Technical Note

Yeasts naturally occurring in sorghum silage

Flávia Oliveira Abrão[1*], Adriana Oliveira Medeiros[2], Carlos Augusto Rosa[3], Luciana Castro Geraseev[4], Norberto Mário Rodriguez[4], Eduardo Robson Duarte[4]

[1]Instituto Federal Goiano, Goiás, Brasil. [2]Universidade Federal da Bahia, Campus Universitário de Olinda, Bahia, Brasil. [3]Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Minas Gerais, Brasil. [4]Universidade Federal de Minas Gerais, Instituto de Ciências Agrárias, Minas Gerais, Brasil. *E-mail: flavia.abrao@ifgoiano.edu.br

ABSTRACT

Few studies have evaluated the natural occurrence of yeasts in forage silages. This study characterized the population of yeasts in sorghum silage produced in northern Minas Gerais State, Brazil. *Sorghum* spp. was harvested at 130 days of culture and biomass were ensiled in surface silos. Four silage samples were collected at 60 and at 120 days. Chemical and pH analysis were conducted, and yeast quantification was obtained using the pour plate technique on Sabouraud agar with chloramphenicol. After incubation for seven days, mean values of 4.7 x 10^5 and 4.3 x 10^6 colony forming unit per gram (CFU.g^{-1}) were observed for silage of 60 and 120 days fermentation, respectively. Thirty-one yeast isolates were identified with *Pichia membranifaciens* as the predominant specie (P<0.05), able to utilize ethanol. Mycelial fungi were not isolated. Considering the chemical composition and pH in evaluated silage and the significant yeast population in the samples, future studies should evaluate their role during the fermentation of forage.

Key words: ensiling, fermentation, forage conservation, *Pichia* spp., semiarid.

Las levaduras de origen natural en el ensilaje de sorgo

RESUMEN

Pocos estudios han evaluado la presencia natural de levaduras en los ensilajes de forraje. En este estudio se caracterizó la población de levaduras en el ensilaje de sorgo producidas en el norte de Minas Gerais, Brasil. *Sorghum* spp. fue cosechado a los 130 días de cultivo y la biomasa fue ensilada en silos de superficie. Se colectaron cuatro muestras de ensilaje a los 60 y 120 días. Se realizó análisis químico y de pH y se obtuvo la cuantificación de levaduras mediante la técnica de vertido en placa en agar de Sabouraud con cloranfenicol. Después de siete días de incubación, se observaron valores medios de 4,7 x 10^5 y 4,3 x 10^6 unidades formadoras de colonias por gramo (UFC.g^{-1}) para el ensilaje de 60 y 120 días de fermentación, respectivamente. Se identificaron treinta y un aislamientos de levaduras, con *Pichia membranifaciens*, como especie predominante (P<0,05), capaz de utilizar etanol. No se aislaron hongos fi lamentosos. Considerando la composición química y el pH del ensilaje evaluado y la significativa población de levaduras en las muestras, se debe evaluar su participación durante la fermentación del forraje en estudios futuros.

Palabras clave: ensilado, fermentación, conservación de forraje, *Pichia* spp., semiárido.

Aprobado: junio 2017

INTRODUCTION

Ensiling of fodder is important in ruminant farming for preserving forage for dry or cold periods and maintaining or minimizing loss of nutritional value. Silage quality depends on the forage and microorganisms present during fermentation. Research has reported involvement of fungi and yeasts in silage deterioration. In sugarcane silage, for example, the occurrence of yeasts is high, promoting the conversion of soluble carbohydrates into ethanol, carbon dioxide and water, leading to energy losses and excessive reduction of lactic and acetic acids. These microorganisms have a high resistance to changes in pH and can release CO_2 via sugar metabolism, with high ethanol production and high disappearance of soluble carbohydrates, resulting in loss of dry matter (Rossi and Delaggio 2007, Carvalho *et al*. 2014, Motta *et al*. 2015).

