Characteristics of Lactobacillus Spp. Isolated From the Infant's Feces As a Potential Probiotic in Vitro

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KEYWORDS
Infants, Probiotics, Lactobacillus Spp, Antimicrobial Activity, Ph Tolerance

ABSTRACT
This study aimed to identify and characterize the potential probiotic Lactobacillus spp isolated from healthy newborns' feces and assessed in vitro. This study examined the strain for resistance to (pH 2, 3 h), and antimicrobial activities against some human pathogenic strains (Escherichia coli and Staphylococcus aureus) of thirty positive isolates were assessed. Lactobacillus spp. showed tolerance properties to acid conditions Furthermore, they exhibited survival at pH 2 in the existence of pepsin. Using the well-diffusion method, all strains demonstrated potent antibacterial activity against enteropathogens. Furthermore, cell-free culture supernatants with low pH had great antimicrobial activity and displayed this effect against S. aureus more than the effect against E,coli only as a result. Thus, the thirty Lactobacillus strains could be considered as potential probiotics, but further in vitro and in vivo studies on these probiotic strains are still required.

1. Introduction

The origin of the child's gut microbiota is diverse, primarily arising from the maternal gut, human breast milk (HBM), vagina, mammary gland, and the environment (1). Breastfeeding is a fundamental factor in influencing the composition of the gut microbiota. Human breast milk plays a crucial role in the formation of the gut microbiota in newborns by consistently supplying bacteria to the newborn's stomach Several weeks postpartum (2). The process of microbial establishment is a carefully coordinated event that leads to the formation of distinct microbial communities in various parts of the gut. Nevertheless, the colonization process can be impacted by many environmental conditions. One of the main factors is the nutrition of newborns, and it is widely acknowledged that human milk (HM) is the best diet for promoting the proper development of the infant's microbiota (3). Before birth, the gastrointestinal system of the fetus is devoid of microorganisms, however, microbial colonization commences promptly following delivery. Various factors, including the method of delivery, premature birth, use of antimicrobial drugs, hygiene conditions, and diet (breastfeeding or formula feeding), influence the presence of microbes in the gastrointestinal tract of infants (4). Immediately following birth, a varied collection of microorganisms colonizes the gastrointestinal tract (GIT) of neonates. This colonization phase is essential for the development of the gut and the immature immune system, and it can have long-term health benefits (5). The strains that provide benefits are referred to as probiotic strains. The use of probiotics as prophylactic agents to augment immunity and mitigate infection is extensively prevalent (6). In 2001, the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO), both of which are part of the United Nations, collaborated. revised this definition and declared that probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (7). These nonpathogenic species are currently being evaluated as potential probiotic microorganisms due to their expected significant benefits for the human population (8). To be categorized as a probiotic, a bacterium must possess the ability to withstand the effects of bile and gastric acid, also being non-pathogenic. do not form spores The genus *Lactobacillus* is widely recognised as being safe for human consumption and demonstrates probiotic characteristics (9). Members of the *Lactobacillus* genus are often classified as "generally regarded as safe" (GRAS) or have Qualified Presumption of Safety (QPS) status (10). However, the microbiota can decrease as a person gets older because of factors including malnutrition, antibiotic treatment, and other external influences. These variables can disrupt the natural balance of the microbiota, leading to an imbalanced ecology. *Lactobacilli* species are Gram-positive bacteria, catalase-negative, non-spore-forming, and aerotolerant or anaerobic

bacteria. These microorganisms are commonly present in dairy, grain, meat, and fish products, as well as in beer, fruits, pickled vegetables, silage, sourdough, and soil. They also reside in the mouth, intestinal tract, and vagina of both humans and various animals as part of their normal flora (4). Diarrheal sickness is a significant contributor to illness and death among newborns in underdeveloped countries. Diarrhoea can be caused by a diverse range of microorganisms, such as bacteria, viruses, and parasites. Diarrheagenic Escherichia coli strains are commonly linked to diarrhoea in children in impoverished nations. Recent investigations have provided evidence of the role of *Lactobacillus* spp. in the prevention and treatment of diarrheal illnesses caused by Shigella and Salmonella (11). *Lactobacillus* can thrive in acidic environments and hinder the colonization and growth of pathogenic bacteria and other dangerous microorganisms by the production of lactic acid (12). There is a growing association between the composition of faecal microbiota and human disease. Likewise, intestinal bacteria often exhibit a strong association with various other characteristics connected to the host, such as genetics and age, as well as environmental factors like nutrition and medication. These correlations have identified that *Lactobacillus* is much more abundant in the distal gut during both healthy and diseased states (13). To achieve this objective, The objectives of the study are to identify *Lactobacillus* strains isolated from infant faeces using biochemical tests and PCR analysis of the 16S rRNA gene. The study evaluates the potential probiotic properties of the *Lactobacillus* strains isolated from the feces of the infant by determining their antimicrobial activity against some enteropathogenic bacteria (E.coli and S, aureus), and evaluating the resistance of isolates to simulated *Lactobacillus* ted gastric acid.

