An Acad Bras Cienc (2022) 94(4): e20200213 DOI 10.1590/0001-3765202220200213
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Encapsulated nitrate replacing soybean meal in diets with and without monensin on *in vitro* ruminal fermentation

ANDRESSA S. NATEL, ADIBE LUIZ ABDALLA, RAFAEL C. DE ARAUJO, TIAGO P. PAIM, ADIBE LUIZ ABDALLA FILHO, PATRÍCIA LOUVANDINI, MELKI K. LIMA & PAOLA PIZA

Abstract: This study assessed the association between encapsulated nitrate product (ENP) and monensin (MON) to mitigate enteric methane (CH_4) *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 CH_4 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 CH_4 reduction, however, no additive effect of ENP and MON was observed for mitigating CH_4 production.

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

INTRODUCTION

Methane (CH_4) production in the rumen is an inherent part of the digestive process of ruminants (Beauchemin et al. 2008). Reduction of the CH_4 production can be achieved by use of feed additives that affect methanogenic microorganisms (Beacon 1988) or allow alternative hydrogen (H) sink, competing with CH_4 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 CH_4 production between 27 and 31% (Guan et al. 2006). MON promotes selection of succinate-producing bacteria, reduces the number of H_2-producing bacteria and stimulates the production of propionate (Chen & Wolin 1979). On the other hand, nitrate (NO_3^-) has a higher affinity for H_2 than CO_2 (Leng 2008). Thus, when NO_3^- is present in the rumen, its reduction to nitrite (NO_2^-) and ammonia (NH_4) is favored over the production of CH_4 (Ungerfeld & Kohn 2006). In a review of studies using NO_3^- in ruminant diets, Lee & Beauchemin (2014) observed that all the reviewed studies reported a significant reduction in CH_4 emissions from animals fed with NO_3^-.

Nitrate and MON have different routes for enteric methane reduction. Capelari et al. (2018) demonstrated an additive effect of NO_3^- plus MON on CH_4 production using a Ruminal

Simulation System. Nevertheless, NO_3^- has been used sparingly for CH_4 reduction because of the possibility of NO_2^- poisoning (Leng 2008). Besides that, the use of MON can decrease the reduction of NO_3^- to NH_3, 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).

Table I. Ingredients and chemical composition of experimental diets (%, DM basis)

Item	Experimental diets+		
	0% ENP	1.5% ENP	3% ENP
Ingredients (%)			
Tifton 85 hay‡	50.0	50.0	50.0
Ground corn¥	35.2	37.2	39.3
Soybean meal£	14.8	11.3	7.7
ENP¶	-	1.5	3.0
Chemical composition§			
Dry Matter (%)	91.2	91.2	91.3
Organic Matter (% of DM)	94.3	94.1	93.9
Crude Protein (% of DM)	15.4	15.4	15.2
Ether Extract (% of DM)	2.7	2.7	2.6
Neutral Detergent Fiber (% of DM)	50.3	50.2	50.2
Acid Detergent Fiber (% of DM)	27.6	27.2	26.8
ENP-N (% of total N)§	-	13.4	27.1
NO_3^-	-	1.07	2.14

+ENP: encapsulated nitrate product; 0% (control) and 1.5% and 3.0% are the inclusion rates of ENP in the experimental diets (DM basis).
‡Tifton 85 hay chemical composition: 92.7% dry matter, 8.97% crude protein, 65.7% neutral detergent fiber, 47.3% acid detergent fiber.
¥Ground corn chemical composition: 89.0% dry matter, 9.79% crude protein, 39.4% neutral detergent fiber, 5.18% acid detergent fiber.
£Soybean meal chemical composition: 91.1% dry matter, 49.7% crude protein, 25.3% neutral detergent fiber, 14.2% acid detergent fiber.
¶Encapsulated nitrate product chemical composition: 85.6% dry matter, 17.6% nitrogen, Ca 19.6% calcium, 71.4% nitrate.
§DM: dry matter; N: nitrogen; ENP-N: estimate of N content of the diet provided by ENP; NO_3^-: nitrate - estimate amount of NO_3^- provided by ENP.

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% NO_3^-. The source of NO_3^- was a double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)_2{\cdot}NH_4NO_3{\cdot}10H_2O$]. The 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 NO_3^- and nitrite (NO_2^-) reducers, and NO_3^- and nitrite (NO_2^-) reducing activities in the ruminal environment. Otherwise, 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 × 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_4P) was calculated according to Longo et al. (2006) according to the following equation CH_4P, mL = (Total gas, mL + Head space, 85 mL) x 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_4P 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% H_3PO_4 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).

