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Lactobacillus rhamnosus as Additive for Maize and Sorghum Ensiling

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The effects of *Lactobacillus rhamnosus* AT195, a potential probiotic microorganism cultured in buffalo "scotta" whey, on chemical and microbiological composition in maize and sorghum ensiling were evaluated. Both crops were harvested, chopped, and treated or not with the selected strain prior to ensiling in fiberglass vertical silos; 90 days after ensiling, silages were sensorially evaluated and sampled. Different chemical components were evaluated both on fresh crops and silages: in particular, the water-soluble carbohydrates content was investigated by high-field NMR spectroscopy and the carbohydrate fermentation profile was performed by GC. Besides phenotypic identification and typing, microbiological studies included *Lb. rhamnosus* genotype typing by RAPD-PCR. All silages, inoculated or not, were well preserved, as their chemical and microbiological data along with the fermentation profiles showed. The selected strain used as inoculum influenced the lactic acid population of silages and evidenced a good survival performance during the ensiling process of both maize and sorghum. Moreover, the use of *Lb. rhamnosus* strain efficiently improved the quality of the multifactorial ensiling process by significantly reducing the ammonia nitrogen content of both maize and sorghum silages.

KEYWORDS: Silage; *Lactobacillus rhamnosus*; lactic acid bacteria; feed additives

INTRODUCTION

In Europe, silages constitute about 55% of the total amount of conserved forages: even though maize (*Zea mays*) is the most popular cereal crop conserved as silage in many parts of the world, sorghum (*Sorghum bicolor*) may have the potential to economically compete with corn silage in many disadvanced areas because of its better adaptation to drought, high temperature, and low pH soil (*1*). However, both ensiling management and nutritional values of sorghum silage have not been completely investigated yet and require further studies.

The quality of silage depends on the interaction among many factors, including forage type and quality of the crops, type of fermentation, rate of pH decrease, and maintenance of anaerobic condition (*2*). Successful ensiling is achieved when lactic acid bacteria (LAB) dominate the fermentation controlling the activity of proteolytic clostridia. In order to improve the forage quality, silage additives, inoculant or not, can also be used mainly to prevent secondary fermentation and to decrease butyric acid production (*3*).

Among silage additives, microbial inoculants can be applied at ensiling to accelerate the decrease of pH by promoting the lactic acid fermentation; they have the advantage of being safe and easy to use, noncorrosive to machinery, and nonpolluting to the environment and are considered as natural products (*4*). Bacterial silage inoculants such as LAB could even contribute to reduce the environmental impact of the dairy food chain; in fact, secondary products of the cheese manufacturing industry may represent an interesting growth medium for LAB.

Notwithstanding the controversial results reported in the literature regarding the effects of microbial silage additives on both the multifactorial ensiling process and the nutritive value of the forage, positive outcomes such as higher lactate:acetate ratio, lower ammonia nitrogen, and lower dry matter losses have been reported (*5*).

Among the LAB inoculants, a strain of *Lactobacillus rhamnosus*, designated as AT195, isolated during the ripening of Parmigiano Reggiano cheese (*6*, *7*) could represent an interesting silage additive according to its sugar fermentation profile and

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Table 1. Composition of Maize and Sorghum Crops at Harvest (mean values ± esm)

	maize	sorghum
DM,[a] g·100 g^{-1}	26.9 ± 0.4	27.3 ± 0.7
CP,[b] g·100 g^{-1} DM	8.5 ± 0.1	7.9 ± 0.2
NDF,[c] g·100 g^{-1} DM	58.2 ± 1.1	57.9 ± 0.8
ADF,[d] g·100 g^{-1} DM	27.6 ± 1.0	39.4 ± 0.3
ADL,[e] g·100 g^{-1} DM	6.5 ± 0.9	9.0 ± 0.6
ash, g·100 g^{-1} DM	5.8 ± 0.4	8.3 ± 0.4

[a] DM = dry matter. [b] CP = crude protein. [c] NDF = neutral detergent fiber. [d] ADF = acid detergent fiber. [e] ADL = acid detergent lignin.

other specific characteristics, such as bile salts and antibiotics resistance, ability to produce H_2O_2, and acidifying capacity (*8, 9*).

The main objective of this study was therefore to evaluate the effects of a selected strain of *Lb. rhamnosus* on chemical and microbiological composition of maize and sorghum ensiled in vertical silos.

MATERIALS AND METHODS

Silage Preparation. Two whole forage crops were used: maize, cultivar *500 GMO free*, variety *midlar*(Sivam, Milan, Italy), harvested at the one-third milk line stage and sorghum, variety *sweet Creek* (SIVAM, MI, Italy), harvested at the milk stage. The two forage crops were harvested from a single field in Southern Italy (Caserta province, latitude 41°22′ N, longitude 14°16′ E; altitude 385 m) with a conventional forage harvester (Claas, mod. Lexion, Harsewinkel, Germany) at a theoretical length of cut of 1.5 cm and at a cutting height of 20 cm. Within 1 h from chopping, the two silages for each crop (C, no inoculant and I, inoculant treated) were packed to achieve a density of 640 (±50, s.d.) kg/m^3 into fiberglass discontinuous vertical silos (diameter 1.04 m × 1.28 m height, ORM srl, Bagnara di Romagna, RA, Italy), equipped with a lid that enables gas release only.

