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RESEARCH ARTICLE

Isolation and Characterization of Antagonistic Bacteria from the Gut of *Lampito mauritii* (Kinberg, 1866)

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Abstract

Objective: To isolate and characterize potentially antagonistic bacteria from the gut of *Lampito mauritii* earthworms. **Methods:** *Lampito mauritii* earthworms were collected and gut bacteria were isolated on nutrient agar. Antibacterial activity of isolates was checked by well diffusion against fish and human pathogens. Potent isolates were characterized biochemically. Antimicrobial metabolites were extracted from *Pseudomonas aeruginosa* isolate TR07 using ethyl acetate and tested by disc diffusion. TR07 was identified by 16S rRNA gene sequencing and phylogenetic analysis. Antibacterial efficacy was assessed statistically. **Results:** Among 38 discrete isolates, 4 exhibited antibacterial activity against *Aeromonas hydrophila* and *Staphylococcus aureus* in well-diffusion assay. These 4 isolates were characterized as *Pseudomonas* sp., *Vibrio* sp., *Aeromonas* sp., and *Bacillus* sp. based on biochemical tests. Antimicrobial metabolites extracted from *Pseudomonas* sp. TR07 using ethyl acetate showed statistically significant inhibition against gram-positive and gram-negative bacterial fish and human pathogens in disc diffusion assay. The 16S rRNA gene sequencing and phylogenetic analysis identified the potent isolate TR07 as *Pseudomonas aeruginosa* with 99.62% sequence homology. **Novelty:** In a fortuitous finding, the antibacterial potency of *Pseudomonas aeruginosa* was dramatically amplified through the utilization of a strikingly minute concentration of the bacterial culture extract.

Keywords: Pseudomonas aeruginosa; Earthworm gut; Antibacterial metabolites; Human pathogens; Fish pathogens

1 Introduction

Earthworms belonging to the annelid phylum inhabit and enrich soil ecosystems across tropical and temperate regions[1]. As ecosystem engineers, earthworms facilitate decomposition, nutrient mineralization, soil aeration, and water filtration through burrowing and feeding activities[2]. Culture-independent metagenomic analysis has revealed functional diversity within the gut microbiome linked to carbohydrate metabolism, defenses against pathogens, and antagonism[3–5].

The anecic earthworm *Lampito mauritii* is widely distributed across the Indo-Malayan region[6]. Metagenomic characterization of *L. mauritii* gut contents has uncovered a rich repository of antimicrobial resistance genes and biosynthetic gene clusters[7,8]. Rapid expansion of the aquaculture industry has been plagued by frequent outbreaks of infectious diseases resulting in substantial economic losses[9,10] A bacterial infection caused by *Aeromonas hydrophila, Aeromonas veronii* and *Aeromonas caviae* that is common in freshwater fish. It can cause bloody spots or ulcers, ragged fins, or enlarged eyes, fluid accumulation in the abdomen. These issues underscore the need for the prospection of novel antimicrobials from relatively unexplored natural sources like the gut microbiome.

This research aimed to isolate and characterize antimicrobial-producing gut bacteria from the *Lampito mauritii* to identify novel leads for therapeutic applications. The pressing need for sustainable alternatives to antibiotics in aquaculture, driven by economic losses and the rise of antibiotic resistance in infectious diseases, underscored the importance of this exploration. The study aimed to contribute to the development of sustainable therapies by thoroughly examining and understanding the antimicrobial properties inherent in the gut symbionts of *L. mauritii*, potentially offering viable solutions for both aquaculture and broader public health concerns.

