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# Determination of the toxicity of *Pseudomonas protegens* CHA0 to *Drosophila suzukii* (Spotted Winged Drosophila) larvae through oral inoculation

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**SUMMARY** *Pseudomonas spp.* are beneficial plant microbes with known insecticidal properties. *Drosophila suzukii* (Spotted Wing Drosophila) is an introduced agricultural pest that is particularly problematic for berries and soft skinned fruits in North America. Although chemical pesticides currently exist, there is a need for more effective and specific methods of combating *D. suzukii*. Currently, SWD populations are managed with a combination of chemical pesticides and cultural control strategies. However, chemical pesticides have negative consequences for beneficial insects, namely bees, and can pose a threat to aquatic life. Recently, research into pest management has shifted towards the use of bioagents to control agricultural pests as an alternative to harmful chemical pesticides. Our study tested the effect of oral inoculation of *D. suzukii* larvae with *Pseudomonas protegens* CHA0. We found that oral inoculation of larvae with *P. protegens* may be associated with increased mortality and fewer larvae developing into adults.

## INTRODUCTION

*Drosophila suzukii* or spotted wing drosophila (SWD) is a newly introduced agricultural pest in North America, and was first identified in British Columbia in 2009 (1). This pest targets soft-skinned fruits, such as blueberries which is a 7-billion dollar industry in British Columbia (1, 2). These flies are particularly problematic to crops because instead of the typical attraction to overripe fruit, the female *D. suzukii* lays eggs inside of unripe fruit (1). When the larvae hatch they eat the fruit from the inside-out before emerging as adults (1). This process makes the fruit soft and unmarketable (1).

Currently there are chemicals available that can manage *D. suzukii* populations such as the organophosphate insecticides including Malathion 8E, pyrethroids such as Ripcord 400 EC, diamides such as Exirel, and spinosyns such as Entrust SC (3, 4). However, the majority of these approved chemicals for *D. suzukii* management cannot be used by organic farmers, and many are highly toxic to bees (3, 5). In addition, many pesticides such as Ripcord 400 EC, and Exirel are toxic to aquatic species and agricultural runoff from these farms poses a risk to adjacent rivers and streams (6, 7). Finally many of these pesticides can cause irritation if they come in contact with human skin and can cause respiratory irritation for agricultural workers (6, 7).

In addition to chemical management strategies, cultural protocols such as minimizing wild host plants near crops such as wild blackberry bushes can help to minimize *D. suzukii* populations near fields (4). Farmers can also implement timely harvest schedules where ripe berries are harvested every other day. This prevents adult *D. suzukii* from emerging from ripe berries and continuing their life cycle (3). However, both of these cultural control methods pose an economic burden on farmers and, minimizing wild host plants can negatively impact

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beneficial insect species (4). For these reasons, there is an interest in developing novel ways of controlling *D. suzukii* populations and protecting crops.

*Pseudomonas spp.* strains such as *P. protegens* CHA0 are known to be beneficial bacteria for plants as they produce insecticidal compounds such as hydrogen cyanide (8). For example, *P. protegens* CHA0 has been found to be toxic to *Musca domestica,* a household fly, through oral inoculation. In addition, various closely related strains such as *Pseudomonas fluorescens* Pf-5 have been associated with developmental delay and lethality to *Drosophila melanogaster* through oral inoculation (9). However, the oral toxicity of *P. protegens* CHA0 to *D. suzukii* larvae has not yet been tested.

The life cycle of *D. suzukii* consists of four stages: egg, larva, pupae, and then adult (10). The length of each life stage is highly temperature dependent; at room temperature, the average development time from egg to adult is between 15 - 20 days and has a success rate between 60 - 100% (10).

The purpose of our study was to determine the effect of oral inoculation of *D. suzukii* larvae with *P. protegens* CHA0 on larval survival and development. We hypothesized that *P. protegens* CHA0 would be toxic to the fly larvae and would delay development of *D. suzukii* as was observed in the closely-related *D. melanogaster*.

## METHODS AND MATERIALS

**Maintaining Flies**. To maintain the flies, a protocol was adapted from Siva-Jothy et al. to make an agar medium that could support the growth and survival of a colony of *D. suzukii* (11). In a 150 mL beaker, 23.39 g of active dry yeast were combined with distilled water to 140 mL of water and 0.25 grams of brown sugar were added. Then in a 2000 mL bottle, 93.78 g of brown sugar was combined with 68.22 g of corn flour, and water was added to 400 mL. The 140 mL activated yeast mixture was then added to the brown sugar and corn flour solution and the bottle was filled to 1 L with water. After adding 6.38 g of agar, the mixture was autoclaved.

