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Research

Effects of Antibiotics on the Dynamic Balance of Bacteria and Fungi in the Gut of the German Cockroach

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Abstract

The German cockroach, *Blattella germanica* (L.) (Blattaria: Blattidae) harbored diverse microorganisms in the digestive tract, including bacteria, fungi, viruses, archaea, and protozoa. This diverse community maintains a relatively stable balance. Some bacteria have been confirmed to play crucial roles in the insect's physiology, biochemistry, and behavior. Antibiotics can effectively eliminate bacteria and disrupt the balance of gut microbiota, but the time-course of this process, the structure of the new microbial community, and the dynamics of re-assemblage of a bacterial community after antibiotic treatment have not been investigated. In the present study, antibiotic (levofloxacin and gentamicin) ingestion reduced bacterial diversity and abundance in the cockroach gut. Within 14 d of discontinuing antibiotic treatment, the number of culturable gut bacteria returned to its original level. However, the composition of the new bacterial community with greater abundance of antibiotic-resistant *Enterococcus* and *Dysgonomonas* was significantly different from the original community. Network analysis showed that antibiotic treatment made the interaction between bacteria and fungi closer and stronger in the cockroach gut during the recovery of gut microorganisms. The study on the composition change, recovery rules, and interaction dynamics between gut bacteria and fungi after antibiotic treatment are helpful to explore gut microbes' colonization and interaction with insects, which contributes to the selection of stable core gut bacteria as biological carriers of paratransgenesis for controlling *Blattella germanica*.

Key words: *Blattella germanica*, antibiotic, gut bacteria, fungi, interaction, niche

The German cockroach, *Blattella germanica* (L.), is a common indoor sanitary pest and can transmit a variety of pathogenic microorganisms and parasites (Graczyk et al. 2005, Salehzadeh et al. 2007, Vazirianzadeh et al. 2014, Zhang and Yang 2019). Because of its rapid development, high fecundity, adaptability to the human environment and pervasive resistance to chemical pesticides, the German cockroach has become an important target for pest control in indoor environments (Zhang et al. 2014, Zhang et al. 2018b, Yang et al. 2019, Pan and Zhang 2020).

Many insects host a large number of microorganisms in the gut, including bacteria, fungi, viruses, archaea, and protozoa (Gijzen et al. 1991, Moya et al. 2009, López-Sánchez et al. 2009, Hongoh 2010, Zhang and Zhang 2018). Bacteria are the most diverse and abundant taxa, and the gut lumen provides suitable conditions for the formation of bacterial biofilm (Parsek and Singh 2003, Kim et al. 2014, Zhang et al. 2020b). Gut bacteria have diverse functions in digestion and absorption of nutrients, including synthesis of vitamins, amino acids, and nitrogen metabolism, as well as in defense against pathogen colonization and reinforcement of the host immune system (Dillon and Dillon 2004, Sabree et al. 2009, Ben-Yosef et al. 2010, Feldhaar 2011, Weiss and Aksoy 2011, Douglas 2015, Zhang and Zhang 2018). Fungi are also abundant in the intestinal tract of insects, where they help the host synthesize amino acids, proteins, and degrade cellulose. For example, yeast-like symbionts in anobiid beetles can synthesize proteins and provide essential amino acids for the host insect and participate in the synthesis of sterols (Noda and Koizumi 2003). *Gloeophyllum sepiarium* can secrete over 20 enzymes such as amylase, cellulase, hemicellulase, and lignase to help termites degrade cellulose and lignocellulose (Noda and Koizumi 2003). And some fungi can also degrade harmful substances such as nicotine (Eberhardt 1995, Frankenburg and Vaitekunas 1995, Wang et al. 2003). A dynamic balance is achieved in the gut between bacteria and fungi through biofilm formation, quorum sensing signaling molecules, and antimicrobials secreted by fungi and bacteria (Kim

et al. 2014, Xiang and Huang 2017). This balance is affected by many external factors, such as food, temperature and especially exogenous antibiotics (Pérez-Cobas et al. 2015).

Several strategies are used to remove gut bacteria in insects (Ohtaka 1991, Prosser and Douglas 1991, Tegtmeier et al. 2015, Rosas et al. 2018), such as sterilizing the egg surfaces and rearing the hatchlings under aseptic conditions (Tegtmeier et al. 2015). This method is not widely used because of the strict aseptic culture conditions for the larvae. In contrast, the gut bacteria are quickly and easily removed by antibiotics, thus antibiotics are commonly used (Chen and Purcell 1997, Raymond et al. 2009, Sharon et al. 2010, Llop et al. 2018, Rosas et al. 2018, Zhang et al. 2018a). Antibiotics are most often used with the method of ingestion, and when used for eliminating bacteria, they usually have many side-effects on insects, including stress, changes in the activity of metabolic enzymes and immune responses (Woo et al. 1999), suppression of growth, development and reproduction, and higher mortality (Chen and Purcell 1997, Raymond et al. 2009, Sharon et al. 2010, Llop et al. 2018). Moreover, antibacterial agents can selectively eliminate bacteria, facilitating the proliferation of fungi and other gut microbes.

Many studies have investigated the composition and community structure of gut bacteria in insects. Few investigations, however, have examined symbiotic gut fungi and their interactions with bacteria, and the resulting dynamic balance represented in species-specific microbial communities. In this article, we analyzed the changes in community composition, recovery rules of gut bacteria and fungi, and the bacterial-fungal interaction during the process of gut bacterial recovery of German cockroaches after antibiotic treatment, which is very useful for studying the microorganism colonization and interaction with insects. Recently, new biological control technologies based on the interaction between insects and symbionts have gradually developed, such as reintroducing genetically modified symbionts to secrete toxic proteins in the hosts (Daily and Johanna 2012). The symbiont *Pantoea agglomerans* in the mosquito midgut was genetically modified to express anti-malaria proteins to disturb malaria development, thereby greatly decreased the plasmodium carrying rate of mosquitoes (Wang et al. 2012). Our study could provide a stable biological carrier for paratransgenesis through the selection of stable gut bacteria for the biological control of *B. germanica*.

Materials and Methods

Insects Collection

German cockroaches were supplied by the Key Laboratory of Animal Resistance Biology of Shandong Province and maintained in a growth chamber. Culture chambers were adjusted to 27 ± 1°C, 60 ± 5% relative humidity (RH), and a photoperiod of 12:12 (L:D) h. The insects fed on rat pellets (Shandong Experimental Animal Center, Jinan, China). All experimental insects were adult males.

Preparation of Gut Samples

In previous studies of our lab, a variety of antibiotics have been studied to remove the gut bacteria of *B. germanica*, among which the combined use of gentamicin and levofloxacin had the best removal effect (Shi et al. 2017). In the experimental group, adult male cockroaches were provided with sterile water fortified with levofloxacin (62 μg/ml) and gentamicin (62 μg/ml) and sterile rat pellets for 4 d in sterile conditions (*B. germanica* was cultivated in a sterilized beaker, and the opening of the beaker was sealed with three sterilized layers of gauze). The control cockroaches were provided with sterile water. In preparation for gut dissection at specific intervals after discontinuing the antibiotics (see below): food was cleared from the digestive tract of cockroaches by starving them for 24 h; their surfaces were disinfected with 75% ethanol for 90 s, and then thoroughly washed with sterile water to remove the disinfectant.

