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ORIGINAL PAPER

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The effects of *Propionibacterium acidipropionici* and *Lactobacillus plantarum*, applied at ensiling, on the fermentation and aerobic stability of low dry matter corn and sorghum silages

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Abstract The aim of this work was to study the effects of applying a strain of *Propionibacterium acidipropionici*, with or without *Lactobacillus plantarum*, on the fermentation and aerobic stability characteristics of low dry matter (DM) corn (*Zea mays* L.) and sorghum (*Sorghum bicolor* L.) silages. Corn at the dent stage and sorghum at the flowering stage were harvested. Treatments comprised control (no additives), *P. acidipropionici*, *L. plantarum* and a combination of *P. acidipropionici* and *L. plantarum*. Fresh forages were sampled prior to ensiling. Bacterial inoculants were applied to the fresh forage at 1.0×10^6 colony-forming units per gram. After treatment, the chopped fresh materials were ensiled in 1.5-l anaerobic glass jars equipped with a lid that enabled gas release only. Three jars per treatment were sampled on days 2, 4, 8, 16 and 60 after ensiling, for chemical and microbiological analysis. At the end of the ensiling period, 60 days, the silages were subjected to an aerobic stability test. The *L. plantarum* inoculated silages had significantly higher levels of lactic acid than the controls, *P. acidipropionici* and combination of *P. acidipropionici* and *L. plantarum* inoculated silages ($P<0.05$). The *P. acidipropionici* did not increase propionic and acetic acid levels of the silages. After the aerobic exposure test, the *L. plantarum* and combination of *P. acidipropionici* and *L. plantarum* had produced more CO_2 than the controls and the silages inoculated with *P. acidipropionici* ($P<0.05$). All silages had high levels of CO_2 and high numbers of yeasts and molds in the experiment. Therefore, all silages were deteriorated under aerobic conditions. The *P. acidipropionici* and combination of *P. acidipropionici* and *L. plantarum* were not able to improve the aerobic stability of fast-fermenting silages, because they could not work well in this acidic environment. The results showed that *P. acidipropionici* and combination of *P. acidipropionici* and *L. plantarum* did not improve the aerobic stability of low DM corn and sorghum silages, which are prone to aerobic deterioration.

Keywords *Propionibacterium acidipropionici* · *Lactobacillus plantarum* · Silage · Fermentation · Aerobic stability

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Introduction

Oxygen is detrimental to silage quality because it enables aerobic spoilage microorganisms such as yeasts, molds and aerobic bacteria to become active [1]. When exposed to air during the feedout phase, silage might also undergo increases in temperature and pH and losses of water-soluble carbohydrates (WSCs) and fermentation end-products, which reduce silage quality and digestibility. The duration of time before the temperature of the silage rises affects the nutrient losses in the silo, the likelihood of toxic effects of fungal growth in silage fed to animals and the degree of management required to minimize exposure to air during the feedout phase [2]. Susceptibility to spoilage is a very important factor determining silage quality and digestibility. Therefore, additives that protect the silage upon exposure to air might be very useful [3].

It is possible to apply bacterial inoculants at ensiling in order to promote adequate fermentation patterns. Inoculants, comprising homofermentative lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, *Enterococcus faecium* and *Pediococcus* species, are often used to control the ensiling fermentation by rapid production of lactic acid and the consequent decrease in pH. However, such inoculants enhance the aerobic spoilage of wheat, sorghum and corn silages [4–7] because in these fermentations, not enough volatile fatty acids (VFAs) are produced to protect the silage against

aerobic yeasts and molds [8]. An explanation for the negative responses to the addition of LAB is that, under anaerobic conditions, the homofermentative LAB inoculants produce mainly lactic acid, which can serve as a substrate for lactate-assimilating yeasts upon exposure to air [9]. Furthermore, only small amounts of VFAs, which inhibit the growth of yeasts and molds, are produced. These observations stimulated the search for bacterial strains that might be suitable as silage inoculants and might also protect the silage upon aerobic exposure.

