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Short Communication

Isolation and Identification of High Lactic Acid Producer Bacteria from Forage and Their Silages Grown in Different Ecologies [1]

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Abstract

In total, 695 Lactic acid bacteria isolation was made from forage crops grown in a wide part of Turkey's rangeland flora and their silages. A big majority of isolated strains (531) could regenerate. All regenerated isolates were incubated in MRS agar media containing CaCO3 in order to determine their total organic acid production. Selected 70 isolates according to their organic acid production were incubated in MRS broth media and their lactic acid productions were determined. High lactic acid producer 10 isolates were selected among treated isolates and they were identified using BIOLOG device in terms of their individual usage of different carbohydrate source during incubation period.

Keywords: *LAB, Identification, Isolation, Silage, Fermentation Products*

Farklı Ekolojilerdeki Yem Bitkilerinden ve Silajlarından Yüksek Laktik Asit Üreten Bakteri İzolasyonu ve Tanımlanması

Özet

Türkiye'nin geniş bir bölümündeki meralarda bulunan yem bitkilerinden ve bunların silajlarından 695 adet LAB izolasyonu yapılmıştır. Elde edilen izolatlardan önemli bir çoğunluğu (531 adet) rejenere olabilmiştir. Bu izolatlar CaCO3 içeren MRS agar besi yerinde inkübe edilmiş ve toplam organik asit üretimleri belirlenmiştir. Toplam asit üretimi yüksek olan 70 adet izolat MRS broth besi yerinde inkübe edilerek, laktik asit üretimleri belirlenmiştir. Bu izolatlar içerisinden laktik asit üretimi yüksek olduğu tespit edilen 10 adet izolat seçilmiştir. Seçilen 10 adet izolatın BIOLOG cihazında inkübasyon süresi boyunca farklı karbonhidrat kaynaklarını kullanma esasına göre tanımlaması yapılmıştır.

Anahtar sözcükler: *İzolasyon, LAB, Silaj, Tanımlama, Fermentasyon Ürünleri*

INTODUCTION

The objective of preserving forage resources is to ensure continuous regular feed for livestock in order to get sustainable growth, fattening or milk production when market prices of forages are highest for the dry season or for winter. One of the most important issues of quality silage making is to achieve a rapid drop of pH to a level of 4.2 in anaerobic phase of silage. Dropping in pH is closely related to lactic acid (LA) production level. The main factors affecting LA production level and speed are epiphytic lactic acid bacteria (LAB) on forage and chemical composition of crop material [1]. Silage quality is largely depends on competition between LAB and other microorganism groups [2]. Enough LA production decreases proteolysis and even can completely stop when pH level comes to below a level of 4 [3]. LAB inoculation has been using widely in recent years instead of inorganic acid or other applications in order to achieve a rapid LA fermentation in silage [4-6].

Due to microbial inoculation in silage making is not a widely used practice, some problems such as insufficient LA fermentation, bad aerobic stability and bad smell of silage are often occurred. Especially silage from legumes such as alfalfa, silage quality problems are getting bigger because of high buffering capacity, low pH level and

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relatively high proteolysis level. Isolation of LAB from rangeland and forage crops flora of some part of Turkey, selecting isolates in terms of their LA production ability and identification of selected stains were main aim of this study.

MATERIAL and METHODS

LAB Isolation Land and Plant Material

Sample collecting for LAB isolation areas were 14 points in Osmaniye, 14 points in Kahramanmaras, 12 points in Goksun, 6 points in Afsin and 2 points in Elbistan, making 48 points in total. Elevation of sample points were ranging from 39 m to 1516 m and samples were taken in different dates in order to extend isolated strain's diversity. Isolations were made not only from the fresh forage, but also from the silage made from the crops in sample points in order to increase possibility of successive strains. Approximately 500 g fresh forage was taken in all sample collecting point and immediately rinsed in ringer's solution in order to keep bacteria alive until they were incubated in MRS in laboratory. An extra 500 g of forage sample was collected and made silage with a portable vacuum machine and these silages were used for further LAB isolation from silage. Total sample replications were 347 of which 211 were from green forage while 136 from the silage made of forages in sample points. The coordinates of the sample points were recorded but not given in this paper.

