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Effect of Tannins and Monensin on Rumen Fermentation and Feed Energy Partitioning of Nellore Cows

Research Article

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ABSTRACT

This study aimed to evaluate the effect of *Acacia mearnsii* tannins and monensin combination on rumen fermentation parameters and feed energy partitioning of Nellore cows. In a 2 × 4 factorial arrangement, 8 cannulated Nellore cows were distributed in 2 contemporary 4 × 4 latin square design and received 8 diets that differed in the level of tannin inclusion (0.00, 0.75, 1.50 and 2.25% of feed dry matter (DM)) and presence of monensin. Monensin was daily administered to each cow in one square (about 32 mg/kg DM). Accordingly, the experiment was conducted in 4 periods of 28 days each. Therefore, daily dry matter intake (DMI), gross energy intake (GEI), rumen solid mass, neutral detergent fibre (NDF) degradability, rumen pH, rumen fermentation products and rumen protozoa were measured and the feed energy partitioning was evaluated. The only interaction (antagonistic) observed between monensin and tannins was on the minimum rumen pH (P=0.0173). Monensin reduced acetate:propionate production ratio in 28.80% (P=0.0007). Tannins linearly reduced DMI, GEI and NDF degradability, but linearly increased rumen solid mass (P<0.05). Tannins had a quadratic effect on the time (min/day) the rumen pH was below 5.8 and 6.0, as well as the pH area (h.pH/day) below 5.8, 6.0 and 6.2. Tannins had neither effect on rumen ammonia nor on protozoa count (P>0.05). Tannins linearly reduced CH_4, acetate, butyrate and total short chain fatty acids (SCFA) production as well as the gross energy release in form of CH_4, but linearly reduced the energy release in intestine and linearly increased feed energy loss in faeces. Monensin and tannins had independent effects on rumen fermentation parameters, as well as on feed energy partitioning, therefore, no synergy was observed. The use of *A. mearnsii* tannins up to 2.25% of feed DM reduced CH_4 production (up to 34.7%), but did not improve feed energy efficiency.

KEY WORDS energy partitioning, feed additives, feed energy, methane, protozoa, short chain fatty acids.

INTRODUCTION

The increase in greenhouse gases (GHG) in the atmosphere has been identified as one of the main causes of climate changes as it increases the potential for global warming. Methane gas (CH_4) is considered the second largest contributor to global warming, with global warming potential 25 times greater than carbon dioxide (CO_2) and lifetime in the atmosphere of 9 to 15 years, with an annual growth rate of 7% (IPCC, 2007). Agricultural soils and livestock production (mainly the enteric fermentation of domesticated ruminants such as cattle, buffalos, sheep and goats) are

some of sectors responsible for the emission of gases (CO_2, CH_4 and nitrous oxide (N_2O)) that cause the increase of greenhouse effect in the atmosphere (Carega and Dantas, 2017).

According to IPCC (2014), global GHG emissions from agricultural production in 2000-2010 period were estimated at 5.0-5.8 $GtCO_2eq$/year. Based on a report by US Environmental Protection Agency (EPA, 2018), global CH_4 emissions from enteric fermentation increased by 7% from 1990 to 2005, from 1764 to 1894 $MtCO_2$-eq, and over this time period, global livestock populations have increased. According to the report, from 2005 to 2030, CH_4 emissions from enteric fermentation are projected to increase 22%, from 1894 to 2320 $MtCO_2$-eq. Despite these data, Lynch (2019) concluded that there are still insufficient data available to fully address important questions regarding the climate impacts of agricultural production.

Global GHG emissions from industrial and waste/wastewater sector grew from 10.4 $GtCO_2eq$ in 1990 to 13.0 $GtCO_2eq$ in 2005 to 15.4 $GtCO_2eq$ in 2010 (IPCC, 2014), showing that the industry-related GHG emissions have continued to increase and are higher than GHG emissions from livestock sector. Therefore, the environmental impact caused by the industrial sector may be higher than that of the livestock sector.

In addition to the environmental problems, CH_4 production from enteric fermentation of ruminants generates feed gross energy losses ranging from 2 to 15% (Wanapat *et al.* 2015), depending on the quantity, quality, and type of feed consumed (EPA, 2018). 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 livestock productivity, improving nutritional management, manipulation of rumen fermentation, changes in diet composition, addition of CH_4 production inhibitors or defaunation (Shibata and Terada, 2010).

Monensin and tannins are two feed additives referenced to promote feed efficiency in ruminants through mechanisms that modulate rumen fermentation, reducing CH_4 production and acetate:propionate ratio (Jayanegara *et al.* 2015; Montano *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 ions across microbial membrane, causing microbial energy depletion (Azzaz *et al.* 2015). Determining the effect of increasing monensin doses, Santos *et al.* (2019) observed improvement in performance of lactating dairy cows.

