Characterizations of Larval Gut Bacteria of *Anopheles subpictus* Grassi (1899) and their Role in Mosquito Development in Hooghly, West Bengal, India

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Abstract

Malaria is a serious vector borne disease transmitted by different species of *Anopheles* mosquitoes. The present study was aimed to isolate and characterize the bacterial flora from the gut of larvae of *An. subpictus* Grassi (1899) prevalent in Hooghly and explore their roles in host survival and development. Mosquito larvae and adults were collected from field and were maintained in laboratory. Bacterial load in the larval mid-gut was determined, and predominant strains were isolated and characterized by polyphasic approach. Role of these bacteria in larval survival and development were assayed. Bacterial load in the gut of larvae was found to vary in field-collected and lab-reared mosquitoes in different seasons. Morphological, bio-chemical, and molecular analyses explored four common bacterial isolates, namely *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus cereus*, and *Proteus vulgaris* in the larval gut throughout the year. Larval survival rate was greatly reduced (0.06) and time of pupation was prolonged (17.8 ± 0.57) [days] in the absence of their gut bacteria. Total tissue protein (7.78 ± 0.56) [µg/mg], lipid (2.25 ± 0.19) [µg/mg] & carbohydrate (16.5 ± 0.79) [µg/mg] contents of larvae, and body weight & wing length of adult male (0.17 ± 0.02 & 1.74 ± 0.43) [mm] & female (0.19 ± 0.02 & 1.99 ± 0.46) [mm] mosquitoes were also found to be greatly reduced in the absence of gut bacteria. Developmental characteristics were restored with the introduction of culture suspension of all four resident gut bacterial isolates. Present study indicates that the mosquitoes largely depend on their gut bacteria for their survival and development. So, manipulation or control of this gut bacterial communities might inhibit survival and development of vector mosquitoes.

Keywords

Malaria · *Anopheles subpictus* · Mosquito · Bacteria · Development · Vector
Introduction

Mosquitoes of different genera like *Anopheles*, *Culex*, and *Aedes* serve as vectors of numerous disease-causing pathogens throughout different parts of the world [1, 2]. Among them, different species of *Anopheles* mosquitoes serve as vectors of malarial disease, which is one of the major cause of world-wide mortality and morbidity [3, 4]. *Anopheles subpictus* Grassi 1899 (sub species A, freshwater breeder) has been reported to act as a potent malarial vector in different regions of the world [5–7] including rural areas of Hooghly, West Bengal, India [8]. Transmission of pathogens by mosquito vectors depends on numerous factors including the climatic condition of the region [9], host-vector contact [10], and physiology of the host [11]. All insects including mosquitoes harbor a large number of microbial communities in their gut environment, some of them have symbiotic associations with their hosts [12, 13]. These microbial communities play different roles in several life history processes of their hosts starting from their survival and development [13, 14] to their reproduction [15]. They are also involved in host nutrition by secreting different enzymes which helps to degrade complex molecules into digestible simpler form [16]. They also produce essential nutrients like vitamins or amino acids for their host [17, 18]. In the blood-sucking insects, gut bacteria play important role in blood digestion by their host [19]. These microbial communities are also a part of host natural immune system [20]. Even they can modulate the vectorial capacity of their hosts [21]. Previously, a large number of bacterial communities have been reported by several researchers from gut of both larval and adult forms of anopheline mosquitoes [22–28]. Some earlier studies have also shown that removal of gut bacteria from mosquito body had several detrimental effects on their host organisms, including their late development, higher mortality, and several adult morphological abnormalities [12–14, 29]. But there is a dearth of literature in the gut bacterial species and their impact on *An. subpictus* mosquitoes prevalent in rural fresh water bodies of Hooghly district, West Bengal.

So, the present study is aimed to characterize the larval gut bacteria of *An. subpictus* mosquitoes of rural areas of Hooghly, West Bengal, by polyphasic approach to confirm their identity and their subsequent role in larval survival and development. Proper identification of beneficial gut bacteria of *An. subpictus* and their subsequent control might generate an avenue of vector management programme in the study areas.

Materials and Methods

**Mosquito Collection and Identification** Larvae of *An. subpictus* were collected from water bodies of some rural areas of Hooghly district (23° 01’ 20” N to 22° 39’32” N and 87° 30’ 20” E to 88° 30’ 15” E) by using standard dipper of 250-mL capacity following standard protocols [30, 31]. Adult mosquitoes were collected within test tubes from cattle sheds and human habitations by hand collection method. All the collections were done once in summer, monsoon, post-monsoon, and winter from March 2017 to February 2018 during early morning or in the afternoon. After collection, the larvae and adult mosquitoes were brought to the Parasitology & Microbiology Research Laboratory, Department of Zoology, The University of Burdwan. Identification of larval and adult forms of mosquitoes were done under microscope following standard keys [32, 33]. Adult *An. subpictus* mosquitoes were maintained in a mosquito rearing cage (30 cm×30 cm×30 cm) in optimum
laboratory condition for egg laying and larval hatching. Some specimens were also sent to Zoological Survey of India, Kolkata, for the reconfirmation of the species identification.

