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Agro-Based Spent Mushroom Compost Substrates Improve Soil Properties and Microbial Diversity in Greenhouse Tomatoes

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Abstract: Spent mushroom compost (SMC) substrates are commonly used as growth media for greenhouse crops and horticulture production. This study aimed to investigate the responses of physiochemical soil properties, enzyme activities, and microbial community compositions to different cultivation durations and SMC soil treatments on tomatoes. The experiment included the following treatments: SMC substrate and the surrounding soil after planting at 1, 3, and 7 years and comparing control treatments including non-planting SMC substrates and continuous mono-cropping soil. The results revealed that the SMC substrates had higher contents of total N P and organic C nutrients than the surrounding soil treatments. The physicochemical soil properties and soil enzyme activities of the SMC substrates were significantly decreased with longer cultivation duration. Microbial alpha diversity was higher in the SMC substrates regardless of cultivation duration than in the control treatments. It was observed that many beneficial microbes, such as bacteria of the *Deinococcus-Thermus*, *Halanaerobiaeota*, and *Nitrospirae* phyla, and the fungi of the *Basidiomycota*, *Mortierellomycota*, and *Chytridiomycota* phyla were enriched in the SMC substrates. The SMC substrate and surrounding soil had enriched several potentially beneficial microorganism genera such as the bacterial *Saccharimonadales*, *Gaiella*, *Bacillus*, and the fungal *Thermomyces*, *Kernia*, and *Mortierella*. Therefore, the agro-based SMC substrate grooved cultivation system is recommended as an environmentally compatible practice for tomato growth in the greenhouse.

Keywords: agro-based SMC substrate; greenhouse tomatoes; physicochemical properties; enzyme activities; microbial community

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1. Introduction

With rapidly intensifying climate change and an increasing global population, food security is a serious global scale issue [1]. The frequent occurrence of extreme weather events and food shortages emergence in a large part of the world exacerbates the challenges that impact economic and social development worldwide [2,3]. To narrow the gap between food supplies and conventional open-field production, greenhouse technologies have been employed for the cultivation of crops and horticulture plants that can improve the production of off-season products [4]. Greenhouse cultivation has the capacity to finely control temperatures and microclimates to facilitate the year-round growth of horticulture plants to

compensate for climatic instabilities in the field [5]. However, greenhouse technologies are a significant energy-intensive agricultural industry [6]. In the pursuit of high crop yields, surplus inputs of fossil-based mineral fertilizers can have deleterious effects on soil, leading to environmental contamination in greenhouse cultivation systems [7]. Hong, et al. [8] suggested that excessive nutrient accumulation in plant root zones and downward leaching via irrigation result in soil and groundwater contamination in greenhouse cultivation systems. Furthermore, increased soil salinity, decreased crop yields, and continuous cropping obstacles (CCO) that arise from continuous mono-cropping in the same field result in bottleneck issues for the development of the vegetable industry in greenhouses [9,10].

In seeking an alternative system to replace conventional greenhouse cultivation, researchers have invested more effort into potential alternatives, for example, the organic cultivation system [11]. The application of agro-based spent mushroom compost substrates has been shown to be a beneficial alternative approach for the cultivation of crops and horticultural plants in greenhouse systems [12]. Spent mushroom compost (SMC) substrates are an effective cultivation medium for vegetable production [13], which can reduce nitrogen loss in greenhouse systems [14]. The spent compost substrate consisted primarily of residual fungal mycelium, disintegrated lignocellulosic biomass, various nutrients, as well as organic matter and enzymes [15]. Further, it had a low bulk density, loose texture, good air permeability, and nutrient retention. Thus, the SMC can be employed to bioremediate contaminated soil, and enhance its health [15] by improving the physical structure of the soil and ecological environment for soil microorganisms [16]. Furthermore, the SMC also contains a high organic matter content, phosphorus, and potassium, as well as trace elements that are required for plant assimilation and utilization [17]. Owing to its low level of toxic elements, strong absorption capacity, and enhanced soil aeration and water retention capacities, SMC has been utilized as a soil amendment that contributes to improving soil quality, agronomic efficiency, and environmental safety. In addition, it contains several biologically active compounds such as therapeutically valuable polysaccharides and antibacterial peptides [18]. These, together with its antibacterial properties protect plants against pathogens; thus, minimizing the incidence of plant diseases [19,20].

Under conventional greenhouse conditions, horticultural systems are subject to reduced soil organic matter, declining soil quality and fertility, and decreased microbial biomass [21–23]. Further, increased soil salinity, nutrient leaching, and soil compaction can result in CCOs, which inhibit the prospects for increased production in greenhouse systems [8,10,24]. Although the use of SMC substrates as a biofertilizer, soil amendment, and for the bioremediation of pollution has attracted much attention [16], its role as a growth media for the cultivation of crops and horticultural plants in greenhouses is not well understood [12]. Unal [13] suggested that the application of SMC as a cultivation media exhibited positive impacts on the quality of tomato seedlings, plant growth, available organic matter, and nutrient uptake of plants [25,26]. Thus, there is a need to determine exactly how SMC influences the growth of tomato plants in greenhouses, inclusive of soil microbial communities and enzyme activities under different cultivation durations.

For this study, we investigated the impacts of SMC under varying cultivation timelines on physicochemical soil properties, soil enzyme activities, and the composition of microbial communities. We hypothesized that under greenhouse conditions: (a) the application of agro-based SMC as growth media enhances the contents of major nutrients, enzyme activities, and microbial alpha-diversity in contrast to continuous mono-cropping soil; (b) soil physicochemical properties and enzyme activities decrease with longer cultivation timelines; (c) the composition of bacterial communities are more similar than fungal communities between the growth substrate and surrounding soil along with longer cultivation timelines; (d) abundant beneficial microbes can be enriched by the SMC substrate and surrounding soil, which contribute to soil amelioration and improved plant growth.

2. Materials and Methods

2.1. Experimental Design and Soil Sampling

This study was conducted in Hongtong County, Shanxi Province, on the Loess Plateau, in Northwestern China (35°87′84″ N, 111°28′91″ E). The annual average temperature is 12.7 °C, with 2079.1 h of sunlight, 441.5 mm of precipitation, and 210 frost-free days (https://www.cma.gov.cn, accessed on 1 January 2021). From May 2013, we started applying agro-based SMC substrate as growth media to cultivate tomatoes (*Solanum lycopersicum* L.) in separate plastic greenhouses. This SMC substrate compost consisted of spent mushroom compost cylinders of oyster mushroom (45~47%), cow manure (45~47%), and fragmented residues of tomato plants (6~10%), and during the composting process, the compost pile was thoroughly turned every 5 days and covered with plastic film [27]. The SMC substrates were completely dried under sunlight and used as the growth media in a groove model system under different greenhouses (Figures S1 and S2). Among them, after planting 1 year of tomato, we regard it as the 1-yr treatment. By analogy, we recognized these after planting 3 years of tomato and after planting 7 years of tomato SMC substrates as the 3-yr treatment and 7-yr treatment, respectively. The SMC substrate prior to planting tomatoes served as the substrate control (Sub CK) in the control greenhouse. Further, the soil of continuous mono-cropping tomato in the same greenhouse conditions was considered as another control treatment (Soil CK), to assess the effects of the SMC substrate amendment. Meanwhile, we selected the surrounding environmental soil which is situated at the bottom and sides of the substrate as the subjects of study. Therefore, we recognized as sampling from substrate under 1-yr treatment (Sub 1 yr); sampling from surrounding soil of 1-yr substrate (Soil 1 yr); sampling from substrate under 3-yr treatment (Sub 3 yr); sampling from surrounding soil of 3-yr substrate (Soil 3 yr); sampling from substrate under 7-yr treatment (Sub 7 yr); sampling from surrounding soil of 7-yr substrate (Soil 7 yr).

