Characterization of bacterial communities associated with the pine wilt disease insect vectors Monochamus alternatus Hope and host trees Pinus massoniana

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Abstract
Background Pine wilt disease (PWD) is a destructive disease caused by the pinewood nematode Bursaphelenchus xylophilus. Monochamus alternatus Hope is the main vector of this disease. The symbiotic microorganisms can play an important role in the transmission cycle mechanism. However, the role of bacterial microorganisms in the transmission of pine wood nematode by M. alternatus is not clear currently. The main purpose of this study is to reveal the composition and diversity of microbial flora in the gut of M. alternatus, as well as healthy and infected Pinus massoniana and its peripheral environment to discover the important microbial flora contributing to the transmission cycle.

Methods In this study, total DNA was extracted from 60 samples, including 20 samples of M. alternatus gut from different larval instars, healthy P. massoniana, nematode-infected P. massoniana and their peripheral environment (needles, bark, phloem, xylem, root, surface soil and rhizosphere soil), by triplicate. Samples were used for 16S rDNA Amplicon sequencing to determine the composition and diversity of microbial flora in each sample.

Results Infection of pinewood nematode resulted in an increase of the microbial community in the nematode-infected P. massoniana and its peripheral environment when compared with healthy P. massoniana, the microbial community in different tissues changed. Among them, Gryllotalpicola and Cellulomonas showed to be endemic microorganisms in nematode-infected P. massoniana, which can be used as indicators to detect the disease. Serratia was shown as an opportunistic pathogen, and was found to be enriched in M. alternatus gut and was also detected in the host plant tissues.

Conclusions This study clarified the change of microbial community in the transmission of pine wilt disease by M. alternatus. An important theoretical basis for the prevention of pine wilt disease was structured by our research.

Background
Pine wilt disease (PWD) is a destructive disease of pine forests worldwide caused by the pine wood nematode (PWN), Bursaphelenchus xylophilus (Steiner & Buhrer) Nickle, transmitted by the beetle Monochamus alternatus Hope, which has caused great environmental and economic losses in China.
and other countries [1]. PWD originated in North America and then spread to Asia and Europe [2, 3]. In Japan, PWD has threatened pine forests since 1905, with the loss of 700 000 m$^3$ of pine trees each year [3, 4]. Since the discovery of PWN in Nanjing Province in China in 1982, the disease has spread rapidly, threatening the safety of nearly 60 million hectares of pine trees. In Asia, PWN mainly infects the stomata during feeding and oviposition of *M. alternatus* adults and continues to spread the disease among pine trees [5, 6]. Therefore, effective prevention and control of *M. alternatus* populations are one of the best approaches to control pine wilt disease.

As were all known, plants were termed the plant microbiota in nature, comprising bacterial, archaeal, fungal and protist taxa [7, 8]. These microbiota has been reported to be a very important connection to the health and adaptability of the host [9]. Some microorganisms are symbiotic with plants, while others are pathogenic to plants, which is related to genotypes of plants and microbes, environmental conditions and other factors [10, 11]. There have studies shown that the PWN, the *M. alternatus*, and associated fungi exhibit a symbiotic relationship in the transmission of pinewood disease [12, 13]. During the pine wilt disease, it is presumed that the PWN and its associated bacteria have mutually beneficial symbiosis, which leads to the establishment of the disease in infected pine trees [14, 15]. The insect-mediated microorganisms also play an important role in the occurrence of pine wilt disease, and these microorganisms have a significant impact on the survival and development time of insects [16]. Symbiotic microorganisms of *M. alternatus* may also affect its life cycle and even oviposition in host plants. The oviposition behavior is an important step for the infection and transmission of the pinewood nematode. Because of the close relationship between pathogens and their vectors, pinewood nematode can acquire the bacterial community from *M. alternatus* [17]. At the same time, the microbial community of *P. massoniana* infected by pinewood nematode can change significantly [18]. Therefore, microorganisms can play an important role in the mechanism of infection of PWD. Through the analysis and identification in the change of microflora about *M. alternatus*, *P. massoniana* and its peripheral environment, which will provide new ideas and methods for biological control of *M. alternatus*.

