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THE EFFECT OF INOCULATION WITH *LACTOBACILLUS* SPP. FOR SELECTED PARAMETERS OF MAIZE SILAGE*

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Abstract

The aim of the study was to investigate the effect of commercial bacterial inoculant CL (Lactobacillus plantarum K KKP/593/p, L. plantarum C KKP/788/p, Lactobacillus brevis KKP 839, Lactobacillus buchneri KKP 907) and M3 (L. buchneri ATCC 4005, Lactobacillus diolivorans LGM 19667, Lactobacillus reuteri ATCC 23272) on selected maize silage parameters. The tested silage was prepared in mini-laboratory silos. The basic composition of the feed was determined by the AOAC method. Aerobic stability was measured as the time required to raise the silage temperature by ≥2°C compared to ambient temperature. The concentration of 1-propanol and 1,2-propanediol in silage was determined using gas chromatography. On the basis of the obtained results, it was found that during 120 days of ensiling, the preparations used significantly ($p<0.05$) reduced the pH, dry matter and water-soluble sugars compared to control silage. The concentration of lactic, acetic and propionic acids in the control samples was significantly lower compared to the samples treated with inoculant CL and M3. The addition of CL and M3 caused a significant increase in the concentration of 1,2-propanediol and 1-propanol in silage, the presence of these compounds was not found in control silage. In addition, the modifiers used reduced the number of mould and yeast fungi and increased the number of lactic bacteria in the silages, as well as prolonged their aerobic stability compared to the control.

Keywords: bacterial inoculants, maize, silage

Introduction

Maize is one of the commonly used silage crops because it produces high yield of starch and dry matter per hectare at low cost (Wilkinson and Rinnie, 2018). The nutritional value of corn silage depends on many factors, the most important of which is the ripeness phase during

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the harvest (Camarasa et al., 2021). Johnson et al. (2002) noted that for the proper maize silage process, the dry matter content should be within 35%, and the main goal in silage production should be to obtain high-quality feed obtained by fermentation by lactic acid bacteria. Bacteria of the genus Lactobacillus are the most numerous group of lactic acid bacteria. They can be widely used as microbiological modifiers to improve the chemical composition of silage (Selwet, 2021). Inoculation with *Lactobacillus* spp. bacteria can affect changes in dry matter content and improve the hygienic quality of silage, which is considered to be an indicator of the abundance of bacteria, molud and yeast (Dorszewski and Grabowicz, 2017). In the initial phase of ensiling, water-soluble sugars are fermented (mainly to lactic acid), lactic homofermentation bacteria acidify the environment relatively quickly, preventing the development of undesirable microorganisms, mainly bacteria of the genus *Clostridium* spp., responsible for butyric fermentation. However, when lactic heterofermentation bacteria metabolize carbohydrates, not only lactic acid is formed, but also short-chain fatty acids, such as acetic and propionic acids, which inhibit the growth of fungi that can cause silage spoilage, especially after the silo is opened (Selvet, 2021). Therefore, bacterial inoculants containing heterofermentation bacteria, such as *Lactobacillus buchneri (Nasci-mento Agarussi* et al., 2022), have been developed to improve the aerobic stability of silage. It was found that *L. buchneri* can metabolize lactic acid to acetic acid and 1,2-propanediol under anaerobic conditions. It has also been shown that 1,2-propanediol as an intermediate metabolite can be transformed to 1-propanol and propionic acid by the species *Lactobacillus diolivorans* (Choińska et al., 2013). This is a very important metabolic pathway, because both acetic and propionic acid have antifungal effects. In addition, another strain of bacteria *Lactobacillus reuteri* has the ability to synthesize cobalamin (vitamin B12), which is a coenzyme of propanediol dehydratase, which converts 1,2-propa-nodiol to 1-propanol and propionic acid (Toraya, 2011). Thus, in order to obtain high-quality silage, the phenomenon of co-fermentation of various substrates by bacteria of the genus *Lactobacillus* may induce the synthesis of desirable metabolites, such as acetic acid, propionic acid and 1,2-propanediol (Selvet, 2021). Therefore, further research is needed on the use of new microorganisms in silage, especially those with antimicrobial activity. They can have a significant impact on fermentation parameters and prolongation of aerobic stability, and thus contribute to improving the quality of the silage obtained (Nascimento Agarussi et al., 2022). The results presented in this work are a continuation of previous research conducted in our laboratory on two varieties of maize: Kresowiak (FAO 240) and San (FAO 240). The aim of the study was to compare the effect of a commercial bacterial preparation (CL) containing four strains of bacteria of the genus *Lactobacillus* and a non-commercial mixture (M3) of three *Lactobacillus* strains on the concentration of 1,2-propanediol and 1-propanol, the chemical composition and population of lactic acid bacteria, yeast fungi and mould in corn silage. The study also focused on determining the synergistic effectiveness of *Lactobacillus* bacterial mixtures on extending the time of aerobic stability of silage.

