Isolation and characterization of lactic acid bacteria from Libyan traditional fermented milk "Laban"

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Abstract

Fermented milk product "Laban" in Libya is one of the most a traditional fermented milk product consumed a refreshing drink, particularly in the warm season. The average values of the physicochemical including titratable acidity, pH, total solids, and fat were 0.73%, 4.16, 8.12%, and 1.54% respectively. Coliform, yeast and mold counts were 21×10^4 , 39×10^4 , and 41×10^3 cfu/ ml., respectively. Most strains of coliform bacteria were *Serratia odorifera*, *Escherichia coli* 1, *E. coli* 2. and *Klebsiella oxytoca*. The average *Lactococcus*, *Streptococcus*, Mesophilic *Lactobacillus* / *Leuconostoc* and Thermophilic *Lactobacillus* counts were 99×10^7 , 96×10^7 , 93×10^7 and 15×10^7 cfu / ml. respectively. A total of 142 lactic acid bacteria (LAB) isolates were identified to the genus level as *Lactobacillus* (48.59%), *Lactococcus* (43.66%), *Streptococcus* (4.93%) and *Leuconostoc* (2.82%). Sugar fermentation tests revealed the most frequent *Lactobacillus* species found to be *Lactobacillus delbrueckii* ssp. *lactis* (62.32%), followed by *Lactobacillus plantarum* (31.88%). Furthermore, other selected LAB isolates were identified by API 50 CH test as *Lactococcus lactis* ssp. *lactics*, *Lactobacillus pentosus*, *Lactobacillus brevis*, and *Leuconostoc mesenteroides* ssp. *cremoris*. Thus, our research documented the lactic acid bacteria strains and will provides fundamental basic and useful information for further studies of strain selection starter culture, with regard to the industrial production of fermented dairy milk products.

Keywords: Lactic acid bacteria, Traditional fermented milk, Isolation, Identification. Characterization.

1. Introduction:

Food fermentation is one of the most traditional procedures for food processing, consuming and preservation, and dates back to thousands of years in many countries within Africa Asia, the Middle East, and Northern and Eastern Europe (Chen et al., 2010; Liu, Han, & Zhou, 2011). Laban is considered part of people's diet because it is low-cost, easy to prepare, and the traditional technique to produce is very simple (Ao et al., 2012; Azam et al., 2017). Libya, a large country in north Africa, has a high percentage of population living in the countryside and depending mainly on livestock and on dairy products. Food fermentation is made mainly from cow, goat, ewe, or camel milks. Furthermore, Libya has warm climate where long storage life of either of pasteurized or raw milk is difficult to reach (Ziyaina, Rasco, Coffey, Mattinson, & Sablani, 2019). These environments climatic conditions make the difficulty of storage, and marketing of dairy products in optimum conditions because of an often-undependable cold chain during the distribution. (Ziyaina, Govindan, Rasco, Coffey, & Sablani, 2018). Thus, food fermentation has played a pivotal role in preserving food and food security. Farmers and householders produce fermented milk by placing the milk in a tanned goat's leather bag container called a *Shakwa* and adding residues of fermented milk from previous batches as a starter culture. The milk is kept in a bag made of animal skin to provide the optimum temperature of lactic acid bacteria growth 20–30 °C (Caplice & Fitzgerald, 1999). During the fermentation and when sour flavors are achieved, milk is soured and churned several times until the fat globules coalesce. After removing the butter granules, the remainder liquid is called Laban or Sour milk.

Laban Lactic acid bacteria (LAB) are extensively distributed in nature and present natural as indigenous bacteria in raw and fermented milk as well. LAB are the most commonly used microorganisms in fermented foods (Silva, Carvalho, Teixeira, & Gibbs, 2002; Sodini, Lucas, Oliveira, Remeuf, & Corrieu, 2002). LAB are crucial to the intrinsic properties of fermented food products and play a major role in the development of the distinctive taste of fermented products such as aroma, texture, and acidity (Hamama & Bayi, 1991; Liu et al., 2011; Olson & Aryana, 2008).

