Turkish Journal of Agriculture - Food Science and Technology, 9(6): 1062-1069, 2021
DOI: https://doi.org/10.24925/turjaf.v9i6.1062-1069.4193

Turkish Journal of Agriculture - Food Science and Technology

Available online, ISSN: 2148-127X | www.agrifoodscience.com | Turkish Science and Technology Publishing (TURSTEP)

The Effects of Lactic Acid Bacteria and Enzyme Mixture Inoculants on Silage Fermentation Characteristics and Feed Values of Silage Prepared from Alfalfa Harvested at Different Maturities[#]

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ARTICLE INFO

[#]*This study was produced from a part of the master's thesis.*

Research Article

Received : 05/01/2021
Accepted : 04/04/2021

Keywords:
Alfalfa
Fermentation
Lactic acid bacteria
Aerobic stability
Feed value

ABSTRACT

This study was carried out to determine the effects of lactic acid bacteria+ enzyme (LAB+E) inoculants on the fermentation characteristics and feed values of silages prepared from alfalfa harvested at three maturity stages. Alfalfa was harvested at the early, middle and late flowering stages. Sil-All (Alltech, UK) were used as LAB+E inoculants. Inoculants were applied to the silages at the rates of 1×10^5, 5×10^5 and 1×10^6 cfu/g levels in 1 liter capacity plastic bags. The bags were stored at 20±2°C under the laboratory conditions. Three bags from each group were sampled for chemical and microbiological analyses on the 45th day after ensiling. The results showed that LAB+E inoculants reduced pH values and ammonia-nitrogen content, whereas increased lactic acid contents and *lactobacillus* count of alfalfa silages. High doses LAB+E inoculant decreased neutral detergent fiber and acid detergent fiber content, increased *in vitro* organic matter digestibility and metabolic energy of alfalfa silages. It has been demonstrated that the most effective application dose of LAB+E inoculant to improve fermentation and feed value of alfalfa silage was 1×10^6 cfu/g, but $1x10^5$ and 5×10^5 cfu/g level can also be considered as effective dose.

Türk Tarım – Gıda Bilim ve Teknoloji Dergisi, 9(6): 1062-1069, 2021

Farklı Olgunluk Dönemlerinde Hasat Edilen Yonca Bitkisinden Hazırlanan Silajlarda Laktik Asit Bakterisi ve Enzim Karışım İnokulant İlavesinin Silaj Fermantasyon Özellikleri ve Yem Değeri Üzerindeki Etkileri

MAKALE BİLGİSİ

Araştırma Makalesi

Geliş : 05/01/2021
Kabul : 04/04/2021

Anahtar Kelimeler:
Yonca
Fermantasyon
Laktik asit bakteri
Aerobik stabilite
Yem değeri

ÖZ

Bu çalışma, üç ayrı vejetasyon döneminde hasat edilen yonca bitkisine farklı düzeylerde laktik asit bakteri+enzim (LAB+E) inokulantı ilavesinin silaj fermantasyon özellikleri ve yem değeri üzerindeki etkilerinin saptanması amacıyla yürütülmüştür. Yonca bitkisi çiçeklenme başlangıcı, çiçeklenme ortası ve çiçeklenme sonu döneminde hasat edilmiştir. Laktik asit bakteri+enzim karışımı inokulant kaynağı olarak Sil-All (Alltech, UK) kullanılmıştır. İnokulant, yonca hasıllarına 1×10^5, 5×10^5 ve 1×10^6 kob/g düzeyinde katılmıştır. Kontrol ve katkı maddeleri ile muamele edilen yonca 1 litre hacimli polietilen torbalarda silolanmıştır. Torbalar laboratuvar koşullarında 20±2°C sıcaklıkta depolanmışlardır. Silolamadan sonraki 45. günde her gruptan 3'er torba açılarak silajlarda kimyasal ve mikrobiyolojik analizler yapılmıştır. Sonuç olarak, LAB+E inokulantı silajların pH ve amonyak azotu içeriklerini azaltırken; laktik asit, asetik asit içerikleri ve *lactobacilli* sayısını artırmıştır. Yüksek dozda LAB+E ilavesi silajların nötr deterjanda çözünmeyen lif ve asit deterjanda çözünmeyen lif içeriğini azaltmış, *in vitro* organik madde sindirilebilirliğini ve metabolik enerji değerlerini artırmıştır. Yoncanın LAB+E inokulantı ilave edilerek silolanmasının fermantasyon özellikleri ve yem değerini iyileştirdiği, en etkili dozun 1×10^6 kob g/kg olmakla birlikte, 1×10^5 kob g/kg ve 5×10^5 kob/g dozlarında da uygulanabileceği belirlenmiştir.

