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Research Article
Molecular Identification of Potential Probiotic Lactic Acid Bacteria Strains Isolated from Egyptian Traditional Fermented Dairy Products

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Abstract
This study aimed to isolate, identify and determine the restrictive criteria for potential probiotic Lactic Acid Bacteria (LAB) strains from artisanal soft cheese (Karish cheese) made from raw buffalo's milk and kishk, traditional Egyptian dried fermented milk wheat based mixture. One hundred strains were isolated and identified by classical phenotypic characteristics including Gram stain, shape, catalase production and biotype protocols using appropriate API system. The ability of selected LAB strains to tolerate acidic pH, resist in bile salts as a crucial criteria for potential probiotic were assessed. The antibacterial activity against food borne pathogens as a preferred capability of probiotic LAB was monitored by agar well diffusion method. Accordingly, ten isolates that were further examined for their ability to endure high osmolarity as a unique technological property needed in lactic starter preferred for processing and ripening domiati cheese, a brined ripened Egyptian soft cheese variety was screened. Results showed that two strains one as *Lactobacillus* spp. and the other as *Enterococcus faecium* spp. were promised in this regard. To confirm the species identity, two selected strains were identified by molecular biology technology based on amplified ribosomal DNA restriction analysis and 16S rDNA sequencing technique. Results indicated that the unique strains were *Lactobacillus paracasei* (Lb 2) and *Enterococcus faecium* (En 4), respectively. The impact of the study was to select probiotic lactic starter culture to be employed in processing probiotic brined soft cheese to satisfy the mounting need of local dairy industry toward production of functional dairy products.

Key words: Molecular identification, functional dairy products, probiotic soft cheese, domiati cheese, LAB

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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

Over centuries, Lactic Acid Bacteria (LAB) have played an essential role in the manufacture and preservation of numerous fermented food products. The interest of LAB in the present industrial food manufacturing is mostly focused on beneficial and health species classified in some lactic acid bacterial genera namely Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Wissella[1]. Some often are found in dairy products[2,3].

Lactic Acid Bacteria (LAB) involved in cheese production not only are responsible mainly for milk and curd acidification but also play relevant roles in the modification of flavour and texture of cheeses and inhibition of spoilage and pathogenic microorganisms[3,4]. In this context, a great number of studies have been focused to the characterization and identification of LAB in artisanal cheeses[5,6], which is considered essential for understanding the contributions of LAB to cheese production.

Recently, consumers are aware of functional and better nutritional aspects of the healthy products and this had led to the increasing trends towards healthy eating and development of functional food, which meet specific nutritional requirements. In this respect, dairy products could be considered to have a great potential of being a functional food. This emerging situation has increased the need to isolate new strains of LAB, which are able to produce beneficial bioactive compounds that have other unique probiotic characteristics[7-10]. Phenotype and biotype protocols for identifying bacteria are considered as classical methods in this regard but discrepancy might occur in differentiation between species of some bacterial genera. Therefore, Ward and Trimmins[11], have developed polymerase chain reaction primers that are specific for the differentiation between closely related hetero fermentative lactobacilli. Actually, most of the available techniques for the confirmed identification of LAB are based on molecular biology[12,13].

One of the most common and faster techniques used for confirmation of bacterial identification in dairy products is partial or total sequencing of the 16S ribosomal RNA (rRNA) gene[14]. This technique is based on the amount of similarity of sequences between different individuals and demonstrative of the variation of their genomes. In general, for practical routine purposes if two organisms present a 16S rRNA gene sequence identity lower than 97%, they are poorly related at the genomic level and thus belong to different species, whereas if the identity values are higher than 97% they can be considered closely related and thus belonging to the same species[15,16].

