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Isolation of *Lactobacillus* spp. from Healthy Infants' Feces and Study of their Antibiotic Susceptibility

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ABSTRACT: This study aimed to isolate *Lactobacillus* spp. bacteria from the feces of healthy newborns and investigate their susceptibility to antibiotic resistance. 120 fecal samples were obtained from infants under 12 months of age and subsequently identified using biochemical tests and molecular identification methods.

A total of 30 isolates, chosen randomly from the 90 positive samples, were selected to conduct a safety assessment. The isolates were analyzed for their hemolytic activity and susceptibility to antibiotics. Using 16S rRNA sequencing, a total of 30 positive -isolates were determined to be *Lactobacillus* spp. The results of the antibiotic resistance test indicated that the majority of the isolates showed susceptibility to tetracycline, chloramphenicol, Gentamicin, clindamycin, and amoxicillin while demonstrating resistance to Vancomycin, nalidixic acid, streptomycin, and Amikacin.

Conclusions: Based on these specific traits, the isolates of *Lactobacillus* spp. demonstrated favorable qualities as probiotics.

Keywords: *Lactobacillus* spp., Probiotics, antibiotic susceptibility, infants.

1. INTRODUCTION

The child's gut microbiota originates from various sources, namely the maternal gut, human breast milk (HBM), vagina, mammary gland, and the environment. (1). Breastfeeding is a fundamental factor in shaping the composition of the gut microbiota. Human breast milk is crucial for the establishment of the neonatal gut microbiota, as it supplies a consistent number of microbes to the infant's stomach a few weeks after birth (2). The transmission of the mother vaginal microbiota to infants is recognised as a means of establishing the first population of microorganisms in the infant's gut. Nevertheless, despite being primarily composed of bacteria belonging to the genus *Lactobacillus* spp (4). The process of microbial colonisation is a carefully coordinated event that leads to the formation of distinct microbial communities in various sections of the gut. Nevertheless, the process of colonisation can be impacted by a multitude of environmental conditions. An important aspect is the newborn diet, and it is widely acknowledged that human milk (HM) is the best diet for promoting proper development of the infant's microbiota (5). Prior to birth, the gastrointestinal system of the foetus is devoid of microorganisms, however microbial colonisation commences promptly following delivery. Various factors, including the method of delivery, premature birth, use of antimicrobial medications, hygiene conditions, and type of food (breast milk or formula), influence the presence and order of microbes in the gastrointestinal tract of infants (6). In 2001, the Food and Agriculture Organisation (FAO) of the United Nations and the World Health Organisation (WHO) revised and clarified the definition of probiotics. They defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (9). In order to be categorised as a probiotic, a bacterium must possess resistance to bile and gastric acid, as well as being non-pathogenic. The technology should

incorporate procedures that result in antimicrobial effects by enabling adherence to intestinal epithelial tissues. The *Lactobacillus* genus is widely recognised as being safe for human ingestion and demonstrates probiotic characteristics (10). In our investigation, our objective was to isolate and identify *Lactobacillus* spp. using biochemical tests and PCR targeting the 16S rRNA gene. Examine the *lactobacillus* isolates for their potential as probiotics by Evaluating their susceptibility to antibiotics.

2. MATERIALS AND METHODS

2.1 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 utilising 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 on the same day. The *Lactobacillus* MRS (De Man Ragosa Sharpe, Merck) broth and solid medium were employed for the cultivation and stimulation of *Lactobacillus* spp. Each sample was cultured in Man, Rogosa, Sharpe broth (MRS broth, 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.

2.2 Molecular Identification of *lactobacillus* spp.

The identification of the *Lactobacillus* spp. strains were confirmed by 16S rRNA sequence analysis. In the present study, the polymerase chain reaction (PCR) primer sequences 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.

2.3 Hemolytic activity

The LAB samples were incubated in an MRS medium at 37°C for a period of 18 to 24 hours. The hemolytic activity on agar plates containing sheep blood (Oxoid, Germany) was measured using streak plate techniques. Subsequently, the dishes were conserved in an incubator set at a temperature of 37°C. The occurrence of α-, β-, or γ-hemolysis was identified based on the presence of clear zones surrounding the bacterial colonies (14).

2.4 Antibiotic Susceptibility

The activated cultures in MRS medium were diluted to a concentration of 0.5 McF (with an absorbance of 0.08-0.1 at 625 nm) using physiological saline. The diluted cultures were then evenly dispersed over sterile MRS agar. The antibiogram discs were positioned on Petri plates at suitable intervals and subjected to incubation at a temperature of 37°C for a duration of 24 hours. The calliper was used to measure the diameters of the zones produced around the antibiotic discs after incubation, in millimetres. The measurements were assessed using the standards established by the National Committee for Clinical Laboratory Standards (NCCLS), categorising them as Resistant (R), Semi-Fine (I), or Sensitive (S).Gentamicin (CN) (10 µg); Tetracycline (T) (30 µg); Vancomycin (VA) (30 µg); Amikacin (AK) (10 µg); nalidixic acid (NA) (30 µg); chloramphenicol(C) (30 µg); streptomycin (S) (10 µg); clindamycin (CD)(2 µg); amoxicillin(AX)(30) discs were used (15).

