Manila Journal of Science 17 (1) 2025, pp. 37-44

Evaluation of *Bacillus amyloliquefaciens* and *Bacillus subtilis* as Biolarvicides Against *Aedes aegypti* (L.)

Jamie C. Camba[1], Shaine Erin L. Lim[1], Shekina Atalaya C. Lunas[1], Alyssa Ashley L. Rosete[1], Kris Lord T. Santos[2,3], Evian Bless B. Matira[3], Divina M. Amalin[2,3,4], and Jose Isagani B. Janairo[2,3,4,*]

[1]Integrated School, De La Salle University, Manila, Philippines
[2]Department of Biology, De La Salle University, Manila, Philippines
[3]Institute of Biological Control, De La Salle University, Manila, Philippines
[4]Ehime University, Ehime, Japan

*Corresponding Author: jose.isagani.janairo@dlsu.edu.ph

ABSTRACT

The excessive use of persistent, nonspecific, synthetic insecticides has resulted in the development of resistance in insect vectors. Alternative methods for controlling vectors have thus been sought to reduce reliance on synthetic insecticides and minimize their impact on the environment and human health. Here, we report the evaluation of *Bacillus subtilis* and *B. amyloliquefaciens* as alternative biolarvicides for the control of *Aedes aegypti,* the primary vector of the dengue virus. Using World Health Organization protocols on laboratory testing of larvicides, treatments with both bacterial isolates showed significant larval mortalities as compared to negative controls. Increasing mortality was likewise observed, which may be attributed to increased production of larvicidal compounds during bacterial growth. Blood agar plate and drop collapse assays confirmed that the two isolates could produce biosurfactants which may be attributed to the observed larvicidal activity. These results feature the potential of these bacterial species as alternative control agents against vector mosquitoes.

Keywords: biological control, dengue, microbial larvicides, mosquito, nature-based solution, vector control

INTRODUCTION

Dengue is a mosquito-borne viral infection that can be transmitted to humans primarily by *Aedes aegypti* mosquitoes that are widely distributed across tropical and subtropical regions (Simmons et al., 2012). It is the most prevalent viral infection that is transmitted by this mosquito species, possibly risking more than 3.9 billion people throughout 129 countries with 96 million people having symptomatic cases and an average of 40,000 annual deaths (World Health Organization [WHO], 2020). The dengue virus has a long history in the Philippines, where the first dengue epidemic in Southeast Asia was recorded in Manila in 1954 (Ong et al., 2022). To this day, dengue plagues the country as the most well-known tropical disease and has since remained endemic. As reported by WHO, the Philippines had a total of 101,778 cases and

382 deaths from January 1 to August 12, 2023. The number of reported cases is 25% lower compared to that reported in the same period in 2022 but remains significant.

The Philippine Department of Health (DOH) strives in its current efforts to mitigate the disease with the national dengue prevention and control program. The main components of the program focus on the surveillance and response to viral infection (DOH, 2012). However, despite the continued efforts of healthcare workers and experts, the spread of dengue remains rampant in the country. DOH's prevention and control program struggled to meet its goal of mitigating dengue cases in the country. Significant barriers to the program's success include the lack of empowerment among its stakeholders and the great challenge of eradicating breeding sites (Ong et al., 2022).

Approach in mosquito control has been almost completely based on the use of synthetic insecticides with persistent residual effects (Mittal & Subbarao, 2003). However, while insecticides have been effective solutions to control the spread of mosquito-borne diseases, their excessive use over the last five decades has caused many insect vector species to develop resistance (Rivero et al., 2010). In addition, they also have harmful effects on nontarget insects, the environment, and human health, especially when exposed for long periods of time. Alternative methods for controlling vectors have thus been sought to reduce reliance on synthetic insecticides and minimize their impact on the environment and human health. One of the promising candidates to substitute synthetic larvicides are bacterial toxins which offer targeted action against the insect pest without harming beneficial organisms thereby reducing the risk of unintended effects on the ecosystem. This makes it a more sustainable vector control practice with reduced environmental impact on agriculture.

