UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS

ALICE HELENA PERES MARQUES ASSUMPÇÃO

The individual and associated use of monensin, tannins and calcium nitrate in the feeding of cattle promote a reduction in the emission of methane gas.

Pirassununga
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Dissertation presented to the College of Animal Science and Food Engineering of the University of Sao Paulo, as part of the requirements for obtaining the Master's degree in Sciences from the postgraduate program in Animal Science.

Area of concentration: Animal Productivity and Quality

Advisor: Prof. Paulo Henrique Mazza Rodrigues, Ph.D.

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Date: _____/_____/_____

Committee Members

Prof. Dr. Paulo Henrique Mazza Rodrigues – The presidente of the committee members – Faculdade de Medicina Veterinária e Zootecnia – Universidade de São Paulo

Advisor

Prof. Dr. Daniel Montanher Polizel – Escola Superior de Agricultura "Luiz de Queiroz" – Universidade de Sao Paulo

Prof. Dr. Garret Suen – University of Wisconsin-Madison

Dr. Juliano Leonel Gonçalves – Faculdade de Medicina Veterinária e Zootecnia – Universidade de Sao Paulo

DEDICATION

I dedicate this study to the most important person in my life.
My greatest example of strength and perseverance.
The biggest supporter of my dreams.
It is all because of you and all for you.
My beloved mom.

Flaviana Vallim Peres Assumpção.

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To God for this achievement.

To my family, for all the support. My mother, Flaviana, for encouraging me, supporting me and believing in myself when I didn't believe I was capable. To my father, Roberto Cézar Assumpção (*in memoriam*), who I believe guides me from another plan. My sister and best friend, Maria Beatriz, who is always by my side. To my stepfather, Marcelo Abnajim, for his unconditional help and encouragement. To my beloved grandparents, Valdemir Peres, Maria Helena Vallim Peres and Hilda Assumpção (*in memoriam*), for their love and affection.

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EPIGRAPH

“I've worked hard for a long time, and it's not about winning. But what it is about not giving up. If you have a dream, fight for it. There is a discipline for passion. And it is not about how many times you get rejected or you fall down, or you're beaten up. It is about how many times you stand up and are brave and you keep on going.”

Lady Gaga

RESUMO

PERES ASSUMPÇÃO, A. H. **O uso individual e associado de monensina, taninos e nitrato de cálcio na alimentação de bovinos promove a redução do gás metano.** 2021. 97 f. Dissertação (Mestrado em Ciências) - Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, São Paulo, 2021.

O objetivo deste trabalho foi avaliar individualmente e de forma combinada o uso de monensina, taninos do extrato de *Acacia mearnsii* e nitrato de cálcio sobre os parâmetros de fermentação ruminal (técnica *ex situ*), microbioma ruminal e a biodigestão anaeróbia de resíduos. Para tanto, foram desenvolvidos dois estudos. No estudo 1, cinco vacas Nelore canuladas, não gestantes e não lactantes, com peso corporal médio de 530 ± 75 kg, foram distribuídas em quadrado latino 5 x 5. Os animais foram alimentados com dieta basal, relação concentrado:volumoso de 60:40 (silagem de milho), por meio do qual foram fornecidos os aditivos; totalizando cinco tratamentos: controle (sem adição de aditivos), monensina (300 mg na dieta), tanino (1,5% da MS), nitrato (3,0% da MS) e 'pool' (combinação das doses dos três aditivos supracitados). Os dados foram analisados usando PROC MIXED do SAS e as diferenças foram consideradas significativas quando o valor de $P<0.05$. Vacas alimentadas com taninos tiveram um aumento de 23% no consumo de matéria seca e energia bruta. Uma redução na produção de CH_4 foi observada quando comparado o tratamento controle com o uso individual ou combinado de todos os aditivos, sendo 9,5% relacionado ao tratamento com monensina, 18,75% no grupo com tanino, 19,8% no grupo com nitrato de cálcio e redução de 28,8% quando as vacas foram alimentadas com o 'pool' (monensina, nitrato e tanino). Em relação à microbiota ruminal, nenhum efeito foi encontrado na contagem total de protozoários quando as vacas foram alimentadas com o 'pool' em comparação com as do controle. Além disso, usando o PCoA, a estrutura dos membros da comunidade bacteriana do rúmen de vacas alimentadas com o 'pool' teve diferenças quando comparada com vacas alimentadas com monensina e tanino, mas a comunidade bacteriana observada no grupo 'pool' foi semelhante àquela das vacas alimentadas com nitrato. No geral, reduções significativas nas emissões de metano no rúmen podem ser alcançadas sem efeitos drásticos na população microbiana do rúmen ou em sua função. No estudo 2, os biodigestores foram dispostos em um delineamento inteiramente casualizado de 5 tratamentos com 5 repetições, totalizando 25 unidades experimentais (as fezes foram a principal variável oriunda das vacas alimentadas com o uso individual ou combinado de todos os aditivos). Os dados foram analisados por meio do software estatístico SAS (SAS 9.3, Institute Inc., 2013). Os biodigestores acrescidos de resíduos das vacas alimentadas com taninos aumentaram em 91% a produção de CH_4 e 52% a produção de CO_2 por

grama de sólidos voláteis. O tratamento ‘pool’ aumentou os nutrientes do biofertilizante em 12,6% de C Total e 35% de N Total, a monensina aumentou 7,5% do N Total, e o K foi aumentado em 35% para o grupo tanino, enquanto 22% foram observados para o grupo nitrato. A monensina não apresentou potencial de produção de CH_4 em relação aos resíduos no meio ambiente. Curiosamente, os taninos apresentaram resultados significativos por meio da digestão anaeróbia, sendo seu uso recomendado na tentativa de reduzir os impactos do esterco não tratado no meio ambiente. Mais estudos são necessários para entender como o nitrato atua no processo de biodigestão.

Palavras-chave: Diversidade bacteriana. 16S rRNA. Microbioma Ruminal. Técnica *ex-situ*. Gases de efeito estufa.

ABSTRACT

PERES ASSUMPÇÃO, A. H. **The individual and associated use of monensin, tannins and calcium nitrate in the feeding of cattle promote a reduction in the emission of methane gas.** 2021. 97 f. Dissertação (Mestrado em Ciências) - Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, São Paulo, 2021.

The aimed of this study was to evaluate the individually and combine use of monensin, tannins of *Acacia mearnsii* extract and calcium nitrate on ruminal fermentation parameters (*ex-situ* technique), rumen microbiome as well as on anaerobic biodigestion of waste. The dissertation was structured in two studies. *In study 1*, five cannulated Nellore cows, non-pregnant and non-lactating, with a mean body weight of 530±75 kg, were distributed into a 5 x 5 Latin square. The animals were fed with a basal diet, concentrate:roughage (corn silage) ratio of 60:40, through which the additives were supplied totaling five treatments: control (without addition of additives), monensin (300 mg in the diet), tannin (1.5% of DM), nitrate (3.0% of DM), and Pool (combination of the doses of the three additives). Data were analyzed using PROC MIXED of SAS and differences were declared significant at 5%. Cows fed with tannins had an increase of 23% on dry matter and gross energy intake. A reduced CH_4 production was observed when the control treatment was compared to the individual or combined use of all additives, being 9.5% related to monensin treatment, 18.75% in tannin, 19.8% in calcium nitrate and a reduction of 28.8% when cows were fed with the pool (monensin, nitrate and tannin). Regarding the ruminal microbiota, no effects were found on the total protozoan count when cows were fed with the pool in comparison to the control. Furthermore, the structure of members using PCoA of the bacterial community of the rumen of cows fed with the pool had differences when compared to cows fed with monensin and tannin, but the bacterial community observed in the pool group was similar to those of cows fed with nitrate. Overall, significant decreases in rumen methane emissions can be achieved without drastic effects on either the rumen microbial population or its function. *In study 2*, biodigesters were arranged in a completely randomized design of 5 treatments with 5 replicates, totaling 25 experimental units (feces were the main variable from cows fed with the individual or combined use of all additives). The data were analyzed by using the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). The biodigesters supplied with waste from cattle fed tannins increased in 91% of CH_4 production and 52% of CO_2 production per gram of volatile solids. The pool treatment increased the biofertilizer nutrient compounds in 12.6% of Total C and 35% of Total N, monensin presented an increase of 7.5% of Total N, and K was increased in 35% to tannins and 22% to nitrate. The monensin did not present the potential for CH_4 production in relation

to the waste in the environment. Interestingly, the tannins showed significant results through anaerobic digestion, being its use recommended with the attempt to reduce the impacts of untreated manure on the environment. More studies are necessary to understand how nitrate acts on biodigesters.

Keywords: Bacterial diversity. 16S rRNA. Rumen microbiome. *Ex-situ* technique. Greenhouse Gas.

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SUMMARY

1. INTRODUCTION

In 2019, the world population was estimated at 7.70 billion people, according to the United Nations (UN), with projections of reaching 9 billion in 2050 and could exceed 10 billion by the end of the century (United Nations, Department of Economic and Social Affairs, 2019). This population expansion, particularly in urban areas, will reflect an increase in per capita income that will result in increased demand for food (SAATH; FACHINELLO, 2018). However, there are restrictions on the expansion of the agricultural frontier, where the availability of arable areas is distributed among a few countries.

Livestock production is associated with concerns about potential environmental impacts that contribute to global warming, depletion of water resources, soil erosion and habitat impairment (FAO, 2013). Among the factors that generate climate change, the emission of Greenhouse Gases (GHG) is intrinsically connected to anthropogenic actions, in which the agricultural sector emits 24% of gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), whose proportions are 76%, 16% and 6%, respectively (IPCC, 2014). It is necessary to understand the sources of emission and implement mitigation strategies (FAO, 2013).

Of the emissions of gases from the agricultural sector, CH_4 gas is the most representative, insofar as its production occurs through the digestive process of cattle, sheep, goats and pigs and the decomposition of manure originating from these animal breeding (NOVAK; FIORELLI, 2010). However, the production of CH_4 in ruminant animals, resulting from the enteric fermentation of carbohydrates in the diet, is not only harmful to the environment, since the removal of H_2 by the ruminal microorganisms causes losses of gross energy for the animal of 2 to 12% (JOHNSON; JOHNSON, 1995; GOEL; MAKKAR, 2012; WANAPAT et al., 2015).

Latin America is relevant (FAO, 2013), and Brazil plays an important role in this agricultural development scenario. In 2012, Brazil's agricultural production area was 246,629 thousand hectares, 28% of which was devoted to agriculture, 69% to livestock and 3% to forest planting. The area suitable for agriculture, including livestock, comprises about three billion hectares, according to Saath and Fachinello (2018). Brazil has the largest cattle herd in the world, with 214.9 million heads (IBGE, 2018), and knowing that an adult bovine can produce

up to 17 L of CH_4 per hour (RUSSELL, 2002), it is essential to use nutritional strategies that reduce CH_4 production, thus making production more efficient and sustainable.

To find solutions that reduce the environmental impact of CH_4 and improve the energy efficiency of cattle, allowing them to increase their production, various strategies can be adopted to achieve this objective. Such as the genetic improvement of herds, management of pastures, increased energy supply of diets, via non-structural carbohydrates, and the use of feed additives such as ionophores, tannins, calcium and ammonium nitrate, essential oils, enzymes, CH_4 inhibitors, among others (GERBER et al., 2013).

Monensin belongs to the class of ionophores (NAGARAJA et al., 1997) and is widely used, because its mode of action favors a group of bacteria that uses H_2 to produce propionic acid, to the detriment of the group that would use it to produce CH_4, thus causing a change in ruminal patterns with increased energy efficiency (McGUFFEY et al., 2001).

Tannins are plant-derived polyphenols (MANGAN, 1988), classified as hydrolysable (TH) or condensed (TC), having desirable or undesirable effects, which will depend on their concentration, source and factors related to animal species, from their physiological state, and composition of the diet (MAKKAR, 2003). More recently, studies have been multiplied using concentrated plant extracts rich in condensed tannins to reduce methanogenesis (BEAUCHEMIN et al. 2008). Two mechanisms of action of tannins on methanogenesis are reported: the first, acts directly on the activity or population of microorganisms producing CH_4 and, the second, indirectly with the reduction of H_2 production resulting from the reduction of the food degradation (TAVENDALE et al., 2005).

The use of agricultural fertilizers in the ruminant's diet is no longer just a source of protein supplementation, but it can also use as a modulator of rumen fermentation (NEWBOLD et al., 2014; VELAZCO et al., 2014). Studies has shown that the inclusion of nitrate in the diet reduces the emission of CH_4 (KLOP et al., 2016; GRANJA-SALCEDO et al.; 2019). This occurs because the pathway of reducing nitrate to ammonia is highly competitive with that of CH_4 formation, in which the two processes use H_2 as intermediary in their reactions (LENG; PRESTON, 2010). In addition to promoting CH_4 mitigation (NOLAN et al, 2010; VAN ZIJDERVELD et al., 2011; EL-ZAIAT et al., 2014; NEWBOLD et al., 2014), nitrate also provides ammonia for microbial protein synthesis in the rumen (LENG, 2008).

The possibilities of using feed additives for reducing CH_4 production are as vast as their mechanisms of action, but there is still a shortage of data in the literature showing the combined action of these additives with *in vivo* evaluation.

Given the context presented, the hypothesis of this study was that the combined use of monensin, tannins of black wattle tree (*A. mearnsii*) and calcium nitrate have a synergistic effect on the reduction of enteric CH_4 production in Nellore cows and in the production of CH_4, CO_2 and N_2O of waste through anaerobic digestion. Thus, the objective was to evaluate the individual and associated effects of monensin, tannins and calcium nitrate on the parameters and products of enteric fermentation, microbiota, and energy partition in cannulated cattle. As well as the production of CH_4, CO_2 and N_2O from wastes by using biodigesters as an alternative for the management and production of biogas.

2. LITERATURE REVIEW

2.1. Greenhouse Gas Emission

According to the System for Estimating Greenhouse Gas Emissions, in Brazil the agricultural activity is pointed out as the main source of GHG, accounting for 74% of emissions (SEEG, 2018), an increase over 2015, when this activity accounted for 69% of emissions. Almost two-thirds comes from the conversion of forest into pasture and crop production, and the other large portion comes from the direct emissions of agriculture, such as enteric fermentation and soil management.

Methane, carbon dioxide and nitrous oxide are gases produced by livestock activity with the potential to retain more heat and raise atmospheric temperature, while allowing visible light from solar radiation to reach the surface. They partially prevent the infrared radiation emitted by the earth's surface from being lost to the atmosphere and thus causing a greenhouse effect (IPCC, 2006). Despite uncertainties in climate variability, the fifth assessment report of the Intergovernmental Panel on Climate Change (IPCC) identified the likely range of the average global surface temperature up to the year 2100, which is between 0.3 and 4.8°C (IPCC, 2013).

Livestock are estimated to be responsible for 14.5% of the total GHG emission from anthropogenic sources (ROJAS-DOWNING et al., 2017), with methane resulting from enteric fermentation the second largest source of anthropogenic GHG, representing 39% of the

livestock sector emissions (GERBER et al., 2013). Therefore, the increasing demand for sustainable animal production is attractive to researchers exploring possible approaches to reduce GHG emissions from livestock (WANAPAT et al., 2015).

Methane is one of the major GHG responsible for at least 14% of total emissions, with a global warming potential of 21-25 times greater than that of carbon dioxide. The CH_4 emission from agriculture is estimated to be 50-60% of the global emission, 14.5 to 33% from livestock production, ruminants referenced as the major producers (WANAPAT et al., 2015; KUMARI et al., 2016).

The production of CH_4 by enteric fermentation of ruminants generates gross energy losses of feeds ranging from 2 to 12% (JOHNSON; JOHNSON, 1995; GOEL; MAKKAR, 2012; WANAPAT et al., 2015). Therefore, considering the importance of ruminant production, it is essential to establish economically viable ways/technologies to reduce CH_4 production (POPOVA et al., 2013) which include increasing productivity, improving nutritional management, manipulation of rumen fermentation, changes in feed composition, addition of CH_4 inhibitors and defaunation (SHIBATA; TERADA, 2010).

2.2.The rumen fermentation and microbiome

The rumen is inhabited by a diversity of microorganisms, including bacteria, archaea, and fungi. The interaction between these microbes is fundamental to promote the breakdown and fermentation of different feed components ingested by the ruminant host (CUNHA et al., 2017). The resulting breakdown products are fermented mainly by bacteria into important nutrients, such as short chain fatty acids (SCFA), proteins and vitamins (KITTELMANN *et al*., 2014). These nutrients are of great importance for the host growth, reproduction and the generation of agriculturally-relevant products like meat and milk (CUNHA *et al.*, 2017).

In addition, the host contributes to the maintenance of physical and chemical conditions for optimal microbial fermentation. Valadares and Pina (2011) described the essential factors for microbial development and growth, including the frequency of feed ingestion, the addition of nutrients and buffers via saliva, the absorption of products from fermentation, the removal of indigestible residues from food and the maintenance of pH, temperature, anaerobiosis and humidity.

During the process of ruminal fermentation, H_2 and CO_2 are liberated, which are utilized by methanogenic archaea to produce CH_4 (STEWART *et al*., 1997). However, the methanogenesis deprives the host of important carbon resources, representing a loss of 2–12% of the total energy ingested by the animal (JOHNSON; JOHNSON, 1995), and contributes to GHE emissions and global warming (IPCC, 2014).

Despite that, methanogenesis represents the largest H_2 sink in the rumen (LAN; YANG, 2019), being an important intermediate produced during the ruminal fermentation process. Through the reducing equivalents released from glycolysis and pyruvate oxidative decarboxylation to acetyl-CoA (RUSSELL; WALLACE, 1997; LOURENÇO et al., 2010), which need to be moved efficiently for the facilitation of further rumen fermentation (MCALLISTER; NEWBOLD, 2008). The methane production contributes to fermentation efficiency by avoiding the increase of H_2 partial pressure that could inhibit the normal function of microbial enzymes involved in electron transfer reactions, particularly NADH dehydrogenase, which would result in accumulation of NADH and reduce rumen fermentation (MORGAVI et al., 2010).

According to Lan and Yang (2019), the interspecies H_2 transfer, from the fermentative community of protozoa, fungi and bacteria to methanogens is of great importance for CH_4 production in rumen. Incorporating the accumulated H_2 into electron sinks that are nutritionally useful to animals is probably an excellent methanogenesis-inhibition strategy, due to its ability to minimize digestible energy (DE) losses from gas production while avoiding fermentation inhibition. In addition to methanogenesis, there are other important H_2 disposal pathways presenting in the rumen such as propionate production, reductive acetogenesis, sulfate reduction and nitrate/nitrite reduction (DRAKE, 1994; NEWBOLD et al., 2005; ZIJDERVELD et al., 2010).

