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Original Article

Mosquito Species-Specific Larvicidal Activity and Detoxification Enzyme Responses to Cell-Free Extract of *Bacillus subtilis* PZ896239

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ABSTRACT

Mosquitoes remain major vectors of malaria, dengue, yellow fever, and lymphatic filariasis, necessitating sustainable larval control strategies. This study evaluated the larvicidal activity of the cell-free extract (CFE) of *Bacillus subtilis* against *Aedes aegypti*, *Anopheles gambiae* s.l., and *Culex quinquefasciatus* larvae. The CFEs were obtained from the bacterial culture incubated for 3 and 8 days, and larval mortality was assessed using bioassays at two concentrations (7.5 and 10.0 %v/v) with five replicates of 25 larvae each. Enzyme activity assays were performed to measure esterase and glutathione S-transferase (GST) responses. Day-3 CFE produced low mortality (≤6.67 %), with *Cx. quinquefasciatus* slightly more affected. By Day 8, mortality increased significantly in *Ae. aegypti* (13.19 ± 7.96 %) and *Cx. quinquefasciatus* (15.64 ± 7.99 %), while *An. gambiae* s.l. remained less responsive. Probit analysis revealed low lethal concentrations, with LC_{50} values of 6.67 % (*Cx. quinquefasciatus*), 6.93 % (*Ae. aegypti*), and 8.83 % (*An. gambiae* s.l.). Enzyme assays showed significant esterase induction across all species, whereas GST activity increased in *Ae. aegypti* and *An. gambiae* s.l. but declined in *Cx. quinquefasciatus*. These findings demonstrated that though larval toxicity of the CFEs to the three mosquitoes was low, toxicity could be enhanced through identification and purification of active metabolites.

Keywords: *Bacillus subtilis,* Esterase, Glutathione S-transferase (GST), Integrated vector management, Vector control

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INTRODUCTION

Mosquitoes are among the most important vectors of human diseases, transmitting malaria, dengue, yellow fever, and lymphatic filariasis. These mosquito-borne illnesses remain a significant global health burden, particularly in tropical and subtropical regions where climatic conditions favour mosquito proliferation and disease transmission [1]. Conventional control strategies of these vectors of disease have relied heavily on chemical insecticides to control mosquito populations and reduce disease incidence [2]. However, the widespread and prolonged use of these chemicals has raised serious concerns, including environmental contamination, adverse effects on non-target organisms, and the rapid development of insecticide resistance in many mosquito populations [3]. These challenges have undermined the long-term effectiveness of chemical-based interventions and highlighted the urgent need for innovative, sustainable, and ecologically sound alternatives.

Microbial cell-free extracts (CFEs) and metabolites, particularly those produced by *Bacillus* species, have shown considerable promise as larvicidal agents. These microbial approaches not only reduce reliance on chemical insecticides but also mitigate the risk of resistance development, with minimal impact on non-target organisms [4], positioning them as vital tools in the sustainable fight against mosquito-borne diseases. *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* are already widely deployed in vector control programs, demonstrating high specificity and effectiveness against mosquito larvae while minimising harm to non-target organisms [3, 5]. These bacterial agents act through the production of crystal (Cry) and binary (Bin) toxins that disrupt larval midgut epithelial cells, leading to mortality.

Recent studies suggest that *B. subtilis* also produces bioactive compounds with larvicidal properties. For instance, CFEs derived from *B. subtilis* have been shown to significantly reduce survival rates of *Ae. aegypti* larvae, indicating potential as a novel biocontrol agent [6, 7]. More recent work has identified cresol derivatives from *B. subtilis* that modulate oviposition behaviour in *Cx. quinquefasciatus*, further expanding its potential role in integrated mosquito management [8]. Additionally, high-throughput screening pipelines have highlighted *B. subtilis* metabolites as candidates for non-live bacterial bioinsecticides, underscoring their relevance in next-generation mosquito control strategies [9].

Despite these promising findings, the efficacy and mode of action of *B. subtilis* metabolites remain less well characterised compared to Bti and *B. sphaericus*. While Cry and Bin toxins have well-established mechanisms, *B. subtilis* metabolites appear to act through diverse biochemical pathways, including interference with larval development, midgut disruption, and behavioural modulation [10]. Further research is needed to elucidate these mechanisms, optimise production, and assess field-level effectiveness. Nonetheless, this growing evidence positions *B. subtilis* as a promising complementary tool in microbial larval control, particularly in regions facing insecticide resistance and ecological concerns.