The autoclaved liquid media was transferred using a pipette into smaller glass tubes with foam lids that hold the fly colonies on the benchtop at room temperature. To transfer the flies from the starter colonies into the holding tubes, the container holding the flies was first transferred to a 4°C fridge for 45 minutes to limit fly movement. We tried to consistently include 5 female and 5 male flies on the first transfer from a starter colony. Flies were then moved onto a paper towel and transferred into a new tube. These new glass tubes were held at room temperature for three days. On the fourth day, the flies were transferred using the same cooling protocol into a new glass tube, while the old holding tube contained larvae that could be used in later phases of the experiment. The flies were transferred up to 3 times

**Generation of the growth curve for *P. protegens* CHA0.** *P. protegens* CHA0 was provided to us by Dr. Cara Haney in the department of Microbiology and Immunology at the University of British Columbia, Vancouver, Canada. The strain was stored in a 50% glycerol solution at -70°C for the duration of the study. Bacteria from glycerol stock was grown in LB overnight in a shaking incubator at 30°C, 200rpm. Three different samples of overnight culture were diluted using LB to 1/100. Each sample had four replicates and a blank (LB) loaded onto a 96-well plate. Using a BioTek microplate reader, the temperature was set to 37°C with the primary wavelength at 600 nm for 20 hours with optical density (OD) measurements taken at 15-minute intervals (Supp. Fig 1).

**Preparing *P. protegens* CHA0 treatment.** An overnight culture was made using bacteria from a glycerol stock grown in 10mL of LB in a shaking incubator at 37°C, 200rpm. 4mL of the overnight culture was added to 36mL of fresh LB in a flask and placed in a shaking incubator at 25°C, 200rpm. OD measurements were taken every 30 minutes until subcultures were in exponential growth phase (after 3-5 hours of growth, or $OD_{600nm} = 0.4$, as determined by our growth curve to be within the log phase of bacterial growth) (Supp. Fig 1). Then, the subculture was pelleted by centrifugation at 4°C, 15 minutes, 2500 x G. After centrifugation, the supernatant was discarded and bacteria were resuspended in 5mL of LB and stored at 4°C to be used for oral inoculation the following day.

**Inoculation of flies.** Agar medium was spread evenly onto petri dishes at a depth of approximately 10-15 mm, and $OD_{600nm}$ = 0.4 bacterial resuspension was transferred with a micropipette onto the medium. Two different protocols were used to inoculate the flies with bacterial solution. For assay 1, 200 uL of bacteria was mixed into the medium. For assay 2, 50 ul of bacterial solution per larvae was spread on the surface of the agar medium to a total of approximately 300 ul (Fig. 1). LB was added to negative control plates. The inoculated larvae were placed onto the surface of the medium and petri dishes were closed with their lids. After around 20 hours, larvae were transferred from petri dishes into new holding tubes (Fig. 1). Larvae were monitored for the next 10-15 days to determine survivorship of larvae to adulthoods under each condition.

