Influence of *Agaricus Bisporus* Establishment and Fungicidal Treatments on Casing Soil Metataxonomy During Mushroom Cultivation

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Abstract

The cultivation of edible mushroom is an emerging sector with a potential yet to be discovered. Unlike plants, it is a less developed agriculture where many studies are lacking to optimize the cultivation. Mushrooms are a source of resources still to be revealed, which have applications not only in food, but in many other sectors such as health, industry and biotechnology. Mushroom cultivation consists of the development of selective substrates through composting where the mushroom grows via solid fermentation process. In case of *Agaricus bisporus*, the compost fully colonized by mycelium hardly produces mushrooms and it is necessary to apply a casing layer with certain physical, chemical and biological characteristics to shift from the vegetative mycelium to the reproductive one, where the native microbiota plays crucial roles. Currently, the industry faces a challenge to substitute the actual peat based casing materials due to the limited natural resources and the impact on the peatlands where peat is extracted.

In this work we have employed high-throughput techniques by next generation sequencing to screen the microbial structure of casing soil employed in mushroom cultivation while sequencing V3-V4 of the 16S rRNA gene for bacteria and the ITS2 region of rRNA for fungi in an Illumina MiSeq. In addition, the microbiome dynamics and evolution (bacterial and fungal communities) in peat based casing along the process of incubation of *Agaricus bisporus* have been studied, while comparing the effect of fungicidal treatment (Chlorothalonil and Metrafenone). Statistically significant changes in populations of bacteria and fungi were observed. Microbial composition differed significantly based on incubation day, changing radically from the original communities to a specific microbial composition adapted to enhance the *A. bisporus* mycelium growth. Chlorothalonil treatment seems to delay casing colonization by *A. bisporus*. *Proteobacteria* and *Bacteroidota* appeared as the most dominant bacterial phyla. We observed a great change in the structure of the bacteria populations between day 0 and the following days. Fungi populations changed more gradually, *A. bisporus* displacing the rest of the species as the cultivation cycle progresses. A better understanding of the microbial communities in the casing will hopefully allow us to increase the biological efficiency during production as well as possibly help us to have a clearer view of the microbial community-pathogen relationships as they are directly related to disease development.

Background

Mushrooms have been cultivated since ancient times especially in the eastern countries [1]. They have been appreciated not just for their flavour but for their nutritional and medicinal value. About 7,000 species of mushrooms are believed to have varying degrees of edibility, and more than 3,000 species of 231 different genera are considered to be major edible mushrooms [2]. Despite this, only about 60 edible mushrooms are grown commercially, and around 10 are produced on an industrial scale. *Agaricus bisporus* is cultivated all over the world for the production of button mushrooms. These carpophores have relative high protein content and contain fiber, vitamins, minerals and bioactive compounds. *A. bisporus* is grown in compost consisting of wheat straw, horse or chicken manure [3]. After the compost is colonized, it is covered with a casing soil, which is necessary for moisture and microbial flora. [4, 5, 6]. This casing soil is prepared with black or white peat and calcium carbonate, to reach pH 7-7.5. Peat is a fossil organic material, coming from deposits of vegetal organic matter (moss) accumulated over thousands of years. The use of peat in *A. bisporus* cultivation is essential, since until now no other material is known to have its characteristics: high water retention but water availability for the mushroom, porous and heavy structure and a specific microbiota [7]. Peat used for mushroom growing accounts for only a small portion of the total volumes of peat extracted worldwide. However, peat is an unsustainably sourced non-renewable resource whose availability is diminishing due to reserve depletion and regulatory restrictions. Many attempts have been made to replace peat by other materials such as pine bark or coconut fibre. However, due to the particular physico-chemical properties and native microbiota, peat is still seen as the only optimal option [8]. Casing soil characteristics may also affect the development of mushroom diseases, such as dry bubble caused by *Lecanicillium fungicola* (Preuss), Zare and Gams, and wet bubble caused by *Mycogone perniciosa* (Magnus) Delacroix [9, 10]. To stop disease appearance, chemical products are applied. These are not specifically developed for mushroom pathogens since

the sector is too small compared with other agricultural sectors. The consequences for the mushroom industry are problems such as non-specificity, resistance of pathogens and limitations in its use.