**Isolation of Bacteria** Both field-collected and laboratory-reared 3rd instar larvae (5–10 in number) of *An. subpictus* were surface sterilized by placing them in 70% alcohol for 3–5 min. Then they were washed with double distilled water and mid-gut of the larvae was dissected carefully in aseptic condition under a binocular (2X magnification). Mid-gut content for laboratory-reared and field-caught larvae were mixed separately with 1 mL sterile distilled water and from this mixture 100 µL was mixed with sterilized moderately cooled nutrient agar media and poured on five sterile Petri plates. The plates were incubated at 37 ± 2 °C temperature in a B.O.D incubator for 24–48 h. On the next day, the total number of colonies that were formed over the nutrient agar plates was counted and bacterial load in the mid-gut of larvae was determined as colony-forming unit (cfu/mL). Colonies that were formed on the nutrient agar plates were characterized by recording different colony characters (size, shape, color, opacity, elevation, and consistency). Pure cultures of the bacterial isolates were maintained on nutrient agar slants in a refrigerator at 4 ± 1 °C temperature for further analysis.

**Characterizations of Larval Gut Bacterial Isolates**

**Phenotypic Characterizations**

Phenotypic properties of bacterial isolates such as Gram staining properties and presence of endospores were evaluated by staining with Gram’s stain and 5% Malachite green solutions respectively. Scanning electron microscopic analysis was performed to observe the surface morphologies of vegetative cells of the bacterial isolates [13].

**Bio-chemical Characterizations**

Several bio-chemical properties of the bacterial isolates such as catalase methyl red; indole; Voges Proskauer test; citrate test; oxidase test; urease test; growth on triple sugar iron (TSI) agar; and starch, protein & lipid hydrolysis test, nitrate reduction test, and fermentation ability of twenty different carbohydrate sources such as dextrose (De), fructose (Fc), galactose (Ga), sucrose (Su), lactose (La), mannitol (Mn), cellobiose (Ce), dulcitol (Du), trehalose (Te), raffinose (Rf), sorbitol (Sb), xylose (Xy), melibiose (Mb), rhamnose (Rh), arabinose (Ar), mannosé (Mo), lactose (La), adonitol (Ad), salicin (Sa), and inulin (In) were performed following standard methodologies [34–36].

**Physiological Characterizations**

Physiological properties of the gut bacterial isolates like sodium chloride (NaCl) tolerance, growth at different pH scale, and different temperature of the culture media were recorded following standard methodologies [34–36].

**Molecular Characterizations**

Genomic DNA were isolated from the overnight grown liquid bacterial cultures following standard method [37] and 16S rDNA sequences were amplified by polymerase
chain reaction by using 27F forward and 1492R primer. The PCR purified products were sequenced bi-directionally using universal primers. The obtained 16S rRNA gene sequences of the bacterial isolates were submitted to NCBI GenBank. Nucleotide percentages of 16S rRNA gene sequences of the bacterial isolates were calculated using Aqua software. Phylogenetic analysis was done by neighbour-joining method using MEGA X software [38].

**Antibiotic Sensitivity of Bacterial Isolates**

Susceptibility of the bacterial isolates to twenty different commercially available standard antibiotics, namely bacitracin (10 µg/disc), nalidixic acid (30 µg/disc), kanamycin (30 µg/disc), ampicillin (10 µg/disc), amoxicillin (10 µg/disc), penicillin (10 µg/disc), levofloxacin (5 µg/disc), chloramphenicol (30 µg/disc), gentamicin (50 µg/disc), neomycin (30 µg/disc), tetracycline (30 µg/disc), ofloxacin (5 µg/disc), norfloxacin (10 µg/disc), rifampicin (5 µg/disc), ciprofloxacin (5 µg/disc), vancomycin (30 µg/disc), azithromycin (30 µg/disc), erythromycin (15 µg/disc), doxycycline (30 µg/disc), and streptomycin (10 µg/disc), were tested on Muller-Hinton agar plates by disc diffusion method [39]. Minimum inhibitory concentration of two broad-spectrum antibiotics, viz., doxycycline and levofloxacin against the bacterial isolates, was determined using MIC strip (Himedia, India).

**Role of Mid-gut Bacteria on Larval Development**

A batch of three hundred 1st instar larvae of *An. subpictus* were collected from the fresh water bodies and they were kept in a plastic tray (30 cm×30 cm) containing 1500 mL of natural habitat water. Out of these total collected specimens, a group of thirty larvae were maintained in a separate tray containing 100 mL natural habitat water. This group of larvae were provided with UV sterilized larval food and without any treatment which served as conventionally reared control group. The rest of the larvae were fed with UV sterilized larval food and a mixture of doxycycline and levofloxacin (1:1 ratio) at their minimum inhibitory concentration for 1 day to clear the gut bacterial communities. Another group comprised of thirty 1st instar larvae were separated in a plastic tray containing sterile distilled water with UV sterilized larval food. This group served as axenic group. The rest of the antibiotic-treated axenic larvae were separated in five trays (30 larvae in each) containing 95 mL sterile distilled water with UV sterilized larval food. Axenic larvae in the 1st to 4th trays were provided with 5 mL suspensions of previously isolated individual gut bacteria for their recolonization within larval gut and in the 5th tray axenic larvae were provided with 5 mL suspension containing a mixture of all gut bacterial isolates. All the larvae were maintained in aseptic condition at 30 ± 2 °C temperature, 75 ± 5% relative humidity, and 12:-12-h (light: dark) photoperiod. Five replicates were done for each of the treatment and control groups. The time of pupa formation for each group of larvae was noted down. After pupal emergence, pupae from each batch were kept in separate mosquito rearing cages (30 cm×30 cm×30 cm) and were maintained at 30 ± 2 °C temperature, 75 ± 5% relative humidity, and 12:-12-h (light: dark) photoperiod. The number of adult mosquitoes emerged from each group was noted down. Adult males and females emerged from each group were kept separately in test tubes with chloroform applied cotton plugging for 6 and 7 days. Dry weight of male and female mosquitoes from each group was measured. Wings of male and female mosquitoes were dissected out and mounted on glass slide with DPX solution.
images of wings were captured by stereo zoom microscope (Olympus) and the length and breadth of each of the wing were measured with M Shot software.

