Effects of Antibiotic Treatment on the Development and Bacterial Community of the *Wolbachia*-Infected Diamondback Moth

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ABSTRACT: Based on the important role of antibiotic treatment in the research of the interaction between *Wolbachia* and insect hosts, this study aimed to identify the most suitable antibiotic and concentration for *Wolbachia* elimination in the *P. xylostella*, and to investigate the effect of *Wolbachia* and antibiotic treatment on the bacterial community of *P. xylostella*. Our results showed that the *Wolbachia*-infected strain was *plutWB1* of supergroup B in the *P. xylostella* population collected in Nepal in this study; 1 mg/mL rifampicin could remove *Wolbachia* infection in *P. xylostella* after 1 generation of feeding treatment and the toxic effect was relatively low; among the 29 samples of adult *P. xylostella* in our study (10 WU samples, 10 WA samples, and 9 WI samples), 52.5% of the sequences were of Firmicutes and 47.5% were of Proteobacteria, with the dominant genera being mainly *Carnobacterium* (46.2%), *Enterobacter* (10.1%), and *Enterococcus* (6.2%); Moreover, antibiotic removal of *Wolbachia* infection in *P. xylostella* and transfer to normal conditions for 10 generations no longer significantly affected the bacterial community of *P. xylostella*. This study provides a theoretical basis for the elimination method of *Wolbachia* in the *P. xylostella*, as well as a reference for the elimination method of *Wolbachia* in other *Wolbachia*-infected insect species, and a basis for the study of the extent and duration of the effect of antibiotic treatment on the bacterial community of the *P. xylostella*.

KEYWORDS: *Plutella xylostella*, antibiotic, *Wolbachia*, the 16S rRNA high-throughput sequencing, the bacterial community

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Introduction

Wolbachia, a member of the Rickettsiaceae (α-Proteobacteria), is a gram-negative bacterium that is widespread in insect species.[1-4] It spreads vertically mainly through the maternal cytoplasm of insect hosts and can alter the sex ratio and reproductive strategy of insect hosts through various regulatory effects, thus increasing its own transmission frequency in host populations.[5,6] *Wolbachia* strains have different types of reproductive manipulation in insect hosts, including cytoplasmic incompatibility, male-killing, parthenogenesis and feminization.[4] Furthermore, *Wolbachia* can also be involved in regulating host nutritional metabolism,[7] resistance to pathogens,[8,9] behavior,[10] developmental duration, and longevity.[11] Currently, it has been demonstrated that the combination of the sterile insect technique (SIT) and the incompatible insect technique (IIT) based on *Wolbachia* has promising applications in the biological control of mosquitoes[12]; transfection of the *w*Stri strain *Wolbachia* into the agricultural pest *Nilaparvata lugens* not only induces high-intensity cytoplasmic incompatibility but also inhibits infection and transmission of the rice ragged stunt virus (RRSV) by *N. lugens*.[13] These findings confirm that *Wolbachia* has good potential for the biological control of arboviruses and agricultural pests.

In the study of *Wolbachia*-host interactions, it is often necessary to eliminate *Wolbachia* infection to obtain control uninfected populations of the same genetic background for subsequent experiments, as the strain and frequency of *Wolbachia* infection varies among different geographic populations.[14-16] Currently, the commonly used method is antibiotic treatment. Antibiotics that have been reported to eliminate *Wolbachia* mainly include: (1) antibiotics that inhibit bacterial cell wall synthesis, including ampicillin and penicillin; (2) antibiotics that inhibit protein synthesis, including gentamicin and tetracycline; (3) antibiotics that interfere with nucleic acid synthesis, including rifampicin; (4) anti-metabolite sulfonamide antibiotics, etc.[15] The commonly used of these antibiotics is tetracycline, which has been widely used for *Wolbachia* elimination in a variety of insect hosts such as Coleoptera, Hymenoptera, Lepidoptera, and Diptera. For example, tetracycline feeding treatment of 0.6 and 2.5 mg/g were effective in eliminating

Wolbachia from *O. furnacalis* and *Lissorhoptrus oryzophilus*, respectively, within 1 generation.[17,18] Raising *Drosophila* on 0.25 mg/mL Tet-HCl containing food for 2 generations is usually enough to get rid of Wolbachia completely from flies.[19] Rifampin may be effective when tetracycline is not effective in eliminating *Wolbachia* infection.[15] In addition, high and low temperature treatment is also a method to eliminate *Wolbachia* infection, and the *Wolbachia* eliminating rate after 6 generations of the *Tetranychus urticae* was 100% when it was treated at 32°C.[20] However, high and low temperature treatments have a large impact on the insect host itself and are subject to many uncertainties, so they are not usually used as the primary means of *Wolbachia* elimination in insect hosts.

Although antibiotic treatment is currently the primary means of *Wolbachi*a elimination in insect hosts, the effects of antibiotics on microorganisms are broad-spectrum, and many researchers still have questions about whether antibiotic treatment has persistent effects on other microorganisms in the host. It has been shown that antibiotic intake reduces host microbial diversity and that it is difficult to fully restore it to its original state.[14,20,21] However, the extent and duration of the effect on the host microbial community after elimination of *Wolbachia* from the host by antibiotic treatment is still poorly understood and deserves further investigation.

