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The Effect of Lyophilized and Frozen Natural Lactic Acid Bacteria on Alfalfa Silage Quality Prepared in Different Ways and Stored for Different Periods of Time

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ABSTRACT Within the scope of this study, it was aimed to determine the effect of the groups with the highest LAB numbers determined as a result of storage for one and three months on alfalfa silage quality by freezing fermented lactic acid bacteria (LAB) liquids prepared with different levels of sucrose addition (5-10%) and incubation (2 and 5 days) for different periods of time in deep freezer and by drying via lyophilization process according to the results obtained from the previous study.In the study, groups consisted of control, 2D5%STsL (lyophilized with TRIS (Ts) after 2 days incubation with 5% sucrose addition), 2D10%SDsL (lyophilized with DMSO (Ds) after 2 days incubation with 10% sucrose addition), 5D10%SDsL (DMSO (Ds) additive lyophilized after 5 days of incubation with 10% sucrose addition), and 5D5%STsD (TRIS (Ts) additive deep freezer after 5 days of incubation with 5% sucrose addition).In the study, LAB count, CO_2, lactic acid (LA) content, acetic acid (AA) content, pH, NH_3-N/TN, and butyric acid (BA) values were statistically significant between the groups at the end of the one-month storage period. Crude protein (CP), pH, LA, and BA values were found to be statistically significant between the groups at the end of the three-month storage period in the study.

Keywords: *Alfalfa, Probiotics, Silage.*

ÖZ

Farklı Şekillerde Hazırlanarak Değişik Sürelerde Depolanan Liyofilize Edilmiş ve Dondurulmuş Doğal Laktik Asit Bakteri Sıvılarının Yonca Silaji Kalitesi Üzerine Etkisi

Bu çalışma kapsamında, önceki çalışmadan elde edilen sonuçlarına göre farklı seviyelerde sükroz ilavesi (%5-10) ve farklı sürelerde inkübasyonla (2 ve 5 gün) hazırlanmış fermente edilmiş laktik asit bakteri (LAB) sıvılarının derin dondurucuda dondurularak ve liyofilizasyon işlemi ile kurutularak bir ve üç ay süre ile depolanması sonucunda belirlenen en yüksek LAB sayılarına sahip grupların yonca silajı kalitesi üzerine etkisi belirlenmesi amaçlanmıştır. Çalışmada gruplar kontrol, 2D5%STsL (%5 sükroz ilavesi ile 2 gün inkübasyon sonrasında TRİS (Ts) katkılı liyofilize), 2D10%SDsL (%10 sükroz ilavesi ile 2 gün inkübasyon sonrasında DMSO (Ds) katkılı liyofilize), 5D10%SDsL (%10 sükroz ilavesi ile 5 gün inkübasyon sonrasında DMSO katkılı liyofilize) ve 5D5%STsD'den (%5 sükroz ilavesi ile 5 gün inkübasyon sonrasında TRİS katkılı derin dondurucu) oluşuyordu.Çalışmada bir aylık depolama süresi sonunda LAB sayısı, CO_2, laktik asit (LA) içeriği, asetik asit (AA) içeriği, pH, NH_3-N/TN ve bütirik asit (BA) değerleri gruplar arasında istatistiksel olarak anlamlı bulunmuştur. Çalışmada üç aylık depolama süresi sonunda ise gruplar arasında ham protein (CP), pH, LA ve BA değerleri istatistiksel olarak anlamlı bulunmuştur.

Anahtar Kelimeler: *Probiyotik, Silaj, Yonca.*

INTRODUCTION

In order to improve silage quality, live bacterial cultures called microbialinoculants have recently been utilized. For this purpose, commercially produced microbialinoculants preparations contain mostly LAB.

Lactic acid bacteria positively improve fermentation by minimizing the growth of aerobic bacteria, yeasts and mold that will compete for the substrate in the environment as a result of the formation of anaerobic conditions in the silo. Lactic acid bacteria inactivate plant protease enzymes by lowering the pH in the silo and reduce the degradation of silage plant proteins, as well as preventing the development of undesirable microorganisms in aerobic silage fermentation (Muck 1996). The desired anaerobic conditions in the silo can be achieved by ensuring that the ensiled material has the

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appropriate dry matter (DM) content, is broken down to the appropriate size, the silo is filled quickly, compacted sufficiently, and closed quickly and airtight. With the formation of anaerobic conditions in the silo, LAB present in the natural microflora of the ensiled product ferment water-soluble carbohydrates (WSC) into various organic acids, mostly lactic acid. These acids, which are the fermentation product of lactic acid bacteria, increase the hydrogen ion level in the silo to a level that inhibits the growth of microorganisms whose activities in the silo are undesirable. As a result, the increase in lactic acid production in the silo and the consequent decrease in pH value inhibits the growth of all remaining microorganisms in the silo (McDonald et al. 2002).

