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Effects of yeasts on ruminal fermentation of various diets: an *in vitro* assessment

Efectos de levaduras sobre la fermentación ruminal de varias dietas: una evaluación *in vitro*

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ABSTRACT

The objective was to evaluate the effects of three different yeast strains on *in vitro* ruminal fermentation of two diets, by measuring ruminal pH, ammonia nitrogen (N-NH_3), volatile fatty acids (VFA), total gas production (TGP), and gas composition (CO_2 and CH_4). A completely randomized design was applied using a 4 × 2 factorial arrangement. *Pichia guilliermondii* (Levica 27), *Candida norvegensis* (Levazoot 15), and a commercial strain of *Saccharomyces cerevisiae* (Levucell® SC 10) were incubated anaerobically for 24 h at 39 °C, using corn stover and a mixed ration (TMR) as substrates. Total gas production was not affected by diet or yeast source. The MR significantly influenced ruminal pH, while N-NH_3 concentration was lower (6.1 µmol) when corn stover was used as the substrate, with no differences among yeast species. However, all three yeasts increased ($P<0.05$) total VFA concentrations. The acetate-to-propionate ratio decreased ($P<0.05$) with Levica 27 and Levazoot 15 in the TMR, whereas no changes were observed with corn stover or Levucell® SC 10. In conclusion, all yeast strains improved *in vitro* ruminal fermentation, as indicated by increased VFA production.

Keywords: probiotics, mixed ration, *Pichia guilliermondii*, *Candida norvegensis*, *Saccharomyces cerevisiae*.

RESUMEN

Se evaluaron los efectos de tres especies de levaduras sobre el pH ruminal, el nitrógeno amoniacal (N-NH_3), las concentraciones de ácidos grasos volátiles (AGV), la producción total de gas y su composición (CO_2 y CH_4) en la fermentación ruminal *in vitro* de dos dietas. Se utilizó un diseño completamente al azar con arreglo factorial 4 x 2. *Pichia guilliermondii* (Levica 27), *Candida norvegensis* (Levazoot 15) y un producto comercial de *Saccharomyces cerevisiae* (Levucell® SC 10) fueron incubados anaeróbicamente durante 24 h a 39 ºC, utilizando como sustratos rastrojo de maíz o una ración integral (RTM). La producción de gas no fue afectada por la dieta ni por la especie de levadura. Las diferencias en pH se produjeron solo con la RTM. La concentración de N-NH_3 fue más baja (6.1 µmol) con rastrojo de maíz, sin mostrar variaciones entre especies de levaduras. Las tres levaduras incrementaron ($P<0.05$) los AGV totales e

individuales. La relación acético:propiónico disminuyó (P<0.05) con Levica 27 y Levazoot 15 en la RTM, pero no cambió con rastrojo de maíz o con Levucell® SC 10. En conclusión, las tres cepas mejoraron la fermentación ruminal *in vitro*, como lo evidenció el aumento en las concentraciones de AGV.
Palabras clave: probióticos, ración integral, *Pichia guilliermondii*, *Candida norvegensis*, *Saccharomyces cerevisiae*.

INTRODUCTION

One of the challenges associated with the increase in human population by the year 2030 is the growing demand for agricultural products (FAO, 2015), which is alarming, especially for developing countries. The generation of new strategies to optimize the production and quality of animal products has become a major research focus (Lara *et al*., 2018).

In ruminants, manipulation of ruminal fermentation is used to improve the utilization of lignocellulosic compounds, thereby enhancing health and productive performance (Arowolo & He, 2018; Liang *et al*., 2020). One strategy is the use of additives such as probiotics, which improve fermentation and therefore the digestibility of fibrous feeds (Pilajun & Wanapat, 2018). It has been reported that these additives promote the growth of beneficial microorganisms in the rumen, particularly cellulolytic bacteria and fungi (Fomenky *et al*., 2017; Liu *et al*., 2022). Furthermore, they help stabilize ruminal pH, reduce methane emissions (Elghandour *et al.,* 2015; Vallejo-Hernández *et al*., 2018; Phesatcha *et al*., 2020), improve fermentation patterns, reduce pathogen concentration, and increase meat and milk production (Leicester *et al*., 2016). However, the available information on this topic is inconsistent (Marrero *et al.,* 2020; Amin & Mao, 2021; Baker *et al*., 2022).

