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SHORT COMMENTARY

Bacterial Community Diversity in Soil Under two Tillage Practices as Determined by Pyrosequencing

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Abstract The ability of soil to provide ecosystem services is dependent on microbial diversity, with 80–90 % of the processes in soil being mediated by microbes. There still exists a knowledge gap in the types of microorganisms present in soil and how soil management affects them. However, identification of microorganisms is severely limited by classical culturing techniques that have been traditionally used in laboratories. Metagenomic approaches are increasingly becoming common, with current high-throughput sequencing approaches allowing for more in-depth analysis. We conducted a preliminary analysis of bacterial diversity in soils from the longest continuously maintained no-till (NT) plots in the world (52 years) and in adjacent plow-till (PT) plots in Ohio, USA managed similarly except for tillage. Bacterial diversity was determined using a culture-independent approach of high-throughput pyrosequencing of the 16S rRNA gene. *Proteobacteria* and *Acidobacteria* were predominant in both samples but the NT soil had a higher number of reads, bacterial richness, and five unique phyla. Four unique phyla were observed in PT and 99 % of the community had relative abundance of <1 %. Plowing and secondary tillage tend to homogenize the soil and reduces the unique (i.e., diverse) microenvironments where microbial populations can reside. We conclude that tillage leads to fewer dominant species being present in soil and that these species contribute to a higher percentage of the total community.

Keywords Pyrosequencing · Soil community analysis · 16S rRNA gene · Long-term tillage · Plow tillage · No-tillage

Introduction

Soil is a complex ecosystem that is part of our biosphere. Soil maintains biogeochemical nutrient cycles and ensures proper functioning of our dynamic ecosystems. The ability of soil to provide ecosystem services is dependent on microbial diversity. Approximately 80–90 % of the processes in soil are mediated by microorganisms including soil structure maintenance, organic matter decomposition, nitrogen fixation, breakdown of toxic compounds, and inorganic compound transformations [1, 2].

The microbial population of soil is diverse. Although the number of microbial species in soil is still being debated, studies have reported ranges that span from 10,000 [3] to a predicted 10^7 per gram of soil, with the upper limit being based on DNA sequencing technologies [4]. A metagenomic approach to estimate microbial diversity predicted about 2000–18,000 bacterial genomes in 1 g of soil [5].

Previous studies have indicated soil properties like structure, pH, water, air and nutrient availability, oxidation-reduction potentials, organic matter availability, etc. all affect bacterial community structure [6]. Because physical disturbances in soil are known to impact soil properties greatly [2], they also impact the abundance, community structure, and activity of soil microorganisms [7]. Thus, land-use management practices, like application of different tillage systems, present themselves as case studies for soil microbial diversity studies.

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Tillage practices (i.e., conventional tillage, conservation tillage) are defined by the amount of crop residue (including straw, stubble, leaves, stalk, etc.) left on the ground after a crop has been harvested. Plow tillage involves soil inversion that buries most of the residues so that the surface is left without cover protection [8]. Conservation tillage retains a minimum of 30 % crop residue on the surface at crop planting time and involves minimal disturbance to soil [9] due to absence of traditional cultivation techniques like plowing and harrowing. Conservation tillage includes processes like no-till, strip-till, minimum-till, and ridge-till. No-till causes the least disturbance of soil due to the avoidance of any mechanical mixing [8]. Long-term tillage practices affect soil characteristics like water content, temperature, aeration, aggregation, organic matter stratification, and nutrient distribution [6, 9] which, in turn, impact microbial diversity. Long-term conservation tillage practices, like no-till, are generally considered beneficial because it results in reduced soil erosion, improved soil structure, increased soil organic matter concentrations, and increased pore-space and water infiltration. All of these factors create an abundance of diverse microhabitats for microorganisms. It has been argued that microbial community richness and diversity are indicators of soil quality [9], with studies proposing the development of high species diversity as one goal of remediating degraded soil [10].

The dynamic microhabitats that exist in soil pose a challenge when it comes to isolating soil microbes by standard culturing techniques. Recreating appropriate soil environmental conditions in the laboratory is limited in its scope and extent. Hence, with the advent of sequencing techniques, soil microbes are being identified using the information stored in their DNA. High-throughput sequencing technologies present a culture-independent and rapid method to determine microbial diversity in different environments.

