CORPUS PUBLISHERS

Corpus Journal of Dairy and Veterinary Science (CJDVS)

Volume 1 Issue 3, 2020

Article Information

Received date : September 15, 2020

Published date: October 16, 2020

*Corresponding author

Vignolo GM and Nader-Macias MEF, Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, Tucumán 4000, Argentina

Keywords

Maize; Sorghum Silages; Homolactic and Heterolactic Lactic Acid Bacteria; Fermentation; Aerobic Stability

Research Article

Effect of Selected and Beneficial Lactic Acid Bacteria as Inoculants for Corn and Sorghum Silages

Uezen JD[1], Di Luca F[1], Mansilla FI[1], Aristimuño Ficoseco C[1], Abeijon Mukdis C[1], Medina R[1], Isla MI[2], Nader-Macías MEF[1]* and Vignolo GM[1]

[1]*Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, Tucumán 4000, Argentina*

[2]*Instituto de Bioprospección y Fisiología Vegetal (INBIOFIV)-CONICET, Tucumán 4000, Argentina*

Abstract

The preliminary effect of *Lactobacillus fermentum* CRL2085, alone or in combination with the homofermentative *Pediococcus acidilactici* CRL2043 on the fermentation and aerobic stability of corn and sorghum silages, was investigated. To be used as silages inoculants, these lactic acid bacteria (LAB) were screened for fibrolytic, amylolytic and antifungal activities. Laboratory silos were prepared, inoculated at 10^5 CFU/g and fermented during 45 days 25 ºC. At the end of fermentation, *Pediococcus acidilactici*-inoculated corn and sorghum silages exhibited the lowest pH and high residual water-soluble carbohydrates (WSC). Forage silages inoculated with *Lactobacillus fermentum* + *Pediococcus acidilactici* showed Lower Dry matter (DM) losses and Neutral Detergent Fiber (NDF) values, while higher residual WSC concentration than *Lactobacillus fermentum*-inoculated silages, thus silage digestibility must be improved. Yeast activity was impaired in silages inoculated with *Lactobacillus fermentum* alone or combined with *Pediococcus acidilactici* and aerobic stability improved. The survival of LAB used as inoculants during fermentation was suggested by PCR. Besides the good performance as silages inoculants, LAB previously selected as having health benefits may be delivered to cattle in feedlot through fermented silage as a vehicle.

Introduction

Maize and sorghum are mostly used for silage in the world, their production leading by Unites States (US) with 370 and 9.3 million tons (MT) respectively, followed by China with 258 and 3.5 MT, European Union (EU) with 61 and 0.7 MT and Argentina with 46 and 2.8 MT, respectively [1]. Whole-crop corn silage is the major source of forage in the US and EU for dairy cows and beef finishing industry; corn destined to silage accounted for near 12 MT in Argentina [2]. Sorghum, as one of the world's crops adapted to a variety of agronomic and environmental conditions, is used more and more for silage. Compared to corn, sorghum shows the advantage of lower production costs and higher production of dry matter [3,4]. In the last years, an increase in both forages crop for milk and meat production occurred in Argentina, agreeing with the evolution towards more intensive meat production systems [5]. These forages are commonly processed as silage for feedlot cattle and dairy cow diets to improve their performance and efficiency by increasing energy density diets [5, 6]. However, depending on the climate, type of soil, and growth conditions, crops can modify their dry matter (DM) content, the proportion and quality of grain in the plant, and consequently the quality of silage. Both plant resources have common characteristics, such as energy concentration that increases with the amount of grain in the plant, protein and water contents, and low digestibility of neutral detergent fiber (NDF), as reported by Di Marco [5].

Preservation of plant material is based on the fermentation under anaerobic conditions by epiphytic and/or added lactic acid bacteria (LAB) that convert water-soluble carbohydrates into organic acids, mainly lactic acid, and as a result the pH decreases and the forage are preserved. Air is detrimental to the ensiling process allowing the activity of aerobic spoilage microorganisms such as yeasts and molds [7]. However, silage is often fully exposed to air during unloading, causing an increase in temperature and spoilage or aerobic deterioration [8, 10]. Inoculants addition to freshly harvested forage can increase the likelihood of obtaining good quality silage, which must be viewed as an insurance policy for silage. Bacterial inoculants are used to obtain more stable ensiling, by accelerating post-ensiling decline of pH and improving nutrient retention, aerobic stability, and silage quality [8, 9, 11]. In addition to forage characteristics and practices at ensiling, inoculants effectiveness depends on the type and viability of the microorganisms used. Thus, different inoculants categories were described according to fermentation patterns [12]. Homolactic lactic acid bacteria (LAB) dominate the fermentation by the rapid production of lactic acid and pH decrease, preventing growth of yeasts/molds helping preserve the forage mass [13, 15], Lactic acid utilization by yeasts/molds during aerobic exposure increases silage pH with a reduction of nutrient value. To overcome this effect, the conversion of lactic acid to acetic acid by heterolactic LAB would enhance yeasts/molds inhibition improving aerobic stability and silage life [9, 16, 18]. Several studies reported the effects of feruloyl esterase (FE)-producing *Lactobacillus buchneri* inoculant on the improvement of silage aerobic stability and digestibility [11, 17, 19]. Moreover, antifungal activity of LAB [20] may also contribute to reduce yeasts/molds during ensiling. Then, the combination of homo and heterolactic LAB strains, added to freshly harvested forage can increase silages efficiency. In previous work, LAB strains isolated from a feedlot industry environment have been characterized [21, 22]. The aim of this study was to use these LAB strains individually or in combination as silage inoculants, to ferment corn and sorghum whole-plants in laboratory silages.

Materials and Methods

Bacterial strains and growth conditions

Thirty LAB strains previously isolated from feedlot environment and selected for their beneficial characteristics [21, 22] were used including *Enterococcus (E.) hirae* (6), *E. faecium* (2), *Lactobacillus (L.) acidophilus* (3), *L. amylovorus* (4), *L. plantarum* (3), *L. rhamnosus* (1), *L. fermentum* (1), *L. mucosae* (8) and *Pediococcus (P.) acidilactici* (2). Inoculum of strains were prepared by transferring freezed-glycerol stock culture to MRS broth (Merck, Darmstadt, Germany) and sub-cultured twice in the same media at 37 ºC for 16 h.

