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GUILHERME ACÁCIO DE SENE

The strategic combination of feed additives aimed at manipulating ruminal kinetics and fermentation to increase energy efficiency and mitigate enteric methane emission and waste in ruminants

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Thesis presented to the College of Animal Science and Food Engineering at the University of Sao Paulo, as part of the requirements for obtaining the title of Doctor of Science.

Area of Concentration: Animal Quality and Productivity

Advisor: Prof. Paulo Henrique Mazza Rodrigues, Ph.D.
Co –Advisor: Flávio Perna Junior, Ph.D

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RESUMO

SENE, G. A. **Combinação estratégica entre aditivos alimentares visando a manipulação da cinética e da fermentação ruminal para aumento da eficiência energética e mitigação da emissão de metano entérico e dos dejetos em ruminantes** 2021. 131 f. Tese (Doutorado em Ciência) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2021.

Este estudo teve como objetivo avaliar o uso de óleos essenciais, enzimas exógenas, monensina sódica e suas associações como estratégia nutricional visando mitigar as emissões de CH_4 ruminal de vacas Nelore e seus impactos sobre a biodigestão anaeróbica dos dejetos. A tese foi estruturada em dois estudos. No estudo 1, foram utilizadas oito vacas Nelore canuladas (480 ± 55 kg), distribuídas em delineamento quadrado latino 4 x 4 duplicado, em esquema fatorial 2 x 2 x 2 (4 períodos de 28 dias) totalizando 4 repetições (32 unidades experimentais). Os animais receberam dieta basal composta por 60% de silagem de milho e 40% de concentrado, sendo o fornecimento diário ajustado em função do CMS. Os fatores foram compostos pela presença ou ausência de enzima exógena (1027 mg/kg MS), óleo essencial (31.7 mg/kg MS) e monensina (30.6 mg/kg MS) na dieta. Os dados foram analisados usando PROC MIXED do SAS e as diferenças foram declaradas significativas a 5%. A associação entre os aditivos promoveu alterações no CMS, se mostrando antagônica sobre a eficiência microbiana, sobre a energia digestível perdida na forma de CH_4 ruminal e sobre energia bruta liberada no intestino. No entanto não foram observados efeitos associativos entre os aditivos sobre a produção de AGCC. No estudo 2, utilizou-se biodigestores anaeróbios experimentais do tipo batelada, em delineamento inteiramente casualisado (32 unidades experimentais) para avaliar a biodigestão anaeróbica dos dejetos de vacas Nelore alimentadas com monensina sódica, enzima exógena, óleo essencial e suas combinações. Os dados também foram analisados usando PROC MIXED do SAS e as diferenças foram declaradas significativas a 5%. A associação entre os aditivos testados não promoveu alterações no processo de biodigestão, no entanto, a utilização de monensina sódica demonstrou reduzir o potência de produção de biogás e a concentração de nutrientes no biofertilizante. Deste modo, a associação entre os aditivos testados como estratégia nutricional não demonstrou ser capaz de reduzir as emissões de CH_4 ruminal, não apresentando efeito sobre a biodigestão anaeróbica dos dejetos.

Palavras-chave: Impactos ambientais, Bovinos de corte, AGCC, Digestibilidade, Biogás.

ABSTRACT

SENE, G. A. **Strategic combination of feed additives aimed at manipulating ruminal kinetics and fermentation to increase energy efficiency and mitigate enteric methane emission and waste in ruminants.** 2021. 131 f. Thesis (PhD in Science) – College of Animal Science and Food Engineering, University of Sao Paulo, Pirassununga, 2021.

This study aimed to evaluate the use of essential oils, exogenous enzymes, sodium monensin, and their associations as a nutritional strategy to mitigate ruminal CH_4 emissions from Nellore cows and their impacts on the anaerobic biodigestion of manure. The thesis was structured in two studies. In study 1, eight cannulated Nellore cows (480 ± 55 kg) were used, distributed in a duplicated 4 x 4 Latin square design, in a 2 x 2 x 2 factorial scheme (4 periods of 28 days) totaling 4 repetitions (32 experimental units). The animals received a basal diet composed of 60% corn silage and 40% concentrate, with the daily supply being adjusted as a function of DMI. The factors were composed by the presence or absence of exogenous enzyme (1027 mg/kg DM), essential oil (31.7 mg/kg DM), and monensin (30.6 mg/kg DM) in the diet. Data were analyzed using SAS PROC MIXED and differences were declared significant at 5%. The association between the additives promoted alterations in the DMI, being antagonistic on the microbial efficiency, on the digestible energy lost in the form of ruminal CH_4, and on the gross energy released in the intestine. However, no associative effects were observed between the additives on the production of SCFA. In study 2, experimental anaerobic batch-type biodigesters were used in a completely randomized design (32 experimental units) to evaluate the anaerobic biodigestion of waste from Nellore cows fed with sodium monensin, exogenous enzyme, essential oil, and their combinations. Data were also analyzed using SAS PROC MIXED and differences were declared significant at 5%. The association between the tested additives did not change the biodigestion process, however, the use of sodium monensin has been shown to reduce the biogas production potency and the concentration of nutrients in the biofertilizer. Thus, the association between the tested additives as a nutritional strategy did not prove to be able to reduce ruminal CH_4 emissions, with no effect on the anaerobic biodigestion of manure.

Keywords: Environmental impacts, Beef Cattle, SCFA, Digestibility, Biogas.

Summary

1. INTRODUCTION

The growth of the world population, which is estimated to rise to 9.8 billion people in 2050 and 11.2 billion in 2100 (UN, 2017), together with socioeconomic issues imposed by modern society, has promoted a sharp increase in world demand for food. Bruinsma (2009) predicted the need to increase agricultural production by 70% by the year 2050, with a large part of this production going to animal feed due to the increase in meat demand, which would come out of the current ones 37 kg per inhabitant to 52 kg in 2050.

This sharp increase in the demand for food, together with environmental issues, poses an enormous challenge to agriculture in the coming years, since, despite the importance of agriculture for food production, job and income generation, the environmental impact caused across the sector has been the focus of major discussions regarding terrestrial climate stability (HRISTOV et al., 2013; IPCC, 2014). This is because the increase in the concentration of greenhouse gases (GHG) would lead to an increase in the temperature of the earth's surface, which could cause everything from changes in rain regimes to an increase in the incidence of extreme weather events.

Within this scenario, the agricultural sector represents a significant source of GHG emissions worldwide, accounting for about 10 to 12% of global anthropogenic GHG emissions (IPCC, 2014), with emphasis on methane emissions (CH_4) and nitrous oxide (N_2O)..Despite having a lower atmospheric concentration than CO_2, it has a global warming potential 25 and 298 (both based on a 100-year projection) greater than CO_2, respectively (IPCC, 2007).

According to the IPCC (2014), analyzing non-CO_2 emissions, enteric fermentation of ruminants is the main emitter and responsible for about 30 to 40% of agricultural emissions, followed by the emission of waste deposited in pastures, which accounts for 15% of agricultural emissions. Analyzing the Brazilian scenario, in 2016 the agricultural sector was responsible for 29% of net GHG emissions, with waste management being responsible for 4% of emissions and enteric fermentation (CH_4) responsible for 65% (SEEG, 2018).

The CH_4 produced by ruminants, through methanogenesis, is not only related to environmental problems, but is also associated with energy losses and, consequently, reductions in the retention and use of ingested energy (MOUMEN et al., 2016). Although methanogenesis is a natural process, intrinsic to ruminants and essential for ruminal metabolism, it has a direct relationship with the efficiency of rumen fermentation due to

the loss of carbon, representing energy loss for the system and consequently influencing animal performance. According to Buddle et al. (2011), 5% to 9% of the diet's gross energy is lost in the form of CH_4.

The problems generated by CH_4 have encouraged researchers to look for alternatives aimed at mitigating CH_4 produced by ruminants worldwide (MARTIN et al., 2010). In addition to efficient production systems, pasture management techniques, and animal genetic improvement, several nutritional strategies, such as ionophores, tannins, calcium nitrate, essential oils, lipids, microbial enzymes, CH4 inhibitors, among others (MOHAMMED et al., 2004; REIS et al., 2006; GERBER et al., 2013) have been used to manipulate the rumen environment and reduce CH_4 emission. However, few have shown a substantial, persistent, and consistent decrease in the level of contemporary expectation of the technical-scientific community, especially when evaluated in vivo.

On the other hand, advances in the area of knowledge about the use of medicated drugs in an associated way proved to be fundamental to assist in the search for solutions to some challenges faced by modern medicine. Simultaneous prescription of several drugs is a practice commonly used in the medical field, to improve the effectiveness of drugs, reduce toxicity or treat coexisting diseases, which may result in a synergistic effect, where the result of the association is greater than the simple sum of the drugs. effects of isolated drugs (SECOLI, 2001). Within this perspective, it is important to emphasize that the different additives used as modifiers of ruminal metabolism do not decrease CH_4 production through a single mechanism of action.

Sodium monensin presents itself as a classic manipulator of the rumen environment, directing the H_2 that would be used for the production of CH_4 for the production of propionic acid, thus causing a change in ruminal patterns with increased energy efficiency (McGUFFEY et al., 2001).

Essential oils such as cinnamaldehyde and garlic oil have antimicrobial properties and the potential to modulate rumen fermentation, being mostly investigated in in-vitro experiments (BUSQUET et al., 2005a, b, c, 2006; CALSAMIGLIA et al., 2007; MATEOS et al., 2013; BLANCH et al., 2016). Among its main advantages, the low risk of microbial resistance stands out, since these compounds present, in most cases, several active principles, which give different modes of action (BROOKER, 2005).

Exogenous enzymes, in turn, appear as alternatives that can promote improvements in digestibility and use of the offered diet; because, even in conditions where the rumen has a high rate of fermentation in the diet, it is still possible to observe the elimination of degradable fibers and starch in the feces. (GALLARDO et al., 2009; TRICARICO et al. 2007). According to Collazoz Paucar (2017), the associated use of different exogenous enzymes showed improvement in rumen fermentation when compared to the use of enzymes used in isolation.

Because the food additives mentioned above do not have a unique and exclusive mechanism of action, nothing prevents their effects from being additive (the result of the combination is equal to the sum of the parts) or even synergistic (the combined result is greater than the sum of the parties). It is of great interest to the scientific community to understand the associative use of these additives and demonstrate that the association of these compounds can increase the mitigation of CH_4 and improve the energy efficiency of animals.

Given this context, it is hypothesized that the association between essential oils, exogenous enzymes, and monensin, can improve rumen fermentation, increase the energy available to the animal and reduce the production of enteric CH_4. Therefore, the present experiment aims to evaluate the association between essential oils, a blend of exogenous enzymes, and sodium monensin as a nutritional strategy aimed at modulating rumen fermentation by increasing the energy available to the animal and reducing the production of enteric CH_4 from Nellore cattle. (Bos taurus indicus). As specific objectives, it is desired to measure other variables, such as food consumption, ingestive behavior, the population of ciliated protozoa, ruminal kinetics and degradability, digestibility and excretion of nutrients, as well as the production of biogas (CH_4, N_2O, and CO_2) from waste through anaerobic digesters.

2. BIBLIOGRAPHIC REVIEW

2.1. Methane production and global warming

The agricultural sector has been the focus of criticism in recent years due to the increase in concentrations of greenhouse gases in the atmosphere and its relationship with the rise in the temperature of the earth's surface. According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC, 2014), agriculture is responsible for about 10 to 12% of global anthropogenic GHG emissions, with emphasis on CH_4 and N_2O. Despite having a lower atmospheric concentration than CO_2, they have

global warming potential 25 and 298 (both based on a 100-year projection) greater than CO_2, respectively (IPCC, 2007).

According to the IPCC (2014), analyzing non-CO_2 emissions, the enteric fermentation of ruminants is the main emitter and responsible for about 30 to 40% of agricultural emissions, reaching, in 2010, average values of 2.1 Gt $C0_2$eq, followed by the emission of waste deposited on pastures, which are responsible for 15% of agricultural emissions. We should highlight the emissions from production systems in Asia and the Americas, which represent 75% of the total CH_4 emissions (IPCC, 2014).

One of the main contributors to GHG emissions cited is Brazil. When we analyze the Brazilian scenario, we observe that in 2016 the agricultural sector was responsible for 29% of the Brazilian net GHG emissions, with waste management being responsible for 4 % of emissions and enteric fermentation (CH_4) responsible for 65% (SEEG, 2018).

Such criticisms of Brazilian cattle farming are based on the size of the national herd, which is the second-largest in the world with 232.35 million head, behind India only (USDA, 2018). Also on slaughter age, it varies between 3 and 3 1/2 years, and in the breeding system predominantly in pastures (PEREIRA E PEDREIRA, 2016).

In 2015, during the UN Summit for Sustainable Development, Brazil made a commitment to reduce GHG emissions by 43% by the year 2030 (MMA, 2015). In this way, it is possible to observe great interest from government agencies, as well as civil society as a whole, in discussing and reducing GHG emissions.

However, a critical aspect for the adoption of mitigation practices by ranchers and farmers is that such practice brings economic benefits to their production activity (HRISTOV et al., 2013). Thus, when we talk about the CH_4 produced by ruminants through rumen fermentation, it, besides being related to environmental problems, also represents energy loss for the system and, consequently, decrease in animal performance, since, according to Buddle et al . (2011), 5% to 9% of the diet's gross energy is lost in the form of CH_4. Thus, it is in the interest of livestock farmers to mitigate the production of such gas.

2.2. Ruminal fermentation and methanogenesis

Ruminants are animals that are able to digest and convert plant materials rich in fiber (cellulose and hemicellulose) into foods of high biological value, such as milk and

meat, without directly representing a form of competition for human consumption (FURLAN et al ., 2011).

Such capacity is due to the main digestive organ of ruminants, the rumen, which is considered a fermentation chamber (DAVIDSON; STABENFELDT, 2014), as well as to the complex microbial ecosystem present in it, composed of bacteria, protozoa, fungi and methanogenic Archeas. (KOZLOSKI, 2002).

During rumen fermentation, due to the metabolism of food eaten, microbial activity is essential, as it allows the conversion of structural (cellulose and hemicellulose) and non-structural (starch) carbohydrates into short-chain fatty acids (SCFA). Mainly acetic acid, propionic, and butyric, used by the ruminant as an energy source, the synthesis of high-quality microbial protein and the production of CH_4 and CO_2, useless for ruminants and which are eliminated through eructation (VAN SOEST, 1994; MARTIN et al., 2009; LI et al., 2014).

Ruminal fermentation, because it involves oxidative processes, ends up generating reduced cofactors (NADH, NADPH and FADH) and, in order for the fermentation process not to be paralyzed, these cofactors need to be re-oxidized ((NAD +, NADP + and FAD +) through reactions dehydrogenation, releasing hydrogen in the rumen (MACHADO et al., 2011).

As an electron acceptor process, methanogenesis is part of the normal digestive process of ruminant herbivores (VAN SOEST, 1994), acting continuously in the removal of H_2 from the medium. Thus, methane formation is essential for the optimal performance of the ruminal ecosystem. It prevents the accumulation of H_2 in the rumen, which could lead to the inhibition of dehydrogenase activity, involved in the re-oxidation of reduced cofactors (MACHADO et al., 2011). The efficient removal of H_2 from the rumen contributes to the increase in the fermentation rate by eliminating its inhibitory effect on the microbial degradation of plant materials (WOLIN, 1979; McALLISTER; NEWBOLD, 2008).

Enteric methane is derived from the activity of methanogenic Archeas, a microbial group distinct from Eukaria (protozoa and fungi) and Bacteria, which use H_2 as an energy source and produce CH4 (McALLISTER et al., 1996). Given the central role of H_2 in metabolism, methanogens are extremely important for the functioning of the rumen and

animal nutrition, although they account for a small part of the ruminal microbial biomass (JANSSEN; KIRS, 2008).

Methanogenesis is highly dependent on the rumen's H_2 balance and, consequently, on the SCFA formed during the rumen fermentation process, since they are not equivalent in terms of H_2 release (MARTIN et al., 2009). In this sense, the formation of acetate and butyrate, which are predominant during the fermentation of structural carbohydrates, results in the release of H_2 and favors methanogenesis (OWENS; GOESTCH, 1993). On the other hand, the formation of propionate, stimulated by non-structural carbohydrates and by mechanisms of manipulation of the rumen environment, is a competitive route in the use of H_2, thus reducing the substrate for methanogenesis (HEGARTY, 2001).

Although methanogenesis is characterized as an essential process for ruminal metabolism, it is directly related to the efficiency of rumen fermentation due to the loss of carbon, representing energy loss for the system and consequently influencing animal performance, since, according to Buddle et al. (2011), 5% to 9% of the diet's gross energy is lost in the form of CH_4.

Given this scenario, research has shown a wide variety of nutritional techniques that can be used to manipulate the rumen environment and reduce CH_4 emissions.

2.3. Modulation tools for ruminal fermentation

In addition to efficient production systems, pasture management techniques and animal breeding, several nutritional strategies have been used and researched to manipulate rumen metabolism and reduce enteric CH_4 emissions.

According to Lascano and Cárdenas (2010), any strategy adopted aiming at the reduction of enteric CH_4 emissions should aim at one or more of the listed objectives. First, the reduction of H_2 production without impairing food digestion. Second, the stimulation of the use of H_2 through the production of alternative products beneficial to the ruminant. Finally, the inhibition of methanogenic Archeas (number and/or activity), with concomitant stimulation of pathways that consume H_2 to avoid the negative effects of increased pressure of H_2 in the rumen.

Among the alternatives to reduce or divert the formation of enteric CH_4 through the manipulation of ruminal metabolism, the use of diets that provide a lower acetate: propionate ratio (C2: C3) and strategies that have direct action on methanogenic microorganisms and / or H_2 producers, such as protozoa, fungi and mainly cellulolytic

bacteria (PATRA, 2012). Thus, the reduction of methanogenesis can be achieved through the inhibition of reactions that release H_2 in the rumen environment or through the promotion of alternative reactions that consume H_2 (BEACH et al., 2015).

In general, the use of diets that provide a high digestion rate reduces the CH_4 emission, since the food does not remain in the rumen for a long time. The amount of forage in the diet, crude protein content, conservation method, the growth stage of the forage plant, particle size and grinding degree, the number of grains in the diet, and the addition of additives are important components that affect and are involved with the production of CH_4 in the rumen (WANAPAT et al., 2013).

Research has shown a wide variety of food additives that can be used to manipulate the rumen environment, including CH_4 production inhibitors, proteolysis inhibitors, ionophores, lipids, tannins, calcium nitrate, essential oils, microbial enzymes, among others (MOHAMMED et al., 2004; REIS et al., 2006; GERBER et al., 2013).

2.3.1. Ionophore

Ionophores, such as Monensin sodium, Salinomycin, Lasalocide and Narasina, are antimicrobial food additives widely used in ruminant herds to improve performance (HOOK et al., 2010) that can reduce methane production by 25% and decrease consumption dry matter (DM) by 4%, without affecting animal performance (TEDESCHI et al., 2003).

According to Austic and Smith (1980), the ionophore is a generic term applied to some compounds in the group of antibiotics called polyesters, containing a carboxylic radical. It facilitates the diffusion of ions through lipid barriers, such as cell membranes, being useful in the control of ruminal acidosis, as they depress or inhibit gram-positive microorganisms that are primary producers of lactic acid, without preventing its use by gram-negative bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium* (NAGARAJA; TAYLOR 1987).

Ionophores are highly efficient in inhibiting Gram-positive microorganisms and have little or no activity against Gram-negative microorganisms, since they have an external lipid layer that contains porins, which do not allow the ionophores to pass through the cell membrane. Gram-positive microorganisms do not have this outer layer, allowing free access of ionophores through the cell membrane (NAGARAJA et al., 1997).

As previously mentioned, the mechanism of action of the ionophores is related to structural factors of the cell wall, which is responsible for regulating the chemical balance between the internal and external environment of the cell, the balance is maintained by a mechanism called the ionic pump. The ionophore, when bound to the cation of greater affinity, transports it through the cell membrane into the cell, which uses the mechanism of the ion pump in an attempt to maintain its osmolarity. The cell uses its energy excessively until it depletes its reserves. In this way, the ionophores affect the growth of Gram-positive bacteria and end up favoring, by decreasing competition for the substrate, the growth of Gram-negative bacteria (RANGEL et al., 2008).

Bacteria that produce lactic, acetic, butyric acids and H_2 are susceptible to ionophores, while bacteria that produce succinic and propionic acids and those that ferment lactate are resistant (MORAIS et al., 2006).

It is possible to notice that the ionophores act by carrying out a microbiological selection in the rumen and, because of this selection, there is a change in the ruminal patterns with an increase in energy efficiency, mainly due to the increase in the production of propionic acid, the reduction of acetate/propionate ratio, and decreased production of methane. In addition to decreased production of lactic acid and reduced losses of amino acids that would potentially be fermented in the rumen (McGUFFEY et al., 2001). Furthermore, the proportions of methane in the rumen are reduced, characterizing lower energy losses, which according to Nagaraja et al. (1997) can reach 10% in a normal feeding system, without the use of additives that improve nutritional efficiency. The set of these changes results in better energy retention by the ruminant with positive effects on its productive performance (SENE, 2017).

The antimethanogenic effect of ionophores is more related to the inhibition of the formation of H_2, a precursor of methanogenesis, than to a direct effect on the population of methanogens, since they are more resistant to ionophores than the bacteria that produce and supply H_2. The reduction in methane precursors would be responsible for only 45% of the effect of ionophores on methane production, the remainder being the consequence of less food intake (NAGARAJA et al., 1997).

However, Rumpler et al. (1986), evaluating some long-term in vivo assays, observed that the inhibition of methanogenesis by monensin did not persist, and the methane production per diet unit returned to the initial levels in two weeks, regardless of whether the animals were fed grains or forage.

The reduction in CH_4 production observed with the use of ionophores can also be associated with inhibition in the growth of ciliated protozoa that produce H2 and are colonized by methanogenic Archeas (McALLISTER et al., 1996; TOKURA et al., 1999). Thus, it is believed that the number and/or activity of methanogenic Archeas is indirectly reduced by ionophores. Therefore, this is pointed out as part of the reason why the reduction of methane is not persistent, since, according to Kobayashi et al. (1988), populations of rumen protozoa depressed by ionophores tend to restore themselves when their use is prolonged.

Guan et al. (2006), who used monensin in diets with high and low, confirmed such information participation of concentrate evaluated the production of methane in steers. For the low-concentrate diet, an initial reduction in methane production of 27% was observed during the initial four weeks, in combination with a decrease in the population of ciliated protozoa of 77%. For the concentrate-rich diet, a 30% reduction in methane production was observed in the first two weeks, together with an 83% reduction in the ciliated protozoan population. However, methane levels and some protozoa returned to baseline levels after six and four weeks, respectively.

2.3.2. Essential oils

In recent years, essential oils have been widely researched as food additives that improve ruminal metabolism, as moderators of starch and protein degradation, aiming at increasing the efficiency of rumen fermentation and inhibiting methanogenesis (CALSAMIGLIA et al., 2007; PATRA and YU, 2012).

Essential oils are complex mixtures of secondary plant metabolites, which are traditionally extracted by steam distillation, being specific to each species and responsible for the characteristic flavor and fragrance of the plant (BENCHAR, 2011). In addition, there may be a wide variation in yield and composition of essential oil between plants of the same species and in different parts of the same plant (COSENTINO et al., 1999; BURT, 2004).

Essential oils have a wide variety of effects, including health effects such as cardiovascular disease, some tumors, inflammatory processes and, in general, diseases in which the uncontrolled proliferation of free radicals is harmful (HARBORNE; WILLIAMS, 2000; REDDY et al. 2003; TROUILLAS et al., 2003). However, the most important activities of these compounds are as antiseptics and antimicrobials, such

properties being known since antiquity (BENCHAR, 2011). However, the first scientific evidence describing its antimicrobial properties did not appear until the beginning of the 20th century (HOFFMANN; EVANS, 1911). Since then, many essential oil compounds with strong antimicrobial activities have been studied (BURT, 2004).

The sensitivity of microorganisms to essential oils is variable. This is the main property of interest to ruminant nutritionists, as it can be used aiming at changes in ruminal fermentation through selection for or against specific groups of microorganisms (BENCHAR et al., 2011), with essential oils being identified as natural alternatives to the use of antibiotic additives that promote growth (CALSAMIGLIA et al., 2007).

What determines the antimicrobial activity of a secondary metabolite is unclear, but the presence of oxygen and sulfur in its chemical structure appears to be important. Hydrocarbons have variable antimicrobial activity (OH et al., 1967; COSENTINO et al., 1999; DORMAN; CRISTANI et al., 2007). However, secondary metabolites containing oxygen, such as phenols, and sulfur, as in sulfides (ROSS et al., 2001; CORZO-MARTÍNEZ et al., 2007), tend to have strong antimicrobial activity. It is believed that the hydroxyl group is fundamental to interrupt the normal transport of ions across the cytoplasmic membrane (ULTEE et al., 2002) and for the inactivation of microbial enzymes (BURT, 2004).

According to Benchar (2011), hydrophobicity seems to have a crucial role in antimicrobial activity, meaning that essential oils start from an aqueous phase for the lipid bilayer of the cytoplasmic membrane, where they accumulate. It is from within the lipid bilayer that essential oils are believed to orchestrate one or more of its antimicrobial effects by altering the permeability of the membrane and thus interrupting ion transport processes and interacting with membrane proteins and/or other cytoplasmic components. This essential oil-mediated response is achieved from within the cytoplasmic membrane or by diffusion into the cytoplasm. Since essential oils are mixtures of many secondary metabolites, there are likely several mechanisms of action.

Cinnamaldehyde and garlic oil are two of the most investigated essential oils in the literature and their antimicrobial properties and potential for fermentation modification have been widely demonstrated, although most of the available research has been conducted in vitro (CALSAMIGLIA et al., 2007; MATEOS et al., 2013).

Cinnamaldehyde (3-phenyl-2-propenol phenol; C_9H_8O) is a phenylpropanoid with antimicrobial activity, presenting itself as the main active component of cinnamon oil (C. verum) and accounting for up to 75% of its composition (CALSAMIGLIA et al ., 2007). Such compound has an effect on Gram-positive and Gram-negative bacteria (OUATTARA et al., 1997; HELANDER et al., 1998; SMITH-PALMER et al., 1998), probably due to the reactivity of its carbonyl group and the inactivation of microbial enzymes (BURT, 2004).

Macheboeuf et al. (2008) showed, through an in-vitro experiment, that the supply of 264 mg / L of cinnamaldehyde slightly decreased the production of CH_4, by 13%, without altering the production of SCFA, acetate or propionate, suggesting that, in this dose, it inhibited rumen methanogenesis, acting directly against rumen methanogens. However, increasing doses, despite providing a decrease in methane production, caused a decrease in SCFA production. In addition, it was observed that in the supply of 661 mg / L, despite almost completely inhibiting CH_4 production (-94%), it also reduced drastically the concentrations of SCFA (-60%), acetate (-55%) and propionate (-92%), indicating that, in high doses, cinnamaldehyde's antimicrobial activity is sufficient to almost completely inhibit rumen microbial metabolism. As for nitrogen metabolism, the results are still inconsistent (CALSAMIGLIA et al., 2007).

Whole garlic contains several sulfur-containing compounds that are converted to thiosulfinates, such as allicin, through enzymatic reactions when raw garlic is cut or crushed (FENWICK; HANLEY, 1985; AMAGASE et al., 2001; AMAGASE, 2006). During oil extraction by steam distillation, allicin degrades to form a variety of fat-soluble organosulfur compounds, including diallyl trisulfide, diallyl disulfide and diallyl sulphide (BLOCK, 1985; LAWSON, 1996). It contains sulfur in its molecules and have activity against a wide range of Gram-positive and Gram-negative bacteria, having their effects against pathogenic bacteria well documented (REUTER et al., 1996; ROSS et al., 2001). However, the potential of garlic oil and its derivatives to inhibit selectively rumen methanogenesis has only recently been explored (BENCHAR, 2011).

Busquet et al. (2005b) were the first to report the effects of garlic essential oil and two of its compounds (ie, diallyl disulfide and allyl mercaptan) on CH_4 production, reporting a reduction in production of up to 74%, a reduction in DM digestibility and decrease in the proportion of acetate, while propionate and butyrate increased. According to Calsamiglia et al. (2007), the increase in butyrate concentrations with the inclusion of

garlic oil in in vitro incubations may indicate that the change in rumen fermentation occurs differently from monensin, which tends to decrease the acetate: propionate ratio and the concentration of butyrate.

Another point to be addressed is the possibility of additive, synergistic or antagonistic effects of essential oils, which have been previously reported (BURT, 2004), with some products that combine essential oils being observed in the market. In this sense, Blanch et al. (2016), evaluating the association of cinnamaldehyde and garlic oil in an in vitro experiment, observed a reduction of 68% in CH4 production and 8% in the proportion of acetate, in addition to an 18% increase in the proportion of propionate.