Understanding the biochemical responses of mosquito larvae to microbial metabolites and/or CFEs is essential for evaluating their potential in vector control. Detoxification enzymes such as esterases and glutathione S-transferases (GSTs) play critical roles in metabolising xenobiotics and mediating insecticide resistance [11]. These enzymes act as the first line of defence against toxic compounds, enabling larvae to survive in environments with chemical stressors. Elevated esterase activity is often associated with enhanced hydrolysis of organophosphates and carbamates, while GSTs contribute to the conjugation of reduced glutathione with electrophilic compounds, facilitating their excretion [12].

Assessing changes in these enzyme activities following exposure to microbial CFEs can provide mechanistic insights into species-specific susceptibility. For instance, exposure to *Bacillus thuringiensis israelensis* (Bti) toxins has been shown to alter GST activity in *Aedes aegypti* larvae, suggesting that microbial metabolites and/or CFEs may interact with detoxification pathways in ways distinct from conventional insecticides [13, 14]. Similarly, studies on fungal metabolites such as those produced by *Metarhizium anisopliae* indicate that larval mortality may be linked to oxidative stress and disruption of detoxification enzyme balance [15]. These findings highlight the importance of biochemical assays in evaluating microbial larvicides, as they not only confirm efficacy but also reveal potential resistance mechanisms and cross-tolerance effects [16].

By integrating biochemical profiling, especially detoxifying enzyme profiles, with larvicidal bioassays, the mechanism of efficacy of microbial larvicides and the identity of biomarkers of susceptibility would be better understood for the design of more effective vector control strategies. Such mechanistic insights are crucial for predicting long-term outcomes, especially in regions where insecticide resistance is widespread and alternative control measures are urgently needed. The present study aimed to evaluate the larvicidal activity of *B. subtilis* CFEs against three major mosquito vectors: *Ae. aegypti*, *An. gambiae* s.l., and *Cx. quinquefasciatus*. Specifically, the objectives were to: (i) determine mortality patterns following exposure to Day 3 and Day 8 CFEs, (ii) estimate lethal concentrations (LC_{50} and LC_{90}) using probit analysis, and (iii) assess changes in detoxification enzyme activity, focusing on esterases and glutathione S-transferases (GSTs). By integrating larvicidal efficacy with biochemical responses, this study provides insights into species-specific susceptibility and explores the potential of *B. subtilis* CFEs as microbial agents for integrated vector management.

MATERIALS AND METHODS

Mosquito Rearing

Larvae of *Ae. aegypti*, *An. gambiae* s.l., and *Cx. quinquefasciatus* were obtained from established laboratory colonies maintained under controlled insectary conditions in the Entomology Laboratory of the Department of Animal Biology, Federal University of Technology, Minna, Nigeria. The colonies were maintained at a temperature of 27 ± 2 °C with relative humidity

between 75–80%, under a 12:12 h light–dark cycle to mimic natural environmental rhythms [17].

Eggs were collected from oviposition cups and transferred into enamel trays containing distilled tap water. Upon hatching, larvae were reared in groups to minimise overcrowding and ensure uniform growth. The water was changed daily to prevent microbial contamination and maintain optimal oxygenation. Larvae were fed finely ground fish food in small quantities twice daily, ensuring adequate nutrition without formation of scum [18]. Larval development was monitored closely, and third instar larvae were selected for bioassays to ensure consistency across experiments. All rearing procedures adhered to WHO guidelines for mosquito larvicide testing, ensuring reproducibility and comparability of results.

Isolation of Bacteria

Water samples (0.1 mL) were collected from rain-pool mosquito breeding habitat within the Bosso Campus of the Federal University of Technology, Minna, Nigeria. The samples were aseptically spread onto 20 cm nutrient agar plates using a sterilised scalpel under laminar flow to minimise contamination [19]. Plates were incubated for 24 hours at 30 °C, and bacterial colonies were isolated according to established microbiological protocols [20]. 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.

Morphological Identification of *Bacillus subtilis*

Morphological identification of *Bacillus subtilis* was carried out using standard microbiological techniques [21]. Colony characteristics were first observed on nutrient agar plates after 24–48 hours of incubation at 30 °C. Colonies appeared circular, opaque, and cream-colored with irregular margins and a dry, rough surface texture, typical of *Bacillus* species. Microscopic examination was performed following Gram staining. Cells were observed as Gram-positive rods arranged singly or in short chains, consistent with the morphology of *B. subtilis* [22].

