Article

A Novel Bacterial-Mediated Method for the Differentiation of *Aedes* spp. Larvae in Mixed-Nursery Assays and Its Application in the Determination of Vegetative *Lysinibacillus sphaericus* Biocontrol Efficiency in Invasive Vector Eradication

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Featured Application: The developed coloration method employed the use of a genetically modified strain of the *Escherichia coli* bacterium, more specifically the pUC19 and red fluorescent protein (mRFP)-containing DH5 α strain. This bacterium was used in a coloration step previous to comparative mortality assays involving two *Aedes* species. This assessment allowed for the quick and easy differentiation between both *Ae. albopictus* and *Ae. aegypti*, and the results obtained shone light onto possible ecological variables to have in mind when applying biocontrollers in the wild. The evaluation of this method applied to differential mosquito lethality served as a proof of concept, and it is theorised that the same coloration method can be employed in similar comparative lethality studies relating to public health and beyond.

Abstract: The establishment of *Aedes aegypti* and the recent invasion of *Aedes albopictus* has put Colombia at risk, as it now harbours two arboviral vector species. Studies have shown the susceptibility of *Aedes aegypti* to the vegetative toxins produced by *Lysinibacillus sphaericus*. This study aims to determine the potential of two *L. sphaericus* strains in the control of both *Aedes* species present in Colombia, in both single and mixed species nurseries. Given the similarities between both species, there exists a need to differentiate them for the development of precise control strategies. Accordingly, mixed nurseries employed a red fluorescent protein (mRFP)-expressing *Escherichia coli* strain as a differentiator. Coloration-aided differentiation facilitated the analysis of mixed nursery treatments and provided deeper scrutiny into the variables at play. This showed an increase in the biocontrol efficiency, pointing to probable ecological variables. Bioassays involving *Ae. albopictus* showed a lower resistance to the one reported for *Ae. aegypti* in single-species nurseries. The study concludes that the use of *E. coli* strains expressing fluorescent proteins are useful tools to be employed in areas like public health entomology. Moreover, it was found that the use of *L. sphaericus* strains for biocontrol of vector mosquitoes is a viable alternative to chemical insecticides.

Keywords: *E. coli*; larvae differentiation; biological control; *Lysinibacillus sphaericus*; vegetative cell; *Aedes* spp.

1. Introduction

Dengue viruses (DENVs) are among the most common arboviral diseases currently affecting humankind, causing between 100 and 400 million infections every year [1]. These diseases have great public health relevance, as they are highly incapacitating, not only leading to economic damages but also generating serious health episodes and in some cases even death [2,3]. Despite interest and research, no specific antiviral treatments or vaccines exist. This means the main control strategy depends on *Aedes* (Stegomyia) vector mosquito management [4]. On account of its arboviral nature, the spread of Dengue

viruses is limited to vector distributions, hence why mosquito density and dispersion are paramount criteria for the prediction and control of Dengue outbreaks [5]. In Colombia, the transmission of DENVs relies mainly on the vector *Aedes aegypti* (Linnaeus, 1762), but it has been shown that *Aedes albopictus* (Skuse, 1894) plays an important role as a secondary vector. This comes as no surprise, as *Aedes* mosquitoes have had a long coevolutionary history transmitting DENVs, which has made them into ideal vectors for these and other arboviral diseases [6]. However, this is not the only shared trait between these mosquito species, as they are practicably indistinguishable from one another during their larval stages, aside from minimal cuticle differences that do not manifest until the last larval instar [7]. The extent of these similarities is enough that there exists evidence of satyrization and even successful mating when their ranges overlap [8]. *Ae. albopictus* is widely known as an important Dengue vector in Southeast Asia and Oceania [9], known also as the Tiger Mosquito, *Ae. albopictus* is native to Asia [10] but, in recent decades, it has been reported as an invasive species in different countries around the world [11,12]. The first American reports of the species date from 1986, when it was discovered in both Texas, USA and Sao Paulo, Brazil [13,14]. A little over a decade later, in 1998, the presence of *Ae. albopictus* in Colombia was first reported in the southern city of Leticia, in the Amazon region [15]. Widespread invasions of *Ae. albopictus* to different countries have been linked to the introduction of larvae and eggs carried accidentally through commerce, namely through imports like tires [11,16] and other suitable nesting materials [17]. Likewise, the main distribution path of the vector within Colombia is thought to be a consequence of terrestrial trade and movement of goods [11,15,17]. Currently, this vector is extensively distributed throughout the national territory [18,19] and is thought to be resistant to many of the broad-spectrum insecticides used in its and *Ae. aegypti*'s eradication [20]. Belonging to the Diptera order, these insects have a holometabolous development, meaning that their maturation process passes through the egg, larval instars, pupa, and adult stages [21]. It is well known that the larval stage of these insects is the easiest to control, does not present a danger of disease transmission and has shown greater susceptibility to various control methods [22]. Nevertheless, given the organisms' developmental speed, it is difficult to implement control projects within an adequate time frame.

