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Different aspects of *Lactobacillus* inoculants on the improvement of quality and safety of alfalfa silage

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There is a significant range of bacterial inoculants for forage ensiling, but there is still a need for formulations to improve the safety of feed. The objective of the study was to evaluate the usefulness of three lactobacilli strains in alfalfa (*Medicago sativa* L.) The following bacterial inoculants were used: *Lactocacillus plantarum* K KKP 593p (LPK), *L. plantarum* C KKP 788p (LPC), *L. buchneri* KKP 907p (LB), and mix of all three strains (LPK+LPC+LB). The application of bacterial inoculants in alfalfa ensiling resulted in a reduction of the total number of molds, *Clostridium perfringens* and *Listeria* sp. (up to 5, 7, and 5 times respectively for LB inoculant in comparison to untreated silage). Total inhibition of *Salmonella* sp. and *Escherichia coli* growth was achieved in silages treated with all inoculants except for LPC. Aerobic stability in the control silage was the lowest (77 h) and doubled under the influence of bacterial inoculants. The most stable according to aerobic stability was silage treated with LB inoculant (175 h), where the highest concentrations of acetic acid (4.8 g kg^{-1}), propionic acid (0.7 g kg^{-1}) and 1,2-propanediol (526 mg kg^{-1}) were reported. The study discussed that it is important to evaluate not only the effect of bacterial inoculants on physicochemical and microbiological silage properties, as the presence and expression of antibiotic resistance genes in lactic acid bacteria have been reported. The results of antimicrobial susceptibility testing of the strains showed that almost all minimum inhibitory concentrations values for eight antibiotics were equal to or below the corresponding breakpoints proposed by the European Food Safety Authority, Additives and Products or Substances used in Animal Feed Panel.

Key words: Aerobic stability, alfalfa, antibiotic susceptibility, *Lactobacillus*, pathogenic bacteria, silage.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a valuable forage plant characterized by a high content of crude protein. Ensiling is one of the best known methods to preserve fodder, but legumes such as alfalfa have long been regarded as difficult to ensile forages because of their low (< 1.5%) content of soluble carbohydrates in the fresh material, low dry matter (DM), and high buffering capacity; therefore, the usage of biological or chemical additives is beneficial (Ohshima et al., 1997; Repetto et al., 2011).

Epiphytic microflora of plants is composed of bacteria, including *Clostridium* sp., *Bacillus* sp., coliform bacteria and lactic acid bacteria (LAB), as well as yeasts and moulds. The role of lactic acid bacteria in the ensiling process is not only to lower pH by converting water-soluble carbohydrates into organic acids, but these bacteria are also responsible for inhibiting the growth of undesirable epiphytic microorganisms by competing for nutrients and synthesizing antimicrobial and antifungal agents. Different lactic acid bacteria species synthesize some metabolites from those mentioned above, such as bacteriocins, hydrogen peroxide, lactate peroxidase or 1,2-propanediol, all of which inhibit the growth of pathogenic and spoilage bacteria, yeast and moulds (Davies et al., 1996; Magnusson and Schnürer, 2005).

The forage before ensiling intended for silages usually contains a low number of lactic acid bacteria - less than 1% of the microflora - so it is necessary to apply starter cultures of selected strains of lactic acid bacteria to stimulate the ensiling process (Danner et al., 2003; Schmidt et al., 2009, Zhang et al., 2009). Therefore, the addition of LAB at more than 10^5 CFU g^{-1} in silage-making has been recommended in order to effectively reduce DM loss and avoid clostridial fermentation (McDonald et al., 1991; Nishino et al., 2003). Ranjit and Kung (2000) recommended a dose of 10^6 CFU g^{-1} for satisfactory improvement. Selected strains of LAB, which are used as silage inoculants, improve the quality and aerobic stability of silages and are often characterized by the probiotic activity in the digestive tract of animals (Oude Elferink et al., 2001; Holzer et al., 2003). Facultative heterolactic acid bacteria such as *Lactobacillus plantarum* synthesize mainly lactic acid and a few volatile

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fatty acids, which are substrates that are important for the protection of silages from the interference of aerobic yeasts and moulds. Since obligate heterolactic acid bacteria, like *L. buchneri*, produce higher levels of acetic acid, they can stabilize silages during aerobic exposure (Zhang et al., 2009). Studies estimating the aerobic stability of silage feed prepared with the addition of bacterial starter cultures, in conjunction with the reduction of the development of aerobic microorganisms, were carried out in a number of research institutes in the field of agriculture (Ranjit and Kung, 2000; Taylor et al., 2002; Schmidt et al., 2009). Currently, there is a significant range of bacterial inoculants for forage ensiling on the market, but there is still the need for formulations to improve not only the quality of feed, but also to decrease the content of pathogenic bacteria and moulds and even to decontaminate the silages of mycotoxins produced by them (Zielinska and Miecznikowski, 2008; Richard et al., 2009).

