The gut microbiota of silkworm are altered by antibiotic exposure

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In recent years, antibiotics have been frequently found in the environment [1], such as soil [2], lakes, and rivers [3]. Antibiotic residues in the environment could alter the microbial structure [4] and cause bacterial resistance [5]. More worryingly, a review proposed that antibiotic residues in the environment could enter the gut through the food chain, and disturb the microbial balance, resulting in dysbacteriosis [6]. But until now, it is still unknown how antibiotics affect the interaction of bacteria and fungi in the intestine.

Microorganisms exist widely in nature, with high richness and diversity, and their interactions are conducive to realize a variety of functions, which plays an important role in maintaining the stability of the ecosystem [7]. Over the past decade, more and more research on microbial interaction attracted much attention, and the microbial cooperative phenotypes played a central role in many functions, including quorum sensing, biofilm formation, antibiotic resistance, and pathogenesis. Rakoff-Nahoum et al. [8] tested the evolved cooperation within Bacteroidales and found that diet was the key factor affecting the bacterial cooperation during growth. Durán et al. [9] investigated the rhizosphere microbial community of Arabidopsis thaliana and detected mostly...
negative correlations between bacteria and filamentous fungi, especially bacterial microbiota was essential for plant survival and protection against root-derived filamentous fungi. However, Xu et al. [10] found that the correlations between the core endophytic bacteria and fungi in *Oxycoccus gracilis* were mainly synergistic. These inconsistent results might be implicated by different hosts. The factors affecting microbial interaction and their underlying mechanism remained poorly understood.

Intestinal microbiota are the largest and most complex microecosystem, affecting a variety of host activities, including digestion, immune response, and pathogen defense [11]. Host genetics, dietary habits, and environmental factors profoundly influence and shape the community structure of gut microbiome [12,13]. A previous investigation showed that the dominant genera were *Streptococcus*, *Enterococcus*, and *Pseudomonas*, which could be distinctly altered by gender, age, state, or living environment [14]. Regarding the abiotic factors, temperature is the most crucial factor among environmental factors. Du et al. [15] found that high temperature (34 °C) obviously changed the gut microbiota of *Bombix mori*, and the most noteworthy point was that the dominant microbiota changed from *Sphingomonas* and *Pseudomonas aeruginosa* to *Clostridium* and *Lactococcus*. In addition, *Methylobacterium* and *Buchner* were observed in the intestine of silkworms eating mulberry leaves, but not found in those fed with an artificial diet [16]. Comparison with silkworms eating mulberry leaves, the abundance of lipase bacteria was lower in the gut of silkworms eating tricuspid cudrania leaves [17]. Furthermore, bacteria and fungi are the main biological factors and active participants in the formation of the microecology. Their relationship includes commensalism, amensalism, mutualism, and competition [18]. However, it is still unclear whether altered bacteria or fungi exert an impact on the other organisms. Jones et al. [19] investigated the factors that influence gut microorganisms of fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*), and found that the host plant (diet) had a greater impact on gut communities than egg source (genetics). Thus, it can be speculated that diet is the key factor affecting the gut microbiota.

To overcome the great challenge of complex interactions among diet, bacteria, and fungi, here we designed a simple experiment on *B. mori*, a kind of lepidopteran insect feeding on mulberry leaves, to explore the association among diet, bacteria, and fungi based on exposure of antibiotics clearing the gut bacteria or fungi. The preliminary findings showed that the clearance of gut bacteria promoted the correlation between diet-derived fungi and gut fungi, which provided a theoretical basis for advanced investigation on the underlying mechanisms of the interaction between gut bacteria and fungi.

**Materials and methods**

**Sample collection**

The silkworm species were “Suchao 2,” which were incubated in the laboratory at 37 °C in a dark incubator. According to the previous literature [20], the worms were divided into three groups: (a) the worms fed with fresh mulberry leaves were referred to as the control group; (b) the worms fed with fresh mulberry leaves treated by 10 μg·mL⁻¹ amphotericin B solution (Sigma Chemical Co.St., Louis, MO, USA; CAS1397-89-3) were regard as the amphotericin group; (c) the worms fed with fresh mulberry leaves treated at 400 U·mL⁻¹ penicillin and 400 μg·mL⁻¹ streptomycin solution (TransGen Biotech Co.St., Beijing, China; FG1010-01) were regard as the penicillin + streptomycin group. In addition, the antibacterial amphotericin B could remove the gut fungi, and the antibacterial penicillin and streptomycin could delete the gut bacteria. In addition, the fresh mulberry leaves were regarded as the mulberry leaf group. After the worms were fed from hatching to the fifth instar larvae, the feces and leaves were sampled for microbial profiling.

