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Amount of lactic acid bacteria in fermented natural lactic acid bacteria liquids prepared with varying sucrose inclusion at different incubation periods

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Summary

Characterization of lactic acid bacteria that naturally occurs during silage formation is important for determining the quality of silage feeds which are required in terms of meeting the need for high quality roughage in cattle breeding. In this study, the aim was to determine the total lactic acid bacteria (LAB) count and pH values of fermented natural LAB liquids as a result of different levels of sucrose addition as microbial silage additive (1%, 3%, 5% and 10%) and different incubation periods (5, 7, 9, 11, 13 and 15 days). Considering the incubation periods of LAB liquids, while the highest LAB count was obtained from the 10% sucrose added groups (3.7×10^{11}, 2.6×10^{11} and 2.53×10^{12} colony forming unit (cfu/ml) at the 7, 9 and 13 days of incubation periods, the highest LAB count was obtained from the 5% sucrose supplemented group (9.13×10^{11} cfu/ml) at the end of the 15-day incubation period. In general, for each incubation period, the total LAB counts in the obtained fluids increased due to the increase in the sucrose level ($P < 0.05$). Depending on the increase in the incubation period, there was an increase in the total LAB counts in the 10% sucrose supplemented group, while a decrease in the total LAB counts occurred after a 15-day incubation period. In general, pH values decreased depending on the increasing with sucrose levels in each incubation period. Considering the pH values of the LAB liquids, there were no differences of pH values depending on the increase the incubation period from the 1% and 3% sucrose added groups ($P < 0.05$). The pH values obtained from the 10% sucrose added group decreased with the increasing incubation periods. The highest pH value was obtained from the 1% sucrose group (4.49) during the 15-day incubation period, whereas the lowest pH value (3.78) was obtained from the 10% sucrose group in the same incubation period. Considering the LAB counts (10^{11-12} cfu/ml) in the study, it has been evaluated that the obtained LAB liquids can be used as silage additives and it has a high potential of commercialization.

Keywords: silage inoculant, silage fermentation, sucrose

The roughage deficit continues to increase in Turkey as well as in the world due to various reasons, such as drought caused by global warming, misuse of meadows, and decrease in the cultivation areas of forage crops. Therefore, silages are an important option in meeting the need for cheap, abundant and high-quality roughage in a short time, particularly in cattle breeding enterprises (12, 14). Apart from technological factors, silage quality may vary depending the dry matter (DM) content of plants, water-soluble carbohydrate (WSC) content, protein content, buffering capacity, microbiological composition, climate, plant variety and ensiling technique. The role of lactic acid bacteria (LAB) in the ensiling process is not only to reduce the pH level of the silage by breaking down WSCs, but also to prevent the proliferation and activity of microorganisms that compete with the nutrients in the plant but are not desired in the silo environment. They achieve this by producing antifungal and antimicrobial products such as bactericine, hydrogen peroxide, lactateperoxidase or 1.2-propanediol (30).

In the use of LAB inoculants as silage additives, it has been reported that LAB inoculants which are homogeneously added to the fresh silage material at a rate of 10^6 cfu/g, are sufficient for the dominant bacterial species in the silage to be LAB (13). LAB inoculants, which are widely used in silage production in developed countries, are used to complete the fermentation

in the silo in a short time and to obtain high quality silage with richer nutritional value. In addition, LAB inoculants used as silage additives provide the opportunity to obtain high quality silage as well as provide the silage stability for a long time after opening without losing its quality (21).

In recent years, the use of fermented natural LAB liquids, which can be easily prepared in a practical and economical way, has attracted attention as an alternative to commercial LAB inoculants that are expensive and sometimes ineffective in terms of improving silage fermentation (5, 24, 33, 36). In the preparation of fermented natural LAB liquids, generally alfalfa, meadow grass, corn, barley and wheat, fresh herbs are used as plant material, while easily soluble carbohydrates sources such as molasses, glucose and sucrose at different levels as a food source have been used for optimum proliferation of LAB in the previous studies (1, 11, 24, 26). Fermented natural LAB liquid for a silage additive has important advantages. Because it is of biological and natural in origin, safe, prepared and used easily and economically, has no toxic effects, does not cause corrosion in the machines used in silage making, does not create environmental pollution (18). This study was carried out to determine the LAB numbers of fermented natural LAB liquids obtained after the addition of different levels of sucrose and incubation at different time periods.