Research on alternative probiotics, other than *Saccharomyces cerevisiae*, has gained momentum. Strains such as *Pichia guilliermondii*, *Issatchenkia orientalis*, and *Candida norvegensis* have shown desirable *in vitro* results, in some cases surpassing the impact on fermentation obtained by pure strains of *Saccharomyces cerevisiae* (Wang *et al*., 2016; Marrero *et al*., 2021; González *et al*., 2023). However, information on the impact of these yeasts on ruminal fermentation, both *in vivo* and *in vitro*, is limited. The objective was to evaluate the effects of pure strains of *Saccharomyces cerevisiae*, *Pichia guilliermondii*, and *Candida norvegensis* on *in vitro* ruminal fermentation using corn stover and a total mixed ration (TMR) as substrates. It was hypothesized that their use would improve *in vitro* fermentation.

MATERIAL AND METHODS

Description of the study area

The study was conducted in 2023 at the Food Biotechnology Laboratory of the Faculty of Animal Science and Ecology of the Autonomous University of Chihuahua (UACH), located at Periférico Francisco R. Almada km 1.0, in Chihuahua city, Chih., Mexico (latitude 28° 35' 10.9'' N; longitude 106° 6' 26.6'' W; altitude 1,440 m a.s.l).

Experimental design and treatments

A completely randomized design with a 4 × 2 factorial arrangement and 4 replications was applied to evaluate the impact of three different yeast strains and a control treatment without yeast on the *in vitro* ruminal fermentation of corn stover and a total mixed ration (TMR). The experimental treatments are shown in Table 1.

Table 1. Experimental treatments

Treatments			
Corn stover		**Total mixed ration (TMR)**	
1	Control	5	Control
2	Levica 27[1]	6	Levica 27
3	Levazoot 15[2]	7	Levazoot 15
4	Levucell® SC 10[3]	8	Levucell® SC 10

[1]Levica 27: *Pichia guilliermondii* at 5.23 x 10^9 ufc mL^{-1}; [2]Levazoot 15: *Candida norvegensis* at 1.32 x 10^{10} ufc mL^{-1}; [3]Levucell® SC 10: *Saccharomyces cerevisiae* at 10^{10} ufc g^{-1}

The yeast strains used in the experiment were: *Pichia guilliermondii* (Levica 27, preserved in the Microorganism Bank of the Institute of Animal Science in Mayabeque, Cuba, registration number 980 at the World Data Center for Microorganisms (WDCM), and registered in GenBank under accession number JF894143.1, Marrero *et al*., 2013), *Candida norvegensis* (Levazoot 15, yeast collection of the Faculty of Animal Science and Ecology of the Autonomous University of Chihuahua, Mexico, GenBank accession number JQ519367.1 GI: 386785959, Ruiz *et al.*, 2016), and *Saccharomyces cerevisiae* (Levucell® SC 10).

In vitro incubations

The part of the research involving the use of animals was carried out in accordance with the regulations of the Institutional Bioethics Committee (case number: CFTZyE-ACTA-101/2015: ACUERDO 4.2).

The *in vitro* trial was conducted in 18 mL tubes, maintaining an effective volume of 10 mL. Corn stover and the TMR used as substrates were previously sun-dried at room temperature (32 °C) and ground through a 1.0 mm sieve before use. Analyses of dry matter (DM), crude protein (CP), and ether extract (EE) of the substrates used for *in vitro* fermentation were determined according to AOAC (2005); while neutral detergent fiber (NDF) and acid detergent fiber (ADF) content were determined following the technique described by Van Soest *et al.* (1991. Table 2). A 0.2 g sample of each substrate was weighed and added to the test tubes for incubation.

