



## Tikrit Journal of Pure Science

ISSN: 1813 – 1662 (Print) --- E-ISSN: 2415 – 1726 (Online)

Journal Homepage: <https://tipsj.org/>Tikrit Journal  
of Pure Science

## The effect of *Lactobacillus reuteri* on the biofilm formation of some bacterial species isolated from children with diarrhea

Zakia Sami Saeed<sup>1</sup> , Sawsan Mohammed Sorchee<sup>2</sup> <sup>1,2</sup>Department of Biology, College of Education, University of Salahaddin, Erbil -Iraq

Received: 21 May 2025. Received in revised form: 27 July 2025. Accepted: 31 July 2025.

Final Proofreading: 6 August 2025. Available online: 25 Oct. 2026.

### ABSTRACT

Biofilm formation is a key factor in bacterial infections and persistence in various settings. This study aimed to assess the antibacterial and antibiofilm properties of both the supernatant and pellet of *Lactobacillus reuteri* against diarrheagenic bacteria. Seventy-six isolates were obtained from 50 children (aged 3 weeks to 9 years) with diarrhea from hospitals in Erbil/Iraq, including *Escherichia coli* 37 (48.68%), enterohemorrhagic *E.coli* 4 (5.26%), *Klebsiella pneumonia* 16 (21.05%), *Enterobacter cloacae* 7 (9.21%), *Proteus mirabilis* 7 (9.21%), *Pseudomonas aeruginosa* 1 (1.32%), *Acinetobacter baumannii* 1 (1.32%), *Shigella sonnei* 1 (1.32%), *Klebsiella oxytoca* 1 (1.32%), and *Enterobacter aerogenes* 1 (1.32%). This is the first report of *A. baumannii* detection in pediatric diarrhea in Iraq. *L. reuteri* was isolated from a single infant formula milk, which may not represent all formula types. Gram staining showed that all pathogens were Gram-negative, while *L. reuteri* was Gram-positive. We confirmed identification by culture on MacConkey, Eosin methylene blue, and MacConkey sorbitol agar, followed by the Vitek-2 system and Molecular diagnosis using 16S rRNA sequencing. Biofilm formation was not detected on Congo red agar, but the microtiter plate method confirmed biofilm production. Both supernatant and pellet of *L. reuteri* significantly inhibited biofilm formation in several diarrheagenic bacteria ( $p < 0.05$ ), while no significant effect was observed against *K. pneumoniae*. These findings support the potential use of *L. reuteri* as an alternative therapy against diarrheagenic bacteria.

**Keywords:** *Lactobacillus reuteri*, biofilms, bacterial species, diarrhea

Name: Zakia Sami Saeed

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## تأثير *Lactobacillus reuteri* على تكوين الأغشية الحيوية لبعض الأنواع البكتيرية المعزولة من الأطفال المصابين بالإسهال

زكية سامي سعيد<sup>1</sup>، سوسن محمد السورجي<sup>2</sup>

<sup>1,2</sup> قسم بايولوجي، كلية التربية، جامعة صلاح الدين، أربيل-العراق

### الملخص

يُعَدُّ تكوين الأغشية الحيوية عاملاً رئيسياً في العدوى البكتيرية واستمرارها في بيئات مختلفة. هدفت هذه الدراسة إلى تقييم الخصائص المضادة للبكتيريا والمضادة للأغشية الحيوية لكلٍ من السائل العلوي والحبوبات من بكتيريا *Lactobacillus reuteri* ضد البكتيريا المسببة للإسهال. تم الحصول على ستة وسبعين عينة من خمسين طفلاً (تتراوح أعمارهم بين 3 أسابيع و9 سنوات مصابين بالإسهال من مستشفيات في محافظة أربيل) بما في ذلك الإشريكية القولونية 37 (48.68%)، الإشريكية القولونية المعوية النزفية 4 (5.26%)، والكلبسيلا الرئوية 16 (21.05%)، والإنتروباكتري كلوكاي 7 (9.21%)، والبروتيس ميرابيليس 7 (9.21%)، والزائفة الزنجارية 1 (1.32%)، والراكدة بومانية 1 (1.32%)، والشيجيلا سوني 1 (1.32%)، والكلبسيلا أوكسيتوكا 1 (1.32%)، والإنتروباكتري أيروجينس 1 (1.32%). علماً من خلال هذه الدراسة تم الكشف عن أول إصابة ببكتيريا الراكدة البومانية من مرضى الإسهال لدى الأطفال في العراق. إضافة إلى ذلك عُزلت بكتيريا *L. reuteri* من حليب الأطفال، والتي قد لا تمثل جميع أنواع الحليب الصناعي. حيث أظهرت الأنواع البكتيرية المرضية سالبة لصبغة كرام، بينما كانت *L. reuteri* موجبة لصبغة كرام. بعد التأكيد التشخيص بالزرع على أجار مأكونكي، وأجار إيوسين ميثيلين الأزرق، وأجار مأكونكي سوربيتول، متبوعاً بنظام فيتيك-2 والتشخيص الجزيئي باستخدام تسلسل الرنا الريبوسومي 16S. إضافة إلى ذلك ينت النتائج عن عدم تكوين الأغشية الحيوية بطريقة أجار الكونغو الأحمر، وتم تأكيده بطريقة اللوحة الدقيقة (microtiter plate)). وبعد المعاملة بالسائل العلوي وحبوبات *L. reuteri* ظهر تثبيط ملحوظ على تكوين الأغشية الحيوية في العديد من البكتيريا المسببة للإسهال (قيمة الاحتمالية  $> 0.05$ )، بينما لم يُلاحظ أي تأثير يُذكر ضد *K. pneumoniae*. تدعم هذه النتائج الاستخدام المحتمل لـ *L. reuteri* كعلاج بديل ضد البكتيريا المسببة للإسهال.

### 1. INTRODUCTION

Diarrhea is one of the most common diseases worldwide, especially in poor countries. It is a prominent cause of death in children, especially young ones <sup>(1)</sup>. Infections can result from parasites, viruses, or bacteria; geographical factors, the host's previous diseases, and the host's immune strength all play a role in determining infection severity <sup>(2)</sup>. A primary factor contributing to bacterial resistance to antimicrobials and infections is the capacity to create biofilms <sup>(3)</sup>. A biofilm is a community of microorganisms encased in a self-produced

matrix. Biofilms exhibit higher resistance to antibiotics, allowing survival in the environment and invasion of other hosts by pathogenic bacteria <sup>(4)</sup>. Probiotics were defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" <sup>(5)</sup>. *Lactobacillus* spp. are among the most ubiquitous probiotics and are found in a wide variety of food products worldwide <sup>(6)</sup>. *Lactobacillus* demonstrated a strong capacity to attach to human epithelial cell lines. Additionally, several *Lactobacillus* strains weakly