In order to improve mushroom cultivation methods and to find alternative peat materials compatible with the current environmental challenges, microbial communities in the casing soil must be determined and analysed. This knowledge could help in the development of artificial peat materials of non-fossil origin, more friendly to the environment and with inhibiting properties of other pathogenic organisms, thus improving both the cultivated mushroom sector and other agricultural sectors that use peat, as nurseries or gardens.

In this work we have employed high-throughput techniques by next generation sequencing (NGS) to screen the microbiome dynamics and evolution (bacterial and fungal communities) in peat based casing soil along the process of fructification of *A. bisporus*, and the impact of fungicidal treatment (Chlorothalonil and Metrafenone) on the microbiome communities. To reach these objectives two commonly used markers were sequenced in an Illumina MiSeq: V3-V4 of the 16S rRNA gene for bacteria and the ITS2 region of rRNA for fungi.

Methods

Casing sampling

Table 2. Samples utilized in the present work.

Cod.	date	Samples
G1	14/01/2019	Casing soil mix (50%blond peat, 50% black peat)
G2	18/01/2019	Casing at day 4*
G3	21/01/2019	Casing at day 7 (Control)
G4	21/01/2019	Casing at day 7 (Chlorothalonil)
G5	21/01/2019	Casing at day 7 (Metrafenone)
G7	30/01/2019	Casing at day 16 (Control)
G8	30/01/2019	Casing at day 16 (Chlorothalonil)
G9	30/01/2019	Casing at day 16 (Metrafenone)

*Samples at day 4 were taken before fungicide application.

Cultivation room was filled with phase III compost from a commercial mushroom compost yard (Germinados de Lodosa, L.S., Lodosa, Spain). The *A. bisporus* strain used was Sylvan A15. The casing soil used was 50% black peat and 50% blond peat from Valimex S.L. Three biological replicates of the casing samples were destructively taken from the cultivation room along the casing incubation process, from the peat-based casing at four different days (Table 2): casing day (D00), fungicides application day (D04), ruffling day (D07) and at beginning of first flush (D16). Fungicide treatments were applied in separated areas. Chlorothalonil treatment concentration was 3 ml/m² and Metrafenone treatment application was 1 ml/m².

Total DNA extraction

Three biological replicates of the casing samples were studied by extracting genomic DNA (n = 3 replicates per sample type). Fresh samples were homogenized in a ceramic mortar with liquid nitrogen. DNA was extracted from up to 0.5 g of casing with NucleoSpin® Soil kit (MACHEREY-NAGEL). DNA quantity and quality were checked using 2 µl of the purified template in a Qubit 2.0 Fluorometer and the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific, MA, USA) and finally it was visualized on a 1.5% agarose gel stained with Midori-Green Advance staining (Nippon Genetics, Tokyo, Japan).

PCR amplification

Library preparation for 16S rRNA and ITS2 gene amplicon sequencing was performed separately following the Illumina (San Diego, CA, USA) recommendations with some modifications. A 2-step amplification procedure was used [19] using paired end universal bacterial primers [20] for the V3-V4 hypervariable region of the 16S rRNA gene. For the hypervariable region ITS2, the primers ITS3 and ITS4 were used [21]. The primers used contained the Illumina sequencing adapters (overhang nucleotide sequences) added to the gene-specific sequences (Table 3).

Table 3. Primers used for bacteria and fungi sequences amplification.

