






Potential Probiotic Properties of *Lactobacillus Sp.* Isolated from Traditional Kurdish Yogurt

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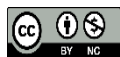
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Abstract:

Lactic acid bacteria are a group of probiotic bacteria that are mostly predominant in fermented foods and have beneficial effects on human health. Traditionally made Kurdish yogurt as undiscoverable food may serve as an important source of these bacteria with potential probiotic properties. Therefore, the current study aimed to determine the probiotic properties of *Lactobacillus* spp. isolates from Kurdish yogurt using different microbiological tests. Results showed that the isolated *Lactobacillus sp.* can grow over a wide range of pH, NaCl concentration, and temperature. Moreover, they could overcome some pathogens like *Staphylococcus aureus*, *Klebsiella pneumonia*, *Bacillus cereus*, and *Salmonella typhi* more than the industrial control strain. While they were susceptible to different commercially used antibiotics, which may add advantages to the safety assessment of the isolates. These results indicate that the *Lactobacillus* sp. strains isolated from local Kurdish yogurt have good probiotic properties, which make them an acceptable strain to be used in the health sector and industry. It may also suggest that Kurdish yogurt is an effective food to fight infections and improve gastrointestinal health in humans. Finally, the current research will pave the way to further investigation in discovering other probiotic strains in Kurdish yogurt.

Keywords: Probiotic; Lactic Acid Bacteria (LAB); Fermented Foods; Yogurt; Mast; *Lactobacillus sp.*

1. Introduction

Probiotics are living microorganisms that have beneficial effects on human and animal health [1–4]. Ingesting these microorganisms can change the immunological response, fight and compete with pathogenic microorganisms, and restore the normal flora of the gut after the use of antibiotics by patients. Therefore, it can be a good agent to treat and prevent various diseases and infections including irritable bowel syndrome, lactose intolerance, allergies, cystic fibrosis, and infections of the urogenital tract. Moreover, it was mentioned in the literature that probiotics can also fight various cancers in the human body [3,5]. Probiotics are introduced into our bodies through water and food, particularly fermented foods, which serves as the best sources for probiotics; including *Lactobacillus sp.* and *Bifidobacterium sp.* [6,7]. They are believed that the production of hydrogen peroxide, lysozyme,

bacteriocins, siderophores, and proteases are the weapons of probiotics used against other microorganisms [8].

One of the most common types of probiotic bacteria are lactic acid bacteria (LAB), they are a group of gram-positive and catalase-negative fastidious bacteria, they can be cocci or rod-shaped and show high levels of tolerance when it comes to low pH [9]. LAB can ferment and transform glucose into lactic acid, which plays an essential part in food fermentation, also aiding in the inhibition of pathogens and spoilage microorganisms [9,10]. LAB members that can be employed as probiotics include *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Oenococcus*, and *Leuconostoc*, in which are also among the gastrointestinal tract natural flora of animals and humans [10,11]. The most common genus among LAB with high probiotic value probiotics is *Lactobacillus sp.* [12,13]

Yogurt as a fermented food is widely consumed in Iraq and the world. it is produced by the action of starter microorganism which consists mainly of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* [14]. The Kurdish yogurt known as Mast, is one of the most popular dairy products in the Kurdistan region. It is a rich source of minerals, protein, fatty acids, essential amino acids, and microbial communities [15,16]. So, it can be a good natural source of probiotics for health promotion when consumed by humans [17]. Unfortunately, studies on microbial constituents and properties of local Kurdish yogurt are limited, therefore, this research aims to isolate and identify Lactobacilli from local Kurdish yogurt and test for their potential probiotic properties for the first time. Helping to get a better understanding of the beneficial effects of this type of yogurt upon consumption by humans and to pave the way for further investigation in this field in the future.

2. Materials and Methods

2.1 Sample Collection

A total of 22 traditionally produced yogurt samples (Mast) originating from rural areas were collected in sterilized containers from different shops in Sulaymaniyah province (Kurdistan region, Iraq), Table 1.

Table 1: Number and location of local yogurt samples collected in this study

Number of samples	Location
2 samples	Bazyan
5 samples	Shadalla
2 samples	Erbil
13 samples	Sharbazher
Total= 22 samples	

2.2 Isolation and Identification of Lactic Acid Bacteria

The obtained samples were serially diluted and cultivated on de Man, Rogosa, and Sharpe (MRS) agar media (HiMedia Laboratories, India) with pH adjusted to ≈ 5 and then incubated under anaerobic conditions at 37°C for (24-48) hours.

