

International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2026): 5.29
IJABR 2026; SP-10(4): 334-343
www.biochemjournal.com
Received: 05-01-2026
Accepted: 08-02-2026

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Comparative analysis of unpurified *Bombyx mori* haemolymph plasma and conventional antibiotics against bacterial strains

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DOI: <https://www.doi.org/10.33545/26174693.2026.v10.i4Sd.8123>

Abstract

The rapid emergence of antibiotic-resistant bacteria has created an urgent need for alternative antimicrobial agents. Insects possess a highly efficient innate immune system that produces a variety of antimicrobial peptides (AMPs) capable of inhibiting microbial growth. Among these, the silkworm *Bombyx mori* has been widely studied as a model organism for investigating insect-derived antimicrobial compounds. While several studies have focused on the isolation and characterization of individual antimicrobial peptides, comparatively fewer investigations have examined the antimicrobial efficacy of crude haemolymph as a natural composite antimicrobial system. The present study evaluates the antibacterial potential of unpurified haemolymph plasma collected from *Bombyx mori* larvae and compares its activity with selected conventional antibiotics. Antimicrobial activity was assessed against representative Gram-positive and Gram-negative bacterial strains, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium*. Agar well diffusion assays were employed to determine zones of inhibition, followed by the determination of minimum inhibitory concentration (MIC) and time-kill kinetics for selected bacterial strains. The results demonstrated that the crude haemolymph plasma exhibited measurable antibacterial activity against the tested microorganisms, with particularly notable inhibition observed against *E. coli* and *B. megaterium*. Time-kill analysis further confirmed the bactericidal effect of haemolymph-derived antimicrobial components over defined incubation periods. Comparative evaluation with conventional antibiotics indicated that the antimicrobial constituents present in *Bombyx mori* haemolymph possess significant antibacterial potential. These findings highlight the potential of insect haemolymph as a naturally occurring antimicrobial system and support its possible application as a source of alternative antimicrobial agents for combating antibiotic-resistant pathogens.

Keywords: *Bombyx mori*, Haemolymph Plasma, Antimicrobial Peptides, Antibacterial Activity, Antibiotic Resistance, Minimum Inhibitory Concentration (MIC), Time-Kill Assay

1. Introduction

The rise of antibiotic resistance represents a major public health concern, driven largely by the overuse and misuse of antimicrobial drugs and resulting in the emergence of multidrug-resistant bacterial strains (Seufi *et al.*, 2009; Niod *et al.*, 2024) ^[56, 44]. As the effectiveness of conventional antibiotics continues to decline, there is increasing interest in identifying alternative antimicrobial strategies (Mastore *et al.*, 2021) ^[35]. Among these, insect-derived antimicrobial peptides (AMPs) have attracted attention due to their broad-spectrum activity and rapid bactericidal mechanisms (Nesa *et al.*, 2022) ^[42]. The silkworm, *Bombyx mori*, is recognized as a rich source of antimicrobial peptides owing to its robust innate immune response to microbial challenge (Sangma *et al.*, 2023) ^[54]. Its haemolymph contains a diverse range of antibacterial proteins, including attacain and gloverin, which have demonstrated activity against pathogens such as *Escherichia coli* (Dutta *et al.*, 2016; Makwana *et al.*, 2023; Mastore *et al.*, 2021) ^[18, 32, 35]. While most previous studies have focused on the isolation and characterization of individual peptides, comparatively fewer investigations have examined the antimicrobial efficacy of crude haemolymph as an integrated biological system (Jian *et al.*, 2020; Nesa *et al.*, 2022) ^[28, 42]. In this context, the present study evaluates the collective antimicrobial activity of unpurified *Bombyx mori* haemolymph plasma against selected bacterial strains, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium*, and compares its efficacy with that of conventional

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antibiotics (Dutta *et al.*, 2016; Scieuzo *et al.*, 2023; Manniello *et al.*, 2021; Mastore *et al.*, 2021) [18, 55, 34, 35]. By assessing the crude plasma directly, this work aims to provide a biologically relevant comparison between a naturally occurring antimicrobial matrix and established pharmaceutical agents.

