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Investigate the impact of probiotics of lactic acid bacteria obtained from various local sources on some pathogenic bacteria.

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ABSTRACT

Probiotics play a crucial role in fighting infectious diseases by significantly impacting their control. They possess the capacity to lower the frequency of contagious illnesses, potentially lessening the dependence on antibiotics and helping to alleviate the development of bacteria that are resistant to various medications. Besides, they hold substantial importance in the realm of food. The objective of this research was to isolate various strains of lactic acid bacteria (LAB) from diverse sources and explore their capabilities in laboratory settings. The efficacy of probiotic strains against some harmful bacteria was assessed through both the agar diffusion method and the agar spot method. Additionally, their resilience under conditions resembling the stomach and intestinal environment, their capacity for auto-aggregation, and antibiofilm were also examined. Our findings suggest that the isolated strains displayed differing levels of probiotic effectiveness, with the HR3 strain, identified as *Lactiplantibacillus plantarum*, demonstrating notable excellence in this regard.

Keywords: Probiotics; Lactic acid bacteria; Biofilm; Antibiotic resistance.

INTRODUCTION

Probiotics encompass live microorganisms that, when consumed adequately, provide health benefits to the host. They influence gut microbiota, strengthen the immune system, thwart infections, and improve metabolic disorders. Commonly present in fermented foods like yogurt, cheese, sauerkraut, kimchi, and kefir, probiotics can also be ingested through dietary supplements or live biotherapeutic products(1-4).

Probiotics include both bacteria and fungi. The beneficial or friendly bacteria are distinguished by their therapeutic and health-enhancing attributes(5). According to (6) a significant proportion of these beneficial bacteria falls within the lactic acid bacteria (7) group, comprising various genera. Among these, the genus Lactobacilli stands out as the largest due to its diverse species. Because the species within this genus are recognized for their safety and numerous health-promoting qualities, several strains have garnered considerable attention (8).

Probiotics exert a significant influence on combating infectious diseases through their impact on the epithelium, the synthesis of antimicrobial substances, and competitive exclusion. They have the potential to reduce the occurrence of contagious infections, thereby reducing the reliance on antibiotics and potentially mitigating the emergence of bacteria resistant to multiple drugs. Individuals

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prone to recurrent infections often face persistent concerns, as antibiotic therapy is frequently ineffective due to drug resistance. Several probiotic strains, tested in a growing number of trials, have demonstrated promising effects in preventing or treating both acute and recurring infectious diseases (9-11).

Traditionally associated with naturally fermented foods, probiotics were not initially recognized for their pharmacological effects(7). However, the landscape has evolved, and now healthy individuals and patients alike incorporate probiotics into their diets through food, nutritional supplements, and medical preparations, marking a departure from their historical association solely with fermented foods(12). The objective of this research was to isolate various strains of lactic acid bacteria (LAB) from diverse sources and explore their capabilities in laboratory settings. This investigation aimed to assess their resistance to stress, their capacity to inhibit various types of pathogenic bacteria, and their ability to impede biofilm formation. The identification of these bacteria was conducted using the 16S rDNA gene for diagnostic purposes.

MATERIALS AND METHODS:

Bacterial pathogens

The bacterial pathogens, namely *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and *Escherichia coli*, were sourced from Maysan's Public Health Laboratory in Iraq. Reconfirmation of their identification was carried out using the VITEK® 2 compact system. All cultures were preserved on Tryptone Soy Agar plates and stored at 4°C for testing purposes. To maintain the viability of the culture stocks, a solution of 20% (v/v) glycerol was used, and these stocks were stored at -20°C.

Collection samples, Cultures, Isolates and Growth Conditions

With parental permission, feces from healthy infants also including artisanal yogurt and cheese were analyzed for Lactobacillus (13). provided a detailed account of the isolation procedure, which was followed with a few tweaks here and there. For the sake of brevity, we homogenized 1 g of each sample in 9 mL MRS broth. MRS agar (de Man-Rogosa-Sharpe, HiMedia) was immediately plated with serially diluted samples. The plates were then placed in anaerobic gas jars and incubated at 37 °C for 48 hours. Colonies were chosen at random to represent each morphology, then sub-cultured until they were pure. Presumptive Lactobacillus spp. included catalase-negative, gram-positive, rod-shaped bacilli. Colonies of bacteria stored in MRS broth with 20% (v/v) glycerol.