2 Methodology

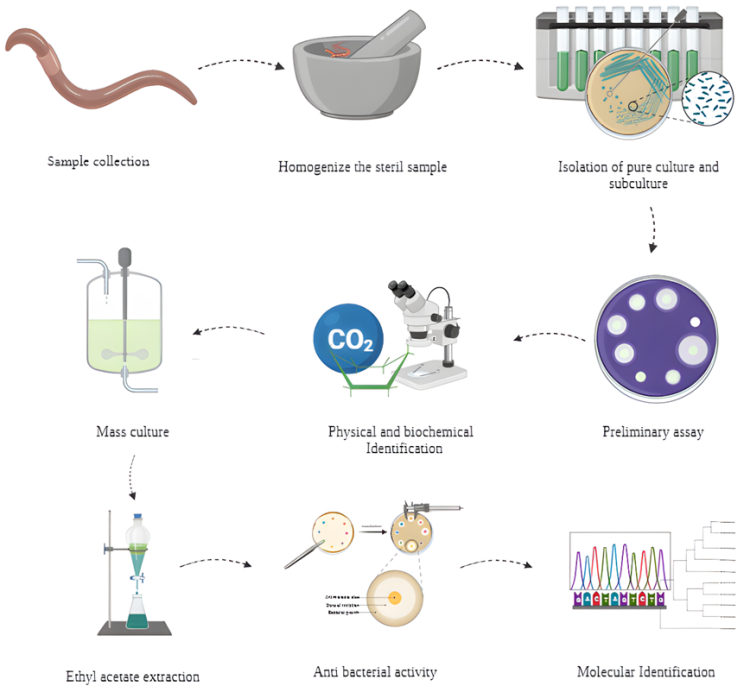

Fig 1. Graphical representation of isolation and characterization of earthworm gut bacteria

2.1 Sample Collection and Earthworm Identification

The *Lampito mauritii* was obtained in sufficient numbers from the Tamirabarani river bank (8°45'39.2" N 77°44'38.4" E) in the Tirunelveli District of Tamil Nadu, India. Samples were collected manually by shoveling and hand sorting the topsoil at a depth of 20 cm[11]. The specimens were taxonomically identified to species level by examining the diagnostic morphological characters such as body pigmentation, the position of the clitellum, the number of segments, and the arrangement of setae using standard taxonomic keys[12].

2.2 Isolation of Gut Bacteria

Five freshly collected earthworms were surface sterilized using 70% ethanol and rinsed in sterile phosphate-buffered saline (PBS). The gut was dissected out aseptically and homogenized in PBS under laminar flow conditions by vigorous vortexing with silica bead. The homogenate was serially diluted up to 10-7 dilutions and 100 μl of each dilution was spread plated on sterile Nutrient agar (Himedia) plates. The plates were incubated aerobically at 37°C for 48 hours. Discrete bacterial colonies exhibiting distinct morphology were picked and purified by quadrate streaking. The axenic cultures obtained were preserved as glycerol stocks at 4 °C for further studies[13]. Isolated strains were given designated codes

2.3 Preliminary Antibacterial screening

To conduct an initial assessment of the antimicrobial capabilities of bacteria isolated from the gut, a colony overlay method, as detailed by[14] was employed. Both the indicator strains and the isolated bacteria were cultured in Nutrient broth (Himedia-M002) and incubated overnight at 27°C. 100 μl of the indicator strain culture at a concentration of 10^5 CFU/ml was evenly spread on Muller Hinton Agar (MHA) plates using a sterile cotton-tipped swab. Following the drying of the culture, drops of the isolated bacterial cultures were carefully dispensed onto the agar plates. After a 24-hour incubation period at 28°C, the assessment of antimicrobial activity was conducted by examining the presence of zones of inhibition surrounding the bacterial colonies. The scoring system outlined by[15] was used, and based on the cumulative scores obtained, the most promising antagonistic bacteria against the indicator strains were selected for further investigation.

2.4 Phenotypic and Biochemical Characterization

The colony morphology of the selected isolates was documented. Gram-staining was performed and observed under a light microscope at 100X magnification. Biochemical tests such as indole production, methyl red, Voges-Proskauer, citrate utilization, catalase, oxidase, nitrate reduction, glucose fermentation, motility, and hydrogen sulfide production were performed as described in Bergey's Manual of Determinative Bacteriology[16].