The presence of endophytic bacteria in their respective *Pinus* host species has been reported, for
examples, the bacterial genera isolated from the needle of *P. flexilis* were *Acetobacter, Gluconacetobacter, Alphaproteobacteria* [19]. The major bacterial genera in the needle, roots, and stem of *P. contorta* were *Bacillus, Kocuria, Paenibacillus, and Pseudomonas* [20]. *Bacillus, Paenibacillus* and *Pseudomonas* were isolated from the roots of *P. Sylvestris, Methylobacterium* and *Pseudomonas* exist in the buds of *P. Sylvestris* [21-23]. In addition, there were many studies about the bacterial genera associated with PWN from different countries and different host *Pinus* spp. In Japan, *Pseudomonas* and *Bacillus* were carried by PWN [14, 24, 25]. *Achromobacter, Steotrophomonas, Ewingella, Buttiauxella, Enterobacter, Pantoea, Peptostreptococcus, Pseudomonas, Rhizobium, Serratia, Stahylococcus* and *Stenotrophomonas* were isolated from PWN from the *Pinus* spp. in China [26-29]. Similarly, *Burkholderia, Brevibacterium, Enterobacter, Ewingella*, and *Serratia* were found in the Republic of Korea associated with the nematode [30]. Bacteria associated with PWN in Portugal were mainly *Pseudomonas, Burkholderia, Serratia, Yersinia* and *Enterobacter* [31, 32]. Also, some researches have characterized the bacteria associated with *Monochamus* spp. The results reported that bacterial communities of *M. galloprovincialis* and *M. alternatus* are mainly composed by Proteobacteria, Firmicutes, and Bacteroidetes [17, 33]. Moreover, the research proposed that nematode-infection of *P. massoniana* causes a reduction in soil bacterial diversity [18]. But, the environmental conditions wherein endophyte of the *P. massoniana* interactions have rarely been investigated.

Therefore, to determine the role of the microflora during the transmission of the PWD, it is necessary to understand the microbial community structure of the vector *M. alternatus*, the host plant *P. massoniana* and its peripheral environment. We analyzed the bacterial diversity in different *M. alternatus* larval instars and adults, as well as in the *P. massoniana* forest and peripheral soil, from nematode-infected and healthy sites. The samples (total 60 samples) collected from different tissues of nematode-infected and healthy *P. massoniana* and different larval instars of *M. alternatus* were sequenced and the dominant microflora of each sample and their occurrence were analyzed by 16S rDNA Amplicon. This study clarified the change of microbial community in the transmission of pine wilt disease by *M. alternatus.*
Methods

Sample collection and characterization

All samples were collected from GuanTou, LianJiang county, Fujian province (N 26.15046°; E 119.59261°). The collected samples were divided into three groups: 1) healthy *P. massoniana* samples (needles, bark, phloem, xylem, root, surface soil and rhizosphere soil), 2) nematode-infected *P. massoniana* samples (needles, bark, phloem, xylem, root, surface soil and rhizosphere soil), and 3) *M. alternatus* samples (gut of II and III instar larvae, pupae, adults, and fecal samples of II and III instar larvae). Three replicates were set for each sample. A total of 60 samples were frozen immediately after collection and stored at -80°C.

DNA extraction

Microbial DNA was extracted from each sample by mechanical lysis in the presence of sodium dodecyl sulfate (SDS), followed by treatment with Hexadecyl Trimethyl Ammonium Bromide (CTAB). Soil samples (0.3 g) or plant samples (1.0 g) were homogenized with liquid nitrogen and mixed with 0.9 mL of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 25 mM sodium EDTA [pH 8.0], 10% SDS, 0.5 M NaCl, 1% CTAB) and 5 μL of proteinase K (10 mg/ml) in 1.5-mL tubes followed by horizontal shaking at 230 rpm for 30 min at 37°C. After incubation, 0.3 mL of 20% SDS was added, and the samples were incubated at 65°C for 2 h with gentle end-over-end inversions every 20 min. Samples were frozen at -70°C for 20 min and incubated at 65°C for 20 min (x3). The supernatants were collected after centrifugation at 12,000 r/min for 10 min at 4°C and transferred into 50-mL centrifuge tubes. Supernatants from the two cycles of extractions were combined and mixed with an equal volume of phenol-chloroform-isoamyl alcohol, (25:24:1, vol/vol/vol). The aqueous phase was recovered by centrifugation and DNA was precipitated with 0.1 volume of sodium acetate and 0.6 volume of isopropanol at room temperature for 1 h. DNA pellet was obtained by centrifugation at 12,000 r/min for 30 min at room temperature, washed with cold 70% ethanol two times, and resuspended in sterile deionized water.
Midgut samples from *M. alternatus* were dissected under a stereoscopic microscope and homogenized in 500 µL of TE buffer. Microbial DNA was extracted from each insect gut sample using the E.Z.N.A.â Bacteria DNA Kit (OMEGA, America). All DNA samples were stored at -20°C until further use.