attach to the epithelial cell surface through noncovalent interactions, which prevent pathogen attachment and biofilm development <sup>(7)</sup>. *Lactobacillus reuteri* provides numerous health advantages to the host, including preventing and mitigating various illnesses <sup>(8)</sup>. It generates reuterin, a powerful antimicrobial substance that suppresses the growth of Gram-positive and Gram-negative bacteria, fungi, and protozoa. *L. reuteri* helps keep the balance of microbiota in the human body and is useful for treating acute watery diarrhea and preventing future diarrhea, even after long courses of antibiotics <sup>(9)</sup>. *L. reuteri* was selected for this study due to its superior probiotic properties, and clinical studies have demonstrated its effectiveness against digestive disorders, including infantile colic, diarrhea, and *H. pylori* infection <sup>(10)</sup>. This study aimed to evaluate the antibacterial and antibiofilm activities of both the supernatant and pellet of *L. reuteri* against bacteria isolated from children with diarrhea. The supernatant and pellet of *L. reuteri* possess antibacterial and antibiofilm properties that can significantly inhibit the growth and biofilm formation of several pathogenic bacteria associated with children's diarrhea.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection

From October 2024 to January 2025, we collected diarrhea samples from 50 patients (aged 3 weeks- 9 years) with diarrhea, with or without blood, at Raparin Pediatric Hospital in Erbil/Kurdistan. We included only patients with acute or chronic diarrhea. We excluded patients who used antibiotics within the previous 2 weeks. We carefully collected data from each patient, including age, sex, geographical area, clinical symptoms, and duration of diarrhea. We collected samples in sterile containers, kept them in a cooler box (4-8°C), and transported them to the laboratory within 3 h for same-day processing without storage.

**Ethical Approval:** The Salahaddin University-Erbil Human Research Ethics Committee (HREC) approved all experimental protocols (Approval Ref No: SU2025HREC).

### 2.2 Isolation of Gram-negative pathogenic bacteria from diarrhea

All samples were inoculated into tubes containing nutrient broth (OXOID, UK) for enrichment at 37°C for 24 h, a method that promotes the growth of pathogenic bacteria <sup>(11)</sup>. The enrichments were streaked onto MacConkey agar, eosin methylene blue agar, and MacConkey sorbitol agar (HI MEDIA, India), and incubated at 37°C for 24 h <sup>(12)</sup>.

### 2.3 Isolation of lactic acid bacteria

A sample of infant milk formula was obtained from a milk center in Erbil city, Kurdistan Region/Iraq, in October 2024. Initially, add one gram of infant formula milk to 4 mL of de Man, Rogosa, and Sharpe (MRS) broth (HI MEDIA, India) as an enrichment medium. The inoculated broth sample was then placed in an anaerobic jar (using a candle) and incubated at 37 °C for 24 to 48 h in the absence of oxygen. A loop containing the proliferating culture broth was used to inoculate MRS agar plates (HI MEDIA, India) <sup>(13)</sup>.

### 2.4 Identification of pathogenic bacteria and lactic acid bacteria

Bacterial isolates were initially identified by evaluating colony morphology (color and shape) through visual observation. Second, Gram stains (Atom Scientific, UK) were used to examine all isolated bacteria under the microscope. A thin bacterial smear was prepared, Gram-stained, and evaluated under a light microscope using an oil-immersion objective lens. <sup>(14)</sup> The Vitek-2 system (Bio-Mérieux, US) was applied to identify the isolated bacteria found in diarrhea. <sup>(15)</sup>

### 2.5 Molecular assay

#### 2.5.1 DNA Extraction

We isolated genomic DNA from pure cultures of *L. reuteri* and *A. baumannii* using the Beta Bayern bacterial DNA extraction kit (Beta Bayern GmbH, 90453 Bayern, Germany). Briefly, bacterial cells were lysed with a lysis buffer and proteinase K, then incubated at 80°C. DNA was then bound to a spin column, washed with buffer solutions, and eluted with preheated elution buffer; the purified DNA was stored at -20°C.

## 2.5.2 Polymerase Chain Reaction (PCR) Amplification of 16S ribosomal RNA

PCR amplification was performed for the 16S rRNA gene, with each reaction conducted in a total volume of 50  $\mu$ l. The reaction mixture included 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhusgervej 22), 10 picomoles (pmol) of 16S-F (5'-CGTTGACTGCCGGTGACAAAC-3') and 16S-R (5'-GCTGTTACGACTTCACCCCA-3') primers, designed using Primer3Plus software based on the reference sequence with GenBank accession number OL336429.1, at <https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>. We used DNase-free water and template DNA, as shown in Table 1, with a Bio-Rad PTC-200 Gradient thermocycler. The temperature profile consists of an initial denaturation at 95°C for 5 minutes. This stage is followed by 35 cycles of denaturation at 95°C for 40 seconds, primer annealing at 58°C for 40 seconds, and extension at 72°C for 1 minute. The final step involves an additional extension at 72°C for 5 minutes. (16).

**Table 1: PCR Amplification Reagents**

| No.   | PCR components   | Concentration | Volume ( $\mu$ l) |
|-------|------------------|---------------|-------------------|
| 1     | Master Mix       | 2x            | 25                |
| 2     | Forward Primer   | 10 Pmol       | 2                 |
| 3     | Reverse Primer   | 10 Pmol       | 2                 |
| 4     | DNase-free water | -             | 18                |
| 5     | Template DNA     | 50ng/ $\mu$ l | 3                 |
| Total |                  |               | 50                |

## 2.6 Antibiotic susceptibility testing

We conducted the antibiotic susceptibility test using the Vitek-2 system (AST-GN) with Gram-Negative Susceptibility cards (AST-N417 and AST-N419) produced by BioMérieux, USA. The test consisted of the following antimicrobial agents: amikacin, gentamicin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefuroxime, ceftriaxone, ceftazidime, imipenem, meropenem, trimethoprim/sulfamethoxazole, ciprofloxacin, nitrofurantoin, fosfomycin, cefazolin, cefepime, cefuroxime axetil, ertapenem, ampicillin/sulbactam, cefotaxime, ceftolozane/tazobactam, colistin, and

tigecycline. The susceptibility test findings are defined as sensitive, intermediate, or resistant based on the Clinical and Laboratory Standards Institute (17) criteria.

## 2.7 Biofilm formation assay

### 2.7.1 Congo red agar method (CRA)

We evaluated phenotypic biofilm formation in *Acinetobacter baumannii*, *enterohemorrhagic Escherichia coli*, *Klebsiella pneumoniae*, *Shigella sonnei*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae* isolates by culturing them on Congo red agar plates, as described earlier. (18). The medium consisted of BHI broth (Merck, Germany) at 37 g/L, 10 g/L agar, sucrose at 50 g/L, and Congo red stain (SDFCL, India) at 0.8 g/L. We prepared Congo red as a concentrated liquid solution, autoclaved it at 121°C for 15 minutes separately from the other medium components, and added it when the agar cooled to 55°C. We incubated the plates for 24 hours at 37 °C. We identified black colonies as strong biofilm producers, whereas nearly black colonies showed weak biofilm formation. In contrast, isolates exhibiting red colonies were categorized as incapable of biofilm formation. (18).