The most broadly used vector control strategy has been the use of different pesticides in large quantities [23,24]. This method was successfully implemented during most of the 20th century [25,26]. Yet, this strategy has been questioned for different reasons, mainly, bioaccumulation of toxins in biomass [27,28], decomposition into even more environmentally toxic compounds [29,30], action on non-target organisms [31,32], and the appearance of resistance in target organisms [20,24,33]. Therefore, both environmental organisations and the scientific community have condemned attempts to control vector mosquitoes through purely chemical means, as the detrimental health effects outweigh the relatively few benefits of the method.

In recent years, biotechnological exploits such as expression vectors have been thoroughly implemented and used across different fields in both industry and research applications, ranging from enabling protein expression in target organisms, to the creation of transgenic organisms [34,35]. Modified bacteria like some *Escherichia coli* strains possess plasmids containing fluorescent reporters like red fluorescent protein (mRFP) and are of great use as they can serve as easily distinguishable colorimetric bioindicators for environmental contaminants, which is the case of leftover antibiotics from both pharmaceutical and agricultural applications [36]. Despite the success expression vectors have had in other areas, they have not yet been employed in the control of arboviral diseases and, more specifically, in the differentiation of public health-relevant mosquito species, as is the case of *Ae. albopictus* and *Ae. aegypti*.

Lysinibacillus sphaericus is a Gram-positive, mesophilic, rod-shaped bacterium commonly found on soil [37]. It can form resistant endospores that are tolerant to high temperatures, chemicals, ultraviolet light, and can remain viable for long periods of time [37,38]. It has shown extensive potential as an insect pathogen and a biocontroller, mostly because

of the Binary (*BinA/BinB*) toxins [37], and previous studies have shown the specificity and little collateral damage caused by the implementation of *L. sphaericus* as a mosquito biocontroller on ecosystems and their dynamics [39]. Extensive research has been conducted on the entomopathogenic properties of the binary toxins present in the parasporal crystal [40–42], concluding that they are generally refractory to *Aedes* mosquitoes [43,44]. However, *L. sphaericus* has other mechanisms of toxicity against mosquitoes, namely the S-Layer protein [45–47] and the Mosquitocidal (*Mtx 1/2/3*) toxins present in the vegetative cell [48]. In the past, assessments with *Mtx* toxins, S-Layer preparations and vegetative *L. sphaericus* cells have been tested against *Ae. aegypti* larvae [40,42,47], but only one report exists on the S-Layer's entomopathogenic action against the novel invader species *Ae. albopictus* [45]. In this study, vegetative cells of *L. sphaericus* III(3)7, and 2362 (WHO reference) were assessed against Rockefeller reference *Ae. aegypti* and Colombian reference *Ae. albopictus* strains in order to assess the effectiveness of the bacterial biocontroller on the novel arboviral vector *Ae. albopictus*. In addition, the study aimed to determine the potential of two *L. sphaericus* strains in the control of the aforementioned *Aedes* species present in the country in both single and mixed species nurseries, employing an mRFP-expressing *Escherichia coli* strain as a differentiator between species.

2. Materials and Methods

The methodology employed and outlined below is a modification of the protocol utilised previously by Rojas-Pinzón and Dussán in 2017 [40].

2.1. *Lysinibacillus sphaericus*, *Aedes aegypti* and *Aedes albopictus* Strains

The *L. sphaericus* III(3)7 strain was isolated from an oak forest soil in Colombia [49]. The WHO reference strain 2362 was isolated from adult *Simulium damnosum* [50] and kindly donated by A. Delecluse from the Pasteur Institute in France. This bacterial mixture was selected as these strains have shown the most lethality in *Ae. aegypti* mosquitoes among the *L. sphaericus* strains present in the CIMIC collection, having a Lethal Dose 50 (LD50) concentration of 10^7 colony-forming units per millilitre (CFU/mL) [40,51,52]. The eggs of the reference *Ae. aegypti* Rockefeller strain and the Colombian reference *Ae. albopictus* strain were kindly donated by the National Institute of Health (INS) in Bogotá, Colombia.