Experimental inoculant was represented by *Lb. rhamnosus* AT195 strain cultured in MRS broth (Oxoid, Milan, Italy) under anaerobic condition for 48 h at 37 °C and for the following 24 h at 37 °C in buffalo "scotta" whey, i.e., the byproduct of ricotta cheesemaking, to achieve a final concentration in scotta of 10^9 cfu g^{-1}. Inoculant was applied to the fresh chopped forages by mixing for 5 min in a vertical mixer wagon to achieve the final microbial concentration of 10^7 cfu g^{-1} of fresh forage. Maize and sorghum chopped fresh crops ($n = 3$) were sampled for chemical and microbiological analyses.

Ninety days after ensiling, odor, color and appearance of maize and sorghum silages were macroscopically and sensorially evaluated at silo opening; each silage was then sampled (5 kg) by drawing three cores at different heights; chemical and microbiological analyses, performed in triplicate, are described below. During the ensiling period, silage temperature was monitored by electronic data loggers (Onset Computer Corp., Hobo H8, Bourne, MA, USA) placed at three different heights in each silo, as already mentioned; the ambient temperature was also continuously monitored during the experiment.

Analytical Methods. Fresh crops and silages samples were analyzed for chemical composition: dry matter, crude protein, and ash content were determined following the official methods (*10*), whereas neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were evaluated according to Van Soest et al. (*11*).

Water extracts were prepared by homogenizing 50 g of silage with 200 mL of water (*12*), and the pH measurement was performed by using a pH-meter Crison Basic (Crison Instruments S.A., Barcelona, Spain). High-field NMR spectroscopy, nonspecific multicomponent analytical technique extensively used for the analysis of mixtures (*13*), was used to study forage crop and inoculated silage of maize and sorghum extracts. Extracts for the NMR study were freeze–dried and then dissolved (10–12 mg) in 700 μL of a phosphate-buffered solution (pH = 6.5). The ^{1}H NMR spectra of the extracts were recorded at 27 °C on a Bruker AVANCE AQS 600 spectrometer operating at the proton frequency of 600.13 MHz. In all the spectra 3-trimethylsilyl-propionic-2,2,3,3-d_4 acid, sodium salt, was used as standard and a soft presaturation of the HOD residual signal was performed.

Ammonia nitrogen, volatile fatty acids, lactic acid, and alcohol concentrations were determined on acid silage extracts: samples of chopped frozen silage were weighed (50 g) in a 400 mL polyethylene bag and extracted with 200 mL of 0.1 N H_2SO_4 at 20 °C for 4 min in

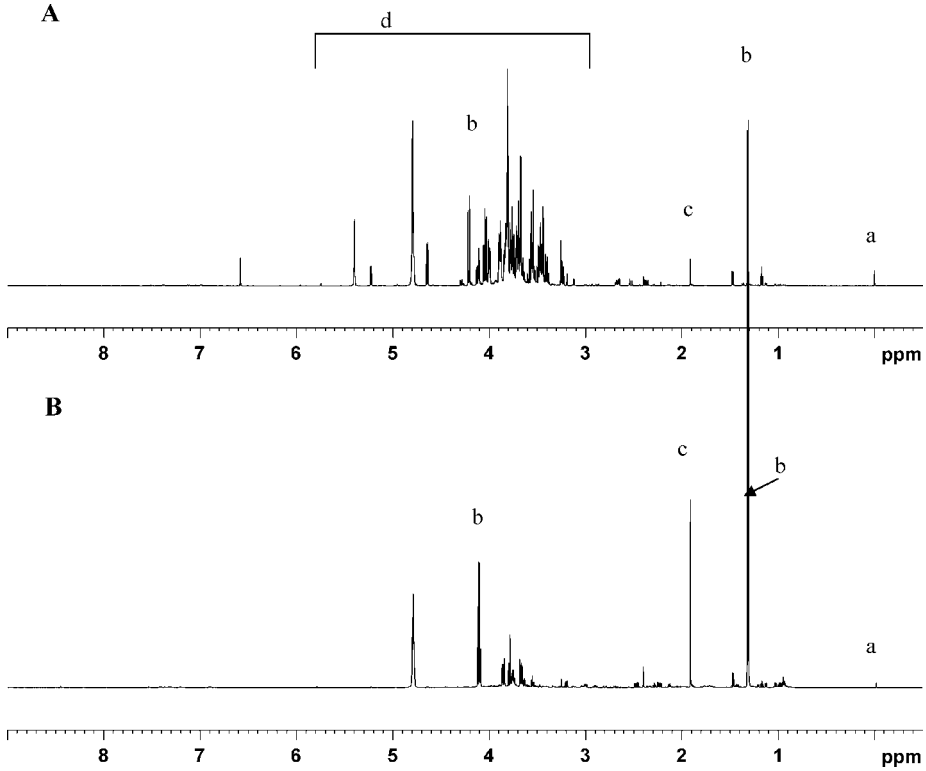

Figure 1. 600.13 MHz, ^{1}H NMR spectrum of extracts dissolved in a phosphate-buffered solution (pH = 6.5) at 27 °C: (**A**) fresh maize crop; (**B**) inoculated maize silage. Resonances are labeled as follows: a = TSP (standard); b = lactic acid, c = acetic acid, d = water-soluble carbohydrates.