2. Methodology

Phenotypic diagnosis of *lactobacillus* spp.

Stool samples were obtained from 120 healthy breastfed infants, aged 1 to 12 months, at AL-zahraa Teaching Hospital in Wasit, Iraq. Faecal samples were obtained by utilizing a sterile swab stick, and the collection of the samples was conducted under sterile conditions. Following the collection process, all the samples were promptly delivered to the laboratory at the College of Sciences Wasit University within one hour. The samples were promptly isolated upon their arrival at the laboratory and assessed immediately. The *Lactobacillus* MRS (De Man Ragosa Sharpe, Merck, Italy) broth and solid medium were employed for the cultivation and stimulation of *Lactobacillus* spp. Each sample was cultured in MRS broth (Man, Rogosa, Sharpe Scharlau, Spain). using approximately one gramme of the sample. The samples were incubated for 48 hours at 37°C under anaerobic conditions using anaerobic jars (Biomérieux, France). Next, the samples were transferred to MRS agar plates (Scharlau Chemie S. A, Barcelona, Spain) and placed in an incubator set at 37 °C for 48 hours under anaerobic conditions. Each plate was examined for three to four questionable colonies. *Lactobacilli* are considered to be Gram-positive rods that are negative for catalase. The selected samples were incubated and then stored in an MRS broth medium containing 30% vol/vol glycerol at a temperature of -18°C until further analysis.

Molecular Identification of *lactobacillus* spp.

The *Lactobacillus* spp. strains were verified using 16S rRNA sequence analysis. The primer sequences used in this investigation for the polymerase chain reaction (PCR) were: forward, 5 -CGTGGGAAACCTACCTCTTA-3, and reverse, 5 -CCCTCAAACATCTAGCAC-3. To prepare a bacterial lysate, transfer bacterial cells to a microcentrifuge tube, centrifuge for 1 minute, discard the supernatant, and add Gram+ve buffer, lysozyme, proteinase K, GB buffer, and absolute ethanol. Transfer the mixture to a GD column, centrifuge for 1 minute, discard the flow-through, and add W1 buffer, wash buffer, and wash buffer. Dry the column matrix for 3 minutes, then transfer it to a clean tube, add elution buffer, and centrifuge for 30 seconds. Store at -20°C until use. The PCR assay was by Primers purchased from ALPHA DNA and precipitated with centrifugation. Deionized sterile distal water was added to each tube to create a stock solution of 100µM. These tubes were stored at -20ºC. A working solution was prepared by adding stock solution to deionized sterile distal water,

forward and reverse primers, master mix, and DNA sample. Then separated by electrophoresis. Agarose gel electrophoresis is a method used to verify amplification processes and DNA specificity. It involves creating a gel by combining agarose powder with TBE Buffer, allowing it to gel, and loading DNA samples. The gel is then filled with TBE buffer, and samples are placed in separate compartments. The DNA is then transferred from the negatively charged electrode to the positively charged electrode, and bands are observed using a UV transilluminator.

Resistance to Low pH (simulated gastric juice)

The Vinderola and Reinheimer (14) method was employed, with slight adjustments, to assess the tolerance of the simulated gastric juice. In order to replicate the composition of stomach juice, a solution was prepared by adding 0.3% w/v of filter-sterilized pepsin (SIGMA-AIDRICH, Germany) and 0.5% w/v of NaCl, with a pH adjustment to 2. The medium was eliminated by performing two washes on the pellets obtained from an overnight cell culture of lactic acid bacteria strains on MRS broth using a 0.85% sterile saline solution (pH 7.0) in a centrifuge. It was subsequently reconstructed in a 3 ml volume of the identical solution. A volume of one millilitre of the purified cell suspension was mixed with ten millilitres of gastric solution with a pH of 2. The enumeration of live lactic acid bacteria (LAB) was conducted on MRS agar medium both before and during 3 hours of incubation at a temperature of 37°C.

