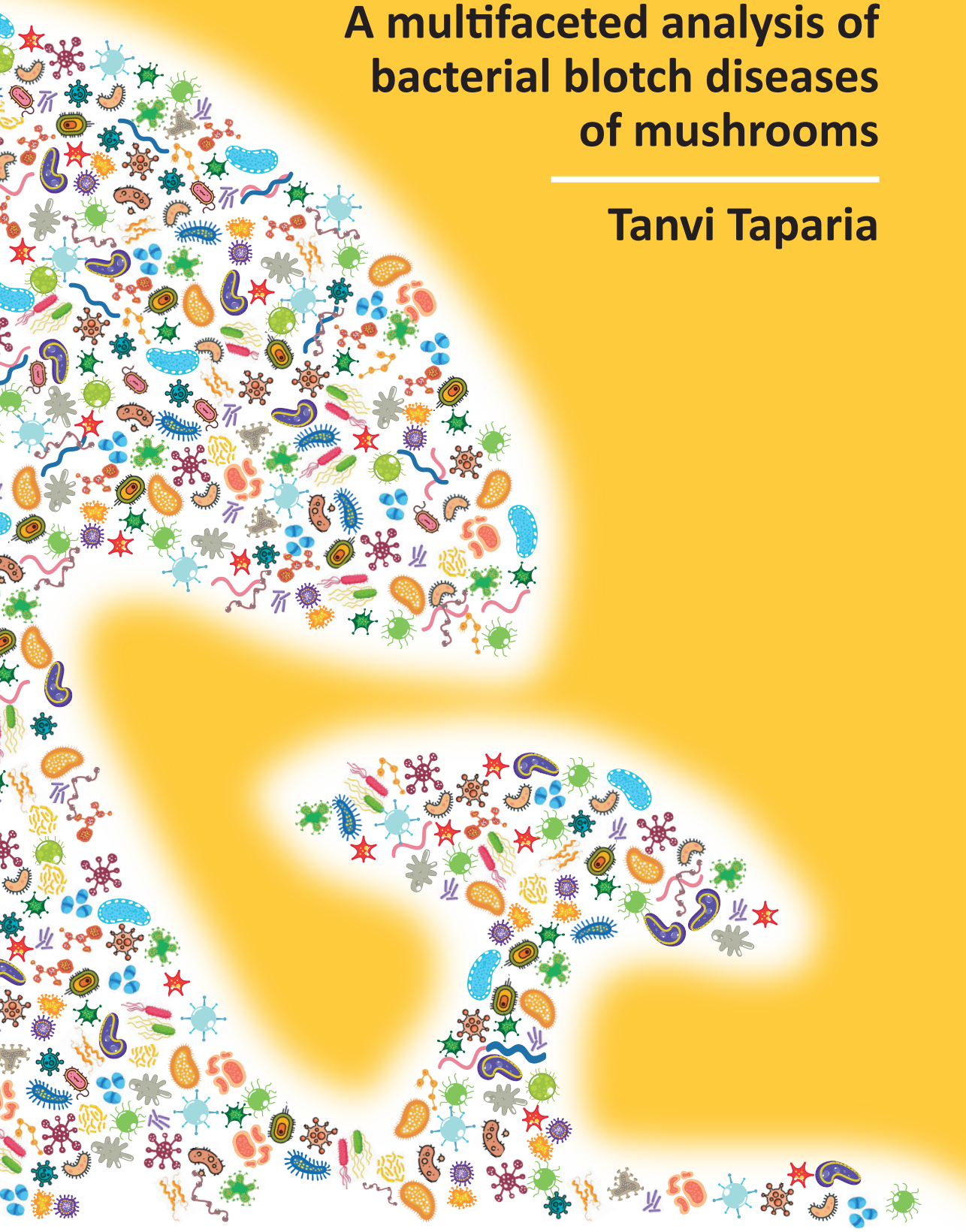
A multifaceted analysis of bacterial blotch diseases of mushrooms

Tanvi Taparia



Propositions

1. Bacterial blotch tracks on mushrooms can lead to different phylogenetic traces (this thesis).
2. The casing soil microbiome mediates suppression of bacterial blotch (this thesis).
3. Translating science to vernacular languages contributes to their preservation.
4. Research should have a mandatory outreach component.
5. Capitalism is a threat to democracies worldwide.
6. Empathy can lead to polarization of the public sphere.
7. We need to strive for meaningfulness and not happiness.
8. Dance is as important as biology.

Propositions belonging to the thesis entitled:

A multifaceted analysis of bacterial blotch diseases of mushrooms

Tanvi Taparia

Wageningen, 9th February 2021

A multifaceted analysis of bacterial blotch diseases of mushrooms

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A multifaceted analysis of bacterial blotch diseases of mushrooms

Tanvi Taparia

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To the strongest women in my life,

Ma, dama and nanima

मेरे जीवन की प्रेरणा-

माँ, दामा और नानीमाँ

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Chapter 1

General Introduction

An overview of mushroom cultivation

The diversity of mushroom-forming fungal species is enormous (>14000 species). Yet, only a handful are commercially produced for human food, of which the white button mushroom, *Agaricus bisporus*, is the most widely cultivated (Royse, Baars, and Tan, 2017). Global mushroom production has increased more than 27-fold in the last 35 years, from about 1 billion kg in 1978 to about 27 billion kg in 2012, which is remarkable specially in comparison to the global human population, which has increased 1.7-fold in the same duration, from about 4.2 billion in 1978 to about 7 billion in 2012 (Royse, 2014). On an average, consumers now enjoy about 5 kg of mushrooms per person per year (Royse et al., 2017). Major white button mushroom producing countries include China, United States of America, the Netherlands, United Kingdom, France and Poland (Chang, 2006).

Commercial cultivation of edible mushrooms represents a unique exploitation of microbial technology for the biological conversion of agricultural, industrial and forestry waste streams, such as compost, into value-added products (Yadav and Tewari, 2008). Mushrooms are a source of high-quality protein (Cheung, 2010), and they produce a range of metabolites that are of interest to the nutraceutical (Rathore et al., 2017) and pharmaceutical industries, such as, anti-tumour, immunomodulation and hypocholesterolaemia agents (Glamočlija and Sokovic, 2017). Mushrooms are also the highest protein source produced per unit area and time (Gupta, 1986). Thus, overcoming challenges in mushroom cultivation presents a promising scope to meet the upcoming global food challenge, with the least pressure on arable land.

The life cycle of the common button mushroom, *A. bisporus,* comprises of three basic stages, basidiospores, vegetative mycelium and reproductive sporophores. Spores germinate to produce mycelium, which are stimulated by environmental conditions to produce sporophores or fruiting bodies, which when developed eject basidiospores back into the environment (Umar and Van Griensven, 1997). Commercial cultivation of *A. bisporus* in developed countries is performed in indoor climate-controlled chambers with regulated environmental conditions such as relative humidity (RH), temperature, ventilation and CO_2 content (Sánchez, 2004). The processes and steps involved in commercial mushroom production are summarized in Figure 1. The primary components of *A. bisporus* cultivation include the compost, spawn and the casing soil (Figure 2).

Growth media and growing conditions

In Western Europe, compost for mushroom cultivation is prepared from a mixture of wheat straw, horse manure and chicken manure, which are stock-piled and allowed to reach temperatures greater than 70°C (Van Griensven, 1988). During this process, thermophilic microorganisms, convert low molecular weight nutrients that can be used by undesired organisms, to complex polysaccharides such as cellulose and lignin, which are specific for growth of *Agaricus* (Chanter and Spencer, 1974; Ross and Harris, 1983; Straatsma et al., 1994). This substrate is referred to as Phase I compost. This compost is then pasteurized in specialized composting tunnels for elimination of remaining mesophilic organisms and removal of ammonia vapours (Sánchez, 2004), and subsequently referred to as phase II compost.

Spawn is the colloquial term for monocultures of *A. bisporus* mycelium cultivated on sterilized cereal grain, sawdust or wood chips under laboratory conditions (Sánchez, 2004). This aseptic monoculture is used as starting material for the inoculation of the growth media. It reduces the risk of transmission of bacterial pathogens, weed fungi and viruses to the mushroom cropping system (Anderson et al., 2000). It is mixed into aerobically fermented compost, and incubated at 25°C for about a week, until the compost is fully colonized with *A. bisporus* mycelium, which is then referred to as phase III compost (Van Griensven, 1988).

The casing soil is a covering layer composed of peat, limestone and chalk (Figure 2). It is applied on top of a bed of phase III compost to facilitate development of the mushroom mycelium into sporophores (Rainey, 1989, 1985). The mushroom bed comprising of compost and casing soil is placed into the climate rooms and incubated at 18°C for 2 weeks, until primordia development of fruiting bodies. The grown sporophores are harvested for up to three consecutive harvest cycles (flushes) (Van Griensven, 1988). After which, the room is steamed at 70°C for 8 hours, by a process called ‘cook-out’, emptied and cleaned. The spent substrate is often used as fertilizer or soil improver.

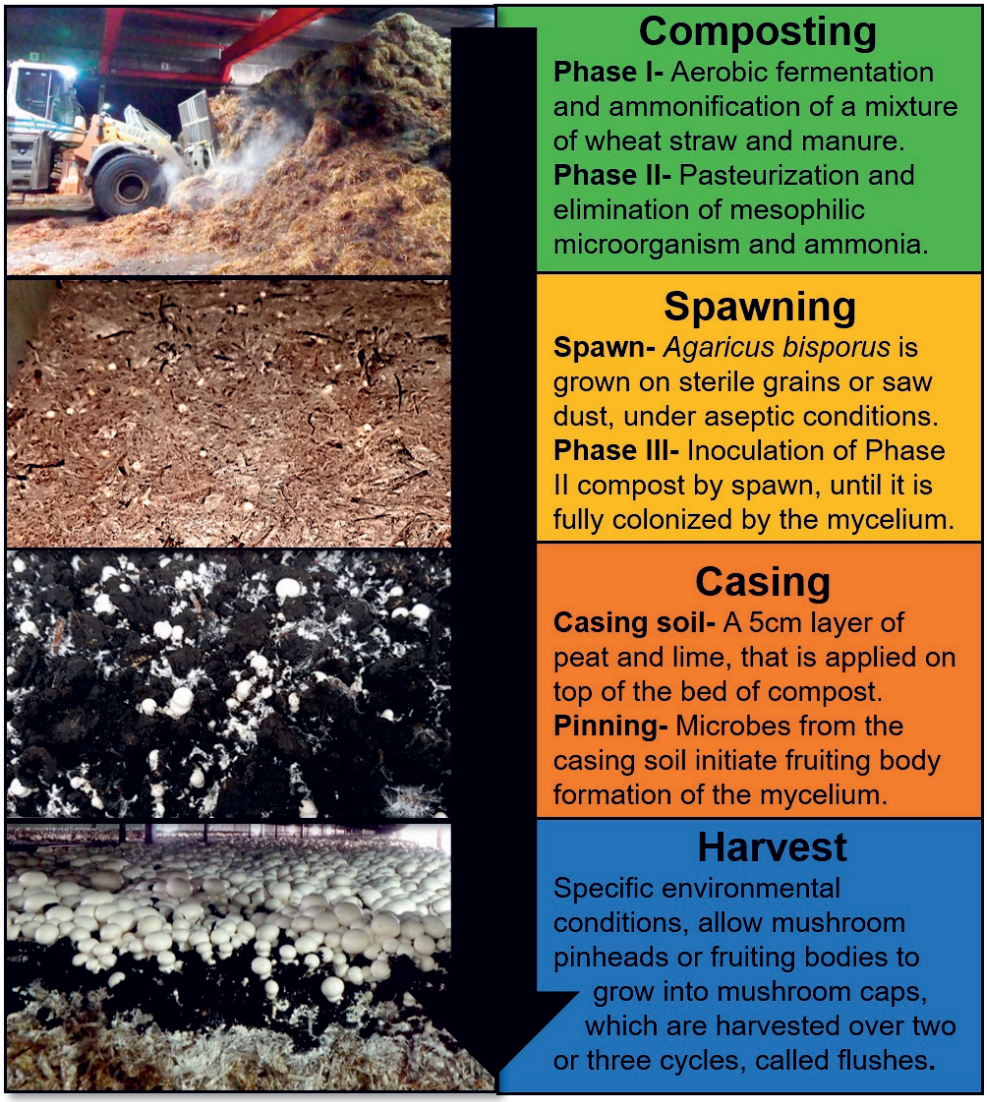

Figure 1. A summary of the steps and materials involved in commercial mushroom cultivation.

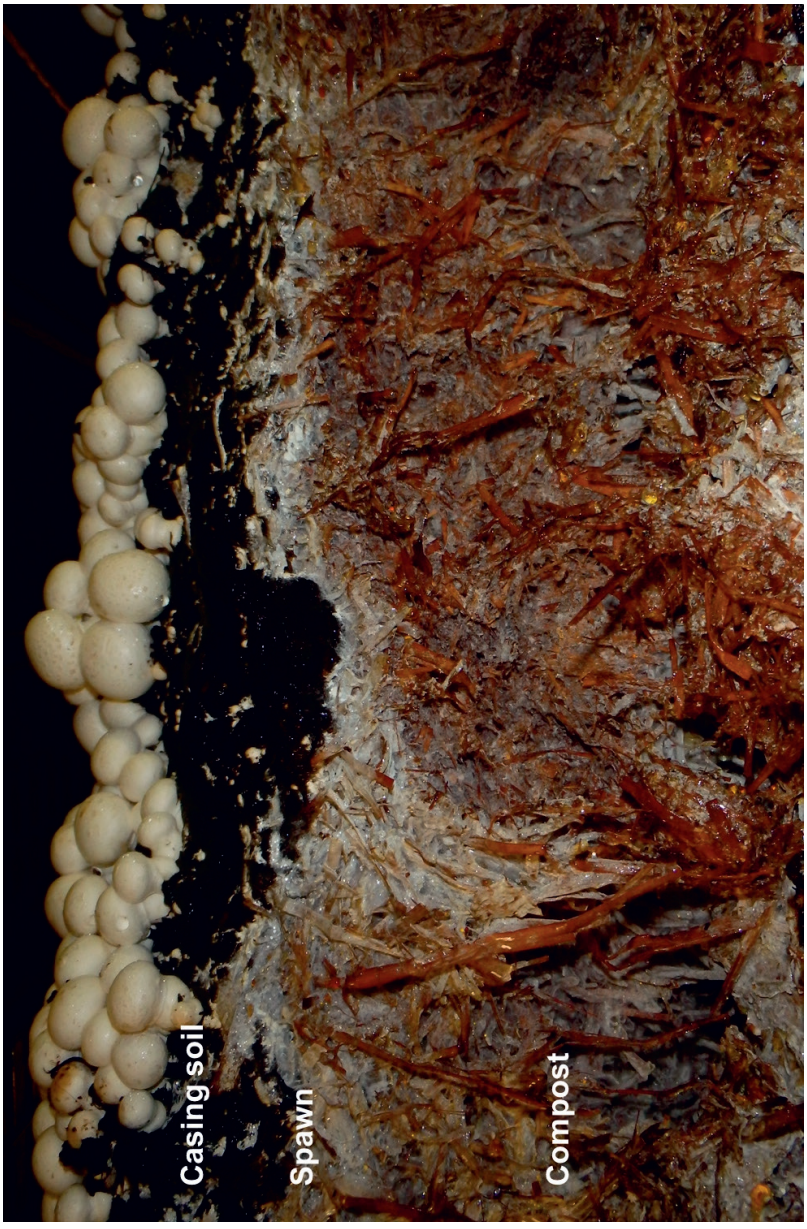

Figure 2. Mushrooms are grown on a bed of compost, over which a layer of peat-based casing soil is applied.

Peat in mushroom casing soils

Role of the casing soil

The casing soil is one of the prime determinants of productivity in mushroom cultivation (Pizer and Leaver, 1947), because it stimulates fruiting body development of the vegetative mycelium and leads to production of the mushroom caps (Rainey, 1989). The casing soil also performs other important functions, such as, the provision of physical support to the developing sporophores, functioning as a water reservoir for the mycelium, prevention of compost desiccation, and resisting structural breakdown due to watering (Noble et al., 1999). Consequently, the casing soil is known to be a major source of variation in the yield, quality and uniformity of commercial mushroom cultivation (Noble and Gaze, 1995).

Physical and chemical properties

Peat has been the primary component of growing media since the 1950s, due to its characteristic physico-chemical properties (Flegg, 1956, 1953). The consistency of its quality, abundant availability, low costs and easy storage conditions has made it an ideal substrate for casing soil and potting soil (Kitir et al., 2018). Due to its low nutrient content, it is also considered to have a low risk as a source of introduction for pests and pathogens (Schmilewski, 2008). Important physical characteristics for mushroom cultivation include high water holding capacity for mycelial growth, high pore fraction to allow gaseous exchange, and low soluble salt content that is non-inhibitory for mycelial growth (Rainey, 1985).

Microbiological interactions

Mushroom cultivation relies heavily on dynamics interactions of *A. bisporus* with the beneficial casing soil microflora to induce transformation of the vegetative mycelium into fruiting bodies (Rainey, 1989). Heat treated casing soil, which lacked a microbial community, failed to produce mushroom caps, and instead, made lumps of mycelial sheets (Eger, 1962; Hume and Hayes, 1972). Many microorganisms endemic to the casing soil are known to induce fructification, including *Bacillus psilocybe, P. putida* (Hayes et al., 1969)*, Bacillus megaterium, Arthrobacter terregens, Rhizobium metiloff* and *Scenedesmus quadricauda* (Stamets and Chilton, 1983). Of these microbes, *P. putida* has received by far the most attention (Arrold, 1972; Rainey, 1989; Baars et al., 2020).

Drawbacks of peat use

Peat use in mushroom casing soils is associated with a high environmental impact (Robinson et al., 2019). Wet peatlands constitute a major source for sequestering carbon (Joosten et al., 2016). They also harbour a high and unique biodiversity (Minayeva et al., 2007). Peat mining and its long-distance transport are hence associated with a high CO_2 footprint (Waddington et al., 2002). EU directives strongly discourage peat extraction. Severe peat supply bottlenecks are expected in the next ten years due to rapidly declining global peat deposits and policies for conservation of peatland biodiversity (Bos et al., 2011). Additionally, enrichment of mushroom pathogens in the casing soil leads to a large chemical input of disinfectants and fungicides to prevent soil-borne diseases. Given the aforementioned concerns (Figure 3), the search for sustainable and circular alternatives to partially or completely replace peat in casing growing media has gained a lot of attention (Noble and Dobrovin-Pennington, 2015; Pardo-Giménez and Pardo-González, 2008; Pardo et al., 2004; Peyvast et al., 2007; Sassine et al., 2005). However, the presence of pests and diseases in these alternative casing soils is often not investigated, and the availability of a suitable alternative to peat in casing soil remains a bottleneck.

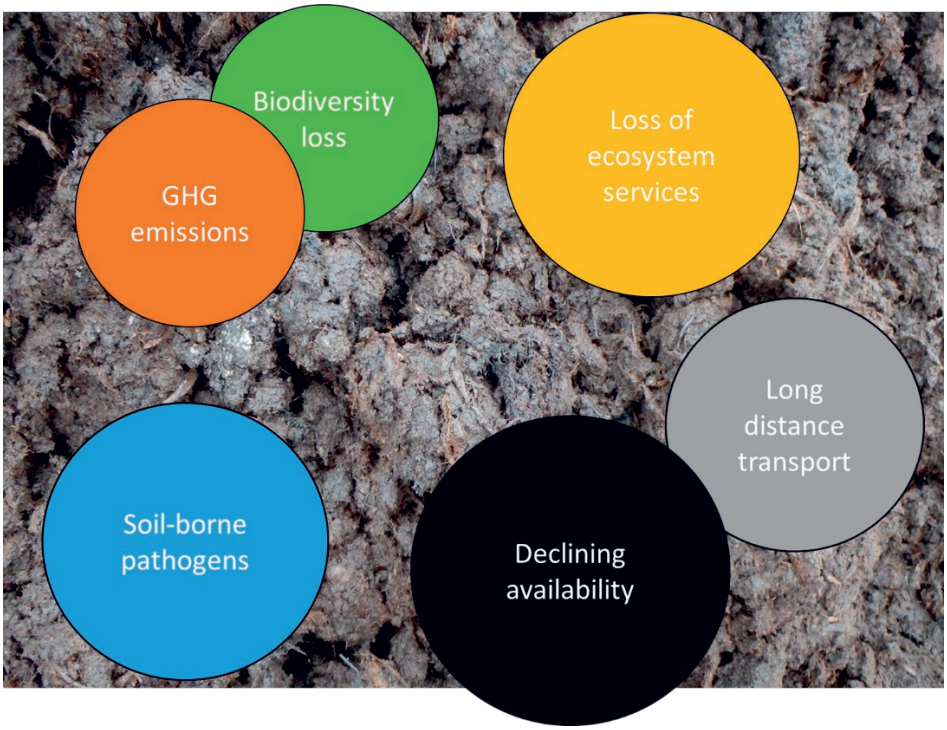

Figure 3. Drawbacks of using peat as casing soil in mushroom cultivation

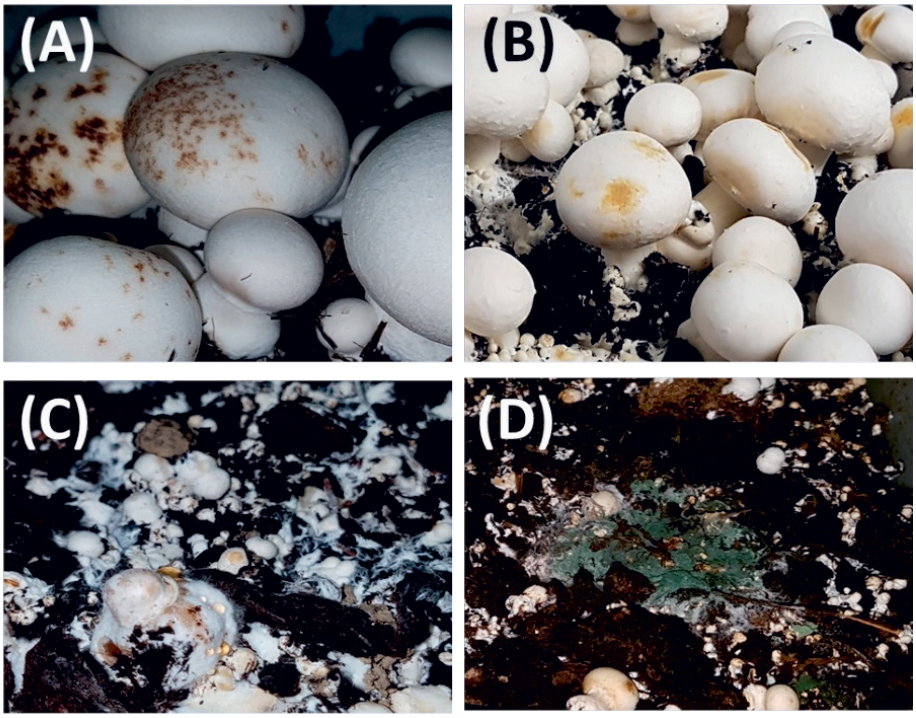

Figure 4. A few casing soil related diseases of white button mushroom, *A. bisporus* are (A) brown blotch, (B) ginger blotch, (C) wet bubble and (D) green mould.

Casing soil microflora

In mushroom beds, which comprise of the compost and the casing soil, fluorescent pseudomonads represent 10% of total aerobic population, which was evaluated as 10^7-10^8 cells/g with plating-based methods. Generally, they increased up to 50%-90% of the total of 10^9 cells during the harvest cycles (Samson et al., 1987). The abundance of the microbial community is estimated to be even higher in the hyphosphere soil surrounding the mycelium (Fermor et al., 2000). These beneficial microflora from the casing soil share a close relationship with the mushroom hyphae and can move upward along longitudinally orientated hyphal strands in the internal stipe of the mushroom (Zarkower et al., 1984).

The casing soil is not only a supplier of beneficial organisms, it is also inhabited by various bacterial and fungal pathogens that can cause diseases on the mushroom cap (Fletcher et al., 1989). Common bacterial diseases of mushroom include, brown blotch, ginger blotch and drippy gill (Paine, 1919; Wong et al., 1982; Young, 1970).

These are caused by closely related *Pseudomonas* sp., which are speculated to be endemic to the casing soil. Some fungi inhabiting the casing soil are also pathogenic to mushrooms, e.g. *Dactylium dendroides* which causes cobweb mould (Fletcher, et al., 1989) and *Mycogone perniciosa* which causes wet bubbles (Stamets and Chilton, 1983). Soil-borne diseases of mushrooms, their pathogens, and management strategies are further described in later.

Recent molecular investigations have further described the casing soil and microflora with culture-independent methods (Pecchia et al., 2014; Carrasco et al., 2019; 2020; Martins et al., 2020). The composition of endemic microbiome of the casing soil has been shown to be influenced by the growth of *A. bisporus* in mushroom beds and increased contact with the compost (Pecchia et al., 2014; Carrasco et al., 2020). The soil microbiome also determined the composition of the mushroom cap microbiome, which plays an important role in disease development (Martins et al., 2020). The casing soil microflora are also implied to naturally suppress the development of fungal diseases in the mushroom beds at low pathogen densities (Carrasco et al., 2019).

Soil-borne diseases of mushrooms

Bacterial blotch

Bacterial blotch is a group of diseases caused by *Pseudomonas* species endemic to the casing soil, that lead to discoloration and disfiguration of the mushroom cap (Fletcher et al., 1989). It leads to large economic losses globally, not only due to loss of marketable crop, but also from reduced shelf-life post-harvest (Wells et al., 1996). Brown blotch is caused by *P. tolaasii*, and produces small, irregular and brown spots on the cap surface, often accompanied by lesions (Paine, 1919). Ginger blotch, caused by '*P. gingeri*', an uncharacterized species, produces orange coloured discolorations that are spread over a larger surface area of the cap (Wong et al., 1982). Other known pathogens of bacterial blotch show large phenotypic variation and genetic diversity, including '*P. reactans', P. agarici, P. marginalis, P. costantinii* and *P. fluorescens* (Abou-Zeid, 2012; van der Wolf et al., 2016). Details about the causative agents of bacterial blotch, their critical densities and thresholds for blotch outbreak, mechanisms of infection and management strategies are described in later sections.

Drippy gill

Drippy gill is a bacterial disease of button mushrooms, caused by *Pseudomonas agarici* (Young, 1970), a close relative of '*P. gingeri*'. The pathogen attacks the gills, after the inner veil breaks down, and the mushrooms do not develop, or their

development is delayed and they become distorted (Young, 1970). Its mechanisms of action include, colonization of the extra hyphal spaces by *P. agarici*, and degradation of the extracellular matrix within affected sporocarps, which reduces the integrity of the sporocarp, and results in splitting of the stipe (Gill and Cole, 2000). Geels et al., (1994) also reported *P. agarici* to produce superficial brown discolorations on *A. bisporus* sporophores, like that of brown blotch (Cantore and Iacobellis, 2004). Drippy gill is a not a common mushroom disease, but when it occurs, it produces high incidence and severity of disease symptoms (Gill, 1994). Additionally, *P. agarici* is also a known pathogen for yellow blotch of oyster mushroom, *Pleurotus ostreatus* (Bessette et al., 1985). No management strategies are available for the control of this pathogen.

Wet bubble

Wet bubble disease is caused by a fungal pathogen, *Mycogone perniciosa* (Stamets and Chilton, 1983). This mycoparasite causes pathological changes in fruit bodies of *A. bisporus*, to form undifferentiated primordia (Kouser and Shah, 2013). It produces large, irregular, confluent lumps of *A. bisporus*, that are hyperplastic and tumorous in character (Umar et al., 2000). *M. perniciosa* is introduced into mushroom farms from the casing soil (Fletcher et al., 1989), although infection through the compost can also occur rarely (Sharma and Kumar, 2000). In severely infected mushroom farms, it leads to 100% crop loss (Kouser and Shah, 2013). Known fungicides prochloraz-Mn (ED_{50} = 0.006–0.064 µg ml^{-1}) and carbendazim (ED_{50} = 0.031–0.097 µg ml^{-1}) have been shown to effectively inhibit the mycelial growth of *M. perniciosa* (Gea et al., 2010). Studies on biological control of wet bubble have also been performed (Najafi et al., 2015; Ozaktan and Bora, 2000).

Green mould

The presence of weed moulds in mushroom cropping systems is usually an indicator for improper hygiene and farm management and is often followed by more diseases and pests (Fletcher et al., 1989). Green mould is caused by several *Trichoderma* species, such as *T. harzianum, T. viride, T. atroviride* and *T. aggressivum* (Samuels et al., 2002), which originate from the compost. Instead of a mycoparasite, *Trichoderma* was generally considered to be a competitor for nutrients or antagonist, as it reduces fruitbody production and inhibits mushroom growth. However, *Agaricus* and *Trichoderma aggressivum* have recently been shown to interact with each other as host-pathogen, not competitors (Kosanovic et al., 2020). A *Trichoderma* infected farm can have a large variation in yield loss from 30 to 100% (Samuels et al., 2002). *Trichoderma sp.* infect both the compost and the casing soil, and outbreaks are encouraged by excessively wet casing soil, high and prolonged humidity in combination with stagnant air and high carbon dioxide levels. Infected mushrooms

are often covered by a fine mildew that eventually becomes greenish due to spore production under light conditions (Krupke et al., 2003). Management of green mould includes extended peak-heating during the compost preparation either in temperature or time (Park et al., 1971), biological control (Pandin et al., 2018), use of fungicides (Kosanović et al., 2015) and the use of resistant spawn (Anderson et al., 2000).

Molecular aspects of bacterial blotch

The causative agents

Pseudomonas strains causing bacterial blotch on mushrooms are known to exist in two variants on King's B agar, as opaque, non-fluorescent smooth colonies for the pathogenic form, and as translucent, fluorescent, rough colonies for the non-pathogenic form (Cutri et al., 1984). In *P. tolaasii*, the switch between these variants is controlled by the *Phen* locus (Grewal, Han, and Johnstone, 1995), the expression of which depends on nutrient conditions and formation of secondary metabolites. This enables the pathogen to colonize multiple environmental substrates, as saprophyte or as a pathogen. Bacterial compounds involved in gaining access to a nutrient source such as the mycelium from *A. bisporus*, include proteinases, lipases and toxins, for disruption of host membranes, and siderophores, for antagonisms against other competing microbes (Soler-Rivas et al., 1999).

Interaction with the host

The presence of *A. bisporus* mycelium in the otherwise nutrient deficient and heavily moistened casing soil, provides a nutrient gradient for the migration of the casing microflora towards the host (Samson et al., 1987). *P. putida* and *P. tolaasii* both show phenotypic variation in their chemotactic response to mycelial exudates from *A. bisporus.* Rough colony forms, that are non-pathogenic, show faster chemotaxis towards the host, than smooth colony forms that are pathogenic (Grewal and Rainey, 1991). Yet the ability to attach itself to the host mycelium is higher in pathogenic variants (Rainey, 1991). Mushrooms generally have defence mechanisms against microbial infections, which involve the production of extracellular bacteriolytic and fungolytic enzymes (Tsuneda, 1992; Tsuneda and Thorn, 1994). *A. bisporus* is known to derive nutrition from heat-killed bacteria, fungi and actinomycetes (Fermor and Wood, 1981). However, this has not been described specifically for the interaction of *P. tolaasii* with *A. bisporus* yet (Soler-Rivas et al., 1999).

Mechanisms of infection

Once attached to the host, pathogenic variants of *P. tolaasii* are known to produce an extracellular pore-forming toxin, tolaasin, which is a major virulence factor for brown blotch (Cho et al., 2007). Tolaasin is a cyclolipodepsipeptide with biosurfactant properties and can produce disease symptoms when inoculated directly on mushrooms (Hutchison and Johnstone, 1993; Nutkins et al., 1991). The gene cluster for biosynthesis of tolaasin has been identified (Rainey et al., 1993). PCR assays for the detection of tolaasin have also been developed (Lee et al., 2002). However, several bacterial blotch pathogens, such as *P. agarici* and '*P. gingeri'* do not produce tolaasin (Lee et al., 2002). The mechanisms of infection of non-tolaasin producing pathogens are not understood yet. The biochemical discoloration of the cap surface, after infection by pathogen is caused by oxidation of phenolic substances by fungal tyrosinases, which are formerly present in latent form (Soler-Rivas et al., 2000). In fungi, tyrosinases have been long-associated with browning and pigmentation (Soler-Rivas et al., 1999), and melanin production constitutes a mechanism of defence against further infection by the pathogen (Bell and Wheeler, 1986).

Critical densities for blotch

Many studies have assessed the minimum bacterial densities required for symptom development on the mushroom cap of *A. bisporus.* These studies have been largely limited to brown blotch pathogen, *P. tolaasii.* Threshold densities of the pathogen on the cap surface for development of blotch symptoms range from $1x10^5$ cfu per cap (Nair and Bradley, 1980) to $6x10^7$ cfu per cap (Wong and Preece, 1982). This can range up to $2x10^{10}$ cfu per cap (Olivier et al., 1997). Fewer studies have investigated pathogen densities in the casing soil. Pathogen populations in the casing soil have been reported in range of 10^7-10^8 cfu/g in diseased beds (Nair and Fahy, 1972), although lower densities have also been reported (Wong and Preece, 1980). In healthy beds, pathogen densities in the range of and 0-10^7 cfu/g have been found based on plating on selective media (Nair and Fahy, 1972). Unfortunately, diagnostic methods such as plating on selective media, colony morphology and phenotypic tests, have proven to be unspecific, considering the vast diversity of blotch-causing organisms and closely related beneficial *Pseudomonas* (Munsch and Alatossava, 2002), leading to largely overestimated pathogen densities. Recently developed PCR assays were only qualitative, and selectively targeted tolaasin-producing pathogens (Lee et al., 2002). Specific and quantitative diagnostic tools need to be developed for the determination of soil inoculum thresholds required for blotch outbreak, and for determining pathogen populations on mushroom caps and in the casing soil.

Management strategies against blotch

Environmental conditions

Brown blotch pathogen, *P. tolaasii*, is endemic to the casing soil and was found to be present on both healthy and diseased mushroom beds (Nair and Bradley, 1980). Under favourable environmental conditions, these endemic populations can trigger a disease outbreak. High relative humidity together with air temperature changes, in the absence of adequate ventilation, leads to condensation of water on the mushroom caps, which induces disease development (Wuest, 1971; Soler-Rivas et al., 1999). However, high relative humidity is also essential for growth and cultivation of *A. bisporus*. Overhead watering of mushroom beds allows further spread of the pathogen and increases the disease severity and incidence of blotch (Sinden, 1971). Abiotic conditions contribute heavily to blotch development, and their proper management is speculated to also drive blotch control.

Multiple studies have been performed to assess the effect of humidity and temperature on the blotch management, although the results are widely inconsistent between reports. Gandy (1967) found that a RH of 82% was favourable for blotch development, and the disease drastically reduced if the RH was maintained at 75%. However, Wong and Preece (1982) reported that neither an increase in temperature from 16 to 19°C, nor an increase in RH from 70-90% affected blotch incidence or pathogen populations. Recently, Navarro et al., (2018) were able to successfully control blotch by increasing the temperature prior to watering and moderating the manoeuvres of the drying process. However, it remains to be understood if such precision control of the climate and drying conditions in mushroom farms is feasible for the majority of growers. In addition, it is unknown what the implications are for post-harvest shelf life of the mushrooms, if the pathogen is not eliminated.

Chemical control

Extensive research has been done on the use of organic and inorganic compounds for control of brown blotch, despite unsuccessful results. Of the tested compounds, chlorine has been most widely used (Bashan and Okon, 1981; Royse and Wuest, 1980), but its efficacy is limited due to the discoloration of mushrooms at higher doses (Royse and Wuest, 1980) and reduced productivity of the crop (Rutjens, 1977). Other chemicals that have been explored include sodium sulphite, bipyridyl, sorbitol, formalin, glutaraldehyde and sodium metasulphite, but both their effectiveness and toxicity are problematic (Godfrey, 2003). Other methods have focused on partial sterilization of the casing soil, such as, by fumigation with ethylene oxide, but this also killed beneficial microbes from the soil. Disinfection with bronopol was found to be most effective due to its slow-acting bactericidal effect (Wong and

Preece, 1985). However, its effect on the beneficial microflora of the casing soil has not been investigated yet. Antibiotics such as kanamycin and streptomycin are effective against blotch at low concentrations (Munjal et al., 1989; Vantomme et al., 1989), without loss of yield. Although antibiotic use in mushroom production is currently illegal. In addition, non-medical use of antibiotics is being severely restricted in the wake of a global crisis of antibiotic resistance (Meek et al., 2015).

Biological control

Mushroom cultivation presents a favourable opportunity for successful deployment of biological control agents for two reasons. Firstly, the stable and mesophilic environmental conditions required for the growth of *A. bisporus*, are ideal for the growth and survival of most microorganisms. Secondly, the casing soil, which is microbiologically poor, is an ideal carrier for antagonist populations. Consequently, biological control of bacterial blotch has received considerable attention in the last years. Microorganisms have been used to combat pathogens in mushroom beds or on caps, on the basis of different antagonist strategies, including nutrient competition with the pathogen, which are reviewed by Godfrey (2003), predatory activity against the pathogen (Saxon et al., 2014), bacteriophage therapy (Kim et al., 2011; Munsch et al., 1991; Sajben-Nagy et al., 2012), detoxification of the toxin tolaasin (Hermenau et al., 2020; Tomita et al., 2018; Tsukamoto et al., 2002), and bacteriocin production (Parret et al., 2005). Despite many successful reports of biocontrol under laboratory conditions, many challenges are yet to be overcome for practical use.

Phage therapy, despite its specific host range, may fail due to the rise of phage-resistance. After a successful round of phage therapy, phage-resistant variants of *P. tolaasii* were found in mushroom farms (Park et al., 2016). For bacterial antagonists, lack of knowledge of the casing soil microbiome and specific physico-chemical characteristics of the soil matrix also contribute to reduced antagonist populations and an inconsistent field performance. Reduced effectiveness *in-situ* has also been documented for gram positive biocontrol agent (Tsukamoto et al., 1998). Additionally, our knowledge of pathogenicity determinants is minimal, and it cannot be predicted if closely related antagonists may also cause damage under specific conditions. For example, *Mycetocola* spp. closely related to those which were used to inactivate tolaasin (Hermenau et al., 2020), were found to induce pitting symptoms on the cut mushroom caps (Osdaghi et al., 2019). Biocontrol strategies against blotch are further complicated by the fact that the genus *Pseudomonas* is both involved in disease expression (Fletcher et al., 1989; Godfrey, 2003) and in fructification of the mushroom pinheads (Rainey, 1989). For instance, predatory bacteria *Bdellovibrio*, despite its effectiveness against brown blotch, were found to also change the beneficial microbiome of the soil due to its broad host range (Osdaghi et al., 2019). Given the importance of endemic and beneficial *Pseudomonas* to mushroom cultivation, application of broad-spectrum antagonists,

suffers from an incomplete understanding of the diversity of blotch causing and beneficial *Pseudomonas* present in the casing soil.

Resistance breeding

Techniques similar to those used in plant breeding and genetic screening can be applied to mushroom breeding for resistance against bacterial blotch. Unfortunately, resistance breeding has received significantly less attention compared to breeding for yield, quality and aesthetic parameters such as pinning time, cap size, and whiteness of the cap (Sonnenberg et al., 2017). Current knowledge on blotch resistance breeding is limited to brown blotch pathogen, *P. tolaasii*. Early screening of mushroom cultivars against brown blotch revealed that wild *Agaricus* strains are significantly less susceptible than the commercial strains (Olivier et al., 1997). A wide range of tolerances and susceptibilities were observed between the cultivars, however, complete resistance to the toxin tolaasin, or the brown blotch pathogen was not found (Moquet et al., 1998). Tolerance to blotch is attributed to the development of an impenetrable layer of dead hyphae below the infection site, which prevents the pathogen from invading the sporophore (Cole and Skellerup, 1986). A quantitative trait locus (QTL) for cap colour formation (pilei pelles colour 1) was also found to be strongly linked to brown blotch resistance (Moquet et al., 1999). However, it could explain only 30% of the variation in sensitivity to blotch between the cultivars. Discoloration of the cap surface after physical damage was also mapped to a QTL identified as sensitivity to bruising (Gao et al., 2015).

A. bisporus lines that are resistant to enzymatic browning were recently generated using CRISPR-Cas 9, by eliminating a group of genes that are involved in the production of PPO's (Waltz, 2016). Even though it is strongly suspected that cap colour, browning activity and resistance to blotch are genetically linked due to the biosynthesis of melanin, it is not clear, if these lines will be resistant to bacterial blotch too. There exists a wide variety of blotch pathogens and their mechanisms of infection and symptom development are largely unknown. Many of these pathogens do not have the gene cluster required for the biosynthesis of known toxin tolaasin (Lee et al., 2002). Traits such as cap colour, bruising sensitivity, resistance to enzymatic browning and blotch resistance appear to be highly heritable, and variations present in wild strains of *Agaricus bisporus* could be useful for mushroom breeding (Sonnenberg et al., 2017). Resistance to tolaasin could be a preliminary assay when screening mushroom strains for bacterial blotch (Osdaghi et al., 2019), but the broad diversity of blotch pathogens should be evaluated as well, in order to study the disease tolerance of a candidate mushroom cultivar.

Aims, research questions and scope

The main aim of this research was to investigate bacterial blotch pathogens in mushroom cultivation, and their related microbial interactions with the host and the soil microbiome, in order to better understand the key factors determining disease outbreaks in mushroom cultivation.

The general research questions addressed in this thesis are:

- Who can cause bacterial blotch?
- How can we detect blotch pathogens?
- Which conditions are favourable for blotch?
- Is peat a source of introduction?
- Can peat-use in casing soil be avoided?
- What role do soil microbes play in blotch outbreaks?

To address these research questions, several genomic, microbial, physico-chemical and phytopathological experiments were performed, the results of which are described and discussed in six experimental chapters of this thesis.

Chapter 2 provides a detailed overview of genetic and regional diversity and the virulence of blotch causing organisms in Western Europe. It describes the molecular characterization of the phylogenetic and phenotypic diversity of bacteria colonizing the caps of diseased mushrooms, using prokaryotic genomics, with large implications in the study of symptomatic disease expression, development of diagnostic tools and design of localized strategies for disease management.

Chapter 3 describes the development of multiplex Taqman^{TM}-qPCR assays for the detection of three blotch pathogens at low densities in environmental samples. It reports the diagnostic sensitivity and specificity, reproducibility and repeatability of the assays. Moreover, it includes successful validation and application of the assays for routine testing of samples associated with mushroom cropping systems, such as water, compost, peat source and casing soil.

Chapter 4 investigates the infection dynamics and population dynamics of newly discovered brown blotch pathogen, *P. salomonii*, with that of ginger blotch pathogen, '*P. gingeri*'. It identifies the soil inoculum thresholds for both pathogens and elucidates the role of abiotic and biotic parameters such as casing soil composition and environmental humidity for their blotch outbreaks. It also studies the endemic and inoculated pathogen populations in various casing soils.

Chapter 5 reports the development of soil suppressiveness to ginger blotch in consecutive mushroom cultivation cycles, despite increasing pathogen populations. It describes changes in the soil microbial community due to pathogen invasion, pathogen establishment and blotch suppression, and identifies specific bacterial and fungal genera that are associated with blotch suppression. It also reports on the transferability of blotch suppressiveness to conducive soils with aqueous soil extracts.

Chapter 6 proposes three circular alternatives for replacement of peat in mushroom casing soils. It evaluates the agronomical performance, physico-chemical characteristics, and the soil microbiome of these peat-reduced growing media. It addresses trade-offs between productivity and disease pressure in these circular cropping systems, with regard to the diversity, composition and interactions within their microbiomes. It also comments on the accessibility and sustainability of these peat-alternatives.

Chapter 7 discusses the main findings presented in this thesis, integrates these results with existing knowledge on bacterial blotch and elaborates on their advantages and limitations, with particular focus on blotch management. Finally, it places this research in context for localized and ecological management of soil-borne diseases in mushroom cropping systems, and the future research required to achieve sustainable and blotch-free mushroom cultivation.

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Chapter 2

Identification of blotch-causing organisms

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Abstract

Bacterial blotch is a group of economically important diseases affecting the cultivation of common button mushroom, *Agaricus bisporus*. Despite being studied for more than a century, the identity and nomenclature of blotch-causing *Pseudomonas* species remains unclear. This research aims to molecularly characterize the phylogenetic and phenotypic diversity of blotch pathogens in Western Europe. In this study, blotched mushrooms were sampled from farms across the Netherlands, United Kingdom and Belgium. Bacteria were isolated from symptomatic cap tissue and tested in pathogenicity assays on fresh caps and in pots. Whole genome sequences of pathogenic and non-pathogenic isolates were used to establish phylogeny via multi-locus sequence alignment (MLSA), average nucleotide identity (ANI) and in-silico DNA:DNA hybridization (DDH) analyses. The known pathogens '*Pseudomonas gingeri*', *P. tolaasii*, '*P. reactans*' and *P. costantinii* were recovered from blotched mushroom caps. Seven novel pathogens were also identified, namely, *P. yamanorum*, *P. edaphica*, *P. salomonii* and strains that clustered with *Pseudomonas* sp. *NC02* in one genomic species, and three non-pseudomonads, i.e. *Serratia liquefaciens*, *S. proteamaculans* and a *Pantoea* sp. Insights on the pathogenicity and symptom severity of these blotch pathogens were also generated. A detailed overview of genetic and regional diversity and the virulence of blotch pathogens in Western Europe, was obtained via the phylogenetic and phenotypic analyses. This information has implications in the study of symptomatic disease expression, development of diagnostic tools and design of localized strategies for disease management.

Introduction

Commercial button mushroom cultivation relies heavily on the dynamic interactions between *Agaricus bisporus* and the casing soil microflora (Hayes et al., 1969). The transformation of vegetative mycelium into a fruiting body is initiated by beneficial microbes in the casing soil (Eger, 1962; Preece and Wong, 1982; Thomas et al., 1964). However, the casing soil also introduces pathogenic microbes into mushroom farms, including blotch causing *Pseudomonas* species (Fletcher et al., 1989; Fletcher and Gaze, 2007; Wong and Preece, 1980). The humid and mesophilic conditions required for mushroom production are highly conducive to the enrichment and spread of such pathogens. Reliable identification and early detection are thus essential to avoid disease outbreaks.

The genus *Pseudomonas* is one of the most complex genera of Gram-negative bacteria due to its large size of 114 species (Gomila et al., 2015). They form a major proportion (~ 40%) of the total culturable bacteria obtained from casing soil in mushroom farms (Godfrey, 2003). While some of these are essential for stimulating the pinning of button mushrooms (e.g. *P. putida)* (Hayes et al., 1969; Hume and Hayes, 1972), others are detrimental to crop health (e.g. *P. tolaasii*) (Paine, 1919; Tolaas, 1915).

Bacterial blotch is a group of diseases that result in discolouration and disfiguration of mushroom caps in *A. bisporus*, due to fungal production of phenols and tyrosinases (Brennan et al., 2000). This reduces the total marketable crop due to compromised aesthetic value, lowers the shelf-life post-harvest, and lessens the overall yields due to pin death. These aspects of bacterial blotch jointly lead to severe economic losses (Burton, 1988; Burton and Noble, 1993; Soler-Rivas et al., 1999). Various *Pseudomonas* species are the main causative agents of blotch diseases on mushroom caps (Fletcher and Gaze, 2007).

Pseudomonas tolaasii causes small sunken dark brown spots or lesions on the mushroom cap that are referred to as “brown blotch” (Paine, 1919; Tolaas, 1915). *‘P. reactans’* is known to cause varying discoloration from dark to light, accompanied by a surface depression (Wells et al., 1996) and *‘P. gingeri’* produces ginger coloured discolorations that are more spread out on the cap surface, called “ginger blotch” (Wong et al., 1982). Both of these species have not been formally described. *P. agarici* is the causative agent of “drippy gill” on *A. bisporus* and “yellow blotch” on oyster mushrooms (*Pleurotus* spp.), where it leads to relatively pale discolorations (Young, 1970). Global reports also indicate the role of other *Pseudomonas*, such as *P. costantinii, P. fluorescens* and *P. marginalis* in bacterial blotch diseases, with large phenotypic variation within and across species (Abou-Zeid, 2012).

Blotch pathogens can be considered as endemic to the casing soil, an artificially prepared growth media composed of peat and lime, that is added on top of the compost (Nair and Bradley, 1980; Wong and Preece, 1980). They have been found on healthy crops at similar densities to that of diseased crops (Godfrey, 2003). It has thus been suggested that not just the pathogen density, but the composition of *Pseudomonas* species in the casing soil, especially the relative abundance of beneficial and disease-causing species, can be an important indicator for disease outbreaks (Godfrey, 2003). A deeper understanding of the beneficials and pathogens within the genus is hence necessary.

Bacterial blotch has been studied for over a century (Elphinstone and Noble, 2018; Osdaghi et al., 2019), despite which the identity and nomenclature of blotch-causing *Pseudomonas* is still unclear. Recent molecular investigations clarify the taxonomy of some blotch pathogens (Godfrey et al., 2001b, 2001a; Munsch and Alatossava, 2002; van der Wolf et al., 2016). However, knowledge on the identity, diversity and pathogenicity of mushroom-associated *Pseudomonas* species at the regional scale is still lacking. This information is instrumental for the development of localized strategies for diagnostics, disease control and breeding of varieties.

In this study, we isolated *Pseudomonas* from blotched mushrooms on farms in the Netherlands, United Kingdom and Belgium. We performed whole genome sequence analyses of pathogenic isolates to develop a deeper understanding of the genetic diversity among the pathogens in Western Europe. Some non-pathogenic isolates were also included in the study. The molecular characterization of these isolates provides insights into the phylogenetic relationships between beneficial and blotch-causing *Pseudomonas* species commonly associated with the button mushroom, *A. bisporus.*

Methods

Bacterial isolations

Blotched mushrooms were sampled from commercial farms in the Netherlands (NL), United Kingdom (UK) and Belgium (BE) for isolation of blotch-causing *Pseudomonas* species. Biopsies from symptomatic tissue of the cap surface (2 cm^2 area) were made in sterilized Ringer's solution (Marino et al., 2008), and homogenized in a polyethylene bag (Bioreba, Switzerland). The extract from each biopsy was dilution plated on King's B medium (King et al., 1954). After incubation at 25 ^oC for 48 h, single colonies were picked and re-plated. In total, 161 single colonies of suspected *Pseudomonas* spp., that were fluorescent under UV light (365nm), were plated to pure cultures by re-streaking on King's B medium. One isolation was also made from

a healthy mushroom that did not display visible blotch symptoms. A list of bacterial isolates is presented as Additional file 1.

Pathogenicity assays

All isolates were tested in an *in-vitro* assay to check their pathogenicity. Bacterial strains were cultured in King's B medium (King et al., 1954) at 25 ºC for 24 h, and tested in the bioassay. Similarly sized cap surfaces (4-5 cm in diameter) of healthy mushrooms were placed on damp filter paper and inoculated with 20 µl of aqueous bacterial suspension of 10^6 colony-forming units (cfu) per ml from the isolate, and tested in replicates of three. The mushrooms were incubated under high humidity conditions for 72 h at 20 °C. The development of blotch symptoms on the cap surface was observed visually and photographed. The isolates were scored, between 0 and 3, with the ascending numbers referring to non-pathogenic, mild, moderate, and severe symptoms for bacterial blotch (van der Wolf et al., 2016). Negative controls consisted of uninoculated mushroom caps and sterile water inoculated mushroom caps.

A selection of isolates was re-tested in pot assays. Mushrooms were grown in plastic pots (230 mm diameter x 220 mm depth) containing 4 kg of Phase III compost, spawn-run with the most commonly cultivated mushroom strain, Sylvan A15. The pots were cased with 1.3 L of casing soil (moist mixture of peat and sugar beet lime). The pots were watered with sterile water and incubated at 25 °C for 7 days. The room was then ventilated, the air temperature was reduced to 18 °C and the relative humidity was maintained at 91-93%, until the end of the cultivation cycle. After 5 days, the casing soil in each pot was inoculated with 50 ml of aqueous bacterial suspension of 10^7 cfu/ml. The development of blotch symptoms on the mushrooms was recorded over two flushes and scored as above. The type of blotch symptoms (brown, ginger or others) was recorded and photographed. Negative controls consisted of casing soil inoculated with sterile water.

