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Unveiling antibiotic resistance and metabolic traits in potential pathogens isolated from Al-asfar Lake

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Abstract

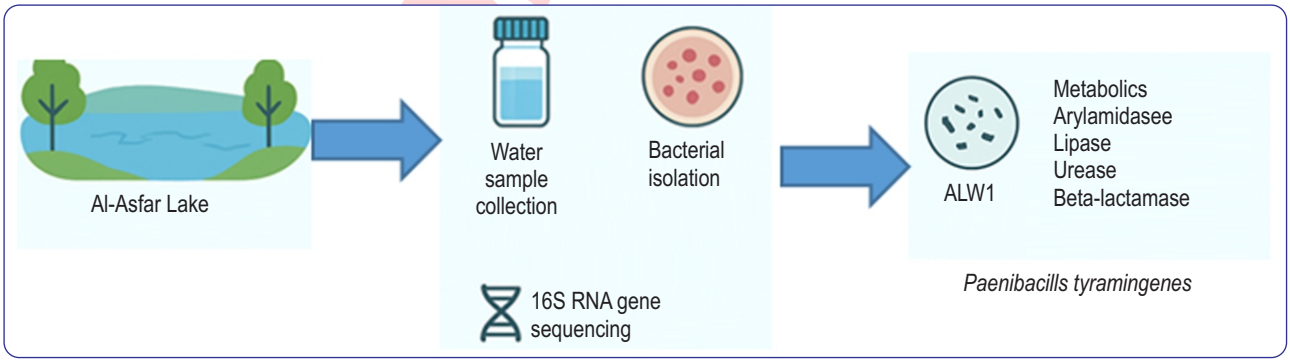
Aim: The aim of this study was to evaluate the antibiotic resistance patterns and metabolic properties of bacteria obtained from Al-Asfar Lake in order to better understand their microbial diversity and possible public health implications.

Methodology: Water samples were collected in sterile containers and processed using serial dilution and culture-based techniques to isolate bacterial strains. Two representative isolates were selected and identified according to their colony morphology and 16S rRNA gene sequencing results. Their hemolytic behavior and biochemical characteristics were further examined using conventional laboratory assays and VITEK analysis.

Results: The two isolates, ALW1 and ALW2, showed high similarity to *Staphylococcus hominis* (99.1%) and *Paenibacillus tyraminigenes* (98.7%), respectively. ALW2 demonstrated beta-hemolytic activity, was able to metabolize a wide range of carbohydrates, and exhibited resistance to bacitracin and polymyxin B. In contrast, ALW1 showed alpha-hemolytic activity, had a more limited metabolic profile, and did not display detectable antibiotic resistance. Both isolates were positive for catalase activity.

Interpretation: The findings indicate that Al-Asfar Lake harbors metabolically diverse bacterial populations, some of which may carry antibiotic resistance traits. Regular monitoring of freshwater ecosystems is therefore important to reduce the potential spread of resistant bacteria and protect public health. These results also contribute to a better understanding of microbial ecology and the possible risks associated with environmental bacterial communities.

Key words: Al-Asfar Lake, Hemolysis, Pathogenic bacteria, *Paenibacillus tyraminigenes, Staphylococcus hominis,* VITEK analysis



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Introduction

Freshwater resources are essential for human and ecosystem sustainability; however, they are increasingly threatened by microbial contamination resulting from inadequately treated wastewater discharged from domestic, agricultural and industrial sources. Such pollution introduces pathogenic bacteria, viruses and protozoa, including *E. coli, Salmonella* and *Vibrio cholerae* (Mishra, 2023). Contaminated water poses serious health risks through drinking, recreation, and indirect exposure. Waterborne diseases remain among the leading causes of mortality worldwide (Boyd, 2020). In addition, aquatic ecosystems are vulnerable, as water facilitates the transmission of diseases among fish and wildlife (Leung *et al.,* 2019). Wastewater discharge into surface water act as a double-edged sword. It alters the physico-chemical parameters, and simultaneously introduces large number of non-indigenous microorganisms, thereby reshaping native microbial communities (Kalinowska *et al.,* 2021). Compared with lotic systems such as rivers and streams, lakes and ponds have longer water residence times. This extended retention enhances interaction among physical, chemical and biological factors, often leading to more pronounced changes in water quality (ElMahmoudi *et al.,* 2008; Loucif *et al.,* 2020).

