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ORIGINAL ARTICLE

Identification of lactic acid bacteria in Moroccan raw milk and traditionally fermented skimmed milk 'lben'

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Keywords
lactic acid bacteria, Moroccan raw milk, phenylalanyl-tRNA synthase (*pheS*) gene sequencing, rep-PCR, SDS PAGE, traditional fermented skimmed milk.

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Abstract

Aims: To identify lactic acid bacteria (LAB) present in Moroccan dairy products to establish and preserve their microbial species diversity.
Methods and Results: Thirty-seven samples were collected from different farms. A total of 146 LAB were isolated and subjected to $(GTG)_5$-PCR analysis. Comparison of the profiles with data available at the Moroccan Coordinated Collections of Micro-organisms allowed identification of 85 isolates. The remaining 61 were subjected to SDS-PAGE analysis of whole cell proteins. Comparison of the profiles with data available at the Belgian Coordinated Collections of Micro-organisms allowed identification of 43 isolates. Several of the remaining 18 isolates exhibited identical protein electrophoretic fingerprints. Therefore, eight representatives of them were subjected to partial *pheS* gene sequencing which allowed identification of all remaining isolates. In raw milk, six genera were found while in 'lben', three were found. This is the first report of *Leuconostoc kimchii* in dairy products.
Conclusions: LAB diversity was established using a stepwise polyphasic identification approach. It used the expertise of both research bodies involved in this study and proved to be cost-effective for the identification of all isolates.
Significance and Impact of the Study: To establish LAB diversity in Moroccan dairy products which could be a source of strains with specific properties.

Introduction

In Morocco, milk production by dairy cows is of great importance in agriculture and it plays a basic role in feeding a growing and increasingly urban population. Milk production has increased from 475 million litres in 1975 to 1 billion 331 million litres in 2002 (Srairi *et al.* 2005). Dairy products made from locally produced raw milk are still a very important part of the daily diet. People living in the countryside use the milk to produce white cheese 'jben', fermented butter 'smen' and fermented skimmed milk 'lben' (Srairi *et al.* 2005). In most cases, raw milk is used and the fermentation process relies on the natural microbiota of milk and the environment. Historically, fermented dairy products have been produced to prolong the shelf life of milk. Backslopping, a process in which a portion of a traditionally prepared product 'lben', 'jben' or 'smen' from a previous batch is used as an inoculum for the new batch, is also practiced sometimes to expedite the fermentation process (Benkerroum and Tamime 2004; Zamfir *et al.* 2006). The stability of the microbial content of these products over time is not well known. However, environmental conditions such as temperature, origin and quality of the milk, processing and sanitary conditions, might have a significant influence on the microbial composition of traditionally made dairy products. Fermentation of milk mainly involves lactic acid bacteria (LAB), but micrococci, coryneforms, yeasts and moulds can also occur (Zamfir *et al.* 2006).

Table 1 Isolates identified in this study

Species	Strain numbers
Enterococcus durans	B520, B526
Enterococcus faecium	B518, B505, B519, B527, B478, B471, B464, B548, B491, B513, B506
Enterococcus gilvus	#B427 (R-31670), #B428 (R-31671), #B430 (R-31672)
Enterococcus hirae	B432, B433, B504
Lactobacillus brevis	B524
Lactobacillus paracasei	B547, B528, B551
Lactobacillus plantarum	B420, B494, B500, B498, B497, B493, B503, B441, B449, B442, B443, B479, B534, B456, B457, B458, B460, B473
Lactobacillus rhamnosus	B523 (R-32689)
Lactococcus garvieae	B508, B514, B522, B530, B431, B435
Lactococcus lactis	B421, B423, B426, B429, B434, B467, B462, B466, B453, B468, B469, B474, B477, B410, B531, B515, B525, B502, B495, B436, B438, B439, B440, B444, B445, B480, B481, B483, B484, B486, B487, B488, B554, B539, B537
Leuconostoc citreum	B546
Leuconostoc kimchii	#B415 (LMG 23786), B416 (LMG 23787)
Leuconostoc mesenteroides	B422, B463, B408, B409, B411, B414, B418, B419, B516, B542, B517, B507, B447, B538, #B412 (R-32721), B413, B417
Leuconostoc pseudomesenteroides	B424, B425, B465, B470, B454, B452, B475, B476, B489, B529, B532, B509, B499, B496, B510, B490, B437, B450, B451, B446 (R-31675), B485, B482, B535, B459, #B540 (R-31690), B552, B553, #B550 (R-31687), B533, B536, B461, B448
Pediococcus pentosaceus	B541
Weissella cibaria	B501, #B521 (R-32690), B512, B544, B549, B455
Weissella confusa	B472
Weissella viridescens	B492, B511
Weissella paramesenteroides	B543

B-numbers refer to in the Moroccan Coordinated Collection of Micro-organisms (CCMM) accession numbers of strains; LMG and R- numbers refer to the duplicates deposited in the BCCM/LMG Bacteria Collection or the research collection of Laboratory of Microbiology (Ghent University) research group, respectively. Strains with (#) were subjected to phenylalanine RNAt synthase gene sequencing.

