Volume 58, January-December 2024, ISSN: 2079-3480

ANIMAL SCIENCE

CUBAN JOURNAL OF AGRICULTURAL SCIENCE

Journal site: https://cjascience.com Cu-ID: https://cu-id.com/1996/v58e18

SACCHAROMYCES CEREVISIAE HYDROLYZATE: ITS EFFECT ON THE *IN VITRO* RUMINAL MICROBIAL POPULATION OF STAR GRASS (*CYNODON NLEMFUENSIS*)

HIDROLIZADO DE *SACCHAROMYCES CEREVISIAE*: SU EFECTO EN LA POBLACIÓN MICROBIANA RUMINAL *IN VITRO* DE PASTO ESTRELLA (*CYNODON NLEMFUENSIS*)

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An experiment was carried out with the hydrolyzate of *Saccharomyces cerevisiae*, registered as PROBIOLEV®, obtained in Cuba from the hydrolysis of distillery cream from the enzymatic crude of *Bacillus subtilis* E-44, to determine its effect on the ruminal microbial population of star grass (*C. nlemfuensis*). The *in vitro* gas production technique was applied, with modifications according to the proposed objective. Three treatments were established: A) star grass control, B) star grass + yeasts cream and C) star grass + yeasts hydrolyzate. The doses of cream and hydrolyzate were 100 mL/kg of concentrate/day, equivalent to 130 mg of (β 1.3) glucan/kg of concentrate. Samplings were done before incubation, and at 3 and 6 hours after incubation. The hydrolyzate activated the total bacterial populations (P=0.0088) by 62 %, which corresponds to 19.36 x 10^{11} CFU/mL more of bacteria, which means an increase in bacterial biomass. Cellulolytic bacteria were more numerous (P=0.0042) and the total number of cellulolytic fungi increased (P=0.0009) with the hydrolyzate. There was no effect of time on the total viable bacteria, cellulolytic bacteria and cellulolytic fungi. It is concluded that the hydrolyzate produces changes in the ruminal population, increasing the population of total viable bacteria, bacteria and cellulolytic fungi, an event that could favor the degradation of the fiber contained in the star grass.

Key words: *cellulolytic, fungi, proteolytic, PROBIOLEV ®, total bacteria*

Se realizó un experimento con el hidrolizado de *Saccharomyces cerevisiae*, registrado como PROBIOLEV®, obtenido en Cuba de la hidrólisis de la crema de destilería a partir del crudo enzimático de *Bacillus subtilis* E-44, para determinar su efecto en la población microbiana ruminal de pasto estrella (*C. nlemfuensis*). Se aplicó la técnica de producción de gases *in vitro*, con modificaciones según el objetivo propuesto. Se establecieron tres tratamientos: A) control de pasto estrella, B) pasto estrella + crema de levaduras y C) pasto estrella + hidrolizado de levaduras. Las dosis de crema e hidrolizado fueron 100 mL/kg de concentrado/día, equivalente a 130 mg de (β 1,3) glucano/kg de concentrado. Los muestreos se hicieron antes de incubar, y a las 3 y 6 horas después de la incubación. El hidrolizado activó las poblaciones totales de bacterias (P=0.0088) en 62 %, lo que corresponde a 19.36 x 10^{11} UFC/mL más de bacterias, lo que significa incremento en biomasa bacteriana. Las bacterias celulolíticas fueron más numerosas (P=0.0042) y el número total de hongos celulolíticos se incrementó (P=0.0009) con el hidrolizado. No hubo efecto del tiempo en las bacterias viables totales, celulolíticas y hongos celulolíticos. Se concluye que el hidrolizado produce cambios en la población ruminal, incrementa la población de bacterias viables totales, bacterias y hongos celulolíticos, evento que pudiera favorecer la degradación de la fibra contenida en el pasto estrella.