**Total DNA extraction, PCR amplification, and high-throughput sequencing**

Total DNA was extracted from the mulberry leaves and feces of *B. mori*, and 1% agarose gel electrophoresis was used to control the quality of DNA. Using the extracted DNA as a template and amplified by polymerase chain reaction (PCR). The V3-V4 region of the bacterial 16S rRNA gene was performed using forward primer 341F (5'-CCTAYGGGGBGCASCAG-3') and reverse primer 806R (5'-GGACTACNNGGGTATCTAAT-3'); the ITS1-ITS2 region was amplified using the forward primer ITS1F (5'-CTTGTTCATTAGGAGGAAGTAA-3') and reverse primer ITS2R (5'-GCTGCGTCTTCTCATAGTGC-3') [21]. The PCR was performed in a 20 μL mixture containing 10 ng DNA extract, 0.8 μL primer (5 μmol·L⁻¹), 0.4 μL FastPfu Polymerase, 4 μL 5× FastPfu Buffer, 2 μL dNTPs (2.5 mmol·L⁻¹), replenished to 20 μL with ddH₂O. All samples were amplified on an ABI GeneAmp 9700 (Applied Biosystems, New York, USA) using the following parameters: initial denaturation at 95 °C for 5 min, 27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s, and an extension at 72 °C for 10 min, then stored at 10 °C [22]. After we purified and quantified the products of amplification, high-throughput sequencing was performed on the Illumina PE250 platform (Shanghai Lingen Biotechnology Co., Ltd, Shanghai, China).
Statistical analysis

The raw data were optimized by removing the low-quality sequences using the qiime software (version 1.17, http://qiime.org), and chimeric sequences were identified and deleted using the UCHIME algorithm. The refined sequences were clustered to the operational taxonomic units (OTUs) by 97% similarity using UPARSE (version 7.1, http://drive5.com/uparse), and the OTUs were assigned according to the SILVA database with a 70% confidence threshold. Alpha and Beta diversity were analyzed based on the original OTUs abundance. After homogenization of OTUs, the relative abundance, community composition, linear discriminant analysis of the effect size (LEfSe), linear discriminant analysis (LDA), and Venn analysis were carried out. In addition, SPSS software (version 21, International Business Machines Corporation, Armonk, NY, USA) was used to conduct the nonparametric test and Spearman correlation analysis. Visualization of the microbial symbiotic was realized by CYTOSCAPE software (https://cytoscape.org/).

Results

Alpha diversity analysis

When the silkworms grew to the fifth instar larvae by feeding the antibiotics-treated mulberry leaves, the fecal samples were collected. Amphotericin B-exposure caused elimination of gut fungi. The PCR amplification and agarose electrophoresis detection results showed that the negative amplification rate of the 18S rRNA gene ITS1-ITS2 region of sil worm feces was 85% (17/20), and six negative fecal samples were randomly selected as the amphotericin group for bacterial sequencing. Meanwhile, penicillin–streptomycin exposure caused the elimination of gut bacteria; the negative rate of the V3-V4 region of 16S rRNA gene in silkworm feces was 90% (18/20), and six negative fecal samples were randomly selected as the penicillin + streptomycin group for fungal sequencing. At the same time, six mulberry leaves were collected as the mulberry leaf group, six fecal samples of silkworms fed the fresh mulberry leaves were selected as the control group, and these samples were conducted for both bacterial and fungal sequencing. Ultimately, the average of 37 830 ± 6702 bacterial sequences and 44 136 ± 9361 fungal sequences were obtained from each sample, and 607 bacterial OTUs and 533 fungal OTUs were obtained according to 97% similarity.

