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ORIGINAL PAPER

Isolation, identification, and technological characterization of wild leuconostocs and lactococci for traditional Raib type milk fermentation

Nahida Bendimerad · Mebrouk Kihal · Françoise Berthier

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Abstract *Raib* (*Rayeb*) is a spontaneously fermented milk primarily acidified and flavored by strains of lactococci and leuconostocs. Raib and its by-products are traditionally consumed in many Mediterranean and sub-Saharan countries. Dedicated ready-to-use starter strains are currently missing. The aim of this study was to isolate and characterize wild strains of lactococci and leuconostocs that could be used to produce these dairy products while preserving their traditional characteristics. Dominant lactic acid cocci were isolated on M17 and MRS-vancomycin plates from Algerian cow's, ewe's, goat's, mare's, and camel's milks, either raw and/or fermented under laboratory conditions. The DNA of the isolates was fingerprinted by Rep-PCR. Strain identification was performed by Rep-PCR combined with specific PCR for genus *Enterococcus* and subspecies *lactis*/*hordniae* and *cremoris* of *Lactococcus lactis*. Isolates were characterized for three phenotypes essential for Raib manufacture: (a) high acidifying activity over the normal associated temperature range, (b) presence of protease, and (c) ability to metabolize citrate. Eighty-four isolates were characterized including four different strains of lactococci and four different strains of leuconostocs. Vancomycin-resistant enterococci were often coisolated. One strain of *Leuconostoc mesenteroides* exhibited an atypical genotype compared to the subspecies-type strains and to other wild *Leuconostoc* strains. Two protease-positive strains of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* isolated from camel's fermented milks and the

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strain *Leuconostoc mesenteroides* subsp. *mesenteroides* isolated from mare's fermented milks had high acidifying potential in milk. These three strains were considered as suitable candidates as acidifying starters to preserve the typical sensory characteristics of traditional Raib while improving its safety and shelf life.

传统Raib型发酵乳中天然明串珠菌和乳球菌的分离、鉴定以及工艺特性

摘要: Raib(Rayeb)是由乳球菌和明串珠菌发酵产酸进而酸化和产生风味的一种自然发酵乳。Raib以及其副产品是许多地中海和撒哈拉以南非洲国家的传统食品。当前尚未有用于发酵Raib的专门发酵剂。本研究目的是分离和鉴定可用于Raib及其副产品的生产, 并能保持自身传统特性的天然乳球菌和明串珠菌菌株。在实验室中, 采用M17和MRS(含万古霉素)培养基, 从新鲜阿尔及利亚牛乳、绵羊乳、山羊乳、马乳、骆驼乳等原料乳或者其发酵乳中, 分离了优势乳酸球菌。提取这些菌株的DNA,并且采用Rep-PCR技术对上述菌株指纹图谱分析。采用Rep-PCR技术结合*Enterococcus*属特异性引物、*lactis/hordniae* 和*cremoris* of *Lactococcus lactis*.亚属特异性引物, 对菌株进行鉴定。分离获得菌株的三个与Raib生产相关的特性被确定, 其中包括a)在正常的相关温度范围内体现的高酸化能力; b)蛋白酶活性;c)柠檬酸代谢能力。 84株菌被定性, 其中包括4株乳球菌和4株明串珠菌。具有抗万古霉素的肠球菌, 常常被共分离。一株*Leuconostoc mesenteroides*菌与亚种模式菌株以及其他天然明串珠菌相比, 显示了非典型的表型特征。两株分离自骆驼发酵乳, 并具有蛋白酶活性的*Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* 和分离自马发酵乳的*Leuconostoc mesenteroides* subsp. *mesenteroides* 在乳中显示高的酸化潜力。以上三株菌适合用作酸化发酵剂, 它们既可保留传统Raib的传统的感官特性, 同时可以改善Raib的安全性和延长货架期。

Keywords Leuconostocs · Lactococci · Enterococci · Raib type milk fermentation · Rep-PCR

关键词 明串珠菌 · 乳球菌 · 肠球菌 · Raib型发酵乳 · Rep-PCR

1 Introduction

There is a need to select novel microbial strains to improve safety of existing traditional fermented dairy products without altering their unique sensory, nutritional, and health-related characteristics; hitherto, strains have mostly been selected for products that have other sensory, nutritional, and health-related characteristics and are manufactured from cow's milk. The selection of strains from dairy ecosystems other than cow's milk and cheeses provides an opportunity to obtain strains which possess unique phenotypes (Drici et al. 2010) and may be useful for new dairy applications as well as traditional ones (Prashant et al. 2009).

Ready-to-use starters dedicated to the manufacture of traditional *Raib* and its by-products are not currently available. The selection of novel strains is crucial to design such starters.

Raib and its by-products are traditional dairy products, still widely produced and consumed in many Mediterranean and sub-Saharan countries (Abou-Donia 2008; Benkerroum and Tamime 2004; Koussou et al. 2007). Raib is consumed directly after fermentation or is skimmed before use. When Raib is churned, *Lben* is obtained from the nonbutter fraction and *Smen* from the butter fraction. *Jben* is a soft white cheese obtained by molding, draining, and salting. The characteristics of Raib and its by-products are broad ranging as they are largely still produced without standardized

procedures. Until recently, they were mostly homemade in rural areas where they make a major contribution to people's diets as well as to the rural economy by promoting local production. Their consumption is increasing in some cities, but has been decreasing among the more prosperous population groups, who have replaced them with industrial-type yoghurts (Khaldi et al. 2006; Koussou et al. 2007). It has been estimated that 30% of Algerian milk output in the 1990s was used to make these products, which were either consumed on-farm or marketed through informal channels (Bencharif 2001).