In Morocco, fermented skimmed milk 'lben' is traditionally made from raw cow milk by spontaneous fermentation. The raw milk is left to sour spontaneously at room temperature until it coagulates. The churning of the fermented milk yields fermented skimmed milk 'lben' and raw butter called 'zebda beldia'. The shelf life of 'lben' is about three days at 4°C. In the countryside, there is sometimes no electricity supply and 'lben' is kept at room temperature, reaching high acidity levels after 2–3 days (Benkerroum and Tamime 2004).

Fresh milk drawn from a healthy cow normally contains a low microbial load (less than 1000 CFU ml^{-1}). The load may increase up to a 100-fold or more once the milk is stored at room temperature (Richter *et al.* 1992). *Lactococcus, Lactobacillus, Leuconostoc, Enterococcus, Streptococcus* and *Micrococcus* species are among the common bacterial species of fresh milk (Richter *et al.* 1992; Kim *et al.* 2000; Chye *et al.* 2004). Traditionally fermented skimmed milk contains the same mesophilic species as the raw milk (Beukes *et al.* 2001; Mathara *et al.* 2004).

In Morocco, few studies have addressed the characterization of the microbiota of raw milk and 'lben' and none of these involved the use of a stepwise polyphasic molecular identification approach. A thorough identification of the micro-organisms present in dairy products from different regions of Morocco is useful in order to: (i) establish and preserve the microbial species diversity of Moroccan traditional products and (ii) select appropriate strains as starter cultures for dairy fermentation (Ouadghiri *et al.* 2005). We started this study by identifying LAB present in indigenous raw milk and fermented skimmed milk samples collected in Spring 2005 from some rural farms situated in Kenitra, Rabat, El-Jadida, Mohammedia and Tetouan, which are widely contributing to milk production in Morocco.