2.2. *Aedes* Mosquito Maintenance

Eggs donated by the INS were kept at 30 °C and 60–70% relative humidity under a 12:12 light/dark photoperiod. Upon hatching, the larvae were fed with pellet food Omega One Natural Protein Formula (OmegaSea, LLC, Painesville, OH, USA) for cichlids, twice a week. The larvae were kept in plastic containers until the third development instar was reached. These conditions were used for both mosquito species.

2.3. Bioassays of *L. sphaericus* against Individual *Aedes* Species Nurseries

In order to determine precise LD50 concentrations for vegetative cells against each species, the two *L. sphaericus* strains were evaluated in several concentrations, which were derived from previous studies regarding *Ae. aegypti* lethality [40,51,52]. The selected concentrations ranged from 10^4 to 10^7 CFU/mL. For the assays, the *L. sphaericus* strains were grown on nutrient agar (NA) at 30 °C. After 15 h, cells were recovered in 1 mL of water, and serial dilutions were then made to calculate the bacterial titre that was added to the bioassays. In order to assess the mosquitocidal potential, the tests were conducted in 15 mL of chlorine-free water with 20 individuals and 15 mL of the bacterial suspension (10^4 to 10^7 CFU/mL). All the trials were conducted in triplicate, and a control without bacteria was included. The number of live larvae were recorded every 24 h until 48 h. This procedure was conducted for both mosquito species. Trials were performed keeping the maintenance parameters for temperature, photoperiod, and relative humidity.

2.4. Preparation, Toxicity Assessment and Coloration of *Ae. aegypti* Using mRFP-Modified *E. coli*

Modified *E. coli* DH5 α cells containing the pUC19 plasmid with mRFP were activated in an overnight culture in Lysogeny Broth (LB) containing 50 μ g/mL of Ampicillin at 30 °C and 150 rpm. Serial dilutions were made, and the bacterial titre was estimated at 10⁸ CFU/mL. A toxicity screen was performed by calculating the LC50. In order to do this, 10 *Ae. aegypti* larvae were subjected to tests in glass containers containing 15 mL of chlorine-free water and 15 mL of the 10⁸ CFU/mL bacterial suspension. This was conducted in duplicate and a control treatment containing 30 mL of chlorine-free water was included. Trials were conducted keeping the maintenance parameters for temperature, photoperiod, and relative humidity. Live larvae were counted every 24 h until 48 h.

2.5. Bioassays of *L. sphaericus* against Mixed *Aedes* Nurseries

To evaluate natural competition nurseries, simulated nurseries containing individuals from both *Aedes* species were set up. The LC50 was determined employing the same procedure used in single-species nurseries, only modifying the number of individuals to 10 larvae per species per assay. Given the extent of the physical similarities between the two larvae species, especially during the first three instar stages, a novel differentiation method based on colour was developed. Through this method, larvae were differentiated employing the aforementioned modified *E. coli* strain, which expressed the fluorescent protein mRFP. Ingestion of this modified bacteria coloured the *Ae. aegypti* larvae pink/red. The colour treatment was carried out during the first 2 instars, before the larvae were placed into the mixed nursery environment. Further differences were employed to aid in the accurate differentiation, namely exoskeleton variations including the lateral comb and thorax hairs. Final bacterial concentrations employed in bioassays against each nursery type are specified in Table 1.

Table 1. Final bacterial concentration ($\pm$ Standard Deviation) in CFU/mL of the Bacterial mixture (III(3)7 + 2362) tested against each *Aedes* species nursery type.

Nursery Type	10 ⁷	10 ⁶	10 ⁵	10 ⁴
<i>Ae. aegypti</i>	$3.26 \times 10^7 (\pm 5.89 \times 10^5)$	$1.91 \times 10^6 (\pm 8.28 \times 10^4)$	$4.9 \times 10^5 (\pm 1.5 \times 10^4)$	$7.87 \times 10^4 (\pm 3.17 \times 10^2)$
<i>Ae. albopictus</i>	$6.39 \times 10^7 (\pm 7.32 \times 10^4)$	$9.24 \times 10^6 (\pm 2.71 \times 10^4)$	$3.76 \times 10^5 (\pm 6.86 \times 10^4)$	$3.22 \times 10^4 (\pm 9.27 \times 10^3)$
Mixed	$3.54 \times 10^7 (\pm 9.51 \times 10^5)$	$6.12 \times 10^6 (\pm 6.42 \times 10^5)$	$9.69 \times 10^5 (\pm 2.91 \times 10^4)$	$4.57 \times 10^4 (\pm 5.93 \times 10^3)$

