Pathogenic fungi-induced susceptibility is mitigated by mutual Lactobacillus plantarum in the Drosophila melanogaster model

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Abstract

Background Animals frequently encounter a variety of harmful fungi in the wild, but their ability to develop sophisticated anti-fungal strategies allows them to flourish across the globe. Extensive studies have highlighted significant involvement of indigenous microbial communities in host health, but the daunting complexity of microflora has hampered our understanding of the intricate relationships among them. In this work, we sought to develop a bacterium-fungus-Drosophila model that offered a model to systematically evaluate the anti-fungal effects of commensal bacteria. Results We isolated a pathogenic fungal strain, Diaporthe FY, that was detrimental to the survival and development of Drosophila upon infection. Using Drosophila as a model system, Drosophila-associated Lactobacillus plantarum functioned as a probiotics, and protected flies from mortality induced by Diaporthe FY. Our results shown that L. plantarum hindered the growth of Diaporthe FY in vitro, and decreased the mortality rate of Diaporthe FY-infected flies in vivo, therefore consequently mitigating the toxicity of Diaporthe FY to hosts. In addition, L. plantarum overrode the avoidance of oviposition on Diaporthe FY-associated substrates. Conclusions Diaporthe FY was identified as a potential pathogen to Drosophila. Commensal L. plantarum mitigated the pathogenic fungi-induced susceptibility in Drosophila, providing an insight into the natural interplays between commensal and pathogenic microbial communities that contribute to animal health and pathogenesis.

Background

Metazoans harbor a plethora of indigenous microbes (collectively referred to as the microbiota) that routinely influence the physiology and fitness of their host[1, 2], while hosts, in turn, shape the gut microbiota. Their forging symbiosis enables hosts to outcompete a variety of pathogens in the environment. In fact, many host phenotypes are largely shaped by the combination of genome and microbiome[3, 4]. Consequently, commensals are critically linked to host fitness, such as development, fecundity, and lifespan. However, host phenotypes were traditionally assessed in the context of their microbiota, and little attention has been devoted to effects of the microbiota on host fitness. In particular, the underlying mechanisms that the microbiota promote their hosts to combat pathogens are largely undefined.

In the wild, the *Drosophila* population mainly feed and breed on rotting fruits that are inhabited by both mutualistic and antagonistic microbes[5, 6]. Owing to their saprophagous foraging behavior, they inevitably ingest many potentially pathogenic fungi from either food resources or the surrounding environment[7, 8]. Although most microbes are not pathogenic[9], pathogens indeed engender the occurrence and severity of infection of the fly. Antagonistic fungi generate an astonishing variety of secondary metabolites that threaten insects[10–12]. In addition, plant thorn injury and ectoparasitic mite biting frequently result in cuticle breaches, which aggravates the fungal infection. However, flies fundamentally employ antifungal strategies in order to flourish in the wild[7]. Extensive studies have revealed that the microbiota properly promote immune system response to pathogenic fungi development, and restrict pathogen colonization[2, 13–14]. However, the roles of specific bacterial

species and/or strains in combating with pathogens are still poorly understood, highlighting the need of model organism to examine the intricate interconnections of hosts, commensals and pathogens.

Drosophila frequently acquires commensals through plant food, and provides amenable insights into the mechanisms that commensals outcompete pathogens, due to genetic tractability and the ease to generate gnotobiotic animals[15, 16]. *Lactobacillus* genusis one of most common bacteria present in appreciable numbers in both mammals and *Drosophila*[6]. Studies show that *L. plantarum* fully recapitulates the beneficial effects of a more complex microbiota, and influences several aspects of host physiology, including behaviors, gut epithelial homeostasis, nutrition and postembryonic development[13, 17–19]. Moreover, *L. plantarum* is required to protect flies against fly infection induced by food-borne bacteria, like *Pectobacterium carotovorum*[20]. Current molecular and genomic studies highlight the opportunities and challenges in studying the interactions of entomopathogenic fungi and fly hosts. However, it remains unknown whether *L. plantarum* could protect hosts from pathogenic fungal infections.

To tackle these biological questions, we developed the *Drosophila* /bacterium/fungus ecological system that afforded the examination of the antagonist of commensals against pathogenic fungi. We found that commensal *L. plantarum* mitigated the pathogenic fungi-induced susceptibility in *Drosophila,* providing an insight into the ecological significance that commensal bacteria could be an integral contributor to *Drosophila* fitness upon infection.

Results

Isolation and identification *Drosophila*-associated fungi

A strain of *Drosophila*-associated fungus was isolated from fly food with molds in accordance to standard protocols. This strain was a typically filamentous fungus with white, velvety-like mycelia and dark grey conidial masses (Fig. 1 A and B). The conidiophores were bi-verticillate with smooth-walled stipes, bearing short conidial chains (Fig. 1C). It promptly grew at the optimal temperature of 28–30° C and filled with the plates (Φ = 90 mm) within 48 h. To confirm the reliability of morphological identification, the strain was subjected to molecular identification based on rDNA ITS sequence analysis. Based on the BLASTn search, it displayed >99% similarity with a published sequence of *Diaporthe sp.* (identities = 555/558) and was relatively close to other *Diaporthe* members. To distinguish our isolate from other strains, it was henceforth nominated as *Diaporthe* FY. For taxonomic reconstructions, the further 12 sequences, including out-group species, were retrieved from GenBank to generate a phylogenetic tree (Fig. 1D). *Diaporthe* species are among the most frequent endophytes of a broad arrange of plants, including grapevines[21, 22]. Owing to the saprophytic foraging behavior, flies might ingest *Diaporthe* from either food or the surrounding resources.

