The Effects of Lactic Acid Bacterial Inoculants on the Fermentation and Aerobic Stability of Sunflower Silages

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This study was carried out to determine the effects of homofermentative and/or heterofermentative lactic acid bacteria inoculants on the fermentation, aerobic stability and *in vitro* organic matter digestibility characteristics of sunflower silages. Sunflower was harvested at the milk stage of maturity. Inoculant 1188 (Pioneer®, USA) was used as homofermentative lactic acid bacteria whereas inoculant 11A44 (Pioneer®, USA) was used as heterofermentative lactic acid bacteria inoculant. Inoculants were applied to the silages at 6.00 $\log_{10}$ cfu/g levels. After treatment, the chopped whole crop sunflower was ensiled in 1.0-litre special anaerobic jars, equipped with a lid enabling gas release only. The jars were stored at 25±2°C under the laboratory conditions. Three jars from each group were sampled for chemical and microbiological analyses 2, 4, 8 and 60 days after ensiling. At the end of the ensiling period, all silages were subjected to an aerobic stability test for 5 days. In addition, *in vitro* organic matter digestibility of those silages were determined. The results revealed that homofermentative lactic acid bacteria inoculants increased the characteristics of fermentation but impaired the aerobic stability of the sunflower silages ($P<0.05$). However, the application of heterofermentative lactic acid bacteria increased the concentration of acetic acid and the aerobic stability ($P<0.05$) of the sunflower silages. *In vitro* organic matter digestibility was numerically increased for treated than control silages ($P>0.05$).

Key Words: Lactic acid bacterial inoculants, silage fermentation, whole plant sunflower, aerobic stability, in vitro organic matter digestibility

Laktik Asit Bakterileri İnokulantlarının Ayçiçeği Silajının Fermantasyon ve Aerobik Stabilite Özellikleri Üzerine Etkileri

Bu çalışma homofermantatif ve/veya heterofermantatif laktik asit bakteri inokulantları ilavesinin, ayçiçeği silajlarında fermantasyon, aerobik stabilite ve in vitro organik madde sindirilebilirliği özellikleri üzerindeki etkilerinin saptanması amacı ile düzenlenmiştir. Araştırmada kullanılan ayçiçeği bitkisi süt olum döneminde hasat edilmiştir. Homofermantatif laktik asit bakterisi olarak inokulant 1188 (Pioneer®, USA) ve heterofermantatif laktik asit bakterisi olarak inokulant 11A44 (Pioneer®, USA) kullanılmıştır. İnokulantlar silajlara 6,00 $\log_{10}$ koloni form ünite/g düzeyinde katılmışlardır. Ayçiçeği hasılları yalnızca gaz çıkışına olanak tanıyan, 1,0 litrelik özel kavanozlara silolanmıştır. Kavanozlar laboratuvar koşullarında 25±2 °C' de depolanmışlardır. Silolamadan sonraki 2, 4, 8 ve 60. günlerde her gruptan 3'er kavanoz açılarak silajlarda kimyasal ve mikrobiyolojik analizler yapılmıştır. Silolama döneminin sonunda açılan tüm silajlara 5 gün süre ile aerobik stabilite testi uygulanmıştır. Ayrıca bu silajların, *in vitro* organik madde sindirilebilirliği saptanmıştır. Sonuç olarak homofermantatif laktik asit bakteri inokulantı ayçiçeği silajlarının fermantasyon özelliklerini arttırmış ancak aerobik stabilitelerini düşürmüştür ($P<0.05$). Bununla birlikte heterofermantatif laktik asit bakteri inokulantı ile muamele edilmiş ayçiçeği silajlarının asetik asit içeriği ile aerobik stabilitesi artmıştır ($P<0.05$). *İn vitro* organik madde sindirilebilirliği üzerine muamelelerin etkisi önemsiz ($P>0.05$) bulunmuştur.

Anahtar Kelimeler: Laktik asit bakteri inokulantları, Fermantasyon, Aerobik stabilite*, in vitro* organik madde sindirilebilirliği

Introduction

Sunflowers have been grown successfully as silage crops in many parts of the world. Compared to corn, high dry matter yield acquired from sunflower is higher while it is more drought resistant and sustains more cold tolerance. In contrast to the former, sunflower silage is known to have higher fibre content, which reduces digestibility of nutrient matters (Demirel et al. 2006, Ozduven et al. 2009). Sunflower is available for ensiling though its ensiling and nutritional quality depend upon the stage of maturity at the harvest time (Koc et al. 2009).