P. xylostella is considered one of the most important pests of cruciferous vegetable crops.[22] At present, chemical control is still the main means of *P. xylostella* control, but due to its high level of resistance, and based on the perspective of environmental friendliness, carrying out effective biological control is the focus of future research on integrated control of *P. xylostella*.[23] *Wolbachia* has good potential for biological control, but research on the interaction between *Wolbachia* and *P. xylostella* and the corresponding mechanisms is still lacking.

In this study, by comparing the elimination efficiency of different types and concentrations of antibiotics on *Wolbachia* and their effects on the survival rate and developmental period of the *P. xylostella* themselves, we identified antibiotics and concentrations that could eliminate *Wolbachia* and have relatively low effects on the *P. xylostella* itself; after eliminating *Wolbachia* infection by antibiotic treatment, the *P. xylostella* were transferred to normal conditions for 10 generations. After 10 generations of rearing under normal conditions, the 16S rRNA high-throughput sequencing was performed on male and female adults of 3 different populations (wild-type naturally uninfected population, wild-type naturally infected population, and antibiotic-treated population), focusing on the effects of different *Wolbachia* infection states and antibiotic treatment on the bacterial community of the *P. xylostella*. Our results provide a theoretical basis for the elimination of *Wolbachia* from the *P. xylostella*, as well as a reference for screening different strains of *Wolbachia*-infected insect species, and further explore the feasibility of antibiotic feeding to eliminate *Wolbachia* infection in the host. In addition, we preliminarily investigated the extent and duration of the effects on the bacterial community of *Wolbachia* in *P. xylostella* after elimination of *Wolbachia* by antibiotic treatment.

Materials and Methods

Sample collection and PCR assay

The population of *Wolbachia*-infected *P. xylostella* (WI population) was initially collected in Nepal (85°38′E, 27°74′N) in October 2019. The collected *P. xylostella* samples were brought back to the laboratory and reared under relative humidity RH = 75% ± 5%, temperature 25 ± 1°C, and photoperiod 14 hours:10 hours (light:dark). The larvae of the *P. xylostella* were fed with radish seedlings grown in our laboratory, and the adults were supplemented with 10% honey water. After the collected *P. xylostella* samples were reared in the laboratory and passed on, all individuals of the G0 generation of *P. xylostella* samples were collected for DNA extraction using the Wizard®SV Genomic DNA Purification System kit from Promega (USA), referring to the kit instructions for the specific steps.

The *Wolbachia* infection status of the *P. xylostella* samples was detected by PCR amplification of the *wsp* gene,[24] and both negative and positive controls were set up in each PCR experiment. In addition, we amplified 5 MLST genes (*gatB, coxA, ftsZ, fbpA*, and *hcpA* genes)[25] of *Wolbachia* by PCR with reference to the PubMLST database (https://pubmlst.org/organisms/wolbachia-spp), and all primers used and PCR systems used are shown in Table 1.

Phylogenetic analysis

All gene sequences obtained by PCR amplification (*wsp, gatB, coxA, ftsZ, fbpA*, and *hcpA* genes) were first confirmed as target sequences by NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and then aligned using the MAFFT plugin of the PhyloSuite software for alignment.[26,27] The nucleotide substitution models were selected using the ModelFinder plugin in the PhyloSuite software.[27,28] The TPM3 + F + R2 model was used for the *wsp* gene, and the TVM + F + I model was used for the tandem sequences of the 5 MLST genes. The phylogenetic tree of *Wolbachia* was constructed based on the maximum likelihood method (ML) using *wsp* genes and the tandem sequences of 5 MLST genes respectively, and bootstrap values were generated with 1000 replicates.[29]

Antibiotic treatment

In this study, tetracycline hydrochloride and rifampicin were used to soak the leaves of radish seedlings fed by *P. xylostella* in different concentrations to simulate the way that *P. xylostella* was exposed to antibiotics during feeding. Briefly, the leaves of radish seedlings were soaked with tetracycline hydrochloride and rifampicin at 0.05, 0.1, 0.5, 0.8, 1, 2, and 5 mg/mL

Table 1. Primer sequences and amplicon information of target genes.

GENE	PRIMERS	SEQUENCE (5′-3′)	AMPLICON LENGTH (BP)	ANNEALING (°C)	REFERENCES
Wsp	wsp_81F	TGGTCCAATAAGTGATGAAGAAAC	602	51	Zhou et al[24]
	wsp_691R	AAAAATTAAACGCTACTCCA			
gatB	gatB_F1adp	TGTAAAACGACGGCCAGTGAKTTAAAYCGYGCAGGBGTT	≈500	57	Baldo et al[25]
	gatB_R1adp	CAGGAAACAGCTATGACCTGGYAAYTCRGGYAAAGATGA			
coxA	coxA_F1adp	TGTAAAACGACGGCCAGTTTGGRGCRATYAACTTTATAG	≈500	57	
	coxA_R1adp	CAGGAAACAGCTATGACCCTAAAGACTTTKACRCCAGT			
ftsZ	ftsZ_F1adp	TGTAAAACGACGGCCAGTATYATGGARCATATAAARGATAG	≈500	50	
	ftsZ_R1adp	CAGGAAACAGCTATGACCTCRAGYAATGGATTRGATAT			
fbpA	fbpA_F1adp	TGTAAAACGACGGCCAGTGCTGCTCCRCTTGGYWTGAT	≈500	57	
	fbpA_R1adp	CAGGAAACAGCTATGACCCCRCCAGARAAAAYYACTATTC			
hcpA	hcpA_F3	ATTAGAGAAATARCAGTTGCTGC	≈500	55	
	hcpA_R3	CATGAAAGACGAGCAARYTCTGG			

