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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

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Research Article
Molecular Characterization of Lactic Acid Bacteria Isolated from Starter Dough of Sudanese Sorghum Fermented Flat Bread (Kissra)

[1]Kawthar, M. Aseel, [2]Hanan, B. Eltahir, [3]Yousif, F. Hamed Elnil and [4]Ahmed, E. Elfaki

[1]Department of Food Technology, Nyala Technological College, Sudan Technological University, Sudan
[2]Department of Biochemistry, Faculty of Medicine and Health Science, University of El Imam El Mahadi, Sudan
[3]Department of Microbiology, College of Medical Laboratory Science, Sudan University of Science and Technology, Sudan
[4]Department of Food Science and Technology, College of Agricultural, Studies, Sudan University of Science and Technology, Sudan

Abstract

Objective: The purpose of this study was to isolate and characterize LAB from fermented kissra dough by both classical and PCR-based molecular methods to identify the isolates to sub-species level which may help to formulate starter culture as well as in the biological preservation of foods. **Materials and Methods:** Both classical and PCR-based molecular methods were used to identify the LAB isolates. A total of 75 lactic acid bacteria (LAB) isolates have been recovered from fermented kissra dough and characterized at strain level with molecular tools. RAPD analysis was performed initially to cluster the isolates using two different primers R2 and M13. Species identification was based on sequence analysis of 16S rRNA gene. Nine cluster of LAB PCR products sequenced and subjected to nucleotide BLAST. **Results:** Four percent (4%) (3 isolates), (Group1L) showed 100% homology towards *Pediococcus acidilactici* and 6.7% (5 isolates), (Group 9L) showed 100% homology towards *Lactococcus lactis* subsp *lactis* strain SFL. Among the rest of the 67 lactobacillus isolates, 1.6% (1 isolate), (Group 2L) showed 100% homology towards *L. murinus*, also same percentage (1.6%) (Group 4L) reported as *L. casei* strain IMAU70007. 2.9% (2 isolates), (Group 5L) showed 100 homology towards *L. plantarum* strain KLAB4. The same percentage (2.9%) (Group 8L) were showed 100% similarity towards *L. fermentum,* 5.9% (4 isolates), (Group 7L) showed 100% homology towards *L. casei* strain SWU30436, 20.9% (14 isolates), (Group 3L) showed 100% similarity towards *L. plantarum* strain 1.0557CGMCC, while the majority of the isolates (64.2%) (43 isolates), (Group 6L) showed 100% homology towards *L. plantarum* strain CSI7. Phylogenetic analysis was performed using software MEGA 6.0. **Conclusion:** Several different species of lactic acid bacteria can be implicated in the fermentation of kissra. The starch-fermenting strains might be important to formulate starter cultures and for use commercially in the production of kissra.

Key words: LAB, kissra dough, fermentation, RAPD PCR, 16s RNA sequence, phylogenetic tree

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Corresponding Author: Kawthar, M. Aseel, Department of Food Technology, Nyala Technological College, Sudan Technological University, Sudan

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lactic acid bacteria (LAB) are constituted of a heterogeneous group of Gram-positive bacteria and are widely consumed along with fermented foods and beverages because of their use as starter cultures in fermentation processes[1]. They colonize the gastrointestinal and urogenital tracts of humans and animals and are present in foods such as dairy products, fermented meats, fruits and vegetables[2]. Some LAB species are classified as "Generally Recognized As Safe" (GRAS) by the United States Food and Drug Administration (FDA) or have the "Qualified Presumption of Safety" (QPS) status by the European Food Safety Authority[2,3]. Accordingly, certain species of these genera are intentionally added to several probiotic products due to their potential health benefits[4].

The genus *Lactobacillus* is the largest group among the *Lactobacteriaceae* and contains over 100 species[5]. They are characterized as Gram-positive rods, anaerobic but aero tolerant, non-sporulating and catalase negative. They are commercially used as starter cultures in the manufacture of dairy products, fermented vegetables, fermented dough, alcoholic beverages and meat products[6].

Like other African countries, the food system in Sudan is classified by its huge and diverse production and consumption of fermented foods[7]. The raw materials from which these foods are prepared include sorghum, pearl millet, dates, honey, milk, fish, meat, wild plants, marginal food crops and even skins, hooves, bones, caterpillars, locusts, frogs and cow urine[8]. These fermented foods are still mainly prepared at the household level under poor sanitary conditions and marketed through informal routes. Consequently, many different contaminating microorganisms and/or indigenous microflora involved in this fermentation processes could be expected.