Being a preservation technology for moist whole-plant forage crops, ensiling is a process of lactic acid fermentation under anaerobic conditions. The process is based on the conversion of water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid. The lactic acid bacteria (LAB) are used as the converting agent during this process, which results in pH decrease, so that forage is preserved for a long time (Filya 2000). The application of silage additives has become the conventional practice to control the ensiling process. Although the main objective in using silage additives is to ensure the fermentation process for well-preserved silages, it is also utilized as an effective method to reduce the ensiling losses and to raise the aerobic stability of silages during the feed-out period (McDonald et al. 1991). In order to improve the ensiling process, various chemical and biological additives have been developed. Among those, biological additives are regarded as advantageous since they are safe and easy to use, non-corrosive to machinery, environment-friendly and naturally produced (Sucu and Filya 2006). Bacterial inoculants, comprising homofermentative (HM) LAB such as *Lactobacillus plantarum*, *Enterococcus faecium* and *Pediococcus* species, generally increase lactic acid and decrease the acetic acid, butyric acid and ammonia-nitrogen (NH_3-N) levels as well as the pH of the silage (Sheperd et al. 1995, Filya 2003, Aksu et al. 2004). Heterofermentative (HT) LAB usually enhances the aerobic stability of silage (Driehuis et al. 1999, Mohammadzadeh et al. 2011) by converting lactic acid to acetic acid under anaerobic conditions. Via this conversion process, fungi are inhibited and silages, which are susceptible to spoilage upon exposure to air, are preserved (Filya et al. 2007, Jatkauskas and Vrotniakiene 2011). *Lactobacillus buchneri* is the main HT LAB inoculant most widely used during the ensiling of forages (Muck 2008).

The aim of this study is to determine the effects of HM and/or HT LAB inoculants on the fermentation aerobic stability and *in vitro* organic matter digestibility characteristics of sunflower silages.

Materials and Methods

During the research, sunflower was harvested by hand at the dough stage (22.89% DM) and was undergone laboratory-type cropping, which was done approximately to 2.0 cm size, before being ensiled in 1.0-litre special anaerobic jars (Weck, Wher-Oftlingen, Germany), equipped with a lid that enables gas release only. There were 48 jars per crop which were stored at ambient temperature (25±2°C). In order to have chemical and microbiological analyses, fresh and ensiled materials were sampled in three jars per each treatment on every ensiling interval after the 2^{nd}, 4^{th}, 8^{th} and 60^{th} days of ensiling. At the end of the whole ensiling period, the silages were subjected to an aerobic stability test for 5 days in a system developed by Ashbell et al. (1991). In this system, the numbers of yeasts and molds change in pH and the amount of CO_2 produced during the test is to be used as an aerobic deterioration indicator.

In the present research, the chopped sunflower was mixed and divided into equal portions for treatment: (1) distilled water, denoted as treatment control; (2) a mixture of LAB consisting of *Lactobacillus plantarum* and *Enterococcus faecium* (Pioneer 1188, USA), treatment HM LAB; (3) a mixture of LAB consisting of *Lactobacillus buchneri*, (Pioneer 11A44, USA), treatment HT LAB; (4) combination of treatment HM+HT LAB. The application rate determined by the manufacturers stated the level of LAB in the products. On the day of the experiment, inoculants were suspended in 20 ml of tap water and the whole suspension was sprayed over 10 kg (wet weight) of chopped forage spread over a 1x4 m area. All inoculants were applied to the forages in a uniform manner with constant mix. The control silage was treated with an equivalent amount of water.

For the application, the pH values and ammonia nitrogen (NH_3-N) content of fresh and silage samples were determined according to the literature (Anonymous, 1986). The WSC content of silages was determined by the spectrophotometer (Shimadzu UV-1201, Kyoto, Japan) after the reaction with an antron reagent (Anonymous 1986). The spectrophotometric method (Koc and Coskuntuna 2003) was utilized to determine the lactic and acetic acid amounts, whereas *lactobacilli*, yeast and mold numbers were obtained through the methods reported by Seale et al. (1990). The microbiological examination included the enumeration of *lactobacilli* on pour plate Rogosa agar (Oxoid CM627 incubated at 30°C for 3 days) while the enumeration of yeast and mold being done on spread plate malt extract agar (acidified with LA to pH 4.0 and incubated at 30°C for 3 days). During this examination, the *lactobacilli,* yeast and mold numbers of the silages were converted into the