V3-V4 16S rRNA	Forward sequence (5'→3')	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGAACGCAGC
	Reverse sequence (5'→3')	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC
ITS2 rRNA	Forward sequence (5'→3')	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
	Reverse sequence (5'→3')	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC

PCR amplification were performed with the KAPA HiFi HotStart ReadyMix PCR Kit in a T100 Thermal Cycler (BioRad) under the following conditions: 95 °C for 120 s, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final cycle of 72 °C for 5min. The presence of the amplified 16S rRNA and the ITS gene bands was verified in 1.5% agarose gels. PCR products were purified using NucleoMag NGS Clean-up and Size Select (Macherey- Nagel Co., Diiren, Germany) and eluted in 50 µL of 10mM Tris pH8.5. After purification with the magnetic beads, 5 µL of DNA was amplified in a second PCR employing Nextera XT Index primers (Illumina). This PCR was run at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final cycle of 72 °C for 5 min. A second purification step with NucleoMag magnetic beads was carried out after the Nextera indexing PCR. The 16S V3-V4 rRNA and the ITS2 gene amplicons containing the Nextera indexes were finally eluted in 25 µL of 10 mM Tris pH 8.5. Resulting libraries were quantified by Qubit® 2.0 (Thermo Fisher Scientific) and qualified by Fragment Analyzer (Advanced Analytical Technologies) [14].

Library pooling and sequencing

Both libraries (one for bacteria and one for fungi samples) were created by pooling 5 µL at 4 nM concentration of each sample. Each pool was sequenced separately. Pools were then diluted with hybridization buffer to 12 pM, using 15% PhiX as a control for low-diversity libraries. Sequencing was performed on an Illumina MiSeq sequencer based in CTICH, using a 2 x 300 pb paired-end run (MiSeq Reagent kit v3 (MS-102-3001) following the manufacturer’s instructions (Illumina, Inc., San Diego, CA, USA).

Sequence analyses

Sequencing data were demultiplexed using Illumina bcl2fastq© program. Demultiplexed paired FASTQ sequences were imported in QIIME2 artifact format and analysed with QIIME2 v2020.8. Used workflow is described in detail in the following link: <https://github.com/Marylou8/Metataxonic-analysis-using-Qiime2-workflow>. Quality control was carried out using the DADA2 pipeline [22] incorporated into QIIME2 [23,24]. The DADA2 program filtered out PhiX reads, removed

chimeric sequences and assigned reads into Amplicon Sequence Variants (ASVs). Taxonomic annotation for bacteria was obtained using SILVA v138 database [25]. Taxonomic annotation for fungi was obtained using UNITE v8.2 2020 database [26]. Chloroplast and mitochondrial contaminants were detected and filtered using the QIIME2 “taxa filter-table” and “taxa filter-seqs” commands.

To filter out low-abundance features, we follow the approach of Morton (<https://forum.qiime2.org/t/ancom-giving-strange-w-values/1002/11>) where those features which do not sum at least 10 sequences among all samples, as well as those that only appear in one sample were filter out (command “feature-table filter-features”). Differential abundance analysis was analysed with ANCOM test [27]. The data-sets will be submitted to National Center for Biotechnology Information (NCBI).

Statistics

Sequencing statistical analyses were done using QIIME2 v2020.8 [28]. Alpha diversity (within-samples) was analysed using Faith’s phylogenetic diversity: bacterial community richness that incorporates phylogenetic relationships between taxa [29], Pielou’s species evenness: bacterial community evenness [30] and Shannon’s index: bacterial community richness and evenness [31]. To determine significance in alpha diversity, non-parametric Kruskal-Wallis comparisons were performed [32]. Box-and-whisker plots for species richness and evenness were generated using QIIME2. Alpha rarefaction curves were analysed, to assess if sampling depth was enough to observe the full community diversity [33]. Beta diversity was analysed using Bray–Curtis distance (abundance without phylogeny) [34] and unweighted UniFrac distance (presence and absence of OTUs with phylogeny) [35]. Principle Coordinate Analysis (PCoA) plots were generated from Bray-Curtis distances and unweighted UniFrac distance using QIIME2. To generate taxonomy heatmaps R-Studio Version 1.3.1093 was used [36]. Two libraries were necessary to plot the heatmaps from QIIME2 artifacts “tidyverse” [37] and “qiime2R” [38].

