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Research Article
Probiotic Activity of Lactic Acid Bacteria Isolated from Rayab Milk (Curdled Skim Milk Made in Lower Egypt)

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Abstract

Background and Objectives: Several infectious diseases became a big threat worldwide after spreading of multidrug resistant pathogens. Rayab milk found to be rich source of lactic acid bacteria with potential probiotics activity. Objective of the study was to evaluate the probiotic activity of LAB strains isolated from commercially fermented Rayab milk and to compare API identification method for some LAB isolates versus 16S rDNA sequencing molecular method. **Materials and Methods:** *Lactobacillus* MRS agar and broth media were applied to selectively isolate LAB from 20 fermented Rayab milk samples. API systems were used for biochemical identification. 16S rDNA sequencing had been used to identify ten randomly selected LAB isolates. Probiotic properties of all isolated LAB were tested, acid tolerability at pH 2.0, 3.0 and ability to grow in the presence of 0.3% bile salts, inhibitory effect of 10 antibiotics and bacteriostasis effect on food pathogens. **Results:** Ninety-five LAB had been isolated from 20 Rayab milk samples, 45 isolates out of them had been identified using API systems and grouped into four main genera previous reports, 62.2% of 45 isolates (28 isolates) showed distinguish resistance for surviving on low pH conditions. Fifteen out of these 28 isolates showed a tolerance to 0.3% bile salt. Moreover, 38 (84.4%) LAB isolates exhibited inhibitory activity against *Staphylococcus aureus* strain, *Listeria monocytogenes* strain (A), *Salmonella typhimurium* strain and *E. coli* O157 strain. Highest inhibition influences against LAB were observed for gentamicin, tobramycin and neomycin. While the lowest inhibition influences were recorded for amoxicillin, ceftriaxone, cefoxitin and nalidixic acid. **Conclusion:** LAB isolates with potential probiotic activity can be used to promote health and to combat food borne diseases.

Key words: Lactic acid, bacteria identification, probiotics, rayab milk, DNA sequencing, antibacterial activity, pathogens

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Competing Interest: The author has 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 heterogenous group of nonsporulating, aerotolerant, gram-positive, microorganisms arranged in rods or cocci[1]. *Pediococcus*, *Streptococcus*, *Leuconostoc*, *Lactococcus*, *Enterococcus* and *Lactobacillus*, are the main identified LAB groups based on morphology and carbohydrates fermentation[2]. However, these biochemical based identification methods are not conclusive as LAB groups have common nutritional and growth requirements. Gene sequencing using 16S rDNA had been the most reliable identification method, despite it sometimes fail to differentiate LAB species[3].

Some LAB are very good examples of probiotics bacteria, which are live microorganisms that are beneficial to health. However, other LAB may have some probiotics potentials or only can be used as fermentation cultures. Probiotic properties are; capability to tolerate acid and bile salt, gastric fluids tolerance; adherence to intestinal mucosa; bacteriostasis on pathogenic bacteria and inhibition by antibiotics[4]. LAB are usually found in many food sources such as dairy products, fermented milk, fish from fresh water and fermented meat or vegetables[5].

Ingestion of probiotic microorganisms with food is one of the approaches to combat the problem of food-borne diseases[6]. Therefore, this study studied the probiotic properties of LAB strains isolated from commercial Rayab milk, as they may have a beneficial role against food-borne pathogens. Moreover, to compare API identification method for some LAB isolates versus 16S rDNA sequencing molecular method.

MATERIALS AND METHODS

Study area: This study was carried during 2 month period from beginning of September to end of October, 2019, samples were collected from local market of Alexandria city, Egypt.

Samples and media: In this investigation, 20 fermented Rayab milk samples (1 L) produced by 4 different manufactures were collected from local market in Alexandria, Egypt. *Lactobacillus* MRS Agar and MRS broth media (HIMEDIA, India) were applied to selectively isolate LAB.

Isolation of LAB: Serial dilutions were prepared from each sample (up to10^5). Pour plate method was done using MRS and M17 agar media, at 37°C for 24-48 h in anaerobic CO_2 incubator (Sysmedical, China Anaerobic Culture Incubator). Bacterial isolates were subcultured twice on fresh MRS plates for purity check[7].

Identification of bacterial isolates

Preliminary phenotypic characterization of isolates: Gram stain reaction, microscopic cell morphology, cellular arrangement and catalase activity were identified for all isolates[8,9].

