Nilaparvata lugens (Hemiptera: Delphacidae) Midgut Microbial Community Responses to Exposure to *Metarhizium flavoviride* (Sordariomycetes: Hypocreales)[1]

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J. Entomol. Sci. 57(4): 587–602 (October 2022)

Abstract Changes to the community of symbiotic bacteria and fungi in the brown planthopper, *Nilaparvata lugens* Stål, were measured at different times after exposure to conidial suspensions of *Metarhizium flavoviride* Gams and Roszypal. Over all concentrations tested, bacterial species associated with *N. lugens* comprised 8 phyla, 17 classes, 22 orders, 26 families, and 31 genera of which the relative proportions of *Arsenophonus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Stenotrophomonas*, and *Bacteroides* were highest. The relative abundance and diversity of bacteria were highest in the carrier control (0.05% Tween-80 only). Fungi comprised 1 phyla, 5 classes, 10 orders, 13 families, and 18 genera, with *Metarhizium* being the dominant taxon in specimens from all treatment groups. *Metarhizium* spp. was greatest in the concentrations of 10^7 and 10^8 conidia/ml at 72 hours after exposure, reaching 94.82% and 93.74% of taxonomic units, respectively. We deduced that *M. flavoviride* competes for nutrition with midgut microorganisms; therefore, exposure to a pathogenic fungus will change the abundance and diversity of bacterial and fungal microorganisms in the midguts of hosts, and pathogens will impact the structure of bacterial communities in the host midgut with an alteration in the bacterial species composition. We observed that following the exposure of *N. lugens* to *M. flavoviride*, *Metarhizium* spp. dominated in the midgut of the host, the abundance and diversity of midgut fungal microorganisms decreased, and the dominant bacterial species in the midgut shifted.

Key Words *Nilaparvata lugens*, *Metarhizium*, symbiont, microbiome

The intestinal environment of insects is a symbiotic system that consists of various microorganisms including bacteria, fungi, viruses, and endophytic fungi (Hussa and Goodrich-Blair 2013) and where a mutually beneficial symbiotic relationship exists between these microorganisms and their hosts (Baumann 2005, Hurst and Jiggins 2000, Mercado et al. 2014, Oliver et al. 2003, Popa et al. 2012, Stouthamer et al. 1999, Wu et al. 2006). That is, insects can provide a sanctuary for microorganisms, whereas the microorganisms may act as regulators for various life activities of hosts, thereby indirectly affecting the health of host insects (Shi et al. 2010). Symbiotic microbial communities contribute to host physiology and evolution, and, moreover, provide substances such as sterols, sugars, amino

[1]Received 14 March 2022; accepted for publication 16 May 2022.
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acids, digestive enzymes, and detoxification enzymes (Crotti et al. 2010). Symbiotic microbial communities play important roles in food digestion, pheromone synthesis, and protection against harmful foreign substances, preventing the invasion of predators, parasites, and pathogens (Engel and Moran 2013, Kaltenpoth and Engl 2014). Previous studies have shown a strong correlation of the physio-biochemical state of host insects with the balance of their symbiotic microbes (Douglas 2011). Changes in the symbiotic microorganism structure, that is, its categorical and proportional membership, are shown to be affected by changes in the host environment, including the invasion of exogenous predators, parasites, and pathogens (Myint Myint Khaing 2017). When the structure of a symbiotic microbial community in a host is ideal, symbiotic microbial community can protect the host against adverse external factors (Kabaluk et al. 2017, Li et al. 2012, Zhou et al. 2019). A nonideal symbiotic microbial community structure can make the host more vulnerable to invasion by adverse external factors such as pathogens (Cox-Foster et al. 2007). Through all stages of their growth, insects are threatened by various external environmental factors that can cause structural disorder in their symbiotic microbial communities (Kaltenpoth and Engl 2014).

According to Kepler et al. (2014), *Metarhizium* spp. and other entomopathogenic fungi penetrate the host cuticle and exoskeleton of insects, consume host nutrients in the hemolymph, interfere with metabolism, secrete toxins, and destroy tissues, thus culminating in death of the host. Therefore, *Metarhizium* spp. exert a remarkable effect on the pest host, especially piercing and sucking insects (Douglas 1998). The migratory brown planthopper *Nilaparvata lugens* Stål is such a pest in rice-growing regions of Asia that causes lodging and "hopperburn" through feeding on the phloem sap of rice plants (Li et al. 1996). In recent years, *N. lugens* has become increasingly resistant to chemical pesticides, and *Metarhizium* spp. have become an alternative to chemical pesticides to protect crops from *N. lugens*. When penetrating the cuticle of *N. lugens*, *M. flavoviride* Gams and Roszypal competes with the host for nutrients (Sánchez-Rodríguez et al. 2016). Because host invasion by a fungus can be accompanied with changing the host microbiome (Kikuchia et al. 2012), it is important to understand the effects of *N. lugens* infected by *M. flavoviride* so that the use of *M. flavoviride* as a microbial control agent can be optimized.