Each experimental plot was established in an independent greenhouse with an area of ca. 0.1 ha under the same fertilizer management with compound fertilizer (N: P_2O_5: K_2O, 28%:9%:63%, *w*/*w*) with about 0.75 t hm^{-2} applied through drip fertigation in the tomato fruiting period, and to prevent plant diseases and insect pest, organic phosphorus and biological pesticides were commonly applied through spraying at an interval of 10–15 days in the whole tomato growing season as well as to achieve high yield sufficient water supply was supported through drip irrigation system [28]. The physicochemical soil properties and soil microbe characteristics, as well as the surrounding soil were analyzed for all samples. Five soil core samples were randomly extracted from each experimental plot (substrates and surrounding soil, respectively) separated by at least 3 m using a soil-drilling sampler (10 cm inner diameter) in a substrate groove on October 30 2020 following the tomato harvest. Five cores from the plots were combined to form four mixed samples among three treatments and two controls, which resulted in a total of sixteen soil samples and sixteen substrate samples. All samples were sifted through a 2 mm mesh sieve, after which each sample was divided in half. One half was stored in a 50 mL centrifuge tube with liquid N and immediately transported to the laboratory pending DNA extraction. The other half was used for the quantification of physicochemical soil properties and soil enzyme activities.

2.2. Measurement of Soil Physicochemical Properties and Enzyme Activities

The soil pH was determined from the supernatant of 0.01 M $CaCl_2$ soil slurries (1:1 (*w*/*v*)) after 10 min of vigorous shaking and soil particle settling [29]. The soil organic matter (SOM, g/kg), total phosphorus (g/kg), and total nitrogen (g/kg) were determined via chromic acid titration, as well as the MADAC and Conway methods, respectively [30–32]. The soil organic carbon (g/kg) was measured using an Isoprime isotope ratio mass spectrometer with a Eurovector elemental analyzer [33]. Further, the soil alkaline phosphatase (ALP, U/g) enzyme activities were determined using a photometric technique with a 660 nm substrate and disodium p-nitrophenyl phosphate hexahydrate, which were incubated for 1 h at 37 °C [34]. The soil protease (Pro, IU/g) and β-glucosidase (β-Glu, U/g) enzyme activities were determined using a solid phase enzyme-linked immunosor-

bent assay (ELISA) method and calculated by comparing the optical density (O.D.) of the samples to the standard curve with a protease ELISA Kit and β-Glucosidase ELISA Kit at a wavelength of 450 nm [35].

2.3. DNA Extraction, PCR, and Amplicon Sequencing

The total microbial genomic DNA was extracted from 0.5 g of a soil sample using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions [36]. Using a NanoDrop NC-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, the quantity and quality, respectively, of extracted DNA were assessed. The PCR amplification of the bacterial 16S rRNA gene V3-V4 region was performed using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) and was amplified by PCR program (initial denaturation at 98 °C for 2 min, followed by 25 cycles comprised of denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final extension of 5 min at 72 °C). The fungal internal transcribed spacer (ITS) gene V1 region was determined using the forward primer (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and the reverse primer (5′-GCTGCGTTCTTCATCGATGC-3′). The PCR components contained 5 μL of buffer (5×), 0.25 μL of Fast pfu DNA polymerase (5 U/μL), 2 μL of 2.5 mM dNTPs, 1 μL (10 μM) of each forward and reverse primers, 1 μL of DNA template, and 14.75 μL of ddH_2O. The thermal cycling program proceeded as follows: initial denaturation at 98 °C for 5 min, followed by 25 cycles comprised of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, with a final extension of 5 min at 72 °C. The PCR amplicons were purified using Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified with a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). The purified amplicons were pooled in equal amounts and paired-end sequenced (2 × 500 bp) using the Illumina MiSeq platform with MiSeq Reagent Kit v3 (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China).

2.4. Data Processing and Bioinformatics Analyses

Microbiome bioinformatics were run in QIIME2 2019.4 (http://docs.qiime2.org/2019.4/tutorials, accessed on 1 November 2022) according to the standardized protocols [37]. Briefly, raw FASTQ data were demultiplexed using the demux plugin followed by primer cutting with the cutadapt plugin [38], which were then quality filtered, denoised, and merged, with chimera removed using QIIME2 with the DADA2 plugin (https://github.com/benjjneb/dada2, accessed on 1 November 2022) [39]. The remaining high-quality amplicon sequence variants (ASVs) were submitted to the SRA (Sequence Read Archive) at the National Center for Biotechnology Information (NCBI) under accession number SUB12292980 (biosample SAMN31710080) for 16S bacteria sequences and number SUB12293105 (biosample SAMN31710019) for ITS fungi sequences. Non-singleton ASV data were aligned with mafft [40] and used to construct a phylogeny with fasttree2 [41]. Alpha-diversity metrics (Chao [42], Shannon [43]), beta-diversity metrics (weighted UniFrac [44], unweighted UniFrac [45], and Bray-Curtis dissimilarity [46]) were estimated using the diversity plugin with samples were rarefied to 35986 and 57261 sequences per sample for bacteria and fungi, respectively. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier of the feature-classifier plugin [47] against the SILVA_132 database for bacteria, and UNITE_8.0 database for fungi [48].

Sequence data analyses were primarily performed using the QIIME2 and R packages (v3.2.0). The ASV-level alpha-diversity indices (Chao and Shannon) were calculated using the ASV table in QIIME2 and visualized as box plots. Beta-diversity analysis was performed to investigate the structural variations in microbial communities using Bray-Curtis metrics [46] and UniFrac distance metrics [44]. These were visualized using principal coordinate analysis (PCoA) and an unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering [49]. The significance of the differentiation (at $\leq$0.05 level)

of microbiota structures between groups was assessed by PERMANOVA (Permutational multivariate analysis of variance) [50], ANOSIM (Analysis of similarities) [51], and Permdisp [52] using QIIME2. The taxonomy compositions and abundances were visualized using MEGAN [53] and GraPhlAn [54]. A Venn diagram was created for visualization using the R package "VennDiagram" (1.7.3) [55]. The relative abundances of taxa were statistically compared between samples or treatments by MetagenomeSeq, and visualized as Manhattan plots [56]. LEfSe (Linear discriminant analysis effect size) was performed to investigate differentially abundant taxa across groups using the default parameters [57]. Random forest analysis was performed to discriminate the samples from different groups using QIIME2 [58,59].

2.5. Statistical Analysis

All of the statistical analyses were performed using the R software package (version 4.2.3), using Two-Way Analysis of Variance (two-way ANOVA) at the 95% confidence level to determine the significance to estimate the effects of tomato cultivation Duration (years), treatments and the Substrate type (substrate or surrounding soil) factors on the soil properties, enzyme activities, and microbial composition and diversity. Post-hoc multiple comparisons were performed using the agricolae package (1.3-6) [60] in Tukey's test for post-hoc multiple comparisons. Since the bacterial Alpha diversity index (Shannon) did not conform to a normal distribution, we used the Kruskal–Wallis test to determine the significance of the difference in this index across treatments at the 95% confidence level; post hoc comparisons were performed using the Kruskal function in the agricolae package when significance was detected at the $p \leq 0.05$ level. We calculated microbial community proximities between different treatments based on the Bray–Curtis distance using the vegan package [61]. Then, we performed statistical tests using Permutation Multivariate Analysis of Variance (PERMANOVA). Finally, we visualized by Principal Co-ordinate Analysis (PCoA) based on dissimilarity Distance in order to facilitate the observation of within-group sample reproducibility and distances between samples between groups. Redundancy analysis (RDA) using the Monte Carlo permutation (999 repetitions) was used to test the relationships among the soil properties, enzyme activities and dominant microbial taxa. Finally, we plotted the graphs by ggplot2 [62] to demonstrate differences in the microbial community composition and diversity between PCoA and RDA analysis results under different treatments.

