Entomotoxigenic Activity of *Lysinibacillus sphaericus* Against Mixed Cultures of *Aedes aegypti* (Diptera: Culicidae) and *Culex quinquefasciatus* (Diptera: Culicidae)

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1. Abstract

Lysinibacillus sphaericus is a spore-forming bacillus used for the biological control of mosquitoes given its entomotoxigenic activity determined by various toxins and layer proteins produced either during sporulation or by the vegetative cell. *Aedes aegytpi* and *Culex quinquefasciatus* are mosquito vectors for several tropical diseases that represent a current public health problem. Both species may coexist in the same hatchery and are sensitive to the larvicidal activity of *L. sphaericus*. In this study, we measure the larvicidal effects of *L. sphaericus* 2362, III (3)7 and OT4b.25 strains against *C. quinquefasciatus* and *A.aegypti* mixed cultures. Our findings show that *L. sphaericus* spores, vegetative cells and the combination of both possess high larvicidal activity against coexisting *C. quinquefasciatus* individuals, whereas only the formulation of *L. sphaericus* vegetative cells was efficient against *A. aegypti* coexisting individuals. We also tested the three strains against field-collected larvae and the same result was observed. We propose that asymmetrical competition between the two species of mosquito is occurring, providing us with a chance to increase mortality rates produced by the treatments. Despite this, we suggest that *L. sphaericus* is a good candidate to control both species due to its highly toxigenic activity found in this study and previous studies evaluating *L. sphaericus* against *A. aegypti* and *C. quinquefasciaus* independently.

Keywords: *Aedes aegypti*; *Culex quinquefasciatus*; *Lysinibacillus sphaericus;* Larvicidal activity

2. Introduction

Aedes aegypti and *Culex quinquefasciatus* Say are mosquito vectors for several tropical diseases that represent a current public health problem: Dengue, malaria, zika fever, yellow fever, chikungunya, West-Nile fever, filariasis, and encephalitis (Morrison, Zielinski-Gutierrez, Scott & Rosenberg, 2008; Hill & Conelly, 2009). These diseases are common in tropical countries where the climate conditions suit the development of these vectors and where the predominant expansion of cities and towns surrounding typical water bodies leads to an increase of transmission cases (Ali, Ravikumar & Beula, 2013).

Despite the highly occurrences of both mosquito species in urban areas, the ecological requirements for each species are different: *C. quinquefasciatus* have a predilection for polluted waters rich in organic matter, whereas *A. aegypti* have a preference for clean and stagnant waters (Rozeendal, 1997). These ecological differences suggest that both species may not breed in the same hatchery, nevertheless Leyva, Marquetti & Montada (2012) found that differences in feeding habits and oviposition modalities between these two vectors lead to coexistence in the same hatchery as long as there is enough food and space for both.

The use of organic insecticides has proven to be a good mechanism to control both mosquito populations, since most of these compounds can be easily recycled and, unlike artificial or chemical insecticides, they are less harmful to the environment and to human health (Ali *et al*., 2013). Biological control is also an efficient solution to insect pests given these benefits, besides it uses natural pest antagonists or predators (Goulson, 2005).

Lysinibacillus sphaericus is an aerobic, mesophilic, spore-forming and Gram-positive bacterium, commonly isolated from soil and water. It is a known fact that some strains are toxic against mosquito larvae and used in commercial products in the biological

control of vectors (P. Baumann, Clark, L. Baumann & Broadwell, 1993). During its sporulation phase, *L. sphaericus* produces a binary toxin that is specific to mosquito larvae. This binary toxin is composed of proteins *BinA* (42 kDa) and *BinB* (51 kDa) that are deposited as a parasporal inclusion body, enclosed with the spore in the exosporium. *BinB* binds to a α-glucosidase receptor in epithelial midgut cells, allowing the entrance of *BinA* and causing cellular lysis. Moreover, *L. sphaericus* toxic strains express three mosquitocidal toxins in vegetative cells: *Mtx1, Mtx2* and *Mtx 3* that contribute to the biological control of mosquitoes. *Mtx1* is a 100kDa protein which is very similar to ADPribosylation-type toxins, whereas *Mtx2* and *Mtx3* contain a domain characteristic of pore form toxins (Berry, 2012).