In order to use biological additives to overcome the problem of aerobic deterioration of silages, it has been suggested that other types of inoculants, such as *Bacillus* species and propionic acid bacteria (PAB) be used [10]. It would be expected that such additives would produce in the silage substances, which have antimycotic properties. These silage substances inhibit the development of yeasts and molds upon aerobic exposure. PAB can ferment sugars and lactate to acetate and propionate; these short-chain aliphatic acids inhibit yeasts and molds [8].

Bolsen et al. [11] reported that the PAB inoculated corn silages were more stable when exposed to air than the control and LAB inoculated silages. In our previous study a PAB inoculant, containing *Propionibacterium acidipropionici*, was tested in wheat, sorghum and corn silages with or without homofermentative LAB. In that study, the PAB inoculant decreased the numbers of yeasts and molds and improved the aerobic stability of all silages [12]. However, Weinberg et al. [13] showed that the addition of PAB had only marginal effect on the aerobic stability of pearl millet and corn silages. In another study by Weinberg et al. [14], the control and PAB inoculated sorghum silages were more stable when exposed to air than the LAB inoculated sorghum silages. In a study by Higginbotham et al. [15], the addition of two concentrations of PAB inoculant had no appreciable effect on the aerobic stability of corn silage. A further study by Higginbotham et al. [16] showed that the addition of PAB inoculant did not affect fermentation characteristics and yeasts, molds and LAB counts of corn silage. Furthermore, the PAB inoculant did not prevent detrimental changes in quality when corn silage was exposed to air.

The objective of this study was to evaluate the effects of a strain of *P. acidipropionici* applied at the time of ensiling, with or without *L. plantarum*, on the fermentation and aerobic stability characteristics of low dry matter (DM) corn and sorghum silages. The *L. plantarum* was used for comparison purposes.

Materials and methods

Experimental

Corn (*Zea mays* L.) at dent stage and sorghum (*Sorghum bicolor* L.) at flowering stage of maturity were harvested and chopped with a laboratory type chopper to about 1.5 cm and ensiled in 1.5-l anaerobic glass jars (Weck®, Wher-Oflingen, Germany) equipped with a lid that enabled gas release only. Each jar was filled with about 850 g (wet weight) of chopped forage, without a headspace. The packing density was 130.9 and 129.2 kg of DM m^{-3} corn and sorghum, respectively. There were 60 jars per crop and they were stored at ambient temperature (25–28°C). Fresh and ensiled materials (on days 2, 4, 8, 16 and 60 after ensiling, three jars per treatment for each time) were sampled for chemical and microbiological analysis. At the end of the ensiling period, 60 days, the silages were subjected to an aerobic stability test at room temperature (27°C), which lasted 5 days, in a ''bottle'' system developed by Ashbell et al. [17]. In this system, CO_2 production, change in pH, numbers of yeasts and molds, and visual appearance serve as spoilage indicators.

Treatments

The following microbial additives were applied to the fresh forages: (1) Control (no additives); (2) *P. acidipropionici* (MA26/4U, Lallemand, Saint-Simon, France; final application rate of 1.0×10^6 colony forming units (cfu) per gram of fresh forage weight); (3) *L. plantarum* (Biomax5; Chr. Hansen Biosystems, Milwaukee, WI, USA; 1.0×10^6 cfu g^{-1}); (4) Combination of *P. acidipropionici* and *L. plantarum* (1.0×10^6 cfu g^{-1}).

The application rate determined by manufacturers stated the level of *P. acidipropionici* and *L. plantarum* in the products. The inoculants were applied as follows: on the day of the experiment, inoculants suspended in 20 ml of deionized water and the whole suspension was sprayed over 10 kg (wet weight) of the chopped forage spread over a 1×4-m area. All inoculants were applied to the materials in a uniform manner with constant mixing. Thus, about 1.0×10^6 cfu per gram of forage were applied. The combined treatment (*P. acidipropionici* + *L. plantarum*) comprised 1.0×10^6 cfu each of *P. acidipropionici* and *L. plantarum* per gram of forage.