**Bio-chemical Profile of Larvae in the Presence and Absence of Gut Bacteria**

The total tissue carbohydrate and protein & lipid content of 4th instar larvae in each of the treated and control groups were evaluated. For that purpose, thirty synchronized 4th instar larvae were selected from each group. The total tissue carbohydrate and lipid were extracted [40]. Carbohydrate estimation was done by anthrone reagent [41]. Lipid quantification was done by vanillin-reagent method [42]. Total tissue protein was extracted [43] and estimated using foline-ciocalteau reagent [44].

**Statistical Analyses**

One-way ANOVA was done to compute any significant differences in pupation time, rate of survival up to adulthood, and body weight and wing length of adult male and female *An. subpictus* mosquitoes and bio-chemical contents of 4th instar larvae among different treatment groups. Dunnett’s multiple comparison tests were performed to evaluate significant difference of each of the treatment group from control group. All the analyses were performed in GraphPad prism 9.0.0.

**Results**

**Bacterial Characterizations of Mosquito Larval Gut**

Bacterial load in the form of colony-forming unit (cfu/mL) in the mid-gut of both field-collected and laboratory-reared 3rd instar larvae of *An. subpictus* was evaluated and is given in Table 1. Bacterial load in all the four seasons was found to be higher in the mid-gut of field-collected larvae than laboratory-reared one and among four seasons, post-monsoon larvae harboured higher bacterial populations in their mid-gut; whereas during winter season, gut bacterial populations of both field-collected and laboratory-reared larvae were found to be lowest. Among seventeen bacterial isolates recorded by colony morphologies, four isolates (HALG2, HALG4, HALG6, & HALG7) were found to be common in all the four seasons in both field-collected and laboratory-reared larvae. So, these four bacterial isolates were assumed to be as the resident bacterial flora of larval mid-gut and further characterizations were done for these four bacterial isolates. Colony characteristics of these

| Season          | Cfu/mL         |            |
|-----------------|----------------|------------|
|                 | Field collected| Laboratory reared |
| Summer          | $10.5 \pm 0.72 \times 10^6$ | $8.4 \pm 0.66 \times 10^4$ |
| Monsoon         | $24.13 \pm 1.3 \times 10^6$ | $12.8 \pm 0.60 \times 10^5$ |
| Post-monsoon    | $16.26 \pm 1.1 \times 10^7$ | $19 \pm 2.5 \times 10^5$ |
| Winter          | $8.6 \pm 0.40 \times 10^5$ | $8 \pm 0.67 \times 10^4$ |

Values are averages (Mean ± SE) of three replicates
isolates have been depicted in Table 2. Gram staining and endospore staining revealed that three isolates (HALG2, HALG4, & HALG6) were rod-shaped Gram-positive bacteria and were able to produce endospores, whereas HALG7 was Gram-negative bacterial isolate and was negative for endospore production (Table 3). Scanning electron microscopic analysis showed that the organisms of four bacterial isolates were rod shaped with single-chain or in short-chain arrangement (Fig. 1a–d).

Bio-chemical characteristics and carbohydrate fermentation abilities of these bacterial isolates have been tabulated in Table 4 and Table 5 respectively. All four bacterial isolates showed positive results for catalase test. None of the isolates were able to produce indole except HALG7. Among four bacterial isolates, two isolates (HALG6 & HALG7) showed positive reaction for methyl red test whereas rest two isolates (HALG2 & HALG4) showed negative reaction for methyl red test. All the four isolates showed negative results for VP test. All the four bacterial isolates were negative for citrate utilization test. Among four bacterial isolates, three isolates (HALG2, HALG6, & HALG7) were able to reduce nitrate. Two of the isolates (HALG4 & HALG6) were able to produce oxidase enzymes, whereas the rest of the two isolates (HALG2 & HALG7) were negative for oxidase production. Except HALG7, none of the isolates were able to produce urease enzyme. All the four isolates were able to degrade starch, lipid, and protein (gelatin) sources of the medium. Motility test showed that all the four bacterial isolates were motile in nature. The growth of the bacterial isolates in TSI agar medium showed that isolate HALG2 and HALG4 were able to utilize both glucose and sucrose or lactose; in addition to that, during fermentation, HALG4 produced gas also, whereas HALG6 and HALG7 were able to utilize glucose only. Among the four bacterial isolates, only one isolate (HALG2) could able to utilize inositol, arabinose, mannitol, sorbitol, raffinose, mannose, and inulin. Sucrose was fermented by all of the isolates except HALG6. Two of the isolates (HALG2 & HALG4) were able to ferment cellobiose; whereas, another two isolates HALG6 & HALG7 could not able to ferment this carbohydrate source. Xylose was utilized as fermentable carbon sources by only one isolate (HALG7), but the rest of the three isolates (HALG2, HALG4, & HALG6) were unable to ferment xylose.

