forests

MDPI

Article

Diversity and Function of Endo-Bacteria in *Bursaphelenchus xylophilus* from *Pinus massoniana* Lamb. in Different Regions

Yan-Mei Fu [1,2,3], Hong-Bin Liu [1,2] and Xiao-Qin Wu [1,2,*]

1 Co-Innovation Center for Sustainable Forestry in Southern China, College of Forestry, Nanjing Forestry University, Nanjing 210037, China; fulif7@126.com (Y.-M.F.); hongbin219@gmail.com (H.-B.L.)
2 Jiangsu Key Laboratory for Prevention and Management of Invasive Species, Nanjing Forestry University, Nanjing 210037, China
3 Zhejiang Zhongtong Testing Technology Co. LTD, Ningbo 315000, China
* Correspondence: xqwu@njfu.edu.cn; Tel.: +86-25-8542-7427

Received: 24 March 2020; Accepted: 24 April 2020; Published: 26 April 2020

Abstract: The pine wood nematode (PWN) *Bursaphelenchus xylophilus* is the pathogen that causes pine wilt disease (PWD), a devastating forest disease. PWN-associated bacteria may play a role in PWD. However, little is known about the endo-bacteria in PWN. We analyzed the diversity of endo-bacteria in nine isolates of PWNs from *Pinus massoniana* Lamb. in nine epidemic areas from three Chinese provinces by high-throughput sequencing of 16S rDNA and isolated and identified culturable endo-bacteria through construction of a 16S rDNA phylogenetic tree and Biolog microbial identification. We also examined the effects of endo-bacteria on PWN fecundity, antioxidant capacity, and virulence using sterile nematodes as a control. While the dominant endo-bacteria in PWNs from different regions exhibited no significant difference in the classification levels of class and genus, their proportions differed. *Pseudomonas* and *Stenotrophomonas* were highly abundant in all PWN isolates. A total of 15 endo-bacterial strains were successfully isolated and identified as six species: *Stenotrophomonas maltophilia*, *Pseudomonas fluorescens*, *Kocuria palustris*, *Microbacterium testaceum*, *Rhizobium radiobacter*, and *Leifsonia aquatica*. We also found that *P. fluorescens* significantly increased the egg production of PWN, and that both *P. fluorescens* and *S. maltophilia* enhanced the mobility of PWN under oxidative stress and reduced the content of reactive oxygen species by increasing antioxidant enzyme activity in PWN. These strains also accelerated the development of PWD, and *P. fluorescens* had a more beneficial effect on PWN than *S. maltophilia*. Diversity exists among the endo-bacteria in PWNs from different regions, and some endo-bacteria can promote PWN infestation by enhancing the fecundity and antioxidant capacity of the nematode. Our study contributes to clarifying the interaction between endo-bacteria and PWN.

Keywords: pine wood nematode (PWN); endo-bacteria; high-throughput sequencing; fecundity; antioxidant capacity; virulence

1. Introduction

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* is a widespread quarantine pest that causes a destructive forest disease, pine wilt disease (PWD), and results in massive ecological and financial losses in numerous parts of Asia and Europe, especially in China [1–6]. Much research has been performed on PWN morphology, life history, parasitical range, and optimal growth media [7]. With the development of molecular biology techniques, recent studies have focused on the genes and proteins related to PWN fecundity, stress resistance, and pathogenesis [8,9]. Huang et al. reported that

a sperm protein BxMSP10 was essential for egg hatching and fecundity in PWN [10]. Autophagy was found to be crucial for the development, fecundity, stress resistance, and pathogenicity of PWN [11–14]. In the early stage of PWN infection, a strong reactive oxygen species (ROS) burst as the main defence response in pine trees was observed [15,16]. However, PWN was found to have an efficient antioxidant system and 12 antioxidant enzymes have been identified in the PWN secretome, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) [8,17]. *Pinus massoniana* Lamb. is an indigenous tree species and a major host of PWN in China [6]. A comparative proteomic study on healthy versus infected *P. massoniana* was conducted to evaluate the pine death mechanisms [18]. In terms of controlling PWN, a *Bacillus pumilus* strain LYMC-3 was shown to have an efficiently nematocidal activity against PWN [19]. However, PWD is highly complicated and also involves interactions amongst many elements, including the PWN, PWN-associated bacteria, the pine host, fungi, the vector beetle, and the environment. Therefore, the pathogenic mechanism of PWD is still unclear and preventing PWD remains very challenging.

The microbial communities associated with organisms have long attracted the attention of researchers, particularly the interaction between bacteria and hosts. The entomopathogenic nematodes *Steinernema* and *Heterorhabditis* are symbiotic with the *Enterobacteriaceae* bacteria *Xenorhabdus* and *Photorhabdus*, respectively, and these bacteria play a key role in the killing of insects by the nematodes [20]. Ever since Oku et al. determined that epiphytic bacteria from PWN could produce toxic metabolites causing pine tree wilting [21,22], many studies have supported the hypothesis that the bacteria associated with PWN play a role in PWD [23,24]. Some bacteria carried by PWN from infected pines were absent from healthy trees and phytotoxins produced by PWN-associated bacteria have been reported to cause symptoms in pine seedlings similar to PWD [25]. Bacteria isolated from the surface of PWNs in different regions and countries show great diversity at the genus level [26–29], and a study proved that some bacteria could promote PWN fecundity while others suppressed the hatching of PWN eggs [30]. Most previous studies have focused on bacteria isolated from the surface of PWN and limited studies of the endo-bacteria of PWN have been reported. Yuan et al. proved the existence of endo-bacteria in PWN by transmission electron microscopy and isolated one bacterial species, *Stenotrophomonas maltophilia*, from PWN [31]. Wu et al. found that the species and carbon metabolism of culturable endo-bacteria in PWN were related to PWN virulence [32]. Furthermore, some endo-bacteria have been reported to influence the development, pathogenesis-related gene expression, and PWN virulence [33,34]. Bacterial diversity and community structure in PWNs with different virulence and from different *Pinus* spp. has been demonstrated [35,36].

However, whether there are any differences among the endo-bacterial communities in PWNs from various regions and the role of PWN endo-bacteria in the development of PWD is still not well understood. Therefore, we first analyzed the diversity of endo-bacteria in PWNs from *P. massoniana* in different regions of China by high-throughput sequencing, and then tested the effects of culturable endo-bacteria on PWN fecundity, oxidative stress resistance, and virulence to determine their function in the PWN. Our results help clarify the relationship between PWN and endo-bacteria, and thus promote understanding of the pathogenesis of PWD.

2. Materials and Methods

2.1. Biological Materials

A total of nine isolates of PWN were isolated from infested *P. massoniana* from three epidemic areas in Jiangsu province (JS12-01, JS12-02, and JS12-03), three in Guangdong province (GD12-01, GD12-02, and GD12-03), and three in Sichuan province (SC12-01, SC12-02, and SC12-03) in China. All the pines used had wilted and were basically the same size, and were felled in September. Each reference number represents a sample from a tree. The isolates were cultured on *Botrytis cinerea* on the potato dextrose agar (PDA) plate at 25 °C for 1 week. Nematodes were obtained using a Baermann funnel [37] and washed with M9 buffer and double-distilled (dd) H_2O prior to use. Two-year-old *P. massoniana*

seedlings were obtained from the greenhouse at Nanjing Forestry University (Nanjing, China) and cultivated at a temperature of 28–32 °C and relative humidity of 65%–75%.

2.2. Surface Sterilization of Nematodes

The nematodes were sterilized with 1% mercuric chloride for 30 min, rinsed with ddH_2O, soaked in a mixture of 2% spectinomycin and 2% gentamicin for 30 min, and rinsed with ddH_2O. Then, the nematodes were transferred to nutrient agar (NA) plates at 28 °C for 48 h and checked for the presence of bacterial colonies.

2.3. High-Throughput Sequencing of Endo-Bacteria from Nematodes

Approximately 50,000 surface-sterilised nematodes from each isolate were frozen in liquid nitrogen and ground into powder. Then, the total genomic DNA of endo-bacteria from the nematodes was extracted with the Bacteria Genomic DNA Extraction Kit Ver. 3.0 (TaKaRa, Shiga, Japan). Polymerase chain reaction (PCR) was performed using the universal primers 515f/806r targeting the bacterial 16S rDNA V4 region [38]. A barcoded-tag including 6 nucleotide bases was added upstream of the primers for distinguishing among the different samples. After quantification and quality assurance, 16S rDNA PCR products were paired-end sequenced through the Illumina MiSeq system (Illumina, San Diego, CA, USA). The reads were spliced by identifying the overlap between paired-end reads using FLASH software [39]. Low-quality data were filtered with QIIME quality filters [40]. The sequences were designated to operational taxonomic units (OTUs) at 97% similarity utilizing UPARSE software application [41]. A representative sequence for every OTU was chosen and then taxonomic information was appointed to every representative sequence with the Ribosomal Database Project classifier [42]. For the analysis of bacterial community structure, the species abundance of samples was counted at the class and genus levels.

