Therefore, tools that help provide proper nutrition, and improve heifer development and health, are essential to reduce disease morbidity and mortality and accelerate the calf development. Additionally, since a calf is born functionally as a non-ruminant, the digestive system, and other organs and tissues, change in several weeks and the microbiota colonization changes to adapt to these transformations [1]. The bacteria in the rumen must start the fermentation of carbohydrates, so the calf can become dependent mostly on volatile fatty acids (VFA) and not on lactose-driven metabolism [4].

For that matter, procedures that reduce the animal's susceptibility to pathogens and stressors, and help this pathway change, may improve future performance and productivity [5].

Since the discovery of the improvement in animal growth due to antimicrobials almost 80 years ago, antimicrobials growth promoters (AGP) have been widely used as a tool to improve both rumen development and animal health [5,6], prevent diseases, and increase performance and feed efficiency [7,8]. However, the use of AGP in animal production for these purposes has been under severe criticism and banned in several countries [9]. The overuse of antimicrobial's concerns human health since there is already a well-established correlation between the increase of bacterial population resistance and the use of AGP, putting both humans and animals at risk [10]. The World Health Organization considers the antimicrobial resistance one of the three major threats to public health [11]. However, the global trends in antimicrobial use show that some countries with the largest share of global antimicrobial consumption in food animals initiated a shift toward a more conservative use [12]. The EU banned the use of AGP since 2006 [13] and the US published the Veterinary Feed Directive in 2015, which limited the use of AGP under the professional supervision of a licensed veterinarian [14] and banned all medically important antimicrobials for humans in 2017 [11]. Other big livestock producing countries, such as China and Mexico, are also changing the acceptability of AGP's use in food animal production [11]. Therefore, there is a motivation for more prudent use of antimicrobials [15] and research for substitutes that can improve animal performance and health. A large number of new additives such as prebiotics and probiotics, organic acids, phytogenic substances, and essential oils have shown good results to improve animal production [4,16] and appear to be a good alternative to decrease the use of AGP and alleviate the antimicrobial resistance [16,17]. One of these alternatives is the phytogenic feed additives, also known as phytobiotics and botanicals, commonly defined as plant secondary compounds [18,19].

Essential oils are one of the additives derived from herbal plant secondary chemical components. They are constituted by volatile or ethereal oils that have been applied as a natural and safe alternative for antibiotics [20]. Some of their properties are antiseptic and antimicrobial activities that interfere with bacterial, fungal, and protozoa cell functioning [16], presenting a similar efficiency to treat some diseases as antimicrobials [21]. They also contribute to the prevention of oxidative stress [22] and help the immune response change leukocyte phagocytic activity and inhibit the complement system [23]. Lastly, essential oils have been shown to function similarly to ionophores, a type of AGP [24]. They

can influence gastrointestinal tract development, rumen microbiological activity, improve feed efficiency, and decrease neonatal diseases [16,25].

Studies focusing on essential oils' action as growth promotors for pigs and poultry show the supplementation's positive effects, generally associated with effects on the gastrointestinal tract (GIT) [26,27]. In those species, essential oil supplementation increased digestibility, improved pancreatic enzymes' activity, changed microbiota, impacted the absorption of amino acids in the intestines, and, consequently, feed conversion rate [27–29]. The supplementation also increases immunoglobulins levels and immune response [30], decreases specify pathogens concentrations in feces [31,32] and presented an insecticidal [33], acaricidal and antioxidant effects [34]. However, there is inconsistent data between other species, probably explained by the complexity of the essential oils' molecules and differences among the many types of GIT [19]. Previous studies have shown that essential oils supplementation in calf's solid starter improves performance [35,36], rumen fermentation [37], and diarrhea severity [38]. However, the effects on liquid diet supplementation are scarce.

This study aimed to evaluate if the supplementation of a commercial blend of essential oils (BEO) in milk replacer (MR) affects feed intake, performance, feed efficiency, body development, blood cells and metabolites, insulin-like growth factor-1 (IGF-1), ruminal parameters, fecal and respiratory scores of dairy heifers during pre-weaning and post-weaning periods. We hypothesized that BEO supplementation in MR during pre-weaning would improve performance and positively influence blood parameters and health scores of dairy heifers.

3.3.Material and methods

Protocols for this study were approved by the Ethics Committee of Embrapa Dairy Cattle (protocol number 9078250118). The experiment was conducted on the Embrapa Dairy Cattle Experimental Farm, located in Coronel Pacheco, Minas Gerais, Brazil, from March to September 2018.

3.3.1. Animals, treatments, and management

Twenty-nine newborn Holstein × Gyr crossbred dairy heifers, with genetic composition of 5/8 or more Holstein and 3/8 or less Gyr and BW at birth of 32.2 ± 5.2 kg, were used and equally distributed among treatments. They were separated from their dams immediately after birth and moved to individual sand-bedded pens (1.25 × 1.75 m, tethered with 1.2 m long chains), allocated in a barn with open sides and end-walls.

All heifers received 10% of their BW of good quality colostrum (Brix > 23%) before 6 h after birth and had their umbilical cord immersed in an iodine solution (10%).

From 2 to 3 d of age, heifers were fed 5 L/d of transition milk divided into two equal meals offered at 0800 and 1600 h, in buckets provided with rubber teats (Milkbar, New Zealand). At 3 d of age, blood samples were collected via jugular venipuncture with a clot activator tube (Labor Import, Osasco, Brazil). They were left at room temperature for 30 min and then centrifuged at 1,800 × *g* for 10 min (22 – 25 °C). The serum was piped into a Brix refractometer (Aichose refractometer, Xindacheng, Shandong, China) to measure the success of the passive immune transfer. Heifers were enrolled only if the Brix was higher than 8.4%.

Water and commercial calf starter (Soymax Rumen pre-inicial Flocculated, Total Alimentos, Três Corações, Brazil, Table 1) were offered in buckets for ad libitum intake (10% orts of solid feed).

At 4 d of age, heifers were assigned to one of two experimental treatments maintaining a balance of the birth month, birth BW, genetic composition, and % Brix value. They were fed at 5 L/d of an MR (Kalvolak, Nutrifeed, Netherlands; Table 1) reconstituted at 15% (dry matter basis), divided into two equal meals (0800 and 1600 h) into buckets provided with rubber teats (Milkbar). The experimental treatments were: Control, no additive (CON; n = 15), and a commercial blend of essential oils additive supplemented at a rate of 1 g/d/calf (BEO, Apex Calf, Adisseo, China; n = 14), as recommended by the manufacturing company. The blend of essential oils is a dry powder that contains a mix of plant extracts derived from anise, cinnamon, garlic, rosemary, and thyme. The amount of the additive for each meal was weighed to have 0.5 g and kept in 15 mL tubes in a dark box. They were then mixed with a 10 mL of MR, homogenized, and incorporated in 0.49 L of MR (0.5 g/calf at morning meal and 0.5 g/calf at afternoon meal) to ensure total ingestion of the product. Immediately after ingesting 0.5 L MR with 0.5 g of the blend of essential oils, the rest of the meal was given. One person was responsible for refilling the milk bucket as soon as the animals had finished, so it would not change the ingestion rate. This person would also evaluate MR acceptance.

Table 1. Nutrient composition (% DM basis ± SD) of milk replacer (MR), starter, and corn silage.

Item	MR[1]	Starter[2]	Corn Silage
DM (%)	96.0 ± 0.4	86.7 ± 0.7	36.1 ± 3.1
CP (% of DM)	19.4 ± 0.5	17.1 ± 0.5	7.9 ± 0.7
Ether extract (% of DM)	14.1 ± 0.6	3.9 ± 1.2	4.3 ± 0.5
Organic Matter (% of DM)	9.7 ± 0.2	7.2 ± 1.5	6.0 ± 1.1
NDF (% of DM)	–	22.1 ± 2.9	46.1 ± 4.1
ADF (% of DM)	–	10.6 ± 0.9	28.9 ± 3.5
Gross Energy (Mcal/kg of DM)	4.5 ± 0.1	4.3 ± 0.1	4.5 ± 0.1

[1]Powder integral milk, wheat isolated protein, acidifying additive, whey, coconut oil, palm oil, vitamin A, Vitamin D3, Vitamin E, Vitamin C (Kalvolak, Nutrifeed, Netherlands).

[2]Basic composition: oats (rolled grains), calcitic limestone, sodium chloride, corn gluten meal, defatted corn germ, wheat bran, soybean meal, rice hulls, kaolin, molasses, flocculated corn, ground corn, corn grain, alfalfa hay, monensin, citrus pulp, dried sugarcane yeast, whole toasted soybean, sodium selenite, copper sulfate, manganese sulfate, cobalt sulfate, iron sulfate, zinc sulfate, calcium iodate, vitamin A, vitamin B1, vitamin B12, vitamin B2, vitamin B6, vitamin C, vitamin D3, vitamin E, vitamin K, niacin, pantothenic acid, folic acid, biotin, propionic acid, caramel aroma, milk aroma, and probiotic additive.

Heifers were weaned abruptly at 60 d of age. During the post-weaning period, from 61 to 90 d of age, all heifers received starter and corn silage (Table 1). The amount of corn silage provided was enough to result in at least 10 % orts, and the starter intake was fixed for a maximum of 3.0 kg calf/d, divided into two meals. All heifers were dehorned at 70 d of age and received local anesthesia (5.0 mL/horn, Lidovet, Bravet, Engenho Novo, Brazil) and 2 d of non-steroid anti-inflammatory treatment (0.025 mL/kg, Maxicam 2%, Ouro fino, Cravinhos, Brazil).

3.3.2. Intake and nutritional composition analysis

Feed intake (MR, starter, water, and corn silage) were measured daily. Samples of MR, starter, and corn silage were collected three times a week to obtain a weekly pool for nutritional analyses. Samples of starter and corn silage were oven-dried at 55 ºC for 72 h and ground in Wiley mill (model 3, Arthur H. Thomas Co., Philadelphia, PA) through a 1-mm screen before analysis. Starter, corn silage, and MR were analyzed to determine DM (Method 934.01), CP (Method 988.05), ether extract (Method 920.39), ash (Method 942.05), according to AOAC [39]. The concentrations of NDF and ADF were determined in sequence using the method described by Van Soest et al. [40]. Gross energy was determined using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL).

3.3.3. Structural growth

Body weight (BW) was measured on the day of birth, 3 d of age, and, after that, every 3 d before the morning meal using a weighing-machine (ICS 300, Coimma, Dracena, Brazil). Wither height (distance from the base of the front feet to the withers), rump height (distance from the base of the rear feet to the rump), rump width (distance between ileus), and heart girth (circumference of the chest) were measured on the day of birth and, after that, every 7 d until the end of the experiment. These measurements were taken on a flat surface using a portable hypometer and a measuring tape. Feed efficiency was calculated using the ADG and DMI ratio [41].

3.3.4. Rumen fermentation

Rumen fluid samples were collected through an oroesophageal tube 4 h after morning feeding at 14, 28, 42, 60, 74, and 90 d of age, and pH was assessed using a portable potentiometer (Phmetro T-1000, Tekna, Araucária, Brazil). Two aliquots of 10 mL of ruminal fluid were separated. One was acidified with 1 mL of 20% metaphosphoric acid, and the other with 2 mL of 50% sulfuric acid. These samples were stored at -20 ºC for further analysis of VFA and nitrogen ammonia. Nitrogen ammonia concentration was quantified using the colorimetric distillation method proposed by Chaney and Marbach [42]. Its absorbance was measured at 630 nm (Thermo Fisher Scientific, Madison, WI, USA) after Kjeldahl distillation with magnesium oxide and calcium chloride according to Method 920.03

[39]. The VFA concentrations were determined in the samples previously centrifuged at 1,800 × *g* for 10 min at room temperature (22 – 25 ° C) by high-performance liquid chromatography (Waters Alliance e2695 Chromatograph, Waters Technologies do Brazil LTDA, Barueri, SP, Brazil).

3.3.5. Blood cell count, metabolites and IGF-1

Jugular blood samples were collected at birth before colostrum ingestion and, 3 h after morning feeding on days 0, 7, 14, 21, 28, 35, 42, 49, 56, 60, 67, 74, 81 and 90, for beta-hydroxybutyric acid (BHB), urea and glucose and, on days 0, 14, 28, 42, 60, 74 and 90, for IGF-1 concentrations. Blood samples were collected into tubes without anticoagulant (for BHB and urea), with sodium fluoride (for glucose), or with heparin for IGF-1 (Labor Import, Osasco, Brazil). They were immediately transported on ice to the laboratory and were centrifuged at 3000 x *g* for 10 min at room temperature (22 – 25 °C). Two aliquots of each metabolite and hormone sample were individually allocated into microtubes and frozen at -20 ºC for further analysis. The serum concentration of BHB and urea were determined by an auto-analyzer (Cobas Mira Plus, Roche Diagnostic Systems, Risch-Rotkreuz, Switzerland) using commercial kits (Ranbut-D-3-Hidroxibutyrate, Randox Laboratories Ltd., Antrim, UK; Urea UV, Kovalent do Brasil Ltda., Bom Retiro São Gonçalo, Brazil). Plasma glucose was measured in a microplate Spectrophotometer EON (Biotek Instruments Inc., Winooski, VT) using the enzymatic colorimetric method (Kovalent do Brasil Ltda., Rio de Janeiro, Brazil). The plasma concentrations of IGF-1 were analyzed using chemiluminescence assay (Immulite2000 Systems 1038144, IGF-1 200, Siemens Healthcare Diagnostics Products Ltd., Llanberis, Gwynedd, UK).

Blood samples were collected for complete blood count during preweaning at 0, 30 and 60 d of age, by jugular vein puncture into EDTA tubes (Labor Import, Osasco, Brazil), and immediately transported on ice to the laboratory. An automatic hematology cell counter (SDH – 3 vet, Labtest Diagnóstica S.A., Brazil) was used to evaluate: red blood cell count (RBC), packed cell volume (PCV), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet and total white blood cell count. Manual white cell blood differential counting was also performed by microscopic examination evaluating 100 leukocytes in a 1,000 x microscopic magnification for total leukocyte count, basophils, eosinophils, neutrophils, band neutrophils, segmented neutrophils, lymphocytes, monocytes. Morphological changes, such as toxic neutrophils,

reactive lymphocytes, and activated monocytes, were calculated [43]. In addition, platelet to lymphocytes ratio (PLR) and neutrophils to lymphocytes ratio (NLR) were calculated.