This study, it was aimed to investigate the effect of different storage methods, incubation times, and preservative-added natural lactic acid bacterial liquid inoculant on the quality of alfalfa silage.

MATERIAL AND METHODS

Ethics committee approval was not required as the study did not include (live or dead animals or their tissues, slaughterhouse materials, procedures with waste fetuses).

Formation of Test groups

In the study, the LAB liquids were frozen and lyophilized and stored for one and three months and the groups with the highest LAB numbers at the end of each storage period were added to the silages prepared from alfalfa plants. In this context, at the end of one month storage period, in addition to the control group, 2D5%STsL, 2D10%SDsL, 5D10%SDsL and 5D5%STsD groups with the highest LAB numbers were prepared as treatment group silages. At the end of three months storage period, 2D5%SDsL, 5D5%STsL, 5D5%STsL,5D10%STsL and 5D10%STsDD groups with the highest LAB numbers were prepared as treatment group silages in addition to the control group.

Activation of Fermented LAB Liquids Lyophilized and Frozen in Deep freezer

Within the scope of the study, lyophilized, dried and deep-frozen LAB fluids were stored for one and three months. The lyophilized groups were left to thaw for 3 hours at room temperature by adding 1 ml of distilled water and the deep-frozen groups were left to thaw for 3 hours at room temperature without adding distilled water, and then microbiological analyses were performed. At the end of each storage period (one and three months), four groups of LAB liquids with the highest LAB numbers were added to the silages prepared from alfalfa plants. In this context, as a result of the microbiological analyzes performed at the end of one month storage, while 2D5%STsL, 2D10%SDsL, 5D10%SDsL and 5D5%STsD groups with the highest LAB numbers constituted the treatment groups, the silage groups were formed so that the alfalfa silage without additives constituted the control group. At the end of the microbiological analysis performed after three months of storage, 2D5%SDsL, 5D5%STsL, 5D10%STsL and 5D10%10STsD groups with the highest LAB numbers constituted the treatment groups, while unamended alfalfa silage constituted the control group.

Preparation of Alfalfa silage

In the silages prepared at the end of one month storage period, the alfalfa plant (*Medicagosativa L*) used as silage material was harvested at the period of second harvest and 20% flowering. In the silages prepared at the end of the three-month storage period, the alfalfa plant, which was used as silage material, was harvested in the period of fifth harvest and when the flowering was 20%. The alfalfa plant, which was used as silage material in the study, was broken into 5-7 cm pieces by mean. The silages prepared at the end of each storage period contained LAB liquids obtained at the end of one month storage period (2D5%STsL, 2D10%SDsL, 5D10%SDsL and 5D5%STsD) according to the results of the control (without additives) and the first test; LAB liquids obtained from frozen and/or lyophilized LAB liquids with the highest LAB count at three months storage period (2D5%SDsL, 5D5%STsL, 5D10%STsL and 5D%10STsD) were prepared by spraying the fresh silage material at a dose of 10^5cfu/ml. At this stage, in order to apply the LAB liquids added to the silage material homogeneously, LAB liquids were added into 10 ml of pure water for each kilogram of fresh silage material and sprayed with hand sprays independently of each other. In the control (no additive) group, the amount of pure water added to the treatment group was sprayed in order to homogenize the DM effect in the treatment groups. The silages were compressed in 1.5 liter glass jars with 4 replicates for control (without additive) and each treatment group and silaged in an airtight manner. Thus, 20 jars of silage were prepared for each storage period (one month and three months). The prepared silages were stored for 60 days at room temperature in a dark environment by covering the jars.

Determination of Silage Composition

Control (without additive) and LAB liquid-added alfalfa silages were opened at the end of the sixty-day ensiling period and the effects of fermented LAB liquid additives on alfalfa silage quality were determined. The silages prepared within the scope of the test (at the end of one and three months storage period) were opened at the end of the fermentation period, and after the 3-5 cm part of the top of the jars was discarded, 100 ml of pure water was added to 25 g of silage sample taken homogeneously and disintegrated with the help of a mixer for 2 minutes and the pH value of the silage liquid obtained was quickly measured with a pH meter (Hanna-HI-9813) (Polan et al. 1998).

Ammonia nitrogen ratio (NH_3-N/TN, %) values in total nitrogen (TN) content of the silages obtained were determined according to the method reported by AOAC (1990). Lactic acid and volatile fatty acids (butyric (BA), acetic (AA) and propionic acid (PA)) concentrations were determined by high pressure liquid chromatography (HPLC) according to the method reported by Suzuki and Lund (Suzuki and Lund 1980). Aerobic stability values of the silages obtained within the scope of the study were determined according to the method reported by Ashbell et al. (1991). In the preparation of the silages, the WSC content of alfalfa plant used as silage material was determined according to the method reported by Dubois et al. (1996) and the buffering capacity (BC) was determined according to the method reported by Playne and McDonald (1966). The part of the silages used in the crude nutrient analysis of the silages evaluated within the scope of the second test was dried at room temperature and ground in a laboratory mill (Şimşek Laborteknik) to pass through a 1 mm of sieve and made ready for analysis. The DM, crude ash (CA) and crude protein (CP) contents of the obtained silages and alfalfa plant used as silage material were determined by the method reported by AOAC (2005). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents were determined according to the method reported by Van Soest et al. (2018).

