Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 27, No. (3–4) : 2025 : 251-260

ISSN-0972-3005

DOI No.: http://doi.org/10.53550/AJMBES.2025.v27i03-04.018
Journal Home Page: www.envirobiotechjournals.com

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SYMBIOTIC MID-GUT BACTERIUM *BACILLUS CEREUS* (PQ324272) OF *CULEX QUINQUEFASCIATUS* LARVAE AND ITS CONTROL BY INDIGENOUS PLANT EXTRACTS

BASANTA SARKAR[1], TUSHAR KANTI DANGAR[2], ABHIJIT MAZUMDAR[3] AND SOUMENDRANATH CHATTERJEE[1*]

[1]Parasitology and Microbiology Research Laboratory, Department of Zoology, The University of Burdwan, Burdwan 713 104, W.B., India
[2]Microbiology Laboratory, Division of Crop Production, National Rice Research Institute, Cuttack 753 006, Odisha, India
[3]Entomology Research Unit, Department of Zoology, The University of Burdwan, Burdwan 713 104, W. B., India

(Received 17 December, 2024; Accepted 19 May, 2025)

Key words: *Culex quinquefasciatus, Gut bacteria,Bacillus cereus, Moringa olieafera, Antimicrobial sensitivity test*

Abstract–Gut of insects harbors a diverse microorganism which can thrive and multiply within the insect, play a crucial role in digestion, nutrition and overall growth. Midgut bacteria significantly influence development and survival of *Culex quinquefasciatus* larvae also. Through this investigation we have identified a bacterium CGB1 from the midgut of third instar larvae of *Culex* mosquito and its symbiotic relationship has been undertaken. Phenotypic and molecular characteristics of CGB1 reveals it is a rod-shaped bacterium that forms round, off-white and flat colonies. It produces spores and utilizes sodium malonate as sole carbon source. It is positive for catalase, methyl-red and urease production; nitrate reduction, citrate utilization and starch and gelatine hydrolysis tests, while negative for lipid and esculin hydrolysis, Voges-Proskauer, indole, ONPG and oxidase tests. It produces acid from trehalose and sucrose, but unable to utilize other twenty carbon sources like lactose,maltose, xylose, fructose, dextrose, mannose, galactose, raffinose, mellibiose, rhamnose, cellobiose, melezitose, a-methyl-d-mannoside, xylitol, d-arabinose, sorbose, inositol, salicin, mannitol and ducital. CGB1 is sensitive to kanamycin, vancomycin, amoxicillin, nalidixic acid, chloramphenicol, levofloxacin, gentamicin, ofloxacin, norfloxacin, tetracycline, ciprofloxacin, rifampicin, fusidic acid, azithromycin and doxycycline but resistant to ampicillin, bacteriocin and penicillin. Antimicrobial potential of *Moringa oleifera* extracts was assessed by well diffusion against the isolate. Inhibition zone (IZ) for fresh leaf juice and aqueous, methanol and ethyl acetate extracts against the bacterium measured 21.33±0.58, 25.0±0.53, 24.0±0.58 and 14.67±0.58 mm, respectively. Elimination of midgut bacteria using antibiotics in the breeding water had an adverse effect on the development of wild mosquito larvae leading to reduced survival and growth rates. Addition of fresh leaf juice extract of *M. oleifera* to the breeding habitat water also lowered larval development and pupal emergence.

INTRODUCTION

Culex quinquefasciatus, commonly called southern house mosquito is a transmission vector of filariasis and West Nile fever in tropical and subtropical regions worldwide. In India also, it is a vector for filariasis and Japanese encephalitis (Mourya *et al.*, 1989; Das *et al.*, 2002). Furthermore, mosquitoes, including *Cx. quinquefasciatus*, have symbiotic associations with various bacterial species in their gut. Removal of these gut microorganisms has been shown to suppress development from larva to adult mosquitoes (Mukhopadhya *et al*., 2016; Roy *et al*., 2010). These gut microbes fulfil crucial functions in nutrition, development and competitive exclusion (Dillon and Dillon, 2004). Microorganisms of mosquito intestine help in digestion of food material by secreting various enzymes (Cambel, 2004; De

Gairo *et al*., 2011). For instance, *Serratia* and *Enterobacter* spp. produce haemolytic enzymes that aid in blood digestion in adult mosquitoes (Gusmao, 2010; De Gairo *et al*., 2011) and symbiotic association of *Asaiabogorens* is in *Anopheles stephensi* provide vitamins to the mosquito host (Crotti *et al*., 2010). Moreover, compounds released by bacteria in the mosquito breeding habitat have been found to impact mosquito larval development e.g. high level of *Pseudomonas aeruginosa* in the breeding medium can enhance growth of *Cx. quinquefasciatus* larvae (Peck and Walton *et al*., 2006). These findings highlight the intricate interactions between mosquitoes and gut bacteria which can influence various physiological processes in mosquitoes like digestion, blood feeding and larval development. Indeed, study of gut microbiota of *Cx. quinquefasciatus* mosquito larvae could envisage a promising approach for reducing the transmission of diseases by inhibiting the larval development of this mosquito species. Exploration and manipulation of the symbiotic gut bacteria could serve as an alternative and effective strategy for vector control (Beard *et al*., 2002; Dillon *et al*., 2000). Various natural agents have been reported to effectively control microbial symbionts that use insects as their hosts (Mickes and Ferguson, 1961; Beard *et al*., 2002). Use of synthetic insecticides against mosquito larvae have detrimental effects on non-target organisms and environment due to their non-biodegradable and non-eco-friendly nature (Chatterjee *et al*., 2023). In contrast, readily biodegradable natural plant extracts are safer for humans and other animals in comparison to synthetic and chemical insecticides (Sharma, 1993; Chatterjee *et al*., 2023). Therefore, exploring potential of the natural agents, manipulation of symbiotic gut bacteria etc. could provide eco-friendly and sustainable approach to control vector-borne diseases transmitted by *Cx. Quinquefasciatus.*

Almost every part of morin gatree (*Moringa oleifera*) is useful as it possess alkaloids and triterpenoids having therapeutic properties such as diuretic, cholagogue, hypoglycemic and tonic effects (Imohiosen *et al*., 2014) and bactericidal agents also (Moura *et al*., 2012). Therefore, the objective of the present work was to evaluate the bio-efficacy of *M. oleifera* leaf extracts on symbiotic mid-gut bacterial isolates of larval *Cx. quinquefasciatus* mosquitoes.

