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Evaluation of yeast-based additives, as an alternative to ionophores, on rumen fermentation of ruminant diets using an *in vitro* gas production system

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Introduction: The study aimed to assess yeast-based additives' effects, as monensin alternatives, on rumen fermentation parameters, greenhouse gas emissions, and ruminal kinetics of ruminant diets using an *in vitro* system. Three experiments were conducted, each individually evaluating escalating levels of three yeast-based additives.

Methods: Three experiments were designed: Experiment 1 evaluated prebiotic blend 1—yeast culture [*Saccharomyces cerevisiae* (Scer)], beta-glucans, fructooligosaccharides, galactooligosaccharides, and mannanoligosaccharides; Experiment 2 investigated prebiotic blend 2—beta-glucan fractions and mannanoligosaccharides from Scer; Experiment 3 examined yeast cells—hydrolyzed, inactivated, and spray-dried yeast (Scer) cells. Uniform experimental design and procedures were employed across the three experiments. Each experiment had six treatments: monensin (Rumensin®, 25 mg/kg DM) as positive control, and yeast additive levels (0, 533, 1,067, 1,600, and 2,133 mg/kg on DM basis) added to ruminant diets (60% corn silage and 40% concentrate). An *in vitro* gas production (GP) system with 50 AnkomRF bottles assessed total GP (at 24 and 48 hours), kinetics, fermentation profiles, methane (CH₄), and carbon dioxide (CO₂) concentrations. Comparison with monensin utilized Dunnett's test (5%). Yeast additive levels were analyzed for linear and quadratic responses.

Results: In Experiment 1, the 1,600 mg/kg yeast additive had lower concentrations of propionate, isobutyrate, valerate, and branched-chain volatile fatty acids (BCVFAs), and a higher acetate concentration and acetate-

to-propionate ratio than monensin. In Experiment 2, the 1,600 mg/kg yeast additive led to lower total VFA and isovalerate concentrations than monensin. Additionally, compared to the 1,067 mg/kg yeast additive, monensin showed lower isovalerate concentration and higher NH₃-N concentration. In Experiment 3, the 533 mg/kg yeast additive resulted in lower valerate and BCVFA concentrations, and higher CH₄ and CO₂ concentrations than monensin. Monensin had lower total VFA, butyrate, and acetate-to-propionate ratio, and higher propionate concentration compared to the 2,133 mg/kg yeast additive.

Discussion: Collectively, these findings suggest yeast-based additives could be monensin alternatives, enhancing animal nutrient utilization efficiency and contributing to improved livestock sustainability.

KEYWORDS

greenhouse gases, methane, monensin, prebiotics, ruminant nutrition

Introduction

Nutrition plays an essential role in the productivity and sustainability of beef and dairy production systems (Montes et al., 2013; Honan et al., 2021). The utilization of feed additives is one of the most widely used nutritional strategies in ruminant nutrition to enhance productivity (Azzaz et al., 2015). These additives modify the ruminal fermentation process to improve feed fermentation, increase host energy supply, reduce enteric methane (CH₄) production, and, consequently, enhance animal feed utilization (Jia et al., 2018; Vyas et al., 2018). In this regard, the ionophore monensin has been used for decades in ruminant nutrition and its effects to improve animal feed efficiency is well documented in the literature (Tedeschi et al., 2003; Azzaz et al., 2015). Monensin improves rumen fermentation efficiency by increasing propionate production at the expense of acetate, resulting in a decrease of substrates [i.e., hydrogen (H₂) and carbon dioxide (CO₂)] for CH₄ production (Rook, 2005; Benedeti et al., 2018). Furthermore, the use of monensin also inhibits the growth of lactic acid-producing bacteria, thus decreasing the acidosis risk (Ipharraguerre and Clark, 2003). However, there is a concern that the use of antimicrobials in animal feed might leave residues in animal products, which in turn leads to some bacterial strains becoming resistant to said antimicrobials (Vikram et al., 2017). Thus, due to government regulations and public concern, since January 2006, the European Union has suspended the use of antibiotic ionophores in animal feed. Therefore, there is an ongoing search for alternative additives with similar efficacy to ionophores, such as yeast-based products.

Yeast-based prebiotics are used with the aim of increasing the rumen's beneficial bacterial population (Chaucheyras-Durand et al., 2012). In addition, these additives can potentially improve rumen fermentation and animal performance by improving feed

degradation and nutrient use by microorganisms without threatening human health (Jia et al., 2018). Furthermore, yeasts do not play an antibiotic role, but provide favorable conditions for the better development of specific bacterial groups. Moreover, yeasts act as oxygen sinks, which favor strictly anaerobic bacteria, such as cellulolytic bacteria (McGinn et al., 2004). In addition, yeast cell walls are composed of polysaccharides, which can interact with bacteria, binding to them and preventing the attachment of harmful microorganisms to the animal gastrointestinal tract (Kogan and Kocher, 2007; Herrera, 2012). Therefore, these natural additives have the potential to be a more secure replacement for monensin when used to improve ruminal fermentation. Indeed, research has demonstrated that yeast-based additives can enhance feed digestibility and nutrient utilization in beef cattle (Batista et al., 2022).

Natural bioactive molecules, such as oligosaccharides and beta-glucans, have also demonstrated the potential to be used in animal nutrition due to their antioxidant, immunostimulant, antibacterial, and metabolic regulatory activities (Krüger and van der Werf, 2018; Li et al., 2018). Thus, these molecules could be used as monensin alternatives to improve ruminal fermentation patterns. Indeed, researchers observed that beta-glucans can improve fiber digestibility and increase volatile fatty acid (VFA) production by modulating rumen fermentation (Grove et al., 2006). However, the additives used in combination with these molecules, with and without yeast cultures, have not been evaluated and compared with monensin; therefore, the ideal dosages of these additives are not well established in ruminant nutrition. Thus, this study aimed to evaluate the effects of yeast-based additives, as alternatives to monensin, on rumen fermentation parameters and ruminal kinetics of ruminant diets using *in vitro* systems. Our hypothesis was that yeast-based additives would enhance nutrient utilization and could potentially serve as a suitable alternative to monensin, acting as beneficial modulators of ruminal fermentation.

Materials and methods

Experimental designs and chemical analysis

We conducted three experiments, aiming that each would serve as an evaluation of three yeast-based additives. The experiments were as follows: experiment 1, prebiotic blend 1—yeast culture [*Saccharomyces cerevisiae* (*Scer*)], beta-glucans, fructooligosaccharides, galactooligosaccharides, and mannanoligosaccharides (Golf[®]; Yessinergy LTDA, Campinas, SP, Brazil). This blend consisted of a mixture of a minimum of 210 g/kg of glucomannans, 150 g/kg of beta-glucans, 120 g/kg of fructooligosaccharides, 72 g/kg of galactooligosaccharides, and 60 g/kg of mannanoligosaccharides. Experiment 2, prebiotic blend 2—beta-glucan fractions and mannanoligosaccharides from *Scer* (GlucanMos[®]; Yessinergy LTDA). This blend consisted of a mixture of a minimum of 440 g/kg of glucomannans, 240 g/kg of beta-glucans, and 140 g/kg of mannanoligosaccharides. Experiment 3, yeast cells—hydrolyzed, inactivated, and spray-dried yeast (*Scer*) cells (BioHydro[®]; Yessinergy LTDA). The experimental design and procedures were identical across the three experiments. Thus, six treatments were evaluated within each experiment, with a monensin-based additive being used as the positive control [Rumensin[®], 25 mg/kg dry matter (DM)] and 0, 533, 1,067, 1,600, and 2,133 mg/kg (DM basis) of yeast additive being included in the finishing beef diets. The experimental diets consisted of a composition of 60% corn silage and 40% concentrate (DM basis), formulated to fulfill the recommended nutrient requirements for beef cattle (Valadares Filho et al., 2016). The ingredients and chemical composition of the basal diet are presented in Table 1.