Diaporthe FY *is the potential pathogen for D. melanogaster*

Drosophila frequently encounter a variety of commensal or pathogenic microbes in the wild. We first asked whether *Diaporthe* FY was beneficial or detrimental to *Drosophila.* To address this question, we examined the developmental timing and survival rate of flies challenged with *Diaporthe* FY. Dechlorinated eggs were used to generate the specific interaction of hosts and specific microbes as previously described[23]. As a result in Fig. 2A, hatched larvae rapidly succumbed to the infection of *Diaporthe* FY in vials with more than 4×10^6 CFU spores. The eclosion duration of flies infected with *Diaporthe* FY was extended compared to conventionally reared (CR) flies (Fig. 2A). This implied that *Diaporthe* FY impeded the normal development of *Drosophila.* Consistent with one previous study[24], *Drosophila* was susceptible to *Aspergillus flavus (*Suppl. 1A), suggesting that pathogenic fungus-induced morbidity of *Drosophila* could be generated in our laboratory. Intriguingly, the developmental time of adults challenged with less than 4×10^6 CFU spores was shorter than that of germ free (GF) flies (Fig. 2A). It could be partly explained by the fact that fungi could produce very low concentrations of toxic secondary metabolites at the exponential phase of nutrition growth, and thus its growth-promoting effects would override the inhibition of host development. Additionally, the survival of CR and GF adults fed with *Diaporthe* FY molds was significantly lower than that of mocked flies (Fig. 2B), suggesting that *Diaporthe* FY reduced the relative survival of flies. Moreover, innate immunity-associated genes weresignificantly triggered in *Diaporthe* FY-infected flies compared to their counterparts (Fig. 2C), indicating that the flies developed a robust immune response to this invader. In agreement with a previous study[25], immune deficient *PGRP-LC* mutant flies were much more susceptible to *Diaporthe* FY than wild-type flies(Fig. 2B), indicating that *Diaporthe* FY was a potentially virulent pathogen to *D. melanogaster.* Hence, we subsequently investigated the survival to septic injury by injecting spores of *Diaporthe* FY into the body cavity of flies. Concomitant to the oral infection, the *Diaporthe* FY-infected flies were more likely to die from infection compared to flies without infection (Fig. 2D), indicating that *Drosophila* was susceptible to *Diaporthe* FY. Collectively, these results suggested that *Diaporthe* FY acted as one of the *Drosophila*-associated pathogens.

L. plantarum undermines the infection of Diaporthe FY *to fly*

Given that pathogenic fungal infections impose morbidity and mortality upon animals in the wild, it was proposed that natural microbiota could promote the survival of flies challenged with *Diaporthe* FY[26]. Previous studies showed that *L. plantarum,* notably due to its vast metabolic repertoire, fostered the development of hosts by accelerating their growth rate[17]. We then examined its antifungal combat against *Diaporthe* FY by simultaneously inoculating them in sterilized *Drosophila* GF eggs. Indeed, the supplement of *L. plantarum* efficiently rescued the lethality of *Diaporthe* FY-infected flies, and ameliorated the delay of pupa formation and adult eclosion (Fig. 2E and F). This result suggested that *L. plantarum* mitigated *Drosophila* susceptibility to *Diaporthe* FY.

L. plantarum suppresses the growth of Diaporthe FY

To validate that *L. plantarum* competes with *Diaporthe* FY, we set out to test the inhibition of *L. plantarum* in the growth of *Diaporthe* FY *in vitro.* Our data showed that *L. plantarum* outcompeted *Diaporthe* FY in a dosage-dependent manner (Fig. 3A). We quantified the effects of suppression by the means of colony growing, mycelia branching and spore forming. Firstly, the colony growth of *Diaporthe* FY was hampered by *L. plantarum* compared to control (Fig. 3B and C). Secondly, the number of mycelia was lowered in the presence of *L. plantarum* (Fig. 3D). In addition, the number of spores was dramatically decreased by *L. plantarum* inoculation (Fig. 3E). Taken together, these results suggested that *L. plantarum* potently reduced the survivability of *Diaporthe* FY.

We further speculated whether *L. plantarum,* after dominating the niche, could thwart colonization by *Diaporthe* FY. To this end, we pre-incubated the diet with *L. plantarum* for different lengths of time (24 h, or 48 h) and then added *Diaporthe* FY onto the "modified" diet. In congruence with the simultaneous competition, the growth of *Diaporthe* FY was hindered in pre-inoculation of the diet with *L. plantarum.* In fact, the longest incubation period completely blocked the growth of *Diaporthe* FY (Fig. 3F). This inhibitory effect was further fortified by the decreased number of mycelia and spores (Fig. 3G and H). Taken together, our current study supported that *L. plantarum* inhibited the growth and dispersal of *Diaporthe* FY.

L*actic acid inhibits the growth of Diaporthe FY*

To further characterize the inhibition, we next sought to identify candidate inhibitory factors derived from *L. plantarum* metabolites. Lactic acid is generated by many lactic acid bacteria, and exerts its antimicrobial effects through disrupting the cytoplasmic membrane or reducing the intracellular pH[27]. Since the strain of *L. plantarum* used in this study typically produced more than 75 mM (~0.7% w/v) of L-lactate at the end of fermentation (Suppl. 2), we therefore focused on the role of L-lactate in inhibiting the growth of *Diaporthe* FY. To determine whether lactic acid could inhibit the growth of *Diaporthe* FY, we scored the fungal growth on the medium supplemented with L-lactate at different concentrations. The result showed that the growth of *Diaporthe* FY was inhibited in a L-lactate dosage-dependent manner (Fig. 4A). *Diaporthe* FY was modestly inhibited by 0.5% lactic acid, and completely inhibited by 1% or more lactic acid. It was unlikely that this antifungal property was derived from the lower pH value, because the comparable pH values adjusted with HCl were unable to inhibit the growth of *Diaporthe* FY (Suppl. 3). These data indicated that the inhibition of *Diaporthe* FY was partly attributed to the properties of lactic acid. The data further showed that the colony growth of lactic acid-treated *Diaporthe* FY was prominently decreased compared to mocked flies (Fig. 4B). Likewise, the numbers of mycelia and hyphae were notably lowered in the case of lactic acid (Fig. 4C and D). Overall, our data suggested that lactate was an important factor that could inhibit the growth of *Diaporthe* FY.

The synergism of Drosophila and L. plantarum to combat Diaporthe FY

Upon pathogenic infection, *Drosophila* initiates innate immune response through the production of reactive oxygen species and antimicrobial peptides. It was assumed that the collaboration of hosts and commensals could be more efficient to resist against pathogenic fungi. To the end, the early third-instar larvae were seeded to fly diet with *Diaporthe* FY and *L. plantarum.* Our data displayed that the colony growth of *Diaporthe* FY wassignificantly obstructed in the presence of larvae compared to that in the absence of larvae (Fig. 5A and C). This result indicated that *Drosophila* and commensals collaborated to antagonize pathogens. Similarly, the number of branching mycelia was declined in the presence of larvae compared to untreated ones (Fig. 5B and C). Intriguingly, *Diaporthe* FY did not form any spores in the case of larvae, partly due to the disrupted configuration of the hypha. These results demonstrated that *Drosophila* synergized with *L. plantarum* to suppress the growth of *Diaporthe* FY, which was critical for host survival against infection.

