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Detoxification Enzyme Modulation and Larvicidal Efficacies of *Klebsiella pneumoniae* (PX652414) and *Proteus mirabilis* (PZ337687) Metabolites in Mosquito Vectors

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Abstract
Mosquitoes of the *Anopheles*, *Aedes,* and *Culex* genera serve as vectors for significant human and animal diseases. The increasing prevalence of insecticide resistance, primarily mediated by detoxification enzymes, highlights the urgent need for innovative biocontrol strategies. This study assessed the larvicidal activity of secondary metabolites produced by *Klebsiella pneumoniae* PX652414 and *Proteus mirabilis* PZ337687, as well as their impact on esterase and glutathione-S-transferase (GST) enzyme activities in three major mosquito species: *An. gambiae* s.l., *Ae. aegypti*, and *Cx. quinquefasciatus*. Metabolites incubated for three and eight days were individually applied to separate cohorts of mosquito larvae. Larval mortality, lethal concentrations (LCs), and enzyme activities were evaluated following 72 hours of exposure. *K. pneumoniae* metabolites induced substantial larval mortality across all mosquito species, demonstrated by low LC50 values and decreased esterase and GST activity, particularly in Cx. quinquefasciatus. Conversely, *P. mirabilis* metabolites resulted in minimal mortality and elevated esterase and GST activity. These results demonstrate enzyme modulation by these metabolites and indicate that *K. pneumoniae* (PX652414) metabolites may serve as promising agents for integrated vector management.

Keywords: larvicidal metabolites, esterase, glutathione-S-transferase, detoxification enzymes, vector control

Declarations

Financial interests

The authors declare they have no financial interests. All authors certify that they have no affiliations with or involvement in any organisation or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. The identified bacterial species have been deposited with the NCBI with accession number PX652414 for *K. pneumoniae* (https://www.ncbi.nlm.nih.gov/nuccore/PX652414) and PZ337687 for *P. mirabilis* (https://www.ncbi.nlm.nih.gov/nuccore/PZ337687).

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Ethical approval

No approval from research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

Authors' Contributions

Conceptualisation: C.C.U, M.E.A., N.U.A., I.K.O, A.C.U; **Methodology:** M.E.A., N.U.A., I.K.O, A.C.U; **Formal analysis and investigation:** C.C.U, A.C.U, I.K.O, I.F.O.; **Writing – original draft:** C.C.U, A.C.U, I.F.O.; **Writing – review & editing:** M.E.A., N.U.A., I.K.O; **Funding acquisition:** C.C.U, M.E.A., N.U.A., I.K.O, A.C.U, I.F.O.; **Resources:** C.C.U, M.E.A., I.K.O, A.C.U; **Supervision:** M.E.A., N.U.A., I.K.O, A.C.U; **Data curation:** C.C.U, A.C.U, I.F.O.; **Investigation:** C.C.U, A.C.U, I.F.O.; **Validation:** M.E.A., N.U.A., I.K.O

Introduction

Mosquitoes belonging to the genera *Anopheles, Aedes*, and *Culex* are among the most important vectors of human and animal diseases, transmitting pathogens responsible for malaria fever, dengue fever, yellow fever, lymphatic filariasis, West Nile virus disease, and other debilitating infections [1]. Despite decades of control efforts, mosquitoes have continued to pose a significant public health threat across tropical and subtropical regions of the world through the burden of these diseases [2]. Conventional chemical (synthetic) insecticides remain the chief control strategy for larval stages of mosquitoes through larval source management (LSM) [3, 4]. However, their widespread use has led to increasing problems of mosquito resistance [5], environmental contamination, and adverse effects on non-target organisms [6]. These pitfalls underscore the urgent need for alternative, sustainable larvicidal strategies [7].

While microbial larvicides such as *Bacillus thuringiensis israelensis* (Bti) and *Lysinibacillus sphaericus* have been widely deployed to control mosquitoes, the diversity of bacterial metabolites with larvicidal potential remains underexplored [8]. Most studies have focused on Gram-positive bacteria [9 – 11], leaving Gram-negative (GN) species relatively neglected despite their rich secondary metabolite reservoir [12 – 14]. Furthermore, little is known about how these metabolites interact with mosquito detoxification enzymes such as esterases and glutathione-S-transferases (GSTs), which play critical roles in xenobiotic metabolism and insecticide resistance [15]. Therefore, there is a need to identify novel microbial products that act through enzyme modulation rather than conventional toxin pathways.

The persistence of insecticide resistance in mosquito populations threatens the sustainability of current vector control programs [1]. Elevated esterase and GST activities are well-documented mechanisms of resistance, enabling mosquitoes to detoxify chemical insecticides and survive exposure [16 – 18]. Without new interventions that can suppress or bypass these detoxification systems, resistance will continue to undermine control efforts [19]. At the same time, deploying microbial metabolites without understanding their mechanistic effects risks ecological consequences, such as inadvertently enhancing detoxification capacity and cross-resistance [12].

Gram-negative bacterial metabolites are reservoirs of highly unexploited compounds with the potential to provide novel modes of action [13]. Several studies have elucidated the toxicity of metabolites from GN bacterial species, either as enhanced or as cell-free extracts [20 – 24]. There are no scientific reports on the influence of these important biocontrol agents on detoxifying enzymes in mosquito larvae. This study was therefore designed to address a critical gap in microbial biocontrol research by focusing on the larvicidal activities of two GN bacteria (*Klebsiella pneumoniae* and *Proteus mirabilis*) and how their metabolites influence esterase and GST activities, providing mechanistic insight into their efficacy and ecological safety. Such knowledge is essential for developing sustainable, enzyme-targeted larvicides that can complement or replace chemical insecticides in integrated vector management (IVM).

In this study, we hypothesised that (1) the metabolites of these GN bacterial species will exert strong larvicidal activities by suppressing detoxification enzymes (esterases and GSTs), (2) the magnitude of enzyme modulation and larvicidal efficacy would vary among three mosquito species (*An. gambiae* s.l., *Ae. aegypti*, and *Cx. quinquefasciatus*), i.e., species-specific metabolic responses. By testing these hypotheses, the study aims to elucidate the mechanistic basis of microbial metabolite activity and assess their ecological implications for vector control.

Materials and Methods

Isolation and Preliminary Identification of Bacteria from Mosquito Breeding Habitats

Water samples (0.1 mL) were collected from selected mosquito breeding habitats (rice fields, rain pools, septic tanks, and drainages) within the city of Minna, Nigeria. The samples were aseptically spread onto 20 cm nutrient agar plates using a sterilised scalpel under laminar flow to minimise contamination [25]. Plates were incubated for 24 hours at 30 °C, and bacterial colonies were isolated according to established microbiological protocols [26]. Distinct colonies were sub-cultured using the streak plate method. A loopful of each colony was streaked onto freshly prepared nutrient agar plates and incubated at 37 °C for 24 hours. Colonies were examined for morphological differences, and streaking was repeated until pure isolates were obtained.

Purified isolates were Gram-stained, morphologically examined and identified using a binocular microscope (Model: Olympus XSZ-107BN) (oil immersion, 100X objective) to determine Gram reaction and cell shape (cocci, bacilli, or rods) [27]. Basic biochemical assays were conducted for preliminary identification of Gram-negative species (Table 1), including coagulase, catalase, indole production, citrate utilisation, H_2S, Voges-Proskauer, methyl red, urease, and sugar fermentation tests (glucose, lactose, sucrose). Results were compared with standard microbiological descriptions to assign species. Phenotypic characterisation of all isolates studied was performed and compared to phenotypic data of known organisms [27], with final authentication by an independent microbiologist.