Traditionally, Raib results from the spontaneous fermentation of full-cream raw cow's, goat's, camel's, or ewe's milks. Cow's milk is also sometimes supplemented with goat's, ewe's, or camel's milk. Fermentation takes place at ambient temperature for 24 to 72 h depending on the temperature. *Lactococcus lactis* and *Leuconostoc mesenteroides* are generally cited as the main lactic acid bacteria (LAB) responsible for its acidification, texture, and aromatization (Benkerroum and Tamime 2004). In that respect, the LAB involved are similar to those used for cultured buttermilk made from cow's milk in developed countries (Vedamuthu 1994). Yeasts are also cited as secondary aroma contributors (Tantaoui-Elaraki and El Marrakchi 1987). All this gives traditional Raib and its by-products specific physicochemical compositions and organoleptic characteristics.

Currently, manufacturing practices are changing for various reasons such as to facilitate large-scale production, production in urban areas, availability of ready-to-use starters and rennet, and hygiene constraints. This results in modification of some essential characteristics such as acidity, levels of solids and volatile compounds, and rheological profiles (Guizani et al. 2001; Samet-Bali et al. 2010). The lactococci that predominated in traditional *Laban* and Jben have been replaced by thermophilic lactobacilli in their commercial counterparts (Benkerroum and Tamime 2004; Guizani et al. 2001). Practices for industrial Laban manufacturing are actually similar to those for conventional yoghurt in Lebanon (Chammas et al. 2006).

The consumption of ripened cream butter has declined in developed countries and with it, research on dedicated *Leuconostoc* strains. Many successful butter cultures containing good aroma-producing leuconostocs have also been lost because of neglect (Vedamuthu 1994). However, for flavor intensification without an objectionable green flavor defect or excessive gas, leuconostocs should be used in preference to citrate-fermenting lactococci in ripened cream butter made from cow's milk (Vedamuthu 1994). In milk, leuconostocs function only in association with lactococci, which initiate their growth (Vedamuthu 1994).

All previous studies of Raib and its by-products have described LAB isolated from ready-made products or from raw milks. With the exception of one recent study (Ouadghiri et al. 2009), LAB have previously been identified by unreliable phenotypic methods. The present study sets out to isolate wild strains of leuconostocs and lactococci that are well adapted to the different environments in which they are likely to grow. Strains of leuconostocs and lactococci were isolated from raw milks and/or spontaneously fermented raw milks of cow, ewe, mare, goat, and camel under laboratory conditions. They were identified by reliable and accurate genotypic methods, i.e., species-specific PCR when available (Wolfgang 2007) and Rep-PCR (Gevers et al. 2001), and were characterized for their potential as acidifiers and flavoring agents of Raib.

2 Materials and methods

2.1 Milk samples

Milks were sampled in spring. To avoid isolating any commercial strains, milks were collected in sterile flasks directly at milking or from the milk can. They were kept at 4 °C until use; all were used within 24 h. To ensure variability among the isolates, samples were taken from 40 different farms.

2.2 Strain isolation

MRS-vancomycin (vancomycin at 20 $mg.L^{-1}$) (Mathot et al. 1994) and M17 (Terzaghi and Sandine 1975) were used to isolate leuconostocs and lactococci, respectively. Strains were isolated directly from raw milks or after spontaneous fermentation of raw milks at 30 °C for 48 h (96 h for camel's milks), mimicking traditional fermentation. The aim was to obtain strains that are active during the first production stages and, therefore, common to Raib and its by-products. Aliquots (0.1 mL) of raw or fermented milks were cultivated in MRS-vancomycin broth; then, aliquots from cultures that produced gas in Durham tubes were streaked on MRS-vancomycin plates. Aliquots (0.1 mL) of raw or fermented milks were also cultivated in M17 broth; then, aliquots from cultures that did not produce gas in Durham tubes were streaked on M17 plates. The cultures were incubated aerobically at 30 °C for 24 h.

Isolated colonies were picked off plates and purified by two successive streakings on MRS-vancomycin or M17 plates. By taking only a few isolates from each sample, redundancy among isolates was avoided, and it can be assumed that only dominant strains were isolated from the milks, with strains well adapted to the different milks that are likely to be fermented. The isolates were stored at −20 °C in sterile milk supplemented with glycerol (15%), glucose (0.5%), and yeast extract (0.25%).

2.3 Type strains

Type strains (see Fig. 1) were obtained from ATCC, American Type Culture Collection (Rockville, MD, USA); CIP, Collection of the Institute Pasteur (Paris, France); and CNRZ, Collection of the Centre National de la Recherche Zootechnique (INRA, Jouy-en-Josas, France) distributed now by CIRM-BIA (INRA, Rennes, France).

2.4 DNA extraction

Each purified isolate and each type strain was streaked on an appropriate agar medium. Total DNA was extracted from one colony by the rapid cold shock method of Gaya et al. (1999).

2.5 Rep-PCR

Rep-PCR was performed as described by Berthier et al. (2001), except that the gel image was captured with a CCD camera, and primer $(GTG)_5$ (5P-GTGGTGGTGGTGGTG-3P) was also used in addition to primers ERIC and REP. Rep fingerprints sharing