2.5 Extraction of Antibacterial Metabolites

The selected antagonistic bacterial isolate (TR07) was grown in Nutrient broth at 37°C for 72 hours to stimulate maximal secondary metabolite production. The culture broth was centrifuged at 10,000 g for 15 minutes and the cell-free supernatant was extracted twice using ethyl acetate at a 1:1 ratio. The organic phase containing the extracted antibacterial metabolites was concentrated by vacuum evaporation and makeup the solution as 25mg/ml of ethyl acetate for antimicrobial assay.

2.6 Antibacterial assay

The disc diffusion method was employed to determine the antibacterial efficacy of the extracted metabolites[17]. Sterile paper discs were impregnated with 100 μl of the extract solution and after complete evaporation of the solvent, placed on Mueller-Hinton agar plates and swabbed uniformly with the 12 h culture of Fish pathogens viz., *Aeromonas hydrophila, Vibrio* sp., and Human pathogens viz., *Staphylococcus aureus, Salmonella typhi*, and *Bacillus cereus*. A standard antibiotic (Streptomycin-10 mcg) was used as the positive control. The plates were incubated at 37°C for 24 hours and the zone of inhibition was measured. The relative percentage inhibitions (PIs) for the ethyl acetate extract with respect to Streptomycin-10 mcg were calculated by using the following formula according to[18].

$$\%\ of\ Inhibition = \frac{Ethyl\ acetate\ extract\ -\ Negative\ control}{Positive\ control\ -\ Negative\ control}$$

Experiments were conducted in triplicate, and results are presented as mean ± standard deviation. Statistical analysis employed SPSS v.22 software, involving one-way ANOVA followed by Tukey's post-hoc test, to discern significant differences among test

samples ($P \leq 0.05$).

2.7 Molecular Identification and Phylogenetic Analysis

Genomic DNA was extracted from the most potent bacterial isolate (TR07) using a commercial kit (Qiagen D Neasy Blood & Tissue kit). Polymerase chain reaction (PCR) was performed using the universal 16S rRNA gene primers 27F (5' AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'TACGGYTACCTTGTTACG ACTT-3') [19] the amplified PCR product was sequenced bidirectionally using an automated sequencer (Applied Biosystems 3500XL). The 16S rRNA gene sequence obtained was analyzed for the closest sequence match by NCBI BLAST analysis. A phylogenetic tree was constructed using the Neighbor-Joining algorithm in MEGAX software [20].

3 Results and Discussion

3.1 Isolation and Preliminary Screening of Earthworm Gut Bacteria

A total of 38 phenotypically diverse bacterial isolates were recovered aseptically from the dissected gut of *Lampito mauritii* on Nutrient agar following aerobic incubation for 48 hours. Out of these 38 isolates, 4 isolates named TR07, TR12, TR22, and TR23 exhibited remarkably potent antibacterial activities based on appreciably large zones of inhibition against the indicator strains *Aeromonas hydrophila* and *Staphylococcus aureus*. These 4 isolates were selected for further morphological and biochemical-based identification. (Table 1, Figure 2)

Table 1. Preliminary Antibacterial screening

Isolates	*Staphylococcus aureus*	*Aeromonas hydrophila*
TR01	++	+++
TR02	-	-
TR03	-	-
TR04	++	+++
TR05	+	-
TR06	-	-
TR07	++	++++
TR08	++	-
TR09	+	+
TR10	-	-
TR11	++	-
TR12	++	++++
TR13	++	-
TR14	+++	+++
TR15	-	-
TR16	+++	-
TR17	++	-
TR18	-	-
TR19	-	-
TR20	++	-
TR21	-	-
TR22	++	++++
TR23	++	+++
TR24	-	-
TR25	+	+
TR26	-	-
TR27	++	-
TR28	-	-
TR29	+	++
TR30	++	-
TR31	-	-
TR32	-	++
TR33	++	+

Continued on next page

Table 1 continued

TR34	-	-
TR35	-	+
TR36	-	-
TR37	+	+
TR38	+	-

+ = <1mm; ++ = 1 to 3mm; +++ = >3mm; (+, ++, +++ indicates zone of inhibition in range); - = No activity

