

Article

Efficacy of 2 commercial formulations of *Bacillus thuringiensis* H-14 in larvae of *Anopheles albimanus* W and *Aedes aegypti* L (Diptera: culicidae).

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Abstract: The bacterial genus and species of *Bacillus thuringiensis* var *israelensis* (*Bti*), is entomotoxic, used in the biological control of mosquito vectors of human diseases, such as malaria and dengue. Following the studies carried out in Mexico and Guatemala with ovillantas, in which the presence of larvae of both species of *Anopheles albimanus* W and *Aedes aegypti* L were observed, and with the intention of continuing to develop an ecologically friendly mosquito control, *Bti* was added to the ovillantas, to improve and already efficient method on the elimination of both types of mosquitoes. The objectives of this work were: to analyze the effectiveness of two commercial formulations of *Bti*, serovar H-14 (Bactimos) wettable powder, 3,500 ITU, from Biochem prods., and Vectobac, 2,000 ITU wettable powder, (Abbot Lab.) on second and third instars larvae of *A. albimanus* and *Ae. aegypti* (Diptera Culicidae). The two formulations were effective against *A. albimanus* W (higher concentrations), while *Ae. aegypti* L was very susceptible to *Bti*, therefore it is proposed for the best control of these genus and vector species of malaria and dengue at adequate concentrations.

Keywords: Mosquito vector, biological control, *Bacillus thuringiensis*, ovillanta, *Bti*, biolarvicide.

1. Introduction

Mosquito control is essential on public health in developing countries due to the negative impact of the illnesses transmitted by these insects, especially in the economy of families. In Latin America in the health sector there are no direct financial support for people infected with dengue or malaria, if the worker gets sick, they are not paid for the day, this causes anxiety, depression and misery in families (1). Herms (2) divided mosquitoes into 2 types: those that only cause nuisance and those that transmit or are vectors of human disease. In 1983, Brown(3) further classified the population of the same mosquito species X into; being a vector with a high rate of infectivity in the transmission of the parasite, while another population of the same species as not infective. This

contradicts the conventional scheme that every mosquito of the same species X is a transmitter of diseases or behaves as the only vector. In Mexico, malaria is a disease transmitted by vector mosquitoes, with the species *Anopheles albimanus* W(4) and *A. pseudopunctipennis* responsible for the mayor transmissions. Four protozoan parasites of the genus *Plasmodium* can be transmitted by *Anopheles* spp. mosquitoes; *P. vivax*, *P. falciparum*, *P. ovale* and *P. malariae*, all can cause malaria illness but *P. falciparum* is by far the most lethal. When a human is infected with malaria it causes a nutritional state that can be disabling to work, with asthenia and even death, if the health center does not provide proper and timely assistance(5). In Latin America, *P. vivax* is dominant and responsible for 77% of the morbidity cases(6). With a territorial extension of 1.5 million km²(7) and before the antimalarial campaign of 1955-1970, Mexico invested 544 million pesos per year to control mosquitoes, but still registered an annual mortality of 24,000 deaths due to malaria. Albeit the resolution signed by PAHO member countries in 2016(8) on the CD55.R7 Action Plan for the eradication of malaria, 609 cases were still reported for the year 2019 in Mexico, a considerable increase in malaria in the Region of the Americas(9).

Dengue is the next most epidemiologically important disease transmitted by mosquito vectors, with no real estimates of morbidity and mortality, as well as of the economic losses. In Mexico, the main transmitters of dengue are the mosquitoes of the genera and species: *Aedes aegypti*, and to a lesser extent *A. albopictus* (the tiger mosquito)(10, 11) infected with the flavovirus serotypes 1 and 2, although 3 and 4 also exist in some parts of the country (Fig. 1). Dengue symptoms are characterized by severe muscle aches, fever with a long period of convalescence, which drastically reduce the effective hours of work, in acute cases of dengue, such as hemorrhagic fever, it can cause hypovolemic shock and even death if medical attention is not provided timely to the patient(12).