For the last 30 years, several species of *Bacillus* particularly *B. sphaericus* and *B. thuringiensis* serovar *israelensis* have been successfully used for controlling insect vectors (Lacey, 2007). In the Philippines, a local strain of *B. thuringiensis* subsp. *morrisoni* (designated as isolated PG-14) was found highly and selectively toxic against *Ae. aegypti* and *Culex molestus* (Padua et al., 1984). However, development of resistance to individual bacterial toxins derived from these species has been reported especially in *B. sphaericus* which produces only a single site action toxin (Wirth, 2013). Development of other bacterial larvicides with varying and multiple modes of action is therefore necessary. Screening other local bacterial isolates is also important as they could serve as potential biolarvicides to replace exotic bacterial isolates that could have unintended effects on the environment where they are introduced. Here, we evaluated local isolates of *B. subtilis* and *B. amyloliquefaciens* for their larvicidal activity against the third-instar larvae of *Ae. aegypti* to determine whether they can be used as biolarvicides against these vector mosquitos. We also screened the local isolates for their capability to produce biosurfactants since certain strains of these species were known to produce lipopeptide biosurfactants that have been recently exploited as larvicides, pupacides, and adulticides against mosquito vectors (Geetha et al., 2011, 2012, 2014; Geetha & Manonmani, 2008, 2010).

MATERIALS AND METHODS

Bacterial Cultures

Pure cultures of *Bacillus amyloliquefaciens* (Accession number: 1130) and *B. subtilis* (Accession number: 1679) were obtained from the Philippine National Collection of Microorganisms (PNCM), National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, Laguna, Philippines. Stock cultures were prepared by inoculation into nutrient agar (NA) slants and stored at 4°C before use. Gram

staining was also performed to ensure the purity of cultures prior to larvicidal testing.

Mosquito Larvae

Aedes aegypti larvae were obtained from the insect rearing facility of the Institute of Biological Control, De La Salle University, Biñan, Laguna, Philippines. Commercially available fish flakes were used as feeding substrate to support larval development.

Larvicidal Activity Evaluation

WHO (2005) guidelines on laboratory testing of mosquito larvicides, as modified by Katak et al. (2021) and Soares-da-Silva et al. (2017), were used for the evaluation of larvicidal activity. Pure cultures of *B. amyloliquefaciens* and *B. subtilis* were inoculated into a 125-mL sterile nutrient broth placed in 250-mL Erlenmeyer flasks. Inoculated broth cultures were incubated for 48 hr at 30°C with shaking at 150 rpm. After incubation, the optical density (OD_{600}) of the broth cultures was adjusted to 0.8 as determined using a NanoDrop One UV-VIS spectrophotometer (ThermoFisher Scientific, Massachusetts, USA). This corresponds to the absorbance of a McFarland standard no. 8 (prepared by mixing 0.8 mL of 1% $BaCl_2$ and 9.2 mL of 1% H_2SO_4) which is about 0.8 when measured using the same spectrophotometer. The dilution allows the standardization of the number of bacterial cells present in the initial inoculum (i.e., approximately 2.4×10^9 bacterial cells per milliliter).

For each isolate, five cups were prepared with each cup containing 45-mL distilled water, 5-mL bacterial culture, and 10 third-instar *Ae. aegypti* larvae. Dead and/or moribund larvae were counted 24, 48, and 72 hr after exposure. No mortality was observed at any time in negative controls without bacteria (i.e., distilled water and uninoculated broth). Permethrin (Pervade 10EC®) at recommended rate was used as positive control. The experiment was repeated three times at different days.

Biosurfactant Screening Methods

The bacterial cultures were screened for biosurfactant production using two different assays. First, cultures were screened for their ability to produce biosurfactants that can lyse red blood cells using the blood agar lysis method. This is confirmed using the drop collapse assay which can detect moderate to high production of biosurfactants. Broth cultures were prepared as previously mentioned.

Blood Agar Lysis Assay

Blood agar plates were prepared by mixing defibrinated sheep blood (5% v/v) with nutrient agar. Bacterial cultures were then streaked on the surface of the plates. Inoculated plates were incubated at 37°C. After 24 hr, the plates were inspected for the presence of clear zones which indicate lysis of blood cells.

Drop Collapse Assay

The method used was adapted from Bodour and Miller-Maier (1998). Two microliters of light mineral oil were placed on the surface of a 96-well plate lid. After equilibration for 1 hr, 5 µL of the broth culture was pipetted onto the surface of the oil. The shape of the drop is inspected using a dissecting microscope after 1 min. Biosurfactant production is indicated by the spreading of the drop. A negative control using uninoculated broth was also included as reference.

