

## Article

# The Biocontrol Strain *Bacillus subtilis* Z-294 Alleviates Pepper *Phytophthora capsici* Disease Associated with Microbial Community Structure

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## Abstract

The soil-borne oomycete pathogen *Phytophthora capsici* poses a serious threat to plant growth and results in substantial economic impact on the pepper industry. Therefore, the isolation of multifunctional biocontrol agents has become a major research priority. In this study, we isolated a biocontrol strain, *Bacillus subtilis* Z-294, which demonstrated strong inhibition of *P. capsici* in both plate and greenhouse experiments. This strain Z-294 exhibited broad-spectrum antagonistic activities as well as plant growth-promoting ability. Phylogenetic analysis based on core-genome and average nucleotide identity (ANI) further identified Z-294 as *B. subtilis*. Importantly, we found that the strain Z-294 alleviates the *P. capsici* disease through mechanisms associated with changes in the plant microbiome. Plants treated with Z-294 showed higher alpha-diversity. The application of *B. subtilis* Z-294 showed that the relative abundance of Alphaproteobacteria, Bacilli, Bacteroidia, Agaricomycetes, and Eurotiomycetes increased, while the relative abundance of Gammaproteobacteria and Sordariomycetes declined. Strain Z-294 holds great promise as a multifunctional biocontrol agent for the sustainable management of diseases and the enhancement of plant health, laying a solid foundation for its future agricultural applications.

**Keywords:** *Bacillus*; plant microbiome; biocontrol; *Phytophthora capsici*



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## 1. Background

Pathogenic microorganisms pose a significant and persistent threat to global food production and ecosystem stability by infecting plant tissues and compromising plant health [1]. With the rise in the human population and the intensification of agricultural practices in recent decades, farmers have increasingly relied on agrochemicals to manage plant diseases [2,3]. However, the overuse of chemical pesticides has resulted in significant detrimental effects on the ecological environment [3]. Consequently, both researchers and farmers are recognizing the necessity for alternatives to chemical pesticides in plant protection, which are steadily increasing and beginning to supplant some chemical pesticides. Biological control is regarded as a sustainable alternative for managing pests and plant pathogens.

Pepper is a significant vegetable crop cultivated globally for both fresh consumption and processing [4]. The soil-borne oomycete pathogen *P. capsici* represents one of the most destructive diseases, potentially leading to yield reductions of up to 100% under hot and humid conditions [5]. *P. capsici* can infect pepper plants at nearly every growth and development stage. Additionally, it affects other crops, such as tomatoes, eggplants, cucumbers, and watermelons [6]. The oospores can persist in the soil for several years without a susceptible host and can initiate infection when a host plant becomes available. The lack of a variety of a range of resistant pepper cultivars, combined with the emergence of new *Phytophthora* strains, complicates disease management [7]. Widely used biocontrol agents include *Bacillus*, *Pseudomonas*, and *Streptomyces*, along with the fungal genera *Trichoderma* and *Penicillium* [8,9]. *Bacillus* species can produce antibiotics that inhibit plant pathogens and play a crucial role in disease management by generating extracellular proteins that trigger plant defense mechanisms and enhance resistance to *P. capsici* [10,11]. Therefore, isolating *Bacillus* strains for the control of *P. capsici* presents substantial economic potential for the development of eco-friendly pesticides.

The microbiome of plants plays a crucial role in determining their health and productivity [12]. Additionally, manipulating the plant microbiome has the potential to reduce occurrences of plant diseases, enhance agricultural yield, and decrease chemical inputs, leading to more sustainable agricultural practices [13,14]. For instance, healthy wine grapevines can attract beneficial bacteria such as *Pseudomonas*, *Bacillus*, and *Streptomyces*, in contrast to grapevines affected by root rot disease [15]. Numerous recent investigations have demonstrated that biocontrol agents can influence the plant microbiome to promote disease suppression. The biocontrol agent *B. velezensis* has been shown to inhibit black spot infection in edible roses by altering the microbial community in the rhizosphere [16]. Results indicated a significant enrichment of two specific *Flavobacterium* amplicon sequence variants following *B. velezensis* BER1 inoculation, as observed in studies utilizing tomato plants, *Ralstonia solanacearum*, and *B. velezensis* to examine biocontrol agent-induced shifts in the microbiome [17]. Furthermore, applying the biopesticide wuyiencin and two chemical pesticides, azoxystrobin and carbendazim, to tomato plants induced varying degrees of change in the bacterial community structure. The most notable alterations were observed with the biopesticide, which led to an increase in *Streptomyces* within the microbiome [18]. Moreover, various studies have illustrated that *Bacillus* biocontrol agents can modify the microbiome. The inoculation of bio-organic fertilizer containing *B. amyloliquefaciens* W19, a highly effective antagonist against banana wilt, notably influenced the resident soil microbial communities, particularly *Pseudomonas* [19]. Nonetheless, it remains uncertain how the relationship between biocontrol strain Z-294 and the microbial community alleviates the *P. capsici* disease.

In the present study, we isolated from soil a biocontrol strain, *B. subtilis* Z-294, which exhibited stronger inhibition of *P. capsici* in both plate and greenhouse experiments. The objectives of this study were: (1) to examine the biocontrol ability of strain Z-294; (2) to conduct the phylogenetic analysis for identification; and (3) to explore the relationship between *B. subtilis* Z-294 and the microbial community in alleviating the *P. capsici* disease. Our results demonstrated that Z-294 exhibited broad-spectrum antagonistic activity and plant growth-promoting traits. The isolate was identified as *B. subtilis* based on the genome sequence. We found that the strain Z-294 alleviates the *P. capsici* disease through mechanisms associated with changes in the plant's microbiome.

## 2. Materials and Methods

### 2.1. Strain Used in This Study and Growth Conditions

The biocontrol strain Z-294 was routinely cultured at 37 °C in Luria–Bertani broth (LB, 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) or solid LB containing 1.5% agar. The College of Plant Protection at China Agricultural University stored all pathogens utilized in this study. The fungal strains were cultured on potato dextrose agar medium (PDA, 200 g potato, 20 g glucose, 15 g agar per liter) for 5–7 days at 28 °C. The fungal strain was kept in paraffin at 4 °C.

