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DEVELOPMENT AND EFFECT OF A *LACTOBACILLUS PLANTARUM* INOCULANT ON QUALITY OF MAIZE GRAIN SILAGE

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Abstract: The main aim of these studies was the characterisation and identification of lactic acid (LAB) bacteria isolated from untreated silage, and the effect of selected bacteria (inoculant was called Silko for maize) on ensiling of maize high-moisture grain. Four isolates of LAB (L1, L2, L3 and L4) were characterised by the use of phenotypic assays and identified by phylogenetic analysis of 16S rRNA as *L. plantarum*. The fresh maize high-moisture grain was ensiled with a Silko for maize inoculant, inoculant available in the market (positive control) and no additive (untreated; negative control). After 60 days of ensiling, the results showed that the chemical composition and fermentation characteristics were better in treated silages with inoculants compared to the negative control. The contents of ash, fat and lactic acid (LA) were significantly higher in the silages treated with inoculants than in negative control. In comparison, the contents of cellulose, acid detergent fibre (ADF), neutral detergent fibre (NDF), NH_3-N/total nitrogen and butyric acids (BA) were considerably lower in silage treated with Silko for maize compared to the positive control. The Silko for maize improve nutritional value and fermentation of maize grain silage and is a competitive product on the market.

Keywords: maize, silage, inoculant, *Lactobacillus plantarum*, chemical composition, fermentation parameters

Introduction

Preparation of quality silage is crucial for the profitability of livestock farms because it is a source of food for the periods of the year when animal nutrition is inadequate in terms of quantity and quality. Maize is a vital crop for world farmers. Maize grain is used to satisfy the energy requirement of livestock. It can be ensiled and used as an animal feed ingredient. Ensiling of moist maize

grains has several advantages, such as savings on drying costs, the high nutritional value of silage, easy use. In the case of low maize grain prices on the market, the valorisation of maize is possible through animal products, and planning of the expected profit improves.

On the other hand, the preparation and storage of silage on farms is a significant problem, since it is necessary to maintain high-quality silage and achieve the maximum profitable production of milk and meat *(Aragón, 2012)*. Silage quality directly affects feed intake and utilisation of nutrients in ruminants, as well as on milk production *(Huhtanen et al., 2003)*. A decrease in pH prevented the loss of nutrients in silage due to higher lactic acid production *(Saarisalo et al., 2007)*. However, when silages are exposed to the air during the opening, it leads to increase the activity of undesirable microorganisms, and that causes the decrease of dry matter and quality *(Borreani et al., 2018)*. Also, during the ensiling process is very important that the forage mass more compacted and that the less oxygen remains in the silage. Accordingly, the inoculants are used to increase the level of lactic acid and the aerobic stability of silage over a more extended period after the opening of the silage. The use of silage inoculants (starter cultures) during silage provides a reduction in pH and the growth of undesired aerobic microorganisms *(Zielińska and Fabiszevska, 2018)*.

The biological additives that are used for conservation of silage include saprophytic, safe bacteria within the *Lactobacillus* sp, which use as consortium as multiple strains (more strains inside same species), or mixed strains including different species *(Jalč et al., 2009)*. In the world market, there are various silage bacteria inoculants for maize, among which are the frequently *Lactobacillus* sp. They are classified into two metabolic categories: homofermentative and heterofermentative bacteria *(Contreras-Gouveia and Muck, 2006)*. Homofermentative bacteria produce about 90% of lactic acid and belonging to various generations of *Lactobacillus* including the most well-known strains of *L. plantarum*. These bacteria dominate inoculants products in the world. One of the reason, because it is highly competitive with epiphytic lactic acid bacteria in silage, produces large amounts of lactic acid, reduces pH and nutritional losses *(Lynch et al., 2012; Đorđević et al., 2017)*. Also, it reduces ammonia nitrogen *(Queiroz et al., 2013)*. In general, silage treated *L. plantarum* has a better fermentation quality than in untreated silage *(Liu et al., 2016)*. However, according to *Muck (2013),* silage inoculants produced in cold regions may or may not be effective when used in hot regions.

The purpose of this study a display of development *L. plantarum* inoculant (Silko for maize) and to investigate the effect on the quality of maize grain silage. The first step in the experimental work was the isolation of a large number of bacteria from different silage samples, their phenotypic and genotypic identification at the level of the species, and prepares the bacterial consortium.

