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Isolation and Identification of Lactobacillus delbrueckii from Cow and Sheep and its Effect on Pseudomonas putida Biofilm Formation

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ABSTRACT:

Lactic acid bacteria (LAB), especially ***Lactobacillus delbrueckii*** subsp. **lactis** and subsp. **Bulgaricus** are well-known for their probiotic and antimicrobial properties. Lactic acid bacteria (LAB) were isolated from yogurt samples and comprehensively characterized using phenotypic and molecular approaches, confirming their classification as ***Lactobacillus delbrueckii*** subsp. **lactis** and subsp. **bulgaricus**. Seventeen pathogenic bacterial isolates were subjected to the same analyses, with seven verified at the molecular level. Notably, ***Pseudomonas putida*** demonstrated multidrug resistance (MDR) against six antibiotics. and demonstrated strong Biofilm formation was assessed using two complementary methods Congo Red agar (CRA) and the microtiter plate assay. The antibacterial effects of Lactobacillus subsp. Lactis cell-free supernatants and bacterial pellets were tested against ***Pseudomonas putida*** using the well diffusion method. Results revealed that both Lactobacilli exerted significant inhibitory effects, with bacterial pellets showing greater activity than supernatants. These findings support the potential use of ***Lactobacillus delbrueckii*** subsp. **Lactis** and **bulgaricus** to combat MDR biofilm-forming pathogens, offering a promising alternative for infection control.

Keywords: Lactic acid bacteria, Pseudomonas putida, Biofilm inhibition, Antibiotic susceptibility.

1 INTRODUCTION

Lactobacillus delbrueckii is recognized as the type species of the redefined genus *Lactobacillus*, following a recent taxonomic revision that divided the original genus into 25 separate genera. This reclassification was supported by comprehensive genetic and phylogenetic studies, along with differences in ecological niches and metabolic characteristics among the species [1]. *Lactobacillus delbrueckii* belongs to the lactic acid bacteria (LAB) group, characterized as Gram-positive, rod-shaped, facultatively anaerobic, and acid-tolerant microorganisms. These bacteria commonly inhabit carbohydrate-rich environments, where they produce lactic acid as the primary end product of fermentation. The species mainly includes two subspecies: *Lactobacillus delbrueckii* subsp. *bulgaricus* and subsp. *lactis*, both of which hold significant industrial importance in the manufacture of fermented dairy products such as yogurt and cheese, as well as in biotherapeutic applications. Several strains of *L. delbrueckii* have demonstrated probiotic potential due to their ability to withstand gastrointestinal stressors, inhibit pathogenic bacteria, and exert anti-inflammatory effects. These probiotic characteristics have been studied in relation to gastrointestinal disorders, including colorectal cancer, ulcerative colitis, and intestinal mucositis. Emerging research also suggests their potential roles in managing systemic conditions such as arthritis, depression, and diabetes [2]. Among these, *L. delbrueckii* subsp. *bulgaricus* is globally recognized for its probiotic attributes and it is widely used as a starter culture in dairy fermentation. This homofermentative bacterium, the first described species within the genus *Lactobacillus*, is notable for its long-standing history of safe use in food production [3]. Probiotic strains of *L. delbrueckii* also contribute to enhancing food quality, extending shelf life, and improving microbiological safety, making them ideal candidates for natural biopreservation strategies [4]. In contrast, numerous pathogenic bacteria continue to be major contributors to nosocomial and opportunistic infections, particularly in healthcare environments. Various multidrug-resistant bacteria obtained from several hospitals due to their relevance to current clinical challenges. *Acinetobacter baumannii* is a Gram-negative, non-motile coccobacillus and a member of