logarithmic coli form unit (cfu/g) and the fermentation losses were evaluated according to weight loss (Filya 2003). The DM content of the fresh and silage materials were determined by drying at 60°C for 72 h in a fan-assisted oven, followed by milling through a 1-mm screen and drying for another 3 h at 103°C. The crude protein (CP) content was determined by following the procedures of the Association of Official Analytical Chemists (1990) whereas the neutral detergent fibre (NDF) and acid detergent fibre (ADF) determinations were performed regarding the instructions by Goering and Van Soest (1983). Furthermore, the three-stage procedure reported by Aufrère and Michalet-Doreau (1988) was proceeded to analyze *in vitro* organic matter (OM) digestibility of the silages as described by the steps of pre-treatment with pepsin in hydrochloric acid (0.2% pepsin in 0.1 N HCl), the starch hydrolysis and the attack by cellulase (Onozuka R 10 from *Trichodermaviride*, Merck), respectively. The statistical analyses of the findings included one-way analysis of variance and Duncan's multiple range tests, which were applied to the data using the Minitab statistical package program (2000).

Results and Discussion

The research findings are discussed considering the four categories initiated by the chemical and microbiological analyses, followed by the aerobic stability test and finalized by *in vitro* OM digestibility of the sunflower silages. At the first stage, the chemical composition of the fresh and ensiled sunflower is given in Table 1. As displayed in the table, the sunflower used for ensiling was characterized by 22.89% DM content, with the concentration of CP by 8.60% and the concentration of WSCs by 41.19 g/kg DM. All silages were well-preserved. The results indicated that, with respect to DM and CP content, there were no significant differences between LAB inoculants and control silages on any of the sampling days. Generally, the addition of LAB inoculants to ensiling is intended to ensure rapid and vigorous fermentation that results in faster accumulation of lactic acid, lower pH values at earlier stages of ensiling, and improved forage conservation. According to the literature, well-preserved sunflower silage is characterized by lower pH, greater lactic acid content and lower contents of NH_3-N (Muck and Kung 1997, Zhang et al. 2009). In the present study, the pH of all inoculated silages decreased faster in 2 and 4 days and to a greater extent as compared with the control silage ($P<0.05$). Moreover, after 60 days of ensiling, sunflower silages treated with HM LAB properly improved the silage fermentation quality with markedly higher lactic acid content as compared with the control silage ($P<0.05$). The concentration of acetic acid was increased ($P<0.05$) in response to the inoculation with HT or HM+HT LAB due to the heterofermentative activity of *Lactobacillus buchneri*. The lower NH_3-N concentration in the HM and/or HT LAB-treated silage (P <0.05) in the present study suggests that the inoculants have reduced proteolysis, which is consistent with Filya (2003b) and Driehuis *et al.* (1999), who reported the reduction of NH_3-N by *Lactobacillus buchneri* + *Lactobacillus plantarum* inoculation as compared to untreated silages. The homofermentative bacteria such as *Lactobacillus plantarum* usually accelerate the drop in pH at the beginning of ensiling (Weinberg and Muck 1996, Driehuis et al. 1997). According to McDonald et al. (1991), this effect arose as a result of the pH reduction with inoculation which inhibited the protein degradation in silages. Moreover, at the end of the 60 days of ensiling, the HM and/or HT LAB -treated silages did not affect the NDF and ADF content of sunflower silage compared to untreated sunflower silage, which is in agreement with past findings (Ranjit and Kung 2000, Filya 2003a,b, Kleinschmit et al. 2005).

The microbiological composition of the silages is revealed in Table 2, which shows the increase in *lactobacilli* numbers during the fermentation period.

Table 1. Results of the chemical analyses of the sunflower silages after 60 days of ensiling