Table 2. Ingredients and chemical composition of corn stover and total mixed ration (TMR) used as substrates for *in vitro* ruminal fermentation

Ingredients (%)	Corn stover	TMR
Corn stover	100	40
Cracked corn	-	35
Molasses	-	9
Cottonseed	-	9
Protein-mineral supplement (Harinolina)	-	4
Urea	-	1.5
Minerals[1]	-	1.0
Ammonium sulfate	-	0.5
Nutrients (% Dry mass)		
Dry mass	91.44	86.75
Crude protein	5.90	15.83
NDF	67.20	42.68
ADF	37.75	25.20
EE (Ether Extract)	3.10	4.60

[1]Minerals: Micro FOS 8 (Phosphorus 8.0 %; Calcium 7.5 %; Magnesium 0.5 %; Potassium 1.2 %; Manganese 1.800 ppm; Zinc 2.400 ppm; Iron 500 ppm; Copper 930 ppm; Iodine 85 ppm; Cobalt 11 ppm; Selenium 7 ppm; Vitamin A 146.500 IU kg^{-1}; Vitamin D_3 16.350 IU kg^{-1}; Vitamin E 20 IU kg^{-1}). NDF: neutral detergent fiber; ADF: acid detergent fiber

Ruminal fluid was collected from three fistulated male Pelibuey sheep (32 kg, 12 months of age), housed in individual pens and fed a total mixed ration (TMR, Table 2) for seven days, with free access to water. Ruminal fluid extraction was performed on day eight, before the first feed offering (9:00 a.m.). Two hundred milliliters of ruminal fluid were collected, placed in a thermos at 39 °C and purged with CO_2, then mixed and immediately transported to the laboratory. The ruminal fluid was filtered through cheesecloth and used to prepare the fermentation medium according to Menke *et al*., (1979). Ten milliliters of fermentation medium were dispensed into each of the four vials containing the substrate, and four tubes without substrate were used as blanks. The tubes were prepared under a constant flow of CO_2 to maintain an anaerobic atmosphere and subsequently sealed for incubation.

Yeast cultures were added to the tubes just before the addition of the fermentation medium. For the preparation of Levica 27 and Levazoot 15 inocula, the strains were activated by two aerobic subcultures in malt extract broth (DIBICO®, Cuautitlán Izcallí, Mexico) at 110 rpm (orbital shaker incubator; New Brunswick Model Innova 4000, Nijmegen, The Netherlands), 30 °C, for 24 hours. From the activated cultures, 10 % (v/v) was used as inoculum for 50 mL of malt extract broth (DIBICO®) in 100 mL flasks, and incubated again at 30 °C and 110 rpm for 24 hours. From these cultures, 0.5 mL

(equivalent to 10 g yeast/adult animal/day) was added to the corresponding tubes for *in vitro* ruminal fermentation. For the control tubes, 0.5 mL of culture medium without yeast was added. Levica 27 and Levazoot 15 cultures had final concentrations of 5.23×10^9 and 1.32×10^{10} CFU mL^{-1}, respectively, in each tube. For Levucell® SC 10, 1 mg of the commercial product (equivalent to 10 g recommended for an adult ruminant) was added to the corresponding tubes. Finally, the tubes were immediately sealed and incubated at 39 °C and 110 rpm (orbital shaker incubator). After 24 hours, the tubes were placed on ice to stop fermentation and prepared for sample analysis.

Variables evaluated

Total gas production was determined using a FESTO® pressure transducer in each experimental unit after 24 hours of fermentation. A 1 mL gas sample was taken from each experimental unit to determine its composition by gas chromatography using a GOW-MAC Series 580 chromatograph equipped with a Carbosphere® 80/100 packed column (5682PC) (GOW-MAC Instrument Company). The carrier gas was nitrogen at a flow rate of 20 mL min^{-1} to determine individual methane and carbon dioxide production after 24 hours of incubation.

The pH was measured using a Hanna Instruments pH meter Model HI 9017, and N-NH_3 concentration was determined following the method of Broderick & Kang (1980).

For volatile fatty acid (VFA) production, a Claurus 400® gas chromatograph (Perkin Elmer) equipped with a flame ionization detector was used. The system used a Varian CP-wax58 (FFAP) CB capillary column (15 m × 0.53 mm, 0.5 µm). Before injection, the sample was treated with 25 % metaphosphoric acid, and a volume of 0.6 µL was introduced for analysis (Galyean, 1980).

Statistical analysis

Analysis of variance was performed using the GLM procedure of SAS (Statistical Analysis System, version 9.3; Cary, NC, USA) fitted to a completely randomized design with a 4 × 2 factorial arrangement. The fitted model equation is as follows:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijk}$$

where y_{ijk} is the measured response variable, μ µ is the overall mean, α_i is the fixed effect of treatment (i = 1, 2, 3, 4), β_j is the fixed effect of diet (j = 1, 2), $\alpha\beta_{ij}$ is the interaction effect between treatment and diet, and e_{ijk} is the random error term. When differences were found ($P < 0.05$), means were separated using Tukey's test.

