



Gut fungal diversity across different life stages of the onion fly *Delia antiqua*

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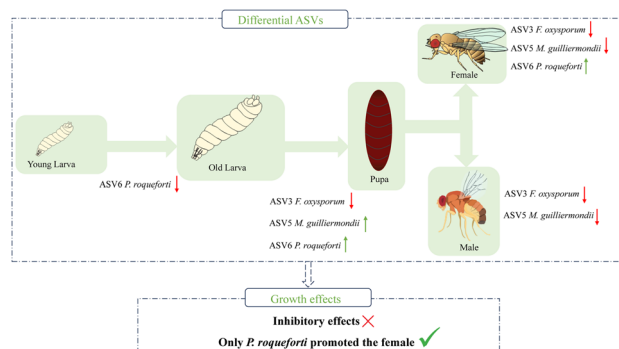
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Abstract

A significant number of microorganisms inhabit the intestinal tract or the body surface of insects. While the majority of research on insect microbiome interaction has mainly focused on bacteria, of late multiple studies have been acknowledging the importance of fungi and have started reporting the fungal communities as well. In this study, high-throughput sequencing was used to compare the diversity of intestinal fungi in *Delia antiqua* (Diptera: Anthomyiidae) at different growth stages, and effect of differential fungi between adjacent life stages on the growth and development of *D. antiqua* was investigated. The results showed that there were significant differences in the α and β diversity of gut fungal communities between two adjacent growth stages. Among the dominant fungi, genera *Penicillium* and *Meyerozyma* and family Cordycipitaceae had higher abundances. Cordycipitaceae was mainly enriched in the pupal and adult (male and female) stages, *Penicillium* was mainly enriched in the pupal, 2nd instar and 3rd instar larval stages, and *Meyerozyma* was enriched in the pupal stage. Only three fungal species were found to differ between two adjacent growth stages. These three fungal species including *Fusarium oxysporum*, *Meyerozyma guilliermondii* and *Penicillium roqueforti* generally inhibited the growth and development of *D. antiqua*, with only *P. roqueforti* promoting the growth and development of female insects. This study will provide theoretical support for the search for new pathogenic microorganisms for other fly pests control and the development of new biological control strategies for fly pests.

Graphical Abstract

Three fungal species were found to differ between two adjacent growth stages. These three fungal species including *Fusarium oxysporum*, *Meyerozyma guilliermondii* and *Penicillium roqueforti* generally inhibited the growth and development of *D. antiqua*, with only *P. roqueforti* promoting the growth and development of female insects.



Keywords Onion maggot · Insect-microbe · Symbiosis · Gut microbiota · Pest control

Extended author information available on the last page of the article

Introduction

The body surface and intestines of animals harbor a number of microorganisms. As one of the largest group of invertebrates, insects establish close symbiotic relationships with microorganisms [1]. Several studies have revealed the role of these microorganisms in improving insect fitness by providing essential nutrients [2, 3], protecting hosts from pathogen invasion [4], aiding in the digestion of indigestible food [5], mediating social behaviors [6], assisting in detoxification processes [7], and defending against predators [8]. Metamorphosis in insects is often accompanied by shifts in microbial communities. How such shifts in turn affect their host's fitness, still remains unclear.

Symbiotic microorganisms play a crucial role in regulating the developmental process of host insects [9]. On one hand, in mutualistic relationships, symbiotic microorganisms play a positive regulatory role in the growth and development of insects. For example, the lack of microorganisms in the mosquito gut can cause significant developmental delays, while the colonization of probiotics can eliminate this effect [10]. Furthermore, symbiotic microorganisms regulate the growth and development of hosts by affecting their nutritional metabolism. For instance, the gut symbiont *Rhodococcus rhodochrous* of *Hermetia illucens* (Diptera: Stratiomyidae) can promote the development of its larvae, leading to larger and more nutritionally efficient mature larvae [11]. On the other hand, in antagonistic relationships, symbiotic microorganisms negatively regulate the growth and development of insects. For example, research by Shin et al. [12] demonstrated that *Acetobacter pomorum* regulated the host's internal microbial homeostasis, growth rate and energy metabolism by controlling insulin-like growth factor signaling in *Drosophila*, with axenic *Drosophila* showing negative regulation of host development and internal metabolic homeostasis.

Delia antiqua (Meigen) is an economically concerned pest of onion and other lily crops globally [13]. The larvae feed on the underground parts of onion plants, including roots and developing bulbs. The adults lay eggs on or near plants, and the larvae move to the root zone to feed on the underground onion tissues. This feeding behavior can cause onion seedlings to rot [14]. *D. antiqua* is found in the Americas, Europe and Asia, and is especially widespread in most temperate onion-growing regions. In the temperate regions, it can reduce onion stands by as much as 55%–80% if left unmanaged [15]. The pest is a destructive underground crop pest, and without timely management, it can lead to extremely serious consequences.

D. antiqua forms a symbiotic relationship with microorganisms present on its body surface or in its gut, and these

microorganisms associated with *D. antiqua* play important ecological functions [16]. For example, it has been discovered that bacteria associated with *D. antiqua* can promote the growth and development of its larvae [17]. Furthermore, the symbiotic bacteria of *D. antiqua* larvae exhibit significant inhibitory effects on the germination of conidia and hyphal growth of the insect pathogen [18]. Organic acids produced by bacteria associated with the body surface of *D. antiqua* larvae are used to protect them from infection by insect pathogens [3, 19]. These research findings indicate that *D. antiqua* forms a symbiotic system with its associated microorganisms, and the microorganisms have a significant impact on the growth and development of *D. antiqua*.

However, the research regarding the diversity and function of *D. antiqua* symbiotic microbiota [3, 16–21] has mostly focused on bacteria, while studies on the fungi are relatively limited. Therefore, this study aims to investigate the diversity and function of fungi associated with *D. antiqua*. Firstly, we detected the diversity shift of *D. antiqua* associated fungi in guts at different developmental stages using a culture-independent method. Secondly, we successfully isolated and identified fungi associated with different growth stages of *D. antiqua* using a culture-dependent method. Thirdly, we systematically analyzed the impact of the differential fungal species between different developmental stages on the growth and development of *D. antiqua*. This study revealed the diversity of fungi in the microbiota associated with *D. antiqua* and thoroughly investigated the influence of these fungi on the ecological functions.

