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Research Article

Lactic Acid Bacteria Isolation and Determination of Their Silage Fermentation Capabilities§

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Abstract

Usage of high quality forage that enhances the production efficiency in the livestock industry is a quite important issue. During a famine period or when it is unavailable to reach fresh forage due to seasonal factors, the ensiled forage becomes a vital role for feeding ruminants. Lactic Acid Bacteria (LAB) is the most important microorganisms in silage for desired fermentation. In this work, LAB isolation in epiphytic flora from diverse field forage crops and ecologies in Turkey were made in order to determine their fermentative capacity and to develop a high-quality silage inoculant. For this purpose, the forage crops were collected from several rangelands from different altitudes of 39 to 1516 m and isolations were made from both fresh forage and ensiled material. Total acid production capacities of isolates were determined and 70 isolates were selected in terms of acid zone creation in growth media containing $CaCO_3$. Then, LA production levels in MRS broth media of isolates were determined and final 10 isolates were selected for inoculation trials. Also, morphological, physiologic, biochemical properties of selected 10 isolates were analyzed and identified by the BIOLOG kit on species level. Isolates that have outstanding properties about lactic acid productivity are left to use further inoculation research work.

Key words: Silage, LAB isolation, selection.

LAB İzolasyonu ve Silaj Fermentasyon Yeteneklerinin Belirlenmesi

Özet

Yüksek kaliteli yem kullanımı, hayvansal üretimi artıran en önemli konuların başında gelmektedir. Yemin kıt olduğu veya iklimsel nedenlerden dolayı kaliteli taze yem elde edilmesinin mümkün olmadığı dönemlerde, silolanmış yemler ruminant beslenmesinde hayati bir öneme sahiptir. Laktik asit bakterileri (LAB), silaj içerisinde arzu edilen fermentasyonun sağlanmasında en önemli rolü üstlenmektedir. Bu çalışmada, Türkiye'de farklı ekolojilerde yetişen yem bitkileri üzerinden LAB izolasyonu yapılmış, izolatların fermentatif özellikleri belirlenmiş ve silaj kalitesini geliştirebilecek izolatların seçimi yapılmıştır. Bu amaçla, 39-1516 m rakımlı bölgelerdeki farklı ekolojilerden yem bitkileri toplanmış ve aynı zamanda silajları yapılmış ve LAB bu materyallerden izole edilmiştir. Tüm izolatların toplam asit üretim kapasiteleri belirlenmiş ve $CaCO_3$ içeren besi yerinde oluşan transparan alan dikkate alınarak, en yüksek oranda asit üreten 70 adet izolat seçilmiştir. Daha sonra, Malt Extract Agar (MRS broth besi yerinde geliştirilen izolatların, besi yeri içerisindeki laktik asit (LA) üretim miktarları belirlenmiş ve en yüksek LA üreten 10 izolat seçilmiştir. Ayrıca, bu 10 izolatın morfolojik, fizyolojik ve biyokimyasal özellikleri incelenmiş ve BIOLOG kiti kullanılarak tanımlamaları yapılmıştır. Seçilen ümit var izolatlar daha sonraki inokulasyon çalışmalarında kullanılmak üzere stok solüsyonda saklanmıştır.

Anahtar kelimeler: Silaj, LAB izolasyonu, seleksiyon.

Introduction

Silage making has been practiced from ancient times (Sifeeldein et al., 2018) and it has been turned into an agricultural discipline since the last few decades. As a fermentative product, silage has many advantageous such as keeping a long time with a high quality, palatability, digestibility and feed intake of ruminants compared to hay. Besides, ensiling forages may help meeting water-rich feed of ruminants in the winter period in which quality feed shortage occurs (Kaplan et al., 2014; Sengul and Aydin, 2019). Microorganism diversity in epiphytic microflora, their counts and activities in the silo (McDonald et al., 1991; Lin et al., 1992; Wang et al., 2017) and their development during the fermentation period has a vital role on determining the quality of resulting silage. The fermentative properties lactic acid bacteria (LAB) on ensiled material and their lactic acid production capacity for lowering the pH and inhibiting the growth of spoilage microorganisms (McDonald et al., 1991) is also important (Wang et al., 2017). In many cases, LAB content in epiphytic microflora or their effectiveness in fermenting silage is poor, and so it is necessary to inoculate rapid-growing and high lactic acid producer LAB isolates in order to obtain successful silage.

