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# Isolation and Characterization of Gut Bacterial Protein from the Earthworm *Drawida pellucida pallida*: A Toxicological Insight Against Pathogenic Bacteria

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## Abstract

It has become more and more popular to use natural products to combat pathogenic bacteria. In this study, the bacterial toxic proteins have been isolated from gut microbiota of a native earthworm species, *Drawida pellucida pallida* using conventional methods. In the study, seven different species of gut bacteria were identified, which were then isolated, characterized, and analyzed for their potential toxic effect on *Aeromonas hydrophila*, a human pathogenic bacterium. It was found that the bacterial isolate designated as D6 presented strong resistance to antibacterial activity and needed to be purified and analysed. The activity fraction of the protein was analysed using SDS-PAGE, which revealed a band at about 33 kDa, indicating a low-molecular-weight protein with toxicity. Molecular identification by 16S rRNA gene sequencing identified the D6 isolate as *Bacillus paramycoides*. According to the phylogenetic analysis, the microbe belongs to the *Bacillus* genus and is closely related to other members of the *Bacillus* genus. *D. pellucida pallida*'s gut is a potential source of bacteria with toxic proteins that can be used against pathogenic bacteria, since *B. paramycoides* and *B. cereus* proteins show antimicrobial activity against *A. hydrophila*.

**Major Findings:** Earthworm gut bacteria were isolated and characterized, molecularly identified by 16S rRNA analysis, and their bioactive proteins were evaluated for toxic antibacterial activity against human pathogenic bacteria.

**Keywords:** Antimicrobial Activity, Earthworms, Gut Microflora, Worm Bioactive Compounds

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## 1. Introduction

The increasing menace of Multidrug-Resistant (MDR) pathogens has emerged as a worldwide public health emergency, prompting the quest for new antimicrobial agents from nonconventional and sustainable sources[1,2]. Bioactive compounds can be found in abundance in natural ecosystems, particularly those characterized by intricate microbial communities. Over the past few years, the invertebrate gut microbiota, particularly earthworms,

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has been in the spotlight due to its potential to provide new bioactive compounds, such as antimicrobial peptides, enzymes, and secondary metabolites[3,4]. Earthworms, commonly known as "ecosystem engineers", are crucial for soil health through the improvement of structure, aeration, decomposition of organic matter, and nutrient cycling. In addition to burrowing activity, these annelids consume and ingest organic matter that greatly impacts soil microbial processes[5,6]. Significantly, earthworms host rich microbial consortia in their gut, and they develop symbiotic associations that not only aid digestion and nutrient metabolism but also play a role in their innate immunity[7].

As soil and organic materials are ingested, earthworm guts act as bioreactors, breaking them down into nutrients that can be absorbed. Microorganisms found in this specialist environment include members of the genera *Bacillus, Pseudomonas, Streptomyces,* and *Enterobacter* - several of which are known producers of antimicrobial proteins, hydrolytic enzymes, and other bioactive metabolites[8]. Additionally, these microbial inhabitants play a critical role in the survival and well-being of the worm, as well as in terms of biotechnology. It has already been established that gut microbes derived from some well-known earthworm species, including *Eisenia fetida*, *Perionyx excavatus*, and *Lampito mauritii*, are biotechnologically valuable[9,10]. Bacterial strains exhibiting antibiosis against human pathogens, protease activity, and biofertilizer properties are identified as a result of these studies. Nevertheless, the microbial communities of less well-studied indigenous species such as *D. pellucida pallida* are still not extensively explored. *D. pellucida pallida*, belonging to the *Moniligastridae* family, is native to tropical and subtropical Asia and commonly inhabits humid, humus soil in which it breaks down organic wastes and ads nutrients.

Due to its host specificity, *D. pellucida pallida* is predicted to possess a unique gut microbiome, possibly containing new strains of antimicrobial bacteria. Investigation of such poorly studied species might result in the discovery of novel biomolecules, such as proteins with strong antibacterial activities. Advances in recent years in the fields of microbiology and molecular biology have allowed for the effective characterization of such microbial populations. In particular, 16S rRNA gene sequencing is a useful technique for taxonomic classification and bacterial phylogenetic analysis of invertebrate guts and other complicated biological matrices[11,12]. For evaluation of bacterial isolates potential for biotechnological applications, functional characterisation is crucial in addition to taxonomic identification. Biomolecules, with which bacterial proteins play a variety of antimicrobial roles, are of particular interest. Crude bacterial proteins can consist of bacteriocins, Antimicrobial Peptides (AMPs), and lytic enzymes, which can suppress the growth of pathogenic bacteria by damaging cell membranes, inhibiting cell wall synthesis, or disrupting nucleic acid and protein metabolism[13]. Agricultural and pharmaceutical industries can make use of bacterial proteins because of these properties.

The investigation of these proteins relies on the integration of biochemical and molecular techniques. Proteins from bacterial cultures are usually precipitated and concentrated using Trichloroacetic Acid (TCA) and acetone[14]. This is sometimes followed by ion exchange chromatography, which classifies proteins according to their charge character, enabling partial purification of bioactive fractions. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) is subsequently employed to identify protein molecular weight and purity, acting as an initial step in the screening of bioactive candidates[15]; after purification, crude bacterial proteins antibacterial activity can be assessed with well-documented *in vitro* methods like agar well diffusion, disk diffusion, or broth microdilution techniques. These assays enable the quantification of the inhibitory activities of bacterial proteins against clinically relevant human pathogens, such as *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* - pathogens often involved in hospital-acquired infections[16]. Successful inhibition by these crude protein preparations not only suggests their potential use as alternative antimicrobial agents but also warrants further purification and structure-function studies.