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Introduction

Alfalfa (*Medicago sativa L.*) is a perennial herbaceous legume. Due to its high adaptability, high yields and high nutritional quality, alfalfa is one of the most important legume roughages in most of the countries in the World. As a major source of protein for livestock, it is a basic component in rations for ruminants and other domestic animals (Radovic et al., 2009). It is cultivated in more than 80 countries in an area exceeding 35 million ha (Zubair et al., 2017). Alfalfa is rather fed to animals in dried form (Canbolat 2013). However, a significant loss of nutrients occurs due to mechanical treatments made during drying and storage (Oktay et al., 1990, Çiftçi et al., 2005, Acar and Bostan 2016). Alfalfa is generally utilized as silage particularly in rainy areas where sufficient drying is not possible (Çerçi 1996, Oten et al., 2016). It is hard to make good quality silage from the alfalfa due to its high buffering capacity and crude protein (CP) levels, lower dry matter (DM) and water-soluble carbohydrate (WSC) content. Therefore, it is necessary to use bacterial inoculants as additive for ensiling alfalfa which are rich in protein and low content of WSCs (Filya 2000). Bacterial inoculants in silage production are defined as products containing lactic acid bacteria (LAB) or bacterial groups at a concentration level that encourages lactic acid (LA) fermentation. LAB, which is used as inoculant, prevents the development of butyric acid (BA) bacteria as a result of increasing acidity (approx. pH: 4) by accelerating LA fermentation in silage. However, since there is not enough WSC during the silage of the alfalfa, LAB cannot proliferate sufficiently, and as a result there is not enough LA production. Thus, LAB inoculants can be used as a silage additive in the form of a mixture of starch-degrading enzyme such as amylase and cell wall degrading enzymes (E), especially cellulase, hemicellulase and pectinase. Indeed, E which are used in conjunction with LAB, while enhancing silage fermentation by releasing an additional substrate for LAB activity in the silages they participate, they reduce the silage's content of neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), hemicellulose and cellulose, and increase dry matter digestibility (DMD) and organic matter digestibility (OMD) (Filya 2001).

This study aimed to determine the effects of lactic acid bacteria+ enzyme (LAB+E) inoculants addition on the fermentation and *in vitro* organic matter digestibility characteristics of silages prepared from alfalfa harvested at three maturity stages.