Material and methods

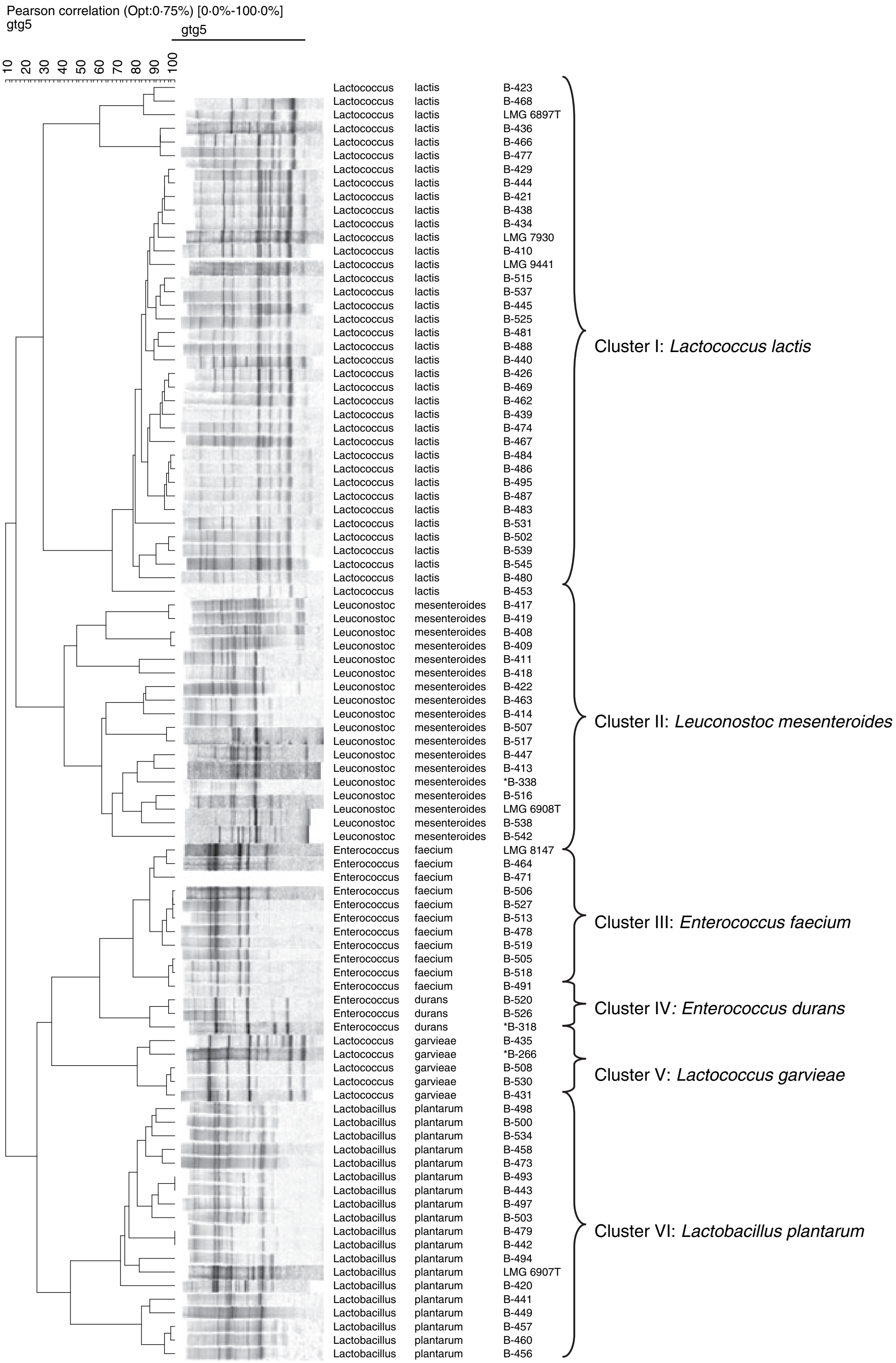
Sampling and isolation of LAB

A total of 37 samples (i.e. 29 raw cow milk samples and eight fermented skimmed milk samples) were collected from farms in rural areas from the regions of El-Jadida/Mohammedia, Kenitra, Rabat and Tetouan. Samples were collected in sterile bottles and kept at 4°C until arrival at the laboratory. The pH was measured using a calibrated pH meter (8521 Hanna Instruments, Amorim, Portugal). The titratable acidity was measured by pipetting

Table 2 Occurrence of LAB in Moroccan raw milk and traditional fermented skimmed milk

		Raw milk from region of				Raw milk further incubated	Pasteurized milk	Fermented skimmed milk from	
		Rabat	El Jadida/ Mohammedia	Kenitra	Tetouan	Kenitra	Rabat	Rabat	Kenitra
Number of farms		3	2	5	5	7	–	1	7
N° of samples (N° of isolates)		5 (20)	4 (9)	6 (22)	5 (8)	9 (42)	1 (6)	1 (16)	7 (23)
pH range		6·63 to 6·8	6·7 to 6·75	6·61 to6·76	6·56 to 6·77	4·50 to 4·89	6·69	4·50	4·25 to 4·57
°D		15·9	17·25	17·42	11·71	85·13	12·15	73·12	84·47
Protein content (g l^{-1})		36·2	34·1	37·8	28·5	N.D·	30	25·7	22 to 26·4
Fat content (g l^{-1})		34	36·4	42·6	32·6	N.D.	30	8	7·5 to 9
CFU ml^{-1}		$1{\cdot}4 \times 10^{5}$	$1{\cdot}1 \times 10^{5}$	$2{\cdot}1 \times 10^{7}$	$8{\cdot}2 \times 10^{3}$	$4{\cdot}2 \times 10^{10}$	2×10^{2}	$4{\cdot}9 \times 10^{9}$	$6{\cdot}4 \times 10^{10}$
Species identified	*Ent. durans* (2)					2			
(number of isolates)	*Ent. faecium* (11)	1		2	1	7			
	Ent. gilvus (3)	3							
	Ent. hirae (3)		2			1			
	Lact. brevis (1)					1			
	Lact. paracasei (3)				1	1			1
	Lact. plantarum (18)	1		1		6	4	4	2
	Lact. rhamnosus (1)					1			
	L. garvieae (6)	2				4			
	L. lactis (35)	8	3	3		5		6	10
	Leuc. citreum (1)				1				
	Leuc. kimchii (2)			2					
	Leuc. mesenteroides (17)	2		10	1	2		1	1
	Leuc. pseudomesenteroides (32)	3	3	3		7	2	5	9
	Ped. pentosaceus (1)				1				
	W. cibaria (6)		1		2	3			
	W. confusa (1)			1					
	W. paramesenteroides (1)				1				
	W. viridescens (2)					2			

°D: Dornic degree (1°D corresponds to 0·1 mg of lactic acid per litre). *Ent: Enterococcus; Lact: Lactobacillus; L: Lactococcus; Ped: Pediococcus, Leuc: Leuconostoc and W: Weissella.* pH, °D and CFU ml^{-1} are given after 16 days of incubation at room temperature (28°C). N.D.: not determined.