Biochemical Characterisation of *Bacillus subtilis*

Basic biochemical assays were conducted for preliminary identification of Gram-positive species following established microbiological protocols [21]. The tests include coagulase, catalase, indole production, citrate utilisation, H_2S, Voges-Proskauer, methyl red, urease, and sugar fermentation tests (glucose, lactose, sucrose) (Table 1). 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 [22], with final authentication by an independent microbiologist.

Table 1. Biochemical Identification of *B. subtilis*

Isolates	Gram Stain	Shape	Coagulase	Catalase	Indole	Citrate	H_2S	Voges-Proskauer	Methyl Red	Urease	Lactose	Glucose	Sucrose	Starch hydrolysis	Oxidase	Suspected organism
NCUBS9	+	rods	-	+	-	+	-	+	-	-	-	+	+	+	+	*B. subtilis*

Molecular Identification of *Bacillus subtilis*

DNA Extraction

Genomic DNA was extracted following an established protocol [29]. Single colonies were inoculated into 1.5 mL of liquid medium and incubated at 28 °C for 48 h with shaking. Cultures were centrifuged at 4600 g for 5 min, and pellets were resuspended in 520 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). To this, 15 µL of 20% SDS and 3 µL of Proteinase K (20 mg/mL) were added, followed by incubation at 37 °C for 1 h. Subsequently, 100 µL of 5 M NaCl and 80 µL of 10% CTAB in 0.7 M NaCl were added, vortexed, and incubated at 65 °C for 10 min before cooling on ice for 15 min. DNA was extracted with chloroform: isoamyl alcohol (24:1), centrifuged at 7200 g for 20 min, and the aqueous phase was precipitated with isopropanol (ratio 1:0.6) at –20 °C for 16 h. DNA was recovered by centrifugation at 13000 g for 10 min, washed with 70% ethanol, air-dried, and dissolved in 50 µL of TE buffer.

PCR Amplification of 16S rRNA Gene

Amplification of the 16S rRNA gene was performed in a 42 µL reaction mixture containing 10 µL of 5X GoTaq buffer, 3 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTPs, 1 µL each of primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5′-AAGGAGGTGATCCAGCC-3′), 0.3 units of Taq DNA polymerase (Promega, USA), and 8 µL of DNA template. PCR was carried out in a GeneAmp 9700 Thermal Cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 50 °C for 60 s, and 72 °C for 90 s; final extension at 72 °C for 10 min; and cooling at 4 °C.

Gel Electrophoresis

PCR products were analysed on 1.5% agarose gels prepared in 1X TAE buffer and stained with ethidium bromide (0.5 µg/mL). Samples were mixed with 10X loading dye and loaded alongside a 100 bp DNA ladder. Electrophoresis was performed at 120 V for 45 min, and bands were visualised under UV transillumination. Product sizes were estimated by comparison with the DNA ladder.

Purification of Amplified Products

PCR products were purified by ethanol precipitation. To 40 µL of PCR product, 7.6 µL of 3 M sodium acetate and 240 µL of 95% ethanol were added, mixed, and incubated at –20 °C for 30 min. Samples were centrifuged at 13000 g for 10 min at 4 °C; pellets were washed with 70% ethanol, centrifuged at 7500 g for 15 min, and air-dried for 10–15 min. DNA was resuspended in 20 µL of sterile distilled water and stored at –20 °C. Purified fragments were confirmed on 1.5% agarose gels run at 110 V for 1 h and quantified using a NanoDrop spectrophotometer (Model 2000, Thermo Scientific).

Sequencing and Phylogenetic Analysis

Purified fragments were sequenced using the Applied Biosystems 3130xl Genetic Analyser with the BigDye Terminator v3.1 cycle sequencing kit. Sequence data were analysed using BioEdit and MEGA6 software. The 16S rRNA gene sequence of *Bacillus subtilis* strain CTK2 was deposited in GenBank under accession number PZ896239 (Table 2)

Table 2: Molecular Identification of Isolate

Species / Strain	Marker Gene	Isolation Source	GenBank Accession
Bacillus subtilis strain CTK2	16S rRNA	Soil	PZ896239

Preparation of Secondary Bacterial Cell-Free Extracts (CFEs)

Pure cultures of *B. subtilis* were incubated at 30 °C in an incubator to promote aeration and optimal bacterial growth. Two incubation periods were selected—3 days and 8 days—to capture differences in metabolite production over time [6].

