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Influence of Physicochemical Parameters on Mosquito Larvicidal Potency of *Bacillus subtilis* Isolated from *Musca domestica* (Linn) Cadavers in Nigeria

F. O. Omoya[1*] and B. E. Boboye[1]

[1]Department of Microbiology, School of Sciences, Federal University of Technology, Akure, Nigeria.

Authors' contributions

This work was carried out in collaboration between both authors. Author FOO designed the study, performed the statistical analysis wrote the protocol and the first draft of the manuscript. Authors FOO and BEB managed the analyses of the study. Author BEB managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Biological control method is one of the methods used in controlling the menace of insect pest because it posed no threat to the human health and less damage to the environment. This study therefore assessed the effect of selected physicochemical parameters on the larvicidal potency of *Bacillus subtilis* (Berg) on *Anopheles arabiensis* (Linn) mosquito larvae.
Study Design: The physicochemical parameters were considered for this study include; pH, concentration, temperature, turbidity and sunlight.
Place and Duration of Study: This research was carried out in the Department of Microbiology Federal University of Technology, Akure, Nigeria, between May 2013 and September 2013.
Methodology: *Bacillus subtilis* were isolated from cadavers of *Musca domestica* and it larvicidal potency on *Anopheles arabiensis* (Linn) mosquito larvae was tested by varying values of each of physicochemical parameters on both the 2nd and 4th instars of laboratory reared *Anopheles arabiensis* using standard methods.

**Corresponding author: E-mail: fomoya@yahoo.com;*

Results: A mortality of 30% was recorded at pH 4.0 while at pH 9.0 higher percentage mortality (81%) was observed on the second instars larvae. Five temperatures of 16℃, 18℃, 24℃, 34℃ and 40℃ representing four major temperature ranges in southwestern Nigeria (night, morning, afternoon and evening) and control temperature (28±2℃) revealed significant variation in the percentage mortality of mosquito larvae. Varying concentrations of *Bacillus subtilis* cells showed that the larvicidal activity of this bacterium was best displayed at higher concentrations. The water turbidity and sunlight also affected the effectiveness of the larvicidal activity.
Conclusion: This study revealed that *Bacillus subtilis* was able to demonstrate high larvicidal activity against *Anopheles arabiensis* larvae despite the varied physicochemical parameters. However, further work may be needed to validate reliability.

Keywords: Physicochemical parameters; Bacillus subtilis; larvicidal activity; Anopheles mosquito larvae.

1. INTRODUCTION

Biological control involves the regulation of pest populations using natural control agents such as predators, parasitoids and pathogens (bacteria, fungi and virus), nematodes and microbial insecticides [1]. The concerns about pesticide tolerance, environmental contamination and human safety resulting from the application of synthetics have enable researchers to look for alternative methods of controlling pest using biological means [2]. Biological control methods are now increasing in number and scientific interest because biological control agents are safer to use and pose less damage to the environment.

Bacillus thuringiensis subsp. Israelensis (Bti) is being widely used in mosquito control programmes [3]. This microorganism has its limitations which include low persistence of the bacterial larvicidal crystal protein in warm environment as a result of sunlight inactivation. In addition, the strain of mosquito existing in a particular region appears to differ from that in another place, BTI may not be effective for use in all regions where mosquitoes are problematic. It is therefore important to investigate into other microorganisms for use as mosquitocidal in relation to the environmental conditions.

Bacillus subtilis is a spore forming pupicidal bacterium which metabolite can kill both the larval and pupal stages of mosquitoes, the production of mosquitocidal toxin could be initiated after the lag phase [4]. The maximum mosquitocidal activity could be obtained at 12 hours. Hence, the mosquitocidal toxins of *B. subtilis* could be produced during the vegetative phase of growth unlike in the case of *B. thuringiensis* and *B. sphaericus* which mosquitocidal toxins production accompanied sporulation [5].

This study focused on some physicochemical parameters such as pH, concentration, temperature, turbidity and sunlight that might influence the larvicidal activity of *Bacillus subtilis* in south western part of Nigeria.

