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*Full Length Research Paper*

# Characterisation of typical Tunisian fermented milk: Leben

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**Traditional Tunisian fermented milk, "Leben" was produced and analysed for it's physicochemical, microbiological and microstructure characteristics and compared with industrial Leben. Traditional Leben was characterized by a higher mineral content and was less fatty and acidic than the industrial Leben. Traditional Leben microstructure revealed an open and a branched structure while a compact structure with small pores was seen in the industrial Leben. Lactic acid bacteria (LAB), especially the genus *Lactococcus* (*Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) followed by *Leuconostoc* were the dominated microorganisms in Traditional Leben. Traditional product contained considerable numbers of yeasts (*Candida lusitaniae*, *Candida tropicalis* and *Candida albicans*) and was contaminated with coliforms and enterococci.**

**Key words:** Fermented cow milk, microstructure, physicochemical, lactic acid bacteria, yeasts.

## INTRODUCTION

Traditional fermented milk products are widely consumed in the entire world. These products are an important supplement to the local diet and provide vital elements for growth, good health and an appreciate flavour. Several of these traditional products were industrially manufactured using selected bacterial strains and a standardized process.

Leben is a fermented milk product traditionally prepared from cow's milk in Tunisia and some Arab countries. It is an accompanying drink at lunch, and also at other meals. Recently, Leben has been produced in pasteurized form by most dairy factories in the region.

Two main kinds of starters are used to manufacture Tunisian Leben: artisanal starters consisting of an unknown number of undefined strains for the production of traditional Leben (TL) and industrial starters characterized by well-defined lactic acid bacteria (LAB) strains for the production of industrial Leben (IL). LAB are employed to produce fermented milk products, including yogurt, Leben, dahi, kefir and koumiss (Belkaaloul et al., 2010). Among the LAB, *Lactococcus lactis* is the primary constituent of many industrial and artisanal starter cultures used for the manufacture of different varieties of fermented dairy products (Taïbi et al., 2011). The yeasts could contribute to the traditional fermentation process of fermented milk products (Mufandaedza et al., 2006). Traditional fermented milks are considered safe because of the low pH and the production of antimicrobial substances by fermenting organisms. However, some pathogens such as *Escerichia coli 0157:H7, Salmonella enteritidis* and *Staphylococcus aureus* have been reported to survive and grow in some traditional fermented milk (Benkerroum and Tamine, 2004; Feresu and Nyathi, 1990).

The dairy industry uses selected industrial cultures in order to obtain safety fermented milk and to provide the product with the desired characteristics that result from the metabolic activity and the technological properties of the strains during their growth in milk. This leads to a standard quality of the products and a high level of reproducibility of the process as it has been reported by

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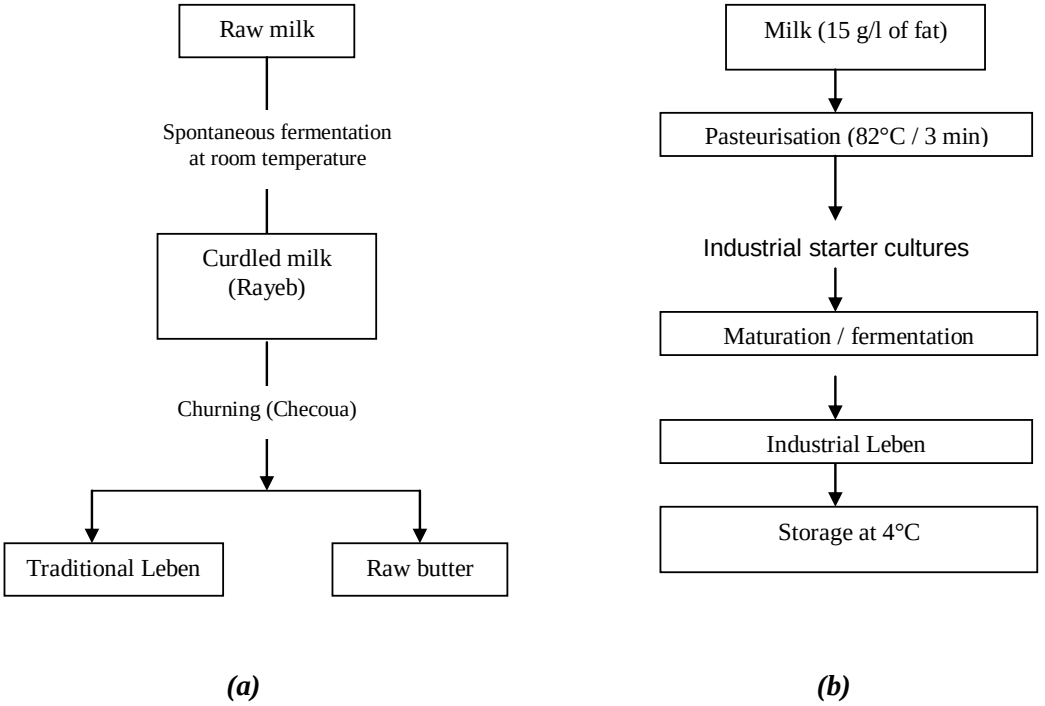


