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Changes in soil bacteria functional ecology associated with *Morchella rufobrunnea* fruiting in a natural habitat

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Summary

Morchella rufobrunnea **is a saprobic edible mushroom, found in a range of ecological niches, indicating nutritional adjustment to different habitats and possible interaction with soil prokaryotic microbiome (SPM). Using the 16S rRNA gene, we examined the SPM of *M. rufobrunnea* that appeared in a natural habitat in Northern Israel. Three sample types were included: bare soil without mushroom, soil beneath young mushroom initials and soil beneath the mature fruiting body. *Morchella rufobrunnea* developmental stage was significantly associated with changes in bacterial populations (PERMANOVA, $p < 0.0005$). Indicator analysis with point-biserial correlation coefficient found 180 operational taxonomic units (OTU) uniquely associated with distinct stages of development. The Functional Annotation of Prokaryotic Taxonomy (FAPROTAX) database helped to infer ecological roles for indicator OTU. The functional ecological progression begins with establishment of a photoautotrophic N-fixing bacterial mat on bare soil. Pioneer heterotrophs including oligotrophs, acidifying nutrient mobilizers and nitrifiers are congruent with appearance of young *M. rufobrunnea* initials. Under the mature fruiting body, the population changed to saprobes, organic-N degraders, denitrifiers, insect endosymbionts and fungal antagonists. Based on this work, *M. rufobrunnea* may be able to influence SPM and change the soil nutritional profile.**

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Introduction

Bacteria are a central component of ecosystems and exhibit a range of harmful to beneficial interactions with other organisms. Some bacteria interact with fungi in ways that impact fungal nutrition and development (Danell *et al*., 1993; De Vries *et al*., 2006; Bonfante and Anca, 2009; Frey-Klett *et al*., 2011; Oh and Lim, 2018; Wagg *et al*., 2019). For example, the microbial community in composted substrates have been shown to be important in acidic mobilization of nutrients that are crucial to *Agaricus bisporus* mushroom development (Kertesz and Thai, 2018). On the other hand, bacteria in mushroom soil may induce fruiting bodies initiation, due to depletion in food sources (Hayes *et al*., 1969; Masaphy *et al*., 1987). In a recent study, Pent *et al*. (2017) concluded that in a boreal setting, bacterial communities within Basidiomycete mushrooms and in soils underneath the fruiting bodies are determined by the specific identity of mushroom host. The structure of bacterial communities inhabiting the fungal fruiting bodies in model species *Cantharellus cibarius* and other orders from Basidiomycota showed significant variation across developmental stages. Certain functional groups, such as those related to nitrogen fixation, were found to persist through maturation, but were then replaced by putative parasites/pathogens (Gohar *et al*., 2020). In nature, bacterial 'biological markets' (Werner *et al*., 2014) may link non-localized transfer of water, carbon and nitrogen between fungi and microbes and contribute to sustaining ecosystem functioning in arid, stressed habitats (Worrich *et al*., 2017).

Morchella spp. (morels) are important edible mushrooms that belong to Ascomycota (Pezizales, Morchellaceae). Efforts to cultivate morels have been ongoing for over 100 years, yet it is only in recent years that successful indoor and outdoor cultivation has been accomplished (Ower *et al*., 1986; Masaphy, 2010; Liu *et al*., 2018). Despite the development of morel cultivation techniques, wild foraged morels remain a significant part of the global morel economic market (Tietel and Masaphy, 2018).

In nature, morel species are hypothesized to have variable trophic states (saprobic and mycorrhizal). This is because morels are known to fruit across a wide range of

habitats in the Northern hemisphere (Pilz *et al*., 2004), from forests to road-sides, to back yard gardens and front lawns. Factors affecting morel growth and reproduction in nature, including their physical environment and nutritional sources, are still not yet well defined. Moreover, their interactions with environmental bacteria are unclear. In a study comparing the interaction between fungi and various bacteria, Pion *et al*. (2013) showed in an in-vitro system that *M. crassipes* mycelium appears to 'farm' species of *Pseudomonas* as nutrition-mobilizing helper bacteria.

Morchella rufobrunnea belongs to a basal lineage of *Morchella* (O'Donnell *et al*., 2011; Taşkın *et al*., 2012) and was one of the first species of morel to be successfully cultivated (Ower *et al*., 1986; Masaphy, 2010). *Morchella rufobrunnea* was originally described from both a disturbed roadside embankment with tree trunks and an urban garden in Mexico (Guzmán and Tapia, 1998). Since then, *M. rufobrunnea* was found in many places around the world in the West Coast of the United States, Israel, Australia, Cyprus, Malta and Switzerland in a range of habitats from natural forest groves (Masaphy *et al*., 2009) to residential yards (Kuo, 2008), and even post-fire (Larson *et al*., 2016). This species is ecologically classified as saprotrophic, since it can grow without the presence of plant hosts or indeed any plant in the vicinity (Tietel and Masaphy, 2018). Basic clayey soils found extensively in Israel (Sandler, 2013) can be detrimental to mineral availability and hence saprobic fungi can benefit from acid-forming microbes (Rashid *et al*., 2016). Similarly, in arid areas, carbon and nitrogen production by microbes may be of importance to fungal nutrition (Oren and Steinberger, 2008; Lohberger *et al*., 2019). However, most studies on the relationship of *M. rufobrunnea* with soil microbes were performed in artificial environments where nutrition is supplied. Consonant with this, Longley *et al*. (2019), have recently shown that the mycobiome and microbiome of *M. rufobrunnea* substrates grown in artificial indoor cultivation system is dynamic over the course of development. They found that *Gilmaniella* and *Bacillus*, respectively, dominated in trays that supported fruiting body production. The ascocarp microbiome of a closely related species, *M. sextelata* (black morel), cultivated in outdoor greenhouses, was recently found to be dominated by *Pedobacter*, *Pseudomonas*, *Stenotrophomonas* and *Flavobacterium* which may be associated to fruiting (Benucci *et al*., 2019). A comparative study of two *Morchella* species (*M. crassipes*, *M. rufobrunnea*) in vitro showed a sympathetic increase of organic-N hydrolysis due to bacterial enhancement of proteolytic enzymes exuded by the fungi and is correlated to organic-C presence (Lohberger *et al*., 2019).