Alpha diversity analysis is presented in Table 1. For bacteria, compared with the mulberry leaf group, the richness index (Ace, Chao, Observed OTUs) and diversity index (Shannon, Simpson) of silkworm intestinal bacteria in the control group were significantly increased ($P < 0.05$), but the bacterial richness and diversity decreased in the amphotericin group, indicating that the loss of fungi decreased the bacterial richness and diversity in the intestine of silkworms. For fungi, compared with the mulberry leaf group, the richness and diversity of the gut fungi were signally decreased in the control group ($P < 0.05$), and no conspicuous alternation of the richness of gut fungi was observed ($P < 0.05$), but the fungal diversity index was remarkably increased in the penicillin + streptomycin group ($P < 0.05$), indicating that clearance of bacteria increases the diversity of fungi in the silkworm’s intestine.

| Table 1. Alpha diversity analysis of bacteria and fungi from the leaf to the gut of Bombyx mori. Compared with the control, the asterisk (*) means a significant difference ($P < 0.05$). |
|---|---|---|---|---|---|---|
| **Bacteria** | **Control (n = 6)** | **Amphotericin B (n = 6)** | **Mulberry leaf (n = 6)** | **H ($P$)** | **Fungi** | **Control (n = 6)** | **Ampicillin + Streptomycin (n = 6)** | **Mulberry leaf (n = 6)** | **H ($P$)** |
| **Ace** | 178.2 ± 139.9 | 115.5 ± 10.9* | 123.3 ± 18.8* | 1.434 (0.488) | 96.5 ± 18.9 | 100.3 ± 57.8 | 253.2 ± 53.6* | 9.018 (0.011) |
| **Chao** | 179.8 ± 139.5 | 115.5 ± 12.4 | 124.1 ± 21.0 | 1.647 (0.439) | 83.5 ± 30.3 | 97.5 ± 61.4 | 251.6 ± 55.1* | 9.029 (0.011) |
| **Shannon** | 2.533 ± 1.429 | 1.845 ± 0.125* | 1.740 ± 0.079* | 6.313 (0.043) | 2.598 ± 0.950 | 3.173 ± 0.629* | 3.497 ± 0.254* | 3.71 (0.156) |
| **Simpson** | 0.194 ± 0.101 | 0.256 ± 0.031* | 0.277 ± 0.030* | 6.918 (0.031) | 0.211 ± 0.262 | 0.089 ± 0.050* | 0.073 ± 0.031* | 2.946 (0.229) |
| **Observed OTUs** | 168.2 ± 144.6 | 105.4 ± 10.4* | 105.4 ± 20.2* | 0.261 (0.879) | 75.0 ± 30.5 | 90.3 ± 56.3 | 212.8 ± 39.9* | 8.404 (0.015) |
between the control group and the mulberry leaf group was obviously detached, suggesting that the gut bacterial community differed distinctly from that of the mulberry leaf. And the bacterial community structure of the amphotericin group was obviously different from that of the control group, indicating that amphotericin B-exposure induced clearance of gut fungi that altered the bacterial community structure (Fig. 1A). On the other hand, the fungal community was distinctly different between mulberry leaf and silk-worm intestine, but the fungal community in the ampicillin + streptomycin group was more similar to the mulberry leaf group than that in the control group, indicating that ampicillin + streptomycin exposure induced clearance of gut bacteria increase the similarity of gut fungi and leaf (diet)-derived fungi (Fig. 1B).

**Exposure of antibiotics distinctly altered the gut microbial composition**

A total of 20 known bacterial phyla were detected, among which the relative abundances of three dominant bacterial phyla (*Firmicutes*, *Cyanobacteria*, and *Proteobacteria*) accounted for 90–96%. The relative abundances of four known fungi phyla (*Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Mucoromycota*) ranked from 81% to 99%. At the genus level, eight dominant bacterial genera included *Bacillus*, *Chloroplast-norank*, *Lactococcus*, *Carnobacterium*, *Mitochondria-norank*, *Streptococcus*, *Exiguobacterium*, and *Enterococcus*. Based on the mulberry leaf group, the gut microbiota obviously altered but conserved some shared microbiota (Fig. 2). Firstly, here did not observe the apparent alteration between the amphotericin group and control group (Fig. 2A), suggesting that amphotericin B-inducing clearance of gut fungi had little effect on the relative abundances of gut bacteria. The five dominant fungal genera including *Cladosporium*, *Nigrospora*, *Tausonia*, *Aureobasidium*, and *Penicillium*. On the other hand, the observed obvious fungal alteration between the penicillin + streptomycin group and the control group (Fig. 2B), indicating that penicillin + streptomycin-inducing clearance of gut bacteria exert deeply effect on gut fungi.

Among the 154 bacterial genera, Venn analysis results presented 56 shared bacterial genera (Fig. 2C), such as *Bacillus* (15.92%), *Chloroplast-norank* (15.70%), *Carnobacterium* (15.53%), and *Lactococcus* (15.26%). Among the 221 fungal genera, the observed 38 shared fungal genera (Fig. 2D), included *Trichosporon*, *Trichomerium*, *Didymella*, and *Ceratocystis*.

