Science of the Total Environment 968 (2025) 178914

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Inclusion of biochar in mushroom substrate influences microbial community composition of the substrate and elemental composition of the fruiting bodies

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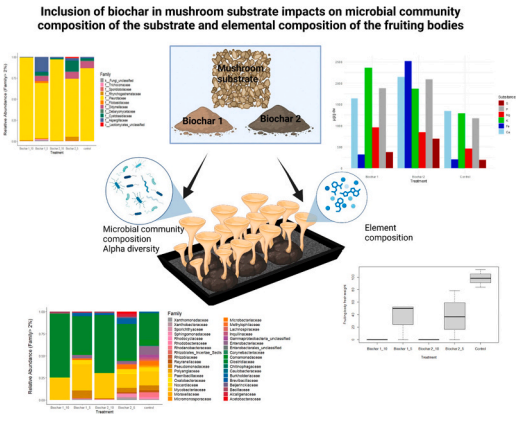
HIGHLIGHTS

- Inclusion of biochar to mushroom substrate was studied
- Fruiting body production was negatively affected by biochar inclusion
- Fruiting body elemental composition was affected by biochar inclusion
- Microbial community diversity was higher when no biochar was added
- Microbial community richness increased when biochar was added to the substrate

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Fang Wang

Keywords:
Fruiting body composition
Metagenomics
Mushroom production
Pleurotus ostreatus
Sludge biochar

ABSTRACT

Due to its structure, biochar makes the soil porous and oxygen-rich, enhancing the water-holding capacity and increasing the cation exchange capacity for a longer duration. These aspects could also be favourable for mushroom production. However, biochar has been considerably less investigated within this context. This study investigated the impact of biochar on mushroom production, quality, and the microbial communities of the substrates. Two different biochar's produced from local feedstocks, plant- or sludge based, were evaluated in the production of oyster mushrooms (*Pleurotus ostreatus*) at two different concentrations (5 % and 10 %). The results showed that inclusion of biochar in the substrate negatively impacted fruiting body production. The elemental composition of the fruiting body was also affected by inclusion of biochar and partly reflected the elemental composition of the biochar. The metagenomics revealed that inclusion of biochar in the substrate altered the microbial community structure. The bacterial diversity based on Shannon indices was higher in the substrate wherein no biochar was added. Bacterial community richness (Chao 1) was higher in samples with biochar compared to the control with no added biochar. Fungal community richness based on Chao 1 indices displayed an increase in samples with an inclusion of biochar. Overall, this study provides novel insights into the impact of biochar in mushroom production regarding its concentration and the effect of the origin material of the biochar.

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https://doi.org/10.1016/j.scitotenv.2025.178914
Received 15 November 2024; Received in revised form 12 February 2025; Accepted 18 February 2025
Available online 20 February 2025

1. Introduction

Biochar is a carbon-dense material produced through pyrolysis, i.e. biomass is heated in an oxygen free environment at high temperatures. The method used for pyrolysis, as well as the added feedstock, will affect the properties of the biochar (Kambo and Dutta, 2015; Lehmann, 2007). Biochar production has received considerable attention for its potential use as a carbon dioxide removal technology because it offers long-term carbon sequestration, thus possibly mitigating climate change. In parallel to this, biochar is receiving increasing attention as a material for industrial, urban, and agricultural applications (Azzi et al., 2021). Common and appreciated features of biochar, aside from its carbon storage capability, are its porosity and high surface area and its cation exchange capacity. In addition, biochar generally has a low bulk density and is typically neutral due to its alkaline pH (Weber and Quicker, 2018). Traditionally, biochar has been produced from wood and forestry residues and hence there are numerous studies that have investigated biochar from those feed stocks. As wood and forestry residues can have other high value implications, there is a growing interest to use alternative feed stocks for biochar production. From a sustainability perspective, it would be an advantage to use feed stocks, such as crop residues and sewage sludge, that are locally produced in agricultural and rural areas (Ghodake et al., 2021). Aside from avoiding long transports of the feed stocks, this would also provide a sustainable solution for usage of the sewage sludge.

In agriculture, biochar has been suggested to improve the physicochemical and biological properties of soils, and its application has been extensively researched within plant production (Kavitha et al., 2018; Khan et al., 2024). However, the inclusion of biochar in mushroom substrate has been investigated to a lesser extent despite the increased interest in mushroom production due to its potential in producing high quality non-meat proteins (Ayimbila and Keawsompong, 2023). Cultivated edible mushrooms such as oyster mushrooms (*Pleurotus* spp.) and shiitake (*Lentinula edodes*) can be directly performed on a wide range of lignocellulosic residues thereby allowing for the development of local and circular food production (Grimm and Wösten, 2018). The substrate is required to be pre-treated to reduce the natural microflora, thus generating both a suitable nutrient composition and physical structure that allows gas exchange and a high water holding capacity (Balan et al., 2022). Considering the inclusion of biochar in mushroom substrate, benefits such as increased retention of moisture and less variation in pH during fungal growth have been demonstrated (Mahari et al., 2020). Mushroom production results in substantial amounts of organic waste. Indeed, spent mushroom substrate (SMS) of approximately 3–5 kg of waste per kg of produced mushrooms have been reported (Zisopoulos et al., 2016). Various types of SMS have therefore been used as a feedstock for biochar production. For instance, Hu et al. (2022) have studied the approach of reusing this type of biochar for mushroom production. Their results suggest that the inclusion of biochar in a concentration of up to 10 % of substrate weight was beneficial for mushroom production.

