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The effect of bacterial inoculant on chemical composition and fermentation of alfalfa silage

Utjecaj bakterijskih inokulanta na kemijski sastav i fermentaciju silaže lucerne

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ABSTRACT

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 study was to estimate effects of commercial bacterial inoculants on chemical composition and fermentation of alfalfa silage, under field conditions in the commercial dairy farm, during 2017. The silage mass was subdivided into five equal parts (control - silage without inoculant) and silages treated with commercial bacterial inoculants (PIO 1 - Pioneer 11H50, PIO2 - Pioneer 11AFT, SIL - Silko and BON - Bonsilage alfa) all ensiled in microsilos. After 90 days of ensiling, silages were analysed for chemical and nutritional composition and fermentation characteristics. Dry matter and crude protein value were higher, lactic acid and acetic acid value were significantly higher in silage treated with bacterial inoculant PIO1, PIO2, SIL and BON compared to control silage. Contrary, alfalfa silage treated with a bacterial inoculant PIO1, PIO2, SIL and BON had lower values of acid detergent fibre, neutral detergent fibre and pH and significantly lower values butyric acid, alcohols and NH_3-N/total nitrogen compared to control silage. Results showed that bacterial inoculant PIO1, PIO2, SIL and BON increases silage quality compared to control silage.

Keywords: alfalfa, chemical composition, fermentation parameters, inoculants, silage

SAŽETAK

Proizvodnja silaže lucerne u stočarstvu predstavlja važno pitanje kako bi se održala kvaliteta silaže i postigla maksimalna profitabilna proizvodnja mlijeka i mesa. Cilj ovog istraživanja bio je da se procijeni učinak komercijalnih bakterijskih inokulanta na kemijski sastav i fermentaciju silaže lucerne, u poljskim uvjetima na farmi mliječnih krava, tijekom 2017. godine. Silažna masa je podeljena na pet jednakih dijelova (kontrola - silaža bez inokulanta) i silaža tretiranih bakterijskim inokulantima (PIO 1 - Pioneer 11H50, PIO2 - Pioneer 11AFT, SIL - Silko i BON - Bonsilage alfa) i silirana u plastične vrečice. Silaža je analizirana 90 dana nakon siliranja. Sadržaj suhe tvari i sirovih proteina je veći, a sadržaj mliječne i octene kiseline je značajno veći u silaži tretiranoj bakterijskim inokulantom PIO1, PIO2, SIL i BON u odnosu na kontrolu. Suprotno, silaža lucerne tretirana sa bakterijskim inokulantom PIO1, PIO2, SIL i BON imala je niže vrijednosti za neutralna deterdžent vlakna, kisela deterdžent vlakna i pH, a značajno niže vrijednosti za maslačnu kiselinu, alkohole i udio amonijskog dušika u ukupnom dušiku u odnosu na kontrolu. Rezultati su pokazali da bakterijski inokulanti PIO1, PIO2, SIL i BON povećavaju kvalitetu silaže u odnosu na kontrolu.

Ključne riječi: inokulanti, kemijski sastav, lucerna, parametri fermentacije, silaža

INTRODUCTION

In Croatia, alfalfa is grown on an area of 23,559 ha with a total annual production of 191,540 tons and an average yield of 8.1 t/ha (Croatian Bureau of Statistics, 2016). As one of the highest quality forages, alfalfa is considered as efficient source of biological nitrogen fixation to maximize protein yield per land area. 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, as less frequently as green food and for livestock grazing. In Croatia, silage is an important livestock feed in winter and early spring when pasture production in reduced. However, alfalfa often is viewed as a difficult crop to ensile, primarily because of its high buffering capacity, low water-soluble carbohydrates (WSC) in the raw material (<1.5%) content and tendency to undesirable secondary clostridial fermentations, especially when ensiled at dry matter (DM) content of less than 300 g/kg (Coblentz and Muck, 2012).

