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Final Report

Optimising nitrogen transformations in mushroom production

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Public summary

Mushrooms are an important source of protein in the human diet. They are increasingly viewed as a sustainable meat replacement, with button mushrooms (*Agaricus bisporus*) the most popular and economically important mushroom in Australia, Europe and North America. Button mushrooms are cultivated on a defined, straw-derived compost, and in Australia the nitrogen (N) required to grow these high-protein foods is provided mainly by the addition of poultry manure. The conversion of straw and poultry manure to productive compost is carried out by a huge range of bacteria and fungi in the compost, but little is known about which microbes in the compost are essential for incorporation of the necessary N, and how reproducible this microbial process is.

Using the correct balance of carbon (C) and N sources to produce mushroom compost is critically important in achieving maximum mushroom yields. This project has examined how changes in the amount and form of N added, the rate and timing of N addition, and the other compost components used can affect the proportion of added N recovered in the mushroom caps, the yield and quality of the mushrooms, and the loss of N as ammonia and nitrogen oxide gases during composting. Over 80% of the added N is recovered in the composting /cropping process, but only about 15% of this N is found as protein in the mushrooms that go to market, while most of the rest remains in the spent compost.

Mushroom composters have relied on chicken manure as their N source for many years, but changes in chicken husbandry have led to a reduction in the availability and the N content of manure, so alternative N sources are urgently required. This project has reviewed alternative N sources for use in Australian composting, evaluating such diverse materials as fish wastes, wool, and vegetable digestate. Recommendations are made to supplement or replace the current use of poultry manure as a sole N source.

The composting process relies on bacteria and fungi to break down the raw materials and incorporate added N into the compost. This project has compared the microbial populations present at different stages of composting in facilities from different Australian states in detail. This has revealed the presence of thousands of different microbial species in the compost, and described how their populations change during composting, and how they affect the incorporation of N into the compost. Although feedstock formulations and composting process differ between producers, the microbial populations in different composts are remarkably similar at the end of the compost conditioning process. This knowledge has been used by isolating samples of the major bacteria in compost and designing mixtures of microbes that can potentially be applied to low-productivity composts in order to produce better yields for the farmer.

Technical summary

Nature of the problem

Mushrooms are an important source of protein in the human diet. They are increasingly viewed as a sustainable meat replacement, with button mushrooms (*Agaricus bisporus*) the most popular and economically important mushroom in Australia, Europe and North America. Button mushrooms are cultivated on a defined, straw-derived compost, and in Australia the nitrogen (N) required to grow these high-protein foods is provided mainly by the addition of poultry manure as a compost feedstock. The conversion of straw and poultry manure to productive compost is carried out by a huge range of bacteria and fungi in the compost, but little is known about which microbes in the compost are essential for incorporation of the necessary N, and how reproducible this microbial process is. The key microbial N transformation processes are ammonification during thermophilic composting and nitrification during the pasteurization/conditioning process, but it is not known which are the main taxa responsible for these processes in compost, and whether this is reproducible in different composting facilities, or varies when different process parameters and feedstocks are used. Importantly, changes in chicken husbandry have led to a reduction in the availability and the N content of manure, so alternative N sources for mushroom compost are urgently required.

Research undertaken

Microbial diversity in mushroom composts from different Australian states was determined with a cultivation-independent method. Total compost DNA was extracted using bead beating/SPRI technology, and bacterial populations were characterized by next-generation amplicon sequencing (MiSeq) of the V3-V4 16S rRNA gene locus. Fungal populations were studied similarly using the ITS1 genomic region. Microbial populations were studied during Phase 1 and Phase 2 composting. In parallel, a representative strain collection of >200 bacterial isolates was obtained from the studied composts, by cultivation at mesophilic (30 °C), thermophilic (50-65 °C) and hyperthermophilic (75-80 °C) temperatures. The most abundant organisms found in the MiSeq dataset were identified in the cultivable strain collection, and these strains were used to construct consortia for compost bioaugmentation experiments. Physical interactions between these organisms in compost and in vitro were studied using cross-plating techniques, co-cultivation in liquid media, and baiting experiments in compost.

Alternative N inputs for composting were identified through a literature review and consultation with composters, focussing on the content of microbe-available N (allowing for moisture content), ease of supply to composting yards, year-round availability, uniformity, and low/zero alternative competitor value as e.g. animal feed or fertiliser. The nitrogen mass balance for mushroom production (composting and cropping) was determined by an extensive survey of growers and measurements of total compost N (combustion) at different stages of composting. This was complemented by measurements of nitrous oxide losses (gas chromatography) and ammonia release (Draeger tubes) during Phase 2, but did not include a reliable assessment of ammonia losses in Phase 1, or N losses as goody water. Nitrous oxide production by key nitrifying bacterial taxa in Phase 2 was measured, as was nitrification during cropping.

Bioaugmentation of Phase 2 compost was performed using a range of bacterial and fungal consortia. These consortia used strains from the compost strain collection, and focussed on the dominant taxa identified in compost in the MiSeq database described above. Stimulation of *Agaricus* growth rates was studied using race-tubes and in bulk compost. A fertigation system was designed and constructed using drip irrigation technology, with driplines installed between compost and casing, and the drippers directed into the compost. Nitrogen-containing additives were added in liquid form through the drippers during pinning and in the period of pin expansion between flushes, and the results were compared with traditional supplementation methods.

Major research findings

The microbial populations in composts from growers in five Australian states differed considerably during Phase 1 composting due to differences in feedstock sources, composting parameters and size of facility. However, the microbial fingerprints converged by the end of Phase 2, and were dominated by the ascomycete fungus *Mycothermus thermophilus* and by strains of nitrifying bacteria, most commonly *Pseudoxanthomonas taiwanensis. P. taiwanensis* produced the greenhouse gas nitrous oxide (N_2O) during growth in vitro, but the measured levels of N_2O release in a Phase 2 tunnel were not of environmental concern, suggesting that other organisms may absorb the released N_2O. The overall N balance for composting and cropping was determined by determining inputs and outputs for a range of Australian compost yards. The average total N inputs were approximately 10 kg /tonne Phase 1 compost (mainly as poultry manure), and measured outputs were approximately 8 kg N/tonne Phase 1 compost, indicating a recovery of ~80% of added nitrogen. However, only about 15% of added nitrogen was incorporated into the final mushroom crop for human consumption, while ~55% of added N was still present in the spent mushroom compost/casing. Losses in the process were largely in Phase 1 as goody water and gaseous ammonia. These could not be accurately quantified, since they differed significantly between yards due to differences in the composting process used.

Baiting experiments using key microbial isolates from Phase 2 compost showed that these appeared to associate with each other into a microbial complex. Bioaugmentation experiments during spawn run with a mixture of *M. thermophilus* and Phase 2 bacteria increased the rate of *Agaricus* hyphal proliferation. The effect was stronger with a mixed inoculum than with individual strains, but the effect on crop yield or quality has not yet been explored.

In a small scale experiment, direct supplementation of cropping compost by a complex nitrogen source (yeast extract) applied immediately before each flush showed an increase in crop yield compared to slow release supplementation, and was accompanied by an increase in the N content of the mushrooms in later flushes. A medium scale fertigation system was used to test this effect with urea or amino acid supplements, but the yield increases could not be confirmed, suggesting that the observed yield stimulation may be unrelated to N supply in the composts used.

Suggestions for future work

This project has provided a great deal of new information on the microbes present in mushroom compost and their activities. More work is needed to explore the range of alternative N sources that have been proposed as substitutes for poultry manure, which is currently the major N source used for composting. This should also examine the role of different microbes in assimilating these alternative N sources during composting, to ensure maximum productivity. The newly designed fertigation system should be used for testing of a broader range of compost supplements during cropping, since it allows a more targeted supplementation than the application of slow release fertilizer. This has the potential to enhance both yield and quality, and could also be applied for biofortification of mushrooms with other nutrients.

Keywords

Compost, mushrooms, microbial diversity, *Mycothermus*, *Agaricus, Pseudoxanthomonas*, fertigation, microbial interactions, nitrous oxide, ammonia

Introduction

Mushrooms are an increasingly important source of nutrition worldwide, and annual production has grown over 30-fold in the last forty years (Royse et al. 2017). Button mushrooms, *Agaricus bisporus*, are one of the most widely cultivated edible mushrooms, and in Europe, the United States and Australia they make up >90% of the mushroom market. Button mushrooms are grown on a composted substrate that is traditionally made from wheat straw, stable bedding, poultry manure and gypsum, though the feedstocks used vary in different parts of the world. Depending on seasonal availability, smaller amounts of other agricultural by-products such as canola meal, soybean meal and cottonseed meal are often added to provide additional nitrogen and stimulate microbial activity at the start of composting.

The production of mushroom compost is a microbially-mediated process in which lignocellulosic waste materials are converted into a nutrient-rich humus-containing medium. The mushroom composting process typically includes a period of wetting to soften the straw and initiate breakdown, a high-temperature composting phase (Phase 1, 70-80 °C) to degrade the structural components of straw and release ammonia through proteolysis, and a period of pasteurization and conditioning (Phase 2, 60 °C, ramping down to 45 °C), in which the breakdown products are incorporated into microbial biomass and humic-lignin products in the final compost. The microbial community in the compost changes continuously during composting, responding to changes in temperature and the progressive assimilation of plant cell components. The microbial dynamics of this process have been studied in detail in recent years (Cao et al. 2019; Carrasco et al. 2020; Song et al. 2021; Thai et al. 2022; Vieira and Pecchia 2021), with the earliest work done as part of Project MU10021 *Improving consistency of mushroom compost through control of biotic and abiotic parameters*, funded by Horticulture Innovation Australia (Kertesz et al. 2016). Although a number of thermophilic fungal and bacterial species have been isolated from mushroom compost using traditional culture-based methodologies (reviewed in Ryckeboer et al. (2003) and Kutzner (2000)), they revealed limited diversity and were primarily related to *Bacillus* and to actinomycetes such as *Streptomyces* and *Thermoactinomyces* (Ryckeboer et al. 2003). The more recent work using molecular sequencing tools has revealed a much wider range of microbial taxa in mushroom composts, with 30,000 – 100,000 different bacterial species observed in Phase 1 and Phase 2 composts (Thai et al. 2022; Vieira and Pecchia 2021).

Within our diet, mushrooms are a source of essential fatty acids and trace elements (Siwulski et al. 2020), and because they contain a high proportion of N they are also regarded as a high protein food (Wang and Zhao 2023). The N contained in the harvested mushrooms is taken up from the microbial biomass in the compost by the *Agaricus* mycelium. The composting process, in turn, relies on microbial activity to incorporate the N provided in the raw materials into the microbial biomass in the compost that feeds the mycelium. However, only about 15% of the total nitrogen added to the composting process is recovered as protein in the harvested mushrooms, largely because most of the added N is used in promoting the composting process itself. Nitrogen is required to stimulate the microbial activity that is important in creating a productive compost, but in the form of ammonia it is also very important in preparing the compost substrate chemically for enzymatic degradation. Proteolysis generates significant quantities of ammonia during Phase 1 composting, and at the elevated pH and temperature conditions present this ammonia helps promote chemical degradation of hemicellulose and lignin (Mouthier et al. 2017).

Australian and New Zealand mushroom farms grow around 60,000 tonnes of button mushrooms (*Agaricus bisporus*) annually (Food and agriculture organisation 2019). This requires around 340,000 tonnes of Phase 1 compost, produced from about 260,000 tonnes of raw materials, almost entirely a blend of wheat straw/poultry manure/gypsum, with minimal additions of other ingredients. The N required for compost production and to produce the mushroom crop is primarily provided through the addition of poultry manure, with occasional

supplementation in forms such as urea or ammonium sulfate. However, in recent years, changes to chicken husbandry techniques in Australia have resulted in considerable reductions in the N content of the poultry manure available to composters from broiler chicken farms, and there is therefore an urgent need to find alternative sources of N to use as compost feedstocks in Australia.

The key nitrogen-transforming activities observed in compost are high levels of proteolysis and ammonification in Phase 1, and high levels of nitrification during Phase 2 conditioning (Caceres et al. 2018). This leads to significant losses of nitrogen as ammonia in the thermophilic phase of composting, compensated by reassimilation of ammonia during conditioning, but it also leads to potential losses through nitrification. The details of these biological processes, and the overall nitrogen balance during composting have not previously been determined.

The current project therefore had the following main aims:

- Survey alternative feedstocks that Australian mushroom composters can use as an N source for composting;
- Develop a detailed understanding of the nitrogen balance in mushroom composting and production, and the microorganisms involved;
- Optimize nitrogen inputs for mushroom crop production to maximize nitrogen use efficiency;
- Develop an optimized microbial inoculum to promote nitrogen retention in mushroom cultivation;
- Develop optimized protocols for nitrogen addition during spawn run and cropping.

This project was closely linked to the 2017-2021 Mushroom Industry Strategic Investment plan. In particular, it was aimed at Outcome 2 of the plan, *Mushroom growers are profitable and sustainable through increased yields, reduced costs and effective risk management,* with a focus on Strategy 1, *Improve production by increasing yield and quality.* At the time the project was designed, the use of alternative composting feedstocks formed a key part of the Mushroom SIP logic (Figure 2 of the 2017-2022 Strategic Investment Plan). This arose from a concern that because the feedstock composition used by the Australian mushroom industry is very consistent through the year, the industry is very dependent on the availability of a small range of raw materials in order to maintain economic viability, and the loss of any of these feedstocks could cause industry failure. However, after this project completed a survey of alternative N-containing feedstocks that are available for composters to use in Australia (see Results), it became apparent that there was very little appetite amongst composters to switch to different feedstocks, even on a supplementary basis. The latter parts of the project therefore focussed on the manner and timing of nitrogen additions during cropping, and not during composting as originally planned.

Methodology

A detailed technical description of methods used is provided in Appendix 1.

Compost sampling from commercial composting facilities

Compost samples used in the project were obtained from nine commercial mushroom composting facilities located in New South Wales, Queensland, Victoria, South Australia and Tasmania. For confidentiality reasons, the composters are not named in this report – several have since gone out of business, and only provided samples for part of the project. Compost samples taken at specific timepoints during the composting process were pooled samples, produced by combining ten independent samples taken from across the face of the compost pile during turning. Sampling was done on site by industry personnel, who were provided with appropriate training by the research team (https://www.youtube.com/watch?v=TZG_QKWlOyA), and the samples were shipped to the University of Sydney for analysis.

Compost physicochemical measurements

Water content of the compost was measured gravimetrically by weighing subsamples of compost before and after oven drying. Moisture content was expressed as a percentage of the fresh weight. Ash content was determined gravimetrically after heating the dried sample for 2 h in a muffle furnace at 550 °C. pH and electrical conductivity of casing and compost extracts were determined using a pH meter and digital conductivity meter in 1:10 water extracts. Total carbon (C), nitrogen (N), and sulfur (S) content of dried and finely ground samples of feedstocks and compost substrates were determined by combustion. Total water-extractable C and N was measured using a TOC analyser. Microbial biomass in compost was estimated using the fluorescein diacetate (FDA) hydrolase assay (Shaw and Burns 2006). The humification index (HIX) in compost was measured by fluorescence spectroscopy of compost extracts (Ohno 2002). Nitrate and ammonium levels in composts were measured in water extracts using anion chromatography and cation chromatography, respectively. Total free amino acids in compost were measured using a ninhydrin-based method (Zhang et al. 2013).

Survey of nitrogen inputs and outputs in composting facilities

The nitrogen balance for the mushroom composting process was determined using process data provided by ten composting facilities around Australia. The data provided included: feedstocks used for composting; mass of typical compost crops; amount of water added; % volume losses during Phase 1, Phase and Phase 3; collection and recycling of goody water; use of supplement; average mushroom crop yield. The total N content of compost from each site was measured at end of Phase 1, end of Phase 2 and end of Phase 3 where applicable, and N-content was also determined for supplement and for casing material. N-contents of mushroom caps/stipes were average values determined by combustion of freeze-dried, ground samples, and N contents of wheat straw and poultry manure were derived from literature values. All values were normalized to 1 tonne of Phase 1 compost, in order to allow comparison of N inputs and outputs at each step between composting yards.

Measurement of nitrous oxide and ammonia release from compost

Release of nitrous oxide and ammonia gases by compost during Phase 2 composting was measured in a conventional commercial Phase 2 tunnel with 70% air recycling rate. Air was sampled from the return air ducting of the tunnel (800 mL samples). Nitrous oxide was quantified using a gas chromatography system equipped with an electron capture detector. Ammonia was sampled at the same time as nitrous oxide and was

quantified using Dräger tubes with detection range 2.5-1500 ppm ammonia.

Nitrous oxide release by specific bacterial strains in compost was measured at laboratory scale in sealed 250 mL bottles containing 5 g compost. Strains of denitrifying bacteria isolated from compost were added to the compost, together with ammonium or nitrite. Gas samples (10 mL) were taken from the bottles at selected timepoints and nitrous oxide release was quantified using gas chromatography.

Enzyme assays

Activity of nine enzymes in water extracts of compost samples was assayed using published colorimetric and fluorometric methods, modified as needed to fit 96-well format (Thai et al. 2022). The enzyme activities tested included protease, amylopectinase, β-glucosidase, cellulase, chitinase, invertase, peroxidase, FDA hydrolase and xylanase. Compost extracts were prepared at room temperature by shaking 0.65 g of ground, frozen sample in 6 mL of sterile ultrapure water for 30 min. Enzyme activity measurements were made using a plate spectrophotometer at times optimized to capture the amounts of product in the linear phase of enzyme activity for each enzyme.

Isolation and characterization of bacterial and fungal strains from compost

Cultivable bacterial and fungal strains present in Phase 1 and Phase 2 compost were isolated by suspending roughly chopped compost in appropriate growth medium, and then plating onto agar plates of the same medium. Bacterial isolates were obtained on R2A medium (Reasoner and Geldreich 1985) and fungal isolates were grown on YpSs medium (Atlas 2010). Cultures were incubated at 30 °C to select for mesophiles, 50 °C for moderate thermophiles and 75 °C for thermophiles. At higher temperatures, agar was replaced by phytagel as solidifying agent. Actinomycetes were enriched by plating on a nylon membrane (0.22 μm) and isolating filamentous organisms that grew through the membrane pores.

Purified individual cultures were stored in 20% glycerol (v/v) at -80 °C. Cultures were identified by PCR-amplification and sequencing of the 16S rRNA gene (for bacteria) and the ITS region (for fungi), followed by sequence comparison with appropriate databases. The roles of bacterial isolates in compost were further characterized by in vitro whole-cell measurements of cellulase, xylanase, laccase and peroxidase activities.

Microbial interaction studies and design of microbial consortia

Physical and biochemical interactions between individual compost strains were studied in vitro using several methods. Direct physical interactions were observed directly using brightfield and phase contrast microscopy. The effects of bacterial and fungal taxa on each other were studied during growth on solid medium by cross streaking, and on liquid media by cultivating a fungal partner on a membrane floating on the bacterial growth medium. Fungal-bacterial interactions in compost were studied by immobilizing the dominant fungal strain (*Mycothermus thermophilus)* on a nylon membrane, and using this as “bait” to enrich and identify bacterial interaction partners in the compost. The effect of such partnerships was studied by evaluating the stimulation of cellulase activity in the resulting microbial consortia.

Mixtures of bacterial isolates (“consortia”) were designed by combining cultures of bacterial isolates representing the dominant taxa present in compost, with or without the dominant fungal species *Mycothermus thermophilus*. These culture mixtures were applied directly to Phase 2 compost at a range of concentrations. The effect of the inoculum addition on *Agaricus* mycelium growth rate was measured using “race-tubes” (Smith et al. 1995), and the effect on overall mushroom crop yield and quality was evaluated after mixing inocula into Phase 2 compost at the start of spawn run in the Mushroom research unit, and cropping mushrooms under standard conditions.

Molecular characterization of microbial diversity in compost

Microbial diversity in compost samples was analyzed by high-throughput amplicon sequencing (Illumina MiSeq) of total compost DNA. Total DNA was extracted from freeze-dried, ground compost samples using a beadbeating method and purified using DNA binding magnetic beads. Bacterial communities were investigated using the V3-V4 region of the 16S rRNA gene, while fungal communities were studied using the ITS1 locus. The resulting sequence files were processed using the error-correcting DADA2 pipeline, and taxonomy was assigned by comparisons with the SILVA database for bacteria and the UNITE database for fungi.

Mushroom cropping in the Marsh Lawson Mushroom Research Unit

Mushroom cultivation from commercial compost was carried out in the University of Sydney Marsh Lawson Mushroom Research Unit. Spawn run compost was provided in standard 20 kg blocks by a local supplier. For growth experiments, these blocks were either cased directly with a mixture of peat, lime and compost, or the compost was repacked into 21-litre plastic boxes containing 7-8 kg compost per box prior to casing. The use of smaller boxes allowed higher replication of different treatments, and avoided pseudoreplication by ensuring that replicates were physically separated from each other. Where required, spawn run was done using end-Phase 2 compost from a commercial supplier, and incubating in plastic boxes until mycelial proliferation was complete. Cropping was done under standard conditions, harvesting three flushes of mushrooms, and these were characterized for yield and also for quality, if required. Compost samples for physicochemical or biological analysis were obtained during cropping by taking 1-cm diameter cores through the full depth of the compost bed, and then separating the cores into casing, top compost and bottom compost samples, as required.

Fertigation of mushroom crops using drip irrigation

Fertigation of mushroom crops was done during cultivation using standard 20 kg blocks (40 x 60 cm) of spawn-run compost. Six rows of drip irrigation piping were laid between compost and casing, with the drippers directed downward into the compost at 30 cm intervals. Nitrogen-containing additives were added in liquid form through the drippers at specific times during cropping, mainly during pinning and in the period of pin expansion between flushes. After addition, the pipes were flushed and emptied, to minimize mycelial proliferation into the drippers. Watering was done using standard overhead spray methods, but the amount of water added was corrected for the additional water input provided with the fertilizer.

Results and discussion

Bacteria and fungi in compost from Australian mushroom compost facilities

Microbial diversity in Australian mushroom composts

How does the total compost microbial diversity vary in mushroom composts from different parts of Australia?

The bacterial diversity in compost from five mushroom compost facilities in Tasmania, New South Wales, Victoria (2 facilities) and South Australia was determined by next-generation 16S rDNA gene sequencing of total DNA isolated from the composts. The populations were analysed in compost taken from the end of Phase 1, the end of Phase 2, and where possible from mid Phase 2 (access to mid-Phase 2 compost was not always possible, depending on how the composting process was managed in each individual yard). Individual compost samples were found to contain an average of 600-700 different bacterial species, though the bacteria found in Phase 1 were almost completely distinct from those found in Phase 2 (where tested, communities found in mid-Phase 2 were very similar to those found at the end of Phase 2). In particular, the genera *Pseudoxanthomonas, Thermus, Sphingobacterium* and *Lysinibacillus* were present in significant numbers in most facilities, with *Thermus* the commonest genus at the end of Phase 1 and *Pseudoxanthomonas* most dominant at the end of Phase 2. However, the proportions of each of these genera varied between yards, and many additional taxa were found in individual yards that were not common in all tested facilities. Interestingly, the bacterial communities were near-identical in successive composting runs from individual yards, suggesting that the precise composting process plays an important role in determining which bacteria are favoured.

The DNA-based method used here measures bacterial presence directly and does not rely on their ability to grow under laboratory conditions, and so it gives a much more reliable indication of which bacteria are active in compost than can be achieved with cultivation-based methods. However, in order to understand what these bacteria are doing in compost it is nevertheless important to cultivate and manipulate them in vitro. The ten most common bacterial species found across the different yards studied are listed in Table 1, and seven of these were successfully identified in the compost bacterial strain collection described below (often with multiple different isolates of each species obtained from different yards). This provides a firm basis for further work both to characterize the activities of compost bacteria, and to design inocula that can be added to composts to ensure reproducibility and consistency of composting and of mushroom crop yields (see below).

Table 1. The ten most abundant bacterial taxa found in Australian mushroom composts

Genus	Species	Are isolates of this species available in the strain collection?
Pseudoxanthomonas	*taiwanensis*	Yes
Chelatococcus	*sp.*	Yes
Chelativorans	*composti*	No
Thermus	*sp.*	Yes
Thermopolyspora	*sp.*	No
Actinotalea	*sp.*	No
Ureibacillus	*sp.*	Yes
Lysinibacillus	*sp.*	Yes
Paenibacillus	*sp.*	Yes
Schlegelella	*sp.*	Yes

Main findings:

- The bacterial communities present in Phase 1 and Phase 2 composts are quite different from each other. Phase 1 composts contains a succession of different bacterial species that change progressively during composting, while Phase 2 is dominated by a relatively stable community of several species, in particular heterotrophic nitrifiers such as *Pseudoxanthomonas* and *Chelatococcus.*
- The diversity of bacteria present in Phase 1 composts varies considerably between compost yards, and appears to depend on feedstocks, composting process factors and the size of the facility concerned. However, the bacterial community in Phase 2 compost is much more conserved between composters, with the result that the microbes present in the compost at the end of Phase 2 are quite similar at different composting facilities.
- Previous work has focussed on actinomycetes as the active microbes in compost, but the current studies has revealed that these generally make up less than 10% of the overall bacterial community in both Phase 1 and Phase 2, and compost bacteria are dominated by Proteobacteria.
- Studies of total bacterial diversity in compost have been carried out using techniques that do not require cultivation of the microbes in the laboratory. This ensures a much greater coverage of all species present. It has nonetheless been possible to also obtain isolates for most of the commonest bacterial species in compost, laying the groundwork for future application of these species in compost bioaugmentation.

Bacterial strain collection from Australian mushroom composts

What are the dominant cultivable bacteria in mushroom composts from different parts of Australia?

Most of the dominant bacterial taxa present in Australian mushroom composts have not previously been characterized in the laboratory. In order to understand the roles these bacteria play it is important to isolate them and characterize their potential activities, since this can help determine which of these strains are likely to play a role in individual environmental functions (e.g. conversion of particular straw carbohydrate polymers during the composting process, or carrying out key N transformations). This knowledge will guide the selection of the most important organisms that could be added to compost to provide more consistency in composting.

In this project, bacterial strains were isolated from Phase 1 and Phase 2 mushroom composts obtained from several Australian composters, to provide a representative Australian compost strain collection. In total, 222 strains have been isolated in pure culture, grown in the laboratory, and stored cryogenically. They represent 65 different species, many of which have been isolated from multiple composting facilities across Australia, and the strain collection therefore now contains many examples of slightly different strains from different states. (Figure 1). These provide additional insights into the importance of strain variation in composts, which will be beneficial in selecting the most appropriate strains when constructing compost inocula for future use. About forty of the isolates remain to be identified, but this is not unexpected with the wealth of unknown bacterial diversity that is present in compost. The isolates obtained from Phase 1 and from Phase 2 composts were distinctly different - the most frequent culturable species from Phase 1 were *Ureibacillus terrenus, Ureibacillus thermosphaericus, Microbacterium maritypicum, Bacillus haynesii* and *Geobacillus thermodenitrificans*, while the most frequent Phase 2 species were *Pseudoxanthomonas suwonensis, Pseudoxanthomonas taiwanensis, Staphylococcus succinus, Sphingobacterium thermophilum, Caulobacter segnis, Enterobacter hormaechei* and *Microbacterium foliorum*.

Pseudoxanthomonas suwonensis
Ureibacillus terrenus
Pseudoxanthomonas taiwanensis
Ureibacillus thermosphaericus
Microbacterium maritypicum
Bacillus haynesii
Thermus thermophilus
Staphylococcus succinus
Geobacillus thermodenitrificans

27, 13, 11, 8, 7, 6, 6, 5, 4, 4, 3, 3, 3, 3, 3, 3, 3, 3

Figure 1. Most frequent compost bacterial strains in the project's strain collection. Each species shown is represented by at least 3 different isolates obtained from different Australian compost yards. Numbers show the number of strains in the collection for each species.

It should be noted, however, that most compost bacteria do not grow well on laboratory media, so the collection only represents a subset of total compost microbial diversity. Strain isolation was done at both mesophilic temperatures (30-40 °C) and at higher temperatures (50 – 75 °C), and with several different growth media, in order to accommodate isolation of a broad range of species. However, isolation of actinomycetes, in particular,

requires specific conditions, and more work is required to expand the collection of these taxa (currently about 20 isolates, most of which have yet to be fully characterized).

One of the key aspects of mushroom compost production is the breakdown of wheat straw polymers (cellulose, hemicelluloses such as xylan, and lignin) into forms that can be used by the microbes in the compost. The compost microbes rely on a complex set of enzymes to access the nutrients in these polymers, including cellulases (both endo- and exocellulases), xylanases, and lignin-degrading enzymes such as laccase and peroxidase. All the bacterial strains in the initial (mesophilic) strain collection were screened for cellulase activity after growth in laboratory medium in the presence of cellulose. Of the 159 strains tested, only twenty strains showed active cellulase activity in vitro, and xylanase activity was detected in only 11 strains. Bacterial strains in the collection more commonly contained enzymes that are thought to be involved in lignin degradation, such as peroxidase (57 strains) or laccase (70 strains). It is therefore notable that many of the dominant compost genera that are represented in this collection (*Pseudoxanthomonas, Lysinibacillus, Sphingobacterium*) do not appear to produce their own cellulase enzymes, despite living in a cellulose-rich environment, and this suggests that successful degradation of polysaccharide polymers is primarily catalyzed by fungi, or by bacterial-fungal consortia.

A selection of the isolates was also tested for the presence of several bacterial genes that are important in nitrogen transformations. These genes included *amoA* (ammonium monooxygenase, important in the conversion of ammonia to nitrate), *nirK* and *nirS* (nitrite reductase, which converts nitrite to nitric oxide), and *nosZ* (nitrous oxide reductase, which converts nitrous oxide to molecular nitrogen). Of the 38 species tested, 10 did not appear to harbour any of the nitrogen transformation genes tested, and they are therefore presumably not actively involved in compost N transformations. The most abundant genes were *nirK* (present in 40% of species tested) and *nosZ* (present in almost 50% of species tested), suggesting that heterotrophic nitrification and denitrification are active processes in many compost isolates.

The dominant fungal species present in Phase 2 compost is *Mycothermus thermophilus (syn. Scytalidium thermophilum)*, which makes up 99% of the fungal biomass by the end of Phase 2 and is often already present at the end of Phase 1. This project has also compiled a collection of *M. thermophilus* strains from compost yards across Australia, which will be invaluable for later comparative studies.

Main findings:

- A bacterial strain collection of cultivable compost bacteria has been established. This currently contains 222 strains of 65 species of bacteria, and it continues to expand as further isolates are added to the collection. The bacteria were isolated from Phase 1 and Phase 2 composts from a range of mushroom composters from different Australian states. The collection provides an important resource for future studies of the microbial physiology of mushroom composts, and will form the basis for standardized microbial inocula that can be used to assure reproducibility of composting efficiency.
- Most of the bacteria in the strain collection are Proteobacteria, in alignment with the overall proportions of compost bacteria found using cultivation-independent techniques. This contrasts with previous studies, which have focussed on *Bacillus* species and Actinomycetes.
- Most of the bacterial isolates in the collection do not degrade cellulose or hemicellulose. In order to grow well in a compost environment they therefore must interact with active cellulose-degraders including fungi. By contrast, most of the bacterial isolates in the collection possess genes for nitrogen transformations such as nitrification and denitrification. The results emphasise the importance of choosing combinations of bacteria with the necessary abilities for use in bioaugmentation applications, rather than using individual strains.

- The bacterial strain collection is complemented by a collection of isolates of the ascomycete fungus *Mycothermus thermophilus* (syn. *Scytalidium thermophilum*), which is the dominant thermophilic fungus in Phase 2 compost. The isolates of this important species were obtained from different composting yards in a range of Australian states, and therefore represent the diversity of this species across the Australian mushroom industry.

Microbial interactions between compost organisms in vivo

Do the dominant microbes in mushroom compost cooperate in symbiotic partnerships to facilitate effective composting?

The dominant microbes in Phase 2 compost are the ascomycete fungus *Mycothermus thermophilus* and the bacterium *Pseudoxanthomonas taiwanensis.* In Phase 2 compost*,* Mycothermus makes up 99% of the fungal population, while *P. taiwanensis* constitutes up to 30% of the bacteria present (Thai et al. 2022). These two species (especially *Mycothermus*) provide the biomass that is the primary source of nutrition for the button mushroom mycelium during spawn run, and growth of the *Agaricus* mycelium is also stimulated by addition of *Mycothermus* in vitro (Op den Camp et al. 1990; Straatsma et al. 1989). Direct and indirect interactions between *Mycothermus* and several strains of compost bacteria were studied in liquid and solid growth media. *Mycothermus* growth was not inhibited by *Pseudoxanthomonas taiwanensis*, though the related *P. suwonensis* did reduce fungal growth significantly. Conversely, growth of *Pseudoxanthomonas* was reduced in the presence of *Mycothermus in vitro*, but this appears to be due to substrate competition rather than direct inhibition. *Mycothermus* hyphae appear to associate directly with *Pseudoxanthomonas* cells in compost, since when *Mycothermus* grown on a membrane filter was incubated in Phase 2 compost, *Pseudoxanthomonas* was subsequently identified in association with the fungal hyphae. This suggests that the two species may function cooperatively in the compost environment, potentially in a symbiotic arrangement relying on the fungal ability to degrade cellulose (*Pseudoxanthomonas* does not possess cellulase activity), and the role of the bacteria as heterotrophic nitrifiers. Interestingly, one of the other key bacterial genera in Phase 2 compost, *Chelatococcus* (Table 1) is also known as a heterotrophic nitrifier, but fungal interactions with this species have not yet been studied. The results have implications for the use of these organisms as compost inocula, since they suggest they may be more effective if the species are applied together.

Mycothermus growth was also strongly inhibited by a novel bacterial species belonging to the *Chitinophagaceae* family that was frequently observed in the Phase 2 microbial community, and has been isolated from two different compost yards. These strains belong to a novel genus and species, which has been named *Mycovorax composti* (Thai et al. 2024). It rapidly degrades *Mycothermus* hyphae in vitro using chitinase activity*,* but fortunately it does not have a similar effect on *Agaricus* hyphae. This species has the potential to reduce the *Mycothermus* population and impact growth of *Agaricus* indirectly, but it appears to be mainly active at elevated temperatures during Phase 2, and so its role is probably mainly to recycle nutrients during the compost conditioning process.

Main findings:

- The dominant fungus and the dominant bacteria in Phase 2 compost appear to interact physically in mushroom compost, and this may play an important role in composting. This suggests that if they are used in bioaugmentation applications, they will be more effective if applied as a combined fungal-bacterial mixture than as either of the components on its own.

- The most frequently occurring bacteria in Phase 2 compost are nitrifiers, which are able to convert ammonia into nitrate and other N-forms. Nitrification is a very important process in Phase 2, as it helps immobilize the ammonia released in Phase 2 into microbial biomass than can be used by *Agaricus* during hyphal proliferation in spawn run.
- The novel genus *Mycovorax* is relatively frequent in late Phase 2 composts, and strains of this genus have been isolated from several compost yards. This novel thermophilic bacterium attacks the dominant fungus *Mycothermus thermophilus* during Phase 2, causing degradation of the fungal hyphae by chitinase activity, leading to cell lysis. It is thought to play an import role in cycling of nutrients during compost conditioning. However, it does not grow well at lower temperatures, and therefore does not attack the *Agaricus* hyphae during spawn run.

Survey of Nitrogen inputs and outputs in Australian compost

N balance for mushroom production

How much of the nitrogen added in composting is recovered in the mushroom crop, and what are the key N losses during the mushroom production process?

Nitrogen inputs by nine different mushroom composting facilities were surveyed. Australian mushroom composters use a wide range of different nitrogen sources for composting, including poultry manure, urea, ammonium sulfate, canola, cotton seed, cotton trash, soybean meal, and feather meal (and a small amount of nitrogen is also provided by the wheat straw itself). In addition, further nitrogen is added to the process as supplement during spawn run, and in the *Agaricus* spawn. In order to compare inputs, all nitrogen additions were normalized to one tonne of P1 compost, correcting for mass loss during the composting process. There is considerable variation in the nitrogen added during the composting process in different Australian composting facilities, with total inputs during composting varying from 6.5 kg N /tonne P1 to 79.2 kg N/tonne P1. There was less variation in the N input as spawn/supplement.

The averaged values of N inputs (Figure 2) indicate a total N input of approximately 10 kg /tonne P1 compost, and outputs of approximately 8 kg N/tonne P1 compost, indicating a recovery of ~80% of added nitrogen. This does not include the impact of goody water, which is anticipated to recycle a considerable proportion of nitrogen from leachate back into the prewet phase (measured values of nitrogen in goody water are about 7.5 g/L (Kertesz and Safianowicz 2015; Safianowicz et al. 2018)), because the rate of application of goody water to straw and the rate of dilution with municipal water or river water varies greatly between yards. Only about 15% of added nitrogen is incorporated into the final mushroom crop for human consumption, and just over 20% is lost from the system, either in discarded leachate or in losses of gaseous N, especially in Phase 1.

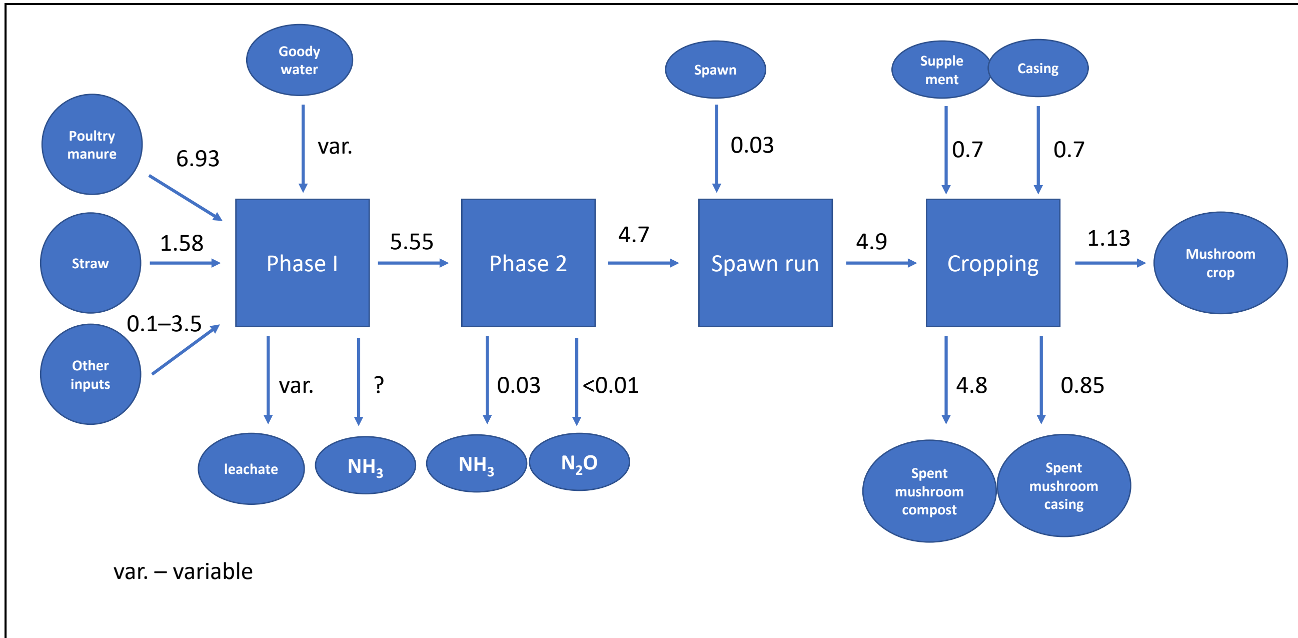

Figure 2. Averaged nitrogen inputs and outputs for the mushroom composting and production process for Australian mushroom composters and farms. Values presented are in kg N, normalized to the equivalent of 1 tonne P1 compost. Losses as leachate and release of ammonia from Phase 1 were not quantified as part of this project (see text), and it should be noted that there is considerable variation between different yards and mushroom farms.

Main findings:

- The main nitrogen inputs to Australian mushroom production are in poultry manure and other raw ingredients such as seeds and meals. Smaller amounts are added as supplement as with spawn. The main losses of N in the process are in Phase 1, with release of ammonia from the compost, but overall about 80% of added N is recovered. However, only about 15% of added N is incorporated into the mushroom crop, and most of the N finishes up in spent mushroom compost or casing. Although some of this N is unavailable to the mushroom mycelium (e.g. as nitrate) it is likely that much of it could be reused by recycling spent compost.
- There is considerable variation between mushroom composters and growers in the types and amounts of N applied to crops. Goody water was found to contain large amounts of N, which can be recycled to the crop is used in wetting. Very little consistency was observed between growers in the use of goody water, and more work is required to optimize its application.
- Supplements provide a small amount of additional N that is added prior to cropping, and are used by most, but not all growers. Given the levels of N that are present in spent mushroom compost, it is not clear how necessary supplementation is with Australian composts. More work needs to be done to quantify and optimize supplement use under Australian conditions.

Gaseous N losses during Phase 1 and Phase 2 composting

Does mushroom composting pose a potential climate risk through release of significant amounts of the greenhouse gas nitrous oxide?

Gaseous losses of N during mushroom composting may take three forms. Ammonia is released in considerable amounts during Phase 1 and Phase 2 composting through conversion of urea and through breakdown of proteins. At the high pH and temperature conditions in Phase 1 this ammonia helps promote chemical degradation of hemicellulose and lignin (Mouthier et al. 2017), playing a very important role in softening straw and making it accessible to further microbial degradation. Some of this ammonia is reincorporated into thermophilic compost microbes as they grow, and a lot of it may react with sulfate (gypsum) to form ammonium sulfate that is retained in the compost. However, much of the produced ammonia is lost as gas. At a few modern compost yards, the ammonia is recovered through scrubbing the off-gas collected from enclosed Phase 1 tunnels, but for most Australian compost yards this option is not available, since Phase 1 composting is done outdoors and exhaust gases are not collected.

A second potential loss of N from composting is molecular nitrogen (N_2), which many microbes produce from nitrate in the absence of air (a process called denitrification). This production of N_2 cannot be easily quantified against the background of atmospheric N_2, (air contains approximately 80% N_2) but could potentially be a sink for added N in the composting process. However, under the highly aerobic conditions found in modern composting systems the rate of denitrification is likely to be extremely low, and the only possible exception is in goody water. This may become anaerobic if not actively aerated, simulating microbes to high rates of denitrification and also to the emission of odoriferous, sulfur-containing gases. However, in well-managed goody water on Australian compost yards the observed denitrification rates are low (Safianowicz et al. 2018), and it is unlikely that this contributes significantly to N losses in composting.

The third form of N that can be released as a gas is nitrous oxide, which can be produced from ammonia or nitrite by a group of compost bacteria called heterotrophic nitrifiers. This is of particular interest because two of the commonest bacterial taxa in Phase 2 compost, *Pseudoxanthomonas* and *Chelatococcus,* (Table 1) belong to this group. It is also of potential concern because nitrous oxide is a potent greenhouse gas, with a global warming potential 265x higher than CO_2, and a lifetime in the atmosphere of >100 years (USEPA Inventory of greenhouse gases and sinks).

Nitrous oxide and ammonia production during Phase 2 composting were quantified by sampling from the recycled air in the return-duct of a Phase 2 tunnel. T A mass balance model (Yalçın et al. 2015) was used to estimate the production rate of the two gases during phase 2 composting, based on varying airflow rates and 55-70% air recycling to maintain compost temperature. Ammonia was initially generated at rate of 15 grams per day per tonne compost, but this decreased rapidly over the 6 days of Phase 2. Nitrous oxide was produced at a much lower rate, initially around 40 milligrams per day per tonne compost, and decreasing to about 15 mg/d/tonne. Summing these modelled values over Phase 2 revealed total losses of 2.5 – 4 kg NH_3-N per phase 2 tunnel and 15 – 30 g N_2O-N.

Main findings:

- The most abundant bacterial strain in Phase 2 compost is commonly the heterotrophic nitrifier *Pseudoxanthomonas taiwanensis*. Strains of this organism isolated from mushroom compost produced the greenhouse gas nitrous oxide from the nitrogenous compound nitrite under aerobic conditions in vitro during growth both in liquid medium and on compost. This suggested that nitrous oxide losses from Phase 2 compost could potentially pose an environmental risk.

- Measurements of nitrous oxide in a commercial Phase 2 tunnel showed that despite the high population of *P. taiwanensis* present in the compost, the amounts of nitrous oxide produced were very low (15-40 milligrams of nitrous oxide per day per tonne compost). This confirmed the presence of an unexpected biological sink for nitrous oxide in the compost, which remains to be identified. Importantly, it also confirmed that Phase 2 composting does not pose an environmental risk through nitrous oxide emissions.
- Gaseous nitrogen losses during Phase 2 as ammonia and nitrous oxide did not constitute a major loss of nitrogen from the composting system.

Nitrification during cropping

Does bacterial nitrification produce sufficient nitrate during cropping to inhibit mushroom production in later flushes?

Previous studies (Project MU10021) have reported a continual rise in the levels of nitrite and nitrate in the casing throughout cropping. This nitrate accumulation during cropping was correlated with the reduced crop yield observed in later flushes, and with increased levels of *Trichoderma* infection. Further work suggested that the accumulation of nitrate might be associated with increased populations of nitrifying bacteria in the casing, since these naturally produce nitrate from released ammonium (Shueh 2017; Thai 2015). To test this further, nitrate accumulation in casing and compost was measured throughout the cropping process, in the absence and presence of a commercial inhibitor of bacterial nitrification (dicyandiamide, DCD), and with periodic addition of nitrate as a control treatment. The DCD treatment led to reduced nitrate concentrations at second and third flush, and also to a slightly enhanced crop yield for these flushes.

However, although DCD has been well characterized as a nitrification inhibitor for research purposes, it cannot be used as a food treatment, due to its toxicity. A series of alternative treatments were therefore applied, including natural essential oils that have been reported to act as nitrification inhibitors, though their mechanism of action is not well characterized (Opoku et al. 2014). Neem oil, spearmint oil and karanj oil were sprayed as aqueous suspensions onto the casing (3 g/m^2) at four time points through cropping. Overall, nitrate levels were slightly reduced for neem oil treatment, but no effect was seen for the other treatments, and a marginal increase in crop yield was observed for neem oil treatment. The proportion of nitrifying bacteria in the casing was assayed throughout the experiment using genetic methods (Levy-Booth et al. 2014), and was found to be significantly reduced with neem oil compared to the control treatment.

Main findings:

- Nitrate accumulates in casing during mushroom cropping, and may affect the yield of third flush mushrooms. The activity of nitrifying bacteria in the casing may contribute to this nitrate accumulation, since the use of dicyandiamide as a nitrification inhibitor reduced nitrate levels later in cropping, and led to a slightly enhanced crop yield.
- Spraying casing with neem oil had a similar effect to using dicyandiamide as a nitrification inhibitor, and gave slightly enhanced crop yields. However, it was not clear that this result was due exclusively to the action of neem oil as a nitrification inhibitor, and this needs to be investigated further.

Alternative N sources for Australian mushroom composting.

What alternative N sources are available and commercially practicable for use by Australian mushroom composters to replace or complement poultry manure?

The main nitrogen-containing feedstock used for production of mushroom compost in Australia is poultry manure (manure/litter from broiler and layer chickens), though smaller amounts of other N sources are also used, including feather meal, cottonseed, soybean meal, canola meal and cotton trash. These organic nitrogen sources are supplemented on occasion by addition of inorganic nitrogen compounds. A comprehensive review of alternative nitrogen sources available to Australian mushroom composters was completed for this project by Dr Ralph Noble (Microbiotech, UK) and is included at Appendix 2. It was also subsequently published in modified form as a journal article (Appendix 8). Possible organic matter N sources were considered on the basis of the following criteria:

- success of similar materials in mushroom cultivation tests in other countries
- content of microbe available N on a weight and bulk volume basis (allowing for moisture content)
- ease of collection and transport from supply to composting yards
- year-round availability and/or storage capability and requirements
- uniformity and absence of physical and chemical contaminants
- wide available and low/zero alternative competitor value such as animal feed and fertiliser.

The key conclusions for the industry are shown below (Table 2), highlighting materials currently used or with potential for use in Australia and New Zealand.

Table 2. Materials currently used or with potential for use as nitrogen sources for mushroom compost in Australia.

N-containing feedstock	N content (% of dry matter)	Dry matter content (%)
Canola/Rape seed meal	3.3	85
Cottonseed meal	6.5	92
Cotton trash	1.5	91
Feather meal	4.9	67
Glasshouse crop haulms	1.8	11
Grape marc	1.8-2	27-32
Horse manure	1.3	37
Paunch grass	3-3.5	15
Poultry manure, caged	1.5-4.7	25-67
Poultry manure, broiler	4.5-5.4	60-66
Poultry manure, deep litter	2.2-2.7	48-79
Soya bean meal	7.1-7.4	91
Vegetable wastes	1.8	13
Wool waste	14	90

Further feedstocks were also investigated in the review but were not regarded as commercially viable as N sources at this stage. These included dried blood meal, dried and fresh brewers' grains, cattle slurry, cocoa meal, poultry manure digestate, fish solubles, guano, dried hop waste, horn meal, dried malt sprouts, pig manure, sea algae meal and sugar cane bagasse.

In preparing this review, Dr Noble accessed a wide range of industry literature sources and consulted in detail with Australian mushroom compost manufacturers to determine their present usage and future needs. One of the key conclusions of the review is that there is a need for an up-to-date inventory of the types, quantities, and localities of by-products from the Australasian agricultural and food production sectors, in order to identify waste streams that can be used as potential compost C and N sources. It was originally planned that Dr Noble would follow this review with a visit to Australia to work with composters and suppliers to help implement use of the most promising alternative N-sources, but this was not possible due to travel restrictions associated with the Covid-19 pandemic, and because of reluctance on the part of composters to make substantial changes to their current compost formulations at this point in time. If desired, this aspect of the project can be investigated further in the future.

Main findings:

- Poultry manure/litter is the most commonly used N source by Australian mushroom composters. A number of alternative N sources are available to Australian composters, including wool and vegetable wastes, glasshouse crop haulms and grape marc, but these have not found general acceptance.
- The application of many other potential N-containing feedstocks is limited by requirements for year-round availability, ease of transport to composting sites, the absence of physical or chemical contaminants and lack of competitor value as e.g. fertilizer or stock feed. Alternative feedstocks that do not meet all of these criteria are unlikely to be accepted by the mushroom industry even as partial substitution for currently used N sources.
- Increased use of inorganic N sources such as urea and ammonium sulphate is not desired, as a literature survey showed that even though a proportion of poultry manure can be replaced by inorganic N sources, replacing 50% or more is likely to result in a reduction in mushroom yield.
- There is an urgent need for an up-to-date inventory of the types, quantities, and localities of by-products from the Australian agricultural and food production sectors that can be used as potential compost C and N sources.

Bioaugmentation/biostimulation of cropping compost to stimulate mushroom production

Addition of microbial inocula during spawn run

Can bioaugmentation with a defined microbial inoculum be applied to stimulate reproducible Agaricus growth during spawn run?

A persistent problem experienced by mushroom growers is the variability of crop yields, with compost "quality" showing significant differences between crops. Part of the reason for this variability may be variation in the microbial load and community in different batches of Phase 2 compost. This was explored by adding defined microbial inocula to Phase 2 composts, and measuring the effect on spawn run. As well as providing more reproducibility, an increase in the mycelial growth rate during spawn run might reduce the time needed for each crop by several days, and therefore provide the potential for more crops per year.

Several of the bacterial strains in the compost collection were investigated in a spawn-run model system, selecting *Pseudoxanthomonas taiwanensis* and *Chelatococcus composti* as examples of Phase 2 bacteria, and *Bacillus licheniformis* and *Thermus composti* as examples of Phase 1 organisms. *Thermus* was later eliminated, as it did not survive well under the chosen conditions. These bacteria were inoculated into Phase 2 compost as individual strains, in mixtures, and in combination with the dominant fungal component of mushroom compost, *Mycothermus thermophilus,* using "race tubes" to study the effect of inocula on the efficiency of *Agaricus* proliferation in a small-scale, reproducible system. The best results were obtained with a mixture of Phase 2 bacteria (*Pseudoxanthomonas* and *Chelatococcus)*, together with the fungus *Mycothermus.* This consortium accelerated hyphal growth by up to 5%*,* confirming the hypothesis that an effective inoculum consists of a combination of fungi and bacteria, rather than bacteria or fungi alone. Bacterial combinations including *Bacillus* appear to have a negative effect on mycelium proliferation, possibly reflecting the source of this organism from Phase 1 compost rather than Phase 2 compost. Overall, the *Agaricus* mycelium growth rate during spawn run was not greatly stimulated by bioaugmentation, but it should be noted that the commercial compost used for these experiments was a high productivity compost, and the effects may be different for less reliable composts.

If bioaugmentation is to be used to accelerate spawn run, it is important to understand whether the added microbes are simply providing a nutrient source for the *Agaricus* mycelium, or whether they are acting in a symbiotic manner to promote mushroom growth. To test this, a range of cell concentrations of the microbial inoculum were applied. Mycelial growth of *Agaricus* was stimulated by addition of even low cell numbers of the added microbes (50 cells/g compost of each strain), and it therefore appears likely that these organisms are acting in a symbiotic role, and not merely providing additional nutrition for the mushroom mycelium. Interestingly, supplementing with the total compost microbial population had a much lesser effect, with 10^5 cells/g compost required for stimulation. This suggests either a specific role of the chosen consortium species, or the presence of growth-inhibitory organisms in the total compost extract. More work is required to define this, using a broader range of microbial strains and strain combinations and combinations of bioaugmentation with nutrient addition (supplementation with N sources or fatty acids).

While an increase in mycelial growth rate during spawn run can potentially decrease the time required for each crop (and hence increase productivity), the desired effect of bioaugmentation is of course an increase in the measured yield or quality of the crop. This was tested several times in the Marsh Lawson Research Unit, and although an increase in the rate of spawn run was again apparent, the crop then became more susceptible to disease, and the yield results were inconclusive. It seems unlikely that the inoculum strains themselves caused

this effect, since they are present in low levels in the compost already, and it will require further work to determine whether this effect was due to the method of inoculum addition, compost-related hygiene issues or unrelated factors.

Main findings:

- Bioaugmentation of compost during spawn run with a mixture of specific Phase 2 bacteria (*Pseudoxanthomonas, Chelatococcus)* and the fungus *Mycothermus* led to an increase in mycelium growth rate. This has the potential to reduce the time required for each crop, but the improvement is unlikely to be commercially significant.
- An increase in mycelial growth rate was observed even at low levels of bioaugmentation, suggesting that these organisms are acting in a symbiotic role, and are not merely providing additional nutrition for the mushroom mycelium.
- It is not yet clear whether bioaugmentation during spawn run has an effect on the crop yield, and more work is required to determine this.

Bio-fertigation with added nitrogen sources during cropping

Can biofertigation with organic N (especially amino acids/protein) be used to increase mushroom crop yield, quality and N content?

In order to maximize crop yield and quality, a nutrient supplement is usually added to commercial composts. These supplements are commonly soybean derived and contain high levels of protein, and they are partially denatured by formaldehyde treatment to provide a slow release formulation. Supplements of this nature are thought to provide additional nitrogen or trace elements for mushroom growth, but they may also yield essential cofactors required for mushroom growth or fructification. Previous work (Kertesz et al. 2015) has described nitrogen speciation changes in compost and casing during cropping, and observed a transient increase in free ammonium in the casing immediately before each flush. This suggested that the *Agaricus* mycelium might have particular nutritional needs at the pin expansion stage. The use of a slow-release supplement has the disadvantage that much of the nutrient is used by other compost microbes, so the effect of time-specific supplementation of *Agaricus* was tested. This involved the use of complex liquid supplement (yeast extract or commercial soy supplement), urea, or ammonium acetate, added either at casing or delayed until the pinning/pin expansion period before each flush.

In initial experiments supplements were manually injected into compost 4 cm below the casing. Crop yields were substantially improved by delayed treatments with complex supplement, with the total yield improved by 7% after delayed supplementation (or 14% compared to the unsupplemented control). Urea supplementation led to a substantial yield increase over unsupplemented compost, but the timing of the urea addition did not further affect the yield obtained. Although ammonium supplementation led to an overall decrease in yield compared to control, delaying the application led to a 13% increase compared to application at casing. For all three supplements tested, delaying the application of supplement led to increased yields of mushrooms in the later flushes, with the largest effect seen for second flush mushrooms.

This approach was explored further by installing a "fertigation" system in the compost to allow large scale addition of liquid supplements at defined times during cropping, using drippers at 30 cm spacings positioned to achieve uniform coverage of the compost blocks and directed down into the compost (Figure 3). Addition of

urea immediately before each flush did not increase crop yield significantly. Further experiments using high levels of a plant-derived amino acid solution were likewise inconclusive. Carefully controlled additions of a combination of traditional supplement and amino acid solution to provide a range of overall nitrogen supplementation levels showed an increase in quality in third flush, but no increase in yields. The results appear to suggest that the compost used did not actually require nitrogen supplementation, and that the yield increases observed in the initial experiments may have been due to some other component of the yeast extract supplement used.

Mushrooms are regarded as a high protein crop, but the work yielded an unexpected finding with respect to the nitrogen content of the mushroom caps themselves. Mushrooms from the second and third flush of cropping had a very much higher content of nitrogen than first flush mushrooms (Figure 4). This effect was independent of moisture content, and suggests that later flush mushrooms, which usually make up a smaller proportion of the overall crop, could profitably be marketed as “super-protein” foods.

Figure 3. Drip fertigation system installed at the MLMRU, showing the drip lines before and after casing. The drippers were approximately 30 cm apart and directed downwards into the compost for nutrient delivery. The drip lines were flushed with water and emptied after each nutrient application. Water to maintain casing moisture content was provided by overhead irrigation.

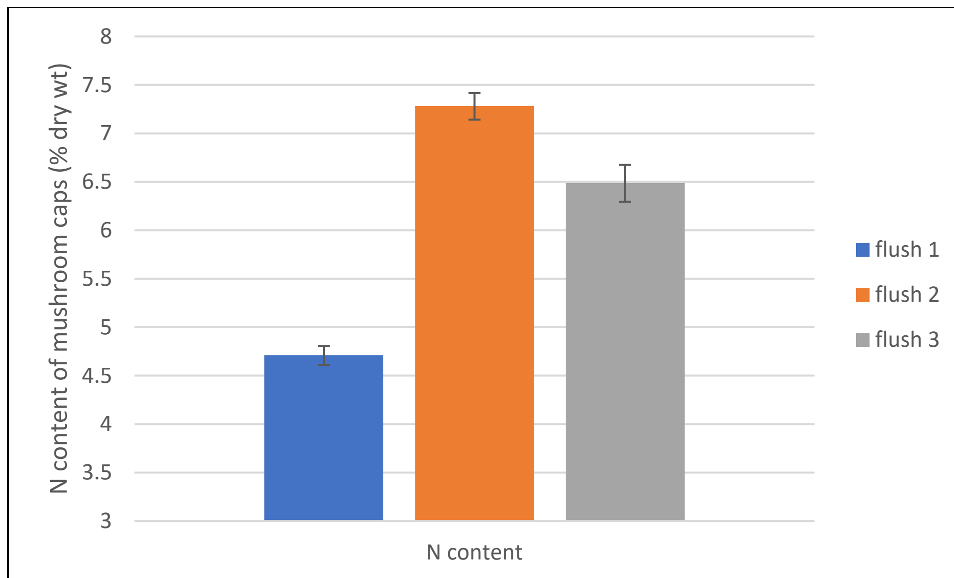

Figure 4. Nitrogen content of mushroom caps for three flushes. Mushrooms were dried and ground before analysis, and data are provided as % of dry weight. Moisture content of the fresh mushrooms were in the range 90-95% for all three flushes Error bars are standard error (n=10)

Main findings:

- The nitrogen content (% dry weight) of mushroom caps and stipes is substantially higher in second and third flush than in first flush mushrooms, with an observed increase of 40-50% in N content. This is not due to a change in moisture content in later flush caps, since this remained almost constant. This suggests the possibility of marketing late flush mushrooms as “super-protein foods”.
- Cropping compost was supplemented by direct injection of complex, protein-containing supplement as yeast extract at the stage of pin expansion immediately before each flush. This led to a 7-14% increase in crop yield, mainly in 2nd and 3rd flush, suggesting a requirement for additional supplement later in cropping.
- A fertigation system was developed using drip irrigation technology to release a defined amount of additional supplement into the compost in liquid form before each flush. The total water supplied to the crop was maintained constant by adjusting overhead watering. The resulting system has the potential to be used for delivery of a wide range of nutrient-enrichments in specialized mushroom crops. In this case, nitrogen was supplied either as urea or as an amino acid solution derived from plant digestate, but the results were variable and no reproducible enhancement to crop yield was observed.