3.3.6. Health measurements

Health measurements (fecal and respiratory scores) were performed daily, in the morning, before other animal management. Fecal scores were graded according to the University of Wisconsin calf health scoring chart [2], as follows: 0 – normal (firm but not hard); 1 – soft (does not hold form, piles but spreads slightly); 2 – runny (spreads readily to about 6 mm depth); and 3 – watery (liquid consistency, splatters). A heifer was considered to have diarrhea if the fecal score was 2 or 3. Severe diarrhea was considered when the fecal score was 3.

Daily respiratory score evaluations were adapted from the University of Wisconsin calf health scoring chart [2], considering rectal temperature score: 0 – temperature between 37.8 and 38.3 ºC, 1 – temperature between 38.4 and 38.8 ºC, 2 – temperature between 38,9 and 39.3 ºC, 3 – temperature above 39.4 ºC; cough score: 0 – none, 1 – induce single cough, 2 – induced repeated or occasional spontaneous coughs, 3 – repeated spontaneous coughs; nose score: 0 – normal serous discharge, 1 – small amount of unilateral cloudy discharge, 2 – bilateral cloudy or excessive mucus discharge, 3 – copious bilateral mucopurulent discharge; eye score: 0 – normal, no discharge, 1 – small amount of ocular discharge, 2 – moderate amount of bilateral discharge, 3 – heavy ocular discharge; ear score: 0 – normal, 1 – ear flick or head shake, 2 – slight unilateral drop, 3 – head tilt or bilateral drop. A final respiratory score was determined by the summation of temperature, cough, nose, eye, and ear scores.

Heifers were treated with non-steroid anti-inflammatory (0.025 mL/kg, Maxicam 2%, Ouro fino, Cravinhos, Brazil) when respiratory score sum was above 4, or if they presented fever for two consecutive days. Fever was considered when the pre-meal morning temperature was ≥ 39.4 ºC. One dose of enrofloxacin antibiotic (0.075 mL/kg, Kinetomax, Bayer, São Paulo, Brazil) was administered when a pulmonary commitment was detected (shortness of breath, edema and/or crepitation detected by auscultation) or an animal had fever combined with diarrhea for 2 d subsequently.

3.3.7. Minimum inhibitory concentration

The broth dilution method was used to evaluate the minimum inhibitory concentration (MIC) of BEO against two relevant enteric bacteria: enterotoxigenic *Escherichia coli* ($K99^+$ strain) and *Salmonella typhimurium* previously isolated from an outbreak in calves [44]. Two different preparations of BEO product were used to perform MIC: a - homogenized in purified water; b - homogenized in a solution with 3.0 g of isopropyl myristate, 8.25 g of propylene glycol, 7.25 g of Tween 80 (Sigma-Aldrich, Santo André, Brazil) and 100 mL of water. Both preparations were submitted to 0.22 µm filtration. A solution with an initial concentration of 1.0 mg/mL was submitted to serial dilutions from 1:2 to 1:256 in 96-wells plates. Thus, 100 µL of a solution containing 5×10^5 CFU/mL of the two selected bacteria. After overnight incubation at 35ºC, microtiter plates were examined for visible bacterial growth evidenced by turbidity and color change.

3.3.8. Statistical analysis

Statistical analysis was conducted utilizing R® (R Core Team, 2019). The data collected was summarized by period (pre-weaning – 4 to 60 d and post-weaning – 61 to 90 d) and per week within each period. A randomized complete block experimental design with repeated measures was implemented to test the hypothesis of the effect of the blend of essential oils on each performance outcome. More specifically, the outcomes analyzed were feed intake, structural growth, ruminal, blood, and health parameters. The control treatment was assigned 15 experimental units (CON), while the blend of essential oils supplementation treatment was assigned 14 (BEO).

The analysis of each outcome was performed independently of all others using linear mixed models (package: nlme). Each independent outcome was modeled as a function of the following fixed effects: treatment, experimental week, the interaction between treatment and week. The genetic composition of the animal was included as a blocking effect. Birth month, birth body weight and Brix value were assessed only to verify if the animals were homogeneously distributed but were not used as a blocking effect. Birth weight and serum Brix value were tested as a covariate but did not improve

statistical significance. Therefore, they were eliminated from the model. The effect of heifer within treatment was included in the models to account for individual variability.

The continuous outcomes such as intakes, structural growth, ruminal, and blood parameters were analyzed with ANOVA. A 95% Confidence Interval was adopted to verify the null hypothesis, and *P*-values were produced with a Fisher test. All outcomes were tested for normality to meet the required assumptions of this model, and a variable transformation was applied to milk replacer intakes to meet that assumption.

The categorical outcomes fecal and respiratory scores were analyzed using a non-parametric aligned rank transformation test, implemented in the R package ARTool. A 95% Confidence Interval was also adopted for the non-parametric tests. Associations between the fecal scores and MR intakes were assessed by using the Spearman correlations.

3.4. Results and discussion

3.4.1. Intake and heifer performance

Most studies evaluate essential oils or a supplement with BEO to dairy calves, feed the additive in the starter to benefit rumen development, and accelerate growth. However, the intake of starter in the first weeks of age is small [45], and the timing of the occurrence of enteric diseases is mainly on the first 30 days of life [2]. Due to the calf's limited capability of ingesting large solid feed amounts in the first days of life, the supplement intake in the starter could be limited, and the desired supplementation level may not be achieved based on intake levels of the starter. Therefore, in this trial, BEO was offered in the liquid diet since the aim was to verify if it would impact on disease morbidity and gut development, and subsequently, on animal's performance.

The supplemented heifers consumed the same amount of liquid diet as the control group, indicating no ingestibility issues of BEO (Table 2). Differences described in the literature between flavor and palatability of BEOs could be due to the delivery method, as well as essential oil plant sources and extraction process [16]. Studies using different supplemented types of essential oils to

other animal species' reported different preferences and acceptability of these essential oils, with changes among animal species and category, juvenile x adults [19]. Previous work with weaned heifers supplemented with cinnamaldehyde essential oil in a total mix ration showed a preference in the taste of the ration without additive. This supplementation caused a change of feed intake, and it was related to palatability problems with the essential oil used in the experiment [46]. However, although cinnamon is an ingredient that is in the mixture in our study, we did not run a palatability test to verify this outcome. It must be point also that the additive was given mixed with a small amount of MR to allow complete ingestion. Visually, the time on ingestion was the same, and all the calves consumed all MR. Therefore, ingestibility of the mixture was not a problem, However, further tests with essential oils palatability to dairy calves are needed.

Although there were no differences between MR intake between treatment and the given amount was fixed, there were a week effect and a week and treatment interaction effect ($P \leq 0.001$, Table 2, Fig 1). From the end of week 1 until week 3, heifers had diarrhea and this event impacted on MR intake, since intake decrease when animals are sick. Differences between treatments were observed in those weeks, with lower intake for the CON. An observed effect between fecal scores and MR intake was found ($P \leq 0.001$), besides a low correlation value (- 0.25). Thus, results revealed a negative association between both parameters, where higher fecal scores reduced MR intake, and vice versa.

Table 2. Pre and post-weaning milk replacer (MR) intake, starter intake, total dry matter intake (DM), total crude protein intake (CP), total gross energy and water intake of heifers of control (CON) and supplemented with blend essential oils (BEO) in milk replacer during pre-weaning.

Intake	Treatment		SEM	*P* – value[3]		
	CON[1] (n =15)	BEO[2] (n =14)		T	W	T x W
Pre-weaning (4 to 60 d)						
MR (kg of DM/d)[4]	0.71 (0.705 - 0.721)	0.71 (0.701 - 0.716)	-	0.30	<0.001	<0.001
Starter (kg of DM/d)	0.30	0.31	0.02	0.92	<0.001	0.82
Total DM (kg/d)	1.00	1.16	0.06	0.58	<0.001	0.31
Total CP (kg/d)	0.19	0.19	0.01	0.58	<0.001	0.31
Total gross energy (Mcal/kg)	4.51	4.59	0.12	0.58	<0.001	0.30
Water (kg/d)	1.39	1.30	0.32	0.98	<0.001	0.64
Post-weaning (61 to 90 d)						
Starter (kg of DM/d)	1.84	2.02	0.28	0.39	<0.001	0.31
Corn Silage (kg of DM/d)	0.12	0.11	0.03	0.51	<0.001	0.26
Total DM (kg/d)	1.97	2.14	0.29	0.39	<0.001	0.32
Total CP (kg/d)	0.44	0.47	0.07	0.36	<0.001	0.72
Total gross energy (Mcal/kg)	8.61	9.35	1.34	0.39	<0.001	0.29
Water (kg/d)	5.41	5.69	0.84	0.61	<0.001	0.10

[1]CON = control; [2]BEO = 1 g/calf/d blend of essential oil.

[3]T = treatment effect; W= week effect, T x W = treatment by week interactions.

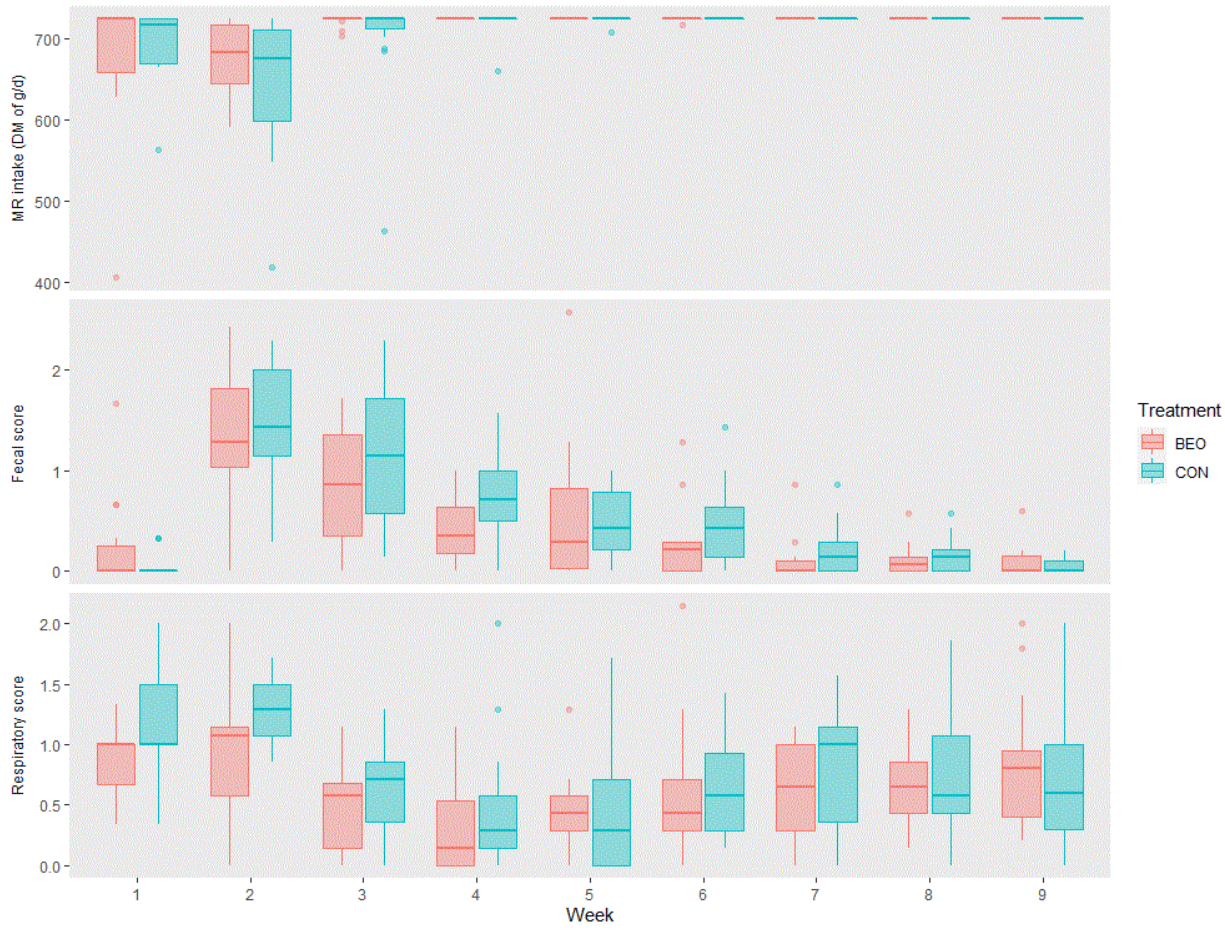

Fig 1. MR intake (g of DM/d), respiratory and fecal scores of control heifers (CON) and heifers supplemented with 1.0 g/calf/ d of the blend of essential oils (BEO) in milk replacer during the pre-weaning period.

Intake of starter, water, total DM, CP, and gross energy, ADG and feed efficiency were not affected by treatment during pre- and post-weaning (Table 2 and 3). A previous study tested a commercial blend of essential oils for dairy calves using two supplementation routes (MR and starter), and had similar results for intake, BW and ADG during preweaning [47]. However, other studies that also used a commercial source of essential oils in the starter found better ADG and feed efficiency during the preweaning period for supplemented calves, as well as higher BW during weaning [36,37]. As for the carry-over effect on post-weaning in those studies, it has been observed that calves

supplemented with essential oils in the starter had higher ADG and lower feed efficiency [48]. In our study, we did not find any carry-over effect on post-weaning for the performance outcomes.

In our study, the lack of differences in evaluated outcomes could be because of the supply route, dosage, or the essential oil plant sources and extraction process. It also must be highlighted that the starter provided contained monensin and other probiotic additives. They are important and efficient additives used not only as a growth promoter but also as coccidiosis control and prevention [49]. However, some studies believe that the combined supplementation of monensin and essential oils could mask the effect of the essential oils or even compete for the same mechanisms of action [50]. In this study, no antagonism between additives was observed, as there were no negative responses for BEO compared to CON. It must also be highlighted that monensin was provided in the starter and the essential oil in the milk replacer. Thus, they would act in different compartments, the rumen and the intestines. To better understand this interaction and a possible effect, it is necessary for other studies to evaluate the impact of the essential oil's supplementation with or without monensin, as also the mechanism of action of the different essential oils.

Table 3. Pre- and post-weaning performance and structural growth of heifers of control (CON) and supplemented with essential oils blend (BEO) in milk replacer during pre-weaning.