All the four bacterial isolates showed NaCl tolerance up to 4% of the growth medium. pH tolerance test revealed that all the isolates were able to grow from pH 5–pH 11 of the media although the bacterial growth increased in the pH range between 7 and 10.5 (Fig. 2). Growth of the bacterial isolates at different temperatures of the environment showed that although they could grow from 15 to 45 °C temperature of the medium, but optimum temperature for their growth ranged between 30 and 35 °C (Fig. 3).

Analysis of nucleotides of 16S rRNA gene sequences of the bacterial isolates revealed that AT & GC content of 16S rRNA gene sequences of HALG2 was 44.15% and 55.84%. The figures were 44.32% and 55.68% for HALG4, 46% and 54% for HAGL6, and 46% and 54% for HAGL7 respectively (Fig. 4).

Phylogenetic tree of the bacterial isolates was constructed by neighbor-joining method which showed that HALG2 (MN894011) had direct branching with Bacillus subtilis (MK367787) with 64% bootstrap value (Fig. 5), HALG4 (MZ363627) branched directly with Bacillus pumilus (KY608827) with 82% bootstrap value (Fig. 6), HALG6 (MZ363632) had direct branching with Bacillus cereus (JX645714) with 40% bootstrap value (Fig. 7), and HALG7 (MZ363637) branched directly with Proteus vulgaris (MG596968) with 60% bootstrap value (Fig. 8). So, after phenotypic, bio-chemical,
Table 2  Colony characteristics of resident gut bacterial isolates of 3rd instar larvae of *Anopheles subpictus* mosquito

| SI no | Name of isolates | Colony characters |
|-------|------------------|-------------------|
|       |                  | Shape | Size (mm) (Mean ± SD) | Opacity | Elevation | Consistency | Margin | Color         |
| 1     | HALG2            | Round | 2.10±0.15             | Opaque  | Flat      | Dry         | Irregular | White         |
| 2     | HALG4            | Round | 1.72±0.28             | Opaque  | Flat      | Moist       | Irregular | Off white     |
| 3     | HALG6            | Round | 2.52±0.39             | Opaque  | Slightly elevated | Moist | Smooth | Off white |
| 4     | HALG7            | Round | 2.78±0.19             | Opaque  | Elevated  | Sticky      | Smooth   | Glossy white |
and molecular analyses, it was confirmed that the four resident gut bacterial isolates (HALG2, HALG4, HALG6, & HALG7) were *Bacillus subtilis, Bacillus pumilus, Bacillus cereus, and Proteus vulgaris* respectively.

Sensitivity of these four resident larval gut bacterial isolates to different commercially available standard antibiotics with their zone diameter inhibition values have been depicted in Table 6. Formation of clear zone surrounding the antibiotic disc on the culture plates indicated their sensitivity to particular antibiotics, whereas the absence of any clear zone surrounding the antibiotic disc indicated their resistant property against that particular antibiotic. Minimum inhibitory concentration of two broad-spectrum
### Table 4  Bio-chemical characteristics of the resident bacterial isolates from the mid-gut of 3rd instar larvae of *Anopheles subpictus*

| Name of the bio-chemical tests | Name of the bacterial isolates |
|--------------------------------|--------------------------------|
|                                | HALG2 | HALG4 | HALG6 | HALG7 |
| Catalase production            | +     | +     | +     | +     |
| Indole production              | −     | −     | −     | +     |
| Methyl red (MR)                | −     | −     | +     | +     |
| Voges Proskauer (VP)           | −     | −     | −     | −     |
| Citrate utilization            | −     | −     | −     | −     |
| Nitrate reduction              | +     | −     | +     | +     |
| Oxidase production             | −     | +     | +     | −     |
| Urease production              | −     | −     | −     | +     |
| Starch hydrolysis              | +     | +     | +     | +     |
| Lipid hydrolysis               | +     | +     | +     | +     |
| Gelatine hydrolysis            | +     | +     | +     | +     |
| Motility                       | +     | +     | +     | +     |
| Triple sugar iron agar (TSI)   | A/A   | A/A, G| K/A   | K/A   |
| H₂S production                 | −     | −     | −     | +     |

+ positive, − negative, K alkaline, A acidic, G gas

### Table 5  Carbohydrate fermentation tests of gut bacterial isolates of 3rd instar larvae of *Anopheles subpictus*

| Carbohydrate source | HALG2 | HALG4 | HALG6 | HALG7 |
|---------------------|-------|-------|-------|-------|
| Dextrose (De)       | +     | +     | +     | +     |
| Sucrose (Su)        | +     | +     | −     | +     |
| Lactose (La)        | −     | −     | −     | −     |
| Trehalose (Te)      | +     | +     | +     | +     |
| Fructose (Fc)       | +     | +     | +     | +     |
| Galactose (Ga)      | −     | −     | −     | −     |
| Inositol (IS)       | +     | −     | −     | −     |
| Arabinose (Ar)      | +     | −     | −     | −     |
| Mannitol (Mn)       | +     | −     | −     | −     |
| Cellobiose (Ce)     | +     | +     | −     | −     |
| Melibiose (Mb)      | −     | −     | −     | −     |
| Dulcitol (Du)       | −     | −     | −     | −     |
| Xylose (Xy)         | −     | −     | −     | +     |
| Adonitol (Ad)       | −     | −     | −     | −     |
| Rhamnose (Rh)       | −     | −     | −     | −     |
| Sorbitol (Sb)       | +     | −     | −     | −     |
| Raffinose (Rf)      | +     | −     | −     | −     |
| Mannose (Mo)        | +     | −     | −     | −     |
| Salicin (Sa)        | −     | −     | −     | −     |
| Inulin (In)         | +     | −     | −     | −     |

+ indicate positive for fermentation, − indicates negative result for fermentation
antibiotics (levofloxacin & doxycycline) against all the four isolates has been given in Table 7.