Results

Data from the trial were divided in three data-sets. The first one (data-set DAYS) represents the differences between incubation days in the control without fungicides application: casing day (D00), fungicides application (D04), ruffling day (D07) and beginning of first flush (D16). Ruffling is an agronomic technique used in mushroom farming that consists of raking the surface of the casing soil in order to aerate it and mix compost with the casing so that mushroom colonization is faster and more homogeneous. The second (data-set TREATMENTS D07) represents the differences between the control and the two fungicide treatments (Chlorothalonil and Metrafenone) at ruffling day (D07). And finally, the third (data-set TREATMENTS D16) represents the differences between the control and the two fungicide treatments in the beginning of the first flush (D16). For these three data-set both bacterial and fungi communities have been analysed.

Sequencing and assembly

Bacterial 16S analysis

For both, the Bacterial 16S analysis and the Fungi ITS2 analysis, all samples (n=24) were sequenced on the Illumina MiSeq. Sample 26 (G9) from the Bacterial 16S analysis, was excluded from the analysis because of the low number of reads obtained (1168 reads). All data from the metataxonomic analysis are collected in Table 1.

Table 1. Metataxonomic analysis data.

Analysis	Bacterial 16S	Fungi ITS2
Number of samples	23	24
Total read pairs	3 935 266	8 407 044
Total read pairs average	178 875	350 293
Total read pairs SD (+/-)	68 328	262 317
Truncation selected (forward/reverse)	260/230	280/200
ASV's classified after DADA2	14 018	2 533
ASV's classified after filtering	3 074	950

Alpha rarefaction curves were analysed (supplementary material Figure 7 and 8), to confirm that sampling depth was enough to observe the full community diversity. Alpha and beta diversity box-and-whisker plots and statistics are collected in Supplementary material Figures 7, 8, 9, 10, 11 and 12).

Analysis data-set DAYS

First we performed a taxonomic analysis to study the different bacterial populations. Phylum and family level were visualized in barplots. Then, we analysed the diversity to check if there were significant differences within samples (alpha diversity) and between samples (beta diversity). Finally, we carried out an ANCOM test to identify different abundances across bacterial samples at genus level.

The most highly represented bacteria phylum were *Proteobacteria* and *Bacteroidota* for all samples in data-set DAYS (Fig. 1A). *Proteobacteria* at D00 appears at 40.34% $\pm$ 1.39 SD (percentage of ASVs classified) and increases along days, with a percentage of 53.70% $\pm$ 0.96 SD at D16. *Firmicutes* phylum presents a 10.88% $\pm$ 0.88 SD the first day (D00), decreasing along the cycle until it reaches a percentage of 1.24% $\pm$ 0.01 SD D16. Other phyla present in smaller percentages, such as *Patensibacteria*, *Verrucomicrobiota*, *Chloroflexi* and *Planctomycetota*, increased during the days. One candidate phylum (WPS-2=*Eremiobacterota*) and one phylum without identification was found.

At family level differences between days can be observed (Fig. 1B). Day D00 appears to have more differences comparing to the other three days. Most abundant family at day D00 was *Bacteroidaceae*, with a 6.70% $\pm$ 1.03 SD. This family decreased deeply during the cycle until D16 with a 0.13% $\pm$ 0.11 SD. In addition, family *Lachnospiraceae* almost disappeared at the end of the cycle. Family *Xanthomonadaceae* was present in all samples with a similar percentage (4-6%). However, the presence of some families increased during days, such as *Sphingomonadaceae* (from 1.36% $\pm$ 0.47 SD to 7.99% $\pm$ 0.70 SD), *Chitinophagaceae* (from 1.45% $\pm$ 0.34 SD to 5.03% $\pm$ 0.14 SD), *Devosiaceae* (from 0.94% $\pm$ 0.07 SD to 2.86% $\pm$ 0.74 SD) or *Spirosomaceae* (0.20% $\pm$ 0.06 SD to 2.25% $\pm$ 0.20 SD).