2. MATERIALS AND METHODS

2.1 Study Area

This study was conducted at the Federal University of Technology, Akure (FUTA), Ondo State, Nigeria. Ondo State, Nigeria is one of the states in South Western Nigeria located at 7° 10' 0" N and 5° 5' 0" E. It covers 15,500 Km^2 with an estimated population of 3,440,000 inhabitants in the 2006 census. The climate condition of this state is classified as tropical, with a mean temperature ranging from 25℃ to 29℃ year round, and minimum and maximum temperatures of 21.6℃ and 33.8℃ respectively. Its mean relative humidity is 85%.

2.2 Insect Rearing

The mosquito larvae were collected from stagnant waters. They were selected and differentiated from other mosquitoes. The A*nopheles arabiensis* larvae were reared in a meshed cage at 25℃ and 70% relative humidity under 14L: 10D photoperiod with slight modifications according to [6]. They were fed daily with Tetramin® fish food in order to attain maturity stage and were offered blood meal. Eggs laid on wet filter papers were transferred to water trays. Larvae were fed and sorted for bioassays.

2.3 Isolation of the Test Bacterium from Insect Cadaver

Houseflies were collected into sterile containers from their natural breeding habitats (around the

refuse dumps) in Akure, Nigeria. In the laboratory, adults' houseflies were placed inside a sterile petri dish containing 10 mL of sterile water each (in triplicate). The petri dish was properly shaken to ensure good washing away of particles that were on the houseflies. One millimeter was taken from the wash water, serially diluted to 10^{-4} and 0.1 mL of the 10^{-4} serial dilution was pour plated using molten nutrient agar. Incubation was done at 37℃ for 24 h and the plates were observed for growth. Identification of the bacterial isolates was done using cultural, morphological and biochemical characteristics according to the methods of [7].

2.4 Cultivation of Bacterium

A sterilized basal medium was used. The medium contained K_2HPO_4 (17.4 g), NH_4SO_4 (1.98 g), $MgSO_4$ (0.48 g), $FeSO_4.7H_2O$ (0.0025 g) and glucose (2.0 g) in 100 mL of distilled water. Each isolate was inoculated into10 mL of the sterile basal medium, incubated at 37℃ for 24 h. The cells were centrifuged at 12,168 X 10^3 g for 15 min (Centrifuge MSE Minor 35) and re-suspended into 2 mL sterile water. The cells were counted and diluted. At inoculation onto mosquito larvae, the diluted cells were pour plated with nutrient agar, incubated and counted using colony counter.

2.5 Assay on the Effect of Physicochemical Parameters on Larvicidal Potency of *Bacillus subtilis* on *Anopheles* Mosquito Larvae

One hundred *Anopheles arabiensis* larvae (2^{nd} and 4^{th} instars) were used for each cell concentration in this experiment. The mosquito larvae were surface sterilized in separate Petri dishes using 75% alcohol and rinsing with sterile water. The sterilization was carried out to kill the bacteria that might have colonized the surface of the larvae. There were four replicates and control per treatments with 25 mosquito larvae in each container. The mosquito larvae were starved for 24 hours prior to inoculation.

2.5.1 Determination of the effect of cell population

Each mosquito larva was inoculated with the cells of the bacterial isolates at varying concentrations of 1.3 X 10^7 to 6.5 X 10^7cfu/mL. The bacterial cells of 5 mL from each concentration were added to the larvae inside a sterile bioassay container containing 20 mL of sterile water. Incubation was carried out for 48 h. The cadavers were enumerated and removed at 24 hours interval.

2.5.2 Evaluation of the effect of turbidity

The effect of turbidity was investigated by conducting the bioassay in sterile distilled water (controlled assay) and water from fish ponds (turbid water) at the highest cell concentrations of 6.5 X 10^7cfu/mL for 48 hours at 28±2℃. Number of dead larvae was sorted at 24 hours interval.

2.5.3 Measurement of the effect of sunlight

The assessment of the effect of sunlight (Ultraviolet radiation) was conducted by exposing the solution containing the bacterial cells and mosquito larvae, directly to sunlight for durations of four, six, eight, and nine hours.

