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THE EFFECT OF BACTERIAL INOCULANT ON CHEMICAL COMPOSITION AND FERMENTATION OF ALFALFA SILAGE

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Abstract: Alfalfa silage is a useful source of protein for feeding ruminants. Therefore, managing alfalfa silage in livestock production systems is an important issue in order to maintain the silage quality and achieve maximum profitable production of milk and meat. The aim of this investigation was to estimate the effects of bacterial inoculant Silko, containing *Lactobacillus plantarum* (strains: LP1, LP2, LP3 and LP4) on chemical composition, energetic characteristics and fermentation alfalfa silage under field conditions in the commercial dairy farm, during the 2016. The first-cut alfalfa in the second year has been conserved in silage form. The silage mass was subdivided into two equal parts (control (silage without inoculant) and silages treated with bacterial inoculant Silko) and ensiled in trench silo. After 60 days of ensiling, the silages were analysed. Dry matter, ash, crude protein, lactic acid, acetic acid, total digestible nutrients value and relative feed value were significantly higher in silage treated with bacterial inoculant Silko compared to control. Contrary, alfalfa silage treated with a bacterial inoculant Silko had lower values of cellulose, acid detergent fibre, neutral detergent fibre, non-nitro extractive matter, pH, butyric acid, soluble nitrogen/total nitrogen and NH_3-N/total nitrogen than untreated silage. Results showed that bacterial inoculant Silko increases silage quality compared to control so that research should be directed toward the use of such prepared silage in ruminant diets and its impact on milk and meat production on farms.

Key words: alfalfa, chemical composition, energetic characteristics, fermentation parameters, inoculant, silage

Introduction

In Serbia, alfalfa is grown on an area of 109230 ha with a total annual production of 481003 tons and an average yield of 4.4 t ha^{-1} *(Statistical Yearbook* *of the Republic of Serbia, 2016)*. Alfalfa is important for the nutrition of all species of domestic animals, and it is used in various forms, such as hay, silage, dehydrated plants, less frequently as green food and for livestock grazing. In modern farming, alfalfa silage is a useful source of protein for feeding to cattle and sheep and a good supplement for maize silage. In Serbia, silage is an important feed for livestock in winter and early spring when reduces pasture production. However, the high protein content and low content of soluble carbohydrates in the fresh material (< 1.5%), low dry matter and high buffering capacity make it difficult to ensile. For these reasons, the application of chemical or bacterial additives is the important factor for ensiling alfalfa *(Repetto et al., 2011)*. The advantage of bacterial inoculants is that they leave no residues and does not adversely affect animal health and product quality and safety. For this reason, everywhere in the world largely suppressed chemical preservatives, regardless of their effectiveness. *McDonald et al. (1991)* stated that the bacterial inoculants safe, easy-to-use and noncorrosive to farm machinery, and do not pollute environment. During ensiling, LAB ferment water-soluble carbohydrates to organic acids, mainly lactic acid which reduce the pH and inhibit the growth of pathogenic and spoilage bacteria, yeast and moulds which influence on heating and spoilage silage and dry matter losses *(Zhang et al., 2009; Čabarkapa et al., 2010a)*. *Pahlow et al. (2003)* stated that LAB which found are silage is members of the genera *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus* and *Leuconostoc*. *Lactobacillus* is a genus of Gram-positive organism which produces lactic acid and acidic environment (pH 5.5-6.5) *(Giraffa et al., 2010)*. *Kizilsimsek et al. (2007)*, *Zhang et al. (2009)* and *Zielińska et al. (2015)* reported that inoculation with LAB of the genus *Lactobacillus* can improve the fermentation of alfalfa silage, quality and aerobic stability. Also, many researches showed beneficial effects of silage inoculant on chemical composition and fermentation alfalfa silage *(Bolsen et al., 1996; Čabarkapa et al. 2010b; Silva et al., 2016; Tian et al., 2016)*. *Đorđević et al. (2011)* reported that addition of homofermentative bacterial inoculants to alfalfa silages reduced the content of NH_3-N and increased the lactic acid and pH compared to untreated silage.