Alpha diversity results showed that there were not significant differences between all days according to Faith's phylogenetic diversity ($p=0.059$) and Pielou's species evenness ($p=0.053$). Bacterial community richness and evenness showed significant differences by Shannon's index ($p=0.027$). However, results showed higher diversity within samples at day 4 (D04) and lower at day zero (D00). Beta diversity results showed significant differences by Bray Curtis: abundance without phylogeny ($p=0.001$) and unweighted UniFrac distance: presence and absence of OTUs with phylogeny ($p=0.001$).

An ANCOM test was also carried out, to compare the composition of microbiomes in the bacterial populations. Results identified 19 different abundances across samples by days at genus level. *Aquicella*, *Halomonas*, *Solitalea*, *Lacihabitans*, *Caulobacter* and *Anaerosporeobacter* had the highest W value (Supplementary material Table S1).

The most identified fungal phyla belong to *Ascomycota* and *Basidiomycota*. Unfortunately, an important number of sequences were not classified in any phylum (Fig. 2A). At day zero (D00) the most abundant phylum was *Ascomycota* (69.13% $\pm$ 0.27 SD). This tendency changes along the cultivation cycle with phylum *Ascomycota* decreasing D04 (57.90% $\pm$ 2.87 SD), D07 (18.68% $\pm$ 7.22 SD) until D16 with 3.86% $\pm$ 1.7 SD. Phylum *Basidiomycota* increases from D00 (22.38% $\pm$ 0.10 SD) until D16 (91.95% $\pm$ 3.29 SD), becoming the controlling phylum from D07. This trend can be also observed at Family level (Fig. 2B), the *Agaricaceae* increasing from D00 (2.44% $\pm$ 0.73 SD) to the D16 (89.43% $\pm$ 4.33 SD).

At family level we can observe clearly the change in the populations of the different fungi families. Family *Agaricaceae* displaced the other families across the cycle while *A. bisporus* is growing. *Helotiaceae* family in the beginning was present in an important percentage (18.74% $\pm$ 4.29 SD) that almost disappears at D16 (0.54% $\pm$ 0.13 SD), as well as other families such as *Piskurozumaceae*, *Exidiaceae* or *Myxotrichaceae*. On the other hand, the presence of the family *Aspergillaceae* increased from D00 (13.54% $\pm$ 6.32 SD) to the D04 (28.84% $\pm$ 1.23 SD) and then it decreased D07 and D16 (1.58% $\pm$ 0.94 SD).

Alpha diversity results showed that there were significant differences between all days using Faith's diversity ($p=0.024$), Pielou's evenness ($p=0.023$) and Shannon's index ($p=0.024$). Beta diversity results showed also significant differences by Bray Curtis: abundance without phylogeny ($p=0.001$) and unweighted UniFrac distance ($p=0.001$).

ANCOM test results identified different abundance across samples by days at genus level in genus *Agaricus* (Supplementary material Table S2).

Analysis data-set TREATMENTS D07

Two different fungicidal treatments were applied in the trial: Chlorothalonil and Metrafenone. Also, samples from a Control without any treatment were analysed. The most representative bacterial phyla were *Proteobacteria* and *Bacteroidota* for all samples in data-set TREATMENTS D07 (Fig 3A). The highest *Proteobacteria* abundance corresponds to Chlorothalonil treatment with 48.51% $\pm$ 1.84 SD. Three candidate phyla were found, WPS-2, WS2 and FCPU426. Most abundant families are common in the three treatments (Fig 3B), such as *Flavobacteriaceae* (8-9%), *Xanthomonadaceae* (6.7-6.9%) and *Sphingomonadaceae* (6.8-5.3%). Family *Chitinophagaceae* showed higher abundance in Metrafenone treatment (5.4% $\pm$ 0.036 SD) and lower in Chlorothalonil treatment (4.51% $\pm$ 0.32 SD). Other families such as *Rhizobiaceae* had a higher abundance in the control (2.64% $\pm$ 0.43 SD) than with the treatments (Chlorothalonil 1.90 $\pm$ 0.70 SD and Metrafenone 1.02% $\pm$ 0.54 SD). Family *Rhodanobacteraceae* presence is 1% higher with Metrafenone treatment 2.42% $\pm$ 0.13 SD.

