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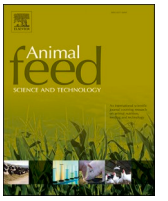

Effects of trace mineral source and exogenous enzymes on ruminal *in vitro* fermentation of roughage-based or concentrate-based simulated diets

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ABSTRACT

Two experiments evaluated the effect of trace mineral (TM) source (inorganic [ITM] and organic [OTM]) and 4 exogenous enzymes treatments [(1) control, without exogenous enzymes; (2) amylolytic enzyme [AMY; 1 g of Amaize®/kg of substrate dry matter (DM)]; (3) fibrolytic enzyme (FIB; 2 g of Fibrozyme®/kg of substrate DM; and (4) a multi-enzyme preparation (SSF; 2 g of Allzyme® SSF/kg of substrate DM] on *in vitro* fermentation parameters of roughage-based or concentrate-based diets (Exp. 1 and 2, respectively). Two roughages were used in each experiment [high- and low-quality tropical grass hay in Exp. 1 (HQH and LQH, respectively), and corn silage and sugarcane bagasse in Exp. 2 (CS and SB, respectively)]. Each experiment was conducted as a randomized complete block (n = 3). Mixed ruminal microorganisms were incubated in anaerobic media containing 300 mg of substrate diet and 50 mL rumen fluid:buffer. Incubations were performed in batch cultures for 48 h (Exp. 1) or 24 h (Exp. 2) at 39°C. There were no three-way interactions observed for Exp. 1 or 2. Roughage × TM source interactions ($P < 0.02$) were verified for total gas production (GP), partioning factor (PF), methane (CH_4) production and yield in both experiments. In Exp. 1, total GP of HQH and CS decreased about 18% with OTM ($P < 0.02$), while for LQH and SB they increased in the same proportion. The PF of HQH and CS were increased ($P < 0.01$), while LQH and SB decreased by 12% ($P < 0.05$) when OTM was added. The CH_4 yield (mL/g DMd) decreased when LQH and SB were incubated with ITM ($P < 0.02$). The HQH have lower pH compared with the LQH ($P < 0.001$; 6.56 vs. 6.72). Additionally, PF presented TM source × enzyme interaction ($P = 0.05$). When ITM was included, PF was higher with addition of AMY than SSF ($P = 0.04$), but they were similar to CON and FIB. When OTM was

Abbreviations: AMY, amylolytic enzyme; CH_4, metano; CON, control; CS, corn silage; DM, dry matter; DMd, DM digested; DMi, DM incubated; FIB, fibrolytic enzyme; GP, gas production; HQH, high-quality tropical grass hay; ITM, inorganic trace mineral; LQH, low-quality tropical grass hay; OTM, organic trace mineral; PF, partioning factor; SB, sugarcane bagasse; SSF, multi-enzyme preparation; TM, trace mineral; VFA, volatile fatty acids.

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supplemented, PF was greater with SSF compared with FIB ($P = 0.04$), but presented similar values to CON and AMY. In Exp. 2, GP was lower when SB and ITM was incubated together ($P = 0.02$; 141 vs. 175 mL/g DM), while for CS there was no difference between ITM sources ($P = 0.17$). The CH_4 yield reduced when OTM was combined with CS or when ITM was added with SB ($P \leq 0.02$). The PF of CS increased in 37% ($P < 0.01$), while SB decreased by 23% ($P < 0.05$) when OTM was added. As verified in Exp. 1, roughage affected pH and CS significantly reduced pH compared with SB ($P < 0.001$; 6.69 vs. 6.81). In conclusion, the *in vitro* treatment of substrates with different roughage-to-concentrate ratios with exogenous enzymes were minorly impacted by the TM source. The incubation of low-quality roughages with ITM source increased PF and decreased GP, which led to a consistent reduction in the CH_4 yields. Additionally, IVDMD increased and lowered greenhouse gas production by OTM inclusion in high-concentrate substrate with CS, resulting in a favorable impact on rumen fermentation efficiency.

1. Introduction

In tropical countries, mineral supplementation is essential due to the typically low concentration of mineral in forages, including trace minerals (**TM**; Little, 1986). The TM (Co, Cu, I, Fe, Mn, Se, and Zn) are required in small concentrations for processes such as cell signaling and enzyme cofactors, and play a role in growth, development, and immune response for the production of healthy cattle (Underwood and Suttle, 1999; National Academies of Sciences, Engineering and Medicine, 2016). Nevertheless, the TM source can affect the mineral absorption and metabolism due to molecular composition and structure of the supplemented form (Kegley and Spears, 1994; Du et al., 1996). In this context, the TM source may produce changes in the ruminal environment, affecting the VFA production, fiber digestibility, and feed digestion.

The use of exogenous enzymes, mainly fibrolytics, holds promise as the increasing forage utilization and improving the productive efficiency of ruminants (Beauchemin et al., 2003). Exogenous enzymes used in ruminant nutrition are characterized into two main categories: fibrolytic and amylolytic enzymes. The exogenous fibrolytic enzyme activity is dependent on cofactors, activators, and inhibitors. For instance, Cu-metalloenzymes are needed for maximal activity of cellulase-enhancing factors (polysaccharide monooxygenases; Quinlan et al., 2011). Some TM that acts as cofactors (i.e., Mn^{2+}, Co^{2+}, Fe^{2+}) could improve the activity of fibrolytic enzymes. Amylolytic enzymes, such as α-amylase, work by cross-feeding mechanisms of ruminal bacteria through oligosaccharides produced by the enzyme, creating modified products of ruminal fermentation (Rojo et al., 2005). In that case, positive effect on ruminal fermentation can be observed (Vargas-Rodriguez et al., 2014; Neumann et al., 2018; Silva et al., 2020).

Beyond the specifics exogenous enzymes effects, a cross-feeding mechanism can improve the digestibility of nutrients not targeted by them (e.g., amylolytic and fibrolytic enzymes; Tricarico et al., 2008). In this scenario, enzymes effects on ruminal fermentation could be dependent of forage source (i.e., chemical composition; Colombatto et al., 2003; Eun and Beauchemin, 2005; Sakita et al., 2020), and by the roughage:concentrate ratio which different substrates and pH changes can affect the enzyme effects. Despite these studies, there is limited literature available on the effects of the exogenous enzymes associated with different TM source on ruminal *in vitro* fermentation.

Based on the above description, ruminal solubility of TM may influence ruminal fermentation and the investigation of the interactions of exogenous enzymes and TM source by using different roughage sources and roughage:concentrate ratio would be interesting. In this context, we hypothesized that dietary supplementation with organic TM could positively affect exogenous enzymes effects in an *in vitro* ruminant fermentation system would be dependent on the dietary characteristics, because the differences in chemical composition and ruminal pH. Therefore, two experiments were conducted to evaluate the effects of TM source (inorganic and organic) and exogenous enzymes (amylolytic, fibrolytic, and a multi-enzyme preparation) on *in vitro* ruminal fermentation parameters of forage- and concentrate-based substrates.

2. Materials and Methods

The experimental protocol was reviewed and approved by the Animal Care Committee of the Universidade Federal de Lavras (Protocols: 007/2019 and 012/2020; UFLA; Lavras, Minas Gerais, Brazil).

2.1. Experimental design and treatments

Both experiments used a randomized complete block ($n = 3$) design experiment with 16 treatments in a $2 \times 2 \times 4$ factorial arrangement, with 2 sources of roughage (high- and low-quality hay at Exp. I, or corn silage and sugarcane bagasse at Exp. 2), 2 sources of trace mineral [inorganic (ITM) or organic (OTM) Co, Cu, Fe, Mn, Se, and Zn at the same levels] and 4 enzymes treatment: (1) control (CON), substrate without exogenous enzymes; (2) amylolytic enzyme (AMY), AmaizeTM (Alltech Inc.) added at 1 g/kg of substrate DM (600 U amylase/kg of diet DM); (3) fibrolytic enzyme (FIB), Fibrozyme® (Alltech Inc.) added at 2 g/kg of substrate DM (200 IU of xylanase activity/kg of diet DM); and (4) a multi-enzymatic preparation (SSF), added Allzyme® SSF (Alltech Inc.) added at 2 g/kg of substrate DM (200 IU of xylanase activity and 120 U of amylase activity, plus 8.000 U of pectinase, 1.400 U protease, 600 U of phytase, 400 U of α-glucanase, 80 U cellulase per kg of DM). According to the manufacturer, Fibrozyme is an extract from *Trichoderma longibrachiatum* fermentation, Amaize consists of an *Aspergillus oryzae* culture extract, and Allzyme SSF is produced from *Aspergillus niger*

fermentation.