The aim of the current study was to characterize the SPM associated with *M. rufobrunnea* fruit body development stages in a natural occurring fruiting site, a residential back yard in Northern Israel, characterized by semi-arid climate and clayey soil. To the best of our knowledge, the relationship between *M. rufobrunnea* fruiting body development in a natural habitat and its SPM has not yet been reported. We hypothesized that *M. rufobrunnea* development would be correlated with changes in SPM since it is a saprotrophic morel that derives nutrition from its environment and could be expected to either enhance or rely on bacterial nutritional mobilization and production.

Results

The developmental stages of *M. rufubrunnea* fruiting bodies included in the study were morphologically distinct and readily identifiable by colour and size (Fig. 1). The initial young fruiting bodies (1–2 cm long) were white, in the early stages of ascocarp development, that is, the ridges and pits have only started to differentiate. The more developed stage of fruiting bodies (7–10 cm long) were coloured, with grey to beige pits and white ridges.

Bacterial community composition of soil underneath and adjacent to developing M. rufobrunnea *fruiting bodies*

A total of 721 of 1388 detected OTUs from the SPM of soil underneath and adjacent to the developing fruiting bodies were included in the study (Table S1). Among

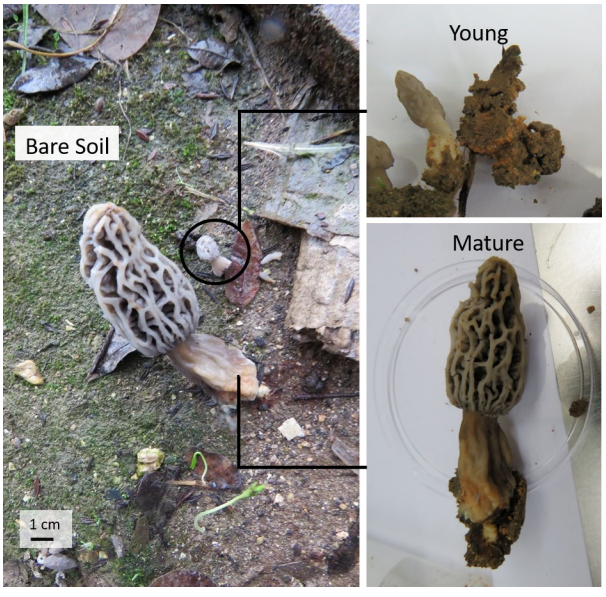

Fig. 1. Samples used in this study: bare soil, young mushroom initials and mature fruiting bodies.

these, Proteobacteria and Bacteroidetes dominated and accounted for between 50%–70% relative abundance across all samples. Other dominant phyla included Gemmatimonadetes, Acidobacteria, Planctomycetes, Verrucomicrobia, Cyanobacteria, Patescibacteria, Chloroflexi and Actinobacteria (Fig. 2A). Phyla that differed significantly between stages at $p < 0.05$ by ANOVA are displayed in Fig. 2B. Relative abundance of Proteobacteria and Bacteroidetes increased in samples from under mature morels relative to the other stages ($p = 0.0081$ and $p = 0.025$, respectively, Fig. 2B). The increase in Proteobacteria was accounted for by family Burkholderiaceae, which increased by a factor of 3 in soil under mature morels compared with other treatments. The orders Flavobacteriales and Sphingobacteriales, each of which accounted for 12% of the relative abundance in soil under mature morels, represented the main increase in phylum Bacteroidetes. Gemmatimonadetes (*Gemmatimonas* sp., Longimicrobiaceae) decreased in soils between developmental stages, with a significant difference between mature relative to bare soil treatments ($p = 0.01$). Acidobacteria (Blastocatellia, Holophagae, Subgroup_6), Patescibacteria (Parcubacteria) and Chloroflexi (Anaerolineae*)* was highest under young morel samples ($p = 0.0031$, 0.03 and 0.02, respectively). Cyanobacteria (Nostocales) and Actinobacteria (Frankiales and Proprionibacteriales) were highest in bare soils lacking morels ($p = 0.0003$ and $p = 0.0073$, respectively).

Species Richenss indices

The number of OTUs sampled in each stage was around 450 (Fig. 3). Bare soil and mature fruiting bodies stages samples had greater variability in alpha diversity compared with young fruiting bodies stages samples. Shannon diversity index increased in young and mature sample treatments compared with bare soil but the difference was not significant by ANOVA.