While the 454 pyrosequencing platform is being slowly phased out, this less-labor intensive and fast method has been used to evaluate microbial diversity in a number of studies [11]. For example, soil bacterial diversity was determined by pyrosequencing in forest and grassland soils in Germany [12], pasture and agriculture systems in Texas [13], agricultural soils from Brazil, Florida, and the Morrow plots in Illinois and a boreal forest in Canada [14], and forest soils from Korea [15].

This study focuses on conducting preliminary analysis of bacterial diversity, using pyrosequencing, in soils from the Triplett-Van Doren long-term tillage plots in Wooster, Ohio. These plots are unique in terms of being part of a long-term agricultural research experiment that was established in 1962 [16]. Thus, the no-till plots have never been disturbed, except for placing of seed into the ground, for more than 50 years. The most dramatic changes in the soil occur near the surface [17], and so, we hypothesize that long-term application of no-till practices will result in a very different microbial community composition at the soil surface compared to plow till (i.e., inversion tillage) when a continuous corn crop is grown. To determine bacterial diversity and community composition (structure), we employed a culture-independent approach of high-throughput pyrosequencing of the 16S rRNA gene.

Materials and Methods

Plots, Experimental Design, and Soil Sampling

The Triplett-Van Doren Long-Term Tillage Plots are located in Wooster, Ohio, USA. The soil at this site is a Wooster silt loam and is classified as a fine-loamy Typic Fragiudalf. The plots are arranged in a randomized manner consisting of three treatments, three rotations, and three replicates. Every crop in the rotation is grown each year so that the total number of plots is 54. The treatment variables include no-till (NT), plow till (PT), and chisel (minimum) till (MT). The rotation variable consists of (i) continuous corn (CC), (ii) corn and soybean in a 2-year rotation (CS), and (iii) corn, oats, and alfalfa or mixed grass meadow in a 3-year rotation (COM) [16]. Three subsamples (0–10 cm) were collected on June 9, 2014 from each of the three replicates of the CC/NT and CC/PT plots before liming (total number of subsamples=18).

Subsamples from the NT and PT replicates were pooled together treatment wise to make two composite samples, one representing NT and the other PT. These pooled samples were transported to the laboratory and divided into two fractions. One fraction was used for measurement of soil properties. The soil was air-dried at room temperature and sieved through a 2-mm sieve. Soil pH was determined with a soil:water ratio of 1:1. Standard soil chemical properties that included lime test index (LTI), available P, exchangeable Ca, exchangeable Mg, exchangeable K, cation exchange capacity (CEC), and organic matter content were determined by STAR Laboratory, Wooster, OH (http://oardc.osu.edu/starlab). Methods used are reported on the STAR Laboratory web page.

DNA Extraction, PCR, and Pyrosequencing

Genomic DNA was extracted from approximately 1 g of field-moist soil immediately after sampling by using the UltraClean® Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions. The extracted DNA was quantified using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The quality of the extracted DNA was confirmed by running the extracts on 1 % agarose gel with 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0).

Purified DNA samples were amplified using phylogenetic markers targeting the V1–V3 regions of the bacterial 16S

rRNA gene (~600 bp). The primer set included the forward primer B16S-F (5′-CCTATCCCCTGTGTGCCTTGGCAG TC-TCAG-AC-GAGTTTGATCMTGGCTCAG-3′) and the reverse primer B16S-R (5′-CCATCTCATCCCTGCGTGTC TCCGAC-TCAG-X-AC-WTTACCGCGGCTGCTGG-3′). The first two primer sections are the adaptor and key, AC is a linker, and underlined sequences are gene specific primers. The barcode primer in B16S-R is marked as X and depends on the sequencing platform or the number of pooling samples. The extracted DNA was amplified in a 50-μl reaction mixture containing 5U Taq DNA Polymerase, 5 μl of 10× ExTaq Buffer (20 mM Mg^{2+}), 2.5 mM dNTP mix, 20 pmol/μl of each primer, and 1 μl of template DNA. The thermocycler conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s; and a final extension at 72 °C for 7 min in a Bio-Rad C1000 Touch (Hercules, CA).

