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Essential oils as rumen fermentation modifier for enteric methane mitigation in ruminants

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Effect of inclusion essential oils as manipulators of rumen fermentation for mitigation of enteric methane production in ruminants

Thesis submitted to the Postgraduate Program in Animal Nutrition and Production of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences

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CERTIFICADO

Certificamos que a proposta intitulada "Óleos essenciais como modificadores da fermentação ruminal para mitigação de metano entérico em ruminantes", protocolada sob o CEUA nº 8453300914 (ID 001375), sob a responsabilidade de **Paulo Henrique Mazza Rodrigues** *e equipe; Roberta Ferreira Carvalho; Eduardo C. O. Cassiano ; Diana Carolina Zapata Vasquez ; Flavio Perna Junior; Lizbeth Collazos Paucar ; Ricardo Galbiatti S. Nogueira ; Tainá Silvestre Moreira* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 17/06/2015.

We certify that the proposal "Essential oils as rumen fermentation modifier for enteric methane mitigation in ruminants", utilizing 8 Bovines (8 females), protocol number CEUA 8453300914 (ID 001375), under the responsibility of **Paulo Henrique Mazza Rodrigues** *and team; Roberta Ferreira Carvalho; Eduardo C. O. Cassiano ; Diana Carolina Zapata Vasquez ; Flavio Perna Junior; Lizbeth Collazos Paucar ; Ricardo Galbiatti S. Nogueira ; Tainá Silvestre Moreira* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 06/17/2015.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de jul/201 a nov/201 Área: Nutrição E Produção Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	a	N:	8
Linhagem:	Holandês e Nelore			Peso:	a		

Local do experimento:

São Paulo, 05 de outubro de 2018

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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RESUMO

CARVALHO, R.F. **Óleos essenciais como modificadores da fermentação ruminal para mitigação das emissões de metano entérico em ruminantes.** [Essential oils as rumen fermentation modifier for enteric methane mitigation in ruminants]. 2018. 95 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

A busca por produtos naturais capazes de aumentar a produtividade animal e diminuir o impacto ambiental tem intensificado. Alguns extratos vegetais, como óleos essenciais, são utilizados como aditivos alimentares capazes de melhorar a fermentação ruminal, através da modulação da produção de ácidos graxos de cadeia curta e inibição da metanogênese. O objetivo deste estudo foi avaliar a produção de metano entérico e das fezes, bem como comportamento alimentar, fermentação e cinética ruminal de bovinos leiteiros e de corte alimentados com óleos essenciais. Foram utilizadas oito vacas não gestantes e não lactantes, fistuladas no rúmen, sendo quatro vacas com aptidão para produção leiteira e quatro para carne. A dieta base foi composta por 70% de volumoso (silagem de milho) e 30% de concentrado (milho grão e farelo de soja), os tratamentos diferiram apenas quanto ao óleo essencial adicionado: CNT: dieta sem nenhum aditivo; OEE: 500 mg/kg de MS do óleo essencial de eucalipto citriodora (*Eucalyptus citriodora*); OEA: 500 mg/kg de MS do óleo essencial de aroeira vermelha (*Schinus terebinthifolius* Raddi) e OEC: 500 mg/kg de MS do óleo essencial de capim cidreira (*Cymbopogon citratus* Stapf). O delineamento experimental utilizado foi o quadrado latino 4x4 contemporâneo em arranjo fatorial 2x4 (referente a duas diferentes aptidões e quatro dietas). Para avaliar a produção de CH_4 dos dejetos utilizou-se biodigestores anaeróbios experimentais do tipo batelada, em delineamento inteiramente casualizado. Os bovinos de corte apresentaram menor ingestão de matéria seca (P = 0,0413) e levaram mais tempo para ruminar e mastigar 1 kg de MS ou FDN (min/kg). Eles também apresentaram maiores valores para produção de acetato, butirato e AGCC total. Os dejetos dos bovinos leiteiros tiveram maior produção de biogás e CO_2 que os dejetos dos bovinos de corte. Os animais que receberam os tratamentos com óleos essenciais tiveram em média 23% mais eventos de ruminação por dia que animais do tratamento controle (P = 0.0201). Dietas contendo óleos essenciais diminuíram a emissão de N_2O nas fezes de animais leiteiros. Os óleos essenciais na dosagem utilizada não alteraram a cinética e fermentação ruminal, bem como a emissão de metano entérico e das fezes.

Palavras-chave: Aditivos naturais. Metano. Fermentação ruminal. Biodigestão anaeróbia.

ABSTRACT

CARVALHO, R.F. **Essential oils as rumen fermentation modifier for enteric methane mitigation in ruminants.** [Óleos essenciais como modificadores da fermentação ruminal para mitigação de metano entérico em ruminantes]. 2018. 95 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2018.

The search for natural products which can increase animal productivity and reduce environmental impact has been intensified. Some plant extracts, such as essential oils, are used as feed additives able of improving ruminal fermentation by modulating the production of short chain fatty acids and inhibiting methanogenesis. The objective of this study was to evaluate the production of enteric and feces methane, as well as feeding behaviour, ruminal fermentation and kinetics of dairy and beef cattle fed with different essential oils. Eight non-pregnant, non-lactating and ruminally canullated cows were used: four were the dairy breed Holstein, and four were the beef breed Nellore. The diet was composed of 70% of roughage (corn silage) and 30% of concentrate (corn grain and soybean meal), the treatments differed only in relation to the essential oil added: CNT, a diet without any additive; EEO, a diet with 500 mg/kg of DM of eucalyptus (*Eucalyptus citriodora*) essential oil; PEO, a diet with 500 mg/kg of DM of Brazilian peppertree (*Schinus terebinthifolius Raddi*) essential oil, and LEO, a diet with 500 mg/kg of DM of lemongrass (*Cymbopogon citratus Stapf*) essential oil. The experimental design was the 4x4 contemporary latin square in a 2x4 factorial arrangement (referring to two specialized cattle breeds and four additives). The evaluation of the CH_4 production of the manure was performed through experimental batch anaerobic digesters, in a completely randomized design. The beef cattle had lower DMI ($P = 0.0413$), they spent more time consuming, ruminating or chewing 1 kg of DM or NDF (min/kg), also had higher values for acetate, butyrate or total SCFA production than dairy cattle. The manure from dairy cattle had higher biogas production (L/gVS add) or CO_2 (liters, percentage and L/gVS add) than the manure of beef cattle. The treatments with essential oils had on average 23% more rumination events per day than the control treatment ($P = 0.0201$). Diet containing essential oils decreased N_2O production of feces from dairy cattle. The essential oils in the dosage used did not affect rumen fermentation and kinetics, as well as the emission of enteric methane and feces.

Keywords: Natural additives. Methane. Ruminal fermentation. Anaerobic biodigestion.

SUMMARY

1 INTRODUCTION

World population increase has led to a sharp rise in demand for animal food and consumers are increasingly demanding for food quality, social responsibility, animal welfare and environmental sustainability. The future challenge for food production will be to reconcile the need for increased production, associated with more efficient distribution, with less waste, and growing concern with environmental preservation.

Brazil occupies a prominent position in livestock production. It is a major supplier of animal protein to the world population. Despite it's importance, Brazilian livestock farming has been criticized for emitting significant amounts of greenhouse gases (GHG). Criticism has been based on the low zootechnics indexes observed in animal husbandry systems based on degraded pastures or below their production potential (IPCC, 2007).

The concept that CH_4 is a byproduct of carbohydrate digestion is widely known. This gas can not be metabolized by the animal or the rumen microbiota. Therefore, most of it is removed from the rumen by expiration or eructation (MOSS, 2000) and released to the environment.

Nutritional techniques such as the use of ionophores, tannins, saponins, essential oils and lipids in addition to pasture management techniques and genetic improvement have been used to manipulate rumen fermentation and reduce the emission of enteric CH_4 gas (MOHAMMED et al., 2004; BERNDT, 2010). Although some dietary strategies have been proposed to decrease the CH_4 production from ruminants, few have shown persistent decrease, mainly *in vivo*.

Based on the precautionary principle, in 2006 the European Union banned antibiotics as animal growth promoters. Studies with products of natural origin have been intensified. Among the products studied, the essential oils showed promising effects on the ruminal fermentation. Compounds present in the essential oils present bactericidal action and the products resulting from fermentation processes are similar to the use of ionophores, despite little scientific research to date.

The objectives of this study were to evaluate the effects of the addition of different essential oils in the diet of cattle, under broader conditions of animal types, on intake and feeding behavior, ruminal kinetics, production of SCFA and enteric CH_4 emission, as well as to evaluate the impact of these feed additives on the production of greenhouse gases from animal manure.

2 LITERATURE REVIEW

2.1 RUMEN FERMENTATION

Rumen microbial population consists essentially of bacteria, *archaea*, protozoa and fungi. The synergism that exists between populations become possible the anaerobic fermentation of feeds, mainly of the fibrous type. Through the action of enzymatic complexes, which act on the cell wall of plants, low quality feeds are converted to high quality protein (VARGA; KOLVER, 1997).

For proper fermentation, some ruminal conditions are of great importance, such as: temperature between 38 and 41 ºC, humidity between 85 and 90%, osmolarity between 260 and 340 mOsm and pH between 5.5 and 7.2 (MARINO et al., 2009).

Feed nutriens, especially energy and protein sources, are fermented and processed to short chain fatty acids (SCFA), microbial mass and gases such as methane (CH_4), carbon dioxide (CO_2) and hydrogen (H_2) (BAKER, 1999). The SCFA can be considered as a fermentation residue for microorganisms. However, for the ruminant, they represent the main source of energy.

High fiber diets result in more alkaline pH and favors the growth of cellulolytic or fibrolitic bacteria. They are able to degrade the cellulose, resulting in higher acetic acid production, lower concentration of total SCFA and higher acetate: propionate ratio. Diets with a higher proportion of concentrate result in a more acidic pH. Values below 6 inhibit the growth of cellulolytic bacteria and favor the growth of amylolytics. Fermentation from amylolytic bacteria produces more propionate than cellulolytics (BERGMAN, 1990; NUSSIO et al., 2006).

The production of SCFA and the proportion in which each acid is produced depends of bacterial species (KOZLOSKI, 2002). The methanogenic microorganisms, mainly a*rchaeas*, eliminate excess of H_2 in the rumen (BAKER, 1999). They preferentially use the CO_2 reduction pathway to produce CH_4 (KOZLOSKI, 2002). According to the stoichiometric balance, the production of acetate and butyrate promotes higher production of CH_4 by the higher H_2 production.

2.2. METHANE PRODUCTION

Methane is a gas composed of carbon and hydrogen, considered the second gas with the greatest responsibility for the greenhouse effect, after CO_2 (PRIMAVESI et al., 2004). It can be formed through the anaerobic fermentation of organic matter in flooded environments, flooded rice fields, enteric fermentation, anaerobic treatment of animal waste and biomass burning.

Methanogenic *archaea* are responsible for the production of CH_4 in enteric fermentation, forming a distinct group of microorganisms, possessing co-factors (coenzyme M, F420, F430) and lipid (isoprenyl ethers glycerol) solids unique (McALLISTER et al., 1996).

In the rumen, *archaea* are found associated with ciliate protozoa and juxtaposed with bacteria. Methanogenic species have great affinity for synthesizing CH_4 from H_2 and CO_2 to generate their energy requirements for growth (MILLER, 1995). They also have the ability to synthesize CH_4 from the format and, to a lesser extent, from methanol, mono-, di- and tri-methylamine, as well as acetate, but the reduction of CO_2 is the preferred route. Anaerobic conversion of organic matter to CH_4 in the rumen involves a consortium of ruminal microorganisms, with the final step performed by the methanogens (McALLISTER et al., 1996).

CH_4 emission also occurs through feces fermentation in the environment, however, this emission is lower than the enteric production. CH_4 production by fecal degradation is variable, since it depends of management made with the manure. Higher emission rates are from feedlot manure than manure deposited directly in the pastures.

The CH_4 contributes about 15% of global warming, and is directly related to the efficiency of ruminal fermentation, causing loss of energy in production systems (COTTON; PIELKE, 1995). It can be responsible for 2% to 12% of the crude energy of the diet that is lost during the fermentation process (JOHNSSON; JOHNSSON, 1995).

2.3 METHANE MITIGATION STRATEGIES

The CH_4 produced by ruminants is one of the few sources that can be manipulated. It is derived from rumen fermentation, which is directly related to feed intake and digestibility

(RIVERA et al., 2010). Thus, it is possible to reduce the production of this gas by modifying the ruminal fermentation, which can be performed by change of composition, digestibility, feed intake, lipid addition and feed additives.

In general, compounds that cause decrease in CH_4 production result in reduced production of acetate and ammonia and increased propionate production (GARCIA-LOPEZ et al., 1996). The manipulation of ruminal fermentation through diet can be performed by balancing the diet with concentrates or by providing better quality pastures (better digestibility) and adding additives.

The most often used additives are antibiotic ionophores and have been very successful in manipulation of rumen fermentation. However, the use of antibiotics in animal feed is facing reduced social acceptance because of the appearance of residues and resistant strains of bacteria (NAGARAJA et al., 1997).

The essential oils are secondary plant compounds, also are indicated as modulatory additives of ruminal fermentation. The use of plant secondary metabolites as rumen modifiers seems to be a better approach since these are natural products that might be environment-friendly and have a better acceptance among consumers (AGARWAL et al., 2009). However, according to Morais et al. (2011), the studies developed with essential oils are still scarce and, therefore, the mechanisms of action on rumen fermentation and the consequences on animal metabolism and production are not well defined.

2.3.1 Use of essential oils

Essential oils are an option in the manipulation of ruminal fermentation. They are extracted from plants and have great potential for use in animal nutrition, since they have scientifically proven antimicrobial activity. Most of them also have GRAS (generally recognized as safe) status for human consumption (FOOD AND DRUG ADMINISTRATION, 2004). Thus, they are natural and safe alternative, with the advantage of lower risk of microbial resistance, because these compounds have, in most cases, several active principles with different modes of action (BROOKER, 2005).

They are not pure substances, they are blend of various volatile organic compounds that are present in different concentrations and vary among vegetal species. According to Cezarotto (2009), the antimicrobial properties of essential oils seem to be associated with high levels of monoterpene hydrocarbons, especially α-pinene. Antimicrobial activities of these compounds are probably due to the ability to destroy cellular integrity, inhibit respiration and the ionic transport process, as well as the ability to increase cell membrane permeability (SIQUEIRA et al., 1985; COX et al., 2000).

According to Calsamiglia et al. (2007) and Benchaar et al. (2008), essential oils are able to decrease ruminal fermentation through changes in the microbial populations of rumen, as confirmed in an experiment of molecular biology (FERME et al., 2004). Screening studies, such as Busquet et al. (2006) and Castillejos et al. (2007), demonstrated that some essential oils were effective in altering rumen fermentation *in vitro*, while others did not show effect. *In vitro* studies show, for the most part, redution or any effect of the essential oils on the total concentration of SCFA. Similarly, dose-dependent effects are clear, since high inclusions usually inhibit ruminal fermentation *in vitro*, evidenced by the decrease in SCFA concentration or substrate degradation. Castillejos et al. (2005) found that 1.5 mg/L of blend of essential oils increased the total concentration of SCFA in continuous flow *in vitro* system, although any effect on organic matter (OM) digestibility was observed. Controversial results were also obtained by Benchaar et al. (2007), these authors observed a tendency of increase SCFA concentration in diets with alfalfa silage, but reduction when fed corn silage, in diets for lactating cows receiving 750 mg/d of blend of essential oils. Effects absence on the total concentration of SCFA can be positive, when changes occur in the SCFA molar ratio (lower ratio acetate: propionate) or by decreases in CH_4 production and ammonia (NH_3) concentration.

Brazil has a wide variety of plants and great cultural use, which present studies on its antimicrobial properties with great potential for use in animal diets, such as Brazilian peppertree (*Schinus terebinthifolius Raddi* - Anacardiaceae), lemongrass (*Cymbopogon citratus Stapf* - Poaceae) and eucalyptus citriodora (*Eucalyptus citriodora*) (SALLAM et al., 2009; ARAUJO, 2010).

2.3.1.1 Brazilian peppertree essential oil

Schinus terebinthifolius is popularly known by many names, as Brazilian peppertree, red aroeira, aroeira-mansa and pink pepper. The essential oil can be extracted from trunks, fruits and leaves. In the present experiment the essential oil was extracted from the fruits. Its main substances mentioned in the literature are: α-pinene, sabinene, β-pinene, α-felandren, Δ-3-carene, β-felandren, terpinen-4-ol, α-copaene, germacrene-D, bicyclogermacrene, β-caryophyllene, σ-cadiene and α-cadiene (BARBOSA et al., 2007; SANTOS et al., 2007).

The effect of Brazilian peppertree essential oil is little studied in the diet of ruminants. However, some studies have shown that its use in methane mitigation can be promising. Araujo (2010) observed a reduction in gas production at all levels (75 and 150 μL/75 mL of buffered ruminal fluid), reduction of methane and acetate at the highest levels (150 μL/75 mL of buffered ruminal fluid) in a study *in vitro*.

Sallam et al. (2011) evaluated the effect of Brazilian peppertree essential oil on the production of gases through the *in vitro* methodology. The authors observed decreased in gas production, organic or dry matter digestibility in higher doses and not any effect on methane production.