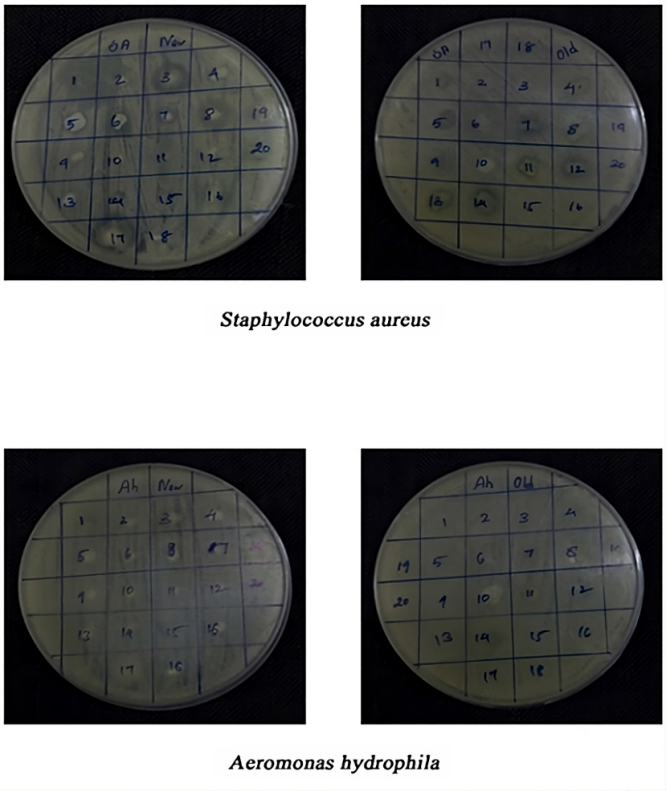

Fig 2. Screening for Anti-bacterial activity

3.2 Phenotypic and Biochemical Profiling

Gram staining performed on fresh cultures showed that isolates TR07 and TR22 were gram-negative bacilli, whereas isolates TR12 and TR23 were gram-positive bacilli. Biochemical tests such as indole production, Voges-Proskauer methyl red, citrate utilization, oxidase, catalase, nitrate reduction, glucose fermentation, motility, and hydrogen sulfide production could characterize isolates TR07, TR12, TR22 and TR23 presumptively as species of *Pseudomonas, Vibrio, Aeromonas* and *Bacillus* respectively based on the observations matched with standard biochemical profiles. (Table 2, Figure 3)

Table 2. Phenotypic and Biochemical Profiling of Selected Isolates

Isolates	**TR07**	**TR12**	**TR22**	**TR23**
		Morphology		
Gram Stain	-	+	-	+
Motility	+	+	-	+
Pigment	Green	White	Green	White
Shape	Rod	Rod	Rod	Rod

Continued on next page

Table 2 continued

Bio-Chemical Test				
Indole	+	-	-	-
Methyl red	-	-	-	+
Voges Poskauer	-	-	-	-
Citrate	+	-	+	+
Oxidase	+	+	+	-
Catalase	+	+	+	+
H2S Production	-	+	-	+
Starch	-	+	-	-
Nitrate Reduction Test	-	-	+	+
Gelatinase	+	-	+	-
Sugar Utilization				
Sucrose	+	+	+	-
Glucose	+	+	+	-
Rhamnose	-	-	-	-
Maltose	+	-	+	-
Lactose	-	+	+	-
Mannitol	+	+	+	-
Identified organisms	***Pseudomonas***	***Vibrio***	***Aeromonas***	***Bacillus***

+ = Presence/Positive result, - = Absence/Negative results

3.3 Anti-bacterial assay

The ethyl acetate extract of isolate TR07 showed statistically significant broad-spectrum antimicrobial activity against all indicator strains tested, including Gram-positive bacteria *Staphylococcus aureus* and *Bacillus cereus* and Gram-negative bacteria *Salmonella typhi, Vibrio* sp. and *Aeromonas hydrophila* ($p > 0.001$ for all strains), with the percent inhibition ranging from 67.55% to 97.87%. The highest mean inhibitions of 24.00 ± 0.25 mm and 23.33 ± 0.39 mm were seen against *B. cereus* and *S. aureus* respectively, while the lowest mean inhibition of 17.16 ± 0.39 mm was seen against *S. typhi* (Figures 4 and 5). Positive controls showed significantly greater inhibition than negative controls for all strains (p=.001 or .005), indicating the ethyl acetate extract has strong antimicrobial potential, especially against *B. cereus* and *S. aureus.* (Table 3)