For all experiments, a 25-bottle automated *in vitro* gas production (GP) system (ANKOM Technology, Macedon, NY, USA) was utilized. The system was equipped with wireless pressure sensors, which were connected to a computer to evaluate the ruminal fermentation pattern of the tested ingredients. The treatments were evaluated through three 48-hour fermentation incubations to assess the *in vitro* GP profiles and ruminal fermentation parameters. Within each incubation, the treatments

were individually incubated in 250-mL bottles, which were randomly arranged in the incubator. Therefore, each fermentation batch consisted of three replicates for each treatment, along with three blanks (consisting only of rumen/mineral/buffer solution), resulting in a total of 63 observations.

All ingredients utilized in these studies were finely ground using a 1-mm screen (Wiley mill; Thomson Scientific Inc.) to ensure consistency in all incubations and analyses. Subsequently, the samples underwent analysis for dry matter (method G-003/1), ash (method M-001/1), crude protein (method N-001/1), and ether extraction (EE; method G-005/1), following the procedures outlined by Detmann et al. (2012). The organic matter (OM) content was calculated as the difference between the DM and ash contents. For the neutral detergent fiber (NDF) analysis, the samples were treated with thermostable alpha-amylase, omitting sodium sulfite, in accordance with the method described by Van Soest et al. (1991), and adapted for use with the Ankom²⁰⁰ Fiber Analyzer (ANKOM Technology).

Ruminal fluid collection and buffer solution preparation

The rumen fluid was collected from three Nellore steers with rumen cannulas (with an average body weight of 640 kg) for this study. The steers were maintained on a total mixed diet comprising 60% corn silage and 40% concentrate, which consisted of ground corn grain, citrus pulp pellet, soybean meal, and a mineral mixture. The rumen fluid samples were collected 2 hours after feeding, with each animal contributing 2,000 mL. The collected rumen fluid was promptly filtered through four layers of cheesecloth, transferred into prewarmed (39°C) thermal bottles, and immediately transported to the laboratory (Yáñez-Ruiz et al., 2016).

The buffer mineral solutions for all experiments were prepared following the method outlined by Menke and Steingass (1988). The buffer solution was maintained at a constant temperature of 39°C in a water bath and continuously purged with nitrogen gas (N₂) for 30 minutes. Resazurin was employed as a color indicator to monitor the pH and oxidation–reduction potential, ensuring proper saturation of the buffer with N₂. The rumen fluid was mixed with the buffer solution in a volumetric ratio of 1:2 (rumen fluid: buffer) under anaerobic conditions, accomplished by flushing with N₂ within a water bath maintained at 39°C.

In vitro gas production

For the kinetics of gas production, VFA, and ammonia-nitrogen (NH₃-N) measurements, the bottle valves of the ANKOM^{RF} gas production system were adjusted to allow venting. Each 250-mL bottle was filled with 0.5 g of the respective diet, and deionized water was added to hydrate the samples and prevent particle dispersion. Subsequently, the bottles were inoculated with 75 mL of rumen/buffer solution, while ensuring continuous flushing of the headspace

TABLE 1 Ingredients and chemical composition of the basal diet.

Item ¹	Proportion
Ingredients (g/kg)	
Corn silage	600
Dry ground corn	280
Soybean meal	120
Chemical composition	
Dry matter (g/kg)	915
Organic matter (g/kg DM)	946
Crude protein (g/kg DM)	170
Ether extract (g/kg DM)	12.8
Neutral detergent fiber (g/kg DM)	164

DM, dry matter.

with N₂. Once inoculated, the bottles were sealed and placed in an air-ventilated shaker incubator (EI-450T; ENGCO, Piracicaba, SP, Brazil) operating at a controlled temperature of 39°C and at an agitation speed of 83 rpm. A data acquisition software (gas pressure monitor; ANKOM Technology) was configured to record the cumulative pressure every 5 minutes, with data recorded at 60-minute intervals for a duration of 48 hours. The valves were programmed to automatically release gas when the pressure reached 3.4 kPa, as described by Tagliapietra et al. (2011).

The cumulative gas pressures at 24 hours and 48 hours were converted into milliliters (mL) using the conversion method described by Tagliapietra et al. (2011), as shown below:

$$GP(\text{mL}) = \left(\frac{P_c}{P_o} \right) \times V_o, \quad (1)$$

where P_c represents the cumulative pressure change (kPa) in the headspace of the bottle; V_o denotes the volume of the bottle's headspace (545 mL); and P_o represents the atmospheric pressure recorded by the equipment at the commencement of the measurement.

The final GP volumes in the bottles were adjusted to account for the contribution of the inoculum by subtracting the final GP value obtained from the blank bottles. To obtain the total GP over time, the cumulative pressure values were adjusted using the dual-pool model proposed by Schofield et al. (1994):

$$V_t = \left[\frac{V_1}{1 + e^{2+4 \times \{K_1 \times (L - \text{Time})\}}} \right], \quad (2)$$

where V_t represents the gas volume produced up to the specific time (mL); V_1 and V_2 denote the maximum gas volumes achieved from the complete digestion of each pool (mL); K_1 and K_2 represent the specific rates of digestion for each pool (h^{-1}); and L signifies the lag time (h). The metabolizable energy (ME) was calculated following the method described by Menke and Steingass (1988), with the lipid content disregarded (Grings et al., 2005). The ME calculation is as follows:

$$ME(\text{MJ}/\text{kg DM}) = 2.20 + (0.1357 \times GP_{200}) + (0.0057 \times GP), \quad (3)$$

where GP_{200} (mL/200 mg of DM incubated) represents the gas production measured at 48 hours. The pH of the solution was measured using an AccumetTM AP61 pH meter (Fisher Scientific, Atlanta, GA) at the initiation and conclusion of each 48-hour incubation period.

The *in vitro* OM digestibility (IVOMD) was determined using the method described by Menke and Steingass (1988), which is expressed as follows:

$$IVOMD(\text{g}/\text{kg DM}) = 31.55 + 0.8343 \times GP_{200}, \quad (4)$$

where GP_{200} represents the net gas production (mL/200 mg DM) measured at 24 hours and 48 hours. At the end of the fermentation batch for each experiment, the bottles were removed from the incubator and were cooled in an ice bath for 15 minutes, to stop the fermentation process. The pH of each bottle was measured (portable pH meter, model TEC-7; Tecnal, Ourinhos, SP, Brazil) and the content of the bottle was filtered through two layers of

gauze. The solid content (residue) was weighed and dried at 60°C in an oven with mechanical air circulation and then weighed to determine the NDF. Subsamples of 10 mL from each bottle's filtered content were added in Falcon tubes (15 mL) containing 0.2 mL of a 50% (v/v; volume to volume) sulfuric acid (H_2SO_4) solution, and centrifugated (4°C, 15 minutes, 1,000 g; centrifuge Avanti JXN-26, Beckman Coulter, Nyon, Switzerland). The supernatant was transferred to Eppendorf Tubes® (2 mL), which were centrifugated (4°C, 30 minutes, 20,000 g; Sorvall ST 8R, Thermo Fisher Scientific, Germany) for the determination of $\text{NH}_3\text{-N}$ and VFA concentrations.