Outputs

Table 3. Output summary

Output	Description	Detail
International research publications	Four reviewed articles appeared in international journals. One additional article is currently in revision, two further articles in preparation	See list below. The published papers are in Appendices 5-8. These papers have already been frequently cited by other authors. The highest impact to date has been achieved by Thai (2018) (Appendix 6), which has been cited approximately once every month since publication (total 74 citations to date).
Webinars and reports in industry journals	One webinar to an international audience. Two publications in the Australian Mushrooms Journal.	See list and links below. The webinar was presented to a total audience of well over 100 registrants from all over the world, with 29 groups and individuals participating live. The Australian Mushrooms Journal articles are in Appendix 3.
Presentations to industry conferences	Regular presentations to the AMGA conferences	Oral presentations in 2018 and 2022. Poster presentations in 2022. Abstracts and posters are in Appendix 4.
Presentations to international mushroom conferences	Regular presentations to the ISMS and NAMC congresses.	Invited oral presentations to the North American Mushroom congress (2019, 2024). Invited oral presentations to the International Society of Mushroom Science congress in 2021 (2 oral presentations, 1 poster) and 2024 (oral presentation). Abstracts and posters are in Appendix 4, links are below, where applicable.
Presentations to other international conferences	Presentation to the Australian Society of Microbiology congress and Australian Microbial ecology conference	Oral presentation to the Australian Society of Microbiology congress (2022), poster presentation to the Australian Microbial ecology conference (2022). Abstracts and posters are in Appendix 4.
PhD thesis	PhD thesis, University of Sydney (148 pp) by Dr Meghann Thai	Available at the University of Sydney Library website https://www.library.sydney.edu.au/

Outcomes

Table 4. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
Australian growers equipped to maximize nitrogen use efficiency in Mushroom production.	Aligned to SIP 2017-2021: Outcome - *Mushroom growers are profitable and sustainable through increased yields, reduced costs and effective risk management;* Strategy - *Improve production by increasing yield and quality;* *KPI - identification of yield productivity increases*	Extensive new information provided to stakeholders concerning compost microbiology, the N balance during composting and mushroom production, and how compost management affects N recovery	Feedback from composters and growers at AMGA conferences, Feedback from composters and growers via the Marsh Lawson Steering Committee, Discussion and advice from the Project Reference Group
Detailed understanding of the microbial interactions between inoculum strains, between inoculum and compost substrate, and between inoculum and the button mushroom mycelium.	Aligned to SIP 2017-2021: Outcome - *Mushroom growers are profitable and sustainable through increased yields, reduced costs and effective risk management;* Strategy - *Improve production by increasing yield and quality;* *KPI - identification of yield productivity increases*	Extensive new information provided to stakeholders concerning compost microbiology, the N balance during composting and mushroom production, and how compost management affects N recovery	Feedback from composters and growers at AMGA conferences, Feedback from composters and growers via the Marsh Lawson Steering Committee, Discussion and advice from the Project Reference Group
An optimized protocol for nitrogen supplementation and microbial augmentation during mushroom spawn run and cropping.	Aligned to SIP 2017-2021: Outcome - *Mushroom growers are profitable and sustainable through increased yields, reduced costs and effective risk management;* Strategy - *Improve production by increasing yield and quality;* *KPI - identification of yield productivity increases*	Although this was a projected outcome for the project, the research results obtained at small/medium scale did not warrant extension to a large scale trial at this stage	See Results and Discussion above. Discussion and advice from the Project Reference Group
Development of fertigation methods to allow phased addition of nutrient to composts during cropping	Aligned to SIP 2017-2021: Outcome - *Mushroom growers are profitable and sustainable through increased yields, reduced costs and effective risk management;* Strategy - *Improve production by increasing yield and quality;* *KPI - identification of yield productivity increases*	Although this was a projected outcome for the project, the research results obtained at small/medium scale did not warrant extension to a large scale trial at this stage	See Results and Discussion above.
Detailed understanding of how the activity of nitrifying bacteria in composting and cropping can be controlled.	Aligned to SIP 2017-2021: Outcome - *Mushroom growers are profitable and sustainable through increased yields, reduced costs and effective risk management;*	The research results showed that the level of nitrification observed during cropping was not sufficient to warrant large scale intervention	See Results and Discussion above.

	Strategy - *Improve production by increasing yield and quality;* *KPI - identification of yield productivity increases*		
Increased rate of adoption of research outcomes.	Aligned to SIP 2017-2021: Outcome - *Mushroom growers are profitable and sustainable through increased yields, reduced costs and effective risk management;* Strategy - *Improve production by increasing yield and quality;* *KPI - identification of yield productivity increases*	Research outcomes only partly adopted. Recommendations concerning alternative N sources were provided to the PRG and to growers directly, but there was no appetite for testing or uptake of the suggestions	Project Reference Group recommended a change of direction to focus on N-supplementation during cropping, rather than during composting.
Increased understanding of the value of industry funded R&D amongst levy payers	Aligned to SIP 2017-2021: Outcome - *Mushroom growers are profitable and sustainable through increased yields, reduced costs and effective risk management;* Strategy - *Improve production by increasing yield and quality;* *KPI - identification of yield productivity increases*	Strong feedback from mushroom industry stakeholders to confirm how much they value the Research information provided.	Extensive discussion and feedback at conferences. Quotes include “the best ever advertisement for the value of research” (AMGA congress), “an outstanding presentation” (NAMC congress)

Monitoring and evaluation

Table 5. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
To what extent has the project achieved its expected outcomes?	The expected outcomes changed considerably during the project, in close consultation with the Project reference Group.	Further work is required to develop the fertigation technology to a point where it can be rolled out to farmers
How relevant was the project to the needs of intended beneficiaries?	The project was highly relevant to stakeholders' information needs. Unfortunately the lack of enthusiasm for trials of alternative composting N sources suggests that these aspects of the project were not well enough user-directed when the project was initially designed and approved.	There is a continuing need for education of composters in the biological aspects of composting, to help optimize the composting process and promote uptake of best practices and innovations.
How well have intended beneficiaries been engaged in the project?	The intended beneficiaries have been closely involved through regular information transfer at AMGA congresses, in the AMGA Journal and through webinars.	
To what extent were engagement processes appropriate to the target audience/s of the project	The engagement processes focussed on information transfer. Demonstration trials and best practice guidelines would have been useful, but were not possible because of changes to project targets, and interruptions caused by the Coviod-19 pandemic.	Best practice guidelines and demo trials will require further work to develop the fertigation technology to a point where it can be rolled out to farmers

Recommendations

- This project has revealed the consistency of microbial populations found in Australian mushroom composts in different geographical locations, and identified a range of microbial taxa that are commonly present in Phase 1 or Phase 2 composts. Further work is required to determine how this microbial diversity changes when different composting substrates are used, and whether bioaugmentation of differing substrates with a standard "compost inoculum" can help provide consistent high-yielding composts from different substrates.
- There is an urgent need for an up-to-date inventory of the types, quantities, and localities of by-products from the Australasian agricultural and food production sectors, in order to identify waste streams that can be used as potential compost C and N sources.
- A number of alternative nitrogen sources for composting have been proposed by this project, but composters have not yet been willing to try out these substrates as substitutes for poultry manure. It is imperative that a small-medium composting system is developed that will allow reproducible trials on these alternative substrates to be carried out without risk to the industry or to individual composters' business plans. A system of this nature will allow trialling of novel composting substrates, but also permit the testing of microbial inocula for Phase 1 and Phase 2 composting.
- This project has described the overall mass balance of nitrogen inputs and outputs to the composting and mushroom cropping process. However, the role of goody water in recycling N at different composting facilities has not been examined in detail. The amount of goody water used for straw wetting can vary greatly between yards, and more work is required to establish best practice for conservation of essential nutrients and microbes by better use of goody water.
- Bioaugmentation of spawn run with specific microbial mixtures has been shown to accelerate *Agaricus* mycelium growth. More work is needed to evaluate whether addition of specific compost-derived microbes also enhances yield and quality of the crop.
- This project has developed a biofertigation system based on drip irrigation during cropping, which allows supplementation of crops with small amounts of readily available nutrients at specific times during cropping. The results suggest that crop production may be limited not by nitrogen supply, but by other nutrients, and that these are required at particular stages of crop production. More work is required to optimize the use of liquid supplements, and their application to compost in a way that does not increase disease pressure.
- The biofertigation system described above also has the potential to be used to enrich mushroom crops at specific times with other compounds such as selenium, iron, iodine and magnesium. All of these elements are priority nutrients for human nutrition, and the use of "biofortified" mushrooms would provide an additional way to market mushrooms to consumers. More research is needed to confirm uptake of these elements into mushrooms and optimize bioenrichment conditions using the biofertigation system.

Scientific publications

Journal articles

Noble, R., Thai, M., and **Kertesz, M. A.,** 2023. Nitrogen balance and supply in Australasian mushroom composts. Applied Microbiology and Biotechnology, *108,* Article 151 (DOI: 10.1007/s00253-023-12933-2); https://link.springer.com/article/10.1007/s00253-023-12933-2

Thai, M., Bell, T.L., and **Kertesz, M. A.,** 2024. *Mycovorax composti* gen. nov. sp. nov., a member of the family *Chitinophagaceae* isolated from button mushroom compost. International Journal of Systematic and Evolutionary Microbiology, *In revision*.

Shamugam, S. and **Kertesz, M. A.** (2022) Bacterial interactions with the mycelium of the cultivated edible mushrooms *Agaricus bisporus* and *Pleurotus ostreatus. J Appl Microbiol 134*, lxac018 (DOI 10.1093/jambio/lxac018); https://doi.org/10.1093/jambio/lxac018

Thai, M., Safianowicz, K., Bell, T. L., and **Kertesz, M. A.,** 2022. Dynamics of microbial community and enzyme activities during preparation of *Agaricus bisporus* compost substrate. ISME Communications 2, Article 88 (DOI: 10.1038/s43705-022-00174-9); https://www.nature.com/articles/s43705-022-00174-9

Kertesz, M. A. and Thai, M., 2018. Compost bacteria and fungi that influence growth and development of *Agaricus bisporus* and other commercial mushrooms. Applied Microbiology and Biotechnology *102*, 1639-1650. (DOI: 10.1007/s00253-018-8777-z); https://link.springer.com/article/10.1007/s00253-018-8777-z

Doctoral thesis, University of Sydney.

Thai, M., 2022. *Microbial dynamics in Australian mushroom compost*. Ph.D. Thesis, University of Sydney, 148 pp.

Mushroom Industry publications

Noble, R. and **Kertesz, M.,** 2021. Putting Nitrogen in mushroom compost: time for a change? *Australian Mushrooms Journal* 2021(1) 34-37.

Kertesz, M., and Thai, M., 2019. Optimisation of nitrogen use in mushroom production. *Australian Mushrooms Journal* 2019(3), 34-35.

Conference presentations and webinars

Webinar:

Kertesz, M. A., and Noble, R., February 21st 2021, as part of the Marsh Lawson Mushroom Research Centre (MLMRC) webinar series. *Can nitrogen be better managed in compost production?* Recording available at https://www.youtube.com/watch?v=P3EN5SXarJg .

Conference presentations:

- 26th North American Mushroom Conference (NAMC), (26th - 29th February, 2024, Las Vegas). Invited keynote presentation (O'Neil lecture) by **M. A. Kertesz**. *The Microbial Matrix: Understanding the influence of microbes on the quality of mushroom compost*
- 20th International Society for Mushroom Science Congress (ISMS), (26th - 29th February, 2024, Las Vegas). Oral presentation by **M. Thai**. Feeding the compost: *Nitrogen supplementation during production of white button (Agaricus bisporus) mushrooms*
- 2022 Australian Microbial Ecology Congress (AusME), (7th – 9th November, 2022, Melbourne). Poster presentation by **M. Thai**. *Feeding Mushrooms: A microbial conversation between bacteria and fungi.*
- 44th Australian Mushroom Growers' Conference, (26th - 28th October, 2022, Adelaide). Invited oral presentation by **M. Kertesz**. *What's new in mushroom compost biology?* Recording available at: https://www.youtube.com/watch?v=1cY80XVap7I).
- 44th Australian Mushroom Growers' Conference, (26th - 28th October, 2022, Adelaide). Poster presentation by **K. T. Kuen, M. Thai & M.A. Kertesz**. *Feeding the compost – nitrogen supplementation during mushroom cropping*.
- 44th Australian Mushroom Growers' Conference, (26th - 28th October, 2022, Adelaide). Poster presentation by **M. Thai & M. Kertesz**. *Feeding Mushrooms: A microbial conversation between bacteria and fungi*.
- 44th Australian Mushroom Growers' Conference, (26th - 28th October, 2022, Adelaide). Poster presentation by **S. Shamugam & M. A. Kertesz**. *Bacterial interactions with* Agaricus bisporus *mycelium*.
- 2022 Australian Society for Microbiology Congress (11th – 14th July, 2022, Sydney) Invited oral presentation by **M. A. Kertesz**. *Using bacteria to grow mushrooms – the microbial ecology of mushroom compost.*
- 19th International Society for Mushroom Science Congress (ISMS), (14th - 17th September, 2021, online e-congress). Invited keynote presentation by **M. Thai**. *Bacterial interactions with Mycothermus thermophilus (syn. Scytalidium thermophilum*). Recording available at: (https://www.isms.biz/Web/Library/Proceedings/eCongresses/eArticles-Members.aspx?doc=6)

- 19th International Society for Mushroom Science Congress (ISMS), (14th - 17th September, 2021, online e-congress). Oral presentation by **M. A. Kertesz**. *Balancing nitrogen supply and demand in button mushroom composting and cropping*. Recording available at: (https://www.isms.biz/Web/Library/Proceedings/eCongresses/eArticles-Members.aspx?doc=84)
- 19th International Society for Mushroom Science Congress (ISMS), (14th - 17th September, 2021, online e-congress). Poster presentation by **M. Thai, M. A. Kertesz, T. Bell**. *Fungal and bacterial diversity in Australian mushroom compost*.
- 25th North American Mushroom Conference (NAMC) (14th – 16th February, 2019, Orlando, Florida). Invited oral presentation by **M. A. Kertesz**. *Compost microbes and nitrogen supply in mushroom production*.
- 43rd Australian Mushroom Growers' Conference, (11th – 13th October, 2018, Sydney). Invited oral presentation by **M. Thai**. *Diversity and activity of bacteria in Australian compost yards*.

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Intellectual property

No project IP or commercialisation to report.

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We are indebted to the late Graham Price and John Foster for invaluable assistance and advice with mushroom cultivation. Graham and John were responsible over many years for managing mushroom cropping experiments at the Marsh Lawson Mushroom Research Unit (MLMRU) at the University of Sydney. The MLMRU is co-funded by the Australian Mushroom Growers Association and Hort Innovation (Project MU21004) and is now managed by Applied Horticulture Research (AHR). We thank Umberto Calvo, Sandra Evangelista and the other members of the AHR team for their assistance, especially with the fertigation experiments.

We are grateful to Dr Malcolm Possell, University of Sydney, for assistance with modelling of nitrous oxide release from Phase 2 composting tunnels.

Appendices

Appendix 1. Detailed technical report, methods and results

Appendix 2. Noble. Review of nitrogen sources in Australasian mushroom composts

Appendix 3. Publications in the AMGA Journal

Appendix 4. Conference presentations (abstracts and posters).

Appendix 5. Thai 2022 ISME Communications

Appendix 6. Kertesz 2018 Appl Microbiol Biotechnol

Appendix 7. Kertesz 2023 J Appl Microbiol

Appendix 8. Noble 2024 Appl Microbiol Biotechnol

Appendix 1. Detailed Technical report

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1 Microbial diversity in Australian mushroom composts

The total bacterial diversity in mushroom composts from different Australian compost yards was determined using molecular, DNA-based methods. Use of these cultivation-independent methods avoids the limitation that many bacteria do not grow well under laboratory conditions with synthetic growth media. Compost samples were taken from Phase 1 and Phase 2 composts, and from mid-Phase 2 composts where this was possible. Reproducibility was assessed by measuring diversity in successive crops from the same yard. The results presented here have been expanded greatly in the ongoing project *MU17006 Developing a database of bio-markers for compost quality control to maximise Mushroom production yield.*

1.1 Methods

1.1.1 Compost sampling

Compost samples used in the project were obtained from nine commercial mushroom composting facilities located in New South Wales, Queensland, Victoria, South Australia and Tasmania. For confidentiality reasons, the composters are not named here. Compost samples taken at specific timepoints during the composting process were pooled samples, produced by combining ten independent samples taken from across the face of the compost pile during turning. Sampling was done on site by industry personnel, who were provided with appropriate training by the research team (https://www.youtube.com/watch?v=TZG_QKWlOyA), and the samples were shipped to the University of Sydney for analysis.

1.1.2 DNA extraction, amplification and sequencing

Total compost DNA was extracted according to Lever et al. (2015) with some modifications. Ground compost samples (200 mg) were suspended in 200 mM sodium hexametaphosphate (100 µL), lysis buffer 1 was added (30 mM Tris/HCl, 30 mM EDTA, 800 mM guanidinium chloride, 0.5% (v/v) Triton X-100, pH 10.0) (500 mL), and the samples were lysed using a homogenizer (MoBio Laboratories Inc.,) at 2000 rpm for 5 min. Lysis buffer 2 (2.5 M sodium chloride, 2% (w/v) cetyltrimethylammonium bromide, 0.1% (w/v) polyvinylpolypyrrolidone) (500 µL) was added, followed by incubation at 65 °C with agitation (500 rpm) for 30 min and centrifugation, Supernatants were extracted once with an equal volume of chloroform:isoamyl alcohol (24:1) and DNA was recovered from the aqueous phase using DNA binding magnetic beads (GE Life Sciences, Australia) in SPRI solution, following the manufacturer's instructions. Bacterial diversity was analysed using primers 341F and 806R (Caporaso et al. 2011; Muyzer et al. 1993) to amplify the V3-V4 16S rRNA gene hypervariable region, with the Illumina MiSeq platform (paired 300 bp read lengths) at the Australian Genome Research Facility (AGRF) (Melbourne, Australia).

1.1.3 Bioinformatics

Raw FASTQ files were processed in R v3.6.1.(R Core Team 2019) Raw read quality was determined using FastQC. Trimming and filtering was determined using the DADA2 function 'filterAndTrim', (Callahan et al. 2016) discarding forward and reverse reads with an expected error score higher than 3 and 4, respectively. Low quality reads were removed during trimming and filtering by setting 'truncLen' parameters to 285 and 240 for the forward and reverse reads, respectively. Forward and reverse primers were trimmed from the 5' end by setting the 'trimLeft' function to 17 and 20, respectively. The sequences were denoised and dereplicated using the 'dada' and 'derep' functions, unique sequences were merged with a minimum overlap of 20 base pairs and a sequence table was constructed with the resulting sequence variants.

Taxonomy was assigned using a pre-trained SILVA Naïve Bayes classifier clustered at 99% identity (SILVA release v132) (Quast et al. 2012). Species assignment was done in a separate step using the SILVA release v132 for species assignment. 16S gene sequences that were affiliated with chloroplasts and mitochondria were removed prior to downstream analysis. Sequence variants which occurred in fewer than three samples and with fewer than three reads in each of these samples were also removed (singletons and doubletons). A phylogenetic tree was constructed using the packages "phangorn" (Schliep 2010) and "DECIPHER", (Wright 2016) using the neighbour-joining method.

Statistical analysis was done using the packages "phyloseq" (McMurdie and Holmes 2013) and "vegan".(Dixon 2003) All graphs and plots were visualized using "ggplot2" (Wickham 2016). Shannon and Simpson alpha-diversity analyses were performed using the 'plot_richness' function from the phyloseq package before singletons and doubletons were removed from the dataset. Differences in the bacterial community (beta-diversity) were analysed in R (R Core Team 2019) using a canonical analysis of principal coordinates with unweighted UniFrac as the distance metric.

DNA sequencing data are available at NCBI Short Read Archive under BioProject PRJNA867030.

1.2 Results

Figure 1.2.1. Species diversity and richness of bacterial communities in five compost yards.

Compost Yard	Compost Phase	Shannon diversity (H)	Simpson diversity (D)
A	End-Phase 1	4.647	0.9645
A	Mid-Phase 2	4.951	0.9790
A	End-Phase 2	3.798	0.8949
A	End-Phase 1	4.534	0.9668
A	Mid-Phase 2	3.795	0.9058
A	End-Phase 2	3.505	0.8847
A	End-Phase 2	3.872	0.9286
B	End-Phase 1	5.686	0.9918
B	End-Phase 2	4.985	0.9812
B	End-Phase 1	5.274	0.9844
C	End-Phase 1	5.394	0.9870
C	End-Phase 2	5.225	0.9851
C	End-Phase 1	5.234	0.9857
C	End-Phase 2	4.851	0.9768
D	End-Phase 1	5.001	0.9781
D	Mid-Phase 2	4.822	0.9811
D	End-Phase 2	4.802	0.9768
E	End-Phase 1	5.750	0.9925
E	Mid-Phase 2	5.269	0.9892
E	End-Phase 2	4.929	0.9842

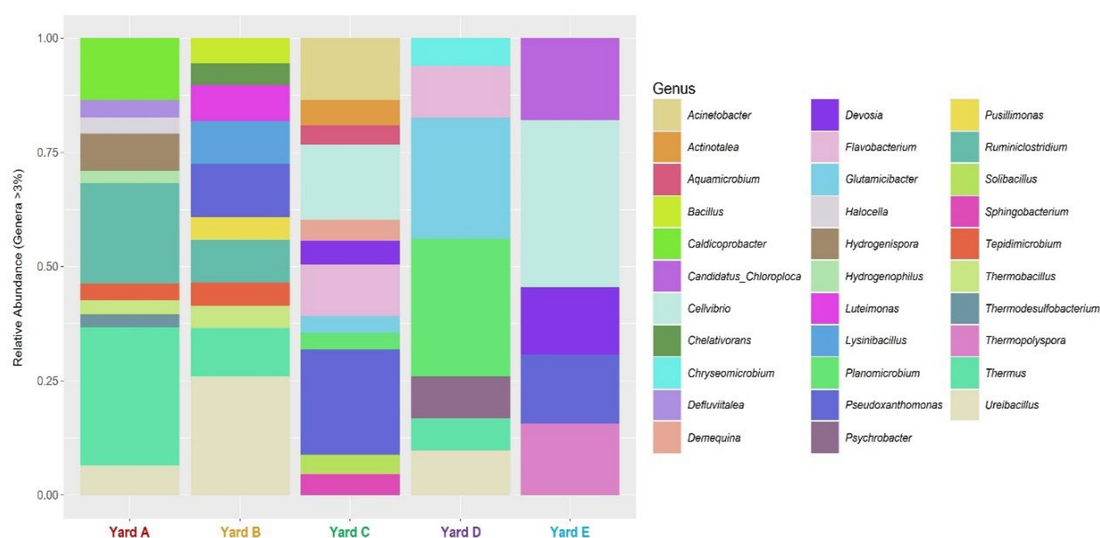

Figure 1.2.2. Bacterial profile of end-Phase 1 compost from all compost yards at genus level. Rare taxa that had a relative abundance of less than 3% and ASVs not classified to genus level are not shown.

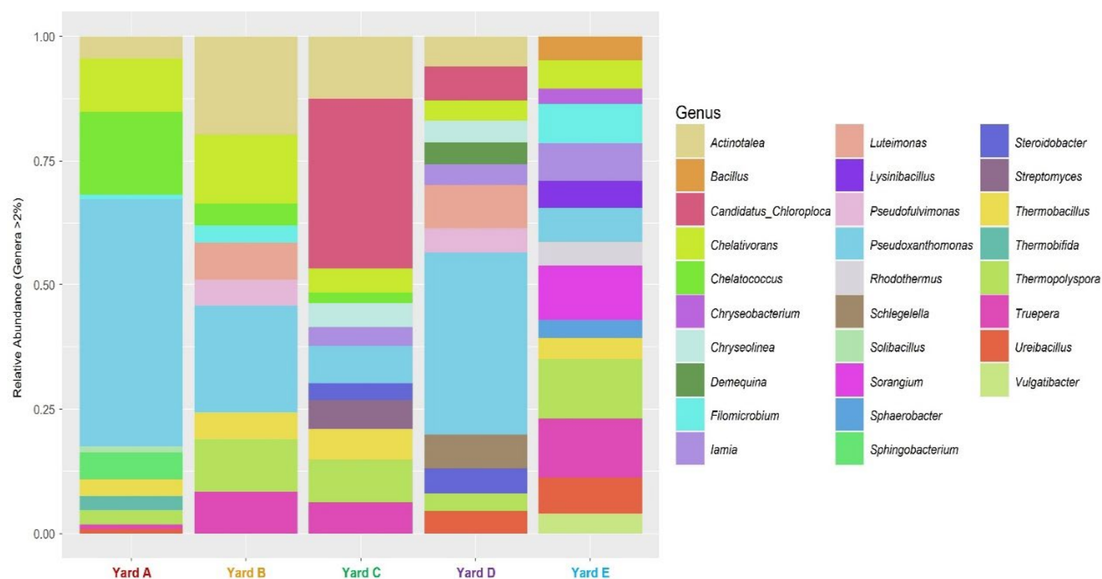

Figure 1.2.3. Bacterial profile at end-Phase 2 for all compost yards at genus level. Rare taxa that had a relative abundance of less than 2% and ASVs that could not be classified to genus level are not shown.

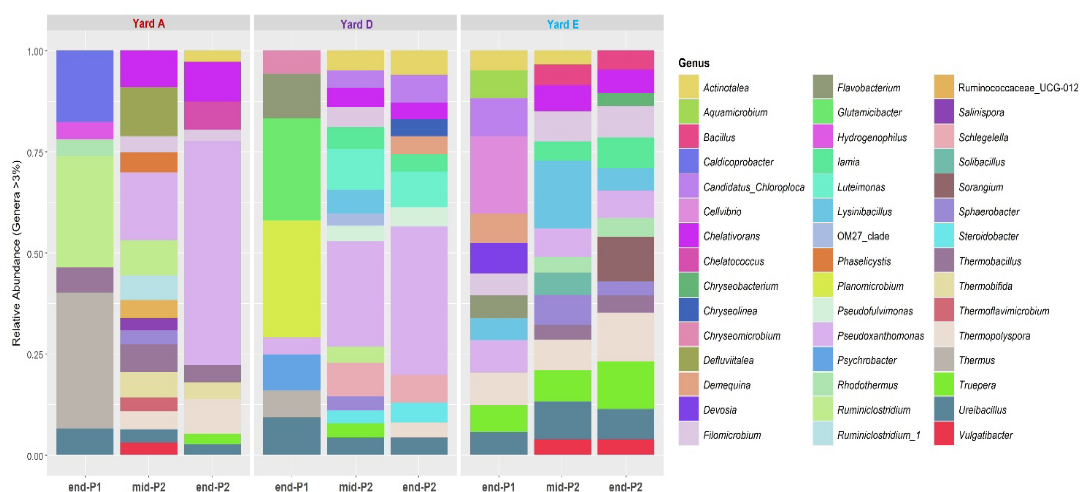

Figure 1.2.4. Bacterial profiles of whole compost crops. Rare taxa that had a relative abundance less than 3% and ASVs that could not be assigned to genus level are not shown. Mid-Phase 2 was sampled after pasteurisation, before the conditioning step. Due to the health risk of entering a Phase 2 tunnel, only these three yards were able to provide mid-Phase 2 compost samples.

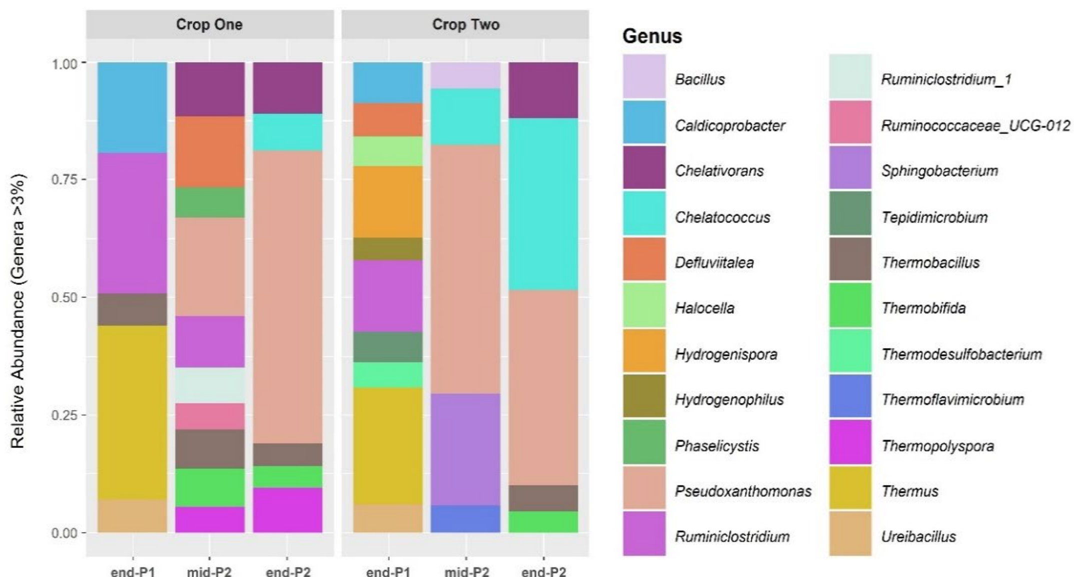

Figure 1.2.5. Bacterial profiles of two successive compost crops. Rare taxa that had a relative abundance of less than 3% and ASVs that could not be classified to genus level are not shown.

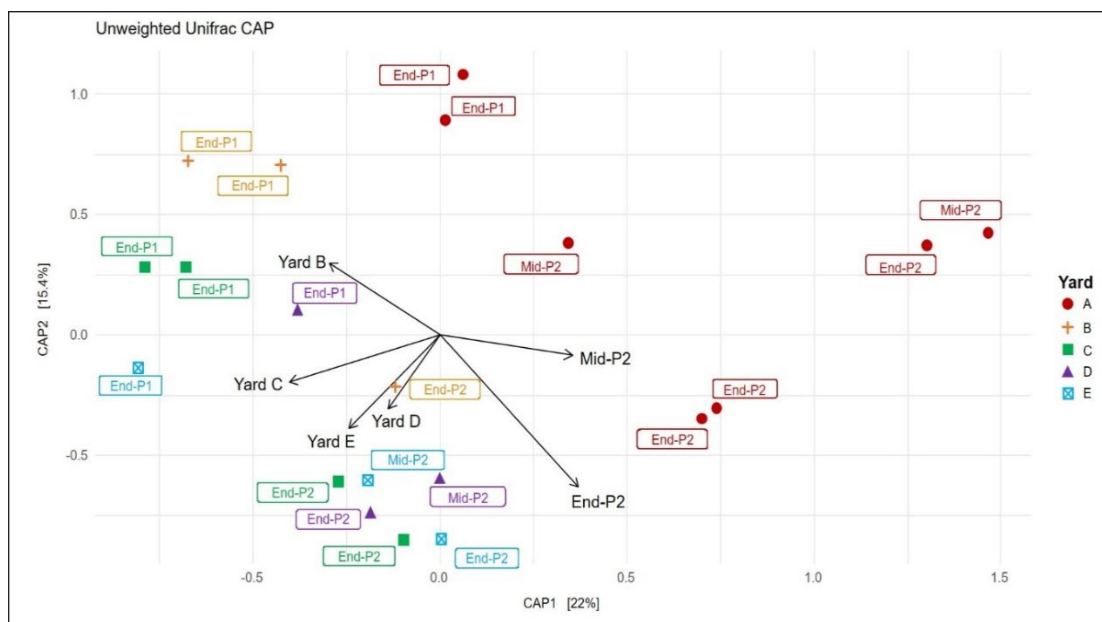

Figure 1.2.6. Unweighted Unifrac Canononical Analysis of Principal Components exploring the similarity in bacterial profiles among compost yards. ANOVA: P <0.05, df = 6. Arrows represent the differences in bacterial communities between compost yards and phases. Compost yard A clustered very differently on the ordination plot from other yards.

2 Bacterial strain collection from Australian mushroom composts

Molecular methods of bacterial diversity analysis provide detailed information on the bacterial taxa present in compost, but in order to study these bacteria in more detail, and potentially to use them as inocula to increase compost quality, it is necessary to cultivate them in the laboratory. A substantial bacterial strain collection was obtained below from a range of different compost yards, selecting for bacterial taxa that grow on different media and at different temperatures, and are present during different phases of composting. The primary aim was to obtain cultivable representatives of the dominant taxa identified in Phase 1 and Phase 2 compost by molecular methods, so that the role of these microorganisms in composting could be studied in more detail.

2.1 Methods

2.1.1 Isolation and identification of compost bacteria

Isolates of compost bacteria were obtained from Phase 2 mushroom compost as follows. Unhomogenised, roughly chopped mushroom compost (1 g) was suspended in 9 mL of liquid nutrient medium (R2A, Hi-Media)(Reasoner and Geldreich 1985) and serial dilutions of the extract were incubated either at 30 °C and 50 °C for 48 h. Extracts were plated onto a solid nutrient medium (R2A agar (1.5%), BactoDifco, Australia) and incubated at 50 °C for 48 h. For bacterial isolates that were unable to grow at 50 °C after 48 h, the plates were incubated at 30 °C for a further 48 h.

To isolate and identify hyperthermophilic organisms, extracts were plated on R2A medium that had been solidified with Phytagel (1.2 % w/v) instead of agar. Incubation was carried out at 75 °C or 80 °C. Filamentous actinomycetes were enriched by placing compost directly onto a nylon filter disc (0.22 µm pore size) that had been laid on a YpSs agar plate and moistened with water (1 mL). The plate was incubated at 45 °C for 307 days, and the filter then carefully removed. Filamentous organisms that had grown through the filter onto the agar plate below were then purified by restreaking directly onto further YpSs agar plates.

The bacterial strains obtained were classified by partial sequencing of the 16S rRNA gene after PCR amplification with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991) in 25 µL reactions using MyTaq™ DNA Polymerase (Bioline, Australia). PCR amplification was done with a thermocycler (BioRad, S1000) using the following program: 5 min initial denaturation at 95 °C, 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, 1 min extension at 72 °C and a final extension for 5 min at 72 °C.

The amplicons were purified (ISOLATE II PCR and Gel Kit, Bioline, Australia) following the manufacturers protocol and Sanger sequencing of the PCR product was done by Macrogen Inc., South Korea. The taxonomic identity of the isolates was determined by comparison of the resulting sequence with the GenBank database using BLAST (Altschul et al. 1990). All isolated strains were preserved in 25% (v/v) glycerol and kept at -80 °C.

A subset of isolates was tested for the presence of several bacterial genes that are important in nitrogen transformations. These genes included *amoA* (ammonium monooxygenase, important in nitrification, the conversion of ammonia to nitrate), *nirK* and *nirS* (nitrite reductase, which converts nitrite to nitric oxide; nirK and nirS are Cu-dependent and haem-dependent versions), and *nosZ* (nitrous oxide reductase, which converts nitrous oxide to molecular nitrogen in the final step in the denitrification process). The isolates were screened using PCR methods targeting the respective genes, applying degenerate primers that are well established for use in soils (Levy-Booth et al. 2014; Petersen et al. 2012).

A further subset of strains was tested for the presence of cellulase, xylanase, laccase and peroxidase enzymes in whole cells. Cellulase and xylanase were tested with carboxymethyl cellulose and beechwood xylanase as substrates, as previously described (Schinner and von Mersi 1990). Laccase was measured using syringaldazine as substrate (Leonowicz and Grzywnowicz 1981), while peroxidase was measured using tetramethylbenzidine (Johnsen and Jacobsen 2008)

2.1.2 Isolation and identification of Mycothermus thermophilus

Several strands of composted wheat straw from Phase 2 compost (2 cm long) were placed onto YpSs agar (per L: 15 g soluble starch, 4 g yeast extract, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 20 g agar, pH 6.5)(Atlas 2010) containing chloramphenicol (35 µg mL^{-1}) and incubated at 50 °C for 4 days. Dark green to black fungal colonies which had chained conidia in the aerial mycelia under light microscopy were isolated and purified from plates inoculated with composts from yards A-J and maintained on solid YpSs agar with 35 µg mL^{-1} of chloramphenicol added to supress bacterial growth. The strains obtained were classified by amplification and sequencing of the ITS regions using primers ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Op De Beeck et al. 2014; White et al. 1990) in 25 µL reactions using MyTaq^{TM} DNA Polymerase (Bioline, Australia). The following PCR protocol was used: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 60 s. A final extension step at 72 °C for 5 min completed the PCR reaction. Amplicons were purified and sent for Sanger sequencing using the same method as the bacterial isolates.

2.1.3 Isolation and identification of the novel genus and species Mycovorax composti

One gram of a Phase 2 compost sample was suspended in nine millilitres of R2A broth (HiMedia Laboratories, Pennsylvania USA) and incubated at 40 °C for 48 hours, with shaking at 200 rpm. A single orange colony that was round, convex with an entire margin was found and purified by routine subculturing at 40 °C for 48 hours on R2A agar plates (Oxoid, Australia). The bacterial strain was designated CP216 and routinely cultured on 350 Emerson's YpSs agar at 40 °C and preserved at -80 °C in glycerol suspension (25% v/v).

Genomic DNA was extracted using the CTAB method, according to the method of Wilson (2001). Briefly, late exponential phase bacterial cells were harvested and resuspended in Tris-ethylenediaminetetraacetic acid (TE) buffer (10mM Tris-HCl, 1 mM EDTA; 100 µL, pH 8.0) with 1 mg/mL lysozyme (Sigma-Aldrich) and incubated at 37 °C for 30 minutes without agitation. *Hexadecyltrimethylammonium* bromide (CTAB) extraction buffer (2% CTAB (w/v), 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl; 500 µL) was added and incubated at 50 °C for one hour without agitation. Chloroform:isoamyl alcohol (24:1) was added in equal volume and the solution was emulsified before centrifuging at 14,000 × g for 10 minutes to separate the two phases. The top aqueous phase (400 µL) was transferred to a fresh tube and cold isopropanol (320 µL) was added. The precipitated DNA was collected by centrifugation (20,000 × g for 20 minutes) and washing twice with 80% (v/v) ambient ethanol. The DNA pellet was resuspended in TE buffer (pH 8.0) and stored at -20 °C.

Whole genome sequencing of the CP216 genomic DNA obtained was done by the Microbial Genome Sequencing Center (Pittsburgh, USA), using their standard Illumina protocols (NextSeq 2000; 2 × 150 bp). The resulting FastQ files were analysed using the Galaxy software package (Afgan et al. 2018). Genome annotation was done in Galaxy using the Prokka tool (Seemann 2014). *DNA G + C contents of strain CP216 was determined from whole genome sequencing.* 16S rRNA sequences of closely related

genera were obtained from the List of Prokaryotic names with Standing in Nomenclature (LPSN) (Parte et al. 2020). The 16S rRNA sequences were aligned using CLUSTAL W (Thompson et al. 1994). A phylogenetic tree was constructed using the software package MEGA-X (version 10.2.2) (Kumar et al. 2018). Distances were determined using the Kimura two-parameter model and clustering was done with the neighbour joining (NJ) method and maximum likelihood (ML) method. The reliability of the trees obtained was confirmed using bootstrap values based on 1000 replicates**.**

Two strains of *Mycovorax composti* has been deposited at the German Collection of Microorganisms (DSMZ), strains DSM114558 and DSM114559. The same two strains have also been deposited with the Belgian Collection of Microorganisms (BCCM), strains LMG32997 and LSM32998. The whole genome sequence is available at the NCBI, accession GCA_028485475.1.

2.1.4 DNA extraction from bacterial and fungal isolates

DNA from the isolated bacteria was extracted using the miniprep method described by Wilson (2001), with some modifications. Briefly, bacterial cells were harvested from late exponential phase (R2A, HiMedia) and resuspended in Tris-EDTA buffer (10 mM Tris-HCl, 1mM ethylenediaminetetraacetic acid (EDTA)) containing 1 mg mL^{-1} lysozyme (Sigma-Aldrich, Australia). The cell suspensions were incubated in a water bath at 37 °C for 30 min. Pre-warmed CTAB buffer (500 µL; 2% hexadecyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl; pH 8.0, 20 mM EDTA, 1.4 M NaCl) was added and incubated in a water bath at 50 °C for 60 min. Chloroform:isoamyl alcohol (24:1) (600 µL) was added and the solutions were emulsified before centrifuging at 14,000 × *g* for 10 min. The top aqueous supernatant (600 µL) was transferred to a fresh tube, 480 µL of isopropanol was added, and the precipitated DNA was collected by centrifugation at 12,000 × *g* for 20 min. The pellet was washed three times with 80% (w/v) ethanol, dried resuspended in 10 mM Tris/HCl, 1mM EDTA, pH 8.0.

For extraction of fungal DNA, fungal material was scraped from a 10 × 10 mm area of a freshly grown plate culture (YpSs) and homogenised in CTAB buffer with zirconium-silicate beads (0.1 mm, Daintree Scientific, Australia) with two soda lime glass beads (3 mm, John Morris Group, Australia). DNA extraction for the fungal isolates was continued following the same chloroform:isoamyl alcohol step that was used for bacterial DNA extraction.

2.2 Results

Table 2.2.1. Bacterial isolate collection from Australian compost yards, as at January 2024. Whole-cell enzyme activities measured for selected isolates are cellulase (C), laccase (L), peroxidase (P) and xylanase (X).

Strain	Species	Obtained from/isolated by:	Enzymes	Isolation Medium
S2241	*Acinetobacter johnsonii*	ScatoPlus End-P2 / M. Thai		R2A
M1296	*Acinetobacter movanagherensis*	SA Mush End-P1 / M. Thai		R2A
C214	*Acinetobacter variabilis*	Mernda End-P2 / M. Thai	P, L	R2A
S1249	*Arthrobacter psychrochitiniphilus*	ScatoPlus End-P1 / M. Thai		R2A
E1106	*Bacillus haynesii*	EFS End-P1 / N. Uddin		R2A
E11014	*Bacillus haynesii*	EFS End-P1 / N. Uddin		R2A
C11	*Bacillus haynesii*	Mernda End-P1 / N. Uddin		R2A
C17	*Bacillus haynesii*	Mernda End-P1 / N. Uddin		R2A
C18	*Bacillus haynesii*	Mernda End-P1 / N. Uddin		R2A
C110	*Bacillus haynesii*	Mernda End-P1 / N. Uddin		R2A
E24518	*Bacillus safensis*	EFS End-P2 / M. Thai	P, L	R2A
E1-591	*Bacillus safensis*	EFS Mid-P2 / M. Thai		R2A
E11015	*Bacillus smithii*	EFS End-P1 / N. Uddin		R2A
C111	*Bergeyella porcorum*	Mernda End-P1 / M. Thai		R2A
E2451	*Bordetella petrii*	EFS End-P2 / M. Thai		R2A
C2111	*Caulobacter segnis*	Mernda End-P2 / M. Thai		R2A
M2299	*Caulobacter segnis*	SA Mush End-P2 / M. Thai	P	R2A
T1-519	*Caulobacter segnis*	Tas Mush Mid-P2 / M. Thai	L	R2A
M22911	*Cellulomonas iranensis*	SA Mush End-P2 / M. Thai		R2A
KFAMC1.8	*Chelatococcus composti*	M.Thai		LB
KFACM1.8.1.4	*Chelatococcus composti*	Maggie Guo		LB
S2244	*Cronobacter condimenti*	ScatoPlus End-P2 / M. Thai		R2A
E193	*Dermacoccus nishinomiyaensis*	EFS End-P1 / M. Thai		R2A
E194	*Dermacoccus nishinomiyaensis*	EFS End-P1 / M. Thai		R2A
E1-599	*Dermacoccus nishinomiyaensis*	EFS Mid-P2 / M. Thai	L	R2A
E24523	*Enterobacter hormaechei*	EFS End-P2 / M. Thai		R2A
E24527	*Enterobacter hormaechei*	EFS End-P2 / M. Thai		R2A
E24529	*Enterobacter hormaechei*	EFS End-P2 / M. Thai		R2A
E11930	*Geobacillus kaustophilus*	EFS End-P1 / N. Uddin	P	R2A
E11932	*Geobacillus stearothermophilus*	EFS End-P1 / N. Uddin		R2A
E1107	*Geobacillus thermodenitrificans*	EFS End-P1 / N. Uddin		R2A
E11018	*Geobacillus thermodenitrificans*	EFS End-P1 / N. Uddin		R2A
E11927	*Geobacillus thermodenitrificans*	EFS End-P1 / N. Uddin		R2A
N113	*Geobacillus thermodenitrificans*	Singleton End-P1 / N. Uddin		R2A
M1291	*Glumacibacter arilaitensis*	SA Mush End-P1 / M. Thai	P	R2A
T215	*Gordonia westfalica*	Tas Mush End-P2 / M. Thai	P	R2A
E1109	*Hyphomicrobium hollandicum*	EFS End-P1 / N. Uddin		R2A
E11012	*Hyphomicrobium hollandicum*	EFS End-P1 / N. Uddin		R2A

M1281	*Leadbetterella byssophila*	SA Mush End-P1 / M. Thai	P, L	R2A
E1-598	*Lysinibacillus acetophenoni*	EFS Mid-P2 / M. Thai		R2A
T1-513	*Lysinibacillus chungkukjangi*	Tas Mush Mid-P2 / M. Thai		R2A
T116	*Lysinibacillus composti*	Tas Mush End-P1 / M. Thai		R2A
T1-514	*Methylobacterium radiotolerans*	Tas Mush Mid-P2 / M. Thai		R2A
E1-597	*Microbacterium foliorum*	EFS Mid-P2 / M. Thai		R2A
C112	*Microbacterium foliorum*	Mernda End-P1 / M. Thai		R2A
M2282	*Microbacterium foliorum*	SA Mush End-P2 / M. Thai		R2A
E191	*Microbacterium maritypicum*	EFS End-P1 / M. Thai		R2A
E196	*Microbacterium maritypicum*	EFS End-P1 / M. Thai		R2A
E1-596	*Microbacterium maritypicum*	EFS Mid-P2 / M. Thai		R2A
S1252	*Microbacterium maritypicum*	ScatoPlus End-P1 / M. Thai		R2A
C213	*Microbacterium maritypicum*	Mernda End-P2 / M. Thai		R2A
M22812	*Microbacterium maritypicum*	SA Mush End-P2 / M. Thai		R2A
T117	*Microbacterium maritypicum*	Tas Mush End-P1 / M. Thai	L	1/10 LB
S1253	*Microbacterium natoriense*	ScatoPlus End-P1 / M. Thai		R2A
E19	*Mycothermus thermophilus*	EFS End-P2/M. Mills		YpSs
C	*Mycothermus thermophilus*	Costa (Mernda) End-P2/M. Mills		YpSs
V	*Mycothermus thermophilus*	Merbien End-P2/M. Mills		YpSs
T	*Mycothermus thermophilus*	Tas Mushrooms End-P2/M. Mills		YpSs
C216	*Mycovorax composti*	Mernda End-P2 / M. Thai		R2A
C2112	*Niabella terrae (93%)*	Mernda End-P2 / M. Thai	L	R2A
M1286	*Nitratireductor lucknowense*	SA Mush End-P1 / M. Thai	L	R2A
T1-5110	*Novosphingobium aromaticivorans*	Tas Mush Mid-P2 / M. Thai		R2A
E2458	*Parapusillimonas granuli*	EFS End-P2 / M. Thai		R2A
E1197	*Pigmentiphaga daeguensis*	EFS End-P1 / N. Uddin		R2A
T1110	*Pseudomonas fluvialis*	Tas Mush End-P1 / M. Thai		R2A
T1114	*Pseudomonas fluvialis*	Tas Mush End-P1 / M. Thai		1/10 LB
T1112	*Pseudomonas sihuiensis*	Tas Mush End-P1 / M. Thai	P, L	1/10 LB
E1193	*Pseudomonas thermotolerans*	EFS End-P1 / N. Uddin		R2A
E11912	*Pseudomonas thermotolerans*	EFS End-P1 / N. Uddin		R2A
M2284	*Pseudoxanthomonas koreensis*	SA Mush End-P2 / M. Thai	L	R2A
M2294	*Pseudoxanthomonas koreensis*	SA Mush End-P2 / M. Thai	P, L	R2A
E24519	*Pseudoxanthomonas suwonensis*	EFS End-P2 / M. Thai		R2A
E24521	*Pseudoxanthomonas suwonensis*	EFS End-P2 / M. Thai		R2A
E24525	*Pseudoxanthomonas suwonensis*	EFS End-P2 / M. Thai		R2A
E24534	*Pseudoxanthomonas suwonensis*	EFS End-P2 / M. Thai		R2A
E1-594	*Pseudoxanthomonas suwonensis*	EFS Mid-P2 / M. Thai		R2A
E1-5910	*Pseudoxanthomonas suwonensis*	EFS Mid-P2 / M. Thai		R2A
S1242	*Pseudoxanthomonas suwonensis*	ScatoPlus End-P1 / M. Thai	P	R2A
S2242	*Pseudoxanthomonas suwonensis*	ScatoPlus End-P2 / M. Thai		R2A
S2245	*Pseudoxanthomonas suwonensis*	ScatoPlus End-P2 / M. Thai		R2A
S2248	*Pseudoxanthomonas suwonensis*	ScatoPlus End-P2 / M. Thai	P, L	R2A

S2249	*Pseudoxanthomonas suwonensis*	ScatoPlus End-P2 / M. Thai	X, P	R2A
S22411	*Pseudoxanthomonas suwonensis*	ScatoPlus End-P2 / M. Thai		R2A
C1-511	*Pseudoxanthomonas suwonensis*	Mernda Mid-P2 / M. Thai		R2A
C211	*Pseudoxanthomonas suwonensis*	Mernda End-P2 / M. Thai		R2A
M2291	*Pseudoxanthomonas suwonensis*	SA Mush End-P2 / M. Thai	P, L	R2A
M2292	*Pseudoxanthomonas suwonensis*	SA Mush End-P2 / M. Thai		R2A
T118	*Pseudoxanthomonas suwonensis*	Tas Mush End-P1 / M. Thai	L	1/10 LB
T1111	*Pseudoxanthomonas suwonensis*	Tas Mush End-P1 / M. Thai		1/10 LB
T1113	*Pseudoxanthomonas suwonensis*	Tas Mush End-P1 / M. Thai		R2A
T1-518	*Pseudoxanthomonas suwonensis*	Tas Mush Mid-P2 / M. Thai	P, L	R2A
E24525-SmR	*Pseudoxanthomonas suwonensis*	EFS End-P2 / Blake		R2A
E1195	*Pseudoxanthomonas suwonensis*	EFS End-P1 / N. Uddin		R2A
E1196	*Pseudoxanthomonas suwonensis*	EFS End-P1 / N. Uddin		R2A
E1199	*Pseudoxanthomonas suwonensis*	EFS End-P1 / N. Uddin		R2A
E11911	*Pseudoxanthomonas suwonensis*	EFS End-P1 / N. Uddin		R2A
N13	*Pseudoxanthomonas suwonensis*	Singleton End-P1 / N. Uddin		R2A
N16	*Pseudoxanthomonas suwonensis*	Singleton End-P1 / N. Uddin		R2A
E2452	*Pseudoxanthomonas taiwanensis*	EFS End-P2 / M. Thai	enzymes	R2A
E2455	*Pseudoxanthomonas taiwanensis*	EFS End-P2 / M. Thai		R2A
E2456	*Pseudoxanthomonas taiwanensis*	EFS End-P2 / M. Thai	P, L	R2A
E2457	*Pseudoxanthomonas taiwanensis*	EFS End-P2 / M. Thai	X, P	R2A
E24522	*Pseudoxanthomonas taiwanensis*	EFS End-P2 / M. Thai		R2A
E24530	*Pseudoxanthomonas taiwanensis*	EFS End-P2 / M. Thai		R2A
S2246	*Pseudoxanthomonas taiwanensis*	ScatoPlus End-P2 / M. Thai		R2A
S2247	*Pseudoxanthomonas taiwanensis*	ScatoPlus End-P2 / M. Thai		R2A
T1-515	*Pseudoxanthomonas taiwanensis*	Tas Mush Mid-P2 / M. Thai	P	R2A
T216	*Pseudoxanthomonas taiwanensis*	Tas Mush End-P2 / M. Thai	L	R2A
E2452-SmR	*Pseudoxanthomonas taiwanensis*	EFS End-P2 / Blake		R2A
E11910	*Schlegelella thermodepolymerans*	EFS End-P1 / N. Uddin		R2A
E2454	*Serratia ureilytica*	EFS End-P2 / M. Thai		R2A
M2297	*Solimonas soli*	SA Mush End-P2 / M. Thai	P	R2A
S1246	*Sphingobacterium alimentarium*	ScatoPlus End-P1 / M. Thai		R2A
E24532	*Sphingobacterium thermophilum*	EFS End-P2 / M. Thai		R2A
E1-592	*Sphingobacterium thermophilum*	EFS Mid-P2 / M. Thai		R2A
M1287	*Sphingobacterium thermophilum*	SA Mush End-P1 / M. Thai		R2A
E11914	*Sphingobacterium thermophilum*	EFS End-P1 / N. Uddin		R2A
E2453	*Staphylococcus epidermidis*	EFS End-P2 / M. Thai		R2A
S1251	*Staphylococcus epidermidis*	ScatoPlus End-P1 / M. Thai	P, L	R2A
E1-5912	*Staphylococcus succinus*	EFS Mid-P2 / M. Thai		R2A
M2283	*Staphylococcus succinus*	SA Mush End-P2 / M. Thai		R2A

M2285	*Staphylococcus succinus*	SA Mush End-P2 / M. Thai	L	R2A
M2286	*Staphylococcus succinus*	SA Mush End-P2 / M. Thai		R2A
M22813	*Staphylococcus succinus*	SA Mush End-P2 / M. Thai		R2A
M22910	*Streptomyces griseorubiginosus*	SA Mush End-P2 / M. Thai		R2A
M2298	*Streptomyces sudanensis*	SA Mush End-P2 / M. Thai		R2A
FW80	*Thermaerobacter sp.*	EFS End-P1 / P.Butterworth		Thermus medium
E11010	*Thermoactinomyces vulgaris*	EFS End-P1 / N. Uddin		R2A
C12	*Thermobacillus composti*	Mernda End-P1 / N. Uddin	X, P	R2A
C13	*Thermobacillus composti*	Mernda End-P1 / N. Uddin		R2A
C115	*Thermobacillus composti*	Mernda End-P1 / N. Uddin		R2A
80-39	*Thermus sp.*	EFS End-P1 / P.Butterworth		Thermus medium
SG0.5JP17-16	*Thermus thermophilus*	EFS End-P1 / P.Butterworth		Thermus medium
E2459	*unidentified*	EFS End-P2 / M. Thai		R2A
E24510	*unidentified*	EFS End-P2 / M. Thai		R2A
E24520	*unidentified*	EFS End-P2 / M. Thai		R2A
E24528	*unidentified*	EFS End-P2 / M. Thai		R2A
E24533	*unidentified*	EFS End-P2 / M. Thai		R2A
E1-593	*unidentified*	EFS Mid-P2 / M. Thai		R2A
E1-5911	*unidentified*	EFS Mid-P2 / M. Thai		R2A
S1244	*unidentified*	ScatoPlus End-P1 / M. Thai		R2A
S1245	*unidentified*	ScatoPlus End-P1 / M. Thai	L	R2A
S1248	*unidentified*	ScatoPlus End-P1 / M. Thai		R2A
S12410	*unidentified*	ScatoPlus End-P1 / M. Thai		R2A
S1254	*unidentified*	ScatoPlus End-P1 / M. Thai		R2A
S1255	*unidentified*	ScatoPlus End-P1 / M. Thai		R2A
S2243	*unidentified*	ScatoPlus End-P2 / M. Thai		R2A
S22413	*unidentified*	ScatoPlus End-P2 / M. Thai		R2A
C219	*unidentified*	Mernda End-P2 / M. Thai		R2A
M1282	*unidentified*	SA Mush End-P1 / M. Thai		R2A
M1283	*unidentified*	SA Mush End-P1 / M. Thai		R2A
M1284	*unidentified*	SA Mush End-P1 / M. Thai	P	R2A
M1285	*unidentified*	SA Mush End-P1 / M. Thai		R2A
M1292	*unidentified*	SA Mush End-P1 / M. Thai		R2A
M1293	*unidentified*	SA Mush End-P1 / M. Thai		R2A
M1295	*unidentified*	SA Mush End-P1 / M. Thai		R2A
M1297	*unidentified*	SA Mush End-P1 / M. Thai		R2A
M2281	*unidentified*	SA Mush End-P2 / M. Thai		R2A
M2295	*unidentified*	SA Mush End-P2 / M. Thai		R2A
M2296	*unidentified*	SA Mush End-P2 / M. Thai		R2A
T119	*unidentified*	Tas Mush End-P1 / M. Thai		1/10 LB
T1-517	*unidentified*	Tas Mush Mid-P2 / M. Thai		R2A
T213	*unidentified*	Tas Mush End-P2 / M. Thai		R2A
T214	*unidentified*	Tas Mush End-P2 / M. Thai		R2A
E11913	*unidentified*	EFS End-P1 / N. Uddin		R2A
E11926	*unidentified*	EFS End-P1 / N. Uddin		R2A
E11928	*unidentified*	EFS End-P1 / N. Uddin		R2A
E11929	*unidentified*	EFS End-P1 / N. Uddin		R2A

E11931	*unidentified*	EFS End-P1 / N. Uddin		R2A
C116	*unidentified*	Mernda End-P1 / N. Uddin		R2A
C123	*unidentified*	Mernda End-P1 / N. Uddin		R2A
C126	*unidentified*	Mernda End-P1 / N. Uddin		R2A
N116	*unidentified*	Singleton End-P1 / N. Uddin		R2A
N117	*unidentified*	Singleton End-P1 / N. Uddin		R2A
N118	*unidentified*	Singleton End-P1 / N. Uddin		R2A
N120	*unidentified*	Singleton End-P1 / N. Uddin		R2A
1A1	*unidentified ascomycete*	J. Bennett/A. Crowe		Actinomycete Agar
1A11	*unidentified ascomycete*	J. Bennett/A. Crowe		Actinomycete Agar
1A111	*unidentified ascomycete*	J. Bennett/A. Crowe		Actinomycete Agar
1B1	*unidentified ascomycete*	J. Bennett/A. Crowe		Actinomycete Agar
1B11	*unidentified ascomycete*	J. Bennett/A. Crowe		Actinomycete Agar
1B111	*unidentified ascomycete*	J. Bennett/A. Crowe		Actinomycete Agar
E1103	*Ureibacillus suwonensis*	EFS End-P1 / N. Uddin		R2A
E1191	*Ureibacillus suwonensis*	EFS End-P1 / N. Uddin		R2A
C125	*Ureibacillus suwonensis*	Mernda End-P1 / N. Uddin		R2A
E11016	*Ureibacillus terrenus*	EFS End-P1 / N. Uddin		R2A
E11019	*Ureibacillus terrenus*	EFS End-P1 / N. Uddin		R2A
E11020	*Ureibacillus terrenus*	EFS End-P1 / N. Uddin		R2A
E11919	*Ureibacillus terrenus*	EFS End-P1 / N. Uddin	L	R2A
E11925	*Ureibacillus terrenus*	EFS End-P1 / N. Uddin		R2A
C14	*Ureibacillus terrenus*	Mernda End-P1 / N. Uddin		R2A
C118	*Ureibacillus terrenus*	Mernda End-P1 / N. Uddin		R2A
C119	*Ureibacillus terrenus*	Mernda End-P1 / N. Uddin		R2A
C124	*Ureibacillus terrenus*	Mernda End-P1 / N. Uddin		R2A
N114	*Ureibacillus terrenus*	Singleton End-P1 / N. Uddin		R2A
N115	*Ureibacillus terrenus*	Singleton End-P1 / N. Uddin		R2A
N119	*Ureibacillus terrenus*	Singleton End-P1 / N. Uddin		R2A
N121	*Ureibacillus terrenus*	Singleton End-P1 / N. Uddin		R2A
E1102	*Ureibacillus thermophilus*	EFS End-P1 / N. Uddin		R2A
E11013	*Ureibacillus thermosphaericus*	EFS End-P1 / N. Uddin		R2A
E1198	*Ureibacillus thermosphaericus*	EFS End-P1 / N. Uddin		R2A
C15	*Ureibacillus thermosphaericus*	Mernda End-P1 / N. Uddin		R2A
C16	*Ureibacillus thermosphaericus*	Mernda End-P1 / N. Uddin		R2A
C19	*Ureibacillus thermosphaericus*	Mernda End-P1 / N. Uddin		R2A
C113	*Ureibacillus thermosphaericus*	Mernda End-P1 / N. Uddin	L	R2A
C114	*Ureibacillus thermosphaericus*	Mernda End-P1 / N. Uddin		R2A
C122	*Ureibacillus thermosphaericus*	Mernda End-P1 / N. Uddin		R2A

Table 2.2.2. Presence of N-transformation genes in selected isolates of bacterial species from the compost bacterial collection.

Species	no. of isolates tested	% of isolates of each species that are positive for N-transformation genes[a]			
		amoA	***nirK***	***nirS***	***nosZ***
Acinetobacter johnsonii	1	0	0	0	0
Acinetobacter movanagherensis	1	0	0	0	0
Acinetobacter variabilis	1	0	0	100	100
Arthrobacter psychrochitiniphilus	1	100	0	0	100
Bacillus safensis	2	100	100	0	50
Bordetella sp.	1	100	100	0	0
Caulobacter segnis	4	0	0	0	75
Cellulomonas flavigena	1	0	0	0	0
Cellulomonas sp.	1	0	0	0	0
Cronobacter condimenti	1	0	0	0	0
Dermacoccus sp.	3	0	33	0	33
Enterobacter cloacae	2	50	50	0	0
Enterobacter sp.	1	100	0	0	0
Glumacibacter arilaitensis	1	0	0	0	0
Gordonia westfalica	1	0	0	0	100
Leadbetterella byssophila	2	0	100	0	0
Lysinibacillus acetophenoni	1	0	100	0	0
Lysinibacillus composti	1	0	0	0	100
Methylobacterium radiotolerans	1	0	0	0	100
Microbacterium foliorum	2	0	50	0	0
Microbacterium maritypicum	8	13	50	13	25
Microbacterium natoriense	1	0	100	0	0
Mycovorax composti	3	33	0	33	100
Nitratireductor lucknowense	1	0	100	0	0
Novosphingobium aromaticivorans	1	0	0	0	100
Pseudomonas fluvialis	2	0	0	0	100
Pseudomonas sihuiensis	1	0	0	100	100
Pseudoxanthomonas koreensis	2	0	0	50	50
Pseudoxanthomonas suwonensis	25	24	48	16	76
Pseudoxanthomonas taiwanensis	13	8	15	15	15
Serratia ureilytica	1	0	0	0	0
Solimonas soli	1	0	0	0	0
Sphingobacterium alimentarium	1	0	100	0	0
Sphingobacterium thermophilum	4	0	100	0	25
Staphylococcus epidermidis	4	0	0	0	0
Staphylococcus xylosus	5	0	40	0	0
Streptomyces griseorubiginosus	1	0	0	0	0
Streptomyces sudanensis	1	0	0	0	100

[a] Isolates were assessed as positive if a PCR amplicon was detected for the respective gene that matched the size of the dominant amplicon for that gene obtained with total compost DNA.