### 2.2. Antagonistic Activity of the Strain Z-294

The plate confrontation assay was performed following the method given [20]. On PDA, the plant pathogens, including *Botrytis conerea*, *Trichothecium roseum*, *Fusarium oxysporum*, *Coniothyrium diplodiella*, *Alternaria alternata*, *Cryptosporella viticola*, *Colletotrichum* sp., *Botryosphaeria* sp., and *Rhizoctonia cerealis*, were activated at 28 °C for 5–7 d. Subsequently, a pathogen plug of about 5 mm in diameter was taken and put in the center of the plate. Subsequently, 2.5 µL of Z-294 strain culture was spotted on the PDA plate, at 2.5 cm from the pathogen plug. The control consisted of plates inoculated with the pathogen alone. The examination of antifungal activity was done by measuring and comparing the mycelial radial growth of the pathogen in treatment and control groups after 5–7 d. All antagonistic activity for each pathogen were performed in triplicate. We performed three independent biological replicates.

### 2.3. The Biocontrol Efficacy of Z-294 Under Greenhouse Conditions

Pepper seeds of cultivar ‘Qicunhong’ were soaked in tap water for 40 min, surface-sterilized for 3 min with 2% sodium hypochlorite and for 3 min with 70% ethanol. The seeds were rinsed with sterile water at least three times after sterilization. For the Z-294-treated group, seeds that had been sterilized were soaked in a suspension of strain Z-294 ( $1 \times 10^8$  CFU/mL) for 3 h. Thereafter, the seeds were placed on moist sterile gauzes in Petri dishes and incubated in the dark at 37 °C for 4 to 6 days to enhance germination. Then, the germinated seeds were put in cultivation substrate, which composed of peat, vermiculite, and nutrient soil at a ratio of 1:1:1 (v/v/v). All pepper plants were cultivated in a growth chamber maintained at 25 °C with 70% relative humidity under a 16 h light/8 h dark photoperiod. Sterile water was added to the plants at three-day intervals.

Mycelial plugs of *P. capsici* were placed at the center of Carrot Agar (CA) plates. The plates were kept in the dark at a temperature of 25 °C for 4 d and in light at this temperature for 5 d. To stimulate the release of zoospores, 15 mL of sterile distilled water was added to each plate. Subsequently, the plates were chilled at 4 °C for 30 min and then incubated at room temperature for 30 min. The concentration of zoospores was quantified using a hemocytometer and adjusted to  $1 \times 10^4$  zoospores/mL with sterile distilled water.

For the growth-promotion experiment, pepper plants were allocated into two treatment groups. At 25 days post-sowing, 15 mL of the biocontrol strain suspension ( $1 \times 10^8$  CFU/mL) was applied to the root zone of each pepper plant, with sterile water serving as the control. The experiment was conducted with three independent biological replicates per treatment, and each replicate consisted of three plots, each containing 15 pepper plants. Growth parameters were subsequently measured at 45 days post-inoculation.

The 1 mL overnight suspension of strain Z-294 was inoculated into 100 mL of LB broth at 37 °C with shaking at 200 rpm for 72 h. Then, the culture was diluted with sterile distilled water to adjust the final bacterial concentration to  $1 \times 10^8$  CFU/mL. Subsequently, each pot (containing approximately 340 g of substrate mixed with peat, vermiculite, and nutrient soil

at 1:1:1 (*v/v/v*) and no fertilizer was added) was subjected to two rounds of root irrigation, each with 15 mL of the Z-294 suspension, while the control group received an equal volume of LB medium each time, after 35 days after planting. After 24 h of biocontrol treatment, 3 mL of *P. capsici* zoospore suspension ( $1 \times 10^4$  zoospores/mL) was introduced to the soil near the roots. Each treatment comprised three replicates in a completely randomized design, each containing three pots. For each treatment, ten healthy and uniform pepper plants were selected for each pot. Then, it was cultivated in a growth chamber under controlled conditions (12 h light/12 h dark, 28 °C) for 40 days. Irrigation was performed every three days using sterile water. Disease severity was assessed using a six-grade scale based on the following criteria: Grade 0, no visible symptoms; Grade 1, slight water-soaked browning on the root or stem base with no wilting or only recoverable wilting; Grade 2, water-soaked browning with a lesion length of 1–2 cm, irreversible leaf wilting, and occasional defoliation of lower leaves; Grade 3, lesions exceeding 2 cm in length accompanied by distinct leaf wilting or significant defoliation; Grade 4, stem constriction (girdling), with almost complete defoliation or whole-plant wilting except for the growing point; and Grade 5, a dead plant. The disease index (DI) and control efficacy (CE) were calculated using the formulae below [21].

$$\text{Disease Index (DI)} = \frac{\sum(\text{Number of diseased plants} \times \text{Disease grade})}{(\text{Total number of plants} \times \text{Highest grade})} \times 100$$

$$\text{Control Efficacy (\%)} = \frac{\text{DI of Control} - \text{DI of Treatment}}{\text{DI of Control}} \times 100\%$$

The greenhouse experiments were conducted with three independent biological replicates (each replicate include three individual plots consisting of 30 plants from independent groups grown in separate batches). Within each replicate, treatments were arranged in a completely randomized design. The individual pot was considered the statistical unit. Following the disease assessment, root, stem, and leaf tissues were collected from each treatment group. For each treatment, 60 randomly selected pepper plants were pooled and thoroughly mixed. The pooled sample was then split into three subsamples (each representing one biological replicate), and each subsample was individually sealed in a sterile plastic bag. Loosely attached soil was gently shaken off the roots. All samples were thoroughly washed with running tap water followed by three rinses with sterile distilled water to remove rhizospheric soil and surface dust. The cleaned tissues were cut into small pieces and immediately snap-frozen in liquid nitrogen. A total of 18 samples were collected in this study, comprising two treatments across three plant parts (leaf, root, and stem) with three biological replicates each.

#### 2.4. Genomic DNA of Z-294 Extraction and Sequencing

The biocontrol strain Z-294 was sequenced on account of its efficient ability to protect pepper from *P. capsici*. A single colony of strain Z-294 was incubated at 37 °C overnight in LB liquid medium. Genomic DNA was extracted using a SteadyPure bacterial genomic DNA extraction kit (Accurate Biotech, Changsha, China) according to the manufacturer's instructions. The DNA's quality and quantity were analyzed through a Nanodrop ONE, 0.38% agarose gel electrophoresis, and a Qubit 3.0 Fluorimeter (Thermo Fisher, Waltham, MA, USA). Afterward, Wuhan Benagen Technology Solutions Co., Ltd., Wuhan, Hubei was engaged for genome sequencing. The utilization of Covaris enabled the shearing of the DNA to 350 bp for library preparation. In addition, the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) was used to verify the DNA quality. A paired-end DNA library was prepared according to the TruSeq DNA Sample Preparation Guide for Illumina kit. Sequencing was performed on an Illumina NovaSeq 6000 with 150 bp reads.