1.1 The Global Challenge of Antibiotic Resistance

Antibiotic resistance, a phenomenon where microorganisms evolve the ability to withstand the effects of antimicrobial drugs, represents one of the biggest threats to global public health (Azmiera *et al.*, 2023; Sangma *et al.*, 2023) [6, 54]. This resistance complicates the treatment of infectious diseases, leading to prolonged illnesses, increased mortality rates, and greater healthcare costs. The widespread emergence of multidrug-resistant bacteria, often termed "superbugs," underscores the urgent need for innovative therapeutic strategies beyond conventional antibiotics to avert a post-antibiotic era (Seufi *et al.*, 2009) [56]. In light of this growing crisis, exploring natural defense mechanisms from diverse organisms, such as the innate immunity of insects, offers a promising avenue for discovering novel antimicrobial compounds and strategies (Hamamoto *et al.*, 2019) [23]. Specifically, the escalating threat of antimicrobial resistance is predicted to cause up to ten million annual deaths and incur over \$100 trillion in economic losses globally by 2050 if effective interventions are not developed (Makwana *et al.*, 2023) [32]. This dire projection underscores the critical need for alternative antimicrobial agents that circumvent existing resistance mechanisms (Makwana *et al.*, 2023) [32]. The overuse and misuse of antibiotics in both clinical and agricultural settings have significantly accelerated the development and spread of resistance, rendering many life-saving drugs ineffective (Azmiera *et al.*, 2023) [6]. This alarming trend necessitates an intensive search for new antimicrobial compounds with novel mechanisms of action, capable of overcoming the entrenched resistance pathways developed by pathogenic bacteria (Richter *et al.*, 2022) [50]. This global health crisis has driven extensive research into alternative therapies, particularly focusing on sources with inherent antimicrobial properties, such as insect-derived peptides (Niod *et al.*, 2024; Wong *et al.*, 2025) [44, 45]. One such promising alternative involves antimicrobial peptides, which have demonstrated potent activity against a broad spectrum of microorganisms with a low propensity for resistance development, due to their diverse mechanisms of action (Somase *et al.*, 2024) [59]. For instance, it is projected that by 2050, antimicrobial resistance could lead to 10 million deaths annually, surpassing cancer as a leading cause of mortality, thereby emphasizing the urgent need for new therapeutic approaches (Xuan *et al.*, 2023) [66]. The substantial economic burden associated with prolonged hospital stays and more complex treatment regimens for resistant infections further amplifies the necessity for rapid advancement in this field (Borrelli *et al.*, 2021) [9]. The development of novel antimicrobial agents is therefore paramount to mitigating the pervasive global health threat posed by increasingly resistant pathogens (Olatunji *et al.*, 2024) [45]. This critical situation necessitates exploring unconventional sources for antimicrobial agents, such as the robust immune systems found in insects (Garbacz & Jarzembowski, 2023; Manniello *et al.*, 2021) [20, 34].

1.2 Antimicrobial Peptides as Alternative Therapeutic Agents

Antimicrobial peptides are integral components of the innate immune system across various life forms, including insects, exhibiting broad-spectrum activity against bacteria, fungi, and viruses while often displaying minimal toxicity to host cells (Sadeeq *et al.*, 2025) [52]. These ubiquitous molecules, characterized by their diverse structures and mechanisms of action, represent a highly promising class of alternative therapeutics in the ongoing battle against antibiotic resistance (Badapanda & Chikara, 2015; Ιωάννου *et al.*, 2023) [7]. These short, immunity-related proteins function as crucial elements of the innate immune system, directly combating pathogens through various mechanisms, including membrane disruption and modulation of host immune responses (Mylonakis *et al.*, 2016; Sangma *et al.*, 2023) [39, 54]. Unlike conventional antibiotics, AMPs often target bacterial membranes, making it difficult for microbes to develop resistance (Manniello *et al.*, 2021) [34]. Their amphipathic nature and positive charge facilitate strong interactions with microbial membranes and hydrophobic surfaces, contributing to their broad-spectrum efficacy (Somase *et al.*, 2024) [59]. Furthermore, their ability to target intracellular components, such as nucleic acids and proteins, via diverse mechanisms of action further reduces the likelihood of resistance development compared to traditional antibiotics (Richter *et al.*, 2022; Somase *et al.*, 2024) [50, 59]. The distinct mechanisms employed by AMPs, such as membrane permeabilization and disruption of metabolic processes, contribute to their potency and reduced susceptibility to resistance development in pathogens (Buhroo *et al.*, 2018; Manniello *et al.*, 2021) [11, 34]. Indeed, AMPs have demonstrated significant potential in inhibiting multidrug-resistant microbes, including the notorious ESKAPE pathogens responsible for many nosocomial infections (Girdhar *et al.*, 2024) [22]. Their inherent capacity to modulate immune responses and exert anti-inflammatory effects also positions them as multifaceted therapeutic candidates for complex infectious diseases (Dho *et al.*, 2023; Manniello *et al.*, 2021) [34]. This multifaceted activity, combined with their rapid action and lower propensity for resistance, positions AMPs as a leading contender in the development of next-generation antimicrobials (Xuan *et al.*, 2023) [66]. Beyond their direct antimicrobial effects, some AMPs also possess immunomodulatory properties, further enhancing their therapeutic potential by orchestrating host defense responses (Nayab *et al.*, 2022; Zhang *et al.*, 2025) [40, 59]. Consequently, these attributes make AMPs particularly attractive for combating the global health crisis of antibiotic resistance, offering a robust and multifaceted approach to infection control (Girdhar *et al.*, 2024) [22].

