Casing microbiome dynamics during button mushroom cultivation: implications for dry and wet bubble diseases

Jaime Carrasco,1,2,⁎ Maria Luisa Tello,2 María de Toro,3 Andrzej Tkacz,1 Philip Poole,1 Margarita Pérez-Clavijo2 and Gail Preston1

Abstract

The casing material required in mushroom cultivation presents a very rich ecological niche, which is inhabited by a diverse population of bacteria and fungi. In this work three different casing materials, blonde peat, black peat and a 50:50 mixture of both, were compared for their capacity to show a natural suppressive response against dry bubble, Lecanicillium fungicola (Preuss) Zare and Gams, and wet bubble, Mycogone perniciosa (Magnus) Delacroix. The highest mushroom production was collected from crops cultivated using the mixed casing and black peat, which were not significantly different in yield. However, artificial infection with mycoparasites resulted in similar yield losses irrespective of the material used, indicating that the casing materials do not confer advantages in disease suppression. The composition of the microbiome of the 50:50 casing mixture along the crop cycle and the compost and basidiomes was evaluated through next-generation sequencing (NGS) of the V3–V4 region of the bacterial 16S rRNA gene and the fungal ITS2 region. Once colonized by Agaricus bisporus, the bacterial diversity of the casing microbiome increased and the fungal diversity drastically decreased. From then on, the composition of the casing microbiome remained relatively stable. Analysis of the composition of the bacterial microbiome in basidiomes indicated that it is highly influenced by the casing microbiota. Notably, L. fungicola was consistently detected in uninoculated control samples of compost and casing using NGS, even in asymptomatic crops. This suggests that the naturally established casing microbiota was able to help to suppress disease development when inoculum levels were low, but was not effective in suppressing high pressure from artificially introduced fungal inoculum. Determination of the composition of the casing microbiome paves the way for the development of synthetic casing communities that can be used to investigate the role of specific components of the casing microbiota in mushroom production and disease control.
INTRODUCTION

Mushrooms provide a healthy contribution to the human diet, and the production of cultivated mushrooms is a dynamic and fast-growing industry worldwide [1, 2]. The commercial cultivation of Agaricus bisporus (Lange) Imbach involves the production of a selective compost, which must be covered with a layer of casing material to achieve a profitable crop [3]. This casing material possesses physical and chemical characteristics that facilitate the shift from the vegetative tissue (hyphae) to the reproductive stage (basidiomes) during mushroom fructification [4]. Some of the natural casing inhabitants have been described as essential for mushroom cropping because of their beneficial impact on the development of the mycelium and fructification [5–7]. Casing composition and the casing microbiota may also affect the development of mushroom crop diseases [8], among the most damaging of which are dry bubble disease (DBD) caused by Lecanicillium fungicola (Preuss) Zare and Gams, and wet bubble disease (WBD) caused by Mycocogue perniciosa (Magnus) Delacroix [8, 9]. The symptoms of DBD and WBD are detected over the casing layer, when the host parasitized by the harmful fungus develops undifferentiated masses of tissue called bubbles (Fig. 1) [10, 11].

Despite the potential importance of the casing microbiota for mushroom fructification and disease control, most recent publications have focused on the characterization of the micro-organisms present in mushroom compost along the different phases of the composting process [12–14]. Techniques that have previously been used to characterize the composition and dynamics of the compost and casing microbiomes include the characterization of the culturable microbiome [15, 16]; the evaluation of enzymatic activity in compost [17, 18]; the phospholipid fatty acid (PLFA) profile [15, 19, 20]; 16S ribosomal deoxyribonucleic acid (rDNA)-based denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) [7, 19, 21, 22]; and the quantification of chitin and laccase activity [20].

Next-generation sequencing (NGS) offers a new spectrum of possibilities to characterize the microbe-rich environmental niche in which A. bisporus grows and develops. Metagenomics conducted through NGS does not rely on enrichment or isolation, and so it is possible to work with crude environmental samples and to detect and quantify non-culturable micro-organisms. Compared to classical techniques such as DGGE and T-RFLP, NGS metagenomics provides a more holistic approach with a more powerful workflow [23].

The formulation of the casing material has been postulated to be a factor conditioning susceptibility to fungal diseases in mushroom crops [24]. In the present work two of the commercial materials most commonly employed by the mushroom industry were evaluated to compare their natural suppressive effect on DBD and WBD. Black peat (mostly employed for the local industry to produce mushrooms for the fresh market), blonde peat (mostly used to produce for the canning industry) and a 50:50 mixture of black and blonde peat were employed as casing layers in a crop trial evaluating disease development and yield losses when crops were artificially inoculated with either L. fungicola or M. perniciosa. During the trial, the uninoculated treatment, cased with the 50:50 mixture, was subjected to metagenomic analysis to analyse the composition and dynamics of the casing microbiome along the crop cycle. Additionally, the microbiome of compost samples and basidiomes was also sequenced.

METHODS

Casing characterization and crop design

Commercially available casing materials were acquired from local providers to run the experiment. C1: black peat, based on peat moss (Euroveen B.V., BVB Substrates, Grubbenvorst, Limburg, The Netherlands); C2: blonde peat, based on Spagnum peat moss (Valimex SL, Valencia, Spain); and C3: a mixture of both casing materials (50 % each) (Euroveen B.V. and Valimex). 