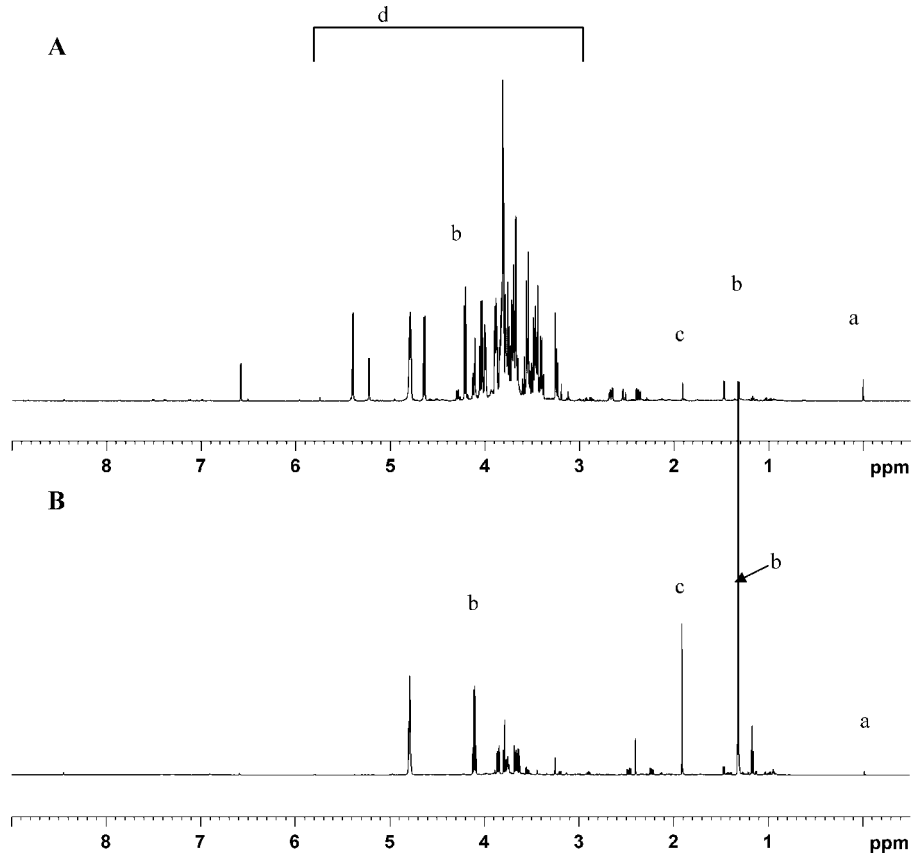

Figure 2. 600.13 MHz, ^{1}H NMR spectrum of extracts dissolved in a phosphate-buffered solution (pH = 6.5) at 27 °C: (**A**) fresh sorghum crop; (**B**) inoculated sorghum silage. Resonances are labeled as follows: a = TSP (standard); b = lactic acid, c = acetic acid, d = water-soluble carbohydrates.

Table 2. Chemical Composition and pH Values of Maize and Sorghum after 90 Days of Ensilage in Silos, Inoculated (I) or Not (C) (mean values ± esm)[a]

	maize		sorghum	
	C	I	C	I
DM,[b] g·100 g^{-1}	24.9 ± 0.5 a	25.5 ± 0.6 a	22.9 ± 0.6 b	23.4 ± 0.3 b
CP,[c] g·100 g^{-1} DM	8.2 ± 0.1 a	8.6 ± 0.4 a	7.4 ± 0.4 b	8.0 ± 0.1 ab
NDF,[d] g·100 g^{-1} DM	53.8 ± 1.4 a	50.0 ± 0.8 a	63.8 ± 1.1 b	63.2 ± 0.5 b
ADF,[e] g·100 g^{-1} DM	26.4 ± 0.8 a	26.1 ± 0.6 a	45.6 ± 0.8 b	43.4 ± 0.6 b
ADL,[f] g·100 g^{-1} DM	5.0 ± 0.6 a	4.9 ± 0.6 a	9.2 ± 1.0 b	7.4 ± 0.4 b
ash, g·100 g^{-1} DM	5.0 ± 0.1 a	4.8 ± 0.1 a	8.9 ± 0.1 b	9.4 ± 0.3 b
pH	3.78 ± 0.13	3.67 ± 0.07	3.69 ± 0.05	3.66 ± 0.02

[a] a, b: different letters in the same row mean significantly different ($P < 0.05$). [b] DM = dry matter. [c] CP = crude protein. [d] NDF = neutral detergent fiber. [e] ADF = acid detergent fiber. [f] ADL = acid detergent lignin.

a Stomacher 400 laboratory blender (Seward Laboratory, London). The mixture was centrifuged for 5 min at 3000*g* and then filtered through Schleicher and Schull membrane filter (BA-83, 0.2 μm). Ammonia nitrogen of the filtrates was determined by distillation and titration, according to AOAC (*10*).