3. RESULTS

3.1 Cultural and Microscopic Characteristics

The appearance of *Lactobacillus* spp. on MRS agar was observed to be spherical, white, shiny, and cream in color (Fig 1.). 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 bad for catalase activity. Positive results were obtained from 90 samples of *lactobacillus* spp. isolates.

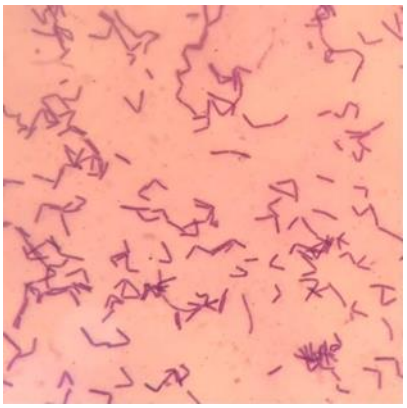

Figure 1. Gram staining results of *Lactobacillus* spp isolated from infants' feces

3.2 Molecular Analysis

The DNA of 30 randomly chosen isolates out of 90 positives from infants' feces samples was extracted. The presence of undamaged DNA fragments was verified by gel electrophoresis, as depicted in (Fig 2). 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.

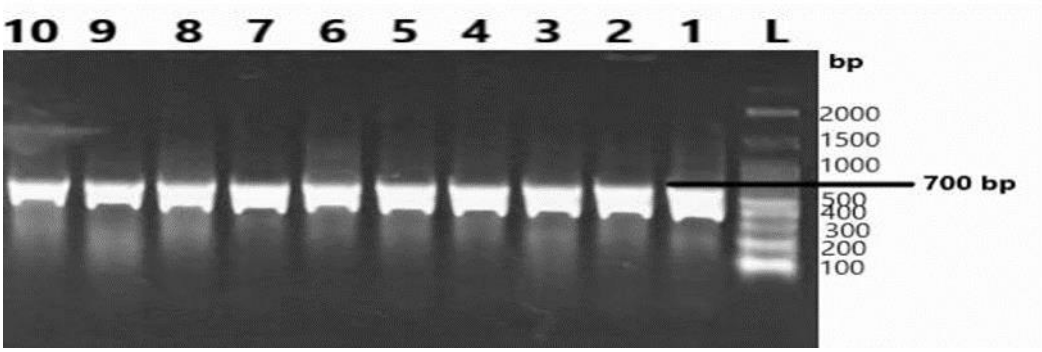

Figure 2. Agarose gel electrophoresis of PCR products PCR was performed with the primer for *Lactobacillus* spp

3.3 Hemolytic Activity:

The isolated strains exhibiting inhibitory activity were determined to be non-hemolytic, as they did not display any distinct or greenish zones surrounding the colonies developed on MRS blood agar plates.

3.4 Antibiotic Susceptibility

The sensitivity profiles of 30 *lactobacillus* spp. isolates against nine antibiotics are displayed in the table. The table displays the susceptibility rates for chloramphenicol (83.33%), Gentamicin (80%), amoxicillin (60%), Tetracycline (53.33%), and clindamycin (73.33%). All strains of *lactobacillus* spp. show resistance to Vancomycin (100%), streptomycin (66.66%), nalidixic acid (53.33%), and amikacin (36.66%).

Table 1. Susceptibility of *lactobacillus* spp. isolated from infants' feces to antibiotics

ISOLATES	VA	C	TE	S	NA	GEN	CD	AK	AX
1	R	S	R	IN	S	R	IN	R	R
2	R	S	R	IN	S	R	IN	R	R
3	R	S	R	IN	S	S	S	R	IN
4	R	S	R	R	S	S	S	R	R
5	R	S	R	R	R	S	S	S	IN
6	R	S	R	R	R	S	S	S	IN
7	R	S	R	R	R	S	S	IN	IN
8	R	S	S	R	R	S	S	R	IN
9	R	S	S	IN	R	S	S	R	IN
10	R	S	S	IN	R	S	S	R	IN
11	R	S	S	R	R	IN	S	R	S
12	R	S	S	R	S	R	S	R	S
13	R	S	S	R	R	IN	R	R	S
14	R	S	S	IN	R	S	R	R	S
15	R	S	S	IN	IN	R	R	S	S
16	R	S	S	IN	IN	S	IN	S	S
17	R	S	S	IN	S	S	IN	S	S
18	R	S	S	R	S	S	S	IN	R
19	R	S	S	R	IN	S	R	IN	IN
20	R	S	S	R	IN	S	S	IN	S
21	R	R	S	R	IN	S	S	IN	S
22	R	R	S	R	IN	S	S	IN	S
23	R	R	S	R	IN	S	S	IN	S
24	R	R	IN	R	R	S	S	IN	S
25	R	R	IN	R	R	S	S	IN	S
26	R	S	IN	R	R	S	S	S	S
27	R	S	IN	R	R	S	S	S	S
28	R	S	IN	R	R	S	S	S	S
29	R	S	IN	R	R	S	S	S	S
30	R	S	IN	IN	R	S	S	S	S