Confirmation by API systems: API systems was used to identify isolates that are gram-positive and catalase negative to species level; API 20 STREP (Bio Mérieux, France) was done for gram positive cocci (*Lactococcus*) On other hand, identification of gram positive rods (*Lactobacilli*) was accomplished by using API 50 CHL micro-identification systems (Bio Mérieux, France).

Molecular confirmatory identification of (LAB) 16S ribosomal DNA: Both biochemical and molecular identifying of 10 randomly selected LAB isolates were done, to compare results of API systems versus 16S rDNA sequencing.

Extraction of genomic DNA: One and half ml overnight LAB culture grown in MRS broth was centrifuged at 12000 rpm. Supernatant was discarded, pellet was used to purify total genomic DNA by Gene JET genomic DNA purification kit (Thermo Fisher, USA). Purified DNA stored at -20°C till use.

16S ribosomal DNA (rRNA) molecular marker identification: LAB isolates were identified through 16S rDNA gene sequenced protocol. DreamTaq Green PCR Master Mix (2X) (K1081, Thermo Fisher, USA) was used for specific gene amplification according to manufacturer protocol through CreaCon (Holland, Inc) Universal 16S rDNA primers were employed to amplify specific amplicons as shown in Table 1. Polymerase chain reaction (PCR) system cycler was used, PCR initiated with 5 min of initial denaturation at 94°C. then, repeated at a total of 30 times (at 94 for 30 sec, at 55 for 30 sec, at 72 for 1 min) and finally at 72 for 7 min[10]. Using DNA ladder (peqGOLD 1 kb DNA-Ladder, Peq (LAB), VWR), 1% agarose gel was used for amplicons visualization via gel documentation system (Geldoc-it, UVP, England).

Table 1: Universal 16S rDNA primers

Primers	Sequences	Target fragment (bp)
16S:F27	5-AGAGTTTGATCCTGGCTCAG-3	1600
16S:R27	5-AAGGAGGTGATCCAGCCGCA-3	

Sequencing and identification of PCR products: Specific DNA bands were eluted from agarose gel. Products of PCR were purified with EZNA ®Gel Extraction Kit, (D2500-01, Omega BIO-TEK, USA). Specific 16S rDNA amplicon were sequenced using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp. Korea). To detect LAB genera, sequence homology analysis was performed for all sequences through submission into www.ncbi.nlm.nih.gov and search in GeneBank database via BLAST (Basic Local Alignment Search Tool).

Construction of phylogenetic tree: 16S rDNA sequences of (LAB) were retrieved from GeneBank database, Clusteral W software analysis was applied to construct Phylogenetic tree based on multiple sequence comparison alignments using Pairwise Distance method.

Probiotic properties

Tolerance to acidity and bile salts

Acid tolerance: Pour plate method was used to determine bacterial count variation. LAB isolates were inoculated onto 5 mol HCL supplemented MRS and M17 media at pH 2.0 and 3.0 and normal MRS and M17 agar media (pH 6.5)[11].

Bile salt tolerance: Bacterial broth cultures were inoculated into MRS broth with and without 0.3% (w/v) bile, in water bath at 37°C. Growth was observed by measuring the absorbance at 600 nm. Delay of growth due to inhibition by the bile salts was calculated as the difference (d) in the length of time between the two samples (with and without bile)[4].

Antibiotic susceptibility: Agar disk diffusion assay was applied to evaluate antibiotic susceptibility of identified LAB, using ten antibiotics discs (ceftriaxone/30 µg, amoxicillin/25 µg, tobramycin/10 µg, penicillin G/10 units, cefoxitin/30 µg, neomycin/30 µg, gentamicin/10 µg, vancomycin/256 nitrofurantoin/300 and nalidixic acid/30 µg (bio TRADING, Netherland)[12].

Hemolytic activity: Hemolysis was evaluated via culturing LAB isolates on Columbia blood agar (Oxoid, Thermo Fisher Scientific, USA), supplemented with 5% (v/v) human blood, for 24 and 72 h periods at 37°C in anaerobic jars[9].