A 0.1 μL aliquot of the acid extracts was injected using an on-column technique with an autosampler (Dani Instruments SpA, GC 1000 DPC, Cologno Monzese, Italy) into a wide-bore capillary column (SGE BP21 25 m × 0.53 mm i.d. and 0.5 μm film thickness; P/N 054474, SGE International, Ringwood, Victoria, Australia) installed in a gas chromatograph (Dani GC 1000 DPC), running in a temperature-programmed mode and equipped with a flame ionization detector and a PTV injection port, used in the split mode, with a split vent flow of 100 mL·min^{-1}. The injector and detector ports were set at 230 and 240 °C, respectively; helium was used as carrier gas, and the oven temperature was programmed from 60 to 200 °C at 5 °C per min and held for 2 min giving a run time of 30 min. The peak area was measured using a Dani Data Station DDS 1000. Each peak was identified and quantified according to pure standards (Sigma Chemical, St. Louis, MO, USA).

For the microbiological studies, 10 g portions of fresh crops and silages were aseptically homogenized in 90 mL of sterile physiological solution (9 g of NaCl L^{-1}) in a Stomacher 400 laboratory blender (Seward Laboratory, London) for 3 min. The extracts were further diluted, and the serial dilutions were inoculated in appropriate media for microbiological analysis. Microbiological evaluation included enumeration of total mesophilic bacteria on Plate Count Agar (Oxoid, Milano, Italy) after incubation at 28 °C for 48 h, LAB on MRS agar (Oxoid, Milano, Italy) after anaerobic incubation (Gas Pack Anaerobic System, BBL, Cockeysville, MD, USA) at both 22 and 45 °C for 72 h, *Enterobacteriaceae* on VRBGA (Oxoid, Milano, Italy) at 37 °C for 24 h, total and faecal coliforms on VRBLA (Oxoid, Milano, Italy) after incubation for 48 h at 37 and 44 °C, respectively, and yeasts and molds on YPD (20 g of peptone ·L^{-1}, 20 g of dextrose·L^{-1}, 10 g of yeast extract·L^{-1}, 20 g of agar·L^{-1}) after incubation at 28 °C for 72 h.

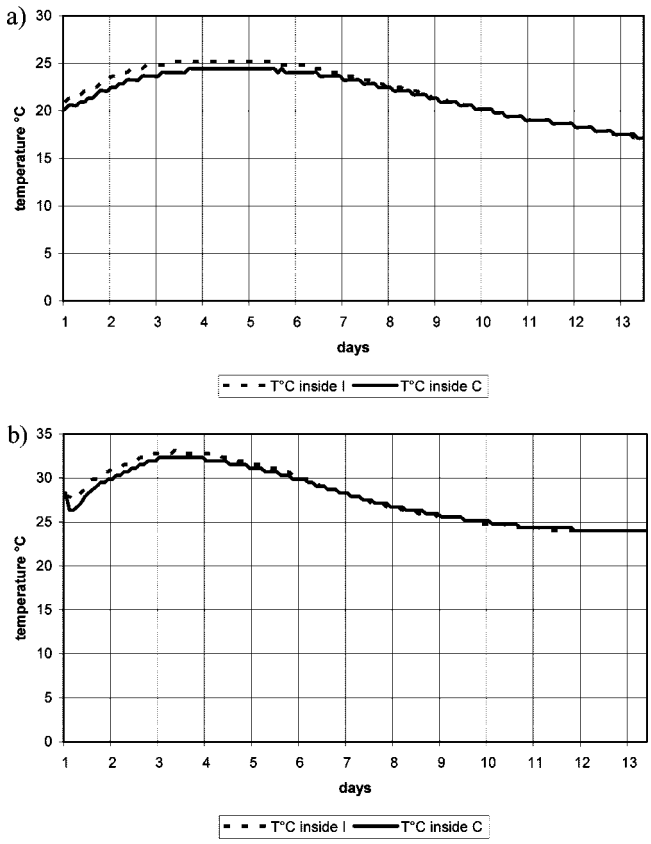

Figure 3. Average silages temperature recorded during the first 14 days of maize (**a**) and sorghum (**b**) ensiling.

Clostridial spores were determined by the most probable number (MPN) technique, according to Annibaldi (*14*).

Colonies were counted directly on the plates at appropriate dilutions containing a minimum of 30 and a maximum of 300 colonies per plate; results are expressed as $\log_{10}$ cfu per gram of fresh materials.

As a further phenotypic identification and typing, colonies of LAB from silages were picked randomly from the plates and identified according to Hammes and Vogel (*15*); Gram-positive and catalase-negative isolates ($n = 315$) were subjected to microscope observation, production of CO_2 from glucose, sugar fermentation pattern using API 50-CHL (bioMérieux Italia S.p.A., Roma, Italy), and growth ability in MRS broth at both 15 and 45 °C.

RAPD-PCR typing was carried out according to Andrighetto et al. (*16*) in order to compare those isolates phenotypically ascribable to *Lb. rhamnosus* with the strain AT195 used as starter. PCR reaction was performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany), using the following primers and amplification conditions: M13, 5′-GAGGGTGGCGGTTCT-3′, 35 cycles of 94 °C for 1 min, 40 °C for 20 s, ramp to 72 at 0.5 °C s^{-1}, 72 °C for 2 min; D8635: 5′-GAGCGGCCAAAGGGACGAGAC-3′, after an initial step of 94 °C for 2 min, 35 cycles of: 94 °C for 1 min, 42 °C for 1 min, 72 °C for 1.5 min were carried out, with a final step at 72 °C for 10 min.

