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Associative effect of tannins and monensin as manipulators of rumen fermentation on the mitigation of methane production in cattle

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Versão corrigida

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

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Dedication

This dissertation is dedicated to the memory of my beloved mother, Joana Simão Bahule, whom I left at home in pursuit of a dream, and could not see her again! May God have given her the best eternal rest!

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RESUMO

Tseu, R. J. **Efeito associativo de taninos e monensina como manipuladores da fermentação ruminal na mitigação da produção de metano em bovinos**. [Associative effect of tannins and monensin as manipulators of rumen fermentation on the mitigation of methane production in cattle]. 2019. 120 f. Dissertação (Mestrado em Zootecnia) - Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2019.

As mudanças climáticas e o aquecimento global são temas de debate científico da atualidade. O aumento de gases de efeito estufa é apontado como uma das principais causas das mudanças. O estudo teve como objetivo avaliar o efeito associativo da monensina (M) com diferentes níveis de inclusão de taninos (T) sobre os parâmetros ingestivo, digestivo e fermentação ruminal (técnica *ex-situ*), bem como sobre a biodigestão anaeróbia de dejetos. Em arranjo fatorial 2 x 4, 8 vacas Nelore, canuladas foram distribuídas em 2 quadrados latinos contemporâneos 4 x 4 e receberam 8 dietas experimentais que diferiram no nível de inclusão de T do extrato da *A. mearnsii* (0,0, 0,75, 1,5 e 2,25% da MS) e M que foi administrada diariamente a cada vaca (cerca de 32 mg/kg de MS) de um quadrado. Para avaliar a produção de biogás foram utilizados biodigestores anaeróbios do tipo batelada, em delineamento inteiramente casualizado. Os dados foram analisados pelo *Statistical Analysis System* (SAS 9.3, Institute Inc., 2013). Os resultados mostraram pouca interação entre M e T. Quanto aos parâmetros ingestivo, digestivo e balanço de N, os T reduziram linearmente o consumo da MS e água, a digestibilidade aparente total da MS, PB, NDT e MO e quadraticamente a da FDN e FDA. Os T reduziram linearmente a taxa de desaparecimento ruminal pela redução linear das taxas de passagem e digestão. Os T reduziram linearmente a excreção de uréia urinária, mas ambos aditivos não tiveram efeito sobre a síntese e eficiência da síntese de proteína microbiana. A M reduziu a proporção de N fecal, mas não teve efeito sobre o balanço do mesmo, enquanto que os T aumentaram linearmente o N fecal e reduziram linearmente o N urinário e retido. Em relação a fermentação ruminal, não foi observado efeito da M sobre a produção de CH_4, mas sim, redução da relação acetato:propionato. Os T reduziram linearmente a produção de CH_4 e AGCC. Em relação a biodigestão anaeróbia, a M e T reduziram a eficiência de remoção de nutrientes. A M e T tiveram efeitos independentes sobre o metabolismo ruminal, porém, o uso de T do extrato da *A. mearnsii* até 2,25% de MS é seguro para bovinos, com potencial para mitigar o CH_4 entérico. O uso combinado de M e T reduziu o potencial de produção de biogás pela redução da eficiência do uso de nutrientes. A M inibiu o efeito dos T sobre a redução da eficiência biodigestiva dos dejetos através da interação antagonista.

Palavras-chave: Aditivos alimentares; Biodigestão anaeróbia; Digestibilidade; Fermentação; Gases de efeito estufa

ABSTRACT

Tseu, R. J. **Associative effect of tannins and monensin as manipulators of rumen fermentation on the mitigation of methane production in cattle**. [Efeito associativo de taninos e monensina como manipuladores da fermentação ruminal na mitigação da produção de metano em bovinos]. 2019. 120 f. Dissertation (Master in Animal Science) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2019.

Climate changes and global warming are topics of scientific debate. The increase of greenhouse gases has been pointed out as one of the main causes of the changes. The study aimed to evaluate the associative effect of monensin (M) with different levels of tannins (T) on feeding, digestive and rumen fermentation (*ex-situ* technique) parameters as well as on anaerobic biodigestion of waste. In a 2 x 4 factorial arrangement, 8 cannulated Nellore cows were distributed in 2 contemporary 4 x 4 Latin squares and received 8 experimental diets which differed in the level of inclusion of T of *A. mearnsii* extract (0.0, 0.75, 1.5, and 2.25% DM) and M which was daily administered to each cow (about 32 mg/kg DM) of one square. To evaluate the production of biogas, experimental batch-type anaerobic biodigesters were used in a completely randomized design. The data were analyzed by the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). The results have shown little interaction between M and T. Regarding the feeding, digestive and N balance parameters, the T linearly reduced DM and water intake, the total apparent digestibility of DM, CP, TDN and OM; for the NDF and ADF the reduction was quadratic. The T linearly reduced rumen disappearance rate by linearly reduce both passage and digestion rates. T also linearly reduced urinary urea excretion, but both additives had no effect on the synthesis and efficiency of microbial protein synthesis. M reduced the proportion of N excreted in feces, but had no effect on N balance, whereas T linearly increased fecal N and linearly reduced urinary and retained N. Regarding the parameters of rumen fermentation, no significant effect of M was observed on CH_4 production, but on the reduction of acetate:propionate ratio. T linearly reduced the production of CH_4 and total SCFA. Regarding anaerobic biodigestion, M and T reduced the nutrient removal efficiency. M and T had independent effects on rumen metabolism, however, the use of T of *A. mearnsii* extract up to 2.25% DM is a safe option for cattle, with potential to mitigate rumen CH_4. The combined use of M and T reduced the potential of biogas production by reducing the nutrient use efficiency. M inhibited the effect of T on the reduction of nutrient use efficiency of the waste through antagonistic interaction.

Keywords: Anaerobic biodigestion; Digestibility; Feed additives; Fermentation; Greenhouse gases

SUMMARY

1. INTRODUCTION

The United Nations (UN, 2013) estimate that the world's population will increase from 7.2 to 9.6 billion by 2050, indicating that there will be a growth of about 33.3% over 37 years. This growth is estimated to be mainly in developing countries, with more than half in Africa. The population growth and purchasing power have promoted a sharp increase in the demand for animal origin food. The livestock sector provides more than one-third of human protein needs and is expected to double by 2050, in addition to be a major livelihood provider in almost all developing countries (SAKADEVAN; NGUYEN, 2017; ROJAS-DOWNING et al., 2017). But while it offers immense benefits to the population, poor management can potentially cause detrimental environmental impacts (SAKADEVAN; NGUYEN, 2017).

Currently, agriculture plays an important role in global environmental problems, such as climate changes, land degradation, water pollution and biodiversity loss (FAO, 2013; ROJAS-DOWNING et al., 2017). Despite the widespread recognition of the importance of agriculture in food production and income generation, livestock, in particular, emits significant amounts of greenhouse gases (GHG), mainly in regions where this activity is based on degraded or seasonal natural pastures and characterized by low productivity and higher GHG production rate (IPCC, 2007).

Among the GHG, carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are the most important in agriculture. Although the concentrations of CH_4 and N_2O in the atmosphere are lower than that of CO_2, these gases have a heating potential of 25 and 298 times more than CO_2, respectively (IPCC, 2007). The animals can contribute directly to the increase of CH_4 concentration through two main forms, (1) enteric fermentation and (2) fermentation of organic waste (NOVAK; FIORELLI, 2010).

Global emissions of enteric fermentation grew from 1.4 to 2.1 $GtCO_2eq$ per year between 1961 and 2010, with average annual growth rates of 0.70 %, whereas global emissions of GHG from manure, as either organic fertilizer on cropland or manure deposited on pasture, grew between 1961 and 2010 from 0.57 to 0.99 $GtCO_2eq$ per year. On average, emissions from manure grew by 1.1% per year (IPCC, 2014). The emission of methane by fecal degradation is variable in function of the manure management, with higher emission rates for waste from confinement in relation to manure deposited directly in pasture (JICONG et al., 2006).

It is widely known that CH_4 is a by-product of carbohydrate digestion, where H_2 is removed by rumen microorganisms generating energy losses to the animal that vary from 2 to 15% of the feed gross energy (JOHNSON; JOHNSON, 1995; GOEL; MAKKAR, 2012;

WANAPAT et al., 2015). An adult bovine can produce up to 17 L of methane per hour (RUSSELL, 2002) and because this gas cannot be metabolized, most is removed from the rumen by expiration or eructation (MOSS, 2000), and then released into the environment.

Animal nutritionists have conducted researches aiming to reduce methane emissions, contributing to the reduction of global warming, and making the food production a more efficient process. According to Grainger et al. (2010), the most successful mitigation strategy should allow a profitable and sustainable increase of livestock production as well as promote a persistent reduction of enteric methane emissions.

Nutritional techniques for the use of different feed additives have been used as strategies to manipulate rumen fermentation and reduce GHG emissions, though few have shown a persistent methane decline.

Monensin and tannins, separately, have shown to reduce enteric CH_4 emission from ruminants. In addition to reducing enteric CH_4 production, monensin affects other processes of rumen fermentation, including protein metabolism. Amongst the major forms by which tannins reduce enteric CH_4 emissions, one leads to depression of fiber digestion in the rumen (PATRA; SAXENA, 2011; CARRASCO et al., 2017). Tannins may also reduce protein digestibility by forming complexes with these macromolecules and make them inaccessible to microbial and enzymatic digestion (NIGRANT et al., 2017; PATRA; SAXENA, 2011). The decreased digestibility of dietary nutrients is expected to increase fermentable organic matter concentration in feces, which can promote a great anaerobic biodigestion for biogas production including CH_4 (FAO, 2013).

In animal nutrition, monensin is largely used to enhance feed efficiency in addition to reduce CH_4 production. Some authors, such as Patra and Saxena (2011), have cited that low to moderate tannin concentrations also improve feed efficiency in addition to reducing CH_4 production. Although much is known about the effects of these additives on rumen fermentation, little is known about their effects on the fermentation of waste. Hence, the overriding question is whether or not the effect of these additives on the reduction of enteric CH_4 production provides conditions for the emission of GHG from waste.

Although there are data regarding the use of the above-mentioned feed additives in cattle that bring some expectancy to reduce enteric CH_4 emissions and increase animal production, in general, they refer to isolate and not combined use.

Given these factors, the hypothesis tested in this study was that the combined use of monensin and tannins would have either a synergy on the reduction of enteric CH_4 production of Nellore cows or on the production of CH_4, N_2O and CO_2 from the waste by means of

anaerobic digestion. Therefore, the objective was to evaluate the associative effect of sodium monensin and tannins of black wattle tree (*A. mearnsii*) on rumen fermentation parameters (through *ex-situ* technique) as well as on feeding behavior, DMI, digestibility, rumen kinetics and nitrogen balance. It was also the aim to evaluate the potential for CH_4, N_2O and CO_2 production from waste by means of biodigesters as an alternative of waste management and biogas production.

2. LITERATURE REVIEW

2.1. **Methane emission and feed efficiency in ruminants**

Agricultural activity is pointed out as the main source of GHG in Brazil, accounting for 74% of emissions, 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 (SEEG, 2018).

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).

It is estimated that animal production contributes with about 14.5% of the global emission of GHG (ROJAS-DOWNING et al., 2017). 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 GHG, 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 15% (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. **Methanogenesis in ruminants**

Ruminants produce methane as an unavoidable by-product of organic matter (OM) fermentation in the rumen (ODONGO et al., 2007) through a process involving microbial fermentation and methanogenesis. In this process, structural carbohydrates, proteins and other organic polymers contained in feeds are degraded and transformed into monomeric units by the primary anaerobic fermenting microorganisms. These monomers are consequently converted (by the primary fermenters and other secondary microorganisms that do not have the ability to hydrolyze complex polymers) into energy for their growth and into final fermentation products which include short chain fatty acids (SCFA), alcohols, H_2 and CO_2 (JANSSEN; KIRS, 2008, MORGAVI et al., 2010; HAMILTON et al., 2010). H_2 and CO_2 are the main electron donor and acceptor, respectively. Methanogenic *Archaea* reduce some of these products (i.e. formate, acetate and CO_2) with H_2 to produce methane (CH_4) and water. Nevertheless, methane production contributes to fermentative 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 finally reduce rumen fermentation (MORGAVI et al., 2010).

The type of diet and the presence of other electron acceptors besides CO_2 have an effect on the presence and activity of H_2 producers and users, since other pathways, in addition to methanogenic, can also use H_2 and, consequently, compete and reduce methanogenesis in the rumen (MORGAVI et al., 2010).

Members of the *Archaea* domain contribute with about 0.3 to 3.3% of the rumen subunit (16S and 18S) rRNA microorganisms. Most of these methanogenic species can grow using H_2 and formic acid as energy sources and use the electrons derived from H_2 (or formate) to reduce CO_2 to CH_4. Some can grow through methyl groups, oxidizing them to CO_2 to produce electrons that are used to reduce other methyl groups to methane. Others may grow with acetate, effectively dissolving the acetate to CH_4 and CO_2, although acetate is not metabolized to CH_4 to a significant extent in the rumen, probably because the passage rate of rumen content through the rumen is greater than the growth rate of acetate methanogen users (LIU; WHITMAN, 2008; JANSSEN; KIRS, 2008).

Archaea are classified into 28 genera and 113 species. Few of these have been isolated from the rumen and those that have already been cultivated correspond to seven (7) species that include: *Methanobacterium formicicum, Methanobacterium bryantii, Methanobrevibacter ruminantium, Methanobrevibacter millerae, Methanobrevibacter olleyae, Methanomicrobium mobile and Methanoculleus olentangyi.* The most abundant in the rumen are *Methanobrevibacter* (61.6%) and *Methanomicrobium* (14.9%) (JANSSEN; KIRS, 2008). These microorganisms are strictly anaerobic and mainly responsible for the production of methane. They can be found closely associated with ciliate protozoa, either adhered on their cell surface or in the intracellular phase. Protozoa that have intracellular methanogenic *archaea* include the species *Dasytricha ruminantium* and *Entodinium spp*. Therefore, intracellular *archaea* are more numerous than superficial ones (FINLAY et al., 1994). For this reason, rumen fermentation manipulation techniques that reduce the total count of protozoa also reduce the concentration of methanogens and, consequently, methane production (PATRA; SAXENA, 2011).

Measurements using indirect respiration calorimetry showed that methane represents losses of ingested gross energy ranging from approximately 2 to 12%, and the higher the digestibility of nutrients, the greater the variability of methane energy losses (JOHNSON; JOHNSON, 1995). There are two primary mechanisms that cause this variation in methane production: (1) amount of fermented dietary carbohydrates in the reticulum-rumen that establish interactions which affect the balance between carbohydrate fermentation and passage rates, (2) mechanism that regulates the hydrogen supply and subsequent production of methane through the ratio of the SCFA produced (JOHNSON; JOHNSON, 1995). According to Wolin and Miller (1997), if the acetate:propionate ratio was 0.5, the methane production would be approximately equal to zero (0) and if the whole carbohydrate was fermented to acetate, methane losses would be about 33%, but because of the variation of acetate:propionate ratio from 0.9 to 4.0, the losses by the corresponding methane also vary.

2.3. **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., 2008ab).

Monensin was originally developed to serve as a coccidiostat for poultry and in the 1970s it was approved for use in cattle in United States of America (RUSSELL; HOULIHAN, 2003). Studies conducted in the same decade by Richardson et al. (1976) and Dinius et al. (1976) demonstrated that the use of monensin in cattle and sheep feeding promoted an increase in feed

efficiency through mechanisms that alter rumen fermentation, reducing methane production and acetate:propionate ratio as well as rumen ammonia resulting in high post-rumen protein availability. Similar results were found by Montano et al. (2015) in an experiment conducted to evaluate the influence of monensin supplementation on finishing diets on the performance and digestive function of steers where monensin was found to ameliorate average daily gain by enhancing feed efficiency associated with changes to greater intestinal digestion of OM and reduced rumen degradation of feed nitrogen and protein synthesis.

The use of monensin in feedlot cattle can improve feed efficiency 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 use of monensin in dairy cows has more evident benefits at the beginning of lactation when the energy balance is generally negative and with greater mobilization of body reserves (RANGEL et al., 2008). In a meta-analysis of the impact of monensin on dairy cattle production by Duffield et al. (2008b), using 36 articles and 77 trials, monensin increased milk production by 0.7 kg and improved milk production efficiency by 2.5%. Although monensin reduced the fat content in milk by 0.13%, it had no effect on daily production; the milk protein content reduced but the production increased 0.016 kg per day; monensin increased body condition score by 0.03 and similarly improved body weight change (0.06 kg/day). In the same study it was found that increasing the protein balance increased the effect of monensin on milk protein production. These data indicate the benefit of monensin in improving efficiency in milk production while maintaining body condition. Results of another meta-analysis of the impact of monensin on dairy cows' metabolism conducted by Duffield et al. (2008a) demonstrated that the use of monensin in these animals also improved energy metabolism.

In a meta-analysis of the impact of monensin on the health and reproduction of dairy cows conducted by Duffield et al. (2008c) in a total of 16 articles in trials with 9500 cows it was verified that monensin reduced the risk of ketosis (relative risk, RR = 0.75), abomasum displacement (RR = 0.75) and mastitis (RR = 0.91) in all the analyzed studies. No significant effect of monensin was found for milk fever, lameness, dystocia, retained placenta or metritis. Monensin had no effect on conception risk at the first service (RR = 0.97) or days for pregnancy (hazard ratio = 0.93). The monensin administration method influenced the incidence of placenta retention and metritis, with lower risk with controlled release capsule treatment. However, the

authors concluded that improvements in ketosis rates, abomasum displacement, and mastitis with monensin were achieved. They noted that exposure to prolonged monensin treatment in the dry period may increase the risk of dystocia and placental retention.

2.3.1. Effect of monensin on rumen metabolism

2.3.1.1. Effect of monensin on the production of short chain fatty acids

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 short chain fatty acids (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 reduced. In the same year, Dinius et al. (1976), studying the effect of monensin administered with forage on the digestion and rumen ecosystem in steers at levels of 0, 11, 22 and 33 ppm, observed a reduction in the molar ratio of acetate from 66.7 to 61.3% and that of propionate increased from 20.1 to 26.1% although there was no change in the total concentration of SCFA as well as cellulose digestibility.

In *in vitro* study by Martínez et al. (2006) about the effects of monensin and essential oils on rumen degradation and SCFA production, monensin did not alter the total production of SCFA but reduced the molar concentration of acetate and butyrate (from 74.9 to 70.9 and from 7.3 to 3.7, respectively) and increased the molar concentration of propionate (from 16.6 to 24.6). Studying the effects of monensin and enramycin on dry matter (DM) intake, rumen fermentation and feeding behavior in cattle fed high concentrate diets, Borges et al. (2008) found that monensin was strong in the increase of total SCFA concentration 12 hours after feeding and decreased acetate:propionate ratio at 0 and 6 hours post feeding. Perry et al. (1976) found significant effect of monensin on the production of SCFA; they observed reduction of acetate and butyrate in the order of 16% and 14%, respectively, while propionate increased in 76%.

Using monensin in forage diets of steers, Crossland et al. (2017) observed a significant reduction in the acetate:propionate ratio compared to the use of bambermycin and the rotational use of monensin and bambermycin. Other studies, such as of Costa et al. (2017) and Ruiz et al. (2001) also demonstrated the potential of monensin in the reduction of acetate and butyrate production and increase of propionate and consequent reduction of the acetate:propionate ratio.

Nonetheless, the change in the SCFA profile may be dependent on the dose of monensin in the diet (ELLIS et al., 2015).

2.3.1.2. Effect of monensin on rumen protein metabolism

Protein degradation in the rumen is nutritionally an expensive process that generally produces more ammonia than microorganisms can use. This excess represents a loss of dietary nitrogen (YANG; RUSSELL, 1993). A review on the efficiency of nitrogen utilization by lactating dairy cows and its relation to environmental pollution, Castillo et al. (2000) found that dairy cows are inefficient in nitrogen use; about 72% of the nitrogen consumed is excreted in feces and urine, a finding also observed by Mills et al. (2009). Haynes and Williams (1993), and Bolan et al. (2004) also observed that much of the nitrogen ingested by cattle (80-95%) is returned to the soil along with urine or feces.

A higher proportion of the protein ingested by ruminants is fermented and transformed into ammonia (and SCFA) by rumen microorganisms as a normal pathway for microbial protein synthesis. Therefore, the production of ammonia in the rumen generally exceeds the capacity of use by microorganisms, resulting in accumulation and subsequent absorption and conversion to urea by the liver (RUSSELL; STROBEL, 1989; RODRIGUES, 2016).

Studies indicate that monensin reduces ammonia production (*in vivo* or *in vitro*) by inhibiting the growth of rumen proteolytic bacteria, although, according to Russell and Strobel (1989), monensin affects deamination more than proteolysis. Studying the effect of monensin on the performance and nitrogen utilization of lactating cows consuming fresh fodder, Ruiz et al. (2001) observed reduction in the production of ammonia in rumen with reduction of fecal nitrogen and increase of total digestibility of nitrogen, and it was obvious that monensin has the potential to improve nitrogen efficiency by protecting amino acids against excessive rumen degradation. In the previous study by Yang and Russell (1993) that aimed to evaluate the effect of monensin on the specific activity of ammonia production by rumen bacteria and rumen disappearance of ammonia nitrogen, the specific use rates of microbial hydrolases in experimental diets reduced in animals which received monensin and it became apparent that this ionophore increased the rumen passage rate of protein nitrogen. These changes were attributed to the reduction or inhibition of ammonia-producing and monensin-sensitive bacteria.

Studying the influence of sodium monensin on intake and digestibility of diets with different protein levels for cattle, Oliveira et al. (2007a) observed a significant reduction of nitrogen loss from feces in animals receiving monensin. Due to the reduction of rumen protein digestion, monensin allows post-rumen digestion, making the process very interesting when

working with feeds rich in protein of high biological value, with adequate balance of lysine and methionine which, according to Rangel et al. (2008), are the first limiting amino acids for dairy cows.

2.3.1.3. Effect of monensin on the production of enteric methane

The use of ionophores (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 methane 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.

Van Vugt et al. (2005) reported reduction in methane production of 9% in cows fed pasture after the administration of monensin capsules and 10.5% in cows fed corn silage. Sauer et al. (1998) also observed reduction in methane production in Holstein cows with the addition of monensin and unsaturated fat in the diet. Studies, such as of Hamilton et al. (2010), Grainger et al. (2008) and McGinn et al. (2004) found no effect of monensin as anti-methanogenic. Using a high dose of monensin (471 mg/animal.day) in confined and grazing animals, Grainger et al. (2010) observed no effect and concluded that high doses did not reduce methane emissions in dairy cows grazing and supplemented with grains.

2.3.1.4. Mechanism of action of monensin in reducing methane production by ruminants

Monensin is a highly lipophilic ionophore with hydrophobic exterior and hydrophilic interior that interacts with ions (K^+, Na^+, Ca^{2+}, H^+ and Mg^{2+}) serving as a transport medium for these ions through microbial membrane (RANGEL et al., 2008).

Rumen bacteria maintain high intracellular concentrations of potassium and low concentrations of sodium and, conversely, the rumen environment (extracellular medium) contains high concentrations of sodium and low concentrations of potassium. Rumen pH is slightly acidic due to SCFA concentrations; however, the intracellular pH of many rumen bacteria is almost neutral, thus creating an inward-directed proton gradient (AZZAZ et al., 2015).

Monensin is a metal/proton antiporter that can exchange H^+ for Na^+ or K^+. 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. The 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).

The most sensitive microorganisms to the action of monensin (or ionophores in general) are Gram-positive bacteria; the Gram-negative bacteria are generally resistant. This difference is due to the fact that 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 and because the ionophores are hydrophobic and with molecular weight above 500 Daltons, this outer membrane serves as a protective barrier. Therefore, Gram-positive bacteria, because they do not have this membrane, are 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.), in relation to Gram-negative bacteria whose final products of fermentation are propionate, succinate, etc., 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).

2.3.1.5. Effect of monensin on dry matter and water intake

The dry matter intake (DMI) is of fundamental importance in nutrition since it establishes the amount of nutrients available for health and production. Low nutrient consumption restricts production and can affect animal health. Excessive nutrient consumption increases the cost of feeding and may result in excessive excretion of nutrients into the environment (NRC, 2001).

Studies show that monensin reduces feed intake, increases or maintains weight gain, and increases feed efficiency in animals fed high concentrate diets. In animals fed grass or forage, monensin does not reduce consumption, but increases weight gain as a consequence of increased feed efficiency (MORAIS et al., 2011). In a meta-analysis of the effects of monensin on feed efficiency of beef cattle, weight gain and DMI, Duffield et al. (2012) observed that the use of

monensin in growth and termination reduces DMI and improves average daily gain as well as feed efficiency. In the meta-analysis of Duffield et al. (2008b) it was observed that the use of monensin in dairy cows significantly reduced DMI in the order of 0.3 kg, although has increased production.

