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Changes in the nutritive value and aerobic stability of corn silages inoculated with *Bacillus subtilis* alone or combined with *Lactobacillus plantarum*

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Abstract. Chemical composition, fermentation characteristics, *in vitro* digestibility and aerobic stability were evaluated in corn silage inoculated with microbial additives in two different experiments. Inoculant treatments (untreated, *Bacillus subtilis* and *B. subtilis* combined with *Lactobacillus plantarum*) were applied to fresh forages. Chopped corn plants (2B655 Hx) were ensiled in laboratory silos for periods of 7, 14, 21 and 63 days to evaluate the fermentation parameters. The experimental silos were weighed to determine gas losses. After the ensiling period, the silage was sampled to determine chemical composition and *in vitro* organic matter digestibility. To evaluate aerobic stability, chopped corn plants (AG-1051) were ensiled in laboratory silos that were opened after 96 days of ensiling. The silage was placed in different buckets containing data loggers. The silage was sampled after 0, 4, 8 and 12 days of exposure to air to evaluate the microbial populations and pH. The data were analysed as a completely randomised design using a mixed repeated-measures model in the MIXED procedure of SAS. To evaluate each treatment relative to the fermentation times, a regression analysis using the PROC REG procedure of SAS was applied. A significance level of $P < 0.05$ was used. Inoculation with both strains increased lactic acid concentration, whereas the use of *B. subtilis* alone or combined with *L. plantarum* improved *in vitro* apparent organic matter digestibility. In the *B. subtilis* and *B. subtilis* combined with *L. plantarum* silages, moulds and yeasts decreased, and aerobic stability was improved. Inoculation with *B. subtilis* alone or combined with *L. plantarum* improved the nutritional value and aerobic stability of corn silage.

Additional keywords: aerobic deterioration, bacterial inoculant, chemical composition, lactic acid bacteria.

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Introduction

Corn silage is the most widely used forage in ruminant feed due to its high nutritional value. According to Jobim *et al*. (2001), the higher the quality of silage, the more oxidation is observed in post-opening due to higher levels of residual soluble carbohydrates and lactic acid.

The use of a combination of homofermentative and heterofermentative inoculants has become common practice for obtaining silage of improved nutritional value that is also less susceptible to deterioration under aerobic conditions. These inoculants are composed of facultative heterofermentative bacteria (for example, *Lactobacillus plantarum*) and obligatory heterofermentative bacteria (for example, *Lactobacillus buchneri*).

The use of microbiological additives has been recommended to overcome the problem of aerobic spoilage of silage under aerobic conditions, and the suggested types of inoculants include *Bacillus subtilis* (Phillip and Fellner 1992; Basso *et al*. 2012). Many species and strains of *Bacillus* produce various antimicrobial substances. These substances include antibiotics that can be used in the biological control of phytopathogens (Lanna Filho *et al*. 2010).

However, *Bacillus* species also produce other compounds, such as lactic acid and acetic acid. Although the growth of such bacteria is not suppressed by these fermentation products or by low pH, these organisms are generally less efficient than lactic acid bacteria (*L. plantarum*) at producing lactic acid (Pahlow *et al*. 2003). Thus, combining *Bacillus* with lactic acid bacteria (*L. plantarum*) may be an alternative for decreasing fermentation losses and protein degradation through a greater production of lactate and additionally enhancing the aerobic stability of silages (Phillip and Fellner 1992).

Several studies have shown that both types of inoculant can improve the efficiency of fermentation and the nutritive value of silages and haylages (McAllister *et al*. 1995; Muck 2004; Zahiroddini *et al*. 2004), but these responses are not consistent (Kung *et al*. 2003; Muck 2004; Baah *et al*. 2011).

Nevertheless, no previous studies have examined the effects of the inoculation of corn silage with *B. subtilis* on

silage fermentation and chemical composition and *in vitro* dry matter (DM) and organic matter (OM) digestibility. The aim of this study is to evaluate the effects of *B. subtilis* alone and in combination with *L. plantarum* on the fermentation, nutritive value and aerobic stability of corn silage.

Materials and methods

Experiment 1: fermentative profile and nutritive value of corn silage

The trial was conducted at Sao Paulo State University – UNESP (Jaboticabal, Sao Paulo, Brazil), located at 21°14′14.04″S and 48°17′27.92″W.

