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# Enumeration and identification of microflora in "Leben", a traditional Tunisian dairy beverage

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**Abstract**

The microflora involved in production of Leben, a Tunisian traditional fermented cow milk product, were enumerated and identified. 15 samples of traditional Leben were analyzed. Total viable microorganisms, lactic acid bacteria (LAB), yeasts and moulds, and coliforms were enumerated. A total of 45 LAB and 30 yeast isolates were isolated from the 15 Leben samples and identified by API 50 CHL and API 20C AUX identification systems, respectively. The LAB counts were 7.8 $\log_{10}$ CFU/mL, while yeast and mould counts were relatively lower (4.7 $\log_{10}$ CFU/ml). Low coliform numbers were encountered (1.8 $\log_{10}$ CFU/ml). The LAB species were identified as *Lactococcus lactis* subsp. *lactis, Lactococcus lactis* subsp. *cremoris* and *Leuconostoc mesenteroides*. The isolated yeasts were identified as *Candida krusei, Candida tropicalis* and *Candida lusitania*. The most frequently isolated species was found to be *Lactococcus lactis* subsp. *lactis* (28% of total isolates), followed by *Lactococcus lactis* subsp. *cremoris* (20%) and *Candida krusei* (18%).



## Introduction

Traditional fermented dairy products are produced throughout the world. Some fermented products obtained as a result of lactic acid fermentation (Liu *et al*., 2011) or lactic acid and yeast fermentations. In Tunisia most milk producers, process the milk into many products such as rayeb (curdled milk), Leben, raw butter (zebda beldi) and smen (clarified butter). Leben is a widely consumed food commodity in Tunisia. It is white coloured traditional lactic acid fermented milk. It can be consumed as a fresh beverage or as an ingredient in some foods such as bread and couscous. Production of Leben in Tunisia takes place in villages at house hold level. Traditionally, raw milk is left spontaneously then fermented overnight or more and then the sour fermented milk is churned in a sac made of goat skin. By churning, the fermentate is separated into aqueous fraction giving Leben and fatty fraction called raw butter.

To standardize product characteristics, selected bacterial cultures composed of *Lactococcus lactis* species are used to produce an industrial Leben in Tunisia. However, Leben is still largely processed using traditional methods because consumers prefer traditional Leben due to its organoleptic quality. Therefore, it is of primary importance to obtain a reliable description of the physiologically active microbial community implemented in the product fermentation. This can be achieved by the enumeration of some microbial groups on a variety of culture media, followed by identification through traditional microbiological methods (Blaiotta *et al*., 2002).

Leben production and consumption are increasing in Tunisia. Its quality is closely related to the microbial ecology, mainly lactic acid bacteria (LAB), of fermentation. As we are aware, there are no available commercial LAB starter cultures isolated from their natural environments to do Leben fermentations. Traditionally, Leben is produced with uncontrolled fermentation which leads to a variation in terms of quality and stability of the product. Therefore, it is important to select the starter cultures for the controlled fermentations to produce a better product.

This study was undertaken to isolate, identify and characterise the microflora present during Leben fermentation. This information can contribute to the development of starter cultures with predictable characteristics, for use in small-scale and commercial production of Leben with stable and consistent quality.

## Materials and Methods

### *Sample collection*

A total of 15 samples of traditionally prepared

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Leben were collected from cow farms in the southern part of Tunisia. The milk was collected in sterile bottles, transported to the laboratory in a cool box and stored at 4–6°C before analysis. Samples were analyzed within 6 h of collection (maximum period between collection and analysis of samples), and were maintained at 4°C during the period between collection and analysis.

