https://doi.org/10.33307/entomon/v48i4/998
Entomon 48(4): 605-610
Short Communication No. ent. 48416

Lysinibacillus fusiformis: A novel mosquitocidal bacterium isolated from Western Ghats, Kerala, India

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ABSTRACT: An extensive field survey was carried out from December 2021 to January 2022 for the collection of soil samples in the Western Ghat region of the Wayanad district of Kerala, India, to isolate potent novel mosquitocidal bacteria which could be used as efficient formulations to control mosquito vectors. Several bacterial colonies were isolated and screened, for mosquitocidal activity. Toxicity assay showed that only one bacterium isolated from forest loamy soil had promising larvicidal activity against *Culex quinquefasciatus* the lymphatic filariasis vector (LC_{50}: 0.03mg L^{-1} and LC_{90}: 0.07mg L^{-1}) and moderate activity was shown against the dengue vector, *Aedes aegypti* (LC_{50}:1.03 mg L^{-1} and LC_{90}: 1.8 mg L^{-1}). The bacterium was identified as *Lysnibacillus fusiformis* by a phylogenetic tree constructed using 16S rRNA genome sequencing. This is the first report that *L. fusiformis* isolated from the forest loamy soil of the Western Ghats, Kerala, which showed proficient mosquitocidal activity against disease-transmitting mosquito vectors. © 2023 Association for Advancement of Entomology

KEYWORDS: *Culex quinquefasciatus*, *Aedes aegypti,* soil, genome sequencing, first report, toxicity assays

Mosquito-borne diseases (MBD), such as dengue, malaria, and Zika virus fever, chikungunya, pose severe risks to the public's health (WHO, 2020). Vector control is crucial for reducing the epidemics of several MBDs, limiting disease transmission, and improving the quality of life. The resurgence of vector-borne diseases as a result of favorable environmental factors, such as rapid urbanization, the development of vector resistance to insecticides, and human lifestyle changes encourage the need for the adoption of safer and more efficient vector control techniques (Chediak *et al.,* 2016; Garcia *et al.,* 2018). The WHO promotes integrated vector management (IVM), which emphasizes the use of long-lasting, eco-friendly alternative vector control measures. Biological control measures, including bacterial pesticides, are found to be a better alternative since they are eco-friendly, cost-effective, and target-specific. To control mosquito vectors, *Bacillus thuringiensis israelensis (Bti)* and *B. sphaericus (Bs)* bacteria are frequently used (Prummongkol *et al*., 2019; Vimala Devi *et al*., 2021). However, the long-term usage of these bio-control agents like *Bs* resulted in the occurrence

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of resistance in vector mosquitoes (Guidi *et al*., 2013; Su *et al*., 2019). This challenging situation prompted the researchers to look for novel mosquitocidal bacteria from natural sources. The Western Ghats in Kerala are renowned for their diverse and unique collection of fauna and flora. Soil is the richest source of microbes and there have been numerous reports of mosquitocidal bacteria isolated from soil in different parts of the world (Nair *et al*., 2020; Iftikhar *et al*., 2023). Whereas the soil of the Western Ghat region of Kerala has not been explored for isolation of mosquitocidal bacteria.Therefore, the present study aimed to isolate novel mosquitocidal bacteria from various soil types collected from the Western Ghats, Wayanad, Kerala, India.

The soil samples were collected from tea plantations, coffee plantations, and forests in the Wayanad district, which includes parts of the Western Ghats (11.68°N; 76.13°E). The soil sampling was done from December 2021 to January 2022. In each sampling spot, the surface layer, 5 and 10 centimeters below the soil, were collected and pooled together in sterile vials. Soil types were documented. Samples were brought into the laboratory and processed by serial dilution and spread plate method. A required amount of soil sample was weighed (1g) and serially diluted (10^{-3}) in 10 ml of sterile water, and 0.1ml of this serially diluted sample was evenly spread on Nutrient Yeast Salt Mineral (NYSM) agar plates (spread plate method) and these plates were incubated for 24 hours at room temperature. Bacterial colonies were inoculated in 10 ml NYSM broth and incubated in an Orbitek shaker at 250 rpm for 72 hours.

