Identification lactobacilli according to the API and PCR techniques isolated from rustic Shanklish

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Summary

The Lactobacilli are the dominant members of the microflora of several types of cheese produced by a natural starters. A total of 82 isolates of Lactobacilli were obtained from 39 samples of Shanklish collected from producing areas of different regions of Syria during 2013 and 2014. The isolates were gram positive and catalase-negative. From these isolates, 62 isolates rods were grown at 37 ºC, from which 20 isolates (24.39 %) were identified as *Lb. plantarum*, 35 isolates (42.68%) were identified as *Lb. paracasei*, while the rest 7 isolates (8.54 %) were identified as *Lb. brevis*. The rest 20 isolates were grown at 45 ºC, from which 18 (21.95%) were identified as *Lb. delbrueckii subsp lactis*, while 2 isolates (2.44%) were identified as *Lactobacillus* sp. Consequently the PCR technique could be efficiently used for identifying and typing the Lactobacilli.

Key words: API technique, Lactobacilli, PCR, Shanklish.

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استخدام تقنيتي API و PCR في تحديد العصيات اللبنية المعزولة من الشنكليش المحلي

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الملخص

تعد العصيات اللبنية من الاحياء الدقيقة المسيطرة على انواع عديدة من الأجبان، حيث تعتبر العصيات اللبنية من الفلورا الطبيعية للأجبان. تم الحصول على 82 عزلة من العصيات اللبنية عزلت من 39 عينة من الشنكليش، حيث تم جمع عينات الشنكليش من مناطق مختلفة في سورية خلال العامين 2013– 2014. جميع العزلات كانت موجبة الغرام سالبة الكاتلاز، من هذه العزلات وجد أن 62 عزلة كانت عصيات نامية على درجة حرارة 37 ْم 20 عزلة (24.39%) تابعة للنوع *Lb. plantarum*، 35 عزلة (42.68 %) تابعة للنوع *Lb. paracasei*، و7 عزلات فقط (% 8.54) تابعة للنوع *Lb. brevis*. وتم عزل 20 عزلة على درجة الحرارة 45 م وجد منها 18 عزلة (21.95%)تابعة للنوع *Lb. delbrueckii subsp lactis* في حين أن 2 عزلة (2.44 %) لم يتم تحدد نوعها وبقيت تتبع الجنس *Lactobacillus*. ومن الممكن استخدام تقنية PCR كتقنية موثوقة في تحديد السلالات Lactobacilli.

الكلمات المفتاحية: نظام API، العصيات اللبنية، PCR، الشنكليش

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Introduction

Lactobacilli are among the most important lactic acid bacteria (LAB) used in food production and are a gaining increasing attention in the area of probiotics (Tannock, 2004 Ortu *et al.*, 2007). The genus *Lactobacillus* represents the largest group of rod shaped, currently, over 60 species has been recognized (Marroki *et al.,* 2011). The identification methods of lactobacilli have been based mainly on fermentation of carbohydrates, morphology and gram staining. However, the characterization of some Lactobacillus to species level by the biochemical methods alone is not reliable (Schleifer *et al.,* 1995), because of the considerable variations in biochemical attributes between strains currently considered to belong to the same species (Marroki *et al.,* 2011). In fact, some species are not readily distinguishable in terms of phenotypic characteristics (Coeuret *et al.,* 2003). In recent years, the development of PCR based molecular techniques for the identification of bacteria (Mannu *et al*., 2002), and this novel taxonomy based on DNA analysis offers a variety of advantages over other more conventional typing procedure, such as the stability of the genomic DNA analysis and the capacity to discriminate bacteria at the strain level (Fitzsimons *et al.,* 1999); (Ortu *et al.,* 2007); (Amran and Abbas, 2011).

Our study focused on Shanklish or Surke, it is a type of cow's milk cheese made in Syria, and it is the mostly produced in "cottage industry" conditions using traditional techniques with little emphasis on hygiene practices. It is typically formed into balls of approximately 6cm diameter, and also sold in much smaller balls, and covered by spices and dried, the most common spice is thyme. Shanklish varies greatly in its texture and flavor. Fresh cheese have a soft texture and mild flavor, while those dried and aged for a longer period become progressively harder and can acquire an extremely pungent odor and flavor.

The aim of this study was to evaluate morphological and biochemical methods in the identification of Lactobacilli isolated from Shanklish (Syrian ripened cheese). Moreover a direct analysis of total Shanklish flora DNA and amplification of 16S rRNA genes encoding by the polymerase chain reaction (PCR) technique were performed.

Materials and Methods

Sample collection:

Seventy samples of Shanklish were collected from different Syrian regions (Damasccus, Rural Area, Daraa, Al-Sweida, Hama, Al-Hasakeh, Aleppo, Tartous, Lattakia,) between 2013 and 2014. The samples into sterile transportation to the laboratory the isolation studies commenced on the same day in Microbilology laboratory in Damascus University.