**16S rDNA gene amplicon sequencing**

The 16S rDNA gene was amplified using the KAPA HiFi Hotstart ReadyMix PCR kit (KAPA, USA) and the universal primers 341F/806R (341F: ACTCCTACGGGRSGCAGCAG, 806R: GGACTACVGGGTATCTAATC) targeting V3-V4 region. PCR amplicons were purified using the AxyPrep DNA kit (AXYGEN, USA) and quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). DNA was pooled for a final concentration of 10 ng/µL concentration. The quality of the amplicon libraries was assessed by using a Thermo NanoDrop 2000 uv spectrophotometer and by agarose gel electrophoresis. The amplicon libraries sequencing was performed using the Illumina Hiseq PE250 platform (Illumina, San Diego, CA).

**Bioinformatics analysis**

The paired-end reads were merged into longer tags and quality filtered to get the high-quality tags by PANDAseq [34]. Amplicon libraries were sequenced by paired-end reads of 425 bp, the high-quality tags were clustered into operational taxonomic units (OTUs) using Usearch with a similarity threshold of 97%. The OTUs were further subjected to the taxonomy-based analysis by RDP algorithm using the Greengenes database (http://greengenes.lbl.gov). Alpha diversity (Shannon) and beta diversity [weighted UniFrac, principal coordinate analysis (PCoA)] were analyzed using QIIME. Linear discriminant analysis (LDA) and effect size (LEfSe) analyses were performed with the LEfSe online tool (http://huttenhower.sph.harvard.edu/galaxy).

**Flat colony counting of Serratia sp. in M. alternatus Larvae Guts**

Three I-V instars of reared indoors and wild *M. alternatus* were selected separately. The intestinal enzyme solution was extracted by dissecting the gut and homogenizing in double-distilled water. The
bacteria were diluted and plated on Serratia Differential Medium (HIMEDIA, India) and LB medium respectively. The bacteria were cultured at 30°C and counted after 24 hours. Data were organized with Microsoft Excel 2016 and analyzed with SPSS 18.0 in one-way ANOVA, a p-value of < 0.05 was considered as statistically significant.

**Results**

Sequencing results of operational taxonomic units (OTUs)

In this study, a total of 9174 OTUs were obtained from 60 samples by cluster analysis of 16S rDNA sequences with a similarity level above 97%. The sequencing data were analyzed by alpha diversity analysis, including the Chao1 index, Goods coverage index, observed species index, Shannon index, Simpson index, and PD_whole_tree index. According to the rarefaction curves, the number of sequences was able to reflect the main bacterial information in each sample (Additional file 1: Figure S1). There were 1573 OTUs shared among all samples. 1778 and 1922 special OTUs were detected in samples from healthy *P. massoniana* and nematode-infected *P. massoniana* respectively, only 195 special OTUs were found in samples from *M. alternatus* (Fig. 1A). Besides, the number of OTUs shared with samples from infected *P. massoniana* and *M. alternatus* was two and a half times of that shared with samples from healthy *P. massoniana* and *M. alternatus* (Fig. 1A). The most abundant OTUs in samples from *P. massoniana* were roots, surface soil and rhizosphere soil (Additional file 1: Figure S2). Of the samples from gut *M. alternatus*, the largest number of specific OTUs was in the bits of infected pine phloem, followed by II instar larvae gut, bits of infected pine xylem, adult gut, III instar larvae gut and pupae gut. Because pupae do not feed, there were few species of microorganisms, including only 12 OTUs (Additional file 1: Figure S2). These results indicated that the microbial species of *P. massoniana* were increased due to PWD.

In the life history of *M. alternatus*, the II instar larvae feed on phloem, thereby the number of OTUs shared by II instar larvae gut and infected pine phloem was the same as II instar larvae gut and healthy pine phloem, 346 and 325 respectively (Fig. 1B). At the process of III instar larvae feeding on xylem, the number of OTUs shared by III instar larvae gut and healthy pine xylem was 233, which is approximately twice of that shared by III instar larvae gut and healthy pine xylem. The number of
specific OTUs of infected pine xylems was 1328, which was far more than 237 of healthy pine xylems (Fig. 1C). There were 84 mutual OTUs of the process of adult feeding on bark. The number of OTUs shared by adult gut and infected pine barks was 137. The number of specific OTUs in infected pine barks was about 2.5 times that in healthy pine barks (Fig. 1D). The results showed that the microbial species in infected wood tissues were higher in number than those in healthy wood tissues in the three stages of feeding by different larva instars especially III instar larvae feeding on xylem. This may be related to the decrease of self-protection ability of infected wood, which makes it is susceptible to various microorganisms from outside.

