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Use of *Lactococci* isolated from Moroccan traditional dairy product: Development of a new starter culture

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Ninety (90) strains were isolated from Moroccan traditional dairy product and identified using biochemical and molecular tests. Among the 20 identified lactic acid cocci, two strains were selected for their important acidifying activity: *Lactococcus lactis* subsp*. lactis* (AML8) and *Lactococcus lactis* subsp*. cremoris* (BML2). Both revealed remarkable acidifying activity, especially when they were mixed in cultures, even in greater values than in the traditional Lben (a Moroccan dairy product). Sensory analysis showed that the so fermented milk has been more appreciated than the Lben. These results show the potential of the two strains as possible culture starter for fermented dairy product.

Key words: *Lactococci*, acidifying activity, fermented milk, Lben.

INTRODUCTION

The isolation of microorganisms from natural habitats is, for the food industry, an inexhaustible source of new and useful strains. Lactic acid bacteria (LAB) are important for the food and dairy industries, because lactic acid, as well as other organic acids produced by these bacteria is a natural preservative (Mohd Adnan and Tan, 2007). Among LAB, *Lactococcus lactis* is the most extensively studied organism with many works dealing with the physiology of growth, carbon and nitrogen metabolism, or regulatory networks. *Lactococcus lactis* metabolism is described as homo-fermentative and produces only L (+) lactic acid (2-hydroxypropanoic acid) with high yield (Kaloyan et al., 2008).

In Morocco, the Lben is one of a large variety of traditional fermented dairy products highly appreciated by consumers. Despite their usual non-conformity to the official regulatory standard they contribute undeniably to urban-rural income (Benkeroum and Tamime, 2004). Reduced costs and low energy requirements of such industries helps stimulate rural and semi rural development. The biodiversity of the microorganisms involved in this process is a fundamental factor for the conservation of the specificity and original characteristics of dairy products. Lactic acid bacteria, belonging especially to the *Lactococcus* and *Leuconostoc* genus, are the dominant microorganisms in traditional Lben (Tantaoui et al., 1983). Lben contains also a considerable amount of yeasts (*Candida lusitaniae, Candida tropicalis* and *Candida albicans*) (Samet-Bali et al., 2012). *Escherichia coli, Salmonella enteritidis* and *Staphylococcus aureus* have also been detected (Benkerroum and Tamine, 2004; Feresu and Nyathi, 1990). This indicates that, despite real efforts in this field, the technology remains often applied without a global understanding of the principles regulating the fermentation process and the conditions required to ensure quality and safety of the product. Such approach

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presents a significant risk for consumer health and leads to marketing damaging (Motarjemi, 2002). In this context, many traditional Moroccan dairy products failed their transfer to the industrial scale which requires a good knowledge of the performances and the technological potentialities of the wild-type lactic bacteria implied in their fermentation.

The objective of the present study was to identify, phenotypically and genotypically, *Lactococci* isolated from traditional Moroccan dairy product samples and to screen these isolates for desirable properties such as acidity. The sensorial characteristics of fermented milk obtained by using these strains will also be compared with those of the traditional Lben.

MATERIALS AND METHODS

Traditional Moroccan dairy product samples

A total of 50 samples of traditional dairy product were collected and aseptically transported in iceboxes (4°C) from different areas of Morocco (northern, north-central and east regions), mainly from small farms located around the cities. The samples were collected in various seasons, over a period of two years.

Isolation of LAB

10 ml of traditional dairy product sample were homogenized with 90 mL of normal saline (9 g/ L) then 10 fold serial dilutions were prepared using autoclaved normal saline and 1 ml of each serially diluted sample was poor-plated in duplicate on MRS and M17 agar media (Biokar, Diagnostics, France) and incubated at the optimal temperature at 35°C for 24 h.

After incubation, the isolated colonies were purified by streaking twice onto appropriate agar media plates. For immediate use, they were maintained at 4°C and streaked every 4 weeks on agar plates. For long-term storage, purified isolates were kept at -20°C in skim milk plus glycerol (85/15 (v/v).