High-throughput sequencing technologies provide a platform that enable the rapid and comprehensive inventory of insect gut microbes and to study the effects of external factors on the structure of microbial symbiotic communities in insects (Engel and Moran 2013, Shi et al. 2010). The Illumina sequencing platform, in particular, generates a high volume of high-quality DNA sequence data in a relatively short period of time, permitting the relatively easy assessment of changes to a host microbiome as represented by genomic operational taxonomic units (OTUs; i.e., structural changes). In this research, we used an Illumina PE250 (Illumina Inc., San Diego, CA; OE Biotech Company, Shanghai, China) to detect structural changes in the community of symbiotic microorganisms (e.g., bacteria and fungi) in *N. lugens* after treatment with *M. flavoviride* to provide foundational information to be considered when improving pest control using entomopathogenic fungi.

Materials and Methods

Source of test materials. *Nilaparvata lugens* were collected from a rice field near Hangzhou city, Zhejiang province, China, and reared for two generations on rice, *Oryza sativa* L. (var. TN1, Taichung native), seedlings in the laboratory. The planthoppers were maintained at 26±0.5°C under a 16-h light:8-h dark photo regime. Fourth-instar nymphs were then selected and used for the experiments. *Metarhizium flavoviride* was provided by the Institute of Subtropical Forestry, Chinese Academy of Forestry, and grown on Petri plates containing potato dextrose agar (PDA) at 28 ± 2°C for 10 d by which time it had fully sporulated. Conidia were scraped into 10 ml of 0.05% Tween-80 to create a stock suspension and vortexed for 30 min, and the number of conidia was determined using a hemocytometer. The stock suspension was serially diluted to create treatment concentrations of 10^7, 10^8, and 10^9 conidia/ml. The control was 0.05% Tween-80 only.

Treatment of *N. lugens* with *M. flavoviride*. Four hundred *N. lugens* fourth-instar nymphs were placed onto 45-d-old rice seedlings grown in each of four pots. Once the nymphs were established, a handheld sprayer was used to uniformly spray each pot of seedlings with 10 ml of its respective treatment concentration after which a glass cover was placed over each pot to maintain humidity and contain the nymphs. The treated seedlings were maintained at 28 ± 2°C, 85% relative humidity (RH), and grown under a 14-h light:10-h dark photo regime. Fifty nymphs were sampled from each pot at 24, 48, and 72 h and coded as follows: Co-24 (control, sampled at 24 h), Co-48 (control, sampled at 48 h), Co-72 (control, sampled at 72 h); 10^7-24 (10^7 *M. flavoviride* conidia/ml, sampled at 24 h), 10^7-48 (10^7 *M. flavoviride* conidia/ml, sampled at 48 h), 10^7-72 (10^7 *M. flavoviride* conidia/ml, sampled at 72 h); 10^8-24 (10^8 *M. flavoviride* conidia/ml, sampled at 24 h), 10^8-48 (10^8 *M. flavoviride* conidia/ml, sampled at 48 h), 10^8-72 (10^8 *M. flavoviride* conidia/ml, sampled at 72 h); and 10^9-24 (10^9 *M. flavoviride* conidia/ml, sampled at 24 h), 10^9-48 (10^9 *M. flavoviride* conidia/ml, sampled at 48 h), 10^9-72 (10^9 *M. flavoviride* conidia/ml, sampled at 72 h).

Illumina PE250 process. Fifty *N. lugens* collected from each treatment at each sampling period were dissected to obtain their midguts, which were then rinsed with sterile water and stored at −20°C for bacterial DNA extraction at a later time. DNA was extracted using a bacterial DNA extraction kit (Omega Bio-Tek Co., Norcross, GA) from whole bodies and dissolved in 200 μl ddH_2O. The concentration and purity of DNA were measured using 1% agarose gel electrophoresis and a Nanodrop 1000 spectrophotometer (Saveen Werner ApS, Jyllinge, Denmark).

For fungal DNA extraction, 50 *N. lugens* were collected from each treatment at each sampling period, sterilized with 75% ethanol, rinsed with sterile water, and stored at −20°C for DNA extraction at a later time. DNA was extracted using a fungal DNA extraction kit (as above) from whole bodies and dissolved in 200 μl ddH_2O. The concentration and purity of DNA were measured using 1% agarose gel electrophoresis and the Nanodrop 1000 spectrophotometer.

The parameters used for polymerase chain reaction (PCR) amplification of the DNA are shown in Table 1. During the amplification process, barcode sequences were added to the 5′ end of forward primers to distinguish samples. Each sample was amplified in triplicate. The PCR products from the same sample were mixed

Table 1. Amplification conditions of midgut microorganisms.

