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Efficacy of *Lysinibacillus sphaericus* against mixed-cultures of field-collected and laboratory larvae of *Aedes aegypti* and *Culex quinquefasciatus*

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Abstract

Lysinibacillus sphaericus (Bacillales: Planococcaceae) is a spore-forming bacillus used for the biological control of mosquitoes (Diptera: Culicidae) due to its larvicidal activity determined by various toxins and S-layer protein produced either during sporulation or by the vegetative cell. *Aedes aegypti* and *Culex quinquefasciatus* are the vectors of arboviruses that cause tropical diseases representing a current public health problem. Both species may coexist in the same larval development sites and are susceptible to the larvicidal activity of *L. sphaericus*. In this study, we compared the larvicidal effects of *L. sphaericus* 2362 (WHO Reference strain) and native strains III(3)7 and OT4b.25 against *Cx. quinquefasciatus* and *Ae. aegypti* in single-species and mixed-culture bioassays. Findings showed that *L. sphaericus* spores, vegetative cells and a combination thereof possessed high larvicidal activity against *Cx. quinquefasciatus* larvae, whereas only the formulation of *L. sphaericus* vegetative cells was effective against *Ae. aegypti* larvae. Similar results were obtained for field-collected larvae. We propose that a formulation of vegetative cells of *L. sphaericus* 2362 or III(3)7 could be a good alternative to chemical insecticides for the *in situ* control of mixed populations of *Ae. aegypti* and *Cx. quinquefasciatus*.

Keywords: Biological control, field-collected strain, vegetative cells, temephos-resistant

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Introduction

Biological insecticides have proven to be effective for the control of mosquito populations since they can be easily recycled, and unlike artificial or chemical insecticides, are less harmful to the environment and to human health (Ali *et al.*, 2013). More specifically, biological control is an efficient solution to insect pests given these benefits, and in addition, it uses natural pest antagonists or predators to regulate other populations (Van Driesche *et al.*, 2009).

Lysinibacillus sphaericus Ahmed (Bacillales: Planococcaceae) is a spore-forming bacterium with entomopathogenic activity. During the final stages of sporulation, *L. sphaericus* produces a binary toxin (BinAB), which is toxic against *Culex* spp. and *Anopheles* spp. (Davidson, 1988; Baumann *et al.*, 1991). After ingestion of BinAB, alkaline pH in the larvae activate the mechanism of each polypeptide: BinB binds to an α-glucosidase receptor identified as Cpm1 in epithelial midgut cells, allowing the entrance of BinA and causing cellular lysis (Davidson, 1988, 1989; Oei *et al.*, 1992; Charles *et al.*, 1996; Silva-Filha *et al.*, 1999; Darboux *et al.*, 2001).

Additionally, a proteinaceous structure found on the surface of several archaea and bacteria identified as the S-layer is expressed in *L. sphaericus* vegetative cells (Peña *et al.*, 2006). This structure contributes to larvicidal activity against *Culex (Linnaeus) quinquefasciatus* Say (Diptera: Culicidae) (Lozano *et al.*, 2011) and have a synergistic effect with the spore (Lozano & Dussán, 2017). Furthermore, *L. sphaericus* toxic strains express three mosquitocidal toxins in vegetative

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cells: Mtx1, Mtx2, and Mtx 3 (Thanabalu *et al.*, 1991; Liu *et al.*, 1996; Thanabalu & Porter, 1996). All three exhibit larvicidal activity but are sensitive to proteases from the sporulation phase (Thanabalu & Porter, 1995) and are not used in commercial products against vectors. Mtx1 is a 100 kDa protein that is very similar to ADPribosylation-type toxins, whereas Mtx2 and Mtx3 contain a domain characteristic of pore form toxins (Thanabalu *et al.*, 1991, 1993; Liu *et al.*, 1996; Thanabalu & Porter, 1996; Marchler-Bauer *et al.*, 2011).

