Pediocin SA-1: A selective bacteriocin for controlling *Listeria monocytogenes* in maize silages

Isabel R. Amado,*¹ Clara Fuciños,*[†] Paula Fajardo,[‡] and Lorenzo Pastrana[§]

*Departamento de Química Analítica y Alimentaria, Facultad de Ciencias de Ourense, Universidad de Vigo, As Lagoas s/n 32004, Spain

[†]Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal

[‡]ANFACO-CECOPESCA, Colegio Universitario 16, 36310 Vigo, Pontevedra, Spain

[§]International Iberian Nanotechnology Laboratory, Avda. Mestre José Veiga s/n, 4715 Braga, Portugal

ABSTRACT

In this study, we assessed the potential as silage additive of a bacteriocin produced by *Pedococcus acidilactici* Northern Regional Research Laboratory (NRRL) B-5627 (pediocin SA-1). Maize was inoculated either with a bacterial starter alone (I) or in combination with the bacteriocin (IP), and untreated silage served as control. We monitored the products of fermentation (ethanol, and lactic and acetic acids), the microbial population, and the presence of the indicator strain *Listeria monocytogenes* Colección Española de Cultivos Tipo (CECT) 4032 (1×10^5 cfu/g) after 1, 2, 5, 8, 16, and 30 d of ensiling. Our results indicated antilisterial activity of the bacteriocin, anticipating the disappearance of *L. monocytogenes* in IP compared with I and control silages. The PCR-denaturing gradient gel electrophoresis analysis revealed the addition of the bacteriocin did not affect the bacterial communities of the spontaneous fermentation, and the inoculant-containing bacteria (*Lactobacillus plantarum*, *Lactobacillus buchneri*, and *Enterococcus faecium*) were found in addition to the bacterial communities of untreated maize silages in I and IP silages. Both treatments increased the concentration of antimicrobial compounds (acetic acid, ethanol, and 1,2-propanediol) and led to lower residual sugar contents compared with the control, which would provide enhanced aerobic stability. The fact that the identified species *L. plantarum*, *L. buchneri*, and *E. faecium* produce some of these inhibitory compounds, together with their persistence throughout the 30 d of fermentation, suggest these bacteria could actively participate in the ensiling process. According to these results, pediocin SA-1 could be used as an additive to control the presence of *L. monocytogenes* in maize silages selectively, while improving their fermentative quality and eventually their aerobic stability.

Key words: silage, lactic acid bacteria, antimicrobial, pathogenic bacteria

INTRODUCTION

Listeria monocytogenes is recognized worldwide as an important foodborne pathogen causing listeriosis. This bacterium, ubiquitous in the environment, has been isolated from different farm-produced foods such as vegetables, meat, and dairy products. *Listeria monocytogenes* is an important microbiological risk at the beginning of the food chain, and concern is increasing about this bacterial pathogen in foods. Moreover, considering *L. monocytogenes* can grow in unfavorable environments (anaerobiosis, pH shifts, high osmolarity, low temperature) and colonize environmental niches such as the gastrointestinal tract, soil, silages, and sludge (Lungu et al., 2009). This contamination has often been related to poorly fermented silage, which is considered one of the main factors leading to cattle infection (Borucki et al., 2005).

Ensilage consists of the fermentation of free sugars to lactic acid irrespective of forage by epiphytic microorganisms, mainly lactic acid bacteria (LAB), varying in number and composition during fermentation (Klocke et al., 2006). Silages are a traditional way to preserve cattle feed for extended periods of time when fresh forage is limited or unavailable. Lactic acid bacteria are traditionally used to improve the nutritional properties of silages and to prevent the growth of undesirable microorganisms (Holzer et al., 2003). In recent years, the use of LAB producing antimicrobial peptides able to inhibit spoilage microorganisms of the silages has been increasingly studied. Gollop et al. (2005) reported the antimicrobial activity of silages treated with different LAB-based inoculants, and Marciňáková et al. (2008) observed positive effects of a bacteriocinogenic strain of *Enterococcus faecium* on the microbiological quality of grass silage. Amado et al. (2012) found the bacteriocinogenic LAB *Lactococcus lactis* Colección Española de Cultivos Tipo (CECT) 539 and *Pedococcus acidilac-*

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¹Corresponding author: sabelara@uvigo.es

tici Northern Regional Research Laboratory (NRRL) B-5627, together with the ensiling strain *Lactobacillus plantarum* CECT 220, were able to control the occurrence of *L. monocytogenes* in grass and maize silages. More recently, Silva et al. (2016) reported the inoculation of alfalfa with a potential bacteriocinogenic *Pediococcus pentosaceus* strain improved the fermentation profile in tropical conditions, although no antimicrobial effect was observed.

