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RESEARCH ARTICLE

Determination of Lactic Acid Bacterial Numbers of Lyophilized or Frozen Natural Lactic Acid Bacterial Liquids Prepared with Different Methods and Stored for Different Times

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ABSTRACT

In this study, it was aimed to determine the lactic acid bacterium (LAB) counts and viability of fermented lactic acid bacteria liquids (pre-fermented juice (PFJ)) prepared with different levels of sucrose (3%, 5% and 10%) addition and incubation at different times (2, 5 and 10 days), using different cryoprotectants (trisodiumcitrate (TRIS) and dimethylsulfoxide (DMSO)), freezing and lyophilization, and at the end of different storage periods (one and three months). In frozen PFJs, the highest LAB counts were obtained in the TRIS and 5% sucrose supplemented group incubated for 5 days in a one-month storage period, and in the TRIS and 10% sucrose supplemented group incubated for 5 days at the end of the three-month storage period ($p<0.01$). At the end of one month of storage, the highest LAB numbers in lyophilized PFJs were obtained in groups incubated for 5 days and supplemented with 10% sucrose with TRIS and DMSO. In lyophilized PFJs subjected to 3-month storage, the highest LAB numbers were determined in the TRIS and 5%-10% sucrose-supplemented groups incubated for 5 days and in the DMSO and 5% sucrose-supplemented group incubated for 2 days ($p<0.01$). When the results obtained in the study were evaluated in general, it was observed that the viability ratios of LAB decreased due to the sucrose level and the prolongation of incubation and storage time. It can be said that cryoprotectant additive has a positive effect on the preservation of LAB and lyophilization process is advantageous compared to freezing in the deep freezer.

Keywords*:* Freezing, lactic acid bacteria, lyophilization

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Farklı Şekillerde Hazırlanarak Değişik Sürelerde Depolanan Liyofilize Edilmiş ve Dondurulmuş Doğal Laktik Asit Bakteri Sıvılarının Laktik Asit Bakteri Sayılarının Belirlenmesi

ÖZ

Bu çalışma kapsamında, farklı seviyelerde sükroz ilavesi (%3, %5 ve %10) ve farklı sürelerde inkübasyonla (2, 5 ve 10 gün) hazırlanmış fermente edilmiş laktik asit bakteri (LAB) sıvılarının (pre-fermented juice (PFJ)), farklı kryoprotektan maddeler (trisodyum sitrat (TRIS) ve dimetil sülfoksit (DMSO)) kullanılarak, dondurma ve liyofilizasyon ile farklı depolama süreleri (bir ve üç ay) sonunda LAB sayıları ve canlılığının belirlenmesi amaçlanmıştır. Dondurulan fermente edilmiş LAB sıvılarında en yüksek LAB sayıları bir aylık depolamada 5 gün inkübe edilen TRIS ve %5 sükroz katkılı grupta elde edilirken, üç aylık depolama süresi sonunda ise 5 gün inkübe edilen TRIS ve %10 sükroz katkılı grupta elde edilmiştir ($p<0.01$). Liyofilize edilmiş PFJ'lerde en yüksek LAB sayıları bir aylık depolama süresi sonunda TRIS ve DMSO katkılı %10 sükroz eklenerek 5 gün inkübe edilen gruplarda elde edilmiştir. Üç aylık depolama uygulanan liyofilize edilmiş PFJ'lerde ise en yüksek LAB sayıları 5 gün inkübe edilen TRIS ve %5-%10 sükroz katkılı gruplarda ve 2 gün inkübe edilen DMSO ve %5 sukroz katkılı grupta belirlenmiştir ($p<0.01$). Çalışmada elde edilen sonuçlar genel olarak değerlendirildiğinde; sükroz seviyesi ile inkübasyon ve depolama süresinin uzamasına bağlı olarak LAB'nin canlılık oranlarında azalmalar görülmüştür. Kryoprotektan katkısının LAB'nin korunmasında olumlu etki yaptığı, liyofilizasyon işleminin ise derin dondurucuda dondurulma işlemine göre avantajlı olduğu söylenebilir.

Anahtar Kelimeler: Dondurulma, laktik asit bakterisi, liyofilizasyon

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INTRODUCTION

Short-term (simple methods) and long-term preservation methods (modern methods) are used for the preservation of lactic acid bacteria inoculants (Haigh et al. 1987). Short-term preservation methods include transfer, preservation under liquid paraffin, preservation in distilled water and drying, while long-term preservation methods include lyophilization and deep freezing. Nowadays, lyophilization and deep-freeze preservation are preferred among long-term drying techniques for the preservation of both commercial lactic acid bacteria (LAB) inoculants used as silage additives and microorganisms used in microbiology (Öztürk and Çakır 2015). Lyophilization is the drying process of biologically derived materials with high moisture content or aqueous solutions under very low pressure by removing (desorption) the frozen free water particles in the material to be lyophilized by creating suitable conditions by sublimation (Meryman 1960). There are many factors affecting microbial viability and activity in the lyophilization process. These factors can be listed as the genus, species, strain, age, medium, cell density before lyophilization, composition of the protective medium used, the atmosphere in which the culture is protected after lyophilization (vacuum, inert gases, etc.), the amount of moisture remaining in the culture, storage conditions (temperature, time, light), the composition and temperature of the solution used in dilution. Apart from these, factors such as the system used in lyophilization, pre-freezing temperature, etc. are also known to be effective on microbial viability and activity. Bacterial cultures can be preserved as frozen and as lyophilized. When frozen cultures are preserved under appropriate conditions, they maintain their viability during storage. When stored under optimum conditions, they can remain viable for years (Tedeschi and De Paoli, 2011; Peiren et al. 2015). The purpose of this study was to ascertain the LAB numbers of fermented LAB liquids that had been made with varying amounts of sucrose addition, incubated for varying lengths of time by freezing in a deep freezer, dried using the lyophilization technique, and stored for one and three months.