Aedes (Stegomyia) aegypti Linnaeus (Diptera: Culicidae) and *Cx. quinquefasciatus* are the vectors of arboviruses that cause tropical diseases representing a current public health problem. Among these diseases, *Ae. aegypti* transmits dengue, Chikungunya, Zika, and yellow fever. Dengue is the most prevalent disease with approximately 390 million infections per year (Bhatt *et al.*, 2013). Chikungunya virus has caused over 2.5 million infections in the last decade and has more recently been spreading in the Americas (Staples & Fischer, 2014) and emerging in Europe (Schaffner *et al.*, 2013). Yellow fever has caused approximately 200,000 severe cases per year in Africa (Garske *et al.*, 2014) and Zika virus approximately 4 million infections in the Americas (Boeuf *et al.*, 2016).

Cx. quinquefasciatus can among other virus transmit West Nile Virus (WNV), Japanese Encephalitis Virus (JEV), and St. Louis Encephalitis Virus (SLEV), pathogenic protozoa and nematode (Bhattacharya & Basu, 2016). WNV is geographically widespread in Africa, Western Asia, the Middle East and recently, East Europe and North America (Petersen & Roehrig, 2001), causing continuously outbreaks during the last decades (Chancey *et al.*, 2015). JEV is the main cause of viral encephalitis in South-East Asia and the Western Pacific, with an estimated 68,000 clinical cases annually (Campbell *et al.*, 2011). SLEV is a reemerging arbovirus in the southern cone of South America (Rocco *et al.*, 2005; Diaz *et al.*, 2016), and a serious public health threat in North America (Reisen, 2003). Another concern is that these diseases are common in the tropics where the climate conditions allow the development of vectors (Rueda *et al.*, 1990) and where the predominant expansion of cities and towns surrounding bodies of water leads to an increase in incidence (Ali *et al.*, 2013). As the mosquitoes seek stagnant water in which they can lay eggs, the chances that they find artificial water containers are high due to increasing human settlement in or near mosquito habitats (Rozendaal, 1997). Therefore, control efforts of *Ae. aegypti* and *Cx. quinquefasciatus* populations have been conducted to reduce the risk of diseases transmission. These control programs, are mostly based on the application of chemical insecticides such as DDT, malation and temephos, and the overuse of these compounds has led to increased resistance in *Ae. aegypti* (Lima *et al.*, 2003; Bisset *et al.*, 2004; Fonseca-González, 2011). *Ae. Aegypti* and *Cx. quinquefasciatus* tend to colonize urban and suburban areas in the tropics and can be found in the same larval development sites (Rios *et al.*, 1978; Obándo *et al.*, 2007; Burke *et al.*, 2010; Leisnham *et al.*, 2014), despite the differences in ecological requirements for each species. *Cx. quinquefasciatus* has a predilection for polluted waters rich in organic matter, whereas *Ae. aegypti* prefers less polluted waters (Rozendaal, 1997) with few exceptions (Barrera *et al.*, 2008; Burke *et al.*, 2010).

Given that *Ae. aegypti* and *Cx. quinquefasciatus* are two species of epidemiological importance sharing larval development sites in the tropics, the objective was to assess the larvicidal activity of *L. sphaericus* spores, vegetative cells and a combination thereof against mixed-cultures of field-collected, temephos-resistant *Ae. aegypti* and field-collected *Cx. quinquefasciatus*. This is in order to evaluate a new alternative to chemical insecticides commonly used.

Materials and methods

Bacterial and mosquito culture conditions

Three *L. sphaericus* strains previously reported as highly toxigenic against laboratory *Cx. quinquefasciatus* (Lozano & Dussán, 2013) were evaluated in the study. *L. sphaericus* III (3)7 originally isolated from soil samples in an oak forest near Bogotá D.C., Colombia and *L. sphaericus* OT4b.25 originally isolated from coleopteran larvae in an oak forest near Bogota D.C., Colombia; both belonging to the CIMIC culture collection; and the WHO reference strain *L. sphaericus* 2362, originally isolated from adult *Simulium damnosum* (Diptera: Simuliidae) samples in Nigeria and obtained from the Pasteur Institute (Charles *et al.*, 1996).