In spite of increasing interest in earthworm-linked microbes, there is limited proteomic profiling of bacteria from the guts of native species such as *D. pellucida pallida*. There is a definite need to push existing knowledge beyond model organisms and investigate microbial diversity in native and ecologically distinct invertebrates. Bioprospecting of new biomolecules is aided by these studies, which aid biodiversity conservation. Considering the critical requirement for novel antimicrobial agents, the current research seeks to biochemically characterise and purify crude bacterial proteins obtained from the midgut of *D. pellucida pallida* with a view to assessing their

antibacterial activity against some human pathogens. The study can entail the isolation of gut-associated bacteria, protein extraction through TCA-acetone precipitation, purification through ion exchange chromatography, and determination of molecular weight through SDS-PAGE while the antibacterial activity of these crude proteins can be determined using standard in vitro antimicrobial assays.

By this method, the research hopes to fill gaps in existing microbial bioprospecting and show the functional significance of microbial communities that live with native invertebrate hosts. The results could eventually lead to the discovery of eco-friendly, biologically sourced alternatives to traditional antibiotics, helping the world combat antimicrobial resistance. Therefore, this study has been aimed: (1) To isolate and identify gut-associated bacteria from the gut of native earthworm *D. pellucida pallida* (2) To determine the molecular identity of the isolates using 16S rRNA sequencing and deposit the sequences in GenBank (3) To purify and characterise crude bacterial proteins using biochemical methods and SDS-PAGE (4) To evaluate the antibacterial activity of the purified crude bacterial proteins against selected pathogens.

# 2. Materials and Methods

## 2.1 Selection of Earthworms

The native earthworm species *D. pellucida pallida*, from family *Moniligastridae*, was chosen for this research based on its ecological importance in tropical soil ecosystems and its comparatively uncharacterized gut microbiome. Individuals were sampled from humus-rich wet agricultural fields of Devakottai, within the Sivaganga district of Tamil Nadu, India (Latitude: 9.95°N, Longitude: 78.82°E). Earthworms were handpicked early in the morning when soil moisture regimes were optimal. Following collection, the earthworms were placed in sterile, pierced plastic boxes lined with wet, sterilized filter paper and taken to the laboratory with minimal disturbance[17].

In the laboratory, the specimens collected were rinsed gently in sterile distilled water to eliminate adherent soil particles and then kept on wet filter paper for 24 h to enable clearance of the gut through depuration[18]. Before microbial isolation, it was imperative that soil-borne pathogens from the intestinal tract were cleared. Morphological identification of earthworm specimens was conducted by an Oligochaetologist employing standard taxonomic keys, taking into account diagnostic characteristics like body coloration, position of the clitellum, segmentation, setal patterns, and shape of the prostomium[19]. The identification was also confirmed through reference descriptions offered by Julka and Paliwal[17]. Healthy, mature specimens with well-developed clitella were chosen for dissection of the gut. A 70% ethanol fixation was used to fix voucher specimens and store them in a departmental repository. Invertebrate specimens were collected and treated in accordance with institutional standards of ethics.

## 2.2 Isolation of Gut Bacteria from *D. pellucida pallida*

Healthy adult earthworms of the native species *D. pellucida pallida* were collected from humus-rich agricultural fields at Devakottai, Tamil Nadu. The worms were washed extensively with sterile distilled water and maintained on moist sterile filter paper for 24 h to depurate and strip the soil particles out of the gut[18]. Subsequently, the earthworms were surface-sterilized with 70% ethanol for 30 seconds and rinsed once more in sterile distilled water to remove microorganisms from the surface[20]. Surface-sterilized worms were anesthetized by immersion in 10% ethanol for a short duration and dissected aseptically on a laminar airflow hood. Under aseptic conditions, with the help of sterile instruments, the midgut was gently removed and transferred to sterile PBS (pH 7.4). The midgut tissue was homogenized gently with a sterile mortar and pestle to release gut-associated microbial contents.

The obtained homogenate was serially diluted to $10^{-6}$ with sterile PBS to facilitate the isolation of discrete bacterial colonies. Aliquots (100 µL) of each dilution were spread plated onto Nutrient Agar (NA) plates and incubated at 30 ± 2° C for 24 to 48 h to favor bacterial growth[19]. In order to obtain pure isolates, morphologically different bacterial colonies were picked after incubation and sub-cultured repeatedly. These isolates were preserved on NA slants at 4° C for temporary use and in 20% glycerol at -20° C for long-term storage. Initial characterization of the bacterial isolates was done based on colony morphology and Gram staining to identify rudimentary cellular characteristics prior to biochemical and molecular[20]. Throughout the process, control plates were kept without inoculation in order to verify sterility and contamination-free conditions.