Gut Microbial Cultures

The digestive tracts of a total of 120 cockroaches were collected: a control (S0), and 2 d (S2), 4 d (S4), 6 d (S6), 8 d (S8), 10 d (S10), 12 d (S12), and 14 d (S14) after stopping antibiotic treatment. Each group consisted of five cockroach guts and replicated three times (8 groups × 5 cockroaches per group × 3 replications = 120 cockroaches). Gut Samples were homogenized in 1 ml of sterile water and ground uniformly under sterile conditions using a vitreous bar with a tapered tip (Jinan Zhongchu Hengtong Biotechnology Co., Ltd., Jinan, China). All samples were vortex-mixed for 1 min and centrifuged at 98 g for 5 min. A series of dilutions were carried out and multiple dilution gradients were selected for the plate culture. The supernatant (200 μl) was collected and streaked on Potato Dextrose Agar (PDA) medium using an applicator. The nystatin was added into the culture medium to inhibit the growth of mycete and yeast. The number of cultured bacterial colony was recorded at 48 h. Bacterial and fungal colonies were distinguished based on differences in the size and morphology of colonies and only the bacterial colonies were counted (Renata et al. 2013).

High-Throughput Sequencing and Analysis of Gut Microbiota

The guts of a total of 360 cockroaches were collected a control (S0) and 1 d (S1), 2 d (S2), 6 d (S6), 10 d (S10), and 14 d (S14) after stopping antibiotic treatment. Each of these six groups consisted of 20 guts and replicated three times (360 cockroaches).

DNA was extracted using the K2306 Karroten Microbial Genomic DNA extraction kit (Novoprotein Scientific Inc., Shanghai, China). The primers for amplification of the 16S rRNA gene were 338F-806R (5′-ACTCCTACGGGAGGCAGCAG-3′ and 5′-GGACTACHVGGGTWTCTAAT-3′). The 16S rRNA PCR was performed in a total volume of 20 μl containing 4 μl of 5 × FastPfu buffer, 2 μl of dNTPs (2.5 mM), 0.8 μl of Forward Primer (5 μM), 0.8 μl of Reverse Primer (5 μM), 0.4 μl of FastPfu Polymerase, 0.2 μl of BSA, 10 ng of DNA template and deionized ultrapure water (to 20 μl). The primers for ITS sequencing were ITS1F-ITS2R (5′-CTTGGTCATTTAGAGGAAGTAA-3′ and 5′-GCTGCGTTCTTCATCGATGC-3′). The ITS PCR was performed in a total volume of 20 μl containing 2 μl of 10 × Buffer, 2 μl of dNTPs (2.5 mM), 0.8 μl of Forward Primer(5 μM), 0.8 μl of Reverse Primer(5 μM), 0.2 μl of Taq Polymerase, 0.2 μl of BSA, 10 ng of template DNA and deionized ultrapure water (to 20 μl). The PCR conditions were as follows: 95°C for 3 min, 95°C for 30 s, 55°C for 30 s and a final elongation step at 72°C for 10 min of 27 cycles.

Raw fastq files were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH (http://ccb.jhu.edu/software/FLASH) according to the following criteria: the reads data were truncated at any site with an average quality score of less than 20 within 50 bp sliding windows, allowing two nucleotides to be mismatched and removing ambiguous bases in primer-matching, splicing sequences overlap longer than 10 bp and removing unspliced sequences. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) and chimeric sequences were identified and removed using UCHIME. The community richness indices (Chao1 and Ace) and diversity indices (Shannon and Simpson) were estimated using

MOTHUR (http://www.mothur.org). We used the UniFrac server for community comparisons.

Network Analysis

In order to study the relationship among the gut flora of *B. germanica* after stopping the antibiotic treatment, the potential relationship between the bacterial and fungal taxa was described by network analysis using the CoNet plug-in in Cytoscape (Shannon et al. 2003, Soffer et al. 2015). In order to reduce the complexity of calculation and ensure the accuracy of taxonomic information, network analysis was conducted at the genus level of these two microbiomes. To explore all the pairwise associations and correlation scores (Spearman correlation, Pearson correlation, Kullback-Leibler dissimilarity, Bray-Curtis dissimilarity, and mutual information) were calculated (Faust and Raes 2012, Faust et al. 2012). The Brown method integrated the *P* values of the five methods, only significant correlations were retained ($P < 0.05$) (Soffer et al. 2015). After the Brown merging *P* values, the Benjamini–Hochberg multiple tests were performed as a correlation (Hu et al. 2017). We import the obtained correlation into the Gephi platform and use the Fruchterma Reingold algorithm for visualization (Bastian et al. 2009). Though the plug-in Network Analyzer in Cytoscape to calculate the network topology parameters, the degree, betweenness centrality, and closeness centrality of each node in the network (Assenov et al. 2008). The clustering coefficient and network density were selected to reflect changes in gut microbial community combination (Barberan et al. 2012), and degree, betweenness centrality, and closeness centrality were used to explore the key hub of the network (Sporns et al. 2007). Network analysis was used to explore the relationship between bacteria and fungi in gut after antibiotic treatment.

Statistical Analysis

The statistical analysis was performed using SPSS version 19.0 for Windows (IBM, Armonk, NY). The independent sample T-test was performed to assess the significant difference in the number of culturable bacteria between each treatment group and control group. Results with $P < 0.05$ between groups were considered statistically significant. Using a one-way ANOVA to examine the significant difference of richness and diversity indices of 18 bacterial samples or 15 fungal samples. $P < 0.05$ is considered a significant difference. The Kruskal–Wallis H test with Benjamini–Hochberg false discovery rate (FDR) correction was used to evaluate the relative abundance differences of bacteria and fungi among multiple groups implemented in the R software.

Availability of Data and Materials

All nucleotide sequences from this study were uploaded to the NCBI SRA (Sequence Read Archive) database, and the accession numbers are PRJNA594114 and PRJNA594155.

Results

Culturable Bacteria After Antibiotic Treatment

The 4 d of continuous antibiotic treatment was effective at reducing the number of culturable bacteria in the gut of German cockroach males from Fig. 1. After stopping the antibiotic treatment, however, the number of culturable gut bacteria increased rapidly and recovered to the pretreatment level on 14 d (Fig. 1). The result of the gut count is not the exact number of gut bacteria, it only shows the number of gut bacteria that can be cultured in vitro.

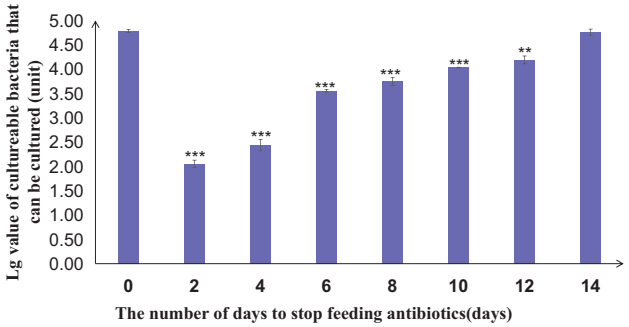

Fig. 1. Number of culturable bacteria (log-transformed) from the guts of German cockroach adult males on different days after discontinuing the availability of antibiotics in their drinking water. 0 indicates males provide sterile water, the other numbers indicate the number of days to stop antibiotic treatment. All treatments were compared to the 0 treatment, with significant differences indicated as follows: ** ($0.001 < P \leq 0.01$), *** ($P \leq 0.001$). Each bar represents the mean of five guts and ±SEM is shown.

