Synergism and Antagonism of *Lysinibacillus sphaericus* consortia against reference and field collected strains of *Aedes aegypti* larvae

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INTRODUCTION

One of the actual problems in public health is the increasing transmission of diseases such as malaria, dengue, filariasis, chikungunya and Zika virus via mosquito vectors (Hafeez et al. 2011). There are several factors related to this reality. On one hand, the predominant expansion of cities and towns surrounding water bodies where mosquito populations are emerging. On the other hand, the indiscriminate use of chemical insecticides contributes to environmental pollution, development of resistance by unspecific organisms and accumulation of recalcitrant compounds harmful to human health (Ali, Ravikumar, & Beula, 2013). Unlike other mosquito vectors, *A. aegypti* is a day-biting feeder with preferences for human blood and this behavior enhances transmission of diseases (Salazar et al., 2013).

To understand such increase in disease transmission it is important to know the life cycle of the mosquito vector *Aedes aegypti*. This vector has two life phases; aquatic phase and terrestrial phase. During the aquatic phase, females deposit eggs in the edge of water bodies slightly above water surface (Wong et al. 2011). It is important to note that *A. aegypti* eggs resist dehydration for periods from days to months (Farnesi et al. 2015). When inundated, eggs hatch and larvae develops into 4 instars in which, initially, feeding is able by filtering and then, by eating organic detritus and microorganisms. After a couple days, larvae develop into a non-feeding stage known as pupae from which the adult emerges (Padmanabha et al., 2012). Once female adults grow, they seek for settled water bodies to deposit their eggs and it is very probable that they find water containers close to houses (Macoris et al., 1997). In consequence, the next generation of mosquitoes will share habitat with humans living in those houses and transmission of tropical diseases keeps spreading.

Chemical insecticides have been a solution used for many years. Unfortunately, vector control programs are facing problems because of the increasing resistance to insecticides by mosquitoes like *A. aegypti* (Marcombe et al., 2012). The continuous use of organophosphate and pyrethroid insecticides has contributed to resistance in two main ways. In the metabolic way, studies showed elevated activities of cytochrome P450 monooxygenase, glutathione S-transferase and cholinesterases in larvae and adults that leads to detoxification. The molecular way, is by target site mutations as, for example, the V1016I in the voltage-gated sodium channel; this point mutations avoids pyrethroids and DDT to bind into the channel which prevents muscular paralysis of adults (Scott 1999; Hardstone et al. 2009). For this reason, biological control might be an alternative option to reduce mosquito-borne diseases.

A promising solution to such disease transmission is the biological control which consists of using living organisms to control populations of other organisms (Goulson, 2005). This control may occur by competition of resources, parasitism and/or predation (Bauer et al. 2008; Martinuz et al. 2012). It is important to mention that eradication is not intended but decrease enough populations to acceptable levels according to governmental institutions.

A recent mosquito control agent is *Wolbachia pipientis*. This is a Gram negative and obligate endosymbiont bacteria which inhabits about to 40-75% of all arthropod species (Joubert et al., 2016). *Wolbachia* is a successful option for control since it is maternally transmitted and it has the ability to manipulate the reproductive organs of mosquitoes. Among these manipulations, there is feminization, parthenogenesis, cytoplasmic incompatibility and male killing. Moreover, previous reports found that *Wolbachia* is even able to limit replication of viruses such as dengue (DENV), yellow fever (YFV) and chikungunya (CHIKV). This limitation occurs by production of interference single stranded RNA (RNAi) that is complementary with some specific viral RNA sequences an preventing protein translation (Moreira et al., 2009). Also, studies found amino acid competition between *Wolbachia* and its host and this results in reduction of egg viability for lack of nutrients (Caragata, Rancès, O'Neill, & McGraw, 2014).

Previous studies found *Lysinibacillus sphaericus* as another biocontrol alternative. This Gram positive bacteria produces spores able to express a protein known as binary toxin which is very effective to control *Culex sp.* and *Anopheles sp.* but not *Aedes sp.* larvae (Chalegre et al., 2009). However, other works showed vegetative cells and S-layer protein to cause larvicidal mortality against *A. aegypti* (Rungrod et al. 2009*;* Lozano and Dussán 2013; Allievi et al. 2014; Silva and Dussán 2015). In addition, another study in Colombia found that strains of *L. sphaericus* isolated from different sources were able to grow, produce spores and binary toxin (Dussán et al 2002). Another study determined *quorum sensing* and *quorum quenching* in *L. sphaericus* (Gómez and Dussán 2016) so it is possible that signaling between strains may occur and bacterial growth may be improved.

Based on these findings, it is possible to mix a *L. sphaericus* consortium effective to decrease both reference and field collected *A. aegypti* populations in order to diminish risk of mosquito-borne disease transmission. If larvicidal activity is enhanced in one of the consortia when compared to mortality of individual strains, then such consortium will be considered as a candidate agent of biological control.

Furthermore, the objective of this study was to evaluate antagonism and synergism of different *L. sphaericus* consortia able to cause mortality against *A. aegypti* larvae and compared the response of larvae to individual bacterial strains as well. The *L. sphaericus* strains used for this study were the World Health Organization (WHO) reference strain 2362, and the Colombian native strains OT4b.25 and III(3)7. These strains were selected for biological control since previous reports obtained acceptable larval mortality against *C. quinquefasciatus* and, also, because these strains contain toxins such as Mtx and *slp*C sequenced in their genomes (Lozano and Dussán 2013; Rey et al. 2016).