2.3.3. Exogenous enzymes

The use of exogenous enzymes as a way to improve the use of food by animals is not recent. This technology is very common in pig and poultry diets. However, in diets for ruminants, its use is relatively recent (the date of the first report from the 1960s). This is because there was a widespread belief that enzymes would be rapidly degraded in the rumen before having any effect (BEAUCHEMIN et al., 1999).

However, some studies have shown improvements in the digestibility of nutrients with the addition of enzymatic preparations in the feeding of ruminants (JUDKINS; STOBART, 1988; BEAUCHEMIN et al., 2000; CRUYWAGEN; GOOSEN, 2004).

According to Guenter (2002), the main goals of enzyme supplementation for animals are to remove or destroy the antinutritional factors of the grains, increase the total digestibility of the feed, enhance the action of endogenous enzymes, and decrease the environmental pollution caused by nutrients excreted in the faeces.

Among the exogenous enzymes used in ruminant nutrition can highlight the Fibrolytic enzymes, amylolytic and proteolytic as the three major categories, with phytase, which is widely used in feed for monogastric animals, it is also becoming popular in ruminant feed (SUJANI; SERESINHE, 2015).

Supplementation of ruminant diets with fibrolytic enzymes has been of interest in many studies in vitro (RODRIGUES et al., 2008; MURAD et al., 2009; AZZAZ, 2009), in vivo (JALILVAND et al., 2008; KRUEGER et al, 2008;.. ARRIOLA et al, 2011) and in situ, since the digestibility of the fiber in the digestive system of ruminants reaches only 65-70% even in ideal conditions (SUJANI; SERESINHE, 2015).

Although research has hypothesized that the digestion of starch and protein is not limited within the rumen, recent research conducted with enzyme supplementation with amylolytic activity (NOZIERE et al., 2014; KLINGERMAN et al., 2009; GENCOGLU et al., 2010) and proteolytic activity (EUN and Beauchemin, 2005; VERA et al, 2012) examined the potential of these enzymes to improve animal performance. Kincaid et al. (2005) conducted research supplementing exogenous phytase in diets of dairy cows with the main focus to reveal its effect on phosphorus digestibility.

Even in conditions where the rumen has a high rate of fermentation in the diet, it is still possible to observe the elimination of degradable fibers, starch and protein in the feces (GALLARDO et al., 2009; TRICARICO et al. 2007). Thus, it is still possible with the use of exogenous enzymes to increase the digestibility of nutrients, improving rumen fermentation parameters, animal performance, production efficiency (TRICARICO et al. 2007) and reducing GHG emissions.

Assessing the effect of using amylase, xylanase, cellulase, and protease on ruminal fermentation, Collazoz Paucar (2017) observed that the association of exogenous enzymes did not alter the consumption of dry matter or the digestibility of the diet, but it did increase the production of acid acetic, propionic acid and consequently the production of SCFA, not changing the production of CH_4. The effect of enzymes used in combination was superior to their effects when used individually.

2.4. Additives association

Despite the wide variety of food additives indicated as capable of modulating rumen fermentation and consequently reducing emissions of enteric methane, few have shown a substantial decrease and in line with the contemporary expectation of the technical-scientific community.

On the other hand, advances in the area of knowledge about the use of medicated drugs in an associated way proved to be fundamental to assist in the search for solutions to some challenges faced by modern medicine. Simultaneous prescription of several drugs is a practice commonly used in the medical field, to improve the effectiveness of drugs, reduce toxicity or treat coexisting diseases. That may result in a synergistic effect, where the result of the association is greater than the simple sum of isolated drugs effects (SECOLI, 2001).

Within this perspective, it is important to note that the different additives used as modifiers of ruminal metabolism (ionophores, essential oils, exogenous enzymes, etc.) do not decrease CH_4 production through a single mechanism of action. Therefore, nothing prevents its effects from being additive (the result of the combination is equal to the sum of the parts) or even synergistic or potentiating (the result of the combination is greater than the sum of the parts). Thus, it is of great interest to the scientific community to understand the associative use of nutritional additives and their effects on CH_4 mitigation.

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3. USE OF DIFFERENT FEED ADDITIVES AND THEIR ASSOCIATION ON DRY MATTER INTAKE, FEEDING BEHAVIOR, NUTRIENTS DIGESTION AND RUMEN QUINETICS OF NELLORE COWS

Abstract

This study aimed to evaluate the use of essential oils, exogenous enzymes, sodium monensin and their associations on consumption parameters, nutrient digestion, ruminal kinetics, as well as microbial protein synthesis in Nellore cows. An experimental design was used, consisting of two 4 x 4 Latin squares, in a 2 x 2 x 2 factorial arrangement (4 periods of 28 days), using 8 cannulated cows (480 ± 55 kg) totaling 4 replications (32 experimental units). The basal diet consisted of 60% corn silage and 40% concentrate with daily-adjusted supply as a function of DMI. The factors were composed by the presence or absence of the tested additives. An interaction between EO and E on the DMI was observed, where the isolated use of these additives provided a reduction in the DMI, impacting ingestive behavior and consumption efficiency, however, when these effects are associated, they are nullified. The different tested additives did not affect ruminal kinetics, but the use of E increased the potentially degradable fraction of NDF. The use of M or E alone promoted an increase in the apparent total digestibility of DM, GE, ADF, NFC and consequently of TDN, however, no associative effect was observed between the additives. The use of M promoted a reduction in the synthesis of microbial N, resulting in a reduction in microbial efficiency when associated with other additives and indicating an antagonistic associative effect. Therefore, the association between the tested additives did not show to promote alterations in the diet digestibility, as well as in the rumen kinetics, being observed an antagonistic effect in the association between M with E and/or EO, reducing the microbial efficiency.

Keywords: Additive association, Ruminal digestion, Microbial protein, Digestibility in beef cattle.

3.1. INTRODUCTION

Problems generated by the emission of CH_4 and the search for greater energy efficiency have stimulated the quest for modulators of ruminal fermentation, which may optimize the use of nutrients by ruminants, such as ionophores, tannins, calcium nitrate, essential oils, lipids, microbial enzymes, inhibitors of CH_4, among others (MOHAMMED et al., 2004; REIS et al., 2006; GERBER et al., 2013).

At the same time, research on ruminal kinetics identifies how the diet and feed additives interact with the degradation time and digestibility of the nutrients as it passes through the gastrointestinal tract (CARVALHO, 2017). Due to this, it is of great importance for the development of efficient feeding programs that promote improvements in the performance of ruminants and reduces the environment impact (LASCANO & QUEIROZ, 1990).

In addition to the effects on ruminal metabolism, many of the additives mentioned above have an effect on other aspects of the digestive metabolism of ruminants, such as sodium monensin, which is capable of reducing dry matter intake and increasing feed efficiency (DUFFIELD et al., 2012). They increase the apparent digestibility of nitrogen (N) and energy in the diet (SPEARS, 1990), as well as exogenous enzymes that improve digestibility when added to the diet of ruminants (JUDKINS; STOBART, 1988; BEAUCHEMIN et al., 2000; CRUYWAGEN; GOOSEN, 2004).

The use of essential oils as feed additives has great potential in the nutrition of ruminants, which was intensively investigated mainly in *in vitro* studies (BUSQUET et al., 2005a, b, c, 2006; CALSAMIGLIA et al., 2007; MATEOS et al., 2013; BLANCH et al., 2016) and there are few studies analyzing its effect on ruminal kinetics. According to Hart et al. (2008), the main effects of essential oils in the rumen are the reduction of protein and starch degradation due to selective action on certain ruminal microorganisms.

As the additives mentioned above do not have a single and exclusive mechanism of action, nothing prevents their effects from being additive or even synergistic in the use of nutrients in the diet. Thus, this study aimed to evaluate the association between essential oils, blend of exogenous enzymes and sodium monensin as a nutritional strategy on nutrient intake and digestion, rumen dynamics, as well as microbial protein synthesis of Nellore cows.

3.2. MATERIAL AND METHODS

3.2.1. Place of experimentation, ethical issue and animals

The current experiment was carried out at the Animal Nutrition and Production Department (VNP) of the College of Veterinary Medicine and Animal Science (FMVZ) of the University of Sao Paulo, Fernando Costa Campus in Pirassununga City, Brazil. The experiment was conducted under the guidelines established by the ethical principles of animal experimentation of the Commission of Ethics in the Use of Animals of the College of Animal Science and Food Engineering (FZEA) – USP, under the protocol number CEUA 4788111017.

Eight Nellore cows, non-pregnant and non-lactating, with a mean body weight (BW) of 480 ± 55 kg and carrying rumen cannula were kept in a barn on individually pens with free accesses to water, individual feed bunks and sand bedding. The barn owned suspended fans that were automatically triggered when the temperature turned up to 28°C to avoid heat stress.

3.2.2. Experimental design and treatments

The animals were assigned into 2 contemporary 4 x 4 Latin squares, in a 2 x 2 x 2 factorial arrangement, the experimental unit was the animal within each experimental period (n = 32). Animals received a basal diet (Table 2), with a concentrate: roughage (corn silage) ratio of 40:60. The experimental diets received the same amount of energy and protein, differing only by the presence or absence of the following factors: (1) essential oil factor; (2) enzyme factor; and (3) monensin factor. Therefore, the animals were randomly assigned to the treatments following described.

The essential oil (EO) factor differed according to the presence or absence of this product in the diet: EO-A (absent essential oil): diet without the addition of essential oil; EO-P (essential oil present): diet added with 31.7 mg/kg DM of a blend of essential oils (43% cinnamaldehyde + 7% garlic oil). The enzyme (E) factor differed depending on the presence or absence of this product in the diet: E-A (absent enzyme): diet without the addition of enzyme; E-P (enzyme present): diet with the addition of 1027 mg/kg DM of the product containing an "enzyme blend" (cellulase, xylanase, amylase, protease, phytase, beta-glucanase, and pectinase). The monensin (M) factor differed with the presence or absence of this product in the diet: M-A (absent monensin): diet without the addition of monensin; M-P (monensin present): diet with the addition of 30.6 mg of sodium monensin/ kg DM.

Next Enhance (43% cinnamaldehyde + 7% garlic oil) (Novus International Inc., Indaiatuba, Brazil) was used for source of essential oil, Allzyme® SSF (Alltech Inc., Nicholasville, USA) was used for source of enzymatic blend and Rumenpac® (Grupo MCassab, São Paulo, Brazil) was used for source of Monensin Sodium.

3.2.3. Feeding management

The feed was offered *ad libitum* (5 - 10% of leftovers), and the animals were fed twice a day, at 8 a.m. and 4 p.m., in the form of total mixed ration (TMR). The software BR-Corte 3.0, claiming an average daily gain (ADG) of 0.950 kg, performed the diet. The proportions of the various ingredients and the chemical composition of the diets are shown in Table 1.

Table 1. Proportion of ingredients and estimated chemical composition of experimental diets

Ingredients (% DM)	Basal diet
Corn Silage	60.00
Dry ground corn grain	25.40
Soybean meal	12.10
White salt	0.50
Mineral premix[1]	2.00
Chemical composition	
Dry matter[2] (%)	58.76
CP[2] (% DM)	13.37
RDP[3] (% CP)	66.40
RUP[3] (% CP)	37.26
NDF[2] (% DM)	36.43
NDFe[3] (% DM)	34.00
ADF[2] (% DM)	20.22
NFC[2] (% DM)	40.55
Starch[2] (% DM)	35.20
MM[2] (% DM)	7.45
Ca[2] (% DM)	0.44
P[2] (% DM)	0.31
EE[2] (%DM)	2.20
TDN[3] (% DM)	64.80

[1]Mineral premix, quantity per kg of product: 200 g of calcium, 70 g of sodium, 60 g of phosphorus, 20 g of sulfur, 20 g of magnesium, , 15 mg of cobalt, 700 mg of cooper, 700 mg of iron, 40 mg of iodine, 1.600 mg of manganese, 19 mg of selenium, 2.500 mg of zinc, 200.000 UI of vitamin A, 50.000 UI of D3 vitamin, 1.500 UI of vitamin E; [2]Quantititaed through chemical analysis; [3]Value estimated by BR-Corte 3.0 software.

3.2.4. Experimental period

The experiment lasted 112 days and was divided into four periods of 28 days each. A wash-out of two days was provided between one period and another. The first 16 days of each period were to adapt the animals to the diets. On the 17th day, the feeding behavior was evaluated for 24 hours. Dry matter intake (DMI) and water consumption were evaluated between the 17th and 21st days of each experimental period, as well the apparent digestibility, excretion of nutrients, and ruminal degradability. On the 24th day, urine was collected every 6 hours (6 a.m., 12 p.m., 6 p.m., and 12 p.m.) to determine urinary parameters and N balance. From the 23rd to the 25th day, rumen content was collected to evaluate the passage rate. On days 25 and 26, it was performed the rumen emptying to

determine the rumen volume and disappearance rate 3 hours after morning feed and before (0 hours) morning feeding, respectively.

3.2.5. Feed intake and water consumption

Dry matter intake (DMI) was evaluated between the 17th and 21st days of each experimental period. The feeders were daily examined, through observation, at 7 a.m. and the feed supply was monitored to ensure daily leftovers in a range of 3% to 5%. During the five days of evaluation, the leftovers from each cow were collected and weighted for the feed intake calculation, which was obtained by the difference between the amount of feed supplied and leftovers and multiplied by the diet DM. The water intake per day was also quantified during those five days by automatic and individual drinking fountains with water meters.

3.2.6. Evaluation of feeding behavior

The feeding behavior was evaluated for 24 hours straight (from 8 a.m. of 16th day up 8 a.m. of 17th day) by visual monitoring. The animals were observed every 5 minutes and the following parameters were evaluated: eating (E), drinking (D), ruminating (R) and idleness (I), according to the methodology described by Maekawa et al. (2002). Each parameter observed was considered to be executed during the entire 5 minutes between observation and called Activity. In order to estimate consumption efficiency it was evaluated the time spent to eat or ruminating a kilogram of DM or neutral detergent fiber (NDF).

The results referring to feeding behavior factors were obtained using equations, where the sum of all feeding events (each event was considered two or more consecutive activities being terminated by another activity other than the current one) represented the daily number of eating events (NEE, events/day). All the other parameters were calculated in the same way, the drinking events (NDE) rumination (NRE), and idleness (NIE).

The total eating time (TET, min/day) was defined as the sum of the times of each of the 5 minutes events in which the animal spent eating. The mean eating time per event (ETE, min/event) was obtained by the TET divided by the NEE. The total rumination time (TRT, min/day) was also defined as the sum of the times of each rumination event, while the mean rumination per event (RTE, min/event) was the ratio between the TRT and the NRE. The total chewing time (TCT, min/day) was calculated by the sum of TET

and TRT, and the daily number of chewing events (NCE, events/day) was obtained by the sum of the NEE and the NRE, while the mean chewing time per event (MCE, min/event) was calculated by the ratio between TCT and NCE. The total idleness time (TIT, min/day) was obtained by the difference between the total period of 24 hours (1440 min) and the TCT.

3.2.7. Evaluation of total apparent digestibility of DM and its fractions

The *in vivo* digestibility of DM and its fractions (CP, EE, NFC, NDF, ADF, and GE) was determined by using an extern marker, titanium dioxide (TiO_2), following the guidelines established by Titgemeyer et al. (2001). Therefore, from day 12th to 21st to each experimental period, the TiO_2 was administrated (15 g/cow days) via a cannula in the same feeding time (8 a.m. and 4 p.m.), splitting the doses into two portions of 7,5 g/cow. The first five days were for the adaptation and the least five ones for feces collection, also twice a day (8 a.m. and 4 p.m.). The feces samples were stored in a freezer at -20°C until the time of analysis according to the methodology described by Myers et al. (2004). The apparent digestibility coefficients of DM (ADCDM) and their nutrients (ADCN) were calculated based on the TiO2 content of the diet and feces using the equations bellow:

$$ADC_{DM} = 100\left(100x\left(\frac{TiO_2\,(\%) in\ the\ diet}{TiO_2\,(\%) in\ feces}\right)\right) \quad (1)$$

$$ADC_N = 100 - \left(100x\left(\frac{TiO_2\,(\%)\ in\ diet}{TiO_2\,(\%)\ in\ feces}\right)\ x\ \left(\frac{\%\ N\ in\ feces}{\%\ N\ in\ diet}\right)\right) \quad (2)$$

Where:

ADC_{DM} = DM apparent digestibility coefficient;

ADC_N = Nutrient apparent digestibility coefficient;

The excretion of DM and nutrients as well as nitrogen (ExN) was determined from the digestibility coefficient data of DM and its fractions multiplying the nutrient intake by the respective digestibility coefficients and divided by 100 according to equation 3.

$$Fecal\ excration\ (kg) = \frac{(100-ADC)x\ Intake\ (kg)}{100} \quad (3)$$

The DM content of the feed feces was determined by drying using a forced air oven at 65°C for 72 hours according to AOAC (1995). After drying, the samples were milled in a willie-type knives mill of 1 mm sieves and stored in properly sealed vials. All

analyzes were corrected for the analytical DM content determined in an oven at 105°C for 4 hours. The MM was obtained by calcination in an oven muffle at 550ºC for 5 hours, and the organic matter (OM) was calculated as the difference between 100 and MM (AOAC, 1990). The CP was determined by the total N content (N x 6.25) using the micro-Kjeldahl technique (method 920.87; AOAC, 1990). The EE was determined with the ANKOM XT15 Extractor® (AOCS, 2005). The NDF and ADF were determined by the method described by Van Soest et al. (1991), where the NDF of the diet was obtained by using thermostable α-amylase. Calcium (Ca) was determined by titration (AOAC, 1995) and phosphorus (P) by colorimetry (AOAC, 1990). The gross energy (GE) was obtained by the samples (feces and diet) total oxidation by a calorimetric bomb (C5000 control, IKA®, Staufen, German). The non-fibrous carbohydrate (NFC) content was obtained by subtracting the amounts expressed in percentage of DM of CP, EE, MM, and NDF from 100.

3.2.8. Urinary parameters, microbial protein

To calculate the production of microbial protein, the urinary volume was determined through creatinine in the urine, according to the methodology described by Valadares et al. (1999). On day 24 of each experimental period, urine samples were collected every 6 hours (at 6 a.m., 12 p.m., 6 p.m., and 12 a.m.) during spontaneous urination or stimulation by vulva massage. At each collection time, 10 mL of urine were taken and diluted in 40 mL of 0.036 N sulfuric acid as a preservative to reduce the pH to below 3 to avoid losses of nitrogen (VASCONCELOS et al. 2010) as well as bacterial destruction, conservation of purine derivatives, and precipitation of uric acid. The samples were stored at -20°C for further analysis of allantoin, uric acid, urea, and creatinine.

Allantoin was determined according to the colorimetric method described by Chen and Gomes (1992) and adapted by Silva and Queiroz (2002). The uric acid was determined by colorimetric enzymatic reaction with Uricase and Peroxidase, through commercial kit (Bioclin® Ref K139). The concentrations of urea and creatinine were determined by using commercial kits (Bioclin® Ref K047 and Bioclin® Ref K067, respectively), through the colorimetric enzymatic reaction and reaction with Alkaline Picrate in buffered medium, respectively. The urine concentration of ureic N was obtained by multiplying the urea concentration by 0.466, the N content in urea.

The daily urinary creatinine excretion (CE) was estimated in relation to animal empty body weight (EBW) using the equation proposed by Costa and Silva et al. (2012), wherein the EBW was estimated -4% of body weight.

$$CE\ (g\ /day) = 0.0345 * EBW^{0.9491} \quad (3)$$

The daily total urinary volume (L/cow) was determined by dividing the daily urinary creatinine excretion by the observed values of urinary creatinine concentration (mg/dL) of the spot samples. This volume was used to calculate the estimated daily excretions of urea, allantoin and uric acid from each cow

The excretion of purine derivatives (PD) in the urine in 24 hours was calculated by multiplying the urine volume in 24 hours by the concentration of PD in the urine sample. The absorbed microbial purines (AP, mmol/day) were calculated from the excretion of purine derivatives in urine (PD, mmol/day) as proposed by Barbosa et al. (2011), by means of the following equation:

$$AP\ (mmol/dia) = [PD – (0.0301 * BW^{0.75}] / 0.8 \quad (4)$$

Where:

AP: absorbed microbial purines;

PD: purine derivatives;

$0.0301 * BW^{0,75}$: excretion of purines of endogenous origin per kg of metabolic weight per day;

0.8: recovery of purines absorbed as urinary derivatives of purines.

The intestinal flow of microbial nitrogen compounds (MicN, g of N/day) was calculated in relation to absorbed microbial purines (AP, mmol/day) using the equation described by Barbosa et al. (2011):

$$MicN\ (g\ N/day) = [(70 * AP)/(0.93 * 0.137 * 1000)] \quad (5)$$

Where:

MicN: microbial nitrogen;

AP: absorbed microbial purines;

70: N content in the purines (mg of N/mmol);

0.93 = digestibility of microbial purines;

0.137 = N-RNA ratio of purine N and total N of rumen microorganisms.

3.2.9. Evaluation of rumen kinetics

The rumen passage rate (kp) of DM, the rate of disappearance of the solid mass in the rumen (kt), the *in situ* rumen degradability and rumen digestion rate (kd) are parameters of rumen kinetics evaluated.

3.2.9.1. Rumen degradability of DM and nutrients

The determination of rumen degradability of DM and nutrients was performed according to the technique proposed by Ørskov et al. (1980). It was conducted between the 17th and 21st days of each experimental period where samples of silage and concentrate were dried at 65°C for 72 hours and milled with Willye-type mills with 2 mm sieves. After grinding, both portions were mixed in proportions of 60:40, then 9 g of this mixture were introduced in 10 x 20 cm nylon bags (with known weight) of 50 μm porosity. These bags were then incubated in rumen via the cannula for 0, 3, 9, 24, 48, and 96 hours. Although they had different incubation times, they were all removed at the same time. After the removal, they were washed with fresh water to ensure the removal of the soluble material. After that, they were dried in a forced-air oven at 65°C for 72 hours and finally weighed. The disappearance of DM was obtained by taking the difference between initial (before incubation) and final (after incubation) weights and calculating the percentage of degraded fraction in the rumen. The zero-time bags (which were not incubated) were introduced in a thermostatic bath at 39^{o}C for 5 minutes and washed with fresh water. Subsequently, they were submitted to the same procedures adopted for the bags of other times. The remaining residues in the bags were analyzed for CP (AOAC, 1990) and NDF (VAN SOEST et al., 1991) to determine the rate of degradation of these fractions.

The potential degradability of DM and CP was calculated according to the model of Ørskov and McDonald (1979) with the aid of SAS NLIN procedure (version 9.3).

$$p = a + b\ (1 - e^{-ct}) \qquad (6)$$

Where:

p: disappearance of nutritive component analyzed at time "t";

a: intercept of the degradation curve when t = 0, which corresponds to the water-soluble and completely degradable fraction of the analyzed nutritive component leaving the nylon bag rapidly;

b: degradation potential of the water insoluble fraction of the nutritive component analyzed;

c = rate of degradation per fermentative action of b;

t: incubation time.

The real effective degradability (RED), which represents the amount of the nutritive component (DM, CP or NDF) that has actually been degraded in the rumen, was calculated according to the method established by Ørskov et al. (1980).

3.2.9.2. Determination of rumen passage rate

The DM passage rate was determined between 23rd and 25th days of each experimental period, where 20 g of chromium oxide (as indicator) were infused in rumen in a single dose. The rumen content samples were collected at zero (0), 8, 10, 12, 24, 36 and 48 hours after the infusion. Then samples were weighed and dried through a forced air oven at 65°C for 72 hours, after which they were weighed again and milled and, finally, analyzed for DM and chromium oxide content. The passage rate was calculated by using the model proposed by Czerkawski (1986).

$$Y = a.e^{-kp \times t} \quad (7)$$

Where:

Y: indicator concentration in time (t);

Kp: passage rate in the rumen (h^{-1});

t: indicator sampling time (h);

a: concentration of the indicator at initial time (t0), assuming instant mixing to the rumen content (ppm);

e: base of the neperian logarithm.

3.2.9.3. Determination of the disappearance rate of rumen solid mass

On the 25th and 26th days of each experimental period the rumen content was emptied, and the disappearance rate was determined. The rumen content was removed manually through the rumen cannula as described by Allen and Linton (2007). On the 25th day, the emptying was performed at 11 a.m., three hours after feeding, when the rumen was theoretically full. The same procedure was performed on the 26th day at 8 a.m. before feeding, when the rumen was, theoretically, at the lowest volume. During the removal of the rumen content, the liquid and solid phases were separated using a 2 mm mesh sieve and buckets, then weighed. Samples of approximately 1 kg of each phase were collected for DM determination. Afterward, both phases of the content were reconstituted and returned to the rumen. The rumen DM and the disappearance rate (kt) were calculated based on the dry weight of each sample. When the consumption is stable, kt of the feed or a feed fraction is equivalent to its intake rate (ROBINSON et al., 1987), so the kt was estimated using the following equations:

$$\text{Kt (\%/h)} = 100 \text{ x } \left(\frac{daily\ DMI\ (kg)}{rumen\ content\ DM\ (kg)}\right)/24 \qquad (8)$$

$$\text{Kt (kg/h)} = \text{Rumen content DM } (kg) \text{ x } \left(\frac{Kt\ (\%/h)}{100}\right) \qquad (9)$$

3.2.10. Statistical analysis

The data were analyzed by using the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). Before analysis, they were evaluated concerning the presence of discrepant information (outliers) and normality of the residues by the Shapiro-Wilk test. The data of DM and water intake, feeding behavior, *in vivo* digestibility, rumen kinetics, and rumen microbial protein production were submitted to analysis of variance which separated, as causes of variation, the monensin effect (also considered as the effect of the

square), the essential oil effect, the enzyme effect, as well the interaction among factors, period effect and animal effect within the square. The main factors were analyzed through analysis of variance using 0.05 as the significance level, whereas the interactions were decomposed using the LSD test using 0.05 as the significance level.

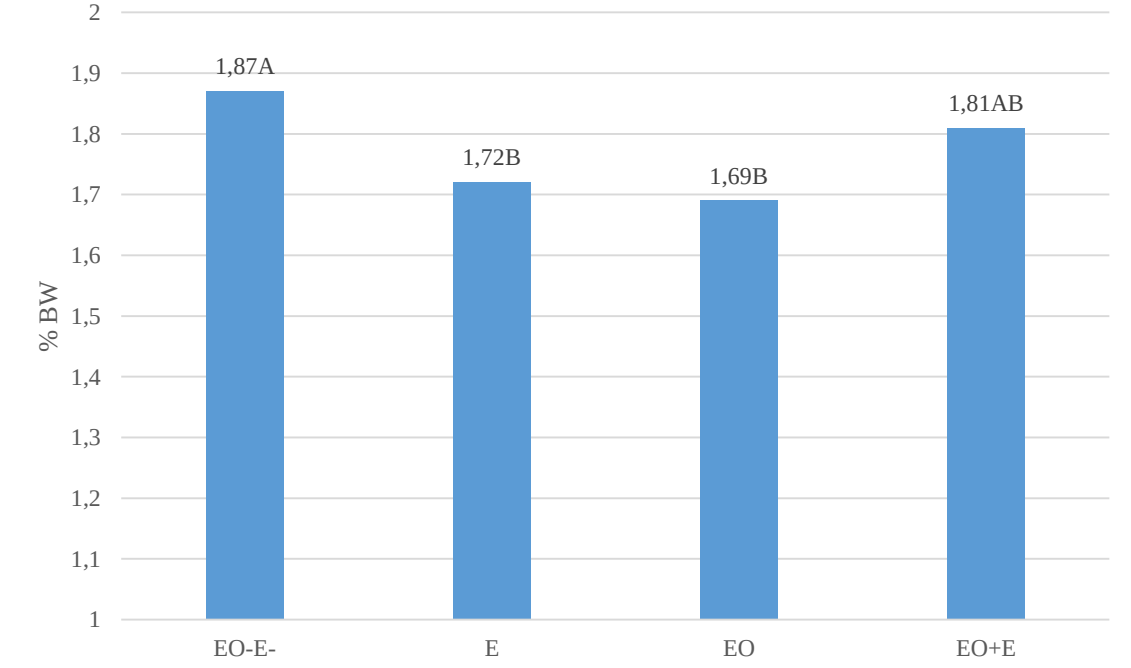
3.3. RESULTS

3.3.1. Consumption and feeding behavior

There was neither significant effect (P > 0.05) among the main factors on DM nor water intake. However, when the factor EO and E were analyzed individually, without interaction, their presence in the diet decreased the DMI in 9.62% and 8.02%, respectively, further, those factors increased the water consumption, as demonstrated in Table 2 and Figures 1 and 2.