The production of pediocin by *P. acidilactici* NRRL B-5627 has long been studied in our group (Guerra and Pastrana, 2002, 2003; Guerra et al., 2005a,b). This bacteriocin (pediocin SA-1) has been isolated and characterized (Anastasiadou et al., 2008), exhibiting a broad spectrum of activity against several foodborne pathogens, including *L. monocytogenes* (Guerra and Pastrana, 2002) and *Listeria* spp. (Anastasiadou et al., 2008). Pediocin SA-1 has high heat stability at acidic pH (3.7; Guerra and Pastrana, 2002) and low temperature (30°C) within a wide range of pH (3.0–12.0; Anastasiadou et al., 2008). Besides, the strain *P. acidilactici* NRRL B-5627 has shown to have a probiotic effect when tested as a feed additive for weaned piglets and has proven to be resistant to acid and bile salts in vitro (Guerra et al., 2007). For these reasons, this bacteriocin has a high potential value for ensiling purposes and as an additive for the animal feed industry.

The aim of this work was to study the use of pediocin from *P. acidilactici* NRRL B-5627 as an additive for maize ensiling. We evaluated the potential of pediocin SA-1 to remove *L. monocytogenes* from maize silages, as well as its effect on the products of fermentation and the naturally occurring microbiota.

MATERIALS AND METHODS

Bacterial Strains and Inoculants

Pediococcus acidilactici NRRL B-5627 and *L. monocytogenes* CECT 4032 were obtained from NRRL (Peoria, IL), and CECT (Valencia, Spain), respectively. The bacteria were maintained as frozen stocks at –80°C in de Man, Rogosa, Sharpe (MRS) and brain heart infusion (BHI), respectively (Cultimed Panreac Química S.A., Barcelona, Spain), containing 30% (vol/vol) glycerol and were propagated twice in appropriate medium before use. All cultures were grown in 250-mL Erlenmeyer flasks with 50 mL of medium, on a rotary shaker at 30°C (or 37°C in the case of *L. monocytogenes*) for 12 h.

We used a commercial inoculant specially formulated to enhance maize fermentation (11C33, Pioneer Hi-Bred International Inc., Johnston, IA), which consisted of a

mixture of *Lactobacillus buchneri* ATCC PTA-2494, *L. plantarum* (ATCC 53187 and ATCC 55942), and *E. faecium* ATCC 55593.

Pediocin Production

Pediocin was produced in cultures of *P. acidilactici* NRRL B-5627 in MRS at 30°C in a laboratory fermentor, under 200 rpm agitation conditions. We followed a fed-batch and re-alkalized cultivation mode that was reported to enhance significantly the synthesis of pediocin (Guerra et al., 2005b). When the growth and the capacity to recover the acidic pH of *P. acidilactici* stopped (92 h of culture), the pH of the medium was adjusted to 3.5 to promote the release of the bacteriocin from the cell surface. Then, the culture broth was heated for 3 min and centrifuged at $27,200 \times g$ for 15 min at 4°C to remove the cells. Finally, the cell-free supernatant containing the total antibacterial activity was subjected to an ultrafiltration-diafiltration process to allow the concentration of the bacteriocinogenic activity while reducing the content of high molecular weight compounds of the culture broth. For this purpose, a Prep/Scale-TFF 6ft² cartridge (Millipore Corporation, Billerica, MA), equipped with a polyethersulfone membrane of 10 kDa was used, and the permeate containing the bacteriocin (2,700 Da) was frozen at –20°C until further use.

