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Role of *Dietzia maris* bacteria in larval development of *Aedes albopictus* (Skuse) (Diptera: Culicidae)

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Abstract
The bacterial species *Dietzia maris* have been recently reported from the midgut of *Aedes albopictus*, however its importance was still unexplored. The present study was conducted to investigate the effect of *D. maris* on egg hatching, development and survival of larvae of *Ae. albopictus*. The eggs of laboratory reared *Ae. albopictus* mosquitoes was exposed to bacterial suspension of *D. maris* and the larval development progress was recorded every 24 hours until they reach to fourth instar larval stage from the eggs. Observations revealed that eggs treated with *D. maris* hatched early and the developmental rate increased significantly with surviving larvae (81.67%) reaching the fourth instar by day-5 in comparison with larvae in control groups (69.67%) where the development duration to reach fourth instar was found to be 7 days (P = 0.021). The number of eggs hatched and survived in the treatment group was slightly but significantly higher compared to control groups. Mortality observed during the development in control group (12.4%) was slightly higher but statistically non-significant in comparison with mortality (4.3%) in treatment group (Sig. 2-tailed = 0.247). In the view of present findings, understanding on the larval ecology could lead to its possible utilization in mosquito rearing and alternatively in the mosquito control by targeting the larval ecology.

Keywords: *Dietzia maris*, *Aedes albopictus*, dengue, larvae

Introduction
Aedes aegypti and *Ae. albopictus* are medically most important vector for the outbreaks of several deadly arboviral diseases such as Dengue virus (DENV), Zika virus (ZIKV), Yellow fever virus (YFV), and Chikungunya virus (CHIKV) [1]. According to the current reported about 390 million dengue infections estimated annually worldwide, of which about 96 million are clinically manifested [2]. Burden of Dengue cases have been dramatically increased in the last decades and it was estimated that about 40% of the world populations in more than 128 countries are affected [2]. Recent study on the midgut microbiota of mosquitoes revealed the presence of a diverse group of microbiota which lives in a symbiotic relationship [3-4] and shows the various important functions including development, fertility and fecundity, providing immunity, reproduction, digestion of food etc [4-9]. Midgut microbiota also affects the host-pathogen interactions, in result significantly influences the disease transmission potential of mosquito vector. Vector competency of the mosquitoes might also be modulated through genetic manipulation of midgut bacteria (paratransgenesis) [10-12].

Several biotic and abiotic factors such as temperature, density-dependent intraspecific competition, dissolved oxygen (DO), food availability and nutrient quality, microbes and microbial concentration plays important role in the regulation of the eggs hatching, rate of larval development and survival [13-14]. Although it has long been known that declined dissolved oxygen (DO) is a sole factor to egg hatching. However later it has been proved that the declining dissolved oxygen (DO) alone cannot stimulate the egg hatching while the bacteria alone can do [13]. Apart from bacteria, bacteria associated compounds also plays important role in the eggs hatching. First time Bacot (1916) and Aitken and Bacot (1917) used the bacteria for the egg hatching and proved that bacteria stimulated the hatching; however the mechanism was unexplored several decades [15-17]. It has been proved that the bacteria lower the oxygen level (hypoxia condition) which serves as egg hatching and growth signal [18-19].

It has also been reported that bacteria are important for larvae as a food sources and is required for the proper growth and development [6, 20-22].

Coon *et al*. 2014 [23] demonstrated that the axenic mosquitoes larvae would not be able to develop beyond the first instar; however their development have been rescued by some bacterial species. Only few midgut bacteria have been reported which plays important role in the egg hatching and larval development [23]. Recently, we have isolated the midgut bacteria *D. maris* form female *Ae. Albopictus* [12], which play an important role in egg hatching and survival and larval development. *Dietzia* strains are widely distributed in nature, the *Dietzia maris* species was originally isolated from halibut by Harrison (1929) and later from soil, skin and intestinal tracts of carp, deepest sea mud, and sediments, blood, human skin, Bone Marrow etc [24-31]. The genus *Dietzia* plays various roles in the various different areas like in the biological therapeutic, synthesis of biocolourant, production of biosurfactant, production of biodemulsifier, biodegradation and Bioremediation, however its importance in the mosquito is still unexplored. Therefore we took a comprehensive study to investigate that the influence and role of *D. maris* on egg hatching, larval development and survival of *Ae. albopictus.*