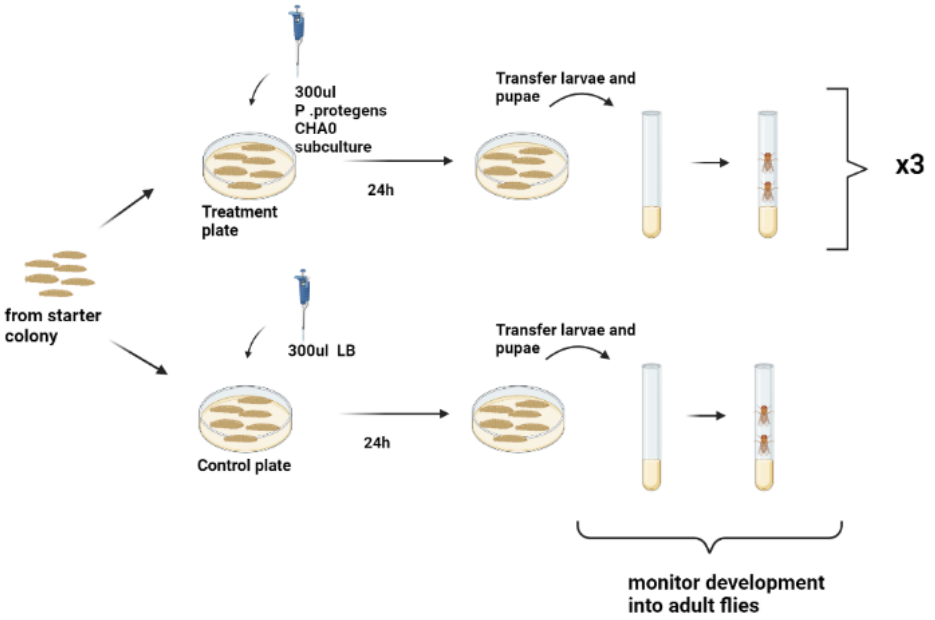


**FIG. 1 Workflow for *D. suzukii* oral inoculation with CHA0.** D. *suzukii* larvae were collected from starter colonies and placed on plates containing CHA0 (treatment) or LB (control). After 24 hours, the larvae and pupae were transferred into holding tubes to monitor their development into adult flies.

## RESULTS

To investigate the toxicity of *P. protegens* CHA0 towards *D. suzukii* larvae, we used a non-invasive assay which mimics the natural route of infection. Briefly, *D. suzukii* larvae were fed media with or without CHA0 and transferred to holding tubes for 14 days to monitor development. After one day, we transferred fly larvae into holding tubes to monitor their development. Our first pilot assay compared the development of *D. suzukii* larvae exposed to concentrated *P. protegens* CHA0 in LB broth to the control group exposed to sterile LB broth alone. In the control group, 62% of larvae developed into adults within 15 days compared to 45 % in the treatment group (Fig 2). This first assay acted as a pilot study and informed decisions made later on in the second assay. However, the results of this assay may not be reliable due to mold contamination in both the control and treatment groups.

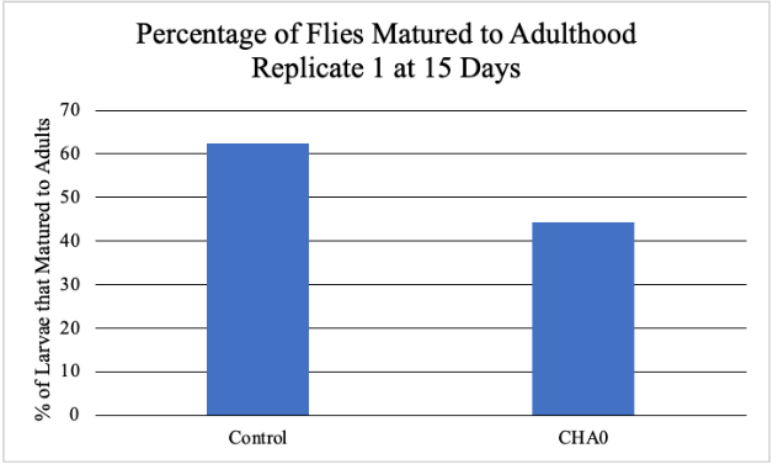


**FIG. 2 Percentage of *D. suzukii* larvae that matured into adult flies at day 15 following infection with *P. protegens* CHA0.** *D. suzukii* larvae were placed on plates containing CHA0 (treatment) or LB (control). For each plate, 200µl of *P. protegens* CHA0 subculture or 200µl of LB was mixed into the fly medium for treatment and control, respectively. The control and treatment had one replicate each. After 24 hours, larvae were transferred into holding tubes to monitor their development into adult flies. On day 15, the number of adult flies were counted in each tube.

We made several modifications when designing our second assay. First, the concentration of bacteria was increased to 50 µl per larvae, increasing the total to 300 µl per plate. Second, the bacteria were spread on top of the fly medium instead of mixing it into the media to ensure contact with larvae. Third, we increased the number of treatment replicates to a total of three. Similarly to the first assay, less larva matured into adults in the treatment group than in the control group. By day 8, 66% of the larvae had matured into adults for the control, whereas only 12% of the larvae had matured for the treatment groups (Fig. 3). This difference in the percentage of flies that matured to adulthood had a p-value of 1.97E-06.