S-layer protein is a proteinaceous structure found on the surface of several archaea and bacteria and it is composed of identical glycoprotein monomers. Its functions involve the interaction between cells and the environment, e.g., cover protection and cell adhesion recognition (Peña *et al*., 2006). This protein is also expressed in *L. sphaericus* vegetative cells and it is known that it contributes to larvicidal activity (Lozano, Ayala & Dussán, 2011).

There are many advantages of using *L. sphaericus* for biological control. First, binary toxin is stable through different temperatures and water quality, and it is UV resistant, thus reducing the costs of distributing the spores *in situ* (Berry, 2012). Furthermore, the spore persists in polluted waters and does not stick to sediments, allowing the binary toxin to float near larval feeding zones. In fact, even when deposited at the bottom, some larvae may still be exposed to this toxin when grazing on shallow mud.

Given that *A. aegypti* and *C. quinquefasciatus* are two species of epidemiological importance, highlighted by their occurrences in urban areas and their possibility to coexist in the same niche, the objective of this study is to assess the entomotoxigenic activity of *L. sphaericus* against mixed cultures of *A. aegypti* and *C. quinquefasciatus.* For this purpose,

we used three strains previously reported as highly toxigenic (Lozano & Dussán, 2013): *L. sphaericus* OT4b.25 and *L. sphaericus* III(3)7 previously isolated in Colombia from coleopteran larvae and oak forest soil, respectively and the WHO bacterial strain *L. sphaericus* 2362 kindly donated by A. Delécluse at the Pasteur Institute (Charles, Nielson-LeRoux, & Delécluse, 1996).

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3. Materials and Methods

Bacterial and Mosquito culture conditions

The three *L. sphaericus* strains: 2362, OT4b.25 and III(3)7 were subjected to a synchronization procedure. To do this, an initial inoculum of each strain was cultivated in an overnight liquid culture of Nutrient Broth (Oxoid) for 16 h. Then, five cycles of cultivation were performed in acetate broth (composed of sodium acetate 5.00 g/l, yeast extract 3 g/l, $MgCl_2$ 1x 10^{-3} M, $CaCl_2$ 7x 10^{-4} M and $MnCl_2$ 5x 10^{-5} M), with incubation at 30°C and subjection to thermal shock (90ºC for 20 min) until 90% of cells were observed (via light microscopy) to have sporulated. This protocol was carried out according to previous studies (Lozano & Dussán, 2013).

The Entomology Laboratory at National Institute of Health of Colombia (INS) kindly donated the mosquito larvae. The colony conditions were 30°C at 70% of relative humidity with light periods of 12 h. The mosquito populations used in this study were *A. aegypti* Rockefeller and *C. quinquefasciatus* Muña. Furthermore, field *A. aegypti* and *C. quinquefasciatus* larvae were collected at La Mesa, Cundinamarca, Colombia (4˚38'02.9"N and 72˚27'43.42"O) and Cordoba wetlands in Bogotá, Colombia (4°42'10.1"N 74°04'07.2"W), respectively, in order to assess the efficiency of laboratory and wild populations of *L. sphaericus*.

Larvicidal bioassays

The larvicidal activity of the strains was assayed against fourth instar larvae of *C. quinquefasciatus* and *A. aegypti* (Laboratory reared and wild populations). To test the toxicity of vegetative cells, the cells from synchronized cultures were grown in Nutrient Agar for 12h at 30°C. The cells were harvested and resuspended in 1 mL of sterile distilled water and then added (10^9 CFU/mL) to 99 mL of chlorine-free tap water with 20 larvae of each species. To test the toxicity of sporulated cultures, we added 1 mL (10^9 CFU/mL) of the synchronized strains grown in acetate broth to 99 mL of chlorine-free tap water with 20 larvae of each species. To test both toxicity of sporulated cultures and vegetative cells, we added 0.5 mL of each of the previously prepared inocula.

Each treatment was carried out by triplicate. The bioassays were incubated at 30°C for 48 h. Larval mortality was recorded after 24 h and 48 h. Our negative control consisted of 100 mL chlorine-free tap water with 20 fourth instar larvae of each species but no bacterial strain.

Statistical analysis

The R v3.1.1 software was used for statistical analysis (R Core Team, 2016). Shapiro-Wilcoxon was used to clarify the normality distribution for the data obtained (Korkmaz, 2015) and the Kruskal-Wallis test was used to validate significant differences between treatments and control with no bacteria.