Table 1: Biochemical Screening of Gram-negative Bacterial Isolates

Isolates	Gram Stain	Shape	Sucrose Starch hydrolys.	Oxidase	Coagulase	Catalase	Indole producti	Citrate utilisatio	H_2S Voges-Proskau	Methyl Red	Urease	Lactose	Glucose	Suspected organism
1	-	rods	+	+	-	-	+	-	+	-	+	-	+	+	+	*Klebsiella pneumoniae*
2	-	rods	+	+	-	+	+	-	+	-	-	+	+	-	+	*Proteus mirabilis*

DNA Extraction and 16S rRNA Gene Amplification by PCR

Bacterial DNA extraction

The bacterial DNA was extracted following an established protocol [28]. Briefly, Single colonies grown on medium were transferred to 1.5 mL of liquid medium and cultured on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600 g for 5 min. The resulting pellets were resuspended in 520 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and 15 µL of 20% SDS and 3 µL of Proteinase K (20 mg/mL) were added. The mixture was incubated for 1 hour at 37 ºC, then 100 µL of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added, and the mixture was vortexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200 g for 20 min. The aqueous phase was then transferred to a new tube, isopropanol (1:0.6) was added, and DNA was precipitated at –20 ºC for 16 h. DNA was collected by centrifugation at 13000 g for 10 min, washed with 500 µL of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µL of TE buffer.

Bacterial Polymerase Chain Reaction (PCR) Analysis

PCR sequencing preparation cocktail consisted of 10 µL of 5X GoTaq colourless reaction, 3 µL of 25 mM $MgCl_2$, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 pmol each 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µL with sterile distilled water 8 µL DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR profile consisting of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles consisting of 94 °C for 30 s, 50 °C for 60 s and 72 °C for 1 minute 30 seconds; and a final termination at 72 °C for 10 mins, and chilled at 4 ºC [29, 30].

Integrity of the amplified gene fragment

The integrity of the amplified gene fragment was checked on a 1% Agarose gel run to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare a

1.5% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3 µL of 0.5 g/mL ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray, and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliters (2 µL) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4 µL of each PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120 V for 45 minutes, visualised by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was run alongside experimental samples in the gel [29, 30].

Purification and sequencing of the amplified product

The amplified fragments were ethanol-purified to remove the PCR reagents. Briefly, 7.6 µL of 3 M sodium acetate and 240 µL of 95% ethanol were added to each about 40 µL PCR-amplified product in a new sterile 1.5 µL Eppendorf tube, mixed thoroughly by vortexing and kept at -20 °C for at least 30 min. Centrifugation was performed for 10 min at 13000 g and 4 °C, followed by removal of the supernatant (invert tube once), after which the pellets were washed by adding 150 µL of 70 % ethanol and mixing, then centrifuged for 15 min at 7500 g and 4 °C. All supernatants were removed, and the tubes were inverted onto paper tissue and allowed to dry in a fume hood at room temperature for 15 minutes, then resuspended with 20 µL of sterile distilled water and kept at -20 ºC in an ultra-freezer before sequencing. The purified fragment was checked on a 1.5% Agarose gel at 110 V for about 1 hour, as previously, to confirm the presence of the purified product and quantified using a NanoDrop (Model: 2000, Thermo Scientific).

The amplified fragments were sequenced using a Genetic Analyser (3130xl sequencer from Applied Biosystems) following the manufacturer's manual with the BigDye Terminator v3.1 cycle sequencing kit. All genetic analyses were performed using BioEdit and MEGA 6 software. Fig. 1 shows the Phylogenetic tree based on 16S rRNA gene sequences, illustrating the placement of isolates SmK (*K. pneumoniae*) and SmP (*P. mirabilis*) relative to reference strains.

>SmK *Klebsiella pneumoniae*

GGGGCGGTAGCACAGAGAGCTTGCTCCCGGGTGACGAGCGGCGGACGGGTGAGT
AATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAAT
ACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGA
TGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATC
CCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAG
GAAGGCGATGAGGTTAATAACCTCGTCGATTGACGTTACCCGCAGAAGAAGCACC
GGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCC
GGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGG
GGTAGAATTCCAGGTGTAGGGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTG
GCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGT
TGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGA

GTACGGCCGCAAGGTTAAAACCCAAATGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCA
CAGAACTTTCCAGAGATGCATTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTATTT

>SmP *Proteus mirabilis*
AAGAGCGGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTG
AATAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTA
ATACCGCATAATGTCTACGGACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGG
ATGAACCCATATGGGATTAGCTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGAT
CTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA
CTCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
CCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAG
GAAGGTGATAAGGTTAATACCCTTGTCAATTGACGTTACCCGCAGAAGAAGCACCG
GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAGGCGTTAATCGGAA
TTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGAAAGCCCCG
AGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGG
GTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTAGAGGTTG
TGGTCTTAACCGTGGCTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTAC
GGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGATGCAATGCGAAGAACCTTACCTACTCTTGACATCCAGCG
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CTTACCC

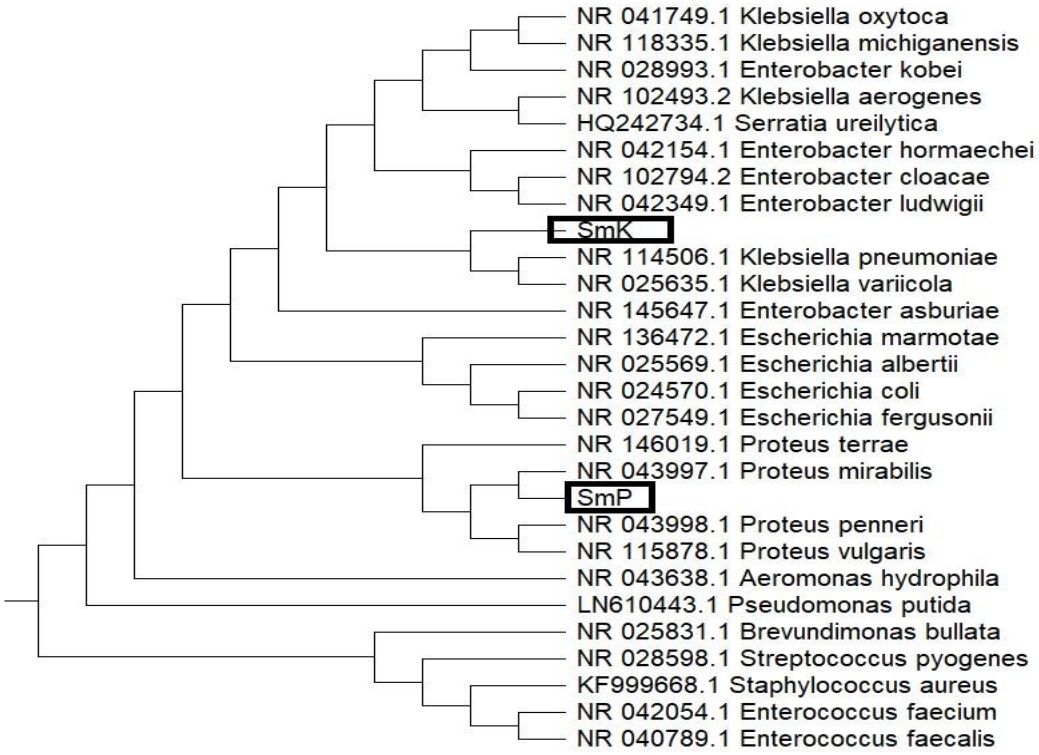


Fig. 1 Phylogenetic tree of the placement of isolates SmK (*K. pneumoniae*) and SmP (*P. mirabilis*) relative to reference strains based on 16S rRNA gene sequences.

Rearing and Colony Maintenance of Experimental Mosquito

The research was carried out at the Centre for Genetic Engineering and Biotechnology, Federal University of Technology, Minna, Nigeria. Day-old larvae of the test mosquito species (*Ae. aegypti, An. gambiae* s.l., and *Cx. quinquefasciatus*) were obtained from laboratory colonies maintained in the Entomology Laboratory, Department of Animal Biology, at the same institution. The mosquito colonies were kept under standard insectary conditions: 28.00±2.00 °C, 86.00 % relative humidity, and a 12:12 light-dark cycle for temperature, humidity, and photoperiod, respectively. Wild stocks were regularly introduced to the colonies to prevent inbreeding depression.

To ensure that there were no elevated resistance markers associated with prior insecticide exposure in the colony, detoxification enzyme activity profiles of the fourth larval instar were assessed [31, 32]. All experimental assays included two controls (a negative and a positive control). The negative control group consisted of larvae reared in sterile distilled water without adding bacterial metabolites or nutrient agar (NA). The positive control group consisted of larvae reared in sterile distilled water with 1 %v/v nutrient agar but no bacterial metabolites. These controls distinguished baseline enzymatic responses, effects from nutritional supplementation, and those specifically caused by bacterial secondary metabolites.