Palabras clave: *bacterias totales, celulolíticas, hongos, proteolíticas, PROBIOLEV®*

Received: December 28, 2023
Accepted: April 03, 2024
Conflict of interest: The authors declare that there is no conflict of interest between them.
CRediT authorship contribution statement: Juana Galindo: **Conceptualization, Data curation, Investigation, Formal analysis, Writing - original draft.** Niurca González: **Data curation, Investigation.** Yoandra Marrero: **Data curation, Investigation.** Marlen Rodríguez: **Data curation.** Magaly Herrera: **Formal analysis**

Introduction

Most studies with yeasts in ruminant feeding have been carried out using live yeasts (Marrero *et al*. 2015 and Marrero *et al.* 2020). However, the use of its hydrolyzates as additives in the ruminant feeding has been little explored, although it constitutes a promising way that make possible the activation of the ruminal microbial population, specifically the cellulolytic one, as well as the main sensitive sites of the complex ruminal ecosystem.

In *S. cerevisiae*, approximately 90 % of the cell wall is composed of polysaccharides, of 5-10 % of proteins and does not exceed 1 % of lipids, although the protein portion is relatively small and approximately 50 % of the cell wall is composed of glycoproteins (Klis *et al.* 2006 and Díaz *et al.* 2018). According to these authors, the main components of the cell wall of these yeasts are mannan proteins and β-glucans, in more or less equal proportions, and a small amount of N-acetylglucosamine.

Among the raw matters available for the production of yeast wall derivatives, the waste from the alcohol industry (distillery creams or yeast creams, vinasses) constitutes a very aggressive pollutant for the environment. Approximately 12-15 L of wastewater/L is produced from distilled alcohol, with an annual production of 2.6 million m^3. Its organic load is 60 - 150 g COD/L, approximately 1000 times greater than that permitted by environmental protection standards. These reasons justify its use to produce animal food through biotechnological procedures, which also constitutes a way to improve the environment and the ecological management of the alcohol industry.

Galindo *et al.* (2010, 2019) suggested the hypothesis that the hydrolyzate obtained from these creams can be used in ruminant feeding under the concept of microbial additives. Microbial additives activate the microbial population that lives in the ruminants' rumen, increasing the digestive use of food and the degradation of fiber (Valenciaga *et al.* 2019). In addition, they can reduce methane production to that level and in that way provide an environmental service in two ways. These antecedents offer the possibility of thinking that the use of an enzymatic hydrolyzate of *S. cerevisiae* could favor the action of ruminal microorganisms in animals that intake fibrous diets. Hence, the objective of this study was to determine the effect of *S. cerevisiae* hydrolyzate on the ruminal microbial population of star grass.

Materials and Methods

The experiment was carried out at Instituto de Ciencia Animal, San José de las Lajas municipality, Mayabeque province, Cuba, at 92 m o. s. l, 22°53' north latitude and 82°02' west longitude. The fercialitic soil, undulating, with 4.84% organic matter, 0.26 of total nitrogen, 40.59 ppm of phosphorus, 4.60 of calcium, 0.46 of magnesium and pH of 6.34.

Experimental treatments: A total of three treatments were evaluated according to a completely random design in a 3x3 factorial arrangement (three treatments and three sampling hours). The treatments were: A) control with star grass, B) star grass + yeast cream and C) star grass + yeast hydrolyzate. The cream and hydrolyzate doses were 100 mL/kg of concentrate/d, equivalent to 130 mg of (β 1.3) glucan/kg of concentrate.

The hydrolyzate and yeast cream were sprayed onto the commercial concentrate for dairy cows at the indicated dose. After homogeneous mixing, the product was ready for it use.

The remainder of the experimental diet consisted on star grass. Its chemical composition was 7.26, 74.57, 10.11, 0.42 and 0.18 for CP, NDF, ash, calcium and phosphorus, % DM, respectively (AOAC 2016).

The yeast cream, raw matter from which the hydrolyzate was obtained, was removed from the Jesús Rabí sugar mill distillery in Matanzas province. The characterization of the chemical composition of the hydrolyzate was carried out according to the techniques described by the AOAC (2016) and DM, OM, ash and TP are indicated. The fibrous fractions were analyzed by the Goering and van Soest (1970) procedure. The crude protein (CP) was determined by the Kjeldahl method. Total reducing sugars (RS) were analyzed according to the 3.5-dinitro salicylic acid (DNS) colorimetric technique, where glucose was used as a standard sugar (Bernfeld 1955). Total carbohydrates quantification (TC) was performed using the phenol-sulfuric acid colorimetric technique (Dubois *et al.* 1956). The values coincide with those reported by Rodríguez *et al.* (2017): 19.39, 17.83, 82.17, 40.28, 38.54, 7.93 and 6.09 % of DM, ash, OM, CP, TPLW, RS, TC. Its pH was 5.56 and the TP/CP ratio was 95.68.