Kissra is the staple Sudanese diet. It is a morsel or piece of bread prepared from fermented sorghum flour[9]. The nutritive value of kissra is basically a discussion of the nutritive value of sorghum or millet, it was found that in Gezira and Managil areas, cereals provided 80% of the protein and together with sugar 84.4% of calories in the diet[7]. The word kissra is Arabic word[10] and together with the word aceda, has been mentioned in the early Arabic books[11]. Literally, the word kissra is a morsel or a piece of bread[12]. Two kinds of kissra can be described based on the method of spreading the dough during baking, kissrat-kass and kissrat-gergriba[7].

Today, with growing urbanization, kissra is becoming a commercial home-based industry in Sudan. Internationally, because of the apparent increase in the incidence of celiac disease and intolerance to wheat, interest in gluten-free cereal products is increasing rapidly[13]. Kissra appears to have considerable potential as the basis for development of a gluten-free sandwich wrap.

Research on kissra is limited. Recently, it has been shown that *Lactobacillus* and *Saccharomyces* cultures can be used to reduce the fermentation time from 19-4 h[14], which would be useful for commercial production. In view of the particularly attractive textural characteristics of the kissra pancake, selection of suitable sorghum cultivars for commercial production and for similar wrap type products is also a critical issue.

In general, the classical protocols of morphological and biochemical characterizations of microbial cultures are in use to identify isolated bacterial culture. The development of PCR-based methods using random amplification of polymorphic DNA (RAPD)[15], analysis of 16S rRNA gene homology, amplified[16] and species specific primers[17], have proved useful for identification of various species of LAB. Thus, the present study was focused on isolation and characterization of LAB from fermented kissra dough by both classical and PCR-based molecular methods to identify the isolates to sub-species level which may help to formulate starter culture as well as in the biological preservation of foods.

MATERIALS AND METHODS

Preparation of the fermented kissra dough: The fermented dough was obtained from the famous women of kissra makers in locality of Bahri, Khartoum State, Sudan. About 100 g of sorghum flour were mixed with 200 mL water in a round plastic container (Khumara). 25 g of previously fermented dough were added to the mixture to act as a starter culture. The dough was allowed to ferment for 3 h at room temperature.

Isolation of LAB: The fermented dough was prepared from sorghum as mentioned above. Ten mL fermented dough was sterilized diluted with saline, plated on De Man Rogosa Sharpe (MRS) agar (Himedia, Mumbai, India) and incubated anaerobically at 37°C for 24-48 h. The dominant colonies on MRS agar which were milky white, circular, convex, elevated and non-pigmented were chosen and further sub cultured. The colonies were streaked on MRS agar to check for purity. The pure cultures were overlaid with glycerol and preserved for further study Pal *et al.*[18].

Classical characterization of LAB isolates: Growth was assayed in MRS broth at room temperature and gram staining test, catalase production, endospore staining test, motility test, oxidase test, oxidative and fermentative test (O/F) and glucose (acid) test were carried out for the isolates as described by Harrigan[19].

DNA isolation: Genomic DNA was isolated by the procedure as described by Moore *et al.*[20].

(RAPD) analysis: RAPD analysis was carried out using the primers R2 5′-GGCGACCACTAG 3′ and M13 5′ GAGGGTGGCGGTTCT-3′[21]. Maxime PCR PreMix Kit (i-Taq) for 20 µL rxn was used to achieve the PCR process with little modification of external addition of 2.5 µL of $MgCl_2$.

The PCR cocktails (20 µL) consisted of 1 µL of the primers, 2 µL of DNA, 2.5U i-Taq DNA polymerase, 2.5 mM of each dNTP, 1 X reaction buffer, 1X gel loading buffer and 2.5 µL of $MgCl_2$. Amplification conditions were initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 1 min, annealing at 38°C for R2 and 40°C for M13 for 45 sec and elongation at 72°C for 1 min, followed by a final elongation at 72°C for 10 min[21]. The PCR products were visualized by running in 1.5% agarose gel electrophoresis with 100 bp DNA ladder (Sigma, Saint Louis, USA). The electrophoresis conditions were 100 V, 60 mA, for 20 min with 1X TBE as the running buffer.