### 2.7.2 Microtiter plate assay

We quantitatively assessed biofilm formation using the microtiter plate method, as in (19). We incubated bacteria in BHI broth medium with 2.5% glucose at 37 °C for 24 hours. Following incubation, a 200  $\mu$ L culture sample was inoculated onto a 96-well plate and incubated at 37 °C for 24 hours. The negative control wells comprised 200  $\mu$ l of BHI and glucose. The plates were subsequently removed, and each well was rinsed three times with phosphate-buffered saline. Each well received 200  $\mu$ L of 1% crystal violet (Scharlau, Spain) for 5 minutes. After the designated time, the wells were rinsed with sterile distilled water and dried. Next, 160  $\mu$ L of 95% ethanol was added to each well, and the plate was read at 570 nm using the ELISA reader (Bioteck, Korea); the optical density (OD) values for each isolate were recorded. Optical density cut-off (ODc) is described as the average optical density of the

negative control plus three times the standard deviation of the negative control <sup>(19)</sup>. Biofilms produced by different strains have been examined and classified according to the absorbance of crystal violet stain associated with adhered cells, as detailed below:  $OD \leq OD_c$  shows the absence of biofilm production,  $OD_c < OD \leq 2 \times OD_c$  shows weak biofilm formation,  $2 \times OD_c < OD \leq 4 \times OD_c$  shows moderate biofilm formation, and  $OD > 4 \times OD_c$  shows strong biofilm formation <sup>(20)</sup>.

## 2.8 Antibacterial test of *L. reuteri* against pathogenic bacteria by agar well diffusion method

The antimicrobial activity of *Lactobacillus reuteri* supernatants and pellets was evaluated using the well diffusion method against isolated bacterial growth. The procedure involved inoculating diluted bacteria onto Mueller-Hinton agar (Merck, Germany). We created 5 mm-diameter wells on the surface of the culture medium. *L. reuteri* were extracted by culturing in MRS broth, incubating at 37 °C for 48 hours under anaerobic conditions, and centrifuging at 6000 rpm for 15 minutes. The supernatants and pellet were used to assess antibacterial activity, with 100 µL of supernatant and the *L. reuteri* pellet placed in the well. The inoculated plates underwent incubation for 24 hours at 37°C, after which the diameter of the inhibition zone was determined in millimeters using calipers <sup>(6)</sup>.

## 2.9 Antibiofilm activity of *L. reuteri* against pathogenic bacteria

The microtiter plate assay was employed to assess the antibiofilm activity of *L. reuteri* against pathogenic bacteria. *L. reuteri* was cultured in MRS broth and thereafter incubated at 37°C for 48 hours. After incubation, *L. reuteri* was centrifuged at 10,000 rpm for 20 minutes. Isolates of pathogenic bacteria were recultured in BHI broth and incubated at 37 °C for 24 hours. After assessing confluency using the OD570 McFarland standard solution, 100 µL of culture was aliquoted into each well of the 96-well plate. After this procedure, 100 µL of *L. reuteri* supernatant or pellet was added and diluted with de

Man Rogosa and Sharpe medium. Ultimately, MRS broth was added to the plates as a negative control. The plates were incubated for 16 hours at 37 °C. Crystal violet was employed to examine biofilm formation. The cultures were removed, and the plates were washed three times with water. Each well received 125 µL of 0.1% crystal violet solution and was incubated at room temperature for 15 minutes. We removed the dye solution and washed the plate three times with sterile water to remove residual stain. After diluting with 125 µL of 30% acetic acid per well and incubating for 15 minutes at room temperature, we assessed the optical density (OD) of the supernatant and pellet at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader <sup>(16)</sup>.

## 2.10 Statistical analysis

We conducted a one-way ANOVA to assess the impact of CFS and *L. reuteri* pellets on biofilm formation in pathogenic bacteria. We then used Tukey's multiple comparisons test to identify significant differences between the OD and supernatant groups and between the pellet groups. The statistical analysis was conducted via GraphPad Prism version 9.

# 3. RESULTS AND DISCUSSION

## 3.1 Isolation and identification of bacteria from diarrhea by Vitek-2 system

We obtained 76 bacterial isolates from 50 diarrhea samples collected from children aged 3 weeks to 9 years, comprising 26 (52%) males and 24 (48%) females. This result is similar to <sup>(21)</sup>, which reported that 52.5% of male and 47.5% of female children had diarrhea in different hospitals in Kurdistan, Iraq, and differs from <sup>(22)</sup>, which reported 60% male and 40% female cases in Diyala, Iraq. We cultured and subcultured the isolates on appropriate media. All clinical samples yielded positive cultures, with no negative cultures observed. We identified the isolates using the Vitek-2 system. According to the result, the predominant species among the 76 isolates was *Escherichia coli*; 37 isolates (48.68%) were identified as *Escherichia coli*, 4 (5.26%) as enterohemorrhagic *E. coli* O157, 16 (21.05%) as

*Klebsiella pneumoniae*, 1 (1.32%) as *Klebsiella oxytoca*, 7 (9.21%) as *Enterobacter cloacae*, 1 (1.32%) as *Enterobacter aerogenes*, 7 (9.21%) as *Proteus mirabilis*, 1 (1.32%) as *Pseudomonas aeruginosa*, 1 (1.32%) as *Acinetobacter baumannii*, and 1 (1.32%) as *Shigella sonnei*, as summarized in Figure 1. In the present study, *E. coli* was the predominant species, consistent with similar studies in Iraq, where *E. coli* was found in 48.38% of diarrhea patients <sup>(2)</sup>. These findings differ from <sup>(23)</sup>, which found *E. coli* in 16 (21.33%) of 75 samples from diarrhea patients in Kirkuk Province, Iraq. Differences in how often *E. coli* O157:H<sub>7</sub> is found across studies may reflect differences in samples, natural sources, study year, methods used, seasonal variation, and laboratory techniques <sup>(8)</sup>. The total

range of *Proteus mirabilis* isolates from diarrhea in this study was 9.21%. These findings agree with study <sup>(24)</sup>, which reported 9.52%, and disagree with study <sup>(25)</sup>, which reported 2.7%. To the best of our knowledge, this is the first study to detect *A. baumannii* in children with diarrhea in Iraq. The results were similar to those of <sup>(26)</sup>, which isolated and identified 5 (1.5%) *A. baumannii* from 187 children with diarrhea in Osun State, Nigeria. Additionally, the results of this investigation agreed with those of other studies, such as <sup>(27)</sup>, which found 55 (0.15%) *A. baumannii* in 7806 stool samples. In the study conducted in Kirkuk city, the rate of *Shigella* spp. was 1 (0.5%), while the rate for *E. coli* was 51.5%.