In plant production, microorganisms have an important role in both soil and substrates and can actively contribute to improved soil health and productivity (Zhou et al., 2019). The addition of biochar to the soil has been shown to alter microbial activity and community structure (Pietikäinen et al., 2000; Steinbeiss et al., 2009). With its porous structure and small cracks, biochar could provide ample living conditions for microorganisms. Its ability to retain nutrients and water may also be advantageous for microorganisms. Studies investigating the impact of biochar on microbial communities in soil show variable results, reporting both increases and decreases in microbial diversity and richness (Chen et al., 2015; Wang et al., 2021). Within mushroom production, it is evident that environmental factors such as changes in temperature, light, and pH are central cues. It is also known that the microbiota of the substrate can play a key role (Carrasco and Preston, 2020). The inclusion of biochar in mushroom substrate has been largely studied from a productivity perspective. Information about the impact of biochar on the microbial community structure in the mushroom substrate has, to the best of our knowledge, not yet been presented. Therefore, the present study examined the impact of biochar inclusion on the microbial community of the mushroom substrate used for cultivation of the common oyster mushroom (*P. ostreatus*). Two different biochars produced from locally obtained feedstocks were evaluated, one based on plant residues and the other based on sludge from a municipal wastewater treatment plant. An additional factor, aside from the impact on the microbial community structure, which has been overlooked in previous studies is the impact of biochar amendment on the elemental composition of the fruiting bodies. Due to the recalcitrant nature of biochar, we hypothesis that (i) inclusion of biochar in the substrate would not affect the elemental composition of the fruiting bodies. (ii) inclusion of biochar will affect microbial communities in the substrate.

2. Materials and methods

2.1. Fungal strain and mushroom substrate

Spawn of oyster mushroom (*Pleurotus ostreatus* M2191) obtained from Mycelia BVBA, Belgium, was used in the experiments. The control substrate was composed of 25 % wheat bran and 75 % sawdust (birch sawdust, 2–4 mm).Two different biochar's were used for the treatments. Biochar 1 was produced from plant residues and is commercially available (Skånefrö, Hammenhög, Sweden). Biochar 2 was produced from sludge from a municipal wastewater plant and is not yet commercially available. Detailed information about the biochar's can be found in the supplementary material (Table S1). Prior to inclusion in the mushroom substrate, both biochar's were filtered through a 2 mm mesh. The biochar's were added to the mushroom substrate at two different concentrations: 5 % and 10 % on a dry weight (dw) basis. Thus, a total of five mushroom substrates were included in the experiment, the control substrate, the control substrate amended with biochar 1 at a concentration of 5 % and 10 %, and the control substrate amended with biochar 2 at a concentration of 5 % and 10 %. The setup of the experiment is shown in supplementary Fig. 1.

2.2. Mushroom production

Distilled water was added to the substrates until a moisture content of 65 % was attained. The substrates were packed in ventilated boxes suitable for mushroom production (Sac O2, Nevele, Belgium), with a total dw of 0.19 kg substrate (corresponding to 0.54 kg wet weight) per box. Three boxes were used for each treatment. The boxes were pasteurised at 65 °C for 8 h and a spawn of *P. ostreatus* was added at a concentration of 10 % (dw/dw) to each box once the substrate had cooled down. The boxes were incubated at 22 °C with closed lids for 15 days, after which the substrate was densely colonized with mycelium. The closed boxes were thereafter incubated at 4 °C for three days to induce fructification, followed by the removal of the lids and incubation in a climate chamber at 22–24 °C with a relative humidity of 85 % until the harvesting of the first flush of fruiting bodies. The fruiting bodies were harvested five days after emergence of the pins.

2.3. Analysis

2.3.1. Mushroom production

The quantity of mushrooms (fresh weight and dw) produced in the first flush was evaluated and dry weight was recorded following lyophilisation. Mushroom production (fresh weight) was related to the amount of substrate (dw), to determine the biological efficiency (BE) of the substrate, calculated as:

BE = (Mushroom (fresh weight)/Substrate (dw)) x 100.

The total protein content in the fruiting bodies was analysed using the Dumas method (Bellomonte et al., 1987), a Vario Max CN, and a conversion factor of 4.38 for total N (Barros et al., 2008). The pH values

of the substrates were determined according to the EN13037 standard.

2.3.2. Elemental composition

To determine the elemental composition of the substrates, they were milled and wet-combusted in HNO_3 (65 %) using a microwave technique (CEN Mars 5) and analysed through inductively coupled plasma optical emission spectrometry (ICP-OES). To determine the elemental composition of the obtained mushrooms, the lyophilised tissue was milled and then analysed as described above.

2.3.3. Molecular analysis of the substrate

Extraction of DNA from the mushroom substrate was carried out using Zymobiomics DNA miniprep Kit (Zymo research) according to the manufacturer's protocol. For each sample 10 g of the mushroom substrate was placed in 50-ml falcon tubes together with a 30 ml PBS buffer (Phosphate buffered saline). The tubes were agitated on a shaker for 2.5 h, transferred to a stomacher bag, and macerated (Smasher; bioMérieux, Inc., 100 Rodolphe Street, Durham, NC 27712, U.S.A.) for 30 s at normal speed. The liquid was poured back into the tube followed by centrifugation at 5000 rpm for 10 min. The supernatant was then discarded, and the pellet was suspended in 750 μl of DNA/RNA shield (Zymo Research). The microbial community composition of the mushroom substrates was analysed using llumina MiSeq, with 300 bp paired end reads, at LGC Genomics GmbH (Berlin, Germany). Bacterial communities were assessed by targeting the 16S ribosomal gene using the primer combination forward primer 799F (5'-AACMGGATTAGATACCCKG-3′) (Chelius and Triplett, 2001) and reverse primer 1115R (5'-AGGGTTGCGCTCGTTRC-3′) (Reysenbach and Pace, 1995). To assess fungal communities, the forward primer ITS1F_Kyo2 (5'-TAGAGGAAGTAAAAGTCGTAA-3′) (Bokulich and Mills, 2013) and the reverse primer (5′- TTCAAAGATTCGATGATTCAG-3′) (Vancov and Keen, 2009) were used to target the ITS region.