DNA extraction and sequencing

For NL and BE isolates, 250 mg of bacterial slime was picked from a pure culture on an agar plate and used as starting material. Total DNA was extracted using Wizard Magnetic DNA Purification System for Food (Promega, United States) according to the manufacturer's protocol, including the DNase-free RNase treatment. Library construction was performed using Illumina Truseq Nano (Illumina, United States) with 1 µg of bacterial DNA. 125 bp paired-end sequencing of the DNA libraries was done using HiSeq2500 (Illumina, United States).

For UK isolates, a single colony was picked from each agar plate and extracted using the Qiagen DNeasy Blood and tissue kit following the manufacturer's protocol. The

DNA was quantified fluorometrically using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, United States) on the Infinite M200 PRO (Tecon, Switzerland) and then stored at −80 °C for downstream processing. Library construction was performed using Illumina Nextera XT library preparation kit (Illumina, United States) with 0.8 ng of bacterial DNA. Sequencing of the DNA libraries was performed on the MiSeq (Illumina, United States) using the V3 Reagent Kit, generating 300 bp paired-end sequences.

The combined dataset included 68 newly generated genome sequences from bacteria isolated from symptomatic cap tissue, 30 sequences of mushroom-associated *Pseudomonas* species from a previous sampling (van der Wolf et al., 2016) and 15 sequences of related strains obtained from NCBI (https://www.ncbi.nlm.nih.gov/). Quality control was performed on the raw reads prior to read mapping using CLC Genomics Workbench (QIAGEN, Germany). Adapter sequences were removed from the raw reads. Bases with a Phred quality score less than 20 based on a modified-Mott algorithm were trimmed. Raw reads greater than 1000 bp and less than 45 bp were discarded. Reads were trimmed to a final length of 125 bp (NL and BE isolates) and 300 bp (UK isolates). Trimmed reads were mapped to the reference genomes without masking. Non-specific matches were randomly mapped.

Determination of prokaryotic taxonomy

Multi-Locus Sequence Alignment (MLSA) with trimmed coding sequences of eleven barcoding genes from 13 reference strains were used to establish phylogeny between the isolates (Zeigler, 2003). Housekeeping genes were chosen as phylogenetic molecular markers based on several criteria. The genes had a single copy number, they coded universally for ubiquitous proteins with housekeeping functions, were likely recalcitrant to the effects of horizontal gene transfer, were long enough (>900 bp) to contain sufficient information and predicted whole-genome relationships (Adékambi et al., 2011). Trimmed reads were mapped to the concatenated sequences of individual barcoding genes from multiple reference strains, using Map Reads to Reference 1.6 with a similarity and length fraction of 0.9 (CLC Genomics Workbench 11.0.2). Consensus DNA sequences were extracted from the mapping and used for making phylogenetic trees with maximum likelihood and maximum parsimony methods (Tamura et al., 2004). Graphics from phylogenetic trees were made in RStudio (Team, 2013) using package *ggtree* (Yu et al., 2017).

Genome assemblies were performed on the trimmed reads using De Novo Assembly 1.4 with a minimum contig length of 200 bp (CLC Genomics Workbench 11.0.2). Legacy BLAST (Goris et al., 2007) based Average Nucleotide Identity (ANI) analysis (Richter and Rosselló-Móra, 2009) was performed on the contig sequences from the

assembled genomes using pyani 0.2.9 (Pritchard et al., 2019). Similarity value of 95% was used as cut-off threshold for identification of a unique taxonomic group. The similarity values were used for phylogenetic analyses and to create graphics in RStudio (Team, 2013). To clarify the taxonomy of isolates that did not cluster together with any of the reference strains in the ANI or the MLSA, a digital DNA: DNA hybridization (Auch et al., 2010) was performed using the Genome-Genome Distance Calculator (GGDC) (Meier-Kolthoff et al., 2013). A threshold of 70% for digital DNA: DNA hybridization and 1% for difference in percentage guanine-cytosine content were used for determination of species and subspecies boundaries via the Type (Strain) Genome Server (Meier-Kolthoff and Göker, 2019). Phylogenetic trees were constructed from the alignment of the whole-genomes and their corresponding 16S rRNA sequences, using a GreedyWithTrimming algorithm on FastME 2.0 (Lefort V, Desper R, 2015).

Results

Pathogenicity of isolates

102 bacterial isolates were tested for their ability to cause bacterial blotch symptoms on fresh mushroom caps (Figure 1). 6 out of the 17 strains belonging to international culture collections could cause blotch symptoms. Out of the 85 bacterial isolates recovered from blotched mushroom tissue, 55 isolates caused mild to severe symptoms in the pathogenicity cap test. From this panel, the pathogenicity of 30 bacterial isolates and strains were also validated in the pot test, by inoculation of the pathogen in the casing soil (Figure 2). The pot test and cap test gave similar results (Additional file 1). The pathogenicity of the isolates is further described in this text with reference to bacterial blotch only.

Whole genome sequences

In total, whole genome sequences from 113 bacteria were analysed. The consortium of sequenced bacteria contained 85 isolates from symptomatic mushroom tissue and 28 reference strains from international culture collections at LMG (Laboratory of Microbiology, Belgium), ATCC (American Type Culture Collection, United States) and NCPPB (National Collection of Plant Pathogenic Bacteria, United Kingdom). The total number of reads per isolate, averaged across the dataset, were 16,075,321 indicating good sequencing depth, with mean Phred score of 38 which suggests high sequence quality and mean GC content of 63%. Additional file 1 describes the sequence identifiers and their metadata.

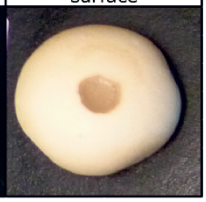
Score	0	1	2	3
Definition	No blotch symptoms. Water mark maybe visible	Mild blotch symptoms. A small ring of discoloration forms along the edges	Moderate symptoms. Distinct discoloration visible on the surface area of inoculation drop	Severe symptoms. Discoloration accompanied by dark lesions and/or sunken spots on surface
Visual characteristics				

Figure 1. *In-vitro* pathogenicity assays to quantify the virulence of an isolate when inoculated on fresh mushroom caps. It describes the visual characteristics used to score the blotch symptoms as none, mild, moderate and severe in a pathogenicity assay.

Figure 2. Pathogenicity bioassays in pots to confirm the virulence of isolates when inoculated in the casing soil. Brown blotch symptoms were caused by (A) *P. salomonii* (IPO3765) and (B) *P. costantinii* (LMG 22119^{T}) and ginger blotch symptoms were caused by *'P. gingeri'* isolates (C) P8018 and (D) IPO3777, in independent pathogenicity bioassays in pots.

Barcoding gene	Description or product	Length variation
atpD	ATP synthase subunit beta	0471- 1380 bp
fusA	Elongation factor G	2106- 2148 bp
glnS	Glutamine--tRNA ligase	1482- 1701 bp
groeL	Chaperonin 1	1620- 1650 bp
gyrB	DNA gyrase subunit B	2379- 2424 bp
ileS	Isoleucine--tRNA ligase	2106- 2832 bp
recA	DNA repair protein A	0612- 1065 bp
recN	DNA repair protein N	1672- 1674 bp
rpoB	DNA-directed RNA polymerase (subunit B)	4074- 4081 bp
rpoD	RNA polymerase sigma factor (sigma-70)	0519- 1851 bp
uvrC	UvrABC system exonuclease (subunit C)	1824- 1839 bp

Table 1. List of individual barcoding genes used for predicting whole-genome relatedness in MLSA

Reference genomes	Accession (Assembly)
P. poae LMG 21465^T	GCA_001439785.1
P. protegens CHAO^T	GCA_000397205.1
P. veronii LMG 17761^T	GCA_001439695.1
P. costantinii LMG 22119^T	GCA_001870435.1
P. putida BIRD 1	GCA_000183645.1
'P. reactans' LMG 5329^T	GCA_000411675.1
P. agarici LMG 2112^T	GCA_900109755.1
P. tolaasii LMG 2342^T	GCA_002813445.1
'P. gingeri' LMG 5327^T	GCA_002895165.1
P. fluorescens LMG 1794^T	GCA_900215245.1
P. syringae DC3000	GCA_000007805.1
P. yamanorum LMG 27247^T	GCA_900105735.1
Pseudomonas sp. *NC02*	GCA_002874965.1

Table 2. List of genomes used for extracting the reference sequences of the barcoding genes for MLSA

Multi-Locus Sequence Analysis

Eleven taxon-specific sequences that are stable with regard to rapid genetic mutations were selected as barcoding genes from known literature reports (Glaeser and Kämpfer, 2015; Gomila et al., 2015; van der Wolf et al., 2016), namely, *atpD, fusA, glnS, groeL, gyrB, ileS, recA, recN, rpoB, rpoD* and *uvrC* (Table 1). Concatenated sequences of the barcoding genes from 13 reference strains of well-known mushroom-associated *Pseudomonas* species (Table 2) were used for MLSA, such that the variability within the barcoding genes predicts the overall whole genome relatedness (Zeigler, 2003). Two of these strains from the *P. fluorescens* group were added to the reference list based on preliminary data exploration. *P. yamanorum* is a psychrotolerant soil bacteria from the *P. fluorescens* group (Arnau et al., 2015), that contains a paralog of the *tolaasin* gene fragment (Basim and Basim, 2018). *Pseudomonas* sp. *NC02* is a recently isolated soil bacterium, which is closely related to *P. yamanorum*.

The largest clusters in the phylogenetic tree are comprised of *'P. gingeri', Pseudomonas* sp. *NC02, 'P. reactans'* and *P. tolaasii,* in decreasing order of size. *'P. gingeri'* and *'P. reactans'* isolates formed multiple clusters within the species (Figure 3). A few isolates also mapped to barcoding genes from *P. putida, P. agarici, P. veronii, P. costantinii* and *P. yamanorum.* With the exception of *'P. gingeri'*, non-pathogenic isolates were also found in species clusters that contained pathogenic isolates.

None of the isolates mapped to reference strains of *P. poae, P. protegens, P. fluorescens* or *P. syringae.* 15 out of 113 isolates mapped non-specifically, with relatively low percentage identity, to multiple reference strains indicating the presence of other *Pseudomonas* species. 5 isolates did not map to any of the references and could potentially be non-pseudomonads. Within species clusters of the phylogenetic tree, the individual isolates had low numbers of substitutions per sequence site, indicating short evolutionary distances within species (Figure 3).

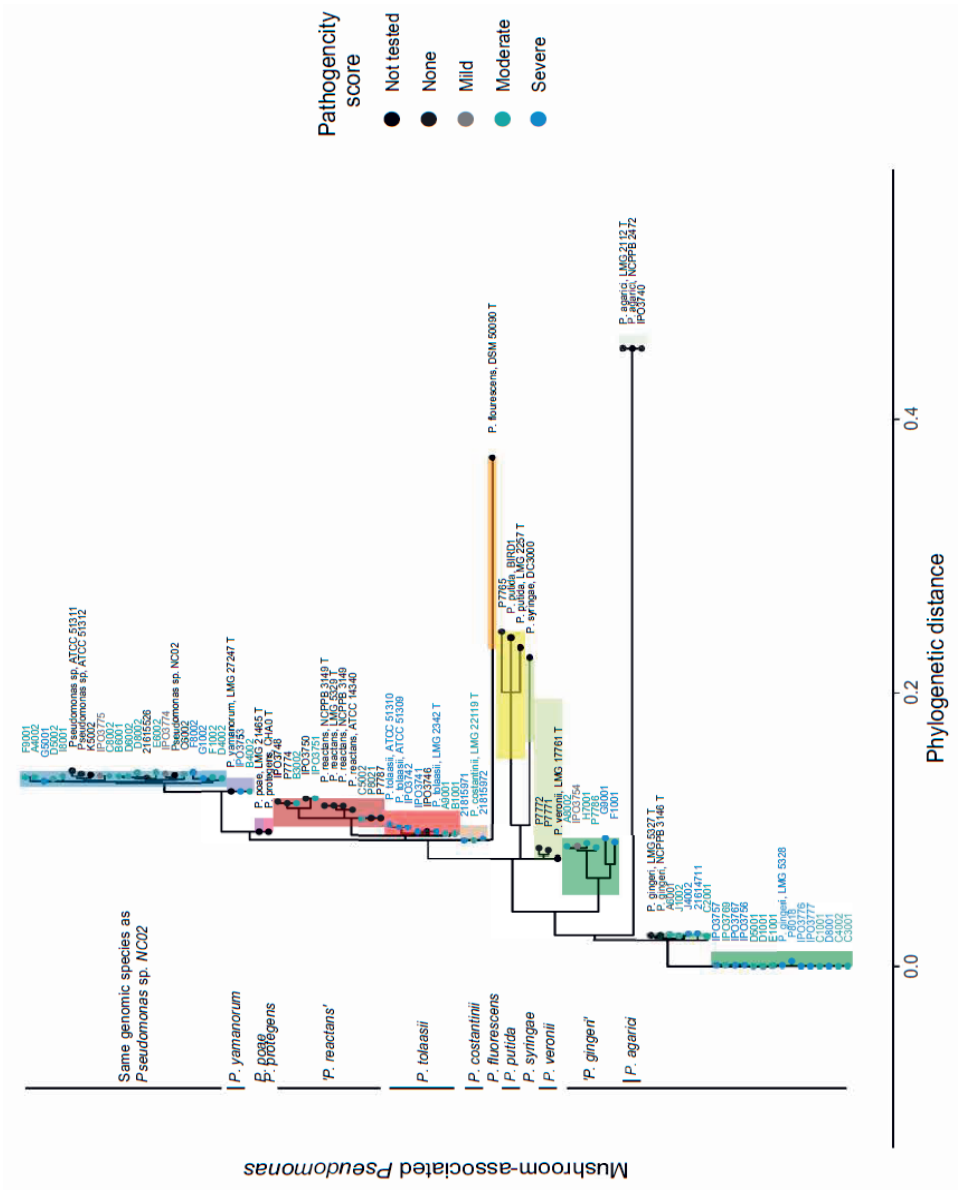


Figure 3. Phylogenetic tree from consensus sequences extracted via Multi-Locus Sequence Alignment of barcoding genes. A maximum-likelihood phylogenetic tree in which weak nodes (<70%) that are not supported by bootstrapping have been collapsed. The colour of the branches indicates the identity of the isolates, based on the mapping of the reference barcoding genes. The colour of the tip labels and tip point indicates the pathogenicity of the isolates on fresh mushroom caps and in pot assays.

Average Nucleotide Identity

Average nucleotide identity analysis recognized 32 unique bacterial phylotypes associated with the cap tissue of blotched button mushrooms in Western Europe (Figure 4). These are phylogenetically distinct whole genome sequences differing at species level, based on a <95% similarity cut-off for delineation. Similar to the MLSA, the largest numbers of blotch pathogens were identified as close relatives of *Pseudomonas* sp. *NC02* or *'P. gingeri',* although pathogenic isolates of *P. tolaasii*, *P. costantinii*, *'P. reactans'* and *P. yamanorum* were also discovered. Isolates that were unable to cause blotch were primarily identified as *'P. reactans', P. agarici, P. veronii* and strains belonging to the same species as *Pseudomonas* sp. *NC02*. Within phylotypes, isolates did not cluster according to geographic region, year of outbreak or pathogenicity scores. Twelve unidentified phylotypes consisted of isolates with varying levels of symptom severity on fresh mushroom caps and did not contain any reference or type strains.

In-silico DNA: DNA Hybridization

Five non-pseudomonad isolates were identified from *in-silico* DDH of the whole genome sequences (Table 3). Two isolates that cause moderate blotch on fresh caps, C2002 (phylotype 15) and C7002 (phylotype 17) were identified as *Serratia liquefaciens* and *Serratia proteamaculans* respectively (Figure 5). Non-pathogenic isolate P7753 (phylotype 30) was closely related to *Brevundimonas bullata*. Non-pathogenic isolate P7760 (phylotype 31) was closely related to *Cedecea neteri*. Moderate blotch causing isolate B9002 (phylotype 13) was related to reference genomes of multiple *Pantoea* species.

Among the *Pseudomonas*, three isolates that cause blotch on fresh caps (phylotype 12) belonged to *P. edaphica* and severe blotch causing isolate IPO3765 (phylotype 25) was identified as *P. salomonii* (Figure 6). Several *Pseudomonas* spp. did not hybridize sufficiently (>70%) with any of the type strains or share a similar %GC content (<1%). Seven of these isolates (phylotypes 4 and 26) were closely related to *P. yamanorum* LMG 27247^{T}. Isolates P7548 (phylotype 9) and B6002 (phylotype 11), clustered together as the same genomic species, and were closely related to *P. fluorescens* DSM 50090^{T} (>60% dDDH). Isolate D2002 (phylotype 21) was also closely related to *P. fluorescens*. Isolate B3002 (phylotype 16) did not map sufficiently with the type strain of *'P. reactans'*, in contrast to the MLSA and ANI results.

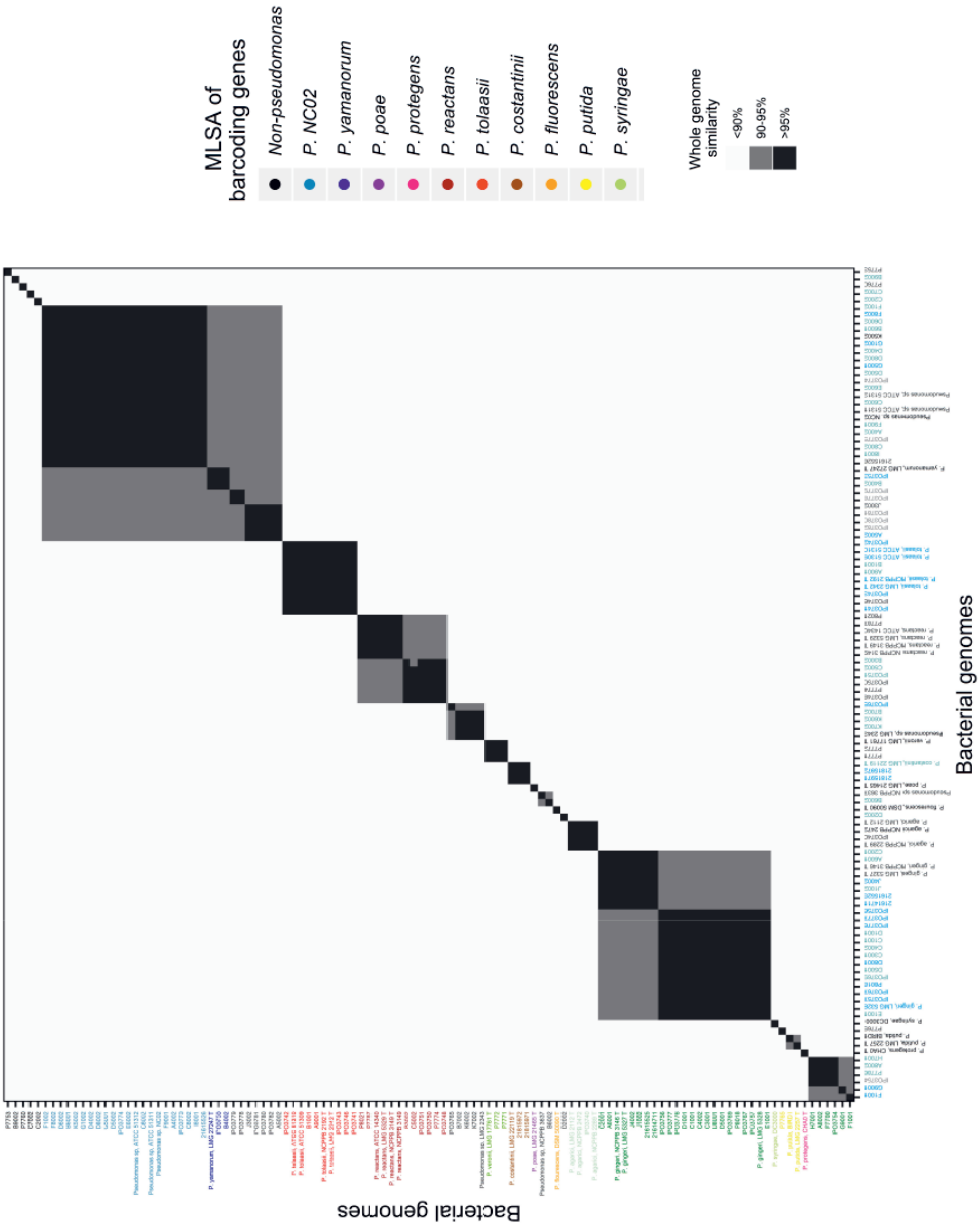


Figure 4. Heatmap from whole-genome similarity values obtained in the Average Nucleotide Identity analysis. Colours of the heatmap indicate the pairwise genome-genome similarity. The labels on the y-axis are coloured according to the identity assumed from the MLSA analyses; the labels on the x-axis are coloured according to the pathogenicity of the isolates on fresh mushroom caps, and in select cases also pot tests.

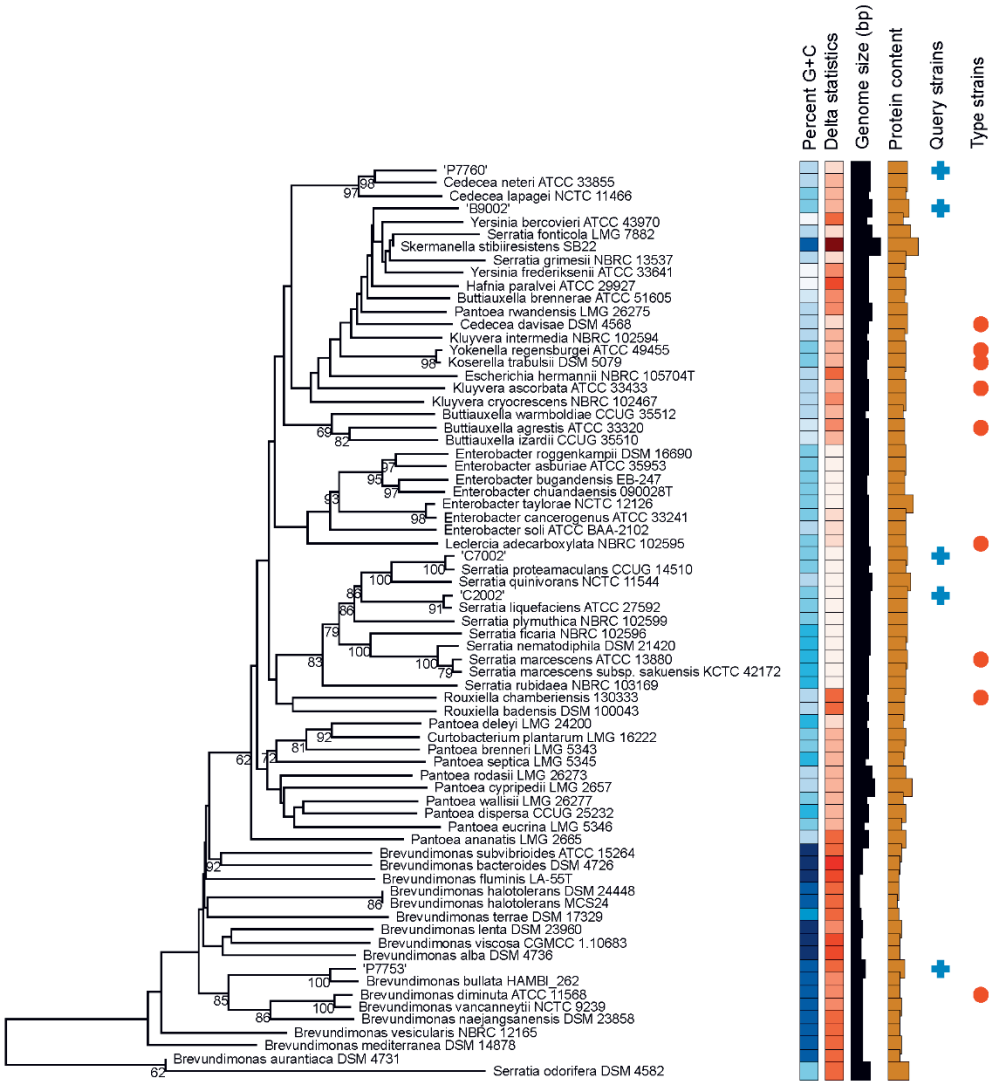


Figure 5. Phylogenetic tree from *in-silico* DNA: DNA hybridization of non-*Pseudomonas* isolates that were unidentified in the MLSA and ANI. A minimum evolution phylogenetic tree rooted at midpoint, in which branch support was inferred from 100 bootstrap replicates each. The genome size, percentage GC content, total protein count and delta statistics are described for 5 query sequences and 9 type strains spanning multiple genera. Low delta statistic values indicate higher accuracy of the phylogeny (Holland et al., 2002).

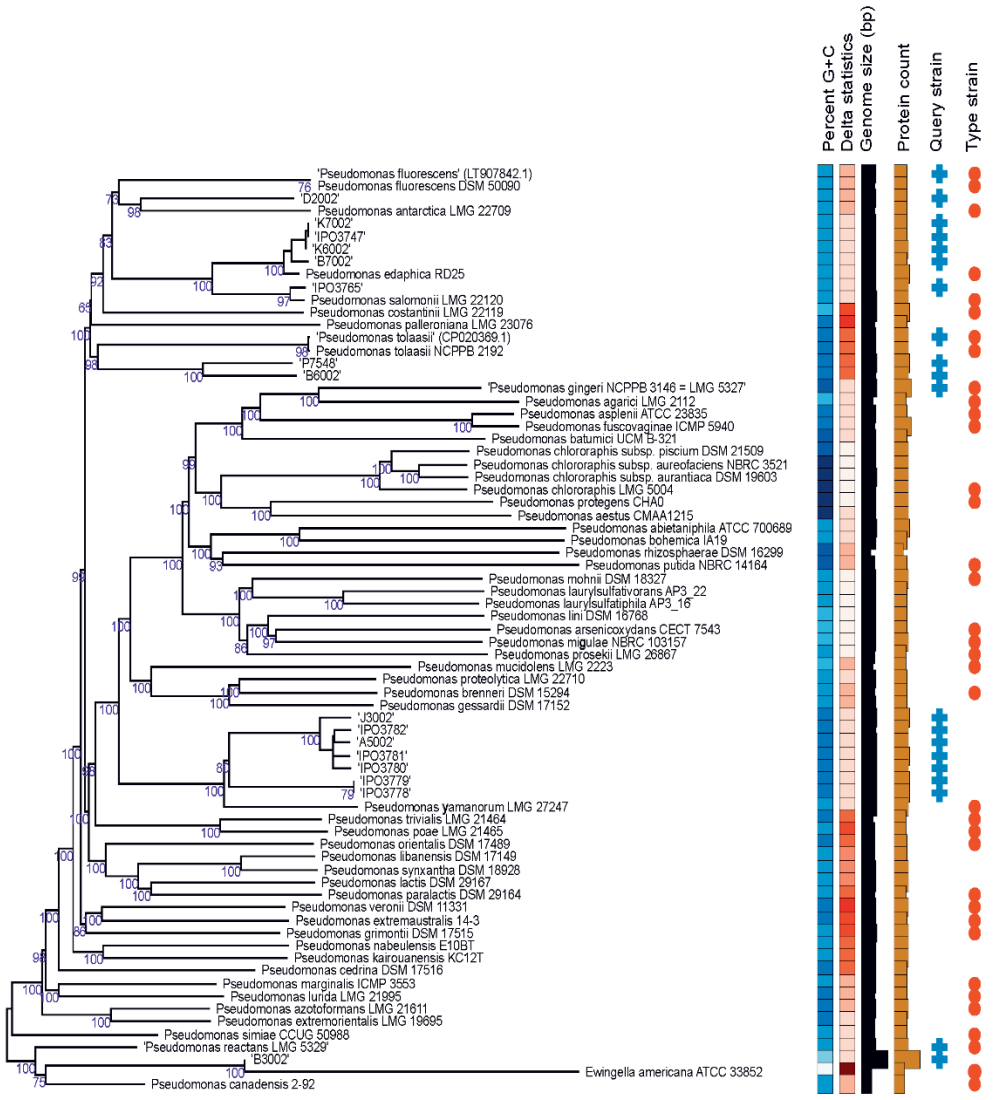


Figure 6. Phylogenetic tree from *in-silico* DNA: DNA hybridization of *Pseudomonas* isolates that were unidentified in the MLSA and ANI. A minimum evolution phylogenetic tree rooted at midpoint, in which branch support was inferred from 100 bootstrap replicates each. The genome size, percentage GC content, total protein count and delta statistics are described alongside the phylogenetic tree for 21 query sequences and 45 type strains from the genus *Pseudomonas*.

Query isolate	Phylotype (ANI)	Reference strain	dDDH value (%)	Confidence intervals (%)	Difference in GC (%) content
Non-*Pseudomonas*					
B9002	13	*Pantoea rodasii* LMG 26273^T	40.5	37.5 - 43.5	0.9
C2002	15	*Serratia liquefaciens* LMG 7884^T	**96.5**	**95.0 - 97.6**	0.14
C7002	17	*Serratia proteamaculans* NCPPB 245^T	**93.4**	**91.1 - 95.1**	0.01
P7753	30	*Brevundimonas bullata* LMG 17157^T	72	68.5 - 75.2	0.22
P7760	31	*Cedecea neteri* LMG 7864^T	63	59.7 - 66.2	0.55
Pseudomonas species					
A5002	4	*P. yamanorum* LMG 27247^T	69.2	65.8 - 72.5	0.63
IPO3780	4	*P. yamanorum* LMG 27247^T	70.3	66.8 - 73.5	0.73
IPO3781	4	*P. yamanorum* LMG 27247^T	68.2	64.8 - 71.4	0.59
IPO3782	4	*P. yamanorum* LMG 27247^T	70.9	67.4 - 74.1	0.76
J3002	4	*P. yamanorum* LMG 27247^T	69.1	65.7 - 72.4	0.67
B6002	9	Isolate P7548 (phylotype 11)	80.9	77.5 - 83.9	0.24
P7548	11	*P. fluorescens* DSM 50090^T	57.3	54.1 - 60.5	0.61
B7002	12	*P. edaphica* LMG 30152^T	**88.2**	**85.2 - 90.6**	0.03
IPO3747	12	*P. edaphica* LMG 30152^T	**89.9**	**87.1 - 92.1**	0.1
K6002	12	*P. edaphica* LMG 30152^T	**89.8**	**87.0 - 92.0**	0.12
K7002	12	*P. edaphica* LMG 30152^T	**89.9**	**87.1 - 92.1**	0.1
B3002	16	*P. reactans* LMG 5329^T	44.4	41.4 - 47.4	2.94
D2002	21	*P. fluorescens* DSM 50090^T	69.4	65.4 - 73.0	0.15
IPO3765	25	*P. salomonii* LMG 22120^T	**87.9**	**84.4 - 90.7**	0.31
IPO3778	26	*P. yamanorum* LMG 27247^T	64.8	61.5 - 68.1	0.59
IPO3779	26	*P. yamanorum* LMG 27247^T	64.8	61.5 - 68.0	0.6

Table 3. *In-silico* DNA: DNA hybridization of unidentified isolates. Only the highest dDDH values from comparing each query strain with multiple reference strains have been reported. Values in bold indicate that the query belongs to the same species as the reference strain, as the confidence intervals meet the 70% threshold for DDH and 1% difference in %GC content. Values that are not highlighted, lie on the border or below the thresholds, and indicate the reference that the query isolate is most closely related to.

Taxonomic corrections

The taxonomy of several reference strains from culture collections have been corrected based on the combined results from MLSA, ANI and dDDH analyses. ATCC 51312, ATCC 51311, LMG 2343 were formerly identified as *P. tolaasii*, but they share less than 95% genome similarity with *P. tolaasii* genome cluster (phylotype 6). They also do not map to barcoding genes of *P. tolaasii* LMG 2342^T. Non-pathogenic strains ATCC 51312 and ATCC 51311 were instead identified close relatives of *Pseudomonas* sp. *NC02* (phylotype 2) in the MLSA and ANI, and LMG 2343 (phylotype 12) was identified as *P. edaphica* in the dDDH. Non-pathogenic isolates P7774, P7753 and P7760 were formerly described as *P. veronii, P. syringae* and *P. agarici,* respectively, in internal culture collections. Based on ANI and dDDH results from this study they were re-identified as *'P. reactans'* (phylotype 16), close relative of *Brevundimonas bullata* (phylotype 30) and close relative of *Cedecea neteri* (phylotype 31) respectively.

Known blotch pathogens

All strains of *P. tolaasii* (phylotype 6) isolated from blotch outbreaks in the Netherlands were found to cause moderate or severe brown blotch symptoms, with the exception of IPO 3746. They also map to barcoding genes of *P. tolaasii* LMG 2342^T in the MLSA. Two pathogenic isolates from the United Kingdom, 21815971 and 21815972 (phylotype 3) that cause severe blotch and pitting in both cap and pot tests, clustered together with the blotch-causing reference strain of *P. costantinii,* LMG 22119^T in the MLSA and ANI. Three isolates from the *'P. reactans'* clusters, B3002, C5002, IPO 3751 (phylotypes 9 and 16), were also pathogenic. All 26 isolates of *'P. gingeri'* obtained from blotch outbreaks in the Netherlands, United Kingdom and Belgium were able to cause blotch, with varying symptom severity in the pathogenicity tests performed. All of them mapped to barcoding genes of *'P. gingeri'* LMG 5327^T. They form 3 MSLA clusters, but 5 distinct ANI clusters (phylotypes 1, 5, 14, 22 and 24), indicating that multiple closely related species can cause ginger blotch.

New blotch pathogens

Several isolates presented in this study are reported as blotch-pathogens for the first time. 19 isolates (phylotype 2) with varying severity of blotch symptoms were identified as belonging to the same species as *Pseudomonas* sp. *NC02*. Two blotch-causing isolates (phylotype 10) from the Netherlands were identified as *P. yamanorum*. They were also closely related to the *Pseudomonas* sp. *NC02* isolates. Three pathogenic isolates (phylotype 12) from Dutch blotch outbreaks were identified as *P. edaphica*. A severely pathogenic isolate (IPO3765) from the

Netherlands was recognized as *P. salomonii*. Four ANI clusters (phylotypes 4, 9, 21 and 26) contained pathogenic *Pseudomonas* isolates without a reference or type strain. Phylotype 4 contained only one severe blotch-causing isolate, A5002. Phylotypes 9 and 21 contained only a single isolate each, both of which (B6002 and D2002) cause brown blotch symptoms. Phylotype 26 contained two unidentified isolates, IPO 3778 and IPO 3779 that cause mild blotch on fresh caps.

Unidentified *Pseudomonas* species

10 isolates demonstrating a range of blotch severity, from non-pathogenic to severe symptoms, remained unidentified. They mapped to barcoding genes from multiple reference strains in the MLSA, formed 7 distinct clusters (phylotypes 4, 9, 11, 21, 26, 30 and 31) in the ANI analysis (Figure 4), and were closely but insufficiently (<70%) related to any other species in the dDDH (Table 3). Their contigs mapped in-specifically to multiple reference strains, when compared via BLAST against the NCBI database. Contamination of these isolates during laboratory work was ruled out by confirming the presence of single copies of the barcoding genes, and low genome duplication in the range of 1.50-2.84% (Additional file 2). These could potentially be new species that have yet to be discovered.

Non-pathogenic *Pseudomonas*

Five non-pathogenic isolates, P7787, P8021, P774, IPO 3748 and IPO3750, mapped to barcoding genes from *'P. reactans'* LMG 5329^{T} in the MLSA, but were split over phylotype 8 and 16 in the ANI analysis. All isolates from phylotype 19 did not cause blotch and mapped to barcoding genes of *P. agarici* LMG 2112^{T}, better known as the causative agent of drippy gill. Fluorescent *Pseudomonas* spp. such as *P. putida* (phylotype 23, 29, 30), *P. veronii* (phylotype 27) and *P. poae* (phylotype 20) that are usually considered as beneficial organisms, were also confirmed to be non-pathogenic.

Discussion

Genetic diversity of blotch-causing *Pseudomonas*

The established phylogeny of bacterial isolates associated to symptomatic cap tissue is largely consistent between the MLSA, ANI and dDDH analyses. This substantiates the conclusions made about the identity of blotch pathogens, and the related taxonomic corrections. *'P. gingeri', P. tolaasii, 'P. reactans' and P. costantinii* are known causative agents of bacterial blotch (Munsch and Alatossava, 2002; Preece and Wong, 1982; Tolaas, 1915; Wells et al., 1996). In addition, several novel blotch pathogens were identified in these analyses, namely *P. yamanorum, P.*

edaphica, P. salomonii and strains clustering together with *Pseudomonas* sp. *NC02,* as the same genomic species.

P. yamanorum is a recently described psychrotolerant member of the *P. fluorescens* group that was found in Argentinian soil under cold and humid climatic conditions (Arnau et al., 2015). In a recent study, the tolaasin gene fragment of a few brown blotch-causing isolates in Turkey were also found to share homology with that of *P. yamanorum* (Basim and Basim, 2018). *Pseudomonas* sp. *NC02* is a recently isolated soil bacterium about which not much is known (Cerra et al., 2018). *P. edaphica* is another newly identified fluorescent *Pseudomonas* that was isolated from rhizosphere soil and is closely related to *P. brenneri* (Ramírez-Bahena et al., 2019). *P. salomonii* was first characterized as a pathogen of 'Café au lait' disease on garlic (Gardan et al., 2002), and was later also recovered from ready-to-eat vegetables (Caldera et al., 2016). This is the first report of the blotch-causing ability of this pathogen on mushrooms. Its pathogenicity in the cap test was also verified by pot tests.

'P. gingeri' and *'P. reactans'* are both invalidly named species known to cause bacterial blotch (Iacobellis and Cantore, 2003; Wells et al., 1996; Wong et al., 1982). In this study, all isolates previously classified as *'P. gingeri'* and *'P. reactans'* map to barcoding genes of their respective reference strain, although they form multiple distinct clusters in the MLSA as observed from the phylogenetic tree (Figure 3). They also split into multiple phylotypes in the ANI analysis instead of clustering together (Figure 4). Delineation of these phylotypes on a genome level (<95% similarity) despite mapping to barcoding references from the same species, perhaps indicates the existence of a species complex with multiple taxonomically related species, as opposed to other blotch pathogens which cluster as single phylotypes.

On the basis of genomic and phylogenetic analyses such as MLSA, ANI and dDDH, this study also reports four blotch-causing *Pseudomonas* spp. (phylotypes 4, 9, 21 and 26) which do not belong to any of the existing species within the genus *Pseudomonas*. These are as yet unidentified and could potentially be new species. The taxonomic assignment of these novel blotch-causing phylotypes as new species requires a polyphasic approach involving additional phenotypic and chemotaxonomic data such as fatty acid methyl esters, polar lipids and respiratory quinones (Kämpfer and Glaeser, 2012).

Pathogenicity and phylogeny

Koch's postulates were evaluated to identify causative agents of bacterial blotch (Fulton, 1981). Bacteria were isolated from symptomatic mushroom tissue and successfully grown to pure culture. In every case, blotch symptoms could be reproduced when isolates were inoculated on mushroom caps. The 4th postulate of

Koch, i.e. re-isolation of pathogen from the experimental host and its identification as originally inoculated pathogen, was not fulfilled. The mushroom cap surfaces also harbour endemic *Pseudomonas* spp. that are closely related to the isolates and have similar characteristics and colony morphology (Cutri et al., 1984; Doores et al., 1987). Due to lack of diagnostic methods, the inoculated isolates cannot be easily differentiated from the endemic microflora. However, the disease development can be attributed to the inoculated pathogens with certainty, due to the presence of multiple mock-inoculated negative controls which did not develop any blotch symptoms.

24, 8, 39 and 28 isolates were confirmed to cause non-pathogenic, mild, moderate and severe blotch symptoms respectively, on fresh mushroom caps in an *in-vitro* pathogenicity test. Bacterial isolates did not cluster together according to their pathogenicity scores in the MLSA or ANI analysis. Often blotch-causing and non-pathogenic isolates were part of the same phylotype or branch (Figures 3 and 4). This elaborates the need for a pan-genomic analysis to identify the pathogenicity determinants of bacterial blotch diseases. Fluorescent *Pseudomonas* share a rather small core genome of 1344-protein coding genes (Garrido-Sanz et al., 2016). The size and diversity of the pan-genome is thus determined by secondary metabolites biosynthesis clusters that can be strain specific and responsible for pathogenicity (Loper et al., 2012). Pan-genomic elements shared exclusively by pathogenic isolates can be ideal targets for the further development of DNA-based diagnostic tools.

Regional diversity of blotch-causing *Pseudomonas*.

Within species, bacterial isolates did not cluster together according to their geographic region or year of outbreak. The two Belgian isolates (IPO 3781 and IPO 3782), both caused mild ginger blotch and clustered together with those of the Netherlands. Two Dutch isolates of *'P. gingeri'* (IPO 3757 and IPO 3756) that caused severe ginger blotch, clustered differently from each other in phylotypes 14 and 24 respectively. The phylogenetic distribution of the common pathogens independently from their region of outbreak indicates that Western Europe shares a well-mixed pathogen pool.

However, a few potentially region-specific blotch pathogens were also recovered in this study. *P. yamanorum, P. edaphica* and *P. salomonii* isolates have so far only been found in the Netherlands. *P. costantinii* isolates in this study, 21815971 and 21815972, which cause brown blotch and pitting, have been found only in the United Kingdom, although other blotch-causing *P. costantinii* have been reported in Finland too (Munsch et al., 2002). It is also important to consider that very few isolates from these species were found in this study. This regional exclusivity could also be an artefact of low sampling due to the low presence of these species.

Blotch causing non-pseudomonads

Two *Serratia* species, *S. liquefaciens, (*C2002, phylotype 15) and *S. proteamaculans, (*C7002, phylotype 17) were recovered from blotched caps in this study. *Serratia* species are present in casing soil, compost, and on mushroom caps (Doores et al., 1987; Jarial et al., 2005; McGee et al., 2017; Reyes et al., 2004). *Serratia liquefaciens* is a known agent of yellow blotch on the caps of *A. bisporus* (Sivanesan, 2003). They cause browning of the *A. bisporus* sporophore (Grewal, 1991) by secreting chitinolytic enzymes and chitin-binding proteins (Watanabe et al., 1997). They also produce a surfactant, serwettin, which enables the bacterium to colonise fresh areas of the mushroom cap, as tolaasin does (Matsuyama et al., 1992, 1986). *P. fluorescens* also uses a similar mechanism to spread on broccoli heads (Hildebrand, 1989).

A moderately pathogenic *Pantoea* isolate (B9002, phylotype 13) was also identified in this study. *Pantoea* species have been previously isolated from caps of *A. bisporus* (Reyes et al., 2004), and are frequently recovered from soil and water environments too (Brenner, 1984; Holt et al., 1994). This Gram-negative bacterium is an agent of soft rot disease on the stipes and pileus of *Pleurotus eryngii* (Kim et al., 2007), although it has not yet been reported to be a pathogen of *A. bisporus*. *Pantoea* species from waste mushroom beds are also used for production of plant auxin, indole-acetic acid, for phosphate solubilization (Walpola et al., 2013).

A non-pathogenic *Cedecea* isolate (P7760, phylotype 31), closely related to *C. neteri,* was also found on a blotched mushroom cap. *Cedecea* were one of the most common genera recovered from blotched tissue of *A. brazilensis* (Jhune et al., 2009) and is often present on *A. bisporus* also (Rossouw and Korsten, 2017). Although *Cedecea neteri* is an agent of soft rot in *Pholiota nameko* (Yan et al., 2018) and yellow sticky disease in *Flammulina velutipes* (Yan et al., 2019), only *Cedecea davisae* strains have been reported to cause blotch in *A. bisporus* (Sivanesan, 2003).

Beneficial bacteria on symptomatic cap tissue

Non-pathogenic and potentially beneficial bacteria were also recovered from cap tissue of blotched mushrooms. *P. putida* (P7771, P7772, phylotype 27) and *P. veronii* (P7765, phylotype 32) isolates were confirmed to be non-pathogenic on fresh mushroom caps. *P. putida, P. poae* and *P. veronii* are known to stimulate primordia formation of *A. bisporus* by the removal of volatile C8 compounds (Fermor et al., 2000; Noble et al., 2009). The relative abundance of these beneficial bacteria on blotched caps is yet to be explored, in relation to disease expression by blotch causing bacteria. *Brevundimonas* isolate, P7753 (phylotype 30), closely related to *B. bullata* was also discovered on symptomatic cap tissue. During the cultivation

cycle, increased *Brevundimonas populations* have been observed in the casing soils from *A. bisporus* and *P. ostreatus* farms (Cho et al., 2008; Mcgee et al., 2017; Nazareth et al., 2010), and *Brevundimonas* sp. can be thus expected to also colonize mushroom caps. It has also been reported as a symbiont on the apothecia of the cup fungus, *Scutellinia scutellata* (Giordano et al., 2013).

Conclusions

This study provides a detailed overview of the regional, genetic and phenotypic diversity of bacterial blotch pathogens in Western Europe. It describes the presence of known pathogens inhabiting blotched mushroom caps: *'P. gingeri', P. tolaasii, P. costantinii* and *'P. reactans'*. It also reports seven new pathogens of bacterial blotch: four *Pseudomonas species*, *P. yamanorum, P. edaphica, P. salomonii* and strains belonging to the same species as *Pseudomonas* sp. *NC02*, and three non-pseudomonads, *S. liquefaciens, S. proteamaculans* and a *Pantoea* sp. Their epidemiology and aetiology deserve further attention. This molecular investigation of bacterial species that colonize blotched mushroom caps allows further study of the mechanisms and microbial ecology of symptomatic disease expression on the *A. bisporus* sporophore. It also highlights the need for development of diagnostic assays against these newly discovered pathogens. Several new blotch-causing phylotypes discovered in this study are as yet unidentified and require additional chemotaxic data for their taxonomic assignment as new species.

Availability of data and materials

The datasets generated and/or analysed during the current study are available in the NCBI repository under BioProject number PRJNA607442.

Authors' contributions

TT and MK sampled and processed the diseased mushrooms; TT performed the pathogenicity assays. TT, MK and EH performed the molecular characterization. TT, MK, EH, JE, RN and JW analysed the results. TT wrote the first draft of the manuscript; JE, RN and JW critically reviewed the draft. All authors contributed to subsequent manuscript revision, read and approved the submitted version.

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Identifier	Sequencing	Label	Pathogenicity score from cap tests	Pathogenicity score from pot tests	Identity from molecular ivestigation		Source
					MLSA consensus	ANI (>95%)	
POWE01	NCBI	P. gingeri, NCPPB 3146 T	0	NT	*P. gingeri*	1	Collection
CP025624	NCBI	Pseudomonas sp. NC02	NT	NT	*P. NC02*	2	Collection
MDDR01	NCBI	P. costantinii, LMG 22119 T	2	2	*P. costantinii*	3	Collection
CP020369	NCBI	P. tolaasii, NCPPB 2192 T	3	3	*P. tolaasii*	6	Collection
PHHD01	NCBI	P. tolaasii, LMG 2342 T	3	3	*P. tolaasii*	6	Collection
AE016853	NCBI	P. syringae, DC3000	NT	NT	*P. syringae*	7	Collection
ASGY01	NCBI	P. reactans, NCPPB 3149	0	0	*P. reactans*	8	Collection
LT629793	NCBI	P. yamanorum, LMG 27247 T	NT	NT	*P. yama*	10	Collection
CP003190	NCBI	P. protegens, CHA0 T	NT	NT	*P. protegens*	18	Collection
CP014135	NCBI	P. agarici, NCPPB 2472	NT	NT	*P. agarici*	19	Collection
FOAR01	NCBI	P. agarici, NCPPB 2289 T	0	0	*P. agarici*	19	Collection
CP034537	NCBI	P. poae, LMG 21465 T	NT	NT	*P. poae*	20	Collection
JYLL01	NCBI	P. veronii, LMG 17761 T	NT	NT	*P. veronii*	27	Collection
LT907842	NCBI	*P. flourescens* , DSM 50090 T	NT	NT	*P. flourescens*	28	Collection
NC017530	NCBI	P. putida, BIRD1	NT	NT	*P. putida*	29	Collection

Supplementary Table 1A. Reference strains whose sequences were obtained from NCBI, with metadata on initial identity, pathogenicity, source, region of outbreak, year of outbreak, MLSA, ANI and dDDH.

Identifier	Sequencing	Label	Pathogenicity score from cap tests	Pathogenicity score from pot tests	Identity from molecular ivestigation			Source	Country
					MLSA consensus	ANI (>95%)	DDH (>70%)		
21614711	FERA 2017	21614711	3	3	*P. gingeri*	1		Industry	UK
21615526	FERA 2017	21615526	0	0	*P. NC02*	2		Industry	UK
P7758	FERA 2017	Pseudomonas sp, ATCC 51311	0	0	*P. NC02*	2		Collection	
P7779	FERA 2017	Pseudomonas sp, ATCC 51312	0	0	*P. NC02*	2		Collection	
P7786	FERA 2017	P7786	2	2	*P. gingeri*	5		Industry	UK
P7757	FERA 2017	P. tolaasii, ATCC 51310	3	3	*P. tolaasii*	6		Collection	
NCPPB3149	FERA 2017	P. reactans, NCPPB 3149 T	0	0	*P. reactans*	8		Collection	
P7759	FERA 2017	P. reactans, ATCC 14340	0	0	*P. reactans*	8		Collection	
P7787	FERA 2017	P7787	0	0	*P. reactans*	8		Industry	UK
P8021	FERA 2017	P8021	0	0	*P. reactans*	8		Industry	UK
P7548	FERA 2017	Pseudomonas sp, NCPPB 3637	0	0	*Pseudomonas sp*	11	*P. fluorescens DSM 50090T*	Collection	
P8018	FERA 2017	P8018	3	3	*P. gingeri*	14		Industry	UK
P7774	FERA 2017	P7774	0	0	*P. reactans*	16		Internal	UK
P7771	FERA 2017	P7771	0	0	*P. veronii*	27		Internal	UK
P7772	FERA 2017	P7772	0	0	*P. veronii*	27		Internal	UK
P7753	FERA 2017	P7753	0	0	*Non-pseudomonad*	30	*Brevundimonas bullata LMG 17157T*	Internal	UK
P7760	FERA 2017	P7760	0	0	*Non-pseudomonad*	31	*Cedecea neteri ATCC 33855*	Internal	UK
P7765	FERA 2017	P7765	0	0	*P. putida*	32		Internal	UK
21615525	FERA 2018	21615525	3	3	*P. gingeri*	1		Industry	UK
21815971	FERA 2018	21815971	3	3	*P. costantinii*	3		Industry	UK
21815972	FERA 2018	21815972	3	3	*P. costantinii*	3		Industry	UK
P7756	FERA 2018	P. tolaasii, ATCC 51309	3	3	*P. tolaasii*	6		Collection	

Supplementary Table 1B. Isolates and reference strains from the United Kingdom that were sequenced, with metadata on initial identity, pathogenicity, source, region of outbreak, year of outbreak, MLSA, ANI and dDDH.