Feruloyl esterase (FE) activity

LAB strains were analyzed for their ability to hydrolyze ethyl-ferulate used as substrate as previously described by Donaghy, Kelly, & McKay [23]. LAB strains were grown overnight in MRS (Britania Lab, Buenos Aires, Argentina) broth at 37 ºC, harvested by centrifugation (10000 g; 10 min) and washed twice with phosphate buffered saline (PBS) pH 7.0. Freshly prepared cells were then resuspended in sterile water to 10^8-10^9 cells/ml. Briefly, FE activity was determined in MRS modified by the replacement of glucose by ethyl-ferulate in methanol 1% (w/v) added in a concentration of 1 g/l (4.5 mM). Plates containing MRS modified medium were streak-inoculated with LAB strains and incubated (37 ºC, 72 h). FE activity was evidenced by the formation of clear zones of hydrolysis around the LAB streaks.

Antifungal activity determination

Aspergillus oryzae and *Penicillium roqueforti* were used for antifungal activity assay by the modified agar diffusion assay [24]. Petri plates containing Sabureaud agar plates (Britania Lab, Buenos Aires, Argentina) were inoculated with the fungus and incubated (25 °C, 48 h). After mycelial colonies development, spores were collected and adjusted to 10^5 per ml of sterile saline solution (0.85% w/v). LAB strains were streak inoculated on MRS agar plates and after incubation (30 °C, 48 h), plates were overlaid with 10 ml of Sabureaud soft agar (0.7% agar) containing fungal spores suspensions (10^4 per ml) and incubated aerobically (30 °C, 48 h). Then, examination for clear zones of inhibition around the LAB streaks was carried out and scored as-(no growth suppression) and + (1-5 mm growth suppression).

Amylolytic and cellulolytic activity

Starch degradation was examined by spot inoculation of active LAB strains (5 ml) on plates containing MRS-starch medium in which glucose was replaced by starch (1%). Inoculated plates were allowed to grow at 30 °C for 48 h and then stored at 4 °C during 24 h before being flooded with iodine solution (4%). Amylase production was indicated by a clear zone around the colonies, while the rest of the plate stained blue-black. *Lactobacillus amylophylus* CECT4133 was used as positive control. For cellulolytic activity determination, LAB cultures were inoculated by a spot in Dubos agar supplemented with cellulose 1.5% as the only hydrocarbon source. The plates were then incubated at 37 °C for 4-5 days and checked for clear zones surrounding the colonies [25].

Forage materials, laboratory silage preparation and LAB inoculation

Whole corn plants of hybrid cultivar harvested at the 1/2 milk line stage (AG7004, AGseed, Argentina) and whole sorghum plants of hybrid BMR cultivar (DK 72-10 VT3P, Bayer, Argentina) were chopped by a conventional forage harvester to 2.5 cm of average size. Laboratory mini silos were prepared with chopped corn and sorghum whole-plant harvested during the summer-autumn season February/March 2016 in central Argentina (31°40′00″S 63°03′00″W; 156 m above sea level) and March/April 2017 northern Argentina (27° 1′ 44.04″ S, 64° 36′ 39.96″ W; 291 m above sea level), respectively. Laboratory mini silos were prepared within 24 h after chopping (Figure 1). Chopped material for each forage was divided into 1.5-kg lots to obtain four laboratory-scale mini silos by packing plant material in tubular plastic structures (polyethylene 80-100 microns thick). Treatments applied to fresh material were (i) non inoculated control, (ii) addition of homolactic LAB strain; (iii) addition of heterolactic FE-producing LAB, and (iv) combined strains addition. Cell pellets (3 x 10^8 CFU) of previously selected LAB strains were diluted in sterile water (30 ml) to 10^7 CFU/ml in sterile sprayer bottles and used to inoculate chopped corn and sorghum whole-plant forages. A similar inoculation level was used when combined LAB strains were inoculated; pellets from both strains were separately diluted in 15 ml of sterile water in bottles at 10^7 CFU/ml each. Both forage mini silos were inoculated with 30 ml suspensions (1 x 10^7 CFU/ml) to obtain a final LAB application of approximately 10^6 CFU/g. The control received 30 ml sterile water. The amount of chopped corn and sorghum for each laboratory mini silo was weighed, uniformly sprayed with appropriate inoculant solution (one sprayer bottle for each treatment), mixed by hand (using sterile gloves), and placed into a polyethylene tubular structure with periodic tamping (using a sterile metal device). The mini silos were then sealed (both sides) by folding the plastic edges and tape closed. To prevent cross-contamination, utensils in contact with inoculated corn and sorghum chopped forage was washed and wiped with ethanol between treatments. The mini silos were weighted before and after filling to determine the actual amount ensiled. Finally, mini silos were stored in a barn at an average ambient temperature of 25 ºC to allow fermenting for 45 days. Gas was not vented from the laboratory silos. The pH values were determined by using a puncture electrode of a portable pH meter (Sartorius PT-10, Gottingen, Germany).

Biochemical analysis

Chemical-nutritional analysis of fresh forages (before ensiling), silages samples, and uninoculated controls after 45 days-fermentation were performed and presented on a DM basis. DM content of the fresh forages and silages was evaluated by drying at 60 ºC for 48 h in a fan-assisted oven. Crude protein (CP) was determined by the Kjeldahl method [26]. Samples (5 g) were analyzed for neutral detergent fiber (NDF) using amylase [27] and the acid detergent fiber (ADF) was analyzed without amylase [28]. In addition, ash was determined by incineration of plant material at 550 ºC for 5 h [29]. For water-soluble carbohydrates (WSC), wet samples stored at -20 ºC were subjected to extraction in distilled water by heating (100 ºC). After 3 min, the suspension was filtered, precipitated with ethanol 96º and maintained 15 min at 4 ºC. The extract is then centrifuged (8000 x g, 10 min) and the supernatant was used to quantify total WSC using the phenol-sulfuric acid method [30]. On the other hand, the precipitate after suspension in distilled water was divided into two aliquots. One of them served to quantify free glucose using glucose oxidase/peroxidase method [31] while the other was hydrolyzed with amylase for 30 min a 37 ºC, precipitated with ethanol 96º, and total glucose (starch) was measured using the same method.