Identification of LAB

Morphological and biochemical identification

The isolates obtained were identified by morphological and biochemical techniques according to the criteria given by Teixeira (2000). All isolates were initially characterized for their morphology by microscopy and Gram staining, and then tested for oxidase and catalase production. Gram positive and Catalase negative cocci were used for further identification. Growth at 10, 35 and 45°C was examined on M17 broth. Carbohydrate fermentations were determined twice with the aid of the API 50 CH medium according to the manufacturer's instructions (BioMerieux, Marcy-l'Etoile, France). Salt tolerance was tested by incorporating 2, 4 and 6.5% of NaCl on M17 broth. Production of CO_2 was also evaluated in M17 broth containing inverted Durham's tube.

Molecular identification

Genetic identification of presumptive *Lactococcus* sp. was carried out by molecular techniques based on the amplification and sequencing of the 16S rRNA gene. This methodology is currently the most used for bacterial phylogeny. It allowed the establishment of large databases. Bacterial DNA extraction from bacteria was carried out according to standard methods (Marmur, 1961). For polymerase chain reaction amplification, universal primers, fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-TACGGCTACCTTGTTACGACTT- 3') were used to amplify the 16S rRNA gene (Weisberg et al., 1991). Direct sequencing of the PCR products was performed on an ABI PRISM sequencing apparatus (ABI Prism 3130 Genetic Analyser, Applied Biosystem) and data analysis was done by sequence analysis software (Sequence analysis 5.2.0, Collection software: foundation data collection version 3.0).

Sample preparation and physico-chemical characterization

Culture conditions

Pure and mixed cultures: Individual strains were pre-grown for 24 h at 35°C in Ultra High Temperature treated milk (UHT). Subsequently, 2% (v/v) of each culture was added to 100 mL UHT milk to obtain approximately 10^7 CFU/ mL and incubated at 35°C. Mixed cultures of two strains were performed at 35°C by inoculating UHT milk with 1% of each strain (pre-grown for 24 h).

Traditional way of manufacturing Lben: The traditional Lben was prepared in the laboratory by spontaneous fermentation of raw milk at the temperature of 35°C until coagulation which may take up to 16 h. For gelation, the product, known as Raib, is churned during approximately 45 min for obtaining the Lben and some amount of raw butter called zebda beldia (Tantaoui-Elaraki et al., 1983; Benkerroum et Tamine, 2004).

Physico-chemical characterization

The dry matter, fat content, pH, titrable acidity (equivalent lactic acid), lactose, enumeration of cells and biomass were followed. Each measurement was performed in quadruplicate.

Dry matter: Dry matter was determined according to standard methods (Afnor, 1993).

Fat content: Fat content in milk was determined by Gerber method as described by FAO (1997). Into butyrometer, 10.94 mL of sample was slowly added to 10 mL of sulphuric acid, then 1 mL of Amyl alcohol. The mixture was thoroughly stirred and centrifuged at 1100 rpm for 5 min. Then, the butyrometer was removed and placed in water bath at 65°C from 3 to 4 min. The fat content (in percent) was established according to the butyrometer scale.

pH: The pH values of inoculated milk were continuously measured by a glass electrode pH-meter (Inolab pH 730) and recorded automatically at 3-min intervals.

Titrable acidity: Titrable acidity, expressed in Dornic Degree (°D), was determined by titration with a standard solution of NaOH 0.1N using phenolphthalein as the indicator.