RESULTS AND DISCUSSION

Total gas production and composition at 24 h

No interaction between diet and yeast strain was observed (P > 0.05) for total gas production (TGP), CH_4, or CO_2 (Figure 1).

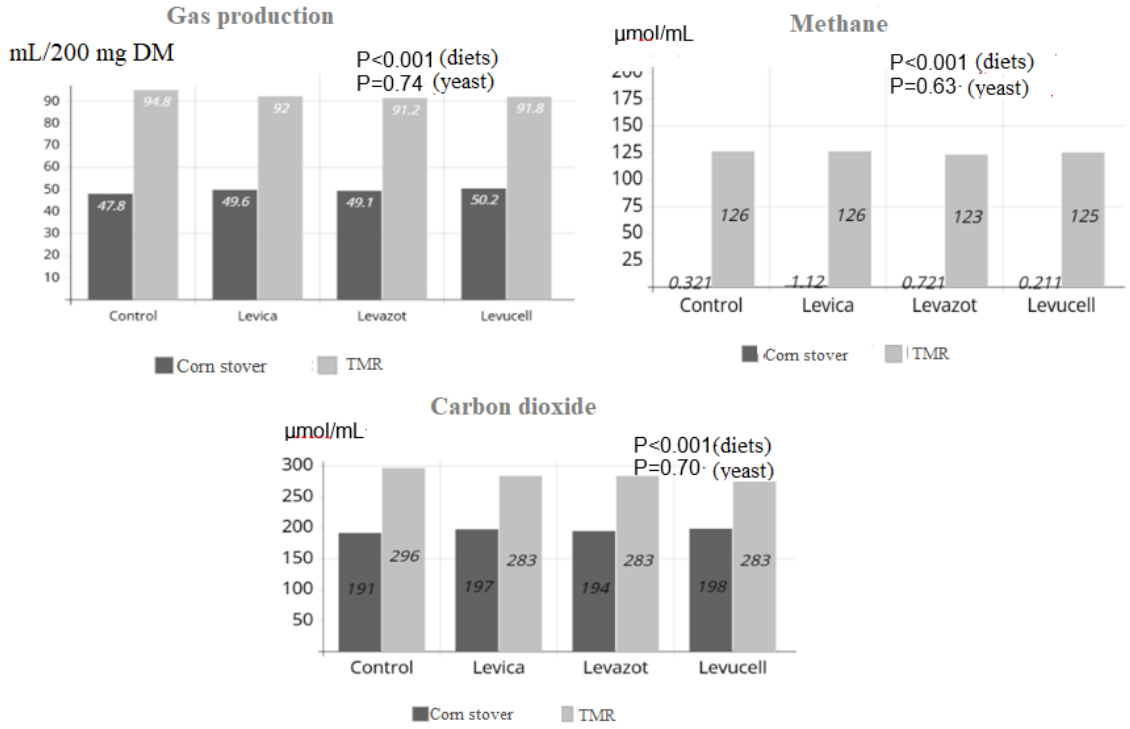


Figure 1. Total gas, methane, and CO_2 production after 24 h of *in vitro* ruminal fermentation of three yeast strains with corn stover and a total mixed ration

Results for diet type were different (P < 0.05) for these variables. Total gas production (TGP) and the molar concentrations of CH_4 and CO_2 were higher with the total mixed ration (TMR) compared to corn stover, highlighting the benefits of balanced diets for optimal ruminal microbial activity.

Corn stover, being low in CP (5.9 g kg^{-1} DM), limited microbial growth and fermentation, which is consistent with Ikhimioya (2008), who reported that this protein value does not provide the required ammonia levels for optimal ruminal microbial activity.

