

Isolation of *Lactobacillus Plantarum* from Human Breast Milk and Study their Susceptibility to Antibiotics

Shaimaa Ghanim Abdullah, Jawad Kadhim Isa*

Department of Biology, College of Science, Wasit University, Wasit, Iraq

ABSTRACT

Probiotics have an assortment of implementations in various domains. Nowadays, probiotics are used as a method to prevent many disorders and treat diseases. In this study, *L. plantarum* was isolated from breast milk with the intention of studying their properties as probiotics in view of their importance to infant health. 18 positive samples out of 108 have been isolated and identified, by using selective media (MRS-VA), biochemical tests and molecular diagnosis using the 16S rRNA gene. This study revealed a correlation between the number of deliveries and the presence of *L. plantarum* (P-Value 0.047), indicating that as the number of births increases, the concentration of *L. plantarum* in breast milk decreases. whereas there is no relationship between the existence of *L. plantarum* and mother age, infant age, or type of delivery. The probiotic potential was assessed by determining antibiotic susceptibility through the disc diffusion method. Among the 18 isolates, resistance was observed against vancomycin, gentamicin, and streptomycin respectively, while intermediate resistance was noted for penicillin and chloramphenicol respectively. Conversely, susceptibility was exhibited towards ampicillin, tetracycline, and clindamycin respectively. Notably, none of the isolates exhibited hemolytic activity. Based on these findings, the isolated *Lactobacillus plantarum* displayed promising probiotic characteristics, highlighting human milk as a potential source of these beneficial bacteria.

Keywords: *Lactobacillus Plantarum*, Susceptibility, Antibiotics, Probiotic

INTRODUCTION

Probiotics are viable microorganisms that reduce the toxic activity of pathogens and exert favorable influences on the microflora of the intestine, consequently bestowing advantageous health outcomes upon the host when introduced into the body in appropriate quantities [1]. As per the World Health Organization and the Food and Agriculture Organization, probiotics are defined as viable microorganisms that, when consumed in adequate amounts, confer beneficial effects on the host. These microorganisms are typically present in specific food products, including cured meats and fermented vegetables, and can also be introduced during dairy production [2]. Consumption of probiotics has been associated with several health advantages in humans, such as enhanced immune function, protection against diarrheal illnesses, reduction in cholesterol levels, defense against hospital-acquired and respiratory infections, mitigation of immune-inflammatory disorders, and potential anticancer effects [3]. Numerous microorganisms have the potential to act as probiotics, *Lactobacillus* and *Bifidobacterium* species being the most commonly utilized [4]. Given the multitude of benefits linked to this genus, extensive research has been conducted on the isolation of *Lactobacillus* species from various origins, with some being evaluated for their probiotic properties [5]. Milk of mother is known as the best nutritional provenance during the first two years of infant life for the reason content of nutritional has evolved together with the requirements of growing infants such as it having a number of bioactive features that support the health of the immune system [6]. Additionally, breast milk can maintain the infant against transferable diseases, largely attributed to the presence of antimicrobial compounds, immunoglobulins, and immunocompetent cells among its constituents [7]. Probiotic bacteria are found in breast milk at levels ranging from 10^2 to 10^7 colony-forming units per mL [8]. The *Lactobacillus* species isolated from breast milk include *L. gasseri*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. reuteri*, *L. fermentum*, *L. brevis*, *L. helveticus*, *L. casei*, *L. paracasei*, *L. delbrueckii*, *L. curvatus*,