Antibacterial Activity Assays

The in vitro antibacterial activity of the pediocin extract was determined both against 12-h cultures of the commercial inoculant and against the strain *L. monocytogenes* CECT 4032. The quantitative antibacterial activity of the ultrafiltered cell-free supernatants was estimated by using a photometric bioassay (Cabo et al., 2001). Briefly, pediocin SA-1 was serially diluted in sterile distilled water, and 1-mL aliquots of the diluted samples were added in sterile culture tubes. Each tube was inoculated with 1 mL of either a culture of the commercial inoculant in MRS or *L. monocytogenes* in BHI, which was diluted to an initial absorbance of 0.2 with sterile buffered culture broth (pH 6.8) just before use. Controls consisted of samples where the bacteriocin was replaced by sterile distilled water. Samples were then incubated at either 30°C (inoculant) or 37°C (*L. monocytogenes*) for 6 h. Dose-response curves were obtained by plotting the absorbance at 700 nm against the inverse of the dilution of the antimicrobial compound. The antibacterial activity was expressed as bacteriocin activity units (BU)/mL, where 1 BU/mL is the amount of antibacterial compound needed to

obtain a 50% of growth inhibition compared the control (Guerra et al., 2005b).

Silage Preparation

Maize was harvested at the Centro de Investigaciones Agrarias de Mabegondo (La Coruña, Spain) and immediately chopped (10 mm length of cut) in the field using a conventional forage harvester. The chopped maize was transported under refrigeration (less than 2 h) to the laboratory and wilted outdoors for approximately 3 h to a final DM content of 435 g/kg. We prepared portions (each treated individually) containing 1.5 kg of wilted maize for each treatment (for a total of 4.5 kg). We sprayed each batch with 15 mL of either deionized water, control; LAB commercial inoculant (**I**), and LAB commercial inoculant + pediocin (**IP**), and thoroughly mixed. The inoculant supplied as a freeze-dried powder, containing a mixture of *L. plantarum*, *L. buchneri*, and *E. faecium*, was suspended in deionized water and applied at a rate of 1.1 mg/kg fresh forage according to the technical sheets, to provide 1×10^6 cfu/g.

One-hundred-gram samples of maize were packed in polyethylene bags designed for food use (dimensions 175 × 240 mm), using a vacuum-packing machine (Elma, Udom SA, Álava, Spain). All bags were heat sealed, leaving a distance of 150 mm from the bottom and cutting the remaining plastic 5 mm above the sealing line. Before sealing, 1 mL of a *L. monocytogenes* CECT 4032 culture was added to the bags to obtain a concentration of approximately 1×10^5 cfu/g and mixed thoroughly.

Triplicate samples for each treatment and sampling time were incubated at 25°C for 30 d. Two bags per treatment were opened at 1, 2, 5, 8, 15, and 30 d after ensiling to perform chemical, microbiological, and PCR-denaturing gradient gel electrophoresis (**DGGE**) analyses.

Chemical Analysis

The DM content of maize and silages was determined by oven drying at 60°C until constant weight. For pH determination and chemical analysis, 20 g of silage sample was mixed with 180 mL of ultrapure water and twice homogenized in a Stomacher (model 400, Seward, London, UK). Aqueous extracts were filtered through Whatman paper grade 1 (11-μm pore size) to remove particulate matter, and centrifuged ($3,700 \times g$ for 10 min at 4°C) for determining the water-soluble reducing sugars (Bernfeld, 1951). The concentration of organic acids (lactic, acetic, butyric, and propionic) and alcohols (ethanol, propane-1,2-diol, and butane-2,3-diol)

was determined by HPLC analysis using an ION-300 Organic Acids column (length, 300 mm; internal diameter, 7.8 mm) with a precolumn longuard (Tecknokröma, Barcelona, Spain). The mobile phase was 3 mM H₂SO₄ at a flow rate of 0.4 mL/min at 65°C, being the peaks detected with a refractive-index detector.