Bacterial diversity

Differences in the bacterial community composition yielded three clusters by principal components analysis, which correlated well with the developmental stage of *Morchella* (Fig. 4). The effect of stage as a clustering variable was confirmed with permutational analysis of variance (PERMANOVA) analysis on Bray–Curtis distance matrix (Table 1). Developmental stage explained 60% of sum of squares variation ($p = 0.0004$). In terms of group dispersion, stage was not a significant factor ($F = 1.31$, $p > 0.05$).

Indicator species

Indicator species analysis using point biserial coefficient found 180 OTUs uniquely associated ($p < 0.05$) to each

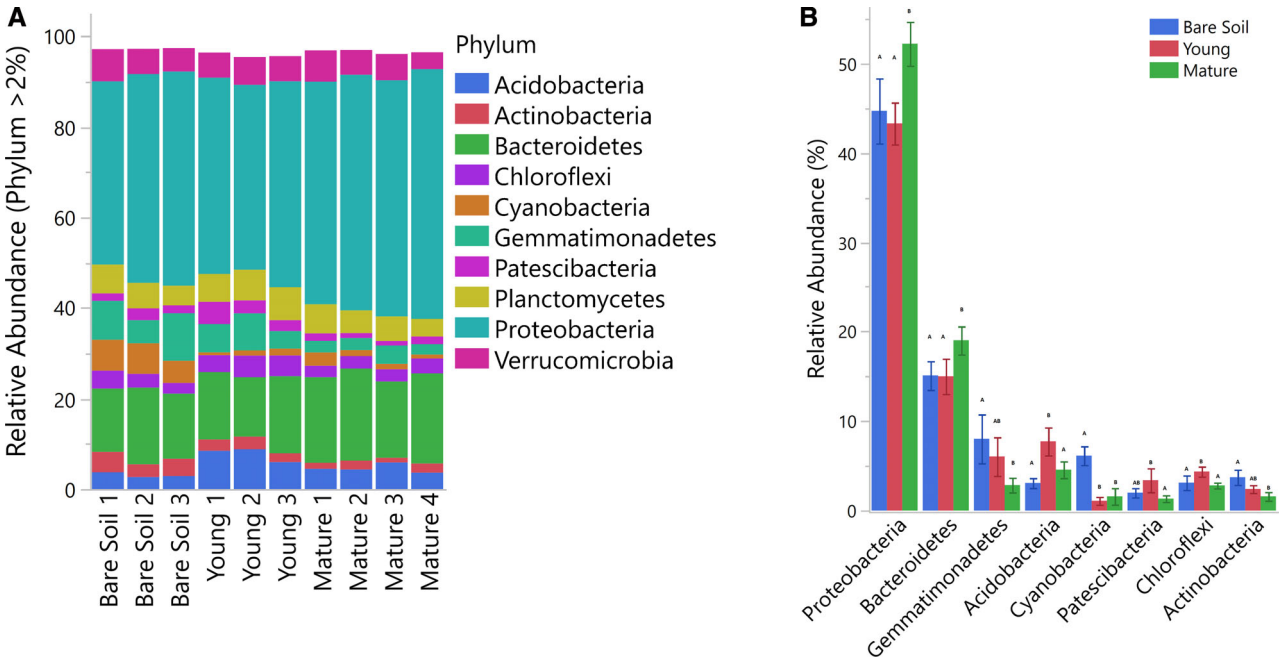

Fig. 2. A. Cumulative relative abundance of phyla >2% in at least one sample. B. Mean ± SD of phyla that changed significantly determined by ANOVA ($p < 0.05$). Different letters above the bars indicate significantly different means determined with Students' *t*-test ($p < 0.05$).

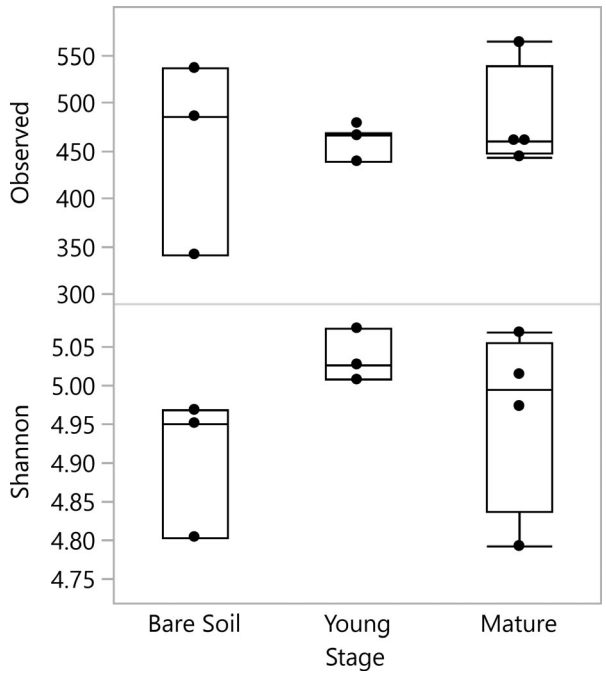

Fig. 3. Observed richness and Shannon diversity index of microbial community in bare soil and beneath two developmental stages of *Morchella rufobrunnea* fruiting bodies.

