DOI: 10.5433/1679-0359.2025v46n3p965

# Evaluation of natural additives on in vitro gas production kinetics and digestibility of Xaraés grass combined or not with dry fiber and solubles

# Avaliação de aditivos naturais na cinética de produção de gases in vitro e digestibilidade do capim-Xaraés combinado ou não com fibra seca e solúveis

Victória Curvo Ormond[1]; Leticia de Assis Calmon Cerisara[2]; Edjane Pereira da Silva[1]; Rafaela Juliana Jardim Cunha[1]; Tayane Barbosa Pereira[1]; Joanis Tilemahos Zervoudakis[3]; Luciano da Silva Cabral[3]; Mozart Alves Fonseca[4]; Ricardo Pereira Manzano[5]; Nelcino Francisco de Paula[3]*

## Highlights

No significant interaction was observed between substrates and additives.
Natural additives showing no difference from the control.
Monensin and Flavomycin reduced dry matter and fiber digestibility.
Flavomycin increased gas production.
DFS improved both dry matter and fiber digestibility compared to forage.
Monensin altered the rumen environment by reducing pH and ammonia nitrogen levels.

## Abstract

This study aimed to assess the impacts of various additives on the kinetic parameters of gas production and *in vitro* dry matter digestibility (IVDMD) and *in vitro* neutral detergent fiber digestibility (IVNDFD) in substrates with high fiber content. The additives evaluated were: control (CON) - without additives; Monensin (MON) - 20 mg/kg DM; Flavomycin (FLAVO) - 4 mg/kg DM; Live yeasts (LY) - *Saccharomyces cerevisiae* - 0.5 g/kg DM; Yeast culture (YC1) - 1.3 g/kg DM; Yeast culture (YC2) - 1.3 g/kg DM; and

[1] Students in the Post-Graduate Program in Animal Science, Universidade Federal de Mato Grosso, UFMT, Cuiabá, MT, Brazil. E-mail: victoriaormond2@gmail.com; edjanep.zoo@gmail.com; jardimrafs@gmail.com; tayanep17@gmail.com
[2] Student in the Post-Graduate Program in Tropical Agriculture UFMT, Cuiabá, MT, Brazil. E-mail: leticiacerisara@gmail.com
[3] Profs. Drs., Post-graduate Program in Animal Science, UFMT, Cuiabá, MT, Brazil. E-mail: joanis.zervoudakis@ufmt.br; lucianoufmt@gmail.com; nelcino.paula@ufmt.br
[4] Prof., Beef Cattle Nutrition & Helth, Departament of Animal and Range Sciences, Clayton, NM, USA. E-mail: mozart@nmsu.edu
[5] Consultant, Catanduva, SP, Brazil. E-mail: rpmanzan@yahoo.com.br
* Author for correspondence

Received: Oct. 10, 2024 Approved: June 10, 2025

Essential Oils (EO) - 3.5 g/kg DM. The substrates used were: forage *Urochloa brizantha* cv. Xaraés (11.5% of CP) alone, Dry Fiber with Solubles (DFS) alone, and a combination of the two (50:50). Ruminal fluid was obtained from two castrated F1 Nellore x Angus (BW = 400 ± 25 kg), fitted with a rumen cannula, grazing *Urochloa brizantha* cv. Marandu, and receiving mineral supplement without additives. Three consecutive incubations were conducted with gas production volume (GP) measured on times 3, 6, 9, 12, 18, 24, 36, and 48 hours using a semi-automatic reader. A total of 67 flasks per incubation (7 additives x 3 substrates x 3 replicates) and four additional flasks as blanks (rumen liquid + buffer solution) were used in each run. Data were analyzed in a 7 x 3 factorial arrangement (7 additives and 3 substrates). No interactions between additives and substrates ($P > 0.05$) were observed for variables analyzed. Asymptotic GP was higher ($P < 0.05$) for combination of the forage + DFS substrate, intermediate for DFS alone, and lower for forage alone. The FLAVO supplementation increased GP ($P < 0.05$) compared to other additives, while MON and FLAVO inclusion reduced ($P < 0.05$) the digestion rate. Lag time was higher ($P < 0.05$) when only forage was used as a substrate, with no differences between additives. The MON and FLAVO decreased ($P < 0.05$) IVDMD, IVNDFD, and $NH_3$-N. The substrate DFS stimulated microbial biomass synthesis ($P < 0.05$), with no significant difference observed between additives. In conclusion, regardless of the substrate, FLAVO inclusion promotes greater *in vitro* gas production, whereas MON and FLAVO had detrimental effects on DM and NDF digestion.
**Key words:** Rumen fermentation. Forage. ionophore. Yeast. non-ionophores. Functional oils.

## Resumo

Este estudo teve como objetivo avaliar os impactos de vários aditivos nos parâmetros cinéticos de produção de gases e na digestibilidade *in vitro* da matéria seca (DIVMS) e digestibilidade *in vitro* da fibra em detergente neutro (DIVFDN) em substratos com alto teor de fibra. Os aditivos avaliados foram: controle (CON) - sem aditivos; Monensina (MON) - 20 mg/kg MS; Flavomicina (FLAVO) - 4 mg/kg MS; Leveduras vivas (LY) - *Saccharomyces cerevisiae* - 0,5 g/kg MS; Cultura de leveduras (YC1) - 1,3 g/kg MS; Cultura de leveduras (YC2) - 1,3 g/kg MS; e Óleos Essenciais (EO) - 3,5 g/kg MS. Os substratos utilizados foram: forragem *Urochloa brizantha* cv. Xaraés (11,5 de PB) isoladamente, Fibra Seca com Solúveis (DFS) isoladamente, e uma combinação dos dois (50:50). O fluido ruminal foi obtido de dois bovinos castrados F1 Nelore x Angus (PV = 400 ± 25 kg), equipados com cânula ruminal, em pastagem de *Urochloa brizantha* cv. Marandu, recebendo suplemento mineral sem aditivos. Três incubações consecutivas foram realizadas com o volume de produção de gás (PG) medido nos tempos de 3, 6, 9, 12, 18, 24, 36 e 48 horas, utilizando um leitor semiautomático. Um total de 67 frascos por incubação (7 aditivos x 3 substratos x 3 réplicas) e quatro frascos adicionais como brancos (líquido ruminal + solução tampão) foram usados em cada incubação. Os dados foram analisados em um arranjo fatorial 7 x 3 (7 aditivos e 3 substratos). Não foram observadas interações entre aditivos e substratos ($P > 0,05$) para as variáveis analisadas. A produção de gás assintótica foi maior ($P < 0,05$) para a combinação de forragem + DFS, intermediária para DFS isoladamente, e menor para forragem isoladamente. A suplementação com FLAVO aumentou a PG ($P < 0,05$) em comparação com outros aditivos, enquanto a inclusão de MON e FLAVO reduziram ($P < 0,05$) a taxa de digestão. O tempo de latência foi maior ($P < 0,05$) quando apenas a forragem foi usada como substrato, sem diferenças entre os aditivos. MON e FLAVO diminuíram ($P < 0,05$) a DIVMS, DIVFDN e NH3-N. O substrato DFS estimulou a síntese de biomassa microbiana (P

< 0,05), sem diferença significativa observada entre os aditivos. Conclui-se que, independentemente do substrato, a inclusão de FLAVO promove maior produção de gás *in vitro*, enquanto MON e FLAVO tiveram efeitos prejudiciais sobre digestão de MS e FDN.
**Palavras-chave:** Fermentação ruminal. Forragem. Ionóforos. Levedura. Não-ionóforos. Óleos funcionais.