Materials and Methods

Procedure for isolation bacteria. For microbiological testing, each sample was taken, about 10 g silage in 300 ml of sterile Erlenmeyer bottle of 300 ml, and 90 ml of purified water was added and incubated with stirring at 120 rpm, 30 min *(Ekundayo, 2014)*. Subsequently, the serial decimal (from 10 times) dilution to 10^{-7} was prepared in sterile phosphate buffer, pH 7.2. After that, 100 µl of each dilution was smeared on the Man, Rogosa, Sharp (MRS) agar plate (Torlak, Serbia), and were cultivated at 37°C in the anaerobic jar (BioMerieux, France), during the 72 h. The separated colonies were inoculated on MRS agar. After cultivation, it was used for phenotypic and genotypic characterization and further checked for treatment silage. All bacterial isolates were stored at 5ºC±3ºC and subculture every two weeks. For long-term storage, stock from overnight cultures was prepared and frozen in cryoprotective agents 20% glycerol and stored at -80 °C.

Preliminary phenotypic characterization. Single, clearly separated colonies were used for morphological characterisation. Initially, preliminarily tested for Gram reaction by Gram staining and catalase enzyme. The following was done sporulation, the growth temperatures range (15°C, 30°C, 37°C and 45°C); in substrates with different osmotic pressure (2%, 4% and 6.5% (w/v) NaCl), growth in aerobic and anaerobic conditions in MRS broth, during the 72h. In the next step, selected isolates were tested by standard API 50CH test, according to the manufacturer's instructions (Bio-Merieux, Montalien-Vecien, France). All experiments were done in triplicate. The isolates were further checked for inoculant on ensiling of maize high-moisture grains.

Isolation of DNA and PCR identification. Total DNA isolation from *L. plantarum* was done with commercial kits according to the manufacturer's protocol (BIOLINE, United Kingdom). Isolated DNA from the samples was used to identify the bacteria. Using PCR, the 16s rRNA gene was amplified using universal primers 27f (AGAGTTTGATCMTGGCTCAG) and 1492 (TACGGYTACCTTGTTACGACTT). PCR reaction was carried out in a reaction mixture of 50 µl according to the manufacturer's protocol (Thermo Fisher Scientific, USA). The PCR reactions were carried out according to the following conditions:

1. One cycle of initial denaturation 95 ºC 5min,
2. 40 cycles of denaturation 95 ºC 30s, annealing 30s 53 ºC and elongation 72 ºC 1min,
3. One cycle of final extension 72 ºC 5min.

The PCR product was checked on 2% agarose gel using electrophoresis and then purified using a commercial kit (Zymo Research, Irvine, USA) and sent to

sequencing in 'MACROGEN' (Netherland) sequencing service. The sequence was bioinformatics processed using the Basic Local Alignment Search Tool (BLAST) on the NCBI.

Agar well diffusion methods. Agar-well diffusion method (AWD) was used *(Harris et al., 1989)* for detection of cross inhibition (antimicrobial activity) between alone strains of the genus *Lactobacillus*.

Preparation of silage. Maize hybrid ZP 684 (FAO 600 maturity group) was grown on a plot at the Research and Development Centre 'Agrounik' (44 ° 52 'N, 20 ° 05' E), Serbia during 2017. Preceding crop was winter wheat. Maize was sown on April 15. The sowing density was 60.000 plants ha^{-1}. The hybrid was harvested with a combine harvester in August when the grains had 26-32% moisture. The grains were ground in a mill to a 3-4 mm particle size. Approximately 100 kg was taken from the field and brought to the laboratory. Three treatments were used:

1. the untreated maize (negative control),
2. positive control (commercial inoculant added at 2 l t^{-1} of grain, contained the *L. plantarum* at total concentration 1 x 10^5 CFU g^{-1} of inoculant),
3. Silko for maize (number of colony-forming units in inoculant is 1 x 10^{10} CFU ml^{-1}; applied at a rate of 5 ml t^{-1} of grain). Maize was packed and compressed in polyethene containers volume 6l and covered with foil and a layer of sand.