Materials and Methods

Insect Sources

In 2023, all *D. antiqua* were collected directly from Lanling (N34°46', E118°4'), Linyi City, Shandong Province, China. To ensure the accuracy of the experiment, five garlic fields were randomly selected to collect *D. antiqua* at one area Lanling and each garlic field was about 2 km apart. In the garlic fields, *D. antiqua* were collected by sweeping the net back and forth such that it scrapes the tops of plants and dislodges the flies that are feeding or resting there. In total, about 50 adults were caught in each field. Subsequently, all *D. antiqua* were rapidly placed in one cage and taken back to the laboratory to be reared according to previous method [22].

Experiment I: Gut Fungal Diversity at Different Developmental Stages of *D. antiqua*

Five groups were set and they were second instar larvae (50 2nd instar larvae were randomly selected and divided

into five repetitive groups, 10 larvae for each repetitive group, young larvae, YL), third instar larvae (15 3rd instar larvae were randomly selected and divided into five repetitive groups, 3 larvae for each repetitive group, old larvae, OL), pupae (10 pupae were randomly selected and divided into five repetitive groups, 2 pupae for each repetitive group, P), males (10 males were randomly selected and divided into five repetitive groups, 2 males for each repetitive group, M) and females (10 females were randomly selected and divided into five repetitive groups, 2 females for each repetitive group, F). Insects in each repetitive group above were used for DNA extraction. The CTAB method [23] was used for the extraction of total genomic DNA. The PCR amplification was performed using the approach of Huang et al. [24]. The 18S rRNA gene was amplified using the ABI GeneAmp[®] 9700 PCR system with primers SSU0817F (5'-TTAGCATGG AATAATTRRAATAGGA-3') and 1196R (5'-TCTGGACCTGGTGAGTTTCC-3'). The total reaction volume is 20 μ L, and it consists of 1 μ L DNA template, 0.8 μ L forward primer, 0.8 μ L reverse primer, 7.4 μ L ddH₂O, and 10 μ L 2X Pro Taq PCR Master Mix. Set the PCR conditions at 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 45 s; followed by 72 °C for 10 min. The PCR products were mixed in appropriate proportions according to the sequencing requirements for each sample. Subsequently, libraries were prepared and subjected to paired-end sequencing with 2 × 250 bp read length using the Illumina NovaSeq platform.

After the Illumina sequencing, the paired-end reads (PE reads) were separated according to sample. Subsequently, the reads underwent quality control (fastp (v0.19.6)) and filtering based on sequencing quality, and were then overlapped (FLASH (v1.2.7)) to obtain optimized data after quality-controlled splicing. Following this, the sequence denoising method (DADA2) was utilized to process the optimized data, resulting in the generation of representative sequences and abundance information for Amplicon Sequence Variants (ASVs). Based on the ASV representative sequences and abundance information, rarefaction curves and α -diversity indices for diversity analysis were calculated using the "alpha_rarefaction.py" function in the QIIME software. Meanwhile, β -diversity was performed using Principal Coordinate Analysis (PCoA) based on weighted and normalized UniFrac distances, as well as Non-metric Multidimensional Scaling (NMDS) based on the Bray–Curtis distance matrix and the displacement number of is 999. Besides, the heatmap of the top 12 ASVs and top 9 genera (relative abundance higher than 1%) was constructed with R to illustrate the dominant ASVs and genera in different growth stages. More detailed information is provided in the Supplementary Methods 3.1.

Experiment II: Fungal Isolation and Identification

Thirty third instar larvae of *D. antiqua* were selected for fungal isolation and identification. Two different isolation media, Potato dextrose agar (PDA) [25] and Yeast malt agar (YMA) [26] were used. Sequences of 18S rRNA gene were aligned using MEGA software version 7.0 [27]. The MEGA software was then also used to construct a maximum-likelihood phylogenetic tree. Detailed information on the isolation and identification of *D. antiqua* gut fungi is provided in the Supplementary Methods 3.2.

Experiment III: Effects of the Differential Fungal Species Between Different Developmental Stages on *D. antiqua* Growth and Development

Based on differential abundance profiles of ASVs, we isolated *Fusarium oxysporum*, *Meyerozyma guilliermondii* and *Penicillium roqueforti*. Subsequently, these strains were selected to validate their effects of these three fungi on the growth and development of *D. antiqua* at different growth stages. For larvae, spraying conidia suspension after treatment of larvae with antibiotic stock solution containing 0.1 mg/mL streptomycin, 0.1 mg/mL cycloheximide, 0.1 mg/mL penicillin, and 0.1 mg/mL lincomycin [19]. For adults, they were fed with a 10% sucrose solution containing antibiotics. In the treatment group, 10% sucrose solution was replaced with conidia suspension. Detailed methods for the effects of the three fungi on *D. antiqua* growth and development are provided in the Supplementary Methods 3.3.

Data Analysis

Prior to statistical analysis, the normality and homogeneity of variances were examined using the Kolmogorov–Smirnov test and Levene's test, respectively. Independent t test was employed to test significant differences in α -diversity of gut fungal communities among different growth stages. Independent t test is also used to compare the differential impact of gut fungi on the growth and development. In cases where variances are unequal, Welch's corrected t test is employed. Non-parametric Mann–Whitney Rank Sum Test was applied to evaluate the significance of differences in the abundance of ASVs in the gut of neighboring growth stages. Statistical analyses were conducted in QIIME for the sequences data or in IBM SPSS 25.0 software for the experiment regarding the effects of fungal strains on *D. antiqua* growth and development, and a significance level threshold of 0.05 was used for statistical analysis.

