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Characteristics of Salt-Tolerant Lactic Acid Bacteria, in Particular Lactobacilli, Leuconostocs and Pediococci, Isolated from Salted Raw Milk

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ABSTRACT

One hundred and fourty five lactobacilli, leuconostocs and pediococci were isolated from salted raw milks incubated at 30°C for 4 to 21 d. Of 126 lactobacilli isolated, mostly from 9 to 12% salted milk, 115 were identified as homofermentative, nonthermophilic lactobacilli-73 were classified as *Lactobacillus plantarum*, 31 *Lactobacillus casei*, 8 strains were motile and 3 *Lactobacillus xylosus*. The remaining 11 isolates were heterofermentative lactobacilli-8 were *Lactobacillus cellobiosus* and 3 *Lactobacillus brevis-buchneri*. Strains of *L. plantarum* fermented many oligosaccharides, produced DL lactate and gas from L(+) but not from D(−) tartrate and their cell wall peptidoglycan was of the mesodiaminopimelic acid type. Eight strains of *L. casei* proved to be subsp. *pseudoplantarum* on the basis of inactive lactic acid production; 8 were subsp. *rhamnosus* and 2 subsp. *alactosus*, according to their pattern of sugar fermentation. *L. xylosus* simulated *L. casei* morphologically but differed from it in fermentation of xylose. The motile strains fermented arabinose and mostly sucrose but not lactose and produced 73.2 to 94 μmoles ml^{-1} inactive lactate from 1% glucose. None of 10 *Leuconostoc* isolates produced dextran from sucrose but they fermented trehalose and were identified with *Leuconostoc paramesenteroides*. Three strains belonged to *Pediococcus* and produced 62 μmoles ml^{-1} of inactive lactate, whereas other six strains were atypical pediococci. Nineteen strains representing *L. plantarum*, *L. casei*, motile strains and *Pediococcus* gave, on examination for isomers of lactic acid, 32.8 to 171 μmoles ml^{-1} inactive lactate; the L(+) enanthiomorph generally predominated.

Lactic acid bacteria represent the most important constituent of the normal microflora of many dairy products. In some of these products, lactobacilli are the predominant part of the flora. That is true not only of the production or ripening stage but also of the consumption stage of these foodstuffs. Lactobacilli are often accompanied by other closely related organisms, e.g. pediococci, leuconostocs and enterococci.

Salt-tolerant lactic acid bacteria are of paramount significance in the ripening and flavor development of white pickled cheese of the Domiati type. Addition of table salt to raw cheese milk before renneting is commonly practiced in Egypt. Up to 15% salted raw milk is occasionally used for the manufacture of this type of cheese (*27*), which is ripened under its own salted whey. The salt inhibits coliform bacteria and imparts to the resultant cheese the desirable salty flavor. In the presence of salt, selective multiplication of salt-tolerant microorganisms occurs, while other salt sensitive organisms are repressed. *Streptococcus lactis* and its subsp. *diacetylactis* are inhibited by 4.0-6.5% NaCl (*26*). The bacterial flora of 8% salted milk consists mainly of staphylococci-micrococci and occasionally also of enterococci, whilst in 10% slated milk staphylococci-micrococci predominate (*17*). Staphylococci-micrococi are enriched in salted raw milk because of their salt-tolerance properties, but they are overgrown by lactobacilli after 4 d (*17*). This work dealt with the characterization and properties of lactobacilli, leuconostocs and pediococci which prevail in salted raw milk after 4 to 21 d of incubation at 30°C. These organisms have many characteristics in common and their growth may cause similar changes in cheese so that their collective detection and study is useful.

MATERIALS AND METHODS

Isolation and characterization of salt-tolerant lactobacilli, leuconostocs and pediococci from salted raw milk

Three to twelve percent salted raw milk samples, incubated at 30°C for 4 to 21 d, were plated on acetate agar (*21*). After incubating the plates at 30°C for 5 d, 10 to 20 colonies were isolated from a countable plate, purified and characterized. Lactobacilli were classified according to the procedures of Abo-Elnaga and Kandler (*1,2*), and of Rogosa (*23,24*). Characteristics investigated were morphology (in the living state), gas production from glucose by the method of Hayward (*16*), fermentation of sugar as reported by Rogosa and Sharpe (*22*), production of gas from L(+) and D(−) tartrate by the method of Gemmell and Hodgkiss (*12*), presence of meso-diaminopimelic acid in the cell wall by the method of Ruhland et al. (*25*) and isomers of lactic acid produced.

