

Full Length Research Paper

Use of the RAPD-PCR fingerprinting and API system for clustering lactic acid bacteria isolated from traditional Sudanese sour milk (Roab)

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One hundred and sixty isolates of lactic acid bacteria (LAB) were isolated from ten samples of traditional soured milk (Roab) obtained from different Sudanese localities using *Lactobacillus*-selective medium. Forty-two representative single colonies were randomly picked (14 rods and 28 cocci). Randomly amplified polymorphic DNA (RAPD) analysis was used for rapid investigation of the genetic diversity of the isolates through un-weighted clustering group method with arithmetic averages. The fourteen rod-shaped isolates formed five clusters based on numerical analysis of the RAPD-PCR profiles. Representative strains from these clusters were identified by the API 50 CHL STREP identification system. These were *Lactobacillus delbreuckii* supsp. *bulgaricus* (one strain), *Lactobacillus rhamnosus* (nine strains), and *Lactobacillus plantarum* (two strains), *Lactobacillus casei* (one strain) and *Lactobacillus pentosus* (one strain). The 28 coccal isolates were separated into nine clusters, the representative strains of which were identified by the API 20 STREP system as *Aerococcus viridians* (four strains), *Enterococcus faecium* (two strains), *Enterococcus gallinarum* (two strains), *Lactococcus lactis* subsp. *lactis* (five strains), *Leuconostoc* sp. (five strains), *Streptococcus acidominimus* (eight strains) and *Streptococcus bovis* (two strains). Some of the isolates produced exopolysaccharides from sucrose (*Leuconostoc* sp., *Lactobacillus delbreuckii* supsp. *bulgaricus*, and *Lactobacillus plantarum* and *Lactobacillus pentosus*).

Key words: Lactic acid, *Lactobacillus*, molecular markers, RAPD-PCR.

INTRODUCTION

The lactic acid bacteria (LAB) are heterogeneous group of bacteria that are generally regarded as safe for use in food and food products (Rodriguez et al., 2000). They are used mainly because of their contributions to flavor, aroma, and increased shelf life of fermented products (Jorgen et al., 1999). Moreover, LAB protects foods from pathogenic microorganisms due to the production of lactic and acetic acids, hydrogen peroxide, diacetyl, fatty acids, phenyllactic acid and/or bacteriocins (Hitchener et al., 1982; Corsetti et al., 1998; Sutherland, 1994).

Traditionally, LAB has been identified by metabolic and

other phenotypic characteristics which are labor-intensive, time-consuming, ambiguous and difficult process (Pot et al., 1993). In addition phenotypic traits can also be affected by environmental conditions (Schleifer et al., 1995). Furthermore, it may be impossible for conventional methods to allow differentiation between phylogenetically related species as had been shown by Hayford et al., 1999. Since the molecular techniques are emerged as major sensitive tools over the traditional culture methods (Leisner et al., 2000), many attempts were developed to be used in rapid strain identification and differentiation (Olubukola, 2003).

Molecular-based methods have shown some promises in genotyping lactic acid bacteria strains (kljin et al., 1991; Le-Bourgeois et al., 1993) and randomly amplified polymorphic DNA polymerase chain reactions (RAPD-PCR)

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assay has become a rapid and reliable tool in Lactobacillus taxonomy and for intra- and inter- species genomic differentiation of various bacteria (Mazurier and Wernars, 1992; Nigatu, 2000). RAPD techniques specific for LAB have been established and successfully applied (Cocconelli et al., 1997; Roushdy et al., 1998).

The objectives of this study were to characterize and identify lactic acid bacteria that occur naturally in Sudanese traditional sour milk (Roab) by using both physiological and biochemical methods. The identities of lactic acid bacteria that are dominant during the fermentation process were also investigated by using RAPD fingerprinting.