L. plantarum reverses ovipositional avoidance to *Diaporthe* FY

Using sensory modalities, animals swiftly respond to certain stimuli in their surrounding environment. In order to enhance the survival and fitness of their offspring, *Drosophila* females select favorable sites to deposit their eggs[28, 29]. Our earlier work showed that commensals, like *L. plantarum,* elicited the oviposition preference of *Drosophila* using the two-choice assay as described[23]. Because *Diaporthe* FY imposed morbidity on both *Drosophila* larvae and adults (Fig. 2), it would be reasonable that *Drosophila* could sense the presence of *Diaporthe* FY in potential egg laying sites. As expected, female adults overwhelmingly avoided egg laying on the food treated with *Diaporthe* FY (Fig. 6A). Many molds produce an extraordinary range of secondary metabolites that repel insects[30, 31]. Indeed, flies were robustly repulsed to lay eggs on the surface of halves containing metabolites of *Diaporthe* FY (Fig. 6B), indicating that secondary metabolites of *Diaporthe* FY alerted flies to the presence of toxic molds. We next wondered whether *L. plantarum* could alter the ovipositional repulsion of females to *Diaporthe* FY. As expected, the addition of *L. plantarum* dosage-dependently increased the oviposition index of females, and even switched to laying eggs in fermented food with the predominance of *L. plantarum* (Fig. 6C), indicating that *L. plantarum* attenuated the ovipositional avoidance to *Diaporthe* FY. We further asked whether *L. plantarum* could abolish the ovipositional aversion to *Diaporthe* FY when it had dominated the community. The diet was pre-incubated with *L. plantarum* for different lengths of time and then exposed to *Diaporthe* FY. We found that flies averted to oviposit in fermented food pre-incubated with *L. plantarum* for 24 h, but overrode it in fermented food pre-incubated with *L. plantarum* for 48 h (Fig. 6D). Hence, our results demonstrated that commensals, if dominating the niche, significantly reversed the oviposition avoidance to pathogenic fungi.

Discussion

Animals are colonized by abundant and diverse microbiota that affect many aspects of host physiology and pathology[32, 33]. Despite advances in sequence-depended microbial profiling, little is known about

the role of mutualistic microbes in antagonizing fungal pathogens for the host animals. Our study has shown that *Drosophila*-associated commensal bacteria exhibited the inhibitory capabilities against fungal infections. Commensal *L. plantarum* suppressed the growth of *Diaporthe* FY *in vitro,* and mitigated the fungal toxicity to *Drosophila in vivo.* Moreover, *L. plantarum* predominantly overrode the egg laying avoidance of *Drosophila* to *Diaporthe* FY. This integrative and synthetic community of *Drosophila,* bacterium and fungus sheds light on fundamental concepts and precise mechanisms involved in animal-commensal-pathogen interactions. The infection-associated *Drosophila* model was previously established by injecting a lethal dose of pathogens into the body cavity of adult flies[34]. However, little attention has been devoted to roles of complex bacterial communities. In fact, a bacterial consortium approach that views the microbiome as a set of functional traits is likely to offer a more comprehensive protection of hosts from threatening pathogens. Consistently, the observed phenomenon supports our hypothesis that the protective traits conferred by natural microbiota were naturally selected to boost host survival in the context of a challenging environmental rife with infectious diseases. Thus, our approach should facilitate the development of animal models that better recapitulate complex natural pathological phenomena.

Entomopathogenic fungi have a pivotal role in regulating insect populations in nature[12]. They have evolved highly diversified lifestyles, and are in competition with insects for natural resources. Metagenetic analysis has unraveled dozens of secondary metabolic gene clusters that encode an astonishing variety of secondary metabolites[10, 30, 31]. Although pathogenic fungi seriously threaten the survival of flies in nature, animals fundamentally develop antifungal strategies to thrive in the world. *Drosophila* possess an innate immune system that induces the production of antimicrobial peptides and reactive oxygen species to prevent fungal infection[35]. Alternatively, the mitigation of fungal toxicity is attributed to complex interactions between hosts and microbes. Commensals and/or probiotics outcompete pathogens through chemical inhibition, physical and nutritional competitive exclusion, and a variety of other adaptive mechanisms[36]. Lactic acid bacteria are widely considered to be natural antifungal tools that can be found in fermented substrates[37, 38]. They hamper the growth of many pathogenic fungi by inhibiting their adherence, establishment, replication, various pathogenic actions, and they decompose mycotoxin to a certain extent[39]. In turn, larvae-derived maintenance factors enhance bacterial population propagation, and override this cost of feeding and gut transit, forming an inextricable holobiont[26]. More importantly, adult *Drosophila* acts as a vector, and promotes ongoing dispersal of bacteria in the environment[40]. Therefore, the synergistic interaction of *Drosophila* and microbiota exerts the antifungal activity against a broad spectrum of molds in the wild.

Hosts exhibit behavioral-immune responses against pathogens, but their important roles remains underappreciated[41]. *Drosophila* females have an innate behavior of selecting favorable oviposition sites to increase the survivability of their offspring, as larvae could be vulnerable to predators due to their restricted mobility Selecting a favorite site to lay their eggs is an innate behavior of females, since larvae are vulnerable to predators and have restricted mobility. The hypothesis of ‘mother-knows-best’ ensures that female egg-laying decisions have evolved to find places with the best survival of offspring[42]. They evaluate nutritional and microbial contents of potential oviposition sites. Previous Studies, including our

work, have shown a general theme that *Drosophila* is robustly allured to lay eggs in fermented food by commensal *Lactococcus, Lactobacillus, Weissella* and *Saccharomyces*[23, 43]. However, they was vigorously repelled by harmful molds, such as *Penicillium expansum*[44]. Females avoid laying eggs on the sites with fungal toxicants, which efficiently protects hatched larvae from infection. Survival and reproduction strategies should be employed in the context of systemic ecology, in which flies need to balance the benefits and threats coming from commensals and pathogens, respectively. Thus, *Drosophila* distinguishes commensals from pathogens, and select favorable sites for egg laying. It is conceivable that females still switch to laying eggs on the fermented food, when nutrition of commensals overrides threats from pathogens. It is consistent with the observation that fermented food with *L. plantarum* and *Diaporthe* FY still attract females to lay eggs, partly because *L. plantarum* has dominated the niche and outcompeted *Diaporthe* FY.

Utilizing the *Drosophila* model system, we revealed an ecological phenomenon whereby indigenous microbiota were required to defend *Drosophila* against pathogenic fungal infection. This model could provide a reductionist approach to disentangle the inherent complexity of host-microbe interactions from the organismic to the molecular level. A more complete understanding of the underlying mechanism of host and bacteria against pathogens would facilitate the discovery of innovative probiotic interventions to foster the fitness of microbe-host holobiont.