To overcome the problem of yeast overgrowing on the MRS media, different procedures were followed; such as heating the samples for 15 minutes at 60°C before inoculating MRS agar plates [15], and using Nystatin as an antifungal agent (10mg/mL).

The grown lactobacilli were initially identified based on their ability to grow under anaerobic condition, colony morphology on MRS agar, Gram staining, and Catalase tests for the purified colonies. Tiny small, flat, white to grey sticky colonies with Gram-positive under the microscope and catalase-negative facultative anaerobic bacteria were considered as *Lactobacillus sp.* The purified strain of *Lactobacillus delbrueckii subsp. bulgaricus* from the yogurt industrial starter (YoFlex® KeepIT 1.1) was used as a control strain in all the tests during this study.

2.3 Hemolytic Activity

To determine the hemolytic activities of the isolates, the fresh and pure lactobacilli isolates grown in MRS broth overnight cultures were streaked on blood agar media (Hi-Media, India) and incubated at 37 °C for 24 h. Observation of clear zones on plates whether (α -hemolysis) alpha, (β -hemolysis) beta, or (γ -hemolysis) gamma hemolysis around the *Lactobacillus* colonies were recorded.

2.4 Long-term Preservation of the Isolates

The purified isolates of *Lactobacillus sp.* were stored for the long term in glycerol (20%), by mixing fresh overnight cultures of the strains in MRS broth with equal volumes of 40% sterilized glycerol and stored at -80o C.

2.5 Probiotic Properties of Lactobacillus Isolates

The potential probiotic characteristics of the isolated *Lactobacillus sp.* were tested using several parameters. All the tests were done in replicates, as the followings:

2.5.1 Acid Tolerance Test

To test the tolerance of the isolates to grow over different pH ranges, 200 μ L of fresh overnight-grown isolates in MRS broth were used to inoculate (10 mL) of MRS broth at different pH (3, 4, and 6) individually. The broth was then incubated at (37°C) under anaerobic conditions. Finally, after 24 h of incubation, the turbidity of cultures was measured using a spectrophotometer at 600nm [10].

2.5.2 Growth at Different Temperatures

This was done by using 200 μ L of fresh and pure *Lactobacillus* strains in MRS broth to seed 10 mL of MRS broth and incubated anaerobically at different temperatures (10°, 20° and 45° C), for 24 h and their turbidity was measured by using spectrophotometer at 600nm [18].

2.5.3 Tolerance for Different NaCl Concentrations

The tolerance of the isolates to different NaCl concentrations was tested by first culturing isolates in MRS broth for one day and inoculating 200 μ L of the broth to 10 mL of different MRS broth containing (%2, %4, and %6.5 NaCl) concentrations. Incubated under anaerobic conditions at 37° C, and their turbidity was measured using a spectrophotometer at 600nm after 24 h of incubation [18].

2.5.4 Antibiotic Sensitivity Test

The isolates were tested for their sensitivity against different types of antibiotics. The antibiotics including Gentamicin (10 μ g), Tetracycline (30 μ g), Erythromycin (15 μ g), Doxycycline Hydrochloride (30 μ g), and Chloramphenicol (30 μ g) (HiMedia, India) by disc diffusion assay. This was done by making a suspension from each isolate from fresh 48-hour-old lactobacillus cultures on MRS agar, standardizing by reading their optical density at 600nm, then inoculating at the surface of Mueller Hinton agar plates (MHA) (Rashmi diagnostics, Bangalore, India) using sterile swabs. After putting the antibiotic discs on the surface of the media, then they were incubated at 37° C under anaerobic conditions. After 24 h, their clear zones were measured using a caliper [18].

2.5.5 Antimicrobial Activity of Isolates

This test was done by well diffusion assay, according to [19] with some modifications (Neutralized step was skipped). The test was started with the cultivation of the isolates in MRS broth for one day under anaerobic conditions at 37° C, then 500 µL of the broth was inoculated to 20 mL of MRS broth and incubated at 37° C under anaerobic conditions. After 48 h, the broth was centrifuged at 6000 rpm for 10 minutes, the condition was kept cold as possible during centrifugation, then the supernatants were filter sterilized through 0.22 µm Millipore filter paper to obtain cell-free filtrate (CFF) of the isolates. Next, they were tested for their antimicrobial activity against different clinically isolated pathogens; including *Escherichia coli*, *Klebsiella*, *Staphylococcus aureus*, *Bacillus cereus*, and *Salmonella typhi* (All are locally isolated from patients and confirmed by Vitek 2 system).