**Figure 1.** Schematic preparation of fermented milk in Tunisia. (a): traditional process; (b): industrial process.

Chammas et al. (2006). Nevertheless, consumers prefer traditional fermented milk since artisanal starters give these products more typical flavours (Wouters et al., 2002).

As we are aware, some traditional technologies for the production of fermented milks and their properties will eventually be lost together with the associated micro-organisms. It is therefore imperative to characterize traditional fermented products, including preservation and characterization of indigenous microbiota. The objective of this work are to study the physicochemical, microbial and microstructure properties of traditional and industrial Leben and then to determine the predominant groups and to identify the LAB and Yeasts present in traditional Tunisian Leben.

## MATERIALS AND METHODS

Cows' milk (Holstein breed) was obtained from a private farm in the South area of Tunisia (Sfax). Samples of cows' milk were collected, kept refrigerated (4°C) and transported to our laboratory within 24 h. Each sample was taken from 20 to 25 animals.

### Fermented milk preparation

Traditional process (Figure 1a): 5 l of raw milk was left spontaneously at 25 ± 2°C until coagulation, which may take up after ~18 h. During the gelation step, the product will be called "rayeb". By churning during 40 min, the "rayeb" is separated into aqueous fraction giving "Leben" (TL) and fatty fraction called "raw butter". Churning takes place in a leather bag called "Checoua". The latter is manufactured from a goat in one piece; the openings of the "Checoua" are subsequently tied up with a string to avoid leakage when filled. The churning is achieved by hanging the "Checoua" filled with "rayeb" to a wooden tripod or to a cottage roof and vigorously shaking it back and forth till the coalescence of the fat globules.

Industrial process (Figure 1b): IL samples were produced by adding mesophilic starter cultures (industrial starter cultures) of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *diacetylactis* and *L. lactis* subsp *cremoris* (Rhodia, France) in pasteurized and standardized milk (15 g/L of fat), during 10 h at 27ºC fermentation temperature.

### Physicochemical analysis

Total nitrogen (TN), non protein nitrogen (NPN) and non casein nitrogen (NCN) contents of the Leben were analysed using the Kjeldahl method (Afnor, 1993) using a Büchi 325 apparatus (Büchi, Flawil, Switzerland). The total casein content was calculated by difference between TN and NCN after separation according to Rowland (1938). Dry matter, ash, lactose and fat contents were determined according to standard methods (Afnor, 1993).

Calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) were measured by atomic absorption spectrometer model Hitachi Z-6100 (Hitachi Instruments Engineering Co., Ibaraki-ken, Japan) in the presence of lanthanum oxide for Ca and Mg and in the presence of cesium chloride for K and Na. The concentration of phosphorus (P) was determined by a colorimetric method with ammonium molybdate. Chloride (Cl) content, expressed in equivalent NaCl, was measured by the Charpentier-volhard method. Titratable acidity, expressed in Dornic degrees, was determined by titration of sample with N/9 sodium hydroxide to

pink endpoint using phenolphthalein as indicator (Afnor, 1993). The pH was determined using a METTLER TOLEDO MP 220 pH meter.