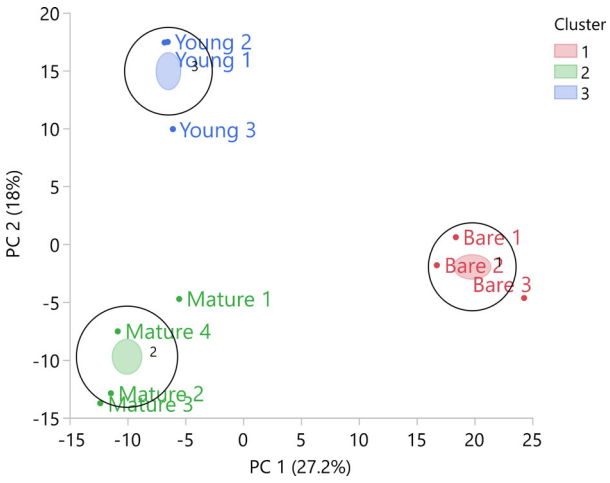

Fig. 4. Principal components bi-plot generated by *k*-means clustering of relative abundance of 721 OTU. Circles are drawn around the cluster centres and the size of the circles is proportional to the count inside the cluster.

developmental stage, with an additional 11, 28 and 3 OTUs common to Bare Soil-Young, Young-Mature and Bare Soil-Mature, respectively (Fig. 5). The relative abundance of indicator genera >1% according to indicator group is displayed as a heat map in Fig. 6. A full list of indicator OTUs is in Table S2.

Putative functional ecology of indicator bacterial community

Figure 7 depicts the average magnitude of ecological functions of indicator OTU exclusively associated with bare soil and two developmental stages of *M. rufobrunnea*. A marked change is evident in the putative functional ecology of the soil bacterial communities unique to each developmental stage. Photoautotrophy and photoheterotrophy are highest in soils lacking *M. rufobrunnea*. As the mushroom fruiting body develops there appears to be a shift in the soil bacterial community from autotrophic N-fixing to heterotrophic NH_3-N oxidizers (nitrification), and finally to organic-N degraders (Fig. 7). In latter stages there is an increase in denitrifiers, sulphate respiration and complex OM degraders.

Discussion

We tracked changes in soil bacteria community underneath *M. rufobrunnea* ascocarps in two developmental stages and nearby soil in one environmental niche. The site was characterized by clayey type of soil, with low concentration of organic matter, and a mat of cyanobacteria covering patches of the soil surface. We attempted to translate the changes in bacterial community, represented by indicators, into ecological functions, such as metabolism, N-fixation/oxidation and sulphur respiration using NGS and the FAPROTAX database (Louca *et al*., 2016).

Overview of bacterial community in the stages of Morchella *development*

In the present study, the bacterial consortium of the bare soil, the soil beneath the mushroom initial and the soil under mature ascocarp formed three distinct communities. Of 11 phyla present at >2% relative abundance, 9 changed significantly between treatments, while the overall alpha diversity remained similar among all treatments. This indicates ecological succession and species turnover where new species are increasing in abundance at the expense of existing species (de Carvalho *et al*., 2016). Whether this is a result of a site effect is not answerable in the current study. On the one hand, it can be argued that fructification took place only in a suitable micro-site due to nutritional stress conditions and associated changes in the soil prokaryote community, or it may be that the presence of the mushroom caused a change in the soil microbiome. Alternatively, it could be a combination of the two processes. Yet, changes in the soil microbiome along with mushroom developmental stages are can be related to the mushrooms biological activity. It is difficult to plan and conduct environmental research to

Table 1. Permutational multivariate analysis of variance (PERMANOVA) of Bray–Curtis distance matrix and multivariate homogeneity of groups dispersions analysis (betadisper) results for microbial community associated with soil adjacent to and beneath *Morchella rufobrunnea* fruiting bodies at different stages of development.

	PERMANOVA				betadisper		
Factor	*df*	SS	*F*-value	R^2	SS	*F*-value	*p*-value
Stage	2	0.40265	5.2636	0.60062	0.0039	1.3117	0.3282
Residuals	7	0.26774		0.39938	0.0104		
Total	9	0.6704		1			

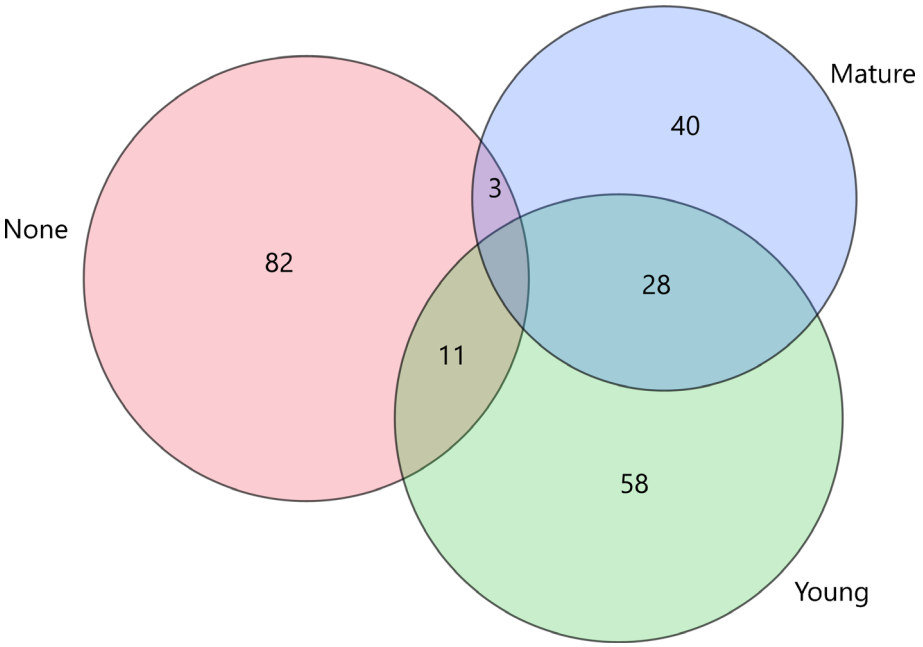

Fig. 5. Venn diagram of indicator OTU with associated with bare soil and two developmental stages of *Morchella rufobrunnea* fruiting bodies.

solve this problem as it is very difficult to predict the precise micro-site in which fructification will take place.