## Introduction

Feed additives are extensively utilized in beef cattle production to enhance ruminal fermentation, improve nutrient utilization efficiency, prevent digestive disorders, and boost overall performance (Marques & Cooke, 2021; Ahmed et al., 2024). Despite these benefits, there is growing public concern regarding the use of antibiotic ionophores due to the potential for residue transfer into animal products and the consequent risk of developing antibiotic-resistant bacterial strains (Morsy et al., 2015; Brutti et al., 2019). In response to these concerns, the European Union has proactively banned the use of ionophores in animal feed since 2006 (Martello et al., 2019). This regulatory shift has spurred interest in identifying and evaluating natural alternatives to conventional antibiotic additives, such as monensin, particularly for cattle on high-concentrate diets, without compromising feed efficiency (Yang et al., 2015; Rezaei Ahvanooei et al., 2023).

Among these natural alternatives, essential oils (EOs) have garnered significant attention within the scientific community, primarily due to their antimicrobial properties (Mutlu-Ingok et al., 2020). The EOs are recognized for their ability to enhance fermentation patterns and positively influence nutrient utilization (Teobaldo et al., 2020). Another promising category of natural additives is yeast products, including live yeasts, yeast cell walls, and yeast cultures, which have demonstrated potential in improving dry matter digestibility and overall animal performance (Maamouri & Ben Salem, 2022; Melo et al., 2023). Strains of *Saccharomyces cerevisiae,* in particular, have shown a capacity to modulate the rumen environment (Öztürk et al., 2015; Zhang et al., 2022; Nzeyimana et al., 2023). Additionally, yeast cell wall components function as prebiotics by fostering the growth of beneficial bacteria, thereby enhancing ruminal fermentation and potentially increasing the degradation and digestibility of fibrous feed fractions (Oeztuerk et al., 2005; Zhang et al., 2022; Xue et al., 2022).

Given the unique mechanisms of action associated with these natural additives, this study aimed to evaluate their potential to induce favorable modifications in the ruminal environment. However, it is important to note that the efficacy of these additives in ruminant diets has produced varied and sometimes contradictory results. Furthermore, a significant limitation in current research is the focus on high-concentrate diets, which are more common in non-tropical regions. In tropical countries, where livestock production predominantly relies on pasture, there is a pressing need to assess the impact of these additives in forage-based diets. Therefore, the objective of this study was to evaluate the inclusion of different natural additives on the kinetic parameters of gas

production, as well as the digestibility of dry matter and fiber in forage-based diets, both with and without the combination of a corn ethanol co-product.

## Materials and Methods

### *Experimental location and ethical approval*

The experiment was conducted at the Beef Cattle Sector of the UFMT's Experimental Farm and in Animal Nutrition Laboratory of the Faculty of Agronomy and Animal Science of the Universidad Federal de Mato Grosso (UFMT), Cuiabá - Mato Grosso, in September 2022.

All experimental procedures conformed to the ethical principles adopted by the National Council for Animal Experimentation (CONCEA) and the protocol was approved by the Ethics Committee in Animal Use of the Universidad Federal do Mato Grosso (CEUA/UFMT) (approval n° 23108.026027/2021-62).

### *Experimental design, treatments, and substrates*

The experiment was carried out in a 3 × 7 factorial arrangement, with three substrates [Forage *Urochloa brizantha* cv. Xaraés (11.5% CP), DFS - *Dry Fiber with Solubles,* and the combination of the two (50:50) – Table 1], and the following additives: **Control (CON)** - no additives; **Monensin (MON) - ionophore** - (Rumensin200™, Elanco, São Paulo, Brazil) - inclusion of 20 mg of monensin/kg DM; **Flavomycin (FLAVO) -- non-ionophore** - (flavomycin® 80, Huverpharma, Porto Alegre, Brazil) - inclusion of 4 mg of flavomycin/kg DM); **Live yeasts (LY)** - (*Saccharomyces cerevisiae,* Aleris, São Paulo, Brazil) - inclusion of 0.5 g of live yeasts/kg DM; **Yeast culture (YC1)** - (Cultron®, Aleris, São Paulo, Brazil; composed based on *S. cerevisiae fermented* in medium with cereal and sugarcane molasses) - inclusion of 1.3 g of yeast culture/kg DM; **Yeast culture (YC2)** - (Cultron X®, Aleris, São Paulo, Brazil; culture-based additive of fermented yeast in cereals with autolyzed sugarcane yeast) - inclusion of 1.3 of yeast culture g/kg DM and **Essential Oils (EO)** - (Essential®, Oligo Basics, Paraná, Brazil; *blend* of cashew nut oil and castor oil) - inclusion of 3.5 g of functional oils/kg DM. The dosages used herein were according to manufacturer's recommendation.

The DFS used was supplied by the company FS Bioenergia, located in Lucas do Rio Verde - MT, Brazil, a product marketed as FS OURO.®. The forage used from *Urochloa brizantha* cv. Xaraés were obtained during the rainy season, in a pasture area of the UFMT Experimental Farm, located in Santo Antônio do Leverger - Mato Grosso. The forage was obtained by hand-plucking method, and then pre-dried in a forced ventilation oven at 55ºC for 72 hours and ground in a Willey mill with a 1 mm sieve (SL32 - Solab®, Piracicaba, Brazil).

**Table 1**
**Chemical composition of the substrates used**

| Item (%) | Forage* | DFSa | Forage+DFS |
|---|---|---|---|
| Dry matter | 93.78 | 93.42 | 93.60 |
| Mineral Matter | 6.74 | 5.16 | 5.95 |
| Crude protein | 11.50 | 21.26 | 15.02 |
| Ether extract | 1.27 | 5.08 | 3.93 |
| NDF | 67.03 | 56.94 | 62.46 |
| Non-Fiber Carbohydrates | 13.46 | 11.55 | 12.63 |

* Urochloa brizantha cv. Xaraés; a DFS = Dry Fiber with Solubles; b NDF = Neutral detergent insoluble fiber.