MATERIALS AND METHODS

Isolation of strains of lactic acid bacteria

Presumptive LAB strains were isolated from 10 samples of Sudanese traditional soured cow's milk (Roab) obtained from different Sudanese localities (Khartoum, Madani, Nayla, El Fashir, Kasala, El Obied, El Gadarif, and Dongola) by plating on MRS agar (Michel et al., 2008), and incubating at 30°C for three days under anaerobic conditions. Pure cultures were maintained in glycerol at -20°C for further investigations (Garciela, 2001).

Phenotypic characterization

The preliminary identification and biochemical tests included gram staining, microscopic examination, catalase and oxidase activity were carried out according to the methods described by Kebede (2007). Growth at 10 and 45°C growth in 6.5% NaCl, production of acid and gas (CO₂) from glucose anaerobically, action on litmus milk and production of exopolysaccharide (EPS) were determined according to Gobbetti et al. (1999).

Carbohydrates fermentation

Ability of the isolates to ferment carbohydrates was studied using the API 50 CHL STREP system for the identification of lactobacilli and API 20 STREP for the identification of lactococci (La Balme les Grottes, 38390, Montalieu Vercieu, France). The selected strains were sub-cultured in MRS broth (incubated at 30°C for 48 h) before the tests were performed. The test was carried out as recommended by the manufacturer and the results were recorded after 24 and 48 h. Patterns of carbohydrates fermentation were identified by using the computer-aided identification program for lactic acid bacteria developed by Cox and Thomsen (1990).

DNA extraction

Genomic DNAs from 42 selected isolates were extracted by using 2 ml samples from overnight cultures that were grown in MRS broth at 30°C, as described by Carozzi et al. (1991). The final concentration of lysozyme used for cell lyses was 2 mg/ml. The concentration and purity of DNA were assessed by determining the optical densities at 260 and 280 nm, (Sambrook et al., 1989) and the DNA concentration of each sample was adjusted to 25 ng/ml.

RAPD-PCR analysis

The amplification reaction was carried out in a volume of 50 µl.

Each reaction mixture contained 10x PCR reaction buffer (Tris 108.0 g, boric acid 55.0 g, EDTA 7.44 g, H₂O (dd) up to 1 liter), 50 mM KCl, 10 mM Tris - HCl (pH 8.3), 2 mM MgCl₂ (0.01%, w/v), 250 µl of each of the four deoxynucleotide triphosphates (dNTPs) (dGTP, dATP, dCTP and dTTP), 2.5 units of *Taq* polymerase (Promega Corp, Madison, USA), 50 pmoles of each primer and 5 µl of the template DNA. A master mix was prepared in a 5 ml tube, according to the number of PCR reactions to be performed. An extra reaction mix was included to compensate for any loss of part of the solution due to frequent pipetting. An aliquot of 45 µl master mix solution was dispensed in each PCR tube (0.2 ml), containing 5 µl of the appropriate template DNA. Accordingly each reaction contained 1.0 µl dNTPs, 2.0 µl MgCl₂, 2.0 µl primers, 1.0 µl *Taq* polymerase, 5.0 µl templates DNA, 5.0 µl 10x PCR buffer and H₂O up to 50.0 µl. The reaction was subjected to 45 cycles consisting of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. This was followed by incubation at 72°C for 5 min, and cooling to 4°C, until samples were retrieved.

The PCR amplification products were visualized by running 20.0 µl of the amplification products on 1.5% agarose gel, which was stained with 3 µl ethidium bromide and examined under UV light and digitized using gel documentation camera (E.A.S.Y.; Herolab). Images were stored as tiff-files and further processed by gel Compare TM software version 3.1 (Applied Maths, Kortrijk, Belgium).