Using low doses of monensin, Polizel *et al.* (2020) observed an improvement in rumen fermentation which resulted in greater growth performance in lambs. Many studies, such as Osorio-Teran *et al.* (2017), Ogunade *et al.* (2018) and Teixeira *et al.* (2020), point out that the higher rumen fermentation efficiency, with a concomitant increase in animal performance, is partly associated with the reduction in the production of CH_4 caused by monensin, which occurs through the reduction of microorganisms (Gram-positive) responsible for the synthesis of substrates used for the production of CH_4 in the rumen. Numerous studies have shown the effect of monensin on reducing CH_4 production in ruminants. Wingard *et al.* (2018), determining effects of direct fed microbials on rumen fermentation of a forage-based diet in the presence and absence of monensin, observed reduction on total gas and CH_4 production. Capelari *et al.* (2018), testing the effect of monensin on rumen fermentation, observed an effective reduction in CH_4 production. Tannins are polymers with the ability to form complexes mainly with proteins and, to a lesser degree, with carbohydrates and minerals due to a greater number of phenolic hydroxyl groups (Addisu, 2016). Tannins may be hydrolysable (HT) or condensed (CT), both with desirable and undesirable effects depending on various factors such as concentration, source, type, composition and molecular weight (Nawab *et al.* 2020a). Among several benefits achieved with the use of tannins, CH_4 mitigation might be the most important for ruminant production (Naumann *et al.* 2017). According to Nawab *et al.* (2020b), tannins have potential to enhance ruminant production through improvement of rumen fermentation, feed energy efficiency (reducing CH_4 emission), and then contributing to minimise the problem of global warming. Using diets including tannins in heifers and mature beef cows, Stewart *et al.* (2019) observed reduction in enteric CH_4 emissions. Aboagye *et al.* (2019) and Aboagye and Beauchemin (2019) also have observed reduction in enteric CH_4 production from cattle fed tannins. To reduce the production of enteric CH_4, tannins act in three ways: (1) direct effect on methanogens (Archaea); (2) direct effect on the reduction of the quantity of archaea associated protozoa and (3) indirectly through depression of fibre digestion (Patra and Saxena, 2011; Carrasco *et al.* 2017). Therefore, by knowing that both monensin and tannins may 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 cows. Specifically, the aim was to evaluate the effect of tannins and monensin combination on rumen fermentation parameters and feed energy partitioning of Nellore cows.

MATERIALS AND METHODS

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 Faculty of Animal Science and Food Engineering of the University of Sao Paulo (Brazil) under the protocol number CEUA 3080240518.

Treatments, experimental design and feeding management

Eight Nellore cows, non-pregnant and non-lactating, carrying rumen cannula and mean body weigth of 582 kg (±96) were kept in a roofed shed in individual pen with free access to sand bedding. They were distributed in 2 contemporary 4 × 4 Latin square design in a 2 × 4 factorial arrangement and received 8 experimental diets that differed in the level of tannin inclusion (0.00, 0.75, 1.50 and 2.25% of feed DM) and the inclusion or not of monensin (Rumensin® 200, Elanco Animal Health, Brazil) which was daily administered to each cow in one square (300 mg, about 32 mg/kg DM). Kaolin was added as the tannin level decreased from 2.25 to 0.00% to equalise the dry matter in all treatments.

The tannins, from a commercial extract, were obtained from the bark of *A. mearnsii* (Seta Natur®-Seta Acacia Tannin Extract). The concentration of total phenols (84.40%) was determined by the Folin-Ciocalteau method (Makkar, 2003) and total tannins (82.30% 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 (32.30% leucocyanidine equivalent) was determined by the HCl-butanol method (Makkar, 2003).

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

Experimental period

The experiment was carried out in 4 periods of 28 days each, but the last two days of each period the cows were kept on pasture. The first 16 days were for diet adaptation. Thereafter, evaluations were recorded at the following times: the DMI and the degradability of NDF between days 17 and 21; rumen pH, rumen fermentation products (CH_4, SCFA and NH_3-N) and rumen protozoa on day 22; the rumen passage rate between days 23 and 25, and finally, the rumen solid mass on days 25 and 26.

Assessment of feed intake and gross energy of the diet and faeces

Cows had a free access to feed 24 hours a day, but the management strategy was to ensure leftovers of approximately 5%. During the 5 days of evaluation, the leftovers from each cow were collected and weighed for intake quantification which was obtained by the difference between the amount of feed supplied and the leftovers. On these days, samples of silage and concentrate were collected to determine the content of feed DM. In parallel, faecal samples were manually collected via the rectum at 8 a.m. and 4 p.m., to form a composite sample for each cow, to determine the faecal gross energy (GE). These samples (feed and faeces) were dried in a forced air oven at 65 ˚C for 72 hours according to AOAC (1995) and ground in a willye type knife mill in 2 mm sieves. Then the real DM content was determined at 105 ˚C for 16 hours. The GE of faeces and diet was determined by complete oxidation in adiabatic calorimetric pump.

Assessment of rumen solid mass

The rumen solid mass was determined by rumen emptying. The rumen content was manually removed through the rumen cannula according to Allen and Linton (2007). On day 25 the emptying was performed three hours after morning feeding and on day 26 the emptying was prior to feed administration. The liquid and solid portions were separated by using a 2 mm sieve, and then separately weighed. Afterwards, samples were collected for DM determination. Finally, both portions were returned to the rumen. The rumen DM was calculated based on the dry weight of the rumen content.

Assessment of NDF real effective degradability

The determination of real effective rumen degradability (RED) of NDF was performed according to the technique proposed by Ørskov *et al*. (1980); Ørskov and McDonald (1979) and Mertens (1993) with the aid of SAS NLIN procedure, version 9.3 (equation 1). Silage and concentrate samples were dried at 65 ˚C for 72 hours and ground with Willye knife type mill with 2 mm sieves. Next, both portions were mixed in proportions of 50:50 (DM basis), then 9 g were put in nylon bags of 50 µm porosity. These were incubated in the rumen for 0, 3, 9, 24, 48, and 96 hours. After the removal, they were washed with fresh water, dried and finally weighed. The DM disappearance was obtained by the difference between initial (before incubation) and final (after incubation) weights and obtained the percentage of degraded fraction. The zero-time bags were put in a thermostatic bath at 39 ˚C for 5 minutes and washed in fresh water.