Molecular characterization by 16S rRNA gene analysis: Amplification of 16S rRNA gene was performed from genomic DNA of the isolates using universal primers fD1 (5′-GAGTTTGATCCTGGCTCA-3′) and rP2 (5′-ACGGCTACC TTGTTACGACTT-3′)[22], with some modification. PCR cocktails (20 µL) consisted of 1 µL of the primers, 2 µL of DNA, 2.5 U i-Taq DNA polymerase, 2.5 mM of each dNTP, 1X reaction buffer, 1X gel loading buffer and 2.5 µL of $MgCl_2$. Amplification conditions were initial denaturation at 94°C for 3 min, 20 cycles of 94°C for 30 sec, annealing at 48°C for 45 sec and elongation at 72°C for 1 min, followed by a final elongation at 72°C for 10 min.

Purified PCR products were sequenced with automated DNA sequencer with specific primers using the facility at Macrogen Inc (Macrogen Inc., Seoul, Korea). Phylogenetic analysis was performed for the isolates using MEGA 6.0 software[23].

Accession number: These sequence data have been submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/gen bank/) under accession number KX430760 to KX430768.

RESULTS AND DISCUSSION

The present study deals with classical and molecular characterization of LAB isolates from fermented kissra dough. This study forms a broader objective to obtain a uniform consortium of strains having many beneficial properties as starter culture for commercial purposes.

Phenotypic characterization of LAB isolates: There were 75 lactobacilli isolates. The results of phenotypic characterization of LAB isolates tested are illustrated in Table 1. The results showed that, all isolates were Gram-positive, catalase negative, non-endospore-forming, non-motile and produced acid without production of gas from glucose. Among them, 89.3% (67 isolates) were rods, which occurred either singly or in pairs, when tested under microscope. These isolates were assigned to the genus *Lactobacillus*. While, 10.7% (8 isolates) were cocci which occurred in pairs or tetrads, 4% (3 isolates) of these cocci were identified as pediococcal strains. Also 6.7% (3 isolates) of these cocci exhibited a well-rounded cell morphology typical of the *lactococcus* when tested under microscope.

RAPD-PCR result: RAPD analysis was performed initially to cluster the isolates using two different primers R2 and M13. Group (1L): Isolates 3, 4 and 7 having similar pattern in the RAPD analysis belonged to a single group. Group (2L) and Group (4L) included isolates 5 and 9, respectively and having different patterns clustered into different groups, while Group (3L): Included isolates 8, 22, 27, 6, 48, 56, 58, 70, 72, 73, 26, 44, 51 and 36. Group (5L): Isolates 17 and 14 having similar patterns. Group (6L): The biggest one included isolates 20, 21, 24, 11, 12, 15, 55, 35, 10, 60, 18, 13, 61, 62, 37, 40, 38, 41, 32, 39, 63, 65, 68, 71, 50, 45, 74, 64, 1, 2, 19, 47, 67, 46, 53, 54, 57, 16, 49, 52, 23, 31 and 75 showed similarity. Group (7L): Isolates 25, 59, 33 and 69 having the same cluster. Group (8L): Isolates 30 and 34 belonged to same group and finally Group (9L): Isolates 42, 66, 43, 28 and 29 having the same cluster (Fig. 1a and b). Thus, nine different clusters were clearly observed based on the RAPD analysis.

16S rRNA result: The 16S rRNA was analyzed for the nine different clusters of isolates. The PCR products were

Table 1: Phenotypic characterization of the isolated LAB from Sudanese kissra fermented dough

	Isolated genera		
	Lactobacillus	lactococcus	*Pediococcus*
Number of isolate	67.0	5.0	3.0
Percentage	89.3	6.7	4.0