Sorghum silage is an important ruminant fodder in semi-arid regions, and their high number of panicles contributes to the nutritional value. However, alterations in the fermentation process can reduce the dry matter digestibility (Rodrigues *et al*. 2002). The predominance of yeast species during ensiling of grass has been associated with oxygen level, with aeration leading to greater diversity of yeasts. Some studies report that yeast and mycelial fungi can contribute to the aerobic deterioration of silage (Pedroso *et al*. 2005).

Studies have demonstrated yeast diversity during ensilage, and assimilation of carbon and nitrogen sources can be observed (Ávila *et al*. 2010, Carvalho *et al*. 2017, Duniere *et al*. 2017). The biochemical and physiological characterization of yeasts in the silage process can provide information about metabolism and products derived and their direct and indirect influence on silage quality (Ávila *et al*. 2010).

Few studies have evaluated the natural occurrence of yeasts in silage of forage crops such as corn, sorghum, and grass to describe their role during the ensilage process (Pedroso *et al*. 2005, Ávila *et al*. 2010, Carvalho *et al*. 2017, Duniere *et al*. 2017). The aim of this study was to characterize the yeast populations naturally present in sorghum silage of good quality.

MATERIAL AND METHODS

The studied sorghum silage was produced in Montes Claros city in northern Minas Gerais, Brazil, (16° 43' S 44° 52´ W). The annual mean temperature of the area is 24.2ºC, with hot dry weather and a dry season from April to October. Ten kilograms of seed per hectare of *Sorghum* spp. var. volumax and 300 kg of 4:14:8 (NPK) fertilizer were used during planting. After 130 days of cultivation, the forage was ensiled in a surface silo with dimensions 15.0 x 4.0 x 1.5 m.

Four samples of 1500 g each were collected at 60 and at 120 days of fermentation. This period (days) was determined according to the period of supply of the feed to the animals. The materials were analyzed for chemical composition according to the procedures described in Van Soest *et al.* (1991) and Silva and Queiroz (2009) with three replications of each sample. Dry matter (DM), ethereal extract (EE), crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined. As recommended, CP was determined following the Kjeldahl method, and the EE was determined after procedures in Goldfisch type extractor with petroleum ether. The pH of the ensiled material was measured in aqueous extract with a digital pH meter (Silva and Queiroz 2009).

For microbiological analysis, four 500 g subsamples of each initial sample were obtained with heat-sterilized forks and stored in Kraft paper bags, sterilized by autoclaving. For inoculation, 50 g of the material was diluted in 450 ml sterile saline solution mixed for 20 min, and sequential decimal dilutions were prepared for the quantification. The colonies were obtained using the pour plate technique in Sabouraud agar medium with chloramphenicol (300 $mg.L^{-1}$), with two replicates for each subsample. After seven days of cultivation at 37°C, the number of $CFU.g^{-1}$ was evaluated for each plate based on morphological characteristics of each yeast type (Kurtzman and Fell 1998).

The selection and re-isolation of each yeast type was carried out on up to eight distinct morphotypes per sample in Sabouraud agar medium. For identification, 31 yeast isolates

were characterized based on morphology and assimilation and fermentation of various carbon sources. The biochemical and physical identification was made according to the taxonomic keys in Kurtzman and Fell (1998). The yeasts were categorized by morphology and biochemical analysis with respect to fermentative capacity and assimilation of carbon and nitrogen (at 10%, respectively). To establish the biochemical profile, growth of the yeasts was evaluated in glucose, galactose, L-sorbose, maltose, sucrose, cellobiose, trehalose, lactose, melibiose, raffinose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, D-ribose, L-rhamnose, ethanol, glycerol, erythritol, adonitol, galactitol, D-mannitol, D-glucitol, salicin, DL-lactate, succinate, citrate, m-inositol, methanol, hexadecane, xylitol, gluconate, isopropanol, ethylacetate, acetone, N-acetylglucosamine, glucosamine, lysine carbonate, nitrite, nitrate and resistance to NaCl, acetic acid, and cyclohexamide. The frequencies of the species type identified were compared by *Chi-squared* test at 5% significance.