S: sensitive, IN: intermediate, R: resistant. Gentamicin (CN) (10 µg); Tetracycline (T) (30 µg); Vancomycin (VA) (30 µg); Amikacin (AK) (10 µg); nalidixic acid (NA) (30 µg); chloramphenicol(C) (30 µg); streptomycin (S) (10 µg); clindamycin (CD) (2 µg); amoxicillin (AX) (30)

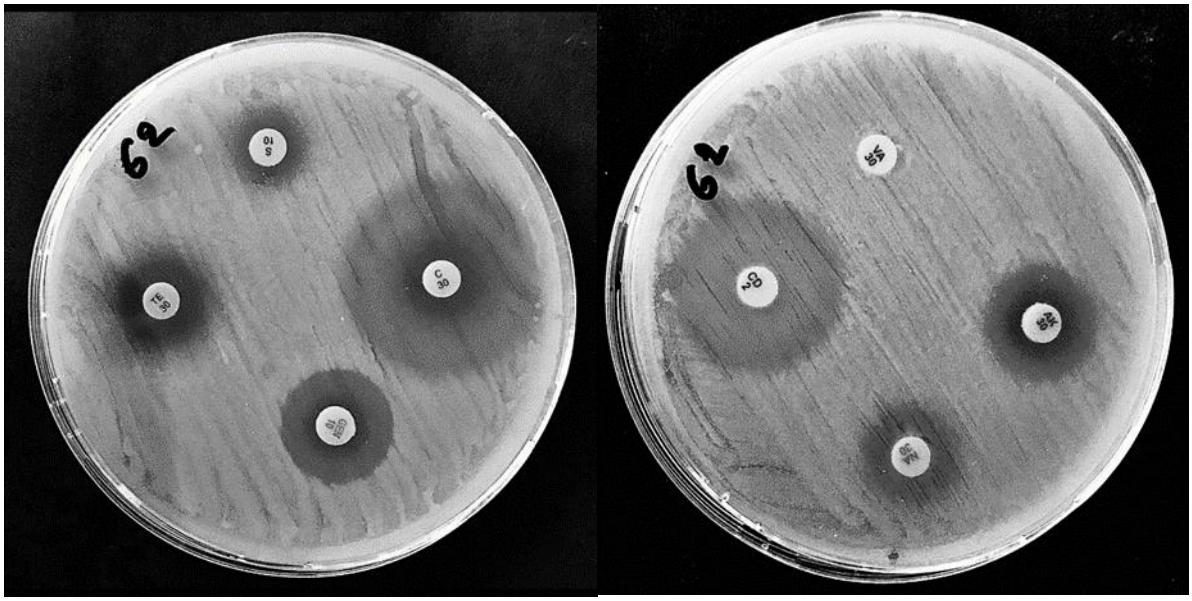

Figure 3. Resistance of *lactobacillus* spp. isolated from infants' feces to antibiotics