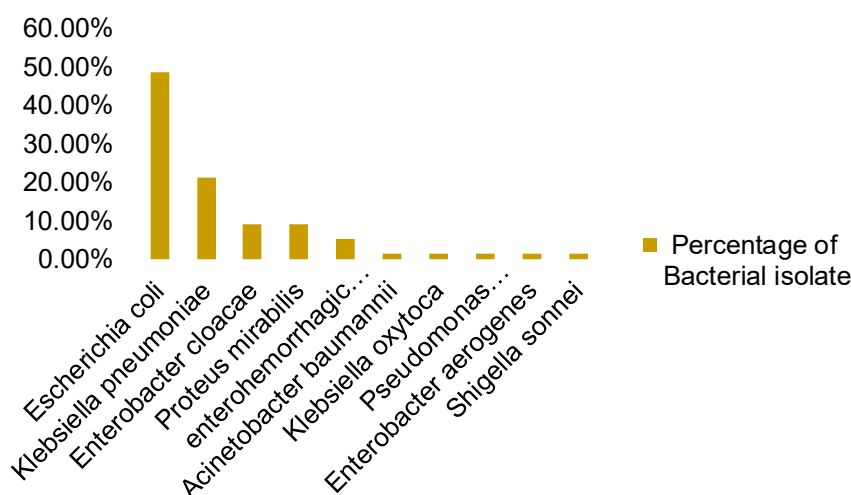


Fig. 1: Percentage of bacterial species isolated

### 3.2 Isolation and characterization of *Lactobacillus reuteri*

*L. reuteri* was isolated from a single infant milk formula (Guigoz 2, Nestlé) using MRS broth and MRS agar. We performed molecular diagnostics to identify *L. reuteri*. Our results showed that *L. reuteri* grew at 37°C for 48 hours under anaerobic conditions. The colonies on MRS agar were small, round, smooth, with a convex surface and a creamy color. These results were similar to those obtained by <sup>(28)</sup> who reported the small, creamy colony shape of the isolate *L. reuteri*.

### 3.3 Gram staining

After Gram staining, all bacteria isolated from diarrhea appeared pink under microscopy, indicating they were Gram-negative, as shown in Figure 2A. Furthermore, isolated *L. reuteri* appeared as Gram-positive, rod-shaped bacteria with a purple color under the microscope and were arranged singly or in pairs, as shown in Figure 2B. This appearance indicates they are Gram-positive bacteria. This result is similar to the study <sup>(29)</sup>. This showed that *L. reuteri* was Gram-positive and rod-shaped under the microscope.

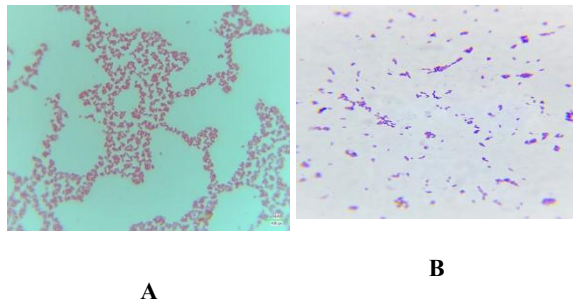


Fig. 2: Gram staining: A) pathogenic bacteria (Gram-negative) and B) *L. reuteri* (Gram-positive).

### 3.4 Molecular Detection of isolate bacteria

#### 3.4.1 Genomic DNA isolated

Genomic DNA was isolated from two distinct pure-culture bacterial species using the Beta Bayern bacterial DNA preparation kit (Beta Bayern GmbH, 90453 Bayem, Germany). The extracted DNA was subjected to electrophoresis in a 1.5% agarose gel. (16).

#### 3.4.2 PCR amplification of partial 16S rRNA genes

Micro-gene Company (South Korea) manufactures the 16S rRNA gene primer specifically for bacteria. These primers were designed to amplify a DNA fragment of the expected size (372 base pairs, bp) using Primer3Plus (<https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). We ran the PCR product on a 1.5% agarose gel and visualized it. (16) As shown in Figure 3. The results show that the 16S rRNA gene can identify bacteria. In this study, we used PCR to identify two isolates: one *Acinetobacter baumannii* from diarrhea and one *Limosilactobacillus reuteri* from infant formula milk, as shown in Table 2. In the study of (30), used polymerase chain reaction to identify *A. baumannii*; 41 (6.8 %) isolates of *A. baumannii* were obtained from 603 clinical samples of burn and sputum in Duhok City. Previous research by (31) reported detecting *L. reuteri* in breast milk using PCR. Similar studies, such as (32), demonstrated the successful identification of *L. reuteri* in breast milk using PCR methods.

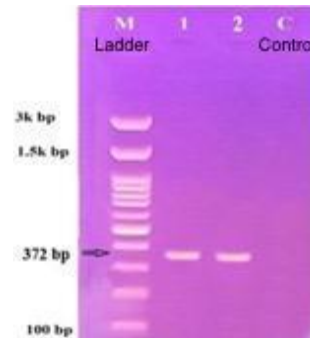


Fig. 3: PCR amplification of the partial 16S rRNA gene from bacteria; wells involving M: ladder (3000-100 bp); wells involving 1: *Acinetobacter baumannii*; wells involving 2: *Limosilactobacillus reuteri*; gene bands of 372 bp amplified; and C shows a negative control without a band.

Table 2: identification of query samples aligned with NCBI GenBank sequences.

| Bacterial Identified                                                | Source       | Query Cover % | Identical Number % | GenBank Accession Number | Bacterial samples Accession Number |
|---------------------------------------------------------------------|--------------|---------------|--------------------|--------------------------|------------------------------------|
| <i>Acinetobacter baumannii</i>                                      | Diarrhea     | 100           | 100                | AP031582                 | PV848980                           |
|                                                                     |              | 100           | 100                | CP169811                 |                                    |
|                                                                     |              | 100           | 100                | CP122365                 |                                    |
|                                                                     |              | 100           | 100                | CP101653                 |                                    |
|                                                                     |              | 100           | 100                | CP142645                 |                                    |
|                                                                     |              | 100           | 100                | CP180031                 |                                    |
| <i>Limosilactobacillus reuteri</i> ( <i>Lactobacillus reuteri</i> ) | Formula milk | 100           | 100                | PP182213                 | PV848981                           |
|                                                                     |              | 100           | 100                | CP095735                 |                                    |
|                                                                     |              | 100           | 100                | EF412976                 |                                    |
|                                                                     |              | 100           | 100                | KR364776                 |                                    |
|                                                                     |              | 100           | 100                | MW367780                 |                                    |
|                                                                     |              | 100           | 100                | LN906634                 |                                    |

#### 3.4.3 Phylogenetic tree

Phylogenetic analysis using MEGA 11, based on 16S rRNA nucleotide sequences, showed that the two investigated bacterial species grouped as expected. Sequence similarity and divergence data showed that species belonging to the same genus clustered closely together, as illustrated in Figure 4.

