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Native *Bacillus* strains from infected insects: a potent bacterial agent for controlling mosquito vectors *Aedes aegypti* and *Culex quinquefasciatus*.

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Abstract

Mosquito-borne diseases are major illnesses caused by pathogens and parasites in humans and animals. Some of the *Bacillus* toxins act as insect control agents and few are potential alternatives to chemical control because they can be innovative and more selective than chemical insecticides. On the other hand, many insects' species may coexist in the larval development sites and are susceptible to the larvicidal activity of *Bacillus* toxins. Therefore, in this study, optimized native *Bacillus* strains from infected insects were identified by 16S rRNA gene sequencing method and to perform experiments on the larvicidal effects of the pathogenicity against third instar larvae of *Culex quinquefasciatus* and *Aedes aegypti*. The maximum larval mortality was observed after 48 hrs of exposure. The LC₅₀ for *Bacillus cereus*, *Bacillus thuringiensis* and *Lysinibacillus sphaericus* against the *A. aegypti* as 5.40×10^8 , 5.33×10^8 and 4.51×10^8 cells and for *C. quinquefasciatus* as 4.70×10^8 , 4.32×10^8 and 3.62×10^8 cells in 24 hrs respectively. Findings showed that optimized native *Bacillus* strains have high larvicidal activity against the larvae of *C. quinquefasciatus* and *A. aegypti* whereas *Bacillus* strains from soil sample was ineffective to the mosquitoes. The outcome of the present study identifies the improved bacterial strains *B. cereus*, *B. thuringiensis* *L. sphaericus* obtained from infected insects that can be used as effective and alternate larvicidal agents against the mosquito larvae of *C. quinquefasciatus* and *A. aegypti*.

Keywords: Mosquitoes, bacillus strains, 16S rRNA gene sequencing and biocontrol agents

1. Introduction

Mosquitoes transmit a wide range of pathogens that cause the life-threatening and devastating diseases like malaria, filariasis, Japanese encephalitis, dengue, chikungunya as well as a variety of arboviruses diseases, West Nile Virus (WNV), Saint Louis Encephalitis Virus (SLEV) and Eastern Equine Encephalitis Virus (EEEV) [1]. The global incidence of these diseases has grown dramatically in recent decades especially in tropical countries. Because of the great reproductive capacity of mosquitoes and its high genomic flexibility make management of these vectors very difficult. Their high genomic flexibility is demonstrated by the resistance of mosquito populations to chemical and biological insecticides as well as by their ability to adapt to different environmental conditions and to climate changes [2, 3, 4]. Nevertheless, effective alternative forms of control methods have proven to be promising strategies for mosquito control.

The mosquitoes control through pathogenic microbes proved to be an efficient solution to control the diseases with specific to the target organisms [5]. Because of the culture-dependent and culture-independent approaches have been analyzed and conducted to survey the bacterial diversity in mosquitoes [6, 7]. This survey is revealed a diverse community of bacteria encompassing several phylogenetic classes. Most abundant bacterial strains are pathogenic and/or inhabitant to many insects. Among the various bacterial pathogens, *Bacillus thuringiensis* and *B. sphaericus* are being widely used to control the mosquito larvae [8, 9, 10, 11]. More than two hundred *Bacillus* species have been described and each species produces a specific set of one or more toxins as a crystal during sporulation [12, 13]. In general, *Bacillus* exhibits a high level of specificity toward an insect phylogenetic group [14, 15]. This adaptation to their host with the selection and conservation of key virulence factors makes *Bt* a pathogen rather than an opportunistic bacterium [16, 17]. Several studies have already shown that a higher tolerance to *Bt* is associated with a difference in bacterial community composition [18, 19].

