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Original Article

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

Mohamed Nahaisi[1], Nadia Almaroum[1] and Mohamed Ziyaina[2,3]

1-Food Science and Technology Department, University of Tripoli, Tripoli Libya

2-School of Food Science, Washington State University, Pullman, WA 99164-6376, USA.

3- Animal Sciences, University of Zawia, Zawia, Libya.

Abstract

Fermented milk product "Laban" in Libya is one of the most traditional fermented milk product consumed as a refreshing drink, particularly in the warm season. The average values of the physicochemical tests on naturally fermented milk samples; 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, this research has documented the lactic acid bacteria isolated from naturally Libyan fermented milk and will provide fundamental basic and useful information for further studies of strain selection starter culture, with regard to the industrial production of fermented dairy products.

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

Introduction

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. Furthermore, Libya has warm climate where long storage life of either of pasteurized or raw milk is difficult to reach (Ziyaina *et al.*, 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

Corresponding Author: Mohamed Nahaisi.Food Sci. and Tech. Dep., Fac. Of Agric., Univ. of Tripoli- Libya

Phone: +218926452587 **Email: m.nahaisi@uot.edu.ly**

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distribution (Ziyaina *et al.*, 2018). 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 and Fitzgerald, 1999). 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. 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 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.

Materials and Methods

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 ice box until they were taken to the laboratory to be immediately examined within 24 hours upon receipt .

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 Bradly *et al.*, (1992).

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 (bioMe´rieux sa) (Christen *et al.*, 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 *et al.* , 1992; Hamama and Bayi,1991). (iii) *Lactococcus* count: Neutral Red Chalk Lactose Agar was used and incubated at 30 ±1 °C for 48 h (An *et al.*, 2004; Harrigan, 1998). (iv) *Streptococcus* count: Neutral Red Chalk Lactose Agar was used and incubated aerobically at 37 ±1 °C for 48 h (Harrigan, 1998). (v) Mesophilic *Lactobacillus*/

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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 anaerobically for 48 h at 42 ±1 °C (Harrigan, 1998).

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 6.5% NaCl was tested in MRS broth at 37 ±1°C for 48 h. Growth at 15 and 45 ±1°C was observed, and production of NH_3 from arginine was evaluated (Harrigon,1998;Guessas and Kihal,2004).

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 identify species level by using the API CH 50 (BioMerieux) technic. Then part of bacterial colonies has been taken and 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 *et al.* , 2001; Guessas and Kihal, 2004).

Results and discussion

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 and Bayi, 1991; Olson and 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 *et al.*, 2009). Laban samples value for total solids was 8.12 ± 0.10 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 was 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 *et al.*, 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 $\times10^3$ 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 *et al.*, 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 $\times10^3$ cfu/ ml. which are a little less than counts found in the present study.

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 *et al.,* 2010; Gadaga *et al.*, 2000; Hamama and Bayi, 1991; Savadogo *et al.,* 2004).

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 $\times10^7$, 96 $\times10^7$, 93 $\times10^7$ and 15 $\times10^7$ cfu / ml. respectively were reported. Those results are close with Abd El Gawad, *et al.*, (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. 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 *et al.*, 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 NH_3 from arginine and production of CO_2 from glucose. The highs 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.

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
Titratable 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 -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 ± standard deviation

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

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

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

Distinguish characteristic	*Lactobacillus*	*Lactococcus*	*Streptococcus*	*Leuconostoc*
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.

Lactobacillus could grow at 15 & 45 °C, and 6.5% NaCl, produce NH_3 from arginine, and ferment glucose, and produce CO_2. 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 NH_3 from arginine, but not CO_2 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, unable to grow at 6.5% NaCl, producing NH_3 and not CO_2 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 but not at 45 °C, growing at 6.5% NaCl, produced gas CO_2 from glucose and could not utilize arginine for NH_3 production.

The illustration of distribution at the genus level of the 146 LABs 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 including: *Leuconostoc*, *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus* from a traditional fermented milk (Abd El Gawad *et al.*, 2010; Harun-ur-Rashid, *et al.*, 2007; Savadogo *et al.*, 2004).

Sugar fermentation tests, including raffinose, arabinose, lactose, and cellobiose, Harrigon (1998) have been applied to find the most frequently-occurring *lactobacillus* species in 69 isolated *Lactobacillus*. The results of this study revealed *Lactobacillus delbrueckii* ssp. *lactis* (62.32%) followed by *Lactobacillus plantarum* (31.88%) and (5.80%) not identified (Table 4).

Furthermore, other selected LAB isolates were identified by API CH 50 test (Table 5) as *Lactococcus lactis* ssp. *lactics*, *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. 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.