Fresh bacterial suspensions from each pathogen were prepared, standardized at 600nm \approx 0.5, and inoculated at the surface of Mueller Hinton agar by sterile swabs. Then 100 µl of the CFF were added to separate wells (7mm in diameter made by using Pasteur pipettes) on the surface of the freshly seeded agar media. Finally, plates were incubated at (37° C) under aerobic conditions and the clear zones were measured after 24 h of incubation.

3. Results

3.1 Isolation and Presumptive Identification of *Lactobacillus sp.*

Despite their specificity, many strains of LAB and yeast species were grown on MRS agar plates. After many attempts and difficulties in optimizing the growth conditions (mentioned in the method briefly), 5 isolates were presumptively identified as *Lactobacillus sp.* out of the 22 different local Kurdish yogurts. The identification was based on the isolate's ability to grow anaerobically, colony morphology, Gram staining, and Catalase tests as well as hemolysis activity on Blood agar compared to the control strain (Table.2 and Fig. 1).

As is clear that the five different isolates present different colonial morphology on MRS agar, while they all were facultatively anaerobic, gram-positive, and catalase negative. Besides, the isolates were mostly γ - hemolytic on Blood agar, no isolates presented β hemolysis activity.

Table 2: Presumptive identification parameters for the isolated *Lactobacillus sp.*

Isolates	Growth	Colony morphology	Gram Stain	Catalase test	Hemolytic activity
K2 A	Facultative anaerobic	Small, Creamy white, Raised and Irregular	+ ve	-ve	γ -hemolytic
K2 B	Facultative anaerobic	Small, Creamy white, Raised and Circular	+ ve	-ve	γ -hemolytic
Z1	Facultative anaerobic	Small, Off white, Flat, and Circular	+ ve	-ve	γ -hemolytic
Z2	Facultative anaerobic	Small, Creamy white, Raised and Circular	+ ve	-ve	γ -hemolytic
Q 2	Facultative anaerobic	Small, Off white, Flat, and Circular	+ ve	-ve	α -hemolytic
<i>Lactobacillus delbrueckii subsp. bulgaricus</i> (Control)	Facultative anaerobic	Small, Creamy white, Raised and Circular	+ ve	-ve	γ -hemolytic

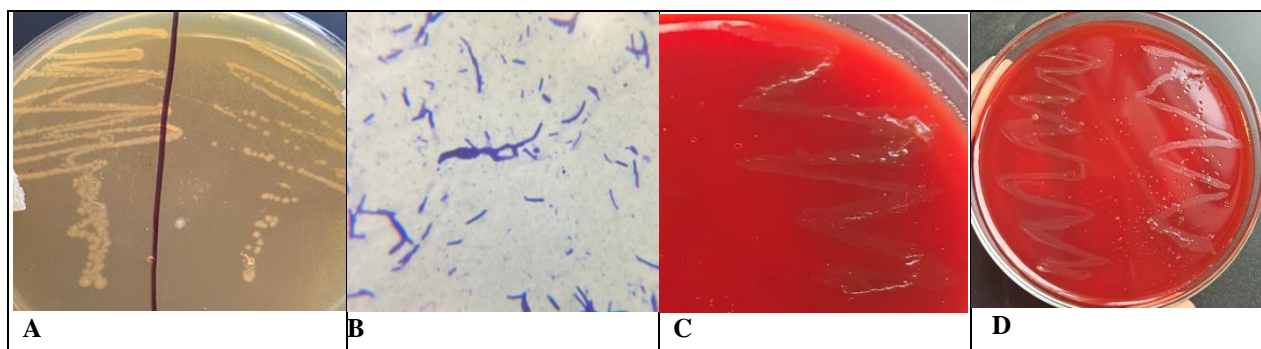


Figure 1: Characteristics of *Lactobacillus sp.* isolated from local Kurdish yogurt. A: Colony morphology on MRS agar. B: Gram +ve *Lactobacillus sp.* under the microscope (Oil emersion). C: Weak α -hemolytic *Lactobacillus sp.* D: γ -hemolytic *Lactobacillus sp.*

3.2 Probiotic Properties of the Isolated *Lactobacillus sp.*

Three out of the five isolated *Lactobacillus sp.* (K2A, K2B, Z2) were chosen to assess their potential probiotic properties compared to the control strain, using several different tests:

3.2.1 Acid Tolerance Test

This test was performed to assess the isolate's ability to grow at three different pH (3,4, and 6). It is obvious from the results that all three isolates (K2A, K2B, Z2) and the control strain were more capable to grow at pH 6 (but less than the control strain) compared to pH 4 and then pH 3 respectively (Fig. 2).