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Nevertheless, whether changes in gut microbiota content are the cause of an increased tolerance or a consequence of *Bt* infection remains unclear. However, there is no information on prevalence of environmentally and locally suitable *Bacillus* strains that it's potential to be used as biocontrol agents. Therefore, the present study was focused on the insecticidal potential of *Bacillus cersus*, *B. thuringensis* and *Lysinibacillus sphaericus* isolated from infected insects and its pathogenicity improved by suitable optimization methods against third instar larvae of *Culex quinquefasciatus* and *Aedes aegypti*.

2. Materials and methods

2.1 Isolation and cultivation of bacteria

The effective bacterial strains were isolated from the naturally infected insects (larvae of *Colocyra cephalonica* and *Bombix mori*, adult of *Gallaria mellonella*) and were collected from Western Ghats of Srivilliputtur region, Tamilnadu. All insect samples were anesthetized by incubating them for 2 min at -20 °C. The surfaces of the samples were sterilized by soaking in 70% ethanol in 2 ml micro-tubes and shaking for 5 min. Dissection was accomplished in sterile conditions under a biological laminar flow hood. Each dissected infected tissues/gut was transferred to a 1.5 ml micro-tube; its contents were suspended in 1 ml of sterile PBS solution and homogenized by an electrical homogenizer. Then, serially these suspensions were diluted and inoculated into the nutrient media by spread plate method. The different form and shape of colonies were identified based on the morphological features.

2.2 DNA extraction from bacterial isolates and amplification of 16S rRNA gene

The bacterial DNA was extracted from prominent and effective bacterial strains by the phenol/chloroform method [20]. Briefly, Pallet of bacterial cells was resuspended in 5 ml solution which contains 2 mg/ml lysozyme and incubated at 37°C for 1h. Then, 0.5 ml of 1% Sodium Dodecyl Sulphate was added and incubated for 15 min at room temperature. The cell lysate was mixed with phenol/chloroform in an equal volume and kept on ice for 5 min, then spinning at 10,000 rpm in 4°C for 20 min. The supernatant contains DNA and washed with an equal volume of phenol/chloroform for 5 min on ice and respinning for 20 min at 10,000 rpm and 4°C for removing the proteins. Then, 0.1 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol added. The pellet was collected through centrifugation and washed with 70% ethanol and air-dried. The DNA pellet mixed with 50 µl TE buffer and mixed well.

Specific forward and reverse primers were designed based on the specific 16S rRNA gene fragments of bacteria using online software as follows: 16s-27F (5' AGAGTTTGATCCTGGCTCAG 3'), 16s-1429R (5' GGTTACCTTGTTACGACTT 3') and obtained from Eurofins Genomics Pvt. Ltd., Bangalore. Reaction mixtures were prepared with a final volume of 25 µl using 1 unit of Taq DNA polymerase (Hi-Media Laboratories Pvt. Ltd., Mumbai), 0.25 mM dNTPs, 1× Taq polymerase buffer, 0.4 µm of each primer, and 20 ng of the extracted DNA as template. Reactions were performed at 94 °C for 5 min and cycled 35 times through a protocol of 1 min at 94 °C, 1 min at 60 °C, and 2 min at 72 °C followed by a final extension step at 72°C for 7 min. Amplicon size was confirmed by agarose gel

electrophoresis on 1% agarose gel. Staining was done with ethidium bromide and amplicons were visualized by an UV trans-illuminator.

2.3 Sequence and phylogenetic analysis

Amplified fragments were purified by the gel purification kit according to the manufacturer's instruction (Qiagen, Duusseldorf, Germany) and PCR products with the expected size of three bacterial strains were directly sequenced by the Eurofins Genomics Pvt. Ltd., Bangalore, India. Sequences were further analyzed for confirmation using the nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and these sequences were submitted to GenBank. The sequences were aligned using the MEGA 10 version software and Clustal W program with the available sequence's representative of *B. cersus*, *B. thuringensis* and *L. sphaericus* for genetic linkage analysis. Additionally, Phylogenetic trees were constructed using the neighbour-joining (NJ) method. The NJ analysis was performed using the Tamura-Nei model with MEGA 10 Version software [21].