Bare soil was characterized by Cyanobacteria and Actinobacteria capable of establishing pioneer, N-fixing microbial mats in nutrient poor semi-arid soils (Hagemann *et al*., 2017; van Bergeijk *et al*., 2020). Soil underneath mushroom fruiting bodies is more moist than surrounding bare soil (Xing *et al*., 2018), which could also enhance competition from other bacteria that are less adapted to dry conditions than Cyanobacteria and Actinomycetes. The genera *Sphingomonas* and *Brevundimonas* indicative of bare soil were also found elsewhere as Cyanobacteria-associated bacteria (Secker *et al*., 2016; Kim *et al*., 2020). Perhaps these bacteria are living off carbohydrates produced by Cyanobacteria.

Soil under young ascocarps contained the highest number of indicator OTUs compared with other treatments which may reflect a diverse transition state. While not directly determined in this study, developing ascocarps undergo biochemical changes, including accumulation of carbohydrates, enzymes, ascorbic acid, tocopherols and other phytochemicals that may influence the surrounding soil microbiome (Sancholle *et al*., 1988; Harki *et al*., 2006; Tietel and Masaphy, 2018; Benucci *et al*., 2019; Carrasco and Preston, 2020). Hence the changing soil conditions under young ascocarps could be expected to include decrease in organic carbon and inorganic nitrogen, and increase in moisture and exudates. These changes may be related to the observed increase in relative abundance of opportunistic heterotrophs in phyla Acidobacteria, Patescibacteria and Chloroflexi in soil under young developing morel fruiting bodies. Similarly, Acidobacteria were found in ectomycorrhiza, ascoma and mycelium of several truffles species (Carrasco and Preston, 2020). Acidobacteria generally thrive in low carbon environments (oligotrophic), have resistance to antibiotics and are capable of extracellular-polymeric substances production which aid in soil stabilization (Kielak *et al*., 2016). This phylum is responsible for maintenance of low-pH in soil, hence facilitating nutrient mobility and uptake to fungal populations, but are rarely isolated in pure culture (Carrasco and Preston, 2020).