Following the protocol of Lozano & Dussán (2013), the three *L. sphaericus* strains: 2362, OT4b.25 and III(3)7 were subjected to a synchronization procedure. An initial inoculum of each strain was cultivated in a liquid culture of Nutrient Broth (Oxoid) for 16 h. This was followed by five cycles of cultivation in acetate broth (composed of sodium acetate 5.00 g l^{-1}, yeast extract 3 g l^{-1}, $MgCl_2$ 1×10^{-3} M, $CaCl_2$ 7×10^{-4} M and $MnCl_2$ 5×10^{-5} M), with incubation at 30 °C and subjection to thermal shock at 90 °C for 20 min until 90% of cells have sporulated. Laboratory strains of *Ae. aegypti* Rockefeller and *Cx. quinquefasciatus* Muña were obtained from the Entomology Laboratory at National Institute of Health of Colombia (INS). *Ae. aegypti* Rockefeller was first collected by the CDC of San Juan, Puerto Rico and *Cx. quinquefasciatus* was collected by the INS from Muña wetlands, Cundinamarca, Colombia. In order to assess the efficiency of *L. sphaericus* against field-collected larvae, *Ae. aegypti* and *Cx. quinquefasciatus* larvae were collected at La Mesa, Cundinamarca, Colombia (4°38′ 02.9″ N and 72°27′43.42″ W) and Cordoba wetlands in Bogotá, Colombia (4°42′10.1″ N and 74°04′07.2″ W), respectively. The field-collected population of *Ae. aegypti* was previously reported resistant to the commonly used larvicide temephos (Santacoloma *et al.*, 2012).

Field-collected larvae were identified based on the identification key compiled by Gualdron (2007). Laboratory- and field-collected larvae were maintained at 30 °C with 70% of relative humidity and a 12-h light/dark photoperiod.

Dose-dependent bioassays

Preliminary bioassays of testing vegetative cells against *Ae. aegypti* showed larvae mortality (data not shown). Therefore, we determined the dose-dependent response of *Ae. aegypti* Rockefeller to bacterial vegetative cells (colony-forming units (CFU) per ml) and estimated the LC_{50}. Four concentrations of 2362 (2.10×10^4 CFU ml^{-1}, 2.10×10^5 CFU ml^{-1}, 2.10×10^6 CFU ml^{-1}, and 2.10×10^7 CFU ml^{-1}) and III(3)7 (7.29×10^4 CFU ml^{-1}, 7.29×10^5 CFU ml^{-1}, 7.29×10^6 CFU ml^{-1}, and 7.29×10^7 CFU ml^{-1}) vegetative cells were tested and mortality at 48 h was recorded.

Larvicidal bioassays

The larvicidal activity of the *L. sphaericus* strains was assayed against early fourth instar larvae of *Cx. quinquefasciatus*

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Table 1. Larvicidal activity of *L. sphaericus* highly toxigenic strains against *Ae. aegypti* fourth larvae.

L. sphaericus strain	LC_{50} (CFU ml^{-1})	95% CI (CFU ml^{-1})	Slope
2362	1.1138×10^7	1.1135×10^7–1.1138×10^7	0.641031
III(3)7	1.04×10^7	1.70×10^6–6.24×10^7	2.99324

and *Ae. aegypti*. In order to determine the toxicity of *L. sphaericus* vegetative cells, cells from synchronized cultures were grown in Nutrient Agar at 30 °C and after 12 h, the cells were resuspended in 1 ml of sterile distilled water obtaining the inocula for the subsequent bioassays. To determine the toxicity of sporulated cultures, the inocula were prepared by separating 1 ml aliquots of the synchronized strains grown in acetate broth.

Single-species bioassays were performed by adding an inoculum of vegetative cells or sporulated cultures to 99 ml of chlorine-free tap water with 10 larvae of *Ae. aegypti* or *Cx. quinquefasciatus*, to have a final exposure of 10^7 CFU ml^{-1}. Mixed-cultures bioassays consisting of a mixture of larvae from each species were performed by adding an inoculum of vegetative cells, sporulated cultures or the mixture of both to 99 ml of chlorine-free tap water with 20 larvae of *Ae. aegypti* and 20 larvae of *Cx. quinquefasciatus*, to have a final exposure of 10^7 CFU ml^{-1}. The bioassays were incubated at 30 °C for 48 h after inoculation and larval mortality was recorded at 24 and 48 h. Negative controls consisted of 100 ml chlorine-free tap water with larvae without bacteria. Each treatment was replicated three times and the procedure was repeated twice for both single-species and mixed-cultures bioassays.