The quality of the PCR products was confirmed by gel electrophoresis, and the amplified products were purified using a QIAquick PCR Purification kit (QIAGEN, Cat. No. 28106). Equimolar amounts of samples were pooled together in a tube and the shorter fragments or dimers (under 300 bp) were removed using a QIAquick Gel Extraction Kit (QIAGEN, Cat. No. 28706) in a subsequent gel electrophoresis step. Pyrosequencing was performed with 454 GS FLX Titanium (454 Life Science, Rosche) in Chunlab, Inc. (Korea) according to the manufacturer's instructions.

Pyrosequencing Data Analysis

Unique barcodes on the reverse primers were used to separate DNA sequences that originated from either the NT or PT sample. The gene-specific primers, sequencing primers, barcodes, and linkers were removed from the original sequencing reads. The resulting sequences were further narrowed down to select for sequences >300 bp. Non-specific sequences with an expectation value of e>−5 in BLASTN search and chimeric sequences were removed. The sequences were then assembled as a way of de-noising.

The taxonomy of the resulting contig sequences were identified using the EzTaxon-e database (http://www.ezbiocloud.net/eztaxon) [18]. In brief, the top five sequences with the highest similarity were extracted from the database using BLASTN. Next, the similarity value of each sequence was obtained using global pairwise sequence alignment. The species were then identified according to their similarity values. The algorithm used was a simple identification scheme where x=sequence similarity to type strain species ($x \geq 97$ %), genus ($97 > x \geq 94$ %), family ($94 > x \geq 90$ %), order ($90 > x \geq 85$ %), class ($85 > x \geq 80$ %), and phylum ($80 > x \geq 75$ %). The operational taxonomic unit (OTU) was used to classify species at 97 % sequence similarity. Clustering of sequence data was done using CD-HIT [19]. Species richness and diversity were estimated by the abundance-based coverage estimator (Ace), Chao 1 estimator (Chao1), JackKnife richness estimator (JackKnife), non-parametric Shannon diversity index (NpShannon), Shannon diversity index (Shannon), and Good's library coverage using Mothur v.1.33.3 [20]. An interactive visualization tool, Krona, was used to visualize quantitative community composition and hierarchical relationships [21]. CLCommunity 3.31 (Chunlab Inc.) was used to generate taxonomic composition graphs, community comparison tables, and diversity indices tables.

Results and Discussion

Soil Characteristics

The soil samples were below the pH optimum for good crop growth with a lower pH recorded in the NT sample as compared to the PT sample (Table 1). Available P concentration in the NT soil was almost double that of the PT soil. pH and available P are often stratified in no-till soils with lower pH values, but higher available P concentrations, at the surface compared to deeper soil layers or compared to surface PT soils. The organic matter content of the NT soil was slightly higher than of the PT soil due to deposition of crop residues on the soil surface. The exchangeable cation concentrations, however, were all less in the NT soil due to the lower soil pH in this sample compared to the PT sample.

Pyrosequencing Analysis and Community Composition

The number of sequence reads obtained for NT (2740) was higher than PT (1894). The maximum number of OTUs for NT soil predicted at 3 % dissimilarity was 1386 while for PT, it was 1177. Each soil sample had OTUs unique to the sample, with 1079 OTUs unique to NT and 873 OTUs unique to PT (Table 1).

Rarefaction curves (Fig. 1), depicting the effect of dissimilarity on the number of OTUs, showed distinct patterns for NT and PT. The curve at 3 % dissimilarity, corresponding to species level diversity, was just starting to plateau at around 1400 OTU for NT. This suggests that a reasonable number of individual species were sequenced and that more intensive sampling is likely to yield several additional species for NT. The curve for PT did not reach a plateau and its steeper slope indicates that a large fraction of the species diversity remains to be discovered. It is however, not uncommon for rarefaction curves to not reach a plateau in soil bacterial communities [12], with both depth of the sequence and number of valid reads determining the OTUs obtained.