respectively to feed the diamondback moth infected with *Wolbachia*. The soaking time of antibiotics at each concentration was 5 minutes. After soaking, the leaves were naturally air-dried on the clean bench with ddH_2O as the control. The antibiotic treatment lasted for the whole larval period, and the number of larval mortality, larval developmental duration, number of pupae, pupae mortality and fecundity were recorded. About 20 larvae were placed in each treatment and 5 replicates were set up. After 24 hours of treatment, the antibiotic-treated radish seedling leaves were replaced. After 1 generation of treatment, the average survival rate of *P. xylostella* in each treatment group to survive to the adult stage was calculated (total number of *P. xylostella* samples that survive to the adult stage in 5 replicates/100). In order to test whether *Wolbachia* was eliminated, DNA of all *P. xylostella* samples was extracted using the method mentioned above, and the infection rate in each treatment group was detected by PCR and qPCR. Both positive and negative controls were set in each PCR assay. The results of positive PCR amplification indicated that *Wolbachia* infection still existed, which was regarded as the sample that had not been eliminated by antibiotics. PCR-negative samples are then tested by qPCR, and both PCR and qPCR-negative samples are considered to have been successfully eliminated by antibiotics. After all *P. xylostella* samples were detected, the antibiotic elimination rate of *P. xylostella* samples in each treatment group was calculated (total number of *P. xylostella* samples that successfully eliminated by antibiotics in 5 replicates/100).

After the elimination efficiency of *Wolbachia* and the development of *P. xylostella* were counted and compared that treated by different kinds and concentrations of antibiotics, we have selected 1 mg/mL rifampicin solution, which could effectively remove *Wolbachia* infection within 1 generation and had low toxic effect on *P. xylostella*, to eliminate *Wolbachia* in *P. xylostella*. The *P. xylostella* treated with 1 mg/mL rifampicin for 1 generation were transferred to normal radish seedlings for propagation and reared for a long period of time for subsequent testing (WA population). Several *P. xylostella* were randomly selected and tested for *Wolbachia* infection per generation to confirm that *Wolbachia* had not returned to infection.

16S rRNA high-throughput sequencing

To assess the effects of *Wolbachia* infection and antibiotic treatment on the bacterial community of the *P. xylostella*, we collected *P. xylostella* samples from the WA population (antibiotic-treated *Wolbachia*-infected *P. xylostella* population, and the 10th generation of non-infected WA population after the first generation treated with 1 mg/mL rifampicin mentioned above), the WI population (100% *Wolbachia*-infected *P. xylostella* population from Nepal), and the WU population (100% *Wolbachia*-uninfected *P. xylostella* population reared in the laboratory), respectively. The WU and WA population were not infected by *Wolbachia*, the WI and WU population were not treated with antibiotics, the WI and WA population had the same genetic background.

The 1-day-old adult *P. xylostella* samples were used for the 16S rRNA sequencing. The total DNA of collected adults was extracted using DNeasy® PowerSoil® Pro Kit (QIAGEN, USA) and tested for quality. The V3-V4 region of the 16S rRNA gene was amplified using primers 338F-806R (338F: ACTCCTACGGGAGGCAGCAG, 806R: GGACTACH VGGGTWTCTAAT).[30] About 5 adult moths were placed

in each sample, 3 PCR replicates were set up, and the PCR products from the 3 replicates were mixed and detected using 2% agarose gel electrophoresis. PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen, USA), and the specific steps were referred to the kit instructions. Libraries were built using NEXTFLEX Rapid DNA-Seq Kit and sequenced using Illumina's Miseq PE300 platform (Shanghai Meiji Biomedical Technology Co., Ltd., China).

Data analysis

The data related to antibiotic treatment were analyzed using the IBM Statistical Package for the Social Sciences (SPSS) version 24.0 (Chicago, IL, USA). The one-way ANOVA were used for comparison between multiple groups of data in the experiment, and Turkey test was used for multiple comparisons of results; *t*-test was used for comparison between 2 groups of data.

The 16S rRNA raw data were quality-controlled using fastp (https://github.com/OpenGene/fastp, version 0.20.0) software,[31] and then spliced using FLASH (http://www.cbcb.umd.edu/software/flash, version 1.2.7) software for splicing.[32] OTU clustering of the spliced sequences was performed based on 97% similarity using UPARSE software (http://drive5.com/uparse/, version 7.1).[33] RDP classifier (http://rdp.cme.msu.edu/, version 2.11)[34] was used for OTU species taxonomy annotation with a confidence threshold of 70% and counted the community composition of each sample. Alpha diversity indices Chao 1, Shannon, ACE, and simpson were calculated based on mothur software (http://www.mothur.org/wiki/Calculators),[35] and intergroup variance analysis of Alpha diversity was performed using the IBM Statistical Package for the Social Sciences (SPSS) version 24.0 (Chicago, IL, USA). PCoA analysis (Principal Component Analysis) based on the bray-curtis algorithm was used to test the similarity of microbial community structure between samples.

Results

Wolbachia *infection in the* P. xylostella

PCR assays based on the *Wolbachia wsp* gene showed 100% *Wollbachia* infection of the *P. xylostella* population collected in Nepal. The phylogenetic tree constructed based on the *Wolbachia wsp* gene showed that the *Wolbachia* infected in this population was the B supergroup *plutWB1* strain reported in a previous study.[36] Consistent with the results of the previous study, the phylogenetic tree we constructed for the *Wolbachia wsp* gene also indicated that the *plutWB1* strain was more closely related to the *Wolbachia* strain in the African butterfly *Acraea pentapolis* (Figure 1A).