Alpha diversity results showed that there were not significant differences between all treatments using Faith's diversity ($p=0.956$), Pielou's evenness ($p=0.875$) and Shannon's index ($p=0.956$). Beta diversity results showed no significant differences by Bray Curtis: abundance without phylogeny ($p=0.149$) and unweighted UniFrac distance ($p=0.237$).

ANCOM test results did not identified different abundances across samples by treatments.

For the fungal phylum level (Fig. 4A) we observe that in the Control without treatment, *Basidiomycota* was the most abundant phylum with 69.71% $\pm$ 10.22 SD. For Chlorothalonil treatment both phyla were in similar proportions: *Basidiomycota* 38.55% $\pm$ 4.95 SD and *Ascomycota* 38.33% $\pm$ 11.88 SD. For this treatment phylum *Mucoromycota* was more abundant than in the other 2 treatments. For Metrafenone treatment the most abundant phylum was *Basidiomycota* with 68.43% $\pm$ 7.20 SD.

At family level there were around 10-20% of sequences unidentified (Fig. 4B). Family *Agaricaceae* in Control and with Metrafenone treatment had a high presence (63.48% $\pm$ 11.15 SD and 61.88% $\pm$ 9.41 SD) while in Chlorothalonil

treatment the percentage of this family was just 26.09% $\pm$ 8.04 SD. The other two families identified with an important presence were *Aspergillaceae* and *Piskurozymaceae*.

Alpha diversity results showed that there were not significant differences between all treatments using Faith's diversity ($p=0.066$), Pielou's evenness ($p=0.060$) and Shannon's index ($p=0.060$). Beta diversity results showed significant differences by Bray Curtis: abundance without phylogeny ($p=0.013$) and unweighted UniFrac distance ($p=0.005$).

ANCOM test results do not identified different abundance across samples by treatment at genus level.

Analysis data-set TREATMENTS D16

Similar phylum abundance was found for the data-set TREATMENTS D16 (Fig. 5A). *Proteobacteria* phylum showed also the highest presence in all samples, representing more than 50% of the bacteria identified. Also for this data-set, *Proteobacteria* was more abundant in Chlorothalonil treatment with 54.78% $\pm$ 2.34 SD. At family level (Fig. 5B) the most common families in all samples were *Sphingomonadaceae* (8.69% $\pm$ 0.84 SD at Control, 9.03% $\pm$ 2.26 SD with Chlorothalonil and 4.54% $\pm$ 1.29 SD with Metrafenone treatment), *Chitinophagaceae*, *Comamonadaceae*, *Flavobacteriaceae* and *Rhizobiaceae*.

Alpha diversity results showed that there were not significant differences between all treatments using Faith's diversity ($p=0.235$), Pielou's evenness ($p=0.367$) and Shannon's index ($p=0.986$). Beta diversity results showed significant differences by Bray Curtis: abundance without phylogeny ($p=0.019$) and unweighted UniFrac distance ($p=0.011$).

ANCOM test results identified different abundances across samples by treatments at genus level. There were 5 genera identified: "*uncultured*", *Pirellula*, *Vicingus*, 37-13 and *Sandaracinus* (Supplementary material Table S3).

In this data-set fungal phylum *Basidiomycota* was the most abundant in all treatments (Fig. 6A). However, differences in the abundance could be observed. In case of Metrafenone treatment, *Basidiomycota* appeared in a 70.72% $\pm$ 22.34 SD and *Ascomycota* at 15.35% $\pm$ 15.29 SD. These standard deviations were higher than in the other treatments. For Chlorothalonil treatment, *Basidiomycota* phylum had a presence of 76.29% $\pm$ 7.91 SD and for the Control *Basidiomycota* had the highest occurrence with 91.95% $\pm$ 3.29 SD.

At family level *Agaricaceae* was the dominant family (Fig. 6B). However, its presence was lower in the treatments (Chlorothalonil 69.37% $\pm$ 13.65SD and Metrafenone 63.28% $\pm$ 28.38 SD) comparing to the Control (89.43% $\pm$ 4.33 SD). In this data-set there were also a high number of unidentified sequences. Other families with an important presence like in the data-set TREATMENT D07 were *Aspergillaceae* and *Piskurozymaceae*.