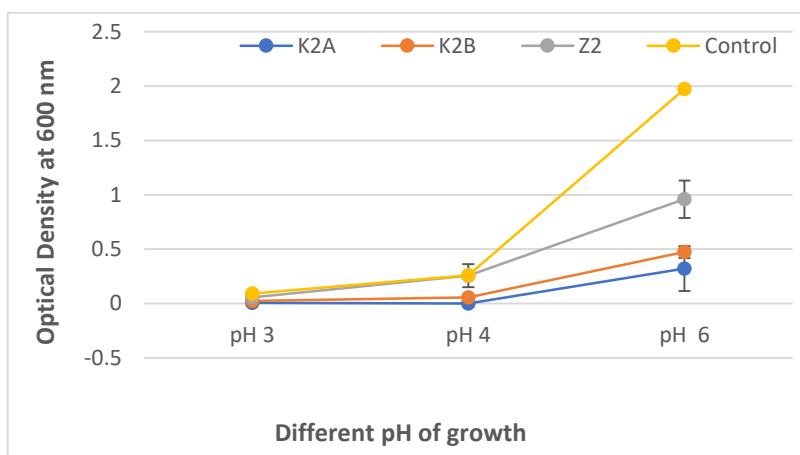


Figure 2: Tolerance of the three isolates (K2A, K2B, Z2) and the control strain to different pH (3, 4, and 6). The optical density was measured by using a spectrophotometer at 600nm. The test was done in replicates, and the bars indicate the stander deviation of means.

3.2.2 Growth at Different Temperatures

The result of the isolate's ability to grow at different temperatures (10°, 20°, and 45° C) is shown in Fig. 3. It's clear that all the three isolates with the control strain were favored to grow at 45 o C compared to other temperature of incubation. K2A had the highest growth rate at 45°C; while the control strain had the lowest growth at 45°C.

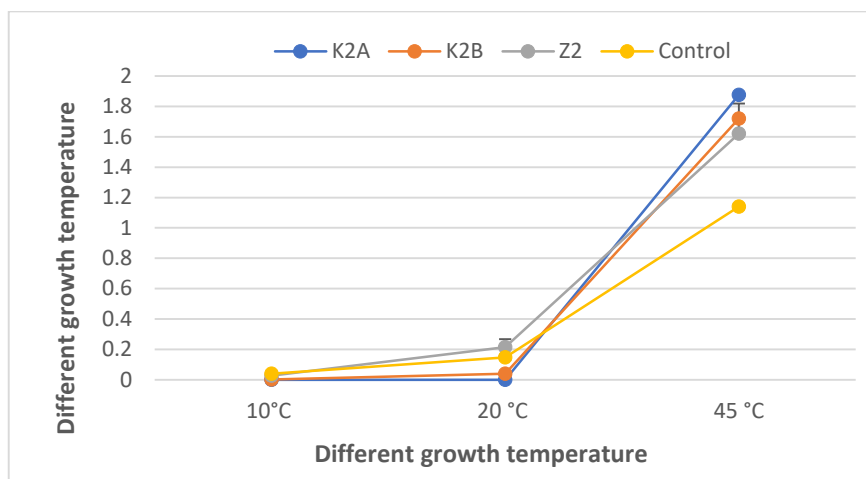


Figure 3: The abilities of the *Lactobacillus sp.* isolates (K2A, K2B, Z2, and the control strain) to grow at different temperatures (10, 20 and 45) oC. The test was done in replicates, and the bars indicate the stander deviation of means.

3.2.3 Tolerance for Different NaCl Concentrations

Different concentrations of NaCl had different effects on the growth of each isolate of *Lactobacillus sp.* (2%, 4%, and 6.5%). As shown in Fig.4. Z2 strain was more similar in its growth ability to the control strain compared to the others.

