Isolation, screening, and molecular identification of new lactic acid bacteria as potential probiotics

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Background and objective
Lactic acid bacteria (LAB) are the most useful microorganisms to society, LAB are used in the production of many fermented food products that stimulate digestion and immunity. Therefore, the objective study aimed to isolate, characterize, and identify new LAB members and determining their potential probiotic properties.
Materials and methods
This study was performed through isolation, selection, and identification of LAB strains morphologically, biochemically, and genetically. Then, to determine their probiotics traits, which include acid resistance, bile-salt tolerance, β-galactosidase activity, heat sensitivity, salinity, cholesterol reduction, and antioxidant activity.
Results and discussion
Eight LAB isolates were isolated and purified from milk and cheeses, out of eight isolates, K_2 showed the highest resistance to acidic pH 2.0, where Log CFU/ml reduced from the initial count 8.79±0.17 at pH 7.0 to 4.57±0.10 at pH 2.0 with 51.99% survival rate. Also, the six bile-tolerant isolates (K_1, K_2, K_3, K_4, A_{11}, and A_5) were found tolerant to 1% bile-salt concentrations for 3 h with 92.50, 71.10, 64.35, 60.72, 71.11, and 72.21% rate of survival, respectively. Also, antibiotic susceptibility of A_{11} isolate was the most sensitive, but isolate A_2 was the most resistant against antibiotics. Besides, heat sensitivity of selected LAB isolates (K_1, K_2, A_{11}, and B_1) were able to survive at temperatures 15, 37, and 45°C, but isolates (K_3, K_4, A_5, and A_2) did not grow at 45°C. Additionally, NaCl tolerance clearly revealed that both isolates (K_2 and A_{11}) were able to tolerate a wide range of NaCl (1–8% w/v), then the growth declined with the increase of salt (10%) concentration. The results showed that the cholesterol assimilation was reduced by 32.66 and 30.20% for A_{11} and K_1 isolates, respectively. Also, K_2 isolate showed the highest antioxidant activity (86.93%), and the best β-galactosidase producer (24.65 U/ml). Therefore, the 16S-rRNA sequencing was carried out to identify four promising LAB isolates that could be employed as a probiotic strain. Phylogenetic analysis showed that isolates belonged to three genera, including *Lactobacillus*, *Lactococcus*, and *Enterococcus*. Strains were *Lactobacillus casei* (A_5) KU510332, *Enterococcus faecium* (A_{11}) KU510333, *Lactococcus lactis* (K_1) KU510334, and *E. faecium* (K_2) KU510335.
Conclusion
Our results presenting four new isolates as promising potential probiotics for further application in processing probiotic products.

Keywords:
16S-rDNA, antioxidant, bile salt, cholesterol, probiotic–lactic acid bacteria, β-galactosidase

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Introduction

Lactic acid bacteria (LAB) are Gram-positive, rods, aerotolerant, nonsporulating, and nonpathogenic organisms, mostly found in nature, its major industrial application in the era of foods and fermented products as Generally Regarded as Safe [1]. They are industrially used as starter cultures in the formation of dairy products, fermented vegetables, and meat products [2].

Probiotic was defined as 'Vital microorganisms that gave critical effects on the human body by increasing its microbial balance' [3]. Also, probiotic bacteria may secrete a large amount of metabolites that prevented the pathogen's growth, and include organic acids and bacteriocins [4,5]. *Lactococcus* also displays antifungal activity [6]. Lactobacilli living as commensal in the intestinal environment might possess probiotic activity; they may have antitumoral activity [7].

LAB have many important nutritional benefits, including increasing the nutritional value of food, preventing gastrointestinal infections, improving

digestion of lactose, and controlling some types of cancer [8,9]. Decreased cholesterol levels [10] help lactose metabolism and stimulate the immune system [11]. These health benefits can be attributed to numerous synergistic mechanisms and biological activities [12]. Also, these lactobacilli species have vital effects on health [13].

Screening of probiotics for their antioxidant potential by different methods like 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays for evaluation of antioxidant activity was performed in vitro [14,15]. β-galactosidase has long been accepted as an important ingredient in food-processing industries. In the dairy industry, β-galactosidase could be used to inhibit lactose crystallization, enhance sweetness, increase milk-product solubility, prepare low lactose-containing food products for low lactose-tolerant people, and recycling of the environmental pollutant, cheese whey [16].

Nowadays, along with the fast development of molecular biology and increasing knowledge on genomic structures of LAB, many molecular techniques, including 16S-rRNA gene-sequence analysis, have been developed [17,18]. The large database of 16S-rRNA gene sequences enables us to identify LAB very easily [19]. The 16S-rRNA gene can be used for studying phylogenetic relationships between microorganisms and provides a more accurate and precise tool for microbial identification [20].

Here we present the isolation and identification of new four LAB strains on species level using the phenotypic, biotypic, and genotypic methods. Our isolates showed promising characteristics as probiotics by expressing acid and bile-salt tolerance, β-galactosidase activity, cholesterol reduction, and antioxidant activity.

Materials and methods

Collection of samples

Isolation of LAB has been carried out from different sources: milk, cheese, and fermented milk.

Isolation of lactic acid bacteria

One gram of sample was immediately processed under aseptic conditions by suspending it in 9 ml of normal-saline NaCl (0.85%) and streaked on the de Mann Rogosa Sharpe (MRS) agar and incubated at 37°C for 48 h under CO_2 5% and observed for the morphology and number of colonies [21].

Identification of selected isolates

Identification of the probiotic isolates was performed based on their morphological and biochemical characterizations, as described in Bergey's Manual of Systematic Bacteriology [22]. Physiological properties were also studied [23].

Evaluation of the potential probiotic properties for lactic acid bacteria bacterial isolates

Tolerance to NaCl

MRS agar plates with different NaCl concentrations (2.0, 4.0, 6.0, 8.0, and 10%) were inoculated with tested isolates and incubated for 24 h at 37°C. The CFU was calculated for each concentration to denote NaCl tolerance [24].

Temperature sensitivity

The isolates were grown on MRS broth at varying temperatures, that is, 15, 25, 37, and 45°C for 48 h. Using the pour-plate method, LAB growth on MRS agar plates was used to denote temperature tolerance [24].

Acid tolerance

The isolates were grown for 24 h in MRS broth at 37°C. An aliquot of 0.1 ml from different cultures incubated for 24 h was inoculated into 10-ml MRS broth with a pH value adjusted to 2, 3, and 4, and incubated for 3 h at 37°C. pH tolerance of the strains was assayed on MRS agar plates by plate-count technique [25].

Bile-salt resistance

Overnight cultures from the tested isolates were inoculated into MRS broth with different concentrations of bile salt (Sigma Chemical Co., USA) (0.3, 0.5, and 1% w/v) for 3 h at 37°C. Then they were plated onto MRS agar plates and incubated for 24 h at 37°C. The MRS without bile salt was used as a control. The viable count was determined by plate count for each [25].

Bile-salt deconjugation

Bile-salt hydrolase (BSH) enzyme was screened. Briefly, 10-μl aliquots of cultures were spotted on MRS agar plates supplemented with 0.3, 0.5, and 1% (w/v) of taurodeoxycholic acid sodium salt (Sigma Chemical Co., USA). The hydrolysis zone surrounding colonies indicated the BSH activity of the tested bacteria [26].