Microbial communities, particularly bacteria, serve as sensitive indicators of environmental health (McCauley *et al.,* 2019). The composition and abundance of bacterial populations reflect ecosystem status. Certain bacteria function as bioindicators, providing insights into contamination levels and ecological conditions (Battin *et al.,* 2007). For example, elevated *E. coli* levels indicate recent fecal contamination and require further investigation (USEPA, 2012). Conversely, beneficial bacteria contribute to organic matter decomposition and nutrient cycling, supporting aquatic food webs (Azam *et al.,* 1998; Xie *et al.,* 2021; Al Mousa *et al.,* 2024). Therefore, studying microbial communities is valuable not only for assessing water quality but also for identifying potential pathogenic species.

Recent evidence indicates that the physico-chemical characteristics of Lake Tonga, Algeria, are key drivers of the spatiotemporal dynamics of its microbial communities, underscoring the strong coupling between environmental variables and microbial community structure. Such findings emphasize the importance of site-specific investigations in freshwater ecosystems. Blood agar, although non-selective, is widely used for isolating potential bacterial pathogens. The presence of red blood cells enhances the growth of fastidious organisms and allow evaluation of hemolytic activity, which reflects the ability of bacteria to lyse erythrocytes (Khalifa *et al.,* (2023a); Van An *et al.,* 2023). Hemolysis patterns (alpha, beta, or gamma) may suggest pathogenic potential; however, they are not definitive indicators. Blood agar, therefore, serves as an important preliminary medium for pathogen screening, while confirmatory identification requires additional phenotypic, biochemical, and molecular analyses (Jain and Nikita, 2023; Khalifa *et al.,* 2023a,c). Within this context, Al-Asfar Lake, located in the Al-Ahsa Oasis of Saudi Arabia, represents a unique freshwater ecosystem for microbial investigation. The lake is shallow and serves as a key stopover site for migratory birds, supporting diverse habitats and complex ecological interactions (Khalifa and Ibrahim, 2023b; Al Mousa *et al.,* 2023). Understanding its biodiversity, including bacterial communities, is essential for assessing ecosystem health and potential environmental risks (Al-Yami *et al.,* 2022; Khalifa *et al.,* 2021). Despite the ecological importance of Al-Asfar Lake, limited studies have focused specifically on isolating and characterizing potentially pathogenic bacteria from its waters. Continuous monitoring is particularly important due to the lake's role as a hub for wildlife, especially migratory birds, which may act as reservoirs or vectors for waterborne pathogens. However, the presence, diversity, and identity of pathogenic bacteria in this ecosystem remain insufficiently explored. This knowledge gap limits accurate risk assessment and evidence-based management strategies.

Therefore, this study aimed to investigate the occurrence of potentially pathogenic bacteria in the water of Al-Asfar Lake. Blood agar was employed for initial isolation. The recovered isolates were subjected to phenotypic and biochemical characterization, followed by molecular identification using comparative analysis of 16S rRNA gene sequencing. This integrated approach provides a clearer understanding of the bacterial diversity in the lake and supports assessment of potential public health and ecological risks.

Materials and Methods

Samples collection: Water samples were collected on August 28th, 2021, from Al-Asfar Lake (25°32'10.5"N, 49°49'00.4"E) using sterile containers.Three water samples were obtained and immediately placed in an icebox at 4 °C, then transported to the laboratory for further analysis. All microbiological experiments were conducted in triplicate to ensure reproducibility and accuracy.

Isolation of bacteria: A serial dilution technique under sterile conditions was used to isolate bacteria from water samples. A 10 ml of water sample was diluted in 90 ml of 0.9% sterile saline diluent. Thereafter, 1ml of each dilution was inoculated onto Blood agar medium plates. All diluted samples were spread evenly on the agar surface using a sterilized L-shaped disposable rod. The inoculated plates were then incubated at 38 °C for 24–72 hrs. Incubation at 37 °C was chosen because it represents the optimal human body temperature, supporting the growth of potential human pathogenic bacteria. After incubation, the number of colony-forming units per ml (CFU ml^{-1}) was calculated from countable plates to estimate the bacterial concentration in the original sample using the following formula:

CFU ml^{-1} = Number of colonies counted ÷ (Dilution factor × Volume plated (ml), where Number of colonies counted = colonies on a countable plate (typically 30–300 colonies). Dilution factor = reciprocal of dilution used. Volume plated = volume

spread or poured onto the agar plate (in ml) (Komariah *et al.,* 2022). Distinct pure colonies were streaked them onto fresh media to obtain pure cultures. These cultures were then preserved at 4°C in agar slants for further analysis.