Antimicrobial Activity

The antibacterial activity of *Lactobacillus* isolates was evaluated by employing the synthesis of bacteriocins, with some slight adjustments based on Balouiri et al (15). The *Lactobacillus* strains were cultivated in MRS broth for a duration of 16 hours in order to produce CFS (cell-free supernatant). The pellet was obtained by centrifuging the cell suspension at 5000 rpm for 30 minutes. The cell-free supernatant, obtained after centrifugation, was then filtered using 0.2 m-size cellulose acetate filters. The nutrient agar plate is contaminated by evenly spreading a volume of pathogenic bacteria (E. coli and S. aureus) suspension, prepared in 5 mL of normal saline solution, across the entire agar surface. The turbidity of the suspension is adjusted to match that of a 0.5 McFarland standard inoculum. The antimicrobial efficacy of microbial extracts is assessed.

Statistical Analysis

The SPSS IBM Version O20 system's Chi-square test was used to provide statistical control on all of the data. Statistical insignificance was defined as a P-value of 0.05 or below Grewal et al (1).

3. Result and Discussion

Cultural and Microscopic Characteristics

The appearance of *Lactobacillus* spp. on MRS agar was observed to be spherical, white, shiny, and creamy. Among the 120 stool samples collected from babies, 90 samples (75%) were positive for *lactobacillus* spp. All of the strains had a blue-purple color upon staining, indicating that they were all Gram-positive bacteria. Microscopic analysis confirmed that they were bacilli. Additionally, it was demonstrated that they were negative for catalase activity. Positive results were obtained from 90 samples of *lactobacillus* spp. isolates.

Molecular Analysis

The DNA of 30 randomly chosen isolates out of 90 positive from infants' feces samples was extracted. The presence of undamaged DNA fragments was verified by gel electrophoresis. The DNA extracted from them was amplified using successive cycles of PCR. The DNA extracted from them and subjected to PCR amplification was found to be 700 base pairs in length, using a particular primer designed for *lactobacillus* spp. This confirms that these isolates belong to the *Lactobacillus* spp. group. Genus refers to a taxonomic rank in the classification of organisms, indicating a group of closely related species.

Resistance to Low pH (simulated gastric juice)

Different *Lactobacillus* strains are more or less tolerant of acidic environments. Table 1 shows the outcomes of a simulation that examined how stomach acid affects the survival of *lactobacillus* species. After three hours of incubation at 37 C and pH 2.0, the mean number of live *lactobacillus* spp. cells in 30 samples dropped from 4.621±0.015 log CFU ml at time zero (before the incubation period) at pH 2 to 4.518±0.018 log CFU ml. The *lactobacillus* isolated sample's viability counts varied significantly ($P \leq 0.01$) throughout each three-hour incubation period at 37 o C and pH 2.0 0. We found that all *lactobacillus* isolates exhibited the ability to develop at low (pH 2.0) after three hours of exposure. The 30 *lactobacillus* isolates were significantly different from one another ($P < 0.001$).

Sample no.	Before incubation 0 time	After incubation (3 hr at 37ºC	P-Value
	Mean(log CFU m-1 ± **SD**	**Mean**(log CFU m-1 ± **SD**	
1	**4.384 ± 0.004 a**	**4.201 ± 0.035 b**	**0.018**
2	**4.421 ± 0.010 a**	**4.332 ± 0.014 b**	**0.018**
3	**4.409 ± 0.017 a**	**4.337 ± 0.007 b**	**0.031**
4	**5.167 ± 0.013 a**	**5.110 ± 0.004 b**	**0.027**
5	**4.431 ± 0.012 a**	**4.257 ± 0.017 b**	**0.007**
6	**5.276 ± 0.016 a**	**5.220 ± 0.022 a**	**0.101**
7	**4.363 ± 0.003 a**	**4.260 ± 0.006 b**	**0.002**
8	**4.390 ± 0.011 a**	**4.269 ± 0.020 b**	**0.017**
9	**4.291 ± 0.011 a**	**4.205 ± 0.010 b**	**0.015**
10	**4.397 ± 0.023 a**	**4.204 ± 0.033 b**	**0.021**
11	**5.204 ± 0.015 a**	**5.105 ± 0.023 b**	**0.036**
12	**4.337 ± 0.007 a**	**4.313 ± 0.011 a**	**0.121**
13	**4.438 ± 0.013 a**	**4.357 ± 0.015 b**	**0.021**
14	**4.378 ± 0.023 a**	**4.230 ± 0.018 b**	**0.019**
15	**4.451 ± 0.012 a**	**4.334 ± 0.011 b**	**0.010**
16	**5.068 ± 0.010 a**	**4.959 ± 0.006 b**	**0.006**
17	**4.444 ± 0.018 a**	**4.320 ± 0.026 b**	**0.031**