2.5.4 Determination of the effect of pH

The pH value used in this study ranged from pH 4.0 to pH 11.0. The pH assessment of the bioassay medium (the solution containing the bacterial cells and mosquito larvae) were adjusted by using an electric pH meter (Easy Way Medical, England 2008E0612) following standardization with appropriate buffers (0.1 M HCL and 0.1M NaOH). The bacterial cells (32.5 X 10^7cfu) and the larvae were added to each solution at various pH values. The cells and the larvae were left at 28±2℃ for 48 hours and the number of died larvae were counted at every 24 hours.

2.5.5 Assessment of the effect of temperatures

Five temperatures of 16℃, 18 ℃, 24℃, 34℃ and 40℃ representing four major temperature regimes in south west Nigeria (night, morning, afternoon and evening) and the control temperature (28±2℃) were applied in this study. Each bioassay container was put inside an insectary with regulated temperatures as specified above for 48 hours.

2.6 Statistical Analysis

All the numerical data obtained were subjected to Analysis of Variance (ANOVA) using SPSS and means were determined using Duncan's multiple range test.

3. RESULTS AND DISCUSSION

3.1 Effect of Cell Concentration (cfu/ml) on the Larvicidal Activity of *B. subtilis* at Both the 2nd and 4th Instars of *Anopheles arabiensis* Mosquito Larvae

The effect of varied concentrations on the larvicidal potency of tested *Bacillus subtilis* on the second and fourth instars *Anopheles* mosquito larvae is shown in Fig. 1. At 1.3 X 10^7 cfu/ml, percentage mortality caused by *B. subtilis* on the 2nd instars were 10% and 22% mortality at 24 and 48 hours treatments respectively. Increase in percentage mortality was observed as the concentration increased as well as increase in hours of treatment. The fourth instars mosquito larvae were also subjected to the same conditions of treatment (concentrations between 1.3 X 10^7 cfu/ml - 6.5 X 10^7 cfu/ml). However, their larvicidal effect at 24 hours and 48 hours of exposure was found to be of lower potency when compared to the 2nd instars treatment. At 1.3 x 10^7cfu/ml, the percentage mortality of 8% and 15% were recorded respectively. The bioassay showed that increase in cells concentration per hour of incubation influenced the percentage mortality. For instance, the onset of death was slower in lower concentrations than in similar larvae inoculated with higher concentrations and under the same conditions. The death of the *Anopheles* mosquito larvae associated with massive numbers of cells/ spores may have been as a result of the release of toxins from the *Bacillus* species. This is supported by the result of [8], who reported that toxins from bacteria are known to kill insects. The delta endotoxin produced by *Bacillus thuringiensis* has the insecticidal property that is effective against blackfly and many mosquitoes, and can be used in malaria control [8]. According to the report of [9], two *B. subtilis* strains are active against third instars larvae of *Culex quinquefacciatus.* Though the mode of action of *Bacillus subtilis* is not known in this work, the activity of *B. subtilis* on larval stage has been reported to be due to the cyclic lipopeptides present [9]. Hence, the ability of the microbes to kill the larvae was dependent on cell toxins concentrations. The higher the cell concentration, the higher the concentration of the toxin produced.

3.2 Effect of Turbidity and Sunlight on the Larvicidal Activity of *B. subtilis* at Both the 2nd and 4th Instars of *Anopheles arabiensis* Mosquito Larvae

Variation in the larvicidal activity of *B. subtilis* in both turbid and clear water treatment was examined by at the 2nd and 4th instars larvae treatments with concentration of 6.5 x 10^7 cfu/ml. Higher percentage mortality was recorded in the clear water than the turbid medium in all the treatments. However, lower % mortality was obtained with the 4th instars of the larvae (Fig. 2). Effect of sunlight is shown in Fig. 3.

