AEM Accepted Manuscript Posted Online 27 March 2020
Appl. Environ. Microbiol. doi:10.1128/AEM.00240-20

A non-live preparation of *Chromobacterium* sp. Panama (*Csp_P*) is a highly effective larval mosquito biopesticide

Running title: Non-live *Csp_P* biopesticide

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Key words: Mosquito control, insecticide, biopesticide, Chromobacterium *Csp_P*, *Aedes aegypti*, *Anopheles gambiae*, *Culex quinquefasciatus*, Semi-field trial

Abstract

Given the continued high prevalence of mosquito-transmitted diseases there is a clear need to develop novel disease and vector control strategies. Biopesticides of microbial origin represent a promising source of new approaches to target disease transmitting mosquito populations. Here we describe the development and characterization of a novel mosquito biopesticide, derived from an air-dried, non-live preparation of the bacterium *Chromobacterium* sp. Panama (Family: *Neisseriaceae*). This preparation rapidly and effectively kills the larvae of prominent mosquito vectors, including the dengue and Zika vector *Aedes aegypti*, and the human malaria vector *Anopheles gambiae*. During semi-field trials in Puerto Rico, we observed high efficacy of the biopesticide against field-derived *Ae. aegypti* populations, and against *Ae. aegypti* and *Culex spp.* larvae in natural breeding water, indicating the suitability of the biopesticide for use under more natural conditions. In addition to high efficacy, the non-live *Csp_P* biopesticide has a low effective dose, a long shelf life, high heat stability, and can be incorporated into attractive larval baits, all of which are desirable characteristics for a biopesticide.

Importance

We have developed a novel preparation to kill mosquitoes from an abundant soil bacterium, *Chromobacterium* sp. Panama. This preparation is an air-dried powder containing no live bacteria, which can be incorporated into an attractive bait and fed directly to mosquito larvae. We demonstrate that the preparation has broad spectrum activity against the larval form of the mosquitoes responsible for the transmission of malaria and the dengue, chikungunya, Yellow Fever, West Nile and Zika viruses, as well as mosquito larvae that are already resistant to commonly used mosquitocidal chemicals. Our preparation possesses many favourable traits: it kills at a low dosage, and does not lose activity when exposed to high

temperatures, all of which suggest it could eventually become an effective new tool for controlling mosquitoes and the diseases they spread.

Introduction

Parasites, viruses and filarial worms that can be transmitted from person to person when mosquitoes feed on human blood represent a clear and present threat to human health around the world. The burden of malaria, a febrile illness caused by *Plasmodium* parasites that are transmitted by mosquitoes from the genus *Anopheles*, is particularly high. Unfortunately, even though unprecedented disease control efforts over the last 15 years have reduced the burden of disease (1), the human cost still remains high. Recent data suggests that there were an estimated 219 million cases of malaria, and approximately 435,000 deaths in 2017, many of which were young children (2).

Mosquito-transmitted arboviral diseases also have a major impact on human health, with key viruses such as dengue virus, transmitted by mosquitoes from the genera *Aedes* and *Culex*, responsible for millions of infections each year (3-6). Critically, the incidence of arboviral disease has risen greatly over the last 20 years (7), as changes in climate, mosquito distribution and human behaviour have brought humans and mosquitoes into contact more frequently (8-10). These factors have also helped to promote the emergence of novel mosquito-transmitted viruses such as chikungunya and Zika (11), which have caused hundreds of thousands of infections during major outbreaks (4, 6).

Unfortunately, there are no effective, commercially available vaccines for most mosquito-transmitted diseases, and crucially, malaria parasites are rapidly developing resistance to drugs (12), while drugs for arboviruses do not currently exist. For this reason, mosquito control has long been the most common strategy employed to limit disease transmission, and historically the most common approach has been to utilize different chemical insecticides to

rapidly, and effectively kill mosquitoes (13). These insecticides can be used to target both larval and adult mosquito stages, and can be used synergistically with mosquito bite prevention strategies (14). Many commonly used insecticides, including pyrethroids and organophosphates, kill by targeting the mosquito central nervous system (15-17). Others, including chitin synthesis inhibitors and juvenile hormone analogues like methoprene, act to prevent development beyond larval stages (17, 18). Concerns about the environmental impacts of chemical insecticides have sharpened focus on the development of environment-friendly mosquitocidals (19). There are also a variety biologically-derived insecticides, or biopesticides, used in mosquito control. These include the bacteria *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and *Lysinibacillus sphaericus*, which produce highly durable spores that form a crystal protein which shreds the mosquito gut after ingestion (20, 21). There are also entomopathogenic fungi, such as *Metarhizium anisopliae*, which can target and kill specific mosquito species (22).

Regular exposure to chemical insecticides has led to genetic resistance becoming increasingly prevalent in mosquito populations (23, 24), complicating mosquito control efforts (25). The implication of insecticide resistance is that no single insecticide will offer perfect, long-term control of any mosquito population. Instead, effective, long-term control will likely come through multi-faceted strategies that exploit synergies between different insecticides, thereby providing a greater chance of limiting or overcoming potential mechanisms of resistance (26). Consequently, novel mosquitocidal chemicals, and biopesticides must continue to be developed, as they will provide new options to improve or supplement existing mosquito control programmes.

Chromobacterium species Panama (*Csp_P)* (Class: *Betaproteobacteria*, Family: *Neisseriaceae*) is a soil bacterium first isolated from *Ae. aegypti* midguts (27), which has a number of unique and useful properties for controlling mosquito-transmitted disease and mosquito populations. When *Anopheles gambiae* mosquitoes are fed on low doses of *Csp_P* they display reduced susceptibility to infection with the human malarial parasite *Plasmodium falciparum* (27), as *Csp_P* produces a depsipeptide called romidepsin, which kills the parasite (28). Similarly, *Csp_P* infection in *Ae. aegypti* reduces susceptibility to dengue virus (27), through production of an aminopeptidase that promotes degradation of the viral envelope protein (29). Critically, higher doses of *Csp_P* have potent adulticidal activity against many mosquito species when provided in sucrose, and are also a very effective larvicide of *Ae. aegypti* and *An. gambiae* (27, 30).

Given these properties, *Csp_P* has great potential as an insecticide. However, to overcome potential regulatory, ecological, and epidemiological concerns about using an insecticide containing live bacteria, we sought to develop a *Csp_P* preparation that contained no live bacteria, which was easily prepared, and had a long shelf life, while retaining insecticidal activity against a range of important mosquito vectors in the laboratory and the field.

Results

Pellet design and attractants

In order to facilitate development of a non-live insecticide based on the bacterium *Chromobacterium* species Panama *Csp_P*, we first developed an attractive larvicidal bait, in the form of a pellet that could be used to deliver *Csp_P* to mosquito larvae. This bait needed to induce feeding amongst larvae, and also maintain structural integrity within an aqueous environment so that mosquitoes could feed on it.

In a series of preliminary experiments (Supplementary File 1), we assessed the attractiveness of fishmeal (Dirty Gardener) and ground tropical fish flakes (Tetramin) when provided to *Ae. aegypti* larvae in pellets made with 20% gelatin as a stabilizing agent. In experiments testing the attractiveness of individual baits, we observed that 97.5% of larvae responded to fishmeal pellets within 30 mins of exposure. In contrast, only 71.7% of larvae responded to pellets containing tropical flakes, over the same time period. When both fishmeal- and tropical flakes-containing baits were offered to larvae over 30 mins in bait choice assays, an average of 80% responded to fishmeal-containing baits, while 13.33% chose tropical flakes-containing baits, and 6.67% did not respond. Consequently, we decided to use fishmeal baits in all subsequent assays.

We then incorporated live *Csp_P* into pellets and fed it to *Ae. aegypti* Rockefeller (ROCK) larvae in order to examine the efficacy of pellets as a larvicide delivery tool. Pellets containing live *Csp_P* killed all larvae in a 300mL container within 6 days, with an average time to death post-exposure of 2.09 (± 0.05) days, and an expected hazard ratio of 83.99 in comparison to

pellets without bacteria (Cox Regression: W = 180.64, df = 1, $Exp\ \beta$ = 83.99, P < 0.0001) (Fig. S1).