RESULTS AND DISCUSSION

We present here the evaluation of *Bacillus amyloliquefaciens* and *B. subtilis* isolates as biolarvicides against the larvae of the mosquito vector *Ae. aegypti*. Isolates were obtained from a culture collection. Gram reaction of the bacterial isolates was determined through Gram staining and used to check the purity of the isolates. Gram staining (Figure 1) revealed the presence of Gram-positive rods which are characteristic of *Bacillus* species and that the cultures were pure.

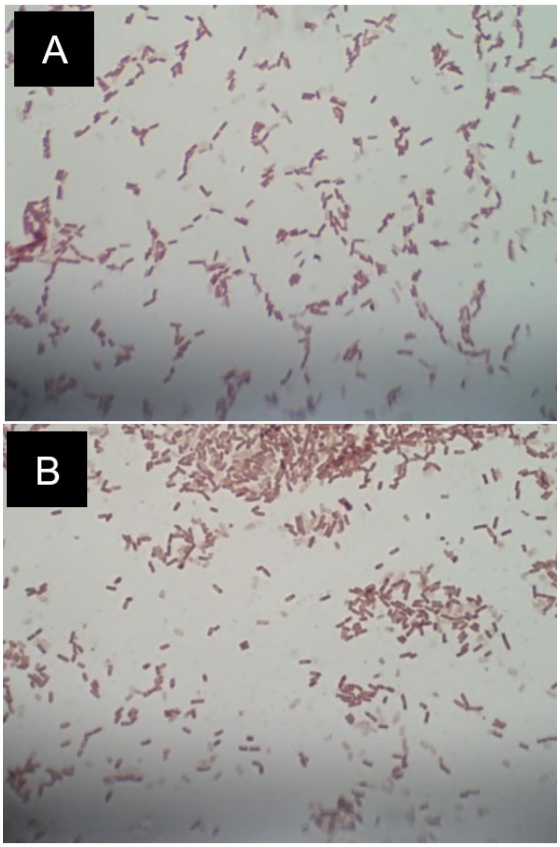

Figure 1. Gram staining of (a) *Bacillus amyloliquefaciens* and (b) *B. subtilis* isolates showed Gram-positive rods.

Using live broth cultures, the cumulative larval mortalities after 24–72 hr were observed and presented in Table 1. Larval mortalities were significant among different treatments and across different time points (two-way repeated measures analysis of variance [RM ANOVA]: treatment effect, $F(4, 10) = 3812$, $p < 0.0001$; time effect, $F(1.779, 17.79) = 340.4$, $p < 0.0001$; interaction, $F(8, 20) = 128.2$, $p < 0.0001$). Further, post hoc comparisons using Tukey's test indicated that larval mortalities due to both *Bacillus* spp. were significantly different to those observed in the negative controls (uninoculated broth and water only) but still significantly lower compared to the synthetic pyrethroid insecticide permethrin.

Increasing larval mortality was also observed for both *Bacillus* spp., which may be attributed to an increase in bacterial population through time. Since the inoculum is a broth culture, this could still contain nutrients enough to support the growth and multiplication of the bacterial cells. The observed larvicidal activities were comparable to the activities of some *Bacillus* strains isolated from soil and water samples in Amazonian microenvironments (Katak et al., 2021). They reported 20 *Bacillus* strains with larvicidal activities against *Ae. aegypti* in which one strain of *B. subtilis* was able to kill 27% and 57% of the larvae after 24 hr and 48 hr, respectively, at an initial concentration of about 2.4×10^9 cells per milliliter.

The increase in mortality may also be due to an increase in larvicidal compound production during bacterial growth. Since certain strains of *B. amyloliquefaciens* (Geetha et al., 2011, 2014) and *B. subtilis* (Geetha et al., 2012; Geetha & Manonmani, 2008) have been reported to produce larvicidal biosurfactants against *Ae. aegypti,* we performed two assays to confirm whether the cultures produce biosurfactants: a preliminary screening assay using blood agar lysis and a confirmatory oil drop collapse assay. The results of the two tests are presented in Figure 2. Both *B. amyloliquefaciens* and *B. subtilis* showed zones of lysis when streaked on blood agar

Table 1. Larvicidal Activity of Local Isolates of *Bacillus amyloliquefaciens* and *B. subtilis* Against Third-Instar *Aedes aegypti* Expressed as Cumulative Mortality After 24, 48, and 72 hr.

