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Manipulating button mushroom casing affects the disease dynamics of blotch and green mold disease

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ABSTRACT

Productive cultivation of the button mushroom (*Agaricus bisporus*) relies on the use of selective substrates and effective disease management. In extending our previous work on manipulating the developmental microbiome (devome), this study employs the strategy of substrate passaging to explore its effects on crop outcomes and disease dynamics. Here we subjected the casing substrate to ten cycles of passaging. This manipulated substrate stimulated early pinning (primordia formation) by at least three days. Passaged casing also altered disease dynamics when challenged with two commercially important *A. bisporus* pathogens, *Pseudomonas tolaasii* (causing bacterial blotch) and *Trichoderma aggressivum* f. *aggressivum* (responsible for green mold). Passaged casing had a suppressive effect on blotch disease and a conducive effect on green mold disease. Blotch suppression resulted in a significantly higher yield of asymptomatic mushrooms in all three mushroom harvests (flushes) and in the overall crop yield. Blotch severity was also significantly reduced in passaged casing compared to standard casing due to a lower yield of mushrooms with the highest degree of blotch disease expression. Green mold disease expression was markedly higher in passaged casing, leading to lower numbers of asymptomatic mushrooms. Zones where no growth of hyphae or mushrooms were also observed in passaged casing due to green mold disease pressure. The stimulating effect of passaged casing on mushroom development and the dynamic outcomes for disease challenge from two distinct, commercially damaging diseases, demonstrates the potential for passaged casing to be used as material to study more sustainable mushroom production and disease management practices.

1. Introduction

Commercial cultivation of the button mushroom, *Agaricus bisporus* (Lange) Imbach, is performed in controlled environments where the fungus colonizes a pasteurized selective composted substrate to form the vegetative mycelium (Sinder and Hauser, 1950; Van Griensven, 1988). This is followed by the addition, on top of the compost layer, of a nutrient-poor casing layer with high water-retention capacity that supports the initiation of mushroom primordia (Sinden and Hauser, 1950; Noble and Gaze, 1996). The casing layer often consists of sphagnum peat moss and lime, which provides optimal physical and chemical properties for the production of fruit bodies (Noble et al., 1998). The casing layer also provides microenvironments that harbor a microbiome pertinent to the development of primordia (Hayes et al., 1969). Species such as *Pseudomonas putida* are attributed to the removal of self-inhibitory volatile compounds during fruit body formation (Noble et al., 2009). The composition of microbial communities in the casing layer have been found to have a greater impact on the developmental microbiome (devome) than those found in the compost layer (Vieira et al., 2023). Composting, spawning, and casing phases in commercial mushroom

Abbreviations: MRC, Mushroom Research Center; CS, standard casing; CFU, colony forming units; KMB, King's Medium B; CI, casing inoculum; SI, spore isolate; PVC, polyvinyl chloride; CP, passaged casing; BS, *P. tolaasii*-inoculated standard casing; BP, *P. tolaasii*-inoculated passaged casing; TS, *T. aggressivum*-inoculated standard casing; TP, *P. aggressivum* -inoculated passaged casing.

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production can be viewed as processes of microbial succession which correlate with crop productivity and yield, but this material may also harbor mushroom pathogens that lead to disease (Siyoum et al., 2016; Vieira and Pecchia, 2022; Carrasco et al., 2019). In fact, the causal agents for most commercially important mushroom diseases are commonly found in compost and casing microenvironments (Fletcher and Gaze, 2007).

The mix of lime and peat that form the casing layer is hypothesized to be the main source of *Pseudomonas tolaasii* in button mushroom cultivation (Wong and Preece, 1980), but the primary inoculum of blotch pathogens has never been conclusively verified. However, blotch pathogens are closely associated with both symptomatic and asymptomatic mushrooms (Martins et al., 2020). Members of the genus *Pseudomonas* such as *P. tolaasii, Pseudomonas agarici,* [*Pseudomonas gingeri*], *Pseudomonas costantinii,* [*Pseudomonas reactans*], and other unnamed species, cause a variety of symptoms resulting in what are known collectively as blotch diseases in the *A. bisporus* cultivation system (Osdaghi et al., 2019). Historically, *P. tolaasii* was considered the primary causal agent of bacterial blotch (Tolaas, 1915), though recently other *Pseudomonas* species have been found to be regionally dominant (Taparia et al., 2021a). Preventative disease measures of blotch include effective control of humidity to help avoid accumulation of moisture on mushroom caps and sometimes the application of an oxidizing agent, for example, sodium hypochlorite (Wong and Preece, 1985). Taparia et al. (2021a) demonstrated that blotch outbreaks can occur when *P. gingeri* (causal agent of ginger blotch) and *Pseudomonas salomanii* (novel bacterial blotch pathogen found in Europe) are applied to peat-based casing at threshold inoculum densities of 10^4 CFU/g and 10^5 CFU/g, respectively. The ginger blotch incidence was shown to decrease over consecutive crop cycles, leading to a disease-suppressive casing, even though the pathogen cell densities did not decrease (Taparia et al., 2021b). Blotch suppressiveness was also shown to be transferable in aqueous extracts. Taparia et al. (2021b) additionally described co-occurrence of certain taxa in blotch suppression such as *Burkholderiaceae* and *Pedobacter*.