Enteric carbon dioxide and methane production

For enteric CO_2 and CH_4 data, the Ankom^{RF} system bottle valves were set to be closed. All other procedures and designs were the same as those used in the total GP essay. After the inoculation process, the bottles were sealed and placed in an air-ventilated shaker incubator set at a temperature of 39°C. On completion of each fermentation batch, which lasted for 48 hours, the production of CO_2 and CH_4 from the headspace was quantified using a gas chromatograph (Nexis GC-2030; Shimadzu) equipped with a GS-CarbonPLOT column (Agilent Technologies, Santa Clara, CA, USA). The carrier gas used was helium (999.9 mL/L). The enteric CH_4 and CO_2 production from the bottles was adjusted for the contribution of the inoculum by subtracting the final gas production obtained from the blank bottles. The pH of the solution was measured at the beginning and end of each 48-hour incubation using an Accumet AP61 pH meter.

Statistical analysis

In this study, we carried out two statistical comparisons: in the first, we conducted a quantitative analysis (regression) of yeast additive levels (0, 533, 1,067, 1,600, and 2,133 mg/kg) to assess the potential linear and quadratic behaviors resulting from increasing these additives; and, in the second, we conducted a qualitative analysis, comparing each additive level against monensin (positive control) to examine the effects of yeast additives relative to the ionophore.

All collected results underwent tests for residual normality and homogeneity of variance (Davis and Stephens, 1989). Data from all experiments were collected and analyzed using a randomized block design, employing mixed models that accounted for additives as fixed factors and incubation as a random factor. The average values obtained from the bottles within each incubation served as the experimental units. The non-linear (NLIN) procedure of SAS 9.4 software was utilized to estimate the fermentation rate and gas pool size. Subsequently, the parameters of the non-linear functions were compared through linear regression analysis, with differences considered statistically significant at a level of $p \leq 0.05$ and trending when $0.05 < p \leq 0.10$. All other variables were analyzed

using the PROC MIXED function in SAS (SAS Institute, Cary, 2013). Analysis of variance was conducted, and in cases of significance, the Dunnett's test (at 5% significance level) was employed to compare the yeast additives with the positive control (monensin). The levels of the yeast additives were examined for linear and quadratic responses using the following model:

$$Y_{ijk} = B_0 + B_1X_i + B_2X_i^2 + P_j + A_k + e_{ijk}, \quad (5)$$

where Y_{ijk} represents the response variable obtained from the i th level of the yeast additive in the diet of the j th incubation and the k th experimental unit. The indices i , j , and k denote the levels of inclusion of the yeast additive, the random factor of incubation, and the random factor of bottle, respectively. The regression parameters of the model are denoted as B_0 , B_1 , and B_2 . The X_i represents the effect of the i th level of the fixed quantitative factor (inclusion of the yeast additive), P_j represents the effect of the level of the random factor incubation, A_k represents the effect of the level of the random factor bottle, and e^{ijkl} represents the residual error, assumed to follow a normal distribution ($0, s^2$).

Results

Experiment 1: prebiotic blend 1

The effects of additive inclusion and monensin on ruminal fermentation variables in GP systems are presented in Table 2 and Figure 1. In comparing the yeast additive with monensin, we found that there was no difference in the production levels of total GP (at 24 hours and 48 hours), ME, IVOMD, CH_4 , and CO_2 , and in the final pH. In addition, the concentrations of total VFAs and butyrate did not differ among treatments ($p > 0.10$). The 1,600 mg/kg (DM basis) of yeast additive included had lower concentrations of propionate ($p = 0.02$), isobutyrate ($p = 0.05$), valerate ($p = 0.01$), and BCFVAs ($p = 0.05$) and a higher acetate concentration ($p = 0.05$), acetate-to-propionate ratio ($p = 0.02$) than monensin. Monensin also had a higher acetate ($p = 0.02$) and lower isovalerate concentration ($p = 0.02$) than the treatment without the additives. The 1,067 mg/kg of yeast included also tended to have a lower NH_3 -N concentration than monensin ($p = 0.07$). Monensin diets had a lower lag time and degradation rate of the second pool than all levels of the yeast additive ($p < 0.05$).

Regarding levels regression, ME (trend, $p = 0.06$), CH_4 production ($p = 0.05$), acetate ($p < 0.01$), propionate ($p = 0.01$), isovalerate ($p = 0.01$), isobutyrate ($p < 0.01$), valerate ($p < 0.01$), and BCFVA ($p < 0.01$) concentrations, and the acetate-to-propionate ratio ($p = 0.02$) responded quadratically. The 1,600 mg/kg (DM basis) of yeast additive included had the lowest concentrations of propionate, isovalerate, isobutyrate, valerate, and BCFVAs and the highest acetate concentration and acetate-to-propionate ratio. In addition, the 1,067 mg/kg (DM basis) of yeast additive included had the lowest CH_4 concentration and ME. The remaining variables were not affected by additive inclusion ($p > 0.05$).

Experiment 2: prebiotic blend 2

The effects of additive inclusion and monensin on ruminal fermentation variables in GP systems are presented in Table 3 and Figure 2. In comparing the yeast additive with monensin, we found that the latter had lower total VFA (trend, $p = 0.08$), isovalerate ($p = 0.03$), and maximum gas volume of the first pool (trend, $p = 0.08$) concentrations than the 1,600 mg/kg (DM basis) of the yeast additive included. In addition, monensin had a lower isovalerate concentration ($p = 0.04$) and higher NH_3 -N ($p < 0.01$) and maximum gas volume of the second pool (trend, $p = 0.08$) concentrations than the 1,067 mg/kg (DM basis) of yeast additive included. The 2,133 mg/kg (DM basis) of yeast additive included had a lower NH_3 -N concentration (trend, $p = 0.06$) and lag time ($p = 0.03$), and higher concentrations of butyrate ($p = 0.06$), isovalerate ($p = 0.08$), and maximum gas volume of the first pool ($p = 0.04$) than monensin. Monensin had higher degradation rates in the first and second pools than all levels of the yeast additive ($p < 0.05$). Finally, the treatment without additives had higher concentrations of butyrate ($p = 0.01$), isovalerate ($p < 0.01$), isobutyrate ($p = 0.04$), valerate ($p = 0.04$), and BCFVAs ($p = 0.05$) and a lower acetate concentration ($p < 0.01$) than monensin. There was no difference in the remaining variables among the treatments ($p > 0.10$).

For levels of regression, the final pH ($p = 0.01$), CH_4 (trend, $p = 0.06$), acetate ($p < 0.01$), butyrate ($p < 0.01$) isovalerate (trend, $p = 0.09$), and NH_3 -N (trend, $p = 0.07$) concentrations responded quadratically. The 1,067 mg/kg (DM basis) of yeast additive included had the lowest final pH, CH_4 , butyrate, and NH_3 -N concentrations. In addition, the 533 mg/kg (DM basis) of yeast additive included had the lowest isovalerate concentration and highest acetate concentration. The maximum gas volume of the first pool linearly increased ($p = 0.04$), and the degradation rate of the second pool linearly decreased ($p = 0.02$) with the inclusion of the yeast additives. The remaining variables were not affected by additive inclusion ($p > 0.05$).