4. Results

The results shown in Figure 1 demonstrate the total mortalities 48 h after larvae exposure to vegetative cells in which there is a significant difference (Kruskal-Wallis test $\alpha < 0.05$, P-value = 0.005228) in the number of dead larvae between the control with no bacteria and the

treatments. Otherwise, Figure 2 again shows significant differences (Kruskal-Wallis test $\alpha < 0.05$, P-value = 0.00265) in the number of dead *C. quinquefasciatus* larvae between the control with no bacteria and the treatment with spores, but in contrast to the vegetative cell bioassay, *L. sphaericus* spores do not show high mortality against *A. aegypti* larvae. Figure 3 shows the bioassay of both larvae exposed to vegetative cells and spores, in which once again there are significant differences (Kruskal-Wallis test $\alpha < 0.05$, P-value = 0.005165) in the number of dead *C. quinquefasciatus* larvae between the control with no bacteria and the treatments. Similarly, the exposure of the larvae solely to spores, both toxicity of sporulated cultures and vegetative cells do not show high mortality against *A. aegypti.*

In order to compare the mortality of *A. aegypti* and *C. quinquefasciatus* laboratory reared larvae against larvae obtained in field, we performed larvicidal bioassays against field-collected larvae of both species. Figure 4, 5 and 6 show the results of these bioassays in which the same pattern could be observed. Figure 4 shows the total mortalities 48h after the exposure of the field larvae to vegetative cells in which there are significant differences (Kruskal-Wallis test $\alpha < 0.05$, P-value = 0.003093) in the number of dead larvae between the control with no bacteria and the treatments. Figure 5 shows high mortality of *C. quinquefasciatus* field collected larvae against *L. sphaericus* spores (Kruskal-Wallis test $\alpha < 0.05$, P-value = 0.003093), but low mortality against field collected *A. aegypti*. For the treatment using only spores, Figure 6 shows both toxicity of sporulated cultures and vegetative cells that do not exhibit high mortality against *A. aegypti*, but do exhibit high mortality against *C. quinquefasciatus* (Kruskal-Wallis test $\alpha < 0.05$, P-value = 0.002752).

All treatments show that laboratory reared and wild populations of *C. quinquefasciatus* are highly sensitive to all formulations of *L. sphaericus*, in contrast to *A. aegypti* populations, which are only highly sensitive to vegetative cells. Furthermore, all

treatments with no bacteria show a mortality rate (26.66%-34.16%) of *C. quinquefasciatus*, which is interestingly lower than the mortality rate of *A. aegypti* (0%-9.16%). This phenomenon will be discussed in the next section.

5. Discussion

Findings in this study show that *L. sphaericus* spores, vegetative cells and the combination of both exert high larvicidal activity against coexisting *C. quinquefasciatus* individuals, whereas only the formulation of *L. sphaericus* vegetative cells was efficient against *A. aegypti* coexisting individuals. These results agree with previous studies showing that the binary toxin present in *L. sphaericus* spores have no toxic effect against *A. aegypti* but that high concentrations of *L. sphaericus* vegetative cells (10^9 CFU/mL) are highly toxic for *A. aegypti* larvae (Silva & Dussán, 2015). *A. aegypti* contain a homologous midgut receptor to the α-glucosidase present in *C. quinquefasciatus* called *Aam1*, but only at very low concentrations, and the binding capacity of any such homologue for the toxin *BinA* and *BinB* is very low (Nielsen-Leroux & Charles, 1992; Lekakarn, Promdonkoy & Boonserm, 2015).

Despite the fact that *A. aegypti* larvae are not susceptible to the binary toxin found in sporulated cultures, we found a low mortality rate in the spores' bioassays. This could be explained by the possibility of *L. sphaericus* spore germination in mosquito larval cadavers (Correa & Yousten, 1995). According to this, we propose that spores would germinate inside the *C. quinquefasciatus* dead larvae, promoting direct contact with either S-layer or Mtx toxins from the vegetative cell stage with *A. aegypti.*

Berry (2012) suggested that preparations with spores and vegetative cells would act synergistically, enhancing the effectiveness of *L. sphaericus.* Our study reveals, that contrary to expectations, there is no synergistic effect in the larvicidal effect of *L. sphaericus* using

the two stages of the bacteria, apparently caused by either proteases produced during the bacteria's stationary phase that could be degrading the *Mtx1, Mtx2* and *Mtx3* toxins present in vegetative cells (Thanabalu & Porter, 1995) and the amount of inoculum applied.