Data obtained in Illumina sequencing was analysed by the bioinformatics service at LGC Genomics GmbH (Berlin, Germany) which also performed quality control on all data included in this study. In brief, the Illumina bcl2fastq v2.20 software was used to demultiplex all libraries for each sequencing lane. The barcode sequence was clipped from the sequence after sorting and reads with missing barcodes, one-sided barcodes, or conflicting barcode pairs were discarded, as were reads with final length of <100 bases. Mothur 1.35.1 was used for community diversity analysis. Clustering of operational taxonomic units (OTUs) of the fungal community was carried out at 97 % identity level, with a cluster representative sequence to the most abundant sequence, instead of the default representative sequence (longest sequence). The prokaryotic sequences were aligned against the 16S Mothur-Silva SEED r 119 reference alignment. The fastTree v2.1.7 method was used to generate de novo phylogenetic trees for both fungal and bacterial communities.

2.4. Statistics

All experiments were set up with three replicates in each treatment. The obtained data was analysed using Minitab 18 for Windows. One-way ANOVA followed by Tukey's multiple comparison test was utilised to test for effects of treatments and the significance level was set to $p < 0.05$. Due to Anova having an assumption of equal variance within treatments, *t*-tests between treatment combinations were performed, in case of large differences in standard deviation. Values presented are mean ± standard deviation (std). R-studio (R Core Team, 2021) was used to analyse the metagenomic data. To estimate changes in the microbial community in the different biochar treatments, Shannon index and Chao1 index were used to estimate alpha diversity using the package phyloseq (McMurdie and Holmes, 2013).

3. Results

3.1. Impact of biochar inclusion in mushroom substrates on microbial community structure

The results from the Illumina sequencing showed that biochar impacted the microbial community of the mushroom substrate. At the bacterial phylum level, the dominating phylum was *Firmicutes* in all biochar treatments and in the control. However, when biochar was included at a concentration of 10 % *Firmicutes* was almost the only phylum present (Fig. 1A). This result was consistent for both biochar's. In the control, wherein no biochar was added, *Proteobacteria* was present at almost the same level as *Firmicutes*. Moreover, *Actinobacteriota* was more prevalent in the control and in the samples treated with 5 % biochar (Fig. 1A). Fungal microbial community at phylum level showed that *Basidomycota* was the dominating phylum in all biochar treatments including the control (Fig. 1B). This finding was expected as the substrate was inoculated with *P. ostreatus*. The relative abundance of *Ascomycota* was highest in the treatment amended with biochar 1 with a concentration of 5 %.

Biochar 1_5 = plant-based biochar with concentration 5 %, Biochar 1_10 plant-based biochar with concentration 10 %, Biochar 2_5 = sludge-based biochar with concentration 5 %, Biochar 2_10 sludge-based biochar with concentration 10 %.

At the family level, the control was more diverse compared to the samples treated with biochar (Fig. 2A). Further, the treatments with a lower concentration of biochar (5 %) were more diverse than the samples with a higher biochar concentration (10 %). In all treatments, the dominating family was *Clostridiaceae* followed by *Peanibacillaceae*. Among the genera exceeding the relative abundance of 2 %, *Clostridium* was the most abundant genus across all treatments followed by *Paenibacillus* in all treatments with biochar. The control exhibited a considerably more diverse pattern with more genera present at lower abundance levels (Fig. 2B). When the relative abundance of the fungal families was analysed, the dominance of *Pleurotus* was confirmed (Fig. 3A). The family *Didymellaceae* was present in the control and in the substrate with the lower inclusion level of biochar (5 %) and was not present in treatment with a high concentration of biochar. At the genus level, the dominating genera was *Pleurotus* with some inclusion of *Penicillum* and *Cytobasidum* (Fig. 3B).

The effect of the different biochar treatments on bacterial community diversity based on Shannon indices indicated higher bacterial community diversity when no biochar was added to the substrate and in the sample treated with 5 % of biochar 2 (Fig. 4). There was only a minor effect of biochar on fungal community diversity, thus indicating slightly higher diversity in the substrate with a 5 % inclusion of biochar (Fig. 4).

Community richness based on Chao1 (Fig. 4) showed a significant positive effect on bacterial community richness when the substrate was amended with 10 % of biochar 1 ($p = 0.03$) and 5 % of biochar 2 ($p = 0.02$). There was also a (non-significant) trend indicating a positive effect of biochar on the community richness of fungi.

3.2. Mushroom production and quality

The fruiting bodies were harvested on day 30 for all the biochar amended substrates whilst the controls were harvested between days 30–32. The inclusion of biochar in the mushroom substrate had a negative impact on fruiting body production (Table 1). Both tested biochar's behaved similarly and the 5 % inclusion significantly lowered the productivity. When the inclusion rate was increased to 10 % of biochar, no fruiting bodies were produced. The protein content of the fruiting bodies recovered from the substrate with inclusion of biochar 1 was significantly higher compared to the control (Table 1). Moreover, for biochar 2, a slightly higher protein content in the fruiting bodies was recorded. This increase was, however, not significant when compared to the control. The moisture content of the fruiting bodies varied between