LAB is known to be Gram-positive, catalase-negative, nonsporing, and microaerophilic, to contain rods and cocci, and to produce lactic acid as the main metabolic result of lactose fermentation (Calo-Mata et al., 2008). Previous studies have reported that LAB has been isolated from different sources such as milk products, fermented foods and vegetation, and the most common food-associated LAB genera are *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus*, and *Pediococcus* (Ao et al., 2012; Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Fox, Guinee, Cogan, & McSweeney, 2017; XIONG, WANG, LU, & XIONG, 2004). Besides LAB there are other microorganisms, including coliforms, yeasts, and molds, that are isolated from fermentation milk; however,

they are present in low numbers and only in the early stages of the fermentation (Ao et al., 2012; Gonfa, Foster, & Holzapfel, 2001).

Presently, studies on LAB isolates from nature, and on the fermentation profile of Libyan Laban, remain scarce. Thus, the purpose of this study is to identify and screen the lactic acid bacteria (LAB) that can be used as starter culture in the manufacturing of Laban, and to evaluate the overall physicochemical and microbiological quality of traditional Laban.

2. Materials and Methods

2.1. Samples Collection and Preparation:

Sixteen naturally fermented cow's milk samples (about one liter) were collected from different cities located in the Northwest of Libya. The samples were put in sterile bottles and kept in an insulated icebox until they were taken to the laboratory to be immediately examined within 24 hours upon receipt.

2.2. Physiochemical Analysis:

The pH of the traditional fermented milk was measured using a digital pH-meter (Jenway pH-meter, Model number 3030). The pH-meter was calibrated before use by using the standard buffer solutions range of pH 4.0 and pH 7.0. Titratable acidity as percentage of lactic acid of Laban samples using 0.1 mol L⁻¹ NaOH was used for the titratable acidity, and expressed as g of lactic acid per 100 g of sample (Bradley et al., 1992). The fat percentage as determined by Gerber method, and the total solids % of Laban samples was determined according to the Standard methods for the examination of dairy products by Bradley *et al.* in 1992.

2.3. Microbiological analyses

Microbial analyses were initiated after 2-8 h sample collection depending on Laban sample's location. Under aseptic conditions, 10 ml Laban sample were diluted to 1:10. Subsequent decimal dilutions were made using sterile 0.1% peptone water, and selected serial dilutions were made and were pour-plated on the following (i) Coliform count: Coliforms were determined on Violet Red Bile Agar (Oxoid) and incubated at 32 ±1 °C for 24 h., and typical Coliform colonies were confirmed using API 20E test kit (bioMérieux sa) (Christen, Davidson, McAllister, & Roth, 1992). (ii) Yeast and Molds count: Potato Dextrose Agar pH 3.5 (Oxoid) was used and incubation was carried out at 25 ±1 °C for 3-5 days (Frank, Hankin, Koburger, & Marth, 1985; Hamama & Bayi, 1991) (iii) *Lactococcus* count: Neutral Red Chalk Lactose Agar (Oxoid) was used and incubated at 30 ±1 °C for 48 h (An, ADACHI, & OGAWA, 2004; Harrigan, 1998). (iv) Streptococcus count: Neutral Red Chalk Lactose Agar (Oxoid) was used and incubated aerobically at 37

± 1 °C for 48 h (Harrigan, 1998). (v) Mesophilic *Lactobacillus/Leuconostoc* count: MRS Agar (Oxoid) was used and aerobic incubation was carried out at 35 ± 1 °C for 48 h (Harrigan, 1998). (vi) Thermophilic *Lactobacillus* count: MRS Agar (Oxoid) was used, then the plates were incubated aerobically for 48 h at 42 ± 1 °C (Harrigan, 1998).

2.4. Identification of LAB to the genus level:

Representative isolates of the different groups were chosen to determine the phenotypic features including morphological, physiological, and biochemical characteristics. Gas production from glucose and growth in 3.0 and 6.5% NaCl was tested in MRS broth at 30 ± 1 °C for 48 h. Growth at 15 and 45 ± 1 °C was observed, and production of NH_3 from arginine was evaluated (Guessas & Kihal, 2004).