2.4. Isolation and Identification of Culturable Bacteria from Nematodes

Culturable bacteria were isolated from surface-sterilized nematodes from each isolate of PWN according to Wu et al. [32]. These bacterial strains were maintained in nutrient broth (NB) liquid medium at 28 °C for 18 h and total genomic DNA was extracted using the EasyPure Genomic DNA Kit (TransGen Biotech, China). Then, the 16S rDNA was amplified by PCR using the primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) and sequenced by Nanjing GenScript (Nanjing, China). The 16S rDNA partial sequences were analyzed through a BLAST search in the NCBI GenBank database. The phylogenetic tree was constructed using MEGA7 with the maximum likelihood method [43]. One thousand replicates were performed for bootstrap analysis. The strains were identified using GEN III MicroPlates (Biolog, Inc., Hayward, CA, USA) according to the Biolog Microbial Identification System [44]. A minimum similarity of 0.5 was used to assign ID.

2.5. Preparation and Treatment of Aseptic PWN

A nematode suspension of the JS12-01 isolate was placed in an aseptic plastic dish and incubated at 25 °C for 5 h for laying eggs. The pure eggs were collected and soaked in a freshly prepared 15% H_2O_2 solution for 1 h under sterile conditions and rinsed five times with ddH_2O. After hatching, the resulting second-stage juveniles were inoculated on PDA plates with *B. cinerea* for 1 week. Then, the nematodes were collected with a Baermann funnel under sterile conditions. A portion of the nematodes was ground to a powder under sterile conditions and cultured on NA plates to check for sterility [33].

The endo-bacteria *S. maltophilia* B.x NS1 and *Pseudomonas fluorescens* B.x NS2 were cultured in 50 mL of NB medium at 28 °C for 24 h, formulated into a 2.0×10^6 CFU bacterial suspension, and sprayed on PDA plates with *B. cinerea*. Plates sprayed with NB medium were used as a control. The sterile JS12-01 isolate was inoculated on these plates and incubated for 1 week. The nematodes

were harvested using a Baermann funnel under sterile conditions and surface sterilization of the nematodes was performed as previously described.

2.6. Determination of Oviposition of PWN

Approximately 100 nematodes from different treatments were placed in an aseptic plastic dish and incubated at 25 °C for 24 h under sterile conditions to lay eggs, and the number of eggs was counted under an optical microscope (Leica DM500; Leica Microsystems, Heerbrugg, Switzerland).

2.7. Measurement of Mobility of PWN under Oxidative Stress

Oxidative stress treatment was performed by soaking the nematodes from different treatments in 200 µL of 15 mM H_2O_2 for 1 h. The mobility of the nematodes in terms of head swinging frequency and body bending frequency was recorded under an optical microscope. The head swinging frequency is defined as the number of times a nematode swings its head from one side to the other and back within 1 min, and the body bending frequency is defined as the number of times a nematode moves its body away from a straight line within 20 s [45].

2.8. Determination of ROS Content and Antioxidant Enzyme Activity in PWN

Following oxidative stress treatment, the nematodes were washed five times with ddH_2O and the ROS content and antioxidant enzyme activity in the nematodes were determined. According to Kampkotter et al. [46], after 100 nematodes were ground into a homogenate, 300 µL of homogenate was added with 100 µM fluorescent probe H2DCFH-DA and incubated at 130 rpm for 30 min in the absence of light at 37 °C. Then, the fluorescence intensity was detected as the ROS content under the conditions of an excitation wavelength of 485 nm and a blocking wavelength of 528 nm using a fluorescence spectrophotometer (CARY-100 PTP-1 Fluorescence Peltier System; Varian, Atlanta, GA, USA). After the nematodes were ground into homogenate and centrifuged, the supernatant was taken for testing and partly used for protein determination using coomassie brilliant blue G-250. The activities of SOD, CAT, and GSH-Px were detected with 0.03mmol/L pyrogallol, H_2O_2, and 5,5′-dithiobis-(2-nitrobenzoic acid) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) at 325, 250, and 422 nm wavelengths, respectively, using a spectrophotometer (Spectronic Helios Gamma; Thermo, Cambridge, UK).

2.9. Virulence Assay

A sterile blade was used to cut a small wound deep into the xylem of *P. massoniana* stems approximately 10 cm near the ground, and a sterile cotton ball was inserted. Then, the incision was covered with a cotton ball and funnel-shaped parafilm. Approximately 10,000 nematodes from different treatments were inoculated into the cotton balls on *P. massoniana* seedlings. The development of PWD was continuously photographed. Seedlings inoculated with only the endo-bacteria *S. maltophilia* B.x NS1 and *P. fluorescens* B.x NS2 were used as blank controls. The infection assay was conducted in triplicate using 18 individual *P. massoniana* seedlings each treatment. Disease severity was classified as follows: 0, all needles were green; I, a few needles had turned yellow; II, approximately half of the needles had turned yellow or brown; III, most of the needles had turned brown; and IV, the entire seedling was withered. The morbidity and disease severity index (DSI) of seedlings were calculated according to Liu et al. [13]:

$$\text{Morbidity of seedlings} = \frac{\sum \text{ number of infected plants with symptoms}}{\text{Total number of plants}} \times 100\% \quad (1)$$

$$\text{Disease severity index (DSI)} = \frac{\sum \text{ number of disease plants} \times \text{symptom stage}}{\text{Total number of plants} \times \text{highest symptom stage}} \times 100 \quad (2)$$

2.10. Statistical Analysis

Three technical replicates and three biological replicates were used for each assay. The mean and standard deviation (SD) values of the replicates were calculated using Microsoft Excel™ software (Microsoft Corp., Redmond, WA, USA). Tukey's HSD test was performed using SPSS software (ver. 17.0; SPSS Inc., Chicago, IL, USA) to determine statistical significance. $p < 0.05$ was considered to indicate a significant difference.

3. Results

3.1. Diversity of Endo-Bacteria in PWNs from P. massoniana in Different Regions

To explore the relationship between PWN endo-bacteria and PWD, we first tested the diversity of endo-bacteria of PWNs from *P. massoniana* in different epidemic areas by high-throughput sequencing. The results showed that the average number of OTUs of the endo-bacteria in the nine PWN isolates was 735. Among them, the average number of OTUs of the endo-bacteria in the three isolates (JS12-01, JS12-02, and JS12-03) from Jiangsu was the largest at 928, followed by that in the three isolates (SC12-01, SC12-02, and SC12-03) from Sichuan (722) and the three isolates (GD12-01, GD12-02, and GD12-03) from Guangdong (619). After analyzing the community structure of these endo-bacteria, we found that most of the endo-bacteria in PWNs from different regions belonged to the following six classes: Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Flavobacteriia, Sphingobacteriia, and Verrucomicrobiae. While Gammaproteobacteria was the dominant class, the proportion in each isolate was different (Figure 1a). At the genus level, the dominant endo-bacteria in PWNs from Jiangsu were *Sphingomonas* (16.38%), *Herbaspirillum* (16.31%), *Stenotrophomonas* (13.5%), and *Pseudomonas* (10.67%), whereas those from Guangdong were *Pseudomonas* (29.23%), *Stenotrophomonas* (13.78%), *Herbaspirillum* (13.49%), and *Achromobacter* (3.09%), and those from Sichuan were *Chryseobacterium* (18.46%), *Pseudomonas* (15.58%), *Stenotrophomonas* (10.54%), and *Sphingomonas* (5.99%). Both *Pseudomonas* and *Stenotrophomonas* were highly abundant in the population of endo-bacteria of PWNs from the three provinces. We clustered the abundances of 35 genera in the endo-bacteria from nine isolates; the first ten genera with higher abundance of the endo-bacteria in the nine isolates were identical but accounted for different proportions (Figure 1b). Furthermore, *Janthinobacterium*, *Sphingomonas*, and *Herbaspirillum* were mainly concentrated in the isolates JS12-01 and JS12-02; *Pseudomonas* was mainly concentrated in GD12-01 and GD12-02; *Sphingobacterium* and *Luteolibacter* were mainly concentrated in SC12-02; *Stenotrophomonas* was mainly concentrated in GD12-02, GD12-03, and SC12-01; *Achromobacter* was mainly concentrated in GD12-03 and SC12-01; and *Sphingobium* and *Chryseobacterium* were mainly concentrated in SC12-01, SC12-02, and SC12-03 (Figure 1b). These results indicate that the community structure of endo-bacteria in PWNs from different regions varies greatly, although there was no difference in the ten dominant genera.