Treatment	Cumulative Larval Mortality* (%, Average ± SD)		
	24 hr	48 hr	72 hr
Bacillus amyloliquefaciens	22 ± 4[b]	40 ± 2[c]	61 ± 4[b]
Bacillus subtilis	19 ± 3[b]	56 ± 2[b]	72 ± 2[b]
Permethrin	100 ± 0[a]	100 ± 0[a]	100 ± 0[a]
Uninoculated broth	0 ± 0[c]	0 ± 0[d]	0 ± 0[c]
Distilled water	0 ± 0[c]	0 ± 0[d]	0 ± 0[c]

*Means followed by the same letter within a column are not significantly different after Tukey's test ($p > .05$); average of mortality from three biological replicates.

plates. This method is based on the ability of surfactants to lyse erythrocytes or red blood cells. The biosurfactant surfactin, produced by *B. subtilis*, was the first to be described to have hemolytic activity (Bernheimer & Avigad, 1970). Since then, the assay has been used to screen other bacterial species for biosurfactant production. However, the assay was reported to produce both false positive and false negative results, so it can only be used as a preliminary test, and other screening methods must be performed to confirm biosurfactant production (Youssef et al., 2004). The second screening method is the drop collapse assay, which can be used to screen bacteria that produce medium to high concentrations of biosurfactants (above 60 mg/L; Youssef et al., 2004). The method is based on the ability of surfactants to lower the surface tension of water resulting in the "collapse" of water droplets when placed on an oiled surface. Results showed the collapse of drops of cultures of *B. amyloliquefaciens* and *B. subtilis* indicating the formation of biosurfactants.

Reports in the literature indicate that certain strains of *B. amyloliquefaciens* and *B. subtilis* produce mosquitocidal toxins that are antagonistic to mosquito vectors. In 2010, this toxin was identified as surfactin, a novel lipopeptide biosurfactant produced by *B. subtilis* ssp. *subtilis* (VCRC B471; Geetha & Manonmani, 2010). This biosurfactant was found effective against the pupae of *Anopheles stephensi*, stable to varied environmental conditions, and biodegradable. Another strain of *B. subtilis* was found lethal against *Ae. aegypti* larvae, and the extracted metabolites significantly reduced the activities of important enzymes (Revathi et al., 2013). In 2011, the first *B. amyloliquefaciens* isolate that exhibited mosquitocidal activity was reported and the active metabolite was identified as a biosurfactant (Geetha et al., 2011). The mode of action of biosurfactants is generally believed to involve interactions with the hydrophobic layers in the respiratory organs. *Aedes aegypti* larvae have specialized respiratory siphons with hydrophobic substances which prevent the entry of water (Christophers, 1960). Biosurfactants can dissolve these substances, which results in the entry of water instead of air and prevents the larvae from staying afloat and receiving oxygen.

The potential of these biosurfactants has been noted since in addition to their stability, they also pose minimal threat to nontarget organisms. For instance, crude lipopeptide extracts from *Bacillus* spp. that were effective in eradicating *Ae. aegypti* had low toxicity to nontargets like *Caenorhabditis elegans, Galleria mellonella,*