Table 3. Antibacterial activity

Indicator strains	**Samples**	**Mean ± S.E**	**F ; p**	**Percentage of inhibition**
Aeromonas hydrophila	S	14.83 ± 0.37	$F_{2,1} = 385.24$; $p = .001$	72.88
	P	20.16 ± 0.68		
	N	00.50 ± 0.26		
Vibrio sp.	S	18.50 ± 0.24	$F_{2,1} = 1099.357$; $p = .005$	80.75
	P	22.83 ± 0.40		
	N	0.333 ± 0.30		
Salmonella Typhi	S	17.16 ± 0.39	$F_{2,1} = 1006.118$; $p = .005$	67.55
	P	25.16 ± 0.41		
	N	00.50 ± 0.25		
Staphylococcus aureus	S	23.33 ± 0.39	$F_{2,1} = 1146.600$; $p = .001$	97.87
	P	23.83 ± 0.40		
	N	0.333 ± 0.14		
Bacillus cereus	S	24.00 ± 0.25	$F_{2,1} = 2402.375$; $p = .005$	96.61
	P	24.83 ± 0.15		
	N	0.333 ± 0.30		

S- Ethyl acetate extract of isolate TR07; P- Positive Control; N- Negative control; S.E – Standard Error, F- Frequency, p – significance