Larval Exposure to Bacterial Metabolites

The larvae were reared in sterile distilled water, until late 3rd and early 4th instar larvae, at the rate of 250 larvae/bowl in 1000 mL of the prepared media in plastic trays (30 cm X 25 cm X 5 cm) and fed standardised diet of finely pulverised fish feed (Coppens®) at a rate of 0.32 mg/larva, sprayed gently onto the surface of the rearing trough [33].

Twenty-five (25) late 3rd and early 4th instar larvae of each mosquito species (*Aedes aegypti*, *Anopheles gambiae* s.l., and *Culex quinquefasciatus*) were exposed separately to either of two lethal concentrations (7.5 or 10.0 %v/v) of secondary metabolites of *K. pneumoniae* and *P. mirabilis,* separately for 72 hours. Feed was presented to all experimental setups (exposed and controls) after 24-hour exposure [34]

The two test concentrations were prepared by diluting the bacterial supernatants in sterile distilled water to obtain 100 mL solutions. Briefly, to obtain 7.5 and 10.0 %v/v test solutions, 7.5 and 10.0 mL of the supernatant were added to 92.50 and 90.00 mL of distilled water, respectively. These concentrations were obtained from a preliminary screening of concentrations (1.0, 2.5, 5, 7.5, 10, and 15 %v/v) of the supernatants following standard protocol [34]. Each test concentration was replicated 8 times, resulting in 200 mosquito larvae per concentration and per monitored mosquito species.

Determination of Activities of Detoxification Enzymes

The exposed and dead mosquito larvae (pools of 5 per replicate) from treatment groups (7.5 or 10.0 %v/v) were analysed by exposure concentration and species. For each replicate, mosquitoes were homogenised in 900 µL of phosphate buffer (0.0625 M potassium phosphate, pH 7.2) using a Pellet Pestle Motor (Kontes Anachem, Mettler Toledo, Luton, Bedfordshire, UK). Homogenates were centrifuged at 10,000 rpm for 10 minutes at 4 °C using a refrigerated centrifuge (Eppendorf Centrifuge 5417R, Motor Parkway, New York, USA). The resulting supernatant served as the crude enzyme extract for subsequent bioassays. Protein concentrations were determined using the Bradford method [35] to normalise enzyme activity.

Esterase activity was measured according to the established protocols and procedures [31], with slight modifications. Briefly, 20 µL of mosquito homogenate was combined with 200 µL of α/β-naphthyl acetate working solution (prepared by diluting 1 mL of 30 mM 1-naphthyl acetate in 99 mL of potassium phosphate buffer, pH 7.2) in microplate wells. Plates were incubated at room temperature for 15 minutes, after which 50 µL of Fast Blue B stain solution was added to each well. Absorbance was read at 570 nm using a Modulus Microplate Reader (Turner Biosystems, Sunnyvale, California, USA). Esterase activity was quantified by comparing absorbance values against standard curves generated with known concentrations of α/β-naphthol. Results were expressed as µmol of product formed per minute per mg protein (µmol/min/mg protein).

Glutathione-S-Transferase (GST) activity was determined following established protocols [31]. In microplate wells, 10 µL of the mosquito homogenate was combined with 200 µL of reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) working solution (prepared by mixing 125 µL of 63 mM CDNB with 2.5 mL of 10 mM GSH solution). The reaction was monitored kinetically at 340 nm for 5 minutes. Blanks were prepared by substituting the homogenate with phosphate buffer. GST activity was expressed as µmol CDNB conjugated per minute per mg protein, using an extinction coefficient of 4.39 mM^{-1} at 340 nm, corrected for path length. Values were normalised to protein content to yield standardised activity units (µmol/min/mg protein). All enzyme assays were performed in triplicate for each treatment group.

Data Analysis

All experimental data were curated, organised, and cleaned in Microsoft® Excel 2019 (Microsoft®, Redmond, WA, USA). Statistical analyses were subsequently performed using R statistical software (version 4.5.3) and RStudio (version 2026.01.2+418) [36]. The Shapiro-Wilk test was used to assess the normality of the data obtained for larval mortality and the enzyme bioassay. The *stats* package was used to test variance homogeneity using Bartlett's

test (for normally distributed data) or the Fligner-Killeen test (for non-normally distributed data).

Descriptive statistics (mean, standard deviation, and range) were applied to summarise key trends in the data using the *BiodiversityR* package [37]. Inferential statistical comparisons in larval mortality and enzyme activity differences between Day 3 and Day 8 metabolites and mosquito cohorts were conducted using Welch's t-test, with significance set at $p < 0.05$. Larval mortality data were subjected to probit analysis for LC_{50}/LC_{90} estimation. Enzyme activity values were reported as mean ± standard deviation of the mean (SD). Results were expressed as mean ± standard deviation. Visualisations of larval mortality (time-series plots) were done in R using the *ggplot2* [38] and *plotly* [39] packages.

Results

Larvicidal activity of the metabolites of bacterial species after 3 and 8 days of incubation period against three mosquito genera

Figs 2 and 3 highlight the larvicidal efficacy of metabolites derived from *K. pneumoniae* (NCUKP3) and *P. mirabilis* (NCUPM4) against three mosquito species (*An. gambiae* s.l., *Ae. aegypti*, and *Cx. quinquefasciatus*) following 3 (D_3) and 8 (D_8) days of bacterial incubation. Independent cohorts of mosquito larvae were exposed to each metabolite (D_3 and D_8) separately for 72 hours.

The crude metabolites of *K. pneumoniae* (NCUKP3) demonstrated pronounced incubation-dependent activity (Fig 2). In *Cx. quinquefasciatus*, larval mortality increased significantly ($p < 0.0001$) from 26.88±10.61 % (after exposure to D_3 metabolites) to 84.38±13.66 % (after exposure to D_8 metabolites) (Fig 2C). A similar pattern was observed in *Ae. aegypti* ($p < 0.0001$) and *An. gambiae* s.l. mosquitoes; with mortality rising respectively from 52.92±12.52 to 82.29±14.90 % (Fig 2B) and 73.95±13.75 to 81.25±14.94 % (Fig 2A).

The metabolite of *P. mirabilis* (NCUPM4) did not elicit a significant increase in larvicidal activity across most mosquito species on both days (D_3 and D_8) of metabolite incubation. Mortality in *Cx. quinquefasciatus* (0.33±1.05 % vs. 1.91±3.46 %; $p = 0.20$) (Fig 3C) and *An. gambiae* s.l. (5.15±4.17 % vs. 8.40±7.82 %; $p = 0.14$) (Fig 3A). Only in *Ae. aegypti,* was larval mortality significantly increase from 1.91±3.46% (in D_3) to 11.24±6.21% (in D_8) ($p = 0.00068$) (Fig 3B).

