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RESEARCH ARTICLE

THE EFFECTS OF LACTIC ACID BACTERIAL INOCULANTS ON THE FERMENTATION, AEROBIC STABILITY AND NUTRITIVE VALUE OF SUNFLOWER SILAGES

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ABSTRACT

This study was implemented to determine the effect of lactic acid bacterial inoculants as silage additives on fermentation, aerobic stability, and *in vitro* organic matter digestibility of sunflower silages. Lalsil®Dry (Lallemand, France), containing water soluble *Lactobacillus buchneri*, *Pediococcus acidilactici* with cellulase and hemicellulase enzymes, was chosen as bacterial inoculants. The inoculants were applied to silages at the rates of 5, 10 and 20 mg kg^{-1} fresh weight levels. After the treatment, chopped whole crop sunflower was ensiled in 1.0-L special vacuum bags. The bags were stored at 25±2 °C under laboratory conditions. Three bags from each group were sampled for chemical and microbiological analysis on the 90th day after ensiling. At the end of the ensiling period, all silages were subjected to an aerobic stability test for five days. In addition, *in vitro* organic matter digestibility of all silages was determined. The results showed that lactic acid bacterial inoculants enhanced the characteristics of fermentation and decreased acid detergent fiber and acid detergent lignin contents of sunflower silages. When compared to the control group, the aerobic stability was found to get improved in Lalsil treatments, as indicated by reduced pH value, carbon dioxide production and yeast populations. Treated silage groups appeared with higher *in vitro* organic matter digestibility than the control group.

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INTRODUCTION

Sunflower is a well-known silage crop which is grown worldwide including Turkey. Compared to corn silage sunflower silage provides higher dry matter yield with better cold and heat resistance and drought tolerance. It is also easier to get sunflower adapted to wide range of climatic conditions since it possesses higher concentrations of crude protein (CP) and ether extract (EE) content (Goes *et al.,* 2012; Gandra *et al.,* 2017). However, these advantages of sunflower silage are restricted by difficulty in ensiling process due to its high-fibre content, which ultimately reduces the digestibility of nutrients and results in lowered dry matter content at maturity period (Demirel *et al.,* 2008; Ozduven *et al.,* 2009; Peiretti and Meineri, 2010). In order to enhance the ensiling process, several additives have been employed either to decrease fermentation and reduce effluent or to improve aerobic stability and raise nutritive value of silage (Filya, 2003b). Biological additives such as lactic acid bacteria (LAB) inoculants are known to be safe, easy to use and non-corrosive to machinery while being away from causing environmental pollution. Most LAB inoculants contain homo-fermentative LAB (e.g. *Lactobacillus plantarum, Enterococcus faecium and Pediococcus* species) rather than hetero-fermentative LAB (e.g. *Lactobacillus buchneri*). Earlier research has revealed that homo-fermentative LAB inoculants usually increase lactic acid while decrease acetic acid, butyric acid and ammonia-nitrogen (NH_3-N) levels and the pH value of the silage (Sheperd*et al.*, 1995; Driehuis *et al.,* 1997; Aksu *et al.,* 2004). On the other hand, homo-fermentative LAB has been reported to enhance aerobic deterioration of silages, presumably due to insufficient volatile fatty acids needed to inhibit fungal growth (Weinberg *et al.,* 1993; Filya *et al.,* 2000). Numerous studies have reported that *Lactobacillus buchneri*, type of hetero-fermentative LAB, increases aerobic stability of silage by enhancing acetic acid release and preventing yeast and mould formation while reinforcing heat resistance of the silage when opened (Kung and Ranjit2001; Holzeret al. 2003; Kleinschmit and Kung 2006; Filya *et al.,* 2006; Tabacco *et al.,* 2009; Reichand Kung, 2010; Mohammadzadeh *et al.,* 2011; Li *et al.,* 2016a). The use of *Lactobacillus buchneri* LAB inoculants has also been characterized with some negative effects including loss of dry matter content, silage intake and higher pH value compared to its relatively lower positive effect on silage