LEfSe (linear discriminant analysis effect size) analysis

The LEfSe analysis further identified the specific microbiota at the phylum, class, order, family and genus levels that were present or abundant in all samples (Fig. 2). The most significant differences were Proteobacteria in samples from M. alternatus and Chloroflexi in samples from healthy P. massoniana. While Bacteroidetes, Armatimonadetes, Actinobacteria, Acidobacteria and Proteobacteria were mainly enriched in samples from infected P. massoniana. It was clear that the main bacterial species in samples from infected P. massoniana were highly similar in Proteobacteria and Acidobacteria to samples from M. alternatus and healthy P. massoniana respectively. It is possible that the ecological niche of various microbiota in P. massoniana may be changed due to PWN spread by M. alternatus. The microbiota in the gut of M. alternatus maybe also infected P. massoniana by feeding and even played an important role in P. massoniana.

Microbial Community Composition in vectors M. alternatus Hope, host trees P. massoniana and Peripheral Environment

Species distribution at the phylum level indicated that the main microorganisms in the gut and peripheral environment of M. alternatus were Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. Moreover, Cyanobacteria/Chloroplast bacteria were dominant in healthy P. massoniana. In contrast, in the soil of P. massoniana, infected P. massoniana, gut and excretion of M. alternatus, the dominant bacteria were Proteobacteria (Additional file 1: Figure S3). Species distribution at the genus level indicated that in the peripheral environment of infected P. massoniana,
Sphingomonas is the most abundant bacterial flora representing 7.66% of the abundance. Burkholderia accounted for 6.51%, which is equal to Gp1. Among the microorganisms in the gut and excretion of M. alternatus, at the genus level, Serratia accounted for 25.25%, Enterobacter 12.42%, Halotalea 8.81% and Stenotrophomonas 6.68% respectively. The total proportion of Gp1, Gp2, and Gp3 in surface soil and rhizosphere soil exceeded 50% of their total microbial abundance respectively, with no differences between the peripheral environment of infected P. massoniana or healthy P. massoniana (Fig. 3). (Additional file 1: Figure S4, S5)

The analysis of life history and coupling mechanism of M. alternatus show that the dominant bacteria in the excretion of II instars larvae was Saccharibacteria after feeding on the phloem of P. massoniana (Additional file 1: Figure S6). Burkholderia was the dominant bacteria in the excretion of III instars larvae after feeding on xylem (Additional file 1: Figure S7). Sphingomonas and Granulicella were the dominant bacteria in the process of adults feeding on the bark of P. massoniana (Additional file 1: Figure S8). It is not surprising to find that the bark, phloem, and xylem of infected P. massoniana contained more pathogenic bacteria than the corresponding tissues of healthy P. massoniana, and the pathogenic bacteria were mainly distributed between the Saccharibacteria, Burkholderia and Granulicella genus. Furthermore, it was found that the intestinal excretion of larvae of all instars has a similar bacterial content than the tissues of infected wood. Therefore, food might affect the intestinal microbial composition of M. alternatus. However, it can also be mentioned that there were significant differences in species composition between infected P. massoniana and healthy P. massoniana, and that the microbial composition of M. alternatus excretions was similar to infected P. massoniana tissues from which M. alternatus fed at corresponding growth stages.

Dominant Microflora in P. massoniana and Its Peripheral Environment

During the infection of PWN, the distribution of some microorganisms in the tissues of P. massoniana would change. Escherichia/Shigella, Pseudomonas and Spartobacteria were mainly distributed in P. massoniana, and the total content of them was identical in healthy and infected P. massoniana. Furthermore, all the samples in the three groups were classified by species. From the classification
tree, it is shown that most of the genera of Proteobacteria include Bradyrhizobium, Sphingomonas, Burkholderia, Rhizobium, Pseudoxanthomonas, Dyella, Mucilaginibacter of Bacteroidetes, Mycobacterium, and Nocardioides of Actinobacteria, Saccharibacteria of Candidatus Saccharibacteria, Terriglobus and Granulicella of Acidobacteria were abundantly distributed in the different tissues of infected P. massoniana but less abundant in healthy wood, and they were also present in the excretion, especially, Pseudoxanthomonas and Dyella which were abundant in the excretion of III instar larvae (xylem). Among them, Bradyrhizobium, Burkholderia, Dyella, Mycobacterium, and Mucilaginibacter were mainly distributed in soil of healthy P. massoniana about 20%, 8%, 4%, 11% and 1% respectively, Rhizobium, Saccharibacteria, Terriglobus, and Nocardioides were mainly distributed in phloem of healthy P. massoniana at 5%, 20%, 3% and 3% respectively. Granulicella and Sphingomonas were mainly distributed in the bark of healthy P. massoniana, the abundance of these bacteria was increased in all tissues of infected P. massoniana. In addition, whether in infected or healthy P. massoniana, Pseudoxanthomonas was mostly distributed in the phloem and root. When compared with the same distribution (14%) in two tissues in healthy P. massoniana, the distribution in phloem increased to 39% and decreased to 2.56% in roots of infected P. massoniana. Interestingly, Cellulomonas only existed in the phloem and its content in infected wood [78%] was higher than that in the healthy wood [22%]. Gryllotalpicola accounted for 82% of the microorganisms in infected P. massoniana including the bark [35%], phloem [27%], xylem [14%] and root [6%] (Fig. 4).