A 2B655 Hx corn hybrid (Dow Agrosciences, Guaíra, SP, Brazil) was sown on 10 January 2009, and harvested on 21 April 2009. The corn plants were harvested manually, using a machete, at a height of 20 cm above the soil. The forage was chopped to achieve a theoretical length of cut of 10 mm in a stationary machine (Penha, Ribeirao Preto, SP, Brazil).

The following treatments were applied to the fresh forage: untreated (Control), *B. subtilis* (BS – 1×10^5 cfu/g) and the combination of BS and *L. plantarum* (BSLP). The combination of microorganisms was applied to fresh forage at a rate of 1×10^5 cfu/g for each inoculant.

The application rate of the inoculants was determined in accordance with Basso *et al.* (2012) for tropical conditions. The correct amount of inoculants for each treatment was weighed to achieve the desired application rates. The inoculants were diluted in distilled water to achieve a ratio of 5 mL/kg of fresh forage and then applied in a uniform manner with a spray to the fresh forage with constant mixing. The Control silage received a similar amount of distilled water.

Immediately after inoculation, samples of fresh forage from all treatments were obtained to determine DM, ash, crude protein (CP), neutral detergent fibre (NDF), ammonia-N content relative to total nitrogen content (NH_3/TN) and pH values.

Chopped forage from each treatment was packed into mini-silos (2.5 L; anaerobic jars) in triplicate, sealed with a lid and stored at room temperature (average 25°C). The mini-silos remained closed for 7, 14, 21 and 63 days.

The mini-silos were weighed after filling and at the end of each fermentation period to determine the gas losses by subtracting the final weights from the initial weights of the mini-silos and then dividing this difference by the dry weight of the ensiled material (Jobim *et al.* 2007).

After each fermentation period, the mini-silos were opened, and the silage was homogenised and sampled to determine the DM content, pH values, NH_3/TN and the concentrations of lactic and acetic acid, as well as ash, OM, CP, NDF, and *in vitro* apparent OM digestibility (IVDOM).

The samples to be used to determine the fermentation characteristics (pH value, NH_3/TN, and concentrations of lactic and acetic acids) were stored at −20°C for the subsequent preparation of an aqueous extract. Samples to be used to determine the chemical composition were stored in dried form.

An aqueous extract was prepared from the wet samples of silage according to Kung *et al.* (1984). The pH was determined using a pH meter (MA522 model, Marconi Laboratory Equipment, Piracicaba, SP, Brazil). The acetic acid was measured using a Shimadzu GC2014 (SHIMADZU Corporation, Kyoto, Japan) gas chromatograph with an HP-INNOwax capillary column (30 m × 0.32 mm; Agilent Technologies, Colorado Springs, CO, USA) at an initial temperature of 80°C and a final temperature of 240°C. Lactic acid was determined by a colourimetric method (Barker and Summerson 1941). Ammonia-N (%TN) was measured by distillation (AOAC 1996; 941.04).

Samples were oven-dried (55°C for 72 h) and processed in a knife mill to pass through 1-mm screen sieves and then analysed for DM (105°C for 12 h) and ash (500°C for 5 h). The OM was calculated. The NDF was analysed using a neutral detergent solution and heat-stable α-amylase without sodium sulfite according to Mertens (2002) and determined in an autoclave at 110°C for 40 min (Senger *et al.* 2008). TN was determined by the Kjeldahl method (AOAC 1996; – ID 954.01), and CP was calculated as TN × 6.25.

The IVDOM was estimated from gas production according to Menke *et al.* (1979) and Mauricio *et al.* (1999). Samples (200 mg) were incubated in serum bottles (115 mL) (Mauricio *et al.* 1999) with an addition of 30 mL of buffered rumen fluid (Menke *et al.* 1979) in a water bath at 39°C. Accumulated head-space gas pressure measurements were performed using a needle attached to a pressure transducer connected to a visual display (Datalogger pressure – pressDATA 800, MPL, Piracicaba, SP, Brazil). Readings were taken at regular intervals throughout the incubation period and at an increased frequency during the initial lag and rapid fermentation phases (for example, 2, 4, 6, 8, 10, 12, 24, 48 and 72 h post-inoculation). Bottles containing buffered rumen fluid without samples were used as blanks. However, the blank correction was omitted because, according to Cone *et al.* (1997), microbial turnover in the blank begins after 1 h and ~30% of the maximum blank reading can be attributed to this turnover in the presence of substrate. As a result, the blank does not accurately reflect what occurs in the sample.