After some promising and controversial results of the action of Brazilian peppertree *in vitro*, further studies are expected to elucidate its efficiency *in vivo* on rumen fermentation manipulation.

2.3.1.2 Lemongrass essential oil

Cymbopogon citratus is a plant native from India, acclimatized in Brazil, popularly known as lemongrass and citrus grass. Some studies have demonstrated its efficiency as a bactericide, antispasmodic, analgesic, insecticide and fungi growth inhibitor (SARGENTI; LANÇAS, 1997). These characteristics are interesting for human industry and for animal feed. Extraction of its essential oil is well known, the citral is main constituent, besides limonene, citronellal, myrcene and geraniol (GUERRA et al., 2000).

Wanapat et al. (2013) evaluated the lemongrass effect, peppermint and pepper on ruminal fermentation in diet to beef cattle. The authors observed increase in propionate production and decrease in acetate, methane production and acetate : propionate ratio in relation to the control treatment. According to the authors, such results are due to the action of the essential oils on the bacterial population, the gGam-positive bacteria were more susceptible to the action of the essential oils than the Gram-negative bacteria.

Samal et al. (2016) observed decrease in methane emission when evaluated a essential oils blend containing 50% of lemongrass in a diet with high proportion of roughage (60:40). The authors observed reduction of 13.3% (L/kg DMI) in the emission of methane compared to the control treatment.

Araujo (2010) observed decrease in total gas production, total short chain fatty acids, acetate, propionate or methane, also observed increase in the acetate: propionate ratio. In this study the author used *in vitro* technique to evaluate effect of essential oils from Brazilian plants.

Kouazounde et al. (2015) studied the effects of essential oils *in vitro*, and observed decrease in methane production of 11.4%, 13.5% and 14.2% for *Eucalyptus citriodora, Ocimum basilicum* and *Cymbopogon citratus*, respectively. No change was found in the short chain fatty acids production.

2.3.1.3 Eucalyptus Citriodora

Eucalyptus citriodora is originally from Australia and is widely distributed in other countries. Essential oil is extracted from branches and leaves. In the present study the essential oil was extracted from the leaves. In the industry, oil is widely used in the manufacture of cleaning products, insect repellents, perfumery and have known bactericidal action (LEE; CHANG, 2000).

Pinski et al. (2016) did not observe effect of eucalyptus oil in dose 500mg/L of culture fluid on the production of methane *in vitro* conditions. Rumen contents used in this study were collected from cannulated Holstein dairy, fed diet containing 55% of roughage.

According to Tatsuoka et al. (2008) eucalyptus oil at the dose of 40mg reduces methane production by 40%, decreasing the protozoal number and increases total VFA and proportion of propionic acid *in vitro* conditions. Rumen fluid in this study was obtained from dairy cattle.

Cobellis et al. (2016) studied the effect of different essential in dose of 1.125 ml/L of culture using *in vitro* methodology. Rumen content were collected from lactating Jersey cow fed with high roughage. The authors observed that eucalyptus essential oil decreased by 6% the total gas production and 18% methane production.

Sallan et al. (2009) evaluated different levels (25, 50, 100 and 150 μl) of eucalyptus essential oil on ruminal fermentation and methane production *in vitro*. The authors observed decrease in the production of methane and ammoniacal nitrogen according to the increase of the dose of the oil, without affecting the degradation of organic matter.

From the controversial results from literature, new studies are expected to define the efficacy of the use of essential oils in fermentation and ruminal kinetics.

2.4 ANAEROBIC DIGESTION OF MANURE AND GREENHOUSE GAS EMISSIONS

Anaerobic digestion is a biological process performed by several microorganisms, in the absence of oxygen, where complex organic compounds are transformed into simpler products such as methane and carbon dioxide (TOERIEN et al., 1969). The process of converting organic matter into methane is broken down into 3 main steps. The first step is the acidogenesis, that converts complex organic matter into intermediary products, primarily SCFA such as acetate, propionate, and butyrate. Acidogenesis is completed by fermentative bacteria (FRANCO et al., 2007). After acidogenesis, acetogenic bacteria convert SCFA with more than 2 carbons to acetate, carbon dioxide and hydrogen gas, this step is known by acetogenesis. Finally, in the last step methanogenic bacteria convert the acetate to methane.

As all biological processes, anaerobic biodigestion depends of several factors, among them can be cited: temperature, pH, inoculum use, solids content and the material composition (MASSÉ et al., 2008; SOUZA et al., 2005). Among all these factors, the material composition directly influences the potential for substrate degradation. Thus, the extension of biogas production from

the manure depends of animals feeding. The potential for biogas production from manure ruminants may vary according to the nutritional quality of the feed supplied to the animals.

CH_4 formation by grazing cattle feces is very low and its quantification is often neglected (JARVIS et al., 1995). However, when stocked, feces may represent 7 to 27% of the total CH_4 emission from ruminants (KULLING et al., 2002; HINDRICHSEN et al., 2006). Therefore, it is globally seen as an important source of CH_4 emission.

In most dietary strategies used to mitigate enteric methane, it is still unclear whether manure-derived methane facilitates mitigation, is neutral or compensates for achievements made in the digestive tract of ruminants. The last scenario would question the usefulness of implementing dietary measures against methanogenesis, unless manure is used for biogas production, where this would even be favorable (KREUZER; HINDRICHSEN, 2006).

Monensin, for example, is widely used in diets for beef cattle, up to 40% of the amount applied gets into feces in an unchanged form (DONOHO et al., 1976). The lasalocid concentration may amount to up to 6ppm in the excrement of factory poultry, the exact amount depending on age, while 20% of the activity of virginiamycin, used in piggeries, is found in liquid manure even after several days of storage (COCITO, 1979).

The addition of lauric acid, which has a known negative effect on methanogenesis, to dairy cows diet had small decrease of enteric methane (KÜLLING et al. 2002). However, methane derived from manure had an increase of approximately nine times. The lauric acid reduced fiber digestibility in the rumen, this resulted in more fermentable fiber in the manure. The use of oat hull concentrate, rich in highly lignified fiber, showed reduction both enteric methane and manure-derived methane (HINDRICHSEN et al., 2005).

Anaerobic biodigestion is an effective technology for the treatment of manure, due to its ability to degrade organic matter with a high level of moisture, carbohydrates, lipids and proteins. In addition, anaerobic digestion can recover the energy content of feces in methane form (ZHANG et al., 2014), avoiding their emission to the environment.

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3 EFFECT OF ESSENTIAL OILS IN DIETS DAIRY AND BEEF CATLLE ON INTAKE, RUMINAL KINETICS AND NITROGEN METABOLISM

Abstract: The use of essential oils to modulate ruminal fermentation has been increasingly studied. Also, it is important to know how they affect the digestion and utilization of nutrients. This knowledge is fundamental to improve diets formulations, and consequently, animal productive performance. The objective of this study was to evaluate the degradability and digestibility of nutrients and also the microbial nitrogen production in specialized dairy breed and specialized beef breed cows fed diets with essential oils. Eight non-pregnant and non-lactating and ruminally cannulated cows were used; four were identified as having specialized dairy and four specialized beef. The diets offered differed only with regard to the essential oil added to each treatment: CNT: contained a diet without any additive; EEO: contained 500 mg/kg of DM of eucalyptus essential oil (*Eucalyptus citriodora*); PEO: contained 500 mg/kg DM Brazilian peppertree essential oil (*Schinus terebinthifolius Raddi - Anacardiaceae*), and LEO: contained 500 mg/kg DM of lemongrass essential oil (*Cymbopogon citratus Stapf - Poaceae*). The experimental design was the 4x4 contemporary latin square in a 2x4 factorial arrangement (referring to two specialized cattle breeds and four additives). The dairy cattle had higher dry matter (P = 0.049) and nutrient intake (P = 0.018), expressed in g/kg MW. The essential oils used did not affect the consumption or digestion of nutrients, nor did they affect the production of microbial protein in either the dairy or and beef animals.

Keywords: Rumen, natural feed additive, ruminal digestion, microbial protein.

3.1 INTRODUCTION

In the search for natural products, ruminal fermentation modulators that optimize the use of nutrients by ruminants have intensified. Research on ruminal kinetics identifies how feeds and feed additives interact with the timing of degradability and digestibility of the diet proceeds as it passes through the gastrointestinal tract. This knowledge is very important for formulating efficient

feeding programs that promote improvements in the performance of ruminants (LASCANO & QUIROZ, 1990).

Among the products researched, essential oils have great potential. They have known antimicrobial action (PASQUA et al., 2007), which may be beneficial to the modulation of ruminal fermentation and in the use of nutrients. They are also a natural product (BUSQUET et al., 2006; CASTILLEJOS et al., 2007). According to Hart et al. (2008), the main effects on rumen are a reduction of protein and starch degradation due to selective action on certain rumen microorganisms.

Molero et al. (2004), found that blends of essential oils reduced the degradation rate of sunflower meal, but not of soybean or fish meals. The structural complexity and diversity of plants is a limiting factor for research progress in this area of knowledge (TEDESCHI et al., 2011).

Most of the data found in the literature about essential oils for ruminants came from *in vitro* studies (MCINTOSH et al., 2003; NEWBOLD et al., 2004; CASTILLEJOS et al., 2007). Nevertheless, some studies *in vivo* were performed, and the results show the potential of the essential oils on modulating ruminal fermentation, which improved nutrient utilization and the performance of dairy cows (BENCHAAR et al., 2007; BENCHAAR et al., 2008).

This study was developed with the hypothesis that essential oils are able to optimize the nutrient utilization in bovines. The objectives of this study were to evaluate the intake and digestion of nutrients, ruminal dynamics and to estimate the microbial nitrogen production in two types of cattle fed different essential oils.

3.2 MATERIAL AND METHODS

3.2.1 Study location and ethical issue

The study was conducted in the Ruminant Nutrition Laboratory from School of Veterinary Medicine and Animal Sience of the University of São Paulo, on *campus* Fernando Costa in Pirassununga, Brazil. The experiment was approved by and complied with the guidelines set out by the Ethics Committee in the Use of Animals of the School of Veterinary Medicine and Animal

Sience, under application number nº 8453300914, in respect to animal experimentation and care of animals used for scientific purposes.

3.2.2 Animal, housing and feeding

Eight cows - ruminally cannulated, not pregnant and non-lactating - were used. Four had specialized dairy breed and four had specialized beef breed. The dairy animals were from the Holstein breed (*Bos taurus taurus*) and had an average weight of de 905±62 kg. The beef animals were from the Nellore breed (*Bos taurus indicus*) and had an average weight of 535±54 kg. It was not the main objective of this study to compare cattle breeds. Instead of this, different cattle breeds were used to study the essential oils effect in broader conditions of animal type. Moreover, these two racial groups represent the prevalent systems of dairy and beef productions.

The cows were housed in individual stalls that had sand beds, individual feed bunkers and drinkers and fans that were automatically turned on at warm hours of the day. Animals were fed twice daily (08h00 and 16h00). Feed was weighed daily and offered to each animal after removing of previous day orts. The amount of essential oil was weighed daily and mixed into the feed concentrate only moments before the diet was offered as a dosage of 500mg/kg DM per animal per day. Orts were recorded once daily, before feeding, and the feeding rate was adjusted to yield orts on the basis of at least 5% of the amount supplied (on an as-fed basis). The animals were weighed individually on the initial and final day of each experimental period.

3.2.3 Experimental design and treatments

Animals were arranged in two contemporary 4 x 4 Latin Square designs with 2x4 factorial arrangements (referring to two specialized cattle breeds and four additives). The diets offered (Table 1) differed only in regard to the type of essential oil added. These were: CNT without any additive, the EEO containing 500 mg/kg of DM of eucalyptus essential oil (*Eucalyptus citriodora,* extracted from the leaves), PEO containing 500 mg/kg of DM of Brazilian peppertree essential oil

(*Schinus terebinthifolius Raddi*, extracted from the fruits) and LEO containing 500 mg/kg of DM of lemongrass essential oil (*Cymbopogon citratus Stapf*, extracted from the leaves).

Table 1. Ingredient proportion and chemical composition of experimental basal diet

Ingredientes (%DM)	Quantity	Corn silage	Concentrate
Corn silage	70.00	-	-
Ground corn grain	14.60	-	-
Soybean meal	12.90	-	-
Essential Oils*			
White salt	0.50	-	-
Mineral[1]	2.00	-	-
Chemical composition			
DM	46.82	28.52	89.55
CP	14.02	8.58	26.72
NDF	35.10	50.60	12.31
ADF	22.74	29.14	7.81
NFC[2]	43.33	33.75	49.16
Ash	6.48	4.85	10.28
EE	2.02	2.22	1.53
TDN[3]	62.90	59.2	71.2
NE_l[3] (Mcal/dia)	1.41	1.28	1.70
Ca	0.51	0.24	1.16
P	0.33	0.15	0.74

*Amount of essential oils added for each treatment was 500 mg/kg of DM

[1]Mineral supplement, quantity per kg of product: 120 g of calcium, 60 g of phosphorus, 12 g of sulfur, 163 g of sodium, 210 mg of cobalt, 1600 mg of copper, 180 mg of iodine, 1,200 mg of manganese, 27 mg of selenium, 5000 mg of zinc.

[2] NFC (non-fiber carbohydrate) = 100 − (% NDF + % CP + % EE + % Ash).

[3]Value estimated by the Spartan Dairy Ration Evaluator/Balancer software, version 3.0.3.

3.2.4 Sampling schedule

The experiment lasted 112 days and was divided into 4 experimental periods of 28 days each. The first 16 days were used for diet adaptation. The ruminal degradability was evaluated from the 17th to the 21st day. Dry matter intake was measured from the 22nd to the 26th day. Fecal collection for digestibility evaluation was performed from the 20th to the 24th day. The 24th day was used for urine collection to evaluate the production of microbial protein. Ruminal emptying to measure the disappearance rate occurred on the 27th and 28th day.

3.2.5 Chemical characterization of essential oils

The characterization of chemical composition of the essential oils (Table 2) was performed in the Multi Users Laboratory of Biochemistry and Instrumental Analysis of the Department of Agroindustry, Food and Nutrition of ESALQ/USP. Analysis was performed through gas chromatography coupled to mass spectrometry GC/MS QP2010 Plus (Shimadzu, Kyoto, Japan) with a diphenyl dimethyl polysiloxane (5% diphenyl and 95% dimethyl polysilozane) capillary column with 30 mx 0.25 mm, 0.25 μm, Rtx®-5MS model (Resteck, Bellefonte, USA). Volatile compounds were identified by comparing Kovats indices (KI) calculated and observed in the literature library (Wiley Library Version 8) and among their mass spectra. The largest total area peaks of the chromatogram were identified.

Table 2 - Main components of the essential oils of Brazilian peppertree, lemongrass and eucalyptus

Compound	Relative Percentage		
	PEO	LEO	EEO
Δ - carene	24.85	-	-
Limoneno	20.42	-	-
α pinene	12.00	-	-
α Phellandrene	16.84	-	-
p-Cymene	5.71	-	-
Geranial or citral A	-	37.67	-
Neral or citral B	-	30.54	-
Geraniol	-	8.77	-
Geranyl acetate	-	6.37	-
β- Caryophyllene	-	2.59	-
Citronellal	-	-	60.97
Citronellol	-	-	13.97
Isopulegol	-	-	9.42
Citronellyl acetate	-	-	3.09
β- Caryophyllene	-	-	1.47
Others	18.18	14.06	11.08

PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil

3.2.6 Dry matter intake

Dry matter intake for each period was determined between days 22 and 26, and the differences in the weights between feeds offered and refused by animal were measured.

During the feed intake determination, feed ingredient samples were collected and stored at −20 °C. Individual feed ingredients were composited in representative samples on an equal-weight basis. A pool of samples was dried at 65°C (forced-air oven) for 48 h and ground to pass a 1-mm Wiley mill screen and analyzed for DM, CP, EE, NDF, ADF, lignin and gross energy.

3.2.7 Total apparent digestibility and excretion of dry matter and nutrients

Total apparent digestibility of nutrients was determined using titanium dioxide. During 15th to 24th days, 15 g/head/day of indigestible marker was placed in the rumen twice daily (08h00 and 16h00 before feeding), via rumen fistula. The first 5 days were used to ensure a stable marker concentration. The last 5 days were used for marker administration and feces collection that was taken rectally twice per day, at 08h00 and 16h00, after feeding. A composite of samples was then analyzed for titanium dioxide concentration according to Pezatto et al. (2002). Nutrient digestibility (%) was calculated as:

$$CD_{(n)} = 100 - \left[100 \left(\frac{\%\ TiO_2d}{\%\ TiO_2f} \right) x \left(\frac{\%\ Nf}{\%\ Nd} \right) \right]$$

Where:

CD (n) = Apparent digestibility coefficient of the nutrient;

% TiO2d = % of titanium dioxide in diet;

% TiO2f = % of titanium dioxide in feces;

% Nd = % of nutrients in the diet;

% Nf = % of nutrients in feces.

The evaluations of DM and nutrient excretions, as well as the excretions of nitrogen (ExN), were calculated based on data from digestibility coefficient of DM and their fractions, then multiplying the consumption of nutrients by the respective coefficients of digestibility and dividing by 100.