Morphological and biochemical characterization

Microorganisms are often characterized using a mix of approaches. In this work, the isolates were separated based on macroscopic morphology on culture media, particularly MRS medium, to find out about the colonies' features and also separated based on certain biochemical properties, in particular Gram staining, Catalase testing, and oxidase testing.

Screening of bacterial strains for probiotic attributes

Blood hemolysis test

Blood agar plates were used for the test. By streaking, a pure culture of the bacterium was inoculated into the blood agar plate. The plate was incubated at 37°C for 24 hours under anaerobic conditions. looked for indications of hemolysis around or under the bacterial colonies on the plate(14).

Antibacterial activity

There are different methods to screen and measure the antibacterial activity of LAB, such as agar diffusion method, broth microdilution method, co-culture method, and Agar spot assay:

A. Agar diffusion method

The diffusion technique published by(15, 16) was utilized with certain modifications. The isolated strains were cultured in MRS broth medium for 24 hours at 37°C before being centrifuged (9000 g for 10 minutes at 4 ºC). Filtering with a 0.22 µm membrane filter yielded cell-free supernatant (CFS). To eliminate the antibacterial action of organic acids, NCFS was made by changing the pH to 7.0 with 6 N NaOH. Fifty microliters of CFS and NCFS were applied to 3 mm wells on Mueller-Hinton agar plates that had been pre-inoculated with pathogenic bacteria. The plates were then incubated for 24 hours at 37 °C. The establishment of a clear zone showed positive antibacterial activity.

B. Agar spot assay

The sensitivity of indicator pathogenic bacteria to the LAB strains bacteria was determined using an agar spot test. One microliter of the LAB's overnight culture (about 106 CFU/mL) was spotted on the surface of MRS agar plates (1.5% agar) and allowed to dry for 10 minutes at room temperature in a sterile environment. After that, the plates were covered with 10 mL of Mueller-Hinton soft agar (1% agar) seeded with about 106 CFU/mL of indicator pathogenic bacteria. Plates were incubated for 24 hours at the optimal temperature for each indicator strain and the inhibition zone was evaluated (17).

Survival under simulated stomach condition

The Survival of LAB under simulated stomach condition experiment was carried out with minor changes as previously published(18). The isolates were grown in MRS broth medium at 37° C for 24 hours. The culture was then centrifuged for 10 minutes at 8000 rpm. After removing the CFS, the bacterial cells were washed twice with pH-neutral PBS. Bacterial cells were resuspended in 5 mL Simulated gastric fluid (SGF). SGF was made by suspending pepsin (3g/lit) in PBS. With 1 M HCl, the solution was adjusted to pH 3, and then filter sterilized through a 0.22 µm filter membrane. The serial dilution was performed, and 1 ml of the seven dilutions was distributed over MRS agar medium, with the remaining incubated for 3 hours. Following the incubation time, 1 ml of the sample was re-spread on MRS agar medium and incubated anaerobically for 48 hours. Following the completion of the incubation period, colonies were counted.

Bile salt tolerance

A probiotic strain should be able to withstand bile salt stress. Oxgall powder was employed to study the bile salt stress of bacterial isolates. The bile salt tolerance of the selected isolates was tested as shown in (19) with minor adjustments. Bile tolerance was tested on overnight-grown cultures. These cells were collected, re-suspended in MRS broth with 0.3% oxgall, and incubated at 37°C. After incubation, samples were taken at 0 and 4 h intervals. After 37°C incubation, viable cell colonies were counted using the plate count technique on MRS agar plates.

Auto-aggregation assay

The experiment was carried out using the approach of (20) with minor changes. The overnight cultures were centrifuged at 4°C (5000 g) for 15 minutes. After centrifugation, the pellet was resuspended in PBS, and the suspension's optical density was measured at 600 nm. The suspension was then incubated for 1 hour at 37°C before the OD was measured again for the upper surface. The following formula was used to compute the percentage of auto-aggregation: Auto-aggregation percentage = [(OD0 – OD1) / OD0] x 100 where OD0 represents the initial OD and OD1 represents the OD after incubation.

