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Kinetic Studies and Sensorial Analysis of Lactic Acid Bacteria Isolated from White Cheese Made from Sheep Raw Milk

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Abstract: Eighteen presumptive isolates of lactic acid bacteria, from local white cheese made from sheep raw milk were isolated and identified. The results of the standard physiological and biochemical tests identified three isolates of *Lactobacillus plantarum*, two isolates of *Lactobacillus buchneri*, three isolates of *Lactobacillus brevis*, six isolates of *Lactobacillus fermentum*, two isolates of *Lactobacillus casei* and two isolates of *Lactococcus lactis.* Results of this study indicated that the presence of heterofermentative *Lactobacillus* in raw milk with variable levels of contamination and frequency. The results classified the isolated strains into three groups according to their rate of coagulation. Glucose, galactose and yeast extract were added to skim milk because it was found that milk has poor acidification process.

Key words: *Lactobacillus*, *Lactococcus*, acidification kinetics, sensorial analysis, white cheese

Introduction

Fermented dairy products are important integral part of the diet consumed in Jordan. The major cheese type produced in Jordan is white cheese (Jeben Balady). It is consumed either fresh or after pickling. In the southern region of Jordan, Karak, white cheese is mainly produced from sheep and goat milk. White brine cheese is soft to semi-hard in texture and has acidic taste. Chemical composition, microbiological qualities and sensorial properties change during ripening in brine (Seçkin and Tosun, 2004). The microorganisms involved in cheese making and cheese ripening can be divided into two principle groups: one is added to the cheese milk as a starter culture after being carefully selected by the starter manufacturer or the cheese making company, and the second group is the nonstarter lactic acid bacteria (Peterson and Marshall, 1990; El Soda *et al*. 2000; Beresford *et al*., 2001; Somers *et al*., 2001).

Lactobacilli are indispensable agents for the fermentation of food and feed, and they exert probiotic effects on human and animal health (Lindgren and Dobrogosz, 1990; Pereira and Gibson, 2002; Ogunbanwo *et al.* 2003; Pereira *et al*., 2003). The lactobacilli usually grow as a heterogeneous population in the cheese matrix, with no single predominating species (Swearingen *et al.*, 2001). Marked quality differences between cheeses made from raw milk and cheeses made from pasteurized milk are thought to be due to the presence of indigenous microflora in the former (McSweeney *et al.*, 1993). To effectively determine the contribution of particular adjunct strains in cheese flavor development, the non starter lactic acid bacteria (NSLAB) must be correctly identified (Peterson *et al.,* 1990). The nonstarter lactic acid bacteria are of interest to researchers and cheese makers because their increase in number as cheese ripens corresponds to the development of positive and negative flavor and texture attributes (Fryer, 1969; Law *et al.,* 1976; Blankenhiem, 1986; Broome *et al.,* 1990; Peterson *et al.,* 1990; Johnson *et al.,* 1990; Broome *et al.*, 1991; Fox *et al.,* 1998; Crow *et al.,* 2001). Generally, *Lactobacillus* species are known to have limited protease activity in the cheese matrix. However, important differences exist between species of *Lactobacillus* in terms of types and quantities of peptidase activities (Peterson *et al.,* 1990; El-Abboudi *et al.,* 1992). Carbohydrate utilization by nonstarter lactic acid bacteria is also an important phenotypic characteristic thought to influence Cheddar quality.

Until quite recently no study has proved the presence of heterofermentative *Lactobacillus* in local white cheese and their contribution to the formation of cheese flavor by production of peptides and amino acids. The objectives of this study were firstly, to reveal the presence of nonstarter lactic acid bacteria (NSLAB) in the Jordanian white cheese (Jeben Balady), secondly, to determine the acidification kinetic behaviors of the presumptive isolated strains on modified sterilized skim milk, thirdly, to assess the technological performance of these isolated strains and finally, the study aimed to evaluate the organoleptic aspects of the fermented milk.