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the ESKAPE group of multidrug-resistant (MDR) pathogens. It is particularly notorious for its ability to survive harsh environmental conditions and form persistent biofilms in clinical settings. It is frequently implicated in ventilator-associated pneumonia and bloodstream infections with high mortality rates [5]. *Burkholderia cepacia* is an environmentally ubiquitous bacterium characterized by exceptional metabolic versatility, a complex genome comprised of three chromosomes, and a remarkable ability to rapidly mutate and adapt. It exhibits intrinsic resistance to a wide range of antibiotics and antiseptics, and is capable of surviving under nutrient-depleted conditions [6]. *Escherichia coli* is an exceptionally versatile microorganism and a prominent member of the normal intestinal microbiota in both humans and animals. While typically a harmless commensal, *E. coli* can acquire a diverse array of mobile genetic elements encoding various virulence factors, thereby transforming into a pathogenic strain capable of causing a broad spectrum of intestinal and extraintestinal diseases [7]. *Klebsiella pneumoniae* is a Gram-negative, encapsulated bacterium and a well-known opportunistic pathogen, predominantly affecting immunocompromised individuals and commonly associated with nosocomial infections. In addition to typical clinical strains, a subset of hypervirulent serotypes has been identified, characterized by excessive production of capsular polysaccharide. These hypervirulent strains are capable of causing severe community-acquired infections in otherwise healthy individuals, such as pyogenic liver abscess, meningitis, necrotizing fasciitis, endophthalmitis, and life-threatening pneumonia. [8]. *Serratia marcescens* is a Gram-negative bacillus recognized as an opportunistic pathogen implicated in respiratory and urinary tract infections, as well as septicemia. Although rarely associated with infective endocarditis, in the uncommon instances where it is involved, the infection typically progresses rapidly and with severe consequences[9]. *Staphylococcus aureus* is a Gram-positive, catalase-positive coccus classified within the genus *Staphylococcus*, family *Staphylococcaceae*, and order *Bacillales*. Cells measure approximately 0.5 to 1.5 µm in diameter and are non-motile, non-spore-forming facultative anaerobes that typically form grape-like clusters. [10]. *Staphylococcus haemolyticus* is a coagulase-negative staphylococcus (CoNS) that constitutes a significant component of the normal human skin microbiota. While typically a commensal organism, it has emerged as an opportunistic pathogen, particularly in healthcare settings, where it is frequently isolated from hospital environments and medical personnel. This species has gained increasing attention due to its role in nosocomial infections and its notable resistance to antibiotics, especially among clinical strains, making it more resilient than other CoNS [11]. The *Pseudomonas putida* group comprises rod-shaped, aerobic, non-spore-forming, Gram-negative bacteria, typically equipped with polar flagella that facilitate motility. This group belongs to the genus *Pseudomonas*, the largest within the family *Pseudomonadaceae*, which includes over 240 validly named species. These bacteria exhibit extensive genetic and metabolic diversity and thrive in a wide range of ecological niches, including soil, water, and both animal and plant tissues [12]. Although many *Pseudomonas* species are environmental, several have been identified as opportunistic pathogens affecting humans, animals, and plants. One of the defining features of *P. putida* is its robust defense mechanisms against oxidative stress. Aerobic metabolism generates reactive oxygen species (ROS), and *P. putida* has evolved sophisticated cellular systems to mitigate their harmful effects. These include stress-sensing proteins, detoxification enzymes, and regulatory networks governed by redox-sensitive transcriptional regulators such as SoxR and OxyR, distinct in function compared to those in *Escherichia coli* and *Salmonella* spp [13]. *P. putida* strains isolated from clinical samples can occasionally cause nosocomial infections. The researchers assessed various virulence factors and biofilm-forming abilities of these strains to understand their pathogenic potential. Although infrequent, it has the capacity to act as an opportunistic pathogen in healthcare settings, particularly in immunocompromised patients[14]. Biofilm formation in *P. putida* is influenced by environmental factors such as temperature and nutrient availability. Under nutrient-rich conditions, biofilms form initially across temperature ranges; however, biofilm detachment occurs at higher temperatures once maturity is reached, whereas detachment is less prevalent at lower temperatures. In nutrient-poor conditions, the effect of temperature is diminished, and detachment becomes evident across all tested temperatures. These observations suggest that nutrient limitation may play a regulatory role not only in biofilm development but also in the timing of biofilm dispersal [15] The present study aimed to isolate and identify *Lactobacillus delbrueckii* strains from locally produced cow and sheep yogurt and to evaluate their potential inhibitory effect on biofilm formation by multidrug-resistant *Pseudomonas putida*. By characterizing the probiotic and antimicrobial properties of the isolated strains, the research sought to assess their possible application as natural, safe, and effective alternatives to conventional antimicrobial agents in controlling pathogenic biofilm-associated infections.

2 MATERIALS AND METHODS

2.1 SAMPLING

A total of fifty clinical samples were collected from various sources, including blood, sputum, cerebrospinal fluid (CSF), vaginal high swabs (VHS), and throat swabs. These samples were obtained from patients at different hospitals in Erbil city, namely Maternity Hospital, Shar Private Hospital, Rozhawa Governmental Hospital, and Raperin Hospital, during the period from July to September 2024. In addition, lactic acid bacteriam (LAB) were isolated from natural *L. delbrueckii* subsp. *Lactis* and *bulgaricus* were obtained from local supermarkets.

2.2 ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA

After adding one gram or one milliliter (according to the type of sample, 1 gram of solid sample or 1 milliliter of liquid sample) to 4 ml of 'de Man, Rogosa, and Sharpe' (MRS) broth (Oxoid, Basingstoke, UK), The mixture was homogenized using a vortex mixer, and this procedure was repeated for each sample. The inoculated broth samples were subsequently placed in an anaerobic jar and incubated at 37 °C for 24 to 48 hours. Following incubation, a loopful of the cultured broth was streaked onto (MRS) agar plates for further growth. The isolates were identified by Gram staining, morphological characterization, and molecular analysis using PCR amplification of the 16S rRNA gene. These tests allowed accurate confirmation of the Lactobacillus strains [16].

2.3 ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA

The bacterial isolates obtained from different clinical sources were streaked onto blood agar, nutrient agar, MacConkey agar, and mannitol salt agar, and incubated at 37 °C for 24–48 hours under aerobic conditions. Subculturing was performed repeatedly to obtain pure colonies. Morphological characteristics of the colonies were assessed, including colony shape, size, color, hemolytic activity on blood agar, lactose fermentation on MacConkey agar, and mannitol fermentation on mannitol salt agar, indicated by color changes in the media [17] . These phenotypic traits were used for initial classification and differentiation of the isolates.

2.4 MOLECULAR IDENTIFICATION

1- DNA Extraction:

Genomic DNA was isolated from nine bacterial isolates, which included seven pathogenic strains and two lactic acid bacteria (LAB). Extraction was performed using the Beta Bayern Tissue and Bacterial DNA Preparation Kit (Beta Bayern GmbH, 90453 Bayern, Germany), depending on the manufacturer's instructions. In brief, 1.5 mL of an overnight bacterial culture grown in Mueller-Hinton Broth for pathogenic isolates and MRS Broth for LAB, was centrifuged at 12,000 × g for 1 min using a bench-top microcentrifuge, and the supernatant was discarded. The bacterial pellet was resuspended in 400 µL of lysis buffer to disrupt the cells, followed by the addition of 12 µL proteinase K (20 mg/mL). The mixture was thoroughly vortexed and then incubated at 80 °C for 5–10 minutes to ensure complete cell lysis before cooling to room temperature. Next, 300 µL of binding buffer (BDB) was added and vigorously mixed to facilitate DNA binding. The lysate was placed on ice for 5 minutes before being transferred into a spin column positioned in a collection tube. The sample was centrifuged at 8,000 × g for 2 minutes, discarding the flow-through. The column was washed sequentially with 300 µL each of wash buffers BDW1 and BDW2, with centrifugation at 10,000 × g for 2 minutes after each wash. The spin column was then moved to a clean 1.5 mL microcentrifuge tube, and DNA was eluted by adding 50 µL of elution buffer (BDE) preheated to 70 °C. After incubation at room temperature for 1 minute, the column was centrifuged at 12,000 × g for 1 minute to collect the purified DNA. The isolated genomic DNA was stored at –20 °C for subsequent analyses.

