Assessment of Some Bacteria from Panteka Stream, Kaduna, Nigeria, for their Larvicidal Activity Against *Anopheles gambiae*

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***Abstract*—It is obvious that malaria is one of the commonest diseases in Africa, hence the need to embark on a study to reduce its transmission by eliminating the vector. Some microorganisms are known to have larvicidal activity leading to destruction of mosquito larvae, thereby, preventing them from metamorphosing into adult mosquitoes that can transmit *Plasmodium spp*. Panteka stream, Kaduna, Nigeria, is a dumping site for refuse and automobile waste and thus, a potential source of bacteria. This present investigation was aimed at screening bacterial isolates for their larvicidal activity against *Anopheles gambiae*. Standard methods were employed in sample collection, isolation, morphological, biochemical identification and protein profiling of these bacteria isolates. Five different types of bacteria were identified; *Bacillus thuringiensis, Staphylococcus aureus, Micrococcus sedentarius, Enterococcus faecalis* and *Streptococcus pneumonia.* Among these bacteria, *B. thuringiensis* exhibited the most larvicidal activity, followed by *M. sedentarius*. On the basis of lethal concentration (LC_{50}), *B. thuringiensis* exhibited the highest lethal activity against *Anopheles gambiae* larvae at 48 hour duration of exposure. Results showed that concentration of bacterial isolates and duration of exposure of larvae to the bacterial isolates, determine the mortality rate of larvae. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) revealed variable bands between *B. thuringiensis* and *M. sedentarius*, which might have accounted for their differences in larvicidal activity. The use of bacteria for the control of mosquito larvae is highly recommended. Further research should be conducted to search for more bacteria and possibly fungi which have potentials for larvicidal activity.**

***Index Terms*—*Anopheles gambiae*; *Bacillus thuringiensis*; Bacteria; Larvicide; *Micrococcus sedentarius*.**

I. Introduction

Mosquitoes are vectors of many medically important pathogens [1]. They transmit pathogens which are the causative agents of human diseases such as malaria, dengue, yellow and chikungunya fever and several other forms of infections [2]. World Health Organization [3] stated that among the various diseases transmitted by mosquitoes, malaria caused by the *Plasmodium spp.*, is the most common parasitic disease in the tropical and sub-tropical regions of the world, where it results in high mortality and morbidity especially among pregnant women and children. *Anopheles gambiae, Anopheles funestus, Anopheles arabiensis and Anopheles melas* are known vectors of human malaria. Among these species, *A. gambiae* was reported to be responsible for the transmission of plasmodium parasites in Sub-Saharan Africa [4]. Malaria has remained endemic in most countries of the African sub-region. Nigeria and Democratic republic of Congo are reported to account for about 40% of total mortality of malaria over the world [5].

Efforts have been made to fight against malaria and other mosquito-transmitted diseases in Nigeria and other malaria endemic countries in the past few years. There was 25% reduction globally in malaria incident rate, and 31% in the African region [4]. Control measures which were adopted include the use of artemisinin-based combination therapy (ACT) as first line drug treatment, employment of long lasting insecticide nets, Intermittent Prevention Treatment (IPT) for pregnant women, and other vector control measures [4]. Long-lasting insecticidal nets reduce malaria parasite transmission mainly by killing or blocking mosquitoes that attempt to feed upon humans under nets. Indoor residual spraying kills mosquitoes and reduces longevity when they rest on insecticide-sprayed surfaces.

Chandra *et al*. [6] reported that the control of mosquitoes in Africa is facing a threat due to the emergence of resistance in mosquitoes to conventional chemicals. There is no doubt that mosquito-transmitted diseases are major cause of morbidity and mortality in endemic areas including Nigeria. In many African countries where mosquito-transmitted diseases are endemic, residual transmission is maintained through a combination of human and vector behaviours; but this strategy has not been totally effective in the control of mosquito in endemic areas, thus necessitating the need for a new vector control strategy that is target-specific and non-toxic to humans. Recently, there has been increasing interest in the discovery of more effective and safer biological control agents against hazardous insects. Panteka stream in Kaduna, Nigeria serves as a dumpsite for waste and wastewater from nearby Army Barrack, residential homes and automobile spare part market, hence making the stream a potential source of bacteria that could be harvested for insect vector control. The use of bacteria as larvicides seem more favourable, safer and target-specific in comparison with chemical insecticide because they are ecosystem-friendly and cheaper [7].