MATERIALS and METHODS

In the study, fermented LAB liquids were prepared by adding sucrose at different levels (3%, 5% and 10%) and incubated for different times (2, 5 and 10 days). At the end of the incubation times, TRIS and DMSO were added to the fermented LAB liquids and the frozen or lyophilized dried LAB liquids were stored for one and three months and LAB numbers were determined.

Preparation of fermented lactic acid bacteria liquid

The fermented LAB liquid was prepared according to the method reported by (Masuko et al. 2002). However, the amount of pure water used was reduced to increase the microbial density. For this purpose, 1000 ml of pure water was added to 3000 g of fresh alfalfa plants and the mixture was disintegrated for 2 minutes with the help of a mixer. The plant liquid mixture obtained after the process was filtered through two layers of cheesecloth. The filtrate was transferred to 50 ml sterile plastic falcon tubes with screw caps and the groups without additive, with 3%, 5% and 10% sucrose addition were formed. The groups without additive, with 3%, 5% and 10% sucrose addition were incubated at 30 °C for 2, 5 and 10 days. After each incubation period, the fermented LAB liquids were centrifuged at 2000 rpm for 4 minutes to increase the LAB density. After centrifugation, 20% of the liquid remaining on top was removed. At the end of each incubation period, 1 ml of fermented LAB liquid was placed in 10 ml sterile bottles for lyophilization and freezing. No cryoprotectant was added to the groups without additives, 20% (v/v) of 50% TRIS solution was added to the TRIS groups, and 0.1% (v/v) DMSO was added to the DMSO groups, and lyophilization as well as deep freezing were performed. Thus, for each incubation period (2, 5 and 10 days), a total of 324 sterile bottles were formed with three replicates for the lyophilization and deep-freezing groups by adding TRIS and DMSO to the groups without additives, with 3%, 5% and 10% sucrose additions.

Lyophilization and deep-freezing process

Sterile special lyophilization bottles were replicated in 15 replicates, and the slit stoppers of the lyophilization bottles were half closed to allow the lyophilization process. In the first stage of the lyophilization process (pre-freezing phase), the lyophilization bottles were frozen at -80 °C for 24 hours and transported under liquid nitrogen vapor to the lyophilization device (Scanvac Coolsafe 55-4) which was started one hour before the start of the lyophilization process and placed on the shelves of the lyophilization device. The vacuum pump of the lyophilization device was operated to start the lyophilization process and the primary drying phase was carried out for 30 hours. At the end of the lyophilization process, the slit lids of the lyophilization bottles were closed without letting air into the system thanks to the mechanism pre-adapted to the lyophilization device. After the completion of the lyophilization process, each sucrose level, incubation time and moisture contents of the lyophilized LAB liquids for the groups without additives, with DMSO and TRIS additives were determined in 3 replicates for each group. The

lyophilized products were preserved in the refrigerator at +4 °C for one and three months.
LAB liquids to be stored using the freezing process were prepared in the same way. Before freezing, the air remaining in the closed bottles was removed with the help of a vacuum motor fitted with a syringe needle. The bottles containing the vacuumed LAB liquids were preserved in a deep freezer at -21 °C for one- and three-months during storage.

Lactic acid bacteria counting
LAB counting of the fermented LAB liquids obtained before incubation, after incubation, and after storage for one and three months was carried out according to the Tempo automatic bacterial counting device test method in 3 replicates for each group. For this purpose, the Tempo LAB device (bioMerieux, MarcyI'Etoile France) was used for LAB counting according to the recommended method (REF-80 071).

Statistical analysis
Statistical analysis was applied according to 3x3x3x2x2 (incubation time x sucrose level x protector x storage time x storage method) factorial experimental design in the evaluation of the data obtained as a result of the study. In this context, the effects of sucrose level, incubation periods, cryoprotectant type, lyophilization and deep-freezing processes and storage periods of one and three months were analyzed. General Linear Model (GLM) was used to determine the significance of the mean of the data and was determined by Duncan's multiple comparison test ($P<0.01$). For this purpose, SAS (1989) package program was used.

RESULTS

The dry matter, crude ash, crude protein, acid detergent fiber (ADF) and neutral detergent fiber (NDF) values of fresh alfalfa plant (*Medicago sativa*) used in the preparation of fermented LAB liquids in the study were determined as 19.21%, 13.19%, 31.11%, 18.70% and 29.59%, respectively; buffering capacity as 670 meq/kg; water-soluble carbohydrate (WSC) content as 64.6 g/kg and LAB number as $1x10^5$ cfu/g. Interaction analyses and sources of interactions for one and three months of storage of LAB liquids incubated at different levels of sucrose addition (3%, 5% and 10%) and for different periods (2, 5 and 10 days), frozen in deep freezer with TRIS and DMSO additives and dried by lyophilization process are presented in Table 1 and Table 2. When Table 1 and Table 2 were examined, sucrose level, incubation time, cryoprotectant type, storage method and storage time showed significant differences ($P<0.01$). It has been observed that incubation time, storage method, storage period and cryoprotectant type affect the survivability of lactic acid bacteria.