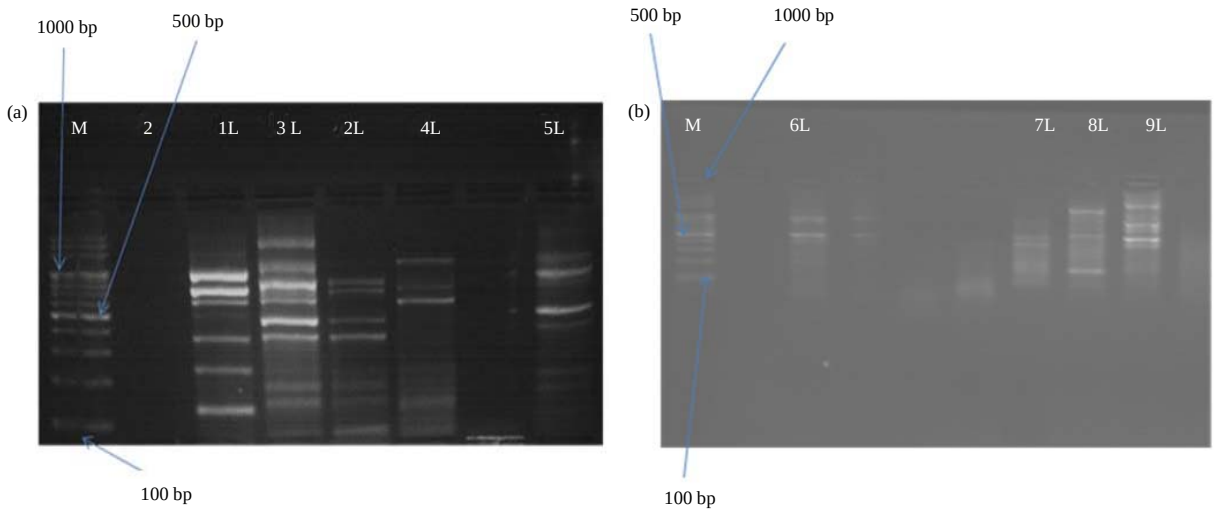

Fig. 1(a-b): RAPD analysis using the primer R2 and M13, (a) Group 1L to 5L and (b) Group 6L to 9L
M = 100 bp marker

sequenced and were subjected to nucleotide BLAST. 4% (Group 1L) showed 100% homology towards *Pediococcus acidilactici* and 6.7% (Group 9L) showed 100 homology towards *Lactococcus lactis* subsp. lactis strain SFL. Among the rest of the 67 *lactobacillus* isolates, 1.6% (Group 2L) showed 100% homology towards *L. murinus*, also same percentage (1.6%) (Group 4L) reported as *L. casei* strain IMAU70007. 2.9% (Group 5L) showed 100 homology towards *L. plantarum* strain KLAB4. The same percentage (2.9%) (Group 8L) showed 100% similarity towards *L. fermentum*. 5.9% (Group 7L) showed 100% homology towards *L. casei* strain SWU30436., 20.9% (Group 3L) showed 100% similarity towards *L. plantarum* strain 1.0557CGMCC, while the majority of the isolates 64.2% (Group 6L) showed 100% homology towards *L. plantarum* strain CSI7.

Multiple sequence alignment was carried out by BioEdit software and later phylogenetic analysis was performed using software MEGA 6.0. All the isolates were phylogenetically closely related to *Lactobacillus plantarum* and *Lactococcus lactis* (Fig. 2).

The population of indigenous LAB tend to dominate sour dough fermentations by the production of acid in the fermented dough[24]. Majority of LAB isolated in this study were homofermenters. This is in agreement with other research workers who reported the predominance of obligately homofermentative LAB in fermented maize meal for the production of sour bread and homofermentative lactobacilli and *Pediococcus* spp. from the final sour dough for production of Swedish rye bread[25-27].

Lactobacillus fermentum, *Lactobacillus plantarum* and *Lactococcus lactis* subsp. *lactis* were isolated from household and laboratory prepared bushera (Ugandan traditional non-alcoholic fermented beverage)[28]. Similarly, in Nigeria, Olasupo *et al.*[29], showed that lactic acid bacteria, notably *L. lactis* was isolated from a dairy product called ''wara''.

The lactic acid bacteria identified in kissra have been reported in other fermented foods. *L. plantarum* has been isolated from the raw material, sorghum powder and also from corresponding fermented and cooked fermented samples[30]. *L. plantarum* has been shown to be the dominant organism at the end of several natural cereal fermentations[31-33], as for instance in maize-derived products like ogi[34-37]. *L. plantarum* has also been identified as the predominant species in most vegetable fermentations[38]. The microbial composition of some African traditional fermented cereals such as ''poto poto'' (a maize dough from the Republic of Congo) and ''de'gue''' (a millet dough from Burkina Faso) has been shown by molecular techniques to include the presence of *L. plantarum*[39]. In some West African countries, the production of ''fufu'' (fermented cassava product), ''ogi'' (fermented maize, sorghum, or millet gruel), ''fura da nunu'' (fresh cow's milk with fermented millet gruel) and ''pito'' and ''burukutu'' (cereal-based alcoholic beverages) are largely brought about by lactic acid bacteria and yeast, with *L. plantarum* predominating[35]. The dominant species *L. plantarum* frequently occurs (spontaneously) in high numbers in most lactic acid fermented foods, especially when the food is based on plant material, for example, in brined olives, makdous and