Results

α -diversity of Gut Fungal Communities of *D. antiqua* Shifted Significantly Among Different Life Stages

The rarefaction curves of all samples exhibited a flat trend, implying an ample sequence depth of data in this experiment, thereby guaranteeing the reliability of subsequent analysis (Supplementary Fig. S1). Furthermore, the coverage rates of YL and M range between 71 and 100%, while those of OL, P and F range between 86 and 100%, and no significant difference were noted among the YL, OL, P and adult (M, F) stages (Fig. 1a, Independent *t*-test, YL vs OL, $t=0.43$, $p=0.68$; OL vs P, $t=1.27$, $p=0.24$; P vs F, $t=0.00$, $p=1.00$; P vs M, $t=0.00$, $p=1.00$). The YL and the OL did not exhibit significant differences in terms of richness and evenness (Independent *t*-test, Sobs, $t=0.4264$, $p=0.6811$; Ace, $t=0.6962$, $p=0.5060$; Chao, $t=0.6325$, $p=0.5447$; Shannon, $t=0.4090$, $p=0.6933$; Simpson, $t=0.3744$, $p=0.7178$). Regarding α -diversity indices, the Sobs, Ace, and Chao indices of fungal communities in the P displayed significantly higher values compared to those in the OL (Independent *t*-test, Sobs, $t=6.532$, $p=0.0028$; Ace, $t=1.998$, $p=0.1117$; Chao, $t=6.532$, $p=0.0028$). Considering the evenness of microbial communities in the samples, there were significant differences in microbial diversity between OL and P, as

indicated by the Shannon and Simpson indices (Independent *t*-test, Shannon, $t=8.121$, $p=0.0008$; Simpson, $t=8.170$, $p=0.0007$). The P and F exhibited significant differences in all four indices, except for the Ace index, indicating differences in microbial diversity between these two stages (Independent *t*-test, Sobs, $t=6.000$, $p=0.0039$; Ace, $t=0.4775$, $p=0.6556$; Chao, $t=6.000$, $p=0.0039$; Shannon, $t=7.900$, $p=0.0008$; Simpson, $t=7.948$, $p=0.0006$). The Ace and Chao indices of microbial communities in the samples from the P exhibited no significant disparities compared to those in the M, but there are differences in the sobs index, indicating a lack of significant differences in species composition between these two stages (Independent *t*-test, Sobs, $t=3.162$, $p=0.0341$; Ace, $t=0.4519$, $p=0.6745$; Chao, $t=1.633$, $p=0.1778$). Additionally, no significant differences were observed between YL and OL among the three indices (Independent *t*-test, Sobs, $t=0.4264$, $p=0.6811$; Ace, $t=0.6962$, $p=0.5060$; Chao, $t=0.6325$, $p=0.5447$). However, the evenness of microbial communities in the P displayed notable distinctions when compared to M, as indicated by the Shannon and Simpson indices (Independent *t*-test, Shannon, $t=3.870$, $p=0.0163$; Simpson, $t=3.584$, $p=0.0209$). Conversely, no significant differences were found in the Shannon and Simpson indices between YL and OL (Independent *t*-test, Shannon, $t=0.4090$, $p=0.6933$; Simpson, $t=0.3744$, $p=0.7178$).

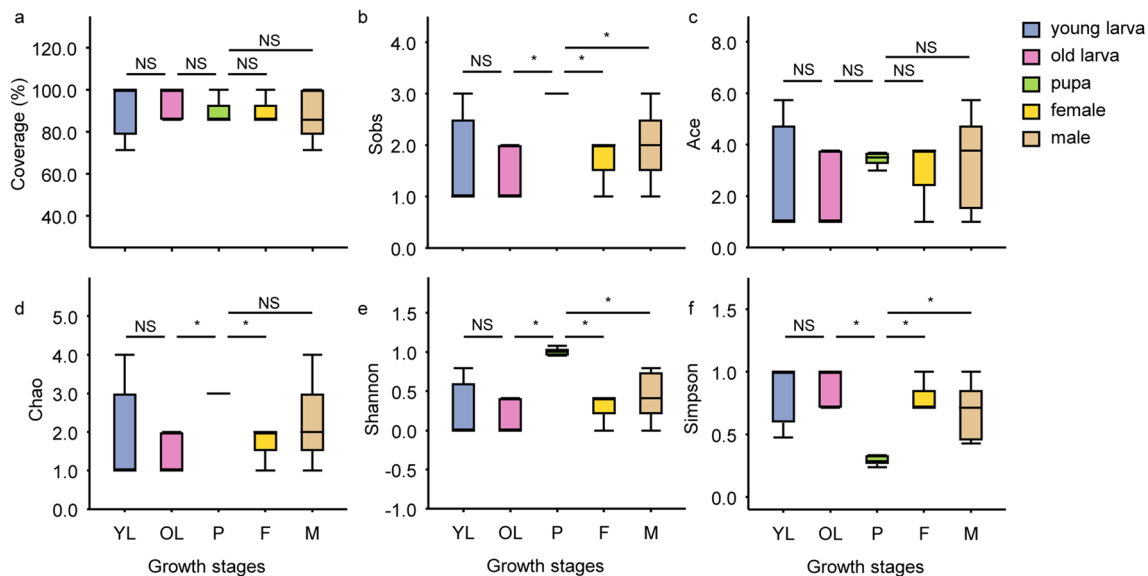


Fig. 1 The α -diversity of fungal microbiota in the gut of *D. antiqua* at different growth stages. The "*" in the figure indicates significant differences between the columns connected by lines (Independent *t*-test, $p < 0.05$), while "NS" indicates no significant difference. a: Coverage

index; b: Sobs index; c: Ace index; d: Chao index; e: Shannon index; f: Simpson index. YL: young larva; OL: old larva; P: pupa; F: female; M: male

β -diversity of Gut Fungal Community Varied Significantly at Different Growth Stages

Based on the similarity or distance measurement between samples, the hierarchical clustering method gradually merges or splits samples at different levels to form a sample hierarchical clustering tree, revealing the overall compositional differences among samples and the distribution of species composition between different samples. Among them, the P stage, OL and YL showed distinct clustering patterns (Fig. 2a). Considering the evolutionary relationships among species and their relative abundance in the microbial community, PCoA revealed distinct clustering patterns among samples of *D. antiqua* gut fungi at different growth stages. Each growth stage formed a statistically separate group (Fig. 2b, ANOSIM, $R=0.4985$, $p=0.001$), indicating significant differences in species composition and abundance. The same pattern was observed in the NMDS analysis, where samples from five different developmental stages showed distinct clustering, forming statistically different groups (Fig. 2c, Adonis, $R=0.4996$, $p=0.001$).

On the other hand, PCoA revealed that the gut fungal samples of *D. antiqua* from the two adjacent growth stages clustered separately, forming statistically different groups respectively (ANOSIM, YL vs OL, Fig. 3a, $R=0.0120$, $p=0.682$; OL vs P, Fig. 3b, $R=0.8880$, $p=0.008$; P vs F, Fig. 3c, $R=0.6760$, $p=0.011$; P vs M, Fig. 3d, $R=0.4740$, $p=0.008$). In the NMDS analysis, a similar trend was observed, with samples from the two adjacent growth stages clustering separately to form statistically distinct groups (Adonis, YL vs OL, Fig. 3e, $R=0.0120$,

$p=0.682$; OL vs P, Fig. 3f, $R=0.8880$, $p=0.008$; P vs F, Fig. 3g, $R=0.6760$, $p=0.011$; P vs M, Fig. 3h, $R=0.4740$, $p=0.008$).