Experiment 3: prebiotic blend 3

The effects of additive inclusion and monensin on ruminal fermentation variables in GP systems are presented in Table 4 and Figure 3. In comparing the yeast additive with monensin, we found that there was no difference in the total GP (at 24 hours and 48 hours), ME, IVOMD, and NH_3 -N concentrations among the treatments ($p > 0.10$). The 533 mg/kg (DM basis) of yeast additive included had lower concentrations of valerate ($p = 0.02$) and BCFVAs ($p < 0.05$), and higher CH_4 ($p = 0.03$) and CO_2 ($p < 0.01$) concentrations than monensin. The 1,067 mg/kg (DM basis) of yeast additive included had a lower propionate concentration (trend, $p = 0.09$) and a higher CO_2 ($p < 0.01$) concentration and acetate-to-propionate ratio (trend, $p = 0.07$) than monensin. In addition, monensin tended to have lower CO_2 production than the 1,600 mg/kg (DM basis) of yeast additive included ($p = 0.09$).

TABLE 2 Effects of yeast additive (prebiotic blend 1¹) inclusion levels and monensin on ruminal fermentation variables in the gas production systems (experiment 1).

Item ²	Additive inclusion (mg/kg DM)					Monensin ³	SEM	Levels <i>p</i> -values	
	0	533	1,067	1,600	2,133			Linear	Quadratic
Total gas production (mL/g DM)									
24 hours	142	126	127	145	144	130	12.8	0.42	0.22
48 hours	172	154	153	176	175	158	14.0	0.39	0.18
CH ₄ (mM/g OM)	10.8	9.00	8.34	8.35	11.2	10.4	1.39	0.99	0.05
CO ₂ (mM/g OM)	27.4	39.7	31.9	31.4	34.4	22.7	4.50	0.63	0.44
ME ⁴ (MJ/kg DM)	7.81	7.02	6.90	7.63	7.78	7.27	0.47	0.59	0.06
IVOMD (g/kg)	601	570	568	607	607	575	24.8	0.37	0.16
pH	6.73	6.71	6.70	6.70	6.71	6.73	0.04	0.25	0.12
Total VFAs (mM/g DM)	158	161	148	149	155	159	8.88	0.43	0.43
VFA profile (mol/100mol)									
Acetate	62.9 ^M	64.7	65.0	65.2 ^M	64.6	64.2	0.55	< 0.01	< 0.01
Propionate	19.7	18.7	18.6	17.7 ^M	19.7	19.4	0.49	0.47	0.01
Butyrate	10.4	10.2	10.1	9.94	10.1	10.0	0.49	0.27	0.40
Isovalerate	1.66 ^M	1.58	1.56	1.44	1.60	1.53	0.06	0.04	0.01
Isobutyrate	1.74	1.61	1.59	1.48 ^M	1.71	1.62	0.06	0.24	< 0.01
Valerate	3.53	3.23	3.17	2.94 ^M	3.42	3.32	0.10	0.13	< 0.01
BCVFAs (mM)	5.27	4.84	4.76	4.42 ^M	5.13	4.94	0.16	0.16	< 0.01
Acetate: propionate ratio	3.20	3.46	3.51	3.88 ^M	3.26	3.31	0.17	0.28	0.02
NH ₃ -N (mg/dL)	20.3	19.5	18.8 ^m	21.3	21.1	21.0	0.83	0.18	0.15

The means with a superscript "M" are different from those for monensin at a *p*-value ≤ 0.05 . The means with a superscript "m" indicate a trend of significant difference from monensin at $0.05 < p \leq 0.10$.

¹Yeast culture (*Saccharomyces cerevisiae*), beta-glucans, fructooligosaccharides, galactooligosaccharides, and mannanoligosaccharides (Golf[®]; Yessenergy LTDA, Campinas, SP, Brazil). This blend consisted of a mixture of at least 210 g/kg of glucomannans, 150 g/kg of beta-glucans, 120 g/kg of fructooligosaccharides, 72 g/kg of galactooligosaccharides, and 60 g/kg of mannanoligosaccharides.

²DM, dry matter; ME, metabolizable energy; IVOMD, in vitro organic matter digestibility; VFAs, volatile fatty acids; BCVFAs, branched-chain volatile fatty acids; SEM, standard error of the mean.

³Monensin inclusion, 25 mg/kg DM.

⁴ME (MJ/kg DM) = $2.20 + (0.1357 \times GP_{200}) + (0.0057 \times CP)$, where GP_{200} is mL/200 mg of DM incubated.

Monensin also had lower total VFA (trend, $p = 0.10$), butyrate (trend, $p = 0.08$), and isovalerate (trend, $p = 0.07$) concentrations, in addition to a higher acetate-to-propionate ratio ($p = 0.01$), and propionate concentration ($p < 0.01$) than the 2,133 mg/kg (DM basis) of yeast additive included. Monensin also had a higher acetate concentration ($p < 0.01$) and acetate-to-propionate ratio ($p = 0.01$), and lower butyrate ($p = 0.01$), isovalerate ($p < 0.01$), isobutyrate ($p < 0.01$), BCVFA ($p = 0.04$), and valerate ($p = 0.04$) concentrations than the treatment without additives. Finally, all the yeast additive treatments had a higher maximum gas volume in the first pool and lower lag time, degradation rate, and maximum gas volume in the second pool than monensin ($p < 0.05$).

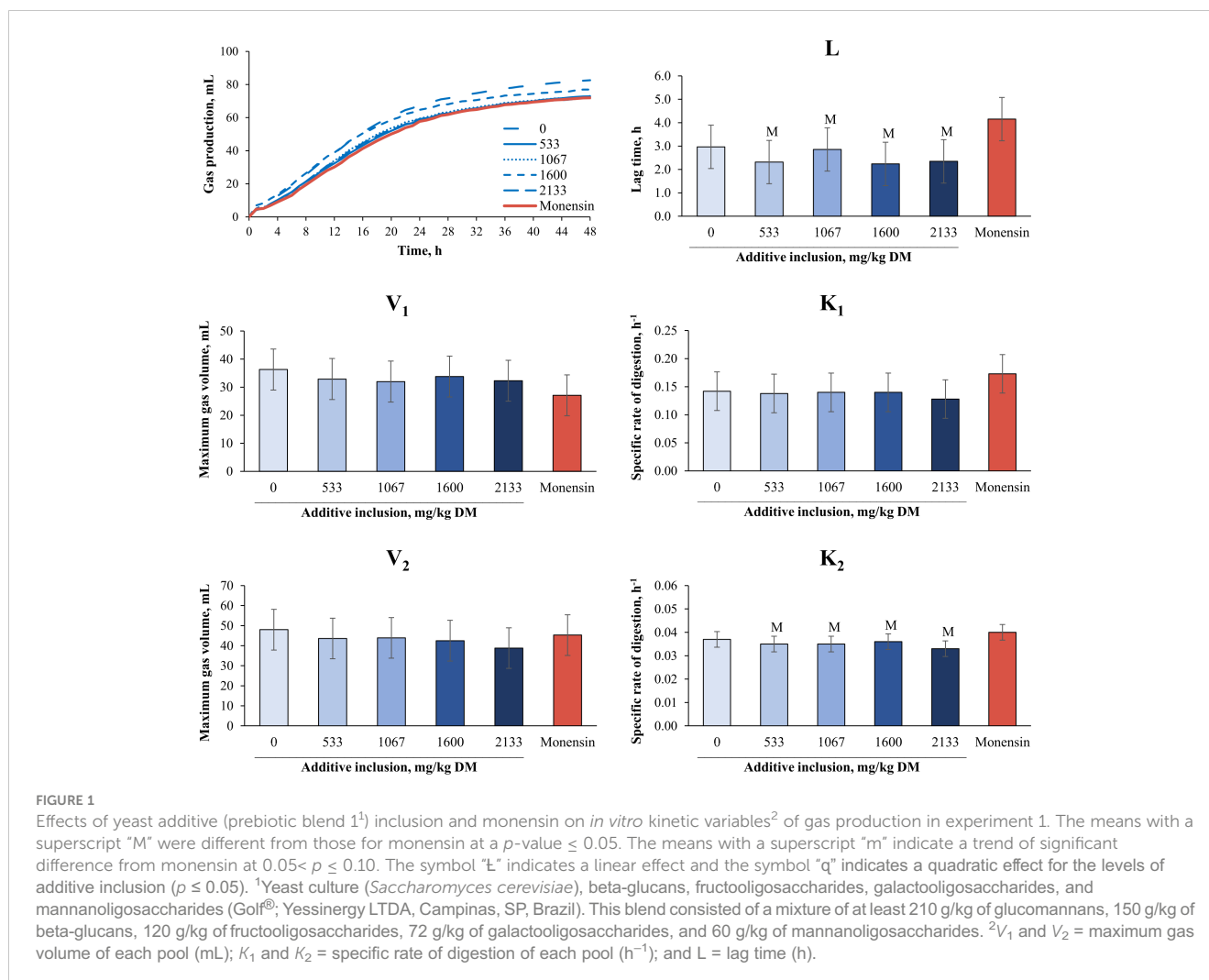
Regarding additive levels, the inclusion of the yeast additive linearly decreased the propionate concentration ($p < 0.01$) and linearly increased the acetate-to-propionate ratio ($p < 0.01$). In addition, the CH₄ (trend, $p = 0.06$), CO₂ ($p < 0.01$), acetate ($p < 0.01$), butyrate ($p = 0.05$), isovalerate ($p < 0.01$), isobutyrate ($p < 0.01$), valerate ($p < 0.01$), BCVFA ($p < 0.01$), and NH₃-N ($p = 0.05$)