METHODS

Field collected mosquito strain

According to the WHO guidelines, two plastic 500 mL cups are needed to attract *A. aegypti* female adults. One of the cups contained a concentrated solution of seven days fermented hay (a proportion of 500 grams of hay per 120 liters of water were recommended) while the other cup contained a 10% diluted solution. Also, all cups contained a folded filter paper surrounding the walls for which the females deposited the eggs. The function of the concentrated solution was, mainly, attract the females while the diluted cup was more preferred for oviposition. Filter papers were changed every three days to prevent some eggs to hatch. Finally, papers with eggs were collected and dehydrated at environmental temperature and placed inside an hermetic bag (Reiter & Nathan, 2001). The hay solution was used because this plant produced compounds like skatole (3-methylindole), *p*-cresol (4-

methylphenol) and phenol which result very attractive to female adults of *A. aegypti* because these volatiles are present in feces and sweat of human (Baak-Baak et al. 2013; Menda et al. 2012). The *in situ* eggs were collected in La Mesa, Cundinamarca by members of the INS (Instituto Nacional de Salud) of Colombia and Cundinamarca Chair and given to the Center for Microbiological Research (CIMIC). La Mesa conditions are: N 4°38'02.94" and W 72°27'43.42", altitude of 1044 m.a.s.l., and average temperature of 35°C. A colony was grown for this strain and its conditions are listed below.

Bacterial and Mosquito growth conditions

L. sphaericus strains were isolated from coleopteran larvae (OT4b.25) and from the native oak tree *Quercus humboldtii* (III(3)7) (Dussán & Lozano 2002); and the WHO reference strain (2362) was kindly donated by A. Delécluse, member of the Pasteur Institute in France (Charles et al. 1996). To obtain bacterial cells at the same phase (mainly spores), bacterial strains were cultured in sporulation medium composed of sodium acetate, yeast extract, $MgCl_2$, $CaCl_2$ and $MnCl_2$ according to a previous study (Lozano & Dussán, 2013). Since this work focuses on vegetative cells instead of spores, synchronized strains were incubated in nutrient agar during 12 hours at 30°C and stored with 20% glycine at -70°C or passed by isolation to nutrient agar.

A. aegypti Rockefeller strain and *Culex quinquefasciatus* Muña strain larvae were gently donated by the Entomology Laboratory at the INS of Colombia. It is important to mention that *C. quinquefasciatus* was used as positive control in bioassays with *A. aegypti* Rockefeller because it is supersensitive to *L. sphaericus* toxins. Mosquito colony conditions were 28 ± 0.3ºC, 60% of relative humidity and 12 hours of dark and 12 hours of light (12D:12L) photoperiod.

Concentrations of individual inoculum and consortia of L. sphaericus

Synchronized cells were passed by isolation into nutrient agar for another 12 hours. After that, bacteria equivalent to $1x10^9$ CFU/mL was resuspended in 1000 µL sterile distilled water containing Eppendorf. Bacterial cells were diluted in sterilized-distilled water into four inocula as follows; 1:1, 1:4, 1:10 and 1:1000. For each bioassay were added 300 µL of inoculum. However, for consortia bioassays of two strains then 150 µL of each strain were added and 100 µL for each strain when testing consortium with all three strains.

In order to name each consortium easier, a codename was assigned to each consortium based on the last number of the strains composing it as shown in Table 1.

Table 1. Nomenclature of the different consortia used for this study.

Bacterial Mixture	Consortium codename
236**2*** + OT4b.2**5**	25
236**2** + III(3)**7**	27
OT4b.2**5** + III(3)**7**	57
236**2** + OT4b.2**5** + III(3)**7**	257

*Numbers in bold and underlined indicate the selected numbers for the codename.

Bioassays

The bioassays were done with a modified protocol of the WHO. Tests were done to 1st - 2nd, and 3th - 4th instars. A volume of 300 µL of the four different inocula of vegetative cells were added to flasks with 30 mL of chlorine-free tap water and 20 larvae per flask. Each concentration was done with 3

replicates. Bioassay conditions were 28 ± 0.3 ºC, 60% of relative humidity and 12 hours of dark and 12 hours of light (12D:12L) photoperiod.

All of consortia tested both against *A. aegypti* Rockefeller and La Mesa. Tests with independent bacteria were done as well. Larval mortality was registered after 48 hours of exposition. No bacteria treatment was the negative control and *C. quinquefasciatus* was the positive control when testing Rockefeller strain. However, Rockefeller strain was the positive control for bioassays with La Mesa strain. All bioassays were done with three replicates and repeated twice. Larvae were considered dead when no response to physical stimuli was observed and could not swim and attach to the surface as well. Inoculum was estimated by plating 30 µL of dilution 1:1000 in nutrient agar.

Statistical analysis

It is important to mention that the level of significance for all statistical analyzes was $\alpha < 0.05$.

Shapiro Wilcoxon Normality Test

This test was used to determine if larval mortalities had a normal distribution. For this test, the null hypothesis assumes that all samples follow a normal distribution (Ghasemi & Zahediasl, 2012).

Multifactorial ANOVA

Despite the non-normality distribution of larval mortalities, this test is able determine statistical differences between factor due to the sample size (a total of 120 larvae per each inoculum including repetitions). In order to evidence statistical differences between testing instars and strains, a multifactorial ANOVA was used. For this test, the factors analyzed were larval instars and bacterial strains.