A previous study shows that ecological niches of *Aedes albopictus* and *Culex pipiens* tend to overlap when food and space are limited, leading to a phenomenon called asymmetrical competition between the two species (Carrieri, Bacchi, Bellini & Maini, 2003). In larvicidal bioassays, space was limited and there was no food supply for the larvae. Thus, we propose that this phenomenon would be occurring for the two species evaluated, encouraging the mortality of *C. quinquefasciatus* in all treatments and clarifying the mortality rate found in treatments with no bacteria.

It is necessary to assess the effect of asymmetric competition in different development conditions for larvae such as food, space and oxygenation, in order to determine whether there is a synergistic effect of a larvicide aimed at a mixed population and natural dynamics of populations to control. This should be carried out in order to avoid biocontrol overestimation and achieve more efficient formulations. Field conditions, which naturally inhabit the populations of both species are, in most cases, characterized as stagnant waters with a large amount of nutrients available, so the larvicide applied to control both populations, should be previously evaluated and its effectiveness should be very high in order to evade the effect of asymmetrical competition that may arise under laboratory conditions.

This study shows that *L. sphaericus* 2362, III(3)7 and OT4b.25 are good candidates to control *A. aegpyti* and *C. quinquefasciatus* coexisting populations *in vitro* and, ultimately, *in situ* while they are vegetative cells but not as spores. Despite the phenomenon of asymmetrical competition, we conclude that *L. sphaericus* has a biological control potential, given that, according our results and the results found in individual bioassays (Silva &

Dussan, 2015; Lozano & Dussán, 2013), *L. sphaericus* vegetative cells are highly toxigenic against both *A. aegypti* and *C. quinquefasciatus* individuals whether they are coexisting or not. Additionally, we strongly recommend the use of vegetative cells, not only because of the low effectiveness of the binary toxin against *A. aegypti*, but also *C. quinquefasciatus'* resistance against *L. sphaericus* binary toxin found in several studies (Chalegre *et al*., 2012; Guo *et al.,* 2013; Nielsen-Leroux, Charles, Thiery & Georghiou, 1995).

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7. References

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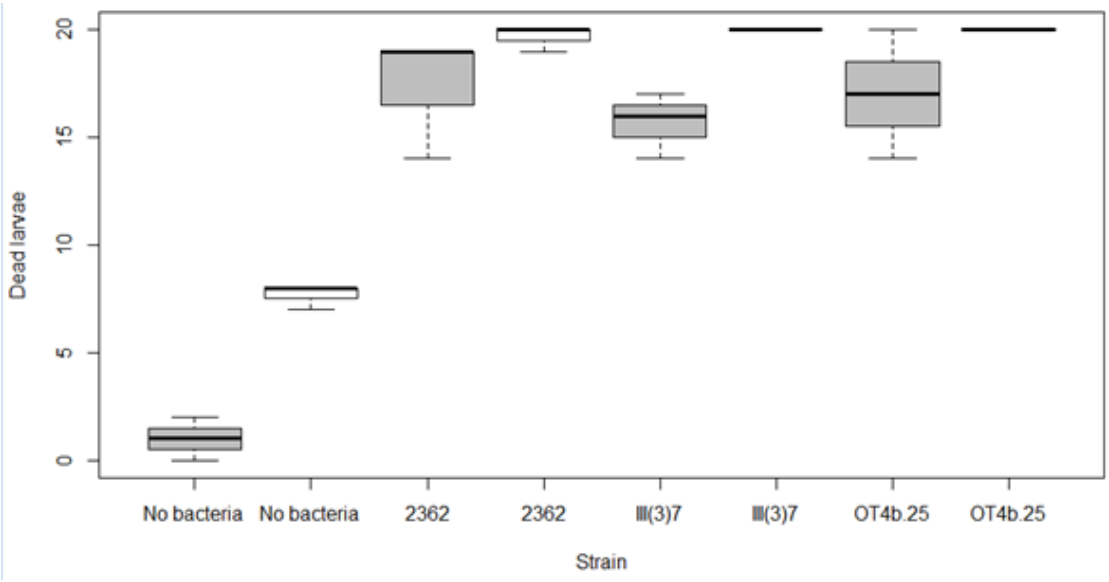

Figure 1. Bioassay of *A. aegypti* (gray) and *C. quinquefasciatus* (white) laboratory reared fourth larvae mixed cultures in presence of *L. sphaericus* vegetative cells (10^9 UFC/mL) after 48 hours of testing.