The ligation sequencing kit (SQK-LSK109, Oxford, UK) was used for the construction of the library for long-read sequencing. The standard protocol provided by Oxford Nanopore Technologies was applied to barcode the DNA library with a native barcoding expansion 1–12 kit. This was performed on a PromethION sequencer after being loaded on r9.4.1 flow cells.

We utilized SOAPnuke v2.1.2 with default parameters to remove and filter low-quality reads and adapters to acquire high-quality reads. The DNA base-calling for third-generation sequencing data was performed using Guppy v5.0.16, which was optimized at default settings for raw read processing. The default settings were used for base-calling, barcode segmentation and adapter sequence removal. The Nanopore platform ultimately produced a total of 165,771 long reads, resulting in 1,594,903,407 bp. The mean read lengths were 9621 bp, with a read length N50 of 17,898 bp. Meanwhile, we obtained 10,629,352 and 10,593,360 raw and clean reads, with a total of 1,594,402,800 bp and 1,522,068,122 bp for Illumina sequencing, respectively. The sequencing coverage for second-generation and third-generation sequencing is  $357.9\times$  and  $374.14\times$ , respectively.

Using the default options in Unicycler v0.4.9, a complete sequence was generated from high-quality short-read and long-read sequences. The annotation step was carried out as indicated [22]. Briefly, Prokka software v1.12 was used to predict the protein-coding genes and functional annotation was performed using the Basic Local Alignment Search Tool (BLAST) against several databases, including the Cluster of Orthologous Group of proteins (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), NCBI NR protein database and InterPro database. The antibiotic's gene cluster was predicted using the AntiSMASH online prediction software (<https://antismash.secondarymetabolites.org/>) with default parameters [23]. To identify putative virulence factors and antimicrobial-resistance genes, we used the database for virulence factors of pathogenic bacteria (VFDB, <http://www.mgc.ac.cn/VFs/>) and the comprehensive antibiotic resistance database (CARD, <https://card.mcmaster.ca>).

### 2.5. Phylogenetic Analysis

All genomes utilized in this study were downloaded in FASTA format from the National Center for Biotechnology Information (NCBI) database. We constructed a maximum-likelihood (ML) phylogenetic tree of *Bacillus* species based on single-copy core proteins among *Bacillus* genomes as well as the *Paenibacillus polymyxa* M1 genome. The following methods were employed in the reconstruction of the tree: (1) multiple alignment of amino acid sequence with MAFFT v7.310; (2) identification of conserved blocks from the multiple alignment using Gblocks; and (3) construction of the ML tree using RAxML software 8.2.13 under the PROTGAMMALGX model with 1000 bootstrap replicates. Molecular Evolutionary Genetic Analysis (MEGA) was used to visualize the tree. The average nucleotide identity (ANI) values between two genome sequences were determined using the online JspeciesWS software (<https://jspecies.ribohost.com/jspeciesws>). The phylogenetic trees of strain Z-294, based on 16S rDNA and *gyrA* gene sequences, were constructed using the neighbor-joining method in MEGA 7.0.

### 2.6. Amplicon Sequencing of 16S rRNA Gene Fragments of Root, Stem, and Leaf Samples and Data Analyses

We extracted total genomic DNA by using the modified cetyltrimethylammonium bromide (CTAB) method of root, stem, and leaf samples (~1 g) for sequencing [24]. The total DNA obtained was used for PCR amplification. Using primers 799F (5'-AACMGGATTAGATACCCCKG-3') and 1193R (5'-ACGTCATCCCCACCTTCC-3'), the V5–V7 region of the bacterial 16S rRNA gene was amplified. The primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3')

were used to amplify the fungal ITS1 region. Each of the PCR reactions prepared was performed with 15 µL of Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 0.2 µM of forward and reverse primers, and approximately 10 ng of template DNA. The thermal cycling conditions were as follows: initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 min. An equal volume of 1× loading buffer was mixed with PCR products and subjected to electrophoresis on a 2% agarose gel. PCR products were pooled in equimolar ratios and subsequently purified using the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). Sequencing libraries were prepared using the TruSeq DNA PCR-Free Sample Preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions, with index codes added. The quality of the library was evaluated using the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and the Agilent Bioanalyzer 2100 system. The Illumina NovaSeq platform was used for the final library, which created 250 bp paired-end reads (Novogene, Beijing, China).

The analysis of amplicon data was performed through the co-association of VSEARCH v2.13.3 and QIIME 1.9.1 software [25,26]. The final sequences were quality-filtered with the use of VSEARCH, which also removed singletons. The sequence reads were clustered into operational taxonomic units (OTUs) at the 97% similarity threshold. Reference-based chimera-checking methods identified and removed chimeric sequences from the data system. Using the blast algorithm and QIIME software (1.9.1), representative sequences were classified with respect to the SILVA (v.13.2) and UNITE v8.0 reference database for bacterial and fungal microbiome, respectively [27,28]. We removed mitochondrial and chloroplastic DNA sequences and OTUs with a total relative abundance of less than 0.00001 across all samples.

For the purpose of alpha-diversity analysis, the OTU table was rarefied to the lowest number of reads (84,413 and 21,739 for bacterial and fungal microbiome, respectively) among the samples. It was normalized with the cumulative sum scaling (CSS) method for beta-diversity analysis. Both alpha-diversity and beta-diversity were computed using QIIME software. UniFrac distance matrices and Bray–Curtis distance matrices were calculated and visualized using non-metric multi-dimensional scaling (NMDS) and Principal Coordinate Analysis (PCoA) ordinations to assess the bacterial and fungal community beta-diversity. The R package, vegan, was used for the statistical tests of permutational multivariate analysis of variance (PERMANOVA) using the adonis function with 1000 permutations with the model:  $\text{bray\_curtis} \sim \text{treatment} * \text{tissue}$  [29]. To test for homogeneity of multivariate dispersions among groups, which is a key assumption of PERMANOVA, we additionally performed PERMDISP (permutational analysis of multivariate dispersions). We used the platform OECloud (<https://cloud.oebiotch.cn>) to analyze the Venn diagram. It showed common and unique OTUs in Z-294-treated and control samples. Then, we performed the Fisher exact test using the annotation results of enriched or depleted OTUs. Meanwhile, statistical analysis of metagenomic profiles (STAMP) software was used to evaluate the significance of differences between Z-294-treated and control samples [30]. The use of the EdgeR generalized linear model (GLM) approach for differential abundance analysis identified differential OTUs with false discovery rate (FDR)-corrected  $p$ -values  $< 0.05$  as indicator OTUs [31]. The linear discriminant analysis effect size (LEfSe) method (Wilcoxon  $p$ -value  $< 0.05$  and logarithmic LDA score  $> 2$ ), which establishes biomarkers of Z-294-treated and control samples, was applied [32]. We used the full dataset to analyze it at the class level or genus level for LEfSe analysis.