Development and assessment of non-live Csp_P preparations

We developed five different culturing methods (non-live_1 through non-live_5) to produce large quantities of non-live, air-dried, powdered *Csp_P* (see Experimental Procedures section for details on the culturing processes). Immediately after air-drying, each of the non-live powders was mixed with 100µL of 1X PBS, and 3 different dilutions of these mixtures were inoculated onto LB agar, and incubated at 30°C for two days. Across three experimental replicates, we did not observe any bacterial colonies for any of the 5 preparations at any dilution.

We compared the larvicidal activity of these preparations across three replicate experiments wherein 100mg of each preparation was incorporated into a gelatin/fishmeal pellet, and then offered to *Ae. aegypti* ROCK larvae. This dose was utilized in all subsequent assays, unless specified. We again observed that all 5 powders killed larvae, however they were not equally effective (Table 1). Based on the results of these experiments we selected the non-live_1 preparation (Fig. 1) for further testing as it produced the shortest average time to death for exposed larvae (2.80 ± 0.10 days), and also produced the highest hazard ratio (Cox Regression: W = 145.83, df = 1, $Exp\ \beta$ = 50.11, P < 0.0001) in comparison to the control treatment. This decision was also based on the consistency of the larvicidal activity that was observed for non-live_1 powder across three replicates, and the high yield of that powder

compared to the other four. In the experiments described below, we refer to the non-live_1 powder as non-live *Csp_P*.

Non-live Csp_P powder does not contain cyanide

We tested each of the five powders for the presence of cyanide using the Cyanide Test Kit, Model CYN-3 (Hach, 2010-02), as cyanide toxicity is considered the likely means through which live *Csp_P* kill larvae when in suspension (30). We observed that the pH of each powder, resuspended in DI water, was between 5-6. At the completion of the test, we did not observe a change in colour for any sample, indicating that there was no evidence of the presence of cyanide species in any of the non-live *Csp_P* powders.

Non-live Csp_P powder has a larvicidal effect against different mosquito species

We examined the larvicidal efficacy of non-live *Csp_P* powder against *Ae. aegypti* Rockefeller (Fig. 2A), pyrethroid-resistant *Ae. aegypti* (Fig. 2B), *An. gambiae* Keele (Fig. 2C), and *Culex quinquefasciatus* (Fig. 2D). In each of these assays, gelatin/fishmeal pellets containing 100mg of non-live *Csp_P* powder were fed to L_2 larvae, and mortality rates compared against groups of larvae fed pellets containing either live *Csp_P* or no-bacteria controls. Rockefeller larvae challenged with live bacteria had an average time to death of 2.48 ± 0.05 days (Cox Regression: $W = 275.42$, $df = 1$, $Exp\ \beta = 67.08$, $P < 0.0001$), while those challenged with non-live *Csp_P* powder had an average time to death of 3.13 ± 0.08 days (Cox Regression: $W = 233.35$, $df = 1$, $Exp\ \beta = 42.68$, $P < 0.0001$). Pyrethroid-resistant

187 *Ae. aegypti* challenged with live *Csp_P* lived 2.52 ± 0.05 days on average post-exposure
188 (Cox Regression: W = 202.04, df = 1, $Exp\ \beta$ = 295.64, P < 0.0001), while larvae from the
189 same line fed on non-live *Csp_P* lived 3.40 ± 0.13 days on average (Cox Regression: W =
190 177.81, df = 1, $Exp\ \beta$ = 183.94, P < 0.0001). *An. gambiae* larvae fed live *Csp_P* had an
191 average time to death of 1.10 ± 0.02 days (Cox Regression: W = 162.63, df = 1, $Exp\ \beta$ =
192 272.37, P < 0.0001), while those challenged with non-live *Csp_P* survived for 2.25 ± 0.09
193 days post-treatment, on average (Cox Regression: W = 122.00, df = 1, $Exp\ \beta$ = 113.30, P <
194 0.0001). Finally, *Cx. quinquefasciatus* larvae challenged with live *Csp_P* lived 1.40 ± 0.03
195 (Cox Regression: W = 199.99, df = 1, $Exp\ \beta$ = 63.02, P < 0.0001) days on average,
196 compared to 1.68 ± 0.05 days on average for those challenged with non-live *Csp_P* (Cox
197 Regression: W = 187.76, df = 1, $Exp\ \beta$ = 51.17, P < 0.0001). During the course of these
198 experiments, no larvae of any species that were exposed to the *Csp_P* biopesticide pupated.
199 In contrast, we observed that pupation amongst larvae fed on control bait occurred from days
200 3 through 7 after exposure to the bait.
201
202 *Larvicidal dose-response of non-live Csp_P powder*
203
204 To assess the efficacy of non-live *Csp_P* at different doses, we performed three experiments
205 where we provided larvae with gelatin/fishmeal pellets containing 100mg, 50mg, 25mg,
206 12.5mg or 6.25mg of non-live *Csp_P* powder (Fig. 3A). We observed that larvae treated with
207 all five doses had significantly greater mortality than the control treatment (Cox Regression: P
208 < 0.0001 for all comparisons). One hundred percent mortality was observed with the 100mg
209 treatment, while greater than 99% mortality was observed in the 50mg treatment at 12 days
210 post-exposure. No pupation was observed in either of these conditions, while control larvae
211 pupated 4-6 days after the start of the experiment. A small number of adults were observed

in the 12.5mg and 6.25mg treatments, with an average mortality of 70.34% and 56.36% observed in those treatments, respectively, at 12 days post-treatment. Based on the results of these experiments, we calculated the LD_{50} of non-live *Csp_P* in our experimental setup (30 larvae in 300mL of water) as 3.40mg of powder. This equated to an LD_{50} of 11.35mg of non-live *Csp_P* powder per litre of water.

Non-live Csp_P powder is highly heat stable

In order to assess the potential shelf life of non-live *Csp_P* powder, we performed accelerated shelf life tests, where we treated the powder at 30°C, 37°C, 54°C (Fig. 3B), or 70°C (Fig. 3C) for two weeks, and then assessed the impact on larvicidal activity by comparing these treatments against powder left at room temperature for two weeks, with powder from each treatment independently incorporated into gelatin/fishmeal pellets. We observed that the mortality of all of non-live *Csp_P* treatments was significantly greater than that of the control treatment (Cox Regression: room temperature - $W = 178.65$, $df = 1$, $Exp\ \beta = 140.13$, $P < 0.0001$; 30°C - $W = 170.88$, $df = 1$, $Exp\ \beta = 126.57$, $P < 0.0001$; 37°C - $W = 185.77$, $df = 1$, $Exp\ \beta = 152.63$, $P < 0.0001$; 54°C - $W = 189.91$, $df = 1$, $Exp\ \beta = 164.63$, $P < 0.0001$). Critically, we observed no difference in activity between the heat-treated powders and the powder left at room temperature (Cox Regression: $P > 0.05$).

Experiments with non-live *Csp_P* powder treated at 70°C were run independently due to incubator availability. In these experiments, we observed that both the room temperature and 70°C treatments had significantly greater mortality than the control treatment (Cox Regression: Room Temperature - $W = 285.91$, $df = 1$, $Exp\ \beta = 44.74$, $P < 0.0001$; 70°C - $W =$

254.31, *df* = 1, *Exp β* = 32.41, *P* < 0.0001). We also observed a slight loss of larvicidal activity with the 70°C treatment, although 99.2% of the larvae that were exposed died within 12 days. The average mortality for the 70°C treatment was 4.03 ± 0.14 days, compared to 3.48 ± 0.09 for the room temperature treatment (Cox Regression: *W* = 12.83, *df* = 1, *Exp β* = 0.72, *P* < 0.0001).