Silage analysis. After 60 days of ensiling, about 450 g samples of the maize grain silage were taken from the containers for analysis. Standard methods according to *AOAC (2000)* were used to determine the contents of dry matter (DM), ash, crude fat (CF), crude protein (CP), acid detergent fibre (ADF) and neutral detergent fibre (NDF). Weende method was used to determine the content of cellulose, while method, according to *Licitra et al. (1996)* for the content of soluble nitrogen/total nitrogen. The content of NH_3-N/total nitrogen was determined using a Kjeltec System 1026. A gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) was used to determine the contents of lactic- (LA), acetic- (AA) and butyric acids (BA). The pH value was measured using an electronic digital pH meter (Hanna Instruments HI 83141 pH meter).

Statistical analysis. One-Way ANOVA was used in the analysis of experimental data, using Statistical software Statistica version 10 (StatSoft, Tulsa, Oklahoma, USA). The randomized complete block analysed the trial with three replicates. Tukey's test ($P \leq 0.05$) was used to compare the results.

Results

Preliminary phenotypic characterization. From 15 silage samples, 62 different colony morphologies were isolated. After the preliminary testing, 17 isolates of *Lactobacillus* and 2 *Pediococcus* were isolated. Four *Lactobacillus* isolates were denoted in laboratory collection bacteria as L1, L2, L3 and L4 and selected based on their high LA production. The morphological, cultural and physiological characteristics of the selected LAB performed in Table 1. According to its fermentative properties (carbohydrate substrate 49), all those which one tested *Lactobacillus* isolates showed the highest similarity to bacteria belonging to *L. planarum* / *L. pentosus*.

Table 1. Morphological, cultural and physiological characteristics selected LAB

Isolate	L1	L2	L3	L4
Gram staining	Gram-positive rods	Gram-positive rods	Gram-positive rods	Gram-positive rods
Colony morphology	White colonies convex, entire, opaque, diameter 3mm	White colonies convex, entire, opaque, diameter 3mm	White colonies convex, entire, opaque, diameter 2mm	White colonies convex, entire, opaque, diameter 3-4mm
Growth in anaerobic conditions	+	+	+	+
Growth in microaerophilic conditions	+	+	+	+
Production of gas from glucose	-	-	-	-
Catalase test	-	-	-	-
Growth temperature				
15 °C	+	+	+	+
30 °C	+	+	+	+
37 °C	+	+	+	+
40 °C	+	+	+	+
45 °C	-	-	-	-

Molecular identification. According to phenotypic and molecular characterisation (complete sequence16S rDNA isolate L1, L2, L3 and L4), the bacterial isolates were identified as *L. plantarum* and signed among our laboratory isolates as *L. plantarum* - L1, *L. plantarum* - L2, *L. plantarum* - L3 and *L. plantarum* - L4. Before forming a consortium of bacteria, we checked whether cross-inhibition occurs among the individual strains using the agar diffusion method. After 24 h cultivation bacteria, cross-inhibition between lactobacilli tested was not detected (Table 2).

Table 2. Testing of antagonism between selected strains *L. plantarum* by AWD methods

Indicator strain	Zone of inhibition growth (mm)			
	Test strain			
	L. p- L1	L. p- L2	L. p- L3	L. p- L4
L. p- L1	/	0	0	0
L. p- L2	0	/	0	0
L. p- L3	0	0	/	0
L. p- L4	0	0	0	/

Values are means of triplicate determinations with standard deviations; 0: do not zone inhibition growth; / do not test

Chemical composition of maize grain sample before ensiling. Chemical composition of maize grain sample before ensiling is shown in Table 3.

Table 3. Chemical composition of the maize sample before ensiling

Parameter	Control
Dry matter (DM) (g kg^{-1})	612.75
Ash (g kg^{-1} DM)	14.41
Crude fat (g kg^{-1} DM)	55.8
Crude protein (g kg^{-1} DM)	101.2
Cellulose (g kg^{-1} DM)	41.9
Acid detergent fibre (ADF) (g kg^{-1} DM)	39.1
Neutral detergent fibre (NDF) (g kg^{-1} DM)	240.0

Table 4. Chemical composition of maize by wet grain silage (untreated silage and silage treated with inoculants).

