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Research Article

Isolation and Identification of LAB from Sudanese Traditional Fermented Camel (*Camelus dromedarius*) Milk Gariss

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Abstract

In present work fermented camel milk gariss collected from different locations in Khartoum and Kordofan regions, in order to isolate and screen lactic acid bacteria dominated gariss traditionally produced or fermented, then Lactic Acid Bacteria (LAB), isolated from the collected gariss samples, were identified genotypically using DNA based technique (PCR-DGGE). The isolation and identification of LAB revealed that the Enterococcus genus was dominant (75%) followed by Lactobacillus (17.5%) and Streptococcus (7.5%). The genotypic identification of 40 strains indicated that there were nineteen different strains in these three genera. These were identified as follows: E. faecium 75(3), E. hirae B3(2), E. faecium G13(3), E. hirae MP1(3), E. faecium NWL(3), E. durans R05 (2), E. dispar DLS3002(2), E. faecium CK1013(2), L. pentosus S2LPO2(1), L. plantarum BJG32(1), S. thermophilus (2), S. acidominimus (1), E. faecium CB6(2), E. durans R03(1), E. sanguinicola UPAA71(2), E. faecalis 45689 (2), E. mundtii MDEYAN(2), L. plantarum BJ6(5) and E. hirae CECT4081(1). The microflora associated with gariss fermentation which was studied in details by phenotypic and genotypic methods, allow an accurate identifications of E.faecium and L.plantarum strains as the predominant strains associated with gariss fermentation, However, more studies and condensed work are needed to be elaborated in future work in order to complete the isolation and characterization of new LAB strains that could be present in camel milk produced in Sudan and to compare the results with reports from other countries and regions rather than Kordofan and Khartoum production sites as recommended point of view.

Keywords: Camel; Fermented camel milk (gariss); LAB; PCR; DDGE; Sudan

Introduction

LAB is of most important microorganisms predominantly found in nutrient-rich habitats or environments. They are part of the normal microbiota of the Gastrointestinal Tract (GIT) of humans, and they constitute an important element of the non-starter microbial communities found in dairy products one of the important of that fresh or fermented milk and also other products rather than milk or dairy products [1]. There are various traditional fermented camel milk products that are produced by camel herders in different parts of the world [2-6]. Fermented milk products such as suusac and gariss are produced from camel milk in Kenya, Somalia and Sudan [2,5]. Camel's milk is produced in certain areas of Sudan, under nomadic conditions, the camel's milk being abundant in remote localities, the camel herders have to prepare gariss, a fermented product, on which they sustain living for several months as the sole source of various nutrients [2,7]. Gariss is a special kind of fermented milk, prepared solely from camel milk under more or less shaking. This product "made in Sudan" is not always available for the family as camels are often driven far away in search of pastures. So the camel boys tending these camels prepare gariss, on which they live for months as the sole source of nourishment. Besides its use as food, camel milk has been used in many regions as a cure for certain diseases [2,7,8]. Now a days studying microflora of traditional fermented dairy products as gariss and creation of starters is very important. The production of gariss of superior quality and to produce this traditionally fermented product on the industrial level with high quality starter cultures should be developed. The first step of such great project is the identification of the main microflora strains available in Sudanese source gariss that is the objective of the present work.

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Methodology

Microorganisms and growth conditions

LAB strains were isolated on the nutritive media M17 and MRS (Biokar Diagnostics, France). The transfers were repeated until to get pure colonies. The pure colony was inoculated in the respective media and conserved at 4°C after incubation at 37°C for 48 hours. For long term maintenance of isolates, stock cultures were stored at -20°C in 40% (v/v) glycerol, with 60% (v/v) M17 and MRS broth.

Preliminary identification of microorganisms

The pure strains were characterized by Gram staining followed by microscopic examination, catalase tests (ID color catalase ID-ASE Biomerieux France) and oxidase tests (Oxidase reagent Biomerieux, France).

