RESEARCH ARTICLE

Marcela J. González [1]
Jorge Olivera [1]
Pablo Chilibroste [2]
Stella Reginensi [1]

Authors' addresses:
[1] Food Technology Unit, Faculty of Agronomy-UdelaR, Garzon 780, Montevideo, Uruguay.
[2] Department of Animal Production and Pastures, EEMAC, Faculty of Agronomy-UdelaR, Paysandú, Uruguay.

Correspondence:
Marcela J. González
Food Technology Unit, Faculty of Agronomy-UdelaR, Garzon 780, Montevideo, Uruguay.
Tel.: +59823547991
e-mail marcejoan@gmail.com

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Effects of reuterin-producing *Lactobacillus reuteri* strain plus glycerol on the quality and aerobic stability of laboratory sorghum silage

ABSTRACT

Aerobic spoilage of silage is one of the main causes of economic losses to the livestock industry. The objective of this study was to evaluate the effect of reuterin-producing *L. reuteri* SO23 plus glycerol on the aerobic stability of laboratory sorghum silage. Chopped sorghum was added with 2% glycerol and inoculated with *L. reuteri* SO23 and/or a commercial silage additive. The treated forage was packed in laboratory silos and stored for 90 days. Microbial inoculation did not affect the chemical parameters and all silages had low pH values (≤3.63). It was determined that the silages inoculated with LAB strains were characterized by higher DM recovery (P<0.001) and aerobic stability (P<0.001), and lower mold and yeast counts (P=0.009). The aerobic spoilage began at day 7 for untreated control silage, and between day 12 and 15 for inoculated silages. The control and treated silages showed some differences in mold counts during the time of exposure to oxygen, but the extent of these differences was dependent on the inoculant applied. In conclusion, the inoculation with *L. reuteri* SO23 plus glycerol improved aerobic stability of sorghum silage, retarded pH increase upon exposure to air, and slowed down the increase of molds and yeasts populations from day 4 of aerobic exposure. The results also showed that the combination of *L. reuteri* SO23 and commercial additive is preferable because this combination reduced fermentation losses, mold and yeast counts, and improved silage aerobic stability.

Key words: *Lactobacillus reuteri*, sorghum silage, aerobic stability

Introduction

Sorghum silage is routinely used in dairy farms worldwide because it minimizes loss of nutrients from harvest through storage and provides the forage/grain base for many dairy feeding systems. Sorghum plants have great production potential and desirable characteristics to produce high-quality silage. Also, these cultures have become an interesting option in semiarid or subtropical regions due to their resistance to harsh weather conditions (Getachew et al., 2016). Pastures constitute the major source for dairy and meat cattle feeding in Uruguay, but as a consequence of seasonality and production intensification, the importance of conserved forages and rations has increased (Bermúdez et al., 2016).

Silage is a forage preservation method based on the conversion of plant water-soluble carbohydrates into organic acids, mainly lactic acid, under anaerobic conditions in the presence of lactic acid bacteria (LAB), which lead to a decrease in pH. Under these conditions, the forage is preserved from spoilage microorganisms (McDonald et al., 1991). As long as anaerobic conditions are maintained, silage remains stable with a limited activity of aerobic microbes (e.g. aerobic bacteria, yeasts and molds) restricted by lactic acid fermentation. However, during unloading or feed out phase, silage is exposed to air, which could result in spoilage and consequent temperature increase (aerobic deterioration). Yeasts are recognized as initiators of aerobic deterioration, consuming sugars and organic acids, causing an increase in pH and temperature of silage (Muck, 2013; Pahlow et al., 2003). Finally, molds, acetic acid bacteria and some *Bacillus* spp. complete the deterioration of the silages (Dolci et al., 2011; Liu et al. 2013; Muck, 2013). This deterioration process involves a decrease in silage nutritional value and negative effects on animal performance (Tabacco et al., 2011), as well as the proliferation of potentially pathogenic (Driehuis, 2013) and undesirable microorganisms like *Clostridium* spp. (Tabacco et al., 2009).

Several researchers have focused on improving aerobic stability by inoculating the plant material with LAB, because they produce antifungal-substances during ensiling (Weinberg and Muck, 1996; Broberg et al., 2007; Muck et al., 2018).