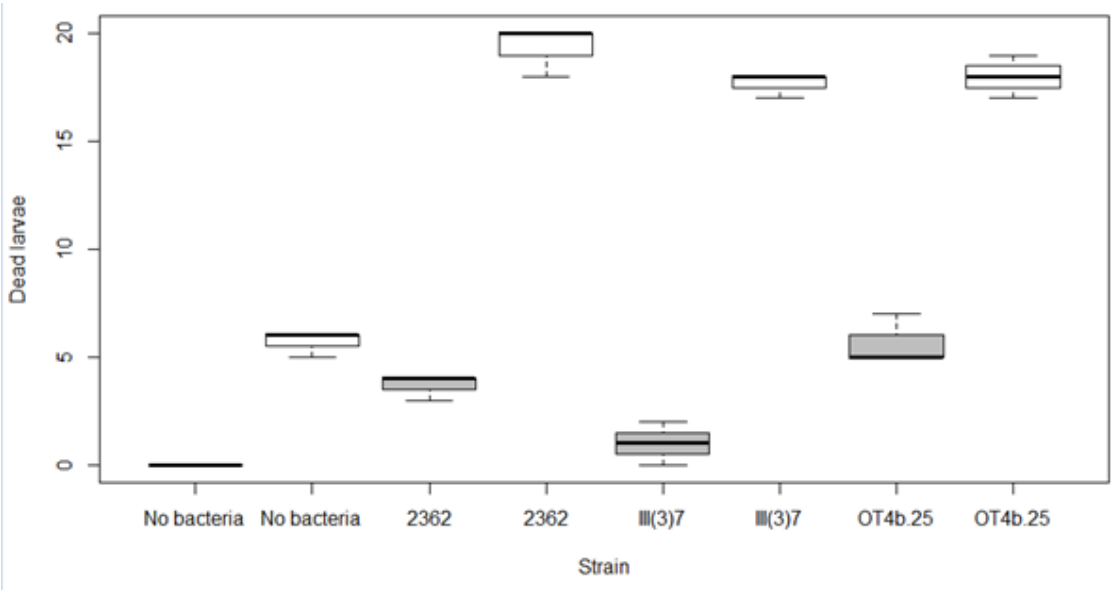

Figure 2. Bioassay of *A. aegypti* (gray) and *C. quinquefasciatus* (white) laboratory reared fourth larvae mixed cultures in presence of *L. sphaericus* spores (10^9 UFC/mL) after 48 hours of testing