The L(+) isomer of lactic acid and total lactate were estimated by optical methods in a broth containing 2% peptone, 1% yeast extract and 1% glucose (PYG) supplemented per liter with 2.5 ml of salt solution containing 11.5% $MgSO_4 \cdot 7H_2O$, 2.8% $MnSO_4 \cdot 4H_2O$ and 0.68% $FeSO_4 \cdot 7H_2O$, distributed in 10-ml quantities in 16 × 160 mm screw-capped test tubes and incubated at 30°C for 1 to 5 d. L(+) lactate was determined enzymatically by the method of Cato and Moore (*5*) at 366 nm, using a Bausch & Lomb spectronic 21 spectrophotometer. The reaction mixture contained

(in 3.0 ml): 1.3 μmoles of glycine and 1.04 μmoles of hydrazine sulfate (adjusted to pH 9.0 with NaOH, 2.5 mol/L); 0.1 ml of the sample deproteinized with 3.5% $HClO_4$; 0.1 ml of lactate dehydrogenase LDH (10 mg of protein ml^{-1}) from bovine heart, (Fa. Fluka AG, Buchs, Switzerland) and 6.3 μmoles of NAD, (Fa. Fluka AG). The total lactate was estimated by the method of Steffen (*28*) at 407 nm.

Leuconostocs were characterized according to the descriptions reported by Garvie (*9,10,11*). Characteristics investigated were morphology (in the living state), gas production from glucose, fermentation of lactose, sucrose and trehalose, and dextran formation.

Pediococci were distinguished from micrococci on the basis of density of surface growth, nitrate reduction, gelatin liquefaction, fermentation of glucose, isomers of lactic acid produced, and catalase production in glucose medium (1% peptone, 1% meat extract, 1% glucose, 1.5% agar), as reported by Breed et al. (*3*) and Kitahara (*18*). On a medium containing 1% glucose, *Pediococcus* strains of weak catalase activity give negative results, whereas only those of high activity are catalase positive (*7*). The methods described by Harrigan and McCance (*13*), were used for carrying out the tests.

RESULTS AND DISCUSSION

It is recognized that acetate agar (*21*) supports growth of a range of lactic acid bacteria, particularly lactobacilli, pediococci and leuconostocs. Identifications were made of 145 isolates obtained with this medium. One hundred and twenty six of these were classified in the genus *Lactobacillus* and further subdivided into 115 isolates of homofermentative, nonthermophilic lactobacilli and 11 isolates of heterofermentative lactobacilli. The identification of the former is shown in Table 1, in comparison with standard description of the species (*24*). Whilst differences exist, they do not seem to justify the creation of new species.

Of the 115 homofermentative, nonthermophilic lactobacilli 73 were *Lactobacillus plantarum,* 31 *Lactobacillus casei,* 8 were motile strains and 3 *Lactobacillus xylosus*. Strains of *L. plantarum* fermented many oligosaccharides, produced DL lactate and gas from L(+) but not from D(−) tartrate and their cell wall peptidoglycan was of the mesodiaminopimelic acid type. Eight strains of *L. casei* proved to be subsp. *pseudoplantarum* on the basis of inactive lactic acid production; 8 were subsp. *rhamnosus* and 2 subsp. *alactosus* according to the pattern of sugar fermentation. *L. xylosus* morphologically simulated *L. casei,* but differed from it in fermentation of xylose. The motile strains differed from *L. casei* and *L. plantarum* in morphology and in their negative or variable reactions on several di- and trisaccharides, pentoses and polyhydric alcohols. They also differed from the motile *L. plantarum* of Harrison and Hansen (*14*), isolated from the cecal feces of turkey, in fermentation of arabinose, in not fermenting lactose, melibiose (only one +) and raffinose and in configuration of lactic acid produced (Table 5). However, the strains closely resembled *L. plantarum* var. *mobilis,* isolated from frozen concentrated orange juice (*15*), in morphology, fermentation of arabinose and sucrose but not lactose. These organisms could have not been identified with *L. plantarum* var. *mobilis* because of their great variation from *L. plantarum* in morphology, in cell wall peptidoglycan, in failure to produce gas from L(+) tartrate (*19*; also this work) and in inability to ferment melibiose and raffinose.

Eight of the isolates of heterofermentative lactobacilli simulated *Lactobacillus cellobiosus* in fermentation of cellobiose; 3 were classified *L. brevis-buchneri* (Table 2). Rogosa and Sharpe (*22*) separated these two species on the basis of fermentation of melezitose. *L. buchneri* ferments melezitose, whereas *L. brevis* does not.

Ten heterofermentative cocci were isolated (Table 3). The strains differed from *Leuconostoc lactis* in their ability to ferment trehalose and from *Leuconostoc dextranicum* and *Leuconostoc mesenteroides* in inability to produce dextran on sucrose agar. They were therefore classified as *Leuconostoc paramesenteroides* (*10,11*).