Lactobacillus buchneri is a species of heterofermentative LAB. Strains of this species are mainly used as silage additives to improve aerobic stability, converting lactic acid to acetic acid and 1,2-propanediol during silage storage (Kleinschmit and Kung, 2006; Muck, 1996; Oude Elferink et al., 2001). The use of inoculants containing *L. buchneri* increases the aerobic stability of sorghum silage since acetic acid and 1,2-propanediol are more effective at reducing yeasts growth than lactic acid (Filya, 2003; Ranjit and Kung, 2000; Tabacco et al., 2011; Weinberg et al., 1999). The focus of the research in this area is to isolate and identify new heterofermentative LAB, or to design combined inoculants, able to improve the aerobic stability of the silage. Other *L. buchneri* group species have been tested on several crops silage, including strains of *Lactobacillus diolivorans* in corn (Krooneman et al., 2002), *Lactobacillus hilgardii* in sugarcane (Ávila et al., 2014) and corn (Polukis et al., 2016), *Lactobacillus brevis* and *Lactobacillus kefiri* (Daniel et al., 2015) in sugarcane, and *Lactobacillus parafarraginis* in oat silage (Liu et al., 2014). In the same way, non-LAB species have been studied as potential silage inoculants, including isolates of *Streptococcus bovis* for tropical grass (Ferreira et al., 2013), *Propionibacterium* for corn and small grains (Filya et al., 2004) and *Bacillus subtilis* for corn (Lara et al., 2016) and sugarcane (Gandra et al., 2016).

LAB inoculants are being readily used by farmers as part of sustainable agriculture. The main mechanisms of microbial inoculants contributing to inhibit the growth of undesired microorganisms are as follows: production of organic acids, competition for nutrients and synthesis of antagonistic compounds (Magnusson and Schnurer, 2005). Then, the ability of a bacterial strain to use different substrates present in the silage and to produce potential inhibitory metabolites can be used as a criterion in the search for novel bacterial inoculant strains (Saarisalo et al., 2007). Some LAB belonging to the genus *Lactobacillus* produce a non-protein antimicrobial compound, soluble in water, resistant to heat, and stable over a wide pH range (2-8), known as reuterin (3-hydroxypropionaldehyde or 3-HPA) (Talarico et al., 1988; El-Ziney and Debevere, 1998). Strains of *L. brevis, L. buchneri, Lactobacillus collinoides, Lactobacillus coryniformis* and *Lactobacillus reuteri*, are able, under anaerobic conditions, to convert glycerol into reuterin as an intermediate step in the conversion of glycerol into 1,3-propanediol (Schütz and Radler, 1984; Claisse and Lonvaud-Funel, 2000; Nakanishi et al., 2002; Gómez-Torres et al., 2014). This low molecular weight compound inhibits growth not only of a wide range of Gram-positive and Gram-negative bacteria (Bian et al., 2011; Spinler et al., 2008) but also of some yeasts and molds (Tanaka et al., 2009; Martin et al., 2005). Hence, reuterin-producing *Lactobacillus* strains are considered as potential bioprotective cultures. Langa et al. (2013) have shown that the application of these bacteria plus glycerol generates a suitable environment for in situ production of reuterin during the manufacture and storage of dairy products (Langa et al., 2013). In the same way, reuterin-producing *L. coryniformis* 394 plus glycerol inhibited butyric fermentation and retarded yeast and mold growth, preventing rice straw silage spoilage (Tanaka et al., 2009).

In our previous study, we isolated from sorghum silages a collection of 30 heterofermentative LAB, of which only two could synthesize reuterin from glycerol under laboratory conditions. An isolate of *L. reuteri* named as SO23, was identified as the best reuterin producer (González et al., 2019). The objective of this study was to investigate the effects of reuterin-producing *L. reuteri* SO23 plus glycerol applied as bacterial silage additive, on the fermentation and aerobic stability in laboratory sorghum silages.

Materials and Methods

Treatments and ensiling

Sorghum (*Sorghum bicolour* L. Moench) sowed in an experimental field of the Faculty of Agronomy (Universidad de la República, UdelaR) (Progreso, Uruguay) was harvested on March 31st , 2017. Then, the material was mechanically chopped at approximately 30% of grain moisture. Prior to ensiling, glycerol was added to the sorghum at a final concentration of 2% referred to fresh material weight, and eight silos (4 L capacity) were prepared, including two silos per treatment. The conditions were: untreated (C), *L. reuteri* SO23 plus SiloSolve™ AS200 (Chr. Hansen Inc. Animal Health and Nutrition, Czech Republic) (RS), *L. reuteri* SO23 (R), and SiloSolve™ AS200 (S). The commercially available additive SiloSolve™ AS200 (*Lactobacillus plantarum* CH6072, *Entrococcus faecium* M74 and *Lactobacillus buchneri* LN1819) was included as reference.