RESULTS AND DISCUSSION

One hundred and sixty LAB isolates were obtained from 10 samples of traditional Sudanese fermented cow's milk (Roab) collected from different locations in the Sudan. Microscopic examination revealed that 32 (20%) isolates were rod-shaped which occurred either singly or in chains and produced lactic acid from glucose anaerobically without production of CO₂ gas. These were considered as obligatory homofermentative lactobacilli, while the others 128 (80%) were cocci which occurred either in pairs or in chains, and produced lactic acid from glucose anaerobically. Among these, 15 isolates (11.72%) produced gas (CO₂) from glucose anaerobically, and they were presumptively identified as *Leuconostoc* sp. The other 113 coccal isolates (88.18%) produce no gas from glucose anaerobically, and they were presumptively identified as aerococci, enterococci, lactococci and streptococci.

On the basis of a number of preliminary tests which are commonly used for the identification of lactic acid bacteria, 42 homo- and heterofermentative isolates were selected for further investigations and characterization. Fourteen of these were rods, five were gas-producing cocci and 23 were non gas producing cocci from glucose anaerobically.

Exopolysaccharide (EPS) production

In this study a few of the tested isolates of lactic acid bacteria were produced EPS on MRS agar plates (in which glucose was replaced with 5%) after 24 h of incubation at 30°C. The isolates included species of the genus *Leuconostoc*, *L. delbrueckii* subsp. *bulgaricus*, *L. plantarum*, and *L. pentosus*, which were able to synthesize EPS.

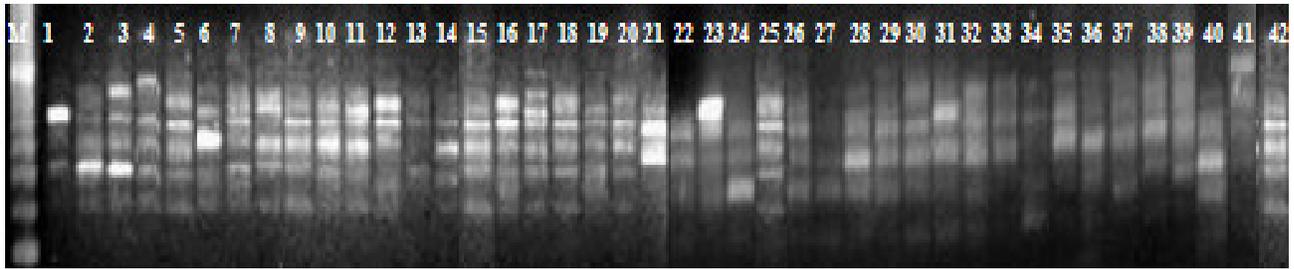


Figure 1. RAPD profiles of the 42 selected LAB isolates generated with primer O10 (70% G+C). Lanes 1 to 28 are coccal-shaped isolates and lanes 29 to 42 are bacillus. M: 1 KB DNA ladder.