While conducting the above experiments, we noticed that our gelatin-based pellets dissolved within 2-3 days after being added to water. Consequently, we developed an agar-based pellet formulation that maintained structural integrity when left submerged in water for 14 days (Fig. S2A). Interestingly, when *Ae. aegypti* larvae were added to containers where the water had been pre-exposed to non-live *Csp_P* agar pellets for 14 days, we observed significantly higher mortality than for larvae treated with freshly-made non-live *Csp_P* agar pellets (Cox Regression: *W* = 31.031, *df* = 1, *Exp β* = 2.07, *P* < 0.0001). However, there was no significant effect on mortality due to pre-exposure for non-bacterial control pellets (Cox Regression: *W* = 0.59, *df* = 1, *Exp β* = 0.67, *P* = 0.442). We then exposed control and non-live *Csp_P* agar pellets to different temperatures (room temperature, 37°C or 54°C) for 7 days in order to see if temperature treatment had an impact on the larvicidal activity of agar pellets (Fig. S2B). We observed no significant influence of temperature on the larvicidal activity of control pellets (Cox Regression: *W* = 0.15, *df* = 2, *P* = 0.929) or non-live *Csp_P* pellets (Cox Regression: *W* = 2.80, *df* = 2, *P* = 0.246).

Effect with other bacteria

We performed the same non-live *Csp_P* culturing and drying procedure described above, with three other mosquito-associated bacteria: *Acinetobacter baylyi*, *Serratia marcescens*, and *Pseudomonas rhodesiae*, in order to demonstrate that the larvicidal effect we observed after feeding on non-live *Csp_P* powder was not due to the culturing and/or drying processes, and was not ubiquitous across all bacteria fed to mosquito larvae in this manner (Fig. 3D). Across three experiments, we observed that only non-live *Csp_P* powder had significantly different mortality to the no bacteria control treatment (Cox Regression: $W = 180.34$, $df = 1$, $Exp\ \beta = 75.20$, $P < 0.0001$). Hazard ratios associated with feeding powders derived from *A. baylyi*, *S. marcescens*, and *P. rhodesiae* cultures were 1.46, 1.56, and 1.51, respectively, compared to the control treatment, indicating that there was no significant larvicidal effect associated with powders derived from these three bacteria.

Non-live Csp_P powder exert larvicidal activity under field conditions

We assessed the efficacy of the non-live *Csp_P* powder in a semi-field setting. These experiments were conducted at a semi-field facility in Gurabo, Puerto Rico. In the first of three trials, we tested the non-live *Csp_P* powder against larvae from the CDC San Juan *Ae. aegypti* colony (Patillas strain) (Fig. 4A). Larvae were moved to small cups containing 300mL of tap water, treated with gelatin/fishmeal pellets containing 200mg of non-live *Csp_P* powder, and the cups left in the semi-field cage, exposed to ambient environmental conditions. We observed that mortality was significantly increased in the treated cups compared to the control cups (Cox Regression: $W = 207.85$, $df = 1$, $Exp\ \beta = 87.59$, $P < 0.0001$), with 100% mortality within 6 days, compared to 2.93% mortality in the control treatment cups over that time. Average time to death for insecticide-treated larvae was 3.03 ±

286 0.05 days. We then performed similar experiments with mosquitoes from the CDC *Aedes*
287 *mediovitattus* colony (Fig. 4B), and observed that all larvae died within four days of treatment,
288 with an average time to death of 2.97 ± 0.08 days (Cox Regression: $W = 64.39$, $df = 1$, Exp β
289 $= 20.64$, $P < 0.0001$).
290
291
292 Next, we collected *Ae. aegypti* eggs using oviposition cups deployed at eleven different sites
293 across Puerto Rico. These egg papers were returned to the laboratory, and hatched in tap
294 water. G_1 larvae from these populations were transferred to the semi-field cage. The larvae
295 from each population were split into two cups, half were fed a control pellet, and half were fed
296 a non-live *Csp_P* pellet. Survival was monitored in these cups for 6 days, at which point
297 100% of the insecticide-treated larvae had died (Fig. 5). Over this time period 6/143 (6.29%)
298 of the control larvae had died (Cox Regression: $W = 112.61$, $df = 1$, Exp $\beta = 49.17$, $P <$
299 0.0001). For *Csp_P*-exposed mosquitoes across all 11 populations, there was an average
300 time to death of 2.73 ± 0.08 days.
301
302
303 We then sought to assess the activity of the non-live *Csp_P* powder against field-collected
304 larvae in their natural breeding habitats. We collected larvae and water from 11 different
305 breeding sites across Eastern Puerto Rico. The sites included a variety of plastic containers,
306 pails and tires (Table 2). Larvae from these breeding sites were taxonomically identified, and
307 fell into three categories: (1) *Aedes aegypti* only, (2) *Aedes aegypti* and *Culex*
308 *quinquefasciatus,* and (3) *Culex quinquefasciatus* only. The water and larvae within these
309 breeding sites were transported to the semi-field facility and then divided into two cups, with
310 half fed a control pellet, and half fed a pellet containing non-live *Csp_P*. In these experiments

(Fig. 6), we monitored adult eclosion rather than larval mortality, as the breeding site water was typically too opaque to perform and accurate assessment of larval mortality. Across all breeding sites, there were 374 larvae involved in each of the control and non-live *Csp_P* treatments. For the control treatment, 342 larvae eclosed as adults (91.44%) and 32 died during the course of the experiment. For non-live *Csp_P* treated cups, 9 adult mosquitoes eclosed (2.41%), 5 females and 4 males, and 365 larvae died (Fisher's exact test: Odds Ratio = 433.4, $P < 0.0001$). Cox Regression analysis indicated that treatment with non-live *Csp_P* was a significant factor affecting the likelihood of adult eclosion, with larvae from a non-live *Csp_P* treated cup 125 times less likely to eclose than untreated larvae (Cox Regression: $W = 179.11$, $df = 1$, Exp $\beta = 0.008$, $P < 0.0001$).

Discussion

We have developed a novel insecticide based on a non-live, air-dried preparation of the bacterium *Chromobacterium* species Panama (*Csp_P*). Previous reports from our group have demonstrated that live *Csp_P* is a highly effective mosquitocidal agent that can rapidly kill the larvae and adults of mosquito vectors of medically important pathogens, including *An. gambiae* and *Ae. aegypti* (27, 30). Given the potential environmental and human/animal health concerns associated with an insecticidal preparation based on live bacteria, as well as complications with storage and shelf-life, we sought to develop a formulation containing non-live *Csp_P* that also retained killing activity against mosquito larvae.

Although the development of a potential mosquitocidal formulation involving non-live *Csp_P* is still in the early stages, the biopesticide that we have developed possesses many desirable properties for an insecticide. Our results confirm that no *Csp_P* cells survive the air-drying process used during preparation, indicating that releasing this product into the field would not spread live bacteria. The current formulation is fast-acting, and capable of killing mosquito larvae within an average of 2-3 days post-exposure. Interestingly, we observed that exposure to non-live *Csp_P* halted larval development, which potentially facilitated a greater window of time for larvae to ingest the biopesticide and be killed. This was in line with what we observed in a previous study, where the larvae of *An. gambiae* females that survived treatment with live *Csp_P* experienced a developmental delay (31). We also determined that the powder had an LD_{50} of 11.36mg per litre of larval rearing water, under laboratory conditions. As mass culturing of bacteria can be quite expensive, such a low effective dose is a highly beneficial trait.

Additionally, the powder had broad larvicidal activity against a broad range of mosquito targets, including those of high epidemiological importance. This included the prominent mosquito vectors *An. gambiae*, *Ae. aegypti*, and *Cx. quinquefasciatus*, and also *Ae. mediovitattus*. The *Csp_P* biopesticide was most effective against *Cx. quinquefasciatus* larvae, where we observed a shorter average time-to-death post-exposure than for the other species. Mosquitoes of the *Culex* genus include prominent vectors of West Nile virus, Japanese encephalitis virus, and the nematodes that cause filariasis. All of these pathogens have significant impacts on human health. Non-live *Csp_P* powder also effectively kills larvae from the pyrethroid-resistant *Ae. aegypti* NR-48830 line, which was expected given that this line had not previously been exposed to our insecticide. Given the widespread usage of pyrethroids in mosquito control programmes around the world, there are high levels of pyrethroid resistance amongst mosquito populations (32, 33), and our data suggest that the biopesticide could prove to be a good candidate compound for a novel insecticide to target these resistant populations.

