Abstract: This study assessed the association between encapsulated nitrate product (ENP) and monensin (MON) to mitigate enteric methane (CH₄) in vitro and possible effects on ruminal degradability, enteric fermentation characteristics, and microbial populations. Six treatments were used in randomized complete design in a 2×3 factorial arrangement with two levels of MON (0 and 2.08 mg/mL of buffered rumen fluid) and three levels of ENP (0, 1.5 and 3.0%). The substrate consisted of 50% Tifton-85 hay and 50% concentrate mixture (ground corn and soybean meal). ENP replaced soybean meal to achieve isonitrogenous diets (15% CP). No ENP×MON interaction was observed for any measured variable (P > 0.05) except for the relative abundance of *F. succinogenes* (P = 0.02) that linearly increased in diets with MON when ENP was added. The ENP addition decreased CH₄ production (P < 0.01) without affecting (P > 0.05) truly degraded organic matter nor the relative abundance of methanogens. Hydrogen production was reduced with MON (P = 0.04) and linearly decreased with ENP inclusion (P = 0.02). We concluded that use of nitrate is a viable strategy for CH₄ reduction, however, no additive effect of ENP and MON was observed for mitigating CH₄ production.

Key words: greenhouse gas, hydrogen, methanogenesis, ruminal bacteria

INTRODUCTION

Methane (CH₄) production in the rumen is an inherent part of the digestive process of ruminants (Beauchemin et al. 2008). Reduction of the CH₄ production can be achieved by use of feed additives that affect methanogenic microorganisms (Beacon 1988) or allow alternative hydrogen (H) sink, competing with CH₄ production (Ungerfeld & Kohn 2006). Ionophores, such as monensin (MON), decrease the concentration of Gram-positive bacteria and protozoa populations (Guan et al. 2006) and can reduce CH₄ production between 27 and 31% (Guan et al. 2006). MON promotes selection of succinate-producing bacteria, reduces the number of H₂-producing bacteria and stimulates the production of propionate (Chen & Wolin 1979). On the other hand, nitrate (NO₃⁻) has a higher affinity for H₂ than CO₂ (Leng 2008). Thus, when NO₃⁻ is present in the rumen, its reduction to nitrite (NO₂⁻) and ammonia (NH₄⁺) is favored over the production of CH₄ (Ungerfeld & Kohn 2006). In a review of studies using NO₃⁻ in ruminant diets, Lee & Beauchemin (2014) observed that all the reviewed studies reported a significant reduction in CH₄ emissions from animals fed with NO₃⁻.

Nitrates and MON have different routes for enteric methane reduction. Capelari et al. (2018) demonstrated an additive effect of NO₃⁻ plus MON on CH₄ production using a Ruminal
Simulation System. Nevertheless, NO$_3^-$ has been used sparingly for CH$_4$ reduction because of the possibility of NO$_3^-$ poisoning (Leng 2008). Besides that, the use of MON can decrease the reduction of NO$_3^-$ to NH$_4^+$, with consequently accumulation of NO$_2^-$ (Capelari et al. 2018) increasing the possibility of NO$_2^-$ toxicity.

Rumen NO$_3^-$ and the reduced intermediate, NO$_2^-$, are toxic to microbes, altering the microbial population and lowering feed digestion (Zhou et al. 2011). Therefore, encapsulated slow-release forms of NO$_3^-$ for ruminants seems to decrease the risk of toxicity (Lee et al. 2017). This occurs because slow release forms provide the possibility of gradual adaptation of microbes to NO$_3^-$ and NO$_2^-$, improving the feed degradation, since NO$_3^-$ metabolism in the rumen can be improved when microbes are acclimatized to NO$_3^-$ (Leng 2008).

Our hypothesis is that NO$_3^-$ can interact with MON manipulating rumen fermentation and reducing CH$_4$ production because of changes to ruminal microbiota. Besides that, the use of an encapsulated form of NO$_3^-$ may reduce the risk of toxicity by NO$_3^-$ and MON interaction. Thus, the aim of this study was to evaluate the in vitro interaction between MON and encapsulated NO$_3^-$ on CH$_4$ mitigation potential and ruminal microbiota.

**MATERIALS AND METHODS**

All animal procedures followed the guidelines recommended by the Internal Commission for Environmental Ethics and Animal Care of the Centre for Nuclear Energy in Agriculture (protocol nº 2013-6; University of São Paulo, São Paulo, Brazil). The experiments were carried out at the Laboratory of Animal Nutrition of the Centre for Nuclear Energy in Agriculture from the University of São Paulo (LANA/CENA/USP), in the city of Piracicaba, São Paulo, Brazil.