The phylogenetic tree constructed based on the 5 MLST genes similarly indicated that the *plutWB1* strain belonged to supergroup B (Figure 1B). In addition, the ST typing of the 5 MLST genes in the PubMLST database was 108 (*gatB*-71, *coxA*-67, *ftsZ*-65, *fbpA*-6, and *hcpA*-74), which was first reported for the Bfel_B strain *Wolbachia* of *Brangas felderi*, a family of Lycaenidae in Lepidoptera.

Effect of antibiotic treatment on the P. xylostella

Wolbachia in different *P. xylostella* individuals were treated with different concentrations of tetracycline hydrochloride and rifampicin solutions, respectively, and the survival rate of *P. xylostella* individuals was found to decrease continuously with increasing antibiotic concentrations, and Figure 2A indicates the average survival rate of *P. xylostella* individuals treated with different antibiotic concentrations. The survival rate of the *P. xylostella* individuals was slightly higher when treated with 0.05 and 0.1 mg/mL of rifampicin solution compared to the control group, while the survival rate of the *P. xylostella* individuals was 56% and 69% when treated with 1 mg/mL of tetracycline hydrochloride solution and rifampicin solution, respectively; the high concentration of antibiotic solution had a high lethality rate for the *P. xylostella* individuals, and the survival rate of the *P. xylostella* individuals was less than 50% when fed with 2 mg/mL of tetracycline hydrochloride solution and rifampicin solution. The survival rate of the *P. xylostella* individuals was less than 50% when fed with 2 mg/mL of tetracycline hydrochloride solution and rifampicin solution.

In order to evaluate the effect of tetracycline hydrochloride and rifampicin treatments on the growth and development of the *P. xylostella* individuals, developmental duration of the *P. xylostella* larvae was recorded during feeding. The development duration of *P. xylostella* larvae after feeding on tetracycline hydrochloride treated radish seedlings was significantly prolonged to 14.00 days when 0.5 mg/mL was treated, about twice that of the control group (Table 2). The larval developmental duration of the *P. xylostella* larvae of radish seedlings fed on rifampicin was also prolonged to some extent, and the average larval developmental duration of the *P. xylostella* larvae was 8.98 days at a concentration of 1 mg of rifampicin, which was significantly higher than that of the control *P. xylostella* larvae at 7.43 days. By comparing the 2 antibiotics, the larval developmental duration of the *P. xylostella* larvae treated with tetracycline hydrochloride was significantly higher than that of the rifampicin treatment for the same concentration of both antibiotics.

Seven different concentrations of tetracycline hydrochloride and rifampicin solutions were used to feed the larvae of the *P. xylostella*, respectively. The results showed that both tetracycline hydrochloride and rifampicin could effectively remove *Wolbachia* from the *P. xylostella* individuals, and the effect of *Wolbachia* treatment is shown in Figure 2B: *Wolbachia* infection rate of *P. xylostella* individuals was not affected when the concentrations of tetracycline hydrochloride and rifampicin were less than 0.5 mg/mL; after feeding rifampicin solution at 0.8 mg/mL for 1 generation, *Wolbachia* infection was 65% of adult *P. xylostella* individuals. *Wolbachia* infection was 65% of

