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Effects of Different Lactic Acid Bacteria Inoculants on Alfalfa Silage Fermentation and Quality

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ABSTRACT

Alfalfa (*Medicago sativa* L.) is a crucial perennial forage plant with high protein and mineral content and may be mowed several times through the vegetation period. Along with having a large cultivation area in Türkiye, it constitutes approximately 61% of the total green forage produced. Silage is the most effective method for preserving herbage and using it as a source of roughage throughout the year. However, ensiling alfalfa, especially with low dry matter (DM) content, is difficult due to its low water-soluble carbohydrate (WSC) and buffering capacity. This study was carried out to improve the alfalfa plant's silage fermentation process by inoculating new lactic acid bacteria (LAB) strains. When the alfalfa plant reached 50% flowering, six different LAB strains were inoculated and compared with the uninoculated alfalfa silage. According to the results obtained, it was determined that LAB inoculants improved the fermentation properties of alfalfa silage in general. All inoculated strains caused a significant decrease in the pH of the resulting silage. The strain *Lactobacillus buchneri* (LS-31-1-4) was superior in terms of DM recovery (96.82%) and protein recovery (94.00%). At the same time, *Lactobacillus brevis* (LS-55-2-2) and *Leuconostoc citerum* (LS-70-6-1) were the most restrictive strains of yeast and enterobacteria growth in silage, respectively.

Keywords: LAB isolates, Microorganisms, Silage quality, Enterobacteria

1. Introduction

The livestock sector is the fastest-growing branch in developing countries' agricultural economies. However, it is known that the most important factor negatively affecting production in this sector remains the animal feeding (Kızılşimşek et al. 2016). The number of cattle units in Türkiye is around 24 million, and their dry matter (DM) needs about 110 million tons/year. The total forage production from rangelands and agricultural areas is 31 million tons, clearly indicating insufficient production, which is far from meeting the quality roughage needs of animals. The alfalfa (*Medicago sativa* L.) plant provides approximately 61% of the total forage field (TÜİK 2022). Alfalfa, with a wide cultivation area, is a perennial plant that can be harvested more than once a year, is rich in protein, and is often used as fodder for animals (Ertekin et al. 2017; Ertekin & Kızılşimşek 2020; Kızılşimşek et al. 2020).

Mowing alfalfa during its appropriate maturity period and preserving its quality is critical for livestock nutrition. Typically, nutrient losses may occur during mowing, negatively affecting quality. There is a potential risk of rain when drying, predominantly in rainy and humid regions, especially for the plant's first and last mowing times (Yakışır & Aksu 2019). For this reason, making silage is the most effective method for preserving herbage quality and enabling the use of forage in animal rations as a source of roughage in all four seasons. The advantages of ensiling are that its less labor intensive has a long supply time, and provides favorable opportunities for using additives to facilitate fermentation (Ding et al. 2020). However, ensiling the alfalfa plant is difficult due to its low DM rate and inadequate water-soluble carbohydrate (WSC) content (McDonald et al. 1991). Lactic acid bacteria (LAB) break down WSCs into acetic acid and CO_2, as well as predominantly lactic acid, accelerating fermentation and contributing to the rapid reduction of the pH in the medium. LAB also inhibits the growth of many aerobic bacteria and thus ensures feed preservation. Lee et al. (2018) noted that

inoculated LAB just before ensiling might support lactic acid fermentation and improve the feed quality of the resulting silage. LAB species associated with silage generally belong to the *Lactobacillus, Pediococcus, Leuconostoc* and *Enterococcus, Streptococcus,* and *Lactococcus* (Pahlow et al. 2003) families.

Due to its low WSC structure, DM content, and high buffering capacity, the alfalfa plant is difficult to ensilage. There is a need for practices and studies to increase the silage quality of the alfalfa plant. One of these applications, LAB inoculant application, is a method that improves the alfalfa plant's silage quality and fermentation properties. This study was carried out to investigate the effects of inoculation of different LAB strains, which were selected within the context of our study group's previous research project, on the fermentation profile and the feed quality parameters of alfalfa silage.

2. Material and Methods

2.1. Material

The Bilensoy-84 alfalfa variety, as a second cut, was used as plant material grown on agricultural fields in Kahramanmaras under irrigated conditions. At sunrise, the alfalfa plants, which are in a 50% blooming period, were harvested and removed from the weeds in them. Six LAB isolates selected among 695 isolates within the scope of the The Scientific and Technological Research Council of Türkiye (TUBITAK) project were used as microorganism material for inoculation. Table 1 shows the LAB isolates' physiological characteristics and the study's isolates numbers. The *Lactobacillus bifermentans, Lactobacillus gasseri, Pediococcus citerum*, and *Leuconostoc citerum* isolates were homofermentative, while *Lactobacillus brevis* and *Lactobacillus buchneri* were heterofermentative.