Dominant Microflora of M. alternatus Hope gut

M. alternatus plays an important role as a vector during the transmission of the pine wilt disease, therefore, it is of great significance to clarify the enteric microorganisms and the role in the transmission for the prevention and control of PWD. The dominant intestinal bacteria of second instar larvae were Serratia, which accounted for 72.11% (Fig. 4). Enterobacter was the most abundant genus in the gut of third instar larvae, which was over 65%, followed by the adult gut (10.30%) (Fig. 4). The abundance of Halotalea in pupae gut was the highest (47.69%). The highest abundance of Granulicella (genus below Acidobacteriaceae) in the excretion of II instar larvae was 12.15%, followed by Sphingomonas (10.11%). Saccharibacteria was the most abundant in the excretion of III instar
larvae (12.57%), followed by *Burkholderia* (11.68%). The abundance of *Pseudoxanthomonas* (5.31%) in the excretion of third instar larvae was higher than in the excretion of second instar larvae and the gut of different instar larvae (average 0.03%) (Fig. 5A). This may be related to the accumulation of multiple wounds, tree weakness and the invasion of pathogenic bacteria when third instar larvae feed on xylem.

Interestingly, *Serratia* was highly enriched in the insect samples, but less abundant in healthy or infected *P. massoniana* and its understory soil. The intestinal microbial distribution of *Serratia* among the different instars of *M. alternatus* was 72.11% in gut of second instar larvae, 23.46% in the gut of third instar larvae, 32.85% in pupae gut and 22.71% in the adult gut. *Serratia* was also found in the excretion of the second and third instar larvae, accounting for 0.01% and 0.61% respectively (Fig. 5B). Although, *Serratia* was not the dominant species in soil and plant tissues, it increased significantly in the gut of all instar larvae.

To confirm the relative concentration of *Serratia* in the gut of *M. alternatus* larvae from different larval instars, the intestinal enzyme solution of *M. alternatus* from reared indoors and wild selected separately were analyzed by flat colony counting. Despite the insects being collected in the field or reared indoors, the content of *Serratia* in the gut of second instar larvae were the highest, while the remaining instars had the lowest amount, and remained relatively stable in I and IV instars. However, the distribution of *Serratia* in the gut of V instar larvae from indoor rearing was higher than the one from the field sampling, this suggests that the food has an effect on the distribution of *Serratia* in the gut of *M. alternatus* larvae, but its distribution pattern and whether it is related to the larval metabolic mechanism still needs further research (Fig. 5C and D).

Also, the network map at the genus level showed that the abundance of *Serratia* was positively correlated with *Lactococcus*, *Variovorax*, *Acinetobacter*, *Stenotrophomonas*, and *Achromobacter*, and negatively correlated with *Inquilinus* and *Burkholderia*. *Stenotrophomonas* and *Achromobacter* had the same abundance trend as *Serratia* in the gut and excretion of *M. alternatus* (Additional file 1: Figure S9). Besides, *Raoultella*, which has ornithine-lysing activity in the samples, was abundant, especially in the gut of insects and sample grouping after II instar. It may be beneficial for *M.*
*alternatus* to feed on crude fibrous tissues such as xylem and bark and promote related digestion and metabolism.

**Discussion**

We provided an in-depth description of the microbial communities in the gut of the *M. alternatus* and its living environment. Using 16S rDNA gene amplicon sequencing, we explored the 60 different samples from *M. alternatus*, *P. massoniana* and soil. We examined the microbial diversity and investigated possible correlations between the *M. alternatus*, *P. massoniana* and its peripheral environment.

