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Biodiversity of lactic acid bacteria in Moroccan soft white cheese (Jben)

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Abstract

The bacterial diversity occurring in traditional Moroccan soft white cheese, produced in eight different regions in Morocco, was studied. A total of 164 lactic acid bacteria were isolated, purified and identified by whole-cell protein fingerprinting and rep-PCR genomic fingerprinting. The majority of the strains belonged to the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Enterococcus*. Sixteen species were identified: *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactobacillus paracasei*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactococcus lactis*, *Lactococcus garvieae*, *Lactococcus raffinolactis*, *Leuconostoc pseudomesenteroides*, *Leuconostoc mesenteroides*, *Leuconostoc citreum*, *Eterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus saccharominimus* and *Streptococcus* sp.

Keywords: Moroccan soft white cheese; Identification; Lactic acid bacteria; Biodiversity; Protein fingerprinting; $(GTG)_5$-PCR genomic fingerprinting; GelCompar II

1. Introduction

Moroccan soft white cheese is a traditional dairy product that has been known and highly appreciated by consumers for centuries. It is widely manufactured and consumed in Morocco, especially in the ''Ramadan'' fasting month. Benkerroum and Tamime [1] have recently reviewed the scientific, technological, and technology transfer aspects of Moroccan traditional dairy products Lben (fermented skimmed milk), Jben (soft white cheese), and Smen (fermented butter). The traditional soft white cheese (Jben) made from non-pasteurized milk, is characterized by a total dry matter of about 35% and a pH lower than 4.5 [2]. Nowadays, Jben is also prepared from pasteurized milk. The final characteristics of a typical Jben are variable and affected by preparation of the cheese. Its production does not conform with official hygiene and other regulatory standards and follows informal marketing routes. The microflora of Jben is dominated by lactic acid bacteria (LAB) present in a range of at least 10^8–10^9 cfu/g [3,4]. No comprehensive identification study on the LAB flora has been performed. Among phenotypic

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methods used, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell proteins has proven to be a useful tool for LAB identification [5–9]. Genotypic techniques have also been applied for the identification of LAB and include plasmid profiling, restriction enzyme analysis, pulsed-field gel electrophoresis, ribotyping, random amplified polymorphic DNA and Rep-techniques [10].

The present study reveals the diversity of LAB in Moroccan soft white cheese traditionally produced in eight different regions. For the identification, the API 50CHL gallery (Biomérieux, Marcy l'Etoile, France), SDS–PAGE of whole-cell proteins [11] and rep-PCR using the $(GTG)_5$ primer [12,13] have been used.

2. Material and methods

2.1. Isolation of LAB and cultivation conditions

Traditional Moroccan soft white cheese was sampled in eight different regions of Morocco (Table 1). Samples were kept refrigerated until arrival in the laboratory for analysis. Tenfold dilutions were plated on Man-Rogosa-Sharpe (MRS) agar (Biolife,Italy) supplemented with sorbic acid (Panreac quimica SA, Spain) and incubated under the following conditions: (1) microaerophilic at 37 °C and (2) aerobic at 30 °C. A total of 164 strains of LAB (Gram positive, catalase negative and oxydase negative) were isolated. All strains were maintained as frozen stocks at −80 °C in MRS broth (Biolife, Italy) supplemented with 15% of glycerol.

2.2. Reference strains

Reference strains used for identification of LAB isolates were obtained from the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm/lmg.htm): *Enterococcus durans* (LMG 12283, LMG 12903, LMG 10746^T); *Enterococcus faecalis* (LMG 7937^T, LMG 7938); *Enterococcus faecium* (LMG 8147); *Lactobacillus brevis* (LMG 11434); *Lactobacillus plantarum* (LMG 6907^T, LMG 18021); *Lactobacillus rhamnosus* (LMG 6400^T); *Lactobacillus sake* subsp. *carnosus* (LMG 17302^T); *Lactococcus lactis* subsp. *lactis* (LMG 9441, LMG 7930); *Lactococcus lactis* subsp. *cremoris* (LMG 6897^T); *Leuconostoc mesenteroides* subsp. *cremoris* (LMG 6909^T); *Leuconostoc mesenteroides* subsp. *dextranicum* (LMG 6908^T).