Table 1 Chemical and microbiological analyses of the fresh forages

Forage type	PH	DM (g kg^{-1})	WSC (g kg^{-1} of DM)	LAB (log cfu g^{-1})	Yeasts (log cfu g^{-1})	Molds (log cfu g^{-1})
Corn	5.9 ± 0.1	231 ± 10	101 ± 17	2.9	2.8	2.1
Sorghum	6.0 ± 0.1	228 ± 8	265 ± 21	2.6	2.6	2.4

Numbers following the "±" are the standard error of the mean
DM dry matter, *WSC* water-soluble carbohydrate, *LAB* Lactic acid bacteria

Table 2 Chemical analyses of the final silages

Forage type	Treatment	pH	WSC (g kg^{-1} of DM)	Lactic acid (g kg^{-1} of DM)	Propionic acid (g kg^{-1} of DM)	Acetic acid (g kg^{-1} of DM)	Butyric acid (g kg^{-1} of DM)	Ethanol (g kg^{-1} of DM)
Corn	Control	3.7 ± 0^{a}	25 ± 6^{a}	55 ± 3^{c}	0^{a}	0.9 ± 0^{a}	0.5 ± 0^{a}	1.6 ± 0.1^{a}
	P. acidipropionici (PA)	3.6 ± 0^{a}	24 ± 6^{a}	54 ± 3^{c}	0.7 ± 0^{a}	0.8 ± 0^{a}	0.6 ± 0^{a}	0.7 ± 0.1^{a}
	L. plantarum (LP)	3.5 ± 0^{a}	19 ± 3^{a}	92 ± 5^{a}	0^{a}	0^{a}	0^{a}	1.3 ± 0.1^{a}
	PA + LP	3.5 ± 0^{a}	20 ± 3^{a}	77 ± 5^{b}	0.5 ± 0^{a}	0^{a}	0^{a}	1.0 ± 0.1^{a}
Sorghum	Control	3.7 ± 0.1^{a}	85 ± 12^{a}	72 ± 4^{c}	0^{a}	0.9 ± 0^{a}	0.8 ± 0^{a}	2.1 ± 0.2^{a}
	P. acidipropionici (PA)	3.7 ± 0.1^{a}	87 ± 15^{a}	70 ± 3^{c}	0.9 ± 0^{a}	1.0 ± 0^{a}	0.8 ± 0^{a}	0.9 ± 0.1^{a}
	L. plantarum (LP)	3.5 ± 0^{a}	70 ± 9^{a}	129 ± 4^{a}	0^{a}	0^{a}	0^{a}	1.8 ± 0.1^{a}
	PA + LP	3.6 ± 0^{a}	72 ± 13^{a}	96 ± 3^{b}	0.7 ± 0^{a}	0^{a}	0^{a}	1.5 ± 0.1^{a}

Numbers following the "±" are the standard error of the mean. Superscript letters within a column and forage type indicate that a value followed by the same letter did not differ significantly ($P > 0.05$) in the Duncan's multiple range test from other values with the same letter*WSC* water-soluble carbohydrate, *DM* dry matter

Analytical procedures

Chemical analyses were performed in triplicate. The DM content of the fresh forages and silages was determined by drying at 60°C for 48 h in a fan-assisted oven. Wet samples stored at −20°C were extracted for 3 min in a blender in water or in ethyl acetate (1:9) for WSCs and fermentation end-product analyses, respectively. WSCs were determined by the phenol sulphuric acid method [18]. Lactic acid was determined by a spectrophotometry method [19]. Ethanol and VFAs were determined in an aqueous extracts using a gas chromatograph with FID detector and a semi-capillary FFAP column (Hewlett Packard, Wardbronn, Germany), over a temperature range of 45–230°C [20].