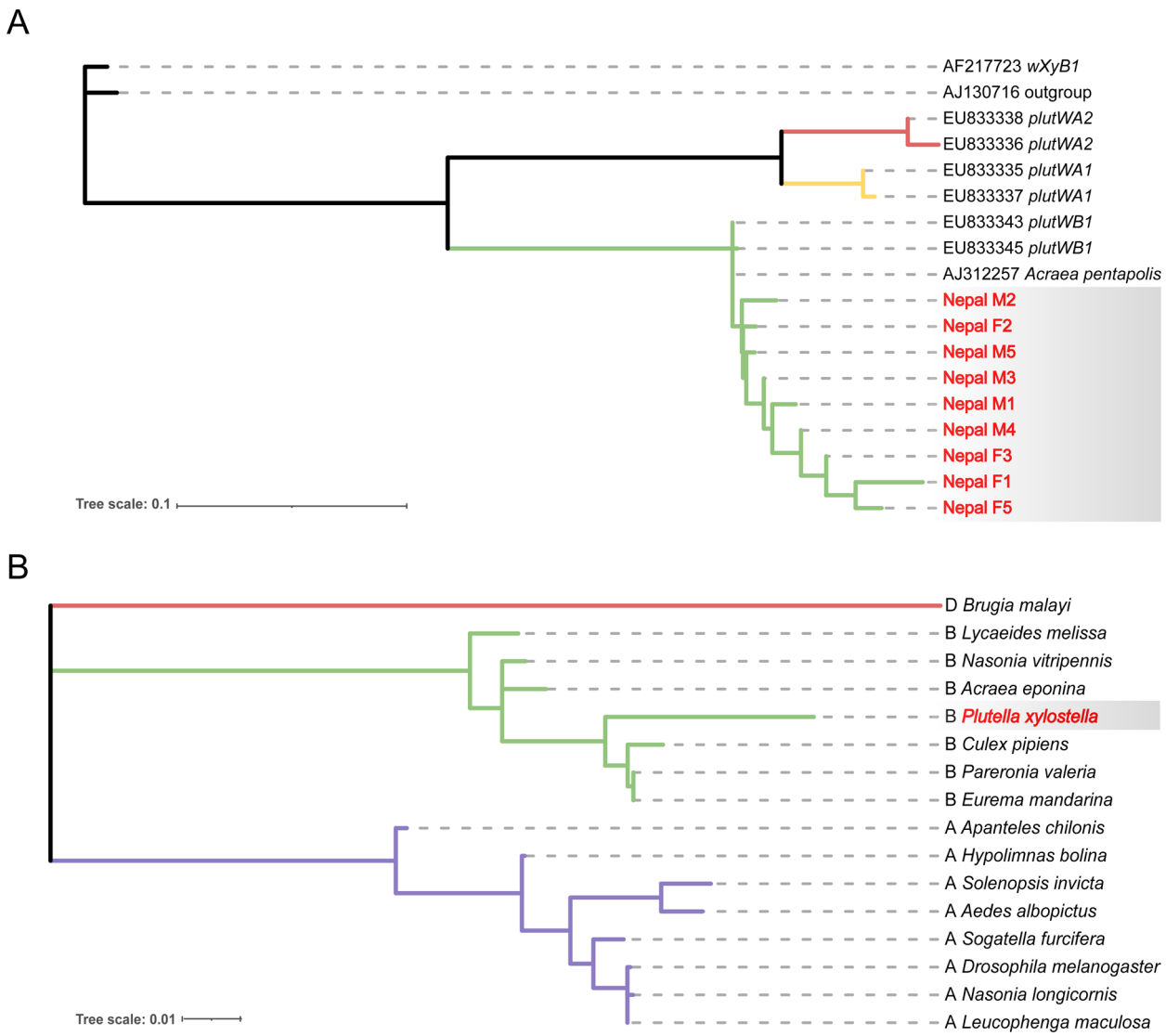

Figure 1. Phylogenetic tree of *Wolbachia*. Maximum-likelihood tree of *Wolbachia wsp* sequences (A) and MLST sequences (B) from *Plutella xylostella*. (A) ML tree constructed from *Wolbachia wsp* sequences of 10 random *P. xylostella* specimens from WI population. Colors represent the different strains (mint green: *plutWB1*, yellow: *plutWA1*, pink: *plutWA2*). (B) ML tree constructed from MLST sequences of *plutWB1* strain *Wolbachia*. Colors represent the different supergroups (purple: supergroup A, mint green: supergroup B, pink: supergroup D).

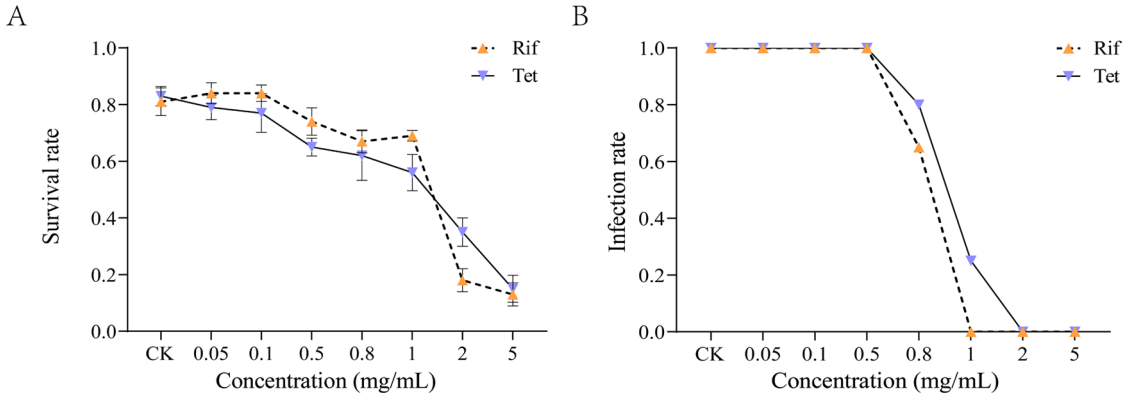

Figure 2. Survival rate and infection rate of *P. xylostella* treated with different concentrations of antibiotics. (A) Survival rate of *P. xylostella* treated with different concentrations of antibiotics. (B) Infection rate of *P. xylostella* treated with different concentrations of antibiotics.

Table 2. Development period of *P. xylostella* larvae treated with different concentrations of antibiotics (mean ± standard error).

CONCENTRATION (MG/ML)	DEVELOPMENTAL PERIOD (D)	
	TETRACYCLINE HYDROCHLORIDE	RIFAMPICIN
CK	7.43 ± 0.15[a]	7.43 ± 0.15[a]
0.05	9.18 ± 0.20[b*]	7.46 ± 0.15[a*]
0.1	9.52 ± 0.21[b*]	7.48 ± 0.15[a*]
0.5	9.95 ± 0.38[b*]	8.05 ± 0.15[b*]
0.8	14.00 ± 0.35[c*]	8.70 ± 0.16[d*]
1	15.10 ± 0.34[d*]	8.98 ± 0.20[de*]
2	16.55 ± 0.42[de*]	9.44 ± 0.25[e*]
5	18.33 ± 0.52[e*]	9.15 ± 0.39[e*]

Different letters indicate significant differences among different concentrations of the same antibiotic.
*Indicates significant differences between different antibiotics ($P < .05$).

the adult *P. xylostella* individuals after 1 generation of rifampicin solution at 0.8 mg/mL treatment; *Wolbachia* infection was completely undetectable after 1 generation of tetracycline hydrochloride at 2 mg/mL and rifampicin at 1 mg/mL.