Identifier	Sequencing	Label	Pathogenicity score from cap tests	Pathogenicity score from pot tests	Identity from molecular ivestigation			Source	Country	Year sampled
					MLSA consensus	ANI (>95%)	DDH (>70%)			
IPO3738	WUR 2016	P. gingeri, LMG 5327 T	0	NT	*P. gingeri*	1		Collection		
IPO3774	WUR 2016	IPO3774	1	NT	*P. NC02*	2		Industry	NL	2015
IPO3775	WUR 2016	IPO3775	1	NT	*P. NC02*	2		Industry	NL	2016
IPO3780	WUR 2016	IPO3780	1	NT	*Pseudomonas sp*	4	*P. yamanorum LMG 27247T*	Industry	NL	2015
IPO3781	WUR 2016	IPO3781	1	NT	*Pseudomonas sp*	4	*P. yamanorum LMG 27247T*	Industry	BE	2015
IPO3782	WUR 2016	IPO3782	1	NT	*Pseudomonas sp*	4	*P. yamanorum LMG 27247T*	Industry	BE	2016
IPO3754	WUR 2016	IPO3754	1	NT	*P. gingeri*	5		Industry	NL	2014
IPO3741	WUR 2016	IPO3741	3	NT	*P. tolaasii*	6		Internal	NL	1989
IPO3742	WUR 2016	IPO3742	3	NT	*P. tolaasii*	6		Internal	NL	1995
IPO3743	WUR 2016	IPO3743	3	NT	*P. tolaasii*	6		Internal	NL	1989
IPO3746	WUR 2016	IPO3746	0	NT	*P. tolaasii*	6		Internal	NL	1989
IPO3749	WUR 2016	P. reactans, LMG 5329 T	0	0	*P. reactans*	8		Collection		
IPO3753	WUR 2016	IPO3753	3	NT	*P. yamanorum*	10		Industry	NL	2014
IPO3747	WUR 2016	Pseudomonas sp, LMG 2343	NT	NT	*Pseudomonas sp*	12	*P. edaphica LMG 30152T*	Collection		
IPO3737	WUR 2016	P. gingeri, LMG 5328	3	NT	*P. gingeri*	14		Collection		
IPO3757	WUR 2016	IPO3757	3	NT	*P. gingeri*	14		Industry	NL	2016
IPO3767	WUR 2016	IPO3767	3	NT	*P. gingeri*	14		Industry	NL	2015
IPO3769	WUR 2016	IPO3769	2	NT	*P. gingeri*	14		Industry	NL	2015
IPO3776	WUR 2016	IPO3776	3	NT	*P. gingeri*	14		Industry	NL	2015
IPO3777	WUR 2016	IPO3777	3	3	*P. gingeri*	14		Industry	NL	2015
IPO3756	WUR 2016	IPO3756	3	NT	*P. gingeri*	14		Industry	NL	2015
IPO3748	WUR 2016	IPO3748	0	NT	*P. reactans*	16		Internal	NL	1989
IPO3750	WUR 2016	IPO3750	0	NT	*P. reactans*	16		Internal	NL	1989
IPO3751	WUR 2016	IPO3751	2	NT	*P. reactans*	16		Internal	NL	1989
IPO3739	WUR 2016	IPO3739	0	0	*P. agarici*	19		Internal		1990
IPO3740	WUR 2016	P. agarici, LMG 2112 T	0	NT	*P. agarici*	19		Collection		
IPO3752	WUR 2016	P. putida, LMG 2257 T	NT	NT	*P. putida*	23		Collection		
IPO3765	WUR 2016	IPO3765	3	3	*Pseudomonas sp*	25	*P. salomonii LMG 22120*	Industry	NL	2015
IPO3778	WUR 2016	IPO3778	1	NT	*Pseudomonas sp*	26	*P. yamanorum LMG 27247T*	Industry	NL	2015
IPO3779	WUR 2016	IPO3779	1	NT	*Pseudomonas sp*	26	*P. yamanorum LMG 27247T*	Industry	NL	2015

Supplementary Table 1C. Isolates and reference strains from Wolf et al., (2016) that were sequenced, with metadata on initial identity, pathogenicity, source, region of outbreak, year of outbreak, MLSA, ANI and dDDH.

Identifier	Sequencing	Label	Pathogenicity score from cap tests	Pathogenicity score from pot tests	Identity from molecular ivestigation			Source	Country	Year sampled
					MLSA consensus	ANI (>95%)	DDH (>70%)			
A6001	WUR 2017	A6001	2	NT	*P. gingeri*	1		Industry	NL	2016
C2001	WUR 2017	C2001	2	NT	*P. gingeri*	1		Industry	NL	2016
A4002	WUR 2017	A4002	2	NT	*P. NC02*	2		Industry	NL	2016
B6001	WUR 2017	B6001	2	NT	*P. NC02*	2		Industry	NL	2016
C6002	WUR 2017	C6002	2	NT	*P. NC02*	2		Industry	BE	2016
C8002	WUR 2017	C8002	2	NT	*P. NC02*	2		Industry	BE	2016
D3002b	WUR 2017	D8002	2	NT	*P. NC02*	2		Industry	NL	2016
D4002	WUR 2017	D4002	2	NT	*P. NC02*	2		Industry	NL	2016
D5002	WUR 2017	D5002	2	NT	*P. NC02*	2		Industry	NL	2016
D6002	WUR 2017	D6002	2	NT	*P. NC02*	2		Industry	NL	2016
E6002	WUR 2017	E6002	2	NT	*P. NC02*	2		Industry	BE	2016
F1002	WUR 2017	F1002	2	NT	*P. NC02*	2		Industry	NL	2016
F8002	WUR 2017	F8002	3	NT	*P. NC02*	2		Industry	NL	2016
F9001	WUR 2017	F9001	2	NT	*P. NC02*	2		Industry	NL	2016
G1002	WUR 2017	G1002	3	NT	*P. NC02*	2		Industry	NL	2016
G5001	WUR 2017	G5001	3	NT	*P. NC02*	2		Industry	NL	2016
I8001	WUR 2017	I8001	2	NT	*P. NC02*	2		Industry	NL	2016
A5002	WUR 2017	A5002	3	NT	*Pseudomonas sp*	4	*P. yamanorum LMG 27247T*	Industry	NL	2016
A8002	WUR 2017	A8002	2	NT	*P. gingeri*	5		Industry	NL	2016
H7001	WUR 2017	H7001	2	NT	*P. gingeri*	5		Industry	NL	2016
A9001	WUR 2017	A9001	2	NT	*P. tolaasii*	6		Industry	NL	2016
B1001	WUR 2017	B1001	2	NT	*P. tolaasii*	6		Industry	NL	2016
B6002	WUR 2017	B6002	2	NT	*Pseudomonas sp*	9	*P7548*	Industry	NL	2016
B4002	WUR 2017	B4002	2	NT	*P. yamanorum*	10		Industry	NL	2016
B7002	WUR 2017	B7002	2	NT	*Pseudomonas sp*	12	*P. edaphica LMG 30152T*	Industry	NL	2016
B9002	WUR 2017	B9002	2	NT	*Non-pseudomonad*	13	*Pantoea rodasii LMG 26273*	Industry	NL	2016
C1001	WUR 2017	C1001	2	NT	*P. gingeri*	14		Industry	NL	2016
C3001	WUR 2017	C3001	2	NT	*P. gingeri*	14		Industry	NL	2016
C4002	WUR 2017	C4002	2	NT	*P. gingeri*	14		Industry	NL	2016
D1001	WUR 2017	D1001	2	NT	*P. gingeri*	14		Industry	NL	2016
D5001	WUR 2017	D5001	2	NT	*P. gingeri*	14		Industry	NL	2016
D8001	WUR 2017	D8001	3	NT	*P. gingeri*	14		Industry	NL	2016
E1001	WUR 2017	E1001	2	NT	*P. gingeri*	14		Industry	NL	2016
C2002	WUR 2017	C2002	2	NT	*Non-pseudomonad*	15	*Serratia liquefaciens LMG 7884T*	Industry	NL	2016
B3002	WUR 2017	B3002	2	NT	*P. reactans*	16	*P. reactans LMG 5329*	Industry	NL	2016
C5002	WUR 2017	C5002	2	NT	*P. reactans*	16		Industry	NL	2016
C7002	WUR 2017	C7002	2	NT	*Non-pseudomonad*	17	*Serratia proteamaculans NCPPB 245T*	Industry	BE	2016
D2002	WUR 2017	D2002	2	NT	*Pseudomonas sp*	21	*P. fluorescens DSM 50090T*	Industry	NL	2016
F1001	WUR 2017	F1001	3	NT	*P. gingeri*	22		Industry	NL	2016
G9001	WUR 2017	G9001	3	NT	*P. gingeri*	24		Industry	NL	2016

Supplementary Table 1D. Isolates and reference strains from the Netherlands and Belgium that were sequenced in 2016-17, with metadata on initial identity, pathogenicity, source, region of outbreak, year of outbreak, MLSA, ANI and

Identifier	Sequencing	Label	Pathogenicity score from cap tests	Pathogenicity score from pot tests	Identity from molecular ivestigation			Source	Country	Year sampled
					MLSA consensus	ANI (>95%)	DDH (>70%)			
J1002	WUR 2018	J1002	2	NT	*P. gingeri*	1		Industry	BE	2018
J4002	WUR 2018	J4002	3	NT	*P. gingeri*	1		Industry	BE	2018
K5002	WUR 2018	K5002	NT	NT	*P. NC02*	2		Industry	NL	2018
J3002	WUR 2018	J3002	0	NT	*Pseudomonas sp*	4	*P. yamanorum LMG 27247T*	Industry	BE	2018
K6002	WUR 2018	K6002	2	NT	*Pseudomonas sp*	12	*P. edaphica LMG 30152T*	Industry	NL	2018
K7002	WUR 2018	K7002	2	NT	*Pseudomonas sp*	12	*P. edaphica LMG 30152T*	Industry	NL	2018

Supplementary Table 1E. Isolates and reference strains from the Netherlands, that were sequenced in 2018, with metadata on initial identity, pathogenicity, source, region of outbreak, year of outbreak, MLSA, ANI and

Copies of barcoding genes present in assembled genomes									Total	Duplicate	% Duplication in genome
atpD	fusA	groeL	gyrB	recA	recN	rpoB	rpoD	uvrC			
1	1	1	1	1	1	1	1	1	7135787	156843	2.2
1	1	1	1	1	1	1	1	1	6681666	104455	1.56
1	1	1	1	1	1	1	1	1	6630667	160112	2.41
1	1	1	1	1	1	1	1	1	7143654	189118	2.65
1	1	1	1	1	1	1	1	1	7151784	203262	2.84
1	1	1	1	1	1	1	1	1	7107288	182219	2.56
1	1	1	1	1	1	1	1	1	7261618	164174	2.26
1	1	1	1	1	1	1	1	1	7042523	144090	2.05
1	1	1	1	1	1	1	1	1	7203935	159301	2.21
1	1	1	1	1	1	1	1	1	7081458	129914	1.83

Supplementary Table 2. Barcoding genes and genome duplication in *Pseudomonas* isolates that map in specifically and remain unidentified. It describes the number of copies of nine barcoding genes found in the

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Chapter 3

Detection of blotch pathogens

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Abstract

Bacterial blotch is a group of economically important diseases of the common button mushroom (*Agaricus bisporus*). Once the pathogens are introduced to a farm, mesophilic growing conditions (that are optimum for mushroom production) result in severe and widespread secondary infections. Efficient, timely and quantitative detection of the pathogens is hence critical for the design of localized control strategies and prediction of disease risk. This study describes the development of real-time TaqMan™ assays that allow molecular diagnosis of three currently prevalent bacterial blotch pathogens: "*Pseudomonas gingeri*," *Pseudomonas tolaasii* and (as yet uncharacterized) *Pseudomonas* strains (belonging to *Pseudomonas salomonii* and *Pseudomonas edaphica*). For each pathogen, assays targeting specific DNA markers on two different loci, were developed for primary detection and secondary verification. All six developed assays showed high diagnostic specificity and sensitivity when tested against a panel of 63 *Pseudomonas* strains and 40 other plant pathogenic bacteria. The assays demonstrated good analytical performance indicated by linearity across calibration curve (>0.95), amplification efficiency (>90%) and magnitude of amplification signal (>2.1). The limits of detection were optimized for efficient quantification in bacterial cultures, symptomatic tissue, infected casing soil and water samples from mushroom farms. Each target assay was multiplexed with two additional assays. *Xanthomonas campestris* was detected as an extraction control, to account for loss of DNA during sample processing. And the total *Pseudomonas* population was detected, to quantify the proportion of pathogenic to beneficial *Pseudomonas* in the soil. This ratio is speculated to be an indicator for blotch outbreaks. The multiplexed assays were successfully validated and applied for routine testing of diseased mushrooms, peat sources, casing soils, and water from commercial production units.

Introduction

Cultivation of the white button mushroom (*Agaricus bisporus*), represents a global economic value of more than 4.7 billion US dollars (Sonnenberg et al., 2011). Bacterial blotch diseases render crops unmarketable by strongly affecting the aesthetic quality of the caps pre-harvest and reducing the shelf life of the mushrooms post-harvest. Economic losses from this disease have been reported globally across Europe (Olivier, Guillaumes, and Martin, 1978; Paine, 1919), North America (Tolaas, 1915), Middle East (Bashan and Okon, 1981; Özaktan and Bora, 1994), Asia (Chen, 1981; Guleria, 1976; Kim, Kwon, and Kang, 1995; Suyama and Fujii, 1993) and Australia (Fahy, 1981; Nair, 1969). In Europe alone, blotch outbreaks can reduce the total yield up to 50% (Soler-Rivas et al., 1999). Bacterial blotch diseases of mushrooms have been well-described for over a century. They are caused by diverse fluorescent *Pseudomonas* species, probably originating from the casing soils in mushroom farms (Wong and Preece, 1980). The casing soil is a 5cm layer of peat and lime that is placed on top of the compost, to facilitate formation of mushroom pinheads.

Pseudomonas tolaasii is the predominant pathogen of “brown blotch”, and produces dark, sunken, brown lesions (Paine, 1919; Tolaas, 1915). It produces pitting and brown lesions on the mushroom caps by secreting the extracellular toxin tolaasin (Soler-Rivas et al., 1997). The biochemical mechanisms of browning, the biosynthesis of tolaasin, and its genetic regulation have been well-studied (Grewal, Han, and Johnstone, 1995; Han, Johnstone, and Grewal, 1994; Rainey, Brodey, and Johnstone, 1993). Non-pathogenic forms of *P. tolaasii*, are unable to produce this toxin. *P. tolaasii* is also a pathogen of specialty mushrooms such as *Pleurotus ostreatus, Flammulina velutipes* and *Pleurotus eryngii* (González, González-Varela, and Gea, 2009; Han et al., 2012; Suyama and Fujii, 1993). *‘Pseudomonas gingeri’* is an invalidly named species documented to produce ginger-coloured superficial lesions. It is the only known causative agent of “ginger blotch” (Wells et al., 1996; Wong et al., 1982). Ginger blotch pathogens do not produce tolaasin (Lee, Jeong, and Cha, 2002) and their symptom development and epidemiology are poorly understood (Fletcher and Gaze, 2007).

Bacterial blotch pathogens are believed to be endemic to the peat component of the casing soil, albeit at low densities. Once infected, secondary infection via insects, water splashing, mushroom pickers and mechanized harvesters is quick (Wong and Preece, 1980). Given the mesophilic and humid conditions required for mushroom cultivation, pathogen densities are soon enriched in the mushroom beds (Godfrey, 2003; Wong et al., 1982). Limited management strategies exist for chemical, environmental or biological control of blotch diseases (Fletcher and Gaze, 2007; Godfrey, 2003; Navarro, Gea, and González, 2018; Osdaghi et al., 2019). Early and

efficient detection of the pathogens is hence critical to predict and prevent blotch outbreaks.

For *P. tolaasii*, identification was formerly performed by a reaction between colonies of *P. tolaasii* and '*P. reactans'* in agar plates, referred to as the white line inducing principle (WLIP) (Goor et al., 1986; Han et al., 1992; Lloyd-Jones et al., 2005; Wells et al., 1996; Wong and Preece, 1979). However, closely related blotch-causing bacteria, such as *P. costantinii,* can also induce the white line precipitate against '*P. reactans'* (Munsch and Alatossava, 2002). WLIP has also been observed in isolates from the species complexes of *P. fluorescens* and *P. putida* (Rokni-Zadeh et al., 2012). Plating and phenotypic methods are thus unspecific for identification of *P. tolaasii* infection.

Recent advances allow qualitative detection of *P. tolaasii* using traditional and nested PCR methods (Lee et al., 2002). However, for *'P. gingeri'*, even qualitative detection methods do not yet exist. There is a need for pathogen-specific quantitative diagnostic assays to track and quantify pathogen populations during the mushroom cultivation cycle and post-harvest chain. Identification of the pathogen, and knowledge of its population dynamics is essential to optimize early measures towards the prevention of blotch outbreaks.

Specific and sensitive molecular detection methods for blotch pathogens will help to solve current inconsistencies in symptom diversity and nomenclature of blotch-causing organisms. Quantitative detection methods will enable fundamental insights into pathogen population structures in the mushroom beds and on the caps, allowing study of the microbial ecology of the pathogens during the mushroom cropping process. The assays can also be used to monitor potential contamination of raw materials such as casing, compost (substrate), spawn (inoculated mycelium) water and environments used for mushroom cultivation.

DNA-based diagnostic methods have gained wider acceptance and reliability than microscopy, phenotypic, immunological and serological techniques due to the higher stability of DNA, its universal presence in cells of all kinds, identification at very low densities and high-throughput detection methods (Schaad and Frederick, 2002; Ward et al., 2004). Formerly time-consuming PCRs have now been replaced with Taqman^{TM}-qPCRs, that not only quantify targets in real-time, but also increase the specificity of detection significantly (Mullis, 1990; Mullis and Faloona, 1989). The use of hydrolysis probes for measurement of sequence-specific amplification also allows detection of multiple targets in one reaction (Elnifroet al., 2000; Liu et al., 2013).

In this study we develop DNA-based diagnostic assays against three causative agents of bacterial blotch, that are currently causing severe disease outbreaks in Western Europe, *'P. gingeri', P. tolaasii* and an unknown *Pseudomonas* species that

was formerly identified as *P. tolaasii*. We design Taqman™-qPCR assays for rapid and specific relative-quantification of multiple blotch pathogen, at low population densities in a variety of environmental samples. The development of the assays and validation of their analytical performance, diagnostic performance, limitations and potential applications are further elaborated.

Materials and Methods

Reference strains

Reference strains for assay development were selected from a previous molecular characterization of blotch pathogens in which whole genomes were sequenced from 30 blotch-associated *Pseudomonas* (van der Wolf et al., 2016). Reference strains for *'P. gingeri'*, include LMG 5327^T, LMG 5328, IPO 3754, IPO 3777, IPO 3776, IPO 3769, IPO 3757, IPO 2767 and IPO 3756. Reference strains for *P. tolaasii* include LMG 2342^T, ATCC 51310 and ATCC 51309. Reference strains for the unknown brown-blotch causing *Pseudomonas* include LMG 2343 (formerly identified as *P. tolaasii*) and IPO 3765. Reference strains for each of the pathogens originate from symptomatic mushroom tissue and have been shown to demonstrate pathogenic behaviour on fresh mushroom caps.

Assay development

Target regions against '*P. gingeri*' and the unknown *Pseudomonas* sp. were selected from a Multi-Locus Sequence Alignment (MLSA) on whole genome sequences of *Pseudomonas* isolated from symptomatic mushrooms (van der Wolf et al., 2016). Reads from reference strains of each pathogen were aligned and 500bp blocks of conserved sequences were selected, that mapped to the pathogen of interest, but not to any other outgroups, and had no single nucleotide polymorphisms (SNPs). The sequence blocks were checked for specificity with NCBI-BLAST. Target regions against *P. tolaasii* were selected on the tolaasin gene fragment (accession numbers AY291584, AY228241, U16024 and AF291753) from LMG 2342^T and other isolates. Target regions were checked for homology to other bacterial species, in particular to other Pseudomonads via NCBI-BLAST. The selected amplicons are described in Supplementary Table 1.

For each pathogen, two amplicons, with length between 80-120 bp, were designed on different loci within their respective target regions. TaqMan™ primers and probes were designed using the PrimerQuest web-tool (Integrated DNA Technologies), or Primer Express (Applied Biosystems) with the oligo size between 18-25 bp (Table 1). All amplicons were subjected to BLAST searches against the *Pseudomonas* database (www.*Pseudomonas*.com) to check for degeneracy. The specificity of the

primers and probes was also checked via NCBI-BLAST. In total, six assays were developed against '*P. gingeri' (Pg2, Pg6), P. tolaasii (Pt1, Pt2)* and the unknown *Pseudomonas* sp. *(Pu4, Pu10)*. The assays were multiplexed to also detect *Xanthomonas campestris* pv. *campestris* (Xcc) (Köhl et al., 2011) and the *Pseudomonas* genus (Pp) (Lloyd-Jones et al., 2005).

Reaction conditions

The detection of bacterial DNA in various samples was performed using TaqMan™ probe technology, on a QuantStudio™ 12K Flex from Applied Biosystems (Thermo Fisher Scientific, United States). Per reaction, the PCR mix included 0.25X ROX dye II, 1X TaKaRa™ Premix Taq™ DNA Polymerase (Takara Bio), 300 nM forward and reverse primers from all three targets, and 100 nM of FAM, VIC and ATTO labelled probes with NFQ-MGB quencher for target pathogens, *X. campestris* pv. *campestris* and generic *Pseudomonas* respectively. The total volume was made up with DNase and RNase free water to 12.5 µl per reaction. Activation of Taq-polymerase was done at 95 °C for 2 minutes. The qPCR amplification conditions consisted of denaturation for 15 seconds at 95 °C and extension for 60 seconds at 60 °C, for 40 cycles. A Threshold cycle (C_T) value lower than 35 was considered a positive reaction. qPCR reactions were performed with 1 ng of target DNA, in duplicates.

Positive and negative controls

For determining the limits of detection, several types of positive controls were tested. The first positive controls consisted of gBlocks® (Integrated DNA Technologies, USA). These synthetic oligonucleotides contain concatenated sequences of all amplicons to be tested within a multiplexed assay. gBlocks® were designed per pathogen, for both target amplicons along with *X. campestris* pv. *campestris* and generic *Pseudomonas* amplicons interspaced with some extra flanking nucleotides from the original gene sequence. The gBlock sequences are described in Supplementary Table 1. A serial dilution of the gBlocks® from 10^8 to 0 copies of target DNA was used to plot the calibration curves. Controls for water samples consisted of sterile water spiked with a pathogen suspension (in Ringers solution) to obtain a 10-fold serial dilution from 10^8 to 0 cfu/ml. Controls for casing soil samples consisted of pure soil DNA, extracted from soil samples spiked with serially diluted pathogen suspension from 10^8 cfu/g to a final concentration of 0 cfu/g of soil. All controls were spiked with three pathogens together. The negative control was DNase and RNase free water.

Assays Pathogen	Forward primer (5' to 3')	Reverse primer (5'to 3')	Probe (5'to 3')	Reporter Dye
Pg2	CACCGGACCGA TGAAGG	CAGTGACTCGGGC TTGC	TCGGCGAAAGCC GTCTGATCACCGT	FAM
Pg6	TCATCCATGCA GTCGGAAAG	ACGCTGAACGCTCA CATT	CAGCGACTTCACG ACAGCGAACAC	FAM
Pt1	TGTTGTGCGCC TCGTTTTTA	AATGCGAGGGTCA CTTTGGT	CCGCCGCACAGG CTCAGGA	FAM
Pt2	AGGCCGAAGGG CAAGGT	TGTCAGCGAGCAG GAGCAT	TGTCGATATCCCC GAGCAACTCGC	FAM
Pu4	GCAGATTGTCG CGTATTCC	ACCTGGCTGACGC CCGCTGC	ACGGTTTACGCGC CAATG	FAM
Pu10	ATGTTGATCACC TCGCCTTC	CGGGTGGAGAAGA TTGCTTT	TTACGCTGTAGCG GGCAT	FAM
Extraction control				
Xcc[1]	GTGCATAGGCC ACGATGTTG	CGGATGCAGAGCG TCTTACA	CAAGCGATGTACT GCGGCCGTG	HEX
***Pseudomonas* population**				
Pp[2]	GGGTGGTGGAA TTTCCTGTGT	TTCCTTGTGGTCAC CGCTTC	GTGAAATGCGTAG ATATAG	ATT0 550

Table 1. Description of TaqMan™ primers, probes, amplicon size and reporter dyes, for quantifying *'P. gingeri', P. tolaasii*, an unknown *Pseudomonas* species, *X. campestris* pv. *campestris* and the generic *Pseudomonas* population, abbreviated as Pg, Pt and Pu, Xcc and Pp respectively. [1] Kohl et. al., 2011 and [2] Llyod-Jones et al., 2005.

Sample processing and DNA extraction

Bacterial isolates were grown on King's B or Tryptic Soy agar for 24 hours at 25 °C before DNA extraction. The entire panel of bacterial strains are described in Supplementary Table 2. Total DNA was extracted from 200 mg of bacterial colonies scraped from a plate for each isolate using Wizard Magnetic DNA Purification System for Food (Promega, United States) according to the manufacturer's protocol, including the DNase-free RNase treatment. All the DNA for the testing panel was quantified fluorometrically using a Quant-iT PicoGreen dsDNA assay Kit (Thermo Fisher Scientific, United States) on the Infinite M200 PRO microplate reader (Tecon, Switzerland).

Assays were also evaluated by testing a variety of environmental samples. Bacterial suspensions were prepared from pure cultures in Ringers solution, and serially diluted from an upper concentration of 10^8 cfu/ml (OD_{280}=0.1). Biopsies from

symptomatic tissue were weighed, homogenized in 1ml of Ringers solution, and the extract was filtered using BIOREBA bags (Bioreba, Switzerland). For water samples, 100 ml of tap-water was centrifuged at 9000 g for 15 minutes at 4 ^{o}C. The supernatant was discarded, and the pellet re-suspended in 1 ml of sterile DNase and RNase free water. The bacterial suspensions, tissue extracts, and water samples were centrifuged at 11200 g, then heated to 100 ^{o}C for 10 minutes, and 1 µl of the supernatant was used for qPCR.

For casing soil samples received from mushroom farms, 250 mg of sampled material was processed for a semi-automated DNA isolation. The PowerMag Soil DNA Isolation kit from MoBio Technologies (Qiagen) was used to perform a total DNA extraction, eluted into 100 µl of Tris-EDTA buffer, using a Kingfisher Flex (Thermo Fisher Scientific) according to manufacturer's protocol. 1 µl of the resultant DNA (concentration as extracted) was used for qPCR.

For diagnostic tests, 10^5 cfu of *X. campestris* pv. *campestris* in 0.01 M phosphate buffer saline was added to water, mushrooms and soil samples before DNA extraction, to allow for relative quantification, by controlling for loss of bacterial DNA during sample processing

Data analyses

Data analyses was performed on RStudio with R version 3.4.0 (R Core Team, 2013). Threshold cycle (C_T) values from TaqMan™ qPCR were imported from the QuantStudio™ 12K Flex Real-Time PCR Software (Thermo Fisher Scientific, United States). When the C_T values were plotted against the copies of template DNA, amplicon efficiency was calculated from the slope of the calibration curve, as $10^{-1/slope}$-1 (Kubista et al., 2006). Pathogen populations in the samples were quantified relative to known concentrations of *X. campestris* pv. *campestris* by the $2^{-\Delta\Delta C}$T method (Livak and Schmittgen, 2001), as a fold change ratio.

Results

Development of real-time TaqMan™ PCRs

In this study, six TaqMan™ qPCR assays were designed, to detect two different loci for each of the three blotch pathogens (Figure 1). Each target-pathogen assay was multiplexed with two additional assays that quantify *Xanthomonas campestris* pv. *campestris* (Xcc) (Köhl et al., 2011) as an extraction control, and the genus *Pseudomonas* (Lloyd-Jones et al., 2005) for the total *Pseudomonad* population (Pp). In total, six triplex assays were developed; two for '*P. gingeri*', (i) Pg2-Pp-Xcc (ii) Pg6-Pp-Xcc; two for *P. tolaasii*, (i) Pt1-Pp-Xcc and (ii) Pt2-Pp-Xcc; and two for the unknown *Pseudomonas* sp., formerly classified as *P. tolaasii*, (i) Pu4-Pp-Xcc (ii) Pu10-Pp-Xcc.

Assay Pt1 and Pt2 amplify two fragments of the tolaasin gene (AF291753, AY291584). Assay Pg2 amplify the LysR transcriptional regulator gene fragment (PNQ94452) whereas assay Pg6 amplifies a hypothetical protein fragment (PNQ88072). Assays Pu4 and Pu10 amplify two unknown gene fragments. The Xcc assays also amplifies a hypothetical protein fragment (QCX70676). The Pp assay amplifies the 16S gene fragment (MK294319). In the reference genome sequences all amplicon targets are single copy. The sequences of primers and probes for the triplex assays are described in Table 1. The amplicons sequences are described in Supplementary Table 1.

Analytical performance

The multiplexed assays for all target pathogens were tested against positive controls comprising of gBlocks®, in replicates of three. The TaqMan™ assays were evaluated according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). The calibration curves showed good linearity, symbolized by the high correlation coefficients (R^2>0.95). The amplification efficiencies (E) estimated from the slope of the curve, varied within 91.6% - 113%. The standard curves are illustrated in Figure 2. Delta Rn values (ΔRn) varied between 2.1 and 3.0 for the assays, suggesting a good magnitude of the amplification signal. The Xcc and Pp control assays both showed high precision, indicated by the low variation observed in qPCR results when tested in replicates of six, within and between experiments. Their repeatability (intra-assay variation) and reproducibility (inter-assay variation) is observed from the confidence intervals in Figure 3.

Figure 1. Symptomatic mushroom tissue naturally infected with bacterial blotch pathogens, A) *P. tolaasii* (LMG2342), (B) unknown *Pseudomonas* species formerly identified as *P. tolaasii* (IPO3765) and (C) '*P. gingeri*' (IPO3777)

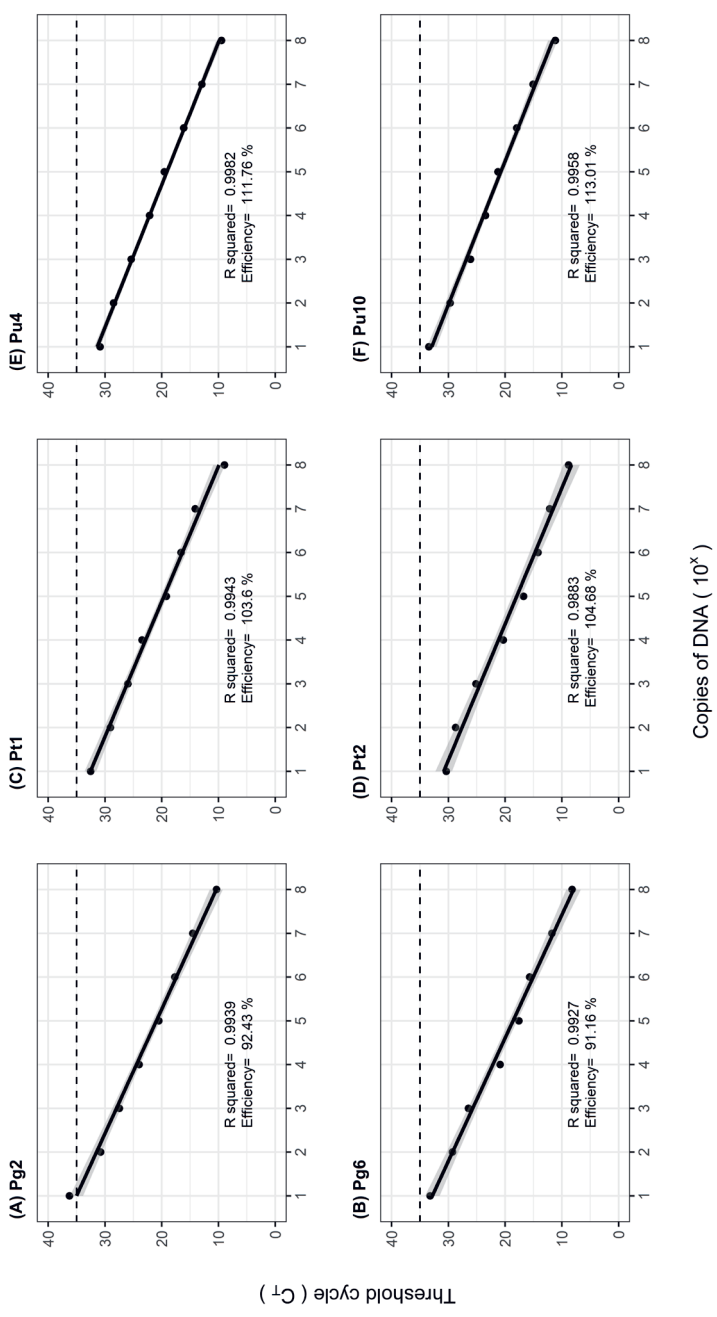

Figure 2. Calibration curves of multiplexed target assays, with amplification efficiencies and adjusted R^2 values, when tested against positive controls comprising of serially diluted gBlocks®. The total copies of target DNA and the C_T values are plotted against the x and y axis respectively. The grey bands indicate a confidence interval of 99%. And the dotted line indicates the cut-off value, C_T=35.

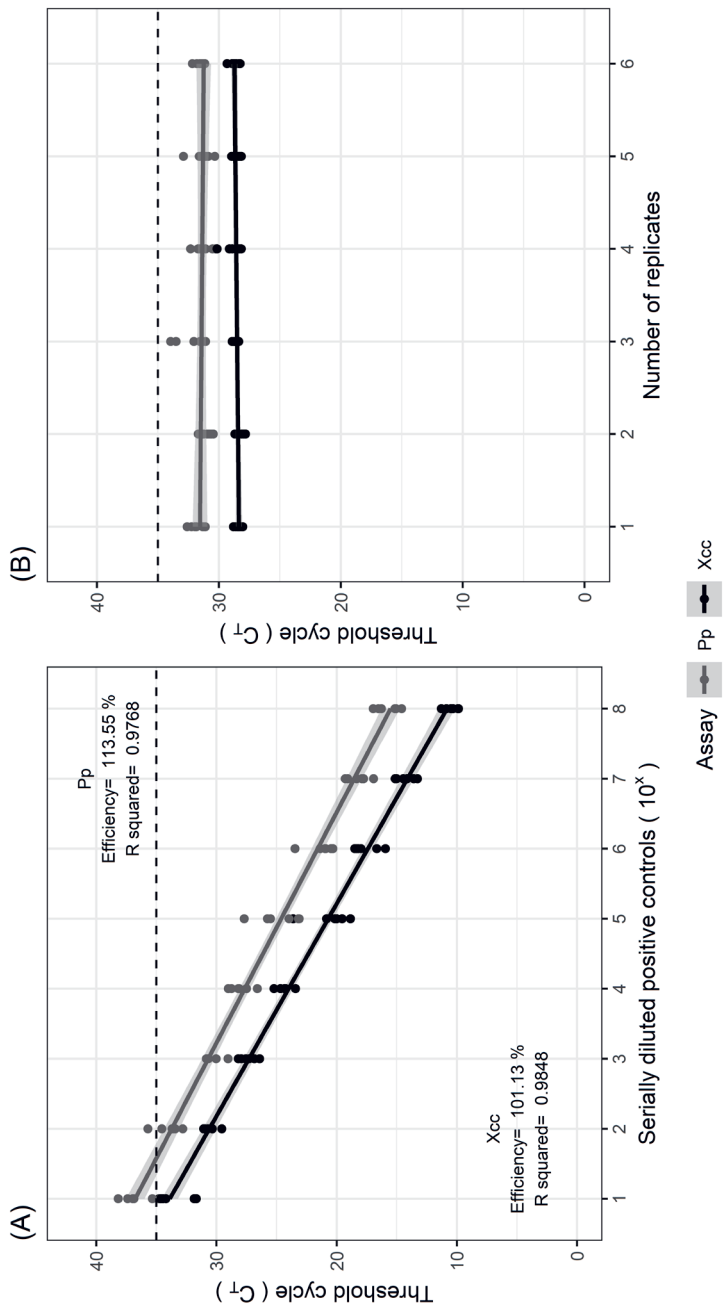

Figure 3. A) Inter-assay (reproducibility) and B) intra-assay (repeatability) variation in multiplexed Xcc and Pp assays, tested on positive controls comprising of serially diluted gBlocks®. Both were tested in replicates of six.

Specificity and sensitivity

A panel to test the diagnostic specificity, inclusivity and sensitivity was assembled from international (LMG, CFBP, ATCC and NCPPB) and local culture collections. This panel included 18 bacterial blotch pathogens, 18 mushroom-associated *Pseudomonas*, 28 plant pathogenic *Pseudomonas*, and 41 other plant pathogenic bacteria (34 species from 14 genera). The assays were tested on 1 ng of total DNA from pure bacterial cultures. The true positive (TP), true negative (TN), false negative (FN) and false positive (FP) results are summarized in Table 2 and 3.

Diagnostic specificity of an assay is a measure of the negative samples that are correctly identified. It is described as TN/(TN + FP). The assays tested negative against the majority of other *Pseudomonas* strains and an assortment of plant-pathogenic bacteria. Very few cross reactions were observed with non-target strains, resulting in an overall specificity of 0.99, 0.99, 1, 1, 0.99 and 1 for Pg2, Pg6, Pt1, Pt2, Pu4 and Pu10 respectively. All non-specific amplifications were characterized by a very late detection of target DNA in one or both replicates, implying a weak positive reaction, where $35>C_T>40$. Therefore $C_T<35$ was considered an unambiguous positive result.

Diagnostic sensitivity of an assay is a measure of the positive samples that are correctly identified. It can be described as TP/(TP + FN). The six target assays, Pg2, Pg6, Put1, Pt2, Pu4 and Pu10, all tested positive for each of their respective reference strains, characterized by an early detection of DNA, where $C_T<25$. No false negatives were detected, implying a high diagnostic sensitivity of 1. The mean C_T values from diagnostic specificity and sensitivity tests are described in Supplementary Table 2.

Detection thresholds

The detection thresholds of all assays increase across target DNA (gBlocks®), spiked water and soil samples, indicated by the mean C_T values (Figure 4). The limit of detection (LOD), is described as the lowest concentration at which 95% of the positive samples are consistently detected, characterized by $C_T<35$. When tested against serially diluted gBlocks®, assays Pg6, Pt1, Pt2, Pu4, Pu10 and Xcc had a LOD of 10 copies of target DNA. Assays Pg2 and Pp had a higher LOD of 100 copies of target DNA. For water samples, the LOD for assays Pt1, Pu4 and Pu10 was 10^3 cfu/ml. Assays Pg2, P6 and Pt2 had a higher LOD of 10^4 cfu/ml. For casing soil samples, assays Pt1 and Pt2 had the lowest LOD of 10^3 cfu/g. Assays Pg2, Pg6, Pu4 and Pu10, had a higher LOD of 10^4 cfu/g. The control assays, Xcc and Pp, gave consistent positive reactions at C_T~28 and C_T~30 for *X. campestris* pv. *campestris* and the total *Pseudomonas* population in the soil respectively.

Diagnostic application

Water, soil and symptomatic tissue from mushroom farms were tested to apply the newly designed assays. For primary detection, assays Pt1, Pg2 and Pu4 were employed, and the results were verified with the secondary assays, namely, Pt2, Pg6, and Pu4. Over the course of 10 independent batches in which samples were processed, diseased mushrooms, casing soil, peat and tap water was sampled from mushroom farms across the Netherlands and Belgium and stored at -20 ^{o}C until tested.

Diseased mushrooms were received from 50 sources varying in mushroom farm, soil, compost, cultivar, environmental conditions, and harvest cycle. Out of 95 symptomatic cap tissue tested, 78% of the samples were strongly positive (C_T < 25) for '*P. gingeri*' with assays Pg2 and Pg6, 42% of mushrooms tested positive for the unknown *Pseudomonas* species with the Pu10 and Pu4 assays, and 20% of the mushroom tested positive for *P. tolaasii* with Pt1 and Pt2 assays. Only 9.4% of the symptomatic samples tested negative against all assays. Several samples also showed a secondary infection with another pathogen, when multiple assays tested positive on the mushroom tissue. Immense variability exists between the pathogen densities on biopsies taken from mushroom caps, ranging from $4.8x10^2$ cfu to $7.9x10^6$ cfu. Pathogen density also varied with the severity of the disease symptoms.

Peat, before being mixed into casing soils in the mushroom farms, was received from 9 geographical sources. Out of 14 samples of peat tested, only 2 were positive for '*P. gingeri*' with assays Pg2 and Pg6, whereas 6 samples tested positive for the unknown *Pseudomonas* species with assays Pu4 and Pu10. These samples were not tested with assays Pt1 and Pt2 for *P. tolaasii*. Pathogen populations were consistently detected in the range of $1.2x10^5$ to $4.2x10^5$ cfu per gram. Co-occurrence of the pathogens was observed in only one peat sample, when it tested positive for multiple assays. 7 peat samples tested negative against all assays. Tap water from all samples tested unanimously negative for the blotch pathogens, characterized by C_T>40.

Bacterial strains tested in panel for specificity and sensitivity	Positive results, where C_T< 35						
	Total	Pg2	Pg6	Pt1	Pt2	Pu4	Pu10
'P. gingeri'	8	8	8	0	0	0	0
P. tolaasii	8	0	0	8	8	0	0
P. unknown	2	0	0	0	0	2	2
Other mushroom-associated *Pseudomonas*	16	1	1	0	0	1	0
Plant pathogenic *Pseudomonas*	28	0	0	0	0	0	0
Other plant pathogenic bacteria	41	0	0	0	0	0	0

Table 2. Description of positive reactions (C_T<35) of all six target assays, against the diagnostic bacterial panel. Weak positive reactions, where C_T is close to the cut-off threshold (C_T~35), are marked in red.

Target assays	True Positive	True Negative	False Positive	False Negative	Diagnostic specificity	Diagnostic sensitivity
Pg2	8	94	1	0	0.99	1
Pg6	8	94	1	0	0.99	1
Pt1	8	95	0	0	1.00	1
Pt2	8	95	0	0	1.00	1
Pu4	2	100	1	0	0.99	1
Pu10	2	101	0	0	1.00	1

Table 3. Summary of diagnostic sensitivity and specificity of target assays. Weak positive reactions, where C_T is close to the cut-off threshold (C_T~35), are marked in red.

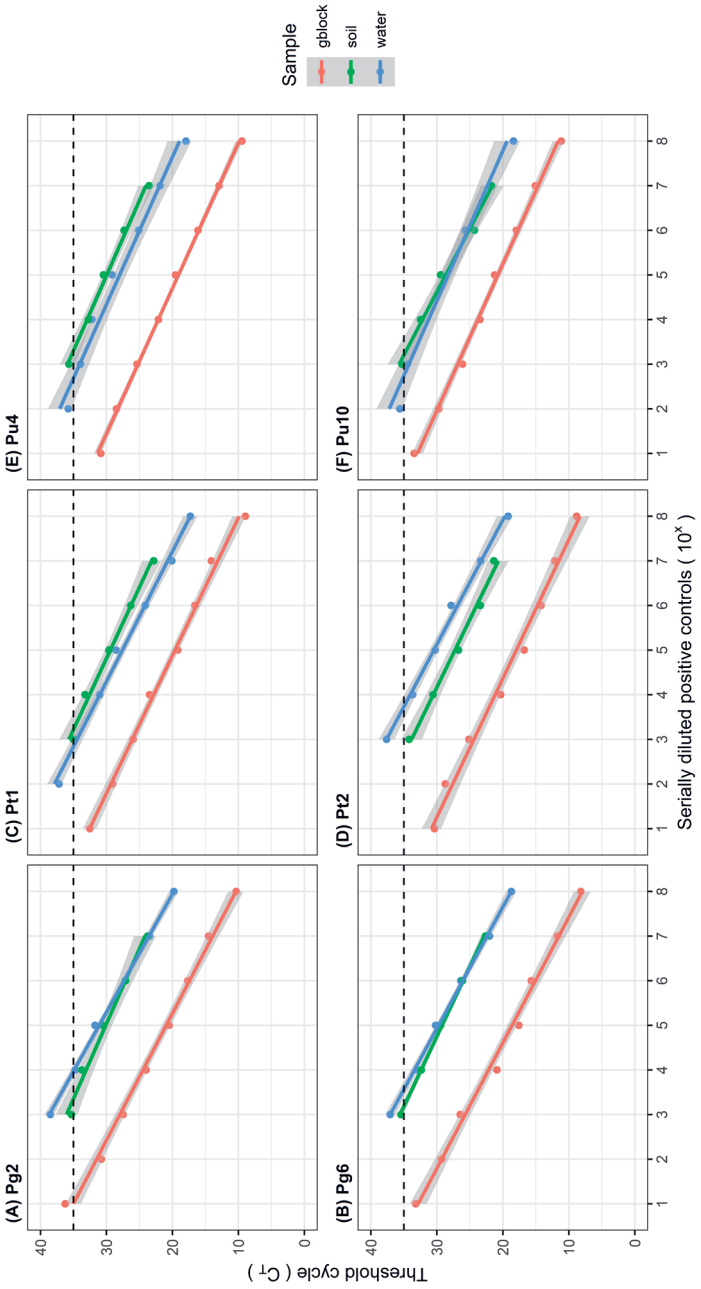

Figure 4. Mean detection thresholds of target-pathogen assays in different sample types spiked with pathogen mixture, as described in the methods. gBlocks®, water samples and soil samples are depicted in red, green and blue color respectively.

Discussion

Overall performance of assays

All assays demonstrated high diagnostic specificity when tested against the bacterial panel, although occasionally weak false positives were detected, as observed in Supplementary Table 2. Multiple negative controls were also tested within the same setup, such as a) no template control b) sterile water control, c) DNA extracted from an uninoculated plate of media. Contamination from positive controls during the qPCR, in reagents, or during DNA extraction is thus ruled out. In such samples, when DNA from pure bacterial cultures is used as template, due to very high DNA concentrations, template DNA can sometimes align with the fluorescent probe despite limited homology, leading to weak false positive reactions. Environmental samples, such as casing soil and water, contain much lower levels of pathogens, with the exception of symptomatic mushroom tissue. Hence these false positives are unlikely to occur when the diagnostic assays are applied during routine screening of raw materials used in mushroom farms. Furthermore, the use of a C_T threshold value, determined from the assay validation data, allows interpretation of true positive and negative samples.

For diagnostic purposes, precise quantification of the very low-density samples by qPCR is critical. All 6 target assays showed good linearity across the dilution range for their calibration curves, even at lower densities. The calibration curves of all assays showed similar slopes across the gBlocks®, water and soil samples, indicating that the qPCR efficiency is uninfluenced by sample type. The mean C_T values for the assays, at a given concentration of pathogen, increased from gBlocks® and water to soils samples. This is potentially due to DNA loss during the extraction process and can be addressed by improving DNA isolation methods for soil samples, which in turn can also reduce the presence of qPCR inhibitors such as humic and fulvic acids.

Practical and industrial applicability

These assays can be successfully applied to identify and quantify pathogens on mushrooms, casing soils and peat sources at much lower densities than previously possible. Former thresholds of *P. tolaasii* density on mushroom caps were in the range of $7.7x10^4$ to $2x10^{10}$ cells per cap (Nair and Bradley, 1980; Nair and Fahy, 1972; Olivier, Mamoun, and Munsch, 1997; Preece and Wong, 1982). In the casing soil, pathogen densities were earlier reported in the magnitude of 10^7 to 10^9 cfu/g of soil (Nair and Fahy, 1972). For both caps and soils, 100-fold lower pathogen densities were observed and reported with the newly developed TaqMan™ assays. This increased sensitivity, makes it now possible to screen raw materials used in

mushroom cultivation, such as spawn, peat, compost and casing soil for presence of bacterial blotch pathogens.

Previously, the use of DNA-based molecular detection methods for routine diagnostics has been demonstrated for agricultural pathogens (Bonants and te Witt, 2017; Ophel-Keller et al., 2008; Ward et al., 2004). However, several considerations and limitations exist in the use of these assays to determine and provide insights into the "health status" of mushroom farms. The relationship between pathogen populations in the casing soil, and a disease outbreak is rather volatile. Blotch outbreaks depend on a variety of other factors also, which include environmental conditions, growing practices, cultivar type, source of compost and type of casing soil (Godfrey, 2003). The direct translation of pathogen population densities retrieved from these assays into prediction of an economic risk requires good knowledge and cautious interpretation of other disease indicators.

Strengths and shortcomings

Previously known diagnostic methods such as microbiology (Wong and Preece, 1979), microscopy (Preece and Wong, 1982; Soler-Rivas et al., 1999), phenotypic tests (Goor et al., 1986) and PCRs (Kwon, Kim, and Go, 2000; Lee et al., 2002) were limited to pathogen identification and qualitative detection. Taqman^{TM}-qPCR assays designed here, provide the first opportunity for quantitative high-throughput detection of blotch pathogens. At high precision, sensitivity and specificity, these assays can be used to detect, monitor and study bacterial blotch pathogens at low population densities in multiple environmental samples. Additionally, the assays ensure repeatability and accuracy in the diagnostic measurements. Despite the many advantages, a major limitation of these assays is that they only offer information on abundance of the pathogens, not its viability (Vincelli and Tisserat, 2008). The active fraction of pathogens cannot be calculated, as DNA-based assays cannot differentiate between dead and living cells (Wolffs, Norling, and Rådström, 2005). An alternative way to quantify viable pathogen densities would be via use of blocking agents that bind to DNA from dead-cells (Fittipaldi, Nocker, and Codony, 2012), selective enrichment on media prior to PCR (Schaad and Frederick, 2002) or reverse-transcriptase PCR to amplify mRNA instead (Kobayashi et al., 2009).

Future prospects

For use of these assays as a monitoring system, an up-scaled sampling strategy needs to be explored for an industrial analysis. The number of samples that are representative of a batch, and the frequency of sampling that takes into account the effect of storage, seasonal variation, and bulk quantities, remain largely undetermined. Selective microbial enrichment of the pathogen or use of a blocking agent, could allow quantification of only the viable pathogen populations. Detailed

information on the population dynamics of the pathogen during the mushroom cropping cycle, and the survival rates of pathogens in various types of raw materials needs to be generated. This knowledge will be essential to place quantitative information about pathogen densities into context. Finally, field trials are also required to understand the variation in the inoculum threshold of the soil for disease outbreaks, based on the type of casing soil, compost, cultivar, watering method and environmental conditions during production, etc.

Conclusions

Six assays have been developed for quick and quantified detection of three aggressive bacterial blotch causing agents. The assays are highly specific and sensitive and can be used to test for pathogen targets in different substrates associated with the mushroom cropping ecosystem, such as mushroom caps, water sources, peat, compost and casing soils. They allow efficient diagnosis of secondary infections within the farms. Direct applications also lie in assessing the efficiency of applied disinfectants and bio-control agents. In case of generic disease symptoms, the assays can be used to verify the pathogen and apply specific disease control measures. Routine industrial application of these assays as a warning system require more insights into sampling strategies, pathogen population dynamics, and both abiotic and biotic disease indicators.

Author Contributions

MK and JH designed the diagnostic assays; TT, MK, JH and MH performed the experiments. TT and JW wrote the first draft of the manuscript; JE critically reviewed the draft. All authors contributed to subsequent manuscript revision, read and approved the submitted version.

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Supplementary Tables

Pp	Xcc	Pu10	Pu4	Pt2	Pt1	Pg6	Pg2	Multiplexed assay
16S rRNA	Hypothetical protein	NA	NA	Tolaasin	Tolaasin	Hypothetical protein	LysR transcription	Target gene (if relevant)
65	66	99	92	80	65	98	112	Amplicon size (bp)
MK294319	QCX70676	NA	NA	AY291584	AF291753	PNQ88072	PNQ94452	Accession
GGTAGAG GGTGGTG GAATTTCC TGTGTAGC GGTGAAAT GCGTAGAT ATAGGAAG GAACACCA GTGGCGA AGGCGAC	GCCAGGG TGCATAGG CCACGATG TTGGCCCA AGCGATGT ACTGCGG CCGTGGT GTAAGACG CTCTGCAT CCGTACC G	CGGATGTT GATCACCT CGCCTTCG TCGGTCCT TACGCTGT AGCGGGC ATCCAGTT GACCGAT GCCATCG GGGCGCA AAAGAAAG CAATCTTC TCCACCCG CCAGGAC	GAAGGCG GGCAGATT GTCGCGTA TTCCGGGT CTGCTGAC GGTTTACG CGCCAATG GTCATGCG GTCGGCT CGGCAGC GGGCGTC AGCCAGG TATTGTTG GCTGCC	ACGTCTTG CAGGCCG AAGGGCA AGGTCGG GGTGTCG ATATCCCC GAGCAACT CGCGGAA GAACGCTT CATGCTCC TGCTCGCT GACACCCA GGCGC	TTGCCGGT CTGTTGTG CGCCTCGT TTTTACCC GCCGCAC AGGCTCA GGATCTGA CCAAAGTG ACCCTCGC ATTCCCCA GCG	GGTCATCC ATGCAGTC GGAAAGC GACAGCG ACTTCACG ACAGCGAA CACTCTCA GCGCCGG GACATCCT GCGGGTA CGCCAGA ATGTGAGC GTTCAGCG TTGCC	GCGATCC GCACCGG ACCGATGA AGGAGTC GAGCCTG GTCCAGC GCCGGCT CGGCGAA AGCCGTCT GATCACCG TGGCGGC GCCGAGC TATATCGC CCGTTTCG GCAAGCC CGAGTCAC TGGAGG	gBlock sequence (Amplicon + extra nucleotides)

Supplementary Table 1. Description of target gene, accession number (if relevant), amplicon sequence and gBlocks® for each target assay.