Antibiofilm activity

The effects of probiotic supernatants on pathogenic bacteria biofilm development were examined using a crystal violet test, using a modified version of the(21) method. Probiotic bacterial strains

were grown and incubated in MRS broth. Pathogenic bacteria were cultivated and incubated in tryptic soy broth with 0.5% (w/v) glucose. After overnight incubation, the probiotic culture supernatants were collected and 100 µL were put into 96-well plates. The 96-well plates were then filled with 100 µL of pathogenic bacterial inoculum (1×106 CFU/mL). The plates were incubated at 37°C for 24 hours. After that, the biofilms were fixed with 200 L methanol for 10 minutes, dyed with 200 L 0.1% crystal violet for 10 minutes, and gently washed three times with water. Crystal violet adhering to the biofilm samples was dissolved in 200 L 33% acetic acid. Inhibitory action was studied by assessing biofilm growth at an optical density of 630 nm. The positive control was pathogenic bacteria cultured on TSB medium with 0.5% (w/v) glucose, whereas the negative control was solely TSB media with 0.5% (w/v) glucose. The relative proportion of biofilm inhibition was calculated by the following formula:

(%) =100–[(630t/A630c) ×100].

Antibiotic susceptibility for isolates of probiotics and indicator pathogenic

Antibiotic susceptibility testing was conducted on both the probiotic strains discovered in this study and indicator pathogenic bacteria. Probiotic bacteria were cultured in MRS broth medium, while MH broth medium was used for the indicator bacteria. After an incubation period, each culture was appropriately diluted to a density of 0.5 using a Densichek. Subsequently, the bacteria were further diluted and spread onto MRS and MH agar media. Culture plates with discs were then incubated at 37°C for 24-48 hours. The zone of inhibition (ZOI) surrounding the discs was utilized to assess the sensitivity and resistance of the screened isolates to antibiotics (22).

Genomic DNA isolation, polymerase chain reaction, and sequencing

Following isolation and identification using normal microbiological techniques, genomic DNA was extracted using a GENEAID, bacterial DNA extraction kit/ Korea. Utilizing NanoDrop, the concentration and purity of the DNA samples were assessed. A concentration of 10-100 ng/µl was used, and a ratio of 1.8-1.9 was regarded as pure. The PCR reaction mixture and all amplification reagents were purchased from iNtRON Biotechnology Corporation in Korea and Universal Primers for 16S rDNA were utilized to amplify. The amplified product was separated for 50 minutes on agarose gels by electrophoretic (1%) in 1X TBE at 80V. Under ultraviolet light, the gels were measured and photographed. The PCR product was sequenced using these primers, and the resulting 16S rDNA sequences were submitted to the National Centre for Biotechnology Information (NCBI) for identification of isolates and comparison with other bacteria (22, 23).

Statistical analysis

One-way ANOVA, paired sample t-test, and Duncan test were used for the statistical analysis, and a p-value of 0.05 was used to determine significance. Every piece of data is expressed as mean S.D.

RESULTS:

Morphological and biochemical characterization

All the bacterial isolates were undergone for the colony morphology on MRS agar plates. The isolated bacterial colonies emerged in white and creamy color with round or elevated shapes. The size, shape, and arrangement of the bacterial isolates, such as rods, bacilli, and cocci in single or in chain form detected at 100X using oil immersion, allowed for their differentiation. The bacterial isolates lacked the catalase enzyme needed to convert hydrogen peroxide into oxygen and water. The bacterial isolates lack cytochrome c oxidase, an enzyme that changes the color of bacterial growth.

Screening of probiotic properties of bacterial isolates

Blood hemolysis

All isolates that exhibited pathogen-inhibitory properties were non-hemolytic against erythrocytes.

Antibacterial activity by agar diffusion

The most prevalent test organisms were used to assess the isolates' antibacterial activity by agar diffusion. *S. aureus, P. aeruginosa, K. pneumonia, E. faecalis*, and *Escherichia coli* were all tested, however only 10 isolates showed a zone of inhibition against them all. Between 10 and 25 mm made up the zone of inhibition. Isolating 3 against all test organisms revealed the highest antagonistic action Table 1.

Antibacterial activity by agar spot

The same pathogenic bacteria were used to assess the isolates' antibacterial activity by Agar spot assay. all 10 isolates showed a zone of inhibition against them all. Between 7 and 25 mm made up the zone of inhibition. Isolating HR3 against all test organisms revealed the highest antagonistic action Table 2.