Phenotypic characterization of bacterial isolates: Morphological characterization of the isolated bacteria were examined by determining the colony color, edge, elevation, and shape on agar plates. Bacterial cells were examined using Gram staining, following the established protocol (Komariah et al., 2022). Bacterial isolates were further investigated using the VITEK 2® COMPACT automated system (BioMérieux) based on their biochemical reactions and nutrient usage. Specific cards were chosen based on Gram stain results: VITEK 2 Gram-negative or Gram-positive cards, each containing 64 wells with various substrates to assess the isolate's metabolic activity. Each isolate was suspended in sterile saline, adjusted to a specific turbidity using a VITEK DensiCHEK, and then loaded into the VITEK 2 system. The instrument's vacuum chamber transferred the suspension into microchannels within the test card, filling each well for analysis.

Biochemical tests of bacterial isolates: Catalase test was performed to determine the ability of the isolates to produce catalase enzyme. A small amount of bacterial culture was placed on a clean glass slide and mixed with 3–5% hydrogen peroxide (H_2O_2). The immediate formation of bubbles indicated a positive catalase reaction (Taylor and Achanzar, 1972). Hemolytic activity was evaluated by streaking the isolates onto blood agar plates, which were then incubated at 37 °C for 24 hr. Hemolysis patterns were interpreted as β-hemolysis (complete clearing around colonies), α-hemolysis (partial greenish discoloration), or γ-hemolysis (no visible hemolysis), as previously described (Shoeb *et al.,* 2015). Antimicrobial susceptibility was evaluated using the Kirby–Bauer disk diffusion assay following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI). Bacterial isolates were uniformly streaked onto Mueller–Hinton agar plates, and antibiotic discs were carefully applied using sterile forceps. The antibiotics tested included erythromycin (15 µg), bacitracin (10 µg), vancomycin (30 µg), novobiocin (5 µg) and cephalothin (30 µg). The plates were incubated at 37 °C for 24 hr. After incubation, the diameters of inhibition zones were measured in millimeters, and all measurements were performed in triplicate. The results were interpreted according to CLSI breakpoint standards (Wayne, 2011).

Molecular identification of isolates using 16S rRNA gene: Genomic DNA was extracted using the boiling method. Bacterial cells were suspended in sterile water and heated at 100 °C for 5 min, followed by centrifugation. The supernatant containing DNA was used for PCR. The supernatant containing DNA was used for PCR. PCR was performed to amplify the 16S rRNA gene using specific primers targeting a conserved region (27F: 5'-AGAGTTTGATCMTGGCTCAG-3'and1492R:5'TACGGYTACCTTGTTACGACTT-3', designed to target the conserved hypervariable regions V3–V4 of the 16S rRNA gene (Oyewusi *et al.,* 2021). PCR mixture consisted extracted DNA, a master mixture containing primers, enzymes, nucleotides, and nuclease-free water. The PCR cycling conditions involved initial denaturation, followed by repeated cycles of denaturation, annealing and extension. Finally, the amplified DNA fragments were visualized on a 1% agarose gel stained with ethidium bromide to confirm successful amplification and estimate their size using a DNA ladder as a reference (Neilson *et al.,* 2013). 16S rRNA gene sequencing was performed for all the selected bacterial isolates. All the sequenced products were resolved using an Applied Biosystems Model 3730XL automated DNA sequencing system.

Phylogenetic analysis: Following DNA sequencing, the obtained sequences were analyzed using computer software with the known sequences in the GenBank Database via the National Center for Biotechnology Information's (NCBI) BLAST algorithm. To visualize the evolutionary relationships between the isolated bacteria and known relatives, a phylogenetic tree was constructed using the MEGA11 program (Tamura, *et al.,* 2021). The tree's branching pattern was inferred using the neighbor-joining method (Saitou and Nei 1987).Evolutionary distances were calculated using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and expressed as the number of nucleotide substitution per site.

Statistical analysis: Statistical analysis was conducted using One-way ANOVA followed by Tukey's post hoc test.