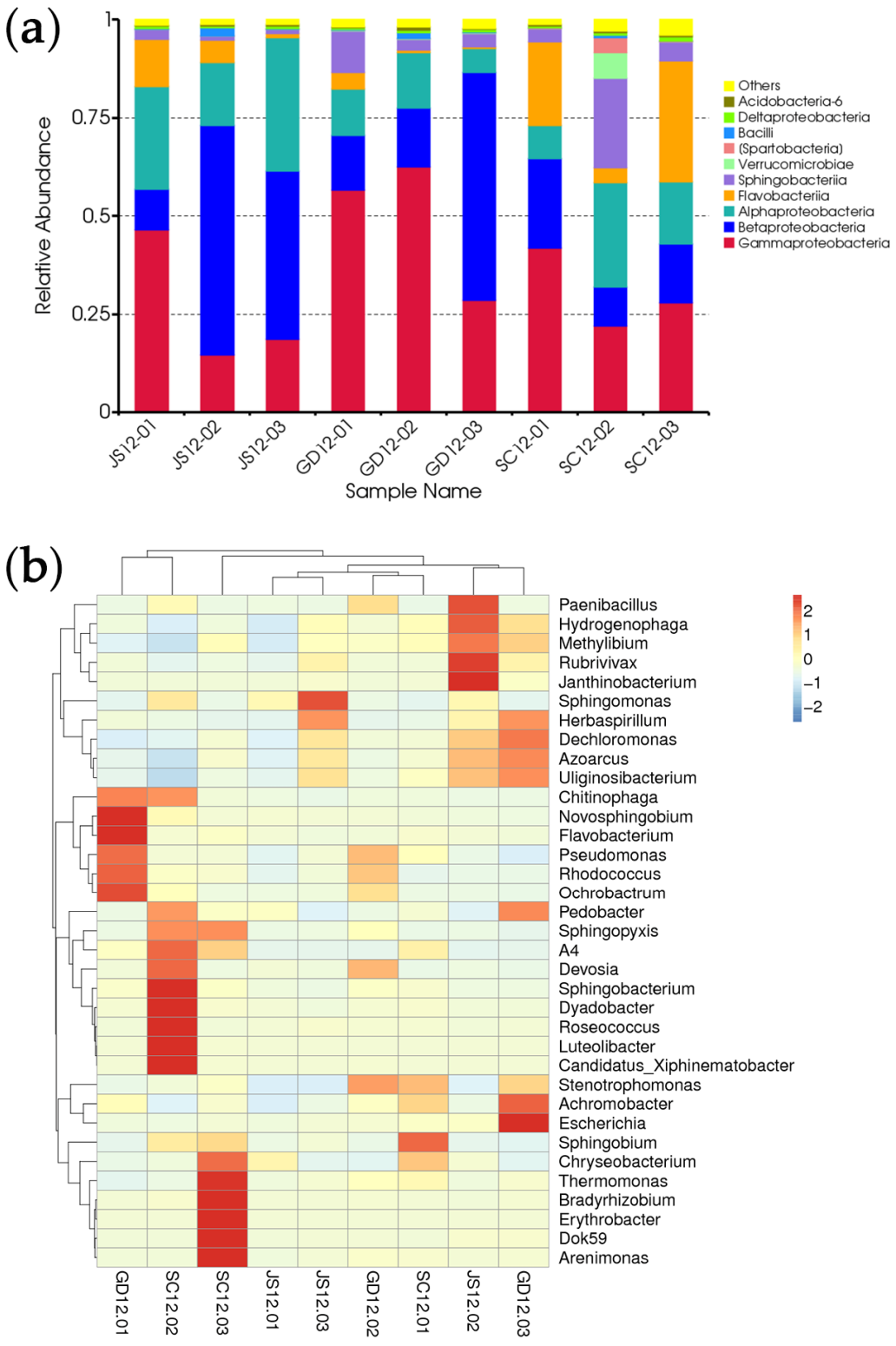

Figure 1. Diversity of endo-bacteria in the pine wood nematode (PWN) *Bursaphelenchus xylophilus* from different regions. Nine reference numbers represent nine PWN isolates from different regions. (**a**) Composition at the class level of the endo-bacterial communities in different PWN isolates. (**b**) Heatmap cluster at the genus level. The range of 2 to −2 indicates the relative abundance level.

3.2. Culturable Endo-Bacteria in PWNs from Different Regions

To further investigate the function of endo-bacteria in PWNs from *P. massoniana* in different regions, we first isolated and cultured the bacteria from the surface-sterilised nematodes and obtained a total of 15 strains (labelled in Table 1). Then, we constructed a phylogenetic tree to identify these endo-bacteria through 16S rDNA sequence analysis (B.x NS1-15 GenBank accession numbers: MT199162-76). The resulting tree showed that the bacteria B.x NS1, B.x NS4, B.x NS5, B.x NS9, and B.x NS12 were clustered with *S. maltophilia*; B.x NS2 and B.x NS6 were clustered with *P. fluorescens*; B.x NS3 was clustered with *Kocuria palustris*; B.x NS7, B.x NS8, and B.x NS10 were clustered with *Microbacterium testaceum*; B.x NS11, B.x NS13, and B.x NS14 were clustered with *Rhizobium radiobacter*; and B.x NS15 was clustered with *Leifsonia aquatica* (Figure 2). We also identified these 15 strains using the Biolog Microbial Identification System and the results were consistent with the above, except that B.x NS5 was identified as *Stenotrophomonas rhizophila* (Table 1). The culturable endo-bacteria from the nine isolates were classified into six species of six genera.

Table 1. Biolog microbial identification of culturable endo-bacteria in different pine wood nematode isolates.

Bacterial Strains	PWN Isolates	Species	Probability (%)	Similarity	Distance
B.x NS1	JS12-01	*Stenotrophomonas maltophilia*	96.5	0.850	2.37
B.x NS2	JS12-01	*Pseudomonas fluorescens*	82.1	0.628	3.97
B.x NS3	JS12-02	*Kacuria palustris*	81.8	0.505	7.33
B.x NS4	JS12-02	*S. maltophilia*	97.4	0.707	4.72
B.x NS5	JS12-03	*S. rhizophila*	84.5	0.679	3.40
B.x NS6	JS12-03	*P. fluorescens*	93.2	0.750	2.56
B.x NS7	GD12-01	*Microbacterium testaceum*	82.5	0.581	3.24
B.x NS8	GD12-02	*M. testaceum*	89.4	0.671	3.14
B.x NS9	GD12-02	*S. maltophilia*	96.9	0.816	2.67
B.x NS10	GD12-03	*M. testaceum*	92.8	0.675	3.64
B.x NS11	SC12-01	*Rhizobium radiobacter*	91.9	0.773	2.99
B.x NS12	SC12-01	*S. maltophilia*	95.8	0.806	2.78
B.x NS13	SC12-02	*R. radiobacter*	93.3	0.749	3.29
B.x NS14	SC12-03	*R. radiobacter*	95.8	0.806	2.78
B.x NS15	SC12-03	*Leifsonia aquatica*	96.1	0.737	3.94

3.3. Effects of Endo-Bacteria on PWN Fecundity

Reproduction is the key to successful infection and colonisation by PWN. Therefore, we examined the effects of the endo-bacteria *P. fluorescens* and *S. maltophilia* isolated from JS12-01 on the fecundity of JS12-01. The results showed that the number of eggs laid by the sterile PWNs (control [CK]) was 1100 per 100. Following treatment with *P. fluorescens*, the spawning capacity of the nematodes increased by 29.1%, but treatment with *S. maltophilia* did not significantly change the fecundity of the nematodes (Figure 3). These results indicate that the endo-bacterium *P. fluorescens* contributes to the reproduction of PWN, while *S. maltophilia* has little effect on nematode spawning.