At the end of each incubation period, cultures were processed to obtain cell-free supernatants containing the metabolites. The broth cultures were first centrifuged at 10,000 rpm for 15 minutes to pellet bacterial cells and other debris. The resulting supernatant was carefully decanted and passed through Whatman No. 1 filter paper to remove residual particulates, yielding a clear cell-free extract solution. This procedure ensured that only extracellular metabolites were retained, eliminating interference from intact bacterial cells. The harvested cell-free supernatants were stored at 4 °C until use in larvicidal bioassays [17].

Larvicidal Bioassays

Third-instar larvae of *Ae. aegypti*, *An. gambiae* s.l., and *Cx. quinquefasciatus* were selected for bioassays [17]. The larvae were carefully transferred into plastic trays containing 100 mL of distilled water. Cell-free extract treatments were prepared by diluting cell-free supernatants of *B. subtilis* to final concentrations of 7.5 and 10 %v/v. Two control groups, positive and negative, were maintained with nutrient broth (1.0 %) and distilled water (0%), respectively. For each concentration and species, five replicates, each consisting of 25 larvae, were set up, with each experimental setup repeated after the first, resulting in the assay of 500 larvae per mosquito species [17].

The larvae of each mosquito species were exposed, independently and continuously, to the treatments, and mortality was recorded at intervals till 24, 48, and 72 hours post-exposure. The larvae were supplied with larval diet (yeast) after the 24th hour post-exposure. Dead larvae were identified by their immobility and failure to respond to gentle probing [17]. All bioassays were conducted at a temperature of 27 ± 2 °C, 75–80% relative humidity, and a 12:12 h light–dark cycle.

Enzyme Activity Assays

Larvae that survived exposure to *B. subtilis* CFEs were collected and immediately processed for biochemical assays. Five larvae per species from each concentration replicate and repeat experiment were used for the assay, resulting in the assay of 50 larvae per concentration per mosquito species. The larvae were

rinsed in cold phosphate buffer (0.1 M, pH 7.4) to remove external contaminants. The larvae were then homogenised using a glass homogeniser on ice to prevent enzyme degradation. The homogenates were centrifuged at 10,000 rpm for 15 minutes at 4 °C, and the resulting supernatants were used as enzyme sources.

Esterase activity was measured using α-naphthyl acetate as substrate, following established methods [23]. Briefly, the reaction mixture contained larval homogenate, phosphate buffer, and α-naphthyl acetate. After incubation, fast blue B salt was added to form a coloured complex with the liberated α-naphthol. Absorbance was read at 570 nm using a spectrophotometer. Enzyme activity was expressed as µmol of substrate hydrolysed per minute per mg of protein (µmol/min/mg protein).

Glutathione S-Transferase (GST) activity was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate [23]. The assay mixture contained larval homogenate, reduced glutathione (GSH), and CDNB in phosphate buffer. The conjugation of GSH with CDNB was monitored spectrophotometrically at 340 nm. Activity was expressed as µmol of conjugated product formed per minute per mg of protein (µmol/min/mg protein).

Protein concentrations in the homogenates were determined using the Bradford method [24]. Aliquots of homogenate were mixed with Coomassie Brilliant Blue dye reagent, and absorbance was measured at 595 nm. A standard curve prepared with bovine serum albumin (BSA) was used to calculate protein concentrations. All enzyme assays were repeated three times. Enzyme activities were normalised to protein content, allowing comparison across treatments and species.

Data Analyses

Data generated from the study were collated in Microsoft Excel 365. All statistical analyses were performed using the R Statistical software (v4.5.3) [25]. Larval mortality data were analysed to compare the efficacy of Day 3 and Day 8 CFEs of *Bacillus subtilis* (NCUBS9) across mosquito species. Before analysis, mortality percentages were corrected using Abbott's formula [26] to account for natural death in control groups. Comparisons between larval mortality due to Day 3 and Day 8 CFE treatments were conducted using Welch's t-test, which does not assume equal variances between groups. This test was applied separately for each mosquito species to determine whether differences in mortality rates were statistically significant at the 95% confidence level.

To estimate lethal concentrations, probit analysis was performed using the established method [27]. Mortality data at 72 hours post-exposure were subjected to regression analysis, generating dose–response curves for each species. From these curves, LC_{50} (concentration causing 50% mortality) and LC_{90} (concentration causing 90% mortality) values were calculated, along with their 95% confidence intervals. Regression equations and coefficients of determination (R^2) were reported to assess the goodness of fit of the probit models. Enzyme activity data (esterase and glutathione-S-transferase) were expressed as

µmol/min/mg protein and analysed using the Kruskal-Wallis non-parametric test to compare activity levels between control and treated groups. Post-hoc comparisons were conducted using Dunn's test to identify significant differences among treatments. Results were considered statistically significant at $p < 0.05$.