Results and Discussion

The pursual of data showed that the number of colony-forming units per milliliter (CFU ml^{-1}) was calculated for all countable water samples. The total CFU in the samples ranged up to 3.1×10^4 CFU ml^{-1} (data not shown), indicating a relatively high abundance of bacterial communities in Al-Asfar Lake. Among the 30 distinct colony morphologies observed, two bacterial isolates, ALW1 and ALW2, were selected for further analysis based on their positive reactions on blood agar plates. The bacterial isolates were characterized based on colony color, margin, elevation and shape as summarized in Table 1.

Both isolates differed in colony color, margin characteristics, and microscopic features; however, they shared same Gram-positive staining. ALW1 orange in colour had a smooth, entire margin and a raised, convex shape (Fig. 1 and Table 1). ALW1 appeared as a coccus-shaped, gram-positive bacterium. ALW2, on the other hand, is beige with a wavy margin and an irregular, convex shape. Microscopically, it appears as a rod-shaped and is also gram-positive. Morphological evaluation revealed clear phenotypic differences: ALW1 formed orange, convex colonies with a smooth entire margin, while ALW2 produced beige, convex colonies with a wavy margin. Microscopically, both isolates were Gram-positive rods, though ALW1 appeared shorter in form (Fig.1; Table 1). This morphological diversity is consistent with the previous findings

Table 1: Morphological characteristics and percentage similarity of bacterial isolates

Characteristics	ALW1	ALW2
Colony color	Pale yellow	Beige
Margin	Entire	Entire
Elevation	Convex	Convex
Shape	Circular	Circular
Microscopic phenotype	Cocci	Rod-shaped
Gram Reaction	Gram-positive	Gram-positive
Hemolytic activity	α hemolysis	β hemolysis
Catalase test	+	+
Closest recognized species	*Staphylococcus hominis* (99.1%) Pz054226	*Paenibacillus tyraminigenes* (98.7%)PZ054086
Total bacterial count (CFU ml^{-1})	$3.1 \times 10^4 \pm 1.55 \times 10^3$	

from various lake environment such as Lonar Lake (Joshi *et al.,* 2008), El-Djerid Salt Lake (Hedi *et al.,* 2009) and Urmia Salt Lake (Kashi *et al.,* 2021). ALW2 showed the ability to use a wider range of carbohydrates than ALW1. ALW2 showed positive reactions for L-lactic acid utilization, indicating its ability to metabolize lactic acid as a substrate. It also exhibited positive enzymatic activities for tyrosine arylamidase, leucine arylamidase, and alanine arylamidase, suggesting an enhanced capacity to break down amino acid-related compounds. In addition, ALW2 was able to utilize several carbohydrates, including sucrose, D-maltose, D-trehalose, D-ribose, D-mannose, and D-sorbitol, reflecting its broad metabolic versatility and ability to adapt to different nutrient sources. In addition, ALW2 was resistant to Novobiocin, Optochin, and Polymyxin B, and it also showed resistance in the O129 test. Both ALW1 and ALW2 were negative for alanine phenylpyruvate transaminase, lipase, glucose amylase activity, alkaline phosphatase, proline aminopeptidase and β-N-acetylglucosaminidase. However, the two isolates differed in several other biochemical traits. ALW1 was positive for citrate utilization, oxidase activity, esculin hydrolysis, urease activity, β-glucuronidase, β-galactosidase, and lactose fermentation, whereas ALW2 was negative for these reactions. The results suggest that ALW2 has greater flexibility in carbohydrate metabolism, while ALW1 is characterized by stronger hydrolytic and enzymatic activities. The detection of arylamidase enzymes is particularly relevant, as these enzymes contribute to nitrogen cycling in aquatic ecosystems (Mwirichia *et al.,* 2010).

Similar carbohydrate assimilation patterns have been reported in microbial studies conducted in Saudi aquatic environments (Alqahtani *et al.,* 2022). Additionally, catalase activity was positive in both isolates, consistent with adaptation to oxidative stress in natural aquatic habitats. Catalase is widely recognized as an indicator of oxidative stress and environmental disturbance (Khalifa *et al.,* 2015, 2016, 2020, 2022).