The significant differences were assessed with two-way ANOVA in GraphPad Prism 8 in this study, with treatment (Z-294-treated and control) and plant organ (root, stem, leaf, and fruit) as fixed factors, including their interaction term (treatment  $\times$  organ).

### 2.7. Nucleotide Sequence Accession Number

The whole genome sequence of Z-294 has been deposited in GenBank under the accession number CP180439. Raw sequence data of bacteria and fungi are available at the NCBI under the accession number PRJNA1466249.

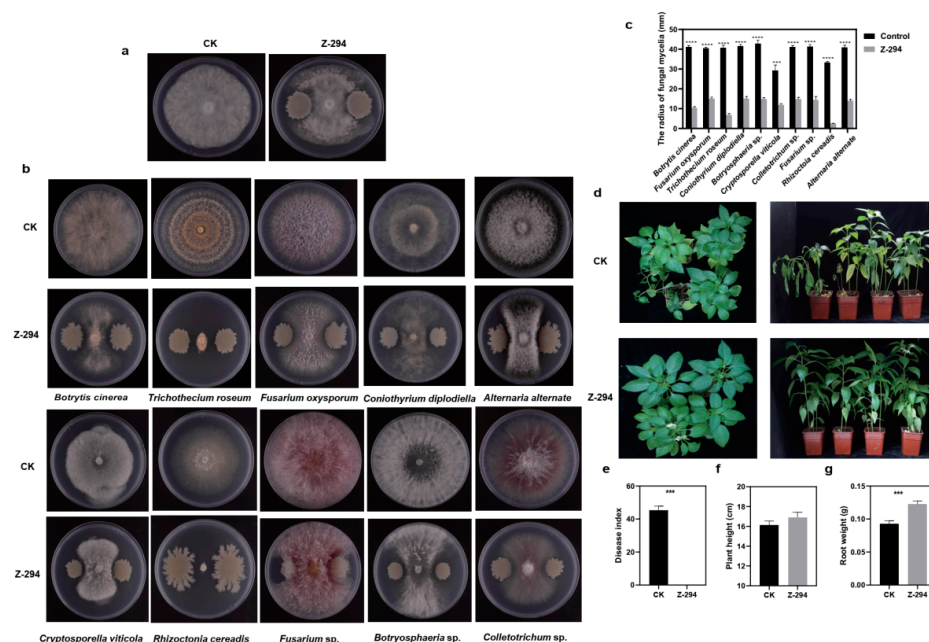
## 3. Results

### 3.1. Strain Z-294 Showed Strong Inhibition Against *P. capsici*

Strain Z-294 inhibited *P. capsici* growth on PDA (Figure 1a). The biological control potential of strain Z-294 against *P. capsici* was evaluated under greenhouse conditions. The disease index of pepper plants treated with Z-294 (0) was significantly lower than that of the LB-treated control group (45.33) (Figure 1d,e). The corresponding control efficacy reached 100%, demonstrating a substantial effect against *P. capsici*. Additionally, strain Z-294 was assessed for its antagonistic effects against various fungal pathogens, including *B. conerea*, *T. roseum*, *F. oxysporum*, *C. diplodiella*, *A. alternata*, *C. viticola*, *Colletotrichum* sp., *Botryosphaeria* sp., and *R. cerealis*. The results indicated that Z-294 markedly inhibited the mycelial growth of these pathogens, as evidenced by the presence of a distinct inhibition zone (Figure 1b,c). The strains demonstrated broad-spectrum antagonistic activity. Z-294 displayed stronger inhibition of other pathogens than of *P. capsici*, such as *T. roseum* and *R. cerealis*. Furthermore, strain Z-294 also exhibited characteristics indicative of a plant growth-promoting agent. The results revealed that the plant height and root weight of Z-294-treated plants were greater than those of the control plants (Figure 1f,g). In conclusion, we have isolated the biocontrol strain Z-294, which demonstrates broad-spectrum antagonistic properties, plant-growth-promoting capabilities, and potential as a beneficial microbe for biotechnological applications.

### 3.2. The General Genome Features of the Biocontrol Strain Z-294

The biocontrol strain Z-294 has a circular chromosome with a GC content of 43.47%, spanning 4,247,419 bp. Notably, this strain does not harbor any plasmids. Genome analysis revealed that the genome sequence of Z-294 comprises 4253 coding sequences (CDSs). Among these predicted CDSs, 2866 were assigned putative functions, while 1387 were predicted to encode hypothetical proteins. The average length of the protein-coding genes is 879 bp, which accounts for 88.02% of the genome size of Z-294. Furthermore, the genome of the biocontrol strain Z-294 predicts 117 RNA genes, including 30 rRNA, 86 tRNA, and 1 tmRNA (Additional File S1: Figure S1). The genomic features are summarized in Table 1. We found that the genome sequence of *B. subtilis* Z-294 contained several gene clusters, including gene clusters for the synthesis of surfactin and fengycin (Additional File S2: Table S1). Their presence in Z-294 suggests this strain could probably be developed as a biopesticide to combat plant pathogens, reducing reliance on chemical fungicides. To assess the biosafety of strain Z-294 for potential biocontrol applications, its genome was screened for antimicrobial-resistance genes and virulence-associated genes (Additional File S2: Tables S2 and S3). The genome of Z-294 contains 17 antimicrobial-resistance genes and 1098 virulence-associated genes.