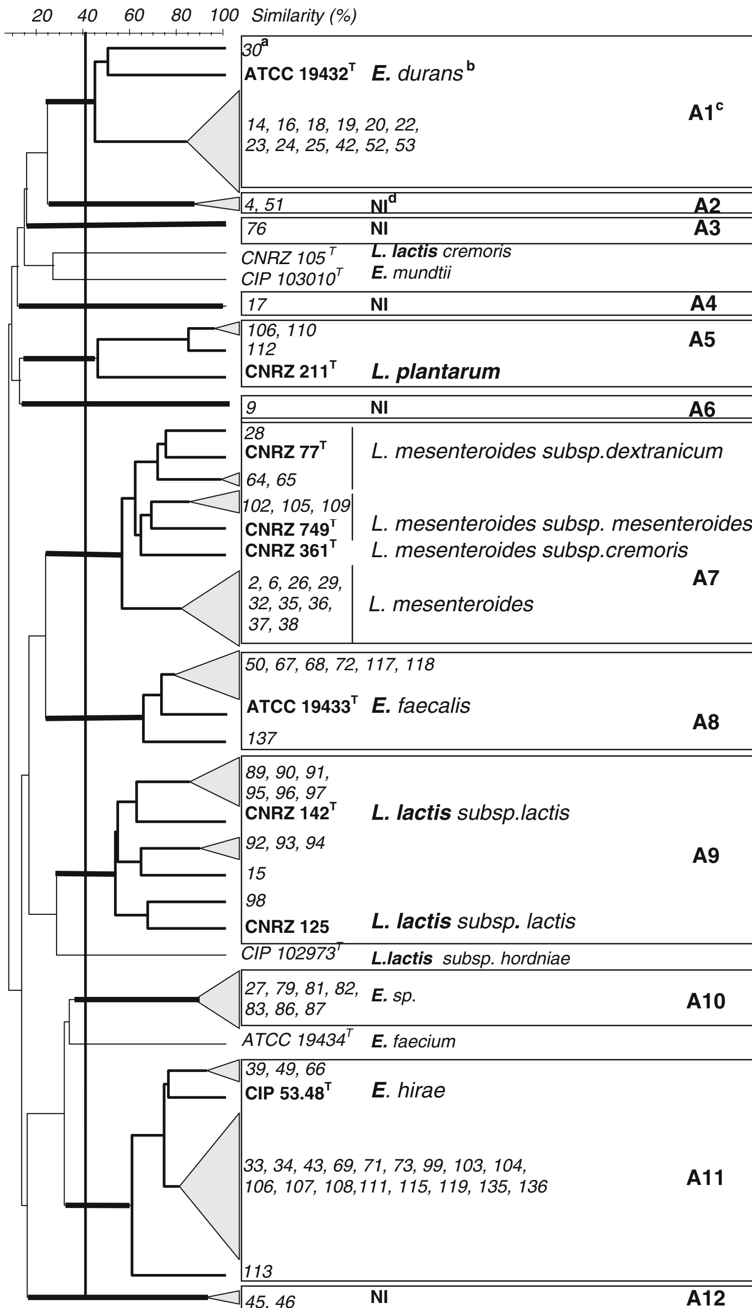

Fig. 1 Dendrogram based on numerical analysis of the 84 concatenated Rep-PCR fingerprints combining ERIC, REP, and $(GTG)_5$ fingerprints. [a]Name of isolate or type strain. [b]Genotypic affiliation by Rep-PCR, *in bold* Rep-PCR affiliation confirmed by specific PCR. [c]No. of the Rep-PCR group. [d]Nonidentified

more than 40% similarity were grouped together. Rep fingerprints were analyzed as described by Berthier et al. (2001). Briefly, isolates with Rep fingerprints sharing

more than 40% similarity with the Rep fingerprint of a type strain were presumptively affiliated to the species that that strain represented. Dissimilar fingerprints were assumed to be fingerprints of different strains. To avoid the erroneous assessment of two dissimilar fingerprints instead of one, similarity between fingerprints was deduced from both clustering analysis and visual inspection. Fingerprints were assumed to be similar when they had the same bands with the same relative intensity. Dissimilar Rep fingerprints within each Rep group were called fingerprint 1, fingerprint 2, …, fingerprint *x*, revealing genotype 1, genotype 2, … genotype *x*.

2.6 Specific PCR

Specific PCR was applied to isolates representative of each Rep-PCR group. The primers used, the microorganisms targeted, the negative and positive controls, and the corresponding references are shown in Table 1.

2.7 Phenotypic characterization

2.7.1 Identification purpose

The ability of lactococci to produce acid from lactose, glucose, or maltose was assessed in M17-lactose, glucose, or maltose (10 g.L^{-1}) broth incubated at 30 °C for 24 h in the presence of bromocresol purple (0.04 g.L^{-1}). The ability of lactococci to grow in the presence of 4% and 6.5% NaCl (gram per kilogram) was tested in M17 broth incubated at 30 °C for 48 h. The ability of lactococci to grow at 10 °C and 40 °C was tested with M17 plates incubated for 10 days and 48 h, respectively. Arginine-positive(negative) lactococci were distinguished by their white (yellow) colonies after growth on M16BCP medium (Thomas 1973) at 30 °C for 24 h.

2.7.2 Technological purpose

The ability of lactococci and leuconostocs to acidify at 25 °C, 30 °C, 37 °C, 42 °C, and 50 °C was tested in 10 mL sterile reconstituted skimmed milk inoculated with 10^6 to 10^7 cfu. mL^{-1}. pH was recorded after 48 h, i.e., at the end of acidification. The experiment was performed in triplicate, and the three recorded pH values were averaged (the standard deviations ranged from 0.01 U to 0.04 U). Protease-positive lactococci were distinguished by the transparent halo around their colonies after growth at 30 °C on milk agar. Citrate-positive (negative) isolates were distinguished by the blue (white) color of KMK liquid medium (Kempler and McKay 1980) after their growth at 30 °C for 48 h. Protease and citrate experiments were performed in triplicate.