Role of Mid-gut Bacteria on Larval Development

Time required for pupa formation from 1st instar larvae varied significantly among different treatment groups (ANOVA: $F (6, 28) = 51.02$, $p < 0.0001$). The longest time for pupa formation was taken by antibiotic-treated axenic group of larvae (average 17.8 days), whereas the conventionally reared larvae (control group) were converted into pupae on an average 7.8 days. One-way ANOVA followed by Dunnett’s multiple comparison test showed that there was significant difference in time of pupa formation between conventionally reared control group and antibiotic-treated axenic group of larvae ($p < 0.0001$), conventionally reared control group and Bacillus subtilis HALG2-treated larval group.
Fig. 4 Nucleotide composition of 16S rRNA gene sequences of resident gut bacterial isolates of larvae of *Anopheles subpictus*.

Fig. 5 Construction of phylogenetic tree on the basis of 16S rRNA gene sequence of HALG2 (MN894011) strain along with the other 16S rRNA gene sequences retrieved from NCBI and RDP.
(\(p<0.0001\)), and conventionally reared control group and Bacillus pumilus HALG4-treated larval group (\(p=0.0019\)), whereas there were no significant differences in time of pupa formation between conventionally reared control group and Bacillus cereus HALG6, Proteus vulgaris HALG7, and all four isolate-treated groups (\(p>0.05\)) (Fig. 9). Survival of antibiotic-treated axenic group of 1st instar larvae and 1st instar axenic larvae that were individually cured with gut bacterial isolates showed significant variation in rate of survival when compared with survival rate of conventionally reared control larvae (\(p<0.0001\)). But the survival rate of 1st instar larvae up to adulthood did not show any significant differences among axenic larvae that were treated with a mixture of all four gut bacterial isolates and conventionally reared control groups (\(p>0.05\)) (Fig. 10).

Body weight of adult male and female An. subpictus mosquitoes that had been developed from conventionally reared control larval groups was found to range between 0.25–0.39 mg and 0.26–0.45 mg respectively. Body weight of adult male An. subpictus was significantly reduced in all treatment groups than conventionally reared control group (\(p<0.01\)). Similarly, a significant difference of body weight of adult female An. subpictus (developed from axenic larvae and axenic larvae that were subsequently colonized by individual gut bacterial isolates) was observed when compared with adult females developed from control larvae (conventionally reared group) (\(p<0.0001\)); only the body weight of adult female developed from mixture of all four gut bacteria-treated axenic 1st instar larval group did not show any significant difference with body weight of adult female An.
subpictus developed from conventionally reared group ($p=0.2452$) (Fig. 11A, B). Wing length of adult male and female An. subpictus mosquitoes developed from conventionally reared control larval groups ranged between 2.91–3.99 mm and 3.08–3.99 mm respectively. One-way ANOVA showed significant variations in wing length of both male ($F(6,135)=54.53, p<0.0001$) and female ($F(6,152)=47.98, p<0.0001$) of different groups of mosquitoes. Dunnett’s multiple comparison test revealed that mean wing length of adult male and female An. subpictus was reduced significantly in all treatment groups than conventionally reared one ($p<0.0001$) (Fig. 11C, D). Correlation between body weight and wing length of adult mosquitoes showed that wing length of both male and female An. subpictus mosquitoes had strong positive correlation with their body weight (Fig. 12A, B).

Total tissue carbohydrate, protein, and lipid contents have been given in Table 8. Average values of total tissue carbohydrate, protein, and lipid content of conventionally reared control larvae (at 4th instar stage) were 32.48±0.88 µg/mg, 16.89±0.94 µg/mg, and 6.38±0.31 µg/mg respectively. One-way ANOVA revealed that all three biomolecular contents varied significantly among different groups of larvae ($p<0.0001$). Axenic larvae (at 4th instar stage) had lowest amount of all the biomolecular compounds (carbohydrate, protein, & lipid) in their body (14.27±0.76 µg/mg, 7.78±0.56 µg/mg, & 2.25±0.19 µg/mg respectively). Total tissue carbohydrate, protein, and lipid contents were found to be
significantly lower in axenic group of larvae that were individually cured with gut bacterial isolates when compared with conventionally reared control group ($p < 0.01$), but all three biomolecules of axenic larvae that were cured with a mixture of all four resident gut bacterial isolates did not show any significant difference when compared with conventionally reared larvae ($p > 0.01$).