The first factor within each experiment was the roughage source (Table 1). The two roughage sources (*Urochloa decumbens* cv. Marandu) in Exp. 1 were high- and low-quality tropical grass hay (HQH and LQH, respectively). The basal diets were formulated to contain a 65:35 ratio of roughage to concentrate (Table 2) and to meet the nutrient requirements of a 300-kg Nellore bull gaining 0.5 kg/d, according to the Brazilian Nutrient Requirements for pure and crossbred Zebu cattle – BR CORTE (Valadares Filho et al., 2016). Concentrate-based diets for Exp. 2 (15:85 roughage:concentrate ratio; Table 2) were formulated based on a 400-kg Nellore bull gaining 1.2 kg/d (BR CORTE; Valadares Filho et al., 2016). The roughage (corn silage and sugarcane bagasse), CP concentration (136 g crude protein/kg) and the roughage: concentrate ratio (15:85), used in the formulation were established based on the inventory of nutritional practices adopted by nutritionists in feedlots in Brazil, prepared by Silvestre and Millen (2021).

Before inclusion into the diets, all ingredients were air dried for at least 48 h and ground to pass a 1-mm screen (except, urea and ammonium sulphate which were ground using mortar and pestle, and mineral supplement which was in a powdered form). Substrates were analyzed for dry matter (DM; dried overnight at 105°C; method INCT-CA number G-003/1), ash (complete combustion in a muffle furnace at 600°C for 4 h; method INCT-CA number M-001/1), N (Kjeldahl procedure; method INCT-CA number N-001/1), and NDF corrected for ash and protein (using a heat-stable α-amylase, omitting sodium sulfite and correcting for residual ash and protein; method INCT-CA number F-002/1) by chemical analytical methods according to the standard analytical procedures of the Brazilian National Institute of Science and Technology in Animal Science (INCT-CA; Detmann et al., 2021). The chemical composition of the simulated diets is provided in Table 2.

Three *in vitro* incubations (run) were conducted for each experiment. In each run, 34 incubation bottles were used. This provided 2 bottles for each diet by TM by ENZ combination, plus two bottles as blanks (i.e., rumen fluid only) per block.

2.2. In vitro incubation conditions and rumen inoculum source

Samples of 300 mg of each diet were accurately weighed into 100-mL serum bottles. The enzymes were dissolved daily (3 mg of AMY, 6 mg of FIB, and 6 mg of SSF) in 10 mL of buffer solution (pH 6.8) and carefully applied directly onto the substrate inside the bottles (1 mL/300 mg DM) before each incubation run. Then, bottles were kept in an incubation room (39ºC ± 0.4) for about 1 h before adding buffered ruminal fluid. The TM source was directly mixed in the diets before incubation.

Inoculum donors were housed at the Metabolism Unit of the Department of Animal Science at UFLA. For Exp. 1, rumen inoculum was collected from 2 ruminally fistulated dry beef cows grazing Marandu palisadegrass (*Brachiaria brizantha*) pasture and receiving 2.0 kg/d of concentrate (DM basis; approximately 77% corn, 5% soybean meal, 8% urea, and 10% mineral mixture) provided once daily. The diets were provided to the cows 2 wk prior to rumen fluid collection to ensure the ruminal microbial ecosystems were adapted to their respective diets. Ruminal contents of each cow were obtained immediately before the morning feeding or supplementation from 5 different rumen locations, mixed and strained through four layers of cheesecloth and then transferred to a preheated 1-L thermos. Final inoculate volume consisted of a mixture (1:1) of the fluid collected from each cow. Anaerobic media was prepared according to the method of Menke and Steingass (1988), omitting the trace element solution. Particle-free ruminal fluid was mixed with the media solution in a proportion 1:2 (v/v) at 39ºC under continuous flushing with CO_2. In Exp. 2, two ruminally fistulated Nellore steers fed a 65:35 forage:concentrate ratio (DM basis; approximately 65% snaplage, 20% corn, 7% cottonseed cake, 5.5% soybean meal, 0.5% urea, and 2% mineral mixture) were used as rumen inoculum donors. The steers were adapted to the diet for 14 d prior to rumen inoculum collections. The ruminal collections, media preparation, and its mixture with ruminal fluid were processed as described above in Exp. 1.

The incubation length was 48 h in Exp. 1 and 24 h in Exp. 2. One run was conducted each week. It must be emphasized that the incubation times for each experiment were designed to be complimentary to realistic rumen fermentation conditions for the given diets. Therefore, these short-term, rather than long-term, incubations were used because long-term (e.g., 96-h) incubations are not representative of the expected residence time of feed in the rumen.

In both experiments, 50 mL of buffered ruminal fluid were added into each bottle under CO_2 flushing. Once each bottle was filled, it was immediately closed with rubber stoppers and aluminum caps, gently shaken and placed in the incubator room at 39ºC (± 0.4). The volume of gas produced was recorded at 2, 4, 6, 8, 10, 12, 16, 20, 24 h (Exp. 1 & 2) and 48 h (Exp. 1 only) using the pressure reading technique (Extech Instruments, Waltham, USA) of Theodorou et al. (1994).

Table 1
Chemical composition of the roughages evaluated at Exp. 1 and 2.

Item	Roughage composition, g/kg DM			
	Experiment 1		Experiment 2	
	High-quality tropical hay	Low-quality tropical hay	Corn Silage	Sugarcane bagasse
Organic matter	941	910	949	945
Crude protein	125	31	72	14
aNDFom[a]	598	805	516	778
Lignin in sulfuric acid	27	86	42	127

[a] Neutral detergent fiber corrected for ash and protein.

Table 2
Ingredients and chemical composition of experimental diets for *in vitro* Exp. 1 (65:35 roughage: concentrate ratio) and Exp. 2 (15:85 roughage: concentrate ratio).

Item	Roughage			
	Experiment 1		Experiment 2	
	High-quality tropical hay	Low-quality tropical hay	Corn Silage	Sugarcane bagasse
Ingredients, g/kg DM				
High-quality hay	650	—	—	—
Low-quality hay	—	650	—	—
Corn Silage	—	—	150	—
Sugarcane bagasse	—	—	—	150
Corn	334	311	631	627
Soyhulls	—	—	150	150
Soybean meal	3	3	37	37
Urea + AS[a]	4	28	12	15
Mineral suplement[b]	9	9	20	20
Calculated chemical composition, g/kg DM				
Organic matter	944	923	945	943
Crude protein	125	124	136	136
aNDFom[c]	438	488	274	325
Non-fiber carbohydrates	372	279	590	337

[a] Urea + AS = 900 g urea + 100 g ammonia sulfate.
[b] Composition: ≥ 160 g/kg Ca, 83 g/kg P, 70 g/kg S, 4 g/kg Mg, 63 g/kg Na, 3.000 mg/kg Zn, 2.000 mg/kg Fe, 875 mg/kg Cu, 1.500 mg/kg Mn, 62.5 mg/kg I, 19 mg/kg Co, 12.5 mg/kg Se. Organic trace minerals source (Co, Cu, Fe, Mn, Se, Zn) was manufactured by Bom Peso Nutrição Animal Ltda, Batayporã, Mato Grosso do Sul, Brazil.
[c] Neutral detergent fiber corrected for ash and protein.

2.3. Collection and chemical analyses

At the end of incubation (48 h in Exp. 1 and 24 h in Exp. 2), fermentation was stopped by placing serum bottles in ice-water bath for 15 min. A 10-mL sample of gas was collected from each bottle using a precise syringe to further CH_4 analysis. After the gas sampling, bottles were then opened, and contents of each serum bottle were filtered under vacuum through glass crucibles with a sintered filter (coarse porosity no. 1, pore size 100–160 μm; Pyrex, Stone, UK). The fluid pH was measured with a pH meter (HI 2221, Portable pH meter; Hanna Instruments) by submerging the probe approximately 3 cm in the fermentation media. Fermentation media (1.2 mL) was then combined with 0.4 mL of 250 g/L metaphosphoric acid and frozen for subsequent analysis of VFA. After that, fermentation residues in glass crucibles were dried at 105°C overnight to estimate DM disappearance with loss in weight after drying being the measure of undegradable DM.

Sample VFA profile was obtained using a gas chromatograph equipped with an FID detector (Vanzant and Cochran, 1994). A GC (EZChrom Elite software interface, Model 7820 A, Agilent Technologies Brasil, Barueri, SP, Brazil) was used for the quantification of CH_4. The GC was equipped with two six-way valves, one being used for the sampler system connected to a loop of 0.5 mL. A split-splitless type injector used in split mode at a ratio of 1:50 at 120ºC. The separation system consisted of two columns. The first column was a HP-Plot/Q 30 m × 0.530 mm × 40.0 mm (Agilent Technologies Brasil). The detection system comprised a thermal conductivity detector at 250ºC, with 25 mL H_2/min as flow reference. The second column was a Molesieve HP-30 m × 0.530 mm × 25.0 mm, using H_2 as carrier gas at a flow rate of 8.3 mL/min, with flame ionization detector (FID) at 270ºC and 15 mL/min H_2 flow rate and 350 mL/min synthetic air flow. The GC was also equipped with a methaniser maintained at 375ºC, which allows detecting very low concentrations of CO_2. The oven temperature was maintained at 55ºC. The calibration curves were performed with reference standards for CH_4 concentrations, as follows: 0%, 5.05%, 10.2%, 14.7% and 20.1%; and for CO2 as follows: 0%, 20.2%, 39.7%, 58.3% and 79.9%.