RESULTS AND DISCUSSION

After 130 days of cultivation, the collected forage showed 33.4% of dry matter and the chemical composition of the silage after 120 days fermentation showed a mean of 32.4% of dry matter with 63.3% neutral-detergent fiber, 38.5% acid detergent fiber, 7.9% crude protein, 2.4% ether extract, and pH of 3.4. These characteristics indicated good quality by sorghum silage standards (Silva and Queiroz 2009). In other studies in similar region, the freshly cut of sorghum cultivar volumax showed, at collection point to silage production (with a cut age of approximately 90 days), 33.3- 34.0% dry matter and 62.3 - 62.5% NDF, 33.7 - 46.5% ADF, 5.9 - 8.0% CP, and 1.9 - 2.8% EE (Avelino *et al*. 2011), suggesting little reduction in chemical composition during silage process.

The growth of yeasts was observed in all samples. The mean yeast CFU.g^{-1} were 4.7 x 10^5 and 4.3 x 10^6 in sorghum silage fermented 60 and 120 days, respectively. These results demonstrated high yeast populations in sorghum silage with good nutritional and physical chemical characteristics (Coutinho 2009; Carvalho *et al*. 2014). Mycelial fungi were not isolated in any samples, suggesting good preservation.

Pichia membranifaciens accounted for 90.4% of the isolates and was the most frequently observed yeast ($P<0.05$). *Candida* sp. and the species *Galactomyces reessii* represented 6.4 and 3.2% of isolates, respectively. At 60 days fermentation, of 19 isolates, 84.2% were *P. membranifaciens*, 10.5% were *Candida* sp., and 5.3% were *G. reessii*. At 120 days of fermentation, all 12 isolates were *P. membranifaciens*, indicating the ability of this species to survive during all period of fermentation and storage.

Biochemical characterization of isolates showed all *P. membranifaciens* samples able to use ethanol, indicating a possible beneficial effect in reducing loss of dry matter in the ensiling process. The presence of ethanol in silages represents energy loss, reducing the animal consumption or by volatilization corresponding to 20 - 30% of the ethanol produced in silages (Alli *et al*. 1982).

Additionally, the microbial mass of this yeast may increase the protein content of the silage. The main components of the yeast biomasses have been the protein (48.5%). Their protein amino acids profile is adequate for human and animal nutrition, supplying all the essential amino acids, being particularly rich in lysine (Caballero-Cordoba and Sgarbieri 2000).

According to Santos and Marquina (2004), the yeast *P. membranifaciens* produces a killer toxin, with maximum effectiveness occurring at pH 4 and temperatures above 20° C. Studies show its antifungal activity against pathogenic fungi in grapevines (Masih *et al*. 2001, Belda *et al*. 2017). Killer toxins are proteins or glycoproteins that are lethal to sensitive strains of the same species and a different variety of other yeast genera. Regardless of certain possible additional effects, the killer toxin of *P. membranifaciens* acts by disrupting plasma membrane electrochemical gradients (Santos *et al*. 2005).

In addition, yeasts must cope with different adverse environmental conditions, including heat

shock, oxidative stress, high osmolarity, extreme pH values, nutrient availability, and toxins from plants or microorganisms, as well as heavy metals and different xenobiotics. Yeasts have therefore adapted to growth under these conditions by developing a variety of protective mechanisms ranging from general stress responses to highly specific regulatory pathways (Santos *et al.* 2005).

The predominance of *P. membranifaciens* in sorghum silage may be explained by these factors, increasing its competitiveness and reducing the number of other yeast species. Studies are needed to assess its role on fermentation process and elimination of other fungi during the ensiling process.

