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Original article

Characterization and evaluation of probiotic strains isolated from different sources

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ABSTRACT

Background: A wide set of safe, industrially important microorganisms known as lactic acid bacteria are mostly utilized as probiotics and starting cultures, but they are also being explored as production systems in industrial biotechnology. The aim of this study was to isolate, identify and characterize lactic acid bacterial (LAB) isolates from dairy products and pickles samples, and evaluate their antimicrobial activity against some pathogenic organisms. **Methods:** Isolates were identified based on morphology, physiology, and biochemical tests. Further analysis of these isolates revealed that they were characterized based on their ability to grow at different pH, temperature, their ability to produce acids, and their tolerance to bile salts. The antibiotic resistance of all the isolates were determined following the method of Kirby Bauer disk diffusion assay. In vitro assessment of antimicrobial activity against pathogenic organisms was also tested. Morphological, physiological, and biochemical identification suggest that 16 of the isolates belong to lactic acid bacteria. The ability of the isolates to grow in different pH and temperatures, to produce acid, and to tolerate bile salts was observed. **Results and Conclusion:** The isolates were resistant to all antibiotics used. The two most active isolates selected showed their potential probiotic traits and demonstrated in vitro antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*.

Introduction

A varied collection of safe microorganisms of industrial significance are lactic acid bacteria. They are frequently employed as probiotics and starting cultures, and they are also being investigated for the potential of bio catalysis to transform renewable feedstocks into useful compounds and pharmaceuticals [1]. In addition to being utilized as feed for animals in silage, LAB is also employed as a culture starter for the fermentation of milk, meat, vegetables, grains, and fish. The most significant items for the economy are the fermented dairy products. Exopolysaccharides, polyols, organic acids, aromatic compounds, and bacteriocins are among the well-known characteristics of LAB that are used to create functional starting cultures. These chemicals are released into the food to give it better qualities with regard to health impacts, shelf life, flavor, texture, and odor [2] .

Inhibiting the growth of pathogen is essential for the manufacture of fermented foods, and this is made possible by the LAB properties of growth, metabolism, nutritional requirements, and generation of antimicrobial chemicals. Making fermented foods requires inhibiting the growth of pathogens and spoilage organisms, which is accomplished by LAB features including nutritional

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requirements, growth, the generation of antimicrobial chemicals, and metabolism [3]. In industrial environments and the gastrointestinal tract, these bacteria use a variety of tactics to combat environmental challenges. These tactics include controlling cellular metabolism, preserving the functioning of cell membranes, expressing stress response proteins, and generating exopolysaccharides [4]. Although the majority of Bifidobacterium species and LAB are considered safe (GRAS) microorganisms, they still need to be tested for antibiotics resistance, hemolytic activity, specific metabolic activities (like bile salt breakdown), and epidemiological surveillance for negative consumer effects. Probiotics also need to make it through the gastrointestinal tract and maybe adhere to the gut's epithelial tissue or form co-aggregations with bacteria in order to continue existing. Utilizing in vitro intestinal models makes it simple to study the adhesion properties, which are thought to be essential for the selection of novel probiotics [5].

The main source of new probiotic bacteria was previously thought to be the gastrointestinal tract (GIT), however attention is now being drawn to the microorganisms found in conventional fermented foods including dairy and fermented vegetables. Because of the variety of native microflora found in fermented vegetables, including different species of Lactobacillus, research on these foods has become more focused. Thus, In order to find lactic acid bacteria with probiotic qualities like acid tolerance, antimicrobial activity, bile salt tolerance, and stability, this study screened a variety of fermented dairy products from Mosul City, Iraq. The ability of potential probiotic bacteria to produce lactic acid during growth was also assessed.

Material and Methods

Sampling

This research was conducted from N 2022 to M 2023 to isolate and characterize LAB from milk and milk products in Mosul/Iraq, samples of dairy products and pickles were gathered from different farms of Mosul city. These samples included raw buffalo milk, yoghurt made from raw buffalo milk, raw goat milk, raw sheep milk, kiri cheese, cheese (Arab), and pickled vegetables.

Procedures for Collecting and Handling Samples

Totally, eight samples of 300–500 ml of raw milk and milk products samples were obtained in a hygienic manner, preserved in airtight containers, and collected from individual households, dairy farms, milk vending shops, and supermarkets by using sterile glass bottles, and 4 pickle samples from local markets. Then, the samples were stored at 4°C before the dilution procedure, as seen in Figures 1(a & b), and the samples were cultivated bacteriologically.

