FEMS Microbiology Letters, 366, 2019, fnz215

doi: 10.1093/femsle/fnz215
Advance Access Publication Date: 11 October 2019
Research Letter

RESEARCH LETTER – Environmental Microbiology

Fungal and bacterial community dynamics in substrates during the cultivation of morels (*Morchella rufobrunnea*) indoors

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One sentence summary: Fungal and bacterial communities present in indoor Morel growth substrates were tracked throughout a cultivation cycle; the results indicated fungal and bacterial genera that may be crucial to Morel fruiting.

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Editor: Stefan Olsson

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ABSTRACT

Morel mushrooms (*Morchella*, Pezizales) are highly prized edible fungi. Approaches to cultivate morels indoors in pasteurized composted substrates have been successful for *Morchella rufobrunnea*. We used DNA amplicon sequencing of the Internal Transcribed Spacer (ITS) ribosomal DNA and 16S rRNA gene to follow bacterial and fungal communities in substrates during indoor morel cultivation. Our goal was to determine changes in microbial communities at key stages of morel cultivation, which included primordia development, fundament initiation, differentiation and maturation. Additionally, we compared microbial communities between trays that successfully fruited to those that produced conidia and primordia but aborted before ascocarp formation (non-fruiting). The prokaryotic community was dominated by Firmicutes belonging to *Bacillus* and *Paenibacillus* with a lower abundance of *Flavobacteria*. At earlier stages, the fungal community was dominated by Pezizomycetes including *Morchella* and other species, whereas, later in the cropping cycle Sordariomycetes dominated. Additionally, differences were observed between trays with successful fruiting, which were dominated by *Gilmaniella*; compared to trays that did not fruit, which were dominated by *Cephalotrichum*. Our findings inform understanding of microbial community dynamics during morel cultivation, and show that fungal genera, such as *Gilmaniella*, and prokaryotic genera, such as *Bacillus*, are abundant in substrates that support *M. rufobrunnea* fruiting.

Keywords: mushroom cultivation; rDNA; microbiome; CONSTAX; USEARCH; amplicon sequencing; *Bacillus*

Received: 3 July 2019; **Accepted:** 10 October 2019

INTRODUCTION

Morchella spp. (morels) are highly prized edible mushrooms in the Morchellaceae (Pezizales). Morels are icons of the fungal world, distinguished by the unique honeycomb-like appearance of the fertile tissues in their fruiting bodies. Morel mushrooms are well known for their enigmatic and ephemeral but productive natural fruiting across the Northern Hemisphere during the Spring. In addition to natural fruiting in the Spring, outdoor farming of various black morel species, such as *Morchella importuna, Morchella sextalata and Morchella eximia*, are becoming increasingly common in China. These outdoor morel farms have demonstrated yields as high as 3000 kg per ha (Liu *et al.* 2017). Morels have been used for centuries as food, and more recently they have been shown to have properties beneficial to human health including antibacterial, antioxidant and anti-inflammatory, and immunostimulation activities (Tietel and Masaphy 2018). Additionally, morels have high economic value as a non-timber forest product given their prized status as an edible mushroom. For these reasons, there is interest in understanding the biology and microbial ecology of morels in order to more efficiently propagate and manage habitats where morels are produced (Li *et al.* 2017).

Morel cultivation was first developed in the early 1980s following a number of papers (Ower 1982; Volk and Leonard 1989) and patents (Ower, Mills and Malachowski 1986, 1988, 1989) related to their biology. Following morel cultivation successes, the life cycle of the garden morel, originally published as *Morchella esculenta* but now known to be *Morchella rufobrunnea*, was elucidated and described (Masaphy 2010). Briefly, the morel life cycle includes a number of distinct phases that are visually recognizable by the differentiation of mycelium into different tissue types. These include compatible mating of monokaryons, sclerotia formation, conidiation, the development of fundaments, primordia and fruiting bodies (Ower 1982; Volk and Leonard 1989; Masaphy 2010). Black morels and *M. rufobrunnea* are cultivated without the presence of plant hosts, thus, they are classified ecologically as saprotrophic fungi.