Figure S1.- Identified Serotypes and Incidences of Confirmed Cases by State; Mexico, 2020

(https://www.gob.mx/cms/uploads/attachment/file/558413/Dengue_24_2020.pdf)

Malaria is an endemic disease in tropical and rural areas(13). Dengue is an urban disease, due to the habits of the *Ae. aegypti* mosquito(14).

Resistance to chemical pesticides by *Aedes* spp species has been widely reported: *Ae. aegypti* has shown tolerance to deltamethrin, permethrin, malathion, chlorpyrifos, temephos and bendiocarb (CARB), while *A. albopictus* to malathion and to a lesser extent to chlorpyrifos, temephos,

permethrin and deltamethrin(15). Since 1970, the 23rd. World Health Assembly, due to the high toxicity, low selectivity of chemical pesticides and the increase resistance of the species, recommended to use new methods of vector control, such as biological(16). In this sense, vector control is no longer based only on applying chemical agents, for the above environmental reasons; a study with ovillantas in 2017 showed that the decrease in *Aedes* spp can be due to the implementation of a simple ovillanta (with no chemicals) and the active participation of the community in the affected area(17). In this study, according to preliminary calculations, the systematic elimination of larvae and eggs in the application area, caused a reduction of up to 77% of *Aedes* spp. The continued use of ovillantas in other sites on the Pacific coast of Mexico has led to the identification of other problems associated with community participation; in Troncones, Mexico a two-year (May 2016-April 2018) study on the use of the ovillantas, showed that in the high season of the second year, the amount of eggs and larvae of *Aedes* spp collected from the ovillantas did not rise to the level of the previous year, which indicated that the vector mosquito was effectively reduced in the region(**Figure. 1**)(18). This demonstrated that the constant destruction of the eggs and larvae of *Aedes* species in the region, induced an evident decrease in the number of eggs surviving from previous years(19,20,21). In **Figure 2**, it is observed that in July 2016 49,600 eggs were collected in the evaluated area, a quantity that decreased during the low season of mosquito reproduction. In July 2017, the same increase in the capture of *Aedes* spp eggs was not recorded, since only 9,200 eggs were detected in that period of the high season, which indicated that the existence of *Aedes* spp was considerably reduced compared to the previous year. However, in the absence of eggs and larvae of *Aedes* spp, other genera and species of vector mosquitoes such as *Anopheles* spp were found and identified in the ovillantas, therefore, it is necessary to find an effective way to control the different species of larvae born in the ovillantas, regardless of the gender and species of mosquito.

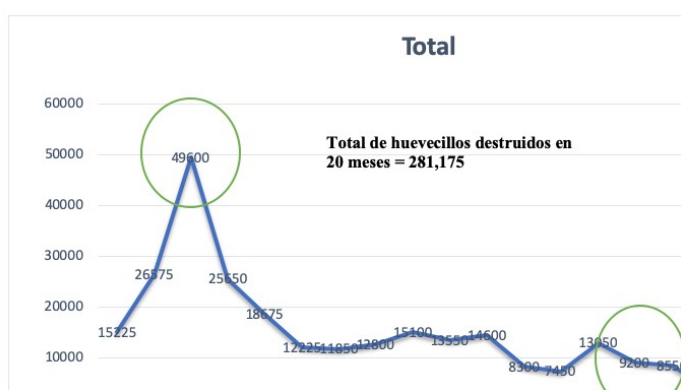


Figure S2- Study on the elimination of eggs and larvae of *Aedes* spp. mosquitoes using ovillantas, Troncones, Mexico (unpublished results G.U. 2018)