Material and methods

Plant material

Corn (Zea mays L.) of the Fortop variety (*FAO 230)* came from Hodowla Roślin Smolice Sp. z o.o. IHAR Group. Trilinear maize (TC), semi flint grain type. The plant density was 95,000 ha-1. Harvesting was carried out in the BBCH 75 phase (full milk maturity). The dry matter content was 38%. The maize was cut at a height of 30 cm and chopped into 6 mm long chaff before ensiling. The cultivation of maize was carried out in monoculture.

Bacterial inoculants

CL – (commercial preparation) contained cultures *L. plantarum* K KKP/593/p, *L. plantarum* C KKP/788/p, *L. brevis* KKP 839 and *L. buchneri* KKP 907. The dose recommended by the manufacturer was 5 g t-1 of ensiled material. The concentration of bacteria in 1 g of the preparation was 10^9 cfu g-1 (Lactosil, Polsil).

M3 – (mixture of 3 cultures) *L. buchneri* ATCC 4005, *L. diolivorans* LGM 19667, *L. reuteri* ATCC 23272 (DSMZ). A dose of 5 ml^{t-1} of ensiled material was used. The concentration of bacteria in 1 ml of the mixture was 10^9 cfu g-1.

Silage method

The silage was prepared in laboratory mini-silos with a capacity of 5 dm^3, made of PVC, with a closure allowing the removal of gas products (the number of repeats for individual combinations was 10). The room temperature during ensiling was on average 20°C ± 2°C. The ensiling time was 120 days.

Microbiological Analysis

The number of lactic bacteria was determined on MRS Agar (Oxoid) in accordance with PN-EN 15787:2022-04. Incubation time 72 h at 37° in conditions with reduced oxygen content. Yeast fungi were cultured on YPD agar medium (Sigma), (YPD, 2006) and mould fungi were determined on OGYE Agar (Oxoid) (Mossel et al., 1970) with oxytetracycline (oxytetracycline-glucose-yeast-
-extract agar). The incubation time was 5–7 days at 24°C.

Chemical Analysis

The concentration of lactic, acetic, propionic acid, ethanol and 1-propanol and 1,2-propanediol was determined using a gas chromatograph equipped with a FID detector, a 2 m long 80/100 Chromosorb WAW (SUPELCO) glass column. 2 mm with GP 10% SP – 1200/1% H_3PO_4 filling and Varian 8200 CX auto-sampler. The carrier gas was hydrogen (flow 30 cm^3 min-1), furnace temperature 120°C, injection temperature 250°C, detector temperature 300°C. Fluka acid standards were standard. The basic composition of the feed was determined in accordance with AOAC (2019).

The pH values were determined using the pH Meter Hann Instruments in suspension prepared from 10 g of silage and 190 cm^3 of distilled water, homogenized for 20 minutes. To determine the dry matter content, silage samples were dried at 65°C for a period of 48h (GZX-9140MBE, Shanghai Boxun Co., Ltd., Shanghai). Crude protein was determined by the Kjeldahl method (Kjeltec 2300 AutoAna- lyzer, FOSS Analytical AB, Hoganas, Sweden). The concentration of water-soluble sugars was determined according to the methodology given by Murphy (2010).