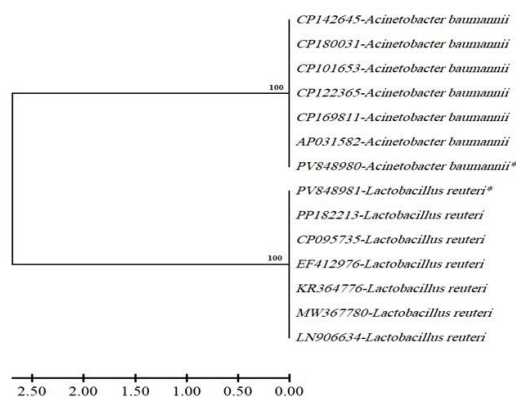


**Fig. 4: Phylogenetic positioning of *A. baumannii* and *L. reuteri* based on similar 16S rRNA GenBank sequences.**

### 3.5 Antibiotic sensitivity test of isolated bacteria

The present study used the Vitek-2 system to evaluate all samples for their susceptibility to the most commonly used antibiotics. The antimicrobial susceptibility test results indicated that the isolated species varied in susceptibility to the tested antimicrobials, as shown in Figure 5. The result of this study showed that *E. coli* showed resistance to amoxicilline/ clavulanic acid and trimethoprime /sulphamethoxazol (45.9%), piperacillin/tazobactam (37.8%), cefuroxime and cefuroxime axetil (75.6%), ceftriaxone (56.7%), ceftazidime and cefepime (18.9%), cefazolin (78.3%), and 2.7 % resistance to ertapenem, nitrofurantoin and fosfomycin. At the same time, amikacin, imipenem, and meropenem were among the most potent antibiotics against *E. coli*. This result agrees with a study conducted in Zakho/Kurdistan. (33). High cefuroxime resistance in *E. coli* complicates treatment because mechanisms such as overproduction of chromosomal beta-lactamases, efflux pumps, and changes in outer

membrane proteins reduce drug effectiveness. Additionally, ESBL production often leads to multidrug resistance, limiting therapeutic options. (34-36). In this study, 16.2% of *E. coli* were resistant to gentamicin, and 32.4% were resistant to ciprofloxacin. This result is similar to that reported by (37), which found that 15% of bacterial isolates were resistant to gentamicin and 37.5% were resistant to ciprofloxacin. Furthermore, this study showed that enterohemorrhagic *E. coli* O157 strains were 100% resistant to cefuroxime, ceftriaxone, cefazolin, and cefuroxime axetil; 75% resistant to ceftazidime; 50% resistant to ciprofloxacin; and 25% resistant to trimethoprim/sulfamethoxazole. In the present study, antimicrobial susceptibility testing showed that *A. baumannii*, *P. aeruginosa*, and *K. oxytoca* isolates were sensitive to the most commonly used antibiotics. These results agreed with the findings of (25), which showed *Pseudomonas* spp. 100% sensitive to gentamicin, amikacin, imipenem, and ciprofloxacin. According to the present study, *K. pneumoniae* showed resistance to amoxicillin/clavulanic acid and nitrofurantoin (31.3%), cefuroxime and cefuroxime axetil (18.8%), cefazolin (43.8%), ceftriaxone, ceftazidime, trimethoprim/sulfamethoxazole, and cefepime (25%). These results differ from a study conducted in Iraq. (38). In this study, *Enterobacter cloacae* showed high sensitivity to amikacin, gentamicin, imipenem, meropenem, trimethoprim/sulfamethoxazole, ciprofloxacin, fosfomycin, ertapenem and cefepime. These results agreed with the research of (39) which recorded high sensitivity to gentamicin, ciprofloxacin, cefepime, meropenem, and amikacin.