3. Results

3.1. Physicochemical Soil Properties

We observed that soil physicochemical characteristics containing organic C, total P, total N, organic matter and pH were all significantly affected by the interaction of Substrate type and Duration (Table S1 and Table 1). The total N, organic matter and organic C content were significantly decreased along with longer cultivation timelines in the substrate; the Sub 7 yr treatment had lower total N (10.81 g/kg), organic matter (37.67 g/kg), and organic C content (21.87 g/kg) than the Sub 1 yr treatment, respectively. The total P and pH were higher in the Sub 1 yr treatment than the Sub 3- and 7 yr treatments, which had no differences between these last two. Only the total P had a significant difference between Sub 1 yr and Sub CK treatments. For the surrounding soil, the Soil 3 yr treatment possessed significantly higher total P than the other treatments. The organic matter and organic C content were both highest under the Soil CK treatment and decreased with the cultivation duration, but showed no significant difference among them. Soil 1 yr treatment exhibited the highest pH over the other treatments, which did not differ among themselves. Moreover, the total P, total N, organic matter, and organic C content were significantly higher for the substrate than in the surrounding soil with the cultivation duration (Table 1).

Table 1. Physicochemical soil properties of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse.

Substrate Type	Duration	Total P (g/kg)	pH	Total N (g/kg)	Organic C (g/kg)	Organic Matter (g/kg)
Substrate (Sub)	CK	2.85 ± 0.06 c	7.81 ± 0.07 a	19.24 ± 0.65 a	38.87 ± 7.83 a	67.02 ± 13.50 a
	1 yr	4.07 ± 0.02 a	7.86 ± 0.13 a	16.92 ± 0.88 a	38.57 ± 3.91 a	66.50 ± 6.75 a
	3 yr	3.11 ± 0.04 bc	7.35 ± 0.05 b	10.65 ± 0.39 b	28.60 ± 4.80 b	49.31 ± 8.28 b
	7 yr	3.22 ± 0.04 b	7.27 ± 0.05 b	6.11 ± 0.38 c	16.70 ± 1.43 c	28.83 ± 2.46 c
Surrounding soil (Soil)	CK	1.34 ± 0.04 e	7.45 ± 0.06 b	1.81 ± 0.02 d	33.97 ± 0.46 ab	58.57 ± 0.80 ab
	1 yr	1.32 ± 0.01 e	8.08 ± 0.07 a	1.29 ± 0.07 d	1.37 ± 0.32 d	2.37 ± 0.55 d
	3 yr	1.74 ± 0.08 d	7.54 ± 0.05 b	2.27 ± 0.09 d	4.35 ± 0.70 d	7.50 ± 1.21 d
	7 yr	0.74 ± 0.04 f	7.39 ± 0.01 b	1.73 ± 0.06 d	4.21 ± 0.52 d	7.25 ± 0.90 d

Data are means ± standard errors (n = 4), different letters means are significantly different at $p \leq 0.05$ (Tukey HSD).

3.2. Soil Enzyme Activities

Soil enzyme activities containing ALP, Pro and β-Glu were also significantly affected by the interaction of Substrate type and Duration (Table S1 and Table 2). Soil ALP, Pro, and β-Glu enzyme activities were highest in the substrate for the sub 1 yr treatment and significantly decreased with the cultivation duration except for ALP in sub 7 yr. For the surrounding soil, the soil ALP enzyme activity was highest in the Soil 3 yr treatment and no difference between the Soil 7 yr treatment and the soil CK. The Soil Pro enzyme activity was highest under the Soil 1 yr treatment and decreased significantly with longer cultivation durations. Conversely, the soil β-Glu enzyme activity was higher in the Soil 7 yr treatment than the other treatments.

Table 2. Enzyme activities of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in a greenhouse.

Substrate Type	Duration	ALP (U/g)	Pro (IU/g)	β-Glu (U/g)
Substrate (Sub)	CK	0.64 ± 0.01 cd	0.78 ± 0.03 c	0.37 ± 0.01 d
	1 yr	0.85 ± 0.02 a	1.61 ± 0.07 a	0.51 ± 0.01 a
	3 yr	0.67 ± 0.03 bcd	1.44 ± 0.06 b	0.40 ± 0.02 cd
	7 yr	0.71 ± 0.05 bc	0.77 ± 0.05 c	0.28 ± 0.02 e
Surrounding soil (Soil)	CK	0.61 ± 0.03 d	0.42 ± 0.02 d	0.30 ± 0.02 e
	1 yr	0.74 ± 0.03 b	1.70 ± 0.07 a	0.45 ± 0.01 b
	3 yr	0.92 ± 0.02 a	0.83 ± 0.08 c	0.44 ± 0.01 bc
	7 yr	0.61 ± 0.03 d	0.49 ± 0.03 d	0.50 ± 0.01 a

Data are means ± standard errors (n = 4), different letters means are significantly different at $p \leq 0.05$ (Tukey HSD). ALP (U/g), soil alkaline phosphatase (Unit per gram). Pro (IU/g), soil protease (Internation unit per gram). β-Glu (U/g), β-glucosidase (Unit per gram).

3.3. Microbial Alpha- and Beta-Diversity

The microbial alpha-diversity Chao1 and Shannon indices were significantly different by the interaction of Substrate type and Duration (Table S2, Figure 1). In particular, the Sub 7 yr treatments had the highest microbial (bacterial and fungal) α-diversity of the Shannon index and were significantly different compared to the Sub CK (p = 0.01 and p= 0.003, respectively). The bacterial α-diversity (Chao1 and Shannon indices) were significantly different between the Sub CK and Sub 3 yr treatments ($p < 0.001$ and $p = 0.001$, respectively) (Figure 1a). Meanwhile, the Sub 1 yr, Sub 3 yr, and Sub 7 yr treatments have significantly higher bacterial diversity in the Shannon index than the Soil CK. For the substrate, fungal alpha diversity (Chao1 and Shannon indices) was increased with the cultivation duration (Figure 1b).

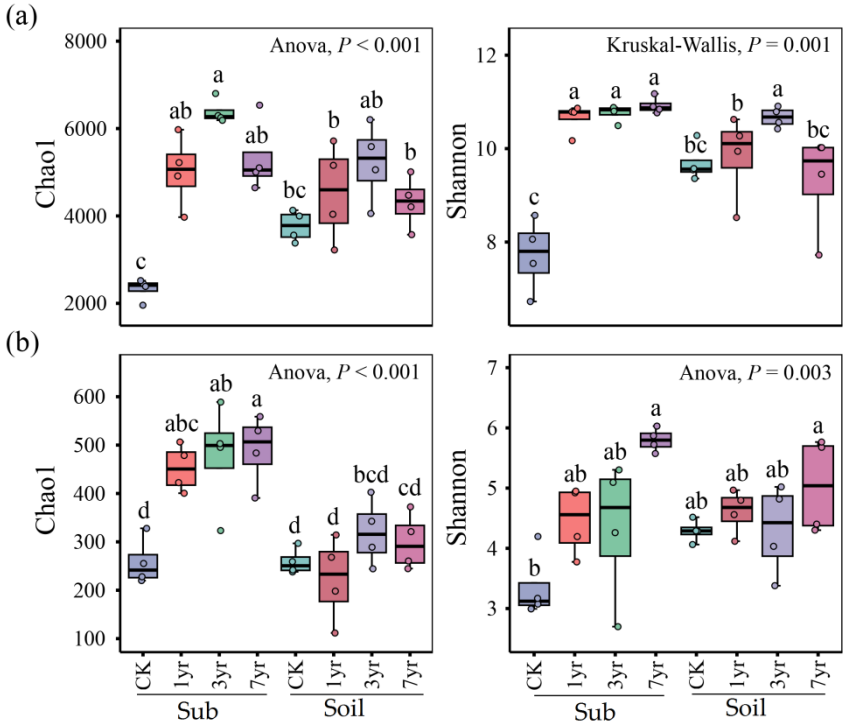

Figure 1. Bacterial (**a**) and fungal (**b**) taxa α-diversity indices of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse. Data are means ± standard errors (n = 4), different letters means are significantly different at $p \leq 0.05$ (Tukey HSD).