In addition to interest in the life cycle of morels, there are still fundamental questions related to the role of microbial ecology on morel fruiting body development and production. Although specific cultivation techniques have shown variable success, the microbial composition of the growth substrate has not yet been explored. It is expected that the microbes in growing substrate are as crucial to morel development and production as has been demonstrated for other mushrooms, such as *Agaricus bisporus*, which are cultivated in composted substrates. The microbial community in composted substrates have been shown to be important in the release of nutrients that are crucial to *A. bisporus* mushroom development (Savoie 1998). As the importance of the microbial component of mushroom substrates continues to be understood, markers for suitable substrates, as well as bioinoculants of particular taxa, have promise in promoting mushroom growth (Carrasco *et al.* 2018).

Previous work on *Ganoderma lucidum*, a species that is grown on sawdust substrates, has shown that physiochemical changes occur in mushroom growing substrates throughout its cultivation cycle (Zhang *et al.* 2018). These changes are thought to correlate with changes in the microbial community due to the creation of different niches that can be exploited by specific microbial taxa (Zhang *et al.* 2018). Due to potential physiochemical changes throughout the cultivation process, we expected that both bacterial and fungal communities would be dynamic throughout the morel life cycle. We expected that significant differences in the microbial communities between fruiting and non-fruiting trays would be evident. We also hypothesized that the pasteurized substrate would be crucial as a source of prokaryote inocula, such as *Pseudomonas* spp., which have been reported as associated with the formation of morel sclerotia and fruiting bodies; consequently, we expected the relative abundance of these taxa to increase during the cultivation cycle (Pion *et al.* 2013; Liu *et al.* 2017). Finally, we expected *M. rufobrunnea* to grow and dominate in trays where it fruits successfully.

MATERIAL AND METHODS

Cultivation beds

Methods for cultivating *M. rufobrunnea* under indoor controlled environments have been patented and published previously (Ower, Mills and Malachowski 1986, 1988, 1989; Masaphy 2010), and have been optimized by Gourmet Mushrooms, Inc. (www.mycopia.com blog 2017). Briefly, this approach starts with the expansion of fertile mycelium on potato dextrose agar, which is used to inoculate the spawn substrate consisting of sterilized nutrient-rich wheat berries covered by an equal volume of nutrient-poor soil media (1:1 v/v leaf-pine bark compost). In this study, carried out at the Gourmet Mushrooms, Inc. facility in Scottville, MI, spawn bags were inoculated with *M. rufobrunnea* GMI 8 to initiate the indoor morel cultivation cycle. Spawn bags were incubated for 35 days, during which abundant sclerotia formed in the top soil media layer. Sclerotia were then harvested and used as inoculum by imbedding them in the pasteurized substrate composed of 1:1 (by volume) composted leaf-pine bark. After 7 days of growth, 18åC and 80% RH, cultures were induced by flooding the cultures with water, which stimulates conidiation and begins the fruiting process. Cultures were then maintained at 18åC and between 88% RH–92% RH. Primordia initiation occured 1 week later. Over the next 10 days, primordia differentiated and developed into mature fruiting bodies 2 weeks thereafter.

Substrate sampling strategy

For this study, we sampled morel cultivation substrates at key developmental times during the cultivation process. Specifically, we sampled during primordia development 9 days after induction, fundament initiation 12 days after induction, morel differentiation 21 days after induction, and fruiting and non-fruiting stage sample 34 days after induction. Samples were taken in triplicate (from different trays) for each sampling time. Sample processing and analysis are described below.

Molecular methods

We extracted DNA from growth substrates using the PowerMag® Soil DNA Kit (Qiagen, Carlsbad, CA) using provided instructions. PCR was performed with ∼20 ng of DNA with DreamTaq Green DNA Polymerase (ThermoFisher Scientific, USA) and the ITS1f-ITS4 primer pair to amplify fungal ITS rDNA and 515F-806R primer pair to amplify prokaryotic 16S rRNA gene. For Illumina library preparation, a protocol was used that employed frameshift primers as developed by Lundberg *et al.* (2013) and later described by Chen and colleagues (2018). PCR products were visualized using ethidium bromide stained agarose gels. Samples were normalized using the SequalPrep Normalization Plate Kit (ThermoFisher Scientific, USA) and combined into a single amplicon pool. Amicon Ultra 0.5 mL 50 K filters (EMD Millipore, Germany) were used to concentrate the libraries at about a 20:1 ratio. Library purification to remove primer dimers and other small fragments was performed with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). Fragment libraries were sequenced on an Illumina MiSeq instrument with v3 chemistry and 600 cycles (Illumina, USA). Sequence reads have been archived at the NCBI SRA repository with the accession number PRJNA510627.