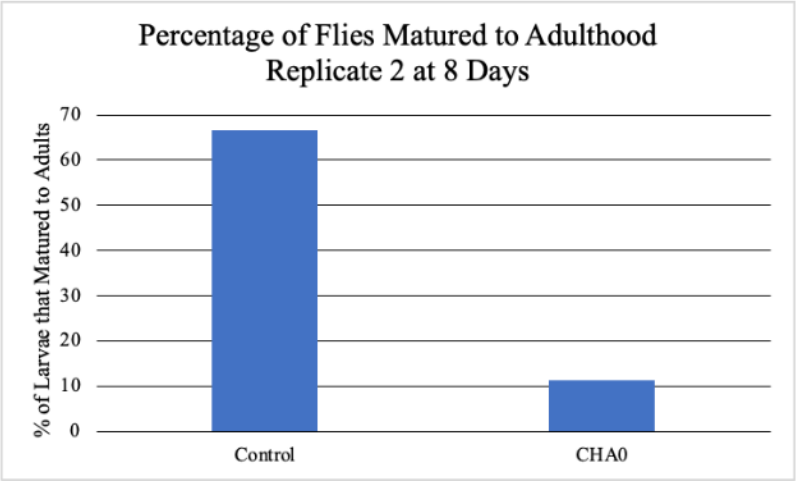


**FIG. 3 Percentage of *D. suzukii* larvae that matured into adult flies at day 8 following infection with *P. protegens* CHA0.** Six *D. suzukii* larvae were initially placed on each plate (n=6), and 300 µl of *P. protegens* resuspension or 300 µl of LB were spread on top of the fly media for treatment and control groups, respectively. One replicate for control and three replicates for treatment were included. After 24 hours, larvae were transferred into holding tubes to monitor their development into adult flies. After 8 days following infection, the number of adult flies were counted in each tube.

In addition to the increased mortality of larvae, another preliminary finding we noticed was that adult flies were slower to emerge for the treatments compared to the control. The emergence of adult flies was first observed in the control group on day 6 (Supp. Fig. 3). In contrast, the first adult fly in the treatment group emerged on day 7, by which time 3 adults had emerged in the control group. This lag in the emergence of adult flies following treatment with CHA0 was consistent throughout the entire period of monitoring and was observed in all three treatment replicates. Our data suggest that infection of *D. suzukii* may not only increase mortality, but also potentially cause developmental delay in *D. suzukii*.

## DISCUSSION

The main question of this experiment was whether oral inoculation of *D. suzukii* larvae with *P. protegens* CHA0 had insecticidal effects on the *D. suzukii*. We found that inoculation with *P. protegens* is associated with increased mortality and fewer larvae developing into adults.

Our results for assay 2, which included three replicates, showed a marked difference in the number of adults that developed in the control and the treatment conditions (Figure 3). This result lends support to our hypothesis that *P. protegens* CHA0 may negatively affect the survival/development of *D. suzukii* larvae. In a previous study on the oral toxicity effects of *P. protegens* on the larvae of Muscoid flies, *Musca domestica* and *Lucilia caesar*, *chitinase D*, *pesticin* and *fitD* were determined as key target genes for virulence (12). However, another study found no significant role of the *P. protegens* Fit toxin in oral infections of *D. melanogaster* (9). Furthermore, the study stated that Orfamide, a cyclic peptide produced by *P. protegens* CHA0, has been shown to be important in oral infections (9). Both studies highlighted that the mechanisms of insecticidal gene activity in *P. protegens* are complex and multifactorial (9, 12). Future studies could further focus on which insecticidal genes in *P. protegens* CHA0 are key for oral toxicity against *D. suzukii* specifically.

Our work has implications for the use of *P. protegens* as a biological insecticide that could be used to prevent the loss of blueberry crops to species such as *D. suzukii*. While other species of bacteria such as *Bacillus thuringiensis* are currently used as biological insecticides in agriculture, these bacteria protect against pests such as the Gypsy moth, *Lymantria dispar dispar*, and to our knowledge there has been no use of biopesticides against *D. suzukii* during the recent infestations in the Pacific Northwest. Additionally, compared to synthetic pesticides that linger in the environment, biopesticides deteriorate quickly after application, offering less exposure and are thus potentially less harmful to humans and the environment

(13). By using a biopesticide for pest management instead of synthetic chemical pesticides, farmers can avoid complications of conventional methods such as development of pesticide resistance (13). Due to the complex nature of biopesticide modes of action, it takes longer for pests to develop resistance to biocontrol agents compared to synthetic pesticides (13) Additionally, biopesticides usually target only a narrow species range, such as *D. suzukii*, and they rarely disrupt the surrounding organisms, compared to synthetic pesticides that frequently impact the ecosystem where they are used (13).