## 2.3 Morphological Identification

Morphological identification of the gut-associated bacterial isolates of the earthworm was initiated with a determination of colony morphology on nutrient agar or other isolation medium. Following incubation, individual colonies of bacteria were investigated for physical features like size, shape, margin, elevation, surface, texture, opacity, and pigmentation. By identifying these characteristics, we can determine bacterial diversity and select isolates for detailed analysis[20]. Microscopically, Gram staining was used to examine cellular morphology after colony observation. Smears of bacteria were heat-fixed, stained, and then viewed with a compound light microscope at 1000× magnification under oil immersion. This facilitates identification of the Gram reaction (positive or negative), as well as cell shape (cocci, bacilli, spirilla) and cell arrangement[21]. Gram staining is instrumental in the initial discrimination between gram-positive and gram-negative bacteria, thus leading on to the subsequent biochemical and molecular identification processes[21].

## 2.4 Biochemical Characterization

Biochemical identification of gut-associated bacteria isolated from *D. pellucida pallida* gives very important information regarding their enzymatic and metabolic characteristics, which are necessary for accurate species-level identification. Standard biochemical tests were used to evaluate multiple physiological functions like carbohydrate fermentation, enzyme activity, and substrate utilization. Tests most commonly utilized were catalase and oxidase tests, which differentiated between aerobic and facultatively anaerobic bacteria on the basis of their capacity to decompose hydrogen peroxide and synthesize cytochrome oxidase, respectively. Other assays, indole production, Methyl Red (MR) test, Voges-Proskauer (VP) test, and citrate utilization form the IMViC series were used mainly for the identification of members of *Enterobacteriaceae*[20]. Triple Sugar Iron (TSI) test, urease test, gelatin liquefaction, nitrate reduction, starch and casein hydrolysis were also performed to assess the ability of bacteria to metabolize certain substrates and form hydrolytic enzymes[22]. The biochemical reactions were referenced against profile standards from Bergey's manual of determinative bacteriology for tentative identification of bacterial genus and species[23]. This step-by-step biochemical evaluation, when employed together with morphological and molecular information, facilitated firm characterization of earthworm gut microbiota.

## 2.5. Molecular Characterization

### *2.5.1 DNA Extraction*

DNA isolation of gut-associated bacterial isolates of *D. pellucida pallida* is an important procedure for molecular identification and entails isolation of high-quality genomic DNA adequate for downstream use in PCR and sequencing. Bacterial cultures from overnight growth on nutrient broth were centrifuged at 10,000 rpm for 10 min to settle the cells. The pellet was resuspended in TE buffer (Tris-EDTA) and subjected to lysozyme treatment (10 mg/mL) to burst the bacterial cell wall, primarily in gram-positive bacteria[24]. A mixture of SDS (1%) and proteinase K (20 mg/mL) were added for breaking the cell membrane and digestion of proteins, respectively. A phenol: chloroform: isoamyl alcohol mixture (25:24:1) was then added to wash away proteins and lipids after incubation for 30 min at 37° C. DNA aqueous phase was centrifuged and precipitated with cold isopropanol. The DNA pellet was washed with 70% ethanol, air-dried, and dissolved in nuclease-free water or TE buffer. Quality and concentration of purified DNA were checked by agarose gel electrophoresis and a spectrophotometer (A260/A280 ratio)[23,24]. This standardized protocol produces intact and pure genomic DNA that is acceptable for 16S rRNA gene amplification and sequencing.

### *2.5.2 16S rRNA Gene Amplification*

The amplification of the 16S rRNA gene is a basic molecular technique for the identification of bacterial isolates from the gut of *D. pellucida pallida*. Genomic DNA from the isolated bacterial cultures acts as the template for PCR amplification. Universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used to amplify the conserved region of the bacterial 16S rRNA gene (Weisburg *et al*., 1991). The reaction mixture for the PCR was generally 1× PCR buffer, 2.5 mM $MgCl_2$, 200 µM each of the four dNTPs, 0.4 µM each of the primers, 1 U Taq DNA polymerase, and 50-100 ng of template DNA in a total volume of 25 µL. The amplification was performed in a thermocycler using the following conditions: The initial denaturation at 94° C for 5 min, 30 cycles of denaturation at 94° C for 30 sec, annealing at 55° C for 30 sec, extension at 72° C for 1 min, and final extension at 72° C for 10 min. The amplified PCR products were then detected by electrophoresis in a 1% ethidium bromide-containing agarose gel and detected

under UV transillumination to verify the ~1500 bp band. If amplification occurs, the quality of the DNA was sufficient for downstream sequencing analysis. This method is used widely in microbial taxonomy to identify unknown bacterial isolates and assess phylogenetic relationship[24,25].

### 2.5.3 Sequencing and Bioinformatics Analysis

The 16S rRNA PCR amplicons were purified and sent to a commercial sequencing center for bidirectional Sanger sequencing with universal primers (27F and 1492R). After receiving the raw nucleotide sequences, quality control was done using software programs like Chromas or BioEdit to remove low-quality ends and validate base calling accuracy[26]. The cleaned sequences were analyzed by BLAST (Basic Local Alignment Search Tool) against the NCBI GenBank database to determine the closest phylogenetic relatives as a function of sequence similarity[27]. Sequences with ≥97% identity were generally assumed to belong to the same species[28]. Bootstrap analysis (usually with 1000 iterations) was conducted to determine the robustness of the branching patterns. This bioinformatics pipeline integration enables accurate taxonomic classification and evolutionary relationship prediction between the gut bacterial isolates of *D. pellucida pallida*.