Bioinformatic analyses

The quality of the ITS and 16S amplicons was analyzed using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing reads were demultiplexed in QIIME based on sample barcodes (Caporaso *et al.* 2010). The Cutadapt program was employed to trim Illumina adapters and primers from reads prior to further processing (Martin 2011). Reads were then filtered at a quality threshold and cut to the same number of nucleotides (Edgar and Flyvbjerg 2015; Edgar 2016). Next, sequences were de-replicated, singleton sequences were excluded and remaining sequences were used to create operational taxonomic units (OTUs) at 97% similarity threshold with the UPARSE algorithm (Edgar 2013). Taxonomy assignments were performed using QIIME and the RDP Naïve Bayesian Classifier (Wang *et al.* 2007) using the Greengenes database (DeSantis *et al.* 2006) version gg_13_8 for 16S rRNA gene, and CONSTAX (Gdanetz *et al.* 2017) using the UNITE fungal ITS rDNA sequence database version 7.1 2016-08-22 (Kõljalg *et al.* 2005).

Statistical analyses

Resulting OTU tables, mapping files containing metadata, taxonomy files and reference sequences for both marker genes were processed in the R statistical environment for remaining analyses (R Core Team 2017). To begin, OTUs that appeared in the extraction or PCR negative controls (i.e. suspected contaminants) were removed across all samples when $\geq$10 reads were present in any single control. The OTU tables were then filtered to remove OTUs with fewer than 10 sequences (Lindahl *et al.* 2013; Oliver, Callaham and Jumpponen 2015). Observed richness (Simpson 1949) and Shannon's diversity (Hill 1973) were determined using the 'plot_richness' function in *phyloseq* (McMurdie and Holmes 2013). Before analyzing β-diversity, sequencing data was normalized using cumulative sum scaling in the *Metagenomeseq* package (Paulson *et al.* 2013). Non-metric multidimensional scaling (NMDS) was employed to probe β-diversity using the 'metaMDS' and 'ordinate' functions of the *vegan* and *phyloseq* R packages. Diversity patterns were tested for statistically significant differences between sites in the *vegan* package with the PERMANOVA function 'adonis'. Differences in community dispersion were assessed using the 'betadisper' function. An indicator species analysis was performed to identify OTUs highly associated to a single developmental stage using the 'multipatt' function in the *indicspecies* package (De Cáceres and Legendre 2009). The R code for generating the figures and performing statistical analyses is available at https://github.com/longleyr/Morel-Indoor-Manuscript-R-Script. All files for producing figures and performing statistical analyses on OTU tables are found in supplementary files 1–8 (Supporting Information).

RESULTS

Morchella fruiting body development

Morchella rufobrunnea progressed as expected through its life cycle, resulting in mature fruiting bodies, but only in some trays. In total, only 57.2% of morel trays fruited, with the yield per tray 46% of anticipated production. As expected, when lower yields are encountered, the ascocarps produced were quite large with the largest produced during this growth trial reaching 116 gm fresh weight (data not shown).

High-throughput sequencing results

Through amplicon sequencing we generated a total 1,448,777 16S rRNA gene and 207,077 ITS rDNA sequences from 15 indoor samples. The 2 communities had average read depths of 96,585 and 13,805 reads/sample, respectively. Following processing and removal of negative controls, contaminants, and the artificial mock, we obtained 1725 OTUs for the prokaryotic communities and 153 OTUs for the fungal communities. We recovered the 12 expected artificial taxa from mock samples, and no other taxa. There were no mock sequences recovered from any other samples, indicating that tag switching did not occur.