The preparation of the inoculant involved the counting of viable bacteria, measured prior to ensiling, using the procedures described for the enumeration of lactic acid bacteria on De Man Rogosa Sharpe agar (MRS)(Oxoid CM361, England). Inoculation dose was 1.2×10^8 CFU/g of fresh forage for *L. reuteri* SO23 and 1.5×10^5 CFU/g of fresh forage for SiloSolve™ AS200. All additives were diluted in distilled sterile water and sprayed onto the forage. Untreated forages were sprayed with sterile distilled water, intending to reach the same level of humidity in all the treatments. Each silo was packed with approximately 3,5 kg of wet forage to achieve a packing density of about 192 kg of DM per m^3. Silos were sealed with lid and water lock, weighed and stored at room temperature (15-23ºC). After 90 days of ensiling, the silos were reweighed to determine DM losses and opened. DM losses were expressed as the proportions of final weight referred to the original weight of each silo. Fresh forage material and silages at the opening, were sampled for chemical and microbiological analysis.

Aerobic stability test

After 90 days of ensiling silages were opened and conditioned for aerobic stability test. Samples of silage (500 g) obtained from each silage treatment were placed into separate insulated polystyrene foam trays, covered with a single layer of aluminum cooking foil to prevent drying and dust contamination, but allowing the circulation of air. Trays were stored at room temperature for 21 days. Room and silage temperatures were monitored for 21 days with a thermometer. Aerobic stability was defined as the number of hours the silage remained stable before the temperature raised more than 2ºC compared to room temperature (Ranjit and Kung, 2000). Aerobic exposed silages were sampled at 0, 4, 9, 14 and 21 days after opening, to evaluate microbial and chemical changes during the exposure to the air.

Chemical and microbial analysis

Fresh forage material prior to ensiling, silage after 90 days of ensiling, and aerobically exposed silage were sampled. Then each sample from each condition was separated into three subsamples. The first subsample corresponded to chemical analysis. Dry matter (DM) content of the fresh material and silages was determined by oven-drying at 60ºC for 72 h (AOAC 967.03 method). Crude protein (CP) content was determined according to AOAC-984.13 methods (1990). The neutral detergent fiber (aNDF) analysis was conducted without sodium sulfite and with the inclusion of heat-tolerant α-amylase, and acid detergent fiber (ADF) was measured sequentially. All fibers were determined according to Van Soest et al., 1991 using an ANKOM200 Fiber Analyzer (ANKOM Technology Corp., Fairport, NY, USA), and expressed in relation to DM. The ash concentration was determined by complete combustion in a muffle furnace at 550ºC for 5 h (AOAC 942.05 method) and then expressed in relation to DM also. Twenty grams from the second subsample were taken and mixed with 180 ml of distilled water. The material was then filtered, and the filtrate was used for determination of pH value measured with a glass electrode pH meter (pH HI98103 Checker 1, Hanna Instruments, USA). The third subsample was used for microbial analysis. Ten grams of silage were homogenized with 90 ml of sterilized peptone-water (1.0g/L peptone and 8.50 g/L NaCl) in a laboratory blender Stomacher 400 Circulator (Seward Ltd., Worthing, UK) for 2 minutes at 260 rpm; serial dilutions were made in the same solution. Lactic acid bacteria (LAB) were counted on MRS agar medium and incubated at 30ºC for 48-72 h under microaerophilic conditions. Total yeast and mold counts (TYMC) were determined by spread plating of serial 10-fold dilutions on malt extract agar (Oxoid CM0059, England), followed by incubation at 28ºC for 72-96 h. For spore-forming bacteria count, homogenized silage samples from each condition were incubated at 80ºC for 10 min to inactivate vegetative cells and to trigger the germination of spores. Anaerobic spore formers concentration was quantified by the most probable number (MPN) procedure using a 3 × 3 scheme. One-milliliter aliquots of the undiluted sample or decimal dilutions were inoculated into each of 3 tubes containing 9 mL of Reinforced Clostridium Media broth (Oxoid, England). The inoculated tubes were then sealed with a sterile layer of paraffin: vaseline (1:5), about 15 mm thick, and heated for 10 min at 80°C to inactivate vegetative cells and to trigger the germination of spores. Tubes were incubated at 37°C for 7 d and observed daily for gas production. Aerobic spore formers count was determined by spread plating of serial 10-fold dilutions of the thermally treated silage on Plate Count Agar (PCA, Oxoid, UK) and subsequent incubation at 37°C for 24-48 h.