Casing characterization and crop design

Commercially available casing materials were acquired from local providers to run the experiment. C1: black peat, based on peat moss (Euroveen B.V., BVB Substrates, Grubbenvorst, Limburg, The Netherlands); C2: blonde peat, based on Spagnum peat moss (Valimex SL, Valencia, Spain); and C3: a mixture of both casing materials (50 % each) (Euroveen B.V. and Valimex).
compost (Germinados de compost SL, Lodosa, Spain) spawned at 1% with the A. bisporus commercial strain Silvan A15M (Silvan, Inc., Kittanning, PA, USA) were cultivated per growing room. The blocks were lined up in metal shelving at three levels in groups of five blocks (crop surface per group: 1 m²). On day 0 of cropping the blocks were covered with a layer of 3–4 cm of the saturated casing materials in a completely randomized block design with six replicates per casing.

Seven days after casing, a conidial suspension of L. fungicola (room 2) or M. perniciosa (room 3) was sprayed onto the surface of the casing layer at a rate of 10⁶ conidia m⁻² or 5×10⁸ conidia m⁻², respectively. The solution employed for the suspension (100 ml m⁻²: 10⁷ for L. fungicola and 5×10⁻⁷ conidia ml⁻¹ for M. perniciosa) consisted of sterile distilled water plus a drop of Tween 80 to avoid sporulation. Disease inoculum was prepared on the day of inoculation as described by Carrasco et al. [24] from strains of L. fungicola L1 and M. perniciosa M1 previously isolated from diseased commercial crops located in La Rioja, Spain. Room 1 was not infected and was used as a negative control (the control blocks were sprayed with water/Tween 80 solution). Commercial-sized healthy mushrooms and diseased mushrooms showing visual symptoms of bubble disease (including undifferentiated masses of diseased tissue; pinheads of sufficient size such that the presence of bubble symptoms could be detected visually on caps or stipes; caps showing pitting; and caps showing stipe blowouts [10, 11]), were harvested daily during two successive flushes. The presence of the pathogen in bubbles was confirmed by recovering the mycoparasite from infected tissue in PDA plates, followed by incubation at 22 °C. Disease incidence was evaluated by comparing the presence of visual symptoms across the three treatments and by comparing the mushroom yield harvested from inoculated and control blocks.

Mushroom production in the different treatments was compared by analysis of variance (ANOVA) using the software package SPSS Statistics 24 (IBM, Armonk, NY, USA). Fisher’s least significant difference (LSD) test, at 5% probability, was used to establish significant differences between means. Non-parametric tests, including the Kruskal–Wallis test and the Mann–Whitney (Wilcoxon) W test, were also implemented to compare medians at the 95% level for non-normal distributions.

**Compost and casing sampling for DNA extraction**

Compost and casing samples were destructively obtained at two different points (G1 and G8) and six different points (G2–G7), respectively, along the crop cycle from the blocks cased with the 50:50 mixed material in the control room (Table 1). Approximately 100–200 g of the complete vertical profile of compost or casing was taken from each of the six replicates assayed with the mixed casing and the samples were homogenized by hand. Two basidiomes (G9) collected from each of the six replicates of the casing mixture were surface-disinfected with a cloth impregnated with a 5% sodium hypochlorite solution and then sliced and homogenized to generate basidiome samples. The beginning and the end of a flush were considered to be the first and last days of mushroom picking, respectively.

**Extraction of DNA from compost and casing**

Three biological replicates of the representative samples were studied by extracting genomic DNA from compost (G1 and G8), casing (G2–G7) and basidiomes (G9) (n=3 replicates per sample type). Fresh samples were homogenized in a ceramic mortar with liquid nitrogen. DNA was extracted from up to 500 mg of compost, casing or sliced fresh mushrooms, respectively, with the DNeasy Power Soil kit and purified using the DNeasy PowerClean Pro Cleanup kit (Quiagen, Hilden, Germany). DNA quantity and quality were checked using 2 µl of the purified template in a Qubit 3.0 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 (Nippon Genetics Co., Tokyo, Japan).

**PCR amplification and purification**

Purified DNA templates were amplified separately for bacteria and fungi by amplifying the regions of interest (Table S2) through a two-step amplification procedure (Table S3) [26]. The V3–V4 region of the bacterial 16S rRNA gene was amplified by PCR using the paired-end universal bacterial primers [27], while the internal transcribed spacer (ITS) was amplified for fungi by employing primers that label the ITS2 region, ITS3/ITS4 (Table S3) [28].

The NucleoMag NGS Clean-up and Size Select (Macherey-Nagel Co., Düren, Germany) was employed to purify the amplicons and libraries (Nextera XT Index kit, Illumina Co., CA, USA) and samples were visualized on a 1.5% agarose gel. The average size and quality of the libraries were evaluated by Fragment Analyzer (Advance Analytical) and the DNA was quantified using Qubit 3.0.