Oxygen stability test

During the aerobic stability test, the test samples were aerated for a period of 180 h at 20 °C ± 2 °C. After this period, changes in the number of microorganisms and selected chemical parameters of silage were studied. Moist samples weighing 100 g were taken after 120 days of ensiling from mini-silos and placed in plastic containers with a volume of 500cm3 with holes with a diameter of 10 mm for air circulation. Temperature changes were measured every 5 minutes at two-hour intervals using a temperature reader (Hotmux DDC Corporation, Pennsauken, NJ, USA). Stability was defined as the time it takes to raise the silage temperature by ≥2°C relative to the ambient temperature. Each combination was performed in 5 repetitions, a total of 15 measurements were made.

Statistical analysis

Statistical calculations were performed using the GLM SAS (2002) package of procedures. The normality of the distribution was checked by the Shapiro-Wolf test. Differences between means were tested using the Tukey test at significance level α=0.05.

Results

Table 1 shows the chemical composition and a number of lactic acid bacteria, yeast fungi and mould fungi in the green mass of maize intended for silage. The content of water-soluble sugars at the level of 90.2 g kg^{-1} dry matter allowed to consider the plant material as good for ensiling.

Table 2 shows the chemical composition and abundance of microorganisms after 120 days of ensiling. Silages treated with bacterial inoculants CL and M3 had significantly lower concentrations of dry matter and water-soluble carbohydrates ($p<0.05$) compared to control samples. Silage treated with modificators containing *Lactobacillus* bacteria (CL, M3) was also characterized by a lower pH. No significant effect of M3 and CL inoculants on changes in crude protein content in the examined silage was demonstrated. The concentration of lactic, acetic and propionic acid was significantly higher in samples treated with microbial inoculants (CL, M3). However, the content of propionic acid was significantly higher in samples treated with M3. No 1,2-propanediol and 1-propanol were found in the control silage. CL and M3 additives

resulted in a significant increase in the concentrations of these substances in experimental silage. Silage samples treated with inoculant M3 were characterized by a marked increase in the concentration of 1,2-propanediol and 1-propanol. Samples with the addition of M3 contained 202% higher concentrations of 1,2-propanediol and 143% higher concentrations of 1-propanol. The mixtures of *Lactobacillus* strains (CL, M3) used in the study resulted in a significant increase in the number of lactic acid bacteria by 27.2% and 28.7% compared to the control and a decrease in the population of yeast fungi relatively by 27.9% and 28.5%. No significant effect of CL inoculation on the overall abundance of mould fungi was observed compared to the control, while the use of M3 reduced the abundance of mould fungi by 23.6%. Table 3 shows the changes in silage chemical composition, microbial counts and the results of the aerobic stability test. A significant effect of the applied CL and M3 preparations on the reduction of silage pH values compared to the control was demonstrated. The concentration of lactic, acetic and propionic acid in samples containing *Lactobacillus* strains was significantly higher than in the control sample. Silage with the addition of M3 preparation was characterized by a significantly higher concentration of these acids compared to samples inoculated with preparation CL. The control samples did not contain 1,2-propanediol and 1-propanol. In combinations of CL and M3, the concentration of these substances was lower than before the aerobic stability test. The samples containing CL showed a reduction of these substances by 40.3 per cent and 59.5 per cent, respectively, and those with M3 by 6.4 per cent and 8.9 per cent. The abundance of lactic acid bacteria in silage containing CL and M3 additives was 45.7% and 47.3% higher, respectively, compared to the control sample. On the other hand, the number of yeast fungi decreased significantly by 18.3% and 31.5% respectively. M3 significantly reduced the number of mould fungi (28.3%). CL and M3 prolonged the aerobic stability of silage by 122 and 179 h, respectively, compared to the control.