Microbiological Analysis

The LAB enumeration was determined in 20-g silage samples, mixed with 180 mL of sterile saline solution, and twice homogenized in a Stomacher (model 400, Seward, London, UK) for 1 min. The number of LAB was obtained by pour plating serial 10-fold dilutions (in sterile saline solution) on MRS agar. The plates were overlaid with a thin layer of bacteriological agar (Cultimed Panreac Química S.A., Barcelona, Spain) and incubated at 30°C for 48 to 72 h, before colony count. Only results between 30 and 300 colonies were considered and expressed as log colony-forming units per gram.

DNA Extraction and PCR Amplification

The DNA was extracted from 10-mL aliquots of the homogenates used for microbiological analysis, centrifuged at $13,000 \times g$ for 5 min at 4°C, following the procedure described elsewhere (Amado et al., 2012). Primers 357F and R518 were used to amplify the V2-V3 variable regions of the total bacterial 16S rRNA gene, which corresponds to the positions 339 of 539 from *Escherichia coli* (Gafan and Spratt, 2005). A region of the *iap* gene of the genus *Listeria* was amplified using the primers List-univ.1 and List-univ.2 (Cocolin et al., 2002). The expected size of amplified fragments, 170 bp for total bacteria and 460 bp for *L. monocytogenes*, was verified on a 2% (wt/vol) agarose gel. A GC clamp was added to the forward primers 357F and List-univ. 1, to improve the sensitivity of the detection of DNA sequence differences by DGGE analysis (Sheffield et al., 1989). The sequences of both pairs of primers and the specific conditions of each PCR are detailed in Table 1. The PCR was performed in a final volume of 50 μL using chemicals and enzymes provided by Bioline (London, UK).

Denaturing Gradient Gel Electrophoresis

The DGGE was performed on a Dcode system (Bio-Rad Laboratories, Hercules, CA). The DNA fragments were loaded onto a polyacrylamide gel (7.8 g/L of polyacrylamide and 0.2 g/L of Bis-acrylamide). According to conditions previously optimized in our laboratory,

Table 1. Primer sequences and PCR conditions used for total bacteria and *Listeria monocytogenes* detection by PCR-denaturing gradient gel electrophoresis in maize silages

Item	Primer	Sequence (5'-3')	Denaturation	Annealing	Extension	Cycles	PCR product (bp)	Reference
Bacteria	F357 R518	GCACGGGGGTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	94°C, 30 s	56°C, 30 s	72°C, 3 min	35	177	Gafan and Spratt, 2005
<i>Listeria monocytogenes</i>	List-univ.1 List-univ.2	ATGTCATGGAATAA GCTTTCCAAAGGTGTTTT	95°C, 1 min	36°C, 2 min	72°C, 3 min	35	457	Cocolin et al., 2002

different denaturing gradients were used for total bacteria (30% to 60%) and *L. monocytogenes* analysis (20% to 40%). The 100% denaturing conditions are defined as 7 M urea containing 40% formamide.

Electrophoresis was run in a 1× Tris-acetate-EDTA buffer at 60°C, for 16 h at 75 V. After electrophoresis, gels were silver stained according to the method of Sanguinetti et al. (1994), scanned, and analyzed using the software Bionumerics 5.1 (Applied Maths, St-Martens-Latem, Belgium).

The amplified fragments of interest were excised from the gel, resuspended overnight in Tris-EDTA buffer and amplified using the primers F357 (without GC clamp) and R518. The PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO) and sequenced using an ABI Prism 3130 DNA sequencer (Applied Biosystems, Foster City, CA). The consensus sequences were obtained after alignment and assembly using the software Geneious v3.6.1 (Drummond et al., 2008), performing the similarity searches within the GenBank databases using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Statistical Analysis

The data of final pH, LAB counts (as log cfu/g), and concentrations of lactic acid, acetic acid, and ethanol were statistically analyzed using a general linear model procedure of the software package SPSS 15.0 for Windows (Release 15.0.1, 2006, SPSS Inc., Chicago, IL). First, Levene's test was used to determine if the variances of each variable were equal. Normal distribution of data between treatments was previously verified (via histograms). Then, a one-way ANOVA with the Tukey (in the case of equal variances) or Games-Howell (for unequal variances) post hoc test ($P = 0.05$) was used to determine whether there were significant differences between the mean final values of the variables.