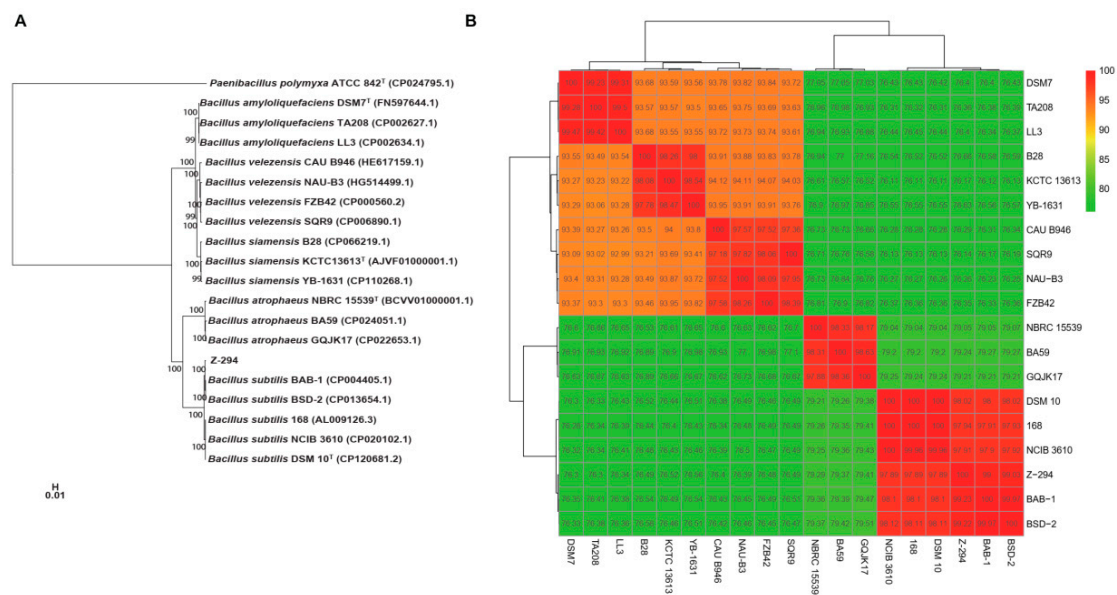
**Figure 1.** Strain Z-294 had the potential to serve as a biocontrol agent against *P. capsici*. (a) The antagonistic activities of Z-294 against *P. capsici* were evaluated on a PDA plate. (b) The antagonistic activity of Z-294 was tested against ten pathogenic fungi on a PDA plate. (c) Quantification of antagonistic activity of Z-294. (d) The biocontrol effect of Z-294 on *P. capsici* was assessed in a greenhouse experiment. (e) The disease index of Z-294 and the control in greenhouse experiments. The plant growth-promoting ability of Z-294 was measured in terms of (f) plant height and (g) root weight. Error bars represent the SD of three replicates. The plant height and root weight were obtained from the plant-growth-promoting experiments. The statistical analysis was performed using GraphPad Prism 8 software with *t*-test. \*\*\*\*,  $p < 0.0001$  and \*\*\*,  $p < 0.001$ .

**Table 1.** General genome features of biocontrol strain Z-294.

| Attribute                                   | Z-294     |
|---------------------------------------------|-----------|
| Genome size (bp)                            | 4,247,419 |
| GC content (%)                              | 43.47     |
| Protein coding genes                        | 4253      |
| RNA genes                                   | 117       |
| rRNA                                        | 30        |
| tRNA                                        | 86        |
| tmRNA                                       | 1         |
| Gene total length (bp)                      | 3,738,528 |
| Average length of protein-coding genes (bp) | 879       |

### 3.3. Phylogenetic Analysis

The phylogenetic analysis based on the 16S rDNA and *gyrA* sequences indicated that strain Z-294 is closely related to *B. subtilis* (Additional File S1: Figure S2). Additionally, a phylogenetic tree was constructed using the 849 single-copy core genes to elucidate the evolutionary relationship of the biocontrol strain Z-294. The results demonstrated that Z-294 clustered with other sequenced *B. subtilis* strains (Figure 2A). Furthermore, the genomic relatedness of strain Z-294 was assessed based on the Average Nucleotide Identity (ANI) values. The ANI values of strain Z-294 against all *B. subtilis* strains were greater than 97%, exceeding the 96% threshold (Figure 2B). Strain Z-294 exhibited a high nucleotide identity of 98.02% with strain DSM 10, the type strain of *B. subtilis*. Clustering analysis based on ANI values among the strains revealed that *B. subtilis* strains form a distinct cluster. Overall, biocontrol strain Z-294 should be classified as *B. subtilis*.

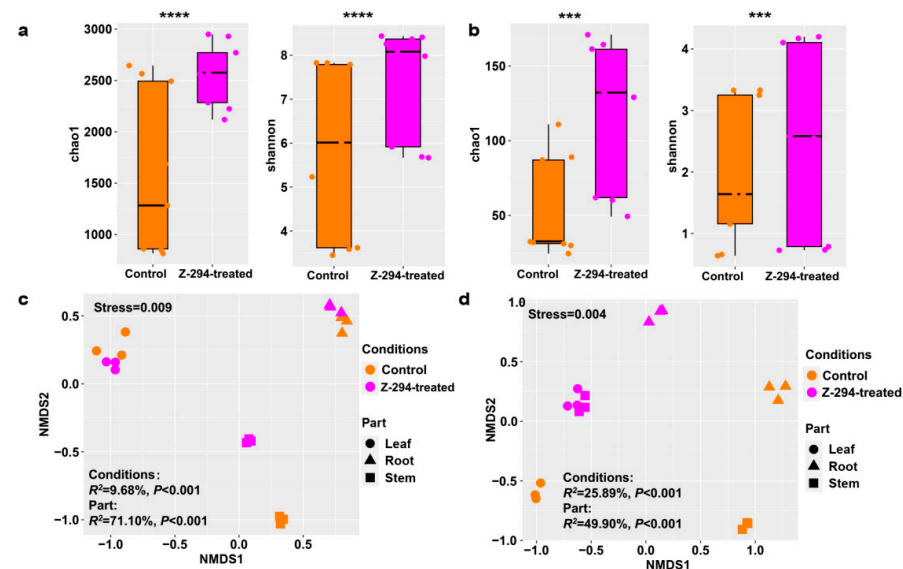


**Figure 2.** Phylogenetic analysis of biocontrol strains of Z-294. **(A)** The maximum-likelihood (ML) tree of various *Bacillus* strains was constructed based on 849 single-copy core genes using RAxML 8.1.24. *P. polymyxa* M1 was used as the outgroup. The percent bootstrap values (from 1000 replicates) are indicated at the nodes. **(B)** A heat map illustrating ANI values among different *Bacillus* strains. The number represented the ANI values.