Days of Ensiling	Treatment	pH	DM, %	WSCs, g/kg DM	CP, % DM	NH_3-N, g/kg TN	LA, % DM	AA, % DM	NDF, % DM	ADF, % DM
0		5.74	22.89	41.19	8.60	-	0.92	-	31.60	28.24
2	Control	4.85±0.04^{a}	23.09±0.27	29.21±1.50^{b}	8.67±0.45	39.98±0.10	1.92±0.05^{b}	0.62±0.08^{b}	31.02±0.70	27.29±0.66
	HM LAB	4.53±0.04^{b}	22.83±0.32	27.22±1.33^{a}	8.69±0.35	32.43±0.21	2.80±0.05^{a}	0.38±0.03^{c}	30.78±0.80	27.25±0.87
	HT LAB	4.58±0.05^{b}	22.92±0.57	29.66±1.25^{ab}	8.48±0.11	36.74±0.19	1.77±0.04^{b}	1.10±0.13^{a}	30.89±0.86	25.92±0.78
	HM+HT LAB	4.55±0.06^{b}	22.74±0.35	26.97±1.33^{a}	8.39±0.44	37.15±0.06	2.53±0.06^{a}	0.57±0.04^{b}	31.02±0.78	27.86±0.23
4	Control	4.73±0.05^{a}	22.87±0.53	24.80±2.58	8.61±0.3	69.60±0.22^{a}	2.66±0.06^{b}	0.74±0.06^{b}	30.31±0.23	27.08±0.29
	HM LAB	4.39±0.03^{b}	22.69±0.23	20.73±0.85	8.50±0.25	49.44±0.18^{b}	3.34±0.07^{a}	0.80±0.06^{b}	31.67±0.58	26.92±1.39
	HT LAB	4.40±0.01^{b}	22.68±0.43	26.18±2.58	8.60±0.18	39.75±0.17^{b}	2.74±0.03^{b}	1.51±0.05^{a}	39.80±0.66	26.33±1.22
	HM+HT LAB	4.52±0.03^{ab}	22.98±0.16	20.14±1.37	8.62±0.08	46.23±0.15^{b}	3.17±0.06^{a}	0.82±0.10^{b}	29.71±0.67	27.12±1.08
8	Control	4. 50±0.02	22.78±0.58	17.65±2.55^{bc}	8.71±0.25	85.41±0.18^{a}	3.69±0.08^{b}	1.10±0.09^{b}	29.49±0.49	26.92±0.63
	HM LAB	4.47±0.02	23.15±0.97	15.22±2.55^{b}	8.69±0.17	54.50±0.10^{b}	4.18±0.08^{a}	1.09±0.19^{b}	30.19±0.16	27.41±0.95
	HT LAB	4.40±0.04	22.40±0.62	24.65±1.73^{a}	8.48±0.34	70.70±0.18^{b}	3.80±0.09^{ab}	1.51±0.05^{a}	29.39±0.55	27.85±1.23
	HM+HT LAB	4.42±0.03	22.58±0.46	13.00±1.13^{c}	8.40±0.34	85.54±0.04^{a}	4.09±0.06^{ab}	1.37±0.10^{ab}	30.84±0.81	26.71±0.82
60	Control	4.41±0.07	22.14±0.67	14.78±1.82	8.31±0.15	113.91±0.17^{a}	5.11±0.14^{b}	1.71±0.13^{ab}	31.23±0.69	28.43±0.89
	HM LAB	4.38±0.02	22.16±0.38	11.56±1.50	8.43±0.45	78.06±0.10^{b}	6.23±0.05^{a}	1.57±0.08^{b}	30.71±0.48	27.19±0.43
	HT LAB	4.30±0.02	22.49±0.31	13.13±1.33	8.58±0.35	91.55±0.21^{b}	5.40±0.05^{ab}	1.90±0.03^{a}	29.62±0.99	26.57±1.04
	HM+HT LAB	4.36±0.03	22.88±0.61	12.31±1.25	8.66±0.11	91.83±0.19^{b}	5.96±0.04^{ab}	2.00±0.09^{a}	29.16±0.94	25.88±0.95

HM: homofermentative; LAB: lactic acid bacteria; HT: heterofermentative; DM: dry matter; WSCs: water-soluble carbohydrates; NH_3-N: ammonia-nitrogen; TN: total nitrogen; LA: lactic acid; AA: acetic acid; CP: crude protein; NDF: Neutral detergent fibre; ADF: Acid detergent fibre,

a-b-c: Within a column means followed by different letter differ significantly ($P<0.05$)

Table 2. Results of the microbiological analysis of the sunflower silages after 60 days of ensiling (log_{10} cfu/g DM)

Days of Ensiling	Treatment	*Lactobacilli*	Yeast	Mold
0		3.05	3.97	ND
2	Control	4,71±0.14^{b}	2,67 ±0.08	ND
	HM LAB	6,20±0.11^{a}	2,66±0.12	ND
	HT LAB	5,45±0.03^{a}	2,24±0.00	ND
	HM+HT LAB	6,06±0.05^{a}	2,45±0.06	ND
4	Control	5,49±0.04^{b}	3,35±0.03^{a}	ND
	HM LAB	6,19±0.16^{a}	3,18±0.05^{a}	ND
	HT LAB	6,38±0.03^{a}	2,52±0.06^{b}	ND
	HM+HT LAB	6,43±0.17^{a}	3,94±0.17^{ab}	ND
8	Control	5,50±0.05^{b}	3,65±0.08^{a}	ND
	HM LAB	6,55±0.03^{a}	3,46±0.04^{a}	ND
	HT LAB	6,23±0.14^{ab}	2,26±0.06^{b}	ND
	HM+HT LAB	6,26±0.03^{ab}	2,45±0.14^{b}	ND
60	Control	5,49±0.04^{b}	3,70±0.01^{a}	ND
	HM LAB	6,78±0.16^{a}	2,92±0.04^{ab}	ND
	HT LAB	5,72±0.01^{ab}	2,35±0.10^{b}	ND
	HM+HT LAB	5,90±0.20^{ab}	2,37±0.06^{b}	ND