In 1976 the genus and bacterial species identified as *Bacillus thuringiensis* (*Bt*) was isolated in Israel, which in the laboratory showed high toxicity against mosquito larvae(22). This *Bt* isolate was sent to the Pasteur Institute in Paris, France for biochemical and serological identification, and was designated as serotype H14 var, israelensis(23). In the last months of the study in Troncones, Guerrero, Mexico, different types of mosquito larvae were observed in the ovillantas, one of the larvae was assumed to be of the genus *Anopheles* due to the horizontal position of the larvae to the

surface of the water. Although the complete identification of the larvae was not carried out, it was assumed that they belonged to the species *A. albimanus* or *A. pseudopuntipennis*, known to exist in the state of Guerrero. Under this observation and with the aim to improving the efficiency of the ovillantas in the destruction of larvae of different species of mosquitoes, a quarter of an imported MosquitoDunks (Summit Chemical Co. Baltimore, USA) donut was installed inside the ovillantas, as means to obtain biological control of these vector mosquitoes. Surprisingly, the MosquitoDunks label does not state the variety of *B. thuringiensis* (*Bt*) used in its manufacture. At the end of several weeks of using *Bt* in the ovillantas, it was observed that in some of the ovillantas there were only dead larvae, while in others, there were living larvae. Our first thought was that the concentration of *Bt* was different among the ovillantas, but it also was observed that the dead larvae were of the species *Aedes*, while the ones that survived were of the species *Anopheles*. These results made it necessary to carry out a study on commercial formulations of *Bti* to determine the minimum concentration necessary to achieve the maximum adequate larval control of all genera and species of vector mosquitoes arriving to the ovillantas. Based on the above, the objective of this work was to analyze the effectiveness of commercial *Bti* products for the larval control of vector mosquitoes of the genera and species of *Aedes* and *Anopheles*.

2. Materials and Methods

2.1 Breeding vector insects

Mosquito colonies of different species are kept at the Malaria Research Center (Centro de Investigación contra el Paludismo (CIP)), in Tapachula, Chiapas, Mexico. To ensure that there is egg production and consequently, that the colony subsists under the same strain, females of the colony are fed with blood from rabbits freshly shaved on the back. The animals are kept in stipulated conditions, by the CIP animal ethics committee, to give them the maximum comfort of life and that they are minimally affected during the feeding of the mosquitoes. If necessary, rabbits are lightly anesthetized under the supervision of the research unit veterinarian.

Adult female and male mosquitoes of each species are kept in a controlled insectary at a constant temperature of 26 ± 1 °C and relative humidity of $70 \pm 10\%$ and light / dark periods of 10h / 14h in folding cages (BioQuip 1450B (30.5 x 30.5 x 30.5 cm)) with expandable polyester feed hose. To obtain the eggs, a plastic container (30 x 25 x 10 cm) is installed inside the cage with a liter of chlorine-free water and cuttings of paper towel (or filter paper) placed in contact with the surface of the water. Adult mosquitoes are generally fed with a 10% sugar solution in cotton balls previously sterilized with alcohol, or with a solution of corn syrup, sugar syrup or fresh cut fruit (grapes) or dried raisins.

2.2. Larvae of *Anopheles albimanus* W.

For the species *A. Albimanus* W, a strain collected in El Gancho, municipality of Suchiate, Chiapas, Mexico was used. The birth of larvae is monitored daily and transferred to different containers marked with the day. First instar larvae were fed with powdered fish, second to fourth instar larvae were fed with a mixture of vegetable protein (incaparin) or with vegetable rabbit food powder (Chowchow, Purina). For the present study, a certain number of second and third instar larvae of *A. albimanus* were transferred to pewter containers and fed exclusively with an incaparin solution.

2.3. Larvae of *Aedes aegypti* L

To create the *Ae. aegypti* colony, fourth instar larvae were initially collected from vases in the municipal cemetery of Tapachula, Chiapas, Mexico. The collected larvae were subjected to the temperature and humidity conditions described above for the CIP insectary. The larvae were placed in pewter trays inside collapsible cages and fed with incaparín until they pupated. Emerging adult females have been kept for several generations, fed rabbit blood for reproduction (under strict CIP rules of ethics on animal care) and fed corn syrup and raisins. For the present study, a certain number of second and third instar *Ae. aegypti* larvae were transferred to pewter containers and fed with an incaparín solution.