Microbial Community Organization Before and After Antibiotic Treatment

Bacteria

After pyrosequencing, 949,839 raw sequences and 2,935 OTUs were obtained from the 18 samples (three replicates of each: a control, and 1, 2, 6, 10, and 14 d after stopping antibiotic treatment).

The rarefaction curves tended to asymptote, showing saturation of the sequencing data, substantial abundance of bacterial reads, and sufficient depth for analysis of the diversity of the bacterial community (Fig. 2). The rarefaction curves also showed variation in OTU density over time after withdrawal of the antibiotics. OTU density of the cockroach gut was highest for the treated cockroaches (S0) and lowest 1 (S1) and 2 (S2) day after the 4-d antibiotic treatment. These curves also indicated that the bacterial richness in the gut lumen of the German cockroach increased with time since discontinuing the antibiotic treatment. Nevertheless, the bacterial richness remained low compared with the controls even in 14th day after stopping the antibiotic treatment (Fig. 2).

Nineteen phyla and 122 genera of bacteria were detected. Sequences that could not be classified were assigned 'no rank'. One-way ANOVA comparing the six treatment groups indicated that the number of OTUs and the bacterial diversity indices were significantly different among the groups ($P < 0.05$) but the bacterial richness indices were not significantly different ($P > 0.05$) (Table 1).

Using Bray-Curtis distance based on pyrosequencing data, we conducted PCA, PCoA, and NMDS analyses. The PCA score map indicated that S0, S10, and S14 were grouped on the left of the graph and separated along PC1, which explained 46.35% of the total variation, from S1 and S2 (Fig. 3). Treatment group S0 separated from S2, S6, S10, and S14 along PC2, which explained 35.19% of the total variation (Fig. 3). Overall, the gut bacterial community soon after the discontinuation of antibiotics (on the 1st and 2nd day) separated from the remaining three groups (on the 6th, 10th, and 14th day), which were more similar to each other, and S0 separated from the latter groups (Fig. 3). This pattern was generally confirmed with PCoA and NMDS analyses (Supp Fig. S1 [online only]).

The relative abundance of 16S rRNA sequences, grouped at the phylum level, are shown in Fig. 4. Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, and Planctomycetes were most represented, with Firmicutes being the most abundant in the control cockroaches (41.4%), followed by Bacteroidetes (35.0%). The most abundant of the gut microbiota on the 1st and 2nd day after

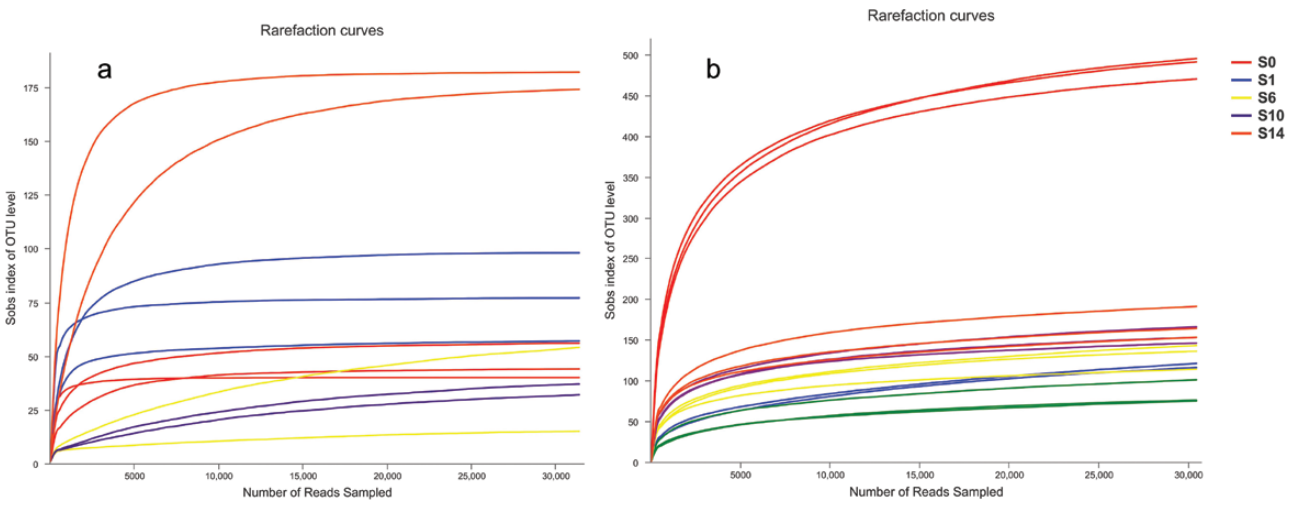

Fig. 2. Rarefaction analysis of the different bacterial (a) and fungal (b) samples, including a control (S0), and 1 (S1), 2 (S2), 6 (S6), 10 (S10), and 14 (S14) d after stopping antibiotic treatment. Sobs represent the observed number of species.

discontinuing the antibiotics were the Firmicutes (65.0 and 60.9%, respectively), but in the last three groups (the 6th, 10th, and 14th day), the Bacteroidetes were most abundant (40.1, 45.3, and 45.9%) and Fusobacteria were second in abundance (30.2, 26.5, and 22.3%) was the second-highest (Fig. 4, Supp Table S1 [online only]).

The relative abundance of the major bacterial genera is shown in Fig. 4. Bacteria in the gut lumen were most diverse in the control cockroaches that were not exposed to antibiotics, as also represented in Table 1. Antibiotic treatment for 4 d dramatically reduced the bacterial diversity, but the diversity of the bacterial community gradually increased with time after stopping the antibiotic treatment; nevertheless, even after 14 d, bacterial diversity did not return to the level of the control cockroaches (Fig. 4). This indicates that antibiotics caused an irreversible change in the gut bacterial community, for at least 14 d.

The relative abundance of the 15 most dominant bacterial genera was subjected to statistical analysis among the six treatment groups (Fig. 5). For 10 genera, there were statistically significant differences ($P < 0.05$) among the six groups. The most common pattern was for some genera that were represented at low relative abundance in the control cockroaches (e.g., *Candidatus_Soleaferrea*, *Anaerotruncus*, *Enterococcus*, *Desulfovibrio*) to undergo dramatic but transient increases or declines in abundance, but return to their original levels after 14 d on antibiotics-free water. Some genera, however, remained at lower abundance after 14 d (e.g., *Tyzzerella_3*, *Parabacteroides*, *Alistipes*), whereas others attained higher relative abundances after 14 d without antibiotics (e.g., *Fusobacterium*, *Dysgonomonas*) (Fig. 5).

Fungi

After pyrosequencing, 698,568 raw sequences and 1,031 OTUs were obtained from the 15 fungal samples. A total of eight phyla and 180 genera of fungi were detected. Sequences that could not be classified were assigned 'no rank'. Using one-way ANOVA for comparison, the number of OTUs and the fungus richness indices were significantly different ($P < 0.05$) (Table 2). The fungus samples on the 2nd day were unqualified and were not sequenced.