Statistical analyses

The R v3.1.1 software was used for statistical analyses (R Core Team, 2012). Shapiro–Wilcoxon test (Korkmaz *et al.*, 2014) was used to test the data for normality. Single-species bioassays and mixed-cultures bioassays results were analyzed by analysis of variance (ANOVA) followed by a Tukey–Kramer test to separate averages among the different strains evaluated and control with no bacteria. If the data were not normally distributed, a Kruskal–Wallis test, followed by a Mann–Whitney *U* test to establish significant differences between different strains and control with no bacteria were used. The Toxicity Relationship Analysis Program (TRAP version 1.30a) was used to determine the LC_{50} of bacterial concentration against *Ae. aegypti* by probit analysis (US EPA, 2015).

Results

Ae. aegypti *Rockefeller dose-dependent bioassays*

Results shown on table 1 suggest that III(3)7 and 2362 strains have similar dose effect on *Ae. aegypti* 4th instar larvae. Subsequent bioassays were therefore performed with doses of 10^7CFU ml^{-1} as described in material and methods.

Ae. aegypti *Rockefeller and* Cx. quinquefasciatus *Muña single-species bioassays*

Single-species bioassays indicated that there was no difference in the percentage mortality of *Ae. aegypti* Rockefeller larvae exposed to spores of *L. sphaericus* III(3)7, OT4b.25, and 2362 and the control with no bacteria (fig. 1; Kruskal–Wallis : $H = 0.2188$, $P = 0.9745$). On the other hand, the *Cx. quinquefasciatus* Muña larvae exposed to spores, showed high mortality with all the bacterial strains when compared with the control with no bacteria (fig. 1; Kruskal–Wallis: $H = 15.22$, $P = 0.0016$).

Regarding vegetative cells after 48 h of exposure, mortality of *Ae. aegypti* Rockefeller larvae showed significant differences between treatments and control with no bacteria (Kruskal–Wallis: $H = 15.25$ $P = 0.0016$, fig. 2). Likewise, *Cx. quinquefasciatus* Muña larvae exposed to vegetative cells after 48 h showed significant differences between treatments and control with no bacteria (Kruskal–Wallis: $H = 16.15$, $P = 0.0010$, fig. 2).

Field-collected larvae of Ae. aegypti *and* Cx. quinquefasciatus *mixed-culture bioassays*

Larvae exposed to *L. sphaericus* spores for 48 h showed significant differences in mortality of *Ae. aegypti* field-collected larvae between treatments and control with no bacteria (ANOVA: $F = 9.611$, $P = 0.0050$; fig. 3a) and *Cx. quinquefasciatus* field-collected larvae between treatments and control with no bacteria (Kruskal–Wallis: $F = 462.2$ $P < 0.0001$; fig. 3). Similar results were obtained for *Ae. aegypti* Rockefeller (ANOVA: $F = 29.79$, $P < 0.0001$; fig. 3) and *Cx. quinquefasciatus* Muña (Kruskal–Wallis: $H = 8.314$, $P = 0.0399$; fig. 3), indicating that spores have similar larvicidal activity against field-collected and laboratory-reared larvae.

Likewise, larvae exposed to *L. sphaericus* vegetative cells for 48 h showed significant differences in mortality of *Ae. aegypti* field-collected larvae between treatments and control with no bacteria (ANOVA: $F = 59.67$, $P < 0.0001$; fig. 4) and *Cx.*