3 Microbial interactions between compost organisms in vivo

Direct and indirect interactions between individual compost taxa were studied, focussing on the most abundant taxa present at the end of Phase 2. This led to the discovery of *Mycovorax,* a novel bacterial genus that attacks *Mycothermus* hyphae in Phase 2 compost. The data provide evidence for the formation of physical interactions between organisms within compost as well as in vitro, and these may be critical for efficient and productive composting.

3.1 Methods

3.1.1 Interaction between M. thermophilus and compost bacteria in vitro

The interactions between *M. thermophilus* and bacterial isolates from compost were tested using both solid and liquid medium. On solid medium (YpSs 1.5% agar), two parallel streaks approximately 10 cm apart were grown to stationary phase at 50 °C or 30 °C in a moist box to prevent the plates from drying. *Mycothermus thermophilus* was subsequently inoculated between the bacterial streaks as an agar plug (5 × 5 mm) taken from a freshly grown plate, and the co-culture was incubated for four days at 50 °C in a moist box. Simultaneous growth of fungi and bacteria with liquid medium was done using a method adapted from van Schöll et al. (2006). Briefly, soda-lime glass beads (5 mm diameter; John Morris Group, Australia) were placed in a single layer in a Petri dish and overlaid with a 45 µm nylon mesh (8.5 cm diameter disc) (SEFAR, Australia) overlaying the glass beads and 15 mL of liquid YpSs medium was added. An agar plug (5 × 5 mm) of *M. thermophilus* from a freshly grown YpSs plate was placed in the middle of the plate and the plate was incubated for four days at 50 °C in a closed moist box to minimise evaporation. Bacterial cultures were grown overnight with shaking (200 rpm) until mid-exponential phase (OD_{600}: 0.4-0.5). An aliquot (4 ml) of the liquid medium was removed and replaced with the same volume of the bacterial cultures to be tested. The plates were incubated statically at 50 °C in a moist box. Growth of the bacteria in the co-culture was monitored by measuring OD_{600}, until the bacterial culture reached stationary phase. Daily growth of the fungus was monitored by measuring radial growth until the fungus fully colonised the nylon membrane or hyphal growth had ceased.

3.1.2 Interaction between M. thermophilus and compost bacteria in compost

Nylon membranes were colonised with *Mycothermus thermophilus* and incubated in Phase 2 compost for one week at 50°C (n =5) (see Figure below). Total DNA of membrane, colonised Mycothermus and any attached organisms was extracted and amplicon sequencing of the 16S rRNA gene was performed using 16S primers 341F and 806R, as described above. Uncolonised membranes were also incubated with Phase 2 compost as “background” community controls (n = 5). The compost from controls was combined into a composite sample and total DNA extracted for NGS (n =1). Taxa that attached to Mycothermus hyphae were identified based on having a higher relative abundance than in the “background” control.

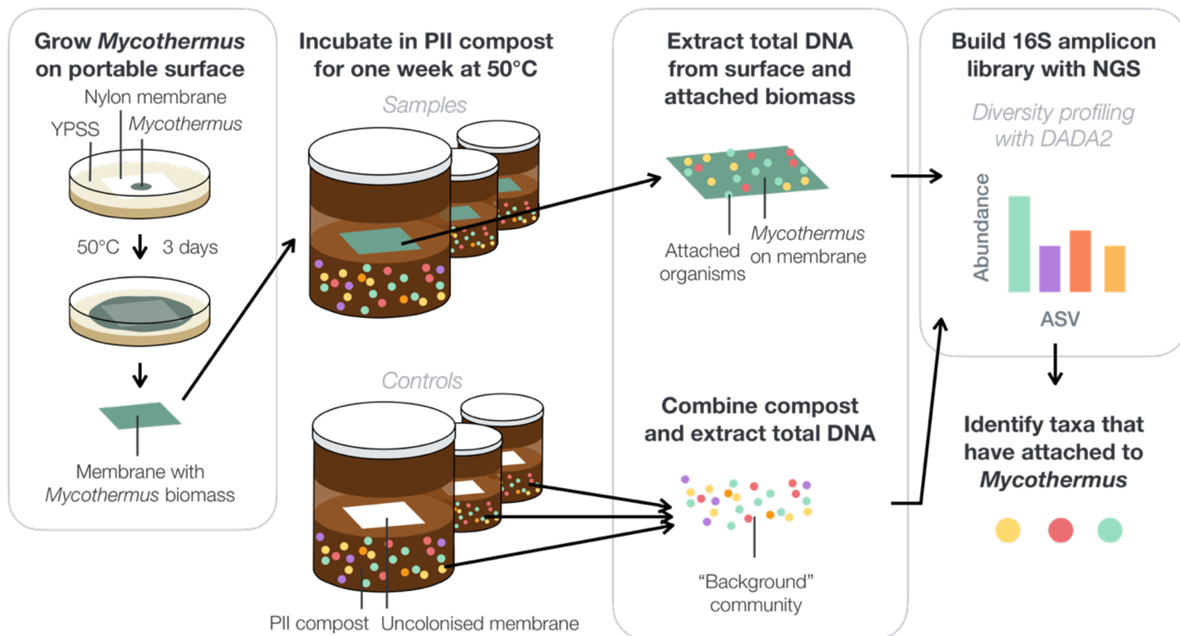

Figure 3.1.1. Experimental design for *in compost* association experiments between *Mycothermus thermophilus* and compost bacteria**.**

3.2 Results

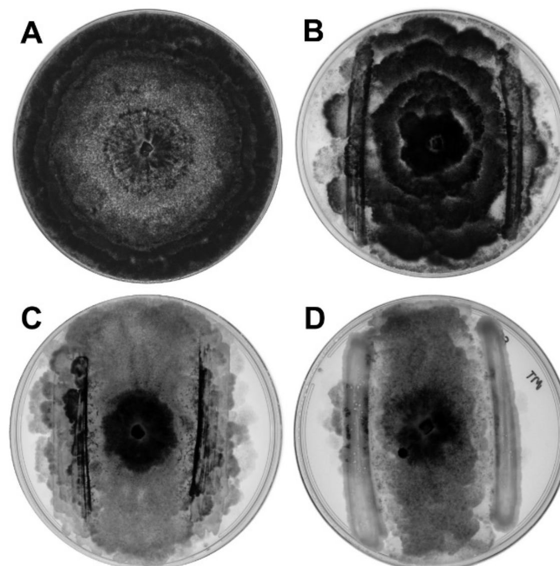

Figure 3.2.1. Interaction of *Mycothermus thermophilus* with *Pseudoxanthomonas* spp. (A) *Mycothermus thermophilus* control, (B) *M. thermophilus* with *Pseudoxanthomonas taiwanensis*, (C) *M. thermophilus* with *Pseudoxanthomonas koreensis*, and (D) *M. thermophilus* with *Pseudoxanthomonas suwonensis*. Bacteria (vertical streaks) were grown to stationary phase before inoculation with *M. thermophilus*. The fungus was inoculated equidistant between the bacterial streaks.

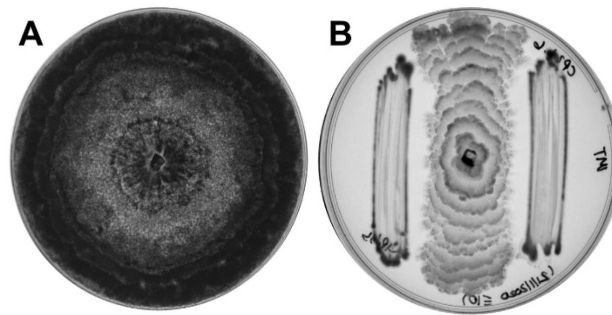

Figure 3.2.2. Interaction of *Mycothermus thermophilus* with *Mycovorax composti*. (A) *M. thermophilus* control, (B) *M. thermophilus* in the presence of *Mycovorax composti*.

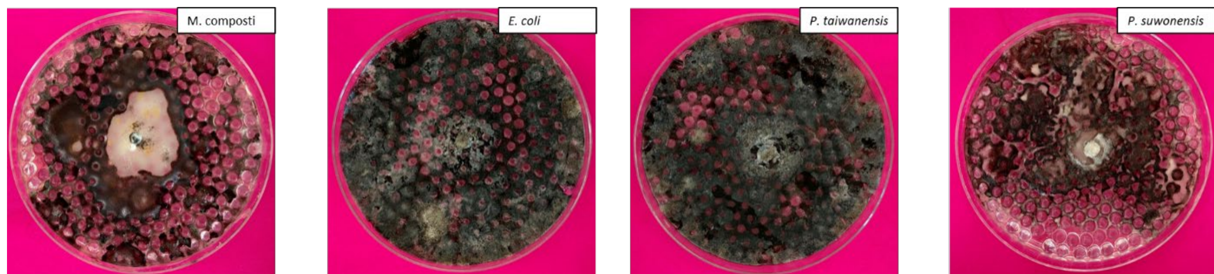

Figure 3.2.3. In vitro interaction between *Mycothermus thermophilus* and four different bacterial strains. Strains were grown on glass beads in a layer of YPSS growth medium, and are presented on a pink background for easy visualization of the black fungal growth. Representative plates are shown. The *Chitinophagaceae* isolate CP21.6 strongly inhibits *Mycothermus* growth, and colonies of this chitin-degrading bacterium can be seen developing on the fungal mycelium (yellow spots). By contrast, *P. taiwanensis* and *E. coli* do not inhibit *Mycothermus* growth, and *P. suwonensis* shows a partial inhibitory effect.

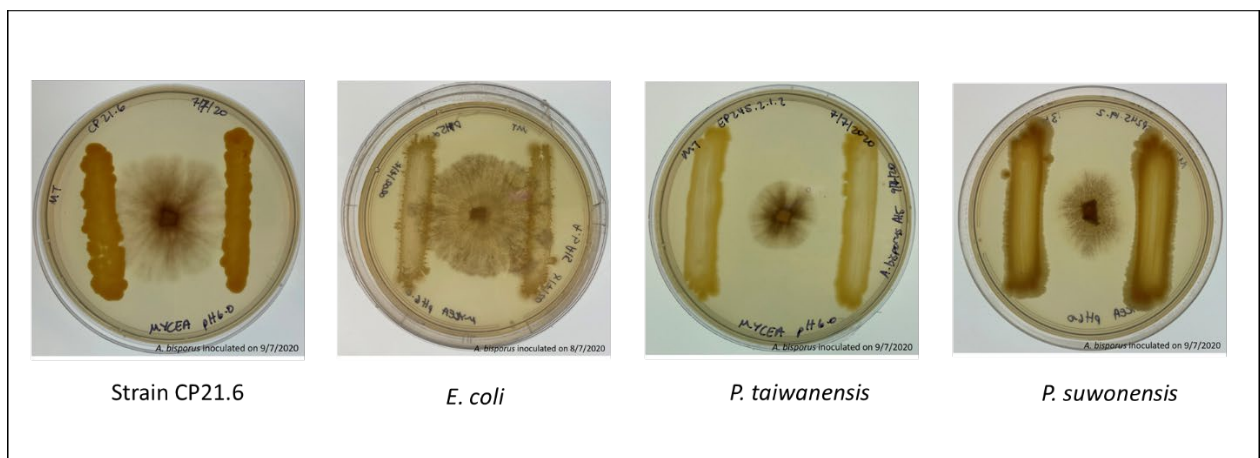

Figure 3.2.4. In vitro interaction between button mushroom mycelium *Agaricus bisporus* A15 (inoculated at the centre of each plate) and bacterial strains (two vertical stripes on each respective plate). Representative plates are shown. *Mycovorax composti* (CP21.6) which strongly inhibits *Mycothermus* has no effect on *Agaricus* growth (similar effect to the control strain of *E. coli*). By contrast, both *P. taiwanensis* and *P. suwonensis* show inhibitory effects on *Agaricus* growth in vitro.

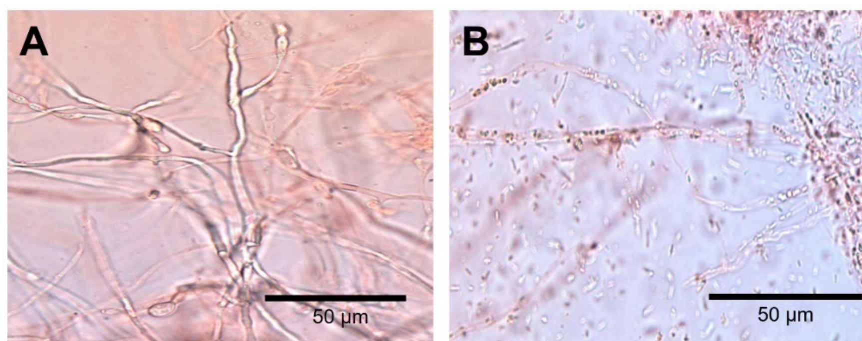

Figure 3.2.5. Light microscopy images of *Mycothermus thermophilus*. (A) Healthy *Mycothermus thermophilus* hyphae. (B) Mycothermus thermophilus hyphae after incubation with *Mycovorax composti*. Stained with 1% (w/v) Congo Red.

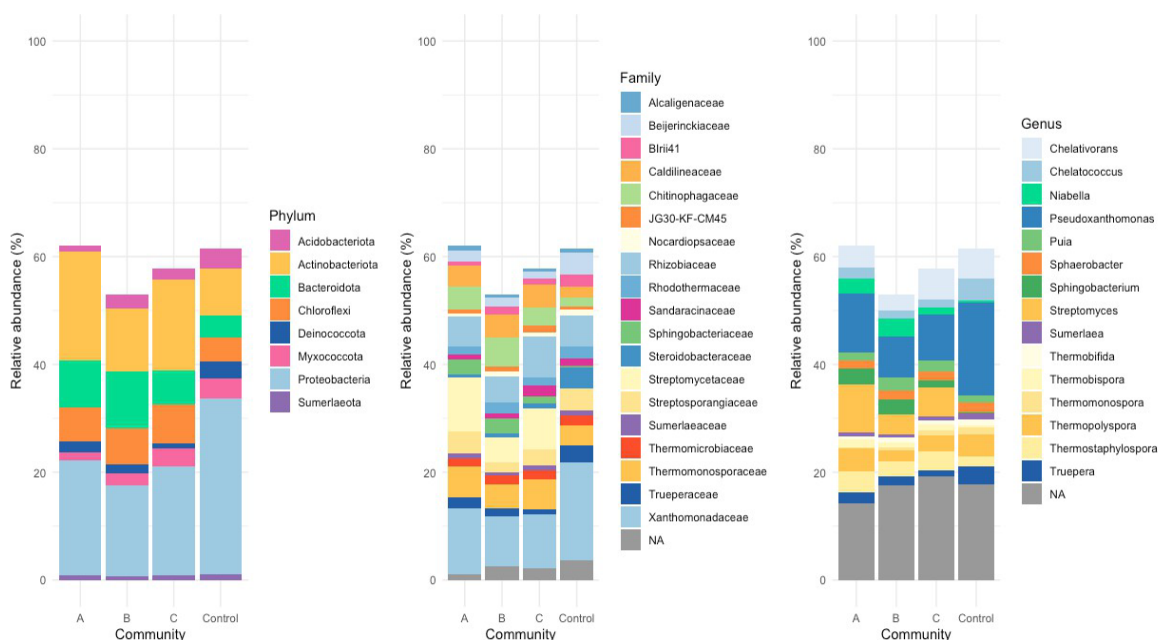

Figure 3.2.6. Bacterial taxa that physically associate with *M. thermophilus*. Communities A, B and C were triplicate samples profiled from DNA of bacteria attached to *Mycothermus* hyphae on nylon membranes (see Fig. 3.1.1). Only the most abundant 20 taxa are shown. Abundance was calculated as a percentage of total reads per sample. NA = unassigned taxa.

4 N balance for mushroom production

The nitrogen mass balance during mushroom composting and cropping was investigated in two parts. Extensive information on inputs, outputs, composting process and mass losses during composting was obtained from individual composters. Samples of raw materials and composts at different stages of composting were analysed, in order to quantify Nitrogen losses at different stages of the composting process. The study was complemented by analysis of spent mushroom compost, spent casing, and mushrooms from three different flushes. Because of variations in process used at different yards, it was not possible to consistently quantify N losses as ammonia during Phase 1 (many yards have an outdoor Phase 1). It was also not possible to accurately measure the recycling of N by use of goody water in straw wetting, since the proportion of goody water used in this process varied dramatically between yards. Losses as gaseous emissions during Phase 2 are described in Section 5. The names of the participating composters have been anonymised for confidentiality reasons.

4.1 Methods

4.1.1 Total Nitrogen

Freeze-dried, finely ground compost, casing or mushroom caps (50 µg) were used for total nitrogen analysis. Total nitrogen was determined by combustion in an Elementar Vario MACRO cube CHNS analyser. The standard used was phenylalanine (30 mg) (Sigma Aldrich, Australia), and all standard, blanks and samples were analyzed in triplicate.

4.1.2 Nitrate and ammonium in compost

Nitrate and ammonium were determined by ion chromatography, using a system a Shimadzu system composed of a CBM-40 controller, DGU-40 degasser, LC-20Ai pump, SIL-20A autosampler and CDD-10A conductivity detector. Compost extract was prepared by suspending compost (4 g) in deionized water (20 mL) and shaking (60 rpm, 15 minutes. The centrifuged extract was diluted 100-fold for analysis.

Ammonium was determined by cation chromatography with the Shimadzu Shim-pack IC-C4 column (150mm L x 4.6 mm D, 7 um particle size), using 2.5 mM oxalic acid as isocratic eluent at a flow rate of 1 ml/min. The column oven temperature was set to 40 °C, and eluting species were detected by conductivity and quantified using appropriate standards.

Nitrate was determined by anion chromatography with the Dionex IonPac AS14 column (250mm L x 4. mm D, 9 um particle size)/IonPac AG14 guard column (250mm L x 4. mm D, 9 um particle size). The column was eluted with 1.0 mM sodium bicarbonate/3.5 mM Sodium Carbonate as isocratic eluent at a flow rate of 1 mL/min. The column oven temperature was set to 40 °C, and eluting species were detected by conductivity after solvent suppression (Shimadzu Anion Suppressor ICDS-40A) and quantified using appropriate standards.

4.2 Results

Table 4.2.1. Variations in the composting process at various yards around Australia.

Compost yard	Average rainfall (mm)	Average temperature (°C)	Yard size* (t per crop)	Raw materials	Prewet▲ (48 h-14 days)	Phase I• (9-21 days)	Phase II♦ (6-10 hrs)
A	700	Min: 11.5 Max: 25.4	Large	Wheat straw Poultry manure Gypsum Added nitrogen supplements	Medium duration Aerated Indoor	Medium duration Aerated indoor bunker Crop turned three times	Medium duration pasteurization Bulk
B	400	Min: 8.7 Max: 22.1	Medium	Wheat straw Poultry Manure Gypsum	Medium duration Aerated Outdoor	Short duration Aerated outdoor bunker Crop turned two times	Long duration pasteurization Bulk
C	390	Min: 11.5 Max: 23.5	Small	Wheat straw Poultry manure Gypsum Added nitrogen supplements	Medium duration Outdoor	Medium duration Aerated outdoor bunker Crop turned five times	Medium duration pasteurization Bulk
D	480	Min: 10.1 Max: 21.3	Large	Wheat straw Poultry manure Gypsum	Short duration Outdoor	Long duration Outdoor concrete slab in windrows Crop turned 10 times	Short duration pasteurization Trays
E	830	Min: 7.7 Max: 16.9	Small	Wheat straw Poultry manure Gypsum Added nitrogen supplements	Long duration Outdoor	Medium duration Outdoor concrete slab in windrows Crop turned six times	Short duration pasteurization Trays

*Yard size – Small: 80-200 tonnes (t) of Phase I compost, medium: 200-800 t of PI, large: >800 t of PI
▲Timing of pre-wet – Short: <3 days, medium: 3-6 days, long: >7 days
•Timing of Phase I – Short: <12 days, medium: 12-18 days, long: >19 days
♦Timing of holding period for Phase II – Short: <7 h, medium: 8-9 h, long: >10 h

Table 4.2.2. Total nitrogen and percentage of nitrogen lost during Phase 1 of mushroom composting at Australian compost yards. Numbers have been normalised to end-Phase I by correcting for mass loss during composting.

	Additional N inputs		Timing of N input addition ***	Summed N in feedstocks▲ (kg N/T P1)	N content at end-P1 (kg N/T P1)	Loss of N during P1 (%)
	Organic*	**Inorganic****				
Yard A	CS, FM	U	3	5.331	4.806	10
Yard B	-	-	2	6.991	5.580	20
Yard C	-	U, AS	2,4	7.591	5.727	25
Yard D	-	-	3	8.790	5.697	35
Yard F	-	-	2	7.379	5.457	26
Yard G	SB	-	3	6.790	4.120	39
Yard H	CS, CT	U	2	7.071	5.469	23
Yard I	RS	AS	2,4	7.416	5.540	25
Yard J	C, CT	U	1,4	6.645	4.741	29

*C, CS, CT, FM, RS, SB – Canola, Cottonseed meal, Cotton trash, Feather meal, Rice straw, Soybean meal
**AS, U – Ammonium sulphate, Urea
***1, 2, 3, 4 – N addition during prewet, N addition at bale break, N addition during P1, N addition after P1
▲– calculated from reported feedstocks

Table 4.2.3. Total nitrogen (N) and proportion of nitrogen lost/gained (%) during Phase 2 composting. Values have been normalised to end-Phase 1 by correcting for mass loss during composting.

Compost Yard	Total N in end-P1 (%DW*)	Total N in end-P2 (%DW*)	N in end-P1 (kg N/T P1)	N in end-P2 (kg N/T P1)	N loss/gain during P2 (%)
Yard A	1.60	2.20	4.806	4.358	-9
Yard B	1.86	2.00	5.580	4.022	-28
Yard C	1.91	1.86	5.727	4.013	-30
Yard D	1.90	2.22	5.697	4.997	-12
Yard F	1.82	1.90	5.457	3.967	-27
Yard G	1.37	2.03	4.120	3.044	-26
Yard H	2.21	2.60	5.469	5.468	-18
Yard I	1.85	1.82	5.540	3.936	-29
Yard J	1.58	2.06	4.741	4.823	2

*** –** dry weight

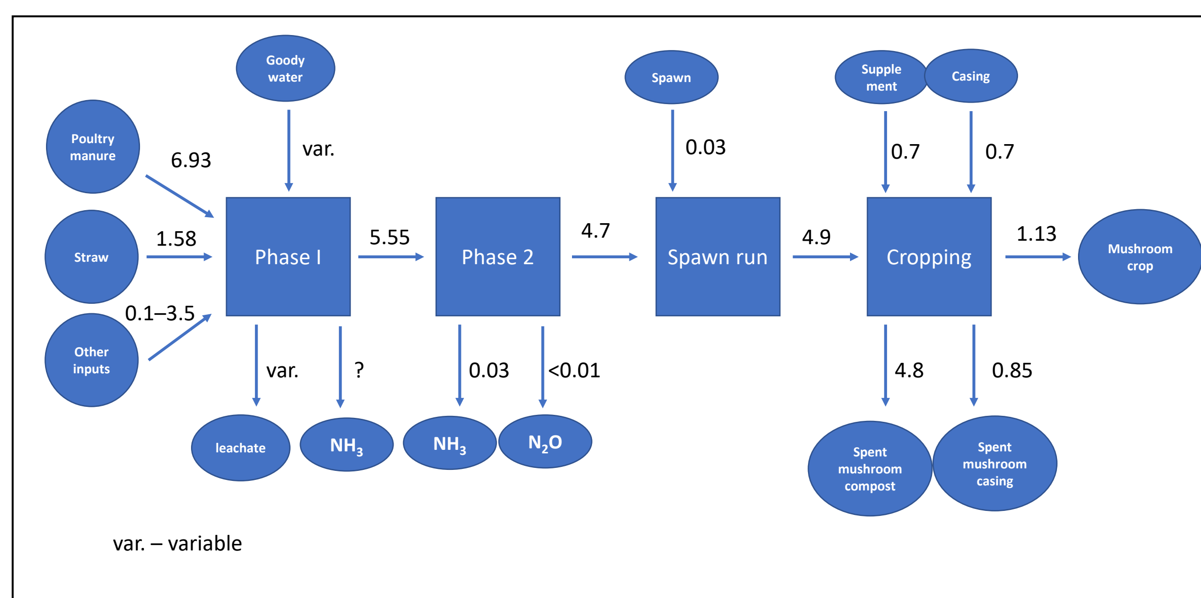

Figure 4.2.1. Averaged nitrogen inputs and outputs for the mushroom composting and production process for Australian mushroom composters and farms. Values presented are in kg N, normalized to the equivalent of 1 tonne P1 compost. Losses as leachate and release of ammonia from Phase 1 were not quantified as part of this project (see text), and it should be noted that there is considerable variation between different yards and mushroom farms (Table 4.2.1).

5 Gaseous N losses during Phase 2 composting

Gaseous nitrogen losses from Phase 2 may take the form of ammonia, nitrous oxide (the product of bacterial heterotrophic nitrification), or potentially molecular nitrogen (the product of bacterial denitrification. The latter is very unlikely under the highly aerobic conditions in Phase 2 composting, but production of nitrous oxide is known from other forms of composting (Sanchez et al. 2015), and mushroom composting is therefore possibly an important source of this potent greenhouse gas.

5.1 Methods

5.1.1 Sampling and analysis of nitrous oxide and ammonia from a Phase 2 tunnel

For analysis of N_2O, air was sampled from the return air ducting of a conventional Phase 2 tunnel at a commercial compost yard near Windsor, NSW (see Figure). Approximately 800 mL of gas was pulled from sampling vent in the return air ducting using a battery-operated handheld vacuum pump at a rate of one L min^{-1}. The gas was cooled using a 500 mL Dreschel flask in an ice bath and stored in a dual-valve one litre aluminium/polyethylene lined gas sampling bag (Hede Tech, China) (Figure 4.3). The whole system was flushed with Phase 2 tunnel gas for 5 min before sampling (i.e., about 5 L). Samples were taken in triplicate with 15-min intervals between each sample.

Ammonia was quantified using the Dräger Accuro handheld pump fitted with Dräger tubes capable of detecting levels of NH_3 between 2.5-1500 ppm (Dräger, Germany). Ammonia gas was sampled from the same sampling point as N_2O. Approximately 500 mL (5 full pumps) of air was sampled per day. If NH_3 was present at the time of sampling, the resulting colour change in the Dräger tube was estimated using the calibrated concentration marked on the tube.

Quantification of N_2O in the gas taken from the tunnel was done by gas chromatography. Gas samples (250 µL) were injected with a 1:1 injection split and 30 °C inlet temperature, onto a 100% divinylbenzene Rt-Q-Bond capillary gas chromatography (GC) column (30 m × 0.53 mm, 20 µm film thickness; Restek Corporation, Bellefonte, PA, USA) coupled to an electron capture detector (ECD; Model 7890A GC; Agilent). Ultra-high purity helium (BOC Ltd, Sydney, NSW) was used as the carrier gas at a flow rate of 3.2 mL min^{-1} and ultra-high purity nitrogen used as the make-up gas for the ECD. The GC oven was held isothermally at 30 °C for 7 min. The GC-ECD system was calibrated using high purity N_2O diluted with ultra-high purity N_2 (BOC Ltd).

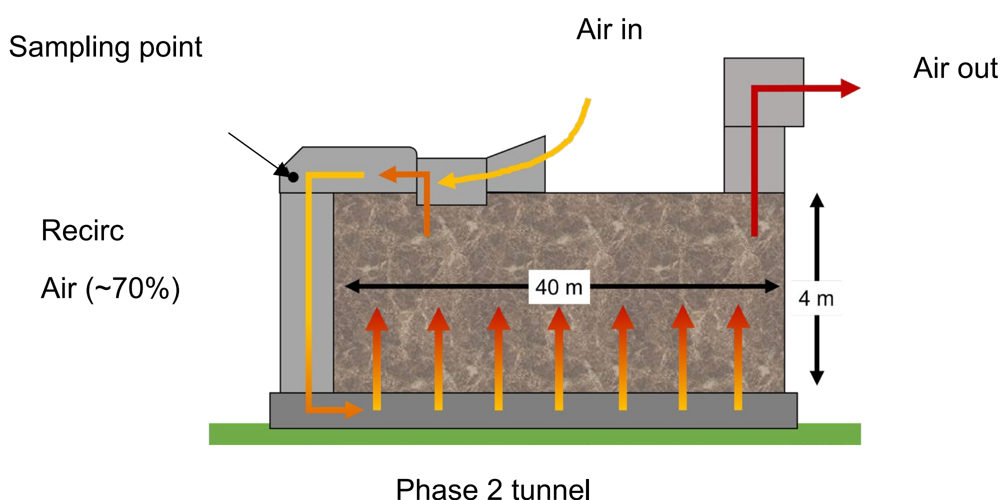

Figure 5.1.1. Sampling of gases from the return air ducting of a conventional Phase 2 tunnel.

5.1.2 Mass balance modelling of nitrous oxide released from compost

A mass balance model for compost tunnel N_2O concentration was adapted from a general mass balance equation developed by Yalçın et al. (2015), using their general mass balance equation:

$$\begin{matrix} mass\ at\ time \\ (t+\ \Delta t) \end{matrix} - \begin{matrix} mass\ at\ time \\ (t) \end{matrix} = \begin{matrix} entered\ mass \\ from\ t\ to\ \Delta t \end{matrix} + \begin{matrix} generated\ mass \\ between\ t\ and\ \Delta t \end{matrix} - \begin{matrix} exited\ mass \\ from\ t\ to\ \Delta t \end{matrix}$$

To do this, the following equations were used:

For a single tunnel, the pollutant mass equation was:

$$V_i \frac{dC_i}{dt} = S_i - L_i C_i \qquad (1)$$

Where C_i was the concentration of N_2O in the tunnel *i* (mg m^{-3}), *t* is time (day, d) V_i is the volume of the tunnel (m^3), S_i is the summed generation rate of N_2O from the compost in the tunnel (mg d^{-1} m^{-3}) and L_i is the summed rate of N_2O losses from the system (d^{-1}). S_i was calculated from the following equation:

$$S_i = \frac{1}{V_i}(C_{OA} P Q_{OA} + C_{RA} Q_{RA} + G_i) \qquad (2)$$

Where C_{OA} is the concentration of ambient outdoor N_2O at the site (mg m^{-3}), *P* is the proportion of N_2O that entered the tunnel from outside the system, Q_{OA} is the volumetric air flow rate that from outside to inside (m^3 d^{-1}), C_{RA} is the concentration of N_2O in the recirculated air (mg m^{-3}), Q_{RA} is the volumetric air flow rate of the recirculated air (m^3 d^{-1}) and G_i is the generation rate of N_2O in the tunnel.

$$Q_T = Q_{OA} + Q_{RA} \qquad (3)$$

The total volumetric flow rate (Q_T, m^3 d^{-1}) is the sum of Q_{OA} and Q_{RA}, which is determined from the proportion of outside air that entered the system and varied daily. L_i is calculated from the following equation:

$$L_i = \frac{1}{V_i}(Q_{OA} + Q_{RA}) \qquad (4)$$

Finally, equations (2) and (4) were substituted into equation (1) and the following equation was used to calculate summed generation rate of N_2O (S_i) from the compost:

$$C_i = C_O e^{-L_i t} + \frac{S_1}{L_i}(1 - e^{-L_i t}) \qquad (5)$$

C_O is the initial concentration of N_2O in the tunnel (mg m^{-3}). In order to calculate S_i, G_i was calculated using the Solver function in Microsoft Excel by changing G_i until the solution of Equation (5) (C_i) was equal to the measured concentration of N_2O in the tunnel.

5.2 Results

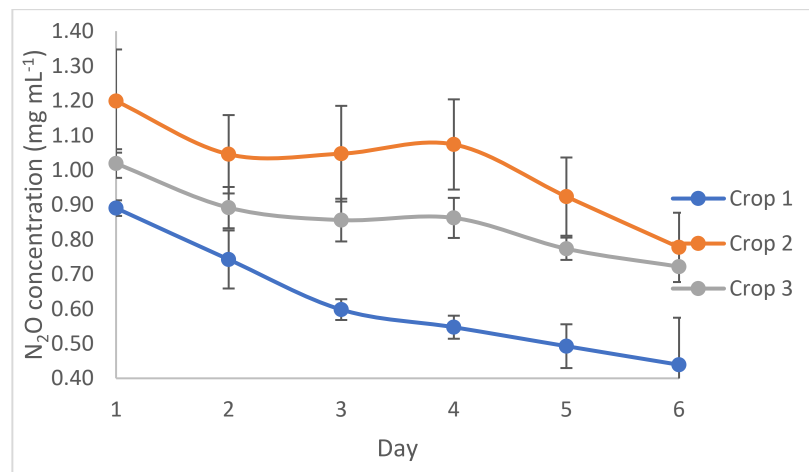

Figure 5.2.1. Nitrous oxide (N_2O) concentration in the airspace of a conventional compost tunnel for three successive crops. Error bars indicate standard deviation of three technical replicates.

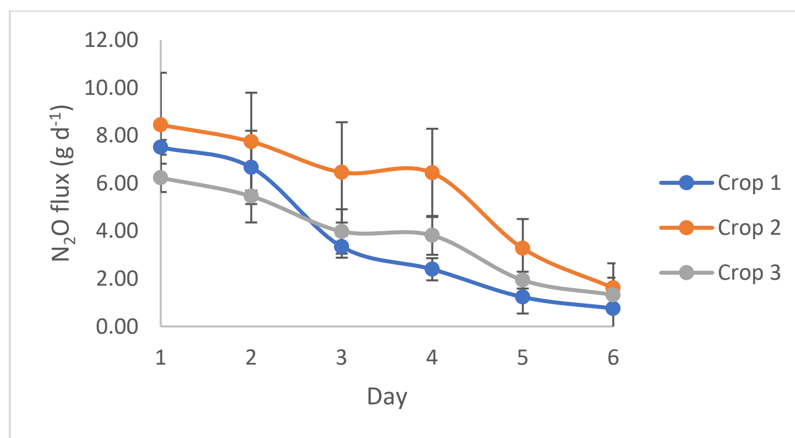

Figure 5.2.2. Calculated emission rate of nitrous oxide (N_2O) for the compost in one Phase 2 tunnel (approximately 200 tonnes) over the duration of Phase 2. Error bars indicate the standard deviation of three technical replicates.

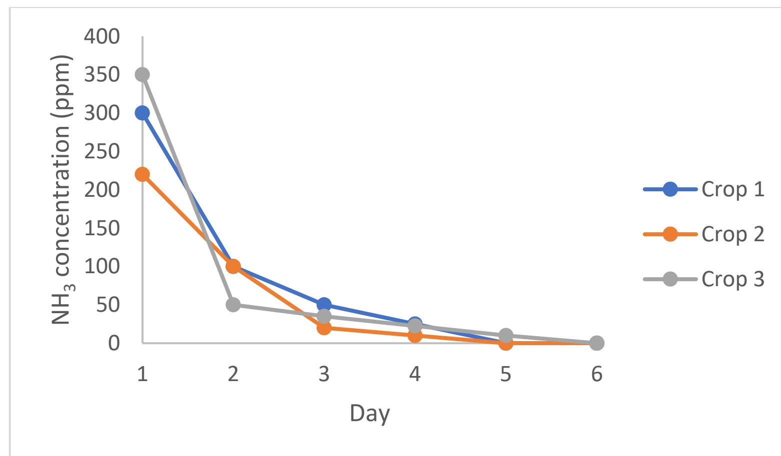

Figure 5.2.3. Ammonia (NH_3) concentration in a conventional compost tunnel measured using Dräger tubes.

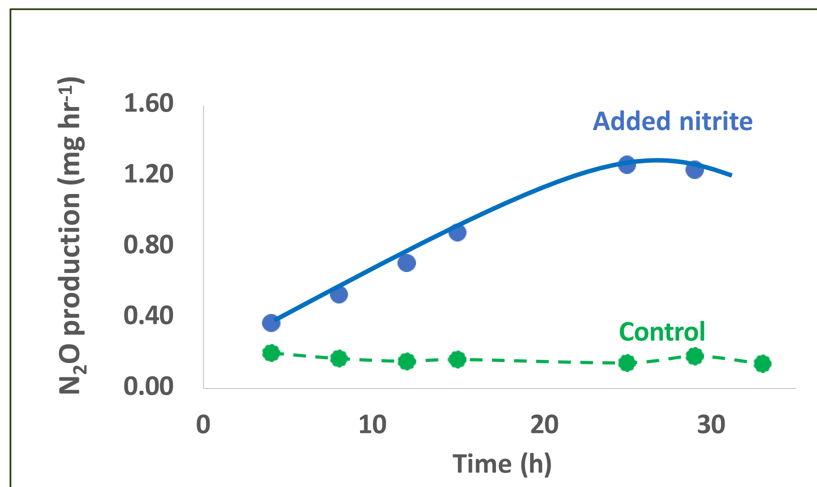

Figure 5.2.4. Nitrous oxide (N_2O) production by a culture of *Pseudoxanthomonas taiwanensis* in vitro. Blue – nitrite (20 mM) added, green – control.

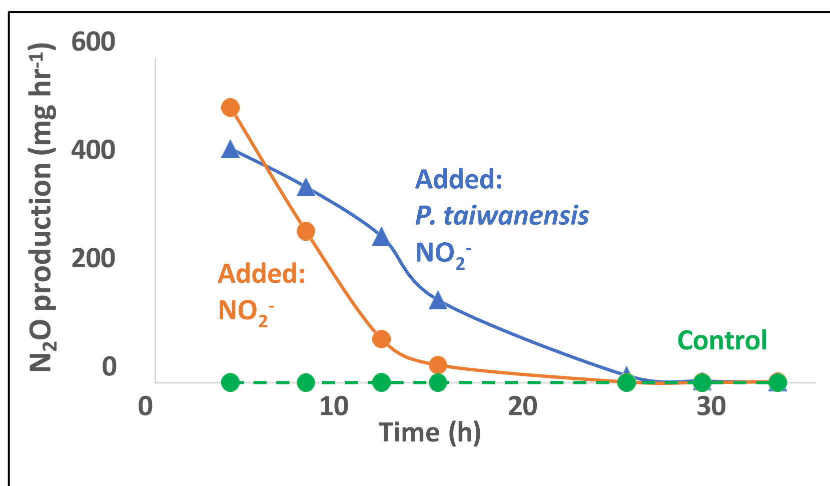

Figure 5.2.5. Nitrous oxide (N_2O) production by compost. Orange – nitrite (20 mM) added. Blue - *Pseudoxanthomonas taiwanensis* and nitrite (20 mM) added. Green - control.

6 Nitrification during cropping

Previous work in project *MU100021 Improving consistency of mushroom compost through control of biotic & abiotic parameters* showed that levels of nitrate in casing increase considerably during cropping, and this may be linked to the decrease in yield observed from first to third flush. This was investigated by a thorough determination of the levels of N metabolites in casing and in two layers of compost during three flushes of cropping. Since nitrate may be produced by bacteria under the aerobic conditions in casing (the process of bacterial nitrification), the populations of nitrifying bacteria were also investigated through quantification of bacteria containing the gene encoding ammonium monooxygenase (*amoA*), and the effect of inhibiting nitrification with specific inhibitors was also studied.

6.1 Methods

6.1.1 Determination of N metabolites in compost

Nitrate and ammonium were determined by ion chromatography as described in Section 4.1.

Nitrite was determined colorimetrically using the Griess reaction (Hood-Nowotny et al. 2010). Compost or casing extracts (25 mL) were treated with 100 μL Griess reagent (equal volumes of 0.77 mM N-napthylethylenediamine dihydrochloride and 58 mM sulphanilamide/3 M HCl), and absorbance measured at 540 nm, using sodium nitrite as a standard.

Free amino acids were determined using a ninhydrin-based method (Zhang et al. 2013). Compost extracts were diluted 1:4 with reaction buffer (100 mM Tris/HCl, pH 7.5, 30% PEG-6000, 50 mM EDTA). To this was added 250 μL tin reagent (80 mg $SnCl_2.2H_2O$ in 50 mL 200 mM citrate pH 5.0, purged with nitrogen immediately before use) and 250 μL ninhydrin solution (0.5 g ninhydrin in 10 mL DMSO). The mixture was mixed, heated at 80 C for 10 minutes, and then cooled and diluted with 500 μL water, and absorbance measured at 570 nM. The absorbance reading was corrected for any ammonium present (determined by ion chromatography above), using ammonium chloride as standard, and the amin acids determined from the residual reading using glycine as standard.

6.1.2 Application of nitrification inhibitors during cropping

Potential nitrification inhibitors were applied to casing during pin expansion immediately prior to each flush (days 7, 18, 29, 39 after casing). Solutions of the respective inhibitor (50 mL) were sprayed evenly onto the surface of the casing (0.5 m^2) using a spray bottle. The inhibitors applied were the following: dicyandiamide (DCD) (23.8 mM), karanj oil, neem oil and spearmint oil (each 1.5 g oil, 3 mL ethanol, 47 ml H_2O). Essential oils were purchased from *New Directions Australia* (Sydney, Aus). Control treatments included water, potassium nitrate (0.6 M), potassium chloride (0.6 M), and ethanol (3 mL in 50 mL H_2O).

6.1.3 Quantitative PCR of bacterial populations in casing

DNA was extracted from casing samples using the Powersoil kit (MoBio), following the manufacturer's instructions. Total bacterial populations and nitrifier populations were determined by quantitative PCR of the 16S and *amoA* genes, respectively. The primer sets used were 341F/806R for the 16S gene (Takahashi et al. 2014) and amoA1F/AmoA2R for the *amoA* gene (Petersen et al. 2012). The qPCR reaction was set up using the SensiFAST SYBR No-ROX Kit (BioLine) and done in the CFX96™ Thermocycler with CFX Manager software (BioRad). The standards used for quantification were amplicons created using casing DNA as template and either 27F/1492 primers for 16S or amoA1F/AmoA2R for *amoA*. Amplicons were purified before use with the Isolate II PCR and Gel Kit (Bioline) and concentrations determined using PicoGreen dye in the QuantiFluor dsDNA System (Promega).

6.2 Results

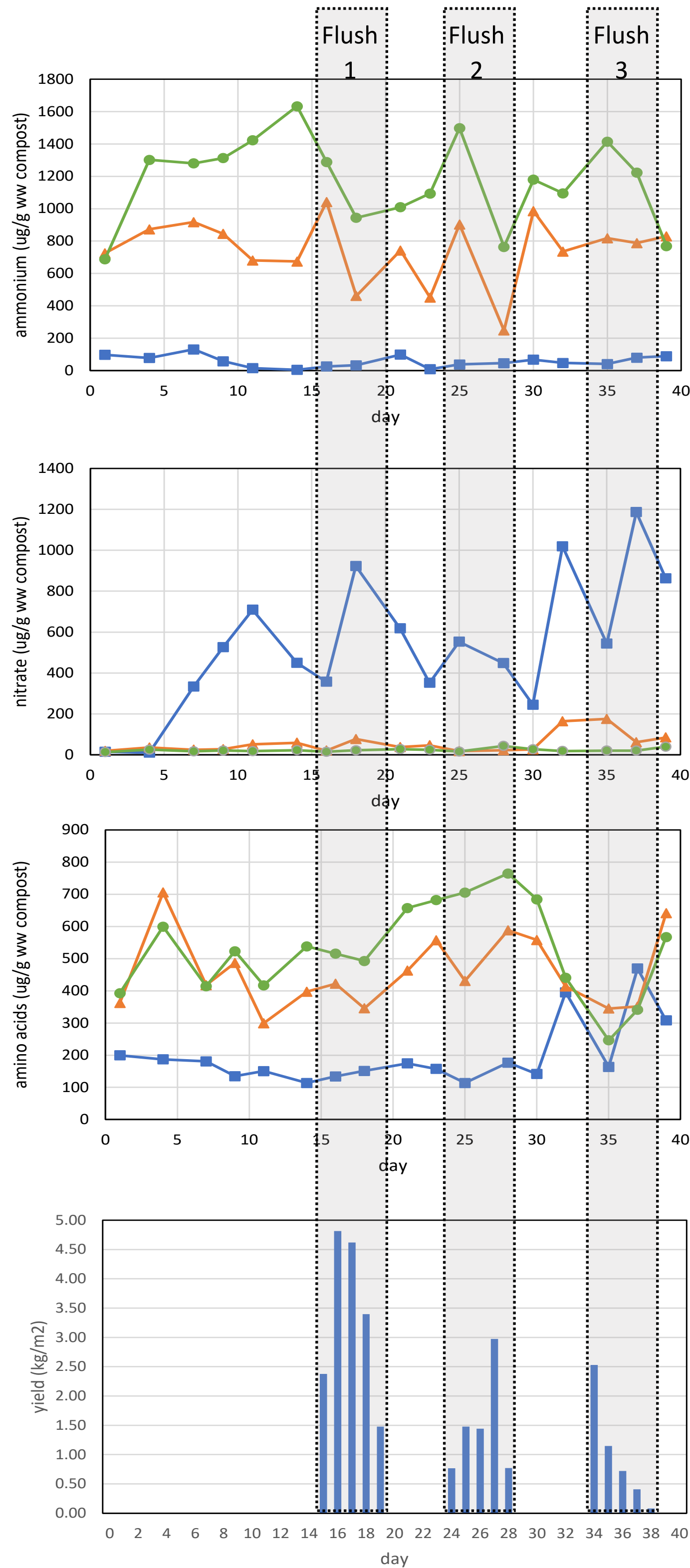

Figure 6.2.1. Nitrogen speciation in compost and casing for three flushes. Mushrooms were cultivated on 24 blocks of supplemented compost (20 kg) in the MLMRU. Data were obtained for triplicate, pooled cores at each time point (1 cm diameter x 15 cm depth). Cores were separated into the casing (blue), the top 5 cm of compost (orange) and the bottom 5 cm of compost (green) before analysis.

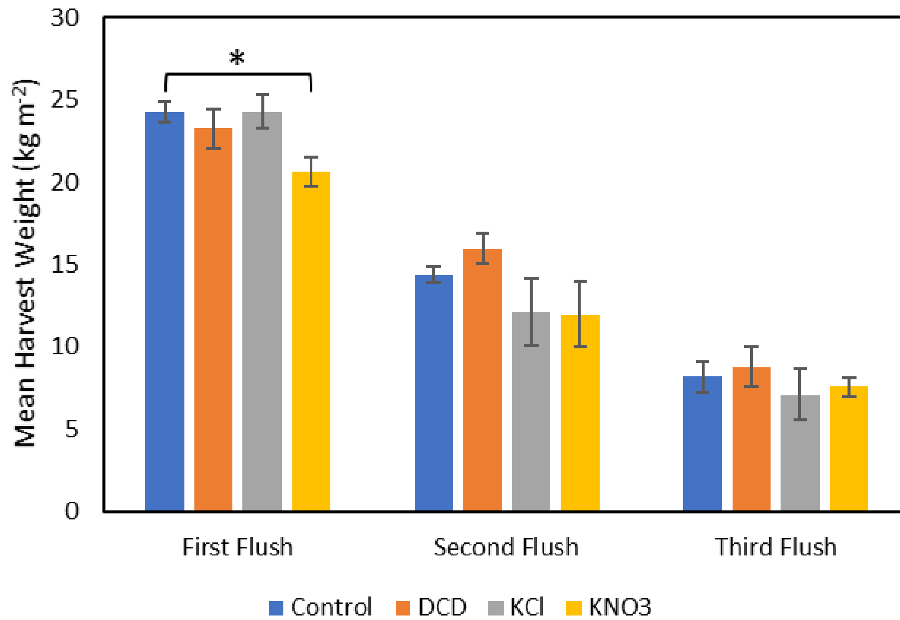

Figure 6.2.2. Yield of mushrooms obtained after treatment with the nitrification inhibitor dicyandiamide (DCD) in the pin expansion period before each flush. The control was treated with water, and further treatments showed the effect of nitrate and chloride addition. Error bars are standard error (n=4).

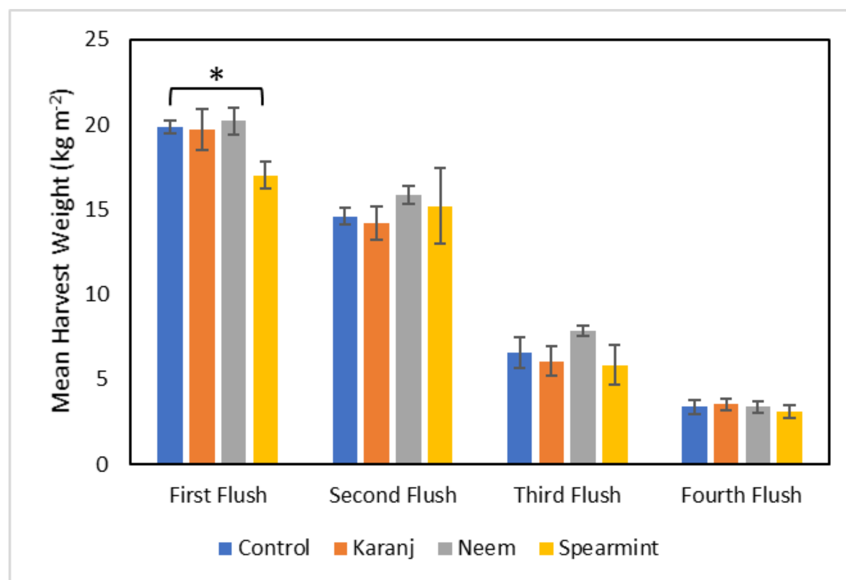

Figure 6.2.3. Yield of mushrooms obtained after treatment with the potential nitrification inhibitors karanj oil, neem oil and spearmint oil in the pin expansion period before each flush. Error bars are standard error (n=4).

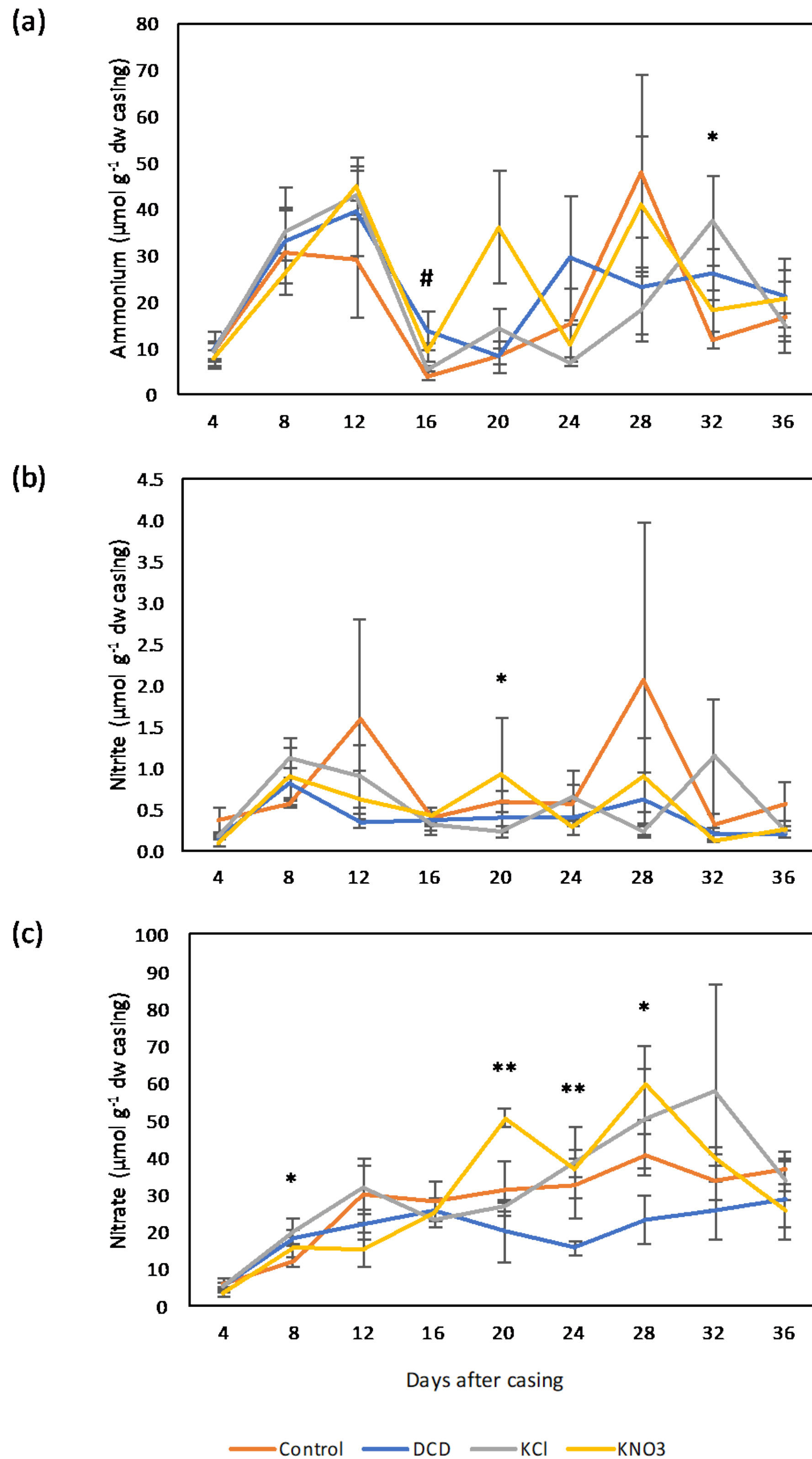

Figure 6.2.4. N Ammonium, nitrite and nitrate levels in casing after treatment with dicyandiamide (DCD) in the pin expansion period before each flush. The control was treated with water, and further treatments showed the effect of nitrate and chloride addition. (a) ammonium; (b) nitrite; (c) nitrate. Error bars are standard error (n=4)

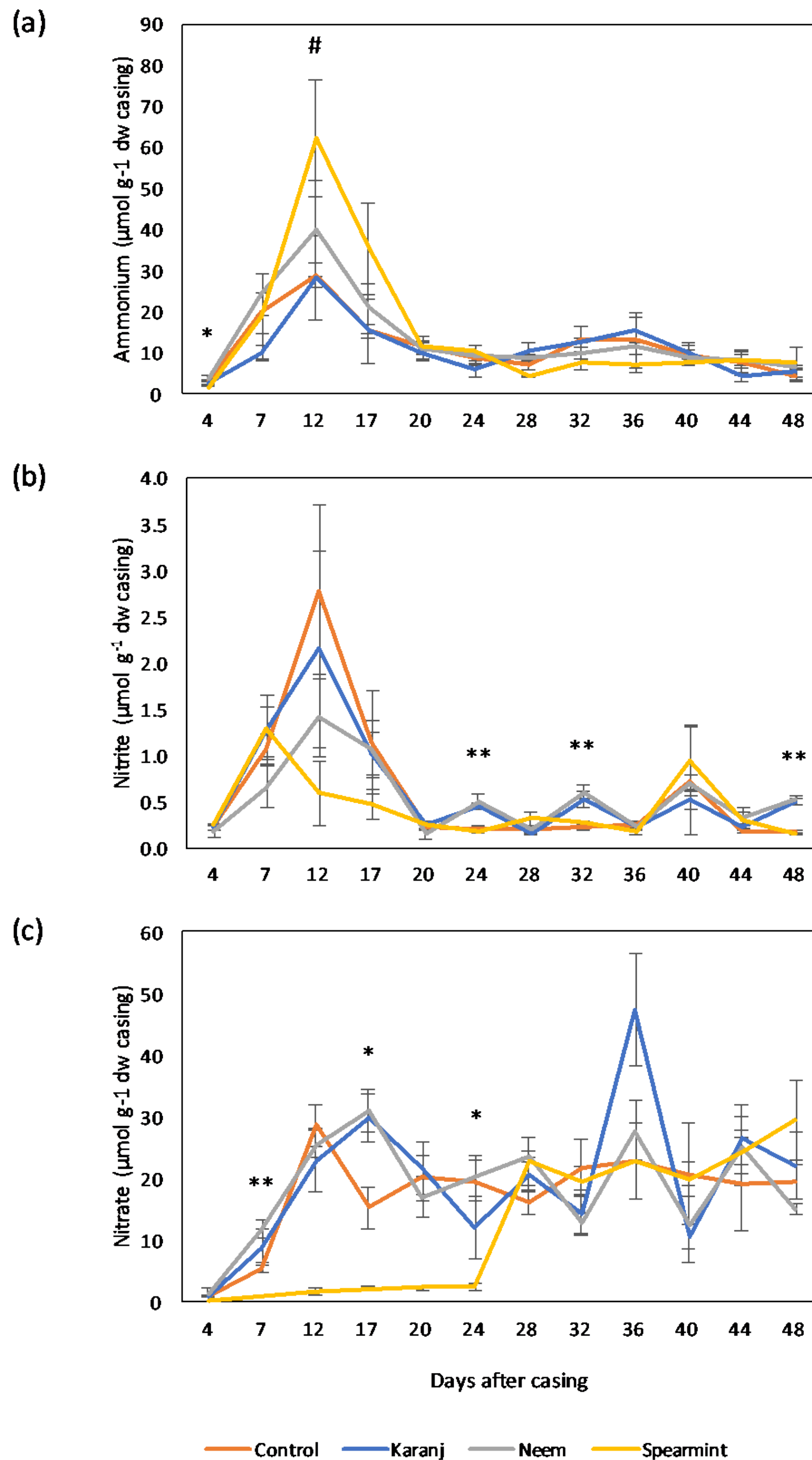

Figure 6.2.5. Ammonium, nitrite and nitrate levels in casing after treatment with the potential nitrification inhibitors karanj oil, neem oil and spearmint oil in the pin expansion period before each flush. (a) ammonium; (b) nitrite; (c) nitrate. Error bars are standard error (n=4).

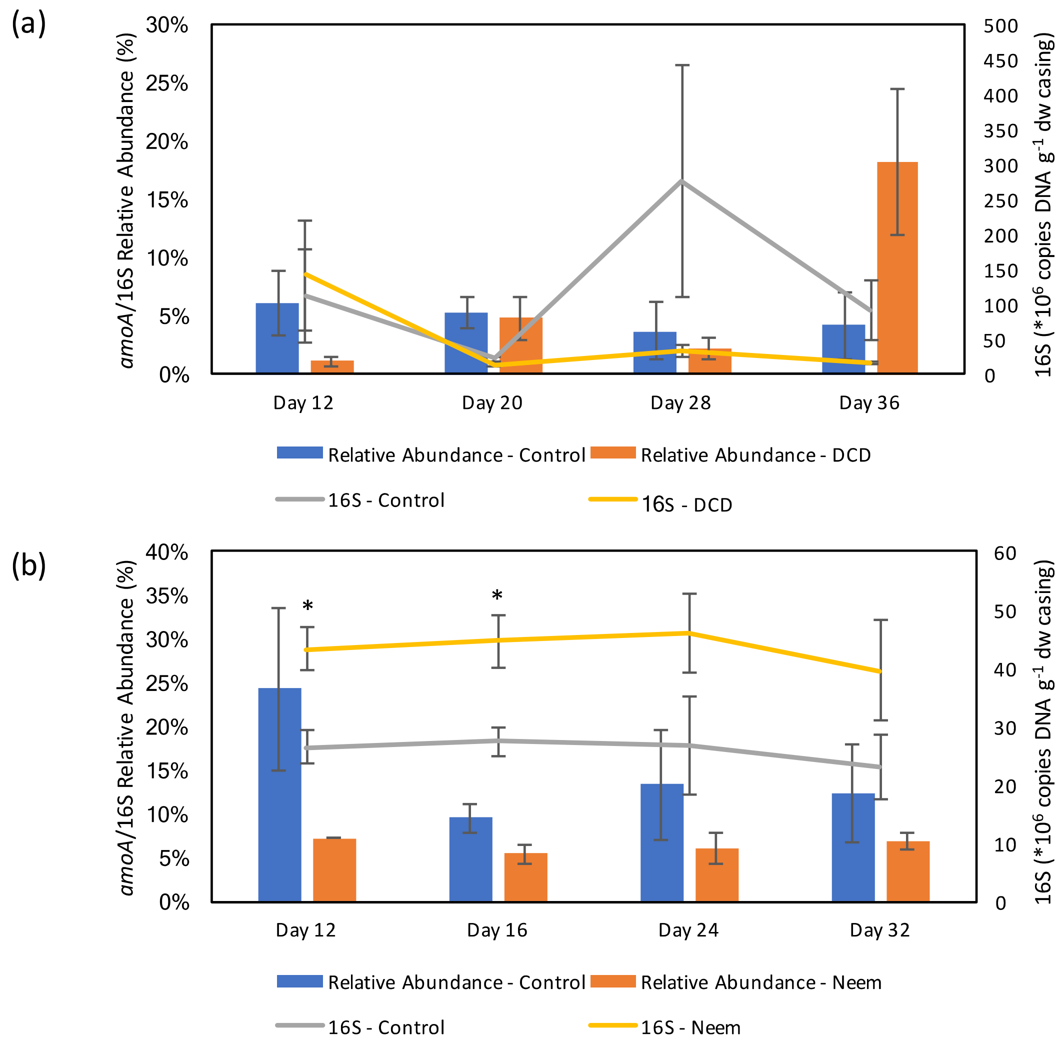

Figure 6.2.6. Nitrifier population density and total bacterial population density in casing at selected days during cropping. (a) treatment with the nitrification inhibitor dicyandiamide (DCD). (b) treatment with the nitrification inhibitor neem oil. Nitrifier population density and total bacterial population density were measured using quantitative PCR of the 16S and *amoA* genes respectively. Lines – total population size. Bars – proportion of the total population containing an *amoA* gene.

7 Addition of microbial inocula during spawn run

Mixtures of desirable microbial taxa isolated from compost have the potential to provide stability in composting and have been used in the past for this purpose (*M. thermophilus* has been marketed as a composting additive by Sylvan in the past). The studies of interactions between taxa (Section 3) suggested that the best effects might be obtained with defined mixtures of *M. thermophilus* and selected bacteria taxa, rather than just with bacteria or just fungi in the inocula. This project had planned to investigate this effect by constructing a medium scale model composting system, but unfortunately support for this was withdrawn during the Covid pandemic. While the most obvious uses of microbial inocula were anticipated for Phase 2 composting, this project has now also studied the effect of adding microbial consortia during spawn run. The inocula used were constructed from the dominant strains identified in Sections 1 and 2.

