Research Article

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Identification of natural pathogens from wild *Drosophila suzukii*

Xiao-Li Bing,[a,b] Jessica Winkler,[a] Joseph Gerlach,[a] Gregory Loeb[c] and Nicolas Buchon[a*]

Abstract

BACKGROUND: *Drosophila suzukii* (Matsumura, 1931) (spotted wing drosophila), an invasive species, has recently become a significant global pest of soft-skinned fruits such as berries. Unlike other *Drosophila* species, female *D. suzukii* have evolved a specialized sharp, serrated ovipositor that pierces and penetrates ripe and ripening fruits, causing them to lose commercial value and preventing their sale. A first step for the development of biological control agents for pest management may be achieved through the identification of microbes infectious for *D. suzukii* in the wild.

RESULTS: We first determined that *D. suzukii* is susceptible to chemicals commonly used to rear Drosophilids in the laboratory and established a diet able to sustain healthy *D. suzukii* growth. Using this diet, we demonstrated that of 25 species of culturable bacteria and fungi isolated from field-collected *D. suzukii*, eight microbes decreased host survival when injected. Three of the eight bacteria (*Alcaligenes faecalis*, *Achromobacter spanius* and *Serratia marcescens*) were acutely pathogenic to both *D. suzukii* and *Drosophila melanogaster* adults by injection. Feeding of these bacteria resulted in susceptibility only in larvae.

CONCLUSION: We successfully identified multiple microbes from field-collected *D. suzukii* that are pathogenic to both larvae and adults through different routes of infection, some of which could be candidates for biocontrol of this species.
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Supporting information may be found in the online version of this article.

Keywords: *Drosophila suzukii*; pathogens; *Alcaligenes faecalis*; *Achromobacter spanius*; *Serratia marcescens*; moldex; diet; preservatives

1 INTRODUCTION

Microorganisms are widespread throughout an insect's habitat and body, playing important roles in their biology. Microbes are either neutral (commensals), beneficial (mutualistic symbionts)[1] or deleterious (pathogens) to the health of their host.[2] For example, the intracellular gut bacterium *Wigglesworthia glossinidia* provides essential nutrients (e.g. B vitamins) to its tsetse fly host[3] and is necessary for optimal fly fecundity and larval immunity.[4] On the other hand, some pathogenic bacteria (such as *Bacillus thuringiensis* or *Serratia* spp.) synthesize toxic proteins that kill insects.[5] This provides the opportunity to use these insect pathogens as biological control agents.[2] However, despite important successes (e.g. *Ba. thurigiensis*, *Beauveria bassiana*) with microbes as biocontrol agents, only few microbes with such pathogenic potential have been identified.

Drosophila suzukii Matsumura (Diptera: Drosophilidae), or spotted wing drosophila (SWD), is an invasive pest of berries and soft-skinned fruits that rapidly spread from its native Asia to Europe and North America.[6] In 2008, reports confirmed *D. suzukii*'s presence in California[7] and over the next 5 years, *D. suzukii* was reported in at least 35 US states and five Canadian provinces.[8, 9] *D. suzukii* females puncture the skin of ripe fruits and insert eggs inside using their serrated and enlarged ovipositor.[10,11] The developing larvae eat (in) the fruit, making it unmarketable and causing substantial economic losses.[12] The economic cost associated with *D. suzukii* damage has been estimated at US $39.8 million for California alone, plus an approximate $8 million spent annually on chemicals in the raspberry industry from 2009 to 2014.[13]

In order to limit the economic damage of *D. suzukii*, the efficiency of several commercial chemical pesticides has been evaluated in laboratory and field settings (such as neonicotinoid insecticides, malathion, organophosphate dimethoate, spinosad, spinetoram, lambda-cyhalothrin and cyantraniliprole).[14-17] Commercially available biological predators such as fungal entomopathogens (*Metarhizium anisopliae*, *Be. bassiana*, *Isaria fumosorosea*, and *Paecilomyces fumosoroseus*) and nematodes (*Heterorhabditis bacteriophora*, *Steinernema feltiae*, *Ste. carpocapsae*, and *Ste. kraussei*) were also tested as potential biological control agents of *D. suzukii*.[18–20] Exposure to both *M. anisopliae* and *I. fumosorosea* suppressed the development of *D. suzukii*

* Correspondence to: Nicolas Buchon, Department of Entomology, Cornell University, Ithaca, NY 14853, USA. E-mail: nicolas.buchon@cornell.edu

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a Department of Entomology, Cornell University, Ithaca, NY, USA

b Department of Entomology, Nanjing Agricultural University, Nanjing, China

c Department of Entomology, Cornell University, Geneva, NY, USA

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populations with a > 40% mortality rate for adult flies.[18] In another study, *M. anisopliae* significantly decreased *D. suzukii* survival but had no effect on *D. suzukii* fecundity.[19] Nematodes such as *H. bacteriophora* were reported to be the most efficient and caused up to 95% mortality in larvae in one study,[18] but did not affect *D. suzukii* survival in another study.[19] Surprisingly, no entomopathogen of larvae has been identified, despite larvae being the stage with the greatest impact on cultures. As a result, identifying and evaluating new entomopathogens for *D. suzukii* is a timely and important endeavor.

In this study, we attempted to identify microbes with potential to be entomopathogens of *D. suzukii*. We hypothesized that natural pathogens of *D. suzukii* would be associated with flies in the wild. We therefore isolated culturable microbes naturally present in wild-caught *D. suzukii* as "candidate" infectious microbes. We next observed that two common *Drosophila* diet supplements, moldex and propionic acid, are toxic to *D. suzukii*. We therefore developed a laboratory diet that optimizes *D. suzukii* health and allows proper evaluation of the pathogenicity of these microbes without added toxicity. Using this diet, we evaluated the survival of *D. suzukii* and another *Drosophila* species, *Drosophila melanogaster*, to infections with our isolated microbes as a measure of their pathogenicity. Differences in pathogenicity between insect species could have important implications for pest management, particularly with the goal of developing species-specific biological control agents.

2 MATERIALS AND METHODS

2.1 Fly strains and rearing

We used two commonly studied wild-type stocks of *D. suzukii* (strain Cali from UC Davis) and *D. melanogaster* (Canton S from Bloomington, stock #64349) for our study. Flies were kept at ~ 24 °C and a 12:12 h light/dark cycle. Flies were maintained in plastic *Drosophila* vials and round-bottomed bottles (VWR International) on an optimized sucrose–yeast diet as described in Section 2.4.