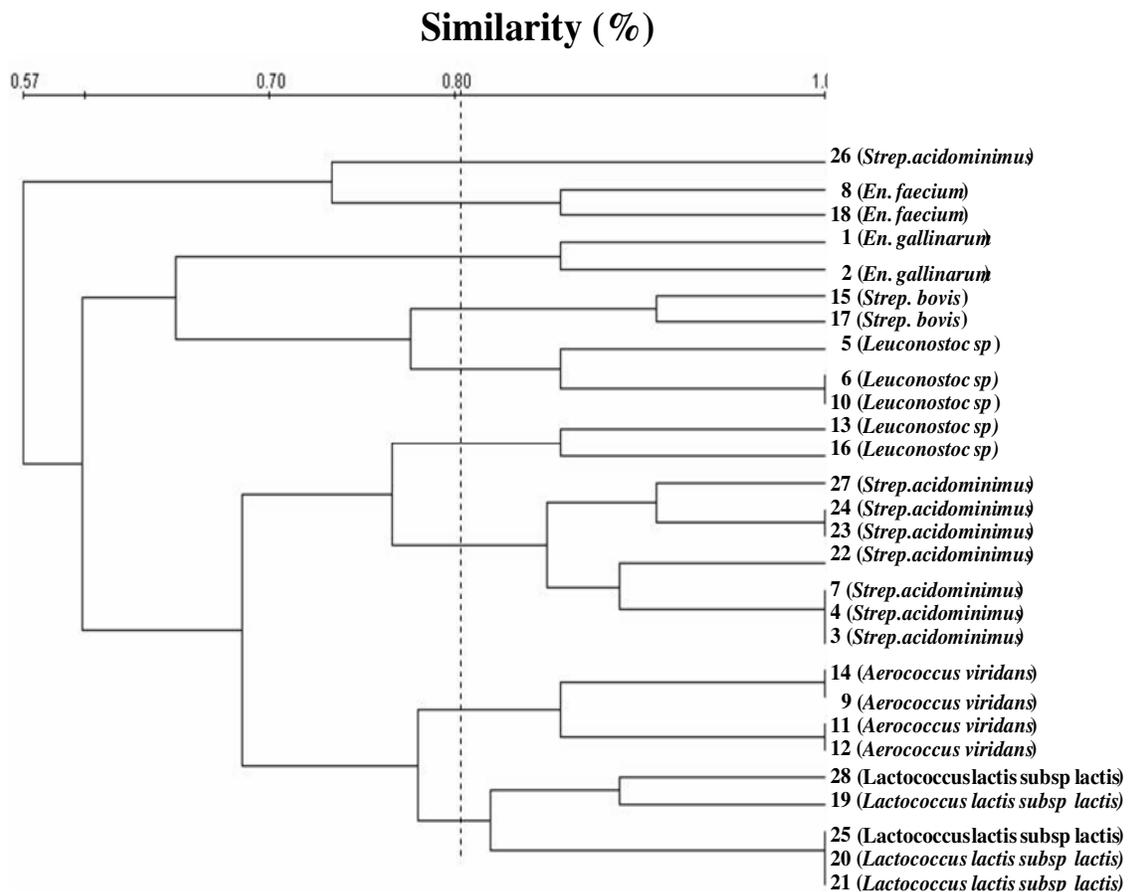


Figure 2. Dendrogram showing the clustering of the 28 coccal-shaped LAB isolates (No. 1 - 28) obtained by numerical analysis from RAPD-PCR profiles. Clustering was obtained by UPGMA program.

RAPD-PCR profiles

In the present investigations, the identification of lactic acid bacteria isolated from Roab, and the differentiation between the isolates has been carried out using the RAPD-PCR fingerprinting technique on 42 representative isolates (Figure 1). In this way specific DNA profiles of species, strains or biotypes can be detected which can elucidate the genetic diversity of this group of bacteria with the aim of confirming the phenotypic identification

and to help in further characterization.

The RAPD-PCR fingerprinting patterns of the selected strains obtained with the four above mentioned primers were analyzed together as a single set of data to obtain single dendrograms as shown in Figure 2 (cocci) and Figure 3 (rods). Numerical analyses of RAPD-PCR profiles were conducted and the similarities between RAPD-PCR profiles were calculated using the Dice coefficient. Cluster analysis was performed by the un-weighted pair-group method using arithmetic averages (UGGMA). The