Microbiological analysis

Corn and sorghum laboratory silos samples were collected at 0, 10, 20, 30, and 45 days. Twenty-five grams of each silage content was diluted in 225 ml of sterile (0.85%/w/v) saline solution, homogenized for 3 min (Stomacher 400, Seward, Worthing, UK) and serially diluted. Decimal dilutions were plated by triplicate in Plate Count Agar (PCA; Britania, Buenos Aires, Argentina) used for total bacteria (TB) and MRS (Biokar, France) media supplemented with cycloheximide (0.1 g l-1) for LAB and incubated aerobically (30 ºC, 48 h). In addition, molds and yeasts were determined on Saboureaud (Britania Lab, Buenos Aires, Argentina) agar medium and incubated in aerobic environment (25 ºC, 3-5 days).

DNA extraction and PCR-based identification of inoculated LAB

To confirm the permanence of the inoculated strains during silages fermentation, homogenized and diluted inoculated sorghum silage samples after 45 days of fermentation (as previously described) were plated on LAPTg (15 g/L peptone, 10 g/L tryptone, 10 g/L glucose, 10 g/L yeast extract, and 1 mL/L Tween-80, pH 6.5) [32]; agar incubated at 37 ºC and 50 ºC to differentiate *L. fermentum* and *P. acidilactici* growth, respectively. Isolated colonies (20) from different dilution plates (between 30 and 300) at each incubation temperature were randomly picked from agar media and transferred to individual tubes containing 5 ml of MRS broth. Cultures were re-streaked onto MRS agar plates and incubated at 37 °C for 48 h until isolated colonies of the same-one morphology were obtained. Pure colonies were preliminarily characterized as Gram positive and catalase negative. Genotypic identification and comparison with a reference culture of inoculated strains were performed by repetitive fingerprint (rep-PCR) using (GTG)5 primer [33]. Genomic DNA was extracted according to Pospiech and Neumann [34]. The master mix contained 4 µl of 5X buffer (Inbio Highway, Argentina), 2 µl of 5mM dNTPs (Promega, Argentina), 2 U of Taq polymerase (Inbio Highway, Argentina), 1 µl of DNA template (50 ng), 2 µl of primer (GTG)5 10 µM (Sigma-Aldrich, Argentina) and 4 µl of MgCl2 (25 mM). The PCR reaction was performed as described by Maldonado et al. [22]. Amplification reactions were carried out in a MyCycler thermocycler (Bio Rad) and the PCR products were separated by electrophoresis in a 1.5% agarose gel.

Aerobic stability

After 45 days fermentation, laboratory mini silos were opened and portions of fermented material were placed in trays (10 cm deep) and exposed to air (22-27 °C) by inserting a thermometer in the geometric center of the silage mass. The temperature was recorded every 12 h for 7 days. Each tray was covered with a double layer of sterile cheesecloth to avoid contamination and drying of the silage, but allowing air to infiltrate the silage mass. Aerobic stability was defined as the number of hours the silage temperature remained stable before increasing more than 2 °C above room temperature [35].

Statistics

The results are expressed as the mean value (or log values) ± standard deviation of the data. All in vitro assays were performed in triplicate. Significant differences between means were determined by Tukey's test after analysis of variance (ANOVA) with Minitab Statistic Program, release 16.1.0 for Windows. P value of <0.05 was considered statistically significant.

Results

LAB selection

Previously characterized LAB based on beneficial/probiotic features [21, 22] were used in this study to select strains as silage inoculants. When they were analyzed for cellulolytic, amylolytic and antifungal activity, *L. mucosae*, *L. plantarum, E. hirae* and *E. faecium* were able to degrade cellulose, starch and exerted inhibitory activity against the assayed fungus (Table 1). In addition, *L. fermentum* strain was the only showing FE activity. From this results *P. acidilactici* CRL2043 (homolactic, high acid producer and inhibitory to fungus) and *L. fermentum* CRL2085 (heterolactic, FE activity) were selected to be used as inoculants.

Table 1: Fibrolytic, amylolytic and antifungal activity of LAB strains.

LAB Strains	FE Activity	Cellulolytic Activity	Amylolytic Activity	Antifungal Activity	
				A. Oryzae	P. Roquefortii
L. acidophilus	0/3	0/3	0/3	0/3	0/3
L. amylovorus	0/4	0/4	0/4	0/4	0/4
L. fermentum	1/1	0/1	0/1	0/1	0/1
L. mucosae	0/8	1/8	1/8	2/8	1/8
L. plantarum	0/3	0/3	0/3	3/3	2/3
L. rhamnosus	0/1	0/1	0/1	0/1	0/1
P. acidilactici	0/2	0/2	0/2	2/2	0/2
E. hirae	0/6	2/6	0/6	1/6	4/6
E. faecium	0/2	0/2	0/2	1/2	1/2

Chemical and microbiological changes during fermentation of inoculated corn and sorghum laboratory silages

As major crops ensiled in Argentina, corn and sorghum laboratory silages were prepared. After preparation/inoculation, silages fermented for 45 days at temperatures between 22-27 ºC (Figure 1), were analyzed. The chemical and microbiological composition of fresh crops are given in Table 2. Initially, chopped corn forage showed lower pH, WSC and NDF values compared with that of sorghum fresh material, whereas DM, starch, and CP contents were higher. LAB and yeast/molds numbers were higher for fresh corn. The effects of *P. acidilactici* CRL2043 (Pa), *L. fermentum* CRL2085 (Lf) and their combination on the fermentation characteristics of corn and sorghum silages after 45 days are shown in Table 3. Uninoculated (control) silages showed final pH values between 4.05 and 4.15, whereas inoculated laboratory corn and sorghum silos reached pH values in the range of 3.45±0.2 - 3.70±0.1 and 3.65±0.2 - 3.90±0.1 respectively, these values reflecting a satisfactory fermentation with a pH reduction. However, Pa-inoculated silages showed the lowest pH (3.45±0.2 and 3.65±0.2) for corn and sorghum respectively, suggesting a higher capacity to restrict contaminants. As expected for carbohydrates-rich crops, WSC concentration in both silages was reduced during the fermentation; corn silage showed higher residual WSC for silos inoculated with heterolactic Lf and its combination (Pa + Lf), suggesting that crop carbohydrates were less extensively fermented by the homolactic LAB strain. When crude protein (CP) was analyzed, a slight decrease was observed, values being less pronounced for Pa-inoculated silages, whereas higher DM losses in silages inoculated with *L. fermentum* were produced. Fermentation losses measured as silage weight loss were higher for Lf-inoculated silages than that for control and Pa- or Pa + Lf-inoculated silages (data not shown). Moreover, NDF (indigestible or slowly digested insoluble fiber), and ADF concentrations were lower for corn and sorghum inoculated silages when compared to the control.