2.4 Formulations used for this study

This study aimed to determine the larvicidal activity of two commercial products available in Mexico, Bactimos (~ 3,000 UTI / mg) and Vectobac (~ 3,000 UTI / mg). Both products are made with *Bti* H-14 (strain 65-52), currently distributed as efficient biological products in larval control of *Aedes* spp. The *Bti* strains used were developed by Abbott Laboratories Inc., and the agriculture division passed into the hands of Sumitomo Chemical Company in 2000, for the formation of Valent Biosciences LLC (VBC), who makes the final formulation of these products.

Studies with similar biological products have been previously reported and have shown activity against *Aedes* vector mosquito species such as *A. canadiensis*, *A. provons* (Walker) and *A. stimulans* (Walker)(24). Although this last study used formulations with 300 UTI (Bactimos) and 200 UTI (Vectobac) impregnated in powdered corn and 600 UTI(25,26). A review of the literature on the application of *Bti* in the control of dengue mosquito vectors was published by Boyce(27).

2.5 Bactimos

Water Granules (WG) Biological larvicide. *Bacillus thuringiensis* subsp. *israelensis* (37.4%), strain AM 65-52, fermentable solids, spores and soluble solids with potency of approximately 3,000 ITU, EPA Reg. No. 73049-504. Valent Biosciences Corp.

(<https://www.valentbiosciences.com/publichealth/wp-content/uploads/sites/4/2017/02/bactimos-wg-specimen-label.pdf>)

2.6 Vectobac

Water Dispersable Granules (WDG) Biological larvicide. *Bacillus thuringiensis*, subsp. *israelensis* (37.4%), strain AM 65-52, fermentable solids, spores and toxins with insecticidal power with a potency of approximately 3,000 ITU, EPA Reg. No. 73049-56. Valent Biosciences LLC.

(<https://www.valentbiosciences.com/publichealth/wp-content/uploads/sites/4/2017/02/vectobac-wdg-specimen-label.pdf>)

2.7 Preparation of bioassays

For each bioassay, a modified technique was used based on the models reported by Georghiou(28) and Van Essen(29). In each experiment 20 larvae of the desired age were placed in 10 ml of distilled water in a plastic cup with a total volume of 50 ml. In a second 150 ml wax paper container, 90 ml of distilled water and enough *Bti* (by weight) of the studied formulation were added to give the desired concentration in 100 ml of total solution. The larvae and 10 ml of solution were transferred with a rubber bulb pipet, to have at the end a total volume of 100 ml.

Each bioassay was reproduced in five-fold at different concentrations (mg / L) of *Bti* in 100 mL of distilled water. The concentrations to be used in each bioassay were predetermined based on Dulmage(30) to calculate the lethal concentration 100 (LC100) and the LC50 of each species of mosquito. Based on this research protocol, the concentration of 11mg / L is the minimum concentration to obtain the CL100 in *A. albimanus*, while the minimum CL100 concentration for *Ae. aegypti* is 1 mg / L. From here, different concentrations were selected to satisfy the requirement of the experiment (see **Tables 1 to 4**).

Table S1: Mortality of *Anopheles albimanus* larvae induced by *Bacillus thuringiensis* var *israelensis* from Bactimos WG.