2.4. Calculations

Gas production was calculated by the equation defined for our laboratory conditions, considering the measured pressure: 6.571 × measured pressure (psi). Total gas production (GP) was calculated as the volume of gas (mL) produced after 48 h and 24 h of incubation (Exp. 1 and 2, respectively), correcting values of gas production for the corresponding blank, and divided by the amount of DM incubated (g).

The fraction of IVDMD for each bottle was calculated by subtracting the dry residue weight (corrected for the blank) from the dry substrate weight and dividing by the dry weight of substrate. The partitioning factor at the end of incubations (PF; a measure of fermentation efficiency) was calculated as the ratio of DM degradaded *in vitro* (DMd, mg) to the volume (mL) of GP (i.e., DMd/total GP), according to Blümmel et al. (1997).

Methane production (mL/g DMi) was calculated as total gas (mL) multiplied by the CH_4 proportion (mL/mL) and divided by the DM incubated. The CH_4 yield, expressed as mL/g DMd, was calculated by correcting values of CH_4 production and DM degradation for the corresponding blank.

2.5. Statistical analyses

All statistical analyses used the MIXED procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC), using the average of two bottles in each batch as the experimental unit (replicas, n=3/treatment; Henry et al., 2021). For each experiment, the data were analyzed by ANOVA with a 2 × 2 × 4, factorial fixed effects treatment structure of roughage (high- and low-quality tropical hay in Exp. 1; and corn silage or sugarcane bagasse in Exp. 2) associated with TM source (inorganic or organic) and enzyme (CON, AMY, FIB or SSF). Each run formed a block and the experimental unit was specified as the treatment combination of substrate within each block. Thus, the random effects blocking structure for the ANOVA was block split for unit, split for bottle. With bottle nested within units in this way, the analysis results are as if the data consisted of 6-bottle averages for each unit. The ANOVA model is given by the following equation:

$$Y_{ijk} = \mu + R_i + M_j + E_k + \beta_l + (R \times M)_{ij} + (R \times E)_{ik} + (M \times E)_{jk} + (R \times M \times E)_{ijk} + U_{ijkl} + \varepsilon_{ijklm}$$

Table 3
Effects of roughage, trace mineral source, exogenous enzymes and their combination on *in vitro* gas and CH_4 production in Exp. 1 (65:35 roughage: concentrate ratio).

Diet[a]	Enzyme[b]	pH	GP[c] (mL/g DMi)	IVDMD[d] (g/kg)	PF[e] (mg DMd/mL)	CH_4[f] (mL/L)	CH_4[g] (mL/g DMi)	CH_4[h] (mL/g DMd)
High-quality tropical hay								
+ ITM	CON	6.59	148	720	4.9	74	11	14
	AMY	6.62	141	739	5.3	70	10	13
	FIB	6.55	149	752	5.2	77	12	16
	SSF	6.51	155	704	4.5	71	11	16
+ OTM	CON	6.67	112	760	6.8	66	8	10
	AMY	6.58	129	772	6.0	69	9	12
	FIB	6.39	149	690	4.6	66	10	13
	SSF	6.59	118	755	6.5	60	7	9
Low-quality tropical hay								
+ ITM	CON	6.78	112	643	6.1	55	6	9
	AMY	6.81	129	703	7.2	65	6	10
	FIB	6.70	118	627	5.9	55	5	8
	SSF	6.59	118	640	5.9	61	8	12
+ OTM	CON	6.65	156	646	4.4	84	16	23
	AMY	6.76	131	661	5.2	81	13	17
	FIB	6.75	109	627	6.0	65	8	11
	SSF	6.67	104	652	6.5	57	6	8
SEM		0.082	15.2	50.7	0.49	10.9	2.8	3.4
Main effect of roughage								
High-quality hay		6.56	138	736	5.5	68	10	13
Low-quality hay		6.72	116	649	5.9	65	9	12
SEM		0.050	9.8	34.4	0.20	6.2	1.7	1.9
Main effect of trace mineral source								
ITM		6.65	128	691	5.6	66	9	12
OTM		6.63	126	695	5.7	68	10	13
SEM		0.050	9.8	34.4	0.20	6.2	1.7	1.9
Main effect of enzyme								
CON		6.67	131	692	5.5	69	10	14
AMY		6.69	125	719	5.9	71	10	13
FIB		6.60	129	674	5.4	65	9	12
SSF		6.60	123	687	5.8	62	8	11
SEM		0.056	10.8	35.2	0.27	7.1	1.9	2.1
P-value (2 × 2 × 4 factorial design)								
Roughage (R)		<0.001	<0.01 01	<0.001	0.11	0.47	0.26	0.63
Trace mineral (M)		0.59	0.83	0.84	0.63	0.64	0.51	0.83
Enzyme (E)		0.16	0.80	0.55	0.47	0.55	0.66	0.64
R × M		0.82	<0.01	0.64	<0.01	0.05	0.01	0.03
R × E		0.33	0.13	0.98	0.12	0.72	0.33	0.31
M × E		0.69	0.25	0.76	0.05	0.56	0.31	0.23
R × M × E		0.22	0.14	0.74	0.07	0.74	0.43	0.40

[a] ITM = inorganic trace minerals; OTM = organic trace minerals.
[b] CON = control, without enzyme; AMY = Amaize®, FIB = Fibrozyme®, and SSF = Allzyme® SSF.
[c] GP = total gas production at 48 h per g of dry matter incubated (DMi).
[d] IVDMD = *in vitro* dry matter digestibility.
[e] Partioning factor (g of dry matter digester per mL of gas).
[f] CH_4 proportion.
[g] CH_4 production per g of dry matter incubated (DMi).
[h] CH_4 yield per g of dry matter digested (DMd).

where Y_{ijk} = is every observation of the ith type of roughage (R_i) when incubated with the jth TM source (M_j) and the kth enzyme (E_l); μ is the general mean; R_i (i = 1–2) is the roughage effect; M_j is the TM source effect (j = 1–2); E_k is the enzyme effect (k = 1–4); βl = blocking factor for run (k = 1–3); $(R \times M)_{ij}$ is the interaction between roughage and TM source; $(R \times E)_{ik}$ is the interaction between roughage and enzyme; $(M \times E)_{jk}$ is the interaction between TM source and enzyme; $(R \times M \times E)_{ijk}$ is the interaction between roughage, TM source, and enzyme; U_{ijkl} = is a unit random effect for treatment within block; and ε_{ijkl} is experimental error. When interaction was observed, differences between the treatments were considered significant at $P<0.05$ using Tukey's procedure for multiple comparisons among means. The relationship between *in vitro* fermentation characteristics was evaluated by a simple correlation analysis using the PROC CORR procedure of the SAS statistical package (version 9.2, SAS Institute, Cary, NC, USA), however the data were not showed.

3. Results

3.1. Experiment 1 – Roughage-based diets (65:35 roughage: concentrate ratio)

There was no three-way interaction observed for the *in vitro* gas and methane production variables, or total VFA concentration and molar proportion (Tables 3 and 4, respectively). Interactions between roughage × TM source ($P \leq 0.05$) was verified on GP, PF, CH_4 proportion, production and yield (Fig. 1). Total GP (Fig. 1A) for HQH was lower with OTM (127 vs. 148 mL; P = 0.02), while for LQH it

Table 4
Effects of roughage, trace mineral source, exogenous enzymes and their combination on *in vitro* total volatile fatty acids (VFA) concentration and individual VFA proportions in Exp. 1 (65:35 roughage: concentrate ratio).