Evaluating the effects of monensin on lactating dairy cows' feeding, Odongo et al. (2007), using a total mixed raction (TMR) in a forage:concentrate ratio of 60:40, found no effect of monensin on DMI. Oliveira et al. (2007a) observed that the inclusion of monensin in diets containing different levels of CP for sheep significantly reduced the intakes of DM, OM, PB, EE, total carbohydrates (TC) and NDF. But the studies of Van Vugt et al. (2005), Gallardo et al. (2005), Benchaar et al. (2006) and Hamilton et al. (2010) found no effect of monensin on consumption. Therefore, these results suggest that the effect of monensin on consumption varies among studies.

2.4. **Use of tannins on ruminant feeding**

Tannins are polyphenolic polymers with the molecular weights in the range of 500-20000 Daltons and ability to form complexes mainly with proteins and, to a lesser degree, with carbohydrates (starch and structural carbohydrates) and minerals because of having a greater number of phenolic hydroxyl groups. They are generally water soluble except for some high molecular weight structures (ADDISU, 2016; PATRA; SAXENA, 2011; AMESA; ASFAW, 2018).

Tannins belong to the phenolic class and all phenolic compounds (primary and secondary) are, in one way or another, formed by the shikimic acid pathway also known as the phenylpropanoid pathway. This pathway leads to the formation of other phenolic compounds, such as isoflavones, coumarins, lignins and aromatic amino acids (tryptophan, phenylalanine and tyrosine) (CORNELL UNIVERSITY, 2018).

The tannins are mainly of two categories: (1) hydrolysable tannins and (2) proanthocyanidins, better known as condensed tannins, resistant to hydrolytic degradation. Hydrolysable tannins (HT) are complex molecules with a polyol as a central nucleus (such as glucose, glucitol, quinic acids, kerocytol and shikimic acid) and their hydroxyl groups are partially or totally esterified with a phenolic group, i.e. gallic acid.

Condensed tannins (CT) are mainly polymers of the flavan-3-ol (epi)catechin and (epi)gallocatechin units which are bound by the C_4-C_8 and C_4-C_6 interflavonoid bonds. They are generally called condensed tannins because of their condensed structure, although HT also undergo condensation reaction. The term proanthocyanidins is derived from oxidation reaction

catalyzed by acid which produces red anthocyanidins by heating proanthocyanidins in alcohol-acid solutions (PATRA; SAXENA, 2011; FRUTOS et al., 2004).

Tannins are synthesized during the secondary metabolism of plants and are widely distributed in nutritionally important forage plants (trees, shrubs and legumes), cereals and grains and are considered as anti-nutritional compounds because of their adverse effects on animal intake and performance. However, its role in the favorable modulation of rumen fermentation, such as the reduction of rumen protein degradation, prevention of bloat, inhibition of methanogenesis, is recognized. The inclusion of tannins in diets has shown to enhance body weight and wool growth in sheep, milk production and reproductive performance (PATRA; SAXENA, 2011; JAYANEGARA et al., 2015; ADDISU, 2016). However, studies have shown that the beneficial effects on rumen modulation and animal performance have not been consistently observed. The discrepancies of responses in different studies are attributed to different chemical structures (such as degree of polymerization, stereochemistry and C-C bonding) and tannin concentrations as well as the type of diets (PATRA; SAXENA, 2011).

Several studies in the use of tannins as feed additives to manipulate rumen fermentation seek to find the best ways of using them to improve their effects in order to increase feed efficiency and consequently improve animal performance, such as reproduction, weight gain, milk and wool production as well as to enhance immunity against gastrointestinal parasites. In addition to aspects related to animal performance, it is also sought to find the best ways of using tannins to minimize environmental problems, improving the efficiency of nutrient use by ruminants and, consequently, reducing methane production and nitrogen excretion.

The beneficial effects of tannins may include better utilization of dietary protein, greater weight gain or growth of wool, higher milk production, reduction of enteric methane production and higher fertility. Tannins enhance animal welfare including health, by preventing bloat and reducing parasitic gastrointestinal load (due to their ability to inhibit egg hatching and motility of gastrointestinal nematode larvae especially in small ruminants) (NAUMANN et al., 2017, JAYANEGARA et al., 2012, MUELLER- HARVEY, 2006). The expression of these benefits depends on the source and tannin content in the diets (JAYANEGARA et al., 2012). In a study with the use of tannins (at 0%, 0.2%, 0.4% and 0.6% DM), Rivera-Méndez et al. (2017) found an increase (6.5%) in weight gain and gain efficiency (5.5%) and trend in diet net energy increase (3.2%) in Holstein steers. In the same study, but in another trial with Holstein heifers fed diets containing tannins up to 0.6% in DM, there was a trend to increase average daily gain (6.8%). A review conducted by Piñeiro-Vázquez et al. (2015) revealed that CT incorporation into the ruminant diet may increase weight gain by 26%, which may be due to increased rumen

protein flow to the small intestine or reduced energy loss in form of methane. Ahnert et al. (2015) reported a linear increase in body weight with increase in the inclusion level of tannins (1, 2, 4, and 6%) in diets of heifers. The toxicity of tannins to some rumen microorganisms, low palatability and digestibility of the diet resulting in reduced DMI and consequent performance reduction are some of the negative effects of tannins (NAUMANN et al., 2017; JAYANEGARA et al., 2012; TIEMANN et al., 2008).

2.4.1. Effect of tannins on rumen fermentation

2.4.1.1. Effect of tannins on DMI, digestibility and degradability

The effects of tannins can be beneficial or harmful depending on the type of tannin consumed, the chemical structure and molecular weight, the amount ingested and the animal species involved. High tannin concentrations reduce voluntary intake and digestibility of nutrients, while low to moderate concentrations may improve the digestive efficiency of the protein, mainly due to the reduction of rumen degradation and subsequent increase in the flow of amino acids to the small intestine resulting in increase of performance (FRUTOS et al., 2004).

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). On the other hand, 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).

Several *in vivo* and *in vitro* studies evaluating the effects of tannins on rumen fermentation do not show regularity in improving digestibility of DM and nutrients. Studying the effects of *Leucaena spp* CT on methane production, rumen fermentation and microbial population *in vitro*, Tan et al. (2011) observed reduction in the digestibility of DM in the order of 7 and 15% when the tannin content was 15 and 30 mg/500 mg DM, respectively. In another *in vitro* study conducted by Jayanegara et al. (2015) with the objective of investigating the effects of purified HT and CT on methane production, rumen fermentation and structure of the microbial population, it was observed a reduction of OM digestibility in both tannin classes. Other *in vitro* studies, such as that of Gemeda and Hassen (2015), also indicate reduced digestibility of OM, but Nigrant et al. (2017), studying the effects of the dosages and different sources of tannins on rumen fermentation *in vitro*, showed an increase in degradability and

fermentability. The digestibility of DM increased from 48.4% (tannin-free diet) to 52.9% (diet with 15% tannin source) and, respectively, the digestibility of OM increased from 51.3% to 57.3%. Jin et al. (2013) also observed a linear increase of the *in vitro* digestibility of DM with the increase of tannin content in the diet after 12 hours of incubation.

The meta-analysis of Jayanegara and Palupi (2010) shows that the *in vitro* and *in vivo* digestibility of OM reduced linearly when CT content was increased. Using 163 or 326 g CT/day (extracted from black wattle – *A. mearnsii*) in grazing dairy cows, Grainger et al. (2009) observed reduction in the energy digestibility from 39% to 26% and 22%, respectively, in addition to the reduction of DMI.

Oliveira et al. (2007b), evaluating the effect of tannin levels on *Sorghum bicolor* silage and concentrate supplementation, also showed increase of total apparent digestibility of OM when concentrate was included in the diet, but there was a reduction in the rumen apparent digestibility of the neutral detergent fiber (NDF). In the study of Tiemann et al. (2008), the total apparent digestibility of OM, NDF and ADF reduced when tannin-rich plants were used in substitution of *Vigna unguiculata* in sheep diets. Most of the studies evaluated in the meta-analysis performed by Jayanegara et al. (2012) on the relationship between the level of dietary tannins and methane production in ruminants from *in vivo* and *in vitro* experiments showed decline in total apparent digestibility of OM and especially fiber.

Evaluating the effect of CT from *A. mearnsii* on *in vivo* digestibility of nutrients and energy values measured in sheep under standard conditions, Gerlach et al. (2018a) did not observe effect of tannins in the digestibility of OM on the concentrate when the tannin content in DM was 1%, but it reduced drastically when the tannin content was 3% (-21%) and 5% (-28%). However, the digestibility of the fibrous fraction decreased when the tannin content was 1% and the concentration of the metabolizable energy of the concentrate, estimated from the digestible nutrients, reduced strongly (-25%) from 12,9 (control diet) to 9,7 MJ/kg DM when the tannin content was 5%. Krebs et al. (2007), feeding sheep with diets containing *Acacia saligna* (high tannin content species), as a control diet, observed increase in DMI when they added polyethylene glycol in diets, a substance that reduces the effect of tannins on the digestion of nutrients. In the study of Aguerre et al. (2016), increasing tannin content of diets, there was a linear reduction of DMI (from 25.5 to 23.4 kg/day), as well as a linear increase in milk production by kg of DMI in Holstein cows. It was also observed reduction in the digestibility of DM, OM, CP and NDF.

Evaluating two diets (one with more forage compared to concentrate (59:41) and one with less forage (41:59)) with the addition of 3% of the extract of quebracho (*Schinopsis spp*.),

Dschaak et al. (2011) observed reduction of DMI regardless of the kind of the diets for dairy cows, but the digestibility of DM and nutrients as well as milk production and its components were not affected, and it was concluded that the negative effect of supplementary tannins in consumption resulted in increased feed efficiency. However, with Holstein heifers fed diets containing CT and HT in the proportion up to 0.6% DM, Rivera-Méndez et al. (2017) observed increase in order of 4% in DMI. Ahnert et al. (2015) found no effect on the apparent digestibility of OM in heifers when they used tannins in the concentration of less than 4%, but was impaired when the tannin content was equal to or higher than 4% and the most pronounced effect was for NDF and ADF.

Tshabalala et al. (2013), hypothesizing that treating *Acacia nilotica* fruits with polyethylene glycol, boiling and other treatments would deactivate the tannins and increase the consumption, digestibility and nitrogen retention observed no effect on the consumption, digestibility of DM, OM, NDF, ADF, and nitrogen retention in goats.

Studies indicate that almost all factors that reduce digestion rate (degradability) as well as digestibility of DM reduce feed intake by limiting rumen physical capacity, hence causing a filling effect (NRC, 2001). Therefore, diets with high levels of tannins generally reduce consumption not only because of reduced rumen digestion, but also because of the reduction of feed palatability due to the astringent effect caused by tannins as well as the development of adverse conditions (ADDISU, 2016; WAGHORN, 2008; FRUTOS et al., 2004). Concentrations of tannins above 50 g/kg DM may negatively affect consumption while low concentrations have not had any effect on consumption by ruminants (PATRA; SAXENA, 2011). However, looking at animal performance, the best that is achieved with diets with moderate to low tannin concentration has been attributed to the protective effect of dietary protein against rumen degradation, which leads to the high flow of essential amino acids to the small intestine and consequently their absorption (MAKKAR, 2003).

2.4.1.2. Effect of tannins on nitrogen metabolism

Tannins have the ability to bind proteins rendering them inaccessible to rumen degradation and favoring post-rumen release, thereby promoting increased meat and milk yield (NIGRANT et al., 2017; KREBS et al., 2007). As noted earlier, several authors point out that despite the adverse effects caused by tannins, low concentrations in diets may have beneficial effects on the use of nitrogen (N) by ruminants (PATRA; SAXENA, 2011; MAKKAR, 2003; SLIWINSKI et al., 2002). 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). Therefore, the amount of protein that flows into the abomasum and small intestine from the rumen and the level of digestion thereof in these compartments are the major determinant of ruminant productivity.

The protein that reaches the abomasum consists of a mixture of dietary protein (which escapes microbial degradation) and microbial protein. However, the increase of protein flux from the rumen depends on the reduction of the proteolytic activity of the microorganisms and the increase on the efficiency of the microbial protein synthesis (PATRA; SAXENA, 2011). Thus, the ability of tannins to reduce protein degradation in the rumen is seen as beneficial because it increases the duodenal supply of non-ammonia N.

The strength of the tannin-protein interaction determines the protein digestibility in ruminants. At the rumen pH, the tannin-protein complexes are difficult to dissociate (mainly when the tannins involved are the condensed), making difficult the microbial proteolytic activity, but they are usually dissociated in the abomasum (pH < 3.5) and in the duodenum (pH > 7). Thus, the enzymatic proteolytic activity at these sites results in a higher rate of amino acid uptake by these animals and then increasing N retention rate (MUELLER-HARVEY, 2006; PATRA; SAXENA, 2011). The *Cistus ladanifer*, *Vitis vinifera* and quebracho (*Schinopsis quebracho-colorado*) tannins have the ability to bind soy protein between pH 6 and 8, reducing the solubility of N; these complexes dissociate at pH 2, releasing the protein. Therefore, these additives can be used to feed ruminants as a way to protect the protein against excessive rumen degradation (DENTINHO; BESSA, 2016).

Assessing the effects of two sources of tannins (tannic acid and tannin extract of pistachio by-product (*Pistacia vera*)) on performance, N utilization and microbial efficiency in protein synthesis in dairy goats, Mokhtarpour et al. (2017) found a tendency ($P < 0.10$) to reduce urinary N loss and increase fecal loss with silage treated with tannin extract compared to silage without treatment. There was also an increase in N retention, however there was no effect on microbial efficiency in protein synthesis. The meta-analysis of Jayanegara and Palupi (2010) shows that the digestibility of CP (*in vitro* or *in vivo*) linearly reduced and there was no significant effect on N retention when the tannin content increased.

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. The study on rumen fermentation and N

balance in steers fed diets containing extracts rich in tannins and saponins developed by Sliwinski et al. (2002) showed that tannins have some potential to reduce rumen concentration of ammonia associated with reduced urinary and fecal excretion of N. Gerlach et al. (2018b) found a tendency to reduce urea in milk of Holstein cows when they included 1% of black wattle extract in the diets and when they included tannins up 3% the milk protein production reduced as well as the efficiency of use of N and the concentration of urea in milk. There was a small reduction in N urine loss and small increase in fecal N, but there was no effect on milk production.

Ahnert et al. (2015), working with diets of up to 6% of tannins (infused directly into the rumen through rumen cannula), found a linear reduction in urinary excretion of N and a linear increase in fecal excretion with increasing tannin content, but regardless of level of tannins in the diets, N retention was higher with than without tannins. The urinary excretion of purine derivatives linearly declined when tannins were increased up to 6% in the diets in the study of Dickhoefer et al. (2016), indicating a reduction in the flow of microbial protein in duodenum. In the study of Aguerre et al. (2016), increasing tannin content in the diets, there was a linear decreasing effect on milk urea nitrogen (from 14 to 12.9 mg/dL), milk protein production as well as the concentration. The tannins also reduced the concentration of ammonia N in the rumen but there was no effect on the efficiency of N utilization when the CP content was high. There was a reduction of the total N excretion in the urine, but the fecal N linearly increased. Dschaak et al. (2011), working with dairy cows, also observed reduction of urea N in milk and rumen ammonia, but the efficiency of N utilization for milk was not affected.

2.4.1.3. Effect of tannins on rumen methanogenesis and production of short chain fatty acids

Among several benefits achieved with the use of tannins, methane mitigation may be the most important for ruminant production (NAUMANN et al., 2017). Studies indicate that forages containing tannins and tannin extracts have the potential to reduce methane production *in vivo* or *in vitro*. The reduction in the production of methane by the tannins is of three forms: (1) by the direct effect on the methanogenic archaea; (2) direct effect on the reduction of the number of protozoa in the rumen and, therefore, reduce the methanogenesis associated with these microorganisms (some archaea remain attached to the protozoa) and (3) indirectly through depression of the digestion of the fiber in the rumen (FINLAY et al., 1994; BHATTA et al., 2009; PATRA; SAXENA, 2011; CARRASCO et al., 2017). On the other hand, the effect of tannins on the reduction of the carbohydrate digestion rate, especially cellulose and

hemicellulose, can reduce the total concentration of SCFA in the rumen by reducing the molar concentration of acetate (PATRA; SAXENA, 2011).

Therefore, despite the recognized effect of tannins on increasing nutrient utilization efficiency, such as N, and consequent increase in average daily gain and milk yield (PIÑEIRO-VÁZQUEZ et al., 2015, AHNERT et al., 2015) as well as reduction in methane production, part of this reduction (in methane production) is questioned, since it occurs throughout reduction in the digestion of nutrients. 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. Carulla et al. (2005) also reported that condensed black wattle tannins in the concentration of 2.5% reduced methane production by about 12% due, in part, to 5% of reduction in NDF digestibility. Several other works, such as the study performed by Animut et al. (2008) and Tiemann et al. (2008) also suggested that part of the reduction in methane production observed when tannins are added in diets is due to reduction of nutrient digestion.

The literature review on the potential of CT in reducing methane emissions conducted by Piñeiro-Vázquez et al. (2015) showed that CT reduce protozoa population and methanogenic archaea by as much as 79% and 33%, respectively. They also found that CT bind proteins and polysaccharides forming complexes that reduce the digestibility of DM and OM as well as the production of H_2 used by archaea to form methane. Therefore, they found studies that indicated that CT can reduce methane production by up to 63% when used *in vitro* and up to 58% *in vivo*.

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. In the study performed by Grainger et al. (2009), there was a reduction in methane emissions in grazing dairy cows of 14 and 29% when they supplied 163 and 326 g/day of black wattle condensed tannins, respectively. Working with tannins from quebracho extract at the concentration of 1, 2, 4 and 6% (infused directly into the rumen of heifers through rumen cannula), Dickhoefer et al. (2016) observed that increasing the tannin content the proportions of propionate and butyrate also linearly increased, while those of acetate reduced.

In *in vitro* study of Tan et al. (2011) there was a decrease (linear and quadratic, respectivily) in the methane and SCFA production with the increase of tannin inclusion levels. Reduction in SCFA concentration was observed by Costa et al. (2018) using CT than when using HT. Jayanegara et al. (2015), using HT and CT *in vitro*, observed that both classes of tannins reduced methane production (linearly or quadraticly), but the magnitude of the reduction was higher for HT relative to CT.

Methane production decreased and the SCFA molar ratio changed from acetate to propionate, reducing the acetate:propionate ratio in the tannin study of Nigrant et al. (2017), and Gemeda and Hassen (2015). Jin et al. (2013) observed a linear increase in SCFA concentration 12 hours after incubation and after 48 hours the increase was quadratic, with the molar ratio of acetate increasing linearly, but that of propionate and butyrate reduced after 12 and 48 hours of incubation. The study by Bhatta et al. (2009) showed a reduction in the protozoa population of 12.3% and 36.2% when CT and HT were used, revealing also a synergy effect between these two types of tannins. These reduced the total concentration of SCFA but increased the molar concentration of propionate and, increasing the tannin levels of the diets, it was observed a decrease in total gas and methane production as well as the population of archaea and protozoa. This confirms the findings that tannins suppress methanogenesis through reduction of methanogenic population by direct effect or by reducing protozoa and, hence reduce the methanogens symbiotically associated with protozoa.

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3. ASSOCIATIVE EFFECT OF TANNINS AND MONENSIN ON FEEDING BEHAVIOR, DRY MATTER INTAKE, DIGESTIVE PARAMETERS AND MICROBIAL EFFICIENCY OF NELLORE COWS

Abstract: The objective of this study was to evaluate the associative effect between monensin and different levels of tannin inclusion in the diet on DM and water intake, feeding behavior, digestive parameters, rumen kinetics, synthesis and efficiency of microbial protein synthesis and nitrogen balance. In a 2 x 4 factorial arrangement, 8 cannulated Nellore cows were distributed in 2 contemporary 4 x 4 Latin squares (4 experimental periods of 28 days each) and received 8 experimental diets that differed only in the level of tannin (extract of *Acacia mearnsii*) inclusion (0.0, 0.75, 1.5 and 2.25% of DM) and presence of monensin which was daily administered to each cow of one square (about 32 mg/kg DM). The data were analyzed by the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). The results have shown no significant interaction between monensin and the different levels of tannins ($P > 0.05$). The different levels of tannins linearly increased the number of events of rumination and mastication as well as the total eating time ($P < 0.05$) but without altering the number of daily meals. Tannins also linearly reduced the DM and water intake. Monensin increased the total apparent digestibility of CP by 6.1% while tannins linearly reduced the apparent total digestibility of DM, CP and OM as well as TDN; for the ADF and NDF the reduction was quadratic. The tannins linearly reduced rumen disappearance rate by linearly reduce both passage and digestion rates. Tannins also linearly reduced urinary urea excretion, but both additives had no effect on the synthesis and efficiency of microbial protein synthesis. Monensin reduced the proportion of N excreted in feces, whereas tannins linearly increased fecal N and linearly reduced urinary and retained N. Monensin and tannins have shown an independent effect on feeding behavior, DM and water intake, DM and nutrient digestibility, rumen kinetics, microbial protein synthesis and N balance. Monensin has shown to have potential to promote N utilization by increasing digestibility of CP and reducing fecal excretion of N, although not significantly affecting the urinary N excretion and N retention. The tannins may play an important role in improving rumen pH by promoting masticatory activity. From the environmental point of view, the tannins may play an important role in reducing the excretion of N in the urine and, consequently, reduce the rate of N_2O emission.

Keywords: Degradability; Digestibility; Feeding additive; Microbial protein; Retention

3.1. INTRODUCTION

Dry matter intake (DMI) is fundamentally important in nutrition by establishing the amount of nutrients to an animal for health and production. Many factors may affect voluntary DMI, either by physical filling of reticulo-rumen or by metabolic-feedback. Feeds of low digestibility are thought to place constraints on DMI because of their slow clearance from the rumen and passage through the digestive tract (NRC, 2001).

The use of feed additives, such as monensin and tannins may affect feeding behavior and, consequently, alter DMI. Monensin is a polyether carboxylic ionophore produced by a natural strain of *Streptomyces cinnamonensis* supplied to the cattle orally as a sodium salt (DUFFIELD et al., 2008ab). The effect of monensin on altering feeding behavior and the consequent reduction of DMI, by metabolic-feedback effect, is attributed to the fact that monensin increases energy efficiency of feeds by promoting the production of propionate in rumen, the precursor of gluconeogenesis (DUFFIELD et al., 2008a).

In a meta-analysis of the effects of monensin on feed efficiency of beef cattle, weight gain and DM intake, Duffield et al. (2012) observed that the use of monensin in growth and termination reduces DMI and improves average daily gain as well as feed efficiency. But other studies such as of Van Vugt et al. (2005), Gallardo et al. (2005), Benchaar et al. (2006) and Hamilton et al. (2010) found no effect of monensin on DMI, showing that the effect of monensin on DMI may vary among studies.

In the rumen protein metabolism, monensin is assigned the role of reducing rumen protein digestion (allowing post-rumen digestion) reducing rumen ammonia production by inhibiting the growth of rumen proteolytic bacteria specially the deamination reactions (RANGEL et al., 2008; RUSSELL; STROBEL, 1989).

Tannins are polyphenolic polymers of relatively high molecular weight with the ability to form complexes, mainly with proteins and, to a lesser degree, with carbohydrates (starch and structural carbohydrates) and minerals (ADDISU, 2016). High tannin concentrations reduce voluntary intake and digestibility of nutrients, while low to moderate concentrations may improve the digestive efficiency of the protein (FRUTOS, 2004). Evaluating the effect of CT from *Acacia mearnsii* on *in vivo* digestibility of nutrients and energy value in sheep, Gerlach et al. (2018a) did not observe any effect of tannins on the digestibility of the concentrate OM when the tannin content in DM was 1%, but it reduced drastically when the tannin content was 3% (-21%) and 5% (-28%).

The effect of tannins on reducing DMI is by physical filling of reticulo-rumen and is attributed to the fact that tannins depress fiber digestion by forming complexes with lignocellulose and, hence preventing microbial digestion (PIÑEIRO-VÁZQUEZ et al., 2015), either by direct inhibition of cellulolytic microorganisms or fibrolytic enzymatic activity or both (PATRA; SAXENA, 2011).