Rumen microorganisms play an important role in host energetic and protein metabolism, and rumen compound detoxification (WEIMER, 1998). The rumen microbiome comprises a diverse symbiotic community of bacteria, archaea, protozoa, and fungi (KRAUSE; RUSSEL, 1996), being assigned to different functional groups, such as cellulolytic, amylolytic, proteolytic, lipolytic which degrade the wide variety of feed components or further metabolize some of the products formed by other microbes (HUNGATE, 1966).

The most abundant groups are the bacteria and protozoa, representing 90% of rumen microbe biomass, but only a small percentage of rumen microorganisms have been cultured and characterized (KIM et al., 2011; MORGAVI et al., 2013). However, knowledge of this ecosystem is quickly accumulating with the development of molecular biology methods such as sequencing of the 16S rRNA genes (EDWARDS et al., 2004; DENG et al., 2008) and the more advanced metagenomic analysis (McCANN et al., 2014).

With a density of $^{10\ -11}$ cells per gram of rumen content (MACKIE et al., 2000), it is estimated that the rumen bacterial community is composed of more than 7000 species of which about 30% are still not identified (McSWEENEY; MACKIE, 2012; PATRA, 2012)

The sequencing of the 16S rRNA gene showed that, regardless of the type of diet, species or age, the phyla Bacteriodetes and Firmicutes are the most abundant, representing around 80% of the total bacterial population of the rumen (MENEZES et al., 2011; HENDERSON et al., 2013; MOHAMMED et al., 2014). In addition, less abundant phyla, such as Proteobacteria, Fibrobacter, Verrucomicrobia, Tenericutes and Spirochaetes are present in a smaller quantity (MENEZES et al., 2011).

At the genus level, were identify seven groups that can be considered the dominant. They are *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as unclassified *Lachnospiraceae*, *Ruminococcaceae*, *Bacteroidales*, and *Clostridiales* (HENDERSON et al., 2015). The genus *Prevotella*, belonging to the phylum Bacteriodetes, is reported as the most abundant of the bacterial community (STEVENSON; WEIMER, 2017; PITTA et al., 2010), being structural polysaccharides as the main substrate source (MATSUI et al., 2000). However, according to Ramask et al. (2000), the *Prevotella* genus has a great genetic variety, leading to the occurrence of a wide functional versatility, not only utilizing structural polysaccharides as substrate.

Through the symbiosis among the rumen microorganisms, it is possible that degradation of a great variety of compounds occurs, highlighting the ability to degrade structural polysaccharides, such as cellulose and hemicellulose, and convert them into energy, essential aminoacids, and vitamin complexes for the animals hosts, in addition to removing harmful molecules, such as ammonia (NH_4^+), H_2, CO_2 and CH_4 (KRAUSE et al., 2003). However, microorganisms that consume primary compounds are not direct producers of CH_4.

Methane is produced exclusively by methanogenic archaea, but all rumen microorganisms participate in methanogenesis in a direct or indirect way (YANG et al., 2016) following the excessive production of H_2, which needs to be removed as CH_4 (MARTIN et al., 2010).

The archaea community contributes 0.3 to 3.3% of rumen subunit 16S rRNA microbes (LIN et al., 1997; SHARP et al., 1998; ZIEMER et al, 2000), being mostly methanogens that belong to phylum Euryarchaeota. The archaeal are classified into 28 genera and 113 species. At the genus level, they are assigned into 3 main groups, which are Methanobrevibacter (61.6%), Methanomicrobium (14.9%) and a large group of uncultured ruminal archaea named as rumen cluster C (RCC) (15.8%) (JANSSEN; KIRS, 2008). However, few of these community have been isolated from the rumen and those already been cultivated correspond to 7 species, including: *Methanobacterium formicicum*, *Methanobacterium bryantii*, *Methanobrevibacter ruminantium*, *Methanobrevibacter millerae*, *Methanobrevibacter olleyae*, *Methanomicrobium mobile* and *Methanoculles olentangyi*.

The ruminal methanogens can be either free-living or associated with protozoa or fungi to improve the exposure to H_2 (LAN; YANG, 2019). The *Methanobacterium* member are free-living methanogens with no sequences discovered from protozoa or fungi (BELANCHE et al., 2014). Approximately 9–25% of ruminal methanogens are associated to protozoa (NEWBOLD et al., 1995), and contribute to nearly 37% of the CH_4 production from ruminants (FINLAY et al., 1994). *Methanobrevibacter* and *Methanomicrobium* are thought to be the first and second largest genera, corresponding to 32.8% and 23.0% of the total protozoa sequences, respectively (JANSSEN; KIRS, 2008; PATRA et al., 2017).

The protozoal community are the second most abundant microbe in the rumen, contributing up to 50% with the biomass, but their roles in rumen microbial ecosystem remain unclear. Molecular techniques are becoming more popular in analyzing ecological status of ruminal protozoa (KARNATI et al., 2003; REGENSBOGENOVA et al., 2004; SYLVESTER et al., 2004; SKILLMAN et al., 2006). Considerable protozoal diversity at different genera and species levels have been found and *Entodinium* is suggested to be the dominant genus (LAN; YANG, 2019).

According to current knowledge, ruminal protozoa themselves are not able to synthesize methane.They are important candidates associated with CH_4 production, especially through their abilities of abundant H_2 production in their hydrogenosomes, their ability to host epi- or

endo-symbiotic methanogens and to protect them from the toxicity of oxygen (FENCHEL; FINLAY, 2006; BELANCHE et al., 2014). For this reason, rumen fermentation techniques that reduce the total count of protozoa also reduce concentration of methanogens and, consequently, methane production (PATRA; SAXENA, 2011).

The characterization of the rumen microbiome presents standards in relation to taxonomic abundance. Nevertheless, the bacterial and archaeal community are sensitive to changes in diet and administration of feed additives, which may favor one group over another. Exploring the possibility of using additives as manipulators for rumen fermentation, it is possible to reduce methane production and increase energy efficiency in diets.

Some feed additives (i.e., monensin) reduce enteric methane emissions in the short-term but lose effectiveness in the long-term because of microbial adaptations to the compounds (LEE; BEAUCHEMIN, 2014).

2.2.1. Use of monensin on ruminant feeding.

Monensin is a polyether carboxylic ionophore produced by a natural strain of *Streptomyces cinnamonensis* and is orally supplied to the cattle as a sodium salt (DUFFIELD et al., 2008). Ionophores are highly lipophilic with a hydrophobic exterior and a hydrophilic interior that interacts with ions (K^+, Na^+, Ca^{2+}, H^+ and Mg^{2+}) serving as a transport medium for these ions through microbial membranes (RANGEL et al., 2008). In this context, monensin is a metal/proton antiporter that can exchange H^+ for Na^+ or K^+ and, once inserted into the membrane it exchanges intracellular ions of potassium by extracellular protons or extracellular sodium by intracellular protons, and as the potassium gradient is greater than that of sodium, the protons accumulate within the bacteria. There is some evidence that rumen bacteria react to this cytoplasmic acidification by activating a reversible ATPase to pump these protons out of the cell; other pumps that also use ATP are activated to restore the ionic gradients of K^+ and Na^+ causing an intracellular energy depletion, leading to cell death (RUSSELL; HOULIHAN, 2003; TEDESCHI et al., 2003; AZZAZ et al., 2015).

Regarding the microorganisms most sensitive to the action of monensin (or ionophores in general) are Gram-positive bacteria; the Gram-negative bacteria are generally resistant. This difference is since the outer membrane of the Gram-negative bacteria is impermeable to most macromolecules and the transit of solutes is mediated by the porins forming

hydrophilic channels with an exclusion limit of about 600 Daltons. Another reason is that the ionophores are hydrophobic and with molecular weight above 500 Daltons, this outer membrane serves as a protective barrier. Gram-positive bacteria do not have this membrane; therefore, becoming sensitive to monensin (AZZAZ et al., 2015).

Gram-positive microorganisms are the major producers of methanogenic substrates as final fermentation products (acetate, formate, butyrate, hydrogen, etc.), while final products of fermentation of Gram-negative bacteria are propionate, succinate, etc. (i.e. little used for the synthesis of methane). Therefore, monensin has little or no direct role in the methanogenic archaea and, methane production is reduced due to the reduction of the amount of methanogenic substrates produced during the fermentation (RUSSELL; HOULIHAN, 2003).

The role of monensin on rumen metabolism has been studied for years. Richardson et al. (1976), in a study about the effect of monensin on rumen fermentation, demonstrated that monensin, *in vitro* or *in vivo*, caused changes in the proportions of SCFA, with increase of up to 50% of propionate and reduction of acetate and butyrate, but with very little or no effect on the total production of these acids. In another subsequent study, with the administration of 100 mg and 500 mg of monensin per animal per day, there was an increase in the molar ratio of propionate from 31.9 to 41.0 and 43.5%, respectively, whereas the molar proportions of the acetate and butyrate were reduced. More recently, Ogunade et al. (2018) showed that feeding monensin altered functional and metabolomic attributes of the rumen microbiota in forage-fed beef cattle by altering the rumen SCFA profiles in favor of propionate production, by reducing amino acid degradation, and by manipulating biohydrogenation of unsaturated fatty acid in the rumen.

In addition, the use of monensin in the bovine diet was proposed as a strategy to mitigate enteric methane emissions (WITTENBERG et al., 2006). According to Johnson and Johnson (1995), monensin has shown to be potent in reducing methane emissions in cattle, although the effect is temporary. However, Appuhamy et al. (2013), performing meta-analysis on the anti-methanogenic effects of monensin in cattle, found inconsistent results. Studying the long-term effects of monensin on CH_4 production in dairy cows, Odongo et al. (2007) had 7% of reduction in methane production, while Guan et al. (2006) found reduction of 30% in the first two weeks of monensin in rotation with lasolacid and 27% of methane reduction six weeks later. Tseu

(2019), comparing the use of monensin and different levels of tannins in the feeding of Nellore cows, did not observe the effect of monensin on the reduction of CH_4; however, there was a reduction of acetate: propionate ratio, while the inclusion of tannins linearly reduced the production of CH_4 and SFCA.

Another effect of the use of monesin is the improvement of feed efficiency (FE), by about 10% (RUSSELL; HOULIHAN, 2003). According to Ellis et al. (2015), one of the benefits associated with the use of monensin in beef cattle is the improvement of energetic metabolism by rumen bacteria, by the animal or both. The best results in beef cattle are obtained when monensin is used with diets rich in concentrates mainly due to the improvement of feed conversion and, consequently, better cost-benefit ratio. The effects of administration of monensin are well relate on literature, and in recent years, microbial characterization via 16S rRNA has been used to better understand how these effects impact directly on ruminal bacterial diversity in dairy and beef cattle.

2.2.2. Use of tannins on ruminant feeding.

Tannins are polyphenolic polymers with the molecular weights in the range of 500- 20,000 Daltons. They are generally water soluble except for some high molecular weight structures (ADDISU, 2016; PATRA; SAXENA, 2011; AMESA; ASFAW, 2018) and are widely distributed in nutritionally important forage plants (trees, shrubs and legumes), cereals and grains (McLEOD, 1974 e PEREVOLOTSKY, 1994).

Due to their solubility, tannins are divided into two categories: hydrolysable tannins (TH) and condensed tannins (TC). The hydrolysable tannins are complex molecules with a polyol as a central nucleus (as well as glucose, glucitol, quinic acids, kerocytol and shikimic acid) and their hydroxyl group are partially or totally esterified with a phenolic group (MÜELLER-HARVEY; MCALLAN, 1992). According to Lewis and Yamamato (1989), HT is abundant in leaves, fruits, and pods of dicotyledonous plants, and can easily undergo hydrolysis by bases, acids and esterase. Hydrolysis of tannic acid, a typical hydrolysable tannin, can happen spontaneously or by the action of enzymes, and results in the glucose and gallic acid (SINGLETON; KRATZER, 1973).

On the other hand, condensed tannins are polymers of flavonoids (SGARBIERI, 1996), mainly formed by flavan-3-ol (epi)catechin and (epi)gallocatechin units which are united

through carbon-carbon bonds, C_4-C_8 and C_4-C_6 interflavonoid bonds. Although many CT are hydrolysable, some with a higher molecular weight are insoluble in water. Normally, CT are more difficult to degrade than HT, and can be toxic to a variety of microorganisms. This may explain the effect of these molecules in delaying biodegradation and decreasing the decomposition of organic matter (BHAT et al., 1998).

Condensed tannins are classically known as an antinutritional factor in ruminant diets, reducing intake and growth performance and negatively altering carcass characteristics (REED, 1995; LARRAIN et al., 2009). One of the main characteristics of tannins is to bind nutrients mainly proteins and form soluble or insoluble tannin-protein complexes, hence reduce protein digestion (ADDISU, 2016; PIÑEIRO-VÁZQUEZ et al., 2015). However, ruminant animals present greater tolerance to tannins, since the action of ruminal microorganisms is capable of degrading several anti-nutritional factors in simpler and non-toxic compounds (SELINGER et al., 1996).

Studies have shown that high concentrations of tannins, in ruminant diets, which remain free after the formation of tannin-protein complexes, can depress fiber digestion by forming complexes with lignocellulose and, thus prevent microbial digestion (PIÑEIRO-VÁZQUEZ et al., 2015) either by direct inhibition of cellulolytic microorganisms or by inhibition of fibrolytic enzymatic activity or both (PATRA; SAXENA, 2011). In addition, previous studies reported that tannins are capable to interact with the cell membrane of bacteria and metal ions (LEINMULLER; KARL-HEINZ, 1991).

Several studies have demonstrated that mitigation of CH_4 production by CTs both *in vitro* and *in vivo* is related to a decrease of methanogenic archaea communities (TAVENDALE et al., 2005; BHATTA et al., 2009; TAN et al., 2011a; TAN et al., 2011b; CIESLAK et al., 2012). Earlier, Tan et al. (2011) found that inclusion of pure unfractionated CT extracts (from 20 to 60 mg/g DM) caused a linear reduction in total methanogens, with corresponding decrease in CH_4 production. Saminathan et al. (2016), found that inclusions of CT fractions of different molecular weights decreased the predominant methanogen genus *Methanobrevibacter* which could contribute to the reduction of CH_4.

The antimicrobial activities of CTs could be attributed to the interaction of CTs with proteins, resulting in both inhibition of extracellular enzymes and substrate unavailability for digestion, leading to deactivation of microbes and subsequently death (SMITH et al., 2005).

Direct interaction of CTs with microbial cell wall has been reported by Patra and Saxena (2009). Condensed tannins exhibit protein-binding affinity through hydrogen bonds and hydrophobic interactions (KUMAR; HORIGOME, 1986; OSBORNE; McNEILL, 2001).

The possible mechanisms of action are the reduction of ammonia (NH_3-N) release by inhibiting the activity of microbial urease and by reducing the rumen protein degradability through formation of tannin-protein complexes as well as by the direct inhibition of proteases (KROBER et al., 2000). The literature review on the potential of CT in reducing methane emissions conducted by Piñeiro-Vázquez et al. (2015) showed that CT reduce methanogenic archaea and protozoa population and by as much as 33% and 79%, respectively. They also found that CT bind proteins and polysaccharides forming complexes that reduce the digestibility of dry matter (DM) and organic matter (OM) as well as the production of H_2 used by archaea to form methane.

In the study of Min et al. (2002), using *Lotus corniculatus*, it was shown that, although CT of this plant might have reduced the population of proteolytic bacteria in sheep, the total microbial protein, and the flow of microbial protein to the abomasum were not affected, but it reduced the digestibility of the rumen N as well as the rumen concentration of ammonia, and increased the flow of undegraded N to the abomasum.

On the other hand, the effect of tannins decreasing the microbial community may reduce the carbohydrate digestion rate, especially cellulose and hemicellulose, and reduce the total concentration of SCFA in the rumen by reducing the molar concentration of acetate (PATRA; SAXENA, 2011). The meta-analysis performed by Jayanegara et al. (2012) on the relationship between dietary level of tannins and methane production in ruminants from *in vitro* and *in vivo* experiments has shown that the reduction of methane production was associated with the reduction of apparent digestibility of OM and, especially, the fiber.

The *in vivo* study of Dschaak et al. (2011), using CT extract, showed increase in the molar ratio of acetate, propionate, and butyrate when the diet contained a higher proportion of forage, but did not increase when the forage was lower. Sliwinski et al. (2002) did not observe any significant differences on the concentration of SCFA and total count of protozoa when they tested two diets with HT in the concentration of 1 or 2 g/kg of DM, but the release of methane increased in the diet with 1g in comparison with the control diet. Beauchemin et al.

(2007) found no significant differences in methane production when they included up to 2% of tannins in the diet.

2.2.3. Use of nitrate on ruminant feeding.

The use of nitrates (NO_3-) in bovine diets has received attention due to the reduction of methanogenesis with the transformation of nitrate to ammonia by ruminal microorganisms (LENG; PRESTON, 2010). Furthermore, nitrates are a non-protein nitrogen sources for cattle, acting as an H_2 sink and adding ammonia-based nitrogen to the rumen (NOLAN et al., 2010; ZHAO et al., 2015). The main route of conversion of nitrate to ammonia goes through two reactions: in reaction-1, nitrate is reduced to nitrite and, in reaction-2, nitrite is reduced to ammonia (reaction equations demonstrated below). These reactions produce more energy than the conversion of CO_2 and water to CH_4, -598 kJ versus -131 kJ, respectively (LENG; PRESTON, 2010)

$$NO_3 + 2H = H_2O + NO_2 \quad (1)$$

$$NO_2 + 7H = 2H_2O + NH_3 \quad (2)$$

Some bacteria species which can reduce nitrate, nitrite or both compounds are present in the rumen naturally. *W. succinogenes* and *S. ruminantium* are the major nitrate-reducing bacteria present in rumen (ASANUMA et al., 2002; YOSHII et al., 2003). However, to compete efficiently with methanogens, the number or activity of nitrate-reducing bacteria needs to be increased due to the number of methanogens in rumen (JEYANATHAN et al., 2011).