Another major disease of button mushroom cultivation is green mold. This fungal-derived disease can cause serious damage to a crop as seen in past epidemic green mold outbreaks with *Trichoderma harzianum* biotypes Th2 in Europe and Th4 in the US. These biotypes were later distinguished as *Trichoderma aggressivum* f. *europaeum* and *T. aggressivum* f. *aggressivum,* respectively (Muthumeenakshi et al., 1994; de la Fuente et al., 1998; Samuels et al., 2002). Green mold disease expression includes the appearance of fast-growing white mycelia which competes with the mycelium of *A. bisporus*. Green spores are often produced within and over the surface of substrates. Necrotic brown spots may also be viewed on the mushroom tissue in some cases. Bare areas where mushrooms are completely absent are also a major symptom of green mold (Fletcher and Gaze, 2007). Hygiene protocols are performed to minimize disease, including room and tool sanitation and physical cleaning. A limited number of fungicides such as thiabendazole (a systemic benzimidazole) and imidazole (prochloraz) have been approved regionally for use against green mold in button mushroom cultivation. Although, these chemical interventions carry the risk of reduced sensitivity to target pathogens, leading to decreased efficacy over time (Potočnik et al., 2015). This situation underscores the need for developing alternative, sustainable management practices to combat such issues. Biological control agents have also been explored, for example, the addition of *Bacillus* spp. to protect *A. bisporus* against this disease (Colavolpe et al., 2015; Gea and Navarro, 2017; Luković et al., 2021; Altaf et al., 2022; Pandin et al., 2018).

Recently, the impact of substrate passaging (combining former crop substrate with fresh crop substrate at a 1:10 ratio and repeating for subsequent cycles) was assessed on the development of *A. bisporus* (Vieira et al., 2023). Passaged casing (made from three passage cycles) exhibited primordia formation 3 days earlier than standard casing (without microbial enrichment). Passaged casing showed a reduction in the relative abundance of *Pseudomonas*, *Pedobacter*, and *Flavobacterium* when compared to standard casing. An overall increase in alpha diversity (richness) was noted in passaged casing communities (Vieira et al., 2023). The manipulation of materials by passaging biomes has also been explored for its plant disease suppressive potential (Ehau-Taumaunu and Hockett, 2022; Morella et al., 2020; Raaijmakers and Weller, 2001; Ramírez et al., 2020). Disease-suppressive attributes are likely more prominent at the biome-level as pathogen antagonism often occurs in multi-species interactions (Garbeva et al., 2011).

This study follows previous work that focused on the manipulation of microbial communities (passaging) to gain new insight into the devome of *A. bisporus* (Vieira et al., 2023). In this study, we aim to further our understanding of the potential roles of passaged casing with a focus on disease dynamics. Crop outcomes of standard and passaged casing were assessed when challenged with two commercially significant crop pathogens, particularly for North American mushroom markets; *P. tolaasii* (bacterial blotch) and *T. aggressivum* f. *aggressivum* (green mold).

2. Materials and methods

2.1. Experimental design

Cropping parameters and processes were carried out as per the standard procedure at the Mushroom Research Center (MRC - https://plantpath.psu.edu/research/centers/mushroom-research-center) at Pennsylvania State University, PA, USA. Details for the preparation of phase I and phase II mushroom compost, inoculation with mushroom spawn (beginning of spawn run) and the procedure for addition of the casing layer (casehold) can be found in Vieira and Pecchia (2022). Phase II compost was inoculated on a dry-weight basis with a ratio of 3 % *A. bisporus* off-white strain 901 Lambert (Lambert 901 SI, PA, USA) spawn and 4 % commercial supplement (Promycel gold [54 % protein], Amycel, CA, USA). Compost was colonized (spawn run) in PVC containers (56.5 cm × 43 cm × 24 cm) housed in growing rooms with substrate temperature maintained at 24–25 °C, with air humidity maintained at 95 %. Spawn run was carried out until the compost substrate was fully colonized by *A. bisporus* (16 days). The casing was prepared with sphagnum peat moss (Scott's sphagnum peat moss, Ontario, CA) adjusted to approximately pH 7.5 using crushed agricultural limestone, mixed with *A. bisporus* mycelium (casing inoculum; CI Lambert 901) and addition of water to achieve ~75 % moisture content. The casehold phase was initiated by adding ~9.1 kg of casing (~4 cm layer) to the colonized compost. Casing moisture was maintained through watering by hand when required. Pinning or primordia formation was triggered by adjusting the growing room parameters as per the MRC standard procedure (Vieira and Pecchia, 2022). The first flush (harvest) began on day 12 after casing (casehold) for passaged casing treatments (passaging process described below) and day 17 after casing (casehold) for standard casing treatments (casehold day numbering is separate from flush day numbering). Mushroom harvest cycles were initiated from the first day of mushroom picking and lasted for a total of 22 days, representing three mushroom breaks (flushes). Mushrooms matured to 2–4 cm in diameter and were picked by hand and weight was recorded. Commercially marketable mushrooms (disease-free) are included towards yield (unless otherwise stated). The paired experimental units (tubs, $n = 8$) consisted of: control standard casing and control passaged casing; *P. tolaasii* -inoculated standard casing and *P. tolaasii*-inoculated passaged casing; *T. aggressivum*-inoculated standard casing and *T. aggressivum*-inoculated passaged casing.

