ENTOMOPATHOGENIC BACTERIA: *XENORHABDUS SPP* AND *PHOTORHABDUS SPP* FROM *STEINERNEMA KARII* AND *HETERORHABDITIS INDICA* FOR THE CONTROL OF MOSQUITO LARVAE

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Abstract
Mosquitoes are vectors of multiple diseases of man through transmission of various pathogens. Malaria, caused by *Plasmodium,* results in over 429,000 deaths annually. Primary methods of mosquito control are demanding, both economically and logistically, necessitating exploration of bio-control agents that are specific, safe, sustainable and easy to produce. The search for and use of entomopathogenic bacteria could have a significant impact on mosquito control. The objective of this study was to determine the virulence of entomopathogenic bacteria *Xenorhabdus spp* and *Photorhabdus spp*, isolated from two entomopathogenic nematodes (EPNs) strains *Steinernema karii* and *Heterorhabditis indica* respectively, against mosquito larvae. Mosquito larvae were collected from set up breeding vessels and maintained in distilled water in the laboratory. Bacterial symbiots from *S. karii* and *H. indica* were isolated and streaked on MacConkey and NBTA plates. Pure colonies of *S. karii*'s *Photorhabdus spp* and *H. indica*'s *Xenorhabdus spp* were obtained and characterization done through colony and cell morphological observation, biochemical and bioluminescence tests, and are preserved in the Jomo Kenyatta University of Agriculture and Technology laboratory. The pure cultures were multiplied in LB medium. Treatment concentrations were prepared from cells plus metabolites (cells/ml), cells-only (cells/ml) and metabolites-only (serial dilutions) for each of the bacterium and a combination of both. Ten 4th instar mosquito larvae were treated with each of the concentration. Highest mortality for each category of treatment; cells-only (70%), metabolites-only (100%) and cells plus metabolites (95%), was achieved by using both bacteria. Within 48 hours, all metabolites-only treatments achieved 50% mortality. There was a significant difference between the various treatments and the mosquito larvae mortality rate (ANOVA; F=11.76, df=2; P=0.000038). To our knowledge, this is the first time that cells, metabolites and combination of both from *Photorhabdus spp*, *Xenorhabdus spp* and both bacteria has been evaluated against mosquito larvae. These bacteria have shown effective virulence thus potential application as an alternative to primary methods of mosquito control in the fight against malaria.

Key words: Entomopathogenic nematodes, *Xenorhabdus*, *Steinernema karii*, *Photorhabdus*, *Heterohabditis indica*, biocontrol, mosquito larvae

1.0 Introduction

Mosquitoes are important vectors of several tropical diseases that suck blood from human and animals (Cholewiński *et al.*, 2015). They are vectors of multiple diseases of man through transmission of pathogenic viruses, bacteria, protozoa and nematodes (Gitonga *et al.*, 2012). From the medical point of view, mosquitoes are among the most important insects due to their capacity to transmit human diseases such as malaria and dengue. WHO (2017) noted that malaria causes approximately 429,000 deaths each year out of more than 212 million annual cases recorded. The African Region records 90% of all cases and 92% of the malaria-related deaths. Specifically, Sub-Saharan Africa shared the highest burden with 76% and 75% of cases and deaths globally respectively. Insecticides such as DDT and treated bed nets have been the primary methods of controlling the vector to reduce the transmission. However, this approach is quite demanding, both economically and logistically.

Use of bacteria as bio-control against insects has gained considerable attention due to their specificity, safety, sustainability and ease of production. Various species of bacteria have been identified and their virulence against various insects' larvae identified. Shahina *et al.* (2004) noted that *Xenorhabdus* and *Photorhabdus* (family Enterobacteriaeae) are unique in the bacterial world and beneficial due to their ability to form a mutualistic symbiosis in one host and mount an aggressive pathogenic attack against a totally different phylum. Sanchez-Contreras and Vlisidou (2008) identified various genes encoding virulent toxins in various genera of bacteria such as *Wolbachia, Pseudomonas, Bacillus* as well as *Xenorhabdus* and *Photorhabdus.* They noted that laboratory-reared insects can be effectively killed by *Xenorhabdus* and *Photorhabdus* but this may be different if the insects were pre-infected by non-pathogenic bacteria. In 2010, Hinchliffe *et al.* elucidated the mechanism through which *Xenorhabdus* and *Photorhabdus* cause death to infected insect larva. They identified how the bacteria effectively evade the host humoral and cellular immune response and the final attack through release of toxins that cause bio-conversion of insect tissues and finally death.