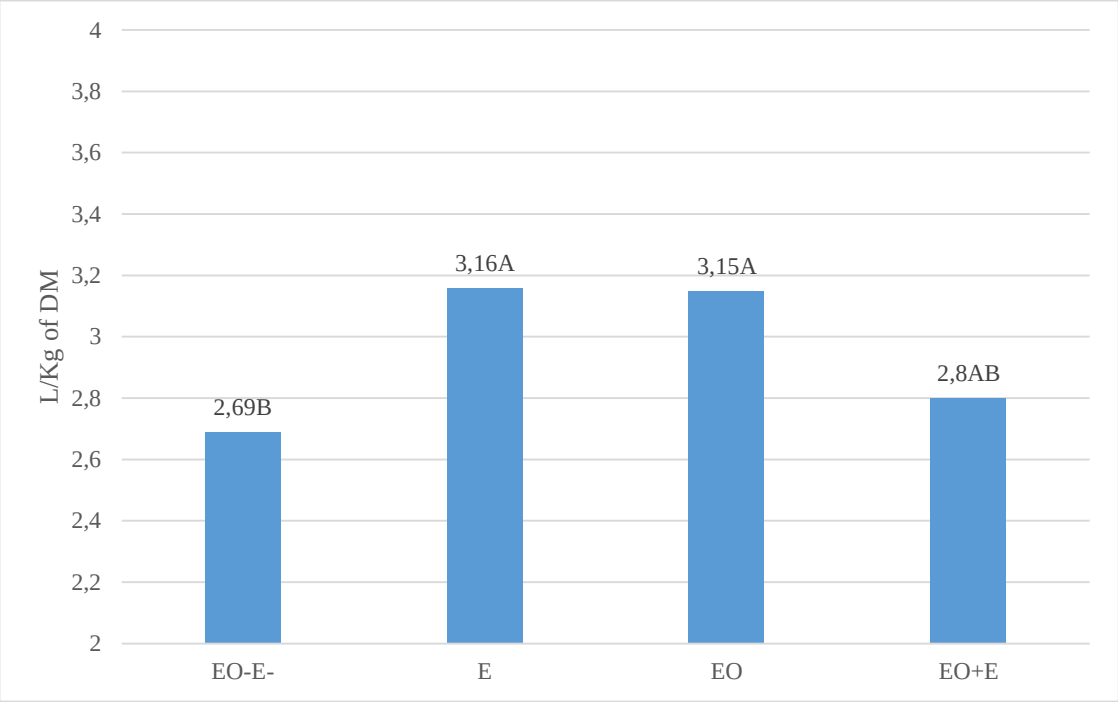
Similar results were observed when the isolated factors, EO and E, and they significant (P < 0.05) increased in 10.79% and 9.74%, respectively, the DM and NDF consumption efficiency (total eating time), as demonstrated in Figure x. However, neither interaction was observed among factors.

Figure 1. Interaction between Essential oil and Enzymes on DMI of BW%



EO-E-: Absence of enzyme and essential oil; E: enzyme presence, EO: essential oil presence; EO+E: enzyme and essential oil presence.

Figure 2. Interaction between Essential oil and Enzymes on water consunption per kg of DMI.



EO-E-: Absence of enzyme and essential oil; E: enzyme presence, EO: essential oil presence; EO+E: enzyme and essential oil presence.

Table 2. Feed intake of Nellore cows fed with essential oils, a blend of exogenous enzymes, sodium monensin and their associations.

Variables	Factors						Mean	SEM	P value						
	EO		E		M				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Presence	Absence	Presence	Absence	Presence									
DMI, day															
kg	9.91	9.47	9.65	9.73	9.57	9.81	9.66	0.284	0.0945	NS	NS	0.0403	NS	NS	NS
BW%	1.80	1.75	1.78	1.77	1.78	1.77	1.77	0.045	NS	NS	NS	0.0115	NS	NS	NS
Water, day															
L	28.1	27.8	27.9	28.0	31.9	24.0	27.96	1.643	NS	NS	NS	NS	NS	NS	0.0639
L/kg DM	2.93	2.98	2.92	2.98	3.37	2.54	2.96	0.188	NS	NS	NS	0.0064	NS	NS	NS

SEM: stanadard error of mean; EO: Essential Oil; E: Enzyme Blend; M: Monensin; EO*E: Interaction between essential oil and enzyme; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzyme and monensin; EO*E*M: Interaction between essential oil, enzyme and monensin.

A similar effect was observed on consumption efficiency, wherein the isolated use of EO and E increased 10.79% and 9.74%, respectively, in time spent to ingest 1 kg of DM (Figure 6), and therefore, increasing the time spent to ingest 1 kg of NDF. However, when the additives were used in association, no effects were observed ($P > 0.05$), the results are shown in table 3. Furthermore, the interaction between EO and E decreased in 7.6% the time spent ruminating ($P = 0.0098$), but no effects were observed on NE and ATE. In addition, animals supplemented with E had a higher number of going to drink fountains ($P = 0.0106$), however, this increase did not affect the time spent in this activity (Table 3).

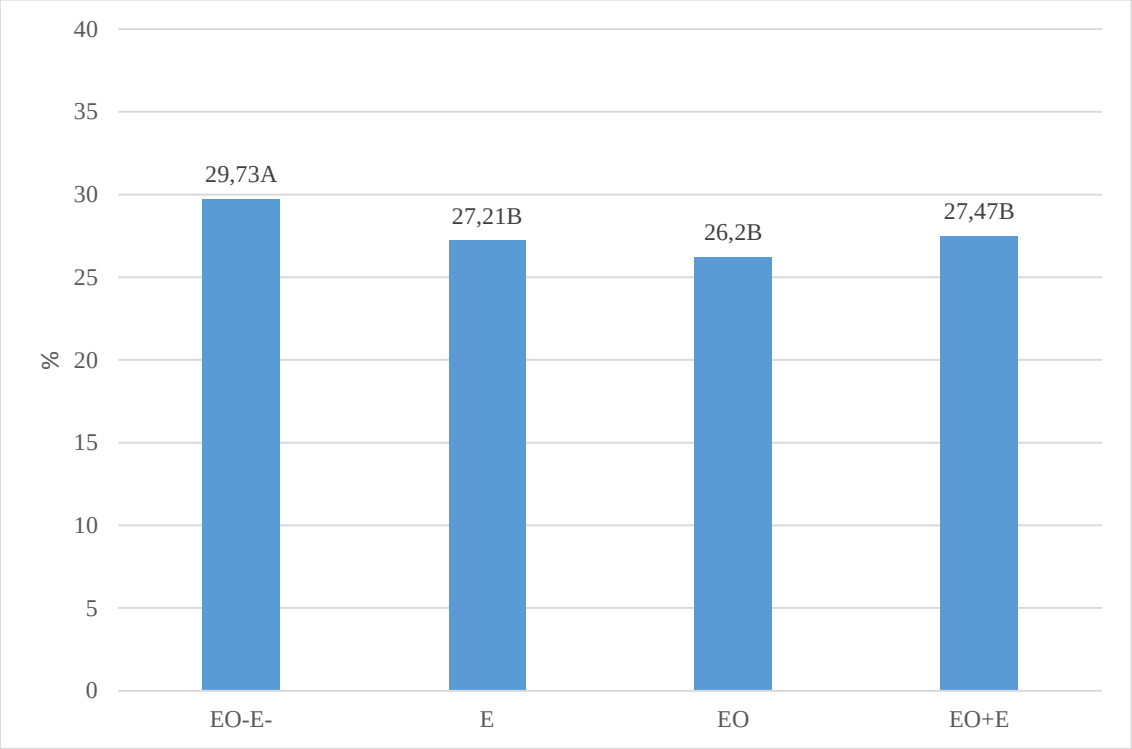
A triple interaction ($P < 0.05$) among factors was observed on chewing ($P = 0.0445$) and idleness time ($P = 0.0097$), where the animals supplemented with the three additives (EO, E and M) presented a decrease in TCT and, therefore, an increase in TIT. The results are presented on table 3 and the interaction on figure 3.

Table 3. Feeding behavior of Nellore cows fed with essential oil, an exogenous enzymes blend, monensin and their associations.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
Eating															
NE	9.00	8.40	8.34	9.06	8.59	8.81	8.77	0.3927	NS	NS	NS	NS	NS	NS	NS
TET (min)	220.0	221.3	221.9	219.4	218.5	222.8	220.5	7.3153	NS	NS	NS	0.093	NS	NS	0.0761
TET (%)	15.28	15.37	15.41	15.23	15.17	15.47	15.31	0.508	NS	NS	NS	0.093	NS	NS	0.0761
ATE (min)	25.17	27.17	27.25	25.08	26.27	26.06	25.98	0.9812	NS	NS	NS	NS	NS	NS	NS
Ruminanting															
NE	13.00	12.53	12.84	12.68	12.41	13.12	12.74	0.4198	NS	NS	NS	NS	NS	NS	NS
TRT (min)	410.0	386.4	402.7	393.7	407.7	388.7	397.6	9.2947	0.0219	NS	NS	0.0098	NS	NS	NS
TRT (%)	28.47	26.83	27.96	27.34	28.31	26.99	27.61	0.6454	0.0219	NS	NS	0.0098	NS	NS	NS
ATE (min)	32.27	31.95	32.55	31.67	33.77	30.44	32.08	1.1968	NS	NS	NS	0.0927	NS	NS	NS
Chewing															
NE	22.00	20.93	21.18	21.75	20.99	21.93	21.51	0.5140	NS	NS	NS	NS	NS	NS	NS
TCT (min)	630.0	607.3	624.1	613.1	625.7	611.6	618.1	12.218	0.0455	NS	NS	NS	NS	NS	0.0445
TCT (%)	43.75	42.17	43.34	42.58	43.45	42.47	42.92	0.8484	0.0455	NS	NS	NS	NS	NS	0.0445
ATE (min)	28.95	29.39	29.74	28.6	29.98	28.37	29.07	0.7145	NS	NS	NS	NS	NS	NS	NS
Drinking water															
NE	2.99	3.08	2.32	3.75	3.20	2.87	3.10	0.2891	NS	0.0106	NS	NS	NS	NS	NS
TDT (min)	20.3	18.5	18.8	20.0	19.7	19.1	19.2	2.5091	NS	NS	NS	NS	NS	NS	NS
TDT (%)	1.41	1.28	1.3	1.39	1.37	1.32	1.33	0.1742	NS	NS	NS	NS	NS	NS	NS
ATE (min)	5.14	4.86	5.07	4.93	4.94	5.06	5.01	0.3485	NS	NS	NS	NS	NS	NS	NS
Idleness															
NE	18.50	17.92	17.98	18.43	18.05	18.37	18.22	0.414	NS	NS	NS	0.0667	NS	NS	NS
TIT (min)	789.7	813.6	796.5	806.9	793.9	809.4	802.7	12.648	0.0118	NS	NS	0.0118	NS	NS	0.0097
TIT (%)	54.84	56.5	55.31	56.03	55.13	56.2	55.74	0.8783	0.0118	NS	NS	0.0118	NS	NS	0.0097
ATE (min)	43.05	46.25	45.02	44.28	44.47	44.83	44.71	1.2196	0.0498	NS	NS	NS	NS	NS	NS

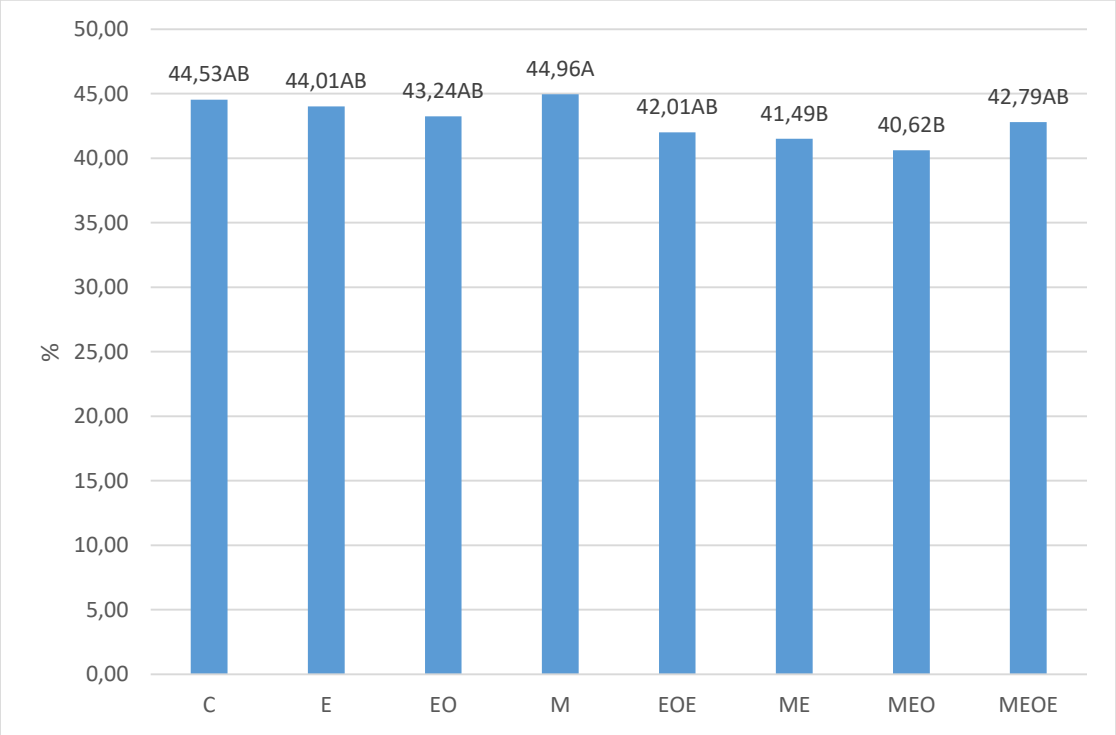
SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin; NE: Number of events; TIT (min): Total idleness time; ATE (min): Average time per event; TRT (min): Total ruminating time; TDT (min): Total water drinking time; TET (min): Total eating time; TMT (min): Total chewing time TCT (min). *Although a significant interaction (P<0.05) was observed, when performing the decomposition, no significant effect was observed.

Figure 3. Interaction between essential oil and enzymes on total ruminanting time.



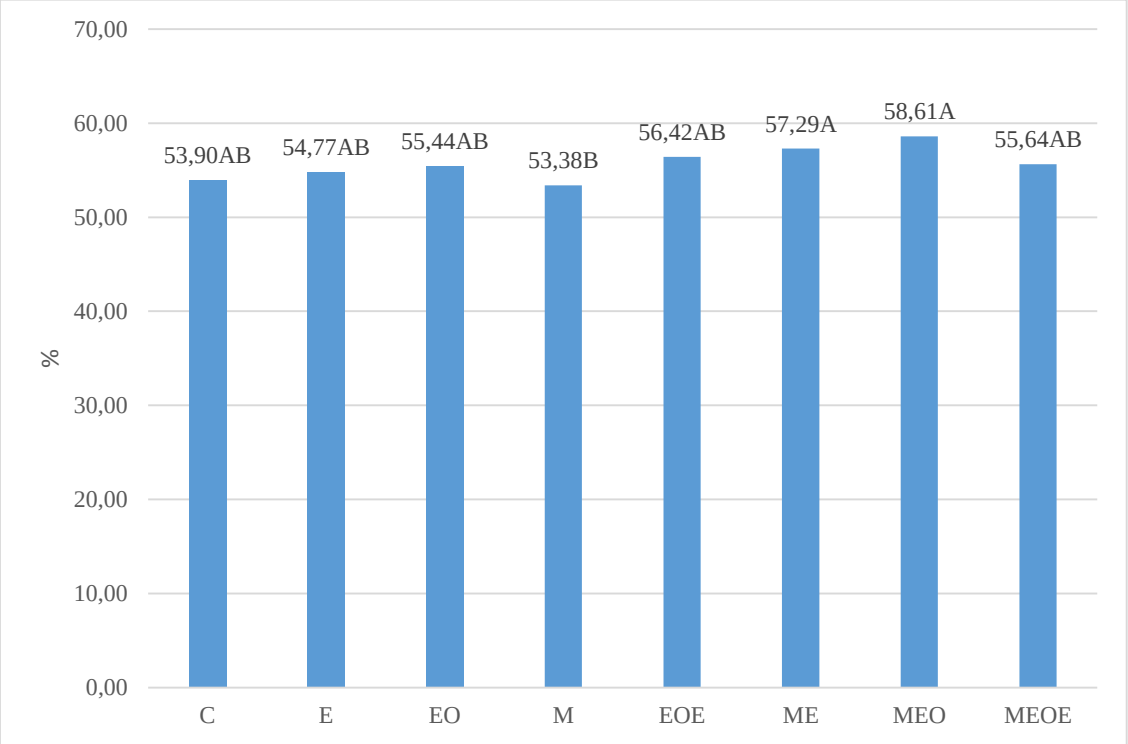
EO-E-: Absence of enzyme and essential oil; E: enzyme presence, EO: essential oil presence; EO+E: enzyme and essential oil presence.

Figura 4. Interaction between enzymes, essential oil and monensin on total chewing time (%).



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

Figure 5. Interaction between enzymes, essential oil, monensin on total idleness time (%).



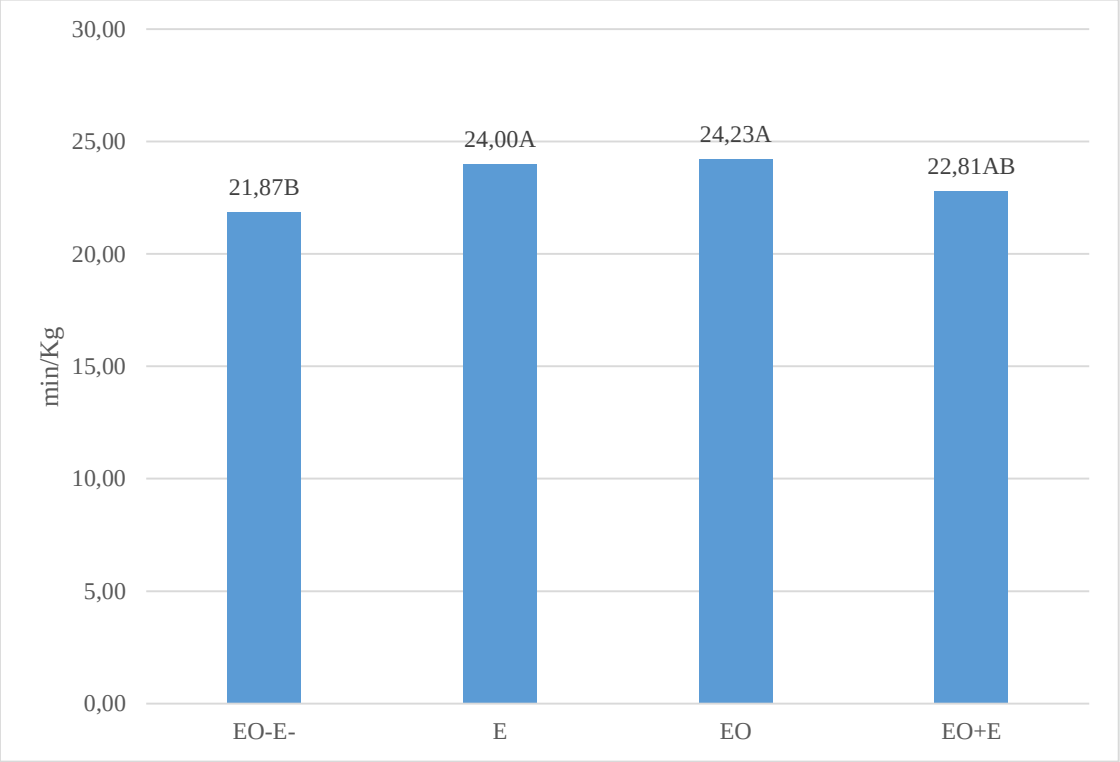
C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

Table 4. Efficiency of consumption, rumination and mastication of Nellore cows fed with essential oil, enzymes blend, monensin and their associations.

Variables	Factors						Mean	SEM	P value						
	Essential Oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
Consumption															
DM, min/kg	22.94	23.52	23.05	23.40	22.77	23.69	23.24	1.0913	NS	NS	NS	0.0101	NS	0.0381*	NS
DM, kg/min	0.047	0.046	0.047	0.046	0.046	0.047	0.046	0.0248	NS	NS	NS	0.0058	NS	0.0126*	NS
DM, kg/NE	1.197	1.239	1.246	1.19	1.216	1.219	1.207	0.0799	NS	NS	NS	NS	NS	NS	0.0886
NDF, min/kg	69.43	71.19	69.77	70.85	68.91	71.7	70.35	3.3028	NS	NS	NS	0.0101	NS	0.0381*	NS
NDF, kg/min	0.015	0.015	0.016	0.015	0.015	0.016	0.015	0.0008	NS	NS	NS	0.0058	NS	0.0126*	NS
NDF, kg/NE	0.395	0.409	0.412	0.393	0.402	0.403	0.399	0.0264	NS	NS	NS	NS	NS	NS	0.0886
Rumination															
DM, min/kg	42.11	41.00	41.59	41.52	42.39	40.71	41.48	1.3799	NS	NS	NS	NS	NS	NS	NS
DM, kg/min	0.024	0.025	0.024	0.025	0.024	0.025	0.025	0.0008	NS	NS	NS	NS	NS	NS	NS
DM, kg/NE	0.785	0.813	0.803	0.795	0.815	0.783	0.797	0.0383	NS	NS	NS	NS	NS	NS	NS
NDF, min/kg	127.5	127.1	125.9	125.7	128.3	123.2	125.5	4.1764	NS	NS	NS	NS	NS	NS	NS
NDF, kg/min	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.0002	NS	NS	NS	NS	NS	NS	NS
NDF, kg/NE	0.259	0.268	0.265	0.262	0.269	0.258	0.263	0.0126	NS	NS	NS	NS	NS	NS	NS
Chewing															
DM, min/kg	65.05	64.44	64.57	64.92	65.09	64.4	64.72	2.1819	NS	NS	NS	NS	NS	NS	NS
DM, kg/min	0.016	0.016	0.016	0.016	0.016	0.016	0.016	0.0005	NS	NS	NS	NS	NS	NS	NS
DM, kg/NE	0.462	0.476	0.472	0.466	0.471	0.467	0.468	0.0229	NS	NS	NS	NS	NS	NS	NS
NDF, min/kg	196.9	195.1	195.5	196.5	197.0	194.9	195.9	6.6040	NS	NS	NS	NS	NS	NS	NS
NDF, kg/min	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.0002	NS	NS	NS	NS	NS	NS	NS
NDF, kg/NE	0.153	0.157	0.156	0.154	0.156	0.154	0.155	0.0075	NS	NS	NS	NS	NS	NS	NS

SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin; DM: dry matter; NDF: neutral detergent fiber; NE: number of events; * Although a significant interaction (P<0.05) was observed, when performing the decomposition, no significant effect was observed.

Figura 6. Interaction between enzymes and essential oil on DMI efficiency.



E-OE-: Absence of enzymes and essential oil; E: Enzyme presence, EO: Essential oil presence; E+OE: Enzyme and essential oil presence.

3.3.2. Ruminal degradability

The use of exogenous enzymes increased (P<0.05) the potentially degradable fraction of NDF (Table 5) by 6.21%.

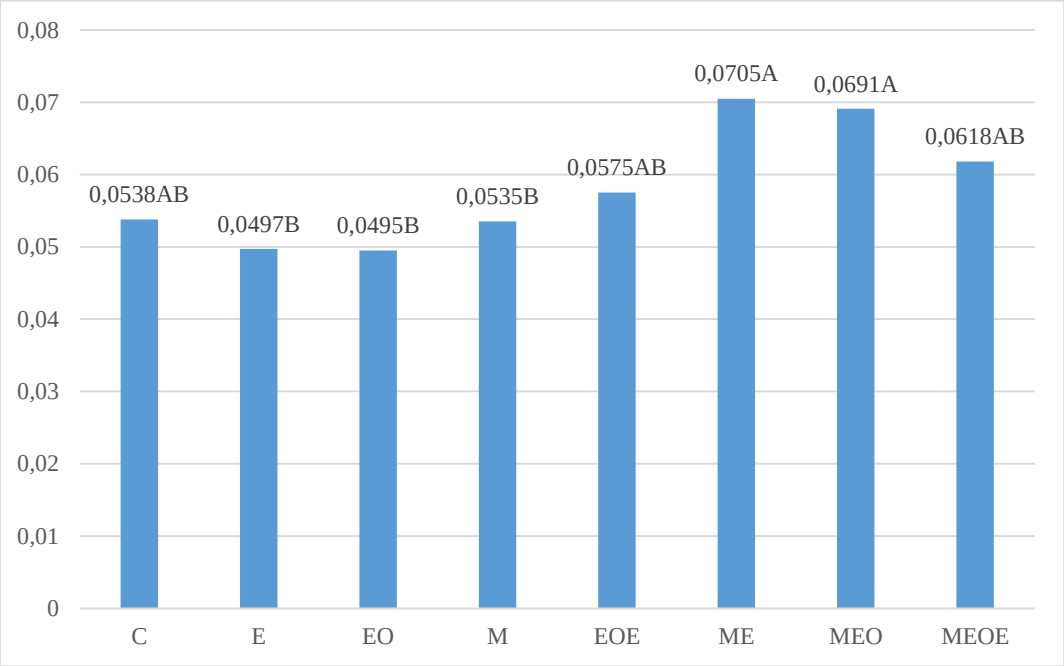
A triple interaction of tested additives was also observed on the degradation rate (C) of DM and CP (Table 5), where the use of additives alone did not change the variable in question, but the association ME and MEO resulted in increased DM degradation rate when compared to the use of additives alone (Figure 7). Regarding CP degradation, the ME association increased the degradation rate when compared to the isolated use of E, EO, and their association (EOE), while the MEO association presented a higher degradation rate than the use of E and EO alone (Figure 8).

Table 5. *In situ* degradability of DM, NDF and CP of Nellore cows fed with Essential oil, enzyme blend, monensin and their interactions.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
DM															
a (%)	32.76	32.42	32.73	32.45	32.67	32.51	32.56	0.2085	NS	NS	NS	NS	NS	NS	0.0756
b (%)	53.37	53.64	53.38	53.64	53.78	53.24	53.51	0.2042	NS	NS	NS	NS	NS	NS	NS
c (h^{-1})	0.057	0.059	0.056	0.060	0.053	0.064	0.058	0.0023	NS	NS	NS	NS	NS	NS	0.0073
RED (%)	59.71	59.75	59.82	59.64	58.34	61.12	59.69	0.6804	NS	NS	NS	NS	NS	NS	NS
PD (%)	86.12	86.06	86.08	86.09	86.46	85.72	86.08	0.1866	NS	NS	NS	NS	NS	NS	NS
Und (%)	13.88	13.94	13.91	13.91	13.54	14.27	13.92	0.1866	NS	NS	NS	NS	NS	NS	NS
NDF															
a (%)	3.26	3.774	3.88	3.15	3.54	3.49	3.50	0.3295	NS	NS	NS	NS	NS	NS	NS
b (%)	70.01	67.048	66.47	70.59	68.69	68.37	68.42	0.9254	0.0902	0.0241	NS	NS	NS	NS	NS
c (h^{-1})	0.029	0.03209	0.032	0.030	0.028	0.033	0.031	0.0014	NS	NS	NS	NS	NS	NS	0.0785
RED (%)	26.45	27.785	27.83	26.40	25.55	28.68	27.48	0.9800	NS	NS	NS	NS	NS	NS	NS
PD (%)	73.26	70.78	70.35	73.69	72.16	71.88	71.93	0.9200	NS	0.0612	NS	NS	NS	NS	NS
Und (%)	26.74	29.219	29.64	26.31	27.84	28.12	28..07	0.9200	NS	0.0612	NS	NS	NS	NS	NS
CP															
a (%)	25.67	25.984	26.15	25.51	26.34	25.31	25.78	0.4708	NS	NS	NS	0.0709	NS	NS	NS
b (%)	65.36	64.83	64.63	65.56	64.82	65.36	65.03	0.5782	NS	NS	NS	NS	NS	NS	NS
c (h^{-1})	0.075	0.0766	0.074	0.078	0.067	0.085	0.076	0.0036	NS	NS	NS	NS	NS	NS	0.0188
RED (%)	62.71	62.761	62.67	62.81	61.11	64.36	62.75	0.8753	NS	NS	NS	NS	NS	NS	NS
PD (%)	90.87	90.814	90.61	91.06	91.16	90.52	90.81	0.3160	NS	NS	NS	NS	NS	NS	0.0884
Und (%)	9.13	9.186	9.38	8.93	8.83	9.48	9.18	0.3160	NS	NS	NS	NS	NS	NS	0.0884

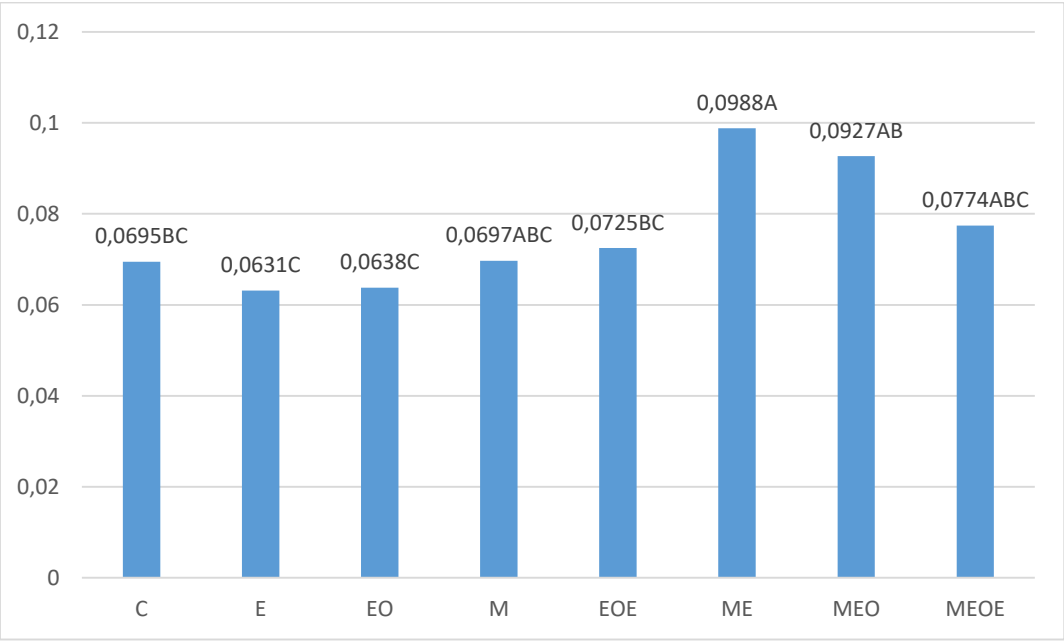
SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin; DM: dry matter; CP: crude protein; NDF: neutral detergent fiber: Interception of the curve at time zero, water-soluble and completely degradable fraction of the analyzed nutritive component leaving the nylon bag rapidly; b: Potentially degradable fraction; c: Rate of degradation of the potentially degradable fraction; lag: time at which the equation derived for a data set equals the actual potentially degradable fraction at zero time; RED: Real effective degradability; PD: Potential degradability (a + b); Und: Undigested fraction (100-PD)

Figure 7. Interaction between enzymes, essential oil and monensin on the degradation rate of the potentially degradable fraction of DM.