L. salivarius, *L. pentosus*, and *L. acidophilus* [9, 10]. *Lactobacillus plantarum* is a versatile species within the *Lactobacillus* genus. This subgroup of lactic acid bacteria (LAB) consists of prokaryotes that live in several niches, as well as the human gut and animal gut, mucosa of the vagina, food products, and breast milk [11]. In the medicinal domain, *L. plantarum* is currently under investigation for an expanding array of applications, for example, the healing of skin injuries and burn infections [12]. treatment of mucosal infections [13]. mitigation of acute and chronic GIT infections [11]. management of gut inflammatory conditions and urinary tract infections, cholesterol-lowering properties [14]. As well as potential effects on colon cancer and cognitive impairments [15, 16]. Antibiotics have been utilized over time as a tool for bacterial eradicating and treating inflammation. Prolonged bacterial exposure to antibiotics has facilitated bacterial adaptation and the development of resistance through genetic modifications within their DNA [17]. The emergence of antibiotic-resistant bacterial strains is a direct consequence of the increased utilization of antibiotics in human and animal populations. Bacteria have evolved various resistance mechanisms to thrive in challenging ecological conditions [18]. Given the valuable health properties of *Lactobacillus*, various studies were disclosed on the isolation of it from many sources and tested it as a probiotic. Since the microbiota of human milk contributes to the microbial composition of infants' intestines, it aids in bacterial colonization and potentially influences immune system improvement [19]. Recent research has demonstrated the bacteria ingested orally by breastfeeding mothers can subsequently be identified within their lacteal secretions [20]. Suggesting a potential "internal pathway" for bacteria to access the mammary gland. Two hypotheses initially proposed by Fernandez [21] are widely recognized as the "retrograde transfer" which refers to bacteria that have an exterior source via the transmission of microbes from the infant's oral cavity to the mammary duct during lactation, and the "entero-mammary pathway" for the migration of inner

bacteria. The entero-mammary " postulates immune cell-mediated bacterial translocation from gut of the mother's to the mammary – glands, whereas certain of these bacteria can well colonized the available site [22]. The primary aim of this study was to isolate *L. plantarum* from human breast milk, conduct characterization of the species using biochemical assays and PCR (16s rRNA gene), and evaluate its resistance to antibiotics.

MATERIALS AND METHODS

Collection of Samples

One hundred and eight breast milk samples were randomly acquired from breastfeeding mothers at AL-Kut Hospital and AL-Zahraa Teaching Hospital in Wasit province, Iraq, between 1 October 2023 and 5 February 2024. The cleaning process involved the application of 70% ethanol alcohol on the nipple and mammary areola, with the initial drops discarded to prevent alcohol contamination.

Sterile breast pumps were employed to collect milk samples, and approximately 5–15 ml of each sample was transferred to a sterile tube. These tubes, containing the samples, were then carefully packed in insulated boxes with dry ice and dispatched to the laboratory within an hour.

L. Plantarum Isolation

L. plantarum was isolated by adding 1 ml of milk samples to 9 ml of sterilized de Man Rogosa Sharp (MRS) broth (Hi-Media, Mumbai, India), thoroughly mixing the solution, and incubating it for 24 hours

Molecular Diagnosis of *L.plantarum*

Sample Preparation and Extraction of DNA

Table 1: Contents of DNA Extraction Kit (Presto™ DNA Extraction Kit)

Component	Volume (μL)
GB Buffer	200 μl
GT Buffer	200 μl
Gram + Buffer	200 μl
Lysozyme	0.8 mg
Proteinase K ²	20 μl
W 1	400 μl
Wash Buffer	600 μl
Elution Buffer	25-50 μl

Initially, all *L.plantarum* isolates were cultured in MRS broth at 37°C for 24 hours. Subsequently, 1×10^9 bacterial cells were transferred into a 1.5 ml microcentrifuge tube, followed by centrifugation at 16000 rpm for 1 minute and removal of the supernatant. Each sample was treated with 200 μl of Gram-positive buffer and 0.8 mg of lysozyme, then incubated at 37°C about 30 minutes. Addition of (20 μl) of K-proteinase was followed by homogenization through pipetting, and the mixture was further incubated at 60°C for a minimum of 10 minutes. The addition of 200 μl of GB buffer was followed by mixing via vortex for 10 seconds and subsequent incubation at 70°C for a minimum of 10 minutes. The sample lysate was then treated with (200 μl) of absolute ethanol and forcefully mixed for ten seconds. All resulting mixtures were carefully transferred into the GD column, which had been assembled in a 2 ml collection- tube provided with the kit. Centrifugation at 16000 rpm for a duration of 1 minute was conducted, after which the flow-through was discarded, and the GD column was moved to a new 2 ml collection tube. Following this, 400 μl of W1 buffer was introduced to the GD- column, and another round of centrifugation (at 16000 rpm) for 30 seconds took place before discarding the flow-through. The GD- column was then reinserted into