3 Results

3.1 Isolation of strains

Eighty-four Gram-positive and catalase-negative isolates, including 81 cocci, were collected from 79 samples representing 40 farms (Table 2). Of the 84 strains isolated,

Table 1 Description of specific PCR used in this study

Organisms targeted	Pair of primers used	Targeted genes	Positive controls	Negative controls	References
Genus	Enc38a	23 S	*E. faecalis*^{T}	*L. lactis*^{T}	Frahm et al. (1998)
Enterococcus	Reverse of 23	rDNA	*E. durans*^{T}		Berthier and Ehrlich (1998)
			E. hirae^{T}		This work[b]
			E. faecium^{T}		
Species	Lpl 16	16 S/23 S spacer rDNA	*L. plantarum*^{T}	*L. paraplantarum*^{T}	Berthier and Ehrlich (1998)
L. plantarum				*L. pentosus*^{T}	
Subspecies	Lhis6R[a]	Histidine biosynthesis operon	*L. lactis* subsp. *lactis*^{T}		Beimfohr et al. (1997)
L. lactis lactis	Lhis5F		*L. lactis* subsp. *cremoris*^{T}		
L. lactis cremoris			*L. lactis* subsp. *hordniae*^{T}		

[a] Could not distinguish between subspecies *lactis* and *hordniae* of *Lactococcus lactis*

[b] The same PCR conditions as *Lactobacillus plantarum* (Berthier and Ehrlich 1998) were applied

38% were obtained from raw cow's, ewe's, and goat's milks, and 62% from laboratory-fermented ewe's, goat's, mare's, and camel's milks. Among the cocci, 62% were isolated on MRS-vancomycin plates and 38% on M17 plates. From two to 16 isolates (average of nine) were available per medium for each source. The enterococci isolates were collected from both MRS and M17 plates, while the lactococci isolates were collected only from M17 plates and the leuconostocs isolates only from MRS plates, which is usual (Mathot et al. 1994). Enterococci that were isolated on MRS-vancomycin plates originated from fermented milks.

3.2 Genotypic characterization

Table 2 summarizes the different genera, (sub)species and genotypes identified among the isolated cocci, according to raw and fermented milks analyzed.

3.2.1 (Sub)species assignation of isolates

Seventy-three percent of the isolates (61 out of 84) were assigned with confidence to seven established mesophilic LAB species or subspecies from four genera, i.e., *Enterococcus* species (41 isolates; *E. hirae* (21), *E. durans* (13), *E. faecalis* (7)), *Leuconostoc* subspecies (six isolates, *L. mesenteroides* subsp. *mesenteroides* (3), *L. mesenteroides* subsp. *dextranicum* (3)), *Lactococcus* subspecies (11 isolates, *L. lactis* subsp. *lactis*), and *Lactobacillus* species (three isolates, *L. plantarum*). Nineteen additional isolates were assigned with confidence to *Enterococcus* sp. or *L. mesenteroides*.

Eighty-one isolates formed nine groups (A1, A2, A5, and A7–A12) and three isolates (9, 17, and 76) were alone (A3, A4, and A6, respectively), according to the clustering of their Rep-PCR fingerprints at 40% similarity or higher (Fig. 1). Seventy isolates shared more than 40% similarity with fingerprints for six established LAB (sub)species, *L. mesenteroides*, *E. hirae*, *E. durans*, *E. faecalis*, *L. lactis* subsp. *lactis*, and *L. plantarum*. The ten isolates of group A10 were related to *E. faecium* and *E. hirae*, but their fingerprints were less than 40% homologous with the fingerprints of either *E. faecium* or *E. hirae*. The *Enterococcus*, *L. plantarum*, and *L. lactis* subsp. *lactis* assignations were confirmed by genus- and (sub)species-specific PCR on 13 isolates representing groups A1 (isolate nos. 2 and 30), A5 (isolate no. 106), and A8–A11 (isolates no. 15, 27, 33, 39, 50, 89, 92, 98, 113, and 137). In so doing, the closely related *L. paraplantarum*, *L. pentosus*, and *L. lactis* subsp. *cremoris* (sub)species were definitively excluded. Six *L. mesenteroides* isolates of group A7 had subsp. *dextranicum* or subsp. *mesenteroides*-type strainlike fingerprints (Fig. 2; fingerprints 1–3). The other nine had a distinct Rep-fingerprint (Fig. 2; fingerprint 4), which presented less similarity to the subspecies-type Rep fingerprints than Rep fingerprints 1–3 of *Leuconostoc* isolates and could not be related to any of the subspecies-type Rep fingerprints. *L. mesenteroides cremoris*-type strain (Fig. 2) had a unique Rep fingerprint. Rep group A9 contained *L. lactis* subsp. *lactis* genotypes (from 11 isolates and the type strain) but not the *L. lactis* subsp. *cremoris*-type genotype, as the *L. lactis* subsp. *lactis*-type strainlike group A obtained from Multilocus Sequence Analysis and $(GTG)_5$-PCR fingerprinting (Rademaker et al. 2007).

Table 2 Occurrence and identity of mesophilic lactic cocci isolated from Algerian raw and fermented milks

Dairy products		Isolates								
Nature[a]	Geographic source	Number of milk/ farm sampled	Number	Medium for isolation	Rep group[b]	Genus[b,c]	Species[b,c]	Subspecies[b,c]	Biovar[d]	Genotype no.[e] (number of isolates)
Cow's raw milk (RCo)	West Algeria, Tlemcen City	15/6	4	MRS-vancomycin	A7	*Leuconostoc*	*mesenteroides*			**4** (2)
					A2, A6	NI				**1** (1), **1** (1)
			11	M17	A9	***Lactococcus***	***lactis***	*lactis diacetylactis*		**4** (1)
					A1	***Enterococcus***	*durans*			**1** (9)
					A4	NI				**1** (1)
Goat's raw milk (RG)	West Algeria, Tlemcen City	14/8	7	M17	A11	***Enterococcus***	*hirae faecalis*			**1** (2), **2** (2)
					A8	***Enterococcus***				**1** (3)
Goat's fermented milk (FG)	West Algeria, Tlemcen City	3/2	2	MRS-vancomycin	A7	*Leuconostoc*	*mesenteroides*	*dextranicum*		**2** (2)
Mare's fermented milk (FM)	West Algeria, Tlemcen City	6/3	10	M17	A9	***Lactococcus***	***lactis***	*lactis diacetylactis*		**1** (6), **2** (3), **3** (1)
		5/3	7	MRS-vancomycin	A10	***Enterococcus***	sp.			**1** (6)
					A3	NI				**1** (1)
	around Tlemcen									
Ewe's raw milk (RE)	West Algeria, around Tlemcen	14/6	10	M17	A11	***Enterococcus***	*hirae*			**1** (1), **2** (2)
					A1	***Enterococcus***	*durans*			**1** (3)
					A8	***Enterococcus***	*faecali*			**1** (1)
					A2, A12	NI				**1** (1), **1** (2)
Ewe's fermented milk (FE)	West Algeria, Tlemcen City	17/9	12	MRS-vancomycin	A7	*Leuconostoc*	*mesenteroides*	*dextranicum*		**1** (1)
					A7	*Leuconostoc*	*mesenteroides*			**4** (7)
					A11	***Enterococcus***	*hirae*			**1** (2)