**Discussion**

The present investigation-noted bacterial load in the mid-gut of field-collected larvae was higher than laboratory-reared group in all the seasons, which indicated that water quality of natural habitat might have some effects in determining bacterial load of mosquito larval gut and that is why gut bacterial load became reduced when cultured in a restricted aquatic environment in laboratory condition. Previous studies by several workers have also indicated that breeding habitats of mosquito play an important role in determining mosquito gut bacterial composition [45, 46]. Bacteria inhabiting in the habitat water enter in the gut of mosquito larvae through feeding [47]. Further, the present study recorded that bacterial load in the mid-gut of both field-collected and laboratory-reared 3rd instar larvae varied in

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**Fig. 8** Construction of phylogenetic tree on the basis of 16S rRNA gene sequence of HALG7 (MZ363637) strain along with the other 16S rRNA gene sequences retrieved from NCBI and RDP.
Table 6  Antibiotic sensitivity of resident gut bacteria 3rd instar larvae of *Anopheles subpictus*

| Antibiotics                  | *B. subtilis* HALG2 |  |  | *B. pumilus* HALG4 |  |  | *B. cereus* HALG6 |  |  | *P. vulgaris* HALG7 |  |  |
|------------------------------|---------------------|---|---|---------------------|---|---|---------------------|---|---|---------------------|---|---|
|                              | Sensitivity        | ZDI (mm) |  | Sensitivity        | ZDI (mm) |  | Sensitivity        | ZDI (mm) |  | Sensitivity        | ZDI (mm) |  |
| Chloramphenicol (C,30)       | S                   | 23 |  | S                   | 18 |  | S                   | 20 |  | S                   | 18 |  |
| Kanamycin (K,30)             | S                   | 24 |  | S                   | 26 |  | S                   | 20 |  | S                   | 25 |  |
| Levofoxacin (LE,5)           | S                   | 40 |  | S                   | 38 |  | S                   | 28 |  | S                   | 35 |  |
| Gentamicin (GEN,50)          | S                   | 32 |  | S                   | 29 |  | S                   | 26 |  | S                   | 31 |  |
| Neomycin (N,30)              | S                   | 28 |  | S                   | 25 |  | S                   | 20 |  | S                   | 27 |  |
| Bacitracin (B, 10)           | S                   | 9  |  | S                   | 12 |  | S                   | 13 |  | S                   | 10 |  |
| Ofloxacin (OF,5)             | S                   | 32 |  | S                   | 34 |  | S                   | 24 |  | S                   |   |  |
| Norfloxacin (NX,10)          | S                   | 36 |  | S                   | 30 |  | S                   | 24 |  | S                   |   |  |
| Tetracycline (TE,30)         | S                   | 30 |  | S                   | 36 |  | S                   | 25 |  | S                   | 27 |  |
| Ciprofloxacin (CIP, 5)       | S                   | 40 |  | S                   | 36 |  | S                   | 29 |  | S                   | 34 |  |
| Vancomycin (VA, 30)          | S                   | 21 |  | S                   | 27 |  | S                   | 19 |  | S                   | 23 |  |
| Rifampicin (RIF,5)           | S                   | 16 |  | S                   | 17 |  | S                   | 13 |  | S                   | 26 |  |
| Azithromycin (AZM,30)        | S                   | 34 |  | S                   | 29 |  | S                   | 23 |  | S                   | 29 |  |
| Erythromycin (E,15)          | S                   | 28 |  | S                   | 23 |  | S                   | 25 |  | S                   | 27 |  |
| Amoxicillin (AMX,10)         | S                   | 40 |  | S                   | 21 |  | R                   | Nil |  | S                   |   |  |
| Ampicillin (AMP,10)          | R                   | Nil |  | R                   | Nil |  | R                   | Nil |  | R                   |   |  |
| Penicillin (P,10)            | R                   | Nil |  | R                   | Nil |  | R                   | Nil |  | R                   |   |  |
| Streptomycin (S,10)          | R                   | Nil |  | S                   | 25 |  | S                   | 22 |  | S                   | 24 |  |
| Doxycycline (DO,30)          | S                   | 30 |  | S                   | 33 |  | S                   | 24 |  | S                   | 30 |  |
| Nalidixic acid (NA,30)       | S                   | 23 |  | S                   | 26 |  | S                   | 20 |  | S                   | 24 |  |

*S* sensitive, *R* resistant
different season throughout the year. Bacterial load was found to be highest in the larval gut during post-monsoon; whereas during winter season, bacterial load in the gut of larvae became reduced to a great extent. These observation gives indication that climatic conditions of the environment have a great influence on the bacterial load in the mosquito larval gut. Variations in several environmental factors in different seasons can affect bacterial diversity in natural habitat water [48, 49] and thus can alter bacterial load and composition in the mosquito larval gut, because breeding habitats serve as one of the major source of larval gut bacterial communities [50]. Similar observation was previously reported in case of *An. gambiae* and *An. coluzzii* mosquitoes from some urban regions of Ghana, where seasons of the year were reported to affect both the diversity and abundances of gut bacteria of mosquitoes [45].