Various species of *Xenorhabdus* and *Photorhabdus* bacteria are as diverse as species of EPNs they form symbiotic relationship with and are distributed according to geographical locations (Thanwisai *et al.*, 2012). A new strain of nematode (*Steinernema. karii*) discovered in Kenya by Waturu *et al.* (1997) remains unexplored for symbiotic bacteria of economic importance. The existence of bacteria community in *Culex* mosquito's larva gut, which include the genera *Lactobacillus, Pseudomonas, Aerobactor* and *Escherichia* has been reported (Duguma *et al.*, 2013). This was associated with their filter-feeding behaviour and hence justifies the ease of getting pathogenic bacteria into the mosquito larvae. Potential of *Xenorhabdus*

and *Photorhabdus* application in insect control was reported by Ruiu *et al.* (2013), and demonstrated by da Silva *et al.* (2013) who studied the use of *Photorhabdus luminescens* and *Xenorhabdus nematophila* in control of Dengue fever with significant mortality rates (>50%) of the *Aedes aegypti* larvae in both strains.

With a wide diversity of *Xenorhabdus* and *Photorhabdus* and the reported applicability in control of larvae stage of insects including mosquitoes, the use of locally available strains against one of the most important insect (mosquito) in the world and especially in Africa, has not been investigated. Various studies have identified the pathogenic relationship between entomopathogenic bacterial strains of *Xenorhabdus* and *Photorhabdus* and insect larvae. Strains from nematodes isolated in Kenya, *Steinernema karii* and *Heterorhabditis indica* were also expected to be virulent to mosquito larvae, which was noted to have potential of significantly reducing the cost of malaria control and provide a sustainable approach to eradicate the use of environmental harmful insecticides in management of malaria vectors.

2.0 Materials and Methods

2.1 Collection of Mosquito Larvae

Mosquito larvae were collected through provision of breeding conditions in areas within the University environment that were identified to have high populations of adult mosquitoes. Bowls of water were placed in damp, cool and shady areas and monitored daily to ensure evaporated water was replaced and no contamination occurred. After two weeks, the mosquito larvae were visible at their 1st instar and were allowed 2 more days for the first bunch to progress to 2nd instars. Using the dipping technique with a white trap as described by Robert *et al.* (2002), larvae on the surface of the bowls were collected and placed in beakers containing distilled water and transferred to the laboratory for maintenance, where they were fed on fish food for 48 hours (to allow them progress to 3rd and 4th instar) prior to the bioassays. The dipping technique was expected to be effective for *Anopheles* larvae that tend to remain at the surface longer than *Aedes* and *Culex* and are usually associated with floating debris and vegetation (Rey *et al.*, 2012). Collected mosquito larvae were maintained in the laboratory in beakers, being fed on fish food. The 4th instar larvae were used in the bioassay.

2.2 Mass Production of Entomopathogenic Nematodes

The nematodes were sourced from the Kenya Agricultural and Livestock Research Organization-Thika and sub-cultured on last instar larvae of *Galleria mellonella*. Ten Petri dishes (five for each nematode) were lined with Whatman filter paper and the nematodes *Steinernema karii* and *Heterorhabditis indica* applied at the rate of 100 infective juveniles (infective stage of nematodes) per dish (9 x 3.5). The nematodes were then applied in 1ml distilled water and given 30 minutes to distribute on the filter. Third instar larvae of *G. mellonella* were placed in ten replicates to petri dishes treated with 100 infective juveniles (ij) of the nematodes. The treatments were left

on laboratory benches at room temperature (18-25°C) and 60% relative humidity for three days. The cadavers from each petri dish were placed in own White traps (Orozco *et al*., 2014) for extraction of emerging entomopathogenic nematodes. Nematodes were harvested and cleaned by sedimentation and decantation. Infective juveniles from each treatment were counted under a binocular microscope. Infective juveniles of *S. karii* were kept in deionized distilled water at a temperature of 5-7°C and those of *H. indica* at 15° C. *Galleria mellonella* larvae maintained on a special diet were used to multiply the nematodes. Infective juveniles were obtained and maintained for isolation of the symbiotic bacteria.