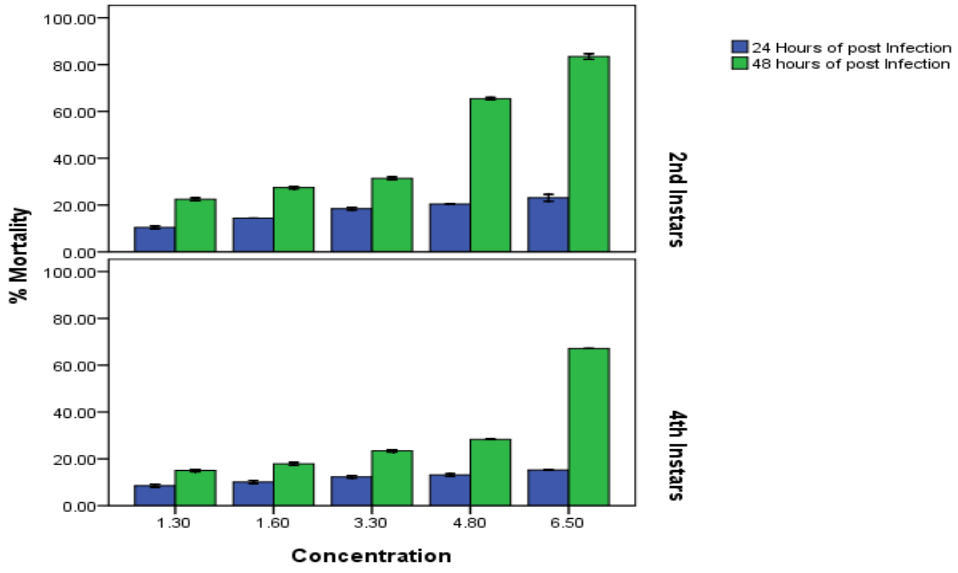

Fig. 1. Effect of cell concentration (cfu/ml) on the larvicidal activity of *B. subtilis* at both the 2nd and 4th instars of *Anopheles arabiensis* mosquito larvae

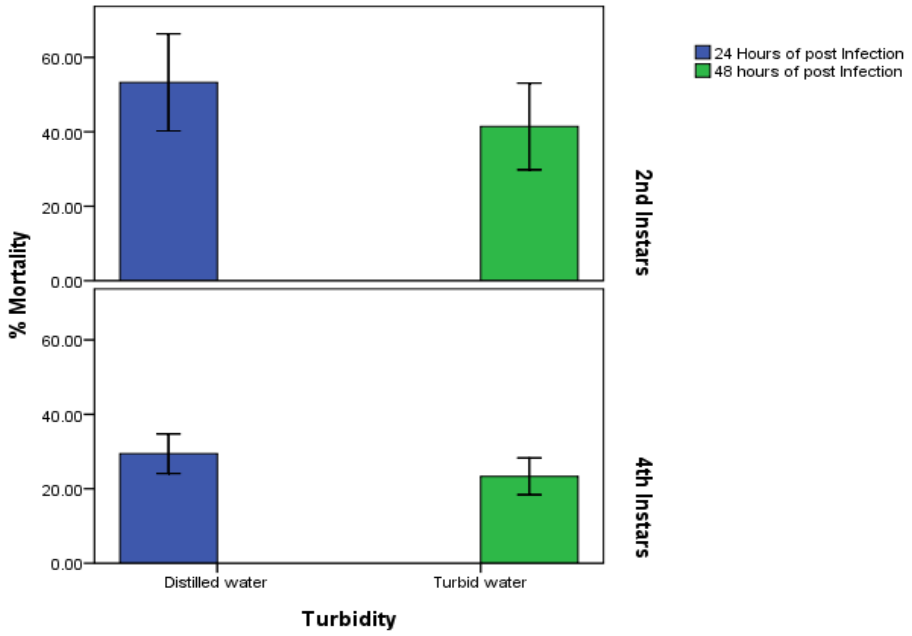

Fig. 2. Effect of turbidity on the larvicidal activity of *B. subtilis* at both the 2nd and 4th instars of *Anopheles arabiensis* mosquito larvae