PCoA and Permanova results showed that the bacterial and fungal beta diversity differed between the substrate and surrounding soil under the respective cultivation durations (Figure 2). The bacterial beta diversity in the Sub CK and Soil CK treatments was significantly lower than in the other treatments. Following 1-, 3-, and 7- years of cultivation, the bacterial structural compositions of the substrates and in the surrounding soil exhibited a similar trend. The bacterial structural composition under the Sub 7 yr treatment was quite similar to the other treatments (Figure 2a). Obviously, the Sub 1-, 3-, and 7-year treatments differed in terms of their fungal community compositions in the substrates and obviously differed from the Sub CK treatment (Figure 2b). Interestingly, the Sub 7 yr treatment was more similar to the Soil 3 yr treatment in fungal structural compositions.

3.4. Microbial Community Composition

For the substrates, the bacterial taxa of relatively highest abundance, ten phyla strongly fluctuated with the cultivation duration (Table 3, Figure S3). The Sub CK treatment possessed a higher abundance of *Chloroflexi*, *Bacteroidetes*, and *Deinococcus-Thermus* phyla than did the other treatments and had a decreased trend with longer cultivation durations. In contrast, the abundances of *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Gemmatimonadetes* phyla showed an increased trend with the cultivation duration, especially the *Acidobacteria* phyla. Further, the predominant fungal taxa of the top-5 most abundant phylum were significantly different between treatments. The abundance of the *Ascomycota* phylum was higher under the Sub 1 yr treatment, while the *Mortierellomycota* phyla were higher under the Sub 3 yr treatment. However, the Sub 7-year treatment exhibited a higher abundance of *Olpidiomycota* phylum than the other treatments.

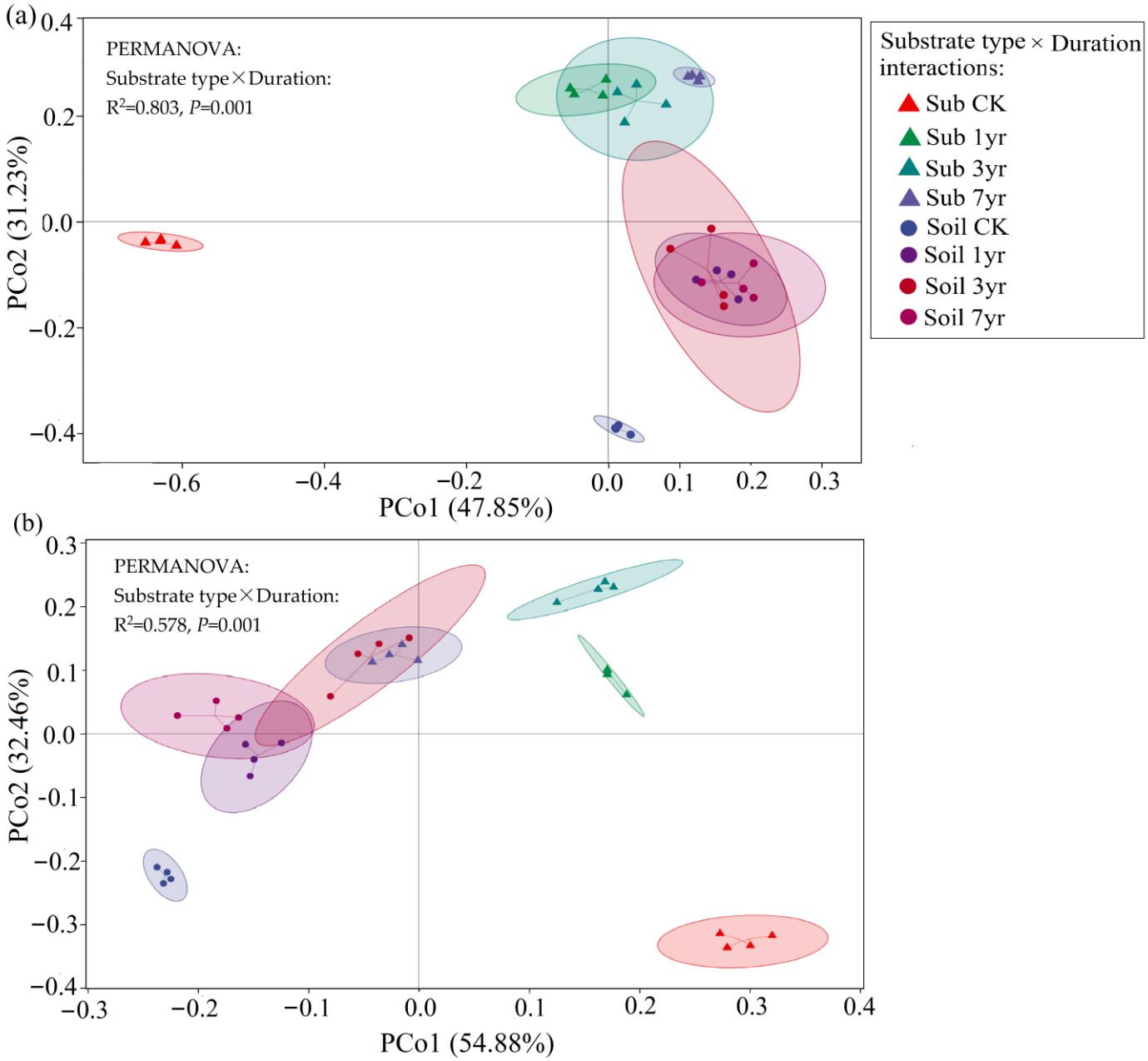

Figure 2. Bacterial (**a**) and fungal (**b**) Beta-diversity of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse.

For the surrounding soil, the abundances of *Proteobacteria*, *Firmicutes*, *Chloroflexi*, *Cyanobacteria*, *Bacteroidetes*, and *Patescibacteria* bacterial phyla varied strongly between treatments (Table 3, Figure S3). The Soil 7 yr treatment had a higher abundance of *Firmicutes*, the Soil 3-yr treatment showed higher abundances of *Chloroflexi* and *Patescibacteria* phyla, and the Soil CK treatment exhibited higher abundances of *Cyanobacteria* and *Bacteroidetes* phyla. The dominant soil fungal taxa of *Ascomycota*, *Mortierellomycota*, and *Olpidiomycota* phyla were significantly different between treatments. The Soil 7 yr treatment showed a higher abundance of *Mortierellomycota*, the Soil 1 yr treatment had a higher abundance of *Olpidiomycota*, and the Soil CK treatment had a higher abundance of *Ascomycota* phylum compared to the other treatments.

Table 3. Bacterial and fungal community composition of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse at the phyla level.