**Experimental design and treatments**

A completely randomized design in a 2 × 3 factorial arrangement with two levels of monensin (MON: 0 and 2.08 mg/mL of buffered rumen fluid) and three levels of encapsulated nitrate product (0, 1.5 and 3.0% in dietary DM) was used. Encapsulated nitrate product (ENP) replaced soybean meal to achieve three isonitrogenous diets (15% Crude Protein, CP) formulated with 50% Tifton-85 hay (Cynodon spp) and 50% concentrate (corn and soybean meal) (Table I). The experimental diets were selected and formulated according to crude protein (CP) requirements for growing and weight gain in lambs (NRC 2007). The forage concentrate ratio aimed at providing adequate substrate for microbial growth and ENP levels was selected according to previous studies from our research group, in which we found that even using an encapsulated form of nitrate, levels higher than 4.5% can cause toxicity and impair the microbial microorganisms (Natel et al. 2019). The diets were ground in a Willey mill (Marconi, Piracicaba, SP, Brazil) to pass through a 1 mm screen while the ENP was incubated in the encapsulated original formula, at doses corresponding to 0; 1.0% and 2.0% NO$_3^-$ in dietary DM (Table I).

For the treatments with MON inclusion, a stock solution of pure MON (M5273; Sigma-Aldrich Co., St. Louis, MO, USA; Molecular Weight 692.850) was prepared by diluting 15.6 mg in 1.0 mL absolute ethanol, stored at -10°C. Then, 10 µL of stock solution was added to each incubation glass flask 15 minutes before incubation, as described by Araujo et al. (2011). The final concentration of MON was 0.156 mg/75mL of buffered rumen fluid (2.08mg/L). This dosage was chosen because it had previously been found to decrease gas and CH$_4$ production, increase propionate, and decrease acetate concentration with minimal effects on OM degradation (Araujo et al. 2009, 2011).
The experimental ENP used in this study is protected by an international patent (submission number #1102284-1) and was manufactured by GRASP Ind. & Com. LTDA (Curitiba, PR, Brazil). The product was composed as follows (% of DM): 85.6% DM in as-fed basis, 17.6% nitrogen (N), 102.0% CP-equivalent), 19.62% calcium (Ca) and 71.38% $\text{NO}_3^-$ . The source of $\text{NO}_3^-$ was a double salt of calcium ammonium nitrate decahydrate $[5\text{Ca(NO}_3)_2\cdot\text{NH}_4\text{NO}_3\cdot10\text{H}_2\text{O}]$. The $\text{NO}_3^-$ release from ENP in buffered rumen fluid was 58% after 24 hours of incubation (Lee et al. 2017).

**Inocula preparation**

Eight rumen cannulated Santa Inês wethers (60 2.8 kg BW) were penned and used as donors of rumen content. Each inoculum was composed of the rumen content of two different wethers, totaling four inocula ($n = 4$) per treatment. Prior to the inoculum collection, the animals were adapted to a basal diet formulated with 50% Tifton hay and 50% concentrate (18% CP) plus ENP at 1% of dietary DM in order to sustain a sufficient population of $\text{NO}_3^-$ and nitrite ($\text{NO}_2^-$) reducers, and $\text{NO}_3^-$ and nitrite ($\text{NO}_2^-$) reducing activities in the ruminal environment. Otherwise, $\text{NO}_3^-$ effects could be underestimated because of a short *in vitro* incubation time. Animals were fed individually *ad libitum* twice-a-day (7:00 and 16:00 h) with free access to water and salt. After fourteen days of adaptation, the inoculum collection was performed: the liquid and solid fractions of ruminal content from each animal were collected separately into thermal bottles and then prepared adopting a 50:50 liquid-to-solid ratio (on a volume basis) (Bueno et al. 2005).

**Incubation conditions and gas production**

An *in vitro* gas production technique (Theodorou et al. 1994) adapted to a semi-automatic system (Maurício et al. 1999) with further modifications (Bueno et al. 2005, Longo et al. 2006) and using
a pressure transducer and data logger (Pressure Press 800, LANA, CENA /USP, Piracicaba, SP, Brazil) was used in this study.

