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Research Article
Molecular Characteristics and Antibacterial Efficacy of Traditional Dairy Isolates of Lactic Acid Bacteria

[1]Amel Ibrahim, [1]Sameh Awad, [2]Reham Madian, [3]Ahmed Noah Badr, [4,5,6]Jianquan Kan, [4,5,6]Muying Du and [1]Khaled Elsaadany

[1]Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt
[2]Department of Genetics, Faculty of Agriculture, Alexandria University, Alexandria, Egypt
[3]Department of Food Toxicology and Contaminants, National Research Centre, Dokki, Cairo 12622, Egypt
[4]College of Food Science, Southwest University, Chongqing 400715, China
[5]Chinese-Hungarian Cooperative Research Center for Food Science, Southwest University, Chongqing 400715, China
[6]Chongqing Key Laboratory of Specialty Food Co-Built by Sichuan and Chongqing, Chongqing 400715, China

Abstract

Background and Objective: Chemical preservatives can be harmful to people's health; their use is becoming less common, so scientists have advised using biological control, with the most important focus being on protective culture. This research aimed to isolate traditional lactic acid bacteria from local dairy products and assess their antibacterial efficacy against *Salmonella typhimurium*. **Materials and Methods:** From traditional Egyptian dairy products, lactic acid bacteria were isolated and characterized based on gram stain, shape and catalase reaction. Technological criteria were assessed, including their capacity to produce acid, autolyze and salt tolerance. The isolates' antibacterial activity against the *Salmonella* pathogen was evaluated. Each isolate's cell-free supernatant (CFS) as crude and after treatment with catalase to remove the effect of hydrogen peroxide and neutralize to pH 7 to remove the influence of organic acid were tested. The promising strains were identified by 16S rRNA. The obtained data was statistically analyzed. **Results:** The most cell-free supernatants had inhibitory activities. The strains with antibacterial activity were tested for safety properties (hemolysis and antibiotic resistance) and for some technological criteria, i.e., acid production, autolysis and flavor potential. Four promising strains (DMCR 309, DMCR 310, DMCR 316 and DMCR 320) were selected according to their technological activities and safety criteria, then molecularly characterized by 16S rRNA sequencing. Three strains, DMCR 309, 310 and 316, have been identified as *Limosilactobacillus fermentum*, while DMCR 320 was identified as *Levilactobacillus brevis*. **Conclusion:** The three *Limosilactobacillus fermentum* and *Levilactobacillus brevis* strains have promising applications for controlling pathogens and increasing dairy food safety characteristics.

Key words: Antibacterial efficacy, food safety, *Limosilactobacillus fermentum*, *Levilactobacillus brevis*, *Salmonella* pathogen

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Corresponding Author: Sameh Awad, Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt

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

INTRODUCTION

There is a global trend to reduce the use of chemical preservatives due to their risk to human health. Therefore, scientists have turned towards biological control, the most important of which is preventive microorganisms[1]. Lactic acid bacteria species are known for controlling a wide range of pathogenic bacteria, including *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus* coagulase-positive and *Escherichia coli*[2]. This LAB-controlling mechanism could be achieved through one of the following mechanisms illustrated in five visions. (I) Lactic acid, the main metabolic byproduct of fermentation, reduces the pH of a medium, making it difficult for *Salmonella* to grow[3]. (II) *Lactobacillus fermentum* can produce organic acids (acetic, lactic, citric and malic acid etc.), lowering pH, increasing the antimicrobial impact and interfering with the operation of bacterial cells[4]. (III) Bacteriocins, produced by *Lb. fermentum* strains, attach to the cell membranes of pathogens, causing cell lysis and cell death that reflects an antibacterial potency[5,6]. (IV) Hydrogen peroxide produced by certain *Lb. fermentum* strains can cause significant oxidative characteristics, leading to pathogen cell death by damaging its biological components such as proteins, lipids and DNA[7]. (V) *Lactobacillus fermentum* restricts the availability of pathogens' growth resources (*S. typhimurium*) by competing for vital nutrients[8]. (VI) To prevent adhesion or colonization of pathogens, *Lb. fermentum* competes with them in adhering to and occupying niches in the food matrix[9].

Lactobacillus species are known for controlling pathogenic bacteria, including *S. typhimurium*, in food products such as cheese. Using particular *Lactobacillus* strains as starter cultures during cheese-making can help guard against *S. typhimurium*. These cultures can be chosen because of their potent antibacterial action and capacity to flourish in the cheese environment[10]. Using *Lactobacillus* as an adjunct culture in addition to the primary starter culture can improve the safety and quality of the cheese. The microbial management strategy's efficacy and the finished product's safety are ensured by routinely testing and monitoring cheese for the presence of pathogens and advantageous *Lactobacillus* strains[11]. This research aimed to isolate and identify promising *Lactobacillus* strains from traditional Egyptian dairy products to control the pathogens in foods, help maintain the food quality and extend the shelf life.

MATERIALS AND METHODS

Study duration and location: The study was carried out at the Laboratory of Dairy Microorganisms and Cheese Research, Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University, Egypt from September, 2022 to June, 2024.

Sample collection: Forty-two samples of traditional Egyptian dairy products (Laban Zeer, Laban Rayeb, Damietta cheese, Karish cheese and Ras cheese) were collected aseptically from different locations in Egypt. Forty-two samples were collected, including 5 samples of Laban Zeer, 7 samples of Laban Rayeb, 12 samples of Damietta cheese, 12 samples of Karish cheese and 8 samples of Ras cheese. The samples were collected in an ice box and immediately transferred into the laboratory for microbiological study.

