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BACTERIAL INOCULANT EFFECT ON QUALITY OF ALFALFA SILAGE AND HYALAGE

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Abstract: Using of silage and haylage of forage legumes in ruminant nutrition and promotion of promoting proper forage conservation techniques should be an important strategy in livestock production in our country. Forage legumes are difficult to ensile, so it is necessary to apply the starter culture of selected strains of lactic acid bacteria that support the ensiling process and prevent bacterial butyric fermentation and thus contribute to the preservation and improvement of silage and haylage quality. In this paper, the influence of bacterial inoculant 'Silko for alfalfa' on the quality of silage and haylage of alfalfa in two separate trials is presented. The inoculant is a combination of homofermentative lactic bacteria *Lactobacillus plantarum* and *Pediococcus* spp. The first-cut alfalfa in the second year was used for silage and haylage. The silage was examined in mini-silos in the laboratory, and the haylage at the cattle farm where the plant material was cuts were collected in experimental silo bags. The treatments were control (untreated silage, i.e. haylage) and silage, i.e. haylage treated with inoculant 'Silko for alfalfa' (rate of 5 ml t^{-1} fresh material). The silages were analyzed after 90 days, and haylage after 40. The inoculant 'Silko for alfalfa' has been found to maintain the nutritive value of silage and haylage and to improve their chemical, energy and fermentation parameters relative to the control. Since 'Silko for alfalfa' positively affects the correct lactic acid fermentation of silage and haylage and contributes to a lesser loss of nutritional value and energy it is expected that it can promote a high level of productivity of ruminants, and thus contribute to the growth of profit in livestock production.

Key words: alfalfa, bacterial inoculant, haylage, silage, quality

Introduction

Profitability of dairy and fattening cattle farms depends significantly on the preparation and use of high quality silage and haylage in combination with a concentrate. In Serbia, legume hay is a widespread form of feed, while silage and haylage are less represented. In our country silage and haylage are used in season when there is no plant production, that is, in the winter and in the early spring when there is no production of feed in the fields, meadows and pastures. Alfalfa is a useful source of protein essential for the nutrition of domestic animals, especially ruminants with high levels of lysine and tryptophan, which are deficient in maize. It is rich in vitamins, primarily carotene and has a high content of mineral substances, especially calcium. Silage and haylage of alfalfa are a very important for combining with maize. In addition to high nutritional value, alfalfa is a very economical plant species due to high yields of green mass and hay, which in the second year of life with 3-4 cuts can be up to 100 t ha^{-1} of green mass and 20 t ha^{-1} hay.