The hydrogen (H_2) produced and utilized (expressed as micromoles per milliliters) as fermentation end products and H_2 consumed to form CH_4 and short chain fatty acids (SCFA) were determined from molar concentration of acetate (C_2), propionate (C_3), butyrate (C_4), isovalerate (Ci_5), valerate (C_5) and CH_4. The H_2 produced ($H_2 = (2xC_2)+C_3+(4xC_4)+(2xC_5)+(2xCi_5)$), H_2 utilized ($H_2U = (2xC_3)+(2xC_4)+(4xCH_4)+Ci_5)$) and H_2 recovery ($H2R = (H_2U/H_2P) \times 100$) were calculated using the equations described by Demeyer & Tamminga (1987), Demeyer (1991), Wolin (1960). The equations do not account for H_2 released in the gaseous form, lactate, microbial mass, and potential acetate produced via reductive acetogenesis. The H_2 recovery was expressed as a percentage.

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 ruminatium* 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).

Table II. PCR primers used in this study.

Target taxon	Primer sequences (5'-3')[+]	Reference
Ruminococcus flavefaciens	F CGAACGGAGATAATTTGAGTTTACTTAGG R CGGTCTCTGTATGTTATGAGGTATTACC	Denman & McSweeney (2006); Denman et al. (2007)
Fibrobacter succinogenes	F GTTCGGAATTACTGGGCGTAAA R CGCCTGCCCCTGAACTATC	
Archaea metanogênicas	F TTCGGTGGATCDCARAGRGC R GBARGTCGWAWCCGTAGAATCC	
Wolinella succinogenes	F CTTCTTGCGAACAGTTAGA R CTCAATGTCAAGCCCTGG	Asanuma et al. (2002)
Selenomonas ruminantium	F TGCGAATAGTTTTTMGCAA R CTCAATGTCAAGCCCTGG	

[+]**F, Forward; R, Reverse.**

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 (CH_4P), 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 mL/g TDOM), resulting in a linear decrease ($y = 12.16 - 1.99 \times (ENP)$; $R^2 = 0.23$, $P < 0.01$) with increasing doses of ENP (0, 1.5 and 3% 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 µmol/mL, y=198.9-0.473*ENP) and H_2 utilization (128.7, 109.8 and 90.8 µmol/mL, y=128.8-12.75*ENP). Consequently, the recovered H_2 decreased by ENP inclusion (64.7, 55.2 and 45.8%, y=64.7-6.29*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 (CH_4P) and hydrogen.

Item	MON		SEM+	ENP+			SEM	*P*-value‡		
	(-)	(+)		0%	1.5%	3%		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
CH_4P (mL/g TDOM)	9.62	8.71	1.209	12.10	9.27	6.12	1.697	0.26	<0.01*	0.40
CH_4 (%)	8.23	7.87	1.237	10.40	8.15	5.50	1.257	0.63	<0.01*	0.52
H_2 Produced (µmol/mL)	199.0	197.5	0.67	198.8	198.7	197.4	0.71	0.048	<0.02*	0.63
H_2 Utilized (µmol/mL)	111.2	108.1	7.01	128.7	109.8	90.5	7.25	0.44	<0.01*	0.47
H_2 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.
[a, b] 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_L = 0.02, y=10.46-0.346*ENP) and isovaleric acids (1.4; 1.3 and 1.2 mmol/L, P_L < 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, R^2 = 0.53, P_L < 0.01) when compared to diets without MON (0.03, 0.20, 0.36%, y = 0.036 + 0.108*ENP, R^2 = 0.36, P_L < 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_L = 0.01) and *S. ruminantium* (0.01, 0.04 and 0.06; y= 0.004+0.0196*ENP; P_L = 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 NO_3^- 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.