Item	Treatment		SEM	*P* – value[3]		
	CON[1] (n = 15)	BEO[2] (n = 14)		T	W	T x W
Performance						
Birth BW (kg)	32.40	31.97	0.59	0.85	–	–
Weaning BW (kg)	64.36	66.66	1.07	0.45	–	–
Final BW (kg)	89.88	93.34	1.57	0.57	–	–
ADG preweaning (kg/d)	0.55	0.53	0.02	0.49	<0.001	0.23
ADG postweaning (kg/d)	0.81	0.84	0.27	0.76	0.001	0.60
Feed efficiency preweaning (kg/kg)	0.62	0.56	0.008	0.06	<0.0001	0.29
Feed efficiency postweaning (kg/kg)	0.44	0.42	0.04	0.50	0.68	0.42
Body measures						
Preweaning (4 to 60 d)						
Withers height (cm)	72.74	72.59	1.25	0.86	<0.001	0.48
Rump height (cm)	75.89	75.90	0.66	0.98	<0.001	0.62
Rump width (cm)	19.03	19.42	0.66	0.23	<0.001	0.94
Heart girth (cm)	80.70	81.50	0.009	0.34	<0.001	0.68
Postweaning (61 to 90 d)						
Withers height (cm)	82.66	82.55	1.06	0.92	<0.001	0.72
Rump height (cm)	86.02	86.64	1.07	0.61	<0.001	0.80
Rump width (cm)	22.59	22.99	0.43	0.28	<0.001	0.40
Heart girth (cm)	96.55	97.85	1.44	0.27	<0.001	0.40

[1]CON = control; [2]BEO = 1 g/calf/d blend of essential oil.

[3]T = treatment effect; W= week effect, T x W = treatment by week interactions.

3.4.2. Structural growth

Structural body growth was not affected by BEO supplementation in MR (Table 3) during pre- and post-weaning. As was also observed for intake and ADG, a week effect ($P \leq 0.001$) was detected in all variables due to healthy animal growth. It was previously suggested that essential oils supplementation could only be effective in structural growth when associated with higher protein concentration in the starter due to an interaction between protein level supplementation and essential oils supplementation [37]. Other studies suggested that feeding essential oils could enhance growth performance if fed at an appropriate rate and in a determined amount [36]. In our study, the calves were fed with protein levels to meet their requirements for optimal growth. However, we did not test different protein levels to see if this interaction could change structural growth. On the other hand, in other species, the increase in structural growth, as well as daily weight gain and feed conversion for supplemented animals, are generally related to a more mature and developed gut. This more developed gut helps the supplement to be absorbed more quickly, improving gut immunity and microbiota, and as a consequence, the animals' body growth [51].

3.4.3. Rumen fermentation

There were no differences in ruminal pH for CON and BEO treatments during the pre-weaning period. Previous studies also did not find changes in ruminal pH for animals supplemented with essential oils [16, 26, 28]. During the post-weaning period, the BEO treatment presented a lower pH ($P = 0.05$, Table 4). Since there were no differences between treatments during pre-weaning, the carry-over effect may not be assumed to be the answer to this difference. Although no differences in intake were observed, heifers' ingestion behavior might justify the difference in post-weaning pH. In other words, the amount of starter consumed before sampling and its impact on ruminal pH. However, this behavior was not evaluated since intake was measure only once every 24 hours.

Table 4. Pre- and post-weaning rumen mean values of rumen pH, ammonia nitrogen (Ammonia-N) and volatile fatty acids (VFA) of control heifers (CON) and heifers supplemented with essential oils blend (BEO) in milk replacer during pre-weaning.

Item	Treatment		SEM	*P* – value[3]		
	CON[1] (n = 15)	BEO[2] (n = 14)		T	W	T x W
Pre-weaning (4 to 60 d)						
Rumen pH	5.99	5.85	0.52	0.37	0.03	0.06
Rumen ammonia-N (mg/dL)	11.40	13.80	0.03	0.15	<0.001	0.37
Rumen VFA (µmol/mL)						
Acetic (C2)	30.80	27.16	8.15	0.24	<0.001	0.14
Propionic (C3)	18.88	20.01	7.11	0.59	<0.001	0.14
Butyric (C4)	0.80	0.80	0.08	0.83	0.005	0.98
C2:C3	1.97	1.69	0.12	0.05	<0.001	0.95
Post-weaning (61 to 90 d)						
Rumen pH	6.19	5.90	0.001	0.05	0.001	0.86
Rumen ammonia-N (mg/dL)	10.97	9.53	9.03	0.17	0.91	0.88
Rumen VFA (µmol/mL)						
Acetic (C2)	38.32	39.03	8.48	0.81	0.006	0.93
Propionic (C3)	28.27	30.69	5.16	0.41	0.003	0.75
Butyric (C4)	5.94	6.16	1.19	0.82	0.95	0.62
C2:C3	1.43	1.23	0.20	0.006	0.74	0.93

[1]CON = control; [2]BEO = 1 g/calf/d blend of essential oil.

[3]T = treatment effect; W= week effect, T x W = treatment by week interactions.

Considering that low pH could enhance essential oils effects, this could benefit younger calves that are supplemented with essential oils in the starter [24]. It is also known that its supplementation is related to antimicrobial and antifungal effects [16,24]. Essential oils cause hydrophobicity and disrupt bacteria membrane, increasing water permeability and causing a toxic effect on the microorganism [7, 12]. This activity could result in inhibition of ruminal deamination and

methanogenesis [25]. This effect on the modulation of nitrogen path would result in a decrease of the ruminal nitrogen ammonia, methane and acetate concentrations and an increase of the propionate and butyrate concentrations [24].

Changes in these profiles in rumen fluid would also alter the acetate:propionate (C2:C3) proportions. Since butyrate and propionate are important for ruminal papillae development, and especially propionate is used in the gluconeogenesis route [5], a smaller C2:C3 ratio is wanted. In this experiment, BEO supplementation did not alter VFA values, but did reduced the C2:C3 proportion during the pre- ($P = 0.05$) and post-weaning phases ($P = 0.006$) (Table 4). Confirming these findings, previous studies registered a lower C2:C3 proportion for calves in both groups supplemented with essential oils in the starter (1.56 and 1.47) compared with two control groups (2.02 and 1.77) [37]. On the other hand, reports are not always constant in the literature, since higher C2:C3 proportion for pre-weaning calves supplemented with thyme essential oils (2.25 x 1.78) were already reported [52]. Despite our findings, it must be highlighted that, in our experiment, essential oils were provided mixed in small amounts of MR to ensure the whole intake of the product. If the BEO was provided in the starter, changes in the rumen would be expected. By providing the BEO in the MR, the treatment should bypass the rumen and have minimal impact on local ruminal microbiota and VFA. Nevertheless, since the MR amount was small and given at the beginning of the feeding, one hypothesis could be that the esophageal groove was still open, permitting essential oils content to arrive at the rumen. Another hypothesis could be a potential communication from the intestines and the forestomach were the nutrients on the lower gut caused adaptations on the upper gut, improving its function and growth, as well as nutrient use and differences in VFA proportions [53]. In monogastric animals, supplementation of essential oils has shown a direct effect on the gut microflora and effects on the gut-associated immune system, causing positive changes in nutrient digestibility and animal performance [54]. A third theory to explain the changes in C2:C3 is that the changes in rumen could not be only by the BEO supplementation, but the interaction between the BEO and the monensin in the starter. They have a similar mechanism of actions and could cause the increase in propionate in the rumen, not enough to be seen when evaluating the VFA alone, but shifting ruminal fermentation and cause differences in C2:C3 proportions [50].

However, despite changes in C2:C3 proportions, nitrogen ammonia concentrations were not affected by BEO supplementation during pre- and post-weaning (Table 4). Previous studies reported

higher nitrogen ammonia for the treated group, suggesting that essential oils could not modulate deamination nor the population of ammonia producing bacteria [47]. One of the characteristics of the essential oils is modulated ruminal microbiota and, consequently, fermentation and nutrient degradation in the forestomach [18,55].

For all ruminal parameters, a week effect during preweaning was observed ($P \leq 0.05$, Table 4). Those findings were expected since ruminal parameters are related to increased starter intake, rumen development, microbiota colonization, and calf development to become a ruminant [4].

3.4.4. Blood cell count, metabolites, and IGF-1

During the pre- and post-weaning periods, all blood metabolites were not altered by BEO supplementation (Table 5). Similar patterns of BHB, glucose [35,47], urea [37], total plasma protein, and IGF-1 [56] were found in both treatments. Nevertheless, BHB and urea increased with age ($P \leq 0.05$, Table 5), since they are directly correlated with fatty acid metabolism and ruminal ammonia concentration, respectively [57]. The IGF-1 concentration increased with age on the preweaning phase ($P \leq 0.001$). Since this hormone is a mitogen and related to cell proliferation and differentiation, it is correlated with BW and animal growth [58].

Table 5. Pre- and post-weaning mean blood concentrations of insulin growth factor type 1 (IGF-1) and metabolites of control heifers (CON) and heifers supplemented with a blend of essential oils blend (BEO) in milk replacer during pre-weaning.

Item	Treatment		SEM	*P* – value[3]		
	CON[1] (n = 15)	BEO[2] (n =14)		T	W	T x W
Pre-weaning (4 to 60 d)						
BHB (mmol/L)	0.17	0.12	0.02	0.43	0.001	0.98
Urea (mg/dL)	24.55	22.69	3.76	0.16	0.02	0.31
Glucose (mg/dL)	100.35	102.97	16.50	0.49	0.15	0.56
IGF-1 (ng/mL)	101.95	93.16	32.4	0.38	<0.001	0.27
Post-weaning (61 to 90 d)						
BHB (mmol/L)	0.36	0.37	0.10	0.70	<0.001	0.13
Urea (mg/dL)	24.57	22.73	4.34	0.16	0.01	0.34
Glucose (mg/dL)	88.45	84.74	8.65	0.29	0.22	0.14
IGF-1 (ng/mL)	160.70	175.94	23.4	0.43	0.31	0.12

[1]CON = control; [2]BEO = 1 g/calf/d blend of essential oil.

[3]T = treatment effect; W= week effect, T x W = treatment by week interactions.

Glucose did not change during the pre-weaning phase and decreased during the post-weaning period (Table 5). Taking into account that calves use glucose as a primary source of energy in the firsts weeks of age, these age-related changes are associated with changes in diet and rumen development [59]. After weaning, calves complete their rumen development and, VFA produced by ruminal microbiota becomes the primary energy source, justifying BHB concentration increase, and glucose concentration decrease [5,60]. However, since there were changes in C2:C3 proportion in the BEO, the increase of propionic acid could consequently impact glucose blood concentration. Since essential oils can increase insulin sensitivity, not finding glucose differences between treatments does not mean that there were no changes in the glucose pathway [38,39]. Therefore, further investigations over these aspects are needed.

All blood cell counts were within normal range based on age and species normality. Changes in blood cell count are typical during heifer growth, and blood cells tend to increase with animal age [61]. These changes corroborate with the week effect on mean corpuscular volume (MCV), basophils, eosinophils, segmented neutrophils, lymphocytes, monocytes, and platelets ($P = 0.04$). There were no differences in erythrogram parameters between BEO and CON (Table 6). Leukogram parameters showed decreased counts of basophil and platelet cells in BEO treatment ($P \leq 0.05$). Basophils and platelets originate from different myeloid precursors and, both play essential roles in inflammation balance and immune response development in mammal [62]. The lower counts of basophil and platelets on BEO treatment may influence and modulate inflammatory response by secretion of immune modulators [63], growth factors, or chemotaxis on a variety of white blood cells [43]. This modulation could help explain an interaction effect found for lymphocytes (Fig 2), where values of d 30 and 60 were different from d 1 with an accentuated increase in BEO. There have been reports of immune response potentiation of piglets supplemented with essential oils. The animals had improved lymphocyte proliferation, phagocytosis rate, and humoral immune response [54].

Table 6. Pre-weaning hematological parameters of control heifers (CON) and heifers supplemented with a blend of essential oils blend (BEO) in milk replacer during pre-weaning.

Item[1]	Treatment		SEM	*P* – value[4]		
	CON[2] (n =15)	BEO[3] (n =14)		T	W	T x W
RBC (x 10^6/µL)	8.02	7.95	0.88	0.86	0.63	0.87
PCV (%)	35.53	35.21	5.05	0.85	0.11	0.69
Hb (q/dL)	11.07	10.94	1.61	0.81	0.14	0.73
MCV (fL)	44.74	44.51	2.94	0.74	<0.001	0.51
MCHC (%)	31.10	31.14	0.76	0.87	0.15	0.99
Total leukocytes (/µL)	10,908.45	11,200.78	2,630.0	0.76	0.19	0.22
Basophils (/µL)	2.14	0.00	1.03	<0.001	<0.001	<0.001
Eosinophils (/µL)	68.40	143.90	0.66	0.24	<0.001	0.36
Band neutrophil (/µL)	31.76	26.22	5.69	0.68	0.83	0.31
Segmented neutrophils (/µL)	5,300.63	5,286.56	1,700.0	0.98	<0.001	0.78
Lymphocytes (/µL)	4,837.40	5,082.82	1,120.0	0.66	<0.001	0.01
Monocytes (/µL)	421.60	466.00	247.0	0.48	0.01	0.29
Platelet (x 10^3/µL)	410.41	353.70	108.0	0.04	<0.001	0.10
Plasmatic protein (g/dL)	6.03	6.03	0.72	1.00	0.17	0.40
PLR	0.08	0.08	0.03	0.91	0.02	0.04
NLR	1.26	1.46	0.03	0.60	<0.001	0.55

[1]RBC: red blood cell, PCV: packed cell volume, Hb: hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, PLR: platelet lymphocyte ratio, NLR: neutrophils lymphocytes ratio.

[2]CON = control; [3]BEO = 1 g/calf/d blend of essential oil.

[4]T = treatment effect; W= week effect; T x W = treatment by week interactions.