2.2 Isolation of microbes from wild *D. suzukii* adults

Field-collected *D. suzukii* adults were trapped live using crushed fresh fruits (raspberry, cherry, and blueberry) as bait from four farms in Geneva, NY, USA in summer 2015 (Table S1). Based on our previous findings,[21] we collected flies from different locations to maximize microbial diversity. Flies were sorted by gender and submerged in sterile distilled water. Approximately half of the captured individuals ($n \sim 20$) were surface sterilized in 70% ethanol, rinsed in sterile distilled water, and finally homogenized in 500 μl phosphate-buffered saline (PBS) (VWR), the others were homogenized directly in PBS to identify both surface and internal microbes. The homogenized PBS solution was then streaked across Luria–Bertani broth (LB) (VWR), De Man, Rogosa and Sharpe (MRS) (HiMedia) and Sabouraud dextrose agar with yeast extract (SDAY) (w/v: 4% glucose, 1% peptone, 2% agar and 1% yeast extract) agar plates, and representative colonies of each morphological type were isolated for further molecular identification.

2.3 Identification of isolated microbes

Microbial DNA was extracted with an alkaline heat-extraction method as described in Takara Fungal rDNA (ITS1) PCR Kit Fast. Microbes were identified by sequencing the amplifications of either the bacterial 16S rDNA gene or the fungal internal transcribed spacer (ITS) region. The bacterial 16S rRNA gene was amplified using the universal primers 27F, 530F and 1495R.[22] The fungal ITS region was amplified with universal primers ITS4 and ITS5.[23] Polymerase chain reactions (PCRs) were performed using GoTaq Green Master Mix (Promega) on a S1000 Thermocycler (Bio-Rad). The PCR procedure consisted of an initial step of 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final step of 72 °C for 10 min. Amplified DNA products were visualized by agarose gel electrophoresis and purified with the Monarch PCR & DNA Cleanup Kit (New England Biolabs). Purified PCR products were sequenced on ABI 3730xl DNA Analyzers (Applied Biosystems). 16S rRNA sequencing results were checked by BLASTN in the NCBI "16S ribosome RNA sequences (Bacteria and Archaea)" database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Fungal ITS sequencing results were checked by BLASTN in the NCBI NR database. The top blast hit (based on the smallest E-value and the highest percentage identity) was assigned to the isolated microbe for identification. Sequences of identified microbes were submitted to the NCBI GenBank database under accession numbers MG198675-MG198696 and MG250487-MG250507.

2.4 Optimizing food for rearing flies

D. suzukii and *D. melanogaster* flies were initially raised on a traditional glucose diet (diet A in Table 1).[24, 25] However, we observed that *D. suzukii* flies had reduced longevity, developmental time and fecundity on such a diet (Figure S1), suggesting nutrient imbalance or diet toxicity.[21, 26] To identify the source of this toxicity, we compared the survival of *D. suzukii* adults on different diets containing various quantities of nutrients and supplemented or not with preservatives (moldex [methylparaben], propionic acid and phosphoric acid). At least three independent replicates each including 20 mated *D. suzukii* female and male flies (4–8 days old, ≥ 60 flies) were tested. Flies were flipped to new vials every 2 days. Mortality was recorded daily for up to 2 weeks. The details of each food diet recipe are listed in Table 1.

TABLE 1. Detailed recipes for different food diets corresponding to Figure 1(A)–(D)[a]

Diet code[b]	A	B	C	D	E	F	G	H
Moldex (w/v)	0.265%	0	0.53%	0.265%	0.265%	0	0.065%	0.13%
Phosphoric acid (v/v)	0.498%	0.498%	0.498%	0.498%	0	0.498%	0.498%	0.498%
Propionic acid (v/v)	4.98%	4.98%	4.98%	0	0	0	0	0

[a] Other ingredients (w/v): glucose, 8.2%; yeast, 8.2%; agar, 0.7%.
[b] Sources of the ingredients: glucose, VWR; yeast, MP Biomedicals; agar, MoorAgar Inc; moldex, propionic acid and phosphoric acid, Sigma-Aldrich Corp.

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2.5 Microbial infection of adults

For infection by septic injury, *D. suzukii* and *D. melanogaster* male adult flies were pricked in the thorax using a 0.15-mm needle (Austerlitz Insect Pins) previously dipped in a concentrated bacterial or fungal suspension ($OD_{600} = 0.1$, 0.5, or 5.0 depending on the experiment. $OD_{600} = 1$ is $\sim 10^8$ bacterial cells per ml or 10^6 fungal cells per ml).[27] Uninfected controls were pricked with the same needle with the microbial solution replaced with sterile PBS.

For adult oral infections, male flies (4–8 days old) were starved for 3 h at room temperature in an empty vial before eating an infection solution containing an equal volume of concentrated pellet from a suspended culture of bacteria or fungi ($OD_{600} = 10$ or 200) with a solution of 5% sucrose (1:1 v/v) as previously described.[28-30] The higher dose ($OD_{600} = 200$, $\sim 2 \times 10^{10}$ bacterial cells per ml or 2×10^8 fungal cells per ml) mimics the high concentration of microbes ingested when insects eat the surface of decomposed food on which microbes grow as biofilms/colonies.[31] The solution was deposited on a round filter disk that completely covered the surface of the sucrose–yeast food. Flies were initially incubated for 1 day on the contaminated filter before being transferred to vials with fresh food. For the unchallenged controls, microbial solutions were replaced with sterile PBS. For fungi that produce spores, flies were coated by fungal spores by direct contact in a Petri dish.[27] Two laboratory strains of fungi, the entomopathogenic fungus *Metarhizium anisopliae* strain ARSEF S249 and *Beauveria bassiana* strain 2963 were used as controls for pathogenicity. OD_{600} values of microbial solutions were adjusted with WPA Biowave CO8000 cell density meter (Biochrom Ltd). In cases where multiple strains were identified, a quick initial screen was performed (survival on ten flies) which did not reveal any massive strain difference (data not shown). Therefore, we randomly selected one strain as representative for further experiments. All experiments were performed at ~24 °C on our optimized sucrose food (see Table 1). Flies were flipped every 2 days and survival was monitored daily for 1 week. At least three independent replicates each of 20 flies (≥ 60 flies tested in total) were tested for each survival experiment.