Tannins have the ability to bind proteins rendering them inaccessible to rumen degradation and favoring post-rumen release, thereby reduce ammonia concentration in the rumen (NIGRANT et al., 2017; KREBS et al., 2007). Using diets of up to 6% of tannins infused directly into the rumen through rumen cannula, Ahnert et al. (2015) found a linear reduction in urinary excretion of N and a linear increase in fecal excretion, but regardless of level of tannins in the diets, N retention was higher with than without tannins. The urinary excretion of purine derivatives declined linearly when tannins were increased up to 6% in the study performed by Dickhoefer et al. (2016), indicating a reduction in the flow of microbial protein to duodenum.

Therefore, the hypothesis tested in this study was that the combined use of monensin and tannins would improve the synchronization of nutrient utilization and microbial protein synthesis on Nellore cows. So, the study aimed to evaluate the interaction effect of monensin and different levels of tannins of *A. mearnsii* on feeding behavior, DMI, total apparent digestibility, rumen kinetics, synthesis and efficiency o microbial protein synthesis, and N balance of Nellore cows.

3.2. MATERIAL AND METHODS

3.2.1. Ethical issue, animals and place of experimentation

The experiment followed 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 University of Sao Paulo (USP) under the protocol number CEUA 3080240518. The experiment was carried out at the Animal Nutrition and Production Department (VNP) of the College of Veterinary Medicine and Animal Science (FMVZ) of USP, Fernando Costa Campus in Pirassununga, Brazil. The analyzes were performed in the Laboratory of Ruminat Nutrition and in the Laboratory of Chromatography of VNP.

Eight Nellore cows, non-pregnant and non-lactating, carrying rumen cannula and mean body weigth of 582 kg (± 96) were kept in a covered shed in individual pen with free access to

water and sand bedding. The shed had suspended fans that were automatically triggered during the hottest hours of the day (> 28ºC) to ease the effects of temperature on animals.

3.2.2. Treatments and experimental design

Animals were distributed in 2 contemporary 4 x 4 Latin square design, in a 2 x 4 factorial arrangement. The experimental unit was the animal within each experimental period (n = 32 experimental units). The animals received experimental diets which differed only in the inclusion of sodium monensin and the level of tannin inclusion. The diets were represented by the following treatments: 1) diet without tannins, 2) diet with 0.75% of tannins in DM, 3) diet with 1.5% of tannins in DM and 4) diet with 2.25% of tannins in DM. The different cores containing the tannin source were prepared prior to the experiment with the addition of caulin as the tannin level decreased from 2.25% to 0%. In addition to tannins, each cow in one square received daily 300 mg (about 32 mg/kg DM) of sodium monensin (Rumensin® 200, Elanco Animal Health, Brazil) from the beginning to the end of the experiment, administered twice a day (150 mg at 8 a.m. and 150 mg at 4 p.m.), mixed with the feed.

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, 2003b) 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, 2003b).

3.2.3. Feeding management

The feed was offered twice a day (at 8 a.m. and 4 p.m.) in the form of total mixed ration (TMR), in a ratio of 50% of corn silage and 50% of concentrate. The proportions of the various ingredients and the chemical composition of the diets are shown in Table 1.

Table 1- Proportions of ingredients and estimated chemical composition of experimental diets

Ingredients (% DM)	Tannin level			
	0.00%	0.75%	1.50%	2.25%
Corn silage	50.00	50.00	50.00	50.00
Dry ground corn grain	32.36	32.36	32.36	32.36
Soybean meal	12.40	12.40	12.40	12.40
White salt	0.50	0.50	0.50	0.50
Mineral mixture[1]	2.00	2.00	2.00	2.00
Tannin extract[2]	--	0.91	1.82	2.74
Caulin	2.74	1.82	0.91	--
Chemical composition				
Dry matter[3] (%)	60.35	60.35	60.35	60.35
CP[3] (%DM)	14.43	14.43	14.43	14.43
RDP[4] (% PB)	65.30	65.30	65.30	65.30
RUP[4] (% PB)	34.70	34.70	34.70	34.70
NDF[3] (%DM)	28.06	28.06	28.06	28.06
NDFe[4] (%DM)	24.47	24.47	24.47	24.47
ADF[3] (%DM)	15.41	15.41	15.41	15.41
NFC[3] (%DM)	47.59	47.59	47.59	47.59
Starch[4] (%DM)	42.58	42.58	42.58	42.58
MM[3] (%DM)	6.73	6.73	6.73	6.73
Ca[3] (%DM)	0.69	0.69	0.69	0.69
P[3] (%DM)	0.40	0.40	0.40	0.40
EE[3] (%DM)	3.19	3.19	3.19	3.19
TDN[4] (% DM)	74.10	74.10	74.10	74.10
E_L[4] (Mcal/kg)	1.50	1.50	1.50	1.50

[1]Mineral mixture, quantity per kg of product: 140 g of calcium, 80 g of phosphorus, 10 g of sulfur, 129 g of sodium, 80 mg of cobalt, 1400 mg of copper, 800 mg of fluorine, 80 mg of iodine, 1 g of manganese, 20 mg of selenium, 3.5 g of zinc; [2]Extract of *Acacia mearnsii* with 82.3% of total tannins, of which 32.3% condensed tannins; [3]Determined through chemical analysis; [4]Estimated. (Own authorship).

3.2.4. Experimental period

The experiment was carried out in 4 periods of 28 days each. At the end of each period the animals spent two days in pasture before the next period began. The first 15 days of each period were to adapt the animals to the diets, but from the 12th day there was administered titanium dioxide (as an external marker for the determination of digestibility) at 8 a.m. and 4 p.m. until the 21st day at 8 a.m.; from 8 a.m. of the 16th day until 8 a.m. of the 17th day, it was evaluated the feeding behavior; from the 17th to the 21st day the evaluations of dry matter intake (DMI) and water consumption were performed as well as the collection of fecal samples to

determine the total and apparent digestibility and excretion of DM and nutrients. In the same period the degradability of DM and nutrients was also determined (96, 48, 24, 9 and 3 and 0 hours).

From the 23rd to the 25th day rumen content was collected to evaluate the passage rate. This activity was preceded by administration (in the rumen via the cannula) of chromium oxide (20 g) at 8 a.m. on day 23. The collections were made obeying times zero (0), 8, 10, 12, 24, 36 and 48 hours after the administration of chromium oxide. On the 24th day urine was collected every 6 hours (6 a.m., 12 p.m., 6 p.m. and 12 a.m.) to determine urinary parameters and N balance. On days 25 and 26 it was performed the rumen emptying to determine the rumen volume and disappearance rate 3 hours after morning feeding and before (0 hour) morning feeding, respectively.

3.2.5. Evaluation of feeding behavior

The feeding behavior was assessed for 24 hours (from 8 a.m. of 16th day up 8 a.m. of 17th day) by visual monitoring. The animals were observed every 5 minutes and during the night observation the environment was maintained with poor artificial light. The following parameters were evaluated: Eating (E), Drinking (D), Ruminating (R) and Idleness (I), according to the methodology described by Maekawa et al. (2002). Each parameter observed was considered to be executed during the entire interval period (5 minutes) between observations and called activity.

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

The total eating time (TET, min/day) was defined as the sum of the times of each of the 5-minute events in which the animal spent eating. The mean eating time per event (ETE; min/event) was obtained by the TET divided by the NEE. The total rumination time (TRT, min/day), in the same way, was defined as the sum of the times of each rumination event, while the mean rumination time per event (RTE, min/event) was obtained by ratio between the TRT and the NRE. The total masticating time (TMT, min/day) was calculated by the sum of TET and TRT. The daily number of masticating events (NME, events/day) was obtained by the sum of the NEE and the NRE, while the mean masticating time per event (MTE, min/event) was

calculated by the ratio between TMT and NME. The total Idleness time (TIT, min/day) was obtained by the difference between the total period of 24 hours (1440 min) and the TMT.

The ingestion rate of DM and NDF (IR_{DM} and IR_{NDF}, respectively, g/min) was calculated by the consumption of DM or NDF divided by TET. The ingestion rate of DM and NDF per event (IRE_{DM} and IRE_{NDF}, respectively, g/event) was calculated as the ratio between DM or NDF intake and NEE. The rumination rates (RR_{DM} and RR_{NDF}, min/kg) and mastication (MR_{DM} and MR_{NDF}, min/kg) of DM and NDF were also determined, where the TRT or TMT were divided by the ingested amounts of these nutrients on the evaluation day.

3.2.6. Assessment of dry matter and water intake

The dry matter intake (DMI) was evaluated from the 17th up 21st day of each experimental period. Therefore, the feeders were examined daily through observation at 7 a.m. and the feed supply was monitored to ensure daily leftovers of approximately 5%. If there were no feed leftovers the amount offered was increased by 5%. If there were leftovers of approximately 5% the amount offered was maintained and if the leftovers were greater than 5% the amount offered was reduced to 5%. During the 5 days of evaluation, the leftovers from each cow were collected and weighed for the exact quantification of the feed intake obtained by the difference between the amount of feed supplied and the leftovers. On the same days, samples (200 g) of the feeds (silage and concentrate) were collected to determine the DM content, mineral matter (MM), crude protein (CP), ethereal extract (EE), calcium (Ca), phosphorus (P), neutral detergent fiber (NDF) and acid detergent fiber (ADF). The daily water intake was also quantified with the use of individual automatic drinking fountains with water meters.

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

The *in vivo* digestibility of DM and its fractions (CP, EE, NFC, NDF, and ADF) were determined by using the external marker, titanium dioxide (TiO_2), according to Titgemeyer et al. (2001). Therefore, from the 12th up 21st day of each experimental period, TiO_2 was administered (15 g/cow.day) directly into the rumen through the cannula twice a day at 8 a.m. (7.5 g) and at 4 p.m. (7.5 g), the first five days for adaptation and the last five ones for fece collection, twice a day (8 a.m. and 4:00 p.m.). The collected feces were stored in a freezer (at -20^{o}C) until the time of analysis according to the methodology described by Myers et al. (2004). The apparent digestibility coefficients (ADC) were calculated based on the TiO_2 content of the diet and feces using the following equations:

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

$$ADC_N = 100 - 100\left[\left(\frac{\%TiO2d}{\%TiO2f}\right) x \left(\frac{\%Nf}{\%Nd}\right)\right] \quad (2)$$

Where:

ADC_{DM} = DM apparent digestibility coefficient;

ADC_N = Nutrient apparent digestibility coefficient;

% TiO_2d = Titanium dioxide content in diet;

% TiO_2f = Titanium dioxide content in feces;

% Nd = Nutrient content in the diet;

% Nf = Nutrient content in feces.

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

$$\text{Fecal excretion (kg)} = [(100 - ADC) \text{ x Intake (kg)}] /100 \quad (3)$$

The DM content of the feed and feces was determined by drying using a forced air oven at 65ºC for 72 hours according to AOAC (1995). After drying, the samples were milled in willye-type knives mill of 1 mm sieves and stored in properly sealed vials. All analyzes were corrected for the analytical DM content determined at 105°C for 4 hours. The MM was obtained by calcination in a muffle furnace at 550ºC for 4 hours, and the organic matter (OM) was calculated as the difference between 100 and MM (AOAC, 1990). The CP was determined by the total N content (N x 6.25) using the micro-Kjeldahl technique (method 920.87; AOAC, 1990). The EE was determined with the ANKOM XT15 Extractor® equipment (method Am 5-04; AOCS, 2005). The NDF and ADF were determined by the method described by Van Soest et al. (1991), the NDF of the diet was obtained by using thermostable α-amylase. Calcium (Ca) was determined by titration (method 968.08, AOAC, 1995) and phosphorus (P) by colorimetry (method 965.17; AOAC, 1990). The non-fibrous carbohydrate (NFC) content was obtained by subtracting the amounts expressed in percentage of DM of CP, EE, MM and NDF from 100.

3.2.8. Evaluation of rumen kinetics

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

3.2.8.1. Determination of rumen degradability of DM and nutrients

The determination of rumen degradability of DM and nutrients was performed according to the technique proposed by Ørskov et al. (1980). It was conducted between the 17th and 21st days of each experimental period where samples of silage and concentrate were dried at 65°C for 72 hours and milled with Willye-type mills with 2 mm sieves. After grinding, both portions were mixed in proportions of 50:50, then 9 g of this mixture were introduced in 10 x 20 cm nylon bags (with known weight) of 50 μm porosity. These bags were then incubated in rumen via the cannula for 0, 3, 9, 24, 48, and 96 hours. Although they had different incubation time, they were all removed at the same time. After the removal they were washed with fresh water to ensure removal of the soluble material. After that they were dried in a forced air oven at 65°C for 72 hours and finally weighed.

The disappearance of DM was obtained by taking the difference between initial (before incubation) and final (after incubation) weights and calculate the percentage of degraded fraction in the rumen. The zero-time bags (which were not incubated) were introduced in a thermostatic bath at 39^{o}C for 5 minutes and washed with fresh water. Subsequently, they were submitted to the same procedures adopted for the bags of other times. The remaining residues in the bags were analyzed for CP (AOAC, 1990) and NDF (VAN SOEST et al., 1991) in order to determine the rate of degradation of these fractions.

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

$$p = a + b\ (1 - e^{-ct}) \qquad (4)$$

Where:

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

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

b = degradation potential of the water insoluble fraction of the nutritive component analyzed; c = rate of degradation per fermentative action of b;

t = incubation time.

After the determination of the coefficients a, b and c, these were applied in the equation proposed by Ørskov and McDonald (1979) to calculate the real effective degradability (RED) (Equation 5), which represents the amount of the nutritive component (DM, CP or NDF) that has actually been degraded in the rumen. The passage rate (kp) was obtained as described below.

$$RED = a + (b \times c)/c + kp \quad (5)$$

Where:

RED = Real effective degradability of the analyzed nutritive component;
kp = Rumen passage rate.

To determine the RED of NDF there was introduced a lag time in the model according to McDonald (1981). Therefore, the potential degradability (p) was calculated according to the following models also with the aid of the SAS NLIN procedure (version 9.3).

$$p = a \text{ if } t \leq lag \quad (6)$$

$$p = a + b\,[1 - e^{-c \times (t - lag)}] \text{ if } t > lag \quad (7)$$

Where: lag is the time at which the equation derived for a data set equals the actual potencially degradable fraction at zero time (MERTENS, 1993).

The RED of NDF was thereafter calculated using the following equation:

$$RED = [b \times c \times e^{(-kp \times lag)}] / (c + kp) \quad (8)$$

3.2.8.2. Determination of rumen passage rate

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

$$Y = a.e^{-kp \times t} \quad (9)$$

Where:

Y = indicator concentration in time (t);

kp = passage rate in the rumen (h^{-1});

t = indicator sampling time (h);

a = concentration of the indicator at initial time (t_0), assuming instant mixing to the rumen content (ppm);

e = base of the neperian logarithm.

3.2.8.3. Determination of the disappearance rate of rumen solid mass

The disappearance rate was determined by total rumen emptying on days 25 and 26 of each experimental period. The rumen content was removed manually through the rumen cannula 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 on 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 using a 2 mm mesh sieve and buckets, then weighed. Samples of approximately 1 kg of each phase were collected for DM determination. Afterwards, both phases of the content were reconstituted and returned to the rumen. The rumen DM and the disappearance rate (kt) were calculated based on the dry weight of each sample. When the consumption is stable, kt of the feed or a feed fraction is equivalent to its intake rate (ROBINSON et al., 1987), so the kt was estimated using the following equations:

$$kt\ (\%/h) = 100 \times [\text{Daily DMI (kg)} / \text{Rumen content DM (kg)}] / 24 \quad (10)$$

$$kt\ (kg/h) = \text{Rumen content DM (kg)} \times [kt\ (\%/h) / 100] \quad (11)$$

The rumen digestion rate (kd) was determined by the difference between the rumen solid mass disappearance rate (kt) and the rumen passage rate (kp) as follows:

$$kd = kt - kp \quad (12)$$

Where:

kd = rumen digestion rate;

kt = rumen solid mass disappearance rate;

kp = rumen passage rate.

3.2.9. Determination of urinary parameters and nitrogen balance

For the calculation of the production of microbial protein, the urinary volume was determined through creatinine in the urine, according to the methodology described by Valadares et al. (1999).

On the 24th day of each experimental period, urine was collected every 6 hours (at 6 a.m., 12 p.m., 6 p.m. and 12 a.m.) during spontaneous urination or stimulation by vulva massage. At each collection time, 10 mL of urine were taken and diluted in 40 mL of 0.036 N sulfuric acid as preservative in order to reduce the pH to below 3 to avoid losses of nitrogen (VASCONCELOS et al. 2010) as well as bacterial destruction, conservation of purine derivatives and precipitation of uric acid. The samples were stored at -20ºC for analysis of allantoin, uric acid, urea and creatinine.

Allantoin was determined according to the colorimetric method described by Chen and Gomes (1992). The uric acid was determined by colorimetric enzymatic reaction with Uricase and Peroxidase, through commercial kit (Bioclin® Ref K139).

The concentrations of urea and creatinine were determined by using commercial kits (Bioclin® Ref K047 and Bioclin® Ref K067, respectively), through the colorimetric enzymatic reaction and reaction with Alkaline Picrate in buffered medium, respectively.

The daily urinary creatinine excretion (CE) was estimated in relation to animal body weight (BW) in kg using the equation proposed by Chizzotti et al. (2004):

$$CE\ (mg/kg\ BW/d) = 32.27 - 0.01093*BW\ (R^2 = 0.70) \quad (13)$$

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

The excretion of purine derivatives (PuD) in the urine in 24 hours was calculated by multiplying the daily urine volume by the concentration of PuD in the urine sample. The absorbed microbial purines (AP, mmol/day) were calculated from the excretion of purine derivatives in urine (PuD, mmol/day) as proposed by Verbic et al. (1990), by means of the following equation:

$$PuD = 0.85 * AP + 0.385 * BW^{0.75} \quad (14)$$

Where:

PuD = purine derivatives;

AP = absorbed microbial purines;

0.85 = recovery of purines absorbed as urinary derivatives of purines;

$0.385*BW^{0.75}$ = excretion of purines of endogenous origin per kg of metabolic weight per day.

The intestinal flow of microbial nitrogen compounds (micN, g N/day) was calculated in relation to absorbed microbial purines (AP, mmol/day) using the equation described by Chen and Gomes (1992):

$$micN = (70 * AP)/(0.83 * 0.116 * 1000) \quad (15)$$

Where:
micN = microbial nitrogen;
AP = Absorbed microbial purines;
70 = N content in the purines (mg N/mmol);
0.83 = digestibility of microbial purines;
0.116 = ratio of purine N and total N of rumen microorganisms.

The efficiency of microbial N synthesis (EMNS) was calculated by the relationship between the production of microbial N (g) and the amount of organic matter (OM) digested.

The consumption of N was determined by dividing the consumption of CP by 6.25 obtaining the quantity (g) of N consumed. The same calculation was carried out with the CP values of the feces, obtaining the total fecal N. The concentration of ureic N in the urine was obtained by multiplying the concentration of urea by 0.466, corresponding to the N content in urea. The retained N was obtained by calculating ingested N minus excreted N (feces + urine). The N balance, in percentage, was obtained by the relation of the N contained in the fractions (feces, urine or retained) by ingested N, using the technique described by Silva and Queiroz (2002).

3.2.10. Statistical analysis

The data were analyzed by using the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). Before they were analyzed they were evaluated in relation to the presence of discrepant information (outliers) and normality of the residues by the Shapiro-Wilk test. When the normality premises were not met the data were transformed. The data of DM and water intake, feeding behavior, *in vivo* digestibility, and rumen kinetics and rumen microbial protein production were submitted to analysis of variance which separated, as causes of variation, the monensin effect (also considered as the effect of the square), tannin level effect, and interaction between monensin and tannin level, period effect and animal effect within the square. The level effect was evaluated by the use of orthogonal polynomials, separating the effects in linear,

quadratic and quadratic deviation. The statistical model used was described according to the equation below:

$$Y_{ijkl} = \mu + L_i + M_j + L_i*M_j + P_k + A_l(S_j) + e_{ijkl}$$

Where:

Y_{ijkl} = observation concerning Level (i) + Monensin (j) + Level (i) * Monensin (j) + Period (k) + Animal (l) within the square (j) + random error associated with each observation (e_{ijkl});

μ = overall mean;

L_i = Level effect (fixed effect);

M_j = Effect of monensin (fixed effect);

L_i*M_j = Interaction between level (i) and monensin (j) (fixed effect);

P_k = Period effect (random effect);

$A_l(S_j)$ = Effect of animal within the square (random effect);

e_{ijkl} = Random error associated with each observation. A significance level of 5% was adopted.

3.3. RESULTS

3.3.1. Feeding behavior, feed intake and total apparent digestibility of DM and nutrients

There was neither significant interaction between monensin and tannins nor significant effect of monensin was observed on feeding behavior ($P > 0.05$). The different levels of tannins had a linear increasing effect ($P < 0.05$) on the number of events (NE) of rumination and mastication as well as on total eating time (TET), but they had no effect on the number of daily meals (eating events) (Table 2). Tannins had linear decreasing effect ($P < 0.05$) on intake and rumination of DM (kg/NE) and, consequently, NDF for each event the animals engaged to consumption or rumination (Table 3), causing the animals have to take more time to consume and ruminate the same amount of DM or NDF (min/kg). The animals consumed and masticated more DM or NDF per minute (DM or NDF, kg/min) in 0.75% tannin inclusion level and consequently spent less time to consume 1 kg of DM or NDF (DM or NDF, min/kg). It made the different levels of tannins have a quadratic effect on these variables.

Table 2 - Feeding behavior of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
Idleness										
NE	20.38	21.44	21.00	21.00	20.38	21.25	0.55	NS	NS	NS
TIT (min)	806.3	814.1	816.3	832.5	808.1	783.8	16.8	NS	NS	NS
TIT (%)	55.99	56.53	56.68	57.81	56.12	54.43	1.16	NS	NS	NS
ATE (min)	40.77	38.55	39.44	40.38	40.93	37.90	1.41	NS	NS	NS
Ruminating										
NE	13.75	14.06	13.38	13.25	13.88	15.13	0.50	NS	0.0242^{L}	NS
TRT (min)	427.7	391.3	408.8	406.3	406.3	416.3	14.3	NS	NS	NS
TRT (%)	29.69	27.17	28.39	28.21	28.21	28.91	0.99	NS	NS	NS
ATE (min)	32.59	28.11	31.22	30.92	30.22	29.04	1.30	NS	NS	NS
Drinking water										
NE	4.69	4.69	4.50	4.50	4.75	5.00	0.44	NS	NS	NS
TDT (min)	23.44	23.44	22.50	22.50	23.75	25.00	2.20	NS	NS	NS
TDT (%)	1.63	1.63	1.56	1.56	1.65	1.74	0.15	NS	NS	NS
ATE (min)	4.69	5.00	5.00	4.38	5.00	5.00	0.15	NS	NS	NS
Eating										
NE	8.31	9.06	8.50	8.00	8.63	9.63	0.42	NS	NS	NS
TET (min)	182.2	211.3	192.5	178.8	201.9	215.0	8.18	NS	0.0263^{L}	NS
TET (%)	12.70	14.67	13.37	12.41	14.02	14.93	0.57	NS	0.0263^{L}	NS
ATE (min)	22.68	23.97	23.29	22.93	24.47	22.60	0.79	NS	NS	NS
Masticating										
NE	22.06	23.13	21.88	21.25	22.50	24.75	0.66	NS	0.0247^{L}	NS
TMT (min)	610.3	602.5	601.3	585.0	608.1	631.3	16.1	NS	NS	NS
TMT (%)	42.38	41.84	41.75	40.63	42.23	43.84	1.12	NS	NS	NS
ATE (min)	28.41	26.32	28.21	27.60	27.77	25.89	0.90	NS	NS	NS

SEM: Standard error of mean; M*L: Interaction between monensin and tannin level; NE: Number of events; TIT (min): Total idleness time; ATE (min): Average time per event; TRT (min): Total ruminating time; TDT (min): Total water drinking time; TET (min): Total eating time; TMT (min): Total masticating time. (Own authorship).

There was no significant effect of monensin ($P > 0.05$) on dry matter intake (DMI) or water consumption. The different levels of tannins linearly reduced the DMI ($P = 0.0034$) (the highest level reduced DMI by 11.13%, when compared to control treatment) and water consumption ($P = 0.0120$), but the differences were not significant when the data were corrected according to animal body weight (% of BW) (Table 4).