The main objective of this study was to isolate and identify bacteria from Panteka stream, Kaduna, Nigeria, and assess them for their larvicidal activity against *Anopheles*

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gambiae.

II. Materials and Methods

A. Sample Collection Site

The sample collection site is Panteka stream, Kaduna, Nigeria (latitude 10°36'21.28"N and longitude 7 °18'57.17"E). Lots of activities such as washing of motor spare parts, fishing, dumping of refuse etc., are carried out in the stream.

B. Sample Collection

Water samples were collected from four locations in Panteka stream using sterile 125ml sample bottles. After the collection of the sample, the sample bottles were sealed immediately, preserved using a zipper bag contained in a cooler with ice packs and taken to Microbiology Laboratory of Nigerian Defence Academy, for analysis.

C. Isolation of Bacteria

Pour plate technique was used for bacteria isolation using nutrient agar (Sigma-Aldrich-70148, USA) in a petri dish, and then incubated for 24 – 48 hours at 25°C. The colonies were sub-cultured in order to obtain pure cultures.

D. Morphological and Biochemical Identification of the Bacterial Isolates

Isolated colonies of each bacteria were observed and identified based on morphological and biochemical characteristics, and confirmed according to Cowan and Steel's Manual for the Identification of Medical Bacteria [8]. This was based on their superficial forms (circular, filamentous and irregular margin), elevation of colonies (flat, convex and umbonate), and shape of bacterial cells (spiral, rod or cocci). Gram's staining was also carried out. Biochemical tests carried out on the bacteria isolates include Triple Sugar Iron Agar (TSI) Test, Motility Test in Motility Indole Ornithine (MIO) Agar, Catalase Test, Oxidase Test, Citrate Test, Methyl Red Test, Urease Test, Nitrate Reduction Test, Hydrogen Sulphide, Coagulase Test and Voges-Proskauer Test.

E. Mosquito Larvae Culture

A 5ml capacity cup was lined with filter paper strips and filled with deionized water up to one-third of the cup. The cup was placed in the open within the Biological Science laboratory of Nigerian Defence Academy, and observed for mosquito oviposition daily. Paper strips with mosquito eggs were collected, dried and identified according to the method of Amadio *et al*. [9]. The paper strips were later immersed in de-chlorinated water in a cup to which larval food was added 24hrs earlier. This was observed until first instars emerged. Larvae hatched were transferred to a shallow pan containing de-chlorinated water with larval food and allowed to stand for 5 days to obtain a homogenous population of late third instar larvae.

F. Exposure of Mosquito Larvae to Bacterial Isolates

The World Health Organization [10] guidelines for laboratory testing of mosquito larvicides was used for testing the larvicidal activity of bacteria isolated from Panteka stream. Four bacterial concentrations (1.25ml, 2.5ml, 5ml and 10ml) were used on 20 late 3rd instar larvae per replicate. The control was sterile distilled water. Percentage mortality was determined after 24hrs and 48hrs by counting the live larvae at each of the specified periods respectively. The strains that killed more than 50% of the larvae were considered as pathogenic [11]. All tests were conducted at room temperature.

G. Determination of Lethal Concentration (LC_{50})

Lethal concentration (LC_{50}) which is the concentration at which 50% of the larvae were immobilized was evaluated. According to McFarland Standard, concentration of the bacteria colony unit (CFU/ml) was determined by using barium sulphate against which the turbidity of the test and inocula were compared.

H. Analysis of Protein Profile

Bacteria isolates showing evidence of pathogenicity against mosquito larvae were cultured in nutrient broth and incubated at 28°C for 4 days. Ten millilitres of the cultured broths was transferred to a centrifuge tube and centrifuged at 10,000rpm for 20 minutes at 4°C and the pellets of each tube washed 3 times in distilled water. To the pellets, each was added 5mM Dithiothrietol (DDT) and protease inhibitors containing 2mg/ml lysozyme and heated for 10mins in a water bath. Protein concentration was measured by the colorimetric method of Porter *et al.* [12] and this was based on the fact that the phenolic group of tyrosine and tryptophan residues (amino acids) in a protein produced blue purple colour complex. The intensity of the colour is dependent on the amount of the aromatic amino acids present and thus varies for different proteins. Bovin Serum Albumin (BSA) was used as standard protein with pH range of 9 – 10.5.