2- Polymerase Chain Reaction(PCR) Amplification :

PCR amplification of the 16S rRNA was conducted in a total volume of 50 µL. Each reaction mixture comprised 2× Taq DNA Polymerase Master Mix (AMPLIQON A/S, Stenhuggervej 22), 10 picomoles (pmol) of each primer, DNase-free water, and template DNA (detailed in Tables 1). Amplifications were performed on a Bioresearch PTC-200 Gradient thermocycler following the specified cycling conditions.

Table 1. PCR Amplification Reagents

No.	PCR components	Concentration	Volume (µl)
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/µl	3
	Total		50

The thermal cycling protocol commenced with a denaturation that initiated at 95 °C for 5 minutes, followed by 35 amplification cycles, each consisting of denaturation at 95 °C for 40 seconds, annealing at 58 °C for 40 seconds, and

extension at 72 °C for 1 minute. A final extension was performed at 72 °C for 5 minutes to complete the amplification process

3- Agarose gel electrophoresis

Agarose gel electrophoresis is a widely used method in molecular biology that enables the separation of DNA fragments based on their size or molecular mass.

4- Preparation of Agarose Gel:

For the preparation of 1% agarose, prepare the agarose gel. Dissolve 1gram of agarose in 1X TBE buffer, then heat the mixture in a microwave until it becomes completely clear. Afterward, the solution was cooled to approximately 37–45°C with gentle swirling to prevent bubble formation. A safety dye (5–10 µL) was added to the molten gel and mixed evenly. The agarose solution was then poured into a gel casting tray and allowed to solidify at room temperature. Once the gel had set, the combs were carefully removed. The gel tray was placed into the electrophoresis tank, and enough 1X TBE buffer was added to submerge the gel with a 2–3 mm layer of buffer above it. For electrophoresis, 5 µL of each DNA sample was combined with 3 µL of 6X loading dye and loaded into the individual wells. A *DNA* ladder (ranging from 1500 to 100 base pairs) was also loaded into a designated well. For *PCR* products, the same loading procedure was used, but additional loading dye was unnecessary. Electrodes were connected to the power supply with correct polarity (red to positive and black to negative), and the voltage was set between 50 and 100 volts. After electrophoresis was complete, the gel was carefully removed using gloves and visualized under ultraviolet (UV) light. The gel was then photographed and documented using a UV transilluminator.

5- Sequencing of DNA

After Electrophoresis of DNA fragments and visualizing it, The PCR products targeting the partial 16S rRNA gene were sequenced using the ABI Prism Terminator Sequencing Kit (Applied Biosystems) at Macrogen Molecular Company, Korea. The resulting chromatograms were edited, and base calls were verified using FinchTV software.

6- Sequence alignment

The gene sequences were analyzed using the Basic Local Alignment Search Tool (BLAST), which is accessible via the NCBI (National Center for Biotechnology Information) website at https://blast.ncbi.nlm.nih.gov/Blast.cgi. BLAST is a sequence alignment tool used to compare a query sequence with known biological sequences stored in public databases. Through this comparison, it identifies regions of similarity and helps determine the closest matching bacterial sequences available in GenBank.

2.5 ANTIBIOTIC SUSCEPTIBILITY TESTING OF PATHOGENIC BACTERIA

Antibiotic susceptibility testing for pathogenic bacteria was conducted using the Kirby–Bauer disk diffusion method, following the standardized procedures outlined by the Clinical and**.** Antibiotic susceptibility testing of the pathogenic isolates was performed using the Kirby–Bauer disk diffusion method, in accordance with the standardized guidelines of the Clinical and Laboratory Standards Institute [18]. Each isolate was cultured on Mueller–Hinton agar, the recommended medium for this assay due to its reproducibility and ability to support the growth of non-fastidious organisms. Bacterial suspensions were adjusted to the 0.5 McFarland turbidity standard and evenly spread over the agar surface using sterile cotton swabs. Commercially prepared antibiotic discs of standard concentrations recommended by CLSI were used. The panel included ten commonly employed antibiotics: Ciprofloxacin (CIP), Imipenem (IPM), Meropenem (MEM), Piperacillin (PIP), Oxacillin (OX), Trimethoprim–Sulfamethoxazole (SXT), Gentamicin (GEN), Levofloxacin (LE), Ampicillin (AMP), and Cefotaxime (CEFO). The discs were aseptically placed on the inoculated plates using sterile forceps, ensuring adequate spacing to prevent overlapping inhibition zones. Plates were incubated at 37 °C for 18–24 hours under aerobic conditions [19].

2.5.1 MODIFIED CONGO RED AGAR METHOD (MCRA)

Phenotypic production of biofilm in selected isolates of *Psuedomonas Putida* was assessed by culture on MCRA plates explained. Colony morphology was evaluated based on pigmentation: colonies that appeared black or very black were considered strong biofilm producers, those with an almost black appearance were classified as weak biofilm producers, and red colonies were regarded as non-biofilm-forming strain [20].

