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

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ABSTRACT: Probiotics have an assortment of implementations in various domains. Nowadays, probiotics are used as a method to prevention many disorders and treat diseases. In this study was isolated L.*rhamnosus* from breast milk with the intention to studying their properties as probiotics in view of important it on infant health. 24 positive samples out of 108 have been isolated and identification by using biochemical tests and molecular diagnosis using the 16S rRNA gene. This research revealed relationship between pregnant period and existence of *L. rhamnosus* (P-Value < 0.05). this relationship refers to the milk of mothers who have completed nine months of pregnancy had high percent of *L.rhamnosus* (75%) compared to mothers who delivery in seventh or eighth month of pregnancy whereas there no relationship (P-Value > 0.05)between existence of *L.rhamnosus* and mother age, infant age and type of delivery. The probiotic potential was assessed by determining antibiotic susceptibility through the disc diffusion method. Among the 24 isolates, resistance was observed to vancomycin and intermediate resistance to penicillin, streptomycin and clindamycin respectively, while susceptibility was shown to ampicillin, tetracycline, chloramphenicol and gentamycin respectively. Notably, none of the isolates exhibited hemolytic activity. Based on these findings, the isolated *Lactobacillus rhamnosus* displayed promising probiotic characteristics, highlighting human milk as a potential source of these beneficial bacteria.

Keywords: *Lactobacillus rhamnosus*, probiotic, breast milk,16S rRNA, antibiotics.

1. INTRODUCTION

Probiotics are viable microorganisms that reduce the toxic activity of pathogens and exert favorable influences on 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]. One of probiotics advantages is alteration of microbiota in gastrointestinal tract, alleviation of lactose intolerance, enhancement of the bioavailability of macronutrients and micronutrients, along with the mitigation of allergic occurrences in predisposed individuals [3*]. Ingestion of probiotics is associated with numerous health benefits, for example enhanced immune systems, support against diarrheal illnesses, anticancer effects, lowering of cholesterol and decrease of immune-inflammatory complaints [4]. There are* many microorganisms that could potentially function as probiotic, of which *Lactobacillus* and *Bifidobacterium* species are the most used [5].

Outstand to numerous benefits gained from this genus, various studies were showed on the isolation of the *Lactobacillus* spp. from many sources and tested it as probiotics [6].

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 the requirements of growing infant such as, it has a number of bioactive features that support the health of immune system [7]. Furthermore, breastfeeding can maintain the infant against transferable diseases This advantage outcome of some components of breast milk such as, diverse antimicrobial combinations, numerous of immunoglobulin and immunocompetent cells [8]. In breast milk probiotic bacteria are present in an amount of 10^2–10^7 colony forming units per mL [9]. The *Lactobacillus* species isolated from breast milk to date are *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* [10,11,].

L. rhamnosus has a number of medical applications, such as: in severe watery diarrhea cases it acts to reduce the duration and frequency of diarrhea, in respiratory and gastrointestinal tract symptoms infections act to decrease the symptoms period and decrease infection risk [12]. *L. rhamnosus* seems to be useful in hindering bacteria growth and act to decrease pH and preventing recurrence of bacterial vaginosis [13]. Moreover, Consumption of *L. rhamnosus* on a methodical rate that leads to decreasing the danger of *Staphylococcus aureus* that existence in the gut. *L. rhamnosus* plays an important role in decreasing the colonization of pathogenic bacteria in the gut by a mechanism known as competitive inhibition. [14]. It protects against allergic illness by reducing the symptoms of eczema, aimed at averting food hypersensitivities and intolerance to bovine milk.[15]

Antibiotics have been utilized over time as a tool for bacterial eradicating and treating inflammation. The continuous of bacterial exposure to antibiotic has facilitated the ability of bacteria to adapt and develop resistance through genetic alterations within the DNA [16]. Antibiotics have been extensively utilized in various domains such as health of human, poultry breeding, and farms of fish [17]. antibiotics effectiveness is attributed to four distinct mechanisms, which include the inhibition of DNA replication, protein synthesis, cell wall formation, and folic acid metabolism [18]. The emergence of resistant bacterial strains is a direct consequence of the rising utilization of antibiotics in both human and animal populations. Microorganisms have evolved an array of resistance strategies to endure extreme environmental circumstances. [19].