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

Figure 8. Interaction between enzymes, essential oil, monensin and their interactions on CP potential degradability rate.



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

3.3.3. Rumen kinetics

Neither effect was observed on rumen dynamics and kinetics on cows fed with EO, E, M and their associations. The results are shown on tables 6 and 7.

Table 6. Rumen dynamics of Nellore cows fed with essential oil, enzyme blend, monensin and their interactions.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
DM%	10.50	10.63	10.88	10.25	11.19	9.34	10.57	0.2452	NS	0.0896	0.0654	NS	NS	NS	NS
Liquid mass															
kg	38.23	36.71	37.22	37.71	3.81	41.13	37.48	1.2118	NS	NS	NS	NS	NS	NS	NS
%BW	6.97	6.87	6.91	6.93	6.3	7.54	6.92	0.2554	NS	NS	NS	NS	NS	NS	NS
Solid mass															
kg	4.51	4.36	4.52	4.35	4.33	4.54	4.43	0.1615	NS	NS	NS	NS	NS	NS	NS
%BW	0.81	0.82	0.84	0.79	0.80	0.83	0.81	0.0317	NS	NS	NS	NS	NS	NS	NS
Total mass															
kg	42.75	41.07	41.74	42.08	38.15	45.67	41.91	1.3372	NS	NS	NS	NS	NS	NS	NS
%BW	7.78	7.68	7.74	7.72	7.1	8.36	7.73	0.2800	0.NS	NS	NS	NS	NS	NS	NS
Disappearance rate (kt)															
kg/h	0.46	0.40	0.42	0.42	0.41	0.41	0.41	0.0135	NS	NS	NS	NS	NS	NS	NS
%/h	8.05	8.31	8.19	8.17	8.19	8.17	8.20	0.2149	NS	NS	NS	NS	NS	NS	NS

SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin; kt: Disappearance rate of solid rumen mass.

Table 7. Rumen kinetics of Nellore cows fed with essential oil, an enzyme blend, monensin and their associations.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Presente	Absence	Presente	Absence	Presente									
kt	8.05	8.31	8.19	8.17	8.19	8.17	8.20	0.2149	NS	NS	NS	NS	NS	NS	NS
kd	2.39	2.42	2.22	2.58	2.64	2.16	2.45	0.3202	NS	NS	NS	NS	NS	NS	NS
kp	5.89	5.90	5.96	5.82	5.78	6.00	5.83	0.0029	NS	NS	NS	NS	NS	NS	NS

SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin; kt: Rate of disappearance of the solid mass in the rumen (kp + kd); kd: Rate of digestion in the rumen ; kp: Rate of passage of undigested residues through the digestive tract.

3.3.4. Digestibility and nutrients excretion

As well as on DMI (Figure 1), it was observed an interaction between EO and E on nutrients consumption (Table 8), but once that the diets received the same amount of protein and energy, there was no effect ($P > 0.05$) on DM excretion and their nutrients (Table 9).

On the other hand, a triple interaction was observed in the aspect of total DM digestibility, as well as OM, GE, FDA, and NFC (Table 10), where the isolated use of monensin and enzymes promoted an increase in total apparent digestibility, DM and, the nutrients previously mentioned ($P < 0.05$), and consequently increasing TDN by 10.82% and 12.26%, respectively when compared to the control group (Figure 9).

Table 8. DM consumption and their nutrients on Nellore cows fed with essential oil, an enzyme blend, monensin and their associations.

Consumption	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	OE*M	E*M	EO*E*M
	Absence	Presente	Absence	Presente	Absence	Presente									
DM (kg)	9.91	9.47	9.65	9.73	9.57	9.81	9.66	0.2840	0.0945	NS	NS	0.0403	NS	NS	NS
CP (kg)	1.35	1.29	1.31	1.32	1.30	1.34	1.32	0.0383	0.0932	NS	NS	0.0409	NS	NS	NS
GE (Mcal)	43.6	41.7	42.5	42.8	42.2	43.2	42.5	1.2526	0.0947	NS	NS	0.0402	NS	NS	NS
NDF (kg)	3.21	3.07	3.12	3.16	3.10	3.18	3.13	0.0931	0.0956	NS	NS	0.0399	NS	NS	NS
ADF (kg)	1.95	1.87	1.9	1.92	1.89	1.93	1.9	0.0569	0.0959	NS	NS	0.0398	NS	NS	NS
EE (kg)	0.26	0.25	0.26	0.26	0.25	0.26	0.26	0.0075	0.0944	NS	NS	0.0403	NS	NS	NS
NFC (kg)	4.33	4.14	4.22	4.25	4.18	4.29	4.22	0.1200	0.0942	NS	NS	0.0404	NS	NS	NS
OM (kg)	9.16	8.75	8.91	8.99	8.84	9.06	8.93	0.2627	0.0946	NS	NS	0.0403	NS	NS	NS
TDN (kg)	7.01	6.45	6.70	6.76	6.58	6.88	6.70	0.1809	0.003	NS	NS	NS	NS	0.0216*	0.0043*

SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin; DM: dry matter; CP: crude protein; GE: gross energy; Mcal; NDF: neutral detergent fiber; ADF: acid detergent fiber; EE: ethereal extract ; NFC: non fiber carbohydrate; OM: organic matter; TDN: total digestive nutrients; * Although a significant interaction (P<0.05) was observed, when performing the decomposition, no significant effect was observed.

Table 9. DM excretion and their nutrients on Nellore cows fed with essential oil, enzyme blend, monensin and their associations.

Excretion	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
DM (kg)	2.78	2.91	2.84	2.85	2.89	2.80	2.84	0.1704	NS	NS	NS	NS	NS	NS	NS
CP (kg)	0.43	0.46	0.44	0.45	0.46	0.43	0.44	0.0226	NS	NS	NS	NS	NS	NS	NS
GE (Mcal)	11.84	12.32	11.98	12.18	12.30	11.86	12.06	0.7346	NS	NS	NS	NS	NS	NS	NS
NDF (kg)	1.21	1.29	1.22	1.29	1.26	1.24	1.25	0.0813	NS	NS	NS	NS	NS	NS	NS
ADF (kg)	0.63	0.65	0.62	0.66	0.65	0.63	0.64	0.0376	NS	NS	NS	NS	NS	0.0866	0.0748
EE (kg)	0.07	0.08	0.07	0.09	0.07	0.08	0.08	0.0070	NS	NS	NS	NS	NS	NS	NS
NFC (kg)	0.67	0.68	0.72	0.63	0.69	0.65	0.67	0.0482	NS	NS	NS	NS	NS	NS	NS
OM (kg)	2.39	2.51	2.45	2.46	2.50	2.41	2.45	0.1511	NS	NS	NS	NS	NS	NS	NS
N (kg)	0.069	0.073	0.070	0.072	0.073	0.069	0.071	0.0043	NS	NS	NS	NS	NS	NS	NS

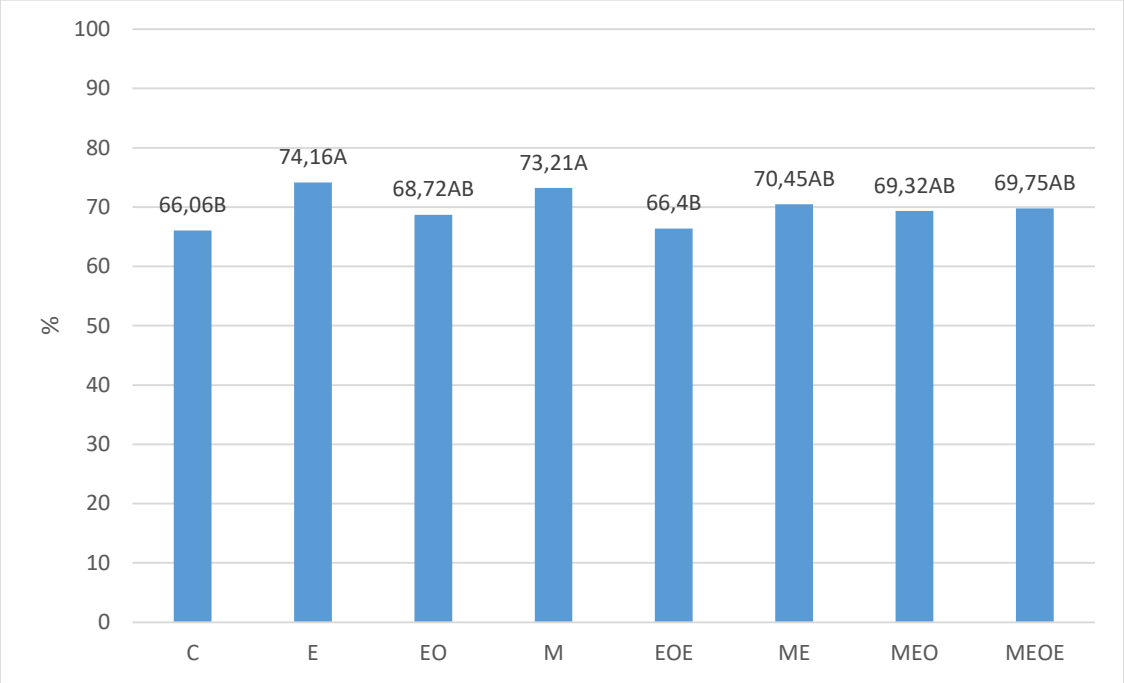
SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin.

Table 10. Total appearance digestibility on Nellore cows fed with essential oil, enzyme blend, monensin and their interactions.

DIG (%)	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
DM	72.23	69.75	70.40	71.59	70.01	72.00	71.00	1.1776	NS	NS	NS	NS	NS	NS	0.0312
CP	68.06	65.21	66.26	67.05	64.95	68.32	66.7	1.4334	NS	NS	NS	NS	NS	NS	0.0517
GE	73.24	70.92	71.71	72.45	71.05	73.11	72.07	1.1561	NS	NS	NS	NS	NS	NS	0.0213
NDF	62.56	58.68	60.76	60.48	59.79	61.45	60.59	1.7693	NS	NS	NS	NS	NS	NS	NS
ADF	67.76	65.48	66.86	66.38	65.69	67.55	66.53	1.2692	NS	NS	NS	NS	NS	0.0185	0.0097
EE	72.16	68.73	72.65	68.24	70.71	70.18	70.52	2.0595	NS	NS	NS	NS	NS	NS	NS
NFC	85.00	83.63	83.01	85.62	83.34	85.29	84.29	0.8782	NS	0.0733	NS	NS	NS	NS	0.0304
OM	74.23	71.73	72.44	73.53	71.97	74.00	72.98	1.1331	NS	NS	NS	NS	NS	NS	0.0442
TDN	70.97	68.55	69.33	70.19	68.84	70.68	69.76	1.0938	NS	NS	NS	NS	NS	NS	0.0407

SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin.

Figure 9. Interaction between enzymes, essential oil and monensin on TDN.



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

3.3.5. Rumen microbial protein

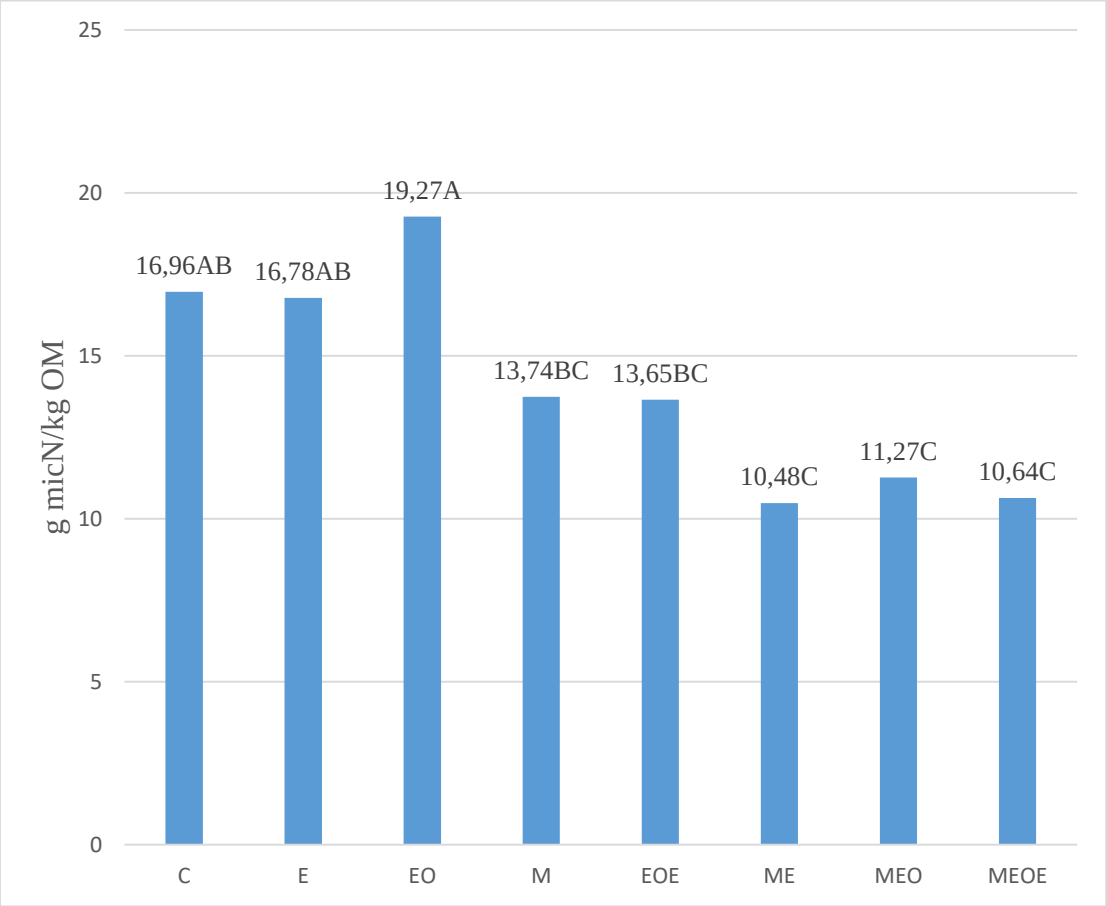
Cows fed with monensin presented an decreased on alantoin and purine derivates excretion. Therefore, a decrease on micN synthesis was observed (P = 0.010). Furthermore, a triple interaction was observed on microbial efficiency, wherein the M in association to the other additives (ME, MEO, MEOE) decreased the efficiency when compared to the control group and isolated use of E and EO (Figure 10).

Table 11. Urinary volume, excretion of urinary compounds, microbial nitrogen synthesis and efficiency of microbial protein synthesis of Nellore cows fed with essential oil, enzyme blend, monensin and their associations.

Variable	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
Urinary volume															
L/day	9.82	10.34	9.72	10.43	7.74	12.41	10.08	0.735	NS	NS	0.095	NS	NS	NS	NS
Urinary compounds															
Urea, mg/kg $BW^{0.75}$	1.83	1.82	1.84	1.81	1.85	1.79	1.822	0.039	NS	NS	NS	NS	NS	NS	0.017
Al, mmol/kg $BW^{0.75}$	1.12	1.05	1.15	1.02	1.21	0.96	1.085	0.044	NS	0.068	0.020	NS	NS	NS	NS
UA, mmol/kg $BW^{0.75}$	0.18	0.16	0.17	0.16	0.20	0.14	0.171	0.009	0.0381	0.082	0.077	0.018	0.0365	NS	NS
DP, mmol/kg $BW^{0.75}$	1.29	1.22	1.33	1.18	1.41	1.09	1.256	0.049	NS	0.062	0.007	NS	NS	NS	NS
Al (%) PuD	86.00	86.44	84,44	86.00	85.61	86.83	86.22	0.628	NS	NS	NS	0.0478*	NS	NS	NS
Sintesys of micN															
mg/kg BW	141.6	130.9	147.0	125.5	158.9	113.5	136.2	7.15	NS	0.059	0.008	NS	NS	NS	NS
mg/kg $BW^{0.75}$	684.1	628.4	707.9	604.6	764.7	547.8	656.2	33.90	NS	0.062	0.007	NS	NS	NS	NS
Microbial efficinecy, kg $PV^{0.75}$															
g micN/kg OM	14.49	13.71	15.31	12.89	16.67	11.53	14.09	0.736	NS	0.017	0.010	NS	NS	NS	0.041

SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin; Al: Allantoin; UA: Uric acid; PuD: Purine derivatives; Al (%) PuD: Allantoin percentage in total purine derivatives; micN: Microbial nitrogen. * Although a significant interaction ($P<0.05$) was observed, when performing the decomposition, no significant effect was observed.

Figure 10. Interaction between enzymes, essential oil and monensin on microbial efficiency.



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

3.4. DISCUSSION

3.4.1. Feed intake and feeding behavior

In the present study, animals that received essential oil or enzyme, individually, showed a 9.62% and 8.02% decrease, respectively, in DMI. In the literature, the effects of the EO under the DMI, especially the cinnamaldehyde and garlic oil, are not completely understood. Despite some studies present no influence on the DMI (BENCHAAR et al., 2008b; CHAVES et al., 2008; BLANCH, 2017), while others (BUSQUET et al., 2003; CARDOZO et al., 2006; YANG et al. 2007) presented a decrease in the DMI, following the results presented in the current study. According to Blanch et al. (2017), is not established how the EO impacts the DMI, but one possible reason is related to the oil palatability, some compounds may not be agreeable, and, therefore, promote a consumption decreasing.

Furthermore, in the current study, beyond the DMI decreases, the consumption efficiency was also decreased, in other words, the animals spent more time eating 1 kg of DM, and this effect can be associated with the additive's palatability. Previous studies reported a decrease in DMI when animals were fed with fibrolytic enzymes (HOUTSHAUSEN et al., 2011) and proteolytic enzymes (EUN and BEAUCHEMIM, 2005); however, no possible reasons were described. The findings in the current study are following previous ones.

Monensin is well reported to reduce feed intake. Duffield et al. (2008; 2012), in a meta-analysis, reported a decrease in DMI when cattle were fed with it. On the other hand, in the present study, cows fed with M does not present a reduction in DMI, similar to results found by Benchaar et al. (2006), Hamilton et al. (2010), Mullins et al. (2012), Perna Junior et al. (2017) and Tseu (2019). Wherein the experimental diets were similar to the used in this study, within a roughage inclusion of 45% to 50%.

Nevertheless, the total chewing time (TCT) presented a triple-interaction between factors. When the M was associated with EO and E, the TCT was reduced when compared with the M isolated. Therefore, the total idleness time (TIT) was increased, driving us to conclude that the feed additives were capable to modify the animal's feeding behavior. This behavior change can harm rumen metabolism, once that the masticating process is responsible to maintain the ruminal pH, through saliva (PERNA JUNIOR et al., 2017). However, no previous studies demonstrate the association between those additives, and how they can modulate the feeding behavior.

3.4.2. Digestibility and excretion of DM and nutrients

The association of ME and MEO increased the DM and CP degradability rate, without modifying the real or potential degradability. However, when the NDF ruminal degradability was analyzed the E supplementation promote an increase of 6.21% on the potentially degradable fraction, resulting in a trend ($P = 0.0621$) to increase the potential degradability of NDF. Nevertheless, the effects of the use of exogenous enzymes on rumen degradability remain inconsistently. Hristov et al. (2000) observed an increase in cellulase and xylanase activity in the rumen of cows fed with exogenous enzymes. In contrast, Colombatto et al. (2003) demonstrated that the addition of exogenous enzymes to the diet can affect degradation at the beginning of the incubation period; however, it affects the degree of degradability of the forage.

When analyzing the digestibility of the diet, Sujani and Seresinhe (2015) observed that both fibrolytic and proteolytic enzymes can increase the digestibility of both NDF and ADF. A similar result was observed in the present study in which the use of E increased the total apparent digestibility of DM, OM, and ADF similarly, resulting in a 12.26% increase in TDN when compared to the control group. Beauchemin et al. (1999), evaluating diets composed of barley silage, found an increase from 58.8 to 61.7% in NDF digestibility with the addition of exogenous fibrolytic enzymes, while Eun & Beauchemin (2005) reported that diets containing proteolytic enzymes (1.25 mL/kg DM) had greater digestibility. In both studies, the amplitude of increase in digestibility was smaller than that observed in this experiment; however, both used a single type of enzyme, whereas this study used an enzyme blend.

Effects of the use of ionophores on the digestion and absorption of nutrients were reported in a meta-analysis carried out by Spear (1990), indicating a 2% increase in the apparent digestibility of dietary energy in cattle, with a maximum increase of 9.2% being reported. Such an increase was lower than that found in the present study, where the use of sodium monensin increased the apparent digestibility of DM, OM, and ADF, resulting in an increase of 10.82% in the TDN of the diet when compared to the control group. This result was remarkable, since the use of sodium monensin did not affect ruminal degradability, nor on ruminal kinetics or dynamics. It is not possible to infer how sodium monensin acted to improve the digestibility of the diet in the present study. According to Spear (1990), the wide variation in the digestible energy response to the addition of ionophores can be explained by the dietary fiber, since the effect on fiber digestibility seems to depend on the diet and the fiber source.

Despite the increase in the total apparent digestibility of DM, OM, ADF, and consequently TND with the isolated use of M or E, no additive or synergistic effect was observed in the association of such additives, indicating that such additives possibly reached the diet digestibility plateau. Similarly, Freire (2018), when analyzing the association of monensin with amylolytic enzyme and a blend of essential oils on the apparent digestibility of DM and its nutrients in a diet composed of 48% corn silage, did not observe an increase in digestibility when associating such additives.

The use of EO did not change the digestibility of DM and its components in the present study. Similarly, Carvalho (2018), evaluating the effect of different essential oils (Eucalyptus, lemongrass, and Aroeira) in a diet composed of 70% corn silage and 30%

concentrate, also did not observe changes in digestibility, as well as in degradability or ruminal kinetics, as well as in the present study. However, according to Hart (2008), essential oils alter the ruminal microbiota, which consequently alters the digestion of degradable food fractions, especially carbohydrates and proteins. Beauchemin and McGinn (2006) observed a decrease in diet digestibility when using a mixture of essential oils (1 g/day of the mixture containing thymol, eugenol, limonene, and vanillin) in beef cattle consuming a diet rich in forage. Benchaar et al. (2007) did not report any change in digestibility in lactating dairy cows supplemented with a mixture of essential oils at doses of 0.75 or 2.0 g/day.

3.4.3. Urinary parameters and microbial protein

The sodium monensin is well know and well related for the ability to decrease the ruminal ammonia concentration due to their action on proteolytic bacteria and the increase of non degraded protein to the small intestine (HAIMOND et al., 1996), and as a result, we can observe a reduction on the ruminal microbial protein flow. Muntifering et al. (1981) observed a reduction on ruminal microbial protein in heifers supplemented with 33 ppm of M in a diet with 90% of whole shelled corn. This modifies on rumen protein metabolism present benefits to the animal, that receives a greater amount of protein due to the outflow of dietary aminoacids to the small intestine, once that the proteolysis and synthesis of microbial protein is decreased (WEDEGAERTENER e JOHNSON, 1983; ZANINE et al., 2006).

The previous found are in agreement with the results observed in the current study, where animals fed with M have shown a decrease in the purine derivatives excretion, and as a result, it has led to a lower microbial N synthesis (micN), as presented on Table 11. However, this result can not be related to decreases on ruminal CP degradability, once that this variable was not affect to the supplementation of M (Table 5). The results that could justify the founds on this chapter are presented on Chapter 2 (i.e., ruminal ammonia concentration).

Furthermore, not only the micN was affect by the use of M, but also the microbial efficiency (Figure 10), reinforcing its great capacity of action on ruminal bacteria (NAGARAJA et al., 1997) decreasing the micN synthesis and consequently the microbial efficiency.

According to Benchar et al. (2011), the M, EO also can affect the rumen microbiota. However, in the present study, the administration of EO did not affect the microbial synthesis. Furthermore, the micN was higher in animals fed with EO in comparison with animals fed with M and may indicate a lower effect of this additive on the rumen microbiome. On the other hand, the association between EO and E caused an antagonist effect by reducing the micN efficiency, but in contrast, when the additives were isolated administrated, this effect was not observed. However, neither previous study can explain why this association causes a negative effect. Therefore, more studies are necessary to understand completely the impacts of EO in association with E in rumen microbiome and microbial efficiency.

3.5. CONCLUSIONS

The isolated use of E or EO proved to promote a reduction in the DMI, influencing ingestive behavior and consumption efficiency; however, such effects are nullified when such additives are associated.

The association between the tested additives did not prove to be an efficient nutritional strategy to improve the digestibility of the diet. An antagonistic effect is observed in the association of M with E and/or EO, reducing microbial efficiency. However, more studies are needed to assess the association of such additives in different diet profiles.

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4. EFFECT OF THREE DIFFERENT FEED ADDITIVES AND THEIR ASSOCIATIONS ON RUMEN ENVIRONMENT AND FEED ENERGY PARTITION OF NELLORE COWS

Abstract

This study aimed to evaluate the use of essential oils, exogenous enzymes, sodium monensin, and their associations as a nutritional strategy on ruminal fermentation parameters and energy fractionation of Nellore cows to mitigate ruminal CH_4. Eight cannulated Nellore cows (480 ± 55 kg) were used, distributed in a duplicated 4 x 4 Latin square design, in a 2 x 2 x 2 factorial arrangement (4 periods of 28 days) totaling 4 replications (32 experimental units). The animals received a basal diet composed of 60% corn silage and 40% concentrate, with the supply adjusted daily as a function of DMI. The factors were composed by the presence or absence of exogenous enzyme, essential oil, or monensin in the diet. No effect of the tested additives on ruminal pH was observed. The use of EO and E reduced the ruminal population of Dasytricha, and antagonism was observed when combined with M. The use of M acted on the rumen bacteria, reducing bacterial diversity. The combination between E and M reduced the production of butyric acid, indicating an associative effect between these additives. The use of M increased the rumen balance of $N\text{-}NH_3$. There was no effect of the tested additives on the total SCFA production or CH_4 production. A triple interaction was observed between the tested additives on energy partition, indicating that the isolated use of E reduces the %DE released in the form of CH_4, increasing the %GE released in the intestine and providing less GE loss in the feces. However, the association between E and EO proved to be antagonistic when analyzing %GE released in the intestine and %GE lost in feces. The isolated use of M also reduced the %GE lost in feces. The use of E proved to reduce losses in the form of CH_4 and increase energy absorption in the intestine of Nellore cows. However, its association with EO proved to be antagonistic, inhibiting such effects. The association between the tested additives did not prove to be a good nutritional strategy for mitigating the production of ruminal CH_4, as it was observed an antagonism with an increase in DE lost in the form of CH_4 and a reduction in GE released in the intestine.

Keywords: Methane, Rumen fermentation, additive association, Exogenous enzyme, *in vivo*

4.1. INTRODUCTION

The increase in the demand for food caused by the growth in the world population and environmental issues have imposed enormous challenges on the agricultural sector in the coming years. Despite its great importance for the generation of jobs, income, and food production, the environmental impact caused by the sector has been the focus of great discussions about the climate stability of the planet (HRISTOV et al., 2013; IPCC, 2014).