2.2. Passaging approach

The process of generating passaged casing was described previously (Vieira et al., 2023). Briefly, casing was collected at the point of primordia formation (day 9–12 of casehold) during the cropping cycle from a selection of pots (30 %) with the highest number of mushroom

pins. This casing was temporarily held at 4 °C prior to each successive crop. Passaged casing was added to standard casing (casing prepared as per standard MRC procedures) at a ratio of 10 % w/w (wet weight) and the process repeated. In this study, the process was done for a total of 10 cycles of passages prior to the full crop. Passaged casing material was homogenized and added to all passaged casing treatments (control and disease-challenged). This passaged casing was used in the full-scale crop described herein.

2.3. Blotch inoculation and measurement

The blotch pathogen inoculum was prepared from overnight cultures of *P. tolaasii* (strain NCPPB 2192^T) grown on King's Medium B agar (KMB; King et al., 1954) suspended in 0.01 M phosphate buffer at 10^6 CFU/mL (adjusted photometrically to 0.6 OD at 600_{nm}). This was added to standard and passaged casing on Day 0 of casehold at a concentration of 10^6 cells/kg of dried casing material by watering the suspension over casing and mixing thoroughly. Standard casing and passaged casing inoculated with blotch were then added to experimental units and casehold was carried out as per standard procedure.

Disease outcomes of bacterial blotch in standard casing and passaged casing were measured by quantifying mushroom yield (kg/m^2 of harvested mushrooms). Blotch disease-suppression was assessed using yield data from both asymptomatic and blotch-symptomatic mushrooms. Calculation of blotch disease severity was made solely from diseased mushroom yield. Disease severity was measured visually during harvest using a grading system; where grade 1 was low severity, grade 2 was moderate, and grade 3 was severe levels of blotch disease. Grades were documented on a per-mushroom basis.

2.4. Green mold inoculation and measurement

Green mold inoculum was prepared using *T. aggressivum* f. *aggressivum* (accession number DC 327, Penn State University mushroom disease collection). Spore suspensions stored at −80 °C in 25 % glycerol were thawed and cultured on potato dextrose agar (PDA) for 5 days at 25 °C. Spores were enumerated using a Levy hemocytometer (Hausser Scientific, Horsham, PA) and diluted to a final volume of 200 μl of sterile saline solution (0.9 %) with a final concentration of 1.5 X 10^4 spores per experimental unit. Inoculation occurred on the day of spawning with a single point inoculation ~2 cm below the surface of the compost layer on one half of the tub surface. Casehold was then implemented under typical practices for standard and passaged casing.

Green mold disease severity was assessed in terms of mushroom yield (kg/m^2) for standard and passaged casing. Disease expression was evaluated by quantifying symptom-free mushroom yield only, as disease intensity is closely related to the inhibition of mushroom growth and development.

2.5. Statistical analysis

The statistical analysis of yield data was performed in pairwise comparisons for control standard casing and passaged casing, standard and passaged casing inoculated with *P. tolaasii*, and standard and passaged casing inoculated with *T. aggressivum*. Treatments were compared first by total yield and by flushes. Flushes typically occur in cycles of 7 – 8-day intervals in *A. bisporus* crops. However, due to the advancement of pinning noted in the passaged casing treatments and with the disease pressure effects of blotch and green mold infection, flushes were considered on a growth-trend scale. Flushes were distinguished by daily yield data, where the yield increases, peaks, decreases and then begins to increase again (signifying the beginning of the next flush). Pair-wise comparisons of standard and passage casing for all treatments were analyzed using non-parametric tests (Wilcoxon rank-sum test) following normality tests (Shapiro–Wilk) and Q–Q plots of the residuals using stats R package v 4.1.2 (R Core Team, 2022). To determine statistical differences in blotch disease progression, disease severity of *P. tolaasii*-inoculated standard casing and *P. tolaasii*-inoculated passaged casing was calculated by transforming the yields of each replicate of Grade 3 blotch-diseased mushrooms into area under the disease progress curve (AUDPC) values. Shapiro–Wilk tests were performed on the data from each day. Independent two-sample t-tests were implemented where data passed normality tests and Mann-Whitney-U tests performed when they did not pass normality tests. Permutation tests were employed as an alternative non-parametric test to the disease progress of blotch in passaged casing versus standard casing over the course of the whole crop.