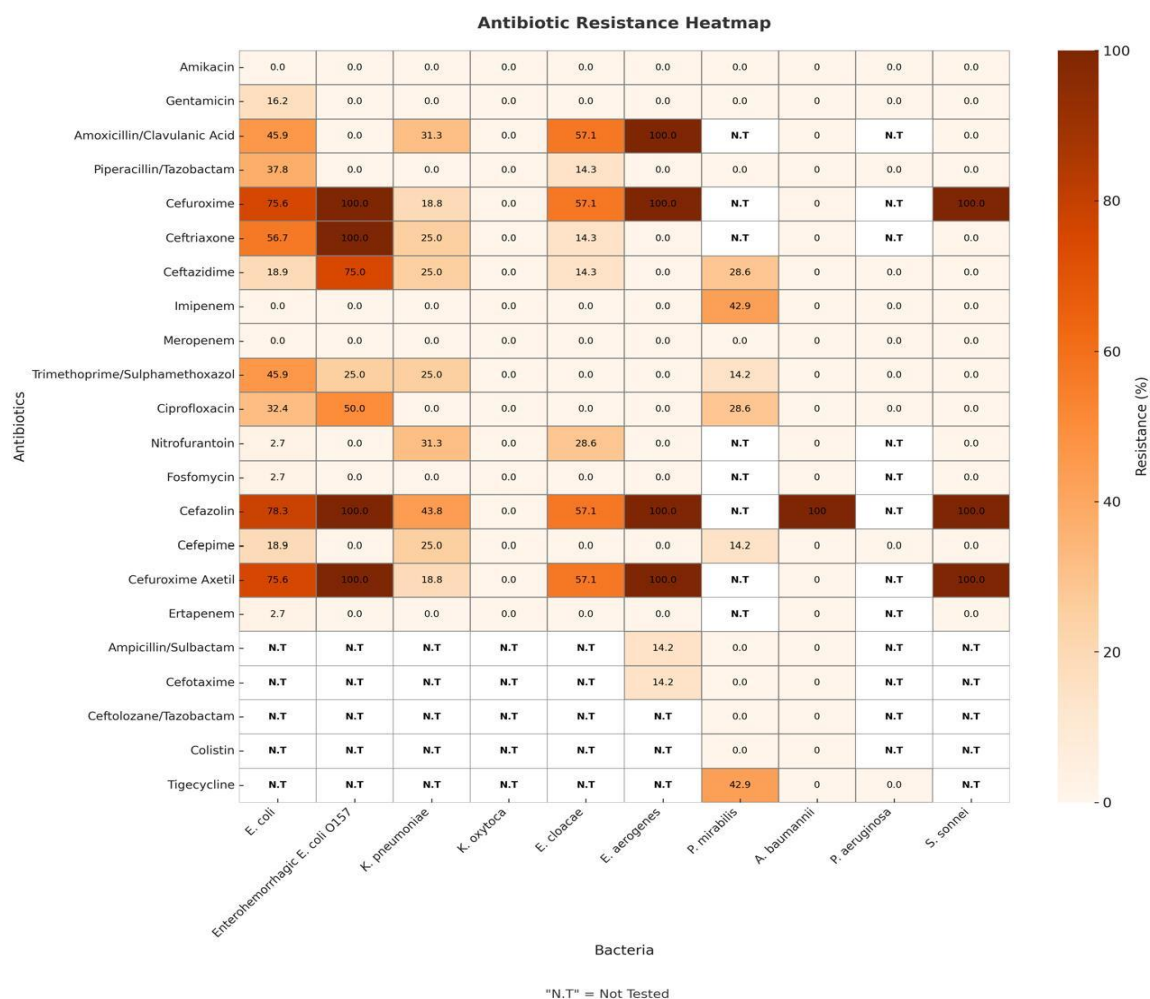


**Fig. 5: Percentage of antibiotic resistance by heatmap**

### 3.6 Biofilm Formation Assay

#### 3.6.1 Congo Red Agar (CRA) Method

The Congo Red Agar method uses a solid medium known as Congo Red Agar. This technique provides results based on color change: black colonies on the medium's surface indicate biofilm, whereas pink-red colonies indicate its absence. This research indicated that none of the isolated bacterial species produced biofilm using the Congo Red Agar method, as shown by the formation of pink colonies. The Congo red agar (CRA) method has limited sensitivity, especially for weak biofilms, causing discrepancies with the more sensitive microtiter plate assay. (19, 40). The findings in this study are similar to those reported in a study in Iraq by (41), which found that when using the Congo red method, 14.28% of isolates were positive for biofilm production and 85.7% were unable to form biofilms.

Additionally, the study by (42) found that 2.7% of *A. baumannii* isolates formed strong biofilm, 3.8% formed moderate biofilm, and 93.4% did not produce any biofilm.

#### 3.6.2 Microtiter Plate Method

The results indicated that each isolated bacterium had a different capacity to produce biofilm under the same conditions; all isolates generated biofilm in the microtiter plate test, as shown in Table 3. All tested bacteria were negative on CRA, but showed varying biofilm formation in the microtiter assay. This is because CRA detects biofilm slime by binding Congo red to polysaccharide intercellular adhesin, whereas not all biofilm producers produce detectable slime, resulting in false negatives (43). Among the tested bacteria, *E. coli*, *E. cloacae*, and *K. pneumoniae* were weak biofilm producers, while *P. mirabilis* and *S. sonnei* were moderate biofilm

producers, and *A. baumannii* and *P. aeruginosa* were strong biofilm producers; both bacteria demonstrated sensitivity to most antibiotics, based on comparison with the ODc, which was calculated as 0.4276; weak biofilm production was defined as  $0.4276 < OD \leq 0.8552$ , moderate as  $0.8552 < OD \leq 1.7105$ , and strong as  $OD > 1.7105$ . The study <sup>(44)</sup> shows that sensitive strains of *A. baumannii* formed strong biofilms for 24 hours, but thereafter their capacity to generate biofilms decreased, resulting in only weak biofilms. This result agrees with findings from <sup>(45)</sup> in Diyala/Iraq, which showed that 100% of *A. baumannii* and *P. aeruginosa* produced strong biofilms. Similarly, <sup>(46)</sup> reported that 100% of *A. baumannii* isolates produced biofilms. The study by <sup>(47)</sup> on 104 *E. coli* isolates showed that 58 (55.8%) were weak biofilm formers and 25 (24%) were moderate or strong biofilm formers. The study by <sup>(48)</sup> found that *Enterobacter cloacae* created weak biofilms in 42% of cases, moderate biofilms in 25%, and strong biofilms in 24%. Additionally, the study by <sup>(49)</sup> found that, of 32 tested *Proteus mirabilis* isolates, 13 (40.6%) formed moderate biofilms, while 8 (25%) produced either strong or weak biofilms.

**Table 3: Biofilm formation assay of isolated bacteria using the microtiter plate method**

| Isolates                         | Optical Density | Mean of ODc values results             |
|----------------------------------|-----------------|----------------------------------------|
| <i>Acinetobacter baumannii</i>   | 1.743           | Strong ( $OD > 1.7105$ )               |
| <i>Pseudomonas aeruginosa</i>    | 1.715           | Strong ( $OD > 1.7105$ )               |
| <i>Proteus mirabilis</i>         | 1.481           | Moderate ( $0.8552 < OD \leq 1.7105$ ) |
| <i>Shigella sonnei</i>           | 0.87            | Moderate ( $0.8552 < OD \leq 1.7105$ ) |
| <i>Klebsiella pneumonia</i>      | 0.5             | Weak ( $0.4276 < OD \leq 0.8552$ )     |
| Enterohemorrhagic <i>E. coli</i> | 0.697           | Weak ( $0.4276 < OD \leq 0.8552$ )     |
| <i>Enterobacter cloacae</i>      | 0.761           | Weak ( $0.4276 < OD \leq 0.8552$ )     |

### 3.7 Antibacterial test of *L. reuteri* by Agar Well Diffusion Method

This study evaluated the antibacterial activity of *L. reuteri* against isolated bacteria using the agar well diffusion method. Both the supernatant and pellet of *L. reuteri* produced a clear zone around the well when compared with the control. We measured the antibacterial activity of the *L. reuteri* supernatant and pellet using an electronic digital caliper. The results showed that the *L. reuteri* supernatant produced clear zones with diameters of 22.9, 17, 7, 25.5, 19.3, 7 and 21.5 against *A. baumannii*, *P. aeruginosa*, *P. mirabilis*, *E. cloacae*, *K. pneumoniae*, enterohemorrhagic *E. coli* and *S. sonnei*, respectively. Additionally, the pellet of *L. reuteri* produced a clear zone with diameters of 24.5, 21, 10.5, 26.6, 20.8, 10.2, and 22.6 against *A. baumannii*, *P. aeruginosa*, *P. mirabilis*, *E. cloacae*, *K. pneumoniae*, enterohemorrhagic *E. coli*, and *S. sonnei*, respectively, as demonstrated in Table 4 and Figure 6. These results agree with research in Iraq <sup>(28)</sup>, which showed that *L. reuteri* from human infant stool samples inhibited *P. aeruginosa* with a 16 mm diameter. In the research conducted by <sup>(50)</sup>, *L. reuteri* exhibited inhibitory activity against *E. coli*, *K. pneumoniae*, and *S. sonnei*, with inhibition zones measuring 8 mm, 9.5 mm, and 10 mm in diameter, respectively. In our study, *Proteus mirabilis* produced smaller inhibition zones. This may be explained by intrinsic and acquired resistance mechanisms, including biofilm formation and antibiotic resistance genes, which can limit antimicrobial activity <sup>(51)</sup>. A previous study by <sup>(52)</sup> revealed that inhibitory activity was observed in the supernatants of *L. reuteri* against most *Proteus mirabilis*, with inhibition zones of 6-15 mm. The antibacterial activity of isolates may mainly arise from pH reduction caused by the synthesis of diverse organic acids by *L. reuteri* isolates during fermentation. In addition to bacteriocin synthesis, lactic acid bacteria (LAB), such as *L. reuteri*, generate lactic acid and acetic acid, which function as effective antibacterial agents against pathogenic bacteria <sup>(53)</sup>. The findings indicate that *L. reuteri*

exhibited strong antagonistic activity against most of the isolated bacteria. This discovery highlights *L. reuteri* as a potential alternative for future treatment strategies in managing bacterial infections.