Doses (mg/L)	Larvae in treatment	T1	T2	T3	T4	T5	χ^2
19.8	20	20	20	20	20	20	1.918
9.99	20	17	15	18	20	9	0.341
5.04	20	19	18	13	10	14	0.003
2.52	20	20	12	11	0	11	0.676
0.99	20	17	10	12	0	8	0.008
0.495	20	16	6	4	0	7	0.033
0.099	20	5	0	4	0	2	0.026
0.055	20	0	0	9	0	3	0.372
Control	20	0	0	0	0	0	SUM:3.377

Table S2: Mortality of *Anopheles albimanus* larvae induced by *Bacillus thuringiensis* var *israelensis* from Vectobac WDG

Doses (mg/L)	Larvae in treatment	T1	T2	T3	T4	T5	χ^2
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19.8	20	20	20	20	20	20	0.683
9.99	20	20	19	20	20	18	0.132
5.04	20	20	19	13	13	10	2.247
2.52	20	20	10	14	19	15	0.123
0.99	20	15	12	8	19	7	0.009
0.495	20	15	1	7	18	2	0.006
0.099	20	5	0	5	1	3	0.004
0.055	20	1	0	6	1	0	0.0016
Control	20	0	0	0	0	0	SUM:3.221

Table S3: Mortality of *Aedes aegypti* larvae induced by *Bacillus thuringiensis* var *israelensis* from Bactimos WG

Doses (mg/L)	Larvae in treatment	T1	T2	T3	T4	T5	χ^2
0.99	20	20	19	20	20	20	0.895
0.495	20	18	20	20	14	20	0.096
0.099	20	16	20	16	10	16	0.538
0.055	20	14	11	14	9	13	0.045
0.009	20	2	6	1	1	0	3.241
0.005	20	1	7	5	0	0	0.228
0.0009	20	2	10	1	0	0	5.560
Control	20	0	0	0	0	0	SUM: 10.562

Table S4: Mortality of *Aedes aegypti* larvae induced by *Bacillus thuringiensis* var *israelensis* from Vectobac WDG.

Doses (mg/L)	Larvae in treatment	T1	T2	T3	T4	T5	χ^2
0.99	20	20	20	20	20	20	0.265
0.495	20	20	20	18	20	20	0.952
0.099	20	13	20	9	5	3	2.782
0.055	20	17	17	6	8	5	0.197
0.009	20	3	4	4	1	0	0.085
0.005	20	0	2	2	0	0	0.190
0.0009	20	0	0	2	0	0	0.016
Control	20	0	0	0	0	0	SUM: 4.487

The appropriate concentrations were prepared following the cascade dilution methodology described by Costa(31) and Briggs(32) based on dilutions of a stock solution. For *A. albimanus* the concentration of the stock solution was 11mg / L and for *Ae. aegypti* the stock solution was 1 mg / L. Each solution was stored for a maximum of 5 days at 5 ° C and was preheated before use in a water bath until reaching 28-29 ° C with cyclical stirring without water. All bioassays were carried out within an atmosphere of 70-75% relative humidity. A small amount of incaparin was added to each bioassay as food for the larvae.

The data obtained was analyzed electronically at the Ohio State University Computer Center in Columbus, Ohio, USA. 4 Probit regression analyzes (using R, SAS, SPSS and Stata software) were performed to obtain the LC50 values, only one value (SPSS) is reported in **Table 5**, with no more data available from the Computer center (we only received comments that all 4 programs gave similar data). From our part, in order to present data analysis in this paper, we used the Excel model created by Lei and Sun (2018)(33) as a comparative method, the average mortality was used for these studies. The results obtained with the Ohio State University system and the Sun model were close and are shown in **Table 5**.

Table S5: Statistical analysis of the effect of Bactimos and Vectobac based on *Bacillus thuringiensis* var. *israelensis*.