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

Item+	MON		SEM+	ENP+			SEM	P-value±		
	(-)	(+)		0%	1.5%	3%		MON	ENP	MON×ENP
pH	6.82	6.83	0.012	6.83	6.82	6.83	0.014	0.62	0.93	0.74
NH_3-N (mg/100 mL)	34.4	34.1	0.91	33.5	34.1	35.1	1.01	0.79	0.33	0.13
Total SCFA (mmol/L)	74.1	73.4	2.48	74.8	74.2	72.3	2.54	0.56	0.16	0.08
Acetate	66.0	65.9	0.67	65.1	65.9	66.9	0.706	0.95	<0,01*	0.94
Proprionate	20.1	20.8	0.60	20.7	20.3	20.1	0.62	0.06	0.31	0.91
Butyrate	10.2[a]	9.7[b]	0.29	10.4	10.1	9.3	0.31	0.02	0.02*	0.24
Isobutyrate	0.66[a]	0.58[b]	0.042	0.64	0.62	0.59	0.047	<0.01	0.09	0.72
Valerate	1.77	1.72[b]	0.087	1.78	1.74	1.71	0.089	0.06	0.09	0.24
Isovalerate	1, 38[a]	1. 24[b]	0.079	1.38	1,31	1,22	0.081	<0.01	0.01*	0.54
C_2:C_3 ratio[C]	3.27	3.30	0.125	3.16	3.27	3.35	0.130	0.21	0.11	0.74

+ 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); 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.
[a, b] Means with different letters in the same row indicate significant treatment effect (Tukey test P < 0.05).

2014). 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, H2 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 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	MON		SEM+	ENP+			SEM	*P*-value‡		
	(-)	(+)		0%	1.5%	3%		MON	ENP	MON×ENP
A. Methanogens (%)	0.116	0.111	0.0673	0.136	0.126	0.081	0.0702	0.92	0.61	0.91
F. succinogenes (%)	0.198	0.446	0.0778	0.037	0.190	0.739	0.0834	0.02	<0.01*	0.02*
R. flavefaciens (%)	0.210	0.241	0.1310	0.022	0.230	0.425	0.1527	0.80	0.054*	0.62
S. ruminantium (%)	0.029	0.036	0.0237	0.001	0.041	0.057	0.0259	0.66	0.02*	0.97
W. succinogenes (%)	0.890	0.694	0.3129	0.002	0.341	2.033	0.3358	0.53	<0.01**	0.77
Protozoa (cel × 10^{-5}/mL)	2.80	2.67	0.244	2.87	2.35	2.41	0.277	0.63	0.12	0.17

[+]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).

observed was 2.82 and 5.98 mg/g TDOM, so the efficiency of CH_4 mitigation (actual CH_4 reduction / theorical CH_4 reduction × 100; Lee et al. 2015) was 83.6 and 92.9% in the 24-hour incubation period, representing the NO_3^- reduced to NH_3 by ruminal microorganisms. The not fully efficient CH_4 mitigation (83.6 and 92.9%) observed in this study could be a result of an incomplete reduction of the total amount of NO_3^- or NO_2^- to NH_3 (Newbold et al. 2014).

Another explanation for the reduction of CH_4 production could be the direct NO_3^- and NO_2^- 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 NO_3^- 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 NO_3^- reduces the exposure to microorganisms because of its slow release rate (Lee et al. 2017) and thus decreases the risk of NO_2^- toxicity. Lee et al (2017), using the same ENP product of this study, observed that 50% of NO_3^- released from encapsulation was metabolized by rumen microbes during the first 6 hours of the incubation period. Consequently, 61% and 93% of NO_3^- released from the encapsulation were metabolized over 12 and 24-hour intervals, respectively, during which no significant NO_3^- accumulation and H_2 production were observed over 24 hours. Besides, our results indicated an increase of NO_3^- and NO_2^- reducing bacteria, *W. succinogenes* and *S. ruminantium*, which contributed to reducing NO_3^- to NH_3 and decreased NO_2^- accumulation.

MON reduces the number of H_2-producing bacteria (Chen & Wolin 1979), promoting, indirectly, an increase in the molar concentration

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. succinogenesis*, nor in CH_4 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. succinogenesis*).

However, reductions in metabolic H_2 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 H_2 per mol of fermented glucose, thereby reducing hydrogen supply (Janssen 2010) and the formation of acetate and butyrate release two moles of CO_2 and four moles of H_2 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 CH_4 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 NH_3-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 NO_3^- plus MON numerically increased the levels of 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 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 NO_3^- in the diet. Possibly because the addition of ENP enabled the increase of NO_3^- substrate, favoring the growth of these bacteria (Lin et al. 2011).

