**Introduction**

* **提出microbial load的重要性**
* **科学问题1：RA 数据分析的局限性之一：组成型数据**
* **科学问题2：真菌和细菌不能关联**
* **解决问题的方法：绝对定量——总结当前绝对定量的研究进展**
* **本研究的研究思路**

Healthy plant roots were colonized by complex and diverse microbes, which perform crucial roles in nutrient acquisition and host immunity【文献】. As many species co-exist, taxa abundance and their interplay vary greatly among individual host plants【文献】. [Particular](javascript:;)ly, the variability in microbial loads itself could be a potentially biological feature reflecting the host status1,2, which remains underutilized in plant microbiome research.

Amplicon high-throughput sequencing allows profiling of the bacterial or fungal community respectively, which then converted sequence counts into the relative abundance or proportions of operational taxonomic units (OTUs) by rarefying or normalization for further analyses. However, microbiome datasets generated by current sequencing-based analyses are compositional，these relative approaches can not reveal the alterations in total microbial load2 or abundance shift of specific taxa which may vary across host samples, failing to discriminate between true and spurious correlations between taxa【Jackson DA. Compositional data in community ecology: the paradigm or peril of proportions? Ecology 1997;78:929e40】(**Fig. 1a**). For example (**Fig. 1b-c**), taxa abundance distributions in sample A and B are different, with the microbial load in sample A is double that of the load in sample B. By rarefying or normalization, the relative abundance profiles assume similar taxa abundance distribution in samples A and B, therefore poorly reflecting the veritable taxa distribution relative to the host plant (false negative). Besides that, large changes in the absolute abundance of one component within compositional dataset would drive apparent changes in the measured abundance (relative abundance) of others and create flawed inference in abundance estimates. As shown in sample A and C, plant A originally contains four OTUs, respectively account for 25%, 12.5%, 12.5% and 50%. In case the increase in amounts of three OTUs including OTU1 (red circle), OUT2 (yellow square) and OTU3 (green triangle), may not influence the abundance of OTU4 (blue rhombus), the real abundance of OTU4 would be expected to be not changed (see sample C, **Fig. 1b**). However, the assumption of true independence could not hold if we use current proportional profiling methods. According to available relative abundance values, we may assume that the number of OTU4 decreased in sample C, thus bringing the erroneous interpretation (false positive) derived from the results.

It is well-recognized that both bacteria and fungi are able to densely colonize the root and form tight associations with plants. So far, most researchers examined individual microbial groups, e.g. separate bacteria- or fungi-only communities, then understanding single group effects on the host. However, as these groups act synergistically in the environment, such experiment design and sequencing data processing limit our understanding to the interactive effects of bacteria and fungi, especially it is still unclear whether the abundance of bacteria and fungi shifted synergistically relative to plant. To overcome this limitation, correlation network analysis were applied to investigate microbial co-occurrence patterns3. Nevertheless, such information should be particularly cautious as the compositional sequencing data may cause false discovery rate. Cross-domain comparison of microbiota is still in challenge (**Fig. 1a**).

Application of quantifying absolute microbial abundances through amplicon sequencing allows to calibrating the bias caused by relative abundance methods. Combining 16S ribosomal RNA (rRNA) sequencing with qPCR or with the flow cytometric ((FCM) to quantify taxon abundances are well established in environmental samples4,5. By integrating microbial cell counting into a sequencing workflow, Vandeputte et al2 improved a new flow cytometry-based approach to quantify microbial abundances in fecal material, showing that the total microbial load varied substantially between individuals (up to tenfold differences) and that this variation is also associated with host status. However, above these methods were not suitable for quantifying the absolute abundance of the root-associated microorganism. Firstly, flow cytometry could not distinguish bacteria existing in rhizoplane or endosphere communities；Furthermore, the total 16S rRNA or ITS copy numbers cannot be quantified by qPCR because host plant-associated chondriosome or ITS DNA fragments can also be amplified using universal 16S rRNA and ITS primers. To address this issue, spike-in based calibration approach seemed to be more feasible and promising. Adding controlled amounts of spike-in materials into samples allows for changing the profile's reference points1, making the spike-in as a new reference to measure the extent of directionality of changes in the total microbial loads and each taxa abundance. So far, spike-in strategies have been well applied in RNA-Seq6 as well as proteomics7 and metabolomics8. Recently, Tourlousse et al9 developed a set of synthetic spike-ins for performing 16S-seq quality control and quantifying absolute microbial abundances in environmental samples. Stammler et al1 added exogenous viable spike-in bacteria to rescale the read counts of endogenous bacteria. Using this method, they reconsidered the emergence of *Enterococcus* as the predominant genus in allogeneic stem cell transplantation (ASCT). Tkacz et al10 used synthetic chimeric DNA spikes that contain universal primer binding sites specific for prokaryotic 16S, eukaryotic 18S and ITS to quantify the microbes in soil samples, and allow compare domain-level shift in microbiota abundance. Yet, these spike-in standards were only be appropriate for gut and environmental samples, but have not been fully explored for plant microbiome which need to consider the host. Currently, quantify the abundance of bacteria and fungi relative to host plant remained a challenge in root-associated microbiome research.