Statistical analysis

All microbial counts were log10-transformed to obtained log-normal distributed data and presented on a fresh matter basis. The data were analyzed by analysis of variance to evaluate the effects of the addition of different microbial inoculum on the chemical and microbial compositions of the silage at the opening. For aerobic stability experiment, analysis of variance was performed using a model that accounted for a factorial arrangement of use of inocoulant (n=4) and time of air exposure (n=5), and their interactions and replication (n=2). A first order autoregressive covariance structure was selected. All procedures were performed with the PROC MIXED procedure of the SAS statistical programme (SAS, 2002-2003). The significance of specific treatment differences was calculated using Tukey's test. P-values <0.05 were considered to be statistically significant, while a tendency towards significance was considered for P<0.10.The least significant difference was used to separate means for treatment comparison within a time point when the inoculant effect was detected, in these cases P<0.1 was considered to be statistically significant.

Results

Characteristics and fermentation quality of sorghum

Nutrient and microbial contents of fresh forage prior to ensiling are presented in Table 1. Whole plant sorghum had a satisfactory chemical composition as shown by its content of crude protein and fiber (ADF and aNDF). DM content was 303 g/kg, suitable for proper compaction and good development of lactic acid bacteria. Epiphytic LAB on fresh forage, estimated prior to ensiling, were more than 1.0×107 CFU/g of fresh material (FM). Mold and yeast counts were about 2.5×107 CFU/g FM, with yeasts as the most abundant organisms. Aerobic spore formers were 2.9×105 CFU/g FM, while

anaerobic spore formers were less than 30 MPN/g FM (Table 1).

Table 1. *Chemical and microbial composition of untreated sorghum forage prior to ensiling.*

Item	Mean ±standard deviation
Dry matter (g/kg)	303.50 ± 3.82
Crude protein (g/kg DM)	58.00 ± 2.83
Ash (g/kg DM)	79.05 ± 0.21
ADF (g/kg DM)	369.95 ± 4.31
aNDF (g/kg DM)	618.65 ± 1.91
LAB (log CFU/g FM)	7.18 ± 0.06
TYMC (log CFU/g FM)	7.40 ± 0.08
Aerobic spore formers (log CFU/g FM)	5.46 ± 0.17
Anaerobic spore formers (log MPN/g FM)	1.34 ± 0.03

Legend: *LAB = lactic acid bacteria, TYMC = total yeast and mold count*

After 90 days of ensiling, the sorghum silages showed good fermentation quality, indicated by low pH values and less counting of spore formers, yeasts and molds than those obtained from sorghum prior to ensiling (Table 2). The silage pH values ranged from 3.52 to 3.63 and no significant differences were observed between the control and treated silages. The control (C) exhibited lower values of feedable DM recovery compared to inoculated silages. Also, the R condition led to the lowest recovery of feedable DM among inoculated conditions. Relative to control silage, the inoculation did not affect the content of CP, ADF and aNDF. Mold and yeast counting showed significant differences between treatments (P=0.009). RS condition led to the lowest mold counting of 7.4x103 CFU/g FM among treatments, while mold counting of R or S condition did not differ compared with the value obtained from C silage. No effect of inoculation was observed in LAB counts and spore formers. The anaerobic spore formers count was below detection limits in all silages (< 3 MPN/g FM). The aerobic stability was similar among the inoculated silages, which were more stable than C (168 h) (P<0.001).The MIC exhibited by chitosan nanoparticles against *S. bongori* are given in Table 2. Obtained results revealed that chitosan nanoparticles are generally more active on *S. bongori* had higher MIC values (99% death).