Isolation and Identification of LAB

Distilled water was used to serially dilute each sample. To create a microbiological suspension, 1 milliliter of a stock solution was suspended in 9 milliliters of distilled water and shaken. 10-1, 10-2, 10-3, 10-4, 10-5, and 10-6 were serially diluted by pipetting 1 milliliter into 9 milliliters of distilled water. 0.2 ml of the dilution was spread on the DeMan, Rogosa, and Sharpe (MRS), pH=5.4, and prepared tomato juice agar (Distilled water 200 ml + Nutrient agar (6g)+ Tomatoes Filtrate (40 ml)+ Glucose (2g)) with pH=4.5 [5]. Plates were kept in microaerophilic (using an anaerobic jar) conditions for 48–72 hours at 37°C. Subcultures are made after the incubation period, the colonies have been taken from the cultures by streaking on MRS agar media and incubated at 37°C for 48-72 hrs under microaerophilic conditions. At the end of incubation, the LAB were selected based on their morphology and selective media such as bio-chemical profiles and colony characteristics.

Morphological and Biochemical Characterization

Morphology

MRS (Man Rogosa and Sharpe) agar [6] and tomato juice agar [7] are the two distinct media that have been employed. The morphological properties of the colony showed similarities with the features of lactic acid bacteria generally. These attributes were used to obtain lab isolates [8].

Identification of lactobacillus strains

Named A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, these isolates seemed to be lactobacilli. They were isolated from MRS agar and Tomato Juice agar and sub-cultured twice, for 48 to 72 hours, at 37 ºC in microaerophilic. Gram staining and catalase tests were carried out in accordance with the available standard methods to confirm and

identify the isolated bacteria [9].Catalase-negative and gram-positive colonies were taken into consideration for further testing.

Acid-Producing Lactic Acid Bacteria (LAB) Selection

On MRS agar medium, acid-producing LAB was isolated by adding 0.3%, 0.5%, and 1% CaCO3 [8]. Two distinct temperatures were used to incubate the plates that were infected by LAB isolates: 37°C for 24 hours and 27°C for 48–60 hours. The development of clear zones around the growing region in the medium is a characteristic of the LAB isolates that possess the potential to produce acid.

Sensitivity to Temperature

The chosen isolates were cultivated for 48–72 hours at three different temperatures: 27°C, 37°C, and 4°C. Temperature-tolerant isolates were identified by the development of LAB on MRS agar plates [10].

Bile salt tolerance

Bile salt (0.3%) by 0.1 cc were added to the MRS broth medium and left for 24 hours. Seven isolates total, from each sample, MRS suspension broth, and 48 hours of 37°C incubation were required. After that, 0.1 ml of the inoculum was added to the agar and left to incubate for 48 hours at 37°C. The process of identifying isolates as bile salt tolerant included the development of LAB cultures on agar [10].

Antibiotic Susceptibility Test

The disk diffusion assay technique was used to perform the antibiotic susceptibility test. Twelve antibiotic discs were carefully put on the agar plates after the newly formed after 24 h, the bacterial isolates were spread out on MRS. Subsequently, the agar plates were incubated for 48 hours at 37°C. Gentamycin (10 µg), cefixime (5 µg), ceftriaxone (10 µg), cephalothin (30 µg), ampicillin (10 µg), streptomycin (25 µg), ceprofloxacin (10 µg), tetracycline (10 µg), nalidixic acid (30 µg), ceftazdime (10 µg), rifampin (30 µg), and nitrofurantoin (100 µg) were among the items present in the antibiotic discs. Lastly, the data was recorded using the rules for reporting established by the Clinical and Laboratory Standards Institute (CLSI) [11].

Antibacterial activity

Salmonella enterica antibiotic activity in vitro Encephalopathy, *Escherichia coli* (O157:H7), *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida cruise* were assessed against the lactic acid isolates. Depending on agar overlay assay, the most active isolates were chosen, and ten microliters of lactic acid bacteria were positioned in the middle of nutrient agar plates. Semisolid Nutrient agar (soft layer) with pathogens isolates were applied on top of the plated samples after a 24-hour incubation period at 37°C, the favorable outcomes were those plates that showed no development on the top of the soft layer [12].