Table 1. Dry matter content, chemical composition, pH, and the number of lactic acid bacteria, yeast-like fungi and mold fungi in maize before ensiling

Sucha masa (g kg^{-1}) Dry matter (g kg^{-1})	392
pH	5,45
Białko surowe (g kg^{-1} s.m.) Crude protein (g kg^{-1} DM)	80,12
Cukry rozpuszczalne w wodzie (g kg^{-1} s.m.) Water-soluble carbohydrates (g kg^{-1} DM)	90,2
Bakterie kwasu mlekowego (log jtk g^{-1}) Lactic acid bacteria (log CFU g^{-1})	6,27
Grzyby drożdżoidalne (log jtk g^{-1}) Yeast-like fungi (log CFU g^{-1})	7,28
Grzyby pleśniowe (log jtk g^{-1}) Mold fungi (log CFU g^{-1})	5,89

Table 2. Characteristics of maize silage at 120 days of fermentation

Parameters	Treatments					
	Control	SEM	CL	SEM	M3	SEM
Sucha masa(g kg^{-1}) Dry matter (g kg^{-1})	387.00 a	1.43	354.00 b	1.29	362.00 b	1.20
pH	4.87 a	0.04	4.04 b	0.09	4.00 b	0.09
Białko surowe (g kg^{-} s.m.) Crude protein (g kg^{-1} DM)	77.80 a	0.71	78.01 a	0.53	78.04 a	0.53
Cukry rozpuszczalne w wodzie (g kg^{-1} s.m.) Water-soluble carbohydrates (g kg^{-1} DM)	53.90 a	3.88	48.20 b	3.12	47.80 b	2.41
Kwas mlekowy (% s.m.) Lactic acid (% DM)	5.21 a	0.49	6.71 b	0.45	7.12 c	0.36
Kwas octowy (% s.m.) Acetic acid (% DM)	1.15 a	0.04	2.81 b	0.04	3.21 c	0.04
Kwas propionowy (% s.m.) Propionic acid (% DM)	0.00 c	…	1.12 b	0.04	1.73 a	0.04
1,2-Propanodiol (% s.m.) 1,2-Propanediol (% DM)	0.00 c	…	0.57 b	0.09	1.72 a	0.04
1-Propanol (% s.m.) 1-Propanol (% DM)	0.00 c	…	0.37 b	0.01	0.90 a	0.01
Etanol (% s.m.) Ethanol (% DM)	0.79 a	0.04	0.81 a	0.004	0.80 a	0.004
Bakterie kwasu mlekowego (log jtk g^{-1}) Lactic acid bacteria (log CFU g^{-1})	7.01 a	0.62	8.92 b	0.18	9.02 b	0.18
Grzyby drożdżoidalne (log jtk g^{-1}) Yeast-like fungi (log CFU g^{-1})	5.73 a	0.58	4.13 b	0.31	4.10 b	0.22
Grzyby pleśniowe (log jtk g^{-1}) Mold fungi (log CFU g^{-1})	5.33 a	0.36	5.42 a	0.18	4.07 b	0.13

*Średnie oznaczone różnymi literami w rzędzie różnią się istotnie statystycznie (p<0,05). SEM – błąd standardowy średniej. CL – *L. plantarum* K KKP/593/p, *L. plantarum* C KKP/788/p, *L. brevis KKP 839, L. buchneri KKP 907; M3 – L. buchneri ATCC 4005, L. diolivorans LGM 19667, L. reuteri ATCC 23272*.

*Means with different letters in a row differ significantly (p<0.05). SEM – standard error of the mean. CL – *L. plantarum K KKP/593/p, L. plantarum* C KKP/788/p, L. brevis KKP 839, *L. buchneri* KKP 907; M3 – *L. buchneri ATCC 4005, L. diolivorans LGM 19667, L. reuteri* ATCC 23272 .