*In vitro incubations*

The ruminal fluid was obtained from two F1 steers (Nellore x Angus), provided with a silicon rumen cannula, with an age of 14 ± 3 months and BW of 400 ± 25 kg kept in grazing. The animals were kept in paddocks with *Urochloa brizantha* cv. Marandu, provided with trough and drinker, receiving mineral supplement without additives.

Incubations were carried out for three consecutive weeks, in which a total of 67 flasks per incubation (7 additives × 3 substrates × 3 replicates) and four additional flasks as blanks (ruminal liquid + buffer solution) were used.

For each 100 mL amber flask, 0.5 g of substrate was weighed, then 0.2 mL of solution containing one of the aforementioned additives was pipetted, except in the control treatment, in which only distilled water was pipetted. The additives MON and EO were macerated and later diluted in ethanol (Ishlak et al., 2015; Martello et al., 2019; Teobaldo et al., 2020). The additives FLAVO, LY, YC1 and YC2 were diluted in water. The additives were diluted to reduce weighing errors and meet the established concentrations. In each flask, 40 mL of buffer solution and 10 mL of rumen fluid were added, resulting in a ruminal fluid: buffer ratio of 1:4 (v/v).

The rumen inoculum was obtained from each animal, gauze-filtered (pore size of 250 µm), and stored in a thermos without empty spaces, as recommended by Yanez Ruiz et al. (2004). The liquid was transported to the Animal Nutrition Laboratory - UFMT, homogenized and filtered again, placed in a glass container to which CO2 was continuously added, and kept in a water bath at 39°C.

After inoculation, the flasks were closed with a rubber stopper sealed with an aluminum seal and randomly placed in a water bath at 39°C, with orbital agitation throughout the incubation period. The pressure caused by the accumulation of gases in the upper part of the flasks was measured utilizing a pressure transducer at the following times: 3, 6, 9, 12, 18, 24, 36, and 48 h, to quantify the cumulative production of gases. Thus, the total production was obtained by the sum of the pressure, which was later converted into volume (mL), since after each reading

performed, the pressure in the flasks was exhausted.

The conversion of pressure (psi) to mL of gas was performed from the regression equation (y= a + bx), where the b coefficient allows the correction and transformation of pressure (psi) into gas volume (psi) corrected for barometric pressure of the day. For this, a known volume of gas was injected into five flasks kept under the same conditions as the incubated samples. The pressures corresponding to the injected volumes were measured and used to obtain the regression equation between the pressure and the volume of the gas.

All flasks were removed from the water bath after the last reading (48 h) and quickly transferred to the refrigerator to cease microbial activity, remaining in this manner for 1 hour.

### *Dry matter and NDF digestibility*

The undigested residues from the substrates of each flask were filtered into non-woven tissue bags (6 cm x 12 cm). The filtered content was used to analyze the digestibility of DM and neutral detergent insoluble fiber (NDF), as well as to quantify the ammonia concentration and the pH of the rumen fluid, following the method G-003/1 as recommended by INCT-CA. Subsequently, the DM residues were subjected to extraction with neutral detergent at 100ºC in an autoclave for 1 hour (INCT-CA - F-002/1). Thus, *in vitro* dry matter digestibility (IVDMD) and *in vitro* neutral detergent fiber digestibility (IVNDFD) were calculated considering the difference between the material initially weighed in each flask and its respective DM and NDF residue.

### *Ammonia nitrogen analysis*

The concentration of ammonia nitrogen was determined by the indophenol-catalyzed colorimetric reaction method (INCT-CA N-006/1). To determine the concentration of $NH_3$-N, 5 mL of the liquid residue from the post-filtration incubation of each bottle was used, which was centrifuged at 10.000 rpm for 10 minutes at 4°C and quantified according to the method described by Chaney and Marbach (1962).

### *Chemical analyses*

The substrates used in the incubations were analyzed for dry matter (INCT-CA n. G-003/1), crude protein (Kjeldahl procedure, INCT-CA method n° N-001/1), ash (complete combustion in muffle at 600°C for 4 h), and organic matter (OM) (INCT-CA n. M-001/1), and NDF using autoclave (INCT-CA - F-002/1). All analyses were performed using the methods proposed by the National Institute of Science and Technology of *Animal Science* (Detmann et al., 2012) except for ether extract which was used (AOCS method Official Procedure Am 5-04).

### *Calculations and statistical analysis*

Microbial protein production (PPM) was calculated according to Blümmel et al. (1997):

**PPM = IVDMD (mg) - (*GP* x 2.2 mg/mL)**

Where GP is the gas production (mL/g DM) at 24 hours of incubation.

The kinetic parameters of in vitro gas production over time were estimated using

the NLIN option of SAS (version 9.4), following the Gompertz function:

$$GP = Vf * \exp(-\exp(1+k*(h-t)))$$

where GP is cumulative gas production (mL); ***Vf*** is the maximum gas production (ml); ***k*** is the rate of digestion (mL/h) that occurs at the inflection point of the curve; ***Lag*** *is the delay time* (h), and t is the incubation time (h).

Data were analyzed using the SAS MIXED procedure (version 9.4). Before the statistical analyses, the averages of the data for each week were obtained and used as the experimental unit (Udén et al., 2012). The statistical model included the fixed effects of substrates, additives the interaction of substrates x additives, and the random effect of incubation. The degrees of freedom and tests were adjusted by the Kenward-Roger option. When the interactions were not significant, the main factors (substrates and additives) were tested separately. It is anticipated that there was no interaction effect for any of the variables evaluated. The LSMEANS option was used to generate individual averages for each treatment. Differences between treatment means were identified using Fisher's Least Significant Difference (LSD) method. In all analyses, differences were considered significant when $P<0.05$.

## Results

No significant interaction was observed between substrates and additives ($P>0.05$). This lack of interaction allowed for the independent assessment of substrate and additive effects on the evaluated variables.

The maximum gas production (*Vf*) was significantly higher ($P<0.05$) for the combination of forage and DFS, intermediate for DFS alone, and lowest for forage (Table 2). Flavomycin (FLAVO) led to significantly higher asymptotic gas production compared to the other additives, with no significant differences observed between the other additives and the control (Table 2).

