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The Microbial Community in a Substrate of Solid-State Fermentation by *Lentinula edodes*: A Preliminary Study

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Abstract: Edible-fungal-based solid-state fermentation holds promise for sustainable food and biofuel production. Understanding the role of microbial communities in fungal substrates is crucial. Birch-based substrates were treated with autoclaving (121 °C, at 2 bar) or hot air pasteurization (75–100 °C), followed by incubation with and without shiitake (*Lentinula edodes*) inoculum. Mycelial growth was monitored by CO_2 release and microbial biomass by phosphate-lipid fatty acid (PLFA). DNA sequencing was used to analyze the microbial communities. Results showed successful colonization of shiitake on all substrates, regardless of pasteurization temperatures and coexisting microbes. Total microbial respiration (CO_2) and PLFA biomass showed no significant differences between pasteurization regimes. However, significant microbial differences were found between shiitake-inoculated and non-inoculated treatments. DNA sequencing revealed the dominance of *Phyllobacterium*, *Sphingomonas*, and *Pelomonas* genera in all inoculated substrates, while non-inoculated substrates were abundant in *Bacillus* spp. and *Paenibacillus* spp. of the *Firmicutes* phylum. This study provides preliminary insights into the microbial community in birch-based shiitake substrates, facilitating further investigation of bacteria involved in shiitake mycelium growth promotion and biochemical conversion for biofuel production.

Keywords: edible fungi; biofuel; hot-air pasteurization; microbial composition; wood substrate; respirator incubation; biorefinery

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1. Introduction

The global situations regarding increasing greenhouse gases and growing populations require actions to develop climate-smart food and energy production systems. Recent studies [1,2] have demonstrated a sustainable approach in fungal biotechnology, where local non-food biomass residues are first used as substrates for growing shiitake (*Lentinula edodes* (Berk.) Pegler) and, after harvesting of shiitake fruit bodies, spent mushroom substrates (SMS) are then recycled as feedstock for production of biofuels such as biogas or cellulosic ethanol. The key underlying process in this system is that the lignin is selectively degraded by the fungi, thereby enabling an efficient bioconversion of the cellulose feedstock to biofuels [3].

Production of shiitake mushroom requires a deactivation of competitive microbes in the growing substrate prior to inoculation. Globally, pressurized steam at 121 °C is often

used for substrate sterilization. However, steam production requires intensive energy input and operation of sterilization requires high costs for pressurized equipment as well as maintenance. Our previous study [2] demonstrated that hot-air pasteurization at 75–100 °C and atmospheric pressure could be an alternative solution. The hot air pasteurization of substrate led to faster growth of mycelia, earlier fructification and higher or comparable yield of shiitake compared with steam-sterilized treatment. It was also observed that hot-air pasteurization at as low as 75–85 °C had a comparable effect to autoclaving with over-pressured steam at 121 °C on substrate lignocellulose degradation by shiitake [4]. Hot air production and operation are much easier and cheaper than steam, for example, it can be obtained from recovered/waste heat, or directly from a hot water boiler. In contrast, steam autoclaving requires 60% more energy compared to 85 °C hot-air pasteurization (784 vs. 341 kJ per kg wet substrate) and additional costs for pressurized equipment [4]. This suggests the use of hot air pasteurization may considerably increase the energy efficiency, cost effectiveness and environmentally friendliness for integrated production of food and biofuel. However, the reasons for why positive effects on mushroom production were observed after hot air pasteurization are unclear. It is known that the mild heat (<100 °C) of pasteurization can deactivate a part microorganisms including vegetative bacteria, but other part and most bacteria spore may survive the process [5]. This contrasts to heat sterilization by saturated steam that can more efficiently destroy microbes including heat-resistant bacteria [5]. Therefore, one hypotheses of this study is that some symbiotic microbes may exist in the substrate and promote the shiitake mycelium growth, possibly due to the low-temperature heat treatment allowing a higher survival rate of certain microbial species. Thus, the effect of different pasteurization regimes on the microbial community in mushroom substrate is the first focus of this study.

Numerous studies have demonstrated symbiotic interactions between edible fungi and bacteria in the growth substrates of, for example, *Agaricus bisporus*, e.g., [6,7], *Pleurotus ostreatus* [8,9] and *P. eryngii* [10,11]. The symbiotic bacteria can promote the fungi by facilitating nutrient uptake, mycelial growth, primordia development and fruiting body formation, and preventing pathogen contamination ([12] and literature reviewed therein). It was suggested that a low temperature (<70 °C) pasteurization would benefit a symbiotic microbial composition in the substrate for *A. bisporus*, in which *Pseudomonas* spp. was found to be responsible for promoting mycelial growth [6,12]. In the substrate used for *Pleurotus* spp. that is often pasteurized at <70 °C, several bacterial species, such as *Bacillus* spp. and *Pseudomonas* spp. were attributed to stimulating mycelial growth and inducing fruiting body formation [11,13]. Similar to plant-growth promoting rhizobacteria, it has been suggested that the capability of bacteria to produce hormone-like compounds, and/or to dissolve bound phosphate and to secrete siderophores present examples of the mechanisms behind the promoting effects [13,14]. Some bacterial genera, such as *Bacillus*, have the ability, through endospore formation, to tolerate high temperature environment [14]. Thus, it is not unlikely that some bacteria can survive from the hot air pasteurization and coexist in the mushroom growing substrates. To the best of our knowledge, studies on the microbial communities in shiitake substrates are so far rarely published.

The main goal of this study was to explore the compositional structure of the microbial community in shiitake growth substrates. For this, an incubation experiment of shiitake mycelia was performed in pretreated substrates and an environmentally-controlled respirator. Microbial respiration, biomass, and species composition in active substrates were investigated. A literature review on three major co-exiting species was also performed to discuss their potential roles in mushroom substrates and implications for future studies.