Antibiotic susceptibility

The susceptibility to antibiotics was tested by the disk-diffusion method [27]. One percent of the test isolates

inoculated in Mueller–Hinton agar (0.8% agar). Antibiotic disk was placed on it to allow the diffusion of antibiotics into the medium and then incubated at 37°C for 24 h. The inhibition zone around each antibiotic disk was measured to check the susceptibility of the isolate.

Antioxidant activity

The chemical assay that includes DPPH was used at a concentration of 0.2 mM in methyl alcohol. The sample was combined with an equivalent volume of freshly DPPH solution (0.2 mM). The mixture was vigorously shaken and left to react for 30 min in the dark. The scavenged DPPH was then monitored by determining the absorbance (Abs) at 515 nm against methanol blank using Agilent Technologies Cary100, UV-VI spectrophotometer [28].

In vitro cholesterol-reduction activity

Overnight isolates were inoculated (1%) into MRS broth supplemented with water-soluble cholesterol (100 mg/dl) and incubated for 24 h at 37°C. The cholesterol content was then measured in the supernatant by quantitative determination of cholesterol kit, 'Spinreact Kits' (Barcelona, Spain) according to the manufacturer's instructions [29].

β-galactosidase activity

β-galactosidase activity in whole cells was determined according to the method of Miller and Brand [30].

Molecular identification of lactic acid bacteria using the 16 S ribosomal RNA gene

Extraction of genomic DNA: Genomic DNA was extracted from the overnight culture of the selected isolates using a Qiagen DNA purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Amplification of 16s-rRNA by PCR and identification by sequence analysis: Genomic DNA of isolates was used as a template in PCR reactions using GoTaq Flexi DNA Polymerase (Promega Company, WI, USA) according to the manufacturer's instructions and two primers, namely, 8 F (5-AGAGTTTGATCCTGGC-TCAG-3) and 1495 R (5-CTACGGCTACCTTGT-TACGA-3) [31]. A mixture of PCR conditions was as follows: 5× Go Taq Flexi buffer, 10 µl, Go Taq Flexi DNA polymerase, 0.5 µl, $MgCl_2$, 25 mM, 2 µl, PCR nucleotide Mix, 10 mM, 1 µl of DNA, and 1.5 µl of double-distilled water mixed in a final volume of 50 µl. The program for PCR was as follows: 95°C for 5 min, 35 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and extension at 72°C for 10 min, +4oC∝. The PCR amplifications were performed by gene amp 9600 thermal cycler (Applied Biosystems, Foster City, CA, US).

Phylogenetic analysis of 16S-rRNA sequences: DNA sequencing of amplified fragments was carried out by the sequencing service of HVD, Germany. The length of 16s-rRNA gene was edited and trimmed with software BioEdit 7.2.5 and corrected sequences were compared with those stored in the GenBank database using BLAST. A phylogenetic tree based on 16S-rRNA genes was also constructed to determine the closest bacterial species by the neighbor-joining method using molecular evolutionary genetics analysis [32].

Statistical analysis

All experimental samples were tested in triplicate, and the resulting values are expressed as the mean±SD.

Results

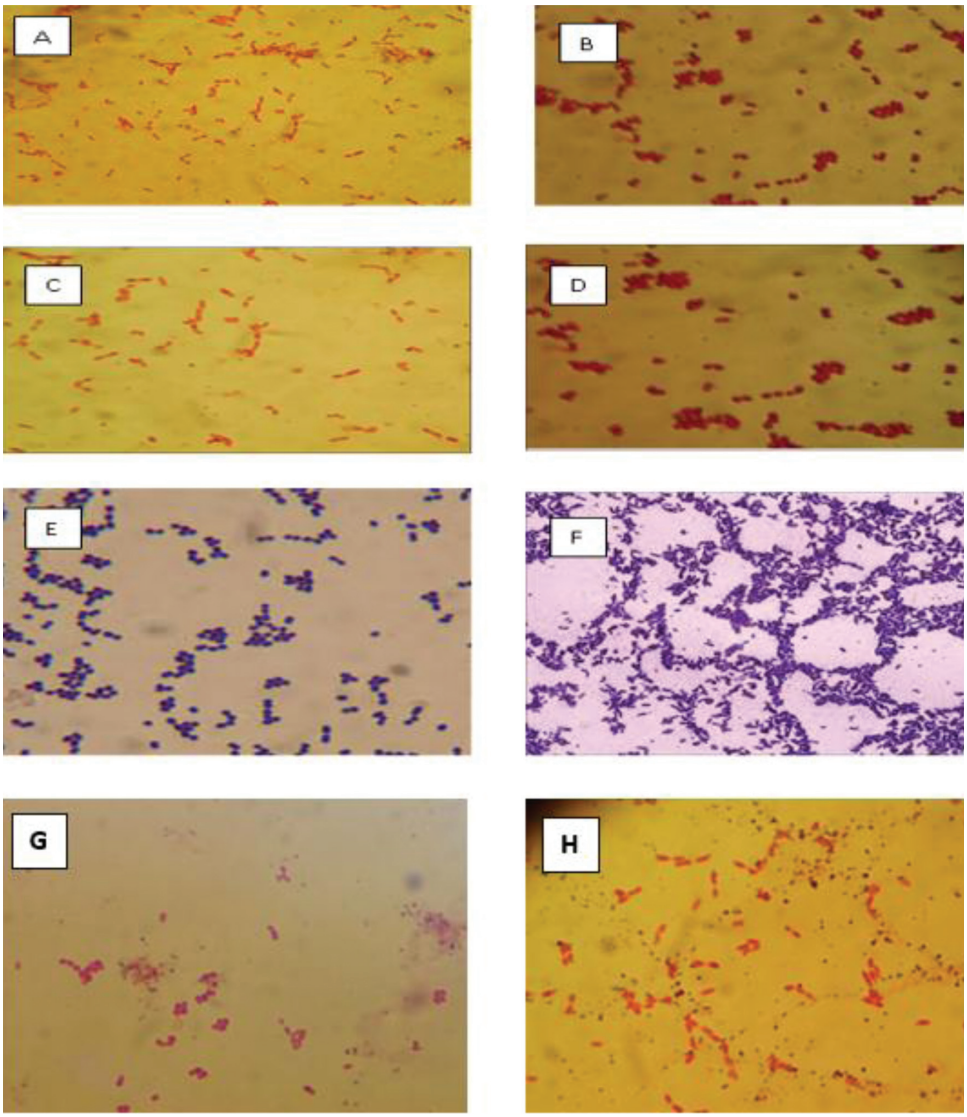
Isolation and identification of lactic acid bacteria isolates

From the samples, eight predominant colonies were isolated. As shown in Table 1 and Fig. 1, the colonial and cell morphology of isolates of LAB were studied. The colonial morphology was either white or cream in color and their shape either circular or pinpoint in shape, and the margin of the colony was entire. All

Table 1 Colonial and cell morphology of the selected isolates from dairy products