Hemolytic assay revealed that ALW2 exhibited β-hemolysis, indicating complete lysis of red blood cells, whereas ALW1 showed α-hemolysis, characterized by partial hemolysis and greenish discoloration around colonies. Hemolytic activity is often associated with potential virulence traits in opportunistic bacteria, suggesting possible health implications in freshwater systems. Similar hemolytic patterns have been reported in lake-derived bacterial isolates from different regions worldwide. In this context, "potentially pathogenic" refers to an organism that possess genetic or phenotypic traits associated with virulence and therefore, has the capacity to cause disease, but lacks direct clinical or experimental evidence of causing infection, whereas "confirmed pathogenic" describes an organism that has verified through clinical cases, epidemiological data, or experimental studieson parhogenes (Hedi *et al*., 2009; Joshi *et al*., 2008).

An antibiotic susceptibility test was performed to determine the effectiveness of five different antibiotics against ALW1 and ALW2. The antibiotic susceptibility test results indicated that the two bacterial isolates, ALW1 and ALW2, exhibited varying degrees of susceptibility to the tested antibiotics. While both isolates formed inhibition zones, suggesting some level of susceptibility, ALW1 displayed a broader spectrum of effectiveness. ALW1 showed higher susceptibility compared to ALW2 for most antibiotics. This is evident from the larger inhibition zone (40 mm) observed for Cephalothin (Kf/30 µg) around ALW1 (Fig. 2). This suggests Cephalothin could be a more effective antibiotic choice for this particular strain. In contrast, ALW2 demonstrated lower susceptibility to Vancomycin (VA/10 µg), Bacitracin (B/10 µg), Erythromycin (E/15 µg) and Novobiocin (NV/5 µg), respectively.

The maximum inhibition zones for these antibiotics in ALW2 were all below 26 mm, demonstrating lesser efficacy against ALW2. Resistance to Bacitracin and Polymyxin B, indicated by positive VITEK reactions (BACI, POLYB), is of particular concern because these antibiotics are commonly used against Gram-positive and Gram-negative pathogens. The presence of antibiotic-resistant bacteria in natural water systems is consistent with global reports linking resistance emergence to pollution, agricultural run-off and wastewater effluents (Ma *et al.,* 2004; Moehario *et al.,* 2021; Habib, *et al.,* 2022). Recent findings from the Al-Ahsa region further support this trend and have identified multiple bacterial strains with strong antimicrobial