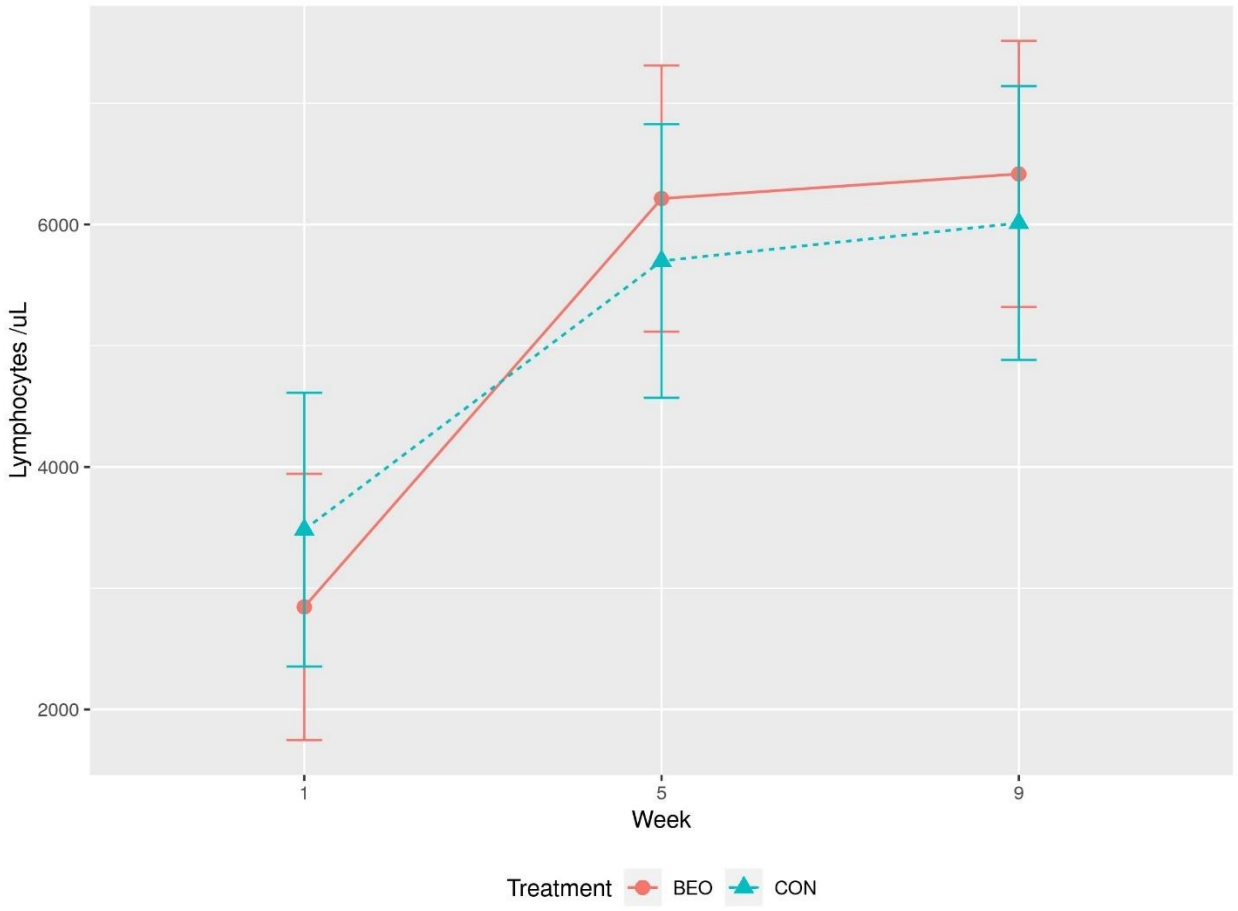

Fig 2. Lymphocytes values of control heifers (CON) and heifers supplemented with 1.0 g/calf/ d of a blend of essential oils (BEO) in milk replacer during the pre-weaning period.

Oregano and thyme oils supplemented to Holstein calves positively influenced erythrogram parameters, lymphocytes, neutrophils, and band neutrophils with higher values for treated calves [64]. For older animals, it has been shown a linear increase in the values for lymphocyte and monocyte counts for heifers supplemented with plant extract containing essential oils [65]. Hence, agents with antioxidant activity, like essential oils, can reduce platelet activation and consequently reduce oxidative stress and inflammation [66]. Platelets also play a central role in the coagulation process. Different essential oils have been used for thrombosis treatment in humans, acting on platelet aggregation and its thromboxane synthesis [67]. Although our results demonstrate a decrease in basophil and platelet counts, it is necessary to perform novel experiments to characterize the effects of BEO on the inflammatory and coagulation process in heifers. Differences between PLR and NLR

were not found (Table 7). These ratios are inflammatory markers and inform disease activity, being a useful tool to understand inflammation pathophysiology and immune response [68].

3.4.5. Health measurements and minimum inhibitory concentration

Diarrhea is the most prevalent disease for calves under one month of age. Causes for juvenile diarrhea include a combination of factors but are generally related to viral, bacterial, or/and protozoa infection [2]. Coronavirus, rotavirus, *Salmonella* spp. and/or *Cryptosporidium parvum* are the most common agents under 14 d of age. *Salmonella* spp., *Eimeria* spp. and/or *Giardia* spp. are the most common pathogens in older calves [2,69].

The supplementation of essential oils has already shown beneficial results for lowering diarrhea and fecal scores in other species with the same efficiency of AGPs [18,31,70]. For piglets, where this is a prevalent disease and caused by similar agents as in calves, it has been shown favorable results with lower diarrhea prevalence for treated animals [70]. In our study, the average age for diarrhea (scores 2 and 3) occurrence was 12.2 ± 3.6 d for BEO and 13.6 ± 3.8 d for CON with no statistical difference ($P = 0.54$). Diarrhea incidence on pre-weaning in BEO treatment was 85% against 93% for CON treatment with no statistical difference ($P = 0.68$). The fecal score was different between treatments ($P = 0.04$), with lower values for BEO, and changed over time ($P \leq 0.001$, Table 7). Days with diarrhea (scores 2 and 3, $P = 0.24$) and days with severe diarrhea (score 3, $P = 0.12$) were not different between treatments (Table 7). Three animals of each treatment were medicated for diarrhea with anti-inflammatories, and the therapy duration was 1.6 ± 0.57 d for BEO and 3.0 ± 1 d for CON. It is noteworthy that this treatment was done outside the hemogram and total cell count evaluation in this study. Besides no differences in the diarrhea prevalence, the lower fecal score in the BEO could point to better gut health and less microbiota disability [54]. However, is important to point out that we did not collect samples to analyze microbiota changes before, during, and after diarrhea, or pathogenic bacteria count in feces.

Table 7. Pre and post-weaning mean values of the fecal score, respiratory score, days with a respiratory score above 4, days with fever, days with diarrhea, days with severe diarrhea of control heifers (CON) and heifers supplemented with a blend of essential oils (BEO) in milk replacer during pre-weaning.

	Treatment		SEM	*P* – value[3]		
Item	CON[1] (n = 15)	BEO[2] (n= 14)		T	W	T x W
Pre-weaning (4 to 60 d)						
Fecal score[4]	0.54	0.45	0.04	0.04	<0.001	0.18
Respiratory score[4]	0.79	0.69	0.02	0.22	<0.001	0.02
Days with respiratory score > 4[5]	0.00	0.14	0.05	0.44	–	–
Days with fever	0.94	0.98	0.20	0.66	–	–
Days with diarrhea	7.87	5.79	0.71	0.24	–	–
Days with severe diarrhea	3.13	1.93	0.37	0.12	–	–
Post-weaning (61 to 90 d)						
Fecal score	0.04	0.04	0.009	0.43	0.68	0.95
Respiratory score	1.10	1.03	0.05	0.59	<0.001	0.74
Days with respiratory score > 4	0.00	0.00	–	–	–	–
Days with fever	0.52	0.90	0.23	0.21	–	–

[1] CON = control; [2]BEO = 1 g/calf/d blend of essential oil.

[3] T = treatment effect; W= week effect, T x W = treatment by week interactions.

[4] Scores were adapted to follow the University of Wisconsin calf health scoring chart [2].

[5]There were no days with respiratory score > 4 during the post-weaning period.

Evaluation of the respiratory score parameters indicated that 2 BEO animals and 1 of CON animals exceeded score 4, indicating respiratory disease on pre-weaning. The average days with a high score were 1.0 ± 0 d for BEO and CON. No effect was found on days with high respiratory score or number of affected animals. However, a week and an interaction week x treatment effect on pre-weaning was observed, with the difference between treatment scores and lower values for the BEO in week 2 (P = 0.02, Table 7, Fig 1). The second week was the period in which animals had a higher

incidence of diarrhea. It is known that diarrhea and respiratory problems are caused by a combination of factors and related to the immunity status, nutrition, type of housing, and season [2]. Herds with respiratory diseases in calves have more diarrheal disease [71]. Thus, in this trial, the respiratory signs could be related to the previous enteric disease. Weeks 5 and 6 showed a lower score difference between treatments and a lower incidence of respiratory signs. The number of treated animals was 2 for BEO only during the preweaning period, with an average of treatment days of 1.3 ± 1.4, and 3 for CON with an average of treatment days of 2.0 ± 0.57. Treatments occurred only in the pre-weaning period using antibiotics and anti-inflammatories.

Pneumonia is usually associated with the post-weaning phase. However, it may affect younger calves [2]. Post-weaning respiratory scores revealed higher mean values when compared with pre-weaning, but no animals had scores above 4. There was a week effect ($P \leq 0.001$), in week 12, probably due to weaning and dehorning stress.

It has been reported that essential oils have an antiseptic and antimicrobial activity that may help balance intestinal microbiota [72]. Gram-positive bacteria are the most sensitive to the essential oils microbial activity [18,23], but Gram-negative bacteria and some types of parasites can also be susceptible [16] to different essential oils. Thus, some essential oils could reduce the incidence and severity of diarrhea syndrome in calves through inhibition of coliform overgrowth [73]. The in vitro test with BEO in 1.0 µg/mL concentration did not inhibit bacterial growth – both *E. coli* and *S. Typhimurium*. Thus, at this concentration, BEO did not have any direct antibacterial effect. However, besides no direct influence found over the bacterial evaluation, BEO calves presented differences on basophil (Table 6) and lymphocyte cell populations (Fig 2), which could be associated with modulation of the inflammatory immune response. Thus, outcomes found on fecal and respiratory scores could be related to indirect changes in hemato-biochemical parameters and not with a direct antibacterial effect.

3.5. Conclusions

Feeding BEO to pre-weaned heifers on MR did not affect intake, performance parameters, blood metabolites, or IGF-1 concentration. However, it changed C2:C3 proportion during pre- and post-weaning periods, showed signs of immunity improvement, and lower fecal scores in the pre-weaning

phase. Therefore, essential oils are a health additive option to modern production systems and could be used as an alternative to improve calf health and performance. Further research is needed to define the best route and dosage, understand the contribution of essential oils to decrease neonatal diseases' morbidity, and verify the possible interaction with other molecules.

3.6. References

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4. CHAPTER IV – PAPER II –

Effects of a blend of essential oils in milk replacer on immunity, health scores, digestibility, organ development, and gene expression in dairy bull calves

4.1. Abstract

The objective of this study was to evaluate blood cells and metabolites, insulin-like growth factor-1 (IGF-1), rumen fermentation, fecal and respiratory scores, digestibility, internal organs weight and histology, gene expression, and a spleen cell proliferation of pre-weaned bull calves supplemented with a blend of essential oils in milk replacer (EO). Sixteen newborn Holstein × Gyr crossbred dairy bull calves, and body weight at birth of 33.3 ± 3.7 kg, were housed in individual sand bedded pens, blocked by genetic composition, and randomly assigned to 1 of 2 treatments in a randomized complete block design: Control (CON, n = 8) and blend of essential oils supplementation (BEO, n = 8, 1 g/d/calf, Apex Calf, Adisseo, China). Animals were fed 5 L of MR/d reconstituted at 15% (dry matter basis), divided into two equal meals. Water and starter were provided *ad libitum*. Feed intake, feed efficiency, fecal and respiratory scores were evaluated daily. Body weight and structural growth were recorded weekly. ß-hydroxybutyrate, urea, and glucose were evaluated weekly, IGF-1 was evaluated biweekly, and total blood cell count was performed every four weeks until the end of the trial at eight weeks of age. Ruminal parameters were measured each 14 d for pH, VFA, ammonia-N, and acetate:propionate proportion (C2:C3). Feed samples were collected three times a week and polled for week analysis. Apparent total nutrient digestibility was determined from d 55 to 60 of age. On d 60±1, animals were euthanized for organ weight, histology, spleen cell proliferation, and intestinal gene expression analysis. Data were analyzed independently using linear mixed models using the REML method in the nlme package in R for continuous outcomes. A non-parametric test was used for ordered categorical outcomes using the Artools package in R. There were no differences between groups for feed intake and efficiency, blood evaluations, health scores, digestibility, gene expression, and a spleen cell proliferation assay. However, BEO calves presented a lower ruminal pH, bigger pancreas, heavier intestines, bigger ileum villi, and higher cecum butyrate levels ($P<0.05$), demonstrating that the essential oil supplementation helped on intestinal development and symbiotic bacteria. Besides no differences in diarrhea and respiratory scores, CON animals had a heavier respiratory tract and a higher

eosinophil count ($P< 0.05$). Therefore, both organs where eosinophils are more active had a better response for BEO animals. No differences were found in the intestinal gene expression in the immune context. These results demonstrate that supplementing an EO in MR could contribute to gut development and immune function. However, more research is needed to understand its impact on body development and define the best dosage and route of administration.
Keywords: Additive, dairy calf, eosinophil, intestinal health, pre-weaned.

4.2. Introduction

The use of antimicrobials as growth promoters in livestock has been questioned lately, particularly because of the possibility of creating bacterial resistance and one health concept [1–3]. Antimicrobials used to treat farm animals, especially neonatal diseases, have been a concern since they are used the same drugs like those used in human medicine [2,4]. Moreover, incorrect use of antimicrobials to prevent or treat diseases could heavy the pathogens' resilience and weaken the host immune system through gut dysbiosis [6,7]. It must also be pointed out that animal welfare correlates with animal health and antimicrobial use in dairy farms, an item measured to assess animal condition and wellbeing [5. Therefore, the politics of antimicrobial use to treat diseases in dairy farms and the rationalization of its use are in constant update by several national veterinary organizations [1].

The pre-weaning period is the phase in a dairy farm with the highest mortality rates [4,8]. The calves still have a naïve immune system and are susceptible to enteric and respiratory diseases [9]. The gastrointestinal tract (GIT) is the largest organ of the immune system [10]. Therefore, since intestinal microbiota has an important role in regulating immune responses outside of the gut, it is important to assure and improve good microbes colonization on this site [11]. The gut microbiome will be crucial to optimize calf performance and health [12]. However, once the ruminal and gut microbiome is settled and complete in an older animal, it is difficult to manipulate this ecosystem [13] permanently. That is why manipulating and developing the calf's gut microbiota at a young age is important since it is a window of opportunity to mediate metabolism and immune response [12,14].