Lactose: Samples preparation and lactose analysis was performed according to the method of Fraysse et al. (2003) using an Agilent HP3DCE capillary electrophoresis instrument (Waldbronn, Allemagne) equipped with a CE LIF/UV cassette. The laser induced fluorescence detector was a Zetalif Discovery (Picometrics, France) coupled with a 488 nm argon laser (26 mW) (Spectra Physics, USA). Capillary (Polymicro Technologies, USA) dimensions were 14 cm effective length and 50 µm internal diameter. Running buffer was aminocaproic acid 40 mM with 0.02% hydroxypropylméthyl-

cellulose at pH 4.5 and the electrophoretic separation was conducted at 20 kV. Calibration was performed by labeling standard lactose solution prepared in M17 medium.

Enumeration of cells: Viable cell counts (CFU/mL) were determined by plating diluted samples in sterile normal saline on M17 agar, and incubating for 48 h at 35°C.

Biomass: Biomass concentration was determined by optical density (OD) measurement using the method of clarification described by Raynaud et al. (2005). One volume of milk culture was mixed with two volumes of a solution of clarification (EDTA 15 mm, NaOH 0.15 mm). The cellular pellets were washed 3 times with normal saline (NaCl 0.9 %) and suspended in the same solution again. The absorbance at 580 nm of the sample can then be measured directly or after dilution, in order to remain the spectrophotometer linearity range.

Sensory analysis

Sensory analyses have been conducted by a panel of five persons, previously trained for the sensory evaluation of manufactured dairy products. They were invited to compare the dairy product obtained by mixed culture to traditional Lben. The evaluation was scored in a 9-point negative to positive scale according to the method of Zamora et al. (2011).

RESULTS AND DISCUSSION

Identification of isolates

A total of 90 strains were obtained from 50 traditional dairy product samples harvested in different regions of Morocco. Twenty (20) isolates was identified as Gram-positive cocci. The colonies observed on M17 plates were small (1 mm diameter), compact and regular. Cells were revealed to be catalase and oxidase negative, non-spore-forming and homofermentative (absence of CO_2 production during glucose and galactose fermentations).

On API 50 CH trays, the 20 isolated cocci produced lactic acid from lactose, glucose, galactose and fructose. They were subdivided into two groups: (1) strains ADH (+) (arginine dihydrolase), acetoin (-) growing at 45°C and with 4% of NaCl, (2) strains ADH (-), acetoin (-) unable to grow at 45°C, sensitive to 4% salt concentrations. These results suggest that the group 1 isolates belong to the *Lactococcus lactis* subsp *lactis* and group 2 to the *Lactococcus lactis* subsp *cremoris*. In fact, according to the study of Badis et al. (2004) these tests distinguished lactococci from other related microorganisms and differentiated between *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris.* Subsequently, the 20 strains were genetically identified. 16S rRNA genes were amplified by PCR. The sequences obtained with fd1 and rp2 primers were 571 bp and 505 bp, respectively. It has been reported that the initial 500-bp sequence provides adequate differentiation for bacterial identification (Clarridge, 2004). Some reports indicate that less sequence was required: 400 bp or even less than 200 bp (Wilck et al., 2001). The sequences were compared to the EMBL, Gen Bank, DDJB and PDB databases, using BLAST NR 2.2.11 software through the National Center for Biotechnology Information (NCBI). The alignment of these sequences with nucleotide databases revealed a very high similarity (99%), with the nucleotide sequences of the genes coding for 16S rRNA bacterial strains of genus *Lactococcus* only. For twelve strains of *Lactococcus* (AML), the percentage of the highest similarity (99%) was obtained with strain of *Lactococcus lactis* subsp. *Lactis* (I 11403). For eight other strains of *Lactococcus* (BML), the percentage of the highest similarity (99%) was obtained with strain *L. lactis* subsp. *cremoris* (MG1363).