Nine cultures were cocci, occurring singly, in pairs and tetrads; non-motile and gram-positive. Their growth was profuse with uniform turbidity in MRS broth, and white along the stab in stab cultures. Three of them (Table 4) were typical *Pediococcus,* showing very faint growth on an agar slant and producing optically inactive lactate from glucose (Table 5). The catalase test was negative in a glucose medium, according to *Bergey's Manual* (*3*). Nitrate was not reduced and gelatin was not liquefied. Placing of these strains in any of the named species in *Bergey's Manual* (*4*) was not, however, an easy task. Six other strains showed, in contrast, abundant surface growth on an agar slant, produced nitrites from nitrates, liquefied gelatin and were catalase-positive; ribose, sucrose and lactose were fermented in MRS fermentation medium recommended by De Man et al. (*6*). No acid was produced from arabinose, xylose, rhamnose, sorbitol, mannitol, raffinose and melibiose, except one strain brought about acid production from sorbitol, mannitol and raffinose. None of the pediococci isolated by Franklin and Sharpe (*8*) from milk and cheese reduced nitrate. All of the typical and atypical pediococci strains examined here were highly salt-tolerant and were isolated from 12% salted raw milk. Reuter (*20*) found the pediococci isolated from meat products but not leuconostocs capable of growing in the presence of 10% NaCl.

Table 5 shows the various amounts of total lactate produced from 1% glucose in PYG broth by different species. Atypical pediococci produced the least amount, 32.8 μmoles ml^{-1}, presumably maximum production occurs during the logarithmic phase. *L. plantarum* gave the largest amount (171 μmoles ml^{-1}). The percentage of L(+) lactate produced by the strains ranged from 20.7 to 52.7. According to Cato and Moore (*5*), this is characteristic of optically inactive lactate-producing species. Stetter and Kandler (*29*) also reported a percentage of L(+) lactate of 52% for *L. casei* subsp. *pseudoplantarum,* DSM No. 20008.

TABLE 1. *Characteristics of homofermentative* Lactobacillus *strains isolated from salted raw milk, compared with standard description of species* (24).

Test	*L. plantarum*		*L. casei*[1)]		*L casei* subsp. *alactosus*		*L. casei* subsp. *rhamnosus*		*L. xylosus*		
	Rogosa	This work	Rogosa	This work	Rogosa	This work	Rogosa	This work	Rogosa	This work	Motile strains
No. of strains examined	NS	73	NS	21	NS	2	NS	8	NS	3	8
Meso-diaminopimelic acid peptidoglycan	+	+(73)	-	-(0)	-	-(0–	-	-(0)	-	-(0)	-(0)
Fermentation of:	d*	+(01)	-	-(0)	-	-(0)	-	-(0)	-	-(0)	+(8)
Arabinose											
Xylose	d*	+(01)	-	-(0)	-	-(0)	-	-(0)	+	+(3)	-(0)
Ribose	+	+(73)	+	+(21)	+	+(2)	+	+(8)	+	+(3)	+(8)
Rhamnose	-	-(0)	-	-(0)	-	-(0)	+	+(8)	-	-(0)	-(0)
Sorbitol	+	+(59)	+	+(11)	+	+(1)	+	-(0)	-	-(0)	+(1)
Mannitol	+	+(55)	+	+(10)	+	-(0)	+	-(0)	+	+(3)	-(0)
Raffinose	+	+(67)	-	-(0)	-	-(0)	-	-(0)	-	-(0)	-(0)
Melibiose	+	+(73)	-	-(0)	-	-(0)	-	-(0)	-	-(0)	+(1)
Sucrose	+	+(71)	(d)	+(20)	(d)	+(2)	(d)	+(8)	+	+(3)	+(7)
Lactose	+	+(72)	+	+(21)	-	-(0)	+	+(8)	∓	+(3)	-(0)
Salt in raw milk (%)		9%		9-12%		3%		9%		9%	9%

+ = Positive reaction by 90% or more strains; d = some strains +, others-(about 89-11% positive); - = negative reaction by 90% or more; +negative or equivocal; ( ) = delayed reaction; * = some strains ferment arabinose and some ferment both arabinose and xylose; NS = not stated; Figures in brackets denote number of strains giving a positive result; [1]8 strains produced DL lactic acid and were considered subsp. *pseudoplantarum*.

TABLE 2. *Characteristics of heterofermentative* lactobacillus *strains isolated from salted raw milk, compared with standard description of species (24).*

Test	*L. cellobiosus*		*L. brevis-buchneri*	
	Rogosa	This work	Rogosa	This work
No. of strains examined	NS	8	NS	3
Fermentation of:	-	+(4)	-	-(0)
Sorbitol				
Mannitol	-	+(5)	(#)	-(0)
Raffinose	+	+(1)	#	-(0)
Melibiose	+	+(6)	+	+(1)
Sucrose	+	+(2)	d	-(0)
Lactose	#	+(8)	#	+(1)
Cellobiose	+	+(8)	-	-(0)
Salt in raw milk (%)		3-6%		3-6%

= weak, slow or negative; ( ) = delayed reaction d = some strains +, others- (about 89-11% positive); NS = not stated.