The total number of taxa observed in each rank is listed in Table 1. Rank-wise, the relative abundance of each taxon was

Table 1 Soil properties and pyrosequencing analysis

Measurements	No-till	Plow-till
Soil properties		
pH	4.84	5.96
Lime test index	65.9	70.0
Organic matter (%)	3.57	3.15
Cation exchange capacity (cmol/kg)	8.80	11.5
P (mg/kg)	44.6	24.8
K (mg/kg)	155	188
Ca (mg/kg)	563	1899
Mg (mg/kg)	85.2	186
Pyrosequencing analysis		
Total number of raw sequence reads	2740	1894
OTUs using CD-HIT	1386	1177
OTUs unique to each soil sample, using CD-HIT	1079	873
Percentage of population in a given rank with relative abundance less than 1 %:		
Phylum (35)[a]	5	5
Class (96)	15	16
Order (186)	25	28
Family (360)	39	42
Genus (739)	56	63
Species (1680)	91	99

[a] Indicates total number of taxa observed in the samples for the respective rank

calculated. The sum of the relative abundances of taxa whose abundance was <1 %, was also calculated for each rank. For phylum, 5 % of the total population was made up of phyla with relative abundance <1 % for both NT and PT soils. The differences in species composition became clearer when the relative abundances for species level was studied (Table 1). Briefly, for NT, 91 % of the total population was comprised of species with relative abundance <1 % while for PT, 99 % of the total population belonged to species with relative abundance <1 %. While the predominance of a few taxa may indicate high species richness, the presence of low abundance taxa also contributes to community diversity.

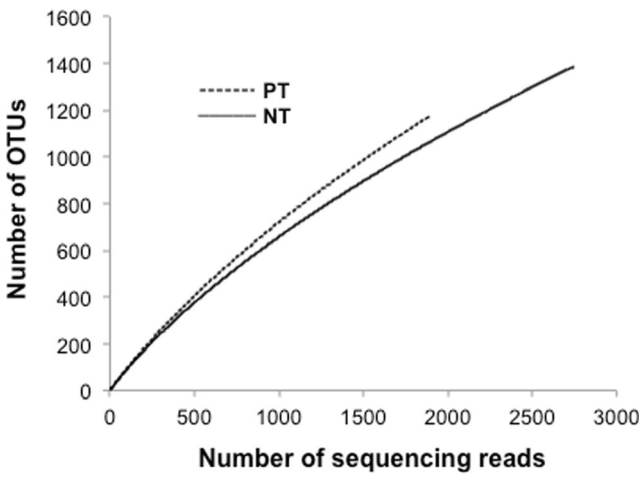

Fig. 1 Rarefaction curves at 3 % sequence dissimilarity

The 10 most abundant phyla in each sample and their relative abundances are shown in Fig. 2. *Acidobacteria* and *Proteobacteria* were the dominant phyla across both soil samples. The relative abundance of *Acidobacteria* in NT (36 %) vs. PT (26 %) can be attributed to the pH difference in the soil, with soil pH being identified as a major contributor of microbial diversity in a number of studies [22–24]. Among the top 20 predominant bacteria in the soil samples, the relative abundances of *Proteobacteria, Bacteroidetes, Actinobacteria, Chloroflexi, Gemmatimonadetes, Planctomycetes, Nitrospirae*, *Verrumicrobia, Armatimonadetes*, and *Cyanobacteria* were lower in NT as compared to PT. Plowing and secondary tillage tend to homogenize the soil and reduces the unique (i.e., diverse) microenvironments where microbial populations can reside. Also, previous studies have suggested that the presence of crop residue and thus higher organic matter content on the surface, favors fungi as primary decomposers [10, 25]. Higher organic matter content in NT suggests the likelihood of a predominantly fungal community favoring organic matter deposition, while bacterial community would be predominant in a tillage management practice like PT. Additionally, acidic soils have been reported to exhibit lower bacterial diversity [24], which is in agreement with lower relative abundance of species in NT as compared to PT.

Five taxa unique to the NT soil included HM187141_p, Bacteria_uc, DQ404828_p, TM6 and *Lentisphaerae*. The PT sample included four unique taxa, namely, *Spirochaetes, Tenericutes*, 10BAV, and DQ833500_p. For a clearer hierarchical view of the relative abundance of taxa, the species were viewed under an interactive web browser (Supplementary Figs. 1 and 2). It should be noted that a number of sequence reads did not exhibit similarity to any of the known and sequenced microbial species, thereby implying the presence of large number of unidentified microbes in soil. Sequences from the NT and PT samples were submitted to Sequence Read Archive (SRA) under accession numbers SRR1610992 and SRR1610991, respectively.