Therefore, some dietary practices and additives could influence nutrient use and commensal microbiota homeostasis and animals' immune response, especially during early life [15–17]. Colostrum and transitional milk supplementation during the first days of age, ruminal transfaunation

inoculation, supplementation of pre and probiotics are potential strategies used to manipulate and improve microbial colonization and gut development of the young calf, and consequently, improve its immune system [12]. Thus, feed additives have been in search as an alternative not only to enhance livestock performance but also for its anti-inflammatory, antimicrobial, ruminal modulation, antioxidant and immunological improvement [18].

Essential oils (EO) are plant metabolites natural extracts with antibacterial, antiviral, antifungal, antioxidant, and anti-inflammatory activities [19,20], beneficial for gut microbiota [21] and calf performance that [22] could be an option for growth promoters use. Different plants are used to obtain EO, as well as different molecules, with different actions, in each of these oils [20,23]. Therefore, additives using a combination of these EO, or blends, have been tested lately to modify ruminal ecosystem and microbiota, improve nutrient utilization, performance, and health during the early stages of life [12].

This study aimed to evaluate the effect of a commercial BEO supplementation in milk replacer (MR) on immunity, health parameters, nutrient digestibility, organ development, and gut gene expression in dairy bull calves during the pre-weaning phase. Performance and carry-over effects were evaluated on our previous work [24] and were demonstrated in the present work with a descriptive purpose. We hypothesized that EO supplementation through a liquid diet could enhance immune response, help gut development, nutrient digestibility, and calf health scores.

4.3.Material and methods

Animal care and use protocol guidelines were strictly followed for this experiment, under protocol number 9078250118 approved by Embrapa Dairy Cattle Ethics Committee.

4.3.1. Animals, management, and treatments

This study was conducted in Embrapa Dairy Cattle facilities (Coronel Pacheco, Brazil) from March to July 2018. Sixteen newborn Holstein and crossbred (Holstein x Gyr) bull calves with an average initial body weight of 33.3 ± 3.7 kg were separated from their mothers immediately after birth and used for this trial. They received 10% of their body weight of good quality colostrum (Brix >23%) during the first six hours of life, and had their umbilical cord immersed in a 10% iodine solution for

the first three days of age. The bull calves were allocated in a barn with open sides, in individual sand-bedded pens (1.25 × 1.75 m) and tethered with 1.2 m long chains. A*d libitum* water and commercial calf starter (Soymax Rumen pre-initial Flocculated, Total Alimentos, Três Corações, Brazil, Table 1) were provided during all experimental period since the first day of life.

A liquid diet was provided twice a day (0800 and 1600 h) in buckets provided with rubber teats (Milbar, New Zeland). At d 2 and 3 of life, calves received 5 L/d of transition milk divided equally into two meals, and from the 4 to 60 days, they were fed with 5 L/d milk replacer divided equally in two meals (MR, Kalvolak, Nutrifeed, Netherlands; Table 1), reconstituted to provide 15% of total solids, 194 g of crude protein and 60 g of fat. The passive immune transfer was checked on d 3, where a serum sample was collected via jugular venipuncture. Tubes were left at room temperature for 30 minutes and then centrifuged at 1,800 × g for 10 minutes (22 – 25 °C). After centrifugation, the serum was evaluated in a Brix refractometer (Aichose refractometer, Xindacheng, Shandong, China). Calves were enrolled only if the Brix was higher than 8.4%.

On day 4, bull calves were randomly assigned into one of two treatments, following: (i) control (CON, no additive; n = 8) and (ii) essential oils supplementation (BEO, 1 g/d/calf, Apex Calf, Adisseo, China; n = 8). The month of birth, weight, and Brix were checked during assignment to ensure that both treatments were balanced. Apex calf is a commercial additive that contains a blend of plant extracts derived from anise, cinnamon, garlic, rosemary, and thyme. This additive was incorporated in the MR during the experiment following manufacture recommendations. The amount of the additive for each meal was weighed previously and kept in 15 mL tubes in a dark box. This amount was mixed with 10 mL of MR, homogenized, and incorporated in 0.49 L of MR (0.5 g/calf at morning meal and 0.5 g/calf at afternoon meal) to ensure total ingestion of the product. As soon as the animal finished ingesting 0.5 L MR with 0.5 g of the additive, the bucket was refilled with the rest of the MR.

Table 1. Nutrient composition (% DM basis ± SD) of milk replacer (MR) and starter.

Item	MR[1]	Starter[2]
DM (%)	96.0 ± 0.4	86.7 ± 0.7
CP (% of DM)	19.4 ± 0.5	17.1 ± 0.5
Ether extract (% of DM)	14.1 ± 0.6	3.9 ± 1.2
Organic Matter (% of DM)	9.7 ± 0.2	7.2 ± 1.5
NDF (% of DM)	–	22.1 ± 2.9
ADF (% of DM)	–	10.6 ± 0.9
Gross Energy (Mcal/kg of DM)	4.5 ± 0.1	4.3 ± 0.1

[1] Powder integral milk, wheat isolated protein, acidifying additive, whey, coconut oil, palm oil, vitamin A, Vitamin D3, Vitamin E, Vitamin C (Kalvolak, Nutrifeed, Netherlands).

[2]Basic composition: oats (rolled grains), calcitic limestone, sodium chloride, corn gluten meal, defatted corn germ, wheat bran, soybean meal, rice hulls, kaolin, molasses, flocculated corn, ground corn, corn grain, alfalfa hay, monensin, citrus pulp, dried sugarcane yeast, whole toasted soybean, sodium selenite, copper sulfate, manganese sulfate, cobalt sulfate, iron sulfate, zinc sulfate, calcium iodate, vitamin A, vitamin B1, vitamin B12, vitamin B2, vitamin B6, vitamin C, vitamin D3, vitamin E, vitamin K, niacin, pantothenic acid, folic acid, biotin, propionic acid, caramel aroma, milk aroma, and probiotic additive.

4.3.2. Intake, performance, and growth

Feed intake (MR, starter, and water), performance, and body frame development were measured between 4 and 60 d of age. The feed intake was calculated daily by subtracting the refusals from the provided amount. Samples of MR and starter were collected three times a week to obtain a weekly pool for nutrient analysis.

The body weight (BW) was measured starting at 4 d of age and after that every 3 d before the morning meal, using a weighing machine (ICS 300, Coimma, Dracena, Brazil). Body frame development (wither height (WH), rump height (RH), rump width (RW), and heart girth (HG)) were measured once a week using a portable hypometer and a measuring tape. The average daily gain (ADG) and dry matter intake (DMI) ratio was calculated to obtain weekly feed efficiency values.

4.3.3. Nutrient apparent digestibility and nutrition composition analysis

Feed digestibility was conducted during the last five days of the trial, between d 55 and 60 of age. A rubber mat (WingFlex, Kraiburg TPE GmbH & Co., Waldkraiburg, Germany) was placed on

each individual tie-stall to allow daily fecal collection. Feces were collected and weighted daily from d 55 to 60 and frozen at -20 ºC for further analysis. On d 59 animals were transferred to metabolic cages (1.5 x 0.8 m, Intergado Ltda., Contagem, Brazil) for 24 h urine collection, and the last day of fecal sampling. The flask that stored the urine during the trial was placed in a cooler covered with ice to avoid bacteria growth and nitrogen loss. After the collection period, the urine's total volume, weight, and density were recorded, and a pooled sample was frozen at -20ºC for further analysis. During the digestibility trial, MR, starter, and refusals samples were collected and pooled for the five days and stored and frozen at -20ºC for further analysis.

Starter and MR samples were collected weekly and during the digestibility trial, as also the feces were oven-dried at 55 ºC for 72 h and ground in Wiley mill (model 3, Arthur H. Thomas Co., Philadelphia, PA) through a 1-mm screen for analysis. They were analyzed to determine DM (Method 934.01), CP (Method 988.05), ether extract (Method 920.39), ash (Method 942.05), according to AOAC [25]. The concentrations of NDF and ADF were determined in sequence using the method described by Van Soest et al. [26]. Gross energy was determined using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL).

Apparent digestibility of each nutrient (%) was determined considering nutrient intake (NUI) and nutrient feces recovery (NFR) using the formula:

$$\frac{NUI - NFR}{NUI} \times 100$$

Nitrogen balance was determined by the difference between nitrogen intake (NI) and fecal (NF) and urinary nitrogen (NU) using the formula:

$$NI - (NFR + NU)$$

Gross energy intake (GEI) was determined by the difference between gross energy (GE) of the diet provided (starter gross energy (GES) and MR gross energy (GEMR)) and refusals gross energy (GER) using the formula:

$$(GES + GEMR) - GER$$

Digestible energy intake (DEI) was determined by the difference between GEI and energy fecal excretion (GEF). To determine metabolizable energy intake (MEI), the energic losses from the urine (GEU) were subtracted from DEI.

4.3.4. Health scores and blood sampling

Health scores were dived in fecal and respiratory scores and previously described [24]. They were evaluated daily before animal management using the University of Wisconsin calf health scoring chart as a reference, where fecal consistency, nasal discharge, eye discharge, ear position, cough, and temperature were evaluated.

To obtain a baseline, jugular blood samples were collected at birth before colostrum ingestion. After that, there was a weekly collection 3 h after morning feeding to obtain the concentrations of beta-hydroxybutyric acid (BHB), urea into tubes without anticoagulant, and glucose with sodium fluoride tubes and, biweekly, for IGF-1 with heparin tubes (Labor Import, Osasco, Brazil). Tubes were centrifuged at 3000 x *g* for 10 min at room temperature (22 – 25 °C), and duplicates of each sample were individually allocated into microtubes and frozen at -20 ºC for further analysis. The serum concentration of BHB and urea were determined by an auto-analyzer (Cobas Mira Plus, Roche Diagnostic Systems, Risch-Rotkreuz, Switzerland) using commercial kits (Ranbut-D-3-Hidroxibutyrate, Randox Laboratories Ltd., Antrim, UK; Urea UV, Kovalent do Brasil Ltda., Bom Retiro São Gonçalo, Brazil). Plasma glucose was measured in a microplate Spectrophotometer EON (Biotek Instruments Inc., Winooski, VT) using the enzymatic colorimetric method (Kovalent do Brasil Ltda., Rio de Janeiro, Brazil). Plasma IGF-1 concentrations were analyzed using chemiluminescence assay (Immulite2000 Systems 1038144, IGF-1 200, Siemens Healthcare Diagnostics Products Ltd., Llanberis, Gwynedd, UK).

At days 0, 30, and 60, blood samples were collected for complete blood count by jugular vein puncture into EDTA tubes (Labor Import, Osasco, Brazil) and immediately transported on ice to the laboratory. An automatic hematology cell counter (SDH – 3 vet, Labtest Diagnóstica S.A., Brazil) was used to evaluate: red blood cell count (RBC), packed cell volume (PCV), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet and total white blood cell count. Manual white cell blood differential counting was also performed by microscopic examination evaluating 100 leukocytes in a 1,000x microscopic magnification for total

leukocyte count, basophils, eosinophils, neutrophils, band neutrophils, segmented neutrophils, lymphocytes, monocytes. Morphological changes, such as toxic neutrophils, reactive lymphocytes, and activated monocytes, were calculated. With the previous results calculated platelet to lymphocytes ratio (PLR) and neutrophils to lymphocytes ratio (NLR). The PRL and NRL are novel inflammatory markers and were chosen to verify if they could be applied as biomarkers to predict inflammation and mortality [27] and balance between inflammation and adaptive immunity to predict disease course as already done in human medicine [28].

4.3.5. Comparative slaughter and histology

All bull calves were euthanized on day 60 ± 1 to compare internal organs development using the procedures recommended by the Brazilian Federal Veterinary Medicine Council [29]. Immediately after stunning and slaughtering, the jugular was cut to drain the body's circulation blood. The abdominal cavity was then opened, and each region of the gastrointestinal tract was isolated and tied off. Internal organs and body parts were removed and weighted following the order: spleen, bladder, all intestinal tract, liver, pancreas, omentum, perirenal fat, kidney, pre stomachs, rumen-reticulum, omasum, abomasum, small and large intestine, tongue, heart, lungs + trachea. After this, the organs with biological content were emptied and weighted again (bladder, rumen-reticulum, omasum, abomasum, small and large intestine). The weight of the organs was evaluated in proportion to the weight of the empty animal; thus, the animal's weight subtracted the fluids' content. The length of small and large intestines was measured using a metric tape. Ruminal and cecal fluid samples were collected to measure pH, VFA, and N-NH_3. After these procedures, some parts were then emptied and then weighted again

Approximately 9 cm^2 area samples were collected for comparative histology: rumen ventral sac, rumen dorsal sac, omasum laminae, abomasum, duodenum (ten centimeters under the abomasum), ileum (40 centimeters before the ileum-cecum junction), and colon (40 centimeters after the ileum-cecum junction). Tissue samples were immediately placed in flasks with formalin for fixation. Forty-eight hours after fixation, formalin was replaced by 70ºGL alcohol and protected from the light. The samples were processed to include paraffin and then sectioned in 5 µm thickness using a manual microtome (Olympus CUT 4055, Tokyo, Japan). For morphometric analysis, sheets were colored using hematoxylin-eosin. Images were captured using a light microscope (Olympus CX31, Tokyo,

Japan), connected to a camera (Olympus OSIS SC30, Tokyo, Japan), using Cell-B software (Olympus, Tokyo, Japan). AxioVision 4.8.2-06/2010 (Carl Zeiss Images Systmes®237, Jena, Germany) was used for morphometric interpretations. For rumen and omasum samples, papilla`s area, height, and mitotic index (MI) of epithelium basal layer were analyzed. For MI determination, 2000 basal layer cells were counted using a light microscope. Estimation considered the ratio between the number of cells in the mitotic division and total counted cell number [30]. The height (μm) and area (μm^2) of villi in the duodenum and ileum regions; the depth (μm) of gastric fossets and crypts in the duodenum, ileum, and colon regions were measured. Cell proliferation was determined by the count of mitotic figures in the epithelium of the gastric and intestinal glands in 10 fields, in an increase of 400x.