Methods

Drosophila andmicrobe husbandry

The Oregon R strain of *D. melanogaster* was used as wild-type flies. PGRP-LC mutant flies were kindly gifted by Dr. Zhai (Hunan Normal University, China). All flies were reared at 25 °C, 60% humidity with a 12 h/12 h light/dark cycle on standard cornmeal-yeast-sucrose food unless otherwise stated[23]. *Drosophila* was cultured with standard cornmeal-sugar-agar medium (1 L) (1350 ml ddH_2O, 13 g agar, 0.83 g $CaCl_2$, 31.6 g sucrose, 63.2 g glucose, 77.7 g cornmeal, 24 g yeast power)[28]. Fungi were cultured using Potato Dextrose Agar (PDA) medium at 25 °C. *L. plantarum* were cultured in selective medium De Man Rogosa Sharpe (MRS) at 35 °C. The mixture of *Diaporthe* FY and *L. plantarum* was cultured in Mueller-Hinton Agar (MHA) medium at 30 °C. *L. plantarum* was isolated from the gut of *Drosophila* with Genbank accession number KY038178. *Aspergillus flavus* (3.3950) was obtained from China General Microbiological Culture Collection Center.

Isolation and Identification of fungi

Fungal strains were isolated from fly food with molds using PDA medium. Mycelium was briefly picked up, transferred to PDA medium, and incubated for 48 h at 25 °C. This procedure was repeated five times for purification. The fungus was grown on liquid PDA medium for 2 d at 25 °C. The mycelium was collected, and genomic DNA was extracted and purified using a DNA isolation kit (Tiangen, Beijing, China). For identification, the internal transcribed spacer regions (ITS1 and ITS2) were amplified using PCR (Thermocycler, Germany) with the universal ITS primers, ITS1 (5’ -TCC GTA GGT GAA CCT GCGG–3’)

and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC–3'). The PCR products were sequenced by a commercial company (Shenggong, Shanghai). The DNA sequences of ITS were aligned against the nucleotide–nucleotide database (BLASTn) of the National Center for Biotechnology Information (NCBI) for final identification of the isolates.

The colony growth, branching mycelium, sporulation assay

One μL extract with 10^6 spores was inoculated into MHA medium. To assess the effect of acids on fungi, L-lactate or chloric acid was directly added to medium in a dose-dependent manner. The diameter of fungal colony was measured at the 6 h interval. Sterile coverslips were inserted into the agar medium at an angle of 45°, and the number of branching mycelia on the coverslip was scored using a microscope. To examine the number of spores on the medium, all mycelia growing on the surface of the medium were transferred to 10 ml PBS. The medium was violently shaken, and 100 μl of medium was transferred to the hemocytometer for counting spores with a microscope.

Germ-free and gnotobiotic flies

The process of generating germ free (GF) embryos was done similarly as previously described[15, 23]. Eggs laid on grape juice agar media were collected within 8 h, and rinsed with ddH2O to remove the yeast paste on the surface. Next, the eggs were sequentially subjected to 1:30 diluted sanitizer walch (Procter & Gamble Co., Cincinnati, OH, USA), 2.5% hypochlorite sodium for 1 min (Sigma Aldrich, St. Louis, MO, USA), 70% ETOH for 1 min, and finally PBS containing 0.01% TritonX–100. The absence of bacteria was confirmed routinely by PCR analysis of 16S rRNA primers (8FE and 1492R) on fly homogenates and by culturing the homogenates in Lysogeny Broth (LB)-agar plates. Sterilized GF eggs were transferred to vials with autoclaved media within a biosafety cabinet. GF flies were supplemented with unknown or known bacteria to generate conventionally reared (CR) or gnotobiotic flies.

Survival rate and developmental timing of flies

For the survival test, 30 eggs within 10 h after egg-laying were transferred to vials with casein-cornmeal-agar medium. Eggs were exposed to fungi or bacteria, and the survival ratio was calculated. The number of pupae and adults formation was recorded, and the formula to calculate developmental timing was expressed as $T = (T1 \times N1 + T2 \times N2 + \ldots + Tm \times Nm)/ (N1 + N2 + \ldots + Nm)$. T, the developmental timing; T_m, the days after egg laying; N_m, the number of pupae and adults on the Tm day[15, 23].

Oral and injury infection

Male and female flies following 5 d after eclosion were collected in vials, with 20 in each vial. For oral infection, flies were transferred to vials that were pre-inoculated with 10^8 and incubated at 25 °C for 48 h. Systemic infections (septic injury) were performed by pricking the thorax of adult females with a thin needle previously dipped into a concentrated pellet of bacterial culture or in a suspension of *Diaporthe* FY spores[45]. All infected flies were incubated at 25 °C. At least three tubes with 20 flies were used for survival experiments and the survival count was scored daily.

Real-time quantitative PCR analysis

Male adult flies were fed with *Diaporthe* FY for 24 h. Fly guts were dissected in cold PBS buffer, and total RNA was extracted with Trizol reagent (Invitrogen, USA). Up to 2 mg of total RNAs were used as templates of reverse transcription with the oligo-dT primer for the real-time quantitative PCR (BioRad). The primer sets for ATT, Dpt and Duox were as previous described[35].The ΔCtmethod was employed to analyze data with *rp*49 as the reference gene. The relative expression value was calculated with formula: $\triangle$Ct = Ct (target gene) - Ct (reference gene), the relative = $2^{-\triangle\triangle Ct}$.

Oviposition preference assay

Two-choice oviposition chambers were constructed similarly to that of previous studies[23, 28]. In each chamber, flies were able to choose their oviposition sites between two types of fermented substrates. To create the fermented substrates, food agar was sterilized by autoclaving for 121 °C, and was plated with either 100 μL of *Diaporthe* FY, *L. plantarum,* and ddH2O for controls. They were then incubated at 25 °C for 48 h.. To assemble the oviposition chamber, a razor blade was used to divide the agar into halves, and two different oviposition substrates were hand-puzzled into a petri-dish. For the assessment of fungal metabolites, liquid fly food (without agar) was inoculated with *Diaporthe* FY and incubated at 25 °C for 48 h. Fermented fly food was centrifuged at 12,000 rpm for 15 min, and supernatant was distributed onto the surface of fly food in the two-choice oviposition chambers. Fifty female flies were collected, and mated for 6 h after transfer to the device. Finally, flies were removed and the number of eggs on each half of the two-choice chamber was counted, and the oviposition index(OI) was calculated: [OI = (no. of eggs laid on experimental food - no. of eggs laid on control food) / total no. of eggs laid].