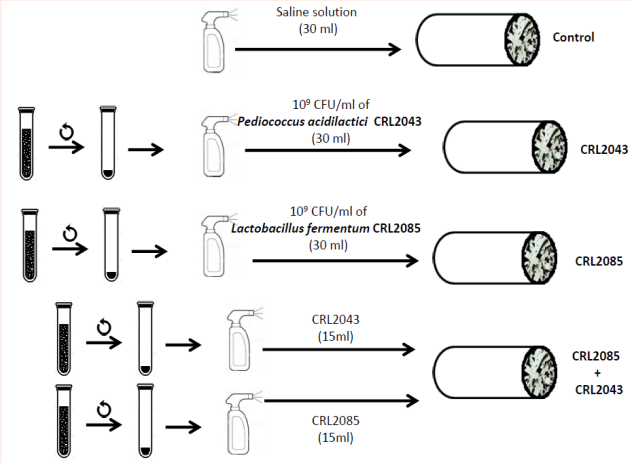

Figure 1: Inoculation scheme of the preparation of silages at laboratory scale.

Table 2: **Chemical and microbiological composition of corn and sorghum fresh forages.**

Forage type	pH	DM (g/kg)	DM (g/kg)					Log CFU/g		
			WSC	Starch	CP	NDF	ADF	Ash	LAB	Yeasts & Molds
Corn	5.8±0.2	360±7	86.2±2	230±12	70.2±2	390±8	215±12	68±2	5.67	3.1
Sorghum	6.1±0.1	295±4	174±3	95±4	58.5±2	457±12	261±16	69±2	5.2	3.5

On the other hand, the microbiological composition of the final corn and sorghum silages showed similar cultivable LAB numbers than control silages (Table 3). Because of bacterial growth during fermentation, inoculated silages reached higher LAB population at 45 days, these being higher for Pa-inoculated silos. When yeasts/molds were evaluated, high counts were found in the presence of *P. acidilactici* CRL2043, while lower numbers were detected in the silages inoculated with *L. fermentum* CRL2085 and its combination with CRL2043.

rep-PCR-based identification of inoculated LAB

Since silage fermentation is a complex process involving a different types of microorganisms, competitive relationships among natural microbiota and inoculated bacteria are established. Although it is known that LAB differ in their crop preference and ability to ferment different substrates, sorghum as a major forage crop in northern Argentina was used to check the permanence of the inoculated LAB strains during fermentation. Thus, the potential for their survival during 45 days of silage fermentation was investigated by rep-PCR fingerprinting using (GTG)5 primer. Colonies from MRS plates and considered as presumptive LAB resulted in rep-PCR band patterns from four (C1-C4) and one (C5) showing to be coincident with the inoculated *L. fermentum* CRL2085 and *P. acidilactici* CRL2043 strains, respectively used as reference (Figure 2).

Table 3: Chemical and microbiological changes of laboratory corn and sorghum silages after 45 days of fermentation

Treatments	pH	DM (g/Kg)	g/Kg DM					log CFU/g	
			WSC	Starch	CP	NDF	ADF	LAB	Yeast & molds
Corn Silage									
Control	4.15±0.1	334±5	11.6±1	210±8	68.2±2	320±10	200±9	6.95	3.90
CRL2043 (Pa)	3.45±0.2	315±3	10.0±2	195±9	67.5±3	280±9	225±10	8.25	3.20
CRL2085 (Lf)	3.70±0.1	261±9	6.00±1	185±7	65.2±3	274±6	230±8	6.5	2.77
CRL2043 + CRL2085	3.60±0.1	301±7	8.50±1	200±4	67.0±1	260±8	221±7	7.45	1.90
Sorghum Silage									
Control	4.05±0.1	281±4	101±6	66.2±2	56.2±1	392±10	ND	6.9	4.1
CRL2043 (Pa)	3.65±0.2	260±5	70.2±2	58.1±2	54.7±1	300±8	ND	7.86	3.78
CRL2085 (Lf)	3.90±0.1	228±7	51.2±2	62.5±1	53.4±2	295±9	ND	6.35	2.80
CRL2043 + CRL2085	3.75±0.2	250±6	68.4±1	63.0±2	55.1±2	280±8	ND	7.15	2.00

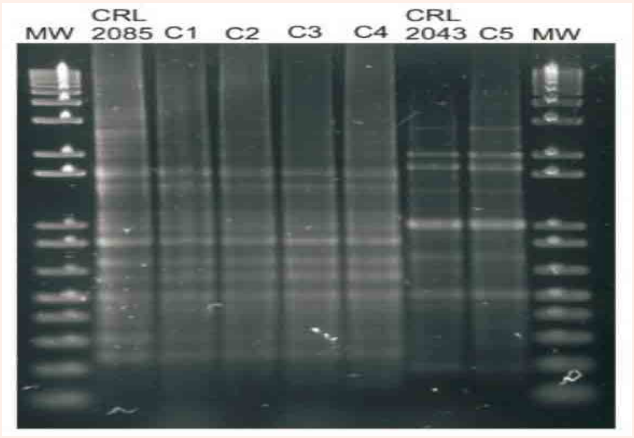

Figure 2: PCR amplification of repetitive bacterial DNA elements fingerprinting using the (GTG)5 primer of lactic acid bacteria from inoculated sorghum silage. Molecular weight marker (1 kb DNA ladder, Invitrogen). *L. fermentum* CRL2085 and *P. acidilactici* CRL2043 (GTG)5-RAPD profiles (controls) and colonies (C1-C5) picked from silages samples.