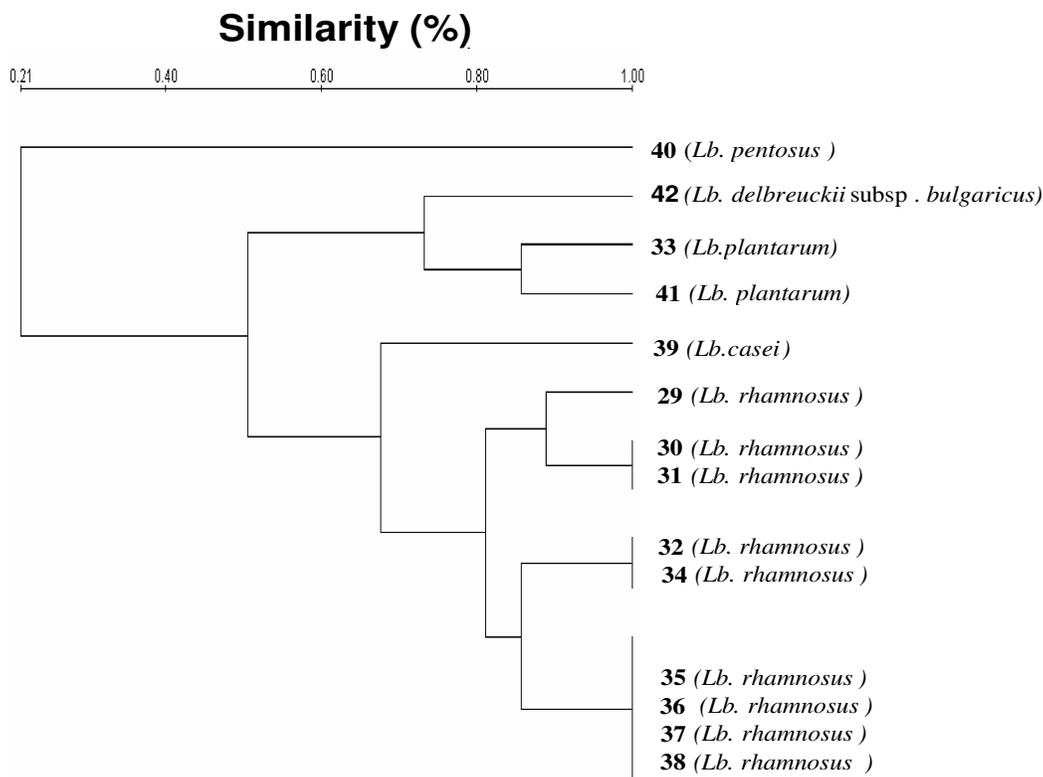


Figure 3. Dendrogram showing the clustering of the 14 Rod-shaped LAB isolates (No. 29-42) obtained by numerical analysis from RAPD-PCR profiles. Clustering was obtained by UPGMA program.

Table 1. Clustering and identification of 14 rod-shaped representative isolates of lactic acid bacteria using API 50 CHL STREP identification system.

Cluster no.	No. of isolates	API identification
1	1	<i>Lactobacillus pentosus</i>
2	1	<i>Lactobacillus delbreukii</i> subsp. <i>bulgaricus</i>
3	2	<i>Lactobacillus plantarum</i>
4	1	<i>Lactobacillus casei</i>
5	9	<i>Lactobacillus rhamnosus</i>
Total	14	

*Based on the results of the cluster analysis of RAPD-PCR as illustrated in Figure 2.

computer program: Diversity Data Base™ Fingerprinting Software (Bio-Rad) was used for fingerprinting.

Different clusters of lactic acid bacteria were observed at the 80% similarity-cut off level. With the use of tenmer primers allowed the delineation of 14 different clusters of selected local LAB isolates (Figures 2 and 3).

Representative strains of each RAPD-PCR cluster were further identified using the API 50 CHL STREP system for the rods (Table 1), and the API 20 STREP system for the cocci (Table 2). A computer program (Cox and Thomsen, 1990) was utilized for analysis of the API STREP identification system which resulted in assignment of the isolates to genera and species. Results of the

biochemical tests confirmed the clustering scheme obtained through the RAPD-PCR profiling system.

Analysis of the representatives of these clusters (42 isolates) showed that 33.33% belonged to the genus *Lactobacillus*, 23.81% to the genus *Streptococcus*, 11.91% to the genus *Lactococcus*, 11.91% to *Leucocostoc*, 9.52% to *Enterococcus* and 9.52% to *Aerococcus*, as can be calculated from the numbers of isolates in the clusters. Based on the RAPD-PCR analysis, cluster five (*L. rhamnosus*) was the predominant species of lactic acid bacteria representing 21.42% of the selected 42 representative isolates.