7.1 Methods

7.1.1 Measurement of mycelial growth rate using race tubes

Liquid bacterial cultures were grown aerobically overnight at 50 °C shaking to an OD600 of 0.3 for exponential phase cultures, and 1.0 for stationary phase cultures. Bacterial cultures were centrifuged for 6 minutes at 5000 RPM at 20 °C and cell pellets were resuspended in Milli Q water to OD600 1.0. Treatments containing two and three bacterial species were prepared by combining equal parts of each bacterial culture. The fungus *Mycothermus thermophilus* was cultured on Malt Yeast Agar (20g/L Malt Extract, 5g/L yeast extract, 15g/L agar) for 3 days, and the spore suspension was prepared by scraping all fungal biomass off a single plate and suspending in 50 mL Milli Q water. The *M. thermophilus* and bacterial combination treatments were made by mixing equal parts of each bacterial culture and resuspending to an OD600 of 2.0, then combining with an equal volume of *M. thermophilus* spore suspension. Microbial consortia were added at the desired concentrations to unspawned end-Phase 2 compost, mixed thoroughly, and incubated at room temperature for 3 hours filing into race tubes. Control treatments were done using MilliQ water and compost extract (15 g Phase 2 compost, 40 mL water, mixed on a rotary wheel for 45 minutes at room temperature)
"Race tubes" were set up by placing 1.15 g of *A. bisporus* spawn (Sylvan strain A15) at the bottom of a test tube (15 cm x 2.5 cm), then filling the tube with compost treatments in 5 replicates. Test tubes were closed with micropore tape and incubated at 22 °C and 90% relative humidity. Mycelium growth was measured every alternate day for 21 days, measuring from the bottom of the tube. Data were analyzed using a one-way ANOVA for significance and Tukey's HSD test for significant differences between treatment pairs.

7.2 Results

Figure 7.2.1. "Race tube" determination of bacterial/fungal inoculum growth effect on *Agaricus* growth. Compost treatments: (1) no addition; (2) water; (3) *Pseudoxanthomonas*; (4) *Chelatococcus* (5) *Bacillus*; (6) *Pseudoxanthomonas/Chelatococcus*; (7) *Bacillus/Chelatococcus*; (8) *Bacillus/ Pseudoxanthomonas*; (9) *Chelatococcus/Pseudoxanthomonas/Bacillus*; (10) *Mycothermus*; (11) *Mycothermus* + all three bacteria. Spawn is added at the bottom of the test tube, and mycelium grows upwards.

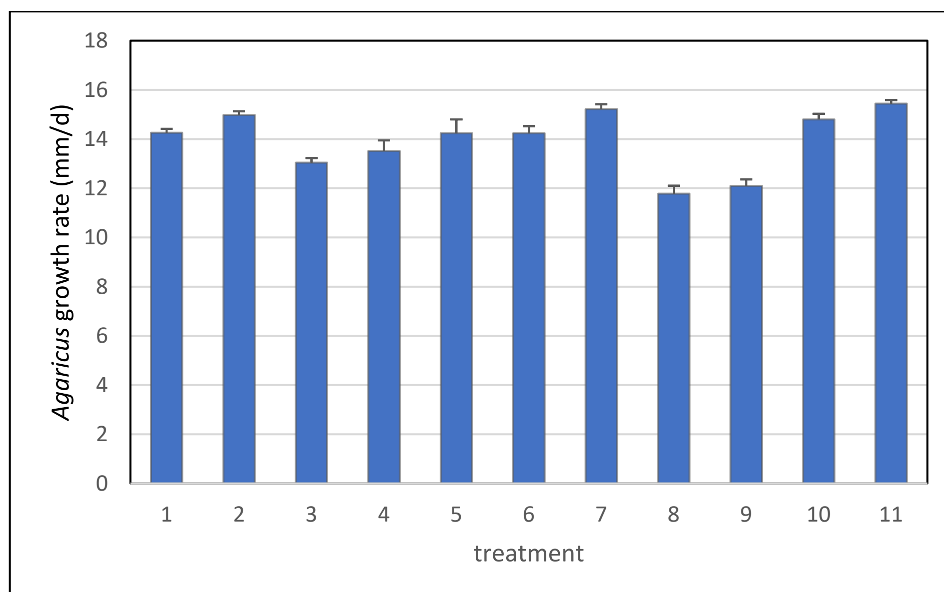

Figure 7.2.2. *Agaricus bisporus* mycelium growth rate after treatment of compost with bacterial and mixed inocula. Compost treatments: (1) no addition; (2) water; (3) *Pseudoxanthomonas*; (4) *Chelatococcus* (5) *Bacillus*; (6) *Pseudoxanthomonas/Chelatococcus*; (7) *Bacillus/Chelatococcus*; (8) *Bacillus/ Pseudoxanthomonas*; (9) *Chelatococcus/Pseudoxanthomonas/Bacillus*; (10) *Mycothermus*; (11) *Mycothermus* + all three bacteria. Error bars are standard error, n=5.

8 Bio-fertigation with added nitrogen sources during cropping

Mushroom composts are currently treated with a slow-release soy-derived supplement, usually at casing, though this is not universal practice. The data obtained in Section 6 revealed the presence of a pulse of free ammonium in compost immediately before each flush, at the time of pin expansion. This suggested that it would be advantageous to omit the slow-release supplement, and instead stimulate mushroom growth at precise times during cropping, using a soluble supplement that is readily available for uptake by the mushroom mycelium.

Supplementation results were obtained from five separate experiments.

1. Small scale supplementation of compost using three different N-supplement forms (complex supplement, ammonium acetate and urea). These were applied either (a) all at casing, (b) divided into three portions and applied immediately before each flush, or 50% applied at casing, and 50% applied as three portions before each flush.
2. Standard mushroom cropping run using overhead watering compared to a cropping run where water was provided via a fertigation system.
3. Supplementation with urea using a fertigation system, with application at pinning.
4. Supplementation with an amino acid supplement applied before each flush. Amino acids applied at twice the rate of commercial supplementation.
5. Supplementation with an amino acid supplement applied before flushes 2 and 3.

8.1 Methods

8.1.1 Small scale supplementation with liquid additives during cropping

Liquid supplements were injected into compost before each flush at pinning. Mushrooms were grown in 21L plastic boxes, containing 7.5 kg unsupplemented spawn-run compost. Three different supplements were applied: yeast extract, urea and ammonium acetate. Total application rate of each supplement was calculated to provide a total addition of 0.47 g N per kg compost over the entire period of cropping, equivalent to the commercial rate of addition of slow-release supplement. The supplementation regime was either (a) addition only at casing; (b) addition in three equal parts immediately before each flush, or (c) 50% of the total added at casing, and the remainer applied in three equal parts immediately before each flush. Supplements were applied to compost below the casing layer, using a long basting needle. The needle was inserted to a depth of 8 cm below the casing surface, and the necessary amount of supplement was delivered in nine 10 mL injections spaced evenly across the surface area of the compost. Watering was done by overhead spray irrigation of the casing.

8.1.2 Large scale fertigation of compost with liquid additives during cropping

Eight identical drip fertigation systems were constructed. Each fertigation system consisted of six 1.8m drip lines, with drippers at 30cm spacings. The position of the drippers was staggered to achieve uniform coverage of the compost blocks. The systems were filled using a hose fitting and drained using a tap. The systems were flushed with tap water after each treatment event.

Compost blocks were placed in the MLMRU, and the drip fertigation system was installed on the top of the compost blocks with the drippers facing down. The compost was then cased with a mixture of black peat, dolomite lime, CAC and water to reach optimal moisture. The release tap was left exposed, along with a single dripper. This was to allow the correct operation of the drip fertigation system to be visually confirmed. The casing layer was applied on the compost on the day of arrival at the unit.

8.2 Results

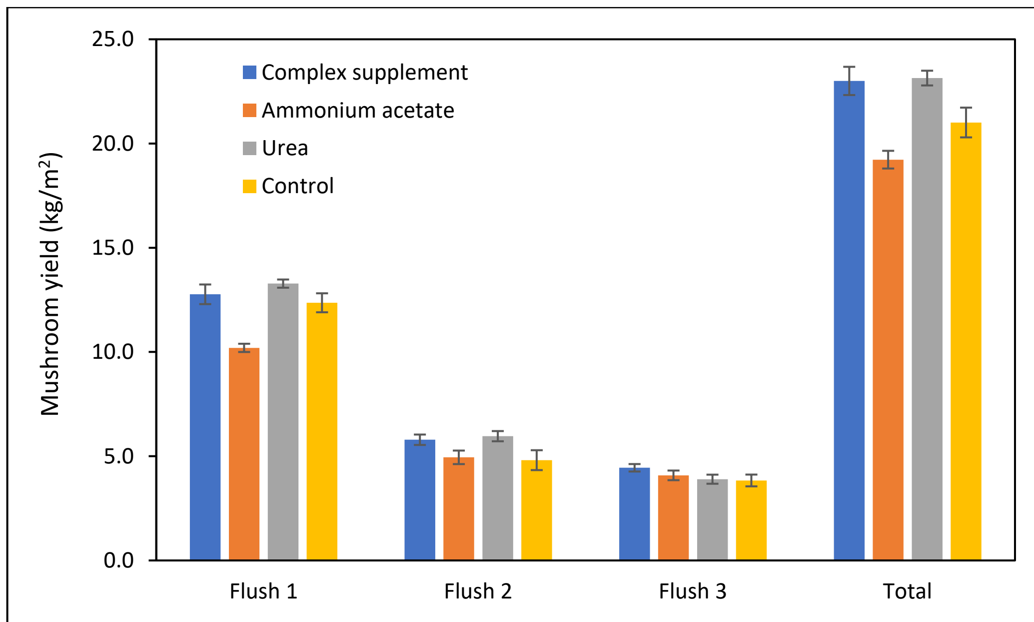

Figure 8.2.1. Mushroom yield for three flushes of small-scale supplementation. The Phase 3 compost was supplemented on small scale (injection) with complex supplement, ammonium acetate (AA) or urea (U), or not supplemented (Control). Supplement was added either at casing, immediately before each flush, or 50% of each, and the values shown are the average for all three treatments. Total N supplied was identical for each treatment. Error bars are standard error (n=5).

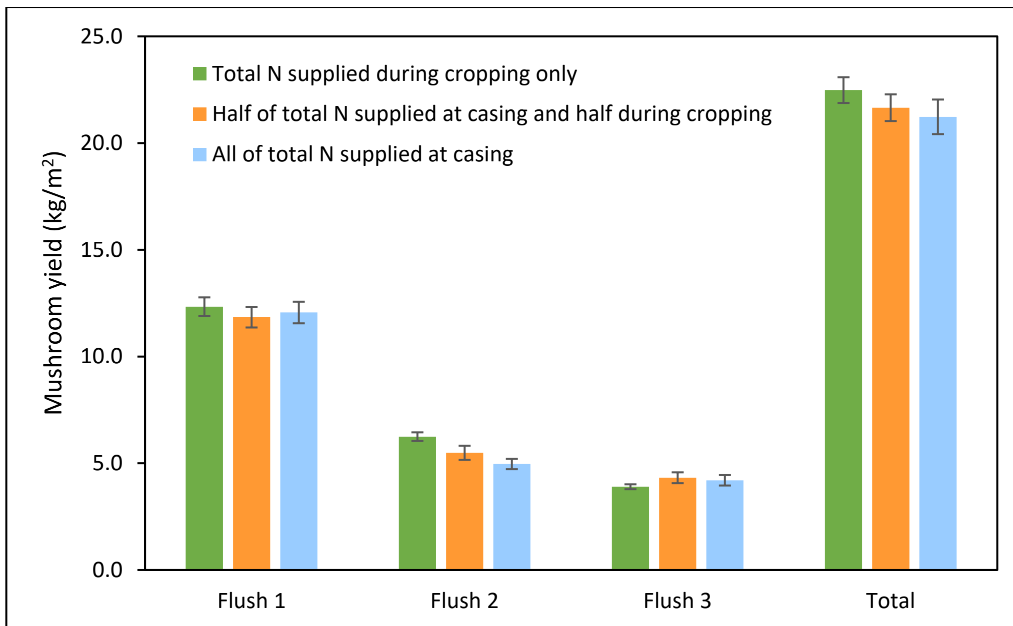

Figure 8.2.2. Mushroom yield for three flushes of small-scale supplementation. Supplements were added either at casing, immediately before each flush, or 50% of each, as shown. The supplements were provided on small scale (injection) with complex supplement, ammonium acetate (AA) or urea (U), and the values shown are the average for all three treatments. Total N supplied was identical for each treatment. Error bars are standard error (n=5).

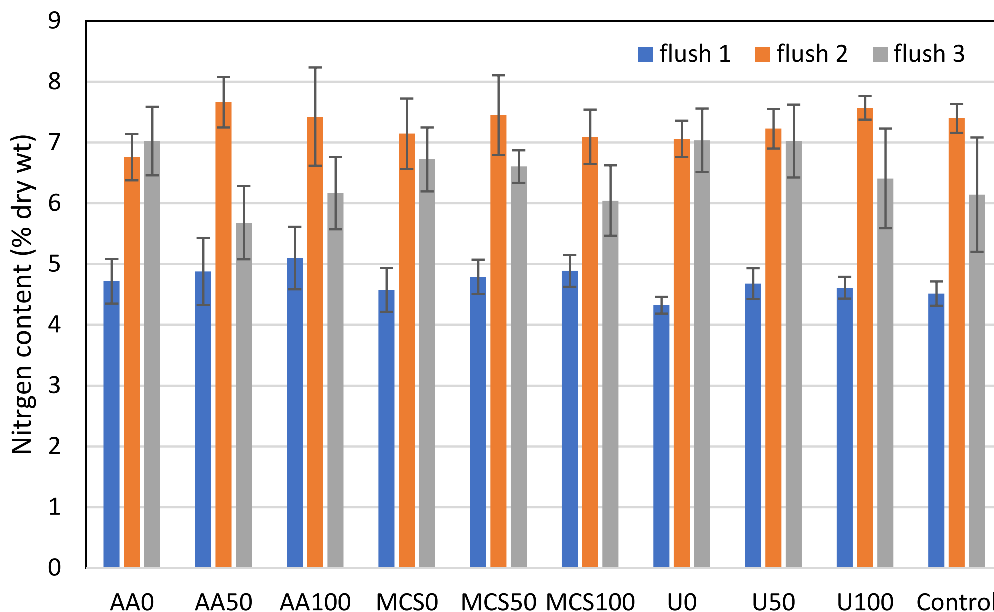

Figure 8.2.3. Nitrogen content of mushroom caps for three flushes of small-scale supplementation. The Phase 3 compost was supplemented on small scale (injection) with complex supplement (MCS), ammonium acetate (AA) or urea (U), or not supplemented (Control). Supplement was added either at casing (100), immediately before each flush (0), or 50% of each (50). Total N supplied was identical for each treatment. Error bars are standard error (n=5)

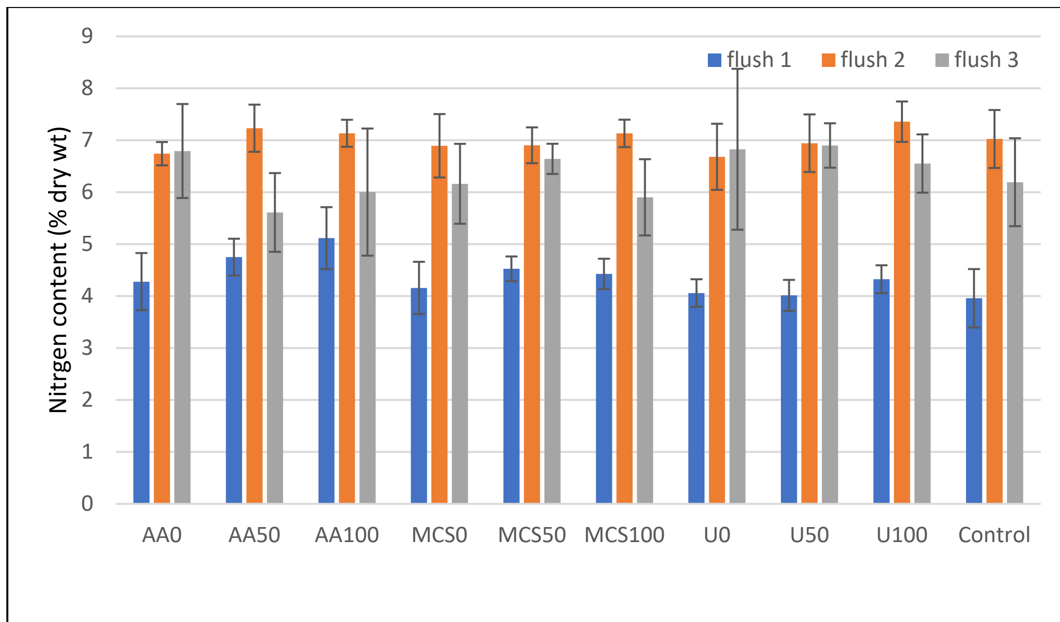

Figure 8.2.4. Nitrogen content of mushroom stipes for three flushes of small-scale supplementation. The Phase 3 compost was supplemented on small scale (injection) with complex supplement (MCS), ammonium acetate (AA) or urea (U), or not supplemented (Control). Supplement was added either at casing (100), immediately before each flush (0), or 50% of each (50). Total N supplied was identical for each treatment. Error bars are standard error (n=5)

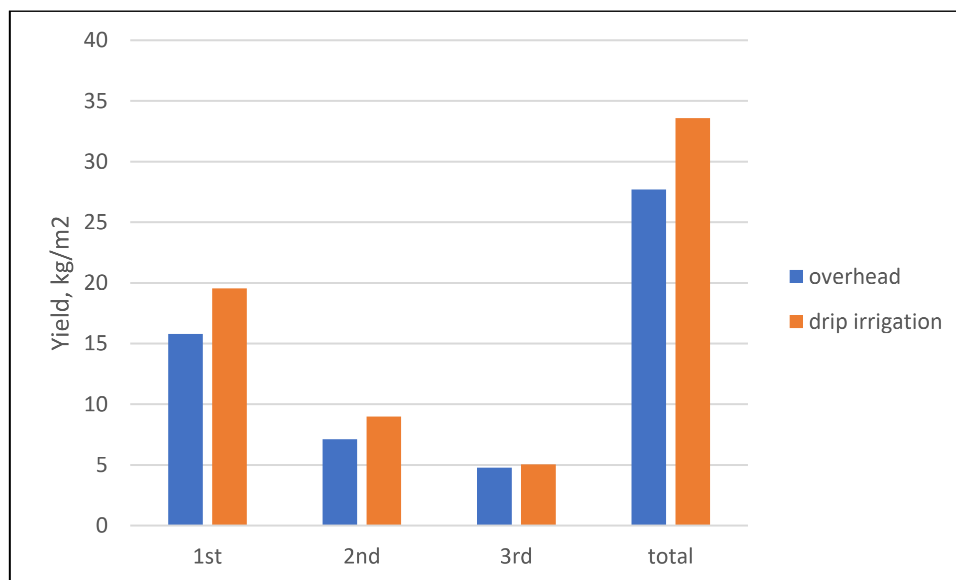

Figure 8.2.5. Yield of mushrooms obtained after overhead irrigation, compared with drip irrigation. Mushrooms were cultivated on 24 blocks of supplemented compost (20 kg) in the MLMRU. Data are average of duplicates.

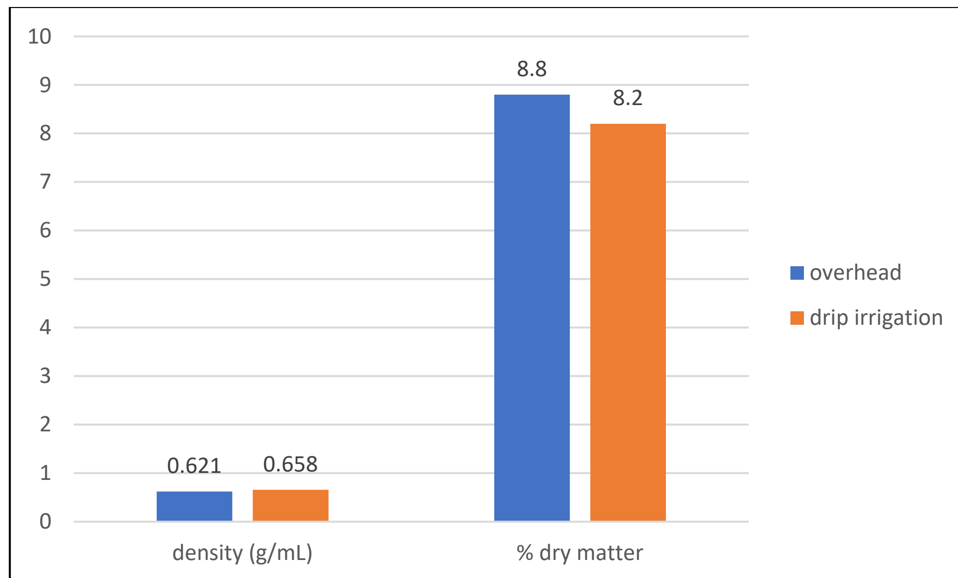

Figure 8.2.6. Quality of mushrooms obtained after overhead irrigation, compared with drip irrigation. Mushrooms were cultivated on 24 blocks of supplemented compost (20 kg) in the MLMRU. Data are average of duplicates.

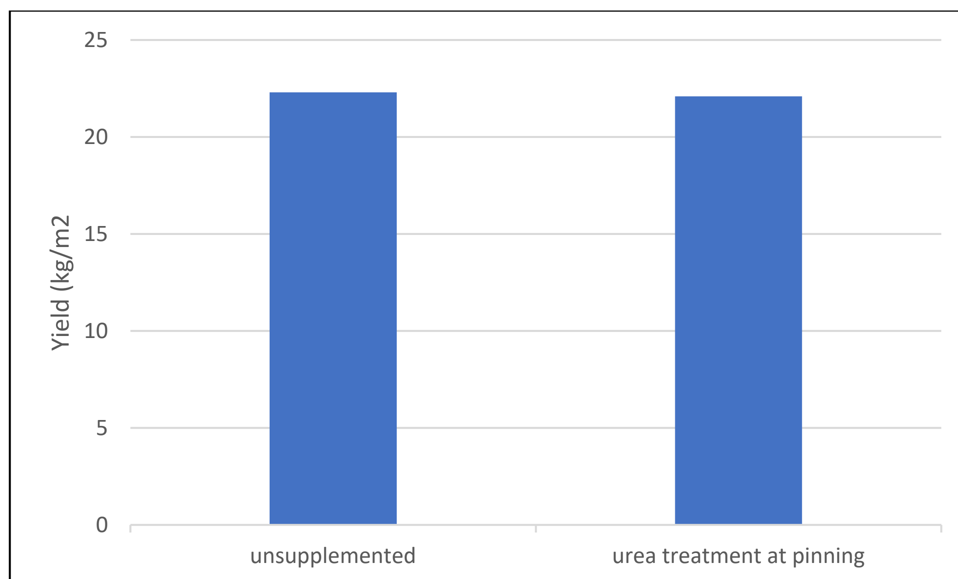

Figure 8.2.7. Yield of mushrooms obtained in first flush after treatment with urea at pinning. Mushrooms were cultivated on 24 blocks of unsupplemented compost (20 kg) in the MLMRU. Urea was applied through drip irrigation rubes at one-third the N-application rate of normal supplementation. The experiment was terminated after first flush due to a *Trichoderma* outbreak.

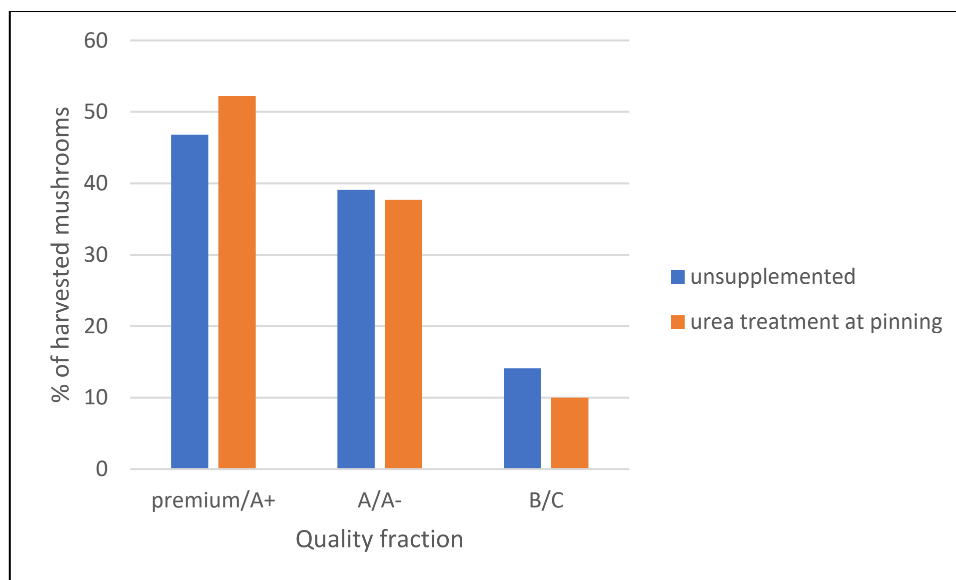

Figure 8.2.8. Quality of mushrooms obtained in first flush after treatment with urea at pinning. Mushrooms were cultivated on 24 blocks of unsupplemented compost (20 kg) in the MLMRU. Urea was applied through drip irrigation rubes at one-third the N-application rate of commercial supplementation. The experiment was terminated after first flush due to a *Trichoderma* outbreak. Quality designations are Premium, A+, A, A-, B and C.

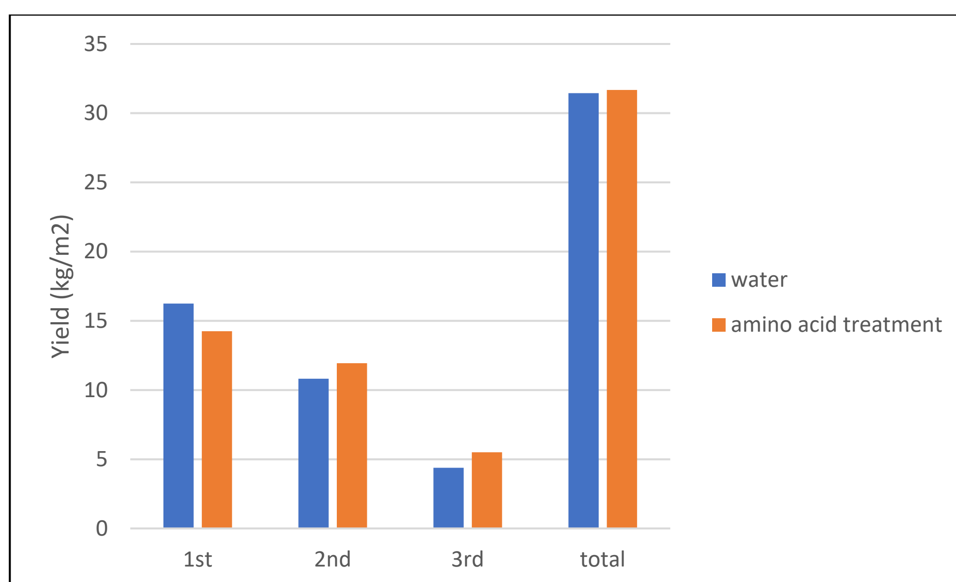

Figure 8.2.9. Yield of mushrooms obtained from three flushes after treatment with amino acid supplement immediately before each flush. Mushrooms were cultivated on 24 blocks of unsupplemented compost (20 kg) in the MLMRU. Amino acid solution was applied through drip irrigation rubes, with one-third the total N applied before each flush. The total N provided was twice commercial rates. Data are average of duplicates.

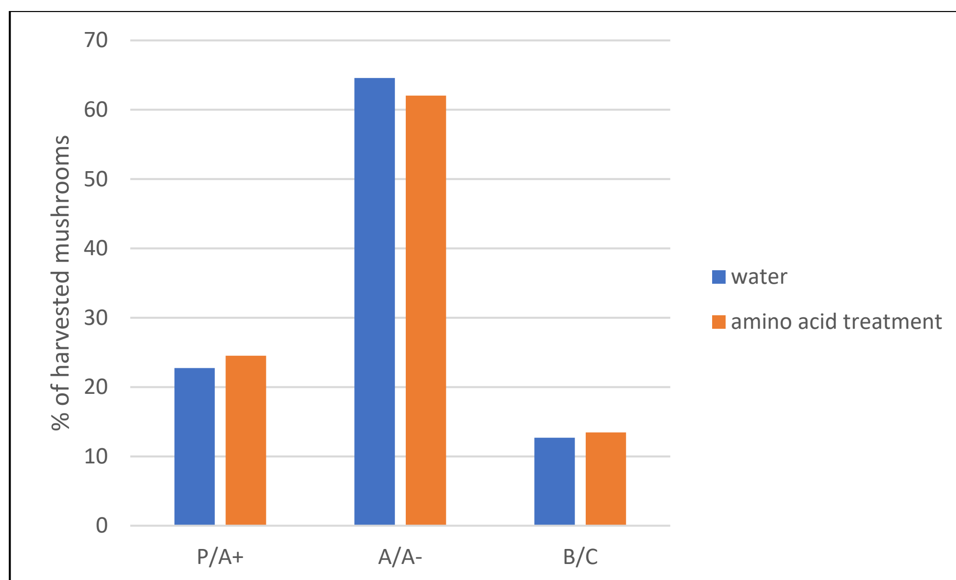

Figure 8.2.10. Quality of mushrooms obtained in first flush after treatment with amino acid supplement at pinning. Mushrooms were cultivated on 24 blocks of unsupplemented compost (20 kg) in the MLMRU. Amino acid solution was applied through drip irrigation rubes, with one-third the total N applied before each flush. The total N provided was twice commercial rates. Data are average of duplicates, quality designations are Premium, A+, A, A-, B and C.

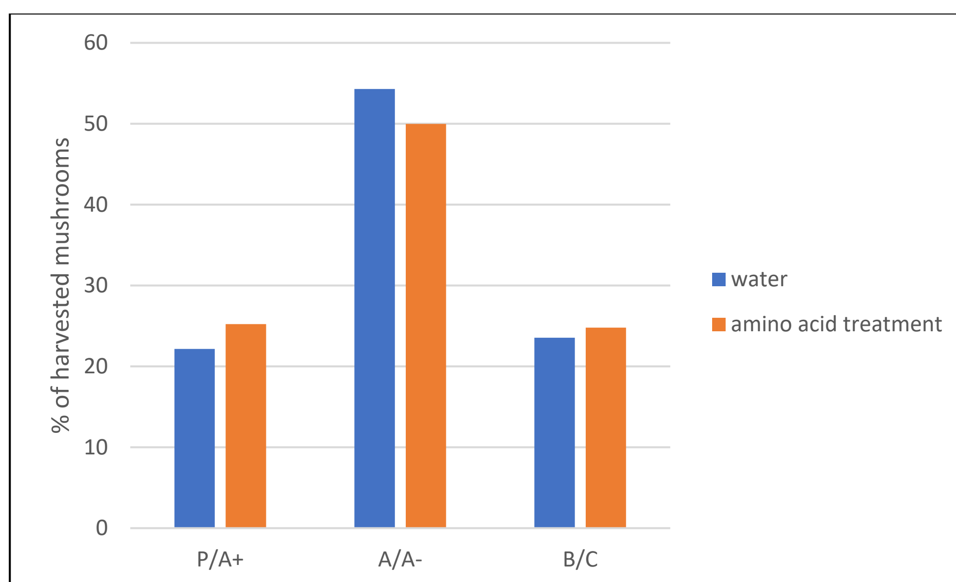

Figure 8.2.11. Average quality of mushrooms obtained from three flushes after treatment with amino acid supplement immediately before each flush. Mushrooms were cultivated on 24 blocks of unsupplemented compost (20 kg) in the MLMRU. Amino acid solution was applied through drip irrigation rubes, with one-third the total N applied before each flush. The total N provided was twice commercial rates. Data are average of duplicates, quality designations are Premium, A+, A, A-, B and C.

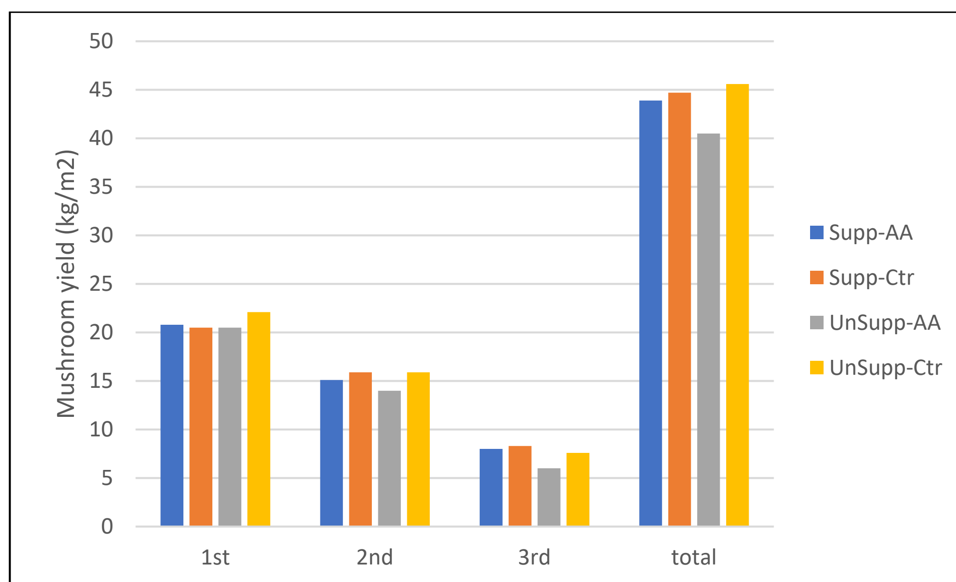

Figure 8.2.12. Yield of mushrooms obtained in second and third flush after treatment with amino acid supplement (AA) before each of these two flushes. Mushrooms were cultivated on 24 blocks of compost (20 kg) in the MLMRU, using either commercially supplemented compost (Supp) or compost without supplement (UnSupp). Amino acid solution was applied through drip irrigation rubes, with half the total N applied before each flush. Data are average of duplicates, quality designations are Premium, A+, A, A-, B and C.

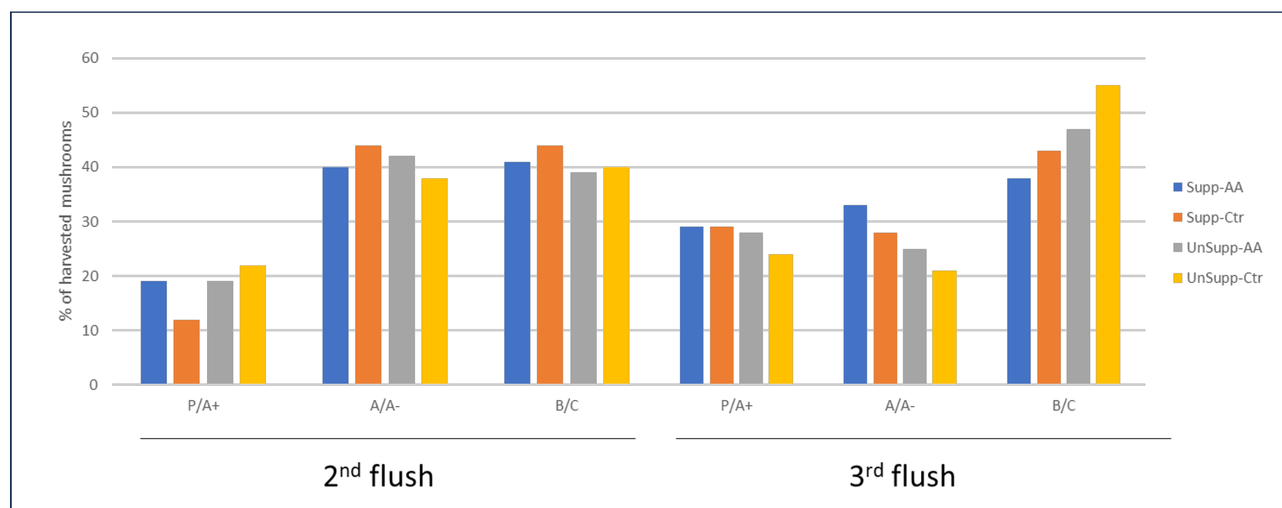

Figure 8.2.13. Quality of mushrooms obtained in second and third flush after treatment with amino acid supplement (AA) before each of these two flushes. Mushrooms were cultivated on 24 blocks of compost (20 kg) in the MLMRU, using either commercially supplemented compost (Supp) or compost without supplement (UnSupp). Amino acid solution was applied through drip irrigation tubes, with half the total N applied before flush 2 and flush 3. Data are average of duplicates, quality designations are Premium, A+, A, A-, B and C.

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Nitrogen balance and supply in Australasian mushroom composts.

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Australian and New Zealand mushroom farms grow around 60,000 tonnes of button mushrooms (*Agaricus bisporus*) annually (Food and agriculture organisation 2019). For this production, around 340,000 tonnes of Phase I compost are manufactured, which requires about 260,000 tonnes of raw materials (den Ouden 2016; Gerrits 1988). For the Australasian mushroom industry to maintain economic viability, the availability of suitable and cheap raw materials, and their efficient conversion into a high yielding substrate are critical. In recent years, there have been substantial changes in mushroom composting, with large, forced-aeration bunker systems with uniformly high temperatures and short composting times replacing slow traditional methods in turned windrows. The aim of this review is to re-examine which raw materials, particularly N sources, are available to the Australasian mushroom industry and how their utilization can be optimized in compost production.

Carbon and nitrogen sources in mushroom compost

Achieving the correct balance of carbon (C) and nitrogen (N) nitrogen sources in mushroom compost is important in achieving maximum yields. During composting, N is used by the compost microbiota to degrade some of the cellulose and hemicellulose in straw, the main C source in compost, into lignin and other high molecular weight polymers, making it a more selective nutrient source for the mushroom and less available for competitor micro-organisms(Fermor et al. 1985). Some of the N is converted by the compost microbiota into proteins, also a nutrient source for the mushroom(Wood and Fermor 1985). Compost formulations deficient in N are therefore less productive than formulations in which there is an adequate supply of N (Gerrits 1988; Noble and Gaze 1994; O'Donoghue 1965) although mushroom yield is unresponsive over a range of compost N contents, between about 2.1 and 2.7% of dry matter (DM) at the time of ‘spawning’, i.e. when mushroom inoculum or ‘spawn’ is added to pasteurized (Phase 2) compost ((Cormican and Staunton 1991); Noble unpublished data). Some loss in N during composting by ammonification is almost inevitable and indicates the availability to microbes of the N source. However, over-supply of N in compost formulations

results in excessive evolution of ammonia and nitrous oxides, and N losses (Noble et al. 2002). Ammonia is also toxic to the mushroom, and composts with a high ammonium-N content, generally above 0.15% of DM at spawning, are less productive ((Cormican and Staunton 1991); Noble unpublished data). This limits the amount of N that can be added into compost formulations for conversion into protein. To increase mushroom yields, additional proteins, normally in the form of soya-based supplements, are therefore usually added to the prepared composted substrate (Gerrits 1988) (see later section on compost supplements).

The description of mushroom compost formulations in terms of the C:N ratio is an over-simplification since it is the available C and N to micro-organisms that are important. However, for most compost ingredients, the total C and N are likely to give an indication of the available C and N (Gerrits 1977b). The optimum C:N ratio for the blended ingredients in a mushroom compost formulation is about 30:1, equivalent to an N (including ammonium-N) content of about 1.5% of dry matter (den Ouden 2016; Gerrits 1977b). At this level, the N losses as ammonia during the first stage or Phase I of composting are almost counterbalanced by the losses in C as carbon dioxide, so that the compost N content increases only slightly. At starting levels of N above 2%, more N is lost as ammonia than C as carbon dioxide, resulting in a decrease in compost N content during composting. When the starting level of compost N is below 1.5%, ammonia losses are small and the compost N content increases during composting (Gerrits 1977b; Gerrits 1988). Materials with N contents of more than 2% of dry matter can therefore be regarded primarily as N sources. Organic matter ingredients with N contents of less than 1% of dry matter can be regarded primarily as C sources, and those with intermediate N contents, such as horse manure, can also be regarded as significant or even sole sources of N.

Straw as a source of C and N

The main component and C source in mushroom composts in temperate regions is wheat straw, used fresh or in horse manure, which may contain proportions of other types of straw such as barley. Noble et al. (2006) found a great diversity in microbial available C in 84 wheat straw samples, even though total C content only ranged from 36 to 39%. For example, soluble carbohydrates varied between 3 and 19% and hemicellulose between 10 and 29%.

Wheat straw contains between 0.3 and 1.09% of N (Atkins 1974; Noble 2006) but it is unclear what contribution this makes to microbe available N during composting. For example, Noble et al. (2002) found that rape straw containing 1.2% N produced a similar amount of ammonia during composting to wheat straw containing 0.5% N when the same amounts of poultry manure were added to each. This indicates that much of the N in straw is unavailable to microbes during composting.

Rye straw degrades and performs similarly to wheat straw for production of mushroom compost (Gerrits 1988; Noble and Dobrovin-Pennington 2007) and in Asia, rice straw is used in place of wheat straw (Kim 1978; Noble et al. 2001). Oat and barley straw degrade more rapidly during composting than wheat straw (Gerrits 1988); this may necessitate shorter composting to avoid loss in structure and aeration. Noble and Gaze (1994) obtained significantly poorer mushroom yields from 'environmentally controlled' composts prepared from barley straw than from wheat straw although (Noble et al. 2002) subsequently obtained at least as good mushroom yields with compost prepared in bunkers from barley straw as from wheat straw. It is possible that the greater digestibility and availability of C in barley straw than in wheat straw may influence the optimum amounts and types of N that can be used for preparing mushroom substrates. Straw from sugarcane, rape, linseed, peas and beans, various grasses and corn cobs have also been used as C sources in mushroom compost formulations although complete replacement of wheat or similar straw has usually resulted in reduced mushroom yields (Noble et al. 1998; Poppe 2000).

To increase the availability of C to compost microbiota and thereby the rate and temperature of mushroom composting, the use of high energy C sources such as molasses were included in compost formulations (Hayes et al. 1969) leading to commercial compost 'activators' such as ADCO Sporavite (Noble et al. 1998). However, the addition of sugars to compost formulations did not shorten the time needed to clear ammonia from the compost or increase mushroom yields (Gerrits 1988). High compost temperatures, rapidly achieved in modern insulated and aerated bunker systems, have also made the use of such compost activators unnecessary.

Organic matter nitrogen sources

Extensive lists of raw materials for mushroom cultivation substrates were made by Stamets (2000) and Poppe (2000) although many of these materials are only available in tropical regions. N sources they listed that may be available in quantity in Australasia include: cow, pig and sheep manures, animal skin, hair, bone, dried blood and horn wastes, feathermeal, fish and shellfish residues, brewery and distillery wastes and grape, citrus and olive fruit wastes. Although Australia and New Zealand produce huge quantities of animal manures, much of this material is widely dispersed and remote from mushroom composting sites. Moisture and total N contents of materials (Table 1) give some indication to their value as an N source, although this will also depend on the microbe available N. An important consideration in the selection of materials is the value for alternative uses. Mushroom composts have been successfully prepared by

Table 1. Organic matter nitrogen sources for mushroom compost. Materials currently used or with potential for use in Australasia are in bold text

N Source	N	Dry matter	References
	% of DM	%	
Blood, dried meal	12.2	100	(Ministry of agriculture fisheries and food 1976)
Brewers' grains, dried	3.4	92	(Beyer and Beelman 1995)
Brewers' grains	3	24	(Noble and Dobrovin-Pennington 2007)
Canola/Rape seed meal	**3.3**	**85**	**(Stamets 2000)**
Cattle slurry	<2.8-10	10, <14	(Dawson 1978; Grabbe 1974)
Cocoa meal	4.2	93	(Noble et al. 2002)
Cottonseed meal	**6.5**	**92**	**(Stamets 2000)**
Cotton trash	**1.5**	**91**	**(Stamets 2000)**
Digestate, poultry manure	3.5	31.3	Noble (unpublished)
Feather meal	**4.9**	**67**	**(Ministry of agriculture fisheries and food 1976)**
Fish solubles	5	50	(Schisler and Patton 1974)
Glasshouse crop haulms	**1.8**	**11**	**(Noble 2005)**
Grape marc	**1.8-2**	**27-32**	**(Noble 2005; Pardo et al. 1995; Stamets 2000)**
Guano	8-15	>94	(Schnug et al. 2018)
Hop waste, dried	3.3	90	(Noble et al. 2002)
Horn meal	14.5	90	(Ministry of agriculture fisheries and food 1976)
Horse manure	**1.3**	**37**	**(Gerrits 1988)**
Malt sprouts, dried	4.3	92.6	(Stamets 2000)
Paunch grass	**3-3.5**	**15**	**(Environment protection authority 2017)**
Pig manure	1.9	23	(van Loon et al. 2004)
Poultry manure, caged	**1.5-4.7**	**25-67**	**(Ministry of agriculture fisheries and food 1976; Wiedemann et al. 2006)**
Poultry manure, broiler	**4.5-5.4**	**60-66**	**(Gerrits 1988; Noble and Gaze 1994; Wiedemann et al. 2015)**
Poultry manure, deep litter	**2.2-2.7**	**48-79**	**(Ministry of agriculture fisheries and food 1976; Wiedemann et al. 2006)**
Sea algae meal	0.7	32	(Ministry of agriculture fisheries and food 1976)
Soya bean meal	**7.1-7.4**	**91**	**(Stamets 2000)**
Sugarcane bagasse	0.2	19	(Kneebone and Mason 1972; Stamets 2000);
Vegetable wastes	**1.8**	**13**	**(Noble and Dobrovin-Pennington 2007)**
Wool waste	**14**	**90**	**(Noble and Dobrovin-Pennington 2007)**

incorporating blood meal, canola meal, cotton seed meal, guano, malt sprouts (Gerrits 1988; MacCanna 1969; Riethus 1962; Rinker 1991) and brewers' grains (Beyer and Beelman 1995; Rinker 1991) but they have a fertilizer or animal feed value. In several experiments, excess application of these materials in compost formulations resulted in poorer mushroom yields than moderate applications (Table 2).

Due to its moderately high N content, widespread availability and low alternative value, poultry manure has been a standard mushroom compost ingredient for many decades (Table 1). Australia produces over 1 million tonnes of poultry litter annually although the composition of the litter and its suitability for mushroom compost production depends on the type of poultry production and the bedding material (Gerrits 1988; Wiedemann et al. 2015; Wiedemann et al. 2006). Poultry manure with readily degradable bedding material such as straw is more suitable

for mushroom composting than manure with sawdust or wood shavings, which can encourage the growth of green moulds (den Ouden 2016). Due to lower moisture content and ease of handling and storage, broiler poultry manure is preferred, although there can still be large variability within similar sources. For example, Cormican and Staunton (1984) recorded a range in N content from <2.1 to >3.6% of DM within Irish sources of broiler manure. Manure from egg laying hens is also used in some countries (Cormican and Staunton 1984; Gerrits 1988), particularly where it is first made into a slurry. Ammonia suppressants are applied to the bedding by some poultry farmers to reduce injurious effects of ammonia on the birds. Although Beyer et al. (2000) found that using poultry manure containing a suppressant based on monocalcium phosphate slightly elevated ammonia levels during composting, it did not affect mushroom cropping performance. They, together with Gonzalez-Matute and Rinker (2006) also found that using poultry litter treated with a suppressant based on sodium hydrogen sulphate did not affect ammonia emissions during composting, compost N or subsequent mushroom cropping performance.

Where straw is the main C source in the compost, there is an optimum inclusion rate of poultry manure, depending on the analysis (Gerrits 1988; Noble and Gaze 1996). Where horse manure is the main C source in the compost, addition of excess poultry manure can readily lead to an over-supply of N and reduced mushroom yield (Gerrits 1989). However, researchers from the 1960s onwards have found that mushroom yields from horse manure composts could be improved by the addition of poultry manure and various other organic N sources, providing that this did not result in residual ammonia in the compost (Table 2).

Ross (1969) obtained mushroom yields comparable with those from horse manure composts using composts prepared from strawy bullock manure or pig slurry and straw. Grabbe (1974) replaced water with liquid cattle slurry in a horse manure-based Phase I but found no effect on mushroom yield. Dawson (1978) obtained mushroom yields at least comparable with straw and poultry manure compost when 70% of the poultry manure was replaced by an equivalent amount of N as cattle manure. Sugarcane bagasse and straw have been used to produce composts with comparable mushroom yields to those obtained from horse manure composts (Kneebone and Mason 1972; Peerally 1981). Digestate fibre from the anaerobic digestion of poultry manure (Table 2), food or crop wastes has been used in the production of mushroom substrates (Noble et al. 2002; Stoknes et al. 2008).

Table 2. Effect of compost organic nitrogen sources on mushroom yield in experiments conducted in medium- and large-scale facilities (>1 tonne compost)

N Source	Inclusion, %*		Yield, % of control		Control compost	Reference
	min	max	@ min	@ max		
Blood, dried meal	0.45-1.25	1.96-3.21	110-126	77-115	Horse manure	(Riethus 1962)
Blood, dried meal	0.27N	0.4N	141	146	Horse manure	(MacCanna 1969)
Brewers' grains	31.25		126		Horse manure	(Riethus 1962)
Brewers' grains	9.15		100		Horse manure	(Schisler and Patton 1974)
Canola meal	12 dm		106		Horse manure	(Rinker 1991)
Cattle slurry	10	40	100	83	Horse manure	(Grabbe 1974)
Cattle slurry	35dw		134		Straw, poultry manure	(Dawson 1978)
Cocoa meal	50N	100N	72	40	Straw, poultry manure	(Noble et al. 2002)
Cotton seed meal	2.5		128		Horse manure	(Bech and Riber Rasmussen 1969)
Cotton seed meal	0.14 N	0.20N	139	123	Horse manure	(MacCanna 1969)
Cotton seed meal	5		109		Horse manure	(Gerrits 1977b)
Digestate, poultry manure	60		88		Straw, poultry manure	Noble (unpublished)
Fish solubles	5	11.8	100	100	Horse manure	(Schisler and Patton 1974)
Grape marc	11		114		Straw, horse and poultry manures	(Pardo et al. 1995)
Guano	1.05-2.09	3.34	72-125	112	Horse manure	(Riethus 1962)
Hop waste, dried	100N		88		Straw, poultry manure	(Noble et al. 2002)
Horn meal	0.89	1.78	62-118	0-92	Horse manure	(Riethus 1962)
Pig manure	81	96	69	0	Straw, poultry manure	(van Loon et al. 2004)
Poultry manure, broiler	9		89-111		Horse manure	(Gerrits 1977b; Gerrits 1989)
Poultry manure, broiler	33	50	100	103	Straw, poultry manure	(Gerrits 1989)
Poultry manure, broiler	20	62.5	100	146	Straw, poultry manure	(Noble and Gaze 1996)
Sea algae meal	2.49	3.13	90	94	Horse manure	(Riethus 1962)
Sugar cane bagasse	100		100		Horse manure	(Kneebone and Mason 1972)
Vegetable wastes	25		105		Straw, poultry manure	(Noble and Dobrovin-Pennington 2007)
Wool waste	5		92		Straw, poultry manure	(Noble and Dobrovin-Pennington 2007)

* % w/w of fresh weight, dry matter (dm) or total nitrogen content of N sources (N)

(Noble et al. 2002; Noble et al. 2006) found that vegetable wastes, dried hop waste and brewers' grains released less ammonia during composting but produced similar mushroom yields to poultry manure when composted with wheat straw. Similarly, crop haulm and residues from glasshouse crops such as peppers and tomatoes contain moderate amounts of available N and could be used in mushroom compost formulations although their availability is seasonal (Noble 2005). Other organic materials (chipboard waste, cocoa meal and shells, wool waste and dried digestate fibre) which had total N contents above 2% of dry matter, only released small amounts of ammonia during composting and resulted in poor mushroom yields (Table 2). However, these materials may be suitable with longer composting periods to enable the release of N. Due to the low price of wool, this is now a significant by-product of the sheep industry, and it is used in the production of horticultural composts (Williams 2020). Paunch grass, the undigested contents of animal carcasses, is a by-product from abattoirs, has moderate N content but it is high in moisture (Table 1). It could be used in mushroom composting where high Phase I bunker temperatures would meet the regulatory requirements of animal waste disposal (Environment protection authority 2017).

Recycling of spent mushroom compost and green wastes into Phase I ingredients was examined by (Noble et al. 2006), reviewed by (Zied et al. 2020) and is being investigated in separate Hort Innovation Projects. Due to changes in the agricultural and food production sectors, an up-to-date inventory of the types, quantities and locations of by-products and wastes from these industries is required. The suitability of these materials for both conventional and organically approved mushroom production should be determined.

Inorganic nitrogen sources

Various chemical fertiliser or inorganic nitrogen sources, including urea, ammonium sulphate and nitrate, and calcium ammonium nitrate, cyanamide and nitrate, have been used in mushroom compost formulations (Table 3). Riber Rasmussen (1965) found an increase in mushroom yield resulting from an addition of 12 kg ammonium sulphate per tonne of compost, providing that this was accompanied by an addition of 35 kg of calcium carbonate. Similarly, MacCanna (1969) found that the addition of ammonium sulphate to Phase I compost to have a beneficial effect on mushroom yield providing it was combined with an application of calcium carbonate. (Gerrits 1977a) found no effect on mushroom yield of replacing a proportion of the poultry manure in compost with ammonium sulphate (without calcium carbonate); however, compost pH was slightly reduced. Ammonium sulphate is a by-product of sulphuric acid scrubbing of composting emissions before biofiltration, and therefore a cheap source of N. If combined with calcium carbonate, it obviates the need for gypsum in mushroom compost (Gerrits 1988; Riber Rasmussen 1965) (see below).

Urea is a more readily available form of nitrogen to compost microbes than ammonium sulphate, and results in a more rapid release of ammonia from compost (Noble et al. 2002). It can be added during the pre-wetting of raw materials where it is less likely to cause odour nuisance than poultry manure (Noble et al. 2002). Bech and Riber Rasmussen (1969) obtained poorer mushrooms yields from composts prepared with urea than with ammonium sulphate. Pardo et al. (1995) added a combination of urea (8.3 kg), ammonium sulphate (4.2 kg) and gypsum (38.9 kg) per tonne of straw and manure. Noble et al. (2002) found that replacing poultry manure with an equivalent amount of N as urea or ammonium sulphate resulted in higher and lower N losses respectively during composting with wheat straw. Composts prepared with either inorganic N source providing 50-100% of the N (remaining N poultry manure) produced lower mushroom yields than poultry manure composts. In other experiments, excess application of inorganic N in compost formulations has usually produced poorer mushroom yields than application of moderate amounts (Table 3).

Table 3. Effect of compost inorganic nitrogen sources on mushroom yield in experiments conducted in medium- and large-scale facilities (>1 tonne compost)

N Source	N	Inclusion, %*		Yield, % of control		Control compost	Reference
	% w/w	min	max	@ min	@ max		
Ammonium nitrate	35	0.37	0.72	99-116	113-129	Horse manure	(Riethus 1962)
Ammonium sulphate	21.2	0.12		136		Horse and poultry manures	(Riber Rasmussen 1965)
Ammonium sulphate		0.27N	0.40N	134	117	Horse manure	(MacCanna 1969)
Ammonium sulphate		0.4		100-105		Horse and poultry manures	(Gerrits 1977a)
Ca ammonium nitrate	27	0.7		108		Horse manure	(Gerrits 1977b)
Calcium nitrate	17	0.27N	0.40N	107	86	Horse manure	(MacCanna 1969)
Urea	46.7	0.17-0.66	0.83	58-112	12	Horse manure	(Riethus 1962)
Urea		0.57		75		Horse manure	(Bech and Riber Rasmussen 1969)
Urea		0.35		100 - 105		Horse manure	(Gerrits 1977b)
Urea		50N		79		Straw, poultry manure	(Noble et al. 2002)
Urea formaldehyde	38.9	0.68		49		Horse manure	(Bech and Riber Rasmussen 1969)
Urea formaldehyde		0.27N	0.40N	107	105	Horse manure	(MacCanna 1969)

* % w/w of fresh weight or total nitrogen content of N sources (N)

Recycled water

Recycled or 'goody' water can be a significant source of compost N if it constitutes a high proportion of the water added during pre-wetting of raw materials. Goody water composition reflects the compost ingredients, wetting and composting procedures and rainfall on compost yards. Goody water samples collected from the storage tanks or pits of 14 mushroom composting sites in Britain and Ireland contained between 3.2 and 6.4 mg N/L, mainly in the form of urea, ammonium N, P-serine and other amino acids (Noble et al. 2006).

Influence of gypsum on compost nitrogen

Gypsum was originally added to mushroom compost to improve the physical structure by flocculating colloids and preventing greasiness and anaerobic conditions; this function has been made unnecessary by shorter and aerated composting systems (Fermor et al. 1985). However, Gerrits (1977a) obtained poorer mushroom yields when gypsum was omitted from compost compared with compost where gypsum was added at 25 kg per tonne. He attributed the effect of gypsum on a reduction in compost pH and a reduction of dissociation of ammonium N into ammonia. Noble (unpublished) obtained normal mushroom yields of 300 kg/t pasteurised compost when gypsum was added at 25 kg/tonne to a straw and poultry manure compost, whereas no mushrooms grew on the same compost without the addition of gypsum. Although Riber Rasmussen (1965) found no effect of adding gypsum to compost, his formulation included both ammonium sulphate and calcium carbonate which would react to form gypsum. Beyer and Beelman (1995) found no effects on compost pH or mushroom yield from increasing the rate of gypsum inclusion from 28 to 84 kg/tonne compost.

The above work indicates that the beneficial effect of gypsum on mushroom compost is due to the sulphate ions reacting with ammonia to form ammonium sulphate, thereby stabilizing compost ammonium N. This effect is partially counteracted by the calcium ion content of gypsum, which would tend to increase compost pH and unstabilise compost ammonium N. Mushrooms do not have a significant calcium requirement (Gerrits 1988) and there is an abundance of calcium from the lime content of the casing material which is used to cover the compost to induce sporophore production. It may therefore be more effective to add dilute sulphuric acid to compost

to stabilize the ammonium N, a technique which is used to remove ammonia from composting emissions before biofiltration. The cost of sulphate ions in sulphuric acid is significantly less than in gypsum, although the cost and safety of spray application of acid would also need to be considered.

Compost supplements

The addition of protein containing supplements to mushroom-colonized or 'spawn-run' (Phase 3) compost to increase mushroom yields and quality is now practiced on most mushroom farms. The benefits are greater than with adding supplements to pasteurized (Phase 2) compost at spawning, where there is more competition for nutrients from other micro-organisms. The mushroom yield benefit of adding supplements increases with 'meagre' composts with low N content, although yields are still increased from composts made with an 'adequate' N supply by the addition of supplements (Gerrits 1988; Noble and Gaze 1994). This indicates that the availability and/or type of protein in such composts is still sub-optimal and restricted by the amount of ammonium-N which can be present in the compost formulation. A wide range of materials of plant and animal origin have been tested for use as supplements. Seed meals and processed products, particularly from cottonseed and soya bean, generally give the best results with performance related to crude protein content (Gerrits 1988; Randle 1985). To reduce the availability of nutrients to competitor molds and a surge in compost temperatures, the substances are usually treated with formaldehyde and/or coated to reduce the immediate availability of protein. Commercial supplements are based on formaldehyde-treated soya bean meal and other biological by-products and are added to Phase 3 compost at 0.5 to 1.6% w/w, with expected mushroom yield increases of 10-30% (Burton and Noble 2015; den Ouden 2016; Gerrits 1988). Randle and Smith (1986) calculated that a mushroom yield increase of at least 10%, without a change in quality, was required to justify the cost of compost supplementation using such materials. A more recent estimate Burton and Noble (2015) put the typical gross value of the additional mushrooms harvested at six times the cost of the supplement, although this did not include the costs of applying the supplement or of harvesting and marketing the extra mushrooms.

Compost nitrogen sources in Australasia

Phase I mushroom compost is produced on around 12 composting yards across Australia and 4 in New Zealand, each site producing between 60 and 1800 tonnes each week. The composts are based on wheat straw as the main C source (Table 4), unlike many Phase I composts in Europe which are partially or entirely based on horse manure and may include other types of straw such as barley, rye and oilseed rape. Phase I compost N contents are typically 1.8 to 2.2% w/w of dry matter (Table 4) which is predominantly supplied by poultry manure. Due to a decline in the rice crop in Australia, broiler poultry bedding material based on degradable rice husks is being replaced by wood shavings, leading to more composting yards using layer hen manure (Wiedemann et al. 2006), and increasing the need for alternative N sources. Other inorganic and organic N sources are used on some composting yards to replace up to 6% and 20% respectively of the N supplied by the poultry manures. To wet the composts, all the composting yards supplement fresh water with at least 50% recycled water.

Table 4. Current formulations used on some Australasian mushroom composting yards, and typical Phase I compost analyses. All sites use wheat straw at 54-61 % w/w of the raw materials and add gypsum at 25-30 kg/t compost.