Aerobic stability

After 45 days of storage, laboratory silos from each treatment were opened and each samples exposed to air. Even when the ambient temperature was not stable, the aerobic stability was significantly improved in corn and sorghum silages inoculated with Lf and Pa+Lf; internal temperature remaining stable up to 168 h of exposure to air (Figures 3a & 3b). On the contrary, uninoculated and Pa-inoculated silages exhibited a temperature increase from 24 h onwards. Results showed that inoculation of corn and sorghum silages with Lf alone or Lf + Pa was effective at improving aerobic stability.

Discussion

Previously selected beneficial LAB [21, 22] were used as inoculants in a preliminary intend to improve silage fermentation and aerobic stability. In animal feeding, forage inoculation with LAB strains possessing amylolytic and fibrolytic (FE and cellulolytic) activities may render the fibrous component of the silage more susceptible to degradation by ruminal polysaccharidases, increasing feeding efficiency [17, 19, 36]. Numerous plant raw materials and agroindustry by-products contain hydroxycinnamates; phenolic acids such as ferulic are found both covalently attached to the cell wall and solubilized in the cytoplasm [37]. In coincidence, *L. fermentum* strain from ensiled corn stoved was reported to hydrolyze ferulate esters [18]. Whereas cellulolytic ability showed by *E. hirae*

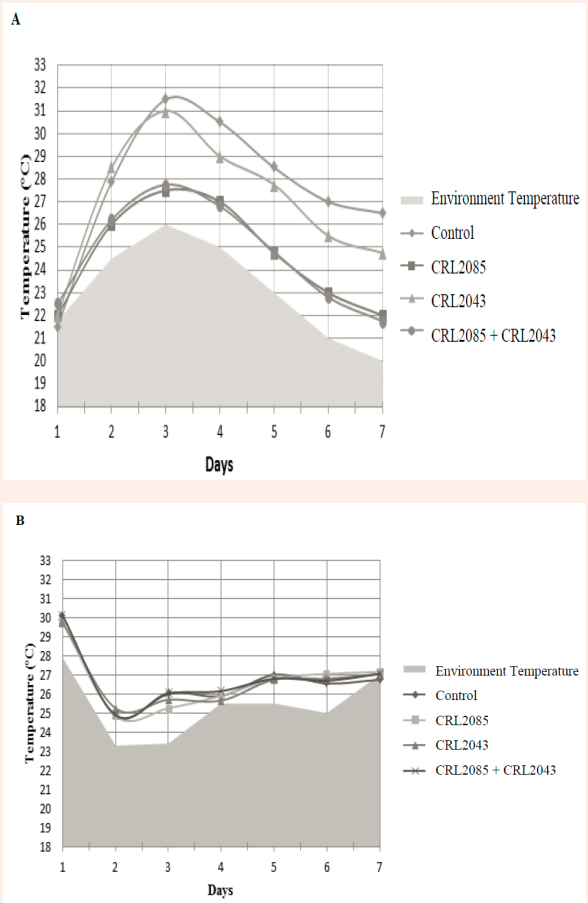

Figure 3: Effect of inoculation of corn (A) and sorghum (B) silages on changes in the temperature after 7 d of aerobic exposure.

and *E. faecium* agrees with the carboxymethil-cellulase activity of *Enterococcus* from bovine rumen [38]. Moreover, cellulose and starch hydrolysis showed by *L. mucosae* strains are in correlation with the cellulose and maltoologosaccharides degradation encoding genes reported for a strain from malnourished children stool [39]. On the

other hand, the antifungal activity detected for *L. mucosae*, *L. plantarum*, *P. acidilactici* and enterococci was also previously reported in lactic acid bacteria strains [24, 40, 41]. Silage inoculation with antifungal LAB strains would contribute both to improve aerobic stability of ensiled forage during fermentation and storage by yeasts/molds reduction or inhibition and to mitigate the risk of mycotoxin poisoning of animals. Based on this characterization, *P. acidilactici* (Pa) CRL2043 and *L. fermentum* (Lf) CRL2085 were selected as corn and sorghum silage inoculants. Chemical-nutritional analysis of fresh forages showed differences in pH when compared to other reports [16, 42, 43]. The lower pH in corn could be explained by its lower buffering capacity due to the higher content of crude protein, a parameter highly dependent on plant constituents mainly attributed to proteins [8]. The high DM, starch, and CP found for fresh corn material are in agreement to that described by other authors [16, 43, 44]. However, wide changes within varieties, maturity at harvest, climatic conditions, and agronomic practices have been reported [5, 45, 46]. In this study, fresh corn forage showed DM, CP, starch, and NDF contents as typical of whole forage harvested at 50% kernel milk line, whereas sorghum showed higher WSC and low starch and CP compared to corn. Similar results were reported for fresh corn and sorghum [16, 43, 47, 48]. The other fractions were observed to vary according to the yield and percentage of grain in crops. CP may decrease with the increase of forages biomass while NDF may rise by increasing the proportion of grain in the plant. However, its value may be diluted by a higher starch content in the silage [5]. In both trials, epiphytic LAB and yeasts/molds numbers in fresh corn were slightly higher than that of fresh sorghum, but lower to that previously reported [16, 42, 49]. At the end of fermentation, the reduction of WSC concentration was produced and consequently, a decrease in pH occurred in LAB-inoculated silages compared to uninoculated controls. Indeed, low pH stabilizes silage fermentation by inhibiting or killing the growth of acid intolerant microbes such as enterobacteria, clostridia, and toxigenic molds [50]. The lower pH for Pa-inoculated corn and sorghum silages compared to those inoculated with the Lf alone or in combination with Pa may be assigned to the high acid production by the homolactic LAB strain. In correlation, a highest residual WSC when Pa was inoculated in both crop silages was found. These results are in agreement with that previously reported using *L. plantarum* alone or in combination with *L.buchneri* [43, 49]. Residual WSC concentration in silages are desirable for an efficient fermentation in the silo and greater availability of energy-yielding substrates for ruminal microbes, although such concentrations can induce spoilage yeast growth as was previously reported [13]. Indeed, silages treated with homolactic LAB inoculants are often lower in pH, acetic acid, butyric acid, and ammonia-N, but higher in lactic acid content exhibiting lower DM loss compared with untreated and heterolactic-inoculated silages [51, 52]. Low DM (< 400 g/Kg and < 300 g/Kg for corn and sorghum, respectively) is optimal for LAB growth because more metabolic water would be available; as DM increases, water starts to become a limiting condition [50].