2.6 Bacterial load assay

To estimate bacterial growth in flies upon infection, we estimated the bacterial load of pathogens from individual flies at 0, 4, 8, 12, and 16 h after inoculation, as previously described.[24, 29] Flies were first briefly washed with ethanol and PBS and then placed in 0.5 ml of PBS in a 1.5-ml Eppendorf microcentrifuge tube containing one metal bead. Samples were homogenized on a FastPrep-24 instrument (MP Biomedicals) and homogenates were plated on LB agar plates using a WASP 2 spiral plater (Microbiology International). Colonies on the plates were counted after 12–16 h of incubation at 29 °C. To estimate the number of colony-forming units (CFU) per fly, colonies were counted using the ProtoCOL 3 Colony Counter plate counting system (Microbiology International).

2.7 Microbial infection of larvae

For oral infection of larvae, 20 late second- or third-instar *D. suzukii* and *D. melanogaster* larvae were placed in a 2-ml tube containing a mixture of 200 μl concentrated bacterial pellet ($OD_{600} \sim 133$) from an overnight culture and ~ 400 μl crushed banana, and were incubated at room temperature for 30 min.[32] The mixture of larvae, bacteria and banana was then transferred to a vial of optimized sucrose fly food and incubated at 24 °C. Emerged adults were considered alive, and the remaining larvae were considered dead. The emergence ratio was calculated by dividing the number of emerged adults from each vial by the initial number of larvae.

2.8 Statistical analyses

Survival curves were analyzed via log-rank test analysis with the R package survival.[33] The pathogenicity of each microbe in *D. suzukii* or *D. melanogaster* was analyzed by comparing survival curves of infected and unchallenged or clean pricked controls. The species-specific pathogenicity of each microbe was estimated by comparing infected *D. suzukii* and *D. melanogaster* flies. Bacterial load results were analyzed with a linear model regression test. The proportion of emerged flies was analyzed with a chi-square test. All analyses were performed with the statistical software R version 3.3.[34]

3 RESULTS

3.1 Identification of microbes from wild *D. suzukii* adults

Under the assumption that some microbes isolated from wild-caught *D. suzukii* could be pathogens, our research started from isolating and identifying culturable microbes that can be found in or on wild *D. suzukii*. We isolated 25 species of microbes including 14 bacterial (eight Gram-negative and six Gram-positive) and 11 fungal/yeast species from wild-caught adult flies collected at four distinct New York locations (Table S1). Among these, nine species of bacteria and nine species of fungi/yeasts were isolated from surface-sterilized flies, suggesting that these microbes are either located in the gut or in the body cavity of flies (see Table S1). Five of the eight identified Gram-negative bacteria were Gammaproteobacteria, while the remaining three were Betaproteobacteria (Table S2). The most frequently detected Gram-negative bacteria were Enterobacteriaceae, including *Enterobacter asburiae*, *Erwinia aphidicola* and *Escherichia hermannii*, while the most prevalent Gram-positive bacteria were Bacillales, including *Bacillus pseudomycoides*, *Paenibacillus taichungensis* and *Staphylococcus saprophyticus*. The most abundant fungi were Saccharomycetales yeasts, including *Geotrichum candidum*, *Hanseniaspora uvarum*, *Candida oleophila*, *Ca. railenensis*, *Ca. cylindracea*, *Metschnikowia* sp., *Pichia kluyveri* and *Torulaspora delbrueckii* (Table S2). Altogether, we identified a diverse new group of culturable microbes that associate with wild *D. suzukii*, some of which could be entomopathogenic microbes.

3.2 Optimizing diet for survival analysis

Next, we aimed to evaluate whether our isolated microbes display some virulence against *D. suzukii*. To that purpose, we chose to evaluate the survival of *D. suzukii* upon microbial exposure as an indicator of microbial pathogenicity. However, we found that laboratory-reared *D. suzukii* showed very limited fitness when reared on a classical *Drosophila* diet (diet A, Table 1). In comparison with no significant mortality of *D. melanogaster* on the classical diet A, more than half of *D. suzukii* flies died after 14 days on this diet (Figure S1A, $P < 0.0001$ for both females and males). In addition, *D. suzukii* flies developed much more slowly than *D. melanogaster* by more than 2 days (average emergence time: 14.7 days for *D. suzukii* compared with 12.1 days for *D. melanogaster*; Figure S1B, $P < 0.0001$) and produced surprisingly few offspring (around 20% of the adult offspring produced by *D. melanogaster*; Figure S1C, $P < 0.0001$). We concluded that classically used *Drosophila* diets do not support proper health in

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FIGURE 1. Survival of *Drosophila suzukii* female and male flies on diets with different supplements. The changing supplements are: (A) moldex, (B) propionic acid, (C) phosphoric acid, (D) moldex and (E) sugar source. Detailed recipes are given in Tables 1 and 2. Survival curves represent average percent survival and gray ribbons represent 95% confidence intervals. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

D. suzukii, which could be a misleading parameter in our survival analyses.

To overcome this limitation, we decided to optimize the diet and generate one that supports *D. suzukii* health and fitness before evaluating microbial pathogenicity. Apart from the nutrient source (sugar and yeast), the "classical" diet (diet A) contained 0.265% (w/v) moldex and a mix of acids (4.98% v/v propionic acid and 0.498% v/v phosphoric acid) as preservatives to prevent the

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overgrowth of fungi and bacteria, respectively (Table 1). At fixed concentrations of propionic acid and phosphoric acid, adult *D. suzukii* survival rate decreased along with increasing moldex concentration (Figure 1A, $P = 0.0095$ for females and $P = 0.0007$ for males). A total of 43.5% of the *D. suzukii* adults died on food containing a standard dose of moldex within 2 weeks (Figure 1A), demonstrating that moldex is toxic to *D. suzukii*. Female and male flies died more on a diet with normal propionic acid relative to one without propionic acid (Figure 1B, $P = 0.0313$ for females and $P < 0.0001$ for males), indicating that the usual concentration of propionic acid was also toxic to *D. suzukii*. There was no significant difference in survival curves between diets with and without phosphoric acid (Figure 1C, $P = 0.119$ for females and $P = 0.298$ for males). Based on the results above, we removed propionic acid and adjusted the concentration of moldex in the food (reduced by half). Moldex was not eliminated from the food, as *D. suzukii* died much faster on a diet completely lacking in moldex (Figure 1D, $P < 0.0001$ for both females and males). This was probably due to microbial overgrowth on food (fungal threads were detected on the surface of the diet), suggesting that moldex is indeed needed to suppress the overgrowth of environmental microbes.[26] We determined that the optimized concentration of preservatives for rearing *D. suzukii* (supporting maximal survival) was 0.13% (w/v) moldex and 0.498% (v/v) phosphoric acid (Table 2). In addition, when placed on this optimized diet, *D. suzukii* developed much faster and produced significantly more adult offspring than when reared on the classical diet A (Figure S1).