2. Materials and Methods

2.1. Materials and Experiment Settings

The initial substrate was composed of downy birch (*Betula pubescens* Ehrh.) sawdust (≤4 mm) at 80% dry mass (DM), wheat (*Triticum aestivum*) grain (3–8 mm) at 10% DM, wheat bran (≤2 mm) at 10% DM and $CaCO_3$ (<0.2 mm) at <0.35% DM. The mixture of all

ingredients was then moistened to 65% water content (wet mass basis). An ultimate analysis on the substrate composition can be found in Xiong et al. (2019) [2]. The mixed substrate had a pH value of 6.34, C/N ratio of 95 and N/P ratio of 3.3. Grain spawn of commercial shiitake strain M3790 (Mycelia BVBA, Brussel, Belgium http://www.mycelia.be/ (accessed on 1 August 2023)) was used as the inoculum.

The main experiment was composed of 8 treatments of the initial substrate, resulting from combinations of 4 pasteurization regimes and 2 options of inoculation (i.e., with vs. without shiitake inoculum) (Table 1). The well-mixed substrate was divided into four parts, each containing 100–150 g in a separate polypropylene plastic bag. One bag was heat-treated in an autoclave (i.e., treatment T121) by saturated steam. Three other bags were hot-air pasteurized in an oven at 100 °C (T100), 85 °C (T85) and 75 °C (T75), respectively. All heat treatments were employed for one hour. After the heat treatments, the substrates were cooled to room temperature. Then, 5 g of each heat-treated substrate was taken to fill in one pre-sterilized polypropylene cups (4.5 cm in diameter and 5 cm in height), with a bulk density of around 600 g/L. Eight replicated cups were prepared for each heat treatment regime, including 4 cups with inoculation of 0.5 g of grain spawn of shiitake per cup and 4 cups without any inoculation. The inoculation was performed under a sterile bench. In total 32 cups of the substrates plus 4 empty cups (control) were installed in the Respicond respirometer (Figure 1; A. Nordgren Innovations AB, Sweden, http://www.respicond.com/ (accessed on 1 August 2023)).

Table 1. Experimental set-ups and performed analyses.

Heat treatment (sterilization/pasteurization)	T121: Steam 121 °C with 2 bar pressure T100: Hot air 100 °C for 1 h T85: Hot air 85 °C for 1 h T75: Hot air 75 °C for 1 h
Shiitake inoculation	Inoculated vs. non-inoculated
Incubation	At 20 °C; in Respicond respirator
Measurement/analysis	CO_2 release ratio (mg/h); CO_2 release accumulated (mg); Profile of microbial community structure and taxonomy by PLFA analysis; and ITS 18S and 16S rRNA gene sequencing.

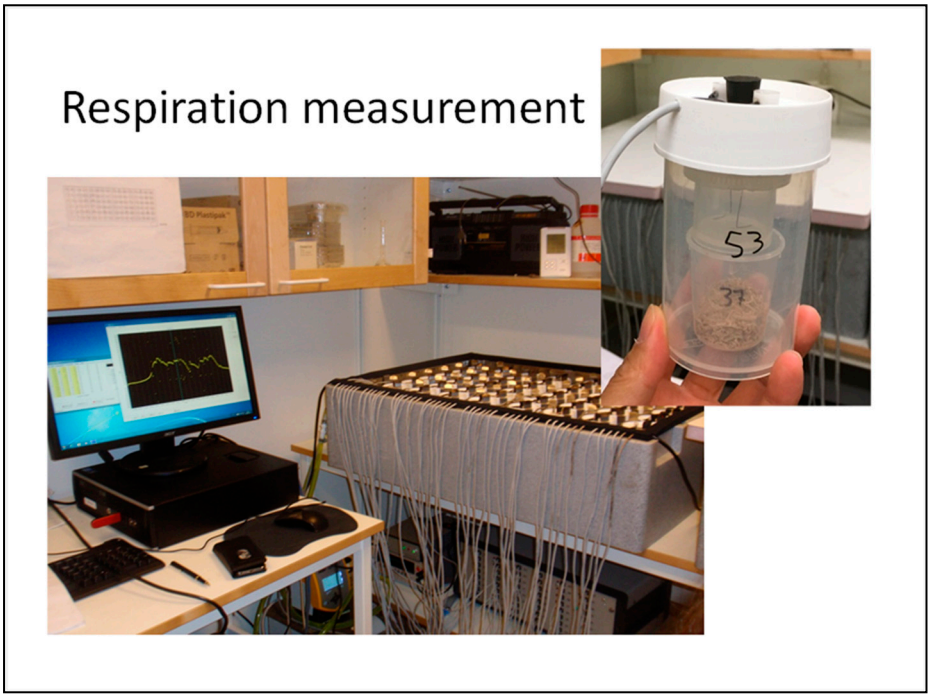

Figure 1. Respicond experiment setting for the estimation of microbial respiration.

2.2. Microbial Incubation and Respiration Measurement

The Respicond respirometer [15] was used to incubate and measure the microbial respiration rate during a period of 24 days. The respiration rate was quantified by CO_2 released from microbial activities that was trapped in KOH solution, which could be calculated by measuring the change in conductance of the KOH traps at regular intervals. The equipment has been previously adapted to soil microbiological studies, which allowed multiple samples (up to 96, one in each vessel), to be measured simultaneously and automatically. Thus, an incubation of microbes in the respirometer allowed a digital measurement of microbial growth. The polypropylene substrate cups (without lid), filled with substrates after inoculation, were randomly distributed inside pre-sterilized vessels (8 cm in diameter and 10 cm in height) of the Respicond respirator (Figure 1). All vessels were placed in a common water bath at 20 °C to maintain homogeneous and constant incubation temperature. Care was taken to avoid external microbial contaminations when handling substrates and inoculation as well as setting up vessels.

The respiration ratio was registered hourly. On 24 days of incubation, when CO_2 emission curves became leveled out and stable, every individual substrate together with the cup was entirely removed from the respirator and lyophilized immediately using Christ Freeze Dryer (Martin Christ, Germany). After drying, each substrate was individually sealed and kept in the same cup and stored at room temperature before further analysis. Accumulated CO_2 was quantified by summing up hour-ratios of CO_2 emission (i.e., respiration ratio), using the formula:

$$Accumlated\ CO_2 = \sum_{d=1}^{24} \sum_{h=1}^{24} CO_2(\mathrm{mgh}^{-1})$$

where d = day and h = hour.