The supplementation of nitrate to the diet of animal is a useful strategy to increase the activity of nitrate-reducing bacteria in rumen, for example, increased number of nitrate-reducing bacteria such as *W. succinogenes* and *Veillonella parvula* were observed in the study of Iwamoto et al. (2002) after the addition of nitrate. The positive effects of nitrate were also shown in the experiments of Zijderveld et al. (2011), Lund et al. (2014) and Klop et al. (2016). However, despite having beneficial effects, the nitrate to ammonia reduction intermediate, nitrite, has a high potential for toxicity when absorbed by the rumen wall. The concern is due to the fact that nitrate, when reduced by the rumen microorganisms to nitrite, when absorbed, causes the oxidation of hemoglobin iron ions, transforming it into methemoglobin, which has no capacity to transport O_2, causing tissue anoxia (LEWIS, 1951).

Interestingly, no clinical effects were observed for poisoning by addition of nitrate in a study conducted by Cassiano et al. (2017), whentesting different levels of inclusion of calcium nitrate (0, 1, 2 and 3% in DM) in the diet of rumen-cannulated bovine femalesof the Nelore and Holstein breed. Besides the addition of calcium nitrate, these authors supplemented the animals with sulfur, as it is considered essential for the prevention of intoxication, since it stimulates the proliferation of the bacterium *M. elsdenii*, which acts by controlling the reduction of nitrate (PERDOK et al., 2011).

Granja-Salcedo et al. (2019), used encapsulated nitrate supplementation in grazing beef cattle and observed a decrease of 18.5% in CH_4 emissions per kg of forage dry matter intake, lower concentrations of NH_3-N and a higher ruminal pH. Steers supplemented with nitrate had a greater ruminal abundance of *Bacteroides*, *Barnesiella*, *Lactobacillus*, *Selenomonas*, *Veillonella*, *Succinimonas*, *Succinivibrio*, and *Duganella* sp., and a lower abundance of *Methanobrevibacter* sp. A strong negative correlation between daily methane emissions and the genus *Proteobacteria* was also observed.

Borges (2018), testing the effect of calcium nitrate levels (0; 1.5; 3 and 4.5% in DM) in comparison and combination of monensin sodium (300 mg), in the diet of cannulated Nellore females, found that the inclusion of nitrate reduced methane production by up to 34.2% without affecting SCFA production, except for butyrate, which decreased by up to 25.7%. The author reported that the interaction between monensin and calcium nitrate did not affect the ruminal pH parameters or the population of ciliated protozoa. However, the addition of calcium nitrate decreased the CMS by up to 10%, with a decreasing linear effect on consumption as nitrate levels increased. This is an expected effect, since the nitrate palatability generally reflects a decrease in consumption (LICHTENWALNER, 1973).

Phommasak et al. (2011) observed a 22% reduction in CH_4 production in a study comparing the inclusion of calcium nitrate (3.8% DM) and urea (2% DM) in diets based on sugarcane and cassava leaves, fresh or dry. The authors concluded that it is possible to substitute urea with nitrate, as this, in addition to contributing to a significant reduction in methanogenesis, is also a source of non-protein nitrogen in the rumen for the synthesis of microbial protein. These results corroborate those found by Silivong et al. (2011), who observed a 23% reduction in CH_4 production in goats supplemented with 3.8% calcium nitrate compared to the control with 2% urea in the DM of the diets.

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3. EFFECT OF MONENSIN, TANNINS AND CALCIUM NITRATE, ALONE OR IN COMBINATION, ON RUMEN ENVIRONMENT AND FEED ENERGY PARTITION OF NELLORE COWS

Abstract: The aimed of this study was to evaluate the effects of monesin, tannins and calcium nitrate on DM and water consumption, daily rumen pH, the products of rumen fermentation through the ex-situ technique, the characterization of the rumen microbiome and the feed energy partition. Five cannulated Nellore cows, non-pregnant and non-lactating, with a mean body weight of 530±75 kg, were distributed into a 5 x 5 Latin square. The animals were fed with a basal diet, concentrate:roughage (corn silage) ratio of 60:40, through which the additives were supplied totaling five treatments: control (without addition of additives), monensin (300 mg in the diet), tannin (1.5% of DM), nitrate (3.0% of DM), and pool (combination of the doses of the three additives). Data were analyzed using PROC MIXED of SAS and differences were declared significant at 5%. Cows fed with tannins had an increase of 23% on DMI and GEI. A reduced CH_4 production was observed when the control treatment was compared to the individual or combined use of all additives, being 9.5% related to monensin treatment, 18.75% in tannin, 19.8% in calcium nitrate and a reduction of 28.8% when cows were fed with the pool (monensin, nitrate and tannin). Regarding the ruminal microbiota, no effects were found on the total protozoan count when cows were fed with the pool in comparison to the control. Furthermore, the structure of members using PCoA of the bacterial community of the rumen of cows fed with the pool had differences when compared to cows fed with monensin and tannin, but the bacterial community observed in the pool group was similar to those of cows fed with nitrate. Overall, significant decreases in rumen methane emissions can be achieved without drastic effects on either the rumen microbial population or its function.

Keywords: Methane. Rumen microbiota. *Streptococcus*.

3.1.INTRODUCTION

The rumen microbiota is composed by several microorganisms that belong to all three domains: Eubacteria (Bacteria), Archaea (Methanogens) and Eukaryote (Protozoa and Fungi). According to Kittelmann (2014), the interaction among those microorganisms promotes the breakdown and fermentation of feed ingested by the animal-host, such as short chain fatty acids (SCFA), proteins and vitamins that are required for animal growth, reproduction, and meat production. The nutrient fermentation also liberates byproducts of fiber degradation including CO_2 and H_2, released by other microorganisms and utilized by methanogenic archaea to produce CH_4 (STEWART; FLYNT; BRYANT, 1997). However, methanogenesis reduces the carbon source to the microbiota, decreasing 2 – 12% of total energy ingested by the animal (JOHNSON; JOHNSON, 1995; GOEL; MAKKAR, 2012; WANAPAT et al., 2015) and is the stronger GHG, having eight-times global warming potential of CO_2 (IPCC, 2014).

In an attempt to reduce enteric methane (CH_4), new studies with the attempt to evaluate options of methane-mitigating additives on the rumen microbiome have been demanded since a total of 14.5% of GHG is released from livestock sector. It is well-established that monensin is an ionophore that increases feed efficiency, increasing the total of SCFA and propionate and, decreasing acetate (RUSSELL; HOULIHAN, 2003). Briefly, this additive inhibits the growth of $Gram^+$ bacteria increasing the membrane permeability and inserting itself through a mechanism that works as an Na^+ / H^+ antiporter, which causes microbial energy depletion (BERGEN; BATES, 1984). Extensive research of individual use of monensin on modulation of rumen microbiome has been performed (VENEAMAN et al., 2015; OGUNADE et al.; 2018), which, by contrast, has encouraged future studies for considering a natural additive source as an option.

In this scenario, tannins are plant secondary compounds with the ability to form complexes with proteins and carbohydrates (BEAUCHEMIN et al., 2007). The mechanisms of tannins action are associated with three main factors: (i) decreasing methanogenic archaea domain; (ii) reducing the number of protozoa in the rumen and, consequently, decreasing methanogenesis and, (iii) decreasing fiber digestion in the rumen (FINLAY et al., 1994; BHATTA et al., 2009; PATRA; SAXENA, 2011; CARRASCO et al., 2017). On the other hand, the methanogenesis process is dependent on CO_2 and H_2 availability (MILLER, 1995) and on

rumen, nitrate (NO_3^-) is reduced to ammonia (NH_3), providing an alternative, and energetically more favorable, pathway for disposal of H_2 generated during fermentation (UNGERFELD; KOHN, 2006). In this context, studies have shown that replacement of urea, as a source of N, by nitrate is also an option (NOLAN et al., 2010; NEWBOLD et al. 2014; VELAZCO et al., 2014).

Overall, the individual use of monensin, tannins and nitrate in the ruminant diet has shown potential for reducing CH_4 through direct action on the ruminal microbiota altering the fermentation and improving feed efficiency (TEDESCHI et al., 2003; JAYANEGARA et al., 2012; LEE; BEAUCHEMIN, 2014). However, to our knowledge, little is known about the effects of additives in combination (e.g. monensin, taninins and nitrate), when working synergistically, on the rumen fermentation and microbiome. Therefore, the hypothesis tested in this study was that the combined use of monesin, tannin and nitrate have an additive or synergistic effect on the reduction of enteric CH_4 production. Thus, this study aimed to evaluate the effect among monensin, tannins of *Acacia mearnsii* and calcium nitrate on water and dry matter intake, rumen fermentation parameters (pH, NH_3-N, SCFA and enteric methane quantifications), microbiota, and energy partition in cannulated Nellore cows.

3.2.MATERIAL AND METHODS

3.2.1. Place of experimentation, ethical issue, and animals

The experiment was conducted at the Animal Nutrition and Production Department (VNP) of the College of Veterinary Medicine and Animal Science (FMVZ) of the University of São Paulo (USP), Fernando Costa *Campus* in Pirassununga, Brazil. Following 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) of the USP, under the protocol number CEUA 4431120419.

Five Nellore cows, non-pregnant and non-lactating, with mean body weight (BW) of 530±75 kg, carrying rumen canula 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

Animals were arranged in a 5x5 Latin Square in which the unit experimental was the animal within each experimental period (n = 25). Cows were offered a basal diet, with a concentrate: roughage (corn silage) ratio of 60:40, differing by the additive's inclusions totalizing five treatments. The following treatments were considered (a) Control without any additive, (b) Monensin including 300 mg (about 32 mg/kg DM) of sodium monensin (Rumensin® 200, Elanco Animal Health, Brazil), (c) Tannin 1.5% of *Acacia mearnsii* extract on DM, (d) Nitrate 3.0% of calcium nitrate on DM and, (e) Pool a blend of all three additives doses.

The tannins were from commercial extract obtained from the bark of the Black Wattle tree (*Acacia mearnsii*) (Seta Natur – Seta Acacia Tannin Extract). The concentration of total phenols (84.4% of extract) was determined by the Folin-Ciocalteau method (MAKKAR, 2003) and total tannins (82.3% tannic acid equivalent) were estimated by the difference in total phenol concentration before and after treatment with insoluble polyvinylpolypyrrolidone (MAKKAR et al., 1993). The concentration of condensed tannins (32.3% leucocyanidine equivalent) was determined by the HCl-butanol method (MAKKAR, 2003).

The calcium nitrate source was the commercial agricultural fertilizer YaraTera™ CALCINIT (Yara Brazil). Determination of nitrate ($N\text{-}NO^{3-}$), ammonium ($N\text{-}NH_4$), Total – N and according with Kjeldahl method and Ca, and the concentrations were 10.61% of $N\text{-}NO_3$, 1.30% of $N\text{-}NH_4$, 11.91% of Total-N and 194.68 g/Kg of Ca.

3.2.3. Feeding management

The experiment lasted 140 days and was divided into five 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. Animals were fed twice a day, at 8 a.m. and 4 p.m., in the form of total mixed ration (TMR). Both treatments, Pool and Mon, received 300 mg of sodium monensin twice a day (150 mg at 8 a.m. and 150 mg at 4 p.m.), mixed with the feed. To ensure that all treatments received the same amount of energy and nitrogen, diets without tannins were supplied with the same proportion of kaolin, an inert clay, and when there

was no calcium nitrate a blend with urea, calcitic limestone and kaolin was including as well. The ingredients proportions and chemical composition of the diets are shown in Table 1.

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

Ingredients (% of DM)	Basal diet	Tannin	Nitrate	Pool
Corn silage	40.00	40.00	40.00	40.00
Ground corn grain	47.60	47.60	47.60	47.60
Soybean meal	6.20	6.20	6.20	6.20
White salt	0.50	0.50	0.50	0.50
Mineral premix[1]	1.00	1.00	1.00	1.00
Sulfur – flower	0.20	0.20	0.20	0.20
Blend[2]	3.00	3.00	-	-
Tannin extract[3]	-	1.50	-	1.50
Calcium nitrate[4]	-	-	3.00	3.00
Kaolin	1.50	-	1.50	-
Chemical composition				
DM[5] (%)	62.20	62.20	62.20	62.20
CP[5] (%DM)	11.70	11.70	11.70	11.70
RDP[6] (%CP)	68.60	68.60	68.60	68.60
RUP[6] (%CP)	31.40	31.40	31.40	31.40
NDF[5] (%DM)	32.0	32.0	32.0	32.0
NDFe[6] (%DM)	23.3	23.3	23.3	23.3
ADF[5] (%DM)	14.1	14.1	14.1	14.1
NFC[5] (%DM)	48.1	48.1	48.1	48.1
Starch[6] (%DM)	44.5	44.5	44.5	44.5
MM[5] (%DM)	5.0	5.0	5.0	5.0
Ca[5] (%DM)	0.96	0.96	0.96	0.96
P[5] (%DM)	0.32	0.32	0.32	0.32
EE[5] (%DM)	3.0	3.0	3.0	3.0
TND[6] (%DM)	67.7	67.7	67.7	67.7

[1]Mineral premix, quantity per kg of product: 140 of calcium, 80 g of phosphorus, 10 g of sulfur, 129 g of sodium, 80 mg of cobalt, 1400 mg of cooper, 800 mg of fluorine, 80 mg of iodine, 1 g of manganese, 20 mg of selenium, 3.5 g of zinc; [2]Blend of calcitic limestone, urea and kaolin per % of DM: 50% of calcitic limestone, 29.25% urea and 20.75% of kaolin; [3]Extract of *Acacia mearnsii* with 82.3% of total tannins, of which 32.3% was condensed tannins; [4]Calcium nitrate with 10.61% of N-NO_3, 1.30% of N-NH_4 and 164.68 g/Kg of Ca; [5]Quantititaed through chemical analysis; [6] Value estimated by NRC (2001).

3.2.4. 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 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.

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.

3.2.5. Assessment of 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 canula, as described by Allen and Linton (2007). On 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 at 26th day at 8 a.m. prior to diet administration, when the rumen was, theoretically, at 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. Afterwards, both phases were reconstituted and returned to the rumen. The rumen DM was calculated based on the dry weight of each sample.

3.2.6. Continuous pH measuring.

The measurement of pH was continuously performed on 22nd day of each experimental period by using a data logger (model T7-1 LRCpH, Dascor, CA). 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).

3.2.7. Evaluation of rumen fermentation products

The evaluation of rumen fermentation products (SCFA, CH_4, NH_3-N and microbiota) was performed using the *ex-situ* technique described by Rodrigues et al. (2012) and Perna Junior et al. (2017). That consisted of collecting rumen samples and putting in flasks (micro-rumen) which are incubated in a thermostatic bath, simulating the rumen conditions for 30 minutes.

3.2.7.1. Sampling of rumen content

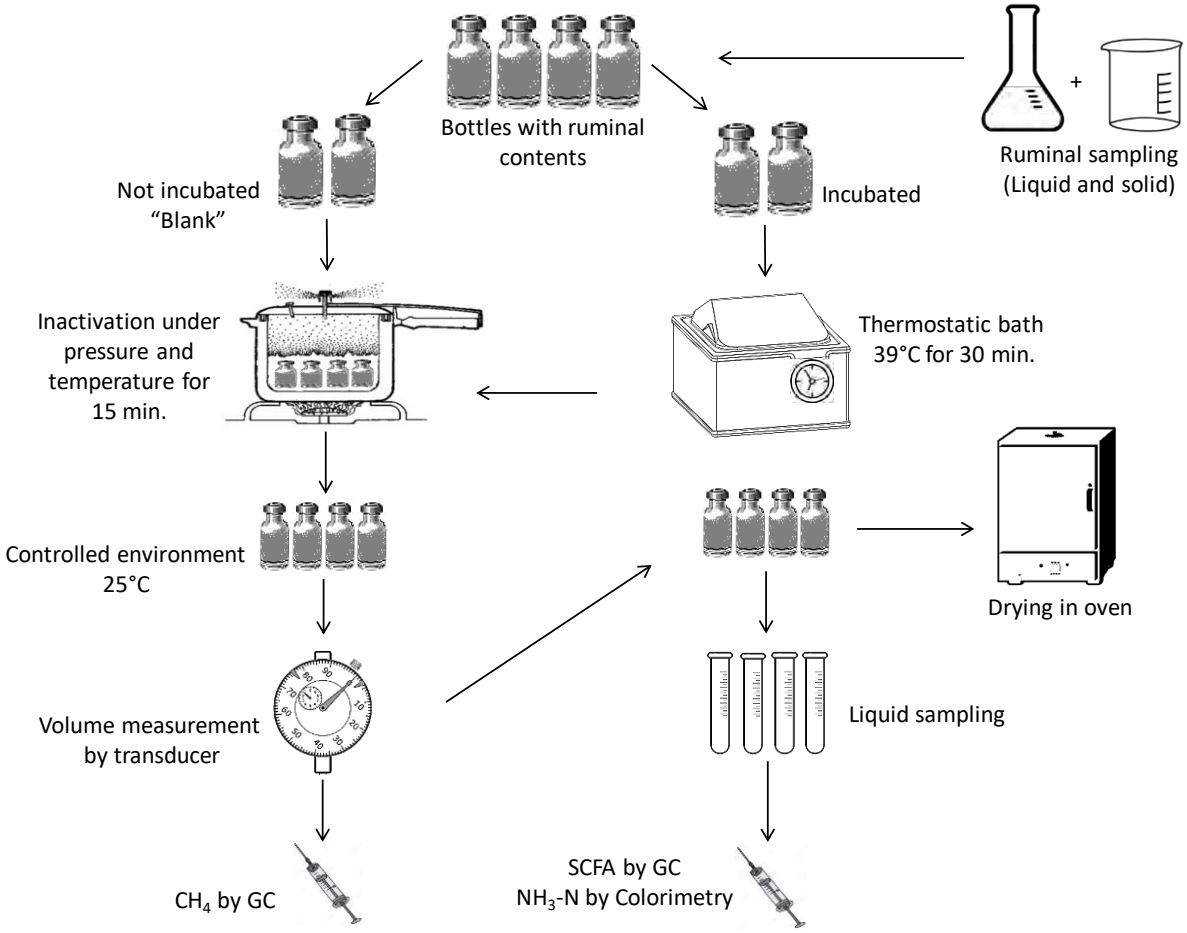
On the 22nd day of each experimental period the rumen content was measured during the day at zero (0), 3, 6, 9 and 12 hours after the morning feeding. The samples were designated for the measuring of SCFA, CH_4, NH_3-N and microbiota. On this day, the cows were fed after the first sampling time, around 8:20 a.m., and after the last sampling time, around 8:30 p.m. The liquid phase was collected with 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 faction, respectively). The flasks were then capped with rubber stoppers and sealed with aluminum sealing wax through specific pilers. Afterwards, they were "washed" with CO_2 by means of two needles for gas inlet and outlet to ensure anaerobiosis.