2.4 Mosquito culture

The eggs raft of *A. aegypti* and *C. quinquefasciatus* were obtained from the Vector Control Research Center, ICMR, Madurai. The larvae were provided with dog biscuit and yeast tablet powder in the ratio 3:1 (W/W) as a source of nutrients. The pupae were collected in plastic cups with the help of a pipette. They were transferred to an enamel bowl containing water and kept inside emergence cages (60 × 60 × 60cm). Adult male mosquitoes were provided with 10% sugar solutions through cotton buds; resins soaked in water were also provided in a petri dish kept inside the cage. Females were provided with blood meal from an immobilized chicken. Small enamel bowls with water were placed inside the cage for facilitating oviposition. Egg rafts deposited by the females were collected on the next morning, hatched in enamel trays and the culture was maintained as described by earlier [22].

2.5 Preparation of bacterial inoculum

The selected isolates were grown in Luria Bertani Broth (LB) medium and tested against third- instar larvae of *A. aegypti* and *C. quinquefasciatus*. The effective bacterial isolates were grown for 30°C. Each bacterial isolate cell was harvested by centrifugation at 10,000×g for 5 min at 4°C and washed twice with sterile DH₂O and resuspended in sterile 100mL of PBS and directly cells number was counted by simple cell counter method. After identification of effective bacterial isolates, each species was individually optimized by their growth rate, pH, temperature, pathogenicity, intake of nutrient sources through different methods. The optimized bacterial cells were harvested and suspended in 100mL of PBS solution were freshly prepared for bioassays. A total of 23×10⁸, 25×10⁸ and 21×10⁸ Cells/dL for *B. cersus*, *B. thuringensis* and *L. fusiformis* respectively. The different concentration to range for each bacterial species was assayed for *B. cersus* [10ml (23×10⁸), 5ml (11.50×10⁸), 2.5ml (5.75×10⁸), 1.25ml (2.87×10⁸), 0.625ml (1.43×10⁸), 0.312ml (0.71×10⁸) and 0.156ml (0.35 ×10⁸)], for *B.thuringensis* [10ml (25×10⁸), 5ml (12.50×10⁸), 2.5ml (6.25×10⁸), 1.25ml (3.12×10⁸), 0.625ml (1.56×10⁸), 0.312ml (0.78×10⁸) and 0.156ml (0.39×10⁸)] and *L. fusiformis* [10ml (21×10⁸), 5ml (10.50×10⁸), 2.5ml (5.25×10⁸), 1.25ml (2.62×10⁸), 0.625ml (1.31×10⁸), 0.312ml (0.65×10⁸) and 0.156ml (0.32 ×10⁸)].

2.6 Estimation larvicidal activity

Freshly moulted larvae of *A. aegypti* and *C. quinquefasciatus* were tested to determine the larvicidal activity of the different bacterial cell volumes of the selected bacterial isolates. According to the previously described experimental design, to 150ml of water taken in plastic cups appropriate different volume live bacterial cells from 100 ml fresh bacterial culture was added. To each dose 20 freshly moulted larvae were released and were provided with larval feed. Two controls (one with 150ml water alone and the other with 150ml of water containing maximum volume of PBS in the test sample) were maintained. The water level in the plastic cups was maintained regularly. Larval mortality, abnormality and deformities were monitored till the larvae moulted into the next instar. Larval mortality in the normal control and PBS control did not significantly differ. The isolates were classified as toxic when mortalities were up to 50% or cause any symptoms. An infected mosquito larva was systematically studied under microscope. Considering the percentage mortality of the larvae after 24 and 48hrs in different doses, LC₅₀ and LC₉₀ (No. of bacterial cells required to kill and/or causes the pathogenicity to treated mosquito larvae) of the selected bacterial isolates were calculated using probit analysis. Differences between the three replicates were analyzed using Chi-square test ($P < 0.05$) in SPSS 16 software package.