Type of commercial larvicide	(Fiducial limits 95%) CL50 (range)		(Fiducial limits 95%) CL95 (range)		Slope (β) (Ohio/Sun)	Intersection (α)
	Ohio	Sun	Ohio	Sun		
<i>A. albimanus</i>	1.216	1.216	19.438	16.978	1.060/1.119	-0.095
BACTIMOS	(0.7462-1.9165)	(0.679-2.181)	(9.775-57.069)	(6.115-47.141)		
<i>A. albimanus</i>	0.691	0.651	6.918	7.043	1.280/1.239	0.231
VECTOBAC	(0.4487-1.023)	(0.369-1.147)	(4.135-14.511)	(3.019-16.428)		
<i>Ae. Aegypti</i>	0.031	0.029	0.372	0.391	1.180/1.137	1.745
BACTIMOS	(0.011-0.082)	(0.015-0.058)	(0.123-4.490)	(0.129-1.189)		
<i>Ae. Aegypti</i>	0.056	0.055	0.373	0.291	1.550/1.771	2.231
VECTOBAC	(0.299-0.106)	(0.032-0.093)	(0.178-1.451)	(0.130-0.648)		

3. Results

In no experiment was larval death observed in the absence (controls) of *Bti*-based biolarvicide (control). In one hundred percent of the experiments, the total lethality of the biolarvicide was shown in the maximum concentration at 24h in second and third instar larvae. In the statistical analysis, the regression line was established between y_i and the logarithm of the dose x_i with the linear regression equation:

$$y_i = \alpha_0 + \beta_0 x_i$$

where the value of i goes from 1 to m , where m is the number of doses of the biolarvicide studied where the corrected proportion was not equal to 1 or 0. The intersection of the linear regression (α_0) and the slope (β_0) was calculated with the least squares function that were recovered with the INTERCEPT (y_i, x_i) and SLOPE (y_i, x_i), respectively, in Excel.

To establish the normality of the mosquito population, Chi-square tests (χ^2) and regression analysis were obtained for each experiment. A statistical analysis with a significant difference of χ^2 may indicate that the population of larvae did not respond independently, compared to the control, to the conditions of the experiment, or that the regression line between Probit vs log (dose) does not adequately describe the relationship dose-mortality of these experiments.

4. Discussion

The application of *Bti* as a larval biological control against vector mosquitoes of different genera and species is effective due to the selective toxicity for insects classified as: Diptera(34,35) The toxicity of *Bti* has been reported against a wide diversity of genera and mosquito species, with multiple formulations available in the international market(36,37). In this case, two commercial products were obtained in Mexico, Bactimos WG and Vectobac WDG, both recommended as biological larvicides for the control of mosquito vectors of human infectious diseases(38,39). Using data from the Lei and Sun template, Probit analysis showed a marked difference between the LC50 of Bactimos and Vectobac against *A. albimanus* (LC50 = 1.216 and LC = 0.651 mg / L) respectively(23,40); With the same trend in the CL95 (CL95 = 19.978 for Bactimos and CL95 = 7.043

mg / L for Vectobac (25,26). While against the genus and species *Ae. aegypti*, the CL50 of both showed a parallel in biological activity of LC50 = 0.029 for Bactimos; and of LC50 = 0.055 mg / L for Vectobac and a similar result in the LC95 of both products LC95 = 0.391 for Bactimos; and of an LC95 = 0.291 mg / L for Vectobac(24). Sun(41), determined with an unspecified formulation, a CL 50 for *A. albimanus* susceptible and resistant to parathion of 0.063 and 0.068 mg / l and a CL 90 of 0.42 and 0.41 mg / l respectively. Although the formulations tested in this work on *Ae. aegypti* had a slightly different effect among themselves, giving the regression line for Vectobac, with a slope of 1.771, lower but with a significant statistical difference, it was registered that both products showed satisfactory results against this genus and species of mosquito according to with the type of toxin that specifically damages the mosquito's midgut(25,26,34). The similarities that exists between the lethal concentrations (CL) registered in this site, with respect to other parts of the world, support the assumption for this genus and species(42). In addition, the similarity that exists between the CL of *Ae. aegypti* with other regions of the world support the assumption that this genus and species has not developed resistance, or does not possess it, due to the constant genetic exchange of groups or individuals that are constantly dispersed, involuntarily, by man.(39)