18	**5.030 ± 0.037 a**	**4.968 ± 0.033 a**	**0.219**
19	**4.358 ± 0.013 a**	**4.290 ± 0.016 b**	**0.043**
20	**4.415 ± 0.012 a**	**4.235 ± 0.018 b**	**0.007**
21	**5.224 ± 0.021 a**	**4.970 ± 0.023 b**	**0.007**
22	**4.291 ± 0.018 a**	**4.221 ± 0.024 a**	**0.081**
23	**4.381 ± 0.019 a**	**4.340 ± 0.035 a**	**0.283**
24	**4.440 ± 0.016 a**	**4.413 ± 0.016 a**	**0.234**
25	**4.413 ± 0.012 a**	**4.371 ± 0.013 a**	**0.078**
26	**4.193 ± 0.016 a**	**4.057 ± 0.011 b**	**0.010**
27	**4.413 ± 0.007 a**	**4.358 ± 0.010 a**	**0.078**
28	**5.197 ± 0.022 a**	**4.970 ± 0.036 b**	**0.017**
29	**5.161 ± 0.021 a**	**5.132 ± 0.016 a**	**0.261**
30	**5.273 ± 0.015 a**	**5.208 ± 0.024 a**	**0.083**
Mean	**4.621 ± 0.015 a**	**4.518 ± 0.018 b**	**0.001**
P-value	**0.001**		

Table 1:(log CFU m-1 ± SD) Mean values of tolerance of *lactobacillus* isolated from infants' feces to simulated gastric juice for each sample.

* Varied letters in the rows represent significant differences ($p \leq 0.01$).

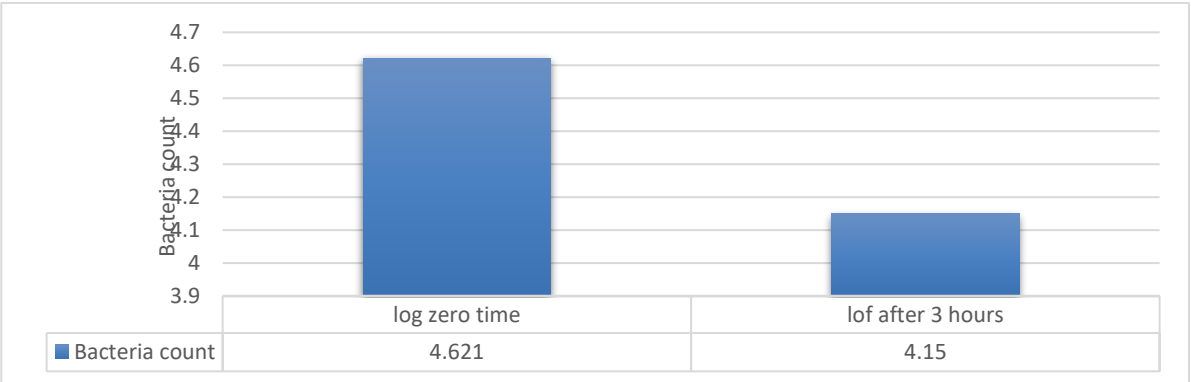

Figure (1): tolerance of *Lactobacillus* spp. separated from infants' feces to simulate gastric juice acidity in zero time and after three hours.

Antibacterial activity

The findings demonstrated a substantial difference (p≤001) in the diameter inhibitory zones of E. coli and Staphylococcus aureus. Table 3.6 and Figure 3.10 show the average antimicrobial effect as measured in the inhibition zone.