Lactic acid strain isolation: Three grams of each sample were cultivated in 30 mL of sterilized skim milk and incubated at specific temperatures (30°C for mesophilic LAB, 42°C for thermophilic LAB and 37°C for both types of the LAB). This incubation (4 hrs) aimed to induce coagulation and facilitate the LAB separation. Various media were used to streak the cultures, including de Man, Rogosa and Sharpe (MRS)[12] agar for *Lactobacilli* isolation, while *Streptococcus thermophilus* isolation Agar (ST agar) was used for isolating of *S. thermophilus*; however, *Enterococci* strains were isolated on the *Streptococcus faecalis* agar media (SF agar; 42°C/48 hrs). As described before, the phenotypic evaluations of isolated strains were examined[12].

Identification of lactic acid bacteria: Pre-identification testing included the catalase test growth at two different temperatures (10 and 45°C). The colonies obtained from MRS plates were transferred into MRS broth (Oxoid) and incubated (30°C/2 days). Cultures with a pH <4.0, Gram-stained and examined for catalase and oxidase activity using the techniques outlined in the practical handbook of microbiology[13]. The classification into phenotypic categories was based on physiological and biochemical parameters[14,15].

The isolates were cultivated using the MRS broth (Oxoid, at 30°C) until the optical density at 600 nm reached a range of 1.5-1.8. The DNA was then extracted[16,17]. Lactic acid bacteria were lysed using a T5 Direct PCR Kit (Beijing Tsingke Biotech Co., Ltd., China) and then the 16S rDNA gene was amplified with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') by Polymerase Chain Reaction (PCR). The PCR cycling conditions were as follows: 98°C for 3 min; 35 cycles of 98°C for 10 sec, 58°C for 10 sec and 72°C for 90 sec; 72°C for 5 min. The analyzed PCR products were sequenced at Beijing Tsingke Biotech Co., Ltd. (Beijing, China). Then, the basic local alignment search

tool (BLAST) was used to align and compare the 16S rDNA sequences with the GenBank database for homology analysis and the identification was constructed with the MEGA 11.0 software.

Acid-producing activity: The acidification activity was measured by monitoring the temporal variation in pH values (pH). During the early stationary growth phase, 2% of the culture was added to 50 mL of Reconstituted Skim Milk (RSM) and the mixture was then incubated. The pH after 0, 2, 4 and 6 hrs was monitored using a pH meter (Milwaukee Mi150, Italy). The change in pH (pH) was utilized to identify the acidification rate. The cultures were categorized as fast acidifying when the pH reached 0.4 U within 3 hrs. They were classed as medium acidifying if it took between 3 and 5 hrs. If it took more than 5 hrs, they were considered slow acidifying[18].

Hemolytic activity: Blood agar base 2 (Oxoide) plates containing 5% (v/v) human blood (EL-Shatby Hospital, Alexandria) were used to measure hemolytic activity. The plates were incubated at 30, 37 and 42°C for 48 hrs. Greenish colonies and a clear zone surrounding them were observed to record α- and β-hemolytic responses, respectively. Two assays were run for the experiment. As a β-hemolytic control strain, *Streptococcus pyogenes* MGAS 15252 was employed[19].

Flavor potential of selected safe strains: Reconstituted skimmed milk (10%) was pasteurized (65°C/30 min) and cooled to 37°C. A single active, tested strain that is safe was added at a level of 3% to pasteurized skimmed milk and then incubated at the ideal temperature until coagulation occurs at pH 4.6. The fermented milk is cooled to 4°C. Panelists from the Faculty of Agriculture, Alexandria University, including postgraduate students and staff members, assessed the final products for organoleptic characteristics (color, taste, smell, texture and appearance) and overall acceptability using a ten-point hedonic scale with excellent (score = 10) and extremely poor (score = 0). Additionally, the panelists were instructed to enumerate any flavor defects.

Screening of antibacterial cell-free supernatant (CFS) against *Salmonella*

Crude cell-free supernatants (CFS): The inhibitory effects of different LAB isolates against *Salmonella typhimurium* were carried out as described before[20]. The positive results were observed in the individual colonies with inhibition zones surrounding them. In the first stage, antibacterial activity was evaluated for the crude type of cell-free supernatant (CFS) extracted by centrifugation (9500 g/10 min/4°C). The results reflect the activity regarding all the CFS components that have antibacterial potency to suppress the pathogen strain of *S. typhimurium*.

Naturalized cell-free supernatants (CFS): In this step, authors were targeted to exclude the effect of organic acids, which may be produced from the applied strains and can interact with the antibacterial impact. Strains were cultured using MRS broth media at optimizing temperature for 24 hrs. To eliminate the antibacterial effect of organic acids, the CFS was extracted by centrifugation (9500 g/10 min/4°C) and the pH value was adjusted to pH 7.0 using 6 N NaOH.

Cell-free supernatants (CFS) treated by catalase: After the strains were cultured using MRS broth media at optimizing temperature for 24 hrs, it was centrifuged (9500 g/10 min/4°C) to collect the CFS. Next, catalase (1 mg/mL) was applied to exclude the hydrogen peroxide's inhibitory activity (Sigma, St. Louis, USA). Again, samples were heated (100°C/10 min) to suppress enzyme activity and to determine the highly stable bacteriocin-like[21].

Antibiotic susceptibility: According to Elsaadany *et al.*[22], the disc diffusion method was used to test antibiotic susceptibility. Ten antibiotic discs (Oxoid, Basingstoke, Hampshire, UK) were tested: Penicillin G (PEN, 10 µg), streptomycin (STR, 10 µg), tetracycline (TET, 30 µg), vancomycin (VAN, 30 µg), ampicillin (AMP, 10 µg), erythromycin (ERY, 15 µg), gentamicin (GEN, 15 µg), kanamycin (KAN, 30 µg), ampicillin (AMP, 10 µg) and clindamycin (CLI, 2 µg). Triplicate testing of each antibiotic was conducted. The discs were placed on MRS agar plates previously inoculated with the various test cultures. The diameters of inhibition were measured after the plates were incubated for 24 hrs at the optimum temperature. An effective agent inhibits bacterial growth and the size of the inhibition zones surrounding the discs is measured. The results were rated as resistant, susceptible and intermediate, respectively.