Parameter	Compost Yard					
	A	B	C	D	E	F
Horse manure	0	0	✓	0	0	0
Poultry manure, laying	✓	✓	✓	✓	0	0
Poultry manure, broiler	0	✓	✓	0	✓	✓
Feather meal	0	0	✓	0	0	0
Canola meal	0	✓	0	0	0	✓
Cotton trash	0	✓	0	0	0	0
Cottonseed meal	0	0	✓	0	0	0
Soya bean meal	✓	0	0	0	0	0
Urea	✓	0	✓	0	✓	0
Ammonium sulphate	0	0	0	✓	0	0
Recycled water, %	50	✓	100	50	✓	90
Dry matter, % w/w	27.0	26.0	24.6	27.0	25.0	26.2
N (g kg^{-1}dry matter)	17.0	18.5	22.4	18.0	22.0	17.6
NH_4^+ (g kg^{-1}dry matter)	n.d.	4.0	4.0	2.0	4.5	1.8
Ash (g kg^{-1}dry matter)	272.4	n.d.	193.9	225.0	n.d.	103.0
pH	8.05	n.d.	8.26	8.25	8.3	8.1

n.d. not determined

Based on the average tonnages of Phase I compost produced and of raw materials, moisture and N contents of the composts (Table 4), and typical moisture and N contents of the raw materials (Table 1), and a dry matter loss of 30% during Phase I composting (den Ouden 2016; Gerrits 1988), the recycled water would account for less than 5% of the N added to the compost formulations. However, this figure would need to be verified with actual measurements of applied water volumes, dry matter losses, N losses due to leaching and ammonification during Phase I and recycled water N content.

The likely suitability of new organic matter N sources for mushroom composting in Australasia depends on the following:

- success of similar materials in mushroom cultivation tests in other countries
- content of microbe available N on a weight and bulk volume basis
- ease of collection and transport from supply to composting yards
- year-round availability and/or storage capability and requirements
- uniformity and absence of physical and chemical contaminants
- wide available and low/zero alternative competitor value such as animal feed and fertiliser.

Organic N sources which are currently used to replace broiler poultry manure in Australasia are highlighted in Table 1. Materials with high moisture contents such as crop haulms, vegetable wastes, grape marc and paunch grass would only be viable if the sources are close to the composting yards, and would require readjustment of the water applications made to the compost. Wool wastes could be used but would need longer composting processes to enable the N content to become available.

The currently most used N source, poultry manure, even if used at an optimum inclusion rate, does not obviate the need for protein supplements in the prepared substrate to increase mushroom yields. The use of alternative N sources to poultry manure, or sulphuric acid to replace gypsum, may enable the compost protein content to be increased without damaging levels of ammonia to be formed. Around 3,000 tonnes of soya protein-based compost supplements are imported by the Australasian mushroom industry annually. This may offer an opportunity for locally produced supplements based on alternatives to soya, depending on the availability and suitability of by-product protein sources.

Conclusions

1. Wheat straw and poultry manure are the main C and N sources in mushroom compost formulations in Australasia, unlike in Europe where many composting yards use horse manure.
2. The reduced availability of rice husks in Australia has forced broiler poultry farmers to use wood shavings for bedding, making the manure less suitable for mushroom compost; this has led to increased use of layer hen manure and increased the need for alternative N sources in compost formulations.
3. Low competitor value, ease of transport to composting sites, analytical uniformity of batches and performance at least comparable with poultry manure are critical in determining the economic viability of N sources.
4. Substitution of N in wheat straw:poultry manure composts is more challenging than in horse manure composts.
5. Up to 20% of the poultry manure addition is substituted with other organic N sources on some composting yards but a wide range of other potential organic matter C and N sources are also available for compost production in Australia and New Zealand; these may require readjustment of the composting process.
6. A proportion of poultry manure can also be replaced by inorganic N sources such as urea and ammonium sulphate but replacing 50% or more is likely to result in a reduction in mushroom yield.
7. The N content of different types and sources of straw can vary but much of this N is probably unavailable to microbes during mushroom composting.
8. Gypsum (calcium sulphate) is added to mushroom compost formulations as a supply of sulphate ions to reduce the dissociation of ammonium-N into ammonia. It may be more effective, and cheaper, to apply ammonium sulphate by-product from an ammonia acid-scrubber (with or without calcium carbonate) or dilute sulphuric acid. This may also enable composts with higher N and protein contents to be produced, thereby obviating, or reducing the need for soya protein-based supplements.
9. An up-to-date inventory of the types, quantities, and localities of by-products from the Australasian agricultural and food production sectors, as potential compost C and N sources is required.

10. Similarly, the availability of locally sourced protein-containing by-products in Australasia should be examined as alternatives to imported soya-based compost supplements.

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OPTIMISATION OF NITROGEN USE IN MUSHROOM PRODUCTION

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Button mushrooms are a nutritious (and delicious!) food source, containing high levels of minerals, vitamins, antioxidants and protein. Although they are sometimes referred to as "meat for vegetarians", mushrooms actually contain slightly lower levels of protein than animal meats, though they rank above most other vegetables in this respect.

Mushrooms normally contain 19-35% protein per gram of dry weight, as compared to 7.3% in rice, 13.2% in wheat, 39.1% in soybean, and 25.2% in milk (Chang and Miles, 2004). These mushroom-derived proteins are also particularly good for us, and their high content of essential amino acids means that button mushrooms are 90-98% as nutritious as most meats, and much more valuable than other vegetables.

NITROGEN INPUTS AND LOSSES DURING THE PRODUCTION PROCESS

So, where does the nitrogen come from that is needed to make these proteins? Button mushrooms are cultivated on a specialised mushroom compost, and the nitrogen present in the mushroom cap on the supermarket shelf is derived entirely from this compost. Wheat straw is the main ingredient of compost, but it only contains small amounts of nitrogen.

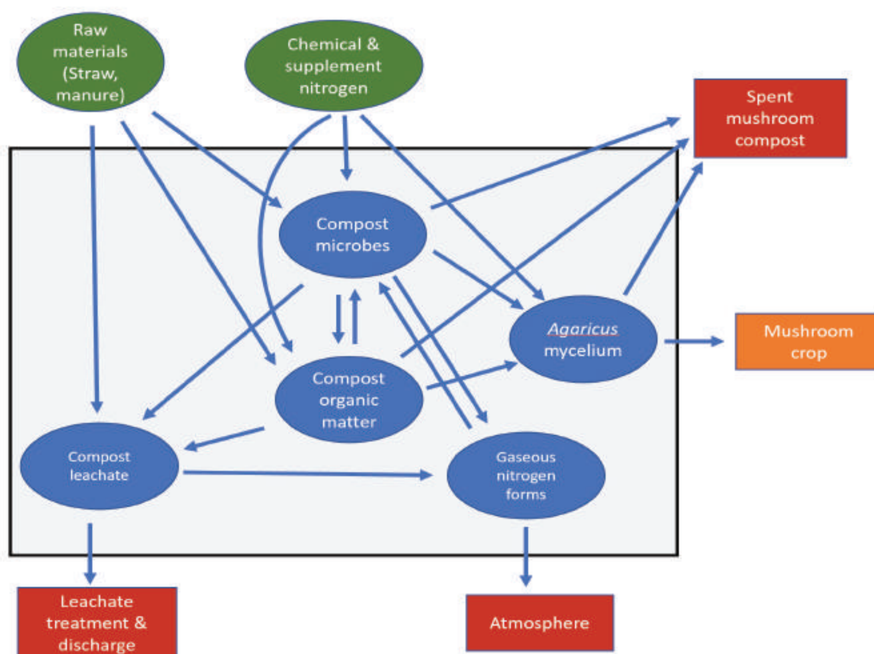
Composters therefore add nitrogen to the mix as manure (usually poultry manure), as chemical fertilisers such as urea, ammonium sulfate or ammonium nitrate, or as other nitrogenous products such as soybean meal. These can be added at the start of composting, or as supplements later in the process, commonly during spawn run or at casing.

Modern poultry farming practices have reduced the nitrogen content of poultry manure, and potential replacements are needed that meet food safety, cost and availability requirements (Noble *et al.*, 2002). The carbon:nitrogen ratio in the starting compost mix is usually set to between 30:1 and 35:1, and achieving this ratio is quite important, since this is optimal for growth of the microbes that carry out the conversion of the straw into compost (Stamets and Chilton, 1983).

Most of these microbes (bacteria and fungi) need the added nitrogen for the same reasons that people do – they use it to build the proteins they need for growth and cell proliferation. Their growth increases the number of active microbial cells in the compost, accelerating the conversion of wheat straw into more productive compost.

However, as the compost heats up, other processes come into play. Chemical reactions cause the breakdown of proteins in the compost, and the nitrogen in these proteins is released as ammonia gas. In many compost yards this ammonia is largely lost to the atmosphere, though some composting facilities collect it from the off-gas and use it to supply nitrogen to the next compost crop.

However, much of the ammonia is reabsorbed into the compost by a range of specialised bacteria and fungi (especially *Mycothermus* – previously called *Scytalidium*) that use it as their nitrogen source for growth during the conditioning (or curing) phase. This phase is necessary because ammonia gas is toxic to *Agaricus* and needs to be removed before the *Agaricus* spawn



NITROGEN TRANSFORMATIONS IN MUSHROOM PRODUCTION – WHAT IS IN THE BLACK BOX?

Nitrogen is supplied to the mushroom production process as manure and chemical nitrogen, and as supplements (green boxes). Significant losses occur as gases (ammonia, nitrous oxide), leachate, and in spent mushroom compost (red boxes). Arrows within the black box indicate processes that are not yet understood or fully quantified. The current project will investigate these processes in order to optimise the yield and nutritional value of the crop (orange box), while minimising losses and optimising inputs.

can be added. As well as taking up the ammonia to build their own cell protein, many of these microbes also use ammonia for respiration – they "breathe" it in a similar way that humans breathe oxygen, though they then release nitrate or nitrous oxide gas instead of carbon dioxide. The previous work has shown that up to 25% of the bacteria present in conditioning phase compost are able to do this, and much of the added nitrogen is therefore potentially lost from the compost in gaseous form. Remarkably, an exact balance of the nitrogen inputs, outputs and transformations in mushroom production (as shown in the Figure) has not yet been done, so it is not yet known how this is influenced by the form and timing of nitrogen addition, or by other aspects of process management.

At the end of the conditioning phase, the carbon:nitrogen ratio in the compost has dropped to about 17:1, which is ideal for growth of the *Agaricus* mycelium. During the first part of spawn run *Agaricus* gets its nutrition mainly by absorbing the large amounts of microbial biomass present in the compost from the previous phase.

Green mould and other disease fungi are unable to do this, and mushroom compost is therefore highly selective for the button mushroom. However, since a lot of the nitrogen added at the start of the composting process has been lost by this stage, growers add nitrogen-rich supplements either during spawn run or at casing to assure productive spawn run and cropping.

These supplements are usually protein-rich soybean derivatives, and although they stimulate *Agaricus* growth, they can also provide nitrogen for growth of fungal weeds and pathogens. To prevent this they are commonly formulated as slow-release fertiliser or treated with biocides to inhibit disease growth (Carrasco *et al*., 2018).

OPTIMISING NITROGEN USE EFFICIENCY – THE CURRENT MUSHROOM LEVY PROJECT

The current research on nitrogen use in mushroom production is supported by Hort Innovation through the Mushroom Industry levy project *Optimise nitrogen transformations in Mushroom production* (MU17004).

For maximum nitrogen use efficiency in mushroom cultivation, composters/growers need to (a) minimise or optimise inputs of nitrogen during composting supplementation (green boxes in the Figure); (b) reduce losses of nitrogen in wastewater and gases (ammonia, nitrous oxide) and the amount of unused nitrogen discarded in spent compost (red boxes in the Figure); (c) optimise uptake of nitrogen into the crop, to create a nutritious, highly marketable product.

The project will seek to optimise this as follows.

1. A detailed survey will be undertaken of current variation in nitrogen use in the Australian mushroom industry, determining nitrogen input/output profiles for typical classes of Australian mushroom producers to determine which producers are most nitrogen-efficient. It is expected that major differences will be found between large and small facilities, between different process types (tunnel/bunker/windrow composters; tray/shelf/block farms) and potentially also between different geographic locations.

2. Potential alternative sources of nitrogen that can be used in Australian composting will be investigated. These alternative N sources will be selected in close consultation with the mushroom industry, and with Dr Ralph Noble, who has pioneered this type of substitution in the United Kingdom. Promising materials (largely agricultural by-products) will be selected based on nitrogen content, cost, and geographic availability for composters in different Australian States. The project is establishing a strain collection of compost bacteria and fungi isolated from a range of different Australian compost yards, and selected combinations of these microbes will be applied to optimise nitrogen release from the alternative substrates tested.

3. Nitrogen use by mushrooms during the cropping process will be investigated. The previous studies have shown that nitrate levels in casing increase during cropping. The study will examine how this nitrate affects crop yield and quality in later flushes, and how timing of supplementation in spawn run and cropping can be used to increase yield and productivity.

The results will be incorporated into an easy-to-read best-practice guide aimed at compost producers and mushroom growers, to provide guidance on how to optimise nitrogen use in mushroom growing. This will be linked to additional resources, including factsheets on improved compost nitrogen management techniques on commercial tray, shelf and block farms, a decision support tool to help compost producers and mushroom growers manage their nitrogen rates, forms and timing, and on-farm grower-hosted demonstration trials.

In order to be successful, this project relies on close exchange of information between the researchers and the mushroom industry. The project team has been in touch with quite a few composting facilities and growers in all Australian states to discuss this project. It would assist with the delivery of the project is farms could share details of the nitrogen management with the research team (on a confidential basis), and growers are encouraged to make contact to discuss this further.

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PUTTING NITROGEN IN MUSHROOM COMPOST: *TIME FOR A CHANGE?*

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Australian mushroom farms produce around 70,000 tonnes of button mushrooms (*Agaricus bisporus*) annually. In order to provide the carbon (C) and nitrogen (N) needed to grow these mushrooms, the industry, therefore, requires about 260,000 tonnes of suitable and cheap raw materials every year. These raw materials are mainly composed of straw and/or stable bedding, manure and gypsum, and their efficient conversion into a high yielding substrate is essential for commercial mushroom production.

Maximum yields are obtained by achieving the correct balance of C and N sources in the raw materials used for composting. These elements are an essential part of the final mushroom crop – about 40% of the dry weight of the mushroom cap is made up of C, and N is required to make the protein in a nutritious mushroom. However, C and N are also essential for the activity of the microbes that carry out the composting process and provide food for the mushroom mycelium.

Compost microbes need energy for growth, and this is provided by the C in the raw materials. More importantly, they use the N in the compost feedstocks to build the proteins in their own cells, which can then convert the cellulose and hemicellulose carbon in straw into forms suitable for mushroom growth. The biomass and high molecular weight humic polymers produced by these microbes provide a selective nutrient source that feeds the button mushroom mycelium but is largely inaccessible for competitor micro-organisms and moulds.

The optimum C:N ratio in a mushroom compost formulation is about 30:1, which is equivalent to an N-content of about 1.5% of dry matter. Most of the C and N becomes microbial biomass during composting, but some of the C is converted to carbon dioxide by microbial respiration, and part of the N content is released as ammonia.

At starting levels of N above 2%, more N is lost as ammonia than C as carbon dioxide, and compost N content therefore decreases during composting. When the starting level of compost N is below 1.5%, ammonia losses are small, and the proportion of N in compost therefore increases during composting.

Feedstocks with N contents of more than 2% of dry matter can consequently be regarded primarily as N sources, while ingredients with N contents of less than 1% of dry matter can be regarded primarily as C sources.

Over-supply of N in compost formulations results in excessive release of ammonia and nitrous oxides and is less productive. Since ammonia is toxic to the *Agaricus* mycelium, this limits the amount of N that can be added into compost formulations at the start of composting. However, mushroom yields can be increased by providing additional N in the form of soy-based protein supplements later in the process.

STRAW AS A SOURCE OF C AND N

The main component of mushroom composts in temperate regions is wheat straw, used fresh or in stable bedding, and this is also the main C source. Wheat straw contains 36 to 39% C, but only between 0.3 and 1.1% of N, and much of the N in straw is probably unavailable to microbes during composting. Rye straw performs similarly to wheat straw for mushroom compost production, and in

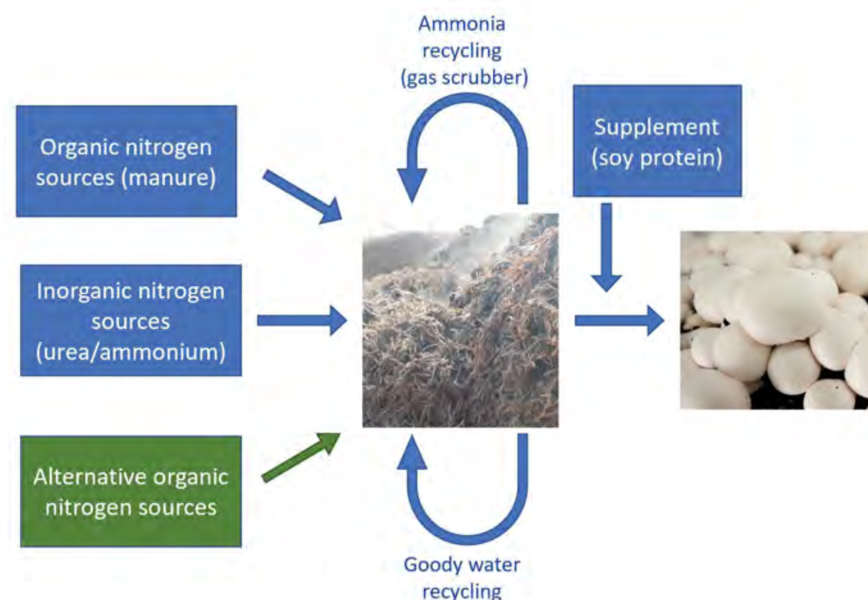

Figure 1. Nitrogen sources added to mushroom compost

Asia, rice straw is used in place of wheat straw. Oat and barley straw degrade more rapidly during composting than wheat straw, though mushroom yields with compost prepared in bunkers from barley straw are as good as those from wheat straw. Straw from sugarcane, canola, linseed, peas and beans, various grasses and corn cobs have also been used as C sources in mushroom compost formulations, but they are usually only part of the mix, since complete replacement of wheat or similar straw results in reduced mushroom yields.

ORGANIC SOURCES OF NITROGEN FOR COMPOSTING

Because straw contains insufficient N to sustain microbial growth in the compost, an additional N source is required. In Australia, this has traditionally been poultry manure, but there is increasing concern that the N content of this feedstock has decreased with changes to poultry farming methods and regulation, and other N sources are needed.

Extensive lists of alternative raw materials for mushroom cultivation substrates have been made by Stamets (2000) and Poppe (2000), although many of these materials are only available in tropical regions (Table 1). N sources that may be available in quantity in Australasia include cow, pig and sheep manures, animal skin, hair, bone, dried blood and horn wastes, feather meal, fish and shellfish residues, brewery and distillery wastes and grape, citrus and olive fruit wastes.

Mushroom composts have also been successfully prepared by incorporating blood meal, canola meal, cottonseed meal, guano, malt sprouts and brewers' grains (Table 1), but these are less available to composters and may be more expensive since they have alternative value as fertiliser or animal feed. In several experiments, excess application of these materials in compost formulations resulted in poorer mushroom yields than moderate applications.

Australia and New Zealand produce huge quantities of manures (especially cow and sheep), but much of this material is widely dispersed and remote from mushroom composting sites. Poultry manure, by contrast, has widespread availability, moderately high N content and low alternative value and has been a standard mushroom compost ingredient for many decades.

N Source	N-content (% dry weight)	Moisture content (%)	Amount used** (% w/w or % N)	Mushroom yield (% of control)
Blood, dried meal	12.2	0	0.45-3.21	77-115
Brewers' grains (dried)	3.7	8	31	126
Canola meal	3.9	15	14	106
Cattle slurry	28-100	90	10-40	100-83
Cocoa meal	4.5	8	50%N	72-40
Cotton trash*	1.6	9		
Cotton seed meal	7.1	8	2.5 - 5	109-128
Digestate, poultry manure	11.2	69	60	88
Feather meal*	7.3	33		
Fish solubles	10	50	5-12	100
Glasshouse crop haulms*	16.4	89		
Grape marc	6-7	68-73	11	114
Guano	8-16	<6	1.1-3.3	72-125
Hop waste, dried	3.7	10	100%N	88
Horn meal	16.1	10	0.9-1.8	62-118
Malt sprouts, dried*	4.6	7		
Paunch grass	22.0	85		
Pig manure	8.3	77	81-96	69
Poultry manure, broiler	6.8-9	31-40	9-63	89-146
Sea algae meal	2.2	68	2.5-3.1	90-94
Sugar cane bagasse	1.1	81	100	100
Vegetable wastes	13.8	87	25	105
Wool waste	15.6	10	5	92

* Yield data not available for large scale tests with these materials as sole N source.
** % fresh weight or % contribution to total nitrogen content of compost ingredients

Table 1. Effect of organic compost nitrogen sources on mushroom yield. Materials currently used or with potential for use in Australasia are in bold text. Yield experiments were conducted in medium- and large-scale facilities (>1 tonne compost). Control composts were based on horse manure/straw/poultry manure.

Australia produces over 1 million tonnes of poultry litter annually, although the composition of the litter and its suitability for mushroom compost production depends on the type of poultry production and the bedding. Poultry manure with readily degradable bedding material such as straw is more suitable for mushroom composting than manure with sawdust or wood shavings, which can encourage the growth of green moulds. Due to lower moisture content and ease of handling and storage, broiler poultry manure is preferred. Ammonia suppressants are applied to the bedding by some poultry farmers, but these do not seem to affect mushroom cropping performance and have only a small effect on compost N or on ammonia emissions during composting.

Where straw is the main C source in the compost, there is an optimum inclusion rate of poultry manure, depending on the N content. Where stable bedding is the main C source in the compost, the addition of excess poultry manure can readily lead to an over-supply of N and reduced mushroom yield. However, researchers from the 1960s onwards have found that mushroom yields from horse manure composts could be improved by adding poultry manure and various other organic N sources, providing that this did not lead to residual ammonia in the compost.

Composts incorporating these other organic N sources can be at least as productive as composts that are made using only horse or poultry manure as available N sources. In early work, composts prepared from strawy bullock manure or pig slurry and straw gave mushroom yields comparable with those from horse manure composts, as did the use of liquid cattle slurry instead of water. In a straw/poultry manure compost, 70% of the poultry manure N could be replaced by an equivalent amount of cattle manure. Digestate fibre from the anaerobic digestion of poultry manure, food or crop wastes has been used in the production of mushroom substrates, and sugarcane bagasse/straw has also afforded good mushroom yields.

Vegetable wastes, dried hop waste and brewers' grains released less ammonia than poultry manure during composting but produced similar mushroom yields when composted with wheat straw. Crop stalks or stems and residues from glasshouse crops such as peppers and tomatoes contain moderate amounts of available N and can be used in mushroom compost formulations, although their availability is seasonal. Paunch grass (the undigested contents of animal carcasses from abattoirs) has moderate N content but is high in moisture and could be used in mushroom composting where high Phase I bunker temperatures meet the regulatory requirements of animal waste disposal.

Other organic materials tested (chipboard waste, cocoa meal and shells, wool waste and dried digestate fibre) have total N contents above 2% of dry matter but only release small amounts of ammonia during composting and result in poor mushroom yields (Table 1). However, these materials may be suitable with longer composting periods to enable the release of N.

N Source	N content [% w/w]	Amount used** [% w/w or %N]	Mushroom yield [% of control]*
Ammonium nitrate	35	0.37-0.72	99-129
Ammonium sulphate	21.2	0.12	136
Ammonium sulphate		27-40% N	134-117
Ammonium sulphate		0.4	100-105
Ca ammonium nitrate	27	0.7	108
Calcium nitrate	17	27-40% N	107-86
Urea	46.7	0.17-0.83	58-12
Urea		0.57	75
Urea		0.35	100 - 105
Urea		50% N	79
Urea formaldehyde	38.9	0.68	49
Urea formaldehyde		27-40% N	107-105

*In several cases, yields are reduced at higher levels of inorganic N source.
**% fresh weight or % contribution to total nitrogen content of compost ingredients

Table 2. Effect of compost inorganic nitrogen sources on mushroom yield in experiments conducted in medium- and large-scale facilities [>1 tonne compost]. Control composts were based on horse manure/straw/poultry manure.

Recycling of spent mushroom compost and green wastes into Phase I ingredients may provide an additional source of N. Recycled compost leachate ('goody' water) can also be a significant source of compost N if it constitutes a high proportion of the water added during pre-wetting of raw materials.

INORGANIC SOURCES OF NITROGEN FOR COMPOSTING

Various chemical fertiliser or inorganic N sources have been used in mushroom compost formulations, but the most common are ammonium sulphate and urea (Table 2). Ammonium sulphate is a by-product of sulphuric acid scrubbing of composting emissions before biofiltration, and therefore a cheap source of recycled N. It usually needs to be added together with calcium carbonate, removing the need for gypsum in mushroom compost. For the compost microbes, urea is a more readily available form than ammonium sulphate and results in a more rapid ammonia release from compost. It can be added during the pre-wetting of raw materials where it is less likely to cause odour nuisance than poultry manure.

Replacing poultry manure with an equivalent amount of N as urea or ammonium sulphate during composting with wheat straw results in higher N loss for urea and lower N loss with ammonium sulphate. Composts where either of these inorganic N sources replaced 50-100% of poultry manure N produced lower mushroom yields than poultry manure compost. Application of excess inorganic N in compost formulations usually produces poorer mushroom yields than application of moderate amounts (Table 2).

FEEDING THE MUSHROOM MYCELIUM - OTHER ASPECTS

In addition to straw and manure, gypsum (calcium sulphate) is the third major component in compost formulations, and it is also important in controlling N supply. Gypsum was originally added to mushroom compost to improve the physical structure and prevent greasiness. This function has been made largely unnecessary today by using shorter and more highly aerated composting systems.

However, gypsum is still needed to obtain good yields because it binds ammonia, stabilising the compost N as the less volatile ammonium sulphate. The effect is partially counteracted by gypsum's high calcium content, which increases compost pH and destabilises ammonium sulphate. It may therefore be more effective to add dilute sulphuric acid to compost. The cost of sulphuric acid is significantly less than gypsum, although the cost and safety of spray application of acid would also need to be considered.

Additional nitrogen can also be provided later in the composting process. Adding protein-containing supplements to 'spawn run' compost before casing increases mushroom yields and quality and is now practised on most mushroom farms. Less benefit is obtained when supplements are added to pasteurised (Phase 2) compost at spawning because there is more competition for nutrients from other micro-organisms at this stage.

The benefit of supplementation is highest for composts with low N content, but increased yields are also obtained with composts made with an 'adequate' N supply. This clearly demonstrates that even in good composts, mushroom nutrition is restricted by the amount of ammonium-N which can be present in the compost formulation.

A wide range of materials of plant and animal origin have been tested for use as supplements. Performance is related to crude protein content, and best results are generally obtained from seed meals and processed products, particularly from cottonseed and soy bean. Commercially available supplements are based on formaldehyde-treated soy bean meal and other biological by-products. These are added to Phase 3 compost at 0.5 to 1.6% w/w, with expected mushroom yield increases of 10-30%.

A mushroom yield increase of at least 10% is required to justify the cost of compost supplementation using such materials. The typical gross value of the additional mushrooms harvested has been estimated at six times the cost of the supplement, although this does not include the costs of applying the supplement or harvesting and marketing the extra mushrooms.

WAYS FORWARD FOR COMPOST NITROGEN IN AUSTRALIA

Phase I mushroom compost is produced on around 12 composting yards across Australia and four in New Zealand, with

each site producing between 60 and 1,800 tonnes per week. The composts are based on wheat straw as the main C source, unlike many Phase I composts in Europe, which are partially or entirely based on stable bedding and may include other types of straw such as barley, rye and oilseed rape.

Phase I compost N contents in Australia are typically 1.8 to 2.2% w/w of dry matter, and this N is predominantly supplied by poultry manure, with broiler poultry bedding material preferred. However, degradable rice husks are increasingly being replaced by wood shavings for poultry bedding, and compost yards are therefore increasingly using layer hen manure and replacing up to 6% and 20% of the poultry manure with alternative inorganic and organic N sources, respectively.

Organic N sources which are currently used or have the potential to replace broiler poultry manure in Australasia are highlighted in bold in Table 1. Materials with high moisture contents such as crop haulms, vegetable wastes, grape marc and paunch grass would only be viable if the sources are close to the composting yards and would require readjustment of the water applications made to the compost. Wool wastes could be used but need a longer composting process to enable the N content to become available. To wet the composts, most Australian composting yards supplement fresh water with at least 50% recycled water, but this accounts for less than 5% of the N added to the compost formulations.

In conclusion, wheat straw and poultry manure are the main C and N sources in mushroom compost formulations in Australasia. However, at least 20% of the poultry manure is already being replaced by other organic N sources on some composting yards. A wide range of other potential organic matter C and N sources are available for compost production in Australia and New Zealand. Their application may require readjustment of the composting process since substitution of N in wheat straw:poultry manure composts is generally more challenging than in horse manure composts.

To facilitate this process, there is an urgent need for an up-to-date inventory of the types, quantities, and localities of by-products from the Australasian agricultural and food production sectors. This will identify novel alternative N sources that have low competitor value, ease of transport to composting sites, analytical uniformity of batches and performance at least comparable with poultry manure.

FURTHER READING

An extended form of this article listing the sources of all the data cited is available on request from the authors.

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Criteria for selecting new Nitrogen sources for Australasian mushroom composting

- success of similar materials in mushroom cultivation tests in other countries.
- content of microbe-available N on a weight and bulk volume basis.
- ease of collection and transport from supply to composting yards.
- wide year-round availability and/or storage capability and requirements.
- uniformity and absence of physical and chemical contaminants.
- low/zero alternative competitor value as e.g. animal feed or fertiliser.

An up-to-date inventory of the types, quantities and locations of by-products and wastes from the agricultural and food production sectors is urgently required. The suitability of these materials for both conventional and organically approved mushroom production should be determined.

Hort Innovation
Strategic levy investment

MUSHROOM FUND

This project has been funded by Hort Innovation using the mushroom research and development levy and funds from the Australian Government. For more information on the fund and strategic levy investment visit horticulture.com.au

26th North American Mushroom Conference (NAMC), (26th - 29th February, 2024, Las Vegas). Invited keynote presentation (O'Neil lecture) by **M. A. Kertesz**. *The Microbial Matrix: Understanding the influence of microbes on the quality of mushroom compost*

Abstract.

The Microbial Matrix: Understanding the influence of microbes on the quality of mushroom compost
Michael A. Kertesz

In recent years, molecular research tools have provided a huge step forward in our understanding of the complex biological changes that occur in compost during compost production and mushroom cropping. This presentation will explore possible ways to translate this new knowledge into practical measures of compost quality that can be used in mushroom production.

20th International Society for Mushroom Science Congress (ISMS), (26th - 29th February, 2024, Las Vegas). Oral presentation by **M. Thai**. *Feeding the compost: Nitrogen supplementation during production of white button (Agaricus bisporus) mushrooms*

Abstract.

Feeding the compost: Nitrogen supplementation during production of white button (Agaricus bisporus) mushrooms

<u>Meghann Thai</u>

Nitrogen uptake by white button mushrooms from the growth substrate can vary from harvest flushes (or breaks) and substrate depth. Nitrogen is a critically important nutrient in mushroom production and is often lost as ammonia during composting. At cropping, nitrogen is added back into the compost using a delayed release soy-based supplement. Using this supplement results in excess nitrogen not being taken up directly by the mushroom and potentially being used by competitive microbial pathogens. During cropping, ammonium concentration has been shown to peak in the growth substrate before each flush and drop rapidly during the flush. Organic and inorganic nitrogen supplementation was directly applied to mushroom compost via needle injection, or in-line watering at the interphase of casing and compost, and was added either at casing, or at pin onset prior to each successive flush. There was an increase in total mushroom yield when N was supplemented throughout cropping, in comparison to N supplemented at casing. Nitrogen content in the mushrooms significantly increased for all flushes when N was applied at pinning prior to each flush, compared to when it was applied at casing. Therefore, applying nitrogen supplement directly to the compost during cropping at pinning for each flush could minimise the dependence on soy-based supplements, while also reducing nitrogen losses from the compost.

44th Australian Mushroom Growers' Conference, (26th - 28th October, 2022, Adelaide). Invited oral presentation by **M. Kertesz**. *What's new in mushroom compost biology?* Recording available at: https://www.youtube.com/watch?v=1cY80XVap7I).

Abstract.

Balancing nitrogen supply and demand in button mushroom composting and cropping

Michael A. Kertesz

Button mushrooms (*Agaricus bisporus*) are an excellent source of protein, containing many of the essential amino acids that humans need. To produce this protein, nitrogen inputs to composting need to be carefully managed. The main nitrogen sources used for Australian mushroom production are poultry manure, synthetic nitrogen, and smaller amounts of other N-containing feedstocks. There total amount of nitrogen added by Australian composters varies considerably, with overall inputs ranging from 6.5-80 kg N /tonne Phase 1 compost. Only about 12% of this nitrogen is recovered in the mushroom crop as protein – the remainder is lost in compost leachate or as gaseous nitrogen compounds, (20-30% of input N) or is discarded with compost/casing after cropping (50-60% of input N). Gaseous emissions consist mainly of ammonia from protein breakdown in the thermophilic phase and nitrous oxide from the activity of nitrifying bacteria during pasteurization and curing.

To increase mushroom cap production, most mushroom yards add a slow-release nitrogen supplement to the compost before cropping. We have tested the effect of replacing this treatment with the addition of different nitrogen sources directly into the compost at the pinning stage before each flush. Depending on the N source used, this increased yields by up to 13%, and led to a significant increase in mushroom protein content. More importantly, second or third flush mushrooms had a much higher N-content (6.7% dry weight) than first flush mushrooms (4.3%). The results demonstrate that optimization of the timing and amounts of nitrogen additions during cropping can improve yield and nutritional value of the crop.

2022 Australian Society for Microbiology Congress (11th – 14th July, 2022, Sydney) Invited oral presentation by **M. A. Kertesz**. *Using bacteria to grow mushrooms – the microbial ecology of mushroom compost.*

Abstract.

Using bacteria to grow mushrooms – the microbial ecology of mushroom compost.

Michael A. Kertesz

Mushrooms are a tasty part of a modern diet and a valuable source of dietary protein. Every year Australians eat nearly 70,000 tons of button mushrooms (*Agaricus bisporus)*, as well as smaller amounts of other fungal species such as oyster and shiitake. Button mushrooms are grown on a highly selective mushroom compost that is produced from recycled agricultural wastes (wheat straw and chicken manure). This controlled, industrial-scale microbial process is an accelerated version of the natural microbial decomposition of lignocelluloses, by which fungi and bacteria return nutrients from leaf litter to soils. The engineered process of mushroom composting includes a mesophilic wetting step, a thermophilic step and a mesophilic conditioning step, with each step characterized by a succession of typical microbial communities. The late thermophilic phase is dominated by *Thermus,* while the conditioning step is dominated by the ascomycete *Mycothermus thermophilus.* This fungus is associated with several specific bacterial species, including heterotrophic nitrifiers from the *Pseudoxanthomonas* and *Chelatococcus* genera. Their combined microbial biomass provides the initial food source for the *Agaricus* mycelium as it proliferates through the conditioned compost. Our work on the specific fungal and bacterial interactions that mediate mushroom composting aims to understand these interactions in detail, while also maximizing output and quality of the mushroom crop and addressing questions of nutrient retention, greenhouse gas release and water conservation.

19th International Society for Mushroom Science Congress (ISMS), (14th - 17th September, 2021, online e-congress). Invited keynote presentation by **M. Thai**. *Bacterial interactions with Mycothermus thermophilus (syn. Scytalidium thermophilum*). Recording available at: (https://www.isms.biz/Web/Library/Proceedings/eCongresses/eArticles-Members.aspx?doc=6)

Abstract.

Bacterial interactions with *Mycothermus thermophilus* (syn. *Scytalidium thermophilum*)

Meghann Thai, Tina L. Bell and Michael A. Kertesz

Australian button mushroom compost is made from wheat straw, chicken manure and gypsum, in a three step process comprising a mesophilic phase, a thermophilic phase, and a pasteurization/curing phase. During the pasteurization/curing phase of compost production, the microbial community was dominated by the fungus *Mycothermus thermophilus* and by three bacterial taxa: *Pseudoxanthomonas taiwanensis*, *Pseudoxanthomonas suwonensis,* and a novel chitin-degrading organism affiliated with the *Chitinophagaceae* family, strain CP21.6. The *Mycothermus* biomass in the pasteurized compost is known to provide essential nutrition for the *Agaricus* mycelium during spawn run, and so its interactions with compost bacteria are important to the compost curing process. Growth of the bacterial strains was unaffected by *M. thermophilus in vitro*, but growth of *Mycothermus* was reduced in the presence of *P. suwonensis*. However, the closely related *P. taiwanensis* did not impede *Mycothermus* growth, and since *P. taiwanensis* is the more thermotolerant of these two bacterial taxa, pasteurization conditions could be used to select against any inhibitory effect during compost production. The chitin degrading strain CP21.6, by contrast, completely inhibited growth of *M. thermophilus* in vitro and actively degraded the fungal hyphae. By degrading *M. thermophilus* on a cellular level it therefore plays an important vital role in nutrient cycling within the compost. It appears unlikely that strain CP21.6 also degrades the *Agaricus* mycelium directly, and as *Agaricus* is able to assimilate both living and dead *Mycothermus* biomass, the activity of strain CP21.6 against *Mycothermus* should not affect *Agaricus* nutrition. However, further study of these bacterial-fungal interactions is called for in order to more fully understand the composting process.

19th International Society for Mushroom Science Congress (ISMS), (14th - 17th September, 2021, online e-congress). Oral presentation by **M. A. Kertesz**. *Balancing nitrogen supply and demand in button mushroom composting and cropping*. Recording available at: (https://www.isms.biz/Web/Library/Proceedings/eCongresses/eArticles-Members.aspx?doc=84)

Abstract.

Balancing nitrogen supply and demand in button mushroom composting and cropping

Meghann Thai, Tina L. Bell and Michael A. Kertesz

Button mushrooms (*Agaricus bisporus*) contain high levels of protein (19-35% of dry weight) and also key minerals, vitamins and antioxidants for human nutrition. Balancing carbon and nitrogen supply is of critical importance in mushroom production, because growth of protein-rich mushrooms requires a consistent supply of nitrogen to the *Agaricus* mycelium. Here, we examined nitrogen management techniques and overall nitrogen balance in ten compost yards across south-eastern Australia, comparing a range of large and small, modern and more traditional facilities. The main initial nitrogen inputs to Australian mushroom production were poultry manure, synthetic nitrogen and other minor feedstocks added at the start of the composting process. Most, but not all, yards supplied additional soy-based nitrogen supplements later in the process, either at spawning or just before cropping (0.70 kg N/tonne Phase I compost). There was considerable variation in total nitrogen added, with overall inputs ranging from 6.5-80 kg N /tonne Phase 1 compost. The main nitrogen losses during composting were in compost leachate and in gaseous nitrogen compounds. Gaseous emissions consisted mainly of ammonia from protein breakdown in the thermophilic phase and nitrous oxide from the activity of nitrifying bacteria during pasteurization and curing. Leachate and gaseous nitrogen losses accounted for about 20-30% of the total input N, even though a considerable amount of leachate was reapplied to the compost as goody water, and most of the ammonia released was directly reassimilated by the thermophilic bacteria and fungi in the compost. The resulting microbial biomass provides a major nutrient source for the *Agaricus* mycelium during spawning. The harvested mushroom crop incorporated 12% of the input nitrogen, with marked differences in N content among individual flushes. About 60% of input nitrogen was still present in the discarded compost and casing, partly as nitrate, but mostly in the *Agaricus* mycelial biomass. Nitrate increased markedly in the casing during cropping and is not used as a nitrogen source by *Agaricus*. The results demonstrate the need to optimize the timing and amounts of nitrogen additions during composting. Aligning these nitrogen additions with microbial activities will minimise nutrient losses and maximize nitrogen yield in the mushroom crop.

25th North American Mushroom conference (NAMC) (14th – 16th February, 2019, Orlando, Florida). Invited oral presentation by **M. A. Kertesz**. *Compost microbes and nitrogen supply in mushroom production*.

Abstract.

Compost microbes and nitrogen supply in mushroom production

Michael A. Kertesz

Research using modern molecular techniques has provided a multifaceted and highly detailed view of the complex biological changes in compost and casing during compost production and mushroom cropping. Decreases in nitrogen content of currently available compost feedstocks are providing challenges to farmers, and new approaches are needed to minimize nitrogen losses during composting and maximize incorporation into the mushroom crop. This presentation will discuss microbial nitrogen transformations in compost and casing during compost production and cropping, and how these can be controlled to optimize the nitrogen use efficiency of the mushroom production process.

43rd Australian Mushroom Growers' Conference, (11th – 13th October, 2018, Sydney). Invited oral presentation by **M. Thai**. *Diversity and activity of bacteria in Australian compost yards*.

Abstract.

No abstract presented.

Poster presentations

- 2022 Australian Microbial Ecology Congress (AusME), (7th – 9th November, 2022, Melbourne). Poster presentation by **M. Thai**. *Feeding Mushrooms: A microbial conversation between bacteria and fungi.*
- 44th Australian Mushroom Growers' Conference, (26th - 28th October, 2022, Adelaide). Poster presentation by **K. T. Kuen, M. Thai & M.A. Kertesz**. *Feeding the compost – nitrogen supplementation during mushroom cropping*.
- 44th Australian Mushroom Growers' Conference, (26th - 28th October, 2022, Adelaide). Poster presentation by **S. Shamugam & M. A. Kertesz**. *Bacterial interactions with* Agaricus bisporus *mycelium*.
- 19th International Society for Mushroom Science Congress (ISMS), (14th - 17th September, 2021, online e-congress). Poster presentation by **M. Thai, M. A. Kertesz, T. Bell**. *Fungal and bacterial diversity in Australian mushroom compost*.

THE UNIVERSITY OF SYDNEY

Feeding Mushrooms: A microbial conversation between bacteria and fungi

Meghann Thai and Michael Kertesz

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Introduction

- Protein content in button mushrooms (*Agaricus bisporus*) depends on the quality of their growth substrate, a selective compost made from wheat straw, poultry manure and gypsum
- The microbial communities in mushroom compost change rapidly during the initial thermophilic stage (Phase I) of composting (80 °C)[1]; but
- Pasteurisation (60 °C) and curing (45 °C) (collectively known as Phase II) leads to a stable consortium of *Pseudoxanthomonas taiwanensis* and *Mycovorax composti*, and one dominant ascomycete fungus, *Mycothermus thermophilus*.[1]
- *Pseudoxanthomonas taiwanensis*, the dominant bacterium in Phase II mushroom compost, is a known heterotrophic nitrifier and can produce nitrous oxide (N_2O), from organic or inorganic N, under aerobic conditions.[2]
- Chitin is the structural polymer found in all fungal cell walls, and *Mycovorax composti*, a novel chitinolytic bacterium from the family *Chitinophagaceae*, is a strong producer of chitin degrading enzymes.

Microbial interactions

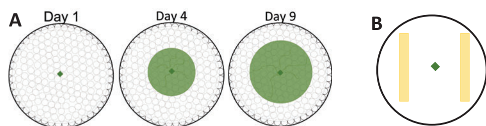


Figure 1. Microbial interactions in co-culture. A) liquid phase co-culture on glass beads plates.[3] Dark green shapes indicate *M. thermophilus* growth on a 45 µm mesh overlay (white translucent circle). B) Solid phase co-culture on solid growth medium. Bacterial streaks are indicated in yellow and *M. thermophilus* is indicated by the green square.

Interactions between *M. thermophilus* and *M. composti* were grown in liquid co-culture on glass bead plates[1] (Fig. 1A) or on solid growth medium (Fig. 1B). For glass bead plates, the fungus was grown in liquid growth medium at 50 °C for 4 days, prior to inoculation with an overnight bacterial culture. The plates were incubated at 50 °C for a further 5 days. Fungal colony diameter and OD_{600} of the bacterial culture were measured daily.
On solid medium, bacterial streaks were grown to stationary before inoculating with *M. thermophilus* equidistant between the bacterial streaks and incubated for 4 days at 50 °C.

N_2O Gas sampling

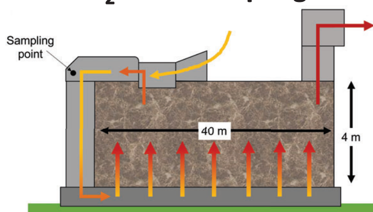


Figure 2. Schematic of a Phase II tunnel. Arrows indicate airflow direction, where yellow arrows represent fresh air, orange arrows represent recycled air and red arrows indicate exhaust off-gas. Gas was sampled daily in the recycled airflow duct.

Gas was sampled from the return air ducting at a commercial composting yard (Fig. 2) into 1 L gas bags. N_2O was measured using GC with an electron capture detector.

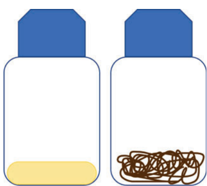


Figure 3. Microcosms of *P. taiwanensis* cells in PBS or Phase II compost.

Gas was sampled in 300 mL microcosms (Fig. 3) of *P. taiwanensis* cells inoculated in 20 mL 50 mM phosphate buffer or 20 g Phase II compost, supplemented with or without 20 mM nitrite. The microcosms were incubated at 45 °C with shaking at 200 rpm and the headspace sampled every 4 hours for 30 hours. N_2O with the same GC method as above.

Conclusions

- **The dominant compost bacterium, *P. taiwanensis,* is not producing N_2O during Phase II composting**
  - *In vitro*, *P. taiwanensis* will produce N_2O aerobically if NO_2^- is available
  - Higher abundance of *P. taiwanensis* will not produce more N_2O unless NO_2^- is present
- **N_2O during Phase II is rapidly transformed by other heterotrophic microbes in compost**
- **The chitin degrading bacterium, *M. composti*, actively degrades the ascomycete fungus, *M. thermophilus***
  - Providing more accessible nutrients for *A. bisporus*

N_2O production rate does not increase during Phase II composting

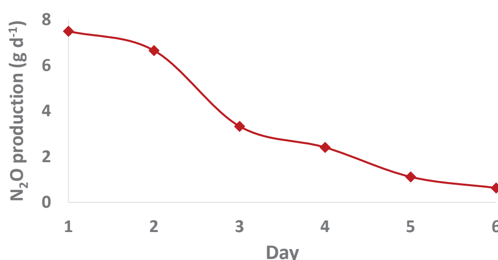


Figure 4. N_2O production rate of one tunnel during Phase II composting at a commercial compost yard.

- Total N_2O produced during Phase II by ~200 T of compost = 21.6 g

P. taiwanensis produces N_2O from NO_2^-, *in vitro*

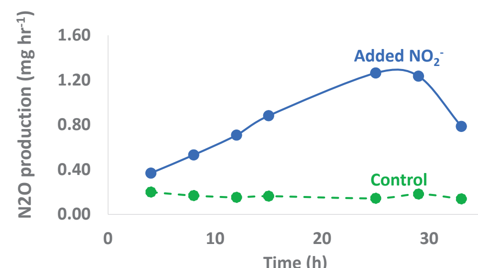


Figure 5. N_2O production rate by *P. taiwanensis* in PBS supplemented with NO_2^- (solid line) and without NO_2^- (dashed line).

- *P. taiwanensis*, the dominant bacterium in Phase II compost, uses nitrite (NO_2^-) as a precursor for N_2O

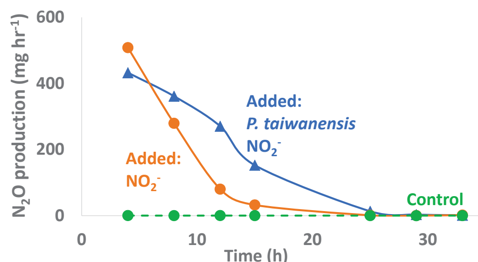
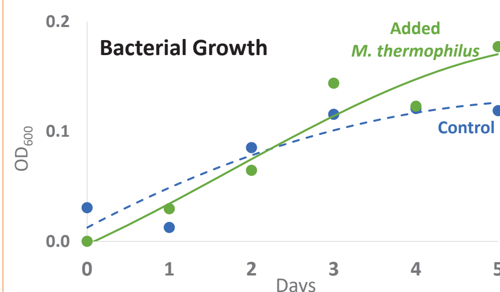


Figure 6. N_2O production rate in Phase II compost spiked with *P. taiwanensis* (Δ) and without *P. taiwanensis* (●), and supplemented with NO_2^- (solid line) and without NO_2^- (dashed line).

- NO_2^- added to Phase II compost will generate N_2O; however
- N_2O in compost is rapidly transformed by other heterotrophic microbes in the compost

Bacterial growth is stimulated by the fungus, *M. thermophilus*



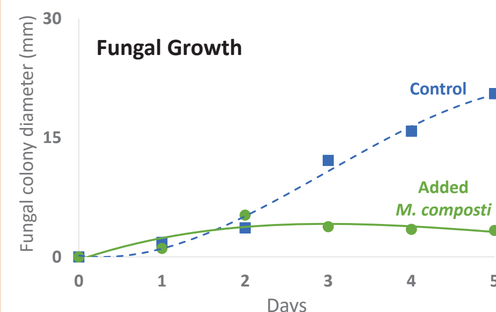


Figure 7. *M. composti* and *M. thermophilus* growth on glass bead plates after bacterial inoculation. Growth of *M. composti* is stimulated in co-culture; however, *M. thermophilus* growth is significantly impacted by *M. composti*. Solid line – growth in co-culture. Dashed line – growth in pure culture.

M. composti degrades *M. thermophilus* cell walls

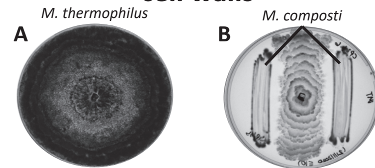


Figure 8. Chitinase activity exhibited by *M. composti* against *M. thermophilus* on solid growth medium. A) M. thermophilus pure culture. B) Co-culture plates (Fig. 1B) of *M. thermophilus* growth with *M. composti* bacterial streaks, showing strong fungal inhibition.

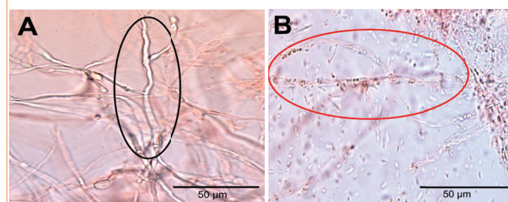


Figure 8. Microscopy of *M. thermophilus* hyphae in liquid culture stained with 1% (w/v) Congo red.[4] A) Healthy *M. thermophilus* hyphae (black circle) without *M. composti*. B) *M. thermophilus* hyphae (red circle) with *M. composti*.

Acknowledgements
We thank the participating mushroom composters and the Australian Mushroom Growers Association (AMGA) for discussions and support. This research was funded by Hort Innovation, using the Australian Mushroom Research and Development Levy and contributions from the Australian Government.

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Feeding the compost – nitrogen supplementation during mushroom cropping

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Background

- Nitrogen (N) is a critically important nutrient in mushroom cultivation. Mushrooms need it to build proteins, genetic material and chitin (cell wall material) in both mycelium and caps. It is added to compost mainly as poultry manure and other compost ingredients (e.g. feathermeal, canola).
- Some of this N is released as ammonia gas during composting and helps pasteurize the compost. Most of the rest is used by compost microbes as they break down the wheat straw. The N in the compost microbial biomass feeds the button mushroom mycelium during spawn run.
- Crop yields are increased by adding slow-release, soy-based supplement before cropping to replace the N that has been lost as ammonia.
- During cropping, we have observed that free N (ammonium) in the compost increases briefly just before each flush, and this N is then absorbed into the caps.

Can we increase the yield and N-content of mushrooms by adding nitrogen into the compost at pinning to mimic the naturally occurring process?

Methods

Mushroom cropping

Unsupplemented spawn-run compost was obtained from a commercial supplier. Compost (7 kg) was filled into 21-litre test boxes, cased (4 cm depth), and three flushes of mushrooms were harvested (total 35 days post casing). Five replicates of all treatments.

Nitrogen additions

All treatments received total added N equivalent to 0.6 % commercial supplement (0.47 g N/kg compost).

Nitrogen was added in three ways:

- Added Nitrogen all mixed with compost at casing.
- Added Nitrogen added in 3 portions by injection into compost before each flush (injection depth 4 cm below casing).
- Half of added Nitrogen mixed with casing, the other half added in three portions by injection before each pinning.

Nitrogen additives used:

- Ammonium acetate.
- Urea.
- Protein Nitrogen (commercial supplement at casing, yeast extract solution at pinning).
- Water (unsupplemented control).

Button mushrooms growing in test boxes (32 x 43 cm) in the Marsh Lawson Research Unit

Conclusions

- Supplementation at pinning rather than casing increases mushroom yields for protein-based supplements and for ammonium, but not for urea.
- Nitrogen content of mushroom caps increases from first flush to third flush.
- Supplementation at pinning rather than casing increases N content of later flushes.
- Possible application for development of in-crop fertigation methods.

Nitrogen supplementation method affects crop yield

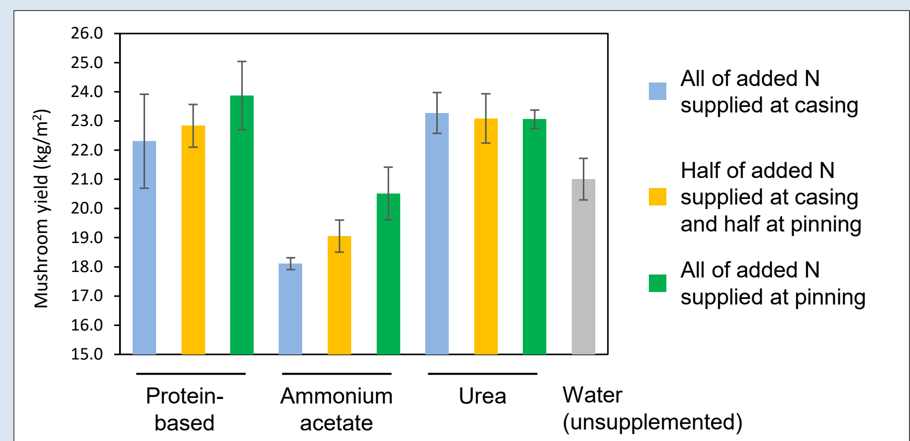

Figure 1. Yield of mushrooms under different N-treatments.

- **Supplementation at pinning rather than casing increases yield for protein-based supplements, but not for urea.**

Nitrogen supplementation method affects N-content

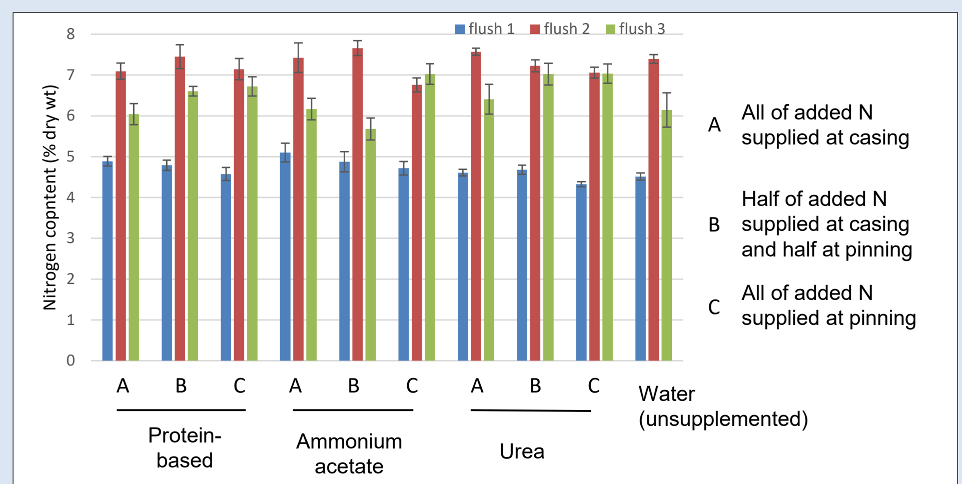

Figure 2. Nitrogen content of mushroom caps (% dry weight)

- **N content much higher in 2nd and 3rd flush mushrooms under all treatments. This is increased by supplementing N at pinning rather than casing**.

Significance

- Supplementing the compost during cropping using fertigation methods avoids the risk of stimulating weed moulds and mushroom disease.

Acknowledgments

This work was supported by Hort Innovation, using the Mushroom research and development levy (Project MU17004 - Optimize nitrogen transformations in mushroom production)

Further reading

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Bacterial interactions with *Agaricus bisporus* mycelium

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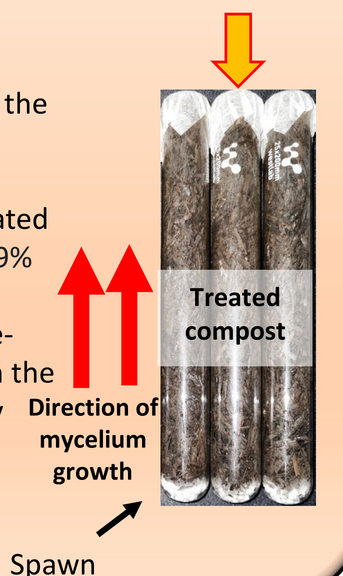
Background

The compost on which the button mushroom (*Agaricus bisporus*) is cultivated is the result of a multi-phase conversion of raw feedstocks to a selective medium for mushroom growth by a succession of bacteria and fungi[1]. *A. bisporus* is introduced as grain spawn into mature compost at the end of Phase 2 composting, and the proliferating mycelium initially degrades the vast microbial biomass, utilizing it as a primary source of nutrition to fully colonize the compost[2].

We hypothesized that boosting the population of naturally abundant bacterial taxa in Australian Phase 2 compost, such as *Pseudoxanthomonas taiwanensis* and *Chelatococcus* composti [3], could promote faster mycelium growth by providing extra nutrition. The common soil bacterium *Bacillus licheniformis* was also investigated as a potential inoculant to determine whether non-native compost bacteria are able to promote mycelium growth. Shortening the time taken for *A. bisporus* to fully colonize compost with microbial inoculants would be of economic importance as it could increase the number of production cycles per year for a compost yard.

Methods

- Bacterial strains were obtained from the laboratory compost strain collection. Inoculum was prepared by culturing in rich growth medium (LB medium). Cell cultures were washed and resuspended in purified water before addition to compost.
- **Individual and combination treatments** of bacterial inoculants (10^6-10^7 cells/g compost) were applied to unspawned end-Phase 2 compost.
- Spawn was added to the bottom of a test tube, and the tube was then filled with compost, covered with micropore tape and incubated for 3 weeks at 22 °C and 99% relative humidity.
- Mycelium growth measure-ments were recorded from the base of the test tube every alternate day.



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This work was supported by Hort Innovation, using the Mushroom research and development levy (Project MU17004 - Optimize nitrogen transformations in mushroom production).

Conclusions and future work

- Inoculation with stationary phase bacterial cultures, particularly *Chelatococcus composti*, reduces the time taken for spawn to start proliferating.
- Inoculation with exponential phase *P. taiwanensis* + *B. licheniformis* leads to strong mycelium growth inhibition.
- Future work should determine bacterial consortia with growth promoting effects and no adverse interbacterial interactions, as well as corresponding cell concentrations with the highest growth rate stimulation.

Growth inhibition by exponential phase bacterial cells (cells in active growth)

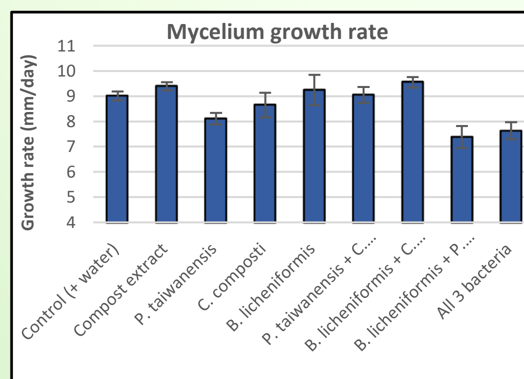

Fig 1. Growth rate of *A. bisporus* on compost inoculated with exponential phase bacterial cells.

- *P. taiwanensis* + *B licheniformis* significantly reduces mycelium growth.
- *B. licheniformis* + *C. composti* demonstrates mild increase of mycelium growth rate.
- Taxon-specific interbacterial interactions either severely inhibit or mildly promote mycelium growth.
- Mycelium growth was slower with *P. taiwanensis* and *C. composti*, both dominant Phase 2 species, but was unaffected by *B. licheniformis*, which is largely absent from Australian mushroom compost.

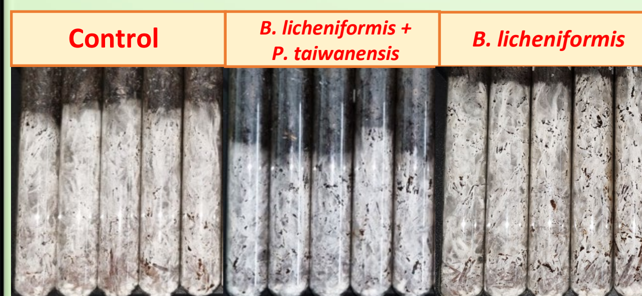

Fig 2. *A. bisporus* mycelium growth on compost with exponential phase bacterial cells.

Growth promotion by stationary phase bacterial cells (resting cells)

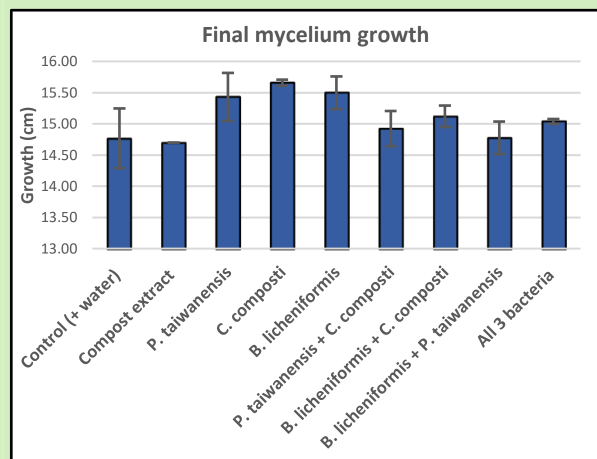

Fig 3. Final growth of *A. bisporus* on compost inoculated with stationary phase bacterial cells.