### 2.5.4 Protein Purification and SDS-PAGE Characterization of Gut Bacteria from D. pellucida pallida

Gut-associated bacterial isolates collected from *D. pellucida pallida* were incubated in nutrient broth at 37° C for 24-48 h with shaking (150 rpm) to initiate maximum protein synthesis[23]. Post-incubation, cultures were centrifuged at 8000 rpm for 15 min at 4° C to remove the bacterial pellet. Crude bacterial proteins were precipitated with a standard TCA-acetone precipitation procedure, in which cold 10% TCA was added to the supernatant, left overnight at -20° C, and centrifuged to obtain protein pellets[29]. The pellets were washed with cold acetone, air-dried, and suspended in Phosphate-Buffered Saline (PBS). Protein concentration was measured by the Bradford assay, using Bovine Serum Albumin (BSA) as a standard[30]. For the separation and purity analysis based on molecular weight, SDS-PAGE (12% resolving gel) was done using the standard method and followed by Coomassie Brilliant Blue staining[15]. Clear bands were present showing the presence of different protein components, and their molecular weights were determined by using a pre-stained protein ladder. This technique allows preliminary characterization of crude protein extracts for further studies[31].

### 2.5.5 Ion-exchange Chromatography for the Purification of Crude Bacterial Proteins

Crude bacterial proteins from gut-associated bacteria of *D. pellucida pallida* were further separated by ion-exchange chromatography to obtain bioactive fractions. The crude protein solution was initially dialyzed against 20 mM Tris-HCl buffer (pH 8.0) at 4° C overnight to eliminate low molecular weight impurities and to condition the sample with the chromatography buffer[32]. A DEAE-cellulose column (diethylaminoethyl cellulose), which was pre-equilibrated with the same buffer, was loaded and washed extensively. Protein sample was loaded onto the column, and excess proteins were eluted by washing with a number of column volumes of Tris-HCl buffer. Bound proteins were eluted by using a linear gradient of NaCl (0-1 M) in Tris-HCl buffer, and fractions were collected at a specific interval[33]. Each fraction was checked for protein content by 280 nm absorbance and on SDS-PAGE to determine purity and molecular weight profiles. Active fractions with clean bands were collected and stored at -20° C for subsequent bioactivity testing, such as antibacterial assays. This purification strategy enriches for functional proteins and allows for identification of bioactive candidates for drug applications.

### 2.5.6 Characterization of Crude Bacterial Proteins by MALDI-TOF Mass Spectrometry

Crude bacterial protein fractions prepared from the gut microbiota of *D. pellucida pallida* were analysed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry in order to ascertain their molecular masses and identify peptide fingerprints. The purified protein samples were preceded by desalting using ZipTip C18 pipette tips in order to remove salts and buffer contents that could cause interference with ionization. A minute aliquot (1 µL) of the protein sample was blended with an equivalent volume of matrix solution, most commonly α-Cyano-4-Hydroxycinnamic acid (CHCA) made in 50% acetonitrile and 0.1% trifluoroacetic acid. The mixture was spotted on a stainless steel MALDI target plate and air-dried[34].

The samples were scanned with a MALDI-TOF mass spectrometer in linear positive mode, and spectra were acquired within a mass range of 1,000-20,000 Da. Protein profiles were matched against reference spectra in accessible microbial or protein databases using BioTyper or Mascot search engines to attribute tentative identities or molecular weights[34,35]. The produced mass spectra gave a specific fingerprint to the bacterial proteins and facilitated initial identification and subsequent functional correlation.

#### *2.5.7 Evaluation of Antibacterial Activity of Bacterial Toxic Proteins*

Toxicity of the bacterial proteins was assessed through antibacterial efficacy by agar well diffusion assay against human pathogenic bacteria, *Aeromonas hydrophila*. The test pathogens were initially grown in nutrient broth at 37° C for 18-24 hours and diluted to 0.5 McFarland standards (~1.5 × $10^8$ CFU/mL). Mueller-Hinton agar plates were uniformly inoculated with sterile cotton swabs, and wells of 6 mm diameter were drilled in the agar using a sterile cork borer. Crude protein samples (50 µL of 1 mg/mL concentration) were filled into each well, and plates were incubated at 37° C for 24 h[36-38]. Clear inhibition zone diameters were recorded in millimeters, and activity was referenced to standard antibiotics (*e.g.*, ampicillin) as a positive control. Sterile distilled water or buffer was employed as the negative control. The procedure is efficient for screening the antimicrobial activity of proteinaceous extracts and identifying their range of inhibition against gram-positive and gram-negative pathogens.

## 3. Results and Discussion

### 3.1 Selection of Earthworms

In this research, *D. pellucida pallida* was selected for its indigenous abundance and ecological importance in tropical soils[39]. As an important aerator and decomposer of organic matter, *D. pellucida pallida* contributes significantly to soil aeration. In its midgut, there is a variety of microorganisms that live in symbiotic relationships and are associated with the gut. Microbes and biochemical potential have not been extensively studied for this species. Therefore, the study presents a new source for the isolation of bioactive bacterial strains.

### 3.2 Isolation of Gut Bacteria from *D. pellucida pallida* - Morphological and Biochemical Characterization

There were seven bacterial isolates resulted in morphological analysis of *D. pellucida pallida* gut (Figure 1). Next to morphological characterization, biochemical characterization of the bacterial isolates (D1-D7) indicated that they were primarily motile and Gram-positive (Table 1)[40]. It was found that some of them were positive for catalase, citrate, and starch hydrolysis. Indole was not produced by any of the isolates and none of them were methyl red positive. In view of these observations, further identification and application of the isolates will be possible based on varying metabolic capacities.