Measurement of the autolysis activity: The examined strains were activated in MRS and the pellet was harvested at the early stationary phase by centrifugation at 4000 g for 10 min at 4°C; the pellet was washed twice with a quarter strength of Ringer's solution. The pellet was re-suspended in potassium phosphate buffer (10 mL, pH 5.5) containing 1 mL NaCl and the O.D at 650 nm was adjusted using the same buffer to 1.0. The autolysis rate was determined using the methodology expounded by Ayad *et al.*[18]. After one round of freezing (-20°C for 24 hrs) and thawing, the cell suspension was incubated at 40°C. The percentage drop in absorbance at 650 nm across various time intervals was used to calculate the autolytic characteristics.

Statistical analysis: Three replications of each treatment were used in the Completely Randomized Design (CRD) experiment. All data were statistically assessed using the Analysis of Variance (ANOVA). Tukey's honestly significant difference (HSD) test was used as a *post hoc* analysis to compare means ($p<0.05$). The results were analyzed using the statistical technique of Principle Component Analysis (PCA)[23].

RESULTS

Isolation of lactic acid bacteria: The results showed that 141 colony isolates could be sourced from Egyptian dairy products. To be concentrated, 98 isolates are selected based on the colony shape during purification, optimum temperature, Gram stain and catalase production. The isolates, characterized as Gram-positive and catalase-negative, are potentially nominated as lactic acid bacteria. The percentage of rods to cocci in isolated lactic acid bacteria (LAB) strains was 47%, classified as *Lactobacillus* Fig. 1a. Oppositely, 53% of isolates are cocci classified as *Lactococcus*, *Enterococcus*, *Pediococcus* or *Streptococcus*.

Acidification activity: The obtained results revealed that the acidifying activity of *Lactobacillus* strains was significantly lower. The obtained results revealed that a low number of the *Lactobacillus* strains can be characterized as fast, as they didn't reach a pH of 0.4 before a time of 3 hrs of incubation at optimum growth temperature. The pH change (pH) reflects the acidification activity of isolates. The results in Fig. 1b show that most isolates (84.4%) are classified as slow acid producers, while only 2.08% were fast acid producers and 14% were medium acid producers. Figure 2a shows the changes in pH values (pH) during the storage time for selected strains encoded as DMCR 306 to DMCR 320. Most selected strains have low acid-producing activity, particularly DMCR 315 and DMCR 320. It is noticed that strains encoded as DMCR 312 and DMCR 316 produce the acids slightly more, which is reflected in rising pH, but they are still classified as low-producing acid strains. However, strains DMCR 315 and DMCR 320 are deficient in acid-producing capacity shown in Fig. 2a-b. With the same trend, strains DMCR 355 and DMCR 379 have high acid-producing capacity, while DMCR 365 and DMCR 396 have low acid-producing capacity shown in Fig. 2c-d.

Hemolytic findings: The hemolytic activity of isolated strains was assessed to evaluate their safety characteristics. The safety assessments expressed that some of the isolates are not safe. Table 1 summarizes the results of the safety features detected in the 33 strains of the isolated LAB. Ten of the investigated strains presented α-hemolysis and three others presented β-hemolysis. The three strains encoding DMCR 303, DMRC 304 and DMCR 354 are recorded as type β-hemolysis, classified as non-safe results and joined with pathogenic strains. These strains are isolated from cheese samples (303 and 304 are sourced from Karish and 354 are sourced from Mesh).

Moreover, the strains with γ-hemolysis results were also isolated from cheese samples. In this regard, further investigations are also needed to emphasize their safety for implementation in food and dairy products. Otherwise, all isolates sourced from milk are classified as γ-hemolysis-type bacteria (hemolysis-absence), which are considered safe. In sum, out of all isolated *Lactobacilli* strains, about 19 can be considered safe.

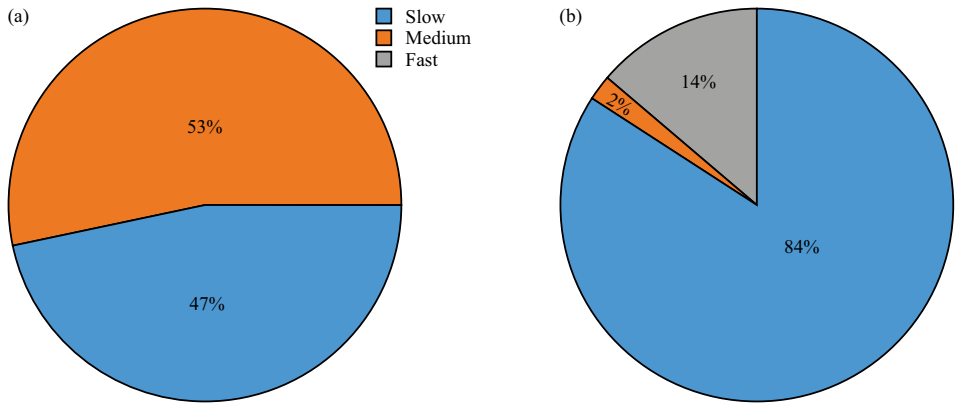

Fig. 1(a-b): Bacterial morphology and acid-producing activity of isolated strains from traditional dairy products, (a) Morphological shape of bacterial isolates and (b) Acidification activity

Isolate's ratio of rods (orange color) to cocci (blue color) represented *Lactobacillus* strains to other lactic acid bacterial types. Acidification activity of isolated strains that possessed antimicrobial activity