Development of the composition of bacterial silage inoculants is a complex but extraordinary aspect from the economic and technological points of view. The precise characteristic of the ensiled plant material is strongly dependent on lactic acid bacteria microflora, both epiphytic and external, introduced with the preparations. The objective of the study was to evaluate the usefulness of three selected lactobacilli strains in alfalfa ensiling when used separately and in combination. To the best of our knowledge, there are plenty of papers describing the effect of bacterial inoculants in meadow grass silages, alfalfa, sugar cane, maize whole crop and maize corn silages. Most authors have focused on the physical and chemical properties of silages and their effect on silage aerobic stability, ruminal fermentation, and dairy and meat production. The authors of this paper focused on the impact of lactic acid selection to prepare the bacterial inoculant in association with the safety and microbiological quality of alfalfa ensilage. This article emphasizes the rarely discussed issues of potentially pathogenic microbes presence in silages as well as antibiotic resistance in bacterial strains used in silage inoculants. Antibiotic resistance is a serious and growing phenomenon in contemporary biological sciences, wrongly pertained only to pathogenic organisms. Some more frequently discussed issues relevant to genetic identification and production of specific metabolites by lactic acid bacteria are described in the paper too.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Three bacterial strains were examined in the study: *Lactobacillus plantarum* K KKP/593/p (Zielinska et al., 1995), *Lactobacillus plantarum* C KKP/788/p (Zielinska et al., 1998) and/or *Lactobacillus buchneri* KKP 907p (Zielinska et al., 2006). All three species were patented and characterized by the different phenotypic features presented in Table 1. The studied strains have been deposited at the Collection of Industrial Microorganisms at the prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology in Warsaw (Poland).

Lactic acid bacteria were cultivated on Lactobacilli MRS broth medium (Difco, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) at 30 °C and under aerobic conditions. Bacterial biomass was centrifuged using a Jouan centrifuge (8000 rpm, 10 min, 5 °C).

Genetic differentiation of strains and evaluation of antibiotic susceptibility

Genomic DNA was purified using bacterial genomic DNA kits (GenElut, Sigma-Aldrich, St. Louis, Missouri, USA) following the manufacturer's instructions. Strain-specific identification was performed using RAPD-PCR with different primers, PRIMO2: 5'-CGGCAAGGAG-3', RP: 5'-CAGCACCCAC-3' and M13: 5'-GAGGGTGGCGGTTCT-3'. The reaction mixture contained 10 μM of primer, 0.2 mM of each dNTP, 3.5 mM $MgCl_2$, 0.625 U *Taq* DNA polymerase, reaction buffer and 100 ng DNA as a template. PCR was performed under one of the following amplification conditions: initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 45 °C for 30 s, 72 °C for 45 s and a final extension step at 72 °C for 7 min (primer M13) or initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 45 °C for 45 s, 72 °C for 2 min and a final extension step at

Table 1. Characteristics of bacterial strains used in the study.

Strain	Source of isolation	Specific features		Reference
		Ability to synthesize lactic acid utilizing different C sources	Content of lactic acid in modified MRS medium after 72 h culture (g kg^{-1})	
Lactobacillus plantarum K KKP/593/p	Raw potato silage	Native potato starch or native cereal starch	19.0-30.0	Zielinska et al., 1995
L. plantarum C KKP/788/p	Alfalfa silage	Cellulose and/or oat xylan native potato starch	13.0-14.0 18.0	Zielinska et al., 1998
		Ability to synthesize 1,2-propanediol	Concentration of 1,2-propanediol during 10 d of culture in MRS medium, mg 100 mL^{-1} medium	
L. buchneri KKP 907 p	Meadow grass silage	Yes	100	Zielinska et al., 2006

72 °C for 5 min (primer RP and PRIMO2). PCR products were separated by electrophoresis on a 1.5% agarose gel (Sigma-Aldrich) stained with fluorescent DNA stain (GelRed, Biotium Inc., Hayward, California, USA). All reactions were performed in three independent studies.

The minimum inhibitory concentrations (MIC) for eight antibiotics (ampicillin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol) were determined using Mueller-Hinton II (Cation-Adjusted) Agar (Becton, Dickinson and Company) supplemented with 5% defibrinated sheep blood. The gradient test method was used for MIC. Evaluator for erythromycin and tetracycline (Oxoid, Basingstoke, Hampshire, UK) and E-test strip for the rest of the antimicrobial agents (bioMérieux, Marcy l'Etoile, France) were used according to the manufacturer's instructions. Inoculum of each strain was prepared by suspending colonies from MRS agar plates in sterile saline to reach a turbidity corresponding to McFarland standard 1. After 24 and 48 h of incubation of plates under microaerophilic condition (5% CO_2) at 35 ºC, MIC values were determined. The susceptibility of strains was established in accordance with the breakpoints proposed by the European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (EFSA FEEDAP, 2012).