There was no interaction between monensin and tannins on digestibility or excretion of DM and nutrients (Table 5), but there was a significant effect of monensin on increasing CP digestibility by 6.1% ($P = 0.0387$). No significant effect of monensin was observed on the other parameters of digestibility and excretion of DM or nutrients. There was no effect of the different levels of tannins on the digestibility or excretion of EE but there was a reduction of digestibility in all other parameters evaluated. The reduction was linear for DM, CP, NFC, OM and TDN, but was quadratic for NDF and ADF (the highest level reduced the digestibility of DM, CP and NDF by 8.54, 16.50 and 23.89%, respectively, when compared to control treatment). The figure

1 presents the effect of tannins on the digestibility of DM, CP, NDF and ADF. The different levels of tannins linearly increased the excretion (kg/day) of DM and nutrients (Table 5).

Table 3 - Efficiency of consumption, rumination and mastication of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
Consumption										
DM, kg/min	0.055	0.046	0.052	0.057	0.050	0.044	0.003	NS	0.0285^{Q}	NS
NDF, kg/min	0.015	0.013	0.014	0.016	0.014	0.012	0.001	NS	0.0285^{Q}	NS
DM, kg/NE	1.254	1.096	1.226	1.291	1.190	0.995	0.072	NS	0.0354^{L}	NS
NDF, kg/NE	0.344	0.301	0.336	0.354	0.327	0.273	0.020	NS	0.0354^{L}	NS
DM, min/kg	20.04	22.52	19.90	19.07	21.53	24.62	0.971	NS	0.0386^{Q}	NS
NDF, min/kg	73.00	82.05	72.49	69.48	78.44	89.70	3.539	NS	0.0386^{Q}	NS
Rumination										
DM, kg/min	0.022	0.025	0.024	0.024	0.025	0.021	0.001	NS	0.0453^{Q}	NS
NDF, kg/min	0.006	0.007	0.007	0.007	0.007	0.006	0.000	NS	0.0453^{Q}	NS
DM, kg/NE	0.745	0.688	0.753	0.743	0.743	0.630	0.043	NS	0.0278^{L}	NS
NDF, kg/NE	0.205	0.189	0.207	0.204	0.204	0.173	0.012	NS	0.0278^{L}	NS
DM, min/kg	46.99	41.38	42.09	43.26	43.10	48.28	1.660	NS	0.0059^{L}	NS
NDF, min/kg	171.2	150.7	153.3	157.6	157.0	175.9	0.049	NS	0.0059^{L}	NS
Mastication										
DM, kg/min	0.015	0.016	0.016	0.017	0.016	0.014	0.001	NS	0.0131^{Q}	NS
NDF, kg/min	0.004	0.004	0.004	0.005	0.004	0.004	0.000	NS	0.0131^{Q}	NS
DM, kg/NE	0.443	0.417	0.460	0.452	0.448	0.360	0.020	NS	0.0028^{L}	NS
NDF, kg/NE	0.122	0.114	0.126	0.124	0.123	0.099	0.005	NS	0.0028^{L}	NS
DM, min/kg	67.03	63.90	61.98	62.34	64.63	72.90	1.966	NS	0.0384^{Q}	NS
NDF, min/kg	244.2	232.8	225.8	227.1	235.4	265.6	7.164	NS	0.0384^{Q}	NS

SEM: Standard error of mean; M*L: Interaction between monensin and tannin level; NE: Number of events; DM: Dry matter; NDF: Neutral detergent fiber. (Own authorship).

Table 4 - Feed and water intake of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*

Intake	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
DM, day										
kg	9.34	9.49	9.80	9.59	9.56	8.71	0.275	NS	0.0034^{L}	NS
% of BW	1.48	1.54	1.54	1.54	1.52	1.44	0.046	NS	NS	NS
Water, day										
L	31.67	24.00	29.25	28.58	27.75	25.75	1.596	NS	0.0120^{L}	NS
L/kg.DM	3.31	2.54	2.99	2.96	2.84	2.93	0.111	0.0533	NS	NS
% of BW	4.93	3.93	4.61	4.52	4.35	4.25	0.232	NS	NS	NS

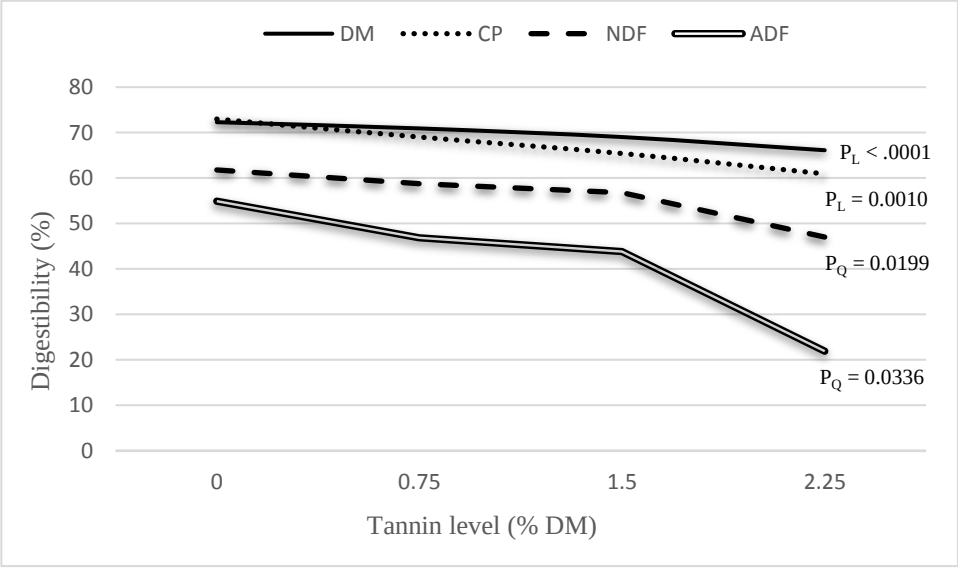
SEM: Standard error of mean; M*L: Interaction between monensin and tannin level. (Own authorship).

Table 5 - Total apparent digestibility and excretion of DM and its fractions of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
Digestibility (%)										
DM	68.21	70.96	72.29	70.92	69.02	66.12	0.862	NS	<.0001^{L}	NS
CP	65.10	69.07	72.97	69.02	65.41	60.93	1.100	0.0387	0.0010^{L}	NS
NDF	56.24	55.93	61.78	58.78	56.77	47.02	1.655	NS	0.0199^{Q}	NS
ADF	41.12	42.64	54.91	46.84	43.85	21.91	3.032	NS	0.0336^{Q}	NS
EE	73.84	74.00	73.96	73.66	74.96	73.10	1.433	NS	NS	NS
NFC	81.40	85.67	85.96	84.71	81.57	81.88	0.819	0.0534	0.0063^{L}	NS
OM	71.03	73.73	76.24	74.09	71.36	67.82	0.928	NS	<.0001^{L}	NS
TDN	69.24	71.76	74.10	72.08	69.59	66.22	0.897	NS	<.0001^{L}	NS
Excretion (kg/day)										
DM	2.74	2.56	2.53	2.57	2.76	2.75	0.098	NS	0.0894^{L}	NS
CP	0.43	0.39	0.35	0.39	0.44	0.45	0.015	NS	<.0001^{L}	NS
NDF	1.05	1.09	0.99	1.02	1.08	1.21	0.044	NS	0.0053^{L}	NS
ADF	0.78	0.78	0.64	0.72	0.77	0.98	0.041	NS	<.0001^{L}	NS
EE	0.070	0.073	0.076	0.072	0.070	0.069	0.004	NS	NS	NS
NFC	0.78	0.61	0.62	0.66	0.79	0.71	0.046	NS	0.0792^{L}	NS
OM	2.33	2.16	2.03	2.14	2.38	2.44	0.095	NS	0.0040^{L}	NS
N	0.067	0.062	0.056	0.063	0.071	0.072	0.002	NS	<.0001^{L}	NS

SEM: Standard error of mean; M*L: Interaction between monensin and tannins. (Own authorship).

Figure 1 - Graph representation of the effect of different levels of tannins on nutrient digestibility.



DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber. (Own authorship).

3.3.2. Rumen kinetics

There was neither significant effect of monensin (P > 0.05) on the solid, liquid and total rumen mass nor on the disappearance rate (kt) of the rumen solid mass as well as on the DM

content of the total rumen mass. The different levels of tannins also did not significantly affect the DM content of the rumen mass, but linearly increased ($P < 0.05$) the liquid, solid and total mass (Table 6). Tannins linearly reduced the rate of disappearance of rumen solid mass ($P < 0.05$), but when the liquid and total mass were expressed as percentage of BW the effect was quadratic ($P < 0.05$), while the solid mass increased linearly ($P = 0.0132$).

Table 6 - Rumen dynamics of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*

Rumen content	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
DM, %	12.92	13.49	12.92	13.04	13.46	13.40	0.211	NS	NS	NS
Liquid mass										
kg	35.69	34.94	33.34	34.77	36.89	36.26	0.979	NS	0.0085^{L}	NS
% of BW	5.62	5.47	5.21	5.53	5.86	5.57	0.107	NS	0.0219^{Q}	NS
Solid mass										
kg	5.36	5.45	5.01	5.23	5.72	5.67	0.203	NS	0.0117^{L}	NS
% of BW	0.84	0.89	0.78	0.83	0.91	0.93	0.030	NS	0.0132^{L}	NS
Total mass										
kg	41.05	40.39	38.34	40.00	42.61	41.93	1.164	NS	0.0072^{L}	NS
% of BW	6.46	6.32	6.00	6.36	6.77	6.42	0.128	NS	0.0207^{Q}	NS
Disappearance rate (kt)										
kg/h	0.395	0.402	0.416	0.405	0.404	0.369	0.011	NS	0.0016^{L}	NS
%/h	7.69	7.45	8.65	7.84	7.10	6.684	0.235	NS	0.0001^{L}	NS

SEM: Standard error of mean; M*L: Interaction between monensin and tannin level; kt: Disappearance rate of solid rumen mass. (Own authorship).

There was neither significant effect of monensin nor interaction ($P > 0.05$) between monensin and tannins on **a**, **b**, **c** and **lag** parameters (Table 7). Consequently, there was neither significant effect of monensin nor interaction between monensin and tannins on the degradability and undigested fractions (Und) of DM, NDF or CP.

The different levels of tannins had no significant effect on "**a**" parameter but linearly reduced ($P < 0.05$) parameters **b**, **c** and **lag**. They also reduced linearly the real effective (RED) and potential (PD) degradability of DM and NDF. Despite the linear reduction of the potential degradability of CP, the RED was not altered. The different levels of tannins linearly increased the rumen undigested fractions of DM, NDF or CP.

Table 7 - *In situ* degradability of DM, NDF and CP of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*.

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
DM										
a (%)	32.84	32.53	32.39	32.87	32.41	33.08	0.155	NS	NS	NS
b (%)	53.29	52.90	55.15	52.77	52.79	51.65	0.500	NS	0.0183^{L}	NS
c (h^{-1})	0.043	0.043	0.051	0.046	0.041	0.035	0.002	NS	$0,0010^{L}$	NS
RED (%)	62.67	61.65	64.00	62.25	61.37	61.03	0.761	NS	0.0816^{L}	NS
PD (%)	86.13	85.58	87.54	85.64	85.52	84.73	0.409	NS	0.0268^{L}	NS
Und (%)	13.87	14.42	12.46	14.36	14.48	15.27	0.409	NS	0.0268^{L}	NS
NDF										
b (%)	65.12	65.05	69.75	66.99	62.93	60.67	1.092	NS	0.0008^{L}	NS
c (h^{-1})	0.030	0.026	0.034	0.025	0.028	0.023	0.002	NS	0.0399^{L}	NS
lag (h)	2.64	3.63	2.59	2.95	2.96	4.04	0.288	NS	0.0301^{L}	NS
RED (%)	27.14	25.17	31.64	24.98	24.88	23.12	1.411	NS	0.0087^{L}	NS
PD (%)	65.12	65.05	69.95	66.99	62.93	60.67	1.092	NS	0.0008^{L}	NS
Und (%)	34.88	34.95	30.25	33.02	37.07	39.33	1.092	NS	0.0008^{L}	NS
CP										
a (%)	33.83	34.52	32.83	34.94	34.05	34.87	0.498	NS	NS	NS
b (%)	60.29	60.54	63.90	59.95	58.74	59.07	0.741	NS	0.0208^{L}	NS
c (h^{-1})	0.042	0.039	0.046	0.045	0.041	0.032	0.002	NS	0.0114^{L}	NS
RED (%)	66.89	65.97	67.93	67.57	66.07	64.16	0.913	NS	NS	NS
PD (%)	94.12	94.99	96.74	94.89	92.80	93.79	0.517	NS	0.0095^{L}	NS
Und (%)	5.88	5.007	3.26	5.11	7.20	6.21	0.517	NS	0.0095^{L}	NS

SEM: Standard error of mean; M*L: Interaction between monensin and level; **a:** Interseption of the curve at time zero, water-soluble and completely degradable fraction of the analyzed nutritive component leaving the nylon bag rapidly; **b:** Potentially degradable fraction; **c:** Rate of degradation of the potentially degradable fraction; **lag:** time at which the equation derived for a data set equals the actual potencially degradable fraction at zero time; RED: Real effective degradability; PD: Potential degradability (a + b); Und: Undigested fraction (100-PD). (Own authorship).

There was neither significant interaction between monensin and tannins nor significant effect of monensin on parameters of rumen kinetics ($P > 0.05$), but it can be seen in table 8 that the above-mentioned effect of the different levels of tannins on the linear reduction of rumen disappearance rate (kt) was through linear reduction ($P < 0.05$) of both digestion (kd) and passage (kp) rates.

Table 8 - Rumen kinetics of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
kt (%/h)	7.69	7.45	8.65	7.84	7.10	6.68	0.235	NS	0.0001^{L}	NS
kp (%/h)	3.45	3.50	3.82	3.73	3.38	2.99	0.153	NS	0.0122^{L}	NS
kd (%/h)	4.24	3.94	4.84	4.11	3.72	3.70	0.226	NS	0.0316^{L}	NS

SEM: Standard error of mean; M*L: Interaction between monensin and level; kt: Rate of disappearance of the solid mass in the rumen (kp + kd); kd: Rate of digestion in the rumen (kt – kp); kp: Rate of passage of undigested residues through the digestive tract (kt – kd). (Own authorship).

3.3.3. Rumen microbial protein, efficiency of microbial nitrogen synthesis and nitrogen balance

It was not observed any significant interaction between monensin and tannins as well as monensin alone (P > 0.05) on the volume of urine (L/day), urinary compounds (mmol/day), microbial protein synthesis (g/day) or on the efficiency of microbial nitrogen synthesis (EMNS). It was also not observed significant effect of the different levels of tannins on urinary volume, microbial protein synthesis or EMNS, but there was a linear decreasing effect on urinary urea (g/day) excretion (P = 0.0051) (when compared to control treatment, the highest-level reduced urea excretion by 22.7%) and uric acid (mmol/day) (P = 0.0092) (Table 9).

Table 9 - Urinary volume, excretion of urinary compounds, microbial nitrogen synthesis and efficiency of microbial protein synthesis of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii.*

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
Urinary volume										
L/day	10.7	7.54	9.38	9.50	9.19	8.32	0.578	NS	NS	NS
Urinary compounds										
Urea, g/day	137.2	138.3	154.7	143.7	132.9	119.6	9.258	NS	0.0051^{L}	NS
Al, mmol/day	127.3	129.0	130.7	130.1	126.7	125.1	4.149	NS	NS	NS
UA, mmol/day	19.38	20.45	22.00	19.59	20.15	17.92	1.200	NS	0.0092^{L}	NS
PuD, mmol/day	146.6	149.6	152.7	149.9	146.7	143.0	4.608	NS	NS	NS
Al (%)PuD	86.89	86.18	85.50	86.77	86.41	87.44	0.695	NS	0.0819^{L}	NS
Synthesis of micN										
g/day	83.91	86.81	89.08	86.92	84.00	81.44	3.878	NS	NS	NS
EMNS, g/kg DOM	14.86	14.10	13.94	13.71	14.02	16.25	0.687	NS	NS	NS

SEM: Standard error of mean; M*L: Interaction between monensin and tannin level; Al: Allantoin; UA: Uric acid; PuD: Purine derivatives; Al (%) PuD: Allantoin percentage in total purine derivatives; micN: Microbial nitrogen; EMNS: Efficiency of microbial nitrogen synthesis; DOM: Digested organic matter. (Own authorship).

No significant differences of monensin were observed on the amount (g/day) of N ingested or excreted (in feces or urine) (P > 0.05), but on the proportion excreted in feces (P = 0.0390) (Table 10).

The different levels of tannins linearly reduced N ingestion (P = 0.0043), urinary N (P = 0.0051) and N retention (P = 0.0027), but linearly increased the fecal N (P < .0001). when compared to control treatment, the highest-level reduced N ingestion, urinary N and N retention by 12.11, 22.69 and 29.27%, respectively, and increased fecal N by 29.01%. Regarding to the ingested N, the different levels of tannins linearly reduced the proportion of N excreted in urine

(P = 0.0276), linearly increased the proportion of N excreted in feces, but linearly reduced the proportion of N retained (P = 0.0228).

Table 10 - Nitrogen balance of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii.*

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
N ing., g/day	205.7	209.9	217.1	211.7	211.5	190.8	6.260	NS	0.0043[L]	NS
N excreted, g/day										
Feces	68.60	62.36	55.99	62.85	70.85	72.23	2.328	NS	<.0001[L]	NS
Urine	63.91	64.45	72.10	66.96	61.94	55.74	4.314	NS	0.0051[L]	NS
N retained, g/day	73.17	83.09	89.00	81.88	78.69	62.95	5.683	NS	0.0027[L]	NS
N balance, % of N ing.										
Feces	33.86	30.03	26.26	30.08	33.54	37.90	1.065	0.0390	<.0001[L]	0.0828
Urine	31.58	30.43	34.58	31.26	29.04	29.12	1.960	NS	0.0276[L]	NS
Retained	34.57	39.54	39.15	38.66	37.42	32.98	2.201	NS	0.0228[L]	NS

SEM: Standard error of mean; M*L: Interaction between monensin and level of tannins; N ing: N ingested; N retained: N ingested – N excreted (feces + urine). (Own authorship).

3.4. DISCUSSION

3.4.1. Feed intake and feeding behavior

The lack of interaction between monensin and tannins on DMI and water consumption may indicate independence of these additives on these parameters.

The meta-analyzes performed by Duffield et al. (2008b), evaluating the impact of monensin on dairy cows, and Duffield et al. (2012), evaluating the effects of monensin on beef cattle, have shown reduction of DMI in both dairy and beef cattle, but the studies performed by Odongo et al. (2007), Van Vugt et al. (2005), Gallardo et al. (2005), Benchaar et al. (2006), Hamilton et al. (2010), Mullins et al. (2012) and Perna Junior et al. (2017) found no effect of monensin. In the present study no significant effect of monensin was observed on DM and water intake (Table 4). These differences may demonstrate the inconsistency of monensin on consumption, reinforcing the idea that the effect of this feed additive on consumption might depend on the study and especially on the energy content of the diet. The lack of significant effect of monensin on water consumption was also observed in pre- and postpartum periods of dairy cows in the study of Mullins et al. (2012).

The linear reduction of DMI caused by the different levels of tannins corroborates with the meta-analysis of Jayanegara and Palupi (2010). The reduction of DMI by using tannins on ruminant feeding was also observed by Aguerre et al. (2016), Grainger et al. (2009) and Dschaak et al. (2011).

Patra and Saxena (2011) stated that concentrations of tannins above 50 g/kg DM may negatively affect consumption while low concentrations usually have no effect. However, taking into a count the highest level of tannins in the present study (22.5 g/kg DM), it is low than the level mentioned by these authors, but even though the DMI was reduced. This difference 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, and not only by the amount ingested and the animal species involved (MAKKAR, 2003a; FRUTOS et al., 2004 and MUELLER-HARVEY, 2006).

Unlike monensin, the different levels of tannins linearly reduced water consumption. This effect was somewhat surprising, since it was thought that with the astringent effect of tannins the animals would consume more water as tannin levels increased. Perna Junior (2018) found no effect on water consumption when included tannins of *A. mearnsii* in Holstein and Nellore cows' diet, but the lack of effect may have been due to the highest level of tannins which was 1.5%.

In the study of Mullins et al. (2012), in transition dairy cows, it was not observed effect of monensin on feeding behavior but reduction of inter-meal interval (min) in pre-partum period and a tendency to reduce inter-meal interval (min) in post-partum period. In the present study it was not observed any effect of monensin on the evaluated feeding behavior parameters (Tables 2 and 3). This may be explaned by the fact that the additive was not able do cause significant differences on DMI or water consumption.

The data in table 2 show that the different levels of tannins linearly increased the number of ruminating and masticating events (NE) although not altering the total ruminating (TRT) or masticating (TMT) time. This effect is considered positive since the stimulation of mastication and rumination elicits saliva production ensuring the maintenance of optimum rumen pH levels. The linear increase of the total eating time (TET), associated with the reduction of DMI (Table 4) might be due to astringent effect of tannins which caused the reduction of consumption rate whenever the animals engaged in consumption activity and consequently reducing the amount of DM and NDF consumed per eating event and increase the time to eat, ruminate and masticate the same amount of DM or FDN (Table 3). Therefore, the effects of tannins observed in the present study corroborate with Lamy et al. (2011), Patra and Saxena (2011),, and Addisu (2016) in asserting that tannins have astringent effect, decrease feed palatability and consequently reduce the amount of feed ingested.

3.4.2. Digestibility and excretion of DM and nutrients

The interaction between monensin and the different levels of tannins was not significant, showing the independence of these two additives on the digestibility and excretion of nutrients.

Ruiz et al. (2001), studying the effect of monensin on performance and N utilization of lactating cows, observed reduction of fecal N and increase of total digestibility of this nutrient. Oliveira et al. (2007) observed a significant reduction of N loss from feces in animals receiving monensin. Although the reduction of fecal N (g/day) was not significant with monensin (compared to control group), the present study corroborates with these authors, as monensin increased the digestibility of CP and reduced the proportion of N excreted (% of N ingested) in feces (Table 10). This may indicate that monensin has improved the efficiency of N utilization. This effect was not observed in the different levels of tannins where the digestibility of CP linearly decreased and, as a consequence, the fecal N linearly increased. Increase in fecal N was also observed by Gerlach et al. (2018b) when they included tannins in Holstein cows' diets. Ahnert et al. (2015) also observed a linear increase of fecal N in diets with up to 6% of tannins.

The reduction of CP digestibility may have been due to formation of protein-tannin complexes in the rumen, reducing protein degradation in this compartment. So, according to Addisu (2016) and Piñeiro-Vázquez et al. (2015), these complexes may also resist post-rumen enzymatic action, culminating in greater fecal N. Tannins may depress the fiber digestion by forming complexes with lignocellulose and, hence 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). This may be the cause of the observed reduction of digestibility in the present study (Table 5) and the consequent linear increase in the excretion of total DM, including fiber (NDF and ADF), OM, NFC and TDN, in addition to the N discussed above.

3.4.3. Rumen degradability and rumen kinetics

There was an independent action between monensin and the different levels of tannins on rumen degradability and rumen kinetics, indicated by the lack of significant interaction.

The lack of effect of monensin on rumen degradability (Table 7) and all parameters of rumen kinetics (Tables 6 and 8) was also observed by Perna Junior et al. (2017) when added monensin (300 mg/cow.day) in the diet of Holstein cows.

The different levels of tannins linearly reduced the RED of DM and NDF (Table 7), while Perna Junior (2018) only observed linear reduction of the ED of NDF and CP and not of DM. Despite the linear reduction of CP digestibility and linear increase in fecal excretion of CP

(Table 5), probably due to the formation of tannin-protein complexes, the different levels of tannins in this study were not able to significantly alter the RED of CP. The linear reduction of RED and PD, and linear increase of rumen undigested (Und) fractions of DM caused by tannins were not observed by Perna Junior (2018). The linear reduction of rumen solid mass disappearance rate was due to the linear reduction of passage (kp) and digestion (kd) rates observed in this study (Table 8). This may have significantly influenced the reduction of DM intake discussed above (Table 4) by increasing the rumen retention time of feed particles. Perna Junior (2018) observed no significant effect on the kp although has observed linear reduction of kt and kd. These differences may be explained by the fact that in the study of this author the highest level of tannins was 1.50% of DM, which may not have been enough to alter these parameters, since he also used tannins from *A. mearnsii.* Feeding up to 4% of quebracho extract for goats, Al-Kindi et al. (2017) also did not observe changes in the kp of the feed particles. Some of the many factors to explain these differences include the type of tannins, the chemical structure and molecular weight, and not only the amount of tannins ingested and the animal species involved (FRUTOS et al., 2004; MUELLER-HARVEY, 2006).

Although the different levels of tannins have reduced DM intake, their effect on the reduction of kp can be seen as beneficial since the long duration of feed particles in the rumen would minimize the effect of low kd caused by these additives and improve the digestibility of DM and nutrients.