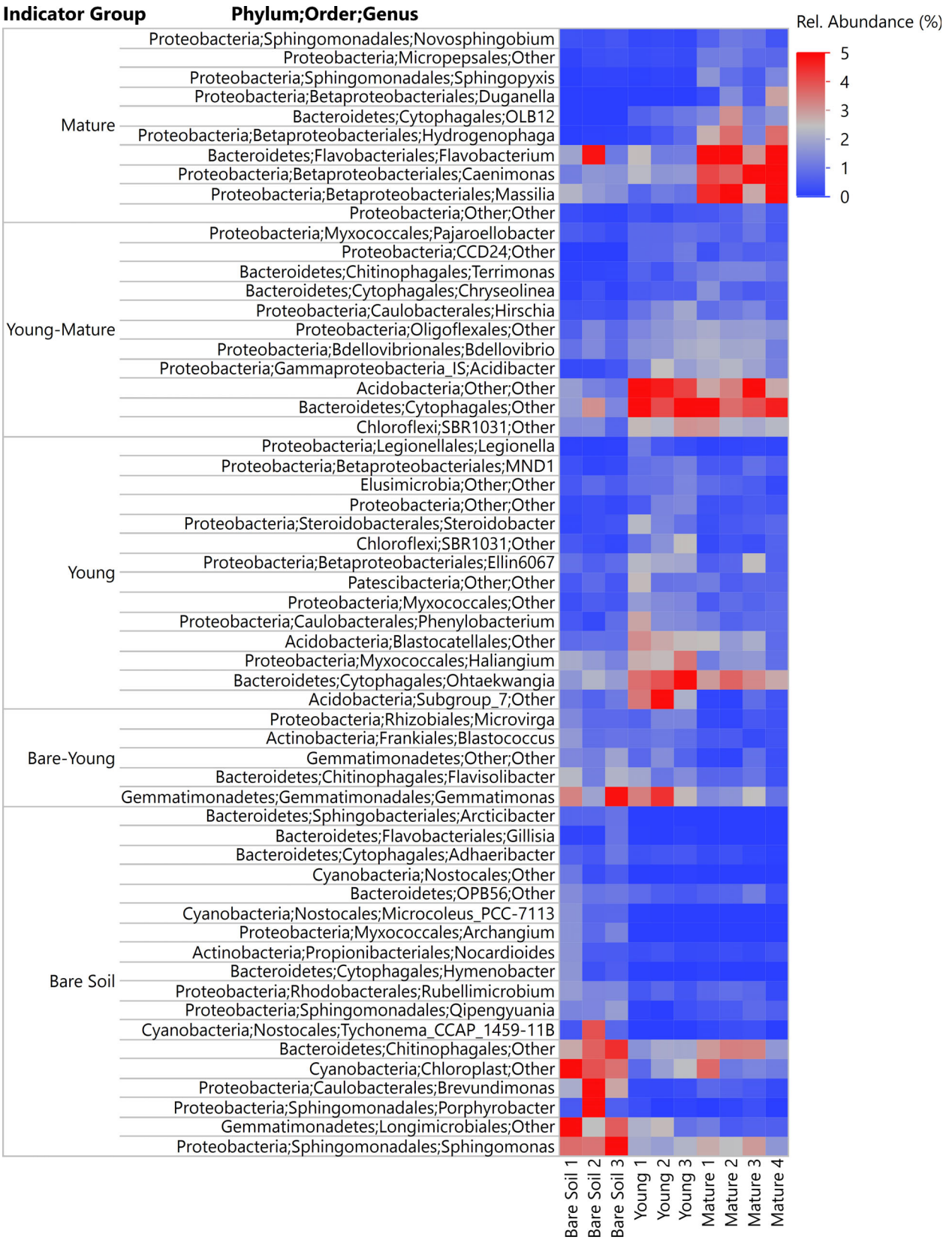

Fig. 6. Indicator OTU (genera) present at >1% relative abundance associated with bare soil and two developmental stages of *Morchella rufobrunnea* fruiting bodies.

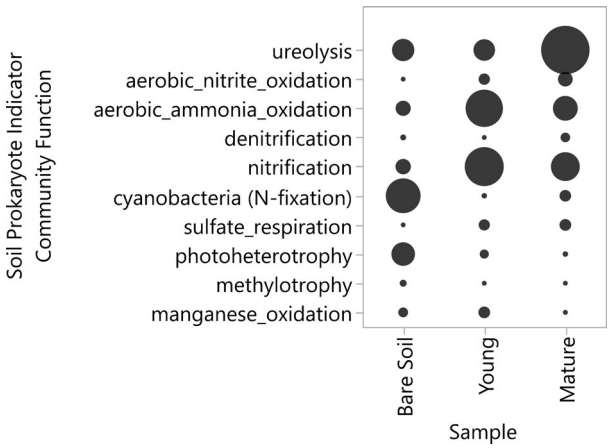

Fig. 7. Putative functional ecology of indicator OTU in soil microbial communities exclusively associated with bare soil and soil under two developmental stages of *Morchella rufobrunnea* fruiting bodies. The circle sizes indicate the average magnitude of the ecological function within the group as predicted by FAPROTAX.

Patescibacteria are small microbes (0.3 μm) with streamlined genomes that may rely on their host community for nutrients or 'public goods' (Tian *et al*., 2020). Their transient increased appearance beneath young developing morel fruiting bodies suggests an exploitation of available metabolites. The Chloroflexi phylum is associated with photoheterotrophic microbial mats (Ward *et al*., 2018). Their unique metabolism uses simple organic carbon compounds such as acetate, glycerol, glucose, pyruvate or glutamate as an electron donor and carbon source, and light as energy source (Overmann, 2008). Very little is known about the species in these phyla, hence the indicator bacteria in soil under the young stage ascocarps were not well defined at genera level.

In soil under mature fruitbodies, the relative abundance of niche-exploiters and organic matter (OM) degraders increased relative to the younger stage and bare soil. Examples include genera in Burkholderiales such as *Caenimonas* (a nitrate reducer) (Ryu *et al*., 2008), *Massilia* (copiotrophic root colonizer) (Ofek *et al*., 2012), *Hydrogenophaga* (hydrogen and recalcitrant amine degrading) (Gan *et al*., 2017) and *Duganella* (antifungal, chitin degrading) (Haack *et al*., 2016). The increase of Burkholderiales may be correlated to accumulation of oxidized nitrogen and OM from degrading soil mycelial biomass which could be expected to be highest under mature *Morchella* fruiting bodies. Burkholderiales include some of the best-known fungal endobacteria (Deveau *et al*., 2018).

The genera *Flavobacterium* (Bacteroidetes) increased around three-fold in soil under mature ascocarps compared with the young stage. Most *Flavobacterium* species are able to degrade a variety of polysaccharide components of algae, plants, fungi and insects such as agar, alginate, chitin, laminarin, pectin, xylan and other organic macromolecules (Bernardet and Bowman, 2006). *Flavobacterium* were abundant in cultured black morel *M. sextelata* fruiting bodies and may contribute to formation of mushroom fruiting bodies (Benucci *et al*., 2019). Sphingobacteriales also increased in mature samples and these are known, along with Burkholderiales, as cellulose decomposers (Eichorst and Kuske, 2012). The relationship between cellulose-degrading microbes and fungi can be either complimentary or competitive depending on edaphic factors such as soil type and OM content (Eichorst and Kuske, 2012).

Interestingly, only three genera were found to be indicators shared between bare soil and under mature *M. rufobrunnea* ascocarps all from order Rhizobiales (*Bosea, Chthonobacter* and *Shinella*). While their presence was only at <1% relative abundance, the role in ecological niche is noteworthy. This order is often referred to as 'helper bacteria' due to their diazotrophic and endophytic abilities (Bates *et al*., 2011), although the genera found in this study appear to be free-living. Since they are typically slow-growing bacteria, perhaps they are overgrown or excluded during the growth of opportunists underneath the young ascocarps but reemerge in the mature stage.