Yeast inclusion did not affect TGP. This agrees with González *et al.* (2023), who observed no effects on *in vitro* digestibility with *Pichia guilliermondii*. The efficacy of yeasts varies depending on diet characteristics, strain, and dose (Chaucheyras-Durand *et al*., 2008; Díaz *et al*., 2017). Some studies (Marrero *et al*., 2014; Castillo *et al*., 2016) have reported increased gas production with yeasts, attributed to greater degradation of structural carbohydrates, while others have found no effect. It is important to mention that yeasts typically act as modulators of the ruminal ecosystem (pH stabilization, improved use of

ammonia nitrogen, promotion of fibrolytic or amylolytic populations) rather than as direct stimulators of gasogenesis. Therefore, they may increase total VFA concentration and improve the fermentative environment without modifying total gas measured *in vitro* (Desnoyers *et al.,* 2009).

Furthermore, in diets with higher energy density, such as the TMR, a ceiling effect is possible where basal fermentation is already efficient, and the detectable improvement in gas attributable to the yeast effect is reduced. In these scenarios, the benefits of yeasts are better observed in VFA profile, pH, and nitrogen utilization than in accumulated gas (Blank & Wolffram, 2009).

Methane production was higher with the TMR, which is related to greater substrate degradation (Benchaar *et al*., 2024). Yeast inclusion had no impact on CH_4, reflecting the variability in the effects of yeasts on methane production across different studies (Wang *et al*., 2016; Mao *et al.,* 2016).

pH

The pH varied among yeast strains within the TMR ($P < 0.05$; Figure 2). The treatment with *Saccharomyces cerevisiae* (Levucell®) had the highest pH compared to the strains *Pichia guilliermondii* (Levica 27) and *Candida norvegensis* (Levazoot 15).

Diets with corn stover maintained pH around 6.4, which is typical of fibrous substrates (Marrero *et al*., 2006; Ruiz *et al*., 2016). These results suggest the activity of methanogenic bacteria, which utilize H_2 and CO_2, thus helping to stabilize ruminal pH (Galindo *et al*., 2010).

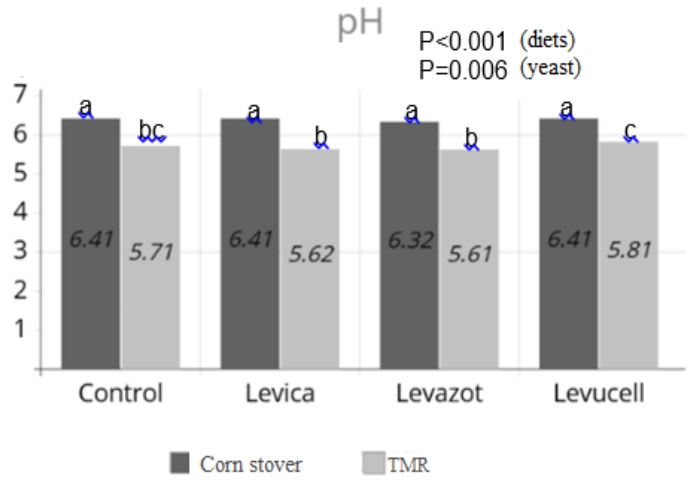


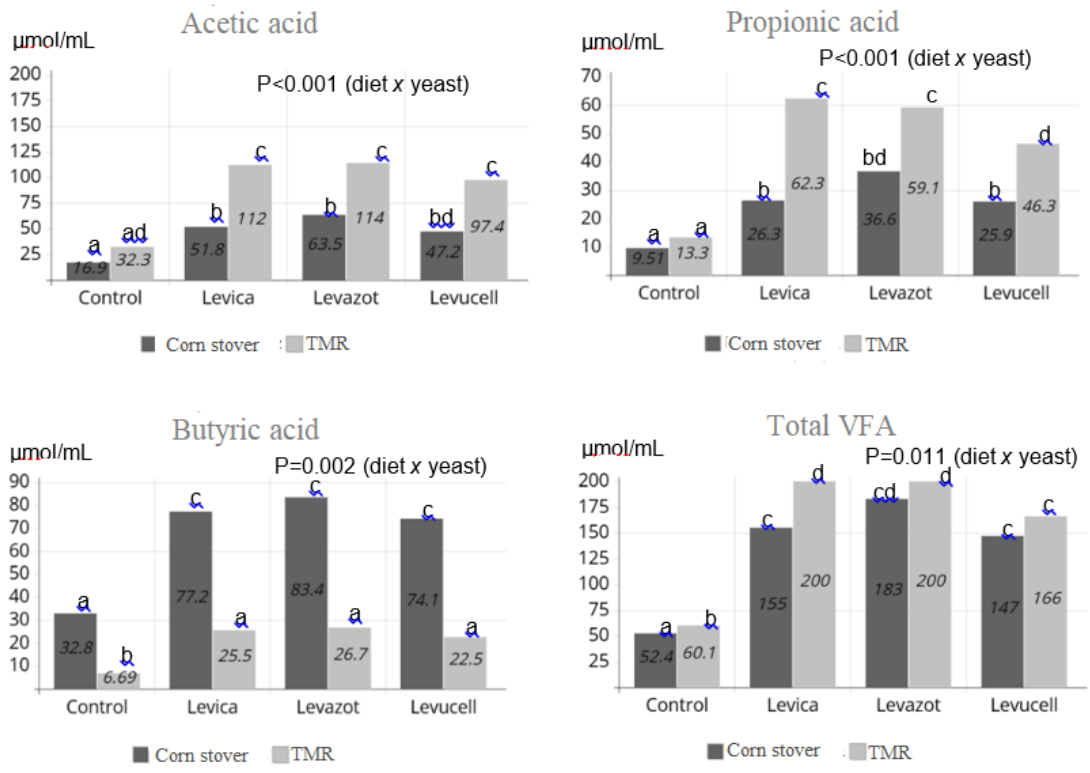
Figure 2. pH at 24 h of *in vitro* ruminal fermentation of three yeast strains with corn stover and a total mixed ration