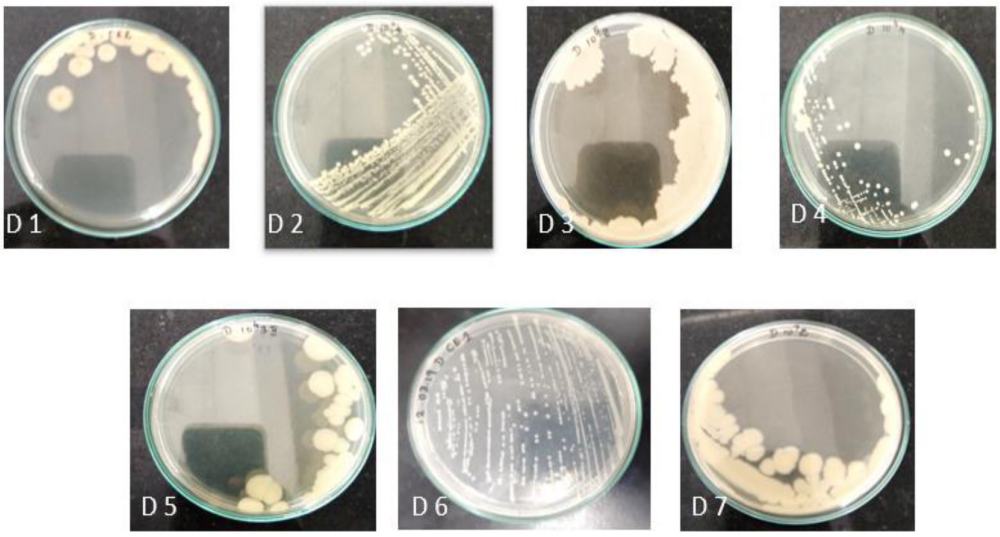



**Figure 1.** Morphological features of bacteria (D1-D7) isolated from the gut of *D. pellucida pallida.*

## 3.3 16S rRNA Gene Amplification

The isolate D6, designated *Bacillus paramycoides*, is firmly embedded in the clade *B. paramycoides* with an extremely close phylogenetic relation to other reference strains of *B. paramycoides* found in NCBI. The 16S rRNA gene sequence of the *B. paramycoides* and *B.cereus* strain determined in this study has been deposited in GenBank under accession number OK602640 and OK602687. The clear-cut phylogenetic separation of this clade from other members of the *Bacillus* genus (*e.g.*, *B. anthracis, B. thuringiensis* and *B. cereus*) is molecular confirmation of species level classification (Figure 2). These findings affirm the isolate's identity as *B. paramycoides*, also revalidating earlier biochemical or molecular characterizations[41-43].

## 3.4 Protein Purification and SDS-PAGE Characterization of Gut Bacteria from *D. pellucida pallida*

Figure 3 effectively illustrates a typical calibration curve for BSA at 700 nm, with a distinct linear correlation between concentration and absorbance. It is possible to use the plot to determine unknown protein concentrations in the test interval with high accuracy (Figure 3). The green and red dashed lines illustrate how experimental absorbance readings are used to pull out approximate protein concentrations from the graph at values of approximately 0.75 mg/ml and 0.82 mg/ml, respectively.

BPF2 (L6) represents a highly enriched protein fraction, likely associated with biological activity (*e.g.*, antibacterial or anticancer potential). Through ion-exchange chromatography, proteins are successfully separated and partially purified using SDS-PAGE (Figure 4). The prominent band around 34-43 kDa in BPF2 may correspond to a bioactive protein.

## 3.5 Ion-exchange Chromatography for the Purification of Crude Bacterial Proteins

A major protein component was successfully isolated around 75 mL. In the early phase of elution, from approximately 0 to 60 mL, small peaks are observed, which likely correspond to unbound or weakly bound

**Table 1.** Biochemical characteristics of bacteria (D1-D7) isolated from the gut of *D. pellucida pallida*

| Sample Name | Motility | Grams Staining | Starch Hydrolysis | Oxidase | Methyl Red | Citrate Utilize | Catalase | Indole |
|---|---|---|---|---|---|---|---|---|
| D1 | + | + | + | - | + | + | + | - |
| D2 | - | + | - | + | + | + | + | - |
| D3 | + | - | + | + | + | + | + | - |
| D4 | + | + | - | - | + | - | + | - |
| D5 | + | + | - | + | + | - | + | - |
| D6 | + | + | + | - | + | - | + | - |
| D7 | + | + | + | + | + | + | + | - |