Half gram of each experimental diet (Table I) was weighted in #F57 ANKOM filter bags (ANKOM, Technology Corporation, Fairport, USA) (Soltan et al. 2017) and put into serum glass flasks (160 mL of total volume and 85 mL of head space) with 50 mL of incubation medium (Menke’s buffered medium) and 25 mL of inoculum. Two incubation flasks per inoculum per treatment served as analytical units and were sealed with 20 mm butyl septum stoppers (Bellco Glass Inc, Vineland, NJ, USA), manually mixed and incubated in a forced air oven at 39°C (Marconi MA35, Piracicaba, SP, Brazil) for 24 hours. In addition, for each inoculum, blank flasks (containing #F57 ANKOM filter bag without substrate, inoculum and medium) were included to correct the values of gas production and degradability, and a laboratory internal standard substrate (Tifton hay) was included to monitor incubation conditions (Soltan et al. 2017).

Head space gas pressure was measured at 2, 4, 8, 16, and 24-hour intervals after the start of incubation. Total volume of gas produced in each flask was determined following the equation $V = (7.365 \times P; n = 500; R^2 = 0.99)$ where: $V =$ gas volume (mL) and $P =$ measured pressure (psi) (Araujo et al. 2011). Total accumulated gas production (TGP) after 24-hour incubation was considered the sum of partial gas production at each time interval and deducting the values of gas production by blanks.

For CH$_4$ determination, 2 mL of gas were sampled and stored in 10 mL vacuum tubes after each gas measurement, resulting in a pool sample of each flask. A 5 mL- surgical syringe (Becton Dickson Indústria Cirúrgica LTDA, Curitiba, Paraná, Brazil) was used for gas sampling. After each gas sampling, flasks were vented, mixed, and returned to air oven. After 24 hours, flasks were placed in cold water (4°C) to cease fermentation and the #F57 ANKOM filter bags were removed. The CH$_4$ concentration in the collected gas was determined in the pool sample of each flask as described in Araujo et al. (2011) using a gas chromatograph (Shimadzu 2014, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1.5875 mm OD x 1.0 mm ID x 1 m length; Ref. n° 19809; Restek, Bellefonte, PA, USA). The temperatures of column, injector, and flame ionization detector were 60, 200, and 240°C, respectively. Helium at 10 mL/min was the carrier gas. CH$_4$ concentration was determined by external calibration using an analytical curve (0, 30, 90, and 120 mL/L) prepared with pure CH$_4$ (White Martins PRAXAIR Gases Industriais Inc., Osasco, SP, Brasil; 99.5 mL/L purity). The production of CH$_4$ (CH$_4$P) was calculated according to Longo et al. (2006) according to the following equation $CH_4P, mL = (Total \ gas, mL + Head \ space, 85 \ mL) \times CH_4$ concentration, mL/mL.

**Ruminal degradability, fermentation characteristics, and microbial populations**

At the end of the incubation period, the #F57 ANKOM filter bags removed from the flasks were treated with neutral detergent solution (NDS) for 1 hour at 90°, washed with hot water, acetone, and DM and ash were determined. The truly degraded organic matter (TDOM) was calculated as the difference between incubated organic matter (OM) and the remaining not degraded OM (Blümmel et al. 1997), and the same was performed with incubated and not degraded DM to determine the truly degraded dry matter (TDDM). Values of TGP and CH$_4$P were expressed in basis of TDOM (mL/g TDOM) and TDDM (mL/g TDDM).

The content of each flask was used for measurements of pH (pHmeter model TEC-2, Tecnal, Piracicaba, Brazil), ammoniacal N (NH$_3$-N)
micro-Kjeldahl steam distillation with sodium tetraborate solution (Preston 1995), short chain fatty acids (SCFA), hydrogen production and microbial populations.