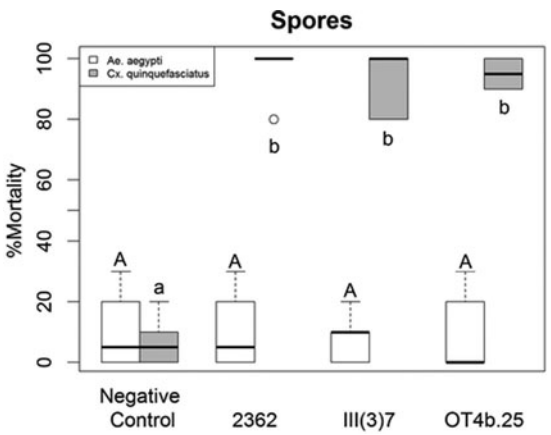

Fig. 1. Mortality of *Ae. aegypti* Rockefeller and *Cx. quinquefasciatus* Muña fourth-instar larvae in presence of *L. sphaericus* spores (10^7 CFU ml^{-1}) in single-species bioassays after 48 h of exposure. Upper or lower-case letters refer to statistical comparisons within the same species. Boxes with the same letter are not significantly different according to Tukey–Kramer test or Mann–Whitney *U* test in case of no normality. Horizontal bars, capped bars, and circles indicate median values, maximum and minimum values and outliers, respectively.

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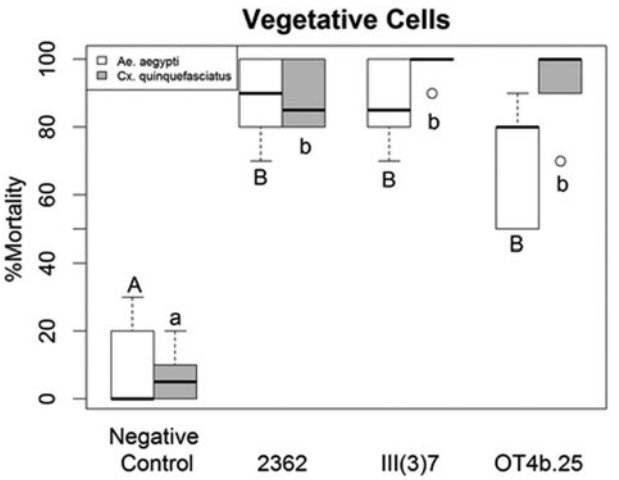

Fig. 2. Mortality of *Ae. aegypti* Rockefeller and *Cx. quinquefasciatus* Muña fourth-instar larvae in presence of *L. sphaericus* vegetative cells (10^7 CFU ml^{-1}) in single-species bioassays after 48 h of exposure. Upper or lower-case letters refer to statistical comparisons within the same species. Boxes with the same letter are not significantly different according to Tukey–Kramer test or Mann–Whitney *U* test in case of no normality. Horizontal bars, capped bars, and circles indicate median values, maximum and minimum values and outliers, respectively.

quinquefasciatus field-collected larvae between treatments and control with no bacteria (Kruskal–Wallis: $H = 9.358$ $P = 0.0248$; fig. 4); and mortality of *Ae. aegypti* Rockefeller larvae between treatments and control with no bacteria (ANOVA: $F = 35.93$, $P < 0.0001$; fig. 4) and *Cx. quinquefasciatus* Muña larvae between treatments and control with no bacteria (Kruskal–Wallis: $H = 8.521$, $P = 0.0228$; fig. 4). In this context, vegetative cells have similar larvicidal activity against field-collected and laboratory-reared larvae, but in contrast to the spores, *L. sphaericus* vegetative cells showed high mortality against *Ae. aegypti*.

Bioassays of larvae exposed to vegetative cells and spores also showed significant differences in mortality of *Ae. aegypti* field-collected larvae between treatments and control with no bacteria (ANOVA: $F = 17.17$, $P = 0.0007$; fig. 5) and *Cx. quinquefasciatus* field-collected larvae between treatments and control with no bacteria (ANOVA: $F = 484$, $P < 0.0001$; fig. 5); and mortality of *Ae. aegypti* Rockefeller larvae between treatments and control with no bacteria (ANOVA: $F = 4.187$, $P = 0.0468$; fig. 5) and *Cx. quinquefasciatus* Muña larvae between treatments and control with no bacteria (Kruskal–Wallis: $H = 8.895$, $P = 0.0307$; fig. 5). As for the exposure of the larvae solely to spores, both toxicity of sporulated cultures and vegetative cells did not show high mortality against *Ae. aegypti*.