Effect of inoculation on aerobic stability

In aerobic spoilage studies, the temperature is recorded as an indicator of microbial activity. After seven days of exposure to the air, C silage deteriorated, indicated by its temperature exceeding ambient temperature by more than 2ºC, while the inoculated silages remained stable for more than 12-15 days (Table 2). The chemical and microbial compositions of sorghum silages at the opening and after 4, 9, 14 and 21 days of air exposure are presented in Table 3. The effects of inoculum and air exposure time, as well as the interaction between both effects, were analyzed. The kind of inoculum affected the profiles of pH and molds population, whereas the time of air exposure affected all the parameters tested, except the anaerobic spore formers count. The DM content tended to slightly decrease with time of air exposure (P<0.1) for all treatments. The silage pH increased after 21 day of air exposure and the addition of bacterial inoculants could have some incidence in the way this increment occurred (P<0.1). LAB population did not differ between treatments at silo

Table 2. *Chemical composition, microbial populations, DM recovery and aerobic deterioration of sorghum silages treated with different microbial inoculants after 90 days of ensiling.*

Item	C	RS	R	S	*P*-value
Dry matter (g/kg)	298	332	324	330	0.441
Crude protein (g/kg DM)	59	62	48	50	0
ADF (g/kg DM)	353	355	365	355	0
aNDF (g/kg DM)	627	627	656	623	0
DM recovery (g/kg)	898^{c}	998^{a}	976^{c}	995^{a}	<0.001
pH	3.63	3.53	3.52	3.59	0.28
LAB (log CFU/g FM)	5.47	5.48	5.59	5.47	0.478
TYMC (log CFU/g FM)	5.47^{a}	3.87^{b}	5.15	5.08^{a}	0.009
Aerobic spore formers (log CFU/g FM)	4.87	3.72	3.99	4.16	0.407
Anaerobic spore formers (log MPN/g FM)	<1.00	<1.00	<1.00	<1.00	0.572
Aerobic stability (h)	168^{b}	360^{a}	336^{a}	312^{a}	<0001

Legend: *C – untreated silage; RS – inoculated with L. reuteri SO23 (1.2x10^{8} CFU/g of fresh forage) and SiloSolve^{TM} AS200 (1.5x10^{5} CFU/g of fresh forage RS); R – inoculated with L. reuteri SO23 (1.2x10^{8} CFU/g of fresh forage); S – inoculated with SiloSolve^{TM} AS200 (1.5x10^{5} CFU/g of fresh forage); DM recovery – dry matter recovery of feedable silage; DM – dry matter; ADF – acid detergent fiber; aNDF – neutral detergent fiber; LAB – lactic acid bacteria, TYMC – total yeast and mold count, FM – fresh matter.*

$^{a, b, c}$ *Means in rows with the same superscript do no differ (P>0.05).*

opening and increased with time in the same way for all silages. The mold population, mainly yeasts, increased along with air exposure, and this increase was dependent on the inoculant applied (P=0.024). The aerobic spore formers population did not differ between treatments and C at silo opening and increased with time in the same way for all silages. However, compared to C silage, RS silage showed a tendency to control these microorganisms (P=0.079) with a difference of 1 log on average between this treatment and C (5 log and 4 log, respectively). The anaerobic spore formers remained at low levels throughout the trial for all treatments, in many cases below the detection limit of the technique (<3 MPN/g FM). Although not significant, some shifts in the population were observed in all treatments throughout the period of exposure to air.

There was no difference in pH between treated silages at silage opening and after 21 days of aerobic exposure, but pH increase profile was not the same for all treatments. The control silage had higher pH values than inoculated silages, specifically when compared to RS treatment, since 24 hours after silo opening until the end of the evaluation period. In turn, the treated silages differed in the pH increase profile depending on the inoculant applied (Figure 1). R and RS treated silages behaved almost in the same way, with pH values generally lower than C along the evaluation period, whereas S treated silage showed fluctuations in pH values being at times equal to those of the control (see Figure 1, days 9 and 21). The control and treated silages showed some differences in mold counts as well, but the extent of these differences was dependent on the inoculant applied and the time of exposure to oxygen (Figure 2). The inoculation led to

Table 3. *Chemical and microbial compositions of untreated (C), inoculated with L. reuteri SO23 and SiloSolve™ AS200 (RS), inoculated with L. reuteri SO23 (R), and inoculated with SiloSolve™ AS200 (S) sorghum silages after 0, 4, 9, 14 and 21 days of air exposure*.