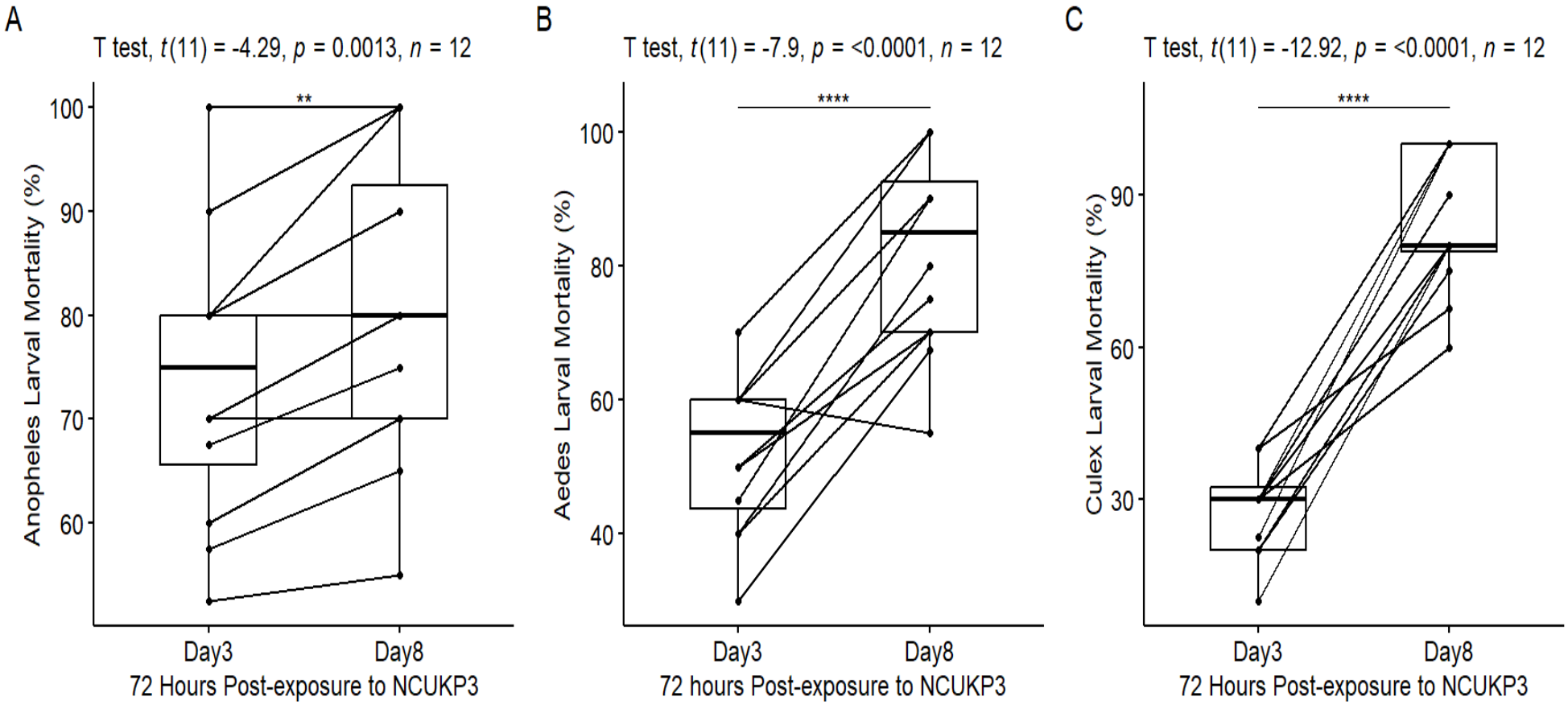


Fig 2: Larvicidal activity of the metabolites of NCUKP3 (*K. pneumoniae*) after 3- and 8-day incubation periods against three mosquito genera. NCUKP3 metabolites produced significant, incubation-dependent increases in mortality, particularly in *Cx. quinquefasciatus* and *Ae. aegypti*, while *An. gambiae* showed high early susceptibility that did not progress further.

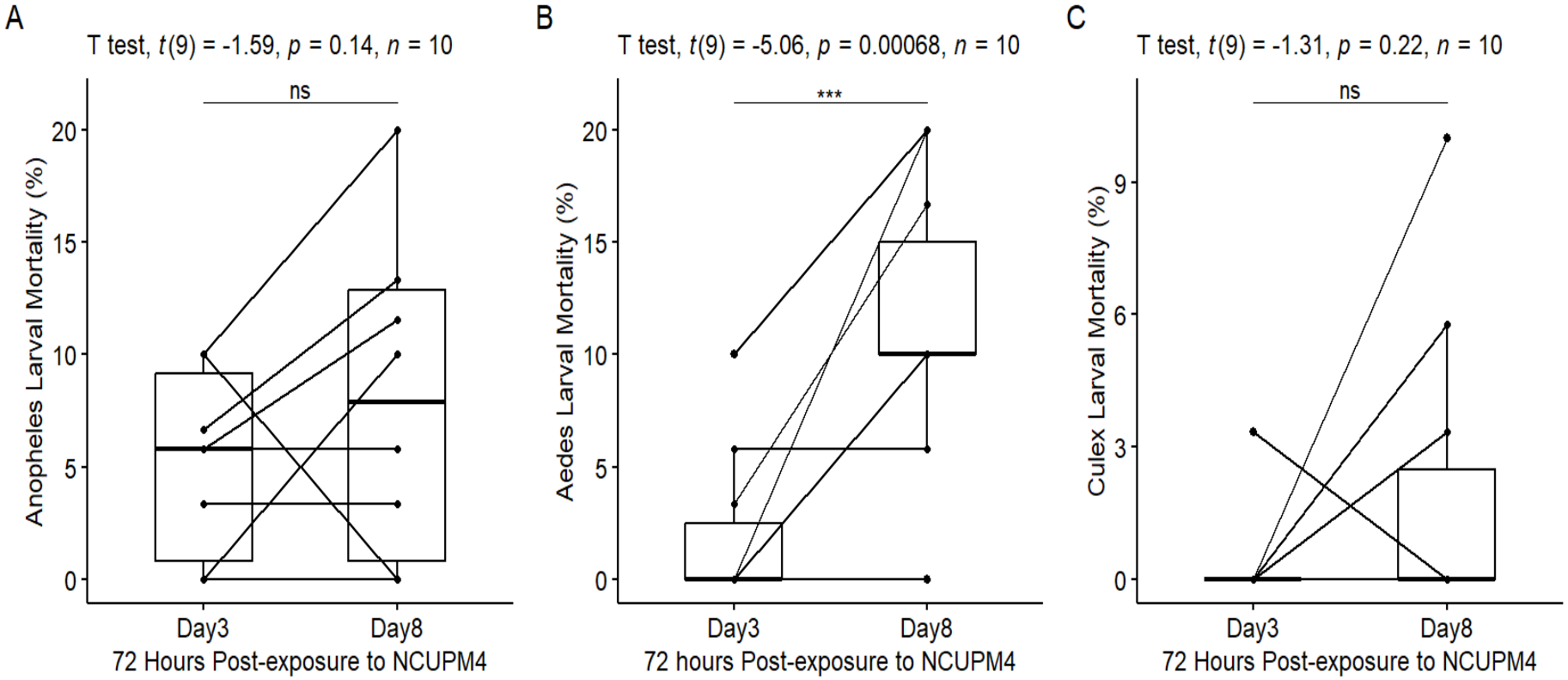


Fig 3 Larvicidal activity of the metabolites of NCUPM4 (*P. mirabilis*) after 3- and 8-day incubation periods against three mosquito genera. NCUPM4 metabolites induced negligible mortality across species, with only a modest effect observed in *Ae. aegypti*.

Time-mortality response of Mosquito larvae to NCUKP3 and NCUPM4 metabolites after 72 hours (4320 minutes) post-exposure

Across all three mosquito species, NCUKP3 metabolites demonstrated strong, time-dependent larvicidal activity that was enhanced by longer incubation and higher concentration**,** while NCUPM4 metabolites consistently lacked efficacy. In *Cx. quinquefasciatus* (Fig. 4), NCUKP3 metabolites displayed clear incubation- and concentration-dependent larvicidal activity. At 10.0 %v/v concentration after 8 days of incubation, mortality increased progressively with exposure time, reaching nearly 80 % by 2880 minutes (48 hours), whereas the 7.5 %v/v metabolite after 3 days produced only ~30 % mortality before plateauing. In contrast, NCUPM4 metabolites at 10.0 %v/v concentration, whether incubated for 3 or 8 days, induced negligible mortality, remaining close to zero throughout the exposure period (Fig. 4). Peak larval mortality of the D_8 metabolite was elicited after 18 hours of exposure.

In *An. gambiae* (Fig. 5), NCUKP3 again showed strong time-dependent efficacy, with the 10.0 %v/v metabolite after 8 days producing sustained increases in mortality over time, while the 7.5 %v/v metabolite after 3 days yielded moderate but lower mortality. Both metabolites (D_3 and D_8) elicited their highest mortality after 48 hours of exposure (2880 minutes). NCUPM4 metabolites, regardless of incubation period, failed to elicit meaningful larvicidal effects, with mortality remaining minimal across the exposure duration. Peak mortality of the D_3 metabolite of NCUPM4 was elicited after 12 hours of exposure, while the same feat for the D_8 metabolite was after 48 hours (Fig. 5).