Survival under simulated stomach conditions and Bile salt tolerance

The acid and pepsin tolerance of each of the ten isolates was tested. The pH 3 environment tested their endurance. The isolates were chosen for additional screening because they were able to survive under these circumstances. Adaptability to acid and pepsin stress varied among isolates. The strain HR3 outperformed other bacterial isolates in terms of its capacity to survive expanded stomach conditions. Following a three-hour incubation period, isolated HR3 displayed a survival rate of 8.03 log CFU/ml at pH 3. Bile salt tolerance testing was done on all 10 isolates. They demonstrated resistance to oxgall 0.3%. The viability of cells is less impacted by salt stress than by a drop in pH 3 Table 3.

Auto-aggregation assay and Antibiofilm activity

The auto-aggregation percentage of the bacterial isolates HR3 and HQM was noticeably higher than that of other isolates. HQM (45.52), which came after HR3 (65.95), had the highest auto-aggregation. It was confirmed that the probiotic supernatant had an impact on biofilm development. The Pseudomonas strain was looked at because, unlike the other harmful strains that had been studied, it could create a biofilm. The outcomes demonstrated that all of the probiotic strains tested could prevent Pseudomonas *aeruginosa* from forming biofilms. The *S. lugdunensis* also was used to study the effect of cell supernatants on Gram-positive bacteria. We found that all strains could inhibit biofilm formation, except for isolate HR5, which had an encouraging effect Table 3.

Genomic DNA isolation, polymerase chain reaction, and sequencing

Genomic DNA extraction was performed using a GENEAID bacterial DNA extraction kit from Korea. Electrophoresis was employed to examine the entire genomic DNA of all isolates. The DNA was visualized as distinct bands under UV light when subjected to 1% agarose gel electrophoresis, as illustrated in Figure 1.

Bacterial isolate HR1 was recognized through *16s rDNA* sequencing as *Lacticaseibacillus* sp strain HR1 and was assigned the GenBank accession number OP935936. Similarly, bacterial isolate HR2 was identified as *Lacticaseibacillus paracasei* strain HR2 with the GenBank accession number OP935936. The remaining eight isolates were determined to be *Lactiplantibacillus plantarum* and their GenBank accession numbers are detailed in Figure 2.

Table 1 illustrates the antimicrobial range of LAB against pathogens using the Agar diffusion test. The mean and standard deviation are representative of three independent experiments.

Probiotic isolates	Indicators				
	S. aureus	***P. aeruginosa***	***K. pneumonia***	***E. faecalis***	***E. coli***
HR1	14.67± 0.57^{a}	14.33± 1.15^{a}	0	14.67± 0.57^{a}	12.33± 0.57^{b}
HR2	16.66± 1.52^{b}	16.33± 1.15^{b}	0	19.00± 1.00^{a}	10.67± 0.57^{c}
HR3	20.67±0.57^{ab}	19.33± 1.15^{b}	0	21.00± 1.00^{a}	15.67± 0.57^{c}
HR4	15.33± 1.52^{a}	13.67± 0.57^{a}	0	14.00± 1.73^{a}	11.67± 0.57^{b}
HR5	13.33± 2.30^{a}	13.67± 0.57^{a}	0	13.00± 1.57^{a}	12.00± 1.73^{b}
HR6	15.33± 0.57^{a}	15.33± 1.52^{a}	0	12.33±1.52^{b}	14.00±1.00^{ab}
HR7	17.67± 1.15^{a}	14.33± 1.52^{b}	0	14.33± 0.57^{b}	13.00± 1.00^{b}
HR8	18.67± 1.15^{a}	16.67± 1.15^{b}	0	16.00± 1.00^{b}	15.33± 0.57^{b}
HQM	17.67± 1. 15^{a}	17.33± 0.57^{a}	0	17.67± 0.57^{a}	12.67± 0.57^{b}
HQ	11.67± 0.57^{a}	11.67± 0.57^{a}	0	10.33± 0.57^{b}	10.66± 0.57^{b}

a-c Different alphabetical letters within one row mean that there are significant differences at the probability level of 0.0 5

Table 2 illustrates the antimicrobial range of LAB against pathogens using the agar spot test. The mean and standard deviation are representative of three independent experiments.