**Table 2**
**Kinetic parameters of gas production overtime on the use of different additives**

| Substrates | Additives | GP Parameters | | | GP *in vitro* (mL/g DM) | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Vf* | *K* | Lag | *Vf* 12 | *Vf* 24 | *Vf* 36 | *Vf* 48 |
| Forage | COM | 230.47 | 0.0924 | 1.86 | 88.21 | 154.10 | 196.79 | 227.84 |
| | MON | 242.83 | 0.0800 | 2.12 | 76.26 | 146.45 | 194.03 | 230.98 |
| | FLAVO | 267.53 | 0.0820 | 2.06 | 88.77 | 164.80 | 216.37 | 257.25 |
| | YC1 | 232.77 | 0.0894 | 1.47 | 89.43 | 155.20 | 197.34 | 228.43 |
| | YC2 | 238.50 | 0.0921 | 1.27 | 95.48 | 164.04 | 206.12 | 236.23 |
| | LY | 230.27 | 0.0894 | 0.80 | 91.70 | 152.79 | 194.12 | 225.64 |
| | EO | 227.00 | 0.0944 | 1.39 | 92.27 | 157.87 | 197.89 | 227.45 |

continue...

continuation...

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| DFS | CON | 244.20 | 0.0886 | 1.18 | 95.01 | 162.22 | 206.89 | 240.43 |
| | MON | 240.43 | 0.0833 | 0.85 | 93.95 | 155.15 | 199.93 | 235.32 |
| | FLAVO | 260.03 | 0.0806 | 1.34 | 93.84 | 160.76 | 211.21 | 250.97 |
| | YC1 | 244.87 | 0.0871 | 0.64 | 96.87 | 163.32 | 208.19 | 239.87 |
| | YC2 | 239.67 | 0.0871 | 1.30 | 92.30 | 160.05 | 203.99 | 235.57 |
| | LY | 267.43 | 0.0876 | 0.99 | 102.98 | 177.18 | 224.91 | 262.43 |
| | EO | 248.53 | 0.0909 | 1.55 | 96.24 | 164.32 | 210.83 | 246.25 |
| Forage + DFS | CON | 256.10 | 0.0859 | 0.46 | 101.84 | 172.27 | 218.23 | 251.59 |
| | MON | 250.00 | 0.0800 | 1.00 | 90.14 | 155.07 | 204.01 | 241.68 |
| | FLAVO | 294.27 | 0.0784 | 1.48 | 98.73 | 179.56 | 237.08 | 280.37 |
| | YC1 | 256.77 | 0.0834 | 0.99 | 94.55 | 165.53 | 213.57 | 247.99 |
| | YC2 | 267.30 | 0.0829 | 0.58 | 101.34 | 172.54 | 223.11 | 258.04 |
| | LY | 260.20 | 0.0849 | 0.75 | 99.47 | 171.77 | 219.28 | 253.59 |
| | EO | 256.73 | 0.0829 | 0.72 | 96.73 | 166.21 | 213.46 | 248.87 |
| SEM | | 11.73 | 0.0069 | 0.31 | 5.62 | 7.98 | 8.49 | 9.70 |
| **Substrates** | | | | | | | | |
| Forage | | 238.48c | 0.08853a | 1.57a | 88.88b | 156.47b | 200.38b | 233.40b |
| DFS | | 249.31b | 0.08646a | 1.12b | 95.88a | 163.29a | 209.42a | 244.41a |
| Forage + DFS | | 263.05a | 0.08265b | 0.85b | 97.54a | 168.99a | 218.39a | 254.59a |
| **Additives** | | | | | | | | |
| CON | | 243.59b | 0.08897a | 1.17 | 95.02 | 162.86 | 207.30 | 239.96b |
| MON | | 244.42b | 0.08110b | 1.32 | 86.78 | 152.22 | 199.33 | 236.00b |
| FLAVO | | 273.94a | 0.08036b | 1.62 | 93.78 | 168.38 | 221.55 | 262.86a |
| YC1 | | 244.80b | 0.08662a | 1.04 | 93.62 | 161.35 | 206.37 | 238.76b |
| YC2 | | 248.49b | 0.08738a | 1.05 | 96.38 | 165.54 | 211.07 | 243.28b |
| LY | | 252.63b | 0.08730a | 0.85 | 98.05 | 167.25 | 212.77 | 247.22ab |
| EO | | 244.09b | 0.08944a | 1.22 | 95.08 | 162.80 | 207.39 | 240.86b |
| *P-value* | | | | | | | | |
| Substrates | | 0.0002 | 0.0011 | 0.0003 | 0.0016 | 0.0069 | 0.0012 | 0.0008 |
| Additives | | 0.0062 | 0.0005 | 0.0860 | 0.0954 | 0.1309 | 0.0924 | 0.0302 |
| Substrates* Additives | | 0.6394 | 0.8757 | 0.2156 | 0.7232 | 0.8054 | 0.7891 | 0.7368 |

Averages on the same line accompanied by different letters differ by Fisher's Minimum Difference. *Vf* - maximum gas production (mL); *k* - digestion rate ($h^{-1}$); Lag - delay time; DFS - dry fiber with solubles; CON - control, without inclusion of additives; MON - monensin; FLAVA - flavomycin; YC1 - yeast culture 1 (Cultron®); YC2 - yeast culture 2 (Cultron X®); LY - live yeast; EO -Essential Oils (Essential®).

The digestion rate (K) was higher (P<0.05) for the forage and DFS substrates individually compared withforage + DFS. The inclusion of monensin (MON) and FLAVO significantly reduced the digestion rate (P<0.05), with no differences observed between the other additives (Table 2).

The lag time was significantly longer (P<0.05) when forage was used as the sole substrate compared with DFS and forage + DFS, that did not differ each other (Table 2). No differences in lag time were observed between the additives (P>0.05; average of 1.18 h). Additionally, the cumulative gas production (Vf) was consistently lower (P<0.05) for forage alone compared with DFS and forage + DFS, that did not differ each other at all evaluated time points (Table 2). On the other hand, no difference was observed for additives inclusions at 12, 24, and 36 h of incubation, with averages of 94.1, 162.91, and 209.4 mL/g DM, respectively. At 48 h FLAVO increased Vf compared with other additives, that did not differ from each other's (Table 2).

Differences in vitro dry matter digestibility (IVDMD) were observed among substrates (P<0.05), with DFS showing the highest IVDMD, the forage + DFS combination yielding intermediate values, and forage alone the lowest (Table 3). The inclusion of MON and FLAVO significantly reduced IVDMD (P<0.05) compared with other additives and the control (CON).