The IVDOM was estimated [Eqn (1)] as follows (Menke *et al.* 1979):

$$\mathrm{IVDOM}(\%) = 14.88 + [(0.889*\mathrm{gas24}) + (0.045*\mathrm{CP}) + (0.065*\mathrm{ash})], \quad (1)$$

where gas24 is the gas production in 24 h (mL/0.2 g DM) and the content of CP and ash is expressed in g/kg DM.

The rumen fluid was collected from two ruminally cannulated beef steers in the morning before feeding. The fluid was filtered through two layers of cheesecloth into pre-warmed thermos flasks, homogenised and mixed with buffering solution. The steers were fed 60% of corn silage without inoculant and 40% of concentrate on a DM basis.

The experiment was performed with a completely randomised design with three replicates. The data were analysed with a mixed model with repeated-measures using the MIXED procedure of SAS (version 9.0 SAS Institute Inc., Cary, NC, USA). The best covariance structure was chosen based on the minimum Akaike information criterion. Differences between means at each time were determined using the DIFF procedure, which differentiates means based on a Fisher's *F*-protected least significant difference test. To evaluate each treatment according to the ensilage times,

a regression analysis was applied using the PROC REG procedure of SAS. A significance level of $P < 0.05$ was used.

Experiment 2: aerobic stability and occurrence of microorganisms in inoculated corn silage

The corn hybrid used was AG-1051 (Monsanto, Barretos, SP, Brazil). The crop was manually cut at a height of 20 cm from the soil and chopped to achieve a theoretical length of 10 mm in a stationary machine (Penha).

The treatments and application rate were the same as those used in Experiment 1 (Control, BS and BSLP). Four mini-silos were used per treatment. The mini-silos remained closed for 96 days.

Immediately after inoculation, samples of fresh forage from all treatments were obtained to characterise the corn plants, as in the previous experiment. Butyric and propionic acids were determined like acetic acid. After mini-silos opening, all spoiled silage was removed, and samples were collected as previously described to evaluate nutritive value as well as yeast and moulds counts and *in vitro* DM and OM digestibility.

For microbiological analyses, 25 g of either the fresh forage or silage sample from each replicate were homogenised in 225 mL of peptone sterile water (1 mg/mL). The yeast and mould counts were performed on a spread-plate of potato dextrose agar acidified with lactic acid (85%) according to Cherney and Cherney (2003), and the plates were grown at 28°C for 3 and 5 days, respectively. All the microbiological data were log-transformed.

In addition, the silage was subjected to an aerobic stability test. Approximately 3 kg of silage from each mini-silo was placed in plastic buckets and kept at room temperature (average on 26°C). The temperature of the silage was measured every half hour for 12 days with a data logger placed in the silage during aerobic exposure. The room temperature was measured with a data logger placed near the mini-silos. The aerobic stability of the silage was defined as the number of hours for which the temperature of the silage remained stable before increasing more than on 2°C above room temperature. Moreover, we evaluated the pH values and the yeast and mould counts (0, 4, 8 and 12 days), as described previously.

The data were analysed using a completely randomised design with four replicates and with a mixed model using the MIXED procedure of SAS (version 9.0 SAS Institute Inc.). The data for aerobic exposure were analysed using the MIXED procedure of SAS with repeated-measures. Unstructured and Toeplitz were the best covariance structures chosen according to the minimum Akaike information criterion. Differences between the means were determined using the DIFF procedure, which differentiates means based on a Fisher's *F*-protected least significant difference test. A significance level of $P \leq 0.05$ was used.

Results

Experiment 1: fermentative profile and nutritive value of corn silage

Silage inoculated with BSLP had a lower DM content than the Control and BS silages up to 21 days after ensilage ($P < 0.05$; Fig. 1). On the 63rd day after ensilage, the DM content was similar among the silages; however, the decrease from the whole-corn plants to the silage was higher in the Control and BS silages than in the BSLP silage (Fig. 1).