Pearson correlation (Opt:0·75%) [0·0%-100·0%]
gtg5
gtg5
10 20 30 40 50 60 70 80 90 100
Lactococcus lactis B-423
Lactococcus lactis B-468
Lactococcus lactis LMG 6897T
Lactococcus lactis B-436
Lactococcus lactis B-466
Lactococcus lactis B-477
Lactococcus lactis B-429
Lactococcus lactis B-444
Lactococcus lactis B-421
Lactococcus lactis B-438
Lactococcus lactis B-434
Lactococcus lactis LMG 7930
Lactococcus lactis B-410
Lactococcus lactis LMG 9441
Lactococcus lactis B-515
Lactococcus lactis B-537
Lactococcus lactis B-445
Lactococcus lactis B-525
Lactococcus lactis B-481
Lactococcus lactis B-488
Lactococcus lactis B-440
Lactococcus lactis B-426
Lactococcus lactis B-469
Lactococcus lactis B-462
Lactococcus lactis B-439
Lactococcus lactis B-474
Lactococcus lactis B-467
Lactococcus lactis B-484
Lactococcus lactis B-486
Lactococcus lactis B-495
Lactococcus lactis B-487
Lactococcus lactis B-483
Lactococcus lactis B-531
Lactococcus lactis B-502
Lactococcus lactis B-539
Lactococcus lactis B-545
Lactococcus lactis B-480
Lactococcus lactis B-453
Leuconostoc mesenteroides B-417
Leuconostoc mesenteroides B-419
Leuconostoc mesenteroides B-408
Leuconostoc mesenteroides B-409
Leuconostoc mesenteroides B-411
Leuconostoc mesenteroides B-418
Leuconostoc mesenteroides B-422
Leuconostoc mesenteroides B-463
Leuconostoc mesenteroides B-414
Leuconostoc mesenteroides B-507
Leuconostoc mesenteroides B-517
Leuconostoc mesenteroides B-447
Leuconostoc mesenteroides B-413
Leuconostoc mesenteroides *B-338
Leuconostoc mesenteroides B-516
Leuconostoc mesenteroides LMG 6908T
Leuconostoc mesenteroides B-538
Leuconostoc mesenteroides B-542
Enterococcus faecium LMG 8147
Enterococcus faecium B-464
Enterococcus faecium B-471
Enterococcus faecium B-506
Enterococcus faecium B-527
Enterococcus faecium B-513
Enterococcus faecium B-478
Enterococcus faecium B-519
Enterococcus faecium B-505
Enterococcus faecium B-518
Enterococcus faecium B-491
Enterococcus durans B-520
Enterococcus durans B-526
Enterococcus durans *B-318
Lactococcus garvieae B-435
Lactococcus garvieae *B-266
Lactococcus garvieae B-508
Lactococcus garvieae B-530
Lactococcus garvieae B-431
Lactobacillus plantarum B-498
Lactobacillus plantarum B-500
Lactobacillus plantarum B-534
Lactobacillus plantarum B-458
Lactobacillus plantarum B-473
Lactobacillus plantarum B-493
Lactobacillus plantarum B-443
Lactobacillus plantarum B-497
Lactobacillus plantarum B-503
Lactobacillus plantarum B-479
Lactobacillus plantarum B-442
Lactobacillus plantarum B-494
Lactobacillus plantarum LMG 6907T
Lactobacillus plantarum B-420
Lactobacillus plantarum B-441
Lactobacillus plantarum B-449
Lactobacillus plantarum B-457
Lactobacillus plantarum B-460
Lactobacillus plantarum B-456
Cluster I: *Lactococcus lactis*
Cluster II: *Leuconostoc mesenteroides*
Cluster III: *Enterococcus faecium*
Cluster IV: *Enterococcus durans*
Cluster V: *Lactococcus garvieae*
Cluster VI: *Lactobacillus plantarum*