Linear discriminant analysis was used to identify the potential microbial markers at the genus level (Fig. 3). Ten bacterial genera were enriched in the amphotericin group, such as *Aerococcus* and *Streptococcus*, belonging to the phylum *Firmicutes* (Fig. 3A). Twelve fungal genera were enriched in the penicillin + streptomycin group, such as *Aspergillus*, belonging to the phylum *Ascomycota* (Fig. 3B). The results indicated that antibiotics exposure induced loss of the corresponding microorganism that deeply alter and shape the host’s gut microbiota.

**Fig. 1.** Effects of antibiotic treatment on intestinal microbiota of *Bombyx mori*. The gut bacteria were obviously altered after exposure to antifungal amphotericin B (A), and the gut fungi were also significantly altered after exposure to antibacterial ampicillin + streptomycin (B).
Exposure of antibiotics deeply altered symbiotic relationships

Among the shared 56 bacterial genera in Fig. 2C and 38 fungal genera in Fig. 2D, bacteria and fungi genera with average relative abundance >0.1% were considered the core microbiota, so 37 bacterial genera and 21 fungal genera were selected to investigate the alteration of symbiotic relationships (Fig. 4). First, the bacterial abundance tendency in the mulberry leaf group (Fig. 4A) was homologous with that in the control group (Fig. 4B). Comparison to the control group, the bacterial abundance tendency did not obviously change in the amphotericin group; however, the tendency of fungal abundances obviously changed in the penicillin + streptomycin group (Fig. 4C). Spearman correlation analysis was conducted between the microbial abundances in the mulberry leaf and that in the gut. The nodal increment in the amphotericin group

Fig. 2. Genus-level analysis of the microbial community structure. Here presented are the genus-level structure of the bacteria treated by antifungal amphotericin (A), and genus-level structure of the fungi treated by antibacterial ampicillin + streptomycin (B). The Venn diagram shows the shared bacterial genera (C) and fungal genera (D).
was lower than that in control group, suggesting that amphotericin B-inducing clearance of gut fungi was weak in the bacterial correlation between the host intestine and the diet leaf (Fig. 4D). On the contrary, the nodal increment in the penicillin + streptomycin group was higher than that in the control group, suggesting that penicillin + streptomycin-inducing clearance of gut bacteria enhances the fungal correlation between the gut and the diet leaf (Fig. 4E). The results suggested exposure of diet-derived antibiotics exerted a different influence on the worm’s gut microbiota.

Finally, based on the 79 fungal genera and 37 bacterial genera, the symbiotic networks were constructed according to the latest literature [23] (Fig. 5). The dominant bacteria and fungi in the mulberry leaf group gathered more closely than that in the control group, suggesting that the intestinal environment obviously dispersed the strong correlation of leaf microbiota (Fig. 5A). In the amphotericin group, the symbiotic network was scattered between gut bacteria and leaf-derived bacteria, suggesting amphotericin B-inducing loss of gut fungi weakens the bacterial relationship between the intestine and the diet leaf (Fig. 5B). However, the symbiotic network between gut fungi and leaf-derived fungi became stronger in the penicillin + streptomycin group, suggesting that penicillin + streptomycin-inducing loss of gut bacteria enhances the fungal relationship between the intestine and the diet leaf (Fig. 5C). These results indicated that exposure of antibiotics distinctly altered the commensal relationships in the worm’s gut.

**Discussion**

The pollution situation of antibiotics in the environment is now getting worse in the world, but little is known about the potential effects on the gut microbiota. As an extremely complex ecosystem, there is a continuous interaction among the microbiota in the human digestive tract [24]. In the foreseeable future, the interaction among the gut microbiota and host health will still be a hotspot [25]. Thus, the silkworm, with the simplest diet, was used as a model to overcome the complexity of diet-driving intestinal microbial interaction in the mammals besides human. The present study used bacterial and fungal sequencing to explore the community structure and interaction of bacteria and fungi from the mulberry leaf to silkworm intestine. The preliminary results showed that the diet exposure of penicillin and streptomycin could cause the loss of gut bacteria, which had a significant impact on the fungi. And vice versa, the diet exposure of amphotericin B could cause the loss of gut fungi, which had a significant impact on the bacteria. Exposure of antibiotics could inhibit or kill the responding microbes, which would significantly alter the correlation between the silkworm’s remaining microbiota in the digestive tract; especially the clearance of gut...
bacteria would promote the fungal correlation between the mulberry leaf and the intestine. In a word, antibiotics can change the diversity of microbial flora and destroy the balance of microecosystem in the gut of silkworm, thus affecting the development of sericulture. Obviously, it is necessary to standardize the use of antibiotics and develop new microecological preparations for silkworm to improve the health of the silkworm.