The 16S rRNA gene sequencing technique is, however, not without limitations due to errors, ambiguous designations or incomplete sequences[16]. More specifically, in the case of recently diverged LAB species, e.g., *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus*, identification may be misled due to that they are closely related species and the 16S rRNA gene sequence analysis can indicate only the belonging to a species group, not to unique species[15,17,18]. Similarly, a high homology (high similarity scores in the 16S rRNA gene, >97%) derived from the comparison of sequences between different Enterococcus strains belonging to a same species line or group has been reported[19]. Moreover, when partial sequences, i.e., few hundreds of nucleotides of the 16S rRNA gene are analysed, the use of different primer sets can result in different identification performance[13], depending on the gene regions considered in the method.

The present study aimed to isolate LAB strains with unique characteristics to be employed for production of probiotic domiati cheese, a brined ripened artisanal soft cheese. The ability of the isolated strains to withstand high acidity, presence of elevated concentrations of bile as a prerequisite for probiotic characteristics and tolerance to high concentration of sodium chloride in domiati cheese as an important technological property suitable for such cheese variety. The literature reflected scarcity if any about application of probiotic lactic acid bacteria in processing domiati cheese, the most consumed brine ripened soft cheese in Egypt, Arabian countries, Turkey, Greece and Bulgaria. The selected strains are identified and confirmed following phenotype, biotype and molecular methods, respectively.

MATERIALS AND METHODS

Isolation of LAB: The LAB strains were isolated from 50 samples of karish soft cheese made from raw buffalo milk and 50 samples of kishk (traditional fermented milk wheat based mixture) according to the method adopted by Lavanya *et al.*[1] with some modification. All samples were procured from the rural area around Giza city. Serial dilutions from each sample up to 10^5 were prepared in sterile saline solution. One milliliter from each sample starting from 10^3-10^5 dilutions were pour plated on de Man, Rogosa and Sharpe (MRS) and M17 agar media (Hi-media, India) and plates were incubated at 37 or 30°C for 48 h, respectively to propagate strains of LAB. One hundred separated colonies, with different morphology and color were picked and inoculated in MRS broth (90 isolates) or M17 broth (10 isolates) and further incubated at 37 or 30°C for 24 h. This step was repeated several times to obtain well

separated purified colonies, which were picked, inoculated in tubes of MRS or M17 broth and incubated as above. Slant tubes of MRS and M17 were inoculated and incubated at the respective temperature and time as above. Such LAB isolates were subjected for phenotype and biotype identification.

Phenotype and biotype identification of bacterial isolates: Phenotype identification of 100 lactic acid bacterial isolates was performed based on Gram stain, shape under microscope and presence or absence of catalase as described by Vos *et al*.[20]. Biotype characterization of 90 isolates of rod shaped LAB was carried out using the API 50 CHL. On the other hand, API 20 strep were applied for the remaining 10 spherical shaped LAB isolates according to instructions of manufacturer (Biomerieux, France). Interpretations of the fermentation results obtained by API 50 CHL and API 20 strep were compared with information from the computer-aided database API LAB plus V.3.2.2. Moreover, the key of identification for *Enterococcus* spp., developed by Manero and Blanch[21] was additionally applied to identify strains within genus Enterococcus.

Potential probiotic characteristics

Acid and bile tolerance: Ten strains of LAB (six lactobacilli and four enterococci) were assayed for their ability to survive in acidic MRS and M17 broth, which were pre adjusted with 5 mol^{-1} hydrochloric acid at pH 2.0 and 3.0. Tubes of previous media were inoculated with the tested strain for 3 h at 30°C or 37°C as described by Zhang *et al*.[6] with modification. The pH tolerance of strains were assayed on MRS or M17 agar plates by pour plate technique. As control, normal MRS and M17 agar (pH 6.5) were parallel inoculated and incubated as aforementioned above. The change in bacterial counts of tested strains indicate the ability to survive in increased acidity. Evaluation of bile tolerance was conducted in presence of 0.3, 0.5 and 1.0% (w/v) bile salts (Oxoid, UK). Briefly, MRS and M17 broth containing the different bile concentrations inoculated with the tested isolates and incubated for 12 h at 37 or 30°C. The MRS and M17 media without bile salts were inoculated and used as control. The viable count was determined by plate count using MRS and M17 agar incubated at 30 or 37°C for 48 h according to the method adopted by Reale *et al*.[10] as CFU g^{-1}.

