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Research Letter

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Soil microbiome transfer method affects microbiome composition, including dominant microorganisms, in a novel environment

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One sentence summary: The transfer of microbial assemblages from soil to commercial potting mix has an impact on microbiome composition.

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ABSTRACT

We show that choice of soil microbiome transfer method, i.e. direct soil transfers and a common soil wash procedure, dramatically influences the microbiome that develops in a new environment, using high-throughput amplicon sequencing of 16S rRNA genes and the fungal internal transcribed spacer (ITS) region. After 3 weeks of incubation in commercial potting mix, microbiomes were most similar to the source soil when a greater volume of initial soil was transferred (5% v/v transfer), and least similar when using a soil wash. Abundant operational taxonomic units were substantially affected by transfer method, suggesting that compounds transferred from the source soil, shifts in biotic interactions, or both, play an important role in their success.

Keywords: microbiome transfer; 16S rRNA gene; fungal ITS, Illumina MiSeq; commercial potting mix

INTRODUCTION

Microorganisms mediate many key processes in soils. However, the extent of functional redundancy among microbes is not fully known (Allison and Martiny 2008; Martiny, Treseder and Pusch 2013), so it can be difficult to disentangle the influence of microbes and the abiotic soil environment on a given process. Although *in vitro* studies provide extensive knowledge of the physiological limits of cultivated microbes, they do not replicate the function and interactions of intact microbiomes (i.e. assemblages of microbes found in a specific environment) in the soil. As an alternative, some research groups have co-opted a common approach from ecology, the reciprocal transplant, to transfer whole microbiomes to new environments and examine their impact on soil processes. For instance, microbiome transplants have been shown to impact the phenotype of plants (Wagner *et al.* 2014; Panke-Buisse *et al.* 2015; Yergeau *et al.* 2015), bioremediation of pollutants (Bell *et al.* 2016), and soil organic matter production (Kallenbach, Frey and Grandy 2016).

Various microbiome transfer approaches have been applied in soils, including direct soil transfer and soil washes. At the high end for soil transfers, Lau and Lennon (2012) transferred 50% of their colonized potting mix to sterile potting mix at the end of three selection generations, although the initial inoculation from field soil represented ~3–4% of the total substrate volume in their pots. Transfers of ~5–10% soil (v/v) have been used in other studies (Tkacz *et al.* 2015; Yergeau *et al.* 2015). At the other extreme, Panke-Buisse *et al.* (2015) and Calderón *et al.* (2016) transferred <1% soil into sterile pots through soil slurries, while Kallenbach, Frey and Grandy (2016) introduced the equivalent of ~10^{-5} g of source soil in the form of a slurry for each

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gram of sterile soil, in order to limit the transfer of soil compounds. The amount of soil transferred also appears to impact function in the inoculated soil; Swenson, Wilson and Elias (2000) varied their inocula by a factor of 10, and found much larger increases in *Arabidopsis* biomass after 11 generations of selection when more inoculum was used. Soil washes have been used in a number of studies aiming to limit the transfer of soil-derived nutrients (Wagner *et al.* 2014), or to isolate the effects of arbuscular mycorrhizal fungi on plants from those of all other soil microorganisms (van der Heijden *et al.* 1998; Glassman and Casper 2012). Whereas direct soil transfers have the disadvantage of transplanting soil compounds in addition to microbes (e.g. nutrients, allelopathic compounds), it is unknown to what extent soil washes alter microbiome structure, since they will likely favour microbes that are less adhered to the soil, and that pass easily through the selected filter.

Due to the wide range of approaches to this important method, we aimed to compare the effectiveness (i.e. compositional similarity to the source soil) of different microbiome transfer methods. The approaches selected were direct soil transfer, using different proportions of the final soil volume (5%, 1%, and 0.1% v/v) and a common soil wash procedure. We transferred the microbiome of a forest soil into a novel environment, which was a commonly used (and sterilized) sphagnum peat moss-based commercial potting mix. Our expectation was that the direct soil transfers would be similar to each other in composition, but with more inter-replicate variability and lower diversity when less soil was transferred as a result of dilution, and that the soil wash composition would be distinct, and less similar to the source soil. We show that both the bacterial and fungal components of the soil wash-treated potting mix were least similar to the source soil after 3 weeks of incubation, but that all microbiome transfer approaches produced distinct microbiomes. Interestingly, abundant microbes were strongly affected by the selected microbiome transfer approach. Our results suggest that consistency in microbiome transfer methods will be essential for inter-study comparison.

MATERIALS AND METHODS

Microcosm establishment

Due to its widespread use in plant studies, we applied sterile potting mix as the recipient matrix for microbiome growth, creating a novel environment for the transferred microorganisms. The potting media, LM-1 (Lambert, Rivière-Ouelle, QC, Canada), is composed of 80–90% sphagnum peat moss (fine particle size) with small amounts of perlite, and was autoclaved three times for 25 min, with 24 h between sterilizations. As a microbial source, we collected soil from an urban forest in Ithaca, NY, USA (42° 27′ 36″ N, 76° 28′ 48″W), which we homogenized by hand after removing rocks and other large debris. Samples of the initial field soil and sterile potting media were collected and frozen for molecular analysis.