LAB Isolation

Dilution series were prepared from all green forage and silage sample replications in order to count microflora (LAB, enterobacteria, yeast and moulds) and LAB isolation. LAB isolations were made from the colonies which are grown decently and well-grown ones in the petri plates. Then isolates were grown in a MRS agar media to get pure isolates. Once pure isolates were gotten, they were kept at -80°C with glycerin (15%) for the further studies. In this study, 695 isolates in total were obtained but 531 of the total isolates could be regenerated from the keeping conditions. Researches were done on these 531 regenerated isolates.

Determination of Total Organic Acid Production of Isolated LABs

Isolates that could be regenerated from the keeping conditions were firstly grown in 1 g/l $CaCO_3$ containing MRS media in order to determine total organic acid production ability of individual isolates. After the incubation period, the colonies formed two circles, one with another. Saturated colored inner circle expresses the colony size while light colored or transparent outer circle expresses the area of total organic acid produced by colony. Both circle diameters were measured by a compass and circle areas were calculated. Net acid production area was calculated by subtraction the area of inner circle from the outer one. The colonies, which have bigger acid production area, were considered as they have higher acid production ability. According to their acid production area and to rate of acid production area to colony area, 70 isolates in total were selected for the further studies and they were identified as morphologically, physiologically and biochemically. 57 of total 70 isolates which have highest acid production area (>150 mm^2) were selected directly. 13 of total isolates which have small both colony and acid production circle diameters but acid production circle is big enough compare to its colony diameter area were also selected in order to eliminate r^2 factor when calculating the area of circles. Directly the rate of two circle diameters was taken into consider when selecting 13 isolates.

Identification of Selected 70 Isolates

Selected 70 LAB isolates in total were identified morphologically (colony morphology), physiologically (catalase test) and cultural (gram reaction) then they were identified biochemically by using BIOLOG identifying system.

Determination of LA Production Ability of Selected LAB Isolates

All isolates were grown in MRS broth media for one night at 32°C then their bacterial densities were determined in spectrophotometer at 600 nm. All isolates were re-incubated for one night more in MRS broth after concentrations of all strains in per volume of MRS broth were equalized in order force all strains to produce LA under the same conditions. Bacteria were separated through filter and extract containing bacterial fermentation's end products were run at HPLC with two parallels to determine organic acids produced by LAB isolates. 10 LAB isolates were selected from 70 isolates considering amount of LA they produced. Selected high LA producer LAB strains were morphologically and physiologically examined as well as they identified by using BIOLOG (BIOLOG, Inc., Hayward, CA, USA) [7] device.

RESULTS

Isolation of LAB

Enterobacteria, yeast and mold counts were at high concentration as much as Log_{10} >7 cfu/g due to high contamination at both green and silage samples taken from the areas on which livestock was grazed in the contrast enterobacteria, yeast and mold counts were as low as Log_{10} <2 cfu/g in the samples taken from forage land and other marginal land at which animal were not grazed. LAB counts in MRS agar plate were between Log_{10} = 3 and Log_{10} = cfu/g in green forage or silage samples. LAB counts in samples taken on early spring were lower, sometimes less than Log_{10} = 2, than the samples taken in

summer time. It has been observed that epiphytic LAB concentration on green forage crops and silages increased (10^{-4}-10^{-5} for green crops and 10^{-8} for silage samples) while enterobacteria, yeast and mold counts decreased as sampling season (shown as sample number in *Table 1*) changed into summer period *(Table 1)*.

Determination of Total Acid Production Ability of LAB Isolates

Regenerated 531 LAB isolates from stock solution were point inoculated in MRS agar containing $CaCO_3$ in order to determine how much the colonies produced total organic acids during the incubation period. The best organic acid producer 70 isolates of all were selected.

Identification of Selected 70 Isolates

Selected 70 isolates according to their higher total acid production ability were identified by BILOG system. Identification probabilities of 33 isolates were higher than 70% and that of 22 isolates were between 50% and 70%. Only 15 isolates were identified with a similarity rates (probability) which are less than 50%. So, 55 of 70 isolates were assumed to be identified successfully. Genus distribution of 70 isolates according to BIOLOG system, were as follows: 43 isolates belong to *Lactobacillus*, 5 isolates belong to *Leuconostoc*, 8 isolates belong to *Enterococcus*, 3 isolates belong to *Brothotrix*, 5 isolates belong to *Corynebacterium*, 4 isolates belong to *Streptococcus*, 2 isolates belong to *Weisella* genus's.