Non-live *Csp_P* powder also appears to be highly temperature stable, with accelerated shelf life assays demonstrating that the larvicidal activity is unaffected by heat treatment at 54°C for two weeks, which is comparable to storage at room temperature for one year. Interestingly, while treatment at 70°C for two weeks did lead to slightly reduced activity, more than 95% of the larvae that were exposed were still killed. Additionally, heat-treatment of whole agar pellets for 7 days had no significant impact on larvicidal activity. All of these results indicate that the as-yet-uncharacterized active ingredient in our insecticide is highly heat stable, and that non-live *Csp_P* powder will likely have a shelf life in excess of one year.

370 Our data demonstrate that non-live *Csp_P* powder is an effective larvicide against laboratory-
371 and field-derived mosquitoes, under semi-field conditions. Experiments were conducted at a
372 semi-field facility in Puerto Rico, mosquito larvae were kept in a contained environment,
373 under a tarpaulin, but otherwise exposed to ambient environmental conditions. We observed
374 that exposure to non-live *Csp_P* powder was highly effective at killing laboratory-reared *Ae.*
375 *aegypti* and *Ae. mediovittatus* larvae that were moved to the field site at 3 days post-
376 hatching. We saw similar efficacy when the biopesticide was trialled against G_1 larvae from
377 11 different field-derived *Ae. aegypti* populations that were collected from around Puerto
378 Rico, where all larvae died within 4 days of exposure. Critically, the *Csp_P* biopesticide was
379 highly effective at preventing the emergence of adult *Ae. aegypti* and/or *Culex* mosquitoes
380 from natural breeding site water, suggesting that it could be successfully deployed to target a
381 range of different mosquito populations and larval habitats in the field.
382
383 The cause of the larvicidal activity observed with the non-live *Csp_P* powder is still unclear.
384 We observed larvicidal activity for fishmeal/gelatin pellets containing either live *Csp_P* or
385 non-live *Csp_P*. However, larvae that were fed on pellets containing live bacteria died
386 approximately 1-2 days sooner on average than those fed on non-live bacterial powder. It is
387 possible that some factor involved in generating larvicidal activity was lost during the air
388 drying process, that there were differences in concentrations of larvicidal factors between the
389 live and non-live treatments, or even that proliferation of live *Csp_P* in the larval gut could
390 have led to increased levels of the insecticidal agent(s). Previous data from our group
391 indicates that live *Csp_P* can mediate mosquito death through the production of hydrogen
392 cyanide (30). However, we did not detect cyanide in any of the 5 non-live, air-dried *Csp_P*
393 powders that we tested. This result was not particularly surprising given that cyanide is a
394 volatile compound that is known to be lost from *Csp_P* cultures during evaporation (30).

These observations may indicate that the non-live *Csp_P* powder might kill mosquito larvae through alternative mechanisms to the live bacteria, or that some factors that mediate mosquito killing in live *Csp_P* are lost during the air-drying process. To fully elucidate these differences, it will likely be necessary to identify the active ingredient associated with larval killing in the non-live *Csp_P* powder.

Our findings suggest that not all bacteria have larvicidal activity when fed to mosquitoes as non-live powders, indicating that the larvicidal effects we observed with the non-live *Csp_P* powder were not simply a by-product of the culturing protocol that we used. At this stage, it is still unclear if this larvicidal active ingredient in the powder is something that is specific to *Csp_P* or something common amongst members of the genus *Chromobacterium*. The latter could be quite likely, given that many *Chromobacterium* species have larvicidal properties when fed live to mosquitoes (30). Interestingly, there is already a commercially-available insecticide Grandevo (Marrone BioInovations), which was developed from a preparation of the bacterium *Chromobacterium subtsugiae*, and is used to target agricultural pest species (34, 35). It is unclear if Grandevo shares a mechanism of action with non-live *Csp_P* powder.

Study limitations and future directions

While our results are encouraging, there are still multiple factors that need to be addressed to determine if the non-live *Csp_P* formulation can become an effective mosquitocidal tool. There is a need to develop a scale-up compatible culturing protocol that minimizes production time and costs, and maximizes yield of the active ingredient without compromising the stability and the efficacy found in the current formulation. The current culturing protocol is unusual in that it involves both liquid and solid media. This method allowed the bacteria to

form a very thick biofilm, which was potentially enriched for our unknown active ingredient(s). However, the culturing process was laborious and would be unlikely to be cost effective or suitable for mass production. Consequently, there is a clear need to develop an optimized formulation for producing the biopesticide. Ideally this should be based solely on liquid media, as this would simplify the mass production of the biopesticide using standard fermentation technology. Identification of the active ingredient and developing a method of quantifying levels of that ingredient in culture could potentially expedite the process, and could allow for improved larvicidal activity. Making these changes could potentially offer scope to improve powder yield, while decreasing time costs associated with production.

We will also need to consider the issue of residual activity of our formulation. In our experiments we observed that gelatin-based pellets dissolved in water quite rapidly, while agar-based pellets had greater structural integrity, and appeared to retain or improve larvicidal activity when left in water for two weeks prior to treatment. An effective larvicidal agent must not persist in the environment for too short a time, as it could necessitate more frequent treatment. Consequently, evaluating the stability and persistence of the biopesticide in water will be a key concern going forward. Ideally it would not persist for too long, as this risks a loss of activity over time, meaning that larvae would get exposed to lower-than-optimal doses and then be more likely to develop resistance. For these reasons, we plan to investigate whether larvae can develop resistance to non-live *Csp_P* powder, and investigate potential mechanisms of resistance in a future study.

We will seek to test an optimized formulation against adult mosquitoes, and against other animal species, particularly agricultural pests and other vectors, as this will indicate whether the non-live *Csp_P* biopesticide has a broader scope for potential use. We will perform

445 rigorous testing of the biopesticide in line with WHO Pesticide Evaluation Scheme guidelines,
446 to determine if the biopesticide affects non-target species, including beneficial insects such
447 as honeybees. We will also investigate potential ecological and health and safety concerns
448 associated with deployments into the environment by testing the biopesticide against
449 mammals. It should be noted that there are attractive toxic baits used to target mosquitoes,
450 which can be developed in a way that prevents non-target insects from feeding (36-38), and
451 we will look at utilizing this technology for future trials with adult mosquitoes.
452
453 Bacteria from the genus *Chromobacterium* are highly abundant in nature, and have been
454 isolated from a wide variety of environments (39-44). As such, any insecticidal compounds
455 they produce are likely already present in nature, although not at the levels that would be
456 found during a field trial of a *Chromobacterium*-based biopesticide. It is important to
457 remember that similar biopesticides are already in use in the field. In the case of Grandevo, it
458 has been demonstrated that toxicity does not occur ubiquitously in all arthropods that are
459 exposed (34), indicating that *Chromobacterium*-based biopesticides may not be universally
460 toxic amongst arthropods.
461
462
463 *Conclusions*
464
465 We have developed a novel biopesticide based on a non-live air-dried preparation of the
466 bacterium *Chromobacterium* sp. Panama, which is highly effective at killing the larvae of
467 multiple mosquito species, including key vectors of malaria, and the dengue and Zika viruses.
468 This insecticide is still in the early stages of development, but displays many beneficial
469 properties for an insecticide, including low effective dosage, and an active ingredient that

appears to be highly heat-stable. Critically, our data demonstrate that the non-live *Csp_P* biopesticide is highly effective at preventing the emergence of adult mosquitoes, under semi-field conditions.

Materials and Methods

Mosquito lines

Multiple mosquito lines were used in the experiments described in this paper. The majority of the laboratory experiments were performed using the *Aedes aegypti* Rockefeller strain. The pyrethroid-resistant *Ae. aegypti* line (BEI resources - NR-48830) was purchased from BEI resources (Manassas, VA, USA). Experiments involving this line were performed using F_{15} generation mosquitoes, one generation post-insecticidal treatment. Additional laboratory experiments were performed using the *Culex quinquefasciatus* JHB strain (originally isolated in Johannesburg, South Africa, BEI resources - NR-43025), and the *Anopheles gambiae* Keele strain, obtained from the Johns Hopkins University Malaria Research Institute Insectary. Laboratory mosquitoes were hatched in DI water mixed with our laboratory's *Aedes* diet (1 part tropical fish flakes (Ken's Fish): 1 part rabbit chow (Nature's Promise): 2 parts liver powder (Now Foods)). At 2 days post-hatching, L_2 stage larvae were thinned to a density of 250 per 1.5L DI water, and then maintained on dry cat food pellets until the start of experiments. All laboratory mosquito strains were maintained in a climate-controlled insectary (temperature – 27°C ± 1°C, RH – 80% ±10%), with a 14:10 hour day-night cycle.