2.3 Isolation of Symbiotic Bacteria

Following the protocol by Rahoo *et al.* (2011), fresh infective juveniles were collected from white-traps and transferred into clean falcon tubes and allowed to sediment. The excess water was removed and the EPNs sediment immersed in 10ml of 0.1% sodium hypochlorite and left for 1 hour. The IJs were then transferred into 10ml fresh 0.1% sodium hypochlorite solution and left for 3 hours. Infective juveniles were rinsed twice with Ringer's solution under a laminar flow hood and carefully suspended in 1ml of sterile nutrient broth in a micro tube and crushed with a sterile motor. The homogenate was aseptically transferred into a sterile Mc Cartney bottles with about 10ml of nutrient broth. Bacteria were then grown in the dark on a shaker for 24-48 hours at 25-28° C. In order to allow colony growth, bacteria from the nutrient broth were streaked on MacConkey and NBTA streak plates and incubated at 25° C for 24hours. Brown colonies were isolated and plated on clean MacConkey and NBTA streak plates. The isolated bacterial colonies were inoculated in liquid LB medium and let to multiply for 16 hrs at 28° C and their concentration adjusted to 10^8 cells per 10µl after counting on a Neubauer chamber (Rahoo *et al.,* 2011).

Gram staining was done to confirm presence of Gram-negative bacteria. The cells were first heat fixed and then stained with a basic dye, crystal violet, which was taken up in similar amounts by all bacteria. The slides were then treated with an I2-KI mixture (mordant) to fix the stain, washed briefly with 95% alcohol (destained), and finally counterstained with a paler dye of different color (safranin). Gram-negative organisms were decolorized by the organic solvent and hence show the pink counter stain. Colony morphology was determined through observation under microscope, while biochemical tests for identification of and differentiation of the two strains included; catalase test, motility test, urease test and lactose fermentation test, bioluminescence, indole test, D-mannose acid production, annular hemolysis of sheep blood agar and nitrate reductase test (Brenner *et al.,* 2005).

2.4 Efficacy of Bacterial Cell Concentrates Against Mosquito Larvae

Serial dilutions were made of the bacterial concentrate, and the appropriate dose used to give the required concentration expressed as cell/ml of water in the test beakers. Bacterial concentrates were centrifuged at 13,000 rpm for 5 minutes to separate cells from metabolites. The cells were then standardized to 10^8 cells/ml and diluted to 10^4, 10^5, 10^6, 10^7 and 10^8 cell/ml using distilled water. Metabolites were serially diluted to 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 0. In each bioassay, 10 larvae of the 4th instar (length 3.0mm to 4.0mm) were added to a 750 ml beaker containing 500 ml of the test concentration of bacterial cells. Each assay was conducted three times. Bioassays were conducted at five different concentrations (10^4, 10^5, 10^6, 10^7 and 10^8 cell/ml) of cells plus metabolites and cells-alone, and five serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 0) of metabolites alone, chosen to produce larval mortalities between 20% and 95% for calculating LC_{50} values. Data was recorded after every 24 hrs for five days.

2.5 Data Analysis Method

Descriptive statistic; frequency distribution and measures of central tendency (mean) were used to describe results from triplicate of experiments. All the data was then subjected to analysis of variance (ANOVA) using Microsoft office excel 2007. A significant level of $P \leq 0.05$ was used in statistical analyses.

3.0 Results

3.1 Isolation of Symbiotic Bacteria

Initial isolation resulted in numerous colonies on NBTA media, where some were; dark blue, cream, white and light blue and had different colony morphology. Cream, white, light brown and brown colonies formed on MacConkey agar. Through sub-culturing, brown colonies were formed on MacConkey agar, while blue colonies were formed on NBTA media as expected for bacteria associated with *S. karii* and *H. indica* respectively as shown in plate 1.

Various morphological and biochemical characterizations were performed and the results compared to known strained of the symbiotic bacteria from *Steinernema spp* and *Heterorhabditis spp*, where they aligned as shown in Table 1.

Isolates from *H. indica* and *S. karii* were all Gram negative and rod shaped. They formed convex raised colonies that had a smooth circular margin and they appeared shinny. Isolates from *H. indica* were positive for catalase, motility, urease and lactose fermentation tests. *S. karii* isolates were positive for motility test and negative for catalase, urease and lactose fermentation tests. This formed the distinguishing basis where *H. indica* isolates are categorized under *Photorhabdus spp* and isolates for *S. karii* under *Xenorhabdus spp*.