DNA isolation

DNA was extracted with the guanidium extraction method [9]. For this method, 5 ml of overnight culture in MRS or M17 broth was centrifuged at 7500 rpm for 5 minutes and the supernatant was discarded. The cells were washed once with 1 × TE buffer (10 mM Tris.Cl, 1 mM EDTA; pH 8.0) containing 0.5% NaCl. After washing, the cell wall was removed by digestion with 100 µl of

TERMLS solution (1 mM Tris.Cl, 10 U/ml mutanolysin, 25 mg/ml lysozyme, 25 µg/ml RNase) at 37°C for 45 minutes. Cells were lysed and the protein denaturated by addition of 500 µl GES solution (5M guanidium thiocyanate, 100 mM EDTA, 0.5% Sarkosyl). Proteins was precipitated by addition of 250 µl 7.5 M ammonium acetate, and protein-containing precipitate was removed by addition of 500 µl of chloroform-pentanol (24:1) and centrifugation at 15000 rpm for 10 minutes. Total DNA was precipitated by addition of 0.6 volume of ice-cold isopropanol. DNA was pelleted by centrifugation at 15000 rpm for 15 minutes, and washed with 0.5 ml 70% ethanol to remove salts. The DNA was dried with vacuum drier for 5 minutes and resuspended in 150 µl of 10 mM Tris.Cl at pH 8.0. The DNA concentration was determined spectrophotometerically at 260 nm, by determining the absorbance and calculating that an absorbance 1 at this wavelength corresponded to 50 µg/ml of double stranded DNA [10]. DNA was adjusted to 10 ng/µl by dilution with 10 mM Tris.Cl pH8.0.

Multiplex-PCR

DNA was amplified in 50 µl volume containing 5 µl template DNA isolation, 2 µl $MgCl_2$, 0.5 µl Taq DNA polymerase, 2 µl DNTPse, 5 µl buffer, 10.5 µl molecular H_2O, 10 µl Lac_1, 10 µl Lac_2 and 5 µl Lac_3. One multiplex- PCR reaction was done for each of the 40 representative samples. The primers used are shown in Table 1.

Conditions of PCR

The conditions of PCR was as follows: Name of program DGGE, number of cycle 20+10 touchdown, the duration 3.5 hours; one cycle in 94°C for 5 min. and in 94°C for one minute. Ten cycle in 65°C for one minute, and in 72°C for 3 min. Twenty cycle in 94°C for one minute; in 55°C for one minute; in 72°C for 3 minutes and one cycle in 72°C for 10 minutes.

PCR products separation and visualization

PCR products were separated by electrophoresis on a 2% (w/v) agarose gel using 1 × TBE [10]. The gels were stained in ethidium bromide and photographed on an UV transilluminator.

PCR- DGGE analysis

PCR products were analyzed on DGGE gels based on the protocol of Muyzer et al. [11]. Polyacrylamide gels (160 mm × 160 mm × 1 mm) consisted of 8% (vol/vol) polyacrylamide (National Diagnostics, Atlanta, GA, USA) in 1 × TAE buffer (Bio-Rad), using a 35% to 70% denaturant gradient (100% denaturing polyacrylamide solution, corresponding to 7 M urea [National Diagnostics] and 40% [vol/vol] Formamide [Sigma-Aldrich]). Electrophoresis was performed for 16.5 h at 70 V in 1 × TAE buffer at a constant temperature of 60°C using the Dcode System (Bio-Rad). Gels were stained with Cyber Gold (solution of 50 × l in 500 ml 1 × TAE buffer) for 1h, followed by visualization of DGGE band profiles under UV light and digital capturing with a charge-coupled device camera.

The PCR for the purified solution of the gel bands was made with the same primer. After the applications of the primers, the PCR products obtained from the DGGE bands and from isolates were purified with Wizard PCR Preps DNA purification resins. Sequence analyses of the purified DNAs were performed using a BigDye terminator cycle sequencing kit (Applied Biosystems, Germany). The primers used for sequencing were same as the primer for reamplification of DNAs from DGGE bands. Sequence similarities were found by BLAST analysis software.

Result and Discussion

DGGE-PCR fingerprinting of LAB isolated from gariss

In this study, designed group of LAB- genus specific primer (Lac_1, Lac_2 and Lac_3) which developed a PCR-DGGE system to monitor the LAB diversity in gariss, which was demonstrated previously by Endo et al. [12] were used as a useful tool to analyze the LAB diversity in fermented food to the species level. The results showing the various bands of LAB can be visualized from (Figures 1-4). From the 149 LAB characterized phenotypically in this study, 40 isolates were selected according to their characteristics (shape after staining, color, size, growth in different NaCl concentrations-data not shown) for DNA extraction and fingerprinting. For the monitoring of LAB diversity isolated from gariss fermentation, 40 isolates and reference strains of *L. plantarum* and *E. coli* were included in the DGGE-PCR analysis using (Lac_1 Lac_2 and Lac_3) primers.