Isolate code	Colonial morphology			Cell morphology	
	Color	Shape	Margin	G+ reaction	Shape and arrangement
K_1	White	Circular and compact	Entire	G+ ve	Cocci
K_2	White	Circular	Entire	G+ ve	Cocci, diplococci
K_3	Cream	Circular and large	Entire	G+ ve	Rods with rounded ends
K_4	White	Circular	Entire	G+ ve	Rods
A_{11}	White	Pinpoint; circular	Entire	G+ ve	Cocci
A_5	White	Circular	Entire	G+ ve	Straight rods
A_2	White	Circular and compact	Entire	G+ ve	Cocci
B_1	Creamish	Circular	Entire	G+ ve	Cocci, streptococci

Figure 1



Microscopic examination of some selected bacterial isolates. (a) Simple stain of isolate A5. (b) Simple stain of isolate K1. (c) Simple stain of isolate K4. (d) Simple stain of isolate K3. (e) Gram stain of isolate K2. (f) Gram stain of isolate A5. (g) Simple stain of isolate B1. (h) Simple stain of isolate A11.

isolates showed a typical appearance of LAB on MRS medium (small, pinpointed colonies). Microscopic examination is the first criteria that provide information about genus level and purity of the isolates. The most important and widely used method is Gram staining. Among eight LAB isolates from cheddar cheese and goat milk, three strains were Gram-positive, rod-a shaped, and catalase-negative, while the remaining five strains were Gram-positive, cocci shaped, and catalase-negative according to their phenotype profile. By determining the cell morphology under the light microscope, all the selected isolates were Gram-positive, catalase-negative, and rod- or cocci-shaped bacteria. Cocci isolates were presumptively identified as *Enterococcus* or *Lactococcus* since they were coccus shaped and isolated from milk. Rod isolates were presumptively identified as the Lactobacilli group. Lactobacilli grow best in slightly acidic media with an initial pH of 6.0–4.0. Growth ceases commonly when pH 3.0–4.0 is reached. Isolates were first classified according to their growth at 45°C and 6.0% NaCl concentration; they were therefore identified as *Enterococcus*. The three isolates (K_2, A_{11}, and B_1) were able to grow at 6.0% NaCl and 45°C and they were identified as *Enterococcus* spp. Whereas the two isolates (K_1 and A_2) could grow at 15 and 40°C, in 4% NaCl, and were able to ferment maltose, salicin, and/or saccharose. Therefore, K_1 and A_2 were identified as *Lactococcus lactis* subsp. *lactis* or *L. lactis*.

Data in Table 2 revealed that all isolates were negative to oxidase, catalase, and nitrate reduction. Only the isolates

Table 2 Biochemical characteristics of the selected lactic acid bacteria isolates from dairy products

Test	Isolates	K_1	K_2	K_3	K_4	A_{11}	A_5	A_2	B_1
Biochemical characteristics	Catalase test	*–	–	–	–	–	–	–	–
	Gas from glucose	**+	+	+	–	+	+	–	+
	Citrate utilization	–	–	–	–	–	+	–	+
	Nitrate reduction	–	–	–	–	–	–	–	–
	Starch hydrolysis	–	–	+	–	–	–	+	–
	Oxidase test	–	–	–	–	–	–	–	–
	Casein hydrolysis	+	–	–	–	–	–	+	–
Carbohydrate fermentation	Glucose	+	+	+	+	+	+	+	+
	Fructose	+	+	+	+	+	+	+	+
	Galactose	+	+	+	+	+	+	+	+
	Lactose	+	+	+	+	+	+	+	+
	D-Lyxose	–	–	–	–	–	–	–	
	Melibiose	–	–	–	–	–		***Nd	–
	Sucrose	+	–	–	+	–	+	+	+
	Mannitol	+	–	+	–	–	–	+	+
	D-Xylose	–	–	–	–	–	–	–	–
	Sorbitol	–	–	–	–	–	+	–	–
	Maltose	+	+	+	+	+	+	+	+
	Salicin	+	+	+	+	+	+	+	+

*+, positive results. **–, negative results. ***Nd, not detected.

(B_1 and A_5) showed the ability to utilize citrate by giving blue color but the other isolates indicating a negative reaction to citrate utilization. According to determination of their abilities to hydrolysis starch, all isolates were unable to hydrolyze starch, except isolates K_3 and A_2 that showed the ability to hydrolyze starch by giving a clear zone as positive results. All the isolates showed no ability to hydrolysis of casein, except the two isolates A_2 and K_1, which showed the ability to hydrolyze casein into simple amino acids by giving a clear zone. The isolates showed different types of sugar-utilization patterns, including glucose, fructose, galactose, lactose, sucrose, mannitol, sorbitol, and maltose. Their ability to ferment the sugar was confirmed by turning the media into yellow in the experimental tubes. Table 2 explains that all isolates are able to ferment all monosaccharides such as glucose, fructose, galactose, and disaccharides (lactose). Furthermore, some of the chosen isolates (K3, A5, A2, and B1) can use sugar alcohol like mannitol and sorbitol, as well as disaccharides, while the other isolates cannot.

Evaluation of the potential probiotic properties for lactic acid bacteria bacterial isolates

Tolerance to NaCl

Salt-tolerant probiotic strains were used in incorporation in probiotic products. Eight selected LAB strains in our study were tested for their growth under different salt concentrations. Data in Table 3 revealed high growth of all isolates in the presence of 2% NaCl, but some isolates were able to grow in the presence of 4.0 and 6.0% NaCl with some differences as the growth was decreased when the concentration of NaCl was increased from 4.0 to 6.0%. The growth pattern at 4% NaCl concentration was also reported and concluded a wide variation in the growth of probiotic isolates. Only two strains showed weak growth at 8% NaCl, while no growth was detected at 10% NaCl concentration.

Table 3 Tolerance of lactic acid bacteria isolates to different concentrations of sodium chloride

LAB isolates	Source	Growth on MRS agar plate (Log CFU/ml)*				
		2%	4%	6%	8%	10%
K_1	Milk	+++	++	+	–	–
K_2	Milk	+++	+++	++	+	–
K_3	Cheese	+++	+	+	–	–
K_4	Milk	+++	+++	+	–	–
A_{11}	Cheese	+++	++	+	+	–
A_5	Milk	+++	+++	++	–	–
A_2	Milk	+++	+	–	–	–
B_1	Milk	+++	++	+	–	–

LAB, lactic acid bacteria; MRS, de Mann Rogosa Sharpe. +++, high growth. ++, moderate growth. +, weak growth. –, no growth.