Table 2 (continued)

Dairy products			Isolates					
					A10	***Enterococcus***	sp.	**1** (1)
					A1	***Enterococcus***	*durans*	**2** (1)
Camel's fermented milk (FCa)	Algerian Sahara, Bechar	5/3	13[f]	MRS-vancomycin	A7	*Leuconostoc*	*mesenteroides mesenteroides*	**3** (3)
					A11	***Enterococcus***	*hirae*	**1** (10)
	Tamanrasset		5	M17	A11	***Enterococcus***	*hirae*	**1** (2)
					A8	***Enterococcus***	*faecalis*	**1** (3)
Total		79/40	81[f]	2	11[f]	3[f]	5 established species[f] 3 established subspecies	19[f] genotypes

NI nonidentified

[a] Abbreviated form

[b] See Fig. 1

[c] Data in bold are as determined by specific PCR

[d] Phenotype on KMK medium

[e] As determined by Rep fingerprinting

[f] In addition, three *Lactobacillus plantarum* isolates (group A5) exhibiting two distinct Rep fingerprints were isolated

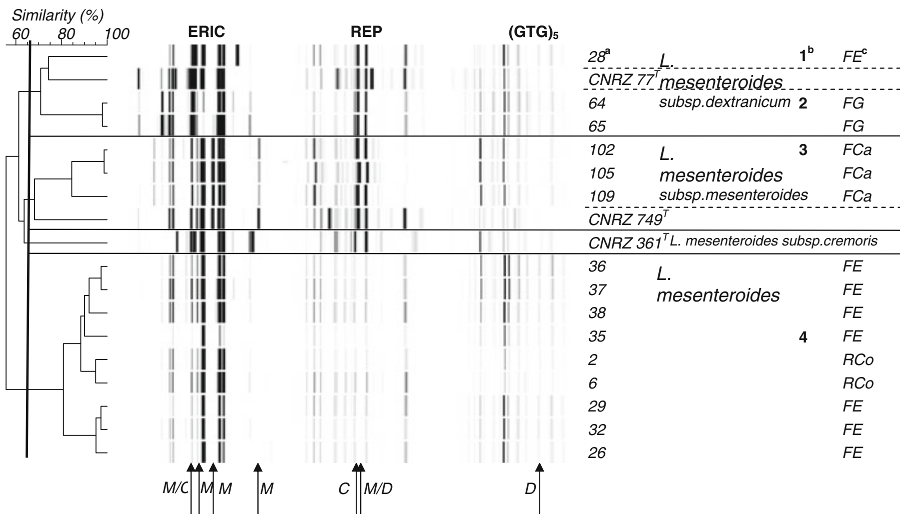

Fig. 2 Dendrogram and Rep fingerprints of *Leuconostoc*. Rep-group=group A7. Clustering was made with concatenated Rep-fingerprints combining ERIC, REP, and $(GTG)_5$ fingerprints. *Solid horizontal lines* separate *Leuconostoc* subspecies. *Dotted horizontal lines* separate dissimilar fingerprints within *Leuconostoc* subspecies. [a]Name of isolate or type strain. [b]Genotypic affiliation by Rep-PCR. [c]See Table 2 for the meaning of RCo, RG, RS, FCa, FE, FG, and FM. *Arrows* indicate specific bands of *Leuconostoc mesenteroides* subsp. *cremoris* (*C*), *Leuconostoc mesenteroides* subsp. *dextranicum* (*D*), and *Leuconostoc mesenteroides* subsp. *mesenteroides* (*M*) fingerprints

The five groups A2–A4, A6, and A12 (seven isolates) had fingerprints with less than 40% similarity with the type fingerprints shown in Fig. 1. Their DNA responded negatively to the *Enterococcus*-specific PCR. Their fingerprints did not cluster (data not shown) with those of cocci, such as *L. garviae*, *Streptococcus thermophilus*, and *S. gallolyticus*, which are starters for some dairy products. They were not further identified because they were rarely isolated and because they were not affiliated to any of the LAB species previously cited as potential starters.

3.2.2 Genotypic diversity within (sub)species

Twenty-one different Rep-PCR fingerprints were distinguished from the 84 isolates according to their clustering. One fingerprint was observed in each of the seven groups A2–A4, A6, A8, A10, and A12; two different fingerprints in each of the three groups A1, A5, and A11 (*E. durans*, *L. plantarum*, and *E. hirae*); and four different fingerprints in groups A9 (*L. lactis* subsp. *lactis*) and A7 (*L. mesenteroides*, Fig. 2), with one in subspecies *mesenteroides* and two in subspecies *dextranicum*.