In *Ae. aegypti* (Fig. 6), NCUKP3 metabolites at 10.0 %v/v concentration were effective, with the 8-day incubation (D_8) producing higher and more progressive mortality compared to the 3-day (D_3) incubation. Both metabolites elicited peak larval mortality after 48 hours post-exposure. By contrast, NCUPM4 metabolites at 10.0 %v/v concentration showed negligible activity under both incubation conditions, with mortality values remaining close to baseline. Peak mortality of the D_3 metabolite was elicited after 12 hours of exposure, and 24 hours of exposure for the D_8 metabolite (Fig. 6).

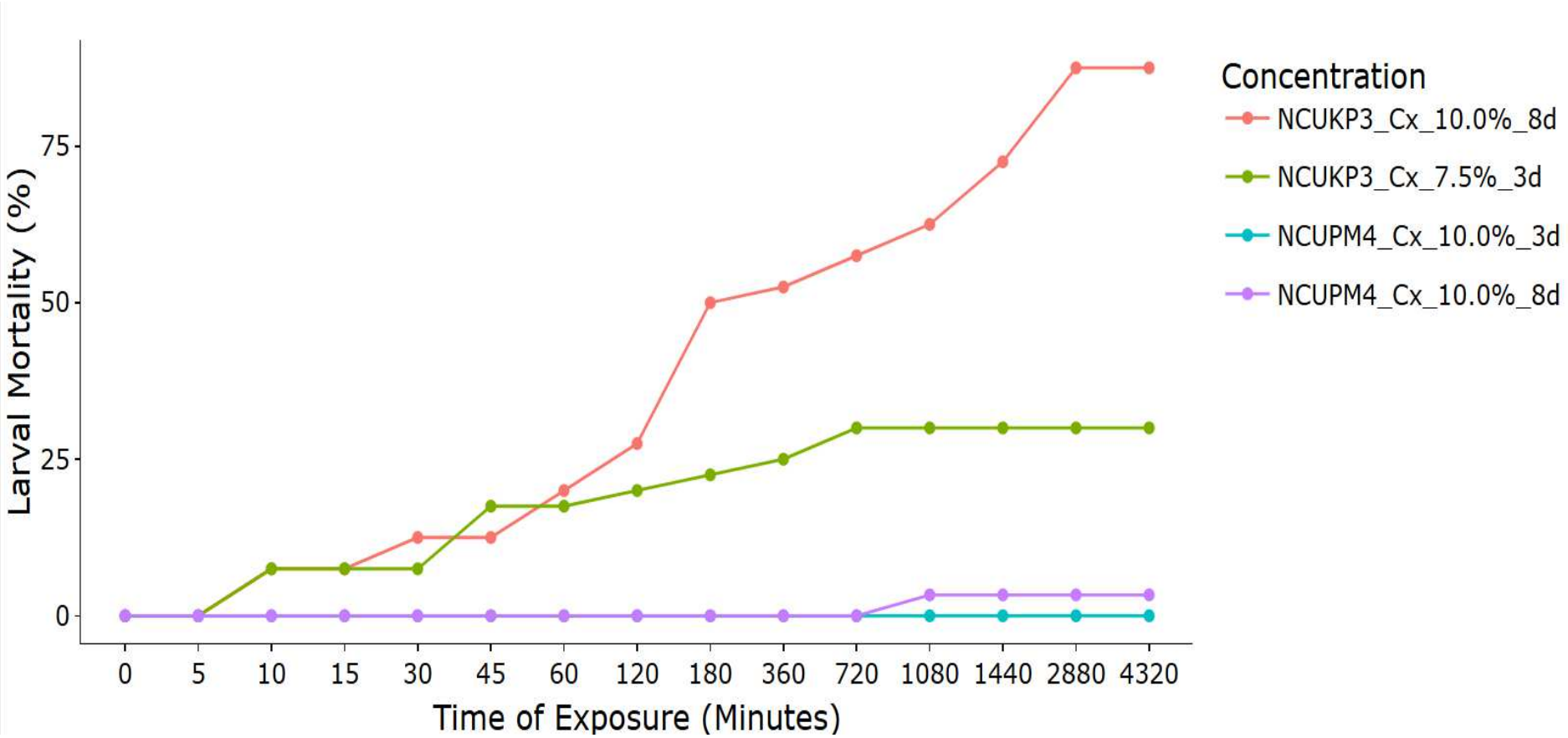


Fig. 4 Time-mortality response of *Cx. quinquefasciatus* larvae to NCUKP3 and NCUPM4 metabolites after 72 hours (4320 minutes) post-exposure. NCUKP3 = *K. pneumoniae*, NCUPM4 = *P. mirabilis*. NCUKP3 showed strong incubation- and concentration-dependent activity, with the 10.0% metabolite at Day 8 producing progressive mortality up to ~80%, while the 7.5% metabolite at Day 3 plateaued at ~30%. NCUPM4 metabolites at 10.0% concentration, whether incubated for 3 or 8 days, induced negligible mortality

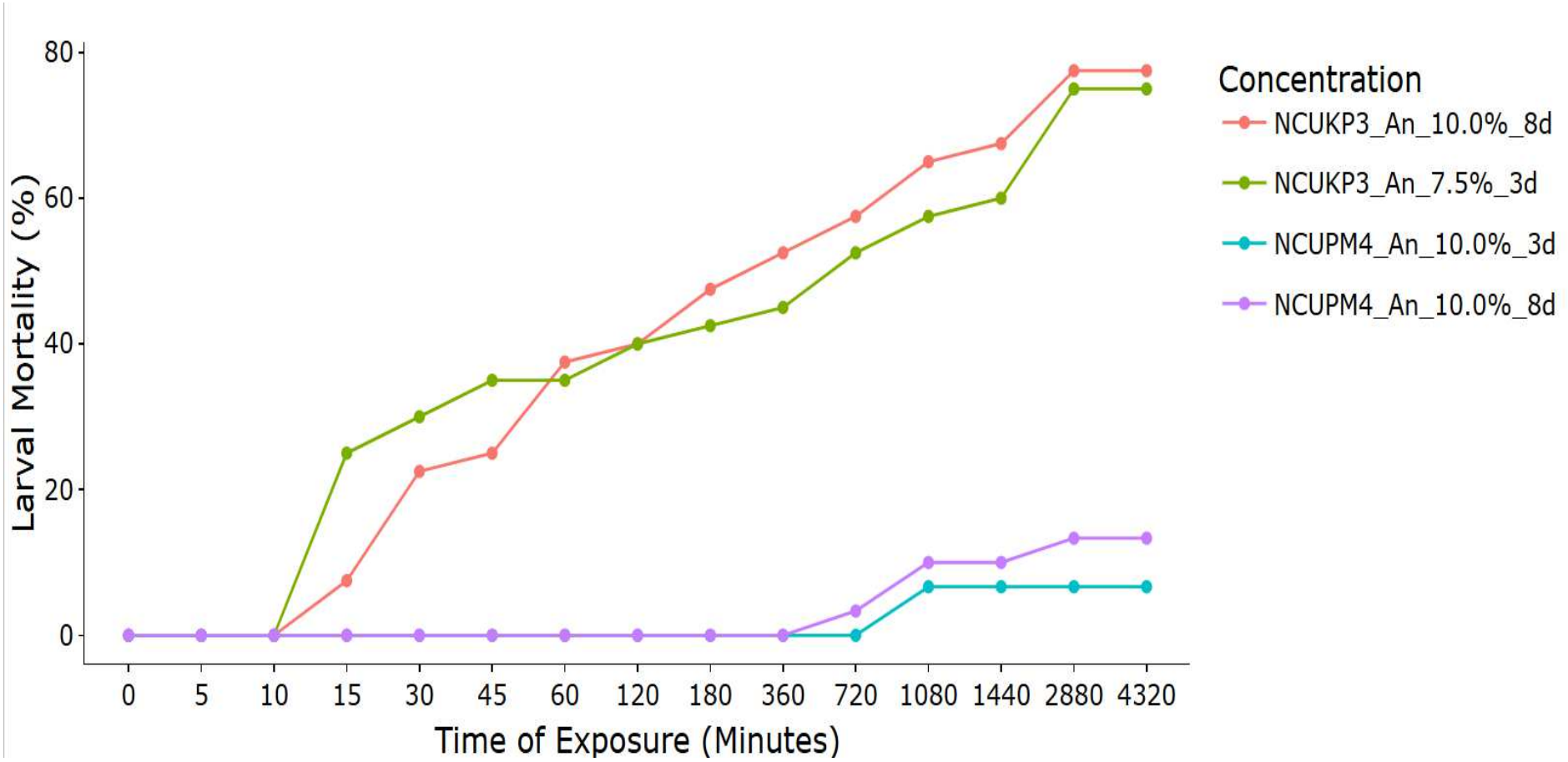


Fig. 5 Time-mortality response of *An. gambiae s.l.* larvae to NCUKP3 and NCUPM4 metabolites after 72 hours (4320 minutes) post-exposure. NCUKP3 = *K. pneumoniae*, NCUPM4 = *P. mirabilis*. NCUKP3 metabolites produced sustained increases in mortality, particularly at 10.0% after 8 days of incubation, while the 7.5% metabolite at Day 3 yielded moderate effects. NCUPM4 metabolites remained ineffective across both incubation periods, with mortality values close to baseline