Statistical Analysis

The effects of LAB liquid addition on the quality of alfalfa silage were evaluated by analysis of variance. The difference between the averages obtained in the test was determined by Duncan multiple comparison test ($p<0.01$) and SAS (1989) package program was used for this purpose.

RESULTS

Alfalfa silages added with lactic acid bacteria liquids stored for one month

In this study, the values of DM, CA, CP, ADF and NDF were determined as 20.30%, 9.95%, 23.70%, 37.96% and 39.14%, respectively; buffering capacity was 620 meq/kg DM; and WSC content was determined as 71.10 g/kg DM, according to DM basis of alfalfa plant used in the preparation of alfalfa silages obtained by adding LAB liquids with the highest LAB values at the end of one month storage period. The effects of LAB liquids with the highest LAB values on the crude nutrient values of alfalfa silage at the end of one month storage period are presented in Table 1.

There was no statistical difference ($p>0.01$) between the LAB liquids with the highest LAB values at the end of one month storage period on the crude nutrient values of alfalfa silage. The effects of LAB liquids with the highest LAB values on the fermentation quality of alfalfa silage at the end of one month storage period are presented in Table 2. When the pH, NH_3-N/TN, CO_2, LA, AA and BA values of the silages obtained were analyzed, the differences between the groups were found to be statistically significant ($p<0.01$).

In addition, propionic acid was not detected in any of the alfalfa silages prepared by adding the LAB liquids with the highest LAB values to the alfalfa plant at the end of one month storage period.

Table 1: The effect of LAB liquids with the highest LAB values on crude nutrient values of alfalfa silage at the end of one month storage period.

Groups	DM	CA	CP	ADF	NDF
Control	17.72	11.57	23.34	30.06	31.57
2D5%STsL	18.33	10.90	24.02	29.99	32.56
2D10%SDsL	18.24	11.05	24.31	29.45	31.65
5D10%SDsL	17.67	11.44	23.36	30.99	32.04
5D5%STsD	18.19	11.25	23.82	32.86	30.83
SEM	0.091	0.085	0.145	0.412	0.310
p	p>0.01	p>0.01	p>0.01	p>0.01	p>0.01

DM: Dry matter %, **CA:** Crude ash (DM%), **CP:** Crude protein (DM%), **ADF:** Acid Detergent Fiber (DM%), **NDF:** Neutral Detergent Fiber (DM%), **2D5%STsL:** lyophilized with TRIS (Ts) after 2 days incubation with 5% sucrose addition, **2D10%SDsL:** lyophilized with DMSO (Ds) after 2 days incubation with 10% sucrose addition, **5D10%SDsL:** DMSO (Ds) additive lyophilized after 5 days of incubation with 10% sucrose addition, **5D5%STsD:** (TRIS (Ts) additive deep freezer after 5 days of incubation with 5% sucrose addition.

Table 2: The effect of LAB liquids with the highest LAB values on the fermentation quality of alfalfa silage at the end of one month storage period.

Groups	pH	NH_3-N/TN	CO_2	LA	AA	PA	BA
Control	5.01^{ab}	50.71^{b}	9.07^{a}	28.41^{b}	13.29^{c}	0.00	5.31^{b}
2D5%STsL	4.75^{b}	40.57^{c}	6.03^{c}	45.89^{a}	18.49^{bc}	0.00	3.10^{c}
2D10%SDsL	4.76^{b}	42.93^{c}	5.42^{c}	35.44^{b}	19.50^{ab}	0.00	3.65^{c}
5D10%SDsL	5.33^{a}	55.07^{a}	7.17^{b}	26.96^{b}	24.25^{ab}	0.00	7.42^{a}
5D5%STsD	5.06^{ab}	53.76^{ab}	6.92^{b}	25.64^{b}	25.31^{a}	0.00	7.51^{a}
SEM	0.066	1.385	0.296	1.946	1.133	-	0.465
p	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01	p<0.01

a-c: Values with different letters in the same column were found to be different ($p<0.01$), **NH_3-N/TN:** Ammonia nitrogen, **CO_2:** Carbondioxide, **LA:** Lactic acid g/kg DM, **AA:** Asetic acid g/kg DM, **PA:** Propionic acid g/kg DM, **BA:** Butyric acid g/kg DM, **2D5%STsL:** lyophilized with TRIS (Ts) after 2 days incubation with 5% sucrose addition, **2D10%SDsL:** lyophilized with DMSO (Ds) after 2 days incubation with 10% sucrose addition, **5D10%SDsL:** DMSO (Ds) additive lyophilized after 5 days of incubation with 10% sucrose addition, **5D5%STsD:** (TRIS (Ts) additive deep freezer after 5 days of incubation with 5% sucrose addition.