Next, the sources of sugar and proteins were evaluated to check whether they were important for *D. suzukii* health. Using the optimized concentration of moldex and phosphoric acid, we tested different diets varying the type of sugar used as well as the protein source (sucrose-based diet, glucose-based diet, and corn syrup-based diet) (Table 2). We found that variation in these nutrient sources had no impact on *D. suzukii* survival (Figure 1E). We chose a sucrose-based diet (diet I) as a viable diet for future survival assays (Table 2) as glucose could influence the pathogenicity of certain bacteria.[35]

3.3 Pathogenicity of isolated microbes to *Drosophila* after systemic infection

Using our optimized diet, we next proceeded to evaluate the pathogenicity of our isolated microbes against both *Drosophila* species. *Drosophila* possess a powerful antimicrobial response and most microbes are rapidly eliminated when injected, leading to no detectable change in survival.[36] A first prerequisite for a microbe to be a potent entomopathogen is for it to be able to overcome these defenses and kill the fly when injected. We therefore decided to measure survival of flies after septic injury as a proxy for pathogenicity. In this study, we considered a microbe to be pathogenic if infection with this microbe was associated with significantly fewer survivors than uninfected controls (threshold of killing >25% flies at day 7 post infection and a *P*-value of 0.05 compared with the control). Survival was estimated in both *D. suzukii* and *D. melanogaster* to determine the specificity (or lack thereof) of microbes that would be pathogenic towards *D. suzukii* (Table 3).

As a first assessment of microbial pathogenicity, we first bypassed all natural barriers and injected our isolated microbes into both *D. suzukii* and *D. melanogaster* adults. Analysis of survival showed that of 25 microbes isolated from *D. suzukii*, only eight

TABLE 3. List of isolated microbes from wild *Drosophila suzukii*

Bacteria (Gram negative)	Bacteria (Gram positive)	Fungi
Achromobacter spanius	*Bacillus pseudomycoides*	*Candida cylindracea*
Alcaligenes faecalis	*Corynebacterium glycinophilum*	*Candida oleophila*
Delftia tsuruhatensis	*Lactococcus lactis* subsp. *hordniae*	*Candida railenensis*
Enterobacter asburiae	*Leucobacter tardus*	*Cladosporium cladosporioides*
Erwinia aphidicola	*Paenibacillus taichungensis*	*Geotrichum candidum*
Escherichia hermannii	*Staphylococcus saprophyticus*	*Hanseniaspora uvarum*
Pseudomonas parafulva		*Metschnikowia* sp.
Serratia marcescens subsp. *sakuensis*		*Penicillium commune*
		Pichia kluyveri
		Rhodotorula glutinis
		Torulaspora delbrueckii

TABLE 2. Detailed recipes of different food diets corresponding to Figure 1(E)[a]

Diet code[b]	H	I	J
Yellow cornmeal (w/v)	0	6%	6.70%
Light corn syrup (v/v)	0	0	7.10%
Soy flour (w/v)	0	0	0.90%
Sucrose (w/v)	0	4%	0
Glucose (w/v)	8.20%	0	0
Yeast (w/v)	8.20%	5%	1.60%

[a] Other ingredients: agar, 0.7%, moldex, 0.13%; phosphoric acid, 0.498%.
[b] Cources of the ingredients: yellow corn meal, Aunt Jemima; light corn syrup, Karo; soy flour, Bob's Red Mill; sucrose and glucose, VWR; yeast, MP Biomedicals; agar, MoorAgar Inc; moldex and phosphoric acid, Sigma-Aldrich Corp.

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FIGURE 2. Survival of *Drosophila melanogaster* (*Dmel*) and *Drosophila suzukii* (*Dsuz*) male flies after septic injury with bacteria isolated from wild-caught *D. suzukii*. Infection with (A) Gram-negative bacteria and (B) Gram-positive bacteria ($OD_{600} = 5$, $\sim 5 \times 10^8$ bacterial cells per ml). Survival curves represent average percent survival and gray ribbons represent 95% confidence intervals. Stars between survival curves indicate a significant difference between *D. melanogaster* and *D. suzukii*. *P*-values on each figure indicate the difference between bacterial infection and aseptic injury (clean prick). *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

were able to act as pathogens when injected. More precisely, three Gram-negative bacteria (*Achromobacter spanius*, *Alcaligenes faecalis* and *Serratia marcescens*) caused complete mortality of all *D. suzukii* and *D. melanogaster* flies within 24 h after systemic infection (Figure 2A). Three Gram-positive bacteria (*Corynebacterium glycinophilum*, *Lactococcus lactis* and *Staphylococcus saprophyticus*) were mildly pathogenic, killing 38–59% of *D. suzukii* and 28–33% of *D. melanogaster* flies after 7 days of infection (Figure 2B). None of the other isolated bacteria affected fly survival. Moreover, of the isolated fungi, only *Candia cylindracea* and *Rhodotorula glutinis* could be considered pathogenic to *D. suzukii*. All *D. melanogaster* flies had a >75% survival rate after fungal infection (Figure 3), suggesting that *D. suzukii* may be more sensitive to infection with fungi than *D. melanogaster*.