20 ml and titrating the acidity against 0·05 mol l^{-1} NaOH to 1% phenolphthalein end point. Total Kjeldahl nitrogen and fat contents were determined according to French standard AFNOR T90-110 (Afnor 1975) and Röse-Gottlieb, respectively. Serial dilutions were plated on Man-Rogosa-Sharp (MRS) agar (Biolife, Milan, Italy) supplemented with sorbic acid (1·4 g l^{-1}) (Panreac Quimica, Barcelona, Spain). The plates were incubated under aerobic conditions at 30°C for 48–72 h. Nine samples of raw milk which did not show any growth after 72 h were further incubated at 28°C and plated on MRS-agar supplemented with sorbic acid after 5, 7, 9 and 16 days of incubation. One sample of pasteurized milk was included in this study as a control. Colonies were chosen randomly or on the basis of their morphology from MRS plates and streaked again for purification. All isolates were initially examined for Gram reaction and production of catalase and oxidase. Only Gram-positive, and catalase and oxidase negative isolates were considered and stored at −80°C in MRS broth (Biolife) with 20% glycerol. These frozen stocks were used for further identification.

A total of 146 isolates were recovered (Table 1).

DNA extraction and $(GTG)_5$-PCR genomic fingerprinting

Total DNA was extracted as described by Versalovic *et al.* (1994). The primer used was $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) (Gevers *et al.* 2001; Svec *et al.* 2005). PCR amplifications were performed with a DNA thermal cycler Gene Amp^R PCR System 2700 (Applied Biosystems, USA). The PCR products were electrophoresed as described by Gevers *et al.* (2001). The rep-PCR profiles were visualized after staining with ethidium bromide under ultraviolet light, followed by digital image capturing using a CCD Camera 570 LTV (GEL SMART, France). The resulting fingerprints were analysed by using Gel Compar II software package (Applied Maths, Sint-Martens-Latem, Belgium). The similarity among the digitized profiles was calculated using the Pearson correlation coefficient, and an average linkage (UPGMA) dendrogram was derived from the profiles. The data for LAB reference strains available at CCMM/LMBM Bacteria collection (Ouadghiri *et al.* 2005) were used for identification.

SDS-PAGE of whole cell proteins

Preparation of protein extracts, SDS-PAGE and computer processing were performed as described by Pot *et al.* (1994). Identification of the isolates was performed by comparison of their protein patterns with the data for LAB reference strains available at the BCCM/LMG Bacteria collections. Pattern storage and database comparison were performed using GelCompar version 4·2 software (Applied Maths).

Phenylalanyl-tRNA synthase (*pheS*) gene sequencing

The primer sequences, amplification conditions and sequencing reactions were performed as described by Naser *et al.* (2005). Raw sequence data were transferred to GeneBuilder (Applied Maths) where consensus sequences were determined. Consensus sequences were imported into BioNumerics 4·0 software (Applied Maths).

The phenylalanyl-tRNA synthase (*pheS*) gene sequences of the strains B427 (R-31670), B428 (R-31671), B430 (R-31672), B550 (R-31687), B540 (R-31690), B521 (R-32690), B412 (R-32721) and B415 (R-32669) were deposited in the EMBL under the accession numbers AM491822, AM491823, AM491824, AM491825, AM491826, AM491827, AM491828 and AM 491829, respectively.

Results

Physico-chemical composition of samples

The physico-chemical composition of raw milk and fermented skimmed milk 'lben' varied slightly between farms. For all raw milk samples analysed, the pH showed an average value of 6·67 and the acidity values ranged from 11·71 to 17·42°D. The protein content ranged from 28·5 to 37·8 g l^{-1} with an average of 34·15 g l^{-1} while the fat content ranged from 32·6 to 42·6 g l^{-1} with an average of 36·4 g l^{-1}. With regards to the pasteurized milk, both fat and protein content were standardized to the value 30 g l^{-1}. Samples of raw milk subjected to further incubation showed, after sixteen days, an average pH of 4·69 and an acidity ranging from 74·25 to 93·25°D with an average of 85·13°D. Fermented skimmed milk showed an average pH of 4·38 and an acidity ranging from 73·12 to 112·5°D with an average of 83·05°D. The average values of protein and fat contents were 24·7 and 8·0 g l^{-1}, respectively (Table 2).

Isolation and identification of LAB

LAB were present in all samples analysed. Their counts on MRS agar varied from $8{\cdot}2 \times 10^3$ to $2{\cdot}1 \times 10^7$ CFU ml^{-1} for raw milk and exceeded 10^9 CFU ml^{-1} for raw milk further incubated and fermented skimmed milk (Table 2).

Figure 1 Dendrogram and $(GTG)_5$-PCR banding patterns of the LAB isolated from Moroccan raw milk and traditional fermented skimmed milk clustered and identified at species level using $(GTG)_5$-PCR. Strains with an asterisk or LMG numbers are reference strains either from CCMM or BCCM/LMG culture collections.