3.3 Phenotype of the isolates

Besides common phenotypic traits, the *L. lactis* and *Leuconostoc* isolates exhibited phenotypic diversity in spite of the small number of isolates analyzed (ten and 15 isolates, respectively). Some phenotypes were atypical. All *L. lactis* isolates grew in the presence of 4.0% NaCl but not 6.5% NaCl and from 10 °C to 40 °C and were

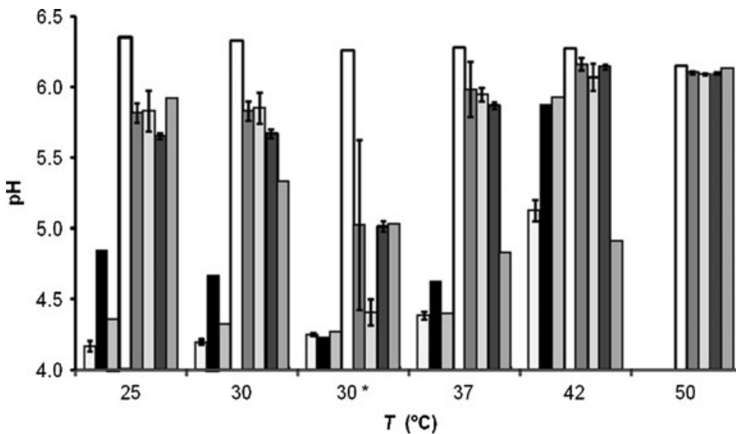

Fig. 3 Acidification potential of the lactococci and leuconostocs isolates in milk. Sterile reconstituted milks were incubated for 48 h at five different temperatures after inoculation either with one out of the isolates or with one type strain. *30** milk incubated at 30 °C and supplemented with yeast extract (0.1%). Isolates and type strains affiliated to the same (sub)species and having similar pH values were grouped. Individual pH when isolates or type strains were not grouped ($N=1$, standard deviations ranged between 0.01 U and 0.04 U), or mean pH and standard deviation of groups ($N>1$), for *Lactococcus lactis* subsp. *lactis* with genotypes 1 or 2 ($N=9$; nine isolates) (*light gray*); *Lactococcus lactis* subsp. *lactis* with genotype 3 ($N=1$; one isolate) (*black*); *Lactococcus lactis* subsp. *lactis* type strain ($N=1$; CNRZ 142^T) (*gray*); noninoculated milk ($N=1$) (*white*); *Leuconostoc mesenteroides* subsp. *dextranicum* ($N=4$; three isolates with *Leuconostoc* genotypes 1 or 2 and CNRZ 77^T) (*gray*); *Leuconostoc mesenteroides* subsp. *mesenteroides* ($N=4$, three isolates with *Leuconostoc* genotype 3, and CNRZ 749^T) (*light gray*); *Leuconostoc mesenteroides* ($N=9$; nine isolates with *Leuconostoc* genotype 4) (*dark gray*); *Leuconostoc mesenteroides* subsp. *cremoris* ($N=1$; CNRZ 361^T) (*gray*)

protease-, maltose-, galactose-, lactose- arginine-, and citrate-positive, which is typical of the *L. lactis* subsp. *lactis* biovar *diacetylactis* phenotype. Isolate no. 98 (genotype *L. lactis* 3) needed a supplementation of milk with yeast extract to acidify to the same extent than the isolates having genotypes 1 and 2 (Fig. 3). The latter isolates were excellent acidifiers from 25 °C to 37 °C, and still good acidifiers at 42 °C which is an atypical *L. lactis* subsp. *lactis* phenotype (Fig. 3). In the temperature range of 25 °C to 37 °C, cow's milk was less acidified by leuconostocs isolates than by lactococci isolates, which is usual (Fig. 3). All *Leuconostoc* isolates produced dextran. Absence of dextran production and the lowest pH distinguished the type strain of *L. mesenteroides* subsp. *cremoris*. Contrary to the *Leuconostoc* isolates and type strains of subspecies *mesenteroides* and *dextranicum*, the type strain of *L. mesenteroides* subsp. *cremoris* showed improving acidification of the milk as the temperature was increased from 25 °C to 37 °C, and still at 42 °C (Fig. 3). However, the type strain had an optimal temperature range of 25 to 30 °C for acidification when incubated in broth as previously described (Cooper and Collins 1978).

KMK liquid medium indicated citrate use for all isolates that coagulated it, i.e., all *E. faecalis* and all *L. lactis*. KMK medium was not coagulated by leuconostocs and other enterococci and indicated a weak citrate use (green color) for *L. mesenteroides* subsp. *cremoris* type strain and other *Enterococcus* isolates.

4 Discussion

In this study, wild strains of leuconostocs (four different strains) and lactococci (four different strains) were isolated from Algerian raw and fermented milks and reliably characterized. Two strains of *L. lactis* subsp. *lactis* and one strain of *L. mesenteroides* subsp. *mesenteroides* are possible primary starters for the traditional production of Raib and its by-products. They were isolated from camel's and mare's fermented milks, respectively. Enterococci were also unintentionally isolated. The isolates were genotyped, and most strains were reliably identified at the (sub)species level.

The major groups of mesophilic lactic cocci isolated in this study from milks spontaneously fermented in the laboratory are similar to those previously isolated on similar media from their traditionally fermented counterparts (Benkerroum and Tamime 2004). *E. hirae* was the main *Enterococcus* species, which is unusual for dairy products (Foulquie Moreno et al. 2006). Isolates assigned to *Enterococcus* sp. may belong to species *Enterococcus lactis*, as these isolates were genotypically related to *E. faecium*. This species, recently proposed for enterococci isolated from different dairy products (Bauer et al. 2009; Morandi et al. 2011a; Sukhodolets et al. 2005), has been recently validated (Morandi et al. 2011b).