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quality (Holzer *et al.,* 2003; Filya *et al.* 2006; Tabacco *et al.* 2009; Basso *et al.* 2012). Besides the use of homo-fermentative and hetero-fermentative LAB inoculants separately, a combination of other bacteria from varying classes such as *Lactobacillus buchneri* and *Lactobacillus plantarum* has been found to be useful during ensiling processes (Driehuis *et al.,* 1999; Kung and Ranjit, 2001; Weinberg *et al.,* 2002; Filya, 2003a; Filya, 2003b; Kleinschmit and Kung, 2006; Hu *et al.,* 2009; Reichand Kung, 2010; Basso *et al.,* 2014;Wang *et al.,* 2014; Li *et al.,* 2016b). Regarding the conceptualization in previous literature as indicated above, this study aims to determine the effect of various concentrations of homo- and hetero-fermentative LAB inoculants containing fibrolytic enzymes (E) on the fermentation characteristics, aerobic stability and *in vitro* organic matter digestibility of sunflower silages.

MATERIALS AND METHODS

Sunflower plant material was obtained at the late flower stage of maturity (31.10 ± 0.81% DM) on the date of August17, 2013 from the Agricultural Research Field of Erciyes University. After that, plants were chopped at about 2- to 4-cm in size and ensiled in 1L special vacuum bags. The ensiled vacuum bags were stored at room temperature (20°C ± 3°C) for 90 days. For the treatment processes, a commercial inoculant LALSIL®DRY (LD) (Lallemand, Montréal, Québec, Canada) containing *Lactobacillus buchneri* (>6×10^{10}colony-forming units [cfu] g^{-1}), *Pediococcus acidilactici* CNCM (>2×10^{10}cfu g^{-1}) as well as cellulase and hemicellulase enzymes (enzyme activity >20 000 UI g^{-1}) was used. The proceeding treatments were applied to fresh forages respectively: (1) Control (C, no additives), (2) with LD inoculants at the levels of 5 mg kg^{-1} fresh weight (FW) (LD low), (3) with LD inoculants at the levels of 10 mg kg^{-1} FW (LD med), (4) with LD inoculants at the levels of 20 mg kg^{-1} FW (LD high). LALSIL®DRY inoculants were sprayed on the chopped sunflower fresh materials after being dissolved in 20 mL distilled water. The control crops were also treated with the same amount of distilled water.

Chemical Analyses

Next, fresh and ensiled crops were sampled for chemical and microbiological analyses in three bags per treatment at each time, just on the 90th day after ensiling. In order to carry out pH measurements, 25 g of silage samples were taken into a beaker and 100 mL distilled water was added. The mixture was first blended for 5 minutes and filter through Whatman filter paper, after that pH measurement was undertaken using a digital pH meter (Akyildiz, 1986). Dry matter (DM, Method 934.01) contents of the silages were determined by drying the samples first at 60°C for 72 h in an oven before being milled via 1-mm screen and re-drying for another 3 h at 103°C. In addition, crude protein (CP) was determined using the method explained in AOAC (1990) where crude ash (CA) was obtained by drying the silage content at 600°C for 4 h. The water soluble carbohydrates (WSCs) content of silages was examined by a spectrophotometer (Shimadzu UV-1201, Kyoto, Japan) concerning its reaction with an antron reagent (Anonymous, 1986). The ammonia nitrogen (NH_3-N) content of silage samples was also determined regarding the above-mentioned method (Anonymous, 1986). The lactic acid (LA) and acetic acid (AA) contents of silages were examined by the spectrophotometric method (Koc and Coskuntuna, 2003). Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL) were analyzed using the sodium sulphite addition method with residual ash (Goering and Van Soest, 1983). In order to determine cellulose (CELL) and hemicelluloses (HEM) content of the silages, NDF, ADF and ADL values were compared so that the difference between NDF and ADF was considered for HEM while ADF and ADL difference was being analysed to identify CELL content. *In vitro* OMD analysis was implemented through a three-stage technique suggested by Aufrère and Michalet-Doreau (1988). The technique was based on pre-treatment of pepsin into hydrochloric acid (0.2% pepsin in 0.1 N HCl), starch hydrolysis and the attack by cellulose (Onozuka R 10 from *Trichodermaviride*, Merck). The aerobic stability test was carried out for five days after the ensiling period according to the procedures reported by Ashbell *et al.* (1991). For this procedure, three variables including the numbers of yeasts and moulds, change in pH and the amount of carbon dioxide (CO_2) released were counted as aerobic deterioration indicators.