Treatment	DM (g/kg)	pH	LAB (log CFU/g FM)	TYMC (log CFU/g FM)	Aerobic spore formers (log CFU/g FM)	Anaerobic spore formers (log MPN/g FM)
0 d of air exposure						
C	298	3.63	5.47	5.47	4.87	<1.00
RS	332	3.53	5.48	3.87	3.72	<1.00
R	324	3.52	5.59	5.15	3.99	<1.00
S	330	3.59	5.47	5.08	4.16	<1.00
4 d of air exposure						
C	287	4.18	6.15	7.99	5.16	<1.00
RS	318	3.62	9.00	7.59	4.43	1.29
R	306	3.76	8.74	8.57	3.97	1.11
S	314	3.49	8.93	7.13	4.05	<1.00
9 d of air exposure						
C	307	4.96	9.75	9.36	4.80	1.26
RS	314	4.03	10.07	7.38	4.87	<1.00
R	309	4.28	9.48	8.91	4.73	<1.00
S	311	4.54	9.88	9.13	4.72	<1.00
14 d of air exposure						
C	257	5.14	11.54	10.21	5.24	1.49
RS	303	4.41	11.76	10.19	4.90	1.49
R	265	4.59	11.59	9.64	5.28	<1.00
S	288	4.60	11.58	10.34	4.70	<1.00
21 d of air exposure						
C	274	5.46	10.73	10.71	4.93	1.26
RS	318	4.73	11.78	10.60	3.80	<1.00
R	276	4.76	10.95	11.09	5.36	<1.00
S	289	5.36	11.46	10.58	5.48	1.26
			P-value			
I	0.1314	0.0965	0.4470	0.0240	0.1110	0.3688
T	0.087	0.0008	<0.0001	<0.0001	0.0374	0.5862
IxT	0.6611	0.1630	0.9490	0.0136	0.2720	0.3617

Legend: *I – inoculum effect; T = air exposure time effect; IxT = interaction effect*

silages with slightly lower mold counts compared with those of C at silo opening, although only RS inoculant effect was significant. No difference was observed between C and treated silages mold counts within the first days of air exposure. After four days of air exposure mold population increased exponentially in C and treated silages up to 3 logs on average (Figure 2). C and S silage mold counts remained the same since silage opening and for the time the aerobic stability trial lasted. Differences observed in mold counts for silage R and RS in relation to the other treatments were dependent on the time of exposure to air. Lowest mold counts were achieved in RS and R treated silages at nine and fourteen days after air exposure, respectively. The exponential and sustained increase in mold counts observed since silo opening and the fourth day of exposure to air was slowed down in R and RS silages, at least until day nine. By the time the aerobic stability trial finished, mold counts in C and treated silages reached $2x10^{10}$ CFU/g FM on average. Despite the high mold and yeast counts recorded in all silages, particularly after day 4, the presence of visible molded areas was evident from day 9 for C, and after day 18 for R and S silages. No molded patches were observed in RS silage despite the high mold count values and the temperature increase registered.

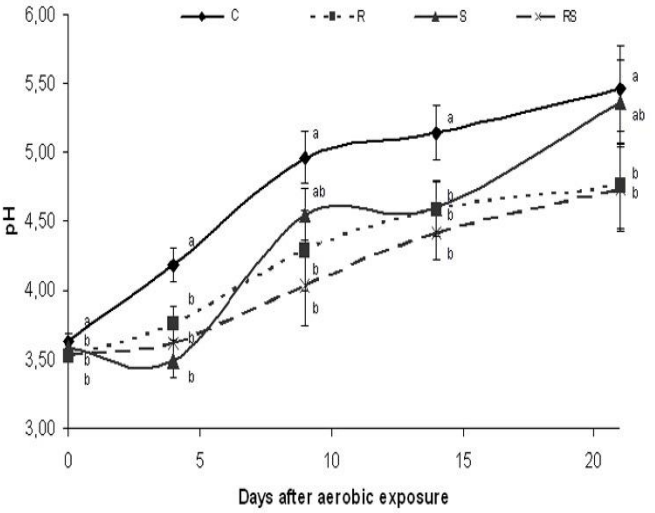

Figure 1. *pH values after aerobic exposure of uninoculated and inoculated sorghum silage.*

Control (C), no exogenous bacterial inoculant applied; R, inoculated with L. reuteri SO23; S, inoculated with SiloSolve™ AS200 (Chr. Hansen Inc. Animal Health and Nutrition, Czech Republic); RS, inoculated with L. reuteri SO23 and SiloSolve™ AS200. [a, b, c] Means in columns with the same superscript do no differ (P>0.1) for treatments comparison within a time point. Errors bars are SD.