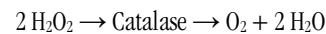
at 37°C in aerobic conditions. Subsequently, 0.1 ml of the solution was plated using the pour plate method on selective de Man Rogosa Sharp (MRS) agar plates containing vancomycin. This process was conducted in duplicate, with the medium supplemented with 0.25% l-cysteine to facilitate the selection for LAB and promote the evolution of *Lactobacillus*. Incubation period was 48 to 72 hours at 37°C in anaerobic conditions. The morphology of the bacteria isolated was checked by microscope [23].

Preparation of Selective Media

Breast milk harbors various lactic acid bacteria that share similarities in morphology, culture requirements, and biochemical tests, necessitating the use of selective media for *L. plantarum* isolation. To prepare the MRS media, a vancomycin solution of 0.5 mg ml⁻¹ was added to 1 L of fluid MRS agar before pouring, achieving a final concentration of 1 mg l⁻¹ [24, 25].

Catalase Test

A catalase assay involved the application of two drops of 3% hydrogen peroxide (H₂O₂) on cultures that had matured for 24 hours on a glass slide. The formation of oxygen bubbles during the catalase assay indicates the presence of the catalase enzyme, which breaks down H₂O₂ into water (H₂O) and oxygen (O₂).



Strains exhibiting no gas bubbles were identified as catalase negative, in contrast to catalase-positive strains like *S. aureus* [26].

the (2 ml) collection tube, 600 μl of wash buffer was added, and centrifugation (at 16000 rpm) for 30 seconds was repeated before discarding the flow-through. The GD- column was reinserted into the 2 ml collection tube once more for the drying of the column matrix, with centrifugation performed for an additional 3 minutes at 16000 rpm. The dried GD -column was carefully moved to a clean 1.5 ml microcentrifuge tube, where 50 μl of elution buffer was added and left at room temperature for a minimum of 3 minutes. Centrifugation (at 1600 rpm) for 30 seconds was done, and the sample was stored at -20 °C until future use.

Detection of Gene Using PCR

The primer pair employed in this investigation consisted of planE forward -AGAGCACTATTAGGTAGTAAATAGCTGTGA- planE reverse -AAATAACATCATACAA GGGGGATTATTT- Primers were purchased from (Macrogen, Korea) [27]. The precipitation of the lyophilized primers was initiated using a centrifuge at 45000 rpm for 1 minute, followed by the addition of deionized sterile distilled water to each tube in a specific size to achieve a solution of (100μM, 100 pmole/μl) as per the manufacturer's guidelines. These tubes were then stored as primer

stock solutions at deep freezing temperatures (-20°C) until required. In order to create a working solution of 100 µl with a concentration of 10 pmole/µl, the stock solution (10 µl) was combined with 90 µl of deionized sterile distilled water following the established dilution formula: C1V1=C2V2.

Subsequently, 1µl of the forward primer and 1µl of the reverse primer, which had been prepared earlier, were introduced along with (12.5 µl) of the master mix plus 5 µl of the samples DNA into a PCR tube. The amplification process was showed in a thermocycler by using the program specified in the provided table 2.