Probiotic isolates	Indicators				
	S. aureus	***P. aeruginosa***	***K. pneumonia***	***E. faecalis***	***E. coli***
HR1	14.33± 0.57^{b}	15.00 ± 0.00^{b}	17.00±0.00^{a}	14.00 ± 1.00^{b}	11.67 ± 0.57^{c}
HR2	15.00± 1.00^{b}	15.33 ± 1.57^{b}	16.33±0.57^{b}	18.33 ± 1.52^{a}	11.33 ± 0.57^{c}
HR3	21.67± 0.57^{b}	20.66 ± 0.57^{b}	25.00±0.00^{a}	24.00 ± 1.00^{a}	16.33± 0.57^{c}
HR4	14.00± 0.00^{a}	13.33 ± 1.52^{a}	9.67±1.15^{b}	13.66 ± 1.52^{a}	10.67 ± 0.57^{b}
HR5	13.67± 0.57^{a}	12.67 ± 1.15^{ab}	11.67±0.57^{b}	11.67 ± 0.57^{b}	12.67 ± 1.15^{ab}
HR6	15.33± 0.57^{b}	14.00 ± 0.00^{bc}	18.00±2.00^{a}	12.00 ± 1.00^{c}	14.33± 0.57^{c}
HR7	18.00± 0.00^{a}	12.67 ± 0.57^{d}	15.67±0.57^{b}	14.00 ± 1.00^{c}	15.00 ± 0.00^{bc}
HR8	19.67± 0.57^{a}	17.33 ± 0.57^{b}	12.00±1.00^{d}	16.33 ± 1.15^{bc}	15.00 ± 0.00^{c}
HQM	16.33± 0. 57^{a}	16.33 ± 0.57^{a}	14.67±0.57^{b}	16.67 ± 1.52^{a}	12.00 ± 0.00^{c}
HQ	11.33 ± 0.57^{a}	11.33 ± 0.57^{a}	8.33±1.57^{c}	10.00 ± 0.00^{ab}	9.33 ± 0.57^{bc}

Table 3 Survival viable count of isolates at PH 3 for 3 hours in the presence of 3 mg/ml of pepsin and for 4 hours when Oxgall (3 mg/ml) was present. The mean and standard deviation are representative of three independent experiments.

Probiotic isolates	PH and pepsin tolerance		Bile salt tolerance	
	0h	3h	0h	4h
	Log CFU/ML	Log CFU/ML	Log CFU/ML	Log CFU/ML
HR1	8.16 ± 0.01	7.98 ± 0.02	8.19 ± 0.02	8.15 ± 0.01
HR2	8.17 ± 0.01	7.71 ± 0.02	8.16 ± 0.01	8.1 ± 0.00
HR3	8.3 ± 0.00	8.03 ± 0.00	8.28 ± 0.01	8.34 ± 0.00
HR4	8.2 ± 0.03	7.24 ± 0.06	8.11 ± 0.00	8.05 ± 0.01
HR5	8.21 ± 0.01	7.33 ± 0.09	8.16 ± 0.01	8.09 ± 0.00
HR6	8.25 ±0.02	7.72 ± 0.04	8.2 ± 0.00	8.14 ± 0.01
HR7	8.29 ± 0.01	8.01 ± 0.06	8.18 ± 0.01	8.17 ± 0.00
HR8	8.29 ± 0.01	7.95 ± 0.00	8.24 ±0.01	8.19 ± 0.00
HQM	8.23 ± 0.02	7.99 ± 0.00	8.2 ± 0.00	8.19 ± 0.00
HQ	8.13 ± 0.02	7.35 ± 0.04	8.11 ± 0.00	8.01 ± 0.01

Table 4 Auto-aggregation and inhibition of biofilm formation percentage. The mean and standard deviation are representative of three independent experiments.

Probiotic isolates	Auto-aggregation %	Inhibition of biofilm formation %	
		P. aeruginosa	*S. lugdunensis*
HR1	25.76 ± 0.04	83.61 ± 0.80	83.28 ± 0.10
HR2	20.93 ± 0.56	80.20 ± 2.82	82.12 ± 0.57
HR3	**65.95 ± 0.95**	86.68 ± 0.11	85.95 ± 0.16
HR4	22.02 ± 0.27	85.32 ± 0.62	79.00 ± 0.32
HR5	26.04 ± 0.68	84.30 ± 0.61	-60.80 ± 2.93
HR6	21.43 ± 0.10	83.61 ± 0.95	28.06 ± 1.78
HR7	19.10 ± 0.51	83.61 ± 0.93	80.91 ± 0.23
HR8	26.68 ± 0.41	84.98 ± 0.77	80.99 ± 0.15
HQM	**45.52 ± 0.22**	85.66 ± 1.07	84.58 ± 0.24
HQ	26.18 ± 1.13	81.91 ± 0.65	78.77 ± 0.15