Microbiological Analyses

The microbiological analysis of the samples was implemented via methods defined by Seale *et al.* (1990) in order to figure out the numbers of *lactobacilli,* yeast and mould in the samples. Microbiological examination comprised the enumeration of lactobacilli on pour plate Rogosa agar (Oxoid CM627 incubated at 30°C for 3 days) while examining yeast and mould on spread plate malt extract agar (acidified with LA to pH 4.0 and incubated at 30°C for 3 days). The acquired numbers concerning the *lactobacilli*, yeast and mould content of silages were converted into logarithmic coli form unit (cfu/g) for the proceeding step of statistical analysis.

Statistical Analysis

Data were analysed using general linear model procedures of the SPSS 15.0 statistical package software (SPSS 15.0© for Windows; SPSS Inc., Chicago, IL, USA). Duncan's multiple range tests were utilized to determine the differences between reported mean scores, which were considered significant at $P<0.05$ level of probability. Results of those statistical analyses were displayed in tables, under the columns of mean scores for experimental and control groups as well as standard errors of the means (SEM), as specified in the next section.

RESULTS AND DISCUSSION

The current research revealed the effect of LD on chemical and microbiological composition, aerobic deterioration tendency and *in vitro* OMD of silages. Comparative analyses of different components of chemical analysis are presented in Table 1. Silage pH is considered as one of the basic criteria revealing the extent of fermentation and quality of ensiled forages. A pH range of 3.7-4.2 is usually accepted as beneficial for whole-crop cereal preservation (Kung and Shaver, 2001). In the current study, after 90 days of ensiling, the pH values slightly increase in all treated groups compared to control group. This result is consistent with the previous findings where it has been shown that the possible increase of pH in sunflower silage caused by the inoculation of LD at ensiling (Driehuis *et al.,* 2001; Kristensen *et al.,* 2010). It is interesting to note that, in case of corn silage inoculation of LD has no significant effect on pH value compared to control (Reich and Kung, 2010) while a decrease in pH was observed in potato hash silage (Nkosi and Meeske, 2010).

Table 1. Results of the chemical analyses of the sunflower silages

Treatment	Control	LD-low	LD-mid	LD-high	P
pH	3.55±0.05[b]	3.89±0.07[a]	3.76±0.04[a]	3.79±0.05[a]	*
DM, %	27.89±0.47[b]	32.03±0.57[a]	31.69±0.17[a]	32.02±0.17[a]	**
CA, DM%	12.70±0.72	14.21±0.33	14.32±0.28	14.49±1.00	NS
WSCs, g/kg DM	13.17±1.40[a]	6.65±0.28[b]	5.74±0.15[b]	5.95±0.43[b]	**
CP, DM%	6.83±0.13[b]	7.41±0.18[a]	7.72±0.10[a]	7.72±0.10[a]	**
NH_3-N, g/kg TN	65.93±3.12[a]	48.51±3.21[b]	46.07±1.41[b]	51.53±5.24[b]	**
LA, g/kg DM	35.02±1.43[b]	48.41±1.64[a]	49.98±1.39[a]	49.20±2.23[a]	**
AA, g/kg DM	20.19±0.82[c]	23.73±0.55[b]	24.64±0.67[ab]	26.68±0.55[a]	**

DM: dry matter; CA: crude ash; WSCs: water-soluble carbohydrates; CP: crude protein; NH_3-N: ammonia-nitrogen; TN: total nitrogen; LA: lactic acid; AA:acetic acid; LD-low: 5 mg LalsiL Dry (LD) kg^{-1} fresh weight (FW) forage; LD-mid: 10 mg LD kg^{-1}FW forage; LD-high: 20 mg LD kg^{-1} FW forage; * P<0.05; ** P<0.01; NS, not significant; [a,b] Values with different superscript in a line differ significantly between treatment groups.