HM: Homofermentative; LAB: lactic acid bacteria; HT: Heterofermentative; ND: No detection

a-b: Within a column means followed by different letter differ significantly (P<0.05)

In the present study, the HM LAB-treated silages were found to increase as compared with the control silage (P<0.05). In contrast, the yeast numbers of HT LAB-treated silages decreased compared with the control silage (P<0.05). Similar to the former, HM LAB inoculants were detected to improve the microbiological composition of sunflower silages as compared with the control silage. It was also seen that HM LAB treatment increased the *lactobacilli* numbers of sunflower silages when compared with the control silage on all sampling days. However, HT and HM+HT LAB treatment decreased mold numbers at the end of the ensiling period. The lack of effects in the present study is in agreement with the findings of previous studies (Weinberg et al. 1995, Sucu and Filya 2006).

Regarding the third stage of the analyses, Table 3 gives the results of the aerobic exposure test, according to which pH change, CO_2 production and an increase in yeast and mold numbers are the indicators of silage deterioration.

In the present study, the HT LAB or HM+HT LAB-treated silages decreased CO_2 production significantly as compared with the HM LAB-treated ones and the control silages (P<0.05). However, the yeast and mold counts were higher in the control silages (P<0.05). When exposed to air for five days, the apparent improvement in the aerobic stability of HT LAB-treated silages may result from the effect of acetic acids. This is because acetic acids are fungicidal agents and enough concentrations of acetate inhibit the growth of yeasts and moulds in the silages (Weinberg et al. 1993, McDonald et al. 1991, Filya and Sucu 2007, Nkosi et al. 2012).

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

Treatment	pH	CO_2, g/kg DM	Yeast, log_{10}cfu/g	Mold, log_{10}cfu/g
Control	4.91±0.14	21.81±0.91^{a}	6.18±0.11^{a}	4.91±0.19^{a}
HM LAB	4.83±0.12	20.63±0.67^{a}	5.77±0.14^{b}	2.99±0.41^{b}
HT LAB	4.71±0.16	11.83±0.38^{b}	4.63±0.12^{b}	2.81±0.23^{b}
HM+HT LAB	4.68±0.10	11.60±0.55^{b}	4.90±0.22^{b}	2.73±0.27^{b}

HM: Homofermentative; LAB: lactic acid bacteria; HT: Heterofermentative; CO_2: Carbon dioxide

a-b: Within a column means followed by different letter differ significantly (P<0.05)

Table 4. *In vitro* OM digestibility of the ensiled sunflower after 60 days of ensiling (% DM)

Treatment	*In vitro* OM Digestibility
Control	44.02±0.65
HM LAB	46.62±0.47
HT LAB	45.55±0.79
HM+HT LAB	46.29±0.50

HM: Homofermentative; LAB: lactic acid bacteria; HT: Heterofermentative; OM: organic matter

Finally, the values for *in vitro* OM digestibility are displayed in Table 4.

Regarding those findings, HM and/or HT LAB treatments did not affect *in vitro* OM digestibility of silages when compared to the control silage (P>0.05). There are various reports indicating that LAB did not affect ruminal OM degradability or the digestibility of silages (Nadeu et al. 2000b, Filya et al. 2001, Hristov and McAllister 2002, Ozduven et al. 2009, Ozduven et al. 2010). On the other hand, in some studies, LAB-treated silage improved the degradability or digestibility (Weinberg et al. 1995, Nadeu et al. 2000a). In the present study, the addition of LAB had no effect on *in vitro* OM digestibility. The lack of effects was in agreement with other studies in which the addition of LAB did not show significant effects on *in vitro* OM digestibility of the silages (Nadeu et al. 2000b, Hristov and McAllister 2002, Ozduven et al. 2009, Ozduven et al. 2010).

In conclusion, the results of this study showed that HM LAB inoculants increased the characteristics of fermentation but impaired the aerobic stability of the sunflower silages. On the other hand, the application of HT LAB alone or in combination with a HM LAB improved the aerobic stability of sunflower silages. *In vitro* OM digestibility was numerically increased for treated than control silages.

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