Four flasks per cow were prepared for each sampling time, two of which were immediately inserted into an autoclave to inactivate the fermentative process was (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 1.

Figure 1. Diagramatic representation of ex situ rumen fermentation technique.



Source: Perna Jr. et. al (2017)

3.2.7.2. 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 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 CH_4 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).

3.2.7.3.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 done 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 Crbopack B-DA/4% (Supelco, Sigma-Aldrich, St. Louis, MO – USA).

3.2.7.4.Production of SCFA and methane, 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 non-incubated flask (equation

1). The individual quantification of SCFA (mmol/L) obtained in the incubated flask, subtracting the production in the non-incubated flask (equation 2).

Subsequently, the production of SCFA and CH_4 were 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. SFAC x Total Liq. Vol.) T_{30} - (Conc. SFAC 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; Consc. 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 in order 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 percentage as well. Thus, theoretical chemical values of the combustion heat were used, if 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 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).

3.2.7.5.Concentration of ammonia nitrogen (NH_3 – N)

The ammonia nitrogen (NH_3-H) concentration was determined by taken 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 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 NH_3-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:

NH_3-N balance (mg/dL.h) = [Conc. 30 min (mg/dL) - Conc. 0 min (mg/dL)] x 2

Where:

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

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

3.2.8. Evaluation and quantification of rumen microbiota

On day 22nd of each experimental period, rumen content (mass and liquid) of each cow was sampled for archaea and bacteria DNA extraction as well for counting total and differential numbers of protozoa. For rumen archaea and 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.

3.2.8.1.Assessment of archaeal and bacterial communities' 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 V3 and V4 regions of the 16S ribosomal RNA gene for bacteria and the V6 through V8 regions of the16S rRNA gene for archaea (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 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 mothur v.1.44.3 (SCHLOSS et al., 2015), following the Standard Operating Procedure previously described (KOZICH et al., 2013). The Chao richness estimator, Simpson index, and Shannon index were used for characterization of alpha diversity at the genus level of 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).

3.2.8.2.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*.

3.2.9. Energy partition

The gross energy intake (GEI) was calculated by multiplication of DMI (kg) and diet GE (Mcal/kg), The energy release 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 release 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 SCFT 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:

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

Where:

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

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

ƐC_2: acetate energy (Mcal/ani.d);

ƐC_3: propionate energy (Mcal/ani.d);

ƐC_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.

3.3.Statistical analysis

The data were analyzed using Statistical Analysis System (SAS 9.4, Institute Inc., 2013). Before the analysis they were evaluated in relation to 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 data of DMI, rumen solid mass, pH and energy partitioning were submitted to analysis of variance which separated, as causes of variation, treatments effect, period effect and animal effect. The statistical model used is described according to the following equation:

$$Yijk = \mu + Ti + Pj + Ak + eijl$$

Where:

Y_{ijk} = observation concerning Treatment_i + Period_j + Animal_j;

μ = overall mean;

T_i = effect of treatment (fixed effect);

P_j = effect of period (random effect);

A_k = animal effect (random effect);

E_{ijl} = random error associated to each observation.

The factor "measures repeated over time" was added for the variables of SCFA, CH_4, NH_3-N productions and differential count of rumen protozoa it, referring to the different sampling hours (0, 3, 6, 9 and 12). The analysis was performed by using the mixed procedure (Proc Mixed). The analysis was performed only when the interactions between time and treatment were significant. For the analyses, 15 different covariance structures were tested, and that which best fit the statistical model was chosen based upon the lowest value of the corrected Akaike information criterion (AICC) (WANG; GOONWARDENE, 2004). The comparisons of means among treatments were performed using the LSD test at 5% of significance.

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 7 most common phyla and 20 most common genera were analyzed using a repeated measures 2-way ANOVA on log-transformed data, considering groups (control, monensin, tannin, nitrate and pool). When a significant effect was present, post-hoc 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) wase compared using the Parsimony (*t* test) and the analysis of molecular variance (AMOVA) tests.

Differences among control and treatments were further explored using linear discriminant analysis effective size (LEfSe, version 1.0) (SAGATA et al., 2011), which uses factorial Kruskal-Wallis sum-rank and a subsequent pairwise test (Wilcoxon rank-sum) to detect features with biological significance by comparing the abundance in all populations, including those with low abundance. As a last step, LEfSe uses linear discriminant analysis (LDA) to estimate the effect size of each differentially abundant feature. Alpha values for the factorial Kruskal-Wallis test among classes and for the pairwise Wilcoxon test between

subclasses were set to .05. Threshold on the logarithmic LDA score for discriminative features was set to 2.0 and the strategy for multiclass analysis was set to all-against-all (stricter).

3.4.RESULTS

3.4.1. Consumption and rumen pH

Interestingly, cows fed with tannins had higher DMI when compared to cows from the control group (Table 2). However, nitrate and monensin cow fed groups had similar DMI in comparison with control treatment. In addition, the treatment named pool (containing monensin, tannin and nitrate) had similar DMI when compared to monensin and control treatments but a lower amount in comparison to tannin and nitrate. Water consumption was not affected by the additive's inclusion.

Table 2. Feed and water intake of Nellore cows fed with monensin, tannins of *A. mearnsii*, Calcium Nitrate and Pool.

Intake	Treatments					SEM	*P value*
	Control	Mon	Tannin	Nitrate	Pool		
DM, day							
kg	7.54^{bc}	8.02^{bc}	9.30^{a}	8.57^{ab}	6.85^{c}	0.5005	0.0095
% of BW	1.37^{bc}	1.44^{ab}	1.64^{a}	1.50^{ab}	1.16^{c}	0.1054	0.0244
Water, day							
L	24.00	23.60	23.60	23.60	21.28	2.4238	0.6399
L/kg BW	3.23	3.02	2.55	2.73	3.17	0.3563	0.2950
% of BW	4.31	4.19	4.20	4.08	3.67	0.4050	0.3881

DM: dry matter; BW: body weight; SEM: standard error of mean;; abc: different letters on the same line differ significantly by the protected LSD test at 5% of significance level.

The overall mean ruminal pH observed in the pool treatment was lower in comparison to the control and other tested groups (Table 3). Furthermore, similar values of ruminal pH were observed when evaluated the isolated usage of monensin, tannin and nitrate. The area per hour and the time that the rumen pH remained below 5.8 had higher values in pool treatment when compared to control group or by looking at the isolated usage of each additives.

Table 3. Rumen pH of Nellore cows fed with monensin, tannins of *A. mearnsii*, Calcium Nitrate and Pool.

Variables	Treatments					SEM	*P value*
	Control	Monensin	Tannin	Nitrate	Pool		
Rumen pH, day							
Minimum	5.17	5.14	5.56	5.29	5.01	0.1221	0.5170
Medium	5.92^{a}	6.07^{a}	6.14^{a}	6.01^{a}	5.36^{b}	0.1455	0.0080
Maximum	6.67	6.98	6.69	6.78	6.18	0.1870	0.0980
Time of pH, min/dia							
<5.8	708.0^{ab}	550.0^{bc}	238.0^{c}	444.0^{bc}	1157.8^{a}	172.40	0.0141
<6.0	892.0^{ab}	684.0^{bc}	412.0^{c}	712.0^{bc}	1238.8^{a}	166.42	0.0117
<6.2	998.0^{ab}	836.0^{b}	750.0^{b}	926.0^{b}	1325.1^{a}	148.24	0.0487
Area, h·(pH/day)							
<5.8	4.46^{b}	3.34^{b}	0.84^{b}	1.95^{b}	11.86^{a}	1.8934	0.0070
<6.0	7.14^{b}	5.39^{b}	1.90^{b}	3.90^{b}	16.95^{a}	2.3361	0.0030
<6.2	10.31^{b}	7.93^{b}	3.81^{b}	6.65^{b}	20.05^{a}	2.7571	0.0050

SEM: standard error of mean; abc: different letters on the same line differ significantly by the protected LSD test at 5% of significance level.

3.4.2. Rumen microbiota characterization

3.4.2.1.Total and differential count of protozoa

Nitrate inclusions increased the total count of rumen protozoa in comparison to other treatment groups. Basically, this increased total count of rumen protozoa can be explained by the higher values observed of *Entodinium* and *Diplodiniinae* when cows were fed with nitrate (Table 4). Considering Protozoa outcomes in percentage, statistical significance was only observed for *Dasytricha* phylum which had higher relative percentage (0.86%) in cows fed with pool treatment in comparison to another groups.

Table 4. Total and differential count of protozoa of Nellore cows fed with monensin, tannins of *A. mearnsii*, Calcium Nitrate and Pool.

Variables	Treatments					SEM	*P value*	
	Control	Monensin	Tannin	Nitrate	Pool		Additive	Time
Protozoa (x10^3/ml)								
Entodinium	1283.6^{b}	1127.3^{b}	1322.1^{b}	1736.5^{a}	1131.2^{b}	124.0	0.0084	<0001
Diplodiniinae	36.00^{b}	33.60^{b}	44.49^{b}	58.42^{a}	41.80^{b}	5.781	<0001	0.0302
Dasytricha	6.57	8.35	4.99	6.00	8.95	1.262	0.0748	0.3971
Isotricha	3.16	4.03	4.32	3.02	2.43	0.929	0.3572	0.0152
Total	1329.3^{b}	1163.9^{b}	1375.9^{b}	1803.9^{a}	1175.9^{b}	123.6	0.0109	0.0005
Protozoa %								
Entodinium	96.43	95.46	96.09	96.32	95.42	0.698	0.4675	0.0212
Diplodiniinae	2.81	3.38	3.20	3.18	3.74	0.577	0.6131	0.2273
Dasytricha	0.50^{bc}	0.82^{ab}	0.38^{c}	0.34^{c}	0.86^{a}	0.135	0.0341	0.1082
Isotricha	0.26	0.45	0.33	0.16	0.20	0.090	0.1213	0.0035

SEM: standard error of mean; abc: different letters on the same line differ significantly by the protected LSD test at 5% of significance level.

3.4.2.2.Bacterial and archaeal community's characterization

A total of 1,210,517 sequences were obtained for bacteria, with an average of 48,420 (varying of 25,055 to 64,909) per sample and 285 OTUs were detected after quality filtering. For archaeal 2,797 were obtained, within an average of 111.84 (varying of 21.00 to 237.00) per sample and 44 OTUs. For all amplicons, good's coverage of all samples was >0.996. The Alpha diversity richness (Chao1), and diversity estimators (Shannon and Simpson) by rumen bacteria and archaea populations were similar among treatments (Table 5).

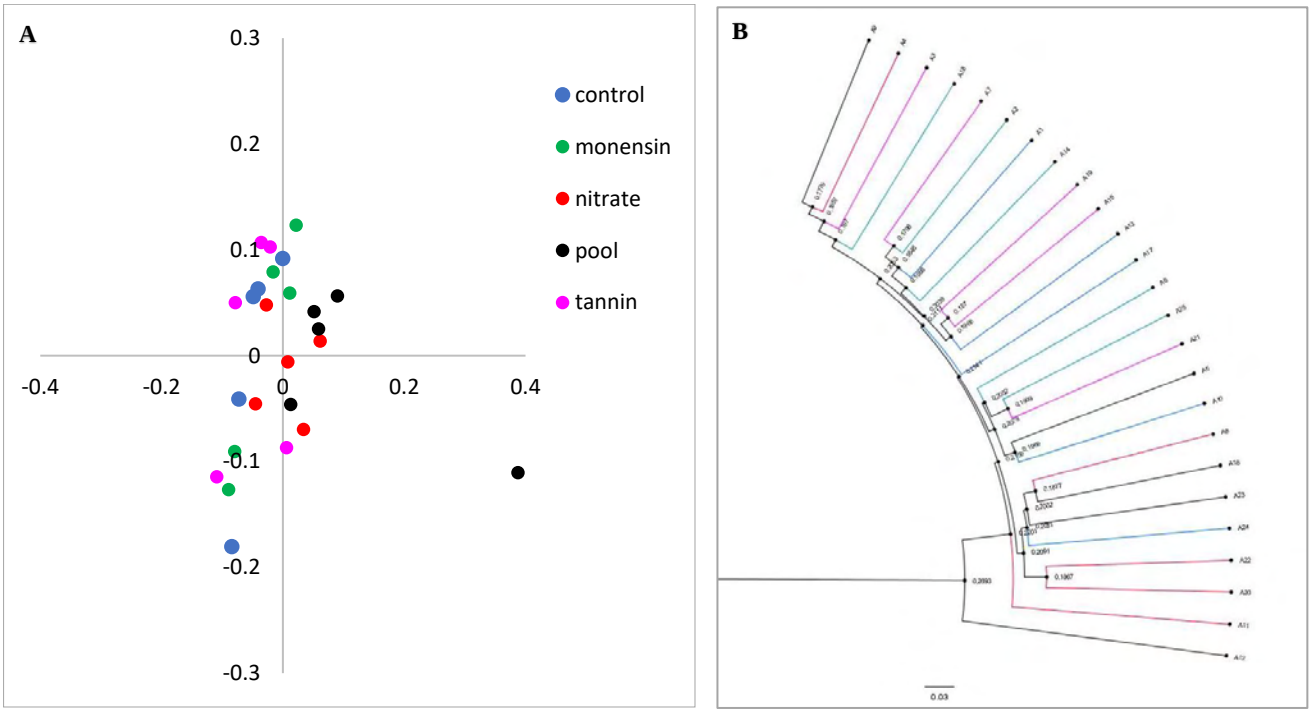
Table 5. Rumen alpha diversity of Nellore cows fed with monensin, tannins of *A. mearnsii*, Calcium Nitrate and Pool.

α Diversity	Treatments					SEM	P-*value*
	Control	Monensin	Tannin	Nitrate	Pool		
Bacterial							
G. Coverage	0.99	0.99	0.99	0.99	0.99	0.0005	0.1425
Chao1	63.9	60.4	58.1	61.5	62.5	2.344	0.4815
Shannon	2.31	2.45	2.52	2.46	2.40	0.0549	0.0582
Archaeal							
G. Coverage	0.99	0.99	0.99	0.99	0.99	0.0005	0.1425
Chao1	6,46	6,86	4,60	5,00	5,65	0,9820	0,4737
Shannon	1,26	1,07	1,12	1,42	1,18	0,1190	0,2967

SEM: standard error of mean; G. Coverage: Good's Coverage.

The principal coordinate analysis (PCoA) of overall diversity, showed that the microbial membership in each of the five treatment groups (all-against-all comparison) are significantly different from each other (AMOVA.J_class, $P < 0.005$). In addition, this analysis showed that the pool treatment had different bacteria community when individually compared with control ($P = 0.01$), monensin ($P = 0.01$) and tannin treatments ($P = 0.0005$) but similarity of bacteria community was observed between pool vs. nitrate treatments ($P = 0.09$) (Figure 2).

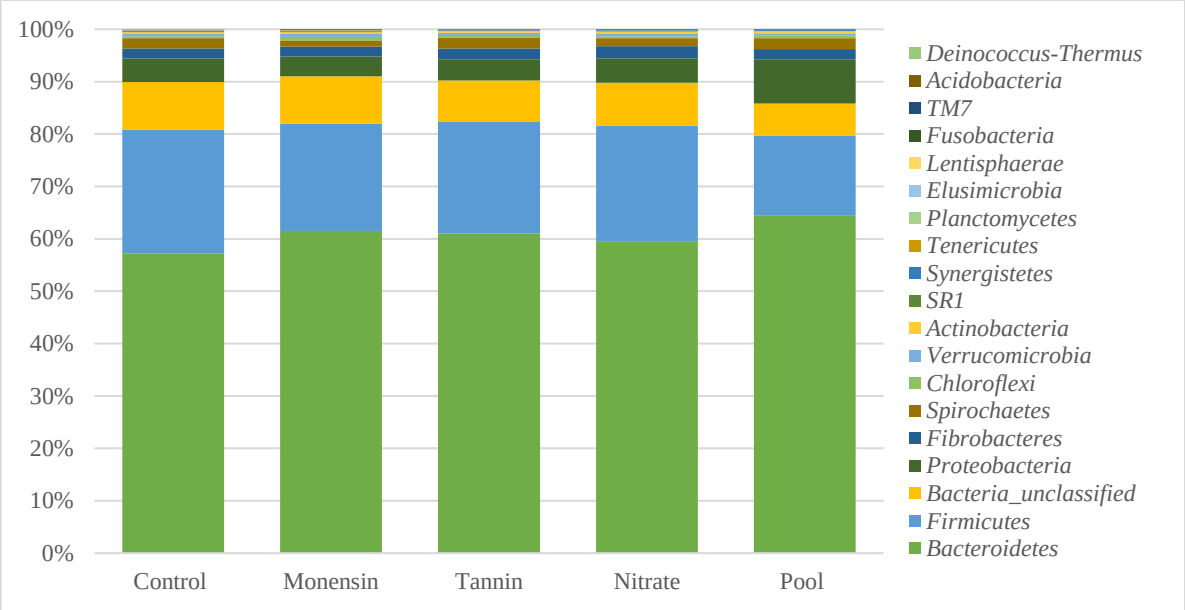
Figure 2. PCoA analyses based on membership evaluation among treatment groups. B. Dendrogram based on parsimony analyses using J_class.