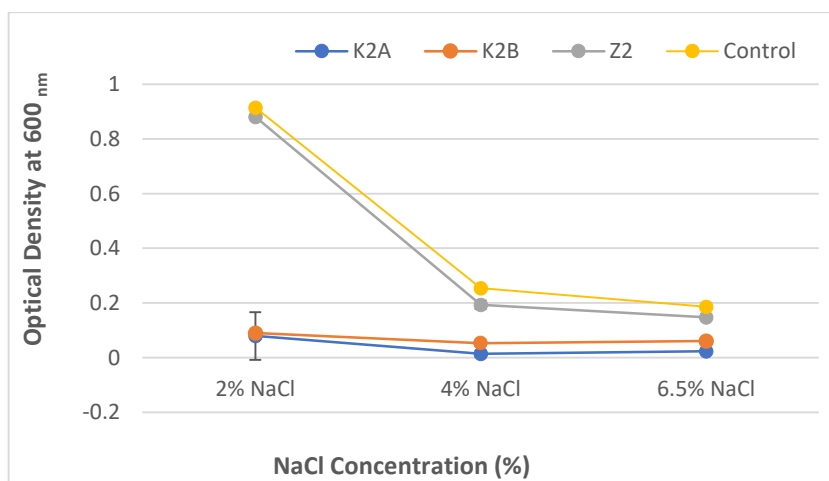


Figure 4: The ability of *Lactobacillus sp.* isolates growth at different NaCl concentrations. The test was performed in a replicate. The bar indicates the stander deviation of the means.

3.2.4 Antibiotic Susceptibility Test

To investigate the response of isolated *Lactobacillus sp.* to different antibiotics, a disc diffusion assay was performed by using five antibiotics against the three isolates of *Lactobacillus sp.* (K2A, K2B, Z2) and the control strain. Fig. 5. shows the differential effects of these antibiotics against the growth of *Lactobacillus sp.* isolates. As it's obvious that almost all the isolates were sensitive to the used antibiotics more than the control strain. The most sensitive isolate to the used antibiotics was K2A, followed by K2B, Z2 then the control strain, the latter showing less sensitivity than the isolated strains. Also, the isolates and the control strain were less sensitive to the used Gentamycin (10mg).

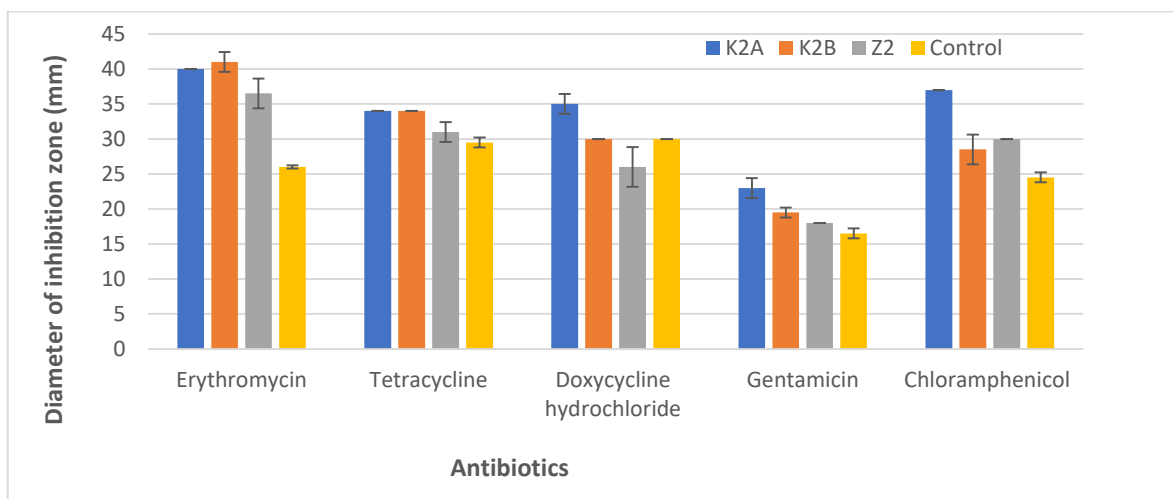


Figure 5: Antibiotic susceptibility test of *Lactobacillus sp.* (K2A, K2B, Z2, and the control) strains. The test was performed by well diffusion assay in replicate. The bar indicates the stander deviation of the means.