Experimental procedure: The experiment was conducted under *in vitro* conditions. The Theodorou *et al.* (1994) technique was used. As described, 100 mL sealed bottles were used to incubate food samples in ruminal fluid and a buffer medium. In each bottle, 0.5 g of the material to be evaluated (star grass), 50 mL of the mixture consisting of rumen fluid and buffer solution in a 1:3 ratio and the concentrate in equivalent quantities with the microbial additive (hydrolyzate or cream) were introduced. The fermentation bottles were previously sterilized at 121 °C and 1.5 a.t.m. for 15 min. The procedure was carried out in a CO_2 atmosphere to ensure strict anaerobic conditions. The ruminal inoculum was obtained from four crossbred cows stabled and rumen cannulated, fed a diet of forage grass and 2 kg/day of commercial concentrate and free access to water. The ruminal fluid sample was collected through the cannula, with the help of a vacuum pump. It was kept in a hermetically sealed thermo until it was transferred to the rumen microbiology and molecular genetics laboratory at Instituto de Ciencia Animal. It was then filtered through

muslin. To form the mixture to be fermented, the total ruminal fluid from the four Holstein crossbred cows was used, with the aim of eliminating the animal effect.

Sampling to determine microbial populations was carried out before incubation and at 3 and 6 hours after incubation (hour 0).

Culture techniques and microorganisms counting: The Hungate (1950) culture technique was used in roll tubes and under strict anaerobic conditions. The inoculation of total viable bacteria and cellulolytic bacteria was carried out in the culture media of Caldwell and Bryant (1966), modified by Elías (1971) and Galindo (1988). For the determination of the fungal population, the Joblin culture medium (1981) was used.

Statistical analysis: For data analysis, the methodology proposed by Herrera *et al.* (2015) was used. The theoretical assumptions of the analysis of variance were tested: normality of errors using the Shapiro and Wilk (1965) test, homogeneity of variance using Levene (1960) test for the variables total bacteria, proteolytic bacteria, cellulolytic bacteria and cellulolytic fungi. All variables failed to fulfill these assumptions.

Subsequently, data transformation was used and did not improve their fulfilling, so analysis of variance was used, according to a non-parametric completely random design in a 3x3 Kruskal-Wallis factorial arrangement. In cases where the interaction was not significant, the main effects were reported and Fisher's LSD (1935) test was applied.

For data processing, the Infostat statistical package (Di Rienzo 2012) was used. In cases where the interaction was not significant, the main effects were recorded.

Results

The yeast hydrolyzate evaluated has glucan oligosaccharide concentrations of 3.34 ± 0.35%. The cream from which it is made has similar concentrations of the above biomolecules, but they are only found in the yeast walls and are not available to the ruminal microorganisms in the product. From these values, the dose of 100 mL/kg of concentrate is equivalent to approximately 130 mg of β (1.3) glucan/kg of concentrate.

From the analysis of the results it can be reported that there was no significant interaction between the populations of total viable bacteria, proteolytic, cellulolytic and cellulolytic fungi with the fermentation time. Table 1 shows the main effects of treatments on these microbial groups. Yeast hydrolyzate activated the total viable bacterial populations of the rumen (P=0.0088) and its population differed from that found when supplementation was not used. The yeast cream produced intermediate values of total bacteria, without showing differences between the control treatments and with hydrolyzate.

In terms of viable rumen bacterial population numbers, the inclusion of doses as small as 100 mL/kg concentrate/d produces increases of 62 % of the total rumen bacterial population, which is equivalent to 19.36 x 10^{11} CFU/mL more bacteria, which means an increase in bacterial biomass.