Table 3. Characteristics of maize silage after the aerobic stability test

Parameters	Treatments					
	Control	SEM	CL	SEM	M3	SEM
pH	6.15 a	0.13	4.78 b	0.04	4.51 b	0.04
Kwas mlekowy (% s.m.) Lactic acid (% DM)	4.71 a	0.18	5.70 b	0.27	6.41 c	0.22
Kwas octowy (% s.m.) Acetic acid (% DM)	1.29 a	0.09	3.31 b	0.13	4.12 c	0.31
Kwas propionowy (% s.m.) Propionic acid (% DM)	0.00 c	…	0.91 b	0.31	1.31 a	0.27
1,2-Propanodiol (% s.m.) 1,2-Propanediol (% DM)	0.00 c	…	0.34 b	0.04	1.61 a	0.13
1-Propanol (%s.m.) 1-Propanol (% DM)	0.00 c	…	0.15 b	0.04	0.82 a	0.04
Bakterie kwasu mlekowego (log jtk g^{-1}) Lactic acid bacteria (log CFU g-	5.71 a	0.03	8.32 b	0.04	8.41 b	0.05
Grzyby drożdżoidalne (log jtk g^{-1}) 1) Yeast-like fungi (log CFU g-	7.47 a	0.04	6.10 b	0.05	5.12 c	0.05
Grzyby pleśniowe (log jtk g^{-1}) 1) Mold fungi (log CFU g-1)	6.99 a	0.09	6.43 a	0.08	5.01 c	0.40
Stabilność tlenowa (h) Aerobic stability (h)	48 a	0.04	170 b	0.01	227 c	0.09

*Means with different letters in a row differ significantly ($p<0.05$). SEM – standard error of the mean. CL – *L. plantarum K KKP/593/p, L. plantarum* C KKP/788/p, L. brevis KKP 839, *L. buchneri* KKP 907; M3 – *L. buchneri ATCC 4005, L. diolivorans LGM 19667, L. reuteri* ATCC 23272 .

Discussion

The aerobic stability of maize silage can be improved by using different strains of bacteria of the genus *Lactobacillus*. The presented results compared the action of the commercially available CL preparation with the action of the preparation (M3) containing strains of *lactic heterofermentation bacteria L. diolivorans, L. buchneri* and *L. reuteri.* Rezende et al. (2011) observed that silage exposed to oxygen underwent significant changes in chemical composition and showed a tendency to heat up faster. The synergistic effect of a combination of different strains of bacteria of the genus *Lactobacillus* on improving the stability of silage can be read in the works of many authors (Zielińska et al., 2015; Muck et al., 2017; Selvet, 2021). The ability of these bacteria to synthesise 1,2-propanediol, which the new *L. buchneri* strain KPP 907 p exhibits, is considered to be a very important characteristic (Zielińska et al., 2014; Zielińska et al., 2017). Strains of *L. diolivorans* sp. nov. capable of metabolising 1,2-propanediol can also be isolated from maize silages (Charley and Kung Jr, 2005), as well as strains conducting cobalamin synthesis, such as *L. reuteri* (Hammes and Hertel, 2009; Toraya, 2011; Sun et al., 2014). In the study of the chemical composition of maize silage samples after inoculation it with *L. buchneri, L. plantarum* and *Lactobacillus rhamnosus* Driehuis et al. (2001), Jungbluth et al. (2017) and Selvet (2021) reported an increase in acetic acid and 1,2-propanediol. This fact is confirmed by the results presented in this work. However, it should be borne in mind that an excessive concentration of acetic acid can negatively affect the palatability of silage and reduce the uptake of this feed by animals. Oliveira et al. (2017) noted different results and found a decrease ($p<0.01$) in acetic acid content and an increase in lactic acid concentration in maize silage samples with the addition of Lactobacillus bacterial strains. Oliveira et al. (2017) and Selvet (2021) noted that treatment of maize silage with a single strain or mixture of *L. plantarum, L. rhamnosus, Pediococcus pentosaceus, L. brevis, L. buchneri, L. diolivorans, L. reuteri* significantly reduced the pH and concentration of water-soluble sugars. This fact is confirmed by the results of current own research. Oliveira et al. (2017) and Selwet (2021) also noted that the inoculation effect may largely depend on the type and stage of development (BBCH) of the ensiled plant. The bacterial inoculants used in my own study did not affect changes in crude protein concentration in silage. Different results were obtained by Silva et al. (2014), who after using two strains of *L. buchneri* in maize silage recorded an increase in crude protein concentration compared to the control sample. Bumbieris et al. (2017) using *L. buchneri* CCT 3746 determined crude protein concentrations of 7.47% in inoculated samples and 6.87% in control samples. The results of own research concerning the aerobic stability test showed an increase in the concentration of acetic acid in samples with microbiological additives (the number of bacteria in 1 g of the preparation was 10^9 cfu g-1). Similar results were obtained by Basso et al. (2012) after inoculation of maize silage with *L. buchneri* strain 40788 9 (bacterial concentration in 1 g of the preparation was 10^5 cfu g^{-1}). Ranjit and Kung Jr (2000) noted that during aerobic exposure of maize silages, acetic acid production by *Lactocacillus* bacteria continues steadily while lactic acid concentration decreases.