2.6. Statistical Analysis

In order to construct the corresponding mortality curves and determine the lethal doses for the different treatments, the Probit model was implemented [53]. The Probit model was constructed in Microsoft Excel [54]. The mortality values were then subjected to further statistical analysis. All statistical tests were performed using the R 3.1.2 statistical package [55], and a significance level of $p < 0.05$ was chosen for every test. Homoscedasticity was evaluated and confirmed through Bartlett's test for homogeneity of variance, and data normality was assessed and verified through a Shapiro–Wilk normality test (Table S1). In order to assess statistically significant differences between mortality per treatment in the *Ae. albopictus* strain, analysis of variance (ANOVA) (one way) tests were performed followed by Tukey's Honestly Significant Difference (HSD) tests as a *post hoc* analysis (Table S2).

3. Results

3.1. Toxicity Assessment and Coloration of *Ae. aegypti* Using mRFP-Modified *E. coli*

The LB culture media turned bright pink past 20 h of incubation (Figure 1A), thus confirming both the presence and activation of the mRFP reporter in the cultured bacteria. Once mRFP activation was confirmed, *Ae. aegypti* larvae were exposed to the bacteria to evaluate colouration and possible antagonism. Past 24 h, no larval mortality was registered, and evidence of ingestion could be seen within the larvae's digestive tract, as it was coloured pink (Figure 1B).

Figure 1. (A) Modified *E. coli* expressing the red fluorescent protein (mRFP) after being exposed to antibiotic. (B) Colouration observed within the larvae's digestive tract after the ingestion of modified *E. coli* expressing mRFP.

3.2. Lethal Doses for and among Different *Aedes* Nurseries

For the *Ae. albopictus* nurseries, LD50 for the mixture of *L. sphaericus* 2362 and III(3)7 was found to be 5.9×10^5 CFU/mL ($R^2 = 0.95$, $y = 14.829 \times -26.566$). *Ae. aegypti* nurseries showed a comparative resistance, having a LD50 value of 1.8×10^7 CFU/mL ($R^2 = 0.82$, $y = 16.455 \times -28.676$). Mixed species nurseries showed a slight decrease in resistance, presenting a LD50 value of 1.45×10^7 ($R^2 = 0.87$, $y = 15.96 \times -27.23$), marginally lower than the one found in *Ae. aegypti* nurseries. All values are reported in Table 2.

Table 2. Lethal *Lysinibacillus sphaericus* bacterial mixture (III(3)7 + 2362) doses for each of the treatment nurseries.

Nursery Type	LD50 (CFU/mL)	LD95 (CFU/mL)
<i>Ae. aegypti</i>	1.8×10^7	6.3×10^7
<i>Ae. albopictus</i>	5.9×10^5	1.23×10^6
Mixed (both species)	1.45×10^7	3.04×10^7
Mixed (<i>Ae. aegypti</i>)	7.23×10^6	3.25×10^7
Mixed (<i>Ae. albopictus</i>)	1.88×10^5	9.74×10^5

3.3. Bioassays against Different Bacterial Inoculum Concentrations

Correlating to the lethal doses found, a dose-dependent effect was observed between *Aedes* larvae mortality and bacterial inoculum dose. Results found fall in line with previous investigations, as *Ae. aegypti* was able to tolerate inoculums as high as 10^7 CFU/mL [51]. A dose-dependent effect was evident among all nursery types (Figure 2), with *Ae. albopictus* showing greater susceptibility to the bacterial treatment, particularly in the 10^5 – 10^6 CFU/mL range. Significant differences were found among the inoculated treatments and the controls for all nursery types except 10^4 CFU/mL at both 24 h (*Ae. albopictus*: ANOVA: $F_{(4,10)} = 548$, $p < 0.0001$. Average larval mortality per inoculum dose: 10^4 : 0%, 10^5 : 55%, 10^6 : 96%, 10^7 : 100%; *Ae. aegypti*: ANOVA: $F_{(4,10)} = 23.54$, $p < 0.0001$. Average larval mortality per inoculum dose: 10^4 : 0%, 10^5 : 15%, 10^6 : 30%, 10^7 : 95%; mixed nurseries: ANOVA: $F_{(4,10)} = 104$, $p < 0.0001$. Average larval mortality per inoculum dose:

10^4 : 0%, 10^5 : 35%, 10^6 : 55%, 10^7 : 100%) and 48 h (*Ae. albopictus*: ANOVA: $F_{(4,10)} = 337.9$, $p < 0.0001$. Average larval mortality per inoculum dose: 10^4 : 10%, 10^5 : 82%, 10^6 : 100%, 10^7 : 100%; *Ae. aegypti*: ANOVA: $F_{(4,10)} = 87.6$, $p < 0.0001$. Average larval mortality per inoculum dose: 10^4 : 7%, 10^5 : 18%, 10^6 : 45%, 10^7 : 100%; Mixed nurseries: ANOVA: $F_{(4,10)} = 257.9$, $p < 0.0001$. Average larval mortality per inoculum dose: 10^4 : 8%, 10^5 : 50%, 10^6 : 70%, 10^7 : 100%) past inoculation. Results indicate the presence of a clear dose-dependent relationship. This relationship is further solidified by the results from the overall mortality across all inoculum concentrations (Figure 3), which again showed statistical significance when compared to the control treatments at both 24 h (ANOVA: $F_{(4,40)} = 45.54$, $p < 0.0001$. Average larval mortality per inoculum dose: 10^4 : 0%, 10^5 : 35%, 10^6 : 55%, 10^7 : 100%) and 48 h (ANOVA: $F_{(4,40)} = 50.07$, $p < 0.0001$. Average larval mortality per inoculum dose: 10^4 : 8%, 10^5 : 50%, 10^6 : 70%, 10^7 : 100%).

Figure 2. Dose-dependent responses of the different *Aedes* nurseries when exposed to the different inoculum concentrations used at the set time intervals. (A) *Aedes albopictus* nurseries (24 h post inoculation). (B) *Aedes albopictus* nurseries (48 h post inoculation). (C) *Aedes aegypti* nurseries (24 h post inoculation). (D) *Aedes aegypti* nurseries (48 h post inoculation). (E) Mixed nurseries (24 h post inoculation). (F) Mixed nurseries (48 h post inoculation).

Figure 3. Overall dose-dependent response observed for the different inoculum concentrations used. (A) 24 h past initial exposure. (B) 48 h past initial exposure.

3.4. Nursery Response to Biocontrol Agent

All three nursery types responded similarly to the biocontrol agent and, as stated above, some instances of resistance and sensitivity were recorded. Nevertheless, these results were not apparent whilst comparing overall mortality between nursery types at the set time intervals (Figure 4). Additionally, no significant difference was found between the species after performing the statistical analysis at neither 24 h ANOVA: $F_{(2,42)} = 1.541$, $p = 0.2261$) nor 48 h (ANOVA: $F_{(4,40)} = 1.611$, $p = 0.2117$). This indicates a similar biocontrol behaviour among all nursery types, pointing to *L. sphaericus* as an adequate biocontrol solution regardless of nursery type.

Figure 4. (A) Overall mortality among all the nursery types tested over all the inoculum concentrations used after (A) 24 h past initial exposure. (B) 48 h past initial exposure.

4. Discussion

4.1. Toxicity Assessment and Coloration of *Ae. aegypti* Using mRFP-Modified *E. coli*

The lack of larval mortality indicated that the colouration treatment would not cause noise during the LD50 calculations involving the biocontroller. In addition, the successful colouring of *Ae. aegypti* larvae greenlit the mixed nurseries treatment, as there was a viable and fast way of differentiating both larval species. The results obtained are congruent with current literature, as it has been reported that not only are gut bacteria innocuous to mosquitoes [56], but they also contribute to their development through a hypoxia-induced growth mechanism [57]. That being said, no size or instar difference was observed between bacteria grown with and without the bacterial colouring agent in this study.