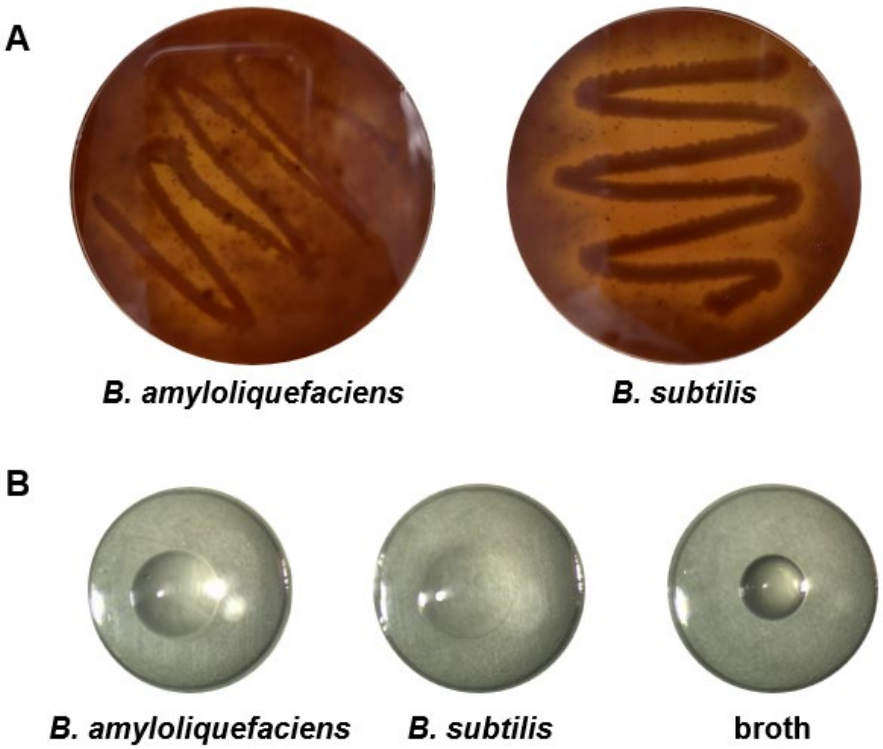

Figure 2. Biosurfactant screening assays reveal production of biosurfactants by local isolates of *Bacillus amyloliquefaciens* and *B. subtilis*: (A) both isolates show zones of lysis on blood agar plates and (B) drop collapse assay display spreading of the droplet (in comparison to the control using uninoculated broth) indicating presence of biosurfactants.

Scenedesmus obliquus, and *Tetrahymena pyriformis* (Falqueto et al., 2021). The results of the screening assays support that the two *Bacillus* species can produce biosurfactants which may account for the observed larvicidal activity; however, since live bacterial cultures were used, the observed larvicidal activities cannot be attributed only to biosurfactant production. In addition, larvicidal activity of the pure biosurfactants must be validated.

CONCLUSION

Through larvicidal bioassays, local isolates of *Bacillus amyloliquefaciens* and *B. subtilis* have been found effective against third-instar larvae of *Aedes aegypti*, the primary vector for the dengue virus. The bacterial isolates showed a slower killing time compared to the synthetic insecticide permethrin which instantly caused mortality to all larvae. An increasing mortality was also observed indicating a possible increase in the production of larvicidal compounds with the increase in bacterial population. Screening assays confirmed the ability of the two isolates to produce biosurfactants which may be attributed to the observed larvicidal activity. The results show the potential of these isolates as alternative biolarvicides to synthetic insecticides and exotic bacterial isolates and as potential sources of larvicidal compounds against vector mosquitoes. Further research is underway to confirm the exact nature and identity of the larvicidal compound/s produced by these bacterial isolates. Field efficacy tests are also necessary to determine their effectiveness and assess their suitability as components of an integrated vector management program.

ACKNOWLEDGEMENT

This study received funding from the Department of Science and Technology (DOST) under one of the projects of the Center for Vector of Diseases of Public Health Importance in the Philippines for Region IV (CVDPhil), a Niche Center in the Regions for R&D (NICER) established under the Science for Change Program (S4CP) of DOST.

REFERENCES

Bernheimer, A. W., & Avigad, L. S. (1970). Nature and properties of a cytolytic agent produced by *Bacillus subtilis*. *Microbiology, 61*(3), 361–369. https://doi.org/10.1099/00221287-61-3-361

Bodour, A. A., & Miller-Maier, R. M. (1998). Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorganisms. *Journal of Microbiological Methods, 32*(1), 273–280. https://doi.org/10.1016/S0167-7012(98)00031-1

Christophers, S. R. (1960). Aedes aegypti *(L): The yellow fever mosquito*. London, UK: Cambridge University Press.

Department of Health. (2012). *Dengue prevention and control program | Department of Health website*. Retrieved October 30, 2023, from https://doh.gov.ph/national-dengue-prevention-and-control-program

Falqueto, S. A., Pitaluga, B. F., de Sousa, J. R., Targanski, S. K., Campos, M. G., de Oliveira Mendes, T. A., Soares, M. A. (2021). *Bacillus* spp. metabolites are effective in eradicating *Aedes aegypti* (Diptera: Culicidae) larvae with low toxicity to non-target species. *Journal of Invertebrate Pathology*, *179*, 107525. https://doi.org/10.1016/j.jip.2020.107525

Geetha, I, Aruna, R., & Manonmani, A. M. (2014). Mosquitocidal *Bacillus amyloliquefaciens*: Dynamics of growth & production of novel pupicidal biosurfactant. *Indian Journal of Medical Research, 140*(3), 427–434.