The determination of SCFA concentration (Nocek et al. 1987, Palmquist & Conrad 1971) was performed in a Gas Chromatograph (Shimadzu 2014, Tokyo, Japan) equipped with a column GP 10% SP – 1200/1% H3PO4 on 80/100 Chromosorb WAW (Cat. n° 11965; 6” x 1/8” stainless steel; Supelco, Bellefonte, PA, USA). The buffered rumen fluid samples were thawed and centrifuged at 11,000 × g (RC 5B plus, Sorvall, Wilmington, DE, USA) for 40 min at 4°C. Then 800 µL of supernatant were added to 100 µL of 2-ethyl-butyric acid (internal standard; MW=116.16; Sigma Chemie Gmbh, Steinheim, Germany) and 200 µL of formic acid. A 1 µL aliquot was injected in the GC with the temperature for the flame ionization detector (FID) at 250°C. The oven heating slope was: 115°C (3.20 min), 123°C (10°C/min; 1.25 min), 126°C (10°C/min; 5 min), with 10.55 min of total analytical time. Helium at 25 mL/min was used as a carrier gas. Hydrogen and synthetic air detectors were kept at 40 and 400 mL/min flow, respectively. An external calibration curve was prepared with a known concentration of a mixed SCFA solution (acetic acid 99.5%, CAS 64-19-97; propionic acid 99%, CAS 04-09-79; isobutyric acid 99%, CAS 79-31-2; butyric acid 98.7%, CAS 107-92-6; isovaleric acid 99%, CAS 503-74-2; valeric acid 99%, CAS 109-52-4; Chem Service, West Chester, PA, EUA).

Protozoa counting was performed according to Dehority et al. (1983): 2 mL of each sample was mixed with 4 mL of methyl green formalin (35 % formaldehyde) saline solution (MFS) and preserved from light at room temperature. The counting procedure used a 0.01 ml aliquot in a modified Neubauer chamber (internal measures 20 mm × 26 mm × 0.4 mm) using a microscope with a 45/66 objective lens (Olympus, model CH 2).

For quantifying the relative abundance of microbial microorganisms, the incubation liquid was collected and stored in frozen condition (-80°C) prior to DNA extraction. The DNA extraction from the buffered rumen fluid samples was performed using a commercial kit PowerLyzerTM PowerSoil (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) and according to the manufacturer recommendations. The quantification of the relative abundance of methanogenic microorganisms as: Archaea, Selenomonas ruminantium and Wolinella succinogenes (nitrate- and nitrite-reducing bacteria), Ruminococcus flavefaciens and Fibrobacter succinogenes was performed using specific primers in real-time PCR (Table II). The relative expression of each microbe was calculated as described by Denman & McSweeney (2006). The quantity of each microbe was expressed as a percentage relative to the total quantity of bacterial 16S rDNA in combined rumen fluid, according to Zhou et al. (2012).
Statistical analyses

Data statistical analyses were performed on SAS software version 9.4 (SAS Institute Inc.). The analytical units (two incubation flasks per inoculum per treatment) were averaged prior to the statistical analysis, and four inocula per treatment \((n=4)\) were used as true statistical repetition. A mixed linear model using restricted maximum likelihood (Restricted Maximum Likelihood: REML) in the MIXED procedure was used to analyze the response variables. The model included the fixed effect of MON, level of ENP, and the interaction between both effects (MON×ENP). Regression analyses (REG procedure) were performed considering ENP levels within diets. The least square means for MON, ENP level, and the interaction between them (MON×ENP) were obtained by LSMEANS procedure and when a significant fixed effect \((P < 0.05)\) was identified, the respective means were compared by F and Tukey-Kramer tests.

RESULTS

In vitro degradability and gas production

No interaction \((P > 0.05)\) between MON and EPN was observed for total gas production (TGP), methane production \((\text{CH}_4)\), degradability of dry matter (DM) and organic matter (OM). These variables were not affected by MON inclusion \((P > 0.05)\) (Table III) either. The TDOM (g/kg), TDDM (g/kg) and TGP (mL/g TDOM) were not affected \((P > 0.05)\) by ENP inclusion. However, ENP inhibited \((P < 0.01)\) methane production \((12.1, 9.3\) and \(6.1 \text{ mL/g TDOM})\) resulting in a linear decrease \((y = 12.16 -1.99 \times \text{ENP}; R^2 = 0.23, P < 0.01)\) with increasing doses of ENP \((0, 1.5\) and \(3\% \text{ DM})\).

The balance of metabolic \(H_2\) was not affected \((P > 0.05)\) by the interaction between ENP and MON (Table III). However, ENP addition \((0, 1.5\) and \(3\%\) ) decreased \((P < 0.05)\) \(H_2\) production \((198.8, 198.7\) and \(197.4 \mu\text{mol/mL}, y = 198.9-0.473\times\text{ENP})\) and \(H_2\) utilization \((128.7, 109.8\) and \(90.8 \mu\text{mol/mL}, y = 128.8-12.75\times\text{ENP})\). Consequently, the recovered \(H_2\) decreased by ENP inclusion \((64.7, 55.2\) and \(45.8\%, y = 64.7-6.29\times\text{ENP})\). MON decreased the amount of \(H_2\) produced when compared to the diet without MON inclusion \((199.0\ vs 197.5, P = 0.048)\).