However, alfalfa is difficult to ensile due to the low content of water-soluble carbohydrates and moisture and high buffering capacity of the fresh mass. Because it does not contain the required sugar minimum for successful lactic acid fermentation, it is necessary to apply chemical or bacterial inoculants in the conservation of alfalfa *(Repetto et al., 2011)*. Otherwise, untreated silage accelerates the activity of *Clostridium butyricum* that uses existing sugars for its activity which leads to produce small amounts of lactic acid and increase in the content of butyric acid and intense degradation of proteins and amino acids *(Pys et al., 2002)*. Epiphytic lactic acid bacteria (<1% microflora) are not sufficient for rapid stimulation of the ensiling process, so it is necessary to apply the starter culture of selected strains of lactic acid bacteria that support the ensiling process and prevent the loss of dry matter and butyric fermentation *(Schmidt et al., 2009)*. Various bacterial inoculants that contain homofermentative microorganisms in a monoculture or a combination of several species, strains of homofermentative and heterofermentative bacteria and bacterial-enzymatic additives are available on the world market *(Đorđević et al., 2009)*. Lactic acid bacteria comprise a heterogeneous group of Gram-positive, non-spore forming, catalase negative microorganisms that synthesize lactic acid as the main product of fermentative metabolism. They belong to the genera *Lactobacillus, Pediococcus, Lactococcus, Enterococcus, Streptococcus* and *Leuconostoc (Pahlow et al., 2003)*. *Jatkauskas et al. (2013)* point out that the use of bacterial and bacterial-enzymatic inoculants is necessary in the ensiling of alfalfa, grass-clover mixtures, grasses and maize as they contribute to faster decrease in pH, inhibit growth of harmful microorganisms, prevent loss of dry matter and increase the aerobic stability of silage. *Kizilsimsek et al. (2007)* find that alfalfa silage treated with a combination of three homofermentative bacteria *Lactobacillus lactis*, *Lactobacillus plantarum* L-54 and *Lactobacillus plantarum* Aber F1 show higher lactic acid content and lower acetic acid, ethanol and ammonium nitrogen content compared to untreated silage. *Zielińska et al. (2015)* find in the alfalfa silages tretated with *Lactobacillus plantarum* K KKP 593p, *Lactobacillus plantarum* C KKP 788p, *Lactobacillus buchneri* KKP 907p and a mixture of all three strains, a smaller total number of molds, *Clostridium perfringens* and *Listeria* spp. and improved fermentation characteristics, quality and aerobic stability in relation to control silage. *Silva et al. (2016)* recommend *Pediococcus pentosaceus* strain 6.16 to inoculate the alfalfa silage as it rapidly reduces pH and increases the concentration of lactic acid. Selected strains of lactic acid bacteria in inoculants, in addition to improving the quality and aerobic stability of silage, also have probiotic activity in the digestive tract of animals *(Holzer et al., 2003)*. Thus, *Han et al. (2014)* conclude that inoculated silage may be a potential agent for the transmission of probiotics because in faeces of cow several species of lactic acid bacteria have been found which have been applied with inoculants in silage. Similarly, silage inoculants increase appetite and daily consumption in animals, thereby contributing to the increase in milk or meat production, higher total gain, lower feed conversion ratio, and low production costs *(Merry et al., 1993)*.

Forage legumes are difficult to ensile because they have low content of soluble carbohydrates and high buffering capacity. Different types of chemical and bacterial additives for ensiling of forage legumes have been developed in the world. 'Silko for alfalfa' is a homemade product intended for silage and haylage of forage legumes. The aim of this research was to examine the influence of inoculant 'Silko for alfalfa' on the quality of silage and haylage of alfalfa in two separate experiments.

Materials and Methods

Bacterial inoculant

Bacterial inoculant 'Silko for alfalfa' is a combination of homofermentative lactic bacteria *Lactobacillus plantarum* and *Pediococcus* spp. which are isolated from maize rizosphere and then identified according to the instructions given in the Bergey's Manual of determinative bacteriology (*Bergey, 2009*). The genotypic identification of bacteria was performed by sequencing the 16S rRNA by PCR method, and the determination of the strain by computer analysis in the Basic Local Aligment Search Tool where the obtained nucleotide sequence with the sequences available in the GenBank database, or NCBI (National Center for Biotechnology Information, Nacional Institutes - http://vvv.ncbi.nlm.nih.gov). Species that are contained in the inoculant are defined. The number of colony forming units in inoculant is $1x10^{10}$ CFU / ml.

Experiment 1
Fresh forage and silages

The influence of ‘Silko for alfalfa’ on the chemical composition and fermentation characteristics of alfalfa silage was tested in laboratory conditions. The cultivar Mirna (Bc Institute Zagreb) was used as the material. The cultivar was grown at the experimental plot of the Bc Institute at Zagreb, Croatia. For the silage, the first-cut in the second year of exploitation was used. The harvesting was done at the beginning of the flowering stage in May 2017. Masses were chopped with chopper harvester at about 20 mm chop length. Samples were taken to a laboratory where two treatments were formed: ‘Silko for alfalfa’, where the mass was treated with inoculant per rate of 5 ml t^{-1} green mass, and control where the mass was treated with distilled water in the same amount as the inoculant so that the moisture content of the silage was the same. A manual sprayer was used to spray liquid on the green mass. Glass jars of 1.5 l volume were mini-silos, with lids with water-valve. The jars were filled with about 850-1150 grams of compacted chopped mass and left in a dark place at room temperature for 90 days after which the silage was analyzed. Each treatment had three replicates. The chemical composition of the starting material before the ensiling was as follows: dry matter (DM) 355.0 g kg^{-1}, crude protein 225 g kg^{-1} DM, ADF 414.4 g kg^{-1} DM and NDF 527.5 g kg^{-1} DM.