Acidifying activity

The lactic acid plays a particularly important role in fermented dairy industry. It acts as a natural preservative and for sensory characteristics of the product (acidity, flavor). Therefore, acidification activity is an important parameter for the selection of lactic starter culture strains. The time required to acidify milk from pH 6.7 to 5.5 (tpH 5.5) was determined at 35°C for 12 *L. lactis* subsp. *lactis* strains (AML) and the eight *L. lactis* subsp. *cremoris* strains (BML). Each strain was inoculated in milk in quadruplicate. As shown in Table 1, the time to reach pH 5.5 (tpH 5.5) by *L. lactis* subsp. *lactis* strains was significantly lower than by *L. lactis* subsp. *cremoris* ($P < 0.05$). The observed differences between both *Lactococci* (BML2 and AML8) may be due the fact that acidifying activity of each strain is linked to its specific proteolytic activity and nutrient transport system (Albenzio et al., 2001). Moreover, following the pH in milk during growth of the *L. lactis*, two strains characterized by low tpH 5.5 values could be highlighted out of the 20 strains; *L. lactis* subsp. *lactis* (AML8; tpH 5.5: 198 ± 12 min) and *L. lactis* subsp. *cremoris* (BML2; tpH 5.5: 237±13 min). These tpH 5.5 are inferior to that obtained in the study of Casalta et al. (1995) by *L. lactis* (416 min) in the same type of milk (cow's milk). Low values for tpH 5.5 reflect a high acidification activity for the considered starter culture (Chammas et al., 2006). A rapid decline of pH during the initial step of dairy fermentation is essential for the milk coagulation and the prevention or reduction of undesired microflora development. Two strains (AML8 and BML2) were retained for further experiments.

The optical density (OD) of the cultures was determined at the beginning of fermentation. They were 0.31 for *L. lactis* subsp. *lactis* and 0.29 for *L. lactis* subsp. *cremoris* in milk. This indicates that the number of cells used in the preparations was very similar, indicating well controlled procedures**.**

Pure culture of L. lactis subsp. lactis (AML8)

The titrable acidity was 56.7°D after 6 h and 64.4°D after

Table 1. Time (min) required to acidify milk from pH 6.7 to 5.5 at 35°C.

Lc. lactis		*Lc. cremoris*	
Strains [a]	**tpH 5.5 (min)** [b]	**Strains** [a]	**tpH 5.5 (min)** [b]
AML1	202± 12	BML1	241±17
AML2	295± 12	BML2	237±13
AML3	216±13	BML3	303±14
AML4	264±12	BML4	266±11
AML5	245±11	BML5	298±12
AML6	226±9	BML6	317±11
AML7	235±12	BML7	323±15
AML8	198±12	BML8	292±11
AML9	236± 17		
AML10	218±11		
AML11	229±13		
AML12	237±18		

[a]AML, *L. lactis* subsp. *lactis* strains ; BML, *L. lactis* subsp. c*remoris* strains. [b]Values ±SD. Inoculation percentages were 2%.