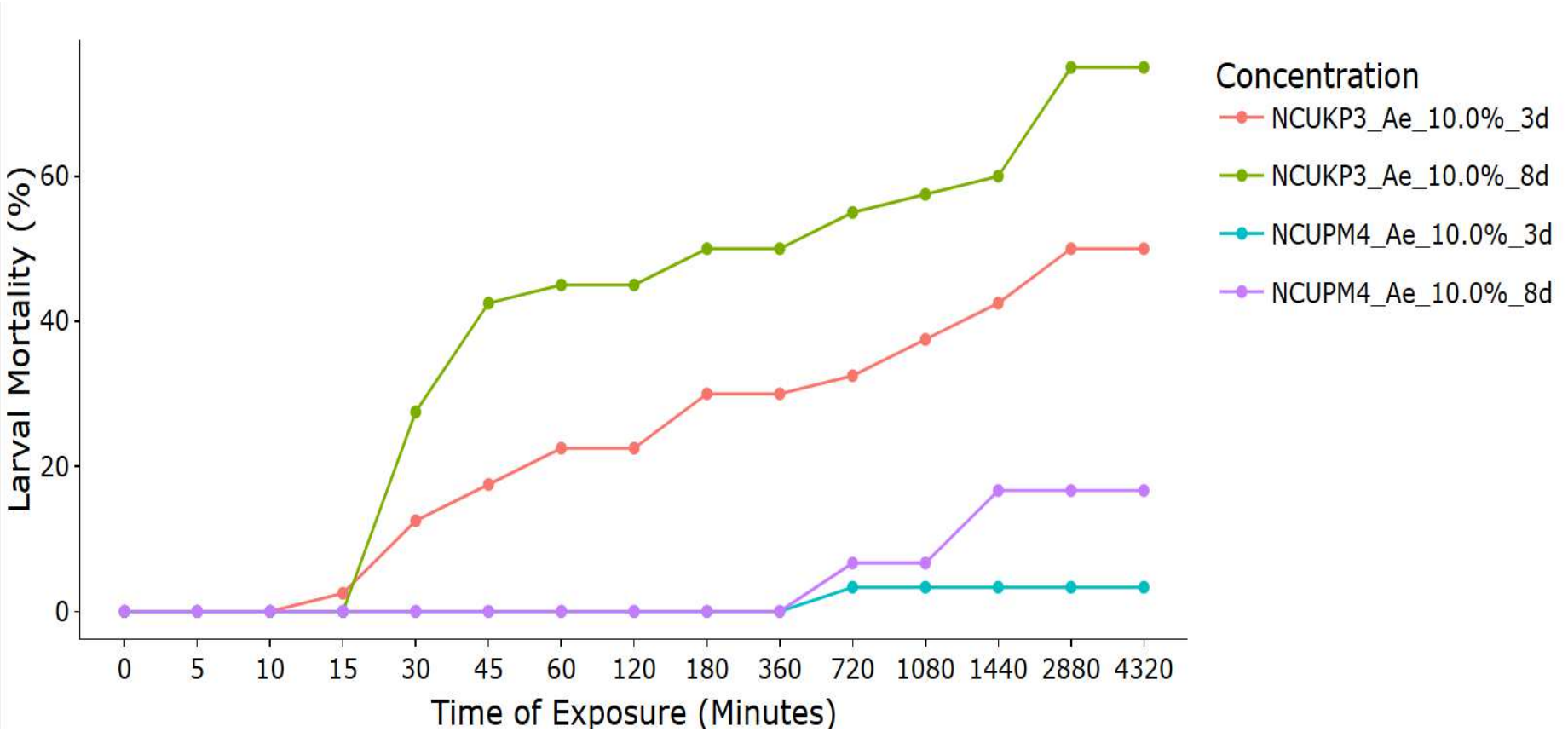


Fig. 6 Time-mortality response of *Ae. aegypti* larvae to NCUKP3 and NCUPM4 metabolites after 72 hours (4320 minutes) post-exposure. NCUKP3 = *K. pneumoniae*, NCUPM4 = *P. mirabilis*. NCUKP3 metabolites at 10.0% concentration were effective, with the 8-day incubation producing higher and more progressive mortality compared to the 3-day incubation. NCUPM4 metabolites at 10.0% concentration showed negligible activity under both incubation conditions, with mortality values remaining near zero

Lethal Concentrations (LC) of the larvicidal activity of the metabolites of bacterial species against mosquito larvae after 72 hours post-exposure

The lethal concentration values revealed clear differences in potency between NCUKP3 (*K. pneumoniae*) and NCUPM4 (*P. mirabilis*) metabolites after 8 days of incubation. Across species, *Cx. quinquefasciatus* was most resistant to NCUPM4 (highest LC values), while *Ae. aegypti* and *An. gambiae* showed relatively lower thresholds, though still far less susceptible compared to NCUKP3 (Table 2).

NCUKP3 exhibited consistently low LC_{50} values across all mosquito species (2.47–2.55 %v/v), with LC_{90} values ranging from 4.19 – 4.26 %v/v (Table 2). NCUPM4, LC_{50} values ranged from 6.89 %v/v in *Ae. aegypti* to 31.33 %v/v in *Cx. quinquefasciatus*, while LC_{90} values extended up to 55.32 %v/v. The regression equations had much shallower slopes (≈1.67–8.33x), underscoring weak potency and slower mortality response (Table 2).

Table 2: Lethal concentrations (LC_{50} and LC_{90}) of NCUKP3 (*K. pneumoniae*) and NCUPM4 (*P. mirabilis*) metabolites after 8 days of incubation against larvae of *Cx. quinquefasciatus*, *An. gambiae s.l.*, and *Ae. aegypti* following 72-hour exposure.

Bacteria Metabolites	Mosquito Species	Lethal concentration after 8 days of incubation			
		LC_{50} (%v/v)	LC_{90} (%v/v)	R^2	Regression Equation
NCUKP3	*Cx. quinquefasciatus*	2.47	4.19	0.77	y = 23.25x - 7.5
	An. gambiae s.l.	2.55	4.26	0.82	y = 23.50x - 10
	Ae. aegypti	2.53	4.21	0.82	y = 23.75x - 10
NCUPM4	*Cx. quinquefasciatus*	31.33	55.32	0.75	y = 1.67x - 2.22
	An. gambiae s.l.	8.67	14.67	0.92	y = 6.67x - 7.78
	Ae. aegypti	6.89	11.69	0.96	y = 8.33x - 7.44

NCUKP3 = *K. pneumoniae*, NCUPM4 = *P. mirabilis.* NCUKP3 metabolites exhibited consistently low LC values across all species, indicating strong potency at low concentrations, while NCUPM4 metabolites required much higher concentrations to achieve comparable mortality, with *Cx. quinquefasciatus* showing the greatest resistance

Effects of the metabolites of Gram-negative bacterial species on the resistance enzyme profile of larval instars of mosquitoes

Esterase Activity

The esterase activity responses differed markedly depending on the bacterial metabolite and mosquito species tested. In NCUKP3, larvae exposed to Day 8 metabolites consistently showed significantly ($p < 0.05$) lower esterase activity compared to those exposed to Day 3 metabolites. This pattern was observed across all species (*Ae. aegypti*: 72.90 → 68.60, $t(7) = 5.94$, $p = 0.0057$, $n = 10$; *An. gambiae* s.l.: 91.50 → 76.60, $t(5.57) = 20.37$, $p < 0.001$, $n = 10$; *Cx. quinquefasciatus*: 93.60 → 87.80, $t(6.51) = 7.05$, $p = 0.00028$, $n = 10$) (Table 3).