The main purpose of inoculating crop material before ensiling with LAB is promoting lactic acid fermentation and improving forage preservation by decreasing pH around 4 as soon as possible. In recent decades, many LAB has been isolated, identified and used as microbial silage inoculant. Isolation from the natural environment and identification of LAB is a routinely employed process (Sifeeldein et al., 2018). Isolation from silage itself may help to discover a really good strain due to isolation among strains that they have already competed with other epiphytic microorganisms.

This study was set out in order to isolate LAB strains that have high lactic acid production capacity and that can lower silage pH rapidly and improve silage quality.

Table 1. Sample collection points, geographic positions and altitutes.

Stop Place*	Latitute	Longitute	Altitute	Stop Place*	Latitute	Longitute	Altitute
Sivircehüyük	37.43318	37.01522	559	Kocalar	37.53984	36.79523	587
Sivircehüyük	37.43274	37.01550	560	Önsen	37.53304	36.86102	449
Kocalar	37.44900	36.96523	601	KSU Kamp.	37.58799	36.81675	525
Kocalar	37.44919	36.96463	605	KSU Kamp.	37.58807	36.81605	523
Kurtlar	37.53945	36.79492	589	Mehmetbey	38.09591	36.46378	1403
Kurtlar	37.53993	36.79542	586	Mehmetbey	38.09618	36.46486	1409
Alahanlı	37.10460	36.18147	70	Mehmetbey	38.09672	36.46884	1402
Alahanlı	37.10714	36.18249	86	Çaglayan	38.04386	36.46706	1353
Cevdetiye	37.13226	36.20511	78	Çaglayan	38.03785	36.4654	1350
Kümbet	37.31991	36.04134	45	Arıtaş	38.33974	36.79556	1340
Orhaniye	37.20502	36.07150	44	Arıtaş	38.33864	36.79456	1342
Cevdetiye	37.12361	36.21626	108	Emirilyas	38.30264	36.88521	1224
Tecirli	37.16460	36.10163	43	Demir	38.22424	37.39218	1279
Sivircehüyük	37.43339	37.01542	560	Demir	38.22862	37.32680	1208
Kocalar	37.44854	36.96599	596	Püren	37.95105	36.54704	1516
Alahanlı	37.10517	36.18203	74	Göksun	37.98116	36.49930	1341
Alahanlı	37.10717	36.18262	88	Göksun	37.98005	36.49945	1342
Alahanlı	37.10033	36.18018	71	Çaglayan	38.04050	36.46621	1351
Cevdetiye	37.13167	36.20474	76	Mehmetbey	38.09607	36.46414	1405
Osmaniye	37.20512	36.07189	45	Arıtaş	38.04326	36.54971	1362
Kümbet	37.32054	36.04581	46	Çardak	38.33937	36.79582	1338
Hemite	37.19930	36.08156	39	Arıtaş	38.31415	36.87013	1244
Tecirli	37.17094	36.09192	43	Emirilyas	38.30285	36.88500	1224
Kocalar	37.53963	36.79515	587	Çardak	38.04326	36.54971	1362

*:Sivricehuyuk, Kocalar, Kurtlar, Kumbet, Onsen, KSU Kampus, Mehmetbey, Caglayan, Aritas, Emirilyas, Demir, Püren, Goksun, and Cardak are belong to Kahramanmaras province, Alahanlı, Cevdetiye, Orhaniye, Tecirli and Hemite are belong to Osmaniye province.

Material and Methods

In this study, lactic acid bacteria (LAB) strains were isolated from rangeland crops, corn and alfalfa fresh plants grown in different ecologies with altitudes from 39 m to 1516 m of Turkey, and from their silages. There were 48 sample points in total and they were visited in order from early spring to late autumn starting from number 1 to

48. First of all, fresh plants were collected from 48 sample collection stops (Table 1), put in a thermos, and then carried to the laboratory. Dilution series were prepared for growing LAB in MRS agar media. Also, some of the collected plants were cut, filled 400±50 g for any individual plastic packets, and ensiled in vacuum packed for 60 days. After the ensiling period, silages were opened, 20 g of silage samples were homogenized in 180 ml Ringer solution by using a blender for 1 minute at high speed. Then, extracts were filtered through 5 layer of cheesecloth and dilution series were prepared. Then LAB were grown in MRS agar. After 48 hours of incubation at 32 °C, 695 isolations were made in total, 423 of which from fresh crop samples while 272 form silage samples.