3. Results

3.1. Comparison of control standard and passaged casing yields in the absence of added pathogen inoculum

The effect of passaged casing on disease dynamics was compared to a standard casing control. The previous crop casing was collected for passaging at the point of primordia formation from the best performing pots, in terms of the highest number of developing primordia. Passaging was repeated for ten cycles with a 1:10 mix of previous crop casing with 'fresh' standard casing prior to comparisons. Disease dynamics were assessed in terms of mushrooms harvested per unit area (kg/m^2) in treatment pairwise comparisons. Mushroom yield was recorded from a 22-day harvest period.

The timing of pinning (primordia formation) and cumulative yield trends of standard casing (CS) and passaged casing (CP) (Fig. 1) were similar to experimental crops reported previously (Vieira et al., 2023). Early pinning was promoted with the use of passaged casing by up to 3 days when compared to standard casing, where day 4 is the first day of harvest (Fig. 1). The cumulative yield values of both treatments were approximately the same around days 11 and 17 (Fig. 1). Differences in the cumulative yield of standard casing and passaged casing were accrued by the variable timing of flushes. Standard casing had an overall higher yield of 22.78 kg/m^2 compared to 20.33 kg/m^2 in passaged casing (Table S1). It is worth noting that the use of ten passages to generate the material for passaged casing potentially resulted in the carry-over of blotch pathogens in this material, as blotch symptoms were noted in a proportion of mushrooms harvested from this material. In passaged casing, the blotch symptoms observed were very mild in severity, but enough for a certain number of mushrooms not to be recorded towards yield (saleable mushrooms). Prevalence of blotch in passaged casing treatments was greater in the 2nd and 3rd flush (days 10–22; Fig. S1A, Table S2). When adjusting for the yield recorded in CP with additional yield of CP Blotch (note that these are non-salable

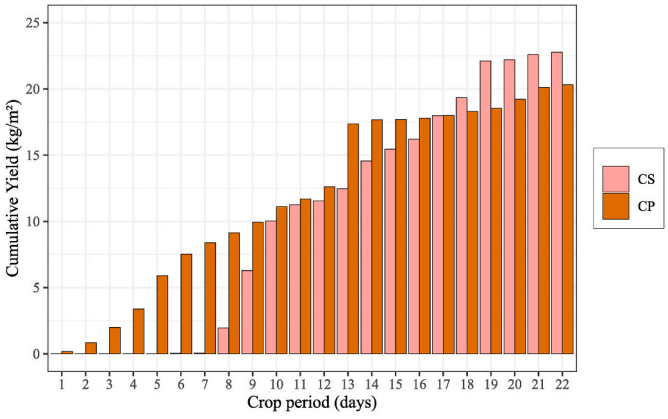

Fig. 1. Cumulative mushroom yield (kg/m^2) for standard casing (**CS**) and passaged casing (**CP**) treatments over a 22-day cropping period (representing mushroom harvest).

mushrooms), the yield in this treatment was higher than CS (Fig. S1B, Table S2). As expected, no blotch was observed in the standard casing treatment. Conversely, no green mold was observed in the compost layer, casing layer or mushrooms of CP experimental units.

3.2. Assessment of bacterial blotch challenge

The disease dynamics of blotch when using passaged casing were evaluated in the presence of added bacterial blotch inoculum. Both asymptomatic (marketable) and blotch-diseased (non-marketable) mushroom yield was measured to assess the impact of passaged casing on blotch disease. Disease pressure was high in both *P. tolaasii*-inoculated standard casing (BS) and *P. tolaasii*-inoculated passaged casing (BP), but notably, a much higher yield of asymptomatic mushrooms was harvested when casing was passaged - BP (Fig. 2A). As seen previously in passaged casing (with no pathogen inoculation), the *P. tolaasii*-inoculated passaged treatment (BP) also had an earlier harvest (pinning stimulation) than the non-passaged treatment. The first day of asymptomatic mushroom harvest in BS was 7 days after the first day of harvest in BP (Fig. 2A–Table S3). When measuring disease severity, a system of grades was established ranging from 1 to 3 for lowest to highest degree of blotch disease (Fig. 2B). The suppression of blotch by passaged casing is more pronounced when considering the degree of disease severity by grade. Blotch disease severity was predominantly in the highest category for mushrooms grown in BS. On the other hand, a more even distribution of grade 1–3 was recorded in BP over the crop period of 22 days (Fig. 2C–Table S4). In BS, grade 1 accounted for only 4 % of blotch-diseased mushroom yield loss, grade 2 at 12 %, and grade 3 as much as 84 % for all blotch-infected mushrooms harvested. In contrast, BP grade 1 was 21 % of the total, grade 2 was 22 %, and grade 3 was just over half the yield at 57 % (Fig. 2D). The disease intensity was significantly different ($P < 0.05$, following an independent two-sample t-test analysis) from day 3 to day 6 in BS and BP due to the promotion of pinning in BP mushrooms leading to the production of grade 3 blotch mushrooms (Fig. 2E–Table S5). Following day 6 (the beginning of yield data for BS), disease intensity was significantly higher ($P < 0.05$, according to an independent two-sample t-test analysis) in BS compared to BP for days 10–12 (proceeding the first flush of BS mushrooms), 20 and 21. The overall grade 3 yield was also significantly different between BS and BP following a permutation test ($P < 0.05$) (Fig. 2E–Table S5).