We created three direct soil transfer treatments by inoculating sterilized potting media directly with field soil at three concentrations, 0.1, 1 and 5% (v/v), using serial dilution. Mixes were homogenized by hand in sterile bins for 5 min each. Separate serial dilutions were prepared for each of five replicates to allow for variability in microbial species transfer. For each replicate, 45 mL of inoculated potting mix was added to 50 mL plastic Falcon tubes. The tubes were incubated in the dark at room temperature for 3 weeks with loosened caps to allow air exchange. As a point of reference, we also placed 45 mL of the initial soil in a 50 mL tube under the same conditions.

To create the soil wash, a 20% (v/v) field soil solution was prepared in sterile 0.85% (w/v) NaCl (aq) in a flask and mixed on a shaker at 180 r.p.m. for 10 min. The mixture was then filtered (11 μm pore size, Whatman #1 filter paper) to remove soil particles. In order to isolate the microbial fraction and remove water-soluble nutrients/chemicals, the filtrate was centrifuged at 3000 *g* for 30 min (Wagner *et al.* 2014) and the supernatant was discarded. The pelleted microorganisms were resuspended in 0.85% NaCl (aq) by vortexing at maximum speed for 30 s. Separate soil washes were prepared for each of five replicates, and were used to inoculate 45 mL of sterile potting media. Although extraction efficiency of microbial cells from soil during the wash would not have been 100%, the amount of wash added was selected to be equal to the 5% (v/v) direct soil transfer treatment if extraction efficiency were 100%. A sample of each soil wash was frozen for molecular analysis.

The moisture of all treatments was adjusted to 17% (v/v), which is roughly the moisture we aim to maintain for plant growth in this media. We also added NaCl (aq) to the direct soil transfer treatments to correct for NaCl used in the soil wash. Microcosms were visually monitored for water loss over the 3-week incubation. Moisture was only observed to decline slightly, and was supplemented with equal amounts of sterile deionized water across microcosms once during the incubation.

DNA extraction, PCR amplification and Illumina MiSeq sequencing

DNA was isolated from 50–150 mg of substrate from each treatment, the sterile potting mix, and the initial soil using the MoBio PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). We used a lower mass of substrate for DNA extraction than is recommended by the manufacturer in order to avoid excess buffer absorption by the potting mix. Using the same kit, we extracted DNA from 300 μL of aqueous initial soil wash inoculant from each of four replicate wash preparations.

Initial 16S rRNA gene and fungal internal transcribed spacer (ITS) PCR reactions were performed on 1:10 DNA dilutions using a Bio-Rad C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). For 16S rRNA gene amplifications, we used the universal bacterial primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Herlemann *et al.* 2011) with overhangs included for index attachment, as described in Bell *et al.* (2016). Reactions occurred in 20 μL volumes, using 8 μL of 5 PRIME HotMasterMix (5 PRIME Inc., Gaithersburg, MD, USA), and 1 μL of each primer from 10 μM solutions. PCR cycling conditions for 16S rRNA gene amplifications were as follows: 94°C for 2 min; 25 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 30 s; with a final elongation at 72°C for 5 min. For ITS amplifications, we used the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 58A2R (5′-CTGCGTTCTTCATCGAT-3′) (Gardes and Bruns 1993; Martin and Rygiewicz 2005) with the required adaptors attached as described above. Reactions occurred in 20 μL volumes, using 8 μL of 5 PRIME HotMasterMix, 0.5 μL of each primer from 10 μM solutions, and 1 μL DMSO. PCR cycling conditions for ITS amplifications were as follows: 94°C for 3 min; 35 cycles of 94°C for 20 s, 45°C for 30 s and 72°C for 45 s; with a final elongation at 72°C for 5 min. Multiple attempts to amplify the ITS and 16S rRNA gene regions from the sterile potting mix extracts were unsuccessful, and so these were not included in our analyses.

Initial amplicons were cleaned with MagBio HighPrep PCR beads (MagBio Genomics, Gaithersburg, MD, USA) in clear 96-well plates. Unique two-barcode index combinations were then attached to the cleaned amplicons by combining in each well of