Therefore, this research was delved to decipher impact of the gut bacteria on development and survival rates of *Cx. quinquefasciatus* larvae, as well as, by oral administration of antibiotics to the larvae, we aimed to suppress gut bacteria to establish functional interplay between these bacteria and their host.

MATERIALS AND METHODS

Mosquito larvae collection and identification

Larvae of *Cx. quinquefasciatus* were collected from organic rich larval breeding habitat of some rural coastal areas of Digha, West Bengal. Standard dipper of 250 ml capacity was used for larvae collection and following standard protocols (Service, 1993). The larvae collected were transported to the Parasitology and Microbiology Research Laboratory, Department of Zoology, The University of Burdwan. Both larval forms and adult mosquitoes were identified by morphological examination under a microscope following established reference keys (Tyagi *et al*., 2015).

Isolation of bacteria from larval mid gut

Third instar larvae (5 to 10 no.) of *Cx. quinquefasciatus* were surface sterilized by immersing them in 70% alcohol for 3-5 min, subsequently rinsed thrice in double distilled water (Chatterjee *et al*., 2010). Midguts of the larvae were dissected out within a laminar airflow cabinet under a binocular dissecting microscope at 4X magnification.

Midguts were macerated in 1 ml of sterilized distilled water. From the extract 100 µL sample was mixed with 100 mL sterilized nutrient agar medium poured onto five sterile petri plates, plates were incubated in a B.O.D. incubator at 37±2 °C and of 48 h. Total colonies were counted to determine bacterial load in midgut and expressed as colony-forming units (cfu/ml). Characteristics of the colonies, such as size, shape, color, opacity, elevation and consistency were documented. Bacterial cultures were maintained on nutrient agar slants in a refrigerator at 4±1 °C for further investigation.

Characterizations of larval gut bacterial isolates

Phenotypic characterizations

Gram staining and presence of endospores in the bacterial isolate were assessed using Gram's stain kit and 5% Malachite green solution, respectively. Surface morphology of vegetative cells of bacterial isolates was recorded under a scanning electron microscope following (Lacey *et al*., 1997).

Bio-chemical characterizations

Various physiological and biochemical tests viz. catalase, methyl red, indole production,Voges-Proskauer, oxidase, ONPG; starch, protein, lipid, esculin hydrolysis; citrate, malonate utilization; and various carbohydrate fermentation tests were conducted using standard methodologies (Pelczar *et al*., 1957; Smibert and Krieg., 1995).

Molecular characterizations

Genomic DNA was extracted from overnight grown bacterial cultures following Janssen *et al*. (1994). Subsequently, 16S rDNA was amplified by polymerase chain reaction (PCR) using 16s rRNA primers through the protocol. The 16S rDNA amplicon was resolved in 1% agarose gel against standard markers observed under a UV transilluminator. Amplicon concentration was determined through a Nanodrop ND800 spectrophotometer.

The amplicons were purified using the GSure extract gel extraction/PCR cleanup kit of Genetix Nucleopore. Ribosomal gene sequences were determined using an ABI 3730xl cycle sequencer against both forward and reverse primers. After sequencing, the forward and reverse sequences were trimmed and the resulting sequences were subjected to a BLAST analysis using the NCBI database to identify similar sequences and assess maximum identity scores.

A subset of the sequences that had the greatest identity scores was chosen and multiple sequence alignment was performed using Clustal Omega software (Thompson *et al*., 1994). Nucleotide percentages of the 16S rRNA gene sequences of the bacterial isolate was assessed through the Aqua software. Lastly, a phylogenetic analysis was conducted using the neighbour-joining method with MEGA X software, following Saitou *et al*. (1987).

Investigation on impact of midgut microbe on development of larval instar and pupal emergence

For each experiment, thirty 1stinstar *Cx. quinquefaciatus* larvae were kept in 200 ml of water with only sucrose solution without antibiotics (control/wild), and other thirty larvae were released in 200 ml habitat water with 10% sucrose solution containing a mixture of tetracycline and doxycycline (1:1) antibiotics treatment for 12 h. Both untreated (wild) and antibiotic-treated (cured) larvae (30 larvae/200 ml water for each set) were released in beakers containing sterile or unsterile habitat water with 5 ml bacterial suspension (10^6 cfu/ml) to assess their beneficial effects on the larvae. To assess effect of the bacterial strain on subsequent development from 1st to 4thinstar and pupal emergence were observed and counted.

Antibiotic sensitivity of bacterial isolates

Susceptibility of the bacterial isolate to eighteen different commercial antibiotics namely ampicillin (10 μg), bacitracin (10 μg), nalidixic acid (30 μg), kanamycin (30 μg), amoxicillin (10 μg), penicillin (10 μg),gentamicin (50 μg), levofloxacin (5 μg), chloramphenicol (30 μg), neomycin (30 μg), tetracycline (30 μg), ofloxacin (5 μg), norfloxacin (10 μg),vancomycin (30 μg), rifampicin (5 μg), ciprofloxacin (5 μg), azithromycin (30 μg) and doxycycline (30 μg) were tested on Muller-Hinton agar plates by the Kirby-Bauer disk diffusion method (Bauer and Kirby, 1966). Minimum inhibitory concentration (MIC, μg/ml) of two broad spectrum antibiotics viz. tetracycline and doxycycline against the bacterial isolate was determined using MIC test strip (Himedia, India).