Link Between Diversity, Richness, and Evenness

The diversity indices for the NT and PT samples (Fig. 3) are dependent both on richness and evenness of a community. These indices provide a link between diversity, richness, and evenness of the communities. Although the rarefaction curves indicated that for a fixed number of reads, bacterial richness seemed to be higher in PT as compared to NT, comparison of species richness indices Chao 1, Ace, and JackKnife showed higher values for NT over PT (Fig. 3a). However, when diversity indices like Shannon and NPShannon were compared (Fig. 3b), PT showed higher values. The real bacterial

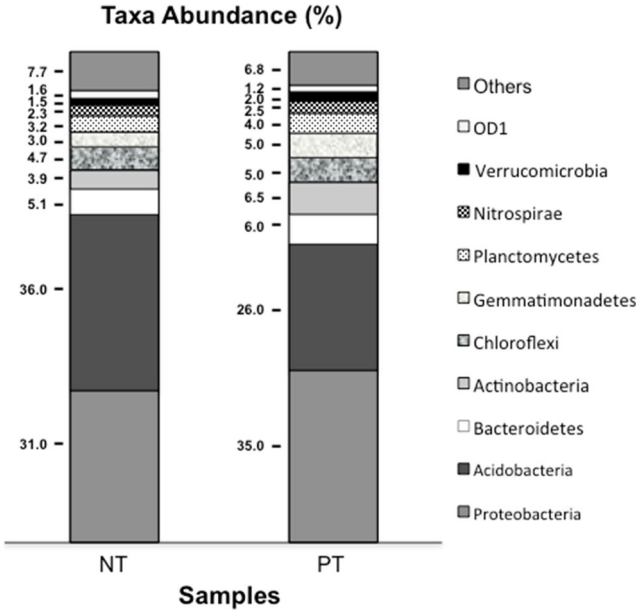

Fig. 2 Relative abundances of the 10 most abundant phyla in the two soil samples. The relative percentages of phyla *Proteobacteria, Acidobacteria*, *Bacteroidetes*, *Actinobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Planctomycetes*, *Nitrospirae*, *Verrumicrobia*, and OD1 are marked

community in PT can thus be said to be less diverse in terms that it is less even but not less rich.

The lower library coverage of PT as compared to NT from Good's Library Coverage (Fig. 3b) values suggests that increasing the sequencing depth of the amplicons might have shown a clearer relation between species diversity in terms of abundance and evenness. Although our samples clearly reflect the tillage differences imposed at our long-term research site, we also recognize Amend et al.'s work about problems associated with read abundance, biological abundance, and inherent biases that the lack of replicates may create [26]. However, we provide preliminary information about the differences that exist in bacterial community diversity under contrasting tillage practices that are continuously maintained on a site for more than 50 years.

Soil Properties and Bacterial Ecology

Soil properties affect the ecology of microbes. While the acidic pH of the soils resulted in dominant *Acidobacterial* population, there is always the possibility of a few abundant taxa skewing the community composition study. Fungal diversity