Isolation of lactobacilli

Ten gram of samples were homogenized with 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, 1000 ml distilled water, pH 7.0). The homogenate was serially diluted and the appropriate dilutions were surface plated on MRS agar media (MERCK, Germany), and then incubated at 37º C and 45° C for 3 days under anaerobic conditions using anaerobic jars.

Physiological and biochemical tests

Isolated bacteria were tested for gram reaction, catalase production, spore formation and cell morphology according to the methods described by Kebede *et al.,* (2007). Growth in the presence of 4% and 6.5% NaCl was observed in MRS broth, and growth at 15 ºC and 45 ºC was also observed in MRS broth after incubation for 2 days. The production of acetoin from glucose was determined using the Voges –Proskauer test (Samelis *et al.,* 1994). Overnight cultures of *Lactobacillus* were inoculated into 10 mL MRS broth containing inverted glass tubes (approximately 6 × 50 mm) and incubated at 30 C. Results were recorded after 48 h, from study gas production. Cultures producing gas within the 48-h incubation period were presumed to be heterofermentative *Lactobacillus*. While cultures that did not produce gas within 48 h were presumed to be homofermentative *Lactobacillus* (Barakat *et al.,* 2011). Fermentation casein in milk agar (Terzic-Vidojevic *et al.,* 2006), Fermentation citrate in Simon citrate agar and analyses fat in Trebucyn agar (Kempler and McKay, 2003).

API assay

The API 50CHL (Biomerieux, Marcy l'Etoile, France) was used to identify the enzymatic and carbohydrate fermentation patterns of *Lactobacillus* isolates. The inoculated strips were incubated at 37ºC and then monitored for changes in the color of medium after 24h.

Discrimination between isolates was based on the principle of a pattern matching manual as described by the manufacturer.

Genomic DNA isolation from bacteria

A single colony from each isolate was inoculated into 10ml of the appropriate medium broth (kept in a 15ml Falcon tubes) and incubated overnight at 37°C. The cultivated culture was harvested by centrifugation at 5,000 rpm for 5min and the genomic DNA was isolated by a modified genomic DNA isolation protocol (Sambrook and Russell, 2001).

PCR experiments

A DNA and 16S RNA regions amplification were performed on a volume of 25µl containing, 1.5 µl PCR reaction buffer, 3 µl $MgCl_2$ (25nm), 0.5 µl of each dNTP, 1 µl of each primer (see Table 1), 2 µl bacterial genomic DNA solution and 1 µl Taq DNA polymerase. The temperature profile was the following: 1 cycle consisting of 94°C for 5min, and 30 cycles consisting of 94°C for 30sec, 50°C for 30sec and 72°C for 30sec. A final extension at 72°C for 10min was performed. All amplification reactions were performed in a GeneAmp® PCR System 9700 (Applied Biosystems). The following amplification, 2µl of product was analyzed at 85V, 150A for 1h by agarose electrophoresis (1.5% agarose gel, 0.2µg ethidium bromide) and imaged under UV light.

Table 1. List of the primers used in this study.

Name	Gene	Oligo Sequence	Fragment
Lactobacillus ssp.	16s RNA	5' TGATCGGCCACATTGGGACT '3	300bp
		5' TCGCCACTGGTGTTCTTCCA '3	

16S RNA sequence determination and phylogenetic analysis

Sequencing of the 16SRNA was performed in collaboration. Sequences were submitted to the National Center for Biotechnology Information (Bethesda, Md.) for similarity searches through BLAST (http://www.ncbi.nlm.nih.gov/blast) and were compared with type strains by multiple alignments.

Statistical analysis

Data were transferred to a Microsoft Excel spreadsheet for analysis. Using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA), a chi-square test and Fisher's exact two-tailed test analysis were performed at the 5% level ($P < 0.05$).

Results

From the collected samples (n = 39), a total of 82 isolates were to else bacilli with rounded ends mostly appeared in chains of 3 - 4 cells, pairs or single cells, gram positive and catalase – negative, and these could tentatively be identified as derivatives of the genus *Lactobacillus* (Table 2 and 3).

Table (2): morphological and simple physiological characterization of Lactobacilli isolates

No. of isolates	Cell Shape	Gram stain reaction	Catalase activity	Pro. acid from lactose	Culture medium	Temperature of isolated
20	Rods	+	-	+	MRS	45C
62	Rods	+	-	+	MRS	37C

Table (3). Morphological, cultural and physiological characteristics of Lactobacilli isolated from Shenglish

Test		Rods
No. of isolates		82
Gram stain reaction		+
Spores formation		-
Catalase activity		-
Fermentation casein		42
Analyses fat		35
Fermentation citrate		9
Gas production		-
Growth in a medium with NaCl (%)	4 %	82
	6.5%	45
Growth at temperature (ºC)	15 C	39
	45 C	70

+ Positive reaction. -: negative reaction.