Material and Method

In this research, alfalfa (*Medicago sativa*) grown in the experimental areas of the Faculty of Agriculture of Tekirdag Namik Kemal University was used as silage material. Alfalfa was harvested at the early flowering (about 10-20% bloom), middle flowering (50% bloom) and late flowering (90-100% bloom) stage. Alfalfa was wilted to approximately 30% DM and chopped to about 1.5-2.0 cm length. In the research, commercial inoculant, Sil-All 4x4, (Alltech, UK) (*Lactobacillus plantarum, Pediococcus acidilactici, Pediococcus pentosaceus* and *Propionibacteria acidipropionici* bacteria together with amylase, cellulase, xylanase and β-glucanase enzymes) was used. Inoculant was added to each silage material at the level of 1.0×10^5, 5.0×10^5 and 1.0×10^6 cfu/g. The first group was the control group, and 10 kg of alfalfa plant was spread on a clean area of 1×4 m, and 20 ml of dechlorinated water was sprayed on it. In the second group, 5 mg of LAB +E inoculant (1.0×10^5 cfu/g) was weighed and thoroughly mixed with 20 ml of chlorine-free water, and then it was sprayed homogeneously on the shredded clover plant. In group 3, 25 mg inoculant (5.0×10^5 cfu/g), in group 4, 50 mg inoculant (1.0×10^6 cfu/g) was applied as described in group 2. After thorough mixing, the alfalfa was ensiled in triplicate for each treatment at approximately 500 g (fresh material), followed in a polythene bag (dimensions 20×25 cm), and sealed by using vacuum packing machine (CAS CVP 260 PD), there were 36 bags (3 maturity stage × 4 treatment × 3 replicates) for each treatment. The bags were kept at 20 ± 2°C in laboratory. On the 45^{th} day after ensiling, the bags were opened and chemical and microbiological analyses were performed. The DM contents of the alfalfa silages were determined by drying the samples first at 60°C for 72 h in a forced-ventilation oven (AOAC 1990). In addition, the total nitrogen (TN) was determined using the Kjeldahl method explained in AOAC (1990), and the CP was calculated by multiplying TN by the factor of 6.25. The ash was determined by incinerating the alfalfa silage content at 600°C for 4 hours (AOAC 1990). The alfalfa silage pH was measured directly from the silage juice using a pH meter (Inolab, WTW, Germany). Ammonia nitrogen (NH_3-N) in the silages was determined by the micro distillation method reported by Anonymous (1986). The content of the WSC in the fresh and silage samples was determined by the antrone-thiourea method reported by the Anonymous (1986) in spectrophotometer (Shimadzu UV-1201, Kyoto, Japan). The LA (Koç and Coskuntuna 2003) contents of silages were determined in the spectrophotometer, while acetic acid (AA) and BA (Supelco 1998) contents were determined in the gas chromatography device. Microbial evaluation included enumeration of lactobacilli on pour-plate MRS, and yeast and moulds on spread plate malt extract agar for 3 days at 30°C of incubation (Seale 1990). Neutral detergent fibre and ADF analyses were performed according to the methods reported by Goering and Van Soest (1970). *in vitro* OMD was carried out based on the enzyme method reported by Naumann and Bassler (1993). For this purpose, Pepsin enzyme (Merck, 0.7 FIP-U / g, Germany) and Cellulase enzyme obtained from *Trichoderma viride* microorganisms (Merck, Onozuka R10; Germany) were used. One-way analysis of variance (ANOVA) was used to evaluate the data obtained from the study, while Duncan multiple comparison test was employed in order to determine significant differences (Soysal 1998). Statistical analyses were performed with SPSS 15.0 (2007) package program.

Results and Discussion

The results of chemical analysis of alfalfa silages are given in Table 1.

In the study, while pH, CP, NH_3-N, WSC and AA contents decreased due to vegetation progression, LA, NDF and ADF content increased ($P<0.001$). The DM and ash were not affected by vegetation period. The DM contents of the silages were between 291.44-322.59 g/kg, no difference was detected between the silages with LAB+E inoculant and the control silage ($P>0.05$).