Diet[a]	Enzyme[b]	Total VFA (mM)	VFA profile[c] (mol/100 mol)						
			Ace (A)	Prop (P)	But	Isobut	Val	Isoval	A:P
High-quality tropical hay									
+ ITM	CON	133	63	23	9	1.2	1.4	2.2	2.8
	AMY	128	61	23	12	1.1	1.3	2.0	2.7
	FIB	148	60	23	13	1.2	1.4	2.0	2.7
	SSF	75	62	22	12	1.1	1.2	1.9	2.8
+ OTM	CON	72	60	24	12	1.1	1.4	1.8	2.9
	AMY	112	60	23	12	1.2	1.3	2.1	3.0
	FIB	95	61	23	12	1.0	1.3	2.0	3.0
	SSF	136	61	22	12	1.1	1.3	2.0	2.8
Low-quality tropical hay									
+ ITM	CON	163	64	22	12	1.0	1.3	2.0	2.9
	AMY	216	61	21	13	1.1	1.3	2.3	2.9
	FIB	103	63	24	13	1.1	1.3	2.1	2.7
	SSF	176	61	22	13	1.0	1.3	1.9	2.8
+ OTM	CON	161	58	23	14	1.0	1.5	2.7	2.7
	AMY	105	64	21	10	1.3	1.5	2.7	3.0
	FIB	131	61	21	13	1.3	1.3	2.2	3.0
	SSF	94	66	24	5	1.4	1.6	2.5	2.8
SEM		39.8	2.2	1.7	2.5	0.32	0.19	0.39	0.22
Main effect of roughage									
High-quality hay		114	61	23	12	1.1	1.3	2.0	2.7
Low-quality hay		144	62	22	11	1.2	1.4	2.3	2.9
SEM		22.6	1.1	1.1	1.0	0.1	0.1	0.3	0.2
Main effect of trace mineral source									
ITM		143	62	22	11	1.1	1.3	2.0	2.8
OTM		115	61	22	11	1.3	1.4	2.2	2.8
SEM		22.6	1.1	1.1	1.0	0.1	0.1	0.3	0.2
Main effect of enzyme									
CON		136	61	23	11	1.1	1.4	2.2	2.7
AMY		140	62	22	12	1.1	1.4	2.3	2.8
FIB		119	61	22	12	1.1	1.3	2.1	2.8
SSF		120	63	22	10	1.2	1.3	2.1	2.8
SEM		26.5	1.3	1.2	1.4	0.1	0.1	0.3	0.2
P-value (2 × 2 × 4 factorial design)									
Roughage (R)		0.11	0.14	0.36	0.32	0.41	0.34	0.08	0.06
Trace mineral (M)		0.14	0.61	0.99	0.99	0.09	0.32	0.20	0.91
Enzyme (E)		0.78	0.75	0.80	0.86	0.99	0.94	0.76	0.84
R × M		0.46	0.76	0.71	0.68	0.04	0.33	0.12	0.54
R × E		0.72	0.76	0.76	0.68	0.91	0.75	0.89	0.76
M × E		0.70	0.14	0.58	0.18	0.80	0.74	0.87	0.45
R × M × E		0.10	0.34	0.67	0.26	0.89	0.96	0.78	0.91

[a] ITM = inorganic trace minerals; OTM = organic trace minerals.
[b] CON = control, without enzyme; AMY = Amaize®, FIB = Fibrozyme®, and SSF = Allzyme® SSF.
[c] Ace = acetate; Prop = propionate; But = butyrate; Isobut = isobutyrate; Val = valerate; Isoval = isovalerate; A:P = acetate:propionate ratio.

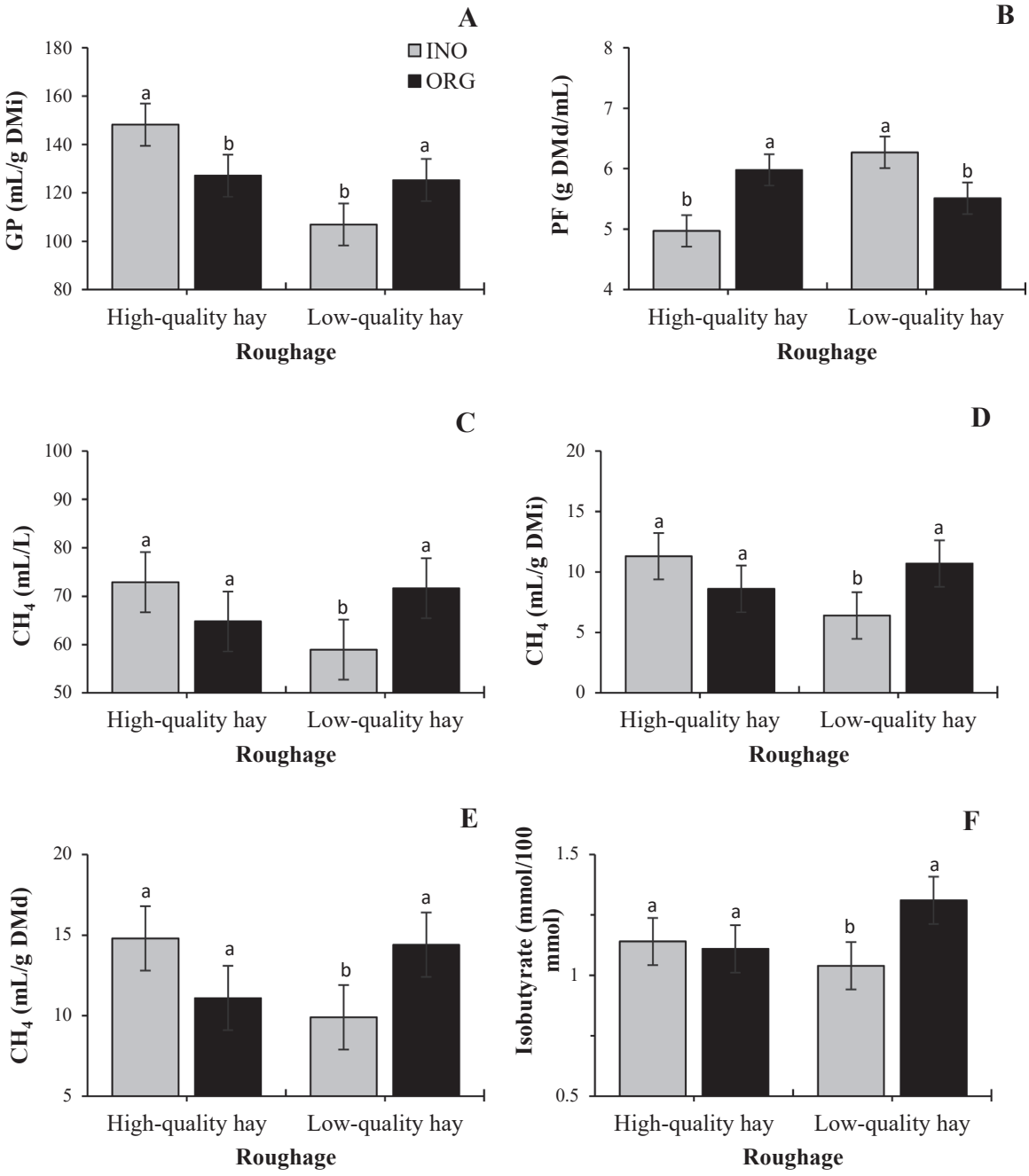

Fig. 1. Effects of roughage and trace mineral type interaction in Exp. 1 (65:35 roughage:concentrate ratio) on total gas production (GP; panel A; SEM = 8.7; $P < 0.01$), partioning factor (PF; panel B; SEM = 0.26; $P < 0.01$), CH_4 proportion in headspace gas (panel C; SEM = 6.7; $P = 0.05$); CH_4 production (panel D; SEM = 1.7; $P = 0.01$); CH_4 yield (panel E; SEM = 2.2; $P = 0.03$); and molar proportion of isobutyrate (panel F; SEM = 0.12; $P = 0.04$). Trace mineral type were: INO, inorganic, and ORG, organic. $^{a-b}$Means in bars with different superscripts differ within roughage source ($P < 0.05$).

was higher (107 vs. 125 mL, $P = 0.04$). In an opposite way contrast, the PF (Fig. 1B) of HQH was 20% greater ($P < 0.01$; 5.0 vs. 6.0 mg DMD/mL GP), while decreased for LQH it was 12% lower ($P = 0.05$; 6.3 vs. 5.5 mg DMd/mL GP) when OTM was added. The use of OTM resulted in a higher CH_4 production and yield (mL/g DMi or DMd, Figs. 1D and 1 E, respectively) when LQH was incubated (10.7 vs. 6.4 mL/g DMi, and 14.4 vs. 9.8 mL/g DMd, respectively; $P \leq 0.05$), but no differences were observed when HQH was used (averaging 10.0 mL/g DMi and 12.9 mL/g DMd; $P \geq 0.13$). Additionally, PF presented source of TM × enzyme interaction ($P = 0.05$; Fig. 2). When ITM was included, PF was higher with addition of AMY than SSF ($P = 0.04$; 6.3 vs 5.2 mg DMd/mL), with CON and FIB