**Table 4: The antibacterial activity of *L. reuteri* (Supernatant and Pellet) using the Well Diffusion Method.**

| Name of pathogenic bacteria     | Mean $\pm$ SD<br>Diameter (mm) of the inhibition zone |                  |
|---------------------------------|-------------------------------------------------------|------------------|
|                                 | Supernatant                                           | Pellet           |
| <i>Acinetobacter baumannii</i>  | 22.9 $\pm$ 0.265                                      | 24.5 $\pm$ 0.519 |
| <i>Pseudomonas aeruginosa</i>   | 17 $\pm$ 0.818                                        | 21 $\pm$ 1.212   |
| <i>Proteus mirabilis</i>        | 7 $\pm$ 0.556                                         | 10.5 $\pm$ 0.888 |
| <i>Enterobacter cloacae</i>     | 25.5 $\pm$ 0.608                                      | 26.6 $\pm$ 0.655 |
| <i>Klebsiella pneumonia</i>     | 19.3 $\pm$ 0.624                                      | 20.8 $\pm$ 0.871 |
| enterohemorrhagic <i>E.coli</i> | 7 $\pm$ 0.916                                         | 10.2 $\pm$ 0.721 |
| <i>Shigella sonnei</i>          | 21.5 $\pm$ 0.871                                      | 22.6 $\pm$ 0.529 |

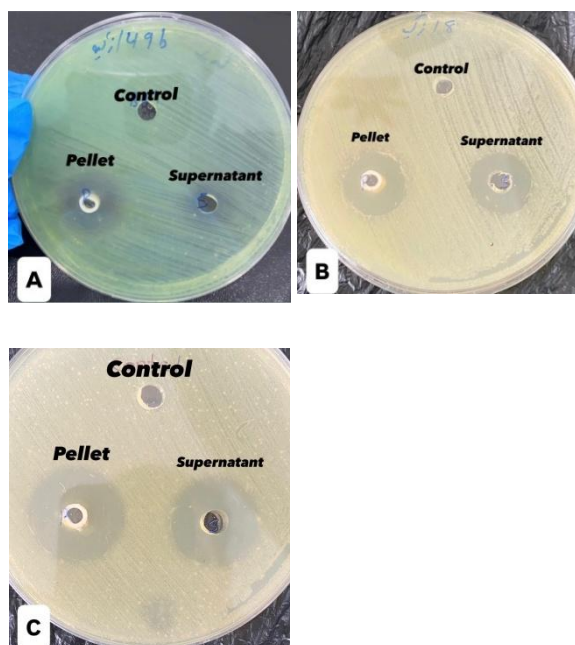


**Fig. 6: Antibacterial activity of *L. reuteri* against isolates: (A) *P. aeruginosa*, (B) *K. pneumoniae*, (C) *A. baumannii*.**

### 3.8 Antibiofilm activity of *L. reuteri* against pathogenic bacteria

We evaluated the *L. reuteri* supernatant and pellet for antibiofilm activity against pathogenic bacteria. Isolated bacteria from diarrhea formed biofilms before being mixed with the *L. reuteri* pellet and supernatant. After co-culturing the supernatant and pellet of *L. reuteri* with each of these bacteria in a microtiter plate, the results showed that *L. reuteri* could prevent biofilm formation, according to comparison with the ODc, which was calculated as 0.423; weak biofilm production was defined as  $0.423 < OD \leq 0.846$ , moderate as  $0.846 < OD \leq 1.692$ , and strong as  $OD > 1.692$ . According to the result, the supernatant of *L. reuteri* exhibited anti biofilm properties against *A. baumannii*, *P. mirabilis*, *E. cloacae*, *K. pneumoniae*, enterohemorrhagic *E.coli* O157, *S. sonnei*, *P. aeruginosa*, with optical density values of 0.502, 0.448, 0.404, 0.355, 0.342, 0.437, and 0.506, respectively, as demonstrated in Table 5 and Figure 7. Similarly, the *L. reuteri* pellet showed antibiofilm activity against *A. baumannii*, *P. mirabilis*, *E. cloacae*, *K. pneumoniae*, enterohemorrhagic *E. coli* O157, *S. sonnei*, and *P. aeruginosa*, with optical density values of 0.414, 0.431, 0.393, 0.446, 0.356, 0.422, and 0.421, respectively. Both the supernatant and pellet of *L. reuteri* significantly inhibited biofilm formation in *A. baumannii*, *P. aeruginosa*, *P. mirabilis*, and *S. sonnei* ( $p < 0.05$ ). In addition, the supernatant significantly reduced biofilm formation in enterohemorrhagic *E. coli* (EHEC) and *E. cloacae* ( $p < 0.05$ ). Figure 8 shows no significant antibiofilm effect of the pellet against *K. pneumoniae*, EHEC, or *E. cloacae*, or of the supernatant against *K. pneumoniae* ( $p > 0.05$ ). Our findings agreed with those of <sup>(54)</sup>, who demonstrated that *L. rhamnosus* exhibited the strongest inhibition of *A. baumannii* biofilms, reducing the optical density at 570 from 3.55 to 2.07, while *L. reuteri* reduced it to 2.64 and *L. acidophilus* to 3.24, indicating that *L. rhamnosus* was more effective than the other probiotics tested. The biofilm-inhibitory activity of supernatants from *L. reuteri*

and *L. lactis* against different pathogenic bacteria was previously shown by (55), who found that *L. reuteri* and *L. lactis* isolated from pineapple exhibited antibiofilm activity against *E. coli*, with inhibition rates of 53% and 73%, respectively. Research in Diyala, Iraq, showed that *L. reuteri* exhibits antibiofilm activity against *S. aureus* (6), *S. Typhimurium* and *S. Enteritidis* (56), and *S. mutans* (57). A study by (7) reported that the supernatants of *L. reuteri* and *L. acidophilus* showed antibiofilm activity against *P. mirabilis* spp. Similarly, (58) demonstrated that *L. reuteri* and *L. casei* reduced biofilm formation by *P. mirabilis*. Nevertheless, no research has examined the antibiofilm efficacy of *L. reuteri* supernatant and pellet against *S. sonnei*, *K. pneumoniae*, and *E. cloacae*. *L. reuteri* produces metabolites like reuterin that disrupt biofilms by reducing bacterial hydrophobicity and auto-aggregation, which limits initial adhesion. It also decreases metabolic activity and extracellular polymeric matrix, weakening mature biofilms (59). This study tested only a single *L. reuteri* strain. It assessed antibacterial and antibiofilm effects only in vitro, without in vivo validation to confirm efficacy and safety.