Nemenyi Test

This is a post-hoc test used to find statistical similarities between different groups based on multiple comparisons by pair-wise test of performance (Saito et al., 2015). In this study, this test was used to determine statistical similarities between the different concentrations of inoculum for the percent larval mortalities.

Probit Model

This model uses a regression in which the dependent variable is binomial, in this case is dead or surviving larvae after 48 hours of exposure to bacterial inoculum. In this study, this model estimates the probability of the bacteria to cause mortality to 50% of the larvae population when the different bacterial inoculum increases. In addition, the inoculum required given a probability of 0.5 to cause mortality is denoted as Lethal Concentration 50 (LC50) (Rath et al., 2011). It is important to mention that the inoculum was transformed into logarithmic scale since the original units were CFU/mL and had exponential values.

Fisher's exact test

This test was used for the contingency tables in order to evaluate statistical significances between observed and expected percent mortalities for each consortium (Arshad et al., 2006).

Calculation

Abbott's formula

Since there was slight mortality (less than 10%) in the negative controls, it was necessary to use Abbott's formula in order to correct observed mortalities to the negative controls (Rosenheim & Hoy, 1989). In the following formula, "T" corresponds to percent mortality of a treatment (in this case, inoculum concentration) and "C" corresponds to the average percent mortality of the negative controls:

$$\text{Corrected Mortality \%} = \frac{(T-C)}{(100-C)} X\ 100$$

Tabashink's formula

With this formula it was possible to estimate the LC50 expected for each consortium based on the LC50s of the individual inoculum. In the formula below, r_a and r_b correspond to the fraction of each bacterial strain added to the bioassays and $LC50_{(a)}$ and $LC50_{(b)}$ are the individual LC50 for each individual strain, respectively (Tabashnik, 1992). This expected LC50 assumes simple independent action for each consortium which means that no interaction between strains is expected to occur:

$$Expected\ LC50 = \frac{1}{\frac{\text{ra}}{LC50(a)} + \frac{rb}{LC50(b)}}$$

Antagonism and Synergism Factors (AF and SF)

After obtaining the expected LC50, the AF was calculated in order to establish antagonism. This factor is the ratio of the observed LC50 over the expected LC50 obtained with Tabashnik's formula. It is important to mention that AF values over 1 indicate antagonism interaction of the bacterial strains, values equal to 1 correspond to additive interaction and values under 1 indicate synergistic interaction (Lemes et al., 2014).

$$\text{AF = Observed LC50} = \frac{Observed\ LC50}{Expected\ LC50}$$

On the other hand, the SF was estimated to evidence synergism between bacterial strains. The Synergism Factor is inversely related to Antagonism Factor (Lemes et al., 2014):

$$\text{SF} = = \frac{1}{AF}$$

RESULTS AND DISCUSSION

Comparison between strains and instars

As an initial approach, a comparison of the response to the presence of bacterial strains against different instars to both Rockefeller and La Mesa mosquito strains was done. In Figure 1, the linear regressions correspond to the relation between number of dead larvae according to different logdoses of the inoculum tested. Moreover, regressions of 1^{st}-2^{nd} instars of both Rockefeller (red lines) and La Mesa (green lines) strains had statistically similar slopes for graphs 1A,1B and 1D. The multifactor ANOVA complemented such results because there is no significant difference between mosquito strains but there were statistical differences between 1^{st} – 2^{nd} to 3^{rd} – 4^{th} instars for the same graphs as mentioned before. However, multifactor ANOVA for graph 1C showed no significant differences between instars nor mosquito strains. As it can be seen, regressions of the 1^{st}-2^{nd} instars intersected the dashed-gray lines in lower logdoses when compared to 3^{rd}-4^{th} instar. In other words, the first instars are more sensitive to bacterial strains than the latest ones because there is 50% of larval mortality for the earliest instars with less inoculum (Berry et al., 1993; Rungrod et al., 2009). For this

reason, the next bioassays were tested against 1st-2nd instars which are more sensitive to bacterial strains.