Amplification products were separated by electrophoresis on 1.5 and 1.8% (w/v) agarose gel; RAPD-PCR profiles were obtained directly using a digital camera Image Master VDS (Amersham Pharmacia Biotech, Milano, Italy).

Statistical Analyses. The general linear model of SAS (SAS Institute, Cary, NC, USA) was used for the analysis of variance of data: in particular, for chemical composition, pH, and fermentation end products of maize and sorghum silages the model included "inoculant" and "type of silage" as main effects, and "inoculant × type of silage" as interaction. Changes in microbial counts during the ensiling process of each crop were analyzed by considering the main effects of "inoculant" and "day of ensiling" and their interaction. Differences among means were determined by the least significant difference method; the significance level was declared at $P < 0.05$.

RAPD-PCR profiles were analyzed with the pattern analysis software package Gel Compar Version 4.1 (Applied Maths, Kortriijk, Belgium). The similarity calculation in the profiles of bands was based on the Pearson product moment correlation coefficient. The dendrograms were obtained by means of the unweighted pair group method, using the arithmetic average clustering algorithm (*17*).

RESULTS AND DISCUSSION

The chemical composition of both maize and sorghum fresh crops at harvest is shown in **Table 1**. Sorghum evidences a lower protein content and a higher ash content than maize; sorghum and maize are also characterized by a different fiber quality: in fact, sorghum shows a lower content of digestible fiber, reported as NDF - ADL difference (maize 51.7% dry matter versus sorghum 48.9% dry matter).

The NMR technique allowed us to study the fresh chopped maize (**Figure 1A**) and sorghum (**Figure 2A**) water extracts: the ^{1}H spectra of both crops show a high amount of soluble carbohydrates, see resonances in the 3.0–5.5 ppm spectral region, and only small amounts both of acetic acid, see the singlet at 1.911 ppm, and lactic acid, see the doublet at 1.318 ppm and the partially overlapped quartet at 4.105 ppm.

Macroscopical and sensorial evaluations of maize and sorghum silages performed at silo openings did not evidence any negative aspect in color, odor, and mixture properties of both control and inoculated silages; control and inoculated silages presented a light-green to green-brown color and a slightly sweet odor of lactic acid.

As shown in **Table 2**, the inoculation of maize and sorghum fresh crops with *Lb. rhamnosus* AT195 did not significantly modify the chemical composition of silages, even though it has

Table 3. Fermentation Products of Inoculated (I) or Untreated (C) Silages (mean ± esm) after 90 Days of Ensilage in Silos[a]

	maize		sorghum	
	C	I	C	I
ammonia N,[b] g·100 g^{-1} TN	6.9 ± 0.9 a	4.1 ± 0.9 b	8.4 ± 0.9 a	5.3 ± 0.9 b
methanol,[c] g·100 g^{-1} DM	0.06 ± 0.03 a	0.11 ± 0.03 a	0.29 ± 0.03 b	0.23 ± 0.03 b
ethanol, g·100 g^{-1} DM	0.32 ± 0.14 a	0.48 ± 0.18 a	1.46 ± 0.16 b	1.17 ± 0.16 b
propanol, g·100 g^{-1} DM	0.05 ± 0.03	0.04 ± 0.04	0.05 ± 0.04	0.07 ± 0.04
lactic acid, g·100 g^{-1} DM	12.44 ± 2.19	13.06 ± 2.83	7.84 ± 2.45	10.04 ± 2.46
acetic acid, g·100 g^{-1} DM	1.82 ± 0.14 a	1.84 ± 0.17 a	1.13 ± 0.15 b	1.19 ± 0.15 b
lactic:acetic ratio	6.89 ± 1.22	7.15 ± 1.58	6.85 ± 1.37	7.12 ± 1.37
propionic acid, g · 100 g^{-1} DM	tr[d]	tr	tr	tr
butyric acid, g·100 g^{-1} DM	tr	tr	tr	tr
isobutyric acid, g·100 g^{-1} DM	tr	tr	0.04 ± 0.01	0.05 ± 0.01
isovaleric, g·100 g^{-1} DM	tr	tr	tr	tr
valeric, g·100 g^{-1} DM	tr	tr	tr	tr

[a] a, b means in the same row with different letters are significantly different ($P < 0.05$). [b] TN = total nitrogen. [c] DM = dry matter. [d] tr, determined in traces.

Table 4. Microbiological Analysis of Maize Fresh Crop (d0) and after Ensiling (d90), Inoculated (I) or Not (C) (mean ± esm, log cfu · g^{-1} fresh matter)[a]

	d0		d90	
	C	I	C	I
total mesophilic bacterial count	7.2 ± 0.6	7.2 ± 0.8	6.2 ± 0.4	6.3 ± 0.5
yeast and molds	4.4 ± 0.7 a	4.5 ± 0.6 a	3.6 ± 0.5 a	1.9 ± 0.5 b
LAB at 22 °C	6.0 ± 0.6 a	7.2 ± 0.5 b	4.6 ± 0.7 a	5.7 ± 0.4 a
LAB at 45 °C	2.4 ± 0.5 a	4.9 ± 0.3 b	2.3 ± 0.7 a	4.0 ± 0.6 b
Enterobacteriaceae	6.3 ± 0.9 a	6.2 ± 1.0 a	<1[b] b	<1 b
total coliform	5.9 ± 0.4 a	5.8 ± 0.7 a	<1 b	<1 b
faecal coliform	3.6 ± 0.4 a	3.5 ± 0.2 a	<1 b	<1 b
Clostridium spp	4.3 ± 0.3	4.4 ± 0.4	4.2 ± 0.5	4.4 ± 0.2

[a] a, b means in the same row with different letters are significantly different ($P < 0.05$). [b] <1 = absent in 1 g.

to be noted that protein content of inoculated sorghum silage tended to be higher ($P > 0.05$) than that of the control, probably due to the LAB activity.