Antibacterial activity: The strains were examined adopt agar well diffusion assay as described by Tulumoglu *et al*.[22]. Ten overnight well grown strains of LAB were centrifuged at 10000×g for 15 min at 4°C and their supernatant was filter sterilized. One hundred microliters each from four indicator food borne pathogenic bacteria (10^7 CFU mL^{-1}) were pour plated on four separated plates of Mueller and Hinton Agar (MHA) and left 4 h to solidify in refrigerator. The agar wells were made with clean sterile cork borer and filled with 100 µL of Cell Free Culture Supernatant (CFCS). Plates were incubated at 37°C for 24 h and the diameter (mm) of inhibition zone was measured. The indicator bacteria used in this study were *E. coli* O157 (ATTC 700728), *Salmonella typhimurium* (ATTC 13311), *Staphylococcus aureus* (NCINB 50080) and *Listeria monocytogenes* (ATTC19111).

Tolerance to NaCl as a technological property: The tolerance of the selected lactobacilli and enterococci isolates to different concentrations of NaCl were performed in MRS and M17 broth containing 4, 6 and 8% (w/v) commercial sodium chloride as described by Reale *et al*.[10] with modifications. Tubes of MRS and M17 broth without NaCl were inoculated with the same strains and considered as control. Both the treatment and control tubes were incubated at 37 and 30°C for 24 h and growth was monitored by streaking on plates of MRS and M17 agar and incubated as above. Results were recorded as + (low growth), ++ (moderate growth), +++ (high growth) and - (negative growth) observed on plates of MRS or M17 agar media after 24 h incubation as above.

Molecular confirmatory identification of LAB 16S ribosomal DNA (rRNA)-based molecular identification

DNA isolation: The LAB isolates were cultivated on MRS and M17 agar plates at 37°C for 18 h. Used QIGENE mini kite.

PCR partial amplification and sequencing of 16S rRNA: According to method described by Adimpong *et al*.[14] with some modifications. Oligonucleotide primers were used to amplify 16S rRNA forward: 8F (5-AGAGTTTGATCCTGGCTCAG3) and reverse: 1492R (CTACGGCTACCTTGTTACGA-3). The 16S rRNA was amplified from the obtained DNA in a reaction mixture of PCR conditions were as follows: 5× GoTaq flexi buffer, 10 µL, GoTaq flexi DNA polymerase, 0.25 µL, $MgCl_2$, 25 mM, 2 µL, PCR nucleotide mix, 10 mM, 1 µL DNA, 1.5 µL double-distilled water mixed in a final volume of 50 µL. The program for PCR was as follows: 95°C for 5 min, 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 2 min and extension at 72°C for 7 min, +4oC∝. The PCR amplification was performed using a PTC-100 thermo cycler (MJ Research Inc., Watertown, Mass.) The PCR products yielded were

analyzed on a 1% (w/v) agarose gel (FMC Bioproducts, Rockland, Maine) after staining with ethidium.

Agarose gel preparation: Agarose was placed in 1X TBE buffer and boiled in water bath, then ethidium bromide was added to the melted gel after the temperature become 55°C. The melted gel poured in the tray of mini-gel apparatus and the comb was inserted immediately, then the comb was removed when the gel become hardened. The electrophoresis buffer (1X TBE), then covered the gel. Fifteen microliters of dsDNA was loaded in each well and 3 µL of 2.5 kbp DNA ladder. The 16S rDNA PCR product was extracted from gel using gel extraction kit QIAquick Qiagen and DNA sequencing was conducted using ABI Prism Big DyeTM Terminator Cycle Sequencing Ready Reaction Kit according to instructions of manufacturer (PE Applied Biosystems) ABI PrismTM 377XL DNA Sequencer (Perkin Elmer).