The LAB numbers of fresh material, non-additive, TRIS and DMSO-additive, deep-frozen and lyophilized and dried LAB liquids obtained by incubating with different levels of sucrose for different periods are presented in Table 3 (cfu/ml) before one- and three-months storage. The highest LAB numbers in fermented LAB liquids frozen in deep freezer with TRIS and DMSO addition were obtained from 10% sucrose added groups ($9.33x10^{10}$ and $4.67x10^9$ cfu/ml) in 2 days incubation. In the lyophilized and dried groups, the highest LAB values were obtained from the groups to which 5% and 10% sucrose were added ($8.67x10^{10}$ and $2.67x10^{11}$cfu/ml) in 5 days of the incubation period. In the fermented LAB liquids frozen in the deep freezer by adding TRIS and DMSO, it was observed that the LAB numbers increased with the increase in sucrose level in 2 days of incubation time, while LAB number decreased with the increase in sucrose level in 5- and 10-day incubation times ($P<0.01$).
In the fermented LAB liquids dried by lyophilization with the addition of TRIS and DMSO, the LAB number decreased with the increase in sucrose level in the 2 days of incubation period. In the 10 days of incubation period, it was observed that the LAB number decreased due to the increase in sucrose level in the TRIS additive groups. In the DMSO additive group, it was observed that there was a decrease in the group with 5% sucrose addition ($P<0.01$).
The LAB viability ratios of lyophilized and dried LAB liquids without additives, with TRIS and DMSO additives, obtained by incubating for different periods of time with different levels of sucrose addition, compared to the freshly prepared group before storage for one and three months are given in Table 4.
The LAB values (cfu/ml) of the fermented LAB liquids made by adding various amounts of sucrose and incubating for various lengths of time after freezing in the deep freezer and lyophilized and stored for one month are presented in Table 5. In the samples stored in the deep freezer for one month, the highest LAB values were obtained with 5% sucrose ($5.33x10^{10}$ cfu/ml) in 5-day incubation time in the group which TRIS was added. In the DMSO added group, it was determined with the addition of 10% sucrose ($1.47x10^9$ cfu/ml) in 2-days incubation time ($P<0.01$). In the samples dried by lyophilization and stored for one month, the highest LAB values were obtained in the group with 10% sucrose in 5-day incubation time in both the TRIS- (9.00×10^{10} cfu/ml) and DMSO-added (1.00×10^{11} cfu/ml) groups ($P<0.01$).
The lowest LAB numbers of lyophilized and dried LAB bacterial liquids after one month of storage were obtained with 10 days of incubation and 3% sucrose ($2.67x10^6$ and $3.00x10^7$ cfu/ml) addition in TRIS and DMSO additive groups ($P<0.01$). Similarly, it was observed that 10 days of incubation period had a negative effect on the number of viable LAB after

one month of storage in LAB liquids frozen in deep freezer with TRIS and DMSO addition.

At the end of one month of storage in deep freezer, the lowest LAB numbers were determined in the group with 3% sucrose ($3.33x10^6$ cfu/ml) addition with TRIS additive in 10 days of the incubation period. In the DMSO additive group, it was obtained as a result of 5% and 10% sucrose additions in 5 and 10 days of incubation periods ($P<0.01$). The highest LAB values were obtained with 5% sucrose addition ($5.33x10^{10}$ cfu/ml) in 5 days of incubation and with TRIS additive, and with 10% sucrose ($1.00x10^{11}$ cfu/ml) addition in 5 days of incubation in the deep-frozen, lyophilized and dried samples. While the values obtained from the samples frozen in deep freezer with DMSO additive were generally low, the LAB values obtained from lyophilized and dried samples in 2 and 5 days of daily incubation and 10% sucrose addition were found to be the highest ($P<0.01$).

The moisture contents of the LAB liquids obtained by lyophilizing and drying the fermented LAB liquids obtained by adding different levels of sucrose (3%, 5% and 10%) and incubating for different periods (2, 5 and 10 days) are presented in Table 6. While the lowest moisture contents of the lyophilized LAB liquids were obtained with 3% sucrose addition and in 2 days of incubation (8.22%, 8.39% and 8.95%), the highest moisture contents were obtained with 10% sucrose addition in 10 days of incubation (15.09%, and 16.64%) in non-additive and DMSO groups ($P< 0.01$). In TRIS groups, the highest moisture content was observed in the 5D10%S group ($P<0.01$).

The LAB viability ratios of TRIS and DMSO additive deep frozen and lyophilized and dried LAB liquids obtained by incubating with different levels of sucrose for different periods of time are given in Table 7.

When the fermented LAB liquids lyophilized and frozen in the deep freezer with TRIS and DMSO additives were compared with the freshly prepared group, the highest LAB viability ratios were obtained from TRIS additive groups. In DMSO additive groups, the microbial viability ratio decreased up to 28% due to the prolonged incubation period. In lyophilized and dried liquids, it can be said that generally the LAB viability ratios obtained from TRIS and DMSO additive groups in 2 and 5 days of incubation were higher than the viability ratios decreased in 10 days of incubation.

The results of the 3-month trial are presented in Table 8 (cfu/ml). In the samples stored in the deep freezer for three months, while the highest LAB value was obtained with 10% sucrose ($8.33x10^9$ cfu/ml) addition in the TRIS additive group in 5 days of incubation, the highest LAB value was obtained with 10% sucrose ($3.67x10^6$ cfu/ml) addition in the DMSO additive group in 2 days of incubation ($P<0.01$).

While the highest LAB values in LAB liquids dried by lyophilization process and stored for three months were obtained with 10% sucrose ($4.60\ x10^{10}$ cfu/ml) addition in the TRIS additive group in 5 days of incubation, the highest LAB values were obtained with 5% sucrose ($7.33x10^{10}$ cfu/ml) addition in DMSO additive group in 2 days of incubation ($P<0.01$). In general, it was observed that 10 days of incubation in TRIS addition as well as 5 and 10 days of incubation in DMSO addition had a negative effect on the number of viable LAB after three months of storage in lyophilized and dried LAB liquids. While the lowest viable LAB numbers after three months of storage in lyophilized and dried LAB liquids were obtained with 10 days of incubation and the addition of 3%, 5% and 10% sucrose ($1.23x10^6$, $1.50x10^6$ and $1.27x10^6$ cfu/ml) in TRIS additive groups, it was obtained with 10 days of incubation and 3% sucrose level ($7.00x10^6$ cfu/ml) in DMSO additive groups. Similarly, it was observed that three months of storage time had a negative effect on LAB number in all incubation times (2, 5 and 10 days) in deep frozen LAB liquids with DMSO addition.