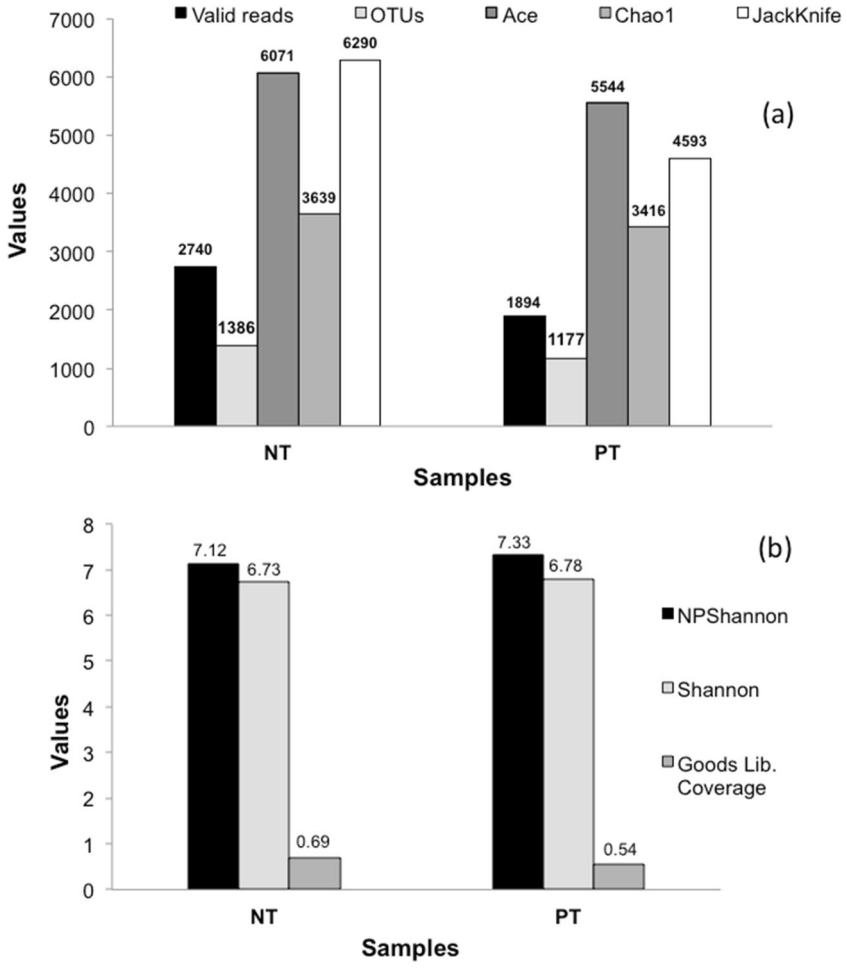

Fig. 3 Diversity indices of samples: **a** graphical representation of valid reads and OTUs obtained, along with the species richness indices Ace, Chao1, and JackKnife. **b** Graphical representation of diversity indices (i.e., NP Shannon and Shannon) in samples along with read coverage

studies by Adams et al. [27] showed that a handful of dominant taxa skews species richness and composition when the sequencing depth is even. While there is a greater chance of the abundant taxa being sequenced and identified, the real challenge of studying soil microbial diversity is in sequencing and identifying the low abundant taxa. Another crucial challenge lies in how one approaches these low abundant taxa. Are the low abundant taxa common soil species or different, but rare species? Indeed, Huse [28] discusses the legitimacy of the occurrence of such rare taxa and proposed the sequences observed could be common biological occurrences or the end-result of sequencing techniques.

An approach to study the low abundant or rare taxa is by using taxa-specific primers. In one of our parallel studies using high-throughput sequencing, targeting methanotrophic bacteria (data not shown), we have used primers and successfully sequenced methanotrophs from the same soil samples. Methanotrophs belong to *Proteobacteria* and aerobically oxidize methane, thereby serving as the sole biological sink of methane in our biosphere. In spite of our samples recording high abundance of *Proteobacteria* in this study, we were able to identify only a single species of methanotroph, *Methylocystaceae_uc* in NT. This strengthens the need to carry in-depth analysis of soil microbial community composition by targeting taxa-specific microbes that might be beneficial to the environment.

Conclusion

Sustenance of life on earth is dependent on the ecosystem services provided by soil and soil microorganisms. The microbial component of soil plays a crucial role in cycling of nutrients in the biosphere. Our study was a preliminary attempt at observing bacterial diversity in soils under two common but contrasting tillage practices that have been maintained for 52 years at a site in Ohio, USA. Our sequencing study reveals a number of uncultured species, many of which are undoubtedly still impossible to study using standard culture techniques. In this study, the results were mixed with some diversity indices favoring NT and some favoring PT, but in general, the PT treatments lead to higher relative abundance of a few species. We conclude that plowing and secondary tillage homogenizes the soil, leading to a reduction in the unique (i.e., diverse) microenvironments where microbial populations can reside.

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Conflict of Interest The authors declare that there are no conflicts of interest.

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