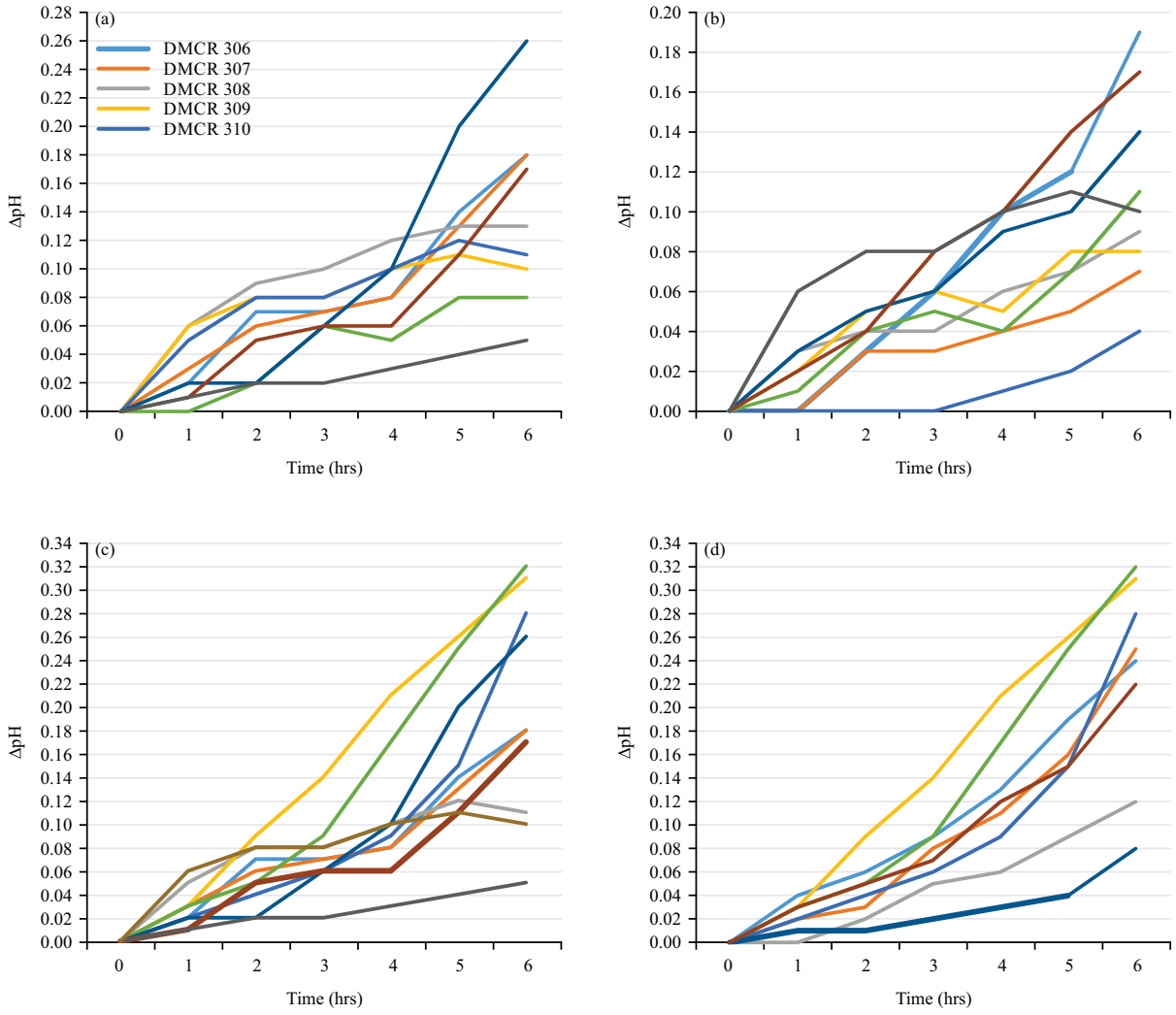

Fig. 2(a-d): Isolates selected strains assessment for their capacity of pH value changes and acid production, (a) pH values changes and acid production of strain number; DMCR 306, DMCR 307, DMCR 308, DMCR 309 and DMCR 310, (b) pH values changes and acid production of strain number; DMCR 316, DMCR 317, DMCR 318, DMCR 319and DMCR 320, (c) pH values changes and acid production of strain number; DMCR 341, DMCR 343, DMCR 354, DMCR 355 and DMCR 358 and (d) pH values changes and acid production of strain number; DMCR 367, DMCR 369, DMCR 378, DMCR 379 and DMCR 382

Cultures were classified as rapid, moderate and slow acidifying as a pH of 0.4 reached by 3, 3-5 or more than 5 hrs, respectively

Selected safe strains flavor potential: Since tested *Lactobacillus* strains were intended to be used for fermented or non-fermented dairy products, which are the most common probiotic foods, the ability of isolated strains to develop desirable aromas was evaluated in formed curds that objective of excluding the non-acceptable samples. Obtained results were presented in Table 1. According to sensory evaluation, most tested *Lactobacilli* strains produced pleasant aromas; only the non-pathogenic ones (negative hemolysis strains) were examined. Only 20 strains were selected for further evaluation based on the intensity of pleasant smell, which varied from low to high. However, strains with a low capacity to reduce acid or are recorded as low-acid producing and utilized in the curd flavor assay are considered the better strains with a preferable aroma. Out of 33 isolated strains, 20 strains are applied for flavoring acceptability by the panelist (Table 1). Six curds prepared using DMCR strains 309, 316, 318, 378, 382 and 316 have higher acceptability (at a degree of 8).