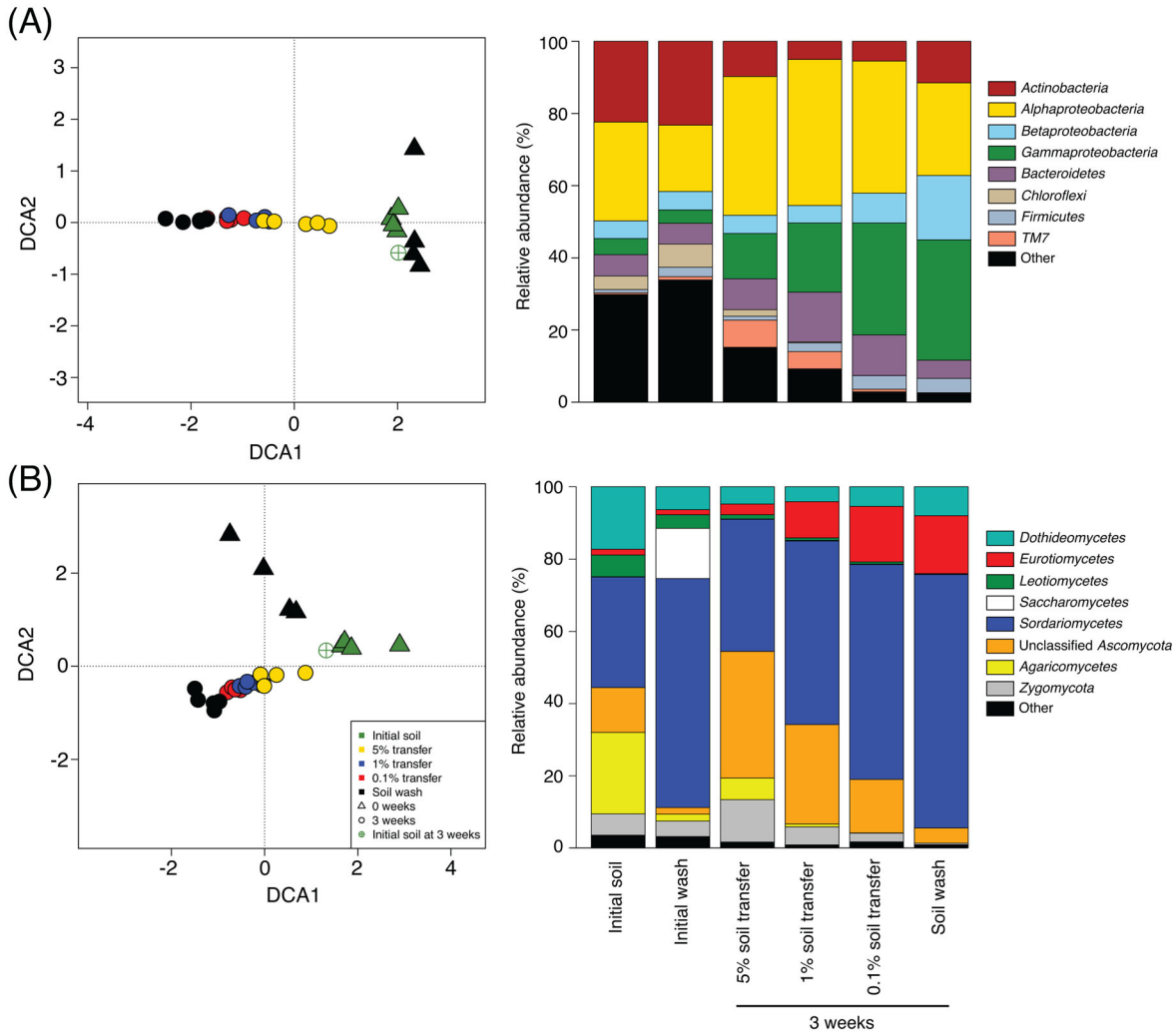

Figure 1. Detrended correspondence analysis (DCA) and 100% plots of the mean relative abundance of dominant phyla/classes of (**A**) bacteria and (**B**) fungi, based on 16S rRNA gene and ITS sequencing, respectively, in the initial soil ($n = 4$), initial soil wash ($n = 4$), and after 3 weeks for each microbiome transfer approach ($n = 5$). The initial soil after 3 weeks of incubation is provided as a reference in the DCA plots, but is not included in future analyses ($n = 1$).

a new 96-well plate: 5 μL of sample, 2.5 μL of forward and reverse primers containing designated barcodes that target the attached overhangs, 2.5 μL of water, and 12.5 μL of Q5 High Fidelity 2X Master Mix (New England Biolabs Inc., Ipswich, MA, USA). PCR cycling conditions for index attachment were as follows: 98°C for 1 min; 8 cycles of 98°C for 15 s, 55°C for 30 s and 72°C for 20 s; with a final elongation at 72°C for 3 min. Barcoded amplicons were then transferred to plates provided with the SequalPrep Normalization Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the manufacturer's instructions were followed to normalize the amount of DNA retained for each sample. We combined 5 μL of each normalized sample into separate pools for 16S rRNA gene and ITS amplicons. Pools were then concentrated and run on 1.2% agarose gels, and bands of the expected size were excised and processed using the PureLink Quick Gel Extraction Kit to give final pool volumes of 30 μL. Pools were sequenced on the Illumina MiSeq at the Cornell Genomics Facility (Ithaca, NY, USA), using a 500-cycle MiSeq Reagent Kit v.2 for the ITS pool, and a 600-cycle MiSeq Reagent Kit v.3 for the 16S rRNA gene pool. A total of 887,783 16S rRNA gene and 648,284 fungal ITS reads were obtained following paired-end merging, primer trimming and singleton removal. For 16S rRNA gene sequences, one of the 1% soil transfer replicates at 3 weeks yielded few sequences and was removed. Otherwise, five replicates were sequenced for all treatments, and four replicates for the initial soil and initial soil wash.

Sequence processing

Initial sequence processing was based on the Brazilian Microbiome Project Pipeline (Pylro *et al.* 2014), with some modifications. In Mothur v.1.36.1 (Schloss *et al.* 2009), paired-end sequences were merged (*make.contigs*), primers trimmed (*trim.seqs*, pdiffs = 2, maxambig = 0), and singleton sequences removed (*unique.seqs* → *split.abund*, cutoff = 1). Clustering of 97% operational taxonomic units (OTUs) and chimera removal (RDP Gold and UNITE databases provided by http://www.brmicrobiome.org/) were performed using USEARCH v.7 (Edgar 2010). In Mothur, representative OTU sequences were classified (*classify.seqs*, cutoff = 80) using the GreenGenes v. 13.8 database for 16S rRNA gene sequences and UNITE v. 7 database for ITS sequences, and OTUs that were suspected to not be of fungal or bacterial origin were removed (*remove.lineage*).