Phylogenetic analysis: The 16S rRNA sequences obtained were added to publically available bacterial 16S rRNA sequences were integrated to the data base with the automatic alignment tool. Phylogenetic tree was generated by performing distance matrix analysis using neighbor joining method. Data base search and comparison were done with the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/NCBI, Bethesda, MD, USA[23].

Statistical analysis: All the numerical data were obtained from three independent experiments and these data analysis was carried out with MS-Excel and SPSS 16 statistical analysis (SPSS Inc., Chicago, IL, USA). The results were presented as Mean±SE. The significant difference between the means were analyzed using one-way ANOVA with a significant level of $p<0.05$.

RESULTS AND DISCUSSION

Identification of Lactic Acid Bacteria (LAB): Table 1 revealed that among 100 LAB isolates from karish cheese and kishk, 90 strains were Gram positive, rod shaped and catalase negative, while the remaining 10 strains were Gram positive, cocci shaped and catalase negative according to their phenotype profile. Results of biotype identification using API 50 CHL indicate that 16 (17.78%), 46 (51.11%) and 28 (31.11%) strains were identical to *L. acidophilus*, *L. casei* group and *L. plantarum*, respectively. On the other hand, biotype identification of the remaining ten cocci isolates according to their fermentation profile using API 20 strep and key of Manero and Blanch[21] were identical to *Enterococcus faecium* (Table 1).

Table 1: Phenotype and biotype identification of Lactobacillus and Enterococcus strains from kishk and karish cheese

	Total No. of examined samples		
Isolated strains	Kishk (n = 50)	Karish cheese (n = 50)	No. of species
L. acidophilus	6	10	16 (17.78%)
L. casei (group)	24	22	46 (51.11%)
L. plantarum	10	18	28 (31.11%)
E. faecium	3	7	10 (100%)

n: Number of dairy product samples examined

Lactobacillus strains play a crucial role in cheese ripening[11]. Mainly, *L. casei*, *L. paracasei* and *L. rhamnosus* constitute a closely related group, which could be easily distinguished from other species in genus Lactobacillus by biotype profile[24]. However, it is not possible or unequivocally to differentiate between these three species according to their fermentation profile alone[11]. Therefore, it is decided to continue with the 46 *L. casei* group and 10 *E. faecium* strains for the two principal tests, which are acid and bile tolerance as restrictive criteria for potential probiotics[6,9,10]. Based on the results obtained six strains of *L. casei* group and four strains of *E. faecium* for their high capability to tolerate acid and bile for further study was screened. Results are shown in Table 2 and 3, respectively. Karish cheese and kishk are traditional Egyptian fermented dairy products processed from raw buffalo milk that expected to contain many bacterial genera specially the LAB group. Therefore, it is choose such dairy products to isolate wild strains needed for the present study. This tendency is in agreement with that reported by other researchers studied on different fermented dairy products and other fermented food[9,14,25-28].

Acid tolerance: Table 2 showed the result of acid tolerance study for six isolates of *L. casei* group and four strains of *E. faecium* strains after exposure for 3 h at pH 2.0 and 3.0, respectively. Exposure to pH 2.0 resulted in drastic reduction

Table 2: Effect of acid on the survival of LAB isolates (Log CFU g^{-1})