Table 1: Morphological characteristics, hemolysis and antibacterial activity of selected strains tested against *S. typhimurium*

Code	Isolation source	Morphology of strains/chain formation	Inhibition zone (mm)			Hemolysis activity	Flavor****
			Crude extract*	Neutralized** (pH 7)	Catalase treated***		
Cheese samples isolation							
303	Karish	Short rods/chains	20.20^{a}	15.15^{c}	17.19^{b}	β	NT
304	Karish	Short rods/chains	18.20^{a}	15.15^{b}	14.16^{c}	β	NT
305	Karish	Very short rods/short chains	19.20^{a}	16.13^{b}	16.17^{b}	γ	7
309	Mesh	Short rods/large-chain	16.15^{a}	13.16^{c}	15.19^{b}	γ	8
310	Mesh	Medium-rods/single	20.22^{a}	16.16^{c}	19.20^{b}	γ	6
311	Mesh	Medium rods/short-chain	19.19^{a}	15.14^{c}	18.22^{b}	α	NT
315	Karish	Very short rods/chains	20.22^{a}	11.13^{c}	17.18^{b}	γ	7
316	Karish	Medium rods-chains	20.20^{a}	15.15^{c}	19.21^{b}	γ	8
317	Karish	Short to long rods/short chains	21.20^{a}	16.17^{c}	18.15^{b}	α	NT
318	Pickle	Medium rods/single	17.19^{a}	13.13^{c}	16.15^{b}	γ	8
319	Pickle	Short rods/chain	17.16^{a}	13.15^{c}	16.18^{b}	γ	7
320	Karish	Very short rods/short chains	19.23^{a}	18.72^{a}	16.02^{b}	γ	7
321	Mesh	Short rods/short chains	17.16^{a}	16.16^{b}	17.18^{a}	γ	7
325	Karish	Very short rods/short chains	21.21^{a}	16.20^{b}	16.16^{b}	α	NT
329	Karish	Very short rods/long chains	22.18^{a}	16.15^{c}	19.18^{b}	α	NT
341	Karish	Short rods/chains	20.00^{a}	16.17^{c}	18.18^{b}	α	NT
345	Mesh	Short rods/chains	21.23^{a}	19.16^{c}	21.00^{a}	α	NT
346	Mesh	Very short rods/short chains	22.00^{a}	19.18^{b}	19.17^{b}	α	NT
348	Pickle	Medium rods/clusters	19.18^{a}	13.16^{b}	13.12^{b}	γ	7
349	Pickle	Very short rods/short chains	23.19^{a}	13.15^{c}	20.17^{b}	γ	7
351	Mesh	Short rods/chains	22.20^{a}	20.18^{b}	15.16^{c}	α	NT
352	Mesh	Short rods/chains	20.20^{a}	18.17^{b}	14.16^{c}	α	NT
353	Mesh	Short-medium rods/short chains	20.20^{a}	18.17^{b}	17.17^{c}	α	NT
354	Mesh	Long rods/single	19.16^{a}	16.17^{b}	16.17^{b}	β	NT
355	Karish	Very short rods/clusters	20.17^{a}	16.15^{b}	14.14^{c}	γ	6
358	Damietta	Short and medium rods/chains	17.16^{a}	16.15^{b}	15.15^{c}	γ	7
378	Low salt	Very short rods/single	21.17^{a}	18.14^{c}	16.15^{b}	γ	8
396	Mesh	Very short rods/short chains	18.18^{a}	17.18^{b}	14.12^{c}	γ	8
Milk samples isolation							
360	Buffalo	Short rods/single	20.16^{a}	17.13^{b}	15.14^{c}	γ	7
361	Buffalo	Very short rods/clusters	12.16^{a}	16.14^{b}	13.14^{c}	γ	7
364	Cow	Medium rods/chains	21.20^{a}	19.16^{b}	16.16^{c}	γ	7
365	Cow	Thin long rods/single	20.16^{a}	18.14^{b}	17.14^{c}	γ	7
382	Cow	Very short thick rods/chains	17.16^{a}	15.15^{b}	14.16^{c}	γ	8

*Crude extract of cell-free supernatant without any treatment, **Neutralized cell-free supernatant treated by NaOH and pH = 7, ***Cell-free supernatant treated by catalase to eliminate H_2O_2 effect, ****Flavor assay by panelists, where (NT) means not tested and $^{a-c}$Means within the same column with no common superscript differ ($p \leq 0.05$)

Assessment of the antibacterial activity: The data represented in the current study (Table 1) illustrate the main factor that controlled the antimicrobial activity of isolated *Lactobacilli* strains against pathogenic *S. typhimurium* ATTC 14028. Isolated strains of *Lactobacilli*, distinguished as short rods/chains, are recorded to have a significant capacity to achieve larger inhibition zones against *S. typhimurium* on the growth media. This previous step evaluated the crude bacterial extract (crude-CFS) as typical characteristics. However, neutralized CFS was also assessed against *S. typhimurium* after the pH value was adjusted to (pH = 7) using a solution of NaOH (6N). Thirty-three strains (91.42%) are recorded to have antimicrobial potency, with a clear inhibition zone formation around the well-diffusion. Again, the CFS was treated using catalase to eliminate the impact of hydrogen peroxide.

Thirteen isolates out of 33 have significantly higher activities after catalase treatment than adjusting the pH to 7. In comparison, 12 isolates out of 33 have significantly higher activities after adjusting the pH to 7 than catalase treatment. There were only 2 strains whose inhibitory activities were not significantly affected after treatment with catalase and there were no significant differences between the inhibitory activities of treatment with catalase or after adjusting the pH to 7.

Table 2: Antibiotic resistance (against 10 antibiotic compounds) and salt tolerance of isolated *Lactobacillus* strains from Egyptian dairy products

Isolate code	STR	CLI	AMP	GEN	VAN	CHL	PEN	KAN	TET	ERY	Salt (%)
305	0	28	38	19	0	30	21	0	31	25	w 6
309	0	30	32	16	0	32	31	10	27	30	6
310	8	27	37	15	0	24	34	7	26	25	-
315	7	30	32	11	9	27	20	0	20	26	6
316	0	36	30	18	0	30	23	0	20	26	8
318	9	29	33	13	0	32	26	6	22	27	w 6
319	9	21	30	16	0	30	24	10	20	26	w 4
320	7	33	37	16	0	31	26	7	23	23	4
321	0	23	30	13	0	25	23	0	21	22	4
331	7	31	34	12	20	28	28	0	26	28	w 8
335	11	37	37	12	0	28	26	0	26	26	w 6
339	0	23	37	19	0	22	31	0	21	24	w 8
340	9	30	32	17	0	27	31	7	21	23	4
343	0	27	32	15	0	25	25	0	22	25	8
354	0	31	39	17	0	24	20	0	22	30	6
355	0	15	35	21	0	31	35	0	29	33	w 4
358	8	32	44	20	0	31	32	0	24	28	w 6
360	8	27	37	14	0	25	30	0	22	27	6
361	14	34	45	18	0	27	29	0	25	31	4
364	0	20	25	15	0	22	22	0	30	27	4
365	15	32	42	43	0	34	33	9	30	33	4
366	16	31	37	16	0	22	27	0	29	30	4
378	7	33	39	21	0	32	36	0	25	31	6
379	0	24	34	19	0	28	29	0	21	29	6
391	20	34	42	19	0	33	36	0	28	29	6
394	14	10	35	14	0	27	27	0	23	30	6
396	14	10	35	14	0	27	29	0	22	27	w 8
398	14	27	36	18	0	31	26	0	20	25	8