3.4 Bacteria that are pathogenic display uncontrolled growth in their host

To understand what explains the acute killing of *D. suzukii* by *Ac. spanius*, *Al. faecalis* and *Se. marcescens*, we first infected flies with multiple doses of each pathogen. The results demonstrate that *D. suzukii* and *D. melanogaster* adults succumbed to infection with all the three pathogens following septic injury, independent of the initial inoculum ($OD_{600} = 0.1$, ~ 150 bacteria per fly) (Figure 4A). However, the dose of bacteria injected influenced the time to death, with the higher dose used for infection associated with a faster death (Figure 4A). In addition, the number of bacteria in individual flies increased continuously from $\sim 10^2$–10^3 at the start of the infection to $\sim 10^5$–10^6 at 16 h after infection, demonstrating that neither *D. suzukii* nor *D. melanogaster*

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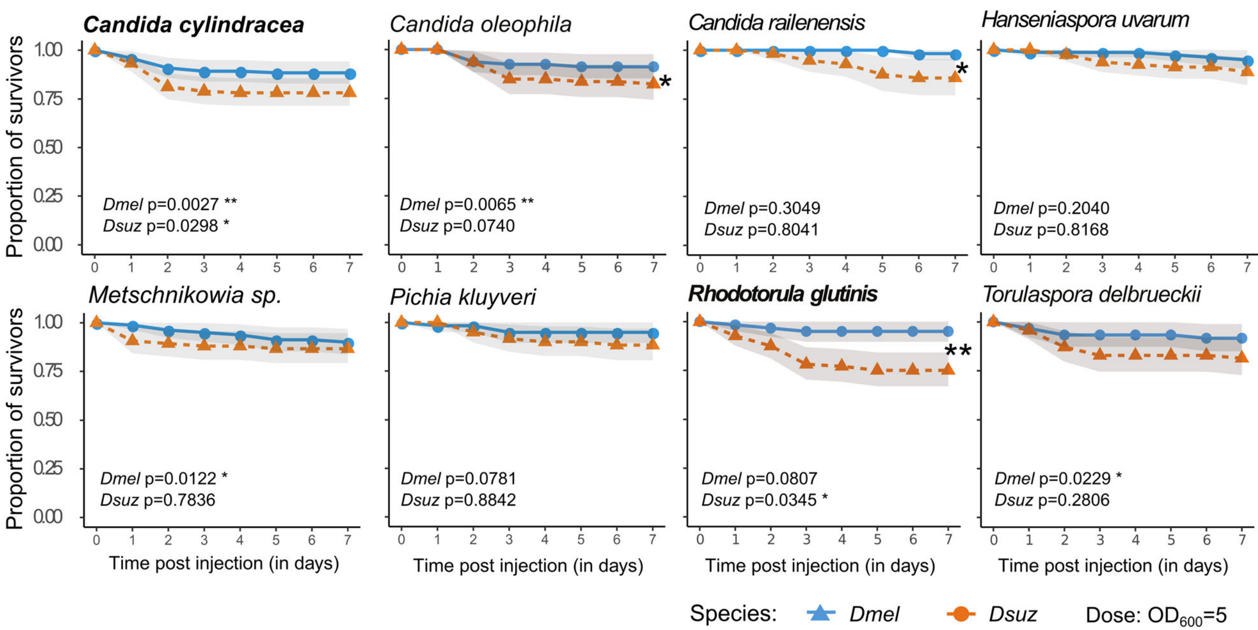

FIGURE 3. Survival of *Drosophila melanogaster* (*Dmel*) and *Drosophila suzukii* (*Dsuz*) male flies after septic injury with fungi isolated from wild-caught *D. suzukii* ($OD_{600} = 5$, $\sim 5 \times 10^6$ fungal cells per ml). Survival curves represent average percent survival and gray ribbons represent 95% confidence intervals. Stars between survival curves indicate a significant difference between *D. melanogaster* and *D. suzukii*. *P*-values on each figure indicate the difference between bacterial infection and aseptic injury (clean pricking). **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001; *****P* ≤ 0.0001.

could suppress bacterial growth (Figure 4B). These results suggest that infection with *Ac. spanius*, *Al. faecalis* and *Se. marcescens* is associated with a massive septicemia that results in the death of *Drosophila* and indicate that these three bacteria are able to grow despite the immune response of *Drosophila*.

3.5 Pathogenicity of microbes to *Drosophila* upon ingestion

For biocontrol strategies to be efficient, microbes must be infectious to hosts through natural routes of infection (feeding or surface exposure for instance). We next tested whether the pathogenic bacteria identified in our screen would behave as pathogens outside the context of a septic injury. Considering that two of the three pathogenic microbes were isolated from surface-sterilized flies, this could suggest that they were residing in the gut compartment. We therefore fed *D. suzukii* and *D. melanogaster* adult flies with two doses of isolated microbes (food contaminated with a bacterial suspension at $OD_{600} = 10$ or 200) and monitored their survival after ingestion. Orally administered bacteria and fungi at $OD_{600} = 10$ were not pathogenic to *D. suzukii* or *D. melanogaster* (Figures S2 and S3). With a higher dose of $OD_{600} = 200$, only *Ac. spanius* was pathogenic and caused 38% mortality in *D. melanogaster* at 7 days after feeding (Figure 5). These results demonstrate that none of the isolated microbes behaved as an oral pathogen to *D. suzukii* adults (Figures 5 and 6).

3.6 Pathogenicity of fungi to *Drosophila* upon spore infection

Three species of spore-forming fungi (*Cladosporium cladosporioides*, *Geotrichum candidum* and *Penicillium commune*) were isolated from *D. suzukii*. Fungi have the ability to infect by crossing the cuticle or peritrophic matrix of flies when germinating. We therefore tested whether exposure to spores of these fungi would lead to a decrease in host survival. None of our three isolated fungi were pathogenic to either *D. suzukii* or *D. melanogaster* after spore infection (Figure 7). To make sure that our infection method was effective, we decided to include a positive control and evaluated the pathogenic impact of fungi that have been reported to behave as entomopathogens on *D. suzukii*. Infection with *M. anisopliae* caused 71% and 56% mortality to *D. suzukii* and *D. melanogaster*, respectively, indicating a strong pathogenicity to both species. In addition, *Be. bassiana* killed ~ 56% of *D. suzukii* adults and 24% of *D. melanogaster* in 7 days after infection (Figure 7). Taken collectively, our data confirmed that *Metarhizium* and *Beauveria* species are pathogenic to *D. suzukii* and validated that none of our isolated spore-forming fungi were pathogenic to either *D. suzukii* or *D. melanogaster* via spore infection.