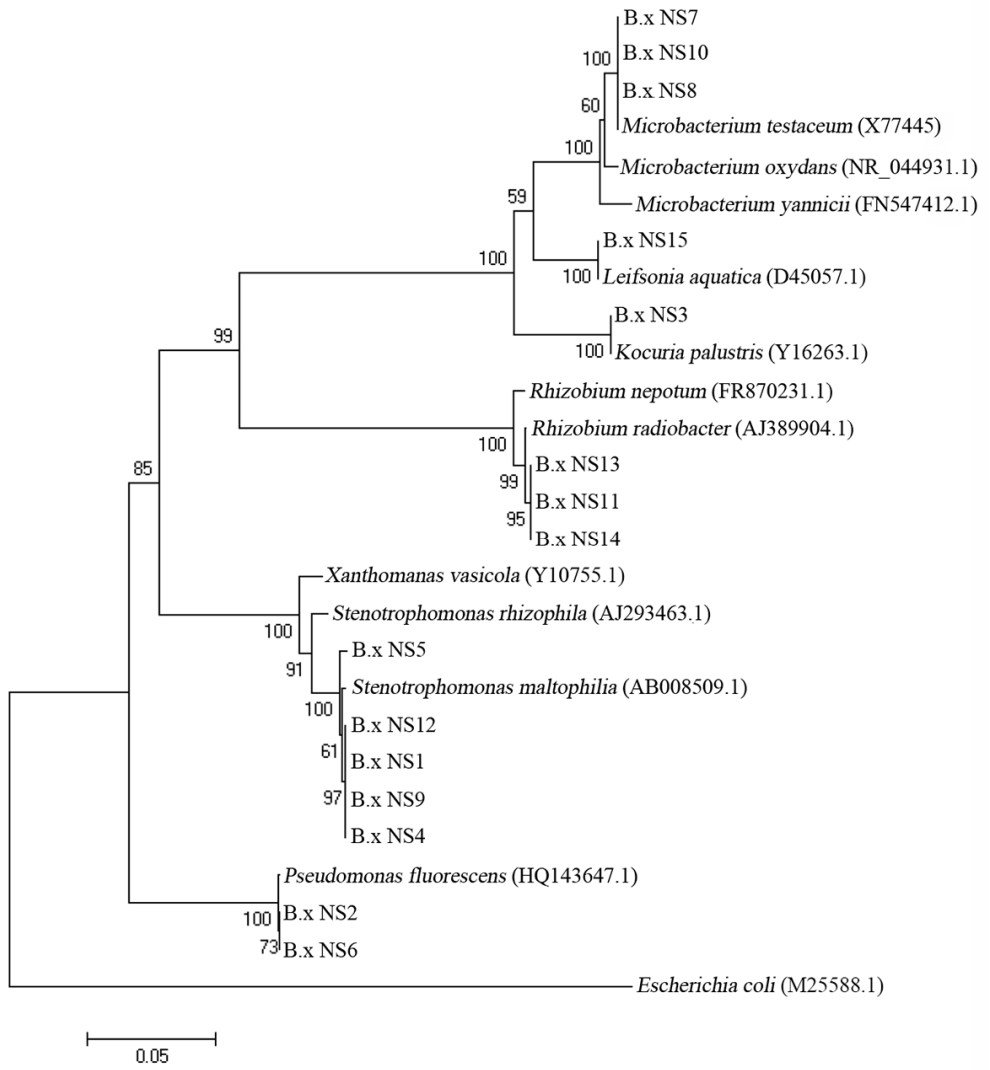

Figure 2. Phylogenetic tree of culturable endo-bacteria in different pine wood nematode isolates based on 16S rDNA. Support values of bootstrap > 50% are indicated at nodes.

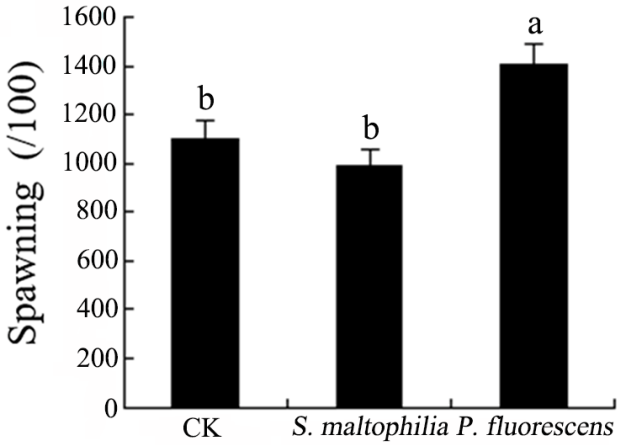

Figure 3. Effects of the endo-bacteria *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia* on the spawning of pine wood nematode (PWN). Sterile PWN was used as the control (CK). Data are the means ± standard deviation, and different letters above the bars indicate significant differences ($p < 0.05$) according to Tukey's HSD test.

3.4. Endo-Bacteria Enhance the Mobility of PWN under Oxidative Stress

PWN is subjected to oxidative stress during the invasion [15,16]. To study whether endo-bacteria are involved in the resistance of the nematode to oxidative stress, we next examined the mobility of nematodes under oxidative stress conditions following treatment with two endo-bacteria. Compared with the sterile nematode (CK), treatment with *P. fluorescens* increased the head swing frequency and body bending frequency of PWN stressed with 15 mM H_2O_2 by 175% and 119%, respectively, and treatment with *S. maltophilia* enhanced these two indicators by 113% and 71.9%, respectively (Figure 4). This reveals that the endo-bacteria *P. fluorescens* and *S. maltophilia* can promote the mobility of PWN under oxidative stress, and that the effect of *P. fluorescens* is greater.

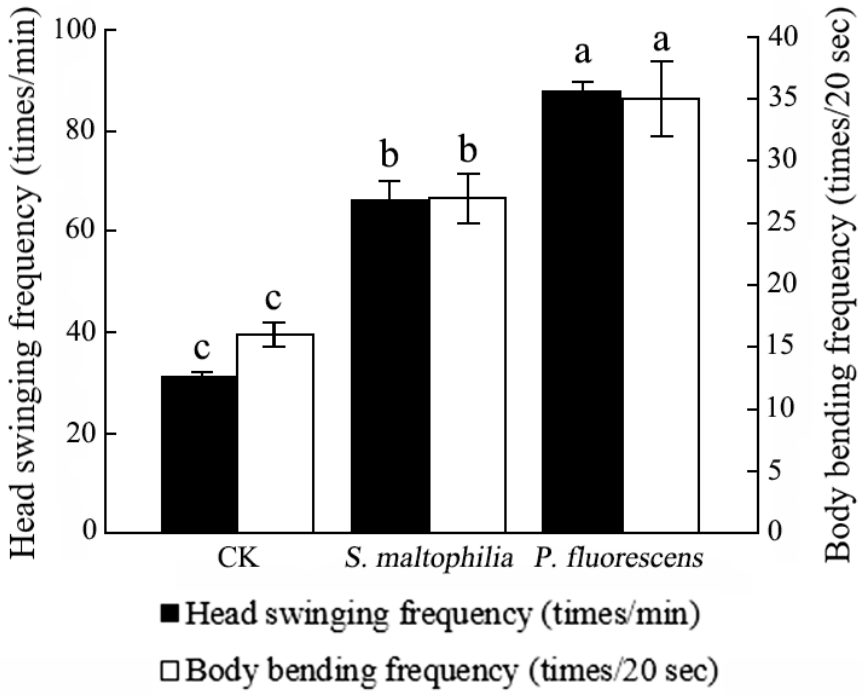

Figure 4. Endo-bacteria *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia* enhanced the mobility of pine wood nematode (PWN) under oxidative stress. The body-bending frequency and head-swinging frequency of PWN were measured and sterile PWN was used as the control (CK). Data are the means ± standard deviation, and different letters above the bars indicate significant differences ($p < 0.05$) among the three treatments according to Tukey's HSD test.

3.5. Endo-Bacteria Increase the Activity of Antioxidant Enzymes in PWN to Reduce ROS Content

To further determine the role of endo-bacteria in the antioxidant activities of PWN, we measured the ROS content and activity of three major antioxidant enzymes in PWN under oxidative stress following treatment with endo-bacteria. Compared with the control sterile nematode (CK), both endo-bacteria *P. fluorescens* and *S. maltophilia* markedly reduced the ROS content of nematodes treated with 15 mM H_2O_2 by 73.5% and 50.9%, respectively (Figure 5a). Furthermore, following treatment with *P. fluorescens* or *S. maltophilia*, the activities of the antioxidant enzymes SOD, CAT, and GSH-Px in PWN stressed by H_2O_2 were significantly enhanced, especially that of GSH-Px, and *P. fluorescens* had a stronger effect than *S. maltophilia* (Figure 5b). These results prove that these two endo-bacteria can enhance the antioxidant capacity of PWN by increasing the activity of antioxidant enzymes to reduce ROS content.