2.3. Profiling of Microbial Community Structure and Composition

Two different methods were used to characterize the microbial communities in a selection of the different experiments as depicted in Table 1: phospholipid fatty acid (PLFA) analysis and DNA sequencing of the overall microbial taxonomy.

PLFA analysis. Phospholipid fatty acid (PLFA) analysis was performed to characterize the structure of the microbial community in the different substrates after 24 days of incubation in the respirator. PLFA has been used for analyzing structure and activities of microbial communities in soils [16] and in composts [7,17], since PLFAs can serve as molecular markers of a selection of groups of living microbes. The samples of lyophilized substrates from Respicond trials, 0.5 g of each replicate, were analyzed on a gas chromatograph from Perkin Elmer with a FID detector and an Elite-5MS column (L 30 m ID 0.25 DF 0.25). For each substrate, the abundance of each fatty acid extracted was expressed as relative nmoles per g of dry substrate mass.

The identification of microbial groups depending on their PLFA profile pattern followed the classification system developed by Frostegård and Bååth [16] and Kroppenstedt [18]. The PLFAs i15:0, a15:0, 15:00, i16:0, 16:1w9, 16:1w7t, 16:1w7c, i17:0, a17:0, cy17:0, 18:1w7, and cy19:0 were used to indicate and calculate the sum of relative bacterial biomass, 18:2w6 was for fungal biomass, and 10Me17:0 and 10Me18:0 were for actinomycete biomass. PLFAs of 16:0, 17:0, and 18:0, previously used as bacterial biomass markers and 16:1w5 and 18:1w9 as fungal biomarkers [19], were not included in the current analysis as they were found in shiitake [20] or other organisms [21]. The PLFAs i-15:0, a-15:0, i-16:0, i-17:0 and a17:00 were included in the calculations for the sum of Gram-positive bacteria, while the PLFAs 16:1w9, 16:1w7c, 16:w7t and 18:1w7 were used for sum of Gram-negative bacteria.

DNA sequencing of the microbial communities. To identify fungal and bacterial species that co-existed in the different substrates after 24 days incubation in the Respicond, DNA sequencing on the lyophilized substrates was performed by the Next-Generation sequencing company Novagene; Hong Kong (https://en.novogene.com/, accessed on 15 May

2023). Four individual samples of each treatment were pooled proportionally (20% each), under a sterile hood, into one mixed sample that was then used for DNA sequencing. For elucidation of the phylogenetic affiliation, 16S rRNA analysis was performed for bacterial species and ITS18S rRNA analysis for fungal species. 16S rRNA/18SrRNA/ITS genes of distinct regions (16SV4/16SV3/16SV3-V4/16SV4-V5, 18S V4/18S V9, ITS1/ITS2, Arc V4) were amplified using specific primer pairs (e.g., 16S V4: 515F-806R, 18S V4: 528F-706R, and 18S V9: 1380F-1510R) with sequencing barcodes. All PCR reactions were carried out with the Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The detailed procedures for DNA extraction, amplification, amplicon library and sequencing can be found in Novogene's website (https://en.novogene.com/services/research-services/metagenome-sequencing/16s-18s-its-amplicon-metagenomic-sequencing/, accessed on 15 May 2022). The amplicon sequencing data were deposited at the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena/browser/home, accessed on 31 May 2022) under the accession PRJEB53156.

2.4. Data Analysis

ANOVA followed by post hoc multiple comparisons (Tukey's test) was conducted to analyze the differences between the heat treatments and between inoculated and non-inoculated substrates in microbial respiration (accumulation CO_2) and biomass (PLFA) using SPSS statistical analysis software (IBM SPSS version 24.0).

3. Results

3.1. Microbial Activities in Shiitake Substrates

After 24 days of incubation in the respirator "Respicond", a full colonization of shiitake mycelia was observed in all inoculated substrates. There was a significant difference between inoculated and non-inoculated substrates in both respiration ratios, measured as CO_2 mg/h (Figure 2a,b), and accumulated CO_2 release (Figure 2c). It was observed that T85 and T75 had a couple of days earlier start of colonization than T100 and T121, which is consistent to the pattern of CO_2 emission during the first week (Figure 2a). However, CO_2 emissions from high-temperature treatment especially T121 exhibited at a higher ratio than T85 and T75. No significant differences were observed for accumulated CO_2 during incubation of 24 days between the heat treatments for the inoculated substrates. In non-inoculated substrates, considerable microbial respiration was measured with treatments of 75 °C and 85 °C in contrast to the treatments of 100 °C and 121 °C that resulted in negligible CO_2 release. This suggests that the high-temperature treatments remained relatively sterile during the entire incubation period. Therefore, non-inoculated T100 and T121 are not included in the following discussion on compositions of the microbial communities.

3.2. Microbial Community Structure Profile Based on PFLA Studies

PLFA analysis indicated that all four inoculated substrates and two non-inoculated substrates contained both fungi and bacteria (Figure 3a–d). The treatments of inoculated versus non-inoculated affected the dominance/existence of the studied organism groups significantly ($p < 0.001$). Fungal species were expectedly dominant in inoculated substrates where shiitake mycelium was growing. Compared with the effects of inoculation, the effects of temperature for pasteurization on fungal biomass was minor and not significant ($p > 0.05$) within inoculated treatments (Figure 3a). In the non-inoculated substrates (thus not supposed to contain shiitake), the PLFA of other fungal species was found in similar quantity (161.84 ± 17.41 vs. 178.48 ± 12.72 nmol g^{-1} DM) in the substrates subjected to 75–85 °C pasteurization.

The bacterial biomass was also more affected by inoculation than pasteurization temperature. There were no significant differences ($p > 0.05$) in bacterial biomass between the heat treatments regardless the inoculated and non-inoculated substrates (Figure 3b). However, the bacterial biomass was significantly higher ($p < 0.05$) than the fungal biomass in the two non-inoculated substrates, where the ratios of fungi to bacteria were 0.61 and 0.69

for 75 °C and 85 °C pasteurizations, respectively. The growth of inoculated shiitake had an inhibitory effect on the bacteria as a whole, which may explain a blooming of bacteria in the non-inoculated and 75–85 °C pasteurized substrates.