**Table 2**
**Effect of different additives on pH, $NH_3$-N digestibility of DM and NDF, and synthesis of microbial biomass**

| Substrates | Additives | PH | $NH_3$-N | IVDMD | IVNDFD | PMM |
|---|---|---|---|---|---|---|
| | CON | 6.70 | 12.48 | 68.56 | 65.67 | 346.52 |
| | MON | 6.40 | 7.04 | 67.05 | 62.66 | 348.32 |
| | FLAVO | 6.70 | 9.58 | 65.36 | 63.40 | 291.07 |
| Forage | YC1 | 6.70 | 10.95 | 67.38 | 64.52 | 332.28 |
| | YC2 | 6.85 | 12.18 | 67.30 | 64.89 | 312.14 |
| | LY | 6.90 | 10.36 | 68.03 | 65.01 | 344.09 |
| | EO | 6.90 | 11.19 | 67.45 | 65.67 | 327.19 |
| | CON | 7.00 | 16.34 | 77.82 | 65.04 | 421.33 |
| | MON | 6.60 | 11.69 | 72.94 | 78.53 | 388.08 |
| | FLAVO | 6.85 | 13.63 | 73.61 | 72.84 | 382.47 |
| DFS | YC1 | 6.70 | 17.59 | 75.11 | 73.36 | 391.78 |
| | YC2 | 6.80 | 15.93 | 77.13 | 77.01 | 419.23 |
| | LY | 6.90 | 13.89 | 75.50 | 77.02 | 365.23 |
| | OF | 6.75 | 14.39 | 76.06 | 76.25 | 399.18 |
| | CON | 6.50 | 15.84 | 72.89 | 74.01 | 349.84 |
| | MON | 6.45 | 8.89 | 72.45 | 74.93 | 383.36 |

continue...

continuation...

| | | | | | | |
|---|---|---|---|---|---|---|
| | FLAVO | 6.80 | 11.62 | 71.44 | 73.51 | 319.38 |
| Forage + DFS | YC1 | 6.75 | 14.65 | 74.04 | 73.30 | 376.28 |
| | YC2 | 6.90 | 14.49 | 73.88 | 74.46 | 359.24 |
| | LY | 6.90 | 15.09 | 74.80 | 74.84 | 370.09 |
| | EO | 6.85 | 13.27 | 73.33 | 75.88 | 367.63 |
| SEM | | 0.1148 | 1.8571 | 1.3640 | 1.1089 | 21.1278 |
| Substrates | | | | | | |
| Forage | | 6.73 | 10.54b | 67.30c | 64.45b | 328.80c |
| DFS | | 6.80 | 14.78a | 75.45a | 75.57a | 395.33a |
| Forage+DFS | | 6.73 | 13.40a | 73.26b | 74.59a | 360.83b |
| Additives | | | | | | |
| COM | | 6.73a | 14.88a | 73.09a | 73.04a | 372.56 |
| MON | | 6.48b | 9.20d | 70.81b | 69.67b | 373.26 |
| FLAVO | | 6.78a | 11.61c | 70.13b | 70.02b | 330.97 |
| YC1 | | 6.72a | 14.40ab | 72.17a | 71.99ab | 366.78 |
| YC2 | | 6.85a | 14.20ab | 72.77a | 72.25a | 363.54 |
| LY | | 6.90a | 13.11bc | 72.78a | 72.38a | 359,80 |
| EO | | 6.83a | 12.95bc | 72.28a | 71.39ab | 364.67 |
| p-value | | | | | | |
| Substrates | | 0.5072 | <.0001 | <.0001 | <.0001 | <.0001 |
| Additives | | 0.0076 | <.0001 | 0.0015 | 0.0022 | 0.1246 |
| Substrates* Additives | | 0.5093 | 0.7726 | 0.4633 | 0.6127 | 0.4834 |

Averages on the same line accompanied by different letters differ by Fisher's Minimum Difference. IVDMD is the in vitro dry matter digestibility (%), IVNDFD is the in vitro digestibility of neutral detergent fiber (%), SEM is thestandard error mean. CON - control. without inclusion of additives; MON - monensin; FLAVA - flavomycin; YC1 - yeast culture 1 (Cultron); YC2 - yeast culture 2 (Cultron X); LY - live yeast; EO - Essential Oi.

For in vitro neutral detergent fiber digestibility (IVNDFD), the inclusion of MON and FLAVO resulted in significantly lower values (P<0.05) compared to the CON, yeast culture 2 (YC2), and live yeast (LY; Table 3). Intermediate IVNDFD values were observed for yeast culture 1 (YC1) and essential oils (EO). For substrates, forage alone resulted in significantly lower IVNDFD (P<0.001) compared to the other substrates (Table 3).

No significant differences (P>0.05) were observed in pH between the substrates (average of 6.75). Among the additives, MON led to a significant reduction (P<0.05) in pH compared with other treatments, that did not differ from each other's. Inclusion of MON and FLAVO reduced ammonia nitrogen ($NH_3$-N) levels, intermediate values were observed for LY and EO, and greater values for CON, YC1, and YC2.

Microbial biomass synthesis was significantly higher (P<0.05) for DFS, intermediate for the 50:50 combination of forage and DFS, and lowest for forage alone. There were no significant differences (P>0.05) in microbial biomass synthesis between the additives tested (average of 361.65 mg/kg).

## Discussion

In the present study, the use of DFS and the DFS-forage combination substrates led to an improvement in in vitro gas production, in vitro dry matter digestibility (IVDMD), and in vitro neutral detergent fiber digestibility (IVNDFD) compared to forage alone. This improvement appears to be directly associated with the lower neutral detergent fiber (NDF) content of the substrates with DFS, which likely reduced the physical barriers to microbial colonization and digestion in the rumen environment. In addition, although DFS and others corn ethanol byproducts present considerably high NDF content, considering that its NDF contains low amount of lignin compared with forage, DFS can be considered as having an NDF highly digestible (Schingoethe et al., 2009; Rosa e Silva et al., 2024). The negative effect of high NDF content on digestibility has been widely reported (Alamouti et al., 2009; Nunes et al., 2016), and the observed results are consistent with this relationship, as also supported by Paciullo et al. (2001), who identified a negative correlation between fibrous components and IVDMD.

Ammoniacal nitrogen ($NH_3$-N) concentration is another critical factor for rumen fermentation and efficient use of fibrous carbohydrates, as it directly influences the growth of fibrolytic microorganisms (Russell, 2002). In our study, the inclusion of monensin, flavomycin, live yeast and essential oils reduced NH3-N concentrations compared to the control treatments. In fact, feed additives might mitigate ruminal proteolysis and reduced N-$NH_3$ concentration (Rogers et al., 1997), although this effect seem more pronounced for monensin. These findings are aligned with earlier studies (Russell & Martin, 1984; Russell & Strobel, 1989; Ishlak et al., 2015; Brutti et al., 2019), which demonstrated monensin's ability to suppress ruminal proteolysis, consequently lowering NH3-N levels. Although the levels remained above the minimum threshold of 8 mg/dL proposed by Detmann et al. (2009) for maintaining fiber degradation, the lower $NH_3$-N availability may have partially limited microbial activity and thus fiber digestibility. However, in the present study, only monensin and flavomycin decreased dry matter and fiber digestibility.