Effect of rifampicin treatment on the bacterial community of the P. xylostella

Using the Illumina Miseq PE300 platform, 1157449 sequences with an average length of 428 bp were obtained using the 16S rRNA gene V3 to V4 variable region as the target. About 68 OTUs were obtained in all 29 samples of the WI, WU, and WA population (IF, IM, UF, UM, AF, AM) according to 97% similarity, belong to 7 phyla, 12 orders, 31 families, 44 families, and 55 genera of bacteria. Among the all 29 *P. xylostella* samples, 52.5% of the sequences were from the Firmicutes, 47.5% were from the Proteobacteria, and the dominant genera were *Carnobacterium* (46.2%), *Enterobacter* (10.1%), and *Enterococcus* (6.2%) (Figure 3). Sequences of *Wolbachia* accounted for 3.9% and 0.3% of the total sequences in the female and male adult samples from the WI population (IF, IM), respectively. Moreover, *Wolbachia* were not detected in other samples from the WA and WU population (UF, UM, AF, AM).

The OTU coverage rate of all samples in our study was above 99.9%, indicating that the sequencing depth was high and could better reflect the true situation of bacterial composition in the samples. From the antibiotic-treated groups, there were no significant differences in ACE index, chao index, shannon index, and simpson index between the antibiotic-treated groups and the non-antibiotic-treated groups; from the infection status groups, there were no significant differences in ACE index, chao index, shannon index, and simpson index between the *Wolbachia*-infected groups and the *Wolbachia*-uninfected groups (Figure 4A–D). Bray–Curtis based PCoA was used to estimate the β-diversity of bacterial communities, but none of the 3 populations clustered significantly separately (Figure 4E).

Discussion

Wolbachia *infections*

In this study, the *P. xylostella* samples were initially collected in Nepal (85°38′E, 27°74′N) in October 2019 and were brought back to our laboratory. After feeding for 1 generation in the laboratory, all *P. xylostella* samples of G0 generation were collected and the *wsp* gene of *Wolbachia* was used for infection detection.[24] The infection rate of *Wolbachia* in this *P. xylostella* population was 100%. A previous study on global *Wolbachia* infection of the *P. xylostella* showed that the natural infection rate of *Wolbachia* in *P. xylostella* was very low, with the global average infection rate only 5%, and the highest infection rate in the population was only 40%.[36] This may be due to the difference of *Wolbachia* infection in different geographic populations. Moreover, this is consistent with the views of many researchers: although some studies have estimated that about 2/3 of insect species are infected with *Wolbachia* at present,[37] this data is still very conservative due to the limitations of sample size and detection methods.[38] The wide distribution of *Wolbachia* in insect species and its various regulatory effects on the host indicate that *Wolbachia* has a promising application prospect in biological control.[37]

The phylogenetic tree constructed based on *Wolbachia wsp* gene and 5 MLST genes showed that *Wolbachia* infected by *P. xylostella* of Nepal population belonged to *plutWB1* of supergroup B (Figure 1). Futhermore, the phylogenetic tree constructed based on the *Wolbachia wsp* gene is consistent with the results of the previous study.[36] Compared with the *Wolbachia* strains reported in *P. xylostella* (*wXylB1*, *plutWA1*, and *plutWA2*),