Since the microbiota of human milk contributes to the microbial composition of infants' intestines it aids in bacterial colonization and potentially influences the immune system improvement [20]. The exact origin of bacteria found in breast milk remains incompletely understood to this day. Recent researches have demonstrated the bacteria ingested orally by breastfeeding mother can subsequently be identified within their lacteal secretions. [21]. Suggesting a possible 'internal' pathway for bacteria to reach the mammary gland. There are two hypotheses, initially suggested by Fernandez [22]

"retrograde transfer" refer to bacteria that has exterior source through transmission of microbes from the infant's oral cavity into the mammary duct during suckling. For the translocation the bacteria from internal, the "entero-mammary pathway" 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 [23*]. Therefore, the aim of this study was to isolate L. rhamnosus from human breast milk, identify L. rhamnosus using biochemical tests and PCR (16s rRNA gene) and determine its antibiotic resistance.*

2. MATERIALS AND METHODS

2.1. Collection of samples:

One hundred and eight breast milk samples were obtained randomly from breastfeeding mothers at AL-Kut Hospital and AL-Zahraa Teaching Hospital in Wasit province, Iraq (from 1 October 2023 to 5 February 2024). The nipple and mammary areola underwent cleaning using 70% ethanol alcohol, with the initial drops being discarded to prevent alcohol contamination. The milk samples were collected using sterile breast pumps, and (5–15 ml) of milk sample was transferred to a sterile tube. The tubes containing the samples were packed in insulated boxes containing dry ice and sent to the laboratory within one hour.

2.2. *L.rhamnosus* isolation:

Added 1 ml of milk samples to 9 ml of sterilized de Man Rogosa Sharp (MRS) broth (Hi-Media, Mumbai, India) that was mixed thoroughly, then inoculated 24 h at 37 °C in anaerobic condition by anaerobic jar, then 0.1 ml of broth spread plated on selective media of de Man Rogosa Sharp (MRS) agar (Hi-Media, Mumbai, India) plates with acetic acid. This was performed in duplicate, and the medium was supplemented with 0.25% l-cysteine to select for LAB and favor the evolution of *Lactobacillus*. Incubation period was 48 to 72 hours at 37°C in anaerobic conditions [24].

2.3. Preparation of selective media:

Breast milk contains a mixture of *Lactobacillus* species that are similar from each other in morphology, culture requirement, and biochemical tests. Therefore, selective media must be used to isolate *L.rhamnosus*. After preparing MRS media, add acetic acid at concentration 99% (Alpha chemmika,India) dropwise until the pH is adjusted by the pH meter to 5.2 [25].

2.4. Catalase test:

A catalase assay was administering two drops of 3% hydrogen peroxide (H_2O_2) on cultures that had undergone a 24-hour maturation period on a glass slide. The production of oxygen bubbles during a catalase assay signifies the existence of the catalase enzyme, which decomposes H_2O_2 into water (H_2O) and oxygen (O_2).

$$2\,O^{-2} + 2\,H^{+} \rightarrow \text{superoxide dismutase} \rightarrow O_2 + H_2O_2$$

$$2\,H_2O_2 \rightarrow \text{Catalase} \rightarrow O_2 + 2\,H_2O$$

The strains that exhibited no gas bubbles were selected in contrast to *S. aureus* that is known as catalase positive. Since, *L.rhamnosus*. are known as catalase negative [26].

2.5. Molecular Diagnosis of *L.rhamnosus*

2.5.1. 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.rhamnosus* isolates were cultured in MRS broth at 37 °C for 24 hours. $1x10^9$bacterial cells were transferred to into a 1.5 ml microcentrifuge tube. Performed centrifugation at 16000 rpm for 1 minute and subsequently removed the supernatant. Added 200 µl of Gram-positive buffer per sample, followed by the addition of 0.8 mg of lysozyme per sample. The mixture was incubated at 37 °C for a duration of 30 minutes. Supplement with proteinase K (20 µl) and homogenize by pipetting. Incubated at 60 °C for a minimum of 10 minutes.