This was followed by cluster 12 (*Streptococcus acido-*

Table 2. Clustering and identification of 28 coccal shaped representative isolates of lactic acid bacteria using API 20 STREP identification system.

Cluster no.	No. of isolates	API identification
6	1	<i>Streptococcus acidominimus</i>
7	2	<i>Enterococcus faecium</i>
8	2	<i>Enterococcus gallinarum</i>
9	2	<i>Streptococcus bovis</i>
10	3	<i>Leuconostoc</i> sp.
11	2	<i>Leuconostoc</i> sp.
12	7	<i>Streptococcus acidominimus</i>
13	4	<i>Aerococcus viridans</i>
14	5	<i>Lactococcus lactis</i> subsp. <i>lactis</i>
Total	28	

*Based on the results of the cluster analysis of RAPD-PCR as shown in Figure 1.

minimus) representing 16.66%, then cluster 14 (*Lactococcus lactis* subsp. *lactis*) representing 11.90%. Identification by the API kit system showed complete agreement with the RAPD-PCR clustering scheme as far as the rod-shaped isolates were concerned. (Table 1) and it generally confirmed the delineation of the coccal forms into clusters by the RAPD-PCR technique. However the API 20 STREP identification system failed to separate between clusters 6 and 12 (both identified as *S. acidominimus*), as well as clusters 10 and 11 (both identified as *Leuconostoc* sp.). Nevertheless, the predominance of *Lactobacillus rhamnosus* (21.42%) and *L. lactis* subsp. *lactis* (11.9%) remains the same in both systems of identification, although the percentage of *S. acidominimus* increases to 19.04% based on the STREP identification system.

Among the other genera found in the present study is the genus *Enterococcus* which represented 9.52 % constituting clusters 7 and 8, as identified by the API 20 STREP identification system as *E. faecium* (cluster 7) and *E. gallinarum* (cluster 8) (Table 2). Although the cluster analysis by the RAPD-PCR profiling and biochemical identification by the API kit showed two distinct clusters (7 and 8). The isolates of both clusters showed very similar phenotypic and physiological characteristics. Most strains in these clusters grew at 10°C, 45°C and in the presence of 6.5% NaCl, produced polysaccharides from sucrose and curdled litmus milk. This indicates the stronger discriminative power of RAPD analysis as supported by biochemical characterization than phenotypic characterization alone.

Five of the 42 representative isolates (11.9%) were identified as *Leuconostoc* spp. (three in clusters 10, and two in cluster 11). The isolates in both clusters showed very similar phenotypic and physiological characteristics. They were differentiated by the RAPD-PCR analysis but could not be differentiated by phenotypic or biochemical

means and this give more confirmation to the higher differentiating power of the RAPD-PCR analysis as compared to phenotypic and biochemical characterization. Similar results were obtained by Cocconcelli et al. (1997) and by Quiberoni and Reinheimer (1998), who found that RAPD-PCR was able to group *Leuconostoc* spp. in accordance with their genetic diversity.

The API identification system has been widely used in the identification of lactic acid bacteria found in milk and fermented milk products, and it gave results agreed with those obtained by using traditional methods (Doliezil and Kirsop 1977; Abdelgadir et al., 2001). In the present study the API 50 CHL and the API 20 STREP identification systems proved very satisfactory, being simple to use and having good reproducibility. However, adoption of the RAPD-PCR profiling system seems to give more subtle differentiation. For instance the RAPD-PCR showed two different clusters (6 and 12), but the API system could not differentiate between representatives of these clusters. It thus appears that RAPD-PCR fingerprinting has more discriminative power and sensitivity as compared to the API system.

In conclusion, considerable variation was found in the LAB isolated from different source of the traditional fermented milk (Roab). Some of these isolates could be potentially useful starter for fermented dairy product. Further evaluation should be undertaken to determine if these isolates are of value in improving the nutritive contents and controlling the growth of spoilage and pathogen in dairy industry.

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