Species Composition and Abundance of Gut Fungal Communities in Different Growth Stages of *D. antiqua* were Significantly Different

Among the dominant ASVs in the gut of *D. antiqua*, ASV3, ASV5 and ASV6 exhibited relatively high abundance (Fig. 4a). Specifically, ASV3 was predominantly enriched in the YL, OL and P, ASV5 was mainly enriched in the P, and ASV6 showed enrichment in the P, F and M. Moreover, hierarchical clustering analysis revealed that the YL, OL, P, F and M formed distinct clusters (Fig. 4a). Among the dominant fungi associated with the gut of *D. antiqua*, Cordycipitaceae, *Penicillium* and *Meyerozyma* exhibited relatively high abundance. Specifically, Cordycipitaceae was mainly enriched in the P, F and M, *Penicillium* showed enrichment in the YL, OL and P, and *Meyerozyma* was enriched in the P. Similarly, each growth stage formed distinct clusters (Fig. 4b).

Specific ASVs Abundance was Significantly Different Among the Adjacent Growth Stage of *D. antiqua*

A differential abundance analysis plot of gut-related ASVs was drawn by selecting the top 18 ASVs ranked by abundance in the gut of *D. antiqua*. Between YL and OL, ASV6 showed significant differential abundance (Mann–Whitney Rank Sum Test, $p=0.019$), with ASV6 in the YL significantly higher than in the OL, while the other ASVs exhibited no

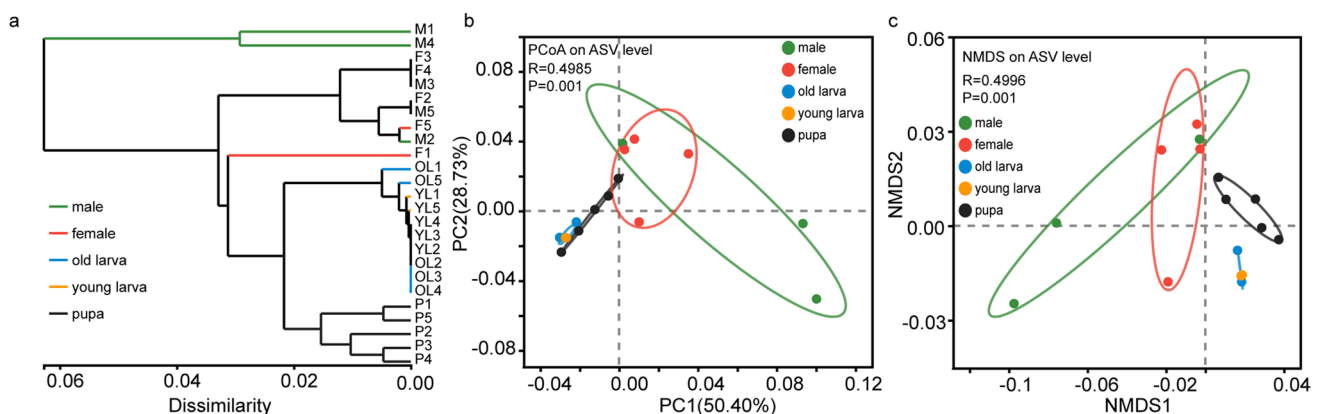


Fig. 2 Cluster analysis and β -diversity of fungal communities in the gut of *D. antiqua* at different growth stages. a: Hierarchical clustering plot; b: Principal coordinate analysis (PCoA) plot. PCoA plot is generated using the weighted UniFrac metric to compare microbial communities. Statistical significance is determined using an analysis of similarity (Adonis, $p < 0.05$) test to assess differences in community composition among different growth stages; c: Non-metric multidimensional scaling (NMDS) plot. The NMDS plot is constructed using a Bray–Curtis distance matrix for microbial communities comprising operational taxonomic units (ASVs) at a 97% similarity level. Significance values are obtained through an analysis of similarity (ANOSIM, $p < 0.05$) test to evaluate differences in community composition among different growth stages. YL: young larva; OL: old larva; P: pupa; F: female; M: male