3. Results

A variety of bacterial colonies were isolated and observed from the infected dead larvae of *C.cephalonica*, *B.mori* and *G. mellonella* by using pure culture method followed by serial dilution technique. Bacterial colonies started to appear on the isolation plates from 1st day onwards. However, plates were incubated for 2-3 days. The number of colonies was increased with increasing incubation duration. A total of 70 colonies were counted in all the petri plates in the selected three samples based on their cultural characteristics. Each bacterial isolate was carefully screened against mosquito larvae of *C. quinquefasciatus*. Preliminary study revealed that these bacterial isolates were more effective for the selected larvae

of *Culex* and deformities also observed.

These preliminary results pointed out that three isolates were more valuable than the other bacterial isolates and hence further assays were carried out only with these bacterial strains. These isolates were also cultivated on Hicrome Bacillus Agar and effective isolates further optimized with different pH, temperature, growth rate. Based on their cultural, morphological, biochemical, carbon sources utilization and 16SrRNA sequences of selected isolates were identified as SZC16 - *Bacillus cersus* (Gen bank No: MN630567), SZB23 - *Bacillus thuringensis* (Gen bank No: MN630562) and SZG12 - *Lysinibacillus fusiformis* (Gen bank No: MN630309). The degree of relatedness of selected isolates was further determined to different species through phylogenetic analysis. It revealed the isolates of SZC12 sequence indicated a 99% similarity with other *L. sphaericus* strains NR_112569.1, NR_112628.1 and BR_042072.1. The NJ tree revealed a close relationship between the strains isolated in the present study and NR134073.1 bacterial strain which also a *L. sphaericus*. The SZB23 is more 99.9% similarity with *B. thuringiensis* (NR_114581.1) and SZC16 is also more similar with *B. cersus* (NR 074540.1) (Figure1).

Toxicity of the third instar larvae of *A. aegypti* and *C. quinquefasciatus* probit regression equations were calculated and presented in table 1 and 2. The assay was carried out using total cell mass in the range of 23×10^8 cells/dL to 0.35×10^8 cells at the interval of 23×10^8 , 10.2×10^8 , 5.8×10^8 , 2.9×10^8 cells. *B. cersus*, *B. thuringiensis* and *L. fusiformis* showed maximum toxicity towards the *A. aegypti* larvae with mortality rate of 80, 85 and 100% respectively, the results are given in table 1 and figure 2. In case of *C. quinquefasciatus* mosquito larvae, least Lc50 was found to be 3.62×10^8 cells. The larval mortality was observed after 24, 48 and 72 hrs of exposure. Slopes of all the regression equations are statistically significant ($P < 0.05$). The adult emergence not recorded within 96 hours of exposures to all of the test bacterial cells and the rates of pupal development also not recorded.