3.2.5 Antimicrobial Activity

One of the main probiotic properties of *Lactobacillus sp.* is to have antibacterial characteristics. To test this property, five of the common clinically isolated bacterial pathogens were used (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Bacillus cereus*, and *Salmonella typhi*). The test was done on Muller Hinton agar, using the isolate's CFF against them.

Results shown in Fig.6 indicate that the isolated *Lactobacillus* strains possess antibacterial activities against all the used pathogens except *E. coli*. It's worth mentioning that the antibacterial activities of local isolates were far more than that of the control strain, which was the most used industrial strain of *Lactobacillus sp.* in yogurt manufacturing plants.

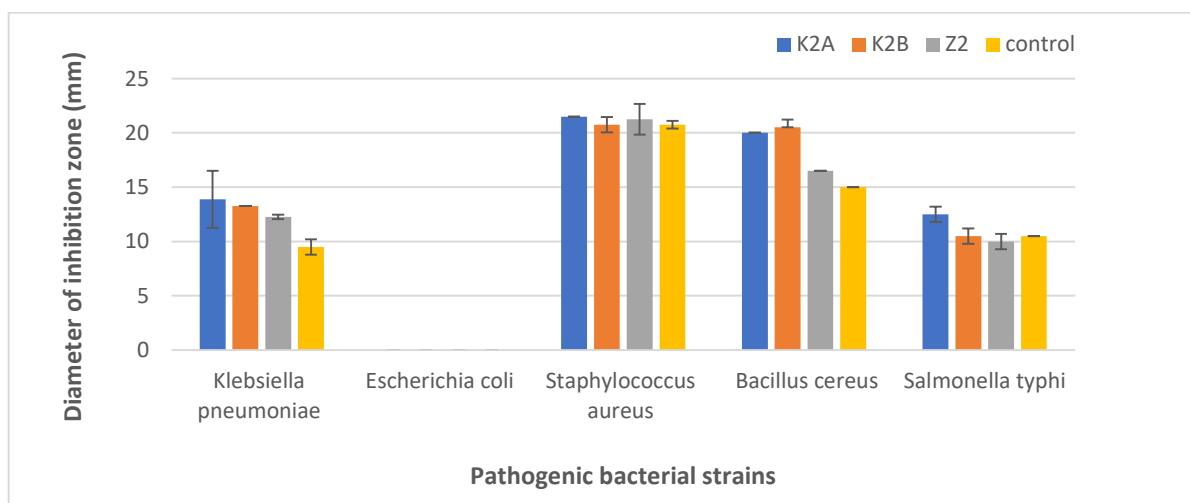


Figure 6: Antimicrobial activity of CFF of the three *Lactobacillus sp.* (K2A, K2B, Z2) and the control strain against the five clinically isolated pathogens. The test was performed by well diffusion assay in replicates. The bar indicates the stander deviation of the means.

4. Discussion

It's for the first time in our region that the probiotic properties of isolated *Lactobacillus sp.* from traditional Kurdish yogurt were investigated. Although we could only test for the isolated *Lactobacillus* strains, still the results suggest that Kurdish yogurt is a rich source of more potential

probiotic strains that could improve human health upon consumption. This may relate to the outcomes of the current investigation as the followings:

When it comes to the tolerance and growth of the isolates at different pHs, one can observe that all of the isolates including the control strain could grow at different pHs with variable rates, and all showed the highest growth at pH 6.0 compared to pH 4 and 3 (shown in Fig. 2). Similar to our finding, a study by [20] observed that the optimum pH for *Lactobacillus sp.* growth was 5.5–6.2. However, *Lactobacillus* is a diverse genus with many different strains, which can grow over a wide range of pH between 4.5 and 6.5 and even lower pH [20]. Again, another study stated that growing best was at pH levels of 5.0-6.0 [1], their ability to tolerate and grow at lower pH may stand with the fact that isolate could also survive gastrointestinal acidity [21].

Moreover, the lactobacillus isolates showed different levels of growth at different temperatures. They were able to grow more at higher temperatures (45° C) than at lower temperatures (10° C and 20° C). In Fig. 3, the K2A isolate showed a higher growth rate at 45° C followed by K2B, Z2 isolate, and the control strain, respectively. Variable growth of *Lactobacillus spp.* at different temperatures was also observed by researchers in Iran [12]. In another study, the isolates of *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Lactobacillus lactis* were able to grow at (42° C) but not at (10° C) [22]. However, it was stated in another study, that the *Lactobacillus* genus can grow in a wide temperature range from (2 to 53 °C) [1]. It is worth mentioning that the pH of the medium, and the temperature of incubation, are among the most influential factors for the activity of probiotic cultures [23].