Regarding the results registered in *A. albimanus*, it was found that the regression lines obtained at the Ohio State University of Bactimos had a slope of 1.06, and of 1.119 using Lei and Sun's template, with a difference without statistical significance. The lethal concentrations (CL) of the statistical analysis indicated for Vectobac a very high CL 95 of 6.918 mg / L, and for Bactimos an extremely high CL 95 of 19.438 mg / L, in comparison with the results registered against this genus and species in other sites the world(42). *A. albimanus* has a different dispersal mechanism and restricted to the tropics and subtropics, they had notable differences in the activity of the formulations on local mosquitoes and in other regions.(34,43)

In the southeastern area of Mexico, vector mosquitoes are permanently subjected to adverse conditions, as a consequence of the social development of the communities; indirect conditions such as the extra frontal that is antivectorial(36). On the other hand, in other countries, such as the United States of America, for example; the fight against the mosquito is done because they are annoying and represent a potential danger to the health of man and breeding animals; these are mosquitoes without diseases, therefore, the fight is not anti-vector but anti-mosquito. The resources for the control they apply are at the level of socioeconomic development and therefore the fight must be a complete integrated control.(26,41)

The Vectobac formulation was more effective against *Ae. aegypti* and *A. albimanus* than Bactimos. For *Ae. Aegypti*, Vectobac is more recommended than Bactimos due to its efficacy, but with respect to *A. albimanus* this genus and species was totally resistant(27) at low concentrations of either formulation. So, it is recommended that in these cases, for practical purposes, insist on testing the susceptibility to biopesticides of mosquito vectors according to each zone(24,34)5.

Conclusions

Given the increase in resistance to pesticides, at the larval level(44) or in the adult stage(45), of the different disease vector species, several formulations of biopesticides have been developed in recent years(46). The type of formulations and support in these biopesticides is of utmost

importance, both for their proper implementation and for their application in an economical and sustainable way.

Here we have shown that both formulations available in Mexico work against the larvae of both mosquitoes, but Vectobac works at a lower concentration than Bactimos against the larvae of *Aedes* spp.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1,

Figure S1: Identified Serotypes and Incidences of Confirmed Cases by State; Mexico, 2020

Figure S2: Study on the elimination of eggs and larvae of *Aedes* spp. mosquitoes using ovillantas,

Troncones Gro., Mexico (unpublished results, 2018)

Table S1: Mortality of *Anopheles albimanus* larvae induced by *Bacillus thuringiensis* var *israelensis* from Bactimos WG.

Table S2: Mortality of *Anopheles albimanus* larvae induced by *Bacillus thuringiensis* var *israelensis* from Vectobac WDG

Table S3: Mortality of *Aedes aegypti* larvae induced by *Bacillus thuringiensis* var *israelensis* from Bactimos WG

Table S4: Mortality of *Aedes aegypti* larvae induced by *Bacillus thuringiensis* var *israelensis* from Vectobac WDG.

Table S5.- Statistical analysis of the effect of Bactimos and Vectobac based on *Bacillus thuringiensis* var. *israelensis*.

Table S6.- Excel template according to Lei and Sun: Calculations of LDs-Bactimos-Vectobac.

Author Contributions: “Conceptualization, J.M.S. and G.U.; methodology, G.U. and V.G.; software, G.U. and V.G.; validation, J.M.S., G.U.; formal analysis, V.G. and G.U.; investigation, V.G.; resources, J.M.S. and G.U.; writing—original draft preparation, J.M.S. and G.U.; writing—review and editing, G.U.; funding acquisition, J.M.S. and G.U. All authors have read and agreed to the published version of the manuscript.”

Funding: This research received no external funding.

Acknowledgments: The authors would like to thank the Coordinación de la Investigación Científica (Project 2.7 (2020)) from the UMSNH and to BIONUTRA, S.A. de CV. Maravatio, Mich. Mexico, for support.

Conflicts of Interest: The authors declare no conflict of interest.

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