### 3.4. The Association Between Biocontrol Strain *B. subtilis* Z-294 and the Microbial Community

After conducting quality control and merging paired-end reads, we obtained a total of 1,811,895 and 1,315,457 high-quality clean reads, which were clustered into 5696 and 268 operational taxonomic units (OTUs) for bacteria and fungi, respectively. Each sample yielded a minimum of 84,413 and 21,739 clean reads for bacteria and fungi, with average counts of 100,661 and 73,081 clean reads, respectively (Additional File S2: Table S4). For the alpha-diversity indices, two-way ANOVA revealed that Z-294 treatment was the dominant factor affecting all measured indices (Chao1,  $F = 210.5$ ,  $p < 0.05$ ; Shannon,  $F = 60$ ,  $p < 0.05$ ) for the bacterial microbiome. While the tissue was the dominant factor affecting fungal Shannon index ( $F = 664.7$ ,  $p < 0.05$ ), it was the Z-294 treatment for the Chao1 index ( $F = 1029$ ,  $p < 0.05$ ). The Shannon index and Chao1 index of Z-294-treated samples were higher than those of control samples (Figure 3a,b). We found there are similar results between Z-294-treated and control samples for each tissue (Additional File S1: Figure S4). Among the different plant parts, leaves exhibited higher fungal diversity (Chao1 and Shannon index) compared to stems and roots. The Chao1 value for roots was higher than that for leaves and stems, whereas the Shannon index for leaves was greater than that for roots and stems in the bacterial microbiome (Additional File S1: Figure S3).

Both bacterial and fungal beta-diversity were primarily influenced by plant parts ( $R^2 = 71.10\%$  and  $49.90\%$ ,  $p < 0.05$ ). The biocontrol strain Z-294 also significantly influenced the pepper bacterial and fungal microbiome (Figure 3c,d and Additional File S1: Figure S5). For different plant parts, the Z-294-treated samples clearly differed from control samples in terms of both bacterial and fungal microbiomes (Additional File S1: Figure S3). PCoA revealed a clear separation between Z-294-treated and control groups for both bacterial and fungal communities (Additional File S1: Figure S5). PERMANOVA confirmed a significant effect of Z-294 treatment on both bacterial ( $F = 27.715$ ,  $p < 0.05$ ) and fungal ( $F = 86.153$ ,  $p < 0.05$ ) community composition. However, the PERMDISP test indicated that the homogeneity of multivariate dispersions was violated for the bacterial dataset ( $F = 13.232$ ,  $p < 0.05$ ), whereas no significant heterogeneity was detected for the fungal dataset ( $F = 0.156$ ,  $p > 0.05$ ).

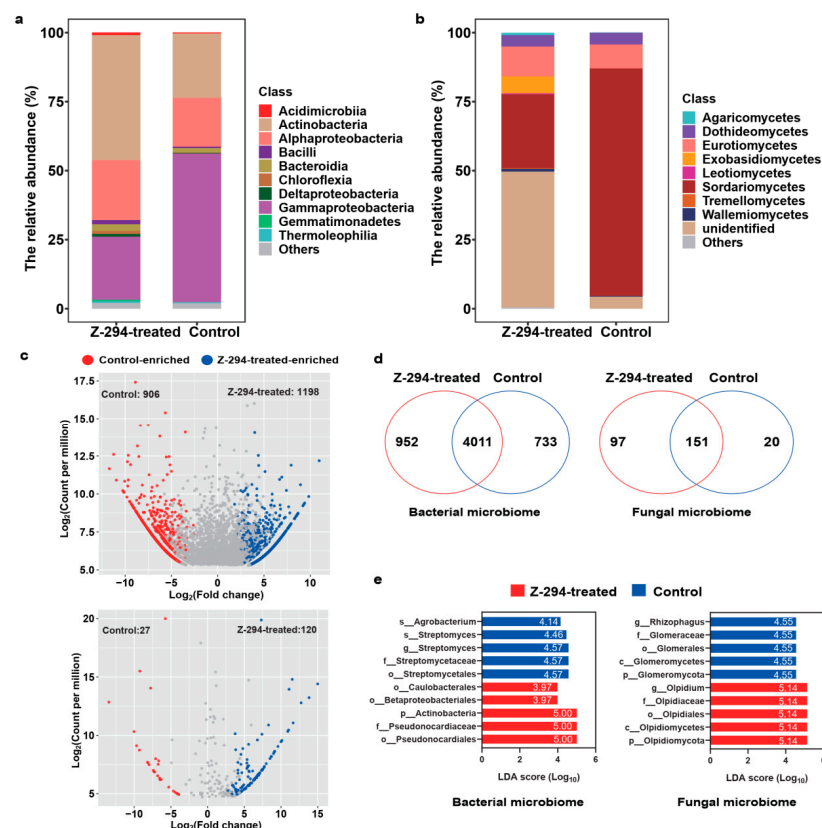


**Figure 3.** The association between biocontrol strain *B. subtilis* Z-294 and the microbial community. Alpha-diversity measurements are based on the Chao1 and Shannon indices for the (a) bacterial and (b) fungal OTUs. Statistical data analyses were performed using two-way ANOVA (\*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ ). The pepper microbiome includes leaf, root, and stem tissue samples. NMSD ordinations based on weighted unifracs distance matrices of (c) bacterial and (d) fungal communities across all samples. The microbiome represents the microbiome of leaf, root, and stem samples.

### 3.5. The Difference in the Composition of the Microbial Community Between Z-294-Treated and Control Pepper Samples

Taxonomic profiling at the class level revealed that the predominant bacterial classes in the pepper (leaf, root, and stem) bacterial microbiome were *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria*, and *Bacteroidia*. The primary fungal divisions included *Sordariomycetes*, *Eurotiomycetes*, *Dothideomycetes*, and *Exobasidiomycetes* (Figure 4a). Notably, substantial differences were observed in the composition of the bacterial and fungal communities under different treatments based on overall relative abundance. Z-294-treated plants exhibited a higher proportion of *Actinobacteria*, *Alphaproteobacteria*, *Bacilli*, *Bacteroidia*, *Deltaproteobacteria*, *Acidimicrobiia*, *Thermoleophilia*, *Chloroflexia*, *Gemmatimonadetes*, *Acidobacteriia*, and *Verrucomicrobiae* at the class level within the bacterial community (Additional File S1: Figure S6). In contrast, the proportion of *Eurotiomycetes*, *Exobasidiomycetes*, *Wallemiomycetes*, *Agaricomycetes*, *Leotiomyces*, *Tremellomycetes*, *Agarocostilbomycetes*, *Cystobasidiomycetes*, *Umbelopsidomycetes*, *Microbotryomycetes*, and *Aphelidiomycetes* was significantly higher in Z-294-treated plants compared to control plants within the fungal community (Additional File S1: Figure S7). The Z-294-treated pepper plants exhibited more enriched and specific OTUs relative to control pepper plants for both the bacterial and fungal microbiomes (Figure 4c,d). OTUs identified as *Alphaproteobacteria*, *Bacteroidia*, *Bacilli*, *Acidimicrobiia*, *Chlamydiae*, and *Acidobacteriia* exhibited greater abundance in Z-294-treated pepper plants. In contrast, *Actinobacteria*, *Gammaproteobacteria*, *Clostridia*, and *Fimriimonadia* were found to be more prevalent in control pepper plants for bacterial microbiome based on enriched OTU analysis (Fisher exact test,  $p < 0.05$ ; Additional File S2: Table S5). There are some discrepancies between the Z-294-treated and control groups for different plant parts (Additional File S1: Figures S8 and S9). Concurrently, OTUs associated with *Agaricomycetes* and *Eurotiomycetes* were more abundant in Z-294-treated pepper plants, while those classified as *Sordariomycetes* were more prevalent in control plants (Fisher exact test,  $p < 0.05$ ; Additional File S2: Table S6). Furthermore, we performed the STAMP analysis and observed a significant increase in the populations of *Gammaproteobacteria*, *Clostridia*, and *Synergistia* at the class level