3.7 Pathogenicity of microbes to *Drosophila* larvae upon ingestion

We identified three microbes that are pathogenic to *D. suzukii* by injection but not when fed to adult *D. suzukii*. However, the developmental stage at which *D. suzukii* are problematic for crops is the larval stage, and most oral pathogens of insects act in the larval digestive tract rather than in adults,[37] partly due to larvae's limited ability to repair the midgut tissue.[38] Therefore, we decided to test whether our candidate microbes would affect larval survival/development following oral infection. All three species of bacteria (*Ac. spanius*, *Al. faecalis* and *Se. marcescens*) caused significantly higher mortality in *D. melanogaster* larvae than uninfected controls. Mortality was 23.3%, 25%, 31.7% and 8.3% for *Ac. spanius*, *Al. faecalis*, *Se. marcescens* and control, respectively (Figure 8). *Al. faecalis* and *Se. marcescens* also showed pathogenicity towards *D. suzukii* larvae, with 43.3% 55%, and 28.3% of mortality for *Al. faecalis*, *Se. marcescens* and control, respectively) (Figure 8). In general, we observed that *D. suzukii* larvae had a higher mortality rate than *D. melanogaster* after infection, as the survival of

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FIGURE 4. Dose response and within-host bacterial growth of three microbes acutely pathogenic to *Drosophila suzukii* after septic injury. (A) Survival of *Drosophila melanogaster* (*Dmel*) and *D. suzukii* (*Dsuz*) male flies after septic injury with different doses of bacterial pathogen (OD_{600} = 0.1, 0.5, 5). Survival curves represent average percent survival and gray ribbons represent 95% confidence intervals. Stars on the bars indicate a significant difference between curves of bacterial doses. (B) Bacterial load (colony-forming units per fly) of acute pathogens in *D. melanogaster* (*Dmel*) and *D. suzukii* (*Dsuz*) flies after septic injury (OD = 0.5, ~ 5×10^7 bacterial cells per ml). The numbers in gray boxes are hours after pricking. n = 15–16. Box plot and dot plot are shown. Stars showed the difference between time post pricking and time 0 by linear model analysis. **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

unchallenged and challenged *D. suzukii* larvae was much lower than that of *D. melanogaster* (Figure 8, $P < 0.0001$). Taken together, our results show that although none of our isolated microbes were pathogenic to adult flies when ingested, oral infection with three new bacterial species led to a decrease in larval survival.

4 DISCUSSION

In this study, multiple bacteria and fungi were isolated from wild populations of *D. suzukii*. In order to evaluate their toxicity towards *D. suzukii*, we developed an optimized diet for fly rearing and identified eight microbes that can decrease *D. suzukii* survival when injected. Three Gram-negative bacteria were strongly pathogenic upon septic injury and decreased survival of both *D. suzukii* and *D. melanogaster* larvae when fed. As these bacteria were found in wild populations of *D. suzukii*, we propose they could be natural pathogens of *D. suzukii*.

Our study agrees with previous research aiming to capture the diversity of microbes associated with *D. suzukii*. Using a culture-dependent method, we confirmed that *D. suzukii* harbors multiple culturable (14 species) bacteria. The fact that *D. suzukii* harbors diverse microbes agrees with studies of microbes associated with *D. suzukii* that were performed using pyrosequencing of 16S rRNA sequences.[39-41] In addition, we observed that Saccharomycetales yeast was prevalent in wild *D. suzukii*. This result is also consistent with a previous independent investigation,[42] which reported that *H. uvarum*, *Pichia* and *Metschnikowia* are dominant fungi in wild

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FIGURE 5. Survival of *Drosophila melanogaster* (*Dmel*) and *Drosophila suzukii* (*Dsuz*) male flies after ingestion of isolated microbes. Infection with (A) Gram-negative bacteria or (B) Gram-positive bacteria ($OD_{600} = 200$, $\sim 2 \times 10^{10}$ bacterial cells per ml). Survival curves represent average percent survival and gray ribbons represent 95% confidence intervals. Stars between survival curves indicate a significant difference between *D. melanogaster* and *D. suzukii*. *P*-values on each figure indicate the difference between bacterial infection and unchallenged control (PBS feeding). $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

D. suzukii adults and larvae. Of note, nine species of the microbes we isolated were identified in surface-sterilized flies, suggesting they were either in the gut or in hemolymph. This suggests that in nature, these microbes have ways to associate with *D. suzukii* naturally, however further work is required to establish this.

We identified an important limitation to laboratory studies that deal with *D. suzukii* development and fitness. Classical fly diets show some toxicity to *D. suzukii*. In particular, the concentrations of moldex and propionic acid used in classical *Drosophila* diets are toxic to *D. suzukii*. It was previously reported that propionic acid could effectively repel and kill wheat weevil (*Sitophilus granarius*) and rice weevil (*Si. oryzae*) adults by fumigation.[43] The combination of propionic acid and diatomaceous earth showed high efficiency in controlling populations of rice weevil and saw-toothed grain beetle (*Oryzaephilus surinamensis*) and maintaining grain conservation and rice quality.[44] Moldex and propionic acid are commonly added as supplements to suppress the overgrowth of bacteria or fungi in *Drosophila* diets.[45, 46] The diversity of *Drosophila* microbes decreased significantly after living on diets supplemented with moldex and propionic acid,[26] which may affect host development and fitness.[47, 48] However, we discard this hypothesis in the case of *D. suzukii*, as we previously observed that *D. suzukii* survived better without microbes on a nutrient rich sucrose-yeast food.[21] Moldex is a relatively safe supplement for humans and is commonly used in alimentary products, suggesting it could be used as a control molecule against insects as it is

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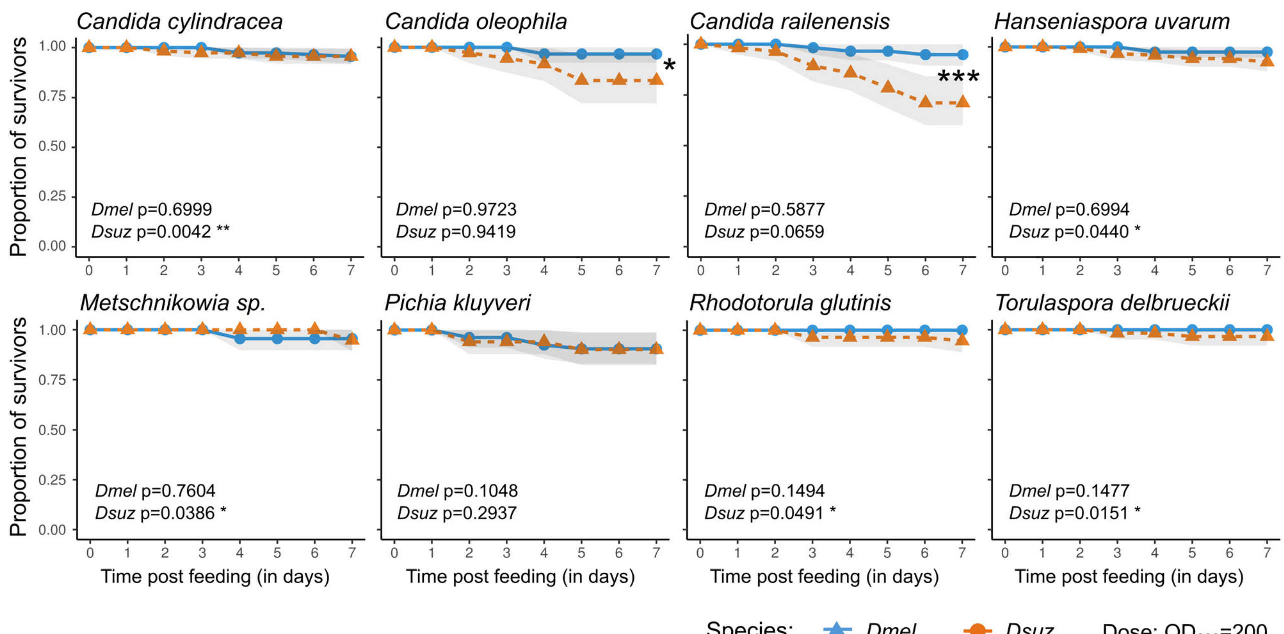