Materials and Methods

Cheese samples: The cheese used in this study was of the traditional Jordanian white cheese type made from sheep raw milk. Cheese samples were collected from local retailers in the region of Karak in southern Jordan. Cheese portions (20 g each), were aseptically sampled, homogenized in 180 ml of sterile isotonic solution ringer (dissolve 2.5 g of ringer powder in 1L of distilled water

and sterilize in the autoclave at 121°C for 15 minutes) using a Stomacher model 400 Seward (London, UK).

Microorganisms: Lactobacilli were isolated using MRS broth (Scharlau Microbiology, Barcelona, Spain). Elliker broth was used for isolation of *Lactococcus* flora. The isolated strains were then kept for further studies. The identification of the isolates was performed according to the criteria of Bergey's manual of determinative bacteriology (Holt *et al.,* 1994) and using the methods and criteria of Sharpe (Sharpe, 1979).

Sugar fermentation profiles of isolates: The ability of the above eighteen isolated strains to produce acid from different carbohydrates was determined by API 50 CHL test kits (bio Mérieux SA). The API test strips were prepared as recommended by the kit supplier and scored after incubation for 24 and 48 hours at 37 or 30°C. The results were communicated to the APIWEB, which used the phenotypic data to predict a species identity for each isolate.

Culture conditions and media: A stock cell suspension of each strain was kept frozen at -30°C. Cells of the stock suspension were inoculated in 10 ml MRS medium. The pre-cultures were incubated overnight at 37°C for *Lactobacillus fermentum* and *Lactobacillus buchneri* and also kept at 30°C for *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactococcus lactis*. The pre-cultures were used to inoculate 90ml of culture media of UHT skim milk modified or not with glucose, galactose and yeast extract. The experiments were carried out in Erlenmeyer flasks of 1L working volume. The flasks were incubated in static condition in regulated water bath temperature. The sterilization of sugars solutions were performed by membrane filtration (0.22 µm), whereas the solution of yeast extract was sterilized at 120°C for 15min. The final concentration of sugars and yeast extract in the culture media of UHT skim milk were 10 gl^{-1} and 5 gl^{-1} respectively. The bacterial suspensions used to inoculate the culture media contained 20 ml l^{-1} of an active microbial subculture. The cultures were conducted in duplicate.

Physicochemical analysis: The technological aptitude of the studied isolated strains was evaluated according to the terms of the acidification rate and coagulation of skim milk under our experimental conditions. A multichannel pH-meter/redox-meter Consort was used to follow the pH value of the milk. The pH reading was registered automatically. The determination of the acidity in degree Dornic (°D) was performed by titration with 9/N NaOH.

Determination of final biomass concentration: Decimal dilutions of culture media were plated on MRS agar or pH 5.7 under anaerobic condition at 37 or 30°C for 72 hours. A solid medium of Elliker broth was used for the determination of *Lactococcus* flora concentration by adding 15 g/l Agar Bacteriological. Samples were analyzed in duplicate.

Proteolytic activity determination: The total nitrogen (TN) and the soluble nitrogen (SN) were determined at the end of the culture using the method of Lowry *et al.* (1951).

Organoleptic analysis: After the inoculation (2%), the milk was divided aseptically in polypropylene packaging materials of a volume of 100 ml each unit, then the pots incubated at 30°C for 24 h, and the pots cooled to 4°C with a view to organoleptic analysis.
The fermented milk in pots of 100 ml was evaluated by a trained test panel composed of 8 persons. The fermented milk was scored qualitatively for attributes grouped into visual aspects, texture, taste, odor and aroma categories.

Results and Discussion

Lactic acid bacteria microflora: Eighteen isolates of Lactic Acid Bacteria (LAB) were isolated from traditional Jordanian cheese made from sheep raw milk. After series of purification on MRS agar, sixteen isolates were found to be Gram-positive, catalase-negative, non-motile bacilli. The series of purification on Elliker medium, two are not motile, Gram-positive cocci isolates were found. The results of the isolation and identification of the standard physiological and biochemical tests were identified the isolates as three isolates of *Lactobacillus plantarum*, two isolates of *Lactobacillus buchnrei*, three isolates of *Lactobacillus brevis*, six isolates of *Lactobacillus fermentum*, two isolates of *Lactobacillus casei* and two isolates of *Lactococcus lactis*. Table 1 presents the results of the best two final identifications for each type of isolates on API gallery.