Experiments in the semi-field facility at Gurabo, Puerto Rico, USA, involved *Ae. aegypti* Patillas strain, and *Ae. mediovitattus*, both derived from previously described CDC San Juan mosquito colonies (45, 46). These colonies were maintained on 10% sucrose, and were kept in an insectary facility at 25–27°C, RH approximately 75%, and a 12-hour light-dark cycle. Eggs from both colonies were hatched in tap water and maintained on rabbit food. At 3 days post-hatching, larvae were transferred to the semi-field facility for experiments. Field-derived

Aedes and *Culex* mosquitoes were also used in experiments conducted at our semi-field facility. Eggs from 11 mosquito populations were collected from different neighbourhoods around Puerto Rico (Bayamon, Catano, Guayanilla, Gurabo, Humacao, Juncos, Loiza, Ponce, Toa Alta, Trujillo Alto, and Yauco). At least 3 oviposition cups containing water or hay infusion and paper as an oviposition medium were left at each site. Cups were left out for approximately 7 days, and then collected. Egg papers were returned to the CDC, dried, hatched, and reared on rabbit food until adulthood. Only *Aedes aegypti* mosquitoes from these collections were used in subsequent experiments. In another set of experiments, larvae and water were collected from breeding sites that were discovered during surveys of different neighborhoods in Eastern Puerto Rico. These mosquitoes were transferred directly to the semi-field facility for experiments. Field collected mosquitoes were identified to the genus and/or species level, where possible.

Larvicidal pellet formulation and laboratory experimental design

For laboratory experiments, groups of 30 L_3 larvae were moved to mosquito breeders (h – 19.5cm, d – 11cm, Bioquip cat: 1425) containing 300mL of DI water, at three days post-hatching. Three replicate breeders were set up for each treatment. Breeders were treated with either a negative control pellet, live bacterial control pellet, or a pellet containing non-live *Csp_P* powder, see below. The control pellets contained 100mg of fishmeal (Dirty Gardener) as an attractant, 500µL of LB broth (Lennox, Sigma-Aldrich cat: L3022), and 500µL of 20% gelatin solution (Sigma-Aldrich cat: 53028) as a stabilizing agent. For control pellets, this formulation provided sufficient nutrients for 30 larvae to pupate and eclose. In place of LB broth, the live bacterial control pellets contained 500µL of *Csp_P* grown in LB broth for 16

hours on a shaker at 30°C, at a speed of 200 rpm. The non-live *Csp_P* pellets had the same composition as the negative control pellet, but also contained 100mg of non-live *Csp_P* powder. After preparation, pellets were allowed to set at 4°C for 1-2 hours and then added to a mosquito breeder. Mosquito survival was then monitored daily for 12 days, with adults provided cotton soaked in 10% sucrose, which was refreshed daily. Each experiment was repeated 3 times and involved 3 replicate cages per treatment.

Non-live Csp_P powder preparation

Five different air-dried, non-live *Csp_P* powders were evaluated for their ability to kill Rockefeller larvae. Three preparations (non-live_1, non-live_2, and non-live_3) were derived from *Csp_P* cultures on sterile 400cm^2 petri dishes (Coning), each containing approximately 200mL of LB agar (Sigma-Aldrich cat: L2897). Each plate was inoculated with 2mL of live *Csp_P* stock (1:1 in 50% glycerol solution, stored at -80°C), and then left to grow for 48 hours in an incubator at 30°C. For the non-live_2 preparation, bacterial cells were removed from the agar using a cell scraper (Sarstedt), and then transferred to a petri dish to dry. For the non-live_1 and non-live_3 preparations, 50mL of LB broth was added to the surface of each plate. Plates were then incubated for a further 120 hours at room temperature. At that point, the liquid on the surface of each plate was decanted and dried down to become the non-live_3 preparation. Plates were left to dry for a further 24 hours, and then the bacterial cells on the surface were removed with a scraper and dried to become the non-live_1 preparation. The final two preparations, non-live_4 and non-live_5, were cultured in sterile 6-well plates (costar cat: 3506). Briefly, each well containing 5mL of LB broth was inoculated with 5µL of live *Csp_P* stock, and the plates were sealed in parafilm and then left to grow for 72 hours at

548 room temperature. For the non-live_5 preparation, biofilm was collected from the surface of
549 each well, mixed with sterile 1X PBS and then dried. The non-live_4 preparation contained
550 the remaining material from the 6-well plate after the surface biofilm was removed. This too
551 was air dried at room temperature. All preparations were dried under continuous air flow in a
552 fume hood. Four preparations, (non-live 2 through 5) were completely dried over the course
553 of 2-3 days. The final preparation (non-live 1) required a longer period to dry completely due
554 to a greater volume of material. After drying, each preparation was manually crushed to a fine
555 powder using a mortar and pestle. Pellets containing the different powders were prepared, as
556 described above, and then fed to Rockefeller larvae to assess their larvicidal activity.
557
558 To validate that each of these powders contained no live *Csp_P* cells, we collected 100mg of
559 each preparation immediately after the air-dried bacteria had been crushed to a powder. The
560 powders were moved to sterile 1.5mL tubes and then mixed with 1mL of sterile 1X PBS.
561 Three dilutions were prepared for each powder (10^0, 10^2 and 10^4) through serial dilution in
562 sterile 1X PBS, and 100µL from each of these tubes was inoculated onto sterile LB agar
563 plates (without antibiotics), and spread using sterile glass beads. The plates were inverted
564 and then placed in an incubator set to 30°C for 48 hours, with these conditions being optimal
565 for culturing live *Csp_P*. These experiments were performed three times, each from
566 independent batches of air-dried powders.
567
568 We assayed for the presence of cyanide containing species in each of the 5 non-live powders
569 using the Cyanide Test Kit, Model CYN-3 (Hach, 2010-02). Each preparation was cultured,
570 air-dried and crushed to powder, as described above, and then used for testing within one
571 week after the air drying process was finished. A total of 10mg of each powder was dissolved
572 in 10mL of MilliQ water in a 15mL tube. These tubes were then mixed by hand until the

573 contents went into suspension. 5mL from each tube was used in the Cyanide test, which was
574 completed according to manufacturer's instructions (30).
575
576
577 *Assaying non-live Csp_P activity*
578
579 Unless specified below, all experiments utilized 100mg of non-live *Csp_P* powder, prepared
580 according to the non-live_1 *Csp_P* protocol, as described above. Pellets containing the
581 biopesticide were prepared and then fed to Rockefeller larvae, pyrethroid-resistant *Ae.*
582 *aegypti* ROCK larvae, *Cx. quinquefasciatus* larvae, and *An. gambiae* larvae, in order to assay
583 whether the powder could kill multiple mosquito vector species, and mosquitoes that were
584 resistant to commonly used insecticides. In further experiments, pellets containing different
585 quantities of non-live_1 *Csp_P* powder (100mg, 50mg, 25mg, 12.5mg and 6.25mg) were
586 offered to ROCK larvae in order to evaluate the larvicidal properties of lower doses, and
587 calculate the LD_{50} of that preparation. To assess whether the larvicidal activity occurred as a
588 result of culturing method, we cultured the mosquito-associated bacteria *Acinetobacter*
589 *baylyi*, *Pseudomonas rhodesiae* and *Serratia marscecens* using the non-live_1 protocol, and
590 fed these to Rockefeller larvae. These species were grown from frozen stocks that were
591 already present in our laboratory, stored at -80ºC in 50% glycerol and LB broth (47).
592
593
594 *Accelerated shelf life testing*
595
596 The EPA guidelines for product development indicate that accelerated shelf life tests be
597 performed to assess stability of the active agents in a product (48). Under the suggested

guidelines, a product treated at 54°C for two weeks is comparable to one year spent at room temperature. Non-live_1 *Csp_P* powder was transferred to 50mL plastic tubes (Falcon) and wrapped in one layer of aluminium foil, with this setup serving as a mock commercial packaging. The tubes of powder were then left at room temperature, 30°C, 37°C, 54°C, or 70°C for two weeks in incubators. Due to incubator availability, the 70°C treatment was performed independently, however, comparisons in those experiments were made using a separate batch of room temperature non-live_1 *Csp_P* powder that was prepared during the same period. Pellets were made from each preparation and fed to Rockefeller larvae.