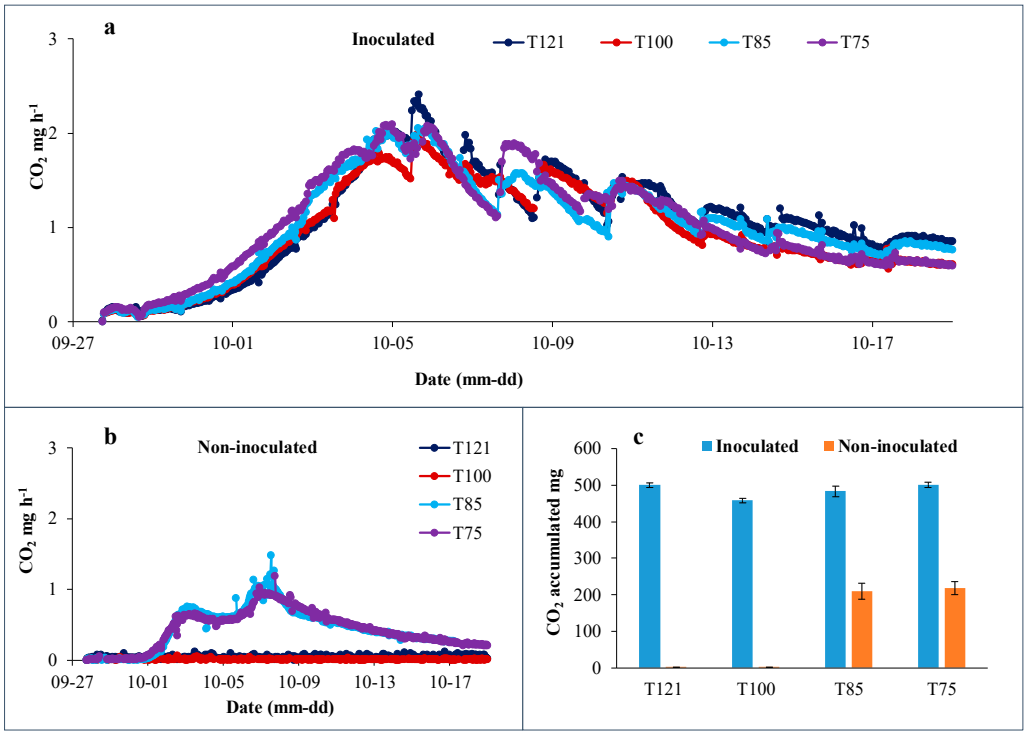

Figure 2. Ratio of microbial respiration in inoculated (**a**) and non-inoculated substrates (**b**); as well as accumulation (**c**) of CO_2 released by microorganism respiration in substrates subjected to different heat treatments during the incubation of 24 days. Data are averages of four replicates for each treatment.

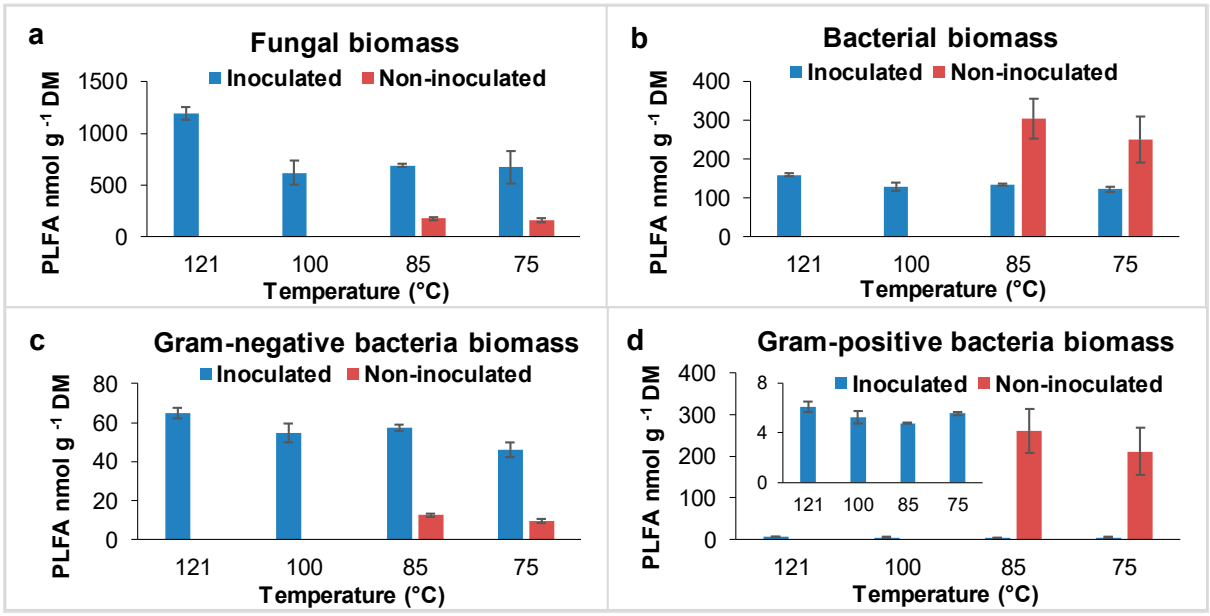

Figure 3. Microbial biomass (measured by phospholipid fatty acid (PLFA) analysis) in the substrates subjected to different heat treatments. Microbial groups were classified based on their PLFA-signatures (**a**–**d**) and grouped based on whether the growth substrate was inoculated with Shiitake fungus or not.

Figure 3c,d demonstrate the observed changes in the subgroups of Gram-negative and Gram-positive bacteria in different treatments. In the inoculated treatments, the weight of Gram-negative bacteria was >10 times higher ($p < 0.001$) than that of Gram-positive species. In contrast, the Gram-positive bacteria dominated in the non-inoculated and 75–85 °C pasteurized substrates.