	E. coli	***S. aureus***	**P-value**
	Mean ± SD	**Mean ± SD**	
Diameter of Inhibition zone (mm)	**14.62 ± 1.08**	**21.60 ± 1.96**	**0.001****

Table 2: Mean of diameter of Inhibition zone mm of *lactobacillus* spp. in contrast to E. coli and S. aureus

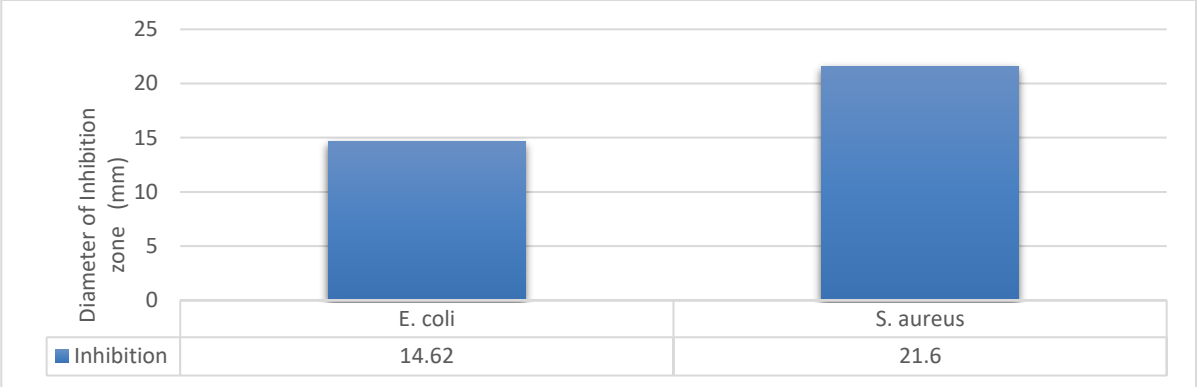

Figure (2) Antimicrobial activity of *Lactobacillus* spp. against E.coli and S.aureus for 30 samples.

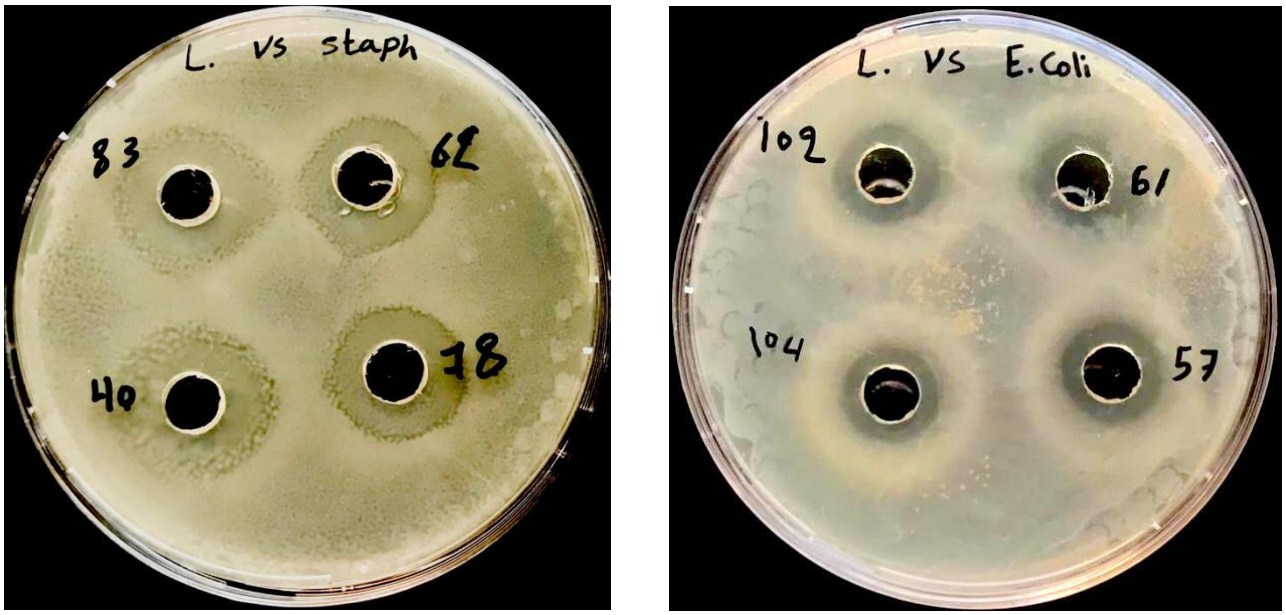

Figure (3) Inhibition zones of the *lactobacillus* isolated from infants' feces against S. aureus and E.coli