Experiment 2
Fresh forage and haylage

In order to determine how inoculant the ‘Silko for alfalfa’ influences the alfalfa haylage quality, an experiment was set up on a commercial cattle farm in the Serbia during 2017. The cultivar Banat ZMS II (Institute of field and vegetable crops, Novi Sad) was used as the material. The first-cut in the second year of exploitation was used for the haylage. Plants were harvested at the beginning of the flowering stage (May) and wilted for 24h. Then the wilted mass was chopped with chopper harvester at about 15-40 mm chop length and packed in experimental silo bags. A special applicator was placed on the chopper harvester by which the chopped mass was treated with a new inoculant which represented the treatment of ‘Silko for alfalfa’ and distilled water for the control. The bacterial inoculant was administered in a rate of 5 ml of t^{-1} of green mass. Each treatment had three replications. The chemical composition of the starting material was as follows: dry matter content (DM) 467.0 g kg^{-1}, crude protein 208.8 g kg^{-1} DM, NDF 494.71 g kg^{-1} DM and ADF 408.6 g kg^{-1} DM. Haylage was analyzed after 40 days by taking three composite samples from each treatment. The composite sample included ten samples taken from different locations in silo bags, and then mixed in a clean plastic bin to form a common sample of about 2.0 kg. On the same day the samples were delivered to the laboratory for chemical analysis.

Determination of chemical, energy and fermetation parameters

The dry matter content was determined as a difference in weight before and after drying at 105 ° C in a oven to a constant mass. Kjeldahl method is used to determine crude protein content *(AOAC, 1990)*. Van Soest method is used to determine neutral (NDF) and acid detergent fiber (ADF) *(Van Soest et al., 1991)*. The silage pH value was determined from silage extract using a pH meter (Hanna Instruments HI 83141 pH meter). The distillation method is used to determine NH_3-N / total nitrogen (TN) using a Kjeltec 1026 analyzer *(Bijelić et al., 2015)*. Gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) is used to determine the amount of milk, acetic and butyric acid (*Faithfull, 2002)*. The energy parameters are calculated according to the following formulas:

Total digestible nutrients (TDN) (%) = (-1,291 × %ADF) + 101.35 according to *Horrocks and Vallentine (1999)*; Relative feed value (RFV) (%) = Digestible Dry Matter (DDM) × Dry Matter Intake (DMI) × 0.775; DDM (%) = 88.9 - (0.779 x % ADF) and DMI (%) = 120 / (% NDF) according to *Horrocks and Vallentine (1999);*Metabolisable energy (ME) (MJ kg^{-1}) = 14.07 + 0.0206 × ether extract (g kg^{-1}) - 0.0147 × crude fibre (g kg^{-1}) - 0.0114 × crude protein (g kg^{-1}) ± 4.5 % according to *Nauman i Bassler (1993)*;Net energy for lactation (NEL) (MJ kg^{-1}) = 9.10 + 0.0098 × ether extract (g kg^{-1}) - 0.0109 × crude fibre (g kg^{-1}) - 0.0073 × crude protein (g kg^{-1}) according to *Baležentienė i Mikulionien (2006)*.

Statistical analysis

The experiments were set by a randomized block system in 3 replications. Experimental data were statistically analyzed by the ANOVA using software SPSS 18 (IBM Corporation). Tukey test was used for the comparison of mean values at the level of $p \leq 0.05$.