STR: Streptomycin (10 µg), CLI: Clindamycin (2 µg), AMP: Ampicillin (10 µg), GEN: Gentamicin (15 µg), VAN: Vancomycin (30 µg), CHL: Chloramphenicol (30 µg), PEN: Penicillin G (10 µg), KAN: Kanamycin (30 µg), TET: Tetracycline (30 µg) and ERY: Erythromycin (15 µg)

Susceptibility of antibiotics: The antibiotic resistance (susceptibility) was investigated after the strains with α or β-hemolysis activity were excluded. The assessment includes an evaluation of the strains that possessed significant antibacterial activity against *S. typhimurium*. Most tested antibiotics are effective against investigated strains shown in Table 2, except for two that reflect no inhibition against several strains (kanamycin and vancomycin). It was reported that two strains (DMCR 315 and DMCR 331) were affected by the vancomycin treatment, where DMCR 331 was the more inhibited strain. Seven strains showed an inhibition response for the kanamycin antibiotic, with high inhibition zones regarding DMCR 309 and DMCR 319 strains.

Moreover, DMCR 365 and DMCR 391 strains are recorded with the highest inhibition zones against 2 antibiotics out of the applied 10 antibiotics. No strain was resistant to more than 3 of the 10 evaluated antibiotics. It was also noticed that 9 strains resist the antibiotic streptomycin.

Salt tolerance: The growth of strains was examined in the presence of salt in the range of 4-8%. The obtained data revealed that 10 strains are resistant in the presence of 4% salt, 12 strains are resistant in the presence of up to 6% salt and 5 strains are resistant in the presence of up to 8% salt (Table 2). It is essential to point out that the higher strains in salt tolerance were mostly sourced from cheese samples. The characteristic of salt tolerance is significant in dairy product manufacturing.

Autolysis: Current results for evaluating the autolysis activity of isolated strains reported a significant difference in autolytic activity among some isolates shown in Fig. 3. The results point out that, after 28 hrs of growth circulation of isolated strains, the autolysis range between 6.35 and 80.98. Strain DMCR 337 demonstrated a high rate of autolysis after 6 hrs of incubation, while the autolysis rates after 24-48 hrs were close and the difference between it and autolysis after 6 hrs was insignificant. Otherwise, some encoding DMCR isolates (such as 309, 316, 378 and 396) are shown to be not significant for the autolysis ratio after 24 and 48 hrs. Again, DMCR 311, 315, 350, 35, 367, 382 and 391 strains have low autolysis activity up to 48 hrs of incubation.

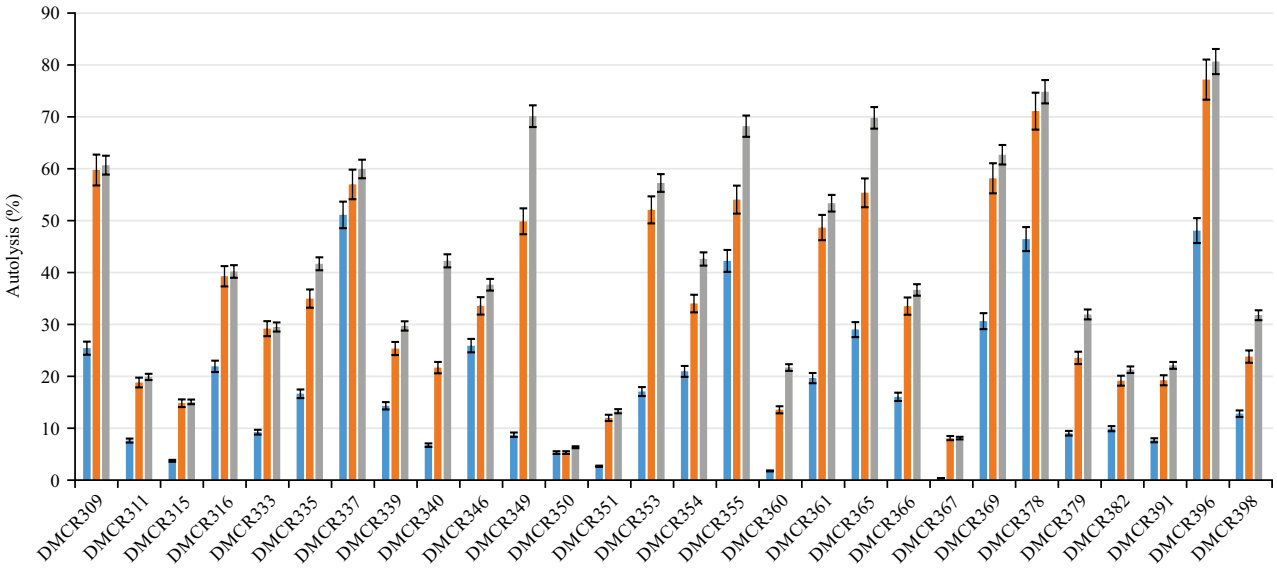

Fig. 3: Autolysis assessment of selected strains of isolated *Lactobacilli* sourced from Egyptian dairy products