Table 2: Thermocycler Amplification Protocol

Steps	Temperature	Time	No. ofCycles
Initial denaturation	94 C°	5 min	1 Cycle
Denaturation	94 C°	30 sec	35 Cycles
Annealing	56 C°	40 sec	
Extension	72 C°	30 sec	
Holding	4 C°	10min	1 Cycle

Gel Electrophoresis Protocol

Agarose gel electrophoresis has been authorized for the purpose of examining the process of amplification and specificity of DNA. The preparation of agarose gel entails the addition of agarose powder to 1X TBE Buffer, in this investigation, 1.5% agarose gel was utilized. Following this, the mixture was placed in a water bath and heated until it attained clarity, after which 5µl of a safe red dye was added once it cooled to 50°C. Subsequently, the agarose was allowed to solidify at room temperature for a period of 30 minutes. Wells for loading DNA samples were established using a comb. The melted agarose is poured carefully into the gel tray and permitted to solidify for 20 minutes. Afterward, the set comb was delicately removed, and the gel plate was positioned in the gel tank. TBE buffer was added gently to the tank until it exceeded the gel surface by 1-2 mm. Following this step, DNA samples were loaded into the designated wells of the gel, and an electrical current of 100 volts was applied for a duration of 40 minutes, enabling the migration of DNA from the cathode to the anode poles. Visualization of the bands within the gel was accomplished through the use of a UV transilluminator emitting light at 350 nm.

Hemolytic Activity of *L. Plantarum*

To evaluate hemolytic activity of the isolates, streak plate technique was applied by culturing pure bacterial isolates on blood agar that contained human blood about 5% V/V and was incubated at 37°C for 24 _ 48 hours.

A greenish discoloration around the bacterial colony refers to alpha hemolysis while a clear zoon around the bacterial colony refers to beta hemolysis. On the other hand, the absence of hemolysis refers to gamma hemolysis. The results of experiment were compared with positive bacteria for hemolytic analysis (*S.aureus*) [28, 29].

Antibiotic Susceptibility

The susceptibility test of *L. plantarum* toward the antibiotics typically employed in medicine of human was assessed in the context of this study by diluting broth cultures overnight. Spectrophotometer

was used to read the solution at (625 nm) of wavelength and the turbidity that refers to absorbance was found about (0.08 to 0.1) that conforming to (0.5) on the McFarland measure. For the sensitivity plates, large (15-cm.) Petri dishes were employed with Mueller-Hinton agar (5 to 6 mm. in depth). The bacterial broth suspension was evenly streaked in 3 planes on the medium's surface using a cotton swab. Once the inoculum had dried (3 to 5 minutes), the disks were positioned on the agar with sterilized forceps that had been subjected to a flame and gently pressed down to ensure contact, then incubated for 24 hours at 37°C. The assay was conducted in triplicate and the average diameter of the inhibition zones surrounding the antibiotic discs was meticulously documented. The susceptibility was expressed in terms of resistance (R), intermediate susceptibility (I), and susceptibility (S) and the criteria for determining drug resistance were taken from the Executive Standard for Antimicrobial Drug Susceptibility Testing [30].

Statistical Analysis

The statistical analyses of all the results were done by using the system SPSS (Statistical Package for the Social Sciences), Chi-square test. A P-value ≤ 0.05 indicates statistical significance [31].

Results and Discussion

Isolation of *L. Plantarum*

Lactobacillus species were isolated from human breast milk obtained from healthy volunteer mothers to serve as the primary source for isolation. The microorganisms underwent cultivation on selective media (MRS-VA) at 37 °C in aerobic conditions. Identification was primarily depended on microscopic and colony morphology, alongside the outcomes of catalase testing. This led to the classification of 18 (16.7%) isolates as Gram-positive, rod-shaped (Figure 1), and catalase negative (Figure 2). Specifically, the colony of *L. plantarum* displayed distinct features such as an ivory and round shape with a protruded center, orderly edges, two blunt round ends, and a short straight stem (Figure 3).