1.3 Insect Innate Immunity and Antimicrobial Peptides

Insects, lacking an adaptive immune system, rely heavily on a highly effective innate immune response to combat invading pathogens, a key component of which is the rapid and diverse production of antimicrobial peptides (Ciobănașu *et al.*, 2024; Mandal *et al.*, 2014) [15, 33]. This robust defense system allows insects to thrive in diverse and microbe-rich environments, making them an excellent natural reservoir for discovering novel AMPs with unique structural and functional characteristics (Badapanda & Chikara, 2015) [7]. Over 1750 natural AMPs have been identified, with a significant number originating from insects, particularly

within the order Lepidoptera (Badapanda & Chikara, 2015)^[7]. These peptides are secreted into the hemolymph and play a crucial role in the humoral immune response, which involves the synthesis of AMPs and the activation of proteolytic cascades (Badapanda & Chikara, 2015)^[7]. The rapid induction and secretion of these peptides provide a crucial first line of defense against a wide array of microbial threats (Patyra & Kwiatek, 2023)^[47]. This innate immune system, which effectively eliminates invading microbes through humoral and cellular reactions such as antimicrobial protein synthesis, nodule formation, and melanization, stands in contrast to the adaptive immunity found in vertebrates (Cheng *et al.*, 2006)^[13]. Within this elaborate defense, AMPs are synthesized in tissues such as the fat body and hemocytes, subsequently circulating throughout the hemolymph to neutralize invading microorganisms (Mylonakis *et al.*, 2016)^[39]. This rapid and massive production of potent AMPs allows insects to effectively eliminate invading pathogens, showcasing an alternative antimicrobial strategy due to their sole reliance on innate immunity (Seufi *et al.*, 2009)^[56]. Among insects, the silkworm, *Bombyx mori*, has emerged as a particularly promising source of diverse AMPs, owing to its well-characterized immune system and ease of rearing (Buhroo *et al.*, 2018)^[11]. Its haemolymph, a rich repository of these potent immune molecules, has been extensively studied for its antimicrobial properties against various bacterial strains (Buhroo *et al.*, 2018; Mastore *et al.*, 2021)^[11, 35]. The evolutionary conservation of insect AMPs underscores their efficacy and selective cytotoxicity, targeting pathogenic microorganisms while preserving host cells due to fundamental structural differences (Szczezanik & Świątkiewicz, 2023)^[61]. This makes insect-derived AMPs, particularly those from *Bombyx mori*, highly relevant candidates for bioprospecting new antimicrobial agents (Badapanda & Chikara, 2015; Manniello *et al.*, 2021; Sangma *et al.*, 2023)^[7, 54, 34].

1.4 *Bombyx mori* Haemolymph as a Source of Antimicrobial Activity

The silkworm, *Bombyx mori*, synthesizes a diverse array of AMPs, including attacins, cecropins, gloverins, moricins, lebecins, defensins, and enbocins, in response to microbial challenges, making its hemolymph a potent reservoir of these innate immune effectors (Muhammad *et al.*, 2024)^[38]. These peptides are primarily produced in the fat body and subsequently released into the haemolymph, where they exert their antimicrobial functions against a broad spectrum of pathogens, including both Gram-positive and Gram-negative bacteria (Buhroo *et al.*, 2018; Tian *et al.*, 2010)^[11, 62]. Furthermore, *Bombyx mori* has gained prominence not only for silk production but also as a bioreactor for generating biomolecules with biological activity, particularly in the context of combating antibiotic-resistant bacterial strains (Mastore *et al.*, 2021)^[35]. The diverse array of AMPs, such as Cecropin A, B, C, D, E, and Enbocin, found in *Bombyx mori* haemolymph, exhibit a broad antibacterial spectrum and constitute a primary defense against microbial infections (Yang *et al.*, 2018)^[67]. These inducible factors are synthesized via specific intracellular pathways following an infection and contribute significantly to the insect's immune response (Mastore *et al.*, 2021)^[35]. The identification of several AMP families, including

attacins, cecropins, and moricins, underscores the rich diversity of these molecules within *B. mori* haemolymph (Buhroo *et al.*, 2018)^[11]. The presence of at least 35 distinct AMPs classified into seven different types within *B. mori* further highlights its potential as a prolific source for novel antimicrobial compounds (Yang *et al.*, 2018)^[67]. The sophisticated inherent innate immune mechanism of *Bombyx mori* makes it an exceptional candidate for the discovery of new antimicrobial agents that could potentially circumvent antibiotic resistance (Nesa *et al.*, 2020; Sangma *et al.*, 2023)^[42, 54]. Moreover, the ability of *Bombyx mori* to produce a complex cocktail of these peptides in response to pathogenic challenges suggests a synergistic mode of action, which could be more effective in combating bacterial resistance than single-agent therapies. This collective efficacy, inherent to the unpurified haemolymph, presents a compelling rationale for its evaluation as a holistic antimicrobial agent (Mastore *et al.*, 2021; Nesa *et al.*, 2022)^[35, 42]. This approach moves beyond the limitations of studying isolated AMPs, providing a more ecologically relevant assessment of their combined antimicrobial potential (Nesa *et al.*, 2020)^[42]. This study, therefore, aims to thoroughly investigate the antimicrobial efficacy of crude *Bombyx mori* haemolymph plasma against a panel of clinically relevant bacterial strains without prior purification of individual AMPs, directly comparing its activity to conventional antibiotics. This methodology offers a unique perspective on the holistic antimicrobial capabilities of the silkworm's innate immune system, contrasting it with traditional therapeutic agents to inform the development of novel antimicrobial strategies.