Microbial community composition of M. rufobrunnea substrate during indoor cultivation

The prokaryotic community associated with *M. rufobrunnea* substrate in the indoor experiment shifted throughout the cultivation cycle, but overall the most abundant phyla were Proteobacteria (34.8%) and Firmicutes (24.7%). In terms of phyla, the substrate prokaryotic community remained mostly consistent throughout morel development, primarily changing in relative abundance instead of presence or absence of particular phyla. The dominant prokaryotic classes were Bacilli, which decreased in relative abundance throughout cultivation and Alphaproteobacteria, which increased in relative abundance throughout cultivation (Fig. 1A). The dominant prokaryotic orders in the indoor substrate were Bacillales (21.9%) and Rhizobiales (9.2%). The most abundant prokaryotic genera were *Bacillus* (8.2%) and *Paenibacillus* (9.6%). In addition to *Bacillus* and *Paenibacillus*, *Pedobacter* was present at low levels (only one Fundament sample and one late fruiting sample had >1% *Pedobacter*). *Pseudomonas* was also present at low levels (1 primordia sample had >1% *Pseudomonas*).

The fungal composition of substrate-associated communities shifted as fruiting bodies matured, yet, the overall community was dominated by Ascomycota (95.6%). The fungal classes varied significantly throughout the morel growth stages. The relative abundance of *Morchella* was low in composted substrates throughout the sampling points, accounting for 0.2% of the total reads in the sampled stages. The relative abundance of *Morchella* was highest in a single fundament sample at 10%. The most distinct shifts in terms of fungal class composition occurred at the differentiation stage and late fruiting/non-fruiting stages. At the differentiation stage, non-*Morchella* Pezizomycetes dominated the community, but at the latest stages, Sordariomycetes dominated (Fig. 1B). Overall, the most abundant fungal orders in the indoor substrate were Microascales (34.7%) and Pezizales (18.9%). The most abundant fungal genera throughout the entire cultivation cycle were *Cephalotrichum* (34.7%) and two unidentified Pezizomycetes (18.8% and 15.8%). In order to assess differences between productive and non-productive trays, the