3.2.8 Rumen dynamics

3.2.8.1 Disappearance rate

On the 27th and 28th day of each period, 3 hours after morning feeding and before morning feeding, respectively, the ruminal digest was manually removed from each cow through rumen cannula to determine the disappearance rate in the rumen (DADO & ALLEN, 1995). Ruminal content was separated manually through a screen into solid and liquid contents. These were weighed to determine total ruminal solid and liquid contents, and then they were sampled. Immediately after this, ruminal content was replaced in the rumen. Solid and liquid samples were dried at 65°C (forced-air oven) for 72 hours to determine the dry matter of each compound. The solid and liquid volume was adjusted by dry matter. The DM disappearance rate was considered equal to intake rate, and they were estimated using the formula (ROBINSON et al., 1987):

$$\text{DM disappearance rate (\%/h)} = 100 \times \frac{\text{Daily DMI (kg)}}{\text{DM Ruminal contents (kg)}} \Big/ 24\text{h}$$

3.2.8.2 Rumen degradability

The degradability of DM, CP and NDF was performed through the technique of the nylon bags according to ØRSKOV et al. (1980). To calculate the degradability of NDF, the model proposed by Mertens (1993) was used. The DM and CP degradability were calculated to according

to Ørskov & McDonald (1979). The determination of the non-degradable fraction (indigestibility %) was done according to Ørskov et al. (1980).

Incubations were performed from the 17th to the 21st day of each experimental period. The samples (5.5 g DM) were submitted to ruminal incubation for periods of 6h, 12h, 24h, 48h, 72h and 96h. The 0h incubation time was performed through 4 nylon bags incubated in a water bath at 39 ºC during 5 minutes.

After the incubation period, the bags were removed and washed in tap water until the water flowed clear. The bags were dried at 65°C for 72 h in a forced ventilation oven.

The rumen degradation curves of the DM and CP were adjusted to a non-linear regression, according to the equation proposed by ØRSKOV and McDONALD (1979):

$$P = a + b\ (1 - e^{-ct})$$

Where:

P = disappearance of the nutrient component analyzed;

a = degradation curve intercept when t = 0, which corresponds the water soluble fraction and completely degradable;

b = degradation potential of the water insoluble fraction of the nutrient component analyzed;

c = rate of degradation per fermentative action of b;

t = incubation time.

The rumen degradation curves of the NDF the model proposed by MERTENS (1993) was used:

$$Y = b^* \exp(-c^* t) + I$$

Where:

Y = time residue "t";

b = potentially degradable fraction;

c = rate of degradation;

t = equivalent to incubation times;

I = non-degradable fraction.

3.2.8.3 Passage rate

The passage rate was determined using the indirect method, which considered that the disappearance rate (Kt) is the sum of the passage rates (Kp) and digestion (Kd) (BOSH; BRUINING, 1995). It was therefore necessary to identify the disappearance rate through ruminal emptying and to identify the digestion rate in the evaluation of ruminal degradability *in situ*. Given this, the Kp was calculated as the difference between the Kt of the ruminal solid mass and the Kd of the potentially degradable fraction in the rumen using the following equation:

$$kp = kt\ (\%/h) - kd\ (\%/h)$$

Where:

kp = passage rate

kt = disappearance rate

kd = digestion rate

3.2.9 Estimation of microbial protein production and nitrogen balance

To calculate microbial protein production, the methodology described by Valadares et al. (1999) was used. Urine samples were obtained through spot collections, performed every 6 hours on the 24th day. The urine samples (spot sample) were filtered and aliquots of 10 mL were immediately diluted in 40 mL of sulfuric acid (0.036 N) to avoid bacterial destruction of the purine derivatives and the precipitation of uric acid or allantoin.

Uric acid was determined by enzymatic colorimetric reaction through uricase and peroxidase by using a commercial kit (Bioclin® Ref k139). The urea concentration was also determined by colorimetric enzymatic reaction, using a commercial kit (Bioclin® Ref k047). Allantoin tests were performed using a colorimetric method according to the technique of Chen and Gomes (1992). The daily creatinine excretion was estimated according to Chizzoti et al. (2004). Purine derivatives excretion in the urine during 24 hours was calculated according to VERBIC et al. (1990). The intestinal microbial nitrogen compounds flow was calculated according to Chen and Gomes (1992).

3.2.10 Statistical analysis

Data were statistically analyzed using the SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Before the actual analysis, the data were analyzed for the presence of disparate information ("outliers") and the normality of residuals (Shapiro-Wilk). The data for intake, digestibility and excretion of nutrients, disappearance rate, ruminal degradability, passage rate and microbial protein evaluation were analyzed and considered as causes of variation in the effect of treatments, period, animal inside square, as well as square effect. The variables were submitted to analysis of variance using the MIXED procedure. The model included the effect of essential oils, specialized cattle breeds (or square) and the interaction of these as fixed factors. Animal effects within square and period were considered random factors. For nutrients digestibility, analysis also used the co-variable intake of DM per kg of metabolic weight that was included in the model. In the presence of an essential oil effect, the comparison of means was performed by the Tukey test. Non-significant (NS) was considered when P value was higher than 10% and statistical significance when P value was less than 5%.

3.3 RESULTS

3.3.1 Intake, digestibility and excretion of nutrients

The essential oils did not affect dry matter intake, digestibility or excretion of nutrients ($P > 0.05$). Animals had an average of 88.5 g/kg of MW for dry matter intake (Table 3). The dairy cattle had higher dry matter intake in g/kg of MW ($P = 0.0413$) or nutrient intake ($P = 0.0018$), as well as higher values of non-fibrous carbohydrates digestibility ($P = 0.046$) in relation to beef cattle.

Table 3 - Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on the intake, excretion and digestibility of nutrients

Variables	Cattle		Essential oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Nutrients intake, g/kg of $BW^{0.75}$										
DM	94.66	82.02	89.16	88.35	89.70	86.15	1.85	0.0413	NS	NS
CP	13.35	11.67	12.60	12.74	12.61	12.10	0.25	0.0483	NS	NS
NDF	36.81	32.28	34.84	35.01	34.88	33.45	0.69	0.0506	NS	NS
ADF	23.85	20.92	22.57	22.69	22.60	21.67	0.46	0.0506	NS	NS
EE	1.90	1.67	1.80	1.81	1.80	1.73	0.04	0.0500	NS	NS
NFC	36.37	31.84	34.36	34.66	34.40	32.99	0.69	0.0491	NS	NS
OM	88.42	77.46	83.60	84.22	83.68	80.26	1.67	0.0496	NS	NS
Nutrients excretion g/kg of $BW^{0.75}$										
DM	18.70	19.51	18.58	18.65	19.78	19.42	0.29	NS	NS	NS
CP	2.51	2.84	2.62	2.67	2.71	2.69	0.06	0.0907	NS	NS
NDF	9.47	9.41	9.13	8.94	10.1	9.63	0.21	NS	NS	NS
ADF	6.92	7.03	6.85	6.77	7.21	7.08	0.15	NS	NS	NS
EE	0.30	0.34	0.36	0.32	0.31	0.30	0.03	NS	NS	NS
NFC	3.59	4.05	3.66	3.93	3.77	3.93	0.12	NS	NS	NS
OM	15.88	16.64	15.77	15.85	16.87	16.55	0.31	NS	NS	NS
N	0.38	0.43	0.42	0.34	0.43	0.43	0.02	NS	NS	NS
Nutrients digestibility, %										
DM	78.96	77.40	79.02	78.97	77.33	77.40	0.57	NS	NS	NS
CP	79.78	76.76	78.97	78.63	77.80	77.68	0.73	NS	NS	NS
NDF	72.29	72.34	73.56	70.22	74.04	71.45	0.84	NS	NS	NS
ADF	69.40	67.46	69.42	69.59	67.48	67.25	0.97	NS	NS	NS
EE	83.55	80.01	79.81	81.99	82.68	82.65	1.66	NS	NS	NS
NFC	89.94	87.38	89.25	88.55	88.95	87.89	0.42	0.0466	NS	NS
OM	80.88	79.40	80.95	80.87	79.32	79.40	0.57	NS	NS	NS

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = Standard error of the mean, C = specialized cattle breed effect, O = Essential oils effect , CxO = Interaction among cattle breeds and essential oils, NS= non-significant ($P > 0.10$).

3.3.2 Rumen kinetics

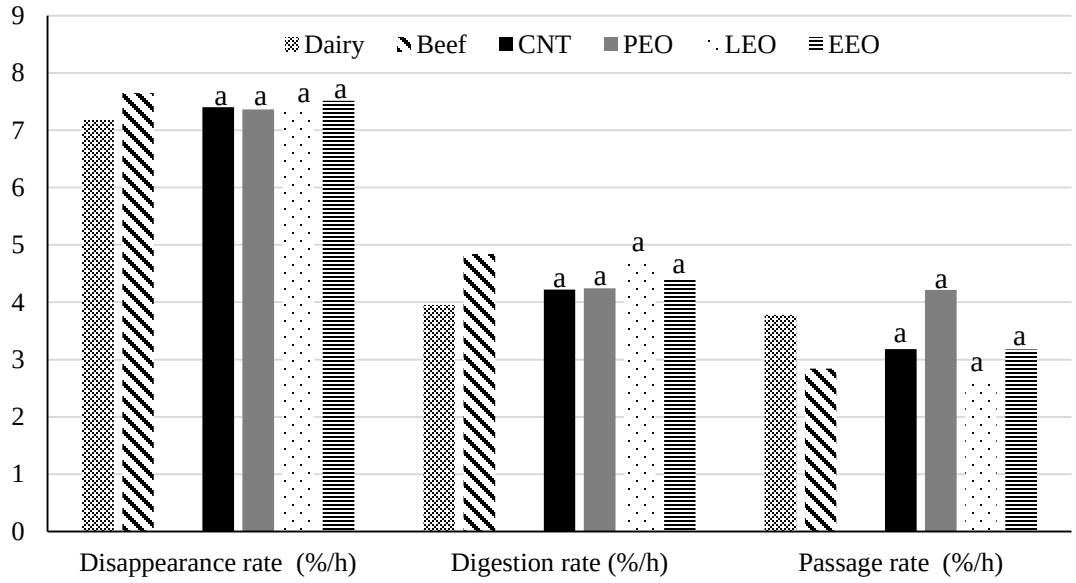
Neither the essential oils nor the different breeds influenced ($P > 0.05$) ruminal kinetics (Table 4, Figure 1). The average of disappearance and passage rates were, respectively, 7.42 and 3.33 %/hour. The real degradability for dry matter, crude protein and neutral detergent fiber was 60.63%, 57.30% and 30.64%, independent of treatment, respectively.

Table 4 – Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on ruminal kinetics

Variables	Cattle		Essential oils				SEM	p value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Ruminal degradability DM										
a, %	27.98	27.71	27.12	27.51	29.13	27.61	0.55	NS	NS	0.0933
b, %	54.95	54.97	56.23	56.29	52.48	54.84	0.85	NS	NS	NS
c, h	0.039	0.048	0.042	0.042	0.047	0.044	0.0021	NS	NS	NS
RD, %	58.07	63.19	60.72	58.71	62.61	60.49	1.84	NS	NS	NS
Ind., %	17.08	17.32	16.65	16.20	18.39	17.55	0.43	NS	NS	NS
Ruminal degradability CP										
a, %	10.47	12.42	11.36	12.47	10.35	11.58	0.57	NS	NS	NS
b, %	77.45	72.73	76.58	71.66	76.43	75.68	1.49	NS	NS	NS
c, h	0.042	0.055	0.043	0.055	0.049	0.047	0.0040	NS	NS	NS
RD, %	55.49	59.11	58.48	54.87	60.28	55.58	2.96	NS	NS	NS
Ind., %	12.08	14.85	12.06	15.87	13.21	12.73	1.39	NS	NS	NS
Ruminal degradability NDF										
b, %	67.96	69.21	69.67	70.027	65.25	69.38	0.94	NS	NS	0.0969
c, h	0.033	0.037	0.033	0.033	0.041	0.034	0.0022	NS	NS	NS
L, h	1.89	1.49	1.99	2.093	1.51	1.16	0.45	NS	NS	NS
RD, %	28.36	32.93	30.85	26.82	34.24	30.67	2.19	NS	NS	NS
Ind., %	32.05	30.79	30.33	29.97	34.75	30.62	0.94	NS	NS	0.0969
Disappearance rate of solid mass										
kg/h	0.68	0.39	0.53	0.56	0.53	0.52	0.03	0.0013	NS	NS
%/h	7.18	7.65	7.401	7.36	7.38	7.52	0.23	NS	NS	NS
Passage rate DM										
%/h	3.14	2.99	3.18	2.94	3.48	3.18	0.44	NS	NS	NS

D = dairy, B = Beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = Standard error of the mean, C = specialized cattle breed effect, O = Essential oils effect, CxO = Interaction among cattle breeds and essential oils, a = rapidly soluble fraction, b = potentially degradable fraction, c = rate of degradation per hour of the potentially degradable fraction, RD = real degradability, Ind = indigestibility, L = lag time, NS= non-significant ($P > 0.10$).

Figure 1 - Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on ruminal dynamics



CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil
Font: Carvalho, (2018).

3.3.3 Microbial protein production and nitrogen balance

The dairy animals had higher production of urine in liters per day (16.32 vs. 10.18 L/day), higher amounts of urea nitrogen (97.98 vs. 64.29 g/day), uric acid (4.37 vs. 2.78 g/day) or allantoin (23.78 vs. 13.83 g/day) in grams per day. When the variables were adjusted for metabolic body weight, the differences disappeared (Table 5). Nevertheless, the dairy animals showed higher nitrogen consumption (2.14 vs. 1.87 g/d/MW) and higher nitrogen retention (1.1 vs. 0.84 g/d/MW), even when the variables were adjusted for metabolic body weight (Table 6).

Table 5 - Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on urine, purine derivatives and microbial nitrogen production

Variables	Cattle		Essential oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Urine production										
L/day	16.32	10.18	13.18	12.36	13.87	13.59	0.89	0.0501	NS	NS
L/day/MW	0.099	0.091	0.095	0.0904	0.098	0.096	0.0045	NS	NS	NS
Urinary Nitrogen in Urine										
g/day	97.98	64.29	84.40	78.27	77.16	84.71	3.93	0.0084	NS	NS
g/kgMW/day	0.59	0.58	0.61	0.57	0.56	0.61	0.015	NS	NS	NS
Uric acid										
g/day	4.37	2.78	3.24	3.75	3.53	3.76	0.22	0.0203	NS	NS
g/kgMW/day	0.026	0.025	0.024	0.027	0.025	0.026	0.0011	NS	NS	NS
Allantoin										
g/day	23.78	13.83	20.62	18.17	19.37	17.06	1.60	0.0139	NS	NS
g/kgMW/day	0.14	0.11	0.14	0.12	0.13	0.11	0.0087	0.0825	NS	NS
Microbial nitrogen production										
g N/ay	96.5	52.3	82.42	71.85	77.36	66.09	8.21	0.0330	NS	NS
g N/d/MW	0.58	0.47	0.60	0.50	0.54	0.46	0.049	NS	NS	NS

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO eucalyptus essential oil, SEM = Standard error of the mean, C = specialized cattle breed effect, O = Essential oils effect, CxO = Interaction among cattle breeds and essential oils, NS= non-significant (P > 0.10).

Table 6 - Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on nitrogen balance

Variables	Cattle		Essential oils				SEM	p value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Nitrogen intake										
g/d	353.5	207.9	280.9	285.8	283.3	272.8	14.6	0.0018	NS	NS
g/kgPM/d	2.14	1.87	2.01	2.04	2.02	1.94	0.04	0.0483	NS	NS
Nitrogen Excretion in Feces										
g/d	66.4	50.4	57.3	58.6	59.0	58.7	1.98	0.0219	NS	NS
g/kgPM/d	0.40	0.45	0.42	0.43	0.43	0.43	0.01	0.0900	NS	NS
Nitrogen Excretion in Urine										
g/d	97.9	64.3	84.4	78.3	77.2	84.7	3.93	0.0084	NS	NS
g/kgPM/d	0.59	0.58	0.61	0.57	0.56	0.61	0.02	NS	NS	NS
Nitrogen Retention										
g/d	189.1	93.3	139.3	148.9	147.1	129.5	10.0	0.0015	NS	NS
g/kgPM/d	1.14	0.84	0.98	1.04	1.03	0.91	0.04	0.0087	NS	NS
% Cons	53.3	44.2	48.7	50.4	50.3	45.7	1.33	0.0047	NS	NS
% Abs	65.8	58.4	61.5	63.8	64.2	58.8	1.36	0.0165	NS	NS

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = Standard error of the mean, C = specialized cattle breed effect, O = Essential oils effect, CxO = Interaction among cattle breeds and essential oils, g = gram, kg = kilogram, MW = metabolic weight, d = day, % Cons. = Nitrogen retention in percentage of consumed, % Abs. = Nitrogen absorbed in percentage, NS= non-significant (P > 0.10).