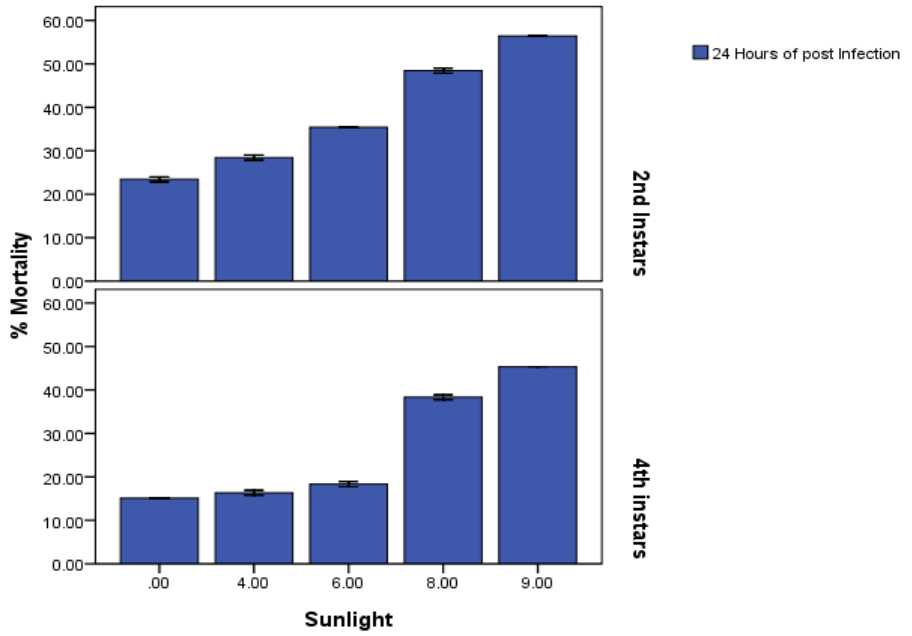

Fig. 3. Effect of sunlight on the larvicidal activity of *B. subtilis* at both the 2nd and 4th instars of *Anopheles arabiensis* mosquito larvae

In the second instars larvae, it was discovered that *Bacillus subtilis* expressed increase potency with increase in the hours of exposure. In the control treatment, 23% mortality was recorded. After 6 hr of exposure 35% mortality was observed. An increase in percentage mortality of 56% at 9 hr of exposure was recorded. Similar pattern of increment in percentage mortality was recorded in the fourth instar mosquito larvae in the presence of the ultra violet ray.

Environmental factors such as cell concentration, turbidity, pH, ultraviolet rays/sunlight, and temperature affected the Larvicidal potency of the *Bacillus* species. These factors have been reported to influence the efficacy of bacterial toxin formulations against mosquito larvae [10]. This study showed that *B. subtilis* was insensitive to ultra violet/sunlight exposure as higher larval susceptibility was observed in the 2nd and 4th instars larvae treatments. This observation might be an added advantage in the use of *B. subtilis* over other *Bacillus* species with larvicidal potency. According to [11], exposure to sunlight for 6 hr reduced the biolarvicidal potency of *Bacillus thuringiensis isrealensis* and *Bacillus sphaericus* to about 50% and 75% respectively of their original activity. This reduction is associated with sunlight irradiation which brings about widespread destruction of indole residues of protein crystals formed by *B. thuringiensis* var. *kurstaki* [12]. In contrast as shown in this study, it seems larvae death inducing factor in *B. subtilis* was not adversely affected by UV rays of the sunlight.

3.3 Effect of pH on the Larvicidal Activity of *B. subtilis* at Both the 2nd and 4th Instars of *Anopheles arabiensis* Mosquito Larvae