FIGURE 6. Survival of *Drosophila melanogaster* (*Dmel*) and *Drosophila suzukii* (*Dsuz*) male flies after ingestion of isolated yeasts ($OD_{600} = 200$, $\sim 2 \times 10^8$ fungal cells per ml). Survival curves represent average percent survival and gray ribbons represent 95% confidence intervals. Stars between survival curves indicate a significant difference between *D. melanogaster* and *D. suzukii*. *P*-values on each figure indicate the difference between bacterial infection and unchallenged control (PBS feeding).

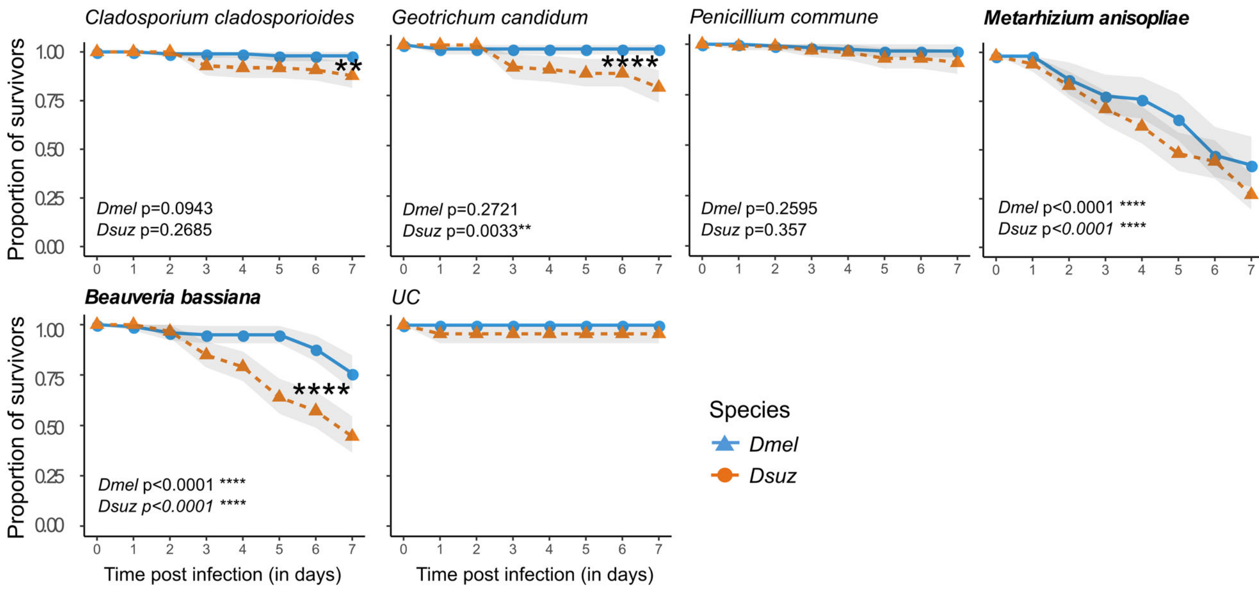

FIGURE 7. Survival of *Drosophila melanogaster* (*Dmel*) and *Drosophila suzukii* (*Dsuz*) male flies after exposure to fungal spores. Survival curves represent average percent survival and gray ribbons represent 95% confidence intervals. Stars between survival curves indicate a significant difference between *D. melanogaster* and *D. suzukii*. *P*-values on each figure indicate the difference between fungal infection and unchallenged control. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

against mold.[49] Further studies on moldex and propionic acid are encouraged to fully elucidate the mechanism behind their action in *D. suzukii* and guide their application as insecticides.

Our screening of microbes by septic injury identified three microbes that can be pathogenic to *D. suzukii*, which are *Ac. spanius*, *Al. faecalis* and *Se. marcescens*. After bacterial inoculation, the number of these bacteria increased by many thousand-fold in 16 h and caused 100% mortality of all flies within 24 h, demonstrating that these bacteria are highly infectious to *D. suzukii*. Considering that these microbes were not pathogenic to adults since they were isolated from field caught adult flies, we propose that they could behave as opportunistic pathogens in the wild. In that case, injuries, mite bites or damage/leaking of the gut epithelium could lead to systemic dissemination of these microbes and disease initiation. *Achromobacter* and *Alcaligenes* bacteria belong to the Alcaligenaceae family and are usually

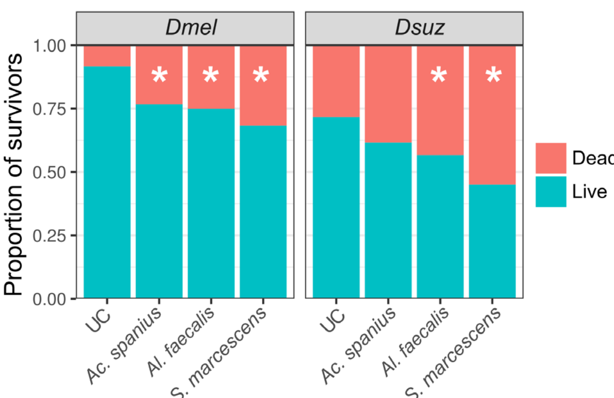

FIGURE 8. Survival of *Drosophila melanogaster* (*Dmel*) and *Drosophila suzukii* (*Dsuz*) larvae to microbial ingestion. Stars on the bars indicate a significant difference between bacterial infection and unchallenged control (UC) in a chi-square test. $^{*}P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$; $^{****}P \le 0.0001$.

found in soils,[50] as well as the sputum of cystic fibrosis patients.[51, 52] In addition, *Achromobacter* has been found in forest cockchafer (*Melolontha hippocastani*), fire ant (*Solenopsis invicta*) and one strain (w1118) of *D. melanogaster*.[53-55] This study demonstrated for the first time that *Achromobacter* can behave as a pathogen of *Drosophila*. However, the mechanism of its pathogenicity is unknown and requires further studies.