Silage quality and nutrient use efficiency are influenced by numerous of factors such as crops, ensiling technologies, machinery and additives used for manipulating fermentation processes (Davies et al., 2005). 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 are largely suppressed chemical preservatives, regardless of their effectiveness. McDonald et al. (1991) stated that the bacterial inoculants are safe, easy-to-use and noncorrosive to farm machinery, and do not pollute environment. Homo-fermentative bacteria convert C6-sugars solely into lactic acid, whereas hetero-fermentative species produce lactic acid and carbon dioxide at equal shares as well as traces of acetic acid or ethanol. Pahlow et al. (2003) stated that lactic acid bacteria (LAB) which are found in silage members of the genera *Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus* and *Leuconostoc*. *Lactobacillus* is a genus of Grampositive 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., 2010; 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. Companies producing inoculants expect that new strains and mixtures will be highly competitive and will improve silage fermentation by reducing pH and by producing largely lactic acid, compared to spontaneously fermented silage. However, some authors reported that homo-fermentative lactic acid bacteria inoculants did not improve the aerobic stability of silages (Sucu and Filya, 2006). After opening of the silo, yeast and moulds can lead to an increase in pH value and temperature of the silage as well as to a reduction of readily available sugars. Loss of carbon dioxide and temperature increase cause dry matter losses and reduce the feeding value of silage (Muck, 2012). During ensiling, lactic acid bacteria (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). Lactic acid should be the primary acid in good silages. This acid is stronger than other acids in silage (acetic, propionic and butyric) and thus usually responsible for decrease in pH value. Lactic acid should (be make) contain at least 65 to 70% of the total silage acids in good silage (Kung, 2010). Excessive amounts of acetic, propionic, or butyric acids as well as ethanol indicate a poorer quality fermentation process as result of other microbes that are not exclusive lactic acid-producing bacteria (Van Saun, 2008). A high concentration of butyric acid (>0.5% of dry matter) indicates that the silage has undergone clostridial fermentation, which is one of the poorest fermentations. High butyric acid can sometimes induced ketosis in lactating cows and because the energy value of silage is low, intake and production can suffer (Gerlach et al., 2014). High concentration of ammonia (>12 to 15% of crude protein) is a result of excessive protein breakdown in the silo, caused by a slow decrease pH value or clostridial action. Usually, silage with high concentrations of ammonia coupled with butyric acid may also have significant concentrations of other undesirable end products, such as amines, that may reduce animal performance (Kung and Shaver, 2001). The present study was conducted in order to assess which mixtures of lactic acid bacteria have a greater potential to improve fermentation pattern of the alfalfa silage.

MATERIAL AND METHODS

The first-cut of alfalfa cultivar Mirna was harvested in the second year at initial flowering stage (May 2017). After 24 h of wilting the silage mass was chopped on about 20 mm (chop) length using chopper harvester. Alfalfa mass was packed into plastic film bags silos (280 x 360 mm) and the bags silos were sealed with a vacuum sealer (SmartVac STATUS SV2000). Each trial had 5 treatments (uninoculated control and 4 inoculants), with 5 micro-silos per treatment. All four inoculants were commercial products and were applied to the forage targeting a dosage as described in Table 1. All inoculants were diluted with distilled water prior to use to obtain a target application rate for each treatment. The suspension obtained was applied at rate 4 ml/kg forage, when untreated control received water at rate 4 ml/kg forage. Subsequently, the additives were sprayed into the fresh forage using sprayapplicators. The forages were thoroughly mixed by hand, and then placed into the silo by hand. The silos were stored for 90 days at a room temperature of about 22 °C, after samples for chemical analyses had been taken. The dry matter was determined as the difference in mass before and after drying to constant mass in an oven at 105 °C. The ash was determined by heating dry samples in an oven at 550 °C for 2 h. Crude fat (CF) content was determined according to Soxhlet method, crude protein (CP) according to Kjeldahl (AOAC, 2000), cellulose according to Weende method, neutral detergent fibre and acid detergent fibre according to Van Soest et al. (1991), 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 the 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).

Silage composition data were subjected to one-way analysis of variance for a 4 (additive) factorial arrangement of treatments within a randomized complete block design by using Proc GLM of SAS, version 8.02 (Statistical Analysis System, 2002). Significance of the differences between the means was determined according to the least significant difference (LSD) at 0.05 probability level.