Table 2. Results of the microbiological analyses of the sunflower silages (log cfu/g DM)

Treatment	Control	LD-low	LD-mid	LD-high	P
Lactobacilli	4.68±0.12[b]	5.10±0.15[a]	5.65±0.19[a]	5.48±0.27[a]	*
Yeast	2.95 ±0.24[a]	2.04±0.08[b]	2.16±0.12[b]	2.01±0.19[b]	**
Mould	2.38±0.16[a]	1.75±0.06[b]	1.81±0.03[b]	1.79±0.05[b]	**

LD-low: 5 mg LalsiL Dry (LD) kg^{-1} fresh weight (FW) forage; LD-mid: 10 mg LD kg^{-1}FW forage; LD-high: 20 mg LD kg^{-1} FW forage; * P<0.05; ** P<0.01; [a,b] Values with different superscript in a line differ significantly between treatment groups.

Table 3. Results of the aerobic stability test (5 days) of the sunflower silages

Treatment	Control	LD-low	LD-mid	LD-high	P
pH	5.31±0.05[a]	4.70±0.08[b]	4.61±0.01[b]	4.82±0.17[b]	**
CO_2, g/kg DM	12.04±0.36[a]	9.03±0.80[b]	7.89±0.44[b]	8.00±0.17[b]	**
Yeast, log_{10}cfu/g	5.49±0.36[a]	4.59±0.30[b]	4.12±0.15[b]	4.46±0.14[b]	*
Mould, log_{10}cfu/g	3.76±0.16	3.37±0.17	3.27±0.14	3.33±0.16	NS

LD-low: 5 mg LalsiL Dry (LD) kg^{-1} fresh weight (FW) forage; LD-mid: 10 mg LD kg^{-1}FW forage; LD-high: 20 mg LD kg^{-1} FW forage; * P<0.05; ** P<0.01; NS: not significant; [a,b] Values with different superscript in a line differ significantly between treatment groups.

Table 4. Cell wall contents and *in vitro* OMD analyses of the sunflower silages (% DM)

Treatment	Control	LD-low	LD-mid	LD-high	P
NDF	39.76±1.66	34.34±1.98	34.82±0.76	34.89±2.29	NS
ADF	33.65±0.67[a]	31.09±0.89[b]	29.01±0.62[b]	29.08±0.62[b]	**
ADL	8.08±0.44[a]	6.50±0.68[ab]	5.39±0.22[bc]	4.71±0.49[c]	**
HEM	6.11±1.95	3.25±1.38	5.81±1.26	5.81±1.97	NS
CELL	25.58±0.23	26.26±1.65	23.90±0.72	24.37±0.71	NS
OMD	47.15±0.44[b]	50.20±0.93[a]	50.44±0.11[a]	50.41±0.98[a]	*

NDF. neutral detergent fiber; ADF. acid detergent fiber; ADL. acid detergent lignin; HEM: hemicellulose; CELL: cellulose; OMD: organic matter digestibility.; * P<0.05; ** P<0.01; NS. not significant.; [a, b, c,] Values with different superscript in a line differ significantly between treatment groups

Crude ash contents in treatment groups were not differ significantly from the control group whereas the CP content was significantly increased after LD treatment. Hargreaves *et al.* (2009) mentioned the crucial feature of CP content in forages by emphasizing its compositional effect on the nutritional quality of silages. The present study complies with the above-mentioned importance as well as the findings of Xu *et al.* (2011), who referred to increased CP content with LD inoculation in erect milkvetch silage compared to relative and inconsistent values of CP content improved by LD inoculation as described in some other studies (Zhang *et al.,* 2013, Nkosi *et al.,* 2016).Another result acquired form LD-treated silages was reduced residual WSCs (P <0.05), which reminds the presumption that residual WSCs in silage may become a substrate for aerobic microbes during the feeding-out phase (Weinberg *et al.,* 1993). High-quality silage is likely to be achieved when LA is the predominant acid produced and recommended LA concentration for high quality silage remains within the range of 40 to 120 g/kg DM (McDonald *et al.,* 1991).