### Scanning electron microscopy (SEM)

Samples of milk, rayeb, TL and IL were prepared using the technique described by Attia et al. (1991) and examined with a Philips XL 30 scanning electron microscope (Philips, Limeil Brevannes, France) after drying to $CO_2$ critical point on a Baltec CPD 030 apparatus and gold-coating on a Baltec MED 20 apparatus (Balzers Union, Balzers, Germany).

### Enumeration of micro-organisms

Duplicate samples of milk, TL and IL (10 ml) were homogenized with 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, 1000 ml distilled water, pH 7.0 ± 0.2). The homogenate was decimal diluted and the relevant dilutions surface plated. The number of viable bacteria was estimated by the IDF Standard method (IDF, 1996). The total count of aerobic bacteria (AB) were enumerated on plate count agar (PCA, Oxoid) after incubation at 30ºC for mesophilic AB and 45ºC for thermophilic AB for 48 h. MRS agar (Oxoid) medium was used for counting mesophilic and thermophilic lactic acid bacteria (LAB). Plates were incubated at 30 and 45ºC for 48 h. Yeasts and moulds were enumerated on Sabouraud dextrose agar after incubation at 22ºC for 5 days. Total coliforms were enumerated on VRBL agar (Oxoid) according to the IDF Standard method (IDF, 1985). Enterococci were counted as described by Tantaoui-Elaraki et al. (1983). *S. aureus* count were determined by surface plating of appropriate sample dilutions on Baird-Parker agar plates and incubated for 48 h at 37ºC (IDF, 1997). *Escherichia coli* and *Salmonella* were counted according to Mufandaedza et al. (2006). TSN Agar (Oxoid) was used for *Clostridium Perfringens* detection.

### Isolation and identification of LAB and yeasts in TL

LAB were isolated in different culture media: MRS agar (Oxoid) was used for *Lactobacilli* isolation and the plates were incubated in anaerobic conditions (BBL Gas-Pack System) at 30ºC for 48-72 h, selective mediums for the isolation of leuconostoc and lactococci were used as recommended by Tantaoui-Elaraki et al. (1983). A total of 120 representative colonies were randomly picked from various culture media plates and confirmed to be Gram-positive and catalase-negative. The LAB isolates were purified by 4 alternate subcultures in MRS agar and MRS broth (Oxoid) before identification. Yeasts (30 representative colonies) were isolated on Sabouraud dextrose agar after incubation at 22ºC for 5 days. Purification and sub-culturing was done using potato dextrose agar (PDA, Oxoid) and yeast extract-malt extract (YM) broth. The purified yeast cultures were stored on PDA slants at 4ºC until required for identification.

The LAB bacteria were characterised by microscopic examination and conventional biochemical and physiological tests. The cultures were examined for colony and cell morphology; motility, cell arrangement, Gram reaction; catalase reaction; growth in broth at 10, 15, 40 and 45ºC, growth in presence of 2, 4 and 5% (w/v) NaCl; production of $CO_2$ from glucose; production of ammonia from arginine; production of dextran from sucrose; and production of carbon dioxide from glucose using Gibson's litmus milk. These tests were done according to procedures described by Harrigan and McCance (1990). LAB bacterial strains were identified using API tests based on the above-mentioned morphological, physiological and biochemical characteristics. The fermentation pattern among carbohydrates was determined by using the API 20 STREP (BioMérieux, Marcy l'Etoile, France) system, which enabled identification of the LAB isolates to species level. Anaerobiosis in the inoculated tubes was obtained by overlaying with sterile paraffin oil. The inoculated galleries were incubated at 30ºC and the observations were made after 4 and 24 h.