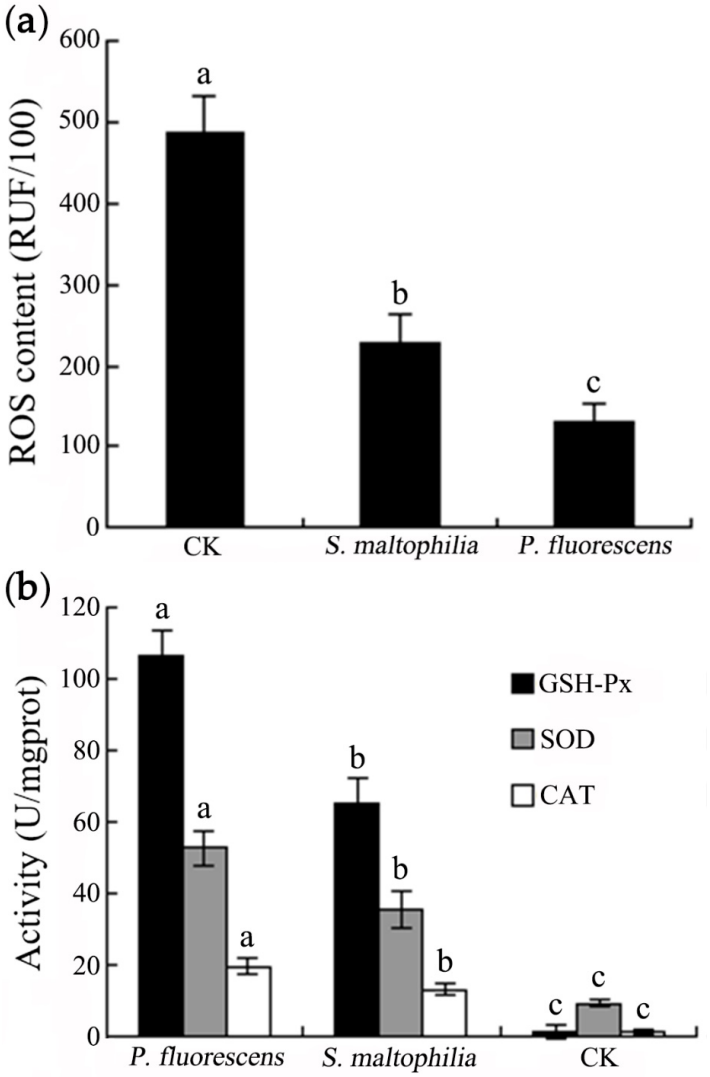

Figure 5. Endo-bacteria *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia* increased the activity of antioxidant enzymes in pine wood nematode (PWN) to reduce reactive oxygen species (ROS) content. (**a**) ROS content in PWN under oxidative stress; (**b**) Activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) in PWN under oxidative stress. Sterile PWN was used as the control (CK). Data are the means ± standard deviation, and different letters above the bars for each enzyme determination indicate significant differences ($p < 0.05$) among the three treatments according to Tukey's HSD test.

3.6. Endo-Bacteria Contribute to PWN Virulence

We finally examined the relationship between endo-bacteria and the pathogenicity of PWN. After inoculating the nematodes into two-year-old *P. massoniana* seedlings, we photographed the development of PWD and calculated the morbidity rate and DSI of the seedlings. The blank controls remained healthy while treatment with *P. fluorescens* or *S. maltophilia* significantly promoted symptom development and increased disease severity compared to the negative control sterile nematodes (Figure 6). On day 10 after inoculation, the pine trees inoculated with positive control wild-type JS12-01 (WT) and *P. fluorescens*-treated nematodes had begun to develop symptoms, while those inoculated with *S. maltophilia*-treated and sterile nematodes had a morbidity rate of zero (Figure 6a,b). By day 13, the pines inoculated with *S. maltophilia*-treated nematodes began to show symptoms, while those inoculated with sterile nematodes maintained a morbidity rate of zero (Figure 6a,b). At day 18, the pines inoculated with sterile nematodes began to develop symptoms (Figure 6a), but the morbidity rate was

much lower than those treated with endo-bacteria; the DSI was only 0.14- and 0.16-fold, respectively, of those in the two treatments (Figure 6b,c). The pines inoculated with WT, *P. fluorescens*-treated, *S. maltophilia*-treated, and sterile nematodes began to die on day 21, 21, 26, and 31, respectively. Therefore, both endo-bacteria play an important role in the pathogenesis of PWN, and *P. fluorescens* contributes more to the virulence than *S. maltophilia*.

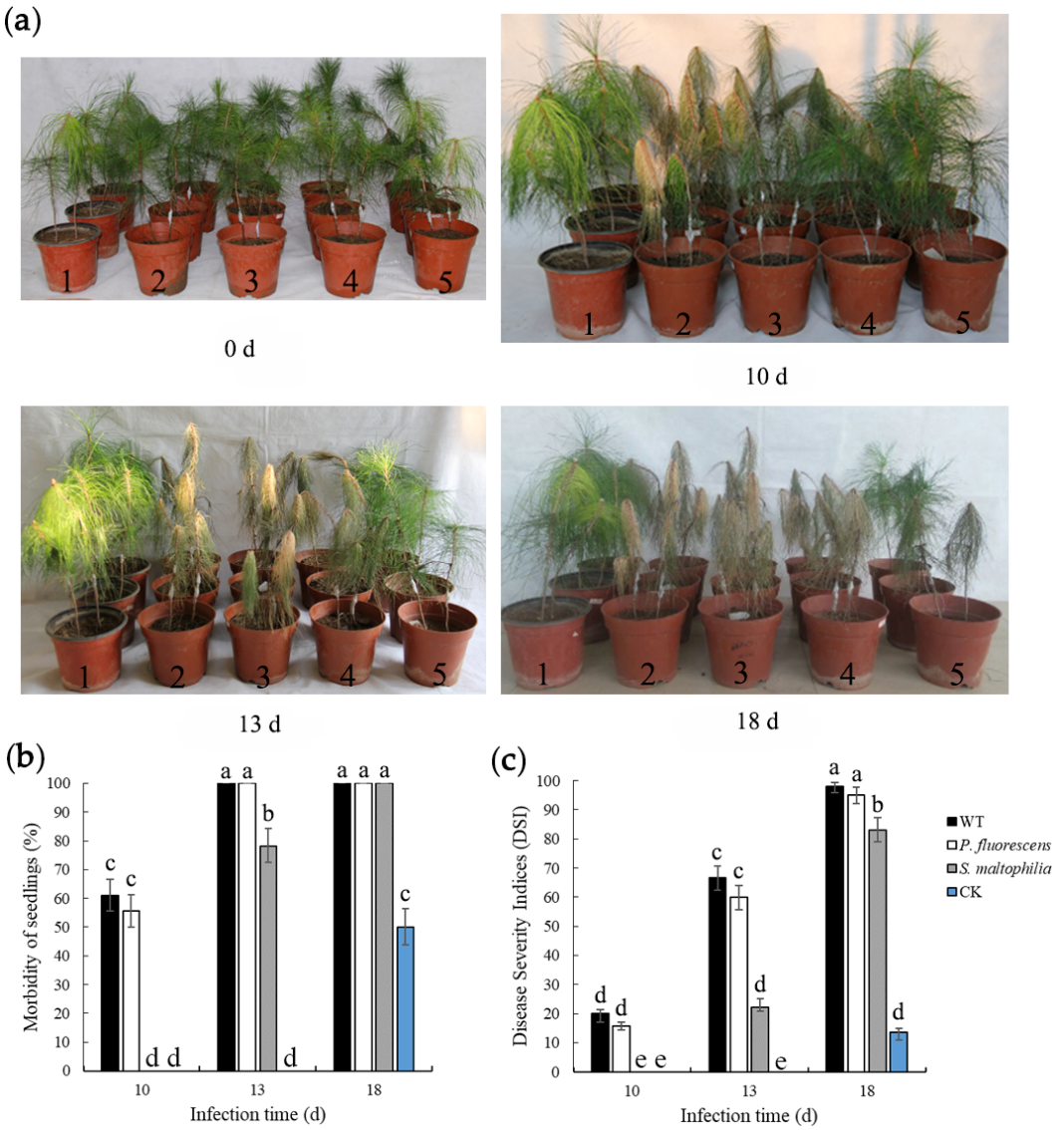

Figure 6. Endo-bacteria *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia* contribute to the virulence of pine wood nematode (PWN). (**a**) Effects of *P. fluorescens* and *S. maltophilia* on the development of PWD. Representative photographs of inoculation assays are presented. 1: *Pinus massoniana* Lamb. seedlings inoculated with *P. fluorescens* and *S. maltophilia* as the blank control. 2: Seedlings inoculated with wild-type (WT) PWN as the positive control. 3: Seedlings inoculated with PWN treated with *P. fluorescens*. 4: Seedlings inoculated with PWN treated with *S. maltophilia*. 5: Seedlings inoculated with sterile PWN as the negative control (CK). (**b**) Morbidity of *P. massoniana* seedlings. (**c**) Disease severity index (DSI) of *P. massoniana* seedlings. Data are the means ± standard deviation, and different letters above the bars indicate significant differences ($p < 0.05$) among all treatments according to Tukey's HSD test.