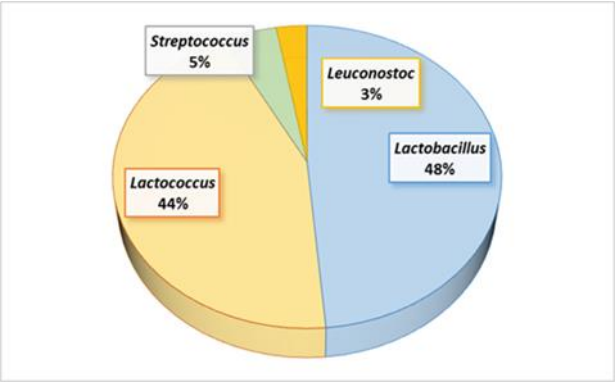

Figure 1. Distribution of LAB at the genus level isolated from traditional fermented milk Laban samples.

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

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

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

Lactococcus lactis ssp. *lactics*
Lactobacillus plantarum
Lactobacillus pentosus
Lactobacillus brevis
Leuconostoc mesenteroides ssp. *cremoris*

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 most frequently isolated genera were *Lactococcus*, *Streptococcus*, Mesophilic *Lactobacillus*, *Leuconostoc*, and Thermophilic *Lactobacillus.* In conclusion, in this study, the LAB strains that was isolated from traditional fermented milk "Laban" can be used on industrial scale as starter components for the manufacture of fermented dairy product, such as traditional buttermilk, and they will play a major role in the development of dairy industries, especially in Libya .

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المجلة الليبية للعلوم الزراعية
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ورقة أصيلة

عزل وتوصيف بكتريا حمض اللاكتيك من اللبن الليبي التقليدي المخمر "لبن"

محمد الهادي النحائسي[1]، نادية المروم[1]، محمد الزياني[2، 3]

1- قسم علوم وتقنية الأغذية ،كلية الزراعة - جامعة طرابلس ، ليبيا

2- قسم علوم الأغذية ، جامعة ولاية واشنطن ، بولمان ، الولايات المتحدة الأمريكية.

3- علوم الحيوان ، جامعة الزاوية ، الزاوية ، ليبيا

المستخلص

يعتبر الحليب المتخمر"اللبن" في ليبيا من أكثر منتجات الألبان المتخمرة التقليدية التي تستهلك كمشروبٍ منعشٍ خاصة في الفصول الحارة.ونظرا للإقبال المتزايد على منتجات الألبان المتخمرة الطبيعية المصنعة وقلة الدراسات البحثية في ليبيا في هذا المجال فقد صممت هذه الدراسة للتعرف على المحتوى الميكروبي للبن المتخمر طبيعيا وعزل بكتيريا حمض اللاكتيك السائدة. بلغت متوسطات القيم الفيزيائية والكيميائية لعينات اللبن المتخمرة طبيعيا والتي شملت حموضة المعايرة والأس الهيدروجيني، المواد الصلبة الكلية، والدهن 0.73٪، 4.16 ، 8.12٪، 1.54٪على التوالي. كما بلغت أعداد بكتيريا القولون والخمائر والأعفان 21× 10^4 و39 ×10^4 و41×10^3 و ت م/ مل على التوالي. كانت معظم أنواع البكتيريا القولونية *Serratia odorifera* و1 *Escherichia coli* و 2 *E.coli* و *Klebsiella oxytoca*. أما متوسط أعداد *Lactococcus* و *Streptococcus* و *Leuconostoc* / *Lactobacillus* المتوسطة المحبة للحرارة و*Lactobacillus* المحبة للحرارة العالية فقد بلغ 99×10^7، 96×10^7،93×10^7، و15×10^7 و ت م/مل على التوالي. كذلك تم تعريف 142 عزلة من بكتيريا حمض اللاكتيك على مستوى الجنس حيث كانت *Lactobacillus* (48.5 %)،*Lactococcus* (43.66%)، *Streptococcus* (4.93%) و *Leuconostoc* (2.82%) . كشفت اختبارات تخمر السكر أن أكثر أنواع *Lactobacillus* شيوعًا هي *Lactobacillus* delbrueckii ssp.lactis (62.32٪)، تليها *Lactobacillus plantarm.* (31.88%) علاوة على ذلك، تم تعريف عزلات LAB مختارة أخرى بواسطة اختبار (API 50 CH) على أنها *Lactococcus lactis* ssp.*lactis* و *Lactobacillus pentosus* و *Lactobacillus brevis* و *Leuconostoc mesenteroides* ssp.*cremoris*. وبالتالي فقد وثق هذا البحث أنواع بكتيريا حمض اللاكتيك المعزولة من اللبن التقليدي وسيوفر معلومات أساسية ومفيدة لمزيد من الدراسات حول اختيار البادئات البكتيرية خاصة المتعلقة بالإنتاج الصناعي لمنتجات الألبان المتخمرة.

الكلمات الدالة: بكتيريا حمض اللاكتيك، الحليب المتخمرالتقليدي، العزل، التعريف، التوصيف.

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هاتف: 218926452587+ البريد الالكتروني: m.nahaisi@uot.edu.ly

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