Added 200 µl of GB buffer and were mixed by vortex for 10 seconds and incubated at 70°C for a minimum of 10 minutes. Introduced absolute ethanol (200 µl) to the sample solution and mixed for ten seconds. All mixture was transported into the GD- column which is assembled into 2 ml assembly tube provided with the kit). Subsequently, were subjected it to centrifugation at 16000 rpm for a duration of 1 minute, after that, the flow-through was discarded and transferred the GD -column was transported to a new 2 ml assembly tube. Introduced 400 µl of W1-buffer to the GD -column and centrifuged once more at (16000 rpm) for thirty seconds before discarding the flow-through. The GD column was reinserted into the (2 ml) assembly tube, then 600 µl of wash -buffer was added to the GD column then, repeated centrifugation (at 16000 rpm) for thirty second and the flow-through was discarded. The GD column was reinserted into the 2 ml collection tube once more. To dry the column matrix, centrifugation was performed for an additional 3 minutes at 16000 rpm. The dried GD column was transferred to a clean 1.5 ml microcentrifuge tube. Added 50 µl elution buffer then left at room temperature for a minimum of 3 minutes. Centrifuge (at 1600 rpm) for 30 seconds, hoard at-20°C until use.

2.5.2. Detection of Gene Utilizing Polymerase Chain Reaction (PCR):

The primer pair employed in this investigation consisted of 27F: AGAGTTTGATCCTGGCTCAG and 1492R: TACGGTTACCTTGTTACGACTT, sourced from Macrogen, Korea [27].

Initially, the lyophilized primers were precipitated by using a centrifuge at 45000 rpm for a duration of 1 minute. Subsequently, sterilized distal water was additional to all tube at a required size to obtain a solution of (100µM, 100 pmole/µl) as per the manufacturer's guidelines and the tube was stored as primer stock solutions at deep freezing temperatures (-20°C) until they were needed.To generate 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 by following the established dilution formula: C1V1=C2V2. Subsequently, introduce 1µl of the forward primer and 1µl of the reverse primer (which have been previously prepared), 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. Of cycles
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

2.4.3. Gel Electrophoresis Protocol:

Agarose gel electrophoresis has been sanctioned for the purpose of scrutinizing the amplification process and specificity of DNA. The formulation of agarose gel involves the addition of agarose powder to 1X TBE Buffer in this study 1% agarose gel was used Subsequently, the solution was placed in a water bath and heated until it achieved clarity, followed by the addition of 5µl of a safe red dye once it was cooled to 50°C. The agarose was then left to solidify at ambient temperature for a duration of 30 minutes. Wells for loading DNA samples were created using a comb. The liquefied agarose is carefully poured into the gel tray and allowed to solidify for 20 minutes. Following this, the fixed comb was meticulously removed, and the gel plate is positioned in the gel tank. TBE buffer was gently added to the tank until it exceeded the gel surface by 1-2 mm. Subsequently, 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, facilitating the migration of DNA from the cathode to the anode poles. Visualization of the bands within the gel was achieved through the utilization of a UV transilluminator emitting light at 350 nm.

2.5. Hemolytic activity of. *L.rhamnnosus*:

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. [28].

2.6. Antibiotic susceptibility:

The susceptibility test of *L.rhamnosus* toward the antibiotics typically employed in medicine of human was assessed in the context of this study. diluted 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 [29]. In the preparation of sensitivity plates, Petri dishes containing Mueller-Hinton agar (Liofilchem,Italy) (5 to 6 mm. in depth) were employed. The bacterial broth suspension was evenly spread in three dimensions on the medium surface using a cotton swab. Following the drying of the inoculum (3 to 5 minute), disks of

antibiotic were meticulously positioned upon the agar utilizing sterilized forceps that had been subjected to a flame, ensuring they were gently pressed to facilitate optimal contact. Subsequently, the samples underwent incubation for a duration of 24 hours at a controlled temperature of 37°C. The experiment was performed in triplicate, and the average diameter of the inhibition zones surrounding the antibiotic discs was meticulously documented. The levels of susceptibility were categorized as resistance (R), intermediate susceptibility (I), and susceptibility (S), with the criteria for determining drug resistance derived from the Executive Standard for Antimicrobial Drug Susceptibility Testing [30].