For the TMR, pH ranged between 5.6 and 5.8, with *S. cerevisiae* showing the highest value (5.8). The effects of yeasts on ruminal pH depend on both the diet and the strain (Vohra *et al*., 2016; Anjum *et al*., 2018). High-concentrate diets generally exhibit lower

lactate accumulation and greater pH stability with yeast supplementation (Chaucheyras-Durand & Fonty, 2008). This is because yeast cultures are rich in organic acids (mainly malic acid) that stimulate the growth of *Selenomonas ruminantium*, a ruminal bacterium that consumes the lactic acid produced in the rumen and thus contributes to pH stabilization in this organ (Elghandour *et al.*, 2015).

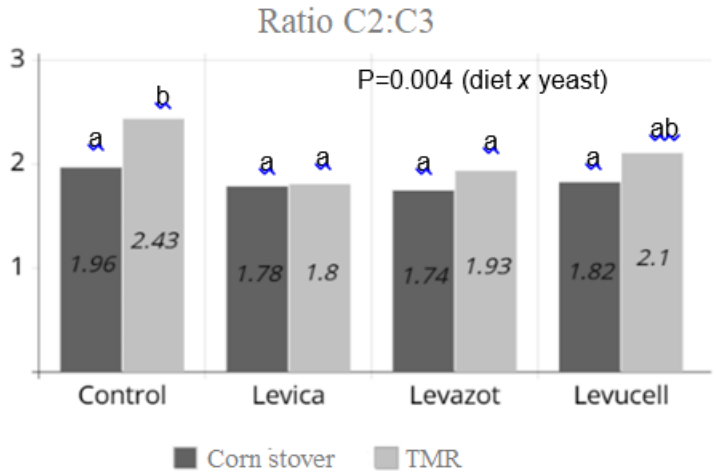
Volatile fatty acids

The interaction between yeast and diet was different (P < 0.05) for total volatile fatty acid (VFA) concentrations (Figure 3). The inclusion of yeasts increased total VFA concentration. Likewise, the molar concentrations of acetate, propionate, and butyrate were higher (P < 0.05) in the presence of yeasts. On the other hand, the acetate:propionate ratio decreased (P < 0.05) with the Levica 27 and Levazoot 15 strains in the TMR but was not modified (P > 0.05) in those treatments with corn stover (Figure 4).