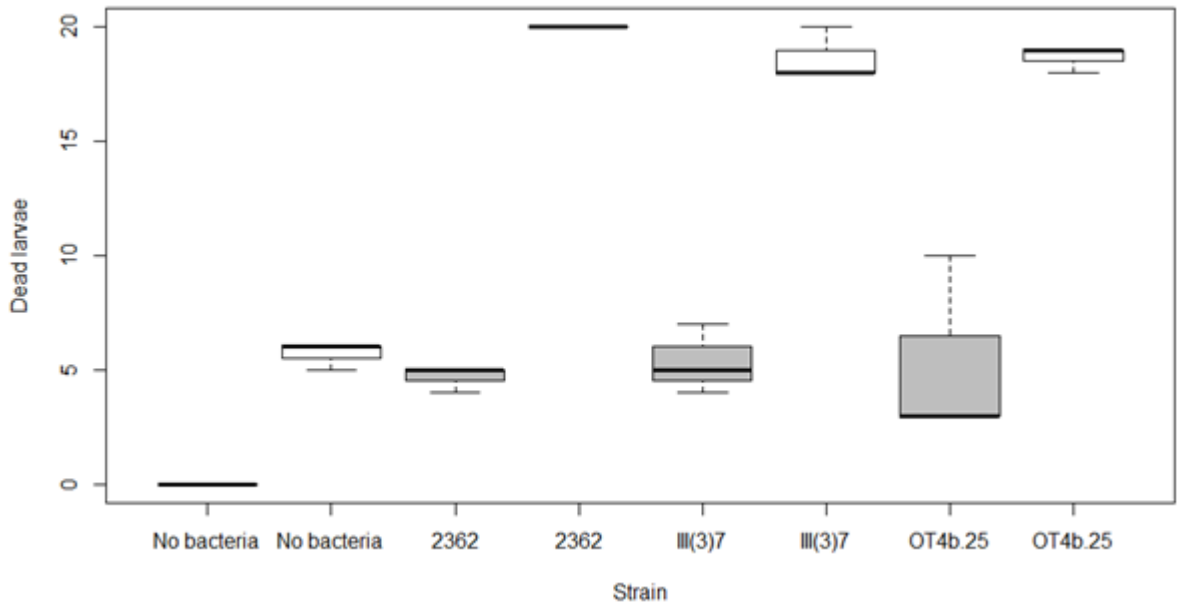

Figure 3. Bioassay of *A. aegypti* (gray) and *C. quinquefasciatus* (white) laboratory reared fourth larvae mixed cultures in presence of both *L. sphaericus* vegetative cells (10^9 UFC/mL) and spores (10^9 UFC/mL) after 48 hours of testing.

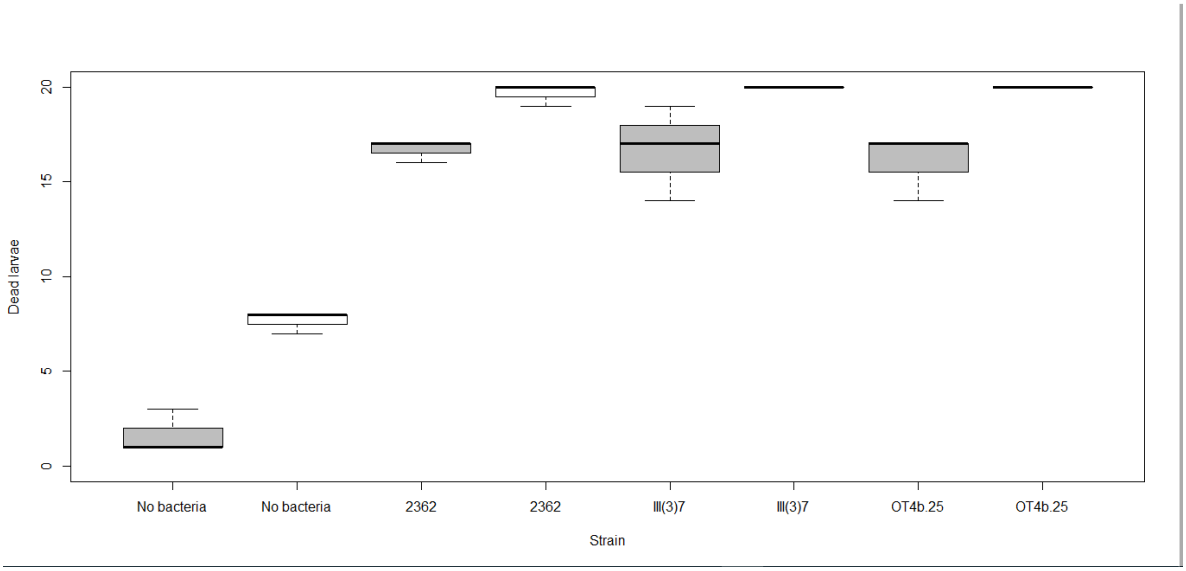

Figure 4. Bioassay of *A. aegypti* (gray) and *C. quinquefasciatus* (white) field collected fourth larvae mixed cultures in presence of *L. sphaericus* vegetative cells (10^9 UFC/mL) after 48 hours of testing