in control pepper plants. Conversely, the levels of *Bacilli*, *Planctomycetacia*, *Deltaproteobacteria*, *Acidobacteriia*, *Thermoleophilia*, and others were significantly reduced in the control pepper plants. At the lower taxonomical level, we found that the abundance of *Pseudolabrys*, *Aeromicrobium*, *Acidovorax*, *Methylothera*, *Gemmobacter*, and *Bdellovibrio* was significantly higher in the Z-294-treated pepper plants (Additional File S1: Figure S10). We observed a higher abundance of *Leotiomyces*, *Malasseziomyces*, *Umbelopsisomyces*, *Aphelidiomyces*, *Saccharomyces*, *Tremellomyces*, *Agaricomycetes*, and *Pezizomyces* in Z-294-treated pepper plants compared to the control pepper plants. Genera such as *Peziza*, *Clitopilus*, *Petriella*, *Naganishia*, *Erysiphe*, *Gjaerumia*, *Umbelopsis*, and *Malassezia* were more abundant in Z-294-treated plants, whereas *Plectosphaerella* was found to be more abundant in control pepper plants at the genus level (Additional File S1: Figure S11). Furthermore, the LEfSe results indicated that *Pseudonocardiales*, *Olpidiomyces* and *Streptomyces*, *Glomeromycota* were the biomarker taxa for the bacterial and fungal microbiome of Z-294-treated and control pepper plants, respectively (Figure 4e). The application of *B. subtilis* Z-294 has an association with the tissue-associated microbiome, enriching *Alphaproteobacteria*, *Bacilli*, *Bacteroidia* and *Agaricomycetes*, *Eurotiomycetes*, while reducing the relative abundance of *Gammaproteobacteria* and *Sordariomycetes* for the bacterial and fungal microbiome, respectively.



**Figure 4.** Comparative analysis of Z-294-treated and control pepper plant microbiome. (a,b) Taxonomic comparison at the class level of the bacterial and fungal microbiome of different treatments. (c) The unconventional volcano plot shows the enriched OTUs in Z-294-treated and control plants for the bacterial and fungal microbiome. Each dot represents a single OTU. Each red dot represents an individual enriched OTU in control samples and each blue dot represents an individual enriched OTU in Z-294-treated samples. The grey node represented the no significant. The x-axis represents the  $\log_2(\text{Fold change})$  and the y-axis represents the  $\log_2(\text{Count per million})$ . Up: Bacterial microbiome; Down: Fungal microbiome. (d) Venn diagrams showing the common and unique bacterial and fungal OTUs among the Z-294-treated and control pepper plant samples. (e) The biomarker taxa of bacterial and fungal microbiome among Z-294-treated and control pepper plants. Only the top five most specific biomarkers are displayed.

#### 4. Discussion

In the present study, we isolated a biocontrol strain *B. subtilis* Z-294, which demonstrated stronger inhibition of *P. capsici* in both plate and greenhouse experiments. This strain exhibited broad-spectrum antagonistic activity and plant growth-promoting traits. We identified this strain as *B. subtilis* based on the genome sequence using single-copy core genes and ANI values. Therefore, strain Z-294 holds great promise as a multifunctional biocontrol agent for sustainable management of diseases and the enhancement of plant health, laying a solid foundation for its future agricultural applications.

In this study, we conducted phylogenetic and ANI analysis for Z-294, which has been identified as *B. subtilis* based on its genome sequence (Figure 2). Importantly, we need to examine and confirm the gene content related to antibiotic and plant–bacteria interaction genes within the genome using molecular biology. Genome mining revealed that the strain contains the gene cluster involved in the synthesis of subtilosin A and bacilysin and mutation of these genes decreased inhibitory activity against *Acidovorax citrulli* [33]. Additionally, the genome sequence of *B. subtilis* 916 contains four nonribosomal peptide synthase gene clusters, including a novel family of lipopeptides known as locillomycins, which contribute to its phenotypic characteristics [34]. The most significant difference between *B. velezensis* and *B. subtilis* strains was identified in the gene cluster encoding the iturin family of lipopeptides, which effectively inhibit *Fusarium* pathogens, as demonstrated by comparative genomics analysis [20]. We need to conduct a more in-depth analysis of the genome sequence, integrating molecular biology and omics technologies, to elucidate the mechanisms of strain Z-294, thereby enabling its effective application in the field. It is important to note that the findings from antiSMASH, CARD, and VFDB are predictive and based solely on sequence homology. While these tools provide valuable hypotheses regarding the strain’s functional capabilities, they do not replace direct experimental evidence.