**Illumina MiSeq sequencing**

The library (including bacteria and fungi samples), normalized at 4 nM and pooled, was denatured with NaOH at room temperature and diluted with hybridization buffer to 12 pM, using 10% PhiX as a control for low-diversity libraries [12, 13]. Sequencing was performed on an Illumina MiSeq sequencer based in CIBIR (Riojasalud, Gobierno de La Rioja, Spain) using a v3 sequencing kit (2×300 cycles) (Illumina Co., San Diego, CA, USA).

**Data analysis**

The quality of the raw reads was assessed using FastQC [29]. The raw reads were trimmed and filtered with a Phred quality score of at least 30, and all adapters removed with Trim Galore [30]. After the pair-end reads were cleaned (the forward R1 and the complementary reverse R2 were assembled at matching regions), operational transcriptomic units (OTUs) were identified using Quime (v1.9.1) [31], following the methodology ‘pick open reference OTUs’ at a 97% threshold of nucleotide identity against the
The allocation of OTUs to their most probable taxonomic levels provides an overview of species diversity and abundance in each sample (alpha diversity) [23]. Alpha-diversity analysis was performed using evenness metrics (distribution accounting for richness, the number of OTUs observed in a sample and the abundance or frequency of OTUs per sample) with the Simpson index as diversity index (a value close to 1 indicates that the reads are distributed over many OTUs and small values indicate that a single large OTU dominates the sample).

Beta-diversity analyses were carried out using two-dimensional principal coordinates analysis (PCoA) by implementing Bray–Curtis index metrics (based on the abundance of OTUs) with the permutational multivariate ANOVA (PERMANOVA) statistical method. Beta-diversity analysis calculates the degree of similarity between samples and/or groups of samples (in the PCoA plot a similarity equal to 0 means that the samples or group of samples share all the OTUs, while dissimilarity is equal to 1–similarity) [23].

The Ward clustering algorithm was used to perform hierarchical clustering analysis and the corresponding heat maps were visualized with the standard Euclidean distance measure. The open-source Metagenomics Core Microbiome Exploration Tool (MetaCoMET) (USDA, USA) was used to conduct a comparative study [36].

The core microbiome, understood as the suite of members shared among microbial consortia from similar habitats, was evaluated by comparing the different samples of casing along the crop cycle, as well as the phase III compost, the casing placed over the compost but still non-colonized by the Agaricus mycelium and basidiomes. Finally, Krona charts [37] summarizing the metagenomics data in interactive charts were constructed.

**RESULTS**

**The use of different casing materials does not affect bubble disease development**

In order to study the effect of casing material on bubble disease development, an experiment was performed in which three different casing materials (C1: black peat; C2: mixed casing, 50% black peat and 50% blonde peat; and C3: blonde peat) were tested in three different experimental rooms (control; artificially inoculated with a conidial suspension of L. fungicola; and artificially inoculated with a conidial suspension of M. perniciosa). The substrates and casing used were physically and chemically characterized, and the effect of disease incidence on mushroom production was evaluated while harvesting the basidiomes daily during two successive flushes.

As expected, the organic matter content and the C/N ratio in the compost decreased along the crop cycle during the trial as a result of the metabolic action of A. bisporus, while the organic matter content of the casing remained relatively constant from the beginning to the end of the trial (Table S1). This was consistent with a previous trial with the same experimental design, in which changes in the three casing materials were analysed in more detail (production data not shown due to contamination of the control with L. fungicola), and in which the organic matter in the casing remained constant from the ruffling point (an agronomical operation that consists of ruffling the surface of the semi-colonized casing to break fungal hyphae and favour better casing colonization) to the end of the trial (Table S1). This supports the accepted hypothesis that the casing is poor in nutrients and therefore does not contribute substantially to feeding of the crop [38]. The three casing materials employed during the trials showed similar physico-chemical parameters (Table S1), and so the environmental conditions for the development of the native microbiome were closely related among the different casing microcosms.

The isolate of L. fungicola employed was highly virulent. Due to the severity of disease symptoms (Fig. 1c, d) only 2 days of the first flush were harvested, with a low production of 2.5 kg m⁻² in C1 (black peat); 3.3 kg m⁻² in C2 (50% black peat: 50% blonde peat); and 2.1 kg m⁻² in C3 (blonde peat). By contrast, two flushes were harvested from the control and the room inoculated with M. perniciosa (Fig. S1), and no disease was detected in the control during the trial.

The 50% black peat : 50% blonde peat mix and the black peat supported the highest level of mushroom production in the control room, with no significant differences being observed between these treatments in the yield of healthy mushrooms (Fig. S1). However, the casing material used had no significant impact on the yield obtained or the disease symptoms observed following infection with either L. fungicola (with heavy losses) or M. perniciosa (which showed a decrease in yield with respect to the control room) (Fig. S1).

**The composition and dynamics of the casing microbiome**

To determine the diversity and dynamics of the communities inhabiting the compost, casing and basidiomes, DNA was isolated from compost, casing and basidiome samples taken from blocks cased with the 50:50 mixture of black and blonde peat along the crop cycle during the trial (Table 1). The V3–V4 region of the 16S rRNA gene was
amplified for bacteria and the ITS2 region was amplified for fungi and sequenced through NGS metagenomics.