Results and Discussion

Experiment 1

The results of the study show that the values of dry matter, crude protein, lactic acid, TDN and RFV were higher, and ADF, NDF, ammonium nitrogen in total nitrogen, acetic and butyric acid and pH were lower in the silage treated with inoculant than in control (Table 1).

Table 1. Chemical, energy and fermentation parameters untreated silage and silage treated with inoculant

Item	Control	'Silko for alfalfa'	F test
Chemical composition			
Dry matter (DM) ($g\ kg^{-1}$)	$325,1^{b}$	$337,1^{a}$	*
Crude protein ($g\ kg^{-1}$ DM)	$202,4^{b}$	$212,2^{a}$	*
Acid detergent fibre (ADF) ($g\ kg^{-1}$ DM)	$355,8^{a}$	$313,1^{b}$	**
Neutral detergent fibre (NDF) ($g\ kg^{-1}$ DM)	$424,7^{a}$	$394,2^{b}$	**
Energy parameters			
Total digestible nutrients (TDN) (%)	55.4^{b}	60.9^{a}	**
Relative feed value (RFV) (%)	134.0^{b}	152.2^{a}	**
Fermentation parameters			
pH	$4,98^{a}$	$4,43^{b}$	*
NH_3-N/TN ($g\ kg^{-1}$ TN)	$65,6^{a}$	$43,9^{b}$	**
Lactic acid ($g\ kg^{-1}$ DM)	$34,1^{b}$	$73,5^{a}$	**
Acetic acid ($g\ kg^{-1}$ DM)	$19,7^{a}$	$6,5^{b}$	**
Butyric acid ($g\ kg^{-1}$ DM)	0.39^{a}	0.08^{b}	**

TN – Total nitrogen; Means followed by the same letter within a row are not significantly different by Tukey's test at the 5% level; ** - significant at 1% level of probability and * - significant at 5% level of probability.

Higher dry matter and protein content and lower content of ADF and NDF indicate that silage treated with inoculant has a better chemical composition compared to untreated silage. The greater loss of dry matter in control is the result of slowed lactic acid fermentation that is regulated only by epiphytic bacteria found on plants, and whose number according to *Schmidt et al. (2009)* <1% microflora. On the other hand, by adding bacterial inoculants, lactic acid fermentation was more intense and less dry matter was lost, while lactic acid synthesis was increased. The content of crude proteins in the investigated silages was over 200 g kg^{-1} DM (control - 202.4 g kg^{-1} DM, treated silage - 212.2 g kg^{-1} DM) which from the aspect of protein content is considered as high quality silage. High protein content in the control silage can be the result of cutting of plants at early flowering stage when the share of leaves in the fodder is greater than the share of the stem. The leaves contain the ¾ of proteins. The content of NDF and ADF was higher in untreated silage which reflects a reduced digestibility. Generally, lower ADF in silage results in higher TDN, while lower ADF and NDF result in higher RFV. Therefore, treated silage has a better energy value compared with untreated silage. According to *Horrocks and Vallentine (1999)*, high quality alfalfa should have a RFV 151% (Standard Prime). Alfalfa with a RFV value of 125% to 140% can be used only in the nutrition of cows in the final lactation phase *(Dunham, 1998)*. *Redfearn et al. (2008)* conclude that alfalfa with a RFV value of 160% can only be used as feed for cows in lactation. In our case, inoculant-treated silage belongs to high quality and can be used for feeding cows at an early stage of lactation since its RFV (152.2%) is more than 151% (Standard Prime). On the other hand, untreated silage has a RFV value of 134% and can be used for feeding cow in the final lactation phase. *Kung and Muck (1997)* have already proved in 47% of their studies that silage treated with inoculants leads to an average increase in milk yield of 1.4 kg per cow per day. According to *Kung et al. (2003)*, silage treated with *Lactobacillus plantarum* MTD/1 gives better results in milk production. Therefore, we can assume that silage treated with inoculant 'Silko for alfalfa' due to its good qualitative and energy properties can improve the performance of animals.