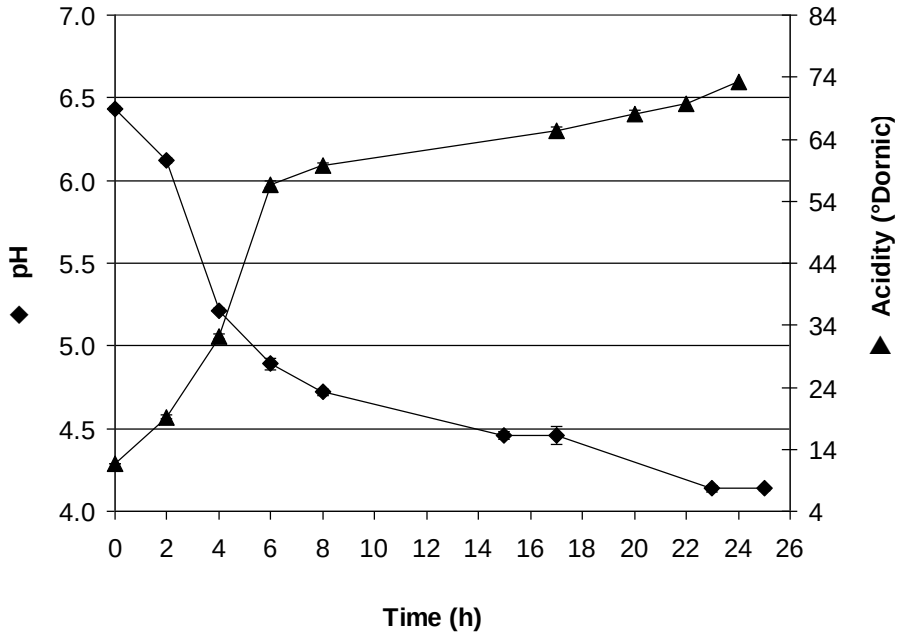

Figure 1. : Mean pH and acidity values of the cultures of *L. lactis* subsp. *lactis* AML8 in milk during 24h. ◆ pH; ▲ acidity.

16 h (Figure 1). These values are similar to those obtained by Alonso-Calleja (2002) who reported approximately 54°D after 6 h for milk inoculated with *L. lactis* subsp. *lactis*.

The pH of *L. lactis* subsp. *lactis* (AML8) cultures correlate well with the titrable acidity (Figure 1). Indeed, his progressive increase is accompanied with a decrease of pH values which reaches the value of 4.46 after about 16 h (Figure 1). The highest value of titrable acidity (73.2°D) was reached after 24 h. At the same time, the pH reached the value of 4.15.

Martinez-Moreno (1976), Nunez and Medina (1979), Mas and Gonzalez-Crespo (1992) and Alonso-Calleja (2001) divided the strains of *L. lactis* subsp. *lactis* into two groups, depending on the acid production rate: fast acid producer (F) strains and slow acid producer (S) strains. They have shown that the difference between fast (F) and slow (S) acid producer strains is the titrable acidity at 6 h: higher than 30°D in F strains. Moreover, they indicate

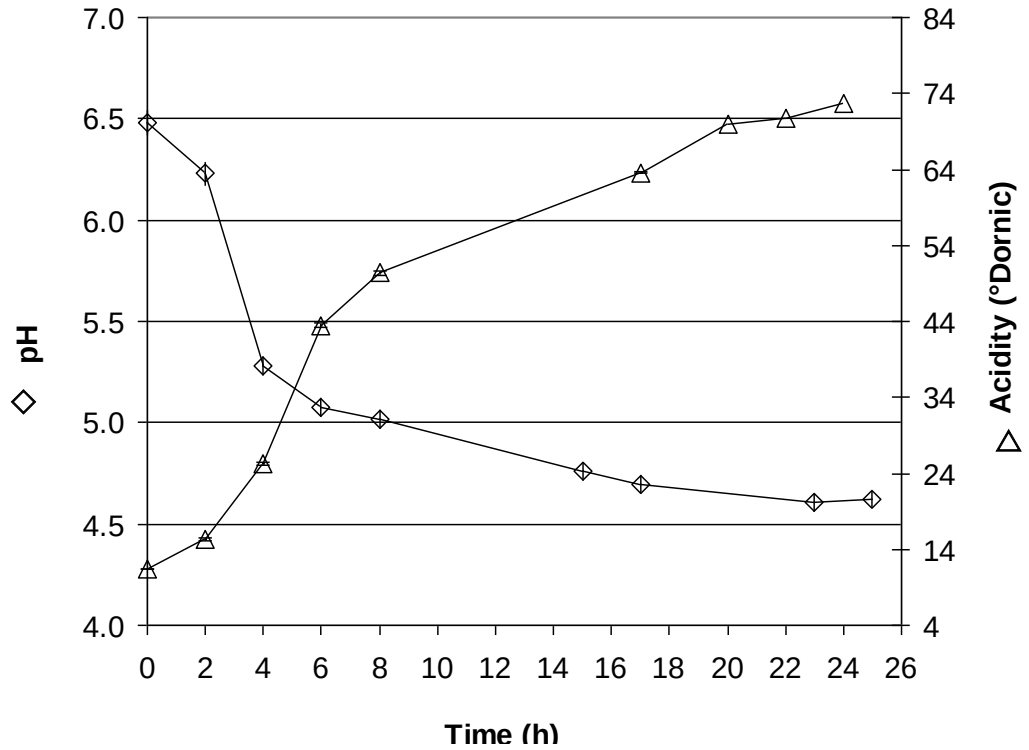

Figure 2. Mean pH and acidity values of the cultures of *L. lactis* subsp. *cremoris* BML2 in milk during 24h. ◇ pH; △ acidity