Table 1- Characteristics of lactic acid bacteria isolates

Bacteria no	***Bacteria name***	***Physiological characteristics***
LS-65-2-1	*Lactobacillus bifermentans*	Homofermentative
LS-51-2-1	*Lactobacillus gasseri*	Homofermentative
LS-55-2-2	*Lactobacillus brevis*	Heterofermentative
LS-8-1	*Pediococcus citerum*	Homofermentative
LS-31-1-4	*Lactobacillus buchneri*	Heterofermentative
LS-70-6-1	*Leuconostoc citerum*	Homofermentative

2.2. Method

The harvested plant material was first cut into 2-3 cm lengths with a chopper machine and divided into seven 2 kg treatment groups, one controlled without inoculation. After the previously determined LAB were revitalized and developed in MRS broth media, they were adjusted to a density of 10^7 cfu/g and diluted in 20 mL of distilled water. A homogeneous distribution was obtained by spraying and mixing the solution on the chopped forage. The control group was sprayed with 20 mL of distilled water without bacteria to manipulate the DM content in the inoculation treatments. Three silage packages containing 400 grams of chopped plant material from each treatment group were ensiled to be opened after 60 days (T_{60}). To determine the microorganism composition and other characteristics in each treatment group at T_0, 20 grams of sample was first blended for 1 minute at high speed with a blender in 180 mL of Ringer's solution. The samples were filtered using Whatman 54 filter paper, and the pH was measured. For the microorganism counts from the LAB strains, 1/10 dilution series were prepared and planted in MRS agar, VRBG agar, and MEA agar media for counting LAB, enterobacteria, yeast, and molds, respectively. The MRS agar media were incubated for 48 hours at 37 °C, VRBG media at 32 °C for 18 hours, and MEA agar at 32 °C for 48 hours. After incubation, microorganism counts were made.

A sample of 70 g silage was taken from the resulting silage and left to dry for 48 hours in an oven set at 78 °C, and the DM content of the samples was determined by weighing with a precision balance; then, the samples were ground in a grinding machine with a 1 mm sieve and made ready for further analysis. The nitrogen content of the feeds was determined using the Kjeldahl method, and the crude protein (CP) ratios were calculated by multiplying with the coefficient of 6.25 (AOAC 1990).

The pH, DM, microorganism counts, and CP ratio analyses mentioned above were performed for T_{60} plant samples by applying the same methods as T_0.

All statistical analysis, variance analysis, and LSD techniques for comparing averages were performed by JMP statistical analysis software.

3. Results and Discussions

The results of the pH value, DM ratios, and CP values of crop material before (T_0) and after (T_{60}) ensiling samples of alfalfa silages strained with different bacterial isolates are given in Table 2.

Table 2- Average values of pH, dry matter, and crude protein of silages belonging to different bacterial inoculants at T_0 and $T6_0$ opening times

Bacteria inoculant	*Dry matter (%)*		*pH*		*Crude protein (%)*	
	(T_0)	*(T_{60})*	*(T_0)*	*(T_{60})*	*(T_0)*	*(T_{60})*
Control	27.32^{bc}	24.01^{d}	6.27	4.87^{a}	21.70^{b}	18.56^{b}
L. bifermentans	27.01^{bc}	24.97^{cd}	6.21	4.54^{b}	21.03^{b}	19.25^{b}
L. gasseri	28.72^{a}	26.64^{a}	6.24	4.52^{b}	21.34^{b}	19.64^{ab}
L. brevis	27.43^{b}	26.19^{ab}	6.21	4.57^{b}	22.88^{a}	20.55^{a}
P. citerum	26.68^{cd}	25.38^{bc}	6.18	4.64^{b}	21.88^{ab}	18.76^{ab}
L. buchneri	26.17^{d}	25.34^{bc}	6.22	4.64^{b}	20.84^{b}	19.59^{b}
L. citerum	26.92^{bc}	25.44^{bc}	6.22	4.52^{b}	20.99^{b}	18.70^{b}
Average	27.18	25.42	6.22	4.62^{b}	21.52	19.29
LSD	0.64**	1.08**	NS	0.20*	1.11*	1.21*

a,b,c: There is a significant difference between the mean values with various symbols.
**p<0.01, *p<0.05 statistically significant
NS: Non-significant, LSD: Least significant difference