2.5. Identification of LAB to the species level:

Sugar fermentation tests including raffinose, arabinose, and lactose or cellobiose were used, and incubation was carried out at 37 ± 1 °C for 24 h. In addition, 10 representative isolates were selected to identification to species level by using the API CH 50 (BioMerieux) technic. Then has been taken part of bacterial colonies and inoculated into tubules of the API according to the manufacturer's instructions. The galleries were incubated at 37 ± 1 °C for 24- 48 h. The API LAB PLUS database (bioMe'rieux sa) was used to interpret the results (Beukes, Bester, & Mostert, 2001; Guessas & Kihal, 2004).

3. Results & Discussion:

3.1. The physicochemical and microbiological parameters of Laban

The average number of the physicochemical and microbiological quality parameters of Laban samples are shown in Table 1. Results indicate that pH of Laban samples ranged between 3.73 to 4.66 with a mean value of 4.16. The low pH value of Laban sample is due to post acidification through milk fermentation process, and, related to the cumulative transformation of lactose into lactic acid by LAB, which is the metabolic activity of LAB growth. The data found are similar to those determined by (Hamama & Bayi, 1991; Olson & Aryana, 2008; Vieira et al., 2021; Ziyaina et al., 2018) who reported the pH of storage and fermented milk samples were 4.2. In this study, the average titratable acidity was 0.73%. The acidity values in fermented milk have a strong correlation with LAB strain activity. Moreover, most lactobacilli in fermented milk are characterized by relatively higher acidification rates and strong post acidification (St-Gelais, Lessard, Champagne, & Vuillemand, 2009). Laban samples value for total solids was 8.12 ± 0.100 as shown in Table 1. Total solids and pH value parameters were different from those reported by Ali, 1987 and Hamama and Bayi 1991, whose results indicated higher total solids content of 10.01 and 10.07%, respectively. The difference in those parameters may be related to several factors, such as differences in milk composition, production method,

microorganisms involved in fermentation, temperature and time of fermentation, and to the water added at the end of the fermentation process to facilitate fat globules separation. Fat (1.54%) content of Laban samples were also low, and this could be related to removal of butter granules, water added during churning process, or differences in milk composition used for preparing Laban Low fat content was also reported by (Samet-Bali, Felfoul, Lajnaf, Attia, & Ayadi, 2016). High coliform and fungi counts were found in Laban samples as presented in Table 1. Coliform, yeast, and mold mean counts were 21×10^4 , 39×10^4 , and 41×10^3 cfu/ ml., respectively. From the results on microbial quality of Laban, the most dominant strains of coliform bacteria were *Serratia odorifera*, *Escherichia coli* 1, *E. coli* 2. and *Klebsiella oxytoca*. These results are in agreement with (Gran, Wetlesen, Mutukumira, Rukure, & Narvhus, 2003), who found *E. coli* and *Klebsiella pneumonia* spp. in fermented soured milk that were produced at small-scale dairies as they reported in their study. Production of Laban from raw milk under poor hygienic conditions is probably the main causes of these high counts. Hamama and Bayi (1991) also reported higher coliform and mold counts of 3.05×10^6 and 4.4×10^4 cfu/ ml. respectively. However, yeast counts were 9.4×10^3 cfu/ ml. which are a little less than counts found in the present study.

Table (1). The average values of the physicochemical and microbiological (cfu/ ml.) parameters of Laban samples.