Table 1 Proportions of ingredients and chemical composition of experimental diets

Ingredients (% dry matter, (DM))	Tannin level (% feed DM)			
	0.00	0.75	1.50	2.25
Maize silage	50.00	50.00	50.00	50.00
Dry ground corn grain	32.36	32.36	32.36	32.36
Soya bean 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]	0.00	0.91	1.82	2.74
Kaolin	2.74	1.82	0.91	0.00
Chemical composition of the diet for all tannin levels				
Dry matter[3] (DM, %)	60.35			
Crude protein[3] (CP, % DM)	14.43			
Ruminally degradable protein[4] (% CP)	65.30			
Ruminally undegradable protein[4] (% CP)	34.70			
Neutral detergent fibre[3] (% DM)	28.06			
Effective neutral detergent fibre[4] (% DM)	24.47			
Acid detergent fibre[3] (% DM)	15.41			
Non-fibre carbohydrates[3] (% DM)	47.59			
Starch[4] (% DM)	42.58			
Ashes[3] (% DM)	6.73			
Calcium[3] (% DM)	0.69			
Phosphorus[3] (% DM)	0.40			
Ether extract[3] (% DM)	3.19			
Total digestible nutrients[4] (% DM)	74.10			
Net energy for lactation[4] (Mcal/kg DM)	1.50			

[1] Mineral mixture, quantity per kg of product: Ca: 140 g; P: 80 g; S: 10 g; Na:129 g; Co: 80 mg; Cu: 1400 mg; Fluorine: 800 mg; I: 80 mg; Mn: 1 g; Se: 20 mg and Zn: 3.50.
[2] Extract of *Acacia mearnsii* with 82.30% of total tannins, of which 32.30% of condensed tannins.
[3] Determined through chemical analysis.
[4] Estimated by the spartan dairy ration evaluator/balancer software, version 3.0.3.

Subsequently, they were submitted to the same procedures adopted for the other bags. The residues were analysed for NDF to determine the rate of degradation.

$$RED_{NDF} = [b \times c \times e^{(-kp \times lag)}]/(c+kp) \quad (1)$$

Where:
RED_{NDF}: real effective degradability of NDF.
b: degradation potential of NDF.
c: rate of degradation per fermentative action of b.
lag: time at which the equation derived for a data set equals the actual potencially degradable fraction at zero time. kp: rumen passage rate.

The rumen passage rate was determined by infusing 20 g of chromium oxide (as indicator) in rumen. Then, rumen content samples were collected at zero (0), 8, 10, 12, 24, 36 and 48 hours after the infusion. Next, they were analysed for chromium oxide content. The passage rate (h^{-1}) was calculated by using the model of Czerkawski (1986) (equation 2).

$$Y = a.e^{-kp \times t} \quad (2)$$

Where:
Y: indicator concentration in time t.
a: indicator concentration at initial time (t_0), assuming instant mixing to rumen content (ppm).
e: base of the neperian logarithm.
kp: rumen passage rate (h^{-1}).
t: indicator sampling time (h).

Rumen pH measuring

The pH measurement was continuously performed by using a data logger (Model T7-1 LRCpH, Dascor, CA). The system consisted of a pH probe enclosed in a protective shield that allowed the rumen liquid percolate freely but preventing the electrode from contacting the rumen epithelium. Weights were attached to each probe to ensure that it remained in the ventral sac of the rumen. The probes were programmed so that the electrodes measured and recorded the rumen pH at every 10 minutes over the measurement period. Each electrode was standardised by using pH 4.0 and 7.0 standards at the beginning and end of each session. The probes were inserted into the rumen of cows to measure the rumen pH (at every 10 minutes) for 24 hours.

The pH data were recorded as mean, maximum, and minimum pH. The area under the curve and duration of time in which pH was below 6.2, 6.0, and 5.8 were also recorded. According to Penner *et al*. (2007), the pH 5.8 indicates the threshold of sub-acute rumen acidosis, and pH 6.0 and 6.2 are thresholds indicative of healthy rumen conditions (Penner and Beauchemin, 2010). 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 and expressed as pH unit per hour according to Moya *et al*. (2011).

Evaluation of rumen fermentation products

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

Sampling of rumen content

Glass flasks of 50 mL capacity (Frascolex, São Paulo, Brazil) were previously identified and weighed. Then, at zero (0), 3, 6, 9 and 12 hours after the morning feeding the rumen content was separately collected in solid and liquid fractions. 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.). Both rumen fractions (solid and liquid) were placed in the flasks (about 10 g of the solid fraction and 20 mL of the liquid fraction). The flasks were then capped with rubber stoppers and sealed with aluminium sealing wax through specific pliers. Afterwards, they were "washed" with CO_2 by means of two needles for gas inlet and outlet to ensure anaerobic environment.

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

After the flasks cooled at room temperature, the volume of gas and the concentration of CH_4, SCFA and ammonia in each flask were measured. The Figure 1 shows the diagram of entire procedure.

Gas volume and CH_4 concentration measurement

In a temperature-controlled environment (25 ˚C) the volume of gas produced in 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 gas in the upper part of the flask using the syringe connected to the transducer until a zero-pressure reading. The volume displaced by the gas produced in the flask was recorded to determine the production of CH_4 gas. The total gas 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 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).

Calculation of liquid volume, solid content and concentration of SCFA

The volume of liquid within incubated and non-incubated flasks was calculated by the difference between the weight of the flask containing the sample after drying (at a 105 ˚C oven) and the weight of the flask containing the sample before drying. The solid content of the flask was obtained by the difference, in weight, between the flask containing the sample after drying and the empty flask (obtained before flasks were filled).

For SCFA (acetate, propionate and butyrate) concentration, 4 mL of rumen fluid content of each flask were taken and centrifuged at 2000 × g for 20 min, and 2 mL of supernatant were added to a test tube containing 0.4 mL of formic acid, then sealed and frozen at -20 ˚C for further analysis, according to Erwin *et al.* (1961). The SCFA were measured through gas chromatography (Focus GC, Thermo Scientific®, Rodano, Milan, Italy) by using a glass column with 1.22 m length and 0.63 cm diameter packed with 80/120 Carbopack B-DA/4% (Supelco, Sigma-Aldrich, St. Louis, MO, USA).