3.4.4. Urinary parameters, microbial protein and nitrogen balance

The lack of significant interaction between monensin and the different levels of tannins on urinary parameters, microbial protein and nitrogen balance may indicate independence between these additives.

Monensin did not significantly affect rumen microbial activity. This was observed by the lack of effect on urinary urea, purine derivatives as well as on microbial N synthesis (micN) and on the efficiency of microbial N synthesis (EMNS). The lack of effect of monensin on urinary volume observed may be explained by the lack of significant effect of this additive on water consumption (Table 4). The linear reduction of water consumption caused by tannins was not accompanied by significant effect on urinary volume, although it may be seen on table 9 that there was a reduction on urinary volume as the levels of tannins increased but the reduction was not statistically significant. Perna Junior (2018) only found a tendency to quadratic effect on the urinary volume.

Unlike monensin, the different levels of tannins may have had a great role in inhibiting rumen protein degradation, reducing the rate of urea production by liver. This effect was observed by the linear reduction of urinary urea excretion besides the linear reduction on uric acid excretion. This shows that tannins may have potential to reduce rumen microbial activity, reducing, as the consequence, the flow of microbial protein in duodenum. Dickhoefer et al. (2016) found linear decline on urinary excretion not only of uric acid but of both allantoin and uric acid. The difference might be due to the level of tannins as these authors included tannins up to 6% of DM. The lack of effect of tannins on micN or EMNS was also observed by Aguerre et al. (2016) and Mokhtarpour et al. (2017), although they found reduction on the concentration of ammonia N in the rumen but Gerlach et al. (2018b) found reduction of EMNS.

Ruiz et al. (2001) and Oliveira et al. (2007) observed a significant effect of monensin on reducing fecal N. This effect was also observed in the present study and along with the increased protein digestibility observed it may be thought that this additive improved the efficiency of N utilization although no effect was observed on the N excreted in urine and the monensin effect on increasing N retention was not significant (Table 10).

In the studies of Mokhtarpour et al. (2017), Aguerre et al. (2016), Ahnert et al. (2015), Gerlach et al. (2018b), Perna Junior (2018) and the meta-analysis of Jayanegara and Palupi (2010) the different levels of tannins linearly reduced the urinary N and linearly increased the fecal N. In the present study it was also observed a linear reduction of urinary N and linear increase of fecal N, and there was a reduction of N retention. The change in N excretion from urine to feces is a widely known tannin effect (THEODORIDOU et al., 2010). There was no significant effect on the RED of CP, but it is also widely known that the formation of tannin-protein complexes in the rumen increases the supply of rumen undegradable protein (RUP) to the duodenum leading to reduction of N losses through urine. Perna Junior (2018) and the meta-analysis of Jayanegara and Palupi (2010) observed no significant effect on N retention, but in the study of Ahnert et al. (2015) the N retention increased. These results show the effect of tannins on the inhibition of protein degradation in the rumen due to the formation of protein-tannin complexes which, besides hindering the microbial degradation of the protein, the complexes may hinder post-rumen enzymatic action culminating in increased fecal excretion of N. Depending on the type of tannins (condensed or hydrolysable) or the level of inclusion in the diets, the fecal excretion of N may be higher or lower resulting in differences in the proportion of N retained (FRUTOS et al., 2004).

The change of the pathway of N excretion caused by the different levels of tannins (from urine to feces) may be considered positive from the environmental point of view, since the fecal

N has a lower N_2O emission factor (0.15%) when compared to the urinary N (0.26%) (SORDI et al., 2013).

3.5. CONCLUSIONS

Monensin and tannins have shown an independent effect on feeding behavior, DM and water intake, DM and nutrient digestibility, rumen kinetics, synthesis and efficiency of microbial protein synthesis. Monensin has shown to have potential to promote N utilization by increasing digestibility of CP and reduce the proportion of fecal N, although not affecting urinary N excretion.

The tannins may play an important role in reducing N_2O emissions through the reduction excretion of N in the urine.

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4. ASSOCIATIVE EFFECT OF TANNINS AND MONENSIN ON RUMEN FERMENTATION AND FEED ENERGY PARTITIONING OF NELLORE COWS

Abstract: The objective of this study was to evaluate the interaction effect between monensin and different levels of tannins of *A. mearnsii* on rumen fermentation parameters and feed energy partitioning of Nellore cows. In a 2 x 4 factorial arrangement, 8 cannulated Nellore cows were distributed in 2 contemporary 4 x 4 Latin square design (4 experimental periods of 28 days each) and received 8 experimental diets that differed in the level of tannin inclusion (0, 0.75, 1.5 and 2.25% of DM) and the inclusion of sodium monensin which was daily administered to each cow of one square (about 32 mg/kg DM). The data were analyzed by the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). The only observed significant effect of monensin in all evaluated parameters was 28.8% of acetate:propionate production ratio reduction. The different levels of tannins linearly reduced DM and gross energy intake, but linearly increased rumen solid mass. A significant interaction between monensin and tannins (M*L) on the daily minimum rumen pH was observed. Tannins had a quadratic effect on the time (min/day) during which the rumen pH was below 5.8 and 6.0 as well as pH area (h.pH/day) below 5.8, 6.0 and 6.2. Tannins had no effect on rumen ammonia concentration or production. No significant effect of tannins was observed on total and differential protozoa count. Tannins linearly reduced CH_4, acetate, butyrate and total SCFA production as well as the gross energy release in form of methane but linearly reduced the energy release in intestine and linearly increased the energy loss in feces. Monensin and tannins had independent effects, thus, there was no synergy on CH_4, SCFA and rumen ammonia production or concentration as well as on energy partitioning of Nellore cows. The use of *A. mearnsii* tannins up to 2.25% DM reduced CH_4 production (up to 34.7%) but did not improve the feed energy efficiency.

Keywords: Ammonia; Feed energy; Methane; Protozoa; Short chain fatty acids

4.1. INTRODUCTION

Rumen fermentation is the result of physical and microbiological activity which converts dietary components into SCFA, microbial protein, CH_4, CO_2, NH_3-N, B and K vitamins and other products (OWEN; GOETSCH, 1993). Methane production by enteric fermentation of ruminants generates feed gross energy losses ranging from 2 to 15% (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 to reduce CH_4 production (POPOVA et al., 2013), which may include increasing productivity, improving nutritional management, manipulation of rumen fermentation, changes in diet composition, addition of CH_4 production inhibitors or defaunation (SHIBATA; TERADA, 2010).

Monensin and tannins are two feed additives referenced to promote feed efficiency in ruminants through mechanisms that alter rumen fermentation, reducing CH_4 production and acetate:propionate ratio as well as reducing the rumen protein degradation leading to high post-rumen protein availability (MONTANO et al., 2015; PATRA; SAXENA, 2011; JAYANEGARA et al., 2015; ADDISU, 2016).

Monensin is mentioned to be a good manipulator of rumen fermentation by reducing acetic acid and H_2 producers (Gram-positive bacteria) and, therefore, reduce CH_4 production rates through a mechanism involving exchange of H^+ for extracellular Na^+ or intracellular K^+ across microbial membrane, causing microbial energy depletion. Monensin is also assigned the role of reducing the concentration of lactate-producing microorganisms and increases lactate-using microorganisms and, consequently, maintain optimal rumen pH levels (RUSSELL; HOULIHAN, 2003).

Tannins are polyphenolic polymers of relatively high molecular weight, with the ability to form complexes mainly with proteins and, to a lesser degree, with carbohydrates (starch and structural carbohydrates) and minerals due to a greater number of phenolic hydroxyl groups. They are generally water soluble, except for some high molecular weight structures (ADDISU, 2016; PATRA; SAXENA, 2011). They are mainly of two categories: (1) hydrolysable tannins (HT) and (2) proanthocyanidins, better known as condensed tannins (CT) and resistant to hydrolytic degradation. They have both desirable and undesirable effects, depending on the concentration, source and other factors, such as the animal species, the physiological state of the animal, type of tannins consumed, the chemical structure, molecular weight and the composition of the diet (MAKKAR, 2003a; FRUTOS et al., 2004; MUELLER-HARVEY,

2006). Among several benefits achieved with the use of tannins, methane mitigation may be the most important for ruminant production (NAUMANN et al., 2017).

Therefore, by knowing that both monensin and tannins reduce CH_4 production in ruminants by different mechanisms, the hypothesis tested in this study was that the combined use of these additives would have a synergy on the reduction of CH_4 production of Nellore cows. So, the study aimed to evaluate the interaction effect between monensin and tannins of *A. mearnsii* on rumen fermentation parameters and feed energy partitioning of Nellore cows.

4.2. MATERIAL AND METHODS

4.2.1. Ethical issue, animals and place of experimentation

The experiment followed 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 of the University of Sao Paulo (USP) under the protocol number CEUA 3080240518. The experiment was carried out at the Animal Nutrition and Production Department (VNP) of the College of Veterinary Medicine and Animal Science (FMVZ) of USP, Fernando Costa Campus in Pirassununga, Brazil. The analyzes were performed in the Laboratory of Ruminat Nutrition and in the Laboratory of Chromatography of VNP.

Eight Nellore cows, non-pregnant and non-lactating, carrying rumen cannula and mean body weigth of 582 kg (± 96) were kept in a covered shed in individual pen with free access to water and sand bedding. The shed had suspended fans that were automatically triggered during the hottest hours of day (> 28ºC) to ease the effects of temperature on animals.

4.2.2. Treatments and experimental design

Animals were arranged in 2 contemporary 4 x 4 Latin square design, in a 2 x 4 factorial arrangement. The experimental unit was the animal within each experimental period (n = 32 experimental units). The animals received experimental diets which differed only in the inclusion of sodium monensin and the level of tannin inclusion. The diets were represented by the following treatments: 1) diet without tannins, 2) diet with 0.75% of tannins in DM, 3) diet with 1.5% of tannins in DM and 4) diet with 2.25% of tannins in DM. The different cores containing the tannin source were prepared prior to the experiment with the addition of caulin as the tannin level decreased from 2.25% to 0%. In addition to tannins, each cow in one square daily received 300 mg (about 32 mg/kg DM) of sodium monensin (Rumensin® 200, Elanco

Animal Health, Brazil) from the beginning to the end of each experiment period, administered twice a day (150 mg at 8 a.m. and 150 mg at 4 p.m.), mixed with the feed.

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, 2003b) 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, 2003b).

4.2.3. Feeding Management

The feed was offered twice a day (at 8 a.m. and 4 p.m.) in the form of total mixed ration (TMR), in a ratio of 50% of corn silage and 50% of concentrate. The proportions of the various ingredients and the chemical composition of the diets are shown in table 1.

4.2.4. Experimental period

The experiment was carried out in four (4) periods of 28 days each. At the end of each period the animals spent two days in pasture before the beginning of the next period. The first 16 days of each period were to adapt the animals to the diets. Between 17th and 21st days the dry matter intake (DMI), the water consumption and the real effective degradability of NDF were evaluated. On 22nd day the rumen pH was measured continuously and rumen content was collected for the quantification of rumen fermentation products (CH_4, SCFA as well as the concentration of NH_3-N) and total and differential counts of protozoa. On days 25 and 26 the rumen was emptied to determine the rumen solid mass 3 hours after the morning feeding and before (0 hour) the morning feeding, respectively.

4.2.5. Assessment of feed intake and gross energy of the diet and feces

The dry matter intake (DMI) was evaluated between 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 weighed for the exact quantification of the feed intake which was obtained by the difference between the amount of feed supplied and the leftovers, then multiplied by the DM content of the diet. The daily water

intake was quantified by the use of automatic and individual drinking fountains with water meters.

Fecal samples were manually collected via the rectum, twice a day (8 a.m. and 4 p.m.) to form a composite sample for each cow, and stored at -20ºC 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 ground in a willye 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 calorimetric pump.

4.2.6. 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 cannula, 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 on 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 mm 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.

4.2.7. Assessment of the real effective degradability of NDF

The determination of the real effective degradability (RED) of NDF was performed according to the technique proposed by Ørskov et al. (1980) and McDonald (1981). It was conducted between the 17th and 21st days of each experimental period. Samples of silage and concentrate were dried at 65°C for 72 hours and milled with Willye knife type mill with 2 mm sieves. After the samples were ground, both portions were mixed in proportions of 50:50; then 9 g of this mixture were put in 10 x 20 cm nylon bags (of known weight) of 50 μm porosity. Next were incubated in the rumen via cannula for 0, 3, 9, 24, 48, and 96 hours. Although they had different incubation time, they were all removed at the same time. Afterwards, they were washed with fresh water to ensure removal of the soluble material, then were dried in a forced air oven at 65°C for 72 hours and finally weighed.

The zero-time bags (which were not incubated) were put in a thermostatic bath at 39ºC for 5 minutes and washed with fresh water. They were subsequently submitted to the same

procedures adopted for the bags of other times. The remaining residues in the bags were analyzed for NDF (VAN SOEST et al., 1991) in order to determine the rate of degradation of this nutritive fraction.

The potential degradability was calculated according to the following models with the aid of the SAS NLIN procedure (version 9.3).

$$p = a \text{ if } t \leq lag \quad (6')$$

$$p = a + b\,[1 - e^{-c \times (t - lag)}] \text{ if } t > lag \quad (7')$$

Where:

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

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

b = degradation potential of the water insoluble fraction of the nutritive component analyzed; c = rate of degradation per fermentative action of b;

t = incubation time;

lag = time at which the equation derived for a data set equals the actual potencially degradable fraction at zero time (MERTENS, 1993).

After the determination of the coefficients **a**, **b** and **c**, these were applied in the equation proposed by Ørskov and McDonald (1979) to calculate the real effective degradability (RED). The passage rate (kp) was obtained as described below. The RED of NDF was thereafter calculated using the following equation:

$$RED_{NDF} = [b \times c \times e^{(-kp \times lag)}]/(c + kp) \quad (8')$$

Where:

RED_{NDF} = Real effective degradability of NDF;

kp = Passage rate.

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

$$Y = a.e^{-kp \times t} \quad (9')$$

Where:

Y = indicator concentration in time (t);

a = concentration of the indicator at the initial time (t_0), assuming instant mixing to the rumen content (ppm);

e = base of the neperian logarithm;

kp = rumen passage rate (h^{-1});

t = indicator sampling time (h).

4.2.8. Continuous pH measuring

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

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

4.2.9. Evaluation of rumen fermentation products

The evaluation of rumen fermentation products was performed using the *ex situ* technique described by Rodrigues et al. (2012) and Perna Junior et al. (2017). The technique consists in putting samples of rumen content in flasks (micro-rumen) which are incubated in a thermostatic bath, simulating the rumen conditions during 30 minutes.

The preparation of all 50 mL capacity flasks (Frascolex, São Paulo, Brazil) consisted of manual washing with fresh water, followed by rinsing with distilled water and finally dried in forced air oven at 65^{o}C. Afterwards, they were identified and weighed in analytical scale, and conditioned in dry place until the moment of use.

4.2.9.1. Sampling of rumen content

The rumen content for the measuring of CH_4, SCFA, NH_3-N as well as total protozoa counting was collected on day 22 of each experimental period, at zero (0), 3, 6, 9 and 12 hours after the morning feeding. On this day the cows were fed after the first collection (about 8:30 a.m.) and after the last collection (about 8:30 p.m.). The liquid phase of the rumen content was collected with the aid of a probe coupled to a vacuum pump. The solid phase was collected by introducing the hand via the rumen cannula, collecting in three distinct points of the rumen. Both fractions were put in the flasks (about 10 g of the solid fraction and 20 mL of the liquid fraction) with the aid of a funnel and a plastic stick. The flasks were then capped with rubber stoppers and sealed with aluminum sealing wax through specific pliers. 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 autoclave to inactivate the fermentative process (under temperature and pressure) for 15 minutes. The other two flasks were immediately incubated for 30 minutes in a thermostatic bath at 39°C. At the end of the incubation time the fermentative process was inactivated under temperature and pressure for 15 minutes.

After the flasks cooled, they were taken to the Gas Chromatography Laboratory of the VNP-FMVZ to measure the volume of gases and concentration of CH_4. The figure 2 shows the diagram of entire procedure described.

4.2.9.2. Quantification of methane

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. This procedure was performed in each of the samples in a temperature-controlled environment (25°C). The volume displaced by the gas produced in the flask was recorded in order to determine the production of methane gas in the sample. The total gaseous volume was obtained by the sum of that 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).

4.2.9.3. Calculation of liquid volume and concentration of SCFA of the rumen content in the micro-rumen

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 flask containing the sample before drying. The amount of solids was obtained by the difference, in weight, between the flask containing the sample after drying and the weight of the empty flask (obtained before flasks were filled).

To determine the SCFA (acetate, propionate and butyrate) concentrations, about 4.0 mL of the liquid portion of the rumen content of each flask were taken and centrifuged for 15 minutes. Then, 2 mL were inserted into a test tube containing 0.4 mL of formic acid, according to Erwin et al. (1961). The SCFA were measured by gas chromatography (Focus GC, Thermo Scientific®, Rodano, Milan, Italy) by using a 1.22 m length and 0.63 cm diameter glass column packed with 80/120 Carbopack B-DA/4% (Supelco, Sigma-Aldrich, St. Louis, MO, USA).

4.2.9.4. Calculation of the production of SCFA and CH_4, as well as the relative energy loss

The quantification of CH_4 production was obtained by multiplying the total volume of the gases (mL) produced in each flask by the concentration of CH_4 in the gas phase (mmol/mL) obtained in the incubated flask, subtracting what was produced in non-incubated flask (equation 16). The individual quantification of SCFA was obtained by multiplying the volume of liquid (mL) by the concentration of each SCFA (mmol/mL) obtained in the incubated flask, subtracting the production obtained in the non-incubated flask (equation 17).

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

$$\text{Prod. } CH_4 = (\text{Conc. } CH_4 \text{ x Total Gas Vol.})T_{30} - (\text{Conc. } CH_4 \text{ x Total Gas Vol.})T_0 \quad (16)$$

Where: Prod. CH_4 = CH_4 Production at the time between 30 minutes (incubated flasks) and zero (0) minute (non-incubated flasks) of incubation; Conc. CH_4 = CH_4 concentration (mmol/mL); Total Gas Vol. = Total volume of gas (obtained 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.

$$\text{Prod. SCFA} = (\text{Conc. SCFA} \times \text{Total Liq.Vol.})T_{30} - (\text{Conc. SCFA} \times \text{Total Liq.Vol.})T_0 \quad (17)$$

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 (obtained by difference of the weight before and after drying (mL)); T_{30} = Incubation time of 30 min; T_0 = Incubation time of 0 min.

After the fermentation products were quantified (CH_4 and SCFA), 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 CH_4 produced and the sum of the energy contained in all fermentation products (CH_4 and SCFA), expressed as percentage. Thus, theoretical chemical values of the combustion heat were used, assuming that 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 kcal per mol, respectively. The REL was calculated using the following equation, according to Rodrigues et al. (2012):

$$\text{REL}\ (\%) = 100 \times [\varepsilon CH_4/(\varepsilon CH_4 + \varepsilon C_2 + \varepsilon C_3 + \varepsilon C_4)] \quad (18)$$

Where:

REL = relative energy loss (%);

εCH_4 = methane energy (kcal/g or kcal/mol);

εC_2 = acetate energy (kcal/g or kcal/mol);

εC_3 = propionate energy (kcal/g or kcal/mol);

εC_4 = butyrate energy (kcal/g or kcal/mol).

4.2.9.5. Determination of ammonia nitrogen concentration

To determine the ammonia nitrogen (NH_3-N) concentration, the liquid fraction was taken from each flask and, after centrifugation, 2.0 mL were taken to a test tube adding 1 mL of 1N H_2SO_4 solution, and then 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 flasks incubated for 30 minutes with the non-incubated flasks. For a better understanding and interpretation, the balance data were estimated per hour, according to the following equation:

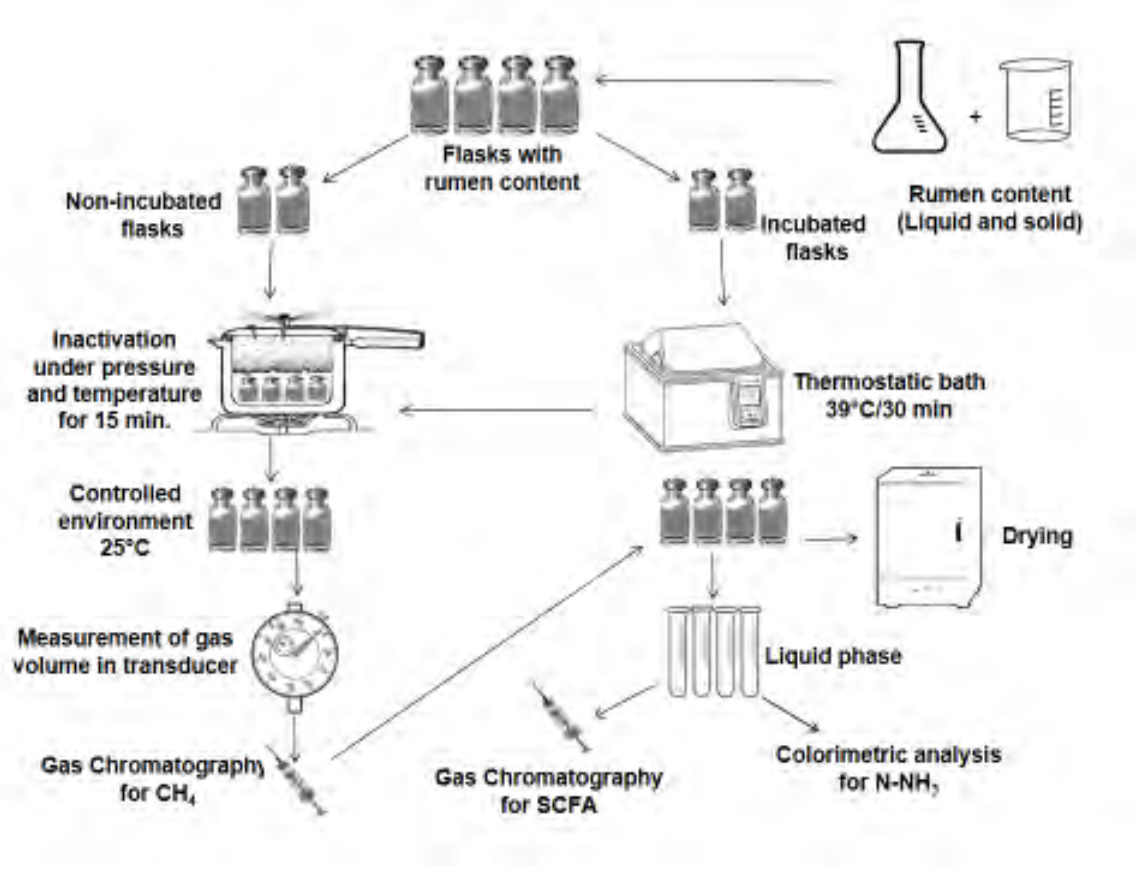
NH_3-N balance (mg/dL.h) = [Conc. 30 min (mg/dL) – Conc. 0 min (mg/dL)] x 2 (19)

Where:

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

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

Figure 2. Diagrammatic representation of *ex situ* rumen fermentation technique.



Source: Perna Junior et al. (2017)

4.2.10. Total and differential count of protozoa

For the total and differential count of rumen protozoa the rumen content used was the same as for the methane measuring (collected on day 22 of each experimental period, at zero (0), 3, 6, 9 and 12 hours after the morning feeding). Equal portions of the solid and liquid fractions (of rumen content) of each cow were mixed and homogenized, then about 10 mL of

this mixture were inserted into flasks containing 20 mL of formaldehyde at 18.5%. Next, 1 mL of this content was stained for 4 hours with 2 drops of 2% brilliant green. Afterwards, 9 mL of glycerol at 30% were added and homogenized, making the aliquot diluted 30 times. Afterwards, the counting chamber (1 mL capacity) was filled with the diluted sample and, coupled to microscope, 100 optical fields were counted through the reticulum with the magnification of 100X.

The identification and counting of ciliate protozoa were performed by using a Neubauer 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 as the subfamily *Diplodiniinae* which included *Diplodinium*, *Eudiplodinium*, *Ostracodinium*, *Metadinium* and *Polysplatron*).

4.2.11. Energy partitioning

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 is produced mainly in the rumen (95%) and, to a smaller extent (5%), in the large intestine. The fermentation heat and microbial ATP were estimated from the ratio among the SCFA produced according to Owens and Basalan (2016).