Results and Discussion

Sampling and isolation of LABs

Sixteen LAB isolates were recovered: two from buffalo milk, two from goat milk, two from sheep milk, two from kiri cheese, two from cheese (arab), two from pickles, and three isolates were from buffalo yoghurt. The findings demonstrated differences in growth density between mediums. **(figure 1).**

We have evaluated the degree of development based on these differences among mediums. Compared to other medium, MRS agar is more selective for LAB, resulting in isolates that are clearer and more pure colonies.

Subcultures were then made from each plate using MRS agar medium alone, and the outcomes revealed differences in the isolates' growth densities (figure 2).

Morphological and Biochemical Characterization

All of the isolates were catalase-negative and Gram-positive, as shown in figure (3) and table (1). All of the isolated bacterial cultures used in this investigation are Gram positive, mostly rod-shaped cells, non-endospore forming, catalase negative, and acid generating, all of which are characteristics of the genus Lactobacillus. Furthermore, the shape of the colony revealed similarities with the features of lactic acid bacteria in general [10].

sensitivity to temperature:

Most of the isolates were able to grow at 37 and 27 °C on MRS agar and tomato juice agar media under microaerophilic conditions, while three strains were not able to grow at 27 °C on MRS agar. All isolates showed survival at refrigeration temperature (4°C), this indicated that they could be the best cultures to use in fermented dairy items that are refrigerated.

Acid- production assay

According to the results, 14 isolates were shown to be capable of producing acid (table 2). The

clear zone showed that LAB is capable of using glucose as an energy source to create acids, which are secondary metabolites. Clear zones appear surrounding LAB growth, indicating that acidic chemicals from LAB may degrade CaCO3 to Ca lactate [13]. Different isolates showed different sizes of clear zones.

The acid production ability showed variations in different temperatures. Since in the 37°C the clear zones were larger than at 27°C and even more clearly, also at 37°C appeared rapidly (after 24 h) while at 27°C taken longer time. Some isolates DO NOT produce acid (have no clear zones around the growth area) depending on the temperature. The study also noticed that the isolates (which incubated at 27°C) showed clear zones around the growth area when we put them in refrigerator temperature for one month (figure 4).

However, the acid production ability showed variations in different concentrations. The clear zones being larger whenever the concentration of CaCO3 is low, since the acid production ability in 0.3% $CaCO_3$ is more clear than 0.5 and 1%.(Figure 5).

The Lactobacillus species differed in terms of growth and acid generation. These results indicated that the various Lactobacillus species displayed different abilities to produce acid, dividing the species into three categories: very, somewhat, and weakly acidogenic. Goat, sheep, and buffalo milk all shown substantial acidogenic properties; pickles, cheese (arab), yogurt, and Kiri cheese, on the other hand, exhibited just mild acidogenic properties.

Bacterial lactic acid is considered the main source of industrial lactic acid (LA) production. LA is produced through glycolysis under anaerobic conditions, and also through LAB metabolic pathways for hexoses and pentoses. Factors such as temperature (5–45 °C), pH (3–9) , the specific LAB strains used, and the availability of nutrients (like nucleotide, vitamin, and peptide) influence the production and efficiency of LA synthesis. Various genera including Streptococcus, Vagococcus, Aerococcus, Carnobacterium, Leuconostoc, Lactococcus, Weissella, Pediococcus, Enterococcus, Tetragenococcus, Oenococcus, and Lactobacillus have been utilized in this process [14].

Bile salt tolerance

It has long been believed that resistance to bile salts is a need for bacterial colonization and metabolic activity in the host's gut. Bile salts have the potential to influence the intestinal microbiota by functioning as antimicrobial agents [15]. Therefore, it's typically crucial to assess LAB's tolerance to bile salts when assessing their potential utility as a probiotic [16]. The findings of the isolates' assessed bile tolerance are shown in figure 6 and Table 3 of our investigation. After 48 hours of incubation, all LAB isolates could grow when cultivated at 0.3% concentrations of bile salt. In the small intestine, Bile salt concentrations typically vary from 0.2% to 0.3%, and depending on the individual and the kind and amount of food they eat, they may even reach 2% (w/v) [17]. According to [18] , The LAB species and the strains themselves differ greatly in their capacity to withstand bile salts. The enzyme activity of bile salt hydrolase (BSH), which aids in hydrolyzing conjugated bile and lessening its impact, is linked to bile resistance in some isolates [19].