Primary classification of colonies from the PDA plates was based on colony characteristics (pigmentation and shape), mode of vegetative reproduction, formation of hyphae or pseudohyphae and ascospore production. The methods described by Harrigan and McCance (1990) were followed. Identification of the yeast isolates to species level was done using the API 20C AUX (BioMérieux, Marcy l'Etoile, France) system of carbohydrate assimilation profiles.

### Statistical analysis

Fermentation process was triplicate and duplicate analyses were performed on each replicate. Values of different tests were expressed as the mean ± standard deviation (x ± SD). SPSS packet program for Windows (SPSS, version 11, USA) was used for the statistical analysis. Significant differences between mean ($P$<0.05) were determined by using a one-way ANOVA (Duncan's test).

## RESULTS AND DISCUSSION

### Physicochemical of TL and IL

The chemical analysis of TL and IL (Table 1) showed similarities in nitrogen and casein composition comparatively to the milk. There was no significant difference ($P$ < 0.05) in proteins between the samples. However, NPN has increased significantly ($P$ < 0.05) to 2.31 and 2.34 g/l in TL and IL, respectively vs. 1.44 g/l in bovine milk showing proteolytic activity of milk during lactic fermentation (Attia et al., 2001). Tunisian Leben can be considered as a product with important nutritional value since it constitutes a high source of digestible protein. The fermentation of milk increases significantly ($P$ < 0.05) the titratable acidity to 65.55 and 71.35 g/l in TL and IL, respectively vs. 16.65 g/l in cow's milk. Consequently, lactose content drops to around 50%. These results were similar to those reported by Tantaoui-Elaraki et al. (1983) for Moroccan Leben. TL was characterized by higher ash content but was less fatty and acidic than IL. The difference in the composition of the two kinds of fermented milks products could be due to the processing conditions employed such as the employment of standardized milk fat for IL (15 vs. 34.5 g/l for TL), and the use of the checoua which has eliminated a large amount of fat during churning operation for the manufacture of TL.

The study of mineral revealed that TL was characterized by higher mineral content, mainly Na and Cl, than IL. This difference could be attributed to the salting of "Checoua" between two successive churning operations (to avoid the unpleasant appearance of smells). Ca content was lower in TL probably due to the passage of Ca in the fatty fraction. Attia et al. (2001) reported that lactic acid fermentation altering casein micelles that progressively lose their surface potential, minerals, caseins and salvation. The results of these

**Table 1.** Physicochemical composition (g/kg; mean [a] ± SD) of raw milk, Traditional Leben (TL) and Industrial Leben (IL).

| Composition | Milk | TL | IL |
|---|---|---|---|
| Dry matter | 117.13 ± 0.28[a] | 70.54 ± 1.46[b] | 78.93 ±0.61[b] |
| Total nitrogen | 33.4 ± 0.65[a] | 32.10 ± 0.40[a] | 31.14 ± 0.36[a] |
| Caseins | 26.71 ±0.53[a] | 25.62 ±1.38[a] | 25.13 ± 0.88[a] |
| Non protein nitrogen | 1.44 ± 0.09[a] | 2.31 ± 0.03[b] | 2.34 ± 0.04[b] |
| Fat | 34.50 ± 0.54[a] | 3.50 ± 0.31[c] | 14.50 ± 0.54[b] |
| Lactose | 41.37 ± 0.48[a] | 25.90 ±0.41[b] | 26.10 ± 0.77[b] |
| Ash | 8.25 ± 0.08[a] | 8.28 ± 0.14[a] | 7.17 ± 0.04[b] |
| Chlorides | 1.54 ± 0.02[a] | 2.10 ± 0.10[b] | 1.52 ± 0.05[a] |
| Calcium | 1.20 ± 0.04[a] | 0.75 ± 0.02[b] | 1.10 ± 0.03[a] |
| Magnesium | 0.11 ± 0.03[a] | 0.10 ± 0.01[a] | 0.10 ± 0.02[a] |
| Sodium | 0.38 ± 0.02[a] | 0.84 ± 0.04[b] | 0.36 ± 0.01[a] |
| Potassium | 1.51 ± 0.01[a] | 1.50 ± 0.02[a] | 1.49 ± 0.02[a] |
| Phosphorus | 1.15 ± 0.02[a] | 1.17 ± 0.06[a] | 1.16 ± 0.02[a] |
| pH | 6.70 ± 0.03[a] | 4.45 ± 0.04 [b] | 4.27 ± 0.10[c] |
| Titratable Acidity (°Dornic) | 16.65 ± 0.03[a] | 65.55 ± 0.10[b] | 71.35 ± 0.54[c] |

[a] Means are average from two independent trials. Different letters indicate significant differences ($P < 0.05$) between samples.