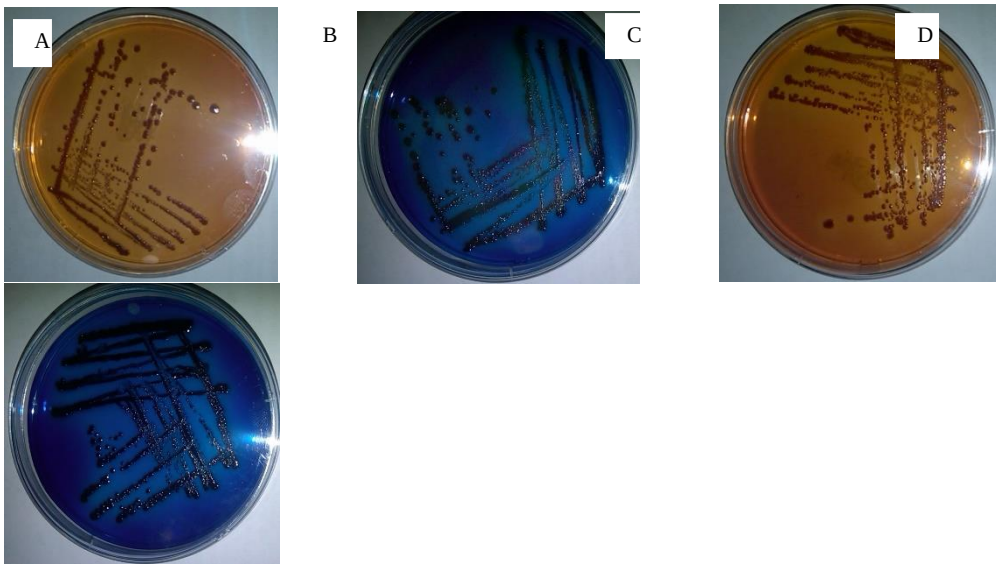


Plate 1: *A and B - S. karii symbiont on MacConkey and NBTA media respectively; C and D - H. indica symbiont on MacConkey and NBTA media respectively*

Table 1: *Characterization of bacteria isolated from H. indica and S. karii respectively*

Tests	Characteristics	*H. indica* isolate	*S. karii* isolate
Selective Media			
	MacConkey agar	Brown colonies	Brown colonies
	NBTA agar	Blue colonies	Blue colonies
Microscopy and colony morphology			
	Gram Staining	Negative	Negative
	Cell shape	Rod	Rod
	Colony margin	Smooth	Smooth
	Colony form	Convex raise	Convex raise
	Colony shape	Circular	Circular
	Color	Shinny	Shinny
Biochemical tests			
	Catalase test	+	–
	Motility test	+	+
	Urease test	+	–

Lactose fermentation	+	+
Indole Test	+	–
Annular hemolysis on sheep blood agar	+	–
D-mannose acid production	+	+
Nitrate reductase	–	–
Bioluminescence	+	–

Symbols: [+] means positive; [–] means negative

3.2. Efficacy of bacterial cell concentrates against mosquito larvae

3.2.1 *Photorhabdus spp* cells plus metabolites

Mortality rates due to different concentration of *Photorhabdus* spp cells plus metabolites were obtained within 120 hours. It was noted that highest concentration (10^8cells/ml) resulted in the highest mortality of mosquito larvae. Mortality of 50% was achieved in 72 hrs and 83% mortality in 96 hours. Concentration of 10^7cells/ml was the only other treatment to have achieved 50% mortality in 96 hours and 73% mortality in 120 hours (Table 2). There were significant differences in mortality rate between control and the *Photorhabdus* cells plus metabolites; among various concentrations of the treatment (ANOVA; F=17.32, df=5; P=0.0000012) and different exposure times (ANOVA; F=9.48, df=4; P=0.00018).