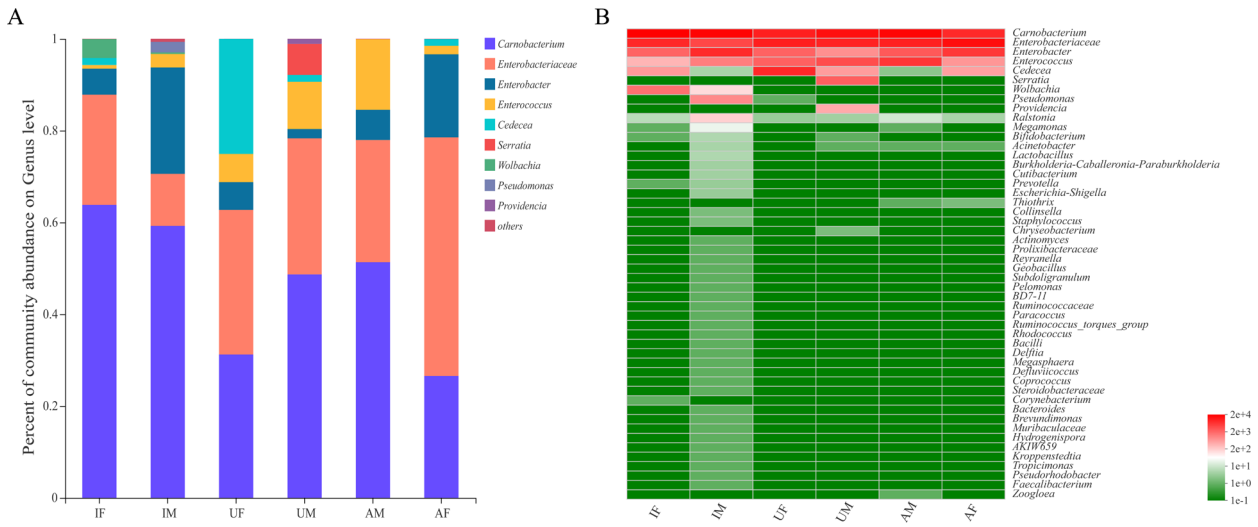

Figure 3. Relative abundance of different samples of *P. xylostella* adults at the genus level. IF: female samples of the 100% *Wolbachia*-infected *P. xylostella* populations; IM: male samples of the 100% *Wolbachia*-infected *P. xylostella* populations; UF: female samples of the 100% *Wolbachia*-uninfected *P. xylostella* populations; UM: male samples of the 100% *Wolbachia*-uninfected *P. xylostella* populations; AF: female samples of the antibiotic-treated *Wolbachia*-infected *P. xylostella* population; AM: male samples of the antibiotic-treated *Wolbachia*-infected *P. xylostella* population. (A) Community barplot of 29 *P. xylostella* specimens, taxonomic composition and relative abundance of bacteria at genus level for all 29 *P. xylostella* specimens. (B) Community heatmap of 29 *P. xylostella* specimens, the relative abundances of the top 50 species with total abundances at the taxonomic level in different samples at the taxonomic level.

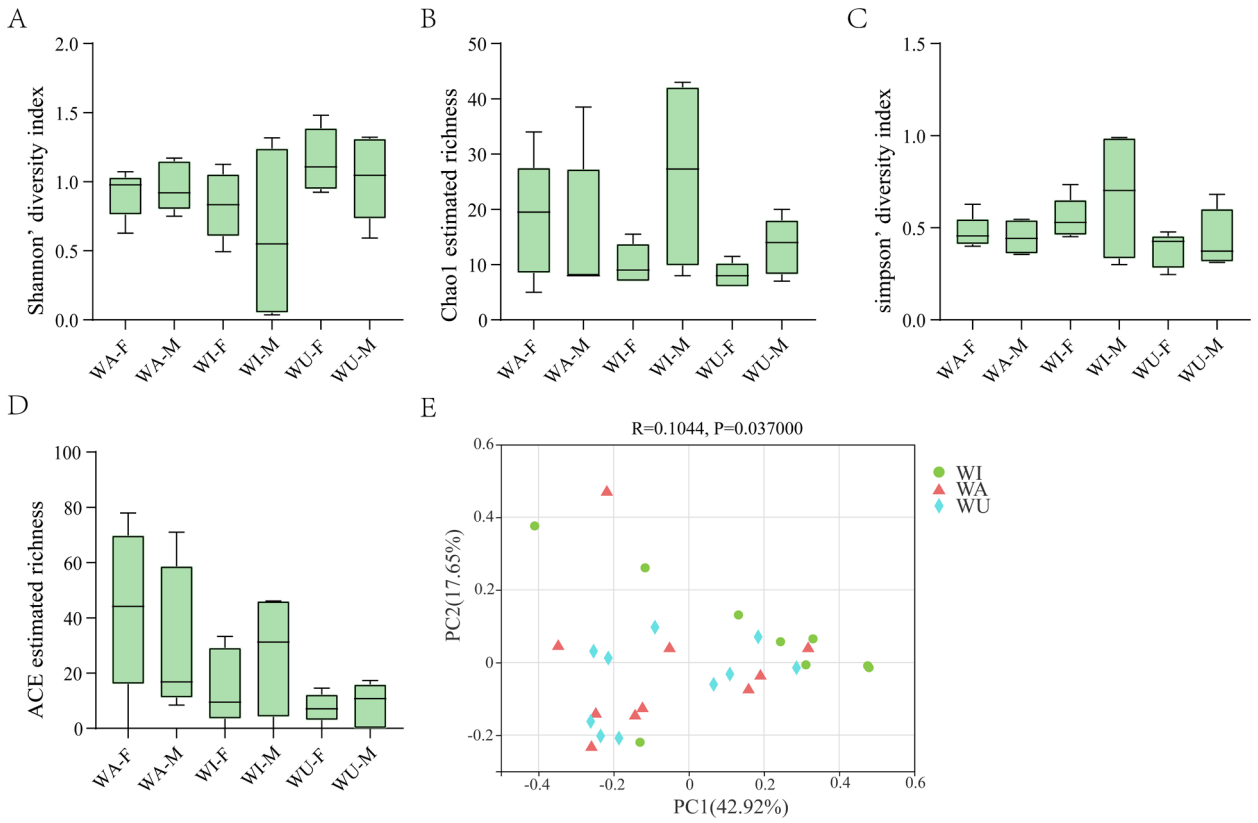

Figure 4 α-Diversity index analysis and β-diversity analysis of *P. xylostella* adults. (A) Shannon' diversity index. (B) Chao1 estimated richness. (C) Simpson' diversity index. (D) Chao1 estimated richness. (E) Principal coordinates analysis (PCoA) plot.

plutWB1 is more closely related to the *Wolbachia* strains in the African butterfly *Acraea pentapolis* (Figure 1A).[3,36] The typing result of 5 MLST genes of *plutWB1* strain *Wolbachia* in PubMLST database was 108 (*gatB*-71, *coxA*-67, *ftsZ*-65, *fbpA*-6, and *hcpA*-74), and the 108 ST was first reported the Bfel_B strain *Wolbachia* of *Brangas felderi*, a family of Lycaenidae in

Lepidoptera.[25] These results may indicate widespread horizontal transmission of *Wolbachia* between lepidopteran butterfly species and moth species, which was also confirmed by the results of a previous study.[38]

Antibiotic treatment

The interaction between *Wolbachia* and the host is currently a hot topic of *Wolbachia*-related research, and due to the different strains and frequency of *Wolbachia* infection in different geographical populations, manual removal of *Wolbachia* from the host is often required to ensure consistent genetic background in control group. Antibiotic treatment is the most widely used method to remove *Wolbachia* from hosts, which has less effect on the hosts themselves and is more stable than other methods. The types and concentrations of antibiotics used to remove different *Wolbachia* strains from different insect hosts vary.