Table 3: 16S rRNA identification of selected strains

Strain number	Identification
DMCR 309	*Limosilactobacillus fermentum*
DMCR 310	*Limosilactobacillus fermentum*
DMCR 316	*Limosilactobacillus fermentum*
DMCR 320	*Levilactobacillus brevis*
DMCR 329	*Lactiplantibacillus plantarum*

Molecular identification of selected strains by 16S rRNA: Four strains were selected based on the antibacterial activities against *Salmonella typhimurium*, safety criteria; hemolysis, antibiotic resistance, technological properties; acidity production, salt tolerance, autolysis and flavor potential, which were identified by 16S rRNA. Three strains, DMCR 309, DMCR 310 and DMCR 316, were identified as *Limosilactobacillus fermentum* (Table 3). The similarity of identification between the strains was 100%, but there was a morphological difference among the three strains (Table 1), as DMCR 309 was short rods, large-chain, DMCR 310 was medium rods, single, DMCR 316 was medium rods, chains. The strain DMCR 320 was identified as *Levilactobacillus brevis* (Table 3), its morphological characteristics were very short rods and short chains.

DISCUSSION

In the current study, 74% of isolates were rods, Gram-positive and catalase-negative, which were considered *Lactobacillus* spp., while 26% of isolates were cocci and considered *Lactococcus*, *Enterococcus*, *Pediococcus* or *Streptococcus*. Out of bacterial isolates, rod-shaped strains (*Lactobacilli*) are selected for the next experimental steps of the study. The results of isolated bacteria showed that most isolated *Lactobacilli* belong to *Lb. fermentum.* These strains include DMCR of 309, 310, 316, 320, 349, 378, 382 and 396. The selected strains were characterized to determine suitable future applications according to the determined characteristics.

The acidification activities of isolates play a good role in selecting the strain for a specific application. The fast-acid-producing strains are promising as primary starter cultures in fermented dairy products. In contrast, strains with poor acidifiers can be considered secondary cultures depending on their antimicrobial activities or flavor formation in cheese[18]. Most of the current isolate strains were classified as low acid producers; in the same way, Ayad *et al.*[18] found that most LAB isolates from the Egyptian environment were classified as low-acid producer strains of the LAB.

Previous isolations of lactic acid bacteria from Egyptian dairy products showed results consistent with those in the current study. Two percent of the *Lactococcus* strains that were isolated were rapidly acidifying strains. In contrast, more than 60% of the isolates were recorded as species of *L. lactis*, which had moderate activity regarding their ability to produce acid[18]. A study by Ayad *et al.*[18] revealed that the acidifying activity of several wild *Lactococci* strains is relatively low. Concerning the *Lactobacillus* isolates from Egyptian dairy milk, a ratio of 13% were classified as fast-acidifying strains. Isolated strains of that study were mainly *Lb. helveticus*, *Lb. delbrueckii* and *Lb. fermentum*[18]. Fast-acidifying strains are ideal candidates for the dairy fermentation process as key starting organisms. Conversely, poor-acidifying strains may be adjunct cultures concerning other significant characteristics, such as food safety applications and shelf life extensions[24].

Scientific knowledge regarding beneficial LAB has advanced significantly in selecting and characterizing new useful cultures, focusing on benefits to consumer health. Some researchers have already reported the absence of hemolytic activity in some LAB strains. Indeed, this characteristic is essential for beneficial and protected candidate strains for food application[25]. Similarly, El Attar[25] studied the hemolysis activity of locally isolated strains belonging to the *Enterococcus* genus to indicate pathogenic activity. El Attar's[25] results indicated that about 82% of the studied strains displayed γ-hemolysis, whereas only a small percentage (17%) produced α-hemolysin. Conversely, a single strain (1%) has the characteristics of β-hemolysis. The earlier studies conducted by de Vuyst *et al.*[26] observed a small proportion of *E. faecium* strains that exhibited β-hemolysis. The β-hemolytic *Enterococci* include strains that can cause disease in humans and animals. The present study attempted to investigate the hemolytic activity of isolated *Lactobacilli* on blood agar, with particular emphasis on these having negative hemolytic activity, for their applications in food safety applications. However, it is significant to note that this activity alone is not a reliable indicator of pathogenicity[25].

For customers, flavor and scent are the primary elements, with consistency, that have the utmost significance in food goods. The distinct taste of fermented dairy products is derived from various aromatic compounds that are either naturally present in raw milk or produced during fermentation. Various fermented dairy products exhibit unique taste qualities, even when comparable[27]. These differences are primarily due to the kind and amount of aroma chemicals present, significantly impacting customers' preferences[28].

The crude bacterial extract (crude-CFS) of 33 isolates had inhibition activity against *S. typhimurium*. In comparison, only 12 isolates have higher activities after neutralizing CFS to pH 7 and then catalase treatment. The neutralizing step is targeted to eliminate any affection for produced organic acids regarding the inhibition zone. In this regard and after the elimination of H_2O_2 and the impact of organic acids, the antibacterial potency seemed to link with amino acids or peptide fractions that could be present in the CFS. Cured cell-free supernatants of most isolates with antimicrobial activities were also active when the pH was adjusted to 7 or after treatment with catalase. This suggested that the antimicrobial components are not only organic acids and/or hydrogen peroxides but could also include other antimicrobial components. In the same manner, the CFS of the *Lactobacillus* strain isolated from Rayeb milk was reported with significant antimicrobial activity and it contained bacteriocin-like substances[29,30].

All the strains, except for 2, have a reduction in their inhibitory activities after either catalase treatment or adjusting the pH to 7. This suggested that the organic acids and/or hydrogen peroxides had been produced by some strains and had antimicrobial activities of the isolates LAB[4,31,32]. Still, inhibitory activities did not refer only to these two components.