All treatments showed that laboratory reared and field-collected populations of *Cx. quinquefasciatus* were highly sensitive to all three formulations of *L. sphaericus*, in contrast to *Ae. aegypti* populations, which were only highly sensitive to vegetative cells. Furthermore, mixed-cultures with no bacteria showed a mortality rate of 18.06 ± 4.89% for *Cx. quinquefasciatus*, which was interestingly higher than the mortality rate of *Ae. aegypti* (3.89 ± 4.39%).

Discussion

This study showed that *L. sphaericus* spores, vegetative cells and the combination of both exerted high larvicidal activity against *Cx. quinquefasciatus* larvae under coexisting and non-coexisting conditions (figs 1–5). These findings are consistent with previous studies showing that *L. sphaericus* III(3)7, 2362 and OT4b.25 are highly toxigenic against *Cx. quinquefasciatus* (Lozano & Dussán, 2013). Therefore, we conclude that sensitivity of *Cx. quinquefasciatus* was not affected by the presence of *Ae. aegypti* in any of the three formulations.

With respect to the formulations of *L. sphaericus* against *Ae. aegypti*, only vegetative cells were toxigenic against both coexisting and non-coexisting *Ae. aegypti* larvae (figs 2 and 4). These results correspond with previous studies (Nielsen-Leroux & Charles, 1992; Lekakarn *et al.*, 2015), which reported that binary toxin present in the spores of *L. sphaericus* has no toxic effect against *Ae. aegypti*. The *Aam1* midgut receptor present in *Ae. aegypti* has been identified as homologous to the *Cx. quinquefasciatus* α-glucosidase midgut receptor, but the former is found at a very low concentration.

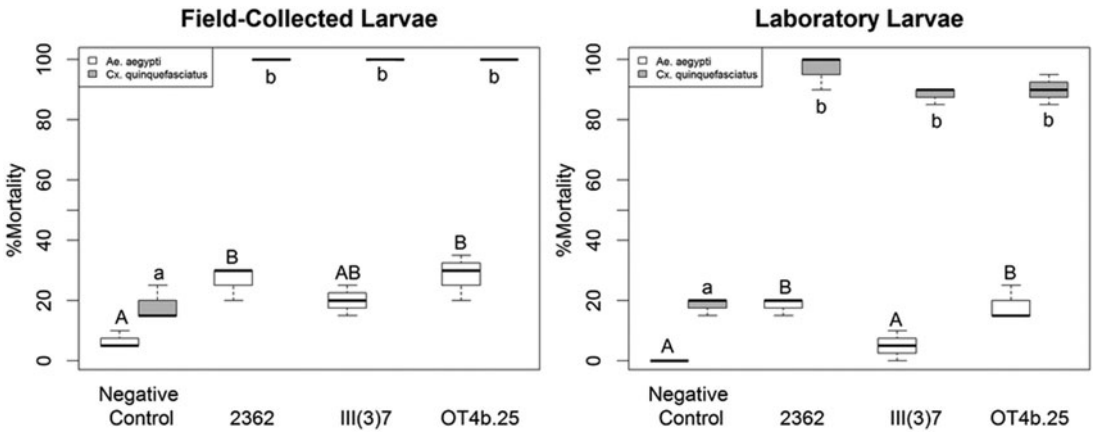

Fig. 3. Mortality of field-collected *Ae. aegypti* and *Cx. quinquefasciatus* and laboratory *Ae. aegypti* and *Cx. quinquefasciatus* fourth-instar larvae in presence of *L. sphaericus* spores (10^7 CFU ml^{-1}) in mixed-culture bioassays after 48 h of exposure. Upper or lower-case letters refer to statistical comparisons within the same species. Boxes with the same letter are not significantly different according to Tukey–Kramer test or Mann–Whitney *U* test in case of no normality. Horizontal bars, capped bars, and circles indicate median values, maximum and minimum values and outliers, respectively.