3.2.2. *Photorhabdus* cells-only and metabolites-only

Mortality due to cells-only was lower compared to metabolites-only or combination of cells plus metabolites. Only 10^8cells/ml achieved 50% mortality after 120 hours. Metabolites-only highest concentration (10^0) treatment achieved 50% mortality in 24 hours and 90% in 96 hours. However, it was only the second highest concentration that achieved 50% mortality after 96 hours (Table 2). Significant difference in mortaity of mosquito larvae was detected in various concentrations of *Photorhabdus* cells-only treatment (ANOVA; F=26.67, df=5; P=0.000000033) and different exposure times (ANOVA;F=8.00, df=4; P=0.00051) as well as *Photorhabdus* metabolites-only treatment concentraions (ANOVA; F=20.50,df=5; P=0.0000003) and different exposure times (ANOVA; F=4.23, df=4; P=0.012). Significant difference between cells, metabolites and cell plus metabolite treatment in mortality rate of mosquito larve was detected (ANOVA; F=5.22, df=2;P=0.0076).

3.2.3. *Xenorhabdus spp* cells plus metabolites

Mortality rate for different concentration of *Xenorhabdus spp* cells plus metabolites was obtained within 120 hours. The highest concentration (10^8cells/ml) achieved the highest mortality rate of 90% with 96 hrs. Moreover, mortality of 50% was achieved within 72 hrs. The concentration of 107 cells/ml was the only other to reach 50% mortality after 96 hrs and a 53% in 120 hrs. The rest achieved mortality that was below 30% as shown in Table 2. There were significant differences in mortaity rate between control and the *Xenorhabdus* cells plus metabolites; among various concentrations (ANOVA; F=11.40, df=5; P=0.000026) and different exposure times (ANOVA; F=4.48, df=4; P=0.0096).

3.2.4. *Xenorhabdus spp* cells-only and metabolites-only

Mortality due to cells-only and metabolites-only of *Xenorhabdus spp* showed that cells-only treatment achieved 50% concentration after 96 hrs and it was the highest in the 10^8cells/ml concentration only. This was different from the metabolite-only assay, as 50% mortality was achieved in 10^0 after 48 hrs and a high of 90% within 96 hrs. Concentration of 10^{-1} achieved a 50% mortality in 96 hrs, which was the highest. The other concentrations did not reach a 50% mortality. Significant differnce in mortaity of mosquito larvae was detceted in various concentarions of *Xenorhabdus* cells-only treatment (ANOVA; F=23.40, df=5; P=0.00000010) and different exposure times (ANOVA; F=7.15, df=4; P=0.00095) as well as *Xenorhabdus* metabolites-only treatment concentraions (ANOVA; F=20.19, df=5; P=0.00000034) and different exposure times (ANOVA; F=4.14, df=4; P=0.013). The three treatment options had no significance difference in mortality (ANOVA; F=1.65, df=2; P=0.20).

3.2.5. *Xenorhabdus spp* and *Photorhabdus spp* cells plus metabolites

Mortality rate for different concentration of *Xenorhabdus spp* and *Photorhabdus spp* cells plus metabolites was obtained within 120 hours. Three concentrations (10^8, 10^7 and 10^6 cells/ml) achieved 50% mortality, with 10^8 and 10^7 after 72 hrs and 106 cells/ml after 96 hrs. 95% mortality was achieved after 96 hrs by the highest concentration of 10^8 cells/ml (Table 2). There were significant differences in mortaity rate between control and the *Photorhabdus* and *Xenorhabdus* cells plus metabolites treatment; among various concentrations (ANOVA; F=15.62, df=5; P=0.0000026) and different exposure times (ANOVA; F=9.50, df=4; P=0.00018).

3.2.6. *Xenorhabdus spp* and *Photorhabdus spp* cells-only and metabolites-only

Mortality due to cells-only and metabolites-only of both *Photorhabdus spp* and *Xenorhabdus spp* showed that only the highest concentration of cells-only treatment that achieved a 50% mortality after 72 hrs and reaching 70% in 96 hrs.

Metabolite-only treatment achieved maximum mortality (100%) within 72 hrs in the highest concentration. Moreover, 10^{-1} concentraton also achieved 50% mortality in 120 hrs (Table 2). Significant differnce in mortaity of mosquito larvae was detceted in various concentarions of *Photorhabdus* and *Xenorhabdus* cells-only treatment (ANOVA; F=27.85, df=5; P=0.000000023) and different exposure times (ANOVA; F=5.56, df=4; P=0.0036) as well as *Photorhabdus* and *Xenorhabdus* metabolites-only treatment concentraions (ANOVA;F=54.16,df=5; P=0.000000000061) and different exposure times (ANOVA; F=4.11, df=4; P=0.014). The three treatment options had a significant difference in mortality of mosquito larvae (ANOVA;F=6.91,df=2;P=0.0018)