Abbreviations

CR: conventionally reared *GF:* germ free

OI: oviposition index *CFU:* colony formation unit

Declarations

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Authors' contributions

WL, WS, and JL conceived and designed the experiments; WS, JL, RP and BM performed the experiments; WS performed the statistical analysis; WL and WS wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

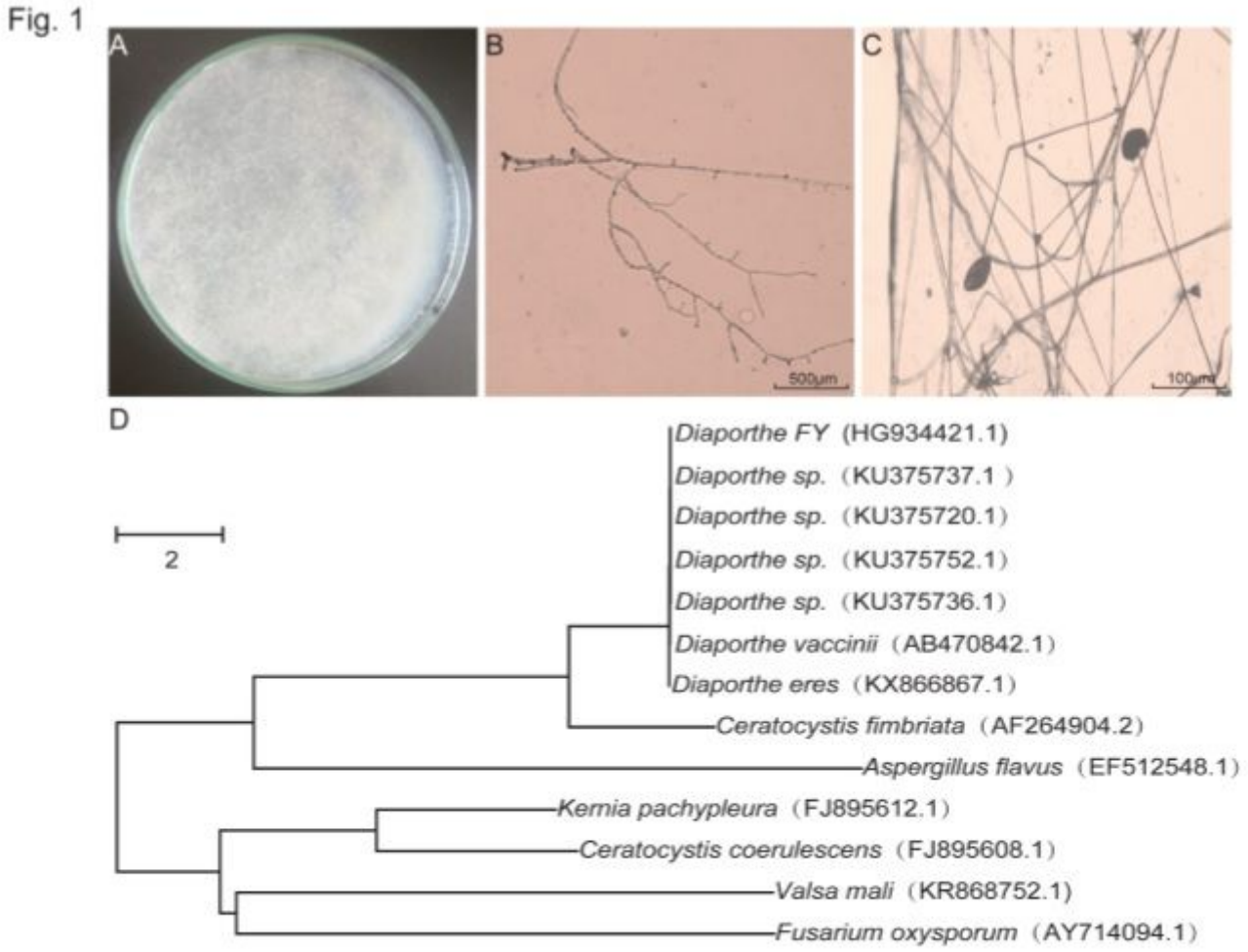

Figure 1

FIG. 1 Morphologies and phylogenetics of Diaporthe FY (A) Colony growth on the yeast-potato medium. Image depicted fungal development after 48 h incubation at 25 °C on nutrient-rich medium. (B-C) Mycelia, conidiophores and conidia of Diaporthe FY. (D) The phylogenetic tree of Diaporthe FY and its homologs constructed with the neighbour-joining method. Bar: Nucleotide divergence; Numbers in the notes present bootstrap percentages; numbers in parentheses are GenBank accession.