In the present study, we improve a spike-in based calibration method **(Fig. 1d)** that permits the quantitative assessment of host plant associated microbiota variation and reveals detection of true associations between bacteria and fungi. For this purpose, we designed and constructed an artificial plasmid as spike-in reference. We added controlled amounts of spike-in into the metagenomic DNA and quantified the selected marker gene through qPCR, enabling us to rescale the read counts and calibrate the relative abundance profile into the absolute abundance profile relative to plant host. A crucial benefit of spike-in is that it simultaneously contain universal primer binding sites specific for bacteria and fungi, and can be amplified both by bacterial and fungal primer sets. Using mock community and designed environmental samples, we tested the accuracy and reproducibility of spike-ins in a series of dilution experiments. Moreover, we applied spike-in based quantitative method to explore the responses of rice root microbiome to drought stress and the total microbial load alterations in wheat root samples with Common root rot disease.

**Results and discussion**

**1. Basic characterization of the spike-in.** We generated artificial spike-in by adding the conserved regions, which acted as universal primer binding sites for both bacteria and fungi PCR amplification into the flanking space of a plant unique marker gene fragment (**Fig. 1d**). The plant marker gene we used in this work was RID1 gene, acting as a master switch from vegetative to floral development in rice11. The spike-in does not exist in the real environment and can be easily distinguishable from the microbial sequences using 16S rRNA or ITS amplicon sequencing.

**2. Design of spike-in based calibration experiments.** Single plasmid BI-12-4 with a series of dilutions was spiked into each of 150 pooled samples at fixed amounts, which can be used to measure the total microbial load and taxa abundance relative to host plant, including bacteria and fungi simultaneously. The whole-synthetic community (mock community) was designed to evaluate the availability of the spike-ins. With equal aliquots of germ-free root DNA samples, twelve different microbial genomic DNA (nine bacteria and three fungi) were pooled in unequal ratios in order to simulate non-constant microbial loads. The semi-synthetic community (disturbed community) was employed to verify the availability of spike-ins in natural plant samples. The input of germ-free plant DNA was to mimic the fold change of the total microbial load relative to host. Upon application of the designed DNA input amounts, we can compare the intra-species ratios or the total load between groups to evaluate the precision of spike-in based quantitative method.

**3. Spike-in based quantification displayed accurate calibration in mock community.** To validate the accuracy of artificial spike-in BI12-4-based calibration, we designed three groups of mock communities with different microbial loads at four gradients (namely E05, E05/5, E05/10, E05/20) and one control experiment (E00) by mixing constant germ-free rice genomic DNA with microbial genomic DNAs at defined ratio, including: (i) Group 1 (G1), a background mock DNA pool with random microbial copy numbers. The input ratio of *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Ascomycota* and *Basidiomycete* was a:b:c:d:e:f; (ii) Group 2 (G2), the input amounts of each taxon doubled relative to G1, but the inter-species ratios within samples did not alter. The input ratio of microbes was 2a:2b:2c:2d:2e:2f; (iii) Group 3 (G3), the input amounts of *Proteobacteria* and *Ascomycota* did not alter compared to a double addition in amounts of *Actinobacteria, Bacteroidetes, Firmicutes* and *Basidiomycete*. The input ratio was 2a:2b:2c:d:e:2f. For each pair of samples between groups, we have expected intra-OTUs ratios (intra-species comparison) defined by the experiment design. Spike-in was kept constant across groups for each dilution gradient.