All silages had similar values of lactic acid content up to the 7th day after ensilage, but the BSLP silage had a higher lactic acid content than the others after the 14th and 21st days ($P < 0.05$; Fig. 1). On the 63rd day after ensilage, the lactic acid content of the BS silage was lower than that of the other treatments. The Control and BSLP silages showed increases in lactic acid over time; however, the lactic acid content of the BS silage remained stable (Fig. 1).

Up to the 7th and 14th days after ensilage, the acetic acid concentration was higher in the Control silage than in the BS and BSLP silages ($P < 0.05$), but it decreased after the 21st and 63rd day. The Control and BS silages had a lower acetic acid content than the BSLP silage on the 63rd day after ensilage ($P < 0.05$; Fig. 1). The acetic acid concentrations of all silages decreased from the 7th to the 63rd day (Fig. 1).

All silages had pH values below 4 on the 7th day after ensilage ($P < 0.05$, Fig. 1) and remained stable until 63 days after ensilage; however, the BSLP silage had higher pH values than the other silages over time ($P < 0.05$, Fig. 1).

The ammonia-N (%TN) content was similar among the silages throughout the time of ensilage ($P > 0.05$). The ammonia-N (%TN) content increased from the ensiling day to the 7th day after ensilage and remained high until 63 days after ensilage.

Gas losses were higher in the BSLP silage than in the Control and BS silages on the 7th and 63rd days after ensilage ($P < 0.05$). The gas losses of all silages increased from the 7th to the 63rd day (Fig. 1).

The OM content of the silages was similar up to the 14th day after ensilage. After the 21st and 63th days of ensilage, the BSLP silage had a lower OM content ($P < 0.01$, Fig. 2). All silages had a greater OM content than the whole-corn plants (evaluated at Day 0) up to the 7th day after ensilage (Fig. 2).

The CP content of the silages was similar up to the 7th day after ensilage. From the 14th day after ensilage, the Control silage had a lower CP content than the BS and BSLP silages ($P < 0.05$, Fig. 2). The CP content of all silages (evaluated at Day 7) was lower than the whole-corn plants (Day 0).

The NDF content was lower in the BS and BSLP than in the whole-corn plants immediately after the inoculation of microorganisms on the day of ensilage ($P < 0.05$, Fig. 2). The NDF content of the silages (Days 7, 21 and 63) was lower than that of the whole-corn plants. During the days following ensilage, the NDF content was lower in both of the inoculated silages.

The IVDOM values were greater in the inoculated silages than in the Control during the days following ensilage ($P < 0.05$, Fig. 2).

Experiment 2: aerobic stability and occurrence of microorganisms in inoculated corn silage

The inoculated silages showed a higher concentration of lactic acid than did the Control (Table 1). The silage inoculated with BS alone had the highest concentrations of acetic acid and butyric acid, followed by the silage inoculated with BSLP. The highest lactic : acetic acid ratio was observed in the Control and BS