RESULTS

Larvicidal activity of the cell-free Extract (CFE) of *B. subtilis* after 3 and 8 days of incubation period against three mosquito genera

The larvicidal efficacy of the cell-free extracts of *B. subtilis* is shown in Figures 1 to 3. The Day 3 CFE of *B. subtilis* produced measurable larvicidal effects against all three mosquito species, though the magnitude of mortality varied with both species and concentration. At the control concentration (0%), no mortality was observed across all species. At 7.5 %v/v, the highest mortality (3.33 %) among the three species was recorded after 24 hours of exposure. At 10 %v/v, mortality increased slightly, with *Cx. quinquefasciatus* showing the highest mortality (6.67 %), while *Ae. aegypti* and *An. gambiae* s.l. each recorded $3.33 \pm 0.00\%$ even after 72 hours (Figure 1).

The Day 8 CFE of *B. subtilis* produced low but measurable larvicidal activity, with variation across species and concentrations. No mortality was observed in the control groups (0 %v/v). At 7.5 %v/v, the highest mortality was recorded at the 24th hour post-exposure in *Ae. aegypti* (16.67 %), and at the 48th hour in *Cx. quinquefasciatus* (16.67 %), while no mortality was recorded in *An. gambiae* s.l. at this concentration. At 10 v/v%, larval mortality was slightly higher, with 72-hour post-exposure mortality of 10.00, 13.33, and 16.67 % in *Ae. aegypti, An. gambiae* s.l. and *Cx. quinquefasciatus* mosquitoes (Figure 2)

Welch's T-test analyses revealed no significant ($t(12.57) = -0.88$, $p = 0.40$, $n = 20$) difference in larval mortality of *An. gambiae* s.l. at D_3 (3.82 ± 3.98 %) and D_8 (6.49 ± 8.76 %) (Figure 3). In *Ae. aegypti* mosquito, larval mortality at D_8 (13.19 ± 7.96 %) was significantly ($t(13.24) = -3.33$, $p = 0.0053$, $n = 20$) higher than at D_3 (3.82 ± 3.98 %) (Figure 3). Larval mortality in *Cx.* mosquitoes exposed to NCUBS9 was also significantly ($t(13.56) = -3.68$, $p = 0.0026$, $n = 20$) higher at D_8 (15.64 ± 7.99 %) than at D_3 (5.15 ± 4.17 %) (Figure 3).

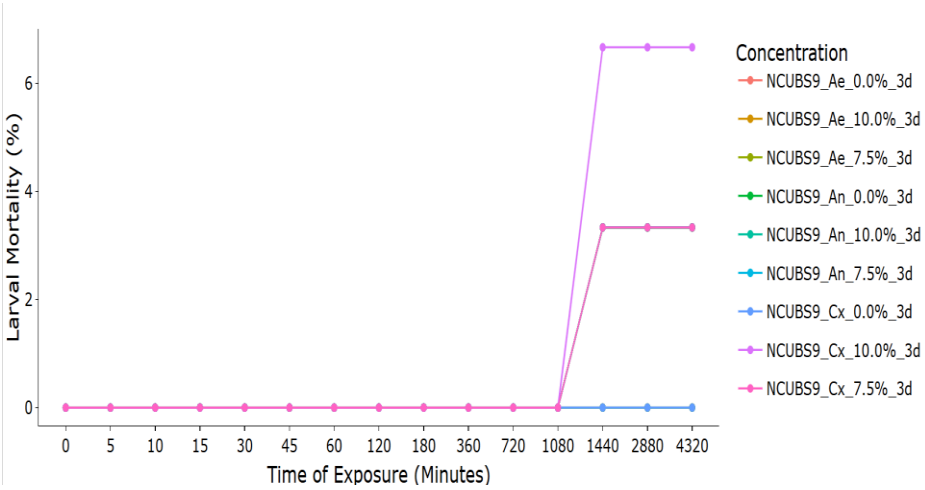

Figure 1: Larvicidal efficacy of Day 3 Cell-free Extract of *Bacillus subtilis* against three mosquito species after 72 hours post-exposure. NCUBS9 – Code name for *B. subtilis, Ae - Ae. aegypti, An – An. gambiae* s.l., *Cx – Cx. quinquefasciatus,* 3d – Day 3.