A total of sixteen isolates were recorded in the mid-gut of field-collected 3rd instar larvae of *An. subpictus*, whereas only seven isolates were recorded to be present in the larval mid-gut of laboratory-reared mosquitoes throughout different seasons of the year. Among these bacterial isolates, only four isolates (HALG2, HALG4, HALG6, & HALG7) were found to be present in the mid-gut of both field-collected and laboratory-reared 3rd instar

| Table 7 | Minimum inhibitory concentration of standard antibiotics against bacterial isolates |
|---------|----------------------------------------------------------------------------------|
| Name of isolates | Doxycycline (µg/mL) | Levofloxacin (µg/mL) |
| HALG2 | 0.125 | 0.32 |
| HALG4 | 0.16 | 0.09 |
| HALG6 | 0.40 | 0.25 |
| HALG7 | 0.75 | 0.30 |

**Fig. 9** Time of pupation from 1st instar larvae of *Anopheles subpictus*. An asterisk (*) above a given bar indicates significant difference of treatment group from the conventionally reared control group as determined by Dunnett’s multiple comparison test (*p* < 0.01)
larvae of *An. subpictus*. So, these isolates were supposed to be as the resident gut bacterial flora of larval forms of *An. subpictus* in the study areas. Identification by polyphasic approach indicated that these isolates were *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus cereus*, and *Proteus vulgaris*. Some of these bacterial species were reported previously from the gut of different insect species including mosquito, such as *Bacillus subtilis* was reported in larval gut of *Aedes aegypti* mosquito [51]; *Bacillus pumilus* was reported as gut-resident of *An. stephensi* larvae [52]. *Bacillus cereus* had been reported as a symbiotic bacterial flora of larval forms of *An. subpictus* Grassi from Sundarbans areas of West Bengal [13]. *Proteus vulgaris* was reported in the gut of *Bombyx mori* larvae [53] and in the hind-gut region of *Blatta orientalis* [54].

In the present study, antibiotic treatment that clear the bacterial communities from the larval mid-gut resulted in reduced survival rate, delayed pupation time, and reduced total tissue carbohydrate, protein, and lipid contents in the final instar larvae. Further, the body weight and wing length became reduced in both adult male and female mosquitoes developed from the axenic group of larvae. Rate of survival up to adult stage and all the development markers (amount of total tissue carbohydrate, protein, and lipid) again became restored only when axenic group of larvae were cured with a mixture of all the resident gut bacteria. No single bacteria could contribute the host development and survival like conventionally reared groups. These results suggested that all the four resident gut bacterial isolates, viz., *Bacillus subtilis* HALG2 (MN894011), *Bacillus pumilus* HALG4 (MZ363627), *Bacillus cereus* HALG6 (MZ363632), and *Proteus vulgaris* HALG7 (MZ363637) of larval forms of *An. subpictus* mosquitoes prevalent in rural areas of Hooghly, together contributed in the survival and normal development of their host. These bacterial isolates also have a great impact on body weight and wing length of their host in adult forms. So, it can be considered that these four bacterial isolates have some symbiotic associations with their host. All these findings have indicated that elimination of these symbiotic bacteria from larval gut could impede the development and survival of their host mosquitoes. Similar observations were previously reported by many workers.
from different regions of the world, such as an ampicillin resistant strain of *Bacillus* sp. was reported from the larval mid-gut of *Anopheles barbirostris* mosquito which played a major role during the larval development of this mosquito [55]. They detected that killing of larval gut bacteria by antibiotic treatment delayed the developmental time of larvae of *Anopheles barbirostris*. Similarly, in the case of *Anopheles subpictus*, larvae could not

Fig. 11 Body weight (a, b) and wing length (c, d) of adult male and female *Anopheles subpictus* mosquitoes (a body weight of male, b body weight of female; c wing length of male, d wing length of female). An asterisk (*) above a given boxplot indicates significant difference from control group (conventionally reared) as determined by Dunnett’s multiple comparison test ($p < 0.0001$)
Fig. 12 Correlation between body weight (mg) and wing length (mm) of adult *Anopheles subpictus* mosquitoes (a male, b female)

Table 8 Total cellular carbohydrate, protein, and lipid content of different groups of 4th instar larvae of *Anopheles subpictus*

| Treatment             | Carbohydrate (µg/mg) | Protein (µg/mg) | Lipid (µg/mg) |
|-----------------------|----------------------|-----------------|---------------|
| Axenic                | 14.27 ± 0.76         | 7.78 ± 0.56     | 2.25 ± 0.19   |
| HALG2                 | 20.06 ± 0.86         | 8.14 ± 0.53     | 3.58 ± 0.29   |
| HALG4                 | 19.91 ± 0.93         | 7.97 ± 0.40     | 4.11 ± 0.21   |
| HALG6                 | 20.81 ± 0.98         | 13.9 ± 0.62     | 4.72 ± 0.26   |
| HALG7                 | 21.26 ± 0.77         | 11.15 ± 0.54    | 3.84 ± 0.16   |
| All four isolates     | 28.08 ± 0.78         | 15.07 ± 0.40    | 5.05 ± 0.42   |
| Conventionally reared | 32.48 ± 0.88         | 16.89 ± 0.94    | 6.38 ± 0.31   |

Values are averages (Mean ± SE) of five replications
develop into adult forms after clearance of symbiotic gut bacteria by antibiotic treatment [13]. Like different species of *Anopheles* mosquitoes, larvae of Japanese encephalitis vector *Culex vishnui* could not survive after removal of one gut bacterial strain (*Pseudomonas fluorescens* Ps1) [14]. Removal of gut bacterial strains by antibiotic treatment caused in the failure of development beyond 1st instar stage in the case of *Aedes aegypti* and *Aedes atropalpus* mosquitoes but recolonization by bacterial strains of breeding habitat water could promote normal development of these two species of mosquitoes [28].