The sequencing analysis results revealed that LAB were identified as *Enterococcus faecium* 75 7.5% (3 isolates), *E. hirae* B3 5% (two isolates), *E. faecium* G13 7.5% (3 isolates), *E. hirae* MP1 7.5% (3 isolates), *E. faecium* NWL 7.5% (3 isolates), *E. durans* R05 5% (two isolates), *E. dispar* DLS3002 5% (two isolates), *E. faecium* CK1013 5% (two isolates), *Lactobacillus pentosus* S2LPO2 2.5% (one isolate), *Lactobacillus plantarum* BJG32 2.5% (one isolate), *Streptococcus thermophilus* 5% (two isolates), *Streptococcus acidominimus* 2.5% (one isolate), *E. faecium* CB6 5% (two isolates), *E. durans* R03 2.5% (one isolate), *E. sanguinicola* UPAA71 5% (two isolates), *E. faecalis* 45689 5% (two isolates), *E. mundtii* MDEYAN 5% (two isolates), *Lactobacillus plantarum* BJ6 12.5% (5isolates) and *E. hirae* CECT4081 2.5% (one isolate). The results indicated that *Enterococcus* isolates (30 out of 40 examined isolates, representing 75%,) was the dominant LAB in gariss samples. *Lactobacillus* and *Streptococcus* was found

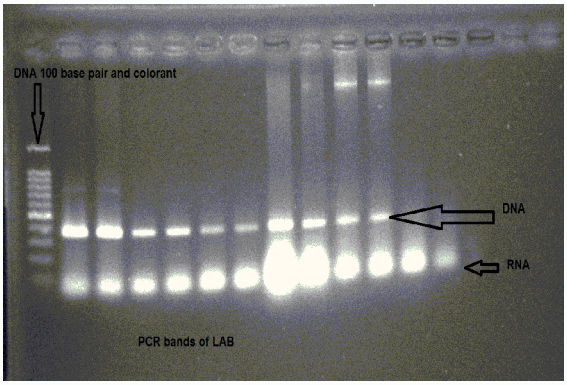

Figure 1: Genus specific multiplex PCR bands of LAB isolated from *gariss*.

Table 1: Genous specific primers of LAB.

Primer	Sequence (5- to 3-)	Target
Lac1	AGCAGTAGGGAATCTTCCA	16S rDNA of a group of LAB (*Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella*)
Lac2	CGCCCGGGGCGCGCCCCGGGCGGCCCGGG GGCACCGGGGGATTYCACCGCTACACATG	16S rDNA of LAB group
Lac3	AGCAGTAGGGAATCTTCGG	16S rDNA of a group of LAB (*Lactococcus*, *Streptococcus*, *Enterococcus*, *Tetragenococcus* and *Vagococcus*)

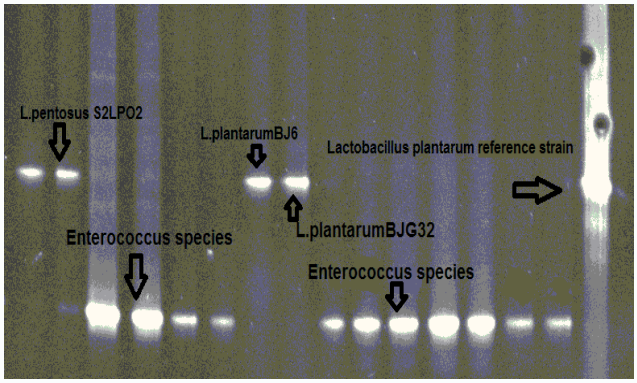

Figure 2: DGGE bands of *Lactobacillus species* isolated from *gariss*.

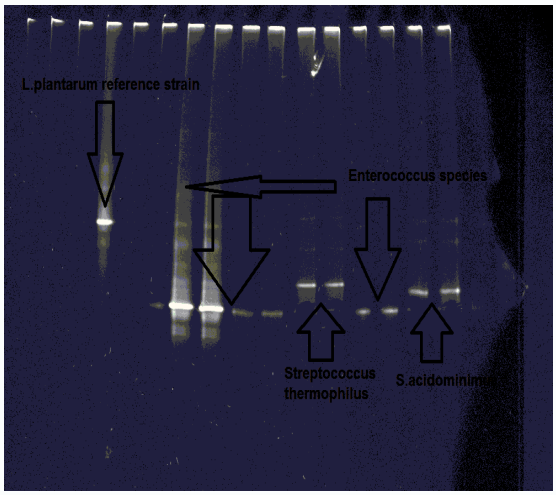

Figure 3: DGGE bands of *Streptococcus* species isolated from *gariss*.