The objective of this research was to determine the effects of bacterial inoculant Silko on chemical composition, energetic characteristics and fermentation alfalfa silage under field conditions in the commercial dairy farm.

Materials and Methods

The first-cut alfalfa cultivar Banat in the second year was harvested at initial flowering stage (May 2016), and after 24h wilting, the silage mass was chopped on about 20 mm chop length using chopper harvester. The silage mass was subdivided into two equal parts (control (silage without inoculant) and silages treated with bacterial inoculant Silko) and ensiled in trench silo. The liquid inoculant was sprayed using a plant sprayer over the course of filling the silos. The inoculant was applied at recommended rate of 5 ml t^{-1} fresh material. The bacterial inoculant Silko contains homofermentative *Lactobacillus plantarum* (strains: LP1, LP2, LP3 and LP4). The number of colony forming units in inoculant is $1x10^{10}$ CFU/ml. After 60 days of ensiling, the silages were analyzed. Three composite samples were collected from each treatment. Composite sample included twelve samples which are collected with different locations in trench silo including from top to bottom and left to right, and were mixed in a clean plastic bucket to form a composite sample weighing about 1.5 kg. The samples were packed into plastic bags to avoid exposure to air and delivered to the laboratory.

The dry matter was determined as the difference in mass before and after the drying to constant mass in an oven at 105°C. The ash was determined heating the dry samples in an oven at 550°C for 2h. Crude fat (CF) content was determined according to Soxhlet method, crude protein (CP) according to Kjeldahl *(AOAC 1990)*, cellulose according to Weende method, neutral detergent fibre (NDF) and acid detergent fibre (ADF) according to Van Soest method, soluble nitrogen/total nitrogen according to *Licitra et al. (1996)*, NH_3-N was determined by the distillation method using a Kjeltec 1026 analyser and the pH value was measured with a Hanna Instruments HI 83141 pH meter. Lactic acid (LA), acetic acid (AA) and butyric acid (BA) were analyzed with a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) according to *Faithfull (2002)*. Non-nitro extractive matter (NEM) was calculated by formula: 100% - % crude protein - % crude fat - % crude fibre - % ash - % moisture. Also, calculated total digestible nutrients value (TDN) and relative feed value (RFV) according to *Horrocks and Vallentine (1999)*, metabolic energy (ME) according to *Nauman and Bassler (1993)* and net energy for lactation (NEL) according to *Baležentienė and Mikulionien (2006)*:

TDN (%) = (-1,291 x ADF) + 101.35;

RFV (%) = Digestible Dry Matter (DDM) x Dry Matter Intake (DMI) x 0.775,

DDM (%) = 88.9 - (0.779 x % ADF) and DMI (%) = 120 / (% NDF);

ME (MJ kg^{-1}) = 14.07 + 0.0206 x crude fat (g kg^{-1}) – (0.0147 x crude fibre (g kg^{-1}) - 0.0114 x crude protein (g kg^{-1}) ± 4.5 %;

NEL (MJ kg^{-1}) = 9.10+0.0098 x crude fat (g kg^{-1}) – 0.0109 x crude fibre (g kg^{-1}) - 0.0073 x crude protein (g kg^{-1}).

Data were subjected to an ANOVA using Statistica version 10, a Randomized Complete Block Design and Duncan's Multiple Range Test was used to compare differences among treatment means ($P < 0.05$).

Results

Chemical composition

Data of ANOVA in Table 1 shows that bacterial inoculant Silko had highly significant effect on content of dry matter, ash, crude protein, cellulose, acid detergent fibre (ADF), neutral detergent fibre (NDF), and non-nitro extractive matter. Values of dry matter (434.4 g kg^{-1}), ash (101.50 g kg^{-1}) and crude protein (202.61 g kg^{-1}) were significantly higher in silage treated with bacterial inoculant Silko than in silage without inoculant (419.9 g kg^{-1}, 86.48 g kg^{-1} and 169.54 g kg^{-1}, respectively). Contrary, values of cellulose (295.70 g kg^{-1}), ADF (351.78 g kg^{-1}), NDF (408.61 g kg^{-1}) and non-nitro extractive matter (404.33 g kg^{-1}) were significantly higher in silage without inoculant than in silage treated with bacterial inoculant Silko (271.03 g kg^{-1}, 314.38 g kg^{-1}, 393.10 g kg^{-1} and 379.90 g kg^{-1}, respectively). The addition of inoculant did not alter crude fat content. Generally, addition of inoculant has improved the chemical composition of alfalfa silage.