The energy release in the intestine (Mcal/ani.d) was calculated from GEI (Mcal/ani.d) subtracting the energy of acetate, propionate, butyrate, rumen CH_4 (Mcal/ani.d) plus feces' GE (Mcal/ani.d), CH_4 release in the cecum and colon (Mcal/ani.d), and fermentation heat (FH), following the equation:

$$ERI = GEI - (\text{Ɛ}C_2 + \text{Ɛ}C_3 + \text{Ɛ}C_4 + \text{feces' GE} + \text{C\&C } CH_4 + FH + mATP) \quad (20)$$

Where:

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

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

$ЄC_2$: acetate energy (Mcal/ani.d);

$ЄC_3$: propionate energy (Mcal/ani.d);

$ЄC_4$: 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 release 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 release in feces, expressed in terms of percentage of GEI, was obtained dividing feces' energy content (Mcal/ani.d) by GEI (Mcal/ani.d) and then multiplying by 100.

4.2.12. Statistical analysis

The data were analyzed using the Statistical Analysis System (SAS 9.3, 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 the data were transformed. The data of DMI, real effective degradability of NDF, rumen solid mass, pH and energy partitioning were submitted to analysis of variance which separated, as causes of variation, the tannin level effect, monensin effect (also considered as the effect of the square), interaction between monensin and the tannin level, period effect and animal effect within the square. The statistical model used is described according to the equation below:

$$Y_{ijkl} = \mu + L_i + M_j + L_i*M_j + P_k + A_l(S_j) + e_{ijkl}$$

Where: Y_{ijkl} = observation concerning Level (i) + Monensin (j) + Level (i) * Monensin (j) + Period (k) + Animal (l) within the square (j) + random error associated with each observation (e_{ijkl}).

μ = overall mean;

L_i = level effect (fixed effect);

M_j = effect of monensin (fixed effect);

L_i*M_j = interaction between level (i) and monensin (j) (fixed effect);

P_k = period effect (random effect);
$A_l(S_j)$ = effect of animal within the square (random effect);
e_{ijkl} = random error associated to each observation.

For the variables of CH_4, SCFA, NH_3-N productions, and total and differential count of rumen protozoa it was added, to the model, the factor "measures repeated over time", referring to the different sampling hours (0, 3, 6, 9 and 12). The analysis was performed by using the mixed model procedure (Proc Mixed). The analysis by the time was performed only when the interactions between time and level were significant. The level effect was evaluated by the use of orthogonal polynomials, separating the effects in linear, quadratic and quadratic deviation.

For the analyzes, among the 15 different covariance structures were tested, and that which best fit the statistical model was chosen based on the lowest value of the corrected Akaike information criterion (AICC) (WANG; GOONEWARDENE, 2004). A significance level of 5% was adopted.

4.3. RESULTS

4.3.1. Dry matter and water intake, real effective degradability of NDF and rumen pH

No interaction was observed between monensin and tannins on DM and water intake, RED of NDF ($P > 0.05$), but significant interaction ($P = 0.0173$) (M^*L) was observed on the minimum rumen pH (Table 11 and Figure 3). It was also not observed significant effect of monensin alone on these parameters.

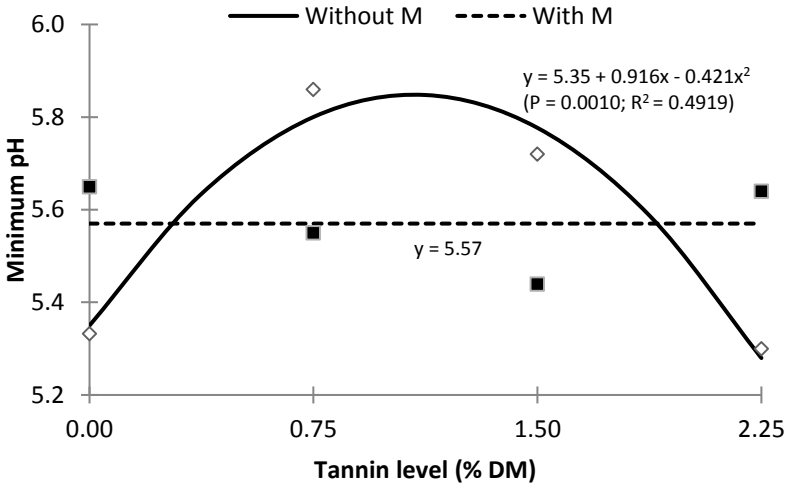
The different levels of tannins linearly reduced the DM and water intake, and RED of NDF ($P < 0.05$). The highest level of tannins reduced DMI by 11.1% when compared to control treatment. Tannins did not affect the minimum, medium and maximum rumen pH, but it was observed a quadratic effect on the time (min/day) during which the pH was below 5.8 and 6.0 as well as pH area (h.pH/day) below 5.8, 6.0 and 6.2.

Table 11 - Ingestion, NDF degradability and rumen pH of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*.

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
Ingestion										
DM, kg/day	9.34	9.49	9.80	9.59	9.56	8.71	0.275	NS	0.0034^{L}	NS
Water, L/day	31.67	24.00	29.25	28.58	27.75	25.75	1.596	NS	0.0120^{L}	NS
NDF degradability										
RED (%)	27.14	25.17	31.64	24.98	24.88	23.12	1.411	NS	0.0087^{L}	NS
Rumen pH, day										
Minimum	5.55	5.57	5.49	5.70	5.58	5.47	0.059	NS	NS	0.0173
Medium	6.21	6.17	6.21	6.26	6.22	6.08	0.038	NS	NS	NS
Maximum	6.65	6.59	6.66	6.68	6.61	6.52	0.030	NS	NS	NS
Time of pH, min/day										
< 5.8	168.3	182.1	196.7	107.5	115.0	281.7	29.85	NS	0.0156^{Q}	NS
< 6.0	385.0	409.2	371.7	230.0	355.0	631.7	58.58	NS	0.0449^{Q}	NS
< 6.2	620.0	710.8	605.0	458.3	686.7	911.7	72.60	NS	NS	NS
Area, h.pH/day										
< 5.8	0.59	0.52	0.81	0.37	0.24	0.81	0.113	NS	0.0107^{Q}	0.0701
< 6.0	1.49	1.34	1.75	0.60	0.98	2.32	0.239	NS	0.0023^{Q}	NS
< 6.2	3.08	3.00	3.38	1.28	2.75	4.76	0.442	NS	0.0049^{Q}	NS

SEM: Standard error of mean; M*L: Interaction between monensin and tannin level; RED: Real effective degradability. (Own authorship).

Figure 3 - Graphic demonstration of interaction between monensin (M) and tannins at the minimum pH.



Graphic demonstration of interaction between monensin (M) and tannins at the minimum pH. The square points in bold represent the means observed in the different tannin levels only for the group of cows which also received M. For this group, the joint effect of M and tannins was not significant, then it was preferred to present the general mean observed in the group (dashed line). The empty square points show the means observed in the different tannin levels for the group which only received tannins (quadratic effect). The continuous line shows the estimated means for the group which received M and tannins if they had not received M (quadratic effect). Therefore, it may be seen that the effect of tannins is observed only when they act alone, but when acting along with M the effect disappears, suggesting an inhibition by M through antagonistic interaction. (Own authorship).

4.3.2. Protozoa and products of rumen fermentation

There was neither significant interaction between monensin and the tannins nor independent effect of both additives on the total and differential count of rumen protozoa (Table 12).

Table 12 - Total and differential count of protozoa of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*.

Variable	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
Protozoa (x10^3/mL)										
Dasytricha	2.63	2.57	2.61	2.46	2.73	2.58	0.25	NS	NS	NS
Entodinium	1193.0	1130.2	1212.7	1160.5	1181.1	1092.4	35.0	NS	NS	NS
Isotricha	1.56	1.59	1.74	1.47	1.74	1.35	0.19	NS	NS	NS
Diplodiniinae	2.70	2.97	2.70	4.23	2.31	2.10	0.44	NS	NS	NS
Total	1199.9	1137.3	1219.7	1168.6	1187.9	1098.3	35.2	NS	NS	NS
Protozoa (%)										
Dasytricha	0.21	0.20	0.20	0.18	0.21	0.23	0.02	NS	NS	NS
Entodinium	99.42	99.39	99.45	99.28	99.44	99.34	0.05	NS	NS	NS
Isotricha	0.14	0.13	0.15	0.14	0.13	0.12	0.02	NS	NS	NS
Diplodiniinae	0.23	0.28	0.20	0.40	0.21	0.21	0.04	NS	NS	NS

SEM: Standard error of mean; M*L: Interaction between monensin and tannin levels. (Own authorship).

No significant interaction between monensin and tannins as well as monensin alone on the concentration and production of rumen ammonia (NH_3-N) was observed (Table 13). It was also not observed any significant effect of the different levels of tannins on the concentration or production (balance) of rumen NH_3-N, although the highest level of tannins caused a negative balance (i.e. instead of producing, there was a consumption). Regardless of tannin level and monensin, the sampling time had an effect ($P < 0.05$) on the concentration of rumen NH_3-N in both non-incubated and incubated flasks.

Table 13 - Concentration and balance of rumen NH_3-N of Nellore cows fed monensin (ppm) and different levels of tannin of *A. mearnsii*.

Variables	Monensin (M)		Level (L)				SEM	Probability		
	0	30	0%	0.75%	1.5%	2.25%		M	L	Time
Concentration										
0 min (mg/dL)	8.15	8.12	8.75	8.33	8.29	7.18	0.34	NS	0.0551^L	<.0001
30 min (mg/dL)	8.28	8.41	9.17	8.53	8.62	7.06	0.38	NS	0.0547^L	<.0001
Balance (mg/dL.h)	0.24	0.59	0.85	0.40	0.66	-0.24	0.30	NS	NS	0.0537

SEM: Standard error of mean; M*T: Interaction between monensin and time; Balance = (30 min – 0 min) x 2. (Own authorship).

No significant interaction was observed between monensin and the different levels of tannins ($P > 0.05$) on the SCFA and CH_4 production or concentration as well as on the relative energy loss (REL) of CH_4 in relation to the other rumen fermentation products ($P > 0.05$). Independently, monensin also did not significantly alter these parameters, but reduced ($P = 0.0007$) the acetate:propionate molar ratio concerning the production by 28.8% (Table 14).

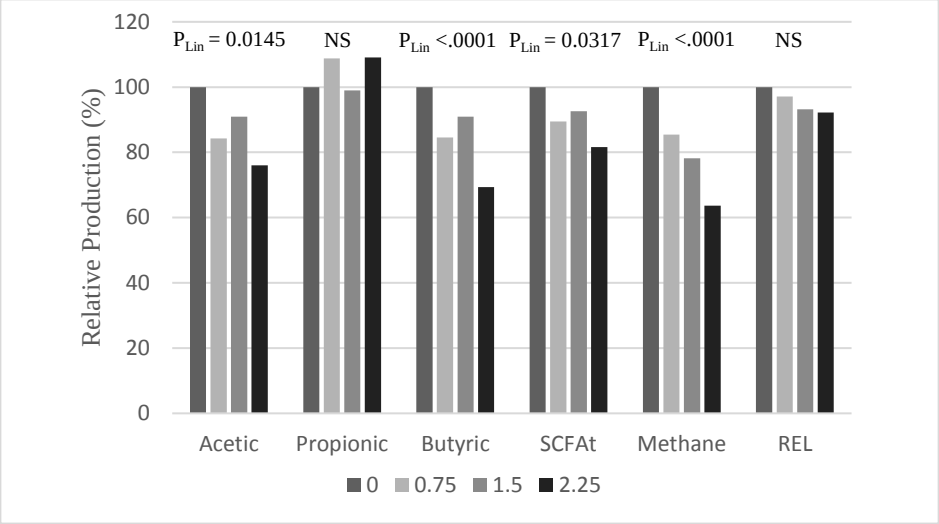
The different levels of tannins linearly decreased the production of CH_4, acetate, butyrate as well as the total SCFA, but had no effect on propionate production or REL. The magnitude of reduction caused by the highest level (g/kg DM.d) was 24% (acetate), 30.6% (butyrate), 18.2% (total SCFA) and 34.7% (CH_4), when compared to control treatment. Consequently, there was a linear reduction of the gross energy (GE) released respective to each of these parameters. The diferent levels of tannins quadraticly increased the concentration of propionate (mmol/L) in both non-incubated and incubated flasks, although not affecting the production. The figure 4 depicts the changes caused by tannins on the relative production of acetate, propionate, butyrate, total SCFA and CH_4 as well as on the REL in relation to the control treatment which was considered as 100%. The different collecting times had a significant effect on the production of these parameters, however no interactions between time and treatment were observed.

Table 14 - SCFA and CH_4 production as well as REL of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*.

Variables	Monensin (M)		Level (L)					Probability		
	0	30	0%	0.75%	1.5%	2.25%	SEM	M	L	Time
Acetic acid										
0 min (mmol/L)	70.80	70.10	70.61	70.69	71.26	69.28	0.529	NS	NS	0.0218
30 min (mmol/L)	75.05	74.39	75.49	75.88	75.81	72.04	0.635	NS	0.0180^{Q}	0.0015
Difference (mmol/L)	4.10	4.09	4.88	4.42	4.39	2.76	0.214	NS	0.0001^{L}	0.0006
Production (mol/kg.d)	2.73	2.60	2.99	2.52	2.84	2.27	0.106	NS	0.0145^{L}	0.0147
Production (g/kg.d)	163.5	155.9	179.3	151.2	170.4	136.2	6.371	NS	0.0145^{L}	0.0147
GE (kcal/kg.d)	557.5	556.0	625.8	537.1	569.2	488.4	21.21	NS	0.0122^{L}	0.0157
Propionic acid										
0 min (mmol/L)	20.02	22.71	18.88	20.74	21.31	24.47	0.412	NS	0.0080^{Q}	<.0001
30 min (mmol/L)	21.79	24.78	20.49	23.86	22.95	26.05	0.458	NS	0.0147^{Q}	0.0002
Difference (mmol/L)	1.62	1.86	1.67	2.06	1.64	1.68	0.080	NS	NS	0.0008
Production (mol/kg.d)	1.00	1.14	1.02	1.11	1.03	1.11	0.047	NS	NS	0.0040
Production (g/kg.d)	73.75	83.98	75.49	82.13	76.14	82.32	3.477	NS	NS	0.0040
GE (kcal/kg.d)	363.3	419.9	375.9	395.3	387.0	410.0	17.24	NS	NS	0.0472
Butyric acid										
0 min (mmol/L)	12.68	12.47	11.87	12.13	13.41	12.82	0.176	NS	0.0069^{L}	0.0003
30 min (mmol/L)	14.01	13.48	13.37	13.41	14.36	13.70	0.197	NS	0.0300^{Q}	<.0001
Difference (mmol/L)	1.28	1.28	1.54	1.38	1.33	0.91	0.050	NS	0.0004^{L}	0.0018
Production (mol/kg.d)	0.81	0.80	0.91	0.81	0.84	0.63	0.029	NS	$<.0001^{L}$	0.0096
Production (g/kg.d)	71.04	70.28	80.38	71.49	74.27	55.77	2.526	NS	$<.0001^{L}$	0.0096
GE (kcal/kg.d)	414.3	411.6	479.0	393.8	442.6	332.4	14.99	NS	$<.0001^{L}$	0.0058
Total SCFA										
0 min (mmol/L)	103.8	105.7	101.4	105.8	105.6	106.6	0.800	NS	0.0169^{L}	0.0005
30 min (mmol/L)	110.4	112.5	109.4	111.9	112.9	111.8	0.907	NS	NS	<.0001
Difference (mmol/L)	6.86	7.23	8.030	7.66	7.37	5.25	0.315	NS	0.0012^{L}	<.0001
Production (mol/kg.d)	4.55	4.54	4.92	4.47	4.73	4.017	0.165	NS	0.0317^{L}	0.0073
Production (g/kg.d)	309.6	310.4	335.2	306.0	322.4	274.3	11.07	NS	0.0317^{L}	0.0073
GE (kcal/kg.d)	1343	1368	1481	1294	1424	1218	48.31	NS	0.0664^{L}	0.0022
Acetate:Propionate	3.37	2.40	3.17	2.40	3.081	2.91	0.133	0.0007	NS	NS
Methane										
0 min (mmol/flask)	0.022	0.020	0.026	0.022	0.020	0.018	0.001	NS	$<.0001^{L}$	<.0001
30 min (mmol/flask)	0.087	0.087	0.105	0.091	0.082	0.068	0.002	NS	$<.0001^{L}$	<.0001
Difference (mmol/flask)	0.065	0.066	0.079	0.069	0.062	0.050	0.002	NS	$<.0001^{L}$	<.0001
Production (mol/kg.d)	1.54	1.51	1.85	1.59	1.47	1.20	0.035	NS	$<.0001^{L}$	<.0001
Production (g/kg.d)	24.80	24.33	29.72	25.38	23.46	19.41	0.551	NS	$<.0001^{L}$	<.0001
GE (kcal/kg.d)	324.5	316.5	389.7	334.0	308.7	251.3	7.286	NS	$<.0001^{L}$	<.0001
REL (%)	20.58	21.41	21.97	21.32	20.47	20.26	0.582	NS	NS	NS

SEM: Standard error of mean; GE: Gross energy, REL: Relative energy loss of methane in relation to the other rumen fermentation products. (Own authorship).

Figure 4 - Graph representing the changes caused by the different levels of tannins of *A. mearnsii* on the SCFA and CH_4 production as well as REL in relation to control treatment (100%).



(Own authorship).

4.3.3. Energy partitioning

There was neither significant interaction between monensin and tannins nor monensin effect ($P > 0.05$) on rumen solid mass, gross energy intake (GEI) as well as on energy partitioning (Table 15).

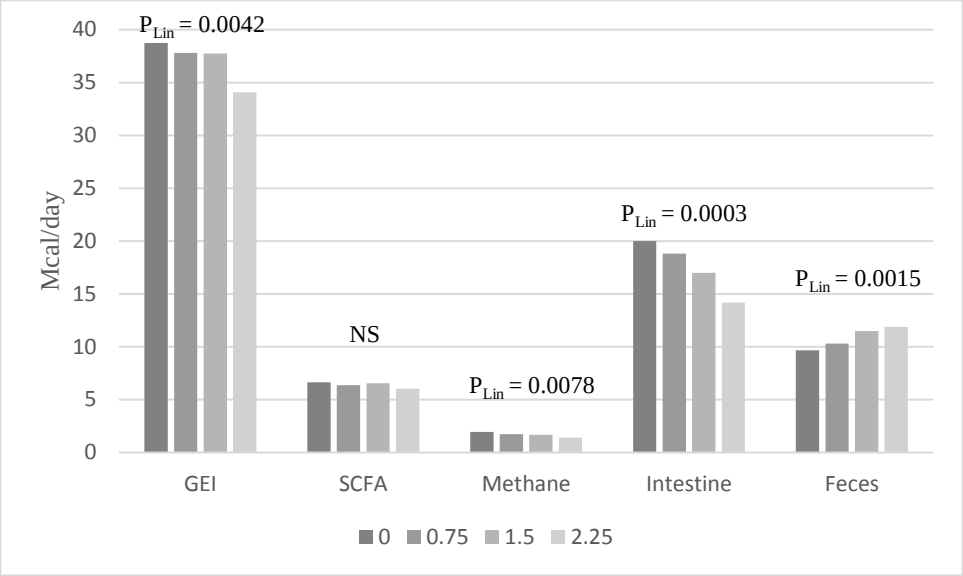
Unlike monensin, the different levels of tannins linearly increased the rumen solid mass, but linearly decreased ($P < 0.05$) the amount of gross energy ingested (Mcal/day). They also linearly increased the digestible energy (DE) released in form of propionate, but linearly reduced the amount of GE released in form of methane and the energy released in intestine. They linearly increased the energy loss in feces, but no effect was observed on the fermentation heat. The figure 5 depicts the changes on GEI and energy partitioning caused by the different levels of tannins.

Table 15 - Estimation of energy released into the gastrointestinal tract of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*.

Variable,	Monensin (M)		Level (L)				SEM	Probability		
day	0	30	0%	0.75%	1.5%	2.25%		M	L	M*L
Rumen mass, kg	5.36	5.45	5.01	5.23	5.72	5.67	0.203	NS	0.0117^{L}	NS
GEI, Mcal	36.72	37.47	38.75	37.79	37.75	34.08	1.117	NS	0.0042^{L}	NS
Energy released into the rumen										
Acetic acid										
Mcal/cow	2.59	2.59	2.82	2.49	2.74	2.34	0.162	NS	NS	NS
GE, %	7.16	6.94	7.26	6.86	7.39	6.77	0.397	NS	NS	NS
DE, %	10.43	9.74	9.82	9.54	10.63	10.47	0.582	NS	NS	NS
Propionic acid										
Mcal/cow	1.63	2.09	1.65	1.94	1.85	2.02	0.124	NS	NS	NS
GE, %	4.61	5.59	4.31	5.16	5.01	5.89	0.315	NS	NS	NS
DE, %	6.70	7.83	5.84	7.12	7.16	8.91	0.447	NS	0.0221^{L}	NS
Butyric acid										
Mcal/cow	1.90	1.99	2.17	1.97	1.98	1.67	0.147	NS	NS	NS
GE, %	5.23	5.30	5.59	5.43	5.32	4.75	0.361	NS	NS	NS
DE, %	7.59	7.41	7.53	7.53	7.62	7.35	0.509	NS	NS	NS
SCFA Total										
Mcal/cow	6.11	6.67	6.64	6.39	6.57	6.025	0.374	NS	NS	NS
GE, %	17.00	17.83	17.15	17.45	17.71	17.42	0.901	NS	NS	NS
DE, %	24.72	24.97	23.19	24.20	25.40	26.73	1.298	NS	NS	NS
Fermentation heat										
Mcal/cow	0.52	0.56	0.57	0.54	0.56	0.50	0.032	NS	NS	NS
GE, %	1.45	1.49	1.48	1.47	1.51	1.45	0.078	NS	NS	NS
DE, %	2.11	2.10	1.99	2.04	2.16	2.23	0.113	NS	NS	NS
Methane										
Mcal/cow	1.70	1.69	1.94	1.74	1.68	1.41	0.086	NS	0.0078^{L}	NS
GE, %	4.64	4.54	4.93	4.78	4.52	4.12	0.186	NS	0.0405^{L}	NS
DE, %	6.74	6.34	6.59	6.65	6.55	6.36	0.276	NS	NS	NS
Mcal/kg DM	0.183	0.179	0.194	0.188	0.180	0.162	0.007	NS	0.0266^{L}	NS
Energy release in the intestine										
Mcal/cow	16.81	18.26	20.01	18.83	16.99	14.18	0.929	NS	0.0003^{L}	NS
GE, %	45.95	48.32	51.61	48.65	45.88	42.10	1.573	NS	0.0004^{L}	NS
DE, %	66.01	66.89	68.51	67.13	65.88	64.14	1.543	NS	NS	NS
Energy release in feces										
Mcal/cow	11.22	10.46	9.684	10.32	11.49	11.87	0.444	NS	0.0015^{L}	NS
GE, %	30.84	28.09	25.06	27.68	30.53	34.58	0.987	NS	<.0001^{L}	NS

SEM: Standard error of mean; M*L: Interaction between monensin and tannin levels; GEI: Gross energy intake; GE: Gross energy; DE: Digestible energy. (Own authorship).

Figure 5 - Graph representing the gross energy intake and energy released in the gastrointestinal tract (Mcal/day) of Nellore cows fed monensin and different levels of tannins of *A. mearnsii*.



GEI: gross energy intake. (Own authorship).

4.4. DISCUSSION

4.4.1. Feed intake and rumen pH

The lack of interaction between monensin and tannins on DMI and water consumption may indicate independent effects of these two additives on these parameters.

Dry matter intake is of fundamental importance in nutrition, since it establishes the amount of nutrients available for health and production (NRC, 2001). The meta-analyzes performed by Duffield et al. (2008), evaluating the impact of monensin on dairy cows, and Duffield et al. (2012), evaluating the effects of monensin on beef cattle, have shown reduction of DMI in both dairy and beef cattle, but the studies performed by Odongo et al. (2007), Van Vugt et al. (2005), Gallardo et al. (2005), Benchaar et al. (2006), Hamilton et al. (2010), Mullins et al. (2012) and Perna Junior et al. (2017) found no effect of monensin. In the present study no significant effect of monensin was observed on DM or water intake (Table 11). These differences may demonstrate the inconsistency of monensin on consumption. The differences observed amongst the studies might, of course, depend on the study and the type of the diet, as well as the amount of monensin per kg of DM. The lack of significant effect of monensin on water consumption was also observed in pre and postpartum periods of dairy cows in the study of Mullins et al. (2012).