Evaluation of antibacterial activity of LAB isolates: Agar well diffusion assay was applied using pour plated MHA plates inoculated with four food borne pathogenic bacteria indicators; *Salmonella typhimurium* strain (ATTC 13311), *Staphylococcus aureus* strain (NCINB 50080), *Listeria monocytogenes* strain (ATTC19111) and *E. coli* O157 strain (ATTC 700728) After solidification, sterile cork borer was applied to made agar wells which filled with 100 µL of filtered LAB supernatant (centrifugation at 10000×g for 15 min). After Plates incubation at 37°C for 24 h, inhibition zones diameter (mm) were measured[13].

Statistical analysis: Data were collected, tabulated and analyzed using statistical software SPSS version 24 (IBM Corp., Chicago, Illinois, USA).

RESULTS

From total 20 fermented Rayab milk products, 95 lactic acid bacteria were isolated. Colonies were circular in shape with varied color ranged from white to creamy. All isolates were catalase negative, 69 of them were Gram positive bacilli and the remaining 26 isolates were Gram positive cocci.

Based on interpretation of the API database, 45 LAB isolates were satisfactorily identified into species level, API 50 CHL identified 22 lactobacilli and another 23 lactococcus were identified using API 20 STREP as shown in Table 2.

The 45 isolates grouped into four main genera; as illustrated in Fig. 1.

Table 2: Data of API50CH/API 20 strep identification methods

Identified species	Number	Total 45 species (%)	Total genus (%)
API 50 strep (22)			
Lactobacillus acidophilus	9	20.00	40.9
Lactobacillus lactis	8	17.80	36.4
Lactobacillus bulgaricus	5	11.10	22.7
API 250 strep (n = 23)			
Enterococcus faecalis	10	22.20	100.0
Aerococcus viridant	7	15.60	100.0
Streptococcus thermophilus	4	8.90	66.7
Streptococcus acidomonas	2	4.40	33.3

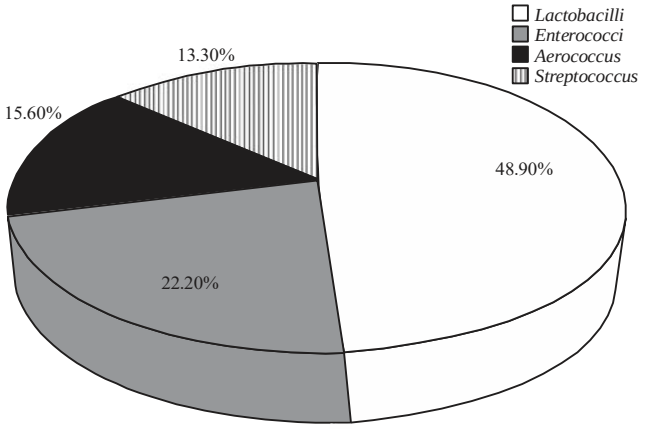

Fig. 1: Distribution of (LAB) at genus level isolated from traditional Rayab milk in Egypt

Table 3: Genotypes of the 10 selected LAB as 16S rDNA gene sequences alignments submitted to the NCBI GeneBank database

Bacterial identification	Accession number	Identity (%)
Lactococcus lactis subsp. cremoris strain NBRC 100676	NR_113925.1	100
Enterococcus faecium strain DSM 20477	NR_114742.1	100
Enterococcus ureilyticus strain CCM 4629	NR_125485.1	100
Enterococcus faecium strain NBRC 100486	NR_113904.1	100
Enterococcus faecium strain NBRC 100486	NR_113904.1	100
Enterococcus faecalis strain NBRC 100480	NR_113901.1	100
Enterococcus crotali strain ETRF1	NR_156980.1	100
Aerococcus vaginalis strain BV2	NR_125468.1	100
Aerococcusurinae hominis strain CCUG 42038b	NR_028922.1	99.94
Streptococcus thermophilus strain DSM 20617	NR_118998.1	100

Table 4: Reliability comparison between API 50CH/API 20 strep and 16S rDNA sequencing identification methods

	Identification method	
Lactic acid bacteria (LAB)	API 50CH/API 20 strep	16S rDNA sequencing
1	*Lactobacillus acidophilus*	*Lactococcus lactis* subsp. cremoris
2	*Lactobacillus acidophilus*	*Enterococcus faecium*
3	*Lactobacillus acidophilus*	*Enterococcus ureilyticus* strain CCM 4629
4	*Lactobacillus acidophilus*	*Enterococcus faecium* strain NBRC 100486
5	*Enterococcus faecalis*	*Enterococcus faecium* strain NBRC 100486
6	*Enterococcus faecalis*	*Enterococcus faecalis* strain NBRC 100480
7	*Enterococcus faecalis*	*Enterococcus crotali* strain ETRF1
8	*Aerococcus viridant*	*Aerococcus vaginalis*
9	*Aerococcus viridant*	*Aerococcusurinae hominis*
10	*Streptococcus acidomonas*	*Streptococcus thermophilus*