Antibiotic susceptibility test

As shown in figure 7, the findings demonstrated that all isolates were resistant in (100%) to every antibiotic that was chosen. Such resistance to a broad range of medicines suggested that isolated probiotics given to patients receiving antibiotic treatment may aid in the patients' quicker recovery since they will quickly develop a favorable microbial flora. Probiotic strains that are resistant to certain antibiotics may be used to treat or prevent intestinal infections [20].In contrast, the isolates in [21]work were sensitive to ampicillin, and some of them were also responsive to ciprofloxacin, rifampin, and gentamycin. The isolates in the current investigation were resistant to all four of these drugs. This might change depending on the strain, its kind, and its suppliers.

Preventing the transfer of antibiotic-resistant traits from probiotics to pathogenic bacteria requires a multifaceted approach, including rigorous screening, genetic modifications, regulatory measures, and innovative technological interventions. By implementing these strategies, the health benefits of probiotics can be harnessed while minimizing the risk of contributing to antibiotic resistance.

Therefore, the presence of resistance genes in LAB does not necessarily mean a high risk, but it requires careful study and understanding of the possible mechanisms of their transmission.

Antibiotic resistance genes may be located on the chromosome or plasmid within bacteria, including lactic acid bacteria (LAB). Antibiotic resistance genes located on the chromosome are fixed and not easily transmitted because they are not designed for horizontal transfer [22].

In short, the lack of transmission of resistance genes is due to the absence of appropriate mechanisms, the genetic location of these genes, the unsuitable environment for gene transfer, in addition to human interventions to select safe strains for use.

Antimicrobial activity

According to our findings, there was no development of some harmful bacteria on the plate's top layer. *S. enterica* and *C. cruise* were not affected by the lactic acid bacteria isolate (O), which exhibited antibacterial action against *E. coli*, *S. aureus*, and *K. pneumoniae*. While the other isolate (E) showed antibacterial activity against all selected pathogenic (figure 8).

As mentioned before, the accumulation of metabolites such lactic acid, ethanol, and carbon dioxide as well as the production of extra antimicrobial compounds like bacteriocins are the main processes behind the inhibitory effect of LAB [23]. The biochemical characteristics of the strains used, as well as the physical and chemical growing conditions, determine the amounts and quantities of these chemicals produced [24]. For a very long time, scientists have been trying to substitute natural items for manufactured drugs [25]. These days, a wide range of natural remedies and methods are used to treat or prevent diseases [26]. Using probiotics is one of them. Lactobacilli and bifidobacteria are examples of normal intestinal flora that are essential for human health because they lower cholesterol, guard against intestinal infections, bolster the immune system and reduce the likelihood of colon cancer. Probiotics work to inhibit the growth of several bacteria, produce organic and lactic acids, and decrease the pH of the surrounding environment. According to Aroutcheva et al. [27], these bacteria create antimicrobial substances such bacteriocin, which may be employed as natural preservatives. Compared to antibiotics, bacteriocins are protein compounds that have the capacity to suppress the development of sensitive pathogenic bacteria and have a distinct mechanism of breakdown in the digestive system [28]. It was discovered in this investigation that the metabolites of these bacteria created might stop the development of the harmful bacteria.

Table 1. Morphological and biochemical characteristics of isolated LABs.

Sample Type	Isolates	Culture characteristic	Gram stain	Morphological characteristic	Catalase Test	Growing at different temperatures 37°	27°
Yoghurt Sample	A	Colonies round, creamy,smooth, white , flat and entire	+	Purple, rod	-	+	-
	B		+	Purple, staph. Purple, rod	Weak	Weak	
	C		+		-	+	+
Kiri sample	D	Colonies small round, creamy, smooth, white , flat and entire	+	Purple, bacillus.	Weak	Weak	
	E		+	Purple, rod, mostly single	-	+	+
Goat milk	F	Large colonies round,	+	Purple, rod, chain	-	+	+
	G		+	Purple, rod	-	+	+
Buffalo milk	H	Colonies round, creamy, smooth, flat and entire, in (H) are small & opaque, while in (I) are larger & white	+	Purple, rod, chain	-	+	+
	I		+	Purple, rod, chain	-	+	+
Sheep milk	J	Colonies round, creamy,smooth,white , flat and entire, in (K&L) are small , while in (J) are larger	+	Purple, coccobacillary , mostly single	-	+	+
	K		+		-	+	+
	L		+	Purple, rod,single	-	+	-
Pickles sample	M	Colonies round,	+	Purple, irregular	-	+	+

	N	creamy,smooth, flat and entire, in (M) are small & opaque, while in (N) are larger & white	+	Purple, cocci, strept	-	+	-
Cheese (arab)	O	Small colonies round, creamy,smooth, entire, and opaque	+	Purple,coccobacillary, chain.	-	+	+
	P		+	Purple, single, filament-shape	-	+	-

Table 2. The variations in acid production ability depending on the concentration.