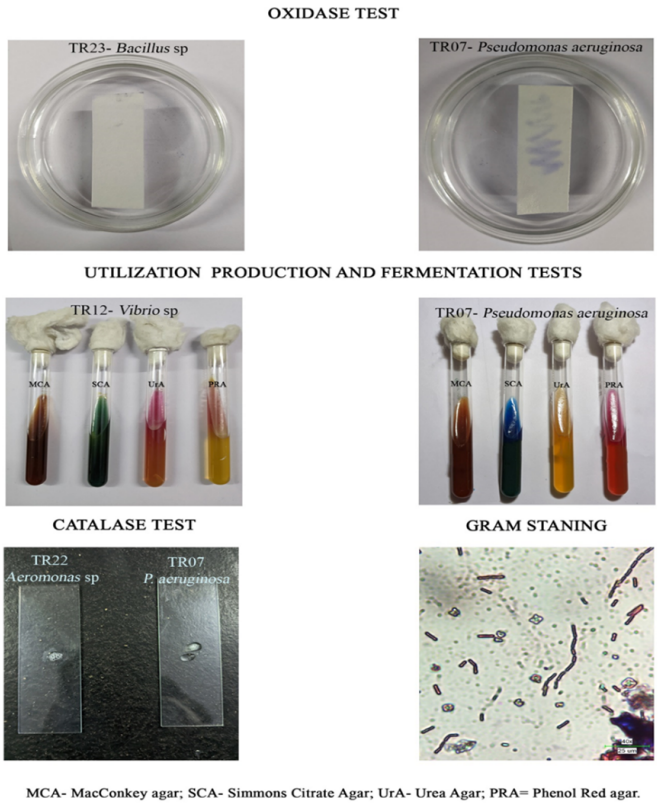

Fig 3. Biochemical characterization

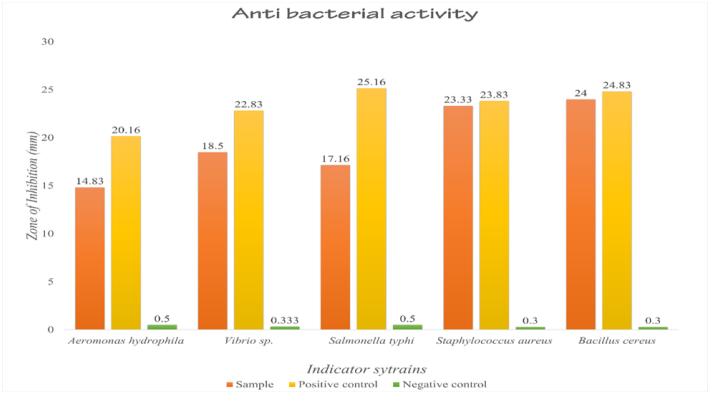

Fig 4. Antibacterial activity of Ethyl acetate extract of the Isolate TR07

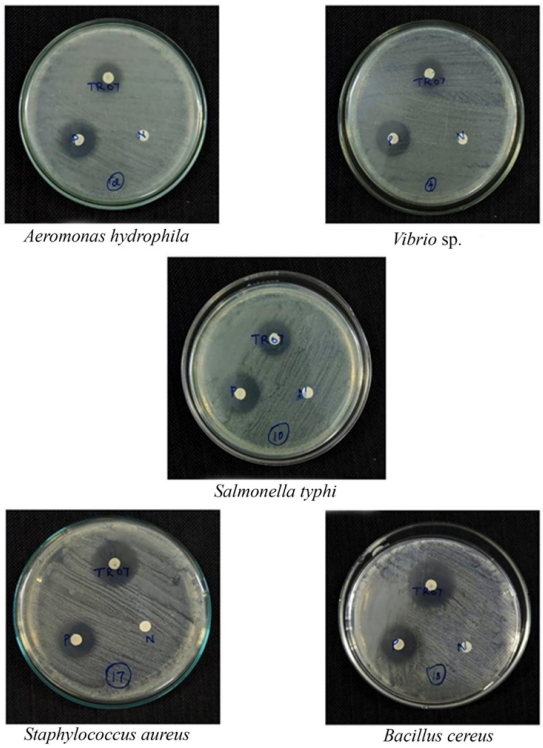

Fig 5. Anti-bacterial activity of Ethyl acetate extract of *Pseudomonas aeruginosa* Strain CAREB01 [TR07 – Ethyl actate extract isolate TR07 (*Pseudomonas aeruginosa Strain* CAREB01); P – Positive control; N-Negative control]

3.4 Molecular Identification and Phylogenetic Analysis

Comparison of the nearly complete 16S rRNA gene sequence (~1450 bp) by BLAST analysis revealed 99.62% sequence similarity and closest homology of TR07 to *Pseudomonas aeruginosa* strains. The construction of a Neighbor-Joining phylogenetic tree with reference sequences also indicated clear taxonomic clustering and grouping of the isolate TR07 within the *P. aeruginosa* clade (Figure 6). Both molecular approaches firmly confirmed the identity of isolate TR07 as *Pseudomonas aeruginosa*. The sequence has been submitted in NCBI GenBank with accession number OQ804675.1 as *Pseudomonas aeruginosa* strain CAREB01.

4 Discussion

Earthworms drive a vital ecological role in soils through burrowing, feeding, and casting activities. These behaviors enhance organic matter decomposition, nutrient cycling, aeration, and water infiltration. Earthworm activities improve soil fertility, porosity, and tilth, leading to increased ecosystem productivity and sustainability.[2]. The gut microbes promote growth, sexual maturation, pathogen inhibition, and overall fitness in their hosts through these metabolic activities[1]. Culture-independent metagenomic analysis has revealed that the microbiota associated in gut is dominated by anaerobic fermentative bacteria from phyla such as Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes[21]. In this study, 38 culturable bacterial isolates were recovered, active isolates were identified as *Pseudomonas aeruginosa, Vibrio, Aeromonas*, and *Bacillus* sp.,