All isolates were kept in 80 °C for the further studies but only 531 of them could regenerate, so the rest were destroyed. Regenerated 531 isolates were grown in MRS media two times for purifying isolates. The purified isolates were grown again in MRS agar containing $CaCO_3$ in order to determine their total acid production abilities. In this procedure, the colony is formed, a transparent area, which is an indicator of acid production, is located all around the colony. The areas of transparent locations were calculated and 70 isolates that have the highest transparent area were selected for the former studies.

Table 2. LAB counts of fresh crops and silages from which LAB isolations were made

Sample No	LAB in Fresh Crop Samples cfu log_{10} g^{-1}	LAB in Silage Samples cfu log_{10} g^{-1}	Silage pH	Silage DM	Sample No	LAB in Fresh Crop Samples cfu log_{10} g^{-1}	LAB in Silage Samples cfu log_{10} g^{-1}	Silage pH	Silage DM
1	2.00	3.70	5.20	15.26	25	<2	5.70	5.47	21.53
2	<2	4.11	4.86	18.36	26	<2	6.11	4.49	23.06
3	<2	3.08	5.12	14.18	27	9.59	5.36	4.49	19.57
4	2.00	3.45	4.94	21.95	28	9.59	3.70	4.47	15.51
5	<2	3.18	5.11	16.74	29	5.08	5.94	5.39	24.96
6	<2	3.41	4.89	17.74	30	3.72	5.45	5.44	36.76
7	2.11	7.34	5.28	13.91	31	6.11	7.04	5.42	26.55
8	2.18	6.36	3.97	22.21	32	5.34	6.15	5.45	31.28
9	<2	8.11	5.29	18.01	33	5.04	6.20	5.40	26.32
10	2.67	6.28	3.59	19.82	34	4.23	4.00	4.88	32.51
11	3.41	7.30	5.28	16.44	35	<2	<2	4.64	25.97
12	2.54	4.83	4.66	23.00	36	3.51	6.60	5.51	26.81
13	3.45	8.74	4.26	22.65	37	4.89	5.97	5.50	31.32
14	3.18	6.48	3.96	24.16	38	4.49	5.53	5.23	27.90
15	2.30	5.71	4.04	18.73	39	<2	4.70	4.99	29.22
16	5.53	5.54	4.81	14.25	40	<2	5.43	5.50	34.32
17	4.94	5.04	5.30	13.03	41	4.56	5.61	5.56	14.94
18	5.15	6.70	4.21	27.16	42	6.63	5.26	5.49	18.15
19	5.40	4.78	5.50	13.39	43	3.75	5.83	5.51	21.08
20	<2	6.71	4.49	11.56	44	5.96	5.51	5.02	16.92
21	5.60	7.46	4.43	21.44	45	6.08	6.74	5.42	16.35
22	3.74	<2	4.06	24.65	46	4.08	4.45	5.49	17.13
23	4.40	6.52	5.36	15.13	47	3.63	6.48	5.48	13.50
24	<2	5.32	5.67	15.09	48	5.69	5.00	5.48	15.96

Biochemical, morphological and cultural test were applied for selected 70 isolates and identifications were made by using the BIOLOG kit (Jones et al., 1993). Selected isolates were inoculated to MRS broth media, incubated for 18 hours at 32 °C and bacterial intensity was calculated by spectrophotometer. Bacteria numbers in unit MRS broth were stabled considering calculated numbers of bacteria, inoculated to a second set of MRS broth media for 18 hours at 32 °C. After the incubation period, bacteria is separated by using filter and the filtrate extract were analyzed for lactic, acetic, butyric and propionic acid by using HPLC, at 41 °C, 0.6 ml/min flow rate and using H_2SO_4 as mobile phase. According to HPLC results, 10 the highest lactic acid producer isolates were selected for silage inoculation studies for alfalfa and corn silage, but the data for silage inoculation studies is not given in this paper.