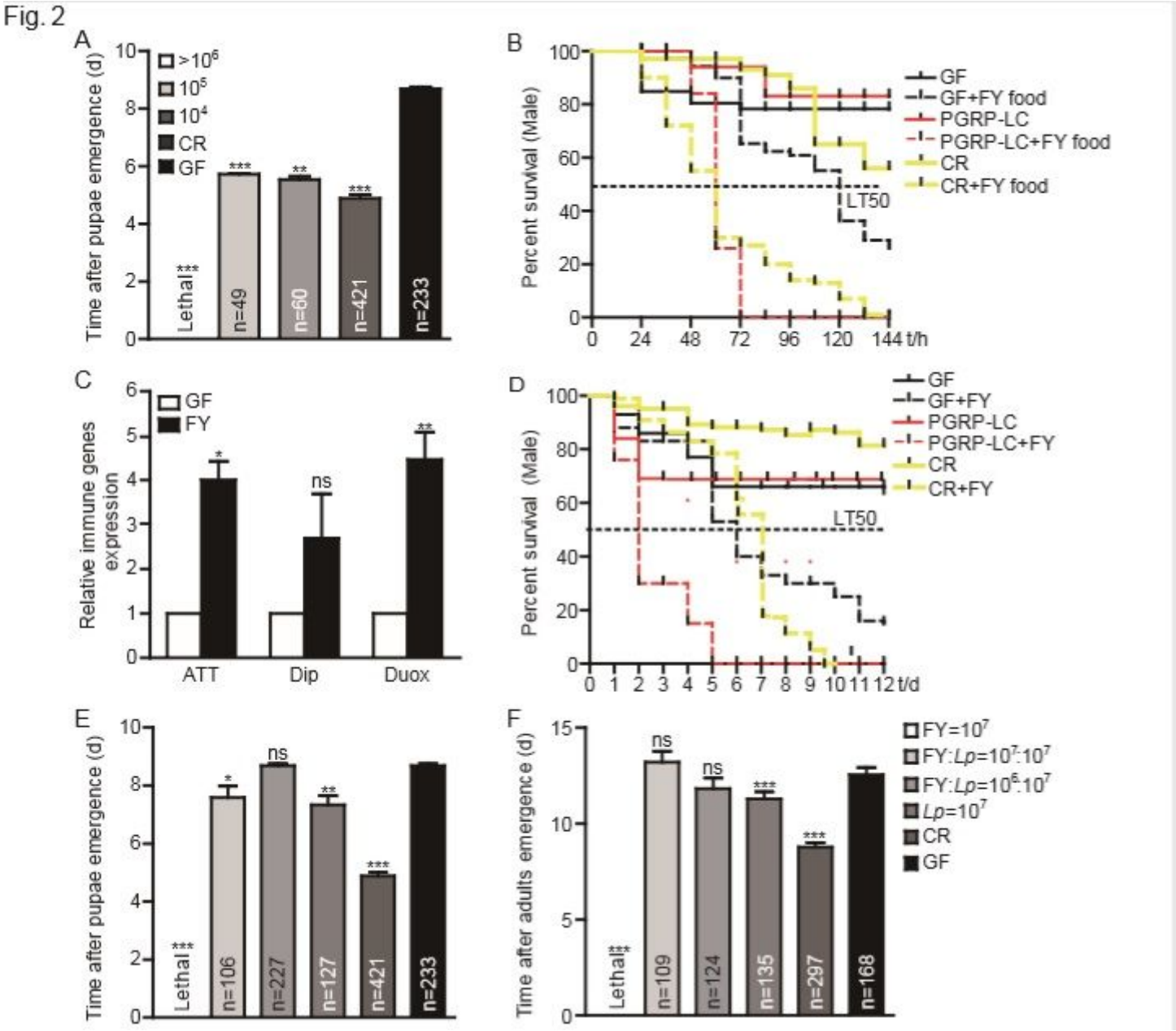

Figure 2

FIG. 2 Commensals alleviated the toxicity of Diaporthe FY to hosts (A) The timing of pupa formation of flies associated with Diaporthe FY. GF larva were inoculated with mixed bacteria, sterile PBS, and Diaporthe FY to generate CR, GF and Diaporthe FY-associated flies, respectively. The number of pupae formation was counted daily. (B) The survival of male adults fed with Diaporthe FY molds in food. Fly food was inoculated with 108 Diaporthe FY spores, and incubated at 25 °C for 48 h. Male adults of conventionally reared- and germ-free wild type and conventionally reared- PGRP-LC mutants were orally

infected by feeding with Diaporthe FY, and the number of dead flies was calculated. (C) Diaporthe FY triggered the innate immune response. RT-qPCR analysis of the gut showed that the relative expression levels of ATT, Dip and Duox were increased upon Diaporthe FY infection. n = 3. (D) The survival rate of conventionally reared- and germ-free wild-type and PGRP-LC mutant male adults infected with Diaporthe FY. Male adults were septically infected by punching flies with Diaporthe FY spores, and the number of dead flies were calculated. (E-F) L. plantarum attenuated the toxicity of Diaporthe FY to flies. The timing of pupa formation and adult eclosion was assessed in the presence of Diaporthe FY, L. plantarum or the mixture of both, respectively. The one-sample t-test, Symbols: *P < 0.05; ** P < 0.01.

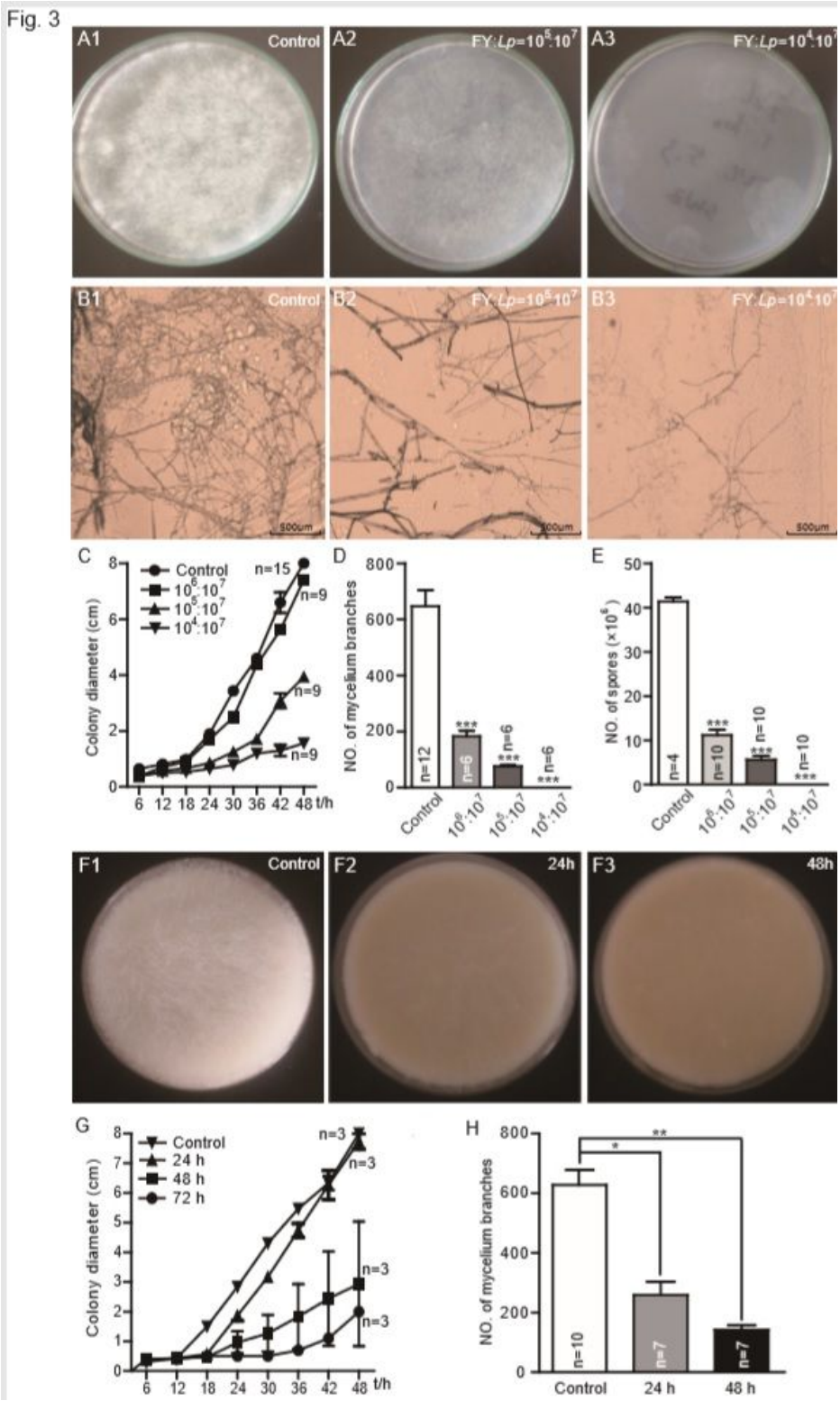

Figure 3

FIG. 3 L. plantarum hindered the growth of Diaporthe FY in vitro (A) L. plantarum dosage-dependently inhibited the growth of Diaporthe FY. Both Diaporthe FY and L. plantarum (with different ratios A1, Diaporthe FY; A2, 1:100; A2, 1:1000) were simultaneously inoculated to nutrient rich medium, and were incubated at 25 °C for 24 h. The growth of Diaporthe FY was shown. (B) The growth of mycelia was inhibited in the case of L. plantarum. (C-E) The quantification of the colony growth rate, the number of

mycelia and spores of Diaporthe FY. (F) The growth of Diaporthe FY in fly food pre-incubated with L. plantarum. White mycelia were observed on the surface of food (F1), while fewer mycelia were observed in L. plantarum-treated food (F2 and 3). (G) The colony growth and the number of mycelium branches of Diaporthe FY in a medium pre-incubated with L. plantarum. The one-sample t-test, Symbols: *P < 0.05; ** P < 0.01.