RESULTS AND DISCUSSION

In Vitro Antimicrobial Activity of Pediocin SA-1

The interest in using bacteriocinogenic strains as inoculants for ensilage purposes focuses on their ability to inhibit the growth of undesirable microorganisms owing to the production of antimicrobial substances. However, one limitation to the application of bacteriocin-producing LAB in this field would be the ability of some of these bacterial strains to synthesize antimicrobial substances active against other LAB (Franz et al., 1996). For this reason, we initially tested the compatibility of pediocin SA-1 and the silage inoculant by performing an in vitro analysis of activity against

a culture of the commercial inoculant (*L. plantarum*, *E. faecium*, and *L. buchneri*) in MRS. The results of the bioassay showed no inhibition of microbial growth at any of the concentrations tested (data not shown), indicating they could be successfully added together as silage inoculants.

Following analysis of dose-response data of the antimicrobial activity of pediocin SA-1 against a culture of *L. monocytogenes* CECT 4032, we found an inhibitory activity of 6,320 BU/mL. This activity is 2-fold higher than that reported against *Carnobacterium piscicola*, the usual indicator microorganism used to test the inhibition of *P. acidilactici* cell-free extracts (Guerra et al., 2005b). This result supports previously published qualitative results of antilisterial activity of pediocin SA-1 using the agar-diffusion method (Guerra and Pastrana, 2002).

Monitoring *L. monocytogenes* in Maize Silages

The genes of peptides with bacteriocin-like activity are found in a high proportion in *Enterococci* isolated from silage (Marciňáková et al., 2005; Strompfová et al., 2008). Indeed, several bacteriocins have been purified from different types of silage (Ohmomo et al., 2000; Eguchi et al., 2001; Sparo et al., 2006; Marciňáková et al., 2008; Bal et al., 2012). Some of these bacteriocins belonging to the pediocin-like family (Aymerich et al., 1996), and a bacteriocin isolated from *Streptococcus bovis* HC5 (Mantovani and Russell, 2003) have shown high antilisterial activity in vitro and bear a great potential as silage additives. However, to the best of our knowledge, this is the first report on the use of pediocin as an additive for controlling *L. monocytogenes* during the ensiling process.

Listeria monocytogenes is an important foodborne pathogen particularly in acidic foods such as silages (Caro et al., 1990) because of its high resistance to acidic pH (Barker and Park, 2001). Traditional detection methods of *L. monocytogenes* are based on enrichment techniques that might outnumber the nonpathogenic *Listeria* species when *L. monocytogenes* is present together with other *Listeria* sp., and might lead to false-negative results (Zittermann et al., 2016). Given the pathogenic potential of *L. monocytogenes*, PCR-based methods provide a more reliable detection of *L. monocytogenes* due to their high sensitivity (Zittermann et al., 2016). Indeed, previous reports have used DNA-based methods to identify *L. monocytogenes* in silages (Torriani and Pallotta, 1994; Amado et al., 2012; Sharifzadeh et al., 2015).

To evaluate the potential of pediocin SA-1 to remove *L. monocytogenes* from the silages, we used the strain *L. monocytogenes* CECT 4032 as an indicator of this

Figure 1. Denaturing gradient gel electrophoresis profiles of the PCR products from DNA extracted from maize silage under different treatments and times of fermentation: 1, 2, 5, 8, 16, and 30 d. I = silages treated with Pioneer 11C33 inoculant; IP = silages treated with Pioneer 11C33 inoculant and pediocin SA-1. Amplification was performed using *Listeria monocytogenes* specific primers, List-univ.1 and List-univ.2. M corresponds to a marker from a pure culture of the *L. monocytogenes* Colección Española de Cultivos Tipo (CECT) 4032 added to the experimental silages.

pathogen. For this purpose, the bacterium was monitored in treated silages (I and IP) and untreated silages (control) during the 30 d of ensiling by comparing the DGGE profiles of the PCR products targeting the *iap* gene encoding a protein related to the virulence of *L. monocytogenes* (Figure 1). This methodology was first reported as a fast screening test to investigate the presence of *Listeria* spp. and *L. monocytogenes* in foods (Cocolin et al., 2002) and successfully applied to monitor the pathogen in ryegrass and maize silages (Amado et al., 2012).