Results and Discussion

LAB isolation

LAB counts, pH and dry matter (DM) contents of fresh crops or silage samples from which LAB isolation was made are given in Table 1. High rate of contamination with molds were determined at the samples that were taken from the places especially on which ruminants were grazed. Besides, enterobacteria and yeast were counted as high as 10^7 and 10^{10} cfu log_{10} g^{-1}, respectively, in many samples; however, they were less than10^2 cfu log_{10} g^{-1} in some samples (Data not given). It may be speculated that epiphytic flora was hypervariable depending on the environment as well as the time point of the season. Similarly, as shown in Table 2, LAB counts on samples were less than 10^2 cfu log_{10} g^{-1} for the fresh samples taken in early spring and higher than 10^9 cfu log_{10} g^{-1} for the samples taken in summer time. This shows clearly that LAB counts in epiphytic microflora are highly depended on the time of the year due to LAB number on crops is affected by temperature. Pahlow et al., (2003) stressed that LAB contents of any plant material is affected by environmental factors. It was determined that LAB counts were stabilized around 10^4-10^6 when the fresh crops were ensiled for 60 days, moreover, these strains were competed with other microorganisms for limited amounts of water soluble carbohydrate (WSC) during ensiling. After counting, LAB colonies were isolated randomly at various numbers for any petri dishes regardless of fresh or ensiled material. Totally 695 LAB colonies were isolated, 423 of them from fresh forage samples which is 61% of total, while 272 from silage samples which is 39%of total isolates. All regenerated 531 isolates were purified twice (Zhang et al., 2015) and kept at -80 °C for further studies. Silage pH values changed from 3.59 to 5.56 depending on the crops botanical composition and epiphytic flora. The contamination rate before ensiling may be the major factor determining the silage acidity level. The DM content of silages were ranged from 13.03 – 34.32%depending on the botanical composition of crop material and collection time points which determine the plants phonological stage.

Determining the total acid production capacity of LAB isolates

The 531 LAB isolates regenerated from the stock solution were point contaminated in MRS growth media containing $CaCO_3$ in order to determine the total acid production capacity of individual isolates. The isolate colonies formed two separate nested circles and the area in between the circles was bright in color as seen in Figure 1.

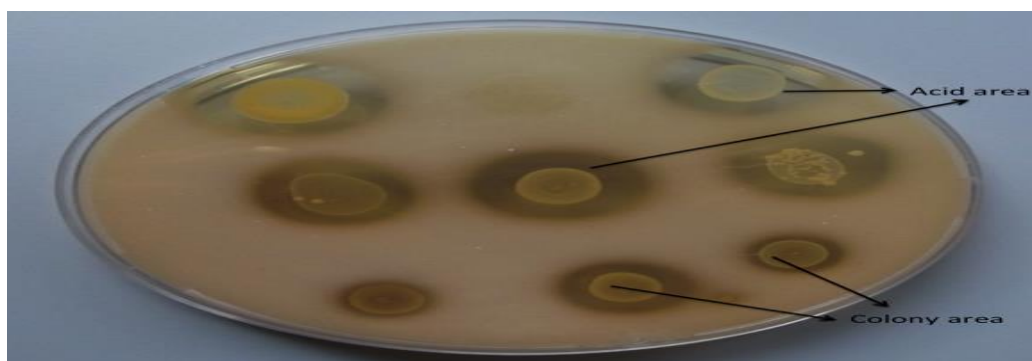

Figure 1. LAB colonies grown in MRS media containing $CaCO_3$ and their acid zones.

All 531 isolates produced acid at low or high volume. The 57 isolates which produced 150 mm^2 acid zone area were selected. However, making selection considering the acid zone only may not be adequate, due to 3.14 pi constant number, for selecting the bacteria which has very low colony

diameter but relatively high acid production area. Therefore, acid zone diameter is directly divided into colony diameter in order to select the isolates, which has lower colony diameter and relatively high acid zone. Another 13 isolates were selected which has small colony size but relatively high acid zone. In total, 70 isolates were selected for further studies.

Table 3. Fermentation profile of selected 70 isolates according to total acid production level.