2.7. Statistical Analysis:

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

3. RESULTSAND DISCUSSION

3.1. Isolation of *L.rhamnosus*

Lactobacillus species was isolated from human breast milk provided by healthy volunteer mothers and served as the source of isolation. The microorganisms were cultured on selective media (MRS-AC) at 37 °C under anaerobic conditions. Identification was based on microscopic and colony morphology, as well as the outcome of catalase testing, determining them to be Gram-positive, rod-shaped (Figure1) catalase negative.Analysis showed that 24 out of 108 samples of breast milk contained *L.rhamnosus*, representing 22.2% of the total isolates. These species exhibited white shiny smooth colonies with a diameter of 1 mm (Figure2) [32],[33].

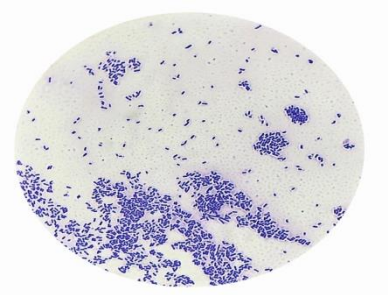

Figure 1. Gram staining result of *L.rhamnosus* isolates.

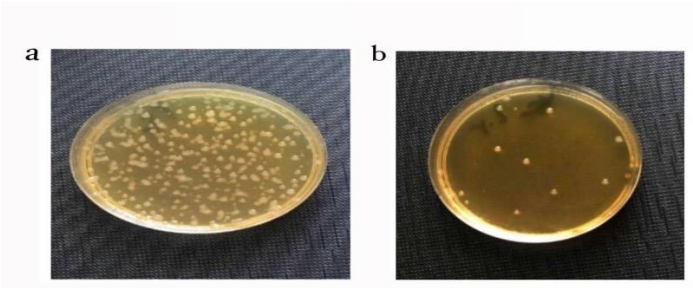

Figure 2. (a) white shiny smooth colonies of *L.rhamnosus(* b) typical colonies of *L.rhamnosus* with a diameter of 1 mm.

Table3. Distribution of *L.rhamnosus* according to age of mothers

		L.rhamnosus		Total
		Negative	Positive	
Mother age (year)	< 20	10	1	11
	20-29	47	16	63
	30-39	21	5	26
	>40	6	2	8
Total		84	24	108

			P-Value **0.651**	

There were no significant differences (*P-Value* >0.05) [Table3] Between the presence of *L.rhamnosus* and mother age.

Table4. Distribution of *L.rhamnosus* according to infant age

		L.rhamnosus		**Total**
		Negative	Positive	
infant age (month)	<6	51	18	69
	6-11	19	2	21
	>12	14	4	18
Total		84	24	108
			P-Value 0.279	

Infant age refers to the duration of breastfeeding. This study reveals no significant variations (*P-Value* >0.05) between the presence of *L.rhamnosus* and the age of the infant[Table4].

Table5. Distribution of *L.rhamnosus* according to pregnant period

		L.rhamnosus		**Total**
		Negative	Positive	
Pregnant period	7	2	2	4
(month)	8	3	4	7
	9	79	18	97
Total		84	24	108
			P-Value **0.024**	

There were highly significant variation differences (*P-Value* < 0.05) between pregnant period and presence of *L.rhamnosus* (*P-Value* 0.024)[Table5].

This possibility arises due to the fact that the stability of the maternal gut microbiota persists throughout the later stages of pregnancy and the initial phases of lactation, which may contribute to the presence of advantageous bacteria in the milk post the nine-month pregnancy period [34]. Moreover, the occurrence of beneficial bacteria in the breast milk of mothers who have completed nine months of pregnancy can be linked to the impact of maternal dietary choices during pregnancy and lactation, alongside the distinct microbial composition found in human breast milk. Research indicates that maternal nutrition, particularly the consumption of nutrients such as vitamin C during pregnancy, acting essential role in the microbiota modulation that present in human breast milk [35]. These results offer support for the theory of the 'entero-mammary pathway'[23].

Table6. Distribution of *L.rhamnosus* according to type of delivery

		L.rhamnosus		Total
		Negative	Positive	
Type of delivery	Caesarean delivery	47	15	62
	Normal delivery	37	9	46
Total		84	24	108
	P-Value 0.567			

There were no significant differences (p>0.05) between presence of *L.rhamnosus* and type of delivery[Table 6].