Type	Identity	International collection	Local collection	Mean CT values					
				Pu10	Pu4	Pg2	Pg6	Pt1	Pt2
Blotch pathogens	*'Pseudomonas gingeri'*	LMG 5328	IPO3737	40.0	40.0	22.0	17.5	40.0	40.0
Blotch pathogens	*'Pseudomonas gingeri'*	LMG 5327 T	IPO3738	40.0	40.0	21.8	20.1	40.0	40.0
Blotch pathogens	*'Pseudomonas gingeri'*		IPO3756	40.0	40.0	21.7	20.2	40.0	40.0
Blotch pathogens	*'Pseudomonas gingeri'*		IPO3757	40.0	40.0	21.4	20.7	40.0	40.0
Blotch pathogens	*'Pseudomonas gingeri'*		IPO3767	40.0	40.0	20.6	19.1	40.0	40.0
Blotch pathogens	*'Pseudomonas gingeri'*		IPO3769	40.0	40.0	20.6	19.1	40.0	40.0
Blotch pathogens	*'Pseudomonas gingeri'*		IPO3776	40.0	40.0	21.1	19.3	40.0	40.0
Blotch pathogens	*'Pseudomonas gingeri'*		IPO3777	40.0	40.0	20.8	19.5	40.0	40.0
Blotch pathogens	*Pseudomonas tolaasii*	ATCC 51309		40.0	40.0	40.0	40.0	18.9	21.7
Blotch pathogens	*Pseudomonas tolaasii*	ATCC 51310		40.0	40.0	40.0	40.0	16.6	19.3
Blotch pathogens	*Pseudomonas tolaasii*	LMG 2342		40.0	40.0	40.0	40.0	18.9	21.7
Blotch pathogens	*Pseudomonas tolaasii*		IPO3741	40.0	40.0	40.0	40.0	17.0	20.0
Blotch pathogens	*Pseudomonas tolaasii*		IPO3742	40.0	40.0	40.0	40.0	17.2	20.5
Blotch pathogens	*Pseudomonas tolaasii*		IPO3743	40.0	40.0	40.0	40.0	17.6	20.7
Blotch pathogens	*Pseudomonas tolaasii*		IPO3744	40.0	40.0	40.0	40.0	18.0	21.1
Blotch pathogens	*Pseudomonas tolaasii*		IPO3746	40.0	40.0	40.0	40.0	19.7	22.7
Blotch pathogens	*Pseudomonas unknown*	LMG 2343	IPO3747	19.1	23.0	40.0	40.0	40.0	40.0
Blotch pathogens	*Pseudomonas unknown*		IPO3765	20.0	21.8	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas agarici*	LMG 2112 T	IPO3739	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas putida*	LMG 2257 T	IPO3752	40.0	35.0	40.0	36.9	40.0	40.0
Other mushroom associated Pseudomonas	*'Pseudomonas reactans'*	LMG 2338		40.0	40.0	40.0	40.0	38.8	40.0
Other mushroom associated Pseudomonas	*'Pseudomonas reactans'*		IPO3748	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*'Pseudomonas reactans'*	NCPPB 3149 T	IPO3749	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*'Pseudomonas reactans'*		IPO3750	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*'Pseudomonas reactans'*		IPO3751	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3754	37.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3753	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3774	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3775	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3778	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3779	40.0	40.0	35.0	33.6	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3780	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3781	40.0	40.0	40.0	40.0	40.0	40.0
Other mushroom associated Pseudomonas	*Pseudomonas* unknown sp		IPO3782	40.0	40.0	40.0	40.0	40.0	40.0

Supplementary Table 2A. Description of the strains that form the bacterial panel for evaluating the diagnostic specificity and sensitivity of the TaqMan™ assays. It describes the names and sources of reference strains from each blotch pathogen and other mushroom-associated *Pseudomonas*. It also describes their reaction (mean C_T) against each assay.

Type	Identity	Local Collection	Mean CT values					
			Pu10	Pu4	Pg2	Pg6	Pt1	Pt2
Plant pathogenic pseudomonas	*Pseudomonas fluorescens*	IPO2280	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas fluorescens*	IPO2281	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. apii*	IPO3351	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. apii*	IPO3352	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. lachrymans*	IPO143	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. lachrymans*	IPO520	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. morsprunorum*	IPO563	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. morsprunorum*	IPO568	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. persicae*	IPO451	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. persicae*	IPO3096	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. persicae*	IPO3346	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. persicae*	IPO3347	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. persicae*	IPO3348	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. persicae*	IPO3349	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. persicae*	IPO3350	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. persicae*	IPO3354	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. phaseolicola*	IPO548	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. pisi*	IPO518	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. pisi*	IPO1630	37.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. porri*	IPO1672	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. porri*	IPO3353	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. primulae*	IPO3097	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. primulae*	IPO3098	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. ribicola*	IPO3100	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. ribicola*	IPO3101	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. syringae*	IPO478	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. syringae*	IPO491	40.0	40.0	40.0	40.0	40.0	40.0
Plant pathogenic pseudomonas	*Pseudomonas syringae pv. viburni*	IPO3099	40.0	40.0	40.0	40.0	40.0	40.0

Supplementary Table 2B. Description of the strains that form the bacterial panel for evaluating the diagnostic specificity and sensitivity of the TaqMan™ assays. It describes the names and sources of plant pathogenic *Pseudomonas*. It also describes their reaction (mean C_T) against each assay.

Type	Identity	Local collection	Mean CT values					
			Pu10	Pu4	Pg2	Pg6	Pt1	Pt2
Other plant pathogens	*Agrobacterium tumefaciens*	IPO2135	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Bacillus caryophylli*	IPO1707	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Clavibacter michiganensis*	IPO3063	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Corynebacterium michiganense pv. sepedonicus*	IPO929	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Corynebacterium michiganensis pv. sepedonicus*	IPO1830	35.9	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Dickeya dadantii*	IPO597	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Dickeya dianthicola*	IPO502	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Dickeya dianthicola*	IPO775	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Dickeya dianthicola*	IPO846	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Dickeya zeae*	IPO648	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Dickeya zeae*	IPO650	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Dickeya zeae*	IPO824	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Erwinia amylovora*	IPO121	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Erwinia amylovora*	IPO212	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pantoae ananatis*	IPO3196	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pantoae citrea*	IPO3197	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pantoae punctata*	IPO3198	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pantoae terrea*	IPO3199	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pectobacterium atrosepticum*	IPO590	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pectobacterium carotovorum*	IPO1949	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pectobacterium carotovorum*	IPO2034	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pectobacterium carotovorum carotovorum*	IPO454	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Pectobacterium wasabiae*	IPO3536	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Rahnella aquaticus*	IPO2233	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Ralstonia*	IPO34	40.0	40.0	40.0	35.0	40.0	40.0
Other plant pathogens	*Ralstonia*	IPO35	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Ralstonia*	IPO37	36.5	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Ralstonia*	IPO39	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Rhodococcus fascians*	IPO1611	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Serratia plymuthica*	IPO3416	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Serratia plymuthica*	IPO3616	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Serratia sp.*	IPO257	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas campestris pv. campestris*	IPO3076	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas campestris pv. phaseoli*	IPO382	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas campestris pv. pruni*	IPO2263	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas euvesicatoria*	IPO3660	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas fragariae*	IPO3058	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas fragariae aardbei*	IPO3085	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas fragariae aardbei*	IPO3089	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas gardneri*	IPO3661	40.0	40.0	40.0	40.0	40.0	40.0
Other plant pathogens	*Xanthomonas perforans*	IPO3662	40.0	40.0	40.0	40.0	40.0	40.0
Negative control	MQ water		40.0	40.0	40.0	40.0	40.0	40.0

Supplementary Table 2C. Description of the strains that form the bacterial panel for evaluating the diagnostic specificity and sensitivity of the TaqMan™ assays. It describes the names and sources of reference strains of other plant pathogens. It also describes their reaction (mean C_T) against each assay.

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Chapter 4

Infection and population dynamics

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Abstract

Bacterial blotch is one of the most economically important diseases of button mushrooms. Knowledge on mechanisms of disease expression, inoculum thresholds and disease management is limited to the most-well known pathogen, *Pseudomonas tolaasii*. Recent outbreaks in Western Europe have been attributed to '*P. gingeri*' and *P. salomonii* for ginger and brown blotch, respectively, although information on their identity, infection dynamics and pathogenicity is largely lacking. In this study, the disease pressure in an experimental mushroom cultivation facility was evaluated for '*P. gingeri*' and *P. salomonii* over varying inoculation densities, casing soil types, environmental humidity and cultivation cycles. The pathogen population structures in the casing soils were simultaneously tracked across the cropping cycle using highly specific and sensitive Taqman™-qPCR assays. '*P. gingeri'* caused disease outbreaks at lower inoculum thresholds (10^4 cfu/g) in the soil than *P. salomonii* (10^5 cfu/g). Ginger blotch generically declined in later harvest cycles, although brown blotch did not. Casing soils were differentially suppressive to blotch diseases, based on their composition and supplementation. Endemic pathogen populations increased across the cultivation cycle although the inoculated pathogen populations were consistent between the 1st and 2nd flush. '*P. gingeri*' and *P. salomonii* had unique infection and population dynamics, that varied over soil types. Their endemic populations were also differently abundant in peat-based casing soils. This knowledge is essential to interpret diagnostic results from screening mushroom farms and design localized disease control strategies.

Introduction

Bacterial blotch is one of the most economically important diseases in the cultivation of the common button mushroom, *Agaricus bisporus* (Osdaghi et al., 2019; Soler-Rivas et al., 1999). Bacterial blotch is a group of diseases that cause rapid and superficial discoloration of the mushroom cap. This causes yield losses during mushroom cultivation due to reduced aesthetic market value and reduced postharvest shelf life (Fermor et al., 1991; Wells et al., 1996). Various *Pseudomonas spp.* from casing soil (a peat-based growing medium) are described to cause bacterial blotch on mushroom caps (Fletcher et al., 1989).

Of these, *Pseudomonas tolaasii* is the most well-known pathogen causing "brown blotch" (Tolaas, 1915). Its phenotypic variation, symptomatic disease expression, biosynthesis of the toxin tolaasin and genetic regulation have been well-studied (Grewal, Han, and Johnstone, 1995; Han, Johnstone, and Grewal, 1994; Rainey, Brodey, and Johnstone, 1993). However, the characterization, epidemiology and regulation of other known blotch pathogens such as *'P. gingeri', 'P. reactans', P. costantinii* and *P. fluorescens*, have received limited attention so far. Recent disease outbreaks in Western Europe led to the discovery of *P. salomonii, P. edaphica* and *P. yamanorum* as other brown blotch causing pathogens (Taparia, Krijger, Haynes, et al., 2020).

'P. gingeri' is one of the only known causative agents of "ginger blotch" (Wong et al., 1982). It produces ginger-coloured discolorations on mushroom caps, that lead to crop loss during the growth cycle and post-harvest (Wells et al., 1996). On King's B medium, *'P. gingeri'* produces mucoid colonies that are fluorescent under UV light. It is characterized by glucose assimilation on HL medium, poor hydrolysis of starch and gelatine, decomposition of distilled citrate, no production levan from sucrose, and is oxidase and catalase positive (Szumigaj-Tarnowska et al., 2010). Biochemically, it differs from brown blotch pathogens in the inability to produce levan and spectrum of carbon source utilization (Wells et al., 1996). *'P. gingeri'* also does not exude the toxin, tolaasin (Lee et al., 2002).

P. salomonii was first identified as a clove-borne pathogen of *'Café au lait'* disease on garlic, which is transmitted via planting material (Gardan et al., 2002; Jacques et al., 2009). It was recently identified as a "brown blotch" pathogen of button mushroom, *A. bisporus,* on the basis of genetic and phenotypic tests (Taparia et al., 2020b, 2020a). On King's B medium, *P. salomonii* forms small, semi-mucoid, opaque and non-fluorescent colonies, with morphological properties similar to that of *P. tolaasii* (Cutri et al., 1984). It is characterised biochemically by Hypersensitive Response on tobacco, hydrolysis of aesculin and gelatine, levan production, and

nitrate reduction and agglutination of a specific polyclonal antiserum (Gardan et al., 2002; Samson, 1982).

In this research, we focus on an aggressive strain of *'P. gingeri'* (IPO3777) and of *P. salomonii* (IPO3765), which were isolated from symptomatic mushroom tissue during a blotch outbreak of 2015 in the Netherlands (Taparia et al., 2020a). They cause bacterial blotch symptoms when inoculated on fresh mushroom caps and in casing soils. Highly specific and sensitive TaqMan™-qPCR assays were recently developed for the molecular detection of *'P. gingeri'* and *P. salomonii.* The assays can detect, 10 copies of pathogen DNA using gBlocks, 10^3 cells/ml of pathogen in water samples and 10^4 cells/g of pathogen in soil samples (Taparia et al., 2020b). In a large-scale sampling of mushroom farms in the Netherlands and Belgium, *'P. gingeri'* and *P. salomonii* were present on 78% and 42% of the blotched mushrooms respectively, indicating large local presence of both pathogens (Taparia et al., 2020b).

In this study, we report the disease prevalence and population dynamics of this newly discovered brown blotch pathogen, *P. salomonii* (IPO3765), in comparison to that of the ginger blotch pathogen, '*P. gingeri'* (IPO 3777), in cultivation experiments which mimic the conditions of commercial mushroom cropping systems. In addition, we studied the relationship between pathogen populations in the casing soil and the bacterial blotch prevalence in the crop using quantitative detection assays. We also explored the role of abiotic and biotic indicators such as environmental humidity and casing soil type on the blotch prevalence.

Materials and Methods

General set-up of cultivation bioassay

In an experimental mushroom cultivation facility (Unifarm, Wageningen University and Research), the conditions of commercial mushroom farms were mimicked. Plastic packaging boxes (dimensions of L*W*H 40x50x23cm) with closed sides and bottom, and a growing surface of $0.2m^2$ were used as mushroom beds. The experiment consisted of 128 boxes, arranged across 2 stands over 5 vertical layers in a randomized block design, with four replicates per treatment.

Two types of casing soils containing different types of peat and amendments were prepared (Supplementary Figure 1). Each box was filled with 8.5 kg of compost fully colonized with *A. bisporus* strain A15 (Sylvan). And 5 L of casing soil inoculated with 100 g per kg of phase III compost (a process called "CAC-ing") was applied on top. The growing chamber was allowed to vent, reducing the temperature from 24 ^oC to 18 ^oC by 1^oC per day until the first pinheads developed. The mushrooms were

allowed to grow further at 18 °C, 1100 ppm of CO_2 and two different conditions, 90% and 95% relative humidity (RH). Weight and numbers of healthy and diseased mushrooms harvested from each unit were registered daily across two cultivation cycles, called flushes. Disease prevalence was calculated as a percentage of the diseased harvest weight to the total harvest weight across all three flushes.

Inoculation of blotch pathogens

Effects of two blotch pathogens on disease pressure and productivity of the cultivation system were observed for a range of biotic and abiotic conditions (Table 1). Pathogenic strains of '*P. gingeri'* (IPO3777) and *P. salomonii* (IPO 3765) (Taparia et al., 2020b) were grown on King's B media (King et al., 1954) for 24 hours at 28 °C. The virulence of the plated isolates was re-tested in an *in-vitro* pathogenicity assay on fresh mushroom caps. Bacterial suspension of 10^8 cells/ml (OD_{600}= 0.1) was made from the pathogen cultures in sterile Ringer's solution (Thermo Fisher Scientific, Germany). For the bioassay, diluted pathogen suspensions were inoculated evenly by watering across the growing surface of the casing soil (after "CAC-ing") at densities of 10^4, 10^5 and 10^6 cfu/g of wet weight of soil. The pathogen suspensions used for inoculation were also dilution plated on King's B medium for colony counting. The negative control (NC) comprised of mushrooms beds supplemented with equivalent amount of Ringer's solution (Thermo Fisher Scientific, Germany).

Bioassay	Description
Experimental factors	
Casing soil type	Two different compositions of casing: soil *A,* soil *B*
Pathogen type	Species of pathogen: '*P. gingeri*', *P. salomonii*
Inoculation density	Inoculation density of pathogen: 0, 10^4, 10^5, 10^6 cfu/g of soil
Environmental humidity	Relative air humidity: 90%, 95%
Flush	Progressive cultivation cycle: 1st flush, 2nd flush
Measurements	
Blotch prevalence	Percentage of diseased harvest/Total harvest (w/w))
Pathogen population	Estimated density from qPCR, relative to *X. campestris (cells/g)*

Table 1. Experimental design of bioassay where blotch prevalence and pathogen populations were recorded over varying biotic and abiotic factors described below.

Soil sampling and DNA extraction

One gram of casing soil was sampled with from the topsoil with a spatula, from each box 4 days after "CAC-ing" (T0), pinhead formation of the 1st flush (T1) and pinhead

formation of the 2nd flush (T2), which respectively correspond to 0, 7 and 14 days post pathogen-inoculation. The mushroom pinheads were avoided during the soil sampling. 250 mg of the homogenized soil was used for DNA extraction using a Soil PowerMag DNA Extraction kit (Qiagen, Germany) according to manufacturer's protocol. A *Xanthomonas campestris* pv. *Campestris* (Xcc) suspension of 10^5 cells was added to each soil sample as an extraction control, before the DNA extraction. The soil DNA was quantified fluorometrically using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, United States) according to the manufacturer's protocol on the Infinite M200 PRO (Tecan, Switzerland) and then diluted to a concentration of 2 ng/µl.

Quantification of pathogen populations

Detection of bacterial DNA in soil samples was performed using the TaqMan™ probe technology, on a QuantStudio™ 12K Flex from Applied Biosystems (Thermo Fisher Scientific, United States). Assays Pg2 and Pg6 were used to detect *'P. gingeri'*, assays Pu4 and Pu10 were used to detect *P. salomonii,* and assay Xcc was used to detect the extraction control *Xanthomonas campestris* pv. *campestris*. The sequence of the primers and probes are described in Taparia et al. (2020b).

qPCR reactions were performed with 2 ng of soil DNA, according to previously reported protocol and reaction conditions (Taparia et al. 2020). A threshold cycle (C_T) value lower than 40 was considered a positive reaction (Supplementary Figure 2). Pathogen populations in the soil were quantified relative to known densities of *X. campestris* pv. *campestris* (Xcc). Fold change ratios were calculated from the C_T values by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Estimated pathogen populations in the soil were reported in cells/g, by multiplying the fold change ratio with inoculation density of Xcc.

Data analysis

Statistical analysis was performed on RStudio with R version 3.4.0 (The R Core Team, 2013). Threshold cycle (C_T) values from Taqman™-qPCR experiments were imported from QuantStudio Software v1.3 (Life Technologies). Bioassay and qPCR observations were organized using *tidyverse* (Wickham et al., 2019). The effect of casing soil type, pathogen type, inoculated pathogen density and harvest cycle on blotch prevalence and pathogen populations was studied using ANOVA and MANOVA (Chambers et al., 2017). Model assumptions such as normality of data and homogeneity of variance were tested using diagnostic plots and statistical tests (Fox et al., 2012). Pathogen population and blotch prevalence were plotted via *ggplot2* (Wickham et al., 2016). Error bars in all figures represent the Standard error of the mean, calculated over a sample size of 20 per treatment (pathogen strain,

pathogen density and soil type) for the bioassay data, and sample size of 27 per treatment for the qPCR data.

Results

Disease prevalence of ginger and brown blotch

In the cultivation bioassay, both pathogens caused visible symptoms that could be distinguished for quantification of blotch prevalence. *'P. gingeri'* produced ginger coloured discolorations that were spread over a large surface area whereas *P. salomonii* produced dark brown discolorations over multiple irregular spots, often accompanied by pitting (Figure 1). Bacterial blotch prevalence in the cropping system was significantly affected by the pathogen type, inoculated pathogen density, casing soil type, the harvest cycle (flush) and their interactions. Increased environmental humidity did not have a significant effect on bacterial blotch in the bioassay. The P values and estimates from the analysis of variance (ANOVA) are described in Table 2. They also met statistical assumptions on normal distribution and homogeneity of variance.

For the control mushroom beds, in the absence of inoculated pathogen, the mean ginger blotch and brown blotch prevalence were similar, at 2.9±0.8% and 2.3±0.7% respectively. In the inoculated mushroom beds, the mean bacterial blotch prevalence increased with inoculation density, but the effect was pathogen-specific (Figure 2). At the lowest inoculation density (10^4 cfu/g), the ginger blotch prevalence (14.3±1.8%) was much higher than the brown blotch prevalence (5.0±0.9%). Despite differences between the pathogens, their blotch prevalence was found to be similar at the higher inoculation densities. At the highest density (10^6 cfu/g), ginger and brown blotch prevalence were observed at 22.3±2.4% and 19.1±2.1% respectively.

The disease prevalence of the pathogens was different for the two casing soils tested (Figure 2). In the control mushroom beds, the inherent blotch prevalence of casing soil *A* (3.5±0.9%) was higher than that of casing soil *B* (1.7±0.5%). In the inoculated mushroom beds, the mean blotch prevalence in casing soil *A* and *B* increased to 11.0±1.1% and 18.8±1.1% respectively. When inoculated in casing soil *A, 'P. gingeri'* and *P. salomonii* had similar blotch prevalence, but in casing soil *B*, the mean ginger and brown blotch prevalence was 21.1±1.4% and 16.5±1.8% respectively.

Bacterial blotch prevalence also followed a temporal pattern across the cultivation cycle which was pathogen specific (Figure 3). In the absence of inoculated pathogen, the inherent blotch prevalence remained low and constant across multiple cultivation cycles. In the inoculated samples, the mean brown blotch prevalence was consistent across both the harvest cycles (flushes) at 14.7±1.8% and 13±1.7%, but the mean

ginger blotch prevalence declined strongly with consecutive harvest cycles, from 20.1±1.6% in the 1st flush to 11.9±1.4% in the 2nd flush.

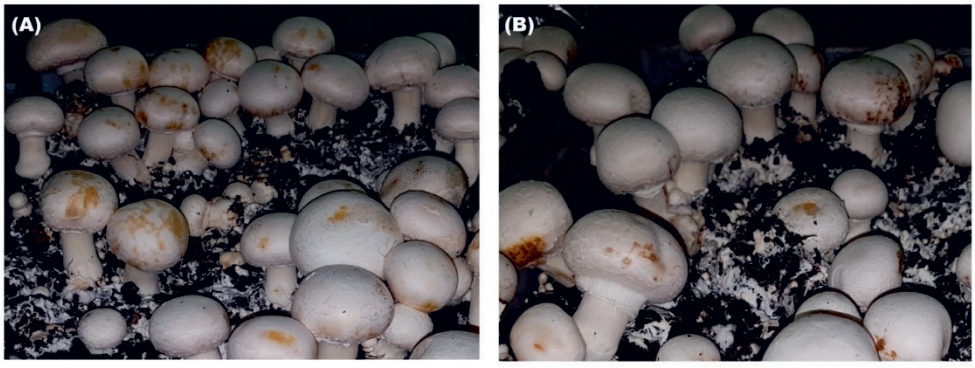

Figure 1. Bacterial blotch symptoms on *Agaricus bisporus* in a bioassay when inoculated with (A) *'P. gingeri'* isolate IPO3777 and B) *P. salomonii* isolate IPO3765, at a density of 10^6 cfu/g of casing soil.

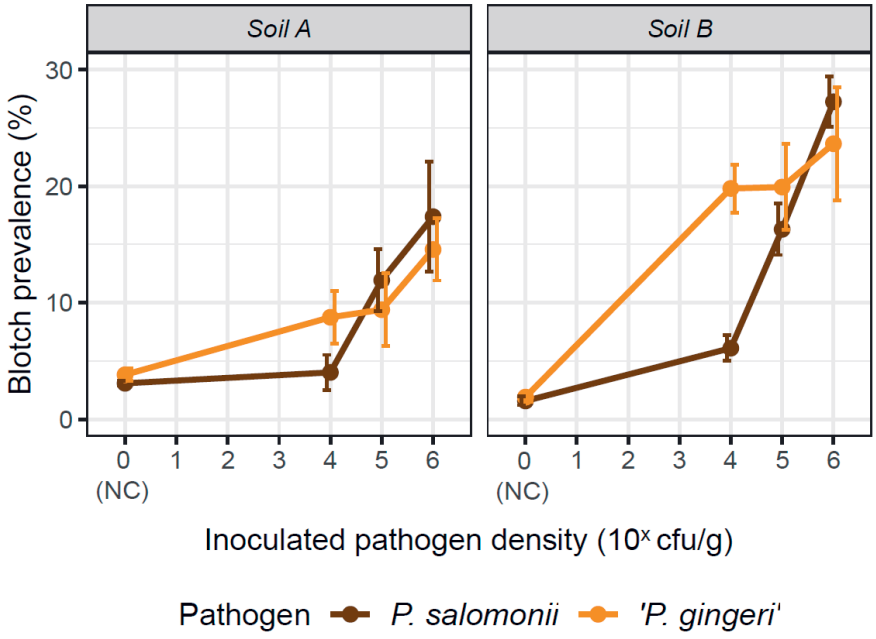

Figure 2. Effect of two different types of casing soils on the mean bacterial blotch prevalence of *'P. gingeri'* and *P. salomonii* on *Agaricus bisporus*, over varying inoculation densities.

Experimental factors	Df	Sum Sq	Mean Sq	F statistic	P (>F)	Sig
Inoculation density	1	1.34	1.34	140.504	0.00000	***
Pathogen type	1	0.09	0.09	9.436	0.00236	**
Flush	1	0.0521	0.0521	5.461	0.02024	*
Casing soil type	1	0.0448	0.0448	4.694	0.03123	*
Casing soil: Flush	1	0.0646	0.0646	6.769	0.00983	**
Casing soil: Inoculation density	1	0.0886	0.0886	9.29	0.00255	**
Pathogen type: Flush	1	0.0359	0.0359	3.763	0.05354	.
Residuals	248	2.3652	0.0095			

Table 2. Univariate analysis of variance (ANOVA) of bacterial blotch prevalence recorded in the bioassay. The degrees of freedom (Df), sum and mean squares (Sq), F test statistic, P values, significance codes (Sig) and residuals from the ANOVA are described.

'P. gingeri' populations						
Experimental factors	Df	Sum Sq	Mean Sq	F statistic	P (>F)	Sig
Time (flush)	2	202.3	101.1	6.18	0.00352	**
Inoculation of pathogen	1	983	983	60.057	0.00000	***
Inoculation density: Time	2	481.7	240.8	14.714	0.00001	***
Casing soil	1	7.2	7.2	0.442	0.50862	
Inoculation density: Casing soil	1	4.2	4.2	0.255	0.61513	
Residuals	64	1047.6	16.4			
P. salomonii populations						
Experimental factors	Df	Sum Sq	Mean Sq	F statistic	P (>F)	Sig
Casing soil	1	161.3	161.3	11.594	0.00115	**
Inoculation of pathogen	1	942.6	942.6	67.765	0.00000	***
Inoculation density: Casing soil	1	137.1	137.1	9.857	0.00256	**
Time (flush)	2	31.9	16	1.147	0.32388	
Inoculation density: Time	2	51.8	25.9	1.863	0.16358	
Residuals	64	890.3	13.9			

Table 3. Multivariate ANOVA of pathogen populations in the casing soil during the bioassay, based on Threshold cycle (C_T) values obtained from diagnostic Taqman™-qPCR assays. The degrees of freedom (Df), sum and mean squares (Sq), F test statistic, P values, significance codes (Sig) and residuals from the MANOVA are described.

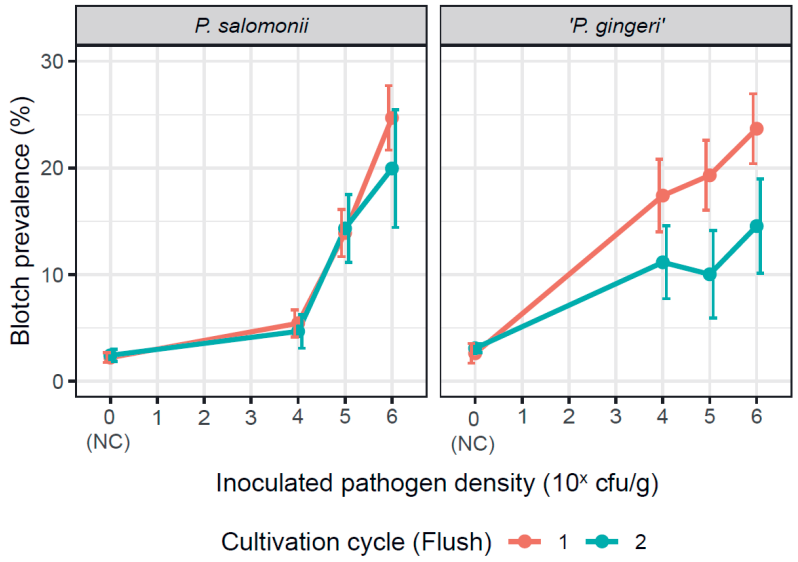

Figure 3. Effect of two consecutive harvest cycles (flushes) on the mean bacterial blotch prevalence of *'P. gingeri'* and *P. salomonii* on *Agaricus bisporus,* over varying inoculation densities.

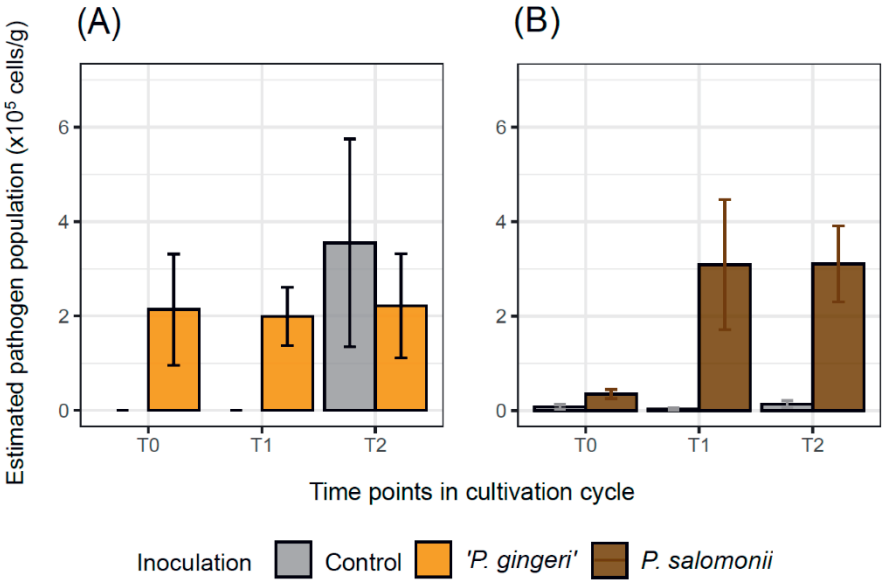

Figure 4. Bar plots describing the estimated pathogen populations of (A) *'P. gingeri'* and (B) *P. salomonii* in control and inoculated mushroom beds across the cultivation

cycle, derived from Taqman^{TM}-qPCR assays on casing soil samples from the bioassay.

Population dynamics of '*P. gingeri*' and *P. salomonii*

Quantitative TaqMan^{TM}-qPCR assays, *Pg2* and *Pg6*, were used to track '*P. gingeri'* populations in the casing soil over three time-points in the cultivation cycle, T0, T1 and T2. Casing soils samples were similarly quantified for presence of *P. salomonii,* using the diagnostic TaqMan^{TM}-qPCR assays *Pu4* and *Pu10*. The estimated populations of '*P. gingeri'* and *P. salomonii* in the soil was significantly affected by inoculation of pathogen, casing soil type and time point of the cultivation cycle (flush). The P values and estimates from the analysis of variance (ANOVA) are described in Table 3. It also met statistical assumptions on normal distribution and homogeneity of variance.

For control mushroom beds, in the absence of inoculated pathogen, *'P. gingeri'* populations were undetectable at T0 and T1. However, at T2, the estimated pathogen population of *'P. gingeri'* in the soil was 3.5±2.2 x10^5 cells/g (Figure 4A. When the mushroom beds were inoculated with 10^6 cells/g of *'P. gingeri'*, the pathogen was detectable immediately. Within the inoculated soils, the estimated pathogen population ranged between 3.1x10^3 and 1.2x10^8 cells/g during the flushes and indicates spatial heterogeneity in the pathogen populations recovered from the casing soil. Despite this high variation, in the soils inoculated with 10^6 cfu/g of pathogen, the estimated population remained largely consistent across T0, T1 and T2 at 2.1±1.1 x10^5, 1.9±0.6 x10^5 and 2.2±1.1 x10^5 cells/g respectively.

In control mushroom beds, that were uninoculated, the estimated population of *P. salomonii* in the soil at T0, was 9.6±7.4 x10^3 and 6.2±6.1 x10^3 cells/g for casing soils *A* and *B* respectively. When *P. salomonii* was inoculated in the mushroom beds using a density of 10^6 cfu/g, the estimated pathogen population increased to 3.0±1.9 x10^4 and 4±0.7 x10^4 cells/g in casing soil *A* and *B* respectively. At T0, T1 and T2, the estimated pathogen population in inoculated soils increased to 3.5±0.9 x10^4, 3.1±1.3 x10^5 and 3.1±0.8 x10^5 cells/g respectively (Figure 4B). The variation in the estimated pathogen population of the inoculated samples ranged between 6.6x10^3 and 4.8x10^6 cells/g during the cultivation cycle. It reaffirms the non-homogenous distribution of pathogen population structures in the soil despite uniform application of inoculum.

Discussion

Soil inoculum threshold

In this study, the disease prevalence and population dynamics of a newly characterized brown blotch pathogen, *P. salomonii,* which is widespread in the Dutch mushroom cultivation ecosystem, have been compared to a ginger blotch pathogen, *'P. gingeri'*, which is responsible for major losses in Western Europe. In cultivation bioassays, *P. salomonii* and *'P. gingeri'* demonstrated different inoculum thresholds for blotch outbreaks. *'P. gingeri'* caused blotch outbreaks at an inoculum density of 10^4 cfu/g of casing soil, while *P. salomonii* caused similar levels of blotch at a ten-fold higher inoculation density of 10^5 cfu/g in the soil.

So far, information on inoculum thresholds of blotch pathogens have been largely limited to studies on the mushroom cap surface. *'P. gingeri'* on mushroom caps has been shown to produce symptoms at a density of 1.7 $x10^7$ cells/cap (Soler-Rivas et al., 2000). For brown blotch from *P. tolaasii*, a minimum inoculum density of 7.7 $x10^4$ cells/cm^2 of mushroom cap surface for symptomatic disease expression was reported (Nair and Fahy, 1972). However, these pathogen detection methods were based solely on plating and colony morphology.

In other bioassays, where *P. tolaasii* was inoculated to the casing soil, a density of $2x10^6$ cells/g of soil caused a similar blotch incidence of 25-30% as in our studies (Grewal, 1991). Application of *P. tolaasii* at 20 cells/cm^2 led to 60% blotch prevalence (Wong and Preece, 1982). However, increasing *P. tolaasii* densities to a higher level such as 10^{10} cells/g, led to a complete crop loss, with >90% blotch prevalence (Royse and Wuest, 1980). So far, only little information is available on threshold levels for other blotch causing *Pseudomonas* species in casing soil.

Role of abiotic and biotic factors

The disease prevalence of the pathogens differed between the two harvest cycles (flushes), when inoculated to the casing soils. Despite reduced ginger blotch prevalence in the second flush, '*P. gingeri'* populations recovered from the soil remain consistent between T1 and T2. In personal communication, Dutch mushroom growers shared similar observations about the reduced susceptibility of later flushes to ginger blotch. This natural suppression of ginger blotch, and its microbial ecology needs further exploration. Interestingly, mushrooms from the second flush have also been reported to have fewer yellow discolorations from bruising than those of the first flush (Burton and Noble, 1993). However, this was attributed to the post-harvest physiology of the mushroom. In case of brown blotch, both flushes were equally

susceptible. This was also reported in bioassays for brown blotch caused by *P. tolaasii* (Royse and Wuest, 1980).

The bacterial blotch prevalence of both pathogens also differed between the two types of casing soils. In uninoculated samples, the blotch prevalence of casing soil *A* was higher than that of soil *B*. The endemic pathogen populations in casing soil *A* were also higher. However, when the pathogens were inoculated, casing soil *B* had slightly higher brown bacterial blotch prevalence compared to soil *A,* which was also reflected in the increased *P. salomonii* populations recovered from casing soil *B*. The ginger blotch prevalence of soil *B* was significantly higher than that of soil *A,* although the estimated density of *'P. gingeri'* was not significantly different between both the casing soil types. It is also interesting to note that at higher pathogen populations, casing soil *A* is more suppressive to bacterial blotch than soil *B*.

Increased environmental humidity did not cause higher bacterial blotch for either pathogens in the bioassay. Blotch development is known to be induced at high environmental humidity due to condensation of water on the mushroom caps, although this effect is rather inconsistent between reports (Navarro, Gea, and González, 2018; Soler-Rivas et al., 1999; Wong and Preece, 1982). The absence of proper ventilation and temperature fluxes influence the rate of condensation and, consequently, blotch development (Wuest, 1971). Thus, in a well-ventilated climate room as in our bioassay chamber, where the air temperature does not change abruptly, blotch prevalence could be unaffected by a higher relative humidity.

Prediction of blotch prevalence depends on a variety of abiotic and biotic factors. It is essential to understand the impact and interaction between these factors as the risk for disease outbreak does not depend only on pathogen populations in the soil but also on casing soil type, harvest cycle (flush), cultivar or variety, harvesting system, environmental conditions during cultivation and interactions with other micro-organisms in the casing soil and on mushroom surfaces.

Pathogen population structures

Quantitative and specific diagnostic qPCRs confirmed the endemic presence of both bacterial blotch pathogens in the casing soil. In uninoculated mushroom beds, *P. salomonii* was consistently detected at T0 in casing soil *A,* which only contained peat from *Friedrichsveen*. In casing soil *B*, which is largely composed of peat from *Holriede,* but also partially *Friedrichsveen*, *P. salomonii* was detected in only a few of the samples. Hence, the endemic population of brown blotch pathogens largely differs between the casing soils based on their peat source. This information supports earlier findings, where raw peat (before it was mixed into a casing soil) was tested positive for presence of multiple blotch pathogens (Taparia et al., 2020b; Wong and Preece, 1980). It confirms the role of peat-based casing soils as a source

of introduction for brown blotch pathogens in mushroom farms. However, other sources of introduction, such as the compost, cannot be excluded.

Both pathogens were detected at higher densities in inoculated soils with high blotch prevalence, than that of uninoculated soils. Similar findings were reported for brown blotch bioassays, where *P. tolaasii* was detected in both, healthy and diseased beds using plating-based methods. With plating on non-selective media, *P. tolaasii* populations were observed at 10^7-10^8 cells/g in diseased beds and 0-10^7 cells/g in healthy beds (Nair and Fahy, 1972). On the basis of colony morphology, *P. tolaasii* was reported at much lower densities in diseased beds (Wong and Preece, 1980). However, the lack of specificity in detection methods suggests that these pathogen populations were likely overestimated. Use of diagnostic qPCRs in this study, enabled detection of *P. salomonii* at much lower densities in the casing soil. In the case of *'P. gingeri'*, the ginger blotch pathogen was undetectable in uninoculated casing soil until the second flush, which suggests that endemic populations of *'P. gingeri'* in these casing soils, if present, are even lower than that of *P. salomonii*.

TaqMan™-qPCR data confirmed the increase of endemic pathogen populations in uninoculated casing soils from T0 to T2 for both *'P. gingeri'* and *'P. salomonii'* during the cultivation cycle. This could be due to the increased nutrient availability in consecutive cultivation cycles, in the form of *A. bisporus* mycelium that grows through the casing soil (Godfrey, 2003). Endemic populations of the brown blotch pathogen, *P. tolaasii*, in the casing soil were previously reported to increase with mycelial colonization of the casing soil (Wong and Preece, 1982). Its chemotaxis towards and attachment to the *A. bisporus* mycelium is also well documented (Grewal and Rainey, 1991; Rainey, 1991). *P. tolaasii* populations have also been reported to increase across the cultivation cycle from $5.6x10^6$ to $45x10^6$ cells/g, although the detection was based on colony morphology only (Royse and Wuest, 1980).

The large variation in the estimated pathogen populations from casing soil samples at T1 and T2, indicates a rather non-homogenous spatial distribution of the pathogens in the casing soils, despite uniform inoculation on the casing surface. This is not unexpected, since the pathogen populations are known to migrate towards food sources such as *A. bisporus* mycelium (Grewal and Rainey, 1991). An extensive sampling strategy can allow more precise and representative measurement of pathogen populations. However, direct translation of estimated pathogen populations in the soil to disease prevalence is not possible. The relationship between pathogen populations and disease outbreaks needs further exploration.

Conclusions

The two blotch causing pathogens demonstrate different infection dynamics in cultivation experiments. *P. salomonii* is able to cause severe brown blotch symptoms on mushroom caps of *A. bisporus* when inoculated in peat-based soils at densities of 10^5 cfu/g or more, while the inoculum thresholds for *'P. gingeri'* are lower, at 10^4 cfu/g. Their blotch prevalence also varies according to the cultivation cycle (flush) and type of casing soil used. Endemic populations of *P. salomonii* in the casing soil are higher than of *'P. gingeri'*, and they vary in density between the casing soil types. *'P. gingeri'* populations in the soil remain undetectable until later in the cultivation cycle. This knowledge on disease prevalence and population dynamics of bacterial blotch pathogens can form a basis for interpreting diagnostic results generated from screening raw materials and processes involved in the mushroom-cropping chain. It generates a better understanding of the microbial ecology of the pathogens in the mushroom cropping cycle, with implications in the design of localized disease control strategies.

Author contributions

TT, EH and JW designed the experiments. TT, ED, MK, and MH performed the bioassay. TT and MH did the molecular analyses. TT, WB and JW wrote the first draft of the manuscript. All authors contributed to subsequent manuscript revision, read and approved the submitted version.

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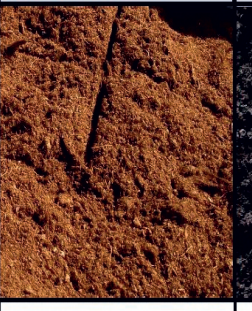
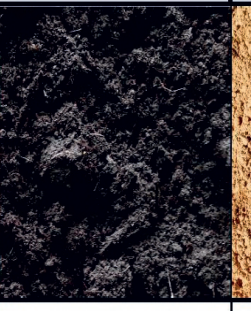
	Peat type			Amendments	
Name	Friedrichsveen	Holriede	Baltic peat	Garden earth	Sugar beet lime
Raw materials					
Soil A	100%				7%
Soil B	20%	75%	2.5%	2.5%	7%

Supplementary Figure 1. Description and illustration of the raw materials used in casing soil composition (by volume), including peat sources and amendments.

Casing soil	Pathogen type	Pathogen density	*'P. gingeri'*			*P. salomonii*		
			T0	T1	T2	T0	T1	T2
A	Control	0 cfu/g	40.0	40.0	29.6	35.0	40.0	29.4
A	Control	0 cfu/g	40.0	40.0	40.0	33.9	40.0	30.6
B	Control	0 cfu/g	40.0	40.0	29.2	40.0	40.0	30.6
B	Control	0 cfu/g	40.0	40.0	34.7	40.0	35.2	40.0
B	Control	0 cfu/g	40.0	40.0	40.0	28.7	29.1	40.0
B	Control	0 cfu/g	40.0	40.0	40.0	35.6	40.0	40.0
B	Control	0 cfu/g	40.0	40.0	22.9	40.0	40.0	40.0
B	Control	0 cfu/g	40.0	40.0	40.0	40.0	40.0	40.0
A	*'P. gingeri'*	10^6 cfu/g	25.6	27.8	35.0	33.3	40.0	40.0
A	*'P. gingeri'*	10^6 cfu/g	24.8	25.7	40.0	29.1	33.5	30.1
A	*'P. gingeri'*	10^6 cfu/g	30.9	31.2	19.7	34.0	40.0	32.9
A	*'P. gingeri'*	10^6 cfu/g	28.5	27.1	40.0	32.5	34.2	27.8
B	*'P. gingeri'*	10^6 cfu/g	28.3	29.5	27.0	40.0	40.0	40.0
B	*'P. gingeri'*	10^6 cfu/g	33.5	26.0	25.4	40.0	40.0	40.0
B	*'P. gingeri'*	10^6 cfu/g	30.0	28.2	40.0	40.0	40.0	40.0
B	*'P. gingeri'*	10^6 cfu/g	28.6	26.6	27.3	40.0	34.3	40.0
A	*P. salomonii*	10^6 cfu/g	40.0	40.0	27.8	28.2	28.3	26.5
A	*P. salomonii*	10^6 cfu/g	40.0	40.0	25.4	31.6	27.3	26.4
A	*P. salomonii*	10^6 cfu/g	40.0	40.0	40.0	29.9	26.5	26.0
A	*P. salomonii*	10^6 cfu/g	40.0	40.0	31.6	40.0	40.0	26.0
B	*P. salomonii*	10^6 cfu/g	40.0	40.0	40.0	30.2	26.2	25.1
B	*P. salomonii*	10^6 cfu/g	40.0	40.0	24.4	28.8	24.4	31.9
B	*P. salomonii*	10^6 cfu/g	40.0	40.0	28.3	29.3	26.2	28.7
B	*P. salomonii*	10^6 cfu/g	40.0	40.0	27.5	29.4	29.5	26.5

Supplementary Figure 2. Threshold cycle (C_T) values from diagnostic Taqman™-qPCRs performed on casing soil DNA during the cultivation cycle in the bioassay. Higher C_T value indicates lower density of pathogen in the soil. Higher C_T value indicates lower density of the pathogen in the soil. C_T>=40 indicates no detection of the pathogen.

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Chapter 5

Casing soil suppressiveness to blotch

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Abstract

Shifts in the soil microbiome during continuous monoculture cropping can coincide with increased suppressiveness against soil-borne diseases, best studied in the take all decline of wheat. Here we report a comparable phenomenon for bacterial blotch of mushrooms, caused by '*Pseudomonas gingeri*', where ginger blotch incidence decreases during consecutive cycles of mushroom cultivation. We explored the infection dynamics of blotch during consecutive cultivation cycles for different casing soil mixtures. We also observed the population dynamics of the pathogen in these casing soils. In addition, the composition of the casing soil microbiome was compared between blotch suppressive and conducive soils. Finally, we studied the transferability of blotch suppressiveness. A consistent decline of bacterial blotch was observed for two consecutive cultivation cycles of mushroom cropping, across ten casing soil mixtures composed of different peat sources and supplements. Blotch suppression occurred without reduction of pathogen populations in the casing soils. Aqueous extracts of suppressive soils reduced blotch incidence in conducive casing soils. Changes in the microbial community composition of the casing soils reflected pathogen invasion, pathogen establishment and disease suppression, in addition to the expected temporal changes across the cultivation cycles. Specific bacterial genera associated with soil suppressiveness to blotch, such as, *Pseudomonas* sp., *Dyadobacter* sp., *Pedobacter* sp., and *Flavobacterium* sp. also clustered together in microbial co-occurrence networks. Their importance in bacterial blotch decline has been further discussed.

Introduction

Management of soil-borne diseases via suppressive soils remains a promising yet significant challenge in intensive cropping systems (Gómez Expósito et al., 2017; Stirling et al., 2016). Studies from diverse soils have attributed disease suppression to the composition and the activity of the soil microbial community (Benítez and Gardener, 2009; Penton et al., 2014; Poudel et al., 2016). Changes in the soil microbiome during consecutive growth of crop monocultures that are susceptible to the disease, or inoculation of high pathogen populations into the soil (Hornby, 1983; Weller et al., 2002) are known to induce soil suppression of fungal pathogens. Best-known examples of such induced specific suppression include take-all decline in continuous monocultures of cereals (wheat, barley) and root rot decline in sugar beet monocultures (Raaijmakers and Mazzola, 2016; Weller et al., 2002). With current developments in -omics technologies, it is now increasingly possible to characterize disease suppressive soils, as well as their microbial composition, networks and activity (Klein et al., 2013; Mendes et al., 2011). A suitable sector for improving our understanding of disease suppressive soils is controlled horticulture, which happens in closed cropping systems, independently of agricultural land and where growth conditions are constantly regulated (Kulak et al., 2013; Marcelis et al., 2007).

Button mushroom, *Agaricus bisporus*, is grown in such climate-controlled indoor farms, on a bed of pasteurized compost over a layer of peat-based casing soil, under standardized cultivation conditions. Bacterial blotch is one of its most important soil-borne diseases, economically and globally (Soler-Rivas et al., 1999). Caused by pathogenic *Pseudomonas* species prevalent in mushroom beds (Fletcher et al., 1989), the disease is responsible for up to 50% yield loss during cultivation, and reduced shelf life post-harvest (Olivier et al., 1978; Wells et al., 1996). Despite being studied for over a century, only few management strategies exist for the control of blotch in button mushrooms (Elphinstone and Noble, 2018; Tolaas, 1915). Management via regulation of environmental conditions in mushroom farms has been widely explored, although the results are inconsistent between studies (Beyer, 2005; Nair and Bradley, 1980; Navarro et al., 2018; Sinden, 1971; Wong and Preece, 1982).

Biological control of bacterial blotch has been explored via nutrient competition, active antagonism, pathogen inactivation, predation, bacteriocin production and phage therapy (Osdaghi et al., 2019). Despite many successful reports under laboratory conditions, no commercial biocontrol products are commercially available. Blotch management is made additionally challenging due to the fact that the genus *Pseudomonas* is involved in both disease expression (Fletcher et al., 1989; Godfrey, 2003) and in formation of the mushroom pinheads (Rainey, 1989). In mushroom beds, fluorescent pseudomonads represent 10% of the culturable cells, which was

found to be 10^7-10^8 cells/g of compost. Generally, pseudomonads increase up to 50%-90% of the total of 10^9 culturable cells during the harvest cycles (Samson et al., 1987). Recently, development of soil suppressiveness to bacterial blotch was reported to occur between consecutive cultivation cycles in mushroom cropping systems. These findings indicate that the disease continues to decline across consecutive harvest cycles even though pathogen populations do not (Taparia et al., 2020a).

Studies on disease suppression of soil-borne plant pathogens have highlighted the importance of exploring the dynamics of the soil microbiome, and better understanding the role of the microbiome in the pathogen-soil-host nexus. The aim of this study was to advance our knowledge on blotch suppressive soils for possible use in disease management strategies. To do so, we explored soil suppression of blotch over consecutive harvest cycles in different casing soil mixtures. We compared the bacterial and fungal fractions of the microbiome of conducive and suppressive soils. We also recorded pathogen population dynamics across the cultivation cycles and pathogen inoculum thresholds in the soil for blotch outbreaks. Finally, we performed soil microbial extract transfers from disease suppressive soils into conducive soils, to study the transferability of blotch suppressiveness. This knowledge is not only instrumental for the development of blotch management strategies, it also improves our fundamental understanding of induced disease suppressiveness in soils.

Materials and Methods

General set-up of cultivation bioassays

Bacterial blotch suppressiveness in mushroom casing soils was studied in an experimental mushroom cultivation facility (Unifarm, Wageningen University and Research) under conditions that resemble that of conventional mushroom farms. Cultivation setup and growing conditions are described in Taparia et al., 2020a. Each experiment comprised of four replicates per treatment. The weight of healthy and diseased mushrooms was registered across three cultivation cycles, called flushes. Disease prevalence (%) was calculated as the proportion of diseased harvest over the total harvest. Mushroom with symptoms unrelated to that of ginger blotch, were categorized as healthy. Mushrooms were categorized according to the intensity of disease symptoms on the cap surface into four severity classes: healthy, mild, moderate and severe symptoms (Supplementary Figure 1) (Geels, 1995). Disease severity (%) was calculated as the proportion of mushrooms belonging to each severity class (Tajalipour et al., 2014).

In bioassay 1, ginger blotch suppression was explored over a range of *'Pseudomonas gingeri'* inoculum densities, in different casing soil mixtures, across two cultivation cycles (flushes) and in three independent replicate experiments (Table 1). Ten types of casing soil were prepared based on peat from three geographic sources in Germany: *Friedrichsveen*, *Holriede*, and *Wiesmoor*, and at two depths: surface peat and deep-dug peat. They were supplemented with Baltic peat, garden peat and sugar beet lime during preparation of casing soil (Supplementary Figure 2). Between the replicate experiments the raw materials were stockpiled and stored at 4° C, up to a period of 12 months. Bioassay 2 was performed to examine if soil suppressiveness to ginger blotch could be transferred to fresh casing soil (Table 2). Suppressive soils were identified as those having less than 5% disease prevalence at the highest inoculated pathogen density, i.e., soils from the second and third harvest cycle. An aqueous soil extract was made from mixed samples of suppressive soils from bioassay 1. The extract was inoculated with and without *'P. gingeri'* in fresh casing soil. Bioassay 2 contained four treatments: casing soil supplemented with (i) water, (ii) soil extract (iii) pathogen or (iv) pathogen + soil extract.