3.4 DISCUSSION

3.4.1 Nutrient intake, excretion and digestibility

The essential oils did not change nutrient digestibility in the present study. However, according to Hart (2008), the essential oils alter the rumen microbiota, which consequently altered the digestion of degradable feed fractions, especially carbohydrates and protein. Beauchemin & McGinn (2006) observed a decrease in diet digestibility when using a blend of essential oils (1 g/day of the blend containing thymol, eugenol, limonene, vanillin) in beef cattle that consumed a diet rich in forage, as corroborated by Hart (2008). Nevertheless, Benchaar et al. (2007) did not report any change in digestibility in lactating dairy cows supplemented with a blend of essential oils at doses of 0.75 or 2 g/day. Another fact must also be considered: essential oils are volatile and can be lost to the gas phase in the rumen and expelled by belching, decreasing its effect on the ruminal microbiota (MISRA et al., 1996; MARÓSTICA JR. & PASTORE, 2007).

The dairy cattle presented greater nutrient intakes, which are directly related to the higher DM intake. Even when the variable was adjusted for metabolic body weight, the difference among the breeds remained. According to Owens et al. (1993), animals with different body compositions demonstrated intake directly proportional to protein mass and inversely proportional to fat deposition. Heavier animals generally have higher feed intake, especially when mature body weight and nutritional requirements are higher. Additionally, as pointed out by Smith & Baldwin (1973), the liver, heart, mammary glands and tissues of the gastrointestinal tract, among the body components, have higher metabolic activity. In dairy cattle, these tissues are larger in size, which would explain their greater maintenance energy requirements per metabolic unit and their higher consumption in relation to beef cattle.

The nutrient digestibility was not different among the cattle breeds, and the digestibility was directly related to the passage rate and the feed quality, which also did not differ among the animals. However, there was a difference in the non-fibrous carbohydrates, which were higher for dairy animals. According to Hegarty (2004), the beef cattle (*Bos indicus* - Nellore breed) have a faster digestion rate than those with dairy cattle (*Bos taurus* - Holstein breed), when fed with low

nitrogen diets. Hunter & Siebert (1985) evaluated the use of low quality forage with and without supplementation in beef cattle (Hereford, taurine and Brahman, zebu) and observed that digestion occurred more rapidly in zebu animals, although when the animals were supplemented with nitrogen, sulfur and minerals, the difference disappeared, as corroborated by Hegarty (2004). In the present study, the diets were not deficient in nitrogen, and thus allowed equal digestive conditions to the animals with both cattle breeds.

3.4.2 Ruminal kinetics

The addition of essential oils did not affect the ruminal kinetic variables. According to Hart (2008), the main effects of essential oils in the rumen are the reduction of protein and starch degradation and the inhibition of amino acid degradation. This is due to the selective action in certain rumen microorganisms, specifically some bacteria. Still, the effect of essential oils depends on chemical composition and dosage, which are not always sufficiently described in the literature. This causes different effects among the vast amount of existing essential oils. The absence of the effect of the essential oils in the present study may be due to the used types, contents of their components or the dosage used. Abdalla et al. (2009) evaluated the inclusion of eucalyptus essential oil levels on ruminal fermentation and degradability of dry and organic matter *in vitro*; the authors also found no effect of essential oil on dry matter degradability.

The passage rate is inversely related to digestion, thus, it was expected that the essential oils could change the passage rate by reducing nutrient digestions, especially starch or protein. The essential oils did not, however, influence the nutrient degradation; consequently there was no change in the rate of passage as expected. Metwally et al. (2016) also did not observe effects from the essential oils (thymol, limonene, guaiacol and eugenol) in a diet with a high proportion of roughage:concentrate (80:20) for dairy cows.

There was no difference between breeds for variables of ruminal kinetics. Differences were expected between animals of different breeds in the passage rate because it is negatively related to dry matter intake, which was higher for dairy cattle. Nevertheless, there was compensation by the beef cattle, which spent more time chewing (Table 7), consequently reducing the particle size of the feed, and also providing a lower rate of passage.

3.4.3 Microbial protein production and nitrogen balance

According to Patra (2011), some essential oils have the ability to inhibit hyper-ammonia producing bacteria, favoring the production of microbial protein. However, in the present study no differences were observed in the purine derivatives, and there was no increase in the microbial protein production for the treatments with the essential oils. Although essential oils inhibit the growth of some hyper-ammonia producing bacteria, other groups are less sensitive (McINTOSH et al., 2003). Its action may be related to diet, because in low protein diets a reduction of hyper-ammonia producing bacteria occurs, but in protein supplemented diets there is no difference. This was corroborated with the present study, where diets were not deficient in protein.

Castillejos et al. (2005) observed that the addition of 1.5 mg/L of a blend of essential oils did not change the rumen ammonia concentration, flow of bacterial N, crude protein degradation or efficiency of microbial protein synthesis. Calsamiglia et al. (2007) observed similar results on nitrogen metabolism, when using the same blend at higher concentrations (5, 50 and 500 mg/L) in an *in vitro* study.

Nitrogenous balance evaluation is very important, because certain conditions and feed additives may interfere with protein metabolism and result in a loss or gain of nitrogen. Protein is the most expensive diet nutrient (Paulino et al., 2001; Valadares Filho et al., 2006). The use of inadequate quantities entails economic loss, besides its derivatives are potential environmental polluters.

The dairy cattle had higher values for urinary volume, urea nitrogen, uric acid and allantoin in urine, which resulted in higher microbial nitrogen production in grams per day; however, when evaluated by kg of metabolic body weight, the differences disappeared, and consequently they were related to the animal's different sizes and not to the different breeds. Nevertheless, dairy animals maintained higher values for nitrogen consumption and nitrogen retention, even when the values were adjusted for metabolic weight. According to Russel et al. (1980), the higher retention is related to higher nitrogen intake. This fact occurred in the present study, where dairy cattle showed higher crude protein and nitrogen intakes.

3.5 CONCLUSIONS

The essential oils at the dosage of 500mg/kg DM do not alter the nutrient intake and digestibility, ruminal dynamics or the microbial protein production. There are some differences between specialized dairy breed and specialized beef breed, but the majority disappeared when the adjustment for metabolic weight is performed.

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4 EFFECTS OF ESSENTIAL OILS ON FEEDING BEHAVIOR AND RUMEN FERMENTATION IN SPECIALIZED DAIRY AND BEEF CATTLE BREEDS

Abstract: Many essential oils have been shown to affect rumen fermentation. The objective of using ruminal fermentation modulating additives is to increase feed efficiency and reduce the environmental impact of ruminants. These effects are of great importance in cattle production. The objective of this study was to evaluate the effects of essential oils on feeding behavior, ruminal fermentation, ruminal protozoa population and energy release in specialized dairy and beef cattle breeds. Eight non-pregnant, non-lactating and ruminally canullated cows were used: four dairy breed Holstein, and four beef breed Nellore. The diets offered differed only with regard to the essential oil added to each treatment: CNT, a diet without any additive; EEO, a diet with 500 mg/kg of DM of eucalyptus (*Eucalyptus citriodora*) essential oil; the PEO, a diet with 500 of mg/kg of DM of Brazilian peppertree (*Schinus terebinthifolius Raddi - Anacardiaceae*) essential oil, and the LEO, a diet with 500 mg/kg of DM of lemongrass (*Cymbopogon citratus Stapf - Poaceae*) essential oil. The experimental design was the 4x4 contemporary latin square in a 2x4 factorial arrangement (referring to two specialized cattle breeds and four additives). The treatments with essential oils had on average 23% more rumination events per day than the control treatment (P = 0.0201). The animals that received lemongrass essential oil (LEO) had lower minimum ruminal pH value (P = 0.0363) than animals that received eucalyptus essential oil (OEE). The beef cattle had lower DMI in kg (P = 0.0018), even when diets were adjusted for metabolic weight (P = 0.0413) and spent more time consuming, ruminating or chewing 1 kg of DM or NDF (min/kg), even when data were adjusted for metabolic body weight. They had higher values for acetate, butyrate or total SCFA production. The essential oils did not change the rumen fermentation. Beef cattle had higher production of total SCFA

Keywords: Ruminants, natural additives, short chain fatty acids production, ruminal protozoa population, energy release.

4.1 INTRODUCTION

The symbiotic relationship between the rumen microorganisms and the ruminant host is related to the animal providing optimal environmental conditions so that the microorganisms can ferment the diet and thus release energy to produce microbial protein for the animal. This symbiotic relationship has energy (methane losses) and protein inefficiencies (losses of ammonia N) (VAN NEVEL AND DEMEYER, 1988). These losses not only reduce production performance, but also contribute to the release of pollutants into the environment (TAMMINGA, 1996).

Essential oils are secondary metabolites, which can be extracted from various parts of a plant, including leaves, flowers, seeds, roots and barks (BENCHAAR et al., 2008). The secondary compounds present in the essential oils can have antioxidant, antimicrobial, analgesic, decongestant, anesthetic, fungicide, among others properties (BURT, 2004). According to Bergen and Bates (1984), the essential oils improve the energy efficiency due to their manipulation of microbiota, and consequently, there is a greater production of propionate and an improvement in the use of nitrogen compounds, which reduce the proteolytic bacteria and also reduce the incidence of ruminal disorders by decreasing the production of lactic acid.

Khiaosa-ard and Zebeli (2013) performed a meta-analysis study about the effects of essential oils and their bioactive compounds on rumen fermentation characteristics and feed efficiency in ruminants. These authors observed that all rumen fermentation parameters presented a linear relationship with essential oil doses, and they reported a decrease of up to 12% in methane production compared to control treatments, at the greatest dose used. The acetate and butyrate proportions were decreased by 10% and 5.6%, respectively, and the molar percentage of propionate increased with increasing doses, which led to decreased acetate to propionate ratio. In this study, the beneficial effects of essential oils were more evident in beef than in dairy cattle or small ruminants.

This study was an extension of the hypothesis that essential oils are able to optimize the rumen fermentation in bovines. The objectives were to evaluate the feeding behavior and rumen fermentation and to estimate the energy release caused by the inclusion of essential oils in diets of different specialized cattle breeds.

4.2 MATERIAL AND METHODS

4.2.1 Study location and ethical issue

The study was conducted in the Ruminant Nutrition Laboratory from School of Veterinary Medicine and Animal Sience of the University of São Paulo, on *campus* Fernando Costa in Pirassununga, Brazil. The experiment was approved by and complied with the guidelines set out by the Ethics Committee in the Use of Animals of the School of Veterinary Medicine and Animal Sience, under application number nº 8453300914, in respect to animal experimentation and care of animals used for scientific purposes.

4.2.2 Animal, housing and feeding

Eight cows, not pregnant and non-lactating, were ruminally cannulated. Four were a dairy breed and four were a beef breed. The dairy cattle were the Holstein breed (*Bos taurus taurus*), with an average weight of 905±62 kg, and the beef cattle were from the Nellore breed (*Bos taurus indicus*), with average weight of 535±54 kg. It was not the main objective of this study to compare cattle breeds; rather, different breeds were used to study the essential oil's effect within broader conditions of animal types. Additionally, these two racial groups represent the prevalent systems of dairy and beef productions.

Cows were housed in individual stalls with a sand bed, individual feed bunkers and drinkers; fans were automatically turned on at warm hours of the day. Animals were fed twice daily (08h00 and 16h00). Feed was weighed daily and offered to each animal after removing of previous day's orts. The essential oils were also weighted daily and mixed in the concentrate to offer a dose of 500mg/kg of DM per animal per day. Orts were recorded once daily, before feeding, and the feeding rate was adjusted to yield orts on the basis of at least 5% and a maximum of 10% of the

amount supplied (on an as-fed basis). The animals were weighed individually on the initial and final day of each experimental period.

4.2.3 Experimental design and treatments

Animals were arranged in two contemporary 4 x 4 Latin Square designs with 2x4 factorial arrangements (referring to two specialized cattle breeds and four additives). The diets offered (Table 1) differed only in regard to the type of essential oil added. These were: CNT without any additive, the EEO containing 500 mg/kg of DM of eucalyptus essential oil (*Eucalyptus citriodora,* extracted from the leaves), PEO containing 500 mg/kg of DM of Brazilian peppertree essential oil (*Schinus terebinthifolius Raddi*, extracted from the fruits) and LEO containing 500 mg/kg of DM of lemongrass essential oil (*Cymbopogon citratus Stapf,* extracted from the leaves). The ingredients and chemical compositions of the experimental diets are presented in Table 1 (chapter 3).

4.2.4 Sampling schedule

The experiment, which lasted 16 weeks, consisted of 4 experimental periods of 28 days each. The first 16 days were designated to adapt the animals to the diets. The 22nd day was used to evaluate feeding behavior. Dry matter intake was measured from the 22nd to the 26th day. A probe for ruminal pH evaluation was inserted into rumen on day 24 and removed on day 26. Ruminal content collection, for the evaluation of SCFA, methane, ammoniac nitrogen production and protozoa count, was performed on day 25.

4.2.5 Feeding behavior

Eating, ruminating and idleness behavior, measured in minutes, were monitored visually over a 24 hours period. The animals were considered to be at eating activity (min/day) when they

had the head in the feed bunk and were in contact with the diet. Rumination activity (min/day) included regurgitation, re-mastication, and re-swallowing. Idles activity (min/day) included periods during which the animals slept, lay down, walked or stood idly.

Activities were noted every 5 minutes and each activity was assumed to persist for the entire 5 min interval. An event of eating was defined as at least 10 min of eating activity followed by at least 10 min by another different activity; rumination events were calculated in the same way. Total eating time was calculated as the sum of spent time during the 24 hours. The same was done for total ruminating time (MAEKAWA et al., 2002). Total time spent chewing was calculated as the total time spent eating and ruminating. To estimate time spent eating, ruminating or chewing per kilogram of DM or NDF intake, the average intake for the period was used. Time spent eating, ruminating or chewing were determined by dividing total time spent eating, ruminating or chewing per kilogram of DM or NDF intake. Correction for metabolic body weight was also performed as explained in the forward.

4.2.6 Dry matter intake

Dry matter intake for each period was determined between days 22 and 26, and the differences in the weights between feeds offered and refused by animal were measured. During the feed intake determination, feed ingredient samples were collected and stored at −20 °C. Individual feed ingredients were composited in representative samples on an equal-weight basis. A pool of samples was dried at 55°C (forced-air oven) for 72 h and ground to pass a 1-mm Wiley mill screen and analyzed for DM according to the methodology AOAC (1995), with the CP according to the method 920.87, AOAC (1990). The EE was performed with equipment ANKOM XT15 Extractor® (methodology Am 5-04; AOCS, 2005). The NDF, ADF and lignin analysis was done following the methodology of Van Soest et al. (1991), gross energy (GE) was determined by the complete oxidation of the samples in adiabatic calorimetric pump (C5000 control, IKA®, Staufen, Germany).

4.2.7 Ruminal pH evaluation

Ruminal pH was continuously measured during 24 h (every 10 min) on day 25 of each experimental period using a pH data logger (Model T7-1 LRCpH, Dascor®, Escondido, CA, USA) (PENNER et al., 2006). Attached on the data logger, 2 weighs of 900 grams each were used to maintain the device in position at rumen ventral sacral. Each electrode was standardized using pH 4.0 and 7.0 standards at the beginning and end of each session. The pH data were recorded as mean, maximum, and minimum pH, this included the area under the curve and duration of time in which pH was below 6.0, 5.8 and 5.6. The area under the curve was calculated by multiplying the absolute value of deviations in pH by the time (min) spent below the established threshold for each measure, and it was then divided by 60 and expressed as pH unit × hour.

4.2.8 Total and differential protozoa counts

For total and differential counts of ruminal protozoa, ruminal contents were collected manually, at 0, 3, 6, 9 and 12 h after the morning, of day 25. Each collected sample (10 mL) was stored in glass vials with 20 mL of 18.5% formaldehyde. Subsequently, the sample was stained with two drops of 2% brilliant green and diluted, and protozoa were identified (genus *Isotricha*, *Dasytricha, Entodinium* and *Diplodiniinae* subfamily) and counted using a Neubauer Improved Bright-Line counting chamber (Hausser Scientific Partnership®, Horsham, PA, USA) using optical microscopy (Olympus CH-2®, Japan) (DEHORITY, 1993).

4.2.9 Short chain fatty acids, methane and ammoniac nitrogen production

Ruminal contents samples were collected through the ruminal cannula at 0, 3, 6, 9 and 12 h, after the morning meal, on 25th day of each period,. The evening meal was offered only after the collection of the 12 h sample. Approximately 300mL of rumen fluid and 300g of solid content

were collected from different parts of the rumen (dorsal sac in the front, middle and back), then they were mixed as proportions of 66% liquid and 33% solid phase.

The samples were prepared and analyzed according to the *ex situ* technique (PERNA Jr. et al., 2017). The principle of this technique involves leaving the ruminal content samples within bottles; half of the bottles are incubated in a water bath (39 °C) and other half are not incubated (are inactivates under pressure and temperature at the time of collection). These bottles simulated the prevailing conditions of the rumen (presence of microorganisms, anaerobic environment, temperature of 39 °C, natural saliva, physiological rumen pH). The quantification of CH_4 production was obtained by multiplying the total volume of gas (mL) and the CH_4 concentration in the gas phase (mmol/mL) obtained in the incubated bottle, and then the result was subtracted from the value that was produced in the bottle not incubated (inactivated). The individual quantification of SCFA was obtained by multiplying the liquid volume (mL) and the concentration of each SCFA (mmol/mL) obtained in the incubated bottle, and this value was also subtracted from the value obtained on the bottle not incubated. The ammoniac nitrogen procedure was performed in the same way. After the quantification of the fermentation products from the sample contained in each bottle, each product was multiplied by its combustion heat in order to express the CH_4 production in relation to the energy from the produced fermentation. Thus, the relative energy loss (REL) was the ratio between the energy in the CH_4 produced and the energy sum in all the quantified fermentation products (CH_4 and SCFA), expressed as a percentage (PERNA Jr. et al., 2017).