	Fungi	Bacteria
Primer	ITS1F:5′-CTTGGTCATTTAGAGGAAGTAA-3′	338F:5′-ACTCCTACGGGAGGCAGCA-3′
	ITS2:5′-GCTGCGTTCTTCATCGATGC-3′	806R:5′-GGACTACHVGGGTWTCTAAT-3′
Amplification system	5×Fast Pfu buffer: 4.0 μl, 2.5 mmol/L/d NTPs: 2 μl, forward primers (5 μmol/L): 0.4 μl, reverse primers (5 μmol/L): 0.4 μl, Fast Pfu polymerase: 0.4 μl, template DNA: 10 ng, ddH_2O add to 20 μl	5×Fast Pfu buffer: 4.0 μl, 2.5 mmol/L/d NTPs: 2 μl, forward primers (5 μmol/L): 0.4 μl, reverse primers (5 μmol/L): 0.4 μl, Fast Pfu polymerase: 0.4 μl, template DNA: 10 ng, ddH_2O add to 20 μl
Cycle	95°C: 2 min; 95°C: 30 s, 55°C: 30 s, 72°C: 30 s, 25 cycles; 72°C extension 5 min	

and identified using 2% agarose gel electrophoresis. The PCR products were then recovered by cutting the gel and diluting the products with Tris-HCl. Finally, the DNA products were detected using 2% agarose gel electrophoresis.

Following the preliminary results derived from electrophoresis, PCR products were quantified using a QuantiFluorTM-ST Blue Fluorescence Quantitation System (Promega Corporation, Madison, WI). Based on the amount required to sequence each sample, the PCR products were mixed in corresponding proportions. Sequencing was performed on an Illumina Miseq with two paired-end read cycles of 300 bases each.

Bioinformatic analysis. Paired-end reads were preprocessed using Trimmomatic software (Bolger et al. 2014) to detect and cut off ambiguous bases (N). It also cut off low-quality sequences with average quality score below 20 using sliding window trimming approach. After trimming, paired-end reads were assembled using FLASH software (Reyon et al. 2012). Parameters of assembly were: 10 bp of minimal overlapping, 200 bp of maximum overlapping, and 20% of maximum mismatch rate. Sequences were performed further denoising as follows: reads with ambiguous, homologous sequences or below 200 bp were abandoned. Reads with 75% of bases above Q20 were retained using QIIME software (version 1.8.0) (Caporaso et al. 2010). Then, reads with chimera were detected and removed using VSEARCH (Rognes et al. 2016). Clean reads were subjected to primer sequences removal and clustering to generate operational taxonomic units (OTUs) using VSEARCH software with 97% similarity cutoff (Rognes et al. 2016). The representative read of each OTU was selected using QIIME package. All representative reads were annotated and blasted against Silva database (Version 123) using RDP classifier (confidence threshold was 70%) (Wang et al. 2007). The microbial diversity in *N. lugens* was estimated using the alpha diversity that include Chao1 index (Chao and Bunge 2002) and Shannon index (Hill et al. 2003). The 16S rRNA gene amplicon sequencing and analysis were conducted by OE Biotech Co., Ltd. (Shanghai).

Results

Structure of bacterial communities in *N. lugens* from different treatment groups. The coverage of sequencing for all 12 samples was greater than 99%, thus providing confidence that the sequences were an accurate representation of the midgut bacterial populations (Table 2). The control group (Co-72 h) had the greatest bacterial diversity, according to Chao, Shannon, and Simpson diversity indices, followed by the group treated with the lowest concentration (10^7) of *M. flavoviride* for 72 h (10^7-72). The average Chao and Shannon values for *N. lugens* treated with the highest concentration (10^9) of *M. flavoviride* for 48 h (10^9-48) were 740 and 2.34, the lowest for all 12 samples. In terms of the Simpson diversity index, the 10^7-72 group showed the lowest value, followed by 10^9-72 group, whereas the 10^9-24 group had the highest value.

Venn plots (Fig. 1) revealed 939 common midgut OTU among the 24-, 48-, and 72-h samples of the total 3,075 OTU identified in the control (Fig. 1a). Of *N. lugens* exposed to 10^7 *M. flavoviride* conidia/ml, 699 common OTUs from a total of 3,258 were identified (Fig. 1b); 365 common OTUs from a total of 2,114 for those exposed

Table 2. Bacterial community richness and diversity indices of *N. lugens* midguts at different concentrations and time intervals following treatment with *M. flavovirides*.