Table 1 Chemical composition of untreated silage and silage treated with bacterial inoculant Silko

Item	Control	Silko	M	F test
Dry matter (g kg^{-1})	419.9^b	434.4^a	427.2	**
Ash (g kg^{-1} DM)	86.48^b	101.50^a	93.99	**
Crude fat (g kg^{-1} DM)	38.27	38.16	38.22	ns
Crude protein (g kg^{-1} DM)	169.54^b	202.61^a	186.07	**
Cellulose (g kg^{-1} DM)	295.70^a	271.03^b	283.36	**
Acid detergent fibre (ADF) (g kg^{-1} DM)	351.78^a	314.38^b	333.08	**
Neutral detergent fibre (NDF) (g kg^{-1} DM)	408.61^a	393.10^b	400.85	**
Non-nitro extractive matter (g kg^{-1} DM)	404.33^a	379.90^b	392.12	**

Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability and ns - not significant

Energy characteristics

Total digestible nutrients value (TDN) (60.76%) and relative feed value (RFV) (152.38%) have significant higher in silage treated with bacterial inoculant than control (55.94% and 139.97%, respectively) (Table 2). Metabolic energy (ME) and net energy for lactation (NEL) were not affected by inoculation treatment.

Table 2. Energy characteristics of untreated silage and silage treated with bacterial inoculant Silko

Item	Control	Silko	M	F test
Total digestible nutrients value (TDN) (%)	55.94[b]	60.76[a]	58.34	**
Relative feed value (RFV) (%)	139.97[b]	152.38[a]	146.18	**
Metabolic energy (ME) (MJ kg^{-1})	8.58	8.56	8.57	ns
Net energy for lactation (NEL) (MJ kg^{-1})	5.01	5.04	5.02	ns

Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability and ns - not significant

Fermentation parameters

Data of ANOVA in Table 3 show that silage inoculant had significant effect on all fermentation parameters. The lactic acid (86.00 g kg^{-1} DM) and acetic acid (8.45 g kg^{-1} DM) were higher in silage treated with inoculant Silko compared to control (79.92 g kg^{-1} and 5.69 g kg^{-1}, respectively). The pH (4.69), butyric acid (0.021 g kg^{-1} DM), soluble N/TN (343.43 g kg^{-1} TN) and NH_3-N/TN (21.83 g kg^{-1} TN) were lower in silage treated with inoculant Silko compared to control (4.80, 0.026 g kg^{-1}, 350.68 g kg^{-1} and 27.90 g kg^{-1}, respectively).

Table 3. Fermentation parameters of untreated silage and silage treated with bacterial inoculant Silko

Item	Control	Silko	M	F test
pH	4.80[a]	4.69[b]	4.74	*
Lactic acid (g kg^{-1} DM)	79.92[b]	86.00[a]	82.96	**
Acetic acid (g kg^{-1} DM)	5.69[b]	8.45[a]	7.07	**
Butyric acid (g kg^{-1} DM)	0.026	0.021	0.024	*
Soluble N/TN (g kg^{-1} TN)	350.68[a]	343.43[b]	347.06	*
NH_3-N/TN (g kg^{-1} TN)	27.90[a]	21.83[b]	24.87	**

Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at the 5% level ($p \leq 0.05$), ** - significant at 1% level of probability and * - significant at 5% level of probability