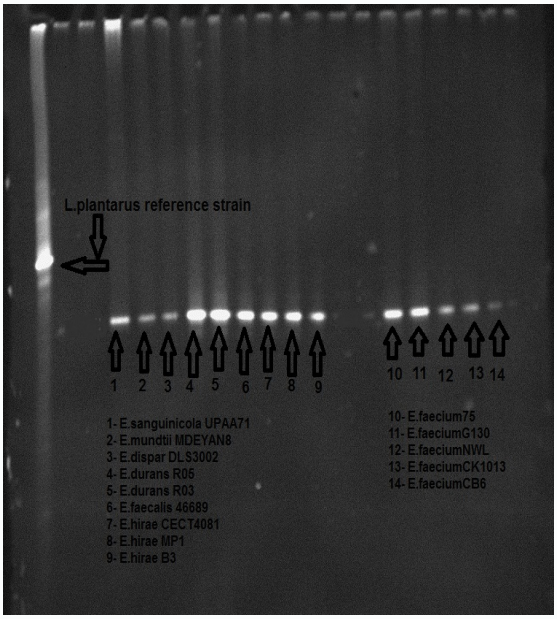

Figure 4: DGGE bands of *Enterococcus* species isolated from *gariss*.

to represent 17.5% and 7.5% of the identified LAB. The species frequency was as follows: *E. faecium* 13/40, *E. hirae* 6/40, *E. durans* 3/40, *E. dispar* 2/40, *E.sanguinicola* 2/40, *E. faecalis* 2/40, *E. mundtii* 2/40, *L.plantarum* 6/40, *L. pentosus* 1/40, *S. thermophilus* 2/40 and *S. acidominimus* 1/40. Estifanos et al. [13] isolated and studied lactic acid bacteria from camel milk found that isolated LAB strains belonged to *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Enterococcus* genera.

Qian et al. [14] found that the dominant isolated lactic acid bacterium using 16sDNA for identification genotypicaly was identified as *Lactobacillus plantarum* in yak yoghurts produced in China, while in Moroccan fermented camel milk microbiological analysis done by Ismaili et al. [15] the results revealed the predominance of lactic acid bacteria. A major proportion of the 93 lactic acid bacteria isolated from these samples was identified as *Lactobacilli* (35%), the other isolates belonged to *Lactococcus* (25%), *Enterococcus* (17%), *Leuconostoc* (13%) and *Streptococcus* (10%). Among the identified lactic acid bacteria, the most dominant species were: *Lactococcus lactis subsp lactis biovar diacetylactis*, *Lactobacillus brevis* and *Streptococcus salivarius subsp.thermophilus*.

Khedid et al. [16] who isolated lactic acid bacteria from one humped camel milk found that from 120 isolates, *Enterococcus* (14 isolates) is only represented with two species *Enterococcus casseliflavus* (9 isolates) and *E. faecalis* (5 isolates). Khedid et al. [16] reported that in camel milk of Morocco among the LAB isolates, rods accounted for 37.5%, cocci 62.5%. Rahman et al. [17] characterized the dominant microflora in shubat, a special fermented product prepared from unheated camel milk. After using phenotypic and molecular methods, LAB isolates were identified as *Lactobacillus sakei*, *Enterococcus faecium*, *Lactobacillus helveticus*, *Leuconstoc lactis*, *Enterococcus feacalis*, *Lactobacillus brevis* and *Weissella hellenica*, with the most frequently isolated LAB being *Lb. sakei*, *Ec. feacium* and *Lb. helveticus*. The microflora involved in production of suusac, a Kenyan traditional fermented camel milk product, were enumerated and identified by Lore et al. [5] who reported that LAB species were identified as *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, *Lactococcus raffinolactis* and *Leuconostoc mesenteroides subsp. Mesenteroides*, but no Enterococcus isolate was found, also *Enterococcus durans* and *E. faecium* were also found in Artisanal yoghurt studied in Cameroon by Moh et al. [18].

Conclusion

In the present work, the dominant lactic acid bacteria responsible for the spontaneous fermentation of the traditional fermented camel milk gariss were identified. In this study the abundance of some species such as *E. faecium NWL*, *E.durans* R03, *E.faecium* 75, *E.hirae* B3, *E.faecium* G130 and others suggests their possible use as starter culture in the manufacture of camel fermented milk products under controlled environment in the future that will be our next work. However, more studies are needed to complete the isolation and characterization of new LAB strains that could be present in camel milk produced in Sudan and to compare the results with reports from other countries and regions rather than Kordofan and Khartoum production sites.

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