3.3. Microbial Community Composition Based on DNA Sequencing

Fungal taxa in different substrates. The DNA sequencing (targeting the ITS within the 18S ribosomal RNA (rRNA) gene) generated 17 different fungal taxa in total from 6 treatments. Seven of the 17 taxa were not identifiable by blasting trials. The remaining 10 strains showed significant affiliations to two fungal phyla: *Basidiomycota* (Figure 4a) in the inoculated treatments and *Ascomycota* in non-inoculated ones of 75 °C and 85 °C. The two phyla covered 87.21% of all retrieved gene sequences across all six treatments. Four families and two genera could be ascertained (Figure 4b,c) by blast hits, representing 83.09% and 82.99% of all sequenced strains, respectively. The taxa that could not be identified with regard to the genus or family taxonomical level are shown as "others" in Figure 4. *L. edodes* was identified as the sole species of genus *Lentinula* in the inoculated substrates, while *Apiospora montagnel* represented the only species of *Apiospora* in both inoculated and non-inoculated substrates.

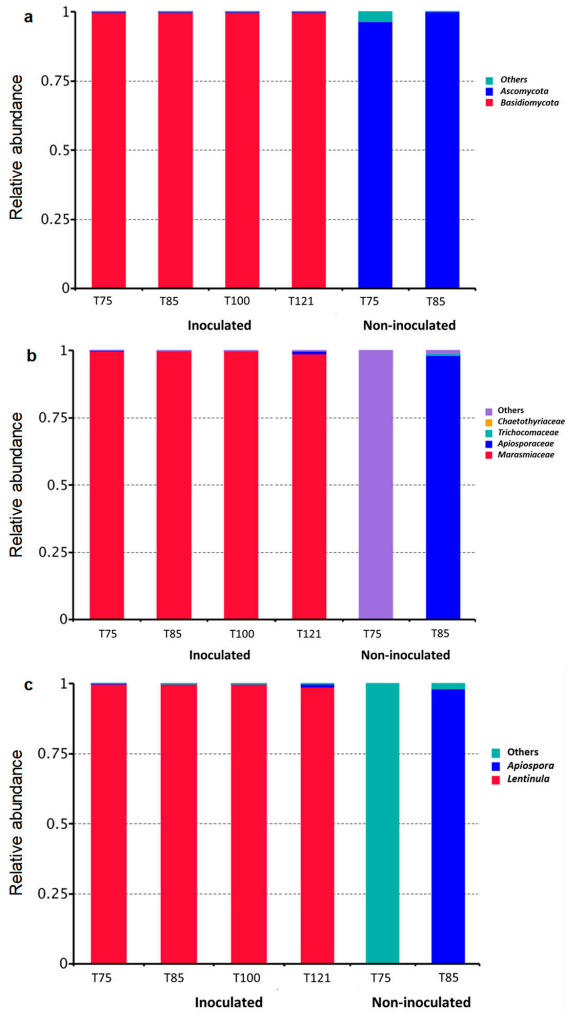

Figure 4. Relative abundance of fungal species belonging to the top 10 fungal phyla (**a**), families (**b**) and genera (**c**) based on sequencing of the ITS 18S rRNA gene. The top 10 taxa in the different taxonomic ranks represent 87.21% of all detected fungal taxa.

Bacterial taxa in different substrates. Across all 6 treatments, more than 238 bacterial taxa affiliated to 23 phyla were detected in the substrates by DNA sequencing of the 16S rRNA genes. The top 10 phyla representing 99.69% of all sequences are listed in Figure 5a. The most dominant bacterial taxa in the inoculated substrates belonged to the phylum *Proteobacteria*. In the non-inoculated 75 °C and 85 °C treatments, however, the most dominant bacterial taxa belonged to the phylum *Firmicutes*.

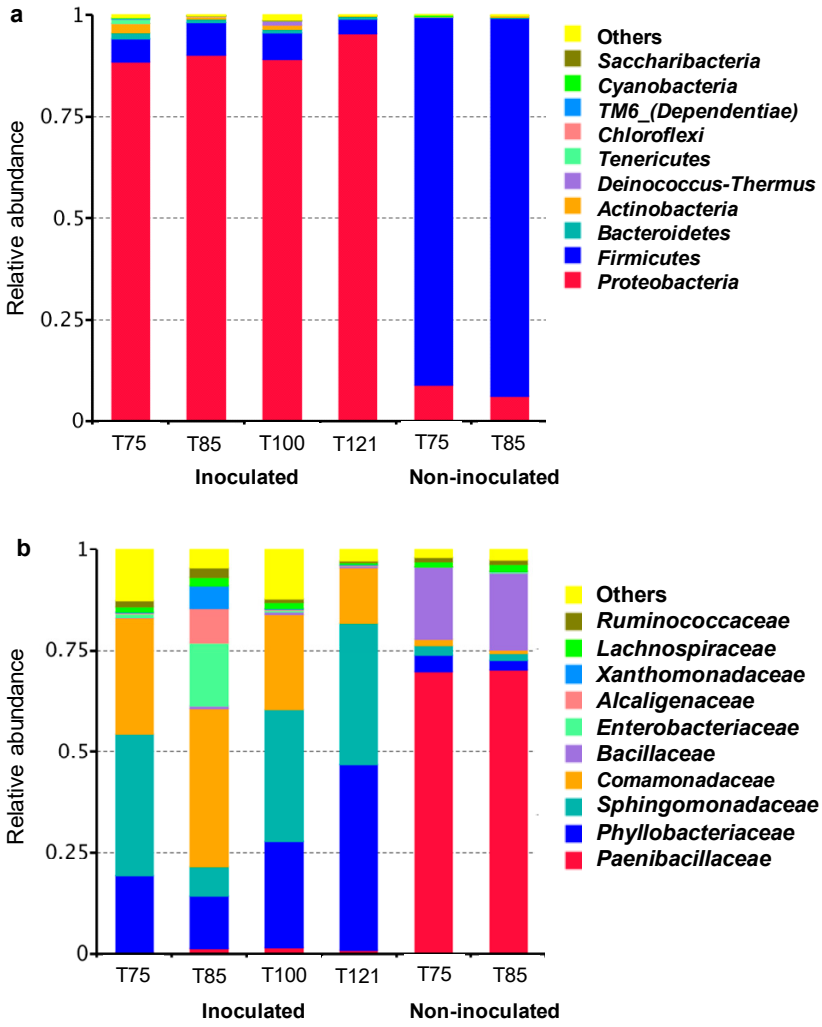

Figure 5. Relative abundance of bacteria grouped in top 10 phyla (**a**) and top 10 families (**b**) in the substrates subjected to different treatments. Data are based on a 16S rRNA gene sequence analysis.