Acid tolerance

Acid tolerance is the second selection criterion for probiotics. Resistance to the acid condition was used as an essential property for probiotic strains. The ability of given LAB isolates to survive at low pH values is a mandatory criterion in the selection of probiotic. Table 5 shows an acid-tolerance study for eight isolates after exposure for 3 h at pH 2.0, 3.0, and 4.0. Strain K_2 was the most resistant to the acid environment among the isolates. The survival rate of selected isolates in acidic buffer (pH 2.0) was examined

Table 4 Effect of acid on the survival of lactic acid bacteria isolates (Log CFU/ml)

LAB isolates	Initial counts (0 h)	pH 4.0 (3 h)	Survival (%)	pH 3.0 (3 h)	Survival (%)	pH 2.0 (3 h)	Survival (%)
K_1	8.32±0.14	8.25±0.14	99.15	6.39±0.12	76.80	3.28±0.23	39.42
K_2	8.79±0.17	8.32±0.07	94.65	6.88±0.09	78.27	4.57±0.10	51.99*
K_3	8.14±0.16	8.12±0.14	99.75	0.00±0.00	0.00	0.00±0.00	0.00
K_4	8.02±0.15	7.45±0.17	92.89	6.83±0.12	85.16	2.19±0.17	27.30
A_{11}	8.87±0.07	7.42±0.11	83.65	5.40±0.08	60.87	3.57±0.19	40.24
A_5	8.15±0.36	7.92±0.12	97.17	4.25±0.10	52.14	3.15±0.35	38.65
A_2	8.02±0.16	7.84±0.12	97.75	4.22±0.10	52.61	0.89±0.45	11.09
B_1	8.35±0.24	7.78±0.24	93.17	4.01±0.10	48.02	2.77±0.18	33.17

LAB, lactic acid bacteria. Values are expressed as the mean±SD. Survival (%) more than 50 resistant isolate. Survival (%) less than 50 not resistant isolate. *This value represents the highest survival and the most tolerance.

Table 5 Effect of bile salt on the survival of lactic acid bacteria isolates (Log CFU/ml)

LAB isolates	Initial counts (0 h)	Oxgall (0.3%) (3 h)	Survival (%)	Oxgall (0.5%) (3 h)	Survival (%)	Oxgall (1%) (3 h)	Survival (%)
K_1	8.54±0.14	8.45±0.26	98.94	8.12±0.20	95.08	7.90±08	92.51*
K_2	8.79±0.17	8.48±0.35	96.47	8.42±0.09	95.79	6.25±11	71.10
K_3	8.14±0.16	7.25±0.12	89.06	7.04±0.13	86.48	5.24±20	64.35
K_4	8.02±0.15	5.92±0.10	73.81	5.25±0.09	65.46	4.87±28	60.72
A_{11}	8.87±0.07	7.96±0.22	89.74	7.24±0.20	81.62	6.31±14	71.11
A_5	8.15±0.36	7.96±0.23	97.66	7.62±0.13	93.49	5.88±13	72.21
A_2	8.02±0.16	4.65±0.08	57.98	3.82±0.09	47.63	0.89±16	11.10
B_1	8.35±0.24	6.25±0.13	74.85	5.85±0.12	70.05	3.45±09	41.13

Values are expressed as the mean±SD. LAB, lactic acid bacteria. Survival (%) more than 50 resistant isolate. Survival (%) less than 50 not resistant isolate. *This value represents the highest survival and the most tolerance.

Table 6 Inhibitory activity of potentially probiotic bacterial isolates against bile salt hydrolase at different concentration (0.3, 0.4, 0.5, 1.0%) and different temperature (15–45°C)

LAB isolates	Bile salt hydrolase (mm)				Growth on a plate (Log CFU/ml)*			
	0.3%	0.4%	0.5%	1%	15°C	25°C	37°C	45°C
K_1	++	++	++	+	–	+	+++	+
K_2	+	–	–	–	+	++	++	+
K_3	+	–	–	–	–	+	++	–
K_4	++	+	–	–	–	++	+	–
A_{11}	+	+	–	–	+	++	+++	+
A_5	++	+	+	–	–	+	++	–
A_2	+	+	–	–	+	+	+++	–
B_1	+	–	–	–	+	+	+	+

LAB, lactic acid bacteria. +, diameter of precipitation zone more than or equal to 3 mm. ++, diameter of precipitation zone more than 5 mm. –, no precipitation zones. +++, high growth. ++, moderate growth. +, weak growth. –, no growth.

by the difference in viable cell counts after 0 min and 3 h of incubation, as shown in Table 4. All the isolates, except isolate k_3, showed better tolerance to acidic pH of 2.0, as compared with normal conditions, isolates k_1 and k_2 showed a high survival rate at pH 4.0, while they showed 39.42 and 51.99% survival rate, respectively, in acidic pH 2.0, and other isolates showed intermediate levels of tolerance.

Bile resistance and bile-salt hydrolase activity

Bile tolerance is the third selection criterion for potential probiotic. In this study, isolates were inoculated in MRS media containing different concentrations of bile salts to check their ability to survive in it. MRS medium lacking bile salt was used as control, whereas 0.3, 0.5, and 1.0% bile-salt concentrations in MRS broth were used to check the tolerance of the isolates. As evident from Table 5, the survival of selected isolates was also examined by the difference in viable cell counts following 0 min and 3 h of incubation in MRS containing 0.3, 0.5, and 1% bile salts and the results were subjected to statistical analysis ($P<0.05$). As given in Table 5, isolate K_1 showed the best survival rate (98.95% at 0.3% bile salt, 92.51% at 1% bile salt) in contrast to standard isolates (A_2) (57.98% at 0.3% and 11.10% at 1% bile salt). Also, other isolates are intermediate between the two. Also, BSH was assayed with MRS agar containing 0.3, 0.5, and 1.0% bile-salt concentrations. According to Table 6,

isolate K_1 was of the highest resistance to bile salt, high activity of BSH enzyme, and the highest clear zone at 1% concentration. While all the isolates showed no clear zone at 1% bile-salt concentration.

Temperature sensitivity

Temperature was considered a selection criterion for probiotic strain that grows at 37°C, which is an essential factor of probiotics to show their effectiveness. Also, growth at high temperatures was used as a technological feature in industries and packing of probiotic products. Therefore, selection of LAB isolates that grow under a broad range of temperature (37–45°C) was the target of our study. The results obtained in Table 6 were positive for growth at the chosen temperature range (15–45°C). All the selected LAB isolates (K_1, K_2, A_{11}, and B_2) were able to survive at temperature ranges 15, 37, and 45°C. But other isolates (K_3, K_4, A_5, and A_2) do not grow at 45°C.

Antibiotic-susceptibility pattern of selected lactic acid bacteria isolates

Antibiotic therapy can significantly affect this microbial balance in the intestine by reducing the viability of indigenous microflora. The result presented in Table 7 showed antibiotic susceptibility of probiotic culture ranging from sensitive (S), intermediate (I), and resistant (R) against different tested antibiotics. Isolate K_1 was sensitive to rifampicin, tetracycline, chloramphenicol, neomycin, gentamicin, and streptomycin and resistant to kanamycin, vancomycin, ciprofloxacin, oxacillin, and cephradine and intermediate to ampicillin, amikacins, and norfloxacin. Isolate K_2 was sensitive to vancomycin, oxacillin, kanamycin, and norfloxacin and resistant to ampicillin, amikacins, ciprofloxacin, and cephradine and intermediate to rifampicin, tetracycline, chloramphenicol, neomycin, streptomycin, and gentamicin. Both isolates (K_3 and K_4) were sensitive to tetracycline, chloramphenicol, and ciprofloxacin and resistant to norfloxacin, gentamicin, neomycin, and streptomycin. Both isolates (A_{11} and A_5) were sensitive to tetracycline, chloramphenicol, and rifampicin and resistant to amikacins and streptomycin.