Microbial inoculations on casing soil

For bioassay 1, a pathogenic isolate of *'P. gingeri'*, IPO3777, was inoculated on the surface of each casing soil at densities of 10^3, 10^4, 10^5 colony-forming units (cfu) per gram of soil, according to the protocol described in Taparia et al., 2020a. Controls were inoculated with water instead of a pathogen suspension. Pathogen-inoculated soils with less than 5% blotch prevalence were defined as suppressive soils. For bioassay 2, 100 g of suppressive soils in the 2nd flush of bioassay 1, were sampled and suspended in 1L of sterile Ringers solution. The soil suspension was filtered through cheesecloth first and then sequentially vacuum-filtered through 270 mm and 80 mm membranes. Out of the resulting filtrate, 200 ml was used as soil extract and inoculated on the surface of fresh casing soil (2.5 L), together with 10^6 cfu/g of *'P. gingeri'* suspension. After inoculation, a series of dilutions of the remaining soil extract was plated on King's B media, to quantify the fluorescent pseudomonads population.

Soil sampling and DNA extraction

One gram of casing soil was sampled with a spatula, from one spot per replicate, at the surface of the casing soil during "CAC-ing" (T0), pinhead formation during the 1st flush (T1), pinhead formation during the 2nd flush (T2) and pinhead formation during the 3rd flush (T3). Mycelium adhering to mushrooms was avoided during soil sampling. Subsequently, 250 mg of the homogenized soil was used for DNA extraction using a Soil PowerMag DNA Extraction kit (Qiagen, Germany) according

to the manufacturer's protocol. A suspension of 10^5 cfu of *Xanthomonas campestris* pv. *campestris* was added to each soil sample as an extraction control. The soil DNA was quantified fluorometrically using a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol on the Infinite M200 PRO (Tecan, Switzerland) and then diluted to a concentration of 2 ng/µl.

Factors	Description
Soil inoculum	Inoculation density of *'P. gingeri'*: *0, 10^3, 10^4, 10^5 cfu/g of soil*
Harvest cycles	Consecutive cultivation cycles: *1st* flush, 2nd flush
Casing soil	Ten casing soil mixtures varying in peat origin, peat depth and supplementation (Supplementary Figure 1).
Storage period	Length of storage period of casing soil between three repetitive experiments (months): *0, 5, 12*
Block	Replicates in randomized block design in all three experiments: *3*
Measurements	Description
Disease prevalence	Proportion of diseased harvest to total harvest
Disease severity	Proportion of mushrooms for each symptom severity class
Pathogen populations	*'P. gingeri'* abundance in soil via quantitative TaqMan-qPCRs (cells/g)

Table 1. Experimental design of bioassay 1

Treatments	Description
Control	Inoculation with tap water
Extract	Inoculation with microbial extract made from suppressive soils
Pathogen	Inoculation with *'P. gingeri'* at 10^6 cfu/g of casing soil
Pathogen + extract	Simultaneous inoculation with pathogen and microbial soil extract
Block	Replicates in randomized block design: *1,2,3*
Measurement	Description
Disease prevalence	Proportion of diseased harvest to healthy harvest

Table 2. Experimental design of bioassay 2

Quantification of pathogen populations

Detection of bacterial DNA in soil samples was performed using the TaqMan™ probe technology, on a QuantStudio™ 12K Flex from Applied Biosystems (Thermo Fisher Scientific, USA). Previously developed qPCR assays Pg2 and Pg6 were used to detect *'P. gingeri'* and assay Xcc was used to detect the extraction and amplification control, *X. campestris* (Taparia et al., 2020b). The sequence of the primers and probes are described in Taparia et al., 2020b. Quantitative PCR reactions were

performed with 2 ng of soil DNA, according to previously reported protocol and reaction conditions (Taparia et al., 2020b). Pathogen populations in the soil were quantified relative to known densities of *X. campestris*, as described in Taparia et al., 2020b.

In-vitro pathogenicity cap test

'Pseudomonas gingeri' was cultured in King's B medium (King et al., 1954) at 25 °C for 24 h. Healthy mushrooms with similarly sized cap surfaces (~4 cm in diameter) were harvested from the first and second flushes. They were placed on damp filter paper and inoculated with 20 µl of aqueous *'P. gingeri'* suspension of 10^6 cfu per ml, and tested in replicates of three. The mushrooms were incubated under high humidity conditions for 72 h at 20 °C. The development of blotch symptoms on the cap surface was observed visually and photographed. Symptoms on the mushroom caps were scored, from 0 to 3, with the ascending numbers referring to non-pathogenic (healthy), mild, moderate, and severe symptoms for bacterial blotch. Negative controls consisted of non-inoculated mushroom caps and mushroom caps inoculated with sterile water.

Sequencing of the soil microbiome

Microbial community composition of the soil was determined from targeted sequencing of the V3-V4 regions of the bacterial 16S rRNA gene and the fungal ITS2 gene (Supplementary Table 1). Target regions were amplified via PCR using gene-specific primers (10 µM), Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, USA) (1x), reaction buffer (1x), dNTP (5mM), 6 ng of template DNA and nuclease-free water up to 30 µl. Reaction conditions for 16S and ITS PCRs included a hot start of 98 °C for 30s, followed by 20 and 25 cycles respectively, of 98 °C for 10s, 55 °C for 30s, 72 °C for 30s and a final extension at 72 °C for 2 min, on a Veriti Thermo Cycler from Applied Biosystems (Thermo Fisher Scientific, USA). PCRs were performed with randomized plate design in replicates of three. Replicate PCR products were pooled and purified with AMPure XP beads (Beckman Coulter, USA). Library construction was performed with Illumina Nextera XT Index kit (Illumina, USA) and KAPA HiFi Hotstart ReadyMix (Roche, Switzerland). The quality and quantity of the libraries were evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). The libraries for 16S and ITS2 respectively, were sequenced on Illumina MiSeq using MiSeq v2 or v3 reagents (Illumina, USA) to generate 250 or 300bp paired-end reads that overlap.

Raw sequences were split according to unique barcode combinations per sample and subsequently analysed with QIIME2 (Bolyen et al., 2019), version qiime2-2020.2. For the ITS2 reads, cutadapt (Martin, 2011) was used as a plugin in QIIME2

to remove primers and possible read-through into opposite primers or adapters. For all data, Dada2 (Callahan et al., 2016) was used to run specific quality control and filtering, merging of paired-end reads and chimera filtering, resulting in a set of unique sequences and an abundance table of amplicon sequence variants (ASVs) (Callahan et al., 2017). Very low abundant ASVs with total abundance below 10 were removed from both datasets. Taxonomy was assigned to the representative unique sequences for each ASV with Naive Bayes classifiers (Bokulich et al., 2018; Pedregosa et al., 2011). The 16S classifier was pretrained on extracted 16S V3-V4 region of the Silva 16S/18S database release 132 (Quast et al., 2012). The ITS2 classifier was pretrained on full length ITS of the Unite database version 8.0, for all Eukaryotes, with dynamic use of clustering thresholds (Nilsson et al., 2019). ASV tables were additionally filtered based on the taxonomy assignments. ASVs that were classified as plant chloroplast or mitochondria were removed, as well as ASVs that lacked assignment at Phylum level. All raw sequences for soil fungi and bacteria were deposited in NCBI under BioProject numbers PRJNA657168 and PRJNA657276 respectively.

Statistical data analyses

The statistical analyses were performed on RStudio with R version 3.4.0 (Team, 2013). Bioassay data was transformed using *tidyverse* (Wickham et al., 2019). Analysis of variance, generalized linear regression and zero-inflated beta regression was performed using packages, *glm* and *betareg* (Cribari-Neto and Zeileis, 2009). Widely applicable information criteria (WAIC) and adjusted R squared values were compared to arrive at a final minimally adequate model (Johnson and Omland, 2004). Model assumption on normality of data and homogeneity of residuals were verified using diagnostic plots and statistical tests (Fox et al., 2012). Sequence data transformation, downstream analysis of ASV tables and compositional graphics were made with packages *vegan* (Oksanen et al., 2007) and *phyloseq* (McMurdie and Holmes, 2013). Core microbiome analysis was performed using *microbiome* (Lahti et al., 2017). Co-occurrence network analysis was performed using packages, *igraph* (Csardi and Nepusz, 2006) and *speic-easi* (Kurtz et al., 2015).

Results

Ginger blotch declined across consecutive cultivation cycles

The disease pressure for ginger blotch in the mushroom cropping system, was determined during two consecutive harvest cycles (flushes) in three independent replicate experiments. In the 1st flushes ginger blotch prevalence (Figure 1A) and blotch severity (Figure 1B) of blotch symptoms strongly increased with the inoculation density of *'P. gingeri'* in the casing soil. However, in the 2nd flushes only

limited effects were observed with increasing inoculation density. In mushroom beds inoculated with *'P. gingeri'*, the mean blotch prevalence declined from 15.3% in the 1st flush to 2.5% in the 2nd flush, and the mean blotch severity also reduced in a similar fashion. In mock-inoculated mushroom beds, blotch prevalence and blotch severity remained very low but consistent between both flushes at 0.8% and 0.3% respectively.

Similar patterns in temporal decline of ginger blotch were observed for all ten different casing soil mixtures (Supplementary Table 2). Longer storage periods of casing soil led to an increase in the overall ginger blotch prevalence ($P= 2x10^{-16}$) and severity ($P= 2x10^{-16}$) of the cropping system (Supplementary Table 2 and Supplementary Table 3). However, this increase in storage period of the raw materials used in casing soil preparation also led to a decrease in the blotch decline in the 2nd flush. To test the difference in susceptibility of mushroom caps from the first and second flush to the pathogen, an *in-vitro* pathogenicity cap test was performed. Freshly harvested mushroom caps from both flushes showed similar blotch symptoms, when inoculated with two strains of *'P. gingeri'* (Supplementary Figure 3).

Endemic and inoculated pathogens had different population dynamics

Endemic and inoculated *'P. gingeri'* populations had unique temporal dynamics ($P=2x10^{-16}$) across the cultivation cycle when tracked via diagnostic Taqman™ assays (Supplementary Table 4). Endemic pathogen populations in uninoculated mushroom beds were undetectable at the beginning of the experiment (T0), but they increased steeply from an estimated $1.8x10^3$ cells/g in the first flush (T1) to $1.5x10^5$ cells/g in the second flush (T2) (Figure 2A). The overall abundance of *'P. gingeri'* was higher in pathogen-inoculated mushroom beds. Inoculated pathogen populations in the casing soil, continued to increase ($P=7x10^{-7}$) between the consecutive flushes, in the three experiments (Figure 2A), despite the decline in disease prevalence. Among the inoculated mushroom beds, *'P. gingeri'* populations increased from $7.5x10^5$ cells/g in the first flush to $2.5x10^6$ cells/g in the second flush. This increase varied with the initial inoculum density of the pathogen in the soil ($P=3x10^{-4}$) (Figure 2B). At densities of 10^3 and 10^4 cells/g, population dynamics of *'P. gingeri'* in inoculated mushroom beds were similar, and the pathogen densities in the soil also remained consistent between the first and second flush. At an inoculum density of 10^5 cells/g, the pathogen populations increased 100x between the flushes. Estimated densities of both endemic and inoculated pathogens differed significantly between casing soil mixtures (P= 0.0007), and the temporal dynamics of the pathogen populations also varied between casing soils (P=0.008) (Supplementary Table 4).

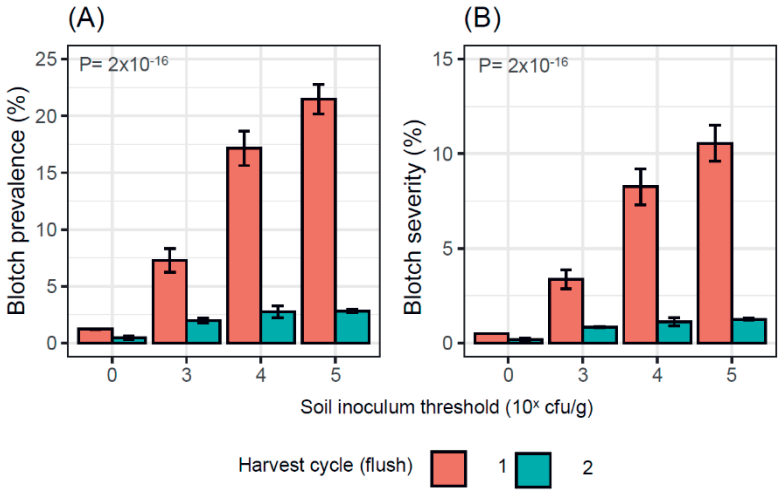

Figure 1. Temporal decline in ginger blotch between the first and second harvest cycles (flushes) in pathogen-inoculated casing soil from bioassay 1. Shown are: (A) disease prevalence and (B) disease severity in the cropping system.

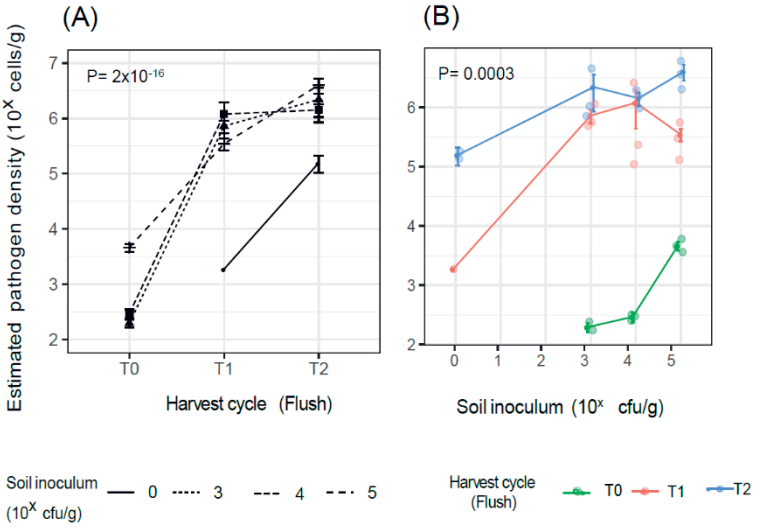

Figure 2. qPCR-based diagnostics of *'P. gingeri'* densities in the mushroom beds. Shown are endemic and inoculated pathogen populations varying over (A) timepoints in the cropping cycle and (B) soil inoculum densities.

Blotch suppressiveness was partially transferable via soil extract

An aqueous microbial extract made from mixed casing soils in the 2nd flush of bioassay 1, was found to moderately suppress ginger blotch when inoculated in fresh casing soils in bioassay 2 (Figure 3). Control mushroom beds with no microbial inoculation had a ginger blotch prevalence of 4.8%. When *'P. gingeri'* was inoculated on the soil at a density of 10^6 cells/g, a blotch prevalence of 51.4% was observed. Addition of a microbial extract made from blotch suppressive soils in previous experiments, together with the inoculated pathogen, reduced the ginger blotch to 34.5% (P=0.0304), suggesting that blotch suppressiveness is transferable. However, mushroom beds inoculated with only microbial extract, in the absence of additional pathogen, also had a blotch prevalence of 15.8%, suggesting that former pathogen populations were also carried over.

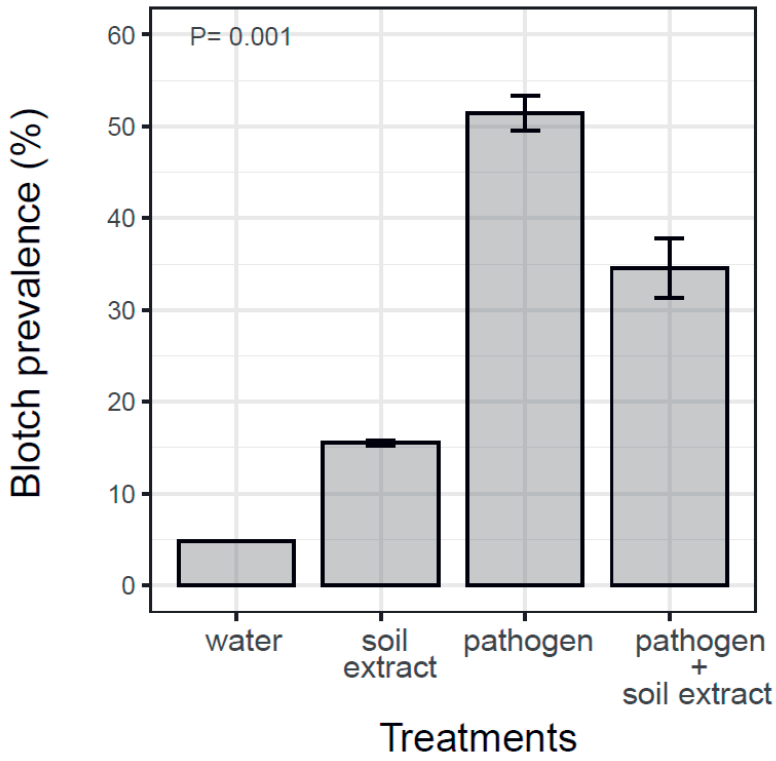

Figure 3. Transferability of ginger blotch decline via microbial extract from suppressive soils to conducive soils, performed in bioassay 2.

Soil microbiome reflects pathogen invasion and establishment

The total bacterial microbiome of casing soils comprised of 10,860 amplicon sequence variants (ASVs) or taxa originating from 42 casing soil samples. The fungal microbiome of the casing soils was less diverse and comprised of only 294 taxa. Both the bacterial and fungal fraction of the microbiome of the casing soil underwent significant temporal changes due to pathogen inoculation, consecutive harvest cycles and casing soil storage period based on non-metric multidimensional scaling analyses (Figure 4A and 4B). The bacterial community composition of the casing soil varied significantly between mock-inoculated and pathogen-inoculated mushroom beds (R^2= 0.022, P= 0.023), based on a PERMANOVA. Overall species richness calculated from inverse Simpson (P= 0.831) and Shannon (P= 0.787) diversity indices, were unaffected by pathogen inoculation. The effect of pathogen inoculation was observed until the end of the experiment, independent of temporal changes across the cultivation cycle, as many taxa were differentially abundant between mock-inoculated and pathogen-inoculated casing soils, across the first and the second flush (Figure 4C). The soil fungal community composition was unaffected by pathogen inoculation. Species richness of the casing soil bacteria also reduced significantly during the year-long storage period between the replicate experiments (P=0.0161).

Specific bacteria and fungi are associated with blotch decline

The alpha diversity of the bacterial fraction of the microbiome varied across different time points in the cultivation cycle (P= $4.1x10^{-6}$). Soils from the second and third harvest cycles (T2 and T3) had higher species richness than that of the first harvest, as indicated by inverse Simpson (P= $4.1x10^{-6}$) and Shannon (P=0.0003) diversity indices (Figure 5A). The bacterial profiles of casing soils from the consecutive flushes also clustered differently from each other, on the basis of Bray-Curtis distances, and had significantly (R^2= 0.435, P=0.001) different beta diversity (Figure 4A). The community composition of the fungal fraction of the microbiome did not vary significantly (P= 0.051) across the harvest cycles based on Bray-Curtis distances, and soils from consecutive flushes did not cluster differently from each other (Figure 4B). More than 200 bacterial taxa that are potentially associated with ginger blotch suppression were identified. These contribute to 70% of the overall differences in beta-diversity between soils from the first and second flush (Supplementary Figure 4). Two fungal taxa, *Saitozyma podzlica* (P= 0.039) and *Trichoderma sp.* (P= 0.029) were found to be associated with ginger blotch decline. Differential abundance between suppressive and conducive soils was also significant for 26 bacterial genera (P<0.05, -2> fold change >2) after correcting for multiple comparisons (Figure 5B). Several of these genera also form clusters in co-occurrence networks of casing soil

microbiome (Supplementary Figure 5), indicating that they are likely to interact with or influence other members of the microbiome.

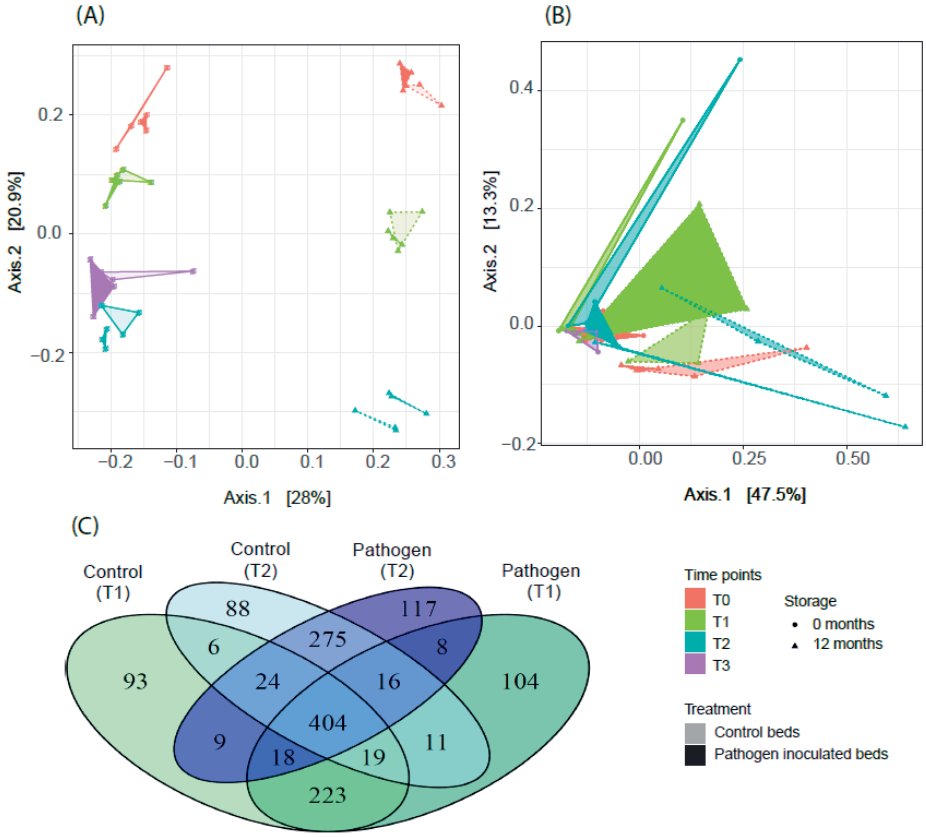

Figure 4. Principal component analyses showing the variation in composition of casing soil microbiomes for (A) bacteria and (B) fungi as affected by pathogen inoculation, time points during cultivation cycles, and storage period of casing soil. (C) Differential presence of bacterial taxa (ASVs) between the cultivation cycles due to pathogen inoculation are shown as a Venn diagram.

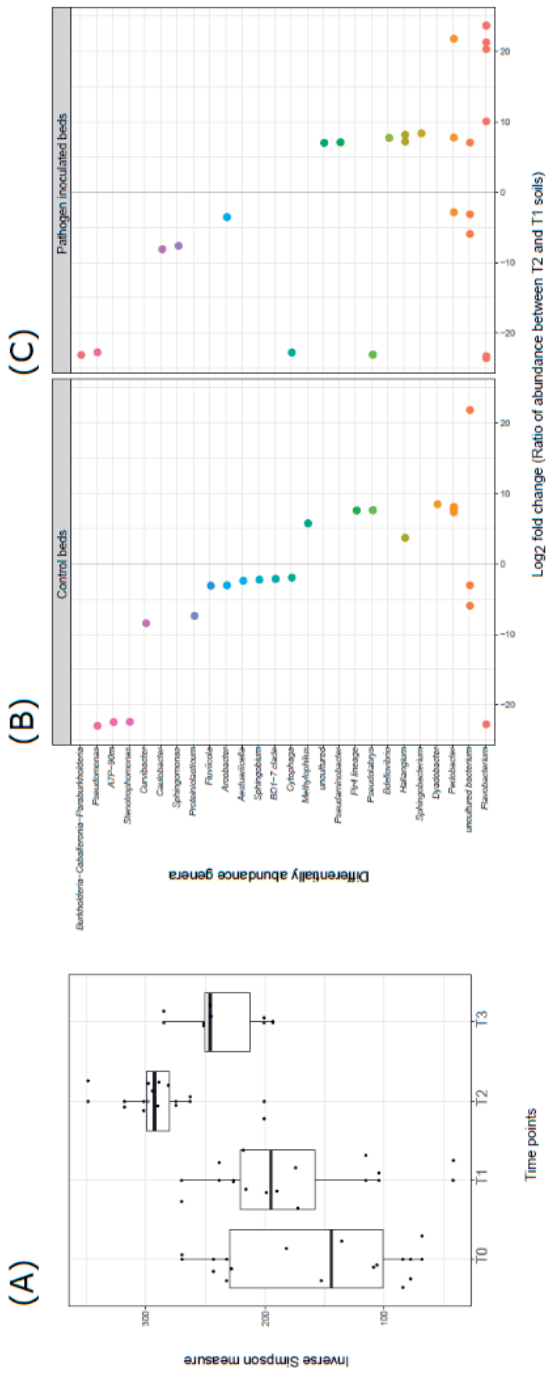

Figure 5. Changes in casing soil microbiome between conducive (T1) and suppressive (T2) soils are visualized as (A) species richness based on Inverse Simpson diversity index. Differentially abundant genera between T1 and T2 with fold-change greater than 2, are described in (B) mock-inoculated beds and (C) pathogen-inoculated beds.

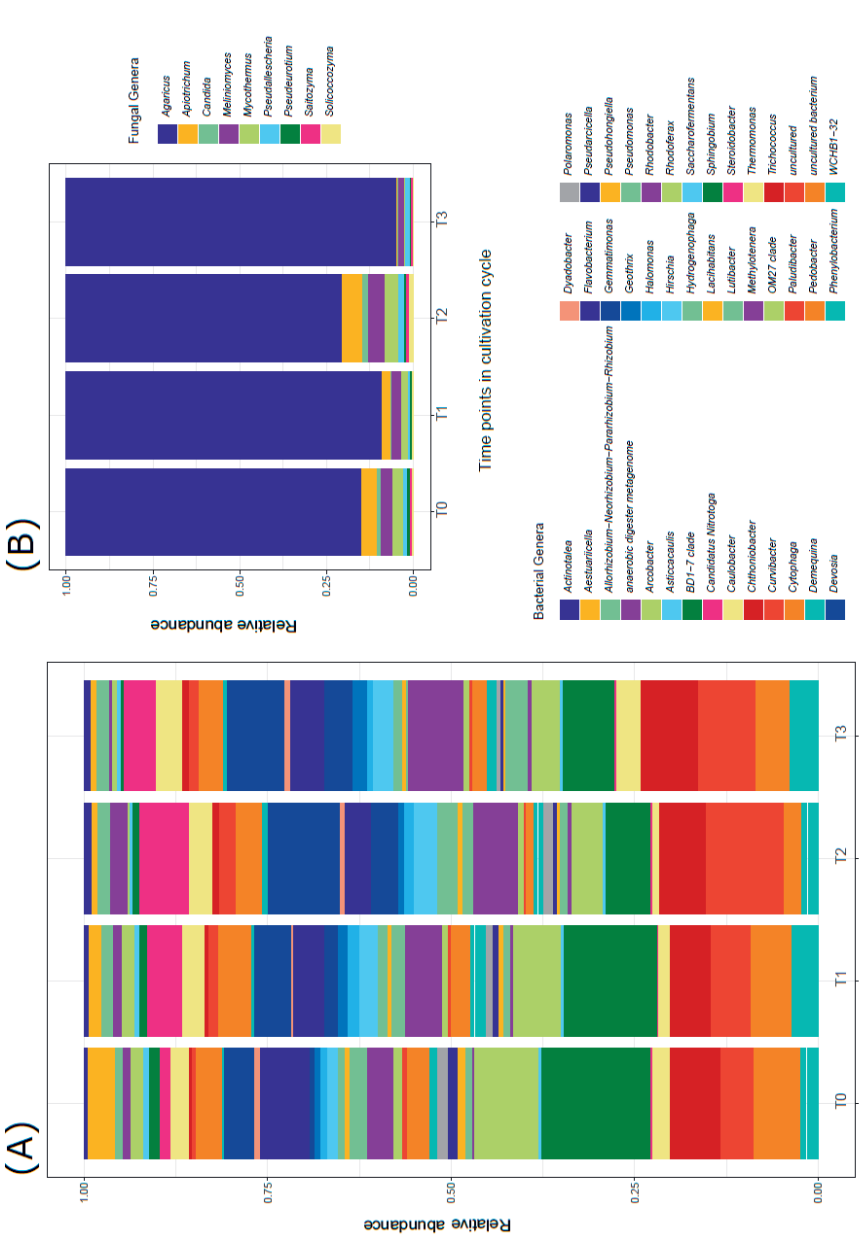

Figure 6. Bar plots representing core microbiome of casing soil across all treatments. Shown are: (A) bacterial and (B) fungal genera, which are present in 99% of the soils above a detection limit of 1%.

The core players of the casing soil microbiome are stable

The core microbiome of the casing soil comprised of 69 bacterial genera and 9 fungal genera (Figure 6A and 6B), that were present in 99% of the soil samples above a detection threshold of 1%. Genera from the core microbiome were relatively conserved between the treatments. Their relative abundances differed between consecutive harvest cycles (R^2= 0.435, P=0.001) and storage period (R^2=0.194, P= 0.001), but they were not influenced by pathogen inoculation (P= 0.056), according to PERMANOVA. The core bacterial community was largely dominated by Proteobacteria. Other phyla in decreasing order of relative abundance included, *Bacteroidetes, Firmicutes, Gemmatimonadetes, Verrucomicrobia, Chloroflexi, Actinobacteria, Epsilonbacteraeota, Acidobacteria* and *Patescibacteria* (Figure 6A). Relative abundances of the core fungal genera differed between storage periods (R^2=0.215, P= 0.001), but not due to pathogen inoculation (P=0.516) or consecutive harvest cycles (P=0.061). The core fungal community in the casing soil was dominated by *A. bisporus,* although it's relative abundance in the casing soil did not increase with consecutive harvest cycles. Other fungal genera, in decreasing order of abundance, included *Apiotrichum, Meliniomyces, Mycothermus, Pseudallescheria, Candida, Pseudeurotium, Solicoccozyma* and *Saitozyma* (Figure 6B).

Discussion

Temporal soil suppressiveness to ginger blotch

A consistent temporal decline in ginger blotch prevalence and severity was observed across consecutive harvest cycles (flushes) for ten different casing soil compositions, in three independent experiments. This reduction in disease pressure cannot be attributed to declining pathogen populations, since there was in fact a relative increase in pathogen populations across consecutive flushes. Similar results were obtained during a preliminary investigation of the pathogen densities in casing soils across multiple flushes (Taparia et al., 2020a). Mushroom caps from the second flush can have fewer yellow discolorations from physical bruising than those of the first flush, due to the post-harvest physiology of the mushroom (Burton and Noble, 1993). However, in our *in-vitro* pathogenicity assay, mushroom caps from first and second flush had the same symptom development and susceptibility to ginger blotch, when tested against two different strains of *'P. gingeri'*. This indicates that the reduced blotch prevalence and severity in the later flushes cannot be attributed to differences in post-harvest physiology or the development of induced resistance in *A. bisporus*. This is in line with findings of another study, where infection of mushrooms with dry bubble pathogen, *Lecanicillium fungicola*, failed to induce systemic resistance in *A. bisporus* (Berendsen et al., 2013).

Filtered soil extract from the second flush, when inoculated into fresh casing soil, led to significantly reduced ginger blotch prevalence. This indicates that the temporal decline in ginger blotch may be attributed to the development of soil suppressiveness to *'P. gingeri'*, and this blotch suppressiveness can be partially transferred to fresh casing soil via a soil microbial extract. Transferability is an important characteristic of biological soil suppressiveness (Baker and Cook, 1974) and has been proven for several soil-borne plant pathogens (Westphal and Becker, 2000). Soil suppressiveness to bacterial blotch decreased across the three repetitions of the experiment. Since, the conditions of the experimental farm were strictly regulated, and did not differ between experiments, this reduced soil suppression may be due to changes in the soil physico-chemical characteristics, or activity and composition of soil microorganisms during the storage period between the experiments. However, it is also possible that this effect is related to differences in the compost microbiomes between the replicates, as freshly prepared compost is required for mushroom cultivation, meaning that different batches of compost were used for the repetition experiments.

Remarkably, similar disease suppression patterns over continuous cultivation cycles, have been observed for plant crops grown in monoculture. Take-all disease caused by soil-borne fungus *Gaeumannomyes* in wheat or barley monocultures, is known to decrease across consecutive harvest cycles, after the onset of disease, a phenomenon known as take-all decline (Schreiner et al., 2010). This suppression is commonly ascribed to the increase of certain antibiotic-producing *Pseudomonas* species (de Souza et al., 2003), but several other rhizosphere bacteria are also speculated to be involved (Sanguin et al., 2009). Another example relates to the continuous cultivation of sugar beet seedlings on soils infested with *Rhizoctonia solani*, which also undergo a temporal decline of root rot (Expósito, 2017). This has been attributed to increased abundance of *Lysobacter* sp. (Gómez Expósito et al., 2015). It is also important to note, that in case of take-all decline, pathogen populations decline over continuous cultivation (Lebreton et al., 2004), but this is not the case for blotch suppression. Hence, different mechanisms may be involved in the decline of ginger blotch in mushroom cultivation. It can be speculated that inhibition of virulence factors such as those controlled by quorum sensing can be responsible, as have been observed for other *Pseudomonas* species (Adonizio et al., 2008; El-Mowafy et al., 2014; Vandeputte et al., 2011). The temporal scale also varies significantly between the cultivation systems, as wheat and barley grow over 7-8 months, and mushroom cropping cycles typically last 4-6 weeks. Despite important differences between the cropping systems and organisms involved, the study of blotch suppressiveness in mushrooms cultivation can elucidate some aspects of disease suppressiveness in plant cropping systems.

Disease suppressive microbiomes, dynamics and targets

The overall bacterial profile of the casing soil was dynamic and varied temporally during the cropping cycle, while the core microbiome remained relatively stable. Changes at the genus level were marginal, although many taxa (ASVs) were differentially abundant across cropping cycles, indicating that more differences are expected at lower taxonomic levels. This is also supported by earlier investigations where the bacterial community of the casing soil changed with the growth of *Agaricus* mycelium into the casing soil along the cropping cycle (Pecchia et al., 2014), although the overall composition remained relatively stable (Carrasco et al., 2019). The mycelium from *A. bisporus* is known to migrate upwards from the compost and colonize the casing soil during the cultivation cycle. However, the relative abundance of *A. bisporus* in the casing soil microbiome did not increase across the cropping cycle. Given that very few fungal genera were detected in the casing soil microbiome, it can be speculated that the absolute abundance of fungi is quite low in peat, and *A. bisporus* was predominantly present from T0 itself, due to inoculation of the casing soil with small amounts of compost by a process called “CAC-ing”. This can also be an artefact of the soil sampling technique, which ensured that bulk soil was collected instead of soil with mycelium adhering to it.

In the current experiments, pathogen inoculation on the casing soil led to an additional shift in the soil microbiome, the effect of which lasted throughout the cultivation cycle. This can be compared with known changes in the rhizosphere microbiome that occur due to pathogen invasion. For instance, invasion by the bacterial plant pathogen *Ralstonia solanacearum*, led to simplification of soil microbiome networks (Wei et al., 2018), whereas inoculation of fungal pathogen *Rhizoctonia solani* coincided with increase of specific bacterial taxa in the soil such as, *Burkholderiaceae*, *Sphingobacteriaceae* and *Sphingomonadaceae* (Chapelle et al., 2016). These changes can also be indirectly mediated by exudate patterns of the plant host itself (Gu et al., 2016). It is speculated that brown blotch pathogen, *P. tolaasii*, can potentially inhibit the growth of other soil microorganisms (Martins et al., 2020), based on the broad-spectrum volatile organic compounds it produces (Lo Cantore et al., 2015). This could be the reason why abundant genera for both the 1st flush and 2nd flush varied between mock-inoculated and pathogen-inoculated mushroom beds. However, it is also important to note that the overall species richness did not vary significantly between mock-inoculated and pathogen-inoculated mushroom beds. On the other hand, blotch suppressive soils of the second flush were found to be more diverse than conducive soils from the first flush. The shift in abundant genera between the 1st and 2nd flush of pathogen-inoculated casing soils could contribute to blotch suppressiveness, as disease suppression is known to be induced by soil-borne pathogens (Alabouvette et al., 1996).

More than 200 taxa were found to be associated with blotch suppression, based on either high relative abundance in 2nd flush soils or high differential abundance from conducive casing soils. Differentially abundant genera between 1st and 2nd flush were specific for pathogen-inoculated mushroom beds. Organisms of interest, that were associated with blotch decline include *Burkholderia* sp., *Metylophilus* sp., *Pir4* lineage, *Pseudomonas* sp., *Pseudaminobacter* sp., *Pseudolabrys* sp., *Bdellovibrio* sp., *Haliangium* sp., *Sphingobacterium* sp., *Dyadobacter* sp., *Pedobacter* sp., and *Flavobacterium* sp. A few of these genera have been previously identified for their biocontrol activity against brown blotch. Non-pathogenic *Pseudomonas* species recovered from casing soil, such as, *P. putida, 'P. reactans', P. fluorescens*, have been shown to significantly reduce blotch incidence in laboratory and commercial trails (Aslani et al., 2018; Fermor et al., 1991; Tajalipour et al., 2014). *Sphingobacterium multivorum* and a *Pedobacter* sp., isolated from wild Agaricales, were also found to strongly suppress brown blotch development *in-vitro* by deactivating the blotch toxin (Tsukamoto et al., 2002). *Burkholderia cepacia* was found to be antagonist to brown blotch pathogen, *P. tolaasii* (Nair, 1974). Predatory bacteria, *Bdellovibrio bacteriovorus*, was found to be effective against bacterial blotch, however, due to its broad host range, it could also affect the endemic beneficial microbiome of mushroom beds (Saxon et al., 2014).

Pathogen-soil interactions for blotch suppression

For successful use of biocontrol agents, a repeated application is often required as populations of the beneficials in soil decline due to competition for space and nutrients, presence of inherent predators and specific physico-chemical features of the soil matrix. Experimental trials of blotch biocontrol agents tested *in-vitro*, resulted in reduced effectiveness *in-vivo* and an inconsistent field performance (Tsukamoto et al., 1998). In this context, blotch management via stable changes of the endemic soil microbiome presents better opportunities, as it preserves community level interactions, and these microorganisms are better adapted to the local conditions (Ab Rahman et al., 2018; Kim et al., 2011; Mueller and Sachs, 2015). Many of the genera that were found to be associated with blotch suppression, also co-occurred with each other in the microbial soil networks. For example, *Burkholderiaceae* interacted with *Pedobacter*, *Flavobacterium* and *Brevundimonas. Dyadobacter* also co-occurred with *Brevundimonas* and *Pedobacter.* Additionally, *Pedobacter* was directly linked with *Pseudomonas.* Co-habitation of *Pedobacter* with other bacterial strains in *in-vitro* assays, was found to suppress several plant fungal pathogens due to interspecific interactions (De Boer et al., 2007). These microbes would hence make good targets for further research on blotch management.

Organic amendments have also been shown to increase disease suppressiveness characteristics of soils in both agricultural and horticultural crops (Postma et al., 2014, 2003; Postma and Schilder, 2015). In our experiments, long-term storage of

the casing soil, between replicate experiments, reduced the bacterial diversity and significantly changed the composition of the casing soil microbiome, which coincided with reduced soil suppressiveness against ginger blotch. The invasion resistance of a microbial community is often attributed to increased diversity, as the endemic microbiome can better exploit available resources (van Elsas et al., 2012), however, this is complicated by the fact that addition of resources can also temporarily make the community more susceptible to invasion (Mallon et al., 2015). This suggests that the casing soil microbiome can also be steered by abiotic factors towards blotch suppression. Better knowledge of the pre-treatment, composition, supplementation, physical and chemical characteristics of the casing soils, is thus required to induce suppressiveness against soil-borne pathogens. A combined effort using a mixture of both biotic (microbial inoculants) and abiotic (soil amendments) factors to induce stable changes in the soil microbiome seems promising for the management of blotch diseases.

Conclusions

This study reported induced soil suppressiveness to bacterial blotch between consecutive cultivation cycles of mushroom cropping systems. Blotch suppression was induced due to high pathogen populations in the soils, and was observed consistently across ten casing soil mixtures. The soil suppressiveness was found to be partially transferable to conducive soils via an aqueous soil extract. This phenomenon is remarkably similar to that of take-all decline in wheat and barley monocultures, although the blotch pathogen populations in the soil continued to increase despite declining disease pressure. Pathogen invasion, pathogen establishment and disease suppression were also reflected in the community composition of the casing soil microbiome. The latter underwent significant changes due to pathogen inoculation. Shifts in the microbiome composition continued to occur until the end of the cropping cycle, in addition to the expected temporal changes across the cultivation cycle. Specific bacterial and fungal genera were associated with blotch suppression and they also clustered with each other in co-occurrence microbial networks. A few of which, have been previously explored as individual blotch control agents. These results generate several fundamental insights on pathogen-induced disease suppression, and also provide a platform for further applied studies on the design of blotch management strategies.

Several questions about ginger blotch suppression remain unexplored, including the functional activity of the casing soil and possible mechanisms behind this induced suppressiveness. The effect of heat treatment on the casing soil or the transferred soil extract, would also verify the role of the microbiome in mediating this soil suppressiveness. The casing soil composition, it’s pre-treatment and physico-chemical characteristics deserves further attention, given that blotch suppression

varied with storage period of casing soils between replicate experiments. Many potential targets to steer the casing soil microbiome towards blotch suppression have been identified. Further research should test the effect of these, as either individual strains or microbial mixtures on soil suppressiveness to blotch. Their survival and efficacy in the casing soil, together with its effect on the composition and activity of the soil microbiome, would require investigation. This knowledge has important implications in successful biological control of bacterial blotch in mushroom cultivation.

Author contributions

TT, EH, and JW designed the experiments. TT, EH, MK, and MH performed the bioassay. TT, MH and EN did the molecular analyses. TT, WB and JW wrote the manuscript. All authors contributed to subsequent manuscript revision, read and approved the submitted version.

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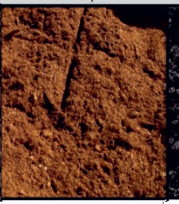
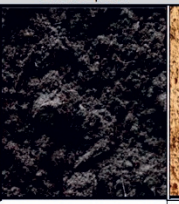
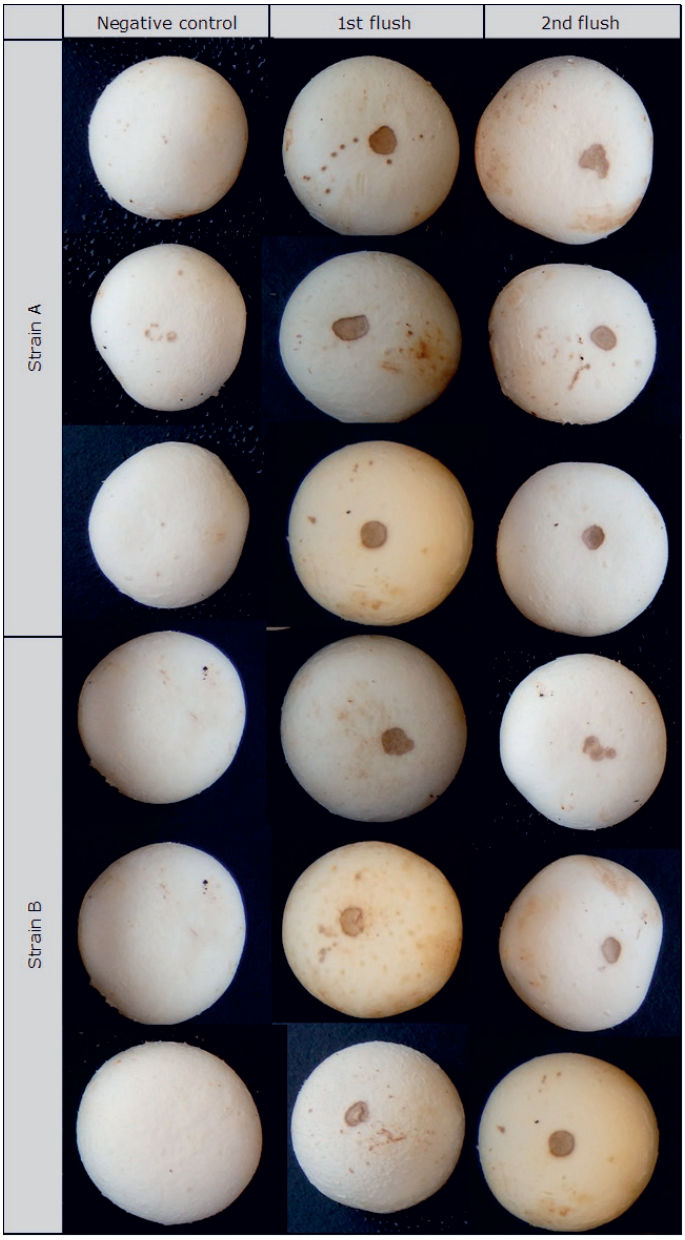
Supplementary Figures

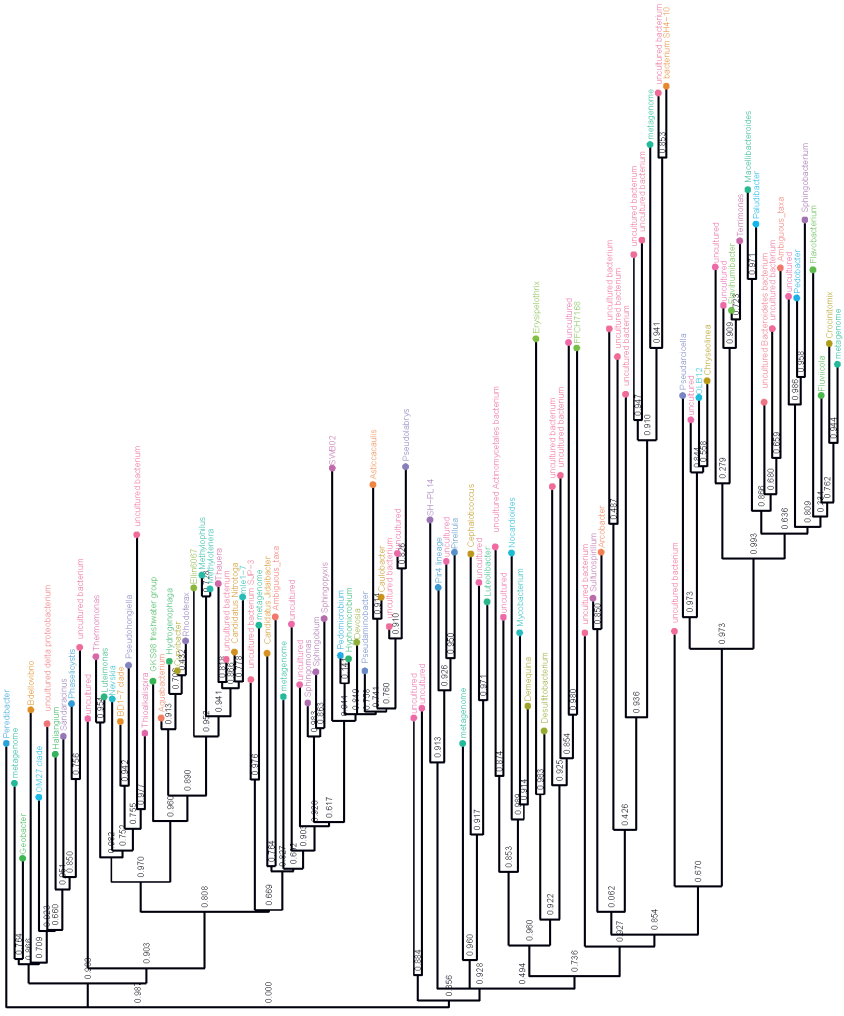
Supplementary Figure 1. Illustration of disease severity scale used for quantification of bacterial blotch

Casing soil composition (by volume) in Bioassay 1								
Raw materials	Surface peat Friedrichsveen	Deepdug peat Friedrichsveen	Surface peat Holriede	Surface peat Wiesmoor	Deepdug peat Wiesmoor	Amendments Baltics peat	Amendments Garden peat	Amendments Sugar beet lime
Casing soils								
1	90							6.3
2		90						6.3
3				90				6.3
4					90			6.3
5	81					4.5	4.5	6.3
6		81				4.5	4.5	6.3
7				81		4.5	4.5	6.3
8					81	4.5	4.5	6.3
9	22		68					6.3
10	20.2		60.8			4.5	4.5	6.3

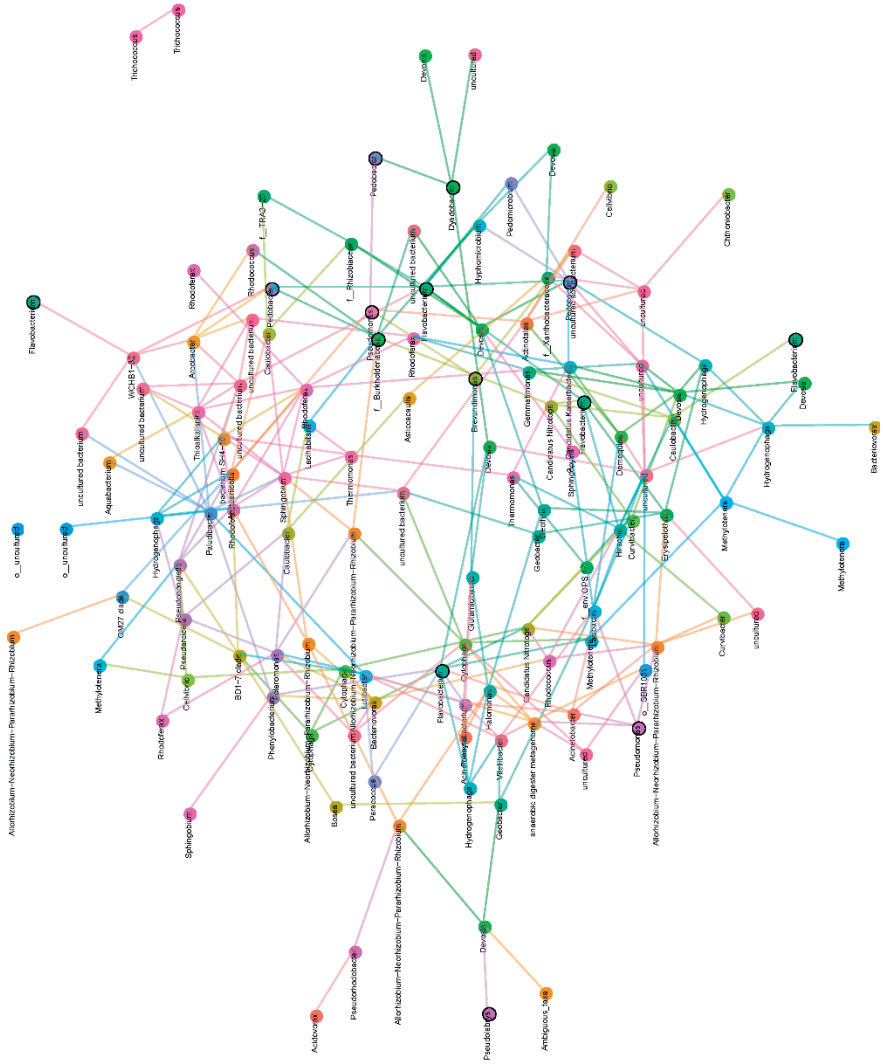
Supplementary Figure 2. Volumetric composition (in Litres) of different casing soil mixtures tested in bioassay 1.



Supplementary Figure 3. *In-vitro* pathogenicity assay on mushroom caps harvested from blotch suppressive soils of the second flush.



Supplementary Figure 4. Phylogenetic tree of various bacterial taxa associated with ginger blotch suppression.



Supplementary Figure 5. Co-occurrence network of bacterial genera in casing soil.