Assessment of technological performance of the lactic isolated strains: The assessment of the technological performance of the isolated strains was based upon the kinetic acidification. The time of skim milk taken to be coagulated was used to classify the isolated strains to be fast, intermediary and slow acidifying agents (Table 2). The results showed that the two isolates of *Lc. Lactis*, *Lb. plantarum* 2 and *Lb. casei* 2 are the fastest isolated strains. The two isolates of *Lc. Lactis* coagulate the milk in less than ten hours and for *Lb. plantarum* 2 and *Lb. casei* 2 coagulated the milk in about 24 hours. However, the two isolates of *Lb. fermentum* 1 and 2, *Lb. plantarum* 1 and *Lb. casei* 1 were classified as intermediary acidification agents, and they coagulated the milk in less than 48 hours. The slowest acidification agents were the isolates of *Lb.*

Table 1: Results of the biochemical tests for the identification of the isolated strains by using API gallery

Isolated strains	Identification	% ID	T- index	Type*
Lb. plantarum 2	Very good identification	99.8	0.9	S
Lb. buchneri 1	Very good identification	99.6	0.89	S
Lb. buchneri 2	Very good identification	99.6	0.89	S
Lb. fermentum 4	Good identification	96.9	0.91	S
Lb. brevis 3	Good identification	90.2	0.86	S
Lb. plantarum 3	Very good identification	99.2	0.86	S
Lb. plantarum 1	Excellent identification	99.9	0.94	S
Lb. brevis 1	Very good identification	99.1	0.56	S
Lb. fermentum 1	Excellent identification	99.9	1	S
Lb. fermentum 6	Good identification	93.7	0.66	S
Lb. casei 1	Very good identification	99.9	0.88	S
Lc. lactis 2	Excellent identification	99.8	0.9	S
Lc. lactis 1	Excellent identification	99.9	1	S
Lb. casei 2	Very good identification	99.6	0.89	S
Lb. fermentum 3	Excellent identification	99.9	0.93	S
Lb. fermentum 2	Excellent identification	99.9	1	S
Lb. fermentum 5	Excellent identification	99.9	1	S
Lb. brevis 2	Good identification	95.4	0.9	S

(*) S = Significant.

Table 2: Classification of the best two isolated strains according to their rate of acidification. The pH of the culture media is less than 5.2

	Time needed for milk coagulation (day)					
Isolated strains	1	2	3	4	5	6
Lc. Lactis 1	**T**					
Lc. Lactis 2	**T**					
Lb. casei 1	**T**					
Lb. casei 2		**T**				
Lb. plantarum 1	**T**					
Lb. plantarum 2		**T**				
Lb. fermentum 1		**T**				
Lb. fermentum 2		**T**				
Lb. brevis 1					**T**	
Lb. brevis 2			**T**			
Lb. buchneri 1			**T**			
Lb. buchneri 2			**T**			

buchneri 1 and 2 and *Lb. brevis* 1 and 2 and they coagulated the milk after three to five days of incubation. The results in Table 3 demonstrate that the classification of the isolated strains into three acidified group is valid. However, there was high degree of heterogeneity in the time required to coagulation due to lactic acid as well as between the isolates within same strain.