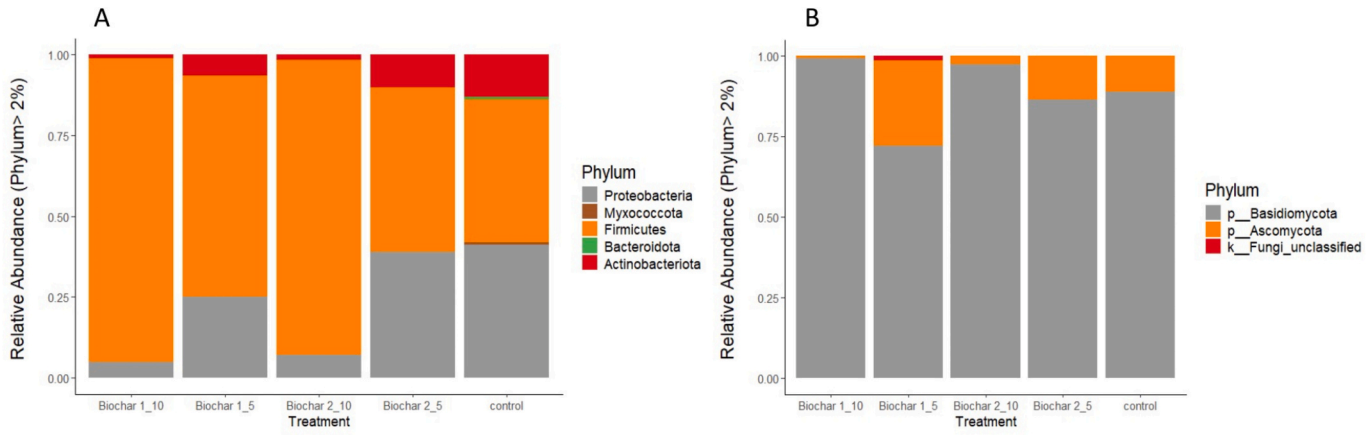

Fig. 1. Relative abundance of taxonomic groups at phylum level for (A) bacteria and (B) fungi.

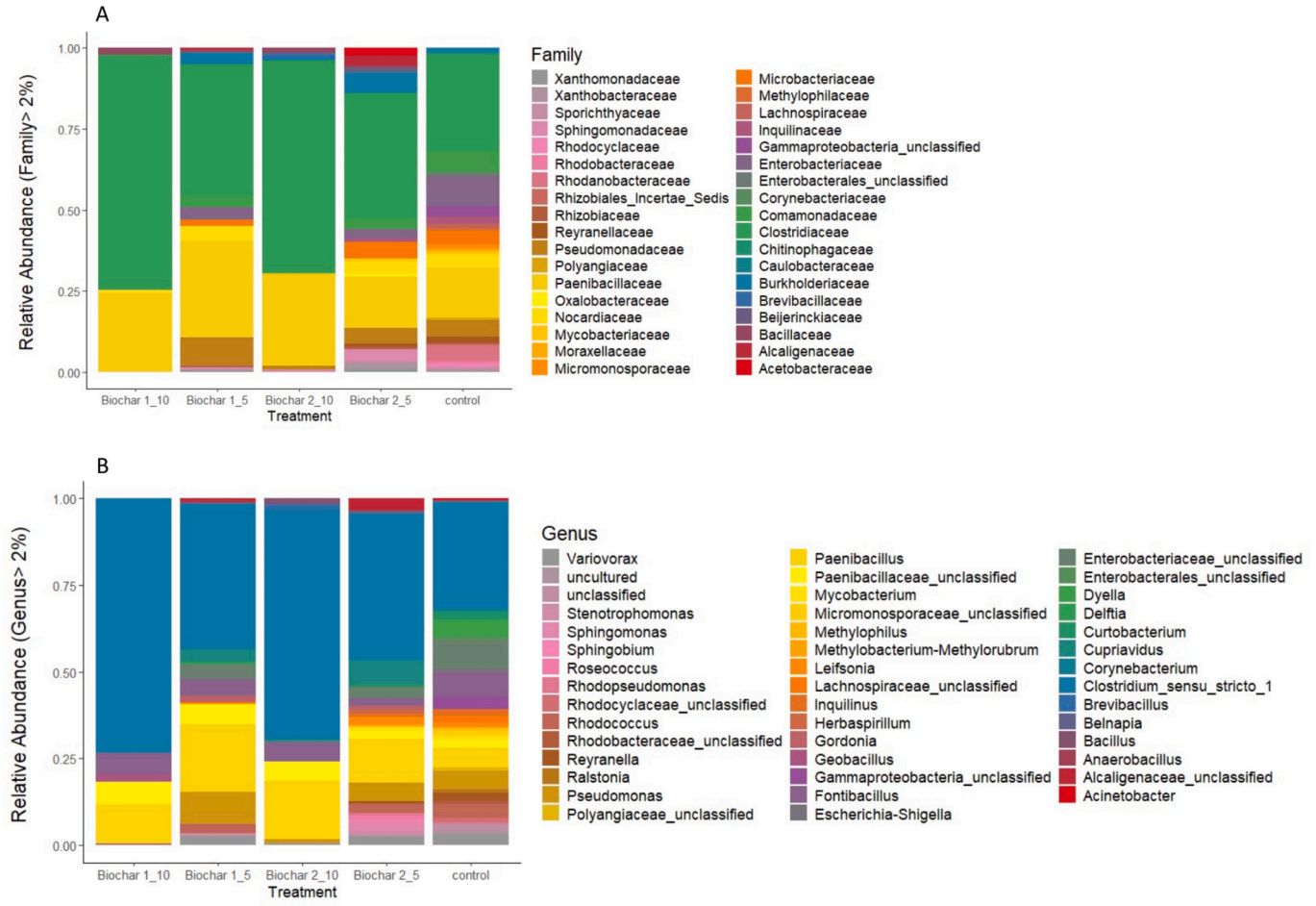

Fig. 2. Relative abundance of bacteria taxonomic group at (A) family level and (B) genus level.

89 and 93 % and no differences were observed between the treatments. The highest pH of the substrates was recorded after the inclusion of 10 % of biochar 1 (Table 1). However, this pH was still neutral (7.0) and suitable for the growth of *P. ostreatus*. When the pH of the substrates was recorded after harvest, a slightly lower pH was observed in the control compared to the biochar supplemented substrates.