The 146 $(GTG)_5$-PCR profiles were clustered and compared with available reference and type strains of established species in the $(GTG)_5$-PCR database allowing identification of 85 isolates at the species level (Fig. 1). The basis of assigning isolates to a particular species was the clustering, the degree of similarity, and the presence of reference or type strains in the same cluster. Six clusters were obtained and were identified as *Lactococcus lactis* (cluster I, 35 isolates), *Lactobacillus plantarum* (cluster VI, 18 isolates), *Leuconostoc mesenteroides* (cluster II, 16 isolates), *Enterococcus faecium* (cluster III, 10 isolates), *Lactococcus garvieae* (cluster V, four isolates) and *Enterococcus durans* (cluster IV, two isolates). Sixty-one isolates remained unidentified and were therefore subjected to SDS-PAGE of whole-cell protein analysis. Although technically demanding, numerous polyphasic taxonomic studies demonstrated that whole cell protein electrophoresis coupled with comparison of the profiles of the unidentified isolates with those of type and reference strains of established LAB species is an excellent tool for species level identification. Comparison of the profiles obtained to the data of LAB reference strains available from previous studies (Svec *et al.* 2006; Vancanneyt *et al.* 2006; De Bruyne *et al.* 2007) revealed the species identity of 43 isolates as *Lactobacillus brevis* (one isolate), *Lactobacillus paracasei* (three isolates), *Lactococcus garvieae* (two isolates), *Leuconostoc citreum* (one isolate), *Leuconostoc pseudomesenteroides* (23 isolates), *Enterococcus faecium* (one isolate), *Enterococcus hirae* (three isolates), *Pediococcus pentosaceus*

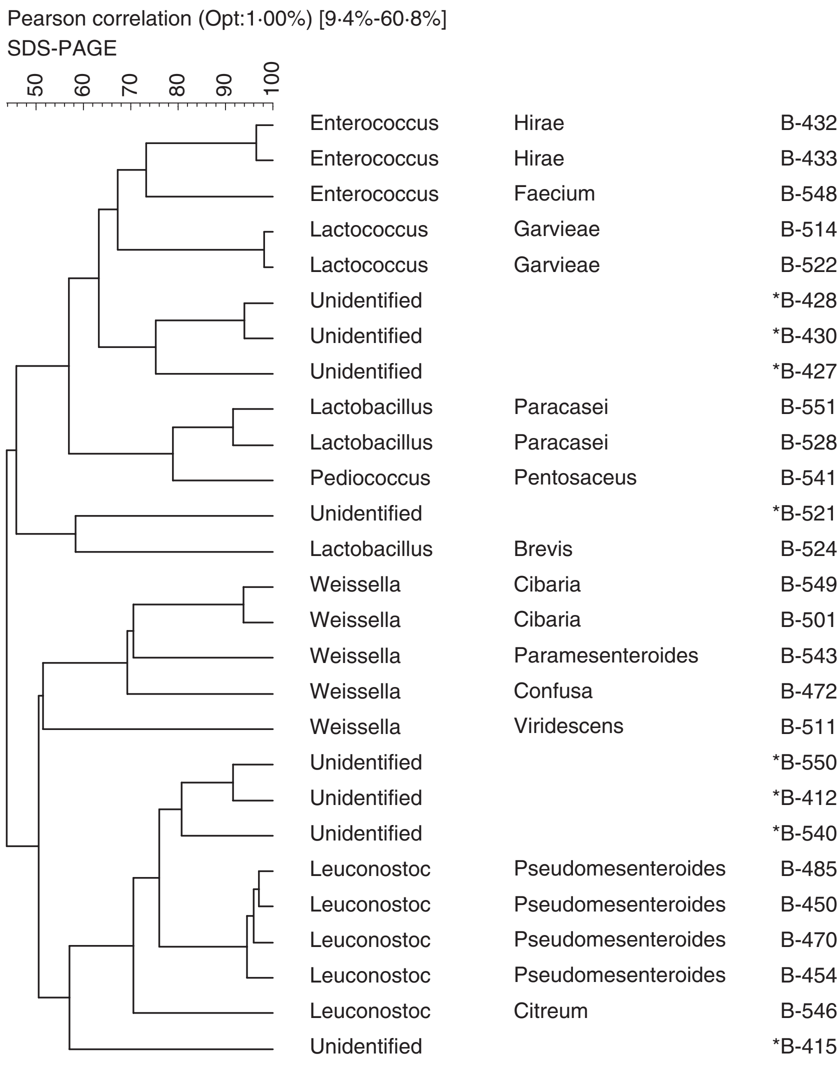


Figure 2 Dendrogram based on numerical analysis of protein banding patterns of representative strains of each observed cluster after SDS-PAGE analysis. Strains marked with an asterisk are representative of the unidentified strains which were subjected to phenylalanine RNAt synthase gene sequencing.