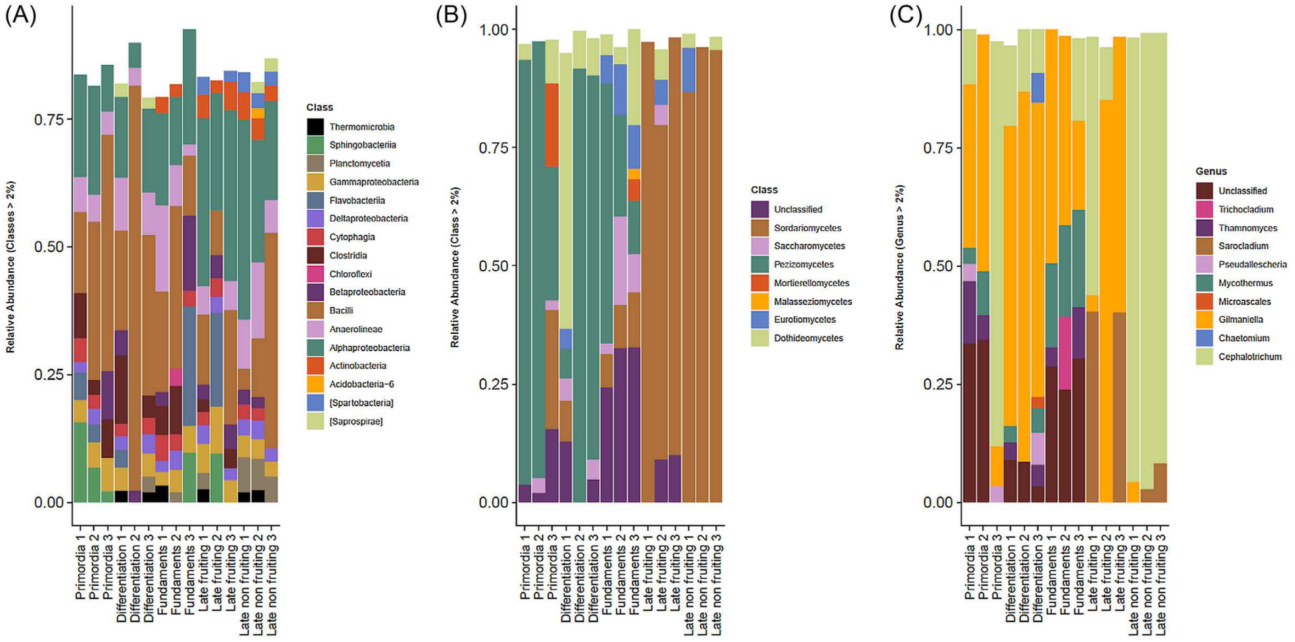

Figure 1 Stacked bar plots showing (A) bacterial classes, (B) fungal classes and (C) genera of Sordariomycetes having a relative abundance ≥2% detected in *M. rufobrunnea* substrate throughout cultivation.

Sordariomycetes class alone was broken down by genus. In substrates where fruiting did not occur, *Cephalotrichum* dominated, whereas, *Gilmaniella* dominated in trays that fruited (Fig. 1C).

Microbial richness in substrates beneath Morchella fruiting bodies

The indoor substrate showed opposite trends in alpha diversity for the prokaryotic community compared to the fungal community. In the prokaryotic community, alpha diversity measurements showed a trend of increasing diversity in the substrate as the morels matured, with the highest alpha diversity values associated with late-stage fruiting and non-fruiting samplings (Fig. 2A). Indoor compost substrates showed an increase in species richness from the primordia to the fundamental stage of growth for fungi. Following a peak in species richness at the fundament stage (over 40 observed OTUs), fungal community diversity dropped sharply in both fruiting and non-fruiting trays (Fig. 2B).

Fungal and bacteria communities β-diversity in Morchella fruiting substrates

In the NMDS ordination obtained with the 16S rRNA gene dataset, prokaryotic communities of the late non-fruiting samples were separate from all the other samples and grouped on the left side of the ordination space (Fig. 3A, stress = 0.09). For the ITS rDNA dataset, NMDS ordination analysis (Fig. 3B, stress = 0.13) showed that substrate samples collected in the earlier stages of development (e.g. primordia, fundaments, differentiation) had similar fungal community structure and clustered together on the left side of the ordination graph compared to the non-fruiting samples, which grouped on the right side of the ordination (Fig. 3B).

PERMANOVA analyses showed a significant effect of the stage of *M. rufobrunnea* fruiting for the fungal and prokaryotic communities. For fungal communities, stage explained 56% of the sum of squares variation. For prokaryotic communities, stage explained 42% of the sum of squares variation. In terms of group dispersion differences, stage was not a significant factor (Table 1).

Indicator species and intersections between stage and site

In substrates where *M. rufobrunnea* was cultivated, 1273 prokaryotic OTUs were shared across every developmental stage of the morel. Overall, there were more unique prokaryotic OTUs associated with the later developmental stages (Fig. 4A). The largest group of OTUs associated to a single developmental stage were OTUs associated with late non-fruiting (246). In the Venn diagram displaying substrate fungal OTUs by morel growth stage, 21 OTUs were shared between all development stages. The largest number of unique OTUs were found in substrates when the mushroom was fruiting (Fig. 4B). An indicator species analysis was performed, but no indicator species associated to a particular developmental stage were detected after *P*-value correction.

DISCUSSION

In this study, we sought to understand the succession of fungal and bacterial taxa in substrates during morel cultivation. Similar to previous work, we found that prokaryotic phyla associated with *M. rufobrunnea* substrates remained mostly consistent with changes in the relative abundance of taxa, but not in the presence and absence of phyla at different growth stage (Zhang *et al.* 2018).