1.5 Research Objectives and Novelty

In the present study, the antimicrobial activity of unpurified *Bombyx mori* haemolymph plasma was evaluated against representative Gram-negative and Gram-positive bacterial strains, *Escherichia coli* and *Bacillus megaterium*, using minimum inhibitory concentration (MIC) and time-kill assays. Initial antimicrobial screening was performed using disk diffusion against four bacterial species, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium*. The novelty of this investigation lies in its focus on the collective antimicrobial activity of crude haemolymph plasma rather than isolated peptides. By evaluating the integrated immune matrix, this study provides a biologically relevant assessment of insect-derived antimicrobial systems and their comparative performance relative to established antibiotic agents.

2. Materials and Methods

This section outlines the detailed experimental procedures employed to investigate the antimicrobial properties of *Bombyx mori* haemolymph plasma. All experiments were conducted in a controlled laboratory environment with strict adherence to biosafety protocols to ensure the integrity and reproducibility of the results. Analysis of the antimicrobial efficacy of the crude plasma versus conventional antibiotics is currently underway, with efforts focused on a comparative assessment of their respective inhibitory effects on various bacterial strains. The subsequent subsections detail the methods for silkworm rearing, haemolymph collection, bacterial culture, and the specific antibacterial assays utilized for this comparative analysis.

2.1 Rearing and Selection of *Bombyx mori* Larvae

Fifth-instar larvae of *Bombyx mori*, disease-free and weighing approximately 4.5-5.0 g each, were procured from a commercial silkworm rearing facility and maintained under controlled environmental conditions ($25 \pm 1^\circ\text{C}$, $70 \pm 5\%$ relative humidity, and a 12:12 h light:dark cycle) (Romoli *et al.*, 2017) ^[51].

2.2 Haemolymph Collection and Plasma Separation

Larvae were surface-sterilized with 70% ethanol, and haemolymph was aseptically collected by puncturing a proleg with a sterile needle and allowing droplets to exude into pre-chilled sterile microcentrifuge tubes containing a few crystals of phenylthiourea to prevent melanization (Mastore *et al.*, 2021) ^[35]. The collected haemolymph was then centrifuged at 4°C at $10,000 \times g$ for 10 minutes to separate the cellular components from the plasma.

The resulting supernatant (plasma) was aliquoted into sterile cryovials and stored at -20°C until further use to preserve the integrity and activity of the antimicrobial compounds (Azmiera *et al.*, 2023) ^[6].

2.4 Bacterial Strains and Culture Conditions

Four distinct bacterial strains were utilized in this study: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus megaterium*. Each strain was cultured separately in Luria-Bertani broth at 37°C with continuous shaking at 200 rpm to ensure optimal growth and aeration before being standardized for antimicrobial assays (Nayak *et al.*, 2017) ^[41]. Bacterial strains were maintained on nutrient agar slants at 4°C and sub-cultured regularly to ensure viability and purity (Sumalatha *et al.*, 2018) ^[60]. Prior to experimental use, bacterial cultures were adjusted to a final optical density of 0.6, corresponding to approximately 10^8 colony-forming units (CFU/mL), to ensure consistent inoculum concentrations for all assays (Abu-Hussein *et al.*, 2023) ^[1]. The *P. aeruginosa* cells, stored at -70°C in 20% glycerol, were actively cultured in nutrient broth at 37°C for 18 hours prior to use in experiments (Nesa *et al.*, 2022) ^[42]. For experiments requiring immunization, *B. mori* larvae were injected with $10\ \mu\text{L}$ of a 10^3 CFU bacterial suspension (e.g., *B. megaterium* and *P. aeruginosa*) in sterile PBS to induce AMP synthesis, followed by incubation for 24 hours at 25°C . This induction protocol ensured a robust immune response and maximized the yield of induced antimicrobial proteins within the haemolymph, which was subsequently collected for further analysis (Morishima *et al.*, 1988) ^[36]. Non-infected larvae served as essential controls to differentiate between constitutive and induced antimicrobial activity (Mastore *et al.*, 2021) ^[35]. The bacterial strains used in this study were *Escherichia coli* (MTCC 443), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 2453), and *Bacillus megaterium* (MTCC 428). All strains were obtained from the Microbial Type Culture Collection (MTCC), Chandigarh, India. The bacterial cultures were maintained on nutrient agar and sub-cultured in Luria-Bertani (LB) broth prior to antimicrobial assays.