Supplementary Tables

Table 1. Primers used for targeted-amplicon sequencing			
Primers	Nucleotide sequence	Target	Other info
MSAf-B-E341degNW-fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	16S	Bacteria (~100%)
MSAr-B-E805degNVW-rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTWTCTAATCC	16S	Bacteria (~100%)
MSAf-F-gITS7ngs-fwd	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGARTCATCRARTYTTTG	ITS	Fungi (~100%), other eukaryotes
MSAr-F-ITS4ngsUni-rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCTSCSCTTANTDATATGC	ITS	Fungi (~100%), plants, protists

Supplementary Table 1. Primers for amplification of 16S and ITS regions

Table 2A. ANOVA on blotch prevalence						
Variables	Df	Sum Sq	Mean Sq	F value	Pr(>F) value	Sig. code
Harvest cycles (Flushes)	1	1.7218	1.7218	484.819	<2e-16	***
Pathogen inoculum density	1	1.2932	1.2932	364.146	<2e-16	***
Storage period	1	0.3219	0.3219	90.653	<2e-16	***
Casing soil mixtures	1	0.0004	0.0004	0.115	0.734	
Residuals	715	2.5393	0.0036			

Table 2B. Beta regression on blotch prevalence					
Variables	Estimate	Std Error	F statistic	Pr(>F) value	Sig. code
Intercept	-3.524038	0.229074	-15.384	< 2e-16	***
Pathogen inoculum density	0.899799	0.053037	16.966	< 2e-16	***
Storage period	-0.082299	0.021049	-3.91	0.0000924	***
Harvest cycles (Flushes)	-0.604208	0.143007	-4.225	0.0000239	***
Soil inoculum: Storage	0.019821	0.003259	6.082	1.19E-09	***
Storage period: Flush	0.068216	0.011281	6.047	1.47E-09	***
Soil inoculum: Flush	-0.425494	0.033193	-12.819	< 2e-16	***
Phi coefficient	27.83				
R squared	0.6728				
Log-likelihood	1936				
Df	8				

Supplementary Table 2. ANOVA and beta regression on blotch prevalence

Table 3A. ANOVA on ginger blotch severity						
Variables	Df	Sum Sq	Mean Sq	F value	Pr(>F) value	Sig. code
Harvest cycles (Flushes)	1	0.4179	0.4179	432.915	<2e-16	***
Pathogen inoculum density	1	0.3091	0.3091	320.217	<2e-16	***
Storage period	1	0.1054	0.1054	109.196	<2e-16	***
Casing soil mixtures	1	0.0003	0.0003	0.359	0.549	
Residuals	715	0.6903	0.001			

Table 3B. Beta regression on ginger blotch severity					
Variables	Estimate	Std Error	F statistic	Pr(>F) value	Sig. code
Intercept	-4.475169	0.220503	-20.295	< 2e-16	***
Pathogen inoculum density	0.85801	0.050467	17.001	< 2e-16	***
Storage period	-0.050182	0.020036	-2.505	0.01226	*
Harvest cycles (Flushes)	-0.436617	0.137449	-3.177	0.00149	**
Soil inoculum: Storage	0.018943	0.003085	6.141	8.21E-10	***
Storage period: Flush	0.046906	0.010689	4.388	0.0000114	***
Soil inoculum: Flush	-0.410713	0.031666	-12.97	< 2e-16	***
Phi coefficient	67.846				
R squared	0.7106				
Log-likelihood	2389				
Df	8				

Supplementary Table 3. ANOVA and beta regression on ginger blotch severity

Table 4. ANOVA on pathogen populations estimated from qPCR						
Variables	Df	Sum Sq	Mean Sq	F value	Pr(>F) value	Sig. code
Pathogen inoculation	1	1.4	1.4	0.184 0	0.66859	
Harvest cycles (Flushes)	2	2425	1212.5	158.119	< 2e-16	***
Casing soil mixtures	9	229.1	25.5	3.32	0.00077	***
Soil inoculum: Flush	2	66.6	33.3	4.34	0.01408	*
Soil inoculum: casing soil	9	90.5	10.1	1.311	0.23186	
Flush: casing soil	18	284	15.8	2.057	0.00804	**
Soil inoculum: flush: casing soil	17	212.7	12.5	1.632	0.05732	.
Residuals	238	1825.1	7.7			

Table 4. Linear regression on pathogen populations estimated from qPCR.					
Variables	Estimate	Std Error	F - statistic	Pr(>F) value	Sig. code
(Intercept)	35.99755	0.91966	39.142	< 2e-16	***
Pathogen inoculation	-0.73617	0.15458	-4.762	0.00000305	***
1st flush	-4.84742	0.51918	-9.337	< 2e-16	***
2nd flush	-7.97663	0.4874	-16.366	< 2e-16	***
Casing soil 10	0.82255	0.77498	1.061	0.2894	
Casing soil 2	-0.4944	0.7656	-0.646	0.5189	
Casing soil 3	-1.22387	0.72791	-1.681	0.0938	.
Casing soil 4	1.72971	0.72782	2.377	0.0181	*
Casing soil 5	0.34179	0.73045	0.468	0.6402	
Casing soil 6	-0.46817	0.75662	-0.619	0.5366	
Casing soil 7	0.05832	0.76289	0.076	0.9391	
Casing soil 8	0.89013	0.70884	1.256	0.2102	
Casing soil 9	-1.0721	0.80533	-1.331	0.1842	
Residual standard error	2.925				
R squared	0.5302				
F statistic	22.73				
Df	282				

Supplementary Table 4. ANOVA and linear regression on pathogen populations in casing soil

Table 5A. ANOVA on transferability of blotch suppression						
Variables	Df	Sum Sq	Mean Sq	F value	Pr(>F) value	Sig. code
Treatments	3	2070	689.8	14.95	0.00199	**
Residuals	7	323	46.1			

Table 5B. Linear regression on transferability of blotch suppression					
Variables	Estimate	Std Error	F statistic	Pr(>F) value	Sig. code
Pathogen	51.426	4.803	10.706	0.0000136	***
Pathogen + soil extract	-16.872	5.547	-3.042	0.018798	*
Soil extract	-35.892	6.793	-5.28E+00	0.001143	**
Control	-46.564	8.32	-5.597	0.000819	***
Residual standard error	6.793				
R squared	0.865				
F statistic	14.95				
Df	7				

Supplementary Table 5. ANOVA and linear regression on transferability of blotch suppression

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Chapter 6

Circular alternatives to peat in casing soils

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Abstract

Peat use in horticulture is associated with a large ecological footprint due to GHG emissions and biodiversity loss. Peat is an essential growth substrate in mushroom cropping systems, in the form of a casing soil. The casing soil is a prime determinant of productivity in mushroom cultivation, as beneficial microorganisms in the peat induce the transformation of vegetative mycelium into fruiting bodies. Yet, in the search for sustainable alternatives to peat-based casing soil, the microbiome of the growing media has often been ignored. In this study we evaluate the physical, chemical and microbiological characteristics of four circular peat-alternatives, when used to proportionally replace peat in the casing soil. We also report the agronomical performance of the alternative casing soils when tested in an experimental mushroom cultivation facility. Grass fibres from agricultural residue streams, peat-moss from degraded peatlands, and spent casing soil recycled from previous cultivation cycles could be used to successfully replace peat in mushroom casing soils. Peat moss and spent casing were expectedly similar to peat in physical, chemical and microbiological properties, but the grass fibres had unique characteristics, such as high organic matter content, low water holding capacity and a diverse and competitive microbiome. However, pre-treatment of the alternative by acidification and steaming significantly reduced the presence of pests, competitive fungi and pathogens in the casing soil. Strong trade-offs existed between the productivity and disease pressure in the cropping system, which are also governed by the accessibility, sustainability, and economic viability of the peat-alternatives. This knowledge on the properties, performance, microbiome and treatment of peat-alternatives is important to transition away from peat use and towards circular and sustainable growing media.

Introduction

Peat has been the primary component of growing media due to its low cost, high availability and unique physico-chemical characteristics (Caron and Rochefort, 2011). Of the total growing media required for horticulture within the European Union, 86% is composed of peat, amounting to 29.3 million m^3 of peat use annually (Altmann, 2008). Wet peatlands are fragile ecosystems with important ecosystem functions such as biodiversity conservation, water purification and climate regulation. They sequester 30% of the global soil carbon despite constituting only 3% of the global terrestrial area (Joosten et al., 2016). Peat excavation is thus associated with a large ecological footprint, and strongly discouraged by EU directives (Owen, 2007). Severe peat supply bottlenecks are expected in the near future due to rapidly declining global deposits and consequent increase in peatland conservation policies (Bos et al., 2011). Increased societal and governmental pressure has fuelled an extensive search for abundant and sustainable alternatives to replace peat in growing media (Alexander et al., 2008).

Peat, in the form of a casing soil, has been the predominant growing media in mushroom cropping systems since the 1950s (Flegg, 1956, 1953). This peat-based covering layer is placed on top of mycelium-containing compost, and induces fructification. The casing soil is a prime determinant of productivity in mushroom cultivation (Pizer and Leaver, 1947) because beneficial microorganisms in the peat induce the transformation of vegetative mycelium into fruiting bodies (Rainey, 1989). However, peat use in casing soils contributes to the highest greenhouse gas (GHG) emissions associated with mushroom cultivation, according to several life cycle assessments (Gunady et al., 2012; Leiva et al., 2015; Robinson et al., 2019). Peat use is also shaped by high environmental impacts, as peat excavation and its long distance transport are associated with global warming and biodiversity loss, while the preparation of the casing soil is associated with eutrophication and water eco-toxicity (Leiva et al., 2015)

In the search for sustainable alternatives, by-products from industrial, forestry or agricultural waste streams have received significant attention as peat-replacement media. Alternatives like coco-peat, fly-ash, paper pulp, pine bark, green waste compost, spent mushroom substrate and recycled rock-wool, have been tested in commercial scale trials for mushroom cultivation (Latinoamericana, 2006; Noble and Dobrovin-Pennington, 2015, 2005; Pardo-Giménez et al., 2011; Pardo et al., 2004, 2003; Peyvast et al., 2007; Sassine et al., 2005; Sharma et al., 1999). However, none of these alternatives have resulted in an industrial application due to poor agronomical performance, either due to introduction of pests and pathogens (competitive weed fungi in paper pulp and recycled coco-coir), inappropriate physical characteristics (insufficient water holding capacity of pine bark, lignite and lump

chalk) or chemical compositions (soluble salt content in spent mushroom substrate and digestates), accumulation of toxic residues (pesticide traces in green waste compost and recycled rockwool), unsustainable sourcing (limited supply of coco-coir, tea wastes, coffee wastes and sugarcane bagasse), unsupportive legislation (EU policy on field disposal of recycled rockwool or paper pulp or spent mushroom compost) or lack of economic viability (prohibitive costs of coir and vermiculite or chemical processing costs of compost).

The microbiomes of alternative growing media have received limited attention (Van Gerrewey et al., 2020; Vandecasteele et al., 2020). Beneficial and harmful microbes co-inhabit growing media, and they increase in diversity and abundance once the growing media is planted (Postma et al., 2008). Most microbes may be benign, such as saprophytic fungi and bacteria, others may offer positive benefits, such as plant growth promotion, and some may be detrimental to crop health, because they compete with the host for nutrients or cause diseases (Carlile and Schmilewski, 2010). Disease management in horticulture has traditionally aimed at keeping the growing media as 'clean' as possible, by using pathogen-free propagation material and substrates and by using disinfestation and other sanitation techniques (Postma et al., 2008). However, circular alternatives to conventional growing media that are derived from agri-residue streams often contain a diverse and competitive microbiome (Carlile and Coules, 2011). This could support disease suppression and plant growth, but its use may also bear a risk for dissemination of potential human pathogens, antibiotic resistance genes and plant pathogens (De Corato, 2020).

In this research, we propose four local, circular and sustainable alternatives to partially replace black peat in mushroom growing media. We attempt to incorporate three principles of circular economy in mushroom cropping systems by utilizing agricultural residues in the casing soil (design out waste); reusing spent casing soil from previous cultivation cycles (keep materials in use), and substituting black peat in the casing soil with peat moss from degraded peatlands (regenerate natural systems). We assess the diversity, composition and interactions within the microbiome of the alternative growing media, before and during the cropping process. The physical and chemical characteristics of the alternative growing media were also evaluated in comparison to black peat. We study the response of the peat-alternatives to ginger blotch disease, which is endemic to the casing soil, and is responsible for recent outbreaks in Western Europe (Taparia et al., 2020a). We evaluate the performance of the peat alternatives in mushroom farms, and discuss the trade-offs between productivity and disease pressure in this circular cropping system. Finally, we comment on the accessibility and sustainability of these peat-alternatives. We believe that this knowledge is essential to transition away from peat use in horticulture. Our research outcomes also enable a better understanding of the advantages and disadvantages of moving from traditional to circular farming practices in horticulture.

Methods

Description of peat alternatives

In this study, peat moss, refers to non-decomposed moss from *Sphagnum* sp. These are grown organically on degraded and drained peatlands that were formerly mined. It has the advantage of being cyclically and renewably harvested every 3-4 years, in comparison to black peat which is conventionally harvested from natural peatlands (Pouliot et al., 2015). As opposed to black peat, which is dug out, peat moss is harvested superficially with minimal damage to below-ground landscape. The moss layer transfer technique allows *Sphagnum* fibres to be used for ecological restoration of cutover bogs (Graf et al., 2012), thus enabling *Sphagnum* farming on degraded peatlands. It consequently reduces negative environmental effects such as peat oxidation, soil subsidence and CO_2 emissions (Joosten et al., 2012). Peat moss was used to proportionally replace 25% of peat in the casing soil.

Grass fibres used in this study, are produced by a patented circular biorefining process that converts non-woody biomass into lignocellulosic fibres (EP2606140B1). It is produced from a mild extraction of various agricultural and horticultural residue streams, the energy demands for which are met by producing biogas from the leftover grass-juice, resulting in zero net emission of CO_2. All the water used in this process is cleaned and recycled within the biorefinery (Newfoss). As an additional step, to assess the effect of a reduced microbial community, some of the grass fibres were acidified via an anaerobic fermentation process that gradually reduces the pH to 4.5 over a year. This acidification is chemical-free and performed with the microbiota in the processing fluids from organic feedstocks. Acidified grass fibres were studied as an independent peat alternative. Both grass fibres and acidified grass fibres were used to proportionally replace 50% of peat in the casing soil.

Spent casing soil is mechanically separated from the compost after a cook-out (steaming) of the growing chambers, at the end of the cropping cycle. Thus, spent casing undergoes pasteurization as part of the previous cultivation cycle, with no extra energy or cost associated to it. The use of disinfectants and salts need to be avoided during cultivation for the re-use of spent casing soil (Noble and Dobrovin-Pennington, 2015). Separation of the casing soil simultaneously reduces the amount of leftover spent mushroom compost (SMC) for waste disposal, which is associated with high costs. The SMC devoid of casing soils, largely contains compost, with very high N and P content, and increased fertilizer value (Noble and Dobrovin-Pennington, 2015). Up to 33% of this spent compost can be re-used in phase I compost for mushroom cultivation with no negative effects on yield (Noble and Dobrovin-Pennington, 2015). Spent casing is a local product, that was used to proportionally replace 30% of peat in fresh casing soil.

Setup of cultivation experiments

To evaluate the performance of peat alternatives in casing soils, two cultivation experiments were performed at an experimental mushroom growing facility (Unifarm, Wageningen University and Research). The generic cultivation setup and growing conditions are described in Taparia et al., 2020b. Peat was proportionally replaced in the casing soil by 25%, 50%, 50%, and 30% of its volume, respectively with (A) *Sphagnum* moss from degraded peat lands, (B) grass fibres from agricultural residue streams, (C) acidified grass fibres and (D) spent casing soil from previous cultivation cycles. The proportions of peat replacement were recommended by the mushroom growers, on the basis of previous experiences with the materials. Each of the materials were steamed at 70^{o}C for 8 hours, similar to a standard cook-out cycle. Both steamed and unsteamed materials were individually used to prepare the alternative casing soils. The experimental design and casing soil compositions are described in Table 1 and 2 respectively.

Ginger blotch pathogen, *Pseudomonas gingeri* (IPO3777) was added to the casing soils at densities of 10^3, 10^4 and 10^5 cfu/g of casing soil. The protocol for pathogen inoculation in the soil is described in Taparia et al., 2020b. Negative controls were mock-inoculated with tap water. Each treatment comprised of three replicates, in a randomized block design. The entire cultivation experiment was repeated twice, with freshly procured alternative materials, peat and compost. Agronomical performance of the peat alternatives was described by the productivity, disease pressure and post-harvest quality of the mushrooms. Weight and numbers of healthy and diseased mushrooms harvested from each unit were registered daily across three cultivation cycles, called flushes. Productivity of the alternative casing soils was measured as the total harvest weight per growing surface (kg/m^2). Disease pressure in the alternative casing soils was measured as the proportion of diseased harvest to total harvest (%). Post-harvest quality was observed by visually inspecting the sliced mushroom caps immediately after harvest.

Table 1. Experimental design	
Factors	
Alternative	Type of alternative used to replace peat in soil
Heat Treatment	Raw materials steamed at 70°C for 8 h before mixing with peat
Pathogen density	Inoculation density of pathogen: *0, 10^3, 10^4, 10^5 cfu/g of soil*
Flush	Progressive harvest cycle: *1st flush, 2nd flush, 3rd flush*
Replicate	Randomized block design within the experiment: *block 1,2,3*
Experiment	Independent replicate experiments, with fresh materials: *exp 1,2*
Measurements	
Yield	Total harvest weight per m^2 of growing surface
Blotch prevalence	Proportion of diseased harvest to total harvest (by weight)

Table 1. Experimental design of cultivation experiments with peat alternatives

Table 2. Casing soil composition			
Casing soil tag	Alternative raw material	% peat replaced	Treatment of raw material
1	Peat	0%	Un-steamed
6	Peat	0%	Steamed
8	Peat moss	25%	Un-steamed
9	Peat moss	25%	Steamed
2	Grass fibres	50%	Un-steamed
3	Grass fibres	50%	Steamed
4	Acidified grass	50%	Un-steamed
5	Acidified grass	50%	Steamed
10	Spent casing	30%	Steamed

Table 2. Description of casing soil composition, including type of alternative material, proportion of peat replaced and steam treatment of raw material.

Physical and chemical properties of alternatives

Five L of peat and alternatives were sampled for the assessment of physical and chemical properties, at three time points, from the raw materials itself, from freshly prepared casing soil at the beginning of the cultivation experiments, and at the end of the third cultivation cycle. Before being mixed with peat in the casing soil, the organic matter content (thermogravimetric), dry weight, moisture content (gravimetric) and respiration were measured from the raw materials itself, according to EN 13039 and EN 13040 protocols. From the freshly prepared casing soils, physical characteristics such as water holding capacity (saturation and drainage), moisture content, soil moisture retention and shrinkage were measured according to EN 13041 protocols. Bulk weight and bulk density were measured according to EN 13040 protocols. Chemical characteristics such as pH (potentiometric) and electrical conductivity (conductimetric) were measured according to EN 13037 and EN 13038 protocols. Structural properties like hardness and adhesiveness were also measured according to in-house protocols in use at BVB-Substrates (Grubbenvorst, the Netherlands). The pH, electrical conductivity and moisture content were measured again at the end of the third flush, to study temporal variation across the cultivation cycle. All measurements were made in replicates of three.

Sequencing the casing soil microbiome

The soil bacterial and fungal community composition of the alternative casing soils was assessed in replicates of three, across three cultivation cycles and over two independent experiments. The soil microbiome was determined from targeted sequencing of the V3-V4 regions of bacterial 16S rRNA gene and the fungal ITS2 gene (as described in Taparia et al., 2020c). The libraries for 16S and ITS2 respectively, were sequenced on Illumina MiSeq using MiSeq v2 or v3 reagents (Illumina, USA) to generate 250 or 300bp paired-end reads that overlap. All raw sequences for soil fungi and bacteria were deposited in NCBI under BioProject numbers PRJNA657168 and PRJNA657276 respectively. Downstream processing of the raw data was performed on QIIME2 version qiime2-2020.2 (Bolyen et al., 2019) using the Dada2 workflow (Callahan et al., 2016), resulting in a set of unique sequences and an abundance table of amplicon sequence variants (ASVs) or taxa (as described in Taparia et al., 2020c).

Statistical data analyses

All statistical analysis from cultivation experiments, physico-chemical assessments and microbiome sequencing was performed on RStudio with R version 3.4.0 (Team, 2013). Cultivation data, such as yield and blotch prevalence were transformed using *tidyverse* (Wickham et al., 2019). Generalized linear regression and zero-inflated

beta regression were performed using packages, *glm* and *betareg* (Cribari-Neto and Zeileis, 2009) respectively. Widely applicable information criteria (WAIC) and adjusted R squared values were compared to arrive at a final minimally adequate model (Johnson and Omland, 2004). Model assumption on normality of data and homogeneity of residuals were verified using diagnostic plots and statistical tests (Fox et al., 2012). Data on physico-chemical properties were assessed with analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA).

Analyses of microbiome sequence data was performed with packages *vegan* (Oksanen et al., 2007) and *phyloseq* (McMurdie and Holmes, 2013). Alpha diversity of the microbiome was calculated by the Inverse Simpson index. Beta diversity was measured using the Bray-Curtis distances. The core microbiome was defined as the taxa present above a detection threshold of 0.1% in 90% of the samples, and performed using package *microbiome* (Lahti et al., 2017). Co-occurrence microbial ecological networks were estimated with inverse covariance, and performed using packages, *igraph* (Csardi and Nepusz, 2006), *speic-easi* (Kurtz et al., 2015) and *ggnetwork* (Briatte, 2016).

Results

Physical and chemical characteristics

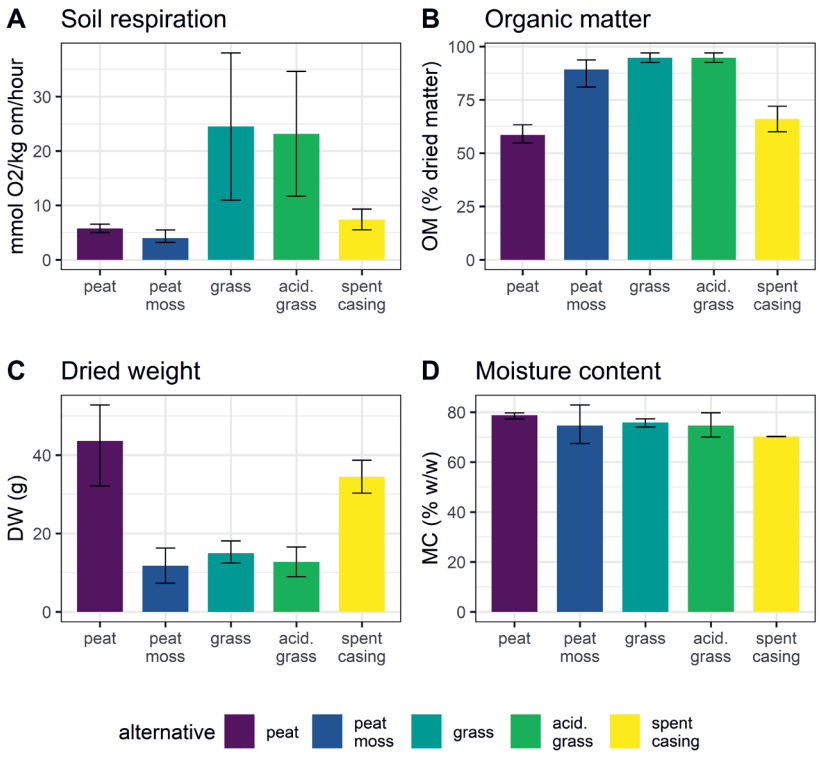

Figure 1. Physical and chemical properties of alternative raw materials before mixing with peat

The individual raw materials, before being used to proportionally replace peat in the casing soil, varied significantly in their physico-chemical characteristics such as soil respiration ($P=0.04$), organic matter content ($P=2x10^{-16}$) and dry weight ($P=2x10^{-16}$), according to a multivariate ANOVA (Supplementary Table 1). Soil respiration for both grass fibres was higher than the other alternatives. Surprisingly, the respiration of spent casing, which was heat treated, was even higher than that of peat (Figure 1A). Both grasses and peat moss had a similarly high organic matter content (Figure 1B). Peat and spent casing had the highest dry weight, which was significantly lower for the grasses (Figure 1C). The moisture content of the raw materials was not significantly different from each other (Figure 1D).

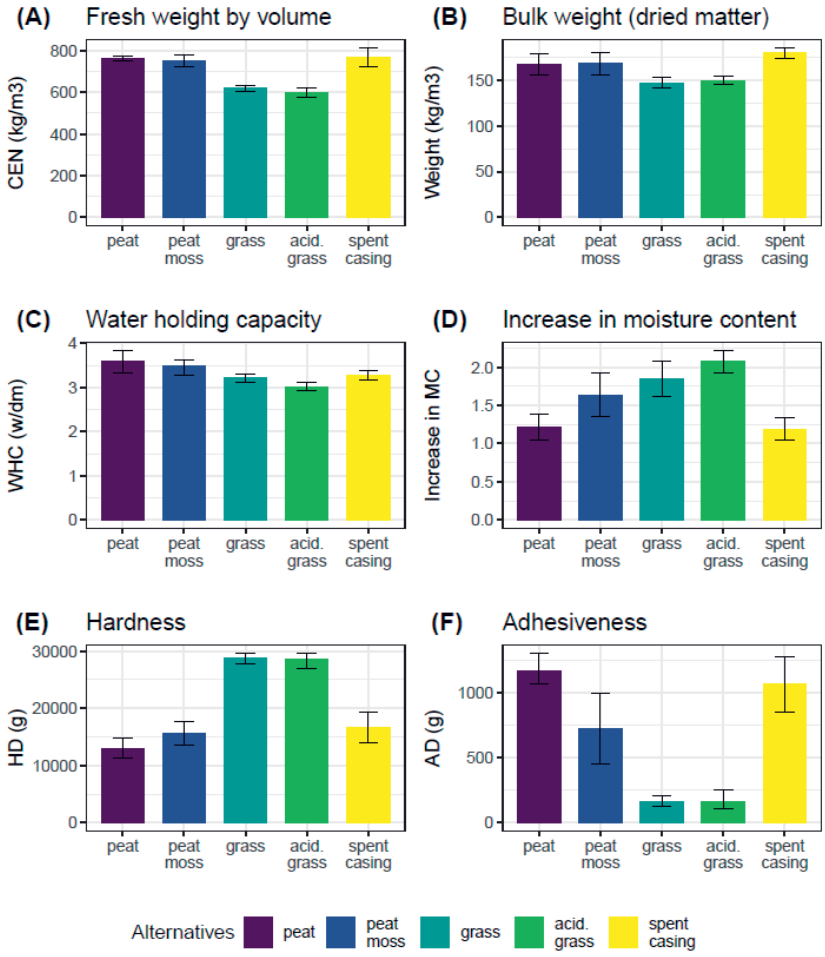

Figure 2. Soil moisture retention and water relation properties of alternative casing soils

After the raw materials were proportionally mixed with peat to constitute casing soils with varying compositions, their water holding capacity (P= 0.05), increase in moisture content when saturated with water (P=0.04), bulk weight (P= $2x10^{-16}$), dry weight (P=0.05), and structure (P= $2x10^{-16}$) differed significantly between the alternative casing soils, according to a multivariate ANOVA (Supplementary Table 2). Heat treatment of the alternatives before preparation of the casing soil, had no impact on these parameters (P<0.05). Both grass-based casings had lowest fresh and dried weight by volume (Figure 2A and 2B). Peat and peat-moss based casing soils had the highest water holding capacity (Fig 2C), whereas both grass-based casing soils absorbed the largest amount of moisture when saturated (Fig 2D). Their physical structure was also significantly different from other casing soils, based on hardness (Fig 2E) and adhesiveness (Fig 2F) measurements by a CT3 texture

analyser. Heat treatment of the raw materials did not affect these parameters (Supplementary Table 2).

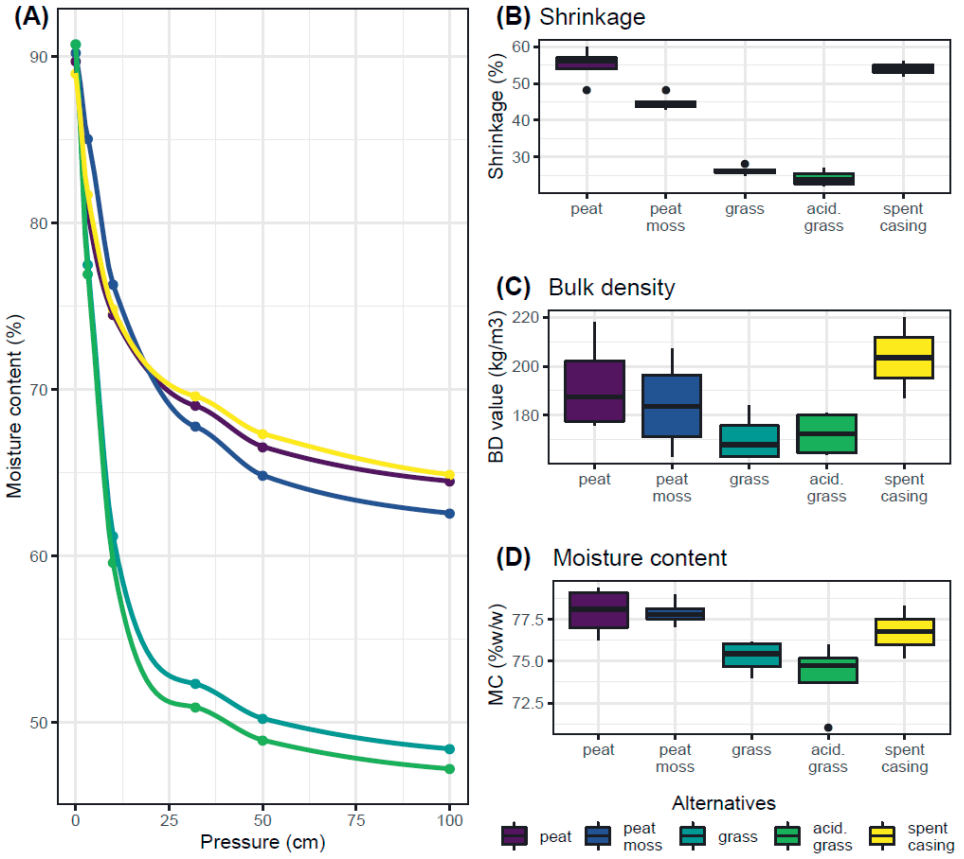

Figure 3. Physical and chemical properties of alternative casing soils during "CAC-ing".

The soil moisture retention curves (Figure 3A) varied significantly between peat alternatives ($P=2x10^{-16}$). Peat, peat moss and spent casing had similar pF curves. Both the grass-based casings were the fastest in losing soil moisture. Related parameters, such as the moisture content (P=0.04), bulk density (P=0.01) and shrinkage ($P=2x10^{-16}$) measured during the pF curves also differed significantly among the peat alternatives (Figure 3B, 3C and 3D), according to a multivariate ANOVA analysis (Supplementary Table 3). Heat treatment of raw materials before preparation of casing soil, had no impact on soil water retention and related properties. The organic matter content of the casing soils did not differ between the alternatives or due to heat treatment.

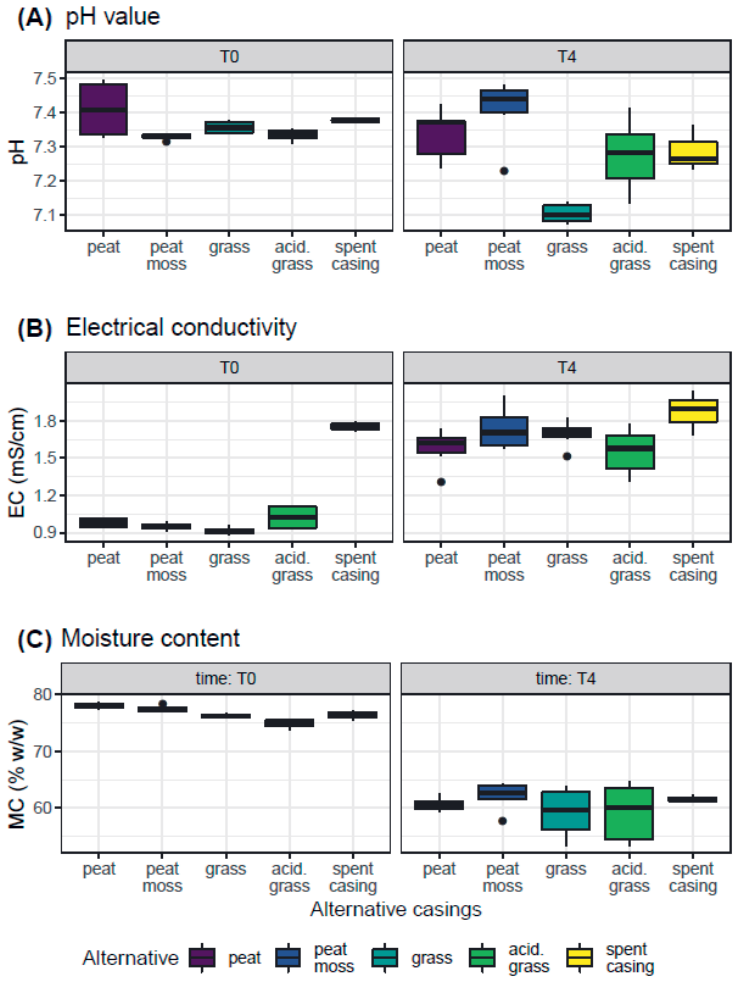

Figure 4. Moisture content, pH and electrical conductivity at the start of the cultivation experiment (T0) and at the end of the 3rd harvest cycle (T4)

The characteristics of the casing soils changed significantly during the cropping cycle (Supplementary Table 4), from being placed onto compost at the start of the experiment (T0) to the end of the third flush (T4). The overall pH varied between 7.1 and 7.5, well within the optimal range, although it differed between peat alternatives ($P=2x10^{-16}$). The pH reduced slightly from T0 to T4 ($P=0.01$), more so for the grass-based casing soil (Fig 4A). The pH of peat and peat moss was similar, as was the pH of acidified grasses and spent casing. Electrical conductivity also differed between peat alternatives ($P=2x10^{-16}$), and varied in the range of 0.8 and 1.8 between the time points ($P=2x10^{-16}$). Spent casing had the highest EC compared to all alternatives at T0. However, at the end of the cultivation cycle, at T4, the EC increased for all the casing soils (Fig 4B). The moisture content of the soils reduced

significantly ($P=2x10^{-16}$) from ~80% at T0 to ~60% at T4 (Fig 4C). Moisture content also varied due to heat treatment of the alternatives ($P=2x10^{-16}$). It was particularly low for both of the steamed grasses.

Microbiological community composition

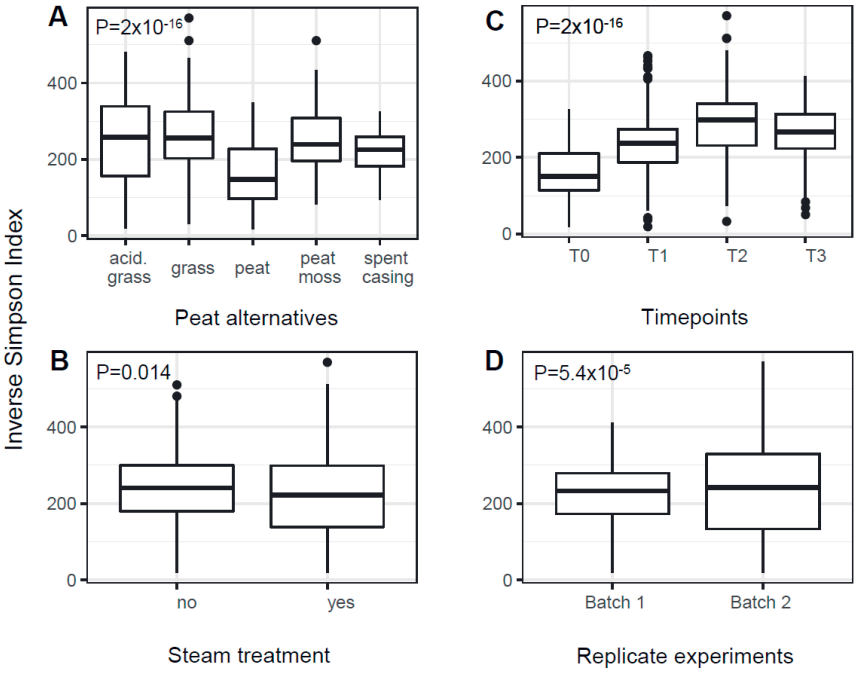

Figure 5. Differences in bacterial species richness of casing soils between (A) peat alternatives (B) steam treatment (C) timepoints of cultivation cycles and (D) independent replicate experiment.

The bacterial and fungal microbiome of the alternative casing soils were also explored throughout the cultivation cycle. The soil bacterial community comprised of 27,666 amplicon sequence variants (ASVs) or taxa originating from 378 casing soil sample. The soil fungal community was less diverse and comprised of only 2116 taxa. The bacterial alpha diversity of the soils, described by the Inverse Simpson index varied significantly between the peat alternatives ($P=2x10^{-16}$) according to a univariate ANOVA (Supplementary Table 5). Casing soil composed of only peat was the least diverse, but when it was supplemented with other alternatives, the casing soil had a comparably higher bacterial diversity (Figure 5A). Grass-based casing soil had the highest alpha diversity. Heat treatment of the raw materials, before being used to proportionally replace peat in the casing soil reduced the alpha diversity ($P=0.014$) of the casing soil significantly (Figure 5B), although the magnitude of the

effect was specific to each alternative. The diversity also increased consistently across the cultivation cycles ($P=2x10^{-16}$), for all casing soil compositions (Figure 5C). The diversity of the alternatives also varied between replicate experiments conducted with freshly procured raw materials ($P=5.4x10^{-5}$), even though the overall differences between the experiments were only marginal (Figure 5D).

The bacterial and fungal community composition of the casing soils differed significantly between the peat alternatives (P=0.001), due to heat treatment (P=0.001), across the cultivation cycles (P=0.001) and between the replicate experiments (P=0.001), according to a PERMANOVA (Supplementary Table 6). The core bacterial profile between peat, peat moss and spent casing was very similar on a genus level, but at lower taxonomic levels, peat was the least diverse as it comprised of only 40 bacterial taxa, whereas peat moss and spent casing comprised of 119 and 108 bacterial taxa respectively. Heat treatment of the grass fibres had a relatively smaller effect on the bacterial community compared to acidification of the grass fibres, which reduced the core bacterial microbiome drastically from 119 taxa to 20 taxa. On a genus level, the core microbiomes between the alternatives may look relatively conserved (Figure 6A), however, most of these changes occur at lower taxonomic ranks, such as the species or strain level.

The core fungal microbiome of peat comprised of only 6 fungal taxa, namely, *Agaricus*, *Apiotrichum, Meliniomyces, Mycothermus, Candida* and *Pseudeurotium* in descending order of abundance (Figure 6B). Heat treatment of peat reduced the abundance of all other fungi, except *Agaricus*. Peat moss additionally comprised of *Pseudallescheria, Saitozyma Solicoccozyma, Trichoderma* and unidentified fungi. Heat treatment of peat moss did not have a significant impact on fungal microbiome of the casing soil. The fungal composition of spent casing, which was steamed, was very similar to that of peat moss. The grass fibres were unique in that, their microbiome was largely dominated by *Pseudeurotium,* most of which was lost from the casing soil after the heat treatment. Acidification of the grass fibres led to reduction of *Pseudeurotium and Dipodascus*, and a relative increase of *Ascobolus* and *Solicoccozyma.*

Figure 6A. Community composition of the core bacterial microbiome of alternative casing soils on a genus level, from combined data across all harvest cycles.

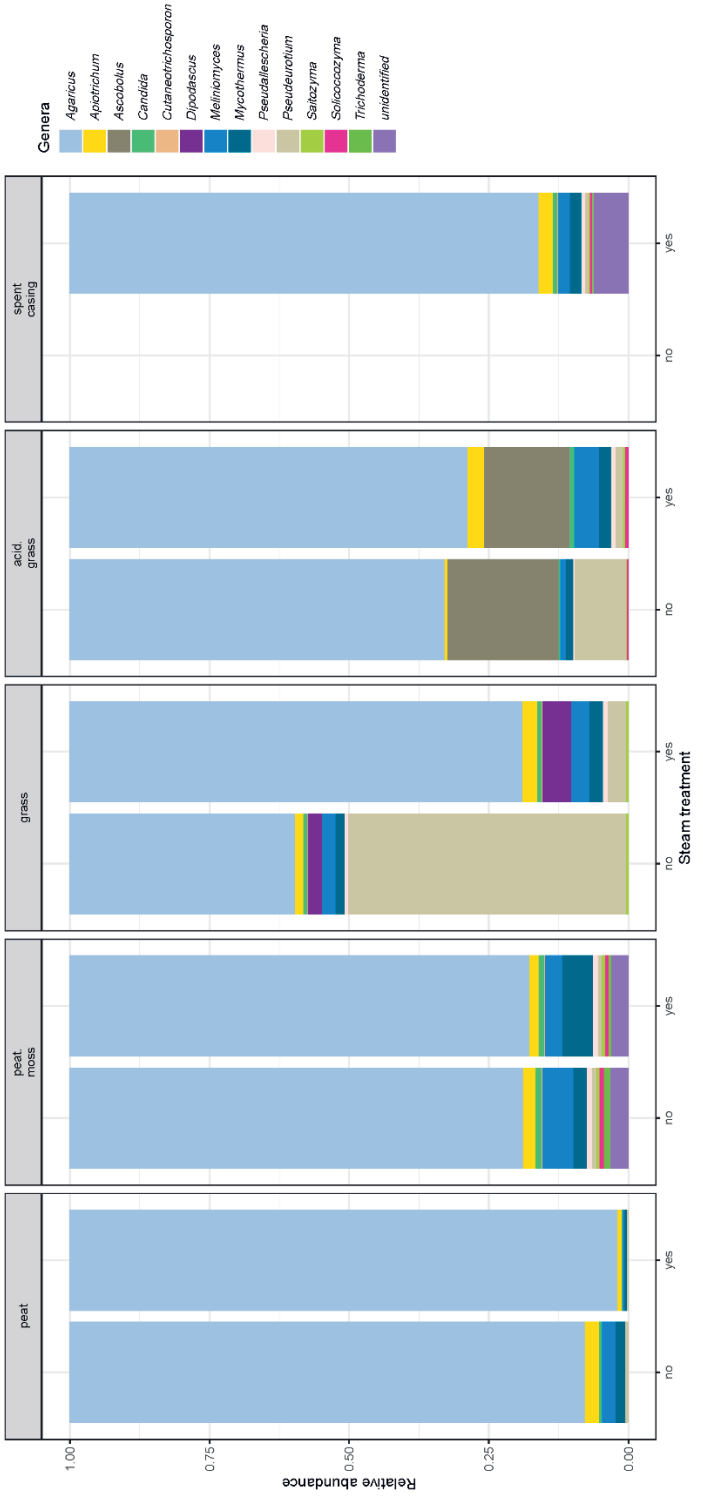

Figure 6B. Community composition of the core fungal microbiome of alternative casing soils on a genus level, from combined data across all harvest cycles.

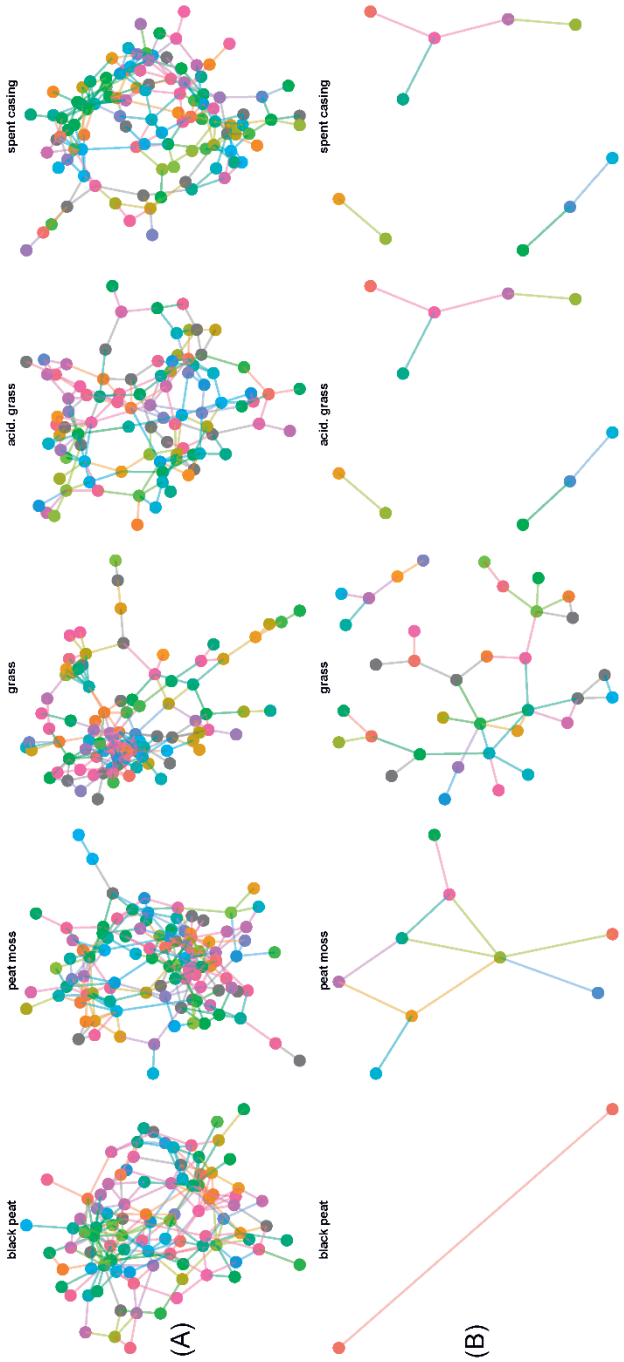

Figure 7. Co-occurrence networks of the core (A) bacteria and (B) fungi in alternative casing soils, from combined data across both harvest cycles.

Interactions within the casing soil microbiome were explored via microbial co-occurrence networks. Significant differences existed in the network topology between the casing soil based on the peat alternatives (Supplementary Table 7). The bacterial network of peat-based casing soil was compact and dense, as indicated by the low average path length, high graph density and high clustering coefficient. It also had a large number of edges (Figure 7A). Peat moss-based casings had a similarly network topology. The bacterial network in spent casing was most modular, as indicated by the highest number of vertices, average path length, modularity and node density. Bacterial networks of grass fibres were also modular, but better connected, as evident from their high clustering coefficient. Acidification of grass, simplified the interactions, as evident from the reduced clustering coefficient and graph density of the network. Fungal interactions within the microbiome were significantly reduced compared to that of the bacterial community (Figure 7B). Peat-based soils lacked a fungal network, whereas peat moss had a minimal but compact fungal network. This was further reduced in heat treated spent casing soil. Grass-based casings had the largest and most complex fungal network, with the highest number of edges, modularity, clustering coefficient, average path length and node density. Acidification of the grass fibres largely reduced the size of the network. High resolution figures with taxonomic labels are available as Supplementary Figure 1 and 2.

With the limitations of amplicon sequencing, the microbiomes of the alternative casing soils were preliminarily screened for presence of genera that could comprise of known human pathogens (*Bacillus cereus, Campylobacter* species*, Clostridium botulinum, Clostridium perfringens, Cronobacter* species*, Enterohemorrhagic Escherichia coli, Escherichia coli O157:H7, Listeria monocytogenes, Salmonella species, Shigella* species*, Staphylococcus aureus* and *Vibrio*). Most of these genera were absent from the alternative casing soils, although a few unidentified species from the genus *Bacillus*, *Clostridium*, *Cronobacter* (ASV223444), *Staphylococcus* (ASV10822), and *Yersinia* (ASV23472 and 23478) were detected. The soil microbiomes were also screened for presence of genera, that could comprise of known plant pathogens (*Pseudomonas fluorescens, Pseudomonas syringae, Agrobacterium tumefaciens, Bacillus caryophylli, Clavibacter michiganensis, Dickeya dadantii, Dickeya dianthicola, Dickeya zeae, Erwinia amylovora, Pantoae ananatis, Pantoae citrea, Pantoae punctata, Pantoae terrea, Pectobacterium atrosepticum, Pectobacterium carotovorum, Pectobacterium wasabiae, Rahnella aquaticus, Ralstonia, Rhodococcus fascians, Serratia plymuthica, Xanthomonas campestris, Xanthomonas euvesicatoria, Xanthomonas fragariae, Xanthomonas gardneri* and *Xanthomonas perforans)*. The majority of these species were absent from the casing soil microbiome, however, a few unidentified taxa found in the casing soil microbiome belonged to genera which could potentially include plant pathogens. These include unidentified taxa from the genus *Erwinia* (ASV ASV23428,

ASV23426, ASV23435), *Pantoea* (ASV23422, ASV23423, ASV23424, ASV23425), *Rahnella* (ASV23450-55), *Ralstonia*, *Rhodococcus, Serratia,* and *Xanthomonas*.

Agronomical performance of the alternatives

The productivity of the casing soil was measured as total harvest across three consecutive cultivation cycles for each peat alternatives, in two independent experiments conducted with fresh batches of raw materials. The productivity of the casing soils differed significantly between peat alternatives ($P=2x10^{-16}$), due to heat treatment ($P=9.2x10^{-5}$) and between replicate experiments ($P=2x10^{-16}$), according to univariate ANOVA (Supplementary Table 8). Peat-based casing soil produced a mean yield of ~31.1 kg/m^2 of growing surface, and heat treatment significantly increased its yield to 34.5 kg/m^2 (Figure 8). When peat moss was used to proportionally replace 25% of peat in the casing, the mean yield was 32.6 kg/m^2. Steamed peat moss had a yield of 33.3 kg/m^2, which was not significantly higher. When grass and acidified grass fibres were used to substitute 50% peat in the casing, their yields were similar at 29.3 and 28.5 kg/m^2 respectively. Heat treatment of the grasses did not increase the yield further. When steamed spent casing replaced 30% of peat in the casing soil, yields were maintained similar to peat at 30.9 kg/m^2. Between the replicate experiments, the mean yield reduced by 5.2 kg/m^2. The productivity of all casing soils decreased from the 1st to the 3rd cultivation cycle.

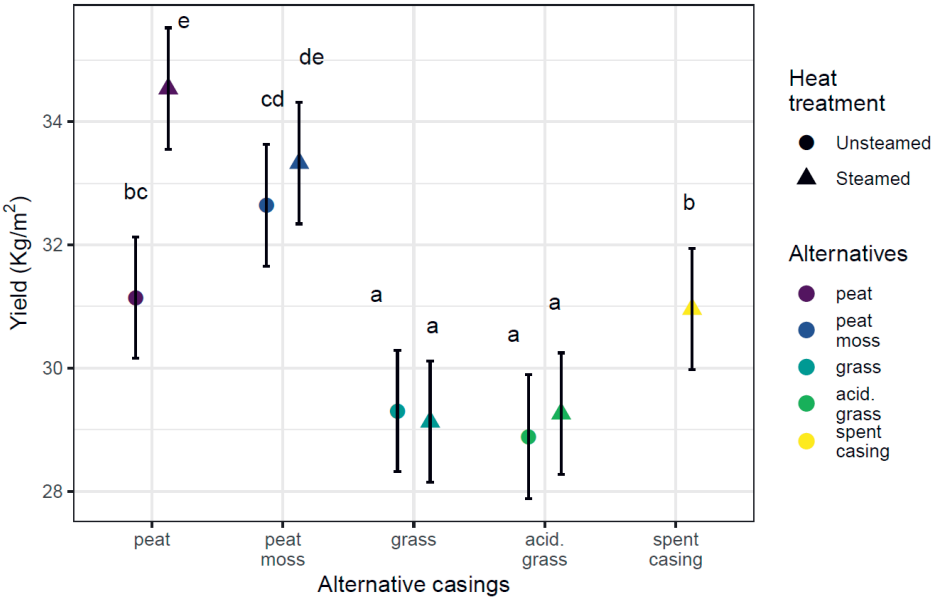

Figure 8. Linear regression on the productivity (yield) of alternative casing soils, and the effect of steaming. Statistically significant comparisons according to Tukey's test are highlighted by different letters.