2.5.2 MICROTITER PLATE ASSAY

Quantitative evaluation of biofilm production was carried out using a modified microtiter plate assay (96 wells). To assess biofilm formation, bacterial cultures were first incubated overnight at 37 °C in tryptic soy broth (TSB; Merck, Germany) supplemented with 0.5% glucose. The resulting cultures were diluted at a 1:40 ratio using fresh glucose-enriched TSB. From each diluted suspension, 200 µL was transferred into separate wells of a sterile, flat-bottom polystyrene microplate. Plates were incubated at 37 °C for 48 hours under static conditions. Wells filled with 200 µL of TSB containing 0.5% glucose, without any bacterial inoculum, served as negative controls.After incubation, each well was rinsed three times

with phosphate-buffered saline (PBS; pH 7.2) to remove planktonic cells, leaving only the attached biomass. The plates were then treated with methanol for 20 minutes to fix the adhered material, followed by air-drying at room temperature. Biofilm residues were stained using 0.1% safranin, and excess stain was washed away. To quantify the biofilm, the bound dye was resolubilized by adding 1 mL of 95% ethanol per well. Absorbance was read at 490 nm using a microtiter plate reader. To interpret the results, the optical density cut-off (ODc) was calculated as the mean absorbance of the negative controls plus three standard deviations.

2.6 DETERMINATION OF ANTIBACTERIAL ACTIVITY OF LACTOBACILLI AGAINST SELECTED PATHOGENIC BACTERIA

2.6.1 WELL DIFFUSION METHOD

Sheep and cow isolates were revived from stock cultures on (MRS) agar and incubated anaerobically at 37 °C for 18 hours [21]. The antimicrobial activity of both subspecies against selected pathogenic isolates was evaluated using the agar well diffusion method, following standardized protocols [22]. Mueller–Hinton agar plates were prepared by pouring the medium to a uniform depth of 4 mm and allowing it to solidify at room temperature. A bacterial lawn of selected pathogenic bacteria was created by uniformly swabbing the agar surface with 100 µL of a suspension adjusted to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). Wells of 7 mm diameter were aseptically punched into the agar using a sterile corkborer. Each well was filled with 60 µL of either the cell-free supernatant (CFS) or the resuspended pellet obtained from the *Lactobacilli* isolates. Sterile brain heart infusion (BHI) broth served as the negative control. The plates were pre-incubated at 4 °C for 30 minutes to promote diffusion, followed by incubation at 37 °C for 18 hours under aerobic conditions. Upon completion, the inhibition zones—including the 7 mm well diameter—were measured in millimeters using a digital caliper.

2.7 STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism version 9.0 (GraphPad Software, USA). Anti-biofilm activity of the probiotic treatments against *Pseudomonas putida* was assessed using OD values measured at 490 nm across five groups: untreated control, cell-free supernatants (CFS) of *Lactobacillus delbrueckii* subsp. *bulgaricus* and subsp. *lactis*, and the corresponding bacterial pellets. Given the small sample size ($n = 3$ per group), normality testing was limited. Therefore, the non-parametric Kruskal–Wallis test was used to compare groups, with Dunn's post hoc test for pairwise comparisons. Results are presented as mean ± standard error (SE), and $p < 0.05$ was considered statistically significant. For antibiotic susceptibility, statistical comparisons were not conducted due to the limited number of isolates; resistance patterns are reported descriptively as percentages.

3 RESULTS

3.1 ISOLATION AND CHARACTERIZATION

A total of 50 clinical samples were collected from various sources including blood, sputum, cerebrospinal fluid (CSF), vaginal high vaginal swabs (VHS), and throat swabs 17 were positive while 33 were negative most of them from CSF source from different hospitals in Erbil city: Maternity Hospital, Shar Private Hospital, Rozhawa Governmental Hospital, and Raperin Hospital. These samples were tested for bacterial identification, antibiotic susceptibility, genomic analysis, and biofilm production (Table 2).

Table 2. Distribution of bacterial isolates by species and sample source

Species	Total Isolates (n)	Sample Sources
Acinetobacter baumannii	6	Blood, Sputum
Burkholderia cepacia	1	Sputum
Escherichia coli	2	Sputum, VHS
Klebsiella pneumoniae	4	Blood, Sputum
Pseudomonas putida	1	Throat Swab
Serratia marcescens	1	Sputum
Staphylococcus aureus	1	VHS
Staphylococcus haemolyticus	1	blood

The distribution of bacterial isolates presented in (Table 3) illustrate that the most frequently isolated organism was *Acinetobacter baumannii*, accounting for 35% of the total isolates. These were predominantly obtained from sputum and blood samples at Rozhawa and Shar hospitals, primarily from male patients. *Klebsiella pneumoniae* represented the

second most prevalent species (23.5%), followed by *Escherichia coli* (12%), which was recovered from both VHS and sputum samples. Other less common isolates, each constituting 6% of the total, included *Burkholderia cepacia*, *Pseudomonas putida*, *Serratia marcescens*, *Staphylococcus aureus*, and *Staphylococcus haemolyticus*. These findings reflect a wide range of opportunistic bacterial pathogens across different anatomical sites and hospital settings, with notable predominance of Gram-negative bacilli in respiratory and bloodstream infections.