Table 1. Results of the chemical composition of the alfalfa silages

Treatment	Maturity	Dose	DM, g/kg	pH	Ash, g/kg DM	CP, g/kg DM	NDF, g/kg DM
1	EF	C	306.82^{bc}	5.18^{a}	90.37	214.40^{a-d}	472.37^{d}
2	EF	I 1	320.31^{a}	4.92^{bc}	90.72	225.84^{a}	459.71^{d}
3	EF	I 2	298.61^{cd}	5.02^{b}	93.43	225.50^{a}	469.84^{d}
4	EF	I 3	307.81^{bc}	4.91^{bc}	94.11	220.46^{ab}	467.61^{d}
5	MF	C	297.47^{cd}	5.20^{a}	88.67	202.36^{d}	581.22^{a}
6	MF	I 1	291.44^{d}	4.87^{bc}	90.60	217.75^{a-c}	558.57^{b}
7	MF	I 2	293.18^{d}	4.89^{bc}	93.76	210.36^{b-d}	561.14^{b}
8	MF	I 3	302.59^{cd}	4.78^{c}	87.65	206.33^{cd}	522.37^{c}
9	LF	C	321.78^{a}	4.91^{bc}	95.58	205.77^{cd}	561.43^{b}
10	LF	I 1	322.59^{a}	4.90^{bc}	92.39	210.11^{b-d}	561.99^{b}
11	LF	I 2	316.52^{ab}	4.87^{bc}	93.29	217.13^{a-c}	560.09^{b}
12	LF	I 3	317.81^{ab}	4.79^{c}	94.33	206.66^{cd}	573.30^{ab}
Standard error of mean			3.95	0.05	2.07	3.96	6.13
Maturity means							
EF			308.39^{b}	5.01^{a}	92.16	221.55^{a}	467.38^{b}
MF			296.17^{c}	4.94^{b}	90.17	209.20^{b}	555.82^{a}
LF			319.67^{a}	4.87^{b}	93.90	209.92^{b}	564.20^{a}
Standard error of mean			1.97	0.02	1.04	1.98	3.07
Dose means							
C			308.69	5.10^{a}	91.54	207.51^{b}	538.34^{a}
I 1			311.45	4.90^{bc}	91.23	217.90^{a}	526.76^{b}
I 2			302.77	4.93^{b}	93.49	217.66^{a}	530.36^{ab}
I 3			309.40	4.83^{c}	92.03	211.15^{a}	521.09^{b}
Standard error of mean			2.28	0.03	1.20	2.28	3.54
Maturity (M)			<0.001	<0.01	0.057	<0.001	<0.001
Dose (D)			0.070	<0.001	0.562	<0.01	<0.05
M×D			<0.001	<0.001	0.238	<0.01	<0.001

Treatment	ADF, g/kg DM	NH_3-N, g/kg TN	WSC, g/kg DM	LA, g/kg DM	AA, g/kg DM	BA, g/kg DM
1	403.05^{ef}	125.63^{a}	12.37^{b}	93.80^{bc}	22.74^{bc}	0.00^{b}
2	380.49^{fg}	101.27^{bc}	15.98^{a}	116.37^{a}	28.32^{ab}	0.00^{b}
3	362.49^{g}	107.82^{b}	10.17^{b}	109.56^{a}	29.97^{ab}	0.00^{b}
4	338.88^{h}	103.74^{bc}	3.41^{de}	112.08^{a}	34.35^{a}	0.00^{b}
5	479.27^{a}	111.64^{b}	3.41^{de}	91.36^{bc}	14.05^{cd}	13.03^{a}
6	455.06^{b}	89.67^{c-e}	1.78^{e}	96.24^{b}	14.23^{cd}	16.61^{a}
7	415.44^{de}	98.66^{b-d}	2.10^{e}	110.11^{a}	12.41^{d}	14.61^{a}
8	392.48^{f}	89.47^{c-e}	2.66^{e}	114.61^{a}	14.65^{cd}	14.48^{a}
9	397.93^{ef}	85.43^{d-f}	1.86^{e}	77.00^{d}	14.33^{cd}	1.33^{b}
10	452.81^{bc}	77.95^{e-g}	6.47^{c}	83.00^{b-d}	13.77^{d}	1.26^{b}
11	447.23^{bc}	71.83^{fg}	7.66^{c}	81.08^{cd}	14.64^{cd}	2.10^{b}
12	431.48^{cd}	71.26^{g}	5.47^{cd}	82.86^{b-d}	12.49^{d}	2.15^{b}
Standard error of mean	7.15	4.47	0.79	4.37	2.66	1.92
Maturity means						
EF	371.23^{b}	109.62^{a}	10.49^{a}	107.96^{a}	28.84^{a}	0.00^{b}
MF	435.56^{a}	97.36^{b}	2.49^{c}	103.08^{a}	13.84^{b}	14.69^{a}
LF	432.36^{a}	76.61^{c}	5.37^{b}	80.99^{b}	13.81^{b}	1.71^{b}
Standard error of mean	3.58	2.23	0.39	2.18	1.33	0.96
Dose means						
C	426.75^{a}	107.56^{a}	5.88^{b}	87.39^{b}	17.04	4.79
I 1	429.45^{a}	89.63^{b}	8.08^{a}	98.54^{a}	18.78	5.96
I 2	408.39^{b}	92.77^{b}	6.65^{b}	100.25^{a}	19.01	5.57
I 3	387.61^{c}	88.15^{b}	3.85^{c}	103.18^{a}	20.50	5.54
Standard error of mean	4.13	2.58	0.45	2.52	1.53	1.11
Maturity (M)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Dose (D)	<0.001	<0.001	<0.001	<0.001	0.479	0.899
M×D	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