*Enumeration and isolation of microorganisms*

The $10^{-1}$ dilution was made by diluting 25 ml of Leben with 225 ml of physiological saline. Further tenfold serial dilutions, ranging from $10^{-2}$ to $10^{-7}$, were prepared and the microbial counts were determined according to the pour plate method of Harrigan and McCance (1986). Total viable counts were determined using plate count agar incubated at 30°C for 2 days. Counts of LAB were determined using de Man Rogosa Sharpe (MRS) agar incubated anaerobically at 30°C for 3 days. The counts of yeasts and moulds were determined using potato dextrose agar (PDA), acidified to pH 3.5 with tartaric acid and incubated at 25°C for 5 days. Coliform numbers were determined using violet red bile glucose agar incubated at 37°C for 48 h.

For the isolation of microorganisms, the serially diluted samples of Leben were plated at different dilutions on to selective media, namely, MRS agar (Becton, Dickinison Co., Sparks, Md., USA) for lactobacilli and leuconostocs, M17 agar (Becton, Dickinison Co., Sparks, Md., USA) for lactococci and acidified PDA for yeasts and moulds. After appropriate incubation, discrete colonies were picked from the plates of the highest dilutions. Either 50% of the colonies were selected or if the plate had less than 10 colonies, all were selected, according to Harrigan and McCance (1986). The isolated colonies were purified by subculturing on fresh agar plates of the isolation medium followed by microscopic examination.

*Identification of lactic acid bacteria*

Colonies from the MRS and M17 agar plates were examined for Gram strain, catalase reaction and cell morphology. Gram-positive, catalase-negative rods and cocci were presumptively identified as LAB. Further classification was done according to the biochemical criteria described by Harrigan and McCance (1986), namely production of $CO_2$ from glucose, production of ammonia from arginine, and growth at 15°C and 45°C. Ability to ferment carbohydrate substrates was studied using the API 50 CHL (BioMérieux, Marcy l'Etoile, France) system, which enabled identification of the LAB isolates to species level.

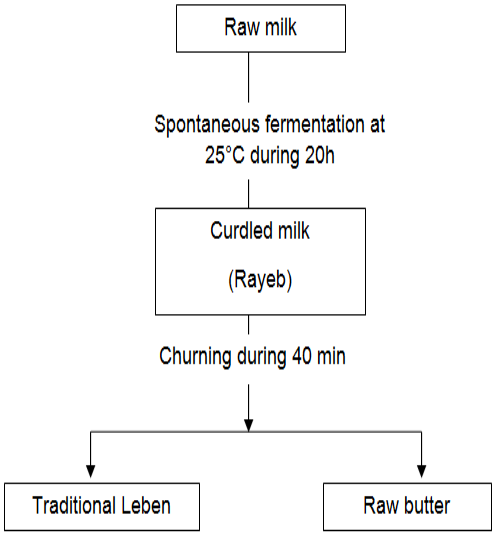


Figure 1. Schematic preparation of traditional Leben

*Identification of yeasts and moulds*

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 (1986) 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.

*Laboratory-based production of Leben*

Based on the traditional method for production of Leben observed in Tunisia (Figure 1), laboratory-based production was carried out in order to study changes in microbial numbers and acidity during spontaneous fermentation of cow milk. Fresh cow milk from a local farm in the South area of Tunisia (Sfax) was collected. Raw milk was left to ferment naturally in an incubator at 25°C until coagulation was occurred during up to 20 h. By churning, the fermentate (curdled milk) was separated into an aqueous fraction called Leben and a fat-rich fraction called raw butter. Traditionally, churning takes place in a skin bag called checoua, which is obtained, from a goat in one piece. The openings of the skin were subsequently tied up with a string to avoid leakage when filled. The churning is achieved after hanging the checoua which is filled with the curdled milk and vigorously shaking it back and forth during 40 min. A total of 15 laboratory-produced Leben samples were prepared and all laboratory experiments were replicated three times. The pH and microbial counts

(total viable count, LAB, yeasts and moulds and coliforms) were determined at 4 h intervals during spontaneous lactic acid fermentation. The pH was measured using a pH meter (744 pH Meter Metrohum, pH meter).