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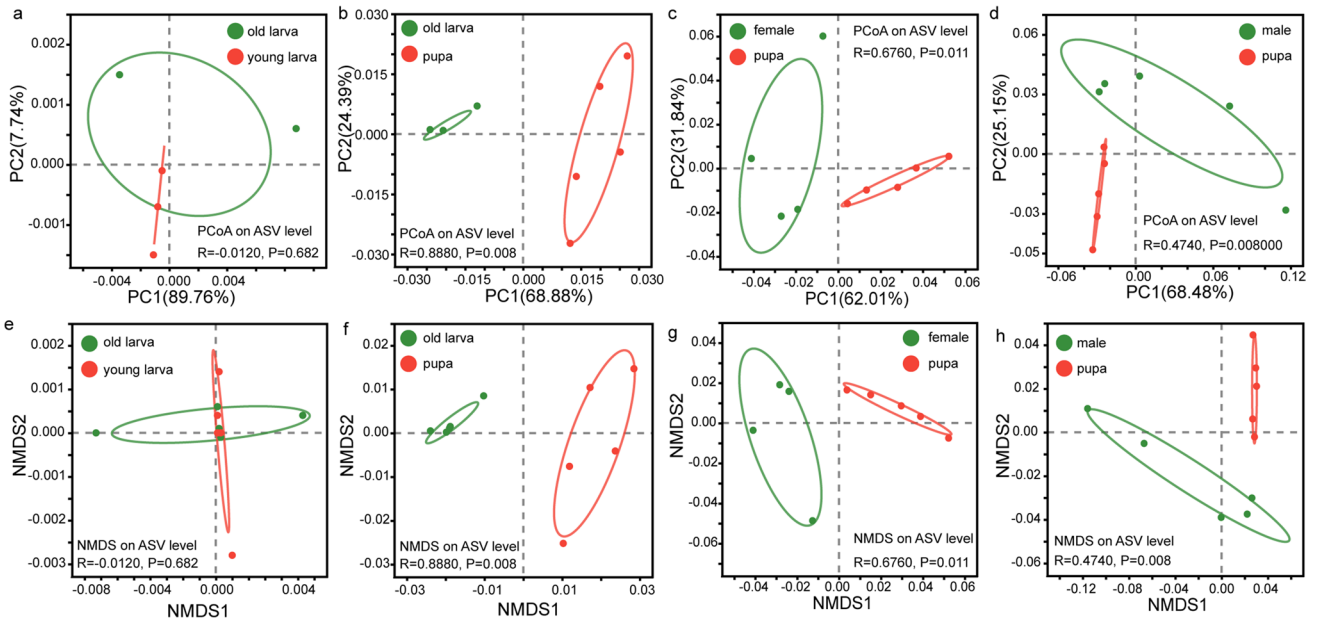
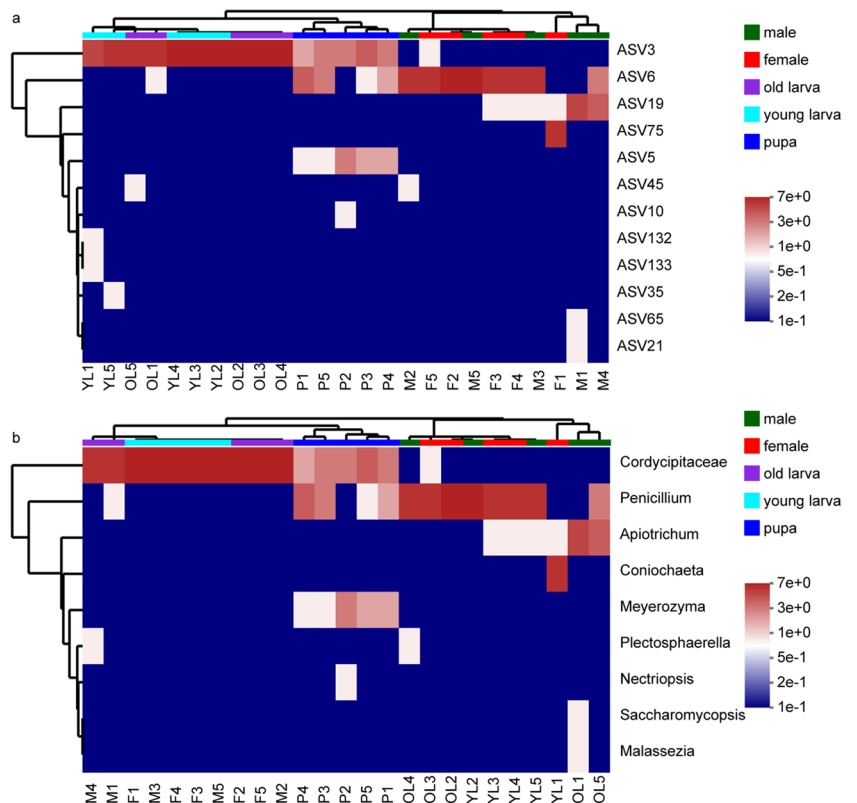


Fig. 3 PCoA and NMDS plots of fungal microbiota in the gut of *D. antiqua* at adjacent growth stages. a: PCoA plot of young larva and old larva gut fungal microbiota; b: PCoA plot of old larva and pupa gut fungal microbiota; c: PCoA plot of pupa and female gut fungal microbiota; d: PCoA plot of pupa and male gut fungal microbiota;

e: NMDS plot of young larva and old larva gut fungal microbiota; f: NMDS plot of old larva and pupa gut fungal microbiota; g: NMDS plot of pupa and female gut fungal microbiota; h: NMDS plot of pupa and male gut fungal microbiota

Fig. 4 Hierarchical clustering heatmap of ASV abundance and species composition in the gut of *D. antiqua* at different growth stages. a: Heatmap of ASV abundance; b: Heatmap of species composition. YL: young larva; OL: old larva; P: pupa; F: female; M: male



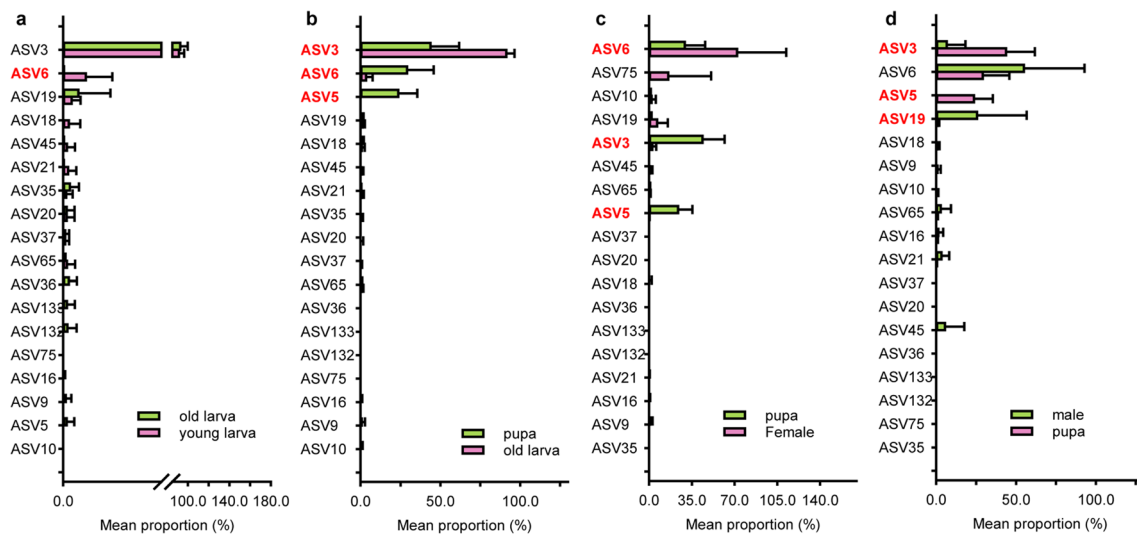


Fig. 5 Comparison of ASV abundance in fungal microbiota of the gut of *D. antiqua* at adjacent growth stages. a: Comparison of ASV abundance in young larva and old larva gut fungal microbiota (Top 18); b: Comparison of ASV abundance in old larva and pupa gut fungal microbiota (Top 18); c: Comparison of ASV abundance in pupa and female gut fungal microbiota (Top 18); d: Comparison of ASV