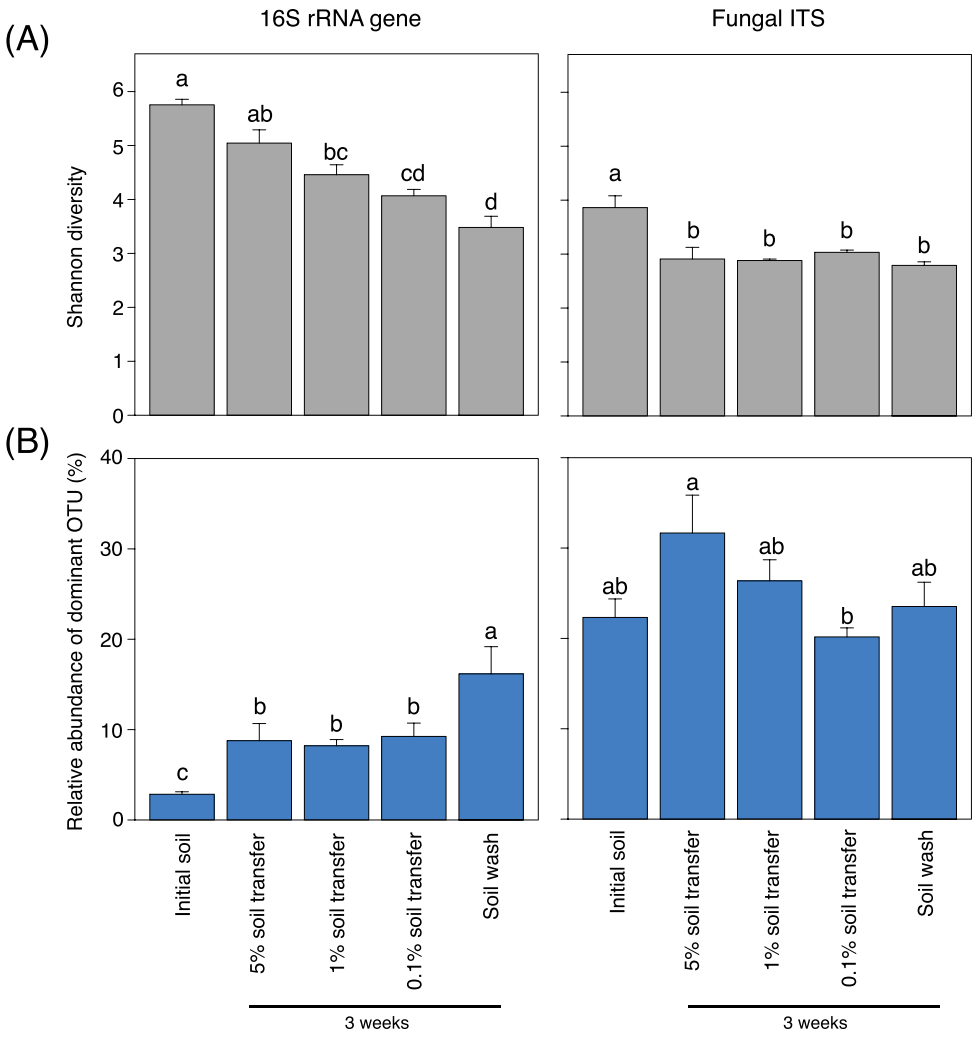

Figure 2. Shannon diversity (**A**) and relative abundance (**B**) of the most abundant OTUs for bacteria and fungi in the initial soil and after 3 weeks for each microbiome transfer approach. Error bars indicate standard error. Columns not connected by the same letter indicate that means were significantly different according to Tukey's HSD test (A) or least squares means tests (B).

OTU tables and taxonomic identifications were uploaded to R v. 3.2.1 (R_Core_Team 2013) for further analyses.

MiSeq data have been deposited in the NCBI Sequence Read Archive and are available under the project number SRP101475.

Statistical analyses

All statistical analyses were performed in R. Data were first randomly subsampled to yield an equal number of sequences for all samples (rarefaction curves are shown in Figs S1 and S2 in the online Supplementary material). Ordinations of bacterial and fungal composition were based on detrended correspondence analysis (DCA) of Bray–Curtis distance values performed using the *decorana* function in the *vegan* package (Oksanen *et al*. 2015) and hierarchical clustering was performed using *hclust* in *stats*. Shannon diversity was calculated using the *diversity* function in *vegan*. We tested for statistical differences in bacterial and fungal composition between microbiome transfer treatments after 3 weeks using the *adonis* function in *vegan* to perform a PERMANOVA (method = bray, perm = 999). We searched for statistical differences in mean diversity using one-way ANOVA, followed by Tukey's HSD post hoc test (functions *lm* and *TukeyHSD* in package *stats*), and compared the mean relative abundance of the most abundant OTU per treatment on our count data using a negative binomial model and least squares means test due to overdispersion in these data (*glm.nb* in the *MASS* package followed by *lsmeans* in the *lsmeans* package). To determine whether there was an obvious difference in the variance in bacterial and fungal composition within treatments (e.g. more inter-replicate variability with lower volumes of soil transferred), we tested for multivariate homogeneity of group dispersions (Anderson, Ellingsen and McArdle 2006) using the function *betadisper* on Bray–Curtis distances in package *vegan*. Shifts in OTU abundance were calculated by subtracting relative abundance at 3 weeks from the mean relative abundance in the initial soil, and visualized using *heatmap.2* in *gplots*. Finally, we determined the 10 most abundant OTUs for each treatment on average and displayed these in a presence–absence heatmap (function *heatmap*, package *stats*).