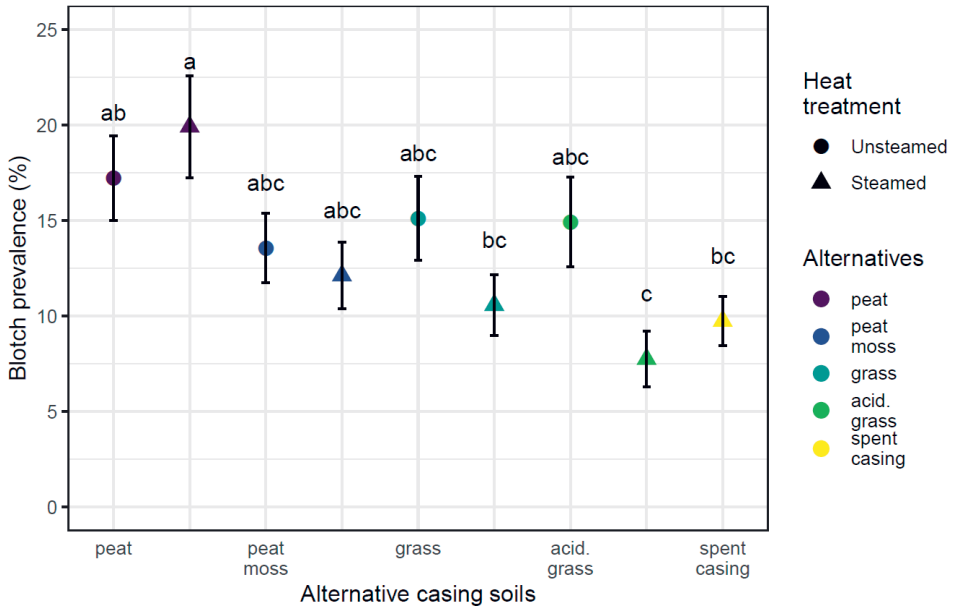

Figure 9. Linear regression on the disease pressure (ginger blotch prevalence) of alternative casing soils, and the effect of steaming. Statistically significant comparisons according to Tukey's test are highlighted by different letters.

The disease pressure was measured as the bacterial blotch prevalence, across three cultivation cycles, over four pathogen densities and two experiments. In mock-inoculated casing soils, without added pathogen, there were no significant differences in the blotch prevalence between the alternatives (P=0.40). Although heat treatment significantly (P=0.00275) reduced the inherent blotch prevalence of the casing soils. In the pathogen-inoculated casing soils, the mean blotch prevalence varied significantly between peat alternatives ($P=6.0x10^{-5}$) and their heat treatment (P= 0.00615), according to univariate ANOVA (Supplementary Table 9). Steamed peat showed the highest susceptibility to blotch with a disease prevalence of 19.94% (Figure 9). Heat treatment of the raw materials reduced the disease pressure in the alternative casings, with the exception of peat, which had higher blotch prevalence when steamed, although it was statistically insignificant. Casing soil composed of steamed acidified grasses had the lowest susceptibility to blotch, with a disease prevalence of 7.7%, which was significantly less compared to that of peat and peat moss based casing soils. Spent casing and steamed grass fibres shared a similarly lower blotch prevalence of 9.7 and 10.5% respectively.

Beta regression revealed that peat alternatives, heat treatment, inoculation density of pathogen and consecutive harvest cycles, all significantly affected the disease

pressure of the cropping system (Supplementary Figure 3). The bacterial blotch prevalence increased with inoculated pathogen density in the soil ($P=2x10^{-16}$), irrespective of the casing soil composition. In the first flush, mock-inoculated soils had 1.5% blotch prevalence, and it increased to 18.5%, 26.9% and 35%, when inoculated with *'P. gingeri'* at 10^3, 10^4 and 10^5 cfu/g of soil respectively. Blotch prevalence declined steeply with consecutive cultivation cycles ($P=2x10^{-16}$), consistently across all peat alternatives. At the highest inoculation density of 10^6 cfu/g of inoculated pathogen, the mean blotch prevalence decreased steeply from 35% in the first flush to 14.8% and 2.5% in the second and third flush respectively.

There were no differences in the post-harvest quality of the mushrooms grown in peat-based and alternative casing soils, when assessed visually for cap size, broken veil, and other deformities (Supplementary Figure 4). However, other pests, pathogens and weeds were observed in the circular cropping system (Supplementary Figure 5). Mushrooms with brown blotch were found throughout the cultivation cycle, in all casing soil compositions. This was confirmed by detection of the causative agent, *Pseudomonas tolaasii*, on the mushroom caps via diagnostic Taqman^{TM}-qPCRs. In the third flush of all replicate experiments, two other competitive fungi from the genus *Peziza* and *Parasola* (formerly *Coprinus*) were also found growing in casing soils composed of unsteamed grasses. Small patches of green mould (*Trichoderma*) were also observed in the third flush, in multiple casing soil types. In the first replicate experiment, towards the end of the third flush, several pests such as mites, gall midges (*Mycophila speyeri*), and scarid flies (*Lycoriella auripila*) also emerged. They could not be associated to a specific casing soil. These pests were absent from the second replicate experiment.

Discussion

The role of microbiomes in casing soils

Mushroom cultivation is different from other horticultural crops in that is it done on a two-component growing media. Firstly, the *A. bisporus* mycelium is grown through a substrate of aerobically fermented compost (Sánchez, 2004). Secondly, a layer of peat-based casing soil is applied on top, which induces fructification of the crop (Rainey, 1989). Other non-microbial functions of the casing soils include, providing physical support to the developing sporophores, acting as a water reservoir for the mycelium and preventing compost desiccation (Noble et al., 1999). Casing soil microbes that stimulate mushroom development include, *Bacillus psilocybe* (Stamets and Chilton, 1983), *Pseudomonas putida* (Hayes et al., 1969), *Bacillus megaterium, Arthrobacter terregens, Rhizobium metiloff* and a blue-green alga, *Scenedesmus quadricauda* (Stamets and Chilton, 1983). Some of them are saprophytic such as *P. putida*, and others are speculated to remove volatile inhibitors

of fruiting body formation. Pseudomonads have received significant attention for their stimulatory effect on sporocarp initiation and development. However, several *Pseudomonas* species can cause bacterial blotch diseases (Taparia et al., 2020a). Some pathogenic microorganisms endemic to the casing soil are *Mycogone perniciosa* (wet bubble disease) and *Trichoderma* species (green mould disease) (Fletcher and Gaze, 2007).

The casing soil microbiome of the peat and alternatives, not only supplies beneficial microorganisms to induce fructification of the mushrooms, but it also determines the invasion resistance of the community in the event of a pathogen introduction. This invasion resistance is often determined by the diversity of the resident community and the complexity and stability of its interaction network (Latz et al., 2012; Mallon et al., 2015). Resistant microbial communities are known to show high modularity and complexity instead of a compact interaction network (Mendes et al., 2018). Modularity in the network suggests diversity in species roles and functionality, and consequently efficient consumption of available resources (Poudel et al., 2016). Thus, a modular microbial network implies ecological robustness and an ability to maintain community-level interactions despite fluctuations in the member species or the environment. Lack of modular interactions in the soil microbiome could thus increase the success of a pathogen invasion (van Elsas et al., 2012; Wei et al., 2015).

The overall species richness, or alpha diversity of the casing soil increased with the ratio of peat substitution. It is expected that addition of organic components like moss (25%), and grass fibres (50%), and spent casing (30%) increased the diversity of the casing soil, as it also increases the nutrient status of the soil. The alpha diversity of the casing soil also increased across the cultivation cycle, in agreement with previous reports of the casing soil microbiome (Carrasco et al., 2019). It can be suspected that many bacterial taxa were present below the detection limit at the beginning of the cultivation cycle, and the increased colonization of the casing soil by the *A. bisporus* mycelium, acts as a nutrient source for the growth of these soil bacteria. This is also supported by previous findings that report both increased alpha diversity and soil suppressiveness to bacterial blotch in later cultivation cycles (Taparia et al., 2020c). Furthermore, it can also be speculated that the bacterial diversity in the casing soil increases from increased contact time with the compost. The casing soil can acquire bacteria from the compost, which has a unique microbiome to that of the casing soil (Carrasco et al., 2019).

The total bacterial microbiome of the casing soil was rich and diverse, and comprised of many rare and low abundance organisms, however, they were not represented in the core microbiome of the casing soils which was relatively small. The core fungal microbiome of the casing soil largely comprised of the mushroom of interest, *Agaricus bisporus*, as expected (Carrasco et al., 2019; Pecchia et al., 2014).

However, several thermophilic species, such as *Meliniomyces* and *Mycothermus* were also abundant in the core microbiome, and were not lost after steam treatment. In circular horticulture, where growing media originate from other agricultural, forestry or industrial waste streams, sufficient attention also needs to be paid to study the propagation of human and plant pathogens. In our experiments, we could not detect the presence of known human or plant pathogens in the microbiome of the casing soil prepared from the alternatives, but several unidentified bacterial taxa were detected belonging to genera which could comprise of human or plant pathogens. Most of these genera include only a few pathogenic variants. Unfortunately, amplicon-targeted sequencing does not allow accurate identification on a species or strain level, as the taxonomic resolution is limited due to the short read length.

Peat as a primary component of casing soil

Peat has been the primary component of casing soils in mushroom cultivation, since the 1950's due to its suitable physico-chemical and microbiological properties (Flegg, 1953). In our experiments, peat-based casing soils had one of the highest yields among the unsteamed soils, which is associated with its high water holding capacity and moisture content (Noble et al., 1999; Rainey, 1985). Peat also had a minimal fungal microbiome, that lacked an ecological network. Peat is generally known to be free of pathogens, and the competitive superiority of *Agaricus*, could also be partially attributed to the low densities of other weed fungi in the casing soil during cultivation (Carrasco et al., 2019). The reduced abundance of- and interactions between- other competing weed fungi, in an otherwise *Agaricus* rich environment, also encourages high productivity. The mean blotch prevalence was highest in casing soils composed of steamed and unsteamed peat. This can be attributed to the composition, interaction and activity of casing soil microorganisms. The core bacterial microbiome was least diverse in peat-based casing soils. Peat had one of the lowest soil respiration rates and organic matter content, which also point towards low microbial activity in the soil. The bacterial interactions in peat were compact and dense, and lacked modular interactions, implying a microbial community that does not occupy different ecological niches, and is likely not invasion resistant.

Heat treatment of the peat, before preparation of the casing soil, led to a large increase in productivity. This is contrary to the well-documented loss of yield in heat sterilized casing soils (Lambert and Humfeld, 1939; Pizer and Leaver, 1947), which led to the important discovery of the role of the casing soil microflora in fructification of mushroom bodies (Arrold, 1972; Hume and Hayes, 1972; Smith and Hayes, 1972). In our experiments, after the peat in the casing soil was heat treated, the casing soil was mixed with fully colonized phase III compost, by a process called "CAC-ing" (MacCanna and Flanagan, 1972). It potentially allowed beneficial bacteria

that are missing from the heat treated casing soil microbiome to be re-supplied via the compost (Reddy and Patrick, 1990) and promotes earlier pinning of the mushroom caps and increased yields (Kertesz and Thai, 2018). Additionally, the *A. bisporus* mycelium was able to colonize the casing soil more easily due to reduced competition from a resident soil microbial community. However, the highest disease pressure was also observed for casing soils composed of heat treated peat. While the blotch prevalence in steamed peat soils without added pathogen was negligible. Once the pathogen was introduced, steamed peat was the most susceptible casing soil to bacterial blotch. Heat treatment further reduced the microbial community composition of the peat, which failed to prevent the establishment of rising pathogen populations, in the event of a pathogen invasion.

Performance of peat alternatives

Sphagnum moss was high in water holding capacity, moisture content and bulk density, similar to black peat, however, it also had high respiration and organic matter content, similar to the grass fibres. In texture, it had intermediate hardness and adhesiveness. Its bacterial and fungal microbiomes were very similar to that of peat, although they were more diverse and relatively abundant, especially at the species level. The co-occurrence network topologies were also alike, indicating similar interaction within microbial community. When peat moss was used to proportionally replace 25% of peat in the casing soil, it had equivalent productivity and disease pressure to that of peat-based casing soil. These high yields can be attributed to its high soil moisture retention curves which are known to encourage fructification (Noble et al., 1999; Rainey, 1989). Heat treatment of peat moss, before preparation of the casing soil, did not have a significant effect on the productivity or disease pressure of the circular cropping system. Steaming of the peat moss significantly reduced the diversity of the bacterial microbiome, although the relative abundance of *Pseudomonas* sp. remained unaffected. Peat moss also had very low dry weight, which implies reduced transportation costs of the raw material.

Grass-based casing soils had lower yields compared to other alternatives, but grasses were used to proportionally replace a much higher amount of peat in fresh casing, equivalent to 50%. The lower productivity can be partially ascribed to their inability to act as a water reservoir (Kalberer, 1990). Casing soil composed of grass fibres had the highest increase in soil moisture when saturated with water, however, they also had the lowest soil moisture retention curves, implying that they are quick to absorb and release soil moisture. Their low water holding capacity may depend on their structure, in which they differ significantly from peat in both hardness and adhesiveness. However, the need for casing soil to be a good buffering system for water, is steadily reducing with automated and frequent watering of mushroom beds in commercial farms (Sánchez, 2004). Genomic and transcriptomic analyses of *A. bisporus* show its adaptation to humic rich and partially decomposed plant material

(Morin et al., 2012). Yet the grass fibres were also a competitive environment for *A. bisporus* mycelium to colonize, due to highly diverse bacterial and fungal resident community. This is also evident from their microbial network which was highly connected and modular. Their soil respiration rates and organic matter content, were also indicative of high microbial activity.

Acidification of the grass fibres did not have a clear effect on the agronomical performance of the casing soil or its physical and chemical properties. The pH of the alternative casing soil was not significantly lower at the start of the experiment, because the acidification was neutralized in the preparation of the casing soil by addition of sugar beet lime. Although there was no overall difference in the microbial diversity, the relative composition of the bacterial and fungal microbiome did change after acidification, mostly at the species and strain level for soil bacteria. Changes in soil fungi were observable at the genus level too, where *Pseudeurotium* sp. and *Dipodascus* sp. decreased in relative abundance, and *Ascobolus* sp. and *Solicoccozyma* sp. increased. Contrary to other reports, acidification did not lead to an increase in *Bacteroidetes* and *Firmicutes* (Abendroth et al., 2017). The microbial co-occurrence network reduced in size after acidification, implying fewer interactions within the microbial community. Heat treatment of the grass fibres had no effect on productivity, although steamed acidified grasses, had the lowest disease pressure overall. While, acidification of the grasses increased the relative abundance of *Dyadobacter* sp. in the casing soil, heat treatment increased the relative abundance of *Saitozyma* sp. Both of these have been previously associated with blotch suppressiveness in the casing soil (Taparia et al., 2020c). Heat treatment also reduced the abundance of other weed and pathogenic fungi, such as *Coprinus* sp., *Peziza* sp., and *Trichoderma* sp. in the casing soil, which is supported by an earlier finding (Park et al., 1971). However, steamed grasses had one of the lowest moisture levels at the end of the cultivation cycle, and require frequent watering. Acidified grass fibres had the lowest dry and fresh weight by volume.

Spent casing, as expected, had similar physical, chemical and structural properties, to that of black peat, namely, water holding capacity, bulk weight, dried weight, organic matter content, soil respiration and texture. At the beginning of the cultivation experiment, spent casing had the highest electrical conductivity, due to soluble salts that were deposited from the previous cultivation cycles (Pardo-Giménez et al., 2011). However, at the end of the cultivation experiment the EC was equivalent for all alternatives. If its proportional use in casing soil needs to be increased, the high EC could pose a problem. Recycling of the same casing soil multiple times, can potentially lead to accumulation of soluble salts, which would need to be leached out (Gonani et al., 2011). Despite the heat treatment of spent casing, its microbial community composition was similar to that of peat, although the bacterial diversity was much higher. It's fungal microbiome additionally comprised of *Saitozyma* sp., *Solicoccozyma* sp., *Trichoderma* sp., and an unidentified fungus, which were largely

absent from peat-based casing soils. Similar findings were reported from spent compost (Eicker and van Greuning, 1989). When spent casing was used to proportionally replace 30% peat in fresh casing soil, the productivity of spent casing was equivalent to that of peat, although the disease pressure was much lower. Spent casing showed a high suppression of ginger blotch, at higher pathogen densities and also in earlier flushes. This can be attributed to the increased abundance of endemic *Pseudomonas* sp. in spent casing, which have a niche overlap with the pathogen. However, in personal communication, farmers are reluctant to reintroduce spent casing soil in their farms over concerns of pests and pathogen management.

Accessibility and sustainability

Four circular and sustainable alternatives, *Sphagnum* moss, grass fibres, acidified grass fibres and spent casing can be used to proportionally replace black peat in growing media. Their agronomical performance depends on their related physical, chemical and microbiological characteristics. However, their future use also depends on their accessibility and sustainability. *Sphagnum* moss in growing media has been shown to perform well on a wide variety of crops (Blievernicht et al., 2012; Emmel, 2005; Oberpaur et al., 2010). However, degraded peat-lands on which *Sphagnum* farming is practised, needs to be constantly water-saturated. Even though the land area is widely available, it requires substantial investment in site preparation and irrigation to support a high enough water table (Gaudig et al., 2014). Several studies show the feasibility of large-scale *Sphagnum* farming, which has been practised in Germany, the Netherlands, Latvia, Georgia and Canada (Gaudig et al., 2017). Economic analyses reveal that the use of *Sphagnum* moss in growing media is currently profitable only for niche markets with high revenues, such as orchid cultivation, but it cannot compete with the low cost of black peat. However, it is predicted that at a surcharge of ~10% to the end-consumers, it is economically viable to completely substitute black peat for *Sphagnum* moss for other horticultural crops as well (Wichmann et al., 2020).

Grass fibres used in this study were produced from agricultural residue streams of mixed origins within the Netherlands, that underwent a patented biorefinery process for conversion to lignocellulose fibres. These can include sugar cane bagasse, sorghum, corn cobs, corn stover, rice straw, nut shells and grass clippings. Europe generates about 700 million tonnes of agricultural waste annually (Pawelczyk, 2005), implying that large amounts of lignocellulosic biomass are available for valorisation. It is thus essential to further develop circular bio-economies with integrated processes (Székács, 2017), to simultaneously reduce both resource consumption and waste generation, thereby, mitigating the environmental impact associated with food production (Commission, 2014; Maina et al., 2017). The major limiting factor in availability of these grass fibres is the biorefinery process, which currently only has one full-scale facility, with a production capacity of 1000 kg fibres per hour. A second

drawback of the use of these waste streams is the inconsistency in substrate quality, resulting in an unreliable agronomical performance. This can be accounted for by partially mixing with standardized growing media like peat.

Spent casing is an abundant and local resource. The production of 1 kg of mushrooms, generates 5 kg of residual material called spent mushroom substrate (SMS) (Lau et al., 2003). In the Netherlands, more than 800,000 tonnes of SMS are produced per year (Oei and Albert, 2012). SMS disposal is a big challenge for mushroom farmers due to regulations from the EU nitrate Vulnerable Zones. An average farm discards about 24 tonnes of SMS per month (Singh et al., 2011), of which 4.8 tonnes comprises spent casing. However, the limiting factor is the separation of the casing soil from the compost. A perforated plastic meshes can be layered between the compost and the casing soil during filling of the room (Farsi et al., 2011; Royse et al., 2008). A MushComb machine can also be used, which allows mechanical recovery of 50-75% of the casing soil (Oei and Albert, 2012). Cost benefit analysis of spent casing soil reveals a significant initial investment, for the casing separator machine and trailer to separate the casing soils, and the hopper and conveyor belt for recycling into fresh casing (Noble and Dobrovin-Pennington, 2015). However, the SMS disposal costs are reduced by 12% and fresh casing soil costs are reduced by 30% (Noble et al., 2015).

Future prospects for circular horticulture

Like most agro-ecological systems, strong trade-offs exist between productivity and disease pressure in mushroom cultivation with the use of peat alternatives. The watering methods, growing conditions and environment currently employed for mushroom cultivation have been optimized for peat-based casing soils over the last 30 years. However, the physical and chemical properties of the alternative casings largely differ from that of peat. Optimization of the growing conditions can play an important role in influencing the productivity and disease pressure of circular cropping systems. It can be speculated that with the complete replacement of peat, currently known soil-borne diseases that are introduced from peat-based casing soil will decline. However, it is also expected that other pests, pathogens and competitive fungi native to the peat-alternatives, may affect the performance of the alternative casing soils, as was observed for unsteamed grass-based casing soils. Steam treatment of the grass fibres resulted in lack of other competitive fungi. Hence, it is also important to explore other methods to manage the microbiome of the peat alternatives, such as biostimulants, peak-heating, disinfection, fermentation, acidification or via storage.

Trade-offs in circular cropping systems are also driven by economics, energy demands and sustainability of the cropping process, which need to be further investigated. Life cycle assessments are required to quantify the environmental

impact of using these peat alternatives. Cost-benefit and supply chain analyses of these peat-reduced growing media are also essential to determine the economic suitability for industrial use. An optimal peat replacement ratio needs to be determined, which balances agronomical performance with environmental impact and economic viability. Common concerns about using circular alternatives in food production systems also involve the accumulation of toxic compounds such as heavy metals, pesticide residues and microplastics. The alternative growing media and its produce need to be screened for presence of these elements, for food safety, but also to meet regulatory limits within the EU for disposal of organic wastes. The identity of and risk from human and plant pathogens needs to be established with the help of genomics and cultivation experiments. Finally, sanitisation of the peat alternatives with methods like steaming, dry heat, composting, irradiation, solarisation, dry or cold storage need to be explored to eliminate the propagation of pests and pathogens that can be detrimental to human health and crop health.

Conclusions

We demonstrated the successful use of three circular alternatives to proportionally replace peat in mushroom casing soils, namely, grass fibres from agricultural residue streams, peat-moss from degraded peatlands, and spent casing soil recycled from previous cultivation cycles. Specific physical and chemical properties of the casing soils that influence productivity varied between the peat-alternatives, namely, water holding capacity, moisture content, pore fraction, soil respiration, electrical conductivity and organic matter content. Peat moss and spent casing were expectedly similar to peat, but the grass fibres had unique structure and physico-chemical characteristics, such as high organic matter content and low water holding capacity. There were no visual differences in the post-harvest quality of the mushrooms grown in peat-based and alternative casing soils.

The disease pressure in the alternative casing soils also varied between the peat-alternatives. Steamed and acidified grass fibres showed the highest suppression of ginger blotch, closely followed by steamed spent casing. The microbial community composition of the alternative casing soils varied significantly from that of peat. Grass fibres had the most diverse microbiomes, and their pre-treatment by acidification and steaming significantly reduced the presence of pests, competitive fungi and pathogens in the casing soil. Strong trade-offs existed between the productivity and disease pressure in the alternative casing soils. These are also governed by the accessibility, sustainability, and economic viability of the peat-alternatives.

Present methods and conditions for mushroom cultivation have been optimized for the use of peat-based casing soils over the last 30 years, and they require to be re-adjusted in order to improve the productivity and decrease the disease pressure in

alternative casing soils. This knowledge on the physical, chemical, and microbiological properties, as well as the agronomical performance of peat-alternatives, is instrumental to transition away from peat use and towards circular and sustainable growing media in horticulture.

Authors contributions

TT, EH, and JW designed the experiments. TT and EH, performed the bioassay. TT, and EN did the molecular analyses. TT wrote the manuscript. All authors contributed to subsequent manuscript revision, read and approved the submitted version.

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Supplementary Tables

Table 1. MANOVA on properties of alternative raw materials before mixing peat						
Respiration	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Sig code
Alternative material	4	1533.97	383.49	3.42	0.04	*
Steam treatment	1	0.46	0.46	0	0.95	
Residuals	12	1344.04	112			
Organic matter						
Alternative material	4	4230.4	1057.59	34.42	< 2e-16	***
Steam treatment	1	10.6	10.56	0.34	0.57	
Residuals	12	368.7	30.72			
Dried Weight						
Alternative material	4	3140.71	785.18	15.4	< 2e-16	***
Steam treatment	1	0.01	0.01	0	0.99	
Residuals	12	611.96	51			
Moisture content						
Alternative material	4	104.14	26.04	0.84	0.53	
Steam treatment	1	1.08	1.08	0.03	0.85	
Residuals	12	372.19	31.02			

Supplementary Table 1. Multivariate ANOVA on physico-chemical properties of alternative raw materials such as soil respiration, organic matter content, moisture content and dried weight.

Table 2. Other casing soil properties during pf measurements						
Moisture content	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Sig code
Alternative material	4	22.49	5.62	11.7	0.04	*
Steam treatment	1	0.24	0.24	0.5	0.53	
Residuals	3	1.44	0.48			
Organic matter						
Alternative material	4	14.88	3.72	0.48	0.76	
Steam treatment	1	3.28	3.28	0.42	0.56	
Residuals	3	23.26	7.75			
Bulk density						
Alternative material	4	1113.35	278.34	26.22	0.01	*
Steam treatment	1	30.03	30.03	2.83	0.19	
Residuals	3	31.84	10.62			
Shrinkage						
Alternative material	4	1562.01	390.5	51.28	< 2e-16	**
Steam treatment	1	2.53	2.53	0.33	0.6	
Residuals	3	22.84	7.61			
pF curve						
Alternative material	4	3522	881	10.95	< 2e-16	***
Steam treatment	1	9	9	0.11	0.75	
Residuals	101	8125	80			

Supplementary Table 2. Multivariate ANOVA on soil water retention and related measurements such as moisture content, organic matter, bulk density, shrinkage and pF curves of alternative casing soils.

Table 3. Soil physico-chemical properties during “CAC-ing”						
Increase in moisture content when saturated with water	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Sig code
Alternative material	4	1.72	0.43	6.62	0.01	**
Steam treatment	1	0.12	0.12	1.78	0.21	
Residuals	10	0.65	0.07			
Water holding capacity						
Alternative material	4	0.67	0.17	3.51	0.05	*
Steam treatment	1	0.04	0.04	0.85	0.38	
Residuals	10	0.48	0.05			
Dried weight						
Alternative material	4	1822	455.5	3.4	0.05	.
Steam treatment	1	38.48	38.48	0.29	0.6	
Residuals	10	1338.37	133.84			
Fresh weight						
Alternative material	4	78095	19523.7	21.06	< 2e-16	***
Steam treatment	1	61	60.6	0.07	0.8	
Residuals	10	9269	926.9			
Hardness						
Alternative material	4	7.00E+08	1.80E+08	35.05	< 2e-16	***
Steam treatment	1	3.50E+06	3.50E+06	0.69	0.43	
Residuals	10	5.00E+07	5.00E+06			
Adhesiveness						
Alternative material	4	2.90E+06	7.30E+05	15.59	< 2e-16	***
Steam treatment	1	5.40E+03	5.40E+03	0.12	0.74	
Residuals	10	4.70E+05	4.70E+04			

Table 3. Multivariate ANOVA on water holding capacity, increase in moisture content, dried weight, fresh weight, hardness and adhesiveness of alternative casing soils immediately after ”CAC-ing” (T0).

Table 4. MANOVA on soil properties at the start of the cultivation cycle (T0) and at the end of the 3rd flush (T4)						
Moisture content	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Sig code
Alternative material	4	52.72	13.18	2.23	0.08	.
Steam treatment	1	64.87	64.87	10.99	< 2e-16	**
Time point	1	2829.17	2829.17	479.52	< 2e-16	***
Residuals	38	224.2	5.9			
pH value						
Alternative material	4	0.19	0.05	6.01	< 2e-16	***
Steam treatment	1	0.01	0.01	0.67	0.42	
Time point	1	0.06	0.06	8.34	0.01	**
Residuals	38	0.29	0.01	8		
Electrical conductivity						
Alternative material	4	0.98	0.24	9.2	< 2e-16	***
Steam treatment	1	0.01	0.01	0.48	0.49	
Time point	1	4.05	4.05	152.8	< 2e-16	***
Residuals	38	1.01	0.03			

Table 4. Multivariate ANOVA on pH, moisture content and electrical conductivity of alternative casing soils across the cultivation cycle. Compared during “CAC-ing” (T0) and at the end of the third flush (T4).

Table 5. ANOVA on bacterial species richness (diversity) of alternative casings						
Variables	Df	Sum Sq	Mean Sq	F value	Pr(>F) value	Sig. code
Alternative material	4	635192	158798	25.97	< 2e-16	***
Heat treatment	1	37214	37214	6.086	1.41E-02	*
Pathogen density	1	1629	1629	0.266	0.606	
Time point	3	1006306	335435	54.857	< 2e-16	***
Internal replicates	1	8103	8103	1.325	0.2504	
Replicate experiment	1	101993	101993	16.68	5.44E-05	***
Residuals	367	2244111	6115			

Table 5. ANOVA on bacterial species richness (diversity) of alternative casings

Table 6A. PERMANOVA on beta diversity of core bacterial community of alternative casings							
Variables	Df	Sum Sq	Mean Sq	F value	R squared	Pr(>F) value	Sig. code
Alternative material	4	6.057	1.5143	5.581	0.04875	0.001	***
Heat treatment	1	1.128	1.1278	4.156	9.08E-03	0.001	***
Pathogen density	1	0.269	0.2686	0.99	0.00216	0.43	
Time point	3	7.738	2.5794	9.506	0.06228	0.001	***
Internal replicates	1	0.265	0.2649	0.976	0.00213	0.497	
Replicate experiment	1	9.219	9.2193	33.978	7.42E-02	1.00E-03	***
Residuals	367	99.579	0.2713	0.80141			

Table 6B. PERMANOVA on beta diversity of core fungal community of alternative casings							
Variables	Df	Sum Sq	Mean Sq	F value	R squared	Pr(>F) value	Sig. code
Alternative material	4	24.447	6.1118	31.091	0.22449	0.001	***
Heat treatment	1	1.262	1.2623	6.421	1.16E-02	0.001	***
Pathogen density	1	0.1	0.0997	0.507	0.00092	0.898	
Time point	3	3.514	1.1714	5.959	0.03227	0.001	***
Internal replicates	1	0.091	0.0913	0.465	0.00084	0.935	
Replicate experiment	1	7.539	7.5393	38.353	6.92E-02	1.00E-03	***
Residuals	367	71.948	0.1966	0.66066			

Table 6. PERMANOVA on beta diversity of core (A) bacterial and (B) fungal community of alternative casings

Table 7A. Co-occurrence network topology of core bacterial community of alternative casings								
Alter-natives	No. of edges	No. of vertices	Transitivity (clustering coefficient)	Average path length	Modularity	Graph density	Node degree	Node density
Peat	211	94	0.1947701	3.424388	0.524831	0.0482727	4.4893617	7
Peat moss	221	100	0.1784081	3.6979798	4.99E-01	0.0446465	4.42	9
Grass fibres	185	92	0.2111732	4.0487339	0.5246311	0.0441949	4.0217391	9
Acidified grass	157	91	0.1621622	4.1472527	0.6019717	0.0383394	3.4505495	9
Spent casing	188	104	0.1683029	4.3149739	0.6098772	0.0351008	3.6153846	10

Table 7B. Co-occurrence network topology of core fungal community of alternative casings								
Alter-natives	No. of edges	No. of vertices	Transitivity (clustering coefficient)	Average path length	Modularity	Graph density	Node degree	Node density
Peat	1	2	NA	1	-0.5	1	1	1
Peat moss	10	9	0.15	2.0555556	2.20E-01	0.2777778	2.2222222	4
Grass fibres	39	35	0.1785714	3.7640449	0.6236029	0.0655462	2.2285714	8
Acidified grass	11	13	0.1764706	1.7142857	0.5289256	0.1410256	1.6923077	3
Spent casing	7	10	0	1.6428571	0.5816327	0.1555556	1.4	3

Table 7. Topological characteristics of (A) bacterial and (B) fungal co-occurrence networks from the core microbiome of alternative casings

Table 8. ANOVA on productivity (yield) of alternative casings						
Variables	Df	Sum Sq	Mean Sq	F value	Pr(>F) value	Sig. code
Alternative material	4	715.7	178.9	44.27	< 2e-16	***
Heat treatment	1	64.3	64.3	15.91	9.21E-05	***
Pathogen density	1	12.9	12.9	3.185	0.0758	
Internal replicates	1	0.1	0.1	0.029	0.8657	
Replicate experiment	1	1469.6	1469.6	363.59	< 2e-16	***
Residuals	207	836.7	4			

Table 8. Univariate ANOVA on productivity (yield) of alternative casing soils.

Table 9. ANOVA on disease pressure (ginger blotch prevalence)						
Variables	Df	Sum Sq	Mean Sq	F value	Pr(>F) value	Sig. code
Alternative material	4	0.29	0.073	6.261	6.05E-05	***
Heat treatment	1	0.088	0.088	7.556	0.00615	**
Flush (harvest cycle)	1	3.575	3.575	308.45	< 2e-16	***
Pathogen density	1	1.175	1.175	101.36	< 2e-16	***
Internal replicate	1	0.001	0.001	0.121	0.72844	
Duplicate experiment	1	0.012	0.012	1.028	0.31091	
Residuals	638	7.395	0.012			

Table 9. Univariate ANOVA on disease pressure (ginger blotch prevalence) of alternative casing soils.

Supplementary Figures

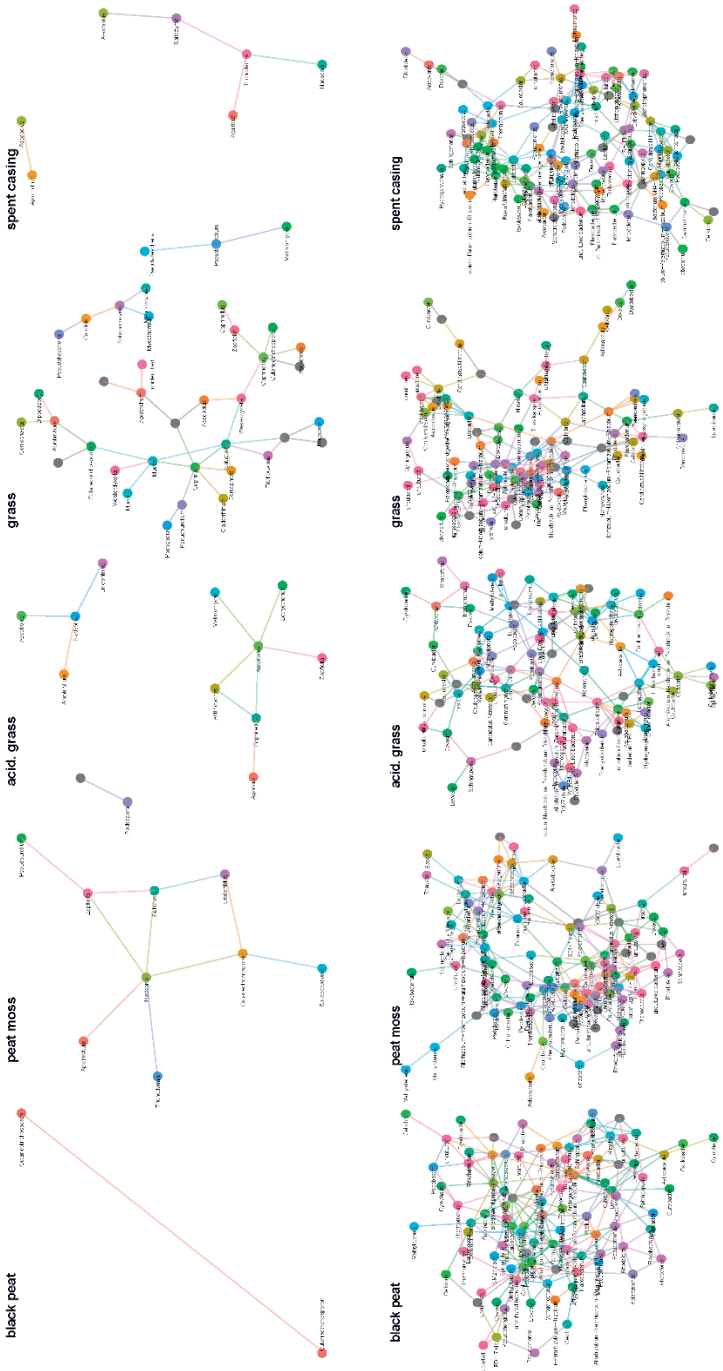

Figure 1. Co-occurrence networks of the core (A) bacteria and (B) fungal microbiomes in alternative casing soils.

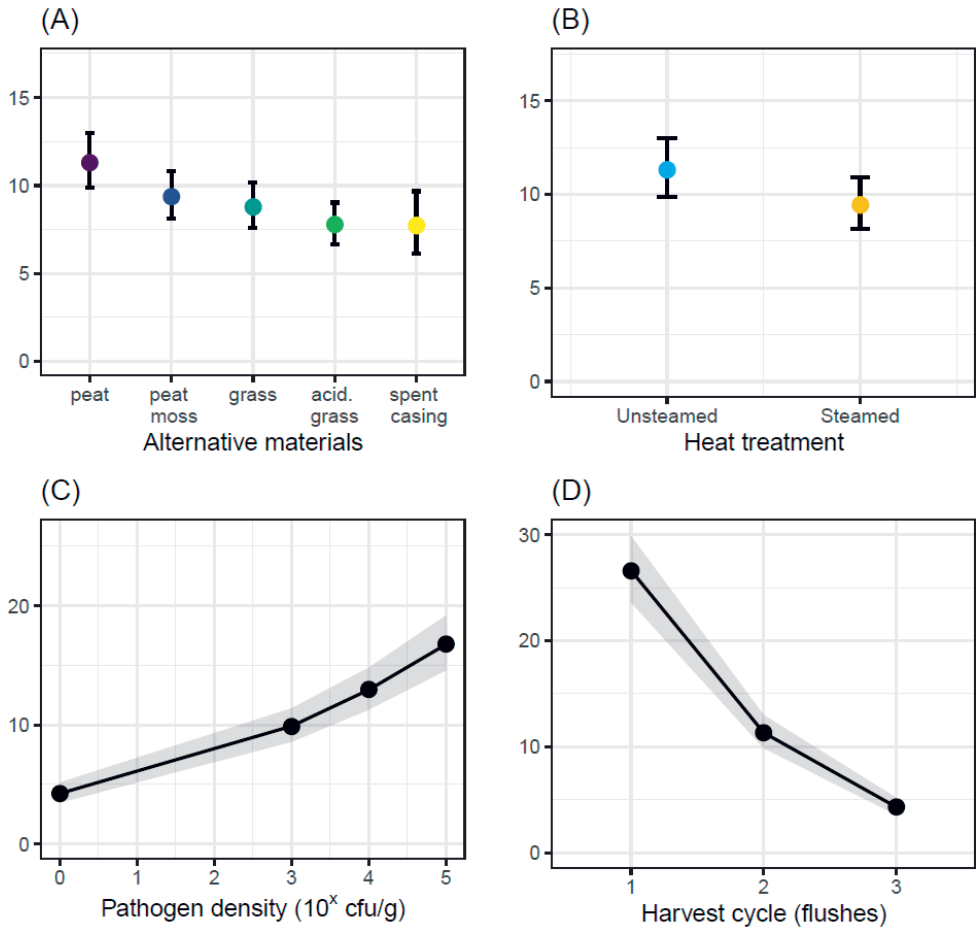

Figure 3. Linear regression on the disease pressure (ginger blotch prevalence) of A) alternative casings and the effect of (B) steaming C) pathogen density in soil D) consecutive harvest cycles.

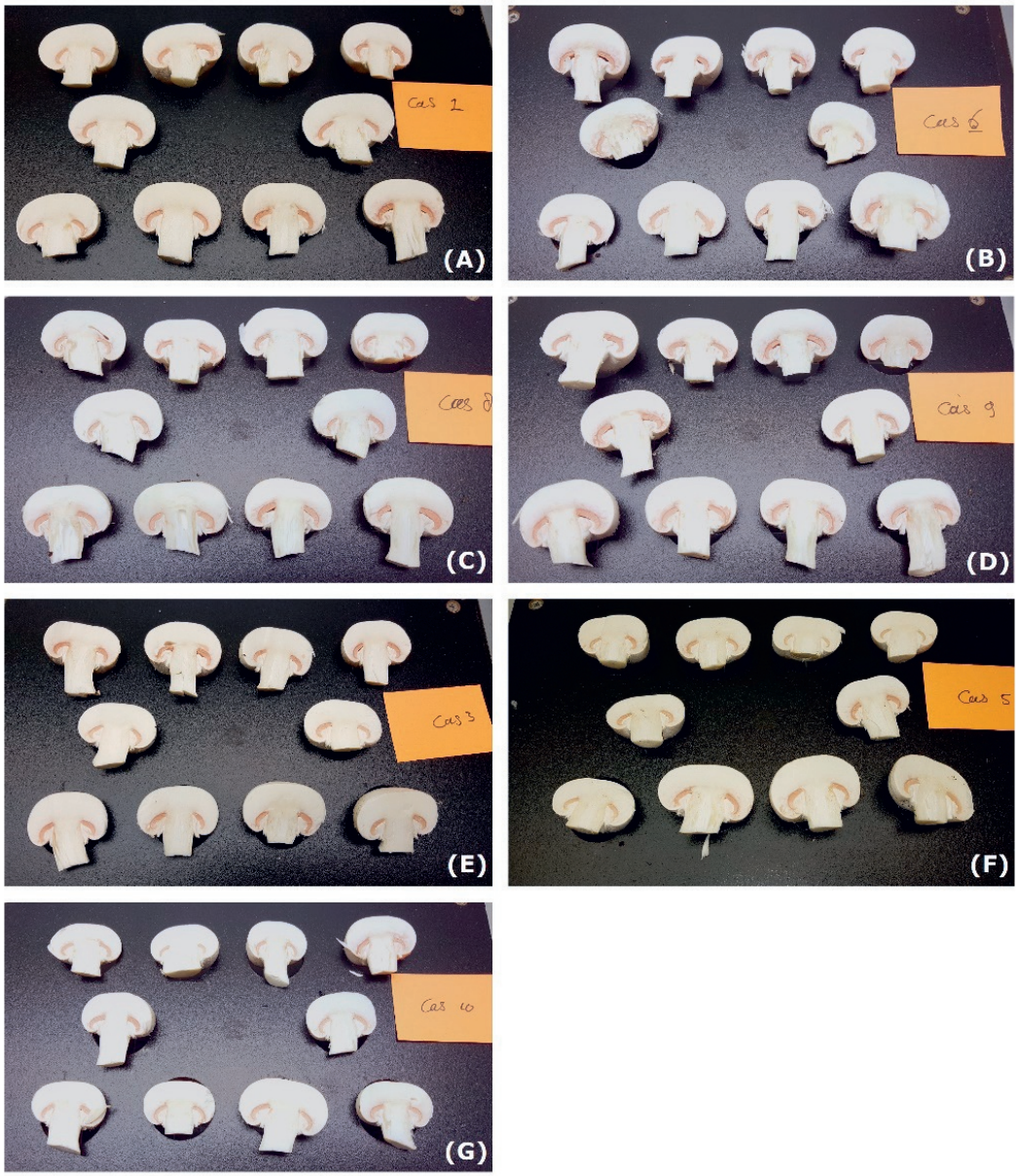

Figure 4. Visual differences in post-harvest quality of mushrooms grown in alternative casing soils.

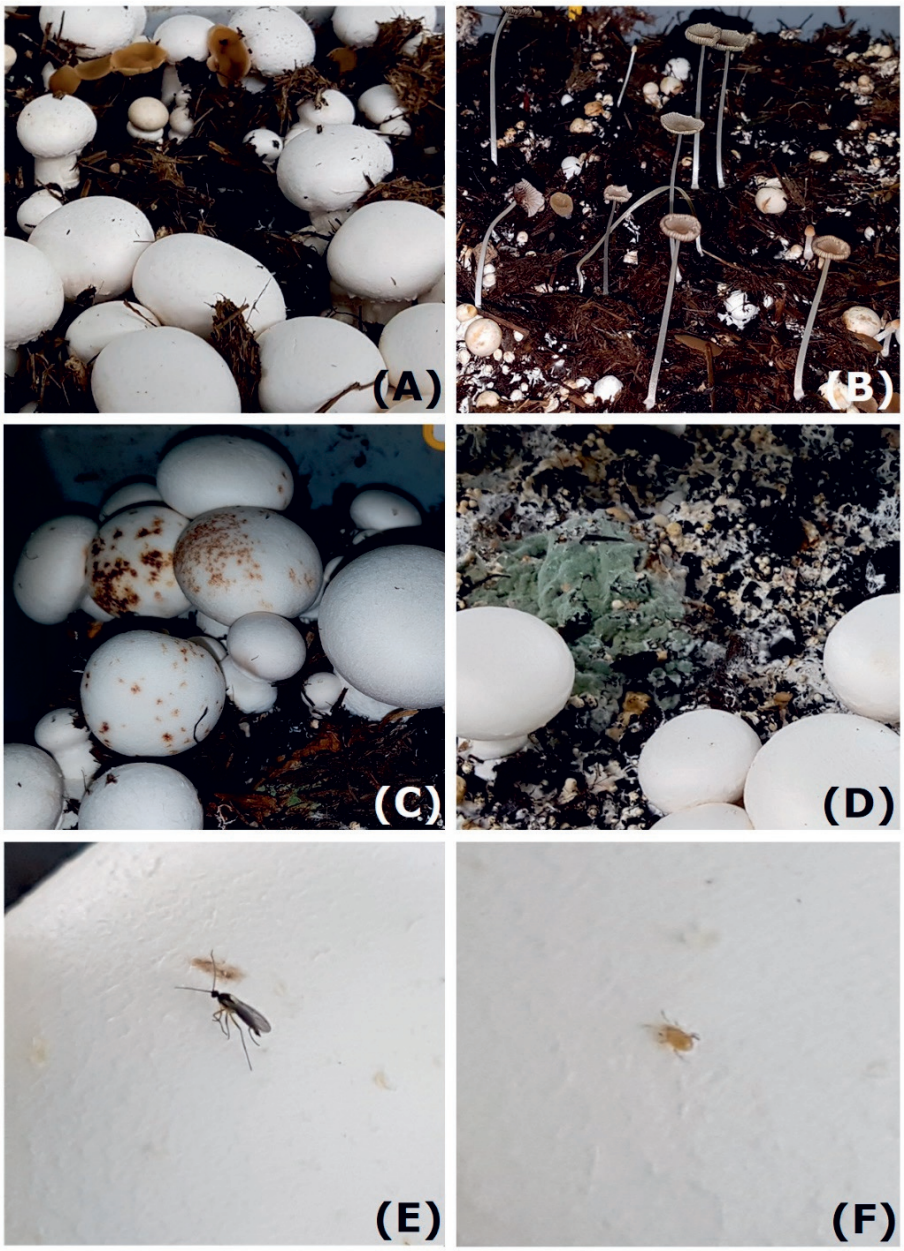

Figure 5. Other pests, pathogens, and competitive fungi growing in casing soil comprising of unsteamed grass fibres.

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Chapter 7

General Discussion

100 years of bacterial blotch

Bacterial blotch diseases of mushrooms are caused by diverse fluorescent *Pseudomonas* species (Fletcher and Gaze, 2007), endemic to the casing soil used in mushroom farms (Wong and Preece, 1980). *P. tolaasii* is the first described bacterial blotch pathogen (Paine, 1919; Tolaas, 1915), and majority of the research on bacterial blotch in the last century has focused on this pathogen. Its mechanism of action involves an extracellular toxin tolaasin (Soler-Rivas et al., 1997), whose biosynthesis and genetic regulation have been well-documented (Grewal et al., 1995; Han et al., 1994; Rainey et al., 1993). A few other blotch pathogens, such as '*P. gingeri', P. agarici, P. costantinii, 'P. reactans', P. marginalis* and *P. fluorescens* have been identified in the meantime (Elphinstone and Noble, 2018). However, the identity, nomenclature and diversity of blotch-causing *Pseudomonas* remained largely unclear.

The advent of -omics technologies and advances in prokaryotic genomics have significantly facilitated the molecular identification of bacterial pathogens. In **Chapter 2** of this thesis, we studied the phylogenetic and phenotypic diversity of blotch-causing organisms in Western Europe using NGS methods. From our molecular characterization of bacteria colonizing diseased mushroom caps, we recovered many known pathogens, *P. tolaasii, 'P. gingeri', P. agarici, P. costantinii,* and '*P. reactans',* but we also discovered seven novel blotch pathogens, namely, *P. yamanorum, P. edaphica, P. salomonii* and strains that clustered with *Pseudomonas* sp*. NC02* in one genomic species, and three non-pseudomonads, i.e. *Serratia liquefaciens, S. proteamaculans* and a *Pantoea* sp. (Taparia et al., 2020a). It also led to several taxonomic corrections of strains from international culture collections (Taparia et al., 2020a). In addition, we also obtained evidence that the ginger blotch pathogen, '*P. gingeri*', is part of a species complex, with multiple taxonomically-related species, as opposed to other brown blotch pathogens which form single phylotypes (Taparia et al., 2020a). This information provided significant advances over taxonomic assessments that only used the 16S rRNA (Godfrey, Harrow, Marshall, and Klena, 2001).

Moreover, we developed a detailed overview of the genetic and regional diversity of blotch-causing organisms in Western Europe. We observed that phylogenetic distribution of common pathogens is independent from their region of outbreak, indicating that Western Europe shares a well-mixed pathogen pool (Taparia et al., 2020a). However, a few potentially region-specific blotch pathogens were also recovered in this study. Yet this regional exclusivity is not certain, since only a few isolates of these species were present. Additionally, we reported the presence of several non-pathogenic and beneficial bacteria on mushroom caps as well (Taparia et al., 2020a). It has been suggested that not just the pathogen density, but also the composition of *Pseudomonas* species, especially the relative abundance of

beneficial and disease-causing species, can be an important indicator for disease outbreaks (Godfrey, 2003). This research on the identification of beneficial and pathogenic *Pseudomonas* also has implications for the study of symptomatic disease expression, development of diagnostic tools and design of localized strategies for disease management.

However, our work is far from finished. We also discovered four blotch-causing *Pseudomonas* spp., which do not belong to any of the existing species within the genus *Pseudomonas*. These are as yet unidentified, and cannot be described as potential new species on the basis of genomic analyses alone. Taxonomic assignment of these novel blotch-causing phylotypes as new species requires additional biochemical, phenotypic and chemotaxonomic experiments (Kämpfer and Glaeser, 2012). Additionally, we found that blotch-causing and non-pathogenic isolates often clustered together with each other (Taparia et al., 2020a). Identification of the pathogenicity determinants of bacterial blotch thus requires pan-genomic analyses, that can select targets for future studies (Freschi et al., 2019). The size and diversity of the pan-genome is often associated with clusters for biosynthesis of secondary metabolites that can be strain specific and responsible for pathogenicity (Loper et al., 2012).

A major leap in diagnostics

After the identification of prevalent regional blotch pathogens, the next important step is their early detection. Once the pathogen is introduced into a mushroom farm, secondary infections via pests, water splashing, mushroom pickers and mechanized harvesters is quick (Wong and Preece, 1980). The mesophilic and humid conditions required for mushroom cultivation further encourage the enrichment of the pathogen in the mushroom beds (Godfrey, 2003; Wong, et al., 1982). Given that management strategies for chemical, environmental or biological control of blotch are very limited (Fletcher and Gaze, 2007; Godfrey, 2003; Navarro, Gea, and González, 2018; Osdaghi et al., 2019) the need for timely, specific and quantitative detection methods is critical to predict and prevent blotch outbreaks.

Detection of *P. tolaasii* previously relied on microbiological methods, such as plating on selective media, colony morphology and phenotypic tests (Goor et al., 1986; Han et al., 1992; Lloyd-Jones et al., 2005; Wells et al., 1996; Wong and Preece, 1979). However, these methods cannot accurately distinguish the different blotch pathogens (Munsch and Alatossava, 2002; Rokni-Zadeh et al., 2012). Recently developed nested PCRs were only qualitative and could only detect tolaasin producing organisms (Lee et al., 2002). To extend the spectrum and increase the sensitivity of bacterial blotch determination, in **Chapter 3** of this thesis, we developed quantitative detection methods for molecular diagnostics of three blotch pathogens, in a variety of environmental samples.

We designed six Taqman™-qPCR assays to detect two loci on *P. tolaasii, 'P. gingeri'* and *P. salomonii* each. These assays demonstrated good analytical performance, including high diagnostic specificity and sensitivity, low limits of detection, and high repeatability and reproducibility (Taparia et al., 2020b). Additionally, these assays were multiplexed with those of *Xanthomonas campestris* (Köhl et al., 2011) as an extraction control for absolute quantification, and non-pathogenic representatives of the genus *Pseudomonas* (Lloyd-Jones et al., 2005) to quantify the pathogens relative to the beneficials (Taparia et al., 2020b). Application of these assays in our survey of mushroom farms revealed that *'P. gingeri'* and *P. salomonii* were the most prevalent pathogens in the Netherlands and Belgium, comprising 78% and 42% of the total infected mushrooms, whereas *P. tolaasii* infections were only 20% (Taparia et al., 2020b).