Material and methods

Fermented natural LAB liquid was prepared using the method described by Masuko et al. (27). However, distilled water was not added to have a more concentrated product in contrast to the method reported by these authors. For this purpose, the fresh alfalfa plant was chopped for 2 minutes with the help of a blender, and the obtained plant liquid mixture was filtered using two layers of cheese cloth. The obtained filtrate was transferred to sterile glass bottles and the bottles were capped after adding 1%, 3%, 5% and 10% (w/v) sucrose. The obtained filtrate was transferred to sterile glass bottles and the caps of the bottles were hermetically capped after the addition of 1%, 3%, 5%, and 10% (w/v) sucrose. The bottles were incubated at 30°C for 5, 7, 9, 11, 13 and 15 days. At the end of different incubation periods, the total LAB count and pH (WTW 7310) values of each sucrose group were determined (16).

Analyses of total lactic acid bacteria. The triplicate LAB counts of the fermented LAB liquids were performed for each group with the Tempo automatic bacteria counter (bioMerieux, MarcyI'Etoile France) according to test method (REF-80 071) at the end of different incubation periods (5, 7, 11, 13 and 15 days). Homogenization was achieved with the help of stomacher under aseptic conditions by adding enrichment solution at a ratio of 1 to 9 MRD (Maximum Recovery Diluent) to the fermented LAB liquids. Twenty minutes before the analysis, 3.9 ml of sterile distilled water was added to each of the ready-to use Tempo LAB medium bottles, after which they were vortexed and then 0.1 ml of fermented LAB liquid homogenized in the MRD enrichment solution was added and the Tempo LAB medium bottles were vortexed again. The medium bottles and incubation cards of the medium were placed in the Tempo Filler device for filling, and the cards taken from the Tempo Filler device after filling were left to incubate at 30°C for 40-48 hours (16). After the incubation period, the cards removed from the incubator were inserted to the Tempo Reader device to determine the microbial density, and the results were counted by the device as coloni forming unit (cfu)/ml.

Statistical method. The data obtained after the addition of different sucrose levels (1%, 3%, 5% and 10%) and different incubation periods (5, 7, 9, 11, 13 and 15 days) were evaluated by one-way analysis of variance. Duncan's multiple comparison test was used to compare group means, and for this purpose, SPSS (1991) package program was used (32).

Results and discussion

In this study, the LAB load of alfalfa plant used in the production of fermented natural LAB was determined as 4.6×10^4 cfu/ml. Total LAB counts of fermented LAB liquids prepared by adding different levels of sucrose (1%, 3%, 5% and 10%) and incubating at 30°C for different time periods (5, 7, 9, 11, 13 and 15 days) are presented in Tables 1 and 2.

Considering the incubation periods in fermented natural LAB liquids, the highest amount of LAB were determined at 3% sucrose level (6.23×10^{10} cfu/ml), at 3% and 10% sucrose levels (1.33×10^{11}cfu/ml and 3.7×10^{11} cfu/ml), at 10% sucrose level (2.6×10^{11} cfu/ml), at 3%, 5% and 10% sucrose levels (7.83×10^{10} cfu/ml, 1.87×10^{11} cfu/ml and 1.8×10^{11} cfu/ml), at 10% sucrose level (2.53×10^{12} cfu/ml) and at 1%, 3% and 5% sucrose levels (5.00×10^{11} cfu/ml, 5.33×10^{11}

Tab. 1. Total LAB Counts (log_{10} cfu/ml) of fermented lab liquids prepared by sucrose addition at different levels (1%, 3%, 5% and 10%) and incubation for different time periods (5, 7, 9, 11, 13 and 15 days)

Incubation period (day)	1% Sucrose	3% Sucrose	5% Sucrose	10% Sucrose	SEM	P
5	10.19 ^{cB}	10.79^{cdA}	10.18 ^{cB}	10.43 ^{cB}	0.083	< 0.05
7	10.52 ^{bcB}	11.10 ^{bcA}	10.32 ^{cC}	11.54 ^{bA}	0.157	< 0.05
9	10.72 ^{bB}	10.52 ^{dB}	10.60 ^{cB}	11.35 ^{bA}	0.124	< 0.05
11	10.39 ^{bcB}	10.83 ^{cdA}	11.24 ^{bA}	11.22 ^{bA}	0.119	< 0.05
13	10.20 ^{cC}	11.93 a ^{AB}	11.44 ^{abB}	12.29 a ^{A}	0.256	< 0.05
15	11.59 a ^{A}	11.58 ^{abA}	11.95 a ^{A}	10.71 ^{cB}	0.161	< 0.05
SEM	0.13	0.13	0.17	0.15		
P	< 0.05	< 0.05	< 0.05	< 0.05		