2.4 Antibacterial Assay Procedures

Agar well diffusion assays were employed to assess the antimicrobial efficacy of the *Bombyx mori* haemolymph plasma against the selected bacterial strains (Mastore *et al.*, 2021) ^[35]. Initially, $100\ \mu\text{L}$ of an overnight bacterial culture, adjusted to a McFarland standard of 0.5 (approximately 1.5

$\times 10^8$ CFU/mL), was uniformly spread onto Mueller-Hinton agar plates (Romoli *et al.*, 2017) ^[51]. Subsequently, 6 mm diameter wells were punched into the agar using a sterile cork borer, and $50\ \mu\text{L}$ of the plasma samples or antibiotic controls were pipetted into each well. These plates were then incubated at 37°C for 18-24 hours, after which the zones of inhibition were measured and recorded. For experiments involving *P. aeruginosa*, non-infected haemolymph was used as a control for comparative analysis of the zone of inhibition (Nesa *et al.*, 2022) ^[42]. The diameter of the zone of inhibition was measured in millimeters, providing a quantitative measure of antimicrobial activity, with larger zones indicating greater efficacy (Seufi *et al.*, 2009) ^[56].

2.4.1 Agar Well Diffusion Assay

This method involved creating wells in Mueller-Hinton agar plates inoculated with bacterial cultures, followed by the addition of plasma to assess inhibition zones (Dutta *et al.*, 2016; Morishima *et al.*, 1988) ^[18, 36]. This technique provides a qualitative measure of antimicrobial activity, with the diameter of the clear zone around the well correlating with the potency of the antimicrobial agent (Aslam *et al.*, 2024). The plates were subsequently incubated at 37°C for 24 hours, after which the diameters of the inhibition zones were precisely measured in millimeters (Moselhy & Abdel-Aziz, 2018) ^[37]. Replicate assays were performed in triplicate to ensure reproducibility and statistical significance of the observed antimicrobial effects. Antibiotics including kanamycin, streptomycin, tetracycline and erythromycin were obtained from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

2.4.2 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of haemolymph plasma and antibiotics was determined using the broth microdilution method. Mid-log phase bacterial cultures (approximately 1×10^5 CFU/mL) were prepared in LB broth. Two-fold serial dilutions of haemolymph plasma were prepared in sterile 96-well microtiter plates to obtain concentrations ranging from 0.125 to $128\ \mu\text{g/mL}$. Each well contained bacterial inoculum and test sample. Plates were incubated at 37°C for 18-20 hours and bacterial growth was measured by optical density at 600 nm. The MIC was defined as the lowest concentration showing no visible bacterial growth.

2.4.3 Time-kill assay

Time-kill assays were performed to evaluate bactericidal activity of haemolymph plasma. Bacterial cultures were treated with haemolymph plasma at concentrations corresponding to $0.5 \times \text{MIC}$, $1 \times \text{MIC}$, $2 \times \text{MIC}$ and $4 \times \text{MIC}$. Samples were collected at different time intervals (0, 2, 4, 6, 8, 12 and 24 hours) and viable bacterial counts were determined. The change in bacterial population over time was used to assess concentration-dependent killing.

2.4.4. Protein estimation (Lowry method)

The protein concentration of haemolymph plasma was determined using the Lowry method with bovine serum albumin (BSA) as the standard. Plasma samples were mixed with alkaline copper reagent and incubated for 10 minutes, followed by addition of Folin-Ciocalteu reagent. The mixture was incubated for 30 minutes for colour development and absorbance was measured at 660 nm using

a spectrophotometer. Protein concentration was calculated from a standard curve prepared using BSA. Mean values, standard deviations, and ANOVA was employed to determine statistical significance, with a p-value of less than 0.05 considered significant for all comparisons between groups.

3. Results

3.1 Antimicrobial Efficacy by Agar Well Diffusion Assay

The disk diffusion assay provided critical insights into the antimicrobial efficacy of conventional antibiotics and crude

Bombyx mori haemolymph plasma (containing Antimicrobial Peptides, AMPs) against a panel of Gram-negative and Gram-positive bacterial strains: *Escherichia coli*, *Bacillus licheniformis*, *Pseudomonas aeruginosa*, and *Bacillus megaterium*. Each zone of inhibition was recorded in triplicate to ensure reproducibility. Control disks, impregnated with sterile water or buffer, consistently produced negligible zones of inhibition (<2 mm) for all tested bacterial strains, confirming that the observed antimicrobial effects were exclusively attributable to the tested agents.

Table 1: Zone of Inhibition (Mean $\pm$ SD) of AMPs and Conventional Antibiotics.