The analysis was conducted with a cluster density of 760 K mm$^2$, with >90% of reads passing filter and an initial average QC of over 30% in >80% of reads. After processing for read quality, 5,694,862 reads remained for 29 bacterial samples from the 9 sample groups (G1–G9) (the sequence data for 1 replicate of the basidiome 16S rRNA gene was excluded due to a sequencing failure), and 4,895,860 reads remained for the 30 fungal samples from the 9 sample groups (Table S4, Figs S3 and S4). After cleaning, excluding singletons, more OTUs were identified for bacteria than for fungi. Three hundred and sixty-six genera and 38 phyla of bacteria and 157 genera and 6 phyla of fungi were identified across all samples. Over 90% of OTUs were not assigned at

---

Fig. 1. Healthy and diseased mushrooms recovered from the trials. (a, b) Healthy crop harvested from room 1 (control) in trial 2. (c, d) Symptoms of dry bubbles observed in room 2, infected with L. fungicola. (e, f) Undifferentiated mass of A. bisporus tissue infected by M. perniciosa (wet bubble) recovered from room 3.
Table 1. Compost, casing and basidiome samples analysed using NGS

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Type of sample*</th>
<th>Point of sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (G1)</td>
<td>Compost</td>
<td>Day 0 (at casing)</td>
</tr>
<tr>
<td>Group 2 (G2)</td>
<td>Casing</td>
<td>Day 0 (at casing)</td>
</tr>
<tr>
<td>Group 3 (G3)</td>
<td>Casing</td>
<td>Day 7 (at ruffling)</td>
</tr>
<tr>
<td>Group 4 (G4)</td>
<td>Casing</td>
<td>Day 16 (Cf flush 1)</td>
</tr>
<tr>
<td>Group 5 (G5)</td>
<td>Casing</td>
<td>Day 20 (end1 flush 1)</td>
</tr>
<tr>
<td>Group 6 (G6)</td>
<td>Casing</td>
<td>Day 25 (beginning flush 2)</td>
</tr>
<tr>
<td>Group 7 (G7)</td>
<td>Casing</td>
<td>Day 29 (end flush 2)</td>
</tr>
<tr>
<td>Group 8 (G8)</td>
<td>Compost</td>
<td>Day 29 (end flush 2)</td>
</tr>
<tr>
<td>Group 9 (G9)</td>
<td>Basidiomes</td>
<td>Day 29 (end flush 2)</td>
</tr>
</tbody>
</table>

Samples obtained in triplicate (n=3) from trial 2 by taking samples from the control room along the crop cycle.

* Casing evaluated was C2, 50:50 mixture of both materials.
† Ruffling, agronomical operation that consists of ruffling the surface of the semi-colonized casing to favor better casing colonization.
‡ The beginning and the end of a flush were considered to be the first and last days of mushroom picking, respectively.

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Type of sample*</th>
<th>Point of sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (G1)</td>
<td>Compost</td>
<td>Day 0 (at casing)</td>
</tr>
<tr>
<td>Group 2 (G2)</td>
<td>Casing</td>
<td>Day 0 (at casing)</td>
</tr>
<tr>
<td>Group 3 (G3)</td>
<td>Casing</td>
<td>Day 7 (at ruffling1)</td>
</tr>
<tr>
<td>Group 4 (G4)</td>
<td>Casing</td>
<td>Day 16 (Cf flush 1)</td>
</tr>
<tr>
<td>Group 5 (G5)</td>
<td>Casing</td>
<td>Day 20 (end1 flush 1)</td>
</tr>
<tr>
<td>Group 6 (G6)</td>
<td>Casing</td>
<td>Day 25 (beginning flush 2)</td>
</tr>
<tr>
<td>Group 7 (G7)</td>
<td>Casing</td>
<td>Day 29 (end flush 2)</td>
</tr>
<tr>
<td>Group 8 (G8)</td>
<td>Compost</td>
<td>Day 29 (end flush 2)</td>
</tr>
<tr>
<td>Group 9 (G9)</td>
<td>Basidiomes</td>
<td>Day 29 (end flush 2)</td>
</tr>
</tbody>
</table>

Samples obtained in triplicate (n=3) from trial 2 by taking samples from the control room along the crop cycle.

* Casing evaluated was C2, 50:50 mixture of both materials.
† Ruffling, agronomical operation that consists of ruffling the surface of the semi-colonized casing to favor better casing colonization.
‡ The beginning and the end of a flush were considered to be the first and last days of mushroom picking, respectively.

the level of species for bacteria, but more than 99.9 % of the OTUs were assigned at the species level for fungi. Rarefaction curves based on the number of observed OTUs and the number of reads obtained per sample reached the plateau, which indicates that the sequencing depth was sufficient for the results to be representative of the whole diversity of the samples, and to provide a reasonable estimation of the relative abundance of individual OTUs in each sample (Figs S3 and S4) [39]. Hierarchical clustering analysis was also performed to compare hierarchies among the different elements conforming to the bacterial phyla and fungal classes identified (the analysis reflects which elements are represented as being ‘above’, ‘below’, or ‘at the same level as’ one another) (Fig. 4), and to study the core microbiome with the aim of establishing shared elements among the different groups (Fig. 5).