Interestingly, treatments in which $NH_3$-N concentrations approached 15 mg/dL exhibited the highest IVDMD and IVNDFD values. This reinforces the idea that the availability of $NH_3$-N is a key modulator of rumen fermentation efficiency, especially for fibrous diets. Detmann et al. (2009) and Leng (1990) previously reported that optimal ruminal degradation and intake of fibrous feeds occur when $NH_3$-N concentrations are maintained between 10 and 20 mg/dL, a range that our data also supports.

In addition to monensin, other feed additives demonstrated a significant influence on ruminal fermentation patterns, particularly functional oils and flavomycin. Both were associated with reduced $NH_3$-N

concentrations, suggesting inhibitory effects on proteolytic and ammonia-producing bacteria. This observation corroborates previous reports describing the antimicrobial action of functional oils and flavomycin (Busquet et al., 2006; Calsamiglia et al., 2007; Teobaldo et al., 2020; Russell et al., 1991; Edwards et al., 2005). The similarities in the effects of monensin and flavomycin on $NH_3$-N concentrations highlight their shared ability to modulate the ruminal microbiota, although through different modes of action.

Regarding ruminal pH, our results confirm that maintaining pH values between 6.40 and 7.00 creates a favorable environment for fibrolytic bacteria, supporting optimal fiber degradation.

The effects of additives on nutrient digestibility are variable. Overall, the literature still is scared about the effects of additives for cattle fed high-forage diets (Bretschneider et al., 2008; Polizel et al., 2020). In our study, despite the $NH_3$-N concentrations, monensin and flavomycin had deleterious effects on dry matter and fiber digestibility. In fact, monensin can inhibit growth of some cellulolytic bacteria (Duffield et al., 2012). Accordingly, the reduction in DM and NDF digestibility with monensin inclusion may be attributed to its inhibitory effect on Gram-positive bacteria, such as *Ruminococcus albus, Ruminococcus flavefaciens,* and *Butyrivibrio fibrisolvens,* which are key fibrinolytic species (Anassori et al., 2012). Corroborating our results, Brutti et al. (2019) observed that in vitro digestibility's of DM and NDF were reduced when monensin was used in nitrogen-fertilized and non-fertilized *Urochloa brizantha* cv. Marandu. In contrast, Flavomycin - despite being a non-ionophore antibiotic - may have exerted selective effects on Gram-negative species like *Fibrobacter succinogenes,* as reported by Edwards et al. (2005). Nonetheless, the potential for compensatory growth of other cellulolytic microorganisms and protozoa may explain the less pronounced effect of flavomycin on fiber degradation compared to monensin.

Our findings also suggest that ionophore supplementation, particularly monensin, may reduce protozoa populations, indirectly limiting fibrolytic activity, as previously reported (Gijzen et al., 1988). While defaunation often impairs fiber digestion and animal performance, the trade-off may include beneficial outcomes such as reduced methane emissions and nitrogen excretion, as demonstrated in the meta-analysis by Newbold et al. (2015).

On the other hand, supplementation with yeast-based products, including live yeast and yeast cell wall polysaccharides, has been associated with enhanced NDF digestibility. This effect is likely explained by the ability of yeast components to stimulate fibrolytic bacterial populations, either by improving rumen pH stability or by supplying essential micronutrients and vitamins that support microbial growth (Oeztuerk et al., 2005). However, in this study, inclusion of yeast products did not result in improvements compared with control. Supporting these results, no significant changes in microbial protein production were observed in yeast-supplemented treatments. This suggests that the effects of yeast products on fiber digestibility and microbial protein synthesis may be limited or influenced by other factors not directly measured in this study.

Another important observation concerns to the fermentation lag time, which was reduced in treatments including DFS. Since DFS has undergone prior fermentation, it likely presented a less lignified and more accessible structure to the ruminal microbiota, facilitating microbial adhesion and colonization. This observation is in line with the concept that the physicochemical characteristics of substrates, particularly their lignin content, are key determinants of lag time and fermentation rate (Krishnamoorthy et al., 1991; Van Soest, 1994; Tomich et al., 2003; Rodrigues et al., 2021).

## Conclusion

This study demonstrates that natural additives, such as LY, YC, and OE, did not show significant differences from the control. In contrast, the additive FLAVO resulted in higher gas production, suggesting its potential to enhance certain aspects of ruminal fermentation. Nonetheless, both MON and FLAVO had a detrimental effect on the digestion of DM and NDF. Among the substrates evaluated, the inclusion of DFS was particularly beneficial, improving DM and NDF digestibility and stimulating microbial protein synthesis. These findings highlight the importance of carefully selecting additives and substrates to optimize ruminal fermentation and nutrient utilization in beef cattle production.

## References

Ahmed, M. G., Elwakeel, E. A., El-Zarkouny, S. Z., & Al-Sagheer, A. A. (2024). Environmental impact of phytobiotic additives on greenhouse gas emission reduction, rumen fermentation manipulation, and performance in ruminants: an updated review. *Environmental Science and Pollution Research, 31*(26), 37943-37962. doi: 10.1007/s11356-024-33664-5

Alamouti, A. A., Alikhani, M., Ghorbani, G. R., & Zebeli, Q. (2009). Effects of inclusion of neutral detergent soluble fibre sources in diets varying in forage particle size on feed intake, digestive processes, and performance of mid-lactation Holstein cows. *Animal Feed Science and Technology, 154*(1-2), 9-23. doi: 10.1016/j.anifeedsci.2009.07.002

Anassori, E., Dalir-Naghadeh, B., Pirmohammadi, R., Taghizadeh, A., Asri-Rezaei, S., Farahmand-Azar, S., Besharati, M., & Tahmoozi, M. (2012). In vitro assessment of the digestibility of forage based sheep diet, supplemented with raw garlic, garlic oil and monensin. *Veterinary Research Forum, 3*(1), 5-11.