The agricultural sector represents a significant source of GHG, pointed out by the IPCC (2014) as responsible for about 10 to 12% of global anthropogenic GHG emissions, emphasizing CH_4 and N_2O. In addition, according to the IPCC (2014), analyzing non-CO_2 emissions, enteric fermentation of ruminants is responsible for around 30 to 40% of global agricultural emissions. When analyzing the Brazilian scenario, the farming sector was responsible for 29% of net GHG emissions in 2016, with enteric fermentation being responsible for 65% of these emissions (SEEG, 2018).

The CH4 produced through methanogenesis by ruminants is not only related to environmental issues, but also energy losses and consequently to reductions in the retention and use of ingested energy (MOUMEN et al., 2016). Although methanogenesis is a natural and essential process for ruminal metabolism, it has a direct relationship with the efficiency of ruminal fermentation, representing energy loss for the system and consequently influencing animal performance, since, according to Buddle et al. (2011), from 5% to 9% of the gross energy in the diet is lost as CH_4.

The CH_4 emissions have stimulated researchers to look for alternatives aimed at mitigating CH_4 produced by ruminants around the world (MARTIN et al., 2010). In addition to efficient production systems - such as pasture management techniques and animal genetic improvement - several nutritional strategies (especially the use of feed additives as ionophores, tannins, calcium nitrate, essential oils, lipids, microbial enzymes, CH_4 inhibitors, among others) (MOHAMMED et al., 2004; REIS et al., 2006; GERBER et al., 2013) have been used to manipulate the ruminal environment and reduce CH_4 emission. However, few have shown a substantial, persistent, and consistent decrease with the level of contemporary expectations of the technical-scientific community, especially when evaluated *in vivo*.

On the other hand, the use of drugs in the association is fundamental to finding solutions to some challenges faced by modern medicine. That has happened with the simultaneous prescription of drugs, being a commonly used practice in the medical field to improve the effectiveness of medications, reduce toxicity or treat coexisting diseases, which may result in a synergistic effect, where the result of the association is greater than the simple sum of the effects of the isolated medications (SECOLI, 2001).

Within this perspective, it is important to emphasize that the different additives used as ruminal metabolism modifiers do not decrease CH_4 production through a single mechanism of action. The use of sodium monensin, which presents itself as a classic manipulator of the ruminal environment, acts by directing the H_2 that would be used for the production of CH_4 for the production of propionic acid, thus causing a change in ruminal patterns with increased energy efficiency (McGUFFEY et al., 2001). Also, essential oils such as cinnamaldehyde and garlic have antimicrobial properties and the potential to modulate ruminal fermentation, being mostly intensively investigated in *in-vitro* experiments (BUSQUET et al., 2005a, b, c, 2006; CALSAMIGLIA et al., 2007; MATEOS et al., 2013; BLANCH et al., 2016). While exogenous enzymes appear as an alternative to promote improvements in the digestibility and use of the diet offered, because, even in conditions where the rumen has a high rate of fermentation in the diet, it is still possible to observe the elimination of degradable fiber and starch in the feces (GALLARDO et al., 2009; TRICARICO et al. 2007).

As the feed additives mentioned above do not have a single and exclusive mechanism of action, nothing prevents their effects from being additive or even synergistic in the ruminal environment and energy partition of the diet. Thus, the present study aimed to evaluate the effects of the association between essential oils, blend of exogenous enzymes and sodium monensin on the ruminal environment and partition of feed energy in Nellore cows.

4.2. MATERIAL AND METHODS

The current experiment was carried out at the Animal Nutrition and Production Department (VNP) of the College of Veterinary Medicine and Animal Science (FMVZ) of the University of Sao Paulo, Fernando Costa Campus in Pirassununga City, Brazil. The experiment was conducted under the guidelines established in accordance with the ethical principles of animal experimentation of the Commission of Ethics in the Use of Animals

of the College of Animal Science and Food Engineering (FZEA) – USP, under the protocol number CEUA 4788111017.

Eight Nellore cows, non-pregnant and non-lactating, with a mean body weight (BW) of 480 ± 55 kg and carrying rumen cannula were kept in a barn on individually pens with free accesses to water, individual feed bunks and sand bedding. The barn owned suspended fans that were automatically triggered when the temperature turned up to 28°C to avoid heat stress.

4.2.1. Experimental design and treatments

The animals were assigned into two contemporary 4 x 4 Latin squares, in a 2 x 2 x 2 factorial arrangement (Table 1), in which the experimental unit was the animal within each experimental period (n = 32). A basal diet (Table 2) with a concentrate: roughage (corn silage) ratio of 40:60 was used. The experimental diets received the same amount of energy and protein, differing only by the presence or absence of the following factors: (1) essential oil factor; (2) enzyme factor; and (3) monensin factor. Therefore, the animals were randomly assigned to the treatments following described:

The essential oil (EO) factor differed according to the presence or absence of this product in the diet: EO-A (absent essential oil): diet without the addition of essential oil; EO-P (essential oil present): diet added with 31.7 mg/kg DM of a blend of essential oils (43% cinnamaldehyde + 7% garlic oil). The enzyme (E) factor differed depending on the presence or absence of this product in the diet: E-A (absent enzyme): diet without the addition of enzyme; E-P (enzyme present): diet with the addition of 1027 mg/kg DM of the product containing an “enzyme blend” (cellulase, xylanase, amylase, protease, phytase, beta-glucanase, and pectinase). The monensin (M) factor differed with the presence or absence of this product in the diet: M-A (absent monensin): diet without the addition of monensin; M-P (monensin present): diet with the addition of 30.6 mg of sodium monensin/kg of DM.

Next Enhance (43% cinnamaldehyde + 7% garlic oil) (Novus International Inc., Indaiatuba, Brazil) was used for source of essential oil, Allzyme® SSF (Alltech Inc., Nicholasville, USA) was used for source of enzymatic blend and Rumenpac® (Grupo MCassab, São Paulo, Brazil) was used for source of Monensin Sodium.

4.2.2. Feeding management

The feed was offered *ad libitum* (5 - 10% of leftovers) and the animals were fed twice a day, at 8 a.m. and 4 p.m., in the form of total mixed ration (TMR). The diet was performed by the software BR-Corte 3.0, claiming an average daily gain (ADG) of 0.950 kg. The proportions of the various ingredients and the chemical composition of the diets are shown in Table 12.

Table 12. Proportion of ingredients and estimated chemical composition of experimental diets

Ingredients (% DM)	Basal diet
Corn Silage	60.00
Dry ground corn grain	25.40
Soybean meal	12.10
White salt	0.50
Mineral premix[1]	2.00
Chemical composition	
Dry matter[2] (%)	58.76
CP[2] (% DM)	13.37
RDP[3] (% CP)	66.40
RUP[3] (% CP)	37.26
NDF[2] (% DM)	36.43
NDFe[3] (% DM)	34.00
ADF[2] (% DM)	20.22
NFC[2] (% DM)	40.55
Starch[2] (% DM)	35.20
MM[2] (% DM)	7.45
Ca[2] (% DM)	0.44
P[2] (% DM)	0.31
EE[2] (%DM)	2.20
TDN[3] (% DM)	64.80

[1]Mineral premix, quantity per kg of product: 200 g of calcium, 70 g of sodium, 60 g of phosphorus, 20 g of sulfur, 20 g of magnesium, , 15 mg of cobalt, 700 mg of cooper, 700 mg of iron, 40 mg of iodine, 1.600 mg of manganese, 19 mg of selenium, 2.500 mg of zinc, 200.000 UI of vitamin A, 50.000 UI of D3 vitamin, 1.500 UI of vitamin E; [2]Quantititaed through chemical analysis; [3]Value estimated by BR-Corte 3.0 software.

Diets with a lower fiber inclusion are associate to decreases on CH_4 emissions (JOHNSON; JOHNSON, 1995), due to a reduction on ruminal H_2 sink (LAN; YANG, 2019). Therefore, in order to keep the enteric CH_4 production through diet and test the additives potentia, a 60%-forage diet was used.

4.2.3. Experimental period

The experiment was carried out in four periods of 28 days each. At the end of each period, the animals spent two days receiving the basal diet without the inclusion of the additive before the beginning of the next period. The first 16 days of each period were to adapt the animals to the diets. Between the 17th and 21st days, the dry matter intake (DMI) and the water consumption were evaluated. On the 22nd day, the rumen pH was measured continuously, and the rumen content was collected to quantify the rumen fermentation products (CH_4, SCFA, the concentration of N-NH_3), total and differential counts of protozoa and bacteria community. On days 25 and 26, the rumen was emptied to determine the rumen solid mass 3 hours after the morning feeding and before (0 hours) the morning feeding, respectively.

4.2.4. Feed intake and gross energy of the diet and feces

Dry matter intake (DMI) was evaluated between the 17th and 21st days of each experimental period. The feeders were daily examined, through observation, at 7 a.m., and the feed supply was monitored to ensure daily leftovers of approximately 5%. During the five days of evaluation, the leftovers from each cow were collected and weighted for the feed intake calculation, which was obtained by the difference between the amount of feed supplied and leftovers, multiplied by the diet DM. The water intake per day was also quantified during those five days by automatic and individual drinking fountains with water meters. The feces were sampled via rectum, twice a day (8 a.m. and 4 p.m.) to form a composite sample for each cow to determine gross energy (GE). They were dried in the oven with constant ventilation and renewal of air at 65°C for 72 hours (AOAC, 1995) and posteriorly ground in a willy type knife mill in 1 mm sieves and stored in properly sealed flasks. The GE of feces and diet was determined by complete oxidation in adiabatic colorimetric pump.

4.2.5. Rumen solid mass

The total solid mass was determined by total rumen emptying on days 25 and 26 of each experimental period. The rumen content was manually removed through the rumen cannula, as described by Dado and Allen (1995). On the 25th day, the emptying was performed at 11 a.m., three hours after diet administration, when the rumen was theoretically full. The same procedure was performed on the 26th day at 8 a.m. before diet administration, when the rumen was, theoretically, at the lowest volume. During the

removal of the rumen content the liquid and solid phases were separated by using a 2 mms sieve and buckets, then weighed, and samples of approximately 1 kg of each phase were collected for DM determination. Afterward, both phases were reconstituted and returned to the rumen. The rumen DM was calculated based on the dry weight of each sample.

4.2.6. Continuous pH measuring.

The pH was continuously measured on the 22nd day of each experimental period by using a data logger (model T7-1 LRCpH, Dascor, CA) according to the methodology described by Penner et al. (2006). The data logger consisted of a pH probe housed in a water-resistant capsule and an electrode protected by a structure that allowed the passage of particles and liquid while protecting the electrode from coming into contact with the rumen epithelium. Two 900 g weights were coupled to each probe to ensure that it remained in the ventral sac of the rumen. Each data logger was programmed to measure the pH every 10 minutes for 24 hours, allowing the calculation of the variables: minimum, medium, and maximum daily pH, time at which pH remained below 5.8, 6.0, and 6.2 as described by Moya et al. (2011).

Before and after introducing the probes in the rumen, they were calibrated in solutions of pH 7.0 and 4.0. The calibration allowed the calculation of a slope and an intercept before and after the test to adjust the measured data. The area under the curve was calculated by multiplying the absolute value of the deviations in pH by the time (min) spent below the threshold established for each measurement and divided by 60, being expressed as pH unit per hour, according to Moya et al. (2011).

4.2.7. Evaluation of rumen fermentation products

The SCFA, CH_4, and N-NH_3 were performed by using the *ex-situ* technique described by Rodrigues et al. (2012) and Perna Junior et al. (2017). This technique consisted of collecting rumen samples and putting in flasks (micro-rumen) which are incubated in a thermostatic bath, simulating the rumen conditions (microbiome, anaerobiosis, 39°C, saliva, and pH) for 30 minutes.

4.2.7.1. Sampling of rumen content

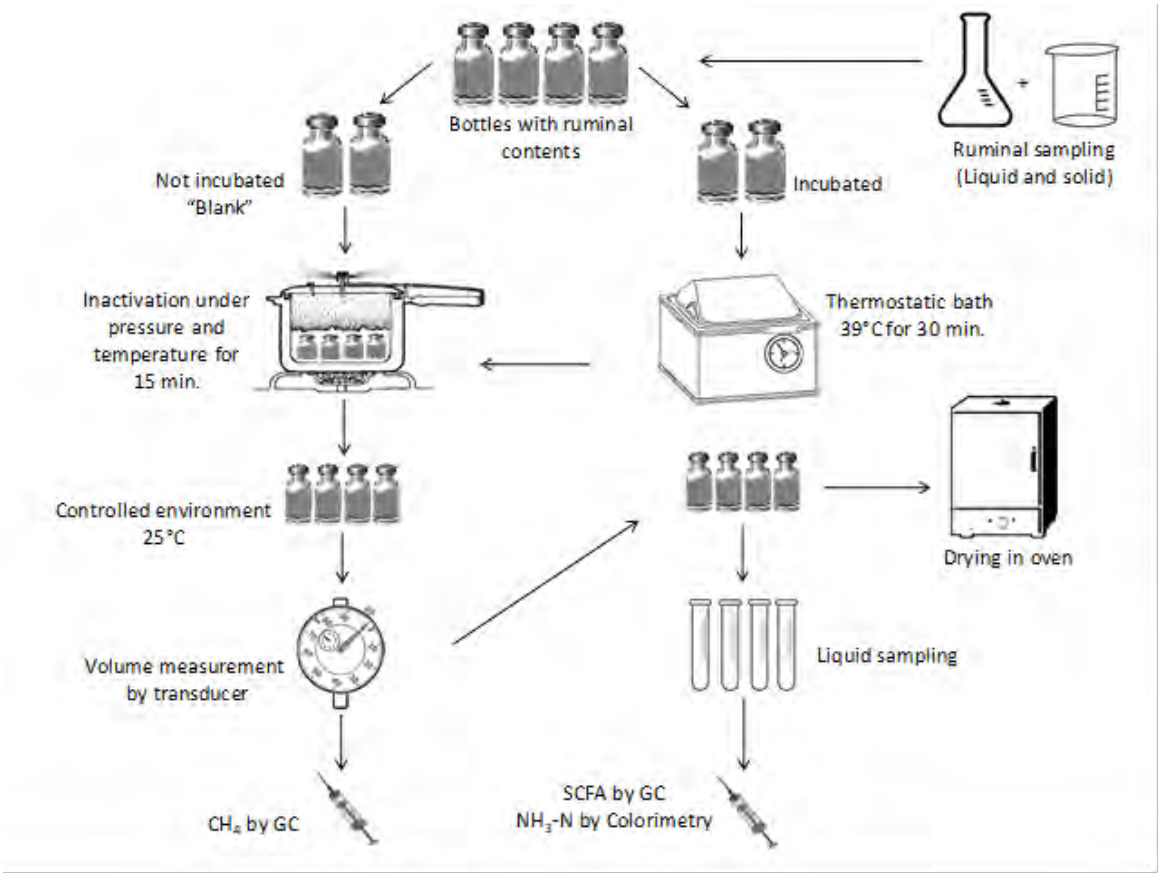
On the 22nd day of each experimental period, the rumen content was measured during the day at zero (0), 3, 6, 9, and 12 hours after the morning feeding. The samples were designated for the measuring of SCFA, CH_4, N-NH_3, and microbiota. On this day, the cows were fed after the first sampling time, around 8:20 a.m., and after the last sampling time, around 8:30 p.m. The liquid phase was collected with the aid of a probe coupled to a vacuum pump. The solid phase was collected by hand via cannula, collecting in three different points of the rumen. Both fractions were put in the flasks (about 10 g and 20 mL of the solid and liquid fraction, respectively). The flasks were then capped

with rubber stoppers and sealed with aluminum sealing wax through specific pilers. Afterward, they were "washed" with CO_2 through two needles for gas inlet and outlet to ensure anaerobiosi.

Four flasks per cow were prepared for each sampling time, two of which were immediately inserted into an autoclave to inactivate the fermentative process (under temperature and pressure) for 15 minutes. The other two flasks were immediately incubated for 30 minutes in a thermostatic bath at 39°C. At the end of the incubation time, the fermentative process was inactivated under temperature and pressure for 15 minutes.

After the flasks cooled, they were taken to the Gas Chromatography Laboratory of the VNP-FMVZ to measure the volume of gases and concentration of CH_4. The whole process is demonstrated in the figure 11.

Figure 11– Diagramatic representation of *ex situ* rumen fermentation technique.



Source: Perna Junior et al. (2017)

4.2.7.2. Methane quantification

The volume of gas produced in the incubated and non-incubated flasks was measured by using a pressure transducer (Data logger Universal AG5000, Genesis SM®, Barueri, SP – Brazil) connected to a reader with a syringe and needle. The volume was measured by dragging the accumulated gases in the upper part of the flask using the syringe connected to the transducer until a zero-pressure reading was obtained in the syringe plus the headspace of the flask. After measuring by the transducer, the determination of CH4 concentration in both incubated and non-incubated flasks was performed by gas chromatography, according to Kaminski et al. (2003), by injecting 0.5 mL of gas into a chromatograph (Trace 1300, Thermo Fisher Scientific®, Rodano, Milan – Italy).

4.2.7.3. Calculation of liquid volume and concentration of SCFA on rumen content

The volume of rumen liquid contained within the flasks was calculated by the difference between the weight of the flask containing the sample after drying in the oven at 105°C, and the weight of the flasks containing the sample before drying. The same procedure was executed to calculate the amount of solids, with the difference, in weight, between the flask containing the sample after drying and weight of the empty flask, obtained before flasks were filed.

The concentrations of SCFA (acetate, propionate and butyrate) were determined by taking about 4.0 mL of the liquid portion of the rumen contents of each flask and centrifuging for 15 minutes. Then, 2 mL of the supernatant was collected and inserted into a tube containing 0.4 mL of formic acid, as described for Erwin et al. (1961). Samples were refrigerated for 24 hours and then the SCFA were measured by gas chromatography (Focus GC, Thermo Scientific®, Rodano, Milan – Italy) by using a 1.22 m length and 0.63 cm diameter glass column packed with 80/120 Carbopack B-DA/4% (Supelco, Sigma-Aldrich, St. Louis, MO – USA).

4.2.7.4. Production of SCFA and CH_4, as well as the relative energy loss

The quantification of CH_4 production was obtained by multiplying the total volume of the gases (mL) produced in each flask by the concentration of CH_4 in the gas phase (mmol/mL) obtained in the incubated flask, subtracting what was produced in a non-incubated flask (equation 1). The individual quantification of SCFA (mmol/L) was

obtained in the incubated flask, subtracting the production in the non-incubated flask (equation 2).

Subsequently, the production of SCFA and CH_4 was expressed based on the solid content contained in the flasks, measured in grams or kilograms. This content was measured by the difference between the weight of the flask containing the sample after drying (105°C) and the weight of the empty flask.

Production of SCFA: (Conc. SCFA x Total Liq. Vol.) T_{30} - (Conc. SCFA x Total Liq. Vol.) T_0

Where: Prod. SCFA = SCFA production at the time between 30 minutes and zero (0) minute of incubation; Conc. SCFA = SCFA concentration (mmol/mL); Total Liq. Vol. = total volume of liquid in the flask; T_{30} = incubation time of 30 min; T_0 = incubation time of 0 min.

Prod. CH_4: (Conc. CH_4 x Total Gas Vol.) T_{30} - (Conc. CH_4 x Total Gas Vol.) T_0

Where: Prod. CH_4 = CH_4 production at the time between 30 minutes and zero (0) minute of incubation; Conc. CH_4 = CH_4 concentration (mmol/mL); Total Gas Vol. = total volume of gas, measured by the sum of the volume determined by the pressure transducer and the headspace (mL); T_{30} = incubation time of 30 min; T_0 = incubation time of 0 min.

Subsequently, for the quantification of fermentation products (SCFA and CH_4), each product was multiplied by its combustion heat to express the CH_4- production as a percentage of the energy from the fermentation produced. Therefore, the relative energy loss (REL) was the ratio between the energy contained in all fermentation products (SCFA and CH_4), expressed as a percentage as well. Thus, theoretical chemical values of the combustion heat were used, as acetate, propionate, butyrate, CH_4, and CO_2 present 3.49, 4.98, 5.96, 13.16, and 0.0 kcal per gram or 209.40, 368.52, 524.48, 210.56 and 0.0 per mol, respectively. The REL was calculated using the following equation, as described by Rodrigues et al. (2012):

$$REL\ (\%) = 100 \times [\text{Ɛ}CH_4/(\text{Ɛ}CH_4 + \text{Ɛ}C_2 + \text{Ɛ}C_3 + \text{Ɛ}C_4)]$$

Where:

REL: relative energy loss (%);

$\text{Ɛ}CH_4$: methane energy (kcal/g or kcal/mol);

$\text{Ɛ}C_2$: acetate energy (kcal/g or kcal/mol);

$\text{Ɛ}C_3$: propionate energy (kcal/g or kcal/mol);

$\text{Ɛ}C_4$: butyrate energy (kcal/g or kcal/mol).

4.2.7.5. Concentration of ammonia nitrogen (N-NH_3)

The ammonia nitrogen (N-NH_3) concentration was determined by taking about 4.0 mL of the liquid portion of the rumen content of each flask and centrifuged for 15 minutes. Then, 2 mL of the supernatant was collected and inserted into a tube containing 1 mL of 1N H_2SO_4 solution. After 24 hours in the fridge, the samples were analyzed through a colorimeter, according to the method described by Kulasek (1972), and adapted by Foldager (1977). The balance was obtained by the difference in the concentration of NH3-N between the flask incubated for 30 minutes with the non-incubated flasks. The balance data were estimated per hour, according to the following equation:

N-NH_3balance (mg/dL.h) = [Conc. 30 min (mg/dL) - Conc. 0 min (mg/dL)] x 2

Where:

Conc. 30 min = N-NH_3 concentration in incubated flasks;

Conc. 0 min = N-NH_3 concentration in non-incubated flasks.

4.2.8. Evaluation and quantification of rumen microbiota

On day 22nd of each experimental period, rumen content (mass and liquid) of each cow was sampled for bacteria DNA extraction as well for counting total and differential numbers of protozoa. For rumen bacteria community sequencing, samples were collected at 3 hours after morning feeding and for protozoa at zero (0), 3, 6, 9 and 12 hours after morning feeding.

4.2.8.1. Assessment of bacterial community sequence

Samples of 50 mL of whole rumen contents were collected on day 22 of each period through the ruminal cannula at 3 hours after the morning feeding and stored at −80◦C. After thawing, samples were separated by phase (liquid and solid). Proportionate amounts of liquid and solid phase rumen content, as determined by when the rumen was completely emptied and phases separated and measured, were combined and then processed to isolate DNA following the procedure detailed in Weimer et al. (2017).

The resuspended pellets were then processed to isolate DNA following the bead-beating method described by Weimer et al. (2017). The DNA was resuspended in 10 mM Tris HCl with 1 mM EDTA (pH 8.0), and was quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, United States), and stored at 4◦C before preparation of the DNA library. A two-step PCR was employed to amplify the V4 region of the 16S ribosomal RNA

gene for bacteria (KITTELMANN et al., 2013). PCR clean-up and sequencing were performed as described (CUNHA et al., 2017).

The PCR reactions contained 25–50 ng of DNA, 10 μM of each primer, 12.5 μL of 2X KAPA HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, United States), and water to a total volume of 25 μL. Cycling conditions were as follows: initial denaturation of 95◦C for 3 min, 25 cycles of 95◦C for 30 s, 55◦C for 30 s, and 72◦C for 30 s, and a final extension at 72◦C for 5 min. Gel electrophoresis was performed using a 1.0% low-melt agarose gel (National Diagnostics, Atlanta, GA, United States), where bands present at ∼380 bp indicated successful amplification.

Bands were excised from the gel and DNA was extracted from the bands using a ZR-96 Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, United States). No-template negative controls were included for each set of PCRs, and the absence of a band in the gel indicated no contamination was present. Extracted DNA was quantified in duplicate on 96-well microplates according to manufacturer's instructions for the Quant-iT dsDNA Broad-Range Assay Kit, using reagents from a Qubit dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, United States), read on a Synergy 2 Multi-Mode Reader (BioTek, Winooski, VT, United States) after a programmed 3-s shaking period and a 2-min incubation at 22◦C.

The extracted DNA was equimolar pooled. The final library was sequenced using a MiSeq v2 2 × 250 kit (Illumina, San Diego, CA, United States), with a final library concentration of 10 pmol/L and 10% PhiX control. Custom sequencing primers as described by Kozich et al. (2013) were used. Sequences were demultiplexed according to their sample-specific indices on the Illumina MiSeq.

Sequence data were processed using the software mother v.1.44.3 (SCHLOSS et al., 2015), following the Standard Operating Procedure previously described (KOZICH et al., 2013). The Chao richness estimator and Shannon index were used for the characterization of alpha diversity at the genus level of the taxonomy. Beta diversity evaluating similarities among samples was addressed by the Jaccard index and the Yue and Clayton index to compare, respectively, community membership (that considers the different taxa) and structure (that considers the different taxa and their distribution within the community). Beta diversity was explored visually using principal coordinate analysis (PCoA).

4.2.8.2. Total and differential count of protozoa

After sampling, 10 mL of the rumen content mixture were inserted into flasks containing 20 mL of formaldehyde at 18.5%. Later, 1 mL of this moisture was stained for 4 hours with two drops of 2% of brilliant green. Following, 9 mL of glycerol 30% were added and homogenized. A counting chamber was filled with the diluted sample and coupled to the microscopy, and 100 optical fields were counted through the reticulum with the magnification of 100X..

Counting and identification of ciliate protozoa were performed by using a Neubauter Enhanced Bright-Line counting chamber (Hausser Scientific Partnership®, Horsham, PA, USA) by optical microscopy (Olympus CH-2®, Japan), according to Dehority (1993). Three genera of protozoa were distinguished: *Isotricha, Dasytricha,* and *Entodinium* as well the subfamily *Diplodiniinae*.

4.2.9. Energy partition

The gross energy intake (GEI) was calculated by multiplication of DMI (kg) and diet GE (Mcal/kg). The energy released as acetate, propionate, butyrate, and CH_4 (Mcal/ani.d) in the rumen was determined by multiplying the productions of these metabolites (g/kg.d) with their respective combustion heat (Mcal/g) and then multiplied by rumen solid mass (kg). The energy released in the rumen, when expressed in terms of percentage of GEI or digestive energy (DE), was obtained by dividing acetate, propionate, butyrate, and CH_4 release (Mcal/ani.d) by GEI (Mcal/ani.d) or DE (Mcal/ani.d) and then multiplying by 100.

Methane release in the cecum and colon (C&C) was considered as 5% of total CH_4 release. Enteric CH_4 in produced mainly in the rumen (95%) and, to a smaller extent (5%), in the low gut. The fermentation heat (FH) and microbial ATP were estimated from the ration among of SCFA produced according to Owens and Basalan (2016).

The energy release in the intestines (Mcal/ani.d) was calculated from GEI (Mcal/ani.d) subtracting the energy of SCFA and CH_4 from rumen (Mcal/ani.d) plus feces GE (Mcal/ani.d), CH_4 release in the cecum and colon (Mcal/ani.d), and FH following the equation:

$$ERI = GEI - (\mathcal{E}C_2 + \mathcal{E}C_3 + \mathcal{E}C_4 + feces'GE + C\&CCH_4 + FH + mATP)$$

Where:

ERI: energy release in the intestine (Mcal/ani.d);

GEI: gross energy intake (Mcal/ani.d);

EC_2: acetate energy (Mcal/ani.d);

EC_3: propionate energy (Mcal/ani.d);

EC_4: butyrate energy (Mcal/ani.d);

Feces GE: energy release in the feces (Mcal/ani.d);

C&C CH_4: CH_4 release in cecum and colon (Mcal/ani.d);

FH: fermentation heat;

mATP: microbial ATP.

The energy released in the intestine, expressed in terms of percentage of GE or DE, was obtained by dividing the energy release in the intestine (Mcal/ani.d) by GEI (Mcal/ani.d) or DE (Mcal/ani.d) and then, multiplying by 100.

The energy released in feces, expressed in terms of percentage of GEI, was obtained dividing feces' energy content (Mcal/ani.d) by GEI (Mcal/ani.d) and then multiplying by 100.

4.2.10. Statistical analysis

The data were analyzed using Statistical Analysis System (SAS 9.4, Institute Inc., 2013). Before the analysis, they were evaluated concerning the presence of discrepant information (outliers) and normality of residues by the Shapiro-Wilk test. When the normality premises were not met; then, the data were transformed. The DM intake, ruminal pH, and energy partition data were subjected to analysis of variance, which separated as causes of variation the effect of factors and their interactions, period effect, animal effect within squared, as well as squared effect. For the variables of production of CH_4, SCFA, N-NH_3 concentration, and total and differential count of ruminal protozoa, the factor repeated measures overtime was added to the model, referring to the different hours of sampling (0, 3, 6, 9, and 12), were analyzed using the mixed model procedure (Proc Mixed). The model included the effect of factors and their interactions as fixed factors and the animal within square and period effects as random factors. For the analyses, 15 different covariance structures were tested, and the one that best fit the statistical model was chosen based on the lowest value of the corrected Akaike information criterion (AICC) (WANG and GOONEWARDENE, 2004). The main factors were analyzed through analysis of variance using 0.05 as the significance level, whereas the interactions were decomposed using the LSD test using 0.05 as the significance level.