Parameter	Range	Mean
pH	3.76 – 4.66	4.16 ± 0.013
Titrateable acidity %	0.48 – 0.90	0.73 ± 0.005
Fat %	0.27 – 3.13	1.54 ± 0.097
Total solids %	4.97 – 9.80	8.12 ± 0.100
Coliform count n= 14	35×10^3 - 13×10^5	21×10^4
Yeast count n= 9	59×10^3 - 11×10^5	39×10^4
Mold count n= 9	8×10^3 - 12×10^4	41×10^3
Mean $\pm$ standard deviation		

From the results, the Laban samples have high counts of coliform and fungi counts for many reasons, including the production of Laban from raw milk, methods of production under poor conditions, lack of hygiene, use of contaminated raw materials, storage conditions, and unstable shelf-life of products. Similar results have been reported in the published studies by (Abd El Gawad, Abd El Fatah, & Al Rubayyi, 2010; Gadaga, Mutukumira, & Narvhus, 2000; Hamama & Bayi, 1991; Savadogo et al., 2004).

3.2. Isolation and identification of LAB:

Table (2) indicates the domination of LAB genera in Libyan traditional fermented milk samples, in which higher mean counts of *Lactococcus*, *Streptococcus*, Mesophilic *Lactobacillus* / *Leuconostoc*, and Thermophilic *Lactobacillus* 99×10^7 , 96×10^7 , 93×10^7 and 15×10^7 cfu / ml. respectively were reported.

Table (2) The bacterial composition of LAB (cfu/ml) genera in traditional fermented milk Labna samples.

Parameter	Range	Mean
<i>Lactococcus</i> count	49×10^6 - 18×10^8	99×10^7
<i>Streptococcus</i> count	59×10^6 - 19×10^8	96×10^7
Mesophilic <i>Lactobacillus</i> / <i>Leuconostoc</i> count	74×10^6 - 19×10^8	93×10^7
Thermophilic <i>Lactobacillus</i> counts	10×10^5 - 51×10^7	15×10^7

Those results are close with El Gawad, 2010 research of traditional Rayeb milk in Egypt, who found the LAB group in Rayeb samples to reach a final population of around 10^6 to 10^7 cfug-1 (Abd El Gawad et al., 2010).

Climate plays a large role in the production and diversity of LAB in traditional fermented milk; therefore, mesophilic bacteria including *Lactococcus* and *Leuconostoc* in traditional

fermented milk have more presence in warmer regions compared to cold climates (Chen et al., 2010; Mathara, Schillinger, Kutima, Mbugua, & Holzapfel, 2004; Watanabe et al., 2008). In our study, Lactic Acid Bacteria (LAB), the predominant microbial group in Laban, was Mesophilic, including *Lactobacillus* and *Leuconostoc* counts.

A total of 142 LAB isolates were first identified to the genus level according to the tests presented in Table 3. The tests were morphological, physiological, and biochemical for the identification of the LAB genus, including cell shape, Gram reaction, catalase reaction, growth at 15, 45°C, and NaCl 6.5%, production of NH₃ from arginine and production of CO₂ from glucose. The high of the isolate (69 isolates from LAB) were *Lactobacillus* with 48.59%. This genus has a short rod shape, positive Gram reaction, and negative catalase. *Lactobacillus* could grow at 15 & 45 °C, and 6.5% NaCl, produce NH₃ from arginine, and fermented glucose, and produce CO₂. A group 62 strains, from a total 142 LAB isolates, were determined, and found to have cocci in pairs, growth at 15 but not at 45°C, and produce NH₃ from arginine, but not CO₂ from glucose. Therefore, this group was considered as the genus of *Lactococcus* (43.66%). A quantity of 7 strains from the total were coccoid to oval in pairs or chains, Gram-positive, absence of catalase, growing at 45 but not 15 °C, also, not able to growing at 6.5% NaCl, producing NH₃ and not CO₂ gas from glucose. Thus, this group belonged to the genus of *Streptococcus* (4.93%). In addition, 4 isolated strains and 2.82% of the total strains were identified as *Leuconostoc* species. Strains displayed positive Gram reaction, catalase negative, grew at 15°C not at 45 °C, growing at 6.5% NaCl, produced gas CO₂ from glucose and could not utilize arginine for NH₃ production.