3.3. Assessment of green mold challenge

The effect of *T. aggressivum*-inoculation and the resulting expression of green mold disease in standard casing (TS) versus passaged casing (TP) was measured in terms of asymptomatic mushrooms harvested per unit area (kg/m^2). The inhibition of *A. bisporus* mycelial colonization in casing due to green mold disease was reflected in a decrease in

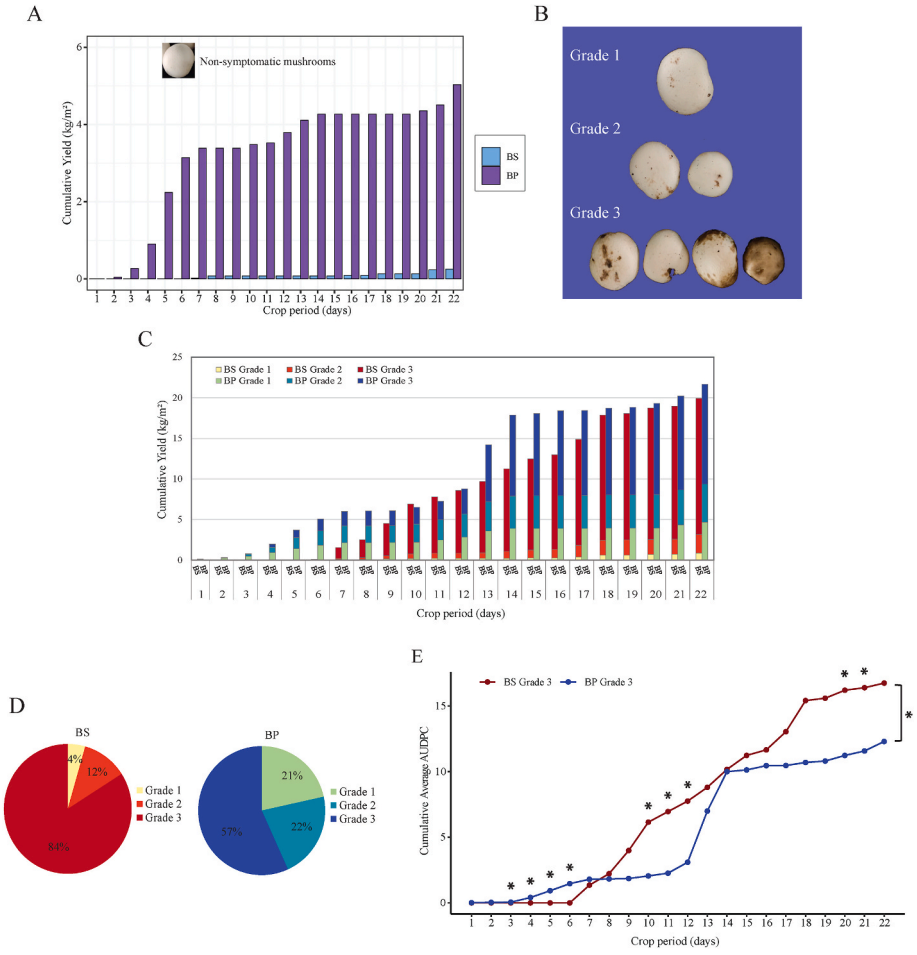

Fig. 2. A – Cumulative asymptomatic mushroom yield (kg/m^2) of standard casing inoculated with *P. tolaasii* (**BS**) and passaged casing inoculated with *P. tolaasii* (**BP**) over a 22-day cropping period (representing mushroom harvest). **B** – Reference guidelines for assigning blotch disease grade for each mushroom during cropping. Grade 1–3 correspond to blotch disease severity, where 1 is low, 2 is moderate and 3 is severe levels of disease. **C -** Cumulative daily yield (kg/m^2) for **BS** and **BP** by grade (1–3), representing blotch-diseased mushrooms. **D -** Blotch severity represented proportionally as a percentage of the total disease yield (kg/m^2) for each grade. **E –** Disease progress curves for grade 3 blotch-diseased mushrooms collected during cropping in **BS** and **BP**. Asterisks indicate significant differences ($p < 0.05$) between the cumulative average AUDPC values per-day and overall, between **BS** and **BP**.