LAB isolates	Initial counts	pH 2.0 (3 h)	pH 3.0 (3 h)
Lb 1	8.52 ± 0.14^{a}	6.39 ± 0.12^{b}	8.25 ± 0.14^{a}
Lb 2	8.59 ± 0.17^{a}	6.88 ± 0.09^{b}	8.32 ± 0.07^{a}
Lb 3	8.54 ± 0.16^{a}	0.00 ± 0.00^{c}	8.12 ± 0.14^{b}
Lb 4	8.02 ± 0.15^{b}	6.83 ± 0.12^{a}	7.45 ± 0.17^{b}
Lb 5	8.37 ± 0.07^{a}	5.40 ± 0.08^{b}	7.42 ± 0.11^{a}
Lb 6	8.15 ± 0.36^{a}	4.25 ± 0.10^{b}	7.92 ± 0.12^{c}
En 1	8.02 ± 0.16^{a}	4.22 ± 0.10^{b}	7.84 ± 0.12^{c}
En 2	8.25 ± 0.24^{a}	4.50 ± 0.08^{b}	8.06 ± 0.13^{a}
En 3	8.07 ± 0.09^{c}	6.26 ± 0.02^{b}	7.78 ± 0.24^{c}
En 4	8.27 ± 0.32^{b}	4.72 ± 0.09^{c}	8.43 ± 0.12^{b}

Means in the same row marked by different superscripts are significantly different ($p<0.05$), Lb: *L. casei* group isolates and En: *E. faecium* isolates

in bacterial counts for all isolates as reflected by plate count. Contrary, most strains were resistant to acid environment of pH 3.0. Obviously, strain Lb 2 was the most resistant to acid environment among *L. casei* group and En 4 was the most acid tolerant among *E. faecium* even it showed slight increase in viable counts at pH 0. 3.

The ability of a given LAB isolate to survive at low pH values is mandatory criterion in the selection of probiotic strain for production of probiotic food and dairy products[29]. The results of acid tolerance showed by most strains isolated in this study are in accordance with that of[10,30,31].

Bile tolerance: Potential probiotic LAB not only should be able of survive passage through the human stomach (high acid environment) but also capable to survive passage in the intestine, where bile juice secreted by gall bladder was added. Therefore, they must resist gastric juices and bile salts during their transition via the digestive canal as requested by FAO and WHO[32]. In this study, LAB strains were assayed for their ability to survive in different concentration of bile salts as shown in Table 3. All ten strains resisted the different concentrations of bile used in the experiment with some fluctuation. However, increasing bile concentrations from 0.3-0.5% up to 1.0% was accompanied by reduction in the ability to survive being clearly observed in the highest bile concentration.

From Table 2 and 3 that Lb 2 and En 4 isolates were promised to be selected for the purpose of this study based on their capability to tolerate the bile exposure under *in vitro* study especially at 1% bile salt concentration.

Obvious from Table 3 that there is a variable distribution between LAB strains under study with their ability to tolerate different concentrations of bile salts. This may be explained as probiotic potentiality is strain specific. Similar reports reported by other researchers are in accordance with this explanation[6,10,30].

Table 3: Effect of bile salt on the survival of LAB isolates (Log CFU g^{-1})

LAB isolates	Initial counts	Oxgall (0.3%)	Oxgall (0.5%)	Oxgall (1%)
Lb 1	8.52±0.14^{a}	8.33±0.22^{a}	8.25±0.23^{b}	7.95±0.12^{b}
Lb 2	8.59±0.17^{a}	8.54±0.26^{a}	8.45±0.20^{a}	8.13±0.08^{c}
Lb 3	8.54±0.16^{a}	8.48±0.35^{a}	8.42±0.09^{a}	8.12±0.09^{b}
Lb 4	8.02±0.15^{a}	7.25±0.12^{b}	7.04±0.13^{b}	6.92±0.10^{c}
Lb 5	8.37±0.07^{a}	5.92±0.10^{b}	5.25±0.09^{b}	4.64±0.20^{c}
Lb 6	8.15±0.36^{a}	7.96±0.22^{b}	7.24±0.20^{b}	6.24±0.30^{c}
En 1	8.02±0.16^{a}	7.96±0.23^{b}	7.62±0.13^{b}	6.22±0.04^{c}
En 2	8.25±0.24^{a}	4.65±0.08^{b}	3.82±0.09^{c}	3.62±0.14^{c}
En 3	8.07±0.09^{a}	6.93±0.23^{b}	5.85±0.12^{c}	5.72±0.13^{c}
En 4	8.27±0.32^{a}	8.22±0.14^{a}	8.27±0.09^{a}	8.22±0.14^{a}