On the other hand, even higher for corn silages the decrease of NDF may be due to a considerable loss of hemicellulose due to the combination of fibrolytic enzymatic activity and acid hydrolysis produced by the acidogenic Pa and the FE-producing Lf, respectively. On the contrary, higher NDF values were reported for *L. buchneri*-inoculated corn silages compared to the uninoculated control [53], while no effect on NDF was described when *L. buchneri* alone or combined with *L. plantarum* were inoculated in corn and sorghum silages [49, 54]. As far as WSC concentration decrease during fermentation, the hydrolase activity is more likely to improve silage quality, positively affecting silage digestibility [55]. The slight CP decrease here observed was more pronounced for Lf-inoculated silages, presumably due to high proteolysis in this silage. Similarly, higher ammonia concentration was reported for corn and sorghum silages inoculated with *L. buchneri* than those inoculated with *L. plantarum* [43]. The combined effects of plant and microbial enzymes resulted in changes in nitrogenous fractions during ensiling. Although proteolytic activity was described for a dairy *L. fermentum* strain [56], an activity on corn and sorghum proteins may be suggested. Although it is known that LAB are low proteolytic organisms, heterolactic strains may generate environmental conditions that benefit proteolytic bacteria (epiphytic and/or inoculated) by converting lactic acid into acetic acid, with a consequent increase of pH [50]. Particularly for corn silages, Junges et al. [57] reported that protein solubilization was mostly assigned to bacterial activity (60%) followed by corn kernel enzymes (30%). During both silages fermentation and under anaerobic conditions, homolactic LAB inoculant such as Pa, produced mainly lactic acid, which can serve as substrate for lactate-assimilating yeasts that increase their numbers, making silage temperature and pH to rise. Due to the lower pKa value of lactic acid compared to acetic and propionic acids, a lower proportion of undissociated acid is available to enter to the microbial cell [40], thus lactic acid by itself is not effective as antimycotic agent. Heterofermentative LAB can ferment sugars and lactate to acetate and propionate, these short-chain acids inhibiting the growth of yeasts/molds as fungicidal agents [43]. However, the antifungal activity showed by *P. acidilactici*, could prevent silages from higher yeast/molds growth during the fermentation.

In view to investigate the survival of *L. fermentum* CRL2085 and *P. acidilactici* CRL2043 used as inoculants in sorghum silage as representative of common forage crop ensiling in northern Argentina, their presence was evaluated by rep-PCR. The band profiles amplified by the rep-PCR fingerprinting method in randomly isolated colonies after the fermentation process were identical to the strains inoculated at the beginning of the assay. This suggests the permanence of inoculated strains, thus the ability of inoculated LAB to persist during 45 days of fermentation was confirmed. This result indicated the high adaptation of selected LAB strains to ensiled forage matrix. On the other hand, the length of silages preservation represent a critical point for their quality and stability. A long conservation period in anaerobiosis determines a decrease in yeasts/molds counts improving the aerobic stability of silages, whatever be the use of inoculants or additives [11]. Because of the changes occurred in the silage, the forage is preserved as long as air does not penetrate inside the silage. Air is a primary cause of spoilage (aerobic deterioration) since it enables respiration and activity of undesirable microorganisms such as yeasts and molds, which result in fermentative nutrients losses [10]. During unloading, silage is normally fully exposed to air, resulting in an increase in temperature due to microorganism's growth. Yeasts are generally the initiators of aerobic deterioration, consumption of sugars and acids produced by fermentation increases the silage temperature and pH. These conditions allowing bacilli and other aerobic bacteria to grow and then molds enhance silage deterioration [51]. In the current study, inoculation with Lf alone or in combination with homolactic Pa improved the aerobic stability of normal DM corn and sorghum silages reaching 168 h, this being in correlation with a high yeasts/molds reduction at 45 days. The Lf- and Lf + Pa-inoculated silages improved aerobic stability by an extensive heterolactic fermentation that resulted in high levels of acetic and propionic acids as well as by the antifungal activity exerted by the homolactic LAB, both causing a reduction of yeasts/molds numbers during the fermentation. Similar results were reported for corn and sorghum silages when heterolactic *L. buchneri* and *L. hilgardii* were used as inoculants, either alone [6, 10, 16] or combined with homolactic LAB strains [9, 47, 58]. However, the fast temperature increase observed for Pa-inoculated silages agrees to that reported for uninoculated and homolactic-inoculated forage silages [8, 43, 59]. Recently, the relationship between yeast counts and aerobic stability has been confirmed; the length of silage conservation increase as yeasts are inhibited or reduced [16, 58].

Conclusion

Preliminary results from this study showed that FE-producer *L. fermentum* CRL2085 alone or in combination with antifungal and acid producer *P. acidilactici* CRL2043 can improve aerobic stability of corn and sorghum silages. Aerobic deterioration was prevented by yeasts/molds reduction/inhibition during fermentation while digestibility was increased. The combination of heterolactic CRL2085 + homolactic CRL2043 accelerates the initial lactic acid fermentation rate, reducing pH, avoiding fermentation losses with lower protein degradation and improving aerobic stability. The survival of LAB inoculants at 45 days of fermentation was suggested by rep-PCR analysis. In addition, beyond their effect on silage conservation, LAB previously selected by their survival and benefits in the gastrointestinal tract of ruminants may enhance animal performance. Thus, the feasibility to use fermented silage as a vehicle for beneficial LAB administration to cattle is under evaluation.