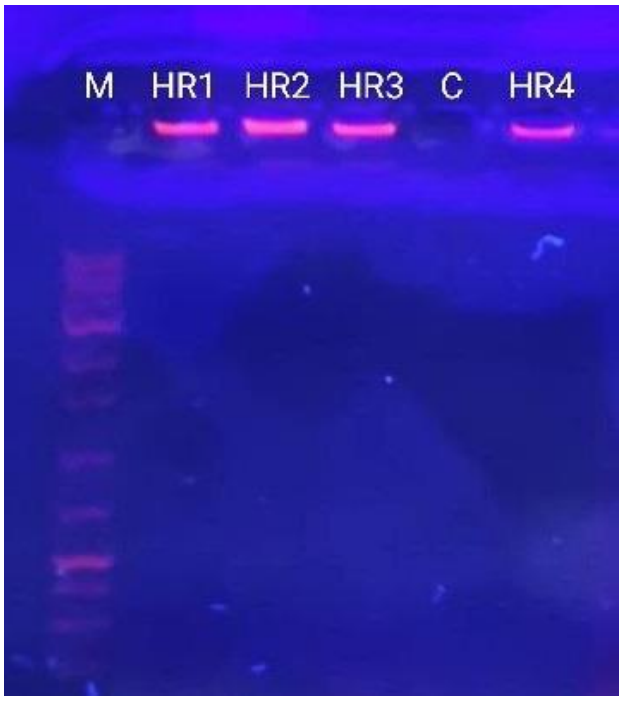

Figure 1 depicts the agarose gel with a 1% concentration, showcasing the total genomic DNA extraction. This gel includes markers, controls, as well as four isolates identified from H1 to H4.