4.3.6. Ruminal and cecum pH and ammonia nitrogen

Ruminal fluid samples were obtained on days 14, 28, 42, and 60, using an esophageal tube four hours after morning feeding. On the day of the euthanasia, samples were collected directly from the rumen and cecum of the animals. Sampling was visually monitored to ensure not to have saliva or other contamination. The samples were then filtered in gauze to separate the liquid fraction. Rumen pH was immediately measured using a pH meter (Phmetro T-1000, Tekna, Araucária, Brazil). Ten milliliters of the filtrated ruminal fluid were acidified with 2 mL of 20% metaphosphoric acid and ten milliliters with 0.2 N 50% sulfuric acid for VFA and N-NH_3 analyses. These samples were stored at -20ºC for further analysis. For N-NH_3 concentration it was used a colorimetric distillation method proposed by Chaney and Marbach [31], where its absorbance was measured at 630 nm (Termo Fisher Scientific, Madison, USA) after Kjedahl destilation with magnesium oxide and calcium chloride. The VFA ruminal concentrations were measured by gas chromatography. They were thawed and centrifuged at 13000 rpm, for 15 minutes, at 13ºC. The supernatant was collected, filtered, and analyzed as previously described [24].

4.3.7. Splenocyte proliferation assay

The spleen function combines the innate and adaptive immune response, and removes older erythrocytes, microorganisms, and cellular debris from the circulation, being the most important organ for antibacterial and antifungal immune reactivity [32]. To evaluate cell proliferation to bacterial

antigens, immediately after the animal slaughter and isolation of the organs, the spleen had its measurements taken and placed into ice to be processed shortly.

Five grams of the tissue were ground at the lab, followed by density gradient centrifugation on a Ficoll-Hypaque solution at 400 g for 30 minutes (Sigma, USA) for mononuclear cell isolation. The splenocyte cells ($5x10^6$ cells/well) were seeded in flat bottomed micro-culture plates and stimulated with lipopolysaccharide from *E. coli* (10ng/mL; Sigma, USA), or Phorbol 12-myristate 13-acetate (PMA; 25ng/mL), or *E. coli* B41 lineage extract (20ng/mL) from streptomycin-resistant derivate of bovine ETEC strains isolated according to Smith and Halls [33]. The isolated colony of *E. coli* B41 lineage were lysate and diluted in PBS buffer added with a cocktail of protease inhibitors (Protease Inhibitors Set, Sigma, USA). *E. coli* and LPS were chosen since *E. coli* is one of the most important mediators of calf diarrhea in the first weeks of life [34]. Therefore, it could be a good choice to visualize the indirect effects of the EO on cell proliferation.

The mononuclear cells from the spleen were then cultured in RPMI 1640 medium (Sigma, USA) with 10% heat-inactivated fetal calf serum (Gibco, USA), 2 mM L-glutamine (Sigma, USA), 100 µg/mL of streptomycin, and 100 U/mL of penicillin (Sigma, USA) at 37 ºC in a 5% humidified CO2. Cell proliferations were analyzed by MTT assay (3-(4,5-dimethylthiazoyl)-2,5diphenyltetrazolium Bromide; Sigma, USA) according to fabricant instructions (Sigma) using non-stimulated cells as a negative control. Briefly, the stimulated splenocytes were incubated at 37 ºC in a 5% humidified CO_2 incubator for 48h. Ten µL of MTT (5 mg/mL) were added to each well afterward, and incubation was carried out for 4h at 37 ºC. The supernatants were aspirated carefully, and 150 µL of DMSO was added to each well. The plates were shaken for an extra 10 min, and the absorbance values were read at 570 nm with an ELISA reader. The absorbance values were compared among stimulated and non-stimulated groups.

4.3.8. Gene expression and RT-Qpcr

Gene expression analyses from buffy coat cells, ilium, and colon biopsies were performed by RT-qPCR. Briefly, peripheral whole blood from CON (n = 8) and BEO (n = 8) groups was collected at days 30 and 60 and centrifuged at 800g for 10 minutes at room temperature for buffy coat isolation. The white blood cells and platelets (buffy coat) formed a layer on red blood cells that were carefully removed with a micropipette. According to the fabricant instructions, red blood cells were then lysate

by Ammonium-Chloride-Potassium Lysing Buffer (ACK; Thermoscientific, Waltham, USA), and only the white layer of cells was frozen at RNA protect reagent (Qiagen, Hilden, Germany) until RNA extraction. The ilium and colon biopsies were obtained from the animal's necropsy and kept on RNAprotect reagent (Qigen, Hilden, Germany) until analysis. RNA extraction from buffy coat and organ samples was performed with RNeasy Mini kit (Qiagen, Hilden, Germany). The obtained total RNA was quantified by the Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, USA) and the cDNA synthesis performed by SuperScript III First-Strand kit (Thermo Scientific, Waltham, USA), all according to manufacturer's instructions [35].

RT-qPCR assays occurred in 7500 Fast Real-Time PCR System (Thermo Scientific, Waltham, USA), using the PowerUp SYBR Green Master Mix (Thermo Scientific, Waltham, USA) to verify the expression of interleukin 6 (*IL-6*) and interleukin 10 (*IL-10*) genes. It was used *β-actin*, *GAPDH*, and *Ubiquitin* as reference genes based on expression stability calculated with RefFinder online software. Each sample calculated the average of Ct values from targets and reference genes using ABI Real-Time PCR 7500 software v.2.3 (Thermo Scientific, Waltham, USA).

4.3.9. Statistical Analysis

Statistical analysis was conducted utilizing R® (R Core Team, 2019). A randomized block experimental design with repeated measures was implemented to test the hypothesis of the effect of the BEO on each performance outcome. The outcomes analyzed were feed intake, structural growth, performance, nutrient digestibility, organ weight, histology, ruminal, blood, health parameters, and gene expression. For each treatment was assigned eight experimental units were assigned.

Each outcome was analyzed independently using linear mixed models (package: nlme). Each independent outcome was modeled as a function of the following fixed effects: treatment, experimental week, the interaction between treatment and week. Birth weight and serum Brix value were tested as a covariate but did not improve statistical significance. Therefore, they were eliminated from the model. The genetic composition of the animal was included as a blocking effect. The effect of bull calf within treatment was included in the models to account for individual variability. All outcomes were tested for homogeneity of variance and normality to meet the required assumptions of this model using residuals versus fits and Q-Q plots, respectively. A variable transformation using

Box-Cox was applied to milk replacer intakes to meet the assumption. A 95% Confidence Interval was adopted for all the tests.

The continuous outcomes such as intakes, structural growth, performance, ruminal, and blood parameters were analyzed with ANOVA. *P*-values were produced with a Fisher test and estimated marginal means and SEM were calculated with the emmeans package. The categorical outcomes fecal and respiratory scores were analyzed using a non-parametric aligned rank transformation test implemented in the R package ARTool.

The outcomes that had a single measure during the study, such as nutrient digestibility, nitrogen balance, energy partitioning organ/viscera weight and size, organ histology, splenocyte proliferation, and gene expression, were analyzed using the linear mixed model (package nlme) were calf was the random effect and treatment was the fixed effect.

4.4.RESULTS

4.4.1. Intake, performance, and body measurements

There were no differences among treatments for feed intake, performance, and body measurements (Table 2). The outcomes were statistically different when the week effect was evaluated, observing higher intake, growth, gain, and efficiency as the animals got older ($P < 0.001$, Table 2). A week and treatment interaction effect for MR intake was observed from weeks 1 to 3 ($P < 0.001$, Table 2). The average intake for the treatments was 4.5% lower for weeks 1 to 3 when compared with the other weeks, with the lowest intake on week 2, where the animals consumed 8.5% less MR. Therefore, it was observed a negative effect between fecal scores and MR intake ($P \leq 0.001$), where higher the fecal score, lower the MR intake. The CON treatment had a 4% lower intake on week 1, 8% on week 2, and 2% on week 3 compared to BEO. The intake of the other weeks was the same for both treatments ($P > 0.05$, Table 2).

Table 2. Intake of milk replacer (MR), starter, total dry matter (DM), total crude protein (CP), total gross energy and water, performance, and body development of bull calves of control (CON, n = 8) and supplemented with blend essential oils (BEO, n = 8) in milk replacer from 4 to 60 days of age.

Item	Treatment[1]		SEM	*P* – value[2]		
	CON (n =8)	BEO (n =8)		T	W	T x W
Intake						
MR (kg of DM/d)	0.71 (0.693-0.722	0.71 (0.701-0.724)	-	0.32	<0.001	<0.001
Starter (kg of DM/d)	0.28	0.26	0.16	0.66	<0.001	0.98
Total DM (kg/d)	1.01	0.97	0.15	0.38	<0.001	0.42
Total CP (kg/d)	0.19	0.18	0.03	0.38	<0.001	0.42
Total gross energy (Mcal/kg)	4.57	4.31	0.70	0.30	<0.001	0.38
Water (kg/d)	1.00	1.31	0.36	0.26	<0.001	0.96
Performance						
Birth BW (kg)	33.55	33.15	0.46	0.86	–	–
Final BW (kg)	68.89	64.38	0.65	0.17	–	–
ADG (kg)	0.61	0.59	0.12	0.15	<0.001	0.56
Feed efficiency	0.44	0.42	0.04	0.35	0.004	0.34
Body measurements						
Withers height (cm)	75.16	73.53	1.23	0.32	<0.001	0.56
Rump height (cm)	78.43	76.56	1.37	0.23	<0.001	0.57
Rump width (cm)	19.45	19.20	0.56	0.46	<0.001	0.90
Heart girth (cm)	82.92	81.00	0.01	0.21	<0.001	0.88

[1]CON = control, BEO = 1 g/calf/d blend of essential oil. [3]T = treatment effect; W= week effect, T x W = treatment by week interactions.

4.4.2. Ruminal and cecum pH, VFA, and ammonia nitrogen

Ruminal pH presented lower values for the BEO treatment when compared to CON ($P = 0.02$, Table 3). A week effect was also observed, with a decrease of 14% on pH values from week 3 to week 9 for both groups. There were no treatment differences for the ruminal ammonia nitrogen and all VFA measured. However, as observed on pH, there was also a week effect for the VFA and C2:C3 proportions ($P < 0.01$, Table 3), with increasing values of all VFA and decreasing values of C2:C3 as the animals were growing older. The C2:C3 proportion also presented a treatment x week interaction, where it was observed 28% and 16% higher values for BEO animals on weeks 3 and 5, respectively. For weeks 7 and 9, those values did to presented differences.

Cecum parameters were evaluated only on the last day of the trial, after euthanasia. There were no differences within treatment groups for all evaluated parameters ($P > 0.05$, Table 3), with an exception for butyric acid values, that presented values 76% higher for the BEO group ($P = 0.05$, Table 3).

Table 3. Rumen and cecum mean values of pH, ammonia nitrogen (Ammonia-N), and volatile fatty acids (VFA) of control bull calves (CON) and bull calves supplemented with essential oils blend (BEO) in milk replacer from 4 to 60 days of age.

Item	Treatment[1]		SEM	*P* – value[2]		
	CON[1] (n = 8)	BEO[2] (n = 8)		T	W	T x W
Rumen pH	6.35	5.91	0.45	0.02	0.05	0.95
Rumen ammonia-N (mg/dL)	13.82	16.61	6.85	0.22	0.47	0.06
Rumen VFA (µmol/mL)						
Acetic (C2)	27.90	23.28	7.94	0.36	0.01	0.59
Propionic (C3)	23.07	18.21	8.12	0.37	<0.001	0.68
Butyric (C4)	3.42	4.18	1.96	0.28	0.006	0.15
C2:C3	1.40	1.55	0.27	0.34	<0.001	0.02
Cecum pH	7.25	7.24	0.27	0.93	–	–
Cecum ammonia-N (mg/dL)	9.7	9.5	1.2	0.89	–	–
Cecum VFA (µmol/mL)						
Acetic (C2)	19.79	24.30	3.74	0.17	–	–
Propionic (C3)	12.26	12.77	2.57	0.81	–	–
Butyric (C4)	1.89	3.33	0.67	0.05	–	–
C2:C3	1.77	1.98	0.35	0.43	–	–

[1]CON = control, BEO = 1 g/calf/d blend of essential oil. [3]T = treatment effect; W= week effect, T x W = treatment by week interactions.

4.4.3. Health scores and blood sampling

There were no differences within treatments for all health parameters ($P > 0.05$, Table 4). Fecal scores differed within the weeks of the trial ($P < 0.001$, Table 4) since the average days for diarrhea occurrence were 17 ± 4.2 d for CON and 15.3 ± 2.5 d for the BEO group. Diarrhea incidence was 100% and did not present differences among the days for both treatment groups ($P = 0.39$, Table 4). Severe diarrhea (fecal score = 3) occurred on 50% of the BEO animals and 87% of CON animals, with

no statistical difference (P = 0.08) and no differences on days with severe diarrhea (*P* = 0.61, Table 4). Two animals of each treatment were medicated for severe diarrhea with non-steroidal anti-inflammatory, with a therapy duration of 2 d. There were not detected any clinical signs of respiratory diseases or pulmonary commitment. Thus, differences between treatments were not observed (*P* = 0.83, Table 4). The respiratory score was higher on weeks 2 and 3, the same weeks of high diarrhea incidence; therefore, a week effect was observed (*P* < 0.001, Table 4). BEO animals had an increase in respiratory scores of 112% on week 2 but normal values on the other weeks. As for CON animals, an increase of 67% and 74 % on weeks 2 and 3 was observed compared to the other weeks.

Table 4. Fecal score, health score, days with health score above 4, days with fever, days with diarrhea, days with severe diarrhea of control bull calves (CON), and bull calves supplemented with a blend of essential oils (BEO) in milk replacer from 4 to 60 days of age.

	Treatment[1]		SEM	*P* – value[2]		
Item	CON (n = 8)	BEO (n = 8)		T	W	T x W
Fecal score[3]	0.04	0.03	0.05	0.21	<0.001	0.59
Respiratory score[4]	0.59	0.67	0.02	0.83	<0.001	0.13
Days with respiratory score > 4	0.00	0.004	0.00	0.84	–	–
Days with fever[5]	0.37	1.00	0.28	0.17	–	–
Days with diarrhea	7.37	10.50	1.44	0.39	–	–
Days with severe diarrhea	3.25	2.25	0.60	0.61	–	–

[1] CON = control, BEO = 1 g/calf/d blend of essential oil; [2]T = treatment effect; W= week effect, T x W = treatment by week interactions.