Figure 5b shows 10 of the 126 detected bacterial families with the highest abundance of sequences across all 6 treatments. The top 10 families represent 93.77% of all bacterial sequences. It is evident that the abundant distribution of the dominant bacterial families in the substrates varied considerably with treatments. Six of the top ten families belonged to *Proteobacteria* phylum and were dominant in the inoculated substrates, while the other four families belonged to *Firmicutes* and dominated in non-inoculated substrates, where *Paenibacillaceae* had a >70% of relative abundance.

Further analysis on the species abundance of the top 10 dominant genera is demonstrated in Table 2, which covers at least 17 species and represents 89.71% of all bacterial taxa found across all treatments. A few phenomena are worth paying attention to. (1) For all 4 inoculated substrates, *Phyllobacterium* spp., *Sphingomonas leidyi*, *Pelomonas* spp. were the most dominant bacterial species among the top 10 genera found. (2) For the non-inoculated substrates, the dominant species were *Cohnella xylanilytica*, *Paenibacillus favisporus* and *Bacillus* spp. in 85 °C pasteurized substrate; while *Paenibacillus* spp., *Bacillus circulans* and

Paenibacillus odorifer dominated in 75 °C pasteurized substrate. In contrast, these species showed a considerable low abundance in the inoculated substrates subjected to the same heat treatments of 75–85 °C, suggesting they might be inhibited by shiitake mycelia. (3) For 75–85 °C pasteurized substrates, the relative abundance of most of the seven genera/species affiliated to *Proteobacteria*, except for *Achromobacter xylosoxidans* at 75 °C, was higher in the shiitake inoculated substrate than in non-inoculated treatments; thus, it is likely that these bacteria were enhanced by shiitake growth. (4) Except for *Bacillus subterraneus*, all other 16 identified bacterial species (Table 2) were common in both inoculated and non-inoculated treatments, despite differences in abundance. This suggests that these 16 species could have an origin in the initial substrate ingredients, e.g., birch sawdust, wheat grain and bran. However, *B. subterraneus* showed an uncertainty of its origin. It could be still originated from substrate but become eliminated by stronger competitors in non-inoculated, although an alternative hypothesis of grain-spawn origin cannot be exclude by this study.

Table 2. Survey of major bacterial species and their relative abundance (%) in the top 10 genera found in different treatments. Data are based on a 16S rRNA analysis.

						Inoculated				Non-Inoculated	
Phylum	**Classs**	**Order**	**Family**	**Genus**	**Species**	**T121**	**T100**	**T85**	**T75**	**T85**	**T75**
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	*B. circulans*	0.20	0.35	0.31	0.11	0.49	18.60
					B. subterraneus	<0.01	<0.01	0.01	<0.01		
					B. spp.	0.18	0.32	0.25	0.09	19.91	0.88
				Cohnella	*C. xylanilytica*	0.30	0.44	0.48	0.15	49.95	0.42
			Paenibacillaceae	*Paenibacillus*	*P. alginolyticus*	0.01	0.01	<0.01	<0.01	0.01	0.20
					P. favisporus	0.12	0.21	0.17	0.09	20.96	3.28
					P. lautus	0.01	0.02	0.01	<0.01	0.03	0.63
					P. odorifer	0.11	0.13	0.36	0.06	0.23	12.02
					P. spp. *i76*	0.01	0.01	0.01	<0.01	0.01	1.48
					P. spp.	0.37	0.78	0.31	0.31	2.36	53.49
Proteobacteria	*α-proteobacteria*	*Rhizobiales*	*Phyllobacteriaceae*	*Phyllobacterium*	*Ph.* Spp.	47.57	30.94	14.32	22.63	2.50	4.32
		Sphingomonadales	*Sphingomonadaceae*	*Sphingomonas*	*S. leidyi*	36.40	38.8	7.73	41.36	1.74	2.76
	β-proteobacteria	*Burkholdderiales*	*Alcaligenaceae*	*Achromobacter*	*A. xylosoxidans*	<0.01	0.09	9.18	0.03	0.04	0.04
			Comamonadaceae	*Delftia*	*D.* spp.	0.16	0.39	26.11	0.17	0.06	0.04
				Pelomonas	*P.* spp.	14.24	27.21	17.46	34.33	1.19	1.54
	γ-proteobacteria	*Enterobacteriales*	*Enterobacteriaceae*	*Serratia*	*S.* spp.	0.10	0.15	17.24	0.33	0.13	0.05
				Pseudomonas	*P.* spp.	0.09	0.07	6.02	0.15	0.03	0.03
Ratio of the identified ones to all bacterial sequences of the top 10 genera %						96.01	84.26	90.36	82.94	92.82	91.84

4. Discussion

4.1. The Microbial Community after Different Heat Treatments

After 24 days of incubation in a respirator at 20 °C, shiitake mycelia colonized well in all inoculated substrates (Figure 2a); however, a significant difference in growth speed could be observed. A faster growth (up to 3–4 days earlier) was observed in the 75–85 °C pasteurized substrates. The respiration patterns in the inoculated substrates (Figure 2a) and the non-inoculated substrates (Figure 2b) differed clearly. In the inoculated substrates there was a steady increase in carbon dioxide emission already during the first days. This pattern agrees well with the colonization of prepared inoculum of white-rot fungi (i.e., shiitake spawn) already adapted for growth on this type of lignocellulosic substrate. In the non-inoculated substrates, a lack of respiration was observed in the high-temperature treatments, evidently demonstrating that the sterilization treatments were applied successfully. For the low-temperature treatments, survival of some heat resistant bacteria (e.g., endospore-forming bacteria) can be expected and this agrees well with the fact that respiration is confirmed in these treatments even in the absence of shiitake inoculum. However, for most of the microorganisms, besides the white-rot fungi, lignocellulosic substrates are too complex to degrade [22]. Thus, as demonstrated in Figure 2b compared to Figure 2a, a longer lag-phase can be expected. Furthermore, the second lag-phase in Figure 2b suggests that a phenomenon similar to diauxic shift occurs. This shift may be explained by metabolization of more easily available carbohydrates in the first step followed by either a change in microbial community structure or induction of lignin-degrading enzymes in the existing microflora. Based on the PLFA analysis, high amounts of Gram-positive bacteria were observed in the non-inoculated low-temperature treatments after 24 days of