concentrations responded quadratically. The 533 mg/kg (DM basis) of yeast additive included had the lowest butyrate, isovalerate, isobutyrate, valerate, and BCVFA concentrations, and the highest CH₄, CO₂, and acetate concentrations. The remaining variables were not affected by additive inclusion ($p > 0.05$).

Discussion

Experiment 1: prebiotic blend 1

Monensin is a common additive used in ruminant nutrition, known for its documented efficacy in enhancing rumen fermentation and feed efficiency (Tedeschi et al., 2003; Azzaz et al., 2015). However, there is a concern about the potential effects of antibiotic ionophores on the resistance of certain pathogenic bacteria (Vikram et al., 2017). On the other hand, the yeast additives might be applied to improve feed degradability,



consequently leading to greater nutrient absorption (Broadway et al., 2015). Moreover, the additives used in this study are based on a mixture of prebiotics intended to increase the populations of beneficial bacteria that carry out feed degradation without causing risks to animal health (Monnerat et al., 2013). In addition, prebiotics can also reduce the levels of pathogens, such as *Escherichia coli*, through competitive exclusion, as both have an affinity for the same activation sites (Cross et al., 2002; Krehbiel et al., 2003). Furthermore, the composition of yeast walls promotes rumen health through the stimulation of lactic acid-utilizing bacteria growth, which creates an environment with improved pH conditions and immunomodulatory effects (Garcia et al., 2017). Therefore, we expected this prebiotic blend to have similar effects to monensin on feed degradability and the rumen fermentation patterns. Indeed, the total GP and VFA, ME, and IVOMD concentrations did not differ among treatments in the first trial. However, our results indicate that the prebiotic (at 1,600 mg/kg DM) promoted VFA profile changes (compared with monensin), with an increase in acetate concentration in exchange for propionate, valerate, and BCVFAs. The different modes of action of the additives could explain these alterations. On the one hand, monensin acts by selecting microorganisms that lead rumen

fermentation to succinate and propionate (Schären et al., 2017). On the other hand, prebiotic yeast provides a ruminal environment with better conditions for anaerobic bacteria by reducing levels of O_2 (Vohra et al., 2016). Anaerobic bacteria are responsible for fiber degradation, leading to higher levels of acetate production (Gharechahi and Salekdeh, 2018). Furthermore, the yeast additive used in this study is a blend rich in amino acids, which some rumen bacteria might use, leading to BCVFA production (Reynolds and Kristensen, 2008). It has also been reported that BCVFAs are related to the increase of fibrolytic bacteria (*Ruminococcus albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, and *Butyrivibrio fibrisolvens*), enzyme activity (carboxymethyl cellulose, cellobiase, and xylanase), and NDF degradation (Wang et al., 2020). These findings are in line with the higher acetate-to-propionate ratio observed in our study. Furthermore, these results suggest that it would be interesting to test these yeast additives in high-forage diets (especially at 1,600 mg/kg DM) and evaluate ruminal NDF fermentation in future studies.

The VFA profile changes are closely correlated with enteric greenhouse gas production (Ku-Vera et al., 2020). The propionate formation pathways in the rumen result in a modification of the overall electron balance, serving as a hydrogen sink and

TABLE 3 Effects of yeast additive (prebiotic blend 2¹) inclusion and monensin on ruminal fermentation variables in the gas production systems (experiment 2).

Item ²	Additive inclusion (mg/kg DM)					Monensin ³	SEM	Levels <i>p</i> -values	
	0	533	1,067	1,600	2,133			Linear	Quadratic
Total gas production (mL/g DM)									
24 hours	134	118	123	126	115	115	15.9	0.34	0.85
48 hours	163	147	151	151	141	143	18.0	0.21	0.85
CH ₄ (mM/g OM)	10.8	10.5	9.30	10.0	11.6	10.4	1.02	0.66	0.06
CO ₂ (mM/g OM)	28.4	33.3	29.8	30.8	30.7	28.3	2.61	0.78	0.52
ME ⁴ (MJ/kg DM)	7.54	7.09	6.94	7.04	6.68	6.79	0.61	0.13	0.77
IVOMD (g/kg)	58.7	55.8	55.6	56.1	53.9	54.9	3.24	0.14	0.78
pH	6.77	6.77	6.68	6.70	6.72	6.71	0.06	< 0.01	0.01
Total VFAs (mM/g DM)	153	147	149	160 ^m	143	145	14.5	0.74	0.56
VFA profile (mol/100mol)									
Acetate	63.0 ^M	65.0	64.5	64.8	64.2	64.7	0.57	0.11	< 0.01
Propionate	19.6	19.2	19.4	19.0	19.3	19.4	0.31	0.27	0.41
Butyrate	10.6 ^M	9.8	9.70	9.75	10.4 ^m	9.8	0.59	0.47	< 0.01
Isovalerate	1.64 ^M	1.54	1.56 ^M	1.56 ^M	1.55 ^m	1.48	0.03	0.06	0.09
Isobutyrate	1.70 ^M	1.66	1.61	1.63	1.57	1.59	0.06	0.02	0.85
Valerate	3.43 ^M	3.09	3.17	3.28	3.19	3.15	0.14	0.33	0.14
BCVFAs (mM)	5.13 ^M	4.75	4.78	4.90	4.66	4.74	0.19	0.07	0.48
Acetate: propionate ratio	3.22	3.31	3.33	3.41	3.34	3.35	0.08	0.10	0.29
NH ₃ -N (mg/dL)	19.4	17.4	15.3 ^M	17.1	16.7 ^m	19.4	1.29	0.07	0.07

The means with a superscript "M" are different from those for monensin at a *p*-value ≤ 0.05 . The means with a superscript "m" indicate a trend of significant difference from monensin at $0.05 < p \leq 0.10$.