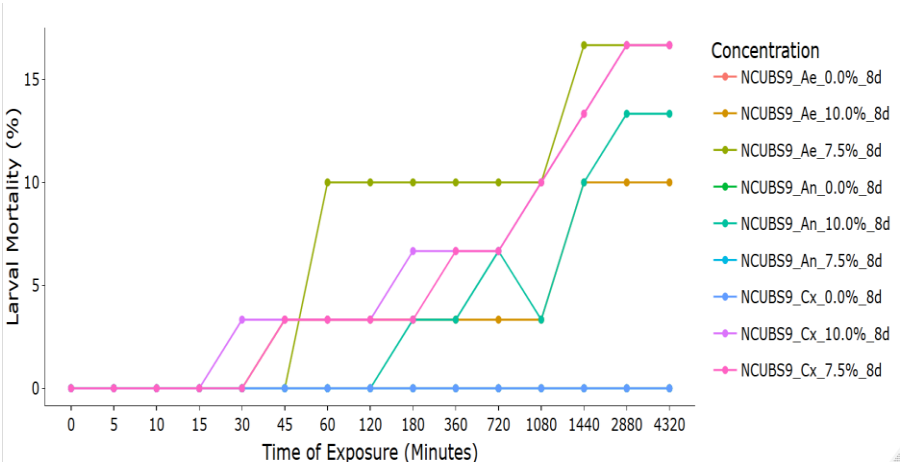

Figure 2: Larvicidal efficacy of Day 8 Cell-free Extract of *Bacillus subtilis* against three mosquito species after 72 hours post-exposure. NCUBS9 – Code name for *B. subtilis, Ae - Ae. aegypti, An – An. gambiae* s.l., *Cx – Cx. quinquefasciatus,* 8d – Day 8.

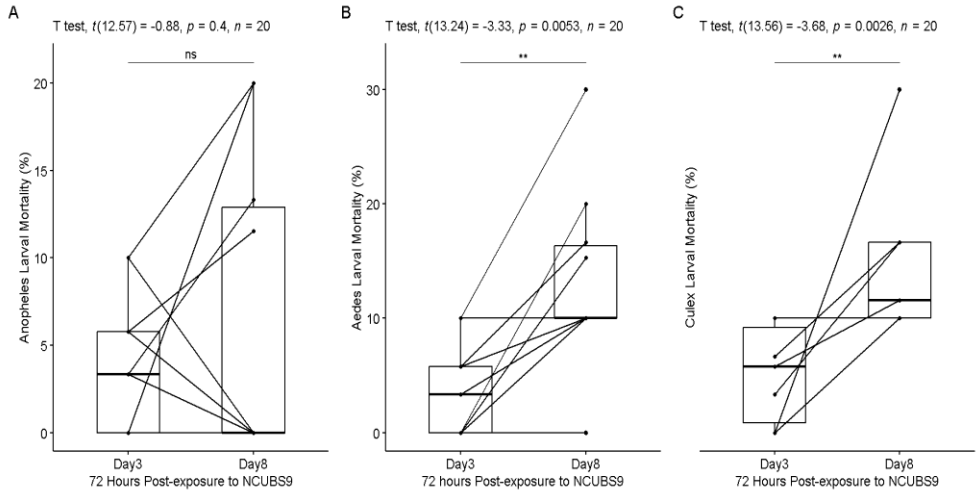

Figure 3: Comparative Larvicidal efficacy of Day 3 and Day 8 Cell-free Extract of *Bacillus subtilis* against three mosquito species after 72 hours post-exposure. NCUBS9 – Code name for *B. subtilis; Ae - Ae. aegypti, An – An. gambiae* s.l., *Cx – Cx. quinquefasciatus*.

Lethal Concentrations of the larvicidal activity of the cell-free extract of *Bacillus subtilis* against mosquito larvae after 72 hours post-exposure

The probit analysis of Day 8 CFE of *B. subtilis* showed that all three mosquito species required low concentrations to achieve 50% and 90% mortality, though the values varied by species (Table 3). For *Cx. quinquefasciatus*, the LC_{50} was 6.67 %v/v, and the LC_{90} was 11.47 %v/v, with a regression equation of y = 8.33x – 5.56 and an R^2 of 0.75. *Aedes aegypti* displayed a similar pattern, with LC_{50} at 6.93 %v/v and LC_{90} at 11.73 %v/v, but with a stronger regression fit ($R^2 = 0.98$). *Anopheles gambiae* s.l. required higher concentrations, with LC_{50} at 8.83 %v/v and LC_{90} at 14.83 %v/v, and a regression equation of y = 6.67x – 8.89 ($R^2 = 0.75$) (Table 3).