(one isolate), *Weissella cibaria* (four isolates), *Weissella confusa* (one isolate), *Weissella paramesenteroides* (one isolate) and *Weissella viridescens* (two isolates). Figure 2 shows representative strains of each species identified by protein electrophoretic analysis. Based on the clusters obtained by numerical analysis of the protein profiles (Fig. 2), eight representatives of the remaining 18 unidentified isolates were further analysed and identified by sequence analysis of partial *pheS* gene as *Enterococcus gilvus* (three isolates), *Leuconostoc pseudomesenteroides* (nine isolates), *Leuconostoc mesenteroides* (one isolate), *Leuconostoc kimchii* (two isolates), *Weissella cibaria* (two isolates) and *Lactobacillus rhamnosus* (one isolate) (Naser *et al.* 2005, 2007; De Bruyne *et al.* 2007; De Vuyst and Vancanneyt 2007).

Based on all these techniques and data, the 146 isolates were grouped into five major species i.e. *Ent. faecium, Lact. plantarum, L. lactis, Leuc. mesenteroides* and *Leuc. pseudomesenteroides.* Species represented by two to six isolates were *Ent. durans*, *Ent. gilvus*, *Ent. hirae, L. garvieae, Lact. paracasei, Leuc. kimchii, W. cibaria* and *W. viridescens.* The species *Lact. brevis*, *Lact. rhamnosus*, *Leuc. citreum*, *Ped. pentosaceus*, *W. confusa* and *W. paramesenteroides* were represented by only one isolate (Tables 1 and 2).

LAB in the different products examined

Raw milk

The most frequent LAB species recovered from raw milk samples analysed in this study belonged to the species *L. lactis*, *Leuc. pseudomesenteroides*, *Leuc. mesenteroides* and *Lact. plantarum* (Table 2). Raw milk of three out of four regions examined comprised seven different species of LAB. Raw milk of Rabat and Kenitra were dominated by a single species (Table 2), *L. lactis* and *Leuc. mesenteroides,* respectively. Raw milk samples of El- Jadida/ Mohammedia and Tetouan had no dominant species. *Leuc. kimchii* was isolated twice from samples from Kenitra. *Ped. pentosaceus* and *Leuc. citreum* were isolated once from samples from Tetouan. In the present study we isolated members of four *Weissella* species, i.e. *W. cibaria, W. confusa, W. paramesenteroides* and *W. viridescens* and members of four *Enterococcus* species i.e. *Ent. durans, Ent. faecium*, *Ent. gilvus* and *Ent. hirae*.

For raw milk that was further incubated, 13 species were recovered, most commonly *Ent. faecium, Lact. plantarum, L. garvieae, L. lactis* and *Leuc. pseudomesenteroides*.

Fermented skimmed milk

Traditionally fermented skimmed milk is typically dominated by *L. lactis* (16 out of 39 isolates), *Leuc. pseudomesenteroides* (14 out of 39 isolates) and *Lact. plantarum* (6 out of 39 isolates). *Leuc. mesenteroides* was found less frequently.

Discussion

Physico-chemical composition of samples

Auldist *et al.* (1998) have reported that the chemical composition of raw milk is mainly influenced by the stage of lactation, time of year, and kind of food. Samples analysed in this study were taken in four different areas recognized for their wide contribution to national milk production, they were collected during spring (March–June) and most milking cows were at the same stage of lactation. The slight variations of the pH, °D, protein and fat contents between regions, mainly between Tetouan and other regions (Table 2), may be related to the kind of food used to feed cows. For fermented skimmed milk 'lben', Benkerroum and Tamime (2004) reported that its chemical composition depends on raw milk quality and varied between different localities, regions and farms. Nevertheless, despite such variation in the chemical composition of 'lben' some parameters like acidity, fat and protein content are considered as good indicators of its quality. They therefore should fall within specific range of the product specification. In the present study, the pH, protein and fat values obtained with all fermented skimmed milk analysed were almost the same as those reported by Tantaoui-Elaraki and El Marrakchi (1987).