The hypothesis that the diet-driving shape of gut microbiota has been gradually confirmed by mounting evidence [26,27], and diet-driving alteration of the gut microbiota has been suspected to be responsible for the increasing prevalence of chronic diseases such as...
obesity and inflammatory bowel disease [28]. For example, our epidemiological investigations showed that the risk of gastric cancer was significantly related to the bacteria but not the fungi in the case of matching the lifestyle, the phenomena were observed both in the oral and fecal samples, which suggested that lifestyle played a key role in driving the alteration of gut microbiota, especially the fungi [21,29]. An animal investigation also showed that the gut mycobiome of healthy mice was shaped by the environment and correlated with metabolic outcomes in response to diet [30]. Comparison to the diet leaf microbiota, the results of LDA showed that 12 bacterial genera and four fungal genera were markedly increased in the intestine of silkworms, two dominant bacterial genera (Bacillus and Enterobacter) were reported in the silkworm’s intestine [31], and exposure of the antibiotics further promoted bacterial or fungal boost, such as Aerococcus and Aspergillus. The results were consistent with the explanation of antibiotics-inducing dysbiotics.

Much evidence has found that bacterial and fungal mutualism play a major role in promoting and maintaining the host health and performance [32], suggesting that the symbiotic relationship between bacteria and fungi reflects the host’s health and disease. Our data showed that the bacterial and fungal abundances in mulberry leaf were significantly different from that in silkworm’s intestine. Interestingly, after exposure of antibiotics, clearance of gut fungi obviously enlarged the differences of bacterial structure between the intestine and the mulberry leaf; however, clearance of gut bacteria could shrink the differences of fungal structure between the intestine and the mulberry leaf.

Since the industrial revolution, many kinds of antimicrobial agents are broadly used for bacteriostasis or sterilization as a natural or artificial synthetic chemical substance and play an essential role in medical treatment and significantly prolong the human life span [33]. However, the abuse of antimicrobial agents, especially the antibiotic, directly or indirectly causes a series of emerging human health problems [34]. Gudda et al. [35] conducted a risk assessment of antibiotic contamination of vegetables on the human intestinal microbiota, pointing out that the presence of dietary antibiotics may destroy some intestinal microbiota, disturb the balance of gut microbiota, and finally promote the abnormal proliferation of opportunistic and pathogenic bacteria. In our study, the clearance of gut bacteria induced by the diet exposure of penicillin + streptomycin promoted the correlation between gut fungi and diet-derived fungi, while the clearance of gut fungi induced by diet exposure of amphotericin B promoted the abnormal proliferation of gut bacteria. The results provided new insight about the balance between gut and diet-derived bacteria/fungi.

Up to now, there are still few studies on the effect of dietary antibiotics and microbiota on the gut microbiota this study used silkworm as a model to reveal the preliminary role of interaction among the bacteria, fungi, and diet exposed to antibiotics. The present data showed that exposure of dietary antibiotics significantly promoted the direct correlation between diet-
Antibiotics alter the gut microbiota of silkworm

derived microbiota and gut microbiota, especially the fungi. Of course, the phenomena were observed in invertebrate, and a validated study is needed in vertebrates and to explore the underlying mechanisms in health and disease. Anyway, the present data provide a simple model to present the underlying mechanisms of cross-talking between diet-derived microbiota, antibiotics, and gut microbiota, which will be an important clue for us to understand the potential hazards of antibiotic abuse.

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Conflict of interest

The authors declare no conflicts of interest.

Data accessibility

The datasets presented in this study have been deposited in Entrez (https://www.ncbi.nlm.nih.gov/sra/PRJNA769808); the submission ID is SUB10504772.

Author contributions

CL performed the data analysis and wrote the article. Shuo Xu and CX conducted the experiment and collected samples. Shixia Xu suggested the constructive comments of the study. QZ designed the study and performed the data analysis. JZ designed and supervised the study, and reviewed and revised the draft.

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Antibiotics alter the gut microbiota of silkworm C. Li et al.

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