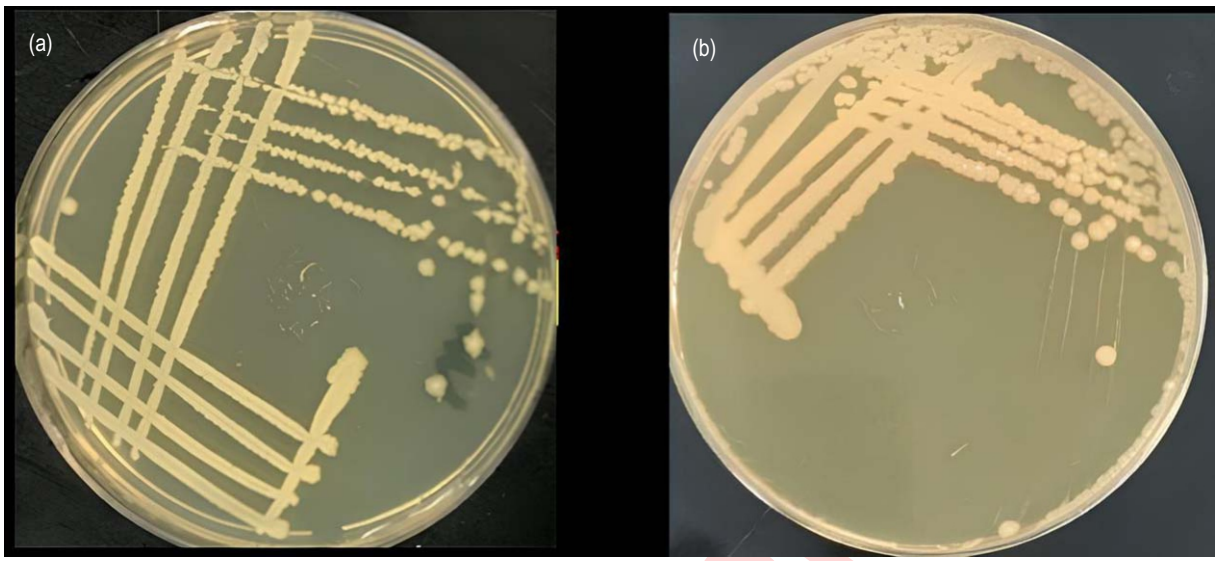

Fig. 1: Bacterial colonies of bacterial isolates, (a) ALW1 and (b) ALW2

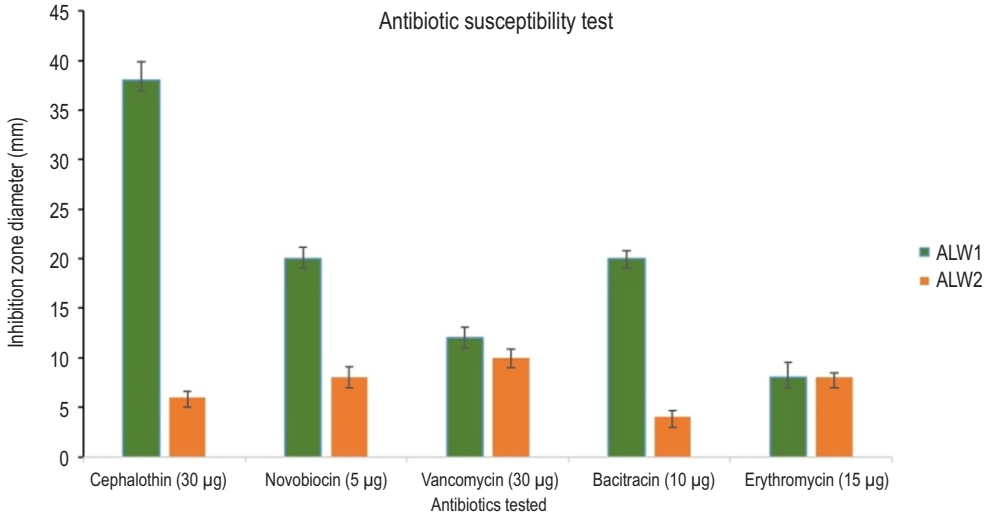

Fig. 2: Average Zone of Inhibition (mm) for Antibiotic Susceptibility of Al-Asfar Lake Bacterial Isolates (Erythromycin (E/15 µg), Bacitracin (B/10 µg), Vancomycin (VA/30 µg), Novobiocin (NV/5 µg), and Cephalothin (Kf/30 µg). Values are presented as mean ± S.D.

potential, highlighting both diversity and clinical relevance of local microbiota (Al-Abdulsalam *et al.* (2025). Comparative sequences of analysis of 16s rRNA gene revealed that the bacterial isolate ALW1 and ALW2 belonged to *Staphylococcus hominis* (99.1%) *Paenibacillus tyraminigenes* to which they exhibited 99.1% and 98.7% sequence homology, respectively. The evolutionary history was inferred using Neighbor-Joining method. Phylogenetic relationships were inferred based on 16S rRNA gene sequences of closely related type strains to determine the taxonomic positions of the isolates using MEGA11 software. The resulting phylogenetic analysis demonstrated that isolates ALW1 and ALW2 clustered distinctly within the *Staphylococcus* and *Paenibacillus lineages* (Fig. 3), thereby confirming the reliability of their taxonomic assignment.

Genotypic identification through 16S rRNA sequencing confirmed ALW1 as *Staphylococcus hominis* (99.1% similarity) and ALW2 as *Paenibacillus tyraminigenes* (98.7% similarity). Phylogenetic analysis using the Neighbor-Joining method placed each isolate within its respective genus with high confidence, corroborating their taxonomic affiliations (Fig. 3). These results align with earlier investigations demonstrating high sequence similarity (>99%) within *S. hominis* (Silva *et al.,* 2020) and comparable clustering patterns among *Paenibacillus* spp. (Zhang

Table 2: Phenotypic traits and antibiotic susceptibility patterns of ALW1 and ALW2 isolates