Means in the same row marked by different superscripts are significantly different ($p<0.05$)

Tolerance to NaCl as a technological property: Domiati cheese is brined ripened soft cheese with 6.0% salt when fresh. Therefore, salt tolerant probiotic strains are needed to be incorporated in production of probiotic domiati cheese[33]. Ten screened LAB isolates in present study were tested for their capability to tolerate under such stress under different salt concentrations. Table 4 revealed high growth of all strains in presence of 2% NaCl but some isolates were able to grow in presence of 4.0 and 6.0% NaCl with some differences as the growth was decreased when concentration of NaCl was increased from 4.0-6.0%. Contrary, at 8% NaCl, only two strains showed moderate growth. The present results are in accordance with the findings[10,34]. In dairy industry, such as domiati cheese manufacture, LAB will exposed to osmotic stress due to high amounts of added salts. According to Ge *et al.*[34], the osmotic stress may cause pronounced inhibition for bacterial growth and delayed ripening of cheese. For this best knowledge there is scarce studies related to the fate of *L. paracasei* and *E. faecium* as probiotics in domiati cheese manufacture. The present study highlighted information on intra-strain variability in osmotolerance in *L. paracasei* and *E. faecium* strains. Therefore, it is concluded that osmotolerance is a crucial factor for the selection of strains for technological application in case of domiati cheese manufacture that contain high salt concentration above 6%. Two screened LAB strains with high osmotolerance to high concentration of NaCl are worth of future investigation for their performance in production of probiotic domiati cheese.

Antibacterial activity: Agar well diffusion assay showed that the antibacterial activity of the ten isolates of LAB against four indicator food borne pathogens widely differ (Table 5). Isolates Lb 2 and En 4 possessed the highest antagonistic effect against all indicator pathogens *E. coli* O157:H7, *S. typhimurium*, *S. aureus* and *L. monocytogenes*. However, their antibacterial activity was most effective towards Gram

Table 4: Tolerance of LAB isolates to different concentrations of sodium chloride

		Growth on plates[2]			
LAB isolates	Source[1]	NaCl (2%)	NaCl (4%)	NaCl (6%)	NaCl (8%)
Lb 1	CH	+++	+++	+++	+
Lb 2	CH	+++	+++	++	++
Lb 3	CH	+++	++	-	-
Lb 4	kl	+++	++	+	-
Lc 5	kl	+++	+++	++	+
Lc 6	kl	+++	++	+	-
En 1	kl	+++	++	+	-
En 2	CH	+++	++	+	-
En 3	CH	+++	++	+	-
En 4	CH	+++	+++	++	++

[1]CH: Cheese, KI: Kishk, [2]Growth on plate, +: low growth (<30 CFU g^{-1}), ++: Moderate growth (<100 CFU g^{-1}), +++: High growth (>100 CFU g^{-1}) and -: No growth (<10 CFU g^{-1})

Table 5: Antibacterial activity of 10 strains of LAB isolated from cheese and kishk

			Inhibition profile (wells test)[2]			
			Indicators pathogens[3]			
Strains	Identifications	Source[1]	I	II	III	IV
Lb 1	*L. casei*	CH	+	+	++	+++
Lb 2	*L. paracasei*	CH	++	++	+++	+++
Lb 3	*L. paracasei*	KI	+	+	++	++
Lb 4	*L. plantarum*	KI	++	-	++	+
Lb 5	*L. acidophilus*	CH	-	-	++	+
Lb 6	*L. plantarum*	CH	+	-	-	+
En 1	*E. faecalis*	KI	+	-	+	+
En 2	*E. faecium*	CH	++	+	+	+
En 3	*E. faecium*	CH	+	+	+	-
En 4	*E. faecium*	CH	++	++	+++	++