M: Maturity, D: Dose, EF: Early flowering, MF: Mid flowering, LF: Late flowering, C: Control, I 1: $1x10^5$ cfu/g LAB, I 2: $5x10^5$ cfu/g LAB, I 3: $1x10^6$ cfu/g LAB, DM: Dry matter, CP: Crude protein, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, WSC: Water soluble carbohydrates, NH3-N: Ammonia-nitrogen, TN: Total nitrogen, LA: Lactic acid, AA: Acetic acid, BA: Butyric acid, a-g Within a column means followed by different letter differ significantly (P<0.05)

The highest DM content was determined at the late flowering stage (P<0.001). The ash contents of alfalfa silage were ranged from 87.65 to 95.58 g/kg DM but there were no differences between the silage groups (Table 1, P>0.05). In this study, the pH values of silages were found between 4.79-5.20 and the highest pH was determined at the early flowering stage (P<0.01). The linear decrease in pH values of alfalfa silages from early to late flowering stage of maturity was in agreement with the findings of Dumlu Gul et al. (2015) and Özduven and Cam Çelebi (2017) who described that pH values of alfalfa silages decreased with advancing growth of fodder. The pH values of silages with LAB+E inoculant were found to decrease significantly compared to the control silage. Due to the high CP of the legumes compared to cereals, it slows down the decrease of pH in fermentation depending on the high buffer capacity (Filya 2005, Dumlu Gul and Tan 2013). This situation was also clearly observed in the current research. Shockey et al. (1985) determined that although LA ingredient of maize silage is nearly two times lower than alfalfa silage, corn silage has lower pH value. In this study, all of the inoculated silages were better fermented and more successfully ensiled.

Harvesting maturity of alfalfa is highly correlated to its nutritive value (Kaiser and Combs, 1989). The CP contents of alfalfa silage was found to decrease significantly from 221.55 to 209.92 g/kg DM at early and full flowering stages, respectively. The reduction of CP contents with maturity of alfalfa is associated to a decrease of leaves and increase of stems in the forage biomass. The CP contents of the alfalfa silage with all LAB+E treatments were higher than that of untreated silage (P<0.01). The most important activity seen after plant harvest is proteolysis. During this event, proteins in the plant are degraded by protease enzymes into peptides and amides, mainly amino acids and ammonia (Filya 2005). High degradation rates of CP into silage NH_3-N contribute usually to increase rumen ammonia concentrations (Givens and Rulquin 2004). Ammonia nitrogen in alfalfa silage has sparked interest as an indicator to evaluate silage quality. The NH_3-N contents was found to be significantly higher in the silages harvested during the early flowering stage (P<0.001). This situation may be attributed to the fact that the CP content of silages in the mentioned period is higher in comparison to other maturity stages (Kung et al., 1986, Çerçi et al., 2002). In this study, the NH_3-N was also recorded as lower in silages treated with LAB+E compared to the control silage. It was remarkable that the levels of NH_3-N in all treated silages were determined to be under the threshold level of 100 g/kg TN per good quality silages (McDonald et al., 1988).