Table 3. Lethal Concentrations (LC) of the CFE (Day 8 of Incubation) of *Bacillus subtili*s against Mosquito Larvae post 72-hour exposure.

Mosquito Species	Lethal Concentration after 8 days of Incubation			
	LC_{50} (%v/v)	LC_{90} (%v/v)	R^2	Regression Equation
Cx. quinquefasciatus	6.67	11.47	0.75	y = 8.33x - 5.56
An. gambiae s.l.	8.83	14.83	0.75	y = 6.67x - 8.89
Ae. aegypti	6.93	11.73	0.98	y = 8.33x - 7.78

Effects of the cell-free extracts of *B. subtilis* on the resistance enzyme profile of larval instars of mosquitoes

Analyses showed a significant increase ($p < 0.001$) in esterase activity in larvae of all three mosquito species following exposure to Day 3 and Day 8 metabolites of *B. subtilis*. In *Ae. aegypti*, activities were 190.28 ± 1.85 and 202.73 ± 1.04 µmol/min/mg protein for Day 3 and Day 8, respectively. In *An. gambiae* s.l., values were 187.91 ± 1.23 and 200.26 ± 1.05 µmol/min/mg protein, while in *Cx. quinquefasciatus,* esterase activities were 169.55 ± 1.93 and 179.67 ± 1.61

µmol/min/mg protein in Day 3 and Day 8 metabolites (Table 4).

GST activity responses differed among the three mosquito species exposed to Day 3 and Day 8 metabolites of *B. subtilis*. In *Ae. aegypti* and *An. gambiae* s.l. larvae, activities increased significantly ($p < 0.05$) at both time points. In contrast, *Cx. quinquefasciatus* larvae showed a significant reduction ($t(6.97) = -14.15$, $p < 0.0001$, $n = 10$), with values of 0.56 ± 0.02 and 0.50 ± 0.02 µmol/min/mg protein for Day 3 and Day 8, respectively. Thus, while GST activity was elevated in *Ae. aegypti* and *An. gambiae s.l.*, it declined in *Cx. quinquefasciatus* (Table 5).

Table 4: Esterase Activities in Larvae exposed to Lethal Concentrations of the Cell-free extract of *B. subtilis* Bacterial Species

Mosquito Species	Esterase activity (µmol/min/mg protein)		*p* value
	Day 3	Day 8	
Ae. aegypti	190.28±1.85[a]*	202.73±1.04[b]	$t(6.27) = -15.10$, $p < 0.001$, $n = 10$
An. gambiae s.l.	187.91±1.23[a]	200.26±1.05[b]	$t(7.8) = -19.68$, $p < 0.0001$, $n = 10$
Cx. quinquefasciatus	169.55±1.93[a]	179.67±1.61[b]	$t(7.74) = -10.38$, $p < 0.0001$, $n = 10$

*Mean values with the same superscript alphabet in a row for a mosquito species are not significantly ($p > 0.05$) different.

Table 5: Glutathione-S-Transferase Activities in Larvae exposed to Lethal Concentrations of the Cell-free extract of *B. subtilis* Bacterial Species

Mosquito Species	Glutathione-S-transferase (GST) activity (µmol/min/mg protein)		*p* value
	Day3	Day8	
Ae. aegypti	0.51±0.04[a]*	0.57±0.05[b]	$t(7.7) = -2.66$, $p = 0.03$, $n = 10$
An. gambiae s.l.	0.51±0.02[a]	0.54±0.02[b]	$t(8) = -3.74$, $p = 0.0057$, $n = 10$
Cx. quinquefasciatus	0.56±0.02[b]	0.50±0.02[a]	$t(8) = 5.13$, $p = 0.00089$, $n = 10$

*Mean values with the same superscript alphabet in a row for a mosquito species are not significantly ($p > 0.05$) different.

DISCUSSION

The larvicidal efficacy of CFEs of *B. subtilis* observed in this study provides insight into mosquito biology and vector control. Day 3 CFE produced low mortality across all three species; *Cx. quinquefasciatus* was slightly more susceptible than *Ae. aegypti* and *An. gambiae s.l.* By Day 8, mortality increased in *Ae. aegypti* and *Cx. quinquefasciatus*, while *An. gambiae s.l.* remained less responsive. Analyses revealed that mortality was significantly higher at Day 8 compared to Day 3 in *Ae. aegypti* and *Cx. quinquefasciatus*, but not in *An. gambiae s.l.* From an entomological perspective, these findings highlight species-specific differences in susceptibility, which may be linked to variations in larval physiology and detoxification enzyme activity [15]. The increase in larval mortality with CFE age suggests that secondary metabolite

accumulation or change over time could have influenced their activity [6].