abundance in pupa and male gut fungal microbiota (Top 18). The red-colored ASVs indicate significant differences between the two growth stages (Mann–Whitney Rank Sum Test, $p < 0.05$). The p -values for non-significant differences between the two groups of adjacent growth stages are provided in Supplementary Table S1

significant differences (Fig. 5a). Compared to the P, ASV3, ASV5 and ASV6 showed significant differential abundance in the OL (Mann–Whitney Rank Sum Test, ASV3, $p = 0.003$; ASV5, $p = 0.011$; ASV6, $p = 0.025$), with ASV3 significantly higher in the OL than in the P, while ASV5 and ASV6 were significantly lower in the OL compared to the P. Specifically, the abundance of ASV3 significantly decreased from OL to P, while the abundance of ASV5 and ASV6 significantly increased (Fig. 5b). The abundance of ASV3, ASV5 and ASV6 showed significant differential levels between P and F (Mann–Whitney Rank Sum Test, ASV3, $p = 0.006$; ASV5, $p = 0.012$; ASV6, $p = 0.076$), with ASV3 and ASV5 significantly higher in the P than in the F, while ASV6 in the P was significantly lower than in the F (Fig. 5c). Compared to the M, ASV3, ASV5 and ASV19 showed significant differential abundance in the P (Mann–Whitney Rank Sum Test, ASV3, $p = 0.006$; ASV5, $p = 0.011$; ASV19, $p = 0.037$), with ASV3 and ASV5 significantly higher in the P than in the M and ASV19 significantly higher in the M than in the P. Specifically, the abundance of ASV3 and ASV5 both significantly decreased from P to M (Fig. 5d).

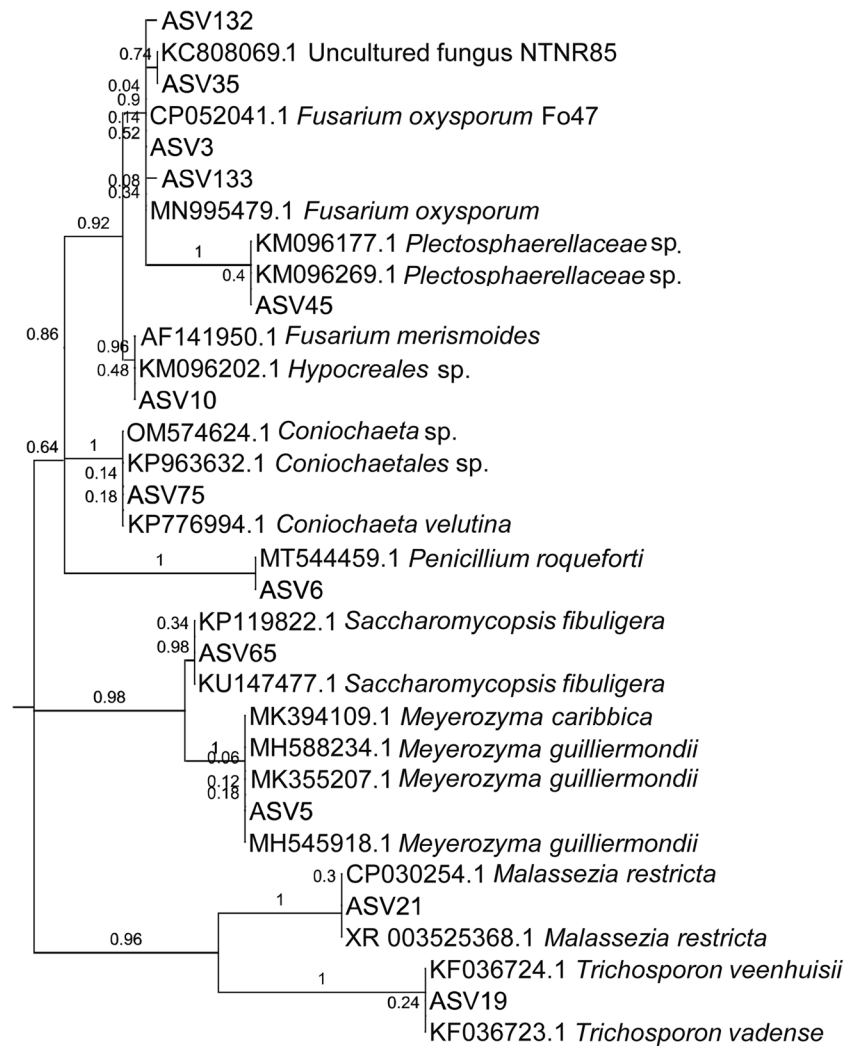
Intestinal Fungi Had Inhibitory Effects on the Growth and Development of Different Stages of *D. antiqua*

The results showed that ASV3, ASV5 and ASV6 had a close relationship with *F. oxysporum*, *M. guilliermondii* and *P. roqueforti* respectively (Fig. 6). *P. roqueforti* ASV6 inhibited

the growth of 2nd instar larvae. Specifically, there was no significant difference in larval weight between control group and treatment group at day 0 (Fig. 7a, Independent t -test, $t = 0.094$, $p = 0.9251$). However, at day 7, there was a significant difference between control group and treatment group, with the control group having a significantly higher larval weight than the treatment group (Fig. 7a, Independent t -test, $t = 28.20$, $p < 0.0001$). Additionally, there were significant differences in larval weight between control group at day 0 and 7 (Fig. 7a, Independent t -test, $t = 58.10$, $p < 0.0001$), as well as between the treatment group at day 0 and 7 (Fig. 7a, Independent t -test, $t = 33.20$, $p < 0.0001$).

F. oxysporum ASV3 and *M. guilliermondii* ASV5 inhibited the growth and development of female adults, while *P. roqueforti* ASV6 had a promoting effect. Specifically, *F. oxysporum* ASV3 and *M. guilliermondii* ASV5 shortened the lifespan of female and prolonged the pre-oviposition period. In contrast, *P. roqueforti* ASV6 extended the lifespan of female and shortened the pre-oviposition period. There was no significant difference in lifespan between control group and 24-h antibiotic treatment group (Fig. 7b, Independent t -test, $t = 0.1419$, $p = 0.8875$). However, there were significant differences in lifespan between the antibiotic group and the three treatment groups, with *F. oxysporum* ASV3 and *M. guilliermondii* ASV5 resulting in significantly shorter lifespan compared to the antibiotic group (Fig. 7b, Independent t -test; ASV3 vs antibiotic group, $t = 27.00$, $p < 0.0001$; ASV5 vs antibiotic group, $t = 10.18$, $p < 0.0001$), whereas the lifespan of female adults in the

Fig. 6 Molecular phylogenetic tree of 18S rRNA gene sequences associated with fungal ASVs using maximum likelihood method. The numbers above the branches represent bootstrap values obtained from 1000 replicates. The numbers preceding the species names correspond to accession numbers in the GenBank



P. roqueforti ASV6 group was significantly longer than that in the antibiotic group (Fig. 7b, Independent *t*-test, $t = 8.046$, $p < 0.0001$).