[abc] Means (n = 32) with different letters in columns indicate a statistically significant difference (P < 0.05). TMR: total mixed ration; Control: fermentation medium without yeasts; Levica: *Pichia guilliermondii*; Levazoot: *Candida norvegensis*; Levucell: *Saccharomyces cerevisiae*

Figure 3. Volatile fatty acid (VFA) production at 24 h of *in vitro* ruminal fermentation of three yeast strains with corn stover and a total mixed ration



[abc] Means (n = 32) with different letters in columns indicate a statistically significant difference (P < 0.05). TMR: total mixed ration; Control: fermentation medium without yeasts; Levica: *Pichia guilliermondii*; Levazoot: *Candida norvegensis*; Levucell: *Saccharomyces cerevisiae*

Figure 4. Acetate:propionate ratio (C_2:C_3) at 24 h of *in vitro* ruminal fermentation of three yeast strains with corn stover and a total mixed ration

Some authors have reported similar results in which yeasts improve substrate degradation and VFA production (González *et al*., 2023; Ruiz *et al*., 2016).

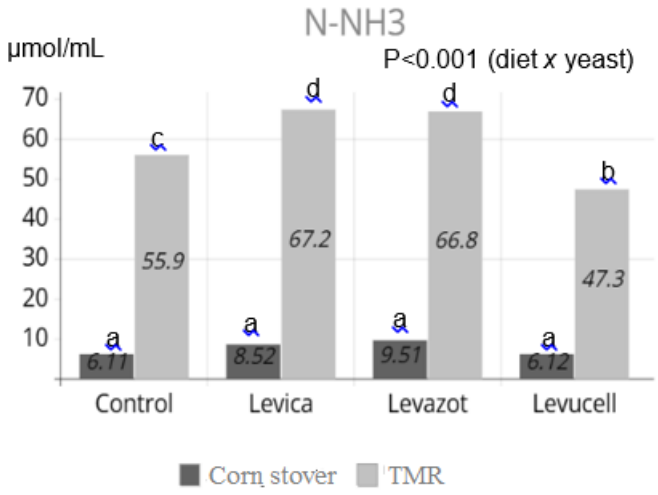
The increase in total and individual VFA reflects greater substrate degradation due to yeast action. In other studies, Fernandes *et al*. (2019) evaluated ruminal fermentation with the inclusion of yeasts isolated from the bovine rumen, and the strains CCMA 933 (*Candida rugosa*) and CCMA 970 (*Candida pararugosa*) showed better survival under ruminal conditions and promoted VFA production during ruminal fermentation. Butyric acid, an essential energy source for enterocytes, was increased, favoring better feed utilization (Miguel *et al.,* 2019). However, some studies have reported variable or null effects of yeast strains on VFA production (Marrero *et al*., 2006; Moya *et al*., 2009).

In the ruminal environment, yeasts have a short lifespan and are believed to be degraded or pass to the lower digestive tract a few hours after supplementation (Kung *et al*., 1997; Jouany, 2006). During their time in the rumen, yeasts contribute to total and individual VFA concentrations, although this contribution was not reflected in gas production (Benchaar *et al*., 2024).

N-NH_3 concentration

In Figure 5, it can be observed that N-NH_3 concentrations were lower (P < 0.05) with corn stover (6.1 µmol mL^{-1}) and increased with the TMR, reaching values of 47–66 µmol mL^{-1}.

Levica 27 and Levazoot 15 raised N-NH_3 concentrations compared to the controls, which is consistent with greater proteolytic activity (Oeztuerk, 2009).



[abc]Means (n = 32) with different letters in columns indicate a statistically significant difference (P < 0.05). TMR: total mixed ration; Control: fermentation medium without yeasts; Levica: *Pichia guilliermondii*; Levazoot: *Candida norvegensis*; Levucell: *Saccharomyces cerevisiae*

Figure 5. Ammoniacal nitrogen (N-NH_3) concentration at 24 h of *in vitro* ruminal fermentation of three yeast strains with corn stover and a total mixed ration

In contrast, S. cerevisiae reduced N-NH_3 concentrations, probably due to an increase in microbial protein synthesis (Anjum *et al*., 2018; Oeztuerk *et al*., 2005). As observed in other studies, the variability in N-NH_3 responses to yeasts depends on the strain and the type of diet (Guedes et al., 2008; Ruiz et al., 2016).

CONCLUSIONS

The inclusion of yeast-based additives (Levica 27, Levazoot 15, and Levucell® SC 10) had no effect on gas composition (CO_2 and CH_4), total gas production, or pH when corn stover and a total mixed ration were fermented *in vitro*. Total VFA production, together with the molar concentrations of acetic, propionic, and butyric acids, increased with the addition of yeasts in both substrates, with the best results observed for the total mixed ration.

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Errata Erratum

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