CONCLUSIONS

The CH_4 reduction by ENP addition reflected the effect of NO_3^- acting as a H_2 sink. However,

the reduction on CH_4 production was lower than expected. The additive effect of ENP and MON was not confirmed on reducing CH_4 nor affecting nitrate- and nitrite-reducing bacteria, but an increase on the relative abundance of gram-negative bacteria (*F. succinogenes*) was observed.

Acknowledgments

The authors are thankful for the technical support of researchers and technicians of the Laboratory of Animal Nutrition from the Centre for Nuclear Energy in Agriculture (CENA-USP). We are also grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, 2012/02592-0) for the scholarship granted to the first author.

REFERENCES

ANDERSON RC, HUWE JK, SMITH DJ, STANTON TB, KRUEGER NA, CALLAWAY TR, EDRINGTON TS, HARVEY RB & NISBET DJ. 2010. Effect of nitroethane, dimethyl- 2-nitroglutarate and 2-nitro-methyl-propionate on ruminal methane production and hydrogen balance in vitro. Bioresour Technol 101: 5345-5349.

ANDERSON RC, KRUEGER NA, STANTON TB, CALLAWAY TR, EDRINGTON TS, HARVEY RB, JUNG YS & NISBET DJ. 2008. Effects of select nitrocompounds on in vitro ruminal fermentation during conditions of limiting or excess added reductant. Bioresour Technol 99: 8655-8661.

ANDERSON RC & RASMUSSEN MA. 1998. Use of a novel nitrotoxin-metabolizing bacterium to reduce ruminal methane production. Bioresour Technol 64: 89-95.

ANDERSON RC, RIPLEY LH, BOWMAN JGP, CALLAWAY TR, GENOVESE KJ & BEIER RC. 2016. Ruminal fermentation of anti-methanogenic nitrate and nitro-containing forages *in vitro*. Front Vet Sci 3: 62. doi: 10.3389/fvets. 2016.00062

ARAUJO RC, PIRES AV, ABDALLA AL, PECANHA MRSR & SALLAM SMA. 2009. Monensin sodium as a positive control on studies about modifiers of rumen fermentation using in vitro gas production technique [In Portuguese]. 46th Annual Meeting of Sociedade Brasileira de Zootecnia, Sociedade Brasileira de Zootecnia, Maringá, Paraná, 14 a 17 de julho de 2009.

ARAUJO RC, PIRES AV, MOURO GB, ABDALLA AL & SALLAM SMA. 2011. Use of blanks to determine in vitro net gas and methane production when using rumen fermentation modifiers. Anim Feed Sci Tecnol 166: 155-162.

ASANUMA N, IWAMOTO M, KAWATO M & HINO T. 2002. Numbers of nitrate-reducing bacteria in the rumen as estimated by competitive polymerase chain reaction. J Anim Sci 73: 199-205.

BEACON SE. 1988. Effect of the feed additives chlortetracycline, monensin and lasalocid on feedlot performance of finishing cattles, liver lesions and tissue level of chlortetracycline. Can J Anim Sci 68: 1131-1141.

BEAUCHEMIN KA, KREUZER M, O'MARA F & MCALLISTER TA. 2008. Nutritional management for enteric methane abatement: a review. Austr J Exp. Agricult 48: 21-27. doi: 10.1071/EA07199.

BERTIPAGLIA LMA. 2008. Suplementação protéica associada a monensina sódica e Sacchatomyces cerevisiae na dieta de novilhas mantidas em pastagem de capim-Marandu. Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinária. Jaboticabal, Brasil 137 p.

BLÜMMEL M, MAKKAR HPS & BECKER K. 1997. In vitro gas production: a technique revisited. J Anim Physiol Anim Nutrit 77: 24-34.

BOŽIC AK, ANDERSON RC, CARSTENS GE, RICKE, SC, CALLAWAY TR, YOKOYAMA MT, WANG JK & NISBET DJ. 2009. Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin and the Hawaiian marine algae Chaetoceros on ruminal fermentation in vitro. Biores Technol 100: 4017-4025. doi:10.1016/j.biortech.2008.12.061.

BROWN EG, ANDERSON RC, CARSTENS GE, GUTIERREZ-BAÑUELOS H, MCREYNOLDS JL, SLAY LJ, CALLAWAY TR & NISBET DJ. 2011. Effects of oral nitroethane administration on enteric methane emissions and ruminal fermentation in cattle. Anim Feed Sci Technol 166-167: 275-281.