The lowest LAB number in deep frozen LAB liquids after three months of storage was obtained in 5 days of incubation and with 3% sucrose ($6.00x10^6$ cfu/ml) in TRIS additive group and in 10 days of incubation with 3%, 5% and 10% sucrose in DMSO additive group ($P<0.01$). LAB values obtained in 5 days of incubation and with 10% sucrose addition ($8.33x10^9$ and $4.60x10^{10}$ cfu/ml) were the highest ($P<0.01$) in the groups prepared with TRIS additive and frozen in deep freezer and lyophilized and stored for three months. While the LAB values obtained from the samples prepared with DMSO additive and frozen in the deep freezer were generally low, the value obtained in 2 days of incubation and with 5% sucrose addition ($7.33x10^{10}$ cfu/ml) was the highest in the lyophilized dried samples ($P<0.01$). When the LAB values obtained at the end of the three-month storage period (Table 8) were examined, it was found that the LAB numbers obtained in 2- and 5-day incubation periods in the groups dried by lyophilization process with TRIS additive were close to the values obtained from freshly prepared LAB liquid. It was also observed that LAB loss was less due to TRIS addition. On the contrary, the values obtained from the TRIS additive groups, which were lyophilized, dried and stored for three months by adding different sucrose levels (3%, 5% and 10%) at the end of the 10 day of the incubation period, were found to be lower than the values obtained from freshly prepared LAB liquid, and LAB loss was observed to be higher in these groups. When the values presented in Table 8 at the end of the three-month of storage period were examined, it was observed that the number of LAB decreased in the groups stored in the deep freezer with the addition of DMSO due to the prolongation of the incubation time ($P<0.01$).

The LAB viability ratios of TRIS and DMSO additive deep-frozen and lyophilized and dried LAB liquids obtained by incubating with different levels of sucrose for different periods are given in Table 9. When the fermented LAB liquids frozen in deep freezer with TRIS and DMSO additives were compared with the freshly prepared group, while the highest LAB viability ratios were obtained from the TRIS additive groups, the viability ratio decreased from 59% to 17% in the DMSO additive groups due to the prolonged incubation period. In lyophilized and dried LAB liquids, while the highest viability ratios were obtained from TRIS and DMSO additive groups (74%-99%) in 2 and 5 days of incubation, the viability ratios decreased in 10 days of incubation.

Table 1. Interaction analyses (Log10) for one- and three-month storage of frozen and lyophilized LAB liquids with different levels of sucrose addition and incubated for different periods of time.

			1 Month Storage Time		3 Month Storage Time	
			Storage Type		Storage Type	
Incubation Time, Days	Sucrose Level %	Protector Type	Freezing	Lyophilized	Freezing	Lyophilized
2	3	TRIS	9.42	10.45	8.60	10.44
		DMSO	8.63	10.00	5.00	9.97
	5	TRIS	9.59	10.71	8.00	10.00
		DMSO	8.90	10.64	5.48	10.62
	10	TRIS	9.80	10.00	8.88	10.10
		DMSO	9.15	10.98	6.56	10.86
5	3	TRIS	7.48	10.48	6.78	10.48
		DMSO	8.20	10.55	3.00	9.90
	5	TRIS	10.73	10.71	9.52	10.60
		DMSO	3.00	10.10	3.00	8.95
	10	TRIS	10.00	10.95	9.92	10.66
		DMSO	3.00	11.00	3.00	8.59
10	3	TRIS	6.52	6.42	6.48	6.09
		DMSO	4.48	7.48	2.00	6.84
	5	TRIS	10.10	6.82	9.52	6.18
		DMSO	3.00	7.75	2.00	7.75
	10	TRIS	10.18	6.63	7.10	6.09
		DMSO	3.00	8.07	2.00	8.00

Incubation Time: 2, 5 and 10 days; Sucrose Level: 3%, 5% and 10%; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide.

Table 2. Interaction sources for one- and three-month storage of frozen and lyophilized LAB liquids with different levels of sucrose addition and incubation for different periods of time.

Interaction Source	F Value	Significance	p
Month	12750.53	0. 000	**
Sucrose	699.23	0. 000	**
Incubation time	40514.93	0. 000	**
Protector	49654.93	0. 000	**
Storage	79540.26	0. 000	**
Month*Sucrose	145.95	0. 000	**
Month*Incubation time	83.27	0. 000	**
Month*Protector	1619.90	0. 000	**
Month*Storage	4236.08	0. 000	**
Sucrose*Incubation time	358.85	0. 000	**
Sucrose*Protector	2440.17	0. 000	**
Sucrose*Storage	158.72	0. 000	**
Incubation time* Protector	5402.52	0. 000	**
Incubation time*Storage	5846.40	0. 000	**
Protector*Storage	62162.50	0. 000	**
Month*Sucrose*Incubation time	278.06	0. 000	**
Month*Sucrose*Protector	923.60	0. 000	**
Month*Sucrose*Storage	414.73	0. 000	**
Month*Incubation time*Protector	424.80	0. 000	**
Month*Incubation time*Storage	701.76	0. 000	**
Month*Protector*Storage	592.70	0. 000	**
Sucrose*Incubation time*Protector	1947.08	0. 000	**
Sucrose*Incubation time*Storage	172.32	0. 000	**
Sucrose*Protector*Storage	3272.60	0. 000	**
Incubation time*Protector*Storage	6270.91	0. 000	**
Month*Sucrose*Incubation time*Protector	197.33	0. 000	**
Month*Sucrose*Incubation time*Storage	595.45	0. 000	**
Month*Sucrose*Protector*Storage	949.65	0. 000	**
Month*Incubation time*Protector*Storage	420.67	0. 000	**
Sucrose*Incubation time*Protector*Storage	666.44	0. 000	**
Month*Sucrose*Incubation time*Protector*Storage	256.95	0. 000	**

Month: Storage period 1 and 3 months; Sucrose: Sucrose levels %3, %5 and %10; Incubation time: 2, 5 and 10 days; Protector: Trisodium citrate (TRIS) and Dimethyl sulfoxide (DMSO), Storage: Storage type Freezing or Lyophilization, **: $p<0.001$

Table 3. LAB numbers (cfu/ml) of LAB liquids prepared in different ways, frozen and lyophilized before one and three months of storage.