Microbial community dynamics during morel cultivation

In the prokaryotic community, the *Bacillus* genus was dominant. *Bacillus* species have been of interest in *Pleurotus* cultivation

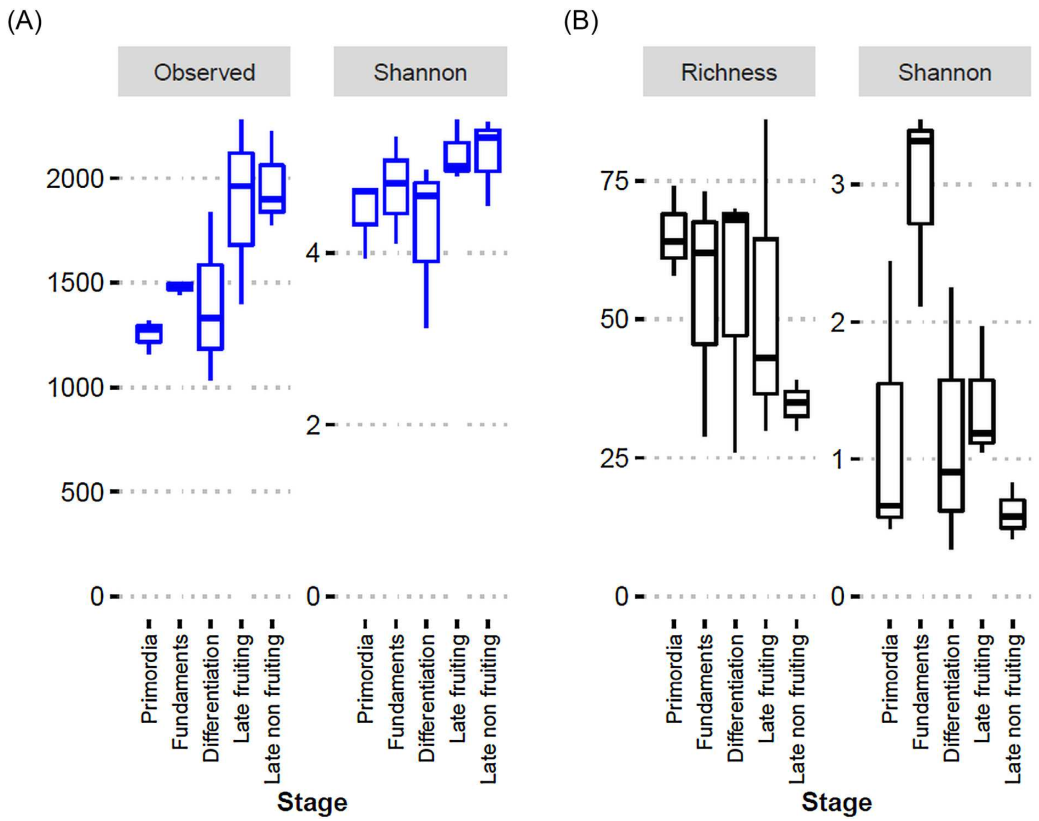

Figure 2 Alpha diversity boxplots for prokaryotic (A) and fungal (B) communities. Richness and Shannon index values associated with *M. rufobrunnea* cultivation substrates at different developmental stages: primordia (9 days after induction), fundament initiation (12 days after induction), morel differentiation (21 days after induction), and fruiting and non-fruiting (34 days after induction). All values were calculated on non-normalized data as suggested by McMurdie and Holmes (2013).