4.2.10 Energy release evaluation

Gross energy intake (kcal/ani/d) was calculated by multiplying DMI (kg) and diet gross energy (kcal/kg). To calculate the energy release of acetate, propionate, butyrate and methane (kcal/ani/d) in the rumen, these metabolite productions (g/kg/d) were respectively multiplied by combustion heat (kcal/g), and then multiplied by ruminal solid mass in kg (the ruminal mass solid was obtained from the disappearance rate analysis, in the previous chapter).

Energy release in the rumen, when expressed in terms of %GEI (gross energy intake) or %DE

(digestible energy), was obtained by dividing the acetate, propionate, butyrate and methane releases (kcal/ani/d) by gross energy intake (kcal/ani/d) or digestive energy (kcal/ani/d) and then multiplying by 100.

Methane release in the cecum and colon (C&C) was considered as 5% of total methane release. According to Dini et al. (2012), enteric methane is produced mainly in the rumen (95%) and, to a smaller extent (5%), in the large intestine. Fermentation heat and microbial ATP was stimated from the rations among the SCFA produced according to Owens and Basalan (2016).

Energy release in the intestine (kcal/ani/d) was calculated from gross energy intake (kcal/ani/d) and subtracting from acetate, propionate, butyrate, methane, fermentation heat (FH) +ATP release in the rumen (kcal/ani/d) plus feces gross energy (kcal/ani/d) and methane release in the cecum and colon (kcal/ani/d), following this equation:

$$ERI = GEI - (C2 + C3 + C4 + \text{feces GE} + C\&C \text{ methane} + FH + ATP)$$

Where:

ERI: energy release in the intestine, kcal/ani/d

GEI: gross energy intake, kcal/ani/d

C2: acetic, kcal/ani/d

C3: propionic, kcal/ani/d

C4: butyric, kcal/ani/d

Feces GE: energy release in the feces, kcal/ani/d (was obtained from the digestibility analyzes, performed in the previous chapter)

C&Cmethane: methane release in cecum and colon (kcal/ani/day)

FH: fermentation heat

ATP: microbial ATP

Energy release in the intestine, expressed in terms of %GE or %DE, was obtained by dividing energy release in the intestine (kcal/ani/d) by GEI (kcal/ani/d) or DE (kcal/ani/d) and then multiplying by 100.

Energy release in feces, expressed in terms of %GEI, was obtained by dividing feces energy content (kcal/ani/d) by gross energy intake (kcal/ani/d) and then multiplying by 100.

4.2.11 Statistical analysis

Data were statistically analyzed using the SAS 9.3 (SAS Institute Inc., Cary, NC, USA). Before the actual analysis, the data were analyzed for the presence of disparate information ("outliers") and normality of residuals (Shapiro-Wilk). The data of feeding behavior, ruminal pH, total and differential count of protozoa, ruminal fermentation and energy release were analyzed with consideration to such causes as the variations of the effect of treatments, period effect, animal effect inside square, as well as the square (or specialized cattle breeds) effect. The variables were submitted to analysis of variance using the MIXED procedure. The model included the effect of essential oils, specialized cattle breeds (or square) and the interaction of these as fixed factors. Animal effects within square and period were considered to be random factors. Ruminal pH analysis was also used the covariable intake of DM per kg of metabolic weight. In the presence of essential oil effects, the comparison of means was performed by the Tukey test. Non-significant (NS) was considered when P value was higher than 10% and statistical significance when P value was less than 5%.

4.3 RESULTS

4.3.1 Dry matter intake and feeding behavior

There was no effect from the essential oils on dry matter intake. Animals presented an average of 12.39 kg for dry matter intake, independent of treatment (Table 7). The dairy animals had higher dry matter intake in kg ($P < 0.01$) or when adjusted for metabolic body weight ($P < 0.05$).

The treatments with essential oils had a greater number of events (17 vs. 14 events) of rumination ($P = 0.0201$) per day and time spent to ruminate1 kg of DM, NDF than the control animals. When the times spent ruminating (min/kg of DM and NDF) were adjusted for metabolic

body weight, there was no difference. The beef cattle spent more time consuming, ruminating and chewing 1 kg of DM or NDF (min/kg), even when data were adjust for metabolic body weight.

Table 7 - Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on dry matter intake and on feeding behavior

Variables	Cattle		Essential oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Dry matter intake										
kg	15.65	9.13	12.46	12.44	12.56	12.10	0.66	0.0018	NS	NS
g/kg $BW^{0.75}$	94.66	82.02	89.16	88.35	89.70	86.15	1.85	0.0413	NS	NS
Eating										
min/day	225.3	220.9	233.1	217.5	215.6	226.3	8.07	NS	NS	NS
event/day	9.81	10.66	11.25	10.13	9.38	10.13	0.35	NS	NS	NS
min/kgDM	15.8	24.13	19.91	18.88	18.77	20.30	1.23	0.0349	NS	NS
min/gDM/$BW^{0.75}$	2.52	2.86	2.64	2.72	2.66	2.73	0.06	0.0435	NS	NS
min/kgNDF	36.73	59.85	49.38	46.84	46.58	50.36	3.04	0.0350	NS	NS
min/gNDF/$BW^{0.75}$	6.24	7.08	6.55	6.75	6.59	6.76	0.16	0.0452	NS	NS
Ruminating										
min/day	472.8	483.7	438.1	505.6	474.4	495.0	14.2	NS	NS	NS
event/day	17.75	15.13	14.00^{b}	17.37^{a}	17.13^{a}	17.25^{a}	0.58	NS	0.0201	NS
min/kgDM	31.61	52.90	37.58^{b}	45.02^{a}	41.06^{a}	43.35^{a}	2.45	0.0043	0.0224	NS
min/gDM/ $BW^{0.75}$	6.07	6.88	6.37	6.55	6.41	6.58	0.14	0.0454	NS	NS
min/kgNDF	75.93	131.2	93.2^{b}	111.7^{a}	101.9^{a}	107.6^{a}	6.07	0.0043	0.0224	NS
min/gNDF/ $BW^{0.75}$	15.06	17.08	15.81	16.26	15.89	16.32	0.35	0.0454	NS	NS
Chewing										
min/day	698.1	705.7	671.3	723.1	690.0	721.3	15.9	NS	NS	NS
event/day	25.36	27.85	26.57	26.55	26.36	26.96	0.65	NS	NS	NS
min/kgDM	45.41	77.03	57.49	63.91	59.83	63.66	3.36	0.0026	0.0521	NS
min/gDM/ $BW^{0.75}$	8.59	9.74	9.01	9.28	9.06	9.30	0.21	0.0455	NS	NS
min/kgNDF	112.7	191.1	142.6	158.5	148.4	157.9	8.32	0.0026	0.0521	NS
min/gNDF/ $BW^{0.75}$	21.30	24.16	22.36	23.01	22.47	23.08	0.51	0.0453	NS	NS

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = Standard error of mean, C = specialized cattle breed effect, O = Essential oils effect, CxO = Interaction among cattle breeds and essential oils, NS= non-significant (P > 0,10).

4.3.2 Rumen pH evaluation

The treatment with eucalyptus essential oil had a higher value for the minimum pH (5.85 vs. 5.51) than the treatment with lemongrass (Table 8). It was possible to observe that the lowest

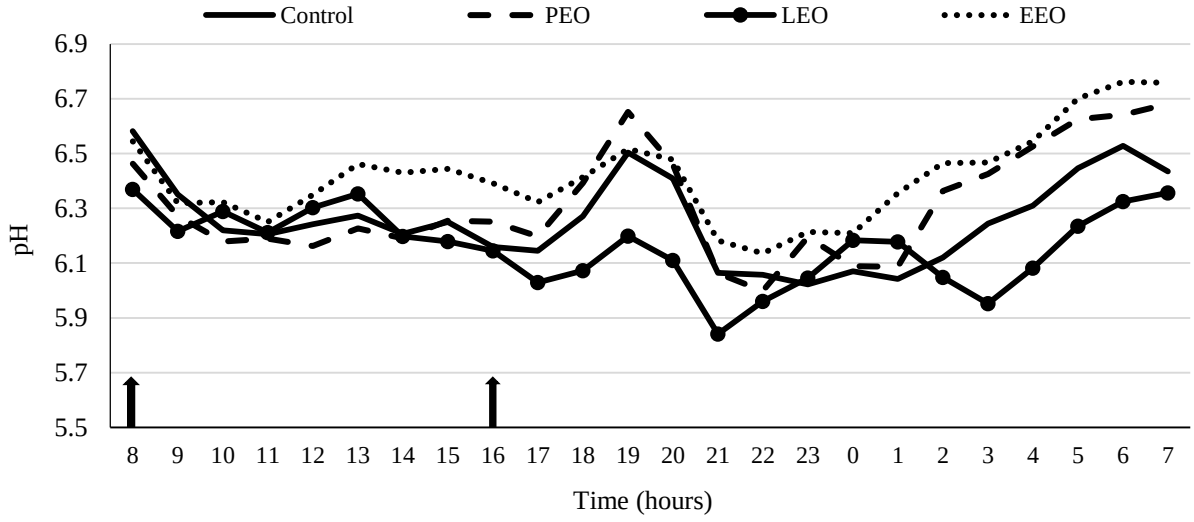
ruminal pH values occurred between 2 and 4 hours after feeding (Figure 2). There was no difference among cattle breeds.

Table 8 - Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on ruminal pH (DMI was included as covariate)

Variables	Cattle		Essential Oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Ruminal pH										
Minimum	5.58	5.76	5.68^{ab}	5.63^{ab}	5.51^{b}	5.85^{a}	0.046	NS	0.0363	NS
Mean	6.24	6.34	6.26	6.32	6.16	6.41	0.044	NS	NS	NS
Maximum	6.87	6.78	6.74	6.96	6.65	6.96	0.058	NS	NS	NS
Time below (minutes)										
pH 5.6	89.48	11.15	35.37	74.99	82.29	8.60	21.71	NS	NS	NS
pH 5.8	224.9	93.8	171.8	171.2	223.1	71.4	43.01	NS	NS	NS
pH 6.0	400.7	224.3	326.7	311.2	397.4	214.8	58.99	NS	NS	NS
Area below (pH deviations x time)										
pH 5.6	0.20	0.01	0.02	0.21	0.20	0.01	0.060	NS	NS	NS
pH 5.8	0.70	0.20	0.39	0.60	0.69	0.12	0.159	NS	NS	NS
pH 6.0	1.73	0.73	1.22	1.40	1.76	0.54	0.328	NS	NS	NS

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = Standard error of the mean, C = specialized cattle breed effect, O = Essential oils effect , CxO = Interaction among cattle breeds and essential oils, NS= non-significant (P > 0.10).

Figure 2 - Effect of the inclusion of essential oils in the diet on ruminal pH during 24 hours



PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil
Font: Carvalho (2018).

4.3.3 Total and differential counts of protozoa

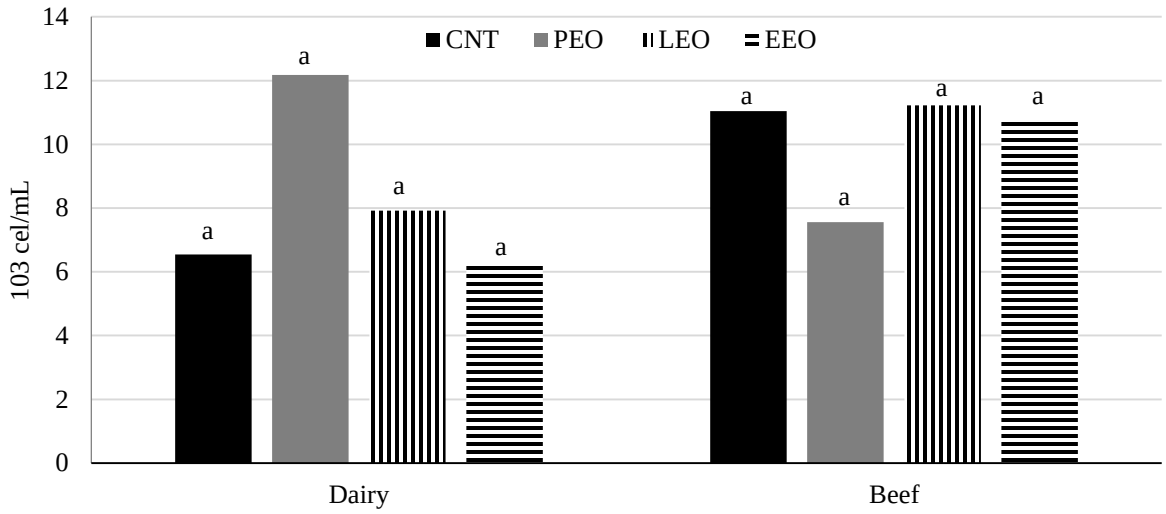
There was an interaction (P = 0.05) between the cattle breeds and essential oils in the count of the *Diplodininae* family. When Brazilian peppertree essential oil was added to the diet of the dairy cows, there was an increase in the *Diplodininae* count, but when it was added to the diet of beef cattle, there was a decrease in the count. The data have been deployed and are presented in Figure 3. The total and differential counts of the other variables were not influence by the essential oils (Table 9). The beef cattle had a higher *Dasytricha* count than dairy cattle (P = 0.05).

Table 9 - Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on total and differential counts of protozoa

Variables	Cattle		Essential Oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Protozoa x 10^3/mL										
Entodinium	402.8	371.3	420.9	372.6	400.8	354.0	10.1	NS	NS	NS
Dasytricha	0.41	0.84	0.45	0.99	0.57	0.48	0.08	0.0406	NS	NS
Isotricha	2.61	3.89	3.78	3.54	2.85	2.82	0.26	NS	NS	0.0987
Diplodininae	8.24	10.2	8.79	9.87	9.57	8.55	0.56	NS	NS	0.0242
Total	414.1	386.2	414.8	387.9	434.0	366.9	10.3	NS	NS	NS
Protozoa %										
Entodinium	97.2	96.1	96.9	96.2	96.6	96.7	0.18	NS	NS	NS
Dasytricha	0.10	0.22	0.12	0.26	0.14	0.12	0.02	0.0452	0.0836	NS
Isotricha	0.66	1.02	0.87	0.89	0.76	0.84	0.06	NS	NS	0.0801
Diplodininae	2.09	2.71	2.05	2.62	2.54	2.38	0.15	NS	NS	NS

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = Standard error of the mean, C = specialized cattle breed effect, O = Essential oils effect , CxO = Interaction among cattle breeds and essential oils, NS= non-significant (P > 0.10).

Figure 3 - Unfolding of specialized cattle breeds and essential oils interaction for counting the family Diplodiniae (103 cells / mL)



CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = OEE = eucalyptus essential oil.
Font: Carvalho (2018)

4.3.4 Short chain fatty acids, methane and ammonia nitrogen production

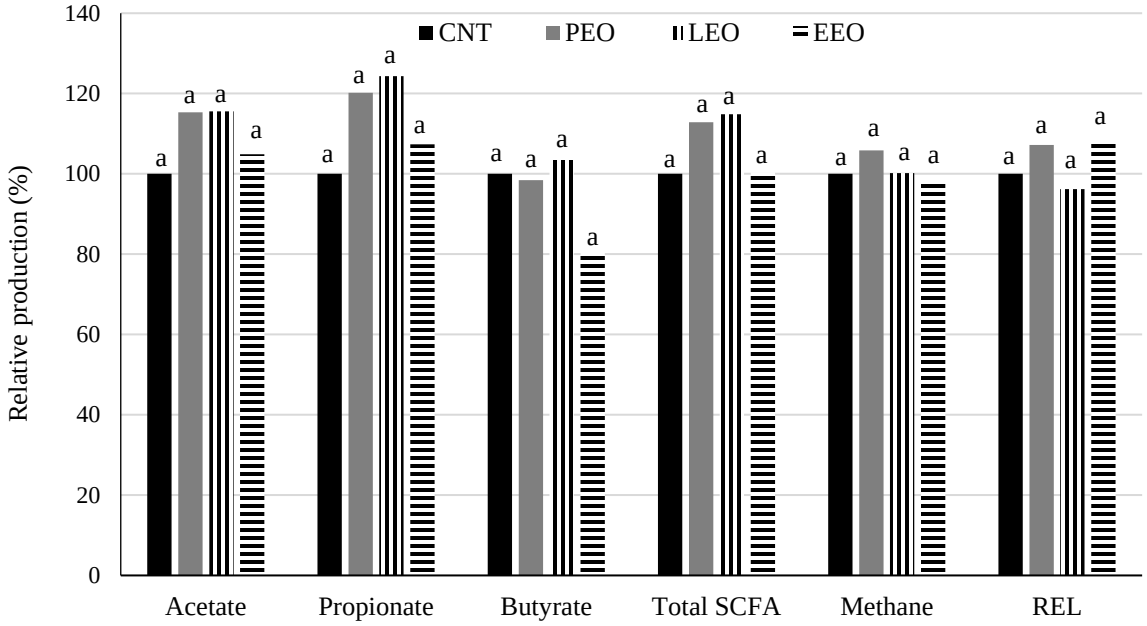
The essential oils did not change the production of SCFA, methane or ammonia nitrogen (Table 10). There was no difference between the specialized cattle breeds on ammonia nitrogen; nevertheless, beef cattle had higher values for acetate, butyrate or SCFA total production. Figure 4 demonstrates the production of the SCFA (acetic, propionic and butyric), methane and the REL in relation to the control treatment (considered as 100%) for the different essential oils.