Table 2 shows that the DM contents of alfalfa before ensiling (T_0) were significantly affected by different bacterial inoculations, and the average DM content was 27.18%. In addition, it was seen that *L. gasseri* (28.72%) is superior to other inoculants in the DM content of alfalfa at T_0. The DM content of resulting silages (T_{60}) varied between 24.01-26.64%, and the highest DM content was obtained from the *L. gasseri* (26.64%) isolate, followed by *L. brevis* (26.19%) isolate (p<0.01). The DM contents of mature silages increased with bacterial isolates compared to the control. Indeed, Agarussi et al. (2019) and Blajman et al. (2020) stated that bacterial inoculants increased the DM content of silage compared to the control. In addition, Silva et al. (2020) reported that silages with low DM content had low LAB numbers and higher pH values. Moreover, it is known that silages with high DM content have relatively better DM preservation. It can be numerically calculated from Table 2 that while the loss in alfalfa silage was 3.17% in the *L. buchneri* isolate, it was 12.11% in control. In other words, 96.83% of DM was recovered by the *L. buchneri* inoculation, while only 87.89% of DM was recovered in control. Filya (2004) reported that fermentation losses, especially ensiling crop material with low DM content, might occur.

The difference between the pH values of alfalfa silage before ensiling (T_0), in which different bacterial inoculants were applied, was not statistically significant. However, when the values are examined, it is observed that the pH value decreased slightly with all LAB inoculations compared to the control (6.27). It is well known that reducing pH values in alfalfa silage is difficult. According to the data from Table 2, the pH values of the control silage were 4.87, which is a satisfying level for alfalfa silage; however, pH values for all inoculated silage were significantly lower than that of the control. All bacterial isolates are statistically in the same group. It can be stated that homofermentative bacteria isolates generally have a positive effect on the decrease in the pH of hard-to-ensilage legume plants such as alfalfa. Filya et al. (2007) reported similar observations in their experiments that homofermentative bacteria are more effective in producing lactic acid than heterofermentative, which ensures low pH values. Likewise, in a study conducted by Zielińska et al. (2015), it was shown that high pH (4.8) in alfalfa silage decreased with bacterial inoculants (4.0-4.2). Furthermore, Uher et al. (2019) demonstrated that commercial lactic acid inoculants lowered the pH compared to the control. In addition, Kuppusamy et al. (2020) reported that the *Lactobacillus plantarum* RJ1 and *Pediococcus pentosaceus* S22 LAB strains decreased the pH in alfalfa silage.

The CP value before ensiling (T_0) was statistically affected by different bacterial inoculants, and the CP value of the *L. brevis* bacterial isolates increased (p<0.05) compared to the uninoculated alfalfa (21.70%). It was observed that other bacterial isolates were statistically in the same group as the control. This was also seen in matured silages. The highest CP content was determined at 20.55% in the *L. brevis* isolate (p<0.05), followed by the *L. gasseri* at 19.64% and the *P. citerum* isolates at 18.76%. Only the *L. brevis* inoculant preserved the CP content of alfalfa silage significantly. All other inoculants and the control were comprised of the same statistic group. The CP content is lower in uninoculated (control) alfalfa compared to the *L. brevis* inoculation, indicating that less proteolysis occurred in inoculated silages. This may be associated with high pH values causing protein degradation (McDonald et al. 1991). Many recent

studies have shown that LAB isolates cause an increase in the CP content of silage compared to the control (Ergin & Gumus 2020; Li et al. 2020). Their study shows that the *L. brevis* isolate is superior to other isolates in terms of the CP content. Similarly, Laslo et al. (2019) applied six different LAB isolates. They reported that the *L. brevis* isolated on the 30th day of ensiling increased CP compared to the control and positively affected the ensiling process.

The results of LAB, yeast, enterobacteria, and mold counts before (T_0) and after 60 days (T_{60}) ensiling samples of alfalfa silages strained with different bacterial isolates are shown in Table 3.

Table 3- Presence of lactic acid bacteria, yeast, and enterobacteria at T0 and T60 opening times of silages belonging to different bacterial inoculants

Bacteria inoculants	*LAB*		*Yeast*		*Enterobacteria*		*Mold*	
	(T_0)	*(T_{60})*	*(T_0)*	*(T_{60})*	*(T_0)*	*(T_{60})*	*(T_0)*	*(T_{60})*
Control	3.70^{bc}	5.19	6.93^{bc}	5.45^{a}	5.87^{bc}	4.52^{a}	5.00	ND
L. bifermentans	4.32^{ab}	5.61	6.87^{c}	5.32^{a}	6.47^{a}	3.39^{bc}	5.10	ND
L. gasseri	4.53^{a}	5.51	7.76^{ab}	3.67^{c}	5.97^{abc}	3.69^{bc}	5.20	ND
L. brevis	3.77^{bc}	5.43	8.48^{a}	3.47^{c}	5.94^{bc}	3.20^{bc}	5.30	ND
P. citerum	4.88^{a}	5.65	7.37^{bc}	5.31^{ab}	6.37^{ab}	3.62^{bc}	5.30	ND
L. buchneri	3.40^{c}	5.52	5.31^{d}	5.11^{ab}	5.48^{c}	3.81^{b}	5.00	ND
L. citerum	3.30^{c}	5.98	5.87^{d}	4.54^{b}	5.74^{c}	3.10^{c}	5.00	ND
Average	3.98	5.56	6.94	4.70	5.98	3.62	5.13	ND
LSD	0.72**	NS	0.85**	0.77**	0.53*	0.68**	NS	ND

a,b,c: There is a significant difference between the mean values with various symbols.
**p<0.01 statistically significant
NS: Non-significant, LSD: Least significant difference, ND: Not detected