Materials and methods

Isolation and characterization of midgut bacteria: The bacterial strains *D. maris*, used in the present study was previously isolated and identified by Yadav and colleagues 2015a [12]. Briefly, the bacterial strain *D. maris* was isolated from the midgut of female *Ae. albopictus* mosquito collected from foot hills of Himalaya, Arunachal Pradesh, India. Female *Ae. albopictus* were dissected out in a sterilized environmental condition and midguts were homogenized in phosphate buffered saline (PBS). The homogenates were pouring plated on nutrient agar media (HiMedia, India) and incubated at 37 ºC for 12-24 h. Purified bacterial colonies were stored in 15% glycerol-supplemented nutrient broth at -80 °C for further experimental work. The bacterial strain was characterized by using various methods like phenotypic characterization, Scanning Electron Microscope (SEM), MALDI-TOFF-MS based analysis, and 16S rRNA gene sequence analysis. The procedure used for the Scanning Electron Microscope (SEM) and MALDI-TOF-MS identification system was described elsewhere (Yadav *et al*. 2015a). For the 16S rRNA gene sequence based analysis, 1.5kb 16S rRNA gene was amplified from the genomic DNA of bacterial strain, which was further, sequenced and analyzed. 16S rRNA gene sequence of bacterial strain was already submitted to GenBank data base under the accession number KT380950 [12].

Bacterial cell suspensions preparation for exploring the eggs: Fresh bacterial culture of *D. maris* on the petri plate of nutrient agar was used for the inoculation of 100ml of nutrient broth in the Erlenmeyer flasks and allowed to incubation for 24h at 120 rpm, 37ºC. After the incubation, broth culture was centrifuged at 1,254g for 10min for the purification of bacterial cell from all the nutritional components of broth culture and re-suspended the bacteria in normal saline (0.85% NaCl) [13]. The process was repeated twice and the final re-suspended bacterial suspension was made of the dilution of 10^7 to 10^9 cells/mL.

Bacterial challenge to the larvae of *Ae. albopictus*: To determine the effect of bacterial species on larval development, the egg of laboratory reared (at 27±2 ºC and 85 ± 5% relative humidity) *Ae. albopictus* mosquitoes was used for the exposure to bacterial cells. 100 number eggs were allowed to rear in the sterilized water in a large size petri plates in the presence of 1ml of purified suspended bacterial cells (~10^6 CFU per ml) in normal saline. The experiment was performed in the triplicate along with control group under sterile conditions to prevent interference of any external bacterial species. Sterilized yeast powder in water was supplied as food in the same quantities to all samples.

Effect of bacterial exposure on larvae: The larval development progress in *D. maris* treated group and control of all samples was observed regularly. The days and number of the emergence of larvae from eggs and their development at various stages were recorded every 24 hours until they reach to fourth instar larval stage.

Effect of bacterial exposure on the total number of midgut bacteria (CFU) of fourth instar larvae: After transformed eggs to the fourth instar larvae stages, 10 larvae were randomly selected from each set of the experiment for isolation of bacteria from their respective midgut. Prior to dissection, all the samples were surface sterilized by washing with 75% ethanol for 5 min followed by phosphate buffered saline (PBS) twice. The isolated midgut from each sample was transferred to a separate 1.5µl micro-centrifuged tube containing 100µl PBS and homogenated with micro-pestle. The homogenates were serially diluted (10 folds) in PBS and pour plated to nutrient agar media (Himedia, India), incubated at 37 °C for 24–48h. Bacterial colonies obtained on the plates were counted using colony counter (Microprocessor Colony Counter -055588). Prior to dissection, all the dissecting apparatus were sterilized by autoclaving and UV treatment, followed by dissecting process under sterilized environment. Negative controls (PBS) were maintained to check the contamination of external bacteria [12].

Statistical analysis: An Independent t-test was performed to estimate the differences in the hatching rate of eggs, larval development and the rate of mortality of the larvae among the bacterial treated and the control groups (P<0.05, 95% confidence interval). Pair t-test was also conducted to analyze the difference between the total number of bacterial colonies (CFU) isolated from midgut of fourth instar larvae of bacterial treated and controls groups at 95% confidence interval and 5% significant level.

Results

D. maris bacterial isolate was identified and characterized from midgut of *Ae. albopictus* in our previous study and the present study was undertaken to investigate the effect of the *D. maris* on the egg hatching, development and survival of *Ae. albopictus* during larval stages. The mean percentage of the observations on day wise larval development from egg hatching to fourth instar is represented in figure 1.