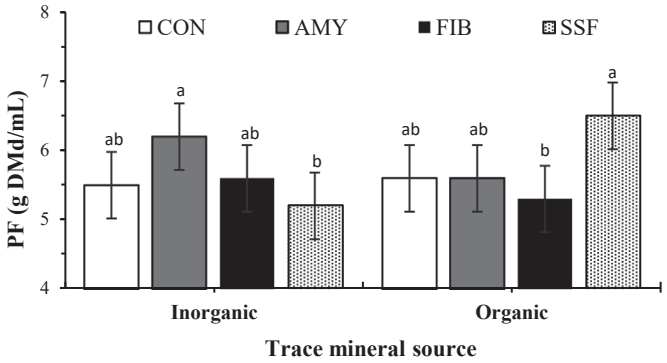

Fig. 2. Effects of trace mineral type and enzyme interaction in Exp. 1 (65:35 roughage:concentrate ratio) on partioning factor (PF; SEM = 0.49; P = 0.05). Exogenous enzymes were: CON, control, without exogenous enzymes; AMY, amylolytic enzyme (Amaize®); FIB, fibrolytic enzyme (Fibrozyme®); SSF, a multi-enzyme preparation (Allzyme® SSF). $^{a-b}$Means in bars with different superscripts differ within trace mineral source ($P < 0.05$).

not differing (5.5 mg/mLin average; $P > 0.46$). When OTM was supplemented, PF was higher with SSF compared with FIB ($P = 0.04$; 6.5 and 5.3 mg/mL, respectively), with CON and AMY intermediate ($P \geq 0.09$; averaging 5.6 mg DMd/mL). No interaction effects of roughage × enzyme or effect of enzyme source alone on *in vitro* gas or VFA production were observed (Tables 3 and 4). For roughage source, the pH was lower by 0.16 units ($P < 0.001$) during HQH incubation. (Table 3). As expected, incubation of HQH resulted in about 13% greater IVDMD ($P < 0.001$) than that of LQH. The pattern of total VFA production and proportions were similar between roughages, source of TM, and exogenous enzymes (Table 4), except for isobutyrate. There was a greater isobutyrate ($P = 0.04$) concentration after fermentation when OTM was included with the LQH compared to the ITM (1.3 vs. 1.0 ± 0.12 mol/mol; Fig. 1F). On the other hand, source of TM did not affect ($P = 0.77$) isobutyrate concentration with HQH (averaging, 1.1 mol/100 mol).

The pH was not correlated with any ruminal *in vitro* fermentation variables ($P > 0.07$; data not presented). On the other hand, GP was positively correlated with IVDMD, CH_4 proportion, CH_4 production, and CH_4 yield ($P < 0.001$; 0.48, 0.72, 0.92, and 0.89, respectively), but negatively to PF and A:P ratio ($P < 0.05$; –0.81 and –0.32, respectively). In addition, there were correlation ($P < 0.05$) between IVDMD and CH_4 proportion (0.48), CH_4 production (0.54), CH_4 yield (0.37), and A:P ratio (–0.32). The CH_4 proportion, production, and yield were also positively correlated ($P < 0.01$) with the molar proportion of isobutyric, valeric, and isovaleric (about 0.67, 0.64, and 0.60, respectively), as well as A:P ratio ($P < 0.05$; averaging 0.40). Neverthless, these CH_4 variables were negatively correlated with molar proportion of propionic acid ($P < 0.05$; about –0.38). Molar proportion of propionic acid was negatively correlated with butyric, isobutyric, and isovaleric ($P < 0.001$; about –0.56). Additionally, molar proportion of isobutyric was strongly correlated with valeric (0.88) and isovaleric acids (0.85), while valeric was also correlated to isovaleric acid (0.89).

3.2. *Experiment 2 – Concentrate-based diets (15:85 roughage: concentrate ratio)*

No three-way interaction ($P \geq 0.11$) of roughage × source of TM × exogenous enzymes was found for *in vitro* gas and methane production variables (Table 5). There were interactions between roughage and source of TM ($P < 0.001$) on GP, IVDMD, PF, CH_4 production, and yield (Table 5 and Fig. 3). The OTM addition compared to ITM resulted in greater ($P \leq 0.02$) total GP (141 vs. 175 mL/g), CH_4 proportion (67 vs. 61 mL/L), production (9 vs. 12 mL/g DMi), and yield (13 vs. 20 mL/g DMd) when SB was used, while these variables were lower when CS was incubated (74 vs. 66 mL/L; 15 vs. 12 mL/g DMi; 27 vs. 18 mL/g DMd for CH_4 proportion and yields, respectively; $P \leq 0.02$), except total GP (averaging 139 mL/g DM; $P = 0.17$). When compared to ITM inclusion, OTM addition with SB did not affect IVDMD (636 g/kg; $P = 0.25$), but PF was lower (5.0–3.9 mg/mL; $P < 0.01$), while IVDMD and PF were higher (582 vs. 680 g/kg and 3.4 vs. 4.7 mg/mL, respectively; $P < 0.02$) when CS was incubated. No interaction effects of roughage × enzyme, TM × enzyme nor effects of enzyme source or TM source on *in vitro* gas and CH_4 production were noted (Table 5). As expected, roughage source affected pH; with CS having significantly lower pH compared with the SB (6.69 vs. 6.81; $P < 0.001$).

There were no effects of treatments and interactions ($P \geq 0.08$) on total VFA, propionate, butyrate, valerate, and isovalerate (Table 6). Effects of enzyme on molar proportion of acetate was influenced by TM source (TM source × enzyme; $P = 0.03$; Table 6). The molar proportion of acetate was higher with OTM when SSF was added (54 mol/100 mol; Fig. 4A), but acetate was lowest with the combination of no exogenous enzymes and OTM (47 mol/100 mol), whereas AMY and FIB were intermediate between them (49 and 50 mol/100 mol, respectively). Exogenous enzyme affected molar proportion of isobutyric acid ($P = 0.03$). The addition of SSF resulted in lower isobutyric proportion than CON (1.26 vs. 1.59 mol/100 mol; $P = 0.03$), while AMY and FIB were not different (averaging 1.5 mol/100 mol; $P \geq 0.15$) (Table 6). There was an interaction effect ($P = 0.04$) between roughage type and source of TM on A:P ratio (Fig. 3F). The A:P ratio averaged 2.15 when SB was incubated with ITM or OTM ($P = 0.87$), but it was higher when OTM was added in CS substrates compared to ITM ($P < 0.01$; 2.3 vs. 2.1 respectively). In addition, it was observed TM source × enzyme interaction for A:P ratio ($P = 0.04$). Within the ITM treatments (Fig. 4B) while inclusion of AMY, FIB and SSF led to lower A:P ratio than the with no enzyme inclusion ($P < 0.01$; 2.13 vs. 2.42, respectively), but addition of OTM did not affected A:P ratio (average of 2.12).

The fluid pH was positively correlated ($P < 0.05$) with PF (0.63), acetate (0.48), isobutyric (0.30), and A:P ratio (0.40). On the other hand, pH was negatively correlated ($P < 0.001$) with the GP (–0.69), CH_4 proportion (–0.58), CH_4 production (–0.67), CH_4 yield

Table 5
Effects of roughage, trace mineral source, exogenous enzymes and their combination on *in vitro* gas and CH_4 production in Exp. 2 (15:85 roughage: concentrate ratio).

Diet[a]	Enzyme[b]	pH	GP[c] (mL/g DMi)	IVDMD[d] (g/kg)	PF[e] (mg DMD/mL)	CH_4[f] (mL/L)	CH_4[g] (mL/g DMi)	CH_4[h] (mL/g DMd)
Corn silage								
+ ITM	CON	6.59	176	588	3.8	77	14	26
	AMY	6.53	218	555	3.0	76	17	34
	FIB	6.75	205	564	3.0	71	15	29
	SSF	6.76	169	620	4.0	72	13	20
+ OTM	CON	6.65	191	674	4.0	69	14	21
	AMY	6.76	170	642	5.2	69	12	16
	FIB	6.75	151	705	6.0	60	15	14
	SSF	6.73	179	679	3.6	67	13	21
Sugarcane bagasse								
+ ITM	CON	6.95	112	681	6.3	60	7	10
	AMY	6.72	154	729	4.9	64	10	13
	FIB	6.81	163	688	4.6	63	11	15
	SSF	6.82	136	538	4.3	56	8	14
+ OTM	CON	6.82	158	620	4.3	66	11	18
	AMY	6.87	184	620	3.7	67	11	13
	FIB	6.80	167	579	3.7	66	13	15
	SSF	6.70	194	632	4.0	70	14	22
SEM		0.156	36.3	58.4	1.12	5.9	3.4	6.9
Main effect of roughage								
Corn silage		6.69	182	631	4.1	64	13	23
Sugarcane bagasse		6.81	158	636	4.4	70	10	17
SEM		0.130	31.3	27.2	0.99	5.0	2.9	5.9
Main effect of trace mineral source								
ITM		6.74	167	620	4.2	67	12	20
OTM		6.76	174	647	4.3	67	12	19
SEM		0.130	31.3	27.2	0.99	5.0	2.9	5.9
Main effect of enzyme								
CON		6.75	159	641	4.6	68	11	19
AMY		6.72	181	655	4.2	69	13	21
FIB		6.77	172	634	4.3	65	12	20
SSF		6.75	170	604	3.9	66	12	19
SEM		0.135	32.1	33.2	1.02	5.1	3.0	6.1
P-value (2 × 2 × 4 factorial design)								
Roughage (R)		0.01	0.02	0.86	0.20	0.001	<0.01	<0.01
Trace mineral (M)		0.71	0.47	0.35	0.83	0.78	0.82	0.58
Enzyme (E)		0.86	0.49	0.62	0.43	0.46	0.61	0.81
R × M		0.32	0.01	0.01	<0.001	<0.001	0.001	<0.001
R × E		0.24	0.50	0.79	0.22	0.32	0.36	0.55
M × E		0.20	0.10	0.96	0.09	0.31	0.13	0.29
R × M × E		0.91	0.85	0.11	0.14	0.85	0.88	0.40