Table 3. Clinical Distribution of Pathogenic Bacterial Isolates According to Source, Hospital Site, Gender, and Relative Frequency

Bacterial ID	Samples source	Samples collection site	Gender	Percentage
Acinetobacter baumannii	Blood	Rozhawa	Male	
Acinetobacter baumannii	Sputum	Rozhawa,	Male	
Acinetobacter baumannii	Sputum	Shar	Female	
Acinetobacter baumannii	Sputum	Rozhawa,	Male	
Acinetobacter baumannii	Sputum	Rozhawa,	Male	
Acinetobacter baumannii	Blood	Rozhawa	Female	35%
Burkholderia cepacia	Sputum	Rozhawa	Male	6%
Escherichia coli	VHS	Maternity	Female	
Escherichia coli	Sputum	Rozhawa	Male	12%
Klebsiella pneumoniae	Sputum	Rozhawa	Female	
Klebsiella pneumoniae	Sputum	Rozhawa	Male	
Klebsiella pneumoniae	Blood	Rozhawa	Male	
Klebsiella pneumoniae	Blood	Rozhawa	Male	23.5%
Pseudomonas putida	Throat swab	Raperin	Male	6%
Serratia marcescens	Sputum	Rozhawa	Female	6%
Staphylococcus aureus	VHS	Maternity	Female	6%
Staphylococcus haemolyticus	Blood	Rozhawa	Male	6%

3.2 GENOMIC DNA ISOLATION AND PCR AMPLIFICATION

Genomic DNA was extracted using the Animal DNA Extraction Kit provided by Beta Bayern GmbH (Germany), in accordance with the manufacturer's recommended protocol. The quality and presence of the extracted DNA were evaluated through electrophoresis on a 1.5% agarose gel. Clear and distinct bands were observed, confirming the integrity of the genomic material (Figure 1). Amplification of the 16S rRNA gene (partial region) was subsequently carried out using species-specific primers obtained from Micro-Gene (South Korea). The PCR produced a clear band at the expected size of 372 bp, indicating successful amplification (Figure 1) Negative controls showed no amplification, ensuring the validity of the PCR (Figure 2).

Genomic DNA isolation

The genomic DNA was isolated by Bioscience Animal DNA preparation Kit Beta Bayern DNA preparation Kit (Beta Bayern GmbH .90453 Bayern, Germany). The isolated DNA was electrophorized in 1.5% Agarose gel (Figure 2).

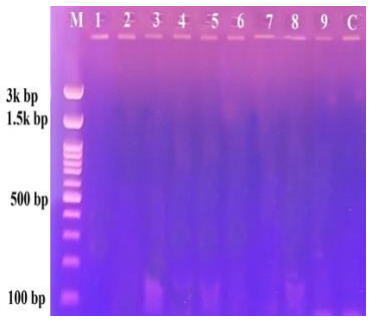

FIGURE 1. Genomic DNA isolated from isolated bacteria PCR amplification of partial genes

Primers specific to the bacterial 16S rRNA gene were synthesized by Micro-Gene Company (South Korea), targeting a 372 bp fragment corresponding to the expected size of the partial gene. The amplified PCR products were resolved on a 1.5% agarose gel, and the resulting bands were visualized using ultraviolet (UV) transillumination. A distinct band of approximately 372 bp confirmed successful amplification (Figure 2).

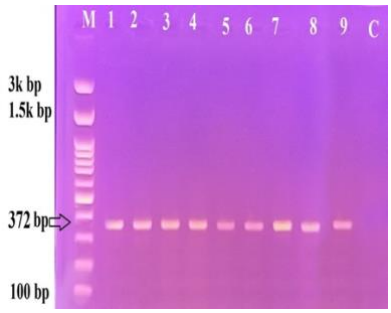

FIGURE 2. PCR amplification of partial 16S rRNA gene from bacteria, wells include M; Ladder (100-3000 bp), lane1-9; gene bands with the size of 372 bp amplified and C indicate negative control without the band.

Partial genes Sequenced

DNA sequencing, using only forward primers of 16S rRNA; (5' CGTTGACTGCCGGTGACAAAC-3') Sequencing was conducted independently using the ABI 3130X Genetic Analyzer (Applied Biosystems). The PCR-amplified products from all samples served as DNA templates for subsequent sequence-specific amplification reactions.

Molecular Identification and BLAST Analysis

Sequencing of the PCR products was done using an ABI 3130X genetic analyzer (Applied Biosystems), and sequences were compared using the NCBI BLAST database. The results revealed high sequence identity (100%) with known reference strains in GenBank.

Molecular Identification of Genus and Species of bacteria depend of 16S rRNA

The partial gene sequences were aligned using the BLAST program from GenBank (http://blast.ncbi.nlm.nih.gov/), which compared the amplified sequences to those stored in the database. The BLAST results showed that the highest query sequence identity was 100% (Table 4).

Table 4. GenBank accession numbers of bacterial identification depend on patrial gene of 16S rRNA gene

sample	Species name	Gen bank Accession No.
1	*Acinetobacter baumannii*	PQ596574
2	*Acinetobacter baumannii*	PQ596575
3	*Acinetobacter baumannii*	PQ596576
4	*Klebsiella pneumoniae*	PQ596577
5	*Klebsiella pneumoniae*	PQ596578
6	*Pseudomonas putida*	PQ596579
7	*Staphylococcus haemolyticus*	PQ596580
8	*Lactobacillus delbrueckii subsp. bulgaricus*	PQ839727
9	*Lactobacillus delbrueckii subsp. lactis*	PQ839728