Bacterial inoculants

The bacterial inoculant contained lactic acid bacteria, carriers and emulsifiers. There were used following carriers: sucrose, glucose, lactose, starch soluble pure and emulsifiers: xanthan gum and lecithin. The preparation was in the dry form of granules characterized by a minimum of 96% DM. One gram of inoculant contained no less than 2×10^{10} colony forming units (CFU) of the strains. Bacterial inoculants were produced by prof. Wacław Dąbrowski Institute of Agriculture and Food Biotechnology in Warsaw, according to the manufacturing process developed by the company and described by Miecznikowski et al. (2008). Four bacterial inoculants were tested: *L. plantarum* K KKP 593p (LPK), *L. plantarum* C KKP 788p (LPC), *L. buchneri* KKP 907p (LB), and a mixture of all three strains (LPK + LPC + LB).

Alfalfa ensiling

Alfalfa was harvested and collected for ensiling. The trial was conducted under farm conditions at the experimental Station at Falenty near Warsaw, Poland. The alfalfa field was cut at the initial flowering in times (two cut). The first cut was not studied. The herbage was mown with a rotary mower-conditioner and before harvest was pre-wilted to a DM about 30%. The material was chopped to 1.5 cm lengths. Following chopping, the plants were ensiled in polyethylene microsilos of 0.01 m^3 capacity, sealed with a rubber stopper allowing the release of gaseous products. Forages were treated and untreated with bacterial inoculants. All experiments were provided using the lowest dose of the bacterial inoculant, which is 10 g t^{-1} of forage before ensiling (alfalfa) or 2×10^5 CFU g^{-1} of raw plant material. Silages untreated with bacterial inoculants were used as a control material. Silos were kept in a room at a temperature of 20 ± 2 °C for 90 d. Once the silo was opened, it was removed from the subsequent experiment. Experiments were made in triplicate, what meant that three silos were prepared for each treatment.

Chemical and microbial analyses of silages

Silage samples were analyzed after 90 d of ensiling. The extract of macerated silage was prepared with distilled water and filtered through two layers of cheesecloth. An adjusted DM content was determined according to ISO standard (PN-ISO 6496: 2002) and pH using the potentiometric method. Determination of L- and D-lactic acid content, acetic and 3-hydroxybutyric acid contents were conducted by enzymatic methods according to the manufacturer's protocols (Boehringer Mannheim, Germany). Ammonia N concentration was determined by the Convay method (Skulimowski, 1974) and the determination of ethanol content was performed by the Weissbach-Laube method (Weissbach and Laube, 1964). To measure the content of soluble carbohydrates, the NIRS method was used (NIRFlex N-500 spectrometer, Büchi Labortechnik, Flawil, Switzerland, the ready-to-use INGOT calibrations were chosen).

Propionic acid and 1,2-propanediol were determined by gas chromatograph with a flame ionization detector (FID) (7890A GC, Agilent Technologies, Santa Clara, California, USA) and a capillary column with a diameter of 0.53 mm and a length of 30 m with phase J&W DB-FFAP column (Agilent). Test samples were previously purified by solid phase extraction on a SPE-C_{18} column and then analyzed using chromatographic techniques. Helium was used as a carrier gas with a flow rate of 85 mL min^{-1} and the following temperature program: 35 °C (0.5 min), with an increase of 20 °C min^{-1} to 90 °C, and an increase of 10 °C min^{-1} to 200 °C (0.5 min). Chemstation Agilent Technologies B.03.01 software was used.

Then, 10 g fresh forage and silages were homogenized in 100 mL sterile peptone water, mixed vigorously for 60 min and used for culturing. Subsequent serial 10-fold dilutions were made, to obtain 15-300 colonies per dish. All microorganisms were determined in triplicate. Lactic acid bacteria were enumerated by pour plating of MRS medium according to ISO standard (PN-ISO 15214:2002). The number of moulds was determined by the plate method according to the ISO standard (PN-ISO 21527-2:2009) and the number of yeasts was determined using an YPG medium with chloramphenicol. *Salmonella* sp. were enumerated by a plate method with the use of Rambach Agar (Merck, New Jersey, USA) and *Clostridium perfringens* by a plate method on selective medium Agar Base 9188 (Neogen, Lansing,

Michigan, USA) according to the ISO standard (PN-EN ISO 7937:2005). Determination of *Escherichia coli* and coliform bacteria was done using a plate method with the use of technical medium (3M Petrifilm *E. coli*/Coliforms count plates, Noack, Vienna, Austria).

Determination of genera and species of yeast was performed after the selection and multiplication of pure cultures, using the API 20 C AUX and mini-API identification tests (bioMérieux).

Aerobic stability

Determination of storage stability was performed according to a temperature method described by Honig (1985). The experiment was conducted for 10 d at 20 ± 1 °C with an excess of oxygen. Silages were kept in plastic containers with a volume of 10 dm^3. Temperature measurements in silages were detected at 1 h and a temperature increase of 3 °C was considered to be an indication of instability.