[a] ITM = inorganic trace minerals; OTM = organic trace minerals.
[b] CON = control, without enzyme; AMY = Amaize®, FIB = Fibrozyme®, and SSF = Allzyme® SSF.
[c] GP = total gas production at 24 h per g of dry matter incubated (DMi).
[d] IVDMD = *in vitro* dry matter digestibility.
[e] Partioning factor (g of dry matter digester per mL of gas).
[f] CH_4 proportion.
[g] CH_4 production per g of dry matter incubated (DMi).
[h] CH_4 yield per g of dry matter digested (DMd).

(–0.58), butyric (–0.32), and isovaleric (–0.35). There was correlation ($P < 0.05$) between GP and PF (–0.87), CH_4 proportion (0.75), CH_4 production (0.97), CH_4 yield (0.87), propionic acid (0.33), and isobutyric acid (–0.46). In addition, PF was strongly correlated ($P < 0.001$) with CH_4 proportion (–0.65), CH_4 production (–0.82), CH_4 yield (–0.80), and A:P ratio (0.53).

4. Discussion

The *in vitro* technique is useful to evaluate the rumen fermentation effects of feedstuffs with different fermentabilities and a wide range of additives, however they have some limitations that must be considered. One of these limitations is the diet composition of donor animal, because it affects both microbial populations in the rumen and microbial activity of rumen inoculum (Mould et al., 2005a, b). With this considered, grazing beef cows were supplemented with a roughage:concentrate proportion close to what was established in the first study (i.e., 65:35), while in the second study we used donor animals from a study in our department, but presented similar fiber and starch concentrations from feedlot diets in Brazil (Silvestre and Millen, 2021). Nevertheless, although it is recommended to fed donor animals with a diet similar in composition to the substrate incubated in vitro (Yán-Ruiz et al., 2016), the

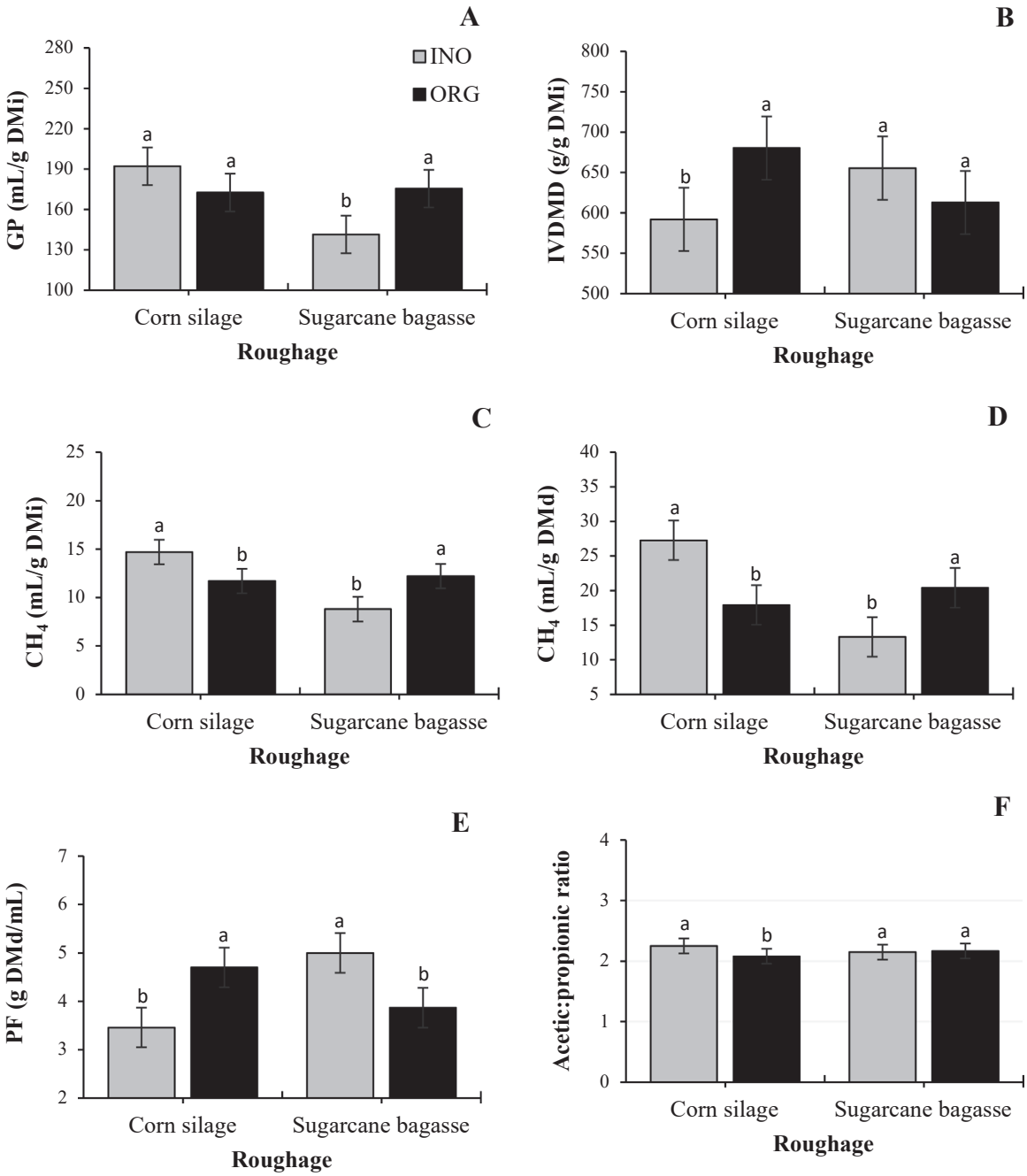

Fig. 3. Effects of roughage and mineral type interaction in Exp. 2 (15:85 roughage:concentrate ratio) on total gas production (GP; panel A; SEM = 13.9; P = 0.01), *in vitro* dry matter digestibility (panel B; SEM = 39.2; P = 0.01), CH_4 production (panel C; SEM = 1.3; P = 0.001); CH_4 yield (panel D; SEM = 2.8; P < 0.001); partioning factor (PF; panel E; SEM = 0.4; P < 0.001), and acetic:propionic ratio (panel F; SEM = 0.07; P = 0.04). Trace mineral type were: INO, inorganic, and ORG, organic. $^{a–b}$Means in bars with different superscripts differ within trace mineral source (P < 0.05).

limitations with the number of cannulated animals' impossibility to use a greater number of animals to receive all the treatments evaluated in this current study. Another limitation of *in vitro* studies is that it requires the use of much greater dosages of exogenous enzymes than those practicable *in vivo* (Calsamiglia et al., 2007). In that case, here the enzymes dosages used in this study were based on the company recommendations to supplement the animals and not to observe the effects on *in vitro* ruminal fermentation. Additionally, as exogenous enzymes could enhance the digestion in the rumen from their effects on the feed prior to consumption (McAllister et al., 2001), *in vitro* studies could not represent a real situation.

We hypothesized that roughage source determines the positive effects of exogenous enzymes supplementation in an *in vitro*

Table 6
Effects of roughage, trace mineral source, exogenous enzymes and their combination on *in vitro* total volatile fatty acids (VFA) concentration and individual VFA proportions in Exp. 2 (15:85 roughage: concentrate ratio).