Sodium Dodecyl Sulphate Polyacrylamide Gel electrophoresis (SDS-PAGE) of proteins was performed as described by Baumann *et al*. [13] using 10% separating and 4% stacking gels. Polyacrylamide Gel Electrophoresis (PAGE), is an analytical method used for separating components of a protein mixture based on their size. The size of the protein was determined by comparing its migration distance with that of a known molecular weight of the ladder (marker).

III. Results

A. Characterization of the Bacterial Isolates

A total of five different types of bacteria were isolated. These isolates were characterized morphologically and biochemically. Table I shows that all the five isolates were gram positive and irregular in their colonial forms. Four of the isolates were cocci, creamy and had convex elevation. Only *Bacillus thuringiensis* appeared rod shape with flat colonial elevation. Microscopic appearances of the bacteria isolates are shown in Fig. 1–5. Biochemical characterizations identified the isolates as *Bacillus thuringiensis, Staphlococcus aureus, Micrococcus sedentarius, Enterococcus faecalis and Streptococcus pneumoniae* (Table II).

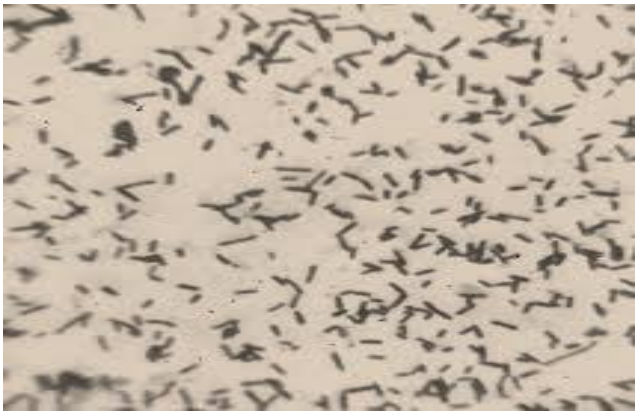

Fig. 1. Microscopic appearance of *B. thuringiensis*

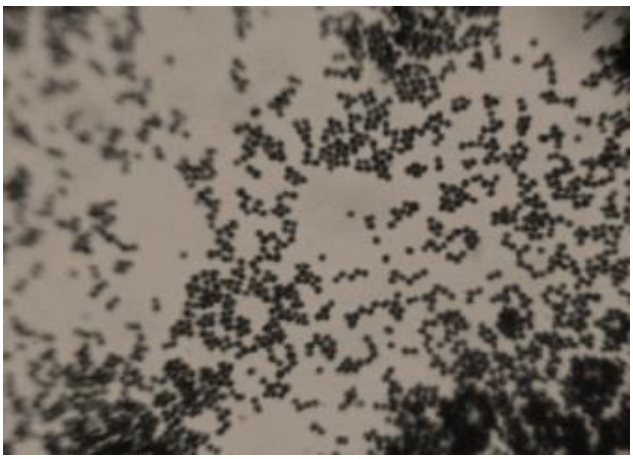

Fig. 2. Microscopic appearance of *Staphylococcus aureus*

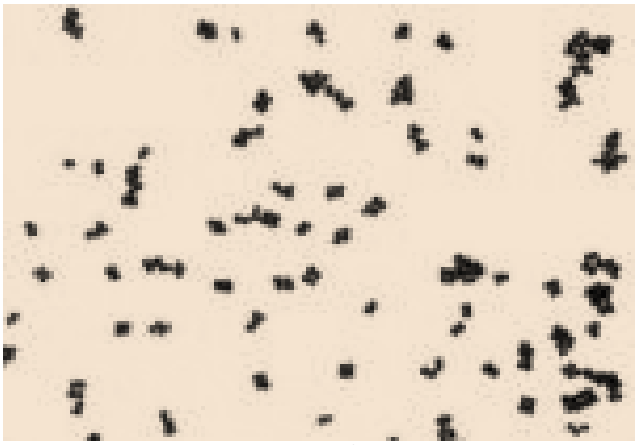

Fig. 3. Microscopic appearance of *Micrococcus sedentarius*

Fig. 4. Microscopic appearance of *Enterococcus faecalis*

Fig. 5. Microscopic appearance of *Streptococcus pneumoniae*

TABLE I: MORPHOLOGICAL CHARACTERISTICS OF THE ISOLATES

Test	**1**	**2**	**3**	**4**	**5**
Gram Staining	+	+	+	+	+
Cell Shape	Rod	Cocci	Cocci	Cocci	Cocci
Colony Colour	Cream	Yellow	Cream	Cream	Cream
Colony Margin	Irregular	Irregular	Irregular	Irregular	Irregular
Colony Elevation	Flat	Convex	Convex	Convex	Convex
Probable Genera	Bacillus	Staphylococcus	Micrococcus	Enterococcus	Streptococcus