Table 10 - Effect of the inclusion of essential oils in the diet of specialized dairy breed and specialized beef breed on ruminal fermentation

Variables	Cattle		Essential oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Acetate										
0 min. mMol/L	79.24	79.28	79.39	80.10	79.22	78.31	0.54	NS	NS	NS
30 min. mMol/L	83.11	84.73	83.85	85.22	83.97	82.65	0.60	NS	NS	NS
Production. Mol/kg/d	2.78	3.87	3.01	3.54	3.53	3.21	0.14	0.0291	NS	NS
Production. g/kg/d	166.8	232.2	180.7	212.5	211.9	192.7	8.59	0.0291	NS	NS
GE, (kcal/kg/d)	582.1	810.3	630.7	741.8	739.6	672.6	29.9	0.0291	NS	NS
Propionate										
0 min. mMol /L	21.34	20.48	20.74	20.80	21.33	20.78	0.30	NS	NS	NS
30 min. mMol/L	22.72	22.29	22.19	22.59	23.01	22.24	0.33	NS	NS	NS
Production. Mol/kg/d	1.01	1.25	1.00	1.22	1.21	1.08	0.05	NS	NS	NS
Production. g/kg/d	74.40	92.75	74.18	90.09	89.75	80.29	3.79	NS	NS	NS
GE, (kcal/kg/d)	370.5	461.9	369.4	448.6	446.9	399.8	18.9	NS	NS	NS
Butyrate										
0 min. mMol/L	11.54	11.82	11.97	11.84	11.74	11.18	0.13	NS	NS	NS
30 min. mMol/L	12.67	13.39	13.43	13.22	13.15	12.32	0.15	NS	NS	NS
Production. Mol/kg/d	0.85	1.12	1.02	1.02	1.06	0.83	0.04	0.0013	NS	NS
Production. g/kg/d	74.69	98.36	89.92	89.81	93.12	73.25	3.56	0.0013	NS	NS
GE, (kcal/kg/d)	445.2	586.2	535.9	535.3	554.9	436.6	21.2	0.0013	NS	NS
SCFA total										
0 min. mMol/L	112.1	111.7	112.1	112.9	112.3	110.3	0.89	NS	NS	NS
30 min. mMol/L	118.5	120.4	119.5	121.1	120.1	117.2	0.99	NS	NS	NS
Production. Mol/kg/d	4.64	6.25	5.06	5.81	5.78	5.13	0.22	0.0376	NS	NS
Production. g/kg/d	316.3	424.1	346.5	394.3	393.6	346.3	14.5	0.0376	NS	NS
GE, (kcal/kg/d)	1400.0	1862.6	1542.4	1733.5	1740.4	1508.9	62.5	0.0376	NS	NS
C_2:C_3 ratio										
0 min	3.83	3.95	3.93	3.95	3.81	3.85	0.04	NS	NS	NS
30 min	3.76	3.87	3.87	3.86	3.74	3.79	0.04	NS	NS	NS
Production	2.96	3.14	3.21	2.99	2.99	3.03	0.09	NS	NS	NS
Methane										
0 min. mMol/L	0.019	0.025	0.023	0.023	0.022	0.021	0.004	0.0092	NS	NS
30 min. mMol/L	0.074	0.093	0.083	0.088	0.084	0.080	0.002	0.0266	0.0705	NS
Production. Mol/kg/d	1.55	1.82	1.65	1.77	1.68	1.65	0.03	NS	NS	0.0765
Production. g/kg/d	24.84	29.15	26.37	28.39	26.86	26.34	0.61	NS	NS	0.0765
GE, (kcal/kg/d)	326.9	383.6	346.9	373.7	353.5	346. 7	8.02	NS	NS	0.0765
REL. %	22.98	19.16	20.69	21.83	19.51	22.24	0.67	NS	NS	NS
N-NH_3. mg/dL/hour										
0 min	7.68	8.09	8.66	7.93	7.42	7.53	0.28	NS	NS	NS
30 min	8.59	9.16	9.63	9.04	8.23	8.60	0.28	NS	NS	NS
Balance	1.74	2.16	1.78	2.23	1.66	2.14	0.20	NS	NS	NS

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = Standard error of the mean, C = specialized cattle breed effect, O = Essential oils effect , CxO = Interaction among cattle breeds and essential oils, NS= non-significant (P > 0.10).

Figure 4 – SCFA production (acetic, propionic and butyric), CH_4 and REL for the different essential oils, in relation to the control treatment



CNT= Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil.
Font: Carvalho (2018).

4.3.5 Energy release estimations

The essential oils did not affect the release of energy in the gastrointestinal tract (Table 11). The dairy animals had higher gross energy intake (427.2 vs. 370.3 kcal/kg $BW^{0.75}$), a higher energy release in the intestine in kcal/kg $BW^{0.75}$, and in relation to GE or DE consumed, also had lower energy release in feces then beef cattle.

Table 11 - Estimates of the energy released in the gastrointestinal tract of specialized dairy breed and specialized beef breed fed different essential oils

Variables	Cattle		Essential oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
R. mass, kg	9.30	5.26	7.59	6.91	7.33	7.29	0.48	0.0134	NS	NS
GEI, kcal/kg $BW^{0.75}$	430.5	370.3	403.9	399.1	410.8	387.8	8.38	0.0288	NS	NS
Energy release in the rumen										
Acetate										
kcal/kg $BW^{0,75}$	34.37	38.38	34.28	37.70	39.76	33.77	2.31	NS	NS	NS
GE %	7.84	10.35	8.48	9.47	9.57	8.87	0.57	NS	NS	NS
DE %	9.57	13.42	10.55	11.86	12.20	11.38	0.74	NS	NS	NS
Propionate										
kcal/kg $BW^{0,75}$	21.98	22.11	19.57	23.05	25.22	20.33	1.52	NS	NS	NS
GE %	5.00	5.94	4.86	5.73	6.03	5.28	0.34	NS	NS	NS
DE %	6.12	7.71	6.08	7.17	7.66	6.76	0.43	NS	NS	NS
Butyrate										
kcal/kg $BW^{0,75}$	25.93	27.94	28.99	26.35	30.44	21.95	1.44	NS	0.0683	NS
GE %	5.99	7.54	7.17	6.65	7.55	5.70	0.36	NS	NS	NS
DE %	7.34	9.77	8.90	8.37	9.65	7.29	0.47	0.0553	NS	NS
Total SCFA										
kcal/kg $BW^{0,75}$	82.17	88.43	82.84	87.10	95.21	76.05	4.82	NS	NS	NS
GE %	18.79	23.84	20.51	21.85	23.04	19.85	1.14	NS	NS	NS
DE %	22.97	30.90	25.53	27.39	29.40	25.43	1.49	NS	NS	NS
Methane										
kcal/kg $BW^{0,75}$	18.27	18.03	18.41	18.34	18.49	17.37	0.68	NS	NS	NS
GE %	4.24	4.88	4.58	4.65	4.51	4.52	0.18	NS	NS	NS
DE %	5.21	6.33	5.69	5.83	5.77	5.80	0.24	NS	NS	NS
Energy release in the intestine										
kcal/kg $BW^{0,75}$	235.5	169.1	210.3	201.1	199.4	198.2	8.39	0.0028	NS	NS
GE %	54.8	45.6	52.0	50.0	48.2	50.6	1.44	0.0175	NS	NS
DE %	67.5	59.0	64.7	62.8	60.9	64.6	1.67	0.0776	NS	NS
Energy release in the feces										
kcal/kg $BW^{0,75}$	79.64	83.84	78.96	79.71	85.25	83.02	1.65	NS	NS	NS
GE %	18.65	22.76	19.60	20.30	21.17	21.77	0.61	0.0317	NS	NS

R. mass = ruminal solid mass, GEI = gross energy intake, Ge= gross energy, DE = digestible energy, D = dairy, B = beef, CNT = Control, OEP = Brazilian peppertree essential oil, OEL = lemongrass essential oil, OEE = eucalyptus essential oil, SEM = Standard error of the mean, C = specialized cattle breed effect, O = Essential oils effect, CxO = Interaction among cattle breeds and essential oils, NS= non-significant ($P > 0.10$).

4.4 DISCUSSION

4.4.1 Dry matter intake and feeding behavior

Animals that received treatments with essential oils had more ruminating events per day and spent more time ruminating. When the data were adjusted to metabolic body weight, the difference disappeared. Note: It is of great importance to properly adjust for metabolic weight in the evaluation when animals are of different types and body weights.

Flavor is one of the five senses that give ruminants and other animals the sense of their environment, especially for feed selection, and it plays a vital biological role in helping animals to regulate the intake of adequate feed and to eschew inadequate intake. According to Segabinazzi et al. (2011), disagreeable palatability would lead to a lower ingestive stimulus; consequently, the animals would ingest more slowly. The absence of the essential oil effect on intake or feeding behavior may be due to the dosages and the types of oils used in the present study.

Tager & Krause (2011) observed no effect with the addition of essential oils (cinnamaldehyde and eugenol) on the feeding behavior of lactating Holstein cows fed diets containing 42% forage and 58% concentrate. Ornaghi (2016) evaluated addition of different levels essential oils (clove and cinnamon) and did not find any influence on feeding behavior of crossbreed cattle (½ Brown Swiss ½ Nellore). Yang et al. (2007) did not find differences in dry matter intake when evaluating the effect of the essential oils of garlic, juniper berry or monensin in diets containing 40% of forage and 60% barley based concentrate diet for Holstein cows.

Few studies have been performed to compare the ingestive behaviors of specialized cattle breeds. In the present study, the beef cattle spent 53% more time ingesting, 67% more time ruminating and 69% more time chewing 1kg of DM than dairy cattle. When values were adjusted for the metabolic body weight, the difference of time spent to ingest, ruminate and chew decreased, but still remained statistically significant, suggesting that such an effect is an animal type characteristic. This disagrees with Jorge et al. (1999), based on the studies of Gonçalves (1988), Peron et al. (1993) and Jorge (1993), and suggests a lower ingestive capacity of zebuine origin animals in relation to taurine and crossbreed, due to their lower digestive tract capacity. In the present study, even with the adjustment for metabolic body weight and removing those proportions

of the interference of the size of the animals, zebu animals that had specialized beef breed still spent more time ingesting, ruminating or chewing than dairy animals.

The beef cattle had lower DMI, this result may be related to their longer time spent ruminating or chewing. This behavior probably increased the degradation surface of the diet and provided a higher productivity of SCFA, as was verified in this work. The higher production of SCFA consequently decreases the dry matter intake of beef, because it provided an energy status adequate to the performance potential of the animals. According to Allen (2014), SCFAs play an important role in eating behavior, as they are responsible for controlling satiety, a fact observed in the present study.

4.4.2 Rumen pH evaluation

Ruminal pH is a variable of great importance, as its value directly affects the ruminal microorganism population. Protozoa and cellulolytic bacteria require pH 6.2 or higher to maintain their activity. The amylolytic bacteria are active at a more acidic pH of around 5.8; therefore, the pH of the ruminal fluid affects nutrient degradation (FURLAN et al, 2011). In the present study, the mean pH of the treatments ranged from 6.16 to 6.41, favoring the degradation of the diet, which had higher roughage proportion (70%).

The addition of the essential oils did not influence the ruminal pH values in relation to the control treatment; however, the animals treated with eucalyptus oil had a higher value of minimum pH than the animals treated with lemongrass oil. Although statistically significant, the treatment with lemongrass oil not presented a decrease in the SCFA production.

There was no difference on ruminal pH among the dairy breed or the beef breed. It was expected that beef cattle would have higher ruminal pH values, because they spent more time eating, ruminating or chewing 1kg of DM or NDF, which could have consequently increased secretion of saliva. According to Santos and Pedroso (2011), the increase of the saliva secretion increases the ruminal pH due to the greater supply of buffers present in this fluid. Nevertheless, the beef cattle had higher SCFA production. As a weak acid, SCFA will dissociate in the rumen and release a proton, thereby decreasing ruminal pH under most circumstances. Thus, when SCFA

production exceeds the animal's ability to neutralize the protons, rumen pH decreases. In this study, however, the increase in SCFA production observed in beef cattle did not exceed buffer rumen capacity, which was also increased by rumination, possibly increasing the release of saliva.

Schären et al. (2017) also did not find any effect of the essential oils on the ruminal pH of dairy cows in the transition period when the average ruminal pH was 7.24. Meyer et al. (2009) evaluated the use of essential oil blends on finishing steer performance, carcass characteristics, liver abscesses, ruminal fermentation, and digestibility of animals for meat production. The authors also did not find any effect of the essential oil blends in relation to the control treatment. This is in agreement with the results found in the present study.

4.4.3 Total and differential counts of protozoa

According to Patra and Saxena (2010), the essential oils have the potential of defaunation in the rumen, because they act on the plasmatic membrane of the protozoa, altering their cellular permeability, and consequently, microbial lysis. There are, however, several studies which indicate that essential oils do not have an effect on protozoa population in the rumen (McINTOSH et al., 2003; NEWBOLD et al., 2004; BENCHAAR et al. 2007).

Khiaosa-ard e Zebeli (2013) performed a meta-analysis study about the effects of essential oils and their bioactive compounds on rumen fermentation characteristics and feed efficiency in ruminants. Theses authors separated the animals by classes of beef or dairy cattle and small ruminants. They did not observe an effect in the classes of the animal; nevertheless, they observed an effect in the levels of the essential oils on the protozoa population, and the greater the dosage resulted in defaunation. The dose effect was even more evident when the authors noted that small dosages (<0.20 g/kg DM) increased the protozoa population relative to the control treatment.

In the present study, the essential oils did not alter the protozoa population. The absence of effects of the essential oils in the present study could be due to the dosage used. When interaction between essential oils and specialized cattle breeds was decomposed, effect of essential oils was not shown ($P > 0.05$).

The use of some essential oils decreased the total number of protozoa, small entodiniomorphs and holotrics, but did not affect large entodiniomorphs (PATRA et al., 2010).

Ando et al. (2003) observed that the addition of 200 mg/day of mentha piperita essential oil decreased *Entodinium, Isotricha and Diplodinium* in dairy cattle; however, Cardozo et al. (2006) reported that the essential oil blend (cinnamaldehyde and eugenol) increased the number of holotrichs and had no effect on entodinomorphs for beef catle.

Diet has a great influence on the population of ruminal microorganisms. The ciliate ruminal protozoa of the holotric subclass mainly use soluble carbohydrates, whereas the entodiniomorphos ingest and ferment fibrous materials (VAN SOEST, 1994; WILLIAMS, 1986). According to Hungate (1966), when there is an inclusion of concentrates in the diet of cattle, the genus *Entodinium* is the predominant protozoan in the total count, reaching 90%, which resembles the data found in this experiment. In addition to diet, the ruminal pH also influences the protozoan population, but in the present study the pH values found favored the population of protozoa, which is 6.2 or higher according to Furlan et al. (2011).

The beef cattle presented a greater population of *Dasytricha*. According to Hegarty (2004), the *Bos indicus* animals (beef cattle) have a larger population of protozoa than *bos taurus* animals (dairy cattle). This difference between the *Dasytricha* populations was not enough to change the total protozoa population, but the genetic predisposition may explain this difference between animals. Hennessy et al. (1995) also observed a difference of protozoa populations, *Bos indicus* had the largest population of the protozoa than *Bos taurus.*

4.4.4 Short chain fatty acids, methane and ammoniac nitrogen production

Diet has great influence on the final products of the ruminal fermentation (BERGMAN, 1990). Diets with higher roughage proportion favor appropriate pH for the growth of cellulolytic bacteria, lower concentration of total SCFA and higher production of acetate and butyrate. Diets with a higher concentrate proportion provide lower pH values, favoring the growth of amylolytic bacteria and higher production of propionate. According to Annison and Armstrong (1970), diets with high roughage provide a acetate : propionate ratio of 4:1. The values observed in the present study were lower, but still maintained a higher proportion of acetate, agreeing with the production of diets with high roughage proportion reported by these authors.

According to Calsamiglia et al. (2007), essential oils can interact with microbial cell membranes and inhibit growth or even cause bacterial death. As a result, this can inhibit deamination and methanogenesis, resulting in lower ammonia N, methane, acetate, and in higher propionate and butyrate concentrations. Nevertheless, some essential oil effects are dependent on conditions such as doseage, pH and diet. For example, capsaicin appears to have small effects in high roughage diets, whereas the changes observed in high concentrate diets can increases dry matter intake, total SCFA, and a reduction in the acetate, propionate ratio or ammonia N concentration.