that the rapid acid producer *L. lactis subsp. lactis* strains have the potential as a starter culture in industry.

These data are very interesting as they indicate that *L. lactis* subsp. *lactis* AML8 strain, whose titrable acidity widely exceeds 30°D at 6 h, is a good candidate for use in fermented dairy product.

Pure culture of L. lactis subsp. cremoris (BML2)

The pH and titrable acidity in pure culture of *L. lactis* subsp. *cremoris* (BML2) behavior were similar to those of *L. lactis* subsp. *lactis* (AML8). However, the titrable acidity obtained was inferior, in particular after 6 h of incubation. The value obtained was 43.5°D (Figure 2). It is nevertheless higher than 30°D; therefore we can also consider the *L lactis* subsp. c*remoris* strain as fast acidifying strain.

Mixed culture of L. lactis subsp. cremoris (BML2) and L. lactis subsp. Lactis (AML8)

In mixed culture, the titrable acidity was compared with those obtained in pure culture. The titrable acidity after 16 h incubation was significantly higher in mixed culture (78.6°D) (Figure 3) than pure culture (64.4°D) ($P < 0.05$). Similarly, after 24 h of incubation, the value of titrable acidity reached in the mixed culture was 91.5°D, whereas it was only 73.2° D for pure cultures.

These results suggest a synergy relationship between the two subspecies involved. Our finding are in agreement with those obtained by Kimoto-Nira et al. (2012), indicating that a mixed culture of two different species, *L. lactis ssp. lactis* strain 54 and *Lactococcus raffinolactis* strain 37; stimulated greater acid production during fermen-tation in milk than occurred with pure culture fermentation.

The pH of milk fermented with mixed cultures and the Lben presented similar behaviors (Figures 3 and 4). Both of them decline from an initial value of 6.8 and 6.7 respectively to reach, after 24 h, the value of 4.5. In the milk fermented with mixed culture, the value of titrabe acidity is correlated with that of pH. The progressive decline of the latter was accompanied by the increase in the titrable acidity which reached 91.5 °D after 24 h. In contrast, in traditional Lben, the value of titrable acidity remained low (around 15°D) in the early hours. It reaches the value of 39°D only after 10 h; even sometimes after 14 h of incubation. Therefore, the decrease in pH observed within the first hours of incubation of traditional Lben (Figure 4), is not correlated to the titrable acidity. This could be explained by the fact that the traditional Lben is produced by an undefined mixture of microorganisms found in the raw milk able to produce a variety of other acids resulting in a pH decrease (Benkerroum and Tamine, 2004; Feresu and Nyathi, 1990).