3.2. Molecular Analysis

According to PCR technique the results of 16s rRNA specific primers revealed that the isolates were diagnosis as *L. rhamnosus* . All the isolates were Gram- positive and catalase negative. (Figure 3) which reveals the bands of PCR product 1534 bp were clearly observed on the 1% agarose gel examination.

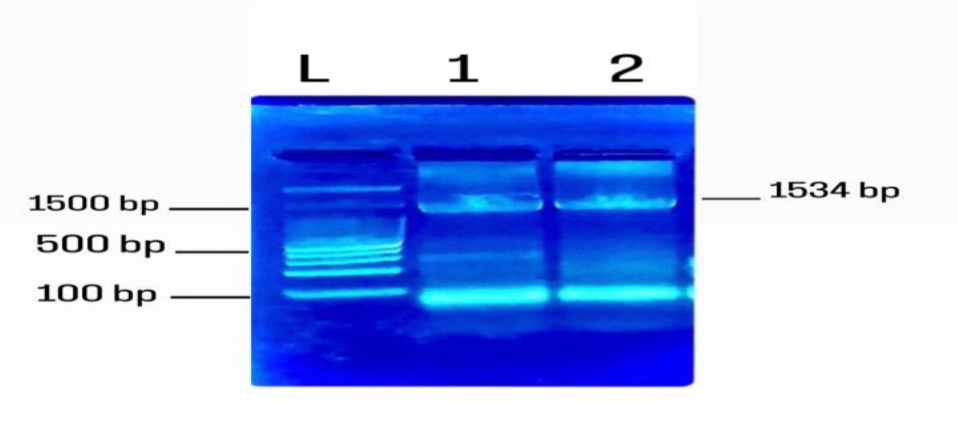

Figure 3. Electrophoresis of agarose gel of 16s-rRNA gene of *Lactobacillus* isolate using Polymerase chain Reaction, that was fractionated on (1%) agarose gel (100 V/40min). Lane[L]:100bp DNA ladder. Lane 1 ,2: *L.rahmnosus*.(1534bp).

3.3. Hemolytic Activity:

The isolates showed no hemolytic activity contrast to *S. aureus* that known hemolytic activity was performed as a control (Figure4). Our results are consistence with results by [36].

L. rhamnosus lacks hemolytic activity, making it suitable as a probiotic due to its safe nature for intestinal health maintenance and potential therapeutic applications [37].

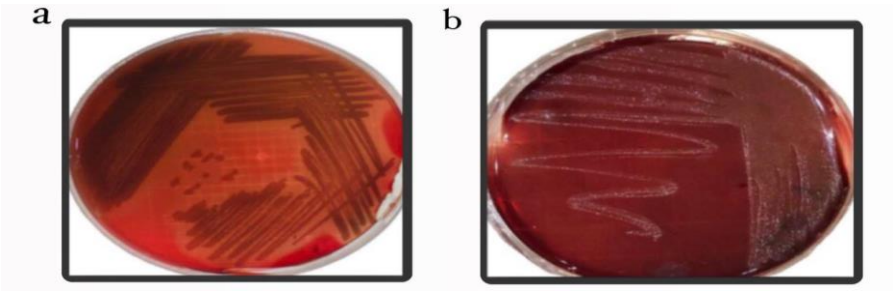

Figure 4. (a)*S. aureus* on blood gar (b) *L.rhamnosus* on blood gar

3.4. Antibiotic susceptibility:

The *L.rhamnosus* isolates exhibited varied degrees of susceptibility and resistance towards the antibiotics used for testing (Table7), (Figure5). Eight antibiotics (Liofilchem,Italy) have been used in the current study, which involve: Ampicillin (AMP), penicillin g (P), vancomycin (va) ,gentamycin (CN) streptomycin(S), chloramphenicol(C) ,clindamycin (CD) and tetracycline (TE).