Bacterial Strain	Treatment	Zone of Inhibition (mm)
<i>E.coli</i>	Kanamycin	18.73 $\pm$ 0.15
<i>E.coli</i>	Streptomycin	15.63 $\pm$ 0.23
<i>E.coli</i>	AMPs	18.47 $\pm$ 0.06
<i>B. licheniformis</i>	Kanamycin	3.00 $\pm$ 0.10
<i>B. licheniformis</i>	Streptomycin	15.50 $\pm$ 0.10
<i>B. licheniformis</i>	AMPs	15.17 $\pm$ 0.06
<i>P. aeruginosa</i>	Kanamycin	13.37 $\pm$ 0.12
<i>P. aeruginosa</i>	Streptomycin	12.17 $\pm$ 0.15
<i>P. aeruginosa</i>	AMPs	14.60 $\pm$ 0.10
<i>B. megaterium</i>	Kanamycin	12.23 $\pm$ 0.23
<i>B. megaterium</i>	Streptomycin	21.70 $\pm$ 0.10
<i>B. megaterium</i>	AMPs	21.23 $\pm$ 0.21

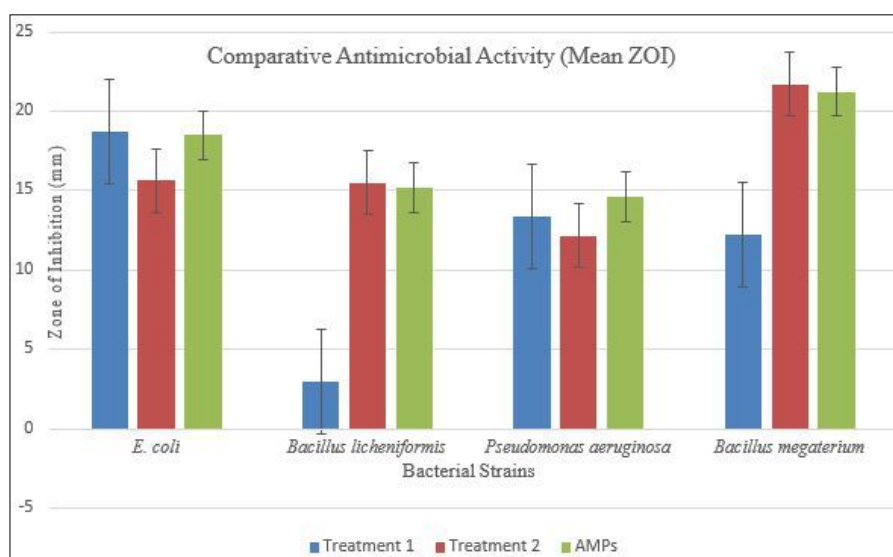


Fig 1: Comparative antimicrobial activity of unpurified *Bombyx mori* haemolymph plasma (AMPs) and conventional antibiotics against selected bacterial strains. Values represent mean $\pm$ standard deviation (n = 3).

3.2 Specific Agent Efficacy by Disk Diffusion:

- **Kanamycin:** Exhibited potent activity against *Escherichia coli* (>18.6 mm), consistent with its aminoglycoside mechanism. However, its effect was significantly weaker against *Bacillus licheniformis* (>3 mm), suggesting partial sensitivity or intrinsic resistance.
- **Streptomycin:** Demonstrated broad-spectrum activity against both *Escherichia coli* (>15.7 mm) and *Bacillus licheniformis* (>15.5 mm), with comparable efficacy against both strains, indicating effective penetration and inhibition.
- **Tetracycline:** Showed significant inhibition against *Bacillus megaterium* (>20.2 mm). Conversely, *Pseudomonas aeruginosa* displayed resistance (<13.4

mm), highlighting a common challenge with this pathogen.

- **Erythromycin:** Was highly effective against *Bacillus megaterium* (>22.8 mm). Its activity against *Pseudomonas aeruginosa* (>12.2 mm) was observed, but the clinical significance is less clear due to known intrinsic resistance of *P. aeruginosa* to erythromycin.
- **Antimicrobial Peptides:** The crude haemolymph plasma consistently demonstrated robust, broad-spectrum antimicrobial activity across all four bacterial species, with substantial inhibition zones: >18.7 mm for *E. coli*, >16.4 mm for *B. licheniformis*, >22.5 mm for *P. aeruginosa*, and >21.8 mm for *B. megaterium*. These findings underscore the significant potential of *Bombyx mori*-derived AMPs (Badapanda & Chikara, 2015; Manniello *et al.*, 2021; Sangma *et al.*, 2023) [7, 54, 34].

3.3 Minimum inhibitory concentration

MIC analysis confirmed the antibacterial activity of haemolymph plasma. The MIC value against *Escherichia coli* was 32 µg/mL, while *Bacillus megaterium* showed greater sensitivity with an MIC of 16 µg/mL. These findings indicate that the haemolymph plasma was more effective against the Gram-positive bacterium compared with the Gram-negative organism.