The preliminary toxicity assay (bioassay) was carried out as recommended by WHO guidelines (WHO, 2005) using 72 hours of bacterial culture to screen for mosquitocidal activity. The bioassays were conducted in paper cups (wax-coated) having 100 ml of tap water (chlorine-free) and 25 late third instar larvae of *Culex quinquefasciatus* and *Aedes aegypti* were released into the cups and acclimatized. A dose of 1µl of 72 hours of bacterial culture was used to treat the larvae in the cups, while controls without the addition of the bacterial culture were maintained. After 24 hours of exposure, the bioassay results were recorded by scoring the live larvae in respective cups. The bacterial isolate with 100 percent mortality of larvae was considered potential bacteria, and stored in a deep freezer as glycerol stock (30%) until further use. The potential mosquitocidal bacterium was subjected to extensive bioassay /detailed toxicity assay to determine the lethal concentration values (LC_{50} and LC_{90}). Bacterial glycerol stocks were inoculated in 10 ml of NYSM broth, kept for incubation overnight at 250 rpm in an Orbitek shaker, and sub-cultured in 100 ml of NYSM broth for 72 hours. After complete sporulation, the bacterial cell pellets were separated by centrifugation (10000g, 20 min) (Hitachi, Japan) and then lyophilized (Christ ALPHA 1-2 LD plus, Germany). A homogenized bacterial stock solution was prepared (5mg/10 ml) using lyophilized powder. Seven different doses of this stock solution were used for conducting the extensive bioassay. Four replicates for each dose were maintained in every experiment with appropriate controls (WHO, 2005). After 24 hours, the result was scored by counting the live larvae. The experiment was repeated three times to ensure reproducibility. The results were finally analyzed by Probit analysis using SPSS.16.0 software.

Table 1. Mosquitocidal activity of *L. fusiformis*

Mosquito species	LC_{50} (mg l^{-1})	LC_{90} (mg l^{-1}) (LCL*-UCL*)	Slope (LCL*-UCL*)	Intercept	X^2
Culex quinquefasciatus	0.03 (0.025-0.039)	0.07 (0.068-0.081)	0.006	-1.160	89.879
Aedes aegypti	1.02 (0.94-1.1)	1.8 (1.69-1.9)	0.002	-1.714	17.864

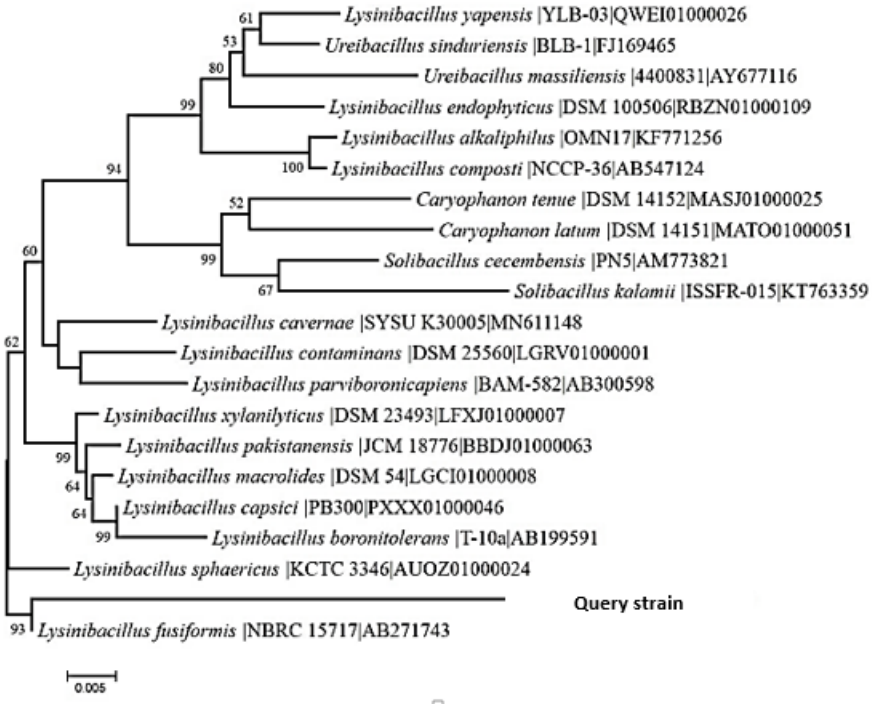

Fig. 1 Phylogenetic tree constructed using 16S rRNA genome of the newly isolated mosquitocidal bacterium (Neighbour joining model)