The rarefaction curves tended to asymptote, suggesting that the depth of the sequencing data was sufficient (Fig. 2). The relative abundances of the fungi at the phylum and genus levels are shown in Fig. 6. The 15 most dominant genera are shown in Fig. 7. The most abundant genus of fungi was *Candida*, which increased from 41.4% in control cockroaches up to 96.7% of all sequences 6 d after stopping the antibiotic treatment; it declined to 19.3% 8 d later (Fig. 6, Supp Table S3 [online only]). Of the 15 fungus genera, only three genera showed statistically significant changes with respect to antibiotic treatment (unclassified_k_Fungi, *Mortierella,* and unclassified_f_norank_o_*Pleosporales*; $P < 0.05$).

Dynamic Network Between Fungi and Bacteria

Five networks represent the interactions among microbes in control cockroaches and during the recovery of the microbial community in the gut of German cockroaches after antibiotic treatment (Fig. 8). These networks differentiate based on the number of bacterial and fungal nodes and the number of edges of microbial interactions. Compared to the control group, the clustering coefficient and network density of the antibiotic treatment groups were respectively increased by 0.039, 0.024, 0.020, 0.039 and 0.055, 0.078, 0.072, 0.063, which indicated that gut microbiome associations were more tightened with the antibiotic treatment (Table 3). The fungi in the networks of the T6 was reduced by 50 nodes; the T10 and the T14 were increased by 19 and 64 nodes than the previous group. The bacteria in the networks of the T6, the T10, and the T14 were increased by 13, 9, and 4 nodes than the previous group. It also displayed that the edges linking bacteria to fungi in the networks of the T6 and T10 were reduced by 13 and 7 edges, the T14 was increased by 294 edges, the edges linking bacteria to bacteria in the networks of the T6, the T10, and the T14 were increased by 38, 1 and 31 edges, the edges linking fungi to fungi in the networks of the T6 was reduced by 355 edges and the T10 and T14 were increased by 371 and 932 edges than the previous group (Table 3).

The network analysis demonstrated that antibiotics interfered with the balance between bacteria and fungi and caused dysbiosis: the percentage of fungi–fungi and fungi–bacteria interactions increased, while bacteria–bacteria interactions were reduced. In terms of overall relationships, antibiotic treatment leads to a closer relationship between gut microflora.

Table 1. Richness and diversity indices of 18 bacterial samples representing three replicates each of six groups

ID	Coverage	Threshold	Number of OTUs	Alpha diversity			
				ACE	Chao	Shannon	Simpson
S0-1	0.999245	0.03	385	394	398	4.37	0.0333
S0-2	0.999344	0.03	392	401	403	4.59	0.0279
S0-3	0.999114	0.03	395	408	410	4.59	0.0227
S1-1*	0.999475	0.03	68	80	80	1.57	0.3292
S1-2	0.998753	0.03	116	207	160	2.37	0.1548
S1-3	0.999081	0.03	104	136	125	2.22	0.1449
S2-1*	0.999442	0.03	74	91	91	1.37	0.4586
S2-2	0.999475	0.03	92	106	103	1.62	0.3281
S2-3	0.999574	0.03	66	78	79	1.74	0.2514
S6-1	0.999344	0.03	129	143	143	2.49	0.1514
S6-2	0.999213	0.03	130	153	153	2.64	0.1350
S6-3	0.999410	0.03	107	121	145	2.44	0.1599
S10-1	0.999344	0.03	138	152	165	2.72	0.1361
S10-2	0.999344	0.03	145	160	156	2.75	0.1288
S10-3	0.999278	0.03	149	167	168	2.95	0.1091
S14-1	0.999475	0.03	169	178	177	3.30	0.0718
S14-2	0.999377	0.03	143	157	159	3.21	0.0774
S14-3	0.999541	0.03	133	143	140	3.17	0.0828
P value			0.018	0.063	0.097	0.015	0.004

OTUs were defined at the 97% similarity level (the threshold is 0.03). The 18 samples represent three replicates each of six treatment groups that include a control (S0), and 1 (S1), 2 (S2), 6 (S6), 10 (S10), and 14 (S14) d after stopping the antibiotics treatment. Sample S1-1 (*) and S2-1 (*) were discarded as an outlier in community composition relative to the other two replicates, and it was also excluded from all subsequent analysis.

Discussion

Antibiotic treatment is a common method to obtain aseptic insects (Woo et al. 1999, Yong-Ming et al. 2006, Ben-Yosef et al. 2008, Rosas et al. 2018). The combination treatment of levofloxacin and gentamicin on German cockroaches for 4 d was effective in removing gut bacteria. Both cultivable bacteria and high-throughput sequencing showed a very low level of bacterial flora. The number of the cultivable gut bacteria had basically recovered to the original level on the 14th day after stopping antibiotic treatment, but there were significantly different in the composition of the gut microbial community, which was consistent with the studies of the rifampicin treatment on German cockroaches. Rifampin treatment experiment showed that bacterial diversity and richness were not completely recovered to the original level after stopping antibiotic for 10 d (Rosas et al. 2018). In addition, the composition of the gut microbiota of cockroaches was different after different antibiotic treatments. Our study showed that after the combined utilization of gentamicin and levofloxacin, the relative abundance of *Candidatus*, *Soleaferrea*, *Fusobacterium*, *Bacteroides*, and *Anaerotruncus* in the gut of cockroaches was increased. After the treatment of rifampicin, the abundance of *Fusobacterium* and *Desulfovibrio* was relatively high (Rosas et al. 2018). After the use of doxycycline, the abundance of Alphaproteobacteria in the gut of cockroaches was relatively high (Pietri et al. 2018). After the use of vancomycin, the abundance of Enterobacteriaceae, Yersiniaceae, Budviciaceae, and Enterobacterales in the gut of cockroaches was relatively high (Domínguez-Santos et al. 2020). Different antibiotics had different effects on the removal of gut microbes of German cockroaches, but the gut microbes of German cockroaches could recover to the original level after stopping antibiotic treatment (Rosas et al. 2018, Domínguez-Santos et al. 2020).