4. DISCUSSION

The probiotic microorganisms must be safe, meaning that the probiotic bacteria, such as *lactobacilli*, should not have the ability to produce hemolysis in the host body. None of the 30 *Lactobacillus* isolates obtained from infants' faeces exhibited α or β hemolysis throughout the immediate examination. These findings are consistent with previous investigations that have demonstrated no hemolytic activity in *lactobacilli* that were obtained from the faeces of babies (16). Furthermore, the probiotic *Lactobacillus* spp. is susceptible to antibiotics, which means it is unable to transmit the resistance trait to other harmful bacteria. The antibiotic resistance of *lactobacilli* can be seen as both advantageous and disadvantageous, making it a complex and contradictory phenomenon. Intrinsic resistance is considered advantageous for probiotic bacteria as it allows them to withstand antibiotic treatment, hence preventing or treating illnesses. Antibiotic resistance can occur through inherent mechanisms, acquired mechanisms, and/or mutations. In LAB (lactic acid bacteria), transposons and conjugative plasmids are commonly found to contribute to antibiotic resistance (17). The present investigation demonstrates that there is a susceptibility rate of 83.33% to chloramphenicol and an intermediate to high susceptibility rate of 53.33% to tetracycline. Previous investigations have also shown that the *lactobacilli* species examined in this study are very susceptible to chloramphenicol and tetracycline, as indicated by (18) and (19). *Lactobacilli* are typically susceptible to antibiotics that hinder protein synthesis, such as chloramphenicol (20). Tetracycline exhibits distinct antimicrobial properties, with both Gram-negative and Gram-positive bacteria being susceptible to its effects. Its antibacterial activity primarily targets the ribosome within bacterial cells. By impeding protein synthesis, it hinders the addition of polypeptide chains to amino acids in this bacterial organelle (21). The research conducted by (22), and (23) indicated that certain species of *Lactobacillus* spp. exhibited significant resistance to vancomycin. In our current study, all tested *lactobacilli* were shown to be completely resistant to vancomycin (100%), consistent with our findings Vancomycin functions by attaching to the D-alanyl-D-alanine (D-ala-D-ala) part of the peptidoglycan precursor, which stops the creation of the cell wall. The enzymatic function of VanX (also known as protein D or D-dipeptidase) leads to the production of atypical peptidoglycan building blocks that end in D-ala-D-lactate instead of D-ala-D-ala, hence eliminating the drug target. This vancomycin resistance mechanism is widely recognized as the most well-defined. It is thought to be an inherent trait, typically encoded in the chromosome, and is not capable of being induced or transferred in *lactobacilli*(24). Several strains of the *Lactobacillus* genus are commonly employed as probiotics for the prevention of Clostridium difficile infection (25). The study revealed that the sensitivity to gentamicin was 80%. The study found that the strains of *Lactobacillus* spp. exhibited comparable resistance to vancomycin, but were extremely susceptible to gentamicin. These results are similar to other studies (2). Gentamicin and Neomycin, which belong to a group of amino acids, have the ability to inhibit protein synthesis in bacteria by binding to a specific ribosomal subunit. Additionally, they possess antibacterial characteristics that are effective against specific types of Gram-positive and aerobic Gram-negative bacteria (26). The *lactobacillus* spp. isolates examined in the study demonstrated a resistance rate of 66.66% to streptomycin. The occurrence of this study was documented by other investigations, such as (27)(28). In addition, they exhibited resistance to aminoglycosides. The majority of *Lactobacillus* species possess inherent resistance to aminoglycosides such as gentamicin, kanamycin, streptomycin, and neomycin (29). *Lactobacilli* often exhibit resistance to Gram-negative spectrum antibiotics such as kanamycin and streptomycin. Strains exhibiting this

form of acquired resistance possess a limited capacity for horizontal transmission (30). Most *Lactobacillus* species are vulnerable to antibiotics that hinder protein synthesis, such as erythromycin, tetracycline, clindamycin, and chloramphenicol (31). The majority of Lactobacillus spp. strains in our investigation exhibited resistance to amikacin. The percentage is 36.33%. *Lactobacilli* are generally vulnerable to antibiotics that hinder the process of protein synthesis. Regarding this matter, *lactobacilli* typically possess inherent resistance to aminoglycosides. The majority of *Lactobacillus* spp. strains in our investigation exhibited resistance to amikacin (24) The isolates showed a significant susceptibility to clindamycin, with a rate of 73.33%. However, it is worth noting that this particular panel of *Lactobacillus* spp. strains exhibited a low degree of resistance to clindamycin (32). The absence of transferrable resistance to therapeutic antibiotics is a crucial factor in choosing the right functional strain. Although clindamycin is one of the most effective antibiotics against Gram-positive microorganisms, (33). Typically, the strains were responsive to amoxicillin, which is an antibiotic that targets Gram-positive bacteria. Amoxicillin exhibited a sensitivity rate of 60%. Furthermore, only four isolates displayed unconventional resistance to amoxicillin. This antibiotic, in combination with clarithromycin and a proton pump inhibitor, is a commonly used first-line triple treatment for treating Helicobacter pylori infection (34). Probiotics can enhance the effectiveness of H. pylori eradication therapy (35). Our findings indicate that *lactobacilli* exhibit a significant degree of susceptibility to amoxicillin. A significant majority of isolates exhibited resistance to quinolones, specifically nalidixic acid (53.33%). Intrinsic resistance mechanisms may contribute to the high resistance to quinolones. This likely contributes to the elevated rates of resistance to nalidixic acid seen in the current investigation. It has been observed that the antibiotic susceptibility of *lactobacilli* may differ depending on where they are obtained from (36).

5. Conclusions

Ultimately, this study centered on the extraction and examination of putative probiotic microorganisms from the faeces of infants. A total of thirty positive strains were found using the process of 16S rRNA sequencing. None of the examined isolates exhibited hemolysis, and they were susceptible to medicines that are crucial for treatment. Our observations may provide insight into the reasons why newborns exposed to antibiotics experience a higher incidence of health issues.

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