Alfalfa silages added with Lactic Acid Bacterial Liquids Stored for Three months

In this study, the values of DM, CA, CP, ADF and NDF were determined as 23.80%, 10.81%, 19.73%, 37.44% and 43.11%, respectively; buffering capacity was determined as 500 meq/kg DM; and WSC content was determined as 76.20 g/kg DM, according to the DM basis of the alfalfa plant used in the preparation of alfalfa silages obtained by adding LAB liquids with the highest LAB values at the end of three months storage period.

The effects of LAB liquids with the highest LAB values on the crude nutrient values of alfalfa silage at the end of three months storage period are presented in Table 3.When Table 3 was examined, it was found that while the differences between the groups in terms of DM, CA, ADF and NDF values of the silages obtained were not statistically significant (p>0.01), the differences between the groups in terms of CP values were statistically significant (p<0.01).

The effect of lactic acid bacteria liquids with the highest LAB values on the fermentation quality of alfalfa silage at the end of three months storage period is presented in Table 4.

When Table 4 is examined, while the differences between the groups in terms of CO_2 and AA values of the silages obtained were not statistically significant (p>0.01), the differences between the groups in terms of pH, NH_3-N/TN, LA and BA values were found to be statistically significant (p<0.01).

Table 3: The effect of LAB liquids with the highest LAB values on crude nutrient values of alfalfa silage at the end of three months storage period.

Groups	DM	CA	CP	ADF	NDF
Control	23.78	11.56	20.07[a]	28.92	35.07
2D5%STsL	23.49	11.50	19.45[b]	29.79	34.83
2D10%SDsL	23.95	11.17	19.22[b]	31.55	35.57
5D10%SDsL	23.75	11.38	19.53[b]	30.59	34.81
5D5%STsD	23.98	11.41	19.23[b]	30.61	35.87
SEM	0.158	0.052	0.087	0.337	0.321
p	p>0.01	p>0.01	p<0.01	p>0.01	p>0.01

[a-c]: Values with different letters in the same column were found to be different (p<0.01), **DM:** Dry matter %, **CA:** Crude ash (DM%), **CP:** Crude protein (DM%), **ADF:** Acid Detergent Fiber (DM%), **NDF:** Neutral Detergent Fiber (DM%), **2D5%STsL:** lyophilized with TRIS (Ts) after 2 days incubation with 5% sucrose addition, **2D10%SDsL:** lyophilized with DMSO (Ds) after 2 days incubation with 10% sucrose addition, **5D10%SDsL:** DMSO (Ds) additive lyophilized after 5 days of incubation with 10% sucrose addition, **5D5%STsD:** (TRIS (Ts) additive deep freezer after 5 days of incubation with 5% sucrose addition.

Table 4: The effect of LAB liquids with the highest LAB values on the fermentation quality of alfalfa silage at the end of three months storage period.

Groups	pH	NH_3-N/TN	CO_2	LA	AA	PA	BA
Control	5.15[a]	29.66[a]	5.12	14.19[b]	14.25	0.00	4.31[a]
2D5%STsL	4.91[c]	25.40[b]	4.55	26.09[a]	18.50	0.00	2.92[b]
2D10%SDsL	4.92[c]	27.08[ab]	4.84	25.41[a]	16.99	0.00	2.98[b]
5D10%SDsL	4.90[c]	26.82[ab]	4.54	23.68[a]	15.02	0.00	2.95[b]
5D5%STsD	5.03[b]	29.12[a]	4.56	12.67[b]	17.57	0.00	4.12[a]
SEM	0.024	0.440	0.121	1.375	0.665	-	0.415
p	p<0.01	p<0.01	p>0.01	p<0.01	p>0.01	-	p<0.01

[a-c]: Values with different letters in the same column were found to be different (p<0.01), **NH_3-N/TN:** Ammonia nitrogen, **CO_2:** Carbondioxide, **LA:** Lactic acid g/kg DM, **AA:** Asetic acid g/kg DM, **PA:** Propionic acid g/kg DM, **BA:** Butyric acid g/kg DM, **2D5%STsL:** lyophilized with TRIS (Ts) after 2 days incubation with 5% sucrose addition, **2D10%SDsL:** lyophilized with DMSO (Ds) after 2 days incubation with 10% sucrose addition, **5D10%SDsL:** DMSO (Ds) additive lyophilized after 5 days of incubation with 10% sucrose addition, **5D5%STsD:** (TRIS (Ts) additive deep freezer after 5 days of incubation with 5% sucrose addition.