Antioxidant activity of selected lactic acid bacteria isolates

Accumulated evidence suggests that LAB strains have good potential for application in functional foods and health-related products. To augment these results, the present study researched the antioxidant activity of these strains. To elucidate the antioxidant activity of the eight strains, DPPH radical scavenging activities were determined. As shown in the results in Fig. 2, the isolate K_2 was of the highest antioxidant activity (86.93%), while isolate K_1 was of the lowest antioxidant activity (61.42%) as compared with all tested isolates.

Anticholesterol activity

Data presented in Fig. 2 indicated that all isolates can remove, and assimilated cholesterol ranged from 16 to 32%. Isolate A_{11} has the highest assimilation of cholesterol with 32.6%, in contrast, isolate K_4 showed 16.5% of cholesterol assimilation. While isolates K_1, K_2, K_3, A_5, A_2, and B_1 have cholesterol-assimilation activity (30.2, 24.3, 27.6, 22.0, 18.7, and 24.9%), respectively, cholesterol assimilation was shown to be the highest for isolates A_{11} and K_1 (up to 32%), whereas isolates K_4 and A_2 showed the least cholesterol removal (16.5 and 18.7%, respectively).

Table 7 Antibiotic susceptibility of the selected probiotic culture

Antibiotic	Concentration (μg/disc)	Antibiotic susceptibility (mm)							
		K_1	K_2	K_3	K_4	A_{11}	A_5	A_2	B_1
Ampicillin	10	I (6.3)	R	R	I (10.0)	I (8.0)	R	I (15.0)	S (27.0)
Rifampicin	10	S (18.0)	R	S (20.5)	R	S (19.0)	S (22.0)	I (14.0)	R
Tetracycline	30	S (22.0)	I (13.0)	S (28.0)	S (20.0)	S (18.0)	S (15.0)	R	S (23.0)
Chloramphenicol	30	S (18.0)	I (11.0)	S (19.0)	S (27.0)	S (15.0)	S (22.0)	I (14.0)	R
Neomycin	10	S (24.0)	I (12.0)	R	R	S (27.0)	I (13.0)	S (17.0)	I (9.0)
Streptomycin	10	S (15.0)	R	R	R	R	R	R	R
Kanamycin	30	R	S (25.0)	R	I (9.0)	S (16.0)	R	R	R
Amikacine	30	I (9.0)	R	I (10.0)	R	R	R	R	I (10.0)
Vancomycin	10	R	S (26.0)	I (13.0)	R (0.00)	S (17.0)	R (2.0)	S (20.1)	R
Gentamicin	10	S (27.0)	I (12.0)	R	R	S (29.0)	R	R	S (21.0)
Ciprofloxacin	5	R	R	S (15.0)	S (17.5)	I (14.0)	I (11.5)	R	S (19.5)
Oxacillin	5	R	S (19.5)	S (22.5)	I (8.5)	I (6.0)	R	R	R
Norfloxacin	10	I (14.5)	S (16.0)	R	R	R	I (8.0)	R	S (28.0)
Cephradine	30	R	R	R	S (18.5)	S (21.0)	I (10.0)	I (11.0)	S (23.0)

R, resistance to antibiotic. No inhibition zones. I, intermediate to antibiotic. Inhibition zones more than or equal to 6.5–15 mm. S, sensitive to antibiotics. Inhibition zones more than or equal to 15 mm.

Figure 2

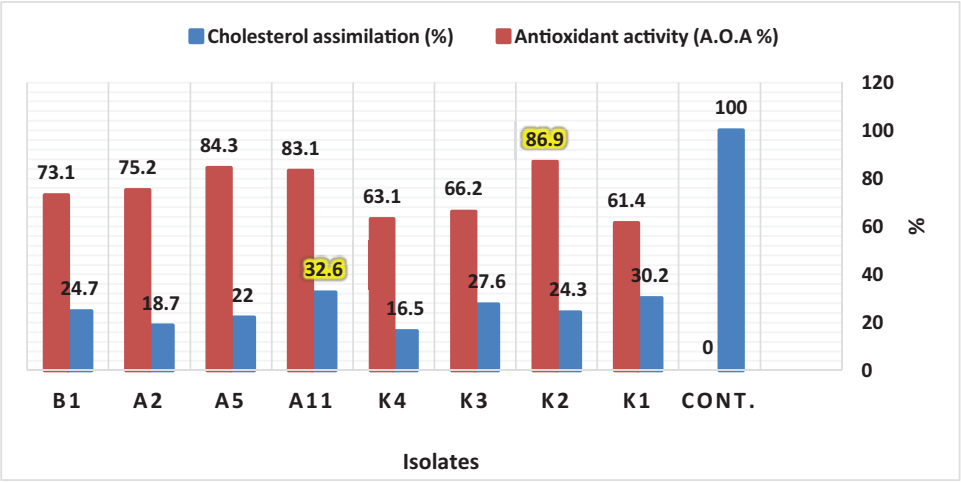


Diagram of cholesterol assimilation and antioxidant activity of the selected isolates.

Figure 3

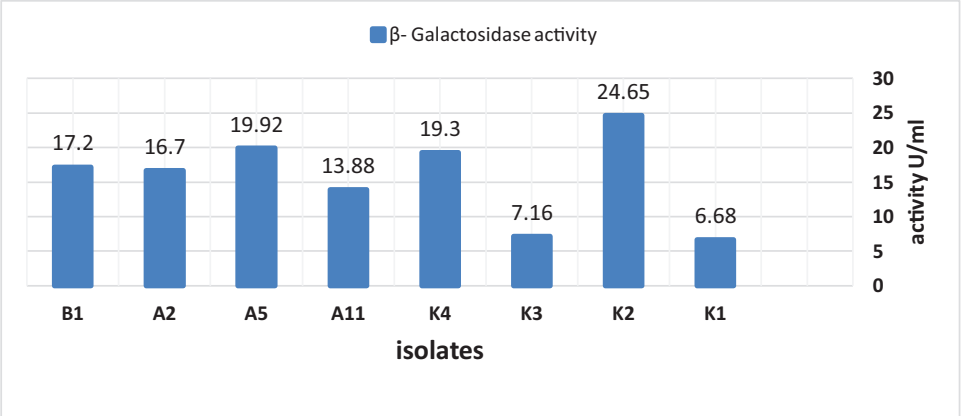


Diagram showing the β-galactosidase-enzyme activity (U/ml) of the selected isolates.

β-galactosidase activity of selected isolates

Data presented in Fig. 3 noted that all tested strains were grown in MRS medium containing 1% lactose as a medium used for colorimetric assay of β-galactosidase activity. When ONPG assay was used, isolate K_2 was the highest enzyme producer of the isolates (24.65 U/ml) and considered to be the best β-galactosidase producer, while isolate K_1 was of the lowest β-galactosidase activity about 6.68 U/ml, isolates K_3, K_4, A_{11}, A_5, A_2, and B_1 have β-galactosidase production (07.16, 19.30, 13.88, 19.92, 16.70, and 17.20 U/ml), respectively.