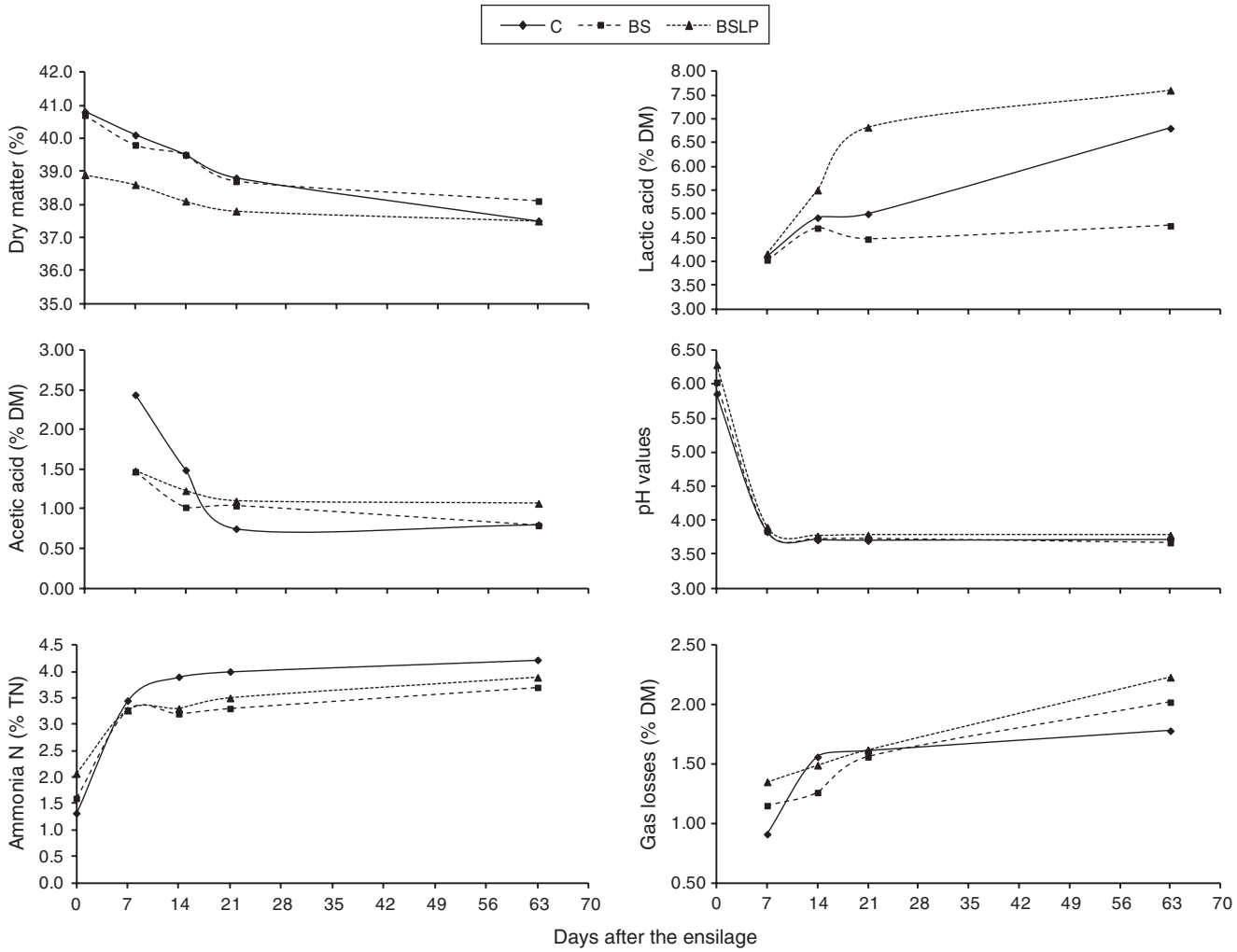

Fig. 1. Changes in fermentation after ensilage. Silages: C, Control; BS, *Bacillus subtilis*; BSLP, *Bacillus subtilis* and *Lactobacillus plantarum*.

silages. Propionic acid was not detected in any of the silages (Table 1).

The DM and ash concentrations were similar among the silages (Table 1). The CP and ether extract concentrations were greater in the BS and BSLP silages than in the Control silage. However, the concentrations of NDF, hemicellulose and lignin were lower in the inoculated silages (Table 1). The acid detergent fibre and cellulose concentration were lowest in the BSLP silage. Silage inoculation improved *in vitro* DM and OM digestibility relative to the corresponding values for the Control silage (Table 1).

All silages showed lower pH values at the opening of the mini-silos compared with the other days (Table 2). After 4 days of aerobic exposure, the silage inoculated with BS alone had a lower pH value than the other silages. The occurrence of yeasts and moulds showed a significant interaction between silages and days of aerobic exposure ($P < 0.0001$). The occurrence of yeasts was lowest in the BS silage on the day when the mini-silos were opened (Day 0). Both inoculated silages had lower yeast concentrations after the 4th and 8th days of aerobic exposure. After 4 days of exposure to the air, the occurrence of moulds was lowest in the BS silage (Table 2).

The silage inoculated with BS had the highest aerobic stability, followed by the BSLP silage (Figs 3 and 4). The BS and BSLP silages were stable for 79 and 54 h, respectively, whereas the Control silage was stable for 23 h.

Discussion

The DM content of whole-crop corn at the time of ensiling (mean of 40%) was above the minimum value of 25% proposed by McDonald *et al*. (1991) as that necessary to lower nutrient losses and maintain forage ensiled under suitable conditions.