BUENO ICS, CABRAL FILHO SLS, GOBBO SP, LOUVANDINI H, VITTI DMSS & ABDALLA AL. 2005. Influence of inoculum source in a gas production method. Anim Feed Sci Technol 123: 95-105. doi: 10.1016/j.anifeedsci.2007.04.011.

CAPELARI MC, JOHNSON KA, LATACK B, ROTH J & POWERS W. 2018. The effect of encapsulated nitrate and monensin on ruminal fermentation using a semi-continuous culture system. J Anim Sci 96: 3446-3459. doi: 10.1093/jas/sky211.

CHEN M & WOLIN MJ. 1979. Effect of monensin and lasalocidsodium on the growth of methanogenic and rumen saccharolytic bacteria. Applied Environm Microbiol 38: 72-77.

DEMEYER DI. 1991. Quantitative aspects of microbial metabolism in the rumen and hindgut. In: Jouany JP (Ed),

Rumen microbial metabolism and ruminant digestion. INRA Editions, Paris, p. 217-237.

DEMEYER DI & TAMMINGA S. 1987. Microbial protein yield and its prediction. In: Jarrige R & Alderman G (Eds), Feed evaluation and protein requirement systems for ruminants. Office for Official Publications of the European Communities, Luxembourg City, Luxembourg, p. 129-141.

DENMAN SE & MCSWEENEY CS. 2006 Development of a realtime PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. FEMS Microbiol Ecolog 58: 572-582. doi: 10.1111/j.1574-6941.2006.00190.x.

DEHORITY AB, DAMRON WS & MCLAREN JB. 1983. Occurrence of the rumen ciliate Oligoisotricha bubali in domestic cattles (*Bos taurus*). Applied Environm Microbiol 45: 1394-1397.

ELLIOT JM & LOOSLI JK. 1959. Relationship of milk production efficiency to the relative proportions of the rumen volatile fatty acids. J Dairy Sci 42: 843-848.

GUAN H, WITTENBERG KM, OMINSKI KH & KRAUSE DO. 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. J Anim Sci 84: 1896-1906.

GUYADER J, UNGERFELD EM & BEAUCHEMIN KB. 2017. Redirection of metabolic hydrogen by inhibiting methanogenesis in the rumen simulation technique (RUSITEC). Front Microbiol 8: 393. doi:10.3389/fmicb.2017.00393.

IWAMOTO M, ASANUMA N & HINO T. 2002. Ability of *Selenomonas ruminantium*, *Veillonella parvula*, and *Wolinella succinogenes* to reduce nitrate and nitrite with special reference to the suppression of ruminal methanogenesis. Anaerobe 8(4): 209-215. DOI: 10.1006/anae.2002.0428.

JANSSEN PH. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim Feed Sci Technol 160: 1-22.

KLOP G, HATEW B, BANNINK A & DIJKSTRA J. 2016. Feeding nitrate and docosahexaenoic acid affects enteric methane production and milk fatty acid composition in lactating dairy cows. J Dairy Sci 99:1161-1172.

KOHN RA & BOSTON RC. 2000. The Role of thermodynamics in controlling rumen metabolism. In: McNamara JP et al. (Eds), Modelling Nutrient Utilization in Farm Animals, New York: CAB International, New York, USA, p. 11-24.

LEE C, ARAUJO RC, KOENIG KM & BEAUCHEMIN KA. 2017. In situ and in vitro evaluation of a slow release form of nitrate for ruminants: nitrate release rates, rumen nitrate metabolism and production of methane, hydrogen, and nitrous oxide. J Anim Sci 231: 97-106. DOI: 10.1016/j.anifeedsci.2017.07.005.

LEE C, ARAUJO RC, KOENIG KM & BEAUCHEMIN KA. 2015. Effects of encapsulated nitrate on enteric methane production, and nitrogen and energy utilization in beef heifers. J Anim Sci 93: 2391-2404. doi: 10.2527/jas2014-8845.

LEE C & BEAUCHEMIN KA. 2014. A review of feeding supplementary nitrate to ruminant animals: Nitrate toxicity, methane emissions, and production performance. Can J Anim Sci 94: 557-570. doi:10.4141/cjas-2014-069.

LENG RA. 2008. Decline in available world resources; implications for livestock production systems in Asia. Livest Resear Rural Develop 20: 8. http://www.lrrd.org/lrrd20/1/leng20008.htm.