Table 5. Percentage distribution of Bacterial species based on partial 16s of rRNA according to blast in GenBank of NCBI

samples	*Bacterial* Identified	Accession Numbers	Query Cover %	Identic Number %	Genbank Accession Number	Genbank Species Identification
1	*Acinetobacter baumannii*	PQ596574	100	100	CP162554	*Acinetobacter baumannii*
			100	100	CP115637	
2	*Acinetobacter baumannii*	PQ596575	100	100	AP031585	
			100	100		
3	*Acinetobacter baumannii*	PQ596576	100	100	CP151704	
			100	100		
4	*Klebsiella pneumoniae*	PQ596577	100	100	CP121612	*Klebsiella pneumoniae*
			100	100	LR133964	
			100	100	CP154203	
			100	100	PQ596577	
5	*Klebsiella pneumoniae*	PQ596578	100	100	CP154072	
6	*Pseudomonas putida*	PQ596579	100	100	MF276644	*Pseudomonas putida*
			100	100	PQ596579	
			100	100	KY074209	
			100	100	KF956613	
7	Staphylococcus haemolyticus	PQ596580	100	100	CP142094	Staphylococcus haemolyticus
			100	100	MK015846	
			100	100	KM877509	
			100	100	MN907478	
8	*Lactobacillus delbrueckii subsp. bulgaricus*	PQ839727	100	100	CP172544	*Lactobacillus delbrueckii subsp. bulgaricus*
			100	100	CP172542	
			100	100	LT899687	
			100	100	CR954253	
9	Lactobacillus delbrueckii subsp. lactis	PQ839728	100	100	CP109946	Lactobacillus delbrueckii subsp. lactis
			100	100	LS991409	
			100	100	CP029250	
			100	100	CP072444	
			100	100	CP031023	
			100	100	CP018215	

Phylogenetic inferences

Phylogenetic analysis was performed using the MEGA 11 software, applying the bootstrap resampling method to assess the reliability of the nucleotide sequence alignments. The resulting phylogenetic tree showed that the bacterial species grouped in accordance with their expected taxonomic relationships. Based on the sequence similarity and divergence data, the constructed phylogeny indicated that species within the same genus clustered closely together. The nine samples of bacteria were grouped in different clusters with high similarity to the same genus and species of NCBI Genbank after the blast (Figure 3).

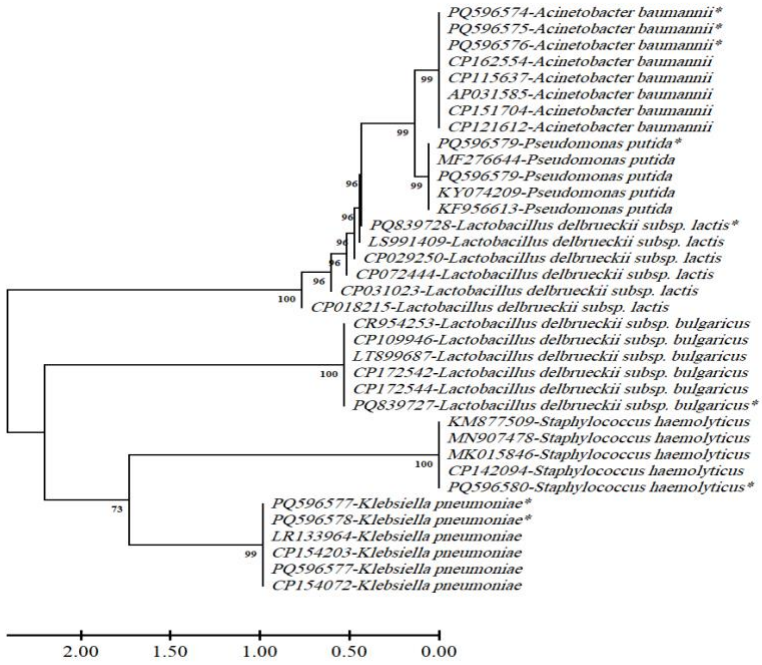

FIGURE 3. Employing Maximum Likelihood with a boost strap of the Mega 11 program shows the phylogenetic positioning of each bacteria species of nine samples with similar GenBank sequences of 16S rRNA partial genes that are available in GenBank. The stars (*) are query samples

3.3 CHARACHTERIZATION OF LACTIC ACID BACTERIA

Two lactic acid bacteria (LAB) isolates were obtained from yogurt samples collected from local supermarkets in Erbil city, Iraq. Following isolation, molecular detection techniques were performed, confirming that both isolates belong to the species *Lactobacillus delbrueckii*. Further subspecies-level identification revealed that one isolate (LAB1) was *L. delbrueckii* subsp. *lactis*, while the other (LAB2) was *L. delbrueckii* subsp. *bulgaricus*. Gram staining confirmed that both isolates were Gram-positive. The relative abundance of each isolate within the respective yogurt samples was estimated at 50%, based on colony morphology and the frequency observed during preliminary culturing on selective media. The details of the isolates are summarized in (Table 6).

Table 6. Characteristics of lactic acid bacteria isolates

Isolate Code	Bacterial Species	Subspecies	Source	Gram Reaction	Relative Abundance (%)
LAB1	*Lactobacillus delbrueckii*	subsp. *lactis*	Cow yogurt	Gram-positive	50%
LAB2	*Lactobacillus delbrueckii*	subsp. *bulgaricus*	Sheep yogurt	Gram-positive	50%

3.4 ANTIBIOTIC SUSCEPTIBILITY TEST OF CLINICAL BACTERIA

Seventeen clinical bacterial isolates from seven pathogenic species were examined for their antibiotic susceptibility. Multidrug resistance was widespread, with *Pseudomonas putida* showing the highest resistance, displaying non-susceptibility to six major antibiotic classes including β-lactams, carbapenems, fluoroquinolones, aminoglycosides, sulfonamides, and penicillins. *Acinetobacter baumannii* also demonstrated resistance to multiple classes, particularly carbapenems and fluoroquinolones, while isolates of *Escherichia coli* and *Klebsiella pneumoniae* showed resistance to β-lactams and aminoglycosides, with additional resistance observed in some strains. Among Gram-positive bacteria, *Staphylococcus aureus* was resistant to oxacillin and ciprofloxacin, confirming methicillin resistance. These findings underline the exceptional resistance profile of *P. putida* and emphasize the urgent need for continuous antimicrobial resistance surveillance and the search for effective alternative therapies (Tables 7&8), (figure 4).