[3]0 – normal (firm but not hard); 1 – soft (does not hold form, piles but spreads slightly); 2 – runny (spreads readily to about 6 mm depth); and 3 – watery (liquid consistency, splatters). A heifer was considered to have diarrhea if a fecal score was 2 or 3, and severe diarrhea was considered severe if a fecal score was 3. Assessments were performed for 21 d.

[4] Respiratory score evaluations considering the sums of rectal temperature score = 0 – temperature between 37.8 and 38.3 ºC, 1 – temperature between 38.4 and 38.8 ºC, 2 – temperature between 38,9 and 39.3 ºC, 3 – temperature above 39.4 ºC; cough score = 0 – none, 1 – induce single cough, 2 – induced repeated or occasional spontaneous coughs, 3 – repeated spontaneous coughs; nose score = 0 – normal serous discharge, 1 – small amount of unilateral cloudy discharge, 2 – bilateral cloudy or excessive mucus discharge, 3 – copious bilateral mucopurulent discharge; eye score = 0 – normal, no discharge, 1 – a small amount of ocular discharge, 2 – moderate amount of bilateral discharge, 3 – heavy ocular discharge; ear score = 0 – normal, 1 – ear flick or head shake, 2 – slight unilateral drop, 3 – head tilt or bilateral drop.

[5]Fever was considered when the temperature was above 39.4ºC.

For blood outcomes, there were no differences within treatments for all metabolic – BHB, urea, and glucose – and hormonal – IGF-1– parameters ($P > 0.05$, Table 5). However, all these parameters presented a week effect ($P < 0.01$, Table 5), increasing concentration values as the animals grew older. As for the hemogram, there was only a difference for red blood cell size through the weeks, with a decrease of MCV from week 1 to 9 ($P = 0.04$, Table 5). A treatment effect was observed for the eosinophils count for the white cell count, with 2.4 times lower values for the BEO group ($P = 0.04$, Table 5). As for the week effect over the white cell count, age impacted eosinophil count, segmented

neutrophils count, lymphocytes count, PLR, and NLR, observing differences from week 1 to 9. There was a significant interaction of treatment x week for segmented neutrophils, where BEO animals had 50% more cells on week 5 when compared to CON animals, but no differences on the other weeks ($P = 0.04$).

Table 5. Blood concentrations of metabolites, insulin growth factor type 1 (IGF-1), and hematological parameters of control bull calves (CON) and bull calves supplemented with a blend of essential oils blend (BEO) in milk replacer from 4 to 60 days of age.

Item[1]	Treatment[2]		SEM	*P* – value[3]		
	CON (n =8)	BEO (n =8)		T	W	T x W
BHB (mmol/L)	0.10	0.06	0.13	0.32	0.001	0.16
Urea (mg/dL)	12.14	12.59	0.10	0.87	0.01	0.48
Glucose (mg/dL)	96.57	96.52	18.40	0.99	<0.001	0.93
IGF-1 (ng/mL)	96.47	92.70	35.8	0.79	<0.001	0.11
RBC (x 10^6/µL)	7.91	7.67	0.81	0.39	0.34	0.06
PCV (%)	34.70	35.29	4.37	0.69	0.79	0.07
Hb (q/dL)	10.73	10.98	1.35	0.57	0.87	0.07
MCV (fL)	44.25	44.28	3.70	0.11	0.04	0.61
MCHC (%)	31.31	31.09	1.45	0.63	0.25	0.51
Total leukocytes (/µL)	9,999.03	11,288.81	3,010.00	0.38	0.15	0.43
Basophils (/µL)	0.00	0.00	0	1.0	1.0	1.0
Eosinophils (/µL)	101.70	42.80	4.97	0.04	0.002	0.47
Band neutrophil (/µL)	21.91	27.08	0.05	0.16	0.71	0.47
Segmented neutrophils (/µL)	4,840.68	5,997.62	2,380.0	0.17	<0.001	0.04
Lymphocytes (/µL)	4,790.32	5,224.41	1,160.0	0.75	<0.001	0.61
Monocytes (/µL)	411.07	505.18	313.0	0.27	0.12	0.57
Platelet (x 10^3/µL)	400.78	396.31	85.9	0.87	0.24	0.16
Plasmatic protein (g/dL)	5.80	6.12	0.66	0.26	0.17	0.42
PLR	0.12	0.09	0.04	0.16	<0.001	0.22
NLR	1.88	1.87	1.65	0.99	<0.001	0.78

[1]RBC: red blood cell, PCV: packed cell volume, Hb: hemoglobin, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration, PLR: platelet lymphocyte ratio, NLR: neutrophils lymphocytes ratio; [2]CON = control, BEO = 1 g/calf/d blend of essential oil; [3]T = treatment effect; W= week effect; T x W = treatment by week interactions.

4.4.4. Nutrient apparent digestibility and nitrogen balance

Total tract apparent digestibility and nitrogen balance were performed at the end of the trial from d 55 to 60. The digestibility of DM, OM, gross energy, CP, and EE did not differ among treatments ($P > 0.05$, Table 6). Outcomes related to nitrogen balance also presented similar values between treatments ($P > 0.05$, Table 6).

Table 6. Apparent nutrient digestibility % and nitrogen balance of control bull calves (CON) and bull calves supplemented with a blend of essential oils (BEO) in milk replacer from 4 to 60 days of age.

Item	Treatment[1]		SEM	*P* – value[2]
	CON (n = 8)	BEO (n = 8)		T
Dry Matter (g/day)	877	892	3.47	0.48
Organic Matter (g/day)	914	926	2.59	0.64
Crude Protein (mg/day)	908	922	1.98	0.64
Ether extract (mg/day)	957	956	1.43	0.96
Ingested nitrogen (g/kg of MW^3/ day)	2.09	2.06	0.02	0.81
Fecal nitrogen (g/kg of MW^3/ day)	0.17	0.15	0.006	0.65
Urine nitrogen (g/kg of MW^3/ day)	0.36	0.37	0.01	0.98
Retained nitrogen (g/kg of MW^3/ day)	1.56	1.55	0.02	0.91

[1]CON = control, BEO = 1 g/calf/d blend of essential oil, [2]T = treatment effect; [3]MW = metabolic weight.

4.4.5. Comparative slaughter and histology

Euthanasia was performed in the morning before feeding not to impact final weight. There were no differences between treatments for empty body weight ($P = 0.12$, Table 7). Most of the evaluated organs were statistically similar between treatments, except for the pancreas, respiratory

tract, and small intestines. The BEO animal's pancreas was 30% heavier when compared to CON (P = 0.05, Table 7). The lungs and trachea were 11% heavier on CON animals when compared to BEO (P = 0.03, Table 7). Moreover, the small intestines were 16% heavier on BEO animals (P = 0.03, Table 7), besides no difference in the intestinal length.

Table 7. Empty body, internal organs weight (% of empty body), and intestinal length of control bull calves (CON) and bull calves supplemented with a blend of essential oils (BEO) in milk replacer from 4 to 60 days of age.

Item	Treatment[1]		SEM	*P* – value[2]
	CON (n = 8)	BEO (n = 8)		T
Empty BW (kg)	55.2	51.5	0.49	0.12
Organ weight (% of empty BW)				
Omental fat	0.29	0.31	0.04	0.40
Mesenteric fat	0.43	0.43	0.08	0.85
Perirenal fat	0.42	0.41	0.07	0.82
Pancreas	0.09	0.12	0.02	0.05
Liver	2.44	2.13	0.36	0.19
Lungs and trachea	2.14	1.91	0.13	0.03
Spleen	0.72	0.67	0.15	0.60
Heart	0.72	0.70	0.06	0.66
Kidneys	0.59	0.50	0.06	0.80
Togue	0.50	0.49	0.04	0.52
Bladder	0.07	0.08	0.02	0.33
Rumen-reticulum	1.67	1.65	0.05	0.84
Omasum	0.29	0.24	0.06	0.28
Small intestine	2.89	3.35	0.33	0.03
Large intestine	0.97	0.92	0.15	0.62
Small intestine length (m)	21.52	22.77	2.79	0.47
Large intestine length (m)	3.59	3.40	0.23	0.18

[1] CON = control, BEO = 1 g/calf/d blend of essential oil, [2] T = treatment effect.

There were no differences among gastrointestinal tract development and histology ($P > 0.05$, Table 8), except for ileum villus height. Animals from BEO presented a 25% higher villus when compared to CON ($P = 0.02$, Table 8).

Table 8. Gastrointestinal tract development of control bull calves (CON) and bull calves supplemented with a blend of essential oils (BEO) in milk replacer from 4 to 60 days of age.

Item	Treatment[1]		SEM	P – value[2]
	CON (n = 8)	BEO (n = 8)		T
Rumen ventral sac				
Cell proliferation	10.8	18.2	0.936	0.10
Total cells	2012	2017	0.810	0.22
Mitotic index	0.005	0.009	0.0004	0.12
Papillae height (mm)	2.07	2.04	0.041	0.31
Papillae area (mm)	6.37	5.80	0.232	0.68
Rumen dorsal sac				
Papillae height (mm)	2.68	2.04	0.048	0.07
Papillae area (mm)	4.51	3.77	0.139	0.28
Omasum				
Cell proliferation	20.1	17.0	1.11	0.61
Total cells	2020	2017	1.98	0.61
Mitotic index	0.01	0.01	0.0005	0.61
Papillae height (mm)	0.39	0.48	0.013	0.25
Papillae area (mm)	0.11	0.17	0.071	0.15
Abomasum				
Fossette depth (mm)	0.27	0.27	0.006	0.96

Glandular depth (mm)	0.15	0.13	0.003	0.24
Cell proliferation	9.11	8.36	0.330	0.70
Duodenum				
Villus height (mm)	0.39	0.38	0.071	0.79
Villus area (mm)	0.57	0.57	0.013	0.94
Crypt depth (mm)	0.31	0.29	0.004	0.19
Cell proliferation	24.40	25.87	1.280	0.83
Ileum				
Villus height (mm)	0.25	0.31	0.006	0.02
Villus area (mm)	0.33	0.40	0.008	0.12
Crypt depth (mm)	0.29	0.29	0.004	0.99
Cell proliferation	44.87	50.05	2.390	0.69
Colon				
Cell proliferation	12.40	18.12	0.650	0.15
Crypt depth (mm)	0.35	0.37	0.002	0.31

[1] CON = control, BEO = 1 g/calf/d blend of essential oil, [2] T = treatment effect.

4.4.6. Splenocytes proliferation and gene expression.

Splenocyte proliferation assay was performed to evaluate supplementation effect over cellular response to bacterial antigens. The spleen cells were stimulated, *in vitro*, with *E. Coli* antigen extract and lipopolysaccharide. To verify potential growth inhibition of EO, PMA was used as a cell proliferation activator via Protein Kinase C (PKC). However, there were no differences between splenocytes proliferation under all tested treatments (Table 9).

Table 9. Splenocytes proliferation assay of control bull calves (CON) and bull calves supplemented with a blend of essential oils (BEO) in milk replacer from 4 to 60 days of age

Item[1]	Treatment[2]		SEM	P – value[3]
	CON (n = 8)	BEO (n = 8)		T
Negative control	0.17	0.22	0.01	0.35
PMA	0.27	0.28	0.02	0.91
ECE	0.15	0.18	0.005	0.56
LPS	0.202	0.205	0.009	0.96
BrEEC	0.21	0.23	0.09	0.76
MTT control	0.12	0.14	0.06	0.70

[1] PMA = *Phorbol 12-myristate 13-acetate*; positive control of the proliferation, ECE = *E. coli* extract, LPS = *E. coli* lipopolysaccharide extract from Sigma), BrEEC = ECE control. [2]CON = control, BEO = 1 g/calf/d blend of essential oil, [3]T = treatment effect.

Gene expression was evaluated on white blood cells (buffy coat) at 30 and 60 days of age and ileum and colon at 60 days of age. These samples were chosen based on the previous statistical results of this paper and our previous results [24], and evaluated genes were interleukin 6 (*IL-6*) and 10 (*IL-10*) since they are related to inflammatory responses and immunity regulation after treatment with BEO [36,37]. There were no differences within treatments for relative gene expression of *IL-6* and *IL-10* in the buffy coat, ileum, or colon ($P > 0.05$, Table 10). The relative gene expression of *IL-6* and *IL-10* increased over time in the buffy coat, but it was not significant ($P > 0.05$, Table 10).

Table 10. Relative gene expression of interleukin 6 (*IL-6*) and interleukin 10 (*IL-10*) in control bull calves (CON) and bull calves supplemented with a blend of essential oils (BEO) in milk replacer from 4 to 60 days of age

Item	Treatment[1]		SEM	P – value[2]		
	CON	BEO		T	W	T x W
	(n = 8)	(n = 8)				
Ileum						
IL-6	31.1	31.0	0.258	0.96	-	-
IL-10	31.4	31.0	0.214	0.69	-	-
Colon						
IL-6	26.8	27.2	0.196	0.68	-	-
IL-10	27.3	27.7	0.114	0.52	-	-
Buffy coat						
IL-6	24.1	24.7	0.69	0.27	0.69	0.81
IL-10	23.6	24.8	0.95	0.15	0.57	0.35

[1] CON = control, BEO = 1 g/calf/d blend of essential oil; [2]T = treatment effect; W= week effect, T x W = treatment by week interactions.

4.5.DISCUSSION

The research and use of alternatives to replace artificial additives have increased widely, especially after the antimicrobials as growth promoters have been a concern for animal production and public health [2,38,39]. The EO exhibit broad-spectrum antimicrobial properties, improving growth and health status in various species [40–42]. However, there is still a small amount of data evaluating EO for young dairy calves and its impact on gut development and immune function. Thus, the present study aimed to take the essential oils research to a further step and quantify the impact of EO supplementation on organ development, gut, and immune function and response. Since the number of experimental units on this paper is small, our previous research quantifies the supplementation of an EO over intake, performance, and health scores [24]. For this reason, those outcomes should be used more to describe the obtained results for viscera development and immunity.