Diet[a]	Enzyme[b]	Total VFA (mM)	VFA profile[c] (mol/100 mol)						
			Ace (A)	Prop (P)	But	Isobut	Val	Isoval	A: P
Corn silage									
+ ITM	CON	159	55	22	14	1.9	2.4	4.7	2.5
	AMY	132	49	22	22	1.4	2.0	4.2	2.2
	FIB	142	48	22	22	1.5	2.0	4.1	2.1
	SSF	146	50	23	21	1.1	1.8	3.7	2.1
+ OTM	CON	155	46	23	23	1.5	2.0	4.3	2.0
	AMY	142	49	23	21	1.5	2.0	4.0	2.1
	FIB	186	47	24	22	1.3	2.0	4.2	2.0
	SSF	101	53	25	14	1.3	2.2	4.1	2.2
Sugarcane bagasse									
+ ITM	CON	204	54	24	15	1.5	2.0	4.1	2.3
	AMY	140	48	23	21	1.3	1.9	3.8	2.1
	FIB	146	53	25	15	1.4	1.8	3.9	2.1
	SSF	91	48	23	21	1.3	2.0	3.8	2.1
+ OTM	CON	216	48	22	22	1.4	2.0	4.0	2.2
	AMY	127	49	24	20	1.6	1.9	3.8	2.1
	FIB	84	53	25	14	1.5	2.1	4.0	2.2
	SSF	123	55	25	13	1.3	2.1	3.9	2.2
SEM		40.7	2.8	1.9	4.4	0.20	0.12	0.39	0.14
Main effect of roughage									
Corn silage		145	50	23	20	1.4	2.0	4.1	2.2
Sugarcane bagasse		141	51	24	18	1.4	2.1	3.9	2.2
SEM		15	1.6	1.3	2.1	0.12	0.05	0.27	0.12
Main effect of trace mineral source									
ITM		144	51	23	19	1.4	2.0	4.0	2.2
OTM		142	50	24	19	1.4	2.1	4.0	2.1
SEM		15	1.6	1.3	2.1	0.12	0.05	0.27	0.12
Main effect of enzyme									
CON		186	51	23	18	1.6	2.1	4.3	2.3
AMY		136	49	23	21	1.4	2.0	3.9	2.1
FIB		131	51	24	17	1.4	2.0	4.0	2.1
SSF		120	51	24	19	1.2	2.0	3.8	2.2
SEM		23	1.9	1.4	2.6	0.13	0.07	0.29	0.13
P-value (2 × 2 × 4 factorial design)									
Roughage (R)		0.84	0.20	0.33	0.35	0.53	0.40	0.11	0.98
Trace mineral (M)		0.88	0.75	0.36	0.90	0.93	0.33	0.95	0.06
Enzyme (E)		0.16	0.48	0.53	0.62	0.03	0.46	0.28	0.16
R × M		0.83	0.51	0.49	0.83	0.38	0.54	0.81	0.04
R × E		0.41	0.45	0.74	0.60	0.42	0.66	0.84	0.62
M × E		0.99	0.03	0.78	0.11	0.22	0.08	0.71	0.04
R × M × E		0.48	0.99	0.97	0.99	0.49	0.32	0.90	0.89

SEM = standard error of mean.
[a] ITM = inorganic trace minerals; OTM = organic trace minerals.
[b] CON = control, without enzyme; AMY = Amaize®, FIB = Fibrozyme®, and SSF = Allzyme® SSF.
[c] Ace = acetate; Prop = propionate; But = butyrate; Isobut = isobutyrate; Val = valerate; Isoval = isovalerate.

fermentation system by organic TM inclusion. Regarding roughage-based diets (Exp. 1), our hypothesis was confirmed because there was mineral × enzyme interaction on PF, which is an indicator of microbial efficiency (Blümmel et al., 1997). However, other *in vitro* fermentation parameters were not altered. With concentrate-based diets (Exp. 2) our hypothesis was also confirmed, because molar proportion of acetate and acetate-to-propionate ratio presented interaction between TM source and enzyme, and tendencies between them were verified on GP, PF, and molar proportion of valerate.

4.1. *Effect of roughage*

It has long been recognized that animals fed low-quality forage generally have a lower DM digestibility than those fed high-quality roughage, resulting in a higher A:P. The lower GP, higher observed A:P, and CH_4 yield in Exp. 1 was possibly a reflection of the lower digestibility of the LQH. In fact, both variables are negatively correlated (–0.32). Although IVDMD and A:P ratio were not correlated to pH in Exp. 1, pH was decreased by the improvement on forage quality (6.72–6.56 when LQH were changed to HQH). On the other hand, pH was positively and negatively correlated to A:P ratio and GP in Exp. 2, respectively, reflecting changes of roughage source quality, although there was no correlation with IVDMD. According to Van Soest (1994), the optimal temperature and pH for assessing enzyme activity are closer to 39°C and 6.0–6.7, respectively. In this study, the range of pH ranges was slightly above the range suggested by Van Soest (1994). It must be emphasized that we considered the recommendations of Navarro-Villa et al. (2011)

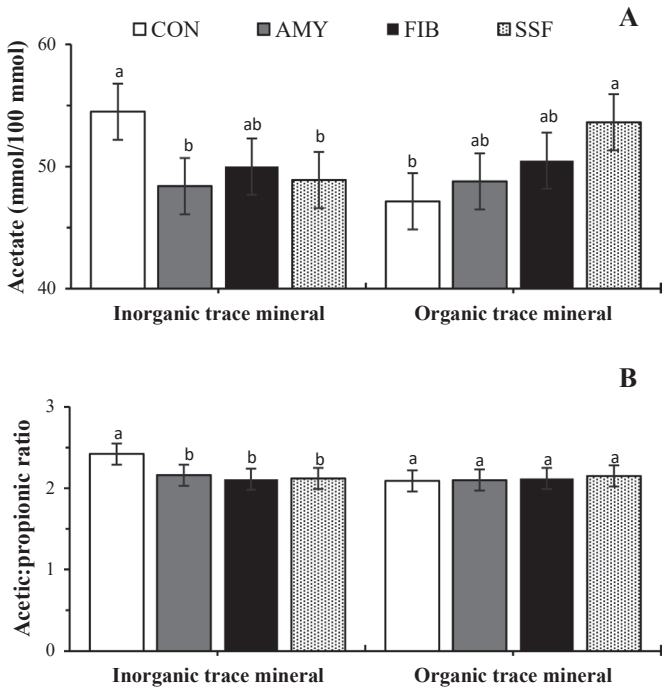

Fig. 4. Effects of trace mineral type and enzyme interaction in Exp. 2 (15:85 roughage:concentrate ratio) on molar proportion of acetate (panel A; SEM = 2.8; P = 0.04) and acetate:propionate ratio (panel B; SEM = 0.09; P = 0.04). Exogenous enzymes were: CON, control, without exogenous enzymes; AMY, amylolytic enzyme (Amaize®); FIB, fibrolytic enzyme (Fibrozyme®); SSF, a multi-enzyme preparation (Allzyme® SSF). [a–b]Means in bars with different superscripts differ within trace mineral source ($P < 0.05$).

regarding the amount of substrate and rumen fluid:buffer mixture (300 mg and 1:2 ratio, respectively) to promote incubation conditions which allowed pH to decline as observed in vivo assays.

4.2. *Roughage × mineral interaction*

In general, positive effects of OTM source were observed when high-quality roughages were incubated (Figs. 1 and 3). According to Blummel and Ørskov (1993), microbial fermentation of organic substrates produces gas as one of the end-products. For that reason, there is a strong correlation between IVDMD digestibility and GP. However, a strong correlation between IVDMD and GP was found only in Exp. 1. The absence of correlation between GP and IVDMD found in Exp. 2 could be due to the high-concentrate inclusion, minimizing the roughage constituents' effects which contributed little to the GP. In both experiments, GP was strong negatively correlated to PF. The OTM supplementation resulted in a higher PF, on average, 27% when high-quality roughage (i.e., HQH and CS in Exp. 1 and 2, respectively) was incubated. Contrary to this, when low-quality roughage (i.e., LQH and SB in Exp. 1 and 2, respectively) was evaluated, PF was lower by ~22%. We can infer with these results that a greater proportion of substrate were assimilated by microbial cells (Blümmel et al., 1997) and a lower proportion of substrate used for VFA and GP when OTM supplementation is associated to high-quality roughages, independent of the inclusion level (i.e., 65% and 15% in Exp. 1 and 2, respectively). It is explained because the fast-growing bacteria present in higher population in the in vitro bottles contained high-quality roughages, which increase the microbial TM requirement when they are degrading non-fibrous carbohydrates (Summers et al., 1957).

As pointed out by Summers et al. (1957), when high levels of starch are provided, ruminal digestion decreases due to TM deficiencies. This may have occurred when high-concentrate diets were incubated in Exp. 2, because fast-growing bacteria increase the microbial TM requirement when they are degrading starch resulting in deficiencies of available TM to be used by slow-growing bacteria that are cellulose digesters. Therefore, the greater microbial efficiency (i.e., PF) found when high-quality roughages were associated with OTM may be attributed to lower lignocellulosic- and starch-metal clusters and higher availability of TM in the organic source for microbial metabolic processes of the fast-growing bacteria, comparing to ITM. Indeed, as mentioned by Tiffany and Spears (2005) and Pino and Heinrichs (2016), amylolytic bacteria use OTM more efficiently and ferment OM more rapidly in the rumen. This is because organic minerals are more similar to the natural forms present in the body and from microorganisms (organic complexes rather than free inorganic ions), especially in reference to fast-growing bacteria strains that degrade non-fibrous compounds in HQH and CS incubation experiments found in Exp. 1 and 2, respectively.