Table 1. Inoculants used in the trials

Products	Active ingredient	Application CFU/g and ml^{-1} forage
CON - control (without the inoculant)		
PIO1 - Pioneer 11H50	Lactobacillus plantarum	4×10^{8}
PIO2 - Pioneer 11AFT	Lactobacillus buchneri, Lactobacillus casei	1.1×10^{11}
SIL - Silko for alfalfa	Lactobacillus plantarum, Pediococcus sp.	1×10^{11}
BON - Bonsilage alfa	Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus buchneri, Lactococcus lactis	1.25×10^{11}

RESULTS AND DISCUSSION

Differences in fermentability between grasses and legumes have been attributed to the major differences in their chemical composition, and particularly the buffering capacity (Playne and McDonald, 1996). Quantitatively, the amount of acid required to decrease the original pH=6 to a stable pH=4 is dependent on the contents of the silages dry matter, contents of the water-soluble carbohydrates and contents of the crude protein. Furthermore, forages with high dry matter content are fermented at a slower rate than forages with low dry matter because of low water activity (Rizk et al., 2005). Based on the water soluble carbohydrates and crude protein content of the herbages prior to ensiling, the alfalfa forage was considered as difficult to ensile (water soluble carbohydrates concentration 15 g/kg fresh forage) according to the European Food Safety Authority (EFSA) opinion on silage additives guidelines (Commission Regulation 429/2008 EC), while perennial ryegrass and red clover/ryegrass/timothy forages were considered to be moderately easy to ensile (water soluble carbohydrates concentration 41 and 31 g/kg fresh forage, respectively). The crude protein content (214 g/kg dry matter) of alfalfa (Table 2) was typical content reported in previous studies (166–225 g/kg dry matter), (Contreras-Govea et al., 2011; Lynch et al., 2014).

Table 2. Chemical composition of alfalfa herbage (cultivar Mirna) prior to ensiling in dry matter

Chemical composition	Mean
Dry matter (g/kg)	361
Crude protein (g/kg)	214
Crude fat (g/kg)	30.6
Crude fibre (g/kg)	259
Crude ash (g/kg)	88
Water soluble carbohydrates (g/kg)	49
Acid detergent fibre (g/kg)	335
Neutral detergent fibre (g/kg)	383
pH	6.4

Moreover, high buffering capacity and crude protein, in combination with low water-soluble carbohydrates concentration of the alfalfa meant that the ensiling properties for the alfalfa were not ideal as suggested by Muck (2012). Some differences in dry matter content, crude protein, neutral detergent fiber (NDF), acid detergent fiber (ADF) and water-soluble carbohydrates concentrations were observed among the silages tested (Table 3). Nutritive value of alfalfa silages is closely related to the phenological stage at harvest (Charmley, 2001). Results showed that values of dry matter and crude protein value were insignificantly higher in treated silage with inoculant PIO1, PIO2, SIL and BON compared to control silages (Table 3).

According to Knotek (1997), in order to make good quality silage it is necessary to produce silage from wilted material which contains dry matter of 320-380 g/kg, or according to Đorđević et al. (2001), the dry matter content of plant material should be above 35% ensure successful fermentation. High quality alfalfa silage has minimum of 200 g crude protein in one kg of dry matter. Only the alfalfa silage treated with inoculant products had a crude protein, neutral and acid detergent fibre value different from the control. Cell-wall content degradation (neutral detergent fiber and acid detergent fiber) during the fermentation improves silages digestibility and animal performance (Bolsen et al., 1996). McDonald et al. (1991) pointed that homofermentative bacteria degrade cellular walls of forage during the ensiling process. Seglar (2003) recommend the optimal content of acid detergent fiber less than 300 g/kg of dry matter in alfalfa silages. According to Mitrík (2010) is the target level of neutral detergent fiber in alfalfa silage is ≤37.5%. Additive treated silages had lower water-soluble carbohydrates concentrations than control silages. Among the inoculated silages the water-soluble carbohydrates remaining after fermentation were the highest in the BON treated alfalfa silage. The water-soluble carbohydrates remaining after fermentation were the lowest ($P<0.05$) in alfalfa silages treated with product PIO1 compared with the SIL, PIO2, BON and control silages. Jatkauskas et al. (2015) reported that bacterial inoculants improve chemical composition