The population of cellulolytic bacteria was significantly higher (P=0.0042) when was supplemented with yeast hydrolyzate compared to the cream from which it originated and to the control treatment without supplementation, which did not differ from each other (table 1). The total number of cellulolytic fungi increased (P=0.0009) with the presence of the hydrolyzate in the animals concentrate, when related to the treatment without supplementation and that supplemented with the *S. cerevisiae* yeast cream. The yeast hydrolyzate and the cream that gave rise to it had no effects on the population of proteolytic bacteria in the rumen.

In the research, there was not recorded effect of fermentation time on the populations of total viable bacteria, cellulolytic bacteria and cellulolytic fungi in the rumen (table 2). However, as shown in the table, proteolytic bacteria showed the lowest populations at 3 h after fermentation start of (P=0.0269).

Table 1. Effect of yeast hydrolyzate and the cream that gave rise to it on some physiological groups of rumen microorganisms

Treatments Variables	Control	Yeasts cream	Yeasts hydrolyzate	Significance
Total viable bacteria, 10^{11} CFU/mL	30.94^{b} (4.48) SD=5.00	41.76^{ab} (7.70) SD=7.69	50.30^{a} (9.70) SD=7.23	P=0.0088
Proteolytic bacteria, 10^{6} CFU/mL	41.06 (14.48) SD=15.79	36.76 (10.56) SD=5.67	41.06 (12.30) SD=5.48	P=0.3974
Cellulolytic bacteria , 10^{6} CFU/mL	3.91^{b} (3.74) SD=3.15	39.35^{b} (5.11) SD=2.70	52.22^{a} (8.48) SD=7.23	P=0.0042
Cellulolytic fungi, 10^{5} TFU/mL	30.80^{b} (2.33) SD=2.66	39.04^{b} (2.52) SD=1.63	53.17^{a} (4.63) SD=3.38	P=0.0009

() Means of the original data

$^{a, b}$: means with different letters in the same row differ at p<0.05

CFU: colony -forming units, TFU: talo-forming units

Table 2. Effect of fermentation time on some physiological groups of rumen microorganisms with yeast hydrolyzate and the cream that gave rise to it as ruminal activators

Indicators	Sampling time, h			Significance
	0	3	6	
Total viable bacteria, 10^{11} CFU/mL	45.83 (8.96) SD=5.79	38.76 (6.85) SD=7.47	38.41 (6.07) SD=5.72	P=0.3934
Proteolytic bacteria, 10^{6} CFU/mL	46.87[a] (13.93) SD=9.71	31.24[b] (11.26) SD=13.76	44.89[a] (12.15) SD=5.63	P=0.0269
Cellulolytic bacteria, 10^{6} CFU/mL	43.31 (4.59) SD=2.63	44.46 (7.41) SD=7.45	35.71 (4.59) SD=3.88	P=0.2913
Cellulolytic fungi, 10^{5} TFU/mL	43.54 (3.59) SD=3.02	45.28 (3.78) SD=3.37	34.19 (2.11) SD=1.53	P=0.1217

Legend: () Means of the original data

[a, b]: means with different letters in the same row differ at $p<0.05$

CFU: colony -forming units, TFU: talo-forming units

Discussion

Many mechanisms have been described about how small doses of yeasts added to the diet of ruminants can stimulate microbial growth in the rumen (Marrero *et al*. 2015 and Marrero *et al.* 2020), a phenomenon that is linked to the quality and type of diet that the animals intake.

It has been reported that yeast cell walls may constitute approximately 30 % of the dry matter of the cell. At the structural level, it is made up of three groups of polysaccharides: mannose polymers or mannoproteins, up to 50 % of the DM; glucose polymers or β-glucans (1.3/1.6), up to 55 % of the DM and, to a lesser extent, N-acetyl-glucosamine or chitin polymers in 6 % of the DM of the cell wall (Díaz *et al.* 2017).

One of the mechanisms that facilitate these biotechnological products is that the activating effect has its origin on the growth factors that yeasts provide for ruminal microorganisms, such as wall polysaccharides, B-complex vitamins, short-chain fatty acids (SCFA) and branched-chain fatty acids, provitamins and micronutrients (Chaucheyras-Durand 2006 and Díaz *et al.* 2017).