The major limitation of sugarcane silage is the high production of ethanol, which is mainly produced by yeast sucrose fermentation (Pedroso *et al.* 2005). However, no correlation between the total yeast population in sugarcane silage and the amount of ethanol produced during fermentation has been observed (Sousa *et al*. 2008).

In other experiments, the presence of yeasts has been evaluated in different crops. In a study of sugarcane silage, Bravo-Martins *et al.* (2006) reported that yeast populations in five varieties of sugarcane silage, without additives, were on average 3.5 x 106 CFU.g-1 of silage, and with the addition of 1% ammonium sulfate and 1% urea, were 5.9 and 3.2 x 105 CFU.g-1 silage, respectively. Ávila *et al.* (2010) evaluated the microorganisms and identified the yeast species present during the ensilage of different sugarcane (Saccharum spp.) cultivars. Lactic acid bacteria predominated during the ensiling process of sugarcane, although yeasts were present at relatively high population levels throughout the fermentation period. The detected yeast species varied according to sugarcane cultivar and time of fermentation. Torulaspora delbrueckii was the predominant yeast, followed by Pichia anomala and Saccharomyces cerevisiae. These species, found in sugarcane silage, differed from those observed in sorghum silage of this study.

Silage of maize, alfalfa, and white clover from various regions of Italy were evaluated and contained only the lactate-assimilating species *Candida apicola*, *Candida mesenterica,* and *Pichia fermentans* (Rossi and Dellaglio 2007). In research with baled grass silage *P. fermentans*, *P. anomala,* and *Geotrichum* spp. were described as the yeasts mostly present (O'Brien *et al*. 2007).

Mycological studies are essential for evaluating possible yeast inoculant effectiveness, under varying production conditions. The selection and identification of potentially beneficial yeast strains in the ensiling process could favor the selection of the most effective inoculants.

CONCLUSIONS

Sorghum silage with good quality and stored 120 days shows yeast population > 10^6 CFU.g^{-1} and its predominant species is *P. membranifaciens* able to use ethanol. Future studies should evaluate the role of this and other yeasts in forage silage to elucidate its possible beneficial or detrimental effects.

LITERATURE CITED

Avelino, PM; Neiva, JNM; de Araújo, VL; Alexandrino, E; Bomfim, MAD; Restle, J. 2001. Chemical composition of silage sorghum hybrids grown at different densities. Revista Ciência Agronômica 42(1):208-215.

Alli, I; Baker, BE; Garcia, G. 1982. Studies on the fermentation of chopped sugarcane. Animal Feed Science and Technology 7(4):411-417.

Ávila, CL; S Bravo Martins, CEC; Schwan, RF. 2010. Identification and characterization of yeasts in sugarcane silages. Journal of Applied Microbiology 109(5):1677-1686.

Bravo-Martins, CEC; Carneiro, H; Castro-Goméz, RJH; Figueiredo, HCP; Schwan, RF. 2006. Chemical and microbiological evaluation of ensiled sugar cane with different additives. Brazilian Journal of Microbiology 37(4):499-504.

Belda, I; Ruiz, J; Alonso, A; Marquina, D; Santos, A. 2017. The Biology of *Pichia membranifaciens* Killer Toxins. Toxins. 9(4):112.

Caballero-Córdoba, GM; Sgarbieri, VC. 2000. Nutritional and toxicological evaluation of yeast (*Saccharomyces cerevisiae*) biomass and a yeast protein concentrate. Journal of the Science of Food and Agriculture 80(3):341-351.

Carvalho, FAL; Queiroz, MAA; Silva, JG; Voltolini, TV. 2014. Características fermentativas na ensilagem de cana-de-açúcar com maniçoba. Ciência Rural 44(11):2078-2083.

Carvalho, BF; Avila, CLS; Pereira, MN; Schwan, RF. 2017. Methylotrophic yeast, lactic acid bacteria and glycerine as additives for sugarcane silage. Grass and Forage Science 72(2):193-368.