Unlike the current study on bacterial communities in natural soil and fruiting bodies, Longley *et al*. (2019) studied the microbiome in artificial cultivation substrates of *M. rufobrunnea*. They did not find any bacterial indicators perhaps due to the supply of ready nutrition to the developing ascocarp, minimizing the need for microbial support. Yet they did find relative dominance of *Bacillus* in trays that supported fruiting body development of *M. rufobrunnea*. In our study, phylum Firmicutes was present at <2% relative abundance in all treatments. Therefore we suggest that in natural conditions, *Bacillus* is not an essential co-inoculant for *M. rufobrunnea* fruiting but in culture conditions may be an antagonistic heterotroph like the genera *Massilia*, *Caenimonas*, *Flavobacterium* and *Hydrogenophaga* in the present study which were highest under mature ascocarps.

The impact of fungal growth on soil bacterial communities was also reported by Xing *et al*. (2018) who found that the mycorrhizal *Floccularia luteovirens* increased N, P, K, soil moisture and pH compared with nearby soil. These changes, especially N content, affect the SPM. Several bacterial strains isolated from the rhizosphere of *F. luteovirens* (e.g., *Pseudomans fluorescens*, *P. koreensis*) promoted hyphal growth, while others (*P. mandelii*, *B. pumilus*) curtailed hyphal growth, suggesting that both mutual and antagonistic interactions are associated with the fruiting process. In the present study, the definitive saprobic nature of *M. rufobrunnea* would suggest a relative depletion of inorganic nutrients

in the soil as the ascocarp matures with a corresponding build-up of complex products suitable for heterotrophic activity. Indeed, the overall community appeared to shift from producer to consumer/degrader as organic byproducts accumulated.

It should be noted that the differences between bare soil and mushroom associated SPM occurred gradually from the initiation stage through the fruit body development. Unlike Basidomycetes that can reach maturation in several days (Kües, 2000), *M. rufobrunnea* fruiting body development takes place over a period of 2 weeks from initiation until young ascocarps develop and a further 1–2 weeks for maturity (Ower, 1982; Masaphy, 2010). This length of time may be enough to cause changes in soil bacterial population. A sample size of 3 can be encountered in environmental studies of fungal-microbe interactions (Xing *et al*., 2018; Longley *et al*., 2019). In the current study the amount of replicates ($n = 3$ for bare soil and young, $n = 4$ for mature samples) were due to incidental conditions in the field, leading to unbalanced groups. In order to assess the impact of the additional replicate in the mature group, separate *k*-means biplot cluster analyses on the SPM community were performed, each time with a different replicate removed from the control group and the clustering did not change (data not shown).The semi-parametric PERMANOVA test used in this study on the beta-diversity dissimilarity matrix is robust to unbalanced designs (Anderson, 2017).

The overall ecological progression of SPM associated with *M. rufobrunnea* development can be summarized as follows. First is the establishment of a mostly photoautotrophic N-fixing microbial mat. Pioneer heterotrophs, including oligotrophs, nutrient mobilizers, nitrifiers are congruent with appearance of young *M. rufobrunnea* initials. As the mushroom cap matures and mycelium senesces, this population gives way to complex OM degraders, denitrifiers and other competitors (Fig. 8). Increase in denitrification and sulphate respiration in latter stages may also indicate negative redox potential and utilization of alternative electron acceptors to atmospheric oxygen which would imply that there is an excess of OM. This correlates well with the abundance of complex organic molecule degrader bacteria found in soils under mature morel fruiting bodies. Whether the change in SPM is a result of niche exploitation by native bacteria or is in some way induced by *M. rufobrunnea* remains to be elucidated with further natural habitat research and studies in controlled conditions.

In summary, the data presented here show as hypothesized, that changes in the SPM under *M. rufobrunnea* are correlated to stages of mushroom development. The changes in SPM and putative microbial function might be promoted by *M. rufobrunnea* or it could be the other way around that the observed changes in SPM induced *M. rufobrunnea* fruiting. The data presented here are from one of *M. rufobrunnea* growth niches in a natural habitat. Since this *Morchella* species can fruit in diverse habitats (road sides, forest, burned sites, etc.), the SPM of other habitats should be studied to defined bacterial types that could be specifically associated with *M. rufobrunnea* species. Further advances in functional ecology assignment of environmental prokaryotes are necessary in order to achieve maximal understanding from metagenomics studies (Nilsson *et al*., 2019).

Experimental procedures

Mushroom and site

M. rufobrunnea mushrooms were located in the back yard of a non-active carpentry shop in the Hula valley, Northern Israel. A wave of mature fruiting bodies (7–10 cm length, brownish head), with another fresh wave of small initials of fruiting bodies (white, 1–2 cm length) emerging in the same site were found in mid-March 2018. The time difference between both waves of fruiting was between 1 and 2 weeks. The average temperature for the month preceding the sampling was 13°C. Precipitation during the month before sampling date (mid-February to mid-March) was 74 mm in February and