Figure 1: Gram Staining Results of *L. Plantarum* Isolated from Breast Milk

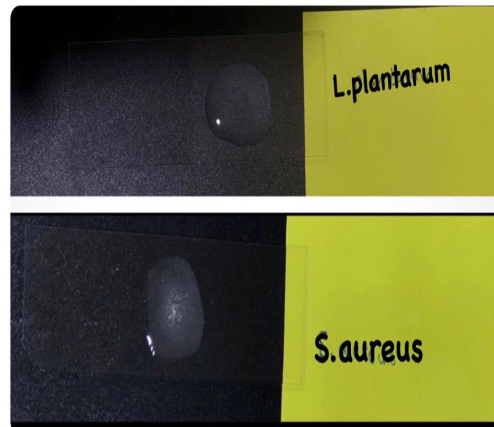


FIGURE 2: Catalase Test Result of *L. Plantarum* Isolates in Contrast to *S. Aureus*.

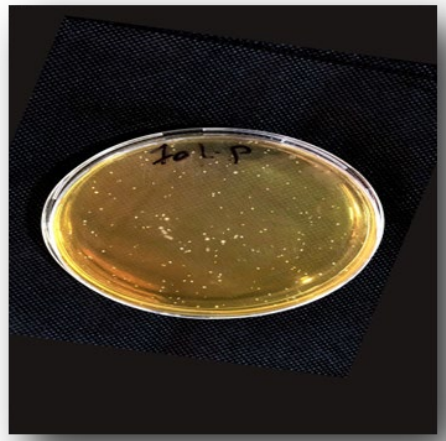


FIGURE 3: Isolation of *L. Plantarum* from Breast Milk on MRS-VA.

Relationship between the Factors that used in this Study and Presence of *L. Plantarum*

TABLE 3: Distribution of *L. Plantarum* according to Age of Mothers

		<i>L. Plantarum</i>		Total
		Negative	Positive	
Mother Age (Year)	< 20	10	1	11
	20-29	52	11	63
	30-39	22	4	26
	>40	6	2	8
Total		90	18	108

P-Value 0.822

There were no significant differences ($p > 0.05$) Between the presence of *L. plantarum* and mother age.

TABLE 4: Distribution of *L. Pantarum* according to Age of Infant

		<i>L. Plantarum</i>		Total
		Negative	Positive	
Infant Age (Month)	<6	59	10	69
	6-11	18	3	21
	>12	13	5	18
Total		90	18	108

P-Value 0.383

There were no significant differences ($p > 0.05$) Between the presence of *L. plantarum* and infant age.

TABLE 5: Distribution of *L. Plantarum* According to Pregnant Period

		<i>L. Plantarum</i>		Total
		Negative	Positive	
Pregnant Period (Month)	7	2	2	4
	8	7	0	7
	9	81	16	97
Total		90	18	108

P-Value 0.100

There were no significant differences ($p > 0.05$) Between the presence of *L. plantarum* and Pregnant period.

Table 6: Distribution of *L. Plantarum* according to Type of Delivery

		<i>L. Plantarum</i>		Total
		Negative	Positive	
Type of Delivery	Caesarean Delivery	51	11	62
	Normal Delivery	39	7	46
Total		90	18	108

P-Value 0.728

There were no significant differences ($p > 0.05$) Between the presence of *L. plantarum* and type of delivery.

Table 7: Distribution of *L. Plantarum* According to Number of Deliveries

		<i>L. Plantarum</i>		Total
		Negative	Positive	
No of Deliveries	1	23	7	30
	2	23	2	25
	3	13	5	18
	4	15	0	15
	5	8	0	8
	6	4	1	5
	7	3	2	5
	8	1	0	1
	9	0	1	1
Total		90	18	108

P-Value 0.047

There existed a notable significant difference ($p < 0.05$) between the number of deliveries and the presence of *L. plantarum* (*P-Value* 0.047).

This discrepancy possibly stemmed from the decline in probiotic bacteria in breast milk with increasing births, which is associated with an altered microbial composition. Such alterations could impact the health of both mothers and newborns due to potential changes in the mammary gland microbiome.