Table 3. Nutritional composition of the alfalfa silages in g/kg dry matter

Products	Dry matter	Crude protein	Neutral detergent fibre	Acid detergent fibre	Water soluble carbohydrates
CON	338[a]	185[a]	399[a]	348[a]	6.65[a]
PIO1	354[a]	203[a]	377[a]	334[a]	2.87[e]
PIO2	348[a]	197[a]	383[a]	341[a]	3.41[c]
SIL	351[a]	200[a]	379[a]	337[a]	3.06[d]
BON	340[a]	192[a]	391[a]	344[a]	4.14[b]

Different letters in the column mean significant difference (P<0.05).

of alfalfa silage by increasing content of dry matter, crude protein and soluble carbohydrates. The use of inoculant additives accelerates the fermentation in alfalfa silages compared to the silages without additives. Spontaneous fermentation in the control alfalfa silages produced lower concentrations of fermentation acids; however, used more sugars available in the herbages with lower pH decrease. The largest reduction in pH from ensiling alfalfa after 90 days was obtained by PIO1, SIL and PIO2 products, whereas BON inoculants produced the smallest reduction in pH in alfalfa silages (Figure 1).

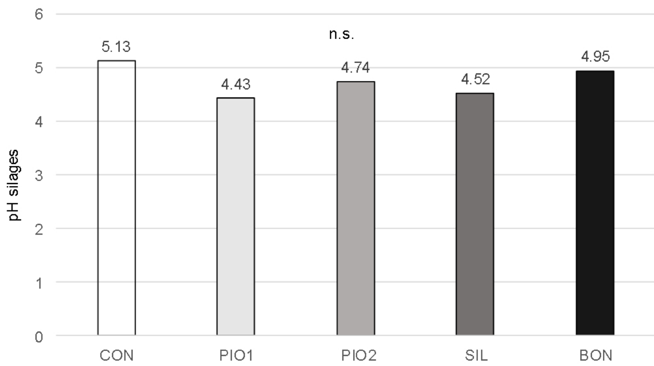

Figure 1. pH silages of alfalfa after 90 days from ensiling (n.s. - non significant)

Well preserved alfalfa silage with dry matter content from 201–300 g/kg has pH value less than 4.3, with dry matter content from 301–400 g/kg has pH value less than 4.5, while a silage with dry matter content from 401–500 g/kg has pH value less than 4.7 (Škultéty, 1999). Using inoculants, products of fermentation are shifted in alfalfa silages, resulting in significantly (P<0.05) higher lactic and acetic acid, and significantly (P<0.05) lower butyric acid, alcohols and ammoniacal nitrogen, compared to control silages, as refereed in the (Table 4).

This indicates that the addition of inoculants allowed a more rapid production of lactic acid which suppresses the buffering effect of legumes and grasses as suggested by Adesogan and Salawu (2004). In alfalfa silages the inoculated treatments produced significantly larger (P<0.05) lactic acid content than the uninoculated treatment. Alfalfa silages inoculated with PIO1, SIL and PIO2 had greater lactic acid content than BON inoculated silages (Table 4). Generaly, the main effect of silage inoculant was the increased production of lactic

Table 4. Fermentation characteristics of the alfalfa silages in g/kg dry matter

Products	Lactic acid	Acetic acid	Butyric acid	Alcohols	Ammoniacal nitrogen
CON	33.2[c]	18.3[c]	5.27[a]	7.13[a]	95.1[a]
PIO1	78.1[a]	22.6[b]	0.94[d]	2.81[c]	47.8[e]
PIO2	67.7[a]	36.2[a]	1.55[c]	2.17[c]	54.6[c]
SIL	72.4[a]	23.1[b]	1.43[c]	2.45[c]	51.5[d]
BON	58.6[b]	30.5[a]	2.35[b]	3.58[b]	64.3[b]

Different letters in the column mean significant difference (P<0.05).