Numerous studies have demonstrated that biocontrol strains could influence microbial communities to suppress diseases. For instance, *B. amyloliquefaciens* PP19 has been shown to prevent imbalances in bacterial diversity and maintain microbial stability by modulating the abundance of *Paenibacillus*, *Actinomycetospora*, and *Spirosoma* on litchi exocarps, thereby suppressing litchi downy blight [35]. Additionally, it has been reported that a bio-organic fertilizer containing *B. amyloliquefaciens* W19 influences the resident soil microbial community, particularly by increasing the abundance of *Pseudomonas*. The application of *Bacillus* and indigenous *Pseudomonas* forms as a plant-beneficial consortium that combats pathogens [19]. Furthermore, *Trichoderma asperellum* FJ035 alters the composition and abundance of microbial communities in the cucumber rhizosphere, effectively suppressing *Fusarium* wilt under continuous cropping conditions [36]. A bio-product comprising *Bacillus* and *Trichoderma* has been found to effectively suppress common scab disease while enhancing tuber yield by fostering a high relative abundance of beneficial bacteria [37]. The combination of *Trichoderma harzianum* and *Arthrobacter ureafaciens* promotes plant growth and reduces *Fusarium* crown rot by decreasing the abundance of phytopathogenic fungi and increasing beneficial microbes [38]. A biocontrol strain, *Rhizobium pusense* TYQ1, is significantly enriched in rhizosphere soil based on microbiome analysis related to root-knot nematode infection. This strain not only directly inhibits root rot nematodes but also induces restructuring of the rhizobacterial community, leading to the enrichment of multiple biomarker species [39]. In this study, we found that the biocontrol strain Z-294 exhibits strong inhibitory effects on plant pathogens in plate assays and influences the microbial community. The results indicate that Z-294 can enhance both alpha-diversity and influence the bacterial and fungal microbiomes (Figures 3 and 4). Furthermore, our analysis revealed that Z-294-treated pepper plants enriched a higher proportion of rare taxa at lower

taxonomic levels, as evidenced by enriched OTU analysis and comparative composition analysis. It was also observed that these rare taxa play an important role in ecosystem functioning [40]. Thus, strain Z-294 holds significant promise as a multifunctional biocontrol agent for the sustainable management of plant diseases.

An increasing body of evidence demonstrates the potential of manipulating leaf and root-associated microbiomes to control plant diseases and enhance yields in cropping systems. The SynComs composed of multiple strains have shown superior performance compared to individual strains. For example, the two-strain consortia were more effective in promoting plant growth and suppressing diseases than a specific strain [41]. The incidence of root rot was significantly reduced in soils with high bacterial diversity. This suppression of pathogens may occur through mechanisms such as nutrient competition and the formation of physical barriers on the root surface [42]. A SynCom constructed with three high-abundance bacteria (*Stenotrophomonas* sp., *Rhizobium* sp., *Ochrobactrum* sp.) and one low-abundance bacterium (*Advenella* sp.) significantly suppressed root rot disease in *Astragalus mongholicus* [43]. Importantly, the infection rate of *Meloidogyne incognita* exhibited an inverse S-shaped decreasing trend with an increase in the number of high-performance functional strains, indicating that six such strains are sufficient to construct a SynCom capable of significantly reducing *M. incognita* infection [44]. Therefore, the optimal number of strains in a SynCom needs to be determined for different plant species to achieve the best possible control efficiency. Additionally, isolating certain strains from Z-294-treated pepper plants is necessary to confirm the biocontrol mechanism of Z-294. The underlying mechanisms, particularly the synergistic interactions between different species, have been reported. The branched-chain amino acid biosynthesis pathways are involved in syntrophic cooperation and metabolic facilitation among bacterial strains, specifically *B. velezensis* SQR9 and *Pseudomonas stutzeri*, in the cucumber rhizosphere [45]. The delivery of LtaE by *Lysobacter enzymogenes*, utilizing T4SS, triggers the production of the antifungal antibiotic 2,3-DAPG through its interaction with the transcriptional repressor PhlF in *Pseudomonas protegens* [46,47]. *Stenotrophomonas maltophilia* can diminish the lipopeptide antibiotics produced by the biocontrol agent *Bacillus*, thereby facilitating pathogen invasion [48]. It is essential to account for the native antibiotic-degrading organisms when applying biocontrol agents in the field. However, the composition of the microbial community, particularly the presence of keystone taxa, is a crucial determinant of disease suppression. The metabolites of the native keystone taxa *Paenibacillus cellulositrophicus* CSC13 induce the production of erythromycin in *Streptomyces* R02, a key compound that directly suppresses *Ralstonia solanacearum* [49]. Therefore, it is important to consider the association with the native soil microbiota following the application of biocontrol agents.

## 5. Conclusions

We isolated a biocontrol strain *B. subtilis* Z-294, which demonstrated strong inhibition of *P. capsici* in both plate and greenhouse experiments. This strain exhibited broad-spectrum antagonistic activity and plant growth-promoting traits. We identified this strain as *B. subtilis* based on the genome sequence using single-copy core genes and ANI values. Additionally, we discovered that the biocontrol strain Z-294 has an association with the microbial community. Therefore, strain Z-294 holds great promise as a multifunctional biocontrol agent for the sustainable management of diseases and the enhancement of plant health, laying a solid foundation for its future agricultural applications.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16151500/s1>, Additional File S1: Figure S1. Genome map of *B. subtilis* Z-294. Figure S2. Phylogenetic analysis of strain Z-294 based on 16S rDNA gene sequence and *gyrA* sequence. Figure S3. Comparative analysis for the plant part microbiome.

Figure S4. Alpha-diversity measurements are based on the Chao1 and Shannon indices for the bacterial and fungal OTUs of leaf, root and stem samples. Figure S5. PCoA ordinations based on Bray–Curtis distance matrices of bacterial and fungal communities. Figure S6. The relative abundance of dominant bacterial class varied among different treatments. Figure S7. The relative abundance of the dominant fungal class varied among different treatments. Figure S8. The relative abundance of the top 15 bacterial classes of the Z-294-treated and control plants for different plant parts. Figure S9. The relative abundance of the top 15 fungal classes of the Z-294-treated and control plants for different plant parts. Figure S10. Differential abundances of bacterial OTUs for pepper plants of Z-294-treated and control samples. Figure S11. Differential abundances of fungal OTUs for pepper plants of Z-294-treated and control samples. Additional File S2: Table S1. The information on antibiotics in the genome of *B. subtilis* Z-294. Table S2. The antimicrobial-resistance genes in the genome of *B. subtilis* Z-294. Table S3. The virulence-associated genes in the genome of *B. subtilis* Z-294. Table S4. The summary data for the bacterial and fungal microbiome. Table S5. Comparison of enriched bacterial OTUs between Z-294-treated and control pepper plants for the bacterial microbiome. Table S6. Comparison of enriched fungal OTUs between Z-294-treated and control pepper plants for fungal microbiome.

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**Conflicts of Interest:** Author Qian Zhou was employed by the company Guizhou Yibaiyi Biotechnology Co., Ltd. Lixia Zhang and Wei Shi were employed by the company Sino Green Agri-Biotech Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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