Statistical analysis

Statistical analyses of the results were performed by repeated measurements with one-way ANOVA in STATISTICA 8.0 (StatSoft, Krakow, Poland), followed by Tukey's multiple comparison test. P-values of $P \leq 0.05$ were considered significant. The Shapiro-Wilk test was used to determine whether the population was normally distributed. Levene's test and Brown-Forsythe test were used to assess the equality of variances for a variable calculated for groups.

RESULTS

Genetic differentiation of strains and evaluation of antibiotic susceptibility

In the present study, the LAB strains were characterized on the basis of genetic diversity and important safety properties. RAPD-PCR analysis with three different primers was used to evaluate the genetic diversity and genetic stability among the studied lactobacilli strains. RAPD-PCR produced patterns with products ranging from approximately 300 bp (primer PRIMO2) or 600 bp (primer RP) to more than 3000 bp (Figure 1). All three strains were distinguished from each other on the basis of specific and reproducible RAPD profiles, indicating a high diversity among isolates (Figure 1). Using this method, the genetic stability of selected strains during a short time period was also confirmed.

The results of antimicrobial susceptibility testing of studied strains using the E-test are shown in Table 2. Almost all MIC values were equal to or below the corresponding breakpoints proposed by EFSA FEEDAP Panel. Only for *L. buchneri* KKP/907/p was an MIC value for one antibiotic above the EFSA recommendation (16 *vs*. 8 μg mL^{-1}).

Effect of bacterial inoculants on physicochemical and microbiological quality of alfalfa silage

The effect of bacterial inoculants on physicochemical and microbiological quality of alfalfa silages was evaluated. The average DM for raw alfalfa was 294.0 g

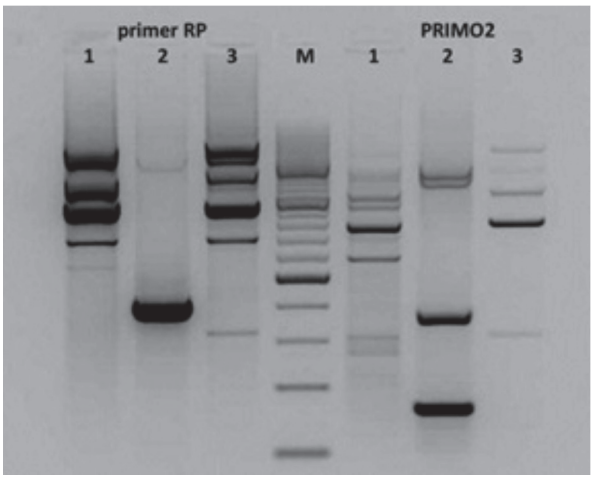

Figure 1. RAPD patterns obtained with primer RP and PRIMO2. Lane 1: DNA template from *Lactobacillus plantarum* KKP/593/p; lane 2: DNA template from *L. plantarum* KKP/788/p; lane 3: DNA template from *L. buchneri* KKP/907/p; lane M: O'RangeRuler 200 bp DNA Ladder (ThermoScientific, the brighter bands at 3000, 2000, and 1000 bp serve as reference bands).

Table 2. Antimicrobial susceptibilities of the tested strains .

	Minimum inhibitory concentrations (MIC) values (mg L^{-1})							
	Lactobacillus plantarum K KKP/593/p		*L. plantarum* C KKP/788/p			*Lactobacillus buchneri* KKP 907p		
Antibiotics	24 h	48 h	24 h	48 h	EFSA[a]	24 h	48 h	EFSA[b]
Ampicillin	0.50	0.75	0.19	0.19	2	ng	0.38	2
Gentamycin	1.00	1.50	1.00	1.00	16	ng	0.125	16
Kanamycin	24.00	32.00	24.00	24.00	64	ng	4.0	32
Streptomycin	16.00	24.00	18.00	24.00	nr	ng	2.0	64
Erythromycin	0.12	0.25	0.12	0.25	1	ng	< 0.015	1
Clindamycin	0.38	0.75	0.25	0.25	2	ng	< 0.016	1
Tetracycline	4.00	8.00	8.00	16.00	32	ng	16.0c	8
Chloramphenicol	6.00	8.00	6.00	6.00	8	ng	2.0	4

nr: Not required; ng: no growth.
[a]European Food Safety Authority recommendation for *Lactobacillus plantarum/pentosus*.
[b]European Food Safety Authority recommendation for *Lactobacillus* obligate heterofermentative.
[c]Value above European Food Safety Authority recommendation.