In the present study, LA contents in all treatments of silages were within the recommended range, indicating well-fermented silages. Compared with control group, increased level of LA was detected in the inoculated sunflower, which is in contrast to decreased LA production in whole plant soybeans with LD treatment, revealed by Nkosi *et al.* (2016) although the results were in consistence with Nkosi *et al.* (2015) in the sense that higher production of acetic acids in LD inoculant silages, which also justified the general findings of several studies concerning the impact of inoculation with *L. buchneri* (Kleinschmidt and Kung, 2006; Kristensen *et al.,* 2010; Nkosi *et al.,* 2012; Nkosi *et al.,* 2015; Nkosi *et al.,* 2016). The latest compositional contents to be analysed were NH_3-N. Ammonia-N in silage is an indicator of the degree of protein degradation which impairs the nutritive value of forages. Regarding the literature, well-preserved silages should not exceed 100 g NH_3-N/kg total nitrogen (TN) (McDonald *et al.,* 2002). In the present study, treated silages were found to have lower levels of NH_3-N in comparison to the untreated control group with no clear dose-response effect. These results

were likely to point out the reductive effect of inoculants on proteolysis, in parallel to the findings of Zhang *et al.* (2013) and Nkosi *et al.* (2015) expressing lowered the amount of NH_3-N in silages due to LD inoculation. Nevertheless, treated silages of the present study produce NH_3-N content at acceptable levels (<100 g NH_3-N/kg TN) for well-preserved silages (McDonald *et al.,* 2002). Next, we examined the microbiological composition of the silages regarding *lactobacilli*, yeast and mould values and are presented in Table 2. In the present study, *lactobacilli* count in all LD-treated silages was significantly increased ($P<0.05$) whereas yeast and mould counts were significantly decreased compared with control silage ($P<0.05$). The microbiological analyses revealed the beneficial effects of LD inoculants on the microbiological composition of sunflower silages, compared with untreated group. These results consistent with those of previous studies (Weinberg *et al.,* 1995; Sucu and Filya 2006; Xu *et al.,* 2011) where they reported similar findings. The impact of LD treatment on the aerobic stability of sunflower silages after exposure to air for five days is shown in Table 3. The aerobic deterioration of silage holds the risk of causing proliferation in potential pathogenic or undesirable micro-organisms, which may decrease the performance of animals. Higher CO_2 production in silage remarks the activity of yeasts and moulds, which raises the temperature and impairs the quality of silage (Woolford, 1990; Ashbell *et al.,* 1991). In the present study, aerobic stability of LD-treated silage was significantly better than that of control silage in terms of intensive CO_2 production, rise in pH value and enlarged yeast population (Table 3). However, no difference was observed within groups in case of moulds production. It has been estimated that the enhancement in aerobic stability of LD-treated silages was due to the effect of acetic acids.