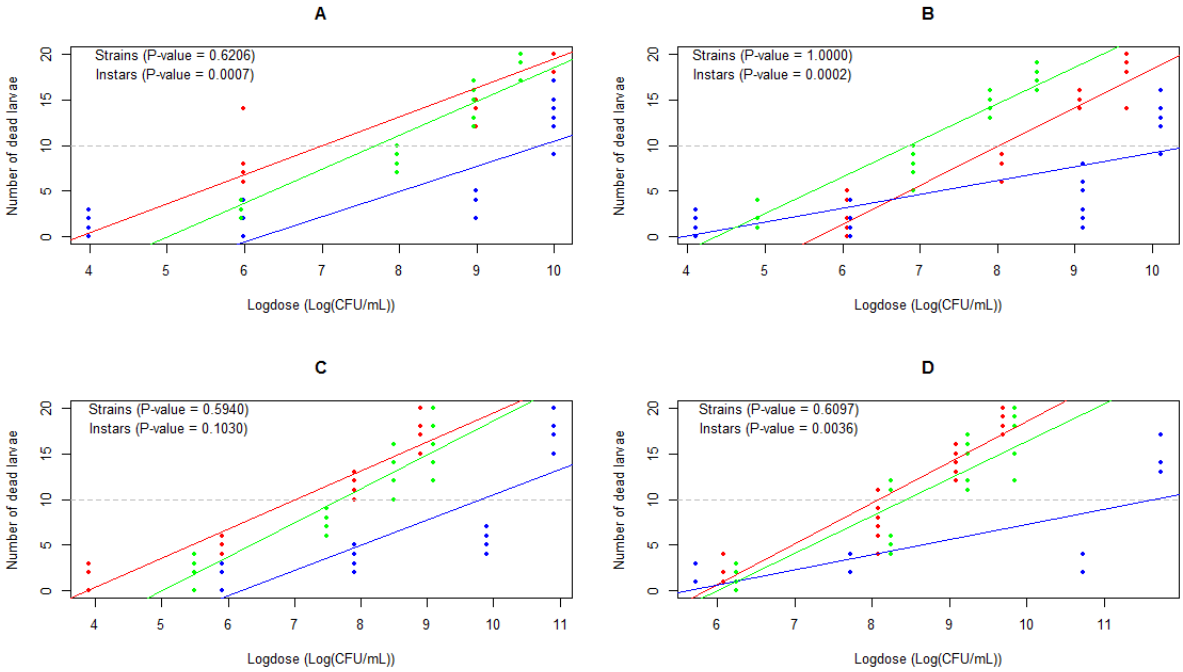

Figure 1. Comparison of mortality and logdose between strains and instars for the different consortia: A) 25, B). 27, C). 57, D). 257. Gray-dashed lines indicate half of the larvae dead. Red line indicates instars 1st-2nd of Rockefeller strain. Green lines indicate instars 1st-2nd of La Mesa strain. Blue lines indicate 3rd-4th instars of Rockefeller strain. Labels in the left upper side of each graph correspond to multifactor ANOVA P values.

Mortality of bioassays

Figure 2 shows that there were larval mortalities of 1st-2nd instars of *A. aegypti* Rockefeller strain against the different consortia. In all graphs, the two highest doses of inoculum are statistically similar to the positive control (*C. quinquefasciatus*) and the two lower doses were similar to the negative control (no bacteria added) according to the Nemenyi test. In addition, it is important to mention that, for the probit model, having two concentrations below and two other above 50% of larval mortality are required to obtain an acceptable regression and thus requirements are shown in Figure 2.

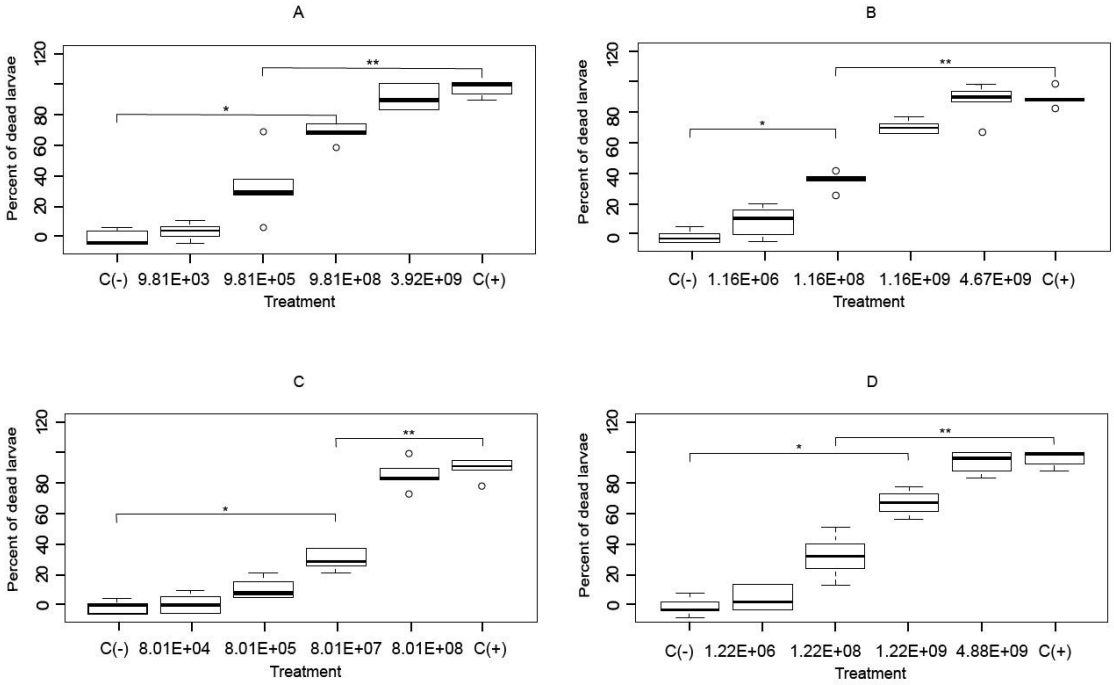

Figure 2. Percent mortalities of *A. aegypti* Rockefeller strain larvae to different inocula for each consortium: A). Consortium 25. B). Consortium 27. C) Consortium 57. Consortium 257. C(-): Negative control and C(+): Positive control. Asterisks indicate levels of significance ($\alpha < 0.05$) according to Nemenyi test.

In Figure 3, the percent mortalities of 1st-2nd instars of *A. aegypti* La Mesa strain against all the consortia are shown. These results are similar to those found in Figure 2 because the highest doses were statistically similar to the positive control (*A. aegpyti* Rockefeller strain) and the lowest doses were similar to the negative control. For the bioassays of the field collected larvae, the positive control was changed from *C. quinquefasciatus* to *A. aegpyti* Rockefeller strain because the interest for these results was to compare the response of the two *A. aegypti* strains directly. Therefore, since the response of La Mesa strains were similar to Rockefeller for the highest doses, this bioassay it was then considered as valid. It is important to note that the levels of significance might be more accurate if more bioassay repetitions are tested and this is a consideration for further studies.