TABLE 3. *Characteristics of* Leuconostoc *strains isolated from salted raw milk, compared with standard description of species (11).*

Test	*L. paramesenteroides*	
	Garvie	This work
No. of strains examined	NS	10
Acid from:Lactose	(d)	+(10)
Sucrose	+	+(10)
Trehalose	+	+(10)
Dextran formation	-	-(0)
Salt in raw milk (%)		3-9%

+ = ≥90% strains positive; d = 10-90% strains positive; - = ≥90% strains negative; ( ) = delayed reaction; NS = not stated.

TABLE 4. *Characteristics of typical and atypical* Pediococcus *strains isolated from salted raw milk.*

Sample no.	Strain	Surface growth	Catalase production in sugar medium	Nitrate reduction	Gelatin liquefaction	Gas from citrate	μmoles ml^{-1} AD from 0.5% pyruvate	μmoles ml^{-1} D. from 0.5% pyruvate	Salt in raw milk (%)
I	I35	poor	-	-	-	-	0.39	0.39	6
	452	poor	-	-	-	-	1.78	0.25	12
II	II28	poor	-	-	-	-	1.38	0.07	9
	I52	abundant	+	-	+	-	0.63	0.20	12
	I53	abundant	+	-	+	-	0.82	0.30	12
III	351	abundant	+	+	+	-	0.26	0.26	12
	354	abundant	+	+	+	-	0.46	0.33	12
	355	abundant	+	+	+	-	0.68	0.11	12
	356	abundant	+	+	+	-	1.98	0.11	12

A = acetoin; D = diacetyl.

TABLE 5. *Amount (μmoles ml^{-1}) and configuration of lactic acid produced in PYG broth by homofermentative lactic acid bacteria isolated from salted raw milk.*

Species	Strain	Assayed after:	L(+) lactate	Total lactate	L(+) lactate %	Configuration
	2310	2 days	35.2	118.0	29.8	DL
	238	5 days	39.4	171.2	23.0	DL
	I 310	5 days	43.6	138.0	31.6	DL
L. plantarum						
	119	5 days	39.4	152.0	25.9	DL
	32	4 days	35.2	142.0	24.8	DL
	I 31	4 days	37.2	131.2	20.7	DL
	333C	5 days	52.0	136.0	38.2	DL
	354C	5 days	52.0	143.2	36.3	DL
	423C	5 days	52.0	158.0	32.9	DL
L. casei ssp.	II 15C	5 days	52.0	131.2	39.6	DL
pseudoplantarum	134C	2 days	50.0	120.0	41.7	DL
	451C	2 days	48.0	91.0	52.7	DL
	II 23C	4 days	48.0	134.0	35.8	DL
	I 315	1 day	21.6	64.8	33.3	DL
Motile	I 311	4 days	39.4	91.2	43.2	DL
	I 17	4 days	40.4	94.0	43.0	DL
Lactobacillus strains	227	4 days	38.4	73.2	52.5	DL
	452	1 day	29.2	62.0	47.1	DL
Pediococcus						
	355	4 days	12.4	32.8	37.8	DL

PYG = peptone-yeast extract-glucose broth.

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The total volatile nitrogen (TVN) content of the swordfish steaks stored in air and the various gas atmospheres is shown in Figure 3. TVN values for different degrees of freshness of fish have been suggested as follows: fresh fish, 12 mg; good quality, 12 to 20 mg; edible, 20 to 25 mg; decomposed and inedible >25 mg TVN-N/100 g tissue (*12*). An initial value of 11.6 mg TVN-N/100 g indicated that the fish was a very good quality. For steaks stored in air, the TVN concentration increased rapidly, obtaining a value of 25 mg TVN-N/100 g after 14 d at 2°C. In contrast, TVN values increased more slowly for samples stored in CO_2-enriched atmospheres. Maximum values for those samples after 22 d of storage did not exceed 25 mg TVN-N/100 g, which is recommended as a cut-off point for an edible product.

Trimethylamine (TMA), produced by the reduction of trimethylamine oxide by common spoilage microorganisms, has also been used as a quality indicator for fish and seafood products. Farber (*4*) stated that sole was spoiled when the TMA value exceeded 4.7 mg TMA-N per 100 g of fish, and Montgomery et al. (*8*) suggested an acceptable limit for shrimp at 5 mg TMA-N per 100 g of shrimp tissue. As shown in Figure 4, the TMA value increased much more rapidly in samples stored in air than in samples sotred under CO_2. However, none of the swordfish steaks, even those that had spoiled, exceeded recommended values for acceptability, indicating that the TMA value may not be a good indicator of quality for fresh swordfish.

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