**Limitations** One issue with both assays carried out in this study was fungal contamination of the fly media. Experiments were carried out in a student laboratory with many moving bodies, increasing the risk that contamination (e.g. mold) could confound results. In our first assay, we observed green mold on the surface of the fly media in our holding tubes a few days after transferring the larvae for both the control and the treatment groups. By day 7 the surface of the media was coated in mold, along with the flies and pupae remaining in the tube. One starter colony of *D. suzukii* also had similar mold growing on the surface of the media, and we believe this to be the source of the contamination. In our second assay, we maintained sterility when transferring the larvae from treatment plates into holding tubes. By day 7, only one out of the four holding tubes had a small amount of mold. Mold could have been a confounding variable, and may be responsible for the differences in results between the first and second assay. Future experiments should maintain aseptic technique when transferring flies to minimize contamination.

Our first assay had one control plate and one treatment plate, each with 8 larvae. Some potential concerns for the first assay included over-dilution of bacteria and whether enough of a bacterial load was used per larvae.

For the second assay, we increased bacterial load and made it scalable, such that 50 µl of bacterial solution of 2.4-2.5 $OD_{600nm}$ was added per larvae. We included one control plate and three treatment plate replicates in the second assay and spread the bacteria solution onto the surface of the medium, putting the bacteria in direct contact with the larvae.

One potential source of error was that flies were counted as adults whether they were alive or dead. This method of measuring development is the most relevant to farmers that would want to prevent maturation of fruit flies to reproductive age to prevent large infestations in their crops. However, by counting dead flies and alive flies as adults, we exclude potential toxic effects on adult flies. Future studies can evaluate differences in the number of dead and alive adults between the control and treatment groups.

Specific larval development stage was not controlled for in this study. Future studies could investigate which larval stage is most susceptible to potential insecticidal effects. Another consideration would be to use non-insecticidal bacteria in addition to LB as a negative control as ingestion of a high concentration of non-insecticidal bacteria may also influence the survival of the flies. Repeating our assay using a non-insecticidal strain of bacteria would allow us to establish a baseline for the adjusted toxicity of *P. protegens* CHA0.

Due to time constraints, the fly treatment and bacterial pelleting steps were not conducted on the same day. Though streaking showed that the bacteria was still viable, this delay could have changed gene expression due to additional stress on the bacteria (Supp. Figure 2). A future study that adjusts the workflow to have these steps occur in the same day, without placing concentrated bacterial solution in a 4ºC condition, could further substantiate results from this study.

**Conclusions** In this paper, the effect of oral inoculation with *P. protegens* CHA0 on the survival and development of *D. suzukii* was examined. The findings from this study indicate that oral inoculation with *P. protegens* CHA0 may increase the mortality and delay the development of *D. suzukii* larvae. However, further research is needed to examine the mechanisms behind developmental delays seen in *D. suzukii* when exposed to *P. protegens* CHA0.

**Future Directions** Though we confirmed the viability of bacterial preparations, due to time constraints we were not able to check for infection of larvae after treatment. One potential future direction may be to validate the infection of the larvae by infecting a group of larvae,

and externally sterilizing and homogenizing the larvae. The homogenized mixture could then be plated on a selection media and observed for growth to ensure that the bacteria were successfully able to infect their hosts.

Empirical analysis of the insecticidal genes of *P. protegens* CHA0, and their potential mechanisms could further aid in effective and efficient application of this strain as a biopesticide. The impact of specific environmental factors, such as temperature on expression of insecticidal genes could also be examined. Continued research into the use of *P. protegens* as a form of agricultural pest control could yield significant benefits for the environment and for agriculture as a whole.

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## CONTRIBUTIONS

The planning and execution of the experiments for this project was a collective effort for the authors. The authors also equally distributed the writing and editing work for this report. Key contributions of each author included: BC: contributed to bacteria subculturing, maintenance and transfer of flies, recovery of larvae after treatment, and sections of the methods, discussion, results, sources of error, and future directions; AD: contributions to bacterial growth and preparations, larvae treatment, fly development monitoring, introduction, section of results, and discussion; AI: contributions to growth curve preparation, pelleting bacteria, larvae treatment steps, sections of methods, discussion, conclusion and future directions; TK: fly maintenance, preparation of bacteria, methods, results, future directions.

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