The microbiota data normality was assessed by visual inspection of the data and use of the Kolmogorov-Smirnov test. Data were log-transformed when indicated. Indices

of alpha diversity and relative abundance of the 17 most common phyla and 20 most common genera were analyzed using a repeated-measures 2-way ANOVA on log-transformed data, considering groups (control, essential oil, exogenous enzyme, monensin, and their combinations). When a significant effect was present, posthoc comparisons were performed among control and treatments, using the Fisher LSD test, without correcting for multiple comparisons. Unless mentioned otherwise, data are reported as the mean ± SD. A *P* value of .05 was used to assign significance. Beta diversity (community membership and structure) were compared using the Parsimony (*t-test*) and the analysis of molecular variance (AMOVA) tests.

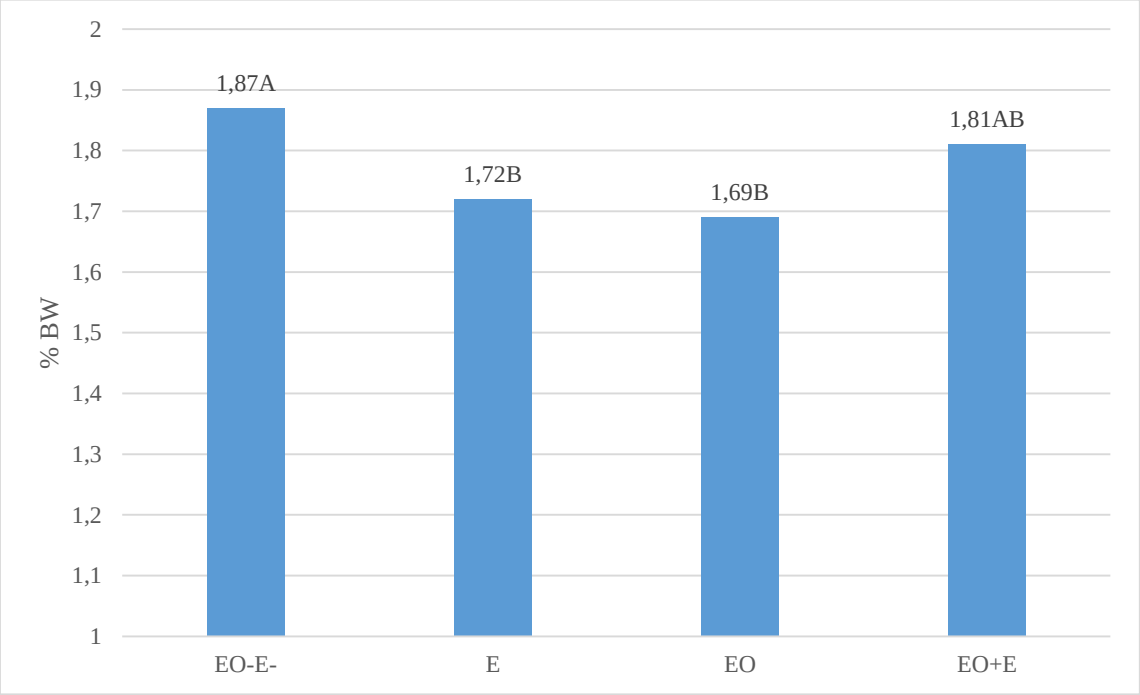
4.3. RESULTS

4.3.1. Intake and rumen pH

There was neither significant effect (P > 0.05) among the main factors on DM nor in water intake. However, when the factors EO and E were analyzed individually, without interaction, their presence in the diet decreased the DMI by 9.62% and 8.02%, respectively further, those factors increased the water consumption, as demonstrated in Table 12 and Figures 12 and 13.

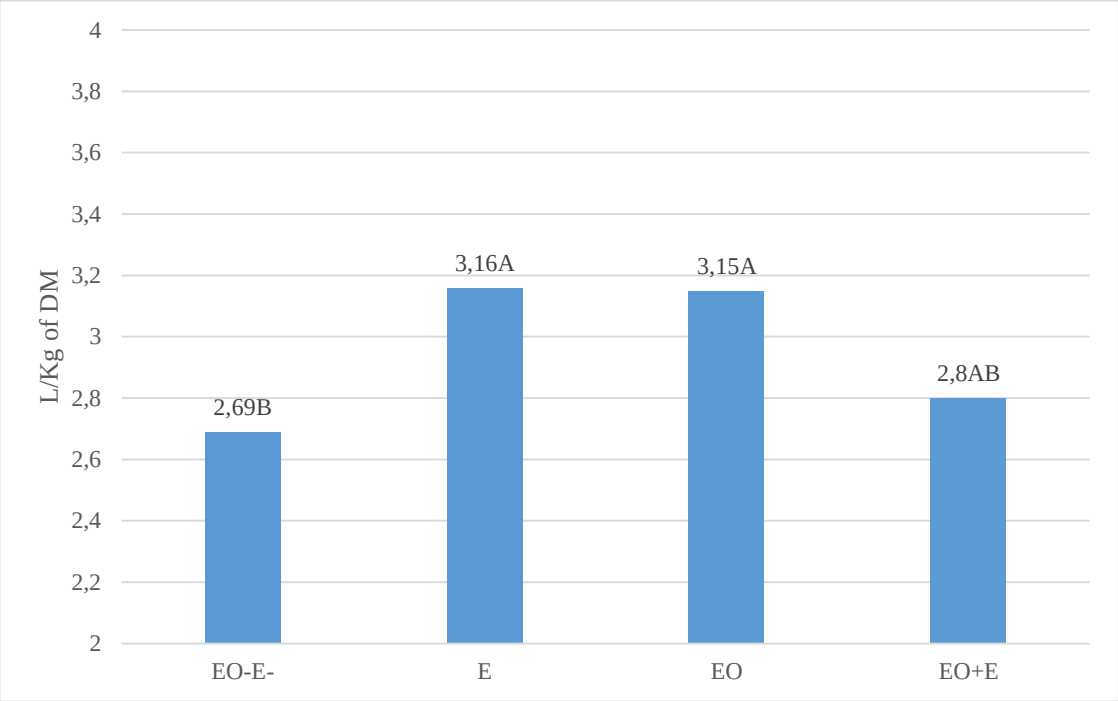
When analyzing the ruminal pH variables, there was no significant difference (P>0.05) for the main factors, as well as no interaction between the factors (Table 13).

Figure 12. Interaction between Essential oil and Enzymes on DMI (BW%)



EO-E-: Absence of enzyme and essential oil; E: enzyme presence, EO: essential oil presence; EO+E: enzyme and essential oil presence.

Figure 13. Interaction between Essential oil and Enzymes on water consumption (L per kg of DMI).



EO-E-: Absence of enzyme and essential oil; E: enzyme presence, EO: essential oil presence; EO+E: enzyme and essential oil presence.

Table 13. DMI, water consumption and rumen pH of Nellore cows fed with essential oil, enzyme blend, monensin and their associations.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
DM, day															
kg	9.91	9.47	9.65	9.73	9.57	9.81	9.66	0.28	0.09	NS	NS	0.04	NS	NS	NS
Water, day															
L/kg BW	2.93	2.98	2.92	2.98	3.37	2.54	2.96	0.188	NS	NS	NS	< 0.01	NS	NS	NS
Rumen pH, day															
Minimum	5.44	5.46	5.49	5.40	5.60	5.30	5.45	0.086	NS	NS	NS	NS	NS	NS	NS
Medium	6.09	6.20	6.21	6.08	6.29	6.00	6.17	0.057	NS	NS	NS	NS	NS	NS	NS
Maximum	6.83	6.79	6.84	6.78	6.87	6.74	6.81	0.051	NS	NS	NS	NS	NS	NS	NS
Time of pH, min/day															
< 5.8	250.6	218.1	194.4	274.4	149.4	319.4	234.4	54.4	NS	NS	NS	NS	NS	NS	NS
< 6.0	406.3	394.4	350.0	450.6	281.3	519.4	400.3	68.1	NS	NS	NS	NS	NS	NS	NS
< 6.2	764.1	668.1	609.4	822.8	517.5	914.7	682.9	77.9	NS	NS	NS	NS	NS	NS	NS
Area, h.(pH/day)															
< 5.8	1.71	1.06	1.33	1.43	0.65	2.12	1.38	0.510	NS	NS	NS	NS	NS	NS	NS
< 6.0	2.78	2.06	2.22	2.63	1.35	3.50	2.42	0.693	NS	NS	NS	NS	NS	NS	NS
< 6.2	4.52	3.81	3.78	4.55	2.64	5.69	4.16	0.901	NS	NS	NS	NS	NS	NS	NS

SEM: standard error of mean; EO: essential oil; E: exogenous enzymes blend; M: monensin; EO*E: interaction between essential oil and enzyme; EO*M: interaction between essential oil and monensin; E*M: interaction between enzyme and monensin; EO*E*M: interaction between essential oil, enzyme and monensin.

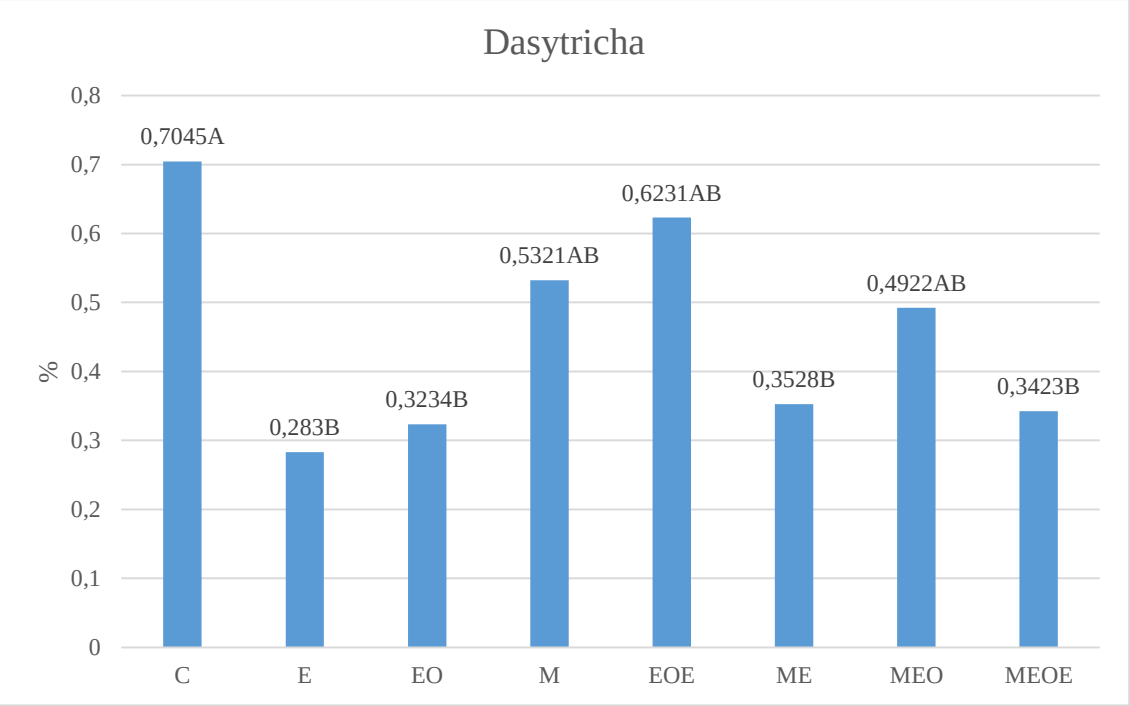
4.3.2. Rumen microbiota characterization

4.3.2.1. Total and differential count of protozoa

A triple interaction (P=0.06) was observed on *Dasytricha* population and population percentage, where animals fed with the pool of additives presented (decrease or increase) of this. However, that (increase or decrease) did not affect the protozoa total population (P>0.05). The results are presented on Table 14.

In addition, the isolated use of EO or E promoted a reduction of 54% and 59.8%, respectively, on *Dasytricha* population (Figure 14), when compared to the control group. Furthermore, the supplementation only with M did not change the protozoa population when compared to the control, as well as when associated with EO. However, the association between M and E, as well the triple association, caused a decrease on *Dasytricha* population of 49.9% and 51.4%, respectively.

Figure 14. Interaction between essential oil, enzyme blend and monensin on *Dasytricha* protozoa.



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.; M+OE: monensin and essential oil present; M+E+OE: monensin, enzyme and essential oil present.

Table 14: Total and differential count of ruminal protozoa from Nellore cows fed essential oils, a blend of exogenous enzymes, sodium monensin and their associations.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
Protozoa (x10^3/ml)															
Entodinium	1477.2	1432.5	1429.1	1480.5	1499.3	1410.3	1454.8	41.42	NS	NS	NS	NS	NS	NS	NS
Diplodiniinae	24.63	28.36	26.31	26.68	28.77	24.22	26.49	2.625	NS	NS	NS	NS	0.08	NS	NS
Isotricha	5.59	6.72	5.97	6.34	5.95	6.36	6.15	0.426	NS	NS	NS	0.05	NS	NS	NS
Dasytricha	5.73	5.79	6.3	5.22	5.86	5.65	5.77	0.353	NS	NS	NS	0.08	NS	NS	0.06
Total	1513.2	1473.5	1467.7	1518.9	1540.1	1446.6	1493.3	42.21	NS	NS	NS	NS	NS	NS	NS
Protozoa %															
Entodinium	97.5	97.1	97.3	97.3	97.1	97.5	97.3	0.181	NS	NS	NS	NS	NS	NS	NS
Diplodiniinae	1.59	1.93	1.71	1.81	1.94	1.58	1.76	0.159	NS	NS	NS	NS	NS	NS	NS
Isotricha	0.40	0.52	0.43	0.49	0.44	0.48	0.46	0.037	NS	NS	NS	NS	NS	NS	NS
Dasytricha	0.46	0.44	0.51	0.40	0.48	0.43	0.46	0.040	NS	NS	NS	0.03	NS	NS	0.04

SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin.

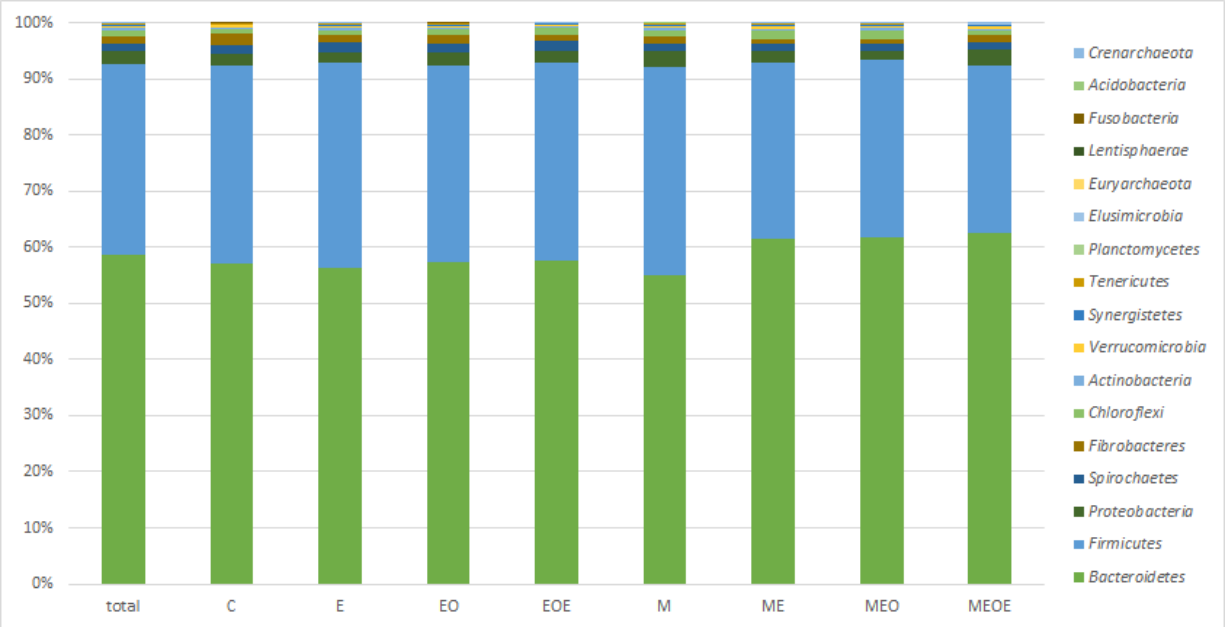
4.3.2.2. Bacterial Community characterization

A total of 1,518,100 sequences were obtained for bacteria, with an average of 47,441 (varying from 33,267 to 86,192) per sample and 287 OTUs were detected after quality filtering. For all amplicons, good's coverage of all samples was >0.992. The Alpha diversity richness (Chao1) of populations were similar among treatments (Table 15), but on the other hand, the isolated use of M decreased (P = 0.02) the diversity estimator (Shannon).

The principal coordinate analysis (PCoA) of overall diversity showed that the microbial membership in each of the factor and their interactions (all-against-all comparison) are not significantly different from each other (AMOVA.J_class, P =0.377).

Further analysis showed that 91.87% of all sequences allowed classification into 17 different phyla (Figure 15). Most of the sequences belonged to the phyla Bacteroidetes, Firmicutes and Proteobacteria.

Figure 15. Relative abundance of the phyla of ruminal bacterial communities observed in Nellore cows fed with essential oil, an enzyme blend, monensin and their associations



Source: own authorship

Table 15: Rumen alpha diversity of Nellore cows fed with essential oil, an enzyme blend, monensin and their associations.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
G.Coverage	0.991	0.991	0.991	0.991	0.991	0.991	0.991	0.0001	NS	NS	NS	NS	NS	NS	NS
Chao1	60.57	60.03	60.38	60.22	59.30	61.30	60.20	0.7127	NS	NS	NS	NS	NS	NS	NS
Shannon	2.597	2.549	2.587	2.559	2.611	2.535	2.576	0.0177	NS	NS	0.02	NS	NS	NS	NS

SEM: standard error of mean; G. Coverage: Good's Coverage; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin.

4.3.3. Products of rumen fermantation

The ruminal fermentation data obtained with the administration of the different additives are shown in tables 16 and 17. The sodium monensin factor had a significant effect ($P<0.05$) in the moments before (0 min) and after incubation (30 min) for acetic acid, butyric acid concentrations, and consequently for total SCFA concentration. However, the production of these variables did not change ($P>0.05$). There was no significant difference ($P>0.05$) for factors and their interactions when the variables related to propionic acid were analyzed, as well as for the acetic: propionic ratio.

An interaction between E and M ($P<0.05$) was observed for the production of butyric acid, indicating that the isolated use of these additives did not change the production of this acid; however, when they were associated, there was a reduction of 18% (Figure 16). The same interaction was observed for CH_4 concentrations before (0 min)(Figure 17) and after incubation (30 min).

There was no significant effect ($P>0.05$) for the variables that measure the production of CH_4, as well as the relative loss (REL). Concentrations at times before incubation (0 min), 30 min after incubation, and the difference between concentrations at times 0 and 30 min had an effect between different sampling times ($P<0.05$), regardless of the factors tested and their interactions for the vast majority of ruminal fermentation variables tested. An exception was observed for butyric acid production variables, total SCFA, and acetic: a propionic ratio that did not show a significant effect over time ($P>0.05$) regardless of the factors tested and their interactions.

Ruminal N-NH_3 concentrations (Table 17) at times before incubation (0 min), 30 min after incubation, and the balance between the concentration at times 0 and 30 min showed an effect between different sampling times ($P<0$ .05), regardless of the factors tested and their interactions. When the factors and their interactions were analyzed, a lower concentration of ruminal N-NH_3 was observed before incubation (0 min) ($P<0.05$) and a greater balance between the concentration at times 0 and 30 min ($P<0.05$) with the addition of sodium monensin, but there was no interaction between the factors ($P>0.05$).

Table 16. SCFA and CH_4 production as well REL of Nellore cows fed with essential oil, an enzyme blend, monensin and their interactions.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
Acetic acid															
0 min (mmol/L)	66.08	63.99	65.26	64.81	69.49	60.58	65.06	0.583	0.04	NS	<0.01	NS	NS	NS	NS
30 min (mmol/L)	71.45	69.05	70.59	69.90	74.85	65.65	70.29	0.643	NS	NS	<0.01	NS	NS	NS	NS
Difference (mmol/L)	5.19	4.64	4.97	4.86	5.04	4.78	4.92	0.173	NS	NS	NS	NS	NS	NS	NS
Production(mol/kg.d)	3.39	3.23	3.40	3.22	3.28	3.34	3.32	0.125	NS	NS	NS	NS	NS	NS	NS
Production(g/kg.d)	203.5	193.9	204.2	193.2	196.8	200.6	199.2	7.524	NS	NS	NS	NS	NS	NS	NS
Propionic acid															
0 min (mmol/L)	17.28	16.57	17.10	16.76	17.68	16.17	16.89	0.280	NS	NS	NS	NS	NS	NS	NS
30 min (mmol/L)	18.96	18.19	18.83	18.31	19.32	17.83	18.58	0.310	NS	NS	NS	NS	NS	NS	NS
Difference (mmol/L)	1.56	1.56	1.64	1.48	1.51	1.61	1.56	0.057	NS	NS	NS	NS	NS	NS	NS
Production(mol/kg.d)	1.02	1.03	1.06	0.98	0.95	1.09	1.02	0.036	NS	NS	NS	NS	NS	NS	NS
Production(g/kg.d)	75.54	76.24	78.56	73.23	70.92	80.86	76.06	2.698	NS	NS	NS	NS	NS	NS	NS
Butyric acid															
0 min (mmol/L)	14.07	13.64	13.93	13.78	15.23	12.48	13.86	0.230	NS	NS	<0.01	NS	NS	NS	NS
30 min (mmol/L)	15.89	15.54	15.84	15.59	17.27	14.15	15.73	0.274	NS	NS	<0.01	NS	NS	NS	NS
Difference (mmol/L)	1.77	1.73	1.80	1.69	1.89	1.60	1.75	0.061	NS	NS	NS	NS	NS	0.01	NS
Production(mol/kg.d)	1.19	1.21	1.25	1.14	1.25	1.14	1.20	0.048	NS	NS	NS	NS	NS	0.01	NS
Production(g/kg.d)	104.8	106.6	110.4	101.0	110.7	100.8	106.2	4.253	NS	NS	NS	NS	NS	0.01	NS
Total SCFA															
0 min (mmol/L)	97.44	94.23	96.35	95.31	102.4	89.2	95.89	0.961	NS	NS	<0.01	NS	NS	NS	NS
30 min (mmol/L)	106.3	102.8	105.3	103.7	111.4	97.61	104.6	1.070	NS	NS	<0.01	NS	NS	NS	NS
Difference (mmol/L)	8.58	8.07	8.58	8.07	8.61	8.04	8.34	0.272	NS	NS	NS	NS	NS	NS	NS
Production(mol/kg.d)	5.62	5.47	5.72	5.37	5.49	5.60	5.55	0.187	NS	NS	NS	NS	NS	NS	NS
Production(g/kg.d)	385.1	376.9	393.4	368.6	378.5	383.4	381.5	12.70	NS	NS	NS	NS	NS	NS	NS
Acetate:Propionic	3.46	3.23	3.33	3.36	3.53	3.16	3.32	0.078	NS	NS	NS	NS	NS	NS	NS
Methane															
0 min (mmol/L)	0.029	0.029	0.030	0.028	0.031	0.028	0.029	0.0005	NS	0.04	NS	NS	NS	<0.01	NS
30 min (mmol/L)	0.121	0.122	0.125	0.118	0.128	0.115	0.122	0.0022	NS	0.04	NS	NS	NS	0.04	NS

Difference (mmol/L)	0.091	0.092	0.094	0.089	0.096	0.087	0.092	0.0019	NS	NS	NS	NS	NS	NS	NS
Production(mol/kg.d)	2.19	2.25	2.26	2.18	2.32	2.12	2.22	0.0438	NS	NS	NS	NS	NS	NS	NS
Production(g/kg.d)	35.04	35.11	36.26	34.89	37.16	33.99	35.62	0.7009	NS	NS	NS	NS	NS	NS	NS
REL (%)	21.84	22.95	22.33	22.46	22.44	22.34	22.41	0.4420	NS	NS	NS	NS	NS	NS	NS

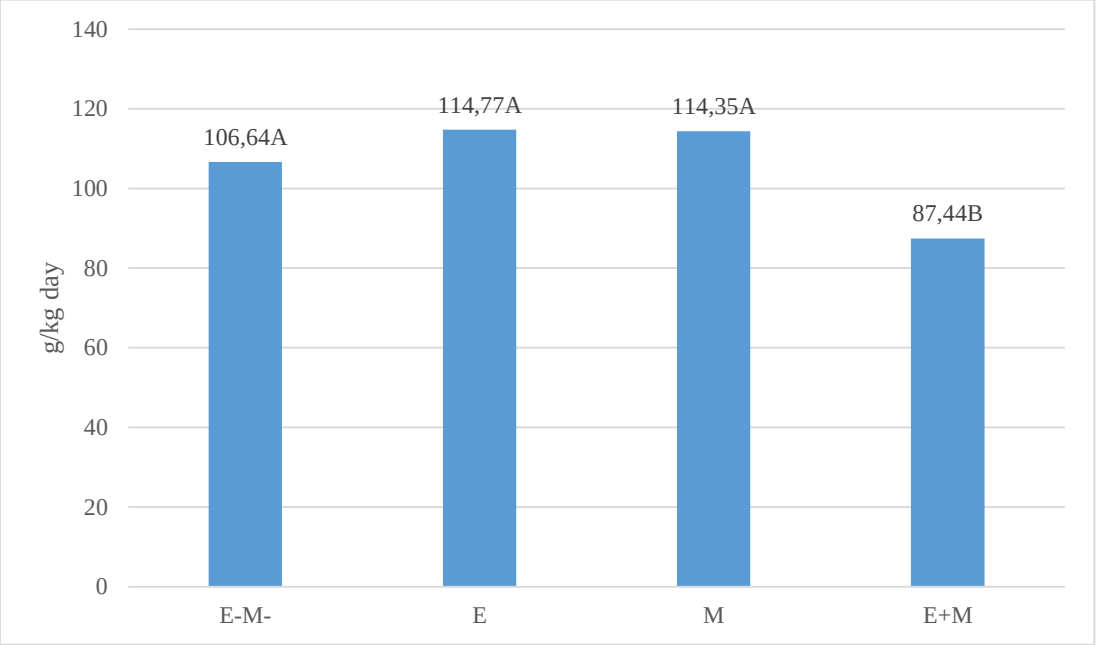
SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin. SCFA: short chain fat acids; REL: relative energy loss of methane in relation to the other rumen fermentation products.

Table 17. Concentration and balance of rumen N-NH_3 of Nellore cows fed with essential oil, an ezyme blend, monensin and their interactions.

Variables	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
Concentration															
0 min (mg/dL)	13.21	12.94	13.08	13.08	14.13	12.02	13.1	0.1685	NS	NS	0.03	NS	NS	NS	NS
30 min (mg/dL)	14.18	13.75	13.93	13.99	14.76	13.16	13.98	0.4128	NS	NS	NS	NS	NS	NS	NS
Balance (mg/dL.h)	1.956	1.429	1.708	1.677	1.078	2.307	1.683	0.2923	NS	NS	0.03	NS	NS	NS	NS

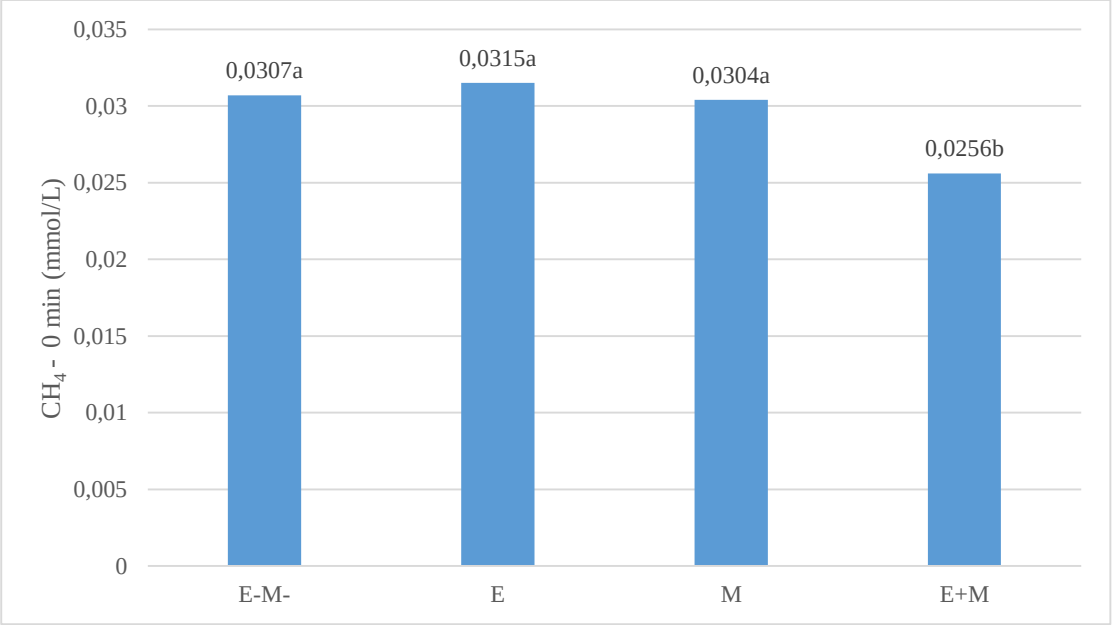
SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin.