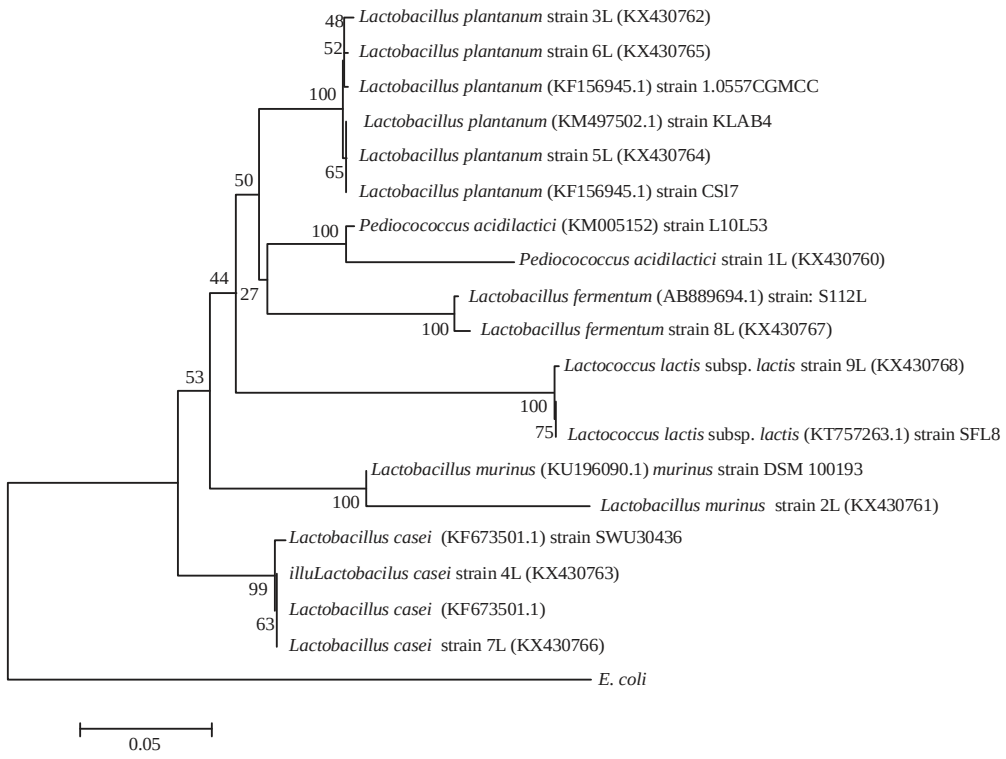

Fig. 2: Phylogenetic tree with the 16S rRNA gene using the MEGA 6.0 program by neighbor-joining (NJ) method
Out rooting done by *E. coli*

fermented vegetables. Thus, individuals consuming these products also consume a large numbers of *L. plantarum* together with *L. casei*, *L. fermentum* and pediococci, which were found in these products[40].

In another study, *L. plantarum* was the dominant lactic acid bacteria isolated in different batches of ''pito'' and ''burukutu'' collected from local producers in Nigeria[41]. The dominance *of L. plantarum* at the late stages of fermentation has been attributed to its high acid tolerance[34,32,38], Hounhouigan *et al.*[42], El Mardi[43], found the bacteria in rob (Sudanese fermented milk) from the suburbs of Khartoum to be *Lactobacillus fermentum* and *Lactococcus lactis.* Abdelgadir *et al.*[44] found in a traditional Sudanese fermented camel's milk product, gariss, *L. fermentum* was the dominant spices. Among LAB, *Lactobacillus plantarum* are the species most widely described in acid-fermented meat products[45].

L. fermentum have been suggested to be the predominating microorganisms during the fermentation of fufu and ogi, two Nigerian foods[46], kenkey, a Ghananian fermented maize dough[47], mawe, a Benin fermented maize dough[42] and agbelima, a Ghananian cassava dough[48]. These species have also been reported to occur in fermented plant materials and sour dough[49,50], in sorghum beer[51] and in togwa[52].

CONCLUSION

The results of this study have indicated that several different species of lactic acid bacteria can be implicated in the fermentation of kissra. Therefore, there is a need for investigation into the selection of the most suitable strains for controlled fermentation of kissra. The starch fermenting strains might be important in the development of the starter cultures and for use in the development of small-scale commercial production of kissra.

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