The number of OTUs showed that the microbial content in the root, rhizosphere soil, surface soil and peripheral environment of healthy and infected *P. massoniana* was abundant. This is consistent with Müller’s study, which found that the vertical microbial content of plants originated from the soil, so the soil microbial content is rich. Furthermore, many studies have suggested that rhizosphere soil is the main source of endophytic bacteria in plants [35, 36]. The gut and excretion of different instars of *M. alternatus*, showed a positive correlation between the microbial content of each instar larvae and their corresponding feeding plant tissues. Because pupae did not feed, there were fewer microbial species identified. The microbial species in infected wood tissues were higher in number than those in healthy wood tissues from the samples involved in the feeding process of II-III instars larvae and adults. This can be explained because *P. massoniana* is attacked by pine wood nematode and *M. alternatus*, resulting in a large number of wounds, followed by a large number of bacteria invading the plant through the wounds. The substances leaking from wounds provide nutrients for bacteria, thus promoting the colonization of a large number of endophytic bacteria [37]. Moreover, the intestinal excretion of larvae had a similar microorganism content than the corresponding infected wood tissues. Therefore, it can be inferred that food affects the intestinal microbial composition of *M. alternatus* as shown in previous studies that the effects of food on the intestinal microorganisms of insects are complex. Broderck et al. found that there are significant differences in the midgut microorganisms of *Lymantria dispar* larvae fed with different diets [38]. The intestinal microbial composition of lower and higher wood-eating termites is also affected by food. When the composition
of food changes, the dominant bacteria changes accordingly [39].

Different organisms have different kinds of endophytic bacteria. *Proteobacteria* is the dominant bacteria in the gut and excretion of infected *P. massoniana* and its peripheral environment. *Proteobacteria* is classified into *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria,* and *Gammaproteobacteria* according to the diversity of morphology, physiology and metabolism. It is of great significance to the cycling of carbon, nitrogen and sulfur in living organisms [40]. Some studies have used 16S rRNA genes to isolate and identify microorganisms in the soil, and found that the dominant microflora is *Proteobacteria* [41, 42]. Besides, for insects, *Proteobacteria* is the resident bacteria in the gut of most insects [29, 43, 44]. *Proteobacteria* is the dominant intestinal bacteria of most insects, such as *Helicoverpa armigera* (Lepidoptera), *Bombyx mori* (Lepidoptera), locust (Orthoptera), and some species of termites (Isoptera) [38, 43, 45]. Previous studies have shown that the dominant bacteria of *M. galloprovincialis* and *M. alternatus* is *Proteobacteria* (mainly *Gammaproteobacteria*) [17, 44], and it has been found that most of the associated bacteria of pine wood nematode is *Gammaproteobacteria* [46].