Table 2: *Mortality of mosquito larvae due to Photorhabdus and Xenorhabdus cells and metabolites*

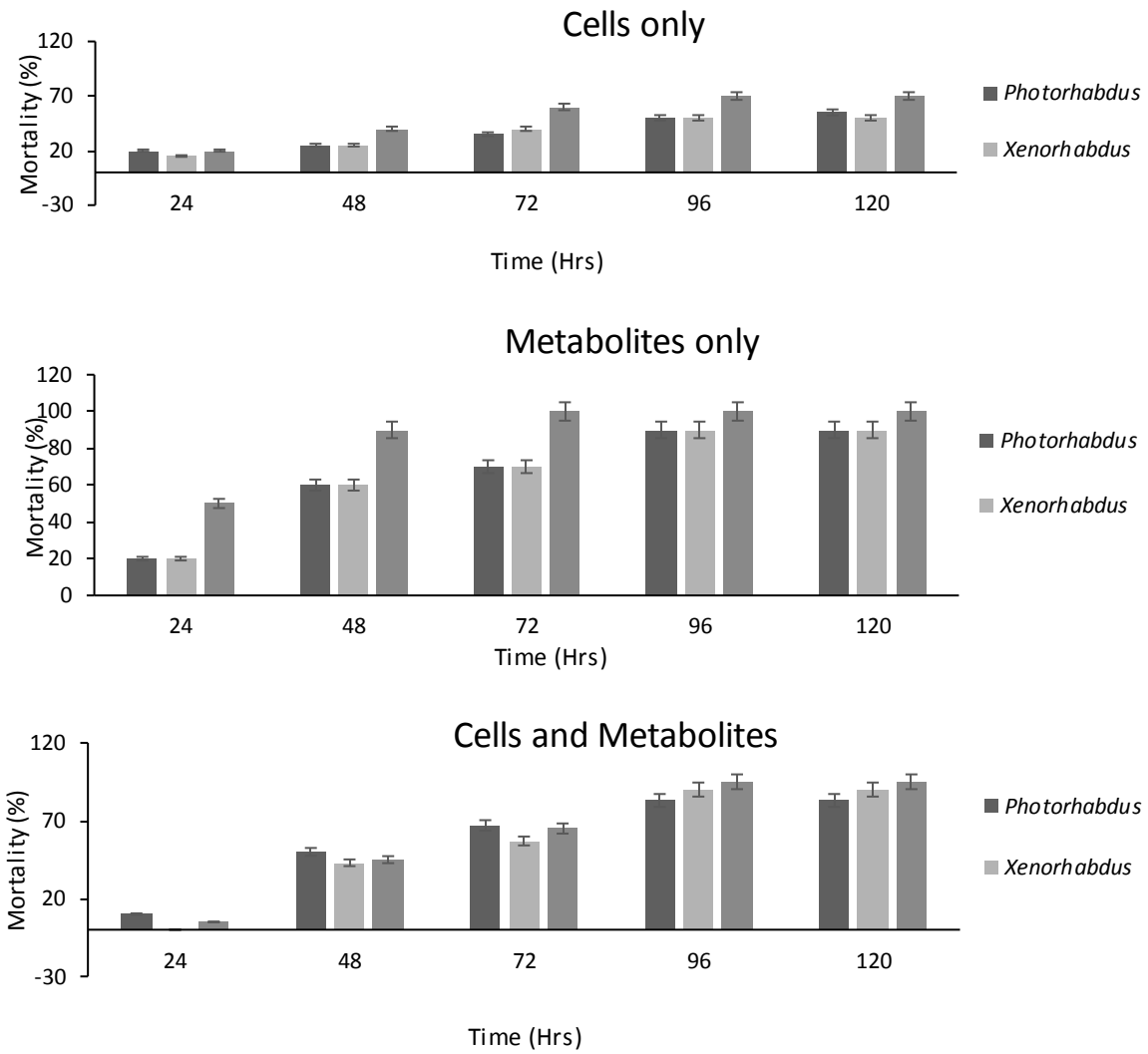
Time (Hours)	***Photorhabdus spp.***						***Xenorhabdus spp.***						***Photorhabdus and Xenorhabdus spp.***					
	Cells only (cells/ml)						Cells only (cells/ml)						Cells only (cells/ml)					
	10^8	10^7	10^6	10^5	10^4	0	10^8	10^7	10^6	10^5	10^4	0	10^8	10^7	10^6	10^5	10^4	0
24	20	0	0	0	0	0	15	5	0	0	0	0	20	10	0	0	0	0
48	25	5	0	0	0	0	25	15	5	5	0	3	40	20	10	0	0	0
72	35	15	10	5	0	3	40	20	10	5	0	3	60	30	20	10	0	0
96	50	20	15	5	5	3	50	25	20	5	5	3	70	30	20	10	0	0
120	55	30	15	5	5	3	50	30	25	5	5	3	70	40	30	10	0	0
	Metabolites only (serial dilutions)						Metabolites only (serial dilutions)						Metabolites only (serial dilutions)					
	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	0	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	0	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	0
24	20	0	0	0	0	0	20	0	0	0	0	0	50	0	0	0	0	0
48	60	20	0	0	0	0	60	20	0	0	0	3	90	30	10	0	0	0
72	70	40	10	10	3	0	70	40	10	10	0	3	100	30	20	0	0	0
96	90	60	10	10	3	0	90	60	10	10	0	3	100	40	30	0	0	0
120	90	60	10	10	3	0	90	60	10	10	0	3	100	50	30	0	0	0