Examined inoculant contributed to the intensification of lactic acid fermentation as it affected the decrease in the pH, the increase in lactic acid content and the reduction in the content of acetic and butyric acid in silage. It preserved the nutritive value of silage compared with control. In the treated silage, the lower NH_3-N/TN content (43.9 g kg^{-1} TN) compared to the control (65.6 g kg^{-1} TN) indicated a lower degradation of the protein. On the other hand, in untreated silage, proteolytic enzymatic activity was enhanced. The pH in the treated silage was lower by 0.55 than in control, which promoted better activity of homofermentative lactic acid bacteria and higher lactic acid content. A higher content of acetic acid was observed in control compared to the treated silage. In both silages the butyric acid content was low (<0.05% of dry matter). Therefore, the treated silage was well preserved due to lower pH and production of a higher amount of lactic acid compared to the control. Our study has shown that used inoculant can improve silage quality and reduce protein degradation in silage. It is precisely the role of inoculants to intensify the production of lactic acid, quickly reduce pH and prevent the development of pathogenic microorganisms *(Wang et al., 2006)*. *Ce et al. (2016)* find that alfalfa silages treated with various lactic acid bacteria inoculants (*Lactobacillus casei*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*) have a higher lactic acid content, lower butyric acid content, and NH_3-N / TN compared to control. *Si et al. (2018)* find that the silage treated with *Lactobacillus plantarum* and *Lactobacillus buchneri* has significantly lower pH, butyric and propionic acid content and NH_3-N / TN and higher content of dry matter and lactic acid in comparison with control.

Experiment 2

Bacterial inoculants should have the same effect in silage and haylage of forage legumes, to quickly reduce pH and support aerobic stability with improved animal performance *(Aragón, 2012)*. Our research has proved that the bacterial inoculant 'Silko for alfalfa' affects the faster fermentation and the creation of larger amounts of lactic acid and in the haylage of alfalfa (Table 2).

Table 2. Chemical, energy and fermentation parameters untreated haylage and haylage treated with inoculant

Item	Control	'Silko for alfalfa'	F test
Chemical composition			
Dry matter (DM) ($g\ kg^{-1}$)	421,4[b]	441,7[a]	**
Ash ($g\ kg^{-1}$ DM)	86,9[b]	98,2[a]	*
Ether extract ($g\ kg^{-1}$ DM)	384,1	388,0	ns
Crude protein ($g\ kg^{-1}$ DM)	177,9[b]	197,3[a]	*
Crude fibre ($g\ kg^{-1}$ DM)	296,6	274,4	ns
Acid detergent fibre (ADF) ($g\ kg^{-1}$ DM)	354,1[a]	316,1[b]	*
Neutral detergent fibre (NDF) ($g\ kg^{-1}$ DM)	419,1[a]	387,9[b]	*
Energy parameters			
Total digestible nutrients (TDN) (%)	55.6[b]	60.5[a]	**
ME ($MJ\ kg^{-1}$)	15.6	15.8	ns
NEL ($MJ\ kg^{-1}$)	8.3	8.5	ns
Relative feed value (RFV) (%)	136.1[b]	154.1[a]	**
Fermentation parameters			
pH	4,27[a]	4,16[b]	*
NH_3-N/TN ($g\ kg^{-1}$ TN)	25,27[a]	20,31[b]	**
Lactic acid ($g\ kg^{-1}$ DM)	65,1[b]	71,57[a]	*
Acetic acid ($g\ kg^{-1}$ DM)	34,9[a]	28,43[b]	**
Butyric acid ($g\ kg^{-1}$ DM)	0	0	ns

TN – Total nitrogen; Means followed by the same letter within a row are not significantly different by Tukey's test at the 5% level; ** - significant at 1% level of probability, * - significant at 5% level of probability and ns – non significant.