*Acidobacteria* is one of the most dominant and abundant phyla in the soil [47]. In this study, *Acidobacteria* was the dominant bacteria in surface soil and rhizosphere soil, in which *Gp1, Gp2,* and *Gp3* accounted for more than 50% of the total microbial abundance. *Acidobacteria* belongs to Acidophilus and studies have shown that *Acidobacteria* plays an important role in the ecosystem, and has a rich diversity of metabolic and genetic functions [48], as well as a great contribution to ecological stability [49]. *Acidobacteria* is the dominant bacteria in most soils because of its low pH value [50]. The soil in *P. massoniana* planting area is acidic. Different subgroups of *Acidobacteria* have different optimum pH values. For example, the subgroup *Gp1* of *Acidobacteria* grows best in the soil environment with pH 4-5.5 [51, 52]. In addition, Cuie Shi et al. studied soil microbial diversity in *P. massoniana* forests which were infected and healthy by pine wilt disease. It was found that the infection of pine wood nematode causes changes in soil physical and chemical properties, bacterial community composition and diversity [18]. The important role of these microorganisms in the mechanism of pine wood nematode infection of *P. massoniana* needs further study.
Due to the infection with pine wood nematode, the dominant bacteria of infected and healthy *P. massoniana* changed significantly. The results indicated that feeding on *P. massoniana* could affect the distribution of these bacteria in different tissues and can be metabolized by *M. alternatus* larvae. According to the distribution of each sample, the transmission routes of these bacteria can be inferred as follows: *Bradyrhizobium, Burkholderia, Dyella, Mycobacterium* and *Mucilaginibacter* spread from the soil (healthy *P. massoniana*) to various tissues of infected *P. massoniana*. *Rhizobium, Saccharibacteria, Terriglobus and Nocardioides* spread from phloem of healthy *P. massoniana* to various tissues of infected *P. massoniana*. *Granulicella and Sphingomonas* spread from the bark of healthy *P. massoniana* to various tissues of infected *P. massoniana*. These microorganisms are related to plant growth. As plant growth-promoting rhizobacteria (PGPR), *Rhizobium* and *Bradyrhizobium* can colonize and survive in plant rhizosphere [53, 54], produce phytohormones and iron spores to promote plant growth and enhance the dissolution of inorganic phosphate [55]. *Burkholderia* occupies a rich niche in nature and has a wide range of functions, including controlling biological growth as a pathogen and promoting plant growth as PGPR [56, 57]. The phylum *Candidate Saccharibacteria* (former candidate division TM7) has been frequently detected in natural environments, the human oral cavities and activated sludge, moreover, the phylum was assigned the *Saccharibacteria* because of their sugar metabolisms [58-60]. *Pseudoxanthomonas* degrades complex organic compounds, so it is also used in environmental governance [61, 62]. Studies have shown that Cellulose-degrading bacteria of *Dyella* genus have been isolated from the gut of Cerambycidae insects [63]. *Mycobacterium* has been found in the gut of necrophagous beetles, suggesting that the bacteria can be transmitted through excretion by the digestive system of insects [64]. Some researchers had hypothesized that *Nocardioides* can degrade refractory organic compounds [65]. In this study, *Gryllotalpicola* was identified only in the tissues of infected *P. massoniana*, including bark, phloem, xylem and root, while *Cellulomonas* was present only in the phloem, and its content in infected wood was higher than that in healthy wood. These two strains can degrade cellulose [63]. The distribution of them in infected and healthy *P. massoniana* can be used as indicator of whether *P. massoniana* is infected with the pine wood nematode.
In a vital dynamic environment, the insect’s gut is associated with feeding, digestion, excretion and other important activities, which are related to the enteric microorganisms [66-69]. Enteric microorganisms are essential to insect growth and development, especially phytophagous insects [66, 70]. Insect intestinal microorganisms play an important role in nitrogen fixation, lignocellulose degradation, amino acid biosynthesis and uric acid degradation [70]. Therefore, the study of microorganisms in insect gut is of great significance to clarify the interaction between insects and plants. Many studies have been conducted on the diversity of enteric microorganisms in insects, such as *Bombyx mori*, *Plutella xylostella*, *Helicoverpa armigera*, *Holotrichia parallela*, etc [45, 71]. With the development of intestinal microbiology, it has been found that enteric microorganisms can be modified by genetic engineering to enrich and express insecticidal genes in the insect gut, which provides a feasible method for the use of microbes to prevent and control *M. alternatus* [72, 73]. The prevention and control of plant diseases and pests by pathogenic bacteria has become a popular research topic and new pathogenic bacteria have been isolated and found continuously, enriching the possibilities of biological control of plant diseases and pests [74-77]. The vector of pine wilt disease and its intestinal microorganisms have a very important influence on host selection and colonization. It can be seen from the results that in the gut and excretion of *M. alternatus*, the dominant microflora was *Serratia* and *Stenotrophomonas* in genus level. *Serratia* was not the dominant species in soil and plant tissues, but its content increased significantly in the gut of II instar larvae, and there was higher abundance of *Serratia* in the gut of different insect states after II instar, which proved that *Serratia* was likely to accumulate in the gut of II instar larvae through feeding pathway. A large number of *Serratia* were also isolated from indoor cultured pine wood nematode, infected *P. massoniana* and *Monochamus galloprovincialis* [46, 78]. Moreover, it has been proved that *S. marcescens* PWN146 can colonize on host plants [79]. *S. marcescens* has multiple roles after colonizing on plants. It can change from beneficial bacteria promoting plant growth to plant pathogenic bacteria under environmental stimulation [60]. Current studies have found that *Serratia* can secrete cellulase and other extracellular enzymes. This indicates that *Serratia* is ubiquitous in the gut of wood-eating insects like *M. alternatus*, and is closely related to the cellulose degradation function of the host. *Serratia* also has
strong stability for rapid adaptation to the environment [80, 81]. If the role of *Serratia* in the mechanism of pine wilt disease transmission by *M. alternatus* can be clarified, a new method for the control of pine wilt disease can be provided.

**Conclusions**

In this study, we analyzed the microbial diversity from each segment of the whole cycle infection mechanism and the dominant and endemic microflora from each part. *Proteobacteria* is the main microorganisms in the gut and peripheral environment of *M. alternatus*. Within the infection of pine wood nematode, the microbial community of infected *P. massoniana* was significantly higher than healthy *P. massoniana*. *Rhizobium*, *Bradyrhizobium*, *Terriglobus*, *Granulicella*, *Sphingomonas*, *Dyella*, *Burkholderia*, *Saccharibacteria*, *Pseudoxanthomonas*, *Mucilaginibacter*, *Mycobacterium*, and *Nocardioides* were dominant bacteria of infected *P. massoniana*. We also found that *Gryllotalpicola* and *Cellulomonas* mainly existed in infected *P. massoniana* but there were a little in healthy *P. massoniana*. *Serratia* and *Enterobacter* were dominant bacteria in the gut of *M. alternatus* (Fig. 6). Flat colony counting results showed that the content of *Serratia* in the gut of II instar larvae was the highest (80%), while that of III instar larvae was the lowest. The results indicated that *Serratia* could not only colonize in the intestinal tract but also enrich in the intestinal tract of *M. alternatus*. However, the role of these microorganisms in the whole cycle mechanism still needs to be further studied. Clarifying the interaction among host, vector and microorganisms in the environment, we can provide a new strategy for the control of pine wilt disease.