	Cells and Metabolites(cells/ml)						Cells and Metabolites (cells/ml)						Cells and Metabolites (cells/ml)					
	10^8	10^7	10^6	10^5	10^4	0	10^8	10^7	10^6	10^5	10^4	0	10^8	10^7	10^6	10^5	10^4	0
24	10	10	0	3	0	0	0	1	0	0	0	0	5	20	20	0	10	0
48	50	23	0	7	0	0	43	13	0	0	0	0	45	40	25	5	10	0
72	67	43	10	10	3	1 0	57	27	7	3	3	0	65	55	40	15	10	5
96	83	67	30	13	13	1 0	90	50	23	7	3	0	95	80	60	25	15	10
120	83	73	33	20	17	1 0	90	53	27	10	3	3	95	85	60	25	20	10

3.3 Comparative analysis

A comparison of the mortality rates for cells plus metabolites of *Photorhabdus*, *Xenorhabdus* and both bacteria combined is showed that at 24 hours, mortality rate for all treatments was below 10%, and 50% mortality was achieved between 48-72 hours. The highest mortality rate was due to combined treatment at 95% followed by *Xenorhabdus* at 90% and *Photorhabdus* at 83% in 120 hours (Figure 1). There was a significant difference between the different treatments and the mosquito larvae mortality rate (ANOVA; F=11.76, df=2; P=0.000038). The mortality rate at different exposure times due to cells of *Xenorhabdus*, *Photorhabdus* and both bacteria was compared and at 24 hours, 15 % mortality for all treatments was achieved, and 50% mortality after was achieved between 72-96 hours. The highest mortality rate was due to combined treatment at 70% followed by *Photorhabdus* at 55% and *Xenorhabdus* at 50% in 120 hours (Figure 2). The combined treatment had higher mortality rate throughout the exposure time. However, there was no significant difference between the three treatment options in mortality rate (ANOVA; F=2.80, df=2; P=0.067). In a comparison between mortality rate of mosquito larvae at different exposure times due to *Photorhabdus*, *Xenorhabdus* and both bacteria metabolites, 20% mortality occurred after 24 hours, while 50% mortality was achieved within 48 hours. The highest mortality rate was due to combined treatment at 100% followed by *Photorhabdus* and *Xenorhabdus* at 85% in 120 hours (Figure 1). The combined treatment had higher mortality rate throughout the exposure time. There was, however, no significant difference between the various treatments and mortality rate (ANOVA; F=0.48, df=2; P=0.62).