The stimulation of microbial growth, according to Chaucheyras-Durand (2006) and Díaz *et al.* (2017), may be associated with the presence of two growth factors, located in the different cellular fractions of the yeast. One of them is thermolabile and has a lipid origin, and the other is thermostable, with a possible peptide origin. Rossi *et al.* (2004) isolated two peptide fractions rich in lysine and histidine from *S. cerevisiae*, which were effective in stimulating the growth of the ruminal bacteria *Megasphaera elsdenii,* the main bacteria that ferments lactate in the rumen.

Other theories were postulated by those who found that malate present in yeasts is capable of stimulating the growth of some Gram-negative bacteria in the rumen. Newbold *et al.* (1996) also found an increase in the population of cellulolytic bacteria and fiber digestion. All this results in an increase in microbial protein in the rumen, which helps to explain the beneficial effects observed when live yeasts or their hydrolyzates are included in the diet of animals.

Chaucheyras-Durand *et al.* (2008) showed the effectiveness of yeasts in influencing the growth and enzymatic activity of rumen fiber-fermenting microorganisms and reported *in vitro* stimulation of the fungus *Neocallimastix frontalis* by the provision by yeasts of thiamine and vitamin required by rumen fungi for zoosporogenesis. Also, these authors showed that yeasts stimulate the growth and enzymatic activity of glucosidase and hydrolase enzymes. The mentioned enzymes are present in fiber-fermenting bacteria, such as *Fibrobacter succinogenes*, *Ruminococcus spp* and *Butyrivibrio fibrisolvens*, due to the supply of nutrients and vitamins they provide to this fibrolytic population.

In general, there are few studies that analyze the effect of yeast hydrolyzates on ruminant animals. Its specific mechanisms of action are not clearly defined. Galindo *et al*. (2010), when evaluating the effect of two levels of enzymatic hydrolyzate of the *S. cerevisiae* yeast on the ruminal microbial population of animals that intake *Cenchrus purpureum* cv. Cuba CT-115, reported increases in the populations of total viable bacteria and cellulolytic bacteria under *in vitro* conditions. The level of 100 mL/kg of concentrate/day was the one that allowed obtaining the greatest increase in the population of total viable bacteria.

Unfortunately, the concentration of short-chain fatty acids (SCFA) could not be determined in this experiment. In this regard, Kettunen *et al.* (2016) and Oeztuerk *et al.* (2016) reported the efficacy of two *S. cerevisiae* yeast hydrolyzates, which stimulated the *in vitro* fermentation of different substrates and increased SCFA production. This aspect, in particular, should be the subject of future studies, since with this same product Díaz *et al.* (2011) obtained increases in the total concentration of SCFA, propionic and butyric acid in sheep.

More recently, Díaz *et al.* (2017) evaluated the effect of supplementation with *S. cerevisiae* hydrolyzate on fermentation indicators in RUSITEC fermenters with alfalfa hay and concentrate in a 1/1 ratio. These authors observed increases in microbial growth in the rumen, especially cellulolytic bacteria, with the addition of the hydrolyzate to the diet. This result corresponds to what has been suggested in the scientific literature about the activating effect of yeast strains on populations of total viable bacteria and cellulolytic bacteria, when these strains are used as additives in diets for ruminants (Herrera 2014 and Casas 2018).

If it is take into account that the enzymatic hydrolyzate of *S. cerevisiae* is mainly composed of low molecular weight peptides, glucan and mannan oligosaccharides, vitamins, amino acids, nitrogenous bases, nucleosides and nucleotides, among other components, then it is evident that this product can exert a stimulating effect on the ruminal microbial population, just like live yeast strains, due to the presence of the mentioned substances present in the enzymatic hydrolyzate. This could directly affect the increase in the microbial population, specifically the cellulolytic one, and, as a consequence, increases in the ruminal degradability of the nutrients in the forage that the animals receive are obtained (Valenciaga *et al*. 2019).

Conclusions

The yeast produces hydrolyzate make changes in the ruminal population, increasing the populations of total viable bacteria, bacteria and cellulolytic fungi, an event that could favor the degradation of the fiber contained in star grass.

Acknowledgments

Thanks to Onidia Moreira, Jorge Hernández, Aned Capó, Lucía Sarduy and Osvaldo Tuero for their valuable contributions to the research.

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