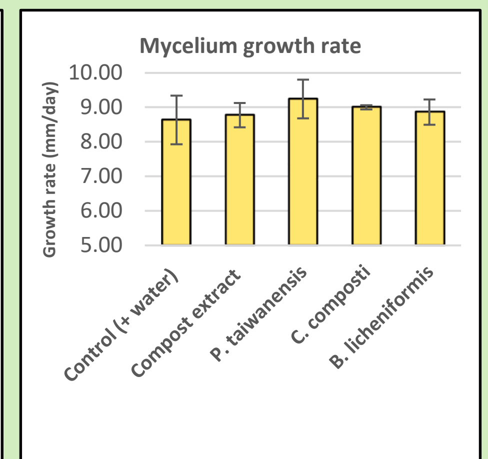

Fig 4. Growth rate of *A. bisporus* on compost inoculated with stationary phase bacterial cells.

- Stationary phase bacterial inoculation causes growth promotion, and eliminates any growth inhibition by the *P. taiwanensis* + *B. licheniformis.*
- Individual bacterial treatments produced the highest mycelium growth.
- Similar growth rates of treatments with different final growth suggests bacterial inoculation shortens time taken for spawn to start proliferating, rather than speeding up growth.

Microbial diversity in Australian mushroom compost

Meghann Thai, Michael Kertesz and Tina Bell

Introduction

Mushroom composting is a process that is largely driven by microorganisms. High throughput amplicon DNA sequencing has been used to explore the microbial succession in mushroom compost since the early 2000s, focusing mostly on bacterial and fungal diversities. Many studies only focus on the bacterial diversity in one compost yard and also typically one compost crop. However, many of these studies have one taxon in common, *Pseudoxanthomonas,* and more recently, *P. taiwanensis*.

Mycothermus thermophilus is the dominant fungus in Phase II button mushroom compost. It has been recognised as a significant thermophilic fungus in mushroom composting. It has growth promoting effects on *Agaricus bisporus* by its ability to incorporate gaseous ammonia as biomass. *In vitro*, *M. thermophilus* has been shown to stimulate vegetative growth of *Agaricus bisporus*, even amongst different strains of *M. thermophilus*.

BOX-PCR is a genomic fingerprinting technique that amplifies repetitive elements in a genome. BOX repetitive elements are found in noncoding regions. The locations of these elements on whole genomes are highly conserved as they are involved with the coordination of gene expression. Amplification of these elements provides a useful tool that can discriminate strains within a species.

This study compares the bacterial diversity and community structure between compost yards and crops particularly focusing on end-Phase II compost. Furthermore, this study also compares the genetic variation of *M. thermophilus* and *P. taiwanensis* isolates from multiple compost yards.

Conclusions

- The compost bacterial diversity at each compost yard can differ between successive composting cycles.
- *Pseudoxanthomonas* is one of the most consistent taxa found in Australian mushroom compost.
- Yard A is significantly different from other composting yards in Australia, probably due to operational differences compared to other yards.
- Isolates of *M. thermophilus* from different composting yards shows no genome variation
- Isolates of P. taiwanensis showed genetic variation, however the variation in bacterial genotypes are most likely due to *P. taiwanensis* adapting to fulfill several functions in composting.

Discussion

- The dominant bacterial taxon in Phase II for most yards sampled was *Pseudoxanthomonas* (Fig. 1).
  - *Pseudoxanthomonas* spp. has been found in many cellulose degrading consortia because it produces β-glucosidase.
- Despite the variability in the bacterial diversity in mushroom compost between compost yards (Fig. 2), the bacterial community structure followed the same trend from the end of Phase I through to the end of Phase II (Fig. 3).
  - This trend indicates that it's not the individual bacterial taxa that are important, but rather the functional groups that develop during composting.
- From 11 isolates of *P. taiwanensis*, seven genotypes were revealed (Fig 4, Gel B)
  - This suggest that compost bacteria adapt to fulfill several functions within the same species.
- Fungal diversity was conserved compared to bacterial diversity.
  - Isolates of *M. thermophilus* from multiple compost yards around Australia showed that there was no genetic diversity between the isolates (Fig. 4, gel A).
  - Suggesting that the composting environment leads to functional selection of a near-clonal line of *M. thermophilus*.

Dominant bacterial genera in Phase II compost

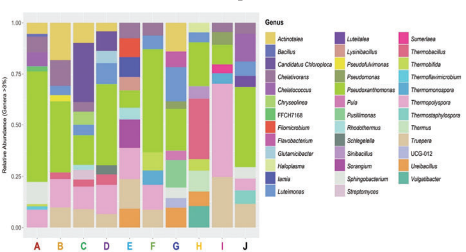

Fig 1. Bacterial taxa in Phase II compost from nine compost yards (bacterial genera > 3%). For most compost yards sampled, *Pseudoxanthomonas* was the most dominant genus.

Bacterial succession during composting

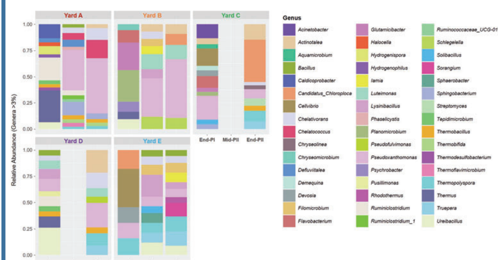

Fig 2. Bacterial taxa in each compost production (Bacterial genera >3%). Phase I bacterial diversity showed the most variability between compost yards. However, the composting environment during the Phase II process shows selection for a functional groups of bacteria in mid-Phase II and end-Phase II. Where Pseudoxanthomonas was not the dominant taxon, other thermophilic taxa were present.

Bacterial community structure during composting from multiple compost yards follow the same trend

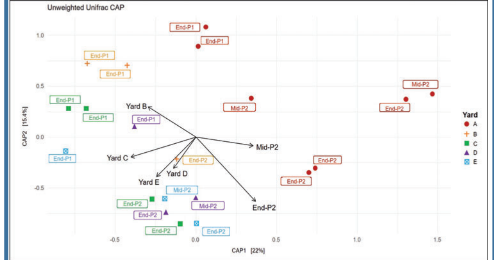

Fig 3. Unweighted Unifrac CAP of the bacterial community characterised by genera (ANOVA: df = 6, P = 0.007). There is clear succession in the bacterial diversity from end-Phase I to end-Phase II compost. Differences in the bacterial communities between compost yards is most likely due to operational differences.

Methods

Sampling
Mushroom compost samples were taken from nine Australian composting yards. Compost was sampled at End-Phase 2 for all yards. End-Phase I compost was sampled for five yards, and Mid-Phase 2 where appropriate. Mid-Phase 2 was sampled after pasteurisation, at the start of conditioning.

***M. thermophilus* isolation**
Phase II compost was plated onto YPSS medium, with chloramphenicol added, and incubated at 50 °C. 15 isolates of dark green to black fungal colonies with chained aerial conidia were purified and maintained on YPSS medium. Identification was confirmed with Sanger sequencing of the ITS region.

DNA extraction and sequencing
DNA was extracted from the compost samples using bead beating for cell lysis and DNA binding with magnetic bead technology (Lever *et al*., 2015). DNA was amplified for paired-end Illumina sequencing of the V3/V4 region of the 16S rRNA gene using primers 341F and 806R. The sequences were filtered, merged and aligned using the DADA2 R package (Callahan *et al*., 2016).

Statistical analyses
Statistical analyses were carried out in R using the phyloseq package (McMurdie and Holmes, 2013).

BOX-PCR
Polymerase chain reaction using BOXA1R primers (Versalovic *et al*., 1994) to amplify repetitive gene regions in genomic DNA.

Fungal genotypes are highly conserved in Phase II compost, whereas bacterial genotypes are more adaptive

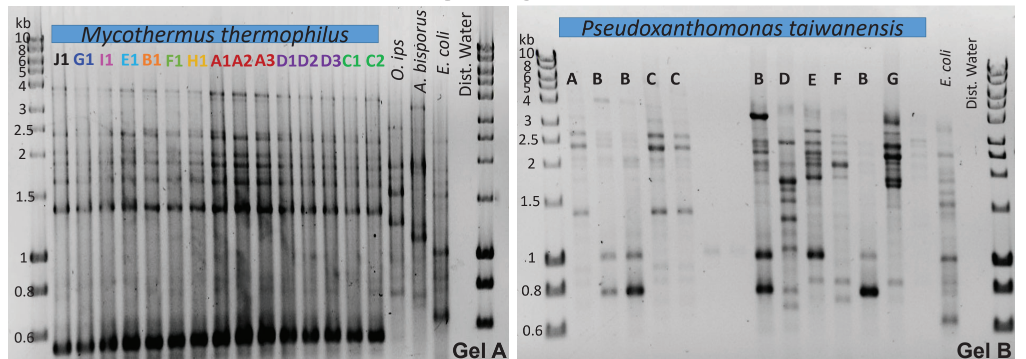

Fig 4. BOX-PCR of *M. thermophilus* strains (Gel A) and *P. taiwanensis* (Gel B). 15 isolates of *M. thermophilus* from different compost yards showed >99% DNA similarity in the ITS region and no whole-genome variation between the isolates. 11 isolates of *P. taiwanensis* showed >99% DNA similarity in the 16S region, but whole-genome fingerprinting revealed seven genotypes.

Acknowledgements

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ARTICLE OPEN

Dynamics of microbial community and enzyme activities during preparation of *Agaricus bisporus* compost substrate

Meghann Thai [1], Katarzyna Safianowicz[1], Tina L. Bell [1] and Michael A. Kertesz [1]✉

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Button mushrooms (*Agaricus bisporus*) are grown commercially on a specialized substrate that is usually prepared from wheat straw and poultry manure in a microbially-mediated composting process. The quality and yield of the mushroom crop depends critically on the quality of this composted substrate, but details of the microbial community responsible for compost production have only emerged recently. Here we report a detailed study of microbial succession during mushroom compost production (wetting, thermophilic, pasteurization/conditioning, spawn run). The wetting and thermophilic phases were characterized by a rapid succession of bacterial and fungal communities, with maximum diversity at the high heat stage. Pasteurization/conditioning selected for a more stable community dominated by the thermophilic actinomycete *Mycothermus thermophilus* and a range of bacterial taxa including *Pseudoxanthomonas taiwanensis* and other Proteobacteria. These taxa decreased during spawn run and may be acting as a direct source of nutrition for the proliferating *Agaricus* mycelium, which has previously been shown to use microbial biomass in the compost for growth. Comparison of bacterial communities at five geographically separated composting yards in south-eastern Australia revealed similarities in microbial succession during composting, although the dominant bacterial taxa varied among sites. This suggests that specific microbial taxa or combinations of taxa may provide useful biomarkers of compost quality and may be applied as predictive markers of mushroom crop yield and quality.

ISME Communications; https://doi.org/10.1038/s43705-022-00174-9

INTRODUCTION

Button mushrooms, *Agaricus bisporus*, are one of the most widely cultivated edible mushrooms, with about 8 billion kg produced per year worldwide [1]. They are grown on a composted substrate that is traditionally made from wheat straw, stable bedding, poultry manure and gypsum. The ingredients used to make mushroom compost varies in different parts of the world; in Europe and the USA, for instance, there is a heavy dependence on stable bedding (horse manure) as the primary carbon and nitrogen source [2–4], in Australia almost no stable bedding is used, and in China rice straw is often used in place of wheat straw [5]. Smaller amounts of other agricultural by-products such as canola meal, soybean meal and cottonseed meal are often added to provide additional nitrogen and stimulate microbial activity at the start of composting, depending on seasonal availability.

Composting is a microbial process in which lignocellulosic waste materials are converted into a nutrient-rich humus-containing medium [6–8]. Details of the mushroom composting process vary between countries, but typically include a wetting phase to soften the straw raw materials and initiate straw breakdown, a thermophilic composting phase (Phase I, 70–80 °C) in which most of the structural components of the straw are degraded, and a pasteurization phase (Phase II, 60 °C) with subsequent conditioning at mesophilic temperatures (45 °C), in which the breakdown products are incorporated into microbial biomass and humic-lignin products in the final compost (Fig. 1). The *Agaricus* mycelium is introduced on a grain-based carrier (referred to as spawn) and allowed to proliferate throughout the compost. Mushroom production is then initiated by application of a low-nutrient layer of mixed peat and lime (referred to as casing), together with lowering the temperature and reducing CO_2 levels in the growing rooms [9, 10]. Because the majority of the easily metabolizable plant metabolites are removed during the composting process and converted to microbial biomass and protein in the compost, the only organisms that can grow effectively on the finished compost are those that can access carbon either from the microbial biomass present or from residual lignin-humic complexes. This provides a nutritional environment that favors basidiomycetes over competing fungal pathogens (typically ascomycetes), yielding a compost that is highly selective for *Agaricus* under the cropping conditions used [11].

The main components of the cell wall of straw are structural carbohydrates, typically cellulose, hemicellulose (mainly xylan) and lignin, with pectin and related molecules providing structural cohesion. Wheat straw typically contains 40% cellulose, 25% hemicellulose, 23% lignin and 3% pectin [12], and these molecules provide the main growth substrate for the microbes present in the composting process. About 50% of the available xylan and cellulose is broken down during the thermophilic phases of composting [3], catalyzed by cellulases and xylanases that are released by the thermophilic bacteria and fungi in the compost. Lignin levels are largely unaffected, and much of the hemicellulose that remains at the end of the thermophilic phase is thought to be bound in lignin complexes. These hemicellulose

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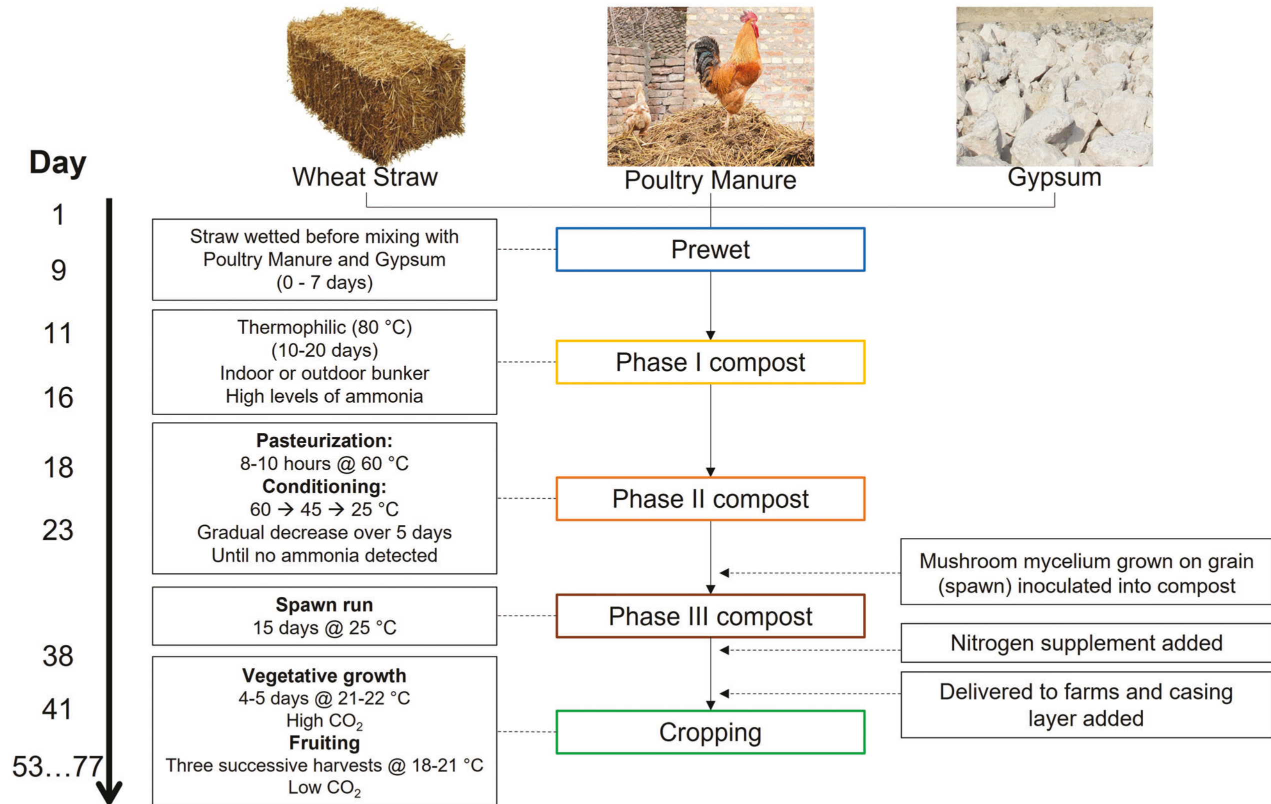

Fig. 1 Summary of the mushroom composting process. Mushroom compost is produced from wheat straw, poultry manure and gypsum. The four phases of mushroom composting are indicated in the centre (Prewet, Phase I, Phase II, Phase III), followed by cropping. The key processes in each phase are shown on the left, together with the approximate number of days required for each phase. The timing and conditions given are typical of those observed in this study, but these may vary between compost yards.

fragments are released by the ligninase activity of *Agaricus* mycelium, but they are often highly substituted and are not well metabolized by *Agaricus* [13].

The succession of microbes that catalyze the composting process in a range of composting applications has been studied previously using traditional culture-based methodologies to isolate organisms on complex growth media (reviewed in Ryckeboer [14] and Kutzner [15]). Although a range of thermophilic fungi and bacteria was described, the cultivable bacteria reported from mushroom composts showed quite limited diversity, and were primarily related to *Bacillus* and to actinomycetes such as *Streptomyces* and *Thermoactinomyces* [14]. More recent work using molecular sequencing tools has revealed a much wider range of taxa [16–19]. In contrast to isolated bacteria, the thermophilic fungi isolated from mushroom compost were slightly more diverse [14]. These thermophilic fungi are essential in Phase II because they convert nutrients from the raw material into microbial biomass and in doing so contribute to the selectivity of mushroom composting. One particularly important thermophilic fungus, *Mycothermus thermophilus* (syn. *Scytalidium thermophilum*/ *Humicola insolens*) aids the reassimilation of ammonia into the compost [2, 20, 21], and stimulates growth of the button mushroom mycelium. In the presence of *M. thermophilus*, hyphal elongation of *A. bisporus* doubles [20, 22] and fungal competitors of *A. bisporus*, such as *Chaetomium globosum*, are suppressed [2, 23]. *Mycothermus thermophilus* is the dominant fungal taxon in Phase II compost and makes up most of the microbial biomass in the compost [18, 22, 24–26], but it is just one player in a multifaceted microbial community.

Previous reports on bacterial and fungal succession in compost during mushroom composting have each studied individual compost yards, while suggesting significant variability in microbial diversity among different compost yards [2, 16, 17, 25–28]. In this study, we provide an in-depth analysis of fungal and bacterial diversity and succession at multiple time points throughout the mushroom composting process, and correlate this with activities of key compost enzymes. Variability among composting facilities was investigated directly by determining the bacterial community diversity in compost from five geographically separated composting yards in south-eastern Australia at three important timepoints in the composting process. This has allowed us to determine how bacterial community structure in mushroom compost varies among facilities, and whether variation can be explained by functional redundancy in species composition.

MATERIALS AND METHODS

Composting process

Compost for button mushroom cultivation was prepared by five commercial composting yards located in the east and southeast of Australia, referred to in this study as Yards A–E to preserve commercial confidentiality. Compost was prepared using the standard three-stage industrial composting process, with minor variations among yards (Table S1). Briefly, wheat straw was soaked for several days with recycled process water and was then mixed with gypsum, chicken manure, and any other additives used by specific yards. The blended raw materials were then subjected to an uncontrolled self-heating step, using underfloor aeration or frequent turning, with temperatures increasing to ~80 °C. The heating step typically lasted for 14–21 days with two to three turns (Phase I). Phase II composting (pasteurization and conditioning) was done either in bulk in a closed tunnel with floor aeration, or in trays, with pasteurization of the compost at 60 °C for 6–10 h followed by conditioning of the compost by gradual cooling to 45 °C over 4–5 days. The compost was further cooled to 25 °C before mixing in button mushroom spawn (*Agaricus bisporus* strain A15), together with a commercial supplement. The *Agaricus* mycelium was allowed to proliferate at 25 °C for 14 days (Phase III)

in a closed tunnel (4 × 4 × 40 m). When required, spawned compost was overlaid with standard casing material (a mixture of peat, lime, and compost) and used for mushroom cropping in the Mushroom Research Unit at The University of Sydney. Three flushes of mushrooms were obtained under standard conditions.

Compost sampling

For the first study, compost was sampled from Yard A in April–May 2012, with samples obtained from feedstocks and at 19 timepoints throughout the composting process over 39 days. Samples were collected at 3 to 4-day intervals throughout pre-wetting and Phase I, three times during Phase II and eight times during Phase III (spawn run) (Table 1). Five-fold replicate samples of ~50 g were collected randomly from different depths and heights within the stack by sampling during the regular compost turning process, as compost was moved between bunkers. Samples were stored in plastic zip-lock bags and immediately frozen at −20 °C. Frozen samples were ground in liquid nitrogen and kept at −80 °C until used.

Comparative data from multiple composting facilities were obtained in a second study, for which compost was sampled from Yards A–E in July–August 2017. Single samples of ~500 g were collected randomly at the end of Phase I, between pasteurization and conditioning in Phase II (where technically possible), and at the end of Phase II. Single samples were taken by hand from the face of the compost pile, stored in plastic zip-lock bags and transported to the laboratory within 1–3 days. Each bulk sample was mixed thoroughly, and five subsamples were taken and stored at −20 °C.

Compost physicochemical measurements

Water content of the compost was measured gravimetrically by weighing subsamples of compost before and after oven drying at 105 °C for 24–48 h. Moisture content was expressed as a percentage of the fresh weight. Ash content was determined gravimetrically after heating the dried sample for 2 h in a muffle furnace at 550 °C. pH and electrical conductivity of casing and compost extracts were determined using a pH meter (pH Cube, TPS, Queensland, Australia) and digital conductivity meter (Model PTI-18, Activon Scientific Products Co, New South Wales, Australia) in 1:10 water extracts (180 rpm shaking, 1 h, room temperature). The tubes were held at room temperature for 2 h to allow particulates to settle before measurements were taken.

Total carbon (C), nitrogen (N), and sulfur (S) content of dried and finely ground samples of feedstocks and compost substrates were determined by combustion (Vario Max CNS, Elementar Analysensysteme GmbH, Hanau, Germany). Total water-extractable C and N was measured using a TOC-analyzer (TOC-V CSH, TNM-1, Shimadzu, Kyoto, Japan). Frozen samples (1.2 g) were extracted with 0.05 M K_2SO_4 (25 ml) at room temperature for 1 h with shaking (200 rpm). Extracts were filtered through filter paper (Whatman Grade 42), and total soluble C and N determined following the manufacturer's instructions. Sodium phthalate and KNO_3 solutions were used as C and N standards.

Enzyme assays

Activity of nine enzymes in water extracts of compost samples was assayed using published colorimetric and fluorometric methods (Table S2), modified as needed to fit 96-well format. Compost extracts were prepared at room temperature by suspending 0.65 g of ground, frozen sample in 6 ml of sterile ultrapure water in 15 ml polypropylene tubes and shaking on an orbital shaker (180 rpm) for 30 min. Particulates were removed by centrifugation at 1500 × *g* (Falcon 6/300, MSE) and the clarified supernatants were stored on ice for up to 4–5 h until used.

Enzyme activity measurements were made using a plate spectrophotometer (BioTek Synergy H1, Agilent, California, USA) at times optimized to capture the amounts of product in the linear phase of enzyme activity. Enzymatic activity was expressed as µg of product generated per g of dry material per h.

DNA extraction, amplification, and sequencing

For the first study, total compost DNA was extracted using a method adapted from Yeates and Gillings [29] Ground compost samples (0.3 g) were suspended in lysis buffer (6X; 1% (w/v) SDS, 1% (w/v) polyvinylpolypyrrolidone, 60 mM EDTA pH 8.0, 300 mM Tris-HCl pH 8.0), and lysed using a homogenizer (MoBio Laboratories Inc., California, USA) at 2000 rpm for 5 min. After protein precipitation with 1.2 M potassium acetate, DNA was recovered with a glass milk Binding matrix solution (MP Biomedicals, California, USA), diluted 1:6 with 6 M guanidine isothiocyanate. Bacterial diversity was analyzed using primers 515F and 806R [30] to amplify the V4 16S rRNA gene hypervariable region, and fungal diversity was measured with primers amplifying the ITS2 region (ITS3F and ITS4R) [31]. Paired-end Illumina sequencing was done using the Illumina MiSeq platform at University of Boulder (Colorado) and at RTL Genomics (Lubbock, Texas, USA).

Quantitative PCR was done with a CFX96 Touch Real-Time System (Bio-Rad, California, USA), using the 16S and ITS primer pairs described above. Purified amplicon standards for quantification were generated from compost DNA.

For the second study, total compost DNA was extracted according to Lever [32] with some modifications. Ground compost samples (200 mg) were suspended in 200 mM sodium hexametaphosphate (100 µl), lysis buffer 1 was added (30 mM Tris/HCl, 30 mM EDTA, 800 mM guanidinium chloride, 0.5% (v/v) Triton X-100, pH 10.0) (500 µl), and the samples were lysed using a homogenizer (MoBio Laboratories Inc.,) at 2000 rpm for 5 min. Lysis buffer 2 (2.5 M sodium chloride, 2% (w/v) cetyltrimethylammonium bromide, 0.1% (w/v) polyvinylpolypyrrolidone) (500 µl) was added, followed by incubation at 65 °C with agitation (500 rpm) for 30 min and centrifugation, Supernatants were extracted once with an equal volume of chloroform:isoamyl alcohol (24:1) and DNA was recovered from the aqueous phase using DNA binding magnetic beads (GE Life Sciences, Australia) in SPRI solution, following the manufacturer's instructions. Bacterial diversity was analyzed using primers 341F and 806R [33, 34] to amplify the V3-V4 16S rRNA gene hypervariable region, with the Illumina MiSeq platform (paired 300 bp read lengths) at the Australian Genome Research Facility (Melbourne, Australia).

Sequencing data for both studies are available at NCBI SRA under BioProject PRJNA867030.

Bioinformatics

Raw FASTQ files were processed in R v3.6.1 [35]. Raw read quality was determined using FastQC. Trimming and filtering was determined using the DADA2 function "filterAndTrim" [36], discarding forward and reverse reads with an expected error score higher than 3 and 4, respectively. Low quality reads were removed during trimming and filtering by setting "truncLen" parameters to 285 and 240 for the forward and reverse reads, respectively. Forward and reverse primers were trimmed from the 5′ end by setting the "trimLeft" function to 17 and 20, respectively. The sequences were denoised and dereplicated using the "dada" and "derep" functions, unique sequences were merged with a minimum overlap of 20 base pairs and a sequence table was constructed with the resulting sequence variants. Rarefaction curves are shown in Fig. S1.

Taxonomy was assigned using a pre-trained SILVA Naïve Bayes classifier clustered at 99% identity (SILVA release v132) [37]. Species assignment was done in a separate step using the SILVA release v132 for species assignment. 16S gene sequences that were affiliated with chloroplasts and mitochondria were removed prior to downstream analysis. Sequence variants which occurred in fewer than three samples and with fewer than three reads in each of these samples were also removed (singletons and doubletons). A phylogenetic tree was constructed using the packages "phangorn" [38] and "DECIPHER" [39], using the neighbor-joining method.

Statistical analysis was done using the packages "phyloseq" [40] and "vegan" [41]. All graphs and plots were visualized using "ggplot2" [42]. Shannon and Simpson alpha-diversity analyses were performed using the "plot_richness" function from the phyloseq package before singletons and doubletons were removed from the dataset. Differences in the bacterial community (beta-diversity) were analyzed in R [35] using a canonical analysis of principal coordinates with unweighted UniFrac as the distance metric.

RESULTS

Compost samples for the initial study were taken from 19 different timepoints during the pre-wet phase (bale-wetting and windrowing), Phase I (thermophilic), Phase II (pasteurization/conditioning), and Phase III (*Agaricus* spawn run) of a standard industrial composting run. The total yield of *Agaricus bisporus* obtained from the studied compost was 61.3 kg m^{-2} (460 g kg^{-1} compost), in four fruiting flushes (yielding 23.2, 15.6, 8.8 and 3.4 kg m^{-2} of mushroom caps, respectively). These yields are comparable or higher than standard yields obtained in the Australian mushroom

Table 1. Sampling day, and enzyme activities in selected compost samples.

Process day	Description	Composting phase	Amylopectinase nmol glucose min^{-1} g^{-1}	ß-glucosidase nmol MUF min^{-1} g^{-1}	Cellulase nmol glucose/min/g	Chitinase nmol MUF min^{-1} g^{-1}	FDA hydrolysis nmol fluorescein min^{-1} g^{-1} g	Invertase nmol glucose min^{-1} g^{-1}	Peroxidase Absorbance @ 450 nm min^{-1} g^{-1}	Protease nmol AMC/min/g	Xylanase nmol glucose min^{-1} g^{-1}
0	Supplement	Feedstocks	11	bdl	1.7	bdl	bdl	bdl	bdl	17.3	bdl
0	Cotton hulls	Feedstocks	949	bdl	30.8	bdl	bdl	0.7	bdl	49.4	bdl
0	Chicken manure	Feedstocks	1450	bdl	11.8	bdl	bdl	4.7	3.7	110.0	192.2
0	Gypsum	Feedstocks	59	bdl	4.0	bdl	bdl	bdl	1.1	5.2	bdl
0	Feather meal	Feedstocks	26	bdl	bdl	bdl	bdl	0.4	bdl	0.8	bdl
0	Brew	Feedstocks	6387	bdl	50.8	51.8	bdl	25.6	7.2	91.4	57.5
1	Bale setup	Pre-wet	1521 ± 1518	47.9 ± 5.7	120.8 ± 61.5	7.0 ± 2.3	40.2 ± 12.3	39.6 ± 10.6	bdl	131.4 ± 10.6	365.6 ± 103.2
2	24 h of wetting	Pre-wet	604 ± 41	47.8 ± 10.1	42.2 ± 25.1	9.4 ± 2.8	43.9 ± 9.3	47.6 ± 9.1	3.9 ± 1.4	121.7 ± 25.4	165.2 ± 6.5
4	72 h of wetting	Pre-wet	239 ± 176	13.5 ± 5.9	24.0 ± 3.5	9.6 ± 3.7	73.0 ± 19.4	7.2 ± 1.7	1.3 ± 1.5	83.8 ± 8.6	44.7 ± 14.0
7	Rick turn start	Pre-wet	411 ± 184	5.0 ± 2.3	15.2 ± 9.0	4.5 ± 1.0	53.3 ± 12.8	2.4 ± 2.4	0.1 ± 0.7	53.1 ± 7.8	31.1 ± 34.9
9	Rick turn end	Pre-wet	bdl	0	2.9 ± 2.8	0.3 ± 0.2	1.3 ± 2.4	0.4 ± 0.3	bdl	14.4 ± 4.1	bdl
11	Phase I tunnel 2	Phase I	41 ± 13	11.0 ± 3.9	8.1 ± 1.7	3.7 ± 0.2	21.2 ± 5.0	0.2 ± 0.1	8.1 ± 1.9	36.7 ± 5.3	62.5 ± 20.7
13	Blend 1	Phase I	46 ± 12	3.4 ± 1.3	3.7 ± 1.5	0.1 ± 0.2	1.0 ± 2.9	0.2 ± 0.1	bdl	12.3 ± 3.1	17.8 ± 10.9
16	Blend 2	Phase I	bdl	6.1 ± 1.8	4.0 ± 1.1	1.1 ± 0.3	7.9 ± 2.1	0	bdl	8.5 ± 1.8	bdl
18	Pasteurization	Phase II	109 ± 34	10.2 ± 2.3	6.6 ± 3.9	1.8 ± 0.3	16.1 ± 2.3	0.3 ± 0.2	0.9 ± 0.5	67.8 ± 5.0	18.2 ± 4.1
20	Conditioning	Phase II	275 ± 61	20.0 ± 3.3	26.4 ± 4.1	1.0 ± 0.1	20.8 ± 6.1	0.6	1.6 ± 1.2	47.4 ± 2.7	249.8 ± 19.2
23	Conditioning end	Phase II	264 ± 104	32.1 ± 3.1	39.0 ± 10.4	1.5 ± 0.4	32.7 ± 12.6	1.1 ± 0.7	1.6 ± 1.3	71.8 ± 16.2	359.0 ± 37.6
25	Spawn run 1	Spawn run	586 ± 57	32.9 ± 8.3	38.0 ± 3.4	3.7 ± 0.6	41.5 ± 8.8	2.0 ± 0.3	2.7 ± 0.6	73.5 ± 14.9	206.4 ± 34.0
27	Spawn run 2	Spawn run	907 ± 233	24.0 ± 5.9	62.1 ± 5.3	1.5 ± 0.4	45.9 ± 14.4	2.9 ± 1.1	0.8 ± 1.3	54.0 ± 13.4	352.0 ± 55.6
29	Spawn run 3	Spawn run	386 ± 53	23.4 ± 5.6	32.8 ± 8.0	2.5 ± 0.2	90.9 ± 20.9	1.6 ± 0.4	5.8 ± 1.3	53.7 ± 13.7	219.1 ± 39.8
31	Spawn run 4	Spawn run	539 ± 97	4.1 ± 1.0	29.0 ± 8.3	bdl	12.1 ± 1.2	3.2 ± 1.0	19.5 ± 5.2	34.6 ± 6.1	296.0 ± 92.9
33	Spawn run 5	Spawn run	323 ± 65	5.8 ± 0.3	39.4 ± 7.7	3.6 ± 0.5	27.6 ± 6.9	3.1 ± 0.4	20.6 ± 9.3	28.6 ± 7.0	158.8 ± 50.3
35	Spawn run 6	Spawn run	605 ± 48	3.1 ± 0.9	9.1 ± 3.5	bdl	2.8 ± 2.1	1.1 ± 0.3	46.9 ± 4.9	11.1 ± 0.4	129.8 ± 44.7
37	Spawn run 7	Spawn run	57.8 ± 1	1.6 ± 0.3	2.4 ± 0.9	2.0 ± 0.2	17.5 ± 4.0	0	43.8 ± 7.1	8.8 ± 2.2	24.5 ± 10.4
39	At casing	Spawn run	118 ± 40	bdl	bdl	bdl	bdl	0.3 ± 0.2	71.3 ± 1.0	3.7 ± 1.1	23.0 ± 5.8

Methods used to measure enzyme activities are outlined in Supplementary Table S1[a].
[a]FDA fluorescein diacetate, MUF methylumbelliferone, AMC 7-amino-4-methylcoumarin, bdl below detection limit.

industry, confirming the high productivity and representative nature of the composting run studied.

Physicochemical changes during the composting process

The initial pH of the compost mix after addition of gypsum to the straw blend was 8.0, and it remained at this level during the pre-wet phase and Phase I, but decreased during pasteurization, conditioning and spawn run to a final value of pH 6.3. The electrical conductivity of the compost was stable at 2.5–3.0 µS throughout (Fig. S1).

The total C content of the compost decreased slowly from 48% (w/w) at bale break to 41% (w/w) when casing was applied at the start of the production phase, due to the loss of C through microbial respiration during composting. The ash content increased correspondingly from 7% (w/w) to 26% (w/w). Interestingly, total N and S content of the compost also increased slowly during the composting process to final values of 2.6% (w/w) for N and 2.2% (w/w) for S, but this increase probably just represents the retention of N and S despite overall loss of C. Extractable C and N levels increased briefly during the pre-wet phase, but then decreased again to base levels of 20 mg g dw^{-1} for carbon and 2–3 mg g dw^{-1} of N (Fig. S2). The moisture content of the compost increased during the initial bale wetting stage to 70% (w/w) and was maintained at 70–80% during Phase I, decreasing to 60–70% in Phase II and during spawn run (Fig. S3).

Extracellular enzyme activities during composting

Conversion of the macromolecular components of compost feedstocks, especially proteins and the structural compounds cellulose, hemicellulose, and lignin, is catalyzed primarily by extracellular enzymes secreted by mesophilic and thermophilic bacteria and fungi. Key changes in the enzymatic profile occurred in the pre-wet phase, during the thermophilic phase, and at the end of spawn run. During initial bale wetting and pre-wet stage (process days 1–5), high activities were observed for most of the enzymes tested (Table 1). The high activities of invertase, amylopectinase and protease enable mesophilic compost organisms rapidly to utilize the soluble sugars, starch and protein made available in the substrate through the wetting process, and these activities decreased to low levels by day 9. Invertase and amylopectinase were not substantially active later in the composting process, possibly in response to depletion of their substrates. However, the enzymes responsible for degradation of cellulose (cellulase, β-glucosidase) and hemicellulose (xylanase) were also highly active at the start of the process and decreased in activity during the pre-wet period. These enzyme activities are therefore likely to be affiliated with mesophilic organisms that initially benefited from increased nutrient availability, but then decreased in activity with increasing temperature in the windrows. This is consistent with the transient increase seen in overall microbial activity (FDA hydrolysis) by day 5, with a subsequent reduction in activity by day 9.

Phase I was a highly aerobic phase, with air actively blown through the compost in large and enclosed tunnels. There was an immediate but transient increase in activity of cellulase/β-glucosidase, xylanase, protease, chitinase and overall microbial activity at the start of Phase I representing rapid growth of thermophilic organisms under these conditions. The transient pulse in enzyme activities was followed by a slow increase in activity of all these enzymes throughout the rest of Phase I, through Phase II (pasteurization and conditioning), and into the spawn run. Protease and β-glucosidase activities were especially high during the conditioning process, as the compost cooled after pasteurization, but the most notable aspect of this period of the process was the profile of xylanase and cellulase activities during the spawn run. Activity of these two enzymes increased to a maximum around day 25 and decreased again as colonization of the compost by *Agaricus* was completed, consistent with displacement of thermophilic cellulose-degrading fungi (especially *Mycothermus*) by the growing *Agaricus* mycelium.

Efficient colonization of the compost by *Agaricus* led to an increase in total microbial activity (FDA hydrolysis) but was also reflected in increasing peroxidase activity from day 30 onwards, corresponding with the onset of *Agaricus*-mediated lignin degradation. Chitinase activity also increased at the end of spawn run, suggesting enhanced activity of organisms producing cell wall degrading enzymes targeting *Agaricus* mycelium.

Microbial populations in mushroom compost

The total population of bacteria in compost (measured as 16S rRNA gene copies per g dry weight of compost) was significantly higher than the fungal population (ITS copies per g dry weight of compost) throughout the composting process (Fig. 2). The size of the fungal population decreased during the pre-wet phase, while bacteria proliferated under these conditions, and the bacteria:fungi ratio reached almost 2500 at day 9. This ratio decreased sharply as temperature increased during Phase I, and mesophilic bacteria were eliminated. The fungal population grew steadily during Phase I and pasteurization/conditioning as thermophilic species proliferated. Interestingly, no substantial increase in fungal numbers was observed during spawn run, consistent with the concept that *Agaricus* obtains most of its nutrition through degradation of the biomass of other fungi such as the thermophilic *Mycothermus*. The bacterial population increased 10-fold during Phase I but decreased again during pasteurization and conditioning and there was always 10–50 times more bacteria than fungi. The size of the bacterial population increased together with *Agaricus* during spawn run, suggesting that the bacteria may have colonized the *Agaricus* hyphae, or otherwise benefited from the presence of this organism.

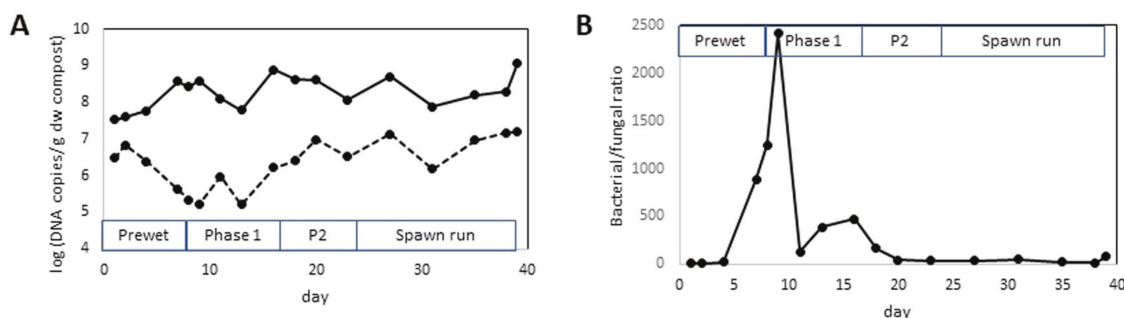

Fig. 2 Total bacterial and fungal populations in compost at selected timepoints during composting. A Bacterial population—solid line; fungal population—dotted line, **B** bacterial/fungal ratio. Microbial populations were measured by qPCR using universal primers 515F and 806R for bacterial populations, and ITS3F and ITS4R for fungal populations.

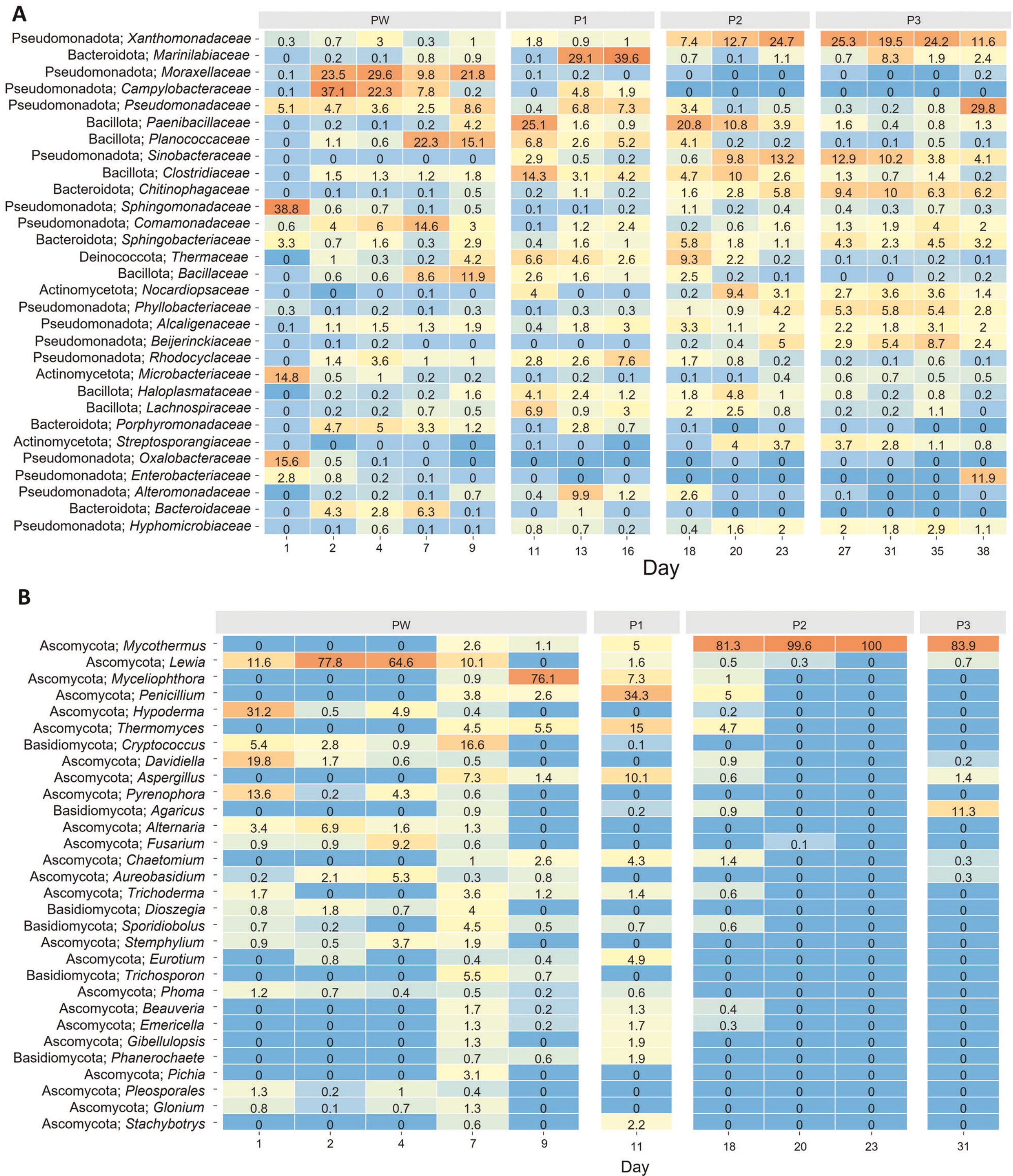

Fig. 3 Relative abundance of microbial taxa in mushroom compost at selected timepoints. A Bacterial diversity (phylum/family). **B** Fungal diversity (phylum/genus). Relative abundances of the 30 most abundant taxa were center log ratio transformed and are displayed as colors ranging from blue (low) to red (high). Numbers indicate relative abundance (%) within each sample. PW prewet, P1, P2, P3 phases 1–3.

Succession of bacterial communities in mushroom compost

Analysis of bacterial diversity in mushroom compost at 19 timepoints during the composting process revealed a total of 3240 different OTUs, with up to 1134 different OTUs present in any given sample (Table S4). Bacterial diversity increased slowly during the pre-wet phase, reaching a maximum at the end of Phase I. Removal of mesophilic organisms during pasteurization led to a steep fall in bacterial diversity; this decrease persisted through the conditioning period and during growth of the *Agaricus* mycelium, suggesting that these conditions were selective for a specific group of organisms.

The dynamics of specific bacterial taxa during production of mushroom compost can be delineated into three main periods (Fig. 3). During the pre-wet phase and early Phase I, the dominant bacteria present changed frequently, with populations growing quickly, and then disappearing equally rapidly as they were

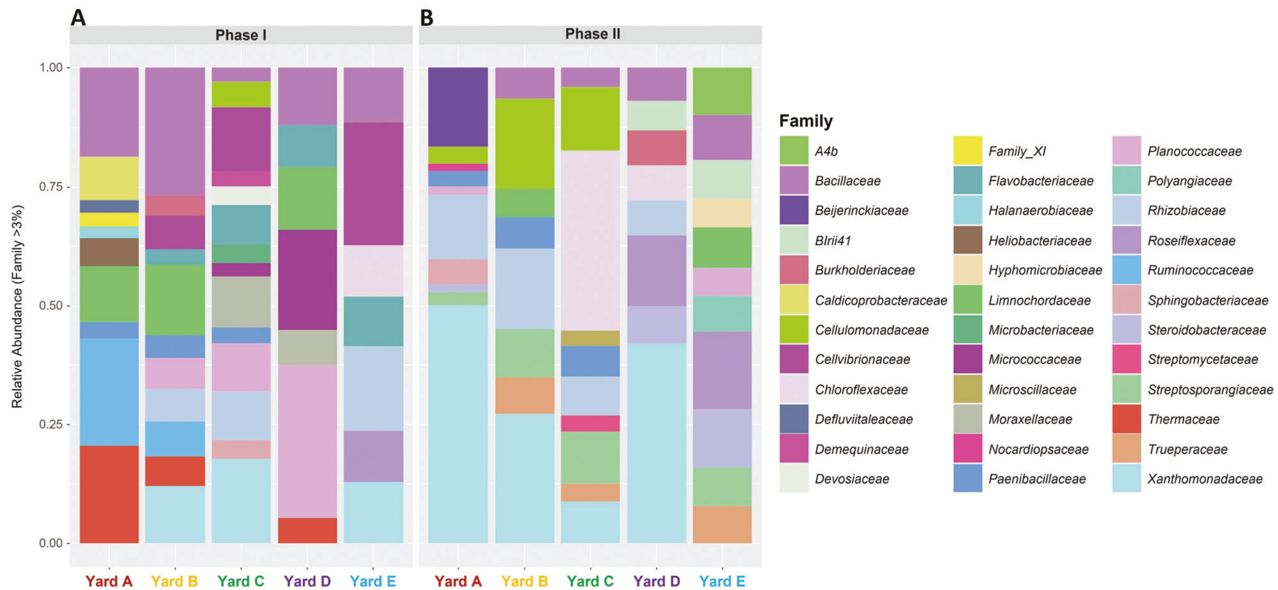

Fig. 4 Relative abundance of bacterial taxa from five geographically distinct compost yards. A End Phase I, **B** end Phase II. Rare taxa with a relative abundance of <3% and taxa that were not classified to family are not shown.

overgrown by other species. *Arcobacter* made up nearly 30% of the total bacterial population at the start of composting, possibly derived from the poultry manure or from the recycled process water used for straw wetting. Several strains of *Acinetobacter* were also dominant (20–30% of overall population) during the early pre-wet phase, while the subsequent dominant taxa were *Solibacillus* and *Comamonas*, followed by *Pseudomonas* and *Bacillus*, largely mesophilic organisms which are characteristic of the straw and field origin of the raw materials. As Phase I progressed, the dominant taxa were *Bacillus, Paenibacillus* and uncharacterized Clostridia and Proteobacteria, followed by *Ruminofilibacter*, which made up to 20% of bacteria present in mid-Phase I. The frequent succession in dominant organisms presumably reflected the depletion of preferred C sources for each species from the compost, and the rapidly changing environmental conditions as the temperature of the compost increased.

The second distinctive period in the composting process was the end of Phase I/start of Phase II. At this point, species evenness increased, and individual organisms became less dominant, reflecting the increase in diversity seen at the end of Phase I. *Thermus* made up 6% of the bacterial community together with a thermophilic *Sphingobacterium* and a species of *Luteimonas*.

The bacterial community changed completely after pasteurization with the rapid variation seen earlier in composting replaced by comparative stability. The dominant organism throughout conditioning and spawn run was the heterotrophic nitrifier, *Pseudoxanthomonas taiwanensis*. The population size of this species increased quickly after pasteurization, and it made up about 15% of the bacterial compost community from this point until almost the end of the spawn run.

Succession of fungal communities in mushroom compost

Fungal community dynamics during composting followed a similar pattern to that found for bacteria, although the overall fungal diversity was much lower (only 340 OTUs found with ITS3-ITS4 amplification) (Table S5). In addition, it was not possible to measure the diversity of compost fungi accurately in later samples than mid-spawn run, because *Agaricus* dominated the amplicon obtained from these timepoints. As with bacteria, the early stages of composting were characterized by a rapid succession of different taxa, responding to changing nutrient availability and temperature conditions. Although the wheat straw feedstock carried a range of different fungi (mainly in the families *Pleosporaceae, Rhytismataceae* and *Davidiellaceae*), the compost in pre-wet was dominated by the pleospore, *Lewia infectoria*, and gradually replaced by an uncharacterized ascomycete and *Myceliophthora* (Fig. 3). During Phase I there was a transient increase in *Thermomyces*, followed by rapid colonization of the compost by the thermophilic fungus, *Mycothermus thermophilus*, which made up more than 80% of the fungal population in the compost from mid-Phase II until it was overgrown by *Agaricus* in mid-spawn run.

Microbial diversity variability among compost yards

All the composting yards sampled used the same fundamental composting process and the same main raw materials (poultry manure, wheat straw and gypsum), but there were differences among them in scale and process details (Table S1). Phase I compost production varied from 80 to 1600 t per crop, and three of the yards provided additional N either as inorganic supplements (urea or ammonium sulfate) or organic materials (e.g., cottonseed meal or soybean meal). The length of the pre-wetting period varied (2–14 days), as did the length of Phase I (9–21 days). The Phase II process, by contrast, was relatively similar at all yards.

To determine the effect of this variation across compost yards, bacterial community composition was measured in end-Phase I and end-Phase II compost at five yards from four Australian states (Fig. 4). At the end of Phase I, *Bacillaceae* was the only taxon consistently present in all compost yards with considerable differences in all other taxa. Yards A and B revealed similar bacterial communities, with a high proportion of *Ruminicoccaceae* and *Limnochordaceae*, and thermophilic bacteria in the *Thermaceae* and *Bacillaceae* families (15–30%). Yard A had a much higher proportion of *Thermus* than Yard B, possibly because it is a larger enterprise, allowing a high temperature to be more easily maintained throughout the compost pile (Table S1). In Yards B, C and E, common families were *Cellvibrionaceae*, *Xanthomonodaceae* and *Flavobacteriaceae* (2–10%), while the bacterial profile for Yard D had a higher proportion of mesophilic taxa such as *Planococcaceae* and *Micrococcaceae* (~7–12%). This may be because Phase I was done outdoors and, as a consequence, lower temperatures were maintained for a longer period than for other yards.

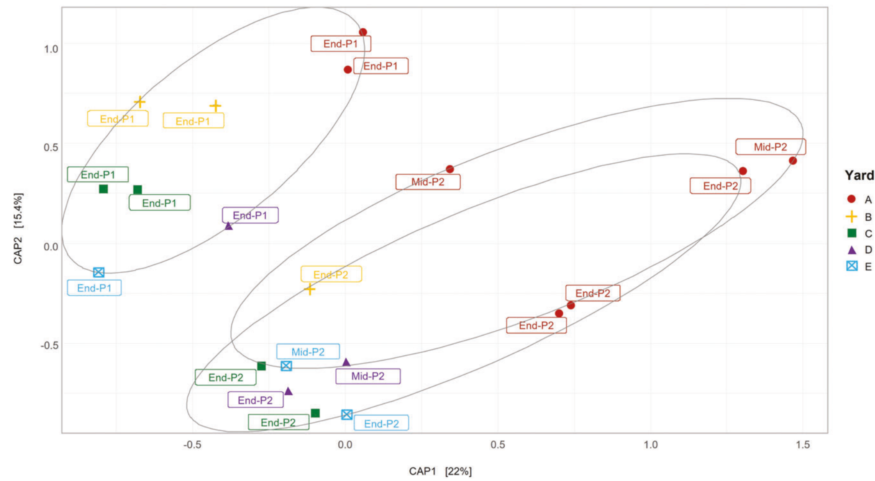

Fig. 5 Canonical analysis of principal components of the bacterial communities from five geographically distinct compost yards. Unweighted Unifrac statistical analysis was used to use to measure the differences in bacterial communities in compost yards (CAP1) and phases (CAP2). Ellipses contain samples from end-Phase 1, mid-Phase 2, and end-Phase 3.

Bacterial profiles were similar in Phase II for all compost yards despite variations in pasteurization time and practice (i.e., pasteurization in bulk or in trays). The common taxa across all yards were *Xanthomonodaceae* (mainly *Pseudoxanthomonas* (2–31%)) and *Streptosporagiaceae* (*Thermopolyspora* (1–2.5%)). In Yards B, C and E, a smaller proportion of *Pseudoxanthomonas* (2–10%) in the bacterial profile corresponded with a larger proportion of other thermophilic taxa such as *Thermobacillus* (1%), *Thermopolyspora* (2–4%) and *Truepera* (2–4%) (Table S3). Although the *Xanthomonadaceae* family was not dominant in Yard E (Fig. 4), *Pseudoxanthomonas* was one of the top 10 genera by the end of Phase II at this yard (Table S3).

Bacterial diversity changes in a similar manner during composting at all yards

An unweighted Unifrac distance metric was used to compare bacterial communities among compost yards and phases (Fig. 5). The succession of different bacterial communities throughout the composting process followed the same general pattern in all compost yards. This was indicated with end-Phase I samples at the top left of the plot, progressing to end-Phase II at the bottom right (Fig. 5). The bacterial community at end-Phase I was significantly different from that in the mid- and end-Phase II (PERMANOVA: $F = 2.4399$, $R^2 = 0.1764$, $p < 0.05$, d.f. = 3). The bacterial communities in Yard A clustered separately from other yards in the ordination plot, presumably due to the combination of large operational scale and indoor Phase I processing (Table S1).

DISCUSSION

Reproducible, commercial yields of button mushrooms can only be achieved if consistent compost quality is guaranteed. Because composting is a microbial process, we hypothesized that the microbial communities responsible for transformation of a uniform composting substrate (wheat straw/poultry manure) into productive compost will also show a degree of consistency. In this study, we examined fungal and bacterial communities present in the compost throughout the composting process (19 time points, from raw materials to commencement of cropping), to determine the succession of microbes present. Importantly, we also compared the compost bacterial communities in five geographically separate composting facilities across south-eastern Australia, to determine whether a consistent composting process is reflected in similar microbial communities. This study extends recent reports which have focussed on individual composting yards [16, 17, 19], and also builds on work done in previous decades, which compared mushroom yields and quality at a large number of facilities over multiple years [43–45].

The detailed timeline study revealed rapid succession of both bacterial and fungal taxa throughout Phase I (Fig. 3). The dominant bacterial taxa included soil and plant-related bacteria like *Acinetobacter* and *Bacillus*, *Arcobacter* (presumably derived from the poultry manure), and several other genera. Fungal diversity also varied, with *Lewia* dominating initially (probably derived from the wheat straw, as it is commonly associated with cereals [46]) followed by *Myceliophthora*, a cellulose-degrading genus that has also been found in *Agaricus subrufescens* compost [47, 48]. In Phase II, the fungal community was entirely dominated by *Mycothermus thermophilus*. The Phase II bacterial community contained high levels of *P. taiwanensis*, which was the dominant taxon not only in the detailed timeline study (Fig. 3), but also in three out of five compost yards tested for comparison (Fig. 4). However, several other taxa were also consistently present in Phase II compost from all compost yards studied, particularly *Chelativorans*, *Pseudoxanthomonas* and *Thermopolyspora*. *Pseudoxanthomonas taiwanensis* has been identified as a key species in other mushroom composts [19, 25], including oyster mushroom compost [49], and in other cellulose degrading consortia [50]. *Pseudoxanthomonas taiwanensis* is also dominant in the thermophilic stage of compost preparation for oyster mushrooms, equivalent to Phase I [49], while Actinobacteria, such as *Thermopolyspora*, and Bacilli, such as *Thermobacillus* and *Ureibacillus*, dominate mature oyster mushroom compost [49].

Much of the cellulose breakdown occurs during Phase II [3, 16] and the dominance of *Pseudoxanthomonas* at this time suggests that it might play a significant role by boosting cellulose degradation. *Pseudoxanthomonas taiwanensis* is known to promote cellulose degradation in consortia used in biofuel production [50–52] but, paradoxically, it does not degrade cellulose when in pure culture, and it does not appear to harbor genes encoding cellulase. It has been suggested that its influence in consortia is due either to its production of β-glucosidase [50] or its contribution to acetate removal and pH control [51], but experiments to confirm this have been inconclusive. The role played by *Pseudoxanthomonas* in cellulose breakdown must therefore be involved with the other microbes and further work is needed to explore this complex relationship.

Where *Pseudoxanthomonas* (2–4%) was not the dominant organism in end-Phase II samples, there was a higher proportion of Actinobacteria (8–12%) and Bacilli (1–1.5%) in the bacterial

profile (Fig. 4). In Yards C and E, *Xanthomonadaceae* appeared in end-Phase I compost and the proportion was higher than in their respective end-Phase II samples (Fig. 4). The dominant actinomycete in Yards C and E was *Thermopolyspora* (Table S3). This pattern has been found in composts that use chemical N or other straw materials (e.g., alfalfa) as their main N source [19, 53]. For mushroom compost produced in China, for example, high throughput sequencing showed that *P. taiwanensis* was the dominant organism in mid- to end-Phase I samples and *Thermobispora*, an actinomycete, was the dominant organism in end-Phase II samples [19], with the proportion of Bacilli being significantly smaller compared to the actinomycete population [19]. Phase I compost for this study in China was done in windrows and details of the temperatures attained during the study were not provided [19], but it would seem likely that temperatures were substantially lower than the 80 °C reached in bunkers in Australian compost yards.

Actinobacteria and Bacilli are important in various composting systems [49, 54, 55]. *Thermobifida, Thermomonospora* and *Thermopolyspora* were among the most abundant of thermophilic Actinobacteria found both in this study and in other studies [19, 56, 57]. These genera are known for their cellulose degrading enzymes; *Thermopolyspora* and *Thermomonospora* produce hemicellulases [57–59] while *Thermobifida cellulolytica* is able to completely degrade cellulose [60]. *Thermopolyspora* dominated the actinomycete community of end-Phase II compost in this study and a similar result was found in end-Phase II compost derived from different straw types [56]. Actinobacteria and Bacilli also dominate in mature oyster mushroom compost [49]. *Geobacillus* and *Ureibacillus* have both been isolated from compost samples in this study (data not shown) as well as from other composts [61, 62]. These taxa are often found in cellulose degrading systems [63], particularly composts, due to their heat resistant, spore-forming nature and their highly active lignocellulolytic enzymes [64].

Another important genus in mushroom compost is the α-Proteobacterium *Chelatococcus* (family *Beijerinckiaceae*). In this study, *Chelatococcus daeguensis* was the most common species of this genus and was found in all yards sampled. *Chelatococcus daeguensis* is able to grow on several C sources, including cellobiose [65], and it has been proposed that *C. daeguensis* aids lignocellulose degradation by activating lignin breakdown [63].

From a technical point of view, the Phase I process was the most variable step among the compost yards studied. Phase I is a partially controlled process that takes place in an enclosed bunker or in ricked windrows. Compost temperatures are initially mesophilic (25–45 °C) and rise to thermophilic conditions (80 °C) [21, 66] as microbial activity increases, and is controlled by the air supply to the compost. Differences among facilities are likely to have occurred because older yards rely on mechanical turning of the compost for aeration, while newer compost yards provide additional aeration to the compost pile through an aerated floor (maintaining at least 5% oxygen concentration in the compost [21, 67, 68]). Phase I is complete when the ammonia concentration reaches levels of 150–800 ppm [69, 70], (due to proteolysis and ammonification) and the time required for this trigger ranged from 9–21 days in this study (Table S1). All these factors influence the succession of microorganisms that degrade the increasingly complex organic matter derived from raw material [17, 25, 26].