Kinetics of the two isolates *Lc. Lactis* 1 and 2: Data in Table 4 showed that there is different kinetic behavior between the two isolates *Lc. Lactis* 1 and 2. The isolated, *Lc. Lactis* 1 was the fastest to coagulate milk, coagulation was obtained in six hours of incubation at 30°C and the acidity was 45 to 48°D when the maximal biomass concentration was 9.6 log. There was stability in the pH value (4.24) between 24 and 48 hours of incubation. After nine hours of incubation the bacterial population started decreasing to reach a biomass concentration of 1.25 log by the end 48 hours incubation. The isolate *Lc. Lactis* 2 was slower than the precedent isolate in terms of acidification. The milk coagulation was observed only after 12 h of incubation and the biomass concentration was 8.5 log. The final pH value (4.3) was identical to that obtained with the isolate *Lc. Lactis* 1 after 48 hours of incubation. The acidification kinetic (Dornic acidity) of the isolate *Lc. Lactis* 1 confirm the weak production of lactic acid, 34°D (10 h), 57°D (24 hours) and 70°D (48 hours), respectively against 80°D (10 hours), 89°D (24 hours) and 78°D (48 hours). The results show that at the end of incubation (48 hours) *Lc. Lactis* 2 had more biomass concentration than *Lc. Lactis* 1. This is because of its low acidification characteristic.

Kinetics of the Lactobacillus isolates on modified skim milk: Cultures on modified skim milk were used to determine the limiting factors for the lactic acid bacterial growth and their acidification power (Table 5). The results showed that the addition of yeast extract had a significant activation effect on the growth and activity of the strains belonging to *Lactobacillus* genus. The results indicated that all the isolates strains had an important nutritional request for easily assimilated nitrogen compounds. The weak proteolytic activities of the genus, *Lactobacillus*, do not permit protein hydrolysis, casein in particular, in order to obtain low molecular weight compounds.

Results of this study demonstrated that the effect of the addition of glucose is observed from 10 hour of incubation; weak effects with the two isolates of *Lb. buchneri* 1 and 2 and the isolate of *Lb. plantarum* 1

Table 3: Evolution of the pH for different inoculation rates of the milk with the isolated strains

Isolated strains	Inoculation rate (%)	Incubation time (h)						
		0	3	6	10	24	48	72
Lb. fermentum 1	2	6.62	6.62	6.66	6.58	6.58	6.56	NS
Lb. fermentum 1	10	6.58	6.49	6.27	6.19	5.57	5.04	NS
Lb. fermentum 2	2	6.58	6.54	6.46	6.41	6.33	5.71	NS
Lb. fermentum 2	10	6.51	6.40	6.30	6.28	5.72	4.87	NS
Lb. buchneri 1	2	6.61	6.58	6.57	6.52	6.47	5.92	NS
Lb. buchneri 1	10	6.53	6.53	6.50	6.50	6.42	5.40	NS
Lb. buchneri 2	2	6.58	6.50	6.56	6.55	6.52	6.15	5.5
Lb. buchneri 2	10	6.52	6.51	6.49	6.50	6.45	5.46	NS
Lb. brevis 1	2	6.57	6.57	6.57	6.54	6.48	6.17	5.34
Lb. brevis 1	10	6.52	6.52	6.50	6.50	6.47	5.34	NS
Lb. brevis 2	2	6.57	6.56	6.57	6.50	6.44	4.89	4.36
Lb. brevis 2	10	6.54	6.52	6.50	6.48	6.38	4.78	NS
Lb. plantarum 1	2	6.55	6.47	6.43	6.38	6.32	4.79	NS
Lb. plantarum 1	10	6.41	6.35	6.35	6.33	6.18	4.52	NS
Lb. plantarum 2	2	6.59	6.46	6.28	6.12	5.33	4.56	NS
Lb. plantarum 2	10	6.43	6.35	6.19	6.04	5.01	4.24	NS
Lb. casei 1	2	6.55	6.46	6.41	6.38	6.35	6.24	NS
Lb. casei 1	10	6.41	6.39	6.38	6.34	6.25	5.38	NS
Lb. casei 2	2	6.59	6.59	6.26	6.12	5.40	4.70	NS
Lb. casei 2	10	6.41	6.33	5.91	5.83	4.86	4.17	NS

(NS) no significant differences in the pH value between 48 and 72 hours of incubation.