RESULTS AND DISCUSSION

In this study, we demonstrate that the approach used for soil microbiome transfer has a large impact on the resulting microbiome in a new environment (sterile commercial potting mix) after 3 weeks of incubation. Interestingly, the

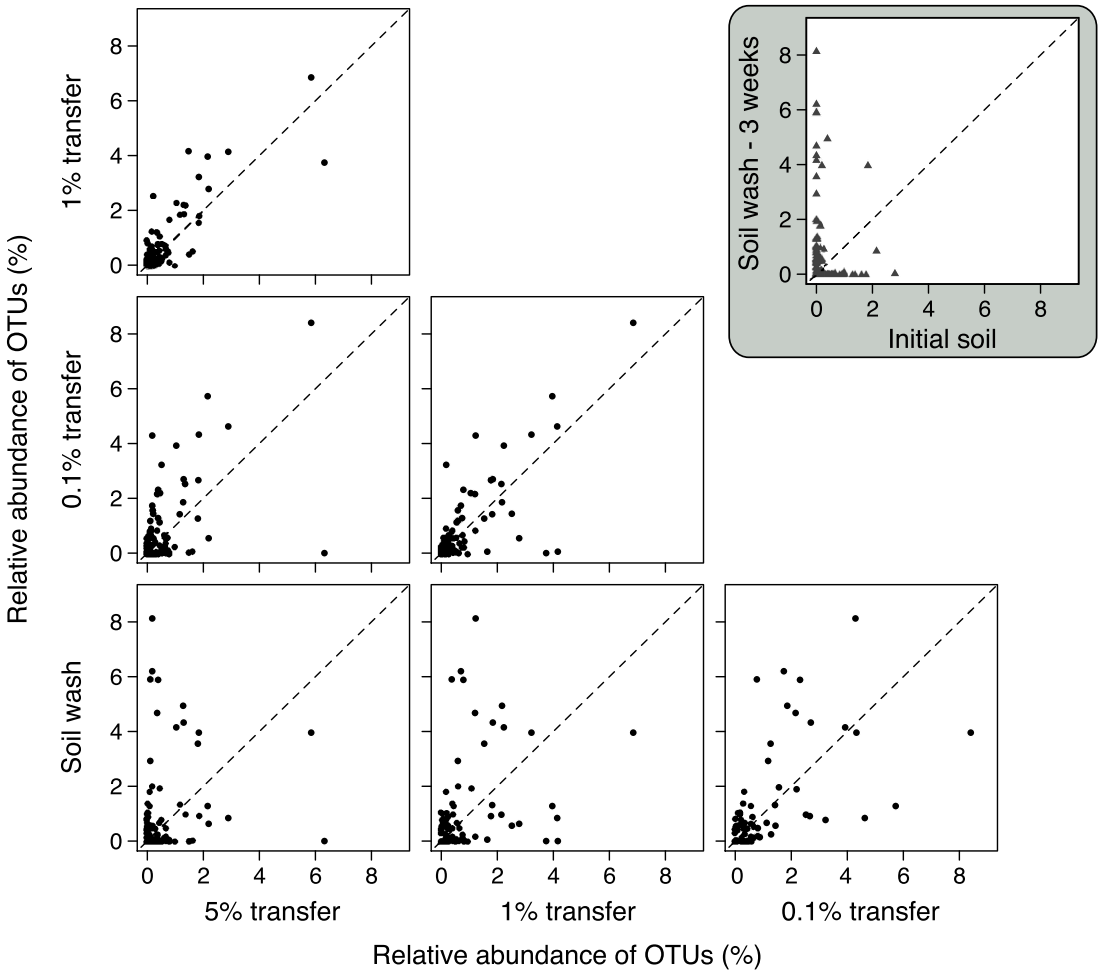

Figure 3. Scatterplots of the mean relative abundance of 16S rRNA gene OTUs across microbiome transfer methods after 3 weeks of incubation. The diagonal line is provided as a reference to indicate OTUs that are more abundant when less soil was transferred or in the soil wash (above the line) or when more soil was transferred (below the line). In the top right is a comparison between the initial soil and the most dissimilar microbiome transfer treatment, which was the soil wash at 3 weeks based on ordination and Pearson/Spearman correlation.

most abundant microorganisms were strongly affected by transfer method.

Both 16S rRNA gene and ITS composition varied significantly by microbiome transfer approach after 3 weeks of incubation based on PERMANOVA (16S and ITS, $P < 0.001$) and all transplanted microbiomes differed substantially from that of the original soil, which was expected based on the major physical differences of potting media substrate *versus* natural forest soil. As expected, bacterial and fungal composition in potting mix treated with the soil wash were least similar to the initial soil after 3 weeks, while increasing the percentage of soil transferred (0.1% → 1% → 5%) led to a resulting microbiome that was more similar to the initial soil (Fig. 1, and Figs S3 and S4 in the online Supplementary material). Yan *et al.* (2015) also show a large bacterial phylum-level difference in different soil dilutions, with 10^{-6} and 10^{-9} dilutions differing substantially from 10^{-1} dilutions. Interestingly, the bacterial component of the initial soil wash more closely resembled the initial soil than did the fungal component, perhaps reflecting a difference in the organisms that were able to pass through the filter. It is also possible that differences in the efficiency of our DNA extraction methods for microbes isolated from soil and the aqueous washes accounted for some of these differences. Substantial shifts in high-level taxonomy (i.e. phylum/class) were visually apparent for both bacteria and fungi (Fig. 1). Although we expected that microbiome composition would be most dissimilar between replicates when less soil was transferred, we did not detect a significant difference in inter-replicate variability between treatments (analysis of multivariate homogeneity of group dispersions; 16S, $P = 0.5189$ and ITS, $P = 0.3434$).