Explanations: a-d – values with different superscripts in the same column were found to be different; A-C – values with different superscripts in the same rows differ significantly; * – P < 0.05; cfu/ml – number of colonies per milliliter; SEM – standard error of means

Tab. 2. Total LAB Counts (cfu/ml) of fermented lab liquids prepared by sucrose addition at different levels (1%, 3%, 5% and 10%) and incubation for different time periods (5, 7, 9, 11, 13 and 15 days)

Incubation period (day)	1% Sucrose	3%Sucrose	5%Sucrose	10% Sucrose	SEM	P
5	$1.70 \times 10^{10\ c\ B}$	$6.23 \times 10^{10\ cd\ A}$	$1.51 \times 10^{10\ c\ B}$	$2.71 \times 10^{10\ c\ B}$	0.083	< 0.05
7	$3.80 \times 10^{10\ bc\ B}$	$1.33 \times 10^{11\ bc\ A}$	$2.37 \times 10^{10\ c\ B}$	$3.70 \times 10^{11\ b\ A}$	0.157	< 0.05
9	$5.73 \times 10^{10\ b\ B}$	$3.83 \times 10^{10\ d\ B}$	$5.00 \times 10^{10\ c\ B}$	$2.60 \times 10^{11\ b\ A}$	0.124	< 0.05
11	$2.47 \times 10^{10\ bc\ B}$	$7.83 \times 10^{10\ cd\ A}$	$1.87 \times 10^{11\ b\ A}$	$1.80 \times 10^{11\ b\ A}$	0.119	< 0.05
13	$1.67 \times 10^{10\ c\ C}$	$8.57 \times 10^{11\ a\ AB}$	$4.40 \times 10^{11\ ab\ B}$	$2.53 \times 10^{12\ a\ A}$	0.256	< 0.05
15	$5.00 \times 10^{11\ a\ A}$	$5.33 \times 10^{11\ ab\ A}$	$9.13 \times 10^{11\ a\ A}$	$5.67 \times 10^{10\ c\ B}$	0.161	< 0.05
SEM	0.13	0.13	0.17	0.15		
P	< 0.05	< 0.05	< 0.05	< 0.05		

Explanations: a-d – values with different superscripts in the same column were found to be different; A-C – values with different superscripts in the same rows differ significantly; * – P < 0.05; cfu/ml – number of colonies per milliliter; SEM – standard error of means

cfu/ml, 9.13×10^{11} cfu/ml) for 5, 7, 9, 11, 13 and 15 days of incubation (P < 0.05), respectively.

Consideringthe sucrose levels in natural fermented LAB liquids, the highest lactic acid bacteria were found in the liquids obtained in 15 days of incubation at a level of 1% sucrose (5×10^{11}cfu/ml), in 13 days of incubation at a level of 3% sucrose supplementation (8.57×10^{11} cfu/ml), in 15 days incubation at a level of 5% sucrose supplementation (9.13×10^{11} cfu/ml), in 13 days of at a level of 10% sucrose supplementation (2.53×10^{12} cfu/ml) (P < 0.05). In general, for each incubation period, the total LAB counts in the obtained liquids increased with increase in sucrose levels (P < 0.05). In the 10% sucrose supplemented group, there was an increase in the total number of LABs with the increasing incubation periods (5, 8, 9, 11, 13), while a decrease was observed in the total number of LABs at the end of the 15-day incubation period (P < 0.05).

The pH values of fermented natural LAB liquids obtained by adding different levels of sucrose (1%, 3%, 5% and 10%) and incubation at different times (5, 7, 9, 11, 13 and 15 days) are presented in Table 3. When the pH values were evaluated, no statistical difference was found in the 1% and 3% sucrose added groups. The pH values decreased (P < 0.05) in the 10% sucrose supplemented group with the increasing incubation period. The lowest pH value was determined as 3.78 at the end of the 15 days of incubation period with 10% sucrose addition. In general, pH values decreased when sucrose levels were increased in each incubation period (P < 0.05).