Casing samples were taken in triplicate at six different points (G2–G7) along the crop cycle, from non-colonized casing (G2) to the end of the second flush (G7). Fig. 2 presents the relative abundance of OTUs at the phylum level, which shows a high degree of consistency within groups. Proteobacteria was the dominant phylum in the casing at the beginning (G2) and remained dominant along the crop cycle (G3, G4, G5, G6, G7) (Fig. 2a), with Flavobacterium being the most abundant genus (Table S5). Conversely, seven of the most abundant genera in G2 lost their prominent status in the later casing groups, which correlates with the statistically significant differences noted between the bacterial community in G2 and those from the other groups, as reflected by alpha- and beta-diversity plots (Figs 3a, b, 4a).

There was a noteworthy increase in the relative abundance of Pseudomonas in the casing during the crop cycle. Although absent among the 10 most abundant genera in non-colonized casing (G2), the relative abundance of pseudomonads increased progressively, with Pseudomonas being the second most abundant genus by the end of the second flush, in G5 (Table S5). However, the relative abundance of the genus Pseudomonas fell and it was only the third most abundant at the beginning of the third flush (G6) and the fourth by the end of the flush (G7), with Flavobacterium consistently being the most abundant genus in all casing samples (Table S5). The genus Bdellovibrio also increased in the casing along the process of cultivation, replacing Pseudomonas as the third most abundant species by the end of the third flush, and the species Bdellovibrio bacteriovorus was annotated as the most abundant taxa among the identified OTUs in G6 (0.55 % of OTUs) and G7 (0.61 % of OTUs) (Table S5). In spite of the different relative abundance among the bacterial communities, the majority of the elements of the casing microbiome were shared among the casing samples (Fig. 5a). Ascomycota was the most abundant phylum in the non-colonized casing, but it was rapidly replaced by the Basidiomycota, from G3 onwards, as A. bisporus colonized the casing. Therefore, once again, the non-colonized casing showed unique features, while there were no statistically significant differences between the host-colonized casing samples (Figs 3c, d, 4b).

Changes in the compost microbiome from the beginning to the end of the crop cycle

Samples from compost at the beginning of the crop cycle (G1) and at the end of the crop cycle (G8) were sequenced to identify changes in the microbiome structure of the phase III compost employed for the cultivation of button mushroom.

Proteobacteria was the dominant group in the phase III compost (G1), but Chelatococcus, Thermobifida, Actinomadura, Hyphomicrobiunm and Bacillus were the most dominant genera, with only two of these (Chelatococcus and Hyphomicrobiunm) belonging to the phylum Proteobacteria (Table S5). Although the relative abundance of Proteobacteria decreased by 30 % in compost by the end of the crop cycle (G8), when Firmicutes and Actinobacteria became dominant, the same seven groups were detected in G1 and G8 among the most representative genera (Table S5, Fig. 3b). The A. bisporus mycelium, which represents 99 % of the Basidiomycota identified, as reflected by the Krona charts (Fig. S2), displaced the Ascomycota from the compost along the crop cycle, from a relative abundance of 11.8 % at the beginning of the crop cycle to one of 1.3 % at the end of the crop cycle (Fig. 2b).

Relation of the compost and casing communities to the configuration of the mushroom microbiome and their influence on it

To investigate the microbial communities that inhabit the mushroom basidiome of A. bisporus, and how they relate to the communities present in the compost (G1) and casing (G2), the microbiome of healthy basidiomes harvested from the end of flush two in the selected blocks was analysed (G9).
Fig. 2. Relative abundance of OTUs at the level of phylum showing composition and dynamics along the crop cycle of the microbiome. (a) 16S rRNA (10 most abundant phyla; >87% OTUs per sample). (b) ITS (100% OTUs per sample). Taxa have been merged based on the sum of their counts across samples and groups. Features with fewer than 10 counts and non-assigned or unidentified OTUs have been removed to help the visualization of major trends. G1, compost day 0; G2, casing day 0; G3, casing day 7; G4, casing day 16; G5, casing day 20; G6, casing day 25; G7, casing day 29; G8, compost day 29; G9, basidiomes harvested on day 29.
Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were the most abundant phyla in compost phase III, casing and basidiomes (Fig. 2a). However, the basidiomes showed a higher number of bacterial components in common with the casing (49.5 %) than with the phase III compost (11.8 %) (Fig. 5b), and so it seems likely that the bacterial diversity in A. bisporus basidiomes is configured more by the casing than by the compost. This similarity was also apparent with the abundance of genera observed, since 6 of the 10 dominant genera in casing samples were detected among the most abundant in basidiomes, while Acinetobacter, Actinomadura and Thermobifida were only detected as major components of the bacterial microbiome in compost (Table S5).

In the case of the fungal communities in the samples of phase III compost, casing and basidiomes, it seems inappropriate to place much emphasis on differences between the fungal communities present in compost and the basidiomes because the predominance of OTUs corresponding to A. bisporus in basidiome samples (Fig. 2) reduced the relative abundance of the remaining OTUs to 10% and below, although significant differences were noted in the composition of the fungal microbiome between the casing, where Ascomycota was dominant, and the rest of the samples (Figs 3c, d, 4b).