Morphological and Biochemical Analyses of Selected 70 Isolates

All selected isolates were found as catalase (-), oksidase (-) and gram (+) concurring with LAB bacteria characters indicated by Hammes and Vogel [8]. Colony morphologies of 36, 22, 9 and 3 isolates were determined as bacillus,

Table 1. *Mean microorganism numbers. pH in silages and DM contents*
Tablo 1. *Silajda ortalama mikroorganizma sayıları. pH ve kuru madde içeriği*

Sample No	Green Forage			Silage Samples					Sample No	Green Forage			Silage Samples				
	Enterobacteria (Log_{10})	Yeast (Log_{10})	LA Bacteria (Log_{10})	Enterobacteria (Log_{10})	Yeast (Log_{10})	LA Bacteria (Log_{10})	pH	DM (%)		Enterobacteria (Log_{10})	Yeast (Log_{10})	LA Bacteria (Log_{10})	Enterobacteria (Log_{10})	Yeast (Log_{10})	LA Bacteria (Log_{10})	pH	DM (%)
1	6.34	8.94	2.00	<2	6.78	3.70	5.2	15.26	25	5.95	7.88	<2	<2	5.00	5.70	5.47	21.53
2	5.48	8.72	<2	5.72	6.73	4.11	4.86	18.36	26	<2	7.38	<2	<2	6.08	6.11	4.49	23.06
3	5.88	8.66	<2	<2	6.71	3.08	5.12	14.18	27	<2	<2	9.59	<2	6.15	5.36	4.49	19.57
4	7.00	8.79	2.00	<2	6.28	3.45	4.94	21.95	28	<2	<2	9.59	<2	4.18	3.70	4.47	15.51
5	7.20	8.68	<2	<2	6.79	3.18	5.11	16.74	29	4.95	6.88	5.08	<2	4.52	5.94	5.39	24.96
6	6.61	8.53	<2	3.70	8.04	3.41	4.89	17.74	30	<2	<2	3.72	<2	6.15	5.45	5.44	36.76
7	6.28	7.67	2.11	3.51	5.45	7.34	5.28	13.91	31	<2	<2	6.11	2.00	6.99	7.04	5.42	26.55
8	7.15	8.00	2.18	<2	5.93	6.36	3.97	22.21	32	<2	<2	5.34	<2	5.91	6.15	5.45	31.28
9	6.93	8.36	<2	5.03	6.04	8.11	5.29	18.01	33	<2	<2	5.04	2.74	6.57	6.20	5.40	26.32
10	6.79	8.40	2.67	<2	6.00	6.28	3.59	19.82	34	<2	<2	4.23	<2	5.89	4.00	4.88	32.51
11	7.30	9.08	3.41	<2	6.41	7.30	5.28	16.44	35	5.74	<2	<2	<2	4.76	<2	4.64	25.97
12	6.94	8.67	2.54	<2	3.70	4.83	4.66	23.00	36	5.41	<2	3.51	2.60	6.91	6.60	5.51	26.81
13	6.66	8.95	3.45	<2	5.74	8.74	4.26	22.65	37	5.74	<2	4.89	2.18	5.67	5.97	5.50	31.32
14	6.18	7.76	3.18	<2	4.18	6.48	3.96	24.16	38	5.67	<2	4.49	3.30	5.48	5.53	5.23	27.90
15	5.40	8.30	2.30	<2	4.00	5.71	4.04	18.73	39	<2	<2	<2	4.18	5.59	4.70	4.99	29.22
16	6.94	7.72	5.53	<2	5.43	5.54	4.81	14.25	40	6.40	7.65	<2	5.89	5.08	5.43	5.50	34.32
17	7.75	8.60	4.94	<2	5.88	5.04	5.30	13.03	41	4.30	<2	4.56	2.85	5.38	5.61	5.56	14.94
18	7.20	7.81	5.15	3.70	6.64	6.70	4.21	27.16	42	4.48	7.60	6.63	2.60	5.81	5.26	5.49	18.15
19	6.81	6.94	5.40	<2	5.00	4.78	5.50	13.39	43	<2	5.70	3.75	<2	6.00	5.83	5.51	21.08
20	7.15	8.34	<2	<2	6.38	6.71	4.49	11.56	44	5.53	6.00	5.96	<2	5.48	5.51	5.02	16.92
21	6.89	7.53	5.60	3.70	7.46	7.46	4.43	21.44	45	6.18	6.93	6.08	<2	5.26	6.74	5.42	16.35
22	7.20	8.15	3.74	<2	4.70	<2	4.06	24.65	46	5.49	5.70	4.08	<2	5.63	4.45	5.49	17.13
23	6.62	7.46	4.40	<2	6.89	6.52	5.36	15.13	47	5.54	<2	3.63	2.90	6.18	6.48	5.48	13.50
24	<2	10.80	<2	<2	5.71	5.32	5.67	15.09	48	5.67	7.27	5.69	<2	6.20	5.00	5.48	15.96