Isolat No	Morphology*	SA (mmol/L)	LA (mmol/L)	AA (mmol/L)	PA (mmol/L)	ETOH (mmol/L)	LA Rate in Total Product (%)	Isolat No	Morphology*	SA (mmol/L)	LA (mmol/L)	AA (mmol/L)	PA (mmol/L)	ETOH (mmol/L)	LA Rate in Total Product (%)
1	B	0.000	43.62	0.74	0.10	0.00	98.10	36	C	0.000	31.67	1.74	0.05	0.00	94.66
2	B	0.433	70.02	6.61	0.01	8.53	81.79	37	B	0.087	30.78	9.98	0.01	35.73	40.19
3	C	0.000	50.16	0.58	0.12	0.84	97.02	38	C	0.000	27.78	9.71	0.02	32.02	39.95
4	C	0.201	43.34	2.20	0.06	4.30	86.50	39	B	0.300	49.49	1.85	0.19	1.33	93.10
5	SB	0.000	32.23	0.00	0.03	0.00	99.90	40	C	0.267	29.94	2.51	0.04	7.16	74.98
6	DC	0.015	28.56	1.88	0.02	0.38	92.57	41	B	0.262	51.10	1.09	0.09	3.01	92.00
7	B	0.000	48.52	0.00	6.81	0.00	87.69	42	B	0.019	54.00	1.64	0.10	1.72	93.94
8	C	0.027	48.03	0.88	0.07	1.40	95.27	43	C	0.293	35.01	3.46	0.04	0.02	90.15
9	B	0.264	53.85	1.71	0.11	1.20	94.24	44	B	0.000	35.30	8.58	0.05	14.36	60.56
10	SB	0.044	52.39	1.54	0.08	0.00	96.93	45	C	0.000	30.37	1.29	0.08	3.07	87.24
11	SB	0.023	51.15	2.20	0.11	3.20	90.24	46	B	0.000	48.39	0.54	0.06	0.00	98.77
12	B	0.060	44.42	4.61	0.11	18.97	65.17	47	B	0.063	48.95	1.94	0.04	0.00	96.00
13	B	0.000	28.13	0.93	0.03	0.00	96.70	48	C	0.000	51.85	1.85	0.14	0.00	96.31
14	SB	0.040	52.96	2.15	0.09	2.35	91.96	49	B	0.245	48.71	1.40	0.13	3.49	90.23
15	B	0.349	49.64	6.00	0.00	11.17	73.91	50	B	0.160	37.47	0.61	0.06	2.27	92.37
16	C	0.063	53.47	4.24	0.06	0.60	91.51	51	C	0.000	20.83	4.45	0.04	21.14	44.83
17	C	0.015	52.69	2.39	0.19	1.95	92.05	52	SB	0.023	27.40	2.87	0.02	14.67	60.92
18	B	0.296	56.65	1.69	0.15	1.07	94.66	53	C	0.013	22.52	2.41	0.03	11.66	61.49
19	C	0.000	18.69	5.62	0.00	15.04	47.49	54	C	0.020	47.40	0.00	0.05	1.18	97.43
20	B	0.080	49.59	2.95	0.16	1.86	90.77	55	B	0.000	51.84	3.72	0.01	4.78	85.89
21	C	0.000	22.11	2.90	0.01	25.81	43.49	56	B	0.032	49.22	1.83	0.09	4.04	89.15
22	B	0.000	42.05	0.00	0.12	0.14	99.37	57	C	0.000	49.33	6.75	0.03	7.27	77.83
23	SB	0.000	50.01	1.56	0.11	1.43	94.17	58	C	0.000	24.13	0.50	0.05	0.00	97.78
24	B	0.000	43.94	0.00	0.10	4.01	91.46	59	SB	0.047	46.49	0.50	0.05	8.44	83.72
25	SB	0.000	25.30	2.06	0.10	4.41	79.39	60	B	0.000	27.60	7.44	0.04	32.18	41.03
26	B	0.000	41.41	4.44	0.04	2.68	85.25	61	B	0.281	59.08	7.78	0.04	2.22	85.12
27	B	0.239	35.30	2.18	0.01	0.06	93.39	62	B	0.000	44.55	0.16	0.07	1.85	95.56
28	C	0.000	51.57	7.17	0.02	5.28	80.53	63	DC	0.059	44.19	1.86	0.01	1.54	92.71
29	DC	0.000	30.65	0.97	0.11	0.00	96.60	64	DC	0.028	51.42	1.28	0.09	3.61	91.13
30	B	0.219	54.59	2.57	0.10	3.01	90.26	65	B	0.175	30.34	9.81	0.03	15.25	54.57
31	B	0.000	35.89	4.38	0.03	28.76	51.97	66	B	0.216	32.49	4.80	0.03	23.51	53.21
32	C	0.000	31.87	2.45	0.01	0.56	91.34	67	B	0.000	49.80	0.34	0.15	2.35	94.62
33	C	0.021	50.03	0.61	0.14	1.31	96.00	68	B	0.060	41.75	0.22	0.12	1.03	96.68
34	B	0.000	30.39	5.69	0.01	30.73	45.48	69	B	0.000	25.47	1.44	0.11	22.29	51.65
35	SB	0.000	30.08	0.72	0.05	0.00	97.52	70	B	0.014	49.39	0.70	0.09	0.00	98.40

*:B: *Bacillus*, C: *Coccus*, SB: Short *Bacillus*, DC: *Diplococcu*s.