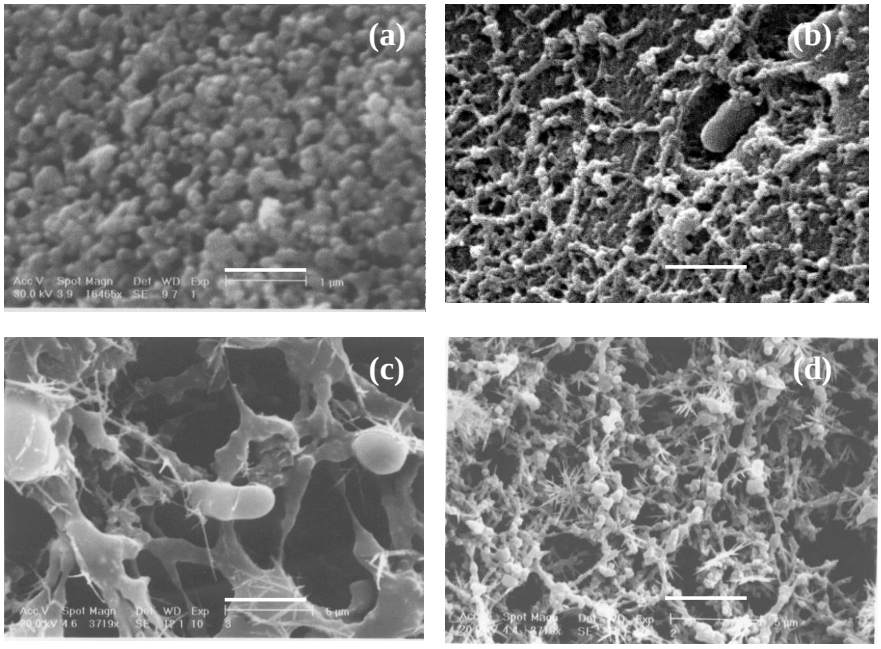


**Figure 2.** SEM micrograph: (a) fresh raw milk; (b) fermented milk ("Rayeb"); (c) Traditional Leben (TL) and (d) Industrial Leben (IL). Scale bars = 1 µm.

modifications are the destruction of the micellar structure and the formation of a three-dimensional network or coagulum which can be visualized by SEM.

## Microstructure of TL and IL

Microscopic structure of milk, spontaneous fermented milk "Rayeb", TL and IL were presented in Figure 2. Microstructure of raw milk at native pH (Figure 2a) shows the existence of individual micelles in a spherical shape (Attia et al., 2000). Figure 2b reveals a protein aggregation and protein network apparition following the pH decrease in "Rayeb". Thus, the structure of the fermented milk consisted of individualized particles that were coalesced in chains leading to relatively

**Table 2.** Counts of the different microbial groups on raw milk, Traditional Leben (TL) and Industrial Leben (IL) (Mean[a] ± SD).

| Microbiol counts (log cfu/ml) | Milk | TL | IL |
|---|---|---|---|
| Aerodic mesophilic counts | 4.40 ± 0.12[a] | 8.40 ± 0.31[c] | 6.60 ± 0.41[b] |
| Mesophilic lactic bacteria | 4.30 ± 0.13[a] | 8.37 ± 0.33[c] | 5.20 ± 0.12[b] |
| Yeats and moulds | 3.20 ± 0.20[a] | 7.30 ± 0.21[c] | 5.17 ± 0.10[b] |
| Total Coliforms | 1.81 ± 0.04[a] | 3.04 ± 0.03[b] | - |
| *Enterococci* | 2.30 ± 0.28[a] | 2.40 ± 0.36[a] | - |