Table 4: Acidification kinetics of the two isolates *Lc. lactis* 1 and 2 on non modified skim milk

Incubation time (h)	*Lc. lactis 1*			*Lc. lactis* 2		
	Cell No. CFU x $10^6 ml^{-1}$	pH	Acidity (ED)	Cell No. CFU x $10^6 ml^{-1}$	pH	Acidity (ED)
0	43	6.51	19.1	33	6.6	19.0
3	367	6.27	19.1	67	6.44	20.6
6	2931	5.20	43.5	361	5.89	25.1
10	3131	4.5	60.2	419	5.51	33.5
24	1302	4.23	69.2	1641	4.47	57.1
48	156	4.23	77.7	1249	4.31	69.8

CFU = Colony Forming Unit.

were observed. Whereas the effects of glucose on the two isolates of *Lb. casei* were more significant. The two isolates of *Lb. casei* and the isolate of *Lb. plantarum* 2 acidified the milk faster in the presence of galactose than in the control cultures. The effect of sugars on the kinetic acidification remained, however, very weak in comparison to the effect obtained by the addition of yeast extract. Glucose utilization by the isolates exceeded that of galactose. The current results suggest, that $-galactosidase is functional only when Glucose is lacking.

Sensory analysis of fermented milk with lactic acid bacteria

Curd texture: The results of sensory analysis were summarized in Table 6. Two textural groups can be established: the first one, which round up curds obtained with the two isolates strains of *Lc. lactis* 1 and 2 and the two isolates strains of *Lb. plantarum* 1 and 2 too. The curds of this group are smooth, homogeneous, firm, compact and does not present or present very low level of syneresis. These two species have interesting textural technological aptitudes, even if *Lb. plantarum* cause a weak syneresis.

In the second group, the curds were inoculated with isolated strains of *Lb. fermentum*, *Lb. casei*, *Lb. buchneri* or *Lb. brevis*. These curds are characterized by an excessive friability, a frothy appearance and a variable syneresis. The gas (invariable) presence is noted with the curds inoculated with *Lb. fermentum* 1 and *Lb. buchneri* 2. These curds seem to be not very interesting from the technological point of view and a priori would be not retained basing on their textural aspect alone.

Table 5: Effect of the addition of glucose, galactose and yeast extract on the acidification kinetic of skim milk by the isolated strains of lactic acid bacteria. (0) =) pH = 0.1 unit, (+) = 0.1 <) pH = 0.5, (++) = 0.5 <) pH = 1.0, (+++) = 1.0 <) pH = 1.5, (++++) =) pH > 1.5

Isolate	Glucose			Galactose			Yeast Extract		
	10h	24h	48h	10h	24h	48h	10h	24h	48h
Lb. plantarum 1	+	+	+	+	+	+	+	++	+++
Lb. plantarum 2	0	0	0	+	+	0	+++	+++	++
Lb. casei 1	+	++	++	+	+	++	+	++	++++
Lb. casei 2	+	+	++	+	+	++	++++	+++	++
Lb. fermentum 1	0	0	0	0	0	0	0	+	++++
Lb. fermentum 2	0	0	0	0	0	0	0	0	++++
Lb. buchneri 1	+	+	+	0	0	+	+	++++	+++
Lb. buchneri 2	0	0	+	0	0	+	0	+	+
Lb. brevis 1	0	0	0	0	0	0	0	0	++
Lb. brevis 2	0	0	0	0	0	0	0	0	++++

Table 6: Classification of the isolated strains according to the sensorial evaluation of the fermented milk