**Detection of the causative agent of bubble disease by NGS**

Interestingly, although the blocks sampled for the characterization of the microbiome remained asymptomatic during the trial (no macroscopic symptoms of bubble disease were detected), the sensitivity of the NGS analysis enabled us to detect the presence of the causative agents of bubble disease in asymptomatic crops.

*Lecanicillium fungicola* (GenBank accession no. FJ810136), which shows >98% base identity with the species *L. fungicola* var. *aleophilum* EF641885 or *L. fungicola* var. *fungicola* AF342784, described as causative agents for dry bubble disease in mushroom crops [40], was detected in the casing in three samples of G2, two of G4, two of G5, two of G6 and three of G7, increasing in relative abundance along the crop cycle to be the fourth most abundant fungal species in the casing layer by the end of the crop cycle (Table S6). In addition, the presence of *L. fungicola* was also detected in the compost, in two of the samples of G1 and three of G8, and even in one of the basidiomes tested that showed none of the macroscopic symptoms of disease. The presence of *M. perniciosa* was also detected, but only residually in one sample of G7 and one of G8.

**DISCUSSION**

Although bubble diseases have been reported as a major cause of yield losses due to biotic agents [9], relatively little is known about the natural suppressive effect of the casing material on the expression of these fungal diseases. The three commercially relevant casing materials used in this study all displayed good agronomical characteristics and supported comparable yield to commercial production under control conditions, with black peat and a 50:50 mixture of black and blonde peat supporting the highest production. However, none of the three casing materials evaluated significantly reduced the severity of bubble diseases when conidial suspensions of the causative agents were inoculated on the casing material. Notably, in a previous trial conducted in the summer of 2017 with the same experimental design and the same facilities, we observed cross-contamination with *L. fungicola* in the control room, associated with a high pressure of flies during cropping (data not shown). In this earlier trial, we also observed that the three casing materials tested were equally inefficient in preventing natural infection by bubble disease carried by flies, the main vectors for the dispersion of bubble diseases [9].

However, interestingly, metagenomics analysis detected the ubiquitous presence of *L. fungicola* in compost, casing and even healthy basidiomes in the control treatment of the trial reported in this study, even though crops remained asymptomatic. The presence of *L. fungicola* in compost has been reported previously through MiSeq sequencing as the fourth most abundant species [13]. However, in this study the authors found a similar relative abundance of *L. fungicola* in compost along the crop cycle. In our experiment the relative abundance of this parasite in compost was very low but detectable at the beginning of the crop cycle, but it increased exponentially by the end (Table S6), which is consistent with the higher impact of DBD observed in mushroom crops when the crop ages [9, 11]. The severity of disease symptoms resulting from DBD has been already directly related to the amount of causative agent applied in the casing [41], therefore we hypothesize that the relative abundance of *L. fungicola* detected in the casing by NGS sequencing was not sufficient to generate detectable outbreaks of the disease. Also, as reported previously, the absence of disease symptoms in the presence of the causative agent could be related to the natural suppressive effect that casing material possesses against mycoparasites, which is associated with the native casing microbiome [8]. Nonetheless, we cannot ignore the possibility that, at low inoculum densities, cultivated mushrooms are also protected by defence mechanisms that may be innate, or linked to the presence of endomicrobes [42, 43], which could include the production of secondary metabolites such as volatile organic compounds that inhibit the germination of *L. fungicola* [44]. The minimum inoculum densities of *L. fungicola* and *M. perniciosa* required to generate macroscopic symptoms of bubble diseases in mushroom crops are unknown, and uncovering the mechanisms that limit bubble disease development at low inoculum densities would be an interesting avenue for future work.

Our sequencing results indicate that *L. fungicola* populations can be present and increase in abundance in both phase III compost and casing, even in asymptomatic crops.
We hypothesize that in our experiment the presence of *L. fungicola* in compost and casing samples reflects external contamination in the context of a mushroom growing environment [45]. As the conditioning stage of phase II compost consists of a treatment of 48°C for 5 to 6 days [14], it seems unlikely that fungal parasites will be present before the cooling down of the mass of compost. However, since flies are attracted to the volatile organic compounds produced by the mycelium of *A. bisporus* [46], phase III of composting, conducted at 25°C for 12–14 days [3], could be vulnerable to infection. To reduce the disease pressure, compost and casing producers, as well as farmers, will benefit from implementing procedures to prevent contamination during substrate management and cropping, including storage of the casing material in clean facilities, pest management, and cleaning of growth rooms and equipment between crops.