Discussion

The low content of soluble carbohydrates (<1.5%) in fresh alfalfa material makes it difficult to ensiling. Various types of chemical or bacterial additives have been developed in order to improve the ensiling process. There are a large number of bacterial inoculants for ensiling on the market. This research showed beneficial effects of bacterial inoculant (Silko) on silage quality. It is believed that the most efficient type of homofermentative lactic acid bacteria *Lactobacillus plantarum* (strains: LP1, LP2, LP3 and LP4) which most effectively transforms water soluble carbohydrates into lactic acid. Inoculant Silko had a positive effect on chemical composition alfalfa silages in terms of higher dry matter content, crude protein and mineral elements, and lower cellulose, ADF, NDF, and non-nitro extractive matter. Generally, inoculant Silko has improved the chemical composition of alfalfa silage. *Jatkauskas et al. (2015)* reported that bacterial inoculants improve chemical composition alfalfa silage by increasing content of dry matter, crude protein and soluble carbohydrates. Dry matter content was higher in silage treated with inoculant Silko. This can be explained by the fact that lactic acid fermentation is slow in control, due to small number of lactic acid bacteria on living plants, even by providing optimal initial conditions. *Doležal et al. (2012)* concluded that optimal dry matter content 350-400 g kg^{-1} for alfalfa silage. In our study, the dry matter content of alfalfa silages was higher than optimal content. High quality alfalfa silage has crude protein minimum 200 g kg^{-1} of dry matter. Crude protein in treated silage (202.61 g kg^{-1}) was higher than untreated silage (169.54 g kg^{-1}) and belongs to a group of high quality silage. In control, the higher crude protein content can be explained by harvesting alfalfa in early phase when share of leaves was equal to or greater than the share of stems. *Bijelić et al. (2015)* reported that crude protein content (179 g kg^{-1}) in alfalfa silage in early harvest phase was higher than phase of late harvest (146.2 g kg^{-1}). The reduction of fractions ADF and NDF in treated silage evidenced favorable anaerobic conditions for a fermentation process. The NDF and ADF are important quality parameters of silage. High contents of NDF and ADF in silage adversely affect the quality and decreased digestibility. *Temel et al. (2015)* reported that the NDF and ADF are undesired structures in fodder crops. Degradation cell-wall content (NDF and ADF) during the fermentation improves silages digestibility and animal performance *(Bolsen et al., 1996)*. *McDonald et al. (1991)* pointed that homofermentative bacteria degrade the cellular walls of forage during the ensiling process.

The chemical composition of treated silage was improved due to a reduction ADF and NDF, as well as increases in energy content. Silage treated with Silko inoculant had higher TDN and RFV than untreated silage. TDN is directly related to digestible energy and is often calculated based on ADF. Higher TDN and RFV values indicate higher forage quality. They are indication of good chemical composition of treated silage. *Horrocks and Vallentine (1999)* reported that the RFV value is greater than 151 is considered prime. In our study the RFV (152.38%) in treated silage was higher than 151. The energy content (ME and NEL) were not affected by the use of the silage inoculant. Contrary, *Sánchez et al. (2014)* reported that ME and NEL contents increased in inoculated alfalfa silage. According to *Juraček et al. (2016)* the average value of NEL in alfalfa silages in Slovakia farms is 4.83 MJ kg^{-1} of DM, while in our study the value of NEL was higher (in average for both silage is 5.04 MJ kg^{-1} of DM).

The lower values of pH indicate that fermentation was initiated effectively by added *Lactobacillus plantarum* strains. The lower pH in inoculated silage is important for conserving of nutrients and promoting homofermentative lactic acid bacteria. Generaly, the main effect of silage inoculant was the increased production of lactic acid with significant reduction of pH *(Jatkauskas and Vrotniakiene, 2011; Hashemzadeh-Cigari et al. 2011; Sánchez et al., 2014)*. The content of lactic acid and acetic acid were significantly higher while soluble N/TN and NH_3-N/TN significantly lower in inoculated silage than control. These indicate efficient fermentation and minimal dry matter loss. Inoculation resulted in lower protein degradation. Many researches showed that silages treated with inoculants containing of *Lactobacillus plantarum* had lower pH and NH_3-N/TN, and higher content of lactic acid than untreated silages *(Saarisalo et al., 2006; Jatkauskas et al., 2013; Jatkauskas et al., 2015)*. In control, the high level of NH_3-N/TN indicating protein degradation from proteolytic enzymatic activity contained within the crop. In treated silage, NH_3-N/TN content decreased due to the lower pH and more lactic acid produced.