When gut bacteria were removed by antibiotics, those bacteria that are not susceptible to antibiotic and fast-growing bacteria may utilize the limited oxygen and other resources to grow rapidly and become dominant in the gut temporarily, such as *Candidatus*, *Soleaferrea*, and *Anaerotruncus* (Wertz and Breznak 2007, Ryu et al. 2008, Hassan et al. 2011, Yogurtcu and Tuncer 2013, Huang et al. 2016, Abdolmaleki et al. 2019, He et al. 2020). The abundance of *Enterococcus*, *Dysgonomonas*, and *Desulfovibrio* were significantly increased in the recovery period and most of them showed resistance to different kinds of antibiotics. *Enterococcus* is well known for resistance to gentamicin, streptomycin, chloramphenicol, tetracycline, and streptomycin; *Desulfovibrio* are resistant to penicillin and rifampicin; *Dysgonomonas* are resistant to various beta-lactams, erythromycin, aminoglycosides, and fluoroquinolones, but was susceptible to clindamycin, minocycline, and chloramphenicol (Dzierzewicz et al. 2001, Hironaga et al. 2008). To better adapt to the gut environment, *Enterococcus* can produce tyramine, which is related to tyrosine metabolism, to enhance the adhesion between bacteria and guts (Ladero et al. 2013, Pérez-Cobas et al. 2013). *Dysgonomonas*, *Fusobacterium*, etc., can rapidly proliferate and occupy a certain proportion, which is related to the basic ecological niche they have realized (Mikaelyan et al. 2016). Interestingly, although *Parabacteroides* is resistant to tetracycline and β-lactam antibiotics, its abundance decreased because these bacteria that possessed the gene ermF and beta-lactamases (BLAs) were more sensitive to the aminoglycoside antibiotic gentamicin (Boente et al. 2010, Brook et al. 2013). In addition, we hypothesized that gut bacteria can not completely recover after treatment with antibiotics, even some taxa that are sensitive to antibiotics may remain in the guts or feces at very low abundance and can colonize their gut niche once again after antibiotic pressure was removed. In fact, similar results had also been showed in *Zootermopsis angusticollis*, which revealed a permanent reduction in bacterial diversity after rifampicin treatment (Rosengaus et al. 2011).

The interaction between bacteria and fungi is mainly antagonistic and achieves a dynamic balance. A variety of bacteria in the gut of *B. germanica* can produce antimicrobial substances that have a broad spectrum for fungi or bacteria, for example, *Bacillus*

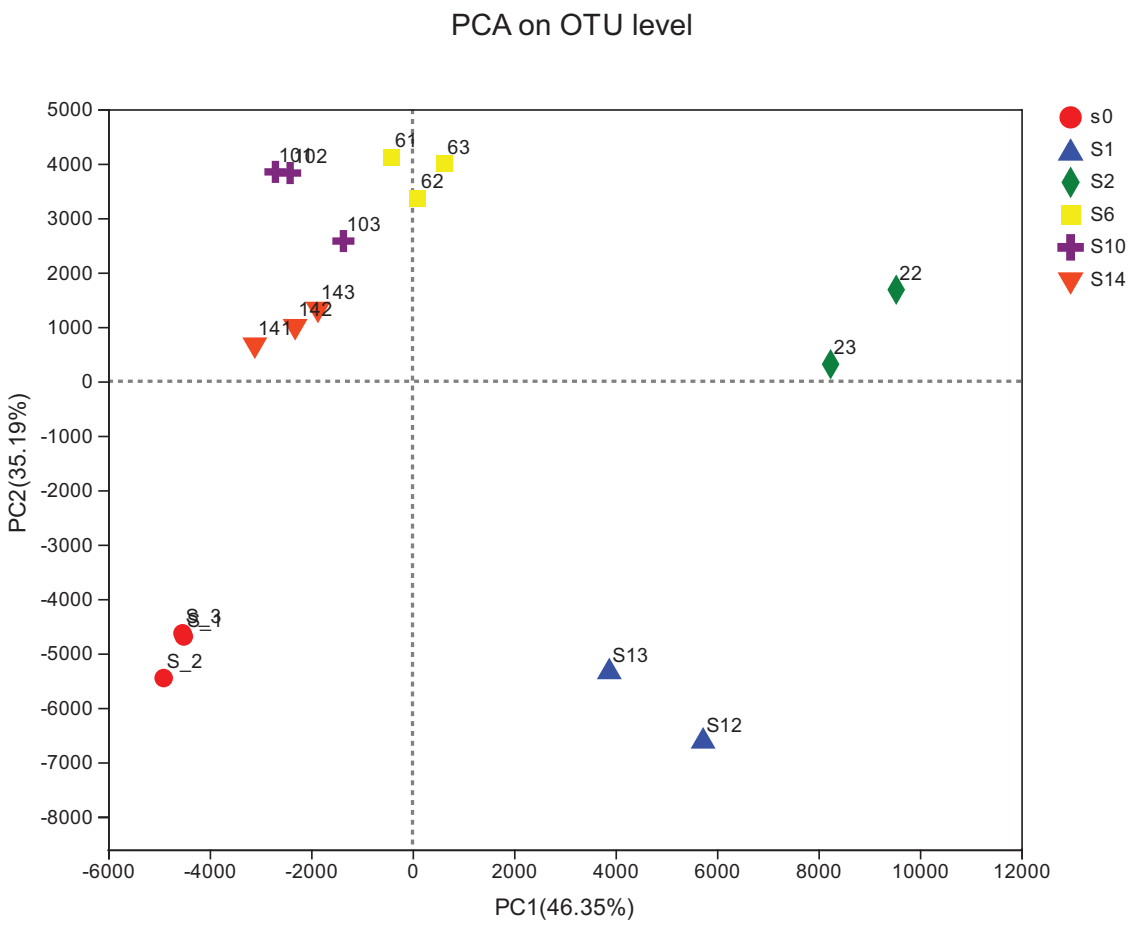

Fig. 3. Principal components analysis showing the similarity of the 16 bacterial communities based on the Bray-Curtis distance. Principal components (PCs) 1 and 2 explained 46.35 and 35.19% of the variance, respectively. The 16 bacterial samples, representing three replicates each of six treatment groups that included a control (S0), and 1 (S1), 2 (S2), 6 (S6), 10 (S10), and 14 (S14) d after stopping antibiotic treatment. One replicate in the S1 and the S2 treatment group was excluded from analysis as an outlier relative to the other two replicates in the same treatment group. In S0, one replicate is obscured by another replicate.

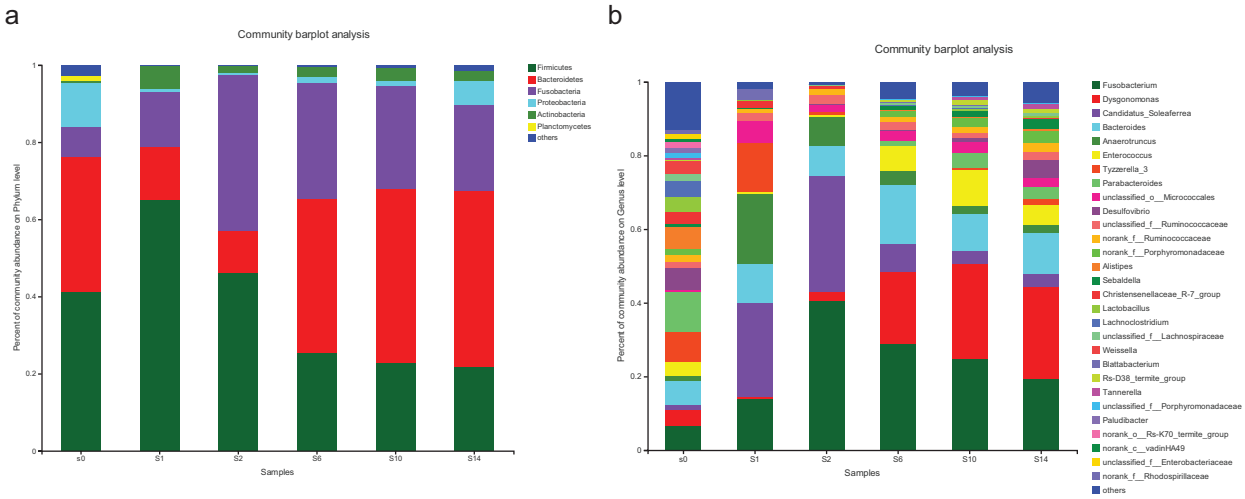

Fig. 4. Bacterial composition of the different communities at the Phylum (a) the Genus (b) level. The relative read abundances of different bacterial genera within the different communities are shown. Taxa with an abundance <1% were included in 'others'. The bacterial samples including a control (S0), and 1 (S1), 2 (S2), 6 (S6), 10 (S10), and 14 (S14) d after stopping the antibiotic treatment.