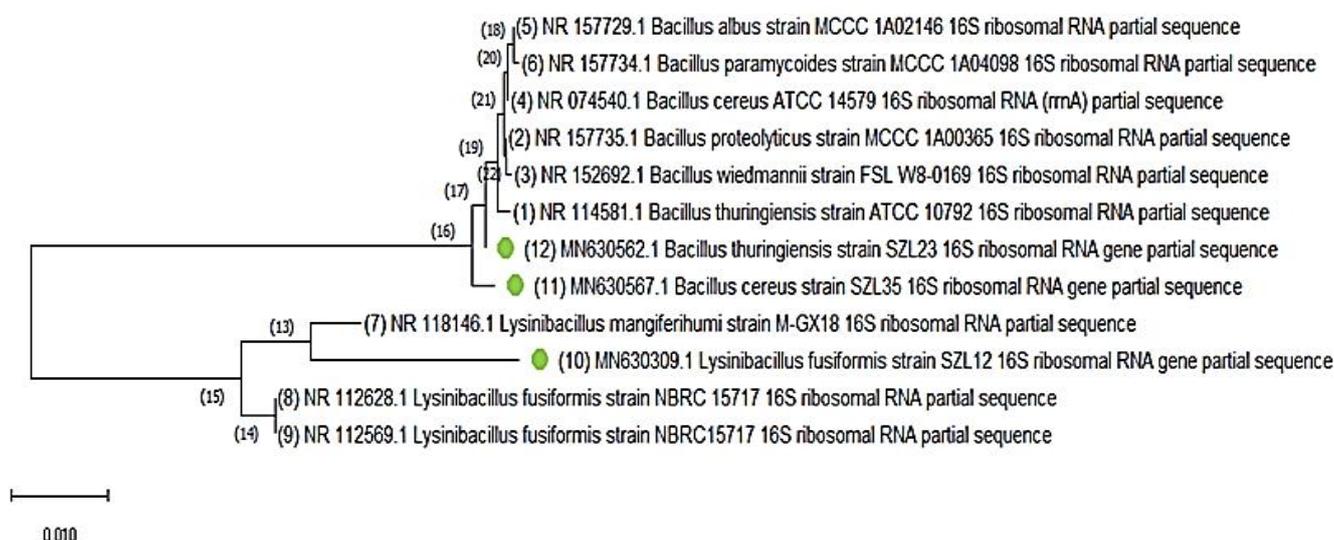


Fig 1: Constructed a Neighbor joining phylogenetic tree by MEGA ver 10 based on its nearly complete 16S rRNA gene of bacterial strains with branch length of 1.0 revealed four distinct groups. Green dot represents the isolated strains MN630309.1, MN630562.1 and MN630567.1.

Table 1: LC₅₀ associated statistics of the effective bacteria tested against early third instar larvae of *A. aegypti*.

Bacteria	Time (Hours)	LC ₅₀	95% LCL-UCL	LC ₉₀	Intercept	Regression equations	R value	X ² Value	PValue df-5
<i>B. cereus</i>	24	5.40×10 ⁸	4.33 ± 6.79	11.22	-1.18	Y= -2.58X+0.78	0.90	8.37	0.13
	48	3.90×10 ⁸	3.05 ± 4.97	8.45	-1.09	Y= -3.36X+0.75	0.86	4.52	0.47
	72	2.01×10 ⁸	1.23 ± 2.80	5.96	-0.65	Y= -6.94X+0.82	0.63	3.14	0.67
<i>B. thuringensis</i>	24	5.33×10 ⁸	4.09 ± 6.92	12.48	-0.95	Y= -4.24X+0.91	0.83	8.17	0.14
	48	3.81×10 ⁸	2.66 ± 5.11	10.33	-0.75	Y= -6.03X+0.92	0.86	5.24	0.38
	72	1.88×10 ⁸	1.05 ± 2.65	5.83	-0.61	Y= -7.72X+0.87	0.59	3.61	0.60
<i>L. fusiformis</i>	24	4.51×10 ⁸	3.60 ± 5.66	9.40	-1.18	Y= -2.39X+0.69	0.86	8.05	0.15
	48	2.81×10 ⁸	1.13 ± 5.49	6.60	-0.95	Y= -3.19X+0.63	0.80	13.05	0.02
	72	1.57×10 ⁸	0.97 ± 2.16	4.49	-0.69	Y= -6.09X+0.71	0.76	2.04	0.84

Table 2: LC₅₀ associated statistics of the effective bacteria tested against early third instar larvae of *C. quinquefasciatus*.