- : abscent; [a] Means are average from two independent trials. Different letters indicate significant differences ($P < 0.05$) between samples.

homogeneous sieve (Attia et al., 2001). In TL (Figure 2c) the structure consisting of irregular aggregates possessed a very loose and open structure. They were probably totally demineralised caseins grouped in clumps by new bonds that were probably electrostatic and hydropholic (Attia et al., 1991). Thus, TL microstructure revealed an open and a branched structure (Figure 2c) while a compact structure with small pores was seen in IL (Figure 2d). The protein networks of traditional Leben were less dense, more open and with more void spaces than that of industrial one. These structure differences could be explained by the pasteurisation and standardisation (15 g/l of fat) operations during the industrial Leben manufacture. Then, microstructure differences were probably due to the lower number of fat globules acting as linking protein agents and the denaturation of protein in the industrial one (Lucey et al., 1998). In addition, a spine shape structure mixed with protein network was observed in TL and IL. According to Hassan et al. (2003) this structure can be identified as exopolysacchrides (EPS) produced by LAB during fermentation. Cerning (1995) reported that EPS were produced from mesophilic lactic acid bacteria: *L. lactis* subsp. *cremoris*. These lead us to suspect the existence of *L. lactis* subsp. *cremoris* in TL.

## Microbiological characteristics of TL and IL

Table 2 summarises the microbial counts obtained from milk, TL and IL. Aerobic mesophilic counts and mesophilic lactic bacteria counts were similar indicating that the microflora responsible for the fermentation of Tunisian Leben was mesophilic and lactic acid bacteria (LAB) were the dominating microorganisms. These results were similar to those reported by Tantaoui–Elaraki et al. (1983) and Guizani et al. (2001) for traditional Moroccan and Omani Leben, respectively. The dominance of mesophilic bacteria may be explained by the fact that the temperature at which the natural fermentation of the samples took place probably favoured proliferation of mesophilic bacteria in fermented milks. Counts of yeasts in TL (7.3 $\log_{10}$ cfu/ml) were higher than in IL (5.17 $\log_{10}$ cfu/ml), which recorded a final pH of about 4.45 and 4.27 in TL and IL, respectively. However, the levels of yeasts in TL were similar to those reported in other traditional fermented milks, where these numbers ranged from 4.64 to 7.32 $\log_{10}$ cfu/ml (Hamama and Bayi, 1991; Mathara, 1999; Abdelgadir et al., 2001). Gadaga et al. (2000) reported that low pH offers a selective environment for yeasts growth, but is unfavourable for most bacteria. The presence of yeasts in fermented milk may be influenced by the age of the product as well as the processing methods used. Benkerroum and Tamime (2004) reported that yeasts were recovered in traditional Moroccan Leben towards the end of the fermentation stage, which may suggest that they play a secondary role in the fermentation process.

Coliforms and enterococci were present in milk and TL. The total counts of these microbial groups increased slowly during traditional fermentation process. Coliforms along with enterococci formed the minority groups in TL in excess of 3.04 and 2.4 log cfu/ml, respectively. *S. aureus*, *E. coli*, *C. perfringens* and *Salmonalla* were not detected in TL. Benkerroum and Tamine (2004) explained the higher counts of indicator micro-organisms (e.g., coliforms, enterococci) and pathogens such as *Salmonalla* spp., *S. aureus* and *E. coli* in Moroccan traditional Leben, by the fact that the methods of production of the various traditional fermented milks are usually primitive compared to modern ways of preparation. Major risk factors are the use of contaminated raw materials, lack of pasteurisation, use of poorly controlled natural fermentations and inadequate storage and maturation conditions (Nout, 1994).