Shannon diversity also varied significantly when comparing across transfer methods and the initial soil for both bacteria and fungi (ANOVA, $P < 0.001$). Diversity decreased consistently with lower soil transfer volumes for bacteria, with the lowest diversity observed in the soil wash and 0.1% transfer. The number of bacterial OTUs identified in the initial soil was 1177, while 1004, 511, 362 and 228 were identified at the 3-week mark in the 5%, 1%, 0.1% and soil wash treatments, respectively. This is not surprising, as serial dilution has been used previously to establish experimental soil microcosms with decreasing bacterial diversity (van Elsas *et al.* 2012). Fungal diversity decreased significantly between the initial soil and transfer treatments for fungi, but not between transfer treatments (Fig. 2A). The number of fungal OTUs identified in the initial soil was 695, while 461, 332, 255 and 241 were identified at the 3-week mark in the 5%, 1%, 0.1% and soil wash treatments, respectively. The relative

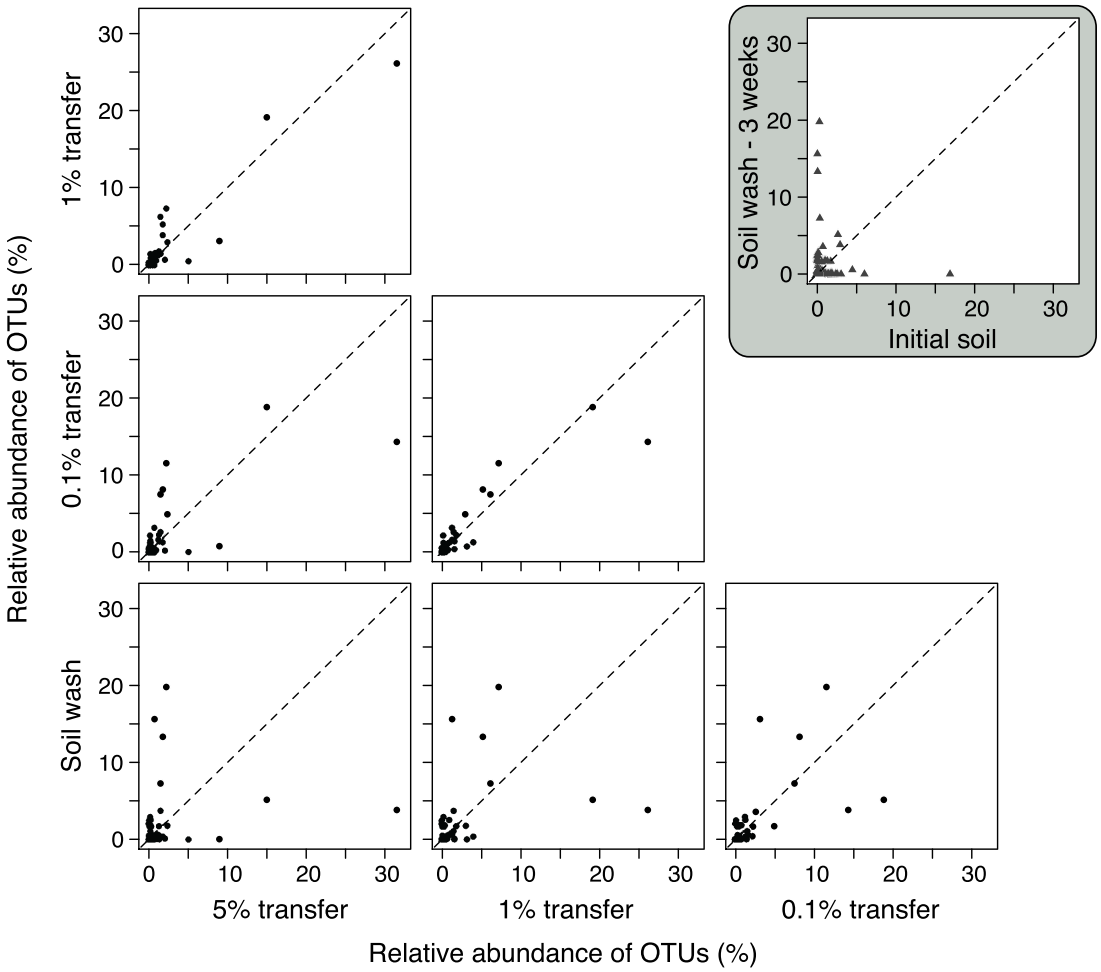

Figure 4. Scatterplots of the mean relative abundance of ITS OTUs across microbiome transfer methods after 3 weeks of incubation. The diagonal line is provided as a reference to indicate OTUs that are more abundant when less soil was transferred or in the soil wash (above the line) or when more soil was transferred (below the line). In the top right is a comparison between the initial soil and the most dissimilar microbiome transfer treatment, which was the soil wash at 3 weeks based on ordination and Pearson/Spearman correlation.

abundance of the most abundant bacterial OTU per sample increased significantly in all treatments after 3 weeks in comparison to the initial soil ($P < 0.0001$), with the highest relative abundance seen in the soil wash treatment, while the relative abundance of the most abundant fungal OTU per sample was significantly higher ($P = 0.017$) in the 5% transfer at 3 weeks than in the 0.1% transfer (Fig. 2B).