Lactobacilli, which are LAB strains, are well recognised as the predominant bacteria used as probiotics. *Lactobacilli*, naturally residing in the human gastrointestinal tract, have a significant impact on the maintenance of the microbial ecosystem in the colon. A total of 120 faecal samples were obtained from newborns, out of which 90 strains were recognised as belonging to the *Lactobacillus* species. A total of thirty *Lactobacillus* isolates were chosen for subsequent assessment and examination of their probiotic capabilities. The human gastrointestinal tract (GIT) is equipped with multiple defence systems that safeguard it against invading pathogens. Gastritis is a medical

condition that is included in a group of similar conditions. In order to be classified as a probiotic, a bacterium must be able to survive in the stomach and maintain its life and metabolic activity in the small intestine. The principal mechanism by which the body defends against most ingested bacteria is the production of stomach acid (17). Probiotics should possess the desirable characteristics of being able to tolerate acid and bile. Following the process of digestion, probiotics need to successfully go through the bile salts and gastrointestinal tract while also providing a positive impact on overall health (9). On a daily basis, the stomach secretes almost 2.5 litres of gastric juice, with a pH of around 2.0, which effectively eliminates most of the bacteria ingested (21). Table 1 demonstrates that *Lactobacillus* exhibited resistance to the simulated circumstances of the gastrointestinal system, since there was no notable reduction in the number of cells. The table displays the survival rates of the isolates at a pH level of 2. The majority of isolates exhibited significant resistance to very acidic conditions after 3 hours of incubation. The data indicate that all strains exhibited significant tolerance to a pH of 2. After 24 hours of incubation, only 10 out of the thirty isolates exhibit no significant variations at pH 2. Comparable findings can be seen in the existing body of literature (18). The pH levels in the human stomach range from 1 while fasting to 4.5 after a meal. The duration of meal digestion can extend for a maximum of 3 hours. Given the ability of *Lactobacillus* strains to endure pH 4.6, we investigated pH values of 1 and 3, which are lower. All of the isolates that were tested showed perfect resistance to pH 2, even after being exposed for 3 hours our results correspond with those findings (19). Several systems regulate the equilibrium of internal pH. The proton-translocating ATPase is of utmost importance for fermentative bacteria. There is no text provided. The enzyme is located in the cellular membrane of L. plantarum and its highest level of activity was seen within the pH range of 5.0-5.5. The plasma membrane's overall proton permeability also plays a role in regulating the internal pH. The membrane permeability of L. plantarum was found to be the lowest at a pH of 4.0, whereas the acid-sensitive organism exhibited its lowest permeability at pH 6.0. Proton-translocating ATPases play a crucial function in expelling protons from cells and decreasing their overall permeability to protons (20). Prior reports have indicated that hydrochloric acid has a significant impact on the biomolecules found in cells, including DNA, proteins, and fatty acids. Metabolism, viability, and proliferation of *Lactobacilli* were dramatically impacted under acidic conditions. Prior research has also demonstrated that when *Lactobacilli* are exposed to gastric acid for 3 hours, there is a significant decrease in the bacterial population (21)(22). The current discovery demonstrated a moderate level of inhibition at this specific pH, suggesting the presence of possible probiotic effects. Consequently, there is typically a high rate of survival. The antimicrobial activity is a crucial factor in choosing probiotic cultures, as they naturally combat dangerous bacteria. *Lactobacilli* exhibited diverse antibacterial activity, which can be related to the release of distinct antimicrobial compounds or metabolites (23). The studied *lactobacilli* demonstrated inhibitory effects on both Gram-positive and Gram-negative bacteria, consistent with a prior study (24). Lactic acid bacteria synthesise a diverse array of antibacterial substances, such as organic acids, bacteriocins, and hydrogen peroxide. The production of organic acids, such as acetic acid, lactic acid, 3-phenyl lactic acid, 4-hydroxyphenyl lactic acid, and benzoic acid, leads to a decrease in pH. This decrease in pH can hinder the proliferation of pathogens (24). The growth of the two examined pathogens (E. coli and S. aureus) was hindered by acidic supernatants due to the existence of organic acids and a low pH. Tharmaraj (25) states that lactic and acetic acids, which are organic acids produced by LAB, contribute to pH reduction and the creation of an inhospitable environment for other species. The author demonstrated that the hydrogen ion was widely believed to be linked to the antibacterial effect for a significant period. The author stated that the bacteriostatic and bactericidal actions of these weak acids are now known to be caused by their undissociated molecules, rather than the hydrogen ion. The undissociated acid molecules harm the pathogens by acidifying the cytoplasm, destroying the proton motive force, and impairing the active transport of nutrients across the membrane, resulting in sub-lethal injury (26). Theron and Lues showed that, in acidic conditions, the non-ionized organic acids are believed to facilitate the passage through the microbial barrier into the cytoplasm (27). As the pH of the cytoplasm decreases, proliferation is inhibited, leading to eventual