Coutinho, HS. 2009. Silagens de milho e sorgo tratadas com inoculante microbiano à base de bactérias homo e heteroláticas (em línea). Dissertacao Mestrado. Viçosa, Brasil, Universidade Federal de Viçosa. Consultado 15 ene. 2017. Disponíble: http://locus.ufv.br/bitstream/handle/123456789/5980/texto%20completo.pdf?sequence=1&isAllowed=y

Duniere, L; Xu, S; Long, J; Elekwachi, C; Wang, Y; Turkington, K; Forster, R; McAllister, TA. 2017. Bacterial and fungal core microbiomes associated with small grain silages during ensiling and aerobic spoilage (em linea). BMC Microbiology 17(1):50. Consultado 15 ene. 2017. http://doi.org/10.1186/s12866-017-0947-0.

Kurtzman, CP; Fell, JW. 1998. The yeast: a taxonomic study. (4° ed). USA, Elsevier. 1076 p.

Masih, EI; Slezack-Deschaumes, S; Marmaras, I; Barka; EA; Vernet, G; Charpentier, C; Adholeya, A; Paul, B. 2001. Characterisation of the yeast *Pichia membranifaciens* and its possible use in the biological control of botrytis cinerea, causing the grey mould disease of grapevine. FEMS Microbiology Letters 202(2):227-232.

Motta, TP; Frizzarin, A; Martins,T; Miranda, MS; Arcaro, JRP; Ambrósio, LA; Pozzi, CR. 2015. Estudo sobre a ocorrência de fungos e aflatoxina B1 na dieta de bovinos leiteiros em São Paulo. Pesquisa Veterinária Brasileira 35(1):23-28.

O'brien, M; O'kiely, M; Forristal, PD; Fuller, HT. 2007. Quantification and identification of fungal propagules in well-managed baled grass silage and in normal on-farm produced bales. Animal Feed Science Technology 132(3-4):283-297.

Pedroso, AF; Nussio, LG; Paziani, SF; Loures, DRS; Igarasi, MS; Coelho, RM; Packer, IH; Horii, J; Gomes, LH. 2005. Fermentation and epiphytic microflora dynamics in sugar cane silage. Scientia Agrícola 62(5):427-432.

Rodrigues, PHM; Senatore, AL; Andrade, SJT; Ruzante, JM; Lucci, CS; Lima, FR. 2002. Effects of microbial inoculants on chemical composition and fermentation characteristics of sorghum silage. Revista Brasileira de Zootecnia 31(6):2373-2379.

Rossi, F; Delaggio, F. 2007. Quality of silages from Italian farms as attested by number and identity of microbial indicators. Journal of applied microbiology 103(5):1707-1715.

Santos, A; Marquina, D. 2004. Ion channel activity by *Pichia membranifaciens* killer toxin. Yeast 21(2):151-162.

Santos, A; Álvarez, MM; Mauro, MS; Abrusci, C; Marquina, D. 2005. The Transcriptional Response of *Saccharomyces cerevisiae* to *Pichia membranifaciens* Killer Toxin. The Journal of Biological Chemistry 280(51):41881-41892.

Silva, DJ; Queiroz, AC. 2009. Análise de Alimentos: Métodos químicos e Biológicos. (3° ed). Viçosa, Brasil, Universidade Federal de Viçosa. 235 p.

Sousa, DP; Mattos, WRS; Nussio, LG; Mari, LJ; Ribeiro, JL; Santos, MC. 2008. Chemical additive and microbial inoculants effects on the fermentation and on the control of the alcohol production in sugarcane silages. Revista Brasileira de Zootecnia 37(9):1564-572.

Van Soest, PJ; Robertson, JB; Lewis, BA. 1991. Methods for dietary fibre, neutral-detergent fibre and non-starch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74(10):3583-3597.