Fermentation characteristics

There was no interaction between MON and ENP \((P>0.05)\) treatments for any of the evaluated enteric fermentation variables (Table IV). The
Table III. Effect of inclusion encapsulated nitrate product (ENP) in diets without (-) or with (+) monensin (MON) on the truly degraded organic matter (TDOM), truly degraded dry matter (TDDM), total gas production (TGP), methane production (CH₄P) and hydrogen.

| Item                      | MON (-) | SEM* | ENP+ 0% | ENP+ 1.5% | ENP+ 3% | SEM       | MON     | ENP     | MON×ENP |
|---------------------------|---------|------|---------|-----------|---------|-----------|---------|---------|---------|
| TDOM (g/kg)               | 540.8   | 516.9| 23.03   | 521.6     | 525.9   | 539.1     | 24.09   | 0.10    | 0.75    | 0.87    |
| TDDM (g/kg)               | 568.2   | 545.2| 21.66   | 549.5     | 553.6   | 566.9     | 22.65   | 0.09    | 0.54    | 0.84    |
| TGP (mL/g TDOM)           | 116.9   | 110.7| 7.82    | 116.3     | 113.7   | 111.2     | 8.16    | 0.19    | 0.67    | 0.94    |
| CH₄P (mL/g TDOM)          | 9.62    | 8.71 | 1.209   | 12.10     | 9.27    | 6.12      | 1.697   | 0.26    | <0.01*  | 0.40    |
| CH₄ (%)                   | 8.23    | 7.87 | 1.237   | 10.40     | 8.15    | 5.50      | 1.257   | 0.63    | <0.01*  | 0.52    |
| H₂Produced (μmol/mL)      | 199.0   | 197.5| 0.67    | 198.8     | 198.7   | 197.4     | 0.71    | 0.048   | <0.02*  | 0.63    |
| H₂ Utilized (μmol/mL)     | 111.2   | 108.1| 7.01    | 128.7     | 109.8   | 90.5      | 7.25    | 0.44    | <0.01*  | 0.47    |
| H₂ Recovered, %           | 55.8    | 54.7 | 3.41    | 64.7      | 55.2    | 45.8      | 3.58    | 0.57    | <0.01*  | 0.49    |

+ 0% (control), 1.5% and 3.0% are the inclusion rates of ENP in the diets (DM basis); SEM: standard error of the mean (MON, ENP, respectively).

MON: fixed effect of monensin (without monensin (-) and with monensin (+)); ENP: fixed effect of encapsulated nitrate product in dietary (0, 1.5 and 3.0% DM); MON+ENP: interaction between monensin and encapsulated nitrate; *linear effect of ENP; **quadratic effect of ENP.

Means with different letters in the same row indicate significant treatment effect (Tukey test P < 0.05).

inclusion of MON reduced (P < 0.05) the molar concentration of butyric (10.2 vs. 9.7), isobutyric acids (0.66 vs. 0.58) and isovaleric acid (1.38 vs. 1.24) when compared to diets without MON.

The increasing levels of ENP inclusion in the diets linearly increased the acetate acid production (65.1, 65.9, 66.9; P <0.01; y = 65.06+0.612*ENP). The inclusion of ENP reduced linearly the production of butyric (10.4; 10.1 and 9.3 mmol/L, P < 0.01; y=10.46-0.346*ENP) and isovaleric acids (1.4; 1.3 and 1.2 mmol/L, P < 0.01; y=1.37-0.039*ENP). However, the addition of ENP did not significantly affect the total SCFA (mmol / L) nor C2:C3 ratio.

Microbial population

Interaction between MON and ENP addition affected the abundance of F. succinogenes (P < 0.02, Table V). Greater population of F. succinogenes was observed in diets with ENP and MON inclusion (0.04, 0.17 and 1.12 %; y = 0.075 + 0.346*ENP, R² = 0.53, P < 0.01) when compared to diets without MON (0.03, 0.20, 0.36%; y = 0.036 + 0.108*ENP, R² = 0.36, P < 0.01).