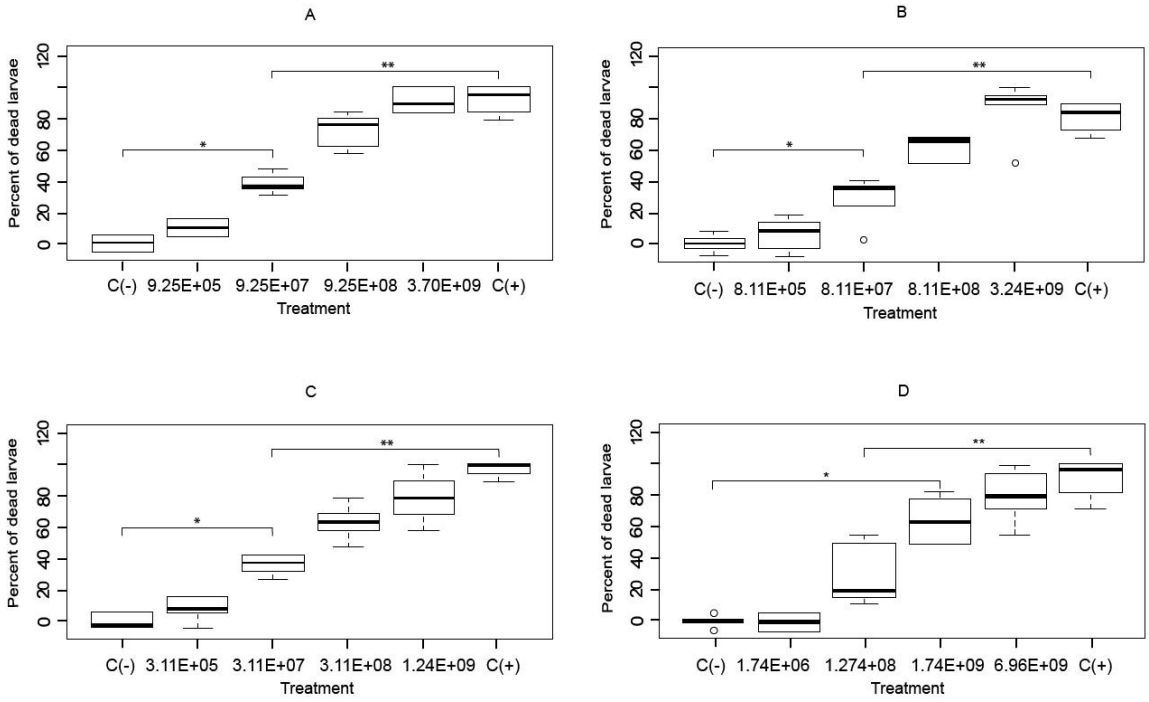

Figure 3. Percent mortalities of *A. aegypti* La Mesa strain larvae to different inocula for each consortium: A). Consortium 25. B). Consortium 27. C) Consortium 57. Consortium 257. C(-): Negative control and C(+): Positive control. Asterisks indicate levels of significance ($\alpha < 0.05$) according to Nemenyi test.

LC50 determination

Table 1 shows the LC50 for each individual bacterial strain as well as consortia against Rockefeller larvae. For each LC50, the corresponding confidence limits are presented and also the respectively logdose value obtained from the probit model and which represents the logarithm of the inoculum required to reach a probability of 0.5 to cause 50% of larval mortality. As it can be seen, all the consortia except the 257 showed lower LC50s when compared to individual strains. For this reason, it is possible to state that consortia composed by two strains are more effective in killing *A. aegypti* Rockefeller strain than individual ones since all of the LC50 values lied below the confidence limits of all the individual strains. On the other hand, the consortium 257 had a LC50 inside the confidence limits of the individual strains and, then, considered similar to these; this may occur because of nutrient competition between all three bacterial strains or due to growth inhibition by *quorum quenching*. Now, with these results and comparing with literature (Allievi et al., 2014) it is considered that using any of the two strains consortia (25, 27 and 57) could be good candidates to control *A. aegypti* Rockefeller strains *in vitro* and, probably, *in situ*.

Table 1. Determination of LC50 for individual bacterial strains and consortia against 1st-2nd instar larvae of Rockefeller strain.

Treatment	LC50 (CFU/mL)	Confidence limits (CFU/mL)	LC50 Log dose (CFU/mL)
2362	$1.36x10^8$	$6.15x10^7 – 8.05x10^8$	8.134
OT4b.25	$6.52x10^8$	$6.15x10^8 – 8.07x10^9$	8.436
III(3)7	$6.61x10^7$	$5.25x10^7 - 6.87x10^8$	7.820

25	$1.56x10^7$	$3.29x10^6$ - $2.92x10^8$	7.888
27	$1.21x10^7$	$1.01x10^7$ - $1.32x10^8$	7.085
57	$1.37x10^7$	$1.31x10^6$ - $4.88x10^7$	7.136
257	$1.39x10^8$	$1.06x10^8$ - $1.39x10^9$	8.145

Similarly, Table 2 shows the LC50 for each individual bacterial strain and consortium against La Mesa larvae. Also, the confidence limits and LC50 logdoses are presented for each LC50. Results from this table are congruent with Table 1 because consortia composed by two bacterial strains are more efficient in causing larval mortality since the LC50s are below confidence limits of individual strains. Once again, consortium 257 is similar to individual strains because the LC50 lied inside the confidence limits of all individual strains. According to Table 2, consortia 27 and 57 were the most effective options control *A. aegypti* La Mesa strain since these two consortia had LC50 values below other consortia and individual strains as well. For this reason, these two consortia are proposed as candidate options to control La Mesa larvae in both *in vitro* and, hopefully, *in situ*.