Calculation of SCFA and CH_4 production

The CH_4 production was obtained by multiplying the total volume of gas (mL) produced in each flask by the concentration of CH_4 in the gas phase (mmol/mL) obtained in incubated flask, and then subtracting what was produced in non-incubated flask (equation 3). The individual quantification of SCFA was obtained by multiplying the volume of liquid (mL) and the concentration of each SCFA (mmol/mL) obtained in the incubated flask, and then subtracting the production obtained in non-incubated flask (equation 4). Subsequently, the CH_4 and SCFA production was expressed based on the solid content of the flasks (g or kg). This content was obtained by the difference between the weight of flask containing dry sample (105 ˚C) and weight of empty flask.

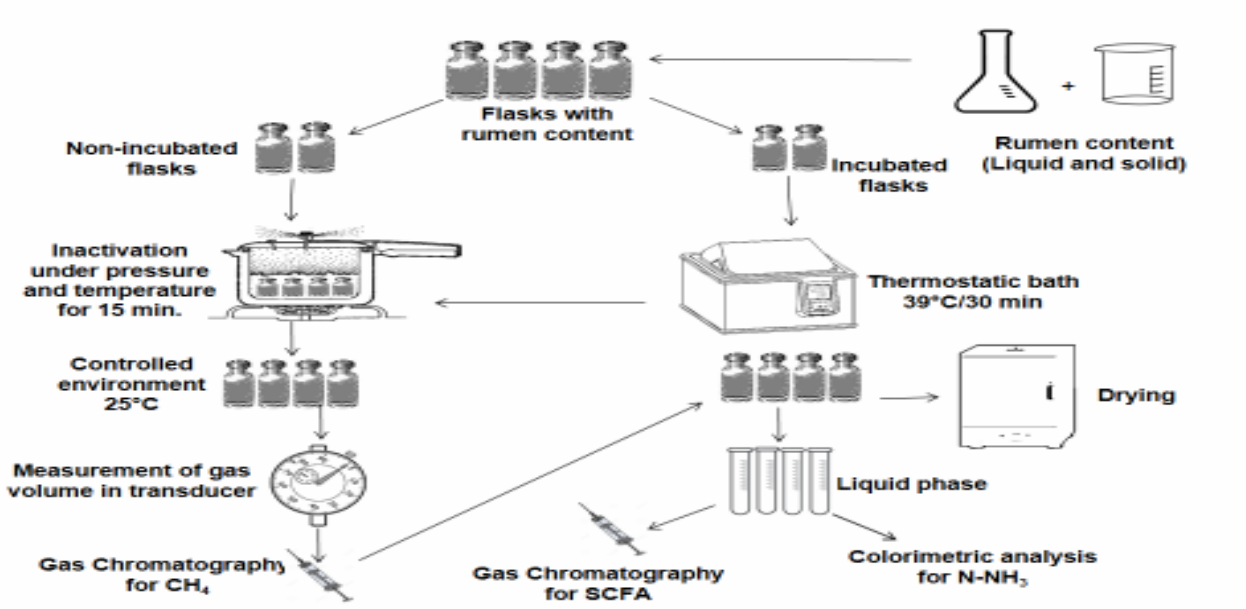

Figure 1 Diagrammatic representation of *ex-situ* rumen fermentation technique
Source; Perna Junior *et al.* (2017)

CH_4 Prod.= (CH_4Conc.×total gas vol.)T_{30} – (CH_4Conc.×total gas vol.) T_0 (3)

Where:
CH_4 Prod.: CH_4 production at the time between rumen content injection in the flask and inactivation.
CH_4 Conc.: CH_4 concentration (mmol/mL).
Total gas vol.: total volume of gas (obtained by the sum of the volume determined by the transducer and the headspace (mL)).
T_{30}: incubation time of 30 minutes.
T_0: incubation time of zero minute.

SCFA Prod.= (SCFA Conc.×total Liq. vol.)T_{30} – (SCFA Conc.×total Liq. vol.) T_0 (4)

Where:
SCFA Prod.: SCFA production at the time between rumen content injection in the flask and inactivation.
SCFA Conc.: SCFA concentration (mmol/mL).
Total Liq. vol.: Total volume of liquid in the flask (obtained by the weight difference before and after drying (mL)).
T_{30}: incubation time of 30 minutes.
T_0: incubation time of zero minute.

Calculation of relative energy loss

After CH_4 and SCFA were quantified, each product was multiplied by the respective combustion heat to express the CH_4 production as a percentage of the energy from the fermentation produced.

Therefore, the relative energy loss (REL) was considered as the ratio between the energy contained in CH_4 produced and the sum of the energy contained in all quantified fermentation products (CH_4 and SCFA), expressed as a 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/gram or 209.40, 368.52, 524.48, 210.56 and 0.0 kcal/mol, respectively. The REL was calculated according to Rodrigues *et al.* (2012), (equation 5).

$$REL\% = 100 \times (\varepsilon CH_4/\varepsilon CH_4+\varepsilon C_2+\varepsilon C_3+\varepsilon C_4)$$

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).

Determination of ammonia concentration and balance

To determine ammonia (NH_3-N) concentration, 2.0 mL of centrifuged liquid of each flask were added to a test tube with 1 mL of 1 N of H_2SO_4 solution, and then analysed through colorimeter, according to Kulasek (1972) and adapted by Foldager (1977). The balance was obtained by the difference of NH_3-N concentration between the 30 minutes incubated flasks with the non-incubated flasks. For a better interpretation, the balance data were estimated per hour (equation 6).

By following this procedure it was possible to evaluate whether the balance of ammonia production in the rumen was positive or negative.

NH_3-N balance (mg/dL.h)= [Conc. 30 min (mg/dL) – Conc. 0 min (mg/dL)] × 2 (6)

Where:
Conc. 30 min: NH_3-N concentration in incubated flasks.
Conc. 0 min: NH_3-N concentration in non-incubated flasks.