When the phenotypic method using API systems compared to the 16S rDNA sequencing results, evidence of non-concomitant result is illustrated in Table 4. 16S rDNA identified higher numbers of *Enterococcus* spp. On the other hand, Lactobacillus genus was mostly identified by API 50CHL. API system correctly identified 6 isolates to genus level (60%), however it showed divergent result in species level identification, where only one isolate (10%) was correctly identified to species level.

Tolerance to acidity and bile salt: In this investigation, 45 LAB reflected varied acid tolerance patterns. Twenty eight (62.2%) out of 45 isolated lactic acid bacteria showed distinguish resistance for surviving on low pH conditions.

At pH 3.0, 19 strains showed survival rates $\geq$90%. On the other hand, 21 strains reflected survival rates $\geq$90% at pH 2.0. Furthermore, only ten strains showed good tolerance at both pH 2.0 and 3.0. Two LAB isolates (6, 25) considered the highest acid tolerant at pH 3.0 and 2.0 with a survival rate of 96 and 94% respectively. Comparing with control LAB isolate, viable counts were decreased at both pH 2.0 and 3.0 after 2 h.

Regarding bile salt tolerance, varied inhibition values were recorded for 15 (53.5%) out of 28 acid tolerant LAB isolates according to Gilliland standards[14]. Only one LAB isolate considered resistant strain, 4 strains reflected tolerant pattern. Furthermore, ten strains classified as bile weakly tolerant. Two LAB isolates (13 and 21) showed bile-sensitive response (Table 5).

Antibiotic susceptibility and hemolytic activity of LAB: Different susceptibility response remarked tested 45 LAB isolates against 10 applied antibiotics. Highest inhibition influences against LAB were observed for gentamicin, tobramycin and neomycin with largest inhibition zones ranges) respectively.

Reduction of inhibition effects against 45 LAB isolates seen with penicillin, nitrofurantoin and vancomycin respectively. Interestingly, lowest inhibition influences were recorded for amoxicillin, ceftriaxone, cefoxitin and nalidixic acid.

Thirty three out of 45 LAB isolates (73.3%) were classified as nonhemolytic (ɣ-hemolysis), while 26.7% reflected α-hemolytic activity as shown in Table 6.

Antibacterial activity of isolated LAB: Variable spectra of inhibition zones of LAB strains against the tested pathogenic bacteria. Thirty-eight (84.4%) out of 45 tested LAB strains showed antibacterial activity against all 4 tested bacteria. Varied antibacterial activity was recorded for LAB isolates against *Salmonella typhimurium* (ATTC 13311), with highest inhibition zones 20.0-30.0 mm for 19 LAB isolates. Against

Table 5: Bile salt tolerance 0.3% (w/v) bile (min)

Lactobacillus strains (LAB) isolates	Resistant (d≤15 min)	Tolerant (15 days 40 min)	Weekly tolerant (40 days 60 min)	Sensitive (days 60 min)
(LAB)1	-	-	+	-
(LAB)3	-	+	-	-
(LAB)4	-	-	+	-
(LAB)5	-	-	+	-
(LAB)8	+	-	-	-
(LAB)9	-	-	+	-
(LAB)11	-	-	+	-
(LAB)12	-	+	-	-
(LAB)13	-	-	-	+
(LAB)17	-	-	+	-
(LAB)18	-	-	+	-
(LAB)19	-	+	-	-
(LAB)20	-	-	+	-
(LAB)21	-	-	-	+
(LAB)22	-	+	-	-
(LAB)23	-	-	+	-
(LAB)28	-	-	+	-

-: No inhibition, +: Inhibition zone 8.0-10.0 mm

E. coli O157 (ATTC 700728) 13 LAB isolates showed highest inhibition zones ranging from 10.1-15.0 mm. Regarding *Staphylococcus aureus* (NCINB 50080), 26 LAB isolates expressed highest inhibition zones ranging from 10.1-15.0 mm. Twenty LAB isolates showed highest inhibition zones from 20.0-30.0 mm of against *Listeria monocytogenes* (ATTC19111) (Table 6).