There was no significant difference in the pre-oviposition period between the control group and 24-h antibiotic treatment group (Fig. 7c, Independent *t*-test, $t = 0.6608$, $p = 0.5103$). However, the pre-oviposition period was significantly longer in the *F. oxysporum* ASV3 and *M. guilliermondii* ASV5 groups compared to the antibiotic group (Fig. 7c, Independent *t*-test; ASV3 vs antibiotic group, $t = 8.519$, $p < 0.0001$; ASV5 vs antibiotic group, $t = 4.833$, $p < 0.0001$), while it was significantly shorter in the *P. roqueforti* ASV6 group compared to the antibiotic group (Fig. 7c, Independent *t*-test, $t = 5.821$, $p < 0.0001$).

For 3rd instar larvae, the three fungi had no significant impact on larval growth. There were no significant differences in larval weight between the control group and the three treatment groups at both day 0 and day 4. Moreover, there were no significant differences among the corresponding groups during the 0 to 4-day period (Fig. 7d).

F. oxysporum ASV3 and *M. guilliermondii* ASV5 inhibited the growth of male adults, resulting in a shorter lifespan for these two fungi. There was significant difference in lifespan between control group and antibiotic group, with the use of antibiotics significantly reducing the lifespan of male (Fig. 7e, Independent *t*-test, $t = 13.02$, $p < 0.0001$). Furthermore, there were significant differences in lifespan between the antibiotic group and the two treatment groups, with both treatment groups having significantly shorter lifespan than the antibiotic group (Fig. 7e, Independent *t*-test; ASV3 vs antibiotic group, $t = 3.684$, $p = 0.0004$; ASV5 vs antibiotic group, $t = 6.557$, $p < 0.0001$).

Discussion

Insect-associated microorganisms can regulate development of host insects [9]. In this study, the diversity of fungal communities in the gut of *D. antiqua* at different growth stages and the effects of differential fungal strains

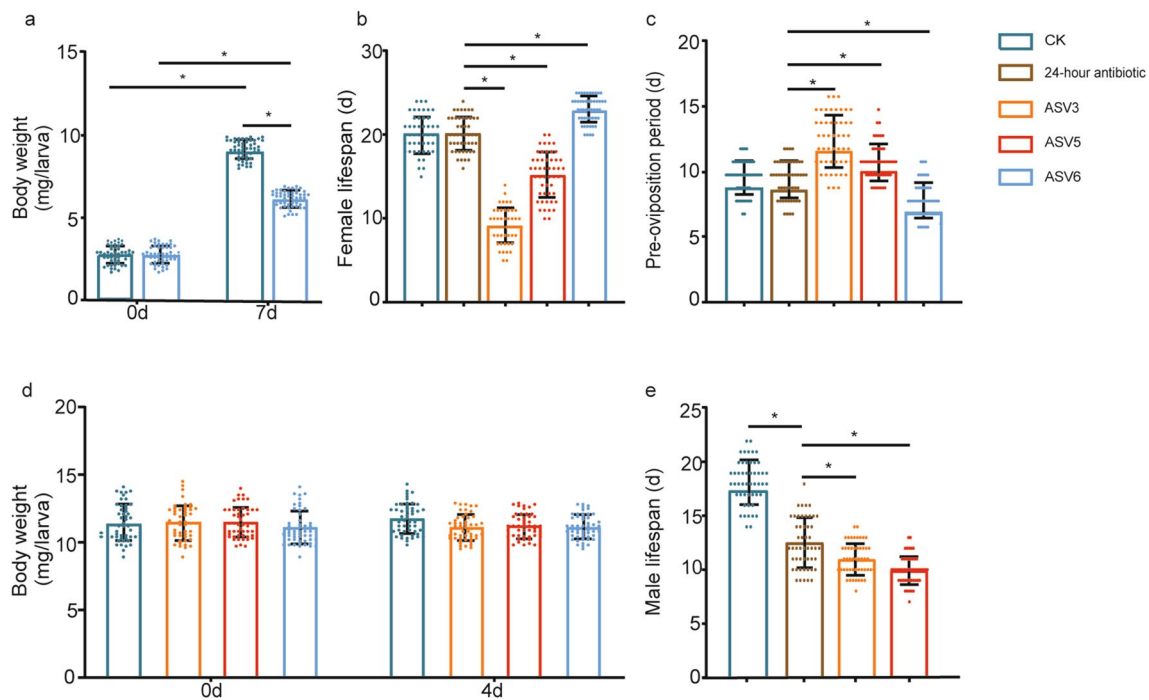


Fig. 7 The impact of three gut fungi on the growth and development of *D. antiqua* at different growth stages. In the figure, "*" denotes significant differences between the bar graphs connected by lines (Independent *t*-test, $p < 0.05$). a: The effect of ASV6 on the body weight of 2nd instar larvae of *D. antiqua*; b: The impact of ASV3, ASV5, and ASV6 on the lifespan of female adults; c: The influence of ASV3,

ASV5, and ASV6 on the pre-oviposition period of female adults; d: The effect of ASV3, ASV5, and ASV6 on the body weight of 3rd instar larvae of *D. antiqua*; e: The impact of ASV3 and ASV5 on the lifespan of male adults. ASV3: *Fusarium oxysporum*; ASV5: *Meyerozyma guilliermondii*; ASV6: *Penicillium roqueforti*

on the growth and development of *D. antiqua* were examined. The results showed that there were significant differences in α diversity indices, except for the coverage and Ace indices (Fig. 1). Whereas, both PCoA and NMDS revealed significant differences in β diversity of *D. antiqua* gut fungal communities at different growth stages (Fig. 3). Among fungal communities associated with *D. antiqua* gut, genera *Penicillium* and *Meyerozyma* and family Cordycipitaceae had higher abundance (Fig. 4), suggesting that they may be the dominant genera of *D. antiqua* gut fungi. The mentioned fungi have been reported in previous studies, for example, *M. guilliermondii* is present in the intestines of *Phoracantha semipunctata* (Coleoptera: Cerambycidae), cerambycid beetles and other insects [28]. A comparison was made to derive three ASVs and their corresponding microbiome with significant differences in abundance in the gut of *D. antiqua* at adjacent growth stages (Fig. 5 and 6). Besides, the effects of these three fungi on the growth and development of *D. antiqua* in different growth stages were examined, which revealed that the three fungi had inhibitory effects on the growth and development of *D. antiqua* at different growth stages, and that only *P. roqueforti* had a promotional effect on females (Fig. 7).