4. Discussion

In recent years, bacteria carried by PWN have been found to be closely related to the development of PWD [23,24], but most studies have focused on bacteria isolated from the surface of PWN. Endo-bacteria play an important role in the physiological and pathological processes of many animals and plants [47]. However, the flora characteristics of endo-bacteria in PWNs from different regions and the functions of endo-bacteria in PWN have not yet been elucidated. Therefore, we investigated the community structure of endo-bacteria in PWN from infested *P. massoniana* in different epidemic areas and explored some of the roles of endo-bacteria in PWN.

First, we obtained nine PWN isolates from nine regions in three provinces of China and performed high-throughput sequencing analysis of endo-bacteria in these nine isolates after sterilisation on the surface of the nematodes. We found that the number of endo-bacteria species in PWN from the three provinces differed and the number of species of endo-bacteria in PWNs from Jiangsu was the greatest. A study by Xiang et al. showed that endo-bacteria in PWN with higher virulence are more abundant [35]. We speculate that PWNs from Jiangsu have higher virulence and ecological adaptability. Surprisingly, the dominant endo-bacteria in PWNs from different regions did not show differences in taxonomic status at the level of class and genus. The classes Flavobacteriia, Sphingobacteriia, and Verrucomicrobiae have not been reported in previous studies. *Pseudomonas* and *Stenotrophomonas* were highly abundant in all isolates. These two genera were also highly abundant in virulent *Bursaphelenchus mucronatus* [35]. Furthermore, *Pseudomonas* carried by PWN has been suggested to be associated with nematode virulence [48], and *Stenotrophomonas* can degrade the pine's main defence substance α-pinene and regulate the expression of pathogenesis-related genes in PWN to affect PWN virulence [23,49]. Interestingly, the proportions of the dominant endo-bacteria genera in PWNs from different regions were different. Endo-bacteria in PWNs from different pine species also exhibited similar diversity [36]. The differences in the predominant flora of PWN endo-bacteria may be caused by the different geographical environment of the host pine tree.

We then successfully isolated and cultivated 15 endo-bacteria strains from nine PWN isolates. Using 16S rDNA sequence analysis, these endo-bacteria were identified as six species of six different genera (*S. maltophilia*, *P. fluorescens*, *K. palustris*, *M. testaceum*, *R. radiobacter*, and *L. aquatica*) and the results of the Biolog microbial identification were largely confirmatory. *S. maltophilia* was most frequently isolated; Wu et al. also found that *S. maltophilia* was the dominant species of culturable endo-bacteria in PWN with different virulence [32]. *S. maltophilia* is generally found in close association with plants and can produce glucanase [50,51]. Glucanase has been reported to degrade the cell wall of parenchymatous cells in plants [52]. *S. maltophilia* was also reported to be a dominant bacterial species in gut flora of *Ips pini* and promotes host intestinal absorption [53]. *Microbacterium* and *Rhizobium* were not annotated at the genus level in high-throughput sequencing analysis, possibly due to a low number of OTUs. For further functional analysis of PWN endo-bacteria, we selected the endo-bacteria *P. fluorescens* and *S. maltophilia*, isolated from JS12-01, as the research subject. Fecundity is necessary for PWN to cause wilting and death of pine trees and PWN virulence has been reported to be positively correlated with its reproductive ability in trees [54]. Oviposition number directly reflects reproductive capacity. Therefore, we first tested the effects of *P. fluorescens* and *S. maltophilia* on the spawning of sterile PWN JS12-01; *P. fluorescens* significantly enhanced PWN fecundity compared to sterile nematodes but *S. maltophilia* did not. Different endo-bacteria have different effects on the reproduction of PWN. Zhao and Lin also found that *P. fluorescens* GcM5-1A and *P. putida* ZpB1-2A isolated from the cuticle of PWN promoted the reproduction of PWN, while *Pantoea* sp. ZM2C inhibited reproduction [55]. Meanwhile, an endosymbiotic bacterium of *Wolbachia* isolated from another plant-parasitic nematode *Radopholus similis* was found to be related to the development and reproduction of the host [56].

Plants can produce ROS to directly destroy pathogens and modulate defence responses against pathogens [57]. Upon PWN infection, pine trees generate an ROS burst as a protection reaction and H_2O_2 is the most abundant ROS in pines [15,16,57]. Thus, tolerance to oxidative stress is a key characteristic of PWN. In our study, we found that the endo-bacteria *P. fluorescens* and *S. maltophilia* can

significantly improve the mobility of PWN under oxidative stress. Vicente et al. showed that *Serratia* spp. LCN-4, *S. proteamaculans* LCN-16, and *S. marcescens* PWN-146 isolated from the surface of PWN could also promote the survival of PWN under strong oxidative stress conditions [58]. Further exploration of the mechanism underlying the ability of endo-bacteria to enhance the antioxidant capacity of PWN revealed that *P. fluorescens* and *S. maltophilia* reduced ROS content by significantly increasing the activities of three main antioxidant enzymes SOD, CAT, and GSH-Px in nematodes. We also found that *P. fluorescens* contributed to the antioxidant capacity of PWN to a greater extent than *S. maltophilia*. Cheng et al. found that PWN-associated bacteria could help the nematode degrade toxic substances [49]. These results suggest that a long-term interaction between PWN and bacteria has evolved a more powerful detoxification system by increasing the expression of detoxification-related enzymes. However, the molecular mechanisms underlying the beneficial and potential beneficial effects of bacteria on PWN require further elucidation.

Finally, we evaluated the contribution of endo-bacteria to PWN virulence. Pine trees inoculated with *P. fluorescens* and *S. maltophilia* remained healthy, although the flagellin of *P. fluorescens* has been reported to destroy suspension cells of *Pinus thunbergii* [59]. We also found that the sterile nematode was pathogenic to pines, which is consistent with previous studies [60,61]. After treating sterile nematodes with *P. fluorescens* and *S. maltophilia* and inoculating the nematodes into pine trees, the symptoms of pine trees appeared significantly earlier, and the morbidity rate and DSI increased greatly. Our results indicate that the endo-bacteria *P. fluorescens* and *S. maltophilia* could enhance the virulence of PWN and that *P. fluorescens* has a greater effect. Tian et al. also found that the endobacterial strain *S. maltophilia* NSBx.14 had a positive effect on the virulence of PWN with low virulence [33]. Some bacteria isolated from the surface of PWN have also been reported to be closely related to the pathogenicity of PWN and development of PWD [23,24]. Further exploration of the molecular mechanism of the interaction between PWN-associated bacteria and nematodes will be important to clarify the pathogenesis of PWD.

5. Conclusions

This study demonstrated the diversity of endo-bacteria in PWNs isolated from *P. massoniana* in different regions and analyzed the functions of two culturable endo-bacteria strains of *P. fluorescens* and *S. maltophilia*. We found that *P. fluorescens*, but not *S. maltophilia*, could promote PWN fecundity, and confirmed that both strains could enhance the antioxidant capacity of PWN by increasing the activity of nematode antioxidant enzymes and had a strong positive effect on PWN virulence. Moreover, *P. fluorescens* had a greater effect than *S. maltophilia*. These results suggest that some endo-bacteria in PWN improve nematode reproduction and resistance to oxidative stress to accelerate the development of PWD. Our findings help clarify the pathogenic mechanism of PWN and deepen our understanding of the interaction between endosymbiotic bacteria and their nematode hosts.