DISCUSSION AND CONCLUSION

At the end of one month storage period, the DM values (18.33%, 18.24% and 18.19%) of the 2D5%STsL, 2D10%SDsL and 5D5%STsD additive groups obtained by adding the LAB liquids with the highest LAB values were not statistically different from the control group, but numerical increases were determined in the treatment groups (Table 1). It is accepted that silages with less than 10-12% DM loss during the ensiling process of the silage material are considered to be fermented in the desired direction, and silages with more than 20% DM loss are considered to have undesired fermentation (Kung 2008). In this study, compared to the control group silage, it was observed that the DM loss of alfalfa plant used as silage material (20.30% DM) was less than 12% in alfalfa silages obtained by adding LAB liquids with the highest LAB values at the end of one month storage period (2D5%STsL, 2D10%SDsL and 5D5%STsD). At the end of the three-month storage period, the DM values (Table 3) of the additive groups prepared by adding LAB liquids with the highest LAB values (2D5%SDsL, 5D5%STsL, 5D10%STsL and 5D10%STsD) were determined as 23.49%, 23.95%, 23.75% and 23.98%, respectively, and there was no statistical difference with the value obtained from the control group (23.78%). At the end of the three-month storage period of this study, it was observed that there was generally no DM loss of the alfalfa plant (23.80% DM) used as silage material in the control and all additive silages prepared at the end of the three-month storage period. In this study, among the alfalfa silages obtained by adding LAB liquids with the highest LAB values at the end of one month storage period, the numerical increase in DM values (18.33% and 18.24%) in the 2D5%STsL and 2D10%SDsL additive groups and the low pH and butyric acid values and high lactic acid values in these groups suggest that the activity of homofermentative LAB was higher in these groups. At the end of one month storage period, the CP value of alfalfa plant used in silage preparation was determined as 23.70% DM. Compared to the control silage, there was no statistical difference in CP values (Table 1) at the end of one month storage period, but numerical increases were determined. At the end of the three-month storage period, the CP value of alfalfa plant used in silage preparation was determined as 19.73% DM. There was no statistical difference between the CP values of the additive groups prepared at the end of the three-month storage period and the control group (Table 3). In this study, the high CP value (23.70% DM) of alfalfa plant used as silage material at the end of one month storage period may be due to the fact that the plant was harvested and ensiled at the beginning of the second harvest and flowering when it was still green. At the end of one- and three-months storage period, it was observed that LAB liquids had no effect on ADF and NDF values (Table 1 and Table 3) of alfalfa silages obtained by adding LAB liquids with the highest LAB values. The results obtained in this study in terms of ADF and NDF parameters were consistent with the report that LAB has little or no effect on the cellulose value of silages due to its lack of degradative effect on cell wall elements (Muck 1996).

The pH values of silages are affected by many factors such as the LAB species used as inoculant source, the buffering capacity of the plant, the WSC content, the structure of mycobialflora present in the plant and the process applied in the preparation of the silage. When the pH values of alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of one month storage period were analyzed, the values obtained from the 2D5%STsL and 2D10%SDsL additive silages (4.75 and 4.76) were numerically lower than the value obtained from the control group silage (5.01), while the values obtained from the 5D10%SDsL and 5D5%STsD additive groups (5.33 and 5.06) were similar to the control group (Table 2). When the pH values of alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of the three-month storage period were analyzed; the values obtained from the additive silages were lower ($p<0.01$) than the value obtained from the control group silage (Table 4).

The pH values (4.75 and 4.76) obtained from the 2D5%STsL and 2D10%SDsL additive groups of the silages prepared at the end of one month storage period were found to be close to Kung and Shaver (2001)'s report that the pH value should be in the range of 4.3-4.7 for quality legume silages. Among the alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of one and three months of storage period, the pH values in the groups with 2D5%STsL and 2D10%SDsL at the end of one month storage period and 2D5%SDsL, 5D5%STsL, 5D10%STsL and 5D10%STsD at the end of three months of storage period were found to be lower than the control and other additive groups, probably because the lactic acid content in these groups was higher than the other groups. The increase in silage pH values with the increase in AA and decrease in LA values can be explained by the fact that acetic acid is a weaker acid than lactic acid (Keleş 2009). When the lactic acid, acetic acid, pH and NH_3-N/TN values of alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of one month storage period of this study were evaluated in general, it was concluded that homofermentative LAB species were more effective in the silo in the 2D5%STsL and 2D10%SDsL groups, and heterofermentative LAB species were more effective in the silo in the 5G%10SDsL and 5D5%STsD groups (Table 2). When lactic acid, acetic acid, pH and NH_3-N/TN values of alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of the three-month storage period of this study were evaluated in general; it is thought that homofermentative LAB species are more effective in the silo in the groups with 2D5%SDsL, 5D5%STsL and 5D10%STsL, and heterofermentative LAB species are more effective in the silo in the group added with 5D10%STsD (Table 4). In this study, the pH values (5.01 and 5.15) of the control silages prepared at the end of one and three months storage period were lower than other studies (Bai et al. 2020; Hu et al. 2020; Li et al. 2020; Yang et al. 2020; Huo et al. 2021; Sun et al. 2021; Wang et al. 2023) and the expected result; this may be due to the number of epiphytic microorganisms carried by the alfalfa plant used in the preparation of silages, its species, vegetation period, withering process and chopping size (Spoelstra and Hindle 1989). In this study, the alfalfa plant used in the preparation of silages was shredded in 3-5 cm length and the plant enzymes released due to this process activated the bacteria that were previously on the plant but not active, especially increasing the LAB population (Lin et al. 1992) and the silage was well compressed.