		Frozen			Lyophilized			
Groups	**Fresh**	**Non-additive**	**TRIS**	**DMSO**	**Non-additive**	**TRIS**	**DMSO**	**SEM**
2D3%S	$3.01x10^{10f\ A}$	$1.00x10^{9c\ D}$	$1.67x10^{10bc\ AB}$	$1.10x10^{9d\ E}$	$2.33x10^{9f\ C}$	$1.33x10^{10c\ B}$	$1.97x10^{10cd\ AB}$	0.164
2D5%S	$5.43x10^{10de\ A}$	$2.33x10^{9b\ D}$	$3.00x10^{10b\ B}$	$2.33x10^{9b\ D}$	$9.33x10^{9cd\ C}$	$4.00x10^{10b\ AB}$	$7.33x10^{9e\ C}$	0.123
2D10%S	$1.20x10^{11bc\ A}$	$2.00x10^{10a\ B}$	$9.33x10^{10a\ A}$	$4.67x10^{9a\ D}$	$6.33x10^{9de\ CD}$	$1.00x10^{10c\ BC}$	$4.00x10^{9e\ D}$	0.131
5D3%S	$7.67x10^{10cd\ A}$	$1.00x10^{8e\ E}$	$4.67x10^{9d\ C}$	$7.00x10^{8c\ D}$	$5.33x10^{9e\ C}$	$5.00x10^{10b\ B}$	$4.00x10^{10c\ B}$	0.221
5D5%S	$6.00x10^{10de\ A}$	$1.00x10^{9c\ D}$	$2.33x10^{9de\ C}$	$4.33x10^{8d\ E}$	$3.00x10^{10b\ B}$	$8.67x10^{10a\ A}$	$2.00x10^{10d\ B}$	0.194
5D10%S	$4.20x10^{11a\ A}$	$9.23x10^{9a\ C}$	$1.17x10^{9e\ D}$	$2.33x10^{7g\ E}$	$6.33x10^{10a\ B}$	$1.00x10^{10c\ C}$	$2.67x10^{11a\ A}$	0.313
10D3%S	$2.83x10^{11a\ A}$	$2.00x10^{9bc\ E}$	$2.33x10^{10bc\ CD}$	$3.00x10^{8d\ F}$	$1.33x10^{10c\ D}$	$3.33x10^{10b\ C}$	$8.00x10^{10b\ B}$	0.214
10D5%S	$1.47x10^{11b\ A}$	$1.33x10^{9bc\ D}$	$3.00x10^{9d\ C}$	$2.00x10^{8e\ F}$	$6.67x10^{8g\ E}$	$1.00x10^{10c\ B}$	$1.67x10^{10d\ B}$	0.203
10D10%S	$4.33x10^{10ef\ A}$	$2.17x10^{8d\ E}$	$1.37x10^{10c\ B}$	$1.00x10^{8f\ F}$	$4.67x10^{8g\ D}$	$1.00x10^{9d\ C}$	$3.00x10^{10cd\ A}$	0.234
SEM	0.071	0.134	0.123	0.132	0.134	0.114	0.103	

a-g: Values with different letters in the same column were found to be statistically different ($p<0.01$); A-E: Values with different letters in the same line were found to be statistically different. ($p<0.01$); 2D: 2 days; 5D: 5 days; 10D: 10 days; %3S: %3 sucrose; %5S: %5 sucrose; %10S: %10 sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide.

Table 4. LAB viability ratios of LAB liquids prepared in different ways, frozen and lyophilized group before storage for one and three months %.

	Frozen				Lyophilized		
Groups	**Fresh**	**Non-additive**	**TRIS**	**DMSO**	**Non-additive**	**TRIS**	**DMSO**
2D3%S	100	86	97	81	89	96	98
2D5%S	100	87	97	87	93	99	92
2D10%S	100	93	99	87	88	90	87
5D3%S	100	74	89	81	89	98	97
5D5%S	100	84	87	80	97	99	95
5D10%S	100	86	78	63	93	86	98
10D3%S	100	81	90	74	88	92	95
10D5%S	100	82	85	74	79	90	91
10D10%S	100	78	95	75	82	85	98

2D: 2 days; 5D: 5 days; 10D: 10 days; %3S: %3 sucrose; %5S: %5 sucrose; %10S: %10 sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 5. LAB values (cfu/ml) at the end of one month storage period of LAB liquids prepared in different ways, frozen and lyophilized.