best note	Texture	Flavor (Odor + taste)
↑	*Lc. lactis* 1 and 2: The curds are smooth, homogeneous, firm, compact and without syneresis	*Lc. lactis* 1: The taste and odor of the curd are pleasant. *Lb. casei* 2: the odor is pleasant and the taste is soft and not very acid.
	Lb. plantarum 1 and 2: The curds are smooth, homogeneous and little syneresis is present.	*Lb. buchneri* 1: The curd is pleasant to the taste, without defect, but not very typed (soft, flat). *Lb. plantarum* 2: The curd is acceptable from taste standpoint, not very acid and rather soft. The odor is pleasant.
	Lb. buchneri 1 and 2, *Lb. brevis* 1 and 2: The curds are soft, smooth and homogeneous. The presence of a strong syneresis is noted with *Lb. brevis* 2.	*Lb. casei 1:* The taste is flat, tasteless therefore without character, but does not present nevertheless any defect. *Lb. buchneri 2:* The odor is strong enough, typically taste, bitterness defect of the curd is present.
Intermediary note		*Lb. plantarum1:* The curd has bitterness and spicy taste. Odor is acceptable.
↑	*Lb. casei* 1 and 2: The curd is flaky with *Lb. casei* 1 and friable with *Lb. casei 2*. A syneresis is observed with the two isolates.	*Lc. lactis* 2: The curd presents marked defects with a strong bitterness and a pricking taste. The odor remains pleasant. *Lb. fermentum* 1: The curd is marked by a not very pleasant taste. Nevertheless, it is little acid and rather flat. *Lb. fermentum* 2: The curd is very unpleasant with a stable odor, and non-acid and flat taste. *Lb. brevis* 2: The odor and the taste are not very pleasant, recall the cowshed, and moreover it has a strong acidity.
	Lb. fermentum 1 and 2: The curd is fragmented, wooly, with an important syneresis. A gas production is revealed with the isolate *Lb. fermentum* 1.	*Lb. brevis* 2: The curd is very bitter, proteolysis and qualified as inedible curd.
Worst note		

Organoleptic quality: This study propose three microbial groups: The first group contains the two isolated strains of *Lc. lactic* 1 and 2, the two isolated strains of *Lb. plantarum* 1 and 2 and the isolated strain of *Lb. casei* 2. These isolates confer to the curd certain pleasant judged odor. The second group made up of *Lb. buchneri* 1, *Lb. brevis* 1 and *Lb. casei* 1. The formed curds had little marked and characteristic odor. The third group composed of the two isolated strains of *Lb. fermentum* 1 and 2, *Lb. buchneri* 2 and *Lb. brevis* 2. The curd had an odor rather strong and little pleasant.

Conclusion: Results of this investigation showed that the presence of heterofermentative *Lactobacillus* in raw milk with variable levels of contamination and frequency. These types of lactic acid bacteria (LAB) are not proposed as starter culture due to their metabolic characteristics and probably to their variable negative impact on dairy products. Their presence in cheese made from raw milk could modify the textural and organoleptic characteristics of cheese. The kinetics acidification of the isolated strains of the group heterofermentatives (*Lactobacillus* belong to the group II and III of Kandler and weiss, 1986) demonstrated that the isolates strains are dependent strains. The results classified the isolated strains into three groups: one which can coagulate milk in 24 hours, and this group contains the isolates of *Lc. lactis* 1 and 2, *Lb. plantarum* 2 and *Lb. casei* 2. The second group which can coagulate milk in 48 to 72 hours includes the two isolates of *Lb. fermentum* 1 and 2 and the isolate *Lb. casei* 1. The third group is composed of the two isolates of the strains of *Lb. buchneri* 1 and 2 and *Lb. brevis* 1 and 2. This group was found to coagulate milk following 4 to 6 days incubation. It was found that the isolates with lower time of coagulation gave the curd with the best sensorial analysis appreciations. In addition, the two isolates *Lb. fermentum* 1 and 2 as well as the isolates of *Lb. brevis* 1 and 2 and *Lb. buchneri* 2 promoted off-flavor development and textural defects in the curd. The other isolates gave only weak acidity to the curd and it appears as insipid. The apparent bitter defects in the curd could be attributed to the weak proteolytic activities of the isolates. In conclusion, the isolated strains which presenting technological aptitudes potentially interesting were *Lc. lactis* 1, *Lb. plantarum* 2, *Lb. casei* 2 and eventually *Lb. buchneri* 1. The isolate of *Lc. lactis* gave the best curd and for this reason this isolate was withheld to be the control isolate during the organoleptic assessment. Different results were obtained within a same species (bitterness, pricking); manufacture accidents are had to *Lc. lactis* 2, *Lb. plantarum* 1, the two isolates of *Lb. fermentum* 1 and 2 and *Lb. brevis* 1 and 2. These isolated strains gave curd with friable texture, flaky aspect and tastes defects.

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