Acknowledgement

This work was granted by CONICET-PIP 545 and 441, and ANPCYT PICT 1187 and 4324. It was performed under a CONICET-Molino TRIGOTUC cooperation project

References

1. (2018) http://www.indexmundi.com/agriculture/
2. www.agroindustria.gob.ar
3. Di Marco ON (2005) Nutritive quality of corn and sorghum silage. Rural Vision Magazine 2.
4. Getachew G, Putnam DH, De Ben CM, De Peters EJ (2016) Potential of sorghum as an alternative to corn forage. American Journal of Plant Sciences 7: 1106-1121.
5. Di Marco ON (2013) Characteristics of corn and sorghum silages. Neither better nor worse, according to the circumstance we must evaluate the best option. Produce XXI 21: 20-28.
6. Richards CJ, Hicks B (2007) Processing of corn and sorghum for feedlot cattle. Veterinarian Clinics of North America: Food Animal Practice 23: 207-221.
7. Wilkinson JM, Davies DR (2013) The aerobic stability of silage: Key findings and recent developments. Grass and Forage Science 68: 1-19.

8. Addah W, Baah J, Groenewegen P, Okine EK, McAllister TA (2011) Comparison of the fermentation characteristics, aerobic stability and nutritive value of barley and corn silages ensiled with or without a mixed bacterial inoculant. Canadian Journal of Animal Science 91: 133-146.

9. Filya I, Sucu E, Karabulutet A (2006) The effect of *Lactobacillus buchneri* on the fermentation, aerobic stability and ruminal degradability of maize silage. Journal of Applied Microbiology 101: 1216-1223.

10. Tabacco E, Piano S, Chion RA, Borreani G (2011) Effect of *Lactobacillus buchneri* LN4637 and *Lactobacillus buchneri* LN40177 on the aerobic stability, fermentation products, and microbial populations of corn silage under farm conditions. Journal of Dairy Science 94: 5589-5598.

11. Comino L, Tabacco E, Righi F, Chion RA, Quarantelli A, et al. (2014) Effects of an inoculant containing a *Lactobacillus buchneri* that produces ferulate-esterase on fermentation products, aerobic stability, and fibre digestibility of maize silage harvested at different stages of maturity. Animal Feed Science and Technology 198: 94-106.

12. Addah W, Baah J, Okine EK, MacAllister TA (2014) Silage inoculants-Are they worth the money? WCDS Advances in Dairy Technology 26: 193-206.

13. Arriola KG, Kim SC, Adesogan AT (2011) Effect of applying inoculants with heterolactic or homolactic and heterolactic bacteria on the fermentation and quality of corn silage. Journal of Dairy Science 94: 1511-1516.

14. Koc F, Aksoy OS, Okur AA, Celikyurt G, Korucu D, et al. (2017) Effect of pre-fermented juice, *Lactobacillus plantarum* and *Lactobacillus buchneri* on the fermentation characteristics and aerobic stability of high dry matter alfalfa bale silage. The Journal of Animal & Plant Sciences 27: 1426-1431.

15. Ni K, Wang Y, Cai Y, Pang H (2015) Natural lactic acid bacteria population and silage fermentation of whole crop wheat. Asian-Australasian Journal of Animal Science 28: 1123-1132.

16. Ferrero F, Piano S, Tabacco E, Borreani G (2018) Effects of conservation period and *Lactobacillus hilgardii* inoculum on the fermentation profile and aerobic stability of whole corn and sorghum silages. Journal of the Science of Food and Agriculture 99: 2530-2540.

17. Nsereko VL, Smiley BK, Rutherford WM, Spielbauer A, Forrester KJ, et al. (2008) Influence of inoculating forage with lactic acid bacterial strains that produce ferulate esterase on ensilage and ruminal degradation of fiber. Animal Feed Science and Technology 145: 122-135.

18. Xu Z, He H, Zhang S, Guo T, Kong J (2017) Characterization of feruloyl esterases produced by the four Lactobacillus Species: *L. amylovorus*, *L. acidophilus*, *L. farciminis* and *L. fermentum*, isolated from ensiled corn stover. Frontiers in Microbiology 8: 941.

19. Addah W, Baah J, Okine EK, MacAllister TA (2012) Use of thermal imaging and the in situ technique to assess the impact of an inoculant with feruloyl esterase activity on the aerobic stability and digestibility of barley silage. Canadian Journal of Animal Science 92: 381-394.

20. Gajbhiye M, Kapadnis B (2016) Antifungal-activity-producing lactic acid bacteria as biocontrol agents in plants. Biocontrol Science and Technology 26: 1451-1470.

21. Ficoseco AC, Mansilla FI, Maldonado NC, Miranda H, Nader Macias MEF, et al. (2018) Safety and growth optimization of Lactic Acid Bacteria isolated from feedlot cattle for probiotic formula design. Frontiers in Microbiology 9: 2220.

22. Maldonado NC, Ficoseco AC, Mansilla FI, Melián C, Hébert EM, et al. (2018) Identification, characterization and selection of autochthonous lactic acid bacteria as probiotic for feedlot cattle. Livestock Science 212: 99-110.

23. Donaghy J, Kelly PF, McKay AM (1998) Detection of ferulic acid esterase production by *Bacillus spp*. and lactobacilli. Applied Microbiology and Biotechnology 50: 257-260.

24. Magnusson J, Ström K, Roos S, Sjögren J, Schnürer J (2003) Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiology Letters 219: 129-135.

25. Chahrour W, Merzouk Y, Henni JE, Haddaji M, Kihal M (2013) Screening and identification of lactic acid bacteria isolated from sorghum silage processes in West Algeria. African Journal of Biotechnology 12: 1703-1709.

26. AOAC (2006a) Method 990.03. Protein (Crude) in Animal Feed. AOAC International, Arlington, VA, USA.

27. AOAC (2005) Method Nº 2002.04. Neutral detergent fiber (NDF) with enzyme Pretreat. AOAC International. Arlington, VA, USA.

28. AOAC (2006b) Method 973.18. Acid Detergent fiber (ADF). AOAC International, Arlington, VA, USA.

29. AOAC (2006c) Method 942.05 (4.1.10). Ash determination in animal feed. AOAC International, Arlington, VA, USA.

30. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28: 350-356.

31. Jorgensen OS, Andersen B (1973) An improved glucoseoxidase-peroxidase-coupled assay for β-fructofuranosidase activity. Analytical Biochemistry 53: 141-145.