Geetha, I., & Manonmani, A. M. (2008). Mosquito pupicidal toxin production by *Bacillus subtilis* subsp. Subtilis. *Biological Control*, *44*(2), 242–247.https://doi.org/10.1016/j.biocontrol.2007.10.007

Geetha, I., & Manonmani, A. M. (2010). Surfactin: A novel mosquitocidal biosurfactant produced by *Bacillus subtilis* ssp. subtilis (VCRC B471) and influence of abiotic factors on its pupicidal efficacy: Abiotic factors and mosquitocidal efficacy of surfactin. *Letters in Applied Microbiology*, *51*(4), 406–412. https://doi.org/10.1111/j.1472-765X.2010.02912.x

Geetha, I., Manonmani, A. M., & Prabakaran, G. (2011). *Bacillus amyloliquefaciens*: A mosquitocidal bacterium from mangrove forests of Andaman & Nicobar Islands, India. *Acta Tropica*, *120*(3), 155–159. https://doi.org/10.1016/j.actatropica.2011.07.006

Geetha, I., Paily, K. P., & Manonmani, A. M. (2012). Mosquito adulticidal activity of a biosurfactant produced by *Bacillus subtilis* subsp. *Subtilis*. *Pest Management Science*, *68*(11), 1447–1450. https://doi.org/10.1002/ps.3324

Katak, R. M., Rocha, E. M., Oliveira, J. C., Muniz, V. A., Oliveira, M. R., Ferreira, F. A. S., Tadei, W. P. (2021). Larvicidal activities against *Aedes aegypti* of supernatant and pellet fractions from cultured *Bacillus* spp. isolated from Amazonian microenvironments. *Tropical Medicine and Infectious Disease*, *6*(2), 104. https://doi.org/10.3390/tropicalmed6020104

Lacey, L. A. (2007). *Bacillus thuringiensis* serovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *Journal of the American Mosquito Control Association*, *23*(sp2), 133–163. https://doi.org/10.2987/8756-971X(2007)23[133:BTSIAB]2.0.CO;2

Mittal, P., & Subbarao, S. K. (2003). Prospect of using herbal product in the control of mosquito vectors. *ICMR Bulletin*, *33*(1), 1–10.

Ong, E. P., Obeles, A. J. T., Ong, B. A. G., & Tantengco, O. A. G. (2022). Perspectives and lessons from the Philippines' decades-long battle with dengue. *The Lancet Regional Health. Western Pacific*, *24*, 100505. https://doi.org/10.1016/j.lanwpc.2022.100505

Padua, L. E., Ohba, M., & Aizawa, K. (1984). Isolation of a *Bacillus thuringiensis* strain

(serotype 8a:8b) highly and selectively toxic against mosquito larvae. *Journal of Invertebrate Pathology*, *44*(1), 12–17. https://doi.org/10.1016/0022-2011(84)90040-5

Revathi, K., Chandrasekaran, R., Thanigaivel, A., Kirubakaran, S. A., Sathish-Narayanan, S., & Senthil-Nathan, S. (2013). Effects of *Bacillus subtilis* metabolites on larval *Aedes aegypti* L. *Pesticide Biochemistry and Physiology*, *107*(3), 369–376. https://doi.org/10.1016/j.pestbp.2013.10.005

Rivero, A., Vézilier, J., Weill, M., Read, A. F., & Gandon, S. (2010). Insecticide control of vector-borne diseases: When is insecticide resistance a problem? *PLOS Pathogens*, *6*(8), e1001000. https://doi.org/10.1371/journal.ppat.1001000

Simmons, C. P., Farrar, J. J., van Vinh Chau, N., & Wills, B. (2012). Dengue. *New England Journal of Medicine*, *366*(15), 1423–1432. https://doi.org/10.1056/NEJMra1110265

Soares-da-Silva, J., Queirós, S. G., de Aguiar, J. S., Viana, J. L., Neta, M. D. R. A. V., da Silva, M. C.,…Tadei, W. P. (2017). Molecular characterization of the gene profile of *Bacillus thuringiensis* Berliner isolated from Brazilian ecosystems and showing pathogenic activity against mosquito larvae of medical importance. *Acta Tropica*, *176*, 197–205. https://doi.org/10.1016/j.actatropica.2017.08.006

Wirth, M. C. (2013). Mosquito resistance to bacterial larvicidal toxins. *The Open Toxinology Journal*, *3*(1), 126–140. https://doi.org/10.2174/1875414701003010126

World Health Organization. (2005). *Guidelines for laboratory and field testing of mosquito larvicides* (No. WHO/CDS/WHOPES/GCDPP/2005.13). World Health Organization. https://apps.who.int/iris/handle/10665/69101

World Health Organization. (2020). *Vector-borne diseases*. https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases

Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R. M., & McInerney, M. J. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal of Microbiological Methods*, *56*(3), 339–347. https://doi.org/10.1016/j.mimet.2003.11.001