Taxon	Phyla	Substrate (Sub)				Surrounding Soil (Soil)			
		CK	1 yr	3 yr	7 yr	CK	1 yr	3 yr	7 yr
Bacteria	Acidobacteria	0.008 c	0.060 b	0.070 ab	0.069 ab	0.056 bc	0.090 a	0.090 a	0.042 bc
	Actinobacteria	0.166 c	0.261 ab	0.221 abc	0.264 a	0.187 abc	0.155 c	0.217 abc	0.172 bc
	Bacteroidetes	0.177 a	0.045 b	0.047 b	0.020 cd	0.046 b	0.018 d	0.026 c	0.017 d
	Chloroflexi	0.348 a	0.149 bc	0.132 cd	0.118 de	0.120 de	0.102 ef	0.193 ab	0.092 f
	Cyanobacteria	0.0002 e	0.001 c	0.0004 de	0.003 b	0.148 a	0.003 b	0.001 cd	0.004 b
	Deinococcus-Thermus	0.042 a	0.004 bc	0.004 bc	0.002 c	0.006 b	0.0002 d	0.004 bc	0.009 b
	Firmicutes	0.074 d	0.107 bcd	0.095 cd	0.159 bc	0.065 d	0.200 ab	0.059 d	0.392 a
	Gemmatimonadetes	0.010 c	0.025 b	0.030 b	0.028 b	0.070 a	0.038 b	0.063 a	0.032 b
	Patescibacteria	0.024 abc	0.034 ab	0.058 a	0.019 bc	0.013 cde	0.008 de	0.017 bcd	0.006 e
	Proteobacteria	0.140 c	0.300 a	0.319 a	0.295 ab	0.261 bc	0.357 a	0.296 ab	0.215 c
	Others	0.012 d	0.015 d	0.026 bc	0.024 bc	0.027 abc	0.029 ab	0.035 a	0.018 cd
Fungi	Ascomycota	0.250 c	0.727 a	0.242 c	0.465 abc	0.593 ab	0.349 bc	0.367 bc	0.327 bc
	Mortierellomycota	0.003 d	0.006 d	0.307 a	0.027 bc	0.012 cd	0.012 cd	0.0124 bc	0.206 ab
	Mucoromycota	0.0001 ab	0.0001 ab	0.001 a	0.0001 ab	0.000 b	0.000 b	0.0004 ab	0.0004 ab
	Olpidiomycota	0.000 c	0.002 ab	0.0001 bc	0.108 a	0.0003 c	0.138 a	0.0004 c	0.007 bc
	Rozellomycota	0.000 b	0.002 a	0.006 a	0.0002 b	0.000 b	0.0002 b	0.0001 b	0.0003 b
	Others	0.734 a	0.256 b	0.432 ab	0.387 b	0.386 b	0.499 ab	0.498 ab	0.450 ab

Data are means ± standard errors (n = 4), different letters in a line means are significantly different at $p \leq 0.05$ (Tukey HSD).

The heat map and clustering analysis of bacterial and fungal community compositions of relatively highest abundance twenty genera (Figure 3) revealed that the four dominant bacterial genera (*AKYG1722* (of *Chloroflexi* phyla), *A4b* (of *Chloroflexi* phyla), *Chryseolinea* (of *Bacteroidetes* phyla), and *SBR1031* (of *Chloroflexi* phyla)) (Figure 3a), and two fungal genera (*Melanocarpus* and *Sodiomyces* of the *Ascomycota* phyla) had significantly higher abundances under the Sub CK treatment (Figure 3b). The Sub 1 yr treatment showed higher abundances of bacterial genera (*KD4-96* (*Chloroflexi* phyla), *Saccharimonadales* (*Patescibacteria* phyla), *67-14* (*Actinobacteria* phyla)), and fungal genera (*Melanocarpus*, *Ascobolus*, *Thermomyces*, and *Iodophanus* (*Ascomycota* phyla)). The Sub 3 yr treatment had higher abundances of bacterial genera (*Saccharimonadales* (*Patescibacteria* phyla), *Nocardioides* (*Actinobacteria* phyla), *Subgroup_6* (*Acidobacteria* phyla), and *Gitt-GS-136* (*Chloroflexi* phyla)) (Figure 3a), and fungal genera (*Kernia* (*Ascomycota* phyla), *Mortierella*, and *Acaulium* (*Ascomycota* phyla)). The dominant bacterial genera (*67-14* (*Actinobacteria* phyla), *Nocardioides* (*Actinobacteria* phyla), *S085* (*Chloroflexi* phyla), and *Gaiella* (*Actinobacteria* phyla)), and fungal genera (*Remersonia*, *Aspergillus*, *Sodiomyces*, *Lophotrichus*, and *Fusarium* (*Ascomycota* phyla)) had significantly higher abundances under the Sub 7 yr treatment (Figure 3b). Furthermore, the Soil CK treatment revealed higher abundances of the dominant bacterial genera (*Dongia* and *Sphingomonas* (*Proteobacteria* phyla)) and fungal genera (*Myceliophthora* and *Acremonium*) of the *Ascomycota* phyla (Figure 3). The Soil 1 yr treatment had a higher abundance of bacterial *Sphingomonas* (*Proteobacteria* phyla), and two fungal genera (*Mycothermus* and *Pseudallescheria*) of the *Ascomycota* phyla. Two dominant bacterial genera (*JG30-KF-CM45* and *JG30-KF-CM66*) belonging to the *Chloroflexi* phyla, and fungal genera (*Remersonia* and *Kernia*) (*Ascomycota* phyla) were in higher abundance under the Soil 3 yr treatment. The Soil 7 yr treatment had higher abundances of bacterial genera (*Paenisporosarcina* (*Firmicutes* phyla), *Bacillus* (*Firmicutes* phyla), *Gaiella* (*Actinobacteria* phyla), and *Dongia* (*Proteobacteria* phyla)) (Figure 3a), and fungal genera (*Aphanoascus*, *Stachybotrys*, *Acremonium*, *Pseudallescheria*, *Aspergillus*, and *Mortierella*) of the *Ascomycota* phyla (Figure 3b).