32. Raibaud P, Caulet M, Galpin JV, Mocquot G (1961) Studies on the microbial flora of the alimentary tract of pigs. II. Streptococci: selective enumeration and differentiation of the dominant group. Journal of Applied Bacteriology 24: 285-291.

33. Gevers D, Huys G, Swingset J (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus species*. FEMS Microbiology Letters 205: 31-36.

34. Pospiech A, Neumann B (1995) A versatile quick-prep of genomic DNA from gram-positive bacteria. Trends in Genetics 11: 217-218.

35. Ranjit NK, Kung L (2000) The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. Journal of Dairy Science 83: 526-535.

36. Aboagye IA, Lynch JP, Church JS, Baah J, Beauchemin KA (2015) Digestibility and growth performance of sheep fed alfalfa hay treated with fibrolytic enzymes and a ferulic acid esterase producing bacterial additive. Animal Feed Science and Technology 203: 53-66.

37. Yu P, McKinnon JJ, Christensen DA (2005) Hydroxycinnamic acids and ferulic acid esterase in relation to biodegradation of complex plant cell walls. Canadian Journal of Animal Science 85: 255-267.

38. Nyonyo T, Shinkai T, Mitsumori M (2013) Improved culturability of cellulolytic rumen bacteria and phylogenetic diversity of culturable cellulolytic and xylanolytic bacteria newly isolated from the bovine rumen. FEMS Microbiology Ecology 88: 528-537.

39. Drissi F, Merhej V, Blanc Tailleur C, Raoultet D (2015) Draft genome sequence of the *Lactobacillus mucosae* strain Marseille. Genome Announcements 3: e00841-e00815.

40. Shnürer J, Magnusson J (2005) Antifungal lactic acid bacteria as biopreservatives. Trends in Food Science & Technology 16: 70-78.

41. Yépez A, Luz C, Meca G, Vignolo G, Mañes J, et al. (2017) Biopreservation potential of lactic acid bacteria from Andean fermented food of vegetal origin. Food Control 78: 393-400.

42. Govea CF, Muck RE, Mertens DR, Weimer PJ (2011) Microbial inoculant effects on silage and *in vitro* ruminal fermentation, and microbial biomass estimation for alfalfa, Bmr corn, and corn silages. Animal Feed Science and Technology 163: 2-10.

43. Filya I, Sucu E (2007) The Effect of bacterial inoculants and a chemical preservative on the fermentation and aerobic stability of whole-crop cereal silages. Asian-Australasian Journal of Animal Science 20: 378-384.

44. Weinberg Z, Khanal P, Yildiz C, Chen Y, Arieli A (2011) Ensiling fermentation products and aerobic stability of corn and sorghum silages. Grassland Science 57: 1-5.

45. Johnson LM, Harrison JH, Davidson D, Mahanna WC, Shinners K (2003) Corn silage management: Effects of hybrid, maturity, inoculation, and mechanical processing on fermentation characteristics. Journal of Dairy Science 86: 287-308.

46. Skonieski FR, Nörnberg JL, Kessler DJ, David BD, Azevedo BE, et al. (2014) Corn plant arrangement and its effect on silage quality. Revista Brasileira de Zootecnia 43: 114-119.

47. Reich LJ, Kung L (2010) Effects of combining *Lactobacillus buchneri* 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. Animal Feed Science and Technology 159: 105-109.

48. Thomas ME, Foster JL, McCuistion KC, Redmon LA, Jessup RW (2013) Nutritive value, fermentation characteristics, and in situ disappearance kinetics of sorghum silage treated with inoculants. Journal of Dairy Science 96: 7120-7131.

49. Filya I (2003) The effect of *Lactobacillus buchneri* and *Lactobacillus plantarum* on the fermentation, serobic stability, and ruminal degradability of low dry matter corn and sorghum silages. Journal of Dairy Science 86: 3575-3581.

50. Kung L, Shaver RD, Grant RJ, Schmidt RJ (2017) Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. Journal of Dairy

Science 101: 4020-4033.

51. Borreani G, Tabacco E, Schmidt RJ, Holmes BJ, Muck RE (2017) Silage review: Factors affecting dry matter and quality losses in silages. Journal of Dairy Science 101: 3952-3979.

52. Muck RE, Nadeau EMG, McAllister TA, Contreras Govea FE, Santos MC, et al. (2018) Silage review: Recent advances and future uses of silage additives. Journal of Dairy Science 101: 3980-4000.

53. Basso FC, Bernardes TF, Roth AP, Lodo NB, Berchielli TT, et al. (2012) Fermentation and aerobic stability of corn silage inoculated with *Lactobacillus buchneri*. R Bras Zootec 41: 7.

54. Filya I (2003) The effect of *Lactobacillus buchneri*, with or without homofermentative lactic acid bacteria, on the fermentation, aerobic stability and ruminal degradability of wheat, sorghum and maize silages. Journal of Applied Microbiology 95: 1080-1086.

55. Huisden CM, Adesogan AT, Kim SC, Ososanya T (2009) Effect of applying molasses or inoculants containing homofermentative or heterofermentative bacteria at two rates on the fermentation and aerobic stability of corn silage. Journal of Dairy Science 92: 690-697.

56. El Ghaish S, Dalgalarrondo M, Choiset Y, Sitohy M, Ivanova I, et al. (2010) Characterization of a new isolate of *Lactobacillus fermentum* IFO3956 from Egyptian Ras cheese with proteolytic activity. European Food Research and Technology 230: 635-643.

57. Junges D, Morais G, Spoto MHF, Santos PS, Adesogan AT, et al. (2017) Influence of various proteolytic sources during fermentation of reconstituted corn grain silages. Journal of Dairy Science 100: 1-4.

58. Burns P, Borgo MF, Binetti A, Puntillo M, Bergamini C, et al. (2018) Isolation, characterization and performance of autochthonous spray dried lactic acid bacteria in maize micro and bucket-silos. Frontiers in Microbiology 9: 2861.

59. Cai Y, Benno Y, Ogawa M, Kumai S (1999) Effect of applying lactic acid bacteria isolated from forage crops on fermentation characteristics and aerobic deterioration of silage. Journal of Dairy Science 82: 520-526.