The increased abundance of bacteria belonging to the genus *Pseudomonas* detected in the casing over the duration of the trial could be partially explained by their well-known ability to colonize substrates while disturbing the native microbial community [47], even showing mycophagy [48] or acting as mushroom parasites (e.g. *Pseudomonas tolaasii* or *Pseudomonas reactans*) [5, 6, 22]. The proliferation of *Pseudomonas* within the casing may play an important role in promoting mushroom fructification [6]. Sterile casing material has been reported to show very limited production of basidiomes [49]. However, in the early 1990s the strain *Pseudomonas putida* PMS118S was shown to promote basidiome morphogenesis [50]. It has since been postulated that fructification inhibitors, such as volatile organic compounds secreted by the vegetative mycelium of *A. bisporus*, are consumed by bacteria belonging to the casing microbiota, thereby allowing fructification to occur (Fig. 1d) [49, 51]. If we accept the hypothesis that the presence of pseudomonads promotes mushroom fructification, we can speculate that the reduction in the relative abundance of *Pseudomonas* observed in the later stages of the trial (G6 and G7, Table S5), also noted while investigating the culturable microbiome in a previous work [52], could be correlated with the agronomical traits of the crop, since after two flushes each successive flush is less productive and mushroom production drops quickly. However, we cannot ignore the likelihood that this reduction in productivity is also likely to be linked to nutrient depletion in the compost [53].

---

**Fig. 3.** Alpha- and beta-diversity analyses to compare the richness and abundance of OTUs among samples and groups. Alpha-diversity analysis was performed for bacteria (a) and fungi (c) using evenness metrics (Simpson measure). Beta-diversity analysis for bacteria (b) and fungi (d) was represented by the two-dimensional PCoA. G1, compost day 0; G2, casing day 0; G3, casing day 7; G4, casing day 16; G5, casing day 20; G6, casing day 25; G7, casing day 29; G8, compost day 29; G9, basidiomes harvested on day 29.
Fig. 4. The hierarchy of microbiome components indicates the good reproducibility of the sample replicates: (a) based on the bacteria phyla and (b) based on fungi classes. The x-axis corresponds to the clustering of the different samples (groups and single samples), while the y-axis corresponds to the clustering of the most abundant OTUs (97% similarity) among reads. G1, compost day 0; G2, casing.
It would be interesting to further investigate the structure and activity of the microbiome when fructification is induced to help understand the factors contributing to fructification.

Although several Pseudomonas sp. strains isolated from casing material have been described as being able to inhibit L. fungicola growth in vitro, to the best of our knowledge no pseudomonads have yet been shown to control bubble disease in crop trials [54]. Consistent with this, our experiment shows that the natural relative increase of Pseudomonas along the crop cycle in the casing did not provide protection against bubble diseases when crops were artificially infected with conidial suspensions. Instead, we observed an increase in pseudomonads alongside an increase in L. fungicola. One potential explanation for this is that the fructification-inhibiting volatile 1-octen-3-ol produced by A. bisporus hyphae, which has been reported to be degraded by pseudomonads, has also been described to inhibit the germination of L. fungicola. Therefore, the consumption of 1-octen-3-ol by Pseudomonas spp. may promote the germination of L. fungicola at the same time as promoting mushroom fructification [2].

The increasing relative abundance of Bdellovibrio spp. within the casing material along the crop cycle could be also related to the increase in pseudomonads and other Proteobacteria. B. bacteriovorus, the most abundant taxa identified in casing in G6 and G7, is a Gram-negative predatory bacterium that was initially isolated from soil, and is known to be able to attack bacteria from a wide range of genera, including several species of Pseudomonas [55, 56]). It has been specifically tested for its ability to limit the proliferation of the brown blotch pathogen P. tulousii on mushrooms post-harvest [57]. Our study is one of the first to report an increasing prevalence of Bdellovibrio along the mushroom crop cycle, and thus it is not yet clear whether this is a common phenomenon or limited to specific batches of casing material. Nevertheless, given the potential importance of pseudomonads as agents promoting both fructification and disease, it is worth noting that the proliferation of predatory bacteria that lyse bacterial cells could either be an additional factor contributing to the decreasing production observed in button mushroom crops throughout the successive flushes of the crop, or could help to suppress bacterial diseases and post-harvest spoilage, and therefore warrants further investigation.

Together with the nutritional content of compost and the physical-chemical characteristics of casing, mycelium growth and fructification appear to be deeply influenced by the native bacteria and fungi cohabiting the substrates [3, 58]. Previous studies have characterized the bacterial and fungal microbiome of compost along the crop cycle [12, 13]; the bacterial and fungal microbiome from the initial mesophilic pre-wet stage to the end of the mushroom production process [22]; the bacterial community in composting phase II [14]; bacterial community changes in casing at the beginning and the end of the crop cycle [22, 59]; and the bacterial dynamics in compost leachates [60]. Our study has characterized the structure and dynamics of the bacterial and fungal microbiome in the casing material along the crop cycle. Additionally, the microbiome structure of compost, casing and freshly harvested basidiomes have been compared using NGS techniques. The number of reads per sample and the quality of the analysis obtained was improved with respect to the above cited works, as reflected in an increase in the genetic diversity of the sequenced pool.