kg^{-1} DM and the average content of soluble carbohydrate was 14.8 g kg^{-1} (Table 3). After 90 d of ensiling, the greatest decrease of silage DM occurred in the control silage prepared without the addition of inoculants (5.4%). The lowest decrease was observed for the silage treated with inoculant containing three *Lactobacillus* strains (LPK+LPC+LB) (4.0%). Alfalfa ensiled without bacterial inoculants was characterized by high pH (4.8), while the tested bacterial preparations had an important impact on pH reduction to 4.0-4.2. Also, other measured physicochemical parameters of alfalfa silage quality, like content of organic acids, amount of ethanol and ammonia N, were the least preferred for silages untreated with a *Lactobacillus* starter culture. In addition, selected strains influenced butyric acid fermentation, which was inhibited in silages treated with bacterial inoculants. In the case of inoculant LB, there was also a noticeable increase in the content of acetic acid (4.8 g kg^{-1}) and propionic acid (0.7 g kg^{-1}) in relation to the other variants of experiment (4.0-4.4 g kg^{-1} acetic acid and 0.4-0.5 g kg^{-1} propionic acid for silages prepared with LC, LK, and mixed inoculants). The addition of LAB starter cultures reduced the ammonia N content (from 97.0 g kg^{-1} in control alfalfa silage to 69.0-75.0 g kg^{-1} for inoculated silages) and increased the content of water-soluble carbohydrates (from 2.2 g kg^{-1} in control alfalfa silage to 3.5 g kg^{-1} for silage prepared with mixed inoculant and 5.2 for silage prepared with LPK inoculant) (Table 3).

The pH value of control silage was lower in all silages treated with bacterial inoculants (4.8 *vs*. 4.0-4.2) (Table 4). Aerobic stability in the control silage was the lowest (77 h) and doubled under the influence of bacterial inoculants. Moreover, the bacterial treated silages characterized by very varied aerobic stability, from 141 h for LC treatment to 175 h for LB treatment.

The results of the physicochemical analysis of ensiled plant material were in accordance with the characteristics of individual bacteria strains. As well as soluble carbohydrates, alfalfa contains starch, cellulose and xylans; therefore, bacteria capable of partial hydrolysis of these compounds may develop more intensively in ensiled plants. The selected bacterial strains have a unique capability to synthesize amylolytic enzymes (*L. plantarum* K KKP/593/p) and cellulolytic enzymes (such as the strain of *L. plantarum* C), and they are also capable of lactic acid synthesis from xylan (*L. plantarum* C KKP 788p) and the efficient synthesis of 1,2-propanediol as the strain of *L. buchneri* (Zielinska et al., 1995; 1998; 2006).

In control silages prepared without the addition of inoculants, the number of *Salmonella* sp., *Clostridium*

Table 3. Chemical composition of alfalfa silages untreated and treated with inoculants of *Lactobacillus* strains.

	Ensiled alfalfa				
		Treated with bacterial inoculants			
	Untreated control	*L. plantarum* K KKP 593 p (LPK)	*L. plantarum* C KKP 788 p (LPC)	*L. buchneri* KKP 907 p (LB)	Lactic acid bacteria mix (LPK+LPC+LB)
Dry matter, g kg^{-1}	278.2 ± 2.0a	281.4 ± 0.7a	280.7 ± 0.6a	280.6 ± 1.3a	282.5 ± 1.6a
pH	4.8 ± 0.1a	4.1 ± 0.1b	4.2 ± 0.1b	4.1 ± 0.1b	4.0 ± 0.1b
Concentration lactic acid, g kg^{-1}	8.2 ± 0.2a	17.5 ± 0.2d	15.2 ± 0.4c	13.3 ± 0.4b	16.5 ± 0.4d
Concentration volatile fatty acids, g kg^{-1}					
Acetic acid	3.8 ± 0.1ab	4.2 ± 0.1bc	4.4 ± 0.1a	4.8 ± 0.2c	4.0 ± 0.1abc
3-Hydroxybutyric acid	1.0 ± 0.2a	0.1 ± 0.0b	0.2 ± 0.0b	0.1 ± 0.1b	0.2 ± 0.1b
Propionic acid	0.2 ± 0.0a	0.5 ± 0.0b	0.4 ± 0.1ab	0.7 ± 0.1c	0.4 ± 0.0ab
Lactate/acetate ratio	2.2 ± 0.1a	4.1 ± 0.1c	3.3 ± 0.1c	2.8 ± 0.1b	4.1 ± 0.1c
Concentration 1,2-propanediol, mg kg^{-1}	No detectable a	92.0 ± 2.0b	84.0 ± 6.0b	526.0 ± 28.0d	230.0 ± 12.0c
Concentration alcohols (ethanol), g kg^{-1}	7.6 ± 0.2a	2.2 ± 0.3b	2.9 ± 0.1b	2.7 ± 0.6b	2.8 ± 0.8b
Concentration ammonia N, NH_3-N/TN, g kg^{-1}	97.0 ± 9.9a	69.0 ± 0.3b	75.0 ± 5.0b	73.0 ± 4.0ab	74.0 ± 5.0b
Concentration of water-soluble carbohydrates, g kg^{-1}	2.2 ± 0.2a	5.2 ± 0.2c	3.9 ± 0.2b	3.6 ± 0.1b	3.5 ± 0.3b

Distinct letters in the row indicate significant differences according to Tukey's test ($P \leq 0.05$).
± Standard deviation.

Table 4. Comparison of silages treated and untreated with bacterial inoculants according to aerobic stability, number of moulds, and number and genus of yeast.