Microbiological analysis was performed on pooled samples of the three replicate silos per treatment per time point except for replicate samples that differed considerably in their appearance. Microbiological evaluation included enumeration of lactobacilli on pour-plate Rogosa agar (Oxoid CM627, Oxoid, Basingstoke, UK), and yeasts and molds on spread-plate malt extract agar (Difco, Detroit, MI, USA) acidified with lactic acid to pH 4.0. The plates were incubated for 3 days at 30°C. Since microbiological analysis was performed on a single sample per time point, no statistical analysis was possible. All microbiological data were transformed to $\log_{10}$.

The statistical analyses of other results included one-way analysis of variance and Duncan's multiple range tests, which were applied to the results using statistical analysis system (SAS, Cary, NC).

Results

The chemical and microbiological compositions of the fresh forages are given in Table 1. With these forages a wide range of chemical compositions and ensiling characteristics was obtained. In general, corn had lower content WSCs than sorghum. Lactobacilli, yeasts and molds were found in small numbers on the fresh forages.

Table 2 gives the fermentation characteristics of corn and sorghum silages. During fermentation, the pH and WSCs levels of the silages were reduced and the concentrations of lactic acid and ethanol were increased. The major fermentation product in all silages was lactic acid. Figure 1 shows the change of pH during the ensiling fermentation of corn and sorghum. In the experiment, the pH decreased very quickly. The pH decreased to below 4.0 in all silages after 4 days. The final pH values were between 3.5 and 3.7 for all silages. Silages inoculated with *L. plantarum* and combination of *P. acidipropionici* and *L. plantarum* had significantly higher lactic acid contents than the controls and the silages inoculated with *P. acidipropionici* ($P < 0.05$). In the experiment, only *P. acidipropionici* and combination of *P. acidipropionici* and *L. plantarum* inoculated silages had measurable levels of propionic acid. No propionic

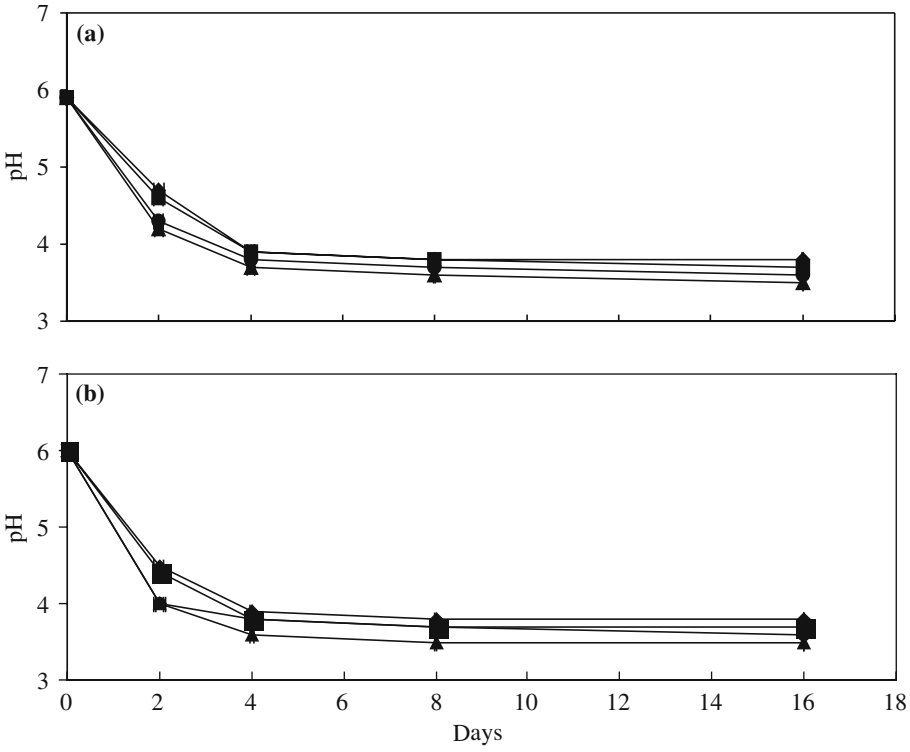

Fig. 1 Change in pH during ensiling of corn (**a**) and sorghum (**b**). *Diamonds* control (no bacteria), *squares P. acidipropionici*, *triangles L. plantarum*, *circles P. acidipropionici* + *L. plantarum*