Author Contributions: Conceptualization, Y.-M.F. and H.-B.L.; methodology, Y.-M.F.; software, Y.-M.F.; validation, Y.-M.F., H.-B.L. and X.-Q.W.; formal analysis, H.-B.L.; investigation, Y.-M.F.; resources, X.-Q.W.; data curation, Y.-M.F. and H.-B.L.; writing—original draft preparation, H.-B.L.; writing—review and editing, H.-B.L.; visualization, H.-B.L.; supervision, X.-Q.W.; project administration, X.-Q.W.; funding acquisition, X.-Q.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Research and Development Program of China (No. 2018YFD0600203), the National Natural Science Foundation of China (NO. 31270683), and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mamiya, Y. Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus*. *Ann. Rev. Phytopathol.* **1983**, *21*, 201–220. [CrossRef] [PubMed]
2. Burgermeister, W.; Braasch, H.; Sousa, E.; Penas, A.C.; Mota, M.; Metge, K.; Bravo, M.A. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* **1999**, *1*, 727–734. [CrossRef]
3. Braasch, H.; Tomiczek, C.; Metge, K.; Hoyer, U.; Burgermeister, W.; Wulfert, I.; Schonfeld, U. Records of *Bursaphelenchus* spp. (Nematoda, Parasitaphelenchidae) in coniferous timber imported from the Asian part of Russia. *Forest Pathol.* **2001**, *31*, 129–140. [CrossRef]
4. Inacio, M.L.; Nobrega, F.; Vieira, P.; Bonifacio, L.; Naves, P.; Sousa, E.; Mota, M. First detection of *Bursaphelenchus xylophilus* associated with *Pinus nigra* in Portugal and in Europe. *Forest Pathol.* **2015**, *45*, 235–238. [CrossRef]
5. Zamora, P.; Rodriguez, V.; Renedo, F.; Sanz, A.V.; Dominguez, J.C.; Perez-Escolar, G.; Miranda, J.; Álvarez, B.; González-Casas, A.; Mayor, E.; et al. First report of *Bursaphelenchus xylophilus* causing pine wilt disease on *Pinus radiata* in Spain. *Plant Dis.* **2015**, *99*, 1449. [CrossRef]
6. Yang, B.J.; Pan, H.Y.; Tang, J.; Wang, Y.Y.; Wang, L.F. *Pine Wood Nematode Disease*; Forestry Publishing House: Beijing, China, 2003; pp. 45–48.
7. Futai, K. Pine wood nematode, *Bursaphelenchus xylophilus*. *Annu. Rev. Phytopathol.* **2013**, *51*, 61–83. [CrossRef]
8. Kikuchi, T.; Cotton, J.A.; Dalzell, J.J.; Hasegawa, K.; Kanzaki, N.; McVeigh, P.; Takanashi, T.; Tsai, I.J.; Assefa, S.A.; Cock, P.J.; et al. Genomic insights into the origin of parasitism in the emerging plant pathogen *Bursaphelenchus xylophilus*. *PLoS Pathog.* **2011**, *7*, e1002219. [CrossRef] [PubMed]
9. Liu, Q.; Wei, Y.; Xu, L.; Hao, Y.; Chen, X.; Zhou, Z. Transcriptomic profiling reveals differentially expressed genes associated with pine wood nematode resistance in masson pine (*Pinus massoniana* Lamb.). *Sci. Rep.* **2017**, *7*, 1–14. [CrossRef]
10. Huang, L.; Wang, P.; Tian, M.Q.; Zhu, L.H.; Ye, J.R. Major sperm protein BxMSP10 is required for reproduction and egg hatching in *Bursaphelenchus xylophilus*. *Exp. Parasitol.* **2019**, *197*, 51–56. [CrossRef]
11. Liu, H.B.; Wu, F.; Wu, X.Q.; Ye, J.R. Differential effects of rapamycin on *Bursaphelenchus xylophilus* with different virulence and differential expression of autophagy genes under stresses in nematodes. *Acta Biochim. Biophys. Sin.* **2019**, *51*, 254–262. [CrossRef]
12. Liu, H.B.; Wu, X.Q.; Feng, Y.Q.; Rui, L. Autophagy contributes to the feeding, reproduction, and mobility of *Bursaphelenchus xylophilus* at low temperatures. *Acta Biochim. Biophys. Sin.* **2019**, *51*, 864–872. [CrossRef] [PubMed]
13. Liu, H.B.; Rui, L.; Feng, Y.Q.; Wu, X.Q. Molecular characterization and functional analysis of three autophagy genes, *BxATG5*, *BxATG9*, and *BxATG16*, in *Bursaphelenchus xylophilus*. *Int. J. Mol. Sci.* **2019**, *20*, 3769. [CrossRef] [PubMed]
14. Liu, H.B.; Rui, L.; Feng, Y.Q.; Wu, X.Q. Autophagy contributes to resistance to the oxidative stress induced by pine reactive oxygen species metabolism, promoting infection by *Bursaphelenchus xylophilus*. *Pest Manag. Sci.* **2020**. [CrossRef] [PubMed]
15. Hirao, T.; Fukatsu, E.; Watanabe, A. Characterization of resistance to pine wood nematode infection in *Pinus thunbergii* using suppression subtractive hybridization. *BMC Plant Biol.* **2012**, *12*, 13. [CrossRef] [PubMed]
16. Santos, C.S.S.; Vascocelos, M.W. Identification of genes differentially expressed in *Pinus pinaster* and *Pinus pinea* after infection with pine wood nematode. *Eur. J. Plant Pathol.* **2012**, *132*, 407–418. [CrossRef]
17. Shinya, R.; Morisaka, H.; Kikuchi, T.; Takeuchi, Y.; Ueda, M.; Futai, K. Secretome analysis of pine wood nematode *Bursaphelenchus xylophilus* reveals the tangled roots of parasitism and its potential for molecular mimicry. *PLoS ONE* **2012**, *8*, e67377. [CrossRef]
18. Zheng, H.Y.; Xu, M.; Xu, F.Y.; Ye, J.R. A comparative proteomics analysis of *Pinus massoniana* inoculated with *Bursaphelenchus xylophilus*. *Pak. J. Bot.* **2015**, *47*, 1271–1280.