TABLE II: BIOCHEMICAL CHARACTERIZATION OF THE BACTERIAL ISOLATES

Biochemical	**Bacteria isolates and their biochemical reactivities**				
Tests	**1**	**2**	**3**	**4**	**5**
Indole test	-	-	-	-	-
Methyl red test	+	-	-	-	-
Voges-proskauer	-	+	-	+	-
Citrase	+	+	+	-	-
Urease	-	-	-	-	-
Catalase	+	+	+	-	-
Oxidase	+	-	+	-	-
Nitrate reduction	+	+	-	-	-
H_2S production	+	-	-	-	-
Glucose fermentation	+	+	-	+	+
Lactose fermentation	+	+	-	+	+
Sucrose fermentation	+	+	-	+	+
Gas production	+	-	-	-	-
Coagulase	-	+	-	-	-
Motility	+	-	-	-	-
Probable identity of Bacteria isolates	*Bacillus thuringiensis*	*Staphylococcus aureus*	*Micrococcus sedentarius*	*Enterococcus faecalis*	*Streptococcus pneumoniae*

+ = Positive, - = Negative

B. Larvicidal Activity of the Bacterial Activity

The percentage mortality of mosquito larvae on treating with different concentration of the bacterial isolates at 24 hours (Table III) and 48 hours (Table IV) revealed that the bacterial isolates had larvicidal activity on the mosquito larvae. From the experimental results obtained of this study, the percentage mortality of the larvae of *A. gambiae* when treated with *B. thuringiensis, S.aureus, M. sedentarius, E. faecalis and S. Pneumoniae*, varied with concentration of

bacteria utilized (Table III and IV), whereas the control were without mortality of *A. gambiae* larvae.

The highest mortality rates were recorded using 10ml concentration of the bacterial isolates at both 24 and 48 hours. *Bacillus thuringiensis* had 100% larvicidal activity on mosquito larvae, followed by *Micrococcus sedentarius* which recorded 50% larvicidal activity within the same length of time. *Stapylococcus aureus, Enterococcus faecalis and Streptococcus pneumoniae* recorded low percentage mortality at both 24 hours and 48 hours of exposure. The mean percentage mortality of mosquito larvae revealed that *B. thuringiensis* had the highest mean mortalities of 48.75% and 83.75% after 24 and 48 hours of treatment respectively. *M. sedentarius* showed 20.00% and 28.75% lethality against the larvae after 24 and 48 hours of treatment respectively.

TABLE III: PERCENTAGE MORTALITY OF MOSQUITO LARVAE AT DIFFERENT CONCENTRATIONS OF BACTERIA ISOLATES AFTER 24 HOURS

Bacterial isolates	No of Exposed larvae	Control	Isolate concentration			
			1.25 ml	2.5 ml	5 ml	10 ml
Bacillus thuringensis	20	0	65	80	90	100
Staphylococcus aureus	20	0	0	10	15	20
Micrococcus sedentarius	20	0	10	20	35	50
Enterococcus faecalis	20	0	0	5	10	15
Streptococcus pneumonia	20	0	0	5	10	15

TABLE IV: PERCENTAGE MORTALITY OF MOSQUITO LARVAE AT DIFFERENT CONCENTRATIONS OF BACTERIA ISOLATES AFTER 48 HOURS

Bacterial isolates	No of Exposed larvae	Control	Isolate concentration			
			1.25 ml	2.5 ml	5 ml	10 ml
Bacillus thuringensis	20	0	25	35	60	75
Staphylococcus aureus	20	0	0	0	5	10
Micrococcus sedentarius	20	0	5	10	25	40
Enterococcus faecalis	20	0	0	0	5	5
Streptococcus pneumonia	20	0	0	0	5	15