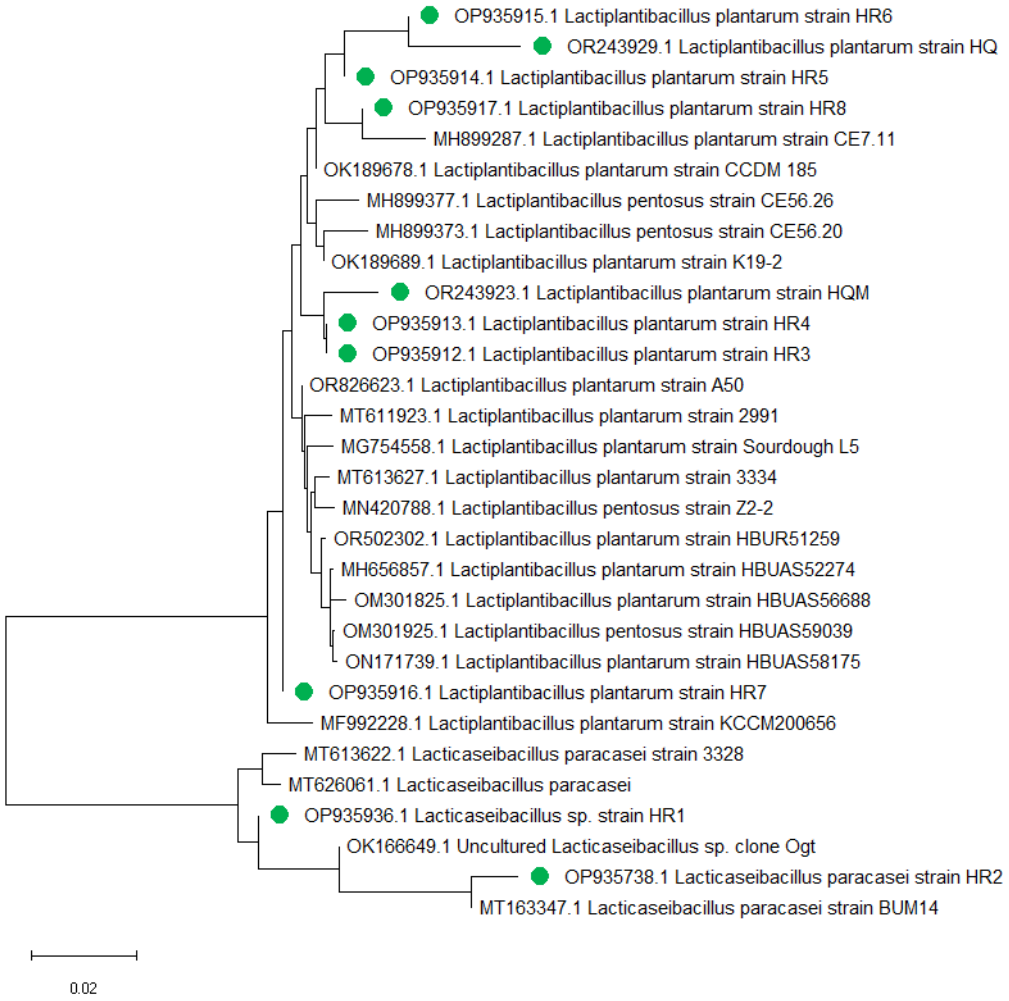

Figure 2 displays the phylogenetic relationships of the isolated strains from this study alongside the most closely related strains available in the Gene Bank. The Maximum Likelihood method coupled with the Tamura-Nei model was utilized to represent these relationships based on the 16s rDNA sequences. The green dots represent the strains that were isolated as part of this study.

DISCUSSION

Probiotics are becoming more popular because people are more aware of how they can help with different health issues, especially those related to the digestive system. People also prefer to use natural ways to prevent diseases and improve their well-being. Moreover, probiotics are added to many kinds of foods and drinks that people consume regularly. Another factor that boosts the demand for probiotics is the research and development of new strains of probiotics. LAB are considered probiotics because of their beneficial effects on human health. Different sources, including dairy products and human guts, can isolate LAB. These microbes also function as probiotics because they can colonize the gut and guard against harmful organisms.

The preliminary selection of probiotic bacteria involves screening for desirable characteristics, such as the ability to produce antibacterial substances that can inhibit the growth of pathogens, Resistance to

antibiotics and metals that may be present in the environment (24), and adaptability to different pH, bile salt, and oxygen levels (25).

The agar well diffusion assay and agar spot test are based on the probiotics' ability to create antimicrobial chemicals that diffuse into solid agar media. Clear zones around the areas or wells where the probiotics or their cell-free supernatants (CFS) are applied are evidence of inhibition (26). The neutral cell-free supernatant (NCFS) did not affect the tested pathogenic bacteria. It is possible that the neutral cell-free supernatant's ineffectiveness on the pathogenic bacteria was caused by denaturation, altered solubility, chemical changes to antimicrobial compounds, changes in pH-dependent activity, neutralization interfering with the combined effects of other factors, or all of the above. The CFS of all isolates did not affect K. pneumonia which may be due to its ability to alter cell surface or metabolism to lessen the uptake or impact of the CFS and may evolve defense mechanisms to thwart or escape the activity of the CFS. Therefore, there are numerous and complex potential explanations for why the CFS failed to inhibit K. pneumonia whereas the identical probiotic strains directly inhibited it(27). To comprehend the processes and circumstances that affect the CFS capability to combat K. pneumonia more study is required.

The contrasting results observed between the lack of inactivation of *K. pneumoniae* by probiotic supernatants in the agar diffusion method and the direct antagonistic effect leading to inactivation in the agar spot method could be due to various factors, such as concentration and diffusion, components affected by diffusion, nature of active agents, and methodological variations. To understand the specific reasons for this discrepancy, further investigation through controlled experiments altering parameters like concentration and nature of contact would be necessary.

The isolate HR3 outperformed other bacterial isolates in terms of their capacity to survive under stimulated stomach circumstances and bile salt stress. According to a study (28), low pH hurts bacterial development. For the survival and colonization of the bacterial isolate in the GIT, the LAB's resilience to such circumstances is advantageous.

The HR1 and HR2 strains were identified by 16S rRNA analysis, which determined their classification as *Lacticaseibacillus* sp and *Lacticaseibacillus paracasei* respectively. This classification aligns with the species often found in traditional fermented food(29). While the other strains isolated in this study were classified as *L. plantarum*. This classification aligns with the species often found in sauerkraut(30).

Currently, comprehensive analyses of the entire genomes of isolates HR3 and HQM are underway to meticulously scrutinize and authenticate the genes responsible for the probiotic properties they exhibit. This investigative approach mirrors the methodology outlined in (31- 34).

Author Contributions

Al-kaabi H. Q. and Chelab R. L. designed the study and performed the experiments. In addition, both authors analyzed the data and wrote the manuscript.

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Conflict of interest:

The authors declare that they have no conflict of interest.

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