The DGGE profiles showed a single band comigrating at the same distance of the *L. monocytogenes* marker strain, although a secondary band of unknown nature appeared whose pattern was not related to the treatment or the time of ensiling. The results of DNA analysis also revealed *L. monocytogenes* CECT 4032 disappeared after 8 d of ensiling in pediocin-treated silages, whereas it was still present after 30 d in the control and in I silages (Figure 1). The pH of silages after 5 d of ensiling (3.7; Figure 2) was reported to maximize the antibacterial activity of pediocin from *P. acidilactici* NRRL B-5627 (Guerra and Pastrana, 2002; Amado et al., 2012), accounting for the observed removal of *L. monocytogenes* after this time of ensiling. Besides, the stability of the bacteriocin at low pH values (3.0) and low temperature (30°C; Anastasiadou et al., 2008) enhances antibacterial activity as the pH of the silage decreases, with the antibacterial activity maximized below a threshold pH of 3.7. This would be an advantage toward the use of Pediocin SA-1 as silage additive because the bacteriocin will maintain its activity in spite of the low pH of the silages.

Effect of Pediocin Addition on the Chemical Composition, LAB Counts, and Bacterial Population of Maize Silages

Genomic DNA from maize silage samples was used to study the effect of the different treatments (I and IP)

on their microbial profile using PCR-DGGE. This technique was reported as a rapid and reproducible tool for the analysis of the bacterial diversity in silages (Wang et al., 2006; Parvin et al., 2010). In agreement with other reports (Li and Nishino, 2011), we observed a significant reduction in biodiversity compared with the

Figure 2. Changes in chemical composition and lactic acid bacteria (LAB) counts during maize fermentation. LAC, lactic acid; WSRS, water-soluble reducing sugars; AcA, acetic acid. Symbols: C, control silage (Δ); I, silages treated with Pioneer 11C33 inoculant (○); IP, silages treated with Pioneer 11C33 inoculant and pediocin SA-1 (●).

unfermented material (Figure 3). The onset of ensiling resulted in the disappearance of some indigenous bacteria (Figure 3), whereas other species such as *P. pentosaceus*, *Pantoea agglomerans*, and *Klebsiella* sp. (bands 10, 12, 13) disappeared in an early stage of fermentation (Table 2, Figure 3). By contrast, some species absent in the pre-ensiled crop appeared as the ensiling process

begun and were identified as *Lactobacillus brevis* (band 1), *Weissella cibaria* (band 3), *Weissella kimchii* (band 4), a nonculturable *Bacillus* (band 5), and *Weissella soli* (band 7). At the same time, *L. plantarum* (band 6) and *L. lactis* (band 8) became more intense, remaining as the major species throughout ensiling, together with *W. cibaria*, *W. kimchii*, a nonculturable *Bacillus*, and

Figure 3. Denaturing gradient gel electrophoresis analysis of PCR-amplified 16S ribosomal DNA fragments from maize silages at different times of ensiling: 1, 2, 5, 8, 16, and 30 d. C, control (untreated); I, silages treated with Pioneer 11C33 inoculant; IP, silages treated with Pioneer 11C33 inoculant and pediocin SA-1. M corresponds to a marker from pure cultures of *L. plantarum*, *L. lactis*, and *P. acidilactici*. The first lane on the left (Maize) corresponds to the untreated forage. The positions of bands discussed in the text are indicated by numbers that correspond to the following species of bacteria (Table 3): 1, *Lactobacillus brevis*; 2, *Lactobacillus pentosus*; 3, *Weissella cibaria*; 4, *Weissella kimchii*; 5, uncultured *Bacillus* sp.; 6, *Lactobacillus plantarum*; 7, *Weissella soli*; 8, *Lactococcus lactis*; 9, *Enterococcus faecium*; 10, *Pediococcus pentosaceus*; 11, *Lactobacillus buchneri*; 12, *Pantoea agglomerans*; and 13, *Klebsiella* sp.