*Statistical analysis*

All analytical determinations were performed at least in triplicate. Values of different tests were expressed as the mean ± standard deviation (x ± SD). SPSS packet program for Windows 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

*Enumeration of microorganisms*

Table 1 shows the counts of total viable microorganisms, LAB, yeasts and moulds, and coliforms in the traditional and laboratory-produced Leben. High total viable counts were observed. Conversely, relatively lower numbers of fungal flora and coliforms were encountered. There was no significant difference ($P<0.05$) in the numbers of total viable microorganisms and coliforms between traditional and laboratory-produced Leben. However, the laboratory-produced Leben had significantly higher numbers of LAB, yeasts and moulds compared to traditional Leben.

*Changes in microbial numbers and pH during spontaneous fermentation*

Figure 2 illustrates the changes in microbial counts during spontaneous fermentation of cow milk. During spontaneous fermentation of cow milk, the pH declined steadily from an initial value of 6.71 to 4.4 after 20 h. Initial counts of coliforms and fungal flora were lower (1.81-3.10 $\log_{10}$ CFU/ml) relative to the LAB (4.70 $\log_{10}$ CFU/ml). Within the first 12 h, there was a steady increase in total viable, LAB and coliforms counts by about 3 log cycles to 8.11, 7.72 and 4.8 $\log_{10}$ CFU/ml, respectively. Between 16 and 20 h of fermentation, coliform numbers reduced sharply from 4.6 to 1 $\log_{10}$ CFU/ml and LAB counts increased from 8.1 to 8.88 $\log_{10}$ CFU/ml. During that time, the pH decreased from 5.2 to 4.6. The yeast counts increase throughout the 20-h fermentation period, with about 2 log cycle from 3.10 to 5.2 $\log_{10}$ CFU/ml.

*Identification of microorganisms*

On the basis of carbohydrate fermentation (for LAB) and assimilation (for yeasts) and other biochemical criteria, 45 LAB and 30 yeast isolates from the 15 traditional Leben samples were identified to species level (Table 2). All results obtained by API were found between good identification-excellent identification. No lactobacilli were detected. The LAB were identified as *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Leuconostoc mesenteroides*. The species of isolated yeasts were identified as *Candida krusei, Candida tropicalis* and *Candida lusitaniae*. The predominant species was *L. lactis* subsp. *lactis* (28% of total isolates), although *L. lactis* subsp. *cremoris* (20%) and *C. krusei* (18%) were also isolated frequently.

Table 1. Average numbers of microorganisms in traditional and laboratory-produced Leben (mean [a] ± SD)

| | Log count ($Log_{10}$CFU/ml) | |
|---|---|---|
| | **Traditional** | **Laboratory-produced** |
| Total viable microorganisms | 9.06 ± 0.38[a] | 9.20 ± 0.22[a] |
| Lactic acid bacteria | 7.7 3± 0.31[a] | 8.88 ± 0.12[b] |
| Yeasts and moulds | 4.61 ± 0.21[a] | 5.11 ± 0.10[b] |
| Coliforms | 1.84 ± 0.04[a] | 1.00 ± 0.02[a] |

[a] Mean are average from 15 samples of each traditional and laboratory-produced Leben
Number of replicates of laboratory-based production of Leben = 3.
Different letters in the same line indicate significant difference between
traditional and laboratory-produced Leben ($P<0.05$).

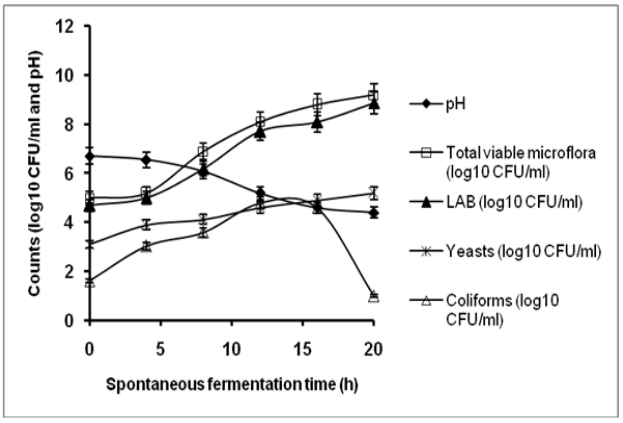


Figure 2. Changes in average microbial numbers ($\log_{10}$ CFU/ml) and pH during spontaneous lactic fermentation of cow milk