Alpha diversity results showed that there were not significant differences between all treatments using Faith's diversity ($p=0.670$), Pielou's evenness ($p=0.193$) and Shannon's index ($p=0.288$). Beta diversity results showed not significant differences by Bray Curtis: abundance without phylogeny ($p=0.324$) and unweighted UniFrac distance ($p=0.641$).

ANCOM test results do not identified differences in abundance across samples by treatment at genus level.

Discussion

The goal of our research is to understand the changes in the microbial dynamics that occur during mycelium development and mushroom (*A. bisporus*) establishment in the casing soil, and the influence of fungicide treatment application on the microbial population changes of this active ecosystem.

The structure of the populations of both bacteria and fungi, at the beginning of the culture (day zero= D00), fluctuate with respect to the other days. These data confirm that the casing soil in contact with the compost colonized by *A. bisporus*,

undergoes changes in the dynamics of its populations. In agreement with other research on *A. bisporus*, we observed that the predominant bacterial phylum identified in all the casing soil samples was *Proteobacteria*, followed by *Bacteroidota* [11, 12]. *Proteobacteria* population increased along the days as seen in other studies [13], and *Firmicutes* phylum decreased throughout the cycle. Other phyla present in smaller percentages, such as *Patescibacteria*, *Verrucomicrobiota*, *Chloroflexi* and *Planctomycetota*, increased during the days. Most abundant family at day D00 was *Bacteroidaceae*, which decreased deeply during the cycle, like family *Lachnospiraceae* that almost disappeared at the end of the cycle. However, the presence of some families increased during days, such as *Sphingomonadaceae*, *Chitinophagaceae*, *Devosiaceae* or *Spirosomaceae*. Results identified different abundances at genus level across samples by days in 19 genera. The genera with highest W value were *Aquicella*, *Halomonas*, *Solitalea*, *Lacihabitans*, *Caulobacter* and *Anaerosporeobacter*. These genera were not present D00 and they started to grow to significant populations during the incubation. Between day D07 and D16 a change in family's abundance was observed. *Flavobacteriaceae* and *Xanthomonadaceae* had a higher presence D07 while *Sphingomonadaceae* and *Chitinophagaceae* at D16. At genus level, *Flavobacterium* was the most abundant in all samples and *Pseudomonas* increase along days as has been suggested in other research [14]. There were 5 genera identified with different abundances between treatments at D16: “uncultured”, *Pirellula*, *Vicingus*, 37-13 and *Sandaracinus*. The most abundant bacterial species identified (Supplementary material Fig. 15) at day D00 was *Bacteroides graminisolvens*, a xylanolytic bacterial strain [15] that could be related with the decomposition of wheat straw in the casing soil development process. This genus almost disappears along the incubation days.

At day D00 the most abundant fungal phylum was *Ascomycota*, but throughout the cultivation cycle, the phylum *Basidiomycota* became dominant as described in other studies [16, 17]. As indicated in these works, *A. bisporus* replace the other species and colonized the casing soil material. For this reason, from day D16 it becomes very difficult to determine the presence or changes in the populations of other fungi. Families *Helotiaceae*, *Piskurozumaceae*, *Exidiaceae* or *Myxotrichaceae* were present in an important percentage at D00 and almost disappears at D16. On the other hand, *Aspergillaceae* family presence increased from D00 to D4 and then it decreased at days D07 and D16. At D07 for the Control without treatment, *Basidiomycota* was the most abundant phylum while for Chlorothalonil treatment both *Basidiomycota* and *Ascomycota* were in similar proportions. For Chlorothalonil treatment phylum *Mucoromycota* was more abundant than in the other 2 treatments. For Metrafenone treatment the most abundant phylum was *Basidiomycota*. Family *Agaricaceae* in Control and with Metrafenone treatment had a high presence while in Chlorothalonil treatment the presence of this family is lower. The other two families identified with an important presence were *Aspergillaceae* and *Piskurozymaceae*. At D07 we observed an effect of Chlorothalonil treatment on the development of *A. bisporus*, being lower its presence in the treated samples (Supplementary material Fig. 19). Beta diversity results confirmed fungicide treatments effect on the bacterial and fungi populations. At D16 phylum *Basidiomycota* was the most abundant in all treatments. Metrafenone treatment had higher standard deviations than other treatments. At family level *Agaricaceae* was the dominant family being the Control treatment the one with the highest presence.