mushroom yield in both treatments when compared to the non-inoculated treatments (Table S6). This loss of yield was pronounced in TP when compared to TS (Fig. 3A). The promotion of early pinning by passaging observed in treatments not inoculated with *T. aggressivum* was also evident in the presence of added *T. aggressivum*. Notably, harvesting for TS occurred 6 days after TP (Fig. 3A). However, the overall cumulative total yield of healthy mushrooms after 22 days was higher in TS at 18.74 kg/m^2 compared to 16.49 kg/m^2 in TP (Table S6). This indicates that disease severity was intensified by passaging (lower healthy yield of mushrooms in TP). Infestations of green mold commonly result in non-productive areas on the surface of casing. Signs and symptoms of green mold were prominently observed as *T. aggressivum* mycelium on the casing surface in passaged TP treatments only. Bare areas with minimal *A. bisporus* growth surrounding the inoculation point were also evident in the passaged casing (Fig. 3B). This effect is absent in the corresponding standard casing treatment (TS). These areas form a distinctive ring of non-productive casing around the point of inoculation (Fig. 3B).

3.4. Global analysis of crop yields

Assessing the yield of healthy (no pathogen inoculation) and asymptomatic mushrooms simultaneously, demonstrates the dynamic relationship of passaged casing versus standard casing across all treatments in terms of total yield and yield by flush (Fig. 4, Table S7). A strong effect is observed for total yield for BP vs BS ($p < 0.001$). Significant differences in yield are captured for TP vs TS in the 1st and 3rd flush ($p < 0.05$). The 2nd flush is the only time where TP is comparable to TS in terms of mushroom yield. BP and BS yield comparisons are significantly different ($p < 0.01$) in all flushes. BP yield was significantly higher than BS yield by total yield and in each of the flushes (Fig. 4, Table S7).

4. Discussion

In general, the use of passaged casing resulted in three outcomes that differed from standard casing: (1) stimulation of early mushroom pinning, confirming findings from our previous work (Vieira et al., 2023), (2) suppressive effect on blotch disease, and (3) conducive effect on green mold disease. The effect on early pinning formation was expected in passaged casing when compared to standard casing control treatments (Fig. 1), as this was recently observed by a similar method using passaged casing made from three cycles of passaging (versus ten in this work) (Vieira et al., 2023). Previously, we also examined the microbial communities present and highlighted a decrease in the relative abundance of certain member genera in the core microbiome of passaged casing such as *Pseudomonas*, *Flavobacterium*, and *Pedobacter*, but an overall greater community diversity when compared to standard casing. Hence, the microbial composition of casing is strongly correlated with mushroom development (Vieira et al., 2023; Wang et al., 2023). The onset of early pinning may be considered a positive strategy towards a more productive cultivation system even if the overall yield has not, hitherto, also increased (Fig. 1, Fig. S1). It is worth noting that early pinning was also a feature of passaged casing even when challenged independently by blotch and green mold pathogens (Figs. 2 and **3**).

Passaged casing served as a suppressive medium against bacterial blotch, more specifically *P. tolaasii*. Although, blotch disease pressure was high in both BS and BP treatments. Especially when we consider the total yield of CS was 22.76 kg/m^2 versus only 0.25 kg/m^2 in BS and CP was 20.33 kg/m^2 where BP was only 5.03 kg/m^2 (Tables S1 and S3). An inoculum density of 10^6 CFU/mL (OD600) manifested a high level of yield loss with many mushrooms being excluded from asymptomatic (saleable) yield records if any level of blotch was observed. Nevertheless, asymptomatic mushroom yields were considerably higher in BP vs BS treatments (Fig. 2A). Visually, the degree of blotch disease on certain mushrooms was striking (Fig. 2B). This made grading disease severity an important element of blotch assessment. The frequency of severe blotch infection (grade 3) was highest in BS (Fig. 2C). Blotch incidence was higher as the crop progressed, namely in 2nd and 3rd flushes (Fig. 2C, E, Table S1). Nearly half of all blotch incidence recorded in BP was of the mid and lower severity scales (Fig. 2D). This differs to findings for ginger blotch where Taparia et al. (2021b) described how increasing [*P. gingeri*] cell density resulted in more blotch disease in the 1st flush but the same was not observed in the 2nd flush. However, the authors go on to describe the increase of pathogen populations from 1st to 2nd flush in casing soils, particularly at an inoculum density of 10^5 CFU/g where a 100x increase was seen between flushes when compared to a smaller inoculum density of 10^3 and 10^4 CFU/g (Taparia et al., 2021b). The virulence of blotch pathogens is likely dependent on multiple factors (cell density, microbial interactions, crop phase, and microbial competition and antibiosis). The suppressive nature of passaged casing material is demonstrated by the combined findings for *P. tolaasii*-inoculated passaged casing treatments; (1) higher asymptomatic mushroom yield; (2) lower yield of blotch diseased mushrooms overall, and (3) lower incidence of blotch disease severity. Using inoculum concentrations of less than 10^6 CFU/mL may allow for a more nuanced assessment of disease suppressiveness in future experiments.