coccus, short bacillus and diplococcus, respectively.

LA and Other Organic Acid Production of Selected 70 LAB Isolates

It has been found that all isolates have produced more or less LA changing from 18.69 mmol/L to 70.02 mmol/L. The highest LA production was obtained from number 2 (LS-55-2-2) while number 61 (LS-31-1-4) produced the highest (7.78 mmol/L) acetic acid. It has been found that the most extended variation among the isolates was in terms of ethanol production ranging from 0 to 35.73 mmol/L. All isolates produced lower propionic acid (<0.2 mmol/L) except for the isolate number 7 (BOLSON-2-3) whose production was 6.81 mmol/L.

The best 10 isolates according to their LA production level among given in *Table 2* is selected *(Table 3)* and used as microbial inoculant for corn and alfalfa silages in order to determine their effects on fermentation profile in both

Table 2. *Fermentation product profile (mmol/l) and rate of LA in total fermentation product (%)*
Tablo 2. *Fermentasyon* ürün *profile (mmol/l) ve toplam fermentasyon* ürünü *içinde laktik asit oranı (%)*

No	Isolat Name	Fermentation End Products						No	Isolat Name	Fermentation End Products					
		Succinic Acid (mmol/L)	Lactic Acid (mmol/L)	Acetic Acid (mmol/L)	Propionic Acid (mmol/L)	Ethanol (mmol/L)	LA Rate in Total Products (%)			Succinic Acid (mmol/L)	Lactic Acid (mmol/L)	Acetic Acid (mmol/L)	Propionic Acid (mmol/L)	Ethanol (mmol/L)	LA Rate inTotal Products (%)
1	L-42-8	0.00	43.62	0.74	0.10	0.00	98.10	36	L-50-8	0.00	31.67	1.74	0.05	0.00	94.66
2	LS-55-2-2	0.43	70.02	6.61	0.01	8.53	81.79	37	LS-30-1	0.09	30.78	9.98	0.01	35.73	40.19
3	L-60-5	0.00	50.16	0.58	0.12	0.84	97.02	38	L-34-1-2	0.00	27.78	9.71	0.02	32.02	39.95
4	L-38-2-2	0.20	43.34	2.20	0.06	4.30	86.50	39	LS-2-2	0.30	49.49	1.85	0.19	1.33	93.10
5	L-44-4-1	0.00	32.23	0.00	0.03	0.00	99.90	40	LS-6-5-1	0.27	29.94	2.51	0.04	7.16	74.98
6	L-70-10-1	0.02	28.56	1.88	0.02	0.38	92.57	41	LS-49-2-1	0.26	51.10	1.09	0.09	3.01	92.00
7	BOLSN-2-3	0.00	48.52	0.00	6.81	0.00	87.69	42	LS-72-2	0.02	54.00	1.64	0.10	1.72	93.94
8	L-61-3-2	0.03	48.03	0.88	0.07	1.40	95.27	43	LS-6-3	0.29	35.01	3.46	0.04	0.02	90.15
9	LS-51-2-1	0.27	53.85	1.71	0.11	1.20	94.24	44	LS-39-1-3	0.00	35.30	8.58	0.05	14.36	60.56
10	LS-71-2-3	0.04	52.39	1.54	0.08	0.00	96.93	45	L-58-6-2	0.00	30.37	1.29	0.08	3.07	87.24
11	L-57-2	0.02	51.15	2.20	0.11	3.20	90.24	46	L-54-10-1	0.00	48.39	0.54	0.06	0.00	98.77