¹Beta-glucan fractions and mannanoligosaccharides from *Saccharomyces cerevisiae* (GlucanMos[®]; Yessenergy LTDA, Campinas, SP, Brazil). This blend consisted of a mixture of at least 440 g/kg of glucomannans, 240 g/kg of beta-glucans, and 140 g/kg of mannanoligosaccharides.

²DM, dry matter; ME, metabolizable energy; IVOMD, in vitro organic matter digestibility; VFAs, volatile fatty acids; BCVFAs, branched-chain volatile fatty acids; SEM, standard error of the mean.

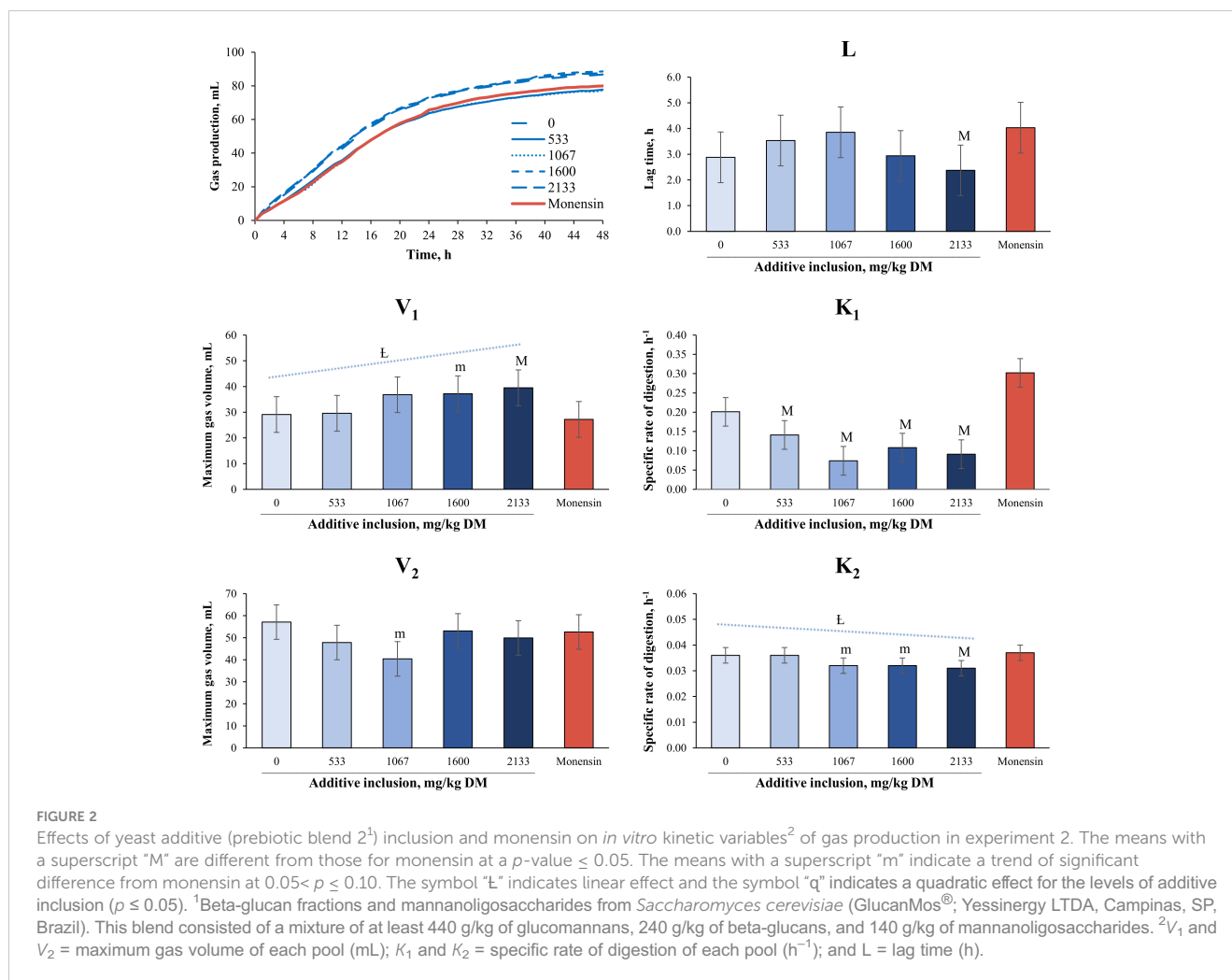
³Monensin inclusion, 25 mg/kg DM.

⁴ME (MJ/kg DM) = $2.20 + (0.1357 \times GP_{200}) + (0.0057 \times CP)$, where GP_{200} is mL/200 mg of DM incubated.

subsequently reducing the availability of hydrogen (Pereira et al., 2022). Furthermore, CO₂ is not produced in any phase of the propionate production pathways (Benedeti et al., 2018). However, the pathways to acetate formation release two CO₂ molecules and eight hydrogens from each hexose (Benedeti et al., 2018). These are substrates for CH₄ production (Pereira et al., 2022). Controversially, the quadratic effects suggested that the yeast additive at levels of 1,067 and 1,600 mg/kg (DM basis) had the highest acetate concentration and the lowest CH₄ production. Greenhouse enteric gas emissions are undesirable from both environmental and productive perspectives. The lost carbons in CH₄ and CO₂ forms could be used for carbohydrate, lipid, or amino acid building (Tiago et al., 2016; Li et al., 2019). As stated by Elghandour et al. (2014), the presence of yeast additives may induce a competition for co-metabolizing H₂ between acetogenic and methanogenic microorganisms. Previous studies also observed CH₄ reduction when the yeast additives were evaluated in rumen fermentation (Polyorach et al., 2014; Pedraza-Hernández et al., 2019). Thus, the

CH₄ reduction in our study could be an indication of better nutrient utilization.

We hypothesized that diets with yeast additives included could have similar rumen kinetics to those with monensin included. Interestingly, we observed that the prebiotic reduced the required time to start fermentation. Others have also observed a reduction in the DM lag time when yeast based on *S. cerevisiae* were tested in both *in vivo* and *in vitro* trials (Williams et al., 1991; Pedraza-Hernández et al., 2019). According to the aforementioned authors, the utilization of *S. cerevisiae*-based additives has the potential to enhance the initial rate of fiber degradation in the rumen. Thus, this effect can be attributed to the presence of macromolecules within the yeast that stimulate the growth and activity of fibrolytic microorganisms. However, contradicting our hypothesis, monensin promoted a higher degradation rate of the second pool, which might be related to the fermentation of slower degraded components, such as fiber. Nevertheless, this higher degradation rate of the second pool for monensin should be the reason for the



similar total GP observed for all treatments at the end of the fermentation baths.

Likewise, in the BCVFA results, part of ruminal $\text{NH}_3\text{-N}$ comes from protein degradation (Refat et al., 2015). Furthermore, some rumen fibrolytic microorganisms can build their amino acids from $\text{NH}_3\text{-N}$ and carbon skeletons (Reynolds and Kristensen, 2008). Hence, we speculate that the reduced $\text{NH}_3\text{-N}$ levels observed in the yeast additive group, at a dose of 1,067 mg/kg (DM basis), indicate that there is a greater conversion of $\text{NH}_3\text{-N}$ into microbial protein when compared with the other doses. Moreover, lower concentrations of $\text{NH}_3\text{-N}$ and higher acetate concentrations (as observed here for yeast) are also indicators of increased fibrolytic bacterial growth. However, we did not evaluate this parameter in our study. Nevertheless, despite the differences in the VFA profile and $\text{NH}_3\text{-N}$ concentration between the monensin and prebiotic blends, these additives promoted similar OM digestibility, ME values, total GP, and concentrations of VFAs. Moreover, the yeast additive also showed the potential to decrease CH_4 enteric production and increase fiber fermentation, which is highly desirable from an environmental perspective.