In contrast, larvae exposed to Day 8 metabolites of NCUPM4 consistently showed significantly higher esterase activity ($p < 0.05$) than Day 3 cohorts (*Ae. aegypti*: 157.77 → 166.26, $t(6.62) = -10.46$, $p < 0.0001$, $n = 10$; *An. gambiae*: 151.63 → 158.29, $t(4.71) = -8.2$, $p = 0.00058$, $n = 10$; *Cx. quinquefasciatus*: 166.74 → 174.69, $t(7.93) = -6.25$, $p = 0.00026$, $n = 10$) (Table 3).

Glutathione-S-Transferase (GST)

The GST enzyme activity responses varied depending on the bacterial metabolite and mosquito species tested. For most species, GST activity did not differ significantly ($p > 0.05$) between larvae exposed to Day 3 and Day 8 metabolites of NCUKP3 (*Ae. aegypti*: 0.42 → 0.41, $t(8) = 1.25$, $p = 0.25$, $n = 10$; *An. gambiae*: 0.45 → 0.43, $t(8) = 1.43$, $p = 0.19$, $n = 10$. However, in *Cx. quinquefasciatus*, GST activity was significantly ($p < 0.05$) reduced (0.46 → 0.36, $t(7.42) = 5.52$, $p = 0.00073$, $n = 10$) (Table 4).

Larvae exposed to Day 8 metabolites of NCUPM4, in contrast, showed significantly higher GST activity ($p < 0.05$) compared to Day 3 cohorts. *An. gambiae* increased from 0.49 → 0.59 ($t(4.59) = -5.24$, $p = 0.0043$, $n = 10$), and *Cx. quinquefasciatus* from 0.41 → 0.44 ($t(6.55) = -2.98$, $p = 0.022$, $n = 10$) (Table 4).

Table 3: Esterase activities in mosquito larvae exposed to lethal concentrations of *K. pneumoniae* and *P. mirabilis* metabolites incubated for 3 and 8 days

Metabolite	Mosquito Species	Esterase activity (µmol/min/mg protein) Day 3	Day 8
NCUKP3	***Ae. aegypti***	72.90±0.90^{b*}	68.60±1.34^{a}
	***An. gambiae* s.l.**	91.50±1.49^{b}	76.60±0.67^{a}
	Cx. quinquefasciatus	93.60±1.60^{b}	87.80±0.95^{a}
NCUPM4	***Ae. aegypti***	157.77±0.95^{a}	166.26±1.55^{b}
	***An. gambiae* s.l.**	151.63±1.77^{a}	158.29±1.60^{b}
	Cx. quinquefasciatus	166.74±0.62^{a}	174.69±2.08^{b}

*Mean values with the same superscript alphabet in a row for a mosquito species are not significantly ($p > 0.05$) different. NCUKP3 = *K. pneumoniae*, NCUPM4 = *P. mirabilis.* Larvae exposed to NCUKP3 metabolites showed significantly lower esterase activity on Day 8 compared to Day 3 cohorts, indicating suppression of detoxification enzymes. In contrast, NCUPM4 metabolites induced significantly higher esterase activity in Day 8 cohorts across all species, consistent with enhanced detoxification and reduced larvicidal efficacy.

Table 4: Glutathione-S-transferase (GST) activities in mosquito larvae exposed to larvicidal concentrations of *K. pneumoniae* and *P. mirabilis* metabolites incubated for 3 and 8 days.

Metabolite	Mosquito Species	GST activity (µmol/min/mg protein) Day3	Day8
NCUKP3	***Ae. aegypti***	0.42±0.22^{a}*	0.41±0.02^{a}
	***An. gambiae* s.l.**	0.45±0.02^{a}	0.43±0.02^{a}
	Cx. quinquefasciatus	0.46±0.03^{b}	0.36±0.02^{a}
NCUPM4	***Ae. aegypti***	0.43±0.02^{a}	0.42±0.04^{a}
	***An. gambiae* s.l.**	0.49±0.04^{a}	0.59±0.01^{b}
	Cx. quinquefasciatus	0.41±0.02^{a}	0.44±0.01^{b}

*Mean values with the same superscript alphabet in a row for a mosquito species are not significantly ($p > 0.05$) different. NCUKP3 = *K. pneumoniae*, NCUPM4 = *P. mirabilis.* NCUKP3 metabolites produced little change in GST activity across most species, except for a significant suppression in *Cx. quinquefasciatus*. In contrast, NCUPM4 metabolites induced significantly higher GST activity in *An. gambiae* and *Cx. quinquefasciatus* Day 8 cohorts, consistent with enhanced detoxification and reduced larvicidal efficacy.

Correlation between Larval Mortality and Detoxifying Enzyme Profiles in *Ae. aegypti*

Correlation analysis revealed a strong negative association between esterase activity (EST_D3) and mortality in *Ae. aegypti* following exposure to day-3 metabolites (r = –0.85). In contrast, glutathione S-transferase activity (GST_D3) showed only a weak, non-significant positive correlation with mortality (*r* = 0.12) (Fig 7A).

Analyses also revealed an extremely strong negative correlation (*r* = -0.97) between esterase activities on Day 8 and larval mortality due to Day 8 metabolites (Fig 7B). A moderate negative correlation (*r* = –0.54) was obtained between Glutathione S-Transferase activity due to Day 8 metabolites and larval mortality (Fig 7B).