The concentration of LA of silages ranged between 77.00-114.61 g/kg DM. The LA content of alfalfa silages had a tendency to decrease with the progression of vegetation. On the other hand, LAB+E inoculants significantly increased the LA content of silages (P<0.001). The AA contents of silages were found between 12.41-34.35 g/kg DM in all maturity stages and applications, which is an acceptable range for silages (Luther 1986, Nursoy et al., 2003). The BA content of alfalfa silages in this study are ranged between 0.00-16.61 g/kg DM values. While the BA content of alfalfa silages in the mid-flowering stage was significantly higher than other maturity stages (P<0.001), LAB+E inoculant did not affect the BA content of silages (P>0.05). Therefore, the treated silage with LAB+E was well preserved due to lower pH and production of a higher amount of LA compared to the control silage. Our study has shown that used LAB+E can improve silage quality and reduce protein degradation in silage. It is precisely the role of inoculants to intensify the production of LA, quickly reduce pH and prevent the development of pathogenic microorganisms (Nadeau et al., 2000). Li et al. (2018) reported that alfalfa silages treated with LAB+E inoculants had significantly lower pH and NH_3-N/TN content, and higher content of LA in comparison with control silage.

The NDF and ADF contents of silages are important quality parameters. A significant increase in NDF and ADF contents of alfalfa silage was observed with advancing stages of maturity (P<0.001). Canbolat et al. (2006), Yari et al. (2012) and Ozduven and Celebi Cam (2017) reported that NDF and ADF contents were lowest in the flowering stage and highest in the late flowering stage. In the present study, NDF (P<0.017) and ADF (P<0.001) contents (except I1 dose for ADF content) of alfalfa silages with the addition of LAB+E in all maturity stages decreased compared to the control silages. This decline in cell wall fractions (ADF and NDF) may have been due to the hydrolytic effect of the fibrolytic enzymes in treated silages. The use of LAB+E inoculants (Chilson et al., 2016, Ozduven and Celebi Cam 2017) lowered the NDF and ADF contents in alfalfa silages. Similar improvements in silage quality following treatment with LAB+E inoculants has been reported in other studies (Nadeau et al., 2000, Filya 2002, Polat et al., 2005, Ozduven et al., 2017). Including cell wall degrading enzymes in silage additives has been practise as a means of increasing the contents of WSCs available to LAB, and as a method to degrade cell wall and subsequently improve the digestibility of OM and fiber (Mc Donald et al., 1991, Xing et al., 2009). The WSCs that are released as a result of the breakdown of the cell wall containing the structural carbohydrates of alfalfa were also used as nutrient by *lactobacilli*. As a result, the alfalfa containing insufficient WSCs for silage fermentation and therefore difficult to ensile was ensiled successfully.

The results of the microbiological analysis of alfalfa silages are given in Table 2. In the present study, the maturity stages and the use of LAB +E inoculant at different levels affected the microbiological compositions of alfalfa silages. As a matter of fact, during the fermentation period, the silages *lactobacilli* count in the mid flowering stage was found higher than the silages in other maturity stages (Table 2, P<0.001). In this study, the *lactobacilli* count of the silages with LAB+E inoculant was significantly higher compared to the control silage (Table 2, P<0.001). In contrast, the yeast count of LAB+E treated silages decreased compared with the control silage (P<0.001). In comparison to the control silage, the low pH levels of silages with LAB+E inoculant was a result of increased *lactobacilli* development and therefore LA production. Similar findings were reported by Koc et al. (2008) and Ozduven and Celebi Cam (2017). In all periods of fermentation, it was found that depending on the dosage used, yeast counts were lower in silages with LAB+E inoculant compared to the control silage (P<0.001). Silages that are well compressed and with low pH and oxygen-free environment are not suitable for mold growth (Filya 2005). In fact, none of the silages developed mold.