Protozoa counting

The rumen content was collected along with that for rumen fermentation products at zero (0), 3, 6, 9 and 12 hours after the morning meal. Equal portions of solid and liquid fractions were mixed and homogenised, then about 10 mL were inserted in 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. Then, the Neubauer Enhanced Bright-Line counting chamber (1 mL capacity) (Hausser Scientific Partnership®, Horsham, PA, USA) was filled and coupled to optical microscope and 100 optical fields were counted according to Dehority (1993). Three genera of protozoa were identified: *Isotricha*, *Dasytricha* and *Entodinium,* as well as the subfamily *Diplodiniinae*.

Feed energy partitioning

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

The energy release in rumen, in percentage of GEI or digestive energy (DE), was obtained by dividing acetate, propionate, butyrate and CH_4 release (Mcal/ani.day) by GEI (Mcal/ani.day) or DE (Mcal/ani.day) and then, multiplied by 100.

Methane release in cecum and colon (C and C) was considered as 5% of total CH_4 release. 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 intestine (Mcal/ani.day) was calculated according to equation 7.

ERI= GEI - (εC_2+εC_3+εC_4+faeces' GE+C and C CH_4+FH+mATP) (7)

Where:
ERI: energy release in intestine.
GEI: gross energy intake (Mcal/ani.day).
εC_2, εC_3, εC_4: energy of acetate, propionate and butyrate (Mcal/ani.day), respectively.
Faeces' GE: energy release in faeces (Mcal/ani.day).
C and C CH_4: CH_4 release in cecum and colon (Mcal/ani.day).
FH: fermentation heat.
mATP: microbial ATP.

The energy release in intestine, in percentage of GE or DE, was obtained by dividing the energy release in intestine by GEI or DE and then, multiplying by 100. The energy release in faeces, in percentage of GEI, was obtained by dividing faeces' energy content by GEI and then multiplied by 100.

Statistical analysis

The data were analysed by using Statistical Analysis System (SAS, 2004). Before the analysis, data 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, NDF degradability, rumen solid mass, rumen pH and feed energy partitioning were submitted to analysis of variance which separated, as causes of variation, tannin level and monensin effect (also considered as the effect of the square), interaction effect between monensin and tannins, period effect and animal effect within the square. The statistical model used was described according to the equation below:

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

Where:
Y_{ijkl}: observed value concerning the tannin level$_i$ + monensin or square$_j$ + interation between tannins$_i$ and monensin$_j$ + period$_k$ + animal$_l$ whithin the square$_j$.
μ: overall mean.
TL_i: tannin level effect (fixed effect).
M_j: monensin or square effect (fixed effect).
$TL_i \times M_j$: interaction effect between tannins and monensin (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.

The data for CH_4 and SCFA production, NH_3-N concentration and balance and rumen protozoa counting were analysed using the mixed model procedure (PROC MIXED) and to the model was added the factor "meas-

ures repeated over time", referring to the different sampling hours. The analysis by the time was performed only when the interactions between time and tannin level were significant. For the analyses, 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) according to Wang and Goonewardene (2004).

Tannin level effect was evaluated by the use of orthogonal polynomials, separating the effects in linear, quadratic and quadratic deviation. The 5% significance level was adopted.

RESULTS AND DISCUSSION

No interaction was observed between monensin and tannins on DMI, RED of NDF (P>0.05), but on the minimum rumen pH (P=0.0173) (Table 2 and Figure 2), where monensin inhibited the negative effect of tannins through antagonistic interaction. There was no effect of monensin on feed intake, NDF degradability and rumen pH. Tannins linearly reduced DMI, and RED of NDF (P<0.05). Quadratic effect of tannins was observed on the time (min/day) the pH was below 5.8 and 6.0, as well as on pH area (h.pH/day) below 5.8, 6.0 and 6.2. There was neither interaction between monensin and tannins nor independent effect of both additives on the total and differential count of rumen protozoa (P>0.05) (Table 3).

No interaction between monensin and tannins as well as monensin alone on concentration or rumen ammonia production (NH_3-N) was observed (Table 4). It was also not observed any significant effect of tannins on NH_3-N balance, although the highest level of tannin inclusion (2.25% feed DM) has caused a negative balance (-24 mg/dL.h). Regardless tannin level and monensin, sampling time had an effect (P<0.05) on NH_3-N concentration in both non-incubated and incubated flasks.

No interaction was observed between monensin and 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. Monensin reduced (P=0.0007) acetate:propionate molar ratio concerning the production by 28.8% (Table 5), although not affecting SCFA and CH_4 production or concentration.

Tannins linearly decreased CH_4, acetate, butyrate, as well as total SCFA production (g/kg.day) and also affected respective concentration (mmol/L). Consequently, there was a linear reduction of the gross energy (GE) released respective to each of these parameters.

Tannins quadraticly increased propionate concentration (mmol/L) in both non-incubated and incubated flasks, although not affecting the production and respective GE release. It was observed a time effect, but no interaction between time and treatment.

There was neither interaction between monensin and tannins nor monensin effect (P>0.05) on rumen solid mass, GEI (Mcal) as well as on energy partitioning (Table 6).

Unlike monensin, the different levels of tannins linearly increased rumen solid mass, but linearly decreased (P<0.05) the amount of GEI (Mcal/day). They also linearly increased digestible energy (DE) released in form of propionate, but linearly reduced the amount of GE released in form of CH_4 (Mcal/kg DM) and the energy released in intestine.

They linearly increased feed energy loss in faeces.

The lack of interaction between monensin and tannins on DMI may indicate independent effects of these two additives on this parameter.