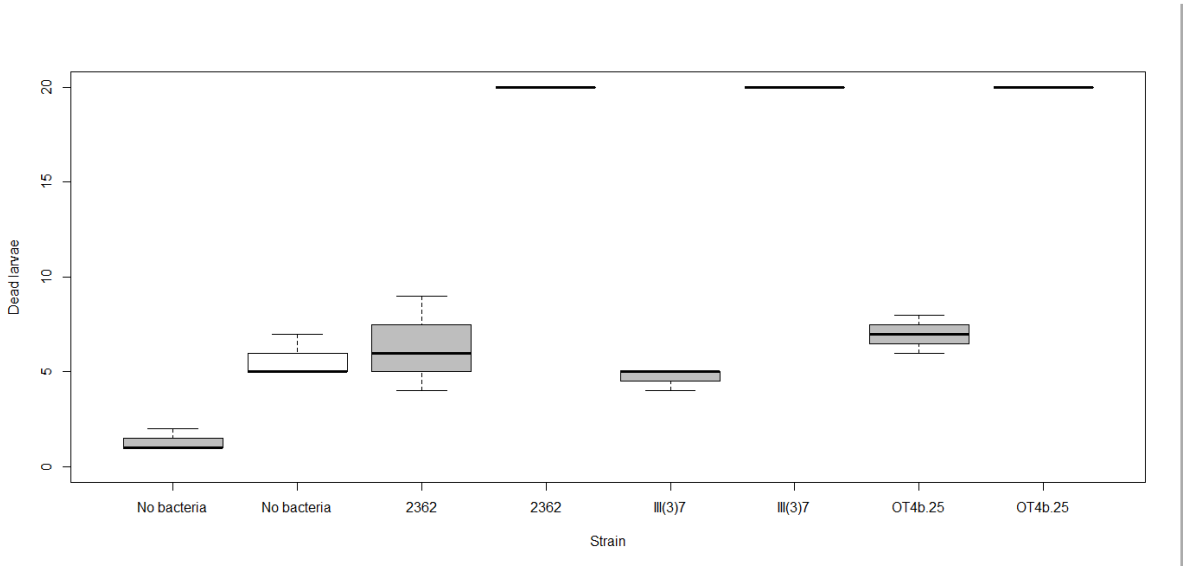

Figure 5. Bioassay of *A. aegypti* (gray) and *C. quinquefasciatus* (white) field collected fourth larvae mixed cultures in presence of *L. sphaericus* spores (10^9 UFC/mL) after 48 hours of testing

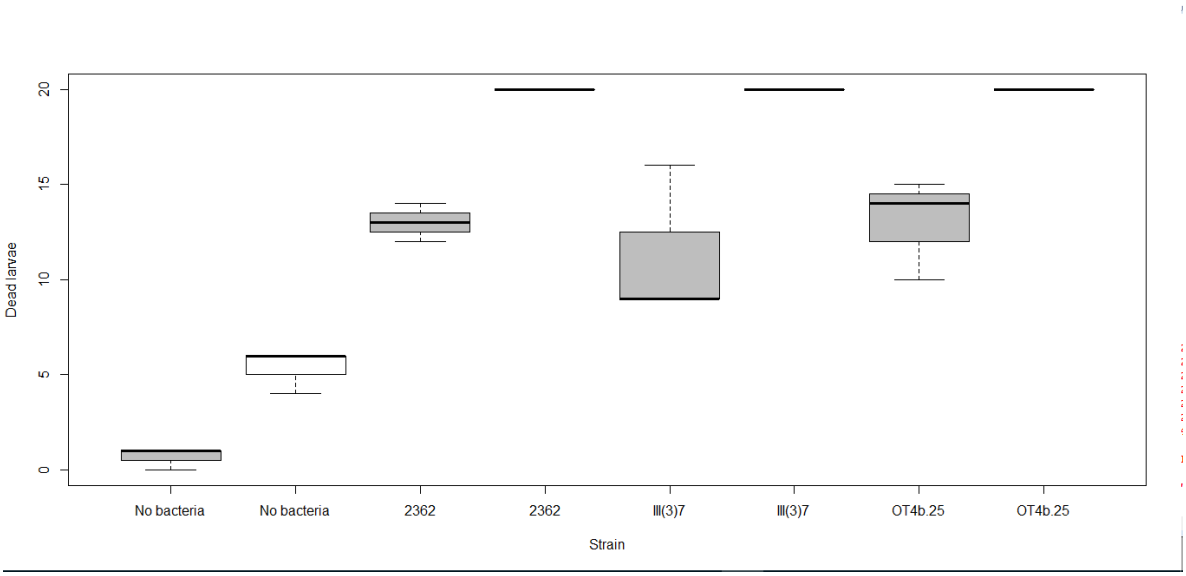

Figure 6. Bioassay of *A. aegypti* (gray) and *C. quinquefasciatus* (white) field collected fourth larvae mixed cultures in presence of both *L. sphaericus* vegetative cells (10^9 UFC/mL) and spores (10^9 UFC/mL) after 48 hours of testing.