DISCUSSION

The inhibitory effect of LAB with probiotic activity against pathogens causing food disease has recommended their use as an alternative to chemical drugs[15]. In the present study two isolates out of 45 LAB tested for acid tolerance at both pH 2.0 and 3.0 for 2 h had a very high survival rate of 94 and 96% respectively. Survival rates ≥90% to pH 2.0, 3.0 were found in 21, 19 LAB isolates respectively. The percentages of tested strains with survival rates ≥90% to pH 2.0, 3.0 were 46.6 and 42.2%, respectively. Tulumoglu *et al.*[16] observed closer percentage of 45%. Also Zhang *et al.*[4] who reported that 21, 17 strains had survival rates ≥90% to pH 2.0, 3.0 for 2 h respectively and lower percentages of tested strains with survival rates ≥90% to pH 2.0, 3.0 which were 30 and 25%, respectively. Similarly, Rajoka *et al.*[17] showed a survival rate above 80% at pH 2 for 3 h. Lower survival rates 55, 49, 65 and 57%, to pH 2.0 for 2 h demonstrated by Mourad and Nour-Eddine[18].

Bile salt tolerability is the criteria by which probiotic bacteria can survive within the intestinal conditions. An important probiotic criterion in order to one out of 28 acid tolerant LAB demonstrated resistant pattern for bile salt (d≤15 min). Similarly, Zhang *et al.*[4] considered only 2 out of 21 isolates to be bile resistant. Higher percentage of resistant isolates was recorded by Jacobsen *et al.*[19] where all the three studied lactic acid bacteria isolated from Ghanaian fermented maize were bile resistant.

The other bile tolerant patterns found in this study were; tolerant pattern (4 isolates) and bile weakly tolerant (10 isolates). Hyronimus *et al.*[20] reported higher percentage of tolerant LAB, where all studied strains isolated from cow excrement were bile tolerant. However more relevant results found by Zhang *et al.*[4] where 6 out of 21 LAB were tolerant strains and 13 out of 21 isolates were weekly tolerant.

Antibiotic resistance can be horizontally transferred from LAB, when used as probiotics, to pathogenic bacteria in the intestine[21]. In the present study, gentamicin, tobramycin and neomycin showed the highest inhibition effects on LAB. Penicillin, nitrofurantoin and vancomycin inhibited 85, 75 and 71% of tested 45 LAB isolates respectively. De Almeida Júnior *et al.*[22] and Dasen *et al.*[23] reported similar high sensitivity to vancomycin 84 and 100%, respectively. These results disagree with the concept of natural resistance of *Lactobacillus* against vancomycin[24].

Probiotics LAB possess a bacteriostasis role by inhibiting pathogenic bacteria and changing the distribution of bacterial community within intestine[16,25].Thirty-eight (84.4%) out of 45 tested LAB strains showed antibacterial activity against all four tested food pathogens. Tadesse *et al.*[26] and Bassyouni *et al.*[27] verified stronger antibacterial effects of LAB where all isolates showed antibacterial effects against *Salmonella typhimurium* and *E. coli*.

Considering LAB isolates identification, several molecular and phenotypic methods are available: Species-specific PCR reaction,16S rDNA sequencing, Biolog and API 50CHL. In this study, 10 selected LAB isolates out of 45 identified by API phenotypic method system were subjected to identification