API assay:

Lactobacilli isolates were screened for their growth characteristics in carbon sources Table (4). Based on phenotypic characteristics and interpretation of the API database, 62 isolates rods were grown at 37 ºC, from which 20 isolates (24.39 %) were identified as *Lb. plantarum*, 35 isolates (42.68%) were identified as *Lb. paracasei*, while the rest 7 isolates (8.54 %) were identified as *Lb. brevis*. The rest 20 isolates were

grown at 45 ºC, from which 18 (21.95%) were identified as *Lb. delbrueckii subsp lactis*, while 2 isolates (2.44%) were identified as *Lactobacillus* sp. (fig1).

Table (4). Fermentation of some carbohydrates by *Lactobacillus* isolated from *Shanklish* by using API system

No. of isolates	bacteria	API system (carbon sources)																						
		RIB	XYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	MAN	SOR	ARB	ESC	LAC	TRE	INU	RAF	AMD	GLYG	TUR	LYX	TAG	FUC
20	*plantar*	+	-	-	-	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
35	*paracas*	+	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	-	+	-
7	*Lb.brevis*	+	+	-	-	+	+	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-
18	*Lb. lactis*	-	-	-	-	+	+	+	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-
2	*cillus*	-	-	-	-	+	+	+	+	-	-	-	+	+	+	+	-	+	-	-	-	-	+	-

+: positive fermentation; -: negative fermentation; ±: partial fermentation.

RIB: ribose; XYL: Lyxose; ADO: Adonitol; MDX: Methyl-D-Xylopyrananoside; GAL: galatosidase; GLU: glucose; FRU: Fructose; MNE: Manose; SBE: Sorbose; MAN: Mannitol; SOR: Sorbitol; ARE: Arbutin; ESC: Esculin; LAC: Lactose; TRE: Trehalose; INU: Inulin; RAF: Rafinose; AMD: Amidon; GLYG: Glocogen; TUR: Turanose; LXY: Lyxose; TAG: Tagatose; FUC: Fucose.

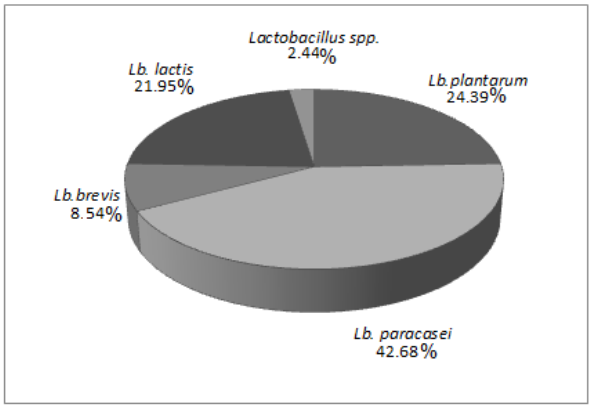

Fig. 1. Percentages of *Lactobacillus* isolated from *Shanklish*.

PCR results

Fig. 2 demonstrate that the length of 16sRNA gene (300 bp) bands (bands 2-7) was similar to that of Hansen's bands (*Lb. delbruckii ssp. bulgaricus*, Hansen® strain which used as a positive reference), which confirmed that the isolates belong to *Lactobacillus* type.

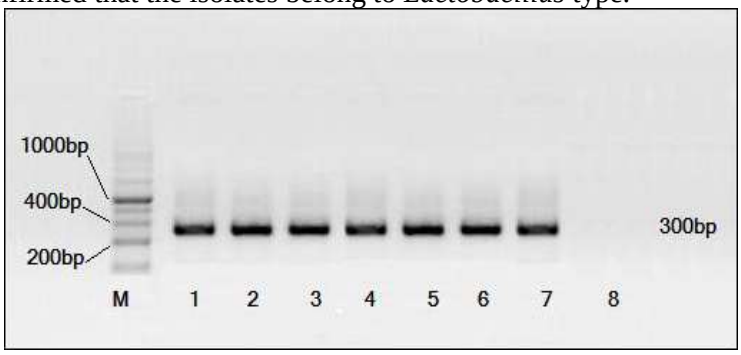

Fig. 2. Electrophoresis on 1.5% agarose gel showing the PCR products and using primers of 16sRNA genes. Bands 1: positive control (*Lb. delbruckii ssp. bulgaricus*, Hansen®). Bands 2-7: fragments of 300bp *Lactobacillus sp.* obtained from Syrian Shenglish. Band 8: negative control (*S.thermophilus* TH-4, Hansen®) Lane MW: molecular weight marker (100-bp DNA ladder).