The preceding studies define acetic acids as fungicidal agent and found to has inhibitory effects on the growth of yeasts and moulds in silages (Weinberg *et al.,* 1993; Filya and Sucu, 2007). Finally, we analysed the cell wall content and *in vitro* OMD and the results are presented in Table 4. There was a significant decrease in ADF and ADL contents in all LD-treated silages compared to the control group ($P<0.05$). Although the difference was not significant, lower trend was observed in almost all LD-treated silages in terms of NDF, HEM and CELL contents. It has been predicted that the utilization of enzymes as silage additives would deteriorate cell wall and subsequently improve the digestibility of silage fibre (McDonald *et al.,* 1991). These results are consistent with the previous studies (Koc *et al.,* 2009) where the authors showed that LAB inoculation significantly lowered the ELL content of sunflower silages. By contrast, there are some empirical data showing the detrimental effect of LAB+E mixture inoculation on cell wall contents of sunflower silages (Demirel *et al.,* 2006). Furthermore, Ozduven *et al.* (2009) demonstrated that LAB+E mixture inoculation also reduced the NDF ratio in sunflower silage. The present study, LD-treatment significantly ($P<0.05$) increases the *in vitro* OMD contents in treated silage compared to control silage. Demirel *et al.* (2006) suggested that a decrease in NDF in silage materials could increase the *in vitro* OMD of LAB inoculants treated silage. In the present study, we found there was a decrease in NDF and ADF contents for all LD-treated silages that may also indicate the improved quality of silage in terms of *in vitro* OMD of silages. These findings are an agreement with previous studies (Ozduven *et al.,* 2009; Sucu and Aydogan Ciftci, 2016) where the authors demonstrated that the LAB inoculation with E increased the OMD of silages. Ozduven *et al.* (2017) reported that LAB+E mixture addition numerically increased *in vitro* OMD of sunflower silages compared to control. The *in vivo* dry matter digestability (IVDMD)and *in vivo* crude protein digestability (IVCPD) of fresh forage were significantly higher than silages – likely due to components, such as WSC and protein that are easy to degrade, being consumed for microbe growth (Nadeau *et al.* 2000). In addition, LB-treated silages had higher IVDMD and IV-CPD than the control. Previous studies (Harrison *et al.* 1989; Aksu *et al.* 2004) showed that inoculation of LAB at ensiling could improve IVDMD and *in vivo* neutral detergent fiber digestibility (IVaNDFD) of grass silage in mixtures with legume or corn silage. In the present study, IVDMD and IVCPD of the LB-treated silage were significantly higher than the control. This may be due to LAB significantly improving the quality of silage fermentation, inhibiting adverse microbial fermentation, in particular, inhibiting protein digestion and hydrolysis, thereby increasing IVDMD and IVCPD.

However, LB treated silages had no effect on IV aNDFD, the reason may be because LAB cannot degrade fiber. The E treatment had lower aNDF, ADF and ADL contents than control, whereas, higher aNDF content than the LB treatment. The concentrations of WSC, sucrose, glucose and fructose in the E and LB treatments were not significantly different compared with control. Furthermore, *in vivo* acid detergent fiber digestibility (IVADFD) in the E treatment was the lower and IVaNDFD higher compared to the control. It can be concluded that some of the CELL was decomposed into HEM, as also found by Van Vuuren *et al.* (1989). The IVDMD and IVCPD of fresh forage were significantly higher than silages – likely due to components, such as WSC and protein that are easy to degrade, being consumed for microbe growth (Nadeau *et al.* 2000). In addition, LB-treated silages had higher IVDMD and IVCPD than the control. Previous studies (Harrison *et al.* 1989; Aksu *et al.* 2004) showed that inoculation of LAB at ensiling could improve IVDMD and IVaNDFD of grass silage in mixtures with legume or corn silage. In the present study, IVDMD and IVCPD of the LB-treated silage were significantly higher than the control. This may be due to LAB significantly improving the quality of silage fermentation, inhibiting adverse microbial fermentation, in particular, inhibiting protein digestion and hydrolysis, thereby increasing IVDMD and IVCPD. However, LB treated silages had no effect on IVaNDFD, the reason may be because LAB cannot degrade fiber.

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IVCPD of the LB-treated silage were significantly higher than the control. This may be due to LAB significantly improving the quality of silage fermentation, inhibiting adverse microbial fermentation, in particular, inhibiting protein digestion and hydrolysis, thereby increasing IVDMD and IVCPD. However, LB treated silages had no effect on IVaNDFD, the reason may be because LAB cannot degrade fiber. The E treatment had lower aNDF, ADF and ADL contents than control, whereas, higher aNDF content than the LB treatment. The concentrations of WSC, sucrose, glucose and fructose in the E and LB treatments were not significantly different compared with control. Furthermore, IVADFD in the E treatment was the lower and IVaNDFD higher compared to the control. It can be concluded that some of the cellulose was decomposed into hemicellulose, as also found by Van Vuuren *et al.* (1989).

Conclusion

This study confirms that all types of LD treatments had an beneficial effect on fermentation characteristics, impaired aerobic stability, decreased ADF and ADL contents of sunflower silages. In addition, it also showed that LD treatments particularly increased *in vitro* OMD of sunflower silages.

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