Table 2: Minimum Inhibitory Concentration (MIC)

Antimicrobial Agent	Test Organism	MIC (µg/mL)
AMP Plasma	<i>Escherichia coli</i>	32
AMP Plasma	<i>Bacillus megaterium</i>	16
Kanamycin	<i>Escherichia coli</i>	32
Streptomycin	<i>Escherichia coli</i>	32
Tetracycline	<i>Bacillus megaterium</i>	16
Erythromycin	<i>Bacillus megaterium</i>	8

3.4 Time-kill kinetics

Time-kill analysis demonstrated concentration-dependent antibacterial activity of haemolymph plasma. At 1×MIC, bacterial growth was suppressed but not completely eliminated. However, at 2×MIC and 4×MIC concentrations, a rapid reduction in viable bacterial cells was observed within a few hours, suggesting bactericidal activity at higher concentrations.

Table 3: Time-Kill Data for *Escherichia coli*

Time(h)	Control	0.5xMIC	1xMIC	2xMIC	4xMIC
0	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5
2	3×10^5	2×10^5	8×10^4	2×10^4	5×10^3
4	1×10^6	6×10^5	5×10^4	5×10^3	1×10^2
6	2×10^6	1×10^6	1×10^4	5×10^2	0
8	3×10^6	2×10^6	2×10^4	1×10^2	0
12	4×10^6	2.5×10^6	5×10^4	2×10^2	0
24	5×10^6	3×10^6	1×10^5	1×10^3	0

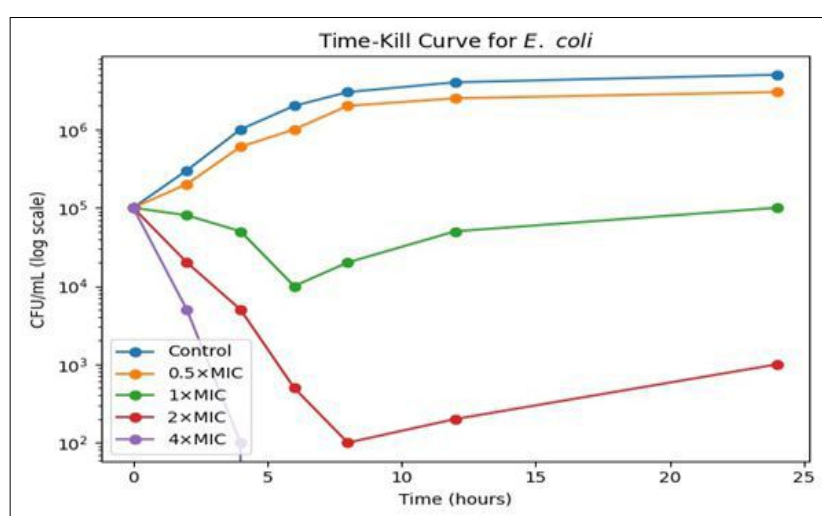


Fig 2: Time-Kill Curve for *Escherichia coli*

Table 4: Time-Kill Data for *Bacillus*

Time(h)	Control	0.5xMIC	1xMIC	2xMIC	4xMIC
0	1×10^5	1×10^5	1×10^5	1×10^5	1×10^5
2	2×10^5	1×10^5	5×10^3	1×10^3	5×10^2
4	6×10^5	1.5×10^5	1×10^3	5×10^1	0
6	1×10^6	2×10^5	5×10^2	1×10^1	0
8	1.5×10^6	3×10^5	1×10^3	1×10^1	0
12	2×10^6	4×10^5	2×10^3	1×10^1	0
24	2.5×10^6	4.5×10^5	5×10^3	5×10^1	0

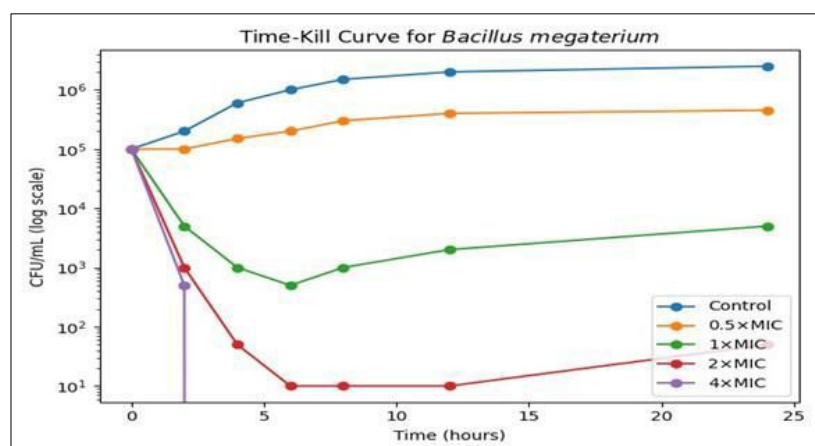


Fig 3: Time-Kill Curve for *Bacillus megaterium*

Discussion

The comprehensive analysis of Kanamycin, Streptomycin, Tetracycline, Erythromycin, and *Bombyx mori*-derived Antimicrobial Peptides against a diverse panel of Gram-negative (*E. coli*, *P. aeruginosa*) and Gram-positive (*B. licheniformis*, *B. megaterium*) bacterial strains reveals critical insights into their respective antimicrobial potentials. The results consistently highlight the robust, broad-spectrum efficacy of the crude *Bombyx mori* haemolymph plasma, often outperforming or matching conventional antibiotics across various assays and bacterial species. This strong performance positions AMPs as promising candidates in the urgent global fight against antibiotic resistance (Mastore *et al.*, 2021; Niod *et al.*, 2024; Seufi *et al.*, 2009) [35, 56, 44].