## Discussion

*Enumeration of microorganisms*

The laboratory-produced Leben had significantly higher numbers of LAB, yeasts and moulds compared

Table 2. Identification of LAB and yeast isolated from Leben

| Strain reference number | API identification |
|---|---|
| B01, B02, B03, B301, B302, B401, B501 | *Lactococcus lactis* subsp. *lactis* (92.6%)[a] |
| B04, B05, B303, B304, B403 | *Lactococcus lactis* subsp. *cremoris* (98.8%)[a] |
| B201, B202, B04 | *Leuconostoc mesenteroides* (99.1%)[a] |
| Y01, Y02 | *Candida lusitaniae* (97.5%)[a] |
| Y101, Y201, Y202 | *Candida tropicalis* (96.6%)[a] |
| Y102, Y301, Y302, Y303, Y304 | *Candida krusei* (98.6%)[a] |

[a](%) Percentage similarity.

to traditional Leben. This was probably because the two Leben products were not made from the same raw milk source, thus variations in microbial composition of the raw milks may have resulted in the observed differences in LAB and yeast counts in the fermented products. Several authors have recorded the predominance of LAB in traditional fermented cow milk products (Mathara, 1999; Abdelgadir *et al*., 2001; Beukes *et al*., 2001). These investigators found the main LAB genera to comprise lactobacilli, lactococci and leuconostocs. The presence of enterococci and pyogenic streptococci has also been reported (Mathara, 1999; Beukes *et al.*, 2001). *Lactobacillus* was not detected in Tunisian Leben. This result can be explained by the fact that the fermentation of milk by lactococci would not be enough moved for allowing the lactobacillus to develop. Benkerroum and Tamime (2004) reported the same explanation to confirm the lower lactobacillus counts in Moroccan Leben. Counts of yeast were lower, relative to the LAB. Since the isolated yeast organisms exhibited limited carbohydrate-assimilation ability, it is likely that they play a role in flavour development in Leben. Narvhus and Gadaga (2003) reported that the proteolytic and lipolytic activity of yeast strains in fermented milk is likely to contribute towards development of flavour compounds and, in the case of kefir and koumiss, the desirable properties of carbon dioxide and ethanol production. 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 not only a secondary role in the fermentation process but also a role in the aroma development in the product.

*Changes in microbial numbers and pH during spontaneous fermentation*

There was an observed sharp decline in coliform counts from 4.6 $\log_{10}$ CFU/ml to almost undetectable levels in the latter stage of the fermentation. This reduction in pH as a result of the production of organic acids (e.g. lactic acid) is likely to be the reasoning behind the suppression of coliform population in Leben. Gran *et al.* (2003) have reported the inhibition of E. coli and other coliforms by low pH caused by the production of organic acids in fermented milk products. Counts of yeasts were relatively low and recorded a 2-log increase during the 20-h fermentation period, which recorded a pH drop from 6.71 to 4.4. Benkerroum and Tamime (2004) studying changes in microflora during fermentation of cow's milk, found that yeasts were recovered towards the end of the fermentation period (due to the favorable effect of acidity developed in the product) and with a final pH of about 4.4. However, the levels of yeasts found in Leben after 20 h (5.11 $\log_{10}$ CFU/ml) were similar to those reported in other traditional fermented milks, ranging from 4.64 to 7.32 $\log_{10}$ CFU/ml (Mathara, 1999; Abdelgadir *et al.*, 2001). Despite the relatively low levels of yeasts compared to LAB in fermenting cow milk, these organisms are likely to be significant in flavour development in Leben, since it has been reported that the proteolytic activity of yeasts contributes to the flavour of fermented products (Samet-Bali *et al*., 2010).