Experiment 2: prebiotic blend 2

The findings of this second trial also confirmed our hypothesis that yeast and monensin have the same total GP, ME, and IVOMD. Moreover, in this experiment we observed that the yeast additive at 1,600 mg/kg (DM basis) had a higher total VFA concentration than monensin. To understand these results, we point out that this additive originates from *S. cerevisiae* strains, which have characteristics of controlling pH and removing O_2 , which maximizes anaerobic bacterial growth (Bach et al., 2007; Broadway et al., 2015). In regard to the VFA profile, only the concentrations for butyrate (at 2,133 mg/kg DM) and isobutyrate (at 1,067, 1,600, and 2,133 mg/kg DM) were higher for the yeast additive than monensin. Butyrate (at 2,133 mg/kg DM) and isobutyrate (at 1,067, 1,600, and 2,133 mg/kg DM) concentrations were higher for the yeast additive than monensin. However, butyrate, isobutyrate, isovalerate, and BCVFA concentrations were reduced, whereas the acetate concentration and acetate-to-propionate ratio increased when the yeast additive was added to the diet. These results are in line with those from experiment 1 and thus

TABLE 4 Effects of yeast additive (yeast cells¹) inclusion and monensin on ruminal fermentation variables in the gas production systems (experiment 3).

Item ²	Additive inclusion, mg/kg DM					Monensin ³	SEM	Levels <i>p</i> -values	
	0	533	1067	1600	2133			Linear	Quadratic
Total gas production (mL/g DM)									
24 hours	145	139	126	143	132	129	8.93	0.46	0.56
48 hours	174	164	152	169	156	156	9.81	0.37	0.54
CH ₄ (mM/g OM)	10.8	14.6 ^M	11.7	12.1	9.60	10.1	1.60	0.23	0.06
CO ₂ (mM/g OM)	27.9	41.9 ^M	39.7 ^M	34.3 ^m	32.0	26.1	5.40	0.95	< 0.01
ME ⁴ (MJ/kg DM)	7.88	7.84	7.33	7.86	7.54	7.18	0.32	0.57	0.70
IVOMD (g/kg)	60.5	58.9	56.9	59.8	57.7	57.0	1.94	0.42	0.58
pH	6.74	6.69	6.71	6.69	6.75	6.69	0.04	0.73	0.09
Total VFAs (mM/g DM)	167	167	172	171	183 ^m	163	10.6	0.18	0.54
VFA profile (mol/100mol)									
Acetate	63.3 ^M	65.3	65.0	64.7	64.8	64.7	0.60	< 0.01	< 0.01
Propionate	19.3	18.8	18.6 ^m	18.7	18.2 ^M	19.0	0.20	< 0.01	0.49
Butyrate	10.4 ^M	9.70	10.1	10.1	10.3 ^m	9.8	0.42	0.85	0.05
Isovalerate	1.65 ^M	1.51	1.54	1.55	1.56 ^m	1.50	0.02	0.12	< 0.01
Isobutyrate	1.76 ^M	1.59	1.63	1.66	1.68	1.68	1.64	0.36	< 0.01
Valerate	3.58 ^M	3.13 ^M	3.24	3.33	3.39	3.36	0.09	0.49	< 0.01
BCVFAs (mM)	5.33 ^M	4.72 ^M	4.88	4.99	5.08	5.00	0.13	0.45	< 0.01
Acetate: propionate ratio	3.29 ^M	3.48	3.51 ^m	3.48	3.56 ^M	3.41	0.14	< 0.01	0.06
NH ₃ -N (mg/dL)	21.0	22.9	22.4	22.8	20.8	21.3	1.13	0.88	0.05

The means with a superscript "M" are different from those for monensin at a *p*-value ≤ 0.05 . The means with a superscript "m" indicate a trend of significant difference from monensin at $0.05 < p \leq 0.10$.

¹Hydrolyzed, inactivated, and spray-dried yeast (*Saccharomyces cerevisiae*) cells (BioHydro[®]; Yessinergy LTDA, Campinas, SP, Brazil).

²DM, dry matter; ME, metabolizable energy; IVOMD, in vitro organic matter digestibility; VFAs, volatile fatty acids; BCVFAs, branched-chain volatile fatty acids; SEM, standard error of the mean.

³Monensin inclusion, 25 mg/kg DM.

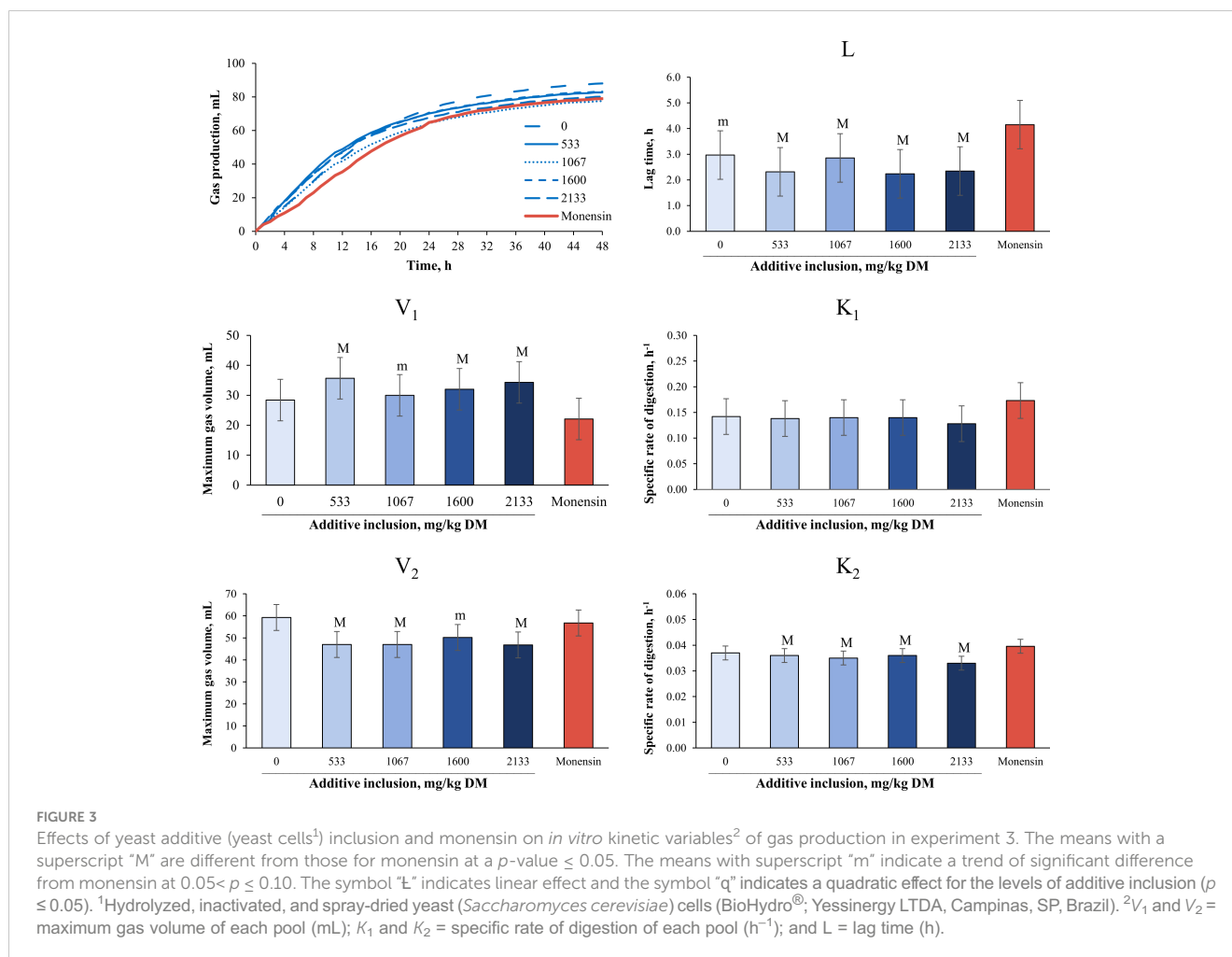
⁴ME (MJ/kg DM) = $2.20 + (0.1357 \times GP_{200}) + (0.0057 \times CP)$, where GP_{200} is mL/200 mg of DM incubated.