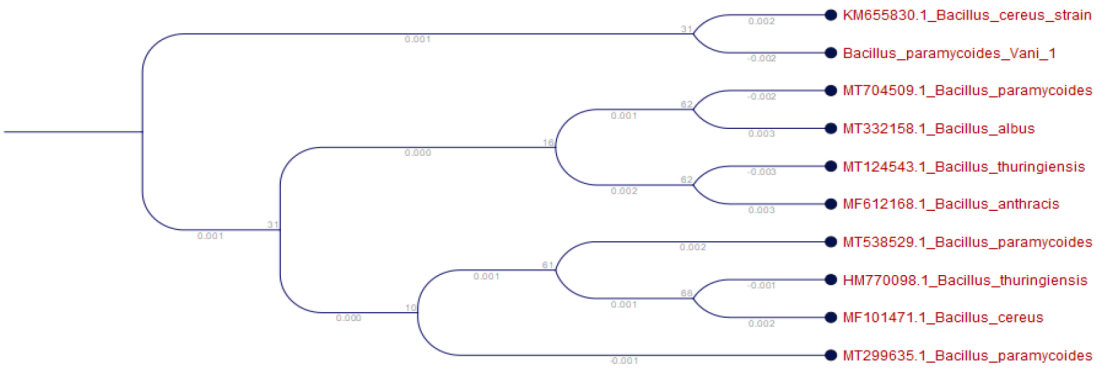



**Figure 2.** Phylogenetic tree based on the 16S rRNA gene sequence showing the phylogenetic position of D6 strain within the *B. paramycoides* complex of *Bacillus* including other *Bacillus* species.

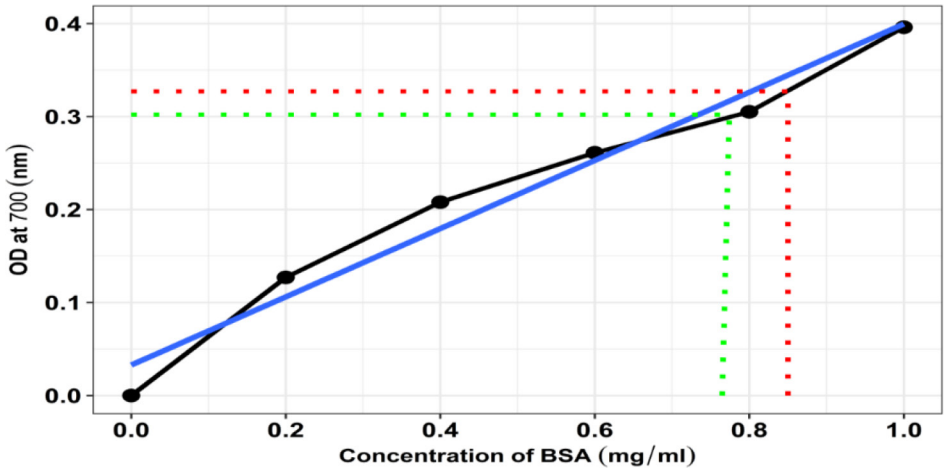



**Figure 3.** BSA standard curve plotted with concentration of BSA (mg/ml) against absorbance at 700 nm.

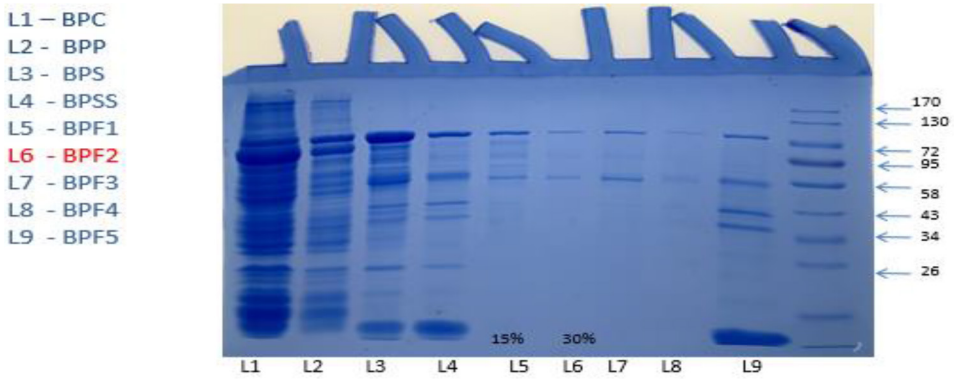



**Figure 4.** Analysis of the active isolate obtained (*B. paramycoides*) after ion-exchange chromatography from the fraction containing proteins using SDS-PAGE electrophoresis.

proteins and impurities that did not interact strongly with the ion exchange resin. A prominent sharp peak appears at around 75 mL, indicating the elution of a major protein component-this peak most likely represents the target protein or the most abundant fraction in the sample. After 80 mL, broad peaks were observed indicating the presence of closely related isoforms, protein aggregates, or moderately retained proteins that eluted later because of variable affinities (Figure 5). Beyond 100 mL, a gradual tailing of the baseline is seen, which may be attributed to weakly interacting components, minor impurities, or degraded protein fragments slowly eluting from the column. The high UV absorbance observed at the main peak, reaching up to ~500 mAU, further supports that a significant quantity of protein was present and successfully eluted. As can be seen from the flat green line representing the flow rate, the chromatographic conditions remained stable throughout the run[44,45].

## 3.6 Characterization of Crude Bacterial Proteins by MALDI-TOF Mass Spectrometry

Crude bacterial proteins from gut-associated bacteria of the earthworm *D. pellucida pallida* were analyzed by MALDI-TOF MS in this research to determine their molecular mass profiles and assess potential bioactivity. The raw bacterial protein extract, obtained from previously purified and cultured gut bacterial isolates, was characterized by MALDI-TOF through a Bruker Autoflex mass spectrometer operating in linear positive mode. Prior to analysis, the samples were mixed with a matrix solution of α-Cyano-4-Hydroxycinnamic Acid (CHCA) or 2,5-Dihydroxybenzoic acid (DHB), which permits the proteins to be desorbed and ionized upon illumination by a laser[34]. An optimal signal intensity was achieved by summing 500 laser shots per sample spot.