The inclusion of MON in the diet did not affect the number of protozoa and the relative abundance of archaea Methanogens and ruminal bacteria (P > 0.05). The nitrate and nitrite-reducing bacteria showed a linear increase following the ENP addition: W. succionogenes (0.02, 0.34 and 2.03; y = 0.0194 + 0.642*ENP; P = 0.01) and S. ruminantium (0.01, 0.04 and 0.06; y=0.004+0.0196*ENP; P = 0.01). No effect of ENP addition (P > 0.05) was observed on the number of protozoa or the relative expression of archaea Methanogens (Table V).

DISCUSSION

The NO₃⁻ addition was effective to reduce methane production linearly, which is in agreement with previous studies in vitro (Anderson et al. 2008, 2010, 2016, Capelari et al. 2018, Natel et al. 2019, Zhang & Yang 2011) and in vivo (Brown et al. 2011, Klop et al. 2016, Lee et al. 2017, Newbold et al. 2018).
There are two major mechanisms in which NO$_3^-$ reduces CH$_4$ production: 1) CH$_4$ is decreased by the competition for H$_2$ between NO$_3^-$ and methanogenesis, in a thermodynamically favorable process to methanogenesis (Lee et al. 2017) the toxicity of NO$_3^-$ and NO$_2^-$ on methanogenic microorganisms (Božic et al. 2009, Iwamoto et al. 2002, Sar et al. 2005, Zhou et al. 2011). In this study, the potential reduction of CH$_4$ was between 21.6% and 47.1% with the addition of 1.5 and 3% ENP (% MS) when compared to control (0% ENP), which agrees with Leng (2010) that showed a decrease of 16-50% with the use of NO$_3^-$ in ruminant diets. This result indicates that NO$_3^-$ reduction (consumption of H$^+$) was the major mechanism for lowering CH$_4$ production because of a reduced availability of H$_2$ to archaea methanogens. This hypothesis is confirmed by the linear reduction in the use of H$_2$ when ENP was added to the diet. In a stoichiometric approach: the complete reduction of NO$_3^-$ to NH$_3$ consumes 4 mol of H$_2$, which is the same number of H$_2$ molecules necessary for methanogens reduce CO$_2$ to CH$_4$ (Capelari et al. 2018). Thus, when NO3 is present in the rumen, H$_2$ is effectively used to reduce NO3 to NO2 and have this reduced to NH3, contributing to reduce CH4 production (because of the lack of H2) (Ungerfeld & Kohn 2006).

The theoretical mitigation potential of NO$_3^-$ assumes that all NO$_3^-$ added is reduced to NH$_3$ (Li et al. 2013) in a way that 1 mol of NO$_3^-$ (62 g) added in ruminant diets reduces 1 mol of CH$_4$ formation (22.4 L). In this study, doses of 5 and 10 mg of NO$_3^-$ in 500 mg of substrate were used, which theoretically should reduce CH$_4$ production around 3.36 and 6.44 mg/g TDOM, respectively. However, the linear CH$_4$ reduction

Table IV. Effect of inclusion encapsulated nitrate product (ENP) in diets without (-) or with (+) monensin (MON) on fermentation characteristics.

| Item* | MON (-) | SEM+ | ENP+ 0% | ENP+ 1.5% | ENP+ 3% | SEM | P-value± | MON | ENP | MON×ENP |
|-------|---------|------|---------|-----------|---------|-----|---------|------|-----|---------|
| pH    | 6.82    | 0.012| 6.83    | 6.82      | 6.83    | 0.014| 0.62    | 0.93 | 0.74 |
| NH$_3^-$N (mg/100 mL) | 34.4    | 0.91  | 33.5    | 34.1      | 35.1    | 1.01 | 0.79    | 0.33 | 0.13 |
| Total SCFA (mmol/L)    | 74.1    | 2.48  | 74.8    | 74.2      | 72.3    | 2.54 | 0.56    | 0.16 | 0.08 |
| Acetate | 66.0    | 0.67  | 65.1    | 65.9      | 66.9    | 0.706| 0.95    | <0,01*| 0.94 |
| Propionate | 20.1    | 0.60  | 20.7    | 20.3      | 20.1    | 0.62 | 0.06    | 0.31 | 0.91 |
| Butyrate | 10.2±   | 0.29  | 10.4    | 10.1      | 9.3     | 0.31 | 0.02    | 0.02*| 0.24 |
| Isobutyrate | 0.66±   | 0.042 | 0.64    | 0.62      | 0.59    | 0.047| <0,01   | 0.09 | 0.72 |
| Valerate | 1.77    | 0.087 | 1.78    | 1.74      | 1.71    | 0.089| 0.06    | 0.09 | 0.24 |
| Isovalerate | 1.38   | 0.079 | 1.38    | 1.31      | 1.22    | 0.081| <0,01   | 0.01*| 0.54 |
| C$_2$:C$_3$ ratio   | 3.27    | 0.125 | 3.16    | 3.27      | 3.35    | 0.130| 0.21    | 0.11 | 0.74 |