When the NH_3-N/TN values of alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of one month storage period were examined (Table 2), the values obtained from 2D5%STsL and 2D10%SDsL additive silages (40.57% and 42.93%) were lower ($p<0.01$) than the value obtained from the control group silage (50.71%). At the end of one month storage

period, the NH_3-N/TN value obtained from the alfalfa silages prepared with the addition of LAB liquids with the highest LAB values from the 5G%10SDsL group (55.07%) was higher ($p<0.01$) than the value obtained from the control group silage (50.71%). When the NH_3-N/TN values of the alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of the three-month storage period were examined (Table 4), the value obtained from 2D5%SDsL-added silage (25.40%) was lower ($P<0.01$) than the value obtained from the control group silage (29.66%). These results were found to be consistent with our study in many studies conducted with the addition of fermented LAB liquid to alfalfa plants, which decreased the NH_3-N values of the silages obtained due to the addition of the additive (Bai et al. 2020; Huo et al. 2021; Sun et al. 2021; Li et al. 2022; Na et al. 2022). It has been reported that the addition of homofermentative LAB inoculant generally decreases the silage NH_3-N value, while the inoculation of *L. buchneri*, which is heterofermentative LAB, decreases the number of yeasts and molds in silages and increases NH_3-N production (Kung and Ranjit 2001; Nsereko et al. 2008). During the proteolysis event occurring in the silo, protease enzymes in the plant break down the proteins in the structure of the plant into peptides and amides, especially amino acids and ammonia. As a result, increases in silage NH_3-N values are formed (Yang et al. 2020). The fact that whether the silo is well compressed or not, the ratio of lactic acid production in the silo, and the DM content of the silage plant are closely related to the silage NH_3-N value. Proteolysis decreases due to the increase in lactic acid in the silo (Davies et al. 1998). In this study, the higher lactic acid values (45.89 and 35.44 g/kg DM) in the 2D5%STsL and 2D10%SDsL additive groups (45.89 and 35.44 g/kg DM) compared to the control group (28.41 g/kg DM) among the alfalfa silages prepared by adding LAB liquids with the highest LAB values at the end of one month storage period can be considered as a reason for the low NH_3-N/TN values in the 2D5%STsL and 2D10%SDsL additive groups. Carpintero et al. (1979) reported that silage can be considered as quality silage if the silage NH_3-N/TN value is 11% or less. In this study, when the NH_3-N/TN values (Tables 2 and 4) of the silages prepared at the end of one- and three-months storage period were evaluated in general, the values obtained were found to be much higher than the values reported by Carpintero et al. (1979) and it was concluded that proteolysis occurred intensively in alfalfa silages prepared in this study.

The number of LAB contaminating the plant before harvesting can vary from $1x10^1$cfu/g to $1.0x10^7$cfu/g and there may be differences in the number and types of LAB contaminating the plants to be silaged. It has been reported that the withering process, hot environmental conditions and the period and number of harvestings have an effect on the number of epiphytic LAB in fresh alfalfa material (Lindgren et al. 1985; Lin et al. 1992). In this study, the plant material used in the silages prepared at the end of the one-month storage period was the second form and was prepared from alfalfa harvested in the period when the air and environmental temperature had not yet risen (May). The plant material used in the silages prepared at the end of the three-month storage period was the fifth form and was prepared from alfalfa harvested during the period when the air and environmental temperature was at its highest (July), and the silage plant was withered before silage preparation in both periods. It is reported that in order for LAB inoculants added to the silage to be effective, the dose value used should be higher than the number of natural LAB in the structure of the plant to be silaged (Pahlow and Honig 1986). The reason why the pH and NH_3-N values of the silages prepared with the addition of LAB liquids stored for one and three months in this study did not reach the expected and desired results may be thought to be due to the fact that the number of epiphytic LAB on the alfalfa plant used as silage material was more than 10^5cfu/g and the application dose of LAB liquids used in this study was insufficient.