Strains of LAB isolated from TL were identified as *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *Leuconostoc* spp. (Table 3). The majority of the bacteria belonged to *L. lactis* group followed by *Leuconostoc* spp. Mathara et al. (2004) reported that the low number of *Leuconostoc* strains isolated from the fermented milk samples could be explained not only by their complex nutritional requirements, but also by their lower adaptation to milk. Lore et al. (2005) noted that *L. lactis* were homofermentative, fermenting glucose via the glycolytic pathway to lactic acid as the major or sole

**Table 3.** Identified isolates from traditional Leben (TL).

| Reference | LAB species |
|---|---|
| $Bl_2$ (n=15) | *Lactococcus lactis cremoris* |
| $Bl_4$ (n=6) | *Lactococcus lactis lactis* |
| $Bl_6$ (n=15) | *Lactococcus lactis cremoris* |
| $Bl_7$ (n=10) | *Lactococcus lactis cremoris* |
| $Bl_8$ (n=10) | *Lactococcus lactis cremoris* |
| $Bl_9$ (n=10) | *Lactococcus lactis lactis* |
| $Bl_{10}$ (n=10) | *Leuconostoc* spp. |
| $Bl_{11}$ (n=11) | *Lactococcus lactis lactis* |
| $Bl_{12}$ (n=8) | *Lactococcus lactis lactis* |
| $Bl_{14}$ (n=12) | *Lactococcus lactis lactis* |
| $Bl_{15}$ (n=13) | *Lactococcus lactis lactis* |

n: number of isolates.

product of fermentation. This suggests their significant role in lactic fermentation of cow milk. *Leuconostoc* are heterofermentative, fermenting glucose via the hexose-monophosphate pathway to produce equimolar amounts of lactic acid, ethanol and $CO_2$ (Jay, 1992). Additionally, members of the genus Leuconostoc are able to convert citrate to aroma compounds such as acetoin and diacetyl (Lore et al., 2005), a characteristic that would be of functional significance towards aroma development in Leben. Several authors have recorded the predominance of LAB in traditional fermented cow milk products (Hamama and Bayi, 1991; Mathara, 1999; Abdelgadir et al., 2001; Guizani et al., 2001; Benkarroum and Tamine, 2004; Miyazaki and Matsuzaki, 2008). These investigators found the main LAB genera to comprise lactobacilli, lactococci and leuconostocs. However, lactobacilli were not detected in TL. Harrati (1974) reported the absence of *lactobacillus* in Algerian Leben by the fact that fermentation of milk by lactococci would not be enough moved for allowing the lactobacillus to develop. Tantaoui-Elaraki et al. (1983) confirm this hypothesis to explain the lower *lactobacillus* counts in Moroccan Leben.

The isolated yeasts were identified as *C. lusitaniae*, *C. tropicalis* and *C. albicans*. Frazier and Westhoff (2001) report that *Candida* species have been used with dairy starter cultures to maintain the activity and increase the longevity of LAB. This could imply a symbiotic association between *Candida* species and the LAB involved in Leben production. Indeed, a symbiosis between yeasts and lactic acid bacteria has been suggested: whereby the bacteria provide the acidic condition favourable for the growth of yeasts. The latter provide vitamins and other growth factors to the bacteria (Gobbetti et al., 1994). Additionally, Alvarez-Martin et al. (2008) reported that yeast growth can be essential to the development of the typical texture and aroma profiles of certain fermented milk products – the outcome of their strong proteolytic and lipolytic activity.

## Conclusion

The main objective of this study was the characterization of Traditional and Industrial Tunisian fermented milk (Leben). Obtained results show that physico-chemical composition of the two products differed slightly. This difference was attributed to the used process and to the starters. Spontaneous starter's presents in TL are responsible of a typical taste appreciated by consumers. Microbiological identification of LAB growth in TL shows that the use of some artisanal strains as starters for the development of industrial Leben is very promising. These strains should first be tested in mixed cultures, in order to obtain fermented milk with flavour characteristics similar to those of artisanal product. Based on these results future works will be dedicated to the elaboration of industrial products using traditional starters and to the identification of aromatic compounds presents in TL and IL processed with Traditional starters.

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