Most active *P. aeroginosa* is a ubiquitous gram-negative proteobacterium and reported elsewhere to secrete a range of bioactive secondary metabolites such as phenazines, quinolones, rhamnolipids, lectins, hydrogen cyanide, siderophores, proteases, and other extracellular enzymes with roles in toxicity, virulence, nutrient acquisition, competition, and defense[21,22]. The liquid-liquid extraction using ethyl acetate could recover broad-spectrum antimicrobial metabolites from *P. aeruginosa* TR07. Previous study had done on *Pseudomonas aeruginosa* had revealed the ability of inhabitance against bacterial pathogens with average zone of inhibition as 16.64 mm while the average zone of inhibition of this current study was 19.56 mm was relatively high with 2.5mg on compare to the 10mg per disc[23]. The extract displayed appreciable antibacterial efficacy against *Bacillus cereus, Staphylococcus aureus* and gram-positive pathogens as well as gram-negative bacteria *Salmonella typhi, Vibrio*

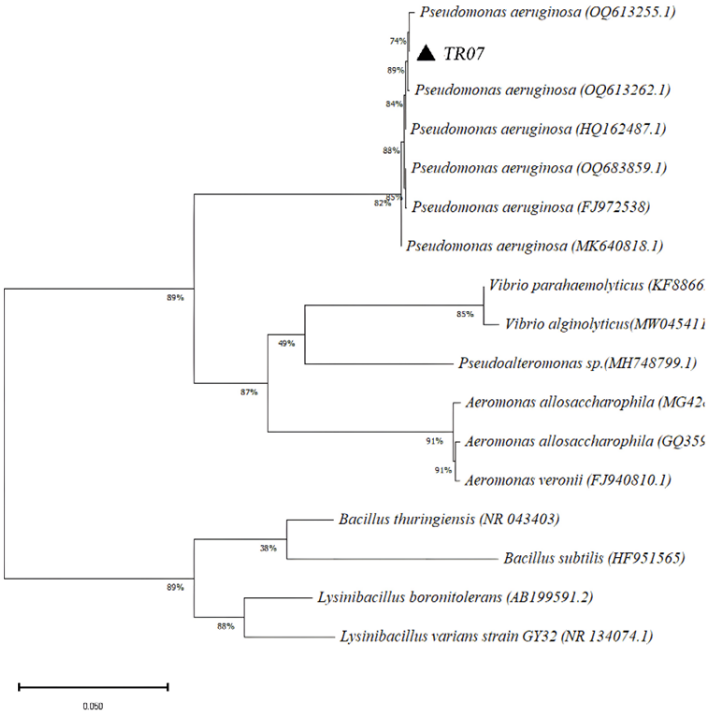

Fig 6. Evolutionary relationship of taxa by a neighbor-joining method

sp, and *Aeromonas hydrophila*. This reveals the antimicrobial potential of earthworm gut microbes, which can be further translated into pharmacological applications. The observed activity is substantiated by Liquid-liquid extraction using ethyl acetate to recover broad-spectrum antimicrobials from *L. mauritii* gut bacteria. Other studies have reported anti-viral, anti-fungal, insecticidal, and probiotic activities of bacteria inhabiting the earthworm intestine[24–26]. Their diverse antimicrobial compounds provide a cheaper and safer alternative to conventional antibiotics used in biomedicine, veterinary practice, and aquaculture[27]. For instance, Phenazine-1-carboxamide from a gut *Pseudomonas* strain exhibited broad-spectrum inhibition against fish pathogens like *Aeromonas hydrophila, Edwardsiella tarda*, and *Streptococcus liniae*[28]. Such examples underscore the merits of exploring this unique intestinal ecosystem through integrated microbiological, metagenomic, metabolomic, and chemical ecology approaches.

5 Conclusion

This study demonstrates the antibacterial potential of earthworm gut symbionts against fish and human pathogens. *Pseudomonas aeruginosa* TR07 recovered from the *Lampito mauritii* exhibited broad-spectrum antagonism mediated by antibacterial metabolites extractable through ethyl acetate. Further purification and characterization of these bioactive compounds can reveal drug leads for treating infectious fish and human diseases. This provides the impetus for further explorations of earthworm gut microbial diversity as a treasure trove of therapeutic phytochemicals and antimicrobials.

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