Sample	Reads	OTUs (97%)	Ace (XX)*	Chao (XX)*	Sequencing Depth, %	Shannon (XX)	Simpson (XX)
Co-24	85,972	2,370	2294 (2260, 2337)	2306 (2262, 2366)	99.60	3.73 (3.71, 3.75)	0.16 (0.16, 0.16)
Co-48	84,197	1,214	1488 (1385, 1615)	1480 (1356, 1641)	99.52	2.89 (2.88, 2.90)	0.14 (0.14, 0.14)
Co-72	78,816	2,389	2931 (2894, 2976)	2919 (2876, 2974)	99.60	5.15 (5.13, 5.17)	0.14 (0.14, 0.14)
10^7-24	83,693	2,125	2533 (2501, 2574)	2533 (2493, 2587)	99.61	3.58 (3.55, 3.60)	0.27 (0.27, 0.27)
10^7-48	82,504	951	1675 (593, 1775)	1570 (1492, 1670)	99.49	2.48 (2.46, 2.49)	0.22 (0.22, 0.22)
10^7-72	94,607	2,738	2767 (2707, 2839)	2739 (2669, 2826)	99.32	4.68 (4.66, 4.70)	0.05 (0.05, 0.05)
10^8-24	83,417	1,767	2308 (2259, 2369)	2305 (2244, 2383)	99.47	2.74 (2.72, 2.76)	0.34 (0.34, 0.34)
10^8-48	84,414	545	2023 (1925, 2141)	1995 (1880, 2139)	99.37	3.05 (3.03, 3.07)	0.15 (0.15, 0.15)
10^8-72	86,067	1,020	1271 (1230, 1325)	1289 (1233, 1367)	99.70	3.19 (3.17, 3.21)	0.13 (0.13, 0.13)
10^9-24	85,058	2,025	2049 (1998, 2112)	2050 (1987, 2132)	99.52	2.49 (2.47, 2.51)	0.37 (0.37, 0.37)
10^9-48	63,794	1,424	785 (725, 866)	740 (680, 825)	99.69	2.34 (2.32, 2.35)	0.19 (0.19, 0.19)
10^9-72	80,907	1,083	1263 (1211, 1329)	1238 (1180, 1316)	99.66	3.51 (3.50, 3.52)	0.07 (0.07, 0.07)

* Mean with minimum and maximum values in parenthesis.

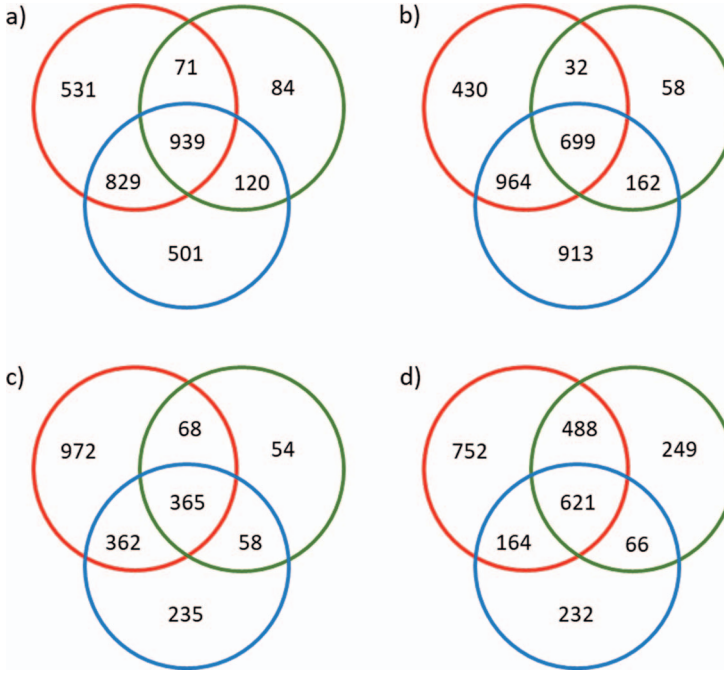

Fig. 1. Venn analysis of the number and commonality of intestinal bacterial taxa found in *N. lugens* exposed to different concentrations of *M. flavoviride* conidia. (a) Control (liquid carrier only; no conidia). (b) 10^7 conidia/ml. (c) 10^8 conidia/ml. (d) 10^9 conidia/ml. Different colors represent different post-treatment *N. lugens* sampling times: red, 24 h; blue, 48 h; green, 72 h.

to 10^8 conidia/ml (Fig. 1c); and 621 from a total of 2,572 exposed to 10^9 conidia/ml (Fig. 1d). Overall, there were fewer common OTUs in bacterial communities from the midguts of *N. lugens* treated with *M. flavoviride* than in the control group.

Analysis of bacterial community structures in the midguts of *N. lugens* after treatment with *M. flavoviride* at various concentrations yielded at the phylum level, prokaryotic microorganisms from seven phyla, that is, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Chloroflexi*, *Acidobacteria*, and *Planctomycetes,* that were taxonomically annotated and 31 taxa at the genus level with *Arsenophonus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Stenotrophomonas*, and *Bacteroides* having the greatest relative abundance (Fig. 2). Among these, the abundance of *Arsenophonus* in the midguts of *N. lugens* treated with *M. flavoviride* for 24 h at all three concentrations were greater than 50% (52.01%, 56.28%, and 59.46%) and greater than the control (Fig. 3). Although the relative abundance of *Arsenophonus* decreased over time, it corresponded to *M. flavorviride* conidia concentration treatments for insects sampled at 72 h (4.76%, 14.10%, and 12.74% for 10^7, 10^8, and 10^9, respectively), which was greater than 0.84% found in the control. *Enterobacter* reached 7.20% and 31.18%, respectively, in the 10^8-48 and