Phenotypic plus genotypic methods distinguished four different strains among 15 *Leuconostoc* isolates. Four genotypes were obtained, genotype 4 having unique phenotypic traits. They were all *L. mesenteroides*, unlike those predominating in Moroccan Lben, which are *Leuconostoc pseudomesenteroides* (Ouadghiri et al. 2009). Three genotypes were related to the genotype of the type strains of subspecies *mesenteroides or dextranicum.* Isolates having these genotypes exhibited the same phenotypic traits as their related subspecies-type strain. The fourth genotype (no. 4), which was shared by nine isolates that produced dextran, clustered with the other *L. mesenteroides* genotypes by Rep-PCR fingerprinting and combined bands characteristic of each *Leuconostoc* subspecies-type fingerprints. The subspecies *mesenteroides* strain isolated from camel's fermented milk could be a useful starter when milk has high levels of nutrients, i.e., ewe's and camel's milks (Abu-Tarboush 1994; Park et al. 2007), as shown by its enhanced acidification of milk in the presence of yeast extract. Lower pH in the presence of yeast extract in milk, especially for the isolates of *L. mesenteroides* subsp. *mesenteroides*, was in accordance with the lower requirements of this subspecies for growth (Garvie 1986). *L. mesenteroides* subsp. *cremoris* is the unique leuconostoc adjunct available in commercial starters. The *cremoris*-type strain did not produce dextran from sucrose, and was also distinguished by its ability to acidify nonsupplemented cow's milk to (a) lower pH at temperatures from 25 °C to 42 °C and (b) improved acidification as the temperature increased. These last two traits have not yet been described for the subspecies *cremoris*. Atypical metabolic features and ribotype profile, but typical SDS-PAGE profile, have previously been described for the *cremoris*-type strain (Cooper and Collins 1978; Villani et al. 1997). Thermotolerance to 50 °C was recently observed for wild strains of dairy lactococci (Drici et al. 2010) although leuconostocs and lactococci are usually mesophilic bacteria. It would be interesting to explore the link between growth/acidification, thermotolerance, and dextran production observed in this study for leuconostocs. The failure in isolating *L. mesenteroides* subsp. *cremoris* strains from raw and fermented

milks in this study has already been noticed for raw milk cheeses (Hemme and Foucaud-Scheunemann 2004). Such strains could prove to be useful starters when LAB growth in milk is poor or when the fermentation temperature is above the usual optimal temperature for leuconostocs, 27 °C (Cooper and Collins 1978), as can be the case during the manufacture of Raib. The strains of leuconostocs isolated in this study were probably capable of metabolizing citrate, as is the case for all leuconostocs described so far (Hemme and Foucaud-Scheunemann 2004); they were recorded as citrate-negative on KMK medium because they grew very poorly on it.

Phenotypic plus genotypic methods distinguished four different strains among 11 lactoccocci isolates. Two of them expressed high acidifying capabilities over a large temperature range of 25 °C to 42 °C. The latter two strains isolated from mare's fermented milks could be useful starters because of their specific phenotype which may be due to the nondairy niche before contaminating milk. They may also exhibit, contrary to lactococci commonly used as dairy starter, fewer amino acid auxotrophies and various additional capabilities such as additional flavor-forming activities and greater stress tolerance (Ayad et al. 1999; Nomura et al. 2006; Smit et al. 2004). These capabilities may be beneficial to milk fermentation and will be interesting to explore. All lactococci isolates exhibited a protease activity, *L. lactis* subsp. *lactis*-like genotypes, and usual *L. lactis* subsp. *lactis* biovar *diacetylactis* phenotype. This subspecies is commonly isolated from Raib by-products (Benkerroum and Tamime 2004).

5 Conclusion

Among the eight wild strains of lactococci and leuconostocs collected in this study, two protease-positive *L. lactis* subsp. *lactis* biovar *diacetylactis* strains, *L. lactis* 1 and 2, and the *L. mesenteroides* subsp. *mesenteroides* strain had high acidifying potential in milk. They were isolated from mare's and camel's fermented milks, respectively. These strains can now be tested in the manufacture of Raib. It would be interesting to investigate whether thermophilic homofermentative LAB also play a part in the spontaneous fermentation for Raib. Efficient strain typing is essential for reliable identification of particular strains and tracking of starter cultures, and this study shows that Rep-PCR combining three independent fingerprints is a reliable and discriminating method for leuconostocs, enterococci, and lactococci strains.

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References

Abou-Donia S (2008) Origin, history and manufacturing process of Egyptian dairy products. Alex J Food Sci Technol 5:51–62

Abu-Tarboush HM (1994) Growth-behavior of Lactobacillus acidophilus and biochemical characteristics and acceptability of acidophilus milk made from camel milk. Milchwissenschaft 49:379–382

Ayad EHE, Verheul A, deJong C, Wouters JTM, Smit G (1999) Flavour forming abilities and amino acid requirements of *Lactococcus lactis* strains isolated from artisanal and non-dairy origin. Int Dairy J 9:725–735