19. Li, L.; Tan, J.; Chen, F. *Bacillus pumilus* strain LYMC-3 shows nematicidal activity against *Bursaphelenchus xylophilus* via the production of a guanidine compound. *Biocontrol. Sci. Technol.* **2018**, *28*, 1128–1139. [CrossRef]
20. Harry, K.K.; Aguillera, M.M.; Alumai, A.; Choo, H.Y.; de la Torre, M.; Fodor, A.; Ganguly, S.; Hazır, S.; Lakatos, T.; Pye, A.; et al. Status of entomopathogenic nematodes and their symbiotic bacteria from selected countries or regions of the world. *Biol. Control.* **2006**, *38*, 134–155.
21. Oku, H.; Shiraishi, T.; Kurozumi, S. Participation of toxin in wilting of Japanese pine caused by a nematode. *Naturwissenschaften* **1979**, *66*, 210. [CrossRef]
22. Oku, H.; Shiraishi, T.; Ouchi, S. Pine wilt toxin, the metabolite of a bacterium associated with a nematode. *Naturwissenschaften* **1980**, *67*, 198–199. [CrossRef]
23. Nascimento, F.X.; Hasegawa, K.; Mota, M.; Vicente, C.S. Bacterial role in pine wilt disease development–Review and future perspectives. *Environ. Microbiol. Rep.* **2015**, *7*, 51–63. [CrossRef] [PubMed]
24. Proença, D.N.; Grass, G.; Morais, P.V. Understanding pine wilt disease: Roles of the pine endo-bacteria and of the bacteria carried by the disease-causing pinewood nematode. *Microbiologyopen* **2017**, *6*, e00415. [CrossRef] [PubMed]
25. Zhao, B.G.; Li, L. Observation of wilting symptoms caused by cell free filtration of the culture in *Pseudomonas fluorescence*. *Acta Agric. Univ. Jiangxiensis.* **2008**, *30*, 575–580.
26. Kawazu, K.; Yamashita, H.; Kobayashi, A.; Kanzaki, H. Isolation of pine wilting bacteria accompanying pine wood nematode, *Bursaphelenchus xylophilus*, and their toxic metabolite. *Sci. Rep. Fac. Agri.-Okayama Univ.* **1998**, *87*, 1–7.
27. Zhao, B.G.; Guo, D.S. Isolation and pathogenicity of a bacterium strain carried by pine wood nematode. *J. Nanjing For. Univ.* **2004**, *26*, 57–60.
28. Proenca, D.N.; Francisco, R.; Santos, C.V.; Lopes, A.; Fonseca, L.; Abrantes, I.M.; Morais, P.V. Diversity of bacteria associated with *Bursaphelenchus xylophilus* and other nematodes isolated from *Pinus pinaster* trees with pine wilt disease. *PLoS ONE* **2010**, *5*, e15191. [CrossRef]
29. Vicente, C.S.L.; Nascimento, F.; Espada, M.; Mota, M.; Oliveira, S. Bacteria associated with the pinewood nematode *Bursaphelenchus xylophilus* collected in Portugal. *Anton. Leeuw.* **2011**, *100*, 477–481. [CrossRef] [PubMed]
30. Zhao, B.G.; Lin, F. Mutualistic symbiosis between *Bursaphelenchus xylophilus* and bacteria of the genus *Pseudomonas*. *For. Pathol.* **2005**, *35*, 339–345. [CrossRef]
31. Yuan, W.M.; Wu, X.; Ye, J.; Tian, X. Observation by transmission electron microscope and identification of endo-bacteria isolated from *Bursaphelenchus xylophilus* and *B. mucronatus*. *Acta Microbiol. Sin.* **2011**, *51*, 1071–1077.
32. Wu, X.Q.; Yuan, W.M.; Tian, X.J.; Fan, B.; Fang, X.; Ye, J.R.; Ding, X.L. Specific and functional diversity of endophytic bacteria from pine wood nematode *Bursaphelenchus xylophilus* with different virulence. *Int. J. Biol. Sci.* **2013**, *9*, 34–44. [CrossRef] [PubMed]
33. Tian, X.J.; Wu, X.Q.; Xiang, Y.; Fang, X.; Ye, J.R. The effect of endobacteria on the development and virulence of the pine wood nematode, *Bursaphelenchus xylophilus*. *Nematology* **2015**, *17*, 581–589. [CrossRef]
34. He, L.X.; Wu, X.Q.; Xue, Q.; Qiu, X.W. Effects of endobacterium (*Stenotrophomonas maltophilia*) on pathogenesis-related gene expression of pine wood nematode (*Bursaphelenchus xylophilus*) and pine wilt disease. *Int. J. Mol. Sci.* **2016**, *17*, 778. [CrossRef] [PubMed]
35. Xiang, Y.; Wu, X.Q.; Zhou, A.D. Bacterial diversity and community structure in the pine wood nematode *Bursaphelenchus xylophilus* and *B. mucronatus* with different virulence by high-throughput sequencing of the 16S rDNA. *PLoS ONE* **2015**, *10*, e0137386. [CrossRef] [PubMed]
36. Xue, Q.; Xiang, Y.; Wu, X.Q.; Li, M.J. Bacterial communities and virulence associated with pine wood nematode *Bursaphelenchus xylophilus* from Different *Pinus* spp. *Int. J. Mol. Sci.* **2019**, *20*, 3342. [CrossRef]
37. Viglierchio, D.R.; Schmitt, R.V. On the methodology of nematode extraction from field samples: Baermann funnel modifications. *J. Nematol.* **1983**, *15*, 438–444.

38. Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-Lyons, D.; Huntley, J.; Fierer, N.; Owens, S.M.; Betley, J.; Fraser, L.; Bauer, M.; et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* **2012**, *6*, 1621–1624. [CrossRef]
39. Magoc, T.; Salzberg, S.L. FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* **2011**, *27*, 2957–2963. [CrossRef]
40. Caporaso, J.G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F.D.; Costello, E.K.; Fierer, N.; Pena, A.G.; Goodrich, J.K.; Gordon, J.I.; et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **2010**, *7*, 335–336. [CrossRef]
41. Edgar, R.C. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* **2013**, *10*, 996–998. [CrossRef]
42. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [CrossRef] [PubMed]
43. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef] [PubMed]
44. Jinxia, C.C.Y.M.L.; Hairong, Y.S.H. Biolog microbial identification system–Study on the operating regulation of bacteria identification. *Food Ferment. Ind.* **2006**, *5*, 50–54.
45. Lu, W.W.; Cao, J.J.; Hu, Y.; Wang, F.; Yu, D.; Liu, A.L. Effect of methylmercury chloride on the movement and sensory function of *Caenorhabditis elegans*. *J. Env. Health* **2015**, *7*, 565–568.
46. Kampkötter, A.; Pielarski, T.; Rohrig, R.; Timpel, C.; Chovolou, Y.; Wätjen, W.; Kahl, R. The *Ginkgo biloba* extract EGb761 reduces stress sensitivity, ROS accumulation and expression of catalase and glutathione S-transferase 4 in *Caenorhabditis elegans*. *Pharmacol. Res.* **2007**, *55*, 139–147. [CrossRef] [PubMed]
47. Moran, N.A.; Wernegreen, J.J. Lifestyle evolution in symbiotic bacteria: Insights from genomics. *Trends Ecol. Evol.* **2000**, *15*, 321–326. [CrossRef]
48. Han, Z.M.; Hong, Y.D.; Zhao, B.G. A study on pathogenicity of bacteria carried by pinewood nematode. *J. Phytopathol.* **2003**, *151*, 683–689. [CrossRef]
49. Cheng, X.Y.; Tian, X.L.; Wang, Y.S.; Lin, R.M.; Mao, Z.C.; Chen, N.; Xie, B.Y. Metagenomic analysis of the pinewood nematode microbiome reveals a symbiotic relationship critical for xenobiotics degradation. *Sci. Rep.* **2013**, *3*, 1869. [CrossRef] [PubMed]
50. Ryan, R.P.; Monchy, S.; Cardinale, M.; Taghavi, S.; Crossman, L.; Avison, M.B.; Berg, G.; Van Der Lelie, D.; Dow, J.M. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. *Nat. Rev. Microbiol.* **2009**, *7*, 514–525. [CrossRef]
51. Zhang, Z.; Yuen, G.Y. Effects of culture fluids and preinduction of chitinase production on biocontrol of bipolaris leaf spot by *Stenotrophomonas maltophilia* C3. *Biol. Control.* **2000**, *18*, 277–286. [CrossRef]
52. Ma, H.B.; Lu, Q.; Liang, J.; Zhang, X.Y. Functional analysis of the cellulose gene of the pine wood nematode, *Bursaphelenchus xylophilus*, using RNA interference. *Genet. Mol. Res.* **2011**, *10*, 1931–1941. [CrossRef] [PubMed]
53. Delalibera, I.; Vasanthakumar, A.; Klepzig, K.D.; Raffa, K.F. Composition of the bacterial community in the gut of the pine engraver, *Ips pini* (Say) (Coloptera) colonizing red pine. *Symbiosis* **2007**, *43*, 97–104.
54. Aikawa, T.; Kikuchi, T. Estimation of virulence of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) based on its reproductive ability. *Nematology* **2007**, *9*, 371–377.
55. Zhao, B.G.; Lin, F. Mutual influences between *Bursaphelenchus xylophilus* and bacteria it carries. *J. Nanjing For. Univ.* **2005**, *29*, 1–4.
56. Haegeman, A.; Vanholme, B.; Jacob, J.; Vandekerckhove, T.T.; Claeys, M.; Borgonie, G.; Gheysen, G. An endosymbiotic bacterium in a plant-parasitic nematode: Member of a new *Wolbachia supergroup*. *Int. J. Parasitol.* **2009**, *39*, 1045–1054. [CrossRef]
57. Vellosillo, T.; Vicente, J.; Kulasekaran, S.; Hamberg, M.; Castresana, C. Emerging complexity in reactive oxygen species production and signaling during the response of plants to pathogens. *Plant Physiol.* **2010**, *154*, 444–448. [CrossRef] [PubMed]
58. Vicente, C.S.; Ikuyo, Y.; Mota, M.; Hasegawa, K. Pine wood nematode-associated bacteria contribute to oxidative stress resistance of *Bursaphelenchus xylophilus*. *BMC Microbiol.* **2013**, *13*, 1–8. [CrossRef] [PubMed]

59. Xu, Z.; Yu, J.; Cui, L.; Li, M.; Li, R.; Guo, D. Effects of *Pseudomonas fluorescens* flagellin on physiological and biochemical characteristics in the suspension cells of *Pinus thunbergii*. *Eur. J. Plant Pathol.* **2013**, *136*, 729–736. [CrossRef]
60. Tamura, H. Pathogenicity of aseptic *Bursaphelenchus xylophilus* and associated bacteria to pine seedlings. *Jpn. J. Nematol.* **1983**, *13*, 1–5.
61. Zhu, L.H.; Ye, J.R.; Negi, S.; Xu, X.L.; Wang, Z.L.; Ji, J.Y. Pathogenicity of aseptic *Bursaphelenchus xylophilus*. *PLoS ONE* **2012**, *7*, e38095. [CrossRef] [PubMed]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).