Identification of LAB

In this study, eight isolates of species already present in the $(GTG)_5$-PCR database (*Leuc. mesenteroides:* one; *L. garvieae*: two; *Ent. faecium*: one; *Lact. rhamnosus*: one; *Lact. brevis*: one; and *Lact. paracasei*: three) were identified by protein electrophoresis or *pheS* gene sequencing. Similarly, 11 strains of established LAB species in the protein electrophoresis database (*Leuc. pseudomesenteroides*: nine and *W. cibaria*: two) were identified using partial *pheS* gene sequencing. In both cases, these strains represent new variants of established LAB species not yet present in the reference profile databases, which is not uncommon as reported by Scheirlinck *et al.* (2007) and as noticed by Zamfir *et al.* (2006). For the species *Ent. gilvus* and *Leuc. kimchii* identified by *pheS* gene sequencing, they represent LAB species not included in the reference profile database of $(GTG)_5$-PCR and protein electrophoretic profiles.

LAB in the different products examined

Raw milk

The present study shows that the biodiversity of Moroccan raw milk is characterized by enterococci, lactobacilli, lactococci, leuconostocs and *Weissella*. The most frequent

LAB species recovered belonged to the species *L. lactis*, *Leuc. pseudomesenteroides*, *Leuc. mesenteroides* and *Lact. plantarum* (Table 2). The variability of the LAB occurrence observed at farm level (data not shown) may reflect the traditional way of milking cows. During sampling, all farmers used hand milking. They further differed by their way of washing the milking equipment, by their premilking and postmilking udder preparation as well as the milk storage conditions. The combination of these practices influences the microbial load and composition of the milk produced as reported by Chye *et al.* (2004) and Lafarge *et al.* (2004). For raw milk that was further incubated, 13 species were recovered, this wide diversity of species is most likely a result of enriching certain bacteria during the long incubation period, and possibly also to a relative oversampling compared to the direct sampling of raw milk.

Leuc. kimchii isolated from raw milk from Kenitra needs to be investigated whether it is a natural component of raw milk or an environmental contaminant since it has thus far only been isolated from kimchi, a traditional Korean vegetable product (Kim *et al.* 2000) and it was never reported as part of dairy products microbiota.

Ped. pentosaceus and *Leuc. citreum* were recovered once. The isolation of this species from dairy products is not common (Beukes *et al.* 2001).

Members of the genus *Weissella* have been isolated from fresh vegetables, sugar cane and meat samples, and also from clinical samples from animals and humans (Björkroth *et al.* 2002). Mathara *et al.* (2004) reported that *Weissella* species were occasionally found in raw milk, but their technological role in fermentation has not been reported. In the present study we isolated members of four *Weissella* species, this is the first report of their occurrence in Moroccan cow's raw milk and they are probably environmental contaminants.

The presence of enterococci in raw milk should be looked at critically, as enterococci might carry virulence factors and antibiotic-resistance genes (Franz *et al.* 1999). Four *Enterococcus* species were isolated from raw milk samples in the present study. According to Giraffa (2003), the presence of these species in food should be considered as part of the normal raw milk microbiota.

Several other LAB isolates identified in Moroccan raw cow milk also represent species that have occasionally been associated with human infection (Avlami *et al.* 2001; Flahetry *et al.* 2003; Martin *et al.* 2005; Vinh *et al.* 2006). Yet, there is currently no evidence to consider this presence truly problematic for public health. There is, therefore, a need to investigate whether all these species are a natural component of raw milk or whether they are environmental contaminants.

Fermented skimmed milk

The species isolated from traditionally fermented skimmed milk were *L. lactis*, *Leuc. pseudomesenteroides*, *Lact. plantarum* and *Leuc. mesenteroides*. The presence of *Leuconostoc* strains could be related to a post preparation contamination as Mathara *et al.* (2004) have reported that *Leuconostoc* strains have complex nutritional requirements and show a weak competitiveness during milk fermentation.

Enterococcus strains were not isolated from 'lben'. Except for enterococci, the dominating LAB species in 'lben' correspond to those reported with traditionally fermented milk and dairy products from South Africa, Kenya and Romania (Beukes *et al.* 2001; Mathara *et al.* 2004; Zamfir *et al.* 2006). The absence of enterococci might be related to the presence of bacteriocin-producing strains (Benkerroum *et al.* 2000) although this characteristic was not verified in the present study.

In a previous study Tantaoui-Elaraki *et al.* (1983a,b) reported that mesophilic LAB dominated by *L. lactis* and *Leuc. mesenteroides* were the main species responsible for lactic acid fermentation and aroma development in 'lben'.

The limited number of species present in 'lben' may be explained by the characteristic of the fermentation process which includes the increase of acidity inhibiting the growth of several species as reported by Wouters *et al.* (2002).

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