(Source: Own authorship)

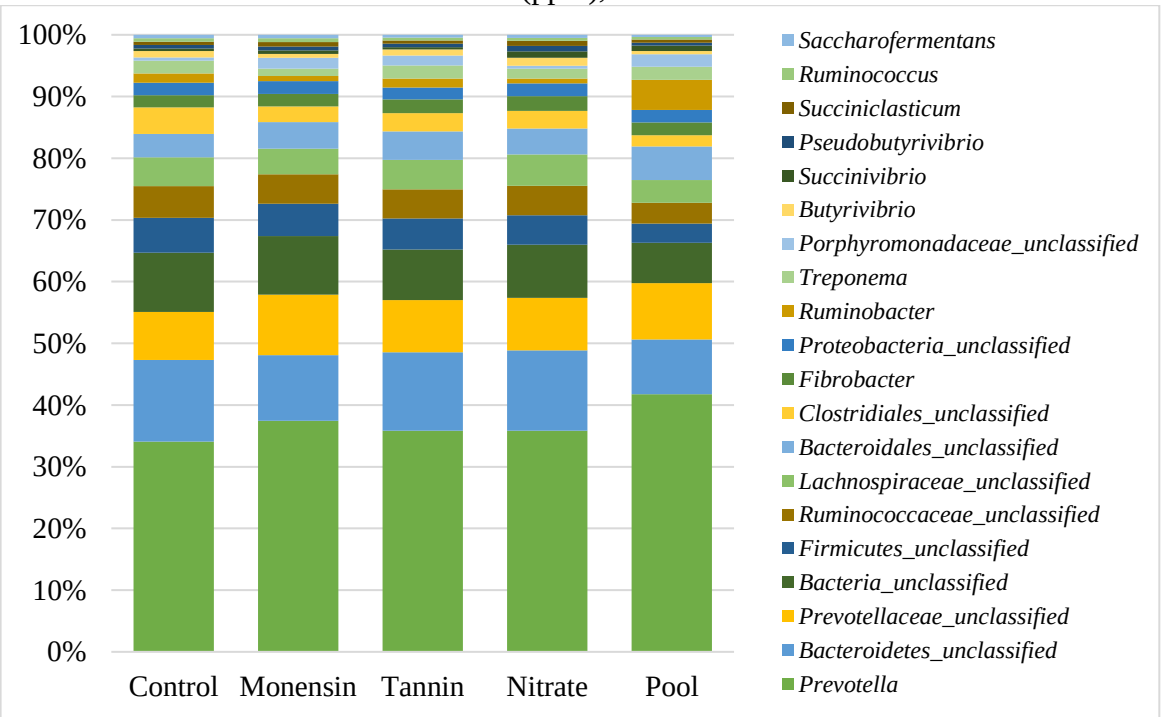
Further analysis showed that 91.87% of all sequences allowed classification into 19 different phyla (Figure 3). Most of the sequences belonged to the phyla Bacteroidetes (60.5%), Firmicutes (20.6%) and Proteobacteria (5.2%). At genera level, most highly included the *Prevotella* (35.05%), *Bacteroidetes unclassifi*ed (11.07%), *Prevotellacea unclassified* (8.22%), *Firmicutes unclassified* (4.56%) *Ruminococcaceae unclassified* (4.30%), *Lachnospiraceae unclassified* (4.27%) and *Bacteroidales unclassified* (4.17%) (Figure 4).

Figure 3. Relative abundance of the phyla of ruminal bacterial communities observed in Nellore cows fed monensin (ppm), tannins of *A. mearnsii* and calcium nitrate.



(Source: Own authorship)

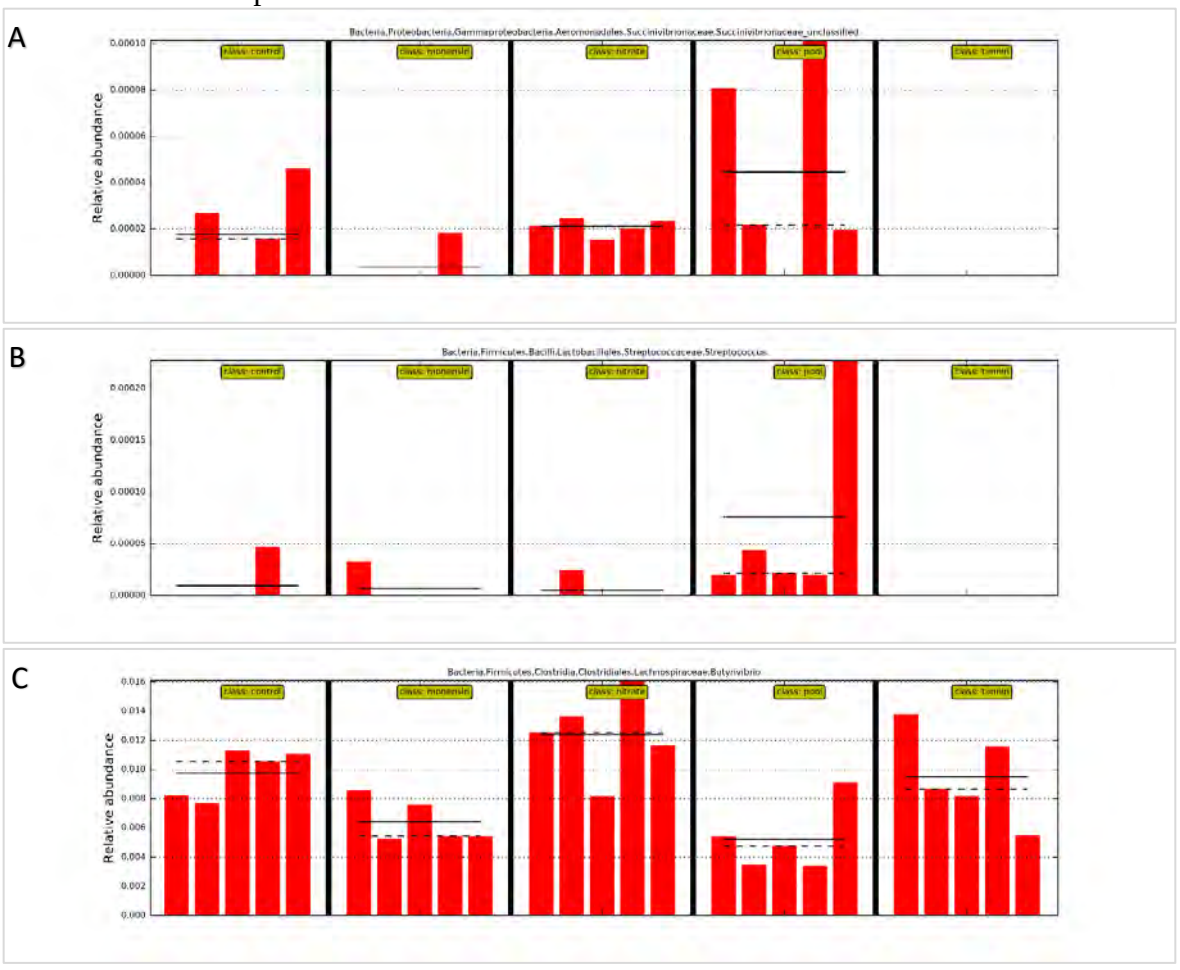
Figure 4. Relative abundance of the top twenty genera of ruminal bacterial communities observed in Nellore cows fed monensin (ppm), tannins of *A. mearnsii* and calcium nitrate.



(Source: Own authorship)

To identify differentially abundant taxa that were mostly affected by treatment with pool, we compared the rumen microbial population of both treatments using LEfSe (OGUNADE et al., 2018). Using LEfSe, the relative abundance of Succinivibrionaceae family and *Streptococcus* genus was enriched in Nellore fed with pool (Figures 5 and 6). Furthermore, using LEfSe to evaluate the treatments effects individually, the tannin increased *Clostridium_VI* genera; nitrate increased *SR1* phylum and *Butyrivibrio* genera; and Monensin increased the *Sinergistetes* phylum (Figure 6).

Figure 5. Relative abundances of (A) Succinivibrionaceae family and, (B) Streptococcus and (C) Butyrivibrio genera of rumen microbiota from Nellore cows fed with monensin, tannins, calcium nitrate and pool.



(Source: own authorship)

Figure 6. Figure 6. A. Linear discriminant analysis effect size (LEfSe) of rumen microbiota of Nellore cows fed with monensin, tannins, calcium nitrate and pool. (A) The LEfSe plot indicates the most differentially abundant taxa found by ranking according to their effect size (≥2.0) at the genus level and (B) and (B) the corresponding taxonomic cladogram obtained by treatment groups.

(Source: own authorship)

3.4.3. Products of rumen fermentation

Neither the concentration nor production (balance) of rumen ammonia (NH_3-N) were modified by the additive's inclusions (Table 6). However, a negative balance (i.e. it was a consumption, instead of production) of NH_3-N was detected for the control and tannin group. Regardless of treatment, it was observed that sampling time affected concentration of rumen NH_3-N in both non- incubated and incubated flasks.

Table 6. Concentration and balance of rumen NH_3-N of Nellore cows fed monensin, tannins of *A. mearnsii*, Calcium Nitrate and Pool.

Variables	Treatments					SEM	P Value	
	Control	Monensin	Tannin	Nitrate	Pool		Additive	Time
Concentration								
0 min (mg/dL)	13.79	15.01	14.64	15.84	11.95	1.568	0.1545	<0001
30 min(mg/dL)	13.70	15.99	14.31	16.37	12.95	1.739	0.4994	<0001
Balance(mg/dL)	-0.18	1.96	-0.66	1.05	1.92	1.958	0.2996	0.0847

SEM: standard error of mean; balance = (30 min – 0 min) x 2.

The production of CH_4 was decreased by the additive inclusions, as well as the GE and the relative energy loss of methane in relation to the other rumen fermentation products. When compared to the control, monensin reduced CH_4 production by 9.5%, tannins reduced by 19%, nitrate by 20%, and when combined in the pool treatment, additives showed a synergistic effect decreasing CH_4 production by 28.8% (Table 7). However, there was no change on the production of acetic acid, propionic acid, butyric acid and total SCFA.

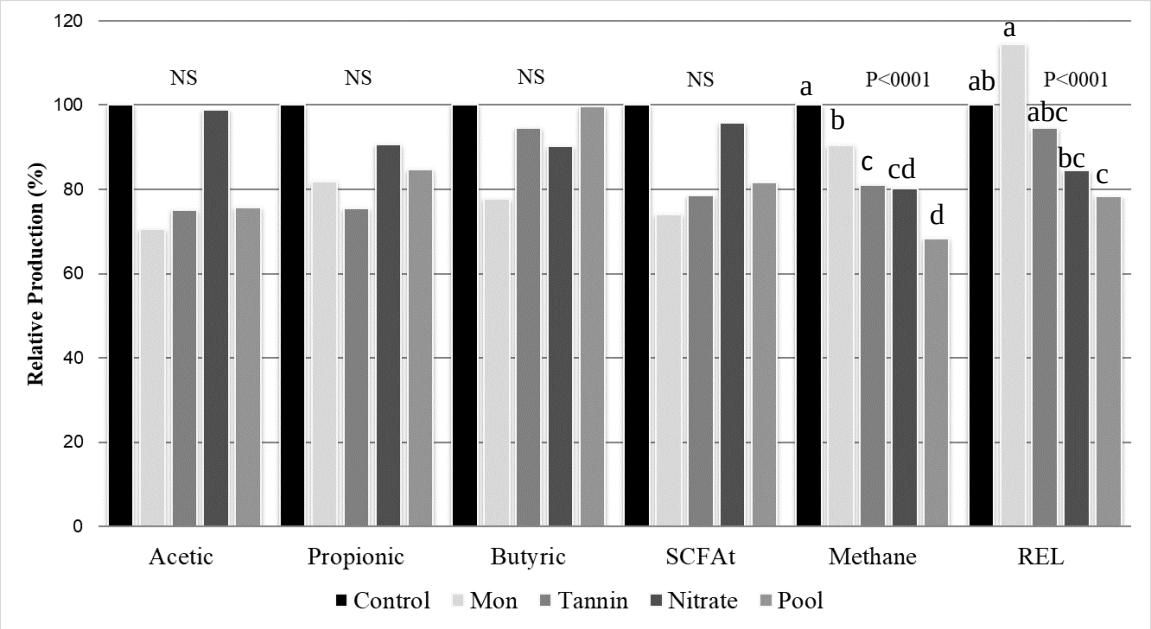
On the other hand, the molar concentration (mmol/L) of acetic acid before and after incubation time (0 and 30 min) was increased by the nitrate treatment when compared to the control group. Furthermore, an increasing of butyric acid molar concentration was found, promoted by the tannin inclusion, differing from the control and other treatments. Thus, the total SCFA molar concentrations were found for tannin and nitrate treatments in comparison to the control. In contrast, monensin decreased total SCFA molar concentration when compared to the control. Overall, the pool treatment reduced the REL%. In the other words, this reduction is related to the lower methane production observed in cows fed with pool (Figure 7).

Table 7.SCFA and CH_4 production as well REL of Nellore cows fed monensin, tannins of *A. mearnsii*, Calcium Nitrate and Pool.

Variables	Treatments					SEM	*P value*	
	Control	Monensin	Tannin	Nitrate	Pool		Additive	Time
Acetic acid								
0 min (mmol/L)	72.14^{b}	69.42^{b}	76.37^{ab}	81.13^{a}	76.90^{ab}	2.411	0.0016	<0001
30 min (mmol/L)	77.42^{b}	73.55^{b}	80.96^{ab}	87.15^{a}	81.70^{ab}	2.638	0.0003	<0001
Difference (mmol/L)	5.22	4.01	4.53	5.99	5.17	0.630	0.0194	0.9509
Production(mol/kg.d)	3.76	2.80	2.82	3.71	2.99	0.465	0.2770	0.8639
Production(g/kg.d)	225.66	168.39	169.35	223.03	179.85	27.92	0.2781	0.8648
GE (kcal/kg.d)	787.5	556.2	591.0	778.3	623.3	98.52	0.2435	0.8772
Propionic acid								
0 min (mmol/L)	19.84	19.55	20.98	19.95	19.94	1.194	0.5312	<0001
30 min (mmol/L)	21.57	21.11	22.52	21.77	21.68	1.394	0.8091	<0001
Difference (mmol/L)	1.73	1.55	1.51	1.80	1.79	0.257	0.6790	0.1893
Production(mol/kg.d)	1.22	1.05	0.92	1.10	1.14	0.156	0.4014	0.1580
Production(g/kg.d)	90.46	77.79	68.24	82.01	84.48	11.59	0.3966	0.1562
GE (kcal/kg.d)	450.5	368.8	339.8	408.4	428.3	61.53	0.3246	0.1480
Butyric acid								
0 min (mmol/L)	12.26^{b}	12.13^{b}	15.30^{a}	11.93^{b}	11.27^{b}	1.092	0.0082	0.0248
30 min (mmol/L)	13.73^{b}	13.36^{b}	16.87^{a}	13.42^{b}	12.96^{b}	1.136	0.0075	<0001
Difference (mmol/L)	1.46	1.23	1.58	1.50	1.56	0.233	0.6203	0.1653
Production(mol/kg.d)	1.02	0.82	0.96	0.92	1.02	0.140	0.7031	0.2425
Production(g/kg.d)	89.91	72.19	85.08	81.15	90.69	12.39	0.6988	0.2460
GE (kcal/kg.d)	535.9	416.3	507.1	483.6	538.4	74.73	0.5993	0.3180
Total SCFA								
0 min (mmol/L)	104.3^{bc}	101.2^{c}	112.7^{a}	113.0^{a}	109.3^{ab}	3.767	0.0042	<0001
30 min (mmol/L)	112.8^{b}	108.1^{c}	120.4^{a}	122.4^{a}	117.7^{ab}	4.369	0.0039	<0001
Difference (mmol/L)	8.51	6.88	7.72	9.39	7.72	0.971	0.1928	0.8859
Production(mol/kg.d)	6.00	4.69	4.81	5.74	5.26	0.629	0.2257	0.8825
Production(g/kg.d)	406.1	319.4	327.9	386.2	360.8	41.52	0.2547	0.7640
GE (kcal/kg.d)	1774.0	1341.5	1437.9	1670.5	1571.3	194.0	0.4141	0.7617
Acetate:Propionate	3.68	3.68	3.69	4.11	4.01	0.189	0.0766	0.0011
Methane								
0 min (mmol/L)	0.03^{a}	0.02^{b}	0.03^{ab}	0.01^{c}	0.01^{c}	0.001	<0001	<0001
30 min (mmol/L)	0.11^{a}	0.10^{b}	0.10^{bc}	0.09^{cd}	0.08^{d}	0.008	<0001	0.0011
Difference (mmol/L)	0.08^{a}	0.08^{ab}	0.07^{b}	0.07^{c}	0.06^{c}	0.007	0.0004	<0001
Production(mol/kg.d)	2.20^{a}	1.98^{b}	1.78^{c}	1.76^{cd}	1.58^{d}	0.139	<0001	<0001
Production(g/kg.d)	35.17^{a}	31.79^{b}	28.57^{c}	28.19^{cd}	25.40^{d}	2.225	<0001	<0001
GE (kcal/kg.d)	462.8^{a}	418.9^{b}	375.3^{c}	371.1^{cd}	334.3^{d}	27.89	<0001	<0001
REL (%)	25.07^{ab}	28.21^{a}	24.01^{abc}	21.16^{bc}	18.65^{c}	2.449	<0001	<0001

SEM: standard error of mean; GE: gross energy; SCFA: short chain fat acids; REL: relative energy loss of methane in relation to the other rumen fermentation products; abcd: different letters on the same line differ significantly by the protected LSD test at 5% of significance level.

Figure 7. Graph representing the changes caused by the additives on the tSCFA and CH_4 production as well as REL in relation to control group.



(Source: Own authorship)

3.4.4. Energy partitioning

The consumption of tannin increased the GEI 23% in comparison to the other treatment groups, as is demonstrated on Table 8 and Figure 8. The energy released from the CH_4 (Mcal/cow) was increased 16% by the monensin and decreased 33% by the pool, when compared to other groups. Furthermore, the amount of gross energy (GE) released in form of CH_4 was decreased in 14.5%, 23%, and 27% by the tannin, nitrate, and pool treatments; only monensin did not differ to the control. In addition, the percentage of digestive energy (DE) was decreased 14.7% by tannin, 23% by nitrate and 26% by pool, comparing to the control group.