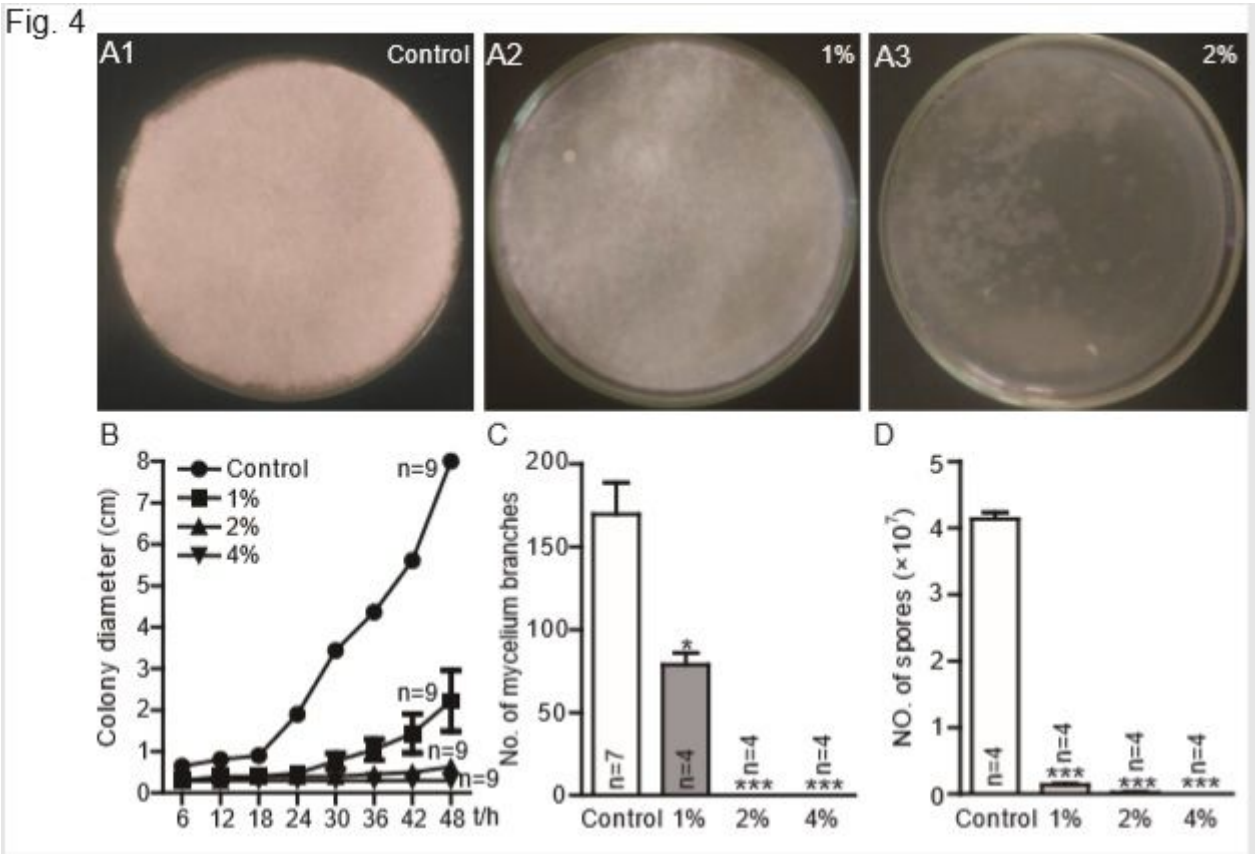

Figure 4

FIG. 4 Lactic acid exerted anti-fungal properties (A) The growth of Diaporthe FY at different concentrations of lactic acid. Diaporthe FY was inoculated in nutrient-rich medium containing different concentrations of L-lactate, and were incubated at 25 °C for 48 h. (B) The colony growth rate of Diaporthe FY treated with lactic acid. (C-D) The number of mycelia and spores in the case of lactic acid. The one-sample t-test, Symbols: NS P > 0.05; *P < 0.05; ** P < 0.01; *** P < 0.001.