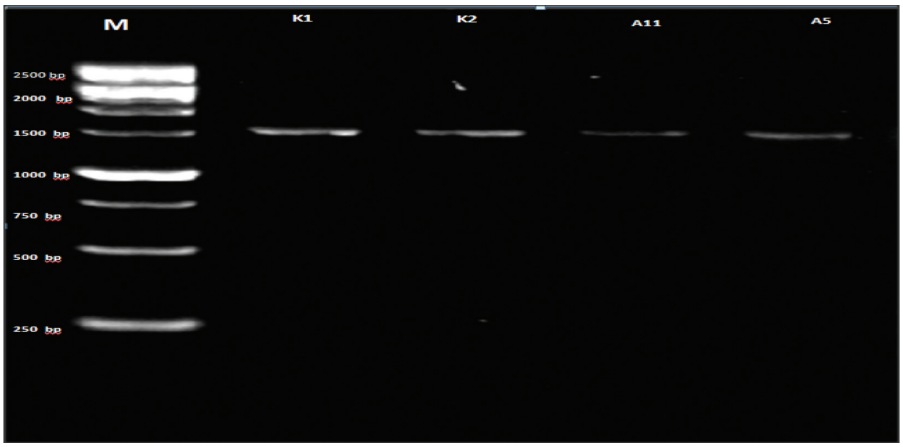
Molecular identification of lactic acid bacteria using 16 S rRNA gene

Molecular identification of the selected isolates (K1, K2, A11, and A5) was carried out by amplification and sequencing their 16 S rRNA gene. The four PCR products were at an expected size 1500 bp (Fig. 4). The amplified PCR products were purified, sequenced, and aligned using a blast with the published sequences of the 16 S rRNA gene of other strains deposited in NCBI databases. According to the blast results of the isolates with more than 99% similarities, they were identified as (K1) *L. lactis*, (A5) *Lactobacillus casei*, and (K2 and A11) *Enterococcus faecium*. Their partial sequences (Figs 5–8) were submitted to the GenBank at NCBI database with accession numbers A5: KU510332.1, A11: KU510333.1, K1: KU510334, and K2: KU510335.1 (Table 8). Their phylogenetic trees are shown in Fig. 9.

Discussion

In the present work, dairy-product samples from milk, fermented milk, and cheese were selected to isolate LAB. The samples were gathered from different sources to get a wide diversity of bacterial strains. Eight isolates were selected randomly and purified. The selection is based on the change in the morphology

Figure 4



About 1.5% agarose-gel electrophoresis of PCR products of 16 s rRNA gene for the isolates. Lane 1, DNA marker 1 Kb; lane 2, K1; lane 3, K2; lane 4, A11; lane 5, A5.

Figure 5

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   1 GCTTCTTTTT CCCCGGAGCT TGCTCCACCG GAAAAAGAAG AGTGGCGAAC GGGTGAGTAA CACGTGGGTA ACCTGCCCAT
  81 CAGAAGGGGA TAACACTTGG AAACAGGTGC TAATACCGTA TAACAATCGA AACCGCATGG TTTTGATTTG AAAGGCGCTT
 161 TCGGGTGTCG CTGATGGATG GACCCGCGGT GCATTAGCTA GTTGGTGAGG TAACGGCTCA CCAAGGCCAC GATGCATAGC
 241 CGACCTGAGA GGGTGATCGG CCACATTGGG ACTGAGACAC GGTCCAAATC CCTACGGGAG GCAGCAGTAG GGAATCTTCG
 321 GCAATGGACG AAAGTCTGAC CGAGCAACGC CGCGTGAGTG AAGAAGGTTT TCGGATCGTA AAACTCTGTT GTTAGAGAAG
 401 AACAAGGATG AGAGTAACTG TTCATCCCTT GACGGTATCT AACCAGAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG
 481 TAATACGTAG GTGGCAAGCG TTGTCCGGAT TTATTGGGCG TAAAGCGAGC GCAGGCGGTT TCTTAAGTCT GATGTGAAAG
 561 CCCCCGGCTC AACCGGGGAG GGTCATTGGA AACTGGGAGA CTTGAGTGCA GAAGAGGAGA GTGGAATTCC ATGTGTAGCG
 641 GTGAAATGCG TAGATATATG GAGGAACACC AGTGGCGAAG GCGGCTCTCT GGTCTGTAAC TGACGCTGAG GCTCGAAAGC
 721 GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT GAGTGCTAAG TGTTGGAGGG TTTCCGCCCT
 801 TCAGTGCTGC AGCTAACGCA TTAAGCACTC CGCCTGGGGA GTACGACCGC AAGGTTGAAA CTCAAAGGAA TTGACGGGGG
 881 CCCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA CGCGAAGAAC CTTACCAGGT CTTGACATCC TTTGACCACT
 961 CTAGAGATAG AGCTTCCCCT TCGGGGGCAA AGTGACAGGT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG AGATGTTGGG
1041 TTAAGTCCCG CAACGAGCGC AACCCTTATT GTTAGTTGCC ATCATTTAGT TGGGCACTCT AGCAAGACTG CCGGTGACAA
1121 ACCGGAGGAA GGTGGGGATG ACGTCAAATC ATCATGCCCC TTATGACCTG GGCTACACAC GTGCTACAAT GGGAAGTACA
1201 ACGAGTTGCG AAGTCGCGAG GCTAAGCTAA TCTCTTAAAG CTTCTCTCAG TTCGGATTGC AGGCTGCAAC TCGCCTACAT
1281 GAAGCCGGAA TCGCTAGTAA TCGCGGATCA GCACGCCGCG GTGAATACGT TCCCGGGCCT TGTACACACC GCCCGTCACA
1361 CCACGAGAGT TTGTAACACC CGAAGTCGGT GAGGTAACCT TTTGGAGCCA GCCGCCTAAG G
```

The 16S-rDNA gene partial sequence of isolate (K_1) *Lactococcus lactis*.

Figure 6

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   1 GCTTCTTTTT CCACCGGAGC TTGCTCCACC GGAAAAAGAA GAGTGGCGAA CGGGTGAGTA ACACGTGGGT AACCTGCCCA
  81 TCAGAAGGGG ATAACACTTG GAAACAGGTG CTAATACCGT ATAACAATCG AAACCGCATG GTTTTGATTT GAAAGGCGCT
 161 TTCGGGTGTC GCTGATGGAT GGACCCGCGG TGCATTAGCT AGTTGGTGAG GTAACGGCTC ACCAAGGCCA CGATGCATAG
 241 CCGACCTGAG AGGGTGATCG GCCACATTGG GACTGAGACA CGGCCCAAAC TCCTACGGGA GGCAGCAGTA GGGAATCTTC
 321 GGCAATGGAC GAAAGTCTGA CCGAGCAACG CCGCGTGAGT GAAGAAGGTT TTCGGATCGT AAAACTCTGT TGTTAGAGAA
 401 GAACAAGGAT GAGAGTAACT GTTCATCCCT TGACGGTATC TAACCAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG
 481 GTAATACGTA GGTGGCAAGC GTTGTCCGGA TTTATTGGGC GTAAAGCGAG CGCAGGCGGT TTCTTAAGTC TGATGTGAAA
 561 GCCCCCGGCT CAACCGGGGA GGGTCATTGG AAACTGGGAG ACTTGACTGC AGAAGAGGAG AGTGGAATTC CATGTGTAGC
 641 GGTGAAATGC GTAGATATAT GGAGGAACAC CAGTGGCGAA GGCGGCTCTC TGGTCTGTAA CTGACGCTGA GGCTCGAAAG
 721 CGTGGGGAGC AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTGCTAA GTGTTGGAGG GTTTCCGCCC
 801 TTCAGTGCTG CAGCTAACGC ATTAAGCACT CCGCCTGGGG AGTACGACCG CAAGGTTGAA ACTCAAAGGA ATTGACGGGG
 881 GCCCGCACAA GCGGTGGAGC ATGTGGTTTA ATTCGAAGCA ACGCGAAGAA CCTTACCAGG TCTTGACATC CTTTGACCAC
 961 TCTAGAGATA GAGCTTCCCC TTCGGGGGCA AAGTGACAGG TGGTGCATGG TTGTCGTCAG CTCGTGTCGT GAGATGTTGG
1041 GTTAAGTCCC GCAACGAGCG CAACCCTTAT TGTTAGTTGC CATCATTCAG TTGGGCACTC TAGCAAGACT GCCGGTGACA
1121 AACCGGAGGA AGGTGGGGAT GACGTCAAAT CATCATGCCC CTTATGACCT GGGCTACACA CGTGCTACAA TGGGAAGTAC
1201 AACGAGTTGC GAAGTCGCGA GGCTAAGCTA ATCTCTTAAA GCTTCTCTCA GTTCGGATTG CAGGCTGCAA CTCGCCTGCA
1281 TGAAGCCGGA ATCGCTAGTA ATCGCGGATC AGCACGCCGC GGTGAATACG TTCCCGGGCC TTGTACACAC CGCCCGTCAC
1361 ACCACGAGAG TTTGTAACAC CCGAAGTCGG TGAGGTACCT TTTTGGAGCC AGCCGCATAA GG
```
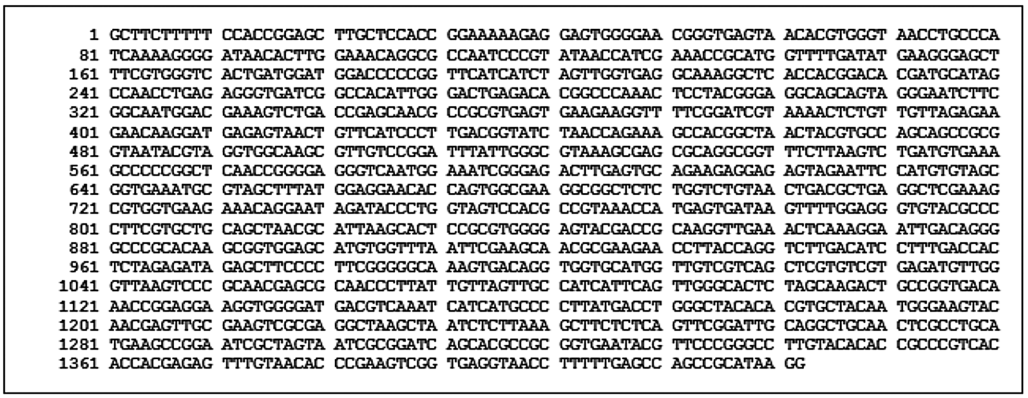