Table 2. Determination of LC50 for individual bacterial strains and consortia against 1^{st}-2^{nd} instar larvae of La Mesa strain.

Treatment	LC50 (CFU/mL)	Confidence limits (CFU/mL)	LC50 Log dose CFU/mL
2362	$2.62x10^8$	$2.46x10^8$ - $1.01x10^9$	8.419
OT4b.25	$5.61x10^8$	$4.29x10^8$ – $5.63x10^9$	8.555
III(3)7	$3.41x10^8$	$3.36x10^8$ - $4.40x10^9$	8.533
25	$9.08x10^7$	$8.08x10^7$ – $1.05x10^9$	7.888
27	$1.02x10^7$	$7.44x10^6$ – $8.84x10^7$	7.010
57	$5.75x10^7$	$2.71x10^7$ - $3.55x10^8$	7.760
257	$3.45x10^8$	$1.51x10^8$ - $1.99x10^9$	8.538

Determination of Antagonism and Synergism

Once observed LC50s were determined, expected LC50 for each consortium were estimated with Tabashnik's formula and collected in Table 3 for bioassays against *A. aegypti* Rockefeller larvae. Based on these LC50s, the next step was to calculate the observed and expected percent mortalities; according to Fisher´s exact test, expected mortalities were statistically lower than observed, and thus, indicating possible synergy between the different strains except when comparing the results for consortium 257. Then, antagonism and synergism factors for each consortium were determined and, in all consortia with two strains, synergism since AF were lower than 1 and SF values higher than 1. In congruence with Table 1, consortium 257 was the only one to show an AF value higher than 1 which indicates antagonism.

Table 3. Determination of Synergism and Antagonism Factors of the different *L. sphaericus* consortia against 1^{st} – 2^{nd} instar larvae of *A. aegypti* Rockefeller strain.

Consortium	Observed LC50 (CFU/mL)	Expected LC50 (CFU/mL)*	Observed Percent Mortality (%)	Expected Percent Mortality (%)**	Antagonism Factor (AF)	Synergism Factor (SF)	X2 (Fisher's exact test P value)***
25	$1.56x10^7$	$3.03x10^8$	49.995	32.356	0.255	3.913	5.715 (0.014)
27	$1.02x10^7$	$2.96x10^8$	52.429	33.413	0.034	29.051	6.624 (0.009)
57	$5.75x10^7$	$3.50x10^8$	49.990	34.978	0.164	6.082	4.017 (0.044)
257	$3.45x10^8$	$3.15x10^8$	48.977	51.021	1.096	0.912	$2.178x10^{-5}$ (1.000)

*Expected LC50 values estimated using Tabashnik's formula

**Expected percent mortality was estimated assuming simple independent action between strains.

***P value corresponds to X^2 between expected and observed LC50 with 1 degree of freedom.

In Table 4, the expected LC50 for the different consortia against La Mesa larvae were estimated with Tabashnik's formula. Also, observed and expected percent mortalities were estimated based on the differences in LC50s. As shown in Table 4, expected LC50 for consortia with only two bacterial strains were higher than observed; this means that more inoculum is needed to reach 50% of larval mortality for the expected values. In other words, observed LC50 values are more efficient killing larvae than the expected and, also, this is represented in the increased percent mortality observed when compared to expected. According to Fisher's exact test, percent mortalities are different between observed and expected. Moreover, SF values were higher than 1 for all consortia with two bacterial strains and AF below 1 and this evidences synergism but no antagonism between bacterial strains when mixed in consortia. On the other hand, consortium 257 had no differences in percent mortalities and obtained AF higher than 1 and SF lower than 1 which was assumed as antagonistic.

Table 4. Determination of Synergism and Antagonism Factors of the different *L. sphaericus* consortia against $1^{st} – 2^{nd}$ instar larvae of *A. aegypti* La Mesa strain.

Consortium	Observed LC50 (CFU/mL)	Expected LC50 (CFU/mL)*	Observed Percent Mortality (%)	Expected Percent Mortality (%)**	Antagonism Factor (AF)	Synergism Factor (SF)	X^2 (Fisher's exact test P value)***
25	$9.08x10^7$	$1.82x10^8$	49,995	33.490	0.085	11.638	4.918 (0.021)
27	$1.21x10^7$	$8.89x10^7$	49.940	35.551	0.136	7.352	3.955 (0.043)
57	$1.37x10^7$	$1.06x10^8$	48.023	33.806	0.128	7.768	4.705 (0.031)
257	$1.39x10^8$	$1.15x10^8$	65.267	63.998	1.210	0.826	0.041 (0.887)

*Expected LC50 values estimated using Tabashnik's formula

**Expected percent mortality was estimated assuming simple independent action between strains.

***P value corresponds to X^2 between expected and observed LC50 with 1 degree of freedom.

LC50 comparison between consortia and individual strains

It is important to mention that LC50 are the x-axis value where each probit regression intersects the proportion of half (value of 0.5 in y-axis) of the larvae are dead (red-dashed line). In Figure 4A, 4B and 4C, each consortia (black lines) had notably lower LC50s when compared to LC50s of individual strains (colored lines). This means that Rockefeller larvae were more sensitive to respond to the presence of consortia that individual bacterial strains. However, consortium 257 showed a mortality effect similar to individual strains and possible reason were discussed before.