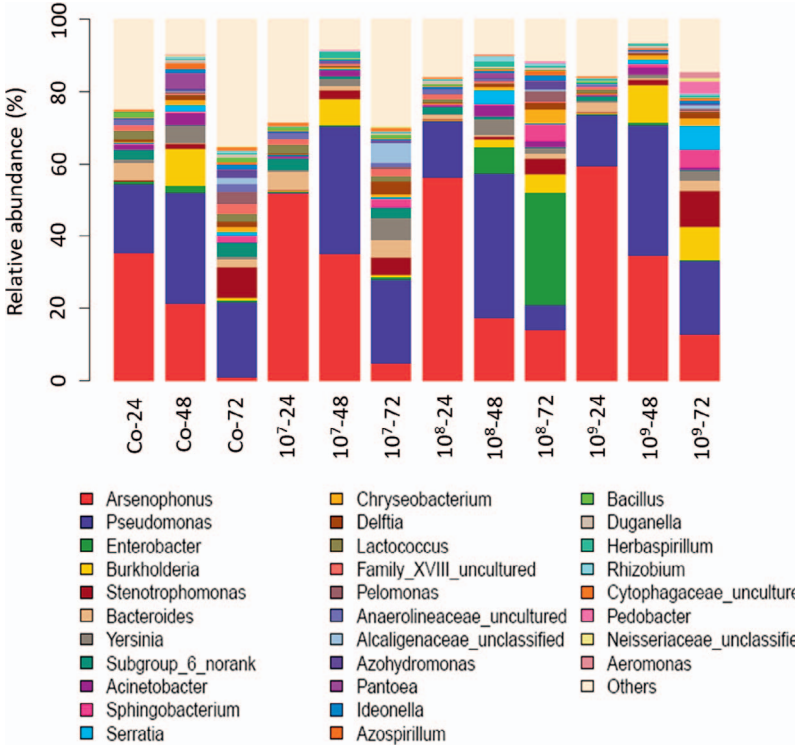

Fig. 2. Genera of intestinal bacteria found in *N. lugens* exposed to different concentrations of *M. flavoviride* conidia and sampled at different post-treatment sampling times (24, 48, 72 h). Co is control (liquid carrier only; no conidia); exponents are number of *M. flavoviride* conidia/ml.

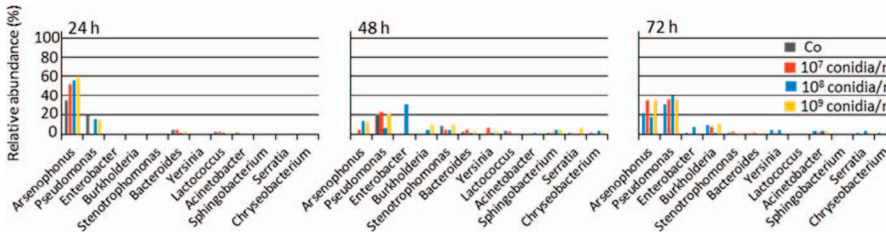

Fig. 3. Changes in selected intestinal bacteria taxa found in *N. lugens* in response to exposure to *M. flavoviride* applied at difference concentrations (see legend; Co is liquid carrier only, no conidia) and sampled at 24, 48, and 72 h after treatment.

10^8-72 treatment groups but was less than 1% in other treatment groups. *Acinetobacter* had the highest relative abundance in control group insects at 24 h. However, at 72 h, *Acinetobacter* in the control group decreased to 0.29%, which was much lower than 3.23% found at 72 h in *N. lugens* treated with 10^9 *M. flavoviride* conidia. The relative abundance of *Stenotrophomonas* and *Sphingobacterium* increased over treatment time, reaching 4.99% and 10.03% in 3-72 insects, exceeding 1.56% and 4.46% found in Co-72.

Structure of fungal communities in *N. lugens* from different treatment groups. The coverage of sequencing for all 12 samples was greater than 99%, providing confidence that the sequences were an accurate representation of the actual midgut fungal populations (Table 3). The control group Co-24 had the highest fungal community diversity according to the Chao and Shannon indices (Table 3). Among the 12 treatment groups, the Shannon index value of the 10^7-72 group was the lowest at 0.36. According to the Simpson index, Co-48 was the least diverse followed by the 10^9-48, whereas 10^7-72 was the most diverse.