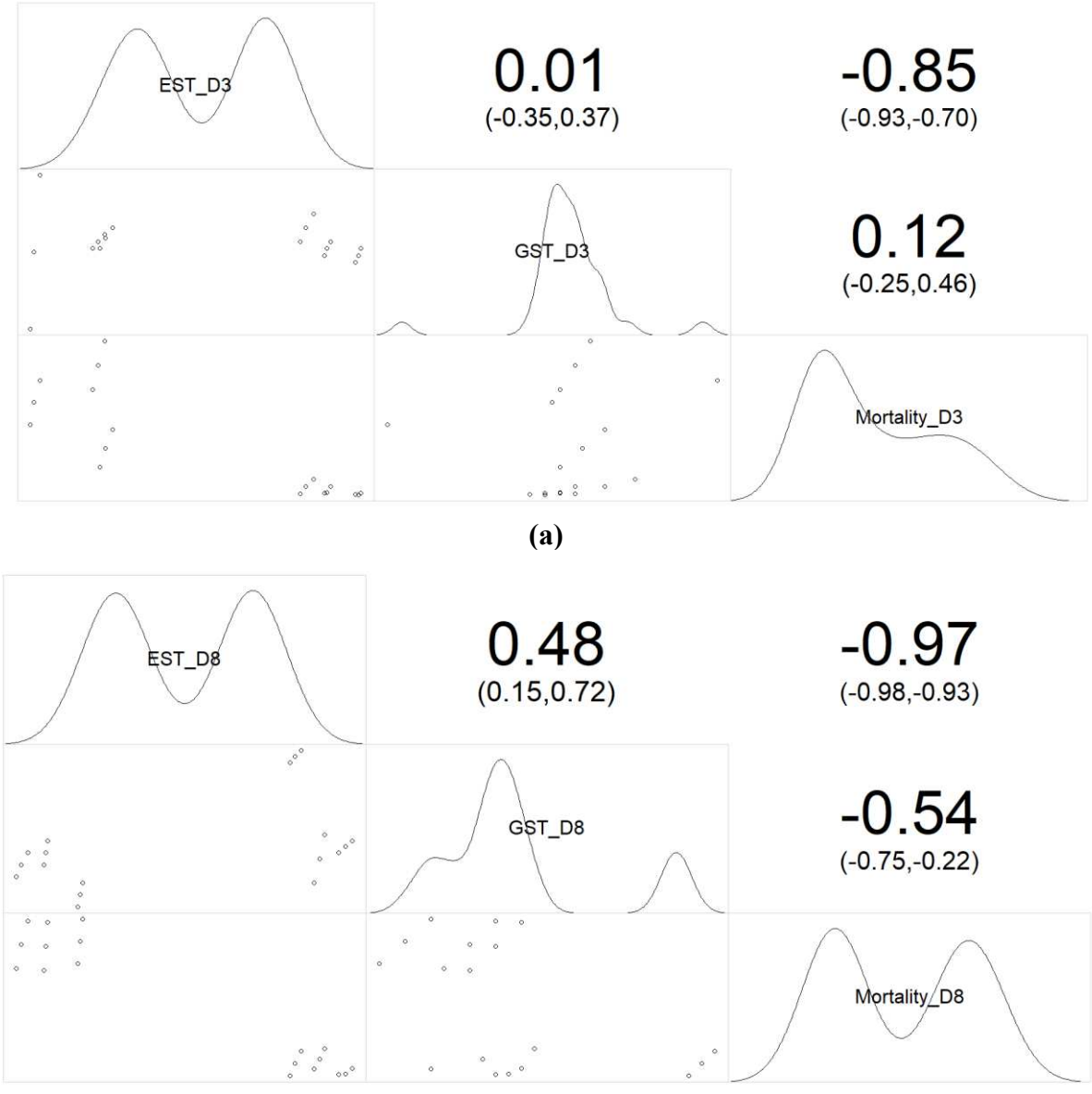


Fig. 7. Correlation between mortality and detoxifying enzyme profiles of *Ae. aegypti* after exposure to the metabolites after (a) Day 3 and (b) Day 8 of incubation. EST = Esterase, GST = Glutathione-S-Transferase, D3= Day 3 metabolites, D8 = Day 8 metabolites. (a) Strong negative correlation (r ≈ –0.85) between esterase activities and mortality due to Day 3 metabolites, and weak, non-significant correlation (r ≈ 0.12) between Glutathione-S-Transferase and larval mortality due to Day 3 metabolites. (b) Extremely strong negative correlation (r ≈ –0.97) between esterase activities and mortality due to Day 8 metabolites. Moderate negative correlation (r ≈ –0.54) between Glutathione-S-Transferase and larval mortality due to Day 8 metabolites

Discussion

The study observed differences in the larvicidal activities of the two bacterial metabolites tested. The metabolites of *K. pneumoniae* showed stronger larvicidal activity than those of *P. mirabilis,* and consistently elicited higher mortality across the three mosquito species (*Cx. quinquefasciatus*, *An. gambiae* s.l., and *Ae. aegypti*), with effects increasing at longer incubation times and higher concentrations. In contrast, *P. mirabilis* metabolites produced comparatively low mortality, even when tested at higher concentrations.

Across all mosquito genera, larval mortality increased with longer metabolite incubation. The higher toxicity of *K. pneumoniae* at Day 8 may reflect the accumulation of secondary metabolites during prolonged incubation, consistent with reports that extended culture periods enhance bacterial metabolite efficacy [40, 41]. The metabolites of *P. mirabilis* also showed greater toxicity at Day 8 compared to Day 3, though their effects varied among species: they were least effective against *Cx. quinquefasciatus* at both time points, more active against *An. gambiae* at Day 3, and most active against *Ae. aegypti* at Day 8.

The study provided evidence that larvicidal activity was associated with modulation of detoxification enzymes. *K. pneumoniae* metabolites were observed to suppress esterase activity and reduce GST activity in *Cx. quinquefasciatus*, suggesting possible inhibition of detoxification pathways that may compromise the larvae's ability to metabolise xenobiotics [15]. In contrast, *P. mirabilis* metabolites tended to induce esterase and GST activity in Day 8 cohorts, a pattern consistent with enhanced detoxification capacity and reduced larvicidal effect. Regression analyses reflected these differences, with steeper slopes for *K. pneumoniae* indicating more rapid increases in mortality, while slopes for *P. mirabilis* were comparatively shallow.

The ecological relevance of these findings lies in the potential use of *K. pneumoniae* metabolites as biocontrol agents. Their activity across three major mosquito species suggests possible inclusion in integrated larval management programs. By suppressing detoxification enzymes, *K. pneumoniae* metabolites may help limit rapid resistance development, which is a common challenge with chemical insecticides [18]. In contrast, *P. mirabilis* metabolites were associated with enzyme induction, raising concerns that they could enhance larval resilience not only to these metabolites but potentially to other control agents. These observations highlight the importance of mechanistic screening to ensure that microbial products selected for vector control do not inadvertently promote cross-resistance [42].

Species-specific responses also carry ecological importance. For example, *An. gambiae* exhibited marked esterase suppression under *K. pneumoniae* metabolite exposure, whereas *Cx. quinquefasciatus* showed strong GST induction with *P. mirabilis*. Such variability highlights the need to tailor interventions to local vector populations, as metabolic responses may differ across ecological contexts [43]. Previous studies on bacterial metabolites have reported variable larvicidal efficacy, often linked to differences in secondary metabolite composition. Work on *B. t. israelensis* (Bti) and *L. sphaericus* has demonstrated enzyme inhibition as a mechanism of toxicity [5, 42]. The suppression of esterase and GST activities by *K. pneumoniae* metabolites is consistent with these findings, supporting the role of detoxification enzyme inhibition in microbial larvicides. In contrast, the enzyme induction observed with *P. mirabilis* metabolites resembles reports of sublethal microbial exposures that enhance detoxification capacity and thereby reduce efficacy [15].

The metabolites of *K. pneumoniae* demonstrated notable larvicidal activity, positioning them as potential candidates for environmentally sustainable mosquito control. Their relatively low lethal concentration thresholds suggest they may pose less ecological risk compared to synthetic insecticides. Incorporating *K. pneumoniae* metabolites into integrated vector management (IVM) strategies could complement existing approaches, reduce reliance on chemical insecticides, and help slow the development of resistance [6].

The findings of this study caution against indiscriminate use of microbial metabolites. The enzyme-inducing effects observed with *P. mirabilis* raise concerns about inadvertently strengthening mosquito detoxification systems. Rigorous screening of microbial candidates is therefore important before field application [44]. While the study provides useful mechanistic and ecological insights, several limitations should be noted. First, the assays were conducted under laboratory conditions, which may not fully capture environmental variability such as temperature, nutrient availability, or microbial interactions. Second, the specific metabolites responsible for enzyme modulation were not identified, highlighting the need for metabolomic profiling to pinpoint active compounds. Third, long-term ecological impacts, including potential effects on non-target organisms, remain to be evaluated.

Future research should include exploring metabolite characterisation to identify bioactive compounds in the *K. pneumoniae* metabolites. Field trials to assess efficacy under natural ecological conditions. Resistance studies to determine whether enzyme suppression by *K. pneumoniae* metabolites reduces the likelihood of adaptive responses, and synergistic applications combining *K. pneumoniae* metabolites with other biocontrol agents to enhance efficacy and sustainability.

Limitation

Despite the larvicidal efficacies of the crude extracts of these Gram-Negative bacteria, several limitations are acknowledged to avoid overgeneralisation of the findings. First, the study was conducted under controlled laboratory conditions, which may not fully reflect natural environmental settings where temperature, humidity, and microbial interactions vary. Only two metabolite concentrations (7.5 and 10.0 % v/v) were tested, limiting dose–response insights; however, higher dosage interactions may be extrapolated. The use of laboratory colonies, though supplemented with wild stocks, may not capture the genetic diversity of field populations of mosquitoes. Specific bioactive compounds responsible for toxicity were not identified. This, however, is a direction for future studies. Another limitation was that the study focused only on esterase and GST, while other detoxification pathways were not assessed. Non-target ecological impacts were not evaluated, and the short 72-hour exposure window may not have captured longer-term outcomes such as delayed mortality or adaptive responses.

Conclusion

The study revealed that the crude metabolite of *K. pneumoniae* exhibits notable larvicidal activity, accompanied by suppression of detoxification enzymes, while those of *P. mirabilis* tend to induce enzyme activity and are associated with lower mortality. These results suggest that modulation of detoxification pathways plays an important role in shaping larval susceptibility to microbial metabolites. The findings point to the potential of *K. pneumoniae* metabolites as candidates for integrated vector management, while also highlighting the need for caution in deploying microbial products that may enhance detoxification and reduce efficacy. Overall, the work contributes to understanding mosquito–microbial metabolite

interactions and provides a basis for further exploration of enzyme-targeted biocontrol strategies.

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