Parameter	Control	Positive control	Silko for maize	M	F test
Dry matter (DM) (g kg^{-1})	605.20	603.50	605.00	604.57	ns
Ash (g kg^{-1} DM)	9.33^{c}	10.29^{b}	11.29^{a}	10.30	**
Crude fat (g kg^{-1} DM)	36.39^{c}	38.96^{b}	41.08^{a}	38.81	**
Crude protein (g kg^{-1} DM)	86.44^{b}	91.51^{a}	89.53^{a}	89.16	*
Cellulose (g kg^{-1} DM)	40.96^{b}	40.34^{b}	39.38^{a}	40.23	*
Acid detergent fibre (ADF) (g kg^{-1} DM)	32.34^{c}	31.81^{b}	30.86^{a}	31.67	**
Neutral detergent fibre (NDF) (g kg^{-1} DM)	222.14^{b}	226.91^{b}	171.37^{a}	206.81	**

Distinct letters in the row indicate significant differences according to Tukey's test ($P \leq 0.05$); ** - significant at 1% level of probability, * - significant at 5% level of probability and ns - not significant.

Effect of the inoculants on maize grain silage quality. Results showed that the ash, crude fat and crude protein were significantly higher, while ADF was significantly lower in silages treated with bacteria inoculants than in control (Table

4). The cellulose and NDF were significantly lower in silage treated with Silko for maize compared to positive control and negative control. The dry matter did not differ among treatments.

Fermentation characteristics of silage are influenced by inoculants (Table 5). The values of pH, NH_3-N/total nitrogen, AC and BA were lower, while LA was higher in treatments with inoculants than in negative control. The pH and AA did not differ among positive control and Silko for maize.

Table 5. Fermentation characteristics of maize by wet grain silage (untreated silage and silage treated with inoculants).

Parameter	Control	Positive control	Silko for maize	M	F test
pH	4.27^{a}	3.77^{b}	3.83^{b}	3.96	**
NH_3 - N/TN (g kg^{-1} TN)	55.27^{a}	37.34^{b}	32.28^{c}	41.63	**
Lactic acid - LA (g kg^{-1} DM)	68.51^{c}	72.10^{b}	73.11^{a}	71.24	**
Acetic acid - AA (g kg^{-1} DM)	5.30^{b}	3.40^{a}	3.40^{a}	4.03	**
Butyric acid - BA (g kg^{-1} DM)	0.11^{c}	0.05^{b}	0.02^{a}	0.06	**

DM – dry matter; TN – total nitrogen; Distinct letters in the row indicate significant differences according to Tukey's test ($P \leq 0.05$), ** – significant at 1% level of probability.

Discussion

Based on LA production, four strains of *L. plantarum* have been selected. Due to the absence of cross-inhibition, they are selected as a consortium of bacteria. Also, strains of *Lactobacillus* belong to the GRAS (General Recognized as Safety). The use of bacterial inoculants in the initial phases of fermentation in grass silage, grass-clover, alfalfa and maize aims to decrease in pH to avoid the rapid growth of harmful microorganisms and losses of dry matter and increase aerobic silage stability *(Jatkauskas et al., 2013).* In generally, *L. plantarum* inoculants used in our study improve fermentation, promoting LA production, decrease pH, NH_3-N/total nitrogen, AA and BA acid contents in silage.

The higher ash and crude fat contents were recorded in silages treated with inoculants. The higher ash content is the result of the metabolism of inoculated strains of bacteria which using soluble components and thus increase the relative ash content *(Đorđević et al., 2017)*.

Crude protein is one of the most critical animal food quality parameters so it is crucial to maintain its high level in silage. Our results showed that the silages treated with *L. plantarum* inoculants have significantly higher crude protein content compared to control. According to *Abdul Rahman et al. (2017)*, addition of *L. plantarum* to silage increases crude protein content due to higher production of protein in the form of nitrogen content. The *L. plantarum* possess reductases that can reduce nitrates and nitrites to ammonia and other ammonia compounds *(Rooke and Hatfield, 2003)*. This contributes to the increase in total protein content,

although these compounds within total proteins mainly occur as non-protein nitrogen. Also, the low pH in treated silages inhibited protein degradation, as evidenced by research of *Vukmirović et al. (2011)*.

The lowest cellulose content was recorded in maize silage treated with Silko for maize. According to *Sadiya and Ibrahim (2015),* the *Lactobacillus* sp. produces enzymes for hydrolysis of cellulose. Therefore, we can assume that the strains of *L. plantarum* produce these enzymes, as indicated by the research of *Đorđević et al. (2017)*. Likewise, *Koc et al. (2009)* found the lowest cellulose content in sunflower silages treated with inoculant containing *L. plantarum* and *Enterococcus faecium*.