The spectra obtained had protein peaks ranging from m/z of approximately 53,202 to 85,365 Da (Figure 6), indicating the presence of high molecular weight proteins that may potentially be bioactive glycoproteins, antimicrobial peptides, or enzymes[32]. Single peaks of high intensity and area values-such as those at m/z 72,380.584 (intensity 168) and m/z 85,365.923 (intensity 108)-suggest the presence of top protein candidates for detailed analysis of structure and function. A list of breast cancer relevant protein candidates derived from earthworm gut bacterial isolates is provided in Table 2.

Analysis of the data was carried out using Flex Analysis and Flex Control software (version 3.4.169.1) for peak recognition, baseline correction, and molecular weight determination. The high resolution of the mass spectra and reproducibility of the peaks indicate the efficiency of MALDI-TOF MS in analyzing crude protein mixtures that are complex[33]. Using this instrumental analysis, proteins can be identified by cross-referencing mass fingerprints with protein databases. Secondarily, the MALDI-TOF profile aids in the selection of specific candidate proteins for subsequent bioassays or sequencing, hence helping towards the general aim of identifying antibacterial proteins from gut bacteria of *D. pellucida pallida*.

## 3.7 Evaluation of Antibacterial Activity of Crude Bacterial Proteins using Plating Technique

Crude protein extracts of *B. paramycoides* and *Bacillus cereus* for their antimicrobial activity against *A. hydrophila* were screened by agar well diffusion

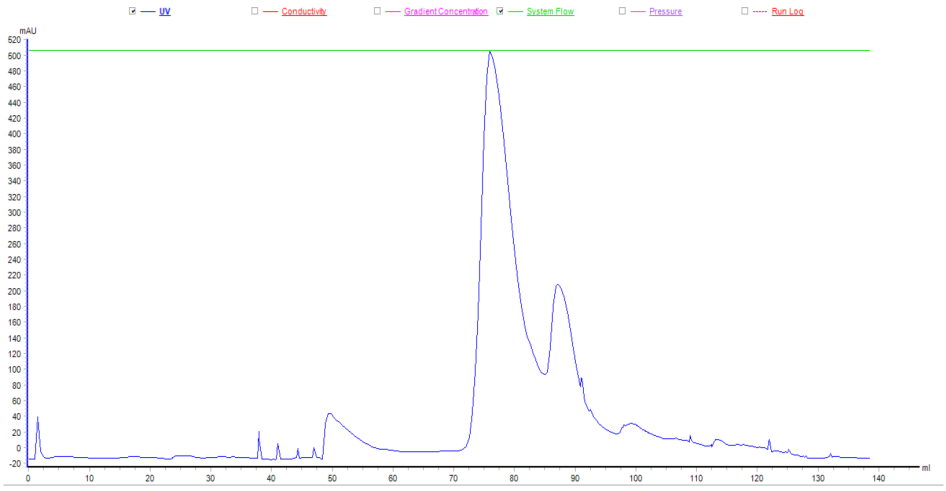



**Figure 5.** Purification of crude bacterial proteins using ion exchange chromatography. X-axis (retention volume or time in mL) shows the volume of eluent (usually in mL) passing through the column. Y-axis (mAU): Absorbance in milli-absorbance units, indicating protein or compound detection via UV absorbance (likely at 280 nm for proteins).