**Abbreviations**

PWD: Pine wilt disease; PWN: Pine wood nematode; OTUs: operational taxonomic units

**Declarations**

**Ethics approval and consent to participate**

There are no specific permits for insect collection in the selected locations. The sampling chosen locations are not privately-owned or natural protected areas. Insects used for the experiments are not considered endangered or protected species, and its collection is legal in China.
Consent for publication

No applicable.

Availability of data and materials

All raw sequences were deposited in the NCBI Sequence Read Archive (BioProject: PRJNA561715).

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

WSQ and ZFP contributed conception and design of the study; GYJ, LQN and CLY collecting metagenome samples; GYJ, LQN and SES performed the data analysis. GYJ performed the flat colony counting of Serratia sp.; GYJ, WSQ, LQN, CLY, CLR, LGH, HX, WR drafted the manuscript; GYJ and WSQ reviewed the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.
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Figures
OTU Venn diagram. (A) H: healthy P. massoniana and its understory soil, D: infected P. massoniana and its understory soil, C: M. alternatus and its intestinal fecal matter, (B) A3: II instar larvae gut, C1: healthy pine phloem, C2: infected pine phloem, F3: bits of infected pine phloem. (C) B3: III instar larvae gut, D1: healthy pine xylem, D2: infected pine xylem, E3: bits of infected pine xylem. (D) D3: adult gut, B1: healthy pine barks, B2: infected pine barks.
Cladogram of samples from M. alternatus, P. massoniana and peripheral environment.

Different colors represent different groups, and different color nodes in the branches represent the microbiota that plays an important role in the group. The blue nodes, red nodes and green nodes represent a group of microbes that play an important role in samples from M. alternatus, infected P. massoniana and healthy P. massoniana respectively.

The yellow nodes represent a group of microbes that do not play an important role.
Figure 3

Profiling bar-plot of samples from M. alternatus, P. massoniana and peripheral environment in genus level. Only the 20 most abundant OTUs are represented. The remaining microbiota are included in the group “other”.
The Heatmap of the main bacteria identified in all samples from M. alternatus, P. massoniana and peripheral environment at genus level. These bacteria are classified into seven groups by different color marked. The bacteria labeled red are mainly abundant in
the gut of M. alternatus. The bacteria labeled green were distributed equally in healthy and infected P. massoniana. The bacteria labeled pink were abundantly distributed in the soil of healthy P. massonianai and various tissues of infected P. massoniana. The bacteria labeled light blue were abundantly distributed in the phloem of healthy P. massonianai and various tissues of infected P. massoniana. The bacteria labeled light blue were abundantly distributed in the phloem of healthy P. massonianai and various tissues of infected P. massoniana. The bacteria labeled yellow were abundantly distributed in the bark of healthy P. massonianai and various tissues of infected P. massoniana. The bacteria labeled blue was present only in the bark, phloem, xylem and root of infected P. massoniana. The bacteria labeled orange was identified only in the phloem and its content in infected wood was higher than that in healthy wood.
Analysis of the distribution and concentration of Serratia in gut of different instar M. alternatus larvae. (A) Profiling bar-plot of M. alternatus samples in genus level. (B) Distribution of Serratia from sequencing data analysis in each sample. (C) Distribution of Serratia in the gut of different instar wild M. alternatus larvae by flat colony counting. (D) Distribution of Serratia in the gut of different instar feeding M. alternatus larvae by flat colony counting. Statistical significance at p < 0.05 are designated by the letters a, b, c and d.
The changes in the distribution of major bacteria during the transmission of pine wilt disease by *M. alternatus*. These bacteria are classified into seven groups by different color marked. The distribution of the seven groups of bacteria in each sample was different, and the content was also different, as shown in figure 4. The bacteria labeled red are mainly abundant in the gut of *M. alternatus*. The bacteria labeled green were distributed equally in healthy and infected *P. massoniana*. The bacteria labeled pink were abundantly distributed in the soil of healthy *P. massoniana* and various tissues of infected *P. massoniana*. The bacteria labeled light blue were abundantly distributed in the phloem of healthy *P. massoniana* and various tissues of infected *P. massoniana*. The bacteria labeled yellow were abundantly distributed in the bark of healthy *P. massoniana* and various tissues of infected *P. massoniana*. The bacteria
labeled blue was present only in the bark, phloem, xylem and root of infected P. massoniana. The bacteria labeled orange was identified only in the phloem and its content in infected wood was higher than that in healthy wood.

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