Monensin significantly interacted with tannins on minimum daily pH (Figure 3) and showed capacity to stabilize minimum rumen pH. The lack of effect of monensin alone on rumen pH was also observed by Perna Junior et al. (2017). Mullins et al. (2012), evaluating the effects of monensin on metabolic parameters, feeding behavior, and productivity of transition dairy cows also observed no effect of monensin on rumen pH. Adequate rumen pH maintenance is a characteristic generally determined by the type of diet. The pH 5.8 indicates the threshold for cases of subacute rumen acidosis (PENNER et al., 2007), pH 6.0 and 6.2 are thresholds indicative of healthy rumen conditions, favoring great cellulolytic activity (PENNER; BEAUCHEMIN, 2010).

The inclusion of tannins of *A. mearnsii* did not impair the rumen pH. In contrast, the inclusion of tannins up to 2.23% improved the minimum daily pH (Figure 3) as well as the time (min/day) during which the pH remained below 5.8 and 6.0, the pH area (pH.h/day) below 5.8, 6.0 and 6.2. Using tannins of *A. mearnsii* in the diet of Holstein and Nellore cows, Perna Junior (2018) found similar results in pH time below 6.0 and 6.2.

The linear reduction of DMI caused by the different levels of tannins, corroborates with the meta-analysis of Jayanegara & Palupi (2010). The reduction of DMI was also observed by Aguerre et al. (2016), Grainger et al. (2009) and Dschaak et al. (2011). The linear increase of rumen solid mass (Table 15) and the linear reduction of the NDF real effective degradability (Table 11) caused by tannins might have been the major causes for the reduction of DMI. The linear reduction of water consumption was somewhat surprising, since it was thought that the astringent effect of tannins would cause increase on water consumption. Perna Junior (2018) found no significant effect on water consumption probably because the highest level of tannin inclusion in his study (1.5%) was not capable to trigger significant alterations.

4.4.2. Rumen fermentation parameters

The ammonia production in the rumen generally exceeds the capacity of use by microorganisms, resulting in accumulation and subsequent absorption and conversion to urea by the liver (RODRIGUES, 2016). The efficiency of microbial protein synthesis is one of the most important factors to reduce the concentration of ammonia in the rumen, which can be improved by diets with high total digestible nutrients (TDN) to supply the energy required for bacterial activity (SEO et al., 2010) or by the use of additives capable to reduce the rumen degradation rate of the protein. Although monensin reduces ammonia production (*in vivo* or *in vitro*) by inhibiting the growth of rumen proteolytic bacteria or deamination process (RUSSELL; STROBEL, 1989; YANG; RUSSEL, 1993ab), this effect was not observed in the

present study (P > 0.05) (Table 13). It was also not observed in the study of Perna Junior et al. (2017). Different results were observed by Ruiz et al. (2001) and Yang and Russel (1993a) who found reduction of rumen ammonia when included monensin in the diets. This difference, in part, may be due to the type of diet used in the present study and the study of Perna Junior et al. (2017) (50% of corn silage and 50% of concentrate) which may have supplied/synchronized the energy required for bacterial activity so that there was no difference among the animals that received monensin with those that did not receive.

Tannins have the ability to bind proteins, rendering them inaccessible to rumen degradation and favoring post-rumen release (NIGRANT et al., 2017). Therefore, their use may partly be as a way to protect the protein against excessive rumen degradation (DENTINHO; BESSA, 2016). Aguerre et al. (2016) and Dschaak et al. (2011), using tannins in diets of dairy cows as well as Min et al. (2002), using tannins in sheep diets, observed reduction of microbial proteolytic activity with the consequent reduction of rumen ammonia concentration. This effect was not observed in the present study (Table 13) and in the study of Perna Junior (2018) as well. Although the reduction of rumen ammonia concentration and the balance were not significant, it may be seen in table 13 that the highest level of tannins (2.25%) had a negative balance (-24 mg/dL.h). This may be the indication that during the 30 minutes of incubation the inhibition of proteolytic activity by tannins was accentuated in the way that the use of ammonia for microbial protein synthesis was greater than the production.

Tannins are also attributed the ability to reduce the number of protozoa in the rumen as one of the mechanisms these additives use to reduce CH_4 production because the *archaea* can be found closely associated with ciliate protozoa, adhering on their cell surface or in the intracellular medium (PATRA; SAXENA, 2011; FINLAY et al., 1994). Although it was neither observed interaction between tannins and monensin nor significant effect of tannins alone as well as monensin on total and differential counts of protozoa (Table 12), it was observed that the most numerous were the protozoa of the genus *Entodinium,* corroborating with Dehority, 2003; Perna Junior et al., 2017 and Perna Junior, 2018.

Using monensin (33 mg/kg DM) in high or low concentrate Angus steers' diets, Guan et al. (2006) observed a reduction of the total ciliate protozoal populations up to the first 4 weeks during which monensin was used, but original ciliate protozoal populations were restored by the fourth and sixth week and no more significant changes were observed thereafter. This suggests that protozoa can develop a mechanism of adaptation to monensin. The only difference observed by Perna Junior et al. (2017), separately using monensin (18 mg/kg DM) and *A. mearnsii* tannins (0.6% DM), was the reduction (in both monensin and tannins) of the number

of *Isotricha* genus, but using *A. mearnsii* tannins up to 1.5% DM, Perna Junior (2018) did not observe any difference. Benchaar et al. (2008), daily using 105 g of quebracho (*Schinopsis spp*.) tannins per cow, also found no effect on total number of rumen protozoa. These results may suggest some effect of tannins on rumen protozoa, but lack consistency.

Monensin and tannins, separately, are referenced to reduce enteric CH_4 emission by ruminants. The lack of interaction between monensin and tannins on CH_4 and SCFA production, as well as on the relative energy loss (REL) of CH_4 in relation to the other rumen fermentation products (Table 14) may indicate independence of these additives on these parameters. Although there was a reduction on acetate:propionate ratio (concerning production), no effect of monensin was observed on CH_4 or SCFA production as well as on the REL. Different observations were reported in many studies, such as of Odongo et al. (2007), Van Vugt et al. (2005) and Perna Junior et al. (2017) who found reduction of CH_4 production, but many other studies, such as of Hamilton et al. (2010), Grainger et al. (2008), McGinn et al. (2004) and Grainger et al. (2010) found no effect of monensin on CH_4 production. Appuhamy et al. (2013), performing a meta-analysis on the anti-methanogenic effects of monensin in cattle, found inconsistent results. In the study of Guan et al. (2006), it was observed reduction of enteric CH_4 by 30% for the first 2 weeks and by 27% for the first 4 weeks in cattle receiving high concentrate and low concentrate diets, respectively, but thereafter, the differences were not significant, suggesting that the monensin sensitive rumen microorganisms may be capable to develop adaptation mechanisms against monensin.

The reduction of acetate:propionate productive ratio by using monensin (Table 14) was also reported by Crossland et al. (2017), Costa et al. (2017) and Guan et al. (2006).

Knowing the three major forms by which tannins reduce CH_4 production, the linear reduction of CH_4 production, by unit of rumen DM, caused by tannins in this study might have been either by reducing *archaea* or depression of fiber digestion in the rumen or both, since it was not observed any tannin effect on ciliate rumen protozoa. Tannins 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). This is why part of reduction of methane production by tannins has been questioned since it occurs by reduction of nutrient digestion. The meta-analysis of Jayanegara et al. (2012) on the relationship between dietary level of tannins and CH_4 production in ruminants from *in vitro* and *in vivo* experiments has shown that the reduction of CH_4 production was associated with the reduction of apparent digestibility of OM and, especially, the fiber. Carulla et al. (2005) also reported that

condensed *A. mearnsii* tannins in the concentration of 2.5% reduced CH_4 by 12% due, in part, to 5% of reduction in NDF digestibility. Animut et al. (2008) and Tiemann et al. (2008) also suggested that part of the reduction of CH_4 production observed when tannins are added to diets is due to reduction of nutrient digestion. In the present study, the RED of NDF linearly reduced (Table 11) up to a magnitude of 26.4% and CH_4 production also linearly reduced up to a magnitude of 34.7% (Table 14). Therefore, it is thought that this reduction of CH_4 production may be greatly related to reduction of NDF rumen degradability.

The reduction of fiber digestion can explain the linear reduction of acetate and butyrate production and the consequent linear reduction of total SCFA (g/kg DM.d). This corroborates with Patra and Saxena (2011) who stated that the effect of tannins on the reduction of carbohydrate digestion rate, especially cellulose and hemicellulose, can reduce the concentration of total SCFA in the rumen by reducing the molar concentration of acetate. So, according to Ellis et al. (2015), the type of SCFA formed in the rumen is essential in mechanistic models that predict enteric methanogens because propionate is a hydrogen sink whereas acetate and butyrate are hydrogen sources, and hydrogen is the major substrate for CH_4 formation.

Despite the linear reduction of acetate and butyrate production and lack of effect on propionate production, tannins did not alter the acetate:propionate ratio. Working with tannins from quebracho extract at the concentrations of 1, 2, 4 and 6% (infused directly into the rumen of heifers through rumen cannula), Dickhoefer et al. (2016) observed a linear increase of propionate and butyrate proportions while those of acetate reduced. On the other hand, the increasing quadratic effect of tannins on the concentraction of propionate (incubated and non-incubated flasks) (Table 14) and the lack of effect on propionate production may explain the fact that tannins generally do not affect propionate production but may increase its concentraction by reducing the production of acetate and butyrate.

4.4.3. Energy partitioning

This is a subject poorly addressed by several researchers, therefore, few are the data available in the literature.

By using the *ex situ* technique, it was possible to estimate the energy release in digestive tract. The lack of interaction between monensin and tannins on the energy partitioning ($P > 0.05$) may suggest independent effects between these additives (Table 15). The transitory effect of monensin on rumen metabolism (discussed in the former section) may have contributed to the lack of effect of this additive on the energy partitioning in gastrointestinal tract.

The linear increase of rumen solid mass (kg/day) caused by tannins might have been due to the linear reduction of both passage and digestion rates (discussed in the former chapter) which led to linear reduction of DMI (Table 11) and, consequently, to a linear reduction on GEI (Mcal/day) (Table 15). Although there was a linear reduction of GE release in form of CH_4 (Mcal/cow.d), the different levels of tannins linearly reduced the GE release in intestine and linearly increased the energy loss in feces. This may have been due to formation of complexes between nutrients and tannins (tannins-proteins or tannins-fiber complexes, for example) which caused the reduction of nutrient digestion and consequent loss of energy in feces. Similar results were observed by Perna Junior (2018).

The proportion of GE loss in form of CH_4 was about 5% of the total GE consumed without tannins (i.e. control treatment) (Table 15), corroborating 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 15%. Including tannins up to 2.25% DM the GE loss significantly decreased to about 4%. This may appear to be slight, but supposing that all cows consumed the same amount of GE (3.94 Mcal/kg DM from the diet used in this study), the loss of GE in the form of CH_4 in 2.25% of tannin inclusion (0.162 Mcal) corresponds to 83.5% of the GE loss without the addition of tannins (0.194 Mcal), representing a decrease of 16.5%. Therefore, the use of tannins to retain feed energy which would be lost as CH_4 appears to have great benefit, the problem, as shown in the study, are the consequences of weak digestibility of the nutrients which lead to high loss of feed energy in feces.

4.5. CONCLUSIONS

Monensin and tannins acted independently, so it was not observed any synergy between these additives on methane, short chain fatty acids or rumen ammonia productions as well as on feed energy partitioning of Nellore cows. The use of *Acacia mearnsii* tannins up to 2.25% DM reduced methane production but also reduced the total short chain fatty acids, reduced the energy release in the intestine and increased the energy loss in feces; therefore, tannins did not improve the feed energy efficiency.

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5. POTENTIAL OF BIOGAS PRODUCTION FROM WASTE OF NELLORE COWS FED MONENSIN AND TANNINS OF *Acacia mearnsii*

Abstract: The intensification of animal production and increased size of animal production units are the tendencies all over the world, and this trend represents a considerable pollution hazard through accumulation of high amounts of animal waste. Anaerobic biodigestion of animal waste appears to offer significant economic and environmental benefits, and generates addtional fossil-free energy from biogas. The present study aimed to evaluate the potential of biogas production from waste of Nellore cows fed monensin and tannins of *A. mearnsii* extract as an alternative to animal waste management and renewable energy production. Eight Nellore cows were arranged in 2 contemporary 4 x 4 Latin square design and received 8 diets that differed in the level of tannin inclusion (0.0, 0.75, 1.5, and 2.25% DM) and sodium monensin, which was daily administered to each cow (300 mg/cow.day) of one square. The feces and urine were then collected for anaerobic biodigestion. Experimental batch type biodigesters were used and were located within a climate chamber (30 to 35ºC). These were arranged in a completely randomized design in a 2 x 4 factorial arrangement of 8 treatments with 4 replicates totaling 32 experimental units. The data were analyzed by using the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). Monensin reduced nutrient removal efficiency and CO_2 production by 18.9%, but increased CH_4, CO_2 and N_2O production per gram of volatile solids (VS) removed. The different levels of tannins had a quadratic effect on total solid (TS) recovery, but the recovery of VS and N linearly increased with a consequent quadratic effect on TS removal efficiency and a linear reduction of the removal efficiency of VS and N. Tannins had a quadratic effect on total biogas and CH_4 production and linearly reduced the production of CO_2 with no effect on N_2O. It was observed an interaction between monensin and tannins on TS and VS recovery and VS removal efficiency. The combined use of monensin and tannins reduced the potential of biogas production from waste by reducing the efficiency of nutrient use. From the environmental point of view these additives may be beneficial in reducing the emission of greenhouse gases from waste, but from the point of view of energy generation there seems to be of no importance. Nevertheless, more studies should be developed to support these findings. Monensin appeared to have the potential to provide more strength to tannins to rapidly reach a significant effect to reduce the efficiency of nutrient use at low concentrations of tannins.

Keywords: Anaerobic biodigestion; Biogas; Carbon dioxide; Methane; Nitrous oxide

5.1. INTRODUCTION

The intensification of animal production and the increased size of animal production units are now tendencies of animal production activity all over the world. This represents a considerable pollution hazard through accumulation of high amounts of animal waste, needing, therefore, a suitable waste management system (HOLM-NIELSEN et al., 2009). The main emissions within the farms include enteric CH_4 from the animals, CH_4 and N_2O mainly in housing facilities during long-term storage and during field application, and N_2O from nitrification and denitrification processes in the soil where feed crops are produced as well as pastures (ROTZ, 2017; SORDI et al., 2013). Although the concentrations of CH_4 and N_2O in the atmosphere are lower than that of CO_2, these gases have a heating potential of 25 and 298 times more than CO_2, respectively. The N_2O from agricultural emission is mentioned to contribute with about 60% of global N_2O emission, principally through deposition of animal excreta into pasturelands and the application of nitrogen fertilizers (IPCC, 2007). Global emissions of GHG from manure, as either organic fertilizer on cropland or manure deposited on pasture, grew between 1961 and 2010 from 0.57 to 0.99 $GtCO_2eq$ per year. On average, emissions grew by 1.1% per year (IPCC, 2014).

Certainly, the efficient treatment of animal waste can therefore support environmental protection in addition to bioenergy management (ACHINAS et al., 2018). Anaerobic digestion is a biological process that can convert organic substrates to biogas (ZHANG et al., 2016), and it is basically characterized by reactions in which biogas is produced from biodegradable products in the absence of oxygen (NESHAT et al., 2017). It is increasingly used worldwide to generate energy from biogas and brings significant economic and environmental benefits (SCARLAT et al., 2018) by being an efficient alternative technology that combines biofuel production with sustainable waste management (ACHINAS et al., 2017). With the rising demand for renewable energy and environmental protection, anaerobic digestion technology has deserved great attention within the scientific community (MAO et al., 2015).

Biogas is mainly comprised of CH_4 and CO_2 and minor amounts of other gases, such as nitrogen, hydrogen sulfide, ammonia and water vapor (NESHAT et al., 2017). Biogas from agricultural waste represents an important way to produce fossil-free energy, allows nutrient recycling and reduces GHG emission (AHLBERG-ELIASSON et al., 2017).

Monensin and tannins, separately, have shown to reduce enteric CH_4 emission from ruminants. The monensin's mode of action is by reducing Gram-positive microorganisms, which are the major producers of methanogenic substrates as the final fermentation products

(CO_2, acetate, formate, butyrate, hydrogen, etc.), (RUSSELL; HOULIHAN, 2003). There are three major forms by which tannins reduce enteric CH_4 emissions. The first is by reducing methanogenic *archaea*; the second is through reduction of *archaea* associated rumen protozoa, and the third is through depression of fiber digestion in the rumen (FINLAY et al., 1994; PATRA; SAXENA, 2011; CARRASCO et al., 2017). Tannins may also reduce protein disgestibility by forming complexes with these macromolecules by making them inaccessible to microbial and enzymatic digestion (NIGRANT et al., 2017; PATRA; SAXENA, 2011). The decreased digestibility of dietary nutrients is expected to increase fermentable organic matter (referenced in this study as volatile solids) concentration in feces, which can promote a great anaerobic biodigestion for biogas production including CH_4 (FAO, 2013).

In animal nutrition, monensin is largely used to enhance feed efficiency in addition to reduce CH_4 production. Some authors, such as Patra and Saxena (2011), have cited that low to moderate concentrations of tannins also improve feed efficiency in addition to reducing CH_4 production. Although much is known about the effects of these additives on rumen fermentation, little is known about their effects on the fermentation of waste. Hence, the overriding question was whether or not the effect of these additives on the reduction of enteric CH_4 production provides conditions for the emission of GHG from waste.

Given these factors, the hypothesis tested in this study was that the combined use of monensin and tannins would have synergy in the production of CH_4, N_2O and CO_2 from the waste of Nellore cows by means of anaerobic digestion. This study's aim was to evaluate the potential of biogas production from the waste of Nellore cows fed monensin and tannins of *A. mearnsii* as an alternative to animal waste management and renewable energy production technique.

5.2. MATERIAL AND METHODS

5.2.1. Ethical issue and place of experimentation

The experiment followed 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 University of Sao Paulo (USP) under the protocol number CEUA 3080240518. The experiment was carried out at the Animal Nutrition and Production Department (VNP) of the College of Veterinary Medicine and Animal Science (FMVZ) of USP, Fernando Costa Campus in Pirassununga, Brazil. The analyzes were

performed in the Laboratory of Ruminat Nutrition and in the Laboratory of Chromatography of VNP.

5.2.2. Treatments and experimental design

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

5.2.2.1. Feeding phase

Eight Nellore cows - non-pregnant and non-lactating, mean body weigth of 582 kg (± 96) - were kept in a covered shed in individual pens with free access to water and feed and sand bedding. The shed had suspended fans that were automatically triggered during the hottest hours of the day (> 28ºC) to ease the effects of temperature on animals.

The animals were arranged in 2 contemporary 4 x 4 Latin square design and received diets which differed in the inclusions of sodium monensin and the levels of tannin. The diets were represented by the following treatments: 1) diet without tannins, 2) diet with 0.75% of tannins in DM, 3) diet with 1.5% of tannins in DM and 4) diet with 2.25% of tannins in DM. In addition to tannins, each cow in one square received daily 300 mg (about 32 mg/kg DM) of sodium monensin (Rumensin® 200, Elanco Animal Health, Brazil) from the beginning to the end of this experimental phase, administered twice a day (150 mg at 8 a.m. and 150 mg at 4 p.m.), mixed with the feed.

The tannins used 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, 2003b), and total tannins (82.3% tannic acid equivalent) were estimated by the difference in total phenol concentration, both 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, 2003b).

The feed was offered twice a day (at 8 a.m. and 4 p.m.) in the form of a total mixed ration (TMR) containing a ratio of 50% of corn silage to 50% of concentrate. The feed supply was monitored to ensure daily leftovers of approximately 5%. The proportions of the various ingredients and the chemical composition of the diets are shown in Table 1.

This phase was carried out in 4 periods of 24 days each; the first 16 were to adapt the animals to the diets, and between the 17th and 21st days the collection of feces for the anaerobic digestion phase was performed. The collection of feces was performed twice per day (8 a.m. and 4 p.m.) by hand. All the feces 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 stimulation by vulva massage. There was no separation of the urine of the same cow.

5.2.2.2. Anaerobic digestion phase

5.2.2.2.1. Substrate preparation, experimental design and treatments

The samples of feces and urine collected and frozen during the feeding phase were 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 analyzes of the substrate (Table 16).

The substrate composition followed these ratios: 40% of waste, 3.3% of inoculum, and 56.7% of water. The sewage sludge from waste treatment had 0.164% of total solids (TS) and was used as inoculum. 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 in a 2 x 4 factorial arrangement of 8 treatments with 4 replicates and totaling 32 experimental units (represented by feces of the animals which received the different levels of tannins and monensin in the diet). The purpose was to evaluate the associative effect of monensin and tannins from four inclusion levels of tannins in the diet (0.0, 0.75, 1.5 and 2.25% of DM). The anaerobic biodigestion was performed in mesophilic conditions (30 to 35ºC), ideal for digestion kinetics (METCALF; EDDY, 2014). The biodigesters were placed inside a climate chamber with electric resistance heating system and digital temperature recorder. The composition of the substrates in the different biodigesters is shown in table 16.

Table 16 - Composition of substrates of anaerobic batch type biodigesters supplied with waste of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii.*

Variable	Monensin (M)		Level (L)					Probability		
	0	30	0%	0.75%	1.5%	2.25%	SEM	M	L	M*L
TS (g/kg)	48.20	47.88	48.32	47.77	48.58	47.48	0.677	NS	NS	NS
VS (g/kg)	40.79	40.19	38.48	39.65	41.83	42.01	0.659	NS	0.0464^{L}	NS
N (g/kg TS)	34.11	36.49	34.17	34.74	35.55	36.75	1.002	NS	NS	NS
NDF (g/kg TS)	386.2	423.2	388.0	395.6	392.1	443.2	7.865	NS	0.0105^{L}	NS
pH	6.45	6.52	6.33	6.37	6.51	6.73	0.0461	NS	0.0010^{L}	NS

SEM: Standard error of mean; ST: Total solids; VS: Volatile solids; N: Nitrogen; NDF: Neutral detergent fiber. (Own authorship).

5.2.2.2.2. Quantitative production of biogas through biodigesters

The batch type biodigesters basically consisted of three straight cylinders with diameters of 15, 10 and 7.5 cm, with a mean capacity to ferment 2 liters of substrate each. The 15 and 7.5 cm cylinders were inserted, one inside the other, so that the space between the outer wall of the inner cylinder and the inner wall of the outer cylinder contained a volume of water (water seal), reaching the depth of 60 cm. The cylinder of intermediate diameter (gas meter) had one of the ends sealed that retained a record for biogas discharge while it was capsized in the water seal to provide anaerobic conditions and to store the produced gas.

After filling, the biodigesters were conditioned in a climate chamber with controlled temperature (between 30 and 35ºC) to guarantee that the test occurred in mesophilic conditions. The temperature was monitored by means of a digital thermometer (in ºC) before biogas reading.

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. The 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 20ºC 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:

$$(V_0P_0)/T_0 = (V_1P_1)/T_1 \quad (21)$$

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 equal to 10273.11 mm H_2O and the pressure conferred by gas meters of 71 mm H_2O, the following expression was obtained to correct the biogas volume:

$$V_0 = (V_1/T_1) \times 293.7703 \quad (22)$$

The biogas sampling was performed whenever the biogas volume was measured. Samples were collected using a 60 mL syringe connected to the gas register at the top of the gas meter. Before the sampling, the biogas was homogenized in the biodigester and the collecting flasks (glass flasks of 50 mL of capacity, Frascolex, Sao Paulo, Brazil) were then washed twice with the gas taken from the biodigester, then 50 mL of biogas were injected for analysis. The gas meters were then emptied to allow a new accumulation of gas. The test was terminated when the biogas production ceased, i.e. there was no more displacement of the gas meter.

The concentration of CH_4, CO_2 and N_2O was determined by 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 chromatograph injector (split/splitless), 4 mL of which were used to wash the injection system and 2 mL were used for analysis. 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 composition of each digester according to the equation:

$$Vol = (Vol_{BIOGAS} \times \%Gas)/100 \quad (23)$$

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 or 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 VS eliminated during the fermentation).