Inversely, the passaged casing led to a more disease conducive material in terms of green mold (Fig. 3). *T. aggressivum* pathogenicity was augmented in passaged casing which was particularly evident by the spatial pattern of visible green spores and bare areas of no-growth on the mushroom bed (Fig. 3). Green mold has caused massive economic damage to the mushroom industry worldwide and there is a push for biological control of the disease over the use of fungicides (Preston et al., 2018). Biocontrol with selective and timely application of *Bacillus* spp. directly into the casing has been shown to reduce green mold disease (Milijašević-Marčić et al., 2017; Pandin et al., 2018). Perturbations from

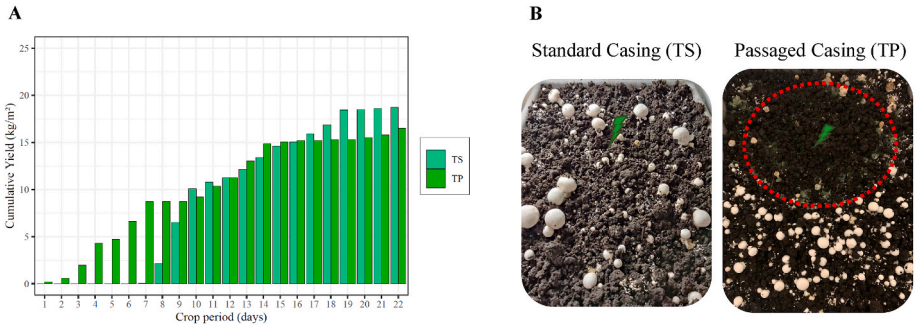

Fig. 3. A - Cumulative mushroom yield (kg/m^2) of standard casing inoculated with *T. aggressivum* (**TS**) and passaged casing inoculated with *T. aggressivum* (**TP**) over a 22-day cropping period (representing mushroom harvest). **B –** Comparison of green mold disease expression in **TS** and **TP**. Markers indicate the point of *T.* aggressivum spore inoculation. Note that in **TP** treatments there is a bare area where few mushrooms have not developed. This is highlighted by a dotted red line. The same bare area is not seen in **TS**.

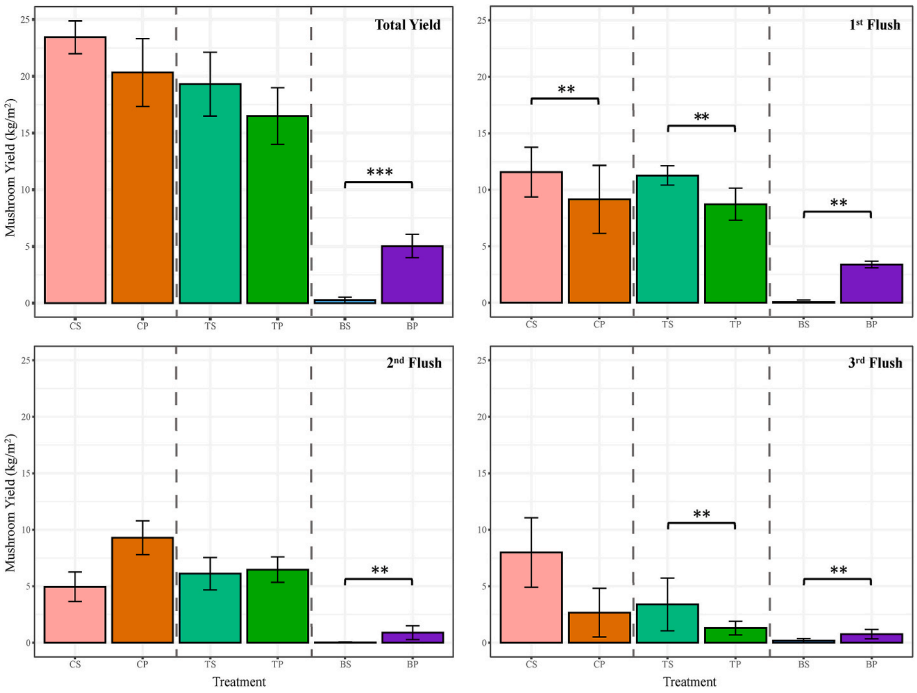

Fig. 4. Cumulative mushroom yield (kg/m^2) for the total yield (entire cropping period), 1st flush, 2nd flush, and 3rd flush. Yield was recorded for standard casing (**CS**), passaged casing (**CP**), standard casing inoculated with *T. aggressivum* (**TS**), passaged casing inoculated with *T. aggressivum* (**TP**), standard casing inoculated with *P. tolaasii* (**BS**) and passaged casing inoculated with *P. tolaasii* (**BP**). Statistical analyses were conducted in treatment pairs only (conveyed by dotted lines). Asterisks denote statistical significance between pairwise comparisons; $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***.