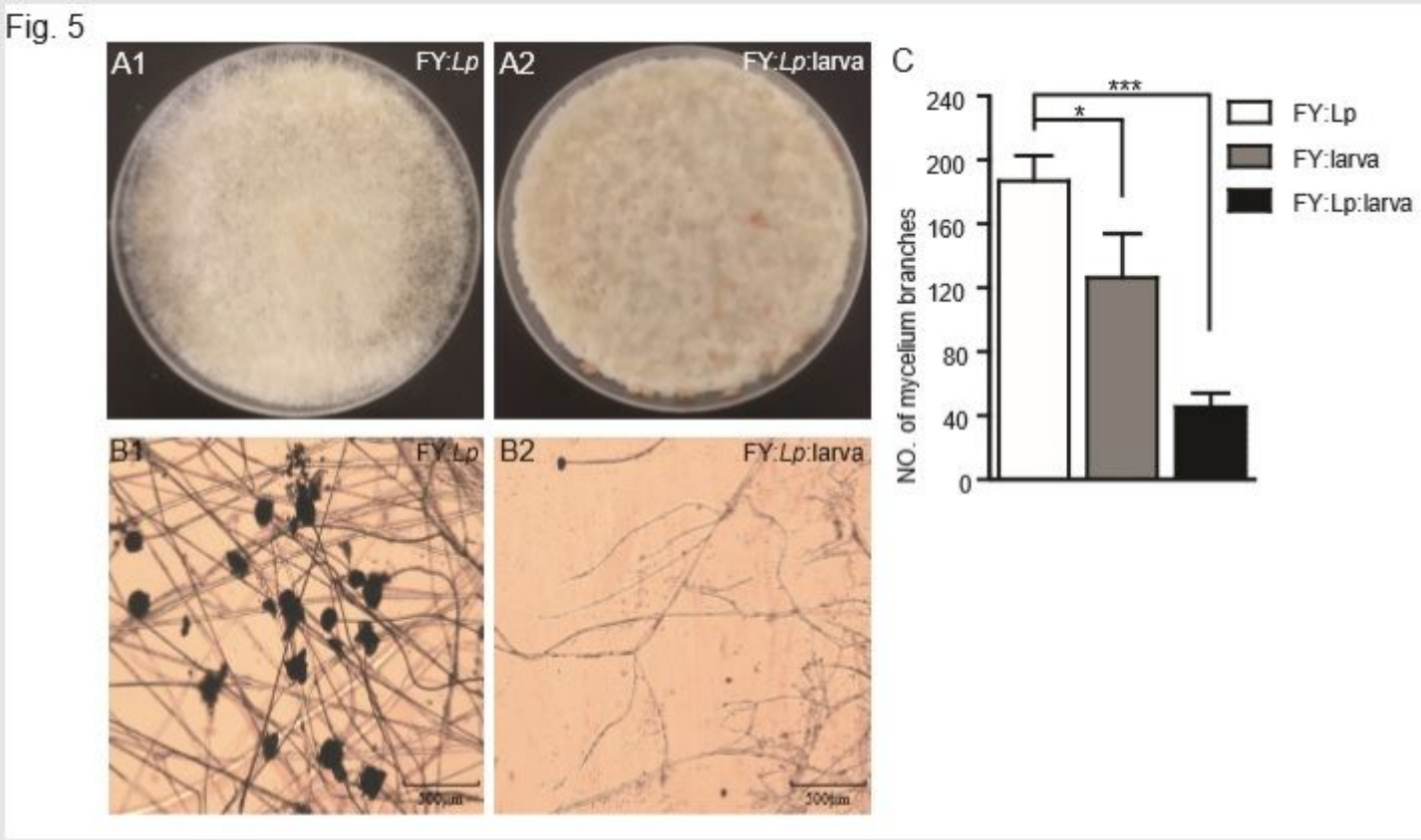

Figure 5

FIG. 5 Drosophila and L. plantarum synergized to defend against Diaporthe FY (A) The colony growth of Diaporthe FY with L. plantarum in the absence or presence of larvae. Twenty five third instar larva were transferred to each plate with fly diet inoculated with Diaporthe FY, L. plantarum or both, respectively. The plates were incubated at 25 °C, and the colony growth was assayed at the time point of 72 h. (B) Mycelia of Diaporthe FY with L. plantarum in the absence or presence of larvae. (C) The number of branching mycelia in the absence or presence of larvae. One-sample t-test, Symbols: *** $P < 0.001$.

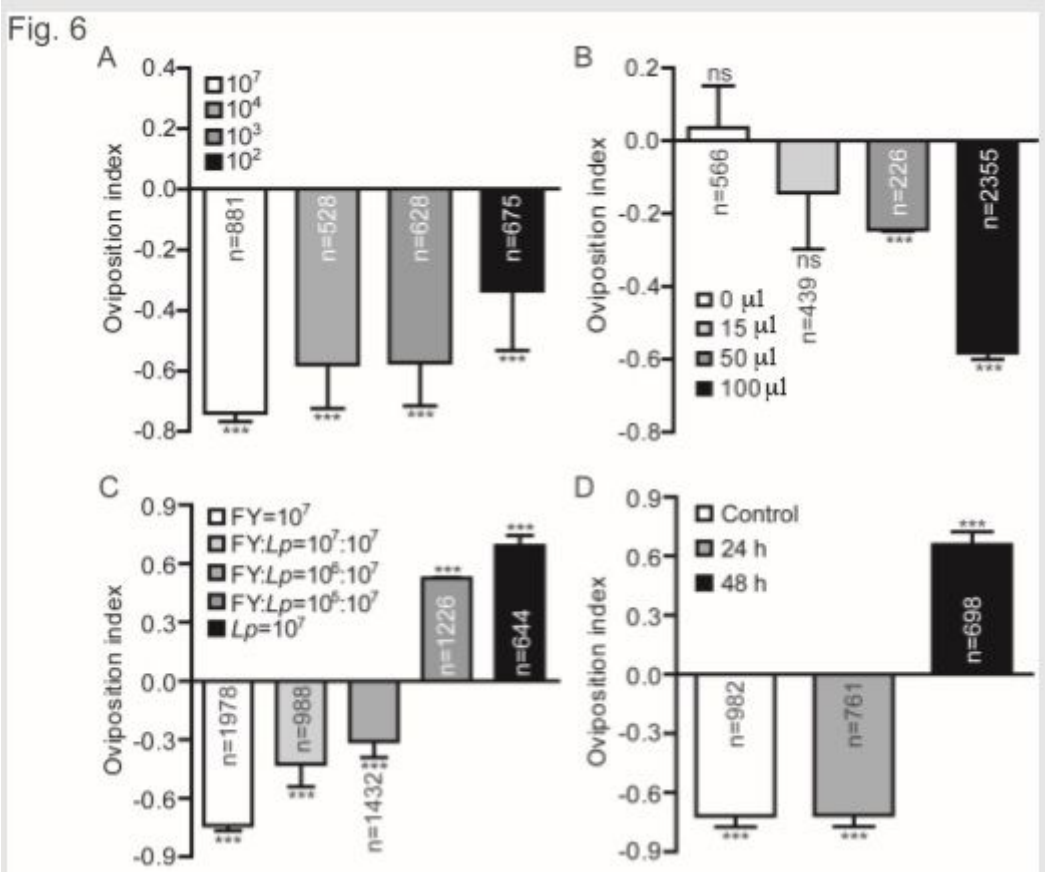

Figure 6

FIG. 6 L. plantarum prevented oviposition avoidance towards Diaporthe FY (A) Quantification of egg-laying avoidance to diet fermented with Diaporthe FY. Egg laying preference was assayed with the two-choice chamber. Mated females were transferred to the chamber and allowed to lay eggs for 8 h. The numbers of eggs were counted on each half, and the oviposition preference was calculated. (B) Ovipositional avoidance towards metabolites of Diaporthe FY. The supernatant of liquid fly food were evenly distributed on the surface of halves in a dosage-dependent manner. (C) L. plantarum reduced oviposition avoidance towards Diaporthe FY. (D) Oviposition preference for Diaporthe FY-treated diet that previously inoculated with L. plantarum. The one-sample t-test was used to assess the mean deviance of each column from 0, n = 6–14. Symbols: NS P > 0.05; *P < 0.05; ** P < 0.01; *** P < 0.001.

Supplementary Files

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