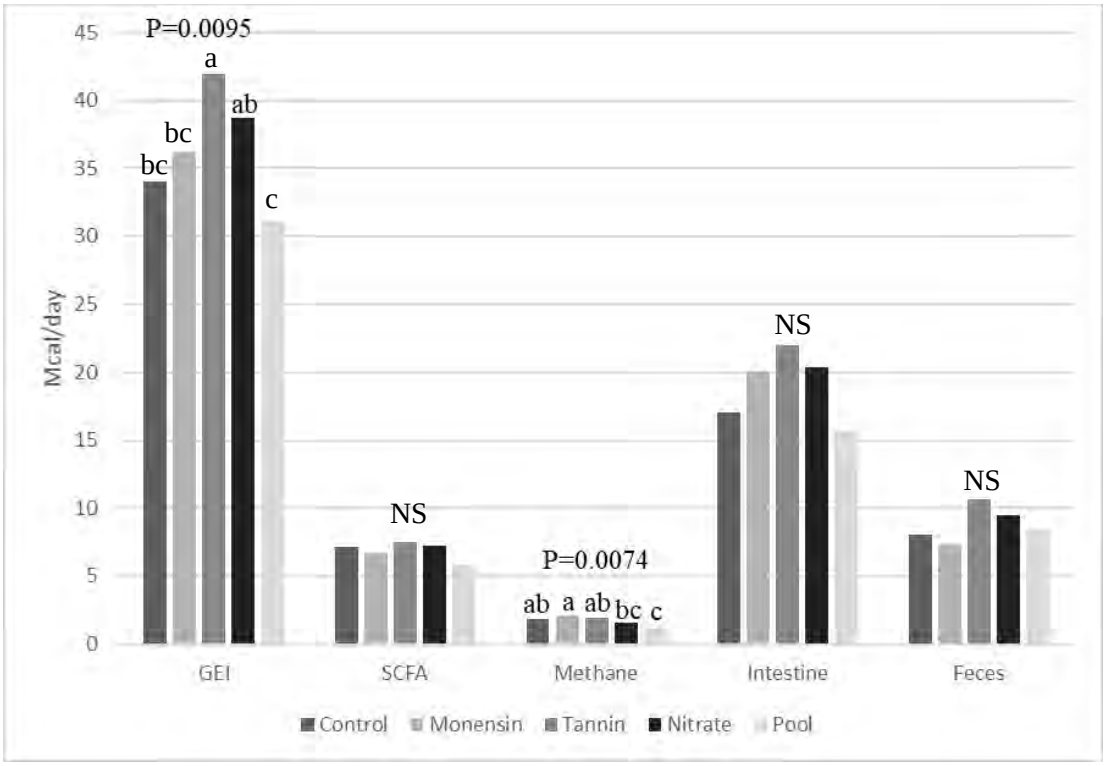
There were no significant effects on rumen mass solids, acetic, propionic, and butyric acids, as well to total SCFA, fermentation heat, energy released in the intestine and energy released in the feces.

Table 8.Estimation of energy released into the gastrointestinal tract of Nellore cows fed monensin, tannins of *A. mearnsii*, Calcium Nitrate and Pool.

Variables, day	Treatments					SEM	P-value
	Control	Monensin	Tannin	Nitrate	Pool		
Rumen mass, kg	2.60	1.95	2.08	2.13	2.25	0.363	0.6864
GEI, Mcal	34.06^{bc}	36.23^{bc}	41.98^{a}	38.70^{ab}	30.98^{c}	2.211	0.0095
Energy released into the rumen							
Acetic acid							
Mcal/cow	3.16	2.76	3.11	3.32	2.38	0.475	0.6495
GE, %	9.53	7.69	7.39	8.67	7.91	1.402	0.6521
DE, %	13.17	9.61	9.98	11.52	10.60	1.957	0.5199
Propionic acid							
Mcal/cow	1.79	1.83	1.73	1.75	1.49	0.266	0.9120
GE, %	5.40	4.92	4.11	4.51	4.68	0.638	0.6756
DE, %	7.45	6.17	5.64	5.98	6.38	0.987	0.7488
Butyric acid							
Mcal/cow	2.22	2.09	2.62	2.17	2.07	0.436	0.8574
GE, %	6.71	5.67	6.18	5.40	6.47	1.039	0.8424
DE, %	9.46	7.09	8.60	6.99	9.06	1.669	0.6758
Total SCFA							
Mcal/cow	7.17	6.69	7.46	7.25	5.88	1.042	0.8452
GE, %	21.64	18.29	17.67	18.59	18.79	2.699	0.7941
DE, %	30.09	22.87	24.22	24.51	25.69	4.169	0.7119
Fermentation heat							
Mcal/cow							
GE, %	23.90	19.98	24.70	24.90	26.04	4.517	0.4897
DE, %	1.87	1.56	1.53	1.62	1.65	0.238	0.7979
Methane							
Mcal/cow	1.80^{ab}	2.10^{a}	1.91^{ab}	1.58^{bc}	1.20^{c}	0.136	0.0074
GE, %	5.32^{a}	5.77^{a}	4.55^{b}	4.08^{b}	3.87^{b}	0.255	0.0005
DE, %	7.18^{a}	7.22^{a}	6.12^{b}	5.53^{b}	5.31^{b}	0.429	0.0053
Mcal/kg DM	0.24^{a}	0.26^{a}	0.21^{b}	0.18^{b}	0.17^{b}	0.0119	0.0005
Energy release in the intestine							
Mcal/cow	16.41	19.51	21.34	19.51	14.58	2.292	0.0577
GE, %	47.02	54.14	51.29	50.56	48.72	5.598	0.7419
DE, %	60.12	67.94	67.56	67.82	66.71	4.877	0.6746
Energy release in feces							
Mcal/cow	8.05	7.35	10.62	9.50	8.45	1.799	0.3335
GE, %	24.14	20.22	24.94	25.13	26.17	4.506	0.4797

SEM: standard error of mean; GEI: gross energy intake; GE: gross energy; DE: digestible energy; abc: different letters on the same line differ significantly by the protected LSD test at 5% of significance level.

Figure 8.Graph representing the gross energy intake and energy released in the gastrointestinal tract (Mcal/day) of Nellore cows fed with monensin, tannins of *A. mearnsii* and calcium nitrate.



(Source: Own authorship)

3.5.DISCUSSION

3.5.1. Feed intake and rumen pH

Surprisingly, in the present study, animals that received the tannin treatment showed a 23% increase in DMI, when compared to the control group. This effect is rarely reported in the literature, where, normally, the addition of tannins in cattle diets reduces the DMI, as reported in a meta-analysis by Jayanegara and Palupi (2010), corroborating to Aguerre et al. (2016) and Grainger et al. (2009). In contrast, Beauchemin et al. (2007) did not obtain effects on intake by using quebracho tannin extract in quantities of 0%, 1% and 2% of diet DM. Perna Junior et al. (2017), also did not obtain effects on DMI on cows fed *A. mearnsii* on a 0.6% inclusion. This difference on DMI may be explained by the fact that the effect of tannins may depend on the type of tannins consumed, the chemical structure and molecular weight, as well as by the amount ingested and the animal species involved (MAKKAR, 2003a; FRUTOS et al., 2004 and

MUELLER-HARVEY, 2006). Thus, the present study reports an unprecedented result in relation to the condensed tannins of *Acacia mearnsii*.

Monensin is well reported to reduce feed intake, increase or maintain weight gain, and increase feed efficiency in animals fed high concentrate diets. In contrast with previous research, in the present study, we did not observe a DMI reduction in animals fed with monensin, wherein the feed intake was similar to control group. Recently, this effect was reported by Perna Junior et al. (2017) and Tseu et al. (2020). Furthermore, in the present study, cattle receiving nitrate in the diet had no effect on DMI, in contrast to Newbold et al. (2014), Guyader et al. (2015) and Cassiano (2017).

The present study did not decrease DMI when the additives were supplied individually; however, cows fed with the pool (monensin, tannin and nitrate) treatment had a DMI reduction of 9%. This result agrees with previous study when the additives were use individually (DUFFIELD et al. 2012; AGUERRE et al. 2016; NEWBOLD et al. 2014).

The change in ruminal pH may explain the reduction of DMI of cow fed with pool treatment. According to Penner et al. (2007), a pH of 5.8 indicates the threshold for cases of subacute rumen acidosis and pH's of 6.0 and 6.2 are thresholds indicative of healthy rumen conditions, favoring great cellulolytic activity (PENNER; BEAUCHEMIN, 2010). The rumen pH of cows fed with pool treatment had shown (Table 3) a lower value of medium daily pH (5.36) and an increase of time and area of ruminal pH bellow 5.8. This value is close to clinical ruminal acidosis, which occurs when excessive levels of organic acids accumulate in the rumen, resulting in a rumen fluid pH of less than 5.2 (NAGARAJA; LECHTENBERG, 2007; OWENS, 1998).

Furthermore, dietary factors can have significant impacts on intake patterns and, therefore, on the incidence of acidosis. Acidosis results when cattle consume fermentable carbohydrates in amounts sufficient to cause a non-physiologic accumulation of organic acids in the rumen, with a concurrent reduction in pH (NAGARAJA; TITGEMEYER, 2007). Organic acids are the products of microbial fermentation of feedstuffs. In a normal situation with normal intake, organic acids do not accumulate in the rumen because ruminal absorption keeps up with production. In addition, ruminal pH impacts microbial populations and fermentation products, and the physiologic functions of the rumen, mainly motility and the absorptive function (NAGARAJA; LECHTENBERG, 2007).

3.5.2. Rumen fermentation and microbiota

According to Lee and Beauchemin (2014), some feed additives (i.e monensin) reduce enteric methane emissions in the short-term but lose effectiveness in the long-term because of microbial adaptations to the compounds. In agreement, the animals fed with monensin in this study, presented a lower reduction of enteric methane, only 9% less than the group without additives (control).

The mechanism of action of ionophore additives, such as monensin, is described as inhibiting Gram-positive bacteria, in detriment to Gram-negative bacteria, not acting directly on the archaeae, but rather reducing the substrate for the formation of CH_4 (H_2 and CO_2). Associated with this, it is reported that monensin also causes a reduction in acetic acid and an increase in propionic acid (RUSSELL; HOULIHAN, 2003; AZZAZ et al., 2015). As matter of fact, this effect was not observed in the current study; despite monensin treatment reduced a total SCFA of either molar concentration and daily production.

A total of CH_4 production of cows fed with tannins was decreased in 19% which was similar to the previous results (25%) observed by Tseu (2019), but slightly lower (30%) than those described by Perna Junior (2018). Although, the CH_4 production was decreased, no impact on the production of SCFA, but an increasing of molar concentration of butyrate and total SCFA was observed in the present study. This result agrees with the study of Dickhoefer et al. (2016) who observed that increasing the tannin content resulting in the molar concentration of propionate and butyrate increased, while those of acetate reduced. Furthermore, Dschaak et al. (2011), using CT extract, showed an increase in the molar ratio of acetate, propionate and butyrate when the diet contained a higher proportion of forage, but did not increase when the forage was lower.

Another positive result from supplementation with tannins was the maintenance of N metabolism, and it is reported in the literature that tannins can bind proteins, rendering them inaccessible to rumen degradation and favoring post-rumen release (NIGRANT et al., 2017), which can have negative effects on the bacterial community and reduce the feed degradation. Although the reductions of rumen ammonia concentration and the balance were not significant, it may be seen in table 6 that the inclusion of tannins produced a negative balance (-66 mg/dL.h). This may be an indication that during the 30 minutes of incubation the inhibition of proteolytic

activity by tannins was accentuated in a way that the use of ammonia for microbial protein synthesis was greater than the production. This was in agreement with similar results found by Tseu (2018) with the inclusion of 2.25% of tannins.

Tannins are also attributed with the ability to reduce the count of number of protozoa in the rumen. One of the mechanisms tannins additive use to reduce CH_4 production is related to the closely association that has been described of ciliate protozoa and archaea. Lower the protozoa, lower the archaea (PATRA; SAXENA, 2011; FINLAY et al., 1994); one explanation of this directly proportional relation is that archaea may adhere to the protozoa cell surface. In this current study, it was not observed a lower count number of protozoa when cows were fed with tannins as similar to Tseu (2019) and Benchaar et al. (2008), neither a reduction of archaea was observed. In summary, the reduction of CH_4 cannot be explained by this pathway (i.e. lower protozoa, lower archaea, lower CH_4).

In agreement with previous studies, nitrate caused a decrease of 20% in daily methane emissions; with the size of the decrease (20– 22%) consistent with that reported for Nolan et al. (2010) and van Zijderveld (2011). In addition, molar concentration of acetic acid was increased by nitrate inclusion. This effect may be explained by the fact that nitrate reduction can compete with propionigenesis for reducing equivalents (UNGERFELD; KOHN, 2006), as it is thermodynamically more favorable, diverting glucose fermentation away from the propionigenesis into acetate.

Butyrivibrio was the most abundant genus in the Firmicutes phylum and was significantly increased in the nitrate treatment when compared to the control and other treatments (Figure 5, C). According to Kelly et al., (2010) the *Butyrivibrio* species contribute to fiber digestion of the animals due to their ability to degrade hemicelluloses (WILLIAMS; WITHERS,1992) and are also involved in protein breakdown and the biohydrogenation of fatty acids (McKAIN et al.,2010; MAIA et al., 2007). A result from a recent study suggested that *Butyrivibrio* species can degraded a variety of substrates using glucose and H_2 for the formation of butyrate (PALEVICH et al., 2019). However, in the present study, it was not observed an increase of butyrate from cows fed with nitrate, although this effect was found in the tannin treatment.

Cows fed with the pool treatment had better performance in comparison to those cows from other individual use of additives. The most striking effect is the reduction of almost 30%

in the production (g / kg / day) of enteric CH_4 in animals receiving pool treatment, which can indicate synergism among additives, in accordance with the proposed hypothesis. The observed results of enteric CH_4 reduction from cows fed with pool treatment are more similar to those fed with tannins and nitrates, with the marked reduction in methanogenesis, maintenance of N metabolism.

Regarding the ruminal microbiota, no effects were found on the total protozoan count when cows were fed with the pool in comparison to the control ones. Furthermore, the structure of members using PCoA of the bacterial community of the rumen of cows fed with the pool had differences when compared to cows fed with monensin and tannin, but the bacterial community observed in the pool group was similar to those of cows fed with nitrate ($P = 0.091$).

In addition, the LefSe analysis showed an increasing in the *Streptococcus* genera in the pool treatment. The most representative specie of these genera is the *Streptococcus bovis*, a largely bacteria found in the rumens of animals adapted to a high-grain diet (RICKE et al., 1996). Currently, when rumen pH is above to 6.0, *S. bovis* produces formate, acetate, and ethanol (CHEN et al., 2016). However, when pH decreases below 5.8, *S. bovis* produces lactate. Due to that, rumen acidosis is associated with an initial overgrowth of *S. bovis* (WANG et al., 2015). As previously reported, in the present study, the ruminal pH of animals receiving the pool treatment, remained below 5.8 for a longer time, but even so the production of SCFA was not impaired, an effect that may explained by the increase in *Streptococcus*.

3.5.3. Energy losses

In the present study, it was possible to estimate the energy release in the digestive tract through the *ex-situ* technique. However, this is a subject poorly addressed by several researchers; therefore, little data is available in the literature. As mentioned before to DMI, cows fed with tannins increased 23% of GEI. This was different in relation to previous results that observed a linear reduction of GEI from cows fed with different levels of tannins (0% to 2.25% of DM) (TSEU, 2019). Interestingly, other results had shown that different levels of tannins inclusion (0% to 1.5% of DM) did not affect the GEI (PERNA JUNIOR, 2018).

The average of GE loss in form of CH_4 was about 4.7% for all five treatments. Cows fed with treatments tannin, nitrate, and pool had GE loss of 4.55, 4.08 and 3.87, respectively (Table 7). The data in the present study agrees with Johnson and Johnson (1995), Goel and

Makkar (2012) and Wanapat et al. (2015) who stated that the production of CH_4 by the enteric fermentation of ruminants generates feed GE losses ranging from 2 to 12%. In addition, the hypotheses of the study were confirmed, since the treatment pool had the better result, decreasing 27% of GE losses from CH_4; against an increasing of 8% of monensin and decreasing of 14.5% and 23% of tannin and nitrate, respectively, when used separately.

Surprisingly, neither an increase nor a decrease was found in the energy release in the intestine and in the feces, in contrast to earlier findings from Perna Junior (2018) and Tseu (2019). However, this may indicate that energy recovered from CH_4 was used in another physiologic function from the cows.

In previous studies, when using monensin in combination with tannins from *Acacia mearnsii*, Tseu (2019) concluded that both additives act separately, with no synergy action. However, there are no previous studies that evaluate the effects of calcium nitrate with monensin and tannins on the energy partition in ruminants. Thus, not understanding how nitrate interacts separately with both additives, we can assume that there is an antagonistic effect, since in the present study the administration of the three additives in the feeding of Nellore cows resulted in a reduction in GEI and a reduction in the efficiency of use of the energy.

3.6.CONCLUSION

This study has demonstrated that significant decreases in rumen methane emissions can be achieved without drastic effects on either the rumen microbial population or its function.

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4. POTENTIAL OF BIOGAS PRODUCTION AND BIOFERTILIZER FROM WASTE OF CATTLE FED WITH MONENSIN, TANNINS OF *Acacia mearnsii* AND CALCIUM NITRATE

Abstract: The present study aimed to evaluate the potential of biogas production and biofertilizer quality from waste of Nellore cows fed with monensin, tannins of *A. mearnsii*, calcium nitrate and a pool (combination of the doses of the three before mentioned additives) as an alternative to animal waste management and renewable energy production. The study was carried out in two steps, the feeding, and the anaerobic digestion. In the feeding (step1), five Nellore cows, with a mean body weight of 530±75 kg, were distributed into a 5 x 5 Latin square and fed with a basal diet, concentrate:roughage (corn silage) ratio of 60:40, supplied by monensin (300 mg in the diet), tannin (1.5% of DM), nitrate (3.0% of DM), pool and a control diet with no additives inclusions. In the anaerobic digestion (step2), feces and urine were then collected from the cows to supply the biodigesters. Biodigesters were arranged in a completely randomized design of 5 treatments with 5 replicates and totaling 25 experimental units (feces were the main variable from cows fed with the individual or combined use of all additives). The data were analyzed by using the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). The biodigesters supplied with waste from cattle fed tannins increased in 91% of CH_4 production and 52% of CO_2 production per gram of volatile solids. The pool treatment increased the biofertilizer nutrient compounds in 12.6% of Total C and 35% of Total N, monensin presented an increase of 7.5% of Total N, and K was increased in 35% to tannins and 22% to nitrate. The monensin did not present the potential for CH_4 production in relation to the waste in the environment. Interestingly, the tannins showed significant results through anaerobic digestion, being its use recommended with the attempt to reduce the impacts of untreated manure on the environment. More studies are necessary to understand how nitrate acts on biodigesters.

Keywords: Batch biodigesters. Greenhouse gases. Bovine.

4.1.INTRODUCTION

Since the last decades, livestock production is being associated with potential environmental impacts that contribute to global warming, depletion of water resources, soil erosion and habitat impairment (FAO STATISTICAL YEARBOOK, 2013). The main factor in animal production is associated to Greenhouse Gas (GHG) emissions, as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Knowing that Brazil has a cattle herd with 214.9 million heads (IBGE, 2018), and the main emissions within the farms include enteric CH_4 from bovines, CH_4 and N_2O from housing facilities during long-term storage and during field application, and N_2O from nitrification and denitrification process in the soil where feed crops are produced as well as pasture (ROTZ, 2017; SORDI et al., 2013). In addition, manure as either organic fertilizer on cropland or deposited on pasture, is a source of GHG emissions as well, and emissions have grown 1.1% per year, on average, from 1961 to 2010 (IPCC, 2014).