The 16S-rDNA gene partial sequence of isolate (A_5) *Lactobacillus casei.*

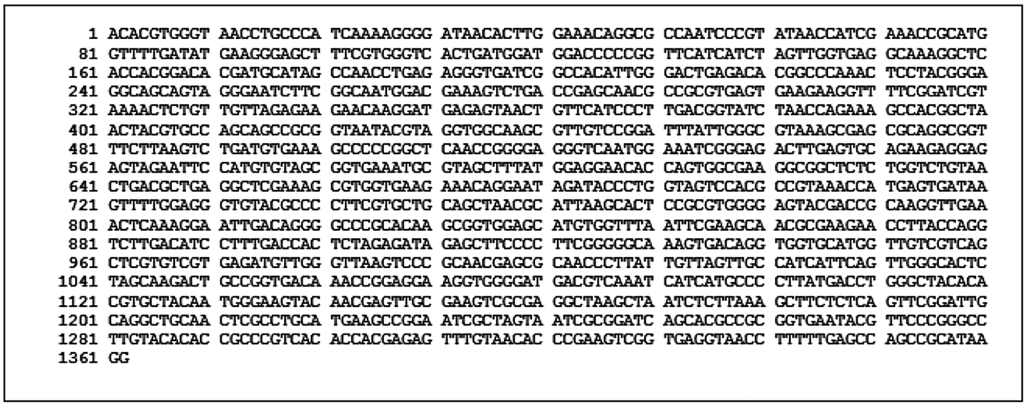
of the colonies, such as color, texture, and margin. Phenotypic characters were used for the characterization of the isolates. For identification, biochemical characterization and molecular techniques were used. These characteristics are in agreement with LAB characteristics obtained by Axelsson [33] and Arasu *et al.* [34]. By comparing the results of the biochemical characterization of the bacterial isolates with Bergey's manual of systematic bacteriology, the isolates were identified as *Enterococcus*

Figure 7



The 16S-rDNA gene partial sequence of isolate (K_2) *Enterococcus faecium*.

Figure 8



The 16S-rDNA gene partial sequence of isolate (A_{11}) *Enterococcus faecium*.

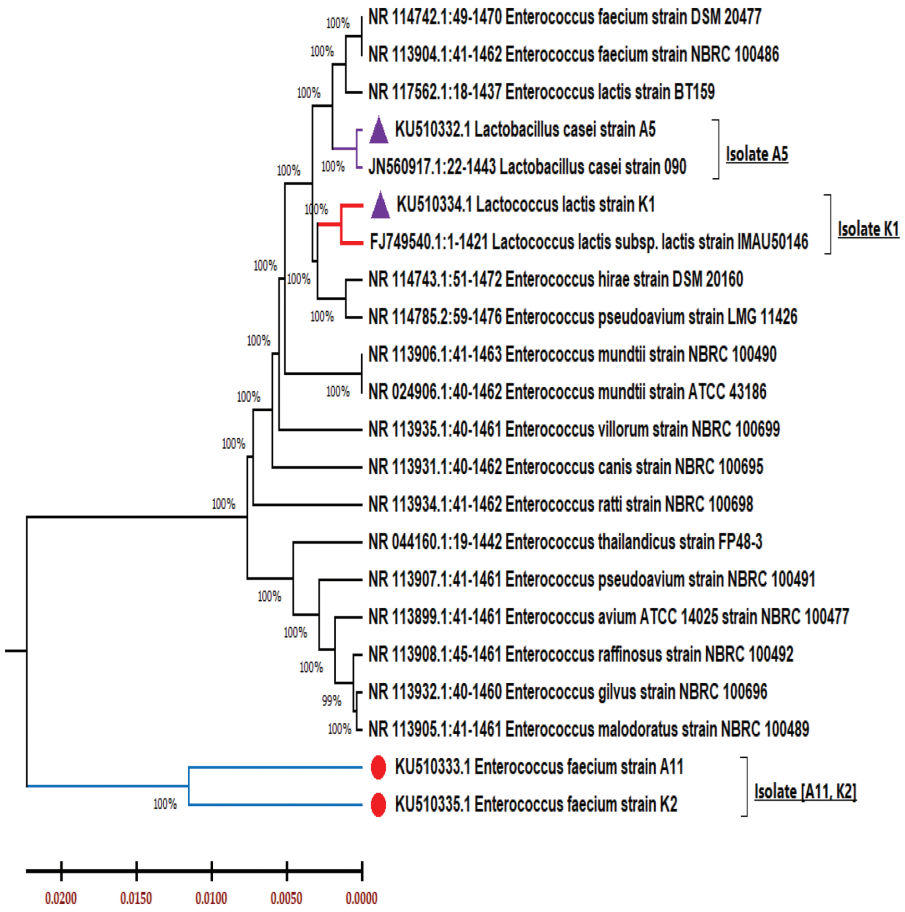
Table 8 Identification percentage between selected potential probiotic bacterial isolates and related species