**Table 5: Antibiofilm activity of *L. reuteri* supernatant and pellet against pathogenic bacteria**

| Name of pathogenic bacteria     | Mean $\pm$ SD<br>Biofilm Optical Density measurement |                   |
|---------------------------------|------------------------------------------------------|-------------------|
|                                 | Supernatant                                          | Pellet            |
| <i>Acinetobacter baumannii</i>  | 0.502 $\pm$ 0.087                                    | 0.414 $\pm$ 0.009 |
| <i>Pseudomonas aeruginosa</i>   | 0.506 $\pm$ 0.022                                    | 0.421 $\pm$ 0.013 |
| <i>Proteus mirabilis</i>        | 0.448 $\pm$ 0.061                                    | 0.431 $\pm$ 0.039 |
| <i>Shigella sonnei</i>          | 0.437 $\pm$ 0.024                                    | 0.422 $\pm$ 0.013 |
| <i>Klebsiella pneumonia</i>     | 0.355 $\pm$ 0.028                                    | 0.446 $\pm$ 0.032 |
| enterohemorrhagic <i>E.coli</i> | 0.342 $\pm$ 0.001                                    | 0.356 $\pm$ 0.036 |
| <i>Enterobacter cloacae</i>     | 0.404 $\pm$ 0.007                                    | 0.393 $\pm$ 0.061 |

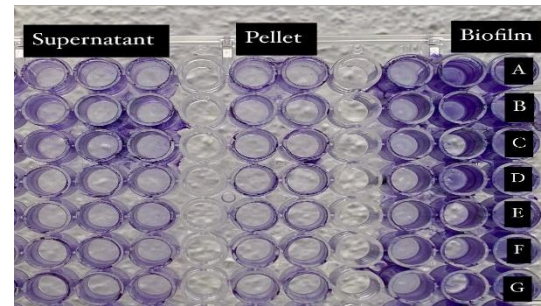
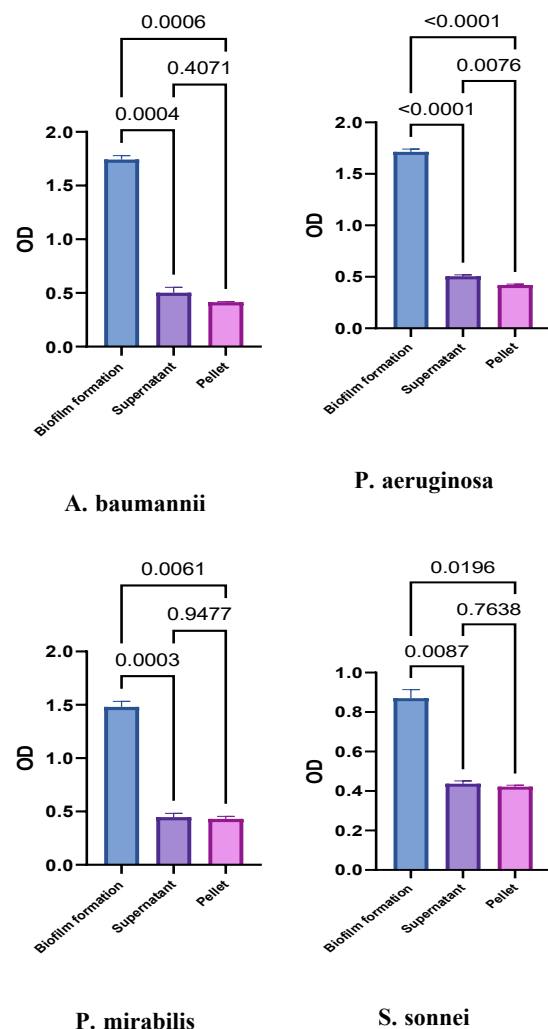


**Fig. 7: Biofilm formation and Antibiofilm activity, A: *A. baumannii*, B: *P. aeruginosa*, C: *P. mirabilis*, D: *S. sonnei*, E: *K. pneumoniae*, F: enterohemorrhagic *E.coli*, G: *E. cloacae***



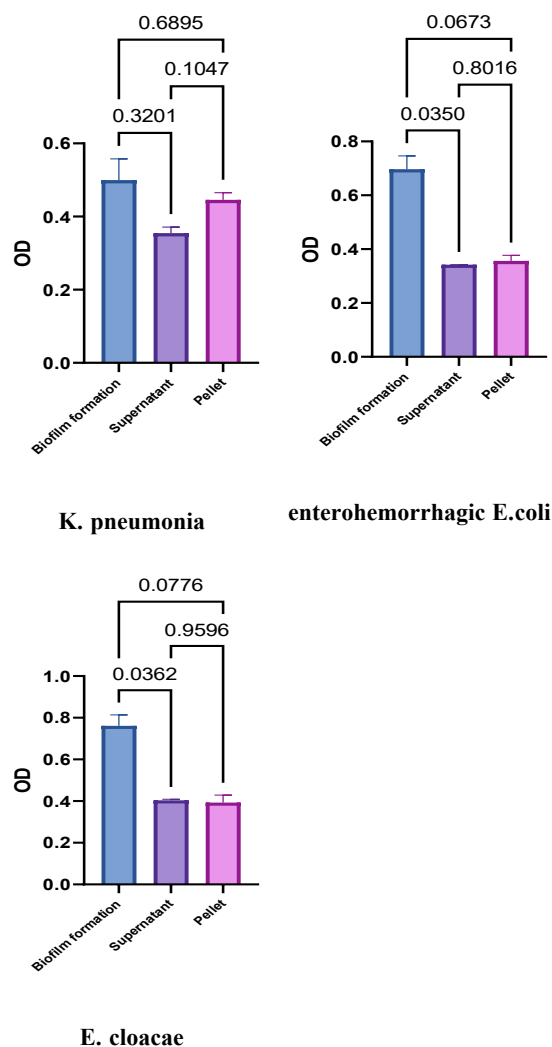


Fig. 8: Pellet and supernatant of *L. reuteri* effect on biofilm formation against pathogenic bacteria.

#### 4. CONCLUSION

This study demonstrates that both the supernatant and pellet of *Lactobacillus reuteri*, isolated from infant formula milk (Guigoz 2, Nestlé), exhibit antibacterial and antibiofilm activity against diarrheagenic bacteria isolated from children with diarrhea. Several pathogens showed significant inhibition of biofilm formation, while no significant effect was observed against *K. pneumoniae*. These findings highlight the potential of *L. reuteri* as an alternative or complementary therapeutic approach to controlling pediatric diarrheal infections, particularly amid increasing antimicrobial resistance. Future studies should include animal trials and formulation optimization to confirm efficacy and develop practical applications.

**Conflict of interest:** The authors declared no conflicts of interest.

**Sources of funding:** This research received no specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Author contributions:** The authors contributed equally to the study.

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