12	L-68-1	0.06	44.42	4.61	0.11	18.97	65.17	47	L-61-8-2	0.06	48.95	1.94	0.04	0.00	96.00
13	L-55-2	0.00	28.13	0.93	0.03	0.00	96.70	48	L-57-4-2	0.00	51.85	1.85	0.14	0.00	96.31
14	LS-2-4-1	0.04	52.96	2.15	0.09	2.35	91.96	49	LS-43-2-3	0.25	48.71	1.40	0.13	3.49	90.23
15	LS-3-4-2	0.35	49.64	6.00	0.00	11.17	73.91	50	LS-4-4	0.16	37.47	0.61	0.06	2.27	92.37
16	L-70-6-1	0.06	53.47	4.24	0.06	0.60	91.51	51	L-61-5-1	0.00	20.83	4.45	0.04	21.14	44.83
17	LS-8-1	0.02	52.69	2.39	0.19	1.95	92.05	52	L-61-2-1	0.02	27.40	2.87	0.02	14.67	60.92
18	LS-65-2-1	0.30	56.65	1.69	0.15	1.07	94.66	53	L-61-6-1	0.01	22.52	2.41	0.03	11.66	61.49
19	L-70-7-1	0.00	18.69	5.62	0.00	15.04	47.49	54	L-53-8-2	0.02	47.40	0.00	0.05	1.18	97.43
20	L-5-9-1	0.08	49.59	2.95	0.16	1.86	90.77	55	L-38-4	0.00	51.84	3.72	0.01	4.78	85.89
21	LS-8-5-1	0.00	22.11	2.90	0.01	25.81	43.49	56	L-51-2-1	0.03	49.22	1.83	0.09	4.04	89.15
22	L-44-2	0.00	42.05	0.00	0.12	0.14	99.37	57	L-55-8-2	0.00	49.33	6.75	0.03	7.27	77.83
23	L-50-5-1	0.00	50.01	1.56	0.11	1.43	94.17	58	L-53-1-1	0.00	24.13	0.50	0.05	0.00	97.78
24	L-57-7	0.00	43.94	0.00	0.10	4.01	91.46	59	L-51-2-2	0.05	46.49	0.50	0.05	8.44	83.72
25	LS-9-3	0.00	25.30	2.06	0.10	4.41	79.39	60	L-54-4	0.00	27.60	7.44	0.04	32.18	41.03
26	LS-8-3	0.00	41.41	4.44	0.04	2.68	85.25	61	LS-31-1-4	0.28	59.08	7.78	0.04	2.22	85.12
27	L-35-1	0.24	35.30	2.18	0.01	0.06	93.39	62	L-58-6-1	0.00	44.55	0.16	0.07	1.85	95.56
28	L-70-5	0.00	51.57	7.17	0.02	5.28	80.53	63	L-60-7	0.06	44.19	1.86	0.01	1.54	92.71
29	L-57-2-1	0.00	30.65	0.97	0.11	0.00	96.60	64	L-61-7-2	0.03	51.42	1.28	0.09	3.61	91.13
30	LS-3-3	0.22	54.59	2.57	0.10	3.01	90.26	65	LS-69-1-2	0.18	30.34	9.81	0.03	15.25	54.57
31	P710-2	0.00	35.89	4.38	0.03	28.76	51.97	66	LS-40-1-2	0.22	32.49	4.80	0.03	23.51	53.21
32	LS-8-4-1	0.00	31.87	2.45	0.01	0.56	91.34	67	L-44-11	0.00	49.80	0.34	0.15	2.35	94.62
33	L-61-9-2	0.02	50.03	0.61	0.14	1.31	96.00	68	L-61-4-1	0.06	41.75	0.22	0.12	1.03	96.68
34	L-44-8-2	0.00	30.39	5.69	0.01	30.73	45.48	69	L-57-4-1	0.00	25.47	1.44	0.11	22.29	51.65
35	LS-3-2-1	0.00	30.08	0.72	0.05	0.00	97.52	70	L-41-1-1	0.01	49.39	0.70	0.09	0.00	98.40

Table 3. *Species name. LA production (mmol/l) and LA rate in total fermentation products (%) and physiological characters of selected and transferred to further inoculation studies 10 isolates*

Tablo 3. İleriki *inokulasyon* çalışmalarında *kullanmak* üzereseçilen *10 adet izolatın tür ismi. LA* üretimi *(mmol/L). toplam fermentasyon* ürününde *LA oranı (%) ve fizyolojik karakterleri*