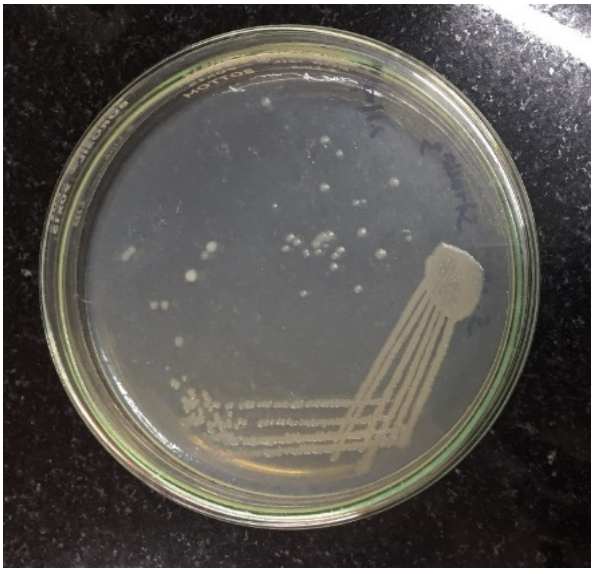

Fig. 2 Colony morphology of *L. fusiformis*

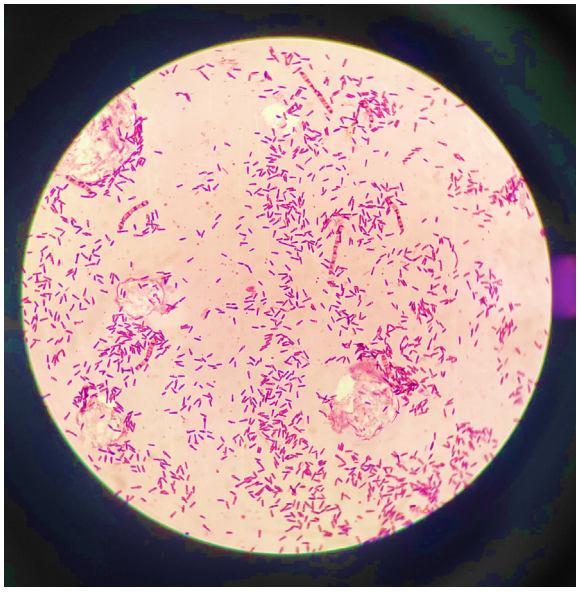

Fig. 3 Vegetative cells of *L. fusiformis* after Gram staining

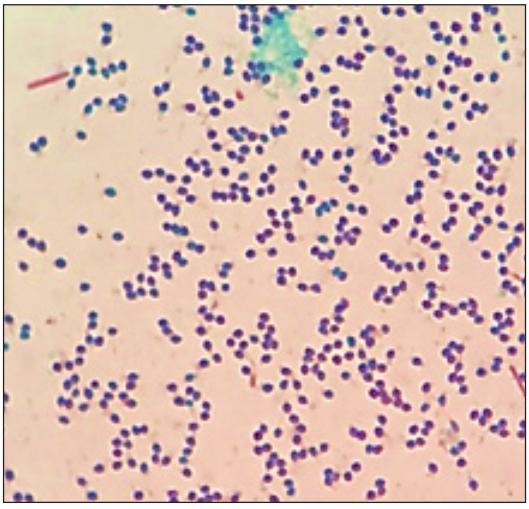

Fig. 4 Spores of *L. fusiformis* after Safranin-Malachite green endo spore staining

The characteristics of bacterial colonies such as the shape, margin, opacity, and color of the bacterial isolate, were observed in the NYSM plate culture. Gram-staining was done to study whether the bacteria were Gram-negative or positive. Spore staining, also known as Schaeffer-Fulton staining, was done to visualize the presence of spores through a microscope (Olympus CX41RF Binocular Microscope, Japan).