Isolates	**Acid production ability** **0.3 0.5% 1%**	**Isolates**	**Acid production ability** **0.3% 0.5% 1%**
A	++ + ++	**H**	++++ ++++ +
B	**Weak**	**I**	++++ ++++ /
C	++++ +++ +		
D	**Weak**	**J**	++++ +++ /
			+++ ++++ ++
E	+++ ++ /	**K**	++++ +++ /
		P	
F	+++ ++ /	**L**	+++ ++++ /
G	++++ ++++ /	**M**	+++ ++ /
N	+++ ++ +	**O**	++++ ++++ /

***++ weak acidogenic, +++ moderate acidogenic, ++++ strong acidogenic, / No produce.**

Table 3. Shows the results of bile tolerance of the isolates evaluated.

Isolate	**Growth density**	**Isolate**	**Growth density**
C	++++	**K**	++
E	++	**L**	++
G	++++	**O**	+++
I	++++		

Figure 1. a) Glass can be used to store milk., b) serial dilutions that made from the stock solution.

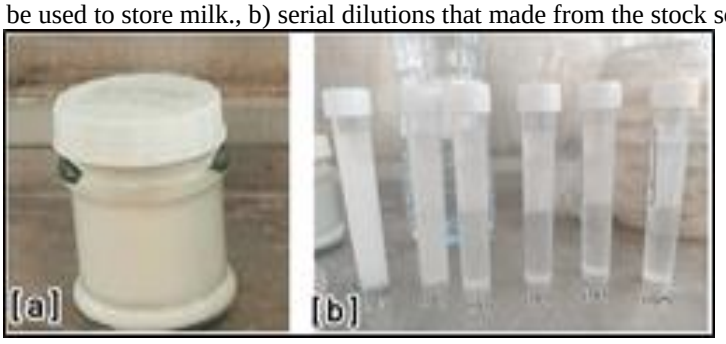
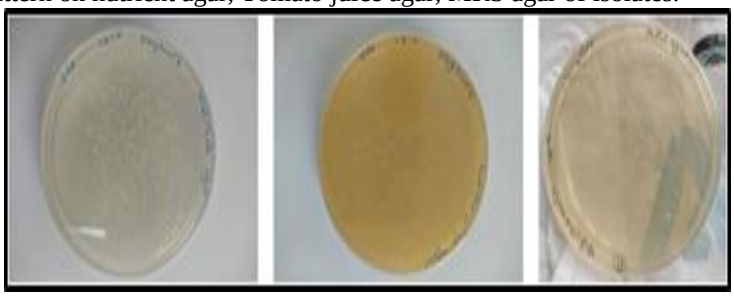


Figure 2. Growth pattern on nutrient agar, Tomato juice agar, MRS agar of isolates.



a) Nutrient agar b) Tomato juice agar c) MRS agar

Figure 3. Showed variations in growth density between isolates

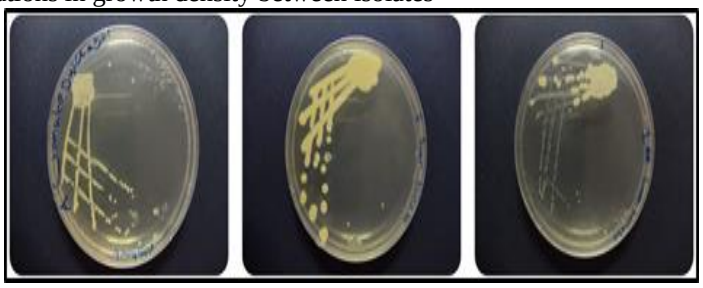


Figure 4. Gram stain and cell shape of LAB isolates: cells under light microscopic at a power of 100x magnification.