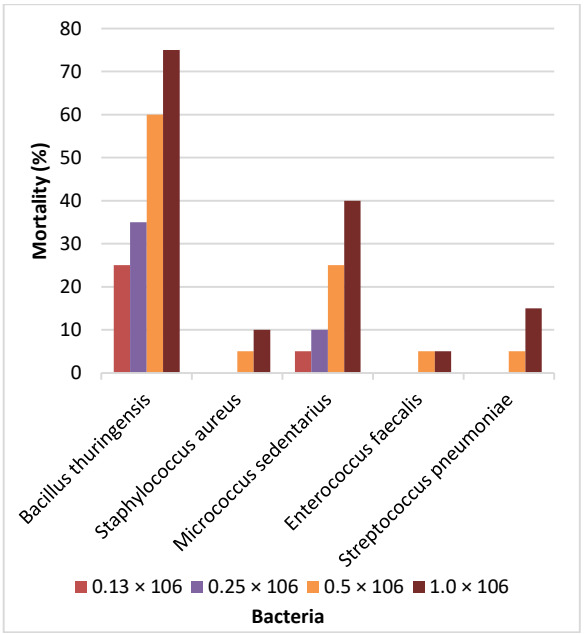

Fig. 6. LC_{50} values of mosquito larvae treated with Bacterial Isolates after 24 hours

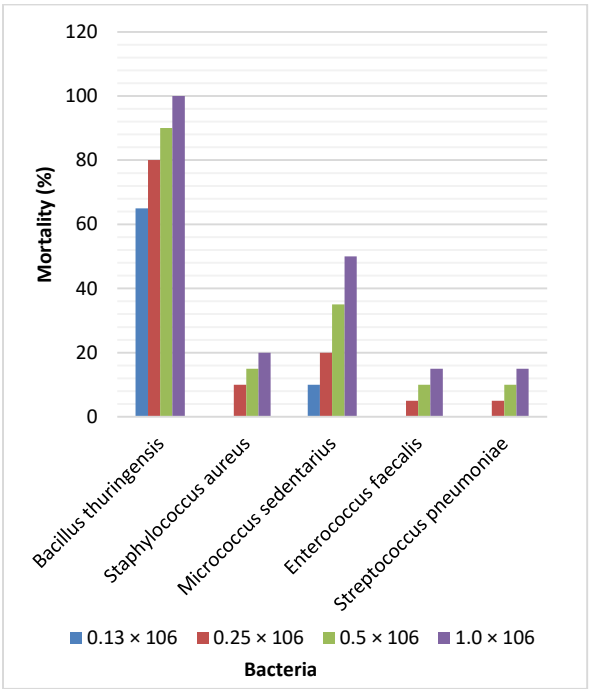

Fig. 7. LC_{50} values of mosquito larvae treated with Bacterial Isolates after 48 hours

C. Protein Profiles of B. thuringiensis and M. sedentarius

The protein profiles of the two bacteria, *Bacillus thuringiensis* and *Micrococcus sedentarius*, which exhibited high larvicidal activity is depicted in Fig. 8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of *Bacillus thuringiensis* and *Micrococcus sedentarius* showed the protein sizes of these organisms as 120KDa and 65KDa respectively.

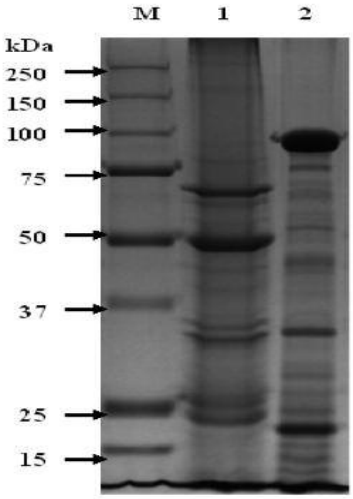

Fig. 8. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Protein Profile of Bacterial Isolates. M – Marker; 1 – *Micrococcus sedentarius*; 2 – *Bacillus thuringiensis*.