Fig. 4 expressed the effect of pH on the larvicidal potency of the *Bacillussubtilis*. As observed in this experiment, the percentage mortality recorded at 24 hours and 48 hours of treatment increased with increase in treatment pH values to a certain value of pH 4.0 to pH 11.0. A gradual decrease in mortality was noticed at higher pH for both the 2nd and 4th instars larvae. At pH 6.0, 14% and 30% mortality were recorded at 2nd instars treatment. At pH 7.0 the percentage mortality value increased to 18 % and 38 % at 24 hr and 48 hr of exposure respectively. The highest mortality percentage recorded is pH 9 (32% and 47% mortality). An opposite trend of decrease in percentage mortality occurred at pH 11.0. The percentage mortality recorded after 24 hr and 48 hr of treatments were 28 % and 41% respectively. For the 4th instars larvae treatment same trend of mortality was obtained but with lower percentage mortality. Other studies have shown that stability, solubility and insecticidal activity of crystal toxins of *B. thuringiensis* var. *aizawai* and *B. thuringiensis* var. *kurstaki* are affected by pH of the medium [13,9]. In the present study, there was a significant ($p<0.05$) reduction in the larvicidal potency of the tested *Bacillus* species at pH 4.0 as compared with pH 7.0. The optimum pH at which this organism exhibited maximum larvicidal efficacy was at pH 9. This study revealed that that the larvicidal potency of our tested bacterium is affected by pH of the medium.

3.4 Effect of Temperature on the Larvicidal Activity of *B. subtilis* at Both the 2nd and 4th Instars of *Anopheles arabiensis* Mosquito Larvae

Difference in larvicidal potency of the tested microorganism at different temperature values was observed. In Fig. 5, the larvicidal potency observed was lower at low temperature (16℃, 18℃ and 24℃) when compared with the 28±2℃ (control). The potency of *B. subtilis* decreased from 24% to 13% in the 2nd instars (24 hour post treatment). However an optimum temperature where the larvicidal potency of the species was highest was at 34℃ with the tested bacterium showing 34% and 68% mortality at 24 and 48 hours respectively. On the other hand, a further increase in temperature (40℃) resulted in a decrease in larvicidal (2nd instar) potency after 24 and 48 hours of the treatment when compared with the optimum temperature (34℃). A similar trend was however recorded for the fourth instars larvae although a lower population of dead larvae was observed at 16℃, 18℃, 24℃ when compared with those recorded at the second instars except at 28℃ and 40℃. This study also revealed that water temperature is a very important factor to be taken into consideration. The *B. subtilis* showed a decreased in the activity against *Anopheles* mosquito larvae in the laboratory at low temperatures (16℃). A low rate of feeding of some larvae, larval diapause and a decrease in metabolic rate could have resulted in this observation. [14] showed that bioassays conducted in the laboratory with second instars larvae of *Aedes vexans* at a low temperature (5℃) yielded 10-fold higher LC_{50} and LC_{90} values compared with those conducted at a higher temperature (25℃). [15] performed bioassays on third instar larvae of *Culex nigripalpus* and *Aedes taeniorhynchus* in the laboratory and found that LC_{50} of both species were 1.4 to 3.0 fold lower at 35℃ than at 15℃. It then means that the optimum temperature for the larvicidal potency of *B. subtilis* is 34℃ for 24 hr and 40℃ for 48 hr.