ADF and NDF were lowest in silage treated with Silko for maize. NDF did not differ between negative and positive controls. The Silko for maize increases digestibility and dry matter intake of silage and can be expected to will have a positive impact on animal performance because of the lower ADF and NDF levels in food increase animal productivity *(López et al., 2018)*.

The pH range 3.77-4.27 indicated of well-preserved silage. The inoculants promoted the silage acidification. The low pH preserves nutrients and promotes homofermentative lactic acid bacteria in silage *(Li et al., 2015)*. In essence, the low pH in silage reduced survival of yeasts, moulds and other undesirable silage microorganisms like Clostridia, prevent heating and spoilage silage and dry matter losses *(Ren et al., 2018)*.

The content of NH_3-N/total nitrogen was lowest in silage treated with Silko for maize. However, the NH_3-N content of treated and untreated silages was <100 g/kg N which suggests successful preservation. Therefore, proteins were degraded to a greater extent in untreated silage where the pH was higher than in silage treated with Silko for maize. Thus, we can be assumed that in silage treated with Silko for maize inoculant, is the higher protein content in an intact form which animals can be utilized directly. According to *Contreras-Govea et al. (2013)* silage treated with *L. plantarum* has more true protein than untreated silage, and has positive effects on milk production.

The studied silages have the higher content of LA and lower contents of AA and BA than reference values for high-quality silage (> 6.5%, < 3-4% and < 0.5%, respectively), indicating proper lactic acid fermentation. According to *Shaver (2003)*, good silage contains from 65 to 75% LA. In our research, all silages have satisfactory LA content. The highest LA and lower BA levels were recorded in silage treated with Silko for maize. The highest AA level was recorded in untreated silage. Generally, the increase in LA content in silage decreased pH and prevents secondary fermentation. The low pH inhibits clostridia growth, resulting in lower BA content in investigated silages. Consistent with our findings, *Muck (2013)* concluded that the homolactic bacteria have greater efficiency of glucose utilisation, produced higher LA in silage, reduced pH, and thus prevent undesirable microbes. According to *Kung and Shaver (2001)*, the ratio of LA to AA in the

silage should be of more than 3:1, indicating the excellent fermentation. In our case, the addition of inoculants increased this ratio compared to control silage and indicated very good fermentation.

Conclusions

Addition of Silko for maize inoculant increased LA concentration and decreased ADF and NDF, NH_3-N/total nitrogen and BA concentration compared to positive control and negative control at day 60 of fermentation. Accordingly, a consortium of bacteria belongs to *L. plantarum* to improve fermentation and preserve the nutritional value of maize grain silage.

Razvoj i uticaj *Lactobacillus plantarum* inokulanta na kvalitet silaže od zrna kukuruza

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Rezime

Cilj ovih istraživanja bio je karakterizacija i identifikacija bakterija mlečne kiseline (BMK) izolovanih iz netretirane silaže, kao i efekat odabranih bakterija (inokulant nazvan Silko za kukuruz) na siliranje vlažnog zrna kukuruza. Četiri izolata BMK (L1, L2, L3 i L4) su okarakterisani upotrebom fenotipskih testova i identifikovani filogenetskom analizom 16S rRNA kao *L. plantarum*. Vlažno zrno kukuruza silirano je sa Silkom za kukuruz, inokulantom koji je dostupan na tržištu (pozitivna kontrola) i bez primene inokulanta (netretirana; negativna kontrola). Nakon 60 dana od siliranja, rezultati su pokazali da su hemijski sastav i fermentacione karakteristike bolji u silažama tretiranim sa inokulantima u poređenju sa negativnom kontrolom. Sadržaj pepela, masti i mlečne kiseline bio je značajno veći u silažama tretiranim sa inokulantima nego u negativnoj kontroli. Sadržaj celuloze, kiselih (ADF) i neutralnih deterdžentskih vlakana (NDF), amonijačnog azota u ukupnom azotu i buterne kiseline (BA) bio je značajno niži u silaži tretiranoj sa Silkom za kukuruz nego u pozitivnoj kontroli. Silko za kukuruz poboljšava hranjivu vrednost i fermentaciju silaže od zrna kukuruza i predstavlja konkurentan proizvod na tržištu.

Ključne reči: kukuruz, silaža, inokulant, *Lactobacillus plantarum*, hemijski sastav, fermentacione karakteristike

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