IV. DISCUSSION

The regular use of chemical insecticides has brought about an increase in mosquito resistance, hence, led to low insecticidal susceptibility. The result of this study showed that bacteria isolated from Panteka stream, Kaduna, can be harvested and used for killing mosquito larvae. *Bacillus thuringiensis, Staphylococcus aureus, Micrococcus*

*sedentarius, Enterococcus faecalis and Streptococcus pneumonia*e were isolated in this study and they showed potential for larvicidal activity against *A. gambiae* larvae. This finding is in agreement with Cetin *et al*. [14] whose study revealed that *Bacillus thuringiensis and Bacillus sphaericus* are highly effective for the control of *Anopheles gambiae* larvae that develop to adult mosquitoes. This finding also showed that the percentage mortality of the larvae of *A. gambiae* mosquito by *B. thuringiensis, S.aureus, M.sedentarius, E. faecalis and S. Pneumonia*e varied at different concentrations.

Bacillus thuringiensis had the highest larvicidal activity against *A. gambiae* mosquito larvae, and this is in consonance with the study of Yadav *et al*. [15], who reported that application of *Bacillus spp.* had good potential for use against disease vectors and mosquito breeding in polluted as well as clean waters. Effective strains of *Bacillus thuringiensis* have been isolated from stream sample against *A. gambiae* [11]. Most of the studies carries out about bacterial larvicides revealed high lethality of *B. thuringiensis* against the larvae of some vectors [16]-[18].

Among the bacteria isolates, only two were effective in the control of *A. gambiae.* The percentage mortality varied with the organisms at different concentrations. It was also observed that the increased concentrations led to increase in mortality rate of the various isolates both in the 24hrs and 48hrs test. Waalwijk [19] established that different concentration of bacteria such as *B. thuringiensis (Bti)* had different ability to control larvae of *A. gambiae* mosquitoes. This study revealed that with the *M. sedentarius* exhibited higher larvicidal activity than the other bacteria isolates which showed low larvicidal activity, with the exception of *B. thuringiensis* which was found to be most effective in killing mosquito larvae.

The bacteria isolates, *Bacillus thuringiensis* and *Micrococcus sedentarius*, had the protein size of 120KDa and 65KDa according to SDS-PAGE analysis. According to Baumann *et al.* [13] and Porter *et al.* [12], protein factors with size range of 60KDa - 150KDa, are responsible for larvicidal activity. The two proteins realized from this study, were assumed to have relationship with their variable toxicities against mosquito larvae. The lethal concentration required to kill 50% of the mosquito larva population was most effective using *B. thuringiensis;* and Armengol *et al*. [18] referred this protein to as Insecticidal Crystalline Proteins, due to its high degree of specificity and their mode of action against insect larvae. Gammon *et al*. [20] reported that the larvicidal activity of *Bacillus spp* especially *B. sphaericus* and *B. thuringiensis subsp*. *israelensis* could be due to the production of toxins during their sporulation and these toxis were active for control of mosquito populations. Furthermore, Aïssaoui and Boudjelida [21] attributed the larvicidal activity of *B. thuringiensis* to the production of protein toxins, Cyt1Aa, Cry47Aa, Cry4Ba, and Cry 11Aa, whose sizes range from 60KDa - 150KDa.

V. Conclusion

The use of chemical insecticides to kill mosquito larvae poses harm to the environment and ecosystem at large, hence the need for an alternative which is environmentally friendlier, cheaper and also more effective. On the basis of this concern, bacteria isolated from Panteka stream showed the ability to kill *A. gambiae* mosquito larvae. This study helped to identify the larvicidal bacteria present in the Panteka stream, and in which case, confirmed and reaffirmed the potential of the bacteria to act as larvicides with variable degree of potencies depending on their concentration and duration of incubation with *A. gambiae* larvae.

In the light of this, Panteka stream can be said to harbour viable bacteria which have larvicidal activity. From this study, *Bacillus thuringiensis* showed the highest larvicidal activity the larvae of *Anopheles gambiae*, followed by *Micrococcus sedentarius.* Concentration of the bacterial larvicides and time of exposure of larvae determine the mortality rate of larvae. The protein profile of bacteria showing larvicidal activity (*Bacillus thuringiensis* and *Micrococcus sedentarius*) were different, thus, suggesting that it must have accounted for their differences in larvicidal activity.

The use of bacteria is highly recommended for the control of mosquito larvae. Further inputs for developing microbial control agents should therefore be diverted to search for new agents, which have not been encountered so far to improve *Bacillus thuringiensis* and *Micrococcus sedentarius* through bioengineering and rDNA techniques on a priority basis.

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