[1]CH: Cheese, KI: Kishk, [2]Antibacterial activity, +: Clear zone around well (1-2 mm), ++: Clear zone ((2-4 mm), +++: Clear zone (>4 mm), -: No inhibition, wells containing 100 µL Cell Free Culture Supernatant (CFCS), [3]Indicators, I: *Escherichia coli* O157: H7 ATCC 700278, II: *Salmonella typhimurium* ATCC 13311, III: *Staphylococcus aureus* NCINB 50080 and IV: *Listeria monocytogenes* ATCC 19111

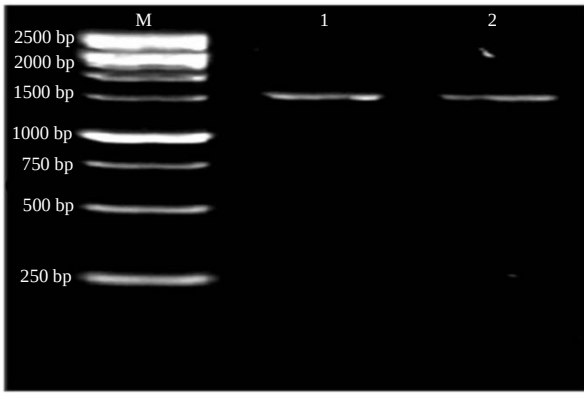

Fig. 1: PCR amplification of 16S rDNA gene (1500 bp), M: ladder 1 Kb, lan 1: Isolate Lb 2, lan 2: Isolate En 2

positive than Gram negative food borne pathogens. The remaining isolates showed low antibacterial effects compared to isolates Lb 2 and En 4. Therefore, it is focused on these two strains in the next step of this study. It is also reported that *L. paracasei* showed strong antibacterial effect, while other lactobacilli tested revealed low antagonistic effect against indicator bacteria[25]. The antibacterial activity is due to the potential of LAB to secret lactic acid or and bacteriocin (s). Also, these bacteria produce different peptides having inhibitory properties[9].

Molecular confirmatory identification of LAB: The species of *L. casei*, *L. paracasei* and *L. rhamnosus* constitute a closely related taxonomic group among hetero fermentative lactobacilli that are difficult to differentiate according to biotype identification only[11]. Therefore, the confirmed identification of *L. casei* group should be based on molecular biology[12]. The API 20 strep were used to identify isolates grew on M17 medium in addition to the biochemical key recommended by Manero and Blanch[21] followed by molecular identification.

PCR amplification of the 16S rRNA gene: The PCR targeting the 16S rRNA has been used extensively to study prokaryote diversity and allows identification of prokaryotes as well as the prediction of phylogenetic relationships[35]. Nucleic acids extracted from the formed bacterial colonies were subjected to primer pair designation to amplify a region of the 16S rRNA gene and all produced an expected size (ca. 1500 bp), as shown in Fig. 1. This result does not indicate that the tested bacteria belong to a specific genus or a particular species, because the primer pair used was a universal eubacterial primer designed to classify related bacteria according to sequencing results[36].