Fungal diseases were found in the samples. *Lecanicillium* genus was detected in samples at D00 and D04, but also in one sample at D07, treated with Chlorothalonil. A small number of sequences belonging to *Mycogone perniciosus* were found just in one sample at D00.

For the metataxonomy analysis with fungal ITS2, many sequences were assigned to “unidentified” taxon. These results were also found in other similar studies [17, 18] although they were focused on the compost dynamics and not the casing soil changes. Also it was not possible to reach species level for both bacteria and fungi, just for some sequences and species. There is still much work to do in taxonomic identification to build better and more complete databases.

Conclusions

The aim of the study was to better understand the changes experienced by different populations of fungi and bacteria in the casing soil used in mushroom production in order to improve mushroom cultivation and find more sustainable alternatives to peat based casing. This study shows that the use of QIIME2 is valid for NGS sequence analysis in this type of samples. However, the current databases do not allow us to reach the species level in most cases, just genus level. Significant differences have been found between the populations of both fungi and bacteria between the days of incubation, being more pronounced between the casing day (D00) and day 4 (D04). Differences have also been found between the fungicide treatments applied. Our improved understanding of the microbiology of casing soil provides renewed potential to design consortia of bacteria and fungi that could be used to optimize alternative casing materials. More detailed studies are also required to explore the relationship between microbial activity and diversity in casing soil and its implications in the mycelium and primordia development. Finally, a more thorough understanding of the pathogens control would have the potential to increase the quality and quantity of mushrooms produced.

Abbreviations

Amplicon Sequence Variant (ASV)

Analysis of Composition of Microbiomes (ANCOM)

Divisive Amplicon Denoising Algorithm (DADA)

National Center for Biotechnology Information (NCBI)

Next Generation Sequencing (NGS)

Polymerase chain reaction (PCR)

Principal Coordinates Analysis (PCoA)

Quantitative Insights Into Microbial Ecology version 2 (QIIME2)

Standard Deviation (SD)

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets analysed during the current study are available in the NCBI SRA repository, with the BioProject accession number PRJNA772891, <https://www.ncbi.nlm.nih.gov/sra/PRJNA772891>

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

MLT and RL performed the experiments and design the study. MLT, AJP, MT performed primary data analysis. MLT carried out bioinformatics analysis and wrote the manuscript. MP and EP participated in the design of the study and helped to draft the manuscript.

All authors read and approved the final manuscript.

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Figures

Figure 1

Barplot with the Bacterial 16S metataxonomical classification. Bacterial 16S metataxonomical classification by days at Phylum level (1A) and at Family level (1B).

Figure 2

Barplot with the Fungal ITS2 metataxonomical classification. Fungal ITS2 metataxonomical classification by days at Phylum level (2A) and at Family level. (2B).

Figure 3

Barplot with the Bacterial 16S metataxonomical classification. Bacterial 16S metataxonomical classification by treatments at Day 7 at Phylum level (3A) and at Family level (3B).

Figure 4

Barplot with the Fungal ITS2 metataxonomical classification. Fungal ITS2 metataxonomical classification by treatments at Day 7 at Phylum level (4A) and at Family level (4B).

Figure 5

Barplot with the Bacterial 16S metataxonomical classification. Bacterial 16S metataxonomical classification by treatments at Day 16 at Phylum level (5A) and at Family level (5B).

Figure 6

Barplot with the Fungal ITS2 metataxonomical classification. Fungal ITS2 metataxonomical classification by treatments at Day 16 at Phylum level (6A) and at Family level (6B).

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