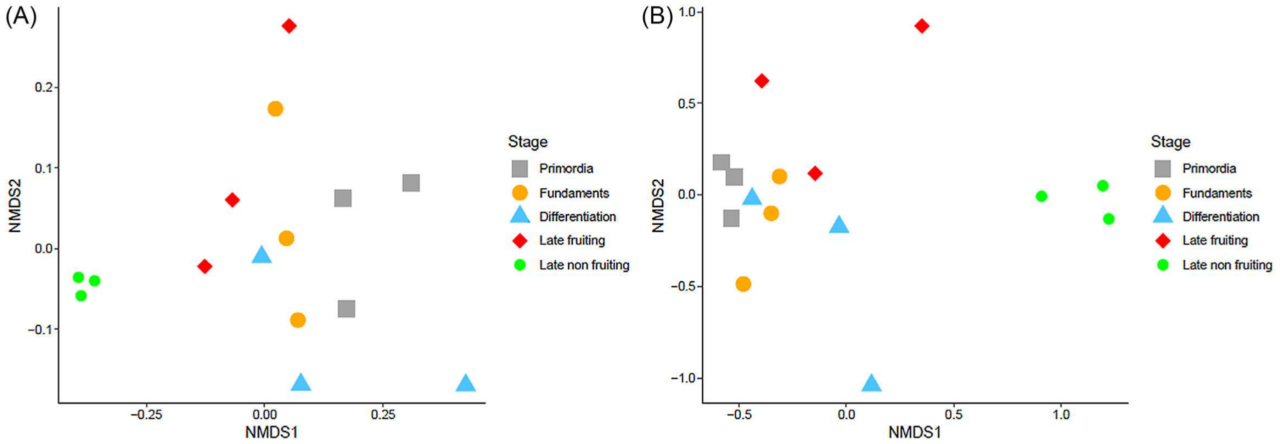

Figure 3 Non-metric multidimensional scaling plots, using Bray–Curtis dissimilarity matrix, of prokaryotic (A) and fungal (B) communities associated with *M. rufobrunnea* substrates.

because they are thought to provide antagonism to *Trichoderma harzianum*, a *Pleurotus* pathogen (Velázquez-Cedeño *et al.* 2008). *Bacillus* taxa have even been considered as potential bioinoculants for promoting growth of *Pleurotus* and *Agaricus* mushrooms (Carrasco *et al.* 2018). If *Bacillus* and *Paenibacillus* play similar roles in *M. rufobrunnea* cultivation, this could explain the consistent abundance of these taxa in our substrates, and in trays that successful fruiting occurred. Interesting associations were found for other bacterial taxa too. While we were not able to directly test the function of bacterial taxa in this study, we determined that fruiting substrates contained approximately twice the *Flavobacterium* reads compared to substrates that did not fruit. Fruiting substrates also contained approximately 1.5 times as many *Pedobacter* reads compared to substrates which did not fruit. Additionally, although *Pseudomonas* had low abundance in this study, substrates which produced morel fruiting bodies had nearly four times the number of *Pseudomonas* reads compared to those that failed to produce.

Table 1. Permutational multivariate analysis of variance (*adonis*) and multivariate homogeneity of groups dispersions analysis (*betadisper*) results for both prokaryotic and fungal communities associated with *Morchella* cultivation substrates. Significant P-values at P ≤ 0.05 are highlighted in bold.

	Factor	*adonis*				*betadisper*	
Prokaryotes		Df	F-value	R2	P-value	F-value	P-value
	Stage	4	1.828	0.422	0.002	0.879	0.510
	Residuals	10		0.578			
	Total	14		1			
Fungi							
	Stage	4	3.140	0.557	0.0001	0.878	0.511
	Residuals	10		0.443			
	Total	14		1			