When the lactic acid values (Table 2) of the silages prepared by adding the LAB liquids with the highest LAB values at the end of one month storage period were examined, the LA value (45.89 g/kg DM) obtained from the 2D5%STsL additive group was higher than the value obtained from the control group silage (28.41 g/kg DM) ($p<0.01$). When the lactic acid values (Table 4) of the silages prepared at the end of three months storage period were examined, the values obtained from 2D5%SDsL, 5D5%STsL and 5D10%STsL additive groups (26.09, 25.41 and 23.68 g/kg DM) were higher than the values obtained from control and 5D10%STsD additive silages (14.19 and 12.67 g/kg DM) ($P<0.01$). In this study, lactic acid values of 2D5%STsL and 2D10%SDsL group silages prepared at the end of one month storage period were found to be compatible with the values obtained from some previous studies (Hu et al. 2020; Sun et al. 2021; Huo et al. 2022). It is thought that the increase in lactic acid value in silages prepared by adding fermented LAB liquids may be due to the decrease in silage pH value due to the fermentation of LAB in the silo (Weinberg et al. 1988). In a quality silage, the lactic acid ratio should be 65-70% of total silage acids (Kung and Shaver 2001). At the end of one month storage period of this study, while the lactic acid ratios of the control, 5D10%SDsL and 5D5%STsD additive groups in total silage acids (60%, 46% and 44%) were below the specified ratio, the lactic acid ratios of the 2D5%STsL and 2D10%SDsL additive groups in total silage acids (62% and 66%) were found to be close. At the end of the three-month storage period of this study, lactic acid ratios (50%, 58%, 60%, 61% and 42%) in total silage acids of the control, 2D5%SDsL, 5D5%STsL, 5D10%STsL and 5D10%STsD additive groups were below the ratios reported by Kung and Shaver (Kung and Shaver 2001).

When the acetic acid values (Table 2) of alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of one month storage period were examined; the AA values obtained from the 2D10%SDsL, 5D10%SDsL and 5D5%STsD additive groups (19.50, 24.25 and 25.31 g/kg DM) were higher than the value obtained from the control group silage (13.29 g/kg DM) ($p<0.01$). Compared to the control group, the increases in acetic acid values of the silages due to the addition of fermented LAB liquid were found to be compatible with the results obtained from some studies on this subject (Bai et al. 2020; Yang et al. 2020; Drouin et al. 2022). When the acetic acid values of the silages prepared at the end of the three-month storage period (Table 4) were examined, the AA values obtained from the additive groups were found to be similar to the value obtained from the control group, although they increased numerically.

When CO_2 values related to aerobic stability parameter of alfalfa silages prepared by adding LAB liquids with the highest LAB values at the end of one month storage period were examined, CO_2 values obtained from all additive groups were lower than the value obtained from the control group silage (9.07 g/kg DM) ($p<0.01$), and although there was no statistical difference in the silages

prepared at the end of three months storage period, a numerical decrease was determined (Table 2, Table 4). There is a direct relationship between silage acetic acid value and aerobic stability values of silage. Acetic acid has an inhibitory effect against microorganisms that cause silage spoilage after the silage is opened and prevents the growth and activity of yeasts (Taylor et al. 2002; Danner et al. 2003). It was observed that the aerobic stability value of silage increased due to the increase in the amount of acetic acid produced by heterofermentative LAB and the aerobic degradation time of silage were prolonged during the feeding process (Kung and Ranjit 2001). When acetic acid and CO_2 values related to aerobic stability parameters of alfalfa silages prepared with the addition of LAB liquids with the highest LAB values at the end of the three-month storage period were examined; acetic acid values obtained from all additive groups increased numerically although there was no statistical difference from the values obtained from the control group silages, while CO_2 values decreased numerically although there was no statistical difference from the value obtained from the control group silage (5.12 g/kg DM) (Table 4). The effect of acetic acid on reducing CO_2 production, i.e. increasing aerobic stability values, was observed significantly in the silages prepared at the end of three months storage period. Heterofermentative LAB increases the production of acetic acid due to the increase in the amount of WSC in the silo. For this reason, lactic:acetic acid ratio in silages varies according to the content of fermentable WSC. In the present study, the DM value of alfalfa plant used for the silages prepared at the end of one month storage period (20.30%) was lower than that of alfalfa plant used for the silages prepared at the end of three months storage period (23.80%). The reason why the CO_2 production values of the control and additive silages prepared at the end of one month of storage were higher than the CO_2 production values of the silages prepared at the end of three months of storage may be due to the fact that the DM value of the alfalfa plant used as silage material was low in the silages prepared at the end of one month storage period.