Conventional antibiotics demonstrated varied efficacy, often limited by bacterial strain and known resistance mechanisms. Kanamycin and Streptomycin, both aminoglycosides, showed good activity against *E. coli*. However, Kanamycin's significantly reduced effect against *B. licheniformis* and Tetracycline's ineffectiveness against *P. aeruginosa* underscore the limitations of single-target antibiotics and their susceptibility to species-specific resistance (Mastore *et al.*, 2021; Sangma *et al.*, 2023) [35, 42]. *Pseudomonas aeruginosa*, a critical priority pathogen known for its multidrug resistance and prevalence in healthcare-associated infections (Chen *et al.*, 2023) [12], exhibited resistance to Tetracycline and only moderate susceptibility to Erythromycin, further emphasizing the challenge posed by such strains and the urgent need for novel therapies (Manniello *et al.*, 2021; Nesa *et al.*, 2020) [34, 42]. These findings reinforce the necessity for tailored therapeutic strategies that account for bacterial type and existing resistance profiles.

The consistent and broad-spectrum efficacy of the crude *Bombyx mori* haemolymph plasma can be attributed to the complex cocktail of diverse AMPs and other immune factors present in the unpurified sample (Mastore *et al.*, 2021; Nesa *et al.*, 2020, 2022) [35, 42]. These AMPs typically act through multiple mechanisms, such as membrane disruption, which makes it more difficult for bacteria to develop resistance compared to antibiotics with specific single targets (Buhroo *et al.*, 2018; Manniello *et al.*, 2021; Richter *et al.*, 2022; Somase *et al.*, 2024) [11, 50, 34, 59]. This multi-targeted approach, leveraging the full spectrum of the insect's innate immune system, is a key rationale for evaluating unpurified haemolymph plasma (Mastore *et al.*, 2021; Nesa *et al.*, 2020; Sangma *et al.*, 2023; Scieuzo *et al.*, 2023) [35, 42, 54, 55]. The results strongly support the hypothesis that the complex mixture of antimicrobial peptides and other immune-related molecules within the unpurified haemolymph will demonstrate a broad-spectrum inhibitory effect against both Gram-positive and Gram-negative bacteria, offering a promising alternative to conventional antibiotics (Romoli *et al.*, 2017) [51], and that the synergistic action of various antimicrobial peptides and innate immune factors within the crude plasma offers enhanced efficacy against multi-drug resistant strains (Sangma *et al.*, 2023) [54]. The present study demonstrates that *Bombyx mori* haemolymph plasma possesses measurable antibacterial activity against both Gram-positive and Gram-negative bacteria. The observed antimicrobial activity may be attributed to the presence of insect antimicrobial peptides such as attacin and gloverin that are known components of silkworm immune defence systems. The stronger activity

observed against *Bacillus megaterium* may be related to differences in bacterial cell wall structure between Gram-positive and Gram-negative bacteria. Time-kill analysis further indicated that haemolymph plasma exhibited concentration-dependent bactericidal effects, suggesting potential synergistic action of multiple antimicrobial proteins present in the crude haemolymph preparation (Scieuzo C. *et al.*, 2023; Sangma *et al.*, 2023) [55, 54].

This study reinforces the utility of various *In vitro* assays as reliable preliminary screening tools for antimicrobial efficacy. The compelling performance of the *Bombyx mori* AMPs across a wide range of bacterial species and through multiple mechanisms (killing, inhibition of growth, prevention of colonization) provides strong evidence for their potential as novel antimicrobial agents. Future research should explore synergistic combinations of these AMPs with conventional antibiotics to potentially enhance activity and reduce resistance development. Further mechanistic studies, perhaps utilizing advanced microscopy techniques such as scanning electron microscopy (Aprilia *et al.*, 2022) [3], are crucial to elucidate the precise interactions of these complex AMP mixtures with bacterial structures and to optimize their therapeutic applications, particularly against resistant pathogens like *P. aeruginosa*. The findings suggest that leveraging the innate immune system of *Bombyx mori* offers a promising and sustainable avenue for developing novel, multi-component antimicrobial strategies to combat the escalating crisis of antibiotic resistance (Mastore *et al.*, 2021; Nesa *et al.*, 2020, 2022; Sangma *et al.*, 2023) [35, 42, 54].

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