Bauer R, Bekker JP, Nv W, du Toit C, Dicks LMT, Kossmann J (2009) Exopolysaccharide production by lactose-hydrolyzing bacteria isolated from traditionally fermented milk. Int J Food Microbiol 131:260–264
Beimfohr C, Ludwig W, Schleifer KH (1997) Rapid genotypic differentiation of *Lactococcus lactis* subspecies and biovar. Syst Appl Microbiol 20:216–221
Bencharif A (2001) Stratégies des acteurs de la filière lait en Algérie: états des lieux et problématiques. Options Méditerranéennes Série B Etudes et Recherches 32:25–45
Benkerroum N, Tamime AY (2004) Technology transfer of some Moroccan traditional dairy products (lben, jben and smen) to small industrial scale. Food Microbiol 21:399–413
Berthier F, Ehrlich SD (1998) Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16 S/23S rRNA spacer region. FEMS Microbiol Lett 161:97–106
Berthier F, Beuvier E, Dasen A, Grappin R (2001) Origin and diversity of mesophilic lactobacilli in Comté cheese, as revealed by PCR with repetitive and species-specific primers. Int Dairy J 11:293–305
Chammas GI, Saliba R, Corrieu G, Beal C (2006) Characterisation of lactic acid bacteria isolated from fermented milk "laban". Int J Food Microbiol 110:52–61
Cooper RK, Collins EB (1978) Influences of temperature on growth of *Leuconostoc cremoris*. J Dairy Sci 61:1085–1088
Drici H, Gilbert C, Kihal M, Atlan D (2010) Atypical citrate-fermenting *Lactococcus lactis* strains isolated from dromedary's milk. J Appl Microbiol 108:647–657
Foulquie Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L (2006) The role and application of enterococci in food and health. Int J Food Microbiol 106:1–24
Frahm E, Heiber I, Hoffmann S, Koob C, Meier H, Ludwig W, Amann R, Schleifer KH, Obst U (1998) Application of 23 S rDNA-targeted oligonucleotide probes specific for enterococci to water hygiene control. Syst Appl Microbiol 21:450–453
Garvie E (1986). Genus *Leuconostoc*. In: Sneath P, Mair J, Sharpe M, Holt J, (eds) Bergey's manual on systematic bacteriology, Baltimore
Gaya P, Babin M, Medina M, Nunez M (1999) Diversity among lactococci isolated from ewes' raw milk and cheese. J Appl Microbiol 87:849–855
Gevers D, Huys G, Swings J (2001) Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol Lett 205:31–36
Guizani N, Kasapis S, Al Ruzeiki M (2001) Microbial, chemical and rheological properties of laban (cultured milk). Int J Food Sci Technol 36:199–205
Hemme D, Foucaud-Scheunemann C (2004) *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int Dairy J 14:467–494
Kempler GM, McKay LL (1980) Improved medium for detection of citrate-fermenting *Streptococcus lactis* subsp. *diacetylactis*. Appl Environ Microbiol 39:926–927
Khaldi R, Haddad M, Padilla M (2006) Attentes et attitudes des consommateurs urbains face aux produits laitiers: cas de la commune de Tunis. Options Méditerranéennes Série A Séminaires Méditerranéens 78:365–375
Koussou M, Duteurtre G, Mopota L (2007) Consommation de lait dans les bars laitiers de la ville de N'Djamena au Tchad. Rev Elev Med Vet Pays Trop 60:39–44
Mathot AG, Kihal M, Prevost H, Divies C (1994) Selective enumeration of *Leuconostoc* on vancomycin agar media. Int Dairy J 4:459–469
Morandi S, Brasca M, Lodi R (2011a) Technological, phenotypic and genotypic characterisation of wild lactic acid bacteria involved in the production of Bitto PDO Italian cheese. Dairy Sci Technol 91:341–359
Morandi S, Cremonesi P, Povolo M, Brasca M (2011b) *Enterococcus lactis* sp. nov. from Italian raw milk cheeses. Int J Syst Evol Microbiol. doi:10.1099/ijs.0.030825-0
Nomura M, Kobayashi M, Narita T, Kimoto-Nira H, Kamoto T (2006) Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. J Appl Microbiol 101:396–405
Ouadghiri M, Vancanneyt M, Vandamme P, Naser S, Gevers D, Lefebvre K, Swings J, Amar M (2009) Identification of lactic acid bacteria in Moroccan raw milk and traditionally fermented skimmed milk 'lben'. J Appl Microbiol 106:486–495
Park YW, Juarez M, Ramos M , Haenlein GFW (2007) Physico-chemical characteristics of goat and sheep milk. Small Ruminant Res 68:88–113
Prashant TSK, Singh R, Gupta SC, Arora DK, Joshi BK, Kumar D (2009) Phenotypic and genotypic characterization of lactobacilli from churpi cheese. Dairy Sci Technol 89:531–540
Rademaker JLW, Herbet H, Starrenburg MJC, Naser SM, Gevers D, Kelly WJ, Hugenholtz J, Swings J, van Hylckama Vlieg JET (2007) Diversity analysis of dairy and nondairy *Lactococcus lactis* isolates, using a novel multilocus sequence analysis scheme and (GTG)5-PCR fingerprinting. Appl Environ Microbiol 73:7128–7137

Samet-Bali O, Bellila A, Ayadi MA, Marzouk B, Attia H (2010) A comparison of the physicochemical, microbiological and aromatic composition of traditional and industrial leben in Tunisia. Int J Dairy Technol 63:98–104

Smit BA, Engels WJM, Bruinsma J, Vlieg JETV, Wouters JTM, Smit G (2004) Development of a high throughput screening method to test flavour-forming capabilities of anaerobic micro-organisms. J Appl Microbiol 97:306–313

Sukhodolets V, Botina S, Lysenko A, Trenina M (2005) The lactic acid enterococci *Enterococcus faecium* and *Enterococcus durans*: nucleotide sequence diversity in 16 S rRNA genes. Microbiology 74:704–708

Tantaoui-Elaraki A, El Marrakchi A (1987) Study of Moroccan dairy products: lben and smen. J Appl Microbiol Biotechnol 3:211–220

Terzaghi BE, Sandine WE (1975) Improved medium for lactic streptococci and their bacteriophages. Appl Microbiol 29:807–813

Thomas T (1973) Agar medium for differentiation of *Streptococcus cremoris* from other bacteria. NZ J Dairy Sci Tech 8:70–71

Vedamuthu ER (1994) The dairy *Leuconostoc*—use in dairy products. J Dairy Sci 77:2725–2737

Villani F, Moschetti G, Blaiotta G, Coppola S (1997) Characterization of strains of *Leuconostoc mesenteroides* by analysis of soluble whole-cell protein pattern, DNA fingerprinting and restriction of ribosomal DNA. J Appl Microbiol 82:578–588

Wolfgang L (2007) Nucleic acid techniques in bacterial systematics and identification. Int J Food Microbiol 120:225–236