Increases in lactic acid content during the days following ensilage were observed in the silage that received the two inoculants in combination. This result is expected for silage that receives LP bacteria (Kung 2009). The silage inoculated with BS alone had a lower content of lactic acid because this compound is not the principal end-product of this fermentation pathway.

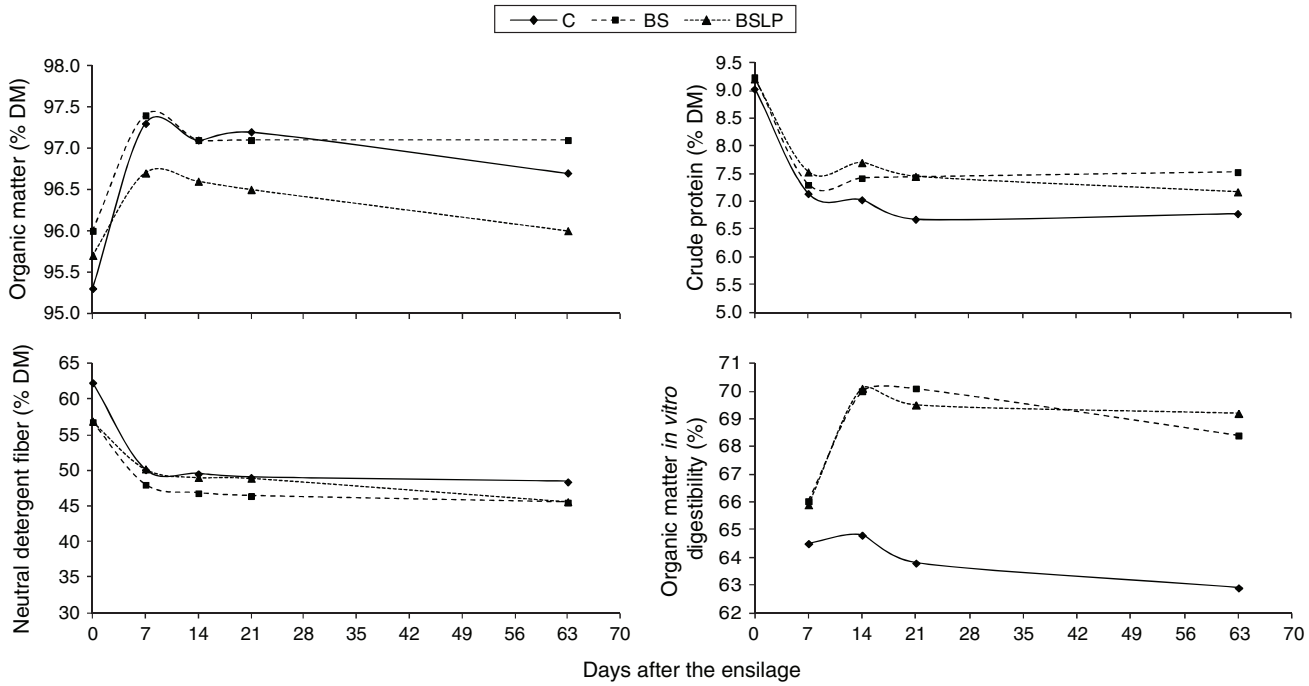

Fig. 2. Changes in chemical composition after ensilage. Silages: C, Control; BS, *Bacillus subtilis*; BSLP, *Bacillus subtilis* and *Lactobacillus plantarum*.

Table 1. Fermentation profile, chemical composition and apparent digestibility of corn silages inoculated with microbial inoculants
Silages: Control = untreated. BS = *Bacillus subtilis* at 1×10^5 cfu/g of fresh forage. BSLP = *B. subtilis* and *L. plantarum* at 1×10^5 cfu/g of fresh forage. Means followed by different letters in a row differ to 5% of significance. These values do not include a comparison with the unensiled crop