2.3. Biochemical and phenotypic identification of LAB

All LAB isolates were identified by SDS–PAGE of whole-cell proteins. Standardized cultivation conditions and extraction procedures were used. Preparation of whole cell extract was performed according to the method described for Gram positive bacteria [14]. Separation of protein extracts using SDS–PAGE was performed as previously described [15]. The digital protein patterns were normalized using the software package GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). The isolates were identified by comparison of their patterns with those in the in-house database available at BCCM/LMG Bacteria Collection.

Thirty four isolates of LAB were additionally identified by API 50 CH strips with API 50 CHL Medium (Biomérieux, Marcy l'Etoile, France). The tests were undertaken following the instructions of the manufacturer, and the results were read after two days of incubation at 30 °C; identification employed the software package API LAB provided by the manufacturer.

2.4. rep-PCR genomic fingerprinting

rep-PCR was applied to all 164 strains. Total DNA was extracted as previously described [13]. The primer used was $(GTG)_5$, (5′-GTGGTGGTGGTGGTG-3′) as previously described [12]. PCR amplifications were performed with a DNA thermal cycler Gene Amp^R PCR System 2700 (Applied biosystems, USA). The PCR products were separated in a 1.5% agarose gel (15 cm by 20 cm) for 16 h at a constant voltage of 2 V cm^{-1} in 1X Tris Acetate EDTA (TAE) at 4 °C. 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 analyzed by GelCompar II software package (Applied Maths, Sint-Martens-Latem, Belgium).

3. Results and discussion

In all analyzed cheese samples, LAB were present in total counts of 108–109 cfu/g. This observation is in accordance with results reported by other authors [3,4]. All isolates were identified by SDS–PAGE and identity was confirmed by (GTG)5-PCR. For the 34 isolates identified by API 50 CHL, only 22 isolates (64.70%) were in agreement with both of the other methodologies (Table 2).

In Casablanca and Rabat, a more extensive sampling was performed, yielding 36 and 44 LAB isolates, respectively, reflecting a broader species spectrum than in other regions. In the case that only one sample of cheese was analyzed, we were able to isolate at least four different LAB species. Grouping based on (GTG)5-PCR, which provided the highest resolution, did not reveal any correlation with the geographic origin of the samples. All identification results of the present study are summarized in Table 1.

Table 1
Biodiversity of LAB in Moroccan soft white cheese

Location	Agadir	Casablanca	Fes	Marrakech	Rabat	Safi	Sefrou	Tetouan	Number of isolates	Identification	
										Species[a]	Genus (number of isolates) (%)
Number of samples	1	3	1	2	6	1	2	1			
Number of isolates	17	36	10	18	44	15	12	12			
cfu/g[b]	2×10^9	5×10^8	10^9	7.5×10^8		2×10^8	7×10^8	8.3×10^8	3×10^9		
	2	6		12	6	2	4	4	36	*Lactobacillus plantarum* (a)	*Lactobacillus* (56) (34%)
		8							8	*Lactobacillus rhamnosus* (b)	
	2	2		1	1				6	*Lactobacillus paracasei* (c)	
				2	1			2	5	*Lactobacillus brevis* (d)	
		1							1	*Lactobacillus buchneri* (e)	
	11	7			16	7	1		42	*Lactococcus lactis* (f)	*Lactococcus* (44) (27%)
					1				1	*Lactococcus garvieae* (g)	
					1				1	*Lactococcus raffinolactis* (h)	
	2	5	3	2	2	2	5	1	22	*Leuconostoc pseudomesenteroides* (i)	*Leuconostoc* (44) (27%)
		3	4		6	1	2	1	17	*Leuconostoc mesenteroides* (j)	
		1	3		1				5	*Leuconostoc citreum* (k)	
		2		1	2			4	9	*Enterococcus durans* (l)	*Enterococcus* (16) (10%)
		1			2	1			4	*Enterococcus faecalis* (m)	
					1				1	*Enterococcus faecium* (n)	
						2			2	*Enterococcus saccharominimus* (o)	
					2				2	*Streptococcus* sp. (p)	*Streptococcus* (2) (1%)
					2				2	Unidentified (q)	Unidentified (2) (1%)

[a] All strains have been deposited in Collections Coordonnées Marocaines de Micro-organismes (CCMM) and in BCCM/LMG. In CCMM Bacteria collection the following numbers have been given: (a) B222 → B257; (b) B258 → B265; (c) B216 → B221; (d) B210 → B214;(e) B215; (f) B267 → B308; (g) B266; (h) B309; (i) B350 → B371; (j) B333 → B349; (k) B328 → B332; (l) B310 → B318; (m)B319 → B322; (n) B323; (o) B208 → B209; (p) B324 → B325; (q) B326 → B327.