16S rDNA gene sequence similarity and phylogenetic analysis: Molecular identification of the isolated strains were carried out based on 16S rRNA sequence analysis. The partial sequences of 16S rRNA obtained from isolates were aligned with all the presently available 16S rRNA sequences in the Gen Bank data base. The 16S rRNA partial sequencing of isolated strains were shown in Fig. 2 and 3. As a result, a phylogenetic tree was mapped using the neighbor joining

GAACGAGTTCTCGTTGATGATCGGTGCTTGCACCGAGATTCAAACATGG
AACGAGTGGCGGACGGGTGAGTAAACACGTGGGTAACCCTGCCCTTAA
GTGGGGGATAACATTTGGAAACAGATGCTAATACCGCATAGAATCCAAG
AACCGCATGGTTCTTGGCTGAAAGATGGGCGTAAGCTATCGCTTTTTGG
ATGGACCCGC

Fig. 2: 16S rDNA gene partial sequence of isolate *Lactobacillus paracasei* (Lb 2)

GGACGAaCGCTGGCGGCGTGCCTaATaCATGCaAGTCGTaCGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAA
AAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAACACTTGGAAACAGG
TGCTAATACCGTATAACAATCGAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGGGTGTCGCTGATGGATGGAC
CCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCCACGATGCATAGCCGACCTGAGAGGGTGAT
CGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGA
AAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAA
GGATGAGAGTAACTGTTCATCCCTTGACGGGATCTAACCAGaAaGCCACGG

Fig. 3: 16S rDNA partial sequences showing the relationship between isolate 1 (LAB 1) and other species

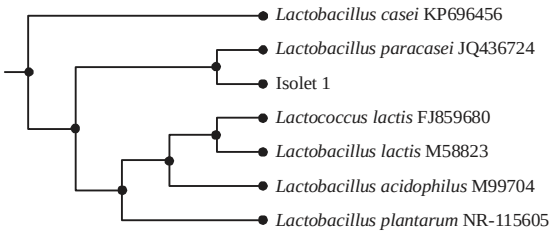

Fig. 4: Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between isolate (Lb 2) and other species belong to the genus Lactobacillus. The tree was constructed using the CLUSTAL-X and neighbor-joining method

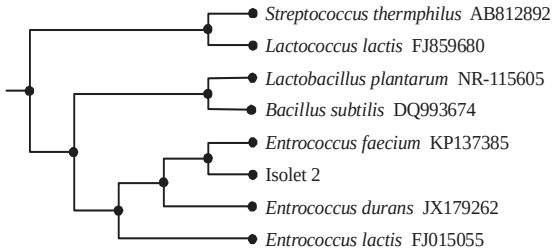

Fig. 5: Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between isolate (En 4) and other species belong to the genus Enterococcus. The tree was constructed using the CLUSTAL-X and neighbor-joining method

method and is shown in Fig. 4 and 5. Phylogenetic analysis using the 16S rRNA sequences indicated that isolate (Lb 2) and isolate (En 4) belonged to the genus Lactobacillus and Enterococcus, according to blast result shown in Table 6 isolate (Lb 2) was identified as *Lactobacillus paracasei*, isolate (En 4) was identified as *Enterococcus faecium*.

Table 6: Identity percentage between bacterial isolates and related species

16S rDNA sequence similarity	Species	Isolates	Source of isolation
99 (%)	*Lactobacillus paracasei* JQ436724	Isolate Lb 2	Karish cheese
99 (%)	*Enterococcus faecium* KP137385	Isolate En 4	Kishk

CONCLUSIONS

In this study, 100 isolates of LAB were isolated, purified, phenotype and biotype identified with the purpose to screen for potential probiotic characteristics among these strains. The ability of the isolates to tolerate acidic pH, resist in bile salts as a prerequisite characteristics for potential probiotics were examined. The ability to show antibacterial activity toward some food borne pathogens as a unique character among LAB and the ability to tolerate high concentration of sodium chloride as a technological property needed in manufacture of domiati cheese, the most popular salt brined ripened soft cheese consumed in Egypt were monitored. We obtained two promised strains as *L. paracasei* (Lb 2) and *E. faecium* (En 4). Molecular biology technology based on amplified ribosomal DNA restriction analysis and 16S rDNA sequencing technique were followed to verify species nomenclature. Two strains isolated were considered as presenting probiotic potential and were selected for further application in processing probiotic domiati cheese.

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