Item	Forage	Control	BS	BSLP	s.e.m.	*P*-value
		Fermentation characteristics (g/kg) of dry matter				
Lactic acid	–	47b	53ab	60a	2.30	0.044
Acetic acid	–	9.0c	17a	13b	0.94	0.001
Butyric acid	–	0.5c	1.5a	1.2b	0.01	0.001
Lactic : acetic ratio	–	5.0a	3.0b	5.0a	0.22	0.001
pH	6.02	3.81b	3.85a	3.84a	0.01	0.017
Ammonia-N (g/kg of total N)	2.15	4.68	4.58	4.73	0.08	0.799
		Chemical composition (g/kg of dry matter)				
Dry matter	354	355	343	351	2.13	0.058
Ash	38	32	30	31	1.03	0.729
Crude protein	103	83c	92a	89b	1.29	0.001
Ether extract	–	22b	28a	32a	1.55	0.076
Neutral detergent fibre	514	499a	435b	393c	13.55	0.001
Acid detergent fibre	314	261a	247ab	226b	5.60	0.014
Hemicellulose	200	238a	188b	167c	9.01	0.001
Cellulose	–	207a	199ab	192b	2.99	0.001
Lignin	–	41a	29b	27b	2.05	0.003
		In vitro *apparent dry matter and organic matter digestibility (g/kg)*				
Dry matter	–	560b	682a	693a	18.35	0.001
Organic matter	–	659b	713a	710a	8.29	0.001

Facultative heterofermentative inoculants produce lactic acid as the main fermentation product and can yield an improvement of 1–2% in DM recovery (Weinberg and Muck 1996). However, the gas losses were unexpectedly greater in the silage containing both inoculants (BSLP).

The decrease in the CP content and increase in the NH_3/TN ratio up to the Day 7 of fermentation occurred due to the action of proteases, which are released into the medium due to rupturing plant cell walls (McDonald *et al*. 1991). After the lowering of the pH (<4), these plant enzymes become inactive,

Table 2. Occurrence of spoilage microorganisms in the corn silages inoculated with microbial inoculants during aerobic exposure

Silages: Control = untreated. BS = *Bacillus subtilis* at 1×10^5 cfu/g of fresh forage. BSLP = *B. subtilis* and *L. plantarum* at 1×10^5 cfu/g of fresh forage. Means followed by different uppercase letters in the same column or different lowercase letters in the same row differ (Tukey test, $P \leq 0.05$)

Aerobic exposure	Control	BS	BSLP
	Yeasts (log_{10} cfu/g of silage)		
0	4,0aC	1.6bC	3.5aC
4	8.9aA	7.8bB	8.0bAB
8	9.3aA	8.5bA	8.5bA
12	8.2B	8.1B	7.9B
	Moulds (log_{10} cfu/g of silage)		
0	3.9C	4.0C	4.2C
4	6.6aB	4.0bBC	6.5aB
8	7.1AB	7.3A	7.5A
12	7.5A	7.5A	7.6A
	pH		
0	3.81C	3.84D	3.83aD
4	4.74aB	4.35bC	4.65aC
8	4.67B	4.85B	4.91aB
12	6.11aA	6.36aA	5.49bA

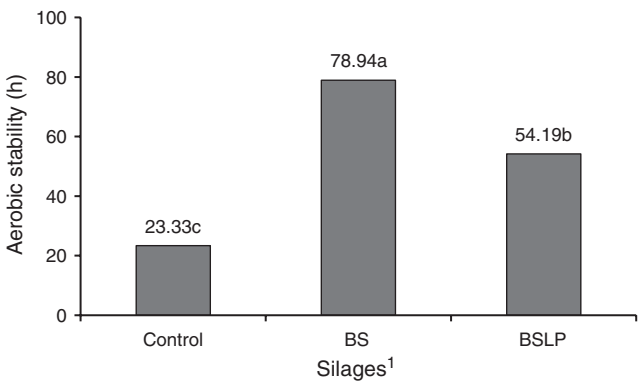

Fig. 3. Aerobic stability of corn silages inoculated with microbial inoculants during aerobic exposure. Silages: Control = untreated. BS = *Bacillus subtilis* at 1×10^5 cfu/g of fresh forage. BSLP = *B. subtilis* and *Lactobacillus plantarum* at 1×10^5 cfu/g of fresh forage.

resulting in stable values of CP and NH_3/TN after the 7th day of fermentation (Muck 1996).