Table (3) Morphological and physiological characterization of LAB genera from traditional fermented milk "Laban"

Distinguish characteristic	<i>Lactobacillus</i>	<i>Lactococcus</i>	<i>Streptococcus</i>	<i>Leuconostoc</i>
Cell shape	Short rods	Cocci in pairs	Cocci to oval, in pairs or chains	Cocci in pairs
Gram stain	+	+	+	+
Catalase reaction	-	-	-	-
Growth at 15 °C	±	+	-	+
Growth at 45°C	+	-	+	-
Growth in 6.5% NaCl	±	-	-	±
Production of NH ₃ from arginine	±	+	±	-
Production of CO ₂ from glucose	±	-	-	+
Number of isolates = 142	69 (48.59%)	62 (43.66%)	7 (4.93%)	4 (2.82%)

(+) all strain positive (-) all strain negative (-/+) number of positive /negative strain.

The illustration of distribution at the genus level of the 146 LAB identified from the Laban' samples are shown in Figure 1. Close and similar results were reported by researchers from

Pakistan, Bangladesh, and Egypt. Where they isolated LAB, genera include: *Leuconostoc*, *Lactococcus*, *Lactobacillus*, *enterococcus*, *streptococcus* from a traditional fermented milk (Abd El Gawad et al., 2010; Harun-ur-Rashid, Togo, Ueda, & Miyamoto, 2007; Savadogo et al., 2004).

Figure (1). Distribution of LAB at the genus level isolated from traditional fermented milk Laban samples.

Sugar fermentation tests, including raffinose, arabinose, lactose, and cellobiose, have used 69 isolated strains from *Lactobacillus* to find the most frequently *Lactobacillus* species (Harrigan, 1998). The results found *Lactobacillus delbrueckii* ssp. *lactis* (62.32%) followed by *Lactobacillus plantarum* (31.88%) and (5.80%) not identified (Table 4).

Table (4) Results of the sugar fermentation tests for the *Lactobacillus* isolates.

Number and % of isolates	Identified Isolates
43 (62.32%)	<i>Lactobacillus delbrueckii</i> ssp. <i>lactis</i>
22 (31.88%)	<i>Lactobacillus plantarum</i>
4 (5.80%)	Not Identified

Furthermore, other selected LAB isolates were identified by API CH 50 test (Table 5) as *Lactococcus lactis* ssp. *lactis*, *Lactobacillus pentosus*, *Lactobacillus brevis* and *Leuconostoc mesenteroides* ssp. *cremoris*. The fermented dairy products manufacturing industry has been using *Lactobacillus* and *Streptococcus* as a starter culture of fermented milk.

Table (5) The identified species of lactic acid bacteria from Laban samples using the API CH 50 (BioMerieuxs).

<i>Lactococcus lactis</i> ssp. <i>lactis</i>
<i>Lactobacillus delbrueckii</i> ssp. <i>lactis</i>
<i>Lactobacillus plantarum</i>
<i>Lactobacillus pentosus</i>
<i>Lactobacillus brevis</i>
<i>Leuconostoc mesenteroides</i> ssp. <i>cremoris</i>

On the other hand, conventionally, the most likely hypothesis is that *Lactobacillus* and *Streptococcus* cooperate metabolically during the milk traditional fermentation process. However, the results reported indicate that these two genera are competitive and reach a dynamic equilibrium during milk fermentation.

4. Conclusion:

Lactic acid bacteria fermented milk constitutes an indispensable beverage of consumption as a cold drink in Libya and is a generous LAB resource. The variety of LAB may be related to the type of fermented dairy product, location origin, and manufacturing process methods. In this study, 146 strains of LAB were identified by a combination of conventional processes. Physiological and biochemical characteristics have been used to identify LAB that was isolated from traditional fermented milk, Laban. The species isolated most frequently were *Lactococcus*, *Streptococcus*, Mesophilic *Lactobacillus*, *Leuconostoc*, and Thermophilic *Lactobacillus* counts. In conclusion, in this study, the LAB strains that was isolated from traditional fermented milk "Laban" can be used in industrial scale as starter components for the manufacture of fermented dairy, such as traditional buttermilk, and they will play a major role in the development of dairy industries, especially in Libya.

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