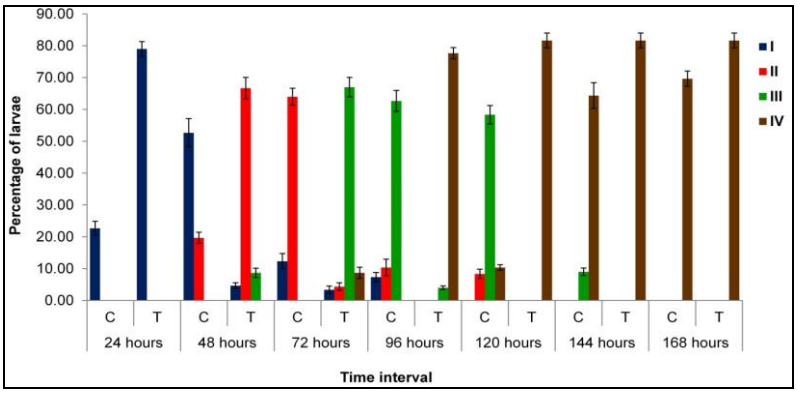

Fig 1: Effect of *D. maris* on *Ae. albopictus* larval development at different time interval from first to fourth instars (C-Control and T-Treatment).

Experiment was initiated (i.e. 0 hour) by introducing the eggs in the treatment and control groups, and the first instar larvae reflecting the percent hatching during the first 24 hours in the treatment group was 79 percent (P<0.00) and was significantly high compared to the control groups with 22.6 percent first instar larvae. In the treatment group the larval development was found to be significantly progressive with more than 65% of the larvae reaching third instar by day 3 (72 hours) compared to control, where most of the larvae were found to be in the development stage in instar II and III reaching third instar stage at 96 hour (Table 1). Overall developmental period of the larvae revealed significant difference between the groups, which was evident in the treatment group, where all the larvae (81%) reached fourth instar by day 5 (120 hours),, whereas the developmental duration observed in control group to reach fourth instar for all the larvae was 168 hours (P = 0.021) (Table-1). The number of eggs hatched and survived in the treatment group was slightly but significantly high compared to control groups. Overall percent mortality observed in the development stages during the experimental period in control group (12.4%) was slightly higher but statistically non-significant in comparison with mortality (4.3%) in treatment group (F = 1.268, Sig. 2-tailed = 0.247).

Table 1: Analysis of larval development from first to fourth instar at different time interval between treatment and control by independent t-test.

Duration (Hours)	F Value; *Sig. (2-tailed) p value			
	Instar-I	Instar-II	Instar-III	Instar-IV
24	0.021; 0.000	-	-	-
48	4.734; 0.000	2.235; 0.000	5.953; 0.004	-
72	2.063; 0.029	1.882; 0.000	12.0; 0.000	7.692; 0.008
96	5.593; 0.007	4.994; 0.017	6.4; 0.000	7.692; 0.000
120	-	5.953; 0.005	5.953; 0.000	2.207; 0.000
144	-	-	4.0; 0.001	0.694; 0.021
168	-	-	-	0.022; 0.023

*Lower value for Sig. 2-tailed (<0.05) indicate significant difference between control and treatment at 95% confidence interval

The effect of *D. maris* bacteria on the overall bacterial load in the midgut region of larvae was observed in treatment and control group. Observations on the isolated bacteria from the midgut of fourth instar larvae in both control and treatment groups revealed significantly high bacterial load (2.42×10^7) in treatment group compared to bacterial load (3.76×10^5) in control group (F = 8.581; P = 0.043).

Discussion

Mosquitoes are well known for its nuisance caused due to its biting behavior for blood feeding and its role in transmission of deadly diseases like dengue, chikungunya, zika, spread by *Aedes* species. Mosquitoes are holometabolous insects that go through complete metamorphosis occupying both aquatic and terrestrial habitat during its development phases. The immature stages (larval/pupal) develop in water and adult is a terrestrial form, thus surviving mosquito traits are influenced by both aquatic environmental conditions during immature development phase and terrestrial conditions. It is important to emphasize that aquatic factors contribute to the larval gut microbiota eventually influencing its development, fitness, survival and its vector potency [32-33]. Recently, it have been reported that the midgut microbiota of mosquitoes significantly influences the interaction of vector and parasite [4, 34]. Apart from parasite-vector interaction the midgut bacteria is also involved in the development, food digestion, and immunity etc [4-9]. In 1916, it was first time reported that bacteria [15-17] and other microorganism stimulates the egg hatching, however its mechanism was explored recently [18].

It has long been known that declining dissolved oxygen (DO) is highly associated with egg hatching and is also currently accepted that it is the principal factor for egg hatching [35-37]. However, there is no sufficient experimental evidence to support that the declining DO is a sole factor for stimulation of egg hatching. Recently, it has been proved that hatching of eggs will not occur only under declining DO concentration conditions until proper conditions are provided. However, the bacteria or bacterial metabolites alone can stimulate the hatching of *Ae. aegypti* eggs irrespective of the DO concentration in the water [13].