Probit analysis of Day 8 CFE further supports these observations. The LC_{50} and LC_{90} values indicate that low concentrations were sufficient to produce measurable mortality, though thresholds varied among species. *Cx. quinquefasciatus* and *Ae. aegypti* required lower concentrations, suggesting greater sensitivity, while *An. gambiae s.l.* required higher concentrations. The regression equations showed positive slopes across species, consistent with increasing mortality at higher concentrations, and the stronger regression fit for *Ae. aegypti* suggests more predictable mortality outcomes compared to the moderate fits observed for *Cx. quinquefasciatus* and *An. gambiae* s.l. These differences reflect species-specific physiological traits, such as detoxification enzyme activity, which influence larval sensitivity to microbial CFEs [6].

From an ecological perspective, microbial CFEs degrade naturally and are less likely to harm non-target organisms compared to synthetic insecticides. Their multiple modes of action may also help slow the development of resistance, which is a growing concern with chemical insecticides [4]. Epidemiologically, reducing larval survival can lower adult mosquito populations, thereby affecting transmission of malaria (*An. gambiae s.l.*), dengue and yellow fever (*Ae. aegypti*), and lymphatic filariasis (*Cx. quinquefasciatus*). Since larval habitats are often localised and predictable, microbial larvicides can be applied in a targeted manner to support public health interventions [28].

The enzyme activity assays provide further mechanistic insight. Esterase activity increased significantly in all three species at both Day 3 and Day 8, with higher values in *Ae. aegypti* and *An. gambiae s.l.* compared to *Cx. quinquefasciatus*. This consistent elevation suggests that esterases are induced in response to exposure, reflecting their role in metabolising xenobiotic compounds. Previous studies have linked esterase upregulation to detoxification and insecticide resistance in mosquitoes [3]. GST activity, however, varied across species. In *Ae. aegypti* and *An. gambiae s.l.*, GST activity increased significantly ($p < 0.05$), consistent with its role in conjugating glutathione to reactive CFEs and reducing oxidative stress [11]. In contrast, *Cx. quinquefasciatus* exhibited a significant reduction, which may indicate reduced capacity for glutathione-mediated detoxification and could be associated with greater susceptibility to microbial CFEs.

The results revealed that esterase activity was elevated across all species, while GST responses differed. The increase in GST activity in *Ae. aegypti* and *An. gambiae s.l.* may support tolerance, whereas the reduction in *Cx. quinquefasciatus* could be linked to higher mortality. These findings highlight the importance of detoxification pathways in shaping species-specific responses and provide a biochemical basis for differences in larvicidal outcomes. Future studies are recommended to evaluate the larvicidal activities of active metabolites of the CFEs of *B. subtilis* for possible efficacy.

CONCLUSION

The study showed that crude *B. subtilis* CFEs produced measurable larvicidal effects, with mortality increasing between Day 3 and Day 8. *Cx. quinquefasciatus* and *Ae. aegypti* were more sensitive to these metabolites than *An. gambiae* s.l., as reflected in lower

LC_{50} and LC_{90} values and stronger regression fits. Resistance enzyme assays indicated consistent esterase induction across species, while GST activity varied—elevating in *Ae. aegypti* and *An. gambiae s.l.* but decreasing in *Cx. quinquefasciatus*. These biochemical differences suggest species-specific responses to this bacterial CFE. Optimisation and purification of the active metabolites of the CFEs of the bacterial species are recommended for future study.

Declarations

Authors Contribution

UAC, UCC, AME, BJD and OIK conceived and designed the experiments. UAC, UCC, LS, IYO, and OFI performed the experiments. UAC, UCC, LS, and OFI analysed the data. UAC, UCC, IYO and OFI wrote the first draft of the manuscript. AME, OIK and BJD corrected the draft copy. All AUTHORS agreed to the final state of the manuscript.

Disclosure of Conflict of Interests

The authors declare that they have no competing interests. This study was funded by a grant from TETFund (Institutional Based Research). However, the funder has not influenced the results or the decision to publish.

Ethics Approval and Informed Consent

This does not involve humans or animals; hence, no ethical approval and informed consent were required.

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