A total of twenty-nine elements were analysed in the substrates and fruiting bodies. The inclusion of biochar resulted in a significant increase in fifteen (biochar 1) and eighteen (biochar 2) of these elements found in the substrate (Table 2). As the samples were subjected to nitric acid digestion prior to analysis this increase is not unexpected and is in line with the high ash content of biochar. A considerable increase was observed for the elements aluminium (Al) and iron (Fe), and this increase was notably high in the substrate that was amended with biochar 2. A steep increase in copper (Cu) was also observed for this substrate. The harmful elements arsenic (As), barium (Ba), cobalt (Co), and mercury (Hg) were below detection limits in all substrates. It should also be highlighted that the addition of biochar did not increase cadmium (Cd) or lead (Pb). The element boron (B) is notable, as it significantly decreased in the biochar amended substrates, and was below detection

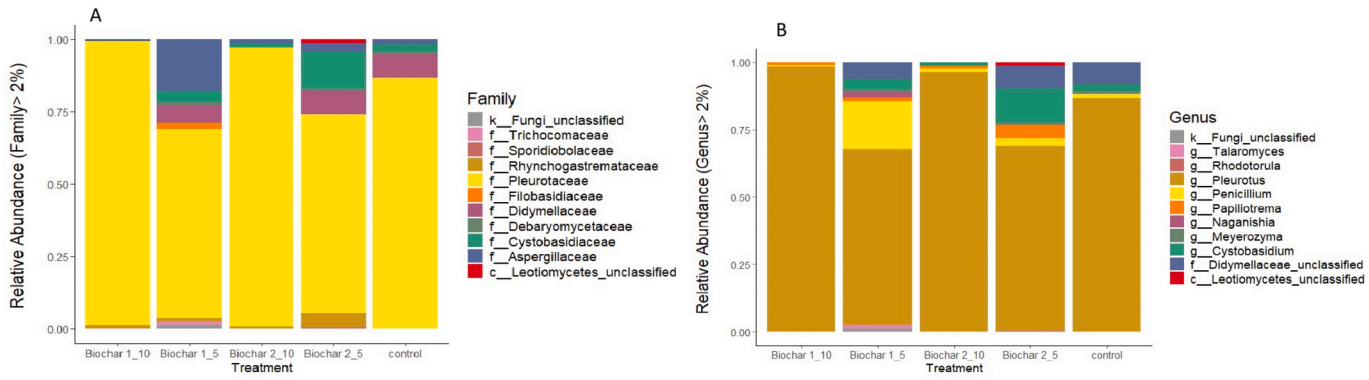

Fig. 3. Relative abundance of fungal (A) families and (B) genus grouped by biochar treatment.

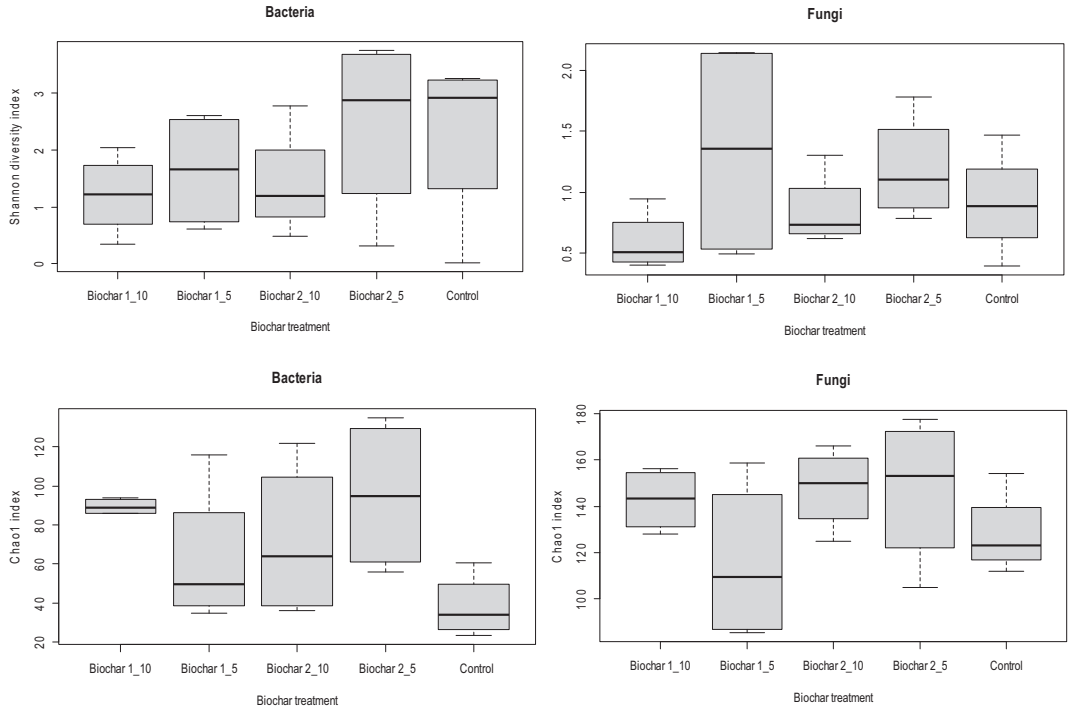

Fig. 4. Boxplot showing alpha diversity indices of bacteria and fungi from mushroom substrate treated with biochar. Biochar 1_5 = plant-based biochar with concentration 5 %, Biochar 1_10 plant-based biochar with concentration 10 %, Biochar 2_5 = sludge-based biochar with concentration 5 %, Biochar 2_10 = sludge-based biochar with concentration 10 %.

Table 1
The biological efficiency (BE) of substrates, protein content of the fruiting bodies (% of dry weight), and pH of the substrates before and after fungal growth. Mean ± std., $n = 3$.

Substrate	BE	Protein content	pH (initial/final)
Control	51.2 ± 7.4a	15.5 ± 1.3a	6.4 ± 0.07/4.7 ± 0.2
Biochar 1 5 %	26.9 ± 1.1b	20.2 ± 1.4b	6.8 ± 0.1/5.0 ± 0.2
Biochar 1 10 %	na	na	7.0 ± 0.02/5.2 ± 0.4
Biochar 2 5 %	26.6 ± 12.9b	17.9 ± 2.8ab	6.4 ± 0.01/5.0 ± 0.3
Biochar 2 10 %	na	na	6.4 ± 0.01/5.1 ± 0.2

levels in the substrate amended with biochar 2.