Figure 16. Interaction between enzyme and monensin on butyric acid production.



E-M-: enzyme and monensin absence; E: enzyme presente; M: monensin present; E+M: enzyme and monensin present.

Figura 17. Interaction between enzyme and monensin on CH_4 concentration at 0 minutes.

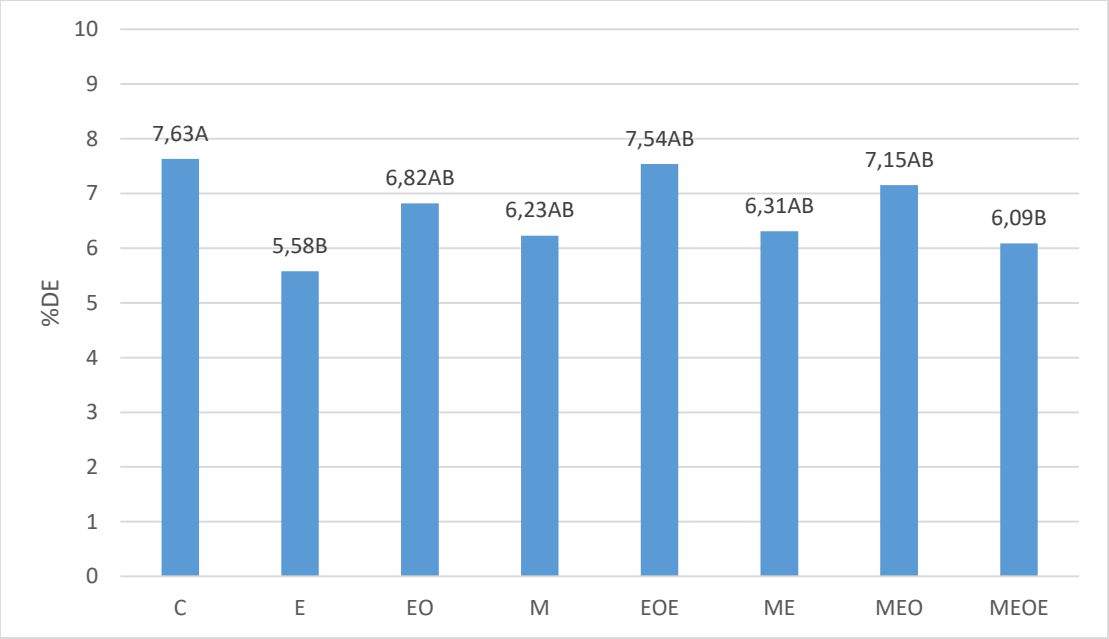


E-M-: enzyme and monensin absence; E: enzyme presente; M: monensin present; E+M: enzyme and monensin present.

4.3.4. Energy partition

As for the DMI (Figure 2), an interaction between EO and E was observed for the GEI (P<0.05) (Table 18). The energy released through SCFA did not change (P>0.05) with the use of tested additives and their associations. However, when analyzing the energy released in the form of CH_4 in the rumen, a triple interaction was observed (P<0 .05) among the tested additives. The use of E reduced by 26.86% the GE released in the form of CH_4 when compared to group C (Figure 18), while M and EO did not promote a significant reduction (P>0.05) when compared to the control group, a result similar to that observed when analyzing their respective associations with E. On the other hand, when analyzing the triple association, a reduction of EG released in the form of CH_4 in the rumen is observed in the order of 20.18% when compared to C.

Figure 18. Interaction between exogenous enzymes, essential oil and monensin on the release of digestible energy as methane in the rumen of Nellore cows.



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

Table 18: Estimation of energy released into the gastrointestinal tract of Nellore cows fed with essential oil, an enzyme blend, monensin and their associations.

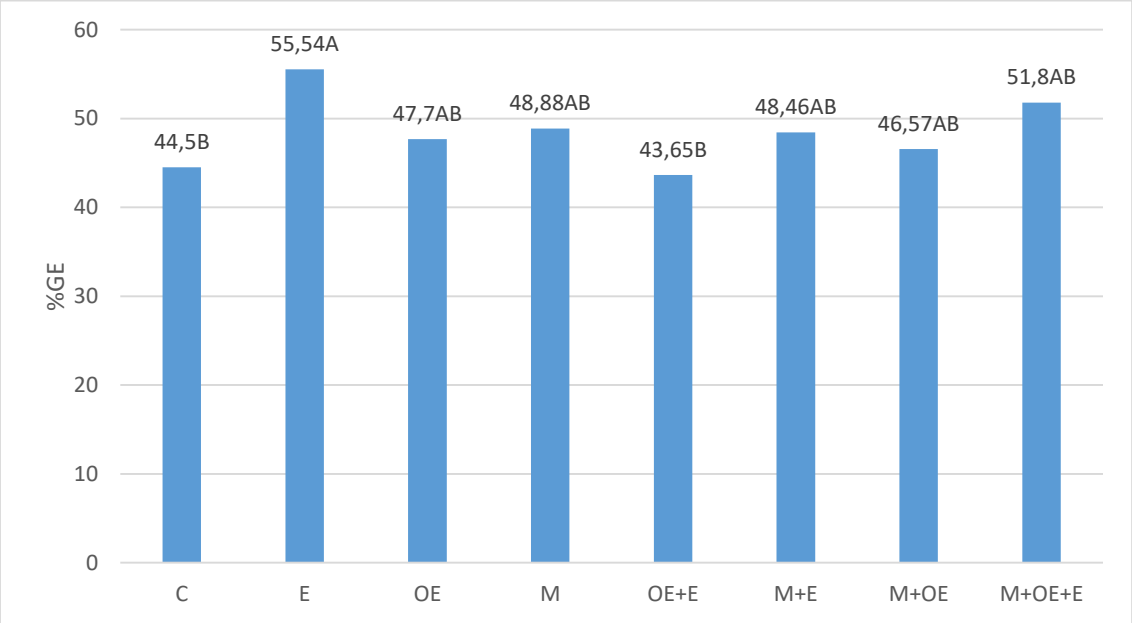
Variable	Factors						Mean	SEM	P value						
	Essential oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Absence	Present	Absence	Present	Absence	Present									
Ruminal mass, kg	4.51	4.36	4.52	4.35	4.33	4.54	4.43	0.161	NS	NS	NS	NS	NS	NS	NS
GEI, Mcal	43.68	41.71	42.5	42.89	42.16	43.23	42.57	1.252	0.09	NS	NS	0.04	NS	NS	NS
Energy released into the rumen															
Acetic acid															
Mcal/cow	3.21	2.89	3.15	2.95	2.88	3.22	3.06	0.184	NS	NS	NS	NS	NS	NS	NS
GE, %	7.41	6.81	7.34	6.87	6.82	7.40	7.11	0.350	NS	NS	NS	NS	NS	NS	NS
DE, %	10.16	9.81	10.29	9.68	9.80	10.17	9.97	0.526	NS	NS	NS	NS	NS	NS	NS
Propionic acid															
Mcal/cow	1.70	1.66	1.77	1.58	1.53	1.83	1.68	0.101	NS	NS	NS	NS	NS	NS	NS
GE, %	3.90	3.95	4.16	3.69	3.63	4.23	3.95	0.201	NS	NS	NS	NS	NS	NS	NS
DE, %	5.33	5.62	5.79	5.15	5.15	5.79	5.51	0.281	NS	NS	NS	NS	NS	NS	NS
Butyric acid															
Mcal/cow	2.74	2.71	2.86	2.58	2.73	2.72	2.72	0.154	NS	NS	NS	NS	NS	0.08	NS
GE, %	6.37	6.40	6.76	6.01	6.44	6.33	6.39	0.341	NS	NS	NS	NS	0.09	NS	NS
DE, %	8.73	9.23	9.50	8.45	9.26	8.69	8.97	0.511	NS	NS	NS	NS	NS	NS	NS
Total SCFA															
Mcal/cow	7.64	7.26	7.80	7.10	7.12	7.77	7.46	0.390	NS	NS	NS	NS	NS	NS	NS
GE, %	17.67	17.17	18.27	16.57	16.87	17.96	17.44	0.754	NS	NS	NS	NS	NS	NS	NS
DE, %	24.24	24.66	25.59	23.31	24.24	24.66	24.45	1.149	NS	NS	NS	NS	NS	NS	NS
Methane															
Mcal/cow	2.03	2.04	2.11	1.96	2.04	2.03	2.04	0.080	NS	NS	NS	NS	NS	NS	NS
GE, %	4.67	4.85	4.95	4.56	4.83	4.69	4.77	0.126	NS	NS	NS	NS	NS	NS	0.07
DE, %	6.44	6.90	6.96	6.38	6.89	6.45	6.69	0.214	NS	NS	NS	NS	NS	NS	0.01
Mcal/kg DM	20.56	21.36	21.81	20.11	21.27	20.66	21.04	0.005	NS	NS	NS	NS	NS	NS	0.07
Energy release in the intestine															
Mcal/cow	21.49	19.47	19.93	21.02	20.05	20.91	20.37	0.699	0.0480	NS	NS	NS	NS	NS	<0.01

GE, %	49.35	47.43	46.91	49.86	47.85	48.93	48.35	1.532	NS	NS	NS	NS	NS	NS	0.04
DE, %	67.23	66.31	65.24	68.3	66.75	66.79	66.76	1.413	NS	NS	NS	NS	NS	NS	NS
Energy release in feces															
Mcal/cow	11.84	12.32	11.98	12.18	12.3	11.86	12.06	0.734	NS		NS	NS	NS	NS	NS
GE, %	26.76	29.07	28.29	27.55	28.95	26.89	27.93	1.156	NS	NS	NS	NS	NS	NS	0.02

SEM: standard error of mean; EO: Essential oil; E: Enzymes; M: Monensin; EO*E: Interaction between essential oil and enzymes; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzymes and monensin; OE*E*M: Interaction between essential oil, enzymes and monensin ; GEI: gross energy intake; GE: gross energy; DE: digestible energy

When analyzing the energy release in the intestine, a triple interaction (P<0.05) was observed in the energy release both in Mcal and as a percentage of GE (Figure 19), demonstrating that the use of E provided an increase of 24.8% in the release of GE in the intestine when compared to group C. However, when associated with EO, the release of GE in the intestine returns to the level of group C.

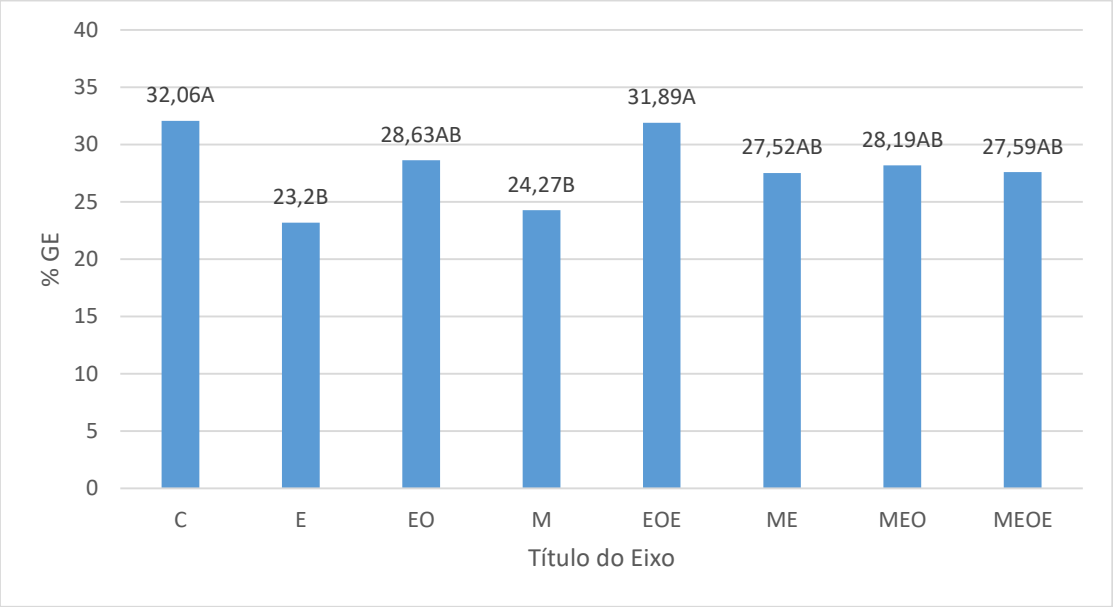
Figure 19. Interaction between exogenous enzymes, essential oil and monensin on the release of gross energy (GE) in the intestine of Nellore cows.



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

A triple interaction was also observed between the tested additives on the energy released in the feces (Figure 20), where the isolated use of E and M reduced 27.63% and 24.29%, respectively, the percentage of GE released in the feces of the animals. However, when they were associated, there was no significant difference (P>0.05) when compared to the control group and its isolated uses. When associating E and EO, there is an increase (P<0.05) in the energy released in the feces when compared to the isolated use of E.

Figure 20. Interaction between exogenous enzymes, essential oil and monensin on energy release in feces of Nellore cows.



C: Control; E: enzyme presence; EO: essential oil presence; M: monensin presence; ME: monensin and enzyme presence; MEO: monensin and essential oil presence; MEOE: monensin, enzyme and essential oil presence.

4.4. DISCUSSION

4.4.1. Rumen pH

The rumen environment is dependent on several factors to keep the fermentation continuously and animal health. One of those factors is the rumen pH and, according to Marino et al. (2009), the rumen pH should stay in a range between 5.5 and 7.2 to work properly. In the present study, the main factors (EO, E, and M), and their associations, presented no influence on rumen pH, where the medium pH stayed between 6.0 to 6.29, in agreement with Penner and Beuchemin (2010), who proposed a range of 6 to 6.2 to indicate health ruminal conditions. Furthermore, a healthy environment increases the rumen microbiome growth, like bacteria, protozoa, and fungi (BERCHIELLI; VAZ PIRES; OLIVEIRA, 2011).

The results observed in the current study were expected, once that the diet supplied had a high inclusion of forage (60%), keeping the pH stable through chemical and physical activities, fermentation, and rumination, respectively.

4.4.2. Rumen microbiota

The ruminal protozoa community represents 40 to 80% of total microbiota biomass (VEIRA, 1986) and constitutes about 31% of the microbial protein (PUNIA; LEIBHOLTS, 1984). The protozoa concentration is mostly influenced by the diet

(HUNGATE, 1966), and in the present study, it was observed an effect on *the Dasytricha* population and percentage on the rumen microbiome of cows fed with E and (And ou or) EO, where those additives decreased the population. A similar result was observed on the *Isotricha* population, where the use of E and EO, decreased this population. Those genera, Dasytricha and *Isotricha*, belong to the same subclass, the *Holotricha*, so we can infer that the association of E and EO directly affects this protozoa subclass.

Interestingly, when the M was in combination with E (ME) or EO (MEO), this effect was canceled, and the *Dasytricha* population was equal to the control group. However, the M loses power when the three additives are in association (MEOE), where the *Dasytricha* population decreases by 51.4%. A possible reason is that maybe the inclusion of E is stronger and overlaps the M effect, but remains unknown how this happens, once that each additive has a different mechanism of action on rumen fermentation. Therefore, we strongly encouraged to carry out specific experiments seeking to confirm and elucidate the mechanism of action of the exogenous enzyme tested here on the population of *Dasytricha*.

Neither effect was observed on alpha and beta diversity on the bacteria community (P>0.05). The PCoA analysis demonstrated that the communities are heterogenous (AMOVA.J_class, P =0.377) and did not change under the influence of the additives. However, when the M was used isolated the diversity estimator, Shanon, decreased, and indicating a diversity reduction on the rumen microbiome. A possible reason for this effect is the direct action of M on Gram+ bacteria, once that M inhibits bacteria growth by primarily increasing the permeability of their cell membrane, and further acting as a Na+/H+ antiporter upon inserting itself into the cell membrane and causing the bacteria death (BERGEN; BATES, 1984). The result found in this study agrees with Shen et al. (2017), who also observed a decrease in diversity populations when cows were fed with M.

4.4.3. Rumen fermentation

Ruminal microorganisms use peptides and amino acids, resulting from the hydrolysis of ingested protein, or ammonia for microbial protein synthesis. When the rate of ammonia synthesis exceeds its use by ruminal microorganisms, there is an increase in the ammonia concentration in the rumen (RUSSEL et al., 1992). In this sense, *in-vitro*,

and *in-vivo* studies indicated that monensin could decrease amino acid deamination and ammonia accumulation in the rumen (DINIUS et al., 1976, VAN NEVEL and DEMEYER, 1977) since it can inhibit rumen bacteria that present a high rate of ammonia production (CHEN and RUSSEL, 1989; RUSSEL et al., 1988).

Ruiz et al. (2001), analyzing dairy cows consuming a diet composed of 25% concentrate and 75% pasture, observed a reduction of 14.28% in the ruminal ammonia concentration with the use of monensin. This reduction was similar to that observed in the present study, where monensin reduced the concentration of $N\text{-}NH_3$ before incubation by 14.93% (Table 5). After the incubation period, there was a balance of $N\text{-}NH_3$ twice as high for animals that received this additive. It indicated that although monensin reduces ruminal deamination, it also reduces its use by ruminal microorganisms resulting in the accumulation of ammonia in the rumen, and consequently lower microbial synthesis, as presented in the previous chapter.

During the process of ruminal fermentation, the nutrients in the diet, mainly energy and protein sources, are transformed into SCFA, microbial mass, and gases such as CH_4, CO_2 and H_2 (BAKER, 1999). SCFA is the main energy sources for ruminants, representing 75-80% of the energy originally present in fermented carbohydrates, contributing 50-70% of the digestible energy in the food (KOZLOSKI, 2002).

Additives such as monensin and essential oils are known for their ability to change the molar concentration of SCFA, increasing the concentration of propionic acid, and decreasing the concentrations of acetic and butyric acids (ELLIS et al., 2012 MATEOS et al., 2013), thus reducing the loss of dietary energy in the form of methane (MACHEBOEUF et al., 2008; APPUHAMY et al., 2013). Although the isolated use of EO and M reduced the acetic acid concentration at time 0 by 3.16% and 12.82% respectively, and the use of M reduced the concentrations of butyric acid and total SCFA by 18.05% at time 0 and 12.89%, respectively (Table 4). However, such additives were not able to promote changes in SCFA and CH_4 production, as demonstrated by Carvalho (2018) evaluating the use of different EO in the diet of cows fed a diet composed of 70% corn silage, as well as Tseu (2019) evaluating the use of M and condensed tannins in the diet of Nellore cows fed 50% corn silage. In addition, the lack of interaction between EO and M both on the production and on the concentration of SCFA and CH_4 may indicate the independence of these additives on these parameters.

Although the use of M and E alone does not affect the production of butyric acid, when they are combined, there is a reduction of 18% in the production of this SCFA in grams/kg.d (Figure 6) when compared to the absence of these additives, also showing a reduction when compared to its isolated uses. As previously mentioned and observed in this study, the ability of M to reduce the concentration of butyric acid in the rumen is known; however, few studies have been able to measure its effects on SCFA production. The use of E in the diet, on the other hand, has among its objectives the promotion of greater digestibility of the diet and potentiation of the action of endogenous enzymes (GUENTER, 2002), with no expected reduction in SCFA production. Collazoz Paucar (2017), evaluating the addition of 30 grams/animal.day of an association of E (amylase, protease, xylanase, and cellulase) on the ruminal fermentation of Holstein cows, observed an increase in the production of acetic and propionic acid and consequently in the production of Total SCFA, indicating the ability of the association of E to increase SCFA production. However, this effect was not observed in this study. A determining factor for ruminal fermentation, the composition of the diet can be considered as influencing the effect of the enzymatic blend on the production of SCFA since in the present study, the diet was composed of 60% corn silage, while in the Collazos Paucar's experiment (2017) used a diet with the highest amount of non-structural carbohydrates, where corn silage represented 30% of the total diet.

References to the association between M and E on the production of SCFA were not found in the literature and, based on the data collected. It is not possible to define which mechanism is present in the association between such additives capable of reducing the production of butyric acid. However, a similar behavior was observed when analyzing the CH_4 concentration before incubation, where the association between such additives reduced the CH_4 concentration by 16.61% when compared to the absence of these additives, with the isolated use not being able to reduce this variable. Thus, further studies are encouraged to confirm and elucidate the possible associative effect between the additives mentioned before on the products of rumen fermentation.

4.4.4. Energy partition

The application of the *ex-situ* technique makes it possible to calculate the daily production of the products of ruminal fermentation (SCFA and CH_4), allowing to estimate the energy released in the digestive tract, a subject poorly discussed by researchers. Therefore, little data is available in the literature. In the present study, a triple interaction

was observed between the additives in energy released in the form of methane, the energy released in the intestine, and the feces (Table 6).

The use of E showed to be able to reduce, when compared to C, by 26.86% the %DE released in the form of CH_4, promoting a 24.8% increase in the %GE released in the intestine and a reduction of 27.63 % in %GE released in feces, but not changing the %DE released by SCFA. This occurrence shows the ability of the enzyme blend to increase the digestibility of the diet without promoting significant changes in rumen fermentation since SCFA production was not altered (Table 4), nor the %DE released through SCFA (Table 6) and showing an increase in energy efficiency. This finding is of great interest to the academic community once it indicates that DE, which would be lost as CH4 during ruminal fermentation, can be absorbed through the intestinal tract of Nellore cows through this enzyme blend.

However, the association between the tested additives proved to be antagonistic, with a total reduction in the effect of E on the %DE released in the form of CH4 associated with both M and EO. When associating the three additives, a reduction of 6 percentage points is observed in the isolated effect of E. As for the %GE released in the intestine, it was observed that when performing an association between E and EO, % GE released was reduced by 21.4% to when E was used alone. Such an antagonistic effect between these additives was also observed on the % of GE released in feces, where the EOE association increased this variable by 37.45% when compared to the use of E alone.

Despite the marked effects and the clear antagonism shown between mainly EO and E regarding the energy partition variables, the data collected in this study do not allow for inferences about possible mechanisms involved, being of great value specific research seeking to elucidate and confirm such occurrences.

4.5. CONCLUSION

The association between the tested additives did not prove to be a good nutritional strategy for mitigating ruminal CH_4, as it did not influence the production of total SCFA or CH_4.

Additionally, an antagonistic effect was observed when E was associated with both M and EO, increasing the DE lost in the form of CH_4 and reducing the GE released in the intestine when compared to the use of E alone.

However, further studies are recommended in order to validate the results obtained, especially in different diet profiles.

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5. GREENHOUSE GASES PRODUCTION POTENTIAL DURING ANAEROBIC BIODIGESTION OF CATTLE FED WITH DIFFERENT FOOD ADDITIVES AND THEIR COMBINATIONS

Abstract

Manure management, in particular, anaerobic digestion is an alternative to reduce the environmental impacts of cattle raising and energy generation. The aim of this study is the production of biogas and biofertilizer from waste from Nellore cows fed with sodium monensin, essential oils, exogenous enzymes, and their combinations. Experimental batch-type biodigesters were used, placed inside a climatic chamber (30 to 35ºC). They were organized in a completely randomized design in a 2 x 2 x 2 factorial arrangement, being tested waste from Nellore cows fed with the presence or absence of factors essential oil, exogenous enzyme, and monensin, with 4 repetitions totaling 32 experimental units (represented by the manure of the animals that received the different additives and their associations). The use of monensin reduced the amount of N inserted in the biodigesters, reduced the nutrient removal efficiency, and reduced the biogas production by 39.26%, in addition to altering the composition of the biofertilizer produced. Other tested additives did not influence the biodigestion process together with the combination of additives. Therefore, the addition of M reduces the nutrient removal efficiency, compromising biogas production, and reducing the concentration of nutrients in the biofertilizer, while EO and E do not affect the biodigestion process. In addition, no associative effect was observed between the tested additives.

Keywords: Greenhouse gases; Biodigesters; Biogas; Additive association, Monensin

5.1. INTRODUCTION

Agriculture represents a significant source of greenhouse gas (GHG) emissions worldwide, accounting for 10 to 12% of global anthropogenic GHG emissions. When analyzing non-carbon dioxide (CO_2) emissions, enteric fermentation of ruminants is the main responsible for about 30 to 40% of agricultural emissions, followed by the emission of waste deposited in pastures, responsible for about 15% of agricultural emissions (IPCC, 2014), which can represent up to 27% of the total emissions of methane gas (CH_4) by ruminants (KULLING et al., 2002; HINDRICHSEN et al., 2006), in intensive production systems, where these wastes end up accumulating in small areas.

Due to this, the correct management of animal waste becomes an important tool to reduce environmental impacts, with anaerobic biodigestion being a promising alternative, as in addition to reducing GHG emissions, it is capable of recycling waste in the form of biofertilizers, in addition to producing biogas, which is considered an alternative source of energy.

According to Côte et al. (2006), anaerobic biodigestion can be defined as a complex interaction of microorganisms that degrade organic components present in the waste, mainly to the form CH_4 and CO_2. In this interaction, the nutrients contained in the waste ensure the survival and reproduction of microorganisms, allowing the degradation of the organic fraction into the form of biofertilizers, as well as the production of biogas (Alvarez & Lidén, 2008).

Bovine waste is characterized as a good substrate for anaerobic biodigestion (AHRING et al., 2001). However, several factors can change the characteristics of the waste, and influence its potential for biogas production, including animal feed (AMON et al., 2007; JARRET et al., 2011). Among all the factors, the composition of the material directly influences its potential for degradation. Therefore, the extent of biogas production is dependent on the feed of the animals.

To increase the feed efficiency of ruminants and reduce the CH_4 emissions, several studies have shown a wide variety of nutritional techniques aimed at manipulating the ruminal environment, highlighting the use of food additives. In addition to acting in the manipulation of the ruminal environment, it can affect the use of nutrients and consequently the characteristics of waste excreted by animals.

According to Spears (1990), the ionophores can increase the apparent energy digestibility by 2%, and the apparent nitrogen digestibility by 3.5%. Some works also

demonstrate the action of essential oils making the digestive process of food more efficient, increasing the digestibility of dry matter (DM) and organic matter (OM) (YANG et al., 2007) and neutral detergent fiber (NDF) (BOYD et al., 2012). Enzyme supplementation, according to Guenter (2002), has as main goal to remove or destroy antinutritional factors and increase the total digestibility of the diet. Furthermore, improvement in nutrient digestibility with the addition of enzyme preparations in ruminant feed has been demonstrated by several studies (JUDKINS; STOBART, 1988; BEAUCHEMIN et al., 2000; CRUYWAGEN; GOOSEN, 2004).

Therefore, it is expected that the increase in the digestibility of nutrients in the diet will result in changes in the composition of waste excreted by the animals and, therefore, influence the anaerobic digestion and, consequently, the production of biogas. Therefore, this study aimed to evaluate the potential for biogas production and the characteristics of the biofertilizer from waste from Nellore cows fed with sodium monensin, essential oils, exogenous enzymes, and their combinations, as an alternative for the management of waste from cattle.

5.2. MATERIAL AND METHODS

5.2.1. Study location and ethical issues

This research project was executed at the College of Veterinary Medicine and Animal Science of the University of Sao Paulo (USP), Campus Fernando Costa – Pirassununga/SP.

The project was approved and authorized in accordance to the norms of the National Council for the Control of Animal Experimentation (CONCEA) by the Ethics Committee in the Use of Animals, Faculty of Animal Science and Food Engineering, University of São Paulo – FZEA/USP (CEUA/ FZEA), filed under CEUA No. 4788111017.

5.2.2. Treatments and experimental design

The experiment was carried out in two phases, the feeding phase, and the anaerobic digestion phase, as follows:

5.2.2.1. Feeding phase

Eight non-pregnant and non-lactating Nellore bovine females, with an average live weight of 480 ± 55 kg, were housed in individual, covered pens with sand beds, cement troughs and automatic drinking fountains.

The animals were divided into two contemporary 4 x 4 Latin squares in a 2 x 2 x 2 factorial arrangement, fed daily (8:00 and 16:00 h) using isoenergetic and isoprotein diets, which differed according to presence or absence of the tested additives. Being: diet without the addition of essential oil (OE-A); diet added with 31.7 mg/kg DM of an essential oil blend composed of 43% cinnamaldehyde and 7% garlic oil (OE-P); diet without added enzyme (EN-A); diet with the addition of 1027 mg/kg DM product containing an "enzyme blend" composed of cellulase, xylanase, amylase, protease, phytase, beta-glucanase and pectinase (EN-P); diet without added monensin (MA); diet with the addition of 30.6 mg of sodium monensin/ kg DM (MP). This phase was divided into 4 periods with 22 days, with 16 days for adaptation to experimental diets, 5 days for feces collection, with urine collections carried out on the 22nd of each period. Feces were collected manually through the rectum at 8:00 am and 4:00 pm, then frozen at -20°C and pooled to form a single composite sample for each animal in each period. Urine samples were obtained every 6 hours during stimulation by vulvar massage and then stored at -20ºC in a single vial, which formed a single composite sample within 24 hours.