Molecular Analysis

Through the utilization of the PCR technique, the results obtained from the 16s rRNA specific primers indicated that 18 out of 108 samples of breast milk contained *L. plantarum*, accounting for 16.67% of the total isolates. All isolates were characterized as Gram-positive and catalase negative. This was further illustrated in Figure 4, where the bands of the PCR product (530 bp) were distinctly visible on the 1.5% agarose gel analysis



Figure 4: Electrophoresis of Agarose Gel of 16s-rRNA Gene of *Lactobacillus* Isolate using Polymerase chain Reaction, that was Fractionated on 1.5% Agarose Gel (100 V/40min). Lane [L]: 100bp DNA Ladder. Lane 1- 5: *L. Plantarum* (530bp)

Hemolytic Activity

The isolates exhibited no hemolytic activity in contrast to *S. aureus*, which is known for its hemolytic activity and was included as a control in the study. The findings of this research align with previous

studies [32]. *L. plantarum*, lacking hemolytic activity, is deemed suitable as a probiotic due to its safe nature, making it beneficial for intestinal health maintenance and potential therapeutic applications [33].

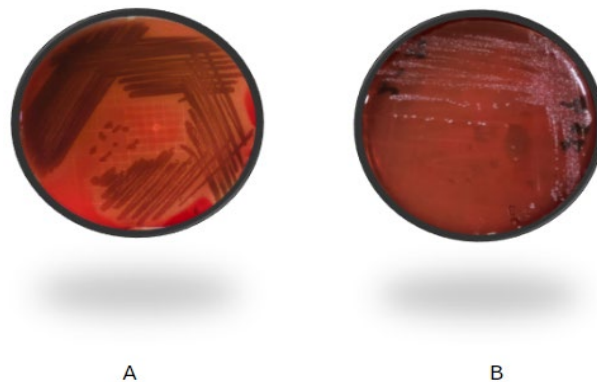


Figure 5: A: Beta Hemolytic Activity of *S. Aureus* on Blood Agar, B: Non-Hemolytic Activity of *L. Plantarum* on Blood Agar

Antibiotic Susceptibility

The *L. plantarum* isolates exhibited varied degrees of susceptibility and resistance towards the antibiotics used for testing (Table8),

(Figure6). Eight antibiotics (Liofilchem,Italy) have been used in current study, which involve: vancomycin (VA)tetracycline (TE), streptomycin(s), gentamycin (CN), Penicillin G (S)chloramphenicol(C), clindamycin (CD)and Ampicillin (AMP).

Table 8: Susceptible of *L. Plantarum* in Breast Milk to Different Antibiotics

Antibiotic	Cod	Clinical Decision			Total %
		Resistance%	Intermediate%	Susceptible%	
Ampicillin	AMP	0	0	100	100%
Penicillin G	P	11.1	66.7	22.2	100%
Vancomycin	VA	100	0	0	100%
Gentamycin	CN	77.8	16.7	5.5	100%
Streptomycin	S	88.9	11.1	0	100%
chloramphenicol	C	0	88.9	11.1	100%
clindamycin	CD	11.1	22.2	66.7	100%
Tetracycline	TE	0	33.3	66.7	100%