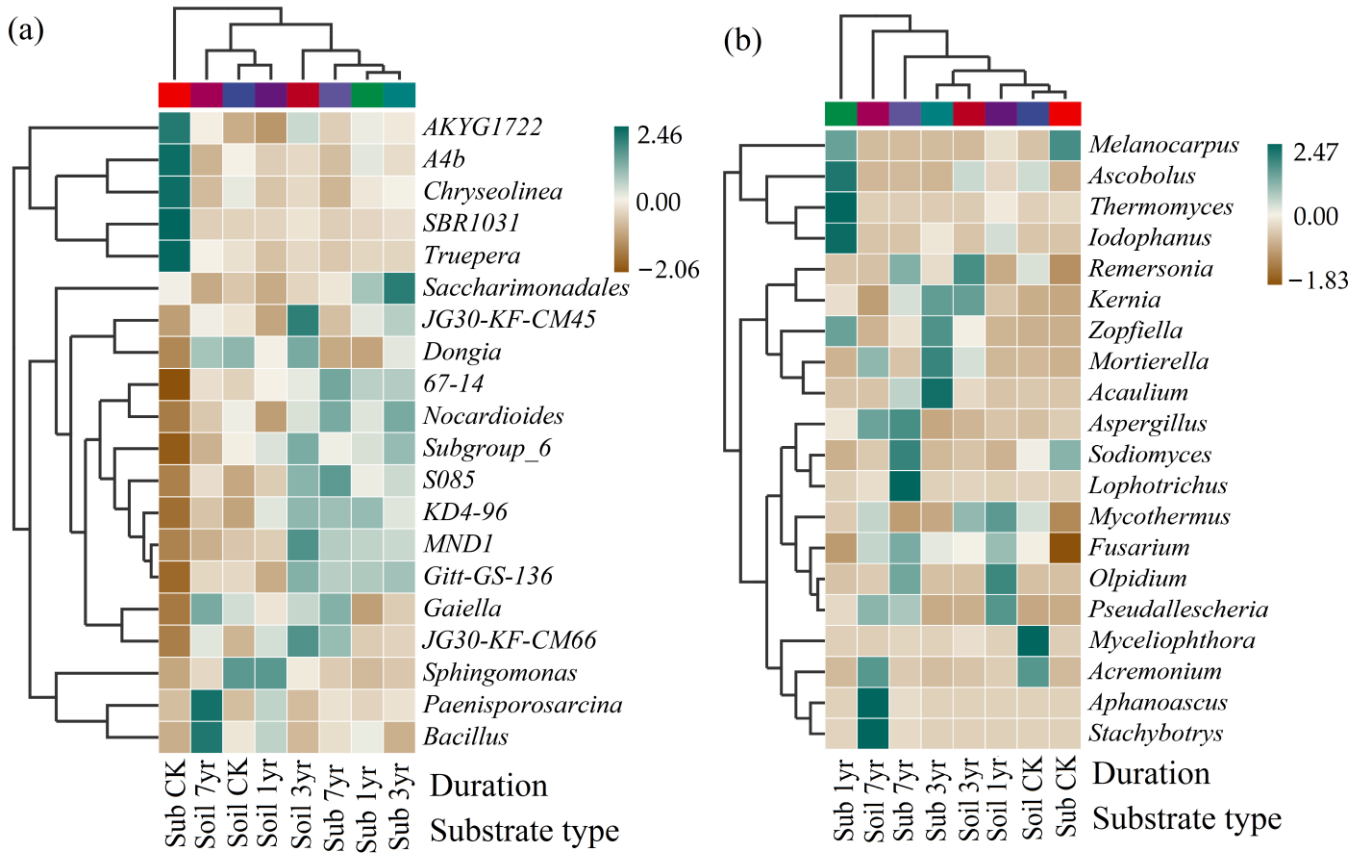

Figure 3. Heat map and clustering analysis of bacterial (**a**) and fungal (**b**) community composition of the top-20 most abundant genera. of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse.

3.5. Most Relevant Taxa Analyses

Random Forests Based Importance Analysis of the relatively highest abundance ten phyla (Figure 4), revealed that bacterial *Deinococcus-Thermus* and *Bacteroidetes* phyla and fungal *Basidiomycota* phyla were identified as the most relevant taxa of the Sub CK treatment with obviously higher relative abundances in the samples. Bacterial *Dependentiae* and *Patescibacteria* phyla, and fungal *Ascomycota* and *Rozellomycota* phyla were the most relevant taxa of the Sub 1 yr treatment. Bacterial *Patescibacteria*, *Dependentiae* and *Nitrospirae* phyla, and fungal *Mortierellomycota*, *Rozellomycota*, and *Mucoromycota* phyla were the most relevant taxa of the Sub 3 yr treatment. The most relevant taxa of the Sub 7 yr treatment were the bacterial *Halanaerobiaeota* phyla and fungal *Chytridiomycota* and *Olpidiomycota* phyla. Moreover, for the surrounding soil, the most relevant taxa of the bacterial *Verrucomicrobia* and *Armatimonadetes* phyla and fungal *Ascomycota* phylum were more highly abundant under the Soil CK treatment. Bacterial *Firmicutes*, *Dependentiae*, *Nitrospirae*, *Rokubacteria*, and *Armatimonadetes* phyla, and fungal *Chytridiomycota* and *Olpidiomycota* phyla were the most relevant taxa of the Soil 1 yr treatment. Bacterial *Rokubacteria*, *Nitrospirae*, and *Dependentiae* phyla, and fungal *Aphelidiomycota* and *Calcarisporiellomycota* phyla were the most relevant taxa of the Soil 3 yr treatment. Bacterial *Firmicutes* phyla and fungal *Blastocladiomycota* and *Mortierellomycota* phyla were the most relevant taxa in the Soil 7 yr treatment.

3.6. Correlations between Soil Properties and Dominant Microbial Phyla

Redundancy analysis (RDA) (Figure 5) showed strong correlations between the tested soil properties and dominant microbial phyla. Based on the length of the arrows, the contents of total N, organic matter, organic C, pH, and β-Glucosidase enzyme activity had extremely significant effects on shaping the microbial community. Moreover, the

β-Glucosidase enzyme had the opposite effect on the dominant microbial community in contrast to Pro and ALP enzymes.

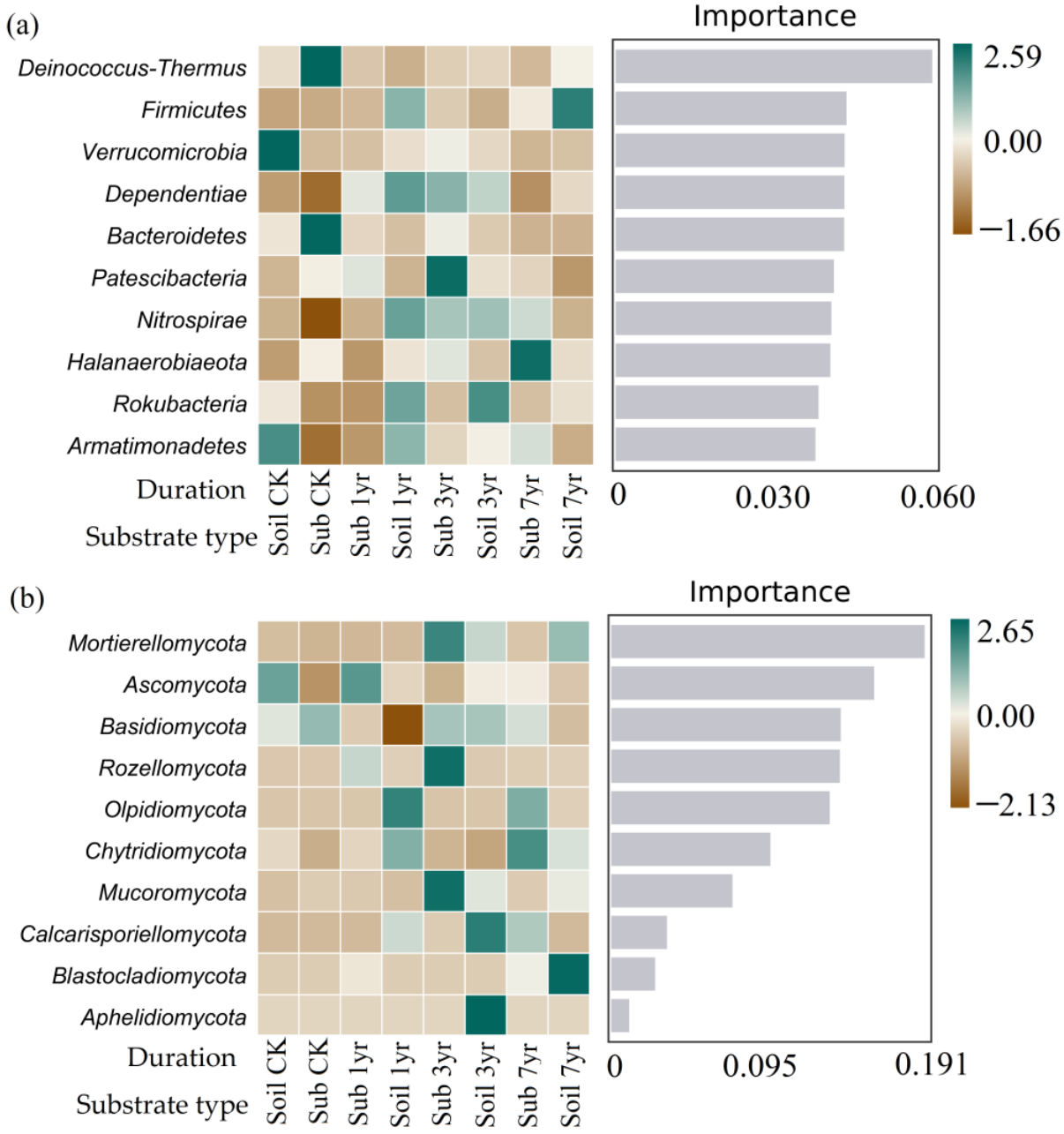

Figure 4. Random forests-based importance analysis of bacterial (**a**) and fungal (**b**) community composition of relatively highest abundance ten phyla of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse.