Table 7. Antibiotic susceptibility test showing resistance patterns of clinical bacterial isolates

Bacterial Species	Antibiotic Resistance
1. *acinetobacter baumannii*	LE, AMP, OX,
2. *acinetobacter baumannii*	AMP, CIP, MEM, IPM, CEFO,
3. *acinetobacter baumannii*	GEN, CIP, AMP, PIP, CEFO,
4. *acinetobacter baumannii*	MEM, IPM, CIP
5. *acinetobacter baumannii*	GEN, CIP, AMP, PIP, MEM,
6. *acinetobacter baumannii*	CIP, GEN, AMP
7. *burkholderia cepacia*	IMP, GEN, CIP, CEFO
8. *Escherichia coli*	AMP, PIP, CEFO, GEN,
9. *Escherichia coli*	AMP, PIP, CEFO, GEN, CIP, SXT
10. *klebsiella pneumonia*	OX, PIP, SXT, IPM
11. *klebsiella pneumonia*	OX, CIP, FA, LE, GEN
12. *klebsiella pneumonia*	LE, CIP, OX, GEN
13. *klebsiella pneumonia*	CIP, AMP, LE
14. *Pseudomonas putida*	PIP, IPM, MEM, GEN, CIP, SXT, LE
15. *serratia marcescens*	LE, CIP, GEN
16. *staphylococcus aureus*	OX, CIP, GEN

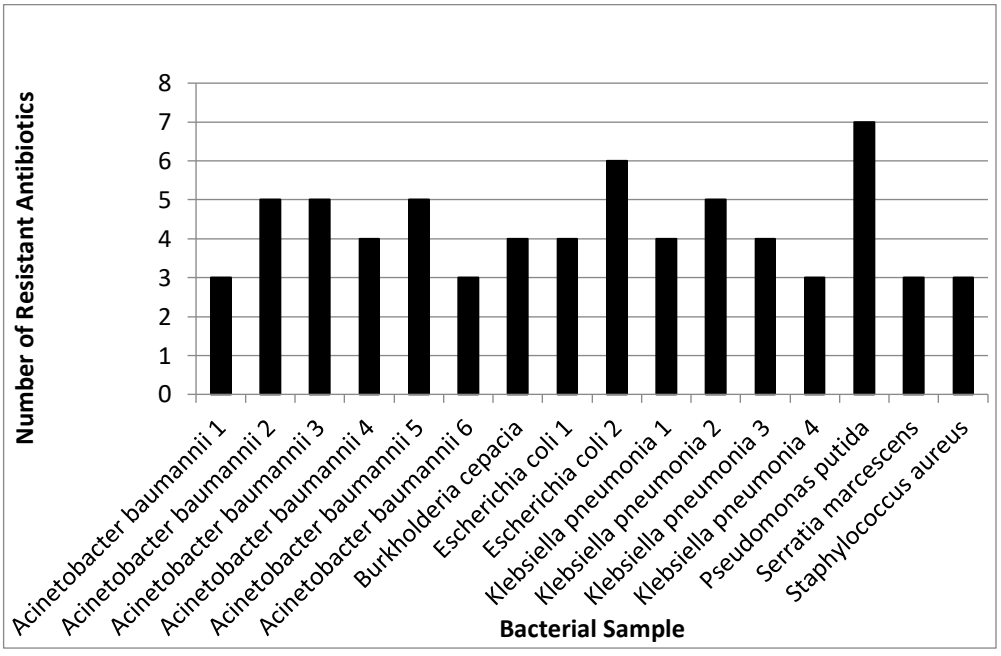

FIGURE 4. Antibiotic resistance per bacterial sample

Table 8. Percentage of resistant clinical bacterial isolates

Bacterial ID (N)	AMP	CEFO	CIP	GEN	IPM	LE	MEM	OX	PIP	SXT
acinetobacter baumannii (6)	83.3%	33.3%	83.3%	50%	33.3%	16.3%	50%	16.3%	33.3%	0%
burkholderia cepacia	0%	100%	100%	100%	100%	0%	0%	0%	0%	0%
Escherichia coli (2)	100%	100%	100%	50%	0%	0%	0%	0%	100%	50%
klebsiella pneumonia(4)	25%	0%	75%	50%	25%	75%	0%	75%	25%	25%
Pseudomonas putida	0%	0%	100%	100%	100%	100%	100%	0%	100%	100%
serratia marcescens	0%	0%	100%	100%	0%	100%	0%	0%	0%	0%
staphylococcus aureus	0%	0%	100%	100%	0%	0%	0%	100%	0%	0%
staphylococcus haemolyticus	0%	0%	100%	100%	0%	100%	0%	100%	0%	0%

Among the isolates, *Pseudomonas putida* and *Burkholderia cepacia* exhibited resistance to 6–8 antibiotics, indicating multidrug resistance, while *Staphylococcus aureus* and *Serratia marcescens* were resistant to fewer antibiotics.