Blümmel, M., Steingaß, H., & Becker, K. (1997). The relationship between in vitro gas production, in vitro microbial biomass yield and 15N incorporation and its implications for the prediction of voluntary feed intake of roughages. *British Journal of Nutrition, 77*(6), 911-921. doi: 10.1079/BJN19970089

Bretschneider, G., Elizalde, J. C., & Pérez, F. A. (2008). The effect of feeding antibiotic growth promoters on the performance of beef cattle consuming forage-based diets: a review. *Livestock Science, 114*(2-3), 135-149. doi: 10.1016/j.livsci.2007.12.017

Brutti, D. D., Paula, N. F. de, Zervoudakis, J. T., Cabral, L. D. S., Fonseca, M. A., Macedo, B. G., & Lima, L. R. (2019). Effects of tannins and monensin on the modulation of in vitro ruminal fermentation and ammonia production of nitrogen-fertilized and non-fertilized *Urochloa brizantha* cv. Marandu. *Grassland Science, 65*(2), 101-108. doi: 10.1111/grs.12221

Busquet, M., Calsamiglia, S., Ferret, A., & Kamel, C. (2006). Plant extracts affect *in vitro* rumen microbial fermentation. *Journal of Dairy Science, 89*(2), 761-771. doi: 10.3168/jds.S0022-0302(06)72137-3

Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., & Ferret, A. (2007). Invited review: essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science, 90*(6), 2580-2595. doi: 10.3168/jds.2006-644

Chaney, A. L., & Marbach, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry, 8*(2), 130-132. doi: 10.1093/clinchem/8.2.130

Detmann, E., Paulino, M. F., Mantovani, H. C., Valadares, S. D. C., Fº., Sampaio, C. B., Souza, M. A. de, Lazzarini, I., & Detmann, K. S. (2009). Parameterization of ruminal fiber degradation in low-quality tropical forage using Michaelis-Menten kinetics. *Livestock Science, 126*(1-3), 136-146. doi: 10.1016/j.livsci.2009.06.013

Detmann, E., Souza, M. A., Valadares, S. C., Fº., Queiroz, A. C., Berchielli, T. T., Saliba, E. O. S., Cabral, L. S., Pina, D. S., Ladeira M. M., & Azevedo, J. A. G. (2012). *Métodos para análise de alimentos - national institute of science and technology - animal science, INCT-CA.* Suprema.

Duffield, T. F., Merrill, J. K., & Bagg, R. N. (2012). Meta-analysis of the effects of monensin in beef cattle on feed efficiency, body weight gain, and dry matter intake. *Journal of Animal Science, 90*(12), 4583-4592. doi: 10.2527/jas.2011-5018

Edwards, J. E., McEwan, N. R., McKain, N., Walker, N., & Wallace, R. J. (2005). Influence of flavomycin on ruminal fermentation and microbial populations in sheep. *Microbiology, 151*(3), 717-725. doi: 10.1099/mic.0.27602-0

Gijzen, H. J., Lubberding, H. J., Gerhardus, M. J., & Vogels, G. D. (1988). Contribution of rumen protozoa to fiber degradation and cellulase activity *in vitro. FEMS Microbiology Ecology, 4*(1), 35-43. doi: 10.1111/j.1574-6968.1988.tb02645.x

Ishlak, A., Günal, M., & AbuGhazaleh, A. A. (2015). The effects of cinnamaldehyde, monensin, and quebracho condensed tannin on rumen fermentation, biohydrogenation, and bacteria in continuous culture system. *Animal Feed Science and Technology, 207*(1), 31-40. doi: 10.1016/j.anifeedsci.2015.05.023

Krishnamoorthy, U., Soller, H., Steingass, H., & Menke, K. H. (1991). A comparative study on rumen fermentation of energy supplements in vitro. *Journal of Animal Physiology and Animal Nutrition, 65*(1-5), 28-35. doi: 10.1111/j.1439-0396.1991.tb00237.x

Leng, R. A. (1990). Factors affecting the utilization of poor-quality forages by ruminants, particularly under tropical conditions. *Nutrition Research Reviews, 3*(1), 277-303. doi: 10.1079/NRR19900016

Maamouri, O., & Ben Salem, M. (2022). The effect of live yeast Saccharomyces cerevisiae as probiotic supply on growth performance, feed intake, ruminal pH, and fermentation in fattening calves. *Veterinary Medicine and Science, 8*(1), 398-404. doi: 10.1002/vms3.631

Marques, R. da S., & Cooke, R. F. (2021). Effects of ionophores on ruminal function of beef cattle. *Animals, 11*(10), 2871. doi: 10.3390/ani11102871

Martello, H. F., Paula, N. F. de, Macedo, B. G., Zervoudakis, J. T., Brutti, D. D., Carvalho, P., Teobaldo, R. W., Cabral, L. S., & Fonseca, M. A. (2019). Effects of tannins and monensin in a feedlot diet on *in vitro* ruminal fermentation. *Semina: Ciências Agrárias, 40*(6, Supl.2), 3223-3232. doi: 10.5433/1679-0359.2019v40n6Supl2p3223

Melo, L. D., Oliveira, P. E. P. D., Plodoviski, D. C., Costa, L. D., Rosa, C. B. D., Pereira, E. L. C., Souza, A. M. de, & Neumann, M. (2023). Supplementation of yeast culture combined with an enzyme complex in the diet for confined steers. *Ciência Animal Brasileira, 24*(1), e-74470. doi: 10.1590/1809-6891v24e-74470E

Morsy, A. S., Soltan, Y. A., Sallam, S. M. A., Kreuzer, M., Alencar, S. M. D., & Abdalla, A. L. (2015). Comparison of the in vitro efficiency of supplementary bee propolis extracts of different origin in enhancing the ruminal degradability of organic matter and mitigating the formation of methane. *Animal Feed Science and Technology, 199*(1), 51-60. doi: 10.1016/j.anifeedsci.2014.11.004

Mutlu-Ingok, A., Devecioglu, D., Dikmetas, D. N., Karbancioglu-Guler, F., & Capanoglu, E. (2020). Antibacterial, antifungal, antimycotoxigenic, and antioxidant activities of essential oils: an updated review. *Molecules, 25*(20), 4711. doi: 10.3390/molecules25204711

Newbold, C. J., De La Fuente, G., Belanche, A., Ramos-Morales, E., & McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. *Frontiers in Microbiology, 6*(1), 1313. doi: 10.3389/fmicb.2015.01313

Nunes, A. T., Cabral, D. L. V., Amorim, E. L. C., Santos, M. V. F. dos, & Albuquerque, U. P. (2016). Plants used to feed ruminants in semi-arid Brazil: a study of nutritional composition guided by local ecological knowledge. *Journal of Arid Environments, 135*(1), 96-103. doi: 10.1016/j.jaridenv.2016.08.015

Nzeyimana, J. B., Fan, C., Tan, L., Butore, J., Zhuo, Z., & Cheng, J. (2023). Meta-analysis of the effect of feeding live yeast (Saccharomyces cerevisiae) on feeding behaviour and lactation performance, rumen fermentation, and rumen microbiota in dairy cattle. *Journal of Animal Behaviour and Biometeorology, 11*(4), 2023028. doi: 10.31893/jabb.23028

Oeztuerk, H. A. K. A. N., Schroeder, B., Beyerbach, M., & Breves, G. (2005). Influence of living and autoclaved yeasts of Saccharomyces boulardii on in vitro ruminal microbial metabolism. *Journal of Dairy Science, 88*(7), 2594-2600. doi: 10.3168/jds.S0022-0302(05)72935-0