The LAB count before ensiling (T_0) varied between 3.30-4.88 ($\log_{10}$ cfu/g silage); the highest LAB count was obtained from *P. citerum* (4.88 $\log_{10}$ cfu/g) and *L.gasseri* (4.53 $\log_{10}$ cfu/g) bacterial inoculants, followed by *L. bifermentans* as (4.32 $\log_{10}$ cfu/g) bacterial inoculants ($p<0.01$). The lowest LAB count was obtained from *L. buchneri* and *L. citerum* isolates inoculations. After ensiling (T_{60}), there was no statistically significant difference between the LAB numbers of different bacterial inoculants. Table 3 shows a slight increase in LAB numbers in LAB inoculations compared to the control (5.19 $\log_{10}$ cfu/g) group. This finding may be related to LA production. Ertekin and Kızılşimşek (2020), in their study investigating the effects of different inoculants on alfalfa silage, reported that *L. citerum* and *L. bifermentans* became prominent in LA production compared to the other isolates. In addition, other studies revealed that inoculant silages increased LAB counts compared to the control (Huo et al. 2021; Silva et al. 2016). Queiroz et al. (2013) reported that the number of the LAB naturally found in the plant is low, and this amount increases with LAB isolates. However, other studies have noted that the number of undesirable microorganisms decreased, and the fermentation process improved with LAB, which became dominant in the silage (Jung et al. 2022; Muck et al. 2018).

In this study, the difference between the yeast numbers before ensiling (T_0) was statistically significant, the yeast numbers ranged between 5.31-8.48 ($\log_{10}$ cfu/g silage), and the highest yeast count was obtained from the *L. brevis* isolate ($p<0.01$). The lowest yeast count was extracted from the *L. buchneri* and *L. citerum* isolates, which were statistically in the same group. Driehuis et al. (1999) reported that the *L. buchneri* isolate inhibits yeast that enhances the silage's aerobic stability. However, this does not match the CP content we detected in the silage. The diversity of isolates can explain this difference between studies. It was observed that the yeast count decreased in silages opened after the 60th day, and the lowest yeast count was obtained at the end of the 60th day, especially in the *L. gasseri* and *L. brevis* isolates, which had the highest yeast count in fresh silage material ($p<0.01$). Therefore, the acid production potential of bacteria rather than the amount of LAB number is debatable. Even though the LAB count was low, especially in the *L. gasseri* isolate, it reduced the presence of yeast and achieved the lowest pH. At the same time, at the beginning of ensiling, the enterobacteria count in silages with the *L. bifermentans* and *P. citerum* inoculants was higher than the control, and the enterobacteria count decreased considerably on the 60th day of silage. These results indicate the inhibitory effects of the two isolates on the growth of enterobacteria. It was found that the number of enterobacteria decreased with all inoculants compared to the control. Therefore, it can be said that LAB inoculation positively affects the presence of undesirable enterobacteria in the silage. The study determined that the mold in the fresh material ranged between 5.00-5.30 ($\log_{10}$ cfu/g silage), and there was no statistical difference. In mature silages, no mold was detected in MEA plantings.

Compared to the control, the LAB isolates used in this study positively affected the fermentation properties and CP content. For quality silage fermentation, it is expected that LAB, the most critical species during silage, will have higher numerically, lower enterobacteria, yeast, and mold numbers.

4. Conclusions

This study investigated the effects of alfalfa plant silage on fermentation properties by inoculating six different LAB strains compared to untreated silage. The *L. brevis* (LS-55-2-2) and *L. citerum* (LS-70-6-1) strains were the most limiting for yeast and enterobacteria growth in silage, respectively. Distinctively, *L. gasseri* (LS-51-2-1) became prominent for recovering DM, and *L. brevis* conserved CP more significantly than the other inoculants and the control. In addition, all strains increased the number of LAB in the obtained silages and control group but did not show any statistical difference. It is concluded that LAB inoculations improved the fermentation properties of alfalfa silage in terms of pH, DM recovery, and protein content, as well as decreasing undesired microorganisms.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: T.G., F.A., M.K., Design: T.G., M.K., Data Collection or Processing: T.G., F.A., S.A., M.K., Analysis or Interpretation: T.G., F.A., S.A., M.K., Literature Search: T.G., S.A., Writing: T.G., M.K.

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