Figure 1: *Mortality of mosquito larvae within 120 hours due to cells plus metabolites of Photorhabdus spp, Xenorhabdus spp and the combination of the two bacteria*



4.0 Discussion and Conclusion

The current study aimed to isolate entomopathogenic bacteria and determine their efficacy against mosquito larvae. In this study, cells plus metabolites and metabolites-only of *Photorhabdus spp* isolated from *H. indica* showed great potential where it caused 50% death of *Anopheles* mosquito larvae after 48 hrs and >80% in 96 hours. The cells-only treatment achieved 50% mortality in 96 hrs and reached a maximum of 55% in 120 hours. The low efficacy of cells-only treatment could be attributed to the fact that cells separated from the metabolites had already reached the stationary phase thus no more division or toxin production occurred. Hinchliffe *et al*. (2010), noted that toxins from *Photorhabdus* and *Xenorhabdus* bacteria have a wide insecticidal properties and their utilization is inevitable. Another study by da Silva *et al*. (2013) found out that these bacteria caused significant mortality to larvae of *Aedes aegypti*, where *P. luminescence* killed 73% of the treated larvae, while *X. nematophila* killed 52%.

Cells plus metabolites and metabolites-only from *Xenorhabdus spp* isolated from *S. karii* achieved 50% mortality in 72 hrs and 48 hrs respectively with a maximum mortality of 90% in 96 hours. The cells-only treatment achieved 50% mortality in 96 hours and this was also the maximum. The findings are consistent with the results reported by da Silva *et al*. (2013). The combination of cells plus metabolites of *Xenorhabdus spp* showed less efficacy compared to *Photorhabdus spp,* but metabolites-only treatment had similar effect on mortality. Moreover, metabolites-only treatment caused 90% mortality compared to 80% caused by *Photorhabdus spp* in 120 hours. Cells plus metabolites and metabolites-only of a combination of *Photorhabdus spp* and *Xenorhabdus spp* achieved 50% mortality in 72 hrs and 24 hrs respectively and a maximum of 95% and 100% in 96 hrs respectively. Their cells-only treatment achieved 50% mortality in 72 hrs and a maximum of 70% in 96 hrs. These three treatments from combination of both bacteria were much more effective than *Photorhabdus spp* or *Xenorhabdus spp* treatments, with the combined metabolites causing 100% mortality in 96 hours.

It is evident that the metabolites of each bacterium are more lethal than the cells or combination of cells plus metabolites. Moreover, the virulence of a combined effect of both *Xenorhabdus* and *Photorhabdus* toxins against mosquito larvae is the highest. The high virulence of cells plus metabolites and metabolite-only treatments compared to cell only treatment can be attributed to the fact that cells after separation from metabolites have no toxins that inhibit growth of other microorganisms as noted by Thanwisai *et al*. (2012) and hence their growth is inhibited by other microbes in the larvae gut. The combined effect of metabolites produced by both bacteria reached a 100% mortality rate. This can be attributed to the difference noted in their biochemical characteristics, which means they may also be producing different toxins targeting different tissues for bioconversion. This leads

to numerous organ failure within the larvae and ultimate death compared to the effect of metabolites from each of the bacteria.
H. indica and *S. karii* harbor *Photorhabdus spp* and *Xenorhabdus spp* respectively.

These bacteria are effective bio-control agents against mosquito larvae. The virulence of both bacteria did not differ significantly, but the combination of their metabolites is the most lethal, followed by cells plus metabolites, while the cells-only is the least effective treatment. It was hypothesized that *Photorhabdus spp* and *Xenorhabdus spp* have no pathogenic relationship with mosquito larvae. At 95% confidence level, significant difference between control and *Photorhabdus spp* concentrates in mosquito larvae mortality was detected (ANOVA; F=17.32, df=5; P=0.0000012), as well as control and *Xenorhabdus spp* concentrates (ANOVA; F=11.40, df=5; P=0.000026). The null hypothesis was therefore rejected and the alternative; there is a significant pathogenic relationship between *Photorhabdus spp* and *Xenorhabdus spp* and mosquito larvae, was adopted.

The experiments were laboratory based but the mosquitoes were collected from the environment. Chances are that some larvae may have harbored nematodes, which are widely dispersed in moist soil, and hence had the test bacteria in their guts resulting in false positive results. The larvae were, however, maintained in the laboratory for more than 48 hours, a period which EPNs are expected to have killed the host, before use to validate their neutrality.

This study, unlike da Silva *et al*. (2013) and the only other similar study so far, show the highest mortality of 100% caused by metabolites of *Photorhabdus* and *Xenorhabdus spp* when used together. The study also reveals that metabolites-only are more lethal than cells-only or combination of cells and metabolites. These results add to the evidence that *Photorhabdus* and *Xenorhabdus spp* have toxins that are virulent to mosquito larvae and thus further studies should be carried out to allow utilization of these bio-resources in the fight against malaria.

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