Öztürk, H., Demirbaş, Y. S., Aydin, F. G., Pişkin, İ., Ünler, F. M., & Emre, M. B. (2015). Effects of hydrolyzed and live yeasts on rumen microbial fermentation in a semicontinuous culture system (Rusitec). *Turkish Journal of Veterinary & Animal Sciences, 39*(5), 556-559. doi: 10.3906/vet-1506-16

Paciullo, D. S. C., Gomide, J. A., Queiroz, D. S., & Silva, E. A. M. D. (2001). Correlações entre componentes anatômicos, químicos e digestibilidade in vitro da matéria seca de gramíneas forrageiras. *Revista Brasileira de Zootecnia, 30*(3 suppl 1), 955-963. doi: 10.35172/rvz.2021.v28.581

Polizel, D. M., Cappellozza, B. I., Hoe, F., Lopes, C. N., Barroso, J. P., Miszura, A., Oliveira, G. B., Gobato, L., & Pires, A. V. (2020). Effects of narasin supplementation on dry matter intake and rumen fermentation characteristics of Bos indicus steers fed a high-forage diet. *Translational Animal Science, 4*(1), 118-128. doi: 10.1093/tas/

Rezaei Ahvanooei, M. R., Norouzian, M. A., Piray, A. H., Vahmani, P., & Ghaffari, M. H. (2023). Effects of monensin supplementation on lactation performance of dairy cows: a systematic review and dose-response meta-analysis. *Scientific Reports, 13*(1), 568. doi: 10.1038/s41598-023-27395-9

Rodrigues, J. A., Abreu, J. G. de, Abreu, M. L. C., Assis, L. M. B., Rocha, N. T. da, Souza, V. B. de, Jr., & Xavier, M. F. N. (2021). Parâmetros cinéticos de produção de gás in vitro de diferentes genótipos de capim-elefante (*Pennisetum purpureum* Schum.). *Veterinária e Zootecnia, 28*(1), 1-8. doi: 10.35172/rvz.2021.v28.581

Rogers, M., Jouany, J. P., Thivend, P., & Fontenot, J. P. (1997). The effects of short-term and long-term monensin supplementation, and its subsequent withdrawal on digestion in sheep. *Animal Feed Science and Technology, 65*(1-4), 113-127. doi: 10.1016/S0377-8401(96)01089-9

Rosa e Silva, P. I. J. L. D., Silva, Y. R. V. B. E., Sousa, D. D. P., Paulino, P. V. R., Possamai, A. J., Freiria, L. B. D., Rolim, H. C. L., Dias Júnior, W. C., Fonseca, A. S. R., Costa, A. C., Negrão, F. M., & Cabral, L. S. (2024). Effect of dried distillers' grains on nutrients digestibility and nitrogen metabolism of Nellore cattle fed non-forage diets. *Revista Brasileira de Zootecnia, 53*(1), e20240045. doi: 10.37496/rbz5320240045

Russell, J. B. (2002). *Rumen microbiology and its role in ruminant nutrition.* Department of Microbiology, Cornell University.

Russel, J. B., & Martin, S. A. (1984). Effects of various methane inhibitors on the fermentation of amino acids by mixed rumen microorganisms *in vitro. Journal of Animal Science, 59*(5), 1329-1338. doi: 10.2527/jas1984.5951329x

Russell, J. B., & Strobel, H. (1989). Effect of ionophores on ruminal fermentation. *Applied and Environmental Microbiology, 55*(1), 1-6. doi: 10.1128/aem.55.1.1-6.1989

Russell, J. B., Onodera, R., & Hino, T. (1991). Ruminal protein fermentation: new perspectives on previous contradictions. In T. Tsuda, Y. Sasaki, & R. Kawashi (Eds.), *Physiological aspects of digestion and metabolism in ruminants* (pp. 681-697). Academic Press.

Schingoethe, D. J., Kalscheur, K. F., Hippen, A. R., & Garcia, A. D. (2009). Invited review: the use of distillers products in dairy cattle diets. *Journal of Dairy Science, 92*(12), 5802-5813. doi: 10.3168/jds.2009-2549

Teobaldo, R. W., De Paula, N. F., Zervoudakis, J. T., Fonseca, M. A., Cabral, L. S., Martello, H. F., Rocha, L. K. J., Ribeiro, I. J., & Mundim, A. T. (2020). Inclusion of a blend of copaiba, cashew nut shell, and castor oil in the protein-energy supplement for grazing beef cattle improves rumen fermentation, nutrient intake, and fiber digestibility. *Animal Production Science, 60*(8), 1039-1050. doi: 10.1071/AN18725

Tomich, T. R., Gonçalves, L. C., Maurício, R. M., Pereira, L. G. R., & Rodrigues, J. A. S. (2003). Bromatological composition and rumen fermentation kinetics of hybrids from crosses of sorghum and sudangrass. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 55*(6), 747-755. doi: 10.1590/S0102-09352003000600012

Udén, P., Robinson, P. H., Mateos, G. G., & Blank, R. (2012). Use of replicates in statistical analyses in papers submitted for publication in Animal Feed Science and Technology. *Animal Feed Science and Technology, 171*(1), 1-5. doi: 10.1016/j.anifeedsci.2011.10.008

Van Soest, P. J. (1994). *Nutritional ecology of the ruminant* (2nd ed.). Cornell University Press.

Xue, L., Zhou, S., Wang, D., Zhang, F., Li, J., & Cai, L. (2022). The low dose of Saccharomyces cerevisiae is beneficial for rumen fermentation (both *in vivo* and *in vitro*) and the growth performance of heat-stressed goats. *Microorganisms, 10*(10), 1877. doi: 10.3390/microorganisms10101877

Yanez Ruiz, D. R., Moumen, A., Martin Garcia, A. I., & Molina Alcaide, E. (2004). Ruminal fermentation and degradation patterns, protozoa population, and urinary purine derivatives excretion in goats and wethers fed diets based on two-stage olive cake: effect of PEG supply. *Journal of Animal Science, 82*(7), 2023-2032. doi: 10.2527/2004.8272023x

Yang, C., Chowdhury, M. K., Hou, Y., & Gong, J. (2015). Phytogenic compounds as alternatives to in-feed antibiotics: potentials and challenges in application. *Pathogens, 4*(1), 137-156. doi: 10.3390/pathogens4010137

Zhang, X., Dong, X., Wanapat, M., Shah, A. M., Luo, X., Peng, Q., Kang, K., Guan, J., & Wang, Z. (2022). Ruminal pH pattern, fermentation characteristics, and related bacteria in response to dietary live yeast (*Saccharomyces cerevisiae*) supplementation in beef cattle. *Animal Bioscience, 35*(2), 184-195. doi: 10.5713/ab.21.0200