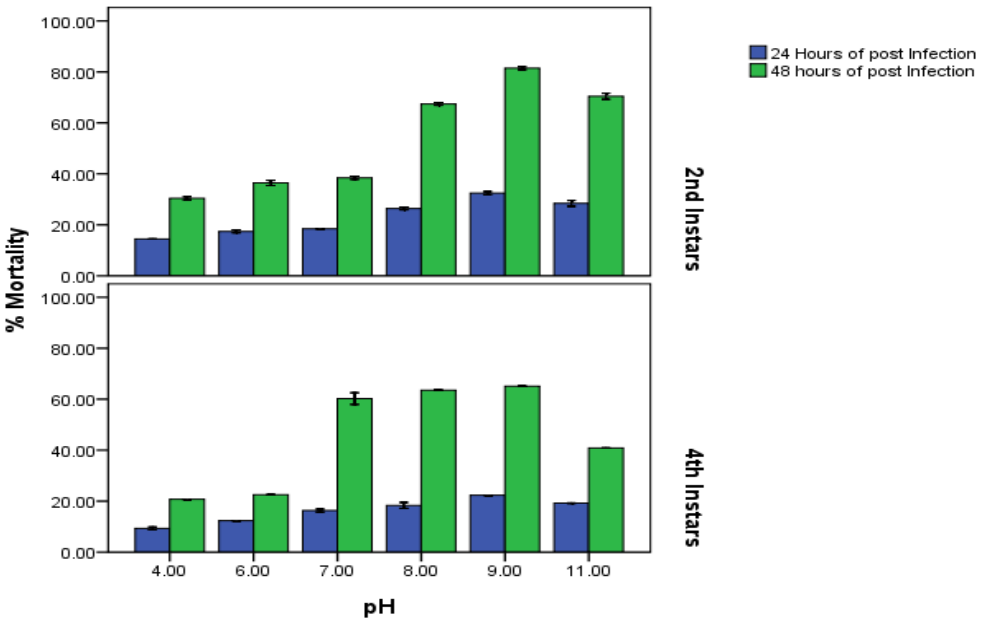

Fig. 4. Effect of pH on the larvicidal activity of *B. subtilis* at both the 2nd and 4th instars of *Anopheles arabiensis* mosquito larvae

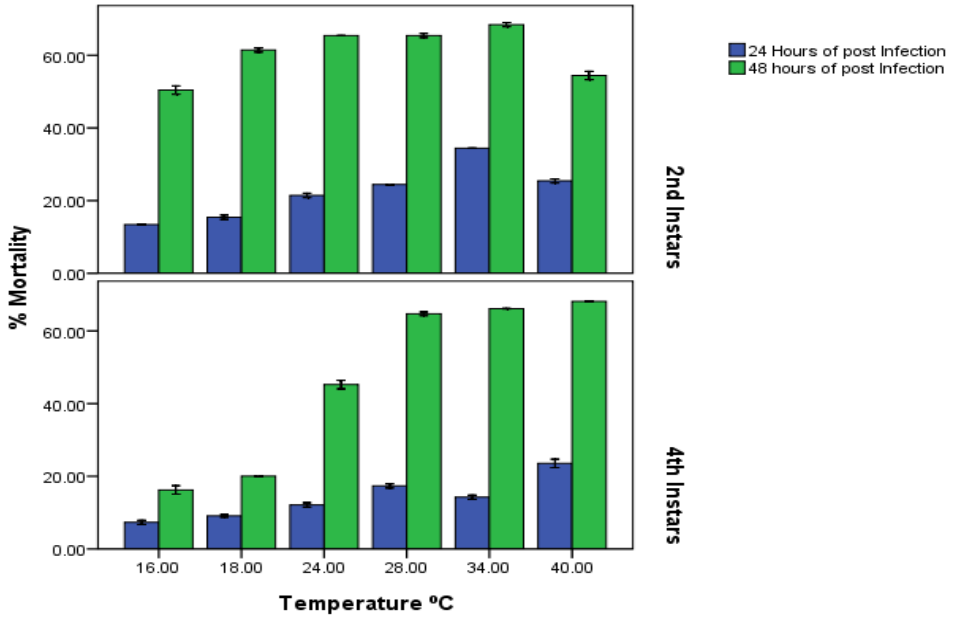

Fig. 5. Effect of temperature on the larvicidal activity of *B. subtilis* at both the 2nd and 4th instars of *Anopheles arabiensis* mosquito larvae

4. CONCLUSION

Summarily, the results obtained in this study showed that the effectiveness of *Bacillus subtilis* against *Anopheles* mosquito larvae is greatly influenced by the assessed physicochemical parameters. Increase in percentage mortality of the *Anopheles* mosquito larvae was observed irrespective of the exposed instars. However, *Bacillus subtilis* was able to demonstrate high larvicidal activity against *Anopheles arabiensis* larvae despite the varied physicochemical parameters. Reduction in mosquito larvae density has been associated with decrease in

malaria prevalence especially in areas where malaria is endemic. Therefore, more studies should be carried out on the use of *Bacillus subtilis* in the tropical countries such as Nigeria where malaria is endemic to reduce the risk of malaria transmission.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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