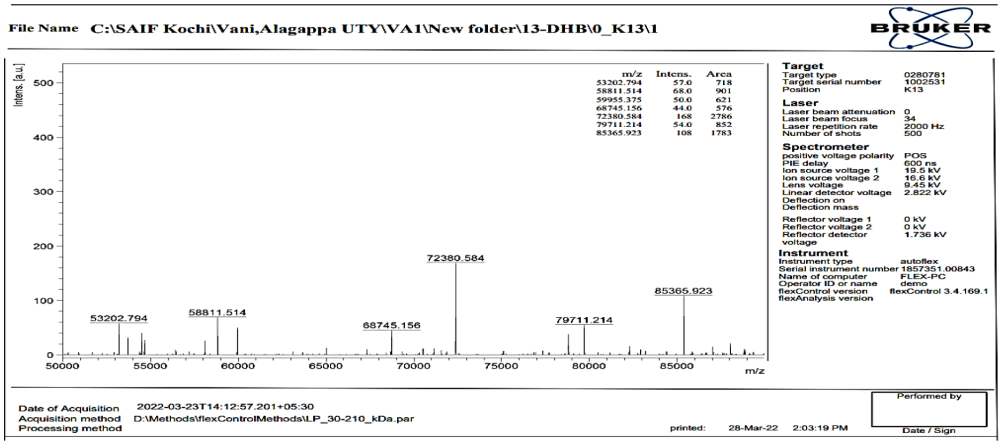


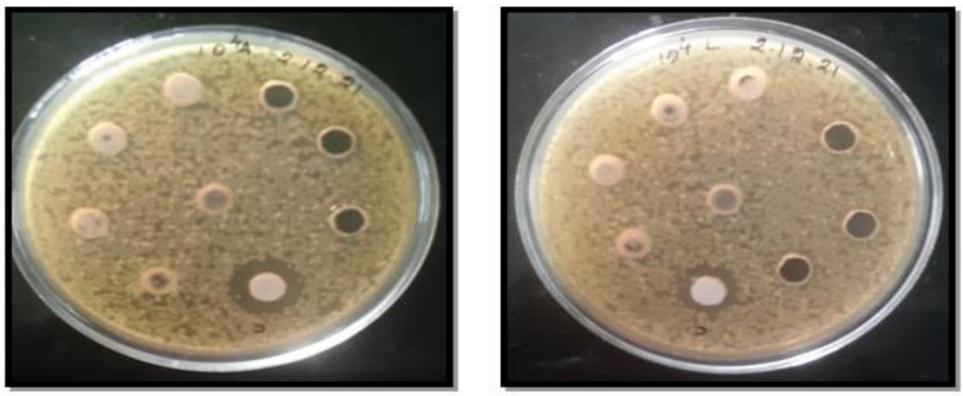

**Figure 6.** Identification of crude bacterial proteins with MALDI-TOF mass spectrometry.

method. Visual observation of zones of inhibition reveals that *B. paramycoides* demonstrated relatively stronger antibacterial activity, as indicated by wider and clearer zones of inhibition (Figure 7). In the plate of *B. paramycoides*, some wells exhibited appreciable zones of inhibition ranging approximately 14 to 20 mm in diameter, particularly at high concentrations. In other wells, there was moderate activity with zones of inhibition ranging 10 to 13 mm, while others exhibited minimal inhibition (<5 mm), most likely due to lower concentrations.

On the other hand, the plate for *B. cereus* exhibited relatively weaker antibacterial activity. The inhibition zones were relatively narrower with moderate clear zones 10 to 13 mm in two or three wells, while the remaining wells exhibited mild inhibition 6 to 9 mm. Minimum or no inhibition, *i.e.*, low protein content or lower activity of the active compounds, were observed in one or more wells. The comparison indicates that *B. paramycoides* contains more active antibacterial proteins against *A. hydrophila* than *B. cereus* proteins, especially with higher crude protein concentration[46].

**Table 2.** Breast cancer-relevant protein candidates of earthworm gut isolated bacteria and their anticancer properties based on MALDI-TOF peaks

| m/z (Da) | Approx. MW (kDa) | Probable Protein Identity | Relevance to Breast Cancer |
|---|---|---|---|
| 53202.794 | ~53.2 kDa | p53 Tumour suppressor, HSP60 | p53 Mutations are common in breast cancer; HSP60 involved in apoptosis regulation. |
| 58811.514 | ~58.8 kDa | GRP78 (BiP), Glucose-regulated protein 78 | Overexpressed in triple-negative breast cancer; promotes chemoresistance. |
| 59955.375 | ~59.9 kDa | PKM2 (Pyruvate kinase M2) | Supports aerobic glycolysis (Warburg effect) in breast tumour cells. |
| 68745.156 | ~68.7 kDa | HSP70 (Heat shock protein 70) | Highly expressed in aggressive breast cancer subtypes; promotes survival under stress. |
| 72380.584 | ~72.4 kDa | Enolase 1 (ENO1), HSP73 | ENO1 plays a role in tumour proliferation; potential target in breast cancer therapy. |
| 79711.214 | ~79.7 kDa | Elongation factor 2 (EF2) | Important in protein synthesis; linked with metastasis and drug resistance. |
| 85365.923 | ~85.4 kDa | Transferrin, or DNA topoisomerase II alpha | Topo IIα is a target of doxorubicin; associated with HER2-positive breast cancer. |



*Bacillus paramycoides* *Bacillus cereus*

**Figure 7.** The antibacterial activity of *B. paramycoides and B. cereus* (crude protein) against *Aeromonas hydrophila* pathogenic bacteria.

## 4. Conclusions

The current research is concerned with the gut-associated bacteria of the indigenous earthworm *D. pellucida pallida* and their antibacterial activity. In this study, 7 isolates of bacteria were recovered and characterized morphologically, biochemically, and molecularly. It was found that the isolate coded D6 was highly resistant to antibacterial activity, and it was purified and further analysed. Crude protein extracts of D6 isolate were prepared by ammonium sulfate precipitation and dialysis. Further purification was performed using ion exchange chromatography, which identified a major peak of protein suggesting an antibacterial compound. SDS-PAGE analysis of the active protein fraction showed a major band at approximately 33 kDa, indicating the presence of a low-molecular-weight antibacterial protein. Molecular identification of the D6 isolate as *B. paramycoides* was carried out by sequencing the 16S rRNA gene. Phylogenetic analysis positioned it solidly within the *Bacillus* genus, well within the group of other members of the *B. paramycoides* complex. The antimicrobial activity of *B. paramycoides* and *B. cereus* proteins against *A. hydrophila* pathogenic bacteria revealed that the gut of *D. pellucida pallida* as a potential source of new antibacterial compounds. There is a potential for natural antibiotics or drugs to be developed from *B. paramycoides* active protein. The discovery emphasizes the biomedical and ecological significance of earthworm-associated microbiota, and the need for more extensive investigation of indigenous species for bioactive metabolites.

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## Author Contributions

Each author made meaningful contributions to the initial discussions that shaped the foundation of this work. Investigation, methodology, writing original draft -V.K., K.K. and N.M.; Methodology, software, revisions - R.B. and V.S.; Methodology, validation and manuscript revision; Revision, software, methodology and revision: J.A.J.P. and B,K.S.; Supervision, investigation, editing and validation - P.S. and M.B; All authors collaborated in reviewing and refining the drafts and have collectively approved the final version for publication.


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