4.2. Lethal Doses for and among Different *Aedes* Nurseries

While corroborating the previously reported lethality values found for *L. sphaericus* interacting with *Ae. aegypti* [40], Probit analysis showed differences between the LD50 and LD95 among both species, pointing at an apparent acute sensibility in *Ae. albopictus*. These results contrast sharply with the current literature, as it has been previously reported that *Ae. albopictus* has a greater expansion potential [58] as well as an important plasticity and adaptative potential [59,60] across different invaded landscapes. The results found seem to support the hypothesis where bacterial communities present at the breeding site have an important influence over intra and interspecific competition outcome, especially affecting *Ae. albopictus* [61,62]. This apparent susceptibility to native bacterial diversity could be an important factor explaining why the invasion of this vector has had a relatively slow spread throughout the Colombian territory [63,64]. The treatment involving mixed nurseries pointed towards *Ae. aegypti* being more resistant to the biocontroller in mixed nurseries, and a slight decrease in resistance was observed, though no significant differences were found in larvae mortality while comparing both species with the single species nurseries treatments. Furthermore, mixed nurseries showed a slight drop in lethal doses, particularly in the LD95 value, pointing at plausible negative ecological interactions between the target species [65] (Table 2). The colorimetric treatment involving the modified mRFP-expressing *E. coli* was a key factor in the visualisation and the detailed determination of larval mortality in the mixed nursery environment, as it helped minimise errors in larval count, providing accurate measures for lethality. This is of special importance while working in enclosed environments, such as the one used in this study, as both inter, and intraspecific competition play key roles in larval development times and survival [66]; however, it has been recently suggested that intraspecific competition may be less detrimental to larvae survival and development than intraspecific competition [67]. The decrease in the LD95 shown between pure *Ae. aegypti* and mixed nurseries points at intraspecific competition as a detrimental factor in a pathogen-laced environment, which considerably reduced the survival of the fittest species. Given how wild mixed nurseries are not as rare as previously thought [68], the use of a bacterial biocontroller such as *L. sphaericus* can benefit from the natural competition between the two vector species, further enhancing its efficiency in the field.

4.3. Bioassays against Different Bacterial Inoculum Concentrations

A clear dose-dependent effect was seen between the bacterial inoculum and the mosquito mortality, regardless of nursery type. The previously reported results for *Ae. aegypti* tolerance were congruent with doses found during the study [40,52]. On the other hand, this study represents the first time *Ae. albopictus*' tolerance to the biocontroller *L. sphaericus* has been tested. In the past, it has only been exposed to the bacterium's S-Layer protein [45], which alone had notable impact on the insect's mortality. Mixed nurseries showed an increase in *Ae. aegypti* susceptibility to the bacterial treatment when compared to pure nurseries, this can correlate to negative ecological interactions between and within the target species, namely intra and interspecific competition for space and nourishment [66,67].

4.4. Nursery Response to Biocontrol Agent

The overall results show no specificity towards a particular *Aedes* nursery, as well as an ample biocontrol potential at the employed inoculum concentrations. With this in mind, the potential use of *L. sphaericus* as a vector control alternative cannot be overlooked, as no specific strategies are being used against *Ae. albopictus* in Colombia [20,33]. Coupled with this, the worrying appearance of resistance [20,24] to chemical control methods, as well as the inherent disadvantages of present chemical control [27–29,31,32] has created an urgent need for the application of novel vector eradication strategies, and since larval stages are especially easy to control [22], the time is ripe for the usage of target-specific and environmentally friendly [39,48] control methods such as the use of *L. sphaericus* as a larvae

biocontroller and *Wolbachia pipientis* as an adult lifespan shortener [69] in the battle against Dengue and every other arboviral disease currently affecting the Colombian population.

5. Conclusions

In summary, the reference *Ae. albopictus* strain was susceptible to the vegetative cells of the *L. sphaericus* strains employed, which further establishes the mosquitocidal activity of this bacterium. The mRFP marking protocol permitted an in-depth evaluation of larval mortality in a mixed nursery environment and permitted the speculation on the different ecological variables at play in such a setting. Delving into the details of mosquito interactions with both the biocontroller and the competing species is paramount for the correct application of control strategies, and such a comparison would have been cumbersome at best without the use of a differentiation method like the one employed. For a better understanding of the vegetative cells' effect on *Ae. albopictus* larvae, future studies involving both field-collected and reference strains should be conducted. Additionally, in order to find out the role of chitinases, hemolysins and other toxic proteins genes annotated in several *L. sphaericus* genomes, further studies are needed. Given that the bacterial mixture was marginally more efficient causing larvae mortality in *Ae. albopictus* rather than *Ae. aegypti*, different formulations should be assessed. The results indicate that the vegetative cells of *L. sphaericus* are a suitable alternative, which can be used to deal with novel secondary vector populations such as that of *Ae. albopictus* in Colombia.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11093970/s1>, Table S1: Statistical analyses of homoscedasticity and normality, Table S2: *Post hoc* analyses of significance between treatments.

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