Average dry matter content of both maize and sorghum silages (**Table 2**) reflected the good stage of maturity of the crops at harvest, as already evidenced by the high amount of carbohydrates in crop extracts, determined by ^{1}H NMR. However, the recovery of DM in silages, as percentage of DM in silage relative to DM at harvest, evidenced a higher DM loss in sorghum silages (DM recovery C: 83.9% vs I: 85.7%) than in maize silages (DM recovery C: 92.6% vs I: 94.8%); from a nutritional point of view, the higher loss of available nutrients due to the ensiling was associated to an increase of the fibrous components NDF and ADF in sorghum silages with respect to the fresh crop.

Notwithstanding the confirmed higher feeding value of maize silages, the use of *Lb. rhamnosus* did not affect the pH values of silages (**Table 2**), resulted in average 3.71 ± 0.10 and 3.68 ± 0.04 for maize and sorghum, respectively. The optimal anaerobic preservation of the two crops suggested by the silage pH values was also confirmed by the temperature values measured during the storage of both inoculated and control maize and sorghum silages, as **Figure 3** shows for the first 14 days, considered crucial in the ensiling process. During this study, the ambient temperature was in average 16.7 °C and ranged between 8.2 and 26.8 °C. All these results suggest that the fiberglass vertical silos used in this study provided optimal experimental and fermentation conditions. From a practical point of view, due to the limited capacity of these silos, their use could be suggested when daily silage consumption is minimal: in this way, well-preserved forage, which is the basis of profitable and safe herbivore nutrition, is guaranteed.

Besides climatic conditions and ensiling technique, many other factors can contribute to the success of an inoculant silage additive, such as epiphytic microflora and type and properties of the crop to be ensiled (*18*). In this regard, the ^{1}H spectra of both maize (**Figure 1B**) and sorghum (**Figure 2B**) inoculated silages showed a drastic reduction of water-soluble carbohydrate content associated to a high concentration of both lactic acid and acetic acid, confirmed also by the quantitative results (**Table 3**), as the major product of fermentations and the major volatile fermentation end-product, respectively.

The use of *Lb. rhamnosus* strain as silage additive significantly reduced by 40% the ammonia nitrogen content of both maize and sorghum silages (**Table 3**). However, it is important to notice that the observed ammonia N levels accounted for less than 6% and 9% of the total N in treated and untreated silages, respectively. The microbial treatment as silage additive did not significantly influence the levels of the other investigated fermentation products (**Table 3**), according to previous studies (*19, 20*).

Many authors reported an increase in lactic acid production and a pH reduction in silage of different crops after inoculation with LAB (*3, 4, 18, 21, 22*); in other studies, instead, inoculation of maize silage with homolactic acid bacteria has failed to increase the lactic acid concentration probably because maize has a low buffering capacity and ensiles rapidly (*23*). A lack of the inoculation effect on the lactic acid content has also been reported for grass (*24, 25*) and barley (*26*) silages, as a possible consequence of both the high numbers of epiphytic LAB already present on crops at ensiling and the chemical characteristics of these forages. From a nutritional point of view, it has to be considered that lactic acid from silage is usually converted by rumen microorganisms to other metabolites; moreover, notwithstanding the low pH, silage intake can contribute to the buffering system in the rumen (*27*).

Lactic acid concentration did not significantly differ between maize and sorghum silages while the acetic acid concentration was significantly lower in sorghum than in maize silages (**Table 3**). Propionic, butyric, valeric, and isovaleric acids were not detected or detected only in traces in all silages; on the other hand, a detectable presence of isobutyric acid was present in sorghum silages, inoculated or not.

Lb. rhamnosus as silage additive did not influence the silage content of alcohols, whose major product was ethanol with values significantly higher in sorghum than in maize. On this regard it has to be considered that a high ethanol concentration in silages is usually associated with a large number of yeasts and a lower aerobic silage stability (*28*). Methanol was also found at higher levels in sorghum silage, while low amount of propanol (<0.1 g·100 g^{-1} dry matter) was detected in all silages.

As far as changes in microbial counts during ensiling are concerned, **Table 4** summarizes the microbial composition of maize fresh crop (d0) and silage, after 90 days of ensiling (d90). The total mesophilic microbial count was not influenced by the treatment both in fresh crop and in silage; the slight decrease observed in silages compared to the fresh crop could be due to the inhibition of bacterial growth following the pH decrease.