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

Treatment	M	D	Lactobacilli	Yeast	Mold
1	EF	C	5.73^{f}	6.09^{a}	ND
2	EF	I 1	5.82^{e}	5.94^{bc}	ND
3	EF	I 2	5.94^{d}	5.86^{de}	ND
4	EF	I 3	6.04^{c}	5.51^{h}	ND
5	MF	C	6.04^{c}	6.00^{b}	ND
6	MF	I 1	6.46^{b}	5.89^{cd}	ND
7	MF	I 2	6.46^{b}	5.82^{e}	ND
8	MF	I 3	6.56^{a}	5.73^{f}	ND
9	LF	C	5.23^{h}	5.59^{g}	ND
10	LF	I 1	5.42^{g}	5.40^{i}	ND
11	LF	I 2	5.35^{g}	5.17^{j}	ND
12	LF	I 3	5.70^{f}	5.11^{j}	ND
Standard error of mean			3.95	0.05	
Maturity means					
EF			5.89^{b}	5.85^{a}	ND
MF			6.38^{a}	5.86^{a}	ND
LF			5.43^{c}	5.32^{b}	ND
Standard error of mean			1.97	0.02	
Dose means					
C			5.67^{c}	5.89^{a}	ND
I 1			5.90^{b}	5.74^{b}	ND
I 2			5.92^{b}	5.61^{c}	ND
I 3			6.10^{a}	5.45^{d}	ND
Standard error of mean			2.28	0.03	
M			<0.001	<0.001	
D			<0.001	<0.001	
M*D			<0.001	<0.001	

M: Maturity, D: Dose, EF: Early flowering, MF: Mid flowering, LF: Late flowering, C: Control, I 1: 1x 10^5 cfu/g LAB, I 2: 5x 10^5 cfu/g LAB, I 3: 1x 10^6 cfu/g LAB, ND: Not detected, $^{a-j}$ Within a column means followed by different letter differ significantly (P<0.05)

Table 3. Result of the aerobic stability of the alfalfa silages

Treatment	M	D	pH	CO_2 g/kg DM	Yeast log_{10} cfu/g DM	Mold log_{10} cfu/g DM
1	EF	C	5.10^{bc}	5.65^{d}	3.51^{d}	ND
2	EF	I 1	5.10^{bc}	8.47^{cd}	4.87^{bc}	ND
3	EF	I 2	5.34^{a-c}	12.92^{c}	5.32^{a-c}	ND
4	EF	I 3	5.57^{a}	25.25^{b}	4.83^{c}	ND
5	MF	C	5.14^{bc}	4.25^{d}	1.52^{f}	ND
6	MF	I 1	5.06^{bc}	4.02^{d}	1.87^{f}	ND
7	MF	I 2	5.39^{ab}	5.68^{d}	2.73^{e}	ND
8	MF	I 3	5.09^{bc}	3.40^{d}	2.53^{e}	ND
9	LF	C	5.08^{bc}	25.40^{b}	5.24^{a-c}	ND
10	LF	I 1	5.23^{a-c}	39.29^{a}	5.39^{ab}	ND
11	LF	I 2	4.98^{c}	25.34^{b}	5.57^{a}	ND
12	LF	I 3	5.04^{bc}	27.04^{b}	5.35^{a-c}	ND
Standard error of mean			0,11	2,15	0.16	-
Maturity means						
EF			5.28	13.07^{b}	4.63^{b}	ND
MF			5.17	4.34^{c}	2.16^{c}	ND
LF			5.08	29.27^{a}	5.38^{a}	ND
Standard error of mean			0,06	1,08	0.08	-
Dose means						
C			5.11	11.77^{c}	3.42^{c}	ND
I 1			5.13	17.26^{ab}	4.04^{b}	ND
I 2			5.24	14.65^{b}	4.54^{a}	ND
I 3			5.24	18.56^{a}	4.24^{b}	ND
Standard error of mean			0,07	1.24	0.09	-
M			0.068	<0.001	<0.001	-
D			0.371	0.004	<0.001	-
M*D			0.037	<0.001	<0.001	-

M: Maturity, D: Dose, EF: Early flowering, MF: Mid flowering, LF: Late flowering, C: Control, I 1: 1x 10^5 cfu/g LAB, I 2: 5x 10^5 cfu/g LAB, I 3: 1x 10^6 cfu/g LAB, ND: Not detected, $^{a-j}$ Within a column means followed by different letter differ significantly (P<0.05)