Regarding the fruiting bodies the inclusion of biochar resulted in a significant increase in two (biochar 1) and six (biochar 2) of the analysed elements compared to the fruiting bodies produced in the control substrate (Table 3). The macronutrients phosphorus (P) and sulphur (S) were increased in fruiting bodies that were produced on both the biochar amended substrates. Moreover, within the substrates a significant increase of both these elements was detected (Table 2). The additional four elements, which were increased only in the fruiting bodies produced on the substrate amended with biochar 2, were aluminium (Al), iron (Fe), strontium (Sr), and titanium (Ti) (Table 3). All of these elements were significantly higher in the substrate amended with biochar 2 (Table 2). Similar to the elemental composition of the substrate, boron stands out with a significant decrease compared to the control for the substrate amended with biochar 2.

4. Discussion

The immense interest in biochar production could be attributed to its high potential to act as a carbon sink. This could in turn help to mitigate climate change. Moreover, its production offers sustainable treatment of waste and biomasses with low value (Mishra and Kaustubha Mohanty, 2022). Thus, biochar production is highly favourable when considering the current societal challenges. With its production expected to increase there is a resulting need to uncover suitable uses for this product. One

Table 2
Elemental composition (μg/g dw) of the mushroom substrates (MS) used in the present study. The control substrate was based on sawdust (birch) and wheat bran (MS control). The substrate MS B1 had a similar composition with an addition of 5 % (dw/dw) of biochar produced from plant residues. The substrate MS B2 had an addition of 5 % (dwt/dwt) of biochar produced from sewage sludge. Mean ± std., $n = 3$ * Values within rows followed by different letters are significantly different ($p \leq 0.05$) **BDL, Below detection limit.

Element	MS control	MS B1	MS B2
Al	3.1 ± 1.0a*	39.4 ± 0.7b	180.6 ± 25.7c
As	BDL**	BDL	BDL
B	14.7 ± 2.5a	10.6 ± 0.7b	BDL
Ba	BDL	BDL	BDL
Ca	1337.6 ± 109.8a	1637.3 ± 62.2b	2145.6 ± 169.0b
Cd	1.3 ± 0.2a	1.3 ± 0.07a	1.3 ± 0.1a
Co	BDL	BDL	BDL
Cr	1.2 ± 0.05a	4.2 ± 0.4b	6.3 ± 0.4c
Cu	2.4 ± 0.3a	7.0 ± 0.5b	30.3 ± 3.7c
Fe	23.1 ± 2.9a	321.6 ± 18.5b	2512.9 ± 341.2c
Hg	BDL	BDL	BDL
K	1287.5 ± 113.9a	2356.5 ± 86c	1864.0 ± 43.4b
Li	0.03 ± 0.003a	0.06 ± 0.01b	0.1 ± 0.01c
Mg	460.7 ± 46.4a	959.4 ± 52.2b	845.5 ± 46.0b
Mn	100.7 ± 16.1a	105.6 ± 7.7a	107.2 ± 4.6a
Mo	0.3 ± 0.1a	0.4 ± 0.02a	0.6 ± 0.06b
Na	38.3 ± 2.4a	67.1 ± 6.2b	78.0 ± 2.7c
Ni	BDL	0.9 ± 0.08a	1.9 ± 0.3b
P	1174.4 ± 86.1a	1877.1 ± 79.2b	2086.8 ± 13.2c
Pb	2.8 ± 0.4a	2.7 ± 0.4a	3.5 ± 0.5a
S	195.5 ± 23.3a	380.8 ± 8.8b	696.1 ± 62.8c
Sb	4.3 ± 0.5a	3.6 ± 0.2a	4.2 ± 0.8a
Se	12.1 ± 2.4a	11.1 ± 1.0a	10.4 ± 1.7a
Si	122.5 ± 5.6a	319.5 ± 33.4b	303.7 ± 17.8b
Sr	1.3 ± 0.1a	1.9 ± 0.05b	3.5 ± 0.4c
Ti	0.5 ± 0.02a	3.4 ± 0.2b	13.8 ± 3.4c
V	5.4 ± 0.2a	6.0 ± 0.3a	6.9 ± 0.3b
Zn	29.2 ± 1.5a	40.5 ± 1.7b	61.3 ± 6.2c
Zr	0.6 ± 0.4a	0.4 ± 0.1a	1.2 ± 0.4a

Table 3
Elemental composition (μg/g dw) of the fruiting bodies produced in the different mushroom substrates. The fruiting bodies used as control (Fruiting body, control) was produced in a substrate based on sawdust (birch) and wheat bran. The fruiting bodies produced in treatment BI (Fruiting body, B1) was produced in a similar substrate but with addition of 5 % (dw/dw) of a biochar produced from plant residues. The fruiting bodies produced in treatment B2 (Fruiting body, B2) was produced in a substrate with an addition of 5 % (dw/dw) of a biochar produced from sewage sludge. Mean ± std., $n = 3$.