Isolate No	Isolate Name	Species	LA Production (mmol/L)	LA/Total Fermentation Products (%)	Physiological Character
2	LS-55-2-2	*Lactobacillus brevis*	70.02	81.79	Heterofermentative
9	LS-51-2-1	*Lactobacillus gasseri*	53.85	94.24	Homofermentative
10	LS-71-2-3	*Lactobacillus plantarum*	52.39	96.93	Homofermentative
14	LS-2-4-1	*Lactobacillus plantarum*	52.96	91.96	Homofermentative
16	L-70-6-1	*Leuconostoc citerum*	53.47	91.51	Homofermentative
17	LS-8-1	*Pediococcus citerum*	52.69	92.05	Homofermentative
18	LS-65-2-1	*Lactobacillus bifermentans*	56.65	94.66	Homofermentative
30	LS-3-3	*Lactobacillus plantarum*	54.59	90.26	Homofermentative
42	LS-72-2	*Lactobacillus plantarum*	54.00	93.94	Homofermentative
61	LS-31-1-4	*Lactobacillus buchneri*	59.08	85.12	Heterofermentative

cereal and legume silages (data not given). All selected as high LA producer isolates were belong to *Lactobacillales* family. 8, 1 and 1 of total 10 isolates scattered *Lactobacillus, Leuconostoc,* and *Pediococcus* genus', respectively. 4 isolates from *Lactobacillus* were belong to *L .plantarum*, and the others to *L. brevis*, *L. gasseri*, *L. bifermentans* and *L. buchneri* species with one each member. Among 10 selected strains, the LA production rate in total organic acid production of *L. brevis* ve *L. buchneri* species were found as 81.79% and 85.12%, respectively. LA proportions in total organic acid productions of all other isolates were higher than the critical level of 90.0% which is assumed as the lowest LA production limit of homofermentative LAB fermentation. Those two isolates which have lower LA proportion level of 90% of total fermentation products were determined as heterofermentative species while the others defined as homofermentative. Hommes and Hertel [9] stated at their Bacteriology book in which they explained widely the morphologic, physiologic and genetic characteristic of LAB, *L. brevis* and *L. buchneri* species were stated as facultative heterofermentative and they have very similar metabolic characters supporting accuracy of our identification results from the BIOLOG device.

DISCUSSIONS

It is well known that pH levels of silages made for getting LAB isolation were affected by crop factors such as DM content of raw material, growing sage of forage crops, botanical composition of forage, microbial factors such as epiphytic LAB and other microbial content of forage, LAB strains effectiveness in silo, and other environmental factors such as silo conditions [10]. pH level of all silages were lower than 5.5 in general and very low pH level values of 3.8 were also reached in some silages. DM contents of silages were varied from 11.56% to 34.32% *(Table 1)*. Incidentally LAB isolations were made in various numbers right after the plate counting then they were kept -80ºC for further use. Total 695 isolates were taken from the plates and kept in -80ºC but 531 of them can regenerate from the stock solution when the time of use. Regenerated isolates were grown at MRS media containing $CaCO_3$ in order to determine total organic acid production and 70 of them were selected.

It is found that 43 of the selected 70 isolates were isolated from green forage samples while 27 of them were from silage samples. This is because of there were much more isolates from green forage sample replications than the isolates from silage samples. Selected 70 isolates in total were transferred to further research for determining the fermentation profiles of isolates after they were identified by BIOLOG microbiological identification system. It is found that *Lactobacillus* was the most common genus (61.4% of total LAB) on epiphytic flora on plant and, as a result, on silage environment.

This is an expectable situation to consider that the most common LAB family found on crop's epiphytic flora is *Lactobacillales* family as all selected isolates were a member of a foresaid family. A great majority of selected strains (9 in 10) were isolated from silage samples while only one isolate (L-70-6-1) was from green forage. This situation indicated that exploring chance of easily grown, high competitive, high LA producer and tolerant to low pH level isolates was clearly increased by isolating directly from matured silages.

Mainly two points from this research can be deduced as follows;

1- Preselection of isolates by using $CaCO_3$ containing media in order to determine their total fermentation acid production is an appropriate method.

2- Bacteria isolation for silage inoculation from matured silage has advantages compare to that of green forage due to the bacteria already grew in silage and competed to other microorganisms. Too many isolate from green forage is needed to get successive results.

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