Increasing pellet stability

To improve pellet stability, we developed a revised pellet formulation with 1.5% agar (Sigma-Aldrich A1296) substituting for gelatin as the stabilizing agent, and LB Broth excluded from the recipe. These agar pellets displayed increased integrity in water, remaining intact for weeks, as opposed to days for gelatin-based pellets. To assess the integrity of the revised formulation we performed two experiments. In these experiments, control pellets contained 100mg of fishmeal as an attractant, and 1mL of 1.5% agar, while non-live *Csp_P* pellets also contained 125mg of non-live_*1 Csp_P* powder. Assay conditions and sample size were as described for gelatin pellet experiments. Each experiment was repeated three times, and these replicates contained two technical replicates of each pellet type/treatment. In the first experiment, we assessed the residual activity of agar pellets. One batch of agar pellets was prepared and immediately placed into individual mosquito breeders, each containing 300mL of DI water. These breeders and pellets were left undisturbed for 14 days at room temperature. After this time, a further batch of pellets was prepared and added to mosquito

623 breeders. *Ae. aegypti* larvae were then added to all breeders, and survival was monitored
624 daily, as described above. Secondly, we assessed the activity of whole agar pellets after heat
625 treatment. Control and non-live *Csp_P* agar pellets were prepared as described above.
626 Pellets were sealed in plastic wrap to prevent moisture loss, and then left at room
627 temperature, 37°C, or 54°C for 7 days. Pellets were then fed to *Ae. aegypti* larvae and
628 survival was monitored daily, as described above.
629
630
631 *Semi-field trials with CDC colony and field-derived mosquitoes*
632
633 For semi-field experiments, pellets were made, as above, at the semi-field site, except that
634 LB broth and gelatin stocks were not prepared under sterile conditions. The dose of *Csp_P*
635 powder in the pellets used in these experiments was increased to 200mg, to account for high
636 larval numbers in some breeding sites. Non-live *Csp_P* powder stocks used in these
637 experiments were prepared at Johns Hopkins University and shipped to Puerto Rico. We first
638 assessed the impact of the powder on *Ae. aegypti* Patillas and *Ae. mediovitattus* colony
639 mosquitoes. Larvae were transported to the field cage at 3 days post-hatching, and then
640 divided into small plastic cups containing 300mL of tap water, 30 larvae to a cup, with four
641 cups of each treatment used per experiment. Larval survival was then monitored daily for 6
642 days. Next, we assessed the efficacy of the non-live *Csp_P* powder on G_1 larvae from 11 *Ae.*
643 *aegypti* populations collected around Puerto Rico. Eggs from each population were hatched
644 and then taken to the field cage 2 days later. As G_1 larval numbers were low, a maximum of
645 15 per cup were used, with one cup per treatment, per population. In these experiments,
646 survival was monitored every 1-3 days.
647

For experiments involving larvae and water collected from breeding sites in the field, larvae and water were transferred to sterile plastic cups using sterile pipettes, and then transported to the semi-field cage where experiments were conducted. The volume of water was measured using plastic measuring cups and divided evenly between two plastic containers. Larvae and pupae from each breeding site were divided into these containers. One cup was fed a negative control pellet, while the other was fed a pellet containing non-live *Csp_P* powder. Cups were then covered in mesh to prevent adults from escaping. In these experiments, adult eclosion was monitored every 1-3 days, by counting and then removing adults in each cup.

Statistical analysis

For all experiments, survival data were compared across replicate experiments using Cox Proportional Hazard Models within SPSS V17 (IBM). For field breeding site experiments, the proportion of mosquitoes that eclosed was compared between treatment and control groups using Fisher's exact test (Prism v6.0h, Graphpad), and Cox Proportional Hazard Models (SPSS V17, IBM). Figures were created using Prism v6.0h (Graphpad), and Microsoft PowerPoint for Mac (v 16.19).

Acknowledgements

The authors wish to thank Manuel Amador and Veronica Acevedo, CDC Dengue branch, Puerto Rico for assistance with mosquito identification. We are also grateful to Chris Kizito

and the JHMRI insectary for providing *Anopheles gambiae* larvae, to Hannah J. McLeod for providing *Culex quinquefasciatus* larvae, and members of the Dimopoulos Group for helpful discussion about the project.

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Tables

Table 1: Larvicidal activity of different non-live *Csp_P* preparations.

Treatment	*N* [a]	Average time to death (± s.e.m.) [b]				Exp. *β* [c]	% Survival [d]
		R_1	R_2	R_3	Overall		
Control	270	5.91 ± 0.39	7.56 ± 0.71	7.00 ± 1.22	6.93 ± 0.44	NA	87.78
Live	260	1.90 ± 0.06	2.36 ± 0.07	2.72 ± 0.09	2.32 ± 0.05	99.17	0.00
Non-live_1	253	2.52 ± 0.20	3.31 ± 0.17	2.55 ± 0.09	2.80 ± 0.10	50.11	0.79
Non-live_2	254	3.97 ± 0.18	6.36 ± 0.34	5.43 ± 0.30	5.19 ± 0.17	31.17	4.72
Non-live_3	262	3.98 ± 0.26	3.73 ± 0.23	3.56 ± 0.18	3.76 ± 0.13	30.44	0.38
Non-live_4	247	3.73 ± 0.22	2.40 ± 0.19	3.88 ± 0.21	3.31 ± 0.13	33.00	0.81
Non-live_5	255	3.09 ± 0.17	4.48 ± 0.30	1.81 ± 0.08	3.08 ± 0.13	44.38	0.39

a - Total larvae counted across three experiments.
b - Average time to death compiled across three replicate cages for replicate experiment 1-3 (R_1-R_3) ± standard error of mean.
c - Exp. *β* = Hazard ratio, calculated using Cox Regression.
d - Percentage of larvae surviving at 12 days post-exposure to pellet.

Table 2: Characteristics of the 11 breeding sites collected around Puerto Rico.

Site	Site type	Collection date [a]	Collection location	Larvae/pupae (*N*)	Mosquito genera
A	Tire	14th Feb 18	Caguas	186	*Aedes*/*Culex*
B	Paint Bucket	15th Mar 18	Cataño	20	*Aedes*
C	Water meter	15th Mar 18	Cataño	52	*Culex*
D	Plastic cup	22nd Mar 18	Cataño	28	*Aedes*
E	Plastic bucket	21st Aug 18	Puerto Nuevo	70	*Aedes*/*Culex*
F	Trash container	22nd Aug 18	Cupey	78	*Aedes*/*Culex*
G	Paint bucket	22nd Aug 18	Cupey	38	*Aedes*
H	Metal pipe	18th Sep 18	Puerto Nuevo	24	*Aedes*
I	Metal bucket	25th Oct 18	Salinas	112	*Aedes*
J	Plastic container	25th Oct 18	Salinas	38	*Aedes*/*Culex*
K	Waste water tank	25th Oct 18	Salinas	38	*Culex*

a - breeding sites were translocated to the semi-field facility on this date and then treated with the non-live *Csp_P* biopesticide.
b - Total larvae and pupae observed in the breeding site water.

825 **Figure Legends**

826

827 **Fig. 1: Workflow for preparing the non-live *Csp_P* biopesticide.** Prepare large plates with
828 200mL of LB agar, then inoculate each with 200µL of *Csp_P* culture **(A)**. Allow plates to grow
829 for 2 days at 30°C, and then inoculate with 200mL of LB broth **(B)**. Grow for 5 days, then
830 decant the liquid phase, let plates sit for 24 hours and then scrape off the bacterial lawn **(C)**.
831 Collect bacterial lawn in a petri dish **(D)**. Air dry preparation in fume hood **(E)**. Once dry,
832 crush preparation to powder with a mortar and pestle **(F)**. Incorporate non-live *Csp_P* powder
833 into gelatin/fishmeal attractive pellets **(G)**. Feed pellets containing 100mg of powder to target
834 mosquito larvae **(H)**.