The variability in process in Phase II was much less than Phase I. Phase II composting is done in enclosed tunnels over 6 days, and temperature and oxygen supply are more closely controlled than during Phase I [71]. In contrast to Phase I composting, Phase II is initially thermophilic (60 °C) during pasteurization and decreases to mesophilic conditions (reduced from 55–25 °C) during conditioning. Following pasteurization, conditioning occurs with a slow decrease in temperature from 55–45 °C (then rapidly cooled to 25 °C for colonization by *A. bisporus*), which is the ideal temperature range for Actinobacteria and fungi to reassimilate free ammonia back into the compost [48, 69, 72]. Although all the yards studied showed very similar management of Phase II, the variation in composting scale (i.e., the size of the facility) and the peak temperatures achieved in Phase I also appear to be important in establishing the bacterial profile in Phase II.

When the abundance of *Pseudoxanthomonas* was low, the abundances of Actinobacteria and Bacilli were greater (Fig. 4). This was most likely due to the temperatures achieved and the process used in Phase I (Table S1). Phase I was done outdoors for Yards B–E and the temperature profiles were therefore more variable than the indoor process of Yard A (Table S1). However, due to the larger scale of Yard D compared to Yards B, C and E, larger compost piles may have been able to reach higher temperatures (Table S1). In studies of a range of composts that did not reach a peak of 80 °C (mushroom compost and partial green waste compost), the bacterial community favored more Actinomycetes, whereas when temperatures were greater than 80 °C, the bacterial profile had more Bacilli and *P. taiwanensis* [25, 54].

Although the dominant bacteria in the five compost yards sampled were clearly variable (Fig. 4), the overall bacterial communities for each phase clustered relatively closely together in the ordination plot (Fig. 5), suggesting a high degree of similarity. Bacterial diversity was clearly different between end-Phase I and Phase II, and all Phase I and Phase II communities followed the same pattern of change (Fig. 5). This suggests that despite the variability in Phase I composting, the Phase II composting process selects for bacteria that fulfill similar roles in transforming raw materials into the desired selective growth substrate for *A. bisporus*.

DATA AVAILABILITY

Sequencing data are available at NCBI SRA under BioProject PRJNA867030.

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AUTHOR CONTRIBUTIONS

MAK and TLB designed the study. MT and KS carried out the experimental work. MAK, MT and TLB prepared the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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MINI-REVIEW

Compost bacteria and fungi that influence growth and development of *Agaricus bisporus* and other commercial mushrooms

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Abstract

Mushrooms are an important food crop for many millions of people worldwide. The most important edible mushroom is the button mushroom (*Agaricus bisporus*), an excellent example of sustainable food production which is cultivated on a selective compost produced from recycled agricultural waste products. A diverse population of bacteria and fungi are involved throughout the production of *Agaricus*. A range of successional taxa convert the wheat straw into compost in the thermophilic composting process. These initially break down readily accessible compounds and release ammonia, and then assimilate cellulose and hemicellulose into compost microbial biomass that forms the primary source of nutrition for the *Agaricus* mycelium. This key process in composting is performed by a microbial consortium consisting of the thermophilic fungus *Mycothermus thermophilus* (*Scytalidium thermophilum*) and a range of thermophilic proteobacteria and actinobacteria, many of which have only recently been identified. Certain bacterial taxa have been shown to promote elongation of the *Agaricus* hyphae, and bacterial activity is required to induce production of the mushroom fruiting bodies during cropping. Attempts to isolate mushroom growth-promoting bacteria for commercial mushroom production have not yet been successful. Compost bacteria and fungi also cause economically important losses in the cropping process, causing a range of destructive diseases of mushroom hyphae and fruiting bodies. Recent advances in our understanding of the key bacteria and fungi in mushroom compost provide the potential to improve productivity of mushroom compost and to reduce the impact of crop disease.

Keywords *Agaricus bisporus* · Button mushroom · *Pleurotus* · Compost · Thermophilic fungi · *Pseudoxanthomonas* · *Mycothermus*

Introduction

Cultivated mushrooms are an important food source for many people around the world, with global production estimated at over 10 million tons per year (Food and Agriculture Organization of the United Nations 2014). Over two thirds of the production of edible mushrooms are harvested in mainland China, where mushrooms form a more traditional role in food and medicine than they do in many Western countries, and where they provide a living for over 25 million mushroom farmers (Zhang et al. 2014b). The most important edible mushroom genus grown commercially is *Agaricus* (mainly *Agaricus bisporus*, the button mushroom), which makes up about 30% of the global market (Royse 2014). Other important edible genera include *Pleurotus* (5–6 species of oyster mushrooms that are cultivated commercially), *Lentinula* (shiitake), *Auricularia* (3–4 species of woodear mushrooms), *Flammulina* (enoki), and *Volvariella* (paddy straw). Edible mushroom production is dominated by a few species where the technology for large-scale industrial cultivation has been optimized (Chang and Miles 2004), but in many countries large numbers of small-scale farms also exist, and there are increasing attempts to domesticate local wild mushrooms for production purposes (Mwai and Muchane 2016).

Cultivated mushrooms are saprophytes which grow by degrading natural lignocellulosic substrates, which are commonly available in large volumes as agricultural or industrial byproducts. The methods for commercial cultivation of

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mushrooms on these substrates can be divided into three broad groups. The first group includes cultivation of many wood-degrading mushrooms that were traditionally grown on wood logs or harvested from trees. Some of these, such as *Lentinula*, are now grown on artificial logs of compacted, sterilized sawdust, while others, such as *Flammulina*, *Pholiota* (nameko), or *Auricularia* are cultivated on a partly composted mixture of sawdust and other components (bran, straw, corncobs), which is sterilized at high temperature (121 °C) before inoculation with mycelium (Chang and Miles 2004; Sanchez 2010). Because of the rigorous sterilization process, these mushrooms are essentially grown in axenic culture. The second group of cultivation methods either uses uncomposted substrates directly, or uses partially composted substrates that have not been subjected to a rigorous sterilization process. This includes methods commonly used for *Pleurotus* and *Volvariella* species, though *Pleurotus* is also sometimes grown on sterilized sawdust substrates. These are fast-growing and adaptable genera capable of rapid bioconversion of a broad range of substrates (e.g., rice straw, bagasse, cornstalks, waste cotton, stalks, and leaves of bananas (Chang and Miles 2004; Thongklang and Luangharn 2016). The substrates are usually not sterilized before inoculation, though a pasteurization step may be included in the process, and the mushrooms therefore grow in competition with other microorganisms on the substrate. The most industrially complex process is the cultivation of *Agaricus*, which is grown on a pasteurized straw-based compost which requires lengthy preparation, but allows selective growth of the *Agaricus* mycelium over competitor organisms.

For mushrooms grown in fully sterilized substrates, such as *Lentinula*, the rate of mycelium growth is dependent on enzymatic degradation of lignocellulose by the mushroom itself and is independent of other microbes. Addition of particular bacteria or other fungi could potentially stimulate either growth or fruiting, but this has not been investigated in any depth. For *Pleurotus* and *Agaricus*, by contrast, growth of the mycelium and production of the commercial fruiting body are dependent not only on the mushroom itself, but also on bacteria and other fungi in the substrate. These bacteria and fungi play critical roles at several different stages of production (Fig. 1), including (i) conversion of the lignocellulose feedstocks into a selective, nutrient-rich compost for mushroom growth; (ii) interactions with the fungal mycelium during hyphal elongation and proliferation through the substrate; and (iii) induction of fruiting body formation during cropping. In addition, several bacterial and fungal taxa act as pathogens of the mushroom crop, causing either a reduction in yield or severe loss of quality.

This review will focus primarily on the importance of bacteria and fungi in mushroom compost during the production of *Agaricus bisporus* and *Pleurotus ostreatus*. Several excellent reviews are available that discuss bacterial-fungal interactions in a range of other environmental, agricultural, and clinical areas (Frey-Klett et al. 2011; Kobayashi and Crouch 2009; Scherlach et al. 2013). A number of other studies provide overviews of the composting of general municipal and agricultural wastes (Chandna et al. 2013; De Gannes et al. 2013a, b; Hultman et al. 2010; Partanen et al. 2010), and highlight the influence of feedstocks and process parameters on fungal and bacterial diversity and succession in the resulting composts (Neher et al. 2013).

Diversity and succession of bacteria and fungi in mushroom compost

A. bisporus is commercially grown on a composted substrate prepared in a thermophilic, microbial process from wheat straw and/or horse stable bedding, nitrogen-containing additives, the most common which are poultry manure, seed meal, or synthetic nitrogen (urea or ammonium nitrate), and gypsum (Chang and Miles 2004; Royse and Beelman 2016; Straatsma et al. 2000) (Fig. 1). The wheat straw is usually soaked for 3–10 days before mixing with the other feedstocks (Noble and Gaze 1996), and then subjected to a period of aerobic, thermophilic composting (phase I) during which the compost temperature rapidly rises to 80 °C due to microbial activity (Straatsma et al. 2000; Zhang et al. 2014a). Phase I can take up to 14 days to complete (Noble et al. 2002) but can be completed as quickly as 6 days (Weil et al. 2013), and serves primarily for growth of the microbial population at the expense of soluble components of the feedstocks, since there is relatively little decrease in the total content of complex carbohydrates (cellulose, hemicellulose) or lignin (Jurak et al. 2015). In phase II, the compost is held at 58–60 °C for 2 days in tunnels that are designed to provide uniform temperature and airflow into the compost (Noble and Gaze 1996), followed by a "conditioning" or "curing" period, in which the compost is maintained at 48–51 °C for 2–3 days (Straatsma et al. 2000). This is a period of intense microbial activity, and by the end of phase II, 50–60% of the cellulose and hemicellulose in the original feedstocks has been degraded (Jurak et al. 2015). During the same period, the excess ammonia released during the thermophilic phase has been assimilated by the microbial biomass in the compost (Miller et al. 1991; Wiegant et al. 1992). The microbial community present at the end of phase II represents a climax community, into which *A. bisporus* is introduced, usually as grain spawn grown on millet or rye. The mycelium of *Agaricus* takes approximately 16 days to fully colonize the mature compost (Jurak 2015; Royse and Beelman 2016), initially utilizing bacterial and fungal biomass as a key source of nutrition and then progressively breaking down over 50% of the lignin (Jurak et al. 2015).

Although it is possible to grow *A. bisporus* mycelium on non-composted wheat straw, yields are low and the process is

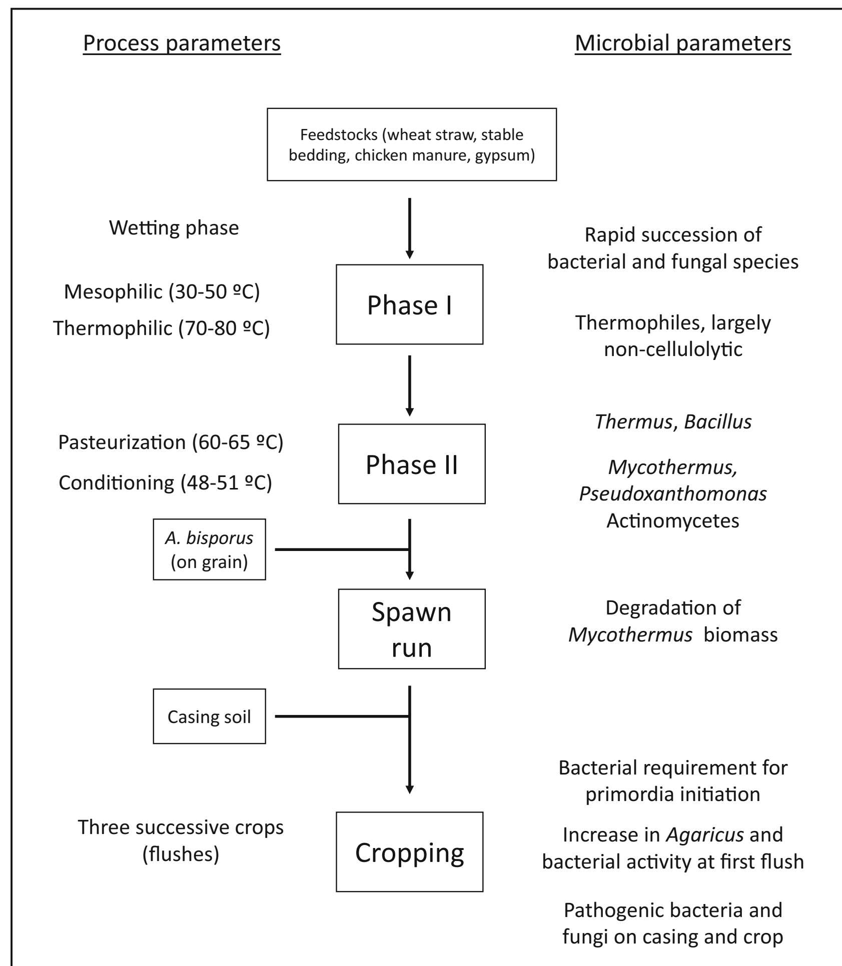

Fig. 1 Overview of composting and cropping for *Agaricus bisporus* highlighting the importance of bacteria and fungi in the process

not used commercially (Mamiro et al. 2007). The composting process provides specificity for *Agaricus* cultivation because it converts the wheat straw into a mixture of microbial biomass and humus-lignin complexes which are not available to competing fungi, but which *Agaricus* can access by degrading living and dead thermophilic fungal and bacterial biomass (Bilay and Lelley 1997; Fermor et al. 1991; Straatsma et al. 1994b; Vos et al. 2017). The selectivity for *Agaricus* can be removed by artificial lysis of phase II compost biomass (chemical treatment or prolonged high temperatures), which allows many other contaminant ascomycetes to grow. However, if the sterilized compost is reconditioned by adding microbes that bind the released nutrients into fresh microbial biomass, then the selectivity for *Agaricus* can be restored (Ross and Harris 1983a; Straatsma et al. 1989).

The microbes in mushroom compost are introduced with the feedstocks, though they can also be enriched by the use of recycled compost leachate (sometimes called "goody water") in the wetting process (Kertesz and Safianowicz 2015). A clear succession is seen in the mushroom compost community, which has been studied by cultivation-based analysis (Hayes 1968; Ryckeboer et al. 2003; Singh et al. 2012; Siyoum et al. 2016) and by a number of cultivation-independent techniques, including fingerprinting methods (Wang et al. 2016) coupled to DNA sequencing methods (Siyoum et al. 2016; Szekely et al. 2009; Vajna et al. 2010, 2012) and, most recently, by metagenomic or amplicon sequencing of bacterial and fungal communities (Kertesz et al. 2016; Langarica-Fuentes et al. 2014; McGee et al. 2017; Souza et al. 2014; Zhang et al. 2014a). Most of these studies have been done on *Agaricus* compost, though partially composted *Pleurotus* compost has also been examined (Vajna et al. 2010, 2012). Cultivation-based analysis of phase I compost has yielded largely actinobacterial and *Bacillus* isolates [reviewed in (Ryckeboer et al. 2003)], but this method provides a very limited window on the microbial diversity present, since many compost bacteria and fungi are not readily cultivable, Many studies have also focused on single time points within the composting process, providing a limited overview. More recent DNA sequencing studies have provided evidence for the presence of a wealth of other microbes in compost and their succession, with a broad range of phyla involved (Fig. 2). The most dramatic changes in microbial populations in compost occur during the initial wetting period

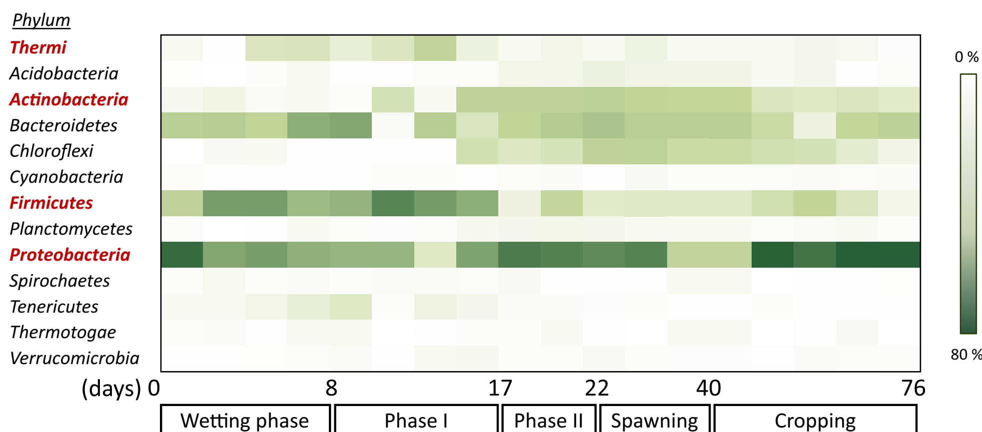

Fig. 2 Bacterial phylum succession in mushroom compost during composting, spawn run, and cropping of *Agaricus bisporus*. Bacterial communities were determined by Illumina Miseq sequencing of the 16S rRNA gene (Kertesz et al. 2016). The heat map shows relative sequence abundance of the indicated phyla at different stages of the process

and during phase I (Kertesz et al. 2016). This period sees rapid assimilation of easily accessible nutrients such as free sugars and amino acids from the compost, and the changes in the most abundant bacteria probably reflect the rapid succession of bacteria and fungi that can use these compounds. Initially, these are mesophilic pioneer organisms such as *Solibacillus*, *Comamonas*, *Acinetobacter*, *Pseudomonas*, and *Sphingomonas* (Kertesz et al. 2016; Vajna et al. 2012).

As temperatures begin to increase in phase I, peaks are seen in populations of thermophilic *Bacillus*, *Paenibacillus*, and uncharacterized Clostridia and Proteobacteria. The most abundant actinobacteria present are *Corynebacterium* and *Streptomyces* (Zhang et al. 2014a) and, although they make up only a small fraction of the total bacterial population, analysis of the cellulase gene diversity reveals that these actinobacteria are cellulolytic (Zhang et al. 2014a). The taxon evenness in the compost increases to reach a maximum at the end of phase I, with an increase in thermophilic taxa. *Thermus thermophilus* is a key member of the bacterial population at this point (Kertesz et al. 2016; Szekely et al. 2009), and Thermi are relatively important, though they disappear again later during composting (Fig. 2).

The dynamics of fungal diversity are similar to the bacteria changes during phase I. Initial pioneers such as *Lewia*, *Rhizomucor*, and *Aspergillus* are overgrown by *Talaromyces*, *Thermomyces*, *Thermus*, and unclassified taxa as temperature and pH rise (Kertesz et al. 2016; Straatsma et al. 1994b). Most of these organisms are not cellulolytic although some of them will break down hemicellulose, and cellulose and hemicellulose are indeed only marginally depleted during phase I (Jurak et al. 2014). Pioneer fungi grow by utilizing other carbon sources in the compost, and once these alternative carbon sources are exhausted, the fungal population is replaced by cellulose degraders such as *Mycothermus thermophilus* and *Chaetomium thermophilum*, with *M. thermophilus* being the climax species in phase II compost (Kertesz et al. 2016; Souza et al. 2014).

Mycothermus (Scytalidium) and other thermophilic microbes—nutrition for *Agaricus*

During phase II, the microbial community dynamics change completely with rapid succession of different genera appearing in phase I being replaced by a comparatively stable microbial community during conditioning. The dominant fungal species during the conditioning process is *M. thermophilus* [previously *Scytalidium thermophilum* or *Torula thermophila* (Natvig et al. 2015)]. This species is a thermophilic, cellulolytic ascomycete which is a dominant component of many composting systems and plays an important role in degradation of polymeric carbohydrates. Isolates of this species from compost secrete a suite of over 60 different cellulases, hemicellulases, and other glycosyl hydrolases (Basotra et al. 2016).

The importance of thermophilic bacteria and fungi in mushroom compost production was recognized very early (Chanter and Spencer 1974; Eicker 1977; Stanek 1972). Thermophilic fungi, in particular, grow rapidly during the conditioning period, removing free nutrients from the compost and assimilating the ammonia released by ammonification (Ross and Harris 1983b). This physiological activity provides a selective environment for growth of *Agaricus* by immobilizing nutrients in a form unavailable to competitor molds (Ross and Harris 1983a). Unlike competing ascomycetes, the *Agaricus* mycelium aggressively decomposes both living and dead bacterial and fungal biomass to obtain the nutrients it requires (Fermor and Wood 1991). This has been shown by growth of *Agaricus* on [^{14}C]-labeled *Bacillus subtilis* cells leading to direct uptake of the isotope into the fungal mycelium (Fermor et al. 1991). *Agaricus* also disintegrated *Mycothermus* cultures on agar medium even through a layer of cellophane, causing complete loss of viability in the latter fungus (Op den Camp et al. 1990). In a cropping setting, the amount of living bacterial biomass in phase II compost (measured as phospholipid fatty acids) decreases by about 75% after addition of *Agaricus* and the

abundance of thermophilic fungal biomass also decreases dramatically (Vos et al. 2017). The mycelium of *Agaricus* seems to be partly selective for *Mycothermus* and does not degrade other thermophilic fungi so effectively (Straatsma et al. 1994b), but the reasons for this are not known.

Bacterial diversity in phase II compost has traditionally been thought to be dominated by cellulolytic actinomycetes and bacilli (Ryckeboer et al. 2003). Recent cultivation-independent studies have shown a peak in *Actinobacteria* at this stage (Fig. 2) and have identified *Thermomonospora*, *Thermobispora*, *Thermopolyspora*, *Thermobifida*, and *Microbispora* as key genera (Silva et al. 2009; Szekely et al. 2009; Vajna et al. 2012; Zhang et al. 2014a). However, the most abundant bacterial taxon in both *Agaricus* and *Pleurotus* compost is *Pseudoxanthomonas taiwanensis*, together with *Thermus* and several bacilli (*Bacillus*, *Geobacillus*, *Ureibacillus*) (Kertesz et al. 2016; Vajna et al. 2012).

P. taiwanensis is only present in low numbers in phase I, because although it is thermophilic, its optimum growth is at 50 °C, and it does not grow above 65 °C (Chen et al. 2002). The population of *P. taiwanensis* increases as the temperature falls during compost conditioning and it is the most abundant bacterial species in mature compost (Kertesz et al. 2016; Szekely et al. 2009; Vajna et al. 2012). As a heterotrophic nitrifier, it is able to convert ammonia into N_2O (Chen et al. 2002), but how important this activity is in removing the abundant ammonia in phase II compost is not yet known. Unexpectedly, *P. taiwanensis* does not appear to break down cellulose, though it produces β-glucosidase and can utilize cellobiose (Chen et al. 2002; Kato et al. 2005). Nevertheless, it has been detected repeatedly as an essential component of stable cellulose-degrading consortia isolated from a variety of sources (Du et al. 2015; Kato et al. 2004, 2005; Wang et al. 2011), and efficient degradation of lignocellulose in bioethanol production, for example, relied on optimization of the *P. taiwanensis* abundance (Du et al. 2015). In one of these consortia, it seems to play its essential role by interacting with a cellulolytic species of *Clostridium* (Kato et al. 2004, 2005), and in mushroom compost, it may well be interacting in a similar way with *Mycothermus.* This is underlined by the fact that its abundance in phase II compost appears to increase in parallel to that of *Mycothermus* (Kertesz et al. 2016), but further studies are required to confirm the relevance of this finding.

Because the presence of thermophilic fungi can promote the growth rate of *Agaricus* mycelium up to two fold (Straatsma et al. 1994a), this fungal group, and in particular *M. thermophilus*, has been studied extensively as an inoculum to promote compost preparation and accelerate *Agaricus* growth (Bilay 2000; Sanchez et al. 2008; Sanchez and Royse 2009; Straatsma et al. 1994a; Wiegant et al. 1992). Although *M. thermophilus* has shown the greatest stimulation of *Agaricus* growth of a range of fungi tested, in its absence several other fungal species had similar effects (Straatsma et al. 1994b). Anecdotal evidence from mushroom farmers suggests that inoculation with *Mycothermus* may stimulate compost productivity on a farm scale, but in general, it is not necessary to add *Mycothermus* unless the compost has been pasteurized at too high a temperature, since naturally occurring strains of the fungus are always present (Ross and Harris 1983b; Straatsma et al. 1994a).

A number of studies have examined the addition of bacterial inocula to increase compost productivity and mushroom yield (Table 1). Inoculation with *Bacillus megaterium* or a thermophilic strain of *Staphylococcus* has been shown to promote mushroom production and advance cropping by several days (Ahlawat and Vijay 2010) and *Pseudomonas putida* also promoted hyphal extension of *Agaricus* in vitro (Rainey 1991). However, in another study, addition of *B. subtilis* or *B. megaterium* to compost along with the *Agaricus* spawn did not affect yield (Ekinci and Dursun 2014). A more thorough search for bacteria that promote compost productivity was also unsuccessful (Straatsma et al. 1994b). It should be noted, however, that these were all small-scale screening experiments using compost that was first sterilized at high temperature to selectively inactivate thermophilic fungi. It seems likely that promotion of *Agaricus* may require an interacting consortium of both bacteria and fungi for effective lignocellulose breakdown.

Bacterial involvement in formation of mushroom primordia and fruiting bodies

Formation of mushroom fruiting bodies is controlled by a range of environmental factors. Fruiting body primordia are initiated in response to a reduction in temperature compared with mycelial growth conditions and greater aeration to reduce levels of CO_2 (Chang and Miles 2004). The optimum ranges of these two factors vary for different species of mushrooms (Stamets and Chilton 1983). Transcriptomic studies of *Agaricus* have shown that a reduction in temperature is essential for further differentiation of primordia, and the level of CO_2 exerts quantitative control on the number of fruiting bodies formed (Eastwood et al. 2013). Many mushroom species also show a requirement for a change in nutrient availability, with high levels of nutrition favoring mycelial growth over primordia formation (Chang and Miles 2004). Nutrient supply governs outgrowth of the primordia, with the appearance of mushroom fruiting bodies in separate flushes governed by depletion of specific nutrients required by the primordia (Straatsma et al. 2013). Some species also require a change in pH or light conditions (Chang and Miles 2004).

For commercial cultivation of *Agaricus*, formation of fruiting bodies is induced by overlaying the colonized compost with a layer of "casing," usually a mixture of peat and

Table 1 Promotion of mushroom growth by bacterial and fungal inocula

Mushroom species	Bacteria/fungi used	Source of inoculum	Applied to casing/compost	Reported effect	Reference
Agaricus bisporus	*Bacillus megaterium*, *Staphylococcus* sp.	Compost	Compost	Increased yield, inhibition of pathogens	(Ahlawat and Vijay 2010)
A. bisporus	*Scytalidium thermophilum*	Compost	Compost	Faster mycelial growth, increased yield	(Coello-Castillo et al. 2009; Straatsma et al. 1994a; Wiegant et al. 1992)
A. bisporus	*B. subtilis*, *B. megaterium*	Compost	Compost	No effect	(Ekinci and Dursun 2014)
A. bisporus	*Pseudomonas putida*	Casing	Casing	Increased primordia formation	(Fermor et al. 2000)
A. bisporus	*P. putida*	Casing	Casing	Primordium induction due to ACC deaminase production	(Chen et al. 2013)
A. bisporus	*P. putida*	Soil	Casing	Yield increase	(Zarenejad et al. 2012)
A. bisporus	*Arthrobacter terregens*, *Rhizobium meliloti*, *Agrobacterium rhizogenes*, *B. megaterium*	Casing	Casing	Increased pinning	(Park and Agnihotri 1969)
A. blazei	Various Actinomycetales	Casing	Casing	Increased mycelial growth and yield	(Young et al. 2013)
A. blazei	*Exiguobacterium* sp., *Microbacterium esteraromaticum*, *P. resinovorans*	Casing	Casing	Increased yield	(Young et al. 2012)
A. bitorquis	*B. megaterium*, *Alcaligenes faecalis*, *B. circulans B. thuringiensis*	Casing	Casing	Increased yield	(Ahlawat and Rai 2000)
A. bisporus	*Mycothermus thermophilus*	n/a	Spawn	Increased mycelial growth	(Bilay 2000)
Pleurotus ostreatus	*Bradyrhizobium japonicum*	n/a	Spawn	Increased mycelial growth	(Zhu et al. 2013)
Pleurotus eryngii	*Pseudomonas* sp. P7014	n/a	Bottle culture	Increased mycelial growth	(Kim et al. 2008)
P. ostreatus	*P. putida*	Spent mushroom compost	In vitro	Accelerated primordial development	(Cho et al. 2003)
A. bisporus	*P. putida*	Casing	In vitro	Increased mycelial growth	(Rainey 1991)

lime. This casing layer contains a diverse bacterial population, and the presence of these bacteria is essential for primordia formation as fruiting does not occur with sterilized casing (Eger 1972; Hayes et al. 1969). Initiation of primordia in autoclaved or fumigated casing can be partially restored by addition of a bacterial inoculum (Eger 1972) with the best studied examples being *P. putida* or a related pseudomonad (Colauto et al. 2016; Hayes et al. 1969; Rainey et al. 1990). Alternatively, addition of adsorptive carbon-based materials such as activated charcoal will also restore fruit body formation (Noble et al. 2003). These results suggested that the stimulatory role of the bacteria is to remove an inhibitor of primordia formation. This compound has been identified as 1-octen-3-ol (Noble et al. 2009), which is a volatile compound produced by the *Agaricus* mycelium, that controls the early differentiation of vegetative hyphae to multicellular knots (Eastwood et al. 2013). A considerable proportion of bacterial isolates from the casing layer are were found to be tolerant to high levels of this compound and many of these isolates were also able to promote mushroom yields by up to 10% (Zarenejad et al. 2012). 1-Octen-3-ol is also important for other mushroom species; it is the dominant flavor component in *Pleurotus* (Misharina et al. 2009; Venkateshwarlu et al. 1999), and bacteria isolated from *Pleurotus* growing on Pangola grass (including *Bacillus cereus*, *B. megaterium*, *Enterobacter asburiae*, *Enterobacter cloacae*, *Kurthia gibsonii*, *Pseudomonas pseudoalcaligenes*, and *Meyerozyma guilliermondii*), were found to grow on 1-octen-3-ol and both

promote mycelial growth and induce fruiting of *P. ostreatus* in vitro (Torres-Ruiz et al. 2016).

Other bacterial metabolic pathways may also be important for stimulation of mushroom growth. For example, an ACC deaminase-producing strain of *P. putida* stimulated primordia formation in *Agaricus* but this property was lost in knockout mutants in the ACC deaminase gene (Chen et al. 2013). This suggests that ethylene, which is produced by *Agaricus* hyphae (Turner et al. 1975), acts as an inhibitor of mycelial growth and primordia formation (Zhang et al. 2016), and that *P. putida* can encourage *Agaricus* fruiting by lowering the levels of ethylene produced by *Agaricus*.

The bacterial population in casing increases dramatically after initiation of the first primordia but decreases during later flushes (Cai et al. 2009; Pardo et al. 2002). The species present are mostly proteobacteria related to *Pseudomonas*, *Pedobacter*, and *Caulobacter* (Fermor et al. 2000; Kertesz et al. 2016; Siyoum et al. 2010). As bacteria are required for initiation and growth of mushroom fruiting bodies, a number of investigators have screened compost or casing for "mushroom growth-promoting bacteria" that promote either mycelial growth or fruiting body formation. Most of the organisms identified have been applied to casing in an attempt to stimulate fruiting, and most of the growth-promoting strains found were *Pseudomonas* or *Bacillus* species (Table 1). The prevalence of these two genera probably reflects the culturing strategies used, since cultivation-independent methods reveal a very high diversity of bacteria present, many of which are uncharacterized (Kertesz et al. 2016; Siyoum et al. 2010). There has been little systematic study of the mechanisms by which inocula stimulate mycelial extension or initiation of primordia, or indeed whether they actively colonize *Agaricus* hyphae or are active on the casing itself.

Not unexpectedly, the fungal community found in button mushroom casing is greater than 90% *A. bisporus* (McGee et al. 2017), but over 200 other fungal species have also been detected, with highest diversity during first flush. *Mycothermus* is not detected at all, though other thermophilic fungi such as *Thermomyces* and *Myceliophthora* are still present, confirming the role of *Mycothermus* as nutrient source for growth of *Agaricus*. The dominant known taxa in casing are *Lecanicillium fungicola*, *Thermomyces lanuginosus*, *Aspergillus* spp., *Myceliophthora* spp., *Sordaria* spp., *Candida subhashii*, *Paecilomyces niveus*, and *Cercophora* spp. (McGee et al. 2017). The overall abundance of unknown fungal taxa is more than 20-fold higher than known taxa (excluding *Agaricus*) (McGee et al. 2017) suggesting that much remains to be learnt about the role of these fungi in this part of mushroom production. Interestingly, *Agaricus* activity (measured as ITS region cDNA abundance) reaches a maximum at first flush and then decreases substantially, and a range of unclassified taxa dominate the cDNA community for the remainder of the cropping period. During this period, the active taxa include *L. fungicola*, the causal agent of dry bubble disease (see below), suggesting that it can co-exist with *Agaricus* without causing disease, and that disease induction may require specific environmental factors (McGee et al. 2017).

Fungal and bacterial pathogens of *Agaricus*

While many of the bacteria and fungi described in the previous section have been shown to promote hyphal elongation or fruiting body formation of *Agaricus*, there has been little work to distinguish whether these organisms are actively colonizing the surface of the hyphae or living in the casing itself. Mushroom fruiting bodies, by contrast, have their own distinctive microflora. The cultivable population of microorganisms on button mushroom fruiting bodies includes pseudomonads, bacilli, and coagulase-negative staphylococci, together with the yeast *Rhodotorula* and several species of actinomycetes (Rossouw and Korsten 2017; Xiang et al. 2017). While the levels of human pathogens found on fruiting bodies are low (Venturini et al. 2011), bacterial contamination can cause postharvest deterioration (Beelman et al. 1989). A specific study of enterobacteria on button mushrooms at the point of harvest has revealed a considerable load of *Ewingella americana* (Reyes et al. 2004), which was confirmed in a recent report on microbial succession on healthy mushrooms at point of harvest (Siyoum et al. 2016). This organism is a potential human pathogen (Hassan et al. 2012), but it is better known as the cause of mushroom stipe necrosis in both *Agaricus* and *Pleurotus* (Gonzalez et al. 2012). The presence of *E. americana* on healthy postharvest mushrooms highlights the susceptibility of edible mushrooms to a variety of microbial diseases that can devastate crop production (Largeteau and Savoie 2010).

The most important of these are three fungal diseases, two of which have been reviewed recently (Berendsen et al. 2010; Carrasco et al. 2017). Cobweb disease (*Cladobotryum dendroides*) grows as a web-like mycelium over the surface of the fruiting bodies and produces large numbers of conidia that are readily dispersed by aerial means (Carrasco et al. 2017). Dry bubble disease (*L. fungicola*) produces severe outbreaks leading to formation of misshapen sporophores (Berendsen et al. 2010), and a similar effect is seen for wet bubble disease (*Mycogone perniciosa*), although this disease is less widespread and has not been studied as extensively (Khanna et al. 2003). Green mold disease (*Trichoderma aggressivum*) caused extensive crop losses in both America and Europe in the 1990s and continues to be a problematic disease worldwide. This disease is caused by two slightly different strains in America and Europe (*T. aggressivum f. aggressivum* and *T. aggressivum f. europaeum*) (Samuels et al. 2002), although the American pathovar has recently also been reported in Europe (Hatvani et al. 2017). *Pleurotus* is

also affected by green mold disease, but the disease is caused by a related but phylogenetically different species of *Trichoderma* (Kredics et al. 2009). Spores of both *L. fungicola* and *T. aggressivum* are sticky in nature, and are therefore mainly transmitted by insect or human vectors or in water (Fletcher and Gaze 2008). Interestingly, spore germination and growth of *L. fungicola*, *M. perniciosa*, and *T. aggressivum* are inhibited by 1-octen-3-ol, the *Agaricus* metabolite described previously that acts as a potent inhibitor of fruiting body formation, possibly allowing the pathogens to time their growth to coincide with appearance of the mushroom fruiting bodies (Berendsen et al. 2013).

Pseudomonas species play a variety of different roles in mushroom casing. As described above, *P. putida* has been identified as the key casing organism that breaks down 1-octen-3-ol and induces fruiting body formation (Noble et al. 2009). *Pseudomonas* isolates have also been shown to antagonize *Lecanicillium* in casing, competing for iron and releasing antifungal compounds, though this is not sufficient to protect *Agaricus* effectively against dry bubble disease (Berendsen et al. 2012). *Pseudomonas* also cause a range of blotch diseases of *Agaricus* which cause severe crop losses. Brown blotch disease is caused by *Pseudomonas tolaasii*, a species which is endemic in compost and induces symptoms through production of an extracellular lipodepsipeptide toxin, tolaasin [see reviews by (Largeteau and Savoie 2010) and (Soler-Rivas et al. 1999)]. *Pseudomonas reactans* causes similar symptoms, releasing a related but distinct toxin to that produced by *P. tolaasii* (Wells et al. 1996). Because *P. tolaasii* is ubiquitous in the compost environment, it is very difficult to control, since it can switch rapidly between nonvirulent and virulent forms in response to environmental changes and possibly also in response to metabolites produced by *Agaricus* (Largeteau and Savoie 2010). *P. tolaasii* also attacks *Pleurotus*, causing yellow discolorations (Lo Cantore and Iacobellis 2014) and *Flammulina* (Han et al. 2012), causing black rot. Several other *Pseudomonas* species also cause commercially important diseases. *Pseudomonas gingeri* is the main causal agent of ginger blotch (Wong et al. 1982), and many different pseudomonads have similar effects, causing a range of discolorations (Godfrey et al. 2001). *Pseudomonas agarici* is responsible for drippy gill disease, degrading the extracellular matrix of the fruiting body and producing droplets of bacterial ooze on the lamellae of *Agaricus* (Gill and Cole 2000). A range of soft rot diseases of mushrooms are also caused by bacterial pathogens. These include *Burkholderia gladioli pv agaricicola*, which attacks a range of different edible mushroom species (Chowdhury and Heinemann 2006; Gill and Tsuneda 1997; Lee et al. 2010), *Janthinobacterium agaricidamnosum*, which causes soft rot in *Agaricus* (Chowdhury and Heinemann 2006; Lincoln et al. 1999), and *Pantoea* species that affect *Pleurotus* (Kim et al. 2015).

Control of these bacterial and fungal mushroom diseases is essential for the mushroom industry, as outbreaks can destroy a large proportion of the crop. Effective prevention has traditionally relied on maintaining good production hygiene, together with the strategic use of biocides and antifungals (Fletcher and Gaze 2008). A major restriction is that only a limited range of products have been approved for application onto mushroom crops. Alternative methods that are being developed include the use of essential oils and antagonistic bacterial species as biocontrol agents (Berendsen et al. 2012; Sokovic and van Griensven 2006), but improved molecular methods for early detection of infection and the selection of resistant mushroom varieties (Savoie et al. 2016) are also important in reducing the impact of these widespread pathogens.

Conclusions and outlook

Research into mushroom compost goes back at least to the work of Waksman in the early 1930s (Waksman and Nissen 1932), with the aim of optimizing microbiological and process parameters to maximize mushroom yields. Most of our present understanding of mushroom compost microbiology has come from cultivating isolates of thermophilic and cellulolytic bacteria and fungi from compost, and it is only recently that sequencing efforts have revealed that some of the most abundant and important organisms in compost have been overlooked by this method. High-yielding sustainable production of edible mushrooms is currently primarily hampered by inconsistency of the compost, caused by variability in the quality and composition of the feedstocks, and by changes in the microbial communities present. Our improved understanding of the microbiology of compost provides renewed potential to design consortia of bacteria and fungi that can be used in bioaugmentation to optimize composting of lower quality feedstocks, and to identify and validate biomarkers that can be used to assess the quality of a compost before cropping commences. More detailed studies are also required to explore the relationship between microbial activity and diversity in compost and casing during cropping. Most of the nutrients in mushroom compost are left untouched by the mushroom crop, illustrated by the fact that spent mushroom compost is a valued soil conditioner. Manipulation of microbial activity and nutrient availability during cropping may allow higher yields of mushrooms in later crop flushes. Finally, a more thorough understanding of the biocontrol of mushroom pathogens has the potential to increase the quality of the mushrooms produced. Mushroom compost is a completely recycled product produced from agricultural wastes, and the fungi and bacteria that define it allow us to enjoy mushrooms as truly sustainable foods.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Review Article

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Bacterial interactions with the mycelium of the cultivated edible mushrooms *Agaricus bisporus* and *Pleurotus ostreatus*

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Abstract

The cultivated edible mushrooms *Agaricus bisporus* and *Pleurotus ostreatus* are valuable food crops and an important source of human nutrition. *Agaricus bisporus* is the dominant cultivated species in the western hemisphere and in Australia, while in Asian countries *P. ostreatus* is more prevalent. These two mushroom species are grown on fermented-pasteurized substrates, and bacteria and fungi play an important role in converting feedstocks into a selective medium for the mushroom mycelium. The mushrooms are usually introduced to the substrate as grain spawn, and the actively growing hyphae form a range of direct interactions with the diverse bacterial community in the substrate. Of these interactions, the most well studied is the removal of inhibitory volatile C8 compounds and ethylene by pseudomonads, which promotes mycelium growth and stimulates primordia formation of both *A. bisporus* and *P. ostreatus*. Bacterial biomass in the substrate is a significant nutrition source for the *A. bisporus* mycelium, both directly through bacteriolytic enzymes produced by *A. bisporus*, and indirectly through the action of extracellular bacterial enzymes, but this is less well studied for *P. ostreatus*. Apart from their role as a food source for the growing mycelium, bacteria also form extensive interactions with the mycelium of *A. bisporus* and *P. ostreatus*, by means other than those of the removal of inhibitory compounds. Although several of these interactions have been observed to promote mycelial growth, the proposed mechanisms of growth promotion by specific bacterial strains remain largely uncertain, and at times conflicting. Bacterial interactions also elicit varying growth-inhibitory responses from *A. bisporus* and *P. ostreatus*. This review explores characterized interactions involving bacteria and *A. bisporus*, and to a lesser degree *P. ostreatus*, and whilst doing so identifies existing research gaps and emphasizes directions for future work.

Keywords: *Agaricus bisporus*, *Pseudomonas*, compost, casing, *Pleurotus ostreatus*, mycelium, volatile organic compound

Introduction

Cultivated edible mushrooms are a significant source of nutrition and an economically important commodity in many regions of the world, but particularly in Asian countries such as China and Korea (Chang and Wasser 2017, Royse et al. 2017). Two genera, *Agaricus* and *Pleurotus*, together represent approximately a third of the world's mushroom supply (Royse et al. 2017). The button mushroom *Agaricus bisporus* is the most widely cultivated species in Europe, America, and Australia (Sonnenberg et al. 2011), but is also widely grown in China, which produces more than half of the global *Agaricus* supply (Royse et al. 2017). *Pleurotus* (oyster mushroom) represents 19% of global mushroom production, with *Pleurotus ostreatus* being one of the two major cultivated species of this genus (Royse et al. 2017). *Pleurotus* is also mainly grown in China (87% of global production; Royse et al. 2017), though both *Pleurotus* and *Agaricus* production is overshadowed in China by mushrooms that use sawdust as growth substrate, including *Lentinula* (shiitake), *Auricularia* (wood ear), and *Flammulina* (enoki). *Agaricus bisporus* and *P. ostreatus*, by contrast, are mainly grown on straw-based substrates that are prepared by a fermentation–pasteurization process in which bacteria and fungi are crucial for converting feedstocks into a selective nutrient medium for mushroom growth, as well as for promoting mushroom mycelium growth and stimulating fructification (Kertesz and Thai 2018, Carrasco et al. 2020).

Bacteria are present at every step of *A. bisporus* and *P. ostreatus* production. A broad range of bacterial taxa is essential at three stages: production of the mushroom growth substrate; growth of the mushroom mycelium; and fruit body production. In recent years, most focus has been placed on understanding the bacterial community that is responsible for production of the mushroom compost, since these interact with *Agaricus* or *Pleurotus* mycelium during hyphal proliferation.

Commercial-scale production of *A. bisporus* compost takes place in three distinct stages: an initial thermophilic Phase I, pasteurization and conditioning in Phase II, and hyphal proliferation in Phase III (Fig. 1). The principal raw materials are wheat straw or stable bedding, poultry manure, and gypsum, and the initial bacterial community of the compost is composed of mesophilic species that are present in the raw materials (e.g. *Acinetobacter*, *Solibacillus*, *Comamonas*, *Pseudomonas*, and *Sphingomonas*), often supplemented by wetting with compost leachate, which contains high numbers of *Thermus* (Safianowicz et al. 2018). The first stage of composting is characterized by a rapid succession of dominant taxa, as these utilize readily accessible carbon sources, and the increase of compost temperature to around 80°C during Phase I then selects for thermophilic bacteria, with a high proportion of Firmicutes and Proteobacteria, followed by Bacteroidetes, *Thermus*, and to a lesser extent Actinobacteria (Straatsma et al. 2000, Thai et al. 2022). During Phase II, the compost is first pasteurized at 58–60°C in a closed tunnel and then con-

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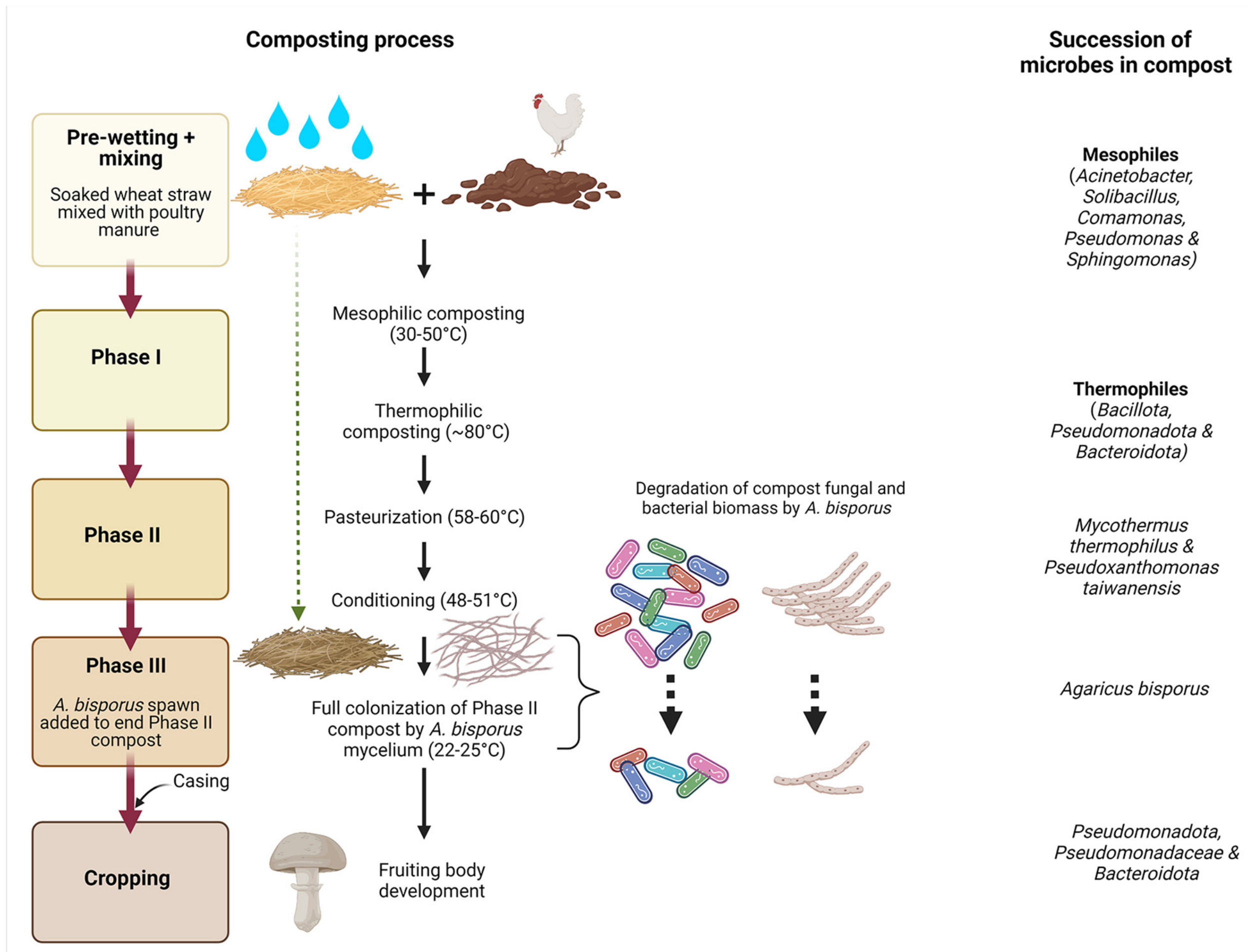

Figure 1. Overview of compost preparation for *A. bisporus* cultivation and the succession of dominant microbial taxa in the compost.

ditioned at 48–51°C, with cooling to ∼25°C before addition of the mushroom spawn (Straatsma et al. 2000). The key roles of bacterial and fungal populations in Phase II compost are to incorporate the cellulose and hemicellulose from the feedstocks into microbial biomass, and to assimilate excess ammonia, which is toxic to *A. bisporus* mycelium (Kertesz and Thai 2018). The most abundant bacterial taxon is often *Pseudoxanthomonas taiwanensis* (Vieira and Pecchia 2018, Thai et al. 2022), though its role in this cellulose-rich environment is unclear, since it lacks cellulase activity (Du et al. 2015). At the end of Phase II, the microbial community in the compost is dominated by the cellulose-degrading ascomycete *Mycothermus thermophilus* and bacterial taxa including *Bacillus* spp., actinomycetes such as *Thermopolyspora* or *Microbispora*, and heterotrophic nitrifiers such as *Chelatococcus* or *Pseudoxanthomonas*. These organisms support the growth of the *Agaricus* mycelium during Phase III. *Agaricus bisporus* is introduced into the compost as grain spawn, and the mushroom mycelium utilizes microbial biomass as its main nutrient source to support mycelium growth during initial colonization of the substrate (Jurak et al. 2015). By the end of Phase III, *A. bisporus* has degraded most of the *M. thermophilus* biomass, as well as a significant portion of bacterial biomass (Vos et al. 2017, Thai et al. 2022).

Substrate preparation for *P. ostreatus* is slightly simpler. *Pleurotus ostreatus* is cultivated on lignocellulosic substrates, most commonly chopped wheat straw or hardwood sawdust supplemented with nitrogen sources such as cereal bran, cottonseed hulls, or urea (Sánchez 2010). The mixed raw materials are subjected to partial composting followed by indoor pasteurization/conditioning before the addition of grain spawn (Bánfi et al. 2021). Proteobacteria (*Pseudomonas* and *Sphingomonas*) are dominant at the start of composting, but these are replaced by *Bacillus*, *Pseudoxanthomonas*, and *Thermobispora* by the end of the partial composting process (Vajna et al. 2012, Bánfi et al. 2021). The mature compost contains several actinobacterial genera, *Thermus* spp., and Firmicutes (*Bacillus*, *Geobacillus*, *Thermobacillus*, *Ureibacillus*) (Vajna et al. 2012). Colonization of the substrate by the *P. ostreatus* mycelium leads to a reduction of *Thermus* spp. and actinobacteria, possibly due to lower temperatures, and an increase in *Bacillales* and *Halomonas* spp., which demonstrate protective effects against competitor moulds (Bánfi et al. 2021).

As the mushroom mycelium grows through the prepared substrate, the hyphae interact with the bacteria in the compost, which colonize the hyphae, mobilize nutrients for hyphal uptake, and stimulate hyphal growth through removal of growth inhibitors. The shift in compost bacterial composition during proliferation of the *A. bisporus* mycelium strongly suggests that *A. bisporus* influences the bacterial population in the substrate through a host of underlying interactions with many different bacterial taxa (Carrasco et al. 2020). By contrast, the

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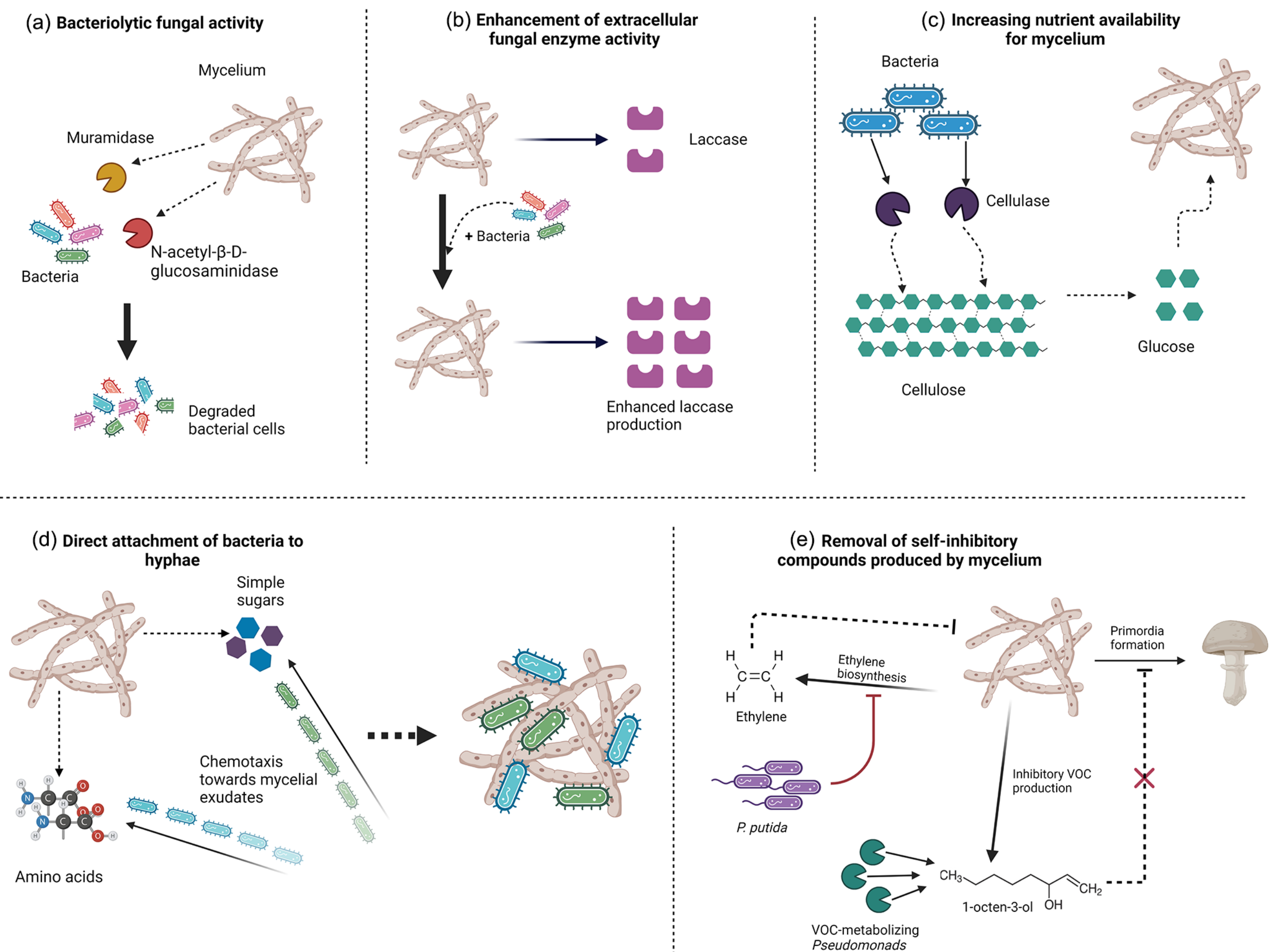

Figure 2. Interactions between bacteria and mushroom mycelium. (a) Bacteriolytic enzyme production by mycelium (*β-N*-acetylmuramidase and *N*-acetyl-*β*-D-glucosaminidase). (b) Enhancement of extracellular fungal ligninolytic enzyme (laccase) activity in the presence of bacteria. (c) Increased nutrient availability for the mycelium through bacterial cellulase secretion. (d) Direct attachment of bacteria to the mycelium through chemotaxis towards hyphal exudates (sugars and amino acids). (e) Removal of self-inhibitory compounds produced by the mycelium: (i) inhibition of the ethylene biosynthetic pathway by *Ps. putida* relieves mycelium growth inhibition by ethylene and (ii) pseudomonads metabolize the volatile C8 compound, 1-octen-3-ol, which inhibits primordia formation.

interactions that are important for fruit body production occur in the casing layer, in which the bacterial community is dominated by Pseudomonadaceae and the relative abundance of *Pseudomonas* spp. increases to very high levels during cropping (Carrasco et al. 2019, Vieira and Pecchia 2021). Most of the studies of these later interactions have therefore been carried out with *Pseudomonas* species, especially *Pseudomonas putida* and *Pseudomonas fluorescens*, and this is reflected in the analysis presented below.

The importance and mechanisms of bacterial–fungal interactions in the environment have been discussed in several excellent reviews (Frey-Klett et al. 2011, Deveau et al. 2018), and the interactions between mushrooms and bacteria that act as mushroom pathogens have also been discussed elsewhere (Largeteau and Savoie 2010, Savoie et al. 2016). This review focuses specifically on key aspects of the interactions between bacteria and the vegetative mycelium of *A. bisporus* and *P. ostreatus*, which are important for mycelial growth and primordia development (Fig. 2). These include (i) the bacteriolytic breakdown of bacterial biomass by the fungal hyphae, (ii) the attachment of bacteria to the mycelium mediated by chemotaxis, (iii) the promotion of hyphal growth through the secretion of extracellular enzymes and exudates by bacteria, or the stimulation of fungal enzyme activities, and (iv) bacterial–hyphal interactions that are critical for primordia development during fructification, mediating the removal of inhibitory compounds or modifying mycelial architecture to promote strand development. This allows us to identify existing research gaps and emphasize directions for future work that will increase our understanding of these essential interactions and increase the productivity of the mushroom industry.

Fungal bacteriolytic activity during growth of the mushroom mycelium

Bacterial biomass in the compost represents a major nutrient source for mycelial growth of both *Agaricus* and *Pleurotus*. This is reflected in the 4-fold decrease in bacterial biomass observed during proliferation of *A. bisporus* (Vos et al. 2017), and a 5–10-fold decrease in bacterial 16S rRNA levels during growth of *P. ostreatus* (Bánfi et al. 2021). *Pleurotus ostreatus* hyphae have been shown to actively penetrate bacterial colonies of *Ps. fluorescens in vitro*, followed by profuse proliferation of short hyphae and lysis of the colonies (Tsuneda

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and Thorn 1995). In similar studies, the mycelium of *A. bisporus* degraded both living and dead *Bacillus subtilis* cells by secreting extracellular enzymes to degrade the peptidoglycan cell wall of bacteria (Fermor and Wood 1981, Fermor et al. 1991). This ability to release essential nutrients from bacterial biomass is one of the proposed reasons for the selective growth in compost of *A. bisporus* over competitor microorganisms such as green moulds, which rely on the availability of free nutrients within the compost (Fermor et al. 1991). In an *in vitro* setting, the cellular components of bacteria provide a sufficient source of carbon and nitrogen for mycelium growth, and are assimilated more efficiently than cellulose (Fermor et al. 1991). The two key extracellular bacteriolytic enzymes produced by *A. bisporus* mycelium are *β*-*N*-acetylmuramidase and *N*-acetyl-*β*-D-glucosaminidase (NAG) (Grant et al. 1984, Lincoln et al. 1997). Muramidase is a glycoside hydrolase that targets muramyl glycosidic linkages unique to peptidoglycan, and is the primary bacteriolytic enzyme produced by *A. bisporus* mycelium (Grant et al. 1984). Maximum activity of *β*-*N*-acetylmuramidase is induced in the presence of bacterial cells and enzyme production is repressed by addition of readily available carbon sources such as glucose and fructose (Grant et al. 1984, Lincoln et al. 1997). Although this repression is not a concern for Phase III where hardly any xylan and cellulose are degraded to free glucose (Jurak et al. 2015), it will be interesting to study whether the breakdown products of xylan and glucan have a similar repressive effect on muramidase activity during fruit body formation, when *A. bisporus* mycelium consumes ∼40% of total carbohydrates in compost (Kabel et al. 2017). If so, this would significantly reduce the ability of *A. bisporus* to continue obtaining any of its nutrition from bacterial biomass at this stage. In contrast to muramidase, fungal production of NAG is lower when *A. bisporus* mycelium is grown solely on bacterial cells as carbon source, and is increased when a combination of glucose and bacterial cells is utilized as the growth medium *in vitro* (Lincoln et al. 1997). NAG is important in recycling nitrogen from chitin and from the products of muramidase action (Rogers et al. 1979), and is therefore in common use as a soil health indicator (Acosta-Martinez et al. 2019). It seems likely that *A. bisporus* senses differences in the chemical composition of the substrate and secretes different concentrations of extracellular enzymes to balance its C and N requirements. This theory could explain why consistently higher levels of *β*-*N*-acetylmuramidase activity in *A. bisporus* has been found in the presence of lower numbers of bacteria (Grant et al. 1984, Lincoln et al. 1997). The *A. bisporus* mycelium also produces extracellular peptidases (though not peptidoglycan-degrading peptidases; Grant et al. 1984), but it is not known whether these attack the secreted muramidase, and they do not have an inhibitory effect on NAG activity (Fermor and Wood 1981, Savoie 1998). The possibility that bacteriolytic enzyme production by the mycelium is regulated through feedback mechanisms involving bacterial metabolites or by-products of bacterial activity should be explored to modify substrate composition for optimal bacterial degradation by *A. bisporus*. Identifying such relationships could improve the ability of *A. bisporus* to utilize bacterial biomass and potentially promote mycelial growth during spawn run.

The preferential degradation of certain bacterial taxa by the *A. bisporus* mycelium is also currently an area of uncertainty. Gram-positive bacterial populations increase and Gram-negative bacteria decrease during mycelial growth in mushroom compost (Vos et al. 2017), indicating either that the compost nutrient environment favours Gram-positive bacterial proliferation, or that the *Agaricus* mycelium preferentially degrades Gram-negative bacteria. The latter may be the case as *A. bisporus* secretes low levels of serine peptidase and metallopeptidase (Grant et al. 1984, Semenova et al. 2017), which could favour the degradation of thinner peptidoglycan cell walls. However, this does not explain the preferential mycelial degradation of both live and dead bacteria from different phyla, e.g. the preferred assimilation of *Priestia megaterium* (Firmicutes) over *Alcaligenes faecalis* (Betaproteobacteria), followed by *Niallia circulans* (Firmicutes) (Ahlawat et al. 2002). Stable isotope measurements of wood-degrading fungi related to *Pleurotus* have shown that the fungal hyphae specifically target diazotrophic bacteria for their nitrogen content (Weisshaupt et al. 2011), and similar methods could be used to examine whether differences in bacterial degradation in compost correspond to differences in nutrient assimilation by the mycelium. Bacterial taxa that are preferentially degraded could be investigated further for use as a compost bioinoculum.