Collection of plant material

Fresh leaves of moringa plant were gathered from the Crop Research and Seed Multiplication Farm (CRSMF) of The University of Burdwan and authenticated taxonomically by the Botanical Survey of India (BSI) in Kolkata. A voucher specimen (BUBS-01) has been archived in the Department of Zoology at the University of Burdwan. These freshly collected leaves were cleaned with distilled water and air dried for 15d at room temperature (27 °C-37 °C) under shade. Subsequently, the dried leaves were powder edinan electronic grinder to prepare the crude extract.

Preparation of plant extracts

The 500 g powder was extracted in methanol, petroleum ether, ethyl acetate and distilled water in a soxhlet apparatus for 8 h. Extracts were collected and stored in a refrigerator at 4 °C for antibacterial sensitivity test (AST), after which it was mixed with dimethyl sulfoxide (DMSO) and kept in an airtight container for an antibacterial sensitivity test.

Yield of extracts were calculated using the formula: Extract yield (%) = R/S×100 (where R is weight of extracted leaf powder residues and S is weight of plant raw sample) (Truong *et al*., 2019).

Agar well diffusion method

Antimicrobial properties of *M. oleifera* leaf fractions were assessed by well diffusion method. To test, 10 µl overnight bacterial cultures containing 10^5 CFU/ml was evenly spread onto Muller Hinton Agar plates. Subsequently, 50 µl each of aqueous, solvent extracts and tetracycline (30 µg) as reference were introduced into separate wells on the agar plates. After incubation for 24 h at 37 °C, antibacterial activity was determined from diameter of the inhibition zones surrounding the wells. Antimicrobial trials were repeated thrice and the diameters of inhibition zones were recorded from each of the independent experiment (Barry, 1986).

RESULTS

Morphological and biochemical properties of bacterial isolates

The bacterial isolate CGB1 formed round, off-white and flat colonies. It exhibited positive results for catalase, methyl-red, nitrate reduction, urease production, citrate utilization, starch hydrolysis and gelatine hydrolysis tests, while showed negative results for lipid hydrolysis, Voges-Proskauer, indole, esculin hydrolysis, ONPG and oxidase tests. CGB1 utilized sodium malonate as a sole carbon source (Table 1). Scanning electron micrograph showed that CGB1 is rod-shaped and produced spores (Fig. 1). CGB1 produced acid from trehalose and sucrose but unable to utilize other twenty carbon sources viz. lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, melibiose, mannose, rhamnose, cellobiose, melezitose, α-methyl-d-mannoside, xylitol, d-arabinose, sorbose, inositol, salicin, mannitol and ducital (Table 1).

Table 1. Carbon source utilization of gut bacterial isolate CGB1 (*Bacillus cereus*).

Carbon source	Result
Lactose, xylose, maltose, melezitose, fructose, dextrose, galactose, raffinose, mellibiose, mannose, rhamnose, cellobiose, α-methyl-D-mannoside, xylitol d-Arabinose, sorbose, inositol, salicin, mannitol, ducital	Negative
Trehalose, Sucrose	Positive

Antibiotic bioassay

The bacteria was sensitive to the recommended doses of kanamycin (30µg/disc), amoxicillin (10µg/disc), nalidixic acid (30µg/disc), chloramphenicol (30 µg/disc), levofloxacin (5 µg/disc), gentamicin (50 µg/disc), ofloxacin (5 µg/disc), norfloxacin (10 µg/disc), tetracycline (30 µg/disc), ciprofloxacin (5 µg/disc), vancomycin (30 µg/disc), rifampicin (5 µg/disc), fusidic acid (10µg/disc), azithromycin (15 µg/disc),doxycycline (30 µg/disc) but resistant to ampicillin (10 µg/disc), bacteriocin (10µg/disc) and penicillin (10 µg/disc) (Table 2).

Phylogenetic analysis

The nucleotide base composition of 16S rDNA of the bacterial isolate CGB1 revealed that the AT and GC content were 47% and 53 %, respectively. The phylogenetic tree revealed that CGB1, *Bacillus cereus* (PQ324272) branched with the cluster containing *B.cereus* (DQ289984) and *B.cereus* (DQ289991) with 90% bootstrap support. The cluster containing *B.cereus* (PQ324272), *B.cereus* (AB 592537) and