Some previous studies had indicated that bio-chemical composition and types of foods taken during the larval stages had several impacts on different life cycle stages of adult mosquitoes [56, 57]. In the present study, adult male and female mosquitoes developed from antibiotic-treated axenic larvae had lowest body weight and smallest wing length. But recolonization of axenic larvae with a mixture of all four resident gut bacterial suspension restored the body weight and wing length of adult male and female mosquitoes like that of the conventionally reared groups. All these findings clearly indicated that these four resident gut bacterial isolates (*Bacillus subtilis* HALG2, *Bacillus pumilus* HALG4, *Bacillus cereus* HALG6, and *Proteus vulgaris* HALG7) played vital role to sustain the adult morphologies. Similar observations were previously reported in case of *Aedes aegypti* mosquitoes where larval diets with different bacterial composition had significant effects on pupation time, rate of adult emergence, and adult morphology in relation to body weight and wing length [58].

Bio-chemical analysis indicated that all these four resident gut bacterial isolates were capable to produce amylase, gelatinase, and lipase enzymes that could degrade starch, gelatin, and lipid content of the medium. Further, the carbohydrate fermentation tests showed that the isolates could ferment a number of different carbohydrate sources. *Bacillus subtilis* HALG2 could ferment twelve carbohydrates, *Bacillus pumilus* HALG4 and *Proteus vulgaris* HALG7 could ferment five carbohydrate sources, and *Bacillus cereus* HALG6 was able to ferment three carbohydrate sources. All these bio-chemical properties of gut bacterial isolates might help their host larvae to degrade carbohydrates, protein, and lipid content of ingested food materials and thus could help in larval digestion. Earlier studies have reported that food consumption and growth of the insects became reduced after removal of gut bacteria by antibiotic treatment, which clearly indicated that bacteria residing in the gut of insects played vital role in consumption and digestion of food of their host [16].

The bio-chemical constraints including protein, lipid, and carbohydrate content of body are important markers for assessing any harmful effects on the insects [59]. The present study recorded that total tissue protein, lipid, and carbohydrate contents of the antibiotic-treated axenic larvae or groups of larvae that were cured with individual bacterial strains were significantly lower than that of the conventionally reared control larvae. But, when the axenic larvae were recolonized with a mixture of all four resident gut bacterial isolates, tissue protein, lipid, and carbohydrate content showed no significant differences with conventionally reared control larval groups. These findings have suggested that all these four gut bacterial isolates are the key players for maintaining the healthy development of their hosts.

Growth of the gut bacterial isolates at different pH of the media indicated that all these bacterial isolates were able to grow 5–11 pH of the media although they could grow well between pH 7 and 10.5, which indicated that alkaline mid-gut environment of mosquito larvae could provide appropriate environment for the growth of these bacterial species. Previously, *Thorsellia anophelis* was reported from the mid-gut of *Anopheles gambiae* mosquito in Kenya, which was adapted to survive in the alkaline gut environment of their host mosquito [24]. Temperature tolerance tests indicated that all the four gut bacterial
isolates could tolerate a wide range of environmental temperature (15–45 °C) and their growth enhanced when the temperature was around 30 °C. Due to poikilothermic nature, the internal temperature of mosquito larvae is not constant and varies depending upon the surrounding environmental temperature [60]. As these bacterial species could tolerate a wide temperature range, they could able to survive even if the temperature of their host got fluctuated. The suitable larval gut microenvironmental conditions in relation to the growth of these bacterial isolates might help them to survive within the host body and to perform a key role in the development of their hosts.

Several research studies have indicated that malarial parasite *Plasmodium* sp. intercats with the gut microbiota of vector *Anopheles* mosquito [26]. Mid-gut microbiota of mosquito vector were reported to play important roles in survival and development of parasites within the host body and thus contribute to disease transmission [61]. Studies by several workers suggested that control or genetic manipulation of beneficial gut bacterial flora of mosquitoes would inhibit their survival and vector competence [62]. Sometimes these gut bacteria are modified genetically to provide anti parasitic molecules which prevent development of disease-causing pathogens within vector body [63].

**Conclusion**

The present study has identified four bacterial species, namely *Bacillus subtilis* HALG2, *Bacillus pumilus* HALG4, *Bacillus cereus* HALG6, and *Proteus vulgaris* HALG7 as symbiotic larval gut bacteria of malarial vector *Anopheles subpictus* in the rural areas of Hooghly district, West Bengal, India. All these four isolates together contributed the larval survival, development, and health of adult male and female mosquitoes. So, management or control of these bacteria from mosquito larval gut could inhibit larval survival and development, which might generate an alternative strategy of vector control program.

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**Author Contribution** Author Madhurima Seal conducted all the experiments, analyzed the data, and wrote the first draft of manuscript. Author Soumendranath Chatterjee designed the work, supervised all the experiments conducted, revised, and edited the manuscript. Both the authors red and approved the final version of the manuscript.

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**Data Availability** Mosquitoes were collected by the authors along with the help of field collectors from rural areas of Hooghly, West Bengal, India. The data were collected from the experiments done by the authors in the Parasitology & Microbiology Research Laboratory, Department of Zoology, The University of Burdwan.

**Declarations**

**Ethics Approval** NA.

**Consent to Participate** All of the authors consent to participate.

**Consent for Publication** The authors hereby give full consent for publication of the data.
Competing Interests  The authors declare no competing interests.

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