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836

837 **Fig. 2: Non-live *Csp_P* effectively kills the larvae of important mosquito vector species,**
838 **including those resistant to common chemical insecticides.** At 3 days post-hatching,
839 larvae from *Ae. aegypti* ROCK strain **(A)**, pyrethroid-resistant *Ae. aegypti* strain NR-48830
840 **(B)**, *An. gambiae* Keele strain **(C)**, and *Cx. quinquefasciatus* JHB strain **(D)** were fed with an
841 attractive pellet containing fishmeal and 20% gelatin. Larvae were treated with one of three
842 different types of pellets: no bacteria controls (black lines), live *Chromobacterium Csp_P* (red
843 lines), or non-live *Chromobacterium Csp_P* (blue lines). Non-live *Csp_P* was an effective
844 larvicide for each line, with an average time to death of 3.13 ± 0.08 days (± s.e.m.) for ROCK,
845 3.40 ± 0.13 days for NR-48830, 2.25 ± 0.09 days for Keele, and 1.68 ± 0.05 days for JHB.
846 Larvae were reared in groups of 25-30 in 300mL de-ionized water. Lines depict the
847 percentage of larvae surviving at each day post-treatment (± s.e.m.) for three experimental
848 replicates, with each containing three cages per treatment.

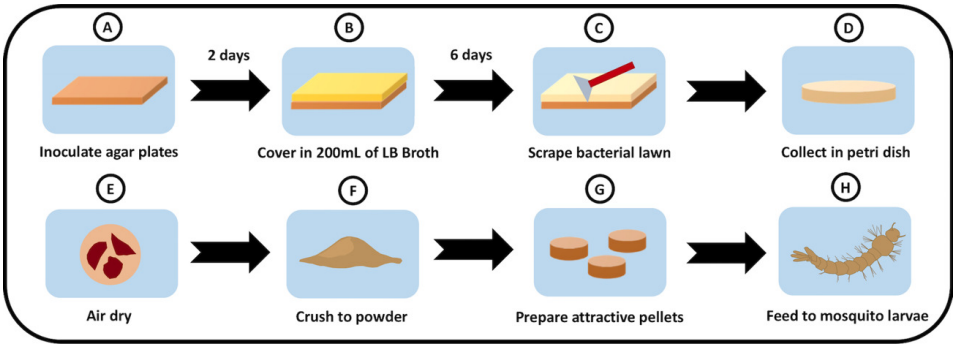
849

Fig. 3: The Non-live *Csp_P* biopesticide has a low effective dose, and a durable active ingredient that is not produced by other common mosquito-associated bacteria. ROCK larvae were fed pellets containing different doses of Non-live *Csp_P* **(A)**. Doses of 100mg and 50mg killed 100% of larvae, while doses of 25mg, 12.5mg and 6.25 had partial mortality and delayed pupation. A LD_{50} was calculated at 11.35mg per liter of water in the larval habitat. Non-live *Csp_P* powder was heat treated at room temperature (22°C), 30°C, 37°C or 54°C **(B)** and in independent experiments, at room temperature (22°C) or 70°C **(C)** in accelerated shelf life tests in order to assess the durability of the active ingredient. The 30°C, 37°C and 54°C treatments did not differ in efficacy from the room temperature treatment (Cox regression; $P > 0.05$), while the 70°C still killed 100% of the larvae exposed but took significantly longer to do so (Cox Regression: $P < 0.0001$), with these results suggesting that the active ingredient was highly heat-stable, and likely to have a long shelf life. Three other common mosquito-associated bacteria were cultured, and dried according to the same protocol used to produce non-live *Csp_P* powder. 100mg of each of these powders was added to attractive pellets and provided to ROCK larvae **(D)**. None of these three preparations caused mortality that was significantly different to larvae treated with no bacteria control pellets (Cox Regression: $P > 0.05$), suggesting that the larvicidal effect we observed in our results was not due to the culturing methods, and not universal amongst all bacteria. In all experiments, larvae were reared in groups of 30 in 300mL de-ionized water. Lines depict the percentage of larvae surviving at each day post-treatment (± s.e.m.) for three experimental replicates, with each containing three cages per treatment.

Fig. 4: Non-live *Csp_P* powder effectively kills mosquito larvae under semi-field conditions. *Ae. aegypti* Patillas **(A)** and *Ae. mediovitattus* **(B)** larvae were reared under

875 insectary conditions for three days, and then transferred to a semi-field facility at Gurabo,
876 Puerto Rico. Larvae were treated with a no bacteria control pellet (black lines), or a pellet
877 containing 200mg of non-live *Csp_P* (blue lines), and then left under ambient environmental
878 conditions. We observed significant mortality induced by Non-live *Csp_P* for larvae from both
879 species (Cox Regression: $P < 0.0001$) indicating that the larvicide performed effectively under
880 semi-field conditions. Larvae were reared in groups of 30 in 300mL of tap water. Lines depict
881 the percentage of larvae surviving at each day post-treatment (± s.e.m.).
882
883
884 **Fig. 5: Non-live *Csp_P* powder effectively kills larvae from different *Ae. aegypti* G_1**
885 **field-derived populations under semi-field conditions.** *Ae. aegypti* eggs were collected
886 from 11 different sites around Puerto Rico using oviposition cups. Egg papers were hatched
887 under insectary conditions, typed for mosquito species, and then transferred to the semi-field
888 cage. Larvae from each population were divided in two, with half fed a control pellet (black
889 lines), and half fed a pellet containing 200mg of non-live *Csp_P* (blue lines), and then left
890 under ambient environmental conditions. 100% mortality for all *Csp_P*-treated larvae was
891 achieved in 2-6 days. Each panel depicts a different *Ae. aegypti* population: Bayamon **(A)**,
892 Catano **(B)**, Guayanilla **(C)**, Gurabo **(D)**, Humacao **(E)**, Juncos **(F)**, Loiza **(G)**, Ponce **(H)**, Toa
893 Alta **(I)**, Trujillo Alto **(J)**, Yauco **(K)**. Larvae were reared in groups of 7-15 in 300mL of tap
894 water. Lines depict the percentage of larvae surviving at each day post-treatment.
895
896
897 **Fig. 6: Non-live *Csp_P* powder effectively prevents adult mosquito emergence from**
898 **translocated breeding sites under semi-field conditions.** Mosquito breeding sites were
899 located at various sites around Eastern Puerto Rico. The water and any larvae and pupae

were removed from the breeding site and moved to the semi-field cage in sterile containers. Larvae and pupae were counted, and along with the water and any detritus, divided evenly between a control treatment (black lines) and a non-live *Csp_P* treatment (blue lines). Adult emergence was monitored as it was too difficult to locate dead L_1 and L_2 larvae in the opaque breeding site water. Across eleven different breeding sites, we observed that 342/374 adults emerged from the control treatment, compared to 9/374 from the non-live *Csp_P* treatment (Fisher's exact test: $P < 0.0001$). Breeding sites contained either *Ae. aegypti* larvae ($N = 5$), *Cx. quinquefasciatus* larvae ($N = 2$), or a mix of both ($N = 4$). Breeding sites were collected from the following recepticles: tire **(A)**, paint pail **(B)**, water meter **(C)**, plastic cup **(D)**, bucket **(E)**, container **(F)**, bucket **(G)**, pipe **(H)**, bucket **(I)**, container **(J)**, waste water tank **(K)**. Lines depict the percentage of adults that had emerged from the breeding site water at different intervals post-treatment.