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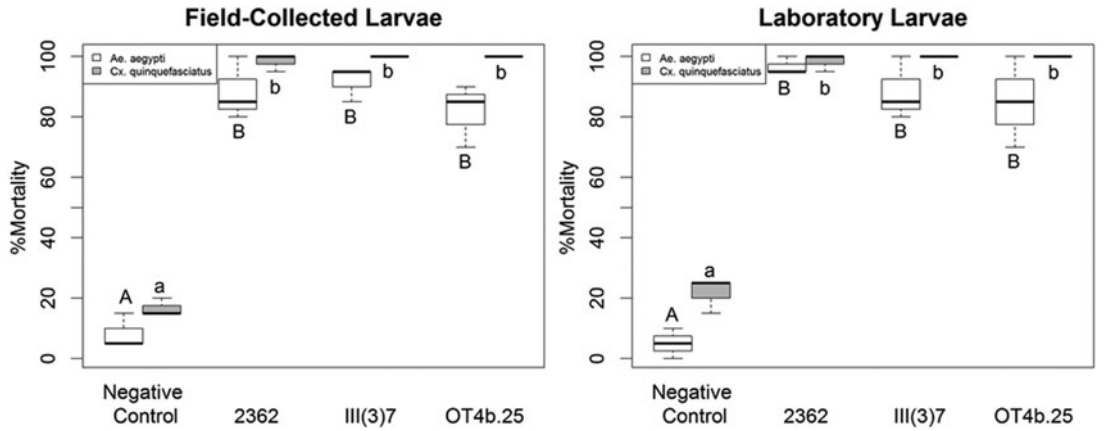

Fig. 4. Mortality of field-collected *Ae. aegypti* and *Cx. quinquefasciatus* and laboratory *Ae. aegypti* and *Cx. quinquefasciatus* fourth-instar larvae in presence of *L. sphaericus* vegetative cells (10^7 CFU ml^{-1}) in mixed-culture bioassays after 48 h of exposure. Upper or lower-case letters refer to statistical comparisons within the same species. Boxes with the same letter are not significantly different according to Tukey–Kramer test or Mann–Whitney *U* test in case of no normality. Horizontal bars, capped bars, and circles indicate median values, maximum and minimum values and outliers, respectively.

Furthermore, the binding capacity of any such homolog of the toxin *BinA* and *BinB* is very low (Nielsen-Leroux & Charles, 1992; Lekakarn *et al*., 2015). However, we found a statistically significant low mortality rate in the mixed-culture bioassay treated with spores (fig. 3), presumably explained by *L. sphaericus* spore germination in mosquito larval cadavers. Correa & Yousten (1995) showed that naturally occurring mosquito toxic strains of *L. sphaericus* are able to recycle in mosquito larval cadavers. Therefore, we suggest that spores might germinate inside the dead *Cx. quinquefasciatus* larvae, which promotes direct contact of either S-layer or *Mtx* toxins from the vegetative cell stage with *Ae. Aegypti* and thus, causing mortality.

As expected, all *Cx. quinquefasciatus* treatments presented high mortality to *L. sphaericus* vegetative cells due to the capacity to produce vegetative mosquitocidal toxins (*Mtx*) and *S-layer* (Thanabalu *et al*., 1993; Liu *et al*., 1996; Thanabalu & Porter, 1996; Promdonkoy *et al*., 2004; Lozano *et al*., 2011). In the case of *Ae. aegpyti*, this finding is interesting because this mosquito is not typically reported as a biocontrol target for *L. sphaericus* by the binary toxin. Instead, *Ae. aegpyti* mortality could be explained by the presence of the other toxins previously mentioned only expressed at vegetative cell stage that presumably exhibit high synergistic activity when co-expressed (Rungrod *et al*., 2009).

Berry (2012) suggested that preparations with spores and a mixture of vegetative mosquitocidal toxins would act synergistically, enhancing the effectiveness of *L. sphaericus*. Our study showed that contrary to expectations, there was no synergistic effect in the larvicidal effect of *L. sphaericus* using the two stages of the bacteria. Presumably, proteases produced during the sporulation phase of the bacteria could be degrading the *Mtx1*, *Mtx2*, and *Mtx3* toxins present in vegetative cells (Thanabalu & Porter, 1995), hence reducing the effectiveness of the formulation. Further investigations are needed to