Bacteria	Time (Hours)	LC ₅₀	95% LCL-UCL	LC ₉₀	Intercept	X ² Value	Regression equations	R value	PValue df-5
<i>B. cereus</i>	24	4.70×10 ⁸	3.68 ± 6.00	10.41	-1.05	6.29	Y= -3.35X+0.79	0.88	0.27
	48	2.97×10 ⁸	2.30 ± 3.83	6.47	-1.08	2.36	Y= -3.93X+0.73	0.68	0.79
	72	1.59×10 ⁸	0.87 ± 2.25	4.93	-0.61	1.81	Y= -7.48X+0.81	0.58	0.87
<i>B. thuringensis</i>	24	4.32×10 ⁸	3.22 ± 5.65	10.56	-0.88	5.52	Y= -4.49X+0.89	0.87	0.35
	48	3.11×10 ⁸	2.28 ± 4.13	7.66	-0.87	2.00	Y= -5.92X+0.88	0.84	0.84
	72	1.35×10 ⁸	0.63 ± 1.96	4.41	-0.57	2.36	Y= -8.34X+0.85	0.72	0.79
<i>L. fusiformis</i>	24	3.62×10 ⁸	2.86 ± 4.61	7.70	-1.13	3.99	Y= -2.90X+0.68	0.74	0.55
	48	2.10×10 ⁸	1.55 ± 2.72	4.91	-0.95	3.39	Y= -4.12X+0.64	0.60	0.63
	72	1.35×10 ⁸	0.80 ± 1.86	3.85	-0.69	2.16	Y= -6.09X+0.69	0.73	0.82

4. Discussion

In the present study, the effective bacterial strains were isolated from the infected insects; *C. cephalonica*, *B. mori* and *G. mellonella* and the potential strains were identified and characterized by 16S rRNA sequencing. Toxicity of selected bacterial strains SZC16, SZB23 and SZG12 were initially tested against larvae of *C. quinquefasciatus*. From the gene sequencing, the selected bacterial strains SZC16, SZB23, and SZG12 were identified as *B. cereus*, *B. thuringensis* and *L. sphaericus* by the 16S rRNA sequence and submitted in NCBI database. The 16S rRNA gene sequence revealed the genus and species identification for isolates based on the “acceptable” or “low likelihood” according to viable systems. According to the cumulative studies, the 16S rRNA gene sequencing afford genus identification in most cases (>90%) [23]. The phylogenetic investigation of bacterial strains reported by several researchers that intact assemblages on categorizing bacterial responses include taxon’s relative abundance changes [24]. Nevertheless, the bacterial isolates from infected insects were confirmed through the phylogenetic analysis. *Bti* toxins are widely used for lepidopteran, coleopteran and dipteran pests with these toxins have been produced for insect

pest control [25]. In fact, because of the structural similarities between *Bti* toxins, cases of resistance and cross-resistance have been identified in field populations of pest worms [26] and in a cell line of *A. aegypti* [27]. However, many researchers reported that an occurrence of different environments is being variation among the species level [28, 29, 10]. From these points of view, the present bacterial strains much more virulent to mosquitoes than previous workers. However, we found a statistically significant high mortality rate in the selected bacterial culture bioassay treated with cell mass (Fig. 2), presumably explained by *L. sphaericus* spore germination in mosquito larval cadavers [30] and also revealed that naturally occurring mosquito toxic strain of *L. sphaericus* is able to recycle in mosquito larval cadavers. Therefore, the present result reveals that optimized strains might germinate inside the *A. aegypti* and *C. quinquefasciatus* larvae, which causes mortalities from the vegetative cell stage. As expected, all treated *A. aegypti* and *C. quinquefasciatus* presented high mortality to *L. sphaericus* vegetative cells due to the capacity to produce vegetative mosquitocidal toxins (Mtx) and S-layer [31, 32, 33].