Table 5. The distribution strains isolated from Syrian regions

	A	*B*	*C*	*D*	*E*
regions	*Lb.paracasei*	*Lb. plantarum*	*Lb. lactis*	*Lb.brevis*	*Lactobacillus spp.*
Southern Region	8	3	4	2	-
Central Region	6	4	2	1	-
Eastern Region	6	1	4	2	1
Coastal Region	8	7	3	1	-
Northern region	7	5	5	1	1
Total	35	20	18	7	2

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	9.116[a]	16	.909
Likelihood Ratio	10.231	16	.854
Linear-by-Linear Association	.101	1	.751
N of Valid Cases	82		

The table shows that the value of Chi - Square is 9.116 and that the degree of df is due to the fact that the number of species 5 and the number of regions 5: (5-1) (5-1) = 16 and the significance level at 0.05 is 0.909, This confirms the independence of the source of sampling from the two regions on the recurrence of the studied species.

Discussion

A understanding of the role which natural Lactobacillus have in a deeper study of the microbial involved in their production and ripening the traditional dairy products. In this study, it was suggested that the phenotypic identification of the isolates would not be positively identified solely by means of microscopic observations of cellular morphology, but must be associated with other methods such as API systems, in particular. However; phenotypic characterization based on sugar fermentation

pattern may not always provide sufficient basis for the reliable identification of *Lactobacillus*, as reported by other researchers (Nigatu, 2000; De Angelis *et al*., 2001; Muyana *et al.*, 2003; Bezeková *et al.*, 2013). Being unable to use PCR for all isolates is related to the absence of their genes sequencing.

In pastoral societies, milk is traditionally consumed in the form of *Shanklish* or *Surke*, it is a type of cow's milk cheese made in Syria, and is the most produced in "cottage industry " conditions using traditional techniques with little emphasis on hygiene practices.

All isolates (82 *Lactobacillus* strains) were rod shaped cells, Gram-positive, catalase-negative, non-motile and facultative anaerobic bacteria. When grown on MRS agar, colonies are small, cream-clouded. Isolates were classified as belonging to the genus Lactobacillus. All isolates were able to grow at 4% NaCl. No gas was produced in MRS broth (Barakat *et al.,* 2011). The *Lactobacillus* was ranging in *Shanklish* most probably because of their higher ability to grow under the low pH conditions and NaCl content (Kasimoglu *et al.,* 2004), and maybe because of the climatic condition of the production regions (Cueto *et al.,* 2007), and perhaps to high acidity. And there are important in the maturation as they are able to ferment citrate and could be involved in proteolysis as well as in other enzymatic processes that occurred during ripening (Crow *et al.,* 2001). Table 4 showed Fermentation of some carbohydrates by Lactobacillus isolated from *Shanklish* by using API system. The analysis of data compared with those of the criteria given by several authors, resulted in five subgroups (A-E).

Subgroup A was the largest one, with 35 strains (42.68%) identified as *Lb. paracasei*. The strains were able to ferment Sorbose, Mannitol, Sorbitol, Arbutin, Esculin, Trehalose, Inulin, Turanose and Tagatose and only *Lb. paracasei* could not ferment Lactose. Subgroup B with 20 strains (24.39%) identified as *Lb. plantarum*. The strains were able to ferment Ribose, Galatosidase, Glucose, Fructose, Manose, Mannitol, Sorbitol, Arbutin, Esculin, Lactose, Trehalose. Subgroup C with 18 strains (21.95%) identified as *Lb. lactis*. The strains were able to ferment Ribose, Lyxose, Galatosidase, Glucose, Fructose, Lactose and Rafinose. Subgroup D with 7 strains (8.54%) identified as *Lb. lactis*. The strains were able to ferment Ribose, Lyxose, galatosidase, glucose and Fructose. Subgroup E with 2 strains (2.44%) identified as Lb. lactis. The strains

were able to ferment Galatosidase, Glucose, Fructose, Manose, Arbutin, Esculin, Lactose, Trehalose; Rafinose, and Tagatose. The same results from fermentation were found by (Marroki *et al.*, 2011); (Pelinescu *et al.*, 2009); (Ortu *et al.*, 2007).

Conclusion

Controlled fermentation using *Lactobacillus* starter culture is a very important strategy for Shanklish processing. Isolates were also identified to the level of species by using fermentation profiles of selected *Lactobacillus* strains after anaerobic growth in MRS broth, Despite differences in sugar fermentation profiles of the strains came with different groups, the initial API identification of strain as Lactobacillus by 16 rRNA gene sequencing, Genetic taxonomy based on phylogenetic data, such as 16S rRNA, was shown the relation ship of the currently recognized and reorganized *Lactobacillus* species.

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