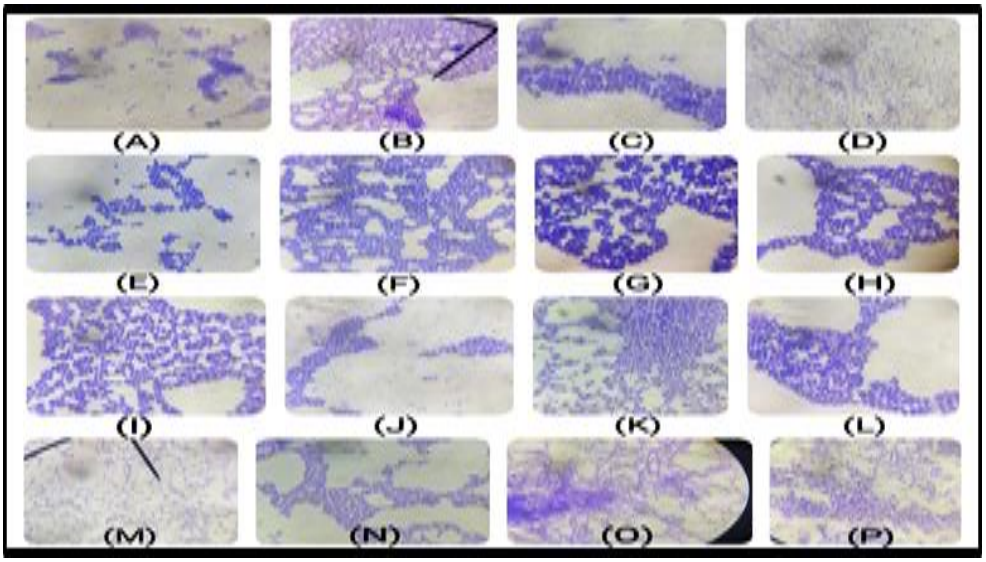


Figure 5. Showing the acid production ability of isolates at different temperatures.

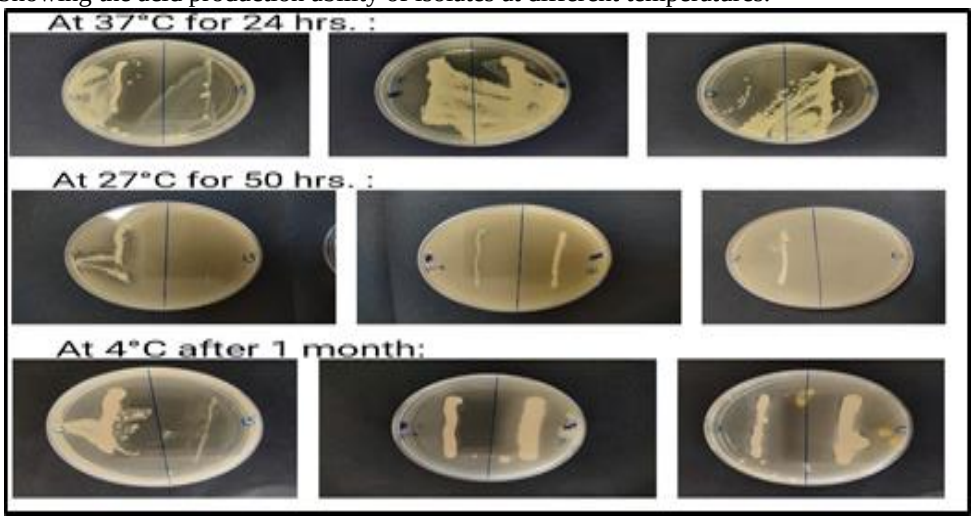


Figure 6. Showing the acid production ability in different concentrations of $CaCO_3$.