	Frozen		Lyophilized		
Groups	**TRIS**	**DMSO**	**TRIS**	**DMSO**	**SEM**
2D3%S	$2.67x10^{9d\ C}$	$4.33x10^{8c\ D}$	$2.83x10^{10c\ A}$	$1.00x10^{10c\ B}$	0.206
2D5%S	$4.00x10^{9d\ B}$	$8.00x10^{8b\ C}$	$5.10x10^{10b\ A}$	$4.43x10^{10b\ A}$	0.228
2D10%S	$6.33x10^{9c\ B}$	$1.47x10^{9a\ C}$	$1.00x10^{10d\ B}$	$9.47x10^{10a\ A}$	0.198
5D3%S	$3.03x10^{7e\ C}$	$1.67x10^{8d\ B}$	$3.00x10^{10c\ A}$	$3.53x10^{10b\ A}$	0.411
5D5%S	$5.33x10^{10a\ A}$	$1.00x10^{3f\ C}$	$5.13x10^{10b\ A}$	$1.33x10^{10c\ B}$	0.984
5D10%S	$1.00x10^{10b\ B}$	$1.00x10^{3f\ C}$	$9.00x10^{10a\ A}$	$1.00x10^{11a\ A}$	1.001
10D3%S	$3.33x10^{6f\ B}$	$3.00x10^{4e\ C}$	$2.67x10^{6g\ B}$	$3.00x10^{7f\ A}$	0.329
10D5%S	$1.33x10^{10b\ A}$	$1.00x10^{3f\ D}$	$6.67x10^{6e\ C}$	$5.67x10^{7e\ B}$	0.772
10D10%S	$1.50x10^{10b\ A}$	$1.00x10^{3f\ D}$	$4.33x10^{6f\ C}$	$1.20x10^{8d\ B}$	0.782
SEM	0.256	0.538	0.366	0.266	

a-g : Values with different letters in the same column were found to be statistically different. ($p<0.01$). A-D: Values with different letters in the same line were found to be statistically different. ($p<0.01$). 2D: 2 days; 5D: 5 days; 10D: 10 days; %3S: %3 sucrose; %5S: %5 sucrose; %10S: %10 sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 6. Moisture Content of LAB Liquids Obtained by Being Dried with Lyophilization Process, %

Groups	**Non-additive**	**TRIS (Ts)**	**DMSO (Ds)**	**SEM**
2D3%S	8.22^{e}	8.39^{e}	8.95^{f}	0.225
2D5%S	$10.97^{bcd\ A}$	$9.20^{e\ B}$	$11.27^{d\ A}$	0.350
2D10%S	$10.02^{d\ B}$	$11.46^{b\ A}$	$9.88^{e\ B}$	0.272
5D3%S	$10.56^{cd\ AB}$	$9.37^{e\ B}$	$11.16^{d\ A}$	0.297
5D5%S	11.44^{bcd}	12.05^{ab}	12.42^{bc}	0.304
5D10%S	12.42^{b}	12.65^{a}	13.06^{b}	0.131
10D3%S	$10.63^{cd\ B}$	$12.23^{ab\ A}$	$12.36^{bc\ A}$	0.289
10D5%S	12.20^{bc}	11.92^{ab}	12.11^{c}	0.122
10D10%S	$15.09^{a\ B}$	$11.59^{ab\ C}$	$16.64^{a\ A}$	0.752
SEM	0.368	0.296	0.407	

$^{a-f}$:Values with different letters in the same column were found to be statistically different. ($p<0.01$). $^{A-C:}$ Values with different letters in the same line were found to be statistically different. ($p<0.01$). 2D: 2 days; 5D: 5 days; 10D: 10 days; 3%S: 3% sucrose; 5%S: 5% sucrose; 10%S: 10% sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 7. LAB viability ratios as a result of freezing and lyophilization of LAB liquids incubated for different periods of time with different levels of sucrose addition and storage for one month, %

		Frozen		Lyophilized	
Groups	**Fresh**	**TRIS**	**DMSO**	**TRIS**	**DMSO**
2D3%S	100	90	82	99	95
2D5%S	100	89	83	99	99
2D10%S	100	88	83	90	99
5D3%S	100	67	75	96	97
5D5%S	100	99	28	99	94
5D10%S	100	86	26	94	95
10D3%S	100	57	39	56	68
10D5%S	100	91	27	61	70
10D10%S	100	96	28	62	76

2D: 2 days; 5D: 5 days; 10D: 10 days; 3%S: 3% sucrose; 5%S: 5% sucrose; 10%S: 10% sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 8. LAB values (cfu/ml) at the end of three months of storage period of LAB liquids prepared in different ways, frozen and lyophilized.

	Frozen		**Lyophilized**		
GRUP	**TRIS**	**DMSO**	**TRIS**	**DMSO**	**SEM**
2D3%S	$4.00x10^{8d\ C}$	$1.00x10^{5c\ D}$	$2.80x10^{10c\ A}$	$9.33x10^{9c\ B}$	0.621
2D5%S	$1.00x10^{8e\ C}$	$3.00x10^{5b\ D}$	$1.00x10^{10d\ B}$	$7.33x10^{10a\ A}$	0.550
2D10%S	$7.67x10^{8c\ C}$	$3.67x10^{6a\ D}$	$1.27x10^{10d\ B}$	$4.13x10^{10b\ A}$	0.469
5D3%S	$6.00x10^{6g\ C}$	$1.00x10^{3d\ D}$	$3.00x10^{10bc\ A}$	$8.00x10^{9c\ B}$	0.756
5D5%S	$3.33x10^{9b\ B}$	$1.00x10^{3d\ D}$	$4.00x10^{10ab\ A}$	$9.00x10^{8d\ C}$	0.770
5D10%S	$8.33x10^{9a\ B}$	$1.00x10^{3d\ D}$	$4.60x10^{10a\ A}$	$4.00x10^{8e\ C}$	0.850
10D3%S	$3.00x10^{6h\ B}$	$1.00x10^{2e\ D}$	$1.23x10^{6e\ C}$	$7.00x10^{6h\ A}$	0.447
10D5%S	$3.47x10^{9b\ A}$	$1.00x10^{2e\ D}$	$1.50x10^{6e\ C}$	$5.67x10^{7g\ B}$	0.632
10D10%S	$1.00x10^{7f\ B}$	$1.00x10^{2e\ D}$	$1.27x10^{6e\ C}$	$1.00x10^{8f\ A}$	0.556
SEM	0.238	0.314	0.396	0.255	

a-h :Values with different letters in the same column were found to be statistically different. ($p<0.01$). A-D: Values with different letters in the same line were found to be statistically different. ($p<0.01$). 2D: 2 days; 5D: 5 days; 10D: 10 days; 3%S: 3% sucrose; 5%S: 5% sucrose; 10%S: 10% sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide

Table 9. LAB viability ratios as a result of freezing and lyophilization of LAB liquids incubated for different periods of time with different levels of sucrose addition and storage for three months, %

		Frozen		Lyophilized	
Groups	**Fresh**	**TRIS**	**DMSO**	**TRIS**	**DMSO**
2D3%S	100	82	48	99	95
2D5%S	100	75	51	93	99
2D10%S	100	80	59	91	96
5D3%S	100	62	28	96	91
5D5%S	100	88	28	98	83
5D10%S	100	85	26	92	74
10D3%S	100	39	17	53	60
10D5%S	100	85	18	55	70
10D10%S	100	67	19	57	75

2D: 2 days; 5D: 5 days; 10D: 10 days; 3%S: 3% sucrose; 5%S: 5% sucrose; 10%S: 10% sucrose; Fresh: Fermented LAB liquid obtained after different sucrose levels and incubation times; Non-additive: No cryoprotectant added; TRIS: Trisodium citrate; DMSO: Dimethyl sulfoxide.

DISCUSSION

The fresh alfalfa plant employed in this study's manufacture of fermented LAB liquids had a LAB number of $1x10^5$ cfu/ml. It was found to be compatible with the reports of Ohshima et al. (1997c) on LAB numbers of alfalfa plants ($3x10^5$ cfu/ml).

Before harvest, the amount of LAB infecting the plant can range from $1x10^1$ cfu/ml to $1.0x10^7$ cfu/ml. There are also discrepancies in the number and types of LAB contaminating the plants that will be used to make silage. These variations can be attributed to a variety of factors, including ambient temperature, UV light, environmental humidity, and other aspects of the plant itself. In this study, in the freshly prepared fermented LAB liquids, the total number of LAB decreased at the end of the 10 days of incubation due to the increase in sucrose level; on the contrary, a general increasing trend was observed in the total number of LAB in 2 and 5 days of incubation (Table 3). Depending on the sucrose level and incubation time, it is thought that the logarithmic increase period of LAB continued in 2, 5 and 10 days of incubation in 3% and 5% sucrose additive groups and in 5 days of incubation in 10% sucrose additive groups. It was observed that there was a decrease in the number of LAB in the 10% sucrose additive group in the 10 days of incubation ($P<0.01$).

The reason for the decrease in the number of LAB during the 10-day incubation period and in the group with 10% sucrose addition may be related to the fact that the environment no longer provided the nutrients that the growing population of LAB required, as well as the rise in the number of toxic substances (lactic acid, acetaldehyde, peroxide, etc.) and the decline in the pH level of the environment. This results in a decrease in the number of bacteria that can survive in the environment and an increase in the number of microorganisms that cease to function (Brock et al. 2003).

The type of freezing process and the rapid cooling process applied in the cryopreservation of microorganisms are important in ensuring maximum cell viability (Tedeschi and De Paoli 2011; Fonseca et al. 2016). It has been reported that damage to the bacterial cell membrane caused by the freezing process may be due to the high solution concentration of the environment in which the cell is located and due to intracellular ice crystals (Dumont et al. 2004; Chang and Zhao 2021). The reason for the use of cryoprotectants in the cryopreservation of microorganisms is that they prevent the formation of large ice crystals inside and outside the cell during the freezing process, allowing frozen microorganisms to reach high microbial viability (Dumont et al. 2004). In 2- and 5-day incubation time, it was observed that the viability ratio increased depending on the increase in sucrose level and the highest viability ratios were obtained from the 10% sucrose additive group. According to Suharman et al. (2021), there were more lactic acid bacteria when the amount of sucrose increased. In the 10-day incubation time, the highest viability ratio was obtained from the 5% sucrose additive group (82%) (Table 4). When the LAB viability ratios of the deep-frozen LAB liquids obtained by adding TRIS and DMSO cryoprotectants to the LAB liquids obtained by adding sucrose at different levels and incubating for different periods (Table 4) before one- and three-month storage were compared with fresh LAB liquid, the highest viability ratios were determined in 2D3%S, 2D5%S, and 2D10%S groups. In the same groups (2D3%S,

2D5%S and 2D10%S), when compared to fresh LAB liquid, it was determined that there were decreases in microbial viability rates due to longer storage periods with the addition of TRIS (Tables 7 and 9). Likewise, it was determined that there were decreases in microbial viability rates as storage time increased in the same groups prepared with the addition of DMSO compared to fresh LAB liquid (Tables 7 and 9).

In this study, the viability ratio of the 5D5%S group decreased from 99% to 88% and the viability ratio of the 5D10%S group decreased from 86% to 85% among the LAB liquids prepared after one- and three-month storage period by freezing in deep freezer by adding TRIS as cryoprotectant. Oluwatosin et al. (2022) reported that sucrose showed the best performance among other cryoprotectant substances for the protection of microorganisms stored frozen. In this study, the high viability ratios determined due to the increase in sucrose level were found to be consistent with the report. In the study conducted by Bâati et al. (2000) regarding storage of *Lb. acidophilus* by being frozen, they reported that *Lb. acidophilus* maintained microbial viability between 24% and 89% depending on the temperature of the freezing process applied to *Lb. acidophilus* and the suspension environment. They stated that a pre-freezing process before the freezing process to be applied to microorganisms is important in increasing microbial viability.