W. soli both in treated and untreated silages (Figure 3). Less intense bands corresponding to *L. pentosus* (band 2) and *L. brevis* (band 1) appeared after the onset of fermentation. These changes in the bacterial population explain the observed increment in the LAB counts (3 log cfu/g) within the early stage (0–5 d) of fermentation (Figure 2). The species *L. plantarum* and *L. lactis* (Parvin and Nishino, 2009; Parvin et al., 2010), *L. brevis* and *L. pentosus* (Wang et al., 2006; Parvin and Nishino, 2009), and various *Weissella* strains (Brusetti et al., 2006; Li and Nishino, 2013) have been identified in different silages.

Inoculation of maize either with or without pediocin did not substantially change the bacterial communities of the spontaneous fermentation. However, as expected, we observed an increase in the intensity of the bands corresponding to the inoculated species, *E. faecium* (band 9) and *L. buchneri* (band 11), which were also weak bands in untreated silages. These results are in agreement with previous findings showing inoculated LAB species (*L. plantarum* and *L. brevis*) detected in addition to bacterial communities in untreated maize silages (Parvin et al., 2010). The fact that inoculant-containing bacteria (*L. plantarum*, *L. buchneri*, and *E. faecium*) were present throughout the 30 d of ensiling (Figure 3) suggest the bacterial inoculants could have an effect on silage quality, which might not occur if better adapted epiphytic microorganisms outcompete the inoculants and dominate the fermentation (Lin et al., 1992).

Within the initial stage of fermentation (0–5 d), our results revealed homolactic fermentation in both untreated and inoculated silages, characterized by lactic acid production and pH decrease (Figure 2). Heterofermentative bacteria are the predominant population in most untreated forages (Lindgren et al., 1983), so the

high production of lactic acid must be due to the development of homofermentative *L. plantarum* and *L. lactis* observed during the initial stage of ensiling (Figure 3). Then, the growth of *L. buchneri* and, to a lesser extent of *E. faecium*, accounted for the higher production of acetic acid and propane-1,2-diol in treated silages than the control (Table 3). *Listeria buchneri* produces high levels of acetic acid in silage through converting lactic acid into acetic acid and propane-1,2-diol under anaerobic and acidic conditions (Oude Elferink et al., 2001). Acetic acid has antimicrobial activity against yeasts and molds (Tabacco et al., 2009) and has been shown to increase the aerobic stability of silages (Danner et al., 2003). Ethanol productions were similar throughout the time course of fermentation (Figure 2), although final compositions showed 38% (I) and 49% (IP) higher ethanol concentrations than untreated silage (Table 3). Hence, inoculation of maize either with or without pediocin increased the concentration of antimicrobial compounds compared with the spontaneous fermentation (Table 3), making them potentially less susceptible to yeast and mold growth as a consequence of aerobic exposure. However, further quantification of the inhibitory concentration of ethanol and propane-1,2-diol against spoilage microorganisms would be needed because the effective concentration of antimicrobial compounds in silages is usually quite high (Danner et al., 2003). The development of LAB observed during the initial phase of fermentation was responsible for the rapid sugar consumption (Figure 2), although final sugar concentrations were lower in inoculated series (1.8 g/kg of DM) compared with the control (7.09 g/kg of DM). The low sugar content also provides an advantage compared with the spontaneous fermentation because a high concentration of residual sugars could support yeast and mold growth (Filya et

Table 2. Identities of denaturing gradient gel electrophoresis bands isolated and purified from maize silages (Figure 3)¹

Band	Closest species	Accession number	% Similarity
1	<i>Lactobacillus brevis</i>	GU295951	98.0
2	<i>Lactobacillus pentosus</i>	AB362751	99.0
3	<i>Weissella cibaria</i>	DQ294964	98.7
4	<i>Weissella kimchii</i>	AY190628	98.0
5	Uncultured <i>Bacillus</i> sp.	FJ95793	99.2
6	<i>Lactobacillus plantarum</i>	FJ867640	99.5
7	<i>Weissella soli</i>	AB485956	99.9
8	<i>Lactococcus lactis</i>	FJ915803	99.4
9	<i>Enterococcus faecium</i>	AF529204	99.8
10	<i>Pediococcus pentosaceus</i>	EU082182	98.7
11	<i>Lactobacillus buchneri</i>	FJ867641	98.5
12	<i>Pantoea agglomerans</i>	FJ756354	99.2
13	<i>Klebsiella</i> sp.	FJ912860	98.4

¹The closest species name, accession number, and similarity percentage from performed Basic Local Alignment Search Tool search are shown.