The impact of LAB+E treatment on the aerobic stability of alfalfa silages after exposure to air for five days is shown in Table 3. Aerobic deterioration of silage is a complex process which depends on many factors. Usually, it is initiated by aerobic yeasts that can use either residual WSCs or LA for their metabolism. Aerobic deterioration usually results in production of CO_2 (Weinberg et al., 2001). In the present study, the LAB+E treated silages had higher CO_2 production and the yeast counts as compared with control silages (P<0.001). Treatment with LAB+E mixture had high contents of residual WSCs and LA and therefore, tended to spoil more upon aerobic exposure, as indicated by more intensive CO_2 production. These results were consistent with those of Chen et al. (1994) who reported reduced aerobic stability with a LAB+E addition in maize silage. Furthermore, there was a slight increase detected in pH values of alfalfa during that 5-day period when silage deterioration occurred.

The ME and *in vitro* OMD values were higher (P<0.001) observed between the maturity and the treatments (Table 4). However, the *in vitro* OMD and ME values at the early flowering stage were higher compared to the mid and full flowering stages (P<0.001). The *in vitro* OMD and ME values in all LAB+E treated silages were found higher compared to control silage (P<0.001).

Ozduven et al. (2017) suggested that a decrease in NDF and ADF in silage materials could increase the *in vitro* OMD of LAB+E inoculants treated silage. In the present study, lower NDF and ADF contents determined for all LAB+E treated silages may also indicate the improved quality of silage fermentation in terms of *in vitro* OMD of silages. The findings obtained in the study on silages *in vitro* OMD are consistent with the findings from previous studies (Ozduven et al., 2009, Denek et al., 2012, Sucu and Aydogan Ciftci 2016).

Table 4. Result of the OMD and ME values of the alfalfa silages

Treatment	M	D	OMD, g/kg DM	ME, MJ/kg DM
1	EF	C	607.15^{b}	8.73^{b}
2	EF	I 1	612.02^{b}	8.81^{b}
3	EF	I 2	608.79^{b}	8.73^{b}
4	EF	I 3	644.50^{a}	9.14^{a}
5	MF	C	521.07^{e}	7.69^{e}
6	MF	I 1	551.19^{cd}	8.06^{c}
7	MF	I 2	540.41^{d}	7.87^{d}
8	MF	I 3	558.26^{c}	8.16^{c}
9	LF	C	487.33^{h}	7.19^{g}
10	LF	I 1	489.50^{g}	7.39^{f}
11	LF	I 2	505.04^{fg}	7.47^{f}
12	LF	I 3	510.03^{f}	7.49^{f}
Standard error of mean			3.72	0.05
Maturity means				
EF			618.11^{a}	8.85^{a}
MF			542.73^{b}	7.95^{b}
LF			500.22^{c}	7.38^{c}
Standard error of mean			1.86	0.03
Dose means				
C			538.51^{c}	7.87^{c}
I 1			553.90^{b}	8.09^{b}
I 2			551.41^{b}	8.02^{b}
I 3			570.93^{a}	8.26^{a}
Standard error of mean			2.15	0.03
M			<0.001	<0.001
D			<0.001	<0.001
M*D			<0.001	<0.001

M: Maturity, D: Dose, EF: Early flowering, MF: Mid flowering, LF: Late flowering, C: Control, I 1: $1x10^5$ cfu/g LAB, I 2: $5x10^5$ cfu/g LAB, I 3: $1x10^6$ cfu/g LAB, DM: Dry matter, OMD: Organic matter digestibility, ME: Metabolic energy, $^{a-g}$Within a column means followed by different letter differ significantly (P<0.05)

Conclusion

From the perspective of feed value, fermentation characteristics and in *vitro* OMD of alfalfa silage, alfalfa harvested at early flowering stage were more suitable for ensiling. The results showed that LAB+E inoculants reduced pH values and NH_3-N content, whereas increased LA contents and *lactobacillus* count of alfalfa silages. High doses LAB+E inoculant decreased NDF and ADF content, increased *in vitro* OMD of alfalfa silages. It has been demonstrated that the most effective application dose of LAB+E inoculant to improve fermentation and feed value of alfalfa silage was $1x10^6$ cfu/g, but $1x10^5$ and $5x10^5$ cfu/g level can also be considered as effective dose.

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