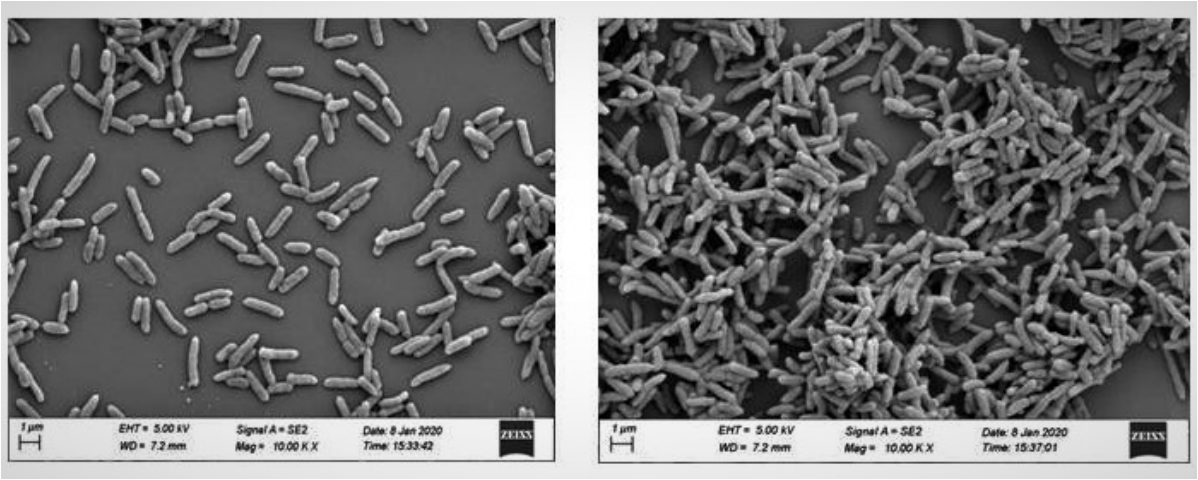

Fig. 1. Scanning electron microscopy photograph showing vegetative cell of the bacterial isolate CGB1 (*Bacillus cereus*).

B.cereus (AB592534) branched with cluster containing *B. cereus* (NR114582), *B.cereus* (NR 113266), *B. cereus* (NR074540), *B. cereus* (NR112630), *B. cereus* (NR 115714), *B. cereus* (NR115526) with 86% bootstrap support (Fig. 2). The restriction map of the 16S rRNA gene sequence of CGB1 is shown in Fig. 3.

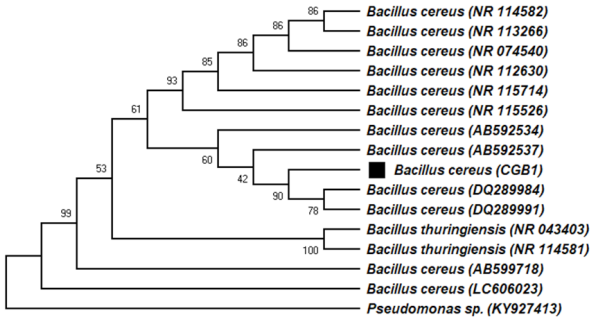

Fig. 2. Phylogenetic tree constructed using partial 16S rRNA gene sequence of *Bacillus cereus* CGB1 (PQ324272) with other neighbouring 16S rRNA gene sequences retrieved from NCBI and RDP database.

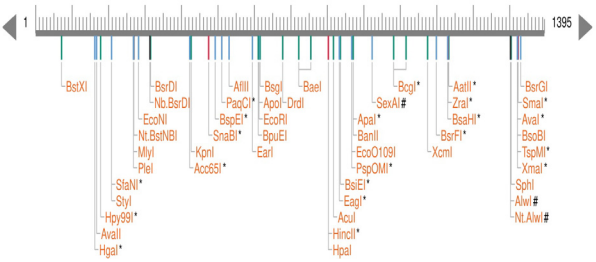

Fig. 3. In silico restriction map of the 16S rRNA gene sequence of the isolate CGB1.

Effect of eradicating mid gut bacteria from *Cx. quinquefaciatus* larvae

The bacterial strain CGB1 was isolated from the midgut of third instar larvae of *Cx. quinquefasciatus* species. This bacterium was a consistent inhabitant during the larval phases of the mosquitoes. To elucidate advantages of CGB1 in the developmental progression of *Cx. quinquefasciatus*, the larvae were treated with different antibiotics including tetracycline and doxycycline which exhibited sensitivity against it. Minimum inhibitory concentrations (MIC) for tetracycline and doxycycline were 0.5 µg/ml and 1.5 µg/ml, respectively against the bacterial strain which were potent to restrain growth of the bacterial isolate Table 3.

In the control, i.e. in breeding habitat water, high percentage of wild larvae developed into 3rd instar (96.66%), 4th instar (95.56%) and pupal stage (95.56%). In sterile breeding habitat water also, wild larvae developed into 3rd instar (95.56%), 4th instar (94.43%) and pupal stage (91.1%). However, when wild larvae were cultured in breeding habitat water with a combination of tetracycline and doxycycline, developmental progress was affected. Only 61.33% wild larvae developed into 2nd instar, 34.43% developed into 3rd instar, 21.1% developed into 4th instar and only 16.66% pupae emerged. When

Table 2. Antibiotic sensitivity of gut bacterial isolate CGB1 (*Bacillus cereus*) against the commercial antibiotics.

Name of the antibiotics	Concentration of antibiotics	Zone diameter of inhibition (mm)	Sensitivity (Sensitive /Resistant)
Kanamycin	(30µg/disc)	27	Sensitive
Amoxicillin	(10µg/disc)	Nil	Resistant
Nalidixic acid	(30µg/disc)	29	Sensitive
Ampicillin	(10µg/disc)	20	Sensitive
Chloramphenicol	(30 µg/disc)	26	Sensitive
Levofloxacin	(5 µg/disc)	26	Sensitive
Gentamicin	(50 µg/disc)	34	Sensitive
Ofloxacin	(5 µg/disc)	23	Sensitive
Norfloxacin	(10 µg/disc)	30	Sensitive
Tetracycline	(30 µg/disc)	27	Sensitive
Ciprofloxacin	(5 µg/disc)	36	Sensitive
Vancomycin	(30 µg/disc)	17	Sensitive
Rifampicin	(5 µg/disc)	18	Sensitive
Fusidic acid	(10 µg/disc)	28	Sensitive
Azithromycin	(15 µg/disc)	24	Sensitive
Penicillin	(10 µg/disc)	Nil	Resistant
Bacteriocin	(10 µg/disc)	12	Resistant
Doxycycline	(30 µg/disc)	29	Sensitive

Table 3. Role of mid gut bacterium CGB1 (*Bacillus cereus*) on the development of larval instar and pupal emergence of *Cx. quinquefasciatus*