Table7. Susceptible of L.rhamnosus in breast milk to different antibiotic

Antibiotic	code	Clinical Decision			Total %
		Resistance%	Intermediate%	Susceptible%	
Ampicillin	AMP	20	0	80	100%
Penicillin G	P	20	66.67	13.33	100%
Vancomycin	VA	100	0	0	100%
Gentamycin	CN	0	20	80	100%
Streptomycin	S	26.67	60	13.33	100%
chloramphenicol	C	6.67	20	73.33	100%
clindamycin	CD	13.33	66.67	20	100%
Tetracycline	TE	0	26.67	73.33	100%

The sensitivity of *L. rhamnosus* to Ampicillin and Penicillin G stands at 80% and 66.67% respectively [38], [39]. where both antibiotics serve as inhibitors of cell wall synthesis. Ampicillin, a β-lactamase inhibitor, exerts its mode of action through interaction with penicillin binding proteins (PBPs), subsequently leading to the disruption of the peptidoglycan layer and cell lysis [40]. Penicillin G, on the other hand, functions by inhibiting the final step of crosslinking of cell wall synthesis, weakening the cell wall by interfering with transpeptidase activity. The mechanism of action of Penicillin G involves acting as a similarity of the L –Ala -Y- D-Ala part of the pentapeptide chains, albeit lacking the essential group of carboxylates necessary for its activity [41]. This study findings reflect 100% resistance of *L. rhamnosus* to Vancomycin, aligning with previous research [42]. Vancomycin resistance in *Lactobacillus* species is chromosomally-mediated [43] The inefficacy of Vancomycin against *Lactobacillus* species can be attributed to their genetic resistance due to the weak affinity of vancomycin binding to their cell walls. This observation emphasizes the importance of exploring 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, with which vancomycin typically interacts [44]. In the context of susceptibility to antibiotics, *L. rhamnosus* exhibits 80% sensitivity and 60% intermediate susceptibility to gentamicin and Streptomycin respectively, this finding is consistent with result by [40],[45]. These antibiotics, together with Neomycin, are classified under a category of amino acids that are capable of

inhibiting protein production through binding to bacterial ribosomal subunits, thereby exhibiting antibacterial properties against certain Gram-positive and aerobic Gram-negative rods [46]. This study results indicate a 73.33% sensitivity of *L. rhamnosus* to Chloramphenicol, approving previous findings [42]. Chloramphenicol antibiotics function by inhibiting the peptidyl transferase that act as essential enzyme found on the (50S ribosomal subunit) crucial for synthesis of protein, thereby disrupting t-RNA binding to the ribosomal A site and impeding protein synthesis [47].

Moreover, this study indicates an intermediate susceptibility of 66.67% to clindamycin, in line with existing literature [45]. Clindamycin, classified under the lincosamides group, influences peptidyl transferase reactions, leading to the premature detachment of incomplete peptide chains [48]. Overall, over 73.33% of the isolates in this study exhibit sensitivity to Tetracycline, a pattern consistent with prior findings [49]. Tetracycline antibiotics target the preserved sequence of (16S rRNA) in the 30S of ribosomal subunit, hindering tRNA binding to the A-site, and thereby impeding the process of protein synthesis [18].

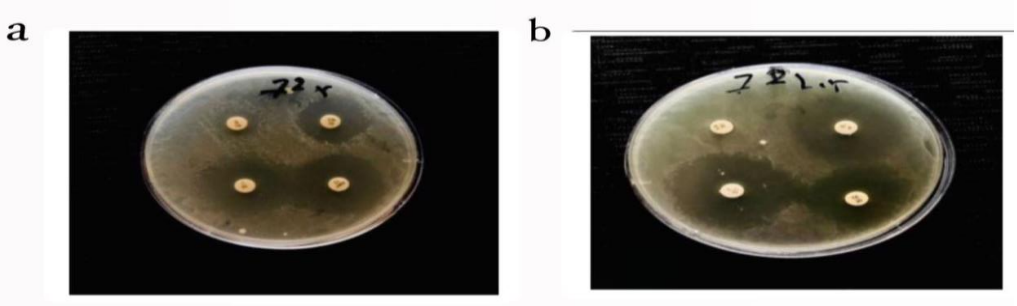

Figure 5.(a) Sensitivity of *L. rhamnosus* to streptomycin, gentamycin, chloramphenicol, ampicillin. (b) Sensitivity of *L.rhamnosus* to vancomycin, penicillin, clindamycin, tetracycline.

4. CONCLUSIONS

Breast milk undoubtedly considered as an important source for the isolation of *L.rhamnosus* 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. rhamnosus* 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.rhamnosus* as a probiotic in formula milk would be advantageous for infants who do not consume breast milk.

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