Dry matter intake is of fundamental importance in nutrition, since it establishes the amount of nutrients available for production and also for health. The studies of Santos *et al.* (2019), Polizel *et al.* (2020), (experiment 2) and the meta-analyses of Duffield *et al.* (2008) and Duffield *et al.* (2012), on monensin effect on cattle feeding, have shown reduction of DMI in both dairy and beef cattle, although many other studies such as Perna Junior *et al.* (2017), Polizel *et al.* (2020), (experiment 1) and Teixeira *et al.* (2020), as well as the present study no effect of monensin was observed on DMI (Table 2). Evaluating monensin effects on lactating dairy cows' feeding, Odongo *et al.* (2007), used a total mixed diets in a ratio of 60% forage and 40% concentrate and found no effect of monensin on DMI. Oliveira *et al.* (2007) observed that monensin inclusion in the diets containing different levels of crude protein (CP) for sheep significantly reduced DMI. Therefore, analysing the results of the different studies shown here (including this study) it may be deduced that the effect of monensin on DMI may depend on the study and type of the diet, as well as the amount of monensin per kg of DM since not all studies used the same amount of monensin, for example, Odongo *et al.* (2007) used 24 mg of monensin/kg DM, Perna Junior *et al.* (2017) used 18 mg of monensin/kg DM and the average concentration of monensin in feed across studies in the meta-analysis by Duffield *et al.* (2012) was 28.1 mg/kg DM.

Monensin interacted with tannins on minimum rumen pH (Figure 2) and has shown to have capacity to inhibit the effect of tannins through antagonistic interaction.

Table 2 Dry matter intake, NDF degradability and rumen pH of cows fed monensin (mg/kg DM) and different levels of *A. mearnsii* tannins

Variables	Monensin (M)		Tannin level (TL, % feed DM)				SEM	P-value		
	0.00	32.0	0.00	0.75	1.50	2.25		M	TL	M × TL
DMI (kg/day)	9.34	9.49	9.80	9.59	9.56	8.71	0.275	NS	0.0034^{L}	NS
NDF 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

DMI: dry matter intake and NDF RED: neutral detergent fibre real effective degradability.
M: monensin; TL: tannins and M × TL: interaction between monensin and tannins.
SEM: standard error of the means.
NS: non-significant; L: linear; Q: quadratic.

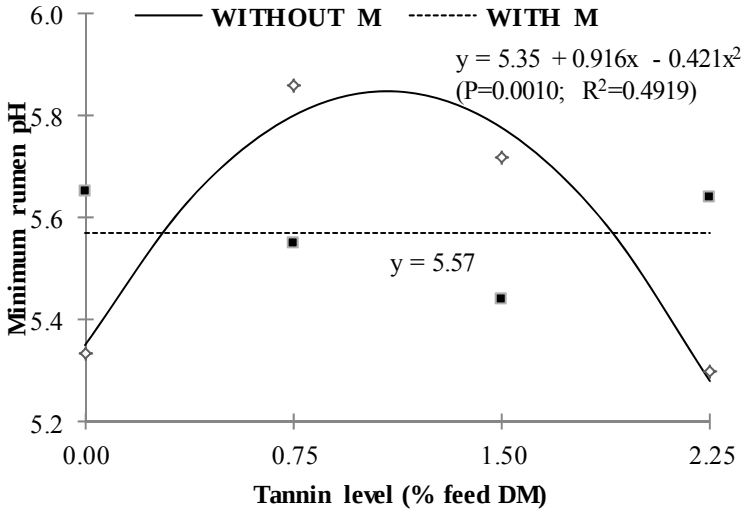

Figure 2 Graphic demonstration of interaction between monensin (M) and tannins at the minimum rumen pH. The square points in bold represent the means observed in the different tannin levels only for the group of cows which also received M. For this group, the joint effect of M and tannins was not significant, 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 cows which received M and tannins if they had not received M (quadratic effect). Therefore, it may be seen that the effect of tannins was observed only when they acted alone, but acting along with M the effect disappeared, suggesting an inhibition by M through antagonistic interaction

Table 3 Total and differential count of protozoa of cows fed monensin (mg/kg DM) and different levels of *A. mearnsii* tannins

Variables	Monensin (M)		Tannins level (TL, % feed DM)				SEM	P-value		
	0.00	32.00	0.00	0.75	1.50	2.25		M	TL	M × TL
Protozoa (×10^3/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

M: monensin; TL: tannins and M × TL: interaction between monensin and tannins.
SEM: standard error of the means.
NS: non-significant.

Table 4 Concentration and balance of rumen NH_3-N of cows fed monensin (mg/kg DM) and different levels of *A. mearnsii* tannins

Variables	Monensin (M)		Tannin level (TL, % feed DM)				SEM	P-value		
	0.00	32.00	0.00	0.75	1.50	2.25		M	TL	Time
NH_3-N concentration										
0 min (mg/dL)	8.15	8.12	8.75	8.33	8.29	7.18	0.34	NS	0.0551^L	< 0.0001
30 min (mg/dL)	8.28	8.41	9.17	8.53	8.62	7.06	0.38	NS	0.0547^L	< 0.0001
NH_3-N balance[1]										
mg/dL.h	0.24	0.59	0.85	0.40	0.66	-0.24	0.30	NS	NS	0.0537

[1] NH3-N balance= (30 min – 0 min) × 2
M: monensin and TL: tannins.
SEM: standard error of the means.
NS: non-significant and L: linear.

Adequate rumen pH maintenance is a characteristic generally determined by the type of diet. The pH 5.8 indicates the threshold for cases of sub-acute rumen acidosis (Penner *et al.* 2007) and the pH 6.0 and 6.2 are thresholds indicative of healthy conditions, favouring a better cellulolytic activity (Penner and Beauchemin, 2010).

The inclusion of *A. mearnsii* tannins did not impair the rumen pH. In contrast, the inclusion of tannins up to 2.23% improved the minimum rumen pH, 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*, Perna Junior (2018) found similar results in time that pH remained below 6.0 and 6.2.