3.5 PSEUDOMONAS PUTIDA BIOFILM FORMATION ASSAY

In this study, the isolate of *Pseudomonas putida* was biofilm negative on Congo red agar while had a moderate (0.805) optical density biofilm on microtiter method Biofilm formation for Pseudomonas putida was assessed using the microtiter plate method. Based on optical density (OD) readings:

Table 9. Biofilm formation for Pseudomonas putida (OD-based).

Sample	OD	Result
P. Putida biofilm	**0.805**	**Moderate**

Table 10. Classification of Biofilm formation abilities result based on optical density by microtiter plate method

Cut-off value calculation	Mean of OD values results	Biofilm formation abilities
OD > 4×ODc	OD > 0.850	Strong
2×ODc < OD ≤4×ODc	0.425< OD ≤ 0.850	Moderate
ODc< OD ≤ 2×ODc	0.212< OD ≤ 0.425	Weak
OD ≤ 0.208	OD ≤ 0.212	None

The antibacterial activity of Lactobacillus isolates was assessed using the agar well diffusion method. The activity of the cell-free supernatant from both cow-derived *Lactobacillus delbrueckii subsp. Lactis* and sheep-derived *Lactobacillus delbrueckii subsp. bulgaricus* showed no inhibition against the tested pathogens (0 mm). In contrast, the cell pellets exhibited measurable antibacterial activity, with inhibition zones of 19 mm for the cow isolate and 23 mm for the sheep isolate. The negative controls, which included culture medium without bacterial inoculum, showed no inhibition (0 mm) (Table 11). These results indicate that the antibacterial effect in this assay is primarily associated with the bacterial cells rather than the cell-free supernatant.

Table11. Probiotic Bacteria Antibacterial Activity

Probiotic bacteria		
	Cow (Lactis)	Sheep (Bulgaricus)
Supernatant	zero	zero
Pellet	19mm	23mm
Control	zero	zero

4 DISCUSSION

4.1 BIOFILM FORMATION AND ANTIBACTERIAL ACTIVITY OF *LACTOBACILLUS DELBRUECKII* AGAINST *PSEUDOMONAS PUTIDA*

The present study demonstrates that the isolated strain of *Pseudomonas putida* exhibited moderate biofilm formation as determined by the microtiter plate assay (OD = 0.805), whereas it appeared negative on Congo Red agar. This discrepancy aligns with previous observations that Congo Red agar may be insufficiently sensitive for detecting moderate biofilm producers, particularly in environmental or opportunistic pathogens such as *P. putida* [23] The capacity of *P. putida* to form biofilms, coupled with its multidrug-resistant (MDR) profile, underscores the urgent need for novel antimicrobial approaches. In this context, *Lactobacillus delbrueckii* isolates demonstrated significant antibacterial and antibiofilm activities, predominantly associated with the pellet fraction. The cow-derived *subsp. lactis* and sheep-derived *subsp. Bulgaricus* showed inhibition zones of 19 mm and 23 mm, respectively, while their cell-free supernatants exhibited no detectable activity. These findings suggest that cell-associated factors, rather than secreted metabolites, play a primary role in suppressing *P. putida* growth and biofilm formation. This is consistent with prior studies indicating that cell-bound components of lactic acid bacteria can exert greater antimicrobial effects than culture filtrates [23]

4.2 COMPARISON WITH PREVIOUS STUDIES AND NOVELTY

Previous research has demonstrated the potential of lactic acid bacteria (LAB) to inhibit biofilm formation of various opportunistic pathogens, including *Pseudomonas* and *Burkholderia* species [24] Previous studies often focused on lactic acid bacteria (LAB) strains of non-dairy origin or did not clearly distinguish between the effects of the pellet and supernatant fractions. In contrast, the present study provides new insights by evaluating both the pellet and supernatant effects of *Lactobacillus delbrueckii* subsp. *lactis* against *Pseudomonas putida*. Additionally, this study reports the first isolation of *P. putida* from a human throat swab in this geographic region and demonstrates the inhibitory potential of LAB against a clinically relevant multidrug-resistant strain. By combining environmental, molecular, and functional data, these findings offer a unique perspective on the potential application of probiotic bacteria to control resistant opportunistic pathogens.

4.3 MECHANISTIC INSIGHTS AND BIOLOGICAL RELEVANCE

The observed preferential activity of the pellet fraction highlights the importance of cell-associated antimicrobial factors, which may include membrane-bound proteins, surface-associated biosurfactants, or other structural components that interfere with pathogen adhesion and biofilm formation [23]. This mechanistic insight is critical, as it indicates that the mere presence of secreted metabolites is insufficient to achieve biofilm inhibition, emphasizing the need to preserve the viability and structural integrity of LAB cells in potential applications.

4.4 PRACTICAL IMPLICATIONS

These findings have several practical implications. First, fermented milk products containing *L. delbrueckii* could serve as a natural source of probiotic bacteria with potential antibiofilm activity, warranting further investigation as dietary supplements or functional foods. Second, the inhibitory effect of LAB against MDR *P. putida* suggests a potential role in developing preventive or adjunctive therapies for infections of the upper respiratory tract, such as gargles or mouthwashes, although in vivo studies are necessary to confirm efficacy and safety. Overall, this work supports the integration of probiotics into strategies targeting biofilm-associated infections, particularly those caused by antibiotic-resistant Gram-negative pathogens.

CONCLUSION

These results indicate that locally isolated *lactobacilli* possess potent anti-biofilm activity and may serve as promising biotherapeutic alternatives to traditional antibiotics. While the study provides promising insights, its in vitro nature limits direct clinical applicability. Future studies should include in vivo models and formulation development for probiotic-based therapies. Nonetheless, this research establishes a scientific foundation for using indigenous *Lactobacillus delbrueckii* strains in controlling pathogenic biofilm formation.

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