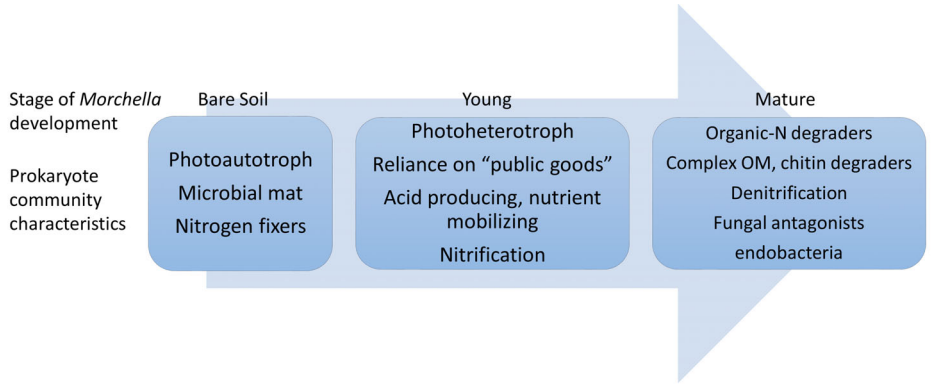

Fig. 8. Summary of ecological progression of microbial community in bare soil and *Morchella rufobrunnea* fruiting bodies development.

7.5 mm in March with the last rain event occurring 5 days prior to sampling. The soil texture (from samples with no mushroom) was classified as clayey with 45.6% clay, 42.8% silt and 11.4% silt (determined with Mastersizer 3000, Malvern Panalytical, Malvern, UK). This soil (no mushroom) had pH 7.85, EC 1.42 mS dm^{-1}, organic matter 8.33%.

Soil sampling

Three sample types were compared in this study: (a) soil with no mushroom (bare soil), (b) soil beneath the young mushroom initials and (c) soil beneath mature fruiting bodies. The mushroom samples appeared to fruit individually and soil samples were separated by a distance of 20–50 cm in a total area of 2 × 1.5 m site. Approximately 10 g of soil from three replicates of bare soil were drawn from the top 5 cm of the soil profile. The mushroom-associated soil samples (3–4 replicates of ~5 g) were taken directly from beneath the mushroom stems. Bare soil samples and mushroom-associated soil samples were collected with sterile spades into sterile plastic cups with lids and transferred on ice to the lab within 2 h. In the lab, samples were cleansed of leaves and debris >2 mm using a sieve and aseptic practices. Subsamples were weighed and kept at −20°C until DNA extraction (within a week).

Molecular methods

DNA extraction. Soil samples were manually homogenized, and two subsamples of 0.25 g wet soil from each replicate were used for DNA extraction from each of the soil samples. DNA was extracted by ZymoBiomics DNA Miniprep kit (Zymo Research, Irvine, California), according to manufacturer instructions. Extracted DNA concentration was determined by NanoDrop spectrophotometer (Thermo Scientific, The United States). DNA extracted from the two subsamples was pooled before next-generation sequencing (NGS) library preparation. Library preparation, pooling and NGS were performed at the Genome Research Core (GRC), Research Resources Center, University of Illinois at Chicago. Genomic DNA was PCR amplified with primers 515F and 926R (Walters *et al*., 2016) targeting the V4 and V5 variable regions of the microbial small subunit ribosomal RNA gene using a two-stage targeted amplicon sequencing (TAS) protocol as described previously (Naqib *et al*., 2018).

Amplicon sequencing. Forward and reverse reads were merged using PEAR (Zhang *et al*., 2014). Merged reads were trimmed to remove ambiguous nucleotides, primer sequences, and trimmed based on quality threshold of $p = 0.01$. Reads that lacked either primer sequence or any sequences less than 225 bp were discarded. Chimeric sequences were identified and removed using the USEARCH algorithm with a comparison to Silva v132 reference sequence database. (Edgar, 2010; Glöckner *et al*., 2017) OTUs were identified using DADA2 (Callahan *et al*., 2016). The representative sequences for each OTU were then annotated taxonomically using the Naïve Bayesian classifier included with DADA2 using the Silva v132 training set (Callahan *et al*., 2016; Glöckner *et al*., 2017).

Statistics and analyses. Data were analysed in R (R Core Team, 2017) and JMP 15.0 (SAS Institute, 2019). Total OTU reads were normalized in all samples to 80,000 in order to minimize bias from differences in sequence depth between samples. OTU having <10 reads were removed. Subsequent analyses generally followed the precedent suggested by Longley *et al*. (2019). Alpha-diversity was determined using the 'plot richness' function in *phyloseq*. Sample clustering due to differences in OTU relative abundance (β-diversity) was visualized with K-means clustering in JMP. The biplot shows the points and clusters in the first two principal components of the data. Circles are drawn around the cluster centres and the size of the circles is proportional to the count inside the cluster (SAS Institute, 2021). Significance of stage to sample grouping and dispersion was tested in package *vegan* with permutational multivariate analysis of variance (PERMANOVA) of the Bray–Curtis dissimilarity matrix using 'adonis' and 'betadisper' functions, respectively. Identification of OTU associated with each sample type was performed with 'multipatt' function in *indicspecies* with the point-biserial correlation coefficient (r_{pb}) (De Caceres and Legendre, 2009). An indicator OTU Venn diagram was prepared with Venn diagram add-in for JMP (Wolfinger, 2014). The Functional Annotation of Prokaryotic Taxonomy (FAPROTAX) database (Louca *et al*., 2016) was used to infer potential ecological roles for OTU, visualized in JMP as a bubble plot sized by relative abundance. The effect of developmental stage on average OTU relative abundance in each group was tested with one-way ANOVA ($df = 2.10$) followed by pairwise comparisons with Student's *t*-test ($df = 2.2$) with *p* values displayed in the text.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Full dataset relative abundance.
Table S2. Full list of indicator OTUs.