The primary goal of rapid fermentation and stabilization of a plant material is to produce higher levels of lactic acid rather than acetic acid. The content of acetic acid was significantly higher in inoculated silage than control. Also, *Zhang et al. (2009)* and *Sánchez et al. (2014)* concluded that the inoculated alfalfa silage had more lactic acid and acetic acid content than the control. Many studies have indicated that acetic acid has anti-fungal properties, reduces aerobic spoilage of silage and growth of moulds and yeasts *(McDonald et al., 1991; Schmidt et al., 2009; Čabarkapa et al. 2010a, b*). Otherwise acetic acid is produced naturally during fermentation, with or without inoculants. *Seglar (2003)* reported that the presence of butyric acid is the result of Clostridial activity. Clostridia spores degrade lactic acid to butyric acid. *Pahlow et al. (2003)* concluded that to prevent Clostridial activity should be reached lower pH value, which was achieved in the treated silage with Silko. Therefore the higher content of butyric acid was detected in control than treated silage, but in both silages concentration of butyric acid is $<0.05\%$ of dry matter. According to the content of butyric acid, the investigated silages are good quality. Generally, fermentation characteristics in treated silage indicate good silage quality.

Conclusions

Results showed that values of dry matter, ash, crude protein, lactic acid, acetic acid, total digestible nutrients value and relative feed value significantly increased in treated silage with inoculant Silko. On the other hand, values of cellulose, acid detergent fibre, neutral detergent fibre, non-nitro extractive matter, pH, butyric acid, soluble nitrogen/total nitrogen and NH_3-N/total nitrogen

significantly decreased in treated silage. Generally, results showed that bacterial inoculant Silko improves chemical, nutritional quality and fermentation quality of alfalfa silage. Adding bacterial inoculant Silko may be a promising management practice to improve fermentation, conserve more nutrients and increase their availability to the ruminants.

Uticaj bakterijskog inokulanta na hemijski sastav i fermentaciju silaže lucerke

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Rezime

Silaža lucerke je koristan izvor proteina za ishranu preživara. Stoga, proizvodnja silaže lucerke u stočarstvu predstavlja važno pitanje kako bi se održao kvalitet silaže i postigla maksimalna profitabilna proizvodnja mleka i mesa. Cilj ovog istraživanja je bio da se proceni efekat bakterijskog inokulanta Silka koji sadrži *Lactobacillus plantarum* (sojevi: LP1 LP2, LP3 i LP4) na hemijski sastav i fermentaciju silaže lucerke u terenskim uslovima na komercijalnoj farmi goveda u 2016. godini. Prvi otkos lucerke u drugoj godini je konzerviran u obliku silaže. Silažna masa je podeljena na dva jednaka dela (kontrola (silaža bez inokulanta) i silaža tretiranih bakterijskim inokulantom Silko) i silirana u rovu silosu. Silaža je analizirana 60 dana nakon siliranja. Sadržaj suve materije, pepela, sirovih proteina, mlečne i sirćetne kiseline, ukupna svarljiva hranljiva materija i relativna hranljiva vrednost značajno su veći u silaži tretiranoj bakterijskim inokulantom Silko nego u kontroli. Suprotno, silaža lucerke tretirana sa bakterijskom inokulantom Silko imala je niže vrednosti za celulozu, ADF, NDF, bezazotne ekstraktivne materije, pH, buternu kiselinu i udeo rastvorljivog i amonijačnog azota u ukupnom azotu nego kontrola. Rezultati su pokazali da bakterijski inokulant Silko povećava kvalitet silaže u odnosu na kontrolu, tako da bi dalja istraživanja trebalo da budu usmerena ka korišćenju ovako pripremljene silaže u ishrani preživara i njen uticaj na proizvodnju mleka i mesa na farmama.

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