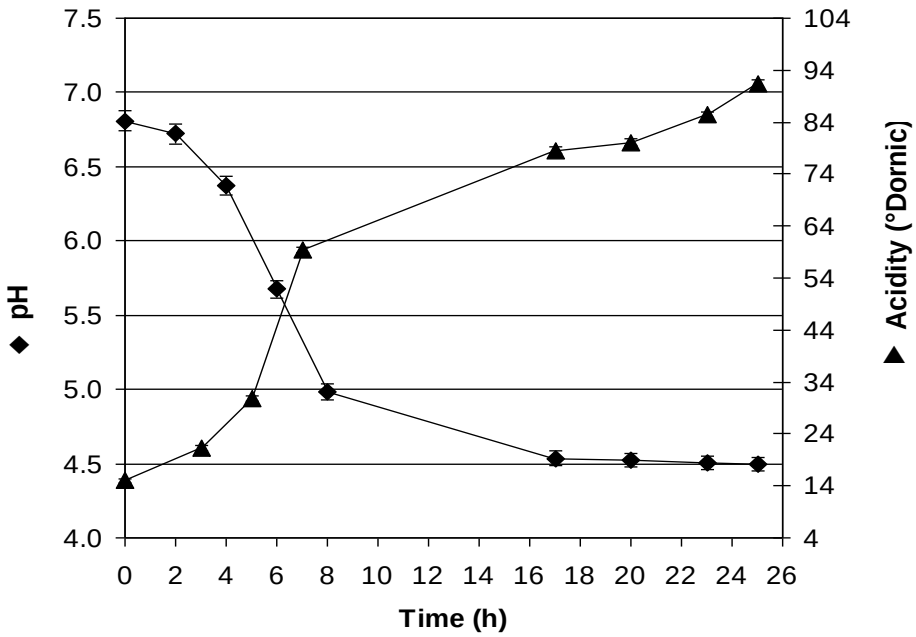

Figure 3. Mean pH and acidity values of the mixed culture in milk during 24h. ◆ pH; ▲ acidity.

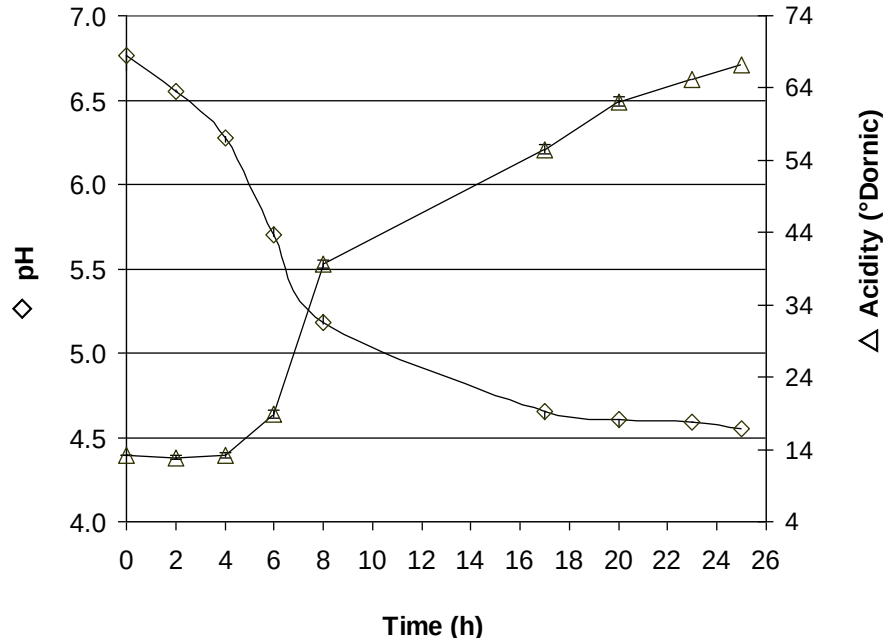

Figure 4. Mean pH and acidity values of the Lben during 24h. ◇ pH; △ acidity.

The progressive acidification of the medium promotes the lactic acid bacteria activity and thus the titrable acidity, but only after 16 h.

Generally, the manufacturers cannot control the natural acidification demonstrating irregular kinetics and therefore results in the heterogeneity of the products obtained. Moreover, bad hygiene results in the presence of hazardous microorganisms in the final product. Therefore, the acidification managed by using well controlled starter cultures is a less risky alternative than natural sowing.

Table 2. Physicochemical analyses of sterilized whole milk, fermented milk, raw milk and traditional Lben.