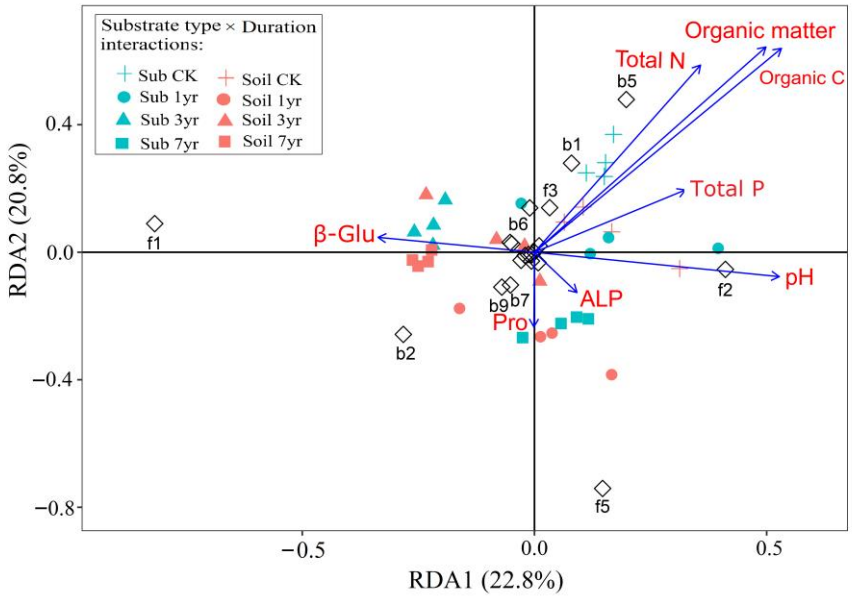

Figure 5. Redundancy analysis (RDA) of soil properties, enzyme activities and dominant microbial taxa of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse. (Details: b1–b10 represent top 10 bacteria phyla, f1–f10 represents top 10 fungi phyla. b1, Deinococcus-Thermus; b2, Firmicutes; b3, Verrucomicrobia; b4, Dependentiae; b5, Bacteroidetes; b6, Patescibacteria; b7, Nitrospirae, b8, Halanaerobiaeota; b9, Rokubacteria; b10, Armatimonadetes; f1, Mortierellomycota; f2, Ascomycota; f3, Basidiomycota; f4, Rozellomycota; f5, Olpidiomycota; f6, Chytridiomycota; f7, Mucoromycota; f8, Calcarisporiellomycota; f9, Blastocladiomycota, f10, Aphelidiomycota).

4. Discussion

Spent mushroom compost (SMC) substrates, which comprise recycled and reutilized waste products in mushroom cultivation, have been recognized as an organic material source and soil amendment for greenhouse vegetable farming [12]. In this experiment, we found that SMC substrates had more major nutrient elements such as total N and total P than continuous mono-cropping soil (soil CK) and the surrounding soil environments of the substrate (Table 1). These results reinforced that the application of SMC substrates can provide a suitable growth medium for horticultural crops [63], and can enhance the crop yields in greenhouse farming [12]. Meanwhile, the physicochemical soil properties of the SMC substrate were significantly decreased along with the duration of cultivation (Table 1). It was proved that nutrient reduction and the depletion of the SMC substrate was accompanied by plant growth with longer cultivation duration [64]. Nevertheless, the physicochemical soil properties were significantly higher in the SMC substrate than in the surrounding soil (Table 1). Lou, et al. [65] proved that the SMC substrate applied to agricultural land enhanced the soil organic matter and nutrient contents, while it reduced the total N leaching. These results demonstrated that the SMC substrate was an efficient alternative growth medium for the cultivation of horticultural crops compared to typical greenhouse soil.

Enzyme activities are an indicator of soil quality and participate in nutrient cycling during plant growth [66], which can be utilized to assess plant growth medium in ecosystems in response to available nutrients and metabolic requirements [67]. Soil alkaline phosphatases [68], oil protease [69], and β-Glucosidase [70] are important enzymes for available P, N, and C cycling processes. The concentrations of these enzymes differ between substrates and surrounding soils under various cultivation durations (Table 2), mainly due to differences in physicochemical soil properties, such as soil organic matter, total N, and pH, and was correlated with the microbial community (Table 1, Figure 5). Sinsabaugh [71] reported that extracellular enzymes are often associated with the acquisition,

transformation, and mineralization of C, P, and N in their growth medium. Among the older cultivated plants, the activities of C, N, and P cycling enzymes were reduced in the substrate medium (Table 2). It was elucidated that crop growth and harvesting can lead to decreased enzyme activities due to the reduction/shortage of nutrients year by year in the growth medium [72]. The β-Glucosidase activity was enhanced with longer cultivation duration, whereas the activities of protease and alkaline phosphatases reached their peak values under the Soil 1 yr and Soil 3 yr treatments compared to the continuous monocropping soil (Soil CK treatment), respectively (Table 2). Furthermore, enzyme activities primarily originated from root secretion and microorganisms [73], and in other studies correlated with microbial communities and structures in the growth media ecosystem [74].

Spent mushroom compost (SMC) substrates, consisting of spent mushroom compost cylinders of oyster mushrooms, livestock manure, and fragmented residues of crops, can partially or completely substitute growth media for horticultural crop production in greenhouses [75]. Meng, et al. [76] reported that SMC substrates were a better alternative than peat-based growth media for greenhouse tomato and pepper seedlings due to higher morphological growth and lower instances of *Fusarium* (of *Ascomycota* phylum) pathogen infections. It is also known that SMC substrates can be employed as an alternative source of organic matter for crop growth, which contributes to increased soil microbiological activities [77]. In this study, following multiple years of tomato cultivation, the SMC substrates still had higher microbial alpha diversity than continuous monocropping soil (Soil CK) and non-planted SMC substrates (Sub CK) treatment (Figure 1). This result confirmed that crop root systems can augment microbial diversity by recruiting more beneficial symbionts by root exudates [78,79]. Undergoing several years of tomato cultivation, the compositions of bacterial and fungal communities showed similar trend in the growth medium (substrate and surrounding soil) (Figure 2). It was revealed that continuous cropping practices altered the microbial community structures and compositions in the rhizospheric soil [80,81], as well as in the spent mushroom substrate [82]. The continuous cropping system is a common practice in greenhouse vegetable farming, which has adverse effects on horticultural crop yields and quality due to pathogenic diseases [83].

Compared with open-field cultivation, plastic-greenhouse cultivation is a popular agricultural production platform on a global scale [9]. With the advantages of a prolonged growing season with a stable hospitable environment, plastic-greenhouse cultivation is preferred in many cases for the production of high-value vegetables and other crops [84,85]. However, long-term intensive plastic-greenhouse cultivation can easily give rise to (CCO), which leads to deleterious changes in the composition of soil microbial communities [9]. Interestingly, this study suggested that the long-term intensive cropping of tomatoes with the SMC substrate growth medium (1-, 3-, and 7-years cultivation) in a plastic-greenhouse had more similar bacterial beta-diversity than the non-planted SMC substrates (Sub CK) (Figure 2). It was demonstrated that long-term greenhouse vegetable cultivation with the SMC substrate growth medium altered the structures of soil microbial communities, as reported by Liu. et al. [28]. Furthermore, the soil fungi beta-diversity of surrounding soil between the different cultivation durations (1-, 3-, and 7-years cultivation) was relatively consistent, in contrast to continuous mono-cropping soil (Soil CK) (Figure 2b). The root systems [86] and root exudates [87] of vegetable crops play critical roles in the compositional restructuring of soil microbial communities in the rhizosphere and surrounding soil environments, particularly under continuous mono-cropping [88]. Unal [13] suggested that SMC substrates were a viable alternative growth media for tomato seedling production in plastic greenhouses. Thus, SMC substrate cultivation might be a feasible strategy for the improved large-scale production of horticultural vegetables and crops in greenhouses worldwide [75]. Continuous cropping obstacles (CCO) induce declines in crop quality and yields and the exacerbation of diseases and pests [89], which may be correlated with the modification of soil enzyme activities and microorganism communities [90]. A reduction in beneficial microbes (i.e., *Bacillus* and *Trichoderma*) and accumulation of fungal pathogens *Fusarium* occurred under various continuous cropping practices in greenhouse soil [91]. *Fusarium*