Given these factors, an alternative is treating the waste (manure and urine) by anaerobic digestion, via biodigesters, which, in addition to support environmental protection, makes it possible to obtain two products: the biogas, being used as an energy source, and the biofertilizer (ACHINAS et al, 2018). The biogas is mainly comprised of CH_4 and CO_2, but also minor amounts of other gases, as nitrogen, hydrogen sulfide, ammonia, and water vapor (NESHAT et al., 2017) and, according to Santos (2001), biofertilizer is the final product form the liquid effluent obtained after the biodigestion ended.

This implementation brings significant economic and environmental benefits (SCAARLAT et al, 2018) by being an efficient alternative technology that combines biofuel production with sustainable waste management (ACHINAS et al, 2018).

However, the diet is the most important factor to determining differences in CH_4 emissions from manure (GONZÁLEZ-AVALOS; RUIZ-SUÁREZ, 2011; ORRICO JÚNIOR et al. 2011). Feed additives have been used in ruminant nutrition to promote feed efficiency in addition to decrease enteric CH_4 emissions. Monensin, tannins and calcium nitrate have shown, separately, the potential to reduce CH_4 from the rumen fermentation (RUSSELL; HOULIHAN, 2003; FINLAY et al., 1994; NOLAN et al., 2010), but the effects after digestion are not known and how these additives may affect biogas production.

Given the context presented, the hypothesis tested in this study was that the bovine manure from animals receiving the same basal diet with an inclusion of monensin, tannins of black wattle tree (*A. mearnsii*) and calcium nitrate used individually, or associate would decrease the production of CH_4, CO_2 and N_2O. This study's aim was to evaluate the administration of different additives from waste of Nellore cows could cause changes in the biodigestion of the manure, the production of biogas and on the chemical characteristics of biofertilizers.

4.2.MATERIAL AND METHODS

4.2.1. Place of experimentation, ethical issue, and animals

The experiment was conducted at the Animal Nutrition and Production Department (VNP) of the College of Veterinary Medicine and Animal Science (FMVZ) of the University of Sao Paulo (USP), Fernando Costa *Campus* in Pirassununga City, Brazil. Following 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) of the USP, under the protocol number CEUA 4431120419.

4.2.2. Experimental design and treatments

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

4.2.3. Feeding phase

Five Nellore cows, non-pregnant and non-lactating, mean BW of 530±75 kg, carrying rumen canula were kept in a barn in individual pens with free accesses to water, individual feed bunks and sand bedding. The barn owned suspended fans that were automatically triggered when the temperature exceeded 28°C to avoid heat stress.

Animals were arranged in a 5x5 Latin Square in which the unit experimental was the animal within each experimental period (n = 25). Was offered a basal diet, with a concentrate: roughage (corn silage) ratio of 60:40, differing by the additive's inclusions totaling five treatments. These were: Control without any additive, Monensin including 300 mg (about 32

mg/kg DM) of sodium monensin (Rumensin® 200, Elanco Animal Health, Brazil), Tannin 1.5% of *Acacia mearnsii* extract on DM, Nitrate 3.0% of calcium nitrate on DM and Pool a blend of all three additives doses.

The tannins were from commercial extract obtained from the bark of the Black Wattle tree (*Acacia mearnsii*) (Seta Natur – Seta Acacia Tannin Extract). The concentration of total phenols (84.4% of extract) was determined by the Folin-Ciocalteau method (MAKKAR, 2003) and total tannins (82.3% tannic acid equivalent) were estimated by the difference in total phenol concentration before and after treatment with insoluble polyvinylpolypyrrolidone (MAKKAR et al., 1993). The concentration of condensed tannins (32.3% leucocyanidine equivalent) was determined by the HCl-butanol method (MAKKAR, 2003).

The calcium nitrate source was the commercial agricultural fertilizer YaraTera™ CALCINIT (Yara Brazil). Determination of nitrate ($N\text{-}NO^{3-}$), ammonio ($N\text{-}NH_4$), Total – N and Ca according with Kjeldahl method, and the concentrations were 10.61% of $N\text{-}NO_3$, 1.30% of $N\text{-}NH_4$, 11.91% of Total-N and 194.68 g/Kg of Ca.

Animals was fed twice a day, at 8 a.m. and 4 p.m., in the form of total mixed ratio (TMR). To ensure that all treatments received the same amount of energy and nitrogen, diets without tannins were supplied with same proportion of kaolin, an inert clay, and when there was no calcium nitrate a blend with urea, calcitic limestone and kaolin was include as well. The ingredient proportions and chemical composition of the diets are shown in Table 1.

The feeding phase was carried out in 5 periods of 28 days each. The first 16 days of each period were to adapt the animals to the diets, and between 17th and 21st days the collection of feces for the anaerobic digestion phase was performed. The feces were collected twice a day (8 a.m. and 4 p.m.) by hand. All the samples corresponding to the same cow were mixed in a single bag. On 24th day of each period urine was collected every 6 hours (at 6 a.m., 12 p.m., 6 p.m. and 12 a.m.) and obtained either during spontaneous urination or simulation by vulva.

4.2.4. Anaerobic digestion phase

4.2.4.1.Substrate preparation, experimental design, and treatments

Samples of urine and feces were frozen during the feeding phase and later thawed and diluted in water. A mixture of feces and urine (waste) was prepared by using a theoretical ratio

of 83%:17%, respectively. Then, this mixture was diluted in water, and finally, the inoculum was added.

Batch type biodigesters were used, and 3 kg of substrate were prepared, 2 kg of which were used to fill the biodigesters and 1 kg to perform the characterization analysis of the substrate (Table 8).

Composition of substrate followed these ratios: 40% of waste, 3.3% of inoculum, and 56.7% of water. The sewage sludge from waste treatment was used as inoculum and had 0.164% of total solids (TS). Accordingly, the substrates were prepared to ensure an estimation of 6% of TS as per Lucas Junior et al. (1993), who found better biogas production in batch type biodigesters when the TS content of the substrate was less than 8%.

The biodigesters were arranged in a completely randomized design of 5 treatments with 5 replicates and totaling 25 experimental units (represented by feces of the animals which received, individual or associative effect of monensin, tannins and Calcium nitrate in the diet). The anaerobic biodigestion was performed in mesophilic conditions (30 to 35°C), ideal for digestion kinetics (METCALF; EDDY, 2014). The biodigesters were placed inside a climate chamber with electric heating system and digital temperature record. Substrates composition in the different biodigesters is shown in table 8.

Table 8. Composition of substrates of anaerobic batch type biodigesters supplied with waste of Nellore cows fed monensin, Tannins of *A. mearnsii*, Calcium Nitrate and pool.

Variable	Treatments					SEM	P-value
	Control	Monensin	Tannins	Nitrate	Pool		
TS(g/kg)	140.80	152.00	143.30	143.73	141.90	3.3876	0.1765
VS (g/kg)	115.46	121.40	125.08	119.10	122.52	2.9671	0.2271
N (g/kg TS)	37.26	38.10	40.01	38.98	39.41	2.0306	0.8836
NDF (g/kg TS)	43.07[b]	43.07[b]	43.08[b]	39.73[b]	57.81[a]	3.8760	0.0322
pH	6.71[b]	6.87[b]	6.74[b]	6.86[b]	7.24[a]	0.1249	0.0510

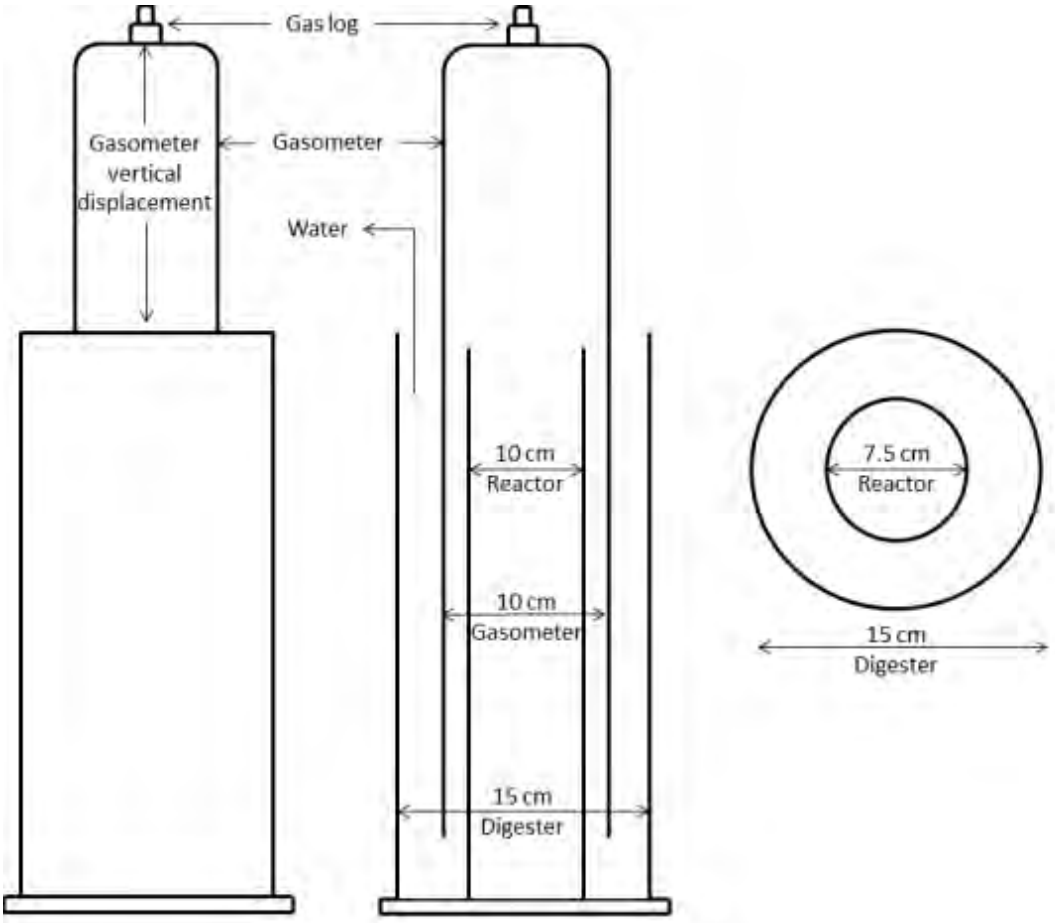
SEM: standard error of mean; TS: total solids; VS: volatile solids; N: nitrogen; NDF: neutral detergent fiber; abc: different letters on the same line differ significantly by the protected LSD test at 5% of significance level. (Own authorship).

4.2.4.2.Quantitative production of biogas

The batch type biodigester consisted of three straight cylinders with diameters of 15, 10 and 7.5 cm, as shown in figure 9, with a mean capacity to ferment 2 liters of substrate each.

After being fueled, the biodigesters were placed in the climate chamber to guarantee that the test occurred in mesophilic conditions. Before every biogas reading the temperature was record.

Figure 9. Layout of bench batch anaerobic digesters



Source: Nogueira (2017).

The reading of biogas production was performed according to the accumulation in the gas meter. It consisted of the height measured by the ruler attached to the gas meter according to the vertical displacement. Then reading value was multiplied by the internal cross-sectional area of the gas meter. After each reading, the gas meters were emptied by using the biogas discharge register. The correction of the biogas volume for the conditions of 1 atm at 20°C was carried out according to the methodology described by Lucas Junior (1994). To correct the biogas volume, the expression resulting from the combination of the laws of Boyle and GayLussac was used:

$$(V_0P_0)/T_0 = (V_1P_1)/T_1$$

Where:

V_0 = corrected biogas volume, m^3 or L;

P_0 = corrected biogas pressure, 10322.27 mm H_2O;

T_0 = corrected biogas temperature, 293.15 K;

V_1 = gas volume in the gas meter;

P_1 = biogas pressure at the time of reading, 10344.11 mm H_2O;

T_1 = biogas temperature, in K, at the time of reading.

Considering the average atmospheric pressure of Pirassununga was equal to 102237.11 mm H_2O and the pressure conferred by the gas meters of 71 mm H_2O, the following expression was obtained to correct the biogas volume:

$$V_0 = (V_1/T_1) \times 293.7703$$

Whenever biogas was measured, the sampling was done as well. Samples were collected using a 60 mL syringe connected to the gas register and the collecting flasks (glass flasks of 50 mL, Frascolex, São Paulo, Brazil) were then washed twice with the gas taken from the biodigester,; after this process 50 mL of biogas were injected for analyses. Afterwards gas meters were emptied to allow a new accumulation of gas. Thereafter the biogas production ceased, i.e. there was no more displacement of the gas meter, the test was finalized.

Concentrations of CH_4, CO_2 and N_2O were determined through gas chromatography (Trace 1300, Thermo Fisher Scientific®, Rodano, Milan, Italy) in controlled temperature (25°C) according to Kaminski et al. (2003). The biogas samples were diluted in glass flasks, with a known volume, 16.78 times in atmospheric air. Then, 6 mL were injected into the chromatography injector (splits/splitless), 4 mL of which were used to wash the injection system and 2 mL were used for analyses. 1 mL was also used for the system with a flame ionization detector (FID), responsible for the measurement of CO_2 and CH_4 and 1 mL for the system with electron capture detector (ECD), 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 that was diluted in atmospheric air. Two gaseous mixtures were used as reference, one with 50% CH_4 to 50% CO_2 and another with 10% N_2O in balance with He (mol/mol). Helium with a flow rate of 30 mL/min was used as the dragging gas.

The volumes of CH_4, CO_2 and N_2O produced (m^3 or L) were calculated using the production data and biogas compositions of each digester according to the equation:

$$Vol = (Vol_{BIOGAS} \times \%Gas)/100$$

Where:

Vol = volume (m^3 or L);

Vol_{BIOGAS} = volume of biogas produced (m^3 or L);

%Gas = content of gas of interest in biogas (%).

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

To study biogas production kinetics and its components was applied the Gompertz model. This model assumes that the gas production rate is proportional to the microbial activity; however according to Lavrencic et al. (1997) the proportion decreases with the incubation time and can be interpreted as a loss of efficiency in the fermentation rate. The mathematical description of the gas production curves allowed the data analysis, the substrate comparison and the performance fermentation. Descriptions of the model are shown in the following equation:

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

Where:

Y_t = gas production (L/g VS added) at time t (day);

A = asymptote of the model, indicates the stabilization value of the production (L/g VS added) in relation to time t;

B = integration constant, with no biological meaning;

kt = maximum growth rate, logarithmic function of the production growth (L/g VS added) per unit of time.

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

$$t_1 = \ln B/k$$

Where: t_1 = time (days) at inflection point; ln = natural logarithm; k = production constant.

The gas production at inflection point was determined as:

$y_1 = A/exp$

Where: y_1 = gas production at inflection point; exp = base of natural logarithm (2.7183).

4.2.5. Nutrients removal

Nutrient removal was calculated by multiplying the substrates weight, added and recovered, in each biodigester by their DM content percentage, so than calculate the DM content in grams. The added and recovered nutrients, expressed in grams, were calculated by multiplying between the added and recovered, and expressed as grams of DM, which then were expressed as a percentage and divided by 100 according to the following equation:

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

The nutrient removal, in percentage, was calculated using added and recovered nutrient content and expressing in g/kg of DM according to the following equation:

$$\text{Removed nutrient (\%)} = \frac{\text{[Added nutrient (g) - recovered nutrient (g)]}}{\text{Added nutrient (g)}} x\ 100$$

4.2.5.1.Biofertilizer characteristics

Samples of the substrate before and after digestion were collected and dried in an oven with ventilation and constant air renewal at 65°C for 72 hours according to AOAC (1995). Then, they were ground with willy type knives in 1 mm sieves and stored in properly sealed vials. To determined DM samples were dried in a oven at 105°C for 4 hours (method 930.15; AOAC, 1995). Sequentially, mineral matter (MM) was obtained by calcination in a muffle oven at 550°C for 5 hours (AOAC, 1990). The TS (TS = 100 – humidity) and VS (VS = TS – MM) content of the substrates were determined with adaptations to the methodology described in APHA (2005). The total N content was determined by micro-Kjedahl technique (method 920.87; AOAC, 1990). Neutral detergent fiber (NDF) was determined according to Van Soest et al. (1991) methodology. And hydrogen ion potential (pH) was measured by portable pH meter (Hanna Instruments®, HI 8424, Italy).

4.2.6. Statistical analysis

The data were analyzed using Statistical Analysis System (SAS 9.4, Intitute Inc., 2013). Before the analysis they were evaluated in relation to the presence of discrepant information (outliers) and normality of residues by the Shapiro-Wilk test. When the normality premises were not met the data were transformed. Data were submitted to analysis of variance which separated treatments, as causes of variation and performed by using the mixed procedure (Proc Mixed). For the comparison of means among treatments were performed the PDIFF test at 5% of significance. The statistical model used was described according to the equation bellow:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where:

Y_{ij} = observation concerning Treatment (i) + random error (e_{ij});

μ = overall mean;

T_i = effect of treatment (fixed effect);

e_{ij} = random error associated to the observation.

4.3.RESULTS

4.3.1. Bio-digestion and nutrient removal

There were no significant effects to added nutrients ($P>0.05$). However, the Pool treatment presented an increase of 34.2% to NDF, when compared to the Control ($P<0.05$). Significant effects were observed for TS, N and NDF recovery (i.e. the amount of nutrients not used during biodigestion) and VS removal efficiency ($P<0.05$).

The TS nutrients not used during biodigestion process were increased by monensin and pool, 15% and 17%, respectively; even with no difference when compared to control group. However, the Tannins and Nitrate decreased in the TS nutrients by 12% and 4%, respectively. The Pool also has showed an increase of 38% of N and 65% of NDF recovered nutrient.

Monensin effluent pH differ significantly (P<0.05) from the control but it was still within the neutral zone as expected.

The Tannins and Nitrate presented an increase of 26% and 14% in removal efficiency, even not differing to the Control group, and the Monensin and Pool decreased 14% and 18%, respectively. Results are shown on the Table 9 below.

Table 9.Biodigestion and removal efficiency of nutrients from anaerobic batch type biodigesters supplied with waste of Nellore cows fed monensin, Tannins of *A. mearnsii*, Calcium Nitrate and Pool.