A total of 367 OTUs were identified comprising 1 phyla, 5 classes, 10 orders, 13 families, and 18 genera. Among sampling times, there were 52 common OTUs from a total of 236 OTUs in *N. lugens* from the control (Fig. 4a), 50 common OTUs from a total of 195 OTUs in those exposed to 10^7 *M. flavoviride* conidia/ml (Fig. 4b), 34 common OTUs from a total of 196 OTUs in *N. lugens* exposed to 10^8 (Fig. 4c), and 43 common OTUs from a total of 194 OTUs in those exposed to 10^9 conidia/ml (Fig. 4d). In pooling sampling periods, the number of common OTU in the 10^8 treatment group was lower than that in the control group, whereas the numbers of common OTUs in the groups treated with 10^7 and 10^9 *M. flavoviride* conidia/ml were similar to the control group.

Analysis of fungal community structures in the midguts of *N. lugens* after treatment with *M. flavoviride* at various concentrations yielded at the phylum level, prokaryotic microorganisms from nine phyla, that is, *Hypocreales*, *Pleosporales*, *Ustilaginales*, *Eurotiales*, *Tremellales*, *Canthellales*, *Mortierellales*, *Trichosporonales*, and *Sebacinales*, which were taxonomically annotated, with 18 taxa at the genus level. *Metarhizium* was dominant in those *N. lugens* treated with *Metarhizium* (Fig. 5). The highest *Metarhizium* levels occurred in *N. lugens* from 10^7-72 and 10^8-72, reaching 94.82% and 93%, respectively (Fig. 5). In addition to *Metarhizium*, the level of *Sarocladium* in 10^9-48 was relatively high (Fig. 6). *Metarhizium* was also naturally present in *N. lugens* from the control (Fig. 5).

Discussion

This study showed that exposure to *M. flavoviride* can change the abundance and diversity of bacterial microorganisms in the midgut of *N. lugens* and that these changes can depend on *M. flavoviride* concentration and postexposure time. *Metarhizium flavoviride* reduced the abundance of bacterial microorganisms in the host, and diversity changed over time. Abundance and diversity of microbial symbionts are indicators of the stability of communities or ecosystems (Glasl et al. 2019). In this context, the invasion of *M. flavoviride* destabilized the microecological environment (Garrido-Jurado et al. 2016), as observed by decreasing bacterial abundance and ultimately, diversity, in the midgut. *Metarhizium flavoviride* affected

Table 3. Fungi community richness and diversity indices of of *N. lugens* midguts at different concentrations and time intervals following treatment with *M. flavovirides*.

Sample	Reads	OTU (97%)	Ace (XX)*	Chao (XX)*	Sequencing Depth, %	Shannon (XX)	Simpson (XX)
Co-24	25,461	145	148 (145, 157)	147 (145, 157)	99.99	2.50 (2.47, 2.52)	0.21 (0.20, 0.21)
Co-48	137,465	125	137 (129, 155)	135 (128, 160)	99.98	2.42 (2.42, 2.43)	0.14 (0.14, 0.14)
Co-72	152,292	123	126 (123, 134)	125 (123, 135)	99.99	1.10 (1.09, 1.10)	0.26 (0.26, 0.26)
10^7-24	148,710	106	109 (107, 120)	112 (107, 133)	99.99	0.60 (0.59, 0.61)	0.78 (0.78, 0.78)
10^7-48	152,368	109	119 (113, 135)	119 (112, 144)	99.98	1.05 (1.04, 1.06)	0.62 (0.62, 0.62)
10^7-72	162,919	118	134 (125, 155)	137 (124, 171)	99.98	0.36 (0.35, 0.37)	0.90 (0.90, 0.90)
10^8-24	130,398	107	111 (108, 121)	125 (111, 182)	99.99	0.59 (0.58, 0.60)	0.81 (0.80, 0.81)
10^8-48	164,927	140	144 (141, 153)	143 (140, 154)	99.99	2.21 (2.20, 2.22)	0.22 (0.22, 0.22)
10^8-72	118,817	71	74 (71, 85)	73 (71, 84)	99.99	0.41 (0.40, 0.42)	0.88 (0.87, 0.88)
10^9-24	145,358	131	133 (131, 141)	132 (131, 142)	99.99	1.41 (1.40, 1.42)	0.49 (0.49, 0.49)
10^9-48	130,471	116	124 (118, 139)	129.00 (119, 163)	99.99	2.39 (2.38, 2.40)	0.15 (0.15, 0.15)
10^9-72	89,190	72	75 (72, 85)	73 (72, 80)	99.99	1.16 (1.15, 1.17)	0.60 (0.59, 0.60)

* Mean with minimum and maximum values in parenthesis.