Interestingly, abundant bacterial and fungal OTUs varied dramatically in abundance between microbiome transfer treatments. This can be seen in Figs 3 and 4, in which OTUs that are not part of the cluster of rare microorganisms (above ~1% relative abundance for bacteria, and ~3% for fungi) tend to be present across treatments, but represent very different proportions of the microbiome. For instance, many of the same bacterial OTUs are abundant in both the 0.1% transfer and soil wash treatments, but most differ substantially in relative abundance (Fig. 3). Taking only abundant OTUs (i.e. representing at least 1% relative abundance in at least one sample), we compared the change in abundance of each at 3 weeks, relative to the initial soil (Fig. 5). Similar changes in rare OTUs (between 0.2% and 1% maximum relative abundance) are shown in Fig. S5 in the online Supplementary material. A majority of the OTUs in this dataset that classified as *Proteobacteria*, *Bacteroidetes* and *Planctomycetes* increased in at least one treatment, while most OTUs that classified as *Acidobacteria* declined. Most abundant OTUs that classified as *Basidiomycota* had declined at 3 weeks. What is important here is that studies using a so-called dilution-to-extinction approach to produce variations in soil microbial diversity (van Elsas *et al.* 2012) are likely also having substantial impacts on the dominant microorganisms in the soil. Thus, soil dilution is not simply an elimination of rare microbes, but a complete reorganization of the soil assemblage. This effect may be enhanced due to the fact that microbes were introduced to a novel environment, with fewer soil compounds transferred at lower dilutions.

We also determined the 10 most abundant bacterial and fungal OTUs on average for each treatment, and found that only one bacterial OTU was within the top 10 for the initial soil and all transfer treatments, and only two were consistently in the top 10 for all transfer treatments (Fig. 6A). For fungi, only two OTUs were in the top 10 for the initial soil and all transfer treatments, while four were shared between transfer treatments (Fig. 6B). Although 10 is an arbitrary cutoff, and a change of only a few organisms within this group may not be particularly meaningful, large changes (e.g. between the 5% and soil wash treatments) indicate substantial reorganization of the assemblages. This suggests that the success of abundant organisms post-transfer is