NGS, in agreement with previous works conducted using other techniques [7, 19, 59], showed higher bacterial diversity (alpha-diversity) in casing and in the basidiomes than in the compost. Our results further suggest that the bacterial diversity and population in basidiomes is highly conditioned by the casing microbiome, and support previous studies showing that bacteria belonging to the genera Pseudomonas and Flavobacterium are abundant in fresh mushrooms [52]. Fungi were also more diverse in non-colonized casing compared to compost or basidiomes, although reads from A. bisporus dominated the fungal OTUs detected in colonized substrates, and the number of reads remaining from the analysis limited our ability to draw conclusions from the fungal microbiome data. Furthermore, as the use of ITS barcodes has been found to present limitations for the identification of closely related Basidiomycota [61], the detection of species from genera such as Pleurotus or Russula among the most abundant fungal OTUs in basidiomes (Table S6) should be interpreted with caution, as it is surprising to detect macrofungi as a component of the microbiome in another macrofungus.

The bacterial dynamics detected in our samples within the compost are similar to those described by McGee et al. [12], with a dominant Proteobacteria population in the beginning that decreased by the end of the compost process to be supplemented by Firmicutes and Actinobacteria. The casing at the beginning of the crop cycle (prior to A. bisporus colonization) showed statistically significant differences in microbiome composition compared to the colonized casing for both bacteria and fungi, however, when colonized by A. bisporus, a conservative pattern was revealed during the crop cycle and no significant differences were detected from G3 (ruffling) onwards. The genera Flavobacterium and Pseudomonas were among the most abundant in casing, which is consistent with some of the individual strains isolated and described previously [16, 52]. In contrast to the non-culturable microbiome described in this paper, where Flavobacterium constantly represents the most abundant genera in colonized casing, the culturable microbiome reported previously was dominated by pseudomonas (54%) and
flavobacteria (10%); however, in agreement with the results discussed above, these authors reported a pseudomonads decrease by the late flushes [52].

Although the metagenomic technique we have implemented does not provide quantitative parameters, according to the rarefaction plots obtained (Figs S3 and S4), the observed bacterial OTUs in Agaricus-free casing (G2) increased by 10% after A. bisporus colonization and the observed fungal OTUs decreased by 5%, although this last observation has to be interpreted carefully, since the predominance of A. bisporus limited the number of reads obtained from other fungal species (Figs S3 and S4). A previous quantitative analysis in which the culturable bacteria were screened and the PLFA profile was analysed reported that the absolute bacterial population increases significantly from casing soil application to primordia formation [15]. Therefore, we can speculate that the higher number of observed bacterial OTUs in our samples after A. bisporus colonization correlates with an increased bacterial diversity, and particularly the gradual increase of the relative abundance of the Pseudomonas genera, could partially explain the breakdown of the fungistatic balance that has previously been described in casing materials, which inhibits the germination of mycoparasites [8, 62]. This explanation is consistent, for instance, with the pathogenesis of WBD, since the presence of opportunistic bacteria, most of them described as Gram-negative, has been reported as a prerequisite for the germination and infection of M. perniciosa [63].

Focusing on the fungal community, it is worth remarking on the relative decrease of Ascomycota in compost by the end of the crop cycle, as previously reported by McGee et al. [13]. This observation is in accordance with the selectivity described for the phase II compost to support the growth of A. bisporus to the detriment of other fungal competitors [14]. Furthermore, the richer fungal diversity (alpha-diversity) observed in non-colonized casing compared with colonized casing indicates the distinctive microbiome of the Agaricus-free material. During casing colonization A. bisporus plays a dominant role while quickly replacing the native mycota.

Agricultural soils suppressive to soil-borne plant pathogens have been classified as showing: (a) general suppression due to the total microbial biomass in soil that is not transferable between soils and (b) specific suppression due to the effects of individual or select groups of micro-organisms, which is transferable [64]. From our results we can conclude that relatively little general suppression exists against DBD or WBD due to the natural microbiota that inhabits the casing materials assayed. Therefore, future prospects should be focused on the identification of specific strains that show a suppressive effect on the conidial germination or the mycelium development of the target diseases in order to develop biocontrol agents to fight these harmful diseases while greening the industry.

Funding information
The project leading to this report received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement no. 742966. The research
was also funded by the ADER project ‘Síntesis de un nuevo aditivo biológico para el cultivo de Agaricus bisporus’, grant no. 2016-IDD-00013.

Author contributions
Data curation: J. C. and G. P.
Formal analysis: J. C., M. L. T., M. T., A. T. and G. P.
Funding acquisition: J. C., P. P., M. P. C. and G. P.
Investigation: J. C. and G. P.
Methodology: J. C., M. L. T., M. P. C. and G. P.
Project administration: J. C., M. P. C. and G. P.
Software: J. C. and M. T.
Supervision: J. C., M. P. C. and G. P.
Validation: J. C., M. T., M. P. C. and G. P.
Visualization: J. C. and G. P.
Writing – original draft: J. C. and G. P.
Writing – review and editing: J. C., M. L. T., M. T., A. T., P. P., M. P. C. and G. P.

Acknowledgements
The database has been uploaded at Oxford Research Archives (ORA) and can be downloaded from this link: https://ora.ox.ac.uk/objects/uuid:603f6945-84f5-41d7-b76c-64ebecd86815. The sequence data for this study have also been deposited in the NCBI Bioproject collection under BioProject ID PRJNA477500 and will be available upon publication.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
No ethical approval was required because the work was accomplished with commercial samples and wild strains from fungal specimens.

References