Regarding the impaired ruminal *in vitro* fermentation where high-quality roughages were incubated with ITM (e.g., higher GP, lower PF, higher CH_4 yield), which has a higher solubility compared to OTM, this potentially must lead to increased formation of lignocellulosic- and/or starch-metal clusters, reducing the availability of minerals to the microorganisms (Moreira et al., 2013; Leonel

et al., 2021). Considering the CS used at the Exp. 1, Leonel et al. (2021) demonstrated that CS has a more selective chemical system for the fixation of Zn, compared to mature roughages. That study demonstrated an increase of the binding of cations, mainly Zn, could have reduced the TM availability. At the Exp. 2, high-concentrate diets were evaluated and starch concentrations increased. Leonel et al. (2021) observed that the electron donor sites of starch must be more accessible to the electrophilic attack by cations as Cu. At pH 6.4, which is closest to that observed when high-quality roughages were incubated at the Exp. 1 and 2 (averaging 6.6), these authors found that retention of the metallic cations in starch is higher when compared with cellulose, hemicellulose, and lignin. Furthermore, basic sites of starch can be associated with a higher structural flexibility, lower supramolecular compaction, and higher exposure of the respective sites, when compared with the lignocellulose. In this context, higher solubility TM from ITM probably increases the formation of starch-metal clusters and reduces the availability of TM to the fast-growing microorganisms.

The low-quality roughages used in this study were mainly composed of cellulose (about 510 and 435 g/kg to LQH and SB, respectively) and lignin (86 g/kg to LQH and 128 g/kg to SB), both of which have the capacity to bind metal cations (Moreira et al., 2013). From this perspective, it is possible to infer that the TM availability for microorganisms could be decreased when low-quality roughages were included in the substrates and consequently, affected ruminal *in vitro* fermentation and microbial populations. In this way, the interaction between fibrous feeds and metallic cations is constituted by strong chemical bonds, as well as weak chemical interactions such as van der Waals (Leonel et al., 2021). Although microorganisms can use both soluble and insoluble forms of mineral elements in the rumen for bacterial metabolism (Cao et al., 2000), ITM are often more soluble in the rumen than OTM sources (Genther and Hansen, 2015). In this sense, when ITM was added together to low-quality roughages the rate of complex-fiber formation probably increased, which may have reduced TM availability to slow metabolism of slow-growth microorganisms (i.e., fiber fermenters). Conversely, when OTM was added in the substrates, may have increase the bioavailability of TM for metabolic processes and reduction of the formation of mineral complexes (e.g., thiomolybdates) or associations with other dietary components that reduce availability for microbial metabolic functions (Durand and Kawashima, 1980). However, in this situation, probably may have a higher TM level for microbial metabolism from slow-growing bacteria which potentially led to depressed cellulose digestion, as verified by Hubbert et al. (1958). Results from the Exp. 1 (roughage-based diet) supported it, because PF (i.e., microbial efficiency) significantly decreased when OTM was added (Fig. 1).

The overall inverse correlation between PF and CH_4 yield (about –0.80) align with previously reported occurrences of *in vitro* rumen fermentation changes. As a higher propionate production tended to be associated with lower CH_4 production (Ellis et al., 2008), we found a negative correlation between CH_4 yield and propionate proportion in Exp. 1 (about 0.37; data not shown). Although treatment effects on propionate proportion were not observed, there was a positive correlation between A:P ratio and CH_4 production and yield. The correlation between pH and CH_4 production and yield were more pronounced when high-concentrate diet was evaluated (Exp. 2), because pH and acetate, and A:P ratio were positively correlated. Consequently, reduced CH_4 production and yield observed for CS incubated with ITM reflects both the reduction in extent of fermentation per g DM incubated and changes in the proportion of fermentation acids (Fig. 3), mainly on A:P ratio. When the A:P ratio is higher it generally indicates proportionally higher digestible NDF in the substrate. As a result, CH_4 production and yield were strongly correlated to PF, meaning a higher microbial efficiency to growth when CH_4 production and yield are lower.

4.3. *Trace mineral source × enzyme interaction*

When ITM source were added to the substrate, AMY presented the more pronounced effect than SSF on ruminal *in vitro* fermentation of low-concentrate diets, which is verified by the higher PF (Fig. 2). As fibrous compounds are the main substrate to fermentation, AMY addition probably supportedd *in vitro* fermentation of non-fibrous carbohydrates (i.e., starch) and enhanced microbial efficiency. Tricarico et al. (2008) explained this as a cross-feeding mechanism, which may improve the digestibility of nutrients not targeted by enzymes. As an example, exogenous enzymes hydrolyze complex carbohydrates into different products (malto-, cello-, and xylo-oligosaccharides), which supports growth of fibrolytic microorganisms. On the other hand, when OTM was included together with SSF, PF was enhanced (Fig. 2), demonstrating that a combination of exogenous enzymes could act indirectly to stimulate ruminal microbial activity through synergistic effects. The results in Exp. 2 also can be corroborated by this interpretation, because the inclusion of either FIB or SSF with OTM addition improved the molar proportion of acetate compared to the control.

The exogenous fibrolytic enzyme activity is dependent on cofactors, activators, and inhibitors such as other compounds or catalytic end products that inhibit further enzymatic hydrolysis. For instance, Cu-metalloenzymes are needed for maximal activity of cellulase-enhancing factors, which are an important group of polysaccharide monooxygenases (Quinlan et al., 2011). In this context, Romero et al., (2015a), b) evaluated the effect of adding exogenous fybrolitic enzymes (sourced from *Trichoderma reesei* and *Aspergillus oryzae*) with or without cofactors on preingestive hydrolysis of NDF and on NDF digestibility of bermudagrass haylage. Some TM that acts as cofactors (i.e., Mn^{2+}, Co^{2+}, Fe^{2+}) could improve the activity of fibrolytic enzymes on pure substrates, the cell-wall saccharification (i.e., water-soluble carbohydrate release) (Romero et al., 2015b). Morgavi et al. (2000) reported that enzymes from *Trichoderma longibrachiatum* promoted higher hydrolysis of soluble cellulose and xylan when ruminal fluid was extracted from high-concentrate diets than high-forage diets. This corroborates our finds regarding the higher PF observed with FIB and OTM in Exp. 2.

Additionally, protease contained in SSF could have played a significant role in fiber degradation of some forages. A possible mechanism by which proteases enhance fiber fermentation is through the attack of some of the cell wall nitrogen compounds that act as physical barriers to degradation (Colombatto et al., 2003). Nevertheless, as fibrolytic enzymes, protease efficacy also depends on the characteristics of the forage utilized (Colombatto et al., 2003; McGinn et al., 2004; Eun and Beauchemin, 2005). Therefore, the higher molar proportion of acetate observed with FIB and SSF with OTM addition could be due the higher IVDMD, due the higher fermentation of fiber, indicating that exogenous enzymes can work synergistically with enzymes from the rumen microbes, which increases

their hydrolytic potential within the rumen (Morgavi et al., 2000). As mentioned above, this process is defined as cross-feeding among rumen microorganisms (Russell, 1985).

5. Conclusions

Our results showed that *in vitro* incubation of low-quality roughages with ITM inclusion resulted in higher PF and lower GP, with a consistent reduction in the CH_4 yields. In a similar way, OTM associated with high-quality roughage improves PF and, when added in high-concentrate substrates, results in higher IVDMD and CH_4 mitigation. Thus, it seems that ITM supplementation should be prioritized in systems which use low-quality tropical roughages, while OTM in systems with high-quality roughages. The results also showed that TM source had little impacts on enzyme addition effects, but there were indications of benefits in certain substrates. It is suggested that further studies are needed to validate the results obtained herein, especially evaluating greater dosages of exogenous enzymes in combination with OTM in other experimental conditions.

CRediT authorship contribution statement

Rayane A. Lino: Writing – review & editing, Visualization. **Javier A. B. Garcia:** Investigation. **Eduardo H. B. K. Moraes:** Writing – review & editing. **Júlia Mara Campos de Souza:** Writing – review & editing. **Jurandy Gouveia Júnior:** Writing – original draft, Investigation. **Luiz F. Costa e Silva:** Writing – review & editing, Conceptualization. **Erick D Batista:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Thierry R. Tomich:** Writing – review & editing, Methodology.

Declaration of Competing Interest

The authors declare that there was no conflict of interest in carrying out this work.

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