Taqman™-qPCR assays designed here, provide the first opportunity for quantitative high-throughput detection of blotch pathogens. We could detect 100-fold lower densities of blotch pathogens (Taparia et al., 2020b), compared to previous studies of the mushroom cap (Nair and Bradley, 1980; Nair and Fahy, 1972a; Olivier et al., 1997; Preece and Wong, 1982) and casing soil (Nair and Fahy, 1972a). This enabled us to now screen raw materials used in mushroom cultivation, such as spawn, peat, compost and casing soil for presence of bacterial blotch pathogens. Despite many advantages, these DNA based methods can only explain the abundance, and do not provide information on the viability of the pathogen cells (Vincelli and Tisserat, 2008; Wolffs et al., 2005). This can be addressed in two ways, either by the use of blocking agents that bind to DNA from dead-cells (Fittipaldi et al., 2012) or by performing selective enrichment on media prior to PCR (Schaad and Frederick, 2002).

Despite successful use of DNA-based detection methods in routine diagnostics for other agricultural pathogens (Bonants and te Witt, 2017; Ophel-Keller et al., 2008; Ward et al., 2004), many challenges need to be overcome before these assays can be used for routine monitoring of bacterial blotch pathogens at low population densities in growth substrates (composts, casing soils) and fungal structures (spawn, mushroom caps) during the different stages of mushroom cultivation cycle and post-harvest supply chain. Sampling strategies need to be developed, which consider the effect of storage, spatial location, seasonal variation, and bulk quantities. The prediction of an economic risk from determined pathogen population densities requires cautious interpretation of other disease indicators, since blotch outbreaks depend on a variety of other factors, which include environmental conditions, growing practices, cultivar type, source of compost and type of casing soil (Godfrey, 2003).

From a casing soil perspective

With the newfound knowledge that *'P. gingeri'* and *P. salomonii* were the most prevalent pathogens in mushroom farms of Western Europe, it was essential to study their infection and population dynamics in the battleground itself, the casing soil! In order to place quantitative information about pathogen densities into context, in **Chapter 4** of this thesis, cultivation trials were performed to obtain more insights on factors causing disease outbreaks by these pathogens. To this end, we determined their inoculum threshold in the casing soil for blotch outbreaks and studied pathogen population dynamics in mushroom beds, including the effect of casing soil type and environmental conditions, during the cultivation cycle.

Cultivation experiments revealed that *'P. gingeri'* caused disease outbreaks at lower inoculum thresholds (10^4 cfu/g) in the casing soil than *P. salomonii* (10^5 cfu/g) (Taparia et al., 2020c). Initially, research on pathogen inoculum thresholds was focused on the cap surface of the mushroom (Nair and Fahy, 1972a; Soler-Rivas et al., 2000), until it was established that recovered pathogen densities did not correlate with overall disease prevalence. Information on the soil inoculum thresholds has so far been limited to the brown blotch pathogen, *P. tolaasii,* which produces equivalent blotch prevalence to *P. salomonii*, but at a 10 fold higher density of $2x10^6$ cfu/g of (Grewal, 1991).

We found that the origin and composition of casing soils had a strong effect on the susceptibility of mushrooms to ginger and brown blotch, even though *'P. gingeri'* populations in the mushroom beds were not significantly different between the casing soils (Taparia et al., 2020c). Endemic pathogen populations of *P. salomonii* differed between the casing soil types, based on their peat source, in support of earlier findings, where raw peat was tested positive for presence of multiple blotch pathogens (Taparia et al., 2020b; Wong and Preece, 1980). This confirms the role of peat as a source of blotch pathogens into farms, although the role of compost cannot be excluded. Yet, it can be suggested that the control of blotch diseases by choosing the 'right' casing soil composition, with a low pathogen pressure, can be a way forward.

The two pathogens demonstrated different temporal dynamics, with ginger blotch generically declining in later harvest cycles, whereas brown blotch did not (Taparia et al., 2020c). In personal communication with mushroom growers, they indicated similar observations regarding ginger blotch decline in later flushes. Mushrooms from later flushes are also less susceptible to discoloration from bruising but this has been related to the post-harvest physiology of the mushroom (Burton and Noble, 1993). The apparent development of ginger blotch suppression in later cultivation cycles needs to be investigated for more casing soil types. Changes in soil microbiome composition and functioning, or in physico-chemical characteristics of

the soil may reveal mechanisms of development of ginger blotch suppressiveness. This could have an interesting perspective for blotch management practices, but requires further examination.

Ginger blotch suppressive soils

A closed cropping system, like mushroom cultivation is an ideal setup to improve our understanding of disease suppressive soils, as it is independent of agricultural land and the environmental conditions can be constantly regulated (Kulak et al., 2013; Marcelis et al., 2007). Due to developments in amplicon sequencing and microbiome research, the characterization of disease suppressive soils, their microbial diversity, composition, interactions and activity is now possible (Klein et al., 2013; Mendes et al., 2011). In **chapter 5** of this thesis, we explored the role of the casing soil composition and soil microbiome in the suppression of ginger blotch.

At first, we found ginger blotch declining with consecutive cultivation cycles consistently across ten casing soil mixtures, comprising of different peat sources and soil amendments. In contrast, the inoculated pathogen populations in casing soils, continued to increase in later cultivation cycles (Taparia et al., 2020d). Mushroom caps from the first and second flush were equally susceptible to ginger blotch in *in-vitro* cap tests, even though they are known to be differently susceptible to bruising (Burton, 1988; Burton and Noble, 1993). This indicates that the reduced blotch prevalence and severity in later flushes cannot be attributed to differences in post-harvest physiology or the development of induced resistance in *A. bisporus*. Induced systemic resistance to dry bubble pathogen, *Lecanicillium fungicola* was also absent in *A. bisporus* (Berendsen et al., 2013).

We suspected that the observed decline in ginger blotch may be attributed to the development of soil suppressiveness to *'P. gingeri'*, which was confirmed, as the effect was found to be partially transferable to conducive soils via an aqueous soil extract. Transferability has been indicated as an important characteristic of biological soil suppressiveness (Baker and Cook, 1974) and has been observed for the suppression of several soil-borne plant pathogens (Westphal and Becker, 2000). The development of ginger blotch suppression with consecutive mushroom harvesting cycles has remarkable similarities with temporal disease suppression patterns observed for plant crops grown in continuous monoculture, such as the decline of take-all disease caused by *Gaeumannomyes* in wheat or barley (Schreiner et al., 2010), or the suppression of root rot caused by *Rhizoctonia solani* in sugar beet seedlings (Expósito, 2017). Both of which have been attributed to the increased abundance of bacteria antagonist to the pathogen (Gómez Expósito et al., 2015).

Our studies revealed that long term and cold-storage of the casing soils, between replicate experiments reduced the soil suppressiveness to ginger blotch. We did not

assess how this affected the physico-chemical composition of the casing soil. But other studies have revealed that changes in soil composition via treatments or amendments affect the disease suppressiveness characteristics in both agricultural and horticultural crops (Postma et al., 2014, 2003; Postma and Schilder, 2015). We suggest that cold-storage reduced the abundance and interactions within the casing soil microbiome, which led to reduced disease suppressive activity.

Into the host-soil-microbiome nexus

In **Chapter 5**, we also attempted to decipher interactions within the host-soil-microbiome nexus to understand the development of this soil suppressiveness to ginger blotch. We found that events such as pathogen invasion, pathogen establishment and blotch suppression, coincided with specific changes in the bacterial and fungal community composition of the casing soil. These changes were in addition to known temporal changes in the casing soil microbiome that are expected across the cultivation cycle. This enabled the identification of specific bacterial and fungal genera that are associated with ginger blotch suppression.

Many of the expected temporal changes in the casing soil microbiome across the cultivation cycle occurred at lower taxonomic ranks. This is supported by earlier investigations where the bacterial diversity of casing soils increased with the colonization of the casing soil by *Agaricus* mycelium along the cropping cycle, although the overall composition of the microbiome remained relatively stable (Pecchia et al., 2014; Carrasco et al., 2019). The mycelium from *A. bisporus* migrates upwards from the compost, and colonizes the casing soil along the cultivation cycle. It acts as a nutrient source for soil bacteria, although it is not clear whether it is due to fungal exudates or the hyphae themselves (Carrasco and Preston, 2020).

Pathogen inoculation onto the casing soil caused additional shifts in the casing soil microbiome, the effect of which lasted until the later cultivation cycles. These were comparable with changes in the rhizosphere microbiome due to pathogen invasion. Invasion by the bacterial pathogen *Ralstonia* have been shown to simplify soil microbiome networks (Wei et al., 2018), and inoculation of the fungal pathogen *Rhizoctonia solani* increases the abundance of specific bacterial taxa in the soil (Chapelle et al., 2016). For plants, these changes are mediated by exudate patterns of the plant host itself (Gu et al., 2016). So far, limited knowledge is available on the response of bacteria to mycelial exudates of *A. bisporus* (Grewal and Rainey, 1991).

Bacterial and fungal genera that were differentially abundant in consecutive flushes, varied between mock-inoculated and pathogen-inoculated casing soils. It has been suggested that the broad-spectrum volatile organic compounds produced by blotch pathogens such as *P. tolaasii* (Lo Cantore et al., 2015) can potentially inhibit the growth of other soil microorganisms (Martins et al., 2020). However, this speculation

contradicts with the fact that the alpha diversity of the casing soils did not decrease between mock-inoculated and pathogen-inoculated casing soils.

Several bacterial taxa that were associated with blotch suppression in our study, have been identified in other reports for their biological control activity against brown blotch, for example, *P. putida, 'P. reactans', P. fluorescens*, (Aslani et al., 2018; Fermor et al., 1991; Tajalipour et al., 2014), *Sphingobacterium multivorum*, *Pedobacter* spp. (Tsukamoto et al., 2002), *Burkholderia cepacia* (Nair and Fahy, 1976, 1972b) and *Bdellovibrio bacteriovorus* (Saxon et al., 2014). Many of the genera associated with blotch suppression co-occurred with each other in microbial soil networks, indicating community-level interactions. Importance of bacterial interactions for suppression of plant pathogenic fungi has been documented in an *in-vitro* study (De Boer et al., 2007). In particular, interspecific interactions between *Pedobacter* and *Pseudomonas* strains increased fungal suppressive activities. These organisms could thus be good targets for future research into biological control.

A quest for peat alternatives

Peat-based casing soil can lead to the introduction of various soil-borne pathogens into mushroom farms (Fletcher et al., 1989; Taparia et al., 2020c; Wong and Preece, 1980). But a multitude of other reasons also require us to transition away from peat-use in mushroom casing soils (Gunady et al., 2012; Kulak et al., 2013; Robinson et al., 2019), including the expected peat-supply bottlenecks in the next years (Bos et al., 2011) and the environmental impact of peat mining and transport (Joosten et al., 2016; Waddington et al., 2002). The search for peat-replacement media is not new. Products from agricultural, forestry or industrial waste streams, have been often tested as peat alternatives (Latinoamericana, 2006; Noble and Dobrovin-Pennington, 2015, 2005; Pardo-Giménez et al., 2011; Pardo et al., 2004, 2003; Peyvast et al., 2007; Sassine et al., 2005; Sharma et al., 1999).

Unfortunately, none of the aforementioned alternatives are applied in practice, either due to emergence of pests and pathogens, unsuitable physical characteristics (water holding capacity) or chemical composition (electrical conductivity), accumulation of toxic residues, unsustainable sourcing, unsupportive legislation or lack of economic viability (Eicker and van Greuning, 1989; Noble and Dobrovin-Pennington, 2015; Pardo et al., 2004). With a two-fold objective to make mushroom cropping systems more sustainable and reduce their disease-pressure, we attempted to replace peat in the casing soil with circular and local alternatives. In **Chapter 6**, we assessed the agronomical performance of four peat-alternatives, peat moss, grass fibres, acidified grass fibres and spent casing soil in casing soils as well as their physico-chemical and microbiological composition.

Moss of *Sphagnum* sp. from degraded peatlands, had similar yield and blotch prevalence as peat. As opposed to black peat, which is dug out, peat moss is harvested superficially, with minimal damage to below-ground landscape, allows ecological restoration of cutover bogs (Graf et al., 2012), and reduces negative environmental effects such as peat oxidation, soil subsidence and CO_2 emissions (Joosten et al., 2012). Grass fibres, produced from agricultural residue streams via a circular biorefining process that converts non-woody biomass into lignocellulosic fibres, had both lower yield and lower blotch prevalence compared to that of peat. This was attributed partially to their inability to act as water reservoir, which is important for mushroom cultivation (Kalberer, 1990; Noble et al., 1999). Spent casing, which was mechanically separated from the compost after a steaming of the growing chambers, at the end of the cropping cycle, had equivalent productivity to that of peat, and lower disease pressure. Expectedly, its physico-chemical properties were very similar to that of peat although it is likely that its high soluble salt content will pose a problem with continuous recycling (Gonani et al., 2011; Pardo et al., 2004).

Peat moss, grasses and spent casing soil could respectively be used to proportionally replace 25%, 50% and 30% of peat in casing soil. Their agronomical performance depended on specific physico-chemical characteristics as well as their microbiological composition. We were able to successfully incorporate three principles of circular economy (Commission, 2014) in mushroom cropping systems by utilizing agricultural residues in the casing soil (design out waste); reusing spent casing soil from previous cultivation cycles (keep materials in use), and substituting black peat in the casing soil with peat moss from degraded peatlands (regenerate natural systems), although strong trade-offs existed between productivity and disease pressure in the circular cropping systems.

The good, the bad and the ugly

The microbial ecology of peat-based casing soils is better understood now (Carrasco et al., 2019; Carrasco and Preston, 2020; Fermor et al., 2000; McGee, 2018; Pecchia et al., 2014). However, microorganisms and their activity in alternative growing media have received rather limited attention (Carlile and Schmilewski, 2010; Van Gerrewey et al., 2020; Vandecasteele et al., 2020). Peat-alternatives that are living material or derived from agri-residue streams, are expected to comprise a more diverse and competitive microbiome (Carlile and Coules, 2011). On one hand these microbiomes could support disease suppression, but it could also compete with crop (fungal) growth, or bear a risk for dissemination of potential human pathogens (De Corato, 2020).

In order to understand the good, the bad and the ugly inhabiting the alternative casing soils, we examined, in **Chapter 6**, the microbiological diversity, activity,

composition and interactions in casing soils composed of peat-alternatives, across the mushroom cultivation cycle. We were able to relate productivity and disease pressure in alternative casing soils with its bacterial and fungal microbiome. Diverse and competitive microbial communities, such as those of the grass fibres, made it difficult for *Agaricus* to colonize the casing soil, despite its adaptation to humic rich and partially decomposed plant material (Morin et al., 2012). In contrast, peat moss and spent casing, which had a relatively reduced microbiome similar to that of black peat, supported higher productivity. This observation agrees with previous reports on the use of spent casing soil (Noble and Dobrovin-Pennington, 2015) and other growing media (Vandecasteele et al., 2020). This can be further confirmed by studying *A. bisporus* growth in sterilized alternatives.

The presence of a diverse microbial community (species richness), with filtered niches (modular interaction networks) and high microbial activity (soil respiration) coincided with lower susceptibility to bacteria blotch. This can be partially explained by the invasion resistance of a community against a new pathogenic colonizer, which depends on the diversity of the endemic microbes and the ecological robustness of their interactions (Mallon et al., 2015; van Elsas et al., 2012; Wei et al., 2015). Spent casing showed ginger blotch suppression, at higher pathogen densities and also in earlier flushes. This might be attributed to the increased abundance of endemic *Pseudomonas* sp. in spent casing, which have a niche overlap with the pathogen (Godfrey, 2003). Casing soil composed of acidified and steamed grass fibres were similarly least susceptible to ginger blotch, which coincided with an increased abundance of *Dyadobacter* sp. and *Saitozyma* sp., both of which are speculated to be involved in blotch suppression (Taparia et al., 2020d).

Weed and pathogenic fungi, such as *Coprinus* sp., *Peziza* sp., and *Trichoderma* sp. that were growing in the casing soil composed of unsteamed grasses, were reduced by the steam treatment of the grasses before being mixed into peat. This is consistent with an earlier report on the effect of heat on competitive fungi in the casing soil (Park et al., 1971). A few thermophilic species, such as *Meliniomyces*, *Mycothermus* were also abundant in the core microbiome of the alternative casing soils, and were not lost after steam treatment. However, we do not know if and how they would interact with *Agaricus*. In circular cropping systems, sufficient caution needs to be paid to the propagation of human pathogens (De Corato, 2020). In our experiments, we could not detect the presence of known human or plant pathogens in the microbiome of the casing soil prepared from the alternatives, but several unidentified species were detected, some of which belong to genera that contain pathogenic variants. A separate investigation needs to be performed to rule out the presence of pathogenic strains, because amplicon-targeted sequencing does not allow us to accurately identify them on a species or strain level (Poretsky et al., 2014).

Looking into the future

The fight against bacterial blotch is difficult, partly because of the genetic and phenotypic diversity of blotch-causing organisms. In this thesis we started out with characterizing this diversity, but in later experiments we chose to focus on pathogens prevalent in Western Europe, to understand their infection dynamics, population dynamics and disease suppression, with the casing soil as the battleground. However, we also discovered several novel pathogens (Taparia et al., 2020b) from European mushroom farms, which deserved further investigation. Given that these pathogens differ not only phylogenetically but also in phenotype (Taparia et al., 2020a), and in their mechanisms for pathogenicity, developing an over-arching management strategy for blotch remains a challenge.

It is said that prevention is better than cure. With that motto, it can be speculated that routine monitoring of the mushroom cultivation and post-harvest supply chains with molecular detection methods can be helpful to minimize risks of introducing pathogens, before the need for blotch management arises (Ophel-Keller et al., 2008). A pan-genomic investigation of pathogenicity determinants that are conserved between pathogens (Freschi et al., 2019), could facilitate the detection of a wide range of blotch-causing organisms. However, with the ability of pathogens such as *P. tolaasii*, to switch from pathogenic to non-pathogenic forms in response to environmental stimuli (Han et al., 1994; Zarkower et al., 1984), it remains to be seen how efficient and economical the identification of a common set of pathogenicity related genes could be for molecular diagnostics. In addition, the lack of an appropriate bulk sampling strategy needs to be addressed.

Our discovery and understanding of ginger blotch suppressive casing soils (Taparia et al., 2020d) is an important step forward. But several questions about ginger blotch suppression remain unexplored, including the functional activity of microbes within the casing soil and possible molecular mechanisms that cause blotch suppressiveness. This is particularly important, because densities of *'P. gingeri'* populations were found to be unaffected in blotch suppressive soils (Taparia et a., 2020d). In addition, the role of casing soil composition, it's pre-treatment and physico-chemical characteristics deserve further attention in the context of blotch suppression. Many potential target organisms were identified, which presents the opportunity to steer the casing soil microbiome towards blotch suppression.

Biological control of blotch has been practiced for many years already (Nair and Fahy, 1972b). Despite many successful laboratory reports, no commercial product for the management of blotch diseases exists. Declining populations of biocontrol agents often result in an inconsistent and reduced field performance (Tsukamoto et al., 1998), which can be due to nutrient competition, predation from native microflora, and physico-chemical characteristics of the casing soil itself. Management of blotch

diseases via stable changes of the endemic soil microbiome presents better opportunities for future research, as it preserves community level interactions, and these microorganisms are better adapted to the casing soil composition.

An essential next step would be the rigorous testing of target organisms identified in this study, which are endemic to the casing soil, as microbial mixtures, for their biocontrol activity against other blotch pathogens. Their population dynamics in the casing soil, together with its effect on the composition and activity of the soil microbiome, also needs to be assessed with the help of functional metagenomics. This information could have large implications in successful biological control of bacterial blotch in mushroom cultivation, as it generates several fundamental insights on pathogen-induced disease suppression, but also provides a platform for further applied studies on the design of blotch management strategies

Our research showed that the casing soil is not just a supplier of beneficial microorganisms for fructification of *A. bisporus*, it also determines disease suppression in the event of a pathogen introduction (Taparia et al., 2020d). The invasion resistance of a microbial community depends on the ability of the microbiome to exploit available resources more efficiently (van Elsas et al., 2012). But it is not as straightforward, as the addition of resources can also temporarily make the community more susceptible to invasion (Mallon et al., 2015). It can be speculated that the casing soil microbiome can also be steered by abiotic factors towards blotch suppression, but it requires better knowledge of the composition, supplementation, physical and chemical characteristics of the casing soils. A joint effort that uses both biotic (microbial inoculants) and abiotic (soil amendments) factors to induce stable changes in the soil microbiome presents a more successful opportunity for the management of blotch diseases.

Another enticing prospect in bacterial blotch management, is the replacement of peat from mushroom casing soils. The last decades have seen an extensive search for peat-alternatives in response to environmental, ecological and supply-chain concerns (Barrett et al., 2016; Bos et al., 2011; Carlile and Coules, 2011). In our research, we found that three circular and sustainable alternatives, *Sphagnum* moss, grass fibres and spent casing can be used to proportionally replace black peat in the casing soil. Specific physical, chemical and structural properties of these alternative casings can differ largely from that of peat. However, the watering methods, climatic conditions and the growing environment currently employed for mushroom cultivation have been optimized for peat-based casing soils over the last 30 years (Sánchez, 2004). Re-optimization of these conditions for alternative casing soils, can play an important role to further increase the productivity and decrease the disease pressure of circular cropping systems.

It can be speculated that with the full replacement of peat, currently known soil-borne diseases that are introduced from peat-based casing soil will decline. However, it is also expected that other pests, pathogens and competitive fungi native to the peat-alternatives, may affect the performance of the alternative casing soils, as was observed for unsteamed grass-based casing soils. Steam treatment of the grass fibres resulted in lack of other competitive fungi. Hence, it is also important to explore other methods to manage the microbiome of the peat alternatives. These could potentially include the use of biostimulants, peak-heating, composting, fermentation, acidification or via storage conditions (Carlile and Schmilewski, 2010; Postma, 2010; Sharma et al., 1999).

Trade-offs in these peat-alternatives are also driven by accessibility, sustainability , economic viability and energy demands of the cropping process, which have not been investigated so far. The environmental impact of using these peat-alternatives requires quantification, by the means of life cycle assessments. An optimal peat replacement ratio needs to be determined that balances agronomical performance with environmental impact and economic viability. They need to be tested for the accumulation of toxic compounds such as heavy metals, pesticide residues and microplastics. Their composition also needs to meet regulatory limits within the EU for disposal of organic wastes. We have a long way to go with peat-alternatives in the casing soil, but they also present a more long-term solution to the sustainability crisis and disease outbreaks threatening mushroom cropping systems.

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Summary

Similar to most agro-ecological systems, cultivation of button mushrooms relies heavily on the dynamic interactions between the host, *Agaricus bisporus,* and soil microorganisms. In **Chapter 1** we introduce mushroom casing soils, as diverse ecosystems, inhabited by good (growth-promoters), bad (mushroom pathogens) and ugly (human pathogens) microbes together. The focus of this thesis is on bacterial pathogens of mushrooms. Bacterial blotch is a group of diseases that result in discoloration and disfiguration of mushroom caps of *A. bisporus.* Blotch not only reduces the total marketable crop due to compromised aesthetic value, it also lessens the overall yields due to pin death and lowers the shelf-life post-harvest, leading to severe economic losses globally. Despite being studied for over a century, the identity and taxonomy of blotch-causing organisms still remains unclear. In **Chapter 2** of this thesis, we ask the question, "Who can cause blotch?". In this chapter, we investigated the bacteria inhabiting diseased mushroom caps using molecular methods, and obtained knowledge on the identity, diversity and pathogenicity of mushroom-associated *Pseudomonas* species in farms in the Netherlands, Belgium and United Kingdom.

On the basis of selective plating, pathogenicity assays on fresh caps and pot tests, and whole genome sequencing, we were able to establish the phylogenetic position of pathogenic and non-pathogenic bacteria colonizing mushroom caps. We found that the genus *Pseudomonas* forms a major proportion of the culturable bacteria obtained from mushroom caps. While some of these are essential for stimulating the pinning of button mushrooms, various others are main causative agents of blotch diseases on mushroom caps. We recovered known pathogens such as, '*Pseudomonas gingeri', P. tolaasii, 'P. reactans' and P. costantinii* from blotched mushroom caps. But we also identified four novel pathogens, including *P. yamanorum, P. edaphica, P. salomonii* and strains that clustered with *Pseudomonas* sp. *NC02* in one genomic species. We also discovered three non-pseudomonads, i.e. *Serratia liquefaciens, S. proteamaculans* and a *Pantoea* sp., which could cause bacterial blotch.

Simultaneously, we developed a detailed overview of the pathogenicity of blotch-causing organisms in Western Europe. Phylogenetic analyses revealed that pathogenic and non-pathogenic bacteria are closely related and cluster together. Thus, a pan-genomic analysis would be required for identifying pathogenicity determinants for blotch. We observed that Western Europe shares a well-mixed pathogen pool, as the phylogenetic distribution of various blotch-causing species is independent from their region of detection and year of disease outbreak. Yet, some region-specific blotch pathogens were recovered in this study. For example, *P. costantinii was* found only in United Kingdom, albeit that only very few isolates of these species were present. This obtained knowledge on the phenotypic and

phylogenetic diversity of pathogenic and beneficial microbes present on mushroom caps, is necessary to develop diagnostic tools and disease management strategies against bacterial blotch.

Bacterial blotch is a big threat to mushroom cropping systems worldwide. Once pathogens are introduced into a farm, the mesophilic and humid conditions required for growth of button mushroom, *A. bisporus*, do also promote the growth and spread of the pathogens, leading to quick and secondary enrichment of pathogen populations in the mushroom beds. Reliable and timely detection of the causative agents is thus essential to avoid disease outbreaks. In **Chapter 3**, we take on the question, "How can we detect blotch pathogens?". Thereto, we developed molecular diagnostic tools that provide the first opportunity for quantitative high-throughput detection of blotch pathogens in various environmental samples at low densities.

We designed six multiplex Taqman™-qPCR assays for the detection of two different loci for three blotch pathogens, *'P. gingeri'*, *P. salomonii* and *P. tolaasii,* on the basis of whole genome sequences. These assays have high diagnostic sensitivity and specificity. Additionally, they allow absolute quantification of pathogen populations with the use of an extraction control. They can also quantify the pathogens relative to the population of beneficial *Pseudomonas*. The assays can detect 100-fold lower pathogen densities compared to previous studies, thus allowing successful routine testing of samples associated with mushroom cropping systems, such as water, compost, peat source and casing soil. We applied these assays in our survey of mushroom farms, and we discovered that *'P. gingeri'* and *P. salomonii* were the most prevalent pathogens in the Netherlands and Belgium, comprising of 78% and 42% of the total infected mushrooms, respectively, whereas *P. tolaasii* infections were the least.

The use of our newly developed assays for prediction of a disease risk is highly contextual and requires careful interpretation of other disease indicators, such as environmental conditions, growing practices, harvest method, cultivar type, source of compost and type of casing soil. Current knowledge on critical densities and disease indicators is limited to one blotch pathogen, *P. tolaasii*. In **Chapter 4**, we ask the question, "What conditions are favourable for other blotch-causing agents?". We performed cultivation experiments and diagnostic assays to explore the infection and population dynamics of the newly discovered brown blotch pathogen, *P. salomonii*, in comparison to that of ginger blotch pathogen, *'P. gingeri'*. We identified the soil inoculum thresholds and abiotic factors for favouring blotch outbreaks.

We discovered that *'P. gingeri'* and *P. salomonii* have unique infection and population dynamics, that vary over soil type. '*P. gingeri'* caused disease outbreaks at lower inoculum thresholds in the soil (10^4 cfu/g) than *P. salomonii* (10^5 cfu/g). Ginger blotch generically declined in later harvest cycles, even though the inoculated

pathogen populations did not decline. However, the brown blotch prevalence and *P. salomonii* populations remained consistent during the harvest cycles. Pathogen-inoculated casing soils were differentially susceptible to ginger and brown blotch diseases, based on their composition and supplementation. Mock-inoculated casing soils, also differed in their endemic pathogen populations. This knowledge on the influence of pathogen type, soil type and threshold densities for blotch outbreaks is very valuable to interpret diagnostic results from screening mushroom farms and for the design of localized disease control strategies.

In **chapter 5** of this thesis, we explored the question "What role do soil microbes play in blotch outbreaks?", by further examining the role of casing soil composition and microbiome in the temporal decline of ginger blotch. With the help of cultivation experiments, diagnostics assays, and microbiome sequencing, we confirmed that ginger blotch declined with consecutive cultivation cycles consistently across ten casing soil mixtures, comprising of different peat sources and soil amendments. In contrast, the inoculated pathogen populations in the casing soil, continued to increase. Reduced blotch prevalence and severity in later flushes could not be attributed to differences in post-harvest physiology or the development of induced resistance in *A. bisporus*.

We suspected that this temporal decline in ginger blotch could be due to the development of soil suppressiveness to *'P. gingeri'*. In support of this, we found that blotch suppressiveness was partially transferable to conducive soils via an aqueous soil extract. Decline in ginger blotch with consecutive harvests showed remarkable similarities to temporal disease suppression patterns of plant crops grown in continuous monoculture, such as the decline of take-all disease caused by *Gaeumannomyes* in wheat or barley, or the suppression of root rot caused by *Rhizoctonia solani* in sugar beet seedlings. Our research revealed that long term and cold-storage of the casing soil, between replicate experiments reduced the soil suppressiveness to ginger blotch. Unfortunately, we did not assess how this affected the physico-chemical composition of the casing soil, but we speculated that cold-storage reduced the abundance of and interactions within the casing soil microbiome, leading to decreased disease suppressive activity.

We attempted to decipher interactions within the host-soil-microbiome nexus in order to better understand the development of this soil suppressiveness to ginger blotch. We found that events such as pathogen invasion, pathogen establishment and blotch suppression, coincided with changes that occurred in the bacterial and fungal community composition of the casing soil. These changes were in addition to the temporal changes in the casing soil microbiome that are expected across the cultivation cycle. We identified specific bacterial and fungal genera that are associated with blotch suppression. Many of which were mentioned in earlier reports for their biological control activity against brown blotch, e.g. *P. putida, 'P. reactans',*

P. fluorescens, *Sphingobacterium multivorum*, *Pedobacter* sp., *Burkholderia cepacia* and *Bdellovibrio bacteriovorus.* Many of the genera associated with blotch suppression also co-occurred with each other in the microbial soil networks. These microbes present good targets for future research into biological control of blotch.

The use of our diagnostic assays allowed detection of blotch pathogens in the peat component of mushrooms casing soils. Routine testing of raw materials used for mushroom cultivation indicated that peat was the primary source of introduction for blotch pathogens. Our results indicate that peat-based casing soils introduce soil-borne pathogens into mushroom farms. This is an argument to transition away from peat-use in mushroom casing soils, which is in addition to other reasons including, expected peat-supply bottlenecks in the next years and the environmental impact of peat mining and transport. In **Chapter 6**, we ask the question, "Is peat-use in casing soils avoidable?". We assessed the potential of select substrates to proportionally replace peat in casing soils, with the help of cultivation experiments, soil physical and chemical measurements, and soil microbiome sequencing.

With a two-fold objective to make mushroom cropping systems more sustainable and reduce their disease-pressure, we attempted to replace peat in the casing soil with circular, local and sustainable alternatives. Four peat-alternatives, namely, peat moss, grass fibres, acidified grass fibres and spent casing soil could respectively be used to proportionally replace 25%, 50% and 30% of peat in casing soil. The productivity of the peat-alternatives could be corelated to select physico-chemical characteristics and the microbial composition of the soil. We were able to successfully incorporate three principles of circular economy in mushroom cropping systems by utilizing agricultural residues in the casing soil (design out waste); reusing spent casing soil from previous cultivation cycles (keep materials in use), and substituting black peat in the casing soil with peat moss from degraded peatlands (regenerate natural systems), although strong trade-offs existed between productivity and disease pressure in the circular cropping systems.

We found that peat-alternatives which are composed of living material or derived from agricultural residue streams, comprise of a diverse and competitive microbiome, that can be altered by acidification and heat treatment. We were able to associate the productivity and disease pressure in peat-alternatives, with their casing soil microbiome and pre-treatment. Diverse and competitive microbial communities, such as those of the grass fibres, made it difficult for *Agaricus* to colonize the casing soil, leading to reduced productivity, but they also showed high suppression of ginger blotch, due to high invasion resistance of the community. Weed and pathogenic fungi were reduced by the steam treatment of the peat-alternative.

The research described in this thesis followed a broad spectrum of topics, from the identification of blotch pathogens, development of diagnostic methods, studying their infection and population dynamics to disease indicators and source of introduction. It also explored the role of the soil-host-microbiome nexus in disease outbreaks, soil suppressiveness to blotch, and the role of the soil composition and microbiome in alternative casing soils. In **Chapter 7**, these results were put into context with current insights on blotch, and they point towards future research required to develop strategies for blotch management in mushroom cultivation.

The fight against bacterial blotch is a never ending race, largely because of the phylogenetic and phenotypic diversity of blotch-causing organisms. In this thesis we started out with characterizing this diversity, but in later experiments we chose to focus on prevalent regional pathogens. We suggest that routine monitoring of the mushroom cultivation chain and the post-harvest chain with molecular detection methods should be done to prevent introduction of the pathogens, and determine the risk for blotch outbreaks, before the need for blotch management arises. However, development of detection methods that cover the diversity of blotch-causing organisms remains a challenge.

Our discovery and understanding of microbiome-mediated ginger blotch suppressiveness, points towards the casing soil as a battleground for future intervention. Many target organisms were identified, which were endemic to the casing soil, and could be used to steer towards blotch suppression, whilst preserving other community level interactions. Unfortunately, several questions remain unanswered, including the functional activity of these microbes and possible molecular mechanisms of action. This is particularly important, because *'P. gingeri'* populations were found to be unaffected in blotch suppressive soils. A joint effort that uses both biotic (microbial inoculants) and abiotic (soil amendments) factors to induce stable changes in the soil microbiome presents a more successful opportunity for the management of blotch diseases.

The removal of peat from mushroom casing soils is another enticing prospect. However, peat alternatives differ in their physical, chemical and structural properties. And watering methods, climatic conditions and the growing environment need to be re-optimized specific to alternative casing soils, to improve the productivity and disease pressure of circular cropping systems. Absolute replacement of peat can also lead to the decline of currently known soil-borne diseases, but other pests, pathogens and competitive fungi that are native to the peat-alternatives may rise instead. Hence, it is important to thoroughly explore the microbiome of the peat alternatives and develop appropriate microbiome management strategies. These could potentially include the use of biostimulants, peak-heating, composting, fermentation, acidification or via storage conditions.

सारांश

पौधों की गुणवत्ता के लिये मिट्टी ज़रूरी होती है। मिटटी में बहुत प्रकार के जीवांणु पाए जाते है। अच्छे जीवांणु पौधों की उत्तमता को बढ़ाते है। बुरे जीवांणु उनमे रोग पैदा करते है। और कुछ जीवांणु उन पौधों को खाने वाले मनुष्यो में रोग फैलाते है। इस शोध के सात भागो में हम रोग जनक जीवांणु की चर्चा करेंगे।

भाग एक में हम कुकुरमुत्तों के रोग जनक जीवांणु से आपका परिचय कराएँगे। कुकुरमुत्तों की फसल कई वजह से ख़राब होती है। इसका प्रमुख कारण है रोग फैलाने वाले जीवाणु, जो खेत में पाए जाते है। 'ब्लोच' एक गंभीर रोग है, जो कई दशकों से दुनिया के देशो में प्रचारित है। इसका खराब असर कृषि की अर्धव्यवस्था पे पड़ता है। इस दृष्टि से इसका अवलोकन करना अत्यंत आवश्यक हो जाता है।

भाग दो में हमने जाना, कौनसे से जीवांणु रोग जनक है? ये कहा पाये जाते है? और उनकी क्या क्षमता है। इसका अध्ययन करने के लिए हमने पश्चिम यूरोप के अनेक खेतों की जांच की। हमने पाया, की विभिन्न प्रकार के जीवांणु पौधों में यह रोग फैला सकते है। साथ ही साथ हमने सात नए जीवांणु ढूँढे जिनका सम्बन्ध रोग से पहली बार किया जा रहा है।

इनकी पहचान के बाद, अब ज़रूरी मुद्दा यह रहा कि इनको कैसे ढूंढा जाये। इसके लिए **भाग तीन** में हमने नए उपकरण इजाद किये। हमने कई मॉलिक्यूलर पीसीआर टेस्ट ईजाद किये, जिनसे इन रोग जनक जीवांणु को कम मात्रा में और तुरंत ढूंढ पाना संभव हो सका। इसके लिए हमने गौर किया पश्चिम यूरोप के तीन आक्रामक और प्रचारित जीवांणु पर। अब हम इन पीसीआर परिक्षण का इस्तेमाल करके, देश और विदेश में खेत, पानी, फसल और मिट्टी की जांच करते है किसानो के लिए।

इन जीवांणु का खेत में होना ही काफी नहीं होता है। अन्य कारण, जैसे, नमी, गर्मी, मिटटी और खेतीबाड़ी के तरीके का भी रोग पर असर पड़ता है। **भाग चार** में हमने ढूँढ़ा, किन हालातो में और किस मात्राओ में खराब जीवांणु फसल में रोग पैदा कर सकते है। हमने यह जांचा की सब जीवांणु पे यह परिस्थियाँ एक जैसी असर नहीं करती है।

भाग पांच में हमने अपने अध्ययनों में पहचाना की 'जिंजर ब्लोच' नाम का रोग पहली फसल को बुरी तरह से ख़राब करता है। परन्तु, आगे आने वाली फसले इससे अपने आप ही रोग मुक्त हो जाती है। इस प्रक्रिया को 'रोग दमन' कहा जाता है। हमने ढूँढा की इस रोग दमन का कारण भूमि में पाए जाने वाले अच्छे जीवांणु की रोग घातक क्षमता है। हम इन जीवांणु का रोग खात्मे में प्रयोग करने की कोशिश कर रहे है।

कृषि में फसल के साथ, भूमि का भी अध्ययन अत्यंत ज़रूरी है। कुकुरमुत्तों के प्रयोग के लिए जिस माध्यम का इस्तमाल किया गया, उसमे हमने रोग जनक जीवांणु को पाया। इस माध्यम का इस्तमाल,

फसल के लिए ही नहीं, वातावरण के लिए भी उचित नहीं है। **भाग छः** में हमने, अलग माध्यमों को जांचा जिनकी रोग घातक क्षमता और सततता अधिक हो। इसके लिए हमने उनके शारीरिक और रासायनिक संगरचना का परीक्षण किया. हमने, इन नए माध्यमों में पाए गए अन्य जीवांणु को भी जांचा. हमने यह निष्कर्ष निकाला की बेहतरीन माधयमो का इस्तेमाल कृषि और वातावरण **दोनों** के लिए उत्तम होगा।

भाग सात में, हमने पहले किये गए सारे अध्ययनों का सारांश निकाला. इनकी तुलना हमने इस क्षेत्र के अन्य प्रचलित अध्ययनो से भी करी। रोग से सम्मिलित जीवांणु की पहचान, खोज और उपचार करना एक अत्यंत जटिल प्रक्रिया है। इसके कई महत्वपूर्ण तत्वों से हम अभी भी अनजान है. परन्तु, अब तक की जानकारी के मुताबिक, हमने रोग थाम के कई मायनों को प्रचार किया है जिससे सतत कृषि उत्पादन हो सके।

Acknowledgements

‘It takes a village to raise a child’, is a common proverb from back-home. And recently, I’ve come to realise that it also takes a village to write a book. In many ways, the writing itself, is a solitary activity, and yet, you will read a plethora of names over the next two pages, people whose contributions have been indispensable for this work to come together.

First and foremost, I would like to thank my supervisor, Jan van der Wolf. I do not have words to express the value of your support, guidance and involvement in every step throughout the process. You assisted me when I needed it, and you gave me freedom to function when I wanted it. I learnt a lot from you, Jan, not just about plant pathology, but also from our many conversations about society, culture and religion. I also wish to thank my promoter, Wietse de Boer, for being such a nice mentor. I really appreciated your clarity of communication, help in keeping my PhD trajectory, professional advice and your witty remarks. It has been a pleasure to work with and learn from the both of you!

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appreciated your help, when I was new, and again, when my hours were few! Also thanks to Leo, Jürgen and Joeke for the lovely discussions on plant health, soil health and biocontrol, which stimulated me to think, read and advance my knowledge. Thanks Willem, for nudging me to write ever so often.

Bea, Lina, Balasz, Marie, Viola and Els, thank you for the many coffee breaks, chair yoga sessions, -omics discussions and hours of troubleshooting in R. I loved that we could jointly vent about the server problems. Stefan, Yvonne, Dirk-Jan, Georgina, Bram, Bea, Marc, Florencia, Stefanie, Daan and Tjasa, you are awesome colleagues at work and outside. I really enjoyed our social interactions, ‘hardcore hiking’, bouldering at Arnhem, ice-skating at Nijmegen, mug curling at Forum, sprinting at WE-Days, falafel lunches and borrels. The Management Team, Willem-Jan and José, thank you also for being incredibly supportive, you are doing a great job! Marion, what can I say about you? I think our department would be defunct without you. To my other colleagues at Biointeractions, thank you for providing such a warm, helpful and cooperative environment to work in. I think I chose the right ‘village’.

To my colleagues at Soil Biology, Carmen, Jana, Joana, Mirjam, Rocio and Guilia, it was so lovely to have a group of people to share my PhD-only questions and moments with. The beers, BBQs, Nijmegen days, outings and PE&RC weekends were all much needed stress-busters. Marnella, thank you so much for your assistance with all things big and small. You are a wonderful bunch of people! To my colleagues at Microbial Ecology, even though my time at NIOO was limited, I loved the scientific feedback and discussions I could have with you. Not to forget, the open days, trips to conferences, borrels and NIOO days. Jos, Anna, Paolo, Maria, thanks for all your help! Lucia and Götz, my salsa amigos. I feel so special to be a part of both these expertise groups, and it lent me such a different perspective on my research.

Getting through this PhD required more than just academic support. I’m especially grateful to my desi gang, Kavya, Siddhuji, Gurnoor, Abhishek, Sanmitra, Aaron, Maulik, Nehal and Priyanka, whom I turned to for Bollywood nights, mad dancing, food cravings, dumb charades and bureaucratic bullshit! To my Salsa friends, I had the best time spinning away at ISOW, International Club, Loburg, Common Barrack and Hemingway's. My terrific friends, Michael, Niamh, Nicolas, Maaike, Robert, Kim and Flo, our joint workouts, treasure-hunts, elaborate dinners, movie nights, night walks, BBQs, picnics at the Rhine and so much more kept me going through this last phase. You are fantastic people! If science doesn’t work out for us, let’s open a restaurant together, please?

As much as I love all the fun we had, there's so much more I need to thank all of you for. Thank you for being there for me, for making me a better person, for teaching me so many things, for the socio-political and scientific discussions, for tolerating my eccentricities, for having hot food ready after long days at work, for the emergency chips, for finding my wallet again and again, and finally, for accepting the spicy! I would like to believe that you are the 'family' I chose for myself. I would not have enjoyed working and being in Wageningen as much as I did, if it was not for your interactions!

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The most instrumental role in my personal development, has been of my parents. Papa and mumma, you were my biggest pillars of support, despite being so far away. Those hours on the phone when you encouraged me, supported me, missed me, corrected me and celebrated with me. I aspire to be more like you! To my brother, Yash, who has never called me back, I love you regardless. Don't grow up too soon! To the rest of my family, loving and crazy, you add so much joy (and confusion) to my life! Thank you, for worrying about me, checking up on me, and taking care of me, even though I've not been around so much. To my 'Lebensabschnittsgefährte', Michael, you are the engine to my car. And I want to drive the world together with you! Need I say more?

It has been such a pleasure to write this note of acknowledgements. It gave me an opportunity to re-live the many warm memories, funny activities and crazy moments created before, during and after this PhD. The list is endless, and I'm sure I missed to thank a few people. But this book is a testimony to the great deal of support and assistance I have received. I will continue to remember these four years as a very special part of my life!

About the author

Tanvi Taparia was born on 23rd November 1992, in Calcutta (India). She moved away from the hustle and bustle of the city, to a quaint little town in Rajasthan for her higher education. She graduated with an Integrated Masters in Biological Sciences from Birla Institute of Technology and Science, Pilani.

During her studies, and various summer internships, Tanvi developed a keen interest in molecular research and all things minute. She started her scientific career by investigating biodiesel production in microalgae. As the recipient of the INSPIRE-SHE fellowship, she moved on to study homologous recombination in bacteria at the Indian Institute of Science, Bangalore.

A tryst with entomology took her to Sweden in 2014, where she explored the host preference of disease vectors using -omics techniques. She continued her research at the Swedish University of Agricultural Sciences, Alnarp for over two years, during the course of which she also met her life partner, Michael.

In 2016, Tanvi received the opportunity to carry out a PhD trajectory in the Netherlands, at Wageningen University and Research and the Netherlands Institute of Ecology. There she studied soil-borne diseases of mushrooms, in close coordination with the stakeholders. Apart from research, she also participated in and organized various outreach activities, like TEDx Wageningen.

During her PhD, she also co-wrote an H2020 Grant for follow-up research, in collaboration with an international consortium, which was recently funded by the European Research Commission. After her PhD, Tanvi is currently busy with transforming her scientific results into practical solutions as part of the new EU project.

In the future, she aspires to use her joint knowledge of microbial ecology, plant pathology and soil ecology to develop sustainable solutions to agro-ecological problems, while also pursuing her interest in popular-scientific communication. She will continue to travel the world together with her partner, and learn from the exchange of cultures.

List of publications

Peer reviewed articles

Tanvi Taparia, Manjari MVSS, Rajesh Mehrotra, Paritosh Shukla, and Sandhya Mehrotra. "Developments and challenges in biodiesel production from microalgae: A review." **Biotechnology and Applied Biochemistry** 63 (2016): 715-726.

Tanvi Taparia, Rickard Ignell, and Sharon Rose Hill. "Blood meal induced regulation of the chemosensory gene repertoire in the southern house mosquito." **BMC Genomics** 18 (2017): 393.

Nasim Sedighian, Marjon Krijger, Tanvi Taparia, S. Mohsen Taghavi, Emmanuel Wicker, Jan M. van der Wolf, and Ebrahim Osdaghi. "Genome Resource of Two Potato Strains of *Ralstonia solanacearum* Biovar 2 (Phylotype IIB Sequevar 1) and Biovar 2T (Phylotype IIB Sequevar 25) Isolated from Lowlands in Iran." **Molecular Plant-Microbe Interactions** (2020): MPMI-02.

Tanvi Taparia, Marjon Krijger, Jennifer Hodgetts, Marc Hendriks, John G. Elphinstone, and Jan van der Wolf. "Six Multiplex TaqMan^{TM}-qPCR assays for quantitative diagnostics of *Pseudomonas* species causative of bacterial blotch diseases of mushrooms." **Frontiers in Microbiology** 11 (2020): 989.

Tanvi Taparia, Marjon Krijger, Edward Haynes, John G. Elphinstone, Ralph Noble, and Jan van der Wolf. "Molecular characterization of *Pseudomonas* from *Agaricus bisporus* caps reveal novel blotch pathogens in Western Europe." **BMC Genomics** 21 (2020): 1-14.

Tanvi Taparia, Ed Hendrix, Marc Hendriks, Marjon Krijger, Wietse de Boer, and Jan van der Wolf. "Comparative studies on the disease prevalence and population dynamics of ginger blotch and brown blotch pathogens of button mushrooms" **Plant Disease** (2020). doi:10.1094/PDIS-06-20-1260-RE.

Sharon Rose Hill, Tanvi Taparia, and Rickard Ignell. "Regulation of the antennal transcriptome of the dengue vector, *Aedes aegypti*, during the first gonotrophic cycle." **BMC Genomics** (2020). (*accepted)*

Tanvi Taparia, Ed Hendrix, Marc Hendriks, Els Nijhuis, Wietse de Boer, and Jan van der Wolf. "Soil microbiome mediated suppression of bacterial blotch of mushrooms during consecutive cultivation cycles" **Soil Biology and Biochemistry** (2020). *(accepted)*

Tanvi Taparia, Ed Hendrix, Els Nijhuis, Wietse de Boer, and Jan van der Wolf. "Circular alternatives to peat in growing media: A microbiome perspective " (*submitted to* **Journal of Cleaner Production***)*

Tanvi Taparia, Els Nijhuis, Johan Baars, Wietse de Boer, and Jan van der Wolf. "Cultivar-specific assembly of the cap and soil microbiome is associated with bacterial blotch susceptibility in mushrooms." (*in prep.)*

Popular scientific articles

Tanvi Taparia, Marjon Krijger, Marc Hendriks, Ed Hendrix, and Jan van der Wolf. "Identification, diagnostics and infection dynamics of bacterial blotch pathogens" **Mushroom Business** (2020).

Tanvi Taparia, Marjon Krijger, Marc Hendriks, Ed Hendrix, and Jan van der Wolf. "Nieuwe inzichten in de ziekteverwekkers van bacterievlekken**" Paddelstoelen** (2020).

Interview by Nicole van 't Wout Hofland. “Enzym uit bacterie voorkomt champignonziekte” NEMO Kennislink (2020).

Tanvi Taparia, Marjon Krijger, Marc Hendriks, Els Nijhuis, Ed Hendrix and Jan van der Wolf. "Management of bacterial blotch diseases of mushrooms: the casing, cultivar and more **" (***in-prep*).

Training and education certificate

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature / writing of project proposal (4.5 ECTS)

- Developments and challenges in the molecular diagnostics of important mushroom diseases (review)
- Molecular investigation of soil-borne diseases of mushrooms (proposal)

Post-graduate courses (13.5 ECTS)

- Basic statistics; PE&RC (2017)
- New frontiers in microbial ecology; PE&RC (2018)
- Soil ecology; PE&RC (2018)
- Advanced statistics using R; RUG (2018)
- Root ecology; PE&RC (2019)

Invited review of journal manuscripts (3 ECTS)

- Plant Disease: review on bacterial blotch of mushroom (2019)
- Plant Disease: first report of mushroom disease called brown pit (2020)
- European Journal of Plant Pathology: interactions of mushroom pathogenic bacteria and fungi (2020)

Competence strengthening / skills courses (3.9 ECTS)

- PhD Workshop carousel; WGS (2017)
- Project and time management; PE&RC (2018)
- Driven leadership; SOUL (2019)

Scientific integrity /ethics in science activities (0.3 ECTS)

- Workshop research integrity; NIOO (2019)
- PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)
- PE&RC First years weekend (2016)
- PE&RC Last years weekend (2020)

Discussion groups / local seminars / scientific meetings (4.7 ECTS)

- Wageningen plant microbiome network (2016-2017)
- KNPV Soil-borne pathogens and soil microbiology (2016-2020)
- KNPV phytobacteriology (2016-2020)
- Plant-soil interactions discussion group (2020)

International symposia, workshops and conferences (10.1 ECTS)

- Federation of European Microbiological Societies; Spain (2017)
- Benelux Bioinformatics Conference; Belgium (2017)
- Wageningen Soil Conference; the Netherlands (2017)
- 15th Symposium on Bacterial Genetics and Ecology; Portugal (2019)
- International Plant Protection Congress; India (2019)

Societally relevant exposure (3 ECTS)

- Volunteer for NIOO open days (2019)
- Organizer TEDx Wageningen (2019)
- Presentation at mushroom days (2019)
- Interview for NEMO Kennislink (2020)
- Wrote an article in farmer magazine: Mushroom Business (2020)
- Wrote an article in farmer magazine: Paddelstoelen (2020)

Lecturing / supervision of practicals / tutorials (4.2 ECTS)

- Ecological aspects of biological interactions (2018)

MSc thesis supervision (3 ECTS)

- Development of Taqman assays for blotch diagnostics

Colophon

The research presented in this thesis was conducted at the Bussiness Unit Biointeractions and Plant Health, Wageningen Plant Research in Wageningen. The research described in this thesis was financially supported by the Topsector ‘Horticulture and propagation material’ of the Dutch Ministry of Economic Affairs. It was conducted under grant number KV1509–050 in collaboration with members of the Dutch and Belgian mushroom supply chain.

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