LENG RA. 2010. Further considerations of the potential of nitrate as a high affinity electron acceptor to lower enteric methane production in ruminants. In: 'International conference on livestock, climate change and resource depletion, p. 9-11. Pakse, Laos: Champasack University.

LI L, SILVEIRA CI, NOLAN JV, GODWIN IR, LENG RA & HEGARTY RS. 2013. Effect of added dietary nitrate and elemental sulfur on wool growth and methane emission of Merino lambs. Anim Produc Sci 53: 1195-1201. doi: 10.1071/AN13222.

LIN M, SCHAEFER DM, GUO WS, REN LP & MENG QX. 2011. Comparisons of *in vitro* nitrate reduction, methanogenesis, and fermentation acid profile among rumen bacterial, protozoal and fungal fractions. Asian-Aust J Anim Sci 24: 471-478. doi: 10.5713/ajas.2011.10288.

LONGO C, BUENO ICS, NOZELLA EF, GODDOY PB, CABRAL FILHO SLS & ABDALLA AL. 2006. The influence of head-space and inoculum dilution on in vitro ruminal methane measurements. In: 'International Congress Series' 1293: 62-65.

MAJAK W. 1992. Further enhancement of nitropropanol detoxification by ruminal bacteria in cattle. Can. J Anim Sci 72: 863-870.

MAURÍCIO RM, MOULD FL, DHANOA MS, OWEN E, CHANNA KS & THEODOROU MKA. 1999. Semiautomated *in vitro* gas production technique for ruminant feedstuff evaluation. Anim Feed Sci Technol 79: 321-330.

MARAIS JP, THERION JJ, MACKIE RI, KISTNER A & DENNISON C. 1988. Effect of nitrate and its reduction products on the growth and activity of the rumen microbial population. Br J. Nutr 59: 301-313. doi:10.1079/ BJN19880037.

NATEL AS, ABDALLA AL, ARAUJO RC, MCMANUS C, PAIM, TP, ABDALLA FILHO AL, LOUVANDINI P & NAZATO C. 2019. Encapsulated nitrate replacing soybean meal changes in vitro ruminal fermentation and methane production in diets differing in concentrate to forage ratio. Asian-Austr J Anim Sci 00:1-12. DOI: 10.1111/asj.13251.

NEWBOLD JR, VAN ZIJDERVELD SM, HULSHOF RB, FOKKINK WB, LENG RA, TERENCIO P, POWERS WJ, VAN ADRICHEM PS, PATON ND & PERDOK HB. 2014. The effect of incremental levels of dietary nitrate on methane emissions in Holstein steers and performance in Nelore bulls. J. Anim. Sci 92: 5032-5040. doi:10.2527/ jas.2014-7677.

NOCEK JE, HART SP & POLAN CE. 1987. Rumen ammonia concentrations as influenced by storage time, freezing and thawing, acid preservative, and method of ammonia determination. J Dairy Sci 70: 601-607.

NRC - NATIONAL RESEARCH COUNCIL. 2007. Nutrient requirements of small ruminants: Sheep, goats, cervids and new world camelids. Washington, DC: National Academic Press, 292 p.

PALMQUIST D & CONRAD H. 1971. Origin of Plasma Fatty Acids in Lactating Cows Fed High Grain or High Fat Diets. J Dairy Sci 54: 1025-1031.

PRESTON TR. 1995. Tropicall animal feeding: a manual for research workers. Rome: FAO, Anim Produc Health Paper 126.

RUSSELL JB & STROBEL HJ. 1988. Effects of additives on in vitro ruminal fermentation: a comparison of monensin and bacitracin, another Gram-positive antibiotic. J Anim Sci 66: 552-558.

SAR C, MWENYA B, SANTOSO B, TAKAURA K, MORIKAWA R, ISOGAI N, ASAKURA Y, TORIDE Y & TAKAHASHI J. 2005. Effect of Escherichia coli wild type or its derivative with high nitrite reductase activity on in vitro ruminal methanogenesis and nitrate/nitrite reduction. J Anima Sci 83: 644-652.

SOLTAN YA, MORSY AS, LUCAS RC & ABDALLA AL. 2017. Potential of mimosine of Leucaena leucocephala for modulating ruminal nutrient degradability and methanogenesis. Anim Feed Sci and Tech 223: 30-41.