On the other hand, varying abilities to grow across different concentrations of NaCl can be seen by the isolates (Fig.4). Again, the K2A isolate showed the least growth followed by K2B, Z2, and the control strain, respectively. Z2 isolate was more similar to the control strain in its growth ability in all three different NaCl concentrations.

It was mentioned in the literature that one of the main characteristics of an effective probiotic microorganism in the human gut is to have the ability to tolerate high NaCl concentrations [24]. Tolerance to NaCl is also important as it is a measuring scale of the ability of bacteria and how much can they tolerate osmotic shock and toxic shock [21]. Similar to our findings, research by [21] also observed that their *Lactobacillus sp.* isolates growth decreased at 6-7% NaCl concentrations, while they showed more growth at lower 1-4% concentrations of NaCl. Furthermore, the results of a study by [25] show that *Lactobacillus plantarum* could survive 1-9% concentrations of NaCl, while [26] findings show that *Lactobacillus helveticus* can survive between 1-7% NaCl concentrations. These findings indicate that different lactobacillus strains have different tolerance to NaCl concentrations.

For the assay for antibiotic susceptibility (Fig.5), the results indicate that all isolates including the control strain were susceptible to the used antibiotics. More clearly, the isolates were more sensitive than the control strain. Also, it's obvious that all the tested strains were less sensitive to the used Gentamycin (10mg), this is due to the concentration of the antibiotic in the disc, which was less than the concentration in the other used discs. These results may reflect the fact that our isolates are safe for administration, it also indicates how irregular, overuse and unnecessary administration of antibiotics can have adverse effects on these beneficial bacteria in our intestinal tract. Disturbing the delicate balance of the microbial communities leads to changes in microbial composition, and reduction of microbial diversity, which in turn may increase the likelihood of infection of the host with pathogens [27]. In contrast to the current results, other studies recorded variable results, as they observed alarming antibiotic resistance among lactobacillus isolates [28–31]. Which may be regarded as a vital tool for evaluating the safety of probiotics for human and animal uses [30,32].

Another beneficial characteristic of good probiotic microorganism is antipathogenic activities. For assaying this property of the isolates, cell-free filtrate (CFF) was used against different clinically

isolated common pathogens (Fig. 6). It was demonstrated that all the isolates were effective against all the used pathogens except *Escherichia coli*, with K2A and K2B isolates scoring larger inhibition zones compared to Z2 and the control strain against the susceptible pathogens. [21] findings also show that the largest clear zones were created against *Staphylococcus aureus* by *Lactobacillus helveticus* and *Lactobacillus plantarum*. Similarly, [25] findings show that their isolates were effective against *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*. It was mentioned that the production of bacteriocins, organic acids, enzymes, cyclic dipeptides, and hydrogen peroxide, could be attributes to the antimicrobial activity of a probiotic [21].

The ability of lactobacillus isolates in the current study to inhibit the growth of different pathogens may suggest that Kurdish yogurt is an effective food to fight infections and improve gastrointestinal health in humans.

5. Conclusion

Our research shows the potential probiotic properties of *Lactobacillus sp.* isolated from the traditional Kurdish yogurt known as Mast. High tolerance to acid, growth at different temperatures and NaCl concentrations, effective antipathogenic activity, and antibiotic sensitivity may make the isolates a good and safe probiotic to be used in the food and pharmaceutical industries. By their variability to grow under different growth conditions, the isolated strains seem to be of variable lactobacillus strains, with the Z2 isolate sharing more similarities with the control strain in different aspects.

Daily consumption of foods supplemented with Kurdish yogurt can improve gastrointestinal health and strengthen the immune system. Finally, it's worth mentioning that it is a preliminary study, still, there are undiscoverable pools of other LAB that may bear powerful probiotic abilities. Besides, it's also important to use a more detailed identification system to specify the strains and testes to determine their properties in future works.

6. Author's Contribution

We confirm that the manuscript has been read and approved by all named authors. We also confirm that each author has the same contribution to the paper. We further confirm that the order of authors listed in the manuscript has been approved by all authors.

7. Conflict of interest

There is no conflict of interest for this paper

8. Acknowledgment

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