Maize lactic acid bacteria count determined at 45 °C was observed significantly higher in inoculated silages than in untreated silages (**Table 4**). This result suggests the good survival performance of *Lb. rhamnosus* AT195, a typically mesophilic microorganism, in the silage fermentation process; in fact, this selected starter, showed growth also at 45 °C.

Besides, the phenotypic test of LAB isolated at 22 °C from control maize at silo openings showed the prevalence of the obligate heterofermentative lactobacilli (82%) ascribable to *Lb. brevis*; the other isolates were facultative heterofermentative lactobacilli identified as follows: 9% *Lb. rhamnosus* and 9% *Lb. paracasei* ssp *paracasei*. Lactobacilli isolated at 45 °C in

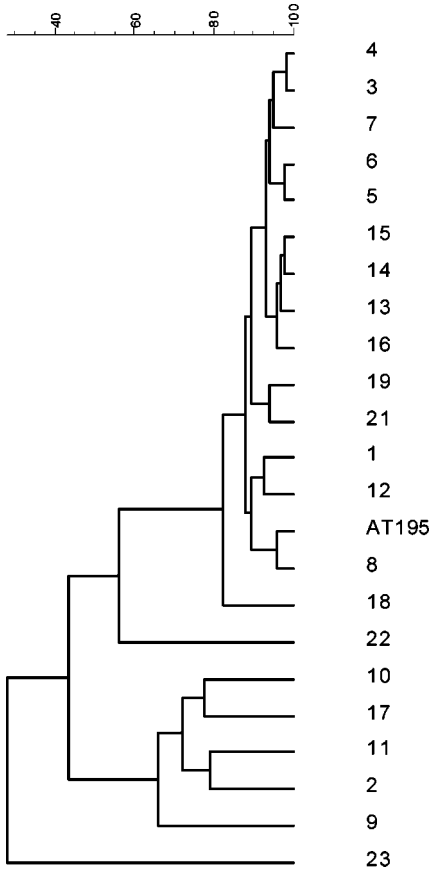

Figure 4. Dendrogram of RAPD-PCR profiles of lactic acid bacteria isolated from inoculated maize silage at the end of fermentation.

control silages resulted for 50% *Lb. paracasei* and 50% *Lb. rhamnosus*. Lactobacilli isolated at 22 °C from inoculated maize silage resulted ascribable to *Lb. rhamnosus* (51%), *Lb. brevis* (31%), *Lb. plantarum* (7%), and *Lb. paracasei* ssp *paracasei* (11%). All lactobacilli isolated at 45 °C in inoculated maize silage were phenotypically identified as *Lb. rhamnosus* (100%).

In this regard it is interesting to note that a microbial inoculant may improve the quality of a silage by increasing the LAB homofermentative activity (*22*), even though the use of heterolactic acid bacteria may enhance the aerobic stability of silages (*23*, *29*).

Figure 4 shows the RAPD-PCR profiles of *Lb. rhamnosus* AT195 and some isolates phenotipically ascribable to *Lb. rhamnosus*: all the isolates at 45 °C from the inoculated maize silage and some of the isolates at 22 °C from the same sample ascribable to *Lb. rhamnosus* showed a coefficient of similarity to the strain AT195 used as inoculum higher than 80%, while those isolated at both temperatures from the control silage (not shown) had a coefficient of similarity less than 50%.

As far as the eumycetes count is concerned (**Table 4**), it has to be noted that yeasts and molds concentration in fresh maize crop presented a significant decrease in inoculated silage compared to untreated maize silage, consistent with the antimicrobial activity of some silage inoculants observed by other authors (*29*, *30*).

Clostridia count was not affected by the experimental treatment both in fresh crops and in silage, while *Enterobacteriacee* counts, total and faecal coliforms, were not detected in maize silages, inoculated or not, after 90 days of ensiling.

The microbial composition of sorghum fresh crops and silages is reported in **Table 5**: total microbial count of fresh crops shows a potentially higher value compared to the silages, consistently with maize; on the other hand, levels of LAB in silages were not influenced by the use of the microbial additive. Phenotypic tests of LAB isolated at 22 °C from control sorghum silage samples showed the prevalence of *Lb. buchneri* (46%), followed by *Lb. rhamnosus* (24%), *Lb. paracasei* ssp *paracasei* (18%), *Lb. plantarum* (6%), and *Lb. collinoides* (6%). Lactobacilli isolated at 22 °C from inoculated sorghum silage were ascribable to *Lb. rhamnosus* (34%), *Lb. collinoides* (18%), *Lb. buchneri* (18%), *Lb. plantarum* (15%), and *Lb. cellobiosus* (12%). As also reported for maize, different results were observed for the lactic acid bacteria isolated at 45 °C: control sorghum silage samples showed the prevalence of *Lb. buchneri* (50%) with *Lb. parabuchneri* (31%) and *Lb. paracasei* ssp *paracasei* (13%) and only a marginal presence of *Lb. rhamnosus* (6%) whereas in the inoculated sorghum samples the predominant species was *Lb. rhamnosus* (73%) with a lower presence of *Lb. buchneri* (27%).

The presence of the starter at the end of the fermentation process was confirmed by the genotypic identification of *Lb. rhamnosus*; **Figure 5** shows the RAPD-PCR profiles of *Lb. rhamnosus* AT195 compared to that of some isolates from the sorghum silage samples, confirming the survival of this microorganism in such substrates.