When the butyric acid values of the silages prepared at the end of one month storage period (Table 2) were examined, the values obtained from 2D5%STsL and 2D10%SDsL additive groups (3.10 and 3.65 g/kg DM) were lower than the value obtained from the control group (5.31 g/kg DM), while the values obtained from 5D10%SDsL and 5D5%STsD additive groups (7.42 and 7.51 g/kg DM) were higher ($p<0.01$). When the butyric acid values of the silages prepared at the end of three months storage period were examined (Table 4), it was determined as 4.31 and 4.12 g/kg DM in the control and 5D10%STsD group silages; compared to the control group, butyric acid values in 2D5%SDsL, 5D5%STsL and 5D10%STsL groups were 2.92, 2.98 and 2.95 g/kg DM lower, respectively ($p<0.01$). The lower butyric acid values obtained from 2D5%STsL and 2D10%SDsL at the end of one month storage period and 2D5%SDsL, 5D5%STsL and 5D10%STsL additive groups at the end of three months storage period compared to the control group can be explained by the low pH values and high lactic acid values in these groups. The fact that butyric acid increased the silage pH value is due to the fact that butyric acid is a weaker acid compared to lactic acid. Clostridial fermentation is reported to occur in silages prepared from silage materials with dry matter content lower than 30-35% (Kendall 1978). In this study, the high CP values (23.70% and 19.73% DM), but low DM (20.30% and 23.80%) and WSC contents (71.10 and 76.20 g/kg DM) of alfalfa plants used as silage material at the end of one- and three-months storage periods are thought to be due to the inadequacy of lactic acid production, which is necessary to inhibit the growth of Clostrodial bacteria (Aydın 2014). In the silages prepared at the end of one and three months of storage period in this study, the low values of the DM and WSC of alfalfa plant used as silage material suggest that saccharolytic Clostridia may have converted the WSC in the plant structure and the organic acids formed in the silage into butyric acid (Mcdonald 1981).

In a quality legume silage, it is preferred to have a DM value of 30-40%, pH value of 4.3-4.7, lactic acid value of 70-80 g/kg DM, acetic acid value of 20-30 g/kg DM, propionic and butyric acid values of 5 g/kg DM, and NH_3-N/TN value of about 10-15% (Kung and Shaver 2001). In this study, DM and lactic acid values of the silages obtained by adding LAB liquids to the silages prepared at the end of one- and three-months storage periods were lower than the specified values and pH values were high. In this study, while the acetic acid values obtained from all of the additive groups obtained by adding LAB liquids to the silages prepared at the end of one month storage period and butyric acid values obtained from 2D5%STsL and 2D10%SDsL groups were found to be compatible with the report of Guo et al. (2020), the acetic and butyric acid values obtained from all of the silages prepared at the end of three months storage period were found to be low.

In the study, the effects on alfalfa silage quality were researched by adding the fermented LAB liquids with the highest LAB values from the frozen and lyophilized LAB liquids for one and three months to the silages prepared from alfalfa plants in four groups for each storage period. At the end of one month storage period, it was observed that CO_2 values decreased, and aerobic stability values increased in alfalfa silages prepared by adding LAB liquids with the highest LAB values. The highest lactic acid content was determined in the group dried by lyophilization process and TRIS (Ts) addition at the end of two days incubation period with 5% sucrose addition (2D5%STsL); the highest acetic acid content was determined in the group dried by lyophilization process and TRIS (Ts) addition at the end of five days incubation period with 5% sucrose addition (5D5%STsD). At the end of the three-month storage period, it was observed that pH values decreased, and lactic acid values increased in alfalfa silages prepared by adding LAB liquids with the highest LAB values. It was observed that the silage fermentation quality was partially improved when the additives obtained by lyophilizing the LAB liquids obtained by incubation at different levels of sucrose and incubation for different periods of time and storage in deep freezer (1 and 3 months) were added to the silages prepared from alfalfa plants. In this study, fermented LAB liquids were generally added to the silages at the dose (10^5cfu/g in fresh silage material) reported for the addition of LAB inoculants. However, considering that alfalfa plants, which were the silage material in this study, had low WSC values and high buffering capacity, the reason why the expected and desired results were not obtained in terms of pH and NH_3-N/TN parameters in this study may be due to the insufficient dose used in the study. It is thought that the additive dose should be higher than 10^5cfu/g to fresh silage material in future studies with legumes.

In this study, considering the high viability rates obtained from the lyophilization and drying of LAB liquids with TRIS (Ts) and DMSO (Ds) cryoprotectants, it is seen that the obtained LAB liquids have a high potential to be used as silage additives and to be commercialized. However, it

was concluded that the lyophilized LAB liquid should be researched in comparison with commercial LAB inoculants in large silos and in silages to be prepared with different silage materials in order to use the application in practice.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Idea / Concept: SSA, ND
Supervision / Consultancy: SSA, ND
Data Collection and / or Processing: SSA, ND
Analysis and / or Interpretation: SSA, ND
Writing the Article: SSA, ND
Critical Review: SSA, ND

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