The linear reduction of DMI caused by tannins corroborates meta-analysis of Jayanegara and Palupi (2010). The reduction of DMI was also observed by Aguerre *et al.* (2016) and Dschaak *et al.* (2011). Patra and Saxena (2011) stated that tannins concentrations above 50 g/kg DM may negatively affect DMI while low concentrations usually have no effect.

However, taking into account the highest level of tannins in the present study (22.5 g/kg DM), is lower than the level mentioned by these authors, even though the DMI was reduced, suggesting that tannins effect depends not only on the amount ingested, but also on the source, type, composition or molecular weight (Nawab *et al.* 2020a).

The linear increase of rumen solid mass (Table 6) and linear reduction of NDF real effective degradability (Table 2) caused by tannins may have been the major causes for DMI reduction.

The ammonia production in rumen generally exceeds the capacity of use by microorganisms, resulting in accumulation and subsequent absorption and conversion to urea by liver (Rodrigues, 2016). Microbial protein synthesis efficiency is one of the most important factors to reduce rumen ammonia concentration, which can be improved by diets with high total digestible nutrients (TDN) to supply energy required for bacterial activity (Seo *et al.* 2010) or by using additives capable to reduce the rumen protein degradation rate.

Although, monensin may reduce ammonia production, the effect was not observed in the present study (P>0.05) as reported by other studies such as Perna Junior *et al.* (2017) and Santos *et al.* (2019). Different results were observed by Ruiz *et al.* (2001) and Wingard *et al.* (2018) who found reduction of rumen ammonia when included monensin in diets. This difference may partially be due to the type of diet used, in the present study and the study of Perna Junior *et al.* (2017), for example, (50% of maize silage and 50% of concentrate) which may have supplied/synchronised the energy required for bacterial activity.

Tannins have ability to bind proteins, rendering them inaccessible to rumen degradation and favouring 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 and Bessa, 2016), although Tseu *et al.* (2020) observed reduced apparent total-tract digestibility of CP. Aguerre *et al.* (2016) and Dschaak *et al.* (2011), using tannins, observed reduction of microbial proteolytic activity with the consequent reduction of rumen ammonia concentration. This effect was not observed in the present study (Table 4), as reported by Perna Junior *et al.* (2017) and Perna Junior (2018). Despite lacking significant effect on this parameter, it is possible to see in Table 4 that highest level of tannins caused a negative balance (-24 mg/dL.h) which may be the indication that during the 30 minutes of incubation the inhibition of proteolytic activity by tannins was accentuated in the way that ammonia use for microbial protein synthesis was greater than the production. Tannins are also attributed to have ability to reduce the number of protozoa in the rumen as one of the mechanisms these additives use to reduce CH_4 production (Patra and Saxena, 2011), but in this study neither tannins nor monensin had effect on this parameter.

Ye *et al.* (2018), determining monensin effects on rumen fermentation characteristics, observed a decreased percentage of protozoa. 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 protozoa populations up to the first 4 weeks during which monensin was used, but original ciliate protozoal populations were restored by the fourth and sixth weeks 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), using 105 g of quebracho (*Schinopsis* spp.) also found no effect. These results may suggest some tannins effect on rumen protozoa but lack consistency.

Monensin and tannins, separately, are referenced to reduce enteric CH_4 emission in ruminants. The lack of interaction between monensin and tannins on CH_4 and SCFA production 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. Different observations were reported in many studies such as Perna Junior *et al.* (2017), Wingard *et al.* (2018) and Capelari *et al.* (2018) who found reduction of CH_4 production, but many other studies such as Hamilton *et al.* (2010) and Grainger *et al.* (2010) found no monensin effect 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% only for the first 2 weeks and by 27% only 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.

Monensin did not affect the total SCFA production or concentration (like in the study of Wingard *et al.* 2018), but significantly reduced acetate:propionate molar ratio concerning production. Similar effect was observed by Crossland *et al.* (2017), Costa *et al.* (2017), Ye *et al.* (2018) and Capelari *et al.* (2018).

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 fibre digestion in the rumen or both, since it was not observed any tannin effect on ciliate rumen protozoa. Tannins can depress fibre digestion by forming complexes with lignocellulose and, thus, prevent microbial digestion (Piñeiro-Vázquez *et al.* 2015; Tseu *et al.* 2020) either by direct inhibition of cellulolytic microorganisms or by inhibition of fibrolytic enzymatic activity or both (Patra and Saxena, 2011).

This is why part of reduction of CH_4 production by tannins has been questioned since it occurs by reduction of nutrient digestion. The meta-analysis of Jayanegara *et al.* (2012), from *in vitro* and *in vivo* experiments with tannins, has shown that reduction of CH_4 production was associated with reduction of apparent fibre digestibility. 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.

Table 5 CH_4 and short chain fatty acids (SCFA) production as well as REL of cows fed monensin (mg/kg DM) and different levels of *A. mearnsii* tannins