In order to identify the most suitable antibiotic and concentration for the elimination of *Wolbachia* in *P. xylostella*, 2 antibiotics, tetracycline hydrochloride and rifampicin, which are currently widely used for the elimination of *Wolbachia* in different insect species,[15] were used and fed at different concentration gradients in the larval stage of *P. xylostella*. The results showed that both antibiotics could obtain *Wolbachia*-uninfected *P. xylostella* after 1 generation of feeding treatment (Figure 2B), but the antibiotic also had some effects on the *P. xylostella* individuals themselves (Figure 2A). Different types and concentrations of antibiotics showed different degrees of toxic effects on the larvae of *P. xylostella* individuals, mainly in the form of a significantly longer larval developmental duration and increased mortality of *P. xylostella* individuals (Table 2). This phenomenon is not surprising considering the mechanism of antibiotics. For example, tetracycline inhibits protein synthesis and can remove *Wolbachia* from *E. formosa* at lower treatment concentrations, but feeding 50 mg/mL of tetracycline can kill all female individuals within 3 days.[39] Therefore, before using antibiotic-treated insect individuals in experiments, a blank treatment period of several generations is usually taken to avoid the effects of antibiotic treatment on the growth and development of the insect hosts themselves. It has been shown in *Drosophila* that selective autophagy contributes to the elimination of antibiotics (Tetracycline) damaged *Wolbachia*.[40] In diamondback moth, whether autophagy can help eliminate damaged Wolbachia remains to be further studied. The results showed that 1 mg/mL rifampicin could remove *Wolbachia* infection after feeding treatment of *P. xylostella* larvae for 1 generation, and the toxic effect was low. Therefore, 1 mg/mL rifampicin was used to feed the *Wolbachia*-infected *P. xylostella* individuals, and after the treatment, the *P. xylostella* individuals was transferred to normal feeding conditions for several generations for subsequent experiments. In addition, we tested the infection status of *Wolbachia* in a random sample of each generation of antibiotic-treated *P. xylostella* populations.

The bacterial community

In this study, the bacterial community of *P. xylostella* were studied and compared for 3 populations of *P. xylostella* (WI, WA, and WU). In general, among the 29 samples of *P. xylostella*, the bacterial sequences were dominated by Firmicutes (52.5%) and Proteobacteria (47.5%); the dominant genera were *Carnobacterium* (46.2%), *Enterobacter* (10.1%), and *Enterococcus* (6.2%) (Figure 3). The bacterial sequences of *Wolbachia* accounted for 3.9% and 0.3% of the total sequences in the female and male *P. xylostella* samples of the WI population, respectively, which is consistent with the transmission characteristics of *Wolbachia*, a maternally transmitted endosymbiotic bacterium that spreads vertically, mainly through the cytoplasm of the eggs.[4] The bacterial sequences of *Wolbachia* were not detected in other *P. xylostella* samples, which further indicated that 1 mg/mL rifampicin was effective in eliminating *Wolbachia* from the *P. xylostella*. In addition, we also confirmed by molecular assays and phylogenetic analysis that the *Wolbachia* strain infecting the WI *P. xylostella* population belongs to the previously reported strain B supergroup *plutWB1*, and that infection by this strain can lead to sex-ratio distortion in *P. xylostella*.[36]

Several studies have shown that the use of antibiotics leads to changes in the microbial community of insects. However, our results showed that there was no significant difference in microbial community diversity of diamondback moth reared for 10 generations under normal conditions after 1 generation of antibiotic treatment (Figure 4). Our research shows that after 10 generations of normal conditions, the impact of antibiotic treatment on the generation has been basically eliminated, and the bacterial abundance and microbial community diversity of diamondback moth have returned to normal. In addition, there was no significant difference in bacterial abundance and diversity among diamondback moth populations with different genetic backgrounds. Although the original sampling sites were located in different geographical locations and genetic backgrounds, they were raised for many generations under the same conditions in the laboratory. The presence or absence of *Wolbachia* has no significant difference in bacterial abundance and diversity within the adult population of diamondback moth. The possible reason for this result is that we only measured the samples of adults. Whether *Wolbachia* has an impact on the bacterial abundance and microbial community diversity of other instars of diamondback moth needs further study.

Conclusions

In conclusion, in this study, we clarified the optimal antibiotic type and concentration for the elimination of *Wolbachia* in the *P. xylostella* by comparing the elimination efficiency of 2 different antibiotics (tetracycline hydrochloride and rifampicin) at different concentration gradients and their effects on the developmental duration of *P. xylostella* larvae. By molecular detection

and the 16S rRNA high-throughput sequencing, we confirmed that 1 mg/mL of rifampicin was effective in eliminating *Wolbachia* of the *P. xylostella*. In addition, we also comparatively analyzed the bacterial community of 3 different populations of the *P. xylostella* (WI, WA, and WU), which can provide a reference for the elimination of *Wolbachia* in other insect species and the effect of antibiotic treatment on the microbial communities of insect hosts.

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