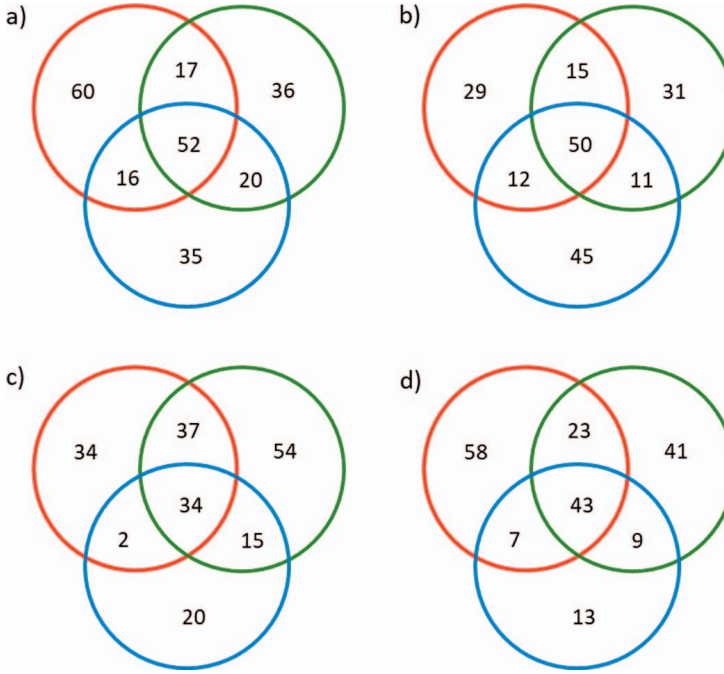

Fig. 4. Venn analysis of the number and commonality of intestinal fungal taxa found in *N. lugens* exposed to different concentrations of *M. flavoviride* conidia. (a) Control (liquid carrier only; no conidia). (b) 10^7 conidia/ml. (c) 10^8 conidia/ml. (d) 10^9 conidia/ml. Different colors represent different post-treatment *N. lugens* sampling times: red, 24 h; blue, 48 h; green, 72 h.

the structure of bacterial communities in the midguts of *N. lugens* by changing bacterial species composition in that the dominant bacterial species in *N. lugens* species shifted after treatment. For example, slight increases in *Arsenophonus* (associated with harmful mutations; Noda et al. 2001), *Acinetibacter* (degrader of imidacloprid; Li 2010), *Stenotrophomonas*, and *Sphingobacterium* (each associated with the degradation of nicotine, imidacloprid, and isotactic polypropylene) were observed (Dai et al. 2010). Further studies are needed to clarify whether the above phenomena occurred in response to the production of secondary metabolites, for example, cyclic carboxyl peptides commonly caused by fungal entomopathogen exposure. Such metabolites often elicited by exogenous or invading substances such as a chemical or a microbial pathogen are known to cause changes in the structure of bacterial communities in host midguts (Brousseau et al. 1996). In support of this, Kabaluk (2017) found that proportions of the bacterial symbionts *Pandoraea pnomenusa* Conye et al., *Pantoea agglomerans* (Ewing & Fife) Gavini et al., *Mycobacterium frederiksbergense* Willumsen et al., and *Nocardia pseudovaccinii* Gordon were linearly related to sublethal concentrations of *Metarhizium* in *Agriotes obscurus* (L.) wireworms.

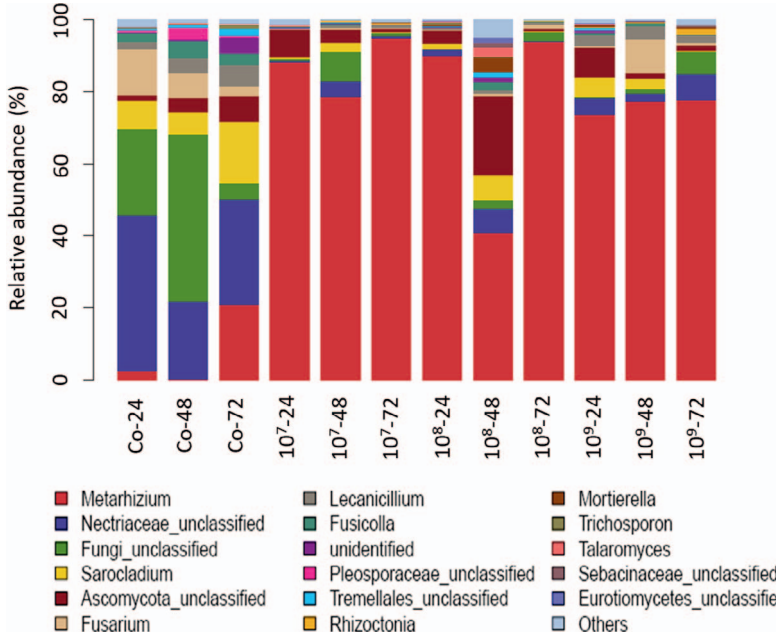

Fig. 5. Genera of intestinal fungi found in *N. lugens* exposed to different concentrations of *M. flavoviride* conidia and sampled at different post-treatment sampling times (24, 48, 72 h). Co is control (liquid carrier only; no conidia); exponents are number of *M. flavoviride* conidia/ml.