±SEM: standard error of the mean (MON, ENP, respectively); SCFA: Short-chain fatty acids; C2:C3: acetate:propionate ratio.
*MON: fixed effect of monensin (without monensin (-) and with monensin (+)); ENP: fixed effect of encapsulated nitrate product in dietary (0, 1.5 and 3.0% DM); MON×ENP: interaction between monensin and encapsulated nitrate; *linear effect of ENP; **quadratic effect of ENP.
*Means with different letters in the same row indicate significant treatment effect (Tukey test P < 0.05).
observed was 2.82 and 5.98 mg/g TDOM, so the efficiency of CH4 mitigation (actual CH4 reduction / theoretical CH4 reduction × 100; Lee et al. 2015) was 83.6 and 92.9% in the 24-hour incubation period, representing the NO3- reduced to NH3 by ruminal microorganisms. The not fully efficient CH4 mitigation (83.6 and 92.9%) observed in this study could be a result of an incomplete reduction of the total amount of NO3- or NO2- to NH3 (Newbold et al. 2014).

Another explanation for the reduction of CH4 production could be the direct NO3- and NO2- toxicity on the methanogens population (Božic et al. 2009, Sar et al. 2005). However, in this study, no reduction was found in the relative expression of methanogenic microorganisms nor in the number of protozoa with ENP addition, indicating that there was no direct effect of NO3- on these populations. On the other hand, studies have shown that at least part of the CH4 that was reduced in the in vitro assays of Capelari et al. (2018), Guyader et al. (2017), Marais et al. (1988) and Natel et al. (2019); and in the in vivo assays of Brown et al. (2011), Lee et al. (2015), Newbold et al. (2014) and Yang et al. (2016), was related to the effect of NO2- on the methanogenic archaeal population. Our hypothesis to explain the lack of effect on these populations is that the encapsulation of NO3- reduces the exposure to microorganisms because of its slow release rate (Lee et al. 2017) and thus decreases the risk of NO2- toxicity. Lee et al. (2017), using the same ENP product of this study, observed that 50% of NO3- released from encapsulation was metabolized by rumen microbes during the first 6 hours of the incubation period. Consequently, 61% and 93% of NO3- released from encapsulation were metabolized over 12 and 24-hour intervals, respectively, during which no significant NO3- accumulation and H2 production were observed over 24 hours. Besides, our results indicated an increase of NO3- and NO2- reducing bacteria, W. succinogenes and S. ruminantium, which contributed to reducing NO3- to NH3 and decreased NO2- accumulation.

MON reduces the number of H2-producing bacteria (Chen & Wolin 1979), promoting, indirectly, an increase in the molar concentration.

Table V. Effect of inclusion encapsulated nitrate product (ENP) in diets without (-) or with (+) monensin (MON) on the abundance relative of microbial populations in the rúmen (% of the total quantity of bacterial 16S rDNA) and protozoa.

| Item                  | **SEM** | **ENP+** | **SEM** | **MON** | **ENP** | **P-value** |
|-----------------------|---------|----------|---------|---------|---------|-------------|
|                       | (-)     | (+)      | 0%      | 1.5%    | 3%      |             |
| A. Methanogens (%)    | 0.116   | 0.111    | 0.0673  | 0.136   | 0.126   | 0.081       |
| F. succinogenes (%)   | 0.198   | 0.446    | 0.0778  | 0.037   | 0.190   | 0.739       |
| R. flavefaciens (%)   | 0.210   | 0.241    | 0.1310  | 0.022   | 0.230   | 0.425       |
| S. ruminantium (%)    | 0.029   | 0.036    | 0.0237  | 0.001   | 0.041   | 0.057       |
| W. succinogenes (%)   | 0.890   | 0.694    | 0.3129  | 0.0002  | 0.341   | 2.033       |
| Protozoa (cel x 10^-5/mL) | 2.80   | 2.67    | 0.244  | 2.87    | 2.35    | 2.41        |

†0% (control), 1.5% and 3.0% are the inclusion rates of ENP in the diets (DM basis); SEM: standard error of the mean (MON, ENP, respectively).
‡MON: fixed effect of monensin (without monensin (-) and with monensin (+)); ENP: fixed effect of encapsulated nitrate product in dietary (0, 1.5 and 3.0% DM); MON×ENP: interaction between monensin and encapsulated nitrate; †linear effect of ENP; ‡quadratic effect of ENP.