		Ensiled alfalfa				
			Treated with bacterial inoculants			
	Raw alfalfa	Untreated control	*Lactobacillus plantarum* K KKP 593 p (LPK)	*L. plantarum* C KKP 788 p (LPC)	*L. buchneri* KKP 907 p (LB)	Lactic acid bacteria mix (LPK+LPC+LB)
Aerobic stability, h	-	77 ± 3a	157 ± 3c	141 ± 3b	175 ± 3d	155 ± 5c
Number of moulds, log CFU g^{-1}	4.40 ± 0.14a	4.89 ± 0.34a	1.02 ± 0.15b	1.56 ± 0.31b	0.86 ± 0.17b	1.53 ± 0.27b
Number of yeast, log CFU g^{-1}	1.37 ± 0.12ab	2.59 ± 0.78a	0.95 ± 0.26b	1.03 ± 0.17b	1.19 ± 0.13ab	0.77 ± 0.56b
Identified genus and yeast species	*Saccharomyces cerevisiae* *Candida pelliculosa*	*S. cerevisiae* *C. pelliculosa* *C. globosa* *C. zeylanoides*	*C. pelliculosa* *C. lambica*	*C. pelliculosa* *C. zeylanoides* *C. krusei*	*C. pelliculosa* *C. lambica*	*C. pelliculosa* *C. lambica*

Distinct letters in the row indicate significant differences according to Tukey's test ($P \leq 0.05$) ± Standard deviation.

perfringens and *E. coli* increased. The number of *Salmonella* sp., *C. perfringens* and *E. coli* in inoculated-treated silages were numerically lower than in control one, but no significant difference was found (Table 5). The number of *Listeria* sp. remained at a similar level in comparison to raw plant material, while the number of coliform bacteria was twice as low (5.42 log CFU g^{-1} for forage before ensiling and 2.40 log CFU g^{-1} for control silage). The application of bacterial inoculants in alfalfa ensiling resulted in a reduction in the total number of undesirable microorganisms ($P < 0.05$), including *Listeria* sp. and *C. perfringens*. Additionally, silage treatment with LPK, LB, and mix inoculants resulted in the elimination of pathogenic bacteria of the genus *Salmonella* and bacteria of the species *E. coli* below detectable levels (Table 5). Silages inoculated with LC preparation were contaminated with *Salmonella* sp. and *E. coli* to a small degree (0.23 and 0.22 log CFU g^{-1}, respectively). There were no significant differences observed regarding the efficiency of particular bacterial inoculants in inhibiting the tested pathogenic bacteria: *C. perfringens*, *Listeria* sp., and coliform bacteria.

Alfalfa silage prepared without the addition of inoculants was characterized by higher pH value, and higher amounts of moulds and yeasts than in the forage before ensiling, while under the action of the tested bacteria, there was a decrease ($P < 0.05$) in the number of those microorganisms (from 0.86 to 1.53 log CFU g^{-1} for moulds and from 0.77 to 1.19 log CFU g^{-1} for yeast in treated silages *vs*. 4.89 and 2.59 log CFU g^{-1} in the control, respectively). The most active in inhibiting the growth of mould and yeast strains proved to be LPK and LB inoculants, while in the case of yeast, a synergistic effect of the three strains in the mix inoculant was observed, but those differences were not significant (Table 4). The following yeast species were present in the forage before ensiling intended for ensiling as well as in control silages prepared without the addition of bacterial inoculants: *Saccharomyces cerevisiae* (yeast which ferment sugars) and *Candida* species including *C. pelliculosa* (Table 4). Meanwhile, the addition of bacterial inoculants to silage resulted in changes in yeast composition. In inoculated silages, yeasts of the genus *Candida* were identified, in particular of the species *C. pelliculosa* and *C. lambica*, both of which are able to ferment lactate.

DISCUSSION

Modern diets of ruminants contain major proportions of roughages on a DM basis. Roughage levels can vary between 30% and 100% in dairy diets and between 10% and 100% in beef diets. Processing roughages by the addition of microbial inoculants can optimize silage quality, and cause an immediate decrease in the pH to prevent growth of undesirable microorganisms; for that reason, the subject is worth considering (Danner et al., 2003; Nikkhah, 2013).

The precise characteristic of the strain is a key step in the selection of the optimal starter cultures. Identification at the intraspecies level is an important issue since it may help to distinguish strains with relevant properties and assess the genetic stability of starter cultures over time. Bacterial identification at the species level was performed by amplification of the 16S rDNA gene using standard PCR amplification conditions (data not shown). In the present study, all three strains were distinguished from each other by their RAPD profiles (Figure 1). RAPD-PCR analysis has been widely presented as an efficient and rapid tool for the evaluation of genetic diversity within lactobacilli strains which are not easy to differentiate using conventional methods (Aymerich et al., 2006; Markiewicz et al., 2010). It can also be used to monitor a selected starter culture in inoculated silage, to follow the growth, survival, and predominance of a strain (Plengvidhya et al., 2004).