Sample	pH[a]	Titrable Acidity (°Dornic) [a]	Lactose (g/L) [a]	Fat content (g/L) [a]	Dry matter (g/L) [a]
Sterilized whole milk	6.08±0.01[b]	15.00±0.14[b]	50.00±0.18[b]	30.54±0.15[b]	104.00±0.67[b]
Fermented milk	4.49±0.01[c]	91.50±0.26[c]	30.00±0.38[c]	28.40±0.42[c]	96.20±0.58[c]
Raw milk	6.76±0.02[b]	13.20±0.25[d]	40.00±0.17[d]	32.50±0.54[d]	110.00±0.45[d]
Traditional Lben	4.55±0.02[c]	67.20±0.32[e]	29.90±0.22[c]	5.20±0.36[e]	87.90±0.81[e]

[a] Mean value±S.D. Means are average from four samples.[b, c, d] and [e] The letters indicate significant differences (P<0.05) between samples.

Table 3. Sensorial parameters.

Paramter	Sensorial parameter	Mean [a]
Odor and flavour	Lacteous	-0.93 ± 0.55
	Yeast	-0.40 ± 0.67
	Fermented	2.00 ± 0.31
Texture and taste	Viscosity	3.00 ± 0.44
	Color	-0.20 ± 0.37
	Acid	1.20 ± 0.37

[a] Mean value±S.E. Mixed culture is compared to Lben. Odor, flavor, texture and taste were scored in a 9-point negative to positive scale (0=no differences with Lben ; ±1=minimal differences; ±2=noticeable differences; ±3=considerable differences; ±4: very considerable differences).

Physico-chemical characterization

During fermentation, the lactose content decreased in fermented milk and in traditional Lben below 30.00±0.38 g/L (40%) and 29.90±0.22 g/L (33%) respectively. Thus, the breakdown of lactose increases the titrable acidity to more than 90°D in fermented milk and 67.20°D in traditional Lben. These results were similar to those reported by Tantaoui-Elaraki et al. (1983) for Moroccan Lben and Samet-Bali et al. (2012) for Tunisian Lben.

The observed difference in fat and dry matter in fermented milk and traditional Lben could be due to different processing conditions. Thus, the use of whole milk in the former case and the elimination of butter granules after churning operation for making traditional Lben significantly reduce its fat content (5.20 ± 0.36 g/L).

Sensory analysis

During growth in milk, the bacteria exert an influence on the microstructure and the sensorial properties of coagulated milk. *L. lactis* subsp. *lactis* strains are resistant to stress and can grow well to provide adequate acidification required for curd formation. *L. lactis* subsp. *cremoris* strains, more sensitive to stress, may contribute to the development of sensory properties (Kim et al., 1999).

For the obtained products, the panel detected a difference in all the sensory attributes (Table 3). The milk fermented with mixed culture has been classified as less yeasty and less lacteous. Generally, the best perception of odor in the Lben could be due to ethanol, volatile compound found in the Lben (Benkerroum and Tamine, 2004).

On the other hand, the values attributed to the viscosity and the acid taste indicated a better perception of the so fermented milk than the Lben. It is very likely that the higher fat content of fermented milk has contributed to its better taste. The correct texture of the fermented milk could be related to the presence of exopolysaccharides (EPS). Gruter et al. (1992) previously reported their production by *Lactococcus lactis* species and we have also detected their synthesis by the species used in this work (results not shown).

Conclusion

This study describes the isolation, identification and acidifying activity of wild strains of lactic acid bacteria from traditional dairy products. It has allowed us to distinguish two interesting strains.The collected data, especially their acidifying capacity, revealed they both have a high potential for future use in the production of fermented dairy products. The mixture culture in milk of these two strains

provided a safe fermented dairy product with the desired sensory features. Additional studies are underway to better determine others technological properties of these strains, especially the production of EPS and aroma. This could lead to the selection of these bacteria to produce a new mixed starter.

On the other hand, they may be used in the artisanal dairies which are very common in Morocco. This would allow a better control of fermentation conditions, and therefore better products quality.

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