acid was detected in the controls and the silages inoculated with *L. plantarum*. Acetic and butyric acid were detected in the controls and *P. acidipropionici* inoculated silages. No acetic and butyric acid were detected in the silages inoculated with *L. plantarum* and combination of *P. acidipropionici* and *L. plantarum*.

The microbiological composition of the corn and sorghum silages is given in Table 3. Lactobacilli, yeasts and molds numbers of the silages increased during the fermentation. After 60 days of ensiling, numbers of lactobacilli were the highest in the *L. plantarum* inoculated silages compared with the controls, *P. acidipropionici* and combination of *P. acidipropionici* and *L. plantarum* inoculated silages. Inoculation with the *P. acidipropionici*, *L. plantarum* and combination of *P. acidipropionici* and *L. plantarum* did not cause big differences in the numbers of yeasts and molds of the silages. After 60 days of ensiling, the numbers of yeasts and molds were between 5.1 and 5.8 cfu g^{-1} for the controls and all inoculated silages.

Table 4 gives the results of the aerobic stability test of the corn and sorghum silages. Silage deterioration indicators are visual appraisal, pH change, CO_2 production, and an increase in yeasts and molds numbers. Under aerobic conditions, the controls and inoculated silages were not stable; all silages deteriorated. After the aerobic exposure test, all silages had high levels of CO_2 production and high numbers of yeasts and molds. The silages inoculated with *L. plantarum* had the highest CO_2 production and yeasts numbers. Therefore, the *L. plantarum* inoculated silages had the highest losses in the experiment. However, the controls and all inoculated silages became moldy under aerobic conditions. Only one sample out of the three was clean in the *L. plantarum* inoculated corn silages.

Discussion

Aerobic deterioration of susceptible silages is a big problem. Spoilage microorganisms in aerobically deteriorated silages include lactate-assimilating yeasts and molds [21]. Whole-crop cereal silages, such as wheat, sorghum and corn are susceptible to aerobic deterioration. Therefore, it is very important to find suitable additives that inhibit fungi and protect the silage upon aerobic exposure. *P. acidipropionici* and *P. shermanii* are PAB, which produce propionic and acetic acid in silage. Some results with these microorganisms in laboratory studies were promising with regard to aerobic stability [11, 12].

Some researchers reported that short-chain VFAs, such as propionic and acetic acid, were fungicidal agents and high concentrations of propionate and acetate inhibited yeasts and molds growth in the slow-fermenting silages [8, 12]. However, Pahlow and Honig [10] showed that the production of propionic acid by PAB ceased below pH 4.8 and PAB were efficient in grass silage only if the decrease in pH was retarded ,e.g., by a delayed filling. Weinberg et al. [13, 14] reported that PAB did not survive the acidic conditions in the whole-crop cereal silages. In the present study, *P. acidipropionici* inoculated silages had trends toward higher propionic acid concentrations than the other silages. But at the end

Table 3 Microbiological analyses of the final silages

Forage type	Treatment	LAB (log cfu g^{-1})	Yeasts (log cfu g^{-1})	Molds (log cfu g^{-1})
Corn	Control	4.6	5.2	5.2
	P. acidipropionici (PA)	4.8	5.4	5.2
	L. plantarum (LP)	6.8	5.1	5.3
	PA + LP	6.1	5.3	5.1
Sorghum	Control	5.1	5.7	5.4
	P. acidipropionici (PA)	5.5	5.8	5.6
	L. plantarum (LP)	6.9	5.6	5.8
	PA + LP	6.2	5.7	5.6