The results of the study showed that the values of dry matter, ash, crude protein, lactic acid, TDN and RFV were higher in haylage treated with inoculant than in the control. In contrast, ADF, NDF, NH_3-N/TN, acetic acid and pH were lower in haylage treated with inoculant than in the control. The pH value in examined haylages was below 4.5 which prevented botulism *(Kenney, 2001)* and listeriosis *(Ryser and Marth, 2007)*. The examined haylages did not differ in the content of ether extract, crude fiber, butyric acid, metabolisable energy and net energy for lactation. In general, the inoculant contributed to achieving optimum pH and better lactic acid fermentation, resulting in a lower loss of organic matter and increased digestibility. It is to be expected that the feeding of ruminants with high quality haylage can improve the production result of animals, as research by *Shirley (1996)* shows that haylage treated with inoculant can increase the dry matter intake, production of milk and weight gain of cows.

Conclusions

The results show that the inoculant 'Silko for alfalfa' preserves the nutritive value of alfalfa silage and haylage and improves chemical, energy and

fermentation parameters. In the treated silage and haylage, the content of dry matter, crude protein and lactic acid was higher than in control. Contrary to this, in the treated silage and haylage the values of ADF, NDF, pH, NH_3-N/TN and acetic acid were lower than in the control. The butyric acid content was lower in the treated silage than in the untreated, while the butyric acid was not detected in the haylage. The inoculant 'Silko for alfalfa' applied in silage and haylage positively influenced on lactic acid fermentation by preventing butyric fermentation and contributing to a reduced loss of nutritional value and energy. Also, it is to be expected that silage and haylage treated with the inoculant 'Silko for alfalfa' can promote a high level of productivity of ruminants, and thus contribute to the profitable livestock production.

Uticaj bakterijskog inokulanta na kvalitet silaže i senaže lucerke

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Rezime

Važna strategija u stočarstvu treba da bude uvođenje silaža i senaža krmnih leguminoza u ishranu preživara i promovisanje pravilnih tehnika siliranja i senažiranja. Krmne leguminoze se teško siliraju pa je neophodno primeniti starter kulture odabranih sojeva bakterija mlečne kiseline koje podržavaju proces siliranja i sprečavaju buternu fermentaciju i time doprinose očuvanju i unapređenju kvaliteta silaža. U radu je prikazan uticaj primene bakterijskog inokulnata 'Silko za lucerku' na kvalitet silaže i senaže u dva odvojena eksperimenta. Inokulant predstavlja kombinaciju homofermentativnih mlečnih bakterija *Lactobacillus plantarum* i *Pediococcus* spp. Za siliranje su korišćeni prvi otkosi lucerke u drugoj godini eksploatacije. Silaža je ispitivana u mini-silosima u laboratoriji, a senaža u silo vrećama na govedarskoj farmi. Tretmani su bili kontrola (netretirana silaža, odnosno senaža) i silaža, odnosno senaža tretirana sa inokulantom 'Silko za lucerku' (doza 5 ml t^{-1} krme). Silaže pripremljene u eksperimentalnim uslovima su analizirane nakon 90 dana, a senaže dobijene u proizvodnim uslovima nakon 40 dana. Ustanovljeno je da korišćeni inokulant čuva nutritivnu vrednost silaže i senaže i da poboljšava njihove hemijske, energetske i fermentacione parametre u odnosu na kontrolu. S obzirom da ispitivani inokulant pozitivno utiče na pravilnu mlečno-kiselinsku fermentacju silaže i senaže lucerke i doprinosi manjem gubitku hranljive vrednosti i energije za očekivati je da može promovisati visok nivo produktivnosti preživara, a samim tim i doprineti rastu profita u stočarstvu.

Ključne reči: lucerka, bakterijski inokulant, senaža, silaža, kvalitet

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