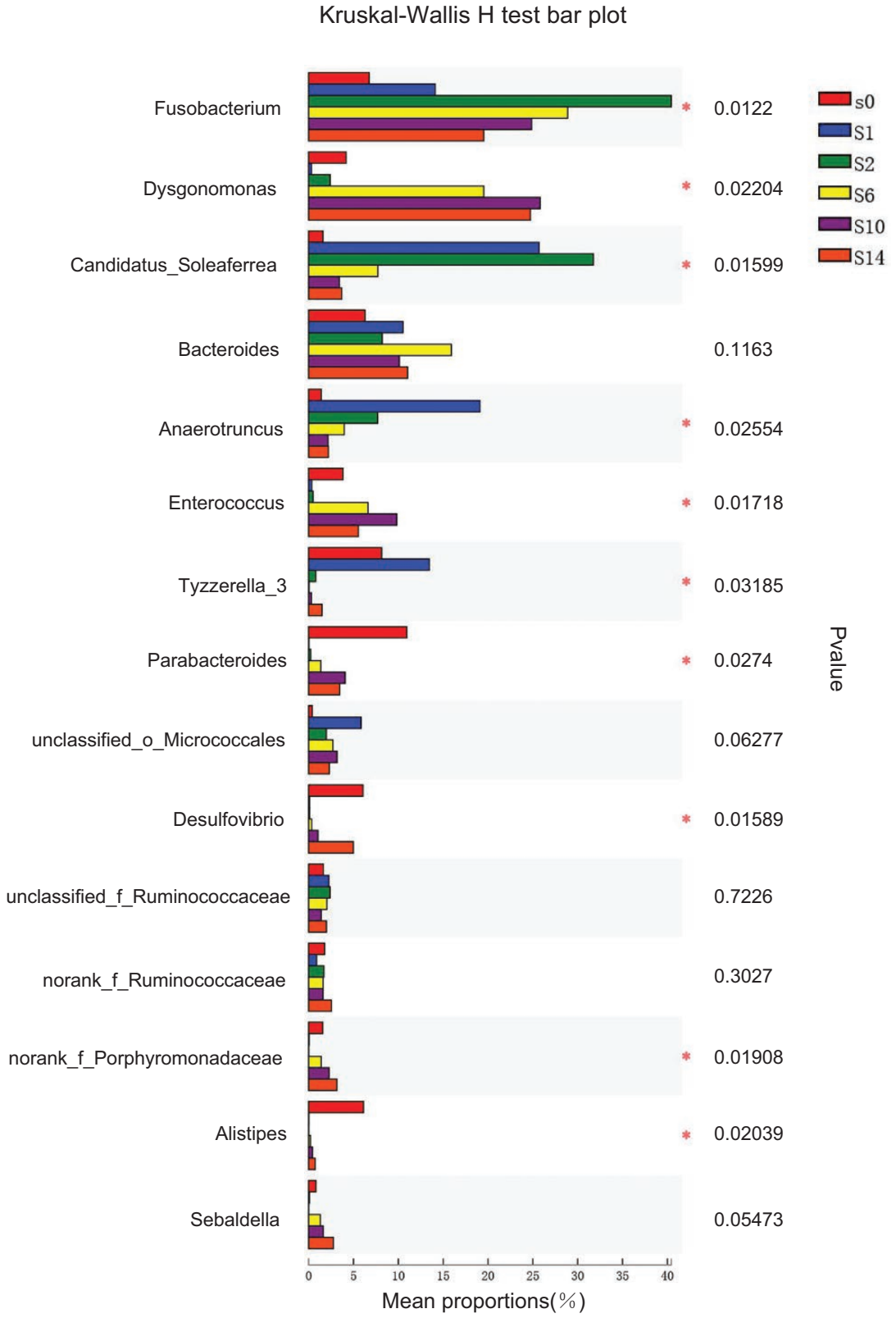

Fig. 5. Differences at the Genus level of bacterial relative abundance across experimental groups after antibiotic treatment was discontinued. The bar length for each genus indicates its average relative abundance in each sample group, and the different colors indicate the six groups (the combined three replicates of each of the six treatment groups included a control (S0), and 1 (S1), 2 (S2), 6 (S6), 10 (S10), and 14 (S14) d after stopping the antibiotic treatment). The *P* value is shown, and * indicates that the six groups are significantly different from each other ($P < 0.05$).

Table 2. Richness and diversity indices of 15 fungal samples representing three replicates each of five groups

ID	Coverage	Threshold	Number of OTUs	Alpha diversity			
				ACE	Chao	Shannon	Simpson
S0-1	1.000000	0.03	55	67	65	0.26	0.9175
S0-2	1.000000	0.03	42	21	19	0.20	0.9315
S0-3	0.999936	0.03	43	39	39	0.98	0.4994
S1-1	1.000000	0.03	77	42	38	0.89	0.4702
S1-2	1.000000	0.03	96	76	76	1.24	0.4695
S1-3	0.999904	0.03	57	43	42	0.90	0.4692
S6-1	0.999458	0.03	55	178	178	1.72	0.3003
S6-2	0.999841	0.03	14	181	181	2.44	0.2585
S6-3*	0.999872	0.03	37	62	62	0.49	0.8178
S10-1	0.999681	0.03	33	55	55	1.99	0.2058
S10-2*	1.000000	0.03	76	42	42	2.12	0.2370
S10-3	0.999681	0.03	36	43	44	1.58	0.3515
S14-1	0.999681	0.03	175	77	77	2.93	0.1267
S14-2	0.999936	0.03	181	96	96	1.39	0.4890
S14-3*	0.999522	0.03	54	59	60	1.51	0.4276
P value			0.008	0.009	0.009	0.084	0.010

OTUs were defined at the 97% similarity level (the threshold is 0.03). The 15 samples represent three replicates each of five treatment groups that include a control (S0), and 1 (S1), 6 (S6), 10 (S10), and 14 (S14) d after stopping the antibiotics treatment. Three samples indicated with * (S6-3, S10-2, and S14-3) were discarded as outliers in community composition relative to the other two replicates in their respective groups, and they were also excluded from all subsequent analysis.