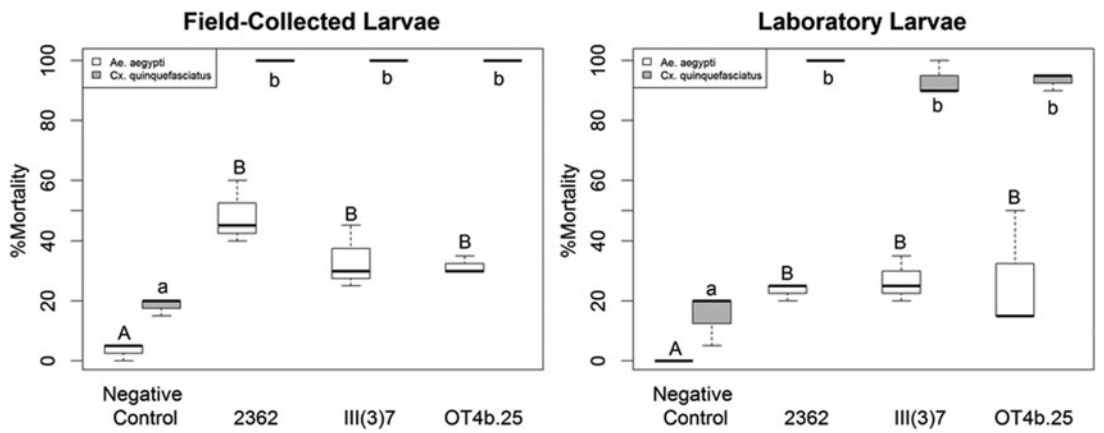

Fig. 5. Mortality of field-collected *Ae. aegypti* and *Cx. quinquefasciatus* and laboratory *Ae. aegypti* and *Cx. quinquefasciatus* fourth-instar larvae in presence of *L. sphaericus* spores and vegetative cells (10^7 CFU ml^{-1}) in mixed-culture bioassays after 48 h of exposure. Upper or lower-case letters refer to statistical comparisons within the same species. Boxes with the same letter are not significantly different according to Tukey–Kramer test or Mann–Whitney *U* test in case of no normality. Horizontal bars, capped bars, and circles indicate median values, maximum and minimum values and outliers, respectively.

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elucidate synergism between BinAB and vegetative mosquitocidal toxins. For this purpose, protease-negative *L. sphaericus* strains expressing Mtx might be used.

Mixed-cultures with no bacteria showed a higher mortality of *Cx. quinquefasciatus*, contrary to *Ae. aegypti* mortality. Santana-Martínez *et al.* (2017), showed that *Ae. aegypti* is a superior resource competitor and appears to be capable of competitively affecting *Cx. quinquefasciatus* under larval coexistence. In this sense, we suggest that a synergistic effect between the larvicide and natural dynamics of populations to control might occurs. However, we conclude that *L. sphaericus* has a biological control potential as a formula intended for mixed populations, due to *L. sphaericus* vegetative cells are highly toxigenic against both *Ae. aegypti* and *Cx. quinquefasciatus* individuals whether they are coexisting or not (figs 2 and 4).

This study shows that *L. sphaericus* 2362, III(3)7 and OT4b.25 are good candidates to control *Ae. aegpyti* and *Cx. quinquefasciatus* coexisting populations *in vitro* and, ultimately, *in situ* while they are vegetative cells but not as spores. The explanation for this phenomenon may be that *L. sphaericus* cells produce several mosquitocidal toxins, other than a binary toxin, that when co-expressed may increase its toxicity. Since *Ae. aegypti* is poorly sensitive to the binary toxin and several studies found the resistance of *Cx. quinquefasciatus* against *L. sphaericus* binary toxin (Nielsen-Leroux *et al.*, 1995; Chalegre *et al.*, 2012; Guo *et al.*, 2013), we recommend further studies on vegetative cells toxins to elucidate mechanisms of action and effectiveness surrounding synergism. Given the low LC_{50} values of *L. sphaericus* 2362 and III(3)7, these strains could be a suitable alternative to control *Ae. aegypti* and *Cx. quinquefasciatus* mixed populations and deal with insecticide resistance, through a formulation of vegetative cells.

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