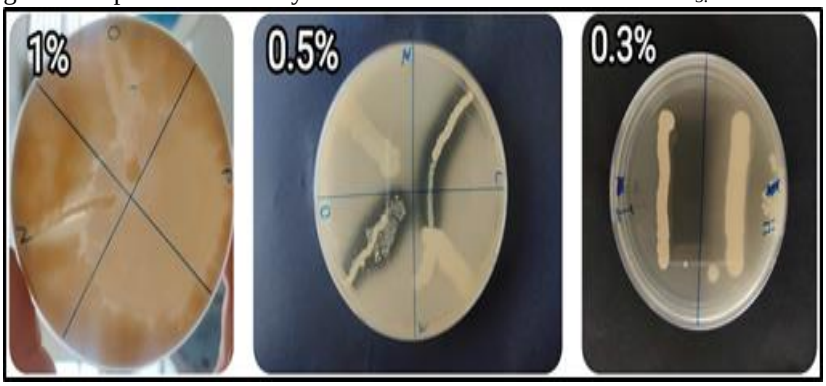


Figure 7. Showing the growth of LAB in MRS broth containing 0.3% of bile salts

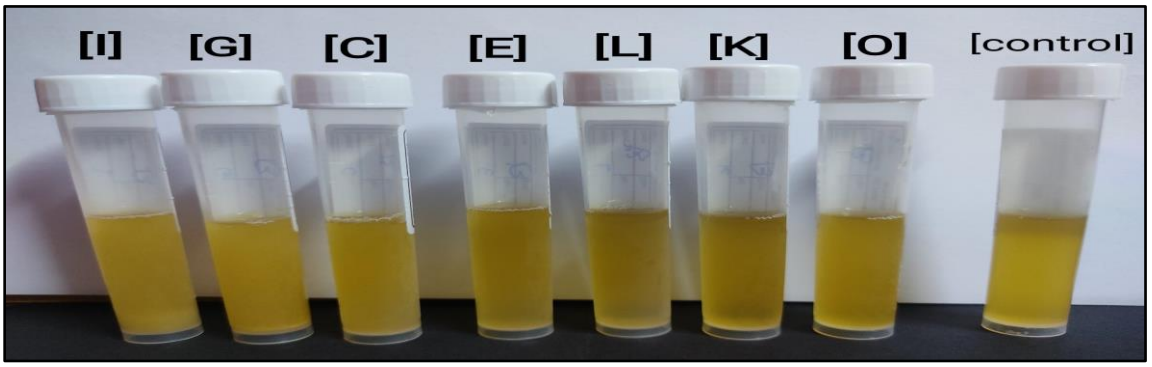
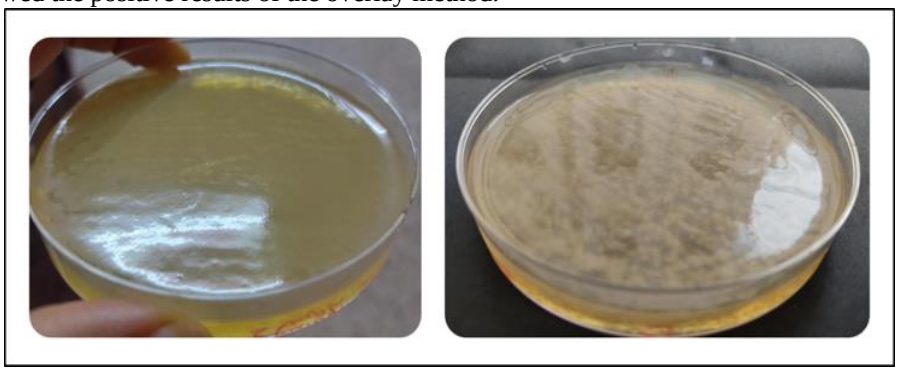


Figure 8. Showed the antibiotic sensitivity on MRS agar inculcated with LAB isolates.

Figure 9. Showed the positive results of the overlay method.



Conclusions

Considering our isolates' beneficial probiotic qualities, the following conclusions may be made,

In Mosul city/ Iraq, raw milk, commercial and natural local milk products, pickles, and other dairy items are appropriate and possible sources for isolating new probiotic strains. The isolates from this investigation, namely O and E, demonstrated comparatively strong antipathogenic activity and survival in challenging environments. As a result, they may be considered as viable "next generation" probiotic candidates that will be beneficial to the pharmaceutical sector. LAB isolates are able to grow in different temperatures (37°C and 27°C) and their activity increases in refrigerator temperature (i.e. 4°C) and in low pH (5.4 and 4.5). The isolates were able to grow on the MRS, Nutrient and Tomato juice agar and the MRS agar was the optimum medium for the isolates with the pH 5.5. All LAB isolates have the ability to produce acid in different temperatures and different concentrations with some differences between isolates. All samples evaluated are tolerance to the bile salts in low concentration (0.3%) and all LAB isolates are resistance to selected antibiotics.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

Data availability

All data generated or analyzed during this study are included in this puplished article.

Authors' contribution

All authors made significant contributions to the work presented, including study design, data collection, analysis, and interpretation. They also contributed to the article's writing, revising, or critical evaluation, gave final approval for the version to be published.

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