The decrease in the NDF content observed after ensiling may be due to the action of hemicellulases from the plant and the action of acid hydrolysis to solubilise the hemicellulose, a natural process that occurs during ensilage (Pahlow *et al*. 2003). Additionally, silages inoculated with BS alone or associated with LP had lower concentrations of NDF. The finding of this study that the lowest NDF content was found in silage inoculated with BS is consistent with the results reported by Basso *et al*. (2011), who noted a decrease in the NDF and hemicellulose concentrations in silages inoculated with BS alone or associated with LP relative to the Control (silage without inoculant).

Silage inoculants were also responsible for the improved *in vitro* DM and OM digestibility. This result implies that the decrease in the NDF content contributed to the improvement in

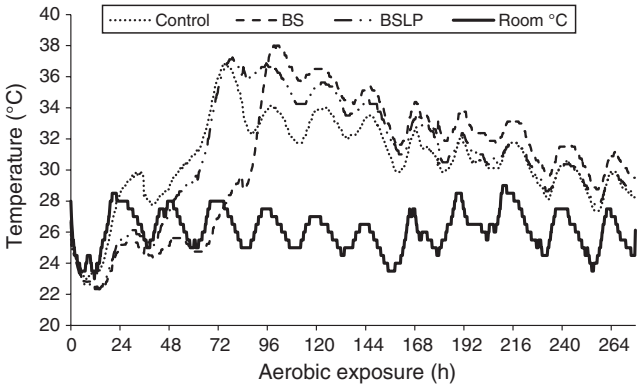

Fig. 4. Changes in the temperature of corn silages inoculated with microbial inoculants during aerobic exposure. Silages: Control = untreated. BS = *Bacillus subtilis* at 1×10^5 cfu/g of fresh forage. BSLP = *B. subtilis* and *Lactobacillus plantarum* at 1×10^5 cfu/g of fresh forage.

the digestibility of OM. In addition, inoculated silages had higher aerobic stability and lower mould and yeast populations, which contributed, along with the reduction of the NDF, to improving the IVDOM. According to the literature, BS can produce bacteriocins and enzymes such as α-amylase and ferulate esterase (Donaghy and McKay 1997). The decrease in the NDF content of inoculated silage may be due to the action of ferulate esterase, which breaks ester bonds between lignin and hemicellulose, allowing the enzymes to act upon hemicellulases and cellulases in the fibre matrix. The results shown in Fig. 2 confirm these hypotheses because the NDF content in the silage Control (nearly 15%) was higher than that in silage treated with inoculants (nearly 13%). Moreover, it is necessary to consider the action of α-amylase produced by BS, as this enzyme can increase the digestibility of starch, thereby increasing digestibility of the inoculated silage.

According to Oba and Allen (1999), increasing the *in vitro* digestibility of NDF by 1 unit results in a potential increase in DM intake of 0.17 kg/day and an increase in milk yield of 0.25 kg/day. Thus, the present study shows that there is a change in the nutritive value of silage inoculated with BS alone or combined with LP. However, the mechanisms responsible for this change are unknown, and the results found in the literature are not consistent. Thus, additional research should be performed to determine whether bacterial inoculants produce ferulate esterases and to validate the effects of bacterial inoculants on intake by animals and their performance.

Corn silage has a residual soluble carbohydrate concentration and a DM concentration of ~30%. These conditions favour the development of moulds and yeasts associated with aerobic stability (Muck *et al*. 1991). Inoculation with heterofermentative microorganisms has been used to control aerobic stability. These inoculants act by inhibiting opportunistic microorganisms after silo opening, either by the increased production of acetic acid (Danner *et al*. 2003; Filya 2003*a*, 2003*b*) or by the production of bacteriocins (Yildirim and Yildirim 2001).

In the present study, silages inoculated with BS had lower occurrences of yeasts and moulds 1 day and 4 days, respectively, after the opening of the mini-silos, promoting greater aerobic

stability in this silage. BS is one of the most important producers of metabolites with antifungal and antibacterial activity (Todovora and Kozhuharova 2009). Basso *et al*. (2012) also found improvements in the aerobic stability of corn silage inoculated with BS.

Conclusions

Silages inoculated with BS alone or combined with LP preserved the fermentation characteristics, decreased the NDF content and increased the *in vitro* digestibility of corn silages, improving their nutritive value. BS decreased the populations of yeasts and moulds and increased aerobic stability.

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