In such silage, a constant decrease in pH is noted, because acetic acid has higher pKa values than lactic acid (Choińska et al., 2013). The results of own research show a clear effect of the CL and M3 preparations used on the reduction of the population of yeast-like fungi and mould fungi both in fresh silage and those subjected to the oxygen exposure. The M3 modifier showed a stronger fungistatic effect. Fabiszewska et al. (2019) noted that the production of fungistatic substances, including acetic acid and propionic acid, can largely depend on the growth phase of the bacteria, the temperature of the medium, the chemical composition and the pH of the environment.

Conclusions

On the basis of the obtained test results, it can be concluded that silage inoculated with strains of bacteria of the genus Lactobacillus was characterized by prolonged oxygen stability compared to control silage. M3 strains (*L. buchneri* ATCC 4005, *L. diolivorans* LGM 19667, *L. reuteri* ATCC 23272) showed a stronger stabilising effect than the commercial CL preparation (*L. plantarum* K KKP/593/p, *L. plantarum* C KKP/788 /p, *L. brevis* KKP 839, *L. buchneri* KKP 907). Silage samples with the addition of M3 preparation contained higher acetic and propionic acid concentrations and lower pH than the control samples, as well as lower numbers of yeast-like fungi and mould fungi. Silage inoculated with heterofermentation strains of *Lactobacillus* (M3) contained higher concentrations of 1,2-propanediol and 1-propanol compared to the control silage. The mixture of M3 strains, compared to CL, seems to be a more effective bacterial inoculant, which can more effectively improve the aerobic stability of silage.

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THE EFFECT OF INOCULATION WITH *LACTOBACILLUS* SPP. ON SELECTED PARAMETERS OF MAIZE SILAGE

SUMMARY

The study aimed to investigate the effect of a commercial bacterial inoculant CL (Lactobacillus plantarum K KKP/593/p, L. plantarum C KKP/788/p, Lactobacillus brevis KKP 839, Lactobacillus buchneri KKP 907) and an M3 preparation (*L. buch- neri* ATCC 4005, Lactobacillus diolivorans LGM 19667, *Lactobacillus reuteri* ATCC 23272) on selected parameters of maize silage. The silage tested in the study was prepared in laboratory mini-silos. The basic composition of the feed was determined using the AOAC method. Oxygen stability was measured as the time needed to in- crease the silage temperature by ≥2°C of the ambient temperature. The concentration of 1-propanol and 1,2-propanediol in silage was estimated using a gas chromatograph. The results indicated that during 120 days of ensilage, the applied preparations significantly (p<0.05) reduced the pH and the content of dry matter, and water-soluble sugars in silages compared to the control silage. The concentration of lactic, acetic, and propionic acids in the control samples was significantly lower compared to the samples containing CL and M3. The addition of CL and M3 caused a significant increase in the concentration of 1,2-propanediol and 1-propanol in the silages, whereas these compounds were not found in the control silage. Furthermore, the applied inoculants reduced the number of mould fungi and yeast-like fungi and increased the number of lactic bacteria in the silages and also extended their aerobic stability compared to the control.

Keywords: bacterial inoculant, maize silage