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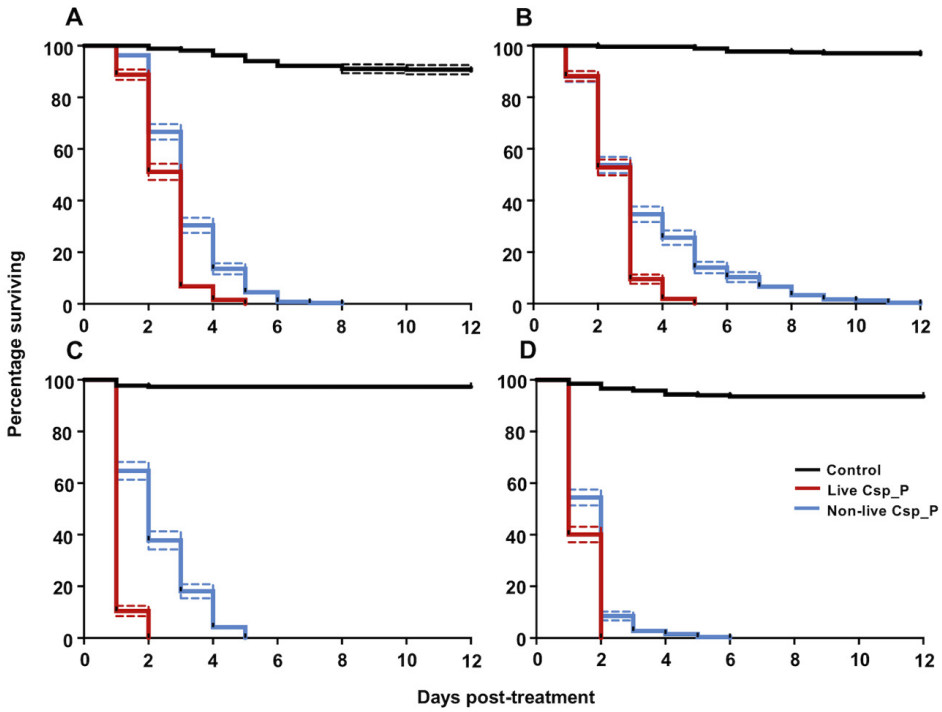

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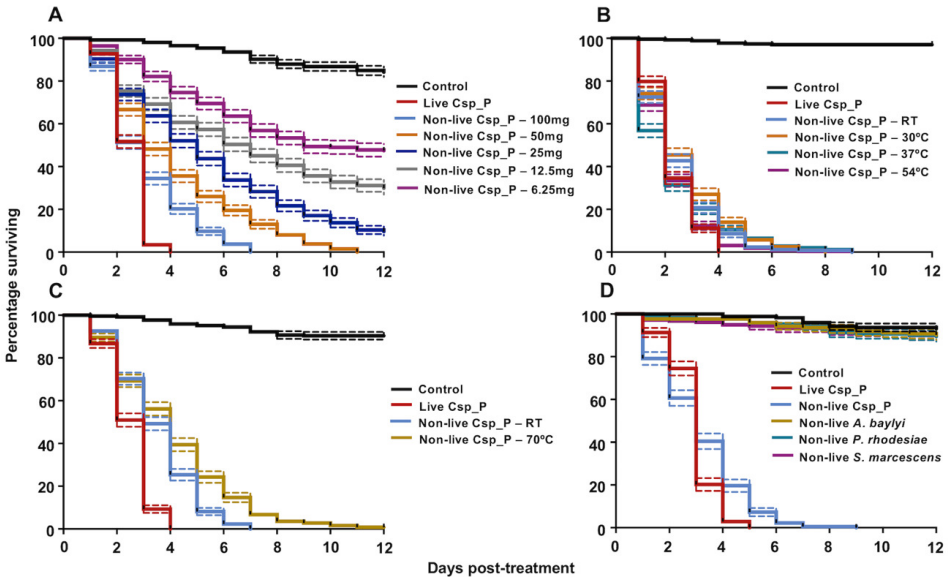

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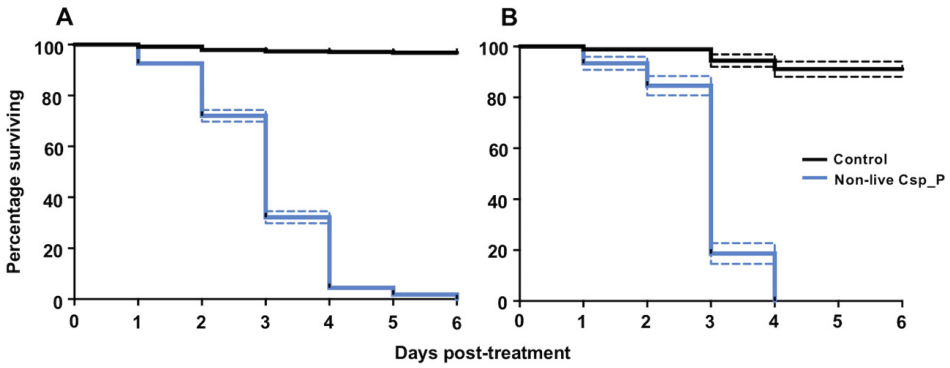

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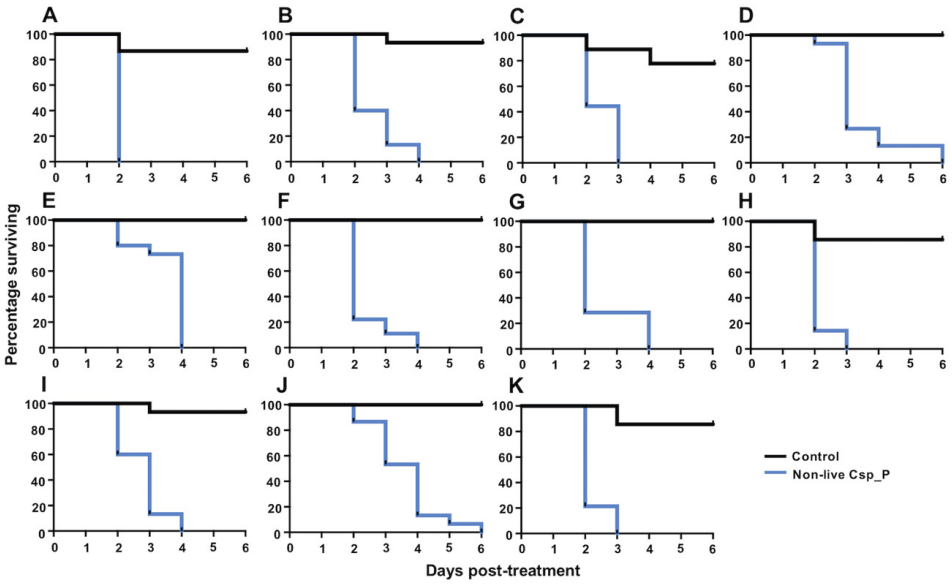

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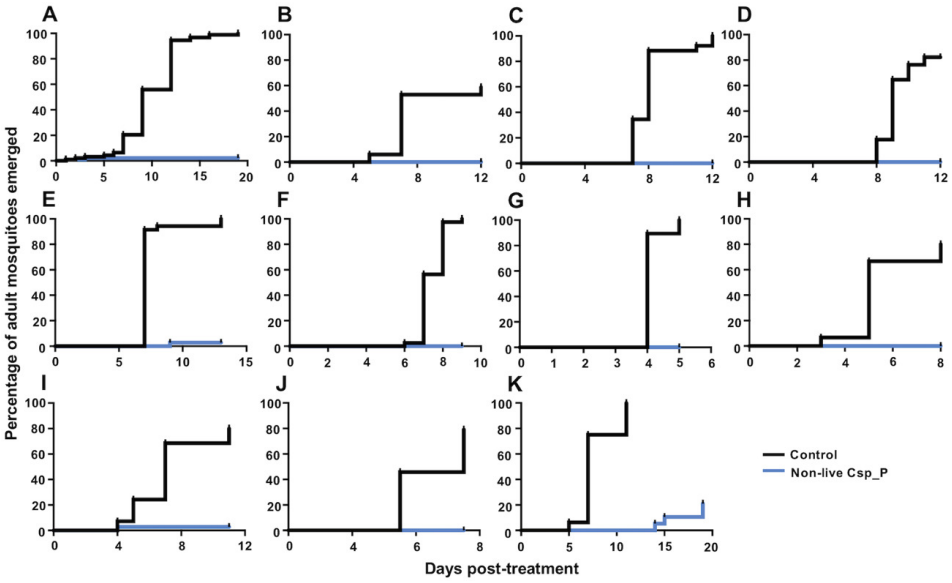

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