LAB lactic acid bacteria

Table 4 The results of the aerobic stability test (5 days) of the silages

Forage type	Treatment	Visual appraisal	pH	CO_2 (g kg^{-1} DM)	Yeasts (log cfu g^{-1})	Molds (log cfu g^{-1})
Corn	Control	Moldy	3.9 ± 0.1^{a}	25.7 ± 1.8^{b}	8.8	4.3
	P. acidipropionici (PA)	Moldy	3.8 ± 0.1^{a}	22.5 ± 1.6^{b}	8.7	5.0
	L. plantarum (LP)	1 Clean, 2 moldy	4.1 ± 0.1^{a}	44.8 ± 2.8^{a}	9.6	3.8
	PA + LP	Moldy	3.8 ± 0.1^{a}	39.9 ± 2.2^{a}	9.3	4.0
Sorghum	Control	Moldy	4.0 ± 0.1^{a}	28.8 ± 2.1^{b}	9.0	4.6
	P. acidipropionici (PA)	Moldy	3.9 ± 0.1^{a}	31.1 ± 2.4^{b}	9.1	5.6
	L. plantarum (LP)	Moldy	4.2 ± 0.1^{a}	49.4 ± 2.8^{a}	9.9	4.0
	PA + LP	Moldy	4.0 ± 0.1^{a}	43.6 ± 2.5^{a}	9.4	4.1

Means of pH and CO_2 are followed by the standard error of the mean. Superscript letters within a column and forage type indicate that a value followed by the same letter did not differ significantly ($P > 0.05$) in the Duncan's multiple range test from other values with the same letter
DM dry matter

of the fermentation, *P. acidipropionici* inoculated silages had still lower amount of propionic acid concentrations, probably because the pH in the silages decreased very quickly to below 4.0 and under such acidic conditions not enough propionic acid was produced by *P. acidipropionici*. Therefore, *P. acidipropionici* did not inhibit yeasts and molds growth in these silages.

After the aerobic exposure test, the controls and the silages inoculated with *P. acidipropionici*, *L. plantarum* and combination of *P. acidipropionici* and *L. plantarum* were not stable aerobically; all silages deteriorated under aerobic conditions. This was evident from the intensive CO_2 production and the development of yeasts. A high level of yeast impaired the aerobic stability of all silages. The results in the present study indicate clearly that inoculation with *P. acidipropionici* and *L. plantarum* did not improve the aerobic stability of low DM corn and sorghum silages. The *P. acidipropionici* and combination of *P. acidipropionici* and *L. plantarum* inoculated silages had low levels of propionic and acetic acid. The *P. acidipropionici* was not able to protect the aerobic stability of both silages because the *P. acidipropionici* did not produce enough propionic acid under acidic conditions and the activity of yeasts was not impaired in both silages. In the experiment, the pH decreased very quickly to below 4.0 in both silages, which might explain why there was no contribution of *P. acidipropionici* to the aerobic stability of low DM and fast-fermenting silages. Weinberg et al. [13, 14] showed that PAB could survive in and improve the aerobic stability of only slow-fermenting silages, which are prone to aerobic deterioration. Higginbotham et al. [16] reported that yeasts and molds counts and pH of corn silage changed with exposure to air but were generally unaffected by PAB treatment.

In conclusion, the results of this study showed that *P. acidipropionici* did not improve the aerobic stability of low DM and fast-fermenting corn and sorghum silages. The pH decreased very quickly in both silages, probably causing an acidic environment that did not favor this microorganism. The addition of *P. acidipropionici* might improve the aerobic stability of silages with slow acidification rates which are prone to aerobic deterioration. Such silages are mainly mature, dry cereals. Therefore, heterofermentative LAB, such as *L. buchneri*, or other types of inoculants and chemicals might be considered to solve the problem of aerobic stability of low DM silages.

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