Similarly, these strains also have antimicrobial activity after eliminating the hydrogen peroxide impact, as they were being treated with catalase. The results support the probability of production of bacteriocin-like from these strains that possess a positive antibacterial impact. In this regard, using the LAB is well-known to guarantee food safety. It was pointed out that LAB has been assessed in several studies as a bio-preservative agent[33,34] for achieving food safety and extending final product shelf life. According to Reis *et al.*[35] and Ünlü *et al.*[36], certain LAB strains can produce antimicrobial compounds such as organic acids, hydrogen peroxide and bacteriocins that can stop the growth of pathogenic and spoilage bacteria.

Regarding the previous investigations, bacteriocins are called bacteriocin-like inhibitory substances (BLIS) when their molecular weight and amino acid sequences are not thoroughly described by Jawan *et al.*[37] and Sidek *et al.*[38], which the authors can utilize to identify and complete the current investigation. However, the fractions of amino acids and small peptides are mentioned as bacteriocin-like inhibitory substances (BLIS) when their molecular weight and amino acid sequences are not thoroughly described by researchers[37,38]. According to Alvarez-Sieiro *et al.*[39] and Piazentin *et al.*[40], bacteriocins and BLIS are peptides or proteins that are produced by ribosomes and excreted in the extracellular environment.

Except for two (vancomycin and kanamycin), which show no inhibition against several strains, most tested antibiotics are effective against the strains under this investigation. It was also noticed that no multiple drug resistance was recorded in most of the tested investigated strains, which is preferred for nomination to apply in safe food production. Several investigations reveal that some *Lactobacillus* species members might create issues including bacteremia, endocarditis and peritonitis, especially in people with a history of pathological illnesses, even though they are considered safe germs. In other situations, one should consider the risk posed by the virulence genes, antibiotic resistance and their possible transmission to harmful microbes. As a result, there have been occasions when their safety has been called into question. For this reason, it is crucial to conduct the necessary evaluations before using them in any capacity[41].

Generally, LABs are needed to ferment cheese, yogurt and milk. LAB produces lactic acid, which lowers product pH, to reduce spoilage and harmful microbes. It also develops fermented dairy products' tastes and aromas. Again, the LAB affects fermented dairy texture and consistency. They help cheese and yogurt coagulate milk proteins. Meanwhile, salt enhances the flavor of several dairy products.

On the other hand, salt-tolerant goods retain texture and consistency. It also controls moisture and hardness in cheese-making. Salt-tolerant lactic acid bacteria are significant for producing salted dairy food's quality, safety, taste, texture and nutritional value, especially those with high salt content. It can survive, populate and exercise more activities to enhance the product quality. Cheese and butter include salt as a preservative. Even when salt is introduced, salt-tolerant LAB maintains similar textural features. With salt tolerance, the product may taste well without sacrificing quality. Also, salt-tolerant LAB protects dairy products even at greater salt levels, improving safety and shelf life. The LAB's presence in dairy products will support the production of vitamins, bioactive peptides and other valuable substances that boost dairy product nutrition, whereas the application of salt-tolerant strains in salted dairy products like cheese can maintain these advantages[42].

It is well known that LAB autolysis contributes to releasing intracellular enzymes and other compounds, which play a crucial role in the ripening process, flavor development and texture modification of cheese. The LAB autolysis can also influence the development of desirable sensory attributes such as creaminess and complexity. The breakdown of LAB cells through autolysis can release bioactive compounds that have health-promoting effects, such as antimicrobial peptides, which can contribute to the safety and shelf-life of the product[18]. Moreover, autolysis leads to the release of nutrients such as peptides, amino acids and vitamins, which can be beneficial for the growth of other microorganisms or for enhancing the nutritional value of the fermented product. Autolysis also manages microbial viability during dairy storage and its functional properties when consumed. Understanding autolysis can help design better formulations and storage conditions to maintain the efficacy of probiotic products[43].

Three strains were selected and identified as *Limosilactobacillus fermentum*; this strain is a significant lactic acid bacterium that exhibits some probiotic properties[44]. It is used as a starter culture for food fermentation and is recognized as safe[45]. In recent years, extensive study has been conducted on the bacterium and its probiotic potentials[44,45]. The strains of this LAB provide probiotic benefits like anti-infectious, anti-inflammatory, immunomodulatory, pro-longevity and damage prevention actions, in addition to technological benefits like improved food product flavor and texture[44,45]. Additionally, the bacteria showed good gut mucosal adhesion ability[46]. However, differences were noted between strains in the morphological characteristics. There was also one strain that had good technological characteristics and safety and was identified as *Levilactobacillus brevis* (DMCR 320). *Levilactobacillus brevis* HL6 demonstrated resilience to low pH levels, bile salts and gastrointestinal conditions and it was recommended to be used as probiotic culture[47].

CONCLUSION

More than 141 colonies recovered from market-handled dairy products are *Lactobacilli*, some linked to *Limosilactobacillus fermentum*. Some strains were antibacterial against dangerous *S. typhimurium* strains. The antimicrobial capability of the crude neutralized and catalase-treated CFSs indicates BLIS's existence. Most chosen LAB isolates produced modest amounts of acid, which is advantageous for dairy products (with little impact on acceptability). Results suggest more than 20 strains are suitable for autolysis and antibiotic susceptibility. Again, 20 strains had negative hemolysis, indicating safety. Eight strains taste well after being applied in simulated curd.

This study recommended that some of the promised strains have good safety criteria and technological properties that can be applied in the production of low-salt soft cheese, hard cheese and yogurt to improve the quality and increase the shelf life of final products.

SIGNIFICANCE STATEMENT

Research on isolating wild strains from natural environments and fermented foods has identified several lactic acid bacteria with notable traits, including salt tolerance, acidity production, flavor enhancement and anti-salmonella activity. Four strains with strong safety profiles were identified, offering potential for use in food protection and flavor improvement. Future work will focus on applying these strains in dairy products to enhance shelf life, quality and safety. This highlights their promise in biological protection and food innovation.

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