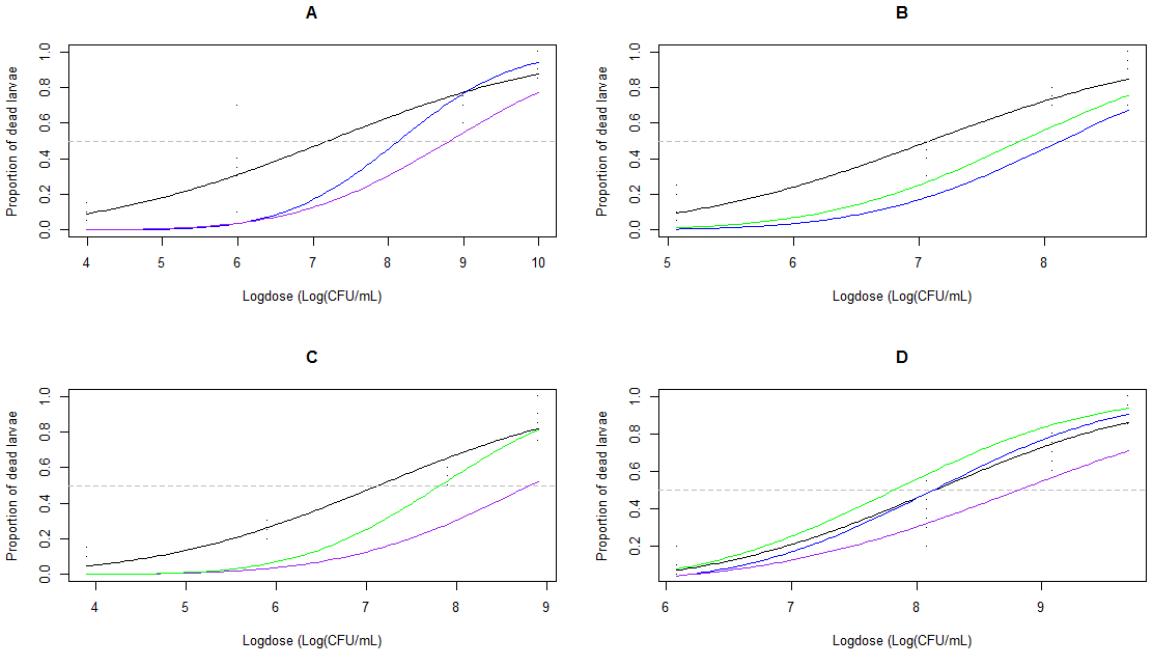

Figure 4. LC50 comparison between *L. sphaericus* individual strains and consortia against 1st-2nd instars of Rockefeller larvae. Black lines correspond to probit regressions of consortia and colored lines correspond to probit regression of individual strains. A). Consortium 25 B). Consortium 27 C). Consortium 57 and D) Consortium 257. Blue line: 2362, Purple line: OT4b.25 and Green line: III(3)7. Gray-dashed line indicates the proportion of 50% of dead larvae.

The same approach was done for the LC50s against La Mesa larvae. Results shown in Figure 5 are similar to those in Figure 4 where bacterial consortia had lower LC50 values when compared to individual strains. As seen in Figure 5B, LC50 of consortium 27 was smaller than the obtained for the same consortium against Rockefeller larvae. The possible biological implication for this finding is that field collected mosquitoes of La Mesa strain were even more sensitive to this consortium than Rockefeller strain.