Table 6: Antibiotic and antibacterial activities for LAB isolates

	No. of antibiotics sensitive to/10		Antibacterial activity			
			E. coli O157	*Salmonella typhimurium*	*Staphylococcus aureus*	*Listeria monocytogenes*
(LAB)1	6	ɣ-hemolysis	++	++++	+++	++++
(LAB)2	6	ɣ-hemolysis	++	++++	++	++++
(LAB)3	7	ɣ-hemolysis	+	++++	+++	++
(LAB)4	8	ɣ-hemolysis	+	++	+++	++++
(LAB)5	8	ɣ-hemolysis	+	+++	++	+++
(LAB)6	5	ɣ-hemolysis	+	+++	++	+++
(LAB)7	6	ɣ-hemolysis	++	+++	+++	++
(LAB)8	6	ɣ-hemolysis	+	+++	+++	+++
(LAB)9	9	ɣ-hemolysis	++	++	++	++++
(LAB)10	8	ɣ-hemolysis	++	+++	+++	++++
(LAB)11	7	ɣ-hemolysis	+	++++	+++	++
(LAB)12	6	ɣ-hemolysis	++	+++	++	+++
(LAB)13	6	ɣ-hemolysis	+	++++	++	+++
(LAB)14	5	ɣ-hemolysis	+	++++	+++	++++
(LAB)15	5	ɣ-hemolysis	+	++++	+++	++++
(LAB)16	4	ɣ-hemolysis	+	+++	+++	+++
(LAB)17	8	α-hemolysis	++	+++	++	++
(LAB)18	6	α-hemolysis	++	++++	+++	++++
(LAB)19	7	α-hemolysis	++	++	++	+++
(LAB)20	5	ɣ-hemolysis	-	+++	+++	+++
(LAB)21	6	ɣ-hemolysis	-	+++	++	++++
(LAB)22	8	ɣ-hemolysis	+	+++	++	+++
(LAB)23	8	ɣ-hemolysis	+	++	++	++
(LAB)24	6	ɣ-hemolysis	-	++++	+++	+++
(LAB)25	7	ɣ-hemolysis	-	+++	++	++++
(LAB)26	5	ɣ-hemolysis	++	+++	+++	+++
(LAB)27	6	ɣ-hemolysis	+	++	+++	++++
(LAB)28	7	ɣ-hemolysis	+	+++	+++	+++
(LAB)29	8	α-hemolysis	-	++++	++	++++
(LAB)30	7	α-hemolysis	+	++++	+++	++++
(LAB)31	7	ɣ-hemolysis	+	++	++	++++
(LAB)32	6	α-hemolysis	+	+++	+++	+++
(LAB)33	5	ɣ-hemolysis	+	++++	++	++
(LAB)34	5	ɣ-hemolysis	+	+++	+++	+++
(LAB)35	6	ɣ-hemolysis	++	++++	+++	++++
(LAB)36	6	α-hemolysis	-	++++	++	++
(LAB)37	6	ɣ-hemolysis	-	++++	+++	++++
(LAB)38	8	ɣ-hemolysis	+	++	++	+++
(LAB)39	7	ɣ-hemolysis	+	++++	+++	++++
(LAB)40	5	α-hemolysis	++	+++	++	+++
(LAB)41	7	α-hemolysis	+	+++	+++	+++
(LAB)42	5	α-hemolysis	+	++++	+++	++++
(LAB)43	6	α-hemolysis	+	++++	++	+++
(LAB)44	5	α-hemolysis	+	++	+++	++++
(LAB)45	6	α-hemolysis	++	++++	+++	++++

-: No inhibition, +: Inhibition zone 8.0-10.0 mm, ++: Inhibition zone 10.1-15.0 mm, +++: Inhibition zone 15.1-20.0 mm, ++++: Inhibition zone 20.0-30.0 mm

using molecular method of 16S rDNA sequencing. API results differed from the molecular reference method for the majority of the tested isolates: 9 (90%) out of 10 isolates showed non concomitant results when compared to 16S rDNA sequencing. Similar result was verified by Moraes *et al.*[28] where 86.2% of isolates showed high divergent results between these two methods. However other studies should compare different identification approaches of LAB using more numbers of LAB isolates. Some LAB isolates from Rayab milk in Egypt had potential probiotic activity, so it can be used to promote health and to combat food borne diseases. LAB identification to species level using conventional biochemical identification method including API systems may be not reliable enough, therefore better to use molecular identification and sequencing to identify LAB species. Limitation of the study was that it needs to compare more numbers of isolates using both biochemical and molecular identification.

CONCLUSION

Lactic acid bacteria can be used as probiotic supplements to treat infectious diarrheal diseases. Rayab milk consumed in lower Egypt is a source of plenty of lactic acid bacteria isolates that had probiotic activity. Use of biochemical method in identification of lactic acid bacteria gave inconsistent results with molecular gold standard method.

SIGNIFICANCE STATEMENT

This study discovered that LAB isolated from Rayab milk can be beneficial for use as probiotics to promote health. This study will help the researchers to uncover the critical areas of probiotic medicine that many researchers were not able to explore. Thus, a new theory to combat food borne diseases may be arrived at.

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