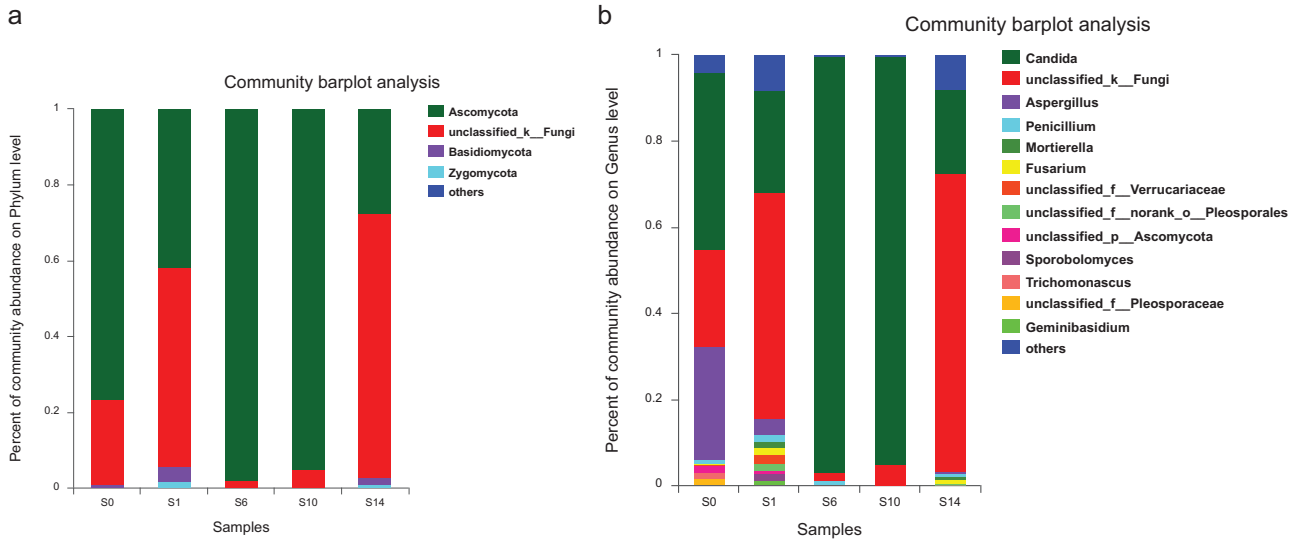

Fig. 6. Composition of the different fungal communities at the Phylum (a) the Genus (b) level. The relative read abundances of different fungus genera within the different communities are shown. Taxa with an abundance <1% were included in 'others'. These samples, including a control (S0), and 1 (S1), 6 (S6), 10 (S10), and 14 (S14) d after stopping the antibiotic treatment.

and *Pseudomonas* had significant inhibitory effects on *Beautalia bassiana* and *Lactobacillus* and *Weissella* can produce bacteriocins, adhesion inhibitors, and organic acids to inhibit the grow of bacteria (Stiles and Holzapfel 1997; Tang et al. 2005; Allaker and Douglas 2009; Zhang et al. 2012, 2013, 2020a; Huang et al. 2013). On the contrary, the fungi can also produce antibiotics such as penicillin and streptomycin to inhibit bacterial growth (Kester et al. 2011). Fungi and bacteria usually need compete adhesion sites to build biofilms in an insect's gut by competing nutrients and secreting substances and evade the host's immune recognition (Peleg et al. 2008, Peters et al. 2010, Huang et al. 2016, Förster et al. 2016). When most bacteria in the gut were removed by antibiotics, fungi could get more adhesion sites to grow and this must be the main reason that the abundance of *Candida* was so high in the early stage of the recovery group. In addition, the balance was also regulated by the immune system of the insect. Innate immunity based on antimicrobial peptides (AMPs) and reactive oxygen (ROS) can effectively eliminate harmful gut bacteria by shaping symbiotic communities (Buchon et al. 2009, Ryu et al. 2010). In fruit flies, the Toll pathway is mainly response to fungi and Gram-positive bacteria. While the Imd pathway, which is similar to the mammalian TNF and TIR-domain-dependent TLR pathway, mainly response to Gram-negative bacteria (Bosco-Drayon et al. 2012, Akira et al. 2006, Lemaitre and Hoffmann 2007, Ferrandon et al. 2007, Royet and Dziarski 2007, Vallabhapurapu and Karin 2009, Neyen and Lemaitre 2016). The increasing of clustering coefficient and

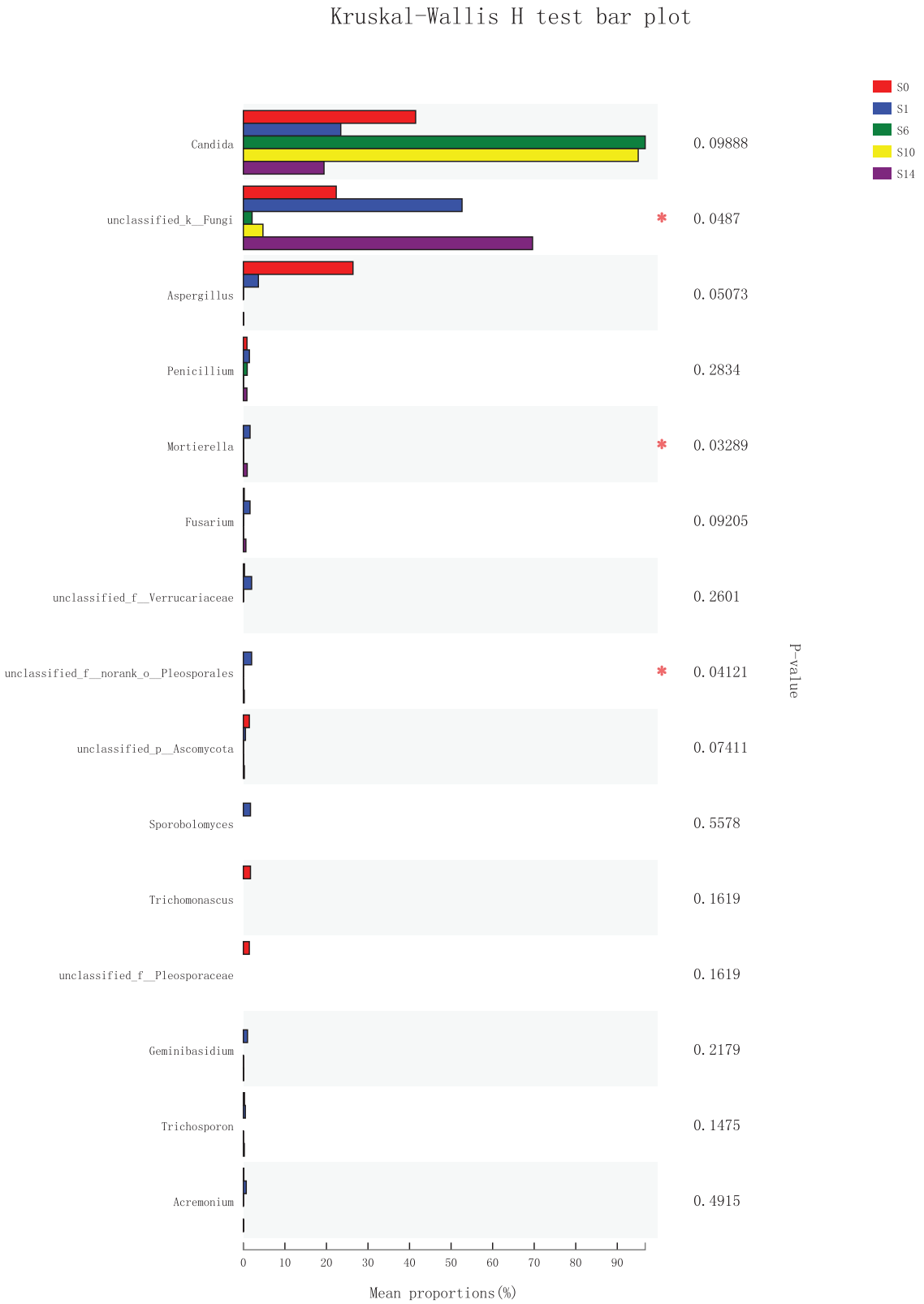

Fig. 7. Differences at the Genus level of fungal relative abundance across experimental groups after antibiotic treatment was discontinued. The bar length for each genus indicates its average relative abundance in each sample group, and the different colors indicate the six groups (including a control (S0), and 1 (S1), 6 (S6), 10 (S10), and 14 (S14) d after stopping the antibiotic treatment). The *P* value is shown, and * indicates that the five groups are significantly different from each other ($P < 0.05$).