In addition to environmental and patient samples, *Alcaligenes* has been reported to be isolated from variegated grasshopper (*Zonocerus variegatus*),[56] honeycomb moth (*Galleria mellonella*),[57] and the entomopathogenic nematode (*Rhabditis blumi*).[58] Quiroz-Castañeda *et al*. showed that 96% of the sixth-instar larvae of honeycomb moths were killed in 24 h by *Al. faecalis* systemic infection,[57] whereas Park *et al*. reported < 30% mortality rate of fourth-instar larva after 48 h.[58] In this study, we observed that *Alcaligenes* was pathogenic to adult *Drosophila* flies. Genome analysis revealed that *Al. faecalis* encodes a protein that is highly similar to the HIP57 protein of *Xenorhabdus nematophila*,[57] an entomopathogenic bacterium symbiotically associated with insect pathogenic nematodes.[59] Injecting the *X. nematophila* HIP57 protein caused dose-dependent mortality of honeycomb moth larvae, activated the phenoloxidase cascade and turned the dead honeycomb moth larvae black.[60] Whether a similar mechanism would be observed in the *Alcaligenes* isolated from *D. suzukii* remains to be explored.

Serratia marcescens belongs to the Yersiniaceae family and is one of the predominant *Serratia* species isolated from insects.[61] *Se. marcescens* secretes various types of extracellular enzymes including chitinases, metalloproteases, serine proteases and Serralysin-like proteins, which are considered as pathogenic factors to insects.[62, 63] *Se. marcescens* causes rapid death upon entering the hemocoel, but only a few strains are pathogenic to insects through the oral route.[64] One exception was the *Se. marcescens* strain DB11, which caused death to *D. melanogaster* adults after they were continuously fed a solution containing only resuspended bacteria and sucrose for 5 days.[64] An explanation of this phenomenon could be that some *Se. marcescens* strains may not be able to cross the peritrophic matrix, a key insect gut barrier. The peritrophic matrix separates immune-reactive epithelial cells from microbes present within the luminal contents and prevents the damaging action of pore-forming toxins on intestinal cells.[65] Bioinformatic analysis showed that the top blast hit of *Serratia* in this study was *Se. marcescens* subsp. *sakuensis*, which was reported to form spores.[66] It is not known whether producing spores changes the oral pathogenicity of *Se. marcescens* subsp. *sakuensis*. We did not observe any high mortality rate of flies after feeding with *Se. marcescens* in our study.

Although the entomopathogenic bacteria identified in this study are not pathogenic via oral infection to adults, some are pathogenic towards larvae. It is noteworthy that all three pathogens isolated from *D. suzukii* in this study are opportunistic human pathogens.[67] However, this should not be a serious obstacle to their potential to be used as pesticides. For example, *Ba. thuringiensis* (Bt) is allegedly an opportunistic pathogen under appropriate conditions in animals and humans,[68] but is still a widely used biocontrol agent.[69] Some genetically modified crops that express Bt toxins have showed great success in controlling pests. Understanding the mechanisms by which these pathogens kill *D. suzukii* may also help identify more insect specific toxins for *Drosophila* pest control.

Yeasts, which are commonly known as food for *Drosophila* adults and larvae,[48, 70] were not pathogenic to *D. suzukii* in this study. Although their oviposition behaviors differ, both *D. suzukii* and *D. melanogaster* are shown to be attracted to yeast-derived volatiles (like *H. uvarum*, *Pichia* sp., *Candida* sp., and *Sa. cerevisiae*).[71] Mori *et al*. reported that mated *D. suzukii* female flies preferred to feed on *H. uvarum* and the presence of *H. uvarum* with insecticide spinosad increased female mortality.[72] Interestingly, adding *Sa. cerevisiae* and cane sugar to the insecticides cyantraniliprole and spinosad reduced *D. suzukii* egg densities.[73] Lewis and Hamby found that *D. suzukii* larvae exhibited a strong attraction to *H. uvarum* though larvae also performed most poorly on diets containing *H. uvarum*.[74] Because *H. uvarum* has a better attractive efficiency than *Sa. cerevisiae* to *D. suzukii*, it is worth testing whether the addition of *H. uvarum* and sugar to pesticides increases pest control efficiency under field conditions.

Finally, we confirmed the strong pathogenicity of entomopathogenic fungi *Be. bassiana* and *M. anisopliae* in our study. Mortality > 50% was observed in *D. suzukii* adults treated with *Be. bassiana* and *M. anisopliae* compared with 13% in controls for *D. suzukii* and 0% in controls for *D. melanogaster*. Previous studies have shown a lot of variation in efficacy between different strains and between different application methods.[18, 19] These variations are likely due to differences in treatment methods applied as well as the strains used, because different isolates of an entomopathogenic fungus may vary in virulence to the same host species. Woltz *et al*. found that after spraying *M. anisopliae*, infected flies continued to lay eggs until they died, resulting in no change in *D. suzukii* fecundity.[19] Therefore, the application of *Be. bassiana* and *M. anisopliae* for *D. suzukii* control requires more optimization before any conclusions can be made. As our studies are based on representative colonies of our isolated microbes, it is also possible that other colonies would show different levels of pathogenicity. Clarifying the responses of *D. suzukii* to those pathogens as well as the mechanisms by which pathogens kill the flies will provide us with good targets for application.

To conclude, we identified three bacterial pathogens (*Ac. spanius*, *Al. faecalis* and *Se. marcescens*), which are naturally infectious to *D. suzukii*. All three bacteria were able to kill adult flies after septic injury and decrease the emergence rate of larvae after ingestion. We also demonstrated the potential of using two preservatives, moldex and propionic acid, as well as the entomological fungal pathogens *Be. bassiana* and *M. anisopliae* for *D. suzukii* control. Future studies will evaluate whether these can be used in control strategies in field scaled studies.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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