[b] The cfu/g correspond to an average number.

Table 2
Discrepancies between SDS–PAGE/$(GTG)_5$-PCR and API 50CHL identification results

SDS–PAGE/$(GTG)_5$-PCR identification result	Number of isolates	API 50CHL identification result
Enterococcus durans	2	*Lactococcus lactis* (very good identification)
Enterococcus faecalis	1	*Lactococcus lactis* (low discrimination)
Enterococcus faecalis	1	*Leuconostoc lactis* (excellent identification)
Enterococcus faecium	1	*Lactococcus lactis* (good identification)
Lactococcus garviae	1	*Lactococcus lactis* (very good identification)
Lactococcus lactis	3	*Lactobacillus paracasei* (unacceptable profile)
Leuconostoc pseudomesenteroide	1	*Lactobacillus acidophilus* (good identification)
Streptococcus sp.	2	*Leuconostoc lactis* (good identification)

The dominant genera isolated from Jben were *Lactobacillus* (34% of the isolates), *Lactococcus* (27%), *Leuconostoc* (27%) and *Enterococcus* (10%). Except for the genus *Enterococcus*, similar results have been reported [7]. At the species level, only *Leuconostoc pseudomesenteroides* occurred in all samples examined. The species *Lactobacillus plantarum* is dominantly present in all samples except one, and contributes to flavor development [16,17] and production of a protective antimicrobial agent in the cheese [18,19]. The latter two species together with *Lactococcus lactis* and *Leuconostoc mesenteroides* constitute the vast majority of the isolates (71%). In a previous study, it has been reported that the dominant microflora of Jben comprises the species *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Lactobacillus casei* [3]. Part of this discrepancy may be explained by the fact that in the latter study mainly classical biochemical and physiological tests were used which often have proven to be unsatisfactory for the identification of LAB [7, Table 2]. Less dominant species were *Lactobacillus paracasei* and *Lactobacillus brevis*, each being isolated in at least three samples. The species *Lactobacillus rhamnosus* and *Lactobacillus buchneri* were even less widely distributed. The presence of *Lactobacillus rhamnosus* raises some health concern because in some reports it has been associated with clinical syndromes, such as endocarditis [20]. The species *Lactococcus garvieae* and *Lactococcus raffinolactis* were found only once. This is also the first report of the occurrence of the newly described species *Enterococcus sacharominimus* in Moroccan Jben [21].

The non-standardized conditions in Jben processing result in a product of variable hygienic quality, which may be a vehicle for pathogens responsible for serious food-borne disease such as *Listeria monocytogenes* [2]. The acidification due to the presence of LAB in soft white cheese will not prevent the development and spread of *Listeria monocytogenes* [2]. According to the results already described [3], pathogens of major health concern including *Salmonella* spp., *Yersinia enterocolitica* and *Listeria monocytogenes* were detected in traditional Jben at frequencies of 10%, 4.1% and 18.1%, respectively. As far as we know, listeriosis has not been associated with Jben in Morocco. Nevertheless, the absence of epidemiological studies concerning the disease in the country could explain the lack of reports on listeriosis cases [2].

The overall presence of *Lactobacillus plantarum*, *Lactococcus lactis*, *Leuconostoc pseudomesenteroides* and *Leuconostoc mesenteroides* in soft white cheese offers the possibility of a standardized use of this set of organisms as inoculum.

In the future, the microbial composition of traditional food matrices such as Jben, Smen or Lben will be further clarified using molecular techniques such as culture-independent denaturing gradient gel electrophoresis (DGGE) analysis [22,23]. Nevertheless, the application of culture-dependent methods remains valuable as it allows isolation of cultures which may be used as starters to improve the technological properties for the preparation of Jben.

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