Biochemical analyses	ALW1	ALW2
Alanine phenylpyruvate transaminase	−	−
Lipase	−	−
Glucose amylase activity	−	−
Alkaline phosphatase	−	−
Proline aminopeptidase	−	−
Beta-N-acetylglucosaminidase	−	−
Antibiotic susceptibility testing	+	−
Citrate utilization	+	−
Oxidase test	+	−
Esculin hydrolysis	+	−
Urease test	+	−
Beta-glucuronidase	+	−
Beta-galactosidase	+	−
L-Lactose fermentation	+	−
L-Lactic acid utilization	−	+
Tyrosine arylamidase	−	+
Leucine arylamidase	−	+
Alanine arylamidase	−	+
Sucrose utilization	−	+
D-Maltose utilization	−	+
D-Trehalose utilization	−	+
D-Ribose utilization	−	+
D-Mannose utilization	−	+
D-Sorbitol utilization	−	+
Novobiocin susceptibility	S	R
Optochin susceptibility	S	R
Polymyxin B susceptibility	S	R
O129 resistance pattern	S	R

Results are expressed as positive (+) or negative (−) reactions.S = Sensitive (susceptible to the antibiotic). R = Resistant (not susceptible to the antibiotic)

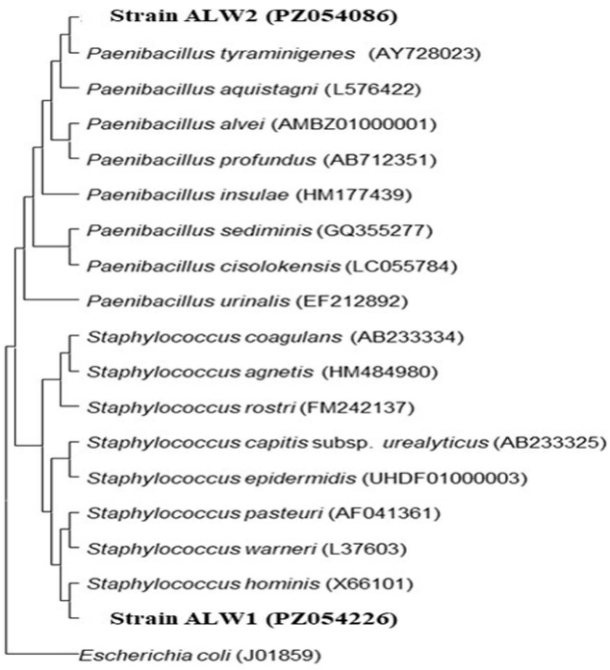

Fig. 3: A Neighbor-Joining phylogenetic tree based on 16S rRNA gene sequences displays the connections between strains ALW1 and ALW2 and known bacterial species that are related in a phylogenetic sense. *Escherichia coli* (GenBank accession number J01859) was used as an outgroup.

et al., 2023; Yan *et al.,* 2023). Collectively, the genetic, biochemical and phenotypic data confirmed the identity and ecological significance of these isolates. The presence of hemolytic, antibiotic-resistant and metabolically versatile bacteria in Al-Asfar Lake highlights a potential public health concern and underscores the need for continuous freshwater monitoring and improved water management strategies. Integration of phenotypic, biochemical and molecular data provides a comprehensive assessment of microbial diversity and health risks in the lake ecosystem, reinforcing the importance of surveillance measures recommended by earlier environmental microbiology studies (Alsaud *et al.,* 2023; Joshi *et al.,* 2008).

Furthermore, the detection of metabolically versatile and antibiotic-resistant strains emphasizes the dynamic interactions between microbial communities and environmental factors in Al-Asfar Lake. Such bacteria may act as reservoirs for resistance genes, potentially facilitating horizontal gene transfer to other microorganisms, including opportunistic pathogens. These findings highlight the lake not only as a unique ecological niche but also as a potential hotspot for the emergence and dissemination of antimicrobial resistance. Continued surveillance, combined with targeted mitigation strategies, is therefore critical for protecting both ecosystem integrity and public health. Integrating molecular, phenotypic and ecological analyses offers a robust framework for assessing microbial risk and guiding evidence-based water management policies. Collectively, potentially pathogenic bacteria were isolated from Al-Asfar Lake, exhibiting distinct metabolic profiles and antibiotic susceptibility patterns that pose public health concerns. These findings enhance monitoring of the lake's ecological health and provide insight into the emergence and spread of antibiotic resistance. The results also inform strategies for effective water treatment and management. Microbial pollution from untreated wastewater remains a significant threat to freshwater resources, highlighting the need for improved wastewater treatment, sustainable agricultural waste practices, and public awareness. Collectively, these measures are essential for safeguarding both aquatic ecosystems and human health.

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