The essential oils in the present study were not able to modify SCFA, ammonia N or methane ruminal production as was initially expected. Also did not influence the REL (loss of energy of CH_4 in relation to the products of the fermentation). Reinforcing the small effect of these oils on ruminal fermentation, the absence of effect may be related to the composition the oils, dosage applied or the diet used as reported by Calsamiglia et al. (2007).

In a study on effects on methane emissions, Meale et al. (2014) evaluated the dietary inclusion of essential oils for dairy cows fed with higher roughage proportion. These authors did not find effects of the essential oils on methane mitigation in relation to the control treatment. Castro-Montoya et al. (2015) evaluated the addition of 0.2 g/d of essential oils in lactating dairy cows and beef heifers. They also did not find any effect of the essential oils on methane production in dairy or beef cattle.

In the present study, beef cattle were more efficient at fermenting the diet, because they produced higher amounts total SCFA (6.25 vs. 4.64 mol/kg/d). The diet fermentative efficiency in these animals may be associated to feeding behavior, because beef cattle spent longer times to ingest, ruminate and chew 1kg of DM or NDF. The longer time spent in these activities may provide greater fractionation of the diet, increasing the area for attachment and fermentation of the ruminal microorganisms. Higher production of acetate of beef cattle may confirm that this effect was predominant in roughage particles.

Gandra et al. (2011) evaluated performance, nutrient digestion and metabolism of dairy cattle (Holstein - *Bos taurus taurus*), beef cattle (Nellore - *Bos taurus indicus*) and Mediterranean Buffaloes (*Bubalis bubalus*) fed with high roughage diets. In this study, the authors did not find a difference on total SCFA production among the animals; however, beef cattle had a lower DMI

and produced the same amount of total SCFA and higher butyric production as that of dairy animals. These results also demonstrate a greater efficiency of beef cattle to ferment the diet.

4.4.5 Energy release estimation

The *ex situ* technique made it possible to calculate the daily production of the ruminal fermentation products (SCFA and CH_4) and to estimate the energy released along the gastrointestinal tract. In general, energy release in the rumen is related to SCFA and methane production, as well as to ruminal solid mass. The essential oils did not change the expected energy release, because they also did not change the SCFA production, DMI or ruminal dynamics. It was expected that beef cattle would have higher energy released in the rumen, because they presented higher SCFA production; however, the dairy cattle had higher DMI, and consequently they had higher DM content in the rumen to fermenter, since the passage rate among the animals did not differ, which allowed an equal release of energy among the cattle of different specialized breeds. Beef cattle had lower energy released in the intestine, which possibly related to their feeding behavior that allowed greater efficiency to ferment the diet in the rumen. They also had greater energy released in the feces. This result may be due to the lower NFC digestibility of these animals (Table 2).

The methane production is of great importance in cattle productivity because, in addition to environmental issues, the production of methane causes energy losses for the animal. Methane production in cattle typically accounts for 5.5 to 6.5% of GEI (JOHNSON and WARD, 1996); however, values of 2 to 12% (JOHNSON and JOHNSON, 1995) have been reported for some diets. The values of energy released in the form of methane found in the present study are in agreement with the literature.

4.5 CONCLUSIONS

Treatments with the essential oils changed the feeding behavior, increasing by 23% the number of rumination events per day; however, this effect did not interfere with the other parameters evaluated. The essential oils did not change the ruminal fermentation; consequently, they were not able to optimize the rumen fermentation in the dosage (500 mg/kg of DM) and experimental conditions used. Beef cattle spent more time eating, ruminating and chewing MS or NDF. This difference on feeding behavior was able to increase total SCFA production and decrease DMI when compared to dairy cattle.

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5 THE POTENTIAL FOR GREENHOUSE GAS PRODUCTION DURING ANAEROBIC BIODIGESTION OF MANURE FROM SPECIALIZED DAIRY AND BEEF CATTLE BREEDS FED ESSENTIAL OILS

Abstract: Many strategies exist to mitigate enteric methane; however, in most of them it is unclear whether the approach to mitigation interferes with the composition and emission of greenhouse gases from manure. The objective of the present study was to evaluate the manure of dairy and beef cattle that are fed with different essential oils for the production of CO_2, CH_4 and N_2O during anaerobic digestion. Experimental batch anaerobic digesters were placed in a climatic chamber (30 to 35ºC). These were arranged in a completely randomized design with a 2x4 factorial arrangement, used manure from animals of two specialized breeds of dairy or beef cattle fed four diets into which different essential oils were added: CNT, a diet without any additive; EEO, an additive of 500 mg/kg of DM of eucalyptus (*Eucalyptus citriodora*) essential oil; PEO, an additive of 500 mg/kg of DM of Brazilian peppertree (*Schinus terebinthifolius Raddi*) essential oil; and LEO, an additive of 500 mg/kg of DM of lemongrass (*Cymbopogon citratus Stapf*) essential oil. The experiment had 8 treatments with 4 replicates totaling 32 experimental units. Diet containing essential oils decreased N_2O production. The manure from dairy cattle had higher biogas production (L/gVS add) or CO_2 (liters, percentage and L/gVS add) than the manure of beef cattle. There was no change in the site of greenhouse gas emissions of enteric origin for manure with the use of essential oils; therefore, essential oils can be added to the diet of the cattle without negatively affecting the biogas production from manure.

Key words: Feed additives, waste, biodigester, biogas, greenhouse gas.

5.1 INTRODUCTION

Recent questions in the agricultural sector about the relationship of climate change and global warming due to the potential effects of greenhouse gas emissions (GHG) from cattle have

been increasing. The livestock sector contributes mainly through methane emissions from enteric fermentation and animal manure management.

Approaches to mitigate enteric methane in ruminants by manipulating diet through the qualities of feed or various feed additives are under investigation. In recent years, studies evaluating the inclusion of natural feed additives, such as essential oils, have increased. Some of these have indicated positive results for the mitigation of enteric methane using essential oils (CASTRO-MONTOYA et al., 2015, LAABOURI et al., 2017, KLEVENHUSEN et al., 2011).

According to Lassey et al. (1997), around 87% of the emission variations of methane are attributed to differences between animals; therefore, the intrinsic characteristics of the animals are an important cause of change. According to Lassey (2002), these variations may occur in zebu, taurine and crossbred animals and may be associated with the different characteristics of the animals, such as rumen volume, feed selection ability, rumen feed retention time, and associations of factors that lead to a greater or lesser degree of feed fiber digestibility. For these reasons, it is important to identify animals with lower methane emission potential.

Several strategies to mitigate enteric methane can be performed; however, in most strategies designed to mitigate enteric methane, it is still unclear whether they also interfere with the composition or emission of greenhouse gases from fecal degradation. According to González-Avalos and Ruiz-Suárez (2001) and Orrico Junior et al. (2011), diet is the most important factor determining differences in methane emission from manure.

Essential oils may have an effect on ruminal degradation of some nutrients, which could alter the composition of the manure and consequently their digestion. Additionally, the presence in manure of the compounds from essential oils can negatively affect the biodigestion process. The objective of the present study was to evaluate the potential for greenhouse gas production and nutrient removal during anaerobic biodigestion of manure from specialized dairy and beef cattle breeds fed essential oils by using batch anaerobic digesters as an alternative to waste treatment and biogas production.

5.2 MATERIAL AND METHODS

5.2.1 Study location and ethical issues

The study was conducted in the Ruminant Nutrition Laboratory from School of Veterinary Medicine and Animal Sience of the University of São Paulo, on *campus* Fernando Costa in Pirassununga, Brazil. The experiment was approved by and complied with the guidelines set out by the Ethics Committee in the Use of Animals of the School of Veterinary Medicine and Animal Sience, under application number nº 8453300914, in respect to animal experimentation and care of animals used for scientific purposes.

5.2.2 Animal feeding, housing and feces collection

Eight cows, not pregnant and non-lactating, were arranged in individual sand bed pens and had free access to water. They were fed *ad libitum* twice daily (08h00 and 16h00). Feed was weighted daily and offered to each animal after removal of the previous day orts. Essential oils were weighted daily and mixed into the concentrate moments before the diet was offered. The dosage was 500mg/kg of DM per animal per day. Four animals were specialized dairy breed and four specialized beef breed. The dairy cattle used were Holstein (*Bos taurus taurus*), with average weight of de 905±62 kg, and the beef cattle were Nellore (*Bos taurus indicus*), with average weight of 535±54 kg. These two racial groups were chosen because they are most representative of the of dairy and meat production systems in Brazil.

For feeding and feces collection, the animals were allocated in two contemporary 4 x 4 latin square design, in 2x4 factorial arrangements (referring to two specialized cattle breeds and four diets). Each experimental period consisted of 24 days. The first 19 days were for adaptation to the diet and the last 5 days for fecal collection. Feces were manually collected via the rectum at 8:00 a.m. and 4:00 p.m., then frozen at -20°C and pooled to form a single sample for each animal in each period. Urine samples were obtained from all cows on the 24nd day of each experimental period. The collection was performed every 6 hours, during urinary stimulation by vulva massage and then stored at -20ºC in a single vial, which formed a single composite sample in 24 hours.

The anaerobic biodigestion was developed in mesophilic conditions (30 to 35ºC) ideal for the digestion kinetics (METCALF; EDDY, 2014), and the digesters were allocated inside a climatic chamber with an electric resistance heating system and digital temperature controller.

5.2.3 Substrates, experimental design and treatments

Samples were composed of a blend of feces and urine and were diluted in water; this sample adopted a total solids content (ST) of 6%. Three kilos of substrate were prepared, of which 2 kg were used to supply the biodigesters and 1 kg to perform the characterization analyzis of the substrates. The composition of the substrates had the following ratio: 39.2% manure (83% of feces and 17% of urine), 11.3% inoculum and 49.5% water. The biodigesters were organized in a completely randomized design, in a 2x4 arrangement, with the two cattle breeds (dairy and beef) and four diets. The diets offered (Table 1) differed only in regards to type of essential oil added. These were: CNT without any additive, the EEO containing 500 mg/kg of DM of eucalyptus essential oil (*Eucalyptus citriodora*, extracted from the leaves), PEO containing 500 mg/kg of DM of Brazilian peppertree essential oil (*Schinus terebinthifolius Raddi*, extracted from the fruits) and LEO containing 500 mg/kg of DM of lemongrass essential oil (*Cymbopogon citratus Stapf*, extracted from the leaves). This totaled 32 experimental units and 4 replicates for each treatment. The treatments and respective characterizations of the substrates are presented in Table 12.

Table 12. Characteristics of substrates used in batch digester test

Nutrients	Cattle		Essential Oils			
	Dairy	Beef	CNT	PEO	LEO	EEO
TS, g/kg	45.50	48.49	47.55	45.55	47.15	47.74
VS, g/kg	38.30	40.66	39.81	38.22	39.71	40.18
N, g/kg TS	38.34	37.94	37.92	37.87	39.76	36.99
NDF, g/kg TS	591.2	534.8	551.3	537.6	610.5	552.5
ADF, g/kg TS	415.5	391.9	409.9	400.9	405.1	398.7
EE, g/kg TS	20.30	19.82	22.60	19.77	19.80	18.07
pH	6.69	6.81	6.75	6.73	6.75	6.78

CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, TS = total solid; VS = volatile solid; N = nitrogen; NDF = neutral detergent fiber; ADF = acid detergent fiber; EE = ether extract

5.2.4 Biogas measurement

In order to evaluate the potential for production of manure gases, bench batch anaerobic digesters were used (Figure 5). Biodigesters were conditioned in a climatic chamber with controlled temperature between 30 and 35ºC, ensuring that the anaerobic biodigestion test occurred in mesophilic conditions.

Frequency of biogas measurement was conducted following gasometer capacity. Biogas volume was calculated using gasometer vertical displacement, which was measured in centimeters. Biogas volume was determined by the displacement and gasometers internal cross-sectional area and corrected to 1 atm and 20ºC according to the methodology of Lucas Junior (1994).

The biogas samples were conducted together with the measurement of the biogas volume. Samples were collected using a 60 mL syringe connected to the gas register at the top of the gasometer. Before the actual sampling, the biogas was collected and used to flush the bottle (twice), after which 50 mL of biogas were injected to analyze its composition. After the biogas sampling, the gasometers were emptied; this allowed a new accumulation of gases. The test was terminated when the biogas production ceased (there was no displacement of the gasometer). This process took place approximately 5 months after the biodigesters were supplied.

The concentrations of CH_4, CO_2 and N_2O were determined by gas chromatography (Trace 1300, Thermo Fisher Scientific®, Rodano, Milan, Italy) under controlled temperature (25°C) conditions according to Kaminski et al. (2003). The biogas samples were diluted in glass flasks with known volume, 16.78 times in synthetic air, and then 6 mL of the diluted sample were manually injected through a syringe into the chromatograph injector (split/splitless). 4 mL of the sample were used to wash the injection system and 2 mL were used for the analysis. The chromatograph was calibrated with 3.1% of CH_4, 3.1% of CO_2 and 0.49% of N_2O, diluted in synthetic air. A gas mixture of 50% CH_4 and 50% CO_2 was used as the reference gas. The carrier gas was helium, and the flow rate was 30 mL $minute^{-1}$. The volumes of CH_4, CO_2 and N_2O produced (m^3 or L) were calculated by multiplication of biogas volume obtained at the gasometer and concentration of each gas.

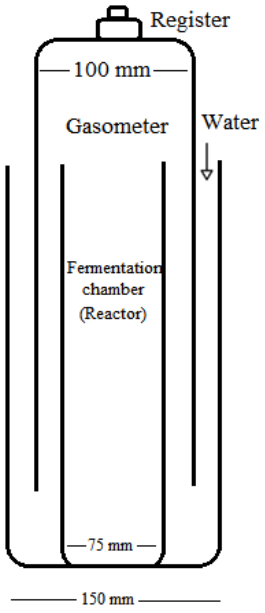
For the study of the kinetics of biogas production and its components, the linear mathematical model of Gompertz was used. This model assumes that the gas production rate is proportional to the microbial activity, but the proportionality decreases with the incubation time,

with the loss of efficiency in the fermentation rate (LAVRENCIC et al., 1997). The following equation describes the model used:

$$y_t = A \exp [-B \exp (-kt)]$$

Where: y_t: gas production (L/g VS added) at time t (day); A: asymptote of the model, indicates the stabilization value of the production (L/g VS added) in relation to time t; B: integration constant, without biological meaning; k: growth rate, logarithmic function of the production growth (L / g SV added) per unit of time (t).

Figure 5. Layout of bench batch anaerobic digesters



Source: Carvalho (2018).

5.2.5 Nutrients removal

The substrates added to and eliminated from each biodigester were weighted and multiplied by their percentage of DM content in order to calculate the DM content in grams. The added and eliminated nutrients, expressed in (g), were calculated by multiplying the added or eliminated values, expressed as grams of DM, with the values of the nutrients added or eliminated; this was then expressed as a percentage and divided by 100 according to the following equation:

$$\text{Nutrient (g)} = \frac{\text{nutrient added or eliminated (\%)x DM (g)}}{100}$$

Nutrient removals, expressed as percentages, were calculated using digestate and ingestate nutrient contents and expressed as grams per kilogram of DM according to the equation:

$$\text{Nutrients removal (\%)} = \frac{\text{nutrient added (g)} - \text{nutrient eliminated(g)}}{\text{nutrient added (g)}} \text{ x } 100$$

5.2.6 Laboratory analysis

Samples of the substrates before and after anaerobic digestion were collected and dried in an oven with ventilation and constant air renewal at 55ºC for 72 hours, according to AOAC (1995). After drying, the samples were ground in a Wiley mill in 1 mm sieves and stored in properly closed vials. The DM concentration was determined at 105°C for 4 h in an oven (method 930.15; AOAC, 1995). Neutral detergent fiber (NDF) and acid detergent fiber (FDA) were determined by the method described by Van Soest et al. (1991), the NDF being obtained with the use of thermostable α-amylase. The total N content was determined by the micro-Kjeldahl technique (method 920.87; AOAC, 1990). The mineral matter (MM) or ash was obtained by calcination in a muffle oven at 550ºC for 5h (AOAC, 1990). The total solid contents (ST at 105ºC = 100 - Humidity) and volatile solids (SV = ST - MM) of the substrates were determined with adaptations to the methodology described in APHA (2005). The hydrogenionic potential (pH) was measured by portable pH meter (Hanna Instruments®, HI 8424, Italy).

5.2.7 Statistical analysis

The experimental design used for the study was a completely randomized design with 32 experimental units (digesters) for 8 treatments (4 diets with essential oils x 2 specialized cattle breeds) and 4 repetitions. Before the actual analysis, the data were analyzed for the presence of disparate information ("outliers") and normality of residuals (Shapiro-Wilk). Statistical data analyses were performed with the Statistical Analysis System software (Version 9.3 SAS Institute Inc., Cary, NC, USA) using mixed model. The data were submitted to analysis of variance, which separated as causes of variation essential oils, specialized cattle breeds and their interaction as fixed effects. In the presence of the essential oil effects, the comparison of means was performed by the Pdiff test. Non-significant (NS) was considered when P value was higher than 10%, and statistical significance was considered when P value was less than 5%.