The efficacy of antibiotics is attributed to four distinct mechanisms, which involve the inhibition of DNA replication, protein synthesis, cell wall formation, and folic acid metabolism [34]. The susceptibility of *L. plantarum* to Ampicillin was found to be 100% consistent with previous findings [35], while it displayed intermediate susceptibility to Penicillin G at 66.7% aligning with previous research [36]. Both antibiotics act as inhibitors of cell wall synthesis. Ampicillin, functioning as a β -lactamase inhibitor, disrupts the peptidoglycan layer and induces cell lysis by interacting with penicillin binding proteins (PBPs) [37]. On the other hand, Penicillin G inhibits the final step of crosslinking of cell wall synthesis by interfering with transpeptidase activity. The mechanism of action of Penicillin G involves acting as an analogue of the L-Ala- γ -D-Ala segment of the pentapeptide chain, albeit lacking the crucial carboxylate groups essential for its effectiveness [38]. This study results demonstrate the complete resistance 100% of *L. plantarum* to vancomycin, consistent with prior research [39]. The chromosomal mediation of vancomycin resistance in *Lactobacillus* species is highlighted [40]. The lack of efficacy of vancomycin against *Lactobacillus* species is attributed to their genetic resistance stemming from the weak binding affinity of vancomycin to their cell walls. This underscores the significance of investigating the peptidoglycan composition in the cell walls of these bacteria, suggesting the presence of a natural ligase that catalyzes the production of D-Ala-D-Lac instead of the target D-Ala-D-Ala that vancomycin typically interacts with [41]. *L. plantarum* shows 77.8%

resistance to gentamicin and 88.9% resistance to Streptomycin, aligning with previous research [40, 42]. *Lactobacillus* species commonly demonstrate resistance to aminoglycoside antibiotics; this resistance is predominantly ascribed to the inactivation of the antibiotic through enzymatic processes involving acetyltransferases, nucleotidyl transferases and phosphotransferases. Nevertheless, alternative mechanisms may also play a role, including a reduction in the uptake of aminoglycosides, diminished cellular permeability, and alterations in ribosome-binding sites, particularly concerning streptomycin [43]. The findings of this study reveal an 88.9% intermediate susceptibility of *L. plantarum* to Chloramphenicol, confirming previous discoveries [44]. Chloramphenicol antibiotics operate by inhibiting peptidyl transferase, a crucial enzyme placed on the 50S- ribosomal subunit that is essential for synthesis of protein, thereby disrupting t-RNA binding to the ribosomal A locate and hindering protein synthesis [45]. Furthermore, this study demonstrates a susceptibility of 66.7% to clindamycin, consistent with established literature [46]. Clindamycin, belonging to the lincosamides group, impacts peptidyl transferase reactions, resulting in the premature detachment of incomplete peptide chains [47]. In conclusion, more than 66.7% of the isolates in this study display susceptibility to Tetracycline, consistent with previous findings [42]. Tetracycline antibiotics target the preserved sequence of 16S- rRNA in the ribosomal 30S subunit, obstructing tRNA binding to the A-site and thereby impeding the protein synthesis process [34].

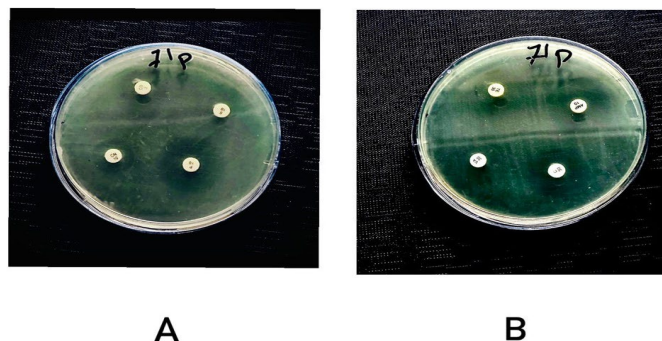


Figure 6: A Sensitivity of *L. Plantarum* to Streptomycin, Gentamycin, Penicillin and Clindamycin.

B Sensitivity of *L. plantarum* to vancomycin, tetracycline. Chloramphenicol and ampicillin.

Conclusion

Breast milk undoubtedly considered as Important source for the isolation of *L. plantarum* which functions as probiotic and significantly contributes to the health of infants. Furthermore,

antibiotic susceptibility results indicated that these isolates have the potential to be utilization as probiotics. Additionally, the *L. Plantarum* isolates did not exhibit any hemolytic activity, thereby indicating its potential application as supplements in the food industry and in functional foods for promoting public health. Finally, the addition of *L. plantarum* as a probiotic in formula milk would be advantageous for infants who do not consume breast milk.

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