In this study, the microbial viability ratios obtained by freezing in deep freezer for one and three months were determined between 74% and 93% in the groups without additives, 85% and 99% in the groups with TRIS additives, and 63% and 87% in the groups with DMSO additives. In the fermented LAB groups frozen in this study, the microbial viability ratios obtained from the TRIS additive groups were found to be higher than the groups without additives and with DMSO additive due to the prolongation of the storage period (1 and 3 months). In the deep-frozen groups, microbial viability decreased in the TRIS and DMSO additive groups due to the prolongation of the storage period in general. Consistent with this study, Strasser et al. (2009) found that the viability of lactic acid bacteria was negatively affected by the extension of storage time. The moisture contents of LAB liquids obtained by lyophilization being dried are presented in Table 6. In general, the moisture content of lyophilized cultures is required to be 2% or less. In addition, a moisture content of 2% or less is considered as an indicator of the success of the lyophilization process (Rambhatla and Pikal 2003). In this study, the moisture contents of lyophilized and dried LAB liquids were found to be between 8.22% and 16.64%. When the moisture contents of LAB liquids obtained as a result of the lyophilization process in this study were compared with previous studies, it was observed that the moisture values obtained were high (Sparkes and Fenje 1972; Avcıoğlu 2013). In this study, the reason for higher moisture values in LAB liquids obtained by lyophilization process can be explained by keeping the secondary drying time short, which is the last step of the lyophilization process and reduces the relative amount of water in the product (Avcıoğlu 2013).

There are many factors affecting microbial viability and activity in the lyophilized drying process of microorganisms and cell cultures. These factors include the genus, species, strain and age of the bacteria, growth medium, cell density before the process, ambient conditions, type of cryoprotectant, ambient conditions in which the culture is preserved after the process, the amount of moisture remaining in the culture, storage conditions (temperature, time, light), composition and temperature of the solution used in dilution. Apart from these factors, the system used during the lyophilization process, excessive sublimation, initial freezing temperature, bacteriophage risk, etc. are also known to be effective on the viability and activity of lyophilized cultures (Halkman and Doğan 2000). In general, while the highest microbial viability ratio was determined in the TRIS additive groups in the deep freezer, the lowest microbial viability ratio was determined in the DMSO additive groups (63%-87%). When evaluated in general, while the highest microbial viability ratio was maintained in the TRIS additive group, the highest microbial loss ratio was determined in the groups without additives in drying by the lyophilization process. In the groups dried by the lyophilization process in this study, the viability ratios obtained from TRIS and DMSO additive groups decreased due to the prolongation of the storage period (one or three months). The highest loss in viability ratio was observed in 10 days of incubation periods (Tables 7 and 9).

In a study conducted by (Ferry 1995), it was stated that 5% to 10% sugar contribution is the most ideal for microbial culture preservation by lyophilization process. Greaves (1964) stated that the sucrose ratio should not exceed 10%, and when the sucrose ratio used is between 10-20%, the viability ratio of the lyophilized culture decreases over time. In this study, the maintenance of high microbial viability of anaerobic and aerotolerant lactic acid bacteria in lyophilized LAB liquids obtained by lyophilization and drying was found to be consistent with the report that it is easier to preserve and maintain anaerobic and especially aerotolerant microorganisms in the field of food microbiology (Halkman and Doğan 2000). In the preservation of lactic acid bacteria, especially in the drying method by applying lyophilization process, high amounts of microbial culture density are maintained. In accordance with the report of Giulio et al. (2005), it was observed that lyophilization method preserved microbial viability more than deep freezing in this study. Pınarkara (2008) researched the effects of sucrose, TRIS and DMSO cryoprotectant additives on microbial viability

ratios of different types of LAB in drying by lyophilization process and reported that the use of TRIS as cryoprotectant in the lyophilization process was effective in maintaining the viability ratios of LAB. Giulio et al. (2005) found that the viability ratios of *Lactobacillus bulgaricus, Streptococcus salivarius ssp. and Thermophilus* strains was 95% after lyophilization with the addition of 32% sucrose, while Morichi (1965) reported that in contrast to this result, the viability ratio of *Lactobacillus bulgaricus* was maintained at 38% with the addition of sucrose. In this study, compared to freshly prepared fermented LAB liquids, the viability ratios of lyophilized LAB liquids with the addition of 3% and 5% sucrose and TRIS as cryoprotectant were found to be compatible with the results obtained by Giulio et al. (2005) and Pınarkara (2008).

CONCLUSION and RECOMMENDATIONS

According to the findings obtained within the scope of the study, it is thought that 2 or 5 days of incubation period with the addition of 10% sucrose will be sufficient to obtain high levels of LAB in fermented LAB liquids to be used fresh without deep freezing and lyophilization and drying. It was determined that lyophilization of fermented LAB liquids in one month storage period preserved their viability ratios better than deep freezing, and the highest LAB values were determined in the groups lyophilized by adding 10% sucrose and DMSO as the cryoprotectant in 2 and 5 days of incubation period. At the end of the three months of storage period, it was determined that the lyophilization process preserved the viability ratios of LAB better than deep freezing and the highest LAB values were determined in the groups lyophilized with 5% and 10% sucrose addition and TRIS as cryoprotectant for 5 days of incubation period, and in the group lyophilized with DMSO addition and 5% sucrose for 2 days of incubation period.

When the results obtained from this study are evaluated in general, it can be said that the viability ratios of LAB liquids decreased due to the prolongation of incubation and storage time, TRIS and DMSO additives had a positive effect on the viability ratios, and lyophilization process was advantageous compared to freezing in deep freezer. Therefore, it may be recommended to dry the fermented LAB liquids by lyophilization with the addition of TRIS or DMSO cryoprotectants for long-term storage. Considering the high viability ratios obtained from the lyophilization and drying of LAB liquids with TRIS and DMSO cryoprotectant additives, it is seen that the LAB liquids obtained in this study have a high potential to be used and commercialized as silage additives.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: SSA and ND contributed to the project idea, design and execution of the study. MK, BS and BD contributed to the acquisition of data. SSA and ND analysed the data. SSA and ND drafted and wrote the manuscript. SSA and ND reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

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