Although midgut microorganisms of sap-sucking insects have been extensively studied in recent years, fungal symbionts have received less attention. However, a recent examination of the microbiome of scale insects revealed no consistently present bacterial species, but instead noted the high prevalence of an Ophiocordyceps-related fungus belonging to a lineage of fungi that are entomo-

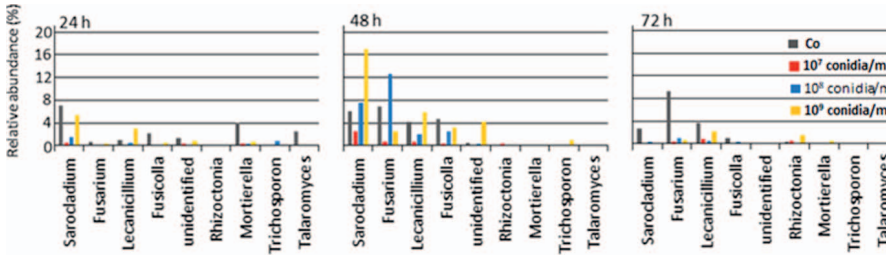

Fig. 6. Changes in selected intestinal fungal taxa found in *N. lugens* in response to exposure to *M. flavoviride* applied at difference concentrations (see legend; Co is liquid carrier only, no conidia) and sampled at 24, 48, and 72 h after treatment.

pathogens (GomezPolo et al. 2017). Presumably, the fungi can enter these insects through feeding and infection **d**uring the growth and development of insects. Yeast-like microorganism symbionts inhabiting various insects (Buchner 1965) have been described from a wide range of insects (Hamby et al. 2012, Urbina et al. 2013). Known essential functions of these symbionts include synthesis of sterols (Noda and Koizumi 2003) and nitrogen recycling (Sasaki et al. 1996). Yeast-like microorganisms and fungi are usually located in fat body tissue and in the digestive tract (Hughes et al. 2011, Urbina et al. 2013).

Our study showed a certain proportion of microorganisms in the midguts of *N. lugens* were fungi, although the number of OTUs was far less than that of bacteria. After treatment with the *M. flavoviride* suspension, *Metarhizium* spp. was dominant in all treatment groups including the control. In some treatment groups, the relative abundance of *Metarhizium* spp. was greater than 90%. *Metarhizium* spp. are one of the most common entomopathogenic fungi in the environment. Because *Metarhizium* spp. resembles the cell wall structure (Duan et al. 2009, Jin et al. 2015, Shang et al. 2015) and is covered by glycogen proteins similar to insect hosts through phosphoketose pathway when it enters the hemocoel to escape the immune recognition and defense of insect blood cells (Wang and Leger 2006). The penetrating hyphae produce granule hyphal bodies by budding or septation, which become distributed throughout all tissues and organs with the circulation of host hemolymph (Wang et al. 2006, Zacharuk 1971). It can be considered that the increase in content of *Metarhizium* spp. undoubtedly arises from the *M. flavoviride* treatments. We hypothesized that *Metarhizium* also existed in the untreated population, as it has been shown to be enzootic in other insects (Kabaluk et al. 2017); for example, *Metarhizium anispoliae* (Metschnikoff) Sorokin was the dominant fungal symbiont in the *Saissetia* sp. population from Cyprus (Priscila Gomez-Polo et al. 2017). The detection of *Metarhizium* spp. in control groups in the study indicates that lineages of these hypocrealean fungi have undergone a shift from insect pathogens to mutualists.

The *M. flavoviride* treatments also decreased the abundance and diversity of midgut microorganisms in *N. lugens***,** and the number of common OTUs in the different treatment groups also decreased as the same time. We speculated that as the conidial spores of entomopathogenic fungi infect their hosts, they come into contact with diverse competing microbes, any of which can cause antagonistic interactions. By comparing the differences in diversity indices after treatment of *N. lugens* with various concentrations of *M. flavoviride* for different periods of time, it was apparent that there were many species of fungi in the midguts of control *N. lugens*, and that the microbial abundance and diversity in the control group were relatively high. Therefore, we could conclude that treatment with *M. flavoviride* decreased the abundance and diversity of fungal microorganisms in the midguts of *N. lugens*. *Metarhizium* spp. dominated in the midguts of *N. lugens*, so differences in the fungal microbial communities in the midguts of *N. lugens* in the various treatment groups were small.

Exposure to *M. flavoviride* will change the abundance and diversity of bacterial and fungi microorganisms in the midguts of *N. lugens*, and *M. flavoviride* affected the structure of bacterial communities in the midguts by changing bacterial species composition in that the dominant bacterial species in *N. lugens* species shifted after treatment. *Metarhizium* spp. dominated in the midguts of *N. lugens* after treatment,

so differences in the fungal microbial communities in the midguts of *N. lugens* in the various treatment groups were small.

Acknowledgments

This research was partly supported by Key R & D projects in Zhejiang Province (Grant 2019C0203003) and the China Agriculture Research System (Grant CARS-24-G-07). The authors thank Todd Kabaluk, Research Biologist from Agriculture and Agri-Food Canada, for reviewing the original manuscript and providing suggestions for its revision.

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