All selected 70 isolates were oxidase-negative, catalase-negative and gram-positive strains, which reflect the general properties of LAB. According to colony morphology, 36 isolates were bacillus, 9 isolates were short bacillus, 21 isolates were coccus and 4 isolates were diplococcus in shape. In order to determine the fermentation profile of the selected 70 isolates, they were grown in MRS broth media for 18 hours then samples were analyzed for succinic acid (SA), lactic acid (LA), acetic acid (AA), butyric acid (BA), propionic acid (PA) and ethanol (ETOH) (Table 4).

Table 4. Selected 10 isolates and their fermentation profile and LA rate in total product.

Isolat No	Isolat Name	LA mmol L^{-1}	AA mmol L^{-1}	PA mmol L	Etoh mmol L^{-1}	LA in total product (%)
2	LS-55-2-2	70.02	6.61	0.01	8.53	81.79
9	LS-51-2-1	53.85	1.71	0.11	1.20	94.24
16	L-70-6-1	53.47	4.24	0.06	0.60	91.51
17	LS-8-1	52.69	2.39	0.19	1.95	92.05
18	LS-65-2-1	56.65	1.69	0.15	1.07	94.66
19	L-70-7-1	18.69	5.62	0.00	15.04	47.49
30	LS-3-3	54.59	2.57	0.10	3.01	90.26
41	LS-49-2-1	51.10	1.09	0.09	3.01	92.00
61	LS-31-1-4	59.08	7.78	0.04	2.22	85.12

The 10 isolates that have the highest LA production level were selected. The isolate numbers, LA production, LA rate in total product and physiological characters of the selected isolates were given in Table 5. Pitt (1990) stressed that the most common bacteria species is *Lactobacillus* and *Streptococcus* genera. *L. plantarum* species are generally grown late, but are the most colony forming species in number compared to others. Eight isolates were belong to *Lactobacillus* while one was *Leuconostoc* and one was *Pediococcus* species. *L. plantarum* species was the most common among selected 10 species with four strains. Only *L. brevis* and *L. buchneri* were heterofermentative while others were homofermentative according to their fermentation profile explained by Bolsen et al. (1996). When isolation sources were taken into consideration, it is clearly seen that the most LA producer bacteria strains were selected from the isolation from silage. At the first isolation, silage source strain numbers were 39% of total isolates while at the final selection, in total, selected 9 isolates in 10 were from silage sources. This indicates that it is good way to isolate LAB strains from fermented silages among the strains that are already competed with each other and other microorganisms in order to catch a really good strain as silage inoculation.

Table 5. Species names, fermentative characters and isolation source of selected 10 isolates.

No	Isolat Name	Species	Physiological Characters	Isolation Source
2	LS-55-2-2	*Lactobacillus brevis*	Heterofermentatif	Silage
9	LS-51-2-1	*Lactobacillus gasseri*	Homofermentatif	Silage
10	LS-71-2-3	*Lactobacillus plantarum*	Homofermentatif	Silage
14	LS-2-4-1	*Lactobacillus plantarum*	Homofermentatif	Silage
16	L-70-6-1	*Leuconostoc citerum*	Homofermentatif	Fresh crop
17	LS-8-1	*Pediococcus citerum*	Homofermentatif	Silage
18	LS-65-2-1	*Lactobacillus bifermentans*	Homofermentatif	Silage
30	LS-3-3	*Lactobacillus plantarum*	Homofermentatif	Silage
42	LS-72-2	*Lactobacillus plantarum*	Homofermentatif	Silage
61	LS-31-1-4	*Lactobacillus buchneri*	Heterofermentatif	Silage

Conclusion

These isolates were transferred for corn and alfalfa silage inoculation studies, but the data have not been given in this paper. The most important result of this study is that isolation should be made from the silage instead of isolation from fresh crop due to increased possibility to select a successful isolate that has already competed the other microorganisms. At the final, %90 of the selected isolates were originally isolated from silage samples, however the rate of isolation from silage was %39 at the beginning. This clearly shows that isolation from silage could be much more successful instead of isolation from epiphytic flora.

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