Variables	Treatments					SEM	*P-value*
	Control	Monensin	Tannins	Nitrate	Pool		
Added nutrients							
TS (g)	140.80	152.00	143.30	143.73	141.90	3.3876	0.1765
VS (g)	115.46	121.40	125.08	119.10	122.52	2.9671	0.2271
N (g)	37.26	38.10	40.01	38.98	39.41	2.0306	0.8836
NDF (g)	43.07^{b}	43.07^{b}	43.08^{b}	39.73^{b}	57.81^{a}	3.8760	0.0322
Recovered nutrients							
TS (g)	93.38^{ab}	108.24^{a}	82.15^{b}	88.85^{b}	109.27^{a}	5.7541	0.0109
VS (g)	57.77	63.12	65.89	68.27	67.12	6.9971	0.8532
N (g)	11.98^{b}	12.94^{b}	13.53^{b}	11.90^{b}	16.56^{a}	0.8332	0.0042
NDF (g)	33.51^{b}	40.00^{b}	34.50^{b}	37.20^{b}	55.32^{a}	0.8333	0.0010
pH	7.68^{a}	7.33^{b}	7.72^{a}	7.77^{a}	7.71^{a}	0.1055	0.0488
Removal efficiency							
TS (%)	33.75^{abc}	28.80^{c}	42.53^{a}	38.18^{ab}	27.60^{c}	3.3711	0.0258
VS (%)	49.56	48.14	47.61	42.68	48.52	5.3390	0.9051
N (%)	66.61	60.67	63.22	63.48	62.38	3.7844	0.9145
NDF (%)	28.97	12.33	26.44	17.68	6.26	6.0250	0.0880

SEM: stanadard error of mean; TS: total solids; VS: volatile solids; N: nitrogen; NDF: neutral detergent fiber; abc: different letters on the same line differ significantly by the protected LSD test at 5% of significance level.

4.3.2. Biogas production

The total volume of biogas production, as well CH_4 and CO_2 presented significant effect (P<0.05). The biodigesters supplied with waste from cattle fed tannins increased in 69% of the total biogas production, 91% of CH_4 production and 52% of CO_2 production per gram of VS removed during the biodigestion process (Figures 10 and 11).

The other treatments did not differ from the control, but presented a decrease in total biogas production, as well to the CH_4 and CO_2, wherein the reductions were 53.5%, 49% and 60% to Monensin, 20%, 6% and 35% to Nitrate, and 9%, 8% and 31% to Pool. There were no significant effects ($P>0.05$) on N_2O gas production or their parameters.

The results are shown on the Table 10 below and the graphs presenting the cumulative CH_4, and CO_2 productions are represented on figures 10 and 11, respectively.

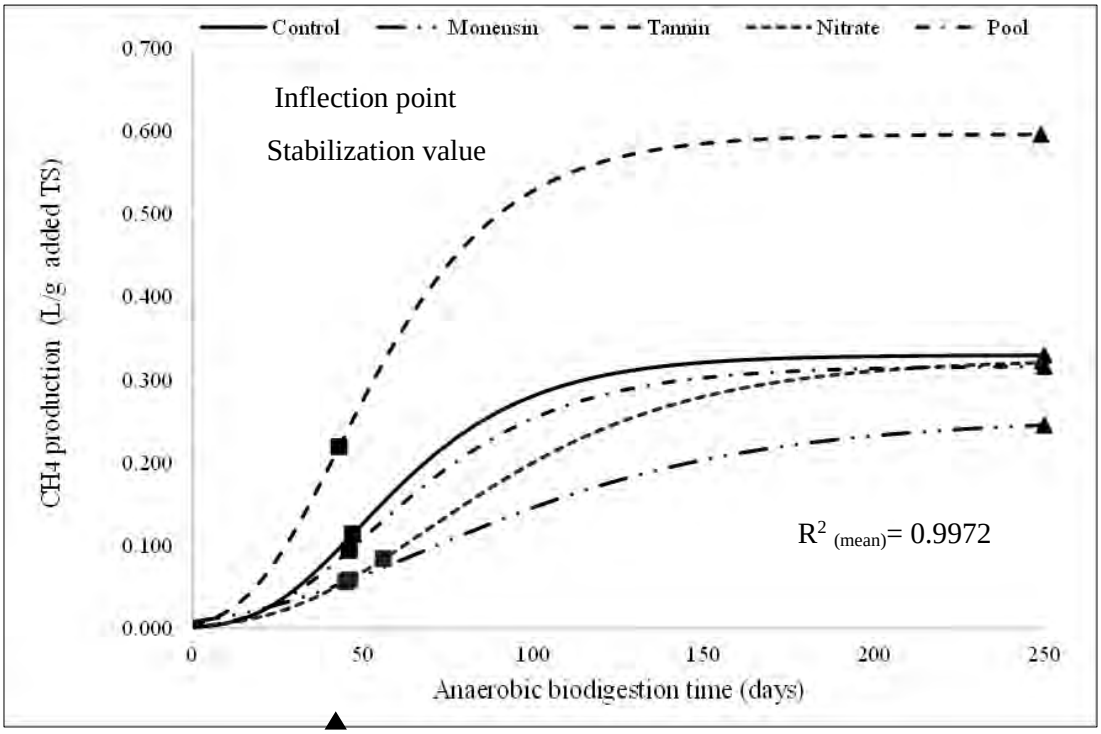
Table 10. Gas production (total biogas, CH4, CO2 and N2O) in batch type biodigesters with waste of Nellore cows fed monensin, Tannins of *A. mearnsii* and Calcium Nitrate.

Variables	Treatments					SEM	*P-value*
	Control	Monensin	Tannin	Nitrate	Pool		
Biogas (L)	27.80[b]	12.90[b]	47.10[a]	22.34[b]	25.34[b]	6.3039	0.0453
CH_4 (L)	16.23[b]	8.22[b]	31.00[a]	15.26[b]	17.60[b]	3.5574	0.0088
CH_4, %	64.30	65.22	62.10	66.05	68.96	1.5975	0.0813
CH_4/feces (L/g)	0.092	0.053	0.146	0.084	0.0917	0.0193	0.0728
CH_4/added VS							
A (L/g)	0.330	0.254	0.597	0.326	0.320	0.0763	0.0568
k (L/g.day)	0.036[a]	0.018[b]	0.037[a]	0.023[b]	0.031[ab]	0.0044	0.0496
t (day)	55.44	49.35	44.32	69.27	51.66	7.9447	0.2032
y (L/g)	0.121	0.094	0.219	0.119	0.112	0.0280	0.0568
CH_4/removed VS (L/g)	0.696	0.410	0.852	0.488	0.657	0.1922	0.5321
CO_2 (L)	10.50[ab]	4.20[b]	16.00[a]	6.85[b]	7.25[b]	2.1228	0.0113
CO_2, %	27.81	20.27	34.00	22.73	25.60	4.1115	0.2055
CO_2/feces (L/g)	0.042	0.016	0.066	0.032	0.045	0.0118	0.1094
CO_2/added VS							
A (L/g)	0.155	0.088	0.263	0.123	0.131	0.0474	0.1325
k (L/g.day)	0.036[a]	0.018[b]	0.037[a]	0.023[b]	0.032[ab]	0.0044	0.0439
t (day)	55.32	52.96	35.74	69.46	51.37	9.1355	0.1100
y (L/g)	0.057	0.032	0.097	0.045	0.048	0.0174	0.1309
CO_2/removed VS (L/g)	0.320	0.143	0.462	0.182	0.275	0.0936	01666
N_2O (mL)	1.06	2.70	8.13	3.53	5.37	2.9237	0.5012
N_2O, %	0.03	0.19	0.06	0.14	0.07	0.0727	0.5970
N_2O /feces (mL/g)	0.03	0.02	0.09	0.04	0.04	0.0238	0.6591
N_2O /added VS							
A (mL/g)	0.13	0.07	0.66	0.05	0.13	0.2057	0.1201
k (mL/g.day)	0.03	0.02	0.09	0.03	0.03	0.0405	0.5780
t (day)	55.8	97.6	44.3	68.4	47.9	15.872	0.4252
y (mL/g)	0.05	0.03	0.24	0.02	0.05	0.0757	0.1209
N_2O/removed VS (mL/g)	0.04	0.08	0.25	0.10	0.22	0.0927	0.4877

SEM: standard error of mean; 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

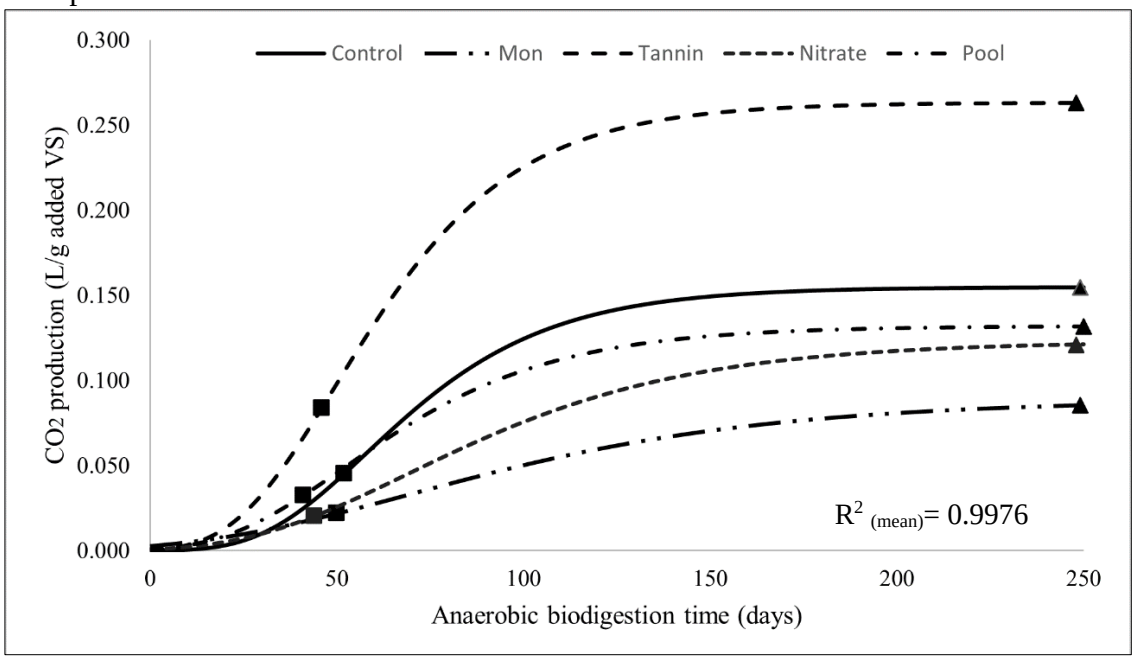
inflection point (L/g added VS; ab: different letters on the same line differ significantly by the protected LSD test at 5% of significance level.

Figure 10. Graph adjusted by the Gompertz model depicting the time (in days) and cumulative CH4 production.



(Source: own authorship)

Figure 11. Graph adjusted by the Gompertz model depicting the time (in days) and cumulative CO2 production.



(Source: own authorship)

4.3.3. Biofertilizer characterization

The Pool treatment increased the biofertilizer nutrient compounds in 12.6% of Total C and 35% of Total N, Monensin presented an increase of 7.5% of Total N, and K was increased in 35% to Tannins and 22% to Nitrate. Results are shown on Table 11.

Table 11.Chemical composition of biofertilizer from batch anaerobic reactors fed bovine manure of Nellore cows fed monensin, Tannins of *A. mearnsii*, Calcium Nitrate and Pool.

Variables	Treatments					SEM	*P-value*
	Control	Monensin	Tannin	Nitrate	Pool		
Minerals							
Total C (g/kg)	417.8^{c}	429.6^{c}	449.0^{b}	422.0^{c}	470.4^{a}	6.5124	<0001
Organic C	167.0	162.6	165.6	162.0	166.7	2.9591	0.6597
Total N (g/kg)	11.90^{b}	12.95^{b}	12.95^{b}	11.83^{b}	16.11^{a}	0.8657	0.0126
P_2O_5 (g/kg)	11.62	10.58	13.52	13.44	13.82	1.0270	0.1415
K (g/kg)	16.00^{b}	16.60^{b}	21.30^{a}	19.50^{a}	15.10^{b}	0.9187	0.0007
C/N	14.31	13.01	11.70	14.03	10.45	1.1134	0.1142
pH	7.68^{a}	7.33^{b}	7.72^{a}	7.77^{a}	7.71^{a}	0.1055	0.0488

SEM: standard error of mean; C/N: Carbon: Nitrogen ratio; abc: different letters on the same line differ significantly by the protected LSD test at 5% of significance level.

4.4.DISCUSSION

According to Ahring et al. (2001), the use of bovine waste as a substrate for the development of anaerobic biodigestion becomes a good alternative, as the waste contains carbohydrates, proteins and fats. However, several factors can alter the characteristics of the waste, influencing its potential for biogas production, with animal feed being an important factor (AMON et al., 2007; JARRET et al.; 2011).

In the present study. Waste from animals that received monensin in their feed showed a reduction in the efficiency of ST removal (Table 9) and a reduction in the levels of total C, total N, K and pH of the effluent (Table 10). However, despite the reduction of the affluent pH, in relation to the control and other treatments, it remained in the ideal range between 7.0 and 8.5 as reported by Rabiu et al. (2014), Mshandete et al. (2006) and Gunaseelan (1995); this is not the factor of change regarding anaerobic biodigestion. Although biogas production (L) did not show any significant effect, there was a 53% decrease in total biogas production, resulting in 49% and 60% reductions in CH_4 and CO_2 production, respectively. In both cases, the rate of gas production (k) was lower for monensin (Table 9), indicating that in addition to causing gas reductions, production was lethargic (Figures 10 and 11).

There is evidence that monensin bioactive metabolites are eliminated in ruminant feces. According to Davidson (1984), most of the consumed monensin is recovered in the feces, however this effect is not observed in the urine and tissues. Herberg et al. (1978) recovered about 95% of the active metabolites of monensin in the feces when determining the pattern of excretion and tissue distribution of monensin in cattle. Corroborating the results of the present study, in which the effects observed by monensin may indicate that the additive has a direct effect on anaerobic biodigestion.

In the other hand, the presence of tannins in the manure increased the production of total biogas (L) by almost 70% and the production of CH_4 and CO_2 by 91% and 52%, respectively, as in Table 10. On the contrary of what was observed for monensin, the rate of gas production (k), for tannins, was higher (Table 10) and making the asymptote faster, because even with no significant difference, there was a trend ($P = 0.0568$) and an increase of 80%.

The results found in the present study are not compatible with those reported by Hao et al. (2011) and Perna Júnior (2018), in which both using condensed tannins from A. mearnsii

did not observe an increase on CH_4 and CO_2 production, however, in both cases, the addition of the tannins did not harm the biogas production. In addition, Hao et al. (2011) found an increase in the agronomic value of manure and compost as a fertilizer; this effect has been observed in the present study, by increasing the K content in the effluent. The inclusion of calcium nitrate had no effect on biogas production, corroborating the results found by Cassiano (2017). However, at the end of the biodigestion process, the biofertilizer analysis showed a reduction in the total C content, but without changing the C / N ratio and with an increase in the K content.

According to Pognani et al. (2009), during the anaerobic biodigestion process, mineralization of OM occurs, resulting in the preservation and concentration of inorganic nutrients, such as P and K and N. In addition, this process can be influenced by several factors. Among them, the C / N ratio is an important factor, since all living organisms need nitrogen to synthesize proteins, and there must be a correct ratio between carbon and nitrogen, otherwise the bacteria will not be able to consume all the carbon present, and the performance of the process will be low (SGORLON et al., 2011). According to Rao and Singh (2004) the optimal C / N ratio is between 20-30 / 1 for the start of treatment and between 10-13 / 1 for the residue to be considered stabilized (MBULIGWE; KASSENGA, 2004). This effect can be observed for both calcium nitrate and tannins. However, there is little data in the literature on the effect of calcium nitrate used as a nutritional additive, on cattle manure in conditions of anaerobic digestion, and further studies are necessary to better understand its effects.

The combined use of the three additives in the Pool treatment, did not show reductions in the production of total biogas (L), CH_4 and CO_2. However, the affluent used to supply the biodigesters contained a higher NDF content (Table 9), possibly due to the reduced digestibility of this nutrient by the animal. The amount of ST, N and NDF eliminated (not used or fermented during biodigestation) was also greater for this treatment and reduced the efficiency of ST removal. Tannins are known for the formation of complexes with the nutrients of the diet, since the formation of complexes occurs with the ingested nutrients (KUMAR; SINGH, 1984) which can result in the reduction of the digestibility of nutrients (FRUTOS et al., 2004) and consequent increase in the excretion of these in the feces.

The increase in nutrients removed leads to a reduction in removal efficiency, an effect also found by Perna Junior (2018) and Tseu et al. (2020) when using different levels of tannins

in association or not with monensin. In the present study, the use of Acacia mearnsii tannins at 1.5% of the DM did not reduce the removal efficiency, on the contrary, the wastes had higher removal efficiency for ST.

Tseu (2020) found similar effects when using monensin and different levels of tannins, in which the interaction between both negatively impacted the efficiency of removing nutrients and reduced the production of biogas. Indicating the possible presence of bioactive monensin metabolites in cattle manure.

The increase in the agronomic quality of the biofertilizer is related to the chemical characteristics of the raw material (TAMBONE et al., 2010) and the transformation of carbon into methane, concentrating the nutrients in the biofertilizer (MOLINUEVO-SALCES et al., 2013). In the present study, there was an increase in the Total C of biofertilizers in the Pool treatment, which may be a factor caused by the low efficiency of ST removal, in addition, there was also an increase in the Total N content, an effect that may be related to the digestibility of CP in the animals' TGI. However, these increases did not affect the C / N ratio of the biofertilizer, with no treatment effect, and keeping in the range indicated by Mbuligwe and Kassenga (2004) of 10-13 / 1.

4.5.CONCLUSION

The use of calcium nitrate did not affect the production of biogas and the quality of the biofertilizer; however, more studies are necessary to understand better its effects on anaerobic biodigestion.

Due to the low production of biogas and the quality of biofertilizer from cattle manure fed with monensin, it may not be recommended for anaerobic biodigestion, as it drastically reduces the production of CH_4, the main component of biogas. On the other hand, the tannins showed good results through anaerobic digestion, increasing the production of biogas by 70% and maintaining the quality of the biofertilizer, being recommended the use of the technique to reduce the impacts of untreated manure on the environment.

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