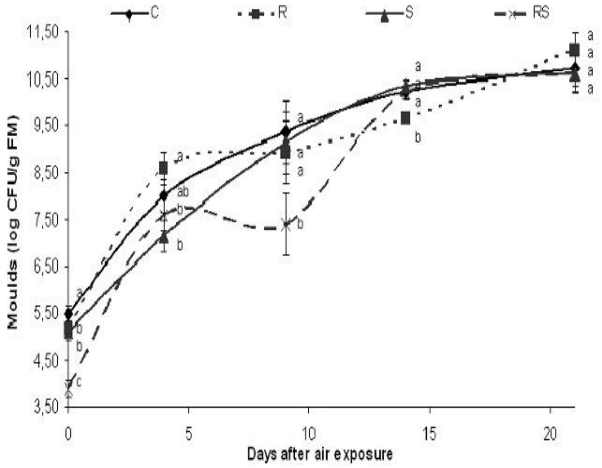

Figure 2. *Total yeast and mold counts (TYMC) after aerobic exposure of uninoculated and inoculated sorghum silage.*

Control (C), no exogenous bacterial inoculant applied; R, inoculated with L. reuteri SO23; S, inoculated with SiloSolve™ AS200 (Chr. Hansen Inc. Animal Health and Nutrition, Czech Republic); RS, inoculated with L. reuteri SO23 and SiloSolve™ AS200. [a, b, c] Means in columns with the same superscript do no differ (P>0.1) for treatments comparison within a time point. Errors bars are SD.

Discussion

Microbial inoculants have become the dominant silage additive in most parts of the world and most of these products include strains of heterofermentative and homofermentative LAB (Muck, 2013). The research here reported was designed to evaluate the efficacy of *L. reuteri* SO23 and glycerol as silage inoculant to enhance aerobic stability and its effects on the chemical and microbial composition of sorghum silage. *L. reuteri* SO23 was examined in contrast and in combination with the silage additive SiloSolve™ AS200. This commercially available additive is a homo and heterofermentative LAB (*Lactobacillus plantarum* CH6072, *Entrococcus faecium* M74 and *Lactobacillus buchneri* LN1819)-based inoculant recommended to improve fermentation process and aerobic stability at feed out. In this experiment, at the end of ensiling, all silages showed good fermentation characteristics. It is well known that silage microbial inoculants can alter different aspects of silage fermentation, but the level of the effect is variable across studies (Muck et al., 2018). The rate of decline in pH during the ensiling, which is important for the production of stable silages, was the same in all silages, even in uninoculated silage (C). In the present study, although inoculant application affected DM recovery, mold and yeast counts, and aerobic stability, no effect was observed in silage pH. Control silage showed a low pH value, comparable to inoculated silages

values, and this may be ascribed to the activity of epiphytic LAB population present in sorghum previous to ensiling ($1.51x10^7$ CFU/g FM). Silages inoculated with *L. reuteri* SO23 or/and SiloSolve™ AS200 showed higher DM recovery compared to untreated silage. Greater dry matter content maintenance as a result of applying microbial inoculant may be attributed to higher fermentation efficiency of inoculant than that of epiphytic LAB. Although *L. reuteri* SO23 inoculated silage showed better DM recovery values than control, these values were lower than those observed in the silages with commercial inoculant (RS and S). *L. reuteri* is an obligate heterofermentative and produces a mixture of acids depending on the type of hexose sugar available, with the production of carbon dioxide and loss of DM in all pathways (McDonald et al., 1991). The heterofermentative pathway is, therefore, less efficient concerning both acidification and preservation of nutrients during the anaerobic storage phase than the homofermentative pathway (Wilkinson and Davies, 2013). This may explain the differences observed in the recovery of DM in R silage compared to those inoculated with SiloSolve™ AS200 containing a mixture of homo and heterofermentative LAB strains.

Aerobic stability of silage is important because it relates to the safety and quality of the preserved forage upon exposure to air during storage and feeding. When silage is exposed to air after opening or removal from the silo, fermentation acids and other substrates are oxidized by aerobic bacteria, yeasts and molds, which results in a temperature increase above ambient, accompanied by nutrient deterioration. The use of inoculants containing *Lactobacillus buchneri*, has been shown to increase the aerobic stability of sorghum silage by controlling yeast growth, primarily responsible for the onset of silage aerobic spoilage (Filya, 2003; Tabacco et al., 2011; Weinberg et al., 1999; Borreani and Tabacco, 2010). It has been demonstrated that *L. buchneri* has a metabolic pathway to degrade lactic acid under anoxic conditions, to acetic acid and 1,2-propanediol; improved aerobic stability is attributed to both products (Oude Elferink et al., 2001). Several methods employed to determine aerobic stability of silage are reported in the literature, but the most commonly used criteria are temperature increases of 2ºC above ambient (Ranjit and Kung, 2000; Wilkinson and Davies, 2013). According to this criterium, aerobic deterioration began at day 7 for untreated control silage, and between days 12 and 15 for inoculated silages. The aerobic stability of inoculated silages was comparable to those reported by Tabacco et al. (2011) for sorghum silage inoculated with *L. buchneri*. There is no background of *L. reuteri* as sorghum silage inoculant but an improvement in aerobic stability of maize silage was reported by Zielińska and Fabiszewska (2018).