cell death. Overall, Gram-negative bacteria exhibited greater susceptibility to these organic acids. The results of our study align with those of Balamurugan, who also observed significant antagonistic activity of curd *Lactobacillus* isolates from India against Salmonella Typhimurium and E. coli (28). Several investigations have indicated that the metabolic process results in the generation of H_2O_2, which effectively inhibits the growth of other dangerous bacteria (29). Goodarzi et al. demonstrated that hydrogen peroxide is a potent antibacterial agent that acts by oxidising sulfhydryl groups and inducing denaturation of enzymes, degradation of cell proteins, and peroxidation of membrane lipids, ultimately resulting in increased membrane permeability. Additionally, it was said that hydrogen peroxide could serve as a precursor for the formation of bactericidal free radicals, including hydroxyl (OH-) and superoxide (O2-) radicals, which have the potential to harm DNA (30). *Lactobacilli* commonly produce bacteriocins, which can help them colonize habitats and gain a competitive advantage over other bacteria. The antibacterial activity of lactic acid bacteria may be attributed to multiple causes (31). The cell wall of Gram-positive bacteria permits the transport of relatively large molecules, making it unlikely that bacteriocin receptors, similar to those present in the outer membranes of Gram-negative cells, are necessary. Teichoic acid and lipoteichoic acid, which are negatively charged polymers on the cell surface, may play a vital role in the initial interaction of positively charged bacteriocins produced by gram-positive bacteria. Bacteriocins are primary metabolites that offer a significant advantage over standard antibiotics due to their easier biosynthesis routes compared to antibiotics, which are secondary metabolites (34). Altuntas proposes that the combination of organic acids with bacteriocins can be successful due to the enhanced positive charge of bacteriocins at low pH, which may aid in the penetration of bacteriocins through the cell wall. Furthermore, the solubility of certain bacteriocins may be enhanced at acidic pH levels, hence promoting their diffusion. The author stated that including two or more bacteriocins can yield promising outcomes, particularly when the bacteriocins come from different categories that target various cellular components (32). Rossland et al. stated that biological models are intricate, and LAB hinders the growth of spoilage and harmful bacteria by producing several antimicrobial compounds and competing for nutrients. The authors noted that when the LAB is present in significantly higher quantities than the pathogenic bacteria in the existing co-cultures (33). The rapid proliferation of a substantial population of LAB has the potential to hinder the growth of other species effortlessly by consuming the most readily assimilated nutrients and co-factors, or even by physically occupying the available space.

4. Conclusion

The findings of this study indicate that it is possible to extract *Lactobacillus* strains with strong probiotic potential from the faecal matter of newborns. *Lactobacillus* strains exhibit antibacterial properties against pathogenic microorganisms and demonstrate enhanced resilience to acidic environments. This study demonstrates a breakthrough in biomedical science by emphasizing the potential of *Lactobacillus* strains derived from infant faeces as probiotics. Human Breast Milk is a source of lactic acid bacteria. However, additional in vitro and in vivo investigations are necessary to fully understand the properties and benefits of these probiotic strains.

Acknowledgments

We gratefully acknowledge the laboratories of the Faculty of Science College, Wasit University, the X Jene Center, and AL-zahraa Teaching Hospital for facilitating our laboratory experiments.

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