Next Enhance (43% cinnamaldehyde + 7% garlic oil) (Novus International Inc., Indaiatuba, Brazil) was used for source of essential oil, Allzyme® SSF (Alltech Inc., Nicholasville, USA) was used for source of enzymatic blend and Rumenpac® (Grupo MCassab, São Paulo, Brazil) was used for source of Monensin Sodium.

5.2.2.2. Anaerobic digestion phase

Substrate preparation, experimental design, and treatments

Fecal and urine samples, collected and frozen in the feeding phase, were thawed and diluted in water. A mixture of feces and urine (waste) was prepared using a theoretical ratio of 83%:17%, respectively. Then, this mixture was diluted with water and, finally, the inoculum was added.

Batch-type benchtop biodigesters were used (Figure 1), and 3 kg of substrate were prepared, of which 2 kg were used to fill the biodigesters and 1 kg to carry out the substrate characterization analyses (Table 19).

Table19: Composition of substrates from anaerobic digesters supplied with waste from Nellore cows fed with essential oils, a blend of exogenous enzymes, sodium monensin and their associations.

Variable	Factor						Average	SEM	P-value						
	Óleo essencial		Enzyme		Monensin				OE	E	M	OE*E	OE*M	E*M	OE*E*M
	Negative	Positive	Negative	Positive	Negative	Positive									
Nutrientes adicionados															
TS, g	117.3	117.1	117.3	117.2	117.3	117.2	117.2	0.084	NS	NS	NS	NS	NS	NS	NS
SV, g	101.1	101.6	101.4	101.4	102	101.8	101.4	0.448	NS	NS	NS	NS	NS	NS	NS
NDF, g	50.8	52.1	50.5	52.5	51.2	51.8	51.5	0.913	NS	NS	NS	NS	NS	NS	NS
N, g	5.72	5.7	5.77	5.65	6.34	5.07	5.74	0.15	NS	NS	<0.001	NS	NS	NS	0.08
pH	6.33	6.32	6.32	6.34	6.32	6.33	6.33	0.061	NS	NS	NS	NS	NS	NS	NS

SEM: standard error of mean; EO: Essential Oil; E: Enzyme Blend; M: Monensin; EO*E: Interaction between essential oil and enzyme; EO*M: Interaction between essential oil and monensin; E*M; Interaction between enzyme and monensin; EO*E*M: Interaction between essential oil, enzyme and monensin; TS: total solids; VS: volatile solids; N: nitrogen; NDF: neutral detergent fiber.

The substrate composition was done with the following proportions: 40% manure, 3.3% inoculum and 56.7% water. The sludge from the bovine manure treatment pond was used as an inoculum, which presented 0.164% of total solids (TS). Thus, the substrates were prepared in order to guarantee an estimate of 6% of TS.

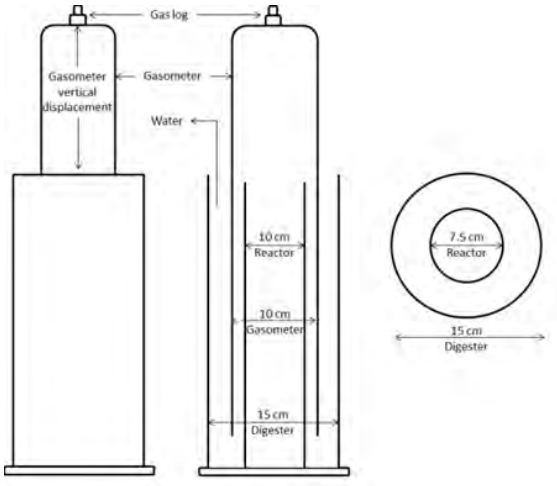
The biodigesters were arranged in a completely randomized design in a 2 x 2 x 2 factorial scheme with 4 replications, totaling 32 experimental units (represented by the manure of the animals that received the different additives and their associations).

Biogas Production

Anaerobic biodigestion was performed under mesophilic conditions (30 to 35ºC), ideal for digestion kinetics (METCALF; EDDY, 2014). The biodigesters were placed inside a climatic chamber with an electrical resistance heating system and a digital temperature controller.

The batch-type biodigesters consisted of three straight cylinders with diameters of 15, 10, and 7.5 cm, with an average capacity to ferment 2 liters of substrate each (Figure 1). The 15 and 7.5 cm cylinders were inserted, one inside the other, so the space between the outer wall of the inner cylinder and the inner wall of the outer cylinder contained a volume of water (water seal), reaching the depth of 60 cm. The intermediate diameter cylinder (gasometer) had one end sealed with a register for biogas discharge, while the other end was in contact with the water seal to provide anaerobic conditions and store the gas produced.

Figure 21. Anaerobic batch-type biodigester shown in front, side, and top views.



Source: Nogueira (2017).

The reading of biogas production was performed according to the accumulation in the gasometer. It consisted of measuring the height with a ruler fixed to the gasometer, according to its vertical displacement. The reading value was multiplied by the internal cross-sectional area of the gasometer. After each reading, the gasometers were emptied using the biogas discharge log. The correction of the biogas volume for conditions of 1 atm at 20ºC was performed according to the methodology described by Lucas Junior (1994). To correct the volume of biogas, the expression resulting from the combination of the Boyle and Gay-Lussac laws was used:

$$(V0P0) /T0 = (V1P1) /T1$$

Where:

V0 = corrected biogas volume, m^3 ou L;

P0 = corrected biogas pressure, 10322.27 mm H_2O;

T0 = corrected biogas temperature, 293.15 K;

V1 = gas volume in the gas meter;

P1 = biogás pressure at the time of reading, 10344.11 mm H_2O;

T1 = biogas temperature, in K, at the time of reading.

Considering the mean atmospheric pressure of Pirassununga equal to 10273.11 mm H_2O and the pressure given by the gasometers of 71 mm H_2O, the following expression was obtained to correct the volume of biogas:

$$V0 = (V1/T1) \times 293.7703$$

Biogas samples were taken 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 sampling itself, the biogas was collected and used to flush the bottle (twice), after which 50 mL of biogas were injected to analyze its composition. After collecting the biogas, the gasometers were emptied; this allowed a new accumulation of gases. The test was terminated when biogas production ceased, which occurred 164 days after filling the biodigesters.

The concentration of CH_4, CO_2, and N_2O was determined by gas chromatography (Trace 1300, Thermo Fisher Scientific®, Rodano, Milan, Italy) in a temperature-controlled environment (25°C), according to Kaminski et al. (2003). The biogas samples

were diluted in glass flasks, with known volume, 16.78 times in atmospheric air. Then, 6 mL was injected into the chromatograph injector (split/splitless), of which 4 mL was used for flushing the injection system, and 2 mL was used for analysis. The system with flame ionization detector (FID) is responsible for measuring CO_2 and CH_4 and the system with electron capture detector (ECD) is responsible for the quantification of N_2O.

The chromatograph was calibrated with 3.1% CH_4, 3.1% CO_2 and 0.49% N_2O diluted in atmospheric air. Two gas mixtures were used as reference, one with 50% CH_4 and 50% CO_2 and the other with 10% N_2O in equilibrium with He (mol/mol).

The volumes of CH_4, CO_2 and N_2O produced (m3or L) were calculated using the biogas production and composition data from each digester according to the equation:

$$Vol = (VolBIOGAS \times \%Gas)/100$$

Where:

Vol = volume (m^3 ou L);

VolBIOGAS = volume of biogas produced (m^3 ou L);

% Gas = contente gas of interest in biogas (%)

The production of CH_4, CO_2 or N_2O was calculated by dividing the total production of each gas by the amount of VS added or removed (difference between SV added in the filling time of the biodigesters and VS eliminated during fermentation).

The Gompertz model was used to study the kinetics of biogas and its components production. The model assumes that the gas production rate is proportional to the microbial activity, but the proportionality decreases with the incubation time, which can be interpreted as a loss of efficiency in the fermentation rate (LAVRENCIC et al., 1997). The mathematical description of the gas production curves allowed data analysis, substrate comparison, and fermentation performance. 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: model asymptote, indicates the production stabilization value (L/g VS added) in relation to time t;

B: integration constant, no biological meaning.

kt: maximum growth rate, logarithmic function of production growth (L/ gVS added) per unit of t.

The time (t) at the inflection point was determined as follows:

t1= ln B/k

Where:

t1: time (days) at the inflection point; ln: natural logarithm; k: production constant.

Gas production at the inflection point was determined as:

y1 = A/exp

Where:

y1: gas production at the inflection point; exp: base of the natural logarithm (2.7183)

Removal of nutrients

The substrates added and recovered in each biodigester were weighed and multiplied by the DM percentage to calculate the DM content in grams. The added and recovered nutrients, expressed in grams, were calculated by multiplying those added or recovered and expressed in DM grams, which were expressed as a percentage and divided by 100 according to the following equation:

Nutrient (g) = Nutrient added or recovered (%) x DM (g)/100

Nutrient removal, in percentage, was calculated from the content of added and recovered nutrients and expressed in g/kg of DM according to the following equation:

Nutrient removed (%) = [Nutrient added (g) – Nutrient removed (g)] x 100 /Added nutrient (g)

5.2.3. Laboratory Analysis

The substrate samples before and after anaerobic digestion were collected, dried in an oven with ventilation and constant air renewal at 65ºC for 72 hours, according to AOAC (1995). Then, they were ground (1 mm) and stored in properly sealed bottles. DM was determined at 105ºC for 4 hours in an oven (method 930.15; AOAC, 1995). Mineral matter (MM) was obtained by calcination in a muffle at 550ºC for 5 hours (AOAC, 1990).

The contents of ST (ST = 100 - moisture) and SV (SV = ST - MM) of the substrates were determined with adaptations to the methodology described in APHA (2005). The total N content was determined by the micro-Kjeldahl technique (method 920.87; AOAC, 1990). Neutral detergent fiber (NDF) was determined by the method described by Van Soest et al. (1991). The hydrogen ion potential (pH) was measured by a portable pH meter (Hanna Instruments®, HI 8424, Italy).

5.2.4. Statistical Analysis

Data were analyzed using the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). Before data analysis, they were evaluated for the presence of discrepant information (outliers) and normality of the residuals using the Shapiro-Wilk test. When the normality premise was not met, the data were transformed. Data were subjected to analysis of variance, which separated as causes of variation the effect of factors and their interactions, period effect, animal effect inside squared, along with squared effect. The effect of factors was analyzed using analysis of variance using 0.05 significance.

5.3. RESULTS

5.3.1. Biodigestion and nutrients removal

The waste added to the biodigesters from animals fed with sodium monensin presented a 20.03% lower amount of N ($P<0.05$) than the waste from animals that did not receive such additive, with no significant difference being observed ($P> 0.05$) for other added nutrients.

The amounts of TS and NDF remaining after the biodigestion process were 25.02% and 66.24% higher for manure from animals that received sodium monensin ($P<0.05$), when compared to manure from animals that did not receive sodium monensin.

The removal efficiency of TS, NDF and N were lower ($P<0.05$) for biodigesters supplied with animal manure fed with sodium monensin, when compared to those supplied with animal manure that did not consume such additive (Table 20).

Table 20. Biodigestion and removal efficiency of nutrients from anaerobic batch type biodigesters supplied with waste of Nellore cows fed with essential oil, an enzyme blend, monensin and their interactions.

Variable	Factors						Average	SEM	P value						
	Essential Oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Negative	Positive	Negative	Positive	Negative	Positive									
Added nutrients															
TS, g	117.3	117.1	117.3	117.2	117.3	117.2	117.2	0.084	NS	NS	NS	NS	NS	NS	NS
VS, g	101.1	101.6	101.4	101.4	102.0	101.8	101.4	0.448	NS	NS	NS	NS	NS	NS	NS
FDN, g	50.82	52.14	50.47	52.48	51.16	51.79	51.53	0.913	NS	NS	NS	NS	NS	NS	NS
N, g	5.72	5.70	5.77	5.65	6.34	5.07	5.74	0.150	NS	NS	<0.001	NS	NS	NS	0.08
Recovered nutrients															
TS, g	68.68	72.86	67.81	73.73	62.9	78.64	70.38	2.158	NS	0.08	<0.001	NS	NS	NS	NS
VS, g	55.41	61.47	55.37	61.51	55.37	61.51	58.41	2.312	NS	NS	NS	NS	NS	NS	NS
NDF, g	30.15	33.43	30.27	33.32	23.88	39.7	31.49	1.747	NS	NS	<0.001	NS	NS	NS	NS
N, g	1.92	1.99	1.87	2.04	1.99	1.92	1.95	0.055	NS	NS	NS	NS	NS	NS	NS
Removal efficiency															
TS, %	41.80	38.25	42.53	37.51	46.69	33.63	40.36	1.83	NS	0.08	<0.001	NS	NS	NS	NS
VS, %	45.11	39.48	45.37	39.21	45.72	38.87	42.36	2.293	NS	NS	NS	NS	NS	NS	NS
NDF, %	40.19	35	39.39	35.8	53.01	22.18	38.24	3.655	NS	NS	<0.001	NS	NS	NS	NS
N, %	65.50	54.09	67.01	62.58	68.3	61.3	65.12	1.545	NS	NS	0.02	NS	NS	NS	0.08

SEM: standard error of mean; EO: Essential Oil; E: Enzyme Blend; M: Monensin; EO*E: Interaction between essential oil and enzyme; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzyme and monensin; EO*E*M: Interaction between essential oil, enzyme and monensin; TS: total solids; VS: volatile solids; N: nitrogen; NDF: neutral detergent fiber.

5.3.2. Biogas Production

Biodigesters supplied with animal waste fed with sodium monensin showed a reduction of 39.26% in biogas production (L). Consequently, lower production of CH_4 and CO_2 in absolute values (liters), 36.17% and 45.08% respectively, and relative values (L/g of feces and L/g of VS) ($P<0.05$) when compared to biodigesters supplied with manure from animals that did not consume this additive. However, there was no change in the composition of the biogas produced ($P>0.05$), which was composed of 71.92% CH_4, 27.55% CO_2, and 0.06% N_2O, using the mean values between the factors analyzed (Table 21).

Manure from animals that consumed sodium monensin had lower ($P<0.05$) production rate (A) and lower ($P<0.05$) production at the inflection point (y) for both CH_4 (Figure 22) and CO_2 variable.

The use of sodium monensin in the diet increased the production rate (A) and production at the inflection point (y) for the N_2O variable ($P<0.05$), however, the total production of this gas was not changed ($P >0.05$) (Table 21).

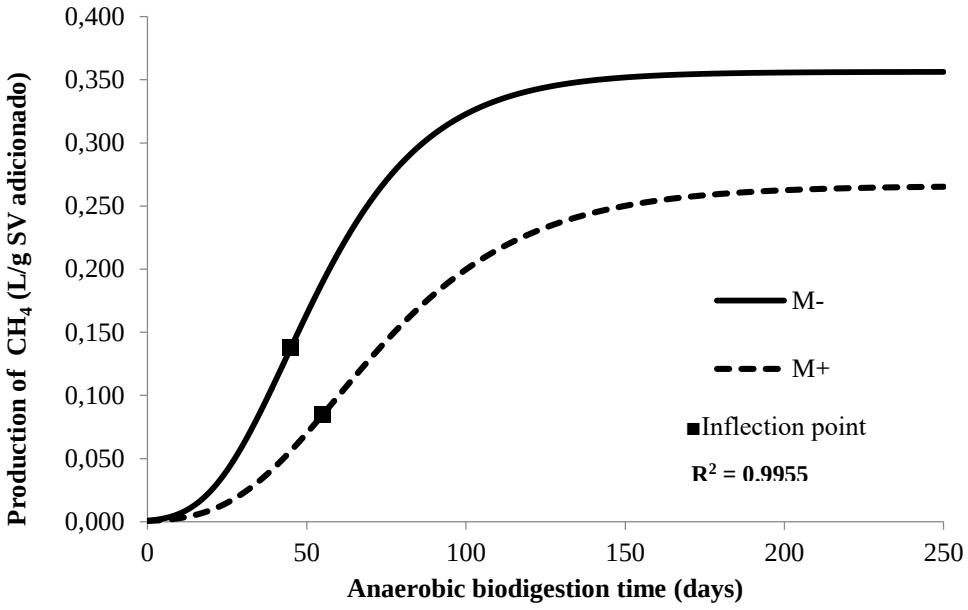
Tabel 21. Gas production (total biogas, CH_4, CO_2 and N_2O) in batch type biodigesters with waste of Nellore cows fed with essential oil, an enzyme blend, monensin and their interactions

Variable	Factors						Average	SEM	P value						
	Essential Oil		Enzyme		Monensin				OE	E	M	OE*E	OE*M	E*M	OE*E*M
	Negative	Positive	Negative	Positive	Negative	Positive									
Biogas, L	40.46	40.29	39.86	40.89	50.24	30.51	40.58	2.40	NS	NS	<0.001	NS	NS	NS	NS
CH_4															
CH_4, L	29.22	28.79	29.11	28.91	35.41	22.6	29	1.65	NS	NS	<0.001	NS	NS	NS	NS
CH_4, %	71.45	72.28	72.73	71.01	70.32	73.41	71.92	0.82	NS	NS	0.07	NS	NS	NS	NS
CH_4/feces, L/g	0.029	0.029	0.029	0.029	0.036	0.023	0.029	0.0016	NS	NS	<0.001	NS	NS	NS	NS
CH_4/added VS	0.28	0.28	0.28	0.28	0.34	0.22	0.28	0.016	NS	NS	<0.001	NS	NS	NS	NS
A, L/g	0.32	0.3	0.3	0.31	0.35	0.26	0.31	0.014	NS	NS	0.001	NS	NS	NS	NS
k, L/g.day	0.04	0.036	0.037	0.034	0.041	0.03	0.036	0.0029	NS	NS	NS	NS	NS	NS	NS
t, day	52.32	47.6	43.56	56.35	44.22	55.69	48.86	3.62	NS	0.09	NS	NS	NS	NS	NS
y, L/g	0.12	0.111	0.113	0.115	0.131	0.097	0.115	0.0052	NS	NS	0.001	NS	NS	NS	NS
CH_4/removed VS, L/g	0.7	0.82	0.7	0.83	0.9	0.62	0.76	0.068	NS	NS	0.05	NS	NS	NS	NS
CO_2															
CO_2, L	11.22	11.01	10.73	11.5	14.35	7.88	11.05	0.83	NS	NS	<0.001	NS	NS	NS	NS
CO_2, %	27.6	27.65	26.31	28.94	28.76	26.49	27.55	0.75	NS	0.09	NS	NS	NS	NS	NS
CO_2/feces, L/g	0.011	0.011	0.01	0.011	0.014	0.08	0.011	0.0008	NS	NS	<0.001	NS	NS	NS	NS
CO_2/added VS	0.11	0.108	0.105	0.113	0.14	0.078	0.108	0.0082	NS	NS	<0.001	NS	NS	NS	NS
A, L/g	0.12	0.116	0.112	0.123	0.143	0.092	0.121	0.0087	NS	NS	0.006	NS	NS	NS	NS
k, L/g.day	0.036	0.034	0.037	0.033	0.042	0.028	0.037	0.0034	NS	NS	0.09	NS	NS	NS	NS
t, day	51	47.58	42.24	56.33	44.14	54.43	47.41	3.92	NS	NS	NS	NS	NS	NS	NS
y, L/g	0.044	0.0426	0.041	0.0455	0.052	0.034	0.044	0.0032	NS	NS	0.006	NS	NS	NS	NS

CO_2/removed VS, L/g	0.27	0.318	0.257	0.331	0.368	0.221	0.294	0.029	NS	NS	0.01	NS	NS	NS	NS
N_2O															
N_2O, mL	18.58	20.99	26.33	13.25	10.08	29.49	19.94	6.21	NS	NS	NS	NS	NS	NS	NS
N_2O, %	0.056	0.061	0.071	0.047	0.017	0.1	0.059	0.02	NS	NS	0.058	NS	NS	NS	NS
N_2O/feces, mL/g	0.018	0.021	0.026	0.013	0.01	0.03	0.02	0.0063	NS	NS	NS	NS	NS	NS	NS
N_2O/added VS	0.184	0.205	0.259	0.13	0.097	0.291	0.196	0.061	NS	NS	NS	NS	NS	NS	NS
A, mL/g	0.318	0.135	0.383	0.071	0.099	0.354	0.189	0.061	0.07	0.005	0.01	NS	NS	NS	NS
k, mL/g.day	0.034	0.035	0.036	0.032	0.04	0.029	0.035	0.0027	NS	NS	0.07	NS	NS	NS	NS
t, day	55.17	47.55	46.22	56.5	44.24	58.48	49.61	3.69	NS	NS	0.06	NS	NS	NS	NS
y, mL/g	0.117	0.049	0.141	0.026	0.036	0.13	0.069	0.022	0.07	0.005	0.01	NS	NS	NS	NS
N_2O/removed VS, mL/g	0.433	0.464	0.57	0.327	0.194	0.702	0.451	0.142	NS	NS	0.1	NS	NS	NS	NS

SEM: stanadard error of mean; EO: Essential Oil; E: Enzyme Blend; M: Monensin; EO*E: Interaction between essential oil and enzyme; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzyme and monensin; EO*E*M: Interaction between essential oil, enzyme and monensin; VS: volatile solids; A: asymptotic production (L/g added VS); k: production constant (L/g added VS per day); t: time at inflection point (day); y: production at inflection point (L/g added VS).

Figure 22. CH_4 production, adjusted by the Gompertz model, in batch-type biodigesters supplied with waste from Nellore cows, fed with or without sodium monensin.



5.3.3. Biofertilizer Composition

Waste from animals fed with sodium monensin resulted in biofertilizers with higher total carbon content ($P<0.05$) and lower levels of N, P_2O_5, and K_2O ($P<0.05$), resulting in a higher C: N ratio without changing the amount of organic carbon (Table 22). Additionally, the biofertilizer obtained from animal waste that received sodium monensin had a lower pH value ($P<0.05$) (Table 22).

Table 22: Composition of biofertilizers obtained in batch type biodigesters supplied with waste from Nellore cows fed with essential oils, a blend of exogenous enzymes, sodium monensin and their associations.

Variable	Factors						Average	SEM	P-value						
	Essential Oil		Enzyme		Monensin				EO	E	M	EO*E	EO*M	E*M	EO*E*M
	Negative	Positive	Negative	Positive	Negative	Positive									
Total C (g/kgMS)	429.3	437.2	429.6	436.9	413.6	452.9	432.4	4.35	NS	NS	<0.001	NS	NS	NS	NS
Organic C (g/kgMS)	163.6	162.6	162.5	163.7	163.8	162.4	163.1	0.84	NS	NS	NS	NS	NS	NS	NS
Total N (g/kgMS)	27.82	27.75	28.02	27.55	31.74	23.83	27.78	0.95	NS	NS	<0.001	NS	NS	NS	NS
P_2O_5 (g/kgMS)	18.21	18.18	18.78	17.6	20.75	15.64	18.31	0.55	NS	0.07	<0.001	NS	NS	NS	NS
K_2O (g/kgMS)	27.00	25.50	26.73	25.77	30.27	22.22	26.40	0.94	NS	NS	<0.001	NS	NS	NS	NS
C/N	15.62	16.32	15.92	16.02	13.22	18.71	15.9	0.60	NS	NS	<0.001	NS	NS	NS	NS
pH	7.70	7.73	7.72	7.71	7.79	7.64	7.72	0.030	NS	NS	0.02	NS	NS	NS	NS

SEM: stanadard error of mean; EO: Essential Oil; E: Enzyme Blend; M: Monensin; EO*E: Interaction between essential oil and enzyme; EO*M: Interaction between essential oil and monensin; E*M: Interaction between enzyme and monensin; EO*E*M: Interaction between essential oil, enzyme and monensin; C/N: Carbon: Nitrogen ratio.

5.4. DISCUSSION

The use of cattle waste as a substrate for anaerobic digestion is a good alternative for containing carbohydrates, proteins, and fat (AHRING et al., 2001). However, some factors can alter the potential for biogas production. According to Orrico Junior et al. (2012), among the factors, the composition of the material directly influences the potential for degradation of the substrate. Thus, the extent of biogas production from manure is dependent on the feed of the animals.

The use of essential oils and exogenous enzymes did not affect the nutrients added in the biodigesters or the biodigestion process. Similarly, Carvalho (2018), analyzing the effect of pepper, eucalyptus, and lemon oils, did not observe the effect of these essential oils on the production of biogas or the composition of the material added in the biodigesters, as did Collazos Paucar (2017), who analyzed the potential for biogas production from cattle manure fed with different exogenous enzymes.

In the present study, sodium monensin did not affect the pH of the material introduced into the biodigesters and the amount of TS, VS, and NDF added. However, it reduced the amount of N added. This reduction justifies by the ability of sodium monensin to increase the use of N in the diet (SPEARS, 1990), influencing the biodigestion process. Since, according to Mendonça (2009), for the biodigestion process to occur under conditions to be satisfactory, nutrients must be present in sufficient quantities, with N being the main one, since during the anaerobic decomposition process, microorganisms use NH3 and organic forms of N for their growth (CHERNICHARO, 2007). Additionally, Sgorlon et al. (2011) indicate the importance of N concentration, since if the C: N ratio is not adequate, bacteria cannot consume all the carbon present, and the process performance will be low.

Despite the lower availability of N with the use of sodium monensin, the efficiency of VS removal was not changed and had an average value of 42.36%, which can be considered within the appropriate range (30% to 45%) cited by Davidsson et al. (2008). The composition of the biogas produced was not altered and within the parameters presented by Coldbella et al. (2006). The mixture of gases was in the proportion of 50-80% of CH_4 and 20-40% of CO_2. In the present study, the biogas produced was composed of 71.92% of CH_4, 27.55% of CO_2, and 0.06% of N_2O, using the mean between all analyzed factors. However, the use of sodium monensin reduced the removal efficiency

of TS and NDF, indicating lower fermentation activity and resulting in a 39.26% drop in biogas production due to the lower production of CH_4 and CO_2.

Peres Assumpção (2021), analyzing the effect of different food additives on anaerobic digestion, also found a reduction in biogas production of about 53.59% with sodium monensin, with no change in the composition of the biogas produced either. However, the author did not find a reduction in the number of nutrients added to the biodigesters, unlike the results found in this study, indicating the possibility that such additive will continue to be active in the manure of cattle fed with it. According to Davison (1984), part of the monensin consumed by the animals can be recovered in the feces. This effect is not observed in the urine and tissues of the animals. Herberg et al. (1978), when determining the pattern of excretion and tissue distribution of monensin in cattle, reported the recovery of 95% of active metabolites of monensin in the feces of animals.

Ribeiro et al. (2007), studying the effect of cattle diet on the environmental impact caused by manure, concluded that the use of ionophores in the diet delayed the start of biogas production and altered its total production. Those results are similar to the ones found in the present study. In addition to the lower biogas production, a lower rate of CH_4 and CO_2 production was observed with sodium monensin in the diet. Also, according to Ribeiro et al. (2007), such change in the biodigestion process is due to monensin being responsible for preventing the growth of acetate-producing bacteria, reducing the availability of C and H for the methanogenic archaea.

In addition to the production of biogas, the biodigestion process makes it possible to use the effluent in fertigation, providing savings on conventional fertilizers and correctives, improving soil fertility, and reducing environmental impacts (MENDONÇA, 2009)

The lower efficiency of TS and NDF removal observed in the biodigesters that received manure from animals fed with monensin resulted in a higher concentration of TS and NDF in the remaining material (Table 20). Consequently, that results in a higher concentration of C Tot in the biofertilizer obtained at the end of the process. It ends up diluting the amounts of N, P_2O_5, and K_2O (Table 22), since the transformation of C into CH4 is responsible for concentrating the nutrients in the biofertilizer (MOLINUEVO-SALCES et al., 2013). Thus, the lower production of CH_4 and CO_2 in these biodigesters (Figure 22, Table 21) justify the higher C Tot concentration and the lower concentrations

of N, P_2O_5, and K_2O in the biofertilizer obtained, consequently changing the C: N and moving away from the optimal ratio of 10-13:1 in the stabilized residue (MBULIGWE; KASSENGA, 2004).

When analyzing the effluent pH, we observed that sodium monensin provided a slight drop (Table 22) but did not remove it from the ideal range between 7 and 8.5 pointed out by Mshandete et al. (2006) and Rabiu et al. (2014), characterizing it in terms of its basic pH as a soil pH corrector.

5.5. CONCLUSION

The addition of sodium monensin reduces nutrient removal efficiency, compromising biogas production and reducing nutrient concentration in the biofertilizer.

The use of EO and E did not affect the anaerobic biodigestion process. In addition, no associative effect was observed between the tested additives on the anaerobic biodigestion process.

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