acid with significant reduction of pH (Hashemzadeh-Cigari et al., 2011; Jatkauskas and Vrotniakiene, 2011; Sánchez et al., 2014). According to Đorđević and Dinić (2003), average 3-7% of lactic acid is contained in good quality silage. Alfalfa silages inoculated with PIO2 and BON had higher acetic acid content than the control and PIO1 and SIL inoculated silages. Acetic acid has strong antifungal influence, and its high concentration was probably the primary reason for improvements in the aerobic stability of silages treated with *Lactobacillus buchneri* (Kung and Ranjit, 2001). If the content of acetic acid is up to 5.5% of the dry matter, it is considered to be a good quality silage (Đorđević and Dinić, 2003). Alfalfa silages inoculated with BON had lower lactic acid content ($P<0.05$) compared with silages inoculated with products PIO1, SIL and PIO2 treated silages, but produced more ($P<0.05$) lactic acid than control silage (Table 4). Lower pH and higher lactic acid content in silage fermentation with homofermentative lactic acid bacteria (PIO1 and SIL) are contents when such inoculants were successful (Kung et al., 2003). Heterofermentative lactic acid bacteria are less efficient in producing lactic acid than homofermentative lactic acid bacteria, usually resulting in more acetic acid, higher pH, higher ethanol and higher dry matter losses (Filya et al., 2007). Furthermore, 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. The largest ratio of lactic acid to acetic acid after ensiling in alfalfa silages was produced by PIO1 and SIL products, whereas PIO2 and BON inoculants produced the smallest ratio of lactic acid to acetic acid in alfalfa silages (Figure 2).

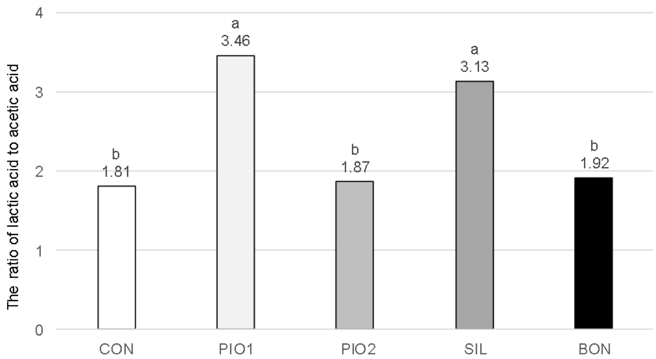

Figure 2. The ratio of lactic acid to acetic acid (different letters in the figure mean significant difference, $P<0.05$)

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., 2010). 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 result in lower pH value, which was achieved in the treated silage with PIO1, SIL and PIO2. Acceptable silages generally contain <3% acetic acid, <0.1% butyric acid, and <0.5% propionic acid (Ward, 2011). According to Škultéty (1999) quality alfalfa silage has lower content of butyric acid under 2.5 g/kg of dry matter. High ethanol concentration of the control silage probably also resulted from a clostridial fermentation, as suggested by Muck (2012). In alfalfa silage, ammonia-N fraction was lower for inoculant treated silages compared to the control silage (Table 4). Higher ammonia indicates protein brake down from proteolytic enzymatic activity (Seglar, 2003). Usually, silage with high concentrations of ammonia coupled with butyric acid may also have significant concentrations of other undesirable end products, such as amines, that may reduce animal performance (Kung and Shaver, 2001). 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 (Richard et al., 2009). 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*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus acidophilus* or *Lactobacillus 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 Schnlirer, 2005).

CONCLUSIONS

Results showed that values of dry matter and crude protein value were insignificantly higher, while lactic and acetic acid value significantly increased in treated silage with inoculant PIO1, PIO2, SIL and BON, compared to control silages of alfalfa cultivar Mirna. Contrary, alfalfa silage treated with a bacterial inoculant PIO1, PIO2, SIL and BON had lower values of acid detergent fibre, neutral detergent fibre and pH and significantly lower values of butyric acid, alcohols and NH_3-N/total nitrogen compared to control silage. Lower pH in inoculated samples probably inhibited protein degradation and therefore concentrations of ammonia−nitrogen were lower in those samples which demonstrates positive effect of PIO1, PIO2, SIL and BON on nutritive value of silage. Adding bacterial inoculant PIO1, PIO2, SIL and BON may be a promising management practice to improve fermentation, conserve more nutrients and increase their availability to the ruminants.

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