In fresh sorghum crops (**Table 5**) the eumycetes counts were in average 10^6 cfu·g^{-1}fresh matter; however, at the end of fermentation period yeast and molds were not recovered in both treated and untreated silages. It is interesting to note that yeast and molds counts observed in both sorghum and maize match the respective alcohol levels (**Table 3**), whose wide variability has been already evidenced. In particular, the initial eumycetes count of maize and sorghum fresh crop seems to be related to the ethanol and methanol concentrations recovered in the respective silages.

As also observed on maize samples, *Enterobacteriaceae*, total and faecal coliform were not detected in both treated and untreated sorghum silages (**Table 5**), while clostridia count, unaffected by the studied inoculant, was observed in sorghum both fresh crops and silages. In this regard, as already mentioned,

Table 5. Microbiological Analysis of Sorghum Fresh Crop (d0) and after Ensiling (d90), Inoculated (I) or Not (C) (mean ± esm, log cfu·g^{-1} fresh matter)[a]

	d0		d90	
	C	I	C	I
total mesophilic bacterial count	8.4 ± 1.1	7.4 ± 0.7	5.9 ± 0.8	5.4 ± 0.9
yeasts and molds	6.5 ± 0.5 a	5.8 ± 0.6 a	<1[b] b	<1 b
LAB at 22 °C	6.0 ± 0.8	6.5 ± 0.5	5.6 ± 0.3	6.7 ± 0.6
LAB at 45 °C	4.7 ± 0.5 a	6.9 ± 0.8 b	5.3 ± 0.7 ab	6.2 ± 0.6 b
Enterobacteriaceae	6.4 ± 1.0 a	6.2 ± 0.8 a	<1 b	<1 b
total coliform	6.5 ± 0.9 a	5.8 ± 0.3 a	<1 b	<1 b
faecal coliform	5.2 ± 0.5 a	4.8 ± 0.6 a	<1 b	<1 b
Clostridium spp	3.5 ± 0.5	3.4 ± 0.6	3.7 ± 0.4	3.3 ± 0.2

[a] a, b means in the same row with different letters are significantly different ($P < 0.05$). [b] <1 = absent in 1 g.

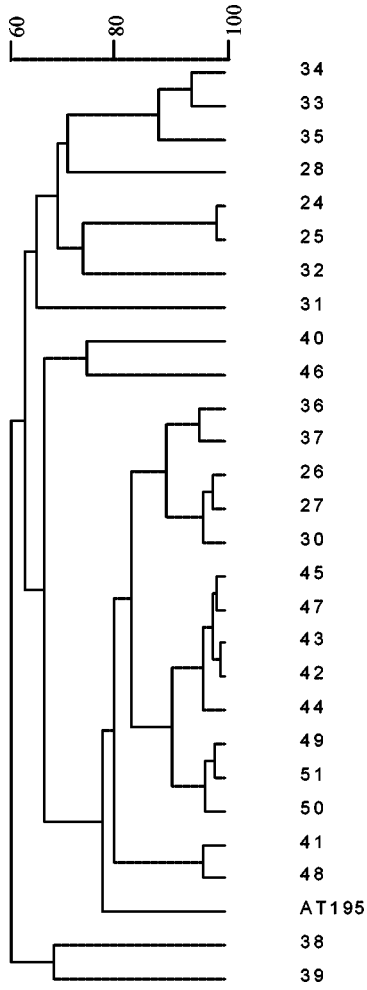

Figure 5. Dendrogram of RAPD-PCR profiles of lactic acid bacteria isolated from inoculated sorghum silage at the end of fermentation.

butyric acid was not detected or detected only in traces in silages inoculated or not, but other indices of the occurrence of the clostridial fermentation could be represented by both the ammonia nitrogen content and the isobutyric acid concentration, formed by the deamination of valine and recovered at low level ($=0.05$ g·100 g^{-1} dry matter) only in sorghum silages, treated or not (**Table 3**). Results therefore may suggest the positive contribute of the addition of *Lb. rhamnosus* AT195 in achieving a more efficient protein utilization, although lactobacilli naturally present produced sufficient lactic acid to control the clostridial activity.

In conclusion, the chemical and microbiological investigations on the effects of *Lb. rhamnosus* strain AT195 as additive for ensiling show that in both control and treated maize and sorghum silage LAB dominate the fermentation; the identification and characterization of LAB colonies isolated on silages pointed out the heterogeneity of lactic microflora population, ascribable to groups II and III of Lactobacilli (heterofermentative or facultative heterofermentative species). In this context, the selected strain of *Lb. rhamnosus* AT195 used as a silage additive showed a good survival performance in the silage fermentation process of both maize and sorghum and improved the fermentation quality of the forages by reducing the ammonia nitrogen content; however, the inoculation did not significantly affect silage chemical composition, pH, and other products of fermentation, probably because of both the good quality of forage crops at harvest and the high-level management of ensiling.

As a practical implication of this study, the collected data on sorghum silage confirm the interest in this forage crop both as a valid substitute of maize in dry areas and as a second crop.

Finally, the scotta whey, a secondary product of cheese manufacturing industry, used in this study as growth medium for *Lb. rhamnosus* strains may contribute in both reducing additives costs and environmental pollution due to animal products.

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