The Gompertz model was used to study biogas production kinetics and its components. The model assumes that the gas production rate is proportional to the microbial activity, but the proportionality decreases with the incubation time which can be interpreted as a loss of efficiency in the fermentation rate (LAVRENCIC et al., 1997). The mathematical description of the gas production curves allowed the data analysis, the substrate comparison, and the performance of the fermentation. The following equation describes the model used:

$$Y_t = A \exp [-B \exp (-kt)] \quad (24)$$

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:

$$t_1 = \ln B/k \quad (25)$$

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

The gas production at inflaction point was determined as:

$$y_1 = A/\exp \quad (26)$$

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

5.2.2.2.3. Nutrient removal

The substrates added and recovered in each biodigester were weighed and multiplied by their DM content percentage to calculate the DM content in grams. The added and recovered nutrients, expressed in grams, were calculated by multiplying between the added or recovered,

and expressed as grams of DM, which then were expressed as a percentage and divided by 100 according to the following equation:

$$\text{Nutrient (g)} = \frac{\text{Added or recovered nutrient (\%) x DM (g)}}{100} \quad (27)$$

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

$$\text{Removed nutrient (\%)} = \left[\frac{\text{Added nutrient (g) – recovered nutrient (g)}}{\text{Added nutrient (g)}}\right] \text{x } 100 \quad (28)$$

5.2.2.2.4. Laboratory analysis

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

5.3. Statistical analysis

The data were analyzed by using the Statistical Analysis System (SAS 9.3, Institute Inc., 2013). Before the data were analyzed they were evaluated in relation to the presence of discrepant information (outliers) and normality of the residues by the Shapiro-Wilk test. When the normality premise was not met, the data were transformed. They were next submitted to analysis of variance, which separated, as causes of variation, the monensin effect, tannin level effect, and the interaction between monensin and tannin level (all as fixed effects). The level effect was evaluated by the use of orthogonal polynomials separating the effects in linear, quadratic, and quadratic deviation. A significance level of 5% was adopted. The statistical model used was described according to the equation below:

$$Y_{ijkl} = \mu + M_i + L_j + M_i*L_j + e_{ijkl}$$

Where:

Y_{ijkl} = observation concerning Monensin (i) + Level (j) + Monensin (i) * Level (j) + random error associated with each observation (e_{ijkl});

μ = overall mean;

M_i = effect of monensin (fixed effect);

L_j = level effect (fixed effect);

M_i*L_j = interaction between monensin (i) and level (j) (fixed effect);

e_{ijkl} = random error associated with each observation.

5.4. RESULTS

5.4.1. Biodigestion and nutrient removal efficiency

There were no significant differences ($P > 0.05$) in the amount of TS, VS or nitrogen (N) on the substrates corresponding to the different treatments (Table 17). Significant interaction was observed between monensin and tannins ($P < 0.05$) either in the TS or VS recovery (i.e. the amount of nutrients not used during biodigestation) and VS removal efficiency (Figures 6, 7 and 8, respectively).

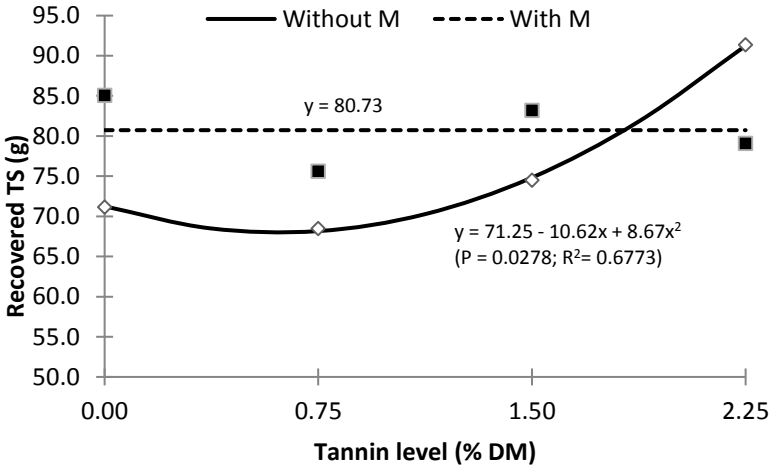
Monensin significantly reduced TS and VS removal efficiency by 29.4 and 29.0%, respectively, but no significant effect was observed for N. The different levels of tannins had a quadratic effect in the TS recovery and consequently in removal efficiency, but on VS and N the recovery linearly increased and the removal efficiency linearly reduced. The increase in VS and N recovery was 28.3% and 41.8%, respectively, for the highest level of tannins compared to the control treatment. Monensin increased pH substrate during biodigestion but although tannins linearly increased the substrate pH before (Table 16), it was quadratically reduced during anaerobic digestion.

Table 17 - Biodigestion and removal efficiency of nutrients from anaerobic batch type biodigesters supplied with waste of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii.*

Variable	Monensin (M)		Level (L)					Probability		
	0	30	0%	0.75%	1.5%	2.25%	SEM	M	L	M*L
Added nutrients										
TS (g)	97.91	95.69	98.75	95.12	96.48	96.84	1.360	NS	NS	NS
VS (g)	81.59	79.67	76.97	79.33	82.98	83.24	1.409	NS	NS	NS
N (g)	3.254	3.434	3.263	3.225	3.446	3.441	0.076	NS	NS	NS
Recovered nutrients										
TS (g)	76.37	80.73	78.09	72.03	78.85	85.22	1.956	NS	0.0639^{Q}	0.0433
VS (g)	57.50	62.70	53.95	55.43	61.77	69.24	1.946	0.0709	0.0003^{L}	0.0346
N (g)	2.18	2.20	1.77	2.17	2.32	2.51	0.067	NS	$<.0001^{L}$	NS
pH after biodigestion	7.53	7.61	7.67	7.55	7.52	7.55	0.023	0.0122	0.0157^{Q}	NS
Removal efficiency										
TS (%)	22.14	15.64	20.85	24.38	18.43	11.91	1.639	0.0102	0.0398^{Q}	0.0754
VS (%)	29.80	21.16	29.63	30.03	25.71	16.55	2.021	0.0028	0.0009^{L}	0.0061
N (%)	32.72	35.44	45.14	32.45	32.27	26.46	1.851	NS	0.0003^{L}	NS

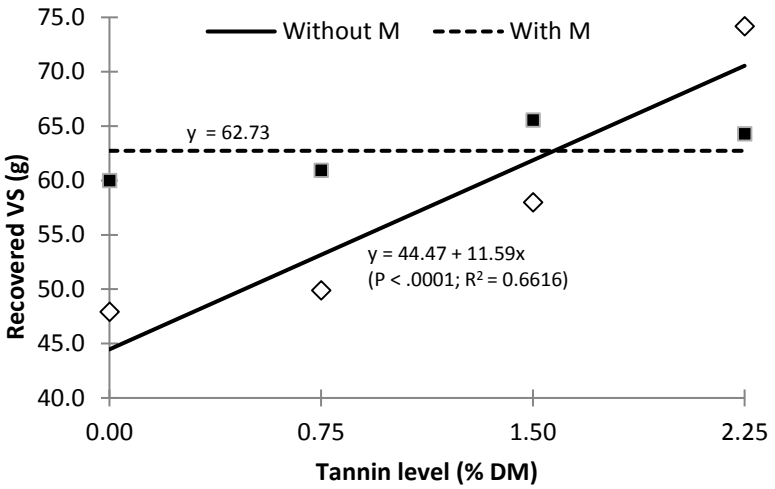
SEM: Standard error of mean; M*L: Interaction between monensin and level; TS: Total solids; VS: Volatile solids; N: Nitrogen. (Own authorship).

Figure 6 - Graph showing the interaction between monensin (M) and tannins on the amount of TS recovered after anaerobic biodigestion.



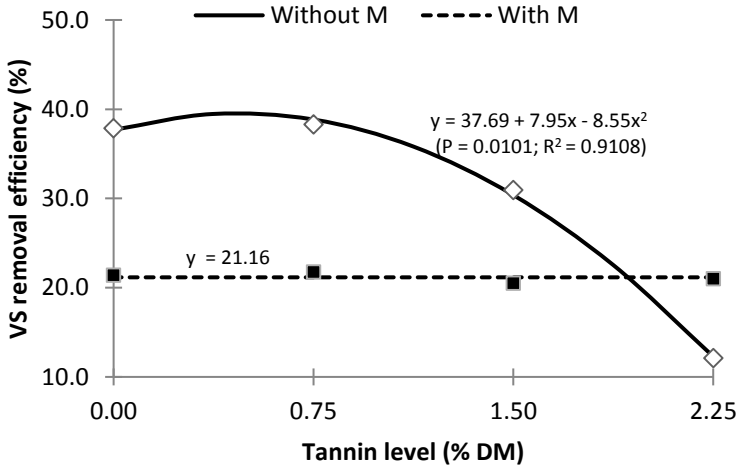
The square points in bold represent the means observed in the different tannin levels only in the biodigesters whose substrates were also treated with M. In these biodigesters, the joint effect of M and tannins was not significant, therefore, it was chosen to present the general mean observed (dashed line). The empty square points show the means observed in the different tannin levels in biodigesters whose substrates were only treated with tannins (quadratic effect). The continuous line passing over the empty squares shows the estimated means for the biodigesters whose substrates received M and tannins if they had not received M (quadratic effect). Then, it may be observed that when M was administered jointly with tannins, the effect of tannins was not observed. This suggests that M blocked the effect of tannins by antagonistic interaction. (Own authorship).

Figure 7 - Graph depicting the interaction between monensin (M) and tannins on the amount of VS recovered after anaerobic biodigestion.



The square points in bold represent the means observed in the different tannin levels only in the biodigesters whose substrates were also treated with M. In these biodigesters, the joint effect of M and tannins was not significant, then it was chosen to present the general mean observed (dashed line). The empty square points show the means observed in the different tannin levels in biodigesters whose substrates were only treated with tannins (quadratic effect). The continuous line shows the estimated means for the biodigesters whose substrates received M and tannins if they had not received M (linear effect). Therefore, it may also be observed that when M was administered with tannins, the effect of tannins was not observed, suggesting inhibition of tannin effect by M through antagonistic interaction. (Own authorship).

Figure 8 - Graph depicting the interaction between monensin (M) and tannins on VS removal efficiency.



The square points in bold represent the means observed in the different tannin levels only in the biodigesters whose substrates were also treated with M. In these biodigesters, the joint effect of M and tannins was not significant, then it was preferred to present the general mean observed (dashed line). The empty square points show the means observed in the different tannin levels in biodigesters whose substrates were only treated with tannins (quadratic effect). The continuous line passing over the empty squares shows the estimated means for the biodigesters whose substrates received M and tannins if they had not received M (quadratic effect). Therefore, it appears to be obvious that when M was administered along with tannins, the effect of tannins was not observed. This also suggests that monensin inhibited the effect of tannins by antagonistic interaction. (Own authorship).

5.4.2. Biogas production

The theoretical, non-significant biogas production (in general) was observed about 160 days after biodigesters were filled; therefore, the biodigestion process was interrupted on day 175. No significant interaction (P > 0.05) was obsereved between monensin and tannins on biogas production parameters (Table 18). Monensin did not significantly alter the total biogas, CH_4 or N_2O production, but it reduced CO_2 production (L) by 18.9%.

Table 18 - Gas production (total biogas, CH_4, CO_2 and N_2O) in batch type biodigesters with waste of Nellore cows fed monensin (ppm) and different levels of tannins of *A. mearnsii*.

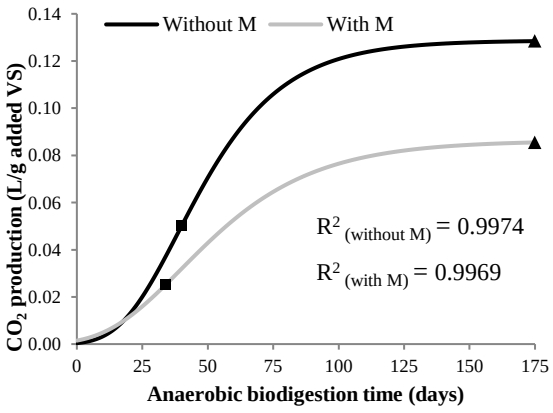
Variable	Monensin (M)		Level (L)					Probability		
	0	30	0%	0.75%	1.5%	2.25%	SEM	M	L	M*L
Biogas (L)	29.50	25.98	30.80	32.22	28.51	19.42	1.420	NS	0.0213^{Q}	NS
CH_4 (L)	20.85	18.96	21.98	22.78	20.81	14.05	0.974	NS	0.0159^{Q}	NS
CH_4, %	72.77	75.36	73.88	73.10	75.43	73.82	0.676	0.0800	NS	NS
CH_4/feces (L/g)	0.031	0.028	0.033	0.034	0.031	0.021	0.0015	NS	0.0160^{Q}	NS
CH_4/added VS										
A (L/g)	0.281	0.248	0.292	0.301	0.272	0.193	0.013	NS	0.0024^{L}	NS
k (L/g.day)	0.037	0.035	0.043	0.038	0.030	0.032	0.002	NS	0.0902^{L}	NS
t (day)	44.96	37.58	41.18	46.77	50.42	26.72	3.144	NS	0.0259^{Q}	NS
y (L/g)	0.103	0.091	0.107	0.111	0.100	0.071	0.005	NS	0.0024^{L}	NS
CH_4/removed VS (L/g)	0.843	1.348	1.108	1.296	1.074	0.905	0.102	0.0182	NS	NS
CO_2 (L)	8.647	7.010	8.818	9.437	6.697	5.362	0.486	0.0482	0.0014^{L}	NS
CO_2 (%)	27.22	24.63	26.10	26.88	24.56	26.16	0.676	0.0797	NS	NS
CO_2/feces (L/g)	0.013	0.011	0.013	0.014	0.012	0.008	0.0007	0.0485	0.0014^{L}	NS
CO_2/added VS										
A (L/g)	0.129	0.086	0.114	0.136	0.109	0.070	0.010	0.0397	0.0389^{L}	NS
k (L/g.day)	0.045	0.035	0.047	0.045	0.037	0.032	0.003	NS	0.0724^{L}	NS
t (day)	40.10	34.82	32.24	40.51	41.30	35.78	3.207	NS	NS	NS
y (L/g)	0.047	0.032	0.042	0.050	0.040	0.026	0.0036	NS	0.0389^{L}	NS
CO_2/removed VS (L/g)	0.352	0.485	0.433	0.464	0.402	0.375	0.0319	0.0591	NS	NS
N_2O (mL)	4.095	3.984	4.219	4.628	4.451	2.859	0.470	NS	NS	NS
N_2O (%)	0.012	0.015	0.013	0.014	0.015	0.013	0.001	NS	NS	NS
N_2O/feces (mL/g)	0.006	0.006	0.006	0.007	0.007	0.004	0.0007	NS	NS	NS
N_2O/added VS										
A (mL/g)	0.052	0.053	0.066	0.062	0.038	0.043	0.006	NS	NS	NS
k (mL/g.day)	0.043	0.042	0.052	0.041	0.033	0.045	0.0037	NS	NS	NS
t (day)	42.65	34.99	27.96	44.21	45.72	37.38	3.090	NS	0.0754^{Q}	0.0759
y (mL/g)	0.017	0.020	0.024	0.023	0.014	0.012	0.0022	NS	0.0391^{L}	NS
N_2O/removed VS(mL/g)	0.149	0.313	0.239	0.289	0.258	0.138	0.036	0.0368	NS	NS

SEM: Standard error of mean; M*L: Interaction between monensin and level; 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). (Own authorship).

Monensin increased the production of CH_4 and N_2O per gram of VS removed during the biodigestion process, but it did not significantly alter the stabilization value (A) for CH_4 or N_2O nor the maximum growth rate (k) or the time to reach the inflection point (t) for CH_4, CO_2 or N_2O production. Monensin did significantly reduce the stabilization value for CO_2 production by 33.3% (Figure 9).

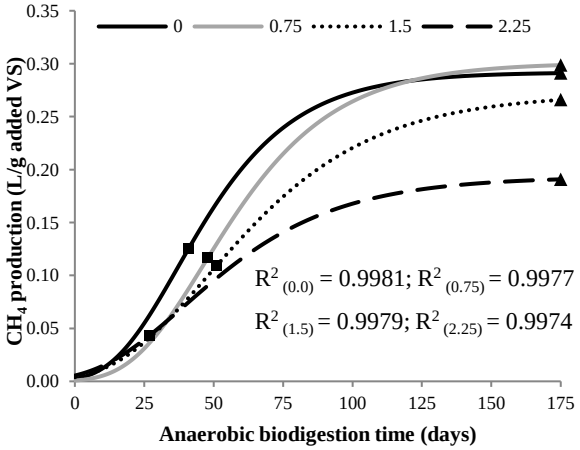
The different levels of tannins had a quadratic effect on total biogas and CH_4 production. The highest level of tannins reduced the total biogas and CH_4 production by 36.9 and 36.1%, respectively, when compared to control treatment. Tannins linearly reduced the stabilization value (A) as well as the maximum growth rate (k) for CH_4 production, but it showed a quadratic effect on the time to reach the inflection point, and the inclusion levels of 0.75 and 1.5% reached the inflaction point singnificantly later than the control and 2.25% treatments (Figure 10). Tannins linearly reduced CO_2 production as well as the stabilization value (A) and the maximum growth rate (k) (Figure 11). They also linearly reduced the production of N_2O (mL) per gram of VS added, but no significant effect of tannins was observed in the production of CH_4, CO_2 and N_2O per gram of VS removed.

Figure 9 - Graph adjusted by the Gompertz model depicting the time (in days) and cumulative CO_2 production.



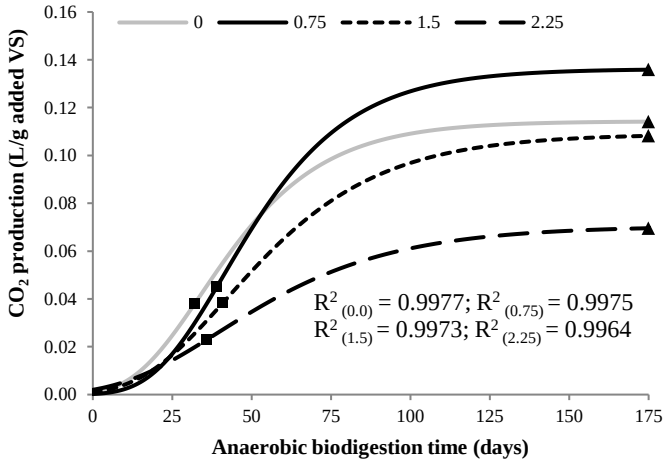
M: monensin; ■ inflection point; ▲stabilization value (Own authorship).

Figure 10 - Graph adjusted by the Gompertz model depicting the time (in days) and cumulative CH_4 production.



■ inflection point; ▲stabilization value (Own authorship).

Figure 11. Graph adjusted by the Gompertz model depicting the time (in days) and cumulative CO_2 production.



■ inflection point; ▲stabilization value (Own authorship).

5.5. DISCUSSION

The substrate pH before anaerobic biodigestion ranged between 6.33 and 6.73 (Table 16). This is a characteristic of ruminant gastrointestinal tract pH, but after anaerobic biodigestion, it ranged between 7.52 and 7.67 (Table 17). This shows a pH increase during biodigestion process that supports Rabiu et al. (2014), Mshandete et al. (2006), and Gunaseelan (1995), who stated that the pH of a normal and healthy anaerobic biodigestion system for CH_4 production is generally in the range of 7.0 to 8.5. Hence, the significant effect of monensin on increasing pH (from 7.53 to 7.61) during biodigestion indicates that this ionophore created good pH conditions for CH_4 production. Unlike monensin, the different levels of tannins reduced biodigestion pH, but it still remained within the optimum range cited by the above authors. Perna Junior (2018), working with waste of Nellore and Holstein cows fed tannins of *A. mearnsii* up to 1.5% DM basis, also observed linear reduction of biodigestion pH.

The increased concentration of VS and NDF in feces, and consequently in the substrates (g/kg) (Table 16), may have occurred because tannins reduce nutrient disgestibility by forming complexes, which make nutrients inaccessible to microbial and enzymatic digestion in gastrointestinal tract (NIGRANT et al., 2017; PATRA; SAXENA, 2011). This effect was also observed by Perna Junior (2018).

The effects of monensin on the reduction of nutrient removal efficiency (Table 17), CO_2 production (Table 18), the increase of CH_4, CO_2 and N_2O production per gram of VS removed, and the effects of tannins on increasing nutrient recovery and reducing nutrient removal

efficiency as well as the reduction of total biogas, CH_4 and CO_2 production may be the indication of direct effect of these additives on anaerobic biodigestion. The hypothesis for this study was that both additives would improve anaerobic biodigestion by increasing the concentration of nutrients in feces. Therefore, the reduction of the performance of biodigestion may suggest that significant concentrations of these additives or their bioactive metabolites might have been present in feces. Hao et al. (2011), adding 25 g/kg of *A. mearnsii* condensed tannins (CT) (i. e. 2.5%) to cattle diets, found increased agronomic value of the manure and compost as fertilizer but found no increase in the production of CH_4, CO_2 and N_2O. Perna Junior (2018) found no differences of CH_4, CO_2 and N_2O production; the only difference he observed was a linear increase on the concentration of CO_2, an effect not observed in this study.

There are some evidences that most of monensin bioactive metabolites are eliminated via feces in ruminants. Davidson (1984), investigating whether or not monensin was absorbed, metabolized and eliminated through the bile of calves and other animal species, found that most of the consumed monensin was recovered in feces but had very little recovery in urine and tissues. Determining the excretion pattern and tissue distribution of [^{14}C] monensin in cattle, Herberg et al. (1978) recovered almost 95% of active monensin metabolites in feces. Hydrolysable tannins may be degraded and metabolized by rumen microorganisms (MCSWEENEY, 2001), but CT are not degraded in the rumen. In addition, the complexes (tannin-protein or tannin-fiber) formed in gastrointestinal tract may not be reversible, and hence eliminated in feces (MAKKAR, 2003a). Therefore, the high recovery rates of monensin and tannins in feces may suggest that these additives affect the fermentation of feces, at least when fresh (HAMILTON et al., 2010). It is not clear how long monensin and CT or their bioactive metabolites remain active to hinder anaerobic biodisgestion and rumen fermentation. Using a rumen simulation technique (RUSITEC), Makkar et al. (1995) exposed rumen microbes to small amounts of quebracho (*Schinopsis spp*) tannins for 8 days to induce enzymes capable to degrade CT, but there was no degradation. Makkar (2003a) reported degradation of purified quebracho and *A. nilotica* CT within 7 days, but this occurred under aerobic conditions in artificial fermenters. To a theoretical imagination, it is difficult to believe that even present in feces, monensin and tannins or their bioactive metabolites, can remain active up to 175 days in order to continually impair the kinetics of biodigestion and consequently reduce the performance of the fermentation, but there must be some explanation whether once the fermentation is disturbed, at the beginning of the process, by the presence of bioactive substances, the future biodigestation performance is also impaired.

The reduction of nutrient utilization by microorganisms is a characteristic of tannins. The reduction of the removal efficiency of TS, VS, and N was the consequence of increased nutrient recovery (Table 17), i.e. the reduced capacity to biodigest nutrient caused by tannins, even though, the VS removal efficiency, which ranged between 16.55 to 30.03%, was only below the range stipulated by Dohányos and Zábranská (2001) (25-50%) when monensin and 2.25% of tannins were included in the diet.

The interactions observed between monensin and tannins (Figures 6, 7 and 8) appear to have been harmful in the use of nutrients (TS and VS) since it can be thought that if monensin had not been used, the tannin effect on the efficiency of nutrient use likely would have been less severe than it really was.

The reduction of the total CO_2 production (L) by monensin was accompanied by the reduction of the stabilization value of the production (A) (Table 18 and Figure 9), but it did not affect the production growth rate (k) and the time to reach inflection point. This may have been due to the fact that monensin reduced microbial capacity to remove nutrients; therefore, it was not possible to reach the production potential. According to IPCC (2006), the specific productivity of fermentation products is measured in terms of removed VS. Besides the reduction of CH_4 production, the tannins also reduced the production growth rate and the stabilization value. This may have been the reason why the average production of CH_4 (0.25 L) per gram of added VS was below the production found by Møller et al. (2004) (0.4 L) and Perna Junior (2018) (0.34 L).

5.6. CONCLUSIONS

According to the results observed in the present study, it was concluded that the combined use of monensin (300 mg/cow.day) and tannins (up to 2.25% DM) in the diet of Nellore cows reduced the potential of biogas production from waste by reducing the efficiency of nutrient use, although tannins have increased the concentration of nutrients in feces. Therefore, the hypothesis, that the use of tannins and monensin as feed additives for cattle can improve the anaerobic biodigestation of the waste, may be mistaken.

The use of these additives may be beneficial in reducing the emission of greenhouse gases from waste, but from the point of view of energy generation there seems to be of no importance. To be sure, more studies on the use of these additives should be developed to further these observations.

Monensin inhibited tannins on the efficiency of anaerobic biodigestion through antagonistic interaction.

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