Variables[1]	Monensin (M)		Tannin level (TL, % feed DM)				SEM[2]	P-value[3]		
	0.00	32.00	0.00	0.75	1.50	2.25		M	TL	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 (g/kg.day)	163.5	155.9	179.3	151.2	170.4	136.2	6.371	NS	0.0145^{L}	0.0147
GE (kcal/kg.day)	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}	< 0.0001
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 (g/kg.day)	73.75	83.98	75.49	82.13	76.14	82.32	3.477	NS	NS	0.0040
GE (kcal/kg.day)	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}	< 0.0001
Difference (mmol/L)	1.28	1.28	1.54	1.38	1.33	0.91	0.050	NS	0.0004^{L}	0.0018
Production (g/kg.day)	71.04	70.28	80.38	71.49	74.27	55.77	2.526	NS	$< 0.0001^{L}$	0.0096
GE (kcal/kg.day)	414.3	411.6	479.0	393.8	442.6	332.4	14.99	NS	$< 0.0001^{L}$	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	< 0.0001
Difference (mmol/L)	6.86	7.23	8.030	7.66	7.37	5.25	0.315	NS	0.0012^{L}	< 0.0001
Production (g/kg.day)	309.6	310.4	335.2	306.0	322.4	274.3	11.07	NS	0.0317^{L}	0.0073
GE (kcal/kg.day)	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	$< 0.0001^{L}$	< 0.0001
30 min (mmol/flask)	0.087	0.087	0.105	0.091	0.082	0.068	0.002	NS	$< 0.0001^{L}$	< 0.0001
Difference (mmol/flask)	0.065	0.066	0.079	0.069	0.062	0.050	0.002	NS	$< 0.0001^{L}$	< 0.0001
Production (g/kg.day)	24.80	24.33	29.72	25.38	23.46	19.41	0.551	NS	$< 0.0001^{L}$	< 0.0001
GE (kcal/kg.day)	324.5	316.5	389.7	334.0	308.7	251.3	7.286	NS	$< 0.0001^{L}$	< 0.0001
REL (%)	20.58	21.41	21.97	21.32	20.47	20.26	0.582	NS	NS	NS

GE: gross energy and REL: relative energy loss of methane in relation to the other rumen fermentation products.
M: monensin; TL: tannins and M × TL: interaction between monensin and tannins.
SEM: standard error of the means.
NS: non-significant; L: linear; Q: quadratic.

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 2) up to a magnitude of 26.4% and CH_4 production also linearly reduced up to a magnitude of 34.7% (Table 5).

Therefore, it is thought that the reduction of CH_4 production may be greatly related to reduction of NDF rumen degradability. The reduction of fibre digestion can explain the linear reduction of acetate and butyrate production and the consequent linear reduction of total SCFA (g/kg DM.day).

Table 6 Estimation of energy released into the gastrointestinal tract of cows fed monensin (mg/kg DM) and different levels of *A. mearnsii* tannins

Variables (day)	Monensin (M)		Tannin level (TL, % feed DM)				SEM	P-value		
	0.00	32.00	0.00	0.75	1.50	2.25		M	TL	M × TL
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
Total SCFA										
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 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 faeces										
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	< 0.0001^{L}	NS

GE: gross energy; GEI: gross energy intake and DE: digestible energy.
M: monensin; TL: tannins and M × TL: interaction between monensin and tannins.
SEM: standard error of the means.
NS: non-significant ans L: linear.

This corroborates Patra and Saxena (2011) who stated that tannins effect on the reduction of carbohydrate digestion rate, especially cellulose and hemicellulose, can reduce total SCFA concentration in the rumen by reducing acetate molar concentration. 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 linear acetate reduction, butyrate production and lack of effect on propionate production, tannins, unlike monensin, did not modulate acetate: propionate ratio. Working with tannins up to 6%, Dickhoefer *et al*. (2016) observed a linear increase of propionate and butyrate proportion while that of acetate reduced.

On the other hand, the increasing quadratic effect of tannins on the concentration of propionate (Table 5) and lack of effect on production may explain the fact that tannins generally do not affect propionate production but may increase its concentration by reducing acetate and butyrate production.

There seems to be few studies that have looked at this topic, as nothing about the feed energy partitioning in cattle, or other type of ruminants, was found. 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 6). The transitory effect of monensin on rumen metabolism may have contributed to the lack of effect of this additive on energy partitioning.

The linear increase of rumen solid mass (kg/day) caused by tannins might have been due to reduction of NDF degradability which led to DMI reduction and, consequently, to a linear reduction on GEI (Mcal/day) (Table 6). Although there was a linear reduction of GE release in form of CH_4 (Mcal/cow.day), the different levels of tannins linearly reduced the GE release in intestine and linearly increased the energy loss in faeces. This may have been due to formation of complexes between nutrients and tannins which caused the reduction of nutrient digestion (Tseu *et al*. 2020) and consequent loss of energy in faeces. Similar results were observed by Perna Junior (2018).

The proportion of GE loss in form of CH_4 was 5.0% of the total GE consumed without tannins (i.e. control treatment), corroborating Goel and Makkar (2012) and Wanapat *et al.* (2015) who stated that CH_4 production generates feed GE losses ranging from 2 to 15%. But including tannins up to 2.25% feed DM, the GE loss significantly decreased to 4.1%.

This may appear to be slight, but supposing all cows consuming the same amount of GE (3.94 Mcal/kg DM, diet used in this study, for example), the loss of GE in form of CH_4 at 2.25% of tannin inclusion (0.162 Mcal) corresponds to 82.2% of the total GE which would be lost without the addition of tannins (0.197 Mcal). In other words this means that addition of 2.25% of tannins (DM basis) may reduce, by 17.8%, the loss of gross feed energy that would be lost in form of CH_4. Therefore, the use of tannins to retain feed energy and increase energy efficiency 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 faeces.

CONCLUSION

Monensin and tannins have shown independent effects on DMI, NDF degradability, CH_4, SCFA, ammonia production or protozoa counting, as well as on feed energy partitioning, therefore, no synergy between these additives was observed on these parameters. Monensin has shown to have capacity to inhibit the negative effect of tannins on the minimum rumen pH through antagonistic interaction. Monensin has shown to have capacity to reduce acetate:propionate molar ratio concerning production, but it had no effect on other parameters evaluated, therefore, it did not impact feed energy efficiency. The use of *A. mearnsii* tannins up to 2.25% feed DM linearly reduced DMI, NDF degradability and CH_4 production, but also linearly reduced the total SCFA, reduced the energy release in intestine and increased the energy loss in faeces; therefore, dispite having reduced methane production, tannins have not improved feed energy efficiency. Nonetheless, the combined use of monensin and *A. mearnsii* tannins did not have significant advantages, but the isolate use of tannins may contribute to reducing the environmental impact by reducing enteric methane production.

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