*Identification of lactic acid bacteria*

As shown in Table 2, *Lactococcus lactis* (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) were the most frequently isolated LAB species from the 15 traditional Leben samples (48% of total isolates). Taïbi *et al.* (2011) reported that among 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. *Lactococcus lactis* are homofermentative, fermenting glucose via the glycolytic pathway to lactic acid as the major or sole product of fermentation. This suggests their significant role in lactic fermentation of cow milk. Itoi *et al.* (2009) reported that *Lactococcus lactis* species have been isolated not only from

milk products but also from various environments suggesting that this bacterium has high adaptability to various environments. *Lactococcus lactis* can also be found in the wild on plants and within the digestive tract of cows. It is believed that in nature, *Lactococcus lactis* stays dormant on plant surfaces awaiting to be ingested along with the plant into animal gastrointestinal tract, where it becomes active and multiplies intensively (Bolotin *et al.*, 2001). The most frequently isolated homofermentative LAB species was *L. lactis* subsp. *lactis* (28% of total isolates) followed by *L. lactis* subsp. *cremoris* (20% of total isolates). The importance of *L. lactis* subsp. *cremoris* is demonstrated by its continual use in food fermentations specifically in the manufacture of fermented milk products. *L. lactis* subsp. *cremoris* strains are preferred over *L. lactis* subsp. *lactis* strains because of their superior contribution to product flavor via unique metabolic mechanisms (Salama *et al*., 1991).

The rest of the isolated LAB species was *L. mesenteroides*. Holzapfel (2002) noted that *L. mesenteroides* frequently dominates the early stages of most spontaneous fermentations. Similar results were also reported for similar fermented milk products (Mathara, 1999; Benkerroum and Tamime, 2004). All members of the genus *Leuconostoc* are heterofermentative, fermenting glucose via the hexose-monophosphate pathway to produce equimolar amounts of lactic acid, ethanol and $CO_2$ (Samet-Bali *et al*., 2010). Additionally, members of the genus *Leuconostoc* are able to convert citrate to aroma compounds such as acetoin and diacetyl, a characteristic that would be of functional significance towards aroma development in Leben.

*Identification of yeasts*

Cultured milk products (fermented milk, sour cream, yogurt, drinking yogurt, cottage cheese, cream cheese, etc.) are ideal medias for the propagation of yeasts, as they exhibit a low pH, which is optimal for yeast growth (Alvarez-Martin *et al.*, 2008). Due to the acidic environment (pH 4.4), there is limited competition from bacteria in Leben. Most of those that can still grow alongside the yeasts are LAB. Yeast growth in milk is attributed to their ability to utilize milk constituents, such as proteins, fat, lactose and citrate. Alvarez-Martin *et al.* (2008) have connected the growth of yeasts in dairy products not with their ability to use lactose but with their capability to metabolize lactic acid. Out of the isolated yeast species, *C. krusei* was isolated most frequently (18% of total isolates and 50% of yeast isolates). Frazier and Westhoff (2001) report that *C. krusei* has been used with dairy starter cultures to maintain the activity and increase the longevity of LAB. This could imply a symbiotic association between *C. krusei* and the LAB involved in Leben production. Additionally, *C. krusei* plays an essential role in flavour development during fermentation of cacao beans, as a result of its proteolytic activity. It may be possible that *C. krusei* plays a similar functional role in flavour development in Leben.

## Conclusion

This work has shown that the microflora involved in production of Leben comprises a combination of LAB and yeasts. The LAB were represented by different Leuconostoc and Lactococcus species, with the most frequently isolated LAB being *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. *C. krusei* was found to be the predominant yeast species. All the isolated LAB species in Leben were lactose-fermenters, an important functional characteristic in fermentation of cow milk. The main functional role of the yeasts is likely to be flavour development and proteolysis, though the precise role of yeasts in Leben requires further study.

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