standard casing microbiome may potentially impact the number or structure of such antagonistic *Bacillus* spp. communities or other uncharacterized antagonists. Loss of bacterial species has been shown to result in loss of antifungal volatiles in previous works (Hol et al., 2015). The passaging approach may cause disruption to bacterial assemblages and thus promote casing conduciveness towards *T. aggressivum* infection. More work is needed to test this hypothesis and the inclusion of a *T. aggressivum*-suppressive casing material would allow for a more holistic way to study these interactions. Another explanation for the increased proliferation of green mold relates to the presence of additional *A. bisporus* hyphae and organic material, which likely originated from the passaged casing material. This material may have conferred a competitive advantage to the growth of *T. aggressivum* in passaged material, as *Trichoderma* spp. are well-known for their robust capacity to biodegrade organic materials (Howell, 2003; Sharma et al., 2012). Alternatively, the process of generating passaging cycles may have incubated *T. aggressivum* cells already present or introduced by contamination and allowed for an increase over time in cell/spore numbers, culminating in the breakout of green mold disease in these materials. However, it is worth noting that green mold was not detected in experimental pots used for the generation of passaged casing material (data not shown) nor was it recorded in the passaged casing treatment without pathogen inoculation. The conducive nature of passaged material for green mold will need further investigation, both in terms of *T. aggressivum* cell enumeration and in the use of a separate stream of casing passages, and an analysis of the microbiome in these systems.

Significant differences ($p < 0.05$) were noted between the treatment pairs when considering the total crop yield and each mushroom flush (Fig. 4). The suppression of blotch led to statistically higher yield of mushrooms in BP when considering the total crop yield. Additionally, there were significant differences in yield between BS to BP in each individual flush. Yields were significantly lower for the 1st and 3rd flush when comparing TP to TS. Examining variability in absolute terms, CP yields were lower in 1st and 3rd flush, but higher in the 2nd flush (Fig. 4). It is worth noting that we do not know what specific physical and chemical changes occur when passaging the casing. Properties such as water content have a strong correlation with disease outcomes in casing (Navarro et al., 2021; Noble et al., 1998) and these variables also need to be considered. We know from our previous work using amplicon sequencing that the abundance of different members of Pseudomonadota are altered in passaged casing (Vieira et al., 2023). *Pseudomonas* is a member of this phylum but without species-level taxonomic resolution, we cannot distinguish non-mushroom pathogenic *Pseudomonas* spp. from those related to blotch disease. More information is needed to decipher if pathogen-bacteria or pathogen-fungi interactions in the context of the casing devome have a role to play in disease conduciveness or suppressiveness. The work described herein adds to the need for microbiome-level investigations on the potential role that the casing devome plays in these disease dynamics.

Much work is needed to expand our understanding of the potential benefits and challenges that the use of passaged casing may have. Future efforts should employ microbiome analysis to understand the potential drivers of disease suppressive and conducive mechanisms found in these treatments. The conceptual framework behind the use of passaged casing is not to propose its use commercially, but to employ this strategy as a model to identify its effects on mushroom development and disease resilience. The aim of such a model is to inevitably draw a microbial cohort from this material that allows for the commercially desirable traits of passaged casing without the potential negative side-effects from passaged casing (for example, potential pathogen carry-over).

5. Conclusions and significance

In this current study, the use of passaged casing was evaluated against two important *A. bisporus* crop diseases, bacterial blotch and green mold. The findings present independent aspects of this manipulated material that may be commercially desirable or undesirable. This is a preliminary study that offers advantages to *A. bisporus* cultivation in terms of shorter cropping cycles (early pinning) and suppression of the virulence of a common crop pathogen (*P. tolaasii*). This work is one part

of a wider effort to improve our understanding of mushroom devomes. Manipulating these systems provides a model to improve our understanding of the role of the casing microbiome in multiple aspects of mushroom cultivation.

CRediT authorship contribution statement

Eoin O'Connor: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Fabricio Rocha Vieira:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Isako Di Tomassi:** Writing – review & editing, Investigation, Formal analysis. **Rachel Richardson:** Writing – review & editing, Resources. **Kevin L. Hockett:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Carolee T. Bull:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **John A. Pecchia:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.11.001.

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