STROBEL HJ & RUSSELL JB. 1989. Non-proton-motiveforce-dependent sodium efflux from the ruminal bacterium Streptococcus bovis: bound versus free pools. Applied Environm Microbiol 55: 2664-2668.

THEODOROU MK, WILLIAMS BA, DHANOA MS, MCALLAN AB & FRANCE J. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim Feed Sci Technol 48: 185-197.

UNGERFELD EM & KOHN RA. 2006. The role of thermodynamics in the control of rumen fermentation. In: Sejrsen K, Hvelplund T & Nielsen MO (Eds), Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress, p. 55-85. Wageningen Academic Publishers: Wageningen, The Netherlands.

WOLIN MJ. 1960. A theoretical rumen fermentation balance. J Dairy Sci 43:1452-1459. doi:10.3168/jds. S0022-0302(60)90348-9.

YANG CJ, ROOKE JA, CABEZA I & WALLACE RJ. 2016. Nitrate and inhibition of ruminal methanogenesis: microbial ecology, obstacles, and opportunities for lowering methane emissions from ruminant livestock. Front Microbiol 7: 132.

ZHANG DF & YANG HJ. 2011. In vitro ruminal methanogenesis of a hay-rich substrate in response to different combination supplements of nitrocompounds; pyromellitic diimide and 2-bromoethanesulphonate. Anim Feed Sci Technol 163: 20-32.

ZHOU ZM, MENG QX & YU ZT. 2011. Effects of methanogenic inhibitors on methane production and abundances of methanogens and cellulolytic bacteria in in vitro ruminal cultures. Appl Environ Microbiol 77: 2634-2639.

ZHOU Z, YU Z & MENG Q. 2012. Effects of nitrate on methane production, fermentation, and microbial populations in in vitro ruminal cultures. Biores Technol 103: 173-179.

How to cite

NATEL AS, ABDALLA AL, ARAUJO RC, PAIM TP, ABDALLA FILHO AL, LOUVANDINI P, LIMA MK & PIZA P. 2022. Encapsulated nitrate replacing soybean meal in diets with and without monensin on in vitro ruminal fermentation. An Acad Bras Cienc 94: e20200213. DOI 10.1590/0001-3765202220200213.

Manuscript received on February 12, 2020;
accepted for publication on September 9, 2020

ANDRESSA S. NATEL[1]
https://orcid.org/0000-0002-8252-1090

ADIBE LUIZ ABDALLA[2]
https://orcid.org/0000-0002-5440-9974

RAFAEL C. DE ARAUJO[3]
https://orcid.org/0000-0003-3484-882X

TIAGO P. PAIM[4]
https://orcid.org/0000-0002-9486-7128

ADIBE LUIZ ABDALLA FILHO[2]
https://orcid.org/0000-0001-5968-5380

PATRÍCIA LOUVANDINI[2]
https://orcid.org/0000-0002-8540-6629

MELKI K. LIMA[1]
https://orcid.org/0000-0002-7192-6981

PAOLA PIZA[1]
https://orcid.org/0000-0002-0759-9760

[1]University "José do Rosário Vellano", Rod. MG 179, Km 0, s/n, Câmpus Alfenas, 37132-440 Alfenas, MG, Brazil

[2]University of São Paulo, Centre for Nuclear Energy in Agriculture, Laboratory of Animal Nutrition, Av. Centenário, 303, São Dimas, 13400-970 Piracicaba, SP, Brazil

[3]GRASP Ind. & Com. LTDA, Division of Research and Development, Av. Juscelino Kubitschek de Oliveira, 4550, Cidade Industrial, 81260-000 Curitiba, PR, Brazil

[4]Instituto Federal de Educação, Ciência e Tecnologia Goiano, Campus Iporá, Av. Oeste, 350, Parque União,76200-000 Iporá, GO, Brazil

Correspondence to: **Andressa Santanna Natel**
Email: andressa.natel@unifenas.br

Author contributions

A. L. ABDALLA: research coordinator. R.C. de ARAUJO. and A.L. ABDALLA designed the study. A.S. NATEL, M.K. LIMA, P. LOUVANDINI and P. PIZA performed the research. T. do P. PAIN analyzed the data. R.C. de ARAUJO, A.L. ABDALA, A.S. NATEL, A.L. ABDALLA FILHO and P. LOUVANDINI contributed to the writing and review of the manuscript. A.L. ABDALLA FILHO and T. do P. PAIM translated the manuscript.