growth (Figure 3d). This may suggest that the endospore-forming bacteria that survived the low temperature regimes also were successful in colonizing and metabolizing the lignocellulosic substrate. Our knowledge about bacterial degradation of lignocellulosic substrates is scarce, compared to the knowledge about the fungal degradation of these substrates, but recent research indicate that lignin degradation can be performed by specific bacterial consortia in different environments [22]. The general 16S rRNA gene sequence data presented in Table 2 suggest that the abundance of *Bacillus* spp. and *Paenibacillus* spp. are high in the non-inoculated treatments. Additionally, *C. xylanilytica*, a Gram-positive and endospore-producing species, belonging to the family *Paenibacillaceae*, was observed. None of the bacteria, which were tentatively identified to species level in these treatments (*B. circulans*, *P. favisporus*, *P. odorifer* and *C. xylanilytica*), have so far been recognized for metabolization of lignin. However, some *Paenibacillus* spp. and *Bacillus* spp. are known for the production of lignin-degrading and lignin-modifying enzymes [23,24] and several of the identified species are known to be able to degrade hemicellulose.

The accumulated CO_2 from microbial respiration (Figure 2c) and PLFA of total fungal and bacterial biomass (Figure 3a,b) in the inoculated media were not significantly different between the pasteurization regimes by the end of 24 days. As the PLFA can generally only be found in living microbial cells, it is evident that many bacteria and few actinomycetes coexisted with shiitake in the growth media, but they did not seem to impair shiitake colonization. However, the heating temperatures had a significant effect on some co-existing microbes; for example, significantly more Gram-negative bacteria ($p < 0.05$) were found in the substrates subjected to high-temperature treatments (Figure 3c). As there was an absence of respiration in the non-inoculated controls of the high-temperature treatments, it can be expected that living cells or spores were rarely present in these treatments at the time of the spawning. Thus, the microbial community that developed might be derived from the spawn. This is supported by the fact that the identified major bacterial species and their relative abundance show high similarity between the two inoculated high-temperature treatments (Table 2). Spawn production is usually a very carefully controlled process, striving for complete sterility, as infection at this stage in practice may risk the whole production [25]. The substrate used for propagation of the spawn, often cereal grain, is generally subjected to prolonged autoclavation. However, since the fungal growth is not compromised, it cannot be ruled out if the fungus, which is added to the carefully sterilized substrate, may carry unrecognized symbiotic bacteria. A new experiment should be helpful to examine this hypothesis in a future study.

4.2. Coexisting Strains of Bacteria in Shiitake Substrates

DNA sequencing of the whole microbial communities demonstrated that *Phyllobacterium* spp., *S. leidyi* and *Pelomonas* spp., all belonging to the Gram-negative *Proteobacteria* phylum, were the most dominant bacterial species in the inoculated substrates where shiitake was the sole dominant fungal species, across all heating treatments. These dominant bacterial species had a much higher relative abundance in the substrates with growing shiitake than without, although there were differences to some extent between the pasteurization temperatures. These traits hint at plausible habitat-specific symbionts and the potential conferred by the microbial genetic makeup. These bacterial species are distributed in diverse ecosystems ranging from the harsh environment to eco-friendly for growth. Our survey of current literature (Table 3) suggests broader beneficial associations of plants with different species of the *Phyllobacterium* spp., *Sphingomonas* spp. and *Pelomonas* spp. These bacteria, especially *Phyllobacterium* spp., have been reported for their significant roles in plant growth promotion (reference in Table 3).

Studies have shown that *Phyllobacterium* spp. are endophytic species that reside within plant tissues [26]. It is widely considered as rhizobacteria, which possess numerous stress tolerant genes, and are adapted to various environmental conditions. Rhizobacteria are well known for their role in promoting plant growth and metabolism. The several beneficial rhizospheric and endophytic bacterial communities improve adaptation and resilience

under changing conditions and promote sustainable systems. The study also revealed that *Phyllobacterium* sp. appears to be linked with a genus of N_2-fixing plant-growth-promoting bacteria [27]. They could establish their role as an N_2-fixing organisms in the roots of land plants and mangrove rhizosphere [28–30]. The recent study performed by Makino et al. [31] on *Pelomonas* strains MRB1 and MRB3 as the first report on plant growth promoting bacteria (PGPB) displayed a strong symbiotic effect concerning the growth, biomass and chlorophyll content of *Lemna minor*. Asaf et al. [32] demonstrated a symbiotic relationship between *Sphingomonas* and plant roots, combined with a fungal association, which promotes the ability to avoid stress and degrades pollutants. Interestingly, *S. leidyi* was described as an endosymbiont harboured in fungus *Stachylidium bicolor* mycelium [33]. All these characteristics are similar to those bacterial promoters of mycelia growth of, e.g., *P. ostreatus* and *A. bisporus* [12–14]. However, no definitive conclusion can be drawn yet from the literature survey on potential symbiotic relationship between most of these species strains and a fungus (Table 3), whilst, *S. leidyi* seems to be the only one that was identified as an endosymbiont in fungal host.

Table 3. Literature survey of the studies on *Phyllobacterium* spp., *Sphingomonas* spp. and *Pelomonas* spp.