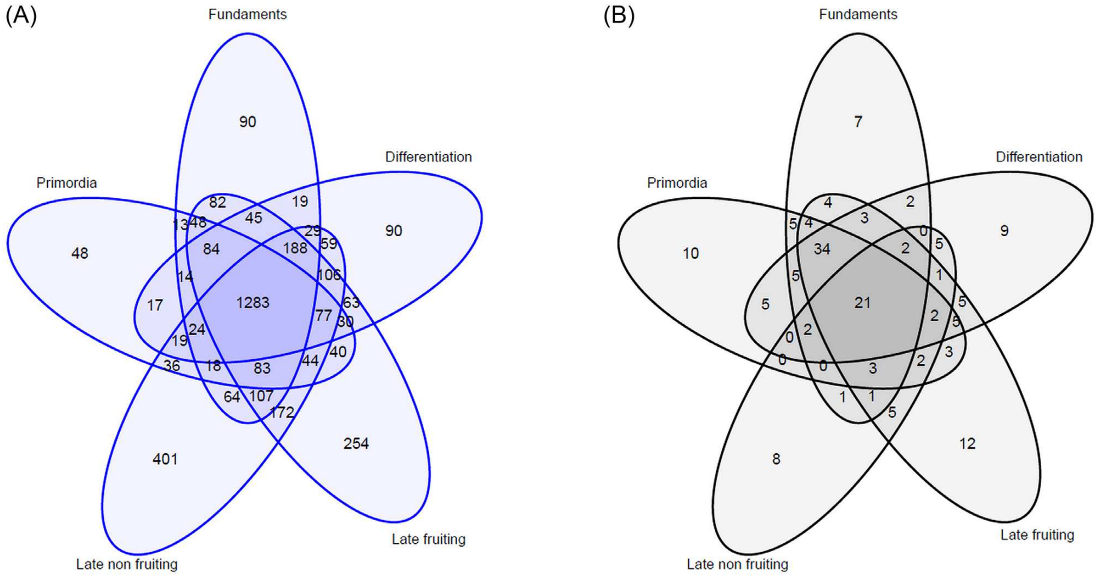

Figure 4 Venn diagrams showing core and unique OTUs among different sample groups. (A) Prokaryotic communities associated *M. rufobrunnea* cultivation substrate; (B) Fungal communities associated with *M. rufobrunnea* cultivation substrate throughout its life cycle.

The trend of increasing prokaryotic diversity during mushroom cultivation has been reported for other mushrooms, including *Ganoderma lucidum*, where prokaryotic diversity increased throughout cultivation until a slight decrease occurred during maturation of the fruiting body (Zhang *et al.* 2018). These changes were related to physiochemical changes in the mushroom substrates. While we did not measure physiochemical changes in the morel substrate, it is probable that changes occurred in relation to the microbial succession, a topic that remains open for future research. The trend of relative consistency was not reflected in fungal communities in *M. rufobrunnea* substrates. Sordariomycetes dominated in later developmental stages (Fig. 1B). However, the genera of Sordariomycetes differed between morel producing and non-producing trays. In trays where fruiting did occur, samples were mostly dominated by *Gilmaniella*, whereas when fruiting did not occur samples were dominated by *Cephalotrichum*. Although less work has been done on the fungal communities associated with mushroom growing substrates (compared to prokaryotes), there are a few examples that deserve mention. *Gilmaniella* has been isolated from substrates used to produce *Agaricus brunnescens*, while *Cephalotrichum* species have been indicated as being potential mycoparasites on cultivated button mushrooms (Fergus 1978; Dugan *et al.* 2012). It is possible that some of the taxa, such as *Cephalotrichum* spp., may outcompete with morels, parasitize morel mycelium, or inhibit morel fruiting. Future studies may directly test these hypotheses on the importance of management of the morel microbiome on morel fruiting body production.

Microbial Communities of Fruiting and Non-Fruiting Trays

Venn diagrams and NMDS ordination results show that microbial communities in substrates where fruiting did not occur were distinct from all other samples in this study. This was reflected in both prokaryotic and fungal NMDS ordinations with a distinct cluster for the three late non-fruiting samples. In the Venn diagrams, this was reflected by a large number of OTUs that were uniquely associated to non-fruiting samples. Additionally, there was a decrease in fungal diversity in the samples where successful fruiting did not occur. Taken together, these results indicate that the underlying microbial community structure in these substrates were different, rather than just a few taxa accounting for the detected differences. This could be due to early colonization by different microorganisms in substrates where *M. rufobrunnea* was slow to colonize. Interestingly, *M. rufobrunnea* was not abundant in the substrates sampled, possibly indicating that nutrient reserves of the plant sclerotia are directed into fruiting bodies, rather than into vegetative mycelial growth through the substrates. Thus, the fruiting substrate appears to be key in facilitating the necessary environmental conditions needed for fruiting to occur (i.e. physical and biological parameters), rather than providing nutrition to the developing fruiting bodies. Future research with soilless media and biological amendments should be able to test this hypothesis. Overall, results from this study highlight the need for further exploration of microbial communities that facilitate or inhibit morel mushroom fruiting. Such knowledge will inform morel cultivation practices, enabling successful large-scale indoor production as has been realized for other cultivated mushroom species.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

FUNDING

RL acknowledges the PBHS Biotechnology Training Program Project NIH T32-GM110523 for graduate research fellowship support.

ACKNOWLEDGEMENTS

GB and GMNB are grateful to Michigan State University AgBioResearch NIFA project MICL02416 and Project GREEEN GR17-083. We acknowledge the contribution of GMI support staff for use of facilities and assistance with sampling.

Conflict of Interest. None declared.

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