Direct attachment of casing bacteria to mushroom mycelium

Because the hyphal density of the cultivated mushroom is at its peak during fructification, most of the bacterial species identified in recent cropping studies (Bánfi et al. 2021, Vieira and Pecchia 2021) are likely to be physically associated with the mushroom mycelium. This applies in principle to all the different bacterial taxa present, but the dominant bacterial species attached to *A. bisporus* hyphae in the casing layer are members of the fluorescent *Pseudomonas* group (Preece and Wong 1982). Pseudomonads comprise up to 80% of the bacteria in casing environments, including the basidiome-initiating bacteria *Ps. putida* and *Ps. fluorescens* and the mushroom pathogen *Pseudomonas tolaasii* (Preece and Wong 1982, Siyoum et al. 2016, Vieira and Pecchia 2021). Because these are easy to study *in vitro*, almost all research on these growth-promoting bacterial–hyphal interactions has concentrated on *Ps. putida* and *Ps. fluorescens*, and the next section therefore focuses very much on these two species. Further work is needed to extend these findings to other taxa that associate with *Agaricus* and *Pleurotus* hyphae in compost and casing.

Bacteria interact with *A. bisporus* mycelium by forming direct attachments to the hyphal surface (Masaphy et al. 1987, Grewal and Rainey 1991, Rainey 1991b). This is actively promoted by bacterial chemotaxis towards hyphal exudates of *A. bisporus*. These exudates consist mainly of simple sugars and free amino acids (Grewal and Rainey 1991, Rainey 1991b), with amino acids playing the major role in eliciting the chemotactic response in *Ps. putida* (Grewal and Rainey 1991). Fibrillar structures and amorphous material, possibly polysaccharide slime, aid in the self-association and anchoring of bacterial cells to the hyphal surface of *A. bisporus* (Rainey 1991b). The attachment of *Ps. putida* is probably enhanced through the addition of $CaCl_2$, due to the importance of calcium as a bridging ion that ensures mechanical stability of extracellular polysaccharide matrices during biofilm formation (Körstgens et al. 2001). This should be confirmed through the addition of calcium compounds to the casing and measuring the effect on attachment rates of *Ps. putida* to mushroom hyphae.

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It is interesting that several phenotypic variants of *Ps. putida* exhibit the ability to respond more quickly to mycelial exudates under nutrient-depleted conditions, and that this improves their attachment rates to *A. bisporus* mycelium (Rainey 1991b). This faster chemotactic behaviour results in greater attachment by these strains and highlights their potential as bioinoculants in the nutrient-depleted casing environment, where *Ps. putida* is generally recognized as important for removing growth-inhibiting compounds produced by the mycelium. This may also explain why a large proportion of bacteria that display mushroom growth-promoting abilities have been isolated from the casing layer and fruiting bodies, and not from the compost (Zarenejad et al. 2012, Xiang et al. 2017).

At present, it is unclear whether bacterial chemotaxis only occurs in response to hyphal exudates, or whether the bacterial cells also recognize gaseous products of the *A. bisporus* mycelium (Baars et al. 2020). *Agaricus bisporus* produces a range of volatile organic compounds (VOCs), most notably ethylene and several C8 compounds, which are metabolized by pseudomonads in the casing layer (Baars et al. 2020). Although several *Pseudomonas* species, including *Ps. putida*, exhibit positive chemotaxis towards ethylene *in vitro* (Kim et al. 2007), in the context of *A. bisporus* mycelium this is unlikely to be important, as several studies have found that the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) serves as the primary chemoattractant for *Ps. putida* (Chen et al. 2013, Li et al. 2019). Chemotactic behaviour towards ACC is not likely to be limited exclusively to *Ps. putida* and other pseudomonads, as 20% of the total culturable bacterial population in casing soil has been found to produce ACC deaminase (AcdS) to utilize ACC as a nitrogen source (Chen et al. 2013). This highlights again the need for more work on bacterial species other than *Ps. putida* in studies of interactions with *A. bisporus* mycelium. During fruiting, ethylene levels in the casing are ~10 times lower than in the compost (Baars et al. 2020). A possibility is that *Pseudomonas* strains in the casing layer might be phenotypic variants with increased levels of chemotaxis towards ACC and ACC degradation, thus interfering with ethylene production more efficiently than their counterparts in the compost. Future efforts directed at bacteria that form direct attachments to *A. bisporus* mycelium should focus on screening for new *Pseudomonas* strains as well as phenotypic variants that exhibit greater chemotactic ability towards ACC, as these bacteria could be important as inoculants for non-peat casing in the future.

Growth-promoting bacterial exudates and extracellular enzymes

Quite a few different bacterial species have been reported to simulate hyphal growth in compost, casing, and *in vitro*, but the most effective mycelium growth-promoting species is probably *Ps. putida* (Kertesz and Thai 2018). A primary mechanism by which this species promotes mycelium growth of *A. bisporus* is by relieving the inhibitory effects of ethylene (Fig. 3) (Chen et al. 2013, Zhang et al. 2016), which is produced from its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) by the mycelium (Turner et al. 1975). After direct attachment to the hyphae, inoculation with strains of *Ps. putida* that can produce the enzyme ACC deaminase (AcdS) leads to a reduction in ethylene production by the mycelium and faster hyphal extension rates (Rainey 1991a, Cho et al. 2003). Interestingly, AcdS-deficient mutants are not only inhibitory to mycelium growth but also unable to attach to *A. bisporus* hyphae (Chen et al. 2013). ACC has been shown to be a strong chemoattractant for AcdS-producing *Ps. putida* (Li et al. 2019), which suggests the involvement of ACC/AcdS in bacterial chemotaxis and attachment to *A. bisporus* mycelium. This was confirmed by transformation of *A. bisporus* with the *Pseudomonas acdS* gene, as the transgenic hyphae not only showed enhanced extension rates but also reduced colonization by *Pseudomonas* cells (Zhang et al. 2019).

Many strains of *Ps. putida* also possess the ability to secrete organic acids, which can solubilize inorganic phosphate and also increase the bioavailability of microelements for assimilation by *A. bisporus* hyphae (Zarenejad et al. 2012). However, it is important to note that phosphorus-solubilizing and nitrogen-fixing bacteria isolated from casing soil of *Agaricus blazei* neither increased the phosphorus and nitrogen content of mushrooms nor promoted mycelium growth (Young et al. 2013). Therefore, caution should be exercised when describing compost bacteria that exhibit these characteristics as 'mushroom growth-promoting', as these abilities may not necessarily benefit mycelium growth. In contrast, 50% of isolates that do promote mycelium growth secreted cellulase (Young et al. 2013). The effect of cellulase secretion by these bacteria on mycelial growth promotion has not yet been directly confirmed, since *A. bisporus* is of course able to degrade cellulose independently (Chow et al. 1994). It is possible that bacterial cellulase could aid in degrading cellulose and hemicellulose in the substrate to increase carbon availability for the mycelium, but cellulase activity may also simply select for bacteria that are able to survive well in compost and promote mycelial growth by other means.

Bacterial growth promotion is also known for *P. ostreatus*. *Glutamicibacter arilaitensis* adheres to the mycelial surface of *P. ostreatus* and exhibits AcdS activity similar to *Ps. putida* (Kumari and Naraian 2021), while *Bradyrhizobium japonicum* is thought to promote mycelium growth by nitrogen fixation (Zhu et al. 2013). The direct attachment of rhizobia to *P. ostreatus* mycelium has not yet been confirmed, but rhizobia are known to attach to hyphae of, e.g. arbuscular mycorrhizal fungi (AMF) through the production of extracellular polysaccharides (Bianciotto *et al.* 2001). Several mechanisms have been proposed to explain growth promotion of *A. bisporus* and *P. ostreatus* mycelium by bacteria, but these have been derived mostly from screening studies that identified growth-promoting bacteria based on cellular characteristics that have not been well studied directly in relation to mushroom mycelium growth *in vitro* or *in situ*.

It is interesting to note that in *P. ostreatus*, bacterial isolates from fruiting bodies exhibited lower enzymatic activities but promoted hyphal extension, whereas bacterial isolates from the vegetative mycelium produced higher levels of hydrolytic enzymes (peptidase and lipase) and inhibited hyphal growth (Suarez et al. 2020). Bacteria that promoted mycelium growth also produced chitinase, while inhibitory bacteria did not, which strongly suggests that growth-promoting bacteria may act by partial degradation of hyphal cell walls to obtain sugars and amino acids from the mushroom mycelium while providing other nutrients in exchange (Suarez et al. 2020). It is unclear what drives the difference in hydrolytic enzyme production between bacteria on fruiting bodies and vegetative mycelium as well as its effect on mycelium growth, and

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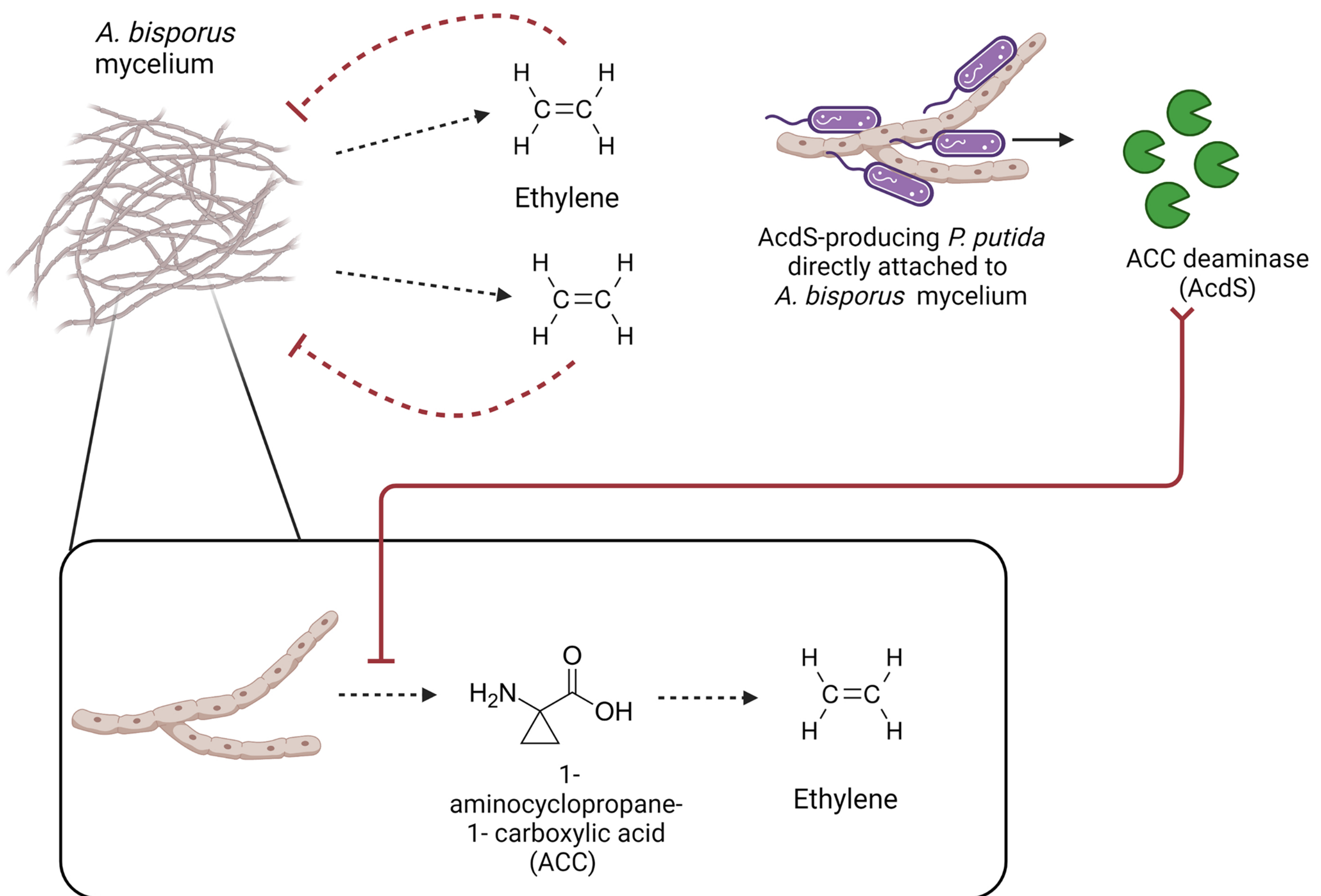

Figure 3. Reduced ethylene production by *A. bisporus* mycelium in the presence of *Ps. putida* strains that produce ACC deaminase (AcdS). Ethylene produced by *A. bisporus* mycelium is self-inhibitory to its growth. AcdS-producing *Ps. putida* cells co-cultured with *A. bisporus* mycelium are capable of direct attachment to *A. bisporus* hyphae and proceed to cleave ACC (immediate precursor to ethylene produced by the mycelium) through bacterial ACC deaminase (AcdS) activity.

whether different nutrient environments elicit different enzymatic responses from bacteria.

There is also uncertainty in the growth-promoting role of bacterial indoleacetic acid (IAA) on *P. ostreatus* mycelium growth. Production of IAA by *Ps. putida* and *Bacillus* spp. compost isolates caused mycelial extension in *P. ostreatus*, whereas bacterial isolates from *P. ostreatus* fruiting bodies (predominantly *Bacillus* and *Paenibacillus*) that promoted mycelial growth did not produce IAA (Suarez et al. 2020). This once again emphasizes the variety of mycelium growth-promoting mechanisms, as well as phenotypic differences between bacteria from nutrient-depleted environments (casing and fruit body) compared with nutrient-rich environments (compost). It is interesting to note that strains of *Ps. fluorescens* isolated from fruiting bodies of *P. ostreatus* are classified as growth promoting due to bacterial IAA production (Xiang *et al.* 2017), while a *Ps. fluorescens* strain from *A. bisporus* casing soil had no effect on the rate of mycelium growth (Mohammad and Sabaa 2015). This suggests that the *A. bisporus* response to IAA may differ from that of *P. ostreatus*, but this does not appear to have been studied in detail.

Enhancement of fungal lignin degradation

Pleurotus ostreatus and *A. bisporus* are lignin-degrading basidiomycetes that produce extracellular phenol oxidase enzymes such as laccase and manganese peroxidase (MnP) that help break down the lignocellulosic substrate on which they are grown (Bonnen *et al.* 1994, Shah and Nerud 2002, Doddapaneni et al. 2013). Genes encoding laccases and MnP are strongly expressed in *A. bisporus* during mycelium proliferation in the compost, and less strongly later in mushroom growth (Patyshakuliyeva et al. 2015). However, these enzymes are not only useful for nutrition, but at least in *P. ostreatus* and related fungi, they play an important interactive role (Baldrian 2004). This has been best characterized for exposure of the *P. ostreatus* mycelium to other fungi (Baldrian 2004), but it is also known for *A. bisporus* (Flores et al. 2009) and indeed co-cultivation of different basidiomycetes is a standard way to enhance industrial production of a range of enzymes and other products (Yu et al. 2021). Exposure of the fungal mycelium to bacteria can also stimulate an increased ligninolytic response. Co-cultivation of *P. ostreatus* with mixed soil bacteria, *Bacillus* spp., *Paenibacillus polymyxa*, or even *Escherichia coli* leads to an increase in laccase activity in the fungal mycelium (Baldrian 2004, Velázquez-Cedeño et al. 2008). This appears to be a defence response, since although *Bacillus* or *Polymyxa* inhibits the growth of *Trichoderma* spp. and other antagonists of *Pleurotus* growth (Velázquez-Cedeño et al. 2008), they also appear to be fungistatic towards *P. ostreatus* itself and lead to reduced fungal colonization of substrate and inhibition of mycelium growth

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in vitro (Velázquez-Cedeño et al. 2004, 2008, Bánfi et al. 2021).

The relationship between mycelium growth inhibition and the ability of certain bacteria to induce ligninolytic enzyme activity may be linked to the production of reactive oxygen species (ROS). The production of hydrogen peroxide (H_2O_2) is positively correlated with the increase in vegetative mycelial biomass of *P. ostreatus* and *A. bisporus* (Savoie et al. 2007), and oxidation reactions catalysed by laccases and ligninolytic peroxidases such as MnP also lead to H_2O_2 production by the mycelium (Martínez et al. 2005, Perna et al. 2019). In higher plants, ethylene and ROS species such as H_2O_2 are closely linked, with H_2O_2 being found to induce the endogenous production of ethylene (Ishibashi et al. 2013, Cui et al. 2019). This suggests that bacteria that increase laccase and MnP activity in *P. ostreatus* may lead to increased H_2O_2 as a by-product, and this could produce positive feedback on the downstream production of growth-inhibiting ethylene by the mycelium. Further work is required to determine the exact mechanism of laccase and MnP regulation of *P. ostreatus* by bacteria, and to identify bacterial strains that enhance ligninolytic enzyme activity without inhibiting mycelial growth.

Interactions affecting primordia development

The presence of bacteria in the casing layer is essential for primordia development by *A. bisporus*, as demonstrated by its inability to form fruiting bodies in sterilized casing (Hayes et al. 1969). The stimulatory effect of bacteria on primordium formation by *A. bisporus* has been studied in most detail for *Ps. putida* (Hayes et al. 1969, Rainey et al. 1990), but a similar effect is seen with many other taxa, including strains of *Alcaligenes*, *Bacillus*, *Rhodopseudomonas*, *Azotobacter*, and *Rhizobium*, and even by an alga (*Scenedesmus*) and a yeast (*Lipomyces*) (Baars et al. 2020). However, the effect of these individual strains is relatively weak compared with the native microbiota on the mushroom beds, and even the most effective pseudomonad tested only affords about a third as many primordia as seen with unsterilized casing (Noble et al. 2009). The ability of *A. bisporus* to produce primordia in axenic casing material after addition of adsorptive materials such as activated charcoal suggests that these microbes stimulate primordia development in a similar manner, through the removal of inhibitory chemicals (Noble *et al*. 2003).

The mycelium of *A. bisporus* produces self-inhibitory volatile C8 compounds, with the most inhibitory VOC identified as 1-octen-3-ol (Noble et al. 2009), produced from linoleic acid by the mushroom hyphae (Husson et al. 2001). Another VOC inhibitory to hyphal growth is 2-ethyl-1-hexanol, and although the source of this compound has been attributed to rye grain substrate (Noble et al. 2009), it is worth noting that several ascomycetes are capable of producing this compound (Ezeonu et al. 1994, Pasanen et al. 1997). The possibility that *M. thermophilus* produces this inhibitory VOC is not known and should be confirmed. Both 1-octen-3-ol and 2-ethyl-1-hexanol are metabolized by pseudomonad populations in the casing, and this process enables primordia formation by *A. bisporus* (Noble et al. 2009). The importance of this interaction between *Ps. putida* and *A. bisporus* mycelium is highlighted by the improved yield obtained from inoculating the casing with *Ps. putida* strains that are highly tolerant to 1-octen-3-ol (Zarenejad et al. 2012). Addition or removal of volatile 1-octen-3-ol to the airstream leads to down- or upregulation of primordia formation (Noble et al. 2009, Eastwood et al. 2013). Future work should identify the genes associated with the ability to remove inhibitory C8 compounds, and conduct knockout experiments to confirm that induction of primordia formation is due to this mechanism.

The attachment of bacteria to the hyphae of *A. bisporus* in casing causes the disappearance of calcium oxalate crystals surrounding the hyphae (Masaphy et al. 1987). Calcium oxalate crystals are thought to interfere with strand formation, which precedes primordial formation in *A. bisporus* (Masaphy et al. 1987). Several *Pseudomonas* species metabolize and utilize oxalate either as a growth substrate (Blackmore and Quayle 1970) or as a siderophore (Dehner et al. 2010). It is possible that the induction of strand formation through oxalate removal by *Ps. putida* provides additional stimulation for *A. bisporus* to commit its resources towards reproductive growth, possibly through the modification of mycelium architecture. This in turn would explain the reduced vegetative mycelial biomass that is observed after inoculation with *Ps. putida* and other fluorescent pseudomonads (Reddy and Patrick 1990, Rainey 1991a).

As discussed earlier, the mycelium of *A. bisporus* produces ethylene, which inhibits mycelium growth (Turner et al. 1975, Zhang et al. 2016), and AcdS-producing *Ps. putida* that bind to the hyphae can reduce ethylene levels and induce primordium formation (Chen et al. 2013). However, there is some uncertainty about the mechanism involved here, since several studies that investigated the effect of increased or reduced ethylene levels on primordia formation have come to contradictory conclusions, with both enhanced and lowered primordia formation observed (Baars *et al*. 2020).

The stimulation of primordia formation of *P. ostreatus* by *Ps. putida* (Cho et al. 2003, 2008) may well be occurring by mechanisms similar to those that are important for *A. bisporus*; however, further confirmation and research into the bacteria–mycelium interactions that induce fruiting body formation are necessary for *P. ostreatus*.

Conclusions and outlook

Interactions between bacteria and the mushroom mycelium can greatly influence hyphal extension and primordia formation. This review makes clear that our current knowledge in this area is largely limited to the interactions between fluorescent pseudomonads and *A. bisporus*, with the majority of studies using *Ps. putida* as a model organism to study these interactions. Although *Ps. putida* has been consistently isolated from the casing, it is not always the dominant bacterial species present in the casing layer, and is sometimes not present at all in either casing or compost (Kertesz et al. 2016, Carrasco et al. 2019, Vieira and Pecchia 2021, Thai et al. 2022). The time taken for primordia formation is relatively consistent across casing layers with varying relative abundance of pseudomonads (Kertesz et al. 2016, Carrasco et al. 2020), and mushroom compost inocula developed on the basis of the growth-promoting abilities of *Ps. putida* may therefore not be effective in increasing yields. This indicates that other native bacterial populations are additionally responsible for removing inhibitory chemicals produced by *A. bisporus* mycelium and inducing primordia development and mycelium growth, and more work is needed to develop effective microbial consortia, rather than using individual strains.

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Apart from *Ps. putida*, there is very little work at present that has identified and characterized growth-promoting or inhibitory interactions between *P. ostreatus* mycelium and bacterial populations present during cultivation, though some progress has been made in the area of wood degradation. Bacteriolytic activity by *P. ostreatus* mycelium is an area of uncertainty, and it is currently unknown whether bacterial biomass is as important a nutrition source for mycelium growth of *P. ostreatus* as it is for *A. bisporus*. The role of bacteria in regulating ligninolytic enzyme production of *P. ostreatus* is also an important bacteria–mycelium interaction that requires further investigation, as increased lignocellulose degradation can optimize substrate productivity and improve nutrient assimilation by the mycelium. A better understanding of the interactions between mushroom mycelium and bacteria during the cultivation process is critical in using microbes as indicators in defining and assessing the 'quality' of the compost. From an industrial standpoint, bacteria–mycelium interactions that promote both vegetative and reproductive growth have the potential to reduce the time needed for mycelium to colonize the substrate, enhance nutrient assimilation, and ultimately improve mushroom yields.

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Conflict of interest

No conflict of interest declared.

Author contributions

S.S. planned and drafted the work. M.A.K. and S.S. wrote the manuscript.

Data availability

Data sharing not applicable to this review article as no datasets were generated or analysed during the current study.

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MINI-REVIEW

Nitrogen balance and supply in Australasian mushroom composts

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Abstract

Mushrooms are an important source of protein in the human diet. They are increasingly viewed as a sustainable meat replacement in an era of growing populations, with button mushrooms (*Agaricus bisporus)* the most popular and economically important mushroom in Europe, Australia and North America. Button mushrooms are cultivated on a defined, straw-derived compost, and the nitrogen (N) required to grow these high-protein foods is provided mainly by the addition of poultry manure and horse manure. Using the correct balance of carbon (C) and N sources to produce mushroom compost is critically important in achieving maximum mushroom yields. Changes in the amount and form of N added, the rate and timing of N addition and the other compost components used can dramatically change the proportion of added N recovered in the mushroom caps, the yield and quality of the mushrooms and the loss of N as ammonia and nitrogen oxide gases during composting. This review examines how N supply for mushroom production can be optimised by the use of a broad range of inorganic and organic N sources for mushroom composting, together with the use of recycled compost leachate, gypsum and protein-rich supplements. Integrating this knowledge into our current molecular understanding of mushroom compost biology will provide a pathway for the development of sustainable solutions in mushroom production that will contribute strongly to the circular economy.

Key points

- *Nitrogen for production of mushroom compost can be provided as a much wider range of organic feedstocks or inorganic compounds than currently used*
- *Most of the nitrogen used in production of mushroom compost is not recovered as protein in the mushroom crop*
- *The sustainability of mushroom cropping would be increased through alternative nitrogen management during composting and cropping*

Keywords Nitrogen · Mushrooms · *Agaricus bisporus* · Ammonia · Nutrient cycling · Circular economy

Introduction

Mushrooms are an increasingly important source of nutrition worldwide, and annual mushroom production has grown over 30-fold in the last 40 years (Royse et al. 2017). Although oyster mushrooms (*Pleurotus*) and shiitake (*Lentinula*) dominate this production globally, in Europe, the USA and Australia the button mushroom (*Agaricus bisporus*) is commercially the most important cultivated mushroom. Button mushrooms are grown on a composted substrate derived mainly from wheat straw, stable bedding (horse manure), poultry manure and gypsum. This varies regionally, however, with horse manure playing a large role in Europe and the USA, and rice straw commonly replacing wheat straw in China (Song et al. 2021). Smaller amounts of other materials are used to provide bulk or nitrogen (N) input, depending on seasonal availability, and although mushroom compost is a more defined substrate than green-waste composts (which may contain woody materials, grasses and leaves at certain seasons), materials such as canola, soybean, cottonseed and sugarcane bagasse are often added to stimulate microbial activity. The composting process varies somewhat between countries, but usually includes an initial period of wetting or soaking of the raw materials, a thermophilic composting

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period (Phase I, 70–80 °C) and a pasteurisation and conditioning step (Phase II, 60 °C; then decreasing to 45 °C).

The composting process relies on microbial activity to break down the lignocellulosic raw materials and incorporate added N into microbial biomass in the compost. The microbial community in the compost changes continuously during composting, responding to changes in temperature and the progressive assimilation of plant cell components, starting with readily available compounds such as lipids and sugars, and progressing to polymers such as cellulose, hemicelluloses and lignin. The microbial dynamics of this process have been studied in detail in recent years, focussing largely on phylogenetic profiling (Cao et al. 2019; Carrasco et al. 2020; Song et al. 2021; Thai et al. 2022; Vieira and Pecchia 2021). Investigations of changes in the functional diversity in mushroom compost have concentrated primarily on enzymes responsible for lignocellulose breakdown to provide carbon for growth (Chang et al. 2022; Kabel et al. 2017; Zhang et al. 2014). Lignocellulose has only a very low N content, so the polysaccharide-degrading bacteria rely on particular strategies for assimilation of available nitrogen while degrading the straw polysaccharides (Gardner and Schreier 2021). For compost in particular, the key nitrogen-transforming activities observed are high levels of proteolysis and ammonification in Phase I and high levels of nitrification during Phase II conditioning (Caceres et al. 2018). This leads to significant losses of nitrogen as ammonia in the thermophilic phase of composting, and conversely, reassimilation of ammonia during conditioning. In Australasian composting facilities, about 30% of the added nitrogen is lost during Phase I and a further 10% during Phase II (Fig. 1) (Thai 2022). Because of these losses, it is usual to add a nitrogenous supplement to the compost before cropping, but this constitutes only a small proportion of the total nitrogen already present in the compost. Within our diet, mushrooms are a source of essential fatty acids (Sande et al. 2019) and major and minor trace elements (Siwulski et al. 2020) and are also regarded as a high protein food (Wang and Zhao 2023). However, only about 15% of the total added nitrogen to the composting process is recovered in the mushroom crop. This highlights that most of the nitrogen that is added in the composting process is not actually required for mushroom fructification, but is important in promoting the composting process itself. Nitrogen is required to stimulate the microbial activity that is important in creating a productive compost, but in the form of ammonia, it is also very important in preparing the compost substrate chemically for enzymatic degradation. Proteolysis generates significant quantities of ammonia during Phase I composting, and at the elevated pH and temperature conditions present this ammonia helps promote chemical degradation of hemicellulose and lignin (Mouthier et al. 2017). This principle applies not only to mushroom composts—the process of general organic waste composting also depends crucially on transformation of individual N fractions in the feedstocks (Estrella-Gonzalez et al. 2020).

Australian and New Zealand mushroom farms grow around 60,000 tonnes of button mushrooms (*Agaricus*

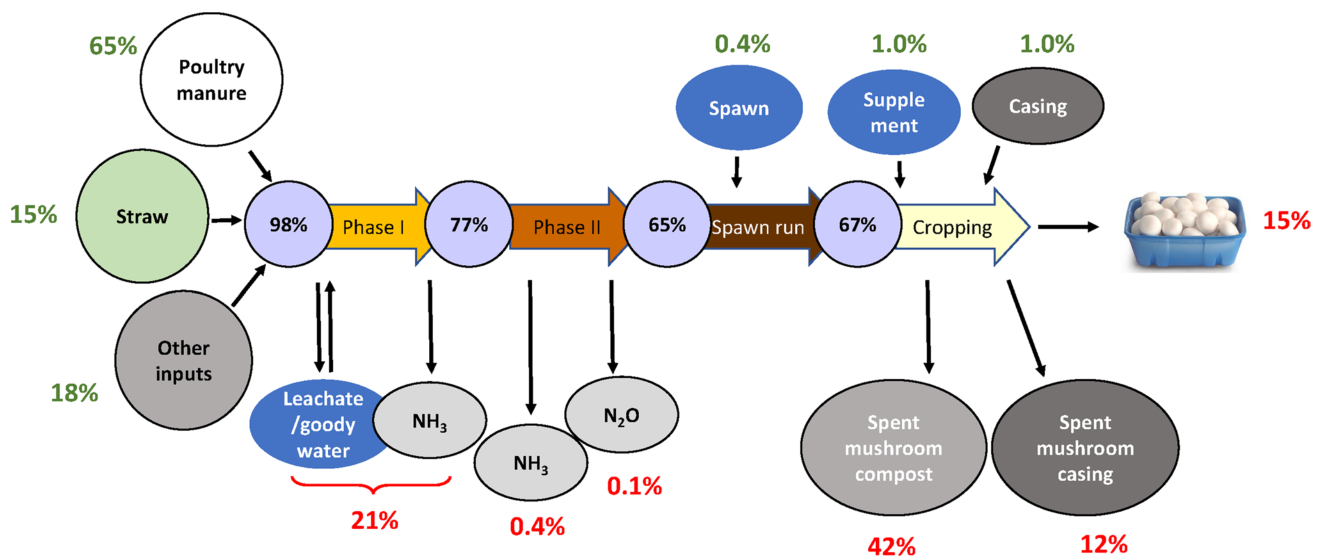

Fig. 1 Nitrogen balance in button mushroom production. Values are shown as percentage of total input nitrogen. Green numbers, N inputs; red numbers, N outputs; purple numbers, derived from compost N content measurements, allowing for volume losses during composting. The data are average values from ten Australian mushroom composting facilities. Losses during Phase 1 are estimated, since NH_3 losses could not be measured and the proportion of recycled leachate used in composting varied greatly between facilities. Data from Thai (2022).

bisporus) annually (Food and agriculture organisation 2019). This requires around 340,000 tonnes of Phase I compost, which is manufactured from about 260,000 tonnes of raw materials (den Ouden 2016; Gerrits 1988). Australasian mushroom compost is almost entirely produced from a wheat straw/poultry manure/gypsum blend, with minimal additions of other ingredients such as horse manure and occasional supplementation with small amounts of N in forms such as urea or ammonium sulphate. The feedstock composition used is very consistent through the year, and in recent years, most Australasian mushroom compost facilities have moved to large, forced-aeration bunker systems with uniformly high temperatures and short composting times, replacing the slow traditional methods of turned windrows. This consistency of feedstocks and process makes Australasian mushroom compost a useful subject for detailed studies of the mushroom composting process, but it also means that the Australasian mushroom industry is very dependent on the availability of a small range of raw materials in order to maintain economic viability.

The 1990s and early 2000s were a 'golden age' of empirical research into composting and particularly the production of *Agaricus*-selective mushroom compost. Extensive studies were done to choose optimal feedstocks of C and N for composting, and to predict crop yields from the inputs and from the physicochemical parameters of compost. Progress has been slower since then, but with the advent of high throughput molecular tools (especially sequencing technologies), there has been a renaissance in studies of compost microbial diversity and development and its impact on mushroom yield and quality. Several reviews have appeared that summarise aspects of mushroom cropping and the biology of *Agaricus bisporus* (Baars et al. 2020; Carrasco et al. 2018; McGee 2018) and review bacterial-fungal interactions in mushroom compost (Braat et al. 2022; Carrasco and Preston 2020; Kertesz and Thai 2018; Shamugami and Kertesz 2023) and mushroom production as part of the circular economy (Grimm and Wosten 2018).

This current report addresses a different critical aspect of composting, and directs the reader's attention back to questions of how best to manage microbial nutrient management in the production of mushroom compost. It aims to summarise and synthesise earlier findings so that modern researchers are able to integrate previous conclusions (including insights from less accessible industry and conference sources) into recent advances in our understanding of mushroom compost biology. This will allow researchers to avoid duplication of previous research and provide a pathway for the pursuit of productive new directions and the development of sustainable solutions that will contribute more strongly to the circular economy.

Carbon and nitrogen sources in mushroom compost

Achieving the correct balance of carbon (C) and N sources in mushroom compost is important in achieving maximum mushroom yields. During composting, N is used by the compost microbiota to degrade some of the cellulose and hemicellulose in straw, the main C source in compost, into microbial biomass and high molecular weight polymers, making it a more selective nutrient source for the mushroom and less available for competitor micro-organisms (Fermor et al. 1985; Wood and Fermor 1985). Compost formulations deficient in N are therefore less productive overall than formulations in which there is an adequate supply of N (Gerrits 1988; Noble and Gaze 1994; O'Donoghue 1965). If adequate N is present when mushroom inoculum or 'spawn' is added to pasteurised (Phase 2) compost, mushroom yield does not change with a further slight increase in compost N contents, e.g. between about 2.1 and 2.7% of dry matter (DM) ((Cormican and Staunton 1991); Noble unpublished data). However, oversupply of N in compost formulations results in excessive evolution of ammonia and nitrous oxides and N losses (Noble et al. 2002). Some loss in N during composting by ammonification is almost inevitable and provides an available N-source for compost microbes, but ammonia is also toxic to the *Agaricus* mycelium, and composts with a high ammonium-N content, generally above 0.15% of DM at spawning, are less productive ((Cormican and Staunton 1991); Noble unpublished data). This limits the amount of N that can be added into compost formulations for conversion into microbial biomass. To increase mushroom yields, additional proteins are therefore usually added to the prepared composted substrate (Gerrits 1988), most commonly in the form of soya-based supplements (see later section on compost supplements).

The total C:N ratio is widely used by composters to determine optimum mushroom compost formulations, but this is an over-simplification, since it is the available C and N to micro-organisms that are important. However, for most compost ingredients, the total C and N are likely to give an indication of the available C and N (Gerrits 1977b). The optimum C:N ratio for the blended ingredients in a mushroom compost formulation is about 30:1, equivalent to an N content (including ammonium-N) of about 1.5% of DM (den Ouden 2016; Gerrits 1977b). At this level, the N losses as ammonia during the first stage or Phase I of composting are almost counterbalanced by the losses in C as carbon dioxide, so that the compost N content increases only slightly. At starting levels of N above 2%, more N is lost as ammonia than C as carbon dioxide, resulting in a decrease in compost N content

during composting. When the starting level of compost N is below 1.5%, ammonia losses are small and the compost N content increases during composting (Gerrits 1977b; Gerrits 1988). Materials with N contents of more than 2% of DM can therefore be regarded primarily as N sources. Organic matter ingredients with N contents of less than 1% of DM can be regarded primarily as C sources, and those with intermediate N contents, such as horse manure, can also be regarded as significant or even sole sources of N.

Straw as a source of C and N

The main component and C source in mushroom composts in temperate regions is wheat straw, used fresh or as horse manure, which may also contain proportions of other types of straw such as barley. The amount of C in wheat straw that is available to microbes varies widely between different straw sources. A study of 84 wheat straw samples from across the UK found that soluble carbohydrates varied between 3 and 19% and hemicellulose between 10 and 29%, even though total C content only ranged from 36 to 39% (Noble et al. 2006).

Wheat straw contains between 0.3 and 1.09% of N (Atkins 1974; Noble 2006) but it is unclear how much of this N can be used by microbes during composting. For example, rape straw containing 1.2% N produced a similar amount of ammonia during composting to wheat straw containing 0.5% N when the same amounts of poultry manure were added to each (Noble et al. 2002). This indicates that much of the N in straw is unavailable to microbes during composting.

Rye straw degrades and performs similarly to wheat straw for production of mushroom compost (Gerrits 1988; Noble and Dobrovin-Pennington 2007) and in Asia, rice straw is commonly used in place of wheat straw (Kim 1978; Noble et al. 2001). Oat and barley straw degrade more rapidly during composting than wheat straw (Gerrits 1988); this may necessitate shorter composting to avoid loss in structure and aeration. Noble and Gaze (1994) obtained significantly poorer mushroom yields from ‘environmentally controlled’ composts prepared from barley straw than from wheat straw although subsequent experiments with compost prepared in bunkers from barley straw yielded comparable yields to those from wheat straw (Noble et al. 2002). It is possible that the greater digestibility and availability of C in barley straw than in wheat straw may influence the optimum amounts and types of N that can be used for preparing mushroom substrates. Straw from sugarcane, rape, linseed, peas and beans, various grasses and corn cobs have also been used as C sources in mushroom compost formulations although complete replacement of wheat or similar straw has usually resulted in reduced mushroom yields (Noble et al. 1998; Poppe 2000).

High energy C sources such as molasses have also been included in compost formulations (Hayes et al. 1969), in order to increase the availability of C to compost microbiota and promote the rate and temperature of mushroom composting. Success with these additives led to the production of commercial compost ‘activators’ such as ADCO Sporavite (Noble et al. 1998). However, the addition of sugars to compost formulations did not shorten the time needed to clear ammonia from the compost or increase mushroom yields (Gerrits 1988). High compost temperatures, rapidly achieved in modern insulated and aerated bunker systems, have also made the use of such compost activators unnecessary.

Organic matter N sources

Extensive lists of raw materials that can be used as substrates for mushroom cultivation were collated by Stamets (2000) and Poppe (2000), but many of these materials are only available in tropical regions and are less appropriate in temperate zones. N sources that may be available in quantity in Australasia include cow, pig and sheep manures; animal skin, hair, bone, dried blood and horn wastes; feathermeal, fish and shellfish residues; brewery and distillery wastes; and grape, citrus and olive fruit wastes. Although Australia and New Zealand produce huge quantities of animal manures, much of this material is widely dispersed and remote from mushroom composting sites, which are mainly located in periurban areas around the major cities. Their value as an N source will depend primarily on the total N contents and the microbially available N, but their practical use is also influenced by the moisture content (Table 1). An important consideration in the selection of materials is whether they are in high demand for alternative uses, since this value may make them prohibitively expensive for composters. Mushroom composts have been successfully prepared by incorporating blood meal, canola meal, cotton seed meal, guano, malt sprouts (Gerrits 1988; MacCanna 1969; Riethus 1962; Rinker 1991) and brewers’ grains (Beyer and Beelman 1995; Rinker 1991) but these materials also have a fertiliser or animal feed value. In several experiments, excess application of these materials in compost formulations resulted in poorer mushroom yields than moderate applications (Table 2).

Due to its moderately high N content, widespread availability and low alternative value, poultry manure has been a standard mushroom compost ingredient for many decades (Table 1). Australia produces over 1 million tonnes of poultry litter annually, though the composition of the litter and its suitability for mushroom compost production depends on the type of poultry production and the bedding material (Gerrits 1988; Wiedemann et al. 2015; Wiedemann et al. 2006). Poultry manure with readily degradable bedding material such as straw is more suitable for mushroom composting

Table 1 Organic matter nitrogen sources for mushroom compost. Materials currently used or with potential for use in Australasia are in bold text

N source	N % of DM	Dry matter %	References
Blood, dried meal	12.2	100	(Ministry of agriculture fisheries and food 1976)
Brewers' grains, dried	3.4	92	(Beyer and Beelman 1995)
Brewers' grains	3	24	(Noble and Dobrovin-Pennington 2007)
Canola/rape seed meal	**3.3**	**85**	**(Stamets 2000)**
Cattle slurry	< 2.8–10	10, < 14	(Dawson 1978; Grabbe 1974)
Cocoa meal	4.2	93	(Noble et al. 2002)
Cottonseed meal	**6.5**	**92**	**(Stamets 2000)**
Cotton trash	**1.5**	**91**	**(Stamets 2000)**
Digestate, poultry manure	3.5	31.3	Noble (unpublished)
Feather meal	**4.9**	**67**	**(Ministry of agriculture fisheries and food 1976)**
Fish solubles	5	50	(Schisler and Patton 1974)
Glasshouse crop haulms	**1.8**	**11**	**(Noble 2005)**
Grape marc	**1.8–2**	**27–32**	**(Noble 2005; Pardo et al. 1995; Stamets 2000)**
Guano	8–15	> 94	(Schnug et al. 2018)
Hop waste, dried	3.3	90	(Noble et al. 2002)
Horn meal	14.5	90	(Ministry of agriculture fisheries and food 1976)
Horse manure	**1.3**	**37**	**(Gerrits 1988)**
Malt sprouts, dried	4.3	92.6	(Stamets 2000)
Paunch grass	**3–3.5**	**15**	**(Environment protection authority 2017)**
Pig manure	1.9	23	(van Loon et al. 2004)
Poultry manure, caged	**1.5–4.7**	**25–67**	**(Ministry of agriculture fisheries and food 1976; Wiedemann et al. 2006)**
Poultry manure, broiler	**4.5–5.4**	**60–66**	**(Gerrits 1988; Noble and Gaze 1994; Wiedemann et al. 2015)**
Poultry manure, deep litter	**2.2–2.7**	**48–79**	**(Ministry of agriculture fisheries and food 1976; Wiedemann et al. 2006)**
Sea algae meal	0.7	32	(Ministry of agriculture fisheries and food 1976)
Soya bean meal	**7.1–7.4**	**91**	**(Stamets 2000)**
Sugarcane bagasse	0.2	19	(Kneebone and Mason 1972; Stamets 2000)
Vegetable wastes	**1.8**	**13**	**(Noble and Dobrovin-Pennington 2007)**
Wool waste	**14**	**90**	**(Noble and Dobrovin-Pennington 2007)**

than manure with sawdust or wood shavings, which can encourage the growth of green moulds (den Ouden 2016). Broiler poultry manure is preferred because of its lower moisture content and easier handling and storage, although there can still be large variability in the quality of poultry manure obtained from apparently similar sources. For example, Cormican and Staunton (1984) recorded a range in N content from < 2.1 to > 3.6% of DM within Irish sources of broiler manure. Manure from egg laying hens is also used in some countries (Cormican and Staunton 1984; Gerrits 1988), particularly where it is first made into a slurry. Ammonia suppressants are applied to the bedding by some poultry farmers to reduce the injurious effects of ammonia on the birds. The presence of these ammonia suppressants does not affect composting or mushroom cropping. For example, use of poultry manure containing a suppressant based on monocalcium phosphate led to only slightly elevated ammonia levels during composting (Beyer et al. 2000), while poultry litter treated with a sodium hydrogen sulphate suppressant did not affect ammonia emissions or compost N during composting (Beyer et al. 2000; Gonzalez-Matute and Rinker 2006). Neither suppressant changed the subsequent mushroom cropping performance.

Where straw is the main C source in the compost, there is an optimum inclusion rate of poultry manure, depending on the C and N analysis (Gerrits 1988; Noble and Gaze 1996). Where horse manure is the main C source in the compost, addition of excess poultry manure can readily lead to an over-supply of N and reduced mushroom yield (Gerrits 1989). However, researchers from the 1960s onwards have found that mushroom yields from horse manure composts were improved by the addition of a range of organic N sources, including poultry manure, providing that this did not result in residual ammonia in the compost (Table 2). Examples include the following. Ross (1969) obtained mushroom yields comparable with those from horse manure composts using composts prepared from strawy bullock manure or pig slurry and straw. Grabbe (1974) replaced

Table 2 Effect of compost organic nitrogen sources on mushroom yield in experiments conducted in medium- and large-scale facilities (> 1 tonne compost)

N source	Inclusion, %*		Yield, % of control		Control compost	Reference
	Min	Max	@ Min	@ Max		
Blood, dried meal	0.45–1.25	1.96–3.21	110–126	77–115	Horse manure	(Riethus 1962)
Blood, dried meal	0.27 N	0.4 N	141	146	Horse manure	(MacCanna 1969)
Brewers' grains	31.25		126		Horse manure	(Riethus 1962)
Brewers' grains	9.15		100		Horse manure	(Schisler and Patton 1974)
Canola meal	12 dm		106		Horse manure	(Rinker 1991)
Cattle slurry	10	40	100	83	Horse manure	(Grabbe 1974)
Cattle slurry	35 dm		134		Straw, poultry manure	(Dawson 1978)
Cocoa meal	50 N	100 N	72	40	Straw, poultry manure	(Noble et al. 2002)
Cotton seed meal	2.5		128		Horse manure	(Bech and Riber Rasmussen 1969)
Cotton seed meal	0.14 N	0.20 N	139	123	Horse manure	(MacCanna 1969)
Cotton seed meal	5		109		Horse manure	(Gerrits 1977b)
Digestate, poultry manure	60		88		Straw, poultry manure	Noble (unpublished)
Fish solubles	5	11.8	100	100	Horse manure	(Schisler and Patton 1974)
Grape marc	11		114		Straw, horse and poultry manures	(Pardo et al. 1995)
Guano	1.05–2.09	3.34	72–125	112	Horse manure	(Riethus 1962)
Hop waste, dried	100 N		88		Straw, poultry manure	(Noble et al. 2002)
Horn meal	0.89	1.78	62–118	0–92	Horse manure	(Riethus 1962)
Pig manure	81	96	69	0	Straw, poultry manure	(van Loon et al. 2004)
Poultry manure, broiler	9		89–111		Horse manure	(Gerrits 1977b; Gerrits 1989)
Poultry manure, broiler	33	50	100	103	Straw, poultry manure	(Gerrits 1989)
Poultry manure, broiler	20	62.5	100	146	Straw, poultry manure	(Noble and Gaze 1996)
Sea algae meal	2.49	3.13	90	94	Horse manure	(Riethus 1962)
Sugar cane bagasse	100		100		Horse manure	(Kneebone and Mason 1972)
Vegetable wastes	25		105		Straw, poultry manure	(Noble and Dobrovin-Pennington 2007)
Wool waste	5		92		Straw, poultry manure	(Noble and Dobrovin-Pennington 2007)

* - % w/w of fresh weight, dry matter (dm) or total nitrogen content of N sources (N)

water with liquid cattle slurry in a horse manure-based Phase I compost and obtained the same mushroom yield. Dawson (1978) obtained mushroom yields at least comparable with straw and poultry manure compost when 70% of the poultry manure was replaced by an equivalent amount of N as cattle manure. Sugarcane bagasse and straw have been used to produce composts with comparable mushroom yields to those obtained from horse manure composts (Kneebone and Mason 1972; Peerally 1981). Digestate fibre from the anaerobic digestion of poultry manure (Table 2), food or crop wastes has been used in the production of mushroom substrates (Noble et al. 2002; Stoknes et al. 2008).

During composting with wheat straw, the use of vegetable wastes, dried hop waste and brewers' grains released less ammonia than poultry manure during composting but produced similar mushroom yields (Noble et al. 2002; Noble et al. 2006). Similarly, crop haulm and residues from glasshouse crops such as peppers and tomatoes contain moderate amounts of available N and could be used in mushroom compost formulations although their availability is seasonal (Noble 2005). Other organic materials (chipboard waste, cocoa meal and shells, wool waste and dried digestate fibre) which had total N contents above 2% of DM, only released small amounts of ammonia during composting and resulted in poor mushroom yields (Table 2). However, these materials may be suitable with longer composting periods to enable the release of N. Due to its current low price, low quality wool is now a significant by-product of the sheep meat industry, and it is used in the production of horticultural composts (Williams 2020). Paunch grass, the undigested contents of animal carcasses, is a by-product from abattoirs, has moderate N content but it is high in moisture (Table 1). It could be used in mushroom composting where high Phase I bunker temperatures would meet the regulatory requirements for animal waste disposal (Environment protection authority 2017).

Recycling of spent mushroom compost and green wastes into Phase I ingredients was examined by Noble et al. (2006) and reviewed recently by Zied et al. (2020). Use of these materials can make a substantial contribution to the circular economy, and an up-to-date inventory of the types, quantities and supply of by-products and wastes from agricultural and food production industries is urgently needed. Such a review should take into account recent changes in these sectors, paying particular attention to whether materials are suitable for both conventional and organically approved mushroom production.

Inorganic N sources

Various chemical fertiliser or inorganic N sources have been used in mushroom compost formulations, including urea, ammonium sulphate and nitrate, calcium ammonium nitrate, calcium nitrate and cyanamide (Table 3). Mushroom yields increased after the addition of ammonium sulphate to Phase I compost, providing that this was accompanied by an addition of calcium carbonate (MacCanna 1969; Riber Rasmussen 1965). When a proportion of the poultry manure in compost was replaced with ammonium sulphate (without calcium carbonate) compost pH was slightly reduced, but there was no effect on mushroom yield (Gerrits 1977a). Ammonium sulphate is widely available since it is a by-product of sulphuric acid scrubbing of composting emissions before biofiltration, and it is therefore a cheap source of N. If combined with calcium carbonate, it also obviates the need for gypsum in mushroom compost (Gerrits 1988; Riber Rasmussen 1965) (see below).

Urea is a more readily available form of N to compost microbes than ammonium sulphate and results in a more rapid release of ammonia from compost (Noble et al. 2002). It can be added during the pre-wetting of raw materials where it is less likely to cause odour nuisance than poultry manure (Noble et al. 2002). However, composts prepared with urea rather than with ammonium sulphate led to reduced mushroom yields (Bech and Riber Rasmussen 1969). Pardo et al. (1995) added a combination of urea (8.3 kg), ammonium sulphate (4.2 kg) and gypsum (38.9 kg) per tonne of straw and manure; replacing poultry manure with an equivalent amount of N as urea resulted in higher N losses during composting with wheat straw; conversely, replacement with ammonium sulphate led to lower losses (Noble et al. 2002). Composts in which either of these two inorganic N sources replaced 50–100% of the poultry manure-N produced lower mushroom yields than when poultry manure provided the sole N source (Noble et al. 2002). This has also been found more generally, with excess application of inorganic N in compost formulations usually producing poorer mushroom yields than application of moderate amounts (Table 3).

Recycled water

Recycled compost leachate (so-called goody water) can be a significant source of compost N if it makes up a high proportion of the water added during pre-wetting of the raw materials. The composition of goody water is influenced not only by the compost ingredients but also by the wetting and composting procedures and rainfall on outdoor compost yards. Goody water samples collected from the storage

Table 3 Effect of compost inorganic nitrogen sources on mushroom yield in experiments conducted in medium- and large-scale facilities (> 1 tonne compost)

N source	N	Inclusion, %*		Yield, % of control		Control compost	Reference
	% w/w	Min	Max	@ Min	@ Max		
Ammonium nitrate	35	0.37	0.72	99–116	113–129	Horse manure	(Riethus 1962)
Ammonium sulphate	21.2	0.12		136		Horse and poultry manures	(Riber Rasmussen 1965)
Ammonium sulphate		0.27 N	0.40 N	134	117	Horse manure	(MacCanna 1969)
Ammonium sulphate		0.4		100–105		Horse and poultry manures	(Gerrits 1977a)
Ca ammonium nitrate	27	0.7		108		Horse manure	(Gerrits 1977b)
Calcium nitrate	17	0.27 N	0.40 N	107	86	Horse manure	(MacCanna 1969)
Urea	46.7	0.17–0.66	0.83	58–112	12	Horse manure	(Riethus 1962)
Urea		0.57		75		Horse manure	(Bech and Riber Rasmussen 1969)
Urea		0.35		100–105		Horse manure	(Gerrits 1977b)
Urea		50 N		79		Straw, poultry manure	(Noble et al. 2002)
Urea formaldehyde	38.9	0.68		49		Horse manure	(Bech and Riber Rasmussen 1969)
Urea formaldehyde		0.27 N	0.40 N	107	105	Horse manure	(MacCanna 1969)

* - % w/w of fresh weight or total nitrogen content of N sources (N)

tanks or pits of 14 mushroom composting sites in Britain and Ireland contained between 3.2 and 6.4 mg N L^{-1}, mainly in the form of urea, ammonium N, P-serine and other amino acids (Noble et al. 2006). Analysis of goody water from an Australian composting site showed much higher levels of 4–11 g L^{-1} of total N, measured over a period of one month (Safianowicz et al. 2018).

Influence of gypsum on compost nitrogen

Gypsum was originally added to mushroom compost to improve the physical structure by flocculating colloids and preventing greasiness and anaerobic conditions, but this function has been made unnecessary by the use of shorter and more aerated composting systems (Fermor et al. 1985). However, better mushroom yields were still obtained with compost where gypsum was added at 25 kg per tonne than when gypsum was omitted (Gerrits 1977a), which the authors attributed to the effect of gypsum in reducing compost pH and decreasing the dissociation of ammonium N into ammonia. Addition of gypsum (25 kg t^{-1}) to a straw and poultry manure compost led to normal mushroom yields of 300 kg t^{-1} pasteurised compost, whereas no mushrooms grew on the same compost without the addition of gypsum (Noble, unpublished). A further study (Riber Rasmussen 1965) found no effect of adding gypsum to compost, though the formulation used included both ammonium sulphate and calcium carbonate, which would react to form gypsum. Removing gypsum from the compost formulation altogether increased the rate of cellulase and xylan degradation, so that the time required to achieve a defined compost quality was reduced by approximately 20% (Mouthier et al. 2017). Increasing the rate of gypsum inclusion from 28 to 84 kg t^{-1} compost did not affect compost pH or mushroom yield (Beyer and Beelman 1995).

The work described above indicates that the beneficial effect of gypsum on mushroom compost is most likely due to the sulphate ions reacting with ammonia to form ammonium sulphate, thereby stabilising compost ammonium N. This effect is partially counteracted by the calcium ion content of gypsum, which would tend to increase compost pH and destabilise the ammonium-N in the compost. Mushrooms do not have a significant calcium requirement (Gerrits 1988) and there is an abundance of calcium from the lime content of the casing material which is used to cover the compost to induce sporophore production. It may therefore be more effective to add dilute sulphuric acid to compost to stabilise the ammonium N, a technique which is used to remove ammonia from composting emissions before biofiltration. The cost of sulphate ions in sulphuric acid is significantly less than in gypsum, although the cost and safety of spray application of acid would also need to be considered.

Compost supplements

The addition of protein-containing supplements to mushroom-colonised or 'spawn-run' (Phase 3) compost to increase mushroom yields and quality is now practised on most mushroom farms and has a considerable effect on yield and quality (Carrasco et al. 2018). The benefits are greater than with adding supplements to pasteurised (Phase 2) compost at spawning, where there is more competition for nutrients from other micro-organisms. In terms of mushroom yield, the benefit of adding supplements increases with 'meagre' composts that have low N content, although yields are still increased by supplementation of composts made with an 'adequate' N supply (Gerrits 1988; Noble and Gaze 1994). This indicates that the availability and/or type of protein in such composts is still sub-optimal and restricted by the amount of ammonium-N which can be present in the compost formulation. A wide range of materials of plant and animal origin have been tested for use as supplements. Seed meals and processed products, particularly from cottonseed and soya bean, generally give the best results, with performance related to crude protein content (Gerrits 1988; Randle 1985). The substances are usually treated with formaldehyde and/or coated to reduce the immediate availability of protein, in order to prevent a surge in compost temperatures, and also reduce the uptake of nutrients by competitor moulds. Commercial supplements are based on formaldehyde-treated soya bean meal and other biological by-products and are added to Phase 3 compost at 0.5 to 1.6% w/w, with expected mushroom yield increases of 10–30% (Burton and Noble 2015; den Ouden 2016; Gerrits 1988). Randle and Smith (1986) have calculated that a mushroom yield increase of at least 10%, without a change in quality, was required to justify the cost of compost supplementation using such materials, while a more recent estimate by Burton and Noble (2015) has put the typical gross value of the additional mushrooms harvested at six times the cost of the supplement, though this did not include the costs of applying the supplement or of harvesting and marketing the extra mushrooms.

Compost N sources in Australasia

Phase I mushroom compost is produced on around 12 composting yards across Australia and 4 in New Zealand, each site producing between 60 and 1800 tonnes each week. The composts are based on wheat straw as the main C source (Table 4), unlike many Phase I composts in Europe which are partially or entirely based on horse manure and may include other types of straw such as barley, rye and oilseed

Table 4 Current formulations used on some Australasian mushroom composting yards and typical Phase I compost analyses. All sites use wheat straw at 54–61% w/w of the raw materials and add gypsum at 25–30 kg/t compost

Parameter	Compost yard					
	A	B	C	D	E	F
Horse manure	0	0	✓	0	0	0
Poultry manure, laying	✓	✓	✓	✓	0	0
Poultry manure, broiler	0	✓	✓	0	✓	✓
Feather meal	0	0	✓	0	0	0
Canola meal	0	✓	0	0	0	✓
Cotton trash	0	✓	0	0	0	0
Cottonseed meal	0	0	✓	0	0	0
Soya bean meal	✓	0	0	0	0	0
Urea	✓	0	✓	0	✓	0
Ammonium sulphate	0	0	0	✓	0	0
Recycled water, %	50	✓	100	50	✓	90
Dry matter, % w/w	27.0	26.0	24.6	27.0	25.0	26.2
N (g kg^{-1}dry matter)	17.0	18.5	22.4	18.0	22.0	17.6
NH_4^+ (g kg^{-1}dry matter)	n.d.	4.0	4.0	2.0	4.5	1.8
Ash (g kg^{-1}dry matter)	272.4	n.d.	193.9	225.0	n.d.	103.0
pH	8.05	n.d.	8.26	8.25	8.3	8.1

n.d. not determined

rape. Phase I compost N contents are typically 1.8 to 2.2% w/w of DM (Table 4), which is predominantly supplied by poultry manure. Due to a decline in the rice crop in Australia, broiler poultry bedding material based on degradable rice husks is being replaced by wood shavings, leading to more composting yards using layer hen manure (Wiedemann et al. 2006) and increasing the need for alternative N sources. Some composting yards substitute some of the N supplied by the poultry manure with other materials, adding up to 6% inorganic N or 20% organic N replacements. To wet the composts, all the composting yards supplement fresh water with at least 50% recycled compost leachate. This addition of recycled compost leachate would theoretically account for less than 5% of the N added to the compost formulations, calculated from the average tonnages of Phase I compost produced, the moisture and N contents of the composts (Table 4), typical moisture and N contents of the raw materials (Table 1), and a DM loss of 30% during Phase I composting (den Ouden 2016; Gerrits 1988). However, this figure still needs to be verified with actual process measurements of e.g. applied water volumes, DM losses, N losses due to leaching and ammonification during Phase I and recycled water N content.

A range of organic matter N sources is available for mushroom composting in Australasia, but not all are equally suitable for widespread use, and their uptake by mushroom composters will depend on a number of factors. The most important of these are their content of microbially available N on a weight and bulk volume basis, and how successful similar materials have been in mushroom cultivation tests in other countries. However, more practical details are also critical, such as their year-round availability and/or tolerance to storage (and requirements for bulk storage), their uniformity and the absence of physical and chemical contaminants. Importantly, commercial viability will also depend on such N sources being widely available and not having significant alternative competitor value for applications such as such as animal feed, fertiliser or biofuels.

Organic N sources which are currently used to replace broiler poultry manure in Australasia are highlighted in Table 1. Materials with high moisture contents such as crop haulms, vegetable wastes, grape marc and paunch grass would only be viable if the sources are close to the composting yards and would require readjustment of the water applications made to the compost. Wool wastes could be used but would need longer composting processes to enable the N content to become available. The currently most used N source, poultry manure, even if used at an optimum inclusion rate, does not obviate the need for protein supplements in the prepared substrate to increase mushroom yields. Around 3000 tonnes of soya protein-based compost supplements are imported by the Australasian mushroom industry annually. This may offer an opportunity to replace these imports with locally produced supplements based on alternatives to soya, depending on the availability and suitability of by-product protein sources.

Conclusion and outlook

Commercial edible mushrooms are cultivated almost entirely on substrates derived from agricultural waste streams, particularly cereal straw and poultry manure, and their

production is therefore rightly viewed as part of the circular economy. In principle, a wide range of alternative agricultural wastes could be used, but the choice is rigidly controlled by the need to achieve the correct balance of available C and N sources in the mix. This balance maximises composting efficiency and balances the rate of ammonia production during composting with the accumulation of compost microbial biomass and with the final mushroom yield. However, changes in broiler chicken husbandry have led to a reduction in the N content of available manure, so alternative N sources are urgently needed. A range of different N sources has already been identified that enable the compost protein content to be increased without forming damaging levels of ammonia, and their use could potentially be improved by the application of sulphuric acid to replace gypsum. Further research is required to optimise the timing of N addition, so that this releases ideal amounts of ammonia for straw softening while retaining sufficient N to support growth and development of the microbial community needed both for lignocellulose breakdown and for subsequent growth of the mushroom mycelium. This research will build on recent advances in our understanding of the microbial dynamics of mushroom composting and allow us to develop processes that optimize feedstock conversion into compost of reproducibly high quality and support the development of microbial communities that maximise mushroom yield.

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Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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