An interesting phenomenon is that the diversity and abundance of intestinal fungal communities are changed during the growth of *D. antiqua*. This may be due to habitat, dietary patterns, physiological characteristics and host phylogeny [29]. First, differences in habitat may lead to changes in the gut microbial community of *D. antiqua*. Females lay eggs in heaps at the base of onion and garlic leaves, and the hatched larvae penetrate into the bulbs of onions and garlic. The 3rd instar larvae pupate in the soil around the plant, while adults move around the plant. Thus, different habitats may lead to changes in the intestinal flora of *D. antiqua* at different growth stages. The study has also shown that the aquatic habitat of mosquito larvae plays a dominant role in the assembly of fungal communities [30]. Second, dietary patterns can largely influence the structure of the host insect microbiota [31]. In this study, neonate larvae of *D. antiqua* fed on decayed garlic or onion, while 3rd instar larvae fed on fresh garlic or onion, and adults fed on flowers around garlic plants [32]. Since insects at different stages have different diets, which may lead to changes in community [33]. This has also been confirmed by the studies of others, Zhang et al. who demonstrated that the gut fungal community structure of *Agilus mali* (Coleoptera: Buprestidae) showed large differences when fed different diets [34]. To minimize selection

bias at the experiments by using diets that may influence the gut microbiome, sterilized garlic were used to feed neonate larvae and 3rd instar larvae and sterilized sucrose solution was used to feed adults. Third, *D. antiqua* larvae empty intestines before entering the pupal stage, the physiological trait that also has the potential to influence changes in the intestinal fungal community. Larvae of *Trypophloeus striatulus* (Coleoptera: Scolytidae) have been reported to empty intestines to complete the act of pupation, thus causing changes in the intestinal flora [35]. Even if pupae do not engage in feeding activities and have reduced metabolic activity, they require energy to accomplish morphological changes that may affect the associated microbial community [36]. Furthermore, the development of the immune system may also influence the colonization of insect gut microbial communities [37]. And other factors such as genetic background and familial transmission (the process by which certain traits or microbes are passed from one generation to another within a family) may also affect the microbial landscape of the insect intestine [37], and these factors need to be further investigated.

In this study, the fungi *F. oxysporum*, *M. guilliermondii* and *P. roqueforti* generally exhibit inhibitory effects, with only *P. roqueforti* promoting the growth and development of females (Fig. 7). A reasonable explanation for this may be that *Penicillium* can play a direct promoting role, namely by promoting the ovarian development of females, thus shortening the pre-oviposition period, while also extending lifespan of females. Many previous studies have also demonstrated the direct promoting effects of *Penicillium* on the growth and development of insects. For example, *Penicillium* can release volatile organic compounds to attract pregnant *Bactrocera dorsalis* (Diptera: Tephritidae) for oviposition, while providing pyridoxine (one of the B vitamins) to promote larval development, shorten their emergence time, and increase emergence rates [38]. The results of Li et al. clarified positive impact of *Penicillium* infection on the growth of *Conogethes punctiferalis* (Lepidoptera: Pyralidae) larvae [39]. These studies all confirm that *Penicillium* can directly have a positive impact on the growth and development of insects.

The gut microbiota of insects plays a crucial role in the health, development, and immunity of hosts [40]. In recent years, insect gut microbes have emerged as key players in the exploration of novel biocontrol strategies against pests and their application in biological control practices. Previous studies have attempted to use fungi as biocontrol agents against bark beetle pests [36]. In this study, both *F. oxysporum* and *M. guilliermondii* exhibited inhibitory effects on the growth and development of both males and females (Fig. 7), suggesting they may have the potential to be useful in controlling *D. antiqua*. Furthermore, the abundance of the two fungi decreased significantly from pupal stage to adult stage (Fig. 5). A possible explanation could be antagonistic

interactions among microbial populations, particularly the interaction between yeasts and other microorganisms [41]. Many insects have close associations with fungi, such as bees, beetles, and ants [42, 43], and yeasts can provide nutrients to insect hosts [44]. However, in this study, *M. guilliermondii* had a negative effect on the growth of *D. antiqua*. This may be attributed to the interaction between *Meyerozyma* and other microbial communities. Study has demonstrated antagonistic interactions between yeasts and filamentous fungi [45]. Also, research has shown that some yeasts significantly promote the hyphal growth of tested fungi [46], and *Fusarium* produces numerous metabolites that are toxic to insects [47]. It is speculated that *M. guilliermondii* may promote the growth of *Fusarium*, and the toxic substances released by *Fusarium* have harmful effects on *D. antiqua*, thereby shortening the lifespan of adults.

Insects and their associated microbiota form a closely related symbiotic relationship, with the microbiota significantly influencing the insects' nutrition absorption, development and immunity [48]. In this study, we investigated the diversity and species composition of fungal communities in the gut of *D. antiqua* at different growth stages. Our findings clarified the impact of differential fungal communities on the growth and development of *D. antiqua*, indicating the existence of potentially crucial interactions between *D. antiqua* and the intestinal microbiota. Based on this research, the elimination of beneficial symbiotic microbiota or the identification of potential pathogenic microbiota can provide resources for pest control and the discovery of novel pest pathogens. However, *D. antiqua* used in this study were all obtained from the same sampling site, resulting in a limited and potentially biased sampling. The sequencing method solely employed DNA dependent sequence, excluding the potential benefits of RNA sequencing. Future work should be focused on elucidating the functional roles of the gut microbiota, exploring potential insecticidal mechanisms, and providing novel strategies for the development of effective biological control agents.

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Data Availability The data will be made available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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