suggest that the yeast additives might lead to greater fibrolytic bacteria proliferation and reduced decarboxylation of branched-chain amino acids (isoleucine, leucine, and valine), which in turn indicates that the addition of the additive at intermediate doses led to a lower degradation of these amino acids (Refat et al., 2015). Furthermore, it is important to emphasize that the tested additive provided an additional source of BCVFAs. Therefore, rumen bacteria did not need to degrade branched-chain amino acids for the formation of BCVFAs. As a result, a larger portion of these compounds could potentially be absorbed in the small intestine, leading to an increase in protein synthesis.

Regarding greenhouse gas production, we observed a trend of quadratic effect for CH₄, with the minimum values reaching 1,067 and 1,600 mg/kg (DM basis). The ruminal CH₄ production can be reduced in different ways: (1) by decreasing H₂ availability (dos Santos et al., 2023); (2) by increasing H₂-consuming pathways, such as the production of compounds that act as H₂ sinks; or (3) by reducing the population of methanogenic *Archaea*. Although we did not observe propionate changes, our results suggest that the

decrease in CH₄ concentration was due to the better efficiency of microbial mass, which decreased the available H₂ for CH₄ production (Mombach et al., 2021).

Changes in fermentation pattern affected the rumen kinetics of yeast-additive diets compared with monensin. Thus, it seems that this prebiotic promoted a faster fermentation initiation that increased the total GP of the first pool (at 1,600 and 2,133 mg/kg DM), even with a lower degradation rate. Moreover, the first pool total fermentation increased with increasing levels of additive inclusion. On the other hand, monensin seems to induce a higher degradation rate of the second pool, which might have been the reason for its second pool maximum gas volume being higher than that of the treatment with 1,067 mg/kg (DM basis) of yeast additive. An interesting advantage of conducting trials that evaluate gas production (kinetics and total volume) together with fermentation parameters (such as VFA, IVOMD, and greenhouse gases) is that it is possible to follow the feed carbon destination (Nelson et al., 2009). This chemical element might be used to form essential nutrients (carbohydrates, lipids, and protein) to provide microbial



energy reserves or structures (Tiago et al., 2016; Li et al., 2019). In the worst scenario, it can be released as a greenhouse gas (CO_2 and CH_4), which is undesirable from both environmental and production perspectives (McAllister et al., 2011). Therefore, the higher total VFA concentration and maximum gas production of the first pool (faster lag time), the reduction of NH_3 -N and CH_4 for the yeast additives, and also the non-differences in total GP (at 24 hours and 48 hours) and fast lag time might suggest that rumen microorganisms more efficiently used carbon from these diets for their growth.

Experiment 3: prebiotic blend 3

The results of experiment 3 are indicative of the same effects as the previous trial, where the yeast additive promoted similar total GP, ME, and OM, and also higher total VFA (at 2,133 mg/kg DM) concentrations than monensin. Moreover, here we also observed changes in the VFA profile, with a higher concentration of butyrate at the expense of propionate and BCVFAs for yeast treatments, which also increased the acetate-to-propionate ratio. We also observed similar results for ruminal kinetics, finding that there was a lower lag time, degradation rate, maximum gas volume of the

second pool, and higher maximum gas volume produced in the first pool for the yeast additive diets than for monensin. Thus, we could see the same fermentation pattern changes for all tested additives. However, we observed contradicting results for CH_4 and CO_2 production in this trial, where the yeast additives produced a greater amount of these gases than monensin. Curiously, these effects were more prominent for the lower levels, especially at 533 mg/kg (DM basis) of additive inclusion. However, these differences diminished with increasing levels of inclusion until the highest level (2,133 mg/kg DM), where greenhouse gas emissions were equivalent to those observed with monensin supplementation.

Therefore, based on our findings, we can draw the conclusion that yeast-based additives might be used as ruminal fermentation modulators to improve nutrient usage. In summary, our results have indicated promising treatment options for each additive examined. Regarding prebiotic blend 1, we highlight the inclusion of 1,600 mg/kg (DM basis). For prebiotic blend 2, 1,600 g of additive included for each kg of DM was the most promising treatment. Concerning yeast cells, the better treatment was the inclusion of 2,133 mg/kg (DM basis). However, in spite of the results found in this study, there is still a gap in the body of knowledge regarding the use of yeast-based products as feed additives to improve ruminal fermentation efficiency in the diets of ruminants. Therefore, future studies evaluating these

additives on rumen fermentation parameters and animal performance are needed. Based on the results presented, we concluded that yeast-based additives possess the potential to modulate ruminal fermentation. This modulation is characterized by an increase in the total concentration of VFAs and a reduction in the concentrations of $\text{NH}_3\text{-N}$ and BCVFAs, and in CH_4 production. Thus, these additives have the potential to be used as alternatives to monensin to improve nutrient utilization from feeds. In addition, the greenhouse gas results observed in this study might also indicate that yeast-based products could potentially be used to improve livestock sustainability. However, we did not observe any effects of additive inclusion on the total gas production, ME, and IVOMD in this study. Therefore, further studies are warranted to validate the findings obtained in this study and assess the effects of these additives on *in vivo* ruminal fermentation parameters, animal performance, and economic feasibility.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Use Ethics Committee of the Instituto de Zootecnia under protocol n° 249-19. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ARC: methodology, investigation, data curation, writing (original draft), and visualization. EM: methodology, investigation, data curation, writing (review and editing), and visualization. FR: methodology, investigation, writing (review and editing), and visualization. KL: methodology, investigation, writing (review and editing), and visualization. ACC: methodology, investigation, writing (review and editing), and visualization. BA: methodology, investigation, writing (review and editing), and visualization. JS: conceptualization, resources, writing (review and editing), visualization, and funding acquisition. VS: conceptualization, resources, writing (review and editing), visualization, and funding acquisition. MM: methodology, validation, formal analysis, data curation, writing (review and editing), and visualization. EP: conceptualization, methodology, validation,

formal analysis, resources, data curation, writing (original draft), visualization, supervision, project administration, and funding acquisition. PB: conceptualization, methodology, validation, formal analysis, data curation, writing (original draft), visualization, supervision, and funding acquisition. RB: conceptualization, validation, resources, writing (review and editing), visualization, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version

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Conflict of interest

Authors JS and VS were employed by the company Yessinergy LTDA.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declare that this study received funding from Yessinergy LTDA. The funder had the following involvement in the study: resources, writing (review and editing), visualization, and funding acquisition.

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