For the identification of the mosquitocidal bacterium the genomic DNA was extracted using the GenEluteTM Bacterial Genomic DNA Kit (Sigma Aldrich, USA), and polymerase chain reaction (PCR) was done to amplify the genomic DNA with universal forward and reverse primers (16S rRNA). The Qiaquick PCR purification kit (QIAGEN, USA) was employed to purify the amplified products, which then served as a template for forward and reverse sequencing. The BigDye Version 3.1 kit from Applied Biosystems was utilized to do the DNA sequencing on the ABI-PRISM 3730 DNA sequencer and the contigs sequences were assembled by Bio-Edit (Version 7.0.9.0) and examined via Mascot Server 2.4. BLAST program (NCBI), which was used to identify the species. The 16S rRNA genome sequence was combined with the closely related sequence, and a next-generation phylogenetic tree was constructed using MEGA 5 using the K2P model and 1000 bootstrapping.

The Western Ghats in the states of Kerala were granted a "heritage tag" from UNESCO as they are the "gene pool" that harbors millions of Millions of species. The soil in this region is an excellent source of numerous unique microorganisms that might be investigated for potential applications. There has been limited research on the isolation of mosquitocidal bacteria from the soils of the Western Ghats region of Kerala. (Nampoothiri *et al*., 2013). In the present study, explorative research was carried out by collecting various soil types from the Western Ghats of Wayanadu District of Kerala for isolating novel mosquitocidal bacteria A total of 180 soil samples were collected, and several bacteria were isolated. Among these 12 bacterial strains showed mosquitocidal activity and among these only one bacterium isolated from forest loamy soil had promising larvicidal activity against *Culex quinquefasciatus* the lymphatic filariasis vector ($LC_{50:}$ 0.03mg L^{-1} and LC_{90}: 0.07mg L^{-1}) and moderate activity was shown against the dengue vector, *Aedes aegypti* (LC_{50}: 1.03mg L^{-1} and LC_{90}: 1.8mg L^{-1}) (Table 2). This potential bacterial strain was identified as *Lysnibacillus fusiformis* by constructing the phylogenetic tree using the 16S rRNA genome sequence (Fig. 1). The colony

morphology of *L. fusiformis* was circular, dry, flat, and rough, with a dull white color (Fig. 2). Microscopic studies on the vegetative stage of the bacterium found it to be rod-shaped and Gram-positive (Fig. 3). The strain was endospore-forming with terminal spherical spores (Fig. 4).

Ramalakshmi and Udayasuriyan (2010) reported different of *Bt* strains from the soil samples of the Western Ghats extension in Tamil Nadu.Similarly, Ganesan and his co-workers (2018) reported *Streptomyces enissocaesilis* (S12-17) from soils of the Western Ghats of Tamil Nadu with effective mosquito larvicidal, ovicidal, and repellent activity (Ganesan *et al.,* 2018).It is the first report of *L. fusiformis* from the soil of Western Ghats, Kerala having mosquitocidal activity. It is a naturally occurring bacterium in the family Bacillaceae and was first discovered in 1901 (Pinheiro *et al.,* 2022). *L. sphaericus* was widely used for mosquito control whereas; the larvicidal activity of *L. fusiformis* has not been reported so far. The underlying mechanism *i.e.,* the toxin (s) responsible for mosquito toxic effect and its mode of action are yet to be studied. In conclusion, this study reports a novel mosquitocidal bacterium, namely, *L. fusiformis* for the first time. It is suggested that the strain may be useful for the control of disease-transmitting mosquito vectors in the current scenario of resistance to some bio-pesticides.

ACKNOWLEDGMENTS

The first author acknowledges the University Grant Commission (UGC), Ministry of Human Resource and Development, Government of India, for the award of a Senior Research Fellowship (Ref: UGC/191620033726). The authors acknowledge the Director, ICMR-Vector Control Research Centre, Puducherry for proving the facilities and RIU approval. This is part of PhD thesis (Ref: PhD/PU/20201129001, 12/04/2021) for the first author under the guidance of the corresponding author (SP).

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(Received June 25, 2023; revised ms accepted September 26, 2023; published December 31, 2023)