5.3 RESULTS

5.3.1 Biodigestion and nutrient removal

The essential oils and specialized cattle breeds did not change the nutrients of the substrate added in the digesters (Table 13), which had on average 93.9g of TS, 78.9g of VS, 22.0g of CP, 52.0g of NDF, 38.1g of ADF and 1.8g of EE per gram of volatile solid added. The manure from beef cattle had higher pH value for substrate and higher amount ($P < 0.05$) of ST, VS, NDF, ADF or EE eliminated (nutrients remaining after the anaerobic biodigestion process) but lower efficiency of removal of NDF or ADF than did the dairy cattle.

Table 13. Efficiency of nutrient removal in batch anaerobic digesters supplied with manure of dairy and beef cattle fed with different essential oils

Variables	Cattle		Essential oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Added nutrients (g/biodigester)										
TS. g	91.71	96.28	94.47	95.84	89.79	95.89	1.98	NS	NS	NS
VS. g	77.22	80.71	79.50	80.06	75.31	80.98	1.68	NS	NS	NS
N. g	3.44	3.61	3.56	3.55	3.47	3.51	0.043	0.0628	NS	NS
NDF. g	53.16	50.99	51.78	50.38	53.70	52.43	0.58	0.0652	NS	NS
ADF. g	38.78	37.56	38.69	37.93	38.03	38.02	0.55	NS	NS	NS
EE. g	1.82	1.91	2.14	1.86	1.74	1.72	0.14	NS	NS	NS
pH	6.69	6.81	6.75	6.73	6.75	6.78	0.0257	0.0328	NS	NS
Eliminated nutrients (g/biodigester)										
TS, g	57.47	64.54	60.90	62.52	61.00	59.58	1.17	0.0026	NS	NS
VS, g	42.12	47.59	44.66	46.04	45.03	43.71	0.94	0.0039	NS	NS
N. g	1.31	1.38	1.39	1.38	1.33	1.29	0.023	NS	NS	NS
NDF, g	18.67	22.24	20.33	21.28	20.05	20.16	0.605	0.0029	NS	NS
ADF, g	14.97	16.99	15.62	17.30	15.91	15.10	0.49	0.0456	NS	NS
EE, g	0.55	0.67	0.57	0.66	0.62	0.59	0.034	0.0961	NS	NS
pH	7.29	7.36	7.33	7.30	7.32	7.35	0.0145	0.0162	NS	NS
Nutrient Removal Efficiency										
TS, %	36.41	32.79	35.49	33.97	31.00	37.94	1.31	NS	NS	NS
SV, %	44.64	40.90	43.78	41.91	39.24	46.15	1.19	NS	NS	NS
N, %	61.51	61.48	60.66	60.86	61.42	63.04	0.86	NS	NS	NS
NDF, %	64.67	56.35	60.60	57.62	62.53	61.29	1.30	0.0008	NS	NS
ADF, %	61.34	54.80	59.53	54.50	57.77	60.47	1.22	0.0051	NS	NS
EE,%	67.86	63.41	67.86	67.14	66.24	61.31	2.65	NS	NS	NS

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = standard error of the mean, C = specialized cattle breeds effect, O = essential oils effect, CxO = interaction among specialized cattle breeds and essential oils, NS = non-significant (P > 0,10), TS = total solid; VS = volatile solid, N = nitrogen, NDF = neutral detergent fiber; ADF = acid detergent fiber EE = ether extract.

5.3.2 Biogas production

Manure from dairy cattle had higher biogas production (L/gVS add), CO_2 (liters, L/gVS add and percentage) and N_2O (mL/gVS add); however, it had lower CH_4 percentage in relation to the manure of beef cattle (Table14).

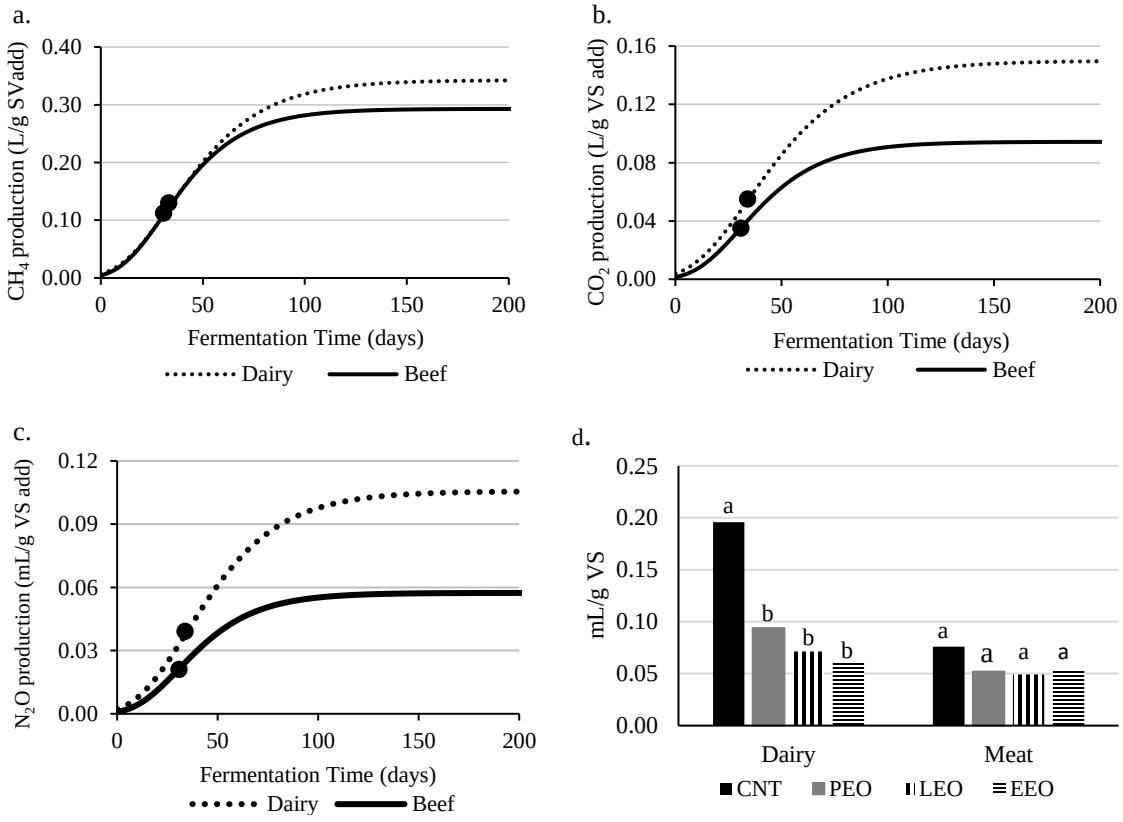
Asymptotic production (a) of CH_4 and CO_2 were higher ($P < 0.05$) for manure of dairy cattle, and it also had higher production of CH_4 and CO_2 at the inflection point (y). The production constants (k) for CH_4, CO_2 and N_2O were higher ($P < 0.05$) for manure of beef cattle (Figure 6).

Table 14. Biogas, CH_4, CO_2 and N_2O production in batch anaerobic digesters supplied with manure of dairy and beef cattle fed with different essential oils

Variables	Cattle		Essential oils				SEM	P value		
	D	B	CNT	PEO	LEO	EEO		C	O	CxO
Biogas, L	39.75	36.89	39.33	39.54	37.15	37.27	0.863	NS	NS	NS
Biogas,L/VSadd	0.524	0.459	0.501	0.534	0.468	0.467	0.016	0.0364	NS	NS
Biogas,L/VSred	1.137	1.073	1.068	1.248	1.061	1.044	0.031	NS	0.0726	NS
CH_4, L	28.33	26.93	28.48	27.91	26.86	27.30	0.545	NS	NS	NS
CH_4, L/VSadd	0.373	0.336	0.363	0.376	0.339	0.341	0.009	0.0772	NS	NS
CH_4, L/VSred	0.811	0.784	0.776	0.880	0.771	0.765	0.020	NS	NS	NS
CH_4, %	72.32	73.93	73.12	72.54	73.19	73.57	0.278	0.0064	NS	NS
CH_4/gVS added										
a, L/g	0.367	0.314	0.339	0.348	0.338	0.335	0.007	0.0026	NS	NS
k, L/g.dia	0.042	0.050	0.046	0.047	0.044	0.047	0.094	0.0074	NS	NS
t, dia	33.45	30.83	30.68	32.77	34.02	31.10	0.792	NS	NS	NS
y, L/g	0.144	0.120	0.147	0.134	0.124	0.123	0.004	0.0046	NS	NS
CO_2, L	10.989	9.619	10.775	10.796	9.833	9.8119	0.307	0.0466	NS	NS
CO_2, L/VSadd	0.144	0.120	0.137	0.146	0.124	0.123	0.005	0.0194	NS	NS
CO_2, L/VSred	0.314	0.277	0.287	0.341	0.280	0.275	0.009	0.0614	0.0734	NS
CO_2, %	27.66	26.08	27.02	27.76	26.43	26.26	0.301	0.0154	NS	NS
CO_2/gVS added										
a, L/g	0.161	0.101	0.161	0.127	0.132	0.103	0.001	0.0062	NS	NS
k, L/g.dia	0.041	0.050	0.048	0.046	0.040	0.047	0.002	0.0169	NS	NS
t, dia	34.08	30.90	30.66	32.94	34.49	31.86	0.86	0.0708	NS	NS
y, L/g	0.059	0.037	0.059	0.047	0.048	0.037	0.004	0.0062	NS	NS
N_2O, mL	6.342	4.780	6.368	6.369	4.778	4.7290	0.421	0.0580	NS	NS
NO_2, mL/VSadd	0.085	0.059	0.081	0.091	0.060	0.059	0.006	0.0373	NS	NS
NO_2, mL/VSred	0.176	0.148	0.188	0.194	0.133	0.132	0.012	NS	NS	NS
N_2O, %	0.016	0.013	0.015	0.016	0.013	0.013	0.001	NS	NS	NS
N_2O /gSV added										
a, mL/g	0.106	0.057	0.136	0.073	0.060	0.057	0.009	0.0005	0.0004	0.0194
k, mL/g.dia	0.039	0.047	0.043	0.0429	0.0413	0.0444	0.001	0.0033	NS	NS
t, dia	33.84	30.83	30.68	33.54	34.03	31.09	0.816	0.0695	NS	NS
y, mL/g	0.039	0.021	0.050	0.027	0.022	0.021	0.003	0.0005	0.0004	0.0191

D = dairy, B = beef, CNT = Control, PEO = Brazilian peppertree essential oil, LEO = lemongrass essential oil, EEO = eucalyptus essential oil, SEM = standard error of mean, C = specialized cattle breeds effect, O = essential oils effect, CxO = interaction among specialized cattle breeds and essential oils, NS = non-significant (P > 0,10), VSadd = volatile solid added, VSred = volatile solid reduced, a = asymptotic production (L/g VS added); k = production constant (L/g of SV added per day); t = time at inflection point (day); y = production at inflection point (L/g SV added).

Figure 6. Cumulative production of CH_4 (a), CO_2 (b), N_2O (c) and N_2O asymptotic production (d) adjusted by the Gompertz model in batch anaerobic digesters supplied with manure of specialized dairy breed and specialized beef breed fed with different essential oils



An interaction between essential oils and specialized cattle breeds was observed for asymptotic production (P = 0.0194) and production value at the inflection point for N_2O (P = 0.0191). The manure of dairy cattle fed with essential oils had a decrease in asymptotic production (Figure 6d) or production at the inflection point (Figure not showed), but this was not observed at beef cattle.

5.4 DISCUSSION

Several studies have quantified diet or feed additive effects on enteric methane production, but few have measured the dietary effects or feed additives on methane from manure. González-Avalos and Ruiz-Suárez (2001) have shown that diet is the most important factor determining

differences in methane emission from manure. According to Orrico Junior et al. (2011), the greater the amount of fiber in the manure, the lower will be the biogas production potential. According to Ruiz and Floats (2016) the presence of essential oils in waste can inhibit anaerobic biodigestion.

The main effects of essential oils in the rumen are the reduction of protein and starch degradation and the inhibition of amino acid degradation (HART et al., 2008), which could increase the presence of these nutrients in manure. In the present study, the diet differed only regarding essential oil, and theses feed additives did not change the nutrients of manure added in the digesters. No difference was observed between specialized cattle breeds.

The efficiency of nutrient removal of manure from dairy cattle was higher; consequently, there was greater fermentation of these nutrients, which generated 12% more biogas (L/VSadd) from their manure. González-Avalos and Ruiz-Suárez (2001) also observed small differences among dairy cattle and beef cattle in different climates; however, these authors observed higher productions of biogas for beef cattle in temperate climates. The temperature in the present study was maintained between 30 to 35 ºC, which is different than the climate temperate observed in that study.

The removal efficiency of the organic matter (represented by the SV) was 26.5%. This value is in agreement with Dohányos and Zábranská (2001), who affirmed that the organic matter removal is between 25 and 50% in mesophilic conditions. According to Davidsson et al. (2008), the removal efficiency of VS in cattle manure is typically between 30-45%, values close to those found in the present study.

Although the pH values of the affluent and effluent were statistically different for dairy and beef cattle, they remained close to 7. This result corroborates those found by El-Mashad and Zhang (2010), when analyzing manure from cattle and food waste. These authors found values of 7.2 for the pH and considered that the internal environment conditions of the biodigesters were enough for the biogas production.

The substrate composition will affect the biogas yield, which has methane (CH_4) as a main component. This gas has a high calorific and energetic power. The manure from a diet with a higher proportion of concentrate will produce larger amounts of biogas and methane. In the present study, the proportion of methane was 68%, and this value is in agreement with the proportion found by Orrico Junior et al (2012) when evaluating manure from diets containing a high proportion of roughage. The methane or biogas production of the manure from diets with the addition of essential

oils was not negatively affected. Some studies have shown that the presence of compounds from essential oils may hinder the anaerobic digestion process (RUIZ et al., 2016; RUIZ; FLOTATS, 2016). The essential oil antimicrobial mechanism is based on cell malfunction and lysis. Once dissolved in the aqueous medium, the essential oil accumulate in the membrane of the microorganisms and changes its structure. The membrane fluidity changes, becomes more permeable and finally leakage of the cell contents occurs. This inhibitory effect is dependent on its composition and concentration of the compounds.

Methane specific productivity was reduced to 0.745 L/gVS, which usually corresponds to the theoretical methane yield (IPCC, 2006). This indicates the integrity of the degradation of organic feces components. The final methane yield was 0.331 L/gVS added, and this will always be less than the theoretical yield because a fraction of the substrate is used to synthesize the bacterial mass, so a fraction of the organic material will be lost in the effluent, and the compounds containing lignin will only be degraded to a graduation limit (FRANCO et al., 2007).

The production asymptotic (a) of CH_4 or CO_2 was higher for the manure from dairy cattle (Table 14); however, production rates (k) of CH_4, CO_2 and N_2O were higher for beef cattle. If there was a higher production rate when production was stabilized - the time needed to reach the inflection point of the curve (Figures 6, 7 and 8) - the production maximum potential could have been anticipated. This would contribute to a reduction of the hydraulic retention time (HRT); however, this fact was not observed in the present study.

The emission of nitrous oxide is dependent of dissolved oxygen concentration in both nitrification and denitrification processes. In nitrification, oxygen maximizes the production of N_2O (KAMPSCHREUR et al., 2008). In denitrification, oxygen inhibits the expression of nitrous oxide reductase, which, because of its greater inhibition by oxygen than the other enzymes involved, causes N_2O to accumulate in the medium (TSUNEDA et al., 2005). The relationship between carbon and nitrogen (C/N) is also an important control parameter for N_2O production. Production rate (k) was higher for beef cattle. It was also possible to observe interaction between the essential oils and the specialized cattle breeds on asymptotic production (Figure 6d) or at inflection point. Inhibition of essential oils was only in dairy cattle and followed the same pattern for asymptotic production or inflection point. Dairy cattle had the N_2O production (mL/gVSadd) decreased in 52% by PEO, 63% by LEO, 69% by EEO, and; beef cattle did not demonstrate these decreases. The presence of N_2O during the anaerobic digestion process is minority among the

gases. In the complete denitrification process, the nitrate is reduced to nitrogen gas; however, this conversion involves some intermediary steps mediated by microorganisms, which give rise to nitric oxide (NO) or nitrous oxide (N_2O). Essential oils may have an inhibitory effect on these intermediate bacteria of dairy cattle by reducing the nitrous oxide production. This different effect between specialized cattle breeds may due to the micribiota that were installed with the feces. It is known that animals of different genetic groups may have a different microbial population in their gastrointestinal tracts (HUNGATE, 1957; MACKIE et al., 1978; FRANZOLIN & DEHORITY 1996). This decrease in the production of N_2O from manure caused by the essential oils is beneficial, because N_2O is considered one of the gases with the highest potential of global warming, being 298 times more powerful than CO_2.

5.5 CONCLUSIONS

The addition of essential oils did not change the composition of the manure or its biodegradation in anaerobic digesters. The manure from dairy cattle had higher biogas production, but lower CH_4 percentage. The manure from beef cattle, however, had higher production rate for CH_4, CO_2 and N_2O. Essential oils interacted with the dairy cattle manure, decreasing the N_2O total and the production at the inflection point, this was observed only with dairy cattle. Biogas production was not negatively affected by the addition of essential oils in the diets of dairy or beef cattle. The biodigestion process of manure in the anaerobic digester is a good alternative to mitigate the emission of greenhouse gases, besides generating energy return for the cattle rancher.

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