*a, b Means with different letters in the same row indicate significant treatment effect (Tukey test P < 0.05).*
of propionic acid with a reduction in acetic, butyric, and lactic acid, in CH4 and CO2 gases, and in ammonium (Bertipaglia 2008). In this experiment there was no influence of MON on the number of bacteria, except \( F.\ succinogenes \), nor in CH4 production. It is possible that the amount of MON used was not able to act on the metabolism of gram-positive bacteria to reduce their number, which would have implied in an increase of gram-negative bacteria (such as \( F.\ succinogenes \)).

However, reductions in metabolic H2 production were observed when MON was added as a consequence of a decrease in butyrate, with tendency to an increase in propionate acid production. Stoichiometrically pyruvate conversion to propionate requires a net input of H2 per mol of fermented glucose, thereby reducing hydrogen supply (Janssen 2010) and the formation of acetate and butyrate release two moles of CO2 and four moles of H2 per mole of fermented glucose. (Kohn & Boston 2000). Thus, volatile fatty acid production rates determine ruminal hydrogen supply, which is used for methane production (Elliot & Loosli 1959).

The MON addition also affected isobutyrate and isovalerate acids production, without changing the total production of SCFA, acetate-to-propionate ratio nor the CH4 production. Since the inhibition of isoacids indicates attenuation of deamination, the reduction of isovaleric production following the addition of MON may be related to the reduction of ruminal deamination and the inhibition of NH3- producing bacteria (Russel & Strobel 1988).

In our study MON inclusion did not affect the nitrate- and nitrite-reducers (\( S.\ ruminantium \) and \( W.\ succinogenes \)). Chen & Wolin (1979) also observed no effect of MON on \( S.\ ruminantium \) population. However, higher dose of MON may affect gram-positive bacteria like \( D.\ detoxificans \), major bacterial groups in the acquisition of tolerance by ruminants that are gradually adapted to nitrotoxins (Anderson & Rasmussen 1998, Majak 1992). According to Capelari et al. (2018) the combination of encapsulated \( \text{NO}_3^- \) plus MON numerically increased the levels of \( \text{NO}_2^- \) in rumen fluid after 24-hour incubation, suggesting a possible undesirable influence of MON on nitrate reduction. Thus, results should be interpreted with care.

An interaction between ENP and MON was observed on the relative abundance of \( F.\ succinogenes \), a gram-negative bacterium, suggesting that through different mechanisms, additives might change the rumen microbiota. Gram-negative bacteria have an outer membrane that prevents MON from reaching the cell membrane and is therefore more resistant to MON than gram-positive bacteria (Strobel & Russell 1989). Although MON did not significantly reduce gram-positive bacteria, it is possible that MON and ENP (more specifically \( \text{NO}_2^- \)) may have inhibited the general activity of gram-positive bacteria in the medium, thereby increasing gram-negative bacteria numbers, such as \( F.\ succinogenes \).

The ENP inclusion did not reduce the number of protozoa nor inhibit archaea methanogens but increased the relative expression of nitrate and nitrite reducing bacteria such as \( S.\ ruminantium \) and \( W.\ succinogenes \). Lin et al. (2011) also observed increased relative abundance of \( W.\ succinogenes \) and \( S.\ ruminantium \) with the addition of \( \text{NO}_3^- \) in the diet. Possibly because the addition of ENP enabled the increase of \( \text{NO}_3^- \) substrate, favoring the growth of these bacteria (Lin et al. 2011).

**CONCLUSIONS**

The CH4 reduction by ENP addition reflected the effect of \( \text{NO}_3^- \) acting as a H2 sink. However,
the reduction on CH₄ production was lower than expected. The additive effect of ENP and MON was not confirmed on reducing CH₄ nor affecting nitrate- and nitrite-reducing bacteria, but an increase on the relative abundance of gram-negative bacteria (F. succinogenes) was observed.

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