No	16 S rDNA sequence (similarity)	Strain	Isolate code	Source of isolation	Accession number
1	99%	*Lactobacillus casei*	A_5	Milk	KU510332.1
2	98%	*Enterococcus faecium*	A_{11}	cheese	KU510333.1
3	99%	*Lactococcus lactis*	K_1	Milk	KU510334.1
4	99%	*Enterococcus faecium*	K_2	Cheese	KU510335.1

spp., *Lactococcus* spp., and *Lactobacillus* spp. The survival under the environmental conditions of the gastrointestinal tract is an important characteristic feature of the isolate for potential use as a probiotic. Thus, the probiotic properties such as bile resistance and acid tolerance were assessed. Our isolates showed the ability to pass the basic criteria for probiotic features as they can tolerate to pH 2 and growth in 0.3% bile salts. Bile-salt tolerance varies significantly among LAB species even between strains themselves. LAB resistance to bile salt can be attributed to the activity of the enzyme BSH, which is responsible for the degradation of bile salts lowering its toxic effect [35].

In our work, data revealed that most of the strains could survive in the presence of different concentrations of bile salts ranging from 0.3 to 1.0% after 3 h of incubation in addition to their ability to hydrolyze the bile acids. The isolates proved their ability to grow at different pH values ranging from 2.0 to 7.0. The results obtained are in agreement with Soliman *et al.* [36] results with three isolates of Lactobacilli from Egyptian-fermented dairy products and showed that they can tolerate acid and bile salts. The ability of the LAB strains to tolerate salt concentration is commonly used as the selection for potential probiotic candidates. Our data indicated that all isolates could tolerate salt concentration (1–8%), while *E. faecium* isolate (K_2) was tolerant to 8% and this result is in agreement with that reported by Adnan and Tan [37]. Also, Hoque *et al.*

Figure 9



Phylogenetic tree based on partial 16S-rDNA sequences, showing the relationship between the isolates A_{11}, K_1, K_2, and A_5 and other LAB. The tree was constructed using the MEGA 6.06 and neighbor-joining method. LAB, lactic acid bacteria.

[24] observed the NaCl (2–10%) tolerance of *Lactobacillus* sp. isolated from yogurts.

The susceptibility of LAB isolate cultures was assessed against common antibiotics to determine the ability of the culture to maintain the microbial balance in the intestine during antibiotic treatment. The results obtained were in agreement with Zhou *et al.* [38] who showed that both *Lactobacillus* and *Bifidobacterium* strains were affected by β-lactam, Gram-positive spectrum, and broad-spectrum antibiotics. Some of the LAB species from *Lactobacillus*, *Leuconostoc*, and *Streptococcus* genera are sensitive to clinically relevant antibiotics such as ampicillin, tetracycline, penicillin, erythromycin, and chloramphenicol [39]. Also, our study was in agreement with Hashemi *et al.* [27] who found that probiotic strains were sensitive to different antibiotics, such as ampicillin, penicillin, chloramphenicol, erythromycin, and rifampicin, but they were resistant to vancomycin and streptomycin.

The antioxidant properties of the selected strains were determined by the DPPH radical scavenging activity method. Among the eight probiotic isolates, *E. faecium* isolate (K_2) showed the highest antioxidant activity (86.9%), while *L. lactis* K1 isolate showed the least antioxidant activity (61.4%). These results are in harmony with Bing *et al.* [40], Wang *et al.* [10], and Shen *et al.* [41] who stated that CFS of *Bifidobacterium* animals gives potent antioxidant activities. Also, Wang *et al.* [10] showed that the filtrate from 35 LAB showed more potent antioxidant activities than bacterial suspensions.Cholesterol assimilation in the intestinal tract decreases the absorption of dietary cholesterol from the digestive tract, multiple mechanisms have been suggested for understanding the cholesterol-lowering activity of probiotic bacteria [42]. The data obtained showed that all the probiotic isolates tested could remove and assimilate cholesterol from media. These results are in harmony with Klaver and Van Der Meer [43], Lee *et al.* [44], and Tsai *et al.* [45].

β-galactosidase is widely used, especially in dairy technologies. As β-galactosidase plays an important role in the digestion of lactose, β-galactosidase-deficient individuals suffer from severe intestinal disorders, including bloating, flatulence, and

abdominal pain. All the isolates in the study showed β-galactosidase activity, which allows them to be used in the dairy technology. *E. faecium* K_2 isolate showed maximum β-galactosidase activity giving 24.65 U/ml. These results are in harmony with Lin *et al*. [46], and LAB probiotics are reported to accelerate lactose digestion lowering lactose-intolerance (LI) symptoms [47]. β-galactosidase possesses both hydrolyzation and transglycosylation activities, it can synthesize oligosaccharides simultaneously with lactose hydrolysis. It is stated that reliance on morphological, physiological, and biochemical characteristics for identification of LAB is often considered as misleading, since many morphological and nutritional requirements may be similar among different species [48]. Also, reliance on sugar-fermentation profile-based phenotypic characterization may not provide reliable identification of LAB; however, molecular-based methods such as 16S-rDNA were used to identify bacteria as an alternative to phenotypic methods [49]. In our study primarily, presumptive identification was done by biochemical assay and sugar-fermentation pattern according to Bergey's Manual of Systematic Bacteriology. Further, the isolates were identified by 16S-rDNA sequencing, such as *E. faecium*, *L. lactis*, and *L. casei*, and these results are in harmony with Saeedi *et al*. [50] and Cibik *et al*. [51] as they showed that 16S-rDNA gene sequencing can be considered as a potent approach that enables both tracing phylogenetic relationships between bacteria and identification of bacterial strains isolated from various environmental sources and fermentation specimens.

Conclusion

Milk products can act as a rich reservoir of LAB strains for their role in probiotic research and be used as probiotics. In this study, among several isolates, eight isolates of LAB were isolated, purified, and identified using morphological, biochemical, and physiological tests such as growth temperature, pH condition, salt concentration, and genotype-based method of 16S-rDNA. Then probiotic criteria, such as acid and bile-salt tolerance, bile-salt deconjugation, cholesterol removal, temperature sensitivity, antioxidant activity, and beta-galactosidase activity, were evaluated. Molecular-biology technology based on the 16S-rDNA-sequencing technique was followed to verify species' nomenclature. We obtained four promising isolates as potential probiotics that showed probiotic characteristics and were identified as *L. casei* (A5), *E. faecium* (K2), *E. faecium* (A11), and *L. lactis* (K1) with accession numbers KU510332.1, KU510335.1, KU510333.1, and KU510334.1, respectively. These selected isolates have good probiotic traits and have many applications in the food industry.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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