The intake and performance showed the same results obtained with de females in our previous work [24], with no differences within treatments. As expected, intake and growth would be impacted with age, i.e., the week. The MR amount given was fixed, but the starter intake increased over the

week's impacted the other outcomes, which explains the week effect. The main goal of calves rearing is to double the weight at weaning, which was achieved in this trial. However, intake and performance results for EO supplemented animals are controversial, and there is a lack of works that supplements natural additives through a liquid diet. Therefore, most of the results for EO supplementation for dairy calves in the literature were obtained by supplementing it though starter [43,44] found beneficial results for ADG and feed efficiency. When comparing the supplementation on liquid and solid diets, no differences were found for performance and intake [21]. However, when different inclusions of EO in MR were tested, animals from the lowest inclusion, 0.5 g/day of BEO, had greater ADG, final BW, body length growth, withers, and hip height gain, and hip-width when compared to control, 1.0 g/day and 1.5 g/day EO inclusion groups. This data also show higher DMI for the 0.5 g/day group when compared to the others, but no differences in feed efficiency [22]. Therefore, the optimum dosage in previous data showed that the lowest EO supplementation had the best calf performance improvement [22]. On our trial, we worked with a 1.0 g/day dosage divided into two meals, by manufacturer recommendation. Thus, the dosage given could be out of the optimum range.

The interaction found for MR intake on treatment and week, where different values were observed on weeks 1 to 3, was also observed in our previous work [24]. Weeks 1 to 3 were the same weeks where diarrhea incidence was high, demonstrating an influence on disease incidence over intake. Other authors reported high health scores on the second and third weeks of age, and lower feed efficiency [22]. Differences in week DMI also explain the week effect over ADG ($P \leq 0.001$), and feed efficiency ($P = 0.004$), where lower values for those variables were observed on weeks 2 and 3. The mean ADG of the calves in this trial of those weeks was 0.05 ± 0.665 kg and 0.36 ± 0.339 kg, respectively. When compared with the other weeks, were the values ranged from 0.59 kg to 0.86 kg, we can observe the disease impact on weight gain. However, besides no differences on ADG between treatments, BEO calves had a higher gain, or lost less, during weeks with higher health scores with a ADG of 0.09 ± 0.79 kg and 0.42 ± 0.36 kg versus 0.04 ± 0.59 kg and 0.31 ± 0.30 kg of the CON calves. It was also observed that BEO animals had a better intestinal development. Therefore, this could be evidence that, over challenge, BEO animals could recover, or lose less, when compared to CON.

When evaluating the GIT, differences found for ruminal and cecum parameters, where BEO animals presented lower pH values and higher butyric acid values, could be correlated with microbiota alteration and changes in ingestion behavior. Changes in behavior could explain higher C2:C3

proportions for BEO animals on weeks 3 and 5 and the ruminal and cecum parameters. Jersey pre-weaned calves treated with green tea extract or oregano extract anticipate the onset of rumination in one week compared to the control group [45]. Additionally, on week 3, the BEO animals had lower fecal and respiratory scores. Besides no statistical difference, a biological difference of these values and microbiota composition, and dehydration due to water loss, could impact feed intake behavior and physiological parameter. The BEO animals had numerically, a higher water intake when compared to CON calves. Therefore, this higher water ingestion could have impacted less dehydration and intestinal tract parameters and explain better ADG values in the weeks with high diarrhea incidence. Our work did not find any statistical differences in production outcomes such as intake, efficiency, metabolism, and growth.

Previous work showed that EO supplemented calves contained more beneficial microorganisms in the intestinal flora [21]. However, it is known that the concentrations of VFA in different parts of the gastrointestinal tract are related to the direct function of the local microbiota. In the ruminant, the peak concentration of VFA happens in the rumen, and the second-highest concentration is found in the cecum, where further digestion of the fiber occurs [46]. Thus, differences in cecum butyrate could indicate a difference in local microbiota. Butyrate is produced by the local microbiota and serves as the primary energetic nutrient to colonocytes. It regulates the multiple functions of gut cells, including its gene expression, cellular differentiation, tissue development, immune modulation, oxidative stress reduction, and diarrhea control. Studies on other species have shown that EO supplementation increased gastrointestinal symbiotic bacteria, known as butyrate producers [47,48]. Therefore, higher content of this AGV could enhance performance, gut development, and modulation of the immune response [49]. Butyrate induces a good response and inhibits inflammatory responses, inducing the naïve T cells to convert to Treg cells, blocking inflammatory cells, and producing *IL-10*, which will turn on secretory IgA production and other antibacterial peptides, helping the GIT defense mechanism [50]. It is also theorized that the lower gut can communicate with the forestomaches, which means that nutrients in the lower gut can cause subsequent adaptations in the forestomaches [51]. This theory could explain why the EO has given in the MR in our trial impact over ruminal outcomes. Technically, since EO was given through MR, its ingestion would pass the esophageal groove and go to the omasum and abomasum. Therefore, it was expected that the EO should impact only gut development; that is why the liquid diet was chosen.

It is also important to mention that the gastrointestinal tract senses the nutrient supply during the first weeks of life and communicates with other organs that contribute to digestion, such as the liver and pancreas [51]. This manipulation will also be important for the animal's future performance on the heard. The gut, especially the ileum, has lymphoid nodules, also called Peyer's patches, that have an important role as an "immune sensor," helping to promote epithelial repair and activating inflammatory sensors, regulation homeostasis, and the presence of innate immunity immune cells in the gut [52]. This connection also explains the integration of gut health and its immune cells and their migration to other body sites, as occurs to the mammary gland through an entero-mammary pathway [53].

Therefore, with theses scientific evidence, it is safe to say that the gut plays an important role in the immune system, microbiota, and disease behavior. It stimulates the immune function and the development of a mucosal layer, facilitating nutrient absorption and microbial activity cross-talk [54]. Alongside with chemical differences in GIT, in our work, the BEO animals had heavier pancreas and intestines, and higher ileum villus height, indicating that supplemented animals could have a better GIT function and nutrient digestibility. A bigger pancreas indicates increased activity in this organ with a higher production of enzymes and a more active metabolism [55]. The dietary effect on pancreas development and function [56], and the microbiota impact on this organ function [57]. Increased secretion of pancreatic enzymes implicates intestine adaptation to use it and corroborates with differences found in cecum butyrate. Additionally, knowing that a heavier intestine could be due to water content or cell proliferation, the histological differences found on the ileum indicate that the heavier intestines were due to higher cell content, impacting a more absorptive tissue. Thus, BEO animals had a dietary effect of the BEO, impacting positively on pancreas size and intestinal development and metabolism. Therefore, differences on nutrient digestibility were expected, but not find.

The digestibility numbers found on this trial were within the normal range preciously reported [44,58,59]. Contrary to what we expected, both groups had similar digestibility. It was previously described that different inclusions rates of oregano EO tested on *in vitro* digestibility showed that high inclusions could be detrimental for digestibility and ruminal parameters. However, median inclusions could beneficially modify ruminal parameters, local microbiota and increase nutrient digestibility [60]. When tested in lactation cows, supplementation of oregano leaves did not change ruminal parameters nor apparent nutrient digestibility but decreased DMI and increased feed efficiency [61]. For young

calves, including a combination of EO and prebiotics in a pelleted calf starter increased total tract digestibility for DM, CP, ADF, NDF, starch, and minerals [44]. Also, when EO was supplemented with monensin in the starter, EO demonstrated a greater impact on total nutrient digestibility, with a synergic effect when supplemented with monensin [58]. The lack of differences could be due to the supplementation route, the dosage, other additives and interactions between them, or because animals were not nutritionally challenged. It is also important to remember that when digestibility was proceeded in this trial – at the 8th week, calves had a more developed rumen, ate more starter, thus nutrient intake proportion was higher from the starter. As shown before, there is an effect on starter form and carbohydrate source on total digestibility tract [59]. The EO in this trial was supplemented via a liquid diet, thus a feed with higher digestibility and passage rate. Besides, no beneficial or detrimental effects over digestibility were found on our trial, to understand better the EO supplementation on GIT development and its impact on nutrient digestion and absorption, digestibility trials should be done around health events, as well animals should be nutritionally challenged.

However, EO supplementation is not only important for nutrient absorption and GIT development. The use of additives that help control and maintain normal gut microbiota would consequently help the calf's development. Previous studies in humans have shown that EO can regulate factors involved in the inflammation pathway, such as tumor necrosis factors (TNF) and *IL-6*, and help treat inflammatory diseases [62]. The *IL-6* is a mediator that contributes to body defense and is produced in response to infections and tissue damage. It stimulates the acute inflammation phase and helps hematopoiesis and immune function, promoting differentiation and proliferation of many non-immune cells [63], which is considered a good response, helping achieve GIT mucosa homeostasis [50]. On the other hand, when the gut microbiome is disrupted due to stress (weaning, transport, and disease), changes in feed intake, dehydration, or use of oral antimicrobials, a dysbiosis occurs, leading to an increase of inflammatory proteins such as *IL-6* and TNF [6,10]. When this dysbiosis is more extensive leading, it leads to severe inflammation and a condition called "leaky gut", where the tight junctions of the enterocytes are not functioning well, leading to a leak of intestinal bacterial to the bloodstream, causing the liver to switch to a metabolic organ to an immune organ, causing a decrease of the animal's growth and performance [7,50].

Thus, developing an adaptive immune system is coordinated by gut microbiota and is important for disease resistance [9,10]. However, animals under chronic or intense stress tend to have differences in immune cell counts with higher eosinophils and lower levels of leukocytes and *IL-6*.

We did find differences in this trial for leukocytes counts. However, our previous work with a large number of calves, observed that the supplemented animals had a higher leukocyte count [24]. This corroborates with other finds where animals submitted to stress had lower leukocyte count [64], evidencing the immunological effect of the EO supplementation. As for the eosinophils, they are granulocyte cells with the same phagocytic and metabolic functions of a neutrophil, but with an important role in killing parasites and dealing with certain types of allergies [65]. The lower eosinophils for the BEO animals that continued through the weeks could also add more evidence of a positive immunological effect of the EO supplementation. It is also important to mention that eosinophils are responsible for local defense, are present in GIT and respiratory tissues, and play an important role in biological functions and maintenance of homeostasis [66]. Observing our results, we see that EO supplementation had a positive impact not only on GIT development but also on the respiratory tract. Previous work has shown a positive impact on plants' secondary components over eosinophil-mediated inflammation [67]. As for the neutrophils, they were withing specie and age normality [68,69], and the evaluation timing wasn't compatible with an acute inflammatory response for neonatal disease occurrence. Therefore, differences in the treatment and week interaction were not biological significant.

Although all calves presented diarrhea and some had positive respiratory scores, especially in the first weeks of age, we did not check lung compromission with ultrasound. It is known that respiratory disease can be silent and misdiagnosed, and ultrasound evaluation could help diagnose it [70]. Thus, some respiratory diseases could have been misdiagnosed in this trial since, during euthanasia, animals from the CON group presented a heavier lung. The consolidation areas were not evaluated and measured during organ evaluation; however, this could be a vestige of previous respiratory disease and replacement of epithelium for connective tissue, thus a heavier and more dense tissue. This difference could correlate to the eosinophil's role in the respiratory tract regulating fibrin accumulation, healing, and remodeling of the organ [66]. Thus, the BEO animals could have better tissue healing, less oxidative stress, and less local inflammation [37]; therefore, lighter respiratory tract.

Additionally, due to eosinophils and organ differences between groups, it was expected to find differences in cytokines and inflammatory responses. It is known that pro-inflammatory cytokines (TNF and *IL-6*) and anti-inflammatory cytokines (*IL-10*) tend to increase with age [10]. However, besides a slight increase over time, no differences were seen in our buffy coat analysis for *IL-6* (*P* =

0.69) and *IL-10* (P = 0.57) between days 30 and 60 of age, and no differences were found between treatments. This could be because samples were collected when the calves were older and only four weeks apart.

As for ileum and colon *IL-6* and *Il-10* gene expression, although similar between treatments (Fig.1), we expected to find differences since there were histological differences in the ileum and higher butyrate content in cecum for BEO animals. We had the statistical power to test these variables, and there is evidence that gut microbiota interacts and influences RNA changes and expression [71]. However, as also cited for the buffy coat results, we might have collected the samples at the wrong time point to find gene expression differences. This can also explain why there were no differences in the proliferation and stimulus of splenocytes. The spleen is also a large immune organ with various immune cells such as lymphocytes and macrophages [32]. Thus, it was expected that the supplemented calves would have boosted and been more responsive to LPS, PMA, and *E. coli* extract induced innate immune response. However, since this response could be correlated with the capacity of *IL-6* secretion, *Il-6* is secreted during the acute phase, other interleukin associated with lymphoproliferation, no differences were found for this parameter [64], both results correlate with each other. The spleen cells in our trial were from 60-day old calves, healthy and not submitted to acute stress. New experiments must be done with varying concentrations of EO and time of sample collection to verify immunomodulation effects promoted by this supplementation. Health and pathological challenges should also be tested to detect significant differences in immunological responses, how the supplementation helps overcome neonatal diseases, the behavior of blood cells, and gene expression around disease time of calves supplemented with EO.

4.6.CONCLUSION

Feeding pre-weaned bull calves with an EO in the MR may be a promising alternative to improve the calf's gut development, especially the lower gut, as well as improve immunological cells response on health challenges during early life. This experimental database improves the results of feeding EO to young calves through a liquid diet. Future work should be done to understand better and evaluate the impact of feeding different EO, optimum dosage, way of providing it, and the impact over gut microbiota of young dairy calves.

4.7.REFERENCES

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5. CHAPTER V – CONCLUDING REMARKS

Nutraceuticals are widely used, especially in folk medicine, due to their anti-inflammatory, antioxidant, and antimicrobials action. Using such substances to improve animals' performance is desirable, especially with few side effects, and if it does not compete with traditional chemical therapeutics. The EO could be an option to modern production systems and could be used as an alternative to improve calf health and performance.

Supplementing a commercial blend of EO via MR to dairy calves caused ruminal manipulation and digestive tract alterations besides no differences in digestibility. Besides subtle, it also improved dairy calves' fecal scores and immune behavior. Considering that preweaning is one of the most important phases of heifer development and performance, EO has already been proved to be a good alternative to antimicrobials growth promoters for neonatal animals. Therefore, EO supplemented calves become more efficient in the rearing phase, improving their performance, accelerating the conversion of pre-ruminants to ruminants, and increasing efficiency and sustainability.

However, future research must be done to understand the mechanisms of action of EO, evaluate interaction with other chemical components, elucidate how it interacts with the host microbiota and how these influence the immunity and impacts over gut microbiota of young dairy calves. We suggest challenging trials to evaluate how the supplemented animals behave under nutritional or pathogenic challenges.