Treatment	Larvae	Total no. of larvae (1^{st} instar	No. of 2^{nd} instar larvae (after 24 h) (Mean±SD)	No. of 3^{rd} instar larvae (after 48 h) (Mean±SD)	No. of 4^{th} instar larvae (after 72 h) (Mean±SD)	No. of pupal Emergence (after 96 h) (Mean±SD)
Breeding habitat water	Wild	100±0	96.66±0	96.66±0	95.56±0.58	95.56±0.58
Sterile breeding habitat water	Wild	100±0	95.55±0.58	95.56±0.58	94.43±0.58	91.1±0.58
Breeding habitat water + tetracycline and doxycycline	Wild	100±0	61.33±2.3	34.43±0.58	21.1±1.15	16.66±1.15
Breeding habitat water + aqueous extracts of *M. oleifera*	Wild	100±0	72±0.54	40±0.3	27±0.13	19±0.13
Sterile breeding habitat water + CGB1	Cured	100±0	95.56±0.58	91.1±0.58	87.76±0.58	87.76±0.58
Sterile breeding habitat water +CGB1 + tetracycline and doxycycline	Cured	100±0	53.43±0.58	31.1±1.53	15.1±1.53	7.76±2.53
Sterile breeding habitat water + aqueous extracts of *M. oleifera*	Cured	100±0	58±0.43	38±0.28	21±0.1	11±0.04

.

aqueous leaf extract of *M. oleifera* was added to the breeding habitat water, lesser wild larvae developed into 2^{nd} instar (72%), 3^{rd} instar (40%) and 4th instar (27%) with 19% of pupal emergence. Moreover, when pure culture suspension of CGB1 was used in sterile breeding habitat water, larval development and pupal emergence rates further improved showing 95.56% wild larvae developed into 2^{nd} instar, 91.1% into 3^{rd} instar and 87.76% into 4^{th} instar, and87.76% of pupal emergence. When cured larvae were cultured in sterile breeding habitat water with aqueous leaf extract of *M. oleifera*, 58% wild larvae developed into 2^{nd} instar, 38% into 3rd instar, 21% into 4^{th} instar and 11% of pupal emergence occurred.

Antibacterial activity of indigenous plant extracts

Antibacterial activity assay against the symbiotic isolate *B. cereus* CGB1 revealed that the aqueous extract of *M. oleifera* exhibited highest sensitivity, followed by methanol and ethyl acetate extracts (Table 4). Zone of inhibition (ZOI) for various extracts viz.fresh leaf juice, aqueous extract, methanol extract and ethyl acetate extract, against the bacterial isolate, measured 21.33±0.58 mm, 25.0±0.53 mm, 24.0±0.58 mm, and 14.67±0.58 mm, respectively. However, chloroform and petroleum ether extracts showed no significant effect on the bacteria. Among the different solvent extracts tested, aqueous extract and fresh leaf juice of *M. oleifera* leaves showed the most effective results (Table 4).

Table 4. Antibacterial sensitivity of *M. oleifera* leaf extracts against the gut bacterial isolate CGB1 (*Bacillus cereus*) of *Cx. quinquefasciatus.*

Name of the extracts	Zone of inhibition (mm±SE)
Fresh leaf juice	21.33±0.58
Aqueous extracts	25.0± 0.53
Methanol extracts	24.0±0.58
Ethyl acetate extracts	14.67±0.58
Chloroform extracts	0
Petroleum ether extracts	0
Tetracycline (control)	25.33±0.33

DISCUSSION

Over the last few years there has been a growing interest in targeting the larval stages of mosquito vectors as the primary vector control strategy because adults are winged and scattered making the applications against them difficult and at times, ineffective. The gut microbiota of mosquitoes plays

a crucial role in their development, survival, and vector competence. Among the diverse microbial communities that inhabit mosquito larvae, symbiotic bacteria have gained significant attention due to their potential impact on host physiology and interactions with pathogens. This bacterium is known for its ability to endure harsh environmental conditions and support the nutritional needs of its host, which may, in turn, influence mosquito growth and resistance to environmental stresses.Recent studies have emphasized the intricate relationship between mosquitoes and their gut bacteria, highlighting the crucial role these bacteria play in regulating larval survival and development, which has increasingly become a focal point for control strategies (Engeland Moran, 2013). In the current study, phenotypic, biochemical, and molecular analyses of the symbiotic bacteria in the mid gut of third-instar *Cx. quinquefasciatus* larvae revealed the presence of *Bacillus cereus* as the predominant species (Table 1, Fig. 1, 2). This finding adds new insights into the symbiotic bacterial community of *Cx. quinquefasciatus* larvae. Notably, *Asaia* sp., which has previously been identified as a symbiont in *Cx. quinquefasciatus* (Suo *et al*., 2022), as well as in *An. stephensi* and *Ae. aegypti* (Gaio *et al*., 2011), was also detected. The biochemical characteristics of the isolate revealed glucose metabolic activity (Table 1), suggesting that *B. cereus* may provide essential nutrients to the larvae, potentially supporting their growth and development. The presence of spores in the CGB1 strain indicates its ability to survive in the harsh environmental conditions within the gut, as previously reported by Gray *et al.* (2019). Previous studies also reported that *B. cereus* was a common inhabitant or symbiotic flora of mosquito larval guts including *Cx. quinquefasciatus* (Luxananil *et al*., 2001; Mukhopadhyay and Chatterjee, 2016; Colvin *et al*., 2020; Seal and Chatterjee, 2022). Maji *et al.* (2013) demonstrated involvement of *B. cereus* in developmental processes of *Drosophila ananassae* also. Similarly, specific species of *B. cereus*, *Acidomonas* sp., *Streptomyces aureus*, *P. fluorescens*, *Enterobacter cloacae* and *Aspergillus niger* were also found in the digestive tracts of *Cx. quinquefasciatus* larvae reared in the laboratory (Wang *et al.,* 2021). Antibiotic sensitivity tests (Table 2) revealed their effects on the bacteria and effective antibiotics which can be used to cure the larvae. Previous studies also suggested that reduction of microbial load in aquatic habitats (Strand, 2018) and antibiotic treatment of larvae ofseveral mosquito species (Wotton *et al.,* 1997; Chouaia *et al*., 2012; Seal and Chatterjee, 2022)can delay development of the larvae into pupae and increase mortality rates. Introduction of CGB1 variant of *B. cereus* in the rearing medium improved development of larvae and pupae, and reduced mortality (Table 3). Similarly, introduction of certain bacteria to antibiotic-treated larvae has been found to promote normal growth of larvae (Mitraka *et al*., 2013; Diaz-Nieto *et al*., 2016). Furthermore, previous research endeavours (Wang *et al*., 2021; Chandel *et al*., 2013; Luxananil *et al*., 2001) also had postulated that *B. cereus* can inhabit in the gastrointestinal tract of the larvae and play a pivotal role in bolstering larval survival and foster their developmental processes. Experimental targeting of the antibiotics towards *B. cereus* CGB1 decreased both larval survival rate and adult emergence (Table 3). Therefore, the results strongly suggest that the presence of CGB1 is crucial for larval survival and development (Table 3) and collective reports suggest that absence of CGB1 can reduce larval survival i.e. interfere with their development. Moreover, presence of commensal gut microbiota within mosquitoes would modulate viability of pathogens or viruses in gastrointestinal tracts, thereby facilitate their transmission via mosquito vectors (Ramirez *et al*., 2014). Consequently, understanding on the role of gut bacteria on delineation of vector competence would pave the way for formulating strategies to manipulate gut microbiota for vector control.