Bacterium	Habitat	Symbiosis	Major Finding/Characteristic	Reference
Phyllobacterium spp.				
Ph. spp. CLE16	Root of *Zostera marina*	PGPB	Nitrogen fixing	[27]
Ph. sophorae spp. CCBAU 3422	Root nodule of *Sophora flavescens*	PGPR	Nodule formation and nitrogen fixation	[34]
Ph. spp. (29-15)	Root of *Brassica napus*	PGPB	Enhanced root length	[35]
Ph. (PEPV15)	Legume nodules of *Phaseolus vulgaris*	PGPB	Increased in citric acid. vitamin C. epicatechin and other bioactive compounds	[36]
Ph. rubiacearum	Black, white, and red mangroves	PGPB	Co-cultivation increased nitrogen fixation and phosphate solubilization.	[30]
Ph. Brassicacearum STM196	Root of *Brassica napus*	PGPR	Increased in biomass. drought resistance and water-use efficiency	[37]
Ph. brassicacearum STM196	Roots of canola plants	PGPR	Stimulating growth	[38]
Ph. strain PETP02	Nodules of *Trifolium pratense*	PGPR	Induced nodules in roots	[39]
***Pelomonas* spp.**				
P. aquatica strain 12868	Soil	–	Remediation of hexahydro-1.3.5-trinitro-1.3.5-triazine (RDX)	[40]
P. spp. MRB1	Roots of aquatic plants	PGPB	Increased in growth. biomass and chlorophyll content	[31]
P. spp. MRB3	Roots of aquatic plants	PGPB	Enhanced in biomass. chlorophyll and high growth-promoting effect	[31]
P. aquatica AIS1S	*Abutilon indicum*	PGPR	Seed germination under salt and heavy metal stress conditions	[41]
P. saccharophila	Mud	–	Hydrogen-oxidizing bacterium	[42]
P. aquatica strain WS2-R2A-65	Nitramine explosive-contaminated effluent	–	Biodegradation of octogen and hexogen	[43]
P. strains CCUG 52769T	Haemodialysis water	–	Presence of *nifH* (nitrogenase) and *hoxG* (hydrogenase) genes	[44]
***Sphingomonas* spp.**				
S. canadensis strain FWC47T	Pulp mill sludge pond	–	Positive towards α-chymotrypsin. β-galactosidase activities and hydrolysed aesculin	[45]
S. spp. strain CHY-1	Soil (heavily contaminated with PAHs from a coal gasification site)	–	Capable of degrading PAHs such as naphthalene. phenanthrene and chrysene	[46]
S. wittichii RW1	Water from the river Elbe, Germany	–	Biodegradation of biaryl ethers dibenzo-p-dioxin and dibenzofuran	[47]
S. paucimobilis GS1	Soil	–	Produce EPS with high mechanical and heat resistance	[48]
S. paucimobilis strain BKK1	Municipal wastewater treatment plant	–	Highly efficient for cadmium removal from solution	[49]
S. spp. FLX-7	Soil	–	Degrades cellulose at low temperatures	[50]

Table 3. *Cont.*

Bacterium	Habitat	Symbiosis	Major Finding/Characteristic	Reference
S. spp. LK11	Leaves of *Tephrosia apollinea*	PGPR	Improve salinity tolerance to tomato crops, increase biomass and growth.	[51]
S. paucimobilis ZJSH1	Root of *Dendrobium officinale*	PGPB	Phytohormone production and nitrogen fixation	[52]
S. spp. Cra20	Root of *Leontopodium leontopodioides*	PGPR	Promotes growth rate under water-deficit	[53]
S. spp. strains BR12245. BR12249. BR12253. BR12195. and BR12200	Washed root of rice plant	PGPR	Nitrogen fixing	[54]
S. spp. (JQ660212.1)	Aerial roots of *Dendrobium moschatum*	PGPR	Promotion of seed germination	[55]
S. spp. strain NSL	Seeds of *Panicum virgatum* cv. Alamo	PGPB	Nitrogen fixation and promote growth under low nitrogen	[56]
S. leidyi	*Stachylidium bicolor* mycelium	Endo-bacterial symbiosis	Secondary metabolites in Ascomycota host	[33]

Not applicable (−); plant growth-promoting bacteria (PGPB); plant growth-promoting rhizobacteria (PGPR).

The DNA sequencing showed that Gram-negative bacteria *Proteobacteria* dominated in the inoculated substrate samples. This is in agreement with the general PLFA analyses (Figure 3c). Plausibly, some of these dominant Gram-negative species (e.g., *Phyllobacterium* spp., *S. leidyi* and *Pelomonas* spp.) in the inoculated substrates might therefore be involved in some kind of symbiotic relationship with shiitake at least during the stage of mycelial colonization, keeping in mind that PLFA can be measured generally only in living organisms. However, for the time being our current data do not allow a deeper interpretation on the role of the identified taxa based on these two methods. There could be inherent biases and discrepancies between DNA-based and PFLA-based databases, due to the fact that DNA based studies can also include dead microbes, since some DNA fractions of dead bacteria may not be completely degraded under high temperature [57,58]. Furthermore, 16S rRNA gene targets are too conservative to allow further speculation about the function of sequenced organisms. This study represents a preliminary attempt to determine the existence of bacterial species within birch-based substrates of shiitake, revealing new information on fungal-bacterial community in shiitake substrates. To complement this information, further studies should be conducted to distinguish the bacterial composition, functions and behavior of these dominant bacterial growths in the presence of shiitake. Additionally, these co-culture models will provide clear insights into microbial dynamics changes in fungi and bacteria relationships.

4.3. Implication for Biorefinery Development

Increased mushroom production holds promise for circular and sustainable production of protein and is of importance for developing a sustainable biobased society. The findings reported in the present study are of interest considering that the common practice in shiitake production is often high-temperature treatment of the substrate before inoculating the spawn. However, the pretreatment of the substrate in high temperature is energy demanding; decreasing this demand will inevitably increase sustainability in production. Our previous study [2,4] demonstrated that low-temperature treatment of the substrate resulted in earlier fructification and higher or comparable yield of shiitake, as well as a similar degree of substrate degradation, compared to the more energy demanding steam sterilization of the substrate. The low temperature hot air pasteurization is apparently beneficial even when considering the potential for developing a circular production system and reusing the substrate for biofuel production. Thus, to understand the mechanisms behind this viable method and increase knowledge on microbial interactions in the mushroom substrate may assist in optimizing mushroom production and consequent biorefinery using spent mushroom substrate.

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