Element	Fruiting body, control	Fruiting body, B1	Fruiting body, B2
Al	10.7 ± 3.8a*	8.2 ± 0.2a	32.8 ± 7.3b
As	BDL**	BDL	BDL
B	4.3 ± 0.4a	4.1 ± 0.3a	2.4 ± 0.4b
Ba	BDL	BDL	BDL
Ca	538.3 ± 66.5a	415.5 ± 80.1a	508.8 ± 84.3a
Cd	1.3 ± 0.1a	1.3 ± 0.3a	1.4 ± 0.2a
Co	BDL	BDL	BDL
Cr	1.07 ± 0.4a	0.8 ± 0.2a	1.3 ± 0.5a
Cu	11.9 ± 4.8a	18.2 ± 5.6a	24.9 ± 11.1a
Fe	88.6 ± 22.9a	55.7 ± 8.2a	316.9 ± 119.5b
Hg	BDL	BDL	BDL
K	8898.6 ± 2027.9a	11,956.9 ± 643a	11,566.7 ± 564.7a
Li	0.03 ± 0.02a	0.03 ± 0.0a	0.04 ± 0.005a
Mg	1267.0 ± 193.4a	1494.2 ± 57.2a	1519.0 ± 167.3a
Mn	9.4 ± 1.1a	10.0 ± 1.3a	12.5 ± 1.4a
Mo	0.1 ± 0.06a	0.2 ± 0.1a	0.2 ± 0.07a
Na	88.0 ± 35.8a	96.1 ± 7.1a	110.5 ± 18.5a
Ni	BDL	BDL	BDL
P	5182.7 ± 497.7a	6294.6 ± 357.7b	6353.4 ± 233.8b
Pb	1.8 ± 0.2a	1.2 ± 0.2a	1.2 ± 0.4a
S	1400.0 ± 101.2a	2018.1 ± 99.3b	2281.6 ± 368.1b
Sb	2.9 ± 0.7a	2.3 ± 1.1a	2.0 ± 0.5a
Se	10.4 ± 0.8a	7.5 ± 3.4a	6.7 ± 0.7a
Si	106.7 ± 22.4a	80.2 ± 11.7a	103.7 ± 10.6a
Sr	0.1 ± 0.06a	0.05 ± 0.02a	0.4 ± 0.2b
Ti	0.5 ± 0.2a	0.3 ± 0.06a	2.3 ± 0.9b
V	5.0 ± 0.4a	4.2 ± 0.7a	3.9 ± 0.2a
Zn	49.5 ± 8.2a	59.0 ± 11.5a	70.6 ± 7.8a
Zr	0.2 ± 0.02a	0.3 ± 0.2a	0.2 ± 0.05a

* Values within rows followed by different letters are significantly different ($p \leq 0.05$).
** BDL, Below detection limit.

potential application is the inclusion of biochar in various primary production systems such as crop and livestock production (Li et al., 2024; Schmidt et al., 2019). In crop production, biochar inclusion has been intensively studied and a recent publication compiling experimental data from >360 peer-reviewed studies suggests a general positive effect on yield (Li et al., 2024).

Concerning mushroom production, the addition of charcoal, a similar form of pyrogenic carbonaceous matter such as biochar (Hagemann et al., 2018), to mushroom substrate is not an uncommon occurrence (da Silva et al., 2020; Menolli jr et al., 2010). In laboratory work on fungi, black agar (agar with an inclusion of activated charcoal) is commonly used to study the growing hyphae. The growth response of two different fungi, including one *Pleurotus* spp., to charcoal has been examined in detail by Ascough et al. (2010). Indeed, that study suggested that charcoal itself does not play a role as a carbon source but could induce an exploratory growth style in the exposed fungus. Thus, when included in mushroom substrate, the charcoal is considered to be a growth supporting substrate, not a nutrient, and is often included in a concentration of 2–10 % of substrate weight. The observed positive effects of shorter time to harvest and increased fruiting body production, can be explained by the increased substrate porosity which increases both water retention and gas exchange (Menolli jr et al., 2010; Zakil et al., 2021). Similar positive effects have been observed in studies investigating the inclusion of biochar in mushroom substrates, as reported for charcoal (Hu et al., 2022; Mahari et al., 2020; Talwar et al., 2023). These findings could, however, not be verified in the present study wherein the inclusion of biochar at 5 % of substrate weight affected fruiting body production negatively when compared to the control without biochar. Further, increasing the biochar concentration to 10 % resulted in a lack of fruiting body production in this study. Interestingly, this result was consistent for both tested biochar's. A recent study by Bhattarai et al., 2024, reported results which are comparable to the present study with a significant decrease in fruiting body production already at an inclusion level of 2 % of biochar in the substrate. In a study by Hu et al. (2022), negative results of inclusion were also reported. In that study, biochar was evaluated at three different concentrations of 5, 10, and 15 % of substrate weight. The two lower concentrations resulted in a considerable increase in total yield and a shorter time to harvest. However, when the concentration was increased to 15 %, a sharp drop in productivity along with a slightly longer period to first harvest was observed. On the other hand, Mahari et al. (2020) tested a considerable amount of biochar, with an addition of 250 g of biochar to a substrate of 500 g and still received a higher amount of fruiting bodies when compared to the control. Thus, variable results have been presented, and this is likely partially linked to the fact that biochar is not clearly defined but rather represents a range of materials (Hagemann et al., 2018). Furthermore, strain variations and different conditions applied during cultivation may play a role. For mushroom producers, the use of an optimised substrate which supports high fruiting body development is vital (Carrasco et al., 2018). Regarding biochar inclusion, it can be concluded that no general advice can be given, instead it must be studied within the specific case.

Within mushroom production, the influence of the elemental composition of the substrate on fruiting body composition as well as species dependent bioaccumulation of certain elements have been shown (Koutrotsios et al., 2020). This is reflected in the broad range of concentrations of different elements reported in *P. ostreatus* when fruiting bodies were collected from several producers and countries

(Mleczek et al., 2018). Including biochar in the substrate will impact the elemental composition of the substrate when measured as total concentration after acid combustion (Table 2). However, due to the high temperature treatment during production, biochar has a stable structure and is widely considered to be resistant to biodegradation and therefore its elements could be expected to be less available for the growing fungus. In our study, the elements P and S significantly increased in both biochar amended substrates and also in the produced fruiting bodies. These elements are important macronutrients in living organisms and a capability for increased uptake when they are present in a higher concentration is unsurprising. A similar, non-significant, trend could also be seen for the macronutrient potassium (K). Bioaccumulation of elements from the biochar in the fruiting bodies was also observed regarding metals. Biochar 2 was produced from wastewater sludge and Al and Fe salts are common additives in the wastewater treatment process. This is clearly reflected in the high concentration of these elements in the substrate amended with biochar 2 and further reflected in the significantly increased levels of these metals in the produced fruiting bodies. Also, for the metals Cu and Zn similar, but non-significant, trends of accumulation from the substrate were observed.