Secondary metabolites produced by plants, such as, tannins, flavonoids, alkaloids and other aromatic compounds play a crucial role ondefence mechanisms of the microorganisms and insects (Mukhopadhyay and Chatterjee, 2016; Chatterjee *et al*., 2023). Several plant species of different families have been found to possess insecticidal properties. *M. oleifera*, for instance, has been reported to exhibit antibacterial properties (Pontual *et al*., 2012) and has been shown to negatively impact growth and development of the mosquito larvae. The results indicate that the application of aqueous leaf extract of *M. oleifera* in the breeding habitat water of wild mosquito larvae led to lower larval development and pupal emergence. Use of *M. oleifera* extracts in breeding waters resulted in lower survival rates and adult emergence of the larvae which suggests that presence of CGB1 did not protect from the negative effects of *M. oleifera* on larval development and survival. However, further research is needed to understand the mechanism of antagonistic effect of

M. oleifera on *Cx. quinquefasciatus* larvae and whether it has specific or broad spectrum effect on all gut bacteria. Presence of trypsin inhibitor activity in *M. oleifera* flowers (Pontual *et al*., 2012) could be responsible for larvicidal activity. Trypsin inhibitors are known to interfere with the activity of trypsin enzyme which is involved in forming complexes with trypsin and inhibiting its activity in digestion. The polypeptide band of 169.9 k Da would bean aggregate of various molecules or complexes formed between the trypsin inhibitor and contaminants from the *M. oleifera* flower extract. It suggests that the larvicidal activity of *M. oleifera* against *A. aegypti* larvae may be mediated by trypsin inhibitor activity which could disrupt larval digestion and ultimately lead to larval mortality. Further research could investigate the specific mechanisms by which the trypsin inhibitor or other compounds in *M. oleifera* exert their larvicidal effects on mosquito larvae. The results of the study correspond with earlier researches suggesting that protease inhibitors, like those present in *M. oleifera* leaf extracts, may have antibacterial properties due to their ability to block microbial enzymes or interact with bacteria's plasma membrane proteins which would damage cellular permeability and ultimately lead to death of the microorganism (Kim *et al*., 2005; Fear *et al*., 2007; Li *et al*., 2007). Significant inhibitory effect of *M. oleifera* leaf extract against *B. cereus* which is a symbiotic normal flora of *Cx. quinquefasciatus* larvae suggests that the extract could be used to control the symbiotic bacterial flora of *Cx. quinquefasciatus* in mosquito control programs. Further research would explore the mechanisms underlying the antibacterial activity of *M. oleifera* leaf extract against *B. cereus* and its potential application in vector control strategies.

CONCLUSION

In conclusion, the research conducted in this study suggests that *M. oleifera* leaf extract possesses larvicidal activity against *Cx. quinquefasciatus* larvae, resulting in larval mortality and arresting of larval development. Furthermore, the antibacterial activity of the protease inhibitors present in the leaf extract could be advantageous for their potential use in strategies for mosquito borne disease control as they exhibit inhibitory effects against the symbiotic gut bacteria *B. cereus* which is associated with *Cx. quinquefasciatus* larvae. Further studies could explore the specific mechanisms underlying the larvicidal and antibacterial activities of *M. oleifera* leaf extract and its potential application as an agent of mosquito vector control programs to suppress vector-borne diseases.

ACKNOWLEDGEMENT

The authors are thankful to the Department of Zoology, The University of Burdwan for providing infrastructural facilities to carry out the work.

Funding

No particular grant from funding organizations in the public, private, or nonprofit sectors was given to this research.

Consent to Participate: All the authors provided their consent to participate.

Consent to Publish: The authors provided full consent for publication of the data.

Authors Contributions: Conceptualization, design and interpretation of the relevant literature were significantly aided by the contributions of authors SNC, TKD, AM and BS. BS carried out the research. The authors read and gave their approval to the final article.

Competing Interests: The authors have no conflict of interests to declare.