Lactic acid bacteria has GRAS status (generally recognized as safe); however, the presence and expression of antibiotic resistance genes in LAB have been reported (Ouoba et al., 2008; Nawaz et al., 2011). Antibiotic resistance genes of lactic acid bacteria can potentially be transferred to the commensal flora of human and animals and to opportunistic and/or pathogenic bacteria. Therefore, it is very important to verify that LAB strains used in the preparation of fermented products which are not heat-treated prior to consumption (including silages) are free of acquired antimicrobial resistance properties and are

Table 5. Inhibition of undesirable bacteria in alfalfa silages treated and untreated with bacterial inoculants.

		Ensiled alfalfa				
			Treated with bacterial inoculants			
	Alfalfa before ensiling	Untreated control	*L. plantarum* K KKP 593 p (LPK)	*L. plantarum* C KKP 788 p (LPC)	*L. buchneri* KKP 907 p (LB)	Lactic acid bacteria mix (LPK+LPC+LB)
			Number of bacteria, log CFU g^{-1}			
Salmonella sp.	0.80 ± 0.58ab	1.26 ± 0.20a	0.0b	0.23 ± 0.20b	0.0b	0.0b
Escherichia coli	0.48 ± 0.34ab	1.08 ± 0.17a	0.0b	0.22 ± 0.31b	0.0b	0.0b
Coliform bacteria	5.42 ± 0.14a	2.40 ± 0.13b	0.62 ± 0.29c	0.88 ± 0.06c	0.92 ± 0.07c	0.66 ± 0.31c
Clostridium perfringens	1.43 ± 0.61ab	2.13 ± 0.15a	0.45 ± 0.10c	0.83 ± 0.04bc	0.31 ± 0.02c	0.36 ± 0.06c
Listeria sp.	3.61 ± 0.18a	3.46 ± 0.04a	0.73 ± 0.08b	1.16 ± 0.09b	1.01 ± 0.15b	0.87 ± 0.22b

Distinct letters in the row indicate significant differences according to Tukey's test ($P \leq 0.05$) ± Standard deviation.

thus safe for humans and animals (Klare et al., 2007). In the present study, the MIC values obtained were situated in the examined concentration range and in most cases did not exceed the breakpoints defined by the European Food Safety Authority (EFSA, Parma, Italy). The exception was tetracycline for *L. buchneri* KKP/907/p, with an MIC value of 16 μg mL^{-1}. Although this is one dilution above the EFSA breakpoint (8 μg mL^{-1}), this value is not considered to be of concern (EFSA FEEDAP, 2011). The obtained results confirmed that all tested bacterial strains can be safely used as technological additives for feed ensiling.

Microbial silage inoculants can alter many parameters of silages, but the strength of the effect of bacterial inoculants is dependent on strain characteristics. It could be seen that all of the tested bacterial inoculants had a positive effect on many physicochemical properties of silages; e.g. lactic acid content, volatile fatty acids content, ammonia N or carbohydrates concentrations, pH and storage stability. Observed effects were in accordance with other authors' results. Muck and Kung (1997) reported that microbial inoculation lowered pH, improved the lactic:acetic ratio, and lowered the ammonia N content in more than 60% of studies. Moreover, it was established that microbial inoculants can alter different aspects of silage fermentation such as volatile fatty acids concentrations, fiber digestibility or DM content (Muck and Kung, 1997). *Lactobacillus plantarum* inoculant preserved more true proteins during silage fermentation than control, which in turn increased *in vitro* ruminal microbial growth. The inoculant had lower concentrations of ammonia-N and alfalfa silage pH and no 3-hydroxy-butyric acid was detected in any of the silages (Contreras-Govea et al., 2013). Jatkauskas et al. (2013) reported that homofermentative and heterofermentative lactic acid bacteria inoculants (*L. buchneri*, *L. buchneri* in combination with *Enterococcus faecium* and *L. plantarum*, *E. faecium* in combination with *L. plantarum* and *Lactococcus lactis*) were able to reduce pH, formation of butyric acid, alcohols and ammonia-N in grass, clover-grass, alfalfa, and maize silages. It was observed that the aerobic stability of silages was improved ($P < 0.05$) (Jatkauskas et al., 2013).

Ohshima et al. (1997) presented the improvement of alfalfa silage quality by the addition of *L. casei* at 20 °C, but the more effective additive was shown to be previously fermented juice. It was quite understandable that in previously fermented juice there was a full composition of LAB strains with different features, which proved its effectiveness in alfalfa ensiling (Ohshima et al., 1997). This is an important conclusion that makes mixed bacterial starter cultures more attractive from technological point of view.

In well-packed silage forages and those prepared with good agricultural practice, oxygen is rapidly depleted and the activities of aerobic microorganisms are inhibited within a few days of ensiling (Levital et al., 2009). The microbiological quality of silage depends largely also on DM content of the plant. If is too low (below 20%), clostridia and *L. monocytogenes* bacteria may develop. In order to improve the quality of roughages, there is a need to eliminate contamination with the spoilage of *Clostridium* sp. and pathogenic bacteria such as *E. coli* and *Salmonella* sp., as well as moulds, which negatively affect the physicochemical quality of forage and the health and welfare of animals (Purwin et al., 2006).