Table 3. Composition of maize silages after 30 d of fermentation¹

Item	pH	LAC (g/kg of DM)	AcA (g/kg of DM)	Ethanol (g/kg of DM)	1,2-Prop (g/kg of DM)	WSRS (g/kg of DM)	LAB (log cfu/g)
C	3.64 ^a	34.6 ^a	6.36 ^a	7.94 ^a	ND	7.09 ^a	6.32 ^a
I	3.74 ^b	34.1 ^a	11.3 ^b	11.1 ^b	18.3 ^a	1.82 ^b	6.72 ^b
IP	3.75 ^b	39.8 ^b	12.2 ^b	11.9 ^b	14.3 ^b	1.80 ^b	6.12 ^a
SEM	0.018	0.939	0.893	0.580		0.889	0.088
<i>P</i> -value	0.002	<0.001	<0.001	<0.001		<0.001	0.002

^{a,b}Means from duplicate samples. Within columns, means followed by different letters are significantly different ($P < 0.01$).

¹LAC, lactic acid; AcA, acetic acid; 1,2-Prop, 1,2-propanediol; WSRS, water-soluble reducing sugars; LAB, lactic acid bacteria. ND, not detected (less than 0.2 g/kg).

al., 2000), which is an issue regarding aerobic stability (Pahlow et al., 2003). In agreement with these results, higher ethanol concentrations and sugar consumptions were found in alfalfa silages inoculated with *L. buchneri*, and with *L. plantarum* in combination with *E. faecium* (Filya et al., 2007), compared with other LAB inoculants and untreated silages. Lactic acid, acetic acid, and ethanol were the main fermentative products, along with propane-1,2-diol detected in treated silages after 30 d of ensiling (Table 3). However, we did not identify any production of propionic acid, butyric acid, and butane-2,3-diol (less than 0.2 g/kg) in any of the samples, which suggests a good fermentation quality.

The DGGE analysis of 16S rRNA PCR products cannot detect active bacteria, but the fermentation products (acetic acid, propane-1,2-diol, and ethanol) coupled with the persistence of the inoculated species suggest these strains, actively participate in the ensiling process. The DGGE profiles also confirmed the results of the in vitro compatibility between the bacteriocin and the microorganisms contained in the commercial inoculant because no inhibition of *L. plantarum*, *E. faecium*, or *L. buchneri* was observed (Figure 3) in IP silages. Pediocin SA-1 did not affect the naturally occurring microbiota of the silages (Figure 3), whereas the removal of *L. monocytogenes* was observed after 8 d of ensiling (Figure 1). This result indicates a narrow spectrum of inhibitory activity that could be useful to control the spoilage microorganisms of silages selectively without affecting the harmless microbiota (Gálvez et al., 2007). This specificity would be an advantage compared with bacteriocins bearing a wider activity spectrum, such as enterocin ON-157 purified from an *Enterococcus faecium* strain isolated from a spoiled silage, and which was bactericidal against *Listeria monocytogenes*, but also enterococci and *Lactobacillus sake* (Ohmomo et al., 2000).

CONCLUSIONS

Pediocin SA-1 could be a potential silage additive that, together with a compatible bacterial inoculant (*L.*

plantarum, *L. buchneri*, and *E. faecium*), selectively controls *L. monocytogenes* without affecting the beneficial microbiota of maize silages. In addition to not inhibiting the naturally occurring microbiota of the silages, higher concentrations of antimicrobial compounds were found in treated silages compared with the control, suggesting the inoculated species were involved in the ensiling process. The stability of pediocin SA-1 at low pH provides this bacteriocin a high potential toward its application as a food preservative and as an additive for the animal feed industry. However, further studies are needed to evaluate the real effects and the safety of this antimicrobial in field silage conditions.

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