Availability of data and materials: This article contains all of the data generated during this investigation.

REFERENCES

Alphey, L., Beard, C.B., Billingsley, P., Coetzee, M., Crisanti, A., Curtis, C., Eggleston, P., Godfray, C., Hemingway, J., Jacobs-Lorena, M. and James, A.A. 2002. Malaria control with genetically manipulated insect vectors. *Science.* 298(5591): 119-121.

Barry, A.L. 1986. Procedure for testing antimicrobial agents in agar media: theoretical considerations. *Antibiotics in Laboratory Medicine*, pp.1-26.

Bauer, A.W.M.M., Kirby, W.M.M. and Sherris, J.C.T., 1966. turck, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology.* 45(4): 493.

Beard, B.C., Cordon-Rosales, C. and Durvasula, R.V. 2002. Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. *Annual Review of Entomology.* 47: 123-141

Chandel, K., Mendki, M.J., Parikh, R.Y., Kulkarni, G., Tikar, S.N., Sukumaran, D., Prakash, S., Parashar, B.D., Shouche, Y.S. and Veer, V. 2013. Midgut microbial community of *Culex quinquefasciatus* mosquito populations from India. *Plos One.* 8(11):

e80453.

Chatterjee, S., Bag, S., Biswal, D., Paria, D.S., Bandyopadhyay, R., Sarkar, B., Mandal, A. and Dangar, T.K. 2023. Neem-based products as potential eco-friendly mosquito control agents over conventional eco-toxic chemical pesticides-A review. *Acta Tropica.* p.106858.

Chatterjee, S.N., Ghosh T.S. and Das, S. 2010. Virulence of *Bacillus cereus* as natural facultative pathogen of *Anopheles subpictus Grassi* (Diptera: Culicidae) larvae in submerged rice-fields and shallow ponds. *African Journal of Biotechnology.* 9(41): 6983-6987.

Colvin, D., Dhuri, V., Samant, M., Verma, H., Lokhande, R. and Kale, A. 2020. Proteomic and metabolomic approach to rationalize the differential mosquito larvicidal toxicity in *Bacillus* sp. isolated from the mid-gut of *Culex quinquefasciatus* mosquito larvae. *Analytical Science Advances*. 1-10. https://doi.org/10.1002/ansa.202000081.

Crotti, E., Rizzi, A., Chouaia, B., Ricci, I., Favia, G., Alma, A., Sacchi, L., Bourtzis, K., Mandrioli, M., Cherif, A. and Bandi, C. 2010. Acetic acid bacteria, newly emerging symbionts of insects. *Applied and Environmental Microbiology.* 76(21): 6963-6970.

Das, P.K., Pani, S.P. and Krishnamoorthy, K. 2002. Prospects of elimination of lymphatic filariasis in India. *Indian Council of Medical Research Bulletin.* 32:41-54

Diaz-Nieto, L.M., D. Alessio, C., Perotti, M.A. and Beron, C.M. 2016. *Culex pipiens* development is greatly influenced by native bacteria and exogenous yeast. *PLoS One*. 11(4): e0153133.

Dillon, R.J., Vennard, C.T. and Charnley, A.K. 2000. Exploitation of gut bacteria in the locust. *Nature.* 403:851

Dillon, R.J. and Dillon, V.M. 2004. The gut bacteria of insects: nonpathogenic interactions. *Annual Review of Entomology*. 49(1): 71-92.

Engel, P. and Moran, N.A. 2013. The gut microbiota of insects–diversity in structure and function. *FEMS Microbiology Reviews*. 37(5): 699-735.

Fear, G., Komarnytsky, S. and Raskin, I. 2007. Protease inhibitors and their peptidomimetic derivatives as potential drugs. *Pharmacology & Therapeutics.* 113(2): 354-368.

Gaio, A.D.O., Gusmão, D.S., Santos, A.V., Berbert-Molina, M.A., Pimenta, P.F. and Lemos, F.J. 2011. Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (Diptera: Culicidae) (L.). *Parasites & Vectors*. 4(1): 1-10.

Gidamis, A.B., Panga, J.T., Sarwatt, S.V., Chove, B.E. and Shayo, N.B. 2003. Nutrient and antinutrient contents in raw and cooked young leaves and immature pods of *Moringa oleifera*, Lam. *Ecology of Food and Nutrition.* 42(6): 399-411.

Gray, D.A., Dugar, G., Gamba, P., Strahl, H., Jonker, M.J. and Hamoen, L.W. 2019. Extreme slow growth as alternative strategy to survive deep starvation in bacteria. *Nature Communications*. 10(1): 890.

Gusmão, D.S., Santos, A.V., Marini, D.C., Bacci Jr, M., Berbert-Molina, M.A. and Lemos, F.J.A. 2010. Culture-dependent and culture-independent characterization of microorganisms associated with *Aedes aegypti* (Diptera: *Culicidae*) (L.) and dynamics of bacterial colonization in the midgut. *Acta Tropica*. 115(3): 275-281.

Imohiosen, O., Gurama, H.H. and Lamidi, T.B. 2014. Phytochemical and antimicrobial studies on *Moringa oleifera* leaves extracts. *Journal of environmental Science, Toxicology and. Food Technology*. 8: 39-45.

Jansen, J.F., de Brabander-van den Berg E.M.M. and Meijer, E.W. 1994. Encapsulation of guest molecules into adendritic box. *Science.* 266(5188): 1226-1229.

Kim, J.Y., Park, S.C., Kim, M.H., Lim, H.T., Park, Y. and Hahm, K.S. 2005. Antimicrobial activity studies on a trypsin-chymotrypsin protease inhibitor obtained from potato. *Biochemical and Biophysical Research Communications*. 330(3): 921-927.