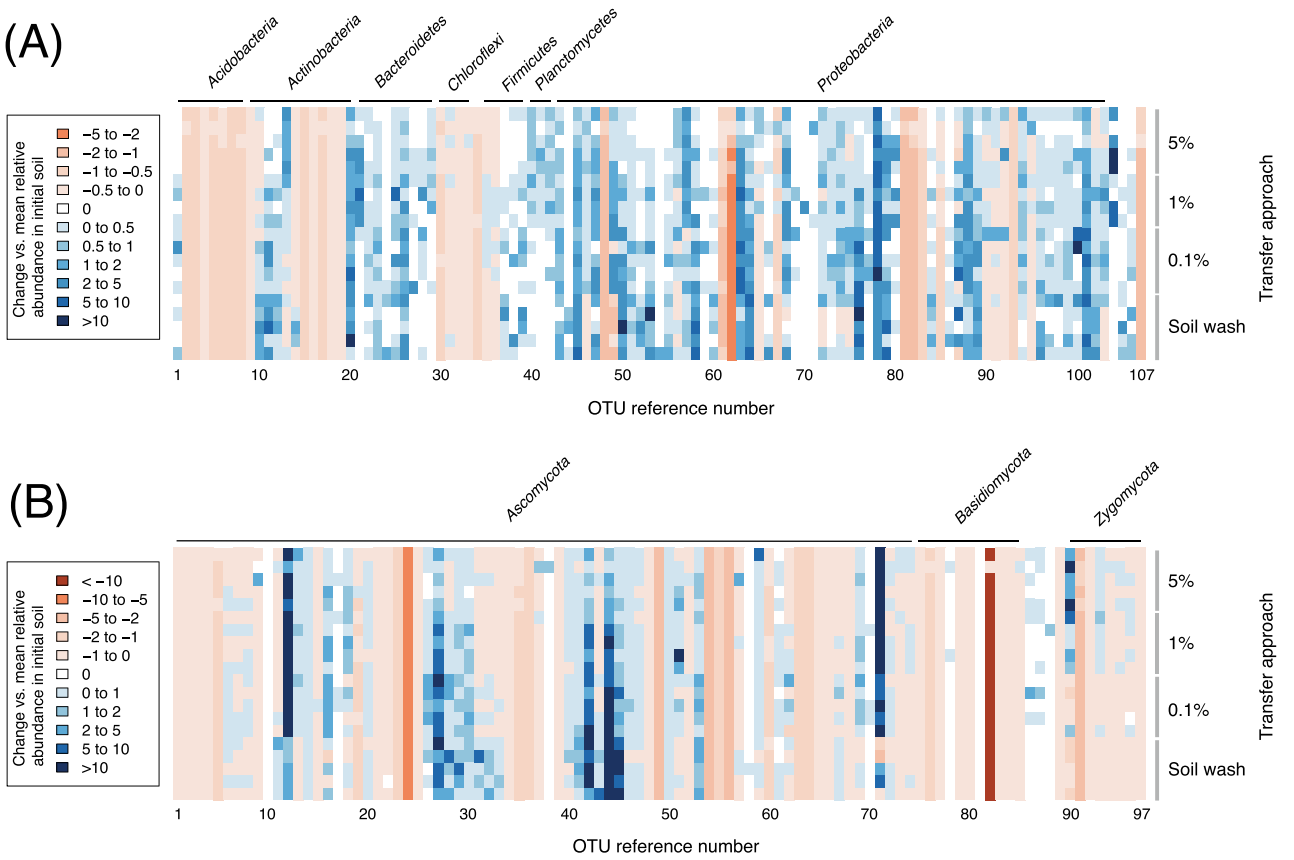

Figure 5. Heatmap displaying percentage change in relative abundance at 3 weeks from the initial soil microbiome for each treatment for OTUs with a maximum abundance (in at least one sample) of over 1% relative abundance. Shown are OTUs obtained from sequencing of the 16S rRNA gene (**A**) and fungal ITS region (**B**). Phylum-level taxonomic affiliations for groups with many OTUs represented are shown at the top, whereas genus-level classifications for OTUs can be found in Table S1 in the online Supplementary material using the OTU reference numbers.

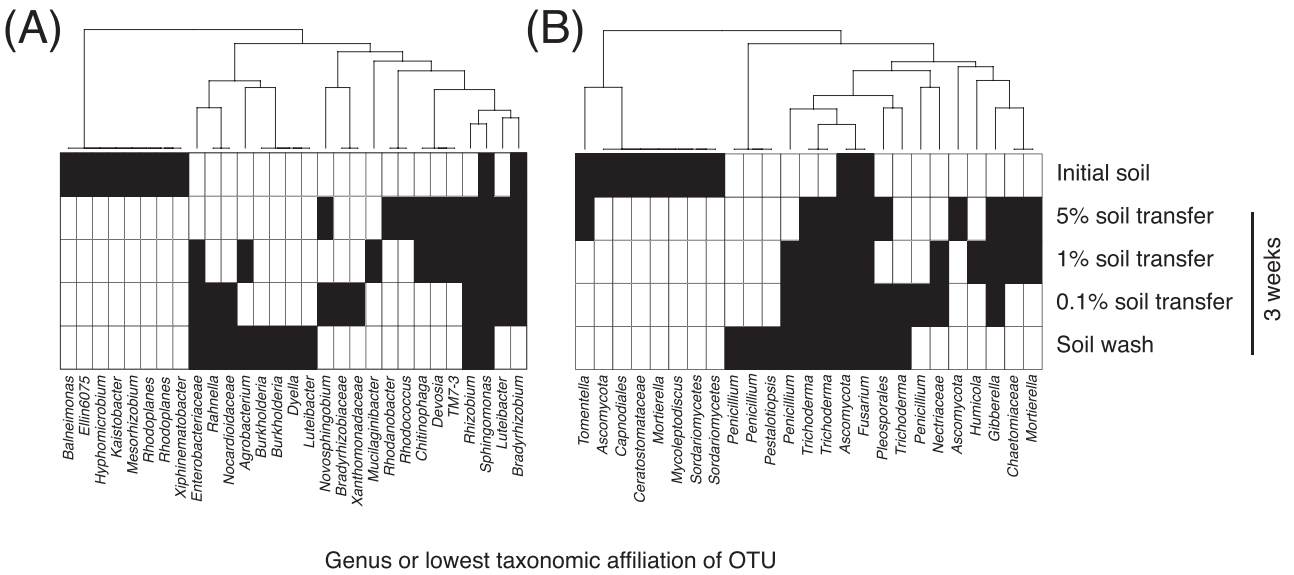

Figure 6. Heatmap of the 10 most abundant OTUs on average for the initial soil ($n = 4$) and each microbiome transfer method after 3 weeks ($n = 5$) for bacteria (**A**) and fungi (**B**), based on sequencing of the 16S rRNA gene and ITS region, respectively. Black indicates that an OTU is one of the 10 most abundant on average for a particular treatment, whereas white indicates that the OTU is not one of the 10 most abundant. OTUs are clustered using average linkage clustering based on Jaccard distances. The genus level or next lowest available taxonomic affiliation for each OTU is provided.

not solely influenced by the characteristics of the new soil environment, but is strongly affected by (i) the presence or absence of other soil organisms, and/or (ii) the concentration of soil compounds transferred. The former possibility is supported by recent studies that indicate that groups of microorganisms that are grown over many generations in combination function differently (typically more productively) than those that are grown alone or in lower diversity combinations (Lawrence *et al*. 2012; Fiegna *et al*. 2015). This is thought to be due, in part, to microbes adapting to use metabolites produced by their neighbours (Lawrence *et al*. 2012), and so the loss of either beneficial or suppressive metabolites could have impacted the success of certain abundant organisms.

CONCLUSIONS

We show that choice of microbiome transfer method has a substantial impact on resulting microbiome composition, and surprisingly, a strong effect on abundant microorganisms. We acknowledge that the transferred microbiomes could converge over time, particularly as transferred soil compounds are degraded, but the stark differences at 3 weeks could still have meaningful effects on target functions under experimental conditions, such as plant establishment. If the goal is to transfer a specific function or group of organisms, we would advise prescreening several methods to ensure that target organisms are promoted. However, if the goal is to transfer the most representative community of the source soil and transferring soil nutrients or other soil compounds is not a major concern, our results indicate that direct soil transfer inoculation is preferred over soil wash methods, especially at higher inoculant concentrations.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

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Conflict of interest. None declared.

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