In this study, the LAB count of the alfalfa plant used in the preparation of fermented natural LAB liquids was determined as 4.6×10^4 cfu/ml. This value was found to be compatible with the findings of Bureenok et al. (9) and Wang et al. (36) who reported LAB counts in alfalfa plant as 1×10^4 cfu/ml and 8.32×10^4 cfu/ml, respectively. In some previous studies, it has been reported that the number of LAB contaminating the plant before harvesting can vary from 1×10^1 cfu/ml to 1.0×10^7cfu/ml, and there are differences in the number and types of LAB on the plants to be silage. Ultraviolet rays, environmental temperature, environmental humidity and many factors related to the plant itself were considered among the reasons for these differences, and it was reported that the decomposition of silage plants increases the number of bacteria on the plant (20, 23).

In this study, the total number of lactic acid bacteria (1.33×10^{10}-2.53×10^{12} cfu/ml) in fermented natural LAB liquids prepared by adding various levels of sucrose at different incubation periods were higher than the ones obtained by many researchers (4, 7, 9, 11, 17, 31), ranging from 1.4×10^7 to 1.85×10^9 cfu/ml, but were found to be similar to the values (3.01×10^{10}--4.2×10^{11}cfu/ml) obtained in the study conducted by Aydın (3).

Aydın (3) has reported that the total number of LAB decreased at the end of 48-hours incubation period depending on the increasing sucrose levels in fermented natural LAB liquids prepared by adding sucrose at different levels (1%, 3%, 5% and 10%) and incubating for different time periods (48, 72 and 96 hours). Contrary to this situation, a general upward trend in the number of LAB at 72 and 96 hours was reported.

Tab. 3. The pH Values of fermented lab liquids prepared by sucrose addition at different levels (1%, 3%, 5% and 10%) and incubation for different time periods (5, 7, 9, 11, 13 and 15 days)

Incubation period (day)	1% Sucrose	3% Sucrose	5% Sucrose	10% Sucrose	SEM	P
5	4.19^{A}	4.05^{B}	$3.98^{bc\ C}$	$3.97^{a\ C}$	0.028	< 0.05
7	4.20^{A}	4.05^{B}	$3.98^{bc\ C}$	$3.97^{a\ C}$	0.028	< 0.05
9	4.27^{AB}	4.45^{A}	$3.89^{d\ B}$	$3.91^{b\ B}$	0.092	< 0.05
11	4.39^{A}	4.03^{B}	$3.93^{cd\ B}$	$3.87^{c\ B}$	0.075	< 0.05
13	4.26^{A}	4.04^{B}	$4.05^{a\ B}$	$3.84^{c\ C}$	0.046	< 0.05
15	4.49^{A}	4.23^{AB}	$4.01^{ab\ BC}$	$3.78^{d\ C}$	0.089	< 0.05
SEM	0.041	0.058	0.014	0.017		
P	–	–	< 0.05	< 0.05		

Explanations: a-d – values with different superscripts in the same column were found to be different; A-C – values with different superscripts in the same rows differ significantly; * – P < 0.05; SEM – standard error of means

In the present study, total LAB counts of fermented LAB liquids prepared by adding sucrose at different levels (1%, 3%, 5% and 10%) and incubating for different periods (5, 7, 8, 11, 13 and 15 days) showed an increase depending on the increases in the sucrose level ($P < 0.0$).

Under optimum conditions, bacteria in the latent phase begin to multiply by dividing at certain intervals within a generation period specific to their species. The important physical factors in the development and reproduction of microorganisms are the osmotic pressure and the amount of nutrients (sugar) in the environment of the microorganism. Increases in LAB counts due to the increase in sucrose level during prolonged incubation periods in fermented natural lactic acid bacterial liquids can be explained by factors such as the stabilization of the ambient osmotic pressure, the more effective use of sucrose by microorganisms after this process, and the continuation of the suitability of the environment adaptation for logarithmic increase (28).