Lacey, L.A. 1997. *Manual of Techniques in Insect Pathology*. Academic press, New York.

Li, J., Zhang, C., Xu, X., Wang, J., Yu, H., Lai, R. and Gong, W. 2007. Trypsin inhibitory loop is an excellent lead structure to design serine protease inhibitors and antimicrobial peptides. *The FASEB Journal.* 21(10): 2466-2473.

Luxananil, P., Atomi, H., Panyim, S. and Imanaka, T. 2001. Isolation of bacterial strains colonizable in mosquito larval guts as novel host cells for mosquito control. *Journal of Bioscience and Bioengineering*. 92(4): 342-345.

Maji, P., Chakrabarti, C. and Chatterjee, S. 2013. Isolation and characterization of symbiotic bacteria from the midgut of natural population of *Drosophila ananassae*. *International Journal of Pharma and Bio Sciences.* 4(3): B697-B707.

Micks, D.W. and Ferguson, M.J. 1961. Microorganisms associated with Mosquitoes. III. Effect of Reduction in the Microbial Flora of *Culex fatigans* Wiedemann on the Susceptibility to *Plasmodium relictum* Grassi and Feletti. *Journal of Insect Pathology*. 3(3): 244-228.

Mitraka, E., Stathopoulos, S., Siden-Kiamos, I., Christophides, G.K. and Louis, C. 2013. *Asaia*accelerates larval development of *Anopheles gambiae*. *Pathogens and Global Health*. 107(6): 305-311.

Moura, M.C., Pontual, E.V., Gomes, F.S., Napoleão, T.H., Xavier, H.S., Paiva, P.M. and Coelho, L.C.B.B. 2011. Preparations of *Moringa oleifera* flowers to treat contaminated water. *Advances in Environmental Research.* 21: 269-285.

Mourya, D.T., Ilkal, M.A., Mishra, A.C., Jacob, P.G., Pant, U., Ramanujam, S., Mavale, M.S., Bhat, H.R. and Dhanda, V. 1989. Isolation of Japanese encephalitis virus from mosquitoes collected in Karnataka state, India from 1985 to 1987. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 83(4): 550-552.

Mukhopadhyay, P. and Chatterjee, S. 2016. Characterization and control of symbiotic Bacillus cereus isolated from the mid gut of *Anopheles*

subpictus Grassi. *Journal of Parasitic Diseases.* 40(4): 1414-1421. DOI 10.1007/s12639-015-0704

Peck, G.W. and Walton, W.E. 2006. Effect of bacterial quality and density on growth and whole-body stoichiometry of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae). *Journal of Medical Entomology.* 43(1): 25-33.

Pelczar, M.J., Bard, R.C., Burnett, G.W., Conn, H.J., Demoss, R.D, Euans, E.E., Weiss, F.A., Jennison, M.W., Meckee, A.P., Riker, A.J., Warren, J. and Weeks, O.B. 1957. *Manual of Microbiological Methods. Society of American Bacteriology.* McGraw Hill Book Company Inc., New York.

Pontual, E.V., Napoleão, T.H., Dias de Assis, C.R., de Souza Bezerra, R., Xavier, H.S., Navarro, D.M.D.A.F., Coelho, L.C.B.B. and Paiva, P.M.G. 2012. Effect of *Moringa oleifera* flower extract on larval trypsin and acethylcholin esterase activities in *Aedes aegypti*. *Archives of Insect Biochemistry and Physiology*. 79(3): 135-152.

Ramirez, J.L., Short, S.M., Bahia, A.C., Saraiva, R.G., Dong, Y., Kang, S., Tripathi. A., Mlambo, G. and Dimopoulos, G. 2014. *Chromobacterium* Csp_P reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and *in vitro* anti-pathogen activities. *PLoS Pathogens.* 10: e1004398. https://doi.org/10.1371/journal.ppat.1004398.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* 4: 406-425

Seal, M. and Chatterjee, S. 2022. Characterizations of Larval Gut Bacteria *of Anopheles subpictus Grassi* (1899) and their Role in Mosquito Development in Hooghly, West Bengal, India. *Applied Biochemistry and Biotechnology*. 1-24.

Service, W.M. 1993. *Mosquito Ecology: Field Sampling Methods.* Chapman and Hall, London, 1-988

Smibert, R. and Krieg, N.R. 1995. Phenotypic testing. Methods for General and Molecular Bacteriology. *American Society for Microbiology.* 607-654.

Strand, M.R. 2018. Composition and functional roles of the gut microbiota in mosquitoes. *Current Opinion in Insect Science*. 28: 59-65.

Suo, P., Wang, K., Yu, H., Fu, X., An, L., Bhowmick, B., Zhang, J. and Han, Q. 2022. Seasonal Variation of Midgut Bacterial Diversity in *Culex quinquefasciatus* Populations in Haikou City, Hainan Province, China. *Biology*. 11(8): 1166.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 22: 4673-4680.

Truong, D.H., Nguyen, D.H., Ta, N.T.A., Bui, A.V., Do, T.H. and Nguyen, H.C. 2019. Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of *Severinia buxifolia*. *Journal of Food Quality.* 2019(1): 1-9.

Tyagi, B.K., Munirathinam, A. and Venkatesh, A. 2015. A catalogue of Indian mosquitoes. *International Journal of Mosquito Research*. 2(2): 50-97.

Wang, Y.T., Shen, R.X., Xing, D., Zhao, C.P., Gao, H.T., Wu, J.H., Zhang, N., Zhang, H.D., Chen, Y., Zhao, T.Y. and Li, C.X. 2021. Metagenome sequencing reveals the midgut microbiota makeup of *Culex pipiens quinquefasciatus* and its possible relationship with insecticide resistance. *Frontiers in Microbiology*. 12: 625539.