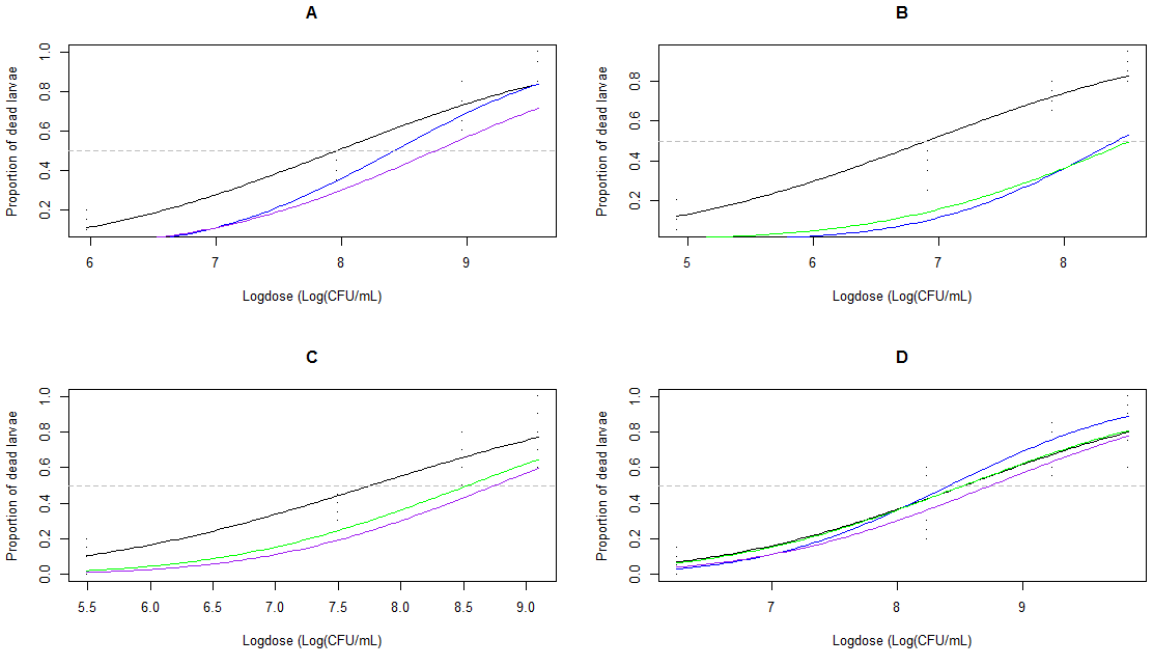

Figure 5. LC50 comparison between *L. sphaericus* individual strains and consortia against 1st-2nd instars of La Mesa larvae. Black lines correspond to probit regressions of consortia and colored lines correspond to probit regression of individual strains. A). Consortium 25 B). Consortium 27 C). Consortium 57 and D) Consortium 257. Blue line: 2362, Purple line: OT4b.25 and Green line: III(3)7. Gray-dashed line indicates the proportion of 50% of dead larvae.

LC50 comparison between consortia

Moreover, probit regressions for all consortia against both Rockefeller and La Mesa strains were compared as shown in Figure 6. All consortia with only two strains against *A. aegypti* Rockefeller larvae are good candidates for biological control than the consortium 257 which containg all three strains as seen in Figure 6A. On the same way, these consortia are good to control *A. aegypti* La Mesa expect consortium 257 as well. However, consortium 27 had a lower LC50 value than other consortia which seems that this mixture of bacteria is even better for La Mesa larvae *in vitro* and, hopefully, *in situ*.

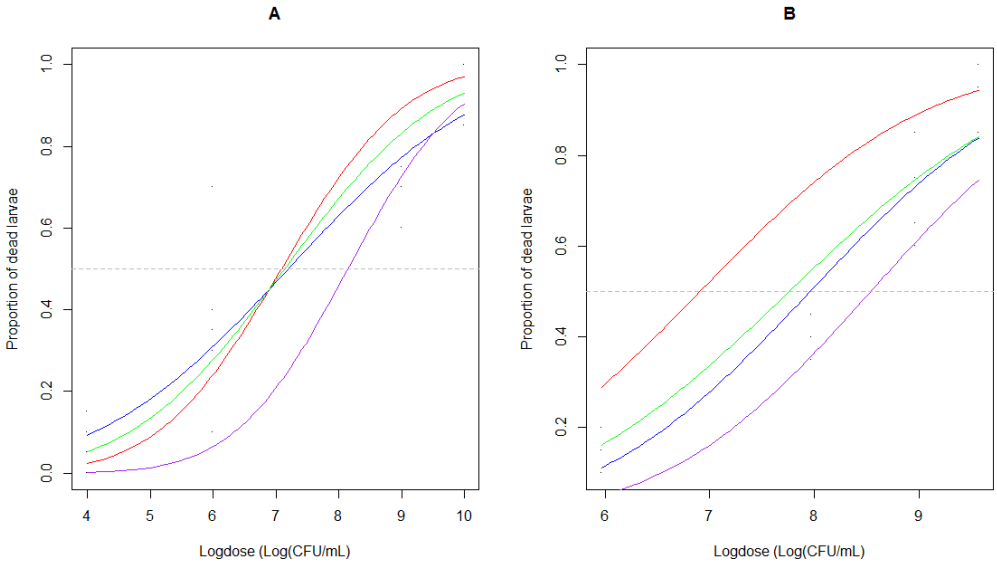

Figure 6. Comparison of the LC50s between the different bacterial consortia against *A. aegypti* larvae A) Rockefeller strain and B). La Mesa strain. The gray-dashed lines correspond to the proportion were half of the larvae were dead. Colored curves correspond to probit regressions for each consortium. Blue line: Consortium 25. Red line: Consortium 27. Green line: Consortium 57. Purple line: Consortium 257.

CONCLUSIONS

According to the results obtained in this study, mortality of *A. aegypti* reference and field collected larvae was obtained when exposed to vegetative cells of three different strains of *L. sphaericus*. Such mortality occurred for both individual bacterial strains as well as consortia and 1st-2nd instars were more susceptible than 3rd-4th instars. Now, it is important to note that consortia composed by two strains (that is consortium 25, 27 and 57) were more effective causing larval mortality than individual strains and consortium 257 against Rockefeller and La Mesa strains. In fact, consortia 27 and 57 were very effective than other consortia and individual bacteria against La Mesa strain and were proposed as candidates to control *in vitro* and *in situ* populations of mosquito. The effectiveness of these consortia was established because of the lower LC50 values and because these values are below confidence limits of the other consortia and individual strains. Such LC50 values are comparable and still higher than those obtained by Allievi when tested spores of *L. sphaericus* 2362 against *A. aegypti* larvae (strain not mentioned) (Allievi et al., 2014). In addition, synergism was determined for consortia 25, 27 and 57 but not for 257. Such synergism may be explained by future studies in *quorum sensing* or another signaling pathways that improve bacterial growth rate and toxin production such as Mtx. In the same way, antagonism was evidenced for consortium 257 and this may occur by nutrient competition between bacterial strains and/or signaling that may block bacterial growth and toxin production. At this point, future studies of toxin production by qPCR are needed to evidence differential expression for individual strains in bioassays as well as in consortia. Further bioassay repetitions and studies *in situ* are required to strengthen the horizon of this work.

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