Bacteria multiply at the maximum level under conditions of appropriate water activity. When the amount of nutrients in the environment increases, the range of water activity in which microorganism live increases (35). The increase in the LAB counts with increasing incubation periods due to the increase in sucrose level in lactic acid bacteria liquids can be explained by the increase in the amount of nutrients depending on sucrose, which expands the water activity limits in favor of microorganisms.

In this study, there was an increase in the number of LAB due to the increase in the incubation period in the 10% sucrose supplemented group (5, 7, 8, 11, 13 days) whereas a decrease was determined in the LAB count at the end of the 15-day incubation period ($P < 0.05$). The reason for this decrease may be explained with the decrease in the nutrients needed by the increasing LAB in the environment, the increase in the amount of toxic substances (lactic acid, acetaldehyde, peroxide, etc.) and the decrease in the pH level of the environment (15). These factors play an important role in the inhibition of the growth of some lactic acid bacteria especially at low pH level (22). Besides, pH changes affect the solubility and ionization of the environment and cell of microorganisms. Therefore, life activities in microorganisms slow down or stop (24). As a result of this situation, there is a decrease in the number of living microorganisms that can remain in the environment and an increase in the number of microorganisms that lose their activity.

In this study, pH values (3.78-4.49) of fermented natural LAB liquids obtained at the end of different incubation periods with different sucrose levels were higher than the values determined by Bureenok et al. (8) (3.45) but they were similar to the values (3.78--3.89) reported by Shao et al. (31), Denek et al. (11), Tao et al. (34), Abebaye et al (1) and Sun et al. (33).

In this study, when the pH values of fermented LAB liquids prepared by adding different levels of sucrose and incubating at different periods were evaluated, a general decrease in pH values was observed depending on the increase in the number of LAB. Similarly, Bureenok et al. (8), Denek et al. (11) and Tao et al. (34) reported the decreases in pH values due to the increase in LAB counts. While homofermentative LAB produces lactic acid as the main product from WSC in the environment, heterofermentative LAB produces secondary products such as ethyl alcohol, acetic acid, diacetyl and carbon dioxide as well as lactic acid (6). Thus, the increased lactic and acetic acid amount due to fermentation lowers the pH value in the medium (23). Moreover, in the present study, the highest pH values were obtained at 1% sucrose level in each incubation period (5, 7, 8, 11, 13 and 15 days), while the lowest pH values were obtained in the 10% sucrose supplemented group. The increase in the total LAB counts and the decrease in pH values is caused by the increase in the sucrose level that are used by acid producing LABs in the environment.

The acid produced by microorganisms as a result of fermentation is affected by the type of water-retaining substances (oligopolysaccharides (sucrose and lactose)) used to regulate water activity (2, 10, 29). After the addition of different levels of sucrose (1%, 3%, 5% and 10%), the differences between pH values based on acid production may be due to different levels of sucrose, which has water-retaining properties for the water activity value.

In this study, although the LAB counts in the group determined at the end of the 15-day incubation period with 1% sucrose addition were higher than in the 10% sucrose supplemented group, the pH value in the 1% sucrose supplemented group was found to be higher than in the 10% sucrose supplemented group ($P < 0.05$). The pH range in which microorganisms can grow may vary depending on the genus, species, strain, growth environment and other environmental factors.

In this study, despite the high LAB count at the end of the 15-day incubation period, high pH values indicated that LAB species in 1% sucrose added groups could be heterofermentative, and 10% sucrose added groups could be homofermentative, thus it can be explained by the fact that and the acetic acid formed by heterofermentative LABs was weaker than lactic acid produced by homofermentative LABs which can lower the pH values of the environment less than homofermentative LAB (20).

In the use of LAB inoculants as silage additives, when the addition of 10^{5-6} cfu/g to the fresh silage material has been evaluated together with the LAB counts (10^{7}-10^{9} cfu/ml) in the fermented natural LAB liquids in the studies carried out to date, it can be seen that the fermented natural LAB liquids makes it difficult to be applied as a silage additive in the field.

In this study, the results obtained from fermented natural LAB liquids prepared by adding different levels of sucrose and incubating at different periods were evaluated. When considered in general the increase in LAB numbers due to the prolonged incubation period with 5-10% sucrose addition was demonstrated by obtaining 10^{11}-10^{12} cfu/ml lactic acid bacteria after a 13-day incubation period. Thus it was concluded that LAB liquids can provide ease of application as a silage additive in the field and has commercialization potential.

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