In the view, there are several studies on the biotic and abiotic factors stimulating hatching of mosquito eggs, however the knowledge about the bacterial species involved in eggs hatching, larval growth rate and rate of mortality is unexplored and need to be explained. The present study was carried out to investigate the effects of bacterial species *D. maris* on the hatching of *Ae. albopictus* eggs, which was previously identified from the midgut of *Ae. albopictus*, vector [12]. We have also observed the effect on the mortality rate of the larvae in the bacterial treated group in comparison with control group.

From any mosquito species, *D. maris* bacterial species was first time reported from the midgut of *Ae. albopictus* mosquito and there are no reports on the presence of *D. maris* in any insects to support the role of this bacterial species in insect fauna [12]. However *D. maris* was reported from various other resources, but from the intestinal track it was previously reported only from the intestinal tracts of carp [25]. *D. maris* has been reported for diverse and important roles from industrial level to human diseases. The *D. maris* have a sources of enzymes which involve in oil degradation and production of biosurfactant, industrial fermentation, and production of canthaxanthin an antioxidant [38]. Recently, it has been reported that this *D. maris* have been implicated in human diseases and emerged as a new group of opportunistic pathogens. It has been isolated from bone marrow [31], blood [28], and human skin [29], hip prosthesis [39] and from pericardial fluid [40]. It has to causes several infections and disease in humans including the bacteremia and septic shock, prosthetic hip infection in immunocompromised patients, aortic disease and the pyrexia [28-29, 31, 39-40].

The *Aedes* mosquitoes are container-breeder and lay their eggs on the side of the water line and directly on the water surface [41]. Post egg laying embryogenesis takes place and the duration varies for couple of days to several days [13]. Mosquito's larvae mostly feeds on the microorganisms and environmental detritus present in the water habitats and diet of the larvae affects the hatching of the eggs, larval growth rate and time to pupation [32]. It has been reported that the axenic larvae of each species of mosquitoes could not able to develop beyond the first instar, however it was successfully colonized and rescued the development by several microbes like *Acinetobacter, Aeromonas, Aquitalea, Chryseobacterium, Microbacterium, Paenibacillus* and *Escherichia coli* [23]. The bacterial species *Aeromonas, Aquitalea, Chryseobacterium, Microbacterium, Paenibacillus* were isolated from the midgut of *Ae. aegypti* larvae, *Acinetobacter* from midgut of *Ae. aegypti* adult and laboratory reared *Escherichia coli* strain was used. Apart from the mosquitoes, the bacteria are also essential for successful development of the larvae of several other Dipterans including house flies, stable flies, face fly and horn flies [42-46] although the basis mechanism are unknown. Till date only few midgut bacteria have been identified and reported which plays important role in the egg hatching. In our study we have also observed that midgut bacterial species *D. maris* significantly stimulated the egg hatching in the treated group (79.0%) compared to the control (22.67%).

Notably, it has been proved that bacteria have the capability to lower the oxygen concentration which acts a signal of egg hatching and development [18-19]. Our observations demonstrated that rearing medium containing *D. maris* not only stimulated egg hatching but also accelerated the rate of development during the larval stages. Also observations on the mortality rate (4.3%) in comparison to mortality observed in control groups (12.4%) indicated that presence of *D. maris* may have led to reduced mortality during juvenile stages leading to higher survival rate. In this study, we have also observed that *D. maris* supported the proliferation of bacterial isolates inside the midgut of larvae. In the case of bacterial treated individuals the midgut bacterial load was much more compared to the individuals of control samples.

Molecular characteristics of *D. maris* was determined however their functional aspect was not explored. *D. maris* shows an interesting characteristics and it can survive in very adverse condition due to having high pH and salt tolerance prosperities [12]. In the present study the role of *D. maris* on the larval development was explored and observations indicated that *D. maris* enhance the development during larval stages. Our results could provide insight on the importance of microbiota in larval development and could possibly contribute to the knowledge on the mosquito larval diet, where technical interest lies in achieving faster development of mosquitoes especially in mass rearing. In contrast, it is also important to emphasize that there are reports suggesting that exposure to differential bacteria during the development of mosquito larvae can have "carry-over" effects in adult stages influencing the ability of the mosquito to be a successful vector of arboviruses and indicating the influence of larval ecology on the vector potency of the emerging adult mosquitoes [47]. In the view, targeting the microbiota of mosquito breeding sites could impact and reduce the vector potency of the adult mosquito and also the survival rate.

Conclusion

In conclusion present findings contribute to the knowledge on effect of larval diet on development and as well as its possible utilization in the alternative mosquito control by targeting the larval ecology. Further investigations to explore on either of the contrasting effect on the mosquito development are necessary.

Abbreviations

CFU- Colony Forming Unit

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