The ability to inhibit the growth of pathogenic bacteria is represented by certain strains of the species, as follows: *Lactococcus lactis*, *Streptococcus lactis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. brevis*, *L. acidophilus*, or *L. buchneri* (Dimova, 2008). Inhibition of the growth of pathogenic bacteria, yeasts and moulds may be the result of the synergistic action of the produced metabolites: bacteriocins, lactic acid, acetic acid, hydrogen peroxide, lactate peroxidase, lysozyme, reuterin, and propylene glycol (Magnusson and Schnürer, 2005). Moreover, these abilities are strain-dependent. A key factor which decides that lactic acid bacteria inhibit the growth of undesirable bacteria in silage is the rapid generation of a low pH by production of organic acids, primarily lactic acid. Nevertheless, other factors may add to this.

The inhibition of undesirable microorganisms of *Salmonella* sp., *E. coli*, and coliform bacteria was confirmed under *in vitro* experiments for the mix inoculant which consisted of LK, LC, and LB starter cultures (Zielinska et al., 2011). According to the results in this paper, those abilities were also acknowledged in silo conditions for a mixed inoculant and for LC, LK, and LB inoculants separately. The use of a starter culture of *L. buchneri* was shown to inhibit the growth of yeast during ensiling (Ranjit and Kung, 2000; Oude Elferink et al., 2001), but in the present study, there was no difference ($P > 0.05$) among other tested inoculants (LC, LK, and mix) in comparison to LB inoculant. Importantly, LB treatment caused the greatest decrease in the number of moulds in alfalfa silage, but the reduction was not different ($P > 0.05$).

Bacteria of the order *Enterobacteriaceae* and genus *Clostridium* are introduced into soil through organic fertilizer. Thus introduced, the microbes multiply in the green fodder and in silages produced from it (Davies et al., 1996; Holley et al., 2006; You et al., 2006). Ruminants are recognized to be a major reservoir of Shiga toxin-producing *E. coli* (Duniere et al., 2011). The combination of proper ensiling techniques and the utilization of selected bacterial inoculants appears to represent a good strategy to guarantee nutritional qualities of cattle feed while at the same time limiting the entry of pathogenic bacteria e.g. *E. coli* into the epidemiological cycle to improve the microbial safety of the food chain (Duniere et al., 2011). The addition of three bacterial inoculants, *Propionibacterium* sp., *L. buchneri*, and *Leuconostoc*

mesenteroides (10^6 CFU g^{-1}), was evaluated by Duniere et al. (2011) for their abilities to control *E. coli* pathogen in corn silages. During ensiling, bacterial treatments did not significantly affect *E. coli* O26 elimination compared with the negative control, except for the *L. buchneri*, which showed *E. coli* strain-dependent inhibition (Duniere et al., 2011).

There can be found a comprehensive literature on pathogenic bacteria elimination (*Salmonella* sp. and *E. coli*) on alfalfa seeds by high pressure treatment (Hudaa et al., 2009). Unfortunately there are no studies on lowering the number of pathogenic bacteria in alfalfa silage by lactic acid bacteria application. Nevertheless the results presented in the study can be confirmed by similar experiments provided in meadow sward silage inoculated with the bacterial-mineral-vitamin, which consisted of the same lactobacilli strains. There were detected bacteria *Salmonella* sp. and *E. coli* accompanied with a high number of coliform bacteria in the silages prepared without the addition of the preparation, while in the silages made with addition of the preparation there were not present any bacteria of the genus *Salmonella*. The number of coliform bacteria and *Escherichia coli* was approximately 100-fold lower in comparison with the silages prepared without the additive (Zielinska et al., 2012).

CONCLUSIONS

Our study showed that it is important to identify and characterize industrial strains for individual features, because the effect of bacterial inoculants is dependent on strain characteristics and varies across the tested preparations. Among the many bacterial features, the most important from a technological point of view seems to be not only the efficiency of lactic acid and volatile fatty acids synthesis, the influence on aerobic stability, but also the antibiotic susceptibility of the strain, genetic stability and the ability to inhibit spoilage microorganisms.

Lactic acid bacteria strains in LK, LC, LB, and mix inoculants showed a positive influence on ensiling process and had effectively inhibited the growth of microorganisms prolonging the aerobic stability of silages. The most stable silage according to aerobic stability was silage treated with LB inoculant. In this silage, the highest concentrations of acetic acid, propionic acid, and 1,2-propanediol were reported. Number of yeast, moulds, *Salmonella* sp., *C. perfringens*, coliform bacteria, *E. coli* and *Listeria* sp. in inoculated-treated silages decreased in comparison to control one or in some cases those pathogens were eliminated. As microorganisms present in a starter culture can dominate the ensiling process and, for that reason, it is quite profitable to compose mixed inoculants consisting of several bacterial strains with different biotechnological features in appropriate proportions.

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