wilt (FW) in strawberry was mainly caused by *Fusarium oxysporum* fungal pathogens in greenhouses [92]. In this study, the continuous cropping soil of tomatoes in greenhouses (Soil CK) exhibited a higher abundance of pathogenic bacteria of the *Cyanobacteria* phyla and fungi of the *Ascomycota* phyla (Table 3, Figure S3). Denikina, et al. [93] reported that *Cyanobacteria* taxa were associated with the diseased endemic sponge *Lubomirskia baicalensis* in Lake Baikal. Challacombe, et al. [94] revealed that *Ascomycota* fungi can exist as latent saprotrophs or pathogens within plant tissues based on genome and secretome analyses in arid ecosystems. In contrast, the SMC substrates had a higher abundance of bacteria (*Deinococcus-Thermus*, *Patescibacteria*, *Dependentiae*, *Halanaerobiaeota*, and *Nitrospirae*) (Table 3, Figure 4a) and fungi (*Basidiomycota*, *Rozellomycota*, *Mortierellomycota*, *Mucoromycota*, *Chytridiomycota*, and *Olpidiomycota*) (Table 3, Figure 4b). *Deinococcus-Thermus* bacteria are highly resistant to extreme environmental stress [95,96]. Tian, et al. [97] demonstrated that the bacteria super-phyla *Patescibacteria* contains ultra-small cells, simple membrane structures and streamlined redundant and nonessential functions to avoid phage predation, and adapt to specific stressed environments. It was reported that the *Halanaerobiaeota* phyla were dominant in extremely haloalkaline environments due to their salt tolerance and anaerobic attributes [98], while *Nitrospirae* phyla were less prevalent and participated in C and N cycling between different ecosystems [99,100]. Further, as an important ectomycorrhizal fungi, the *Basidiomycota* phyla can undergo symbiosis with host plants, which become their C sources and habitats [101]. Both *Rozellomycota* and *Chytridiomycota* fungi belong to the Zoosporic phyla with motile spores, which typically play critical ecological roles in the recycling of energy and matter in food webs [102]. As fast growing saprotrophic fungi, the *Mortierellomycota* phyla are potentially influenced by soil temperature [103] and have important biological functions for the protection of plants against pathogens [104]. Interestingly, *Mucoromycota* fungi may be utilized as a biorefinery that employs fungi for its highly versatile metabolic system, which can generate several valuable bioproducts including of pigments, polyphosphates, ethanol, organic acids, enzymes, as well as low- and high-value lipids [105,106]. Therefore, it was revealed in this study that the greenhouse SMC substrate was more enriched with several beneficial microbes than the continuous cropping soil in greenhouse.

Meanwhile, we found that the dominant taxa *Dongia* and *Sphingomonas* (*Proteobacteria* phyla) were highly abundant in the continuous mono-cropping soil (Soil CK) (Figure 3a). Pathogenic bacteria in the *Sphingomonas* genus commonly cause brown spot disease on yellow Spanish melon fruits [107], and rase Panax ginseng with rusty root disease that seriously affects its production [108]. Yet, several beneficial bacterial genera (i.e., *Saccharimonadales*, *Nocardioides*, *Gaiella*) were significantly dominant in the SMC substrate growth media for tomato cultivation in the greenhouse (Figure 3a). *Saccharimonadales* (*Patescibacteria* phylum) were influenced by soil sugar concentrations, the high abundance of which can enhance soil alkaline phosphatase activities [109]. *Nocardioides* (*Actinobacteria* phylum) was responsible for the degradation of vinyl chloride (VC), which is carcinogenic to humans [110]. Zhao, et al. [111] reported that *Nocardioides* and *Gaiella* were important beneficial bacteria for the suppression of *Fusarium* wilt in a long-term tomato monoculture soil. Even the *Bacillus* genus of important beneficial bacteria exhibited a significantly higher abundance in the surrounding soil of the 7-year cultivation (Figure 3a) [91]. Fungi were enriched in the SMC substrate and surrounding soil environment, which included several potentially beneficial fungal taxa of *Thermomyces* that secrete glycoside hydrolase and proteases [112] and coprophilous fungi of *Kernia* genus [113]. Further, the biodegradation fungi of the *Kernia* and *Mortierella* genera [24], the core fungus of *Remersonia* genus involved in humification processes [114], bioremediation microbiomes of contaminated *Aspergillus* genus environments [115], and alkaliphilic fungus of *Sodiomyces* genus were present [116], (Figure 3b). In contrast, the *Ascobolus* [117] and *Fusarium* [118] taxa of potential pathogens were significantly decreased during long-term cultivation with the SMC growth medium under greenhouse conditions.

5. Conclusions

The application of agro-based spent mushroom compost (SMC) substrates using a groove model system in greenhouses is a novel approach to the cultivation of horticultural plants. It was concluded that as an alternative growth media, SMC substrates had higher contents of total N, total P and organic carbon nutrients than the surrounding soil environments. In conjunction with longer cultivation durations, the physicochemical soil properties and soil enzyme activities were significantly reduced. However, the microbial alpha-diversity was considerably higher in the growth media of the groove model regardless of cultivation duration, in contrast to the continuous monocropping soil (Soil CK) and non-planted SMC substrate (Sub CK) treatment. The composition of bacterial communities had more similarities than fungal communities in both the SMC growth substrate and surrounding soil environment along with longer duration cultivation. Compared with the greenhouse continuous monocropping soil (Soil CK), the SMC substrate recruited more beneficial microbes, such as bacteria (*Deinococcus-Thermus*, *Halanaerobiaeota*, and *Nitrospirae* phyla) and fungi (*Basidiomycota*, *Mortierellomycota*, and *Chytridiomycota* phyla). Several potential beneficial bacterial genera (*Saccharimonadales*, *Gaiella*, *Bacillus*) and fungal genera (*Thermomyces*, *Kernia*, and *Mortierella*) were enriched in both the SMC substrate and surrounding soil. In general, the application of agro-based SMC substrates is recommended as a suitable growth media in the groove model for horticulture plant production under greenhouse conditions in North-western China.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13092291/s1, Figure S1: Schematic of SMC substrate cultivation of greenhouse tomatoes; Figure S2: Photographs of main SMC substrate cultivation processed in greenhouse, (A) Soil preparation and substrate stuffing, (B) Laying of drip irrigation pipe and black film mulching, (C) Seedling stage, (D) Harvesting period; Figure S3: Bacterial (a) and fungal (b) community composition at the phyla level of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse; Table S1: Results of two-way ANOVA for soil parameters and microbial community α-diversity of different cultivation duration (CK, 1 yr, 3 yr, 7 yr) of the substrate and the surrounding soil cultivated on tomato in greenhouse.

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Data Availability Statement: Nucleotide sequence data [The nucleotide data have been submitted and deposited to the SRA (Sequence Read Archive) at the National Center for Biotechnology Information (NCBI) under accession number SUB12292980 (biosample SAMN31710080) for 16S bacteria sequences and number SUB12293105 (biosample SAMN31710019) for ITS fungi sequences. The review links as below: https://dataview.ncbi.nlm.nih.gov/object/PRJNA901255?reviewer=9n9qumsq784i9klabfct48k0db, accessed on 14 November 2022]: The nucleotide data have been submitted and deposited in the GenBank databases.

Conflicts of Interest: The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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