In this study, the number of molds and yeasts decreased after ensiling relative to untreated sorghum forage, even in control silage. Molds are usually present in lower numbers during storage due to their higher susceptibility to the absence of oxygen, so the counts accounted almost exclusively for yeast. Neither the inoculation of silages with *L. reuteri* SO23 nor SiloSolve™ AS200 was sufficient to decrease the yeast population compared to control. Competition for nutrients with epiphytic LAB and the need for higher inoculant doses should be tested. The combination of *L. reuteri* SO23 and SiloSolve™ AS200 (RS treatment) was the only treatment capable of reducing the yeast population during the fermentation phase and slow down yeast increase after aerobic exposure. LAB starter cultures addition to silage ensures an immediate decrease in the pH and prevents the growth of undesirable microorganisms and further aerobic deterioration. The aerobic stability of silages is enhanced by many low mass organic acids, such as acetic, lactic and propionic acid, and some other metabolites like 1,2-propanediol, reuterin and/or bacteriocins, which occurrence in silages is associated with certain LAB strains growth (Magnusson and Schnurer, 2005).

The results in the current study indicate that inoculation with *L. reuteri* SO23 alone or in combination with other LAB like *L. buchneri*, improved the aerobic stability of sorghum silages. Inoculation with *L. reuteri* SO23 plus glycerol improved aerobic stability of sorghum silage, retarded pH increase upon exposure to air, and slowed down molds and yeast population increase from the fourth day of aerobic exposure. This result would provide indications that reuterin may be produced in glycerol added silage although the detection of reuterin in the silage should be examined in a future study. The results also showed that the combination of *L. reuteri* SO23 and SiloSolve™ AS200 bacterial strains is preferable because the combination reduced fermentation losses and mold and yeast counts, and improved aerobic stability. As mentioned above, *L. buchneri* is able to ferment lactate to acetate, 1,2-propanediol, CO_2 and traces of ethanol under anoxic conditions at low pH values. However, the ability of this lactic acid bacteria to degrade 1,2-propanediol further to propionic acid is limited and strain-dependent (Zielińska et al., 2017). Conversely, *L. reuteri* expresses two isofunctional related enzymes (glycerol and propanediol dehydratase) also involved in reuterin production, able to carry out a disproportionation reaction converting 1,2-propanediol to 1-propanol and propionic acid under conditions that prevail in silage (Sriramulu et al. 2008). In theory, *L. reuteri* may have a role in the formation and accumulation of propionic acid from 1, 2-propanediol, but this should be proven in the silage matrix. Volatile fatty acids such as propionic acid and acetic acid are much better inhibitors of yeasts than is lactic acid and mixtures of lactic acid and propionic or acetic acid have a synergistic inhibitory effect (Moon, 1983). This synergistic effect has been reported in maize silage inoculated with strains of *L. reuteri* and *L. buchneri*, where the presence of different

organic acids had a positive result on the increase of aerobic stability, regardless of the addition of other LAB strains to the starter culture (Zielińska and Fabiszewska, 2018).

Tanaka et al. (2003) were the first to suggest an inhibitory effect on butyric fermentation and aerobic spoilage of rice straw silage inoculated with a reuterin producing LAB strain plus glycerol. However, the presence of reuterin in the silage was detected in later studies (Tanaka et al., 2009). In order to confirm the hypotheses raised above, the results here presented should be complemented with a more detailed study of the fermentation profile (e.g. volatile fatty acids), as with the detection and quantification of reuterin in *L. reuteri* SO23 inoculated silages.

Conclusion

In conclusion, the results of this study showed that *L. reuteri* SO23 applied at 1.2×10^8 CFU/g of fresh forage was capable of improving the aerobic stability of sorghum silages. The results also showed that the inoculation with *L. reuteri* SO23 and a commercial silage additive containing *L. buchneri* is preferable as this combination reduced mold and yeast counts and improved aerobic stability at a laboratory scale.

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