network density indicated that bacteria and fungi in the cockroach gut established a closer relationship after antibiotic treatment. We concluded that it was the consequence of multi-factors by antibiotics to tighten the core microbes and lose the low-abundance of microbes that temporarily host in the gut.

Conclusions

In summary, antibiotic treatment significantly reduced the diversity and abundance of gut bacteria, changed the composition of the gut microbiota, and made the relationship of gut microorganisms closer, which is useful for studying the gut microbes'

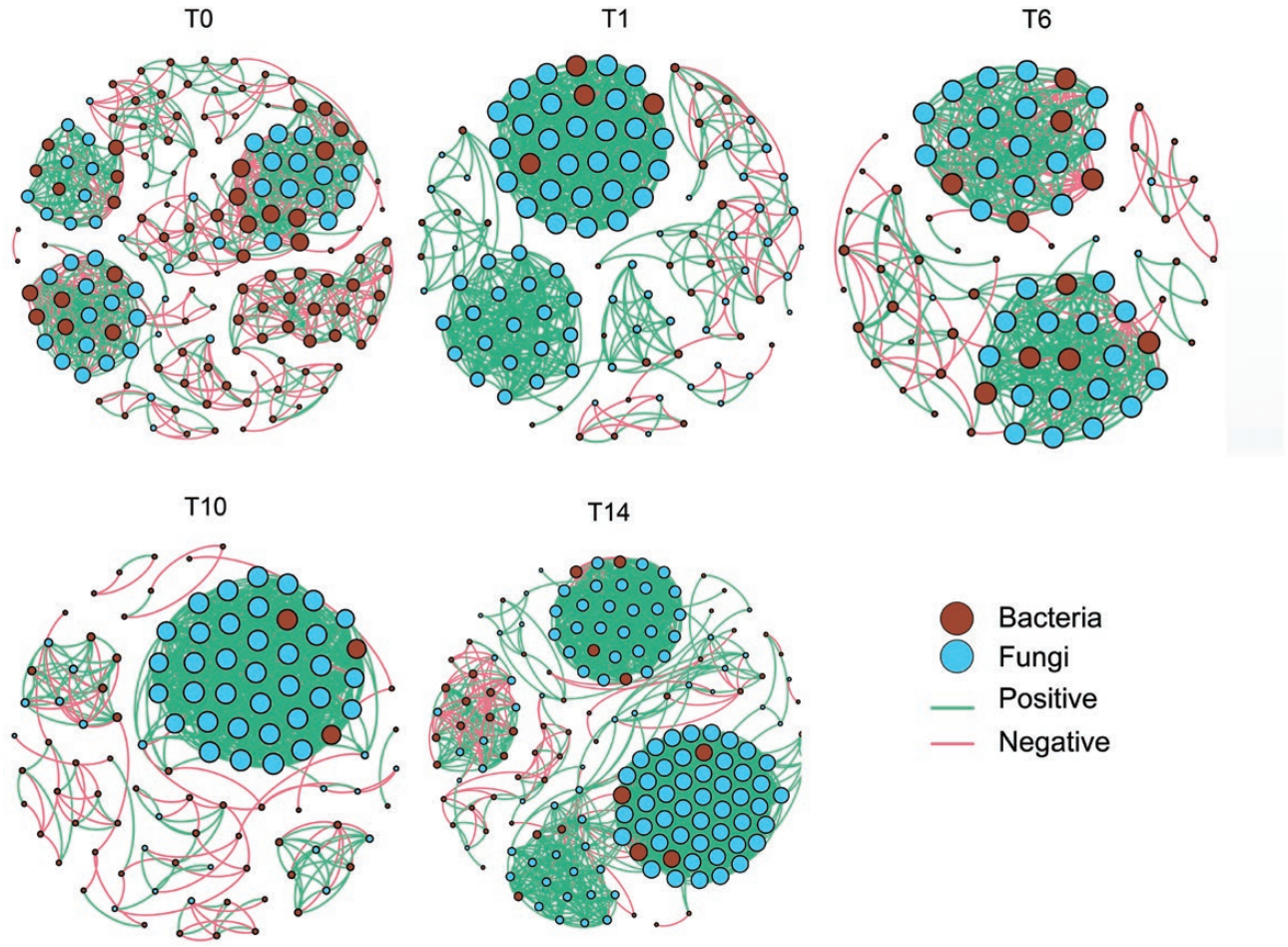

Fig. 8. The networks visualize antibiotic treatment effects on the co-occurrence pattern between bacteria and fungi in the intestines of German cockroach. The node size is proportional to the abundance of taxa, and the nodes filled in red are bacterial taxa and in blue are fungi taxa. The edges are colored according to interaction types; positive correlations are labeled with green and negative correlations are colored in red. The five groups that include a control (T0), and 1 (T1), 6 (T6), 10 (T10), and 14 (T14) d after stopping the antibiotic treatment.

Table 3. Topological indices of each network in Fig. 8

	T0	T1	T6	T10	T14
Clustering coefficient	0.804	0.843	0.828	0.824	0.844
Network density	0.072	0.127	0.150	0.144	0.135
Number of nodes	156	114	81	109	177
Number of edges	869	815	485	850	2,107
Number of fungal nodes	47	82	36	55	119
Number of bacterial nodes	109	32	45	54	58
Number of edges linking bacteria to fungi	332	183	170	163	457
Number of edges linking fungi to fungi	212	596	241	612	1,544
Number of edges linking bacteria to bacteria	325	36	74	75	106

T0 indicates feeding sterile water, the other numbers indicate the number of days to stop antibiotic treatment.

colonization and interaction with host insects. Meanwhile, our study could also lay the foundation for the exploitation of new control strategies based on core gut flora. It can help find potential core microbes from cockroach guts as stable biological carriers of paratransgenesis for the biological control of *B. germanica*.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

Fig. S1 Sample sorting analysis. Study on bacterial community composition of 16 samples by PCoA analysis based on Bray-Curtis distance. Principal components (PCs) 1 and 2 explained 45.7% and 33.72% of the variance, respectively. NMDs showing the difference of bacterial communities according to Bray-Curtis distance. These samples, including a control (S0), and 1 (S1), 2 (S2), 6 (S6), 10 (S10) and 14 (S14) days after stopping antibiotic treatment.

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