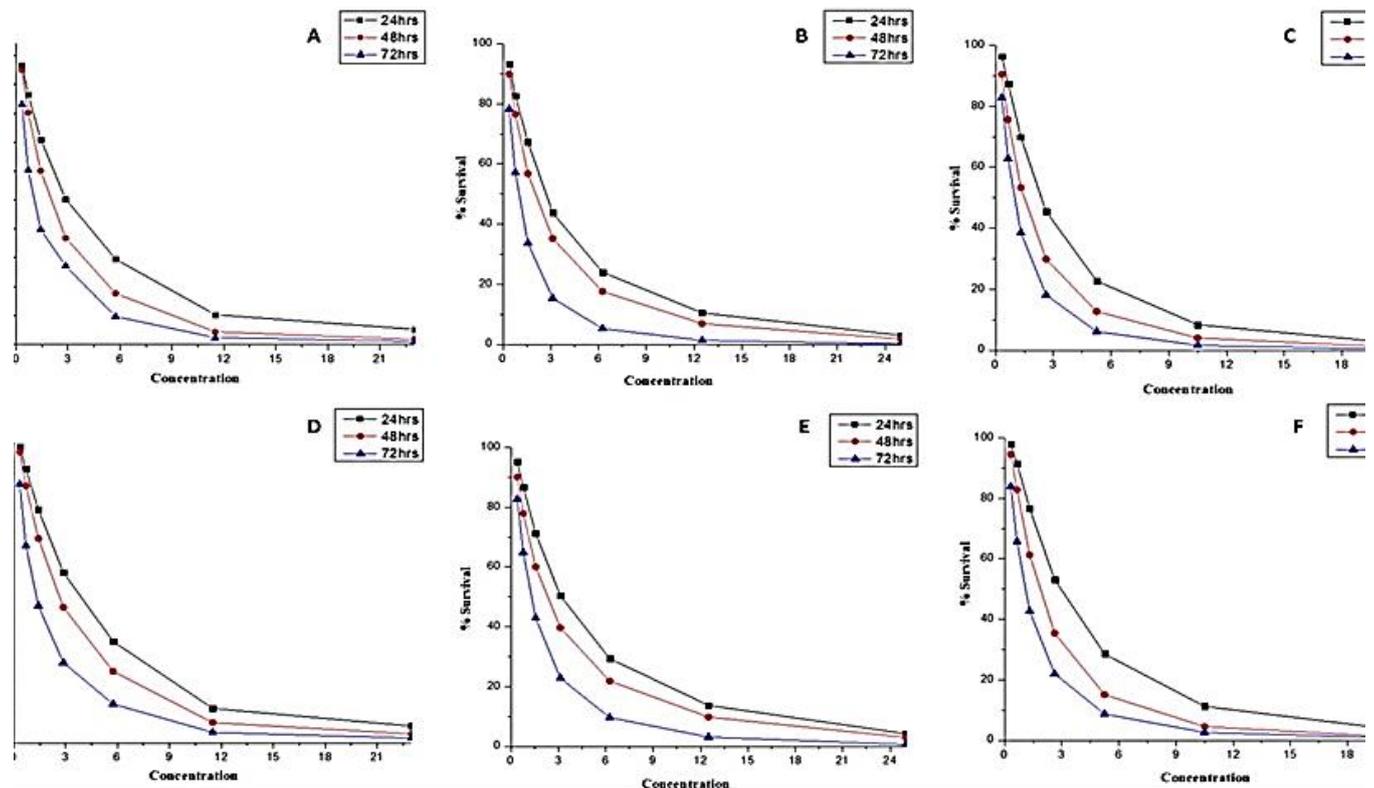


Fig. 2: Survival rate of mosquitos' in different concentrations in exposure of 24, 48 and 72 hrs. where a- *B. cersus*, b-*B. thuringensis* and c-*L. sphaericus* is against *A. aegypti* and d- *Bacillus cersus*, e-*B. thuringensis* and f-*L. sphaericus* against *C. quinquefasciatus*.

5. Conclusion

The selected bacterial strains *B. cersus*, *B. thuringensis* and *L. sphaericus* had the highest percentages for larval mortality at the highest cell density with the least exposure time because the sustained existences of optimized bacteria were released toxins that may be highly toxic to larvae of mosquitoes. Altogether, our results suggest that improved *Bacillus* strains from naturally infected insects could be used as effective alternative bio-control agents especially larvae of mosquitoes without environmental impact.

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Conflicts of interest

The authors confirm that the content of this article does not present any conflict of interest.

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