The resemblance of the elemental composition of the substrate and of the fruiting bodies described above suggests that the elements enclosed in the biochar structure were available for the fungus during growth. Depolymerisation of biochar by wood-decay fungi has been observed by Placido et al. (2016) over a period of 24 days. Estimations of the mean resident time of biochar varies widely between studies and can be attributed to both the fact that biochar represents a range of materials and the experimental set-up. A meta-study based on a wide range of studies suggested a mean resident time of approximately 500 years (Wang et al., 2016). The study by Placido et al. (2016) showed a correlation between the depolymerisation and the fungal production of ligninolytic enzymes such as laccase and manganese peroxidase. In this context it should also be highlighted that two different studies have demonstrated that an addition of biochar impacts the fungal production of the ligninolytic enzymes (Gibson et al., 2016; Taskin et al., 2019). The impact of biochar addition on enzymatic activity varies in these studies, depending on the fungal species and on the type and condition of the biochar but, in most cases, exposure resulted in increased ligninolytic enzyme activity. The fungal species used in the present study, *P. ostreatus*, is a white-rot fungus with a high production of oxidoreductase enzymes, particularly laccases, when cultivated on lignocellulosic residues (Fernández-Fueyo et al., 2016).

Compared to a cropping system focusing on plant production, mushroom cultivation can be considered a significantly more oxidative environment due to the high concentration of ligninolytic enzymes. This could potentially lead to a faster depolymerisation of the biochar and thereby increase availability to its elements as discussed above. Our study also indicates an impact of biochar addition on the microbial community structure with a trend of decreasing diversity when increasing the biochar concentration. A change in the relative abundance of bacteria was also observed and was more pronounced in samples that were amended with 10 % of biochar. The abundance of *Actinobacteriota* was reduced in all biochar treated samples and *Bacteroidota* and *Myxococcota* were not present at all, as was the case for the control. This trend was similar for both biochar's, despite the biochar's considerable difference in origin and elemental composition. Thus, it is unlikely that a single element, such as Fe which was present in excessive concentrations in biochar 2, is primarily responsible for the biochar's impact of the microbial community structure. It could be hypothesised that depolymerisation of the biochar, when exposed to excessive amounts of fungal ligninolytic enzymes, may have increased the concentration of degradation products with an impact on the community structure. This may also partly explain the negative impact of fruiting body formation that was observed in the present study.

To the best of our knowledge, the impact of biochar on the microbial community structure in mushroom substrate has not yet been reported. However, as previously mentioned, biochar has been extensively studied for its use in plant production. The current interest in soil health emphasises the need to gain greater knowledge about the impact of biochar on soil microbial communities and the addition of biochar to soil has been shown to alter the abundance and composition of soil microbial communities (Wang and Ni, 2024; Yin et al., 2021; Yan et al., 2022; Yang and Wu, 2020). The results obtained in the present study suggest that this is also the case in mushroom substrate. However, our finding of a trend of decreased diversity of bacteria, based on Shannon indices, contrasts to other studies that show an increase in α-diversity bacterial communities in the soil rhizosphere (Yan et al., 2022; Cao et al., 2021). Nonetheless, our observation was not consistent, and this trend was not observed at a lower input of biochar 2 to the substrate. Thus, the amount of biochar added, and the origin of the material used when producing the biochar may be of importance. Microbial community richness estimated by Chao1 indices showed a positive effect of biochar on community richness. Elemental composition, humidity, and pH are all important factors for the growth and survival of microorganisms. The water and nutrient holding properties of biochar may be of greater importance as opposed to the porous structure of biochar. The overrepresentation of spore-forming bacteria may be caused by early toxicity of the substrate, which inhibits the growth of the native microbial community. However, spores will be able to survive and begin to grow after a certain period.

From an applied perspective, it can be concluded that in instances wherein biochar is included in a mushroom substrate, it is of interest to know its elemental composition. Our results demonstrate that the elements in the biochar are available for the growing fungus. This may not be an issue, as the concentration of different elements measured in the fruiting bodies in this study were within the previous values reported by Mleczek et al. (2018). Also, in the used biochar the concentration of harmful elements such as Cd, Pb, and Hg was very low. However, depending on the feedstock and processes used for the biochar production, this may not always be the case. Another situation which should be considered is that SMS has a common use as a soil conditioner (Grimm and Wösten, 2018). Amending a soil with a SMS containing biochar of a similar type as biochar 2, sludge-based from a wastewater treatment process, may increase the concentration of bioavailable Fe considerably and resultingly impact plant growth. Thus, when SMS with biochar inclusion is used as a soil conditioner, or included in a growing substrate, increased bioavailability of certain elements must be considered.

5. Conclusion

To conclude, this study explored the impact of biochar inclusion on the microbial community of the mushroom substrate and quality of the produced fruiting bodies in regard to their elemental composition. Two different biochar's, plant- or sludge-based, were evaluated and both were observed to decrease fruiting body production. The addition of biochar altered the abundance and composition of the microbial communities of the substrates with a trend of decreased diversity of bacteria, based on Shannon indices, and with a dominance of spore-forming bacteria. Furthermore, bioaccumulation of elements from the biochar in the fruiting bodies was observed and this was most evident for the sludge-based biochar which had a high concentration of ash. Potentially, the oxidative environment within a mushroom substrate, with a high concentration of ligninolytic enzymes, impacts the biochar and increases availability to its elements. From an applied perspective, it can be concluded that in instances wherein biochar is included in a mushroom substrate, it is of interest to know its elemental composition.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.178914.

CRediT authorship contribution statement

Maria Karlsson: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Helene Larsson Jönsson:** Writing – review & editing. **Malin Hultberg:** Writing – original draft, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Maria Karlsson reports was provided by Swedish University of Agricultural Sciences. Maria Karlsson reports a relationship with Swedish University of Agricultural Sciences that includes: employment. There are no conflict of interest If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge the financial support provided by Partnership Alnarp. Lilja Bergquist is acknowledged for taking part in the initial trials. We also acknowledge Maria Hellström for reviewing the final draft of this manuscript.

Data availability

Data will be made available on request.

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