Understanding the signal response in relation to input amount is critical for spike-in based quantification, as it allowed us to determine the relationship between spike-in reads and known inputs12. The dilution experiments of spike-in BI-12-4 were used to determine the dynamic range of this method. For detected spike-in BI12-4 in the 16S-seq libraries, the total spike-in reads number increased with the input spike-in amounts and Pearson’s correlation coefficient was 0.981(*P* < 0.001). Spike-in reads ranged from 8.75% (E05/20) to 57.0% (E05) in a given group (G2). The constant relationship between spike-in amounts and read counts was further linear quantification by a Poisson general linear regression model (GLM)13, which allowed us to demarcate a dynamic range of the spike-in where we should expect a linear response. As showin in **Fig. 2a**, the distribution of points verified that quantitative detection of spike-in BI-12-4 was reliable across a dynamic range ranging from 3.9×104 and 7.9×105 copies per reaction, validating further use as quantification standards in 16S-seq.

To validate that spike-in BI12-4 did not affect detection efficiencies of mock bacterial community members, we compared E00 libraries with those spiked libraries (E05, E05/5, E05/10, E05/20). **Fig. 2b** showed the distribution of relative abundance of each reference bacterial strain after filtering the spike-in sequences based on G2 dataset. The relative abundances of individual OTUs in spiked samples kept consistent with control samples (E00), suggesting spike-in BI12-4 did not affect bacterial PCR amplification and measurement. The distribution profiles based on G1 and G3 dataset were shown in **Supplementary Fig. 1.** Two-sided Dunn’s adjusted test verified no significant differences between E00 and other spiked communities (*P* > 0.05).

Focusing on our analysis within designed comparison 1 (G1 vs G2), we found the proportional abundances of each strain are almost the same using relative profiling method, which ignored the shift of the total microbial load and did not reflect the actual taxa abundance relative to plant. On the contrary, an average of 2.1 fold variation in strain abundance between G1 and G2 was observed using spike-in based quantitative approach (QA) at E05/20 (**Fig. 2c**), approximately in agreement with expected ratio as 2 fold. The similar results were also observed substantially under other three gradient dilution experiments, all of which were able to link to the variations in the total microbial load, on average from 1.55 fold (E05/5) to 1.86 fold (E05/10) (**see Supplementary Fig. 2**). 此处小宁需要补充统计学分析，统计没有显著性变化。

We next analyzed the ratio for designed comparison 2 (G1 vs G3) at E05/20, shown in **Fig. 2d**. The effect of constituent data remained significant in relative approach (RA). We observed a misleading reduction of relative abundances for four proteobacteria strains，which in fact was associated with obvious increasing in relative abundances of Bac-186 and Fir-11. The relative abundances for three actinobacteria strains (Act-101, Act-135 and Act-140) had almost no change. For calibrated results, we observed approximately 2 fold differences in abundance of Act-Bac-Fir strains. Calibrated approach also bypassed compositionality effects and no significant absolute decreases in abundance of four proteobacteria strains were detected. The quantitative performance of BI12-4 in other dilution gradients was shown in **Supplementary Fig. 3.**

When true differences in taxa abundance exist between samples by design, these differences should be detected; where no differences exist, no differences should be detected14. The bias between the expected ratio (design ratio) and true ratio (actual ratio) were defined as error value and used to assess the measurement performance of relative and quantitative approach. The ratio between QA induced error and RA induced error were calculated and plotted in **Fig. 2e**, where the line with the slop =1 corresponds to the bias caused by quantitative calibration is equivalent to relative profiling method. Dots that fall above that line were identified as outliers, meaning unsuccessful calibrations. For the bacterial mock community data, almost all dots fell below that line (ratio < 1), indicating better precision based on QA. Only one purple dot at E05/5 (看下哪个菌) and three green dots at E05/20 (看下哪个菌) were as close as possible to the identity line or above this line, which might mostly be attributed to subtle variations in amplification efficiency due to certain strains (eg., Pro-672 and Pro-670).

Spike-in coverage also appeared to affect the calibration accuracy【文献】. 在RAN数据中，给一个比较合理的spike-in abu%. In this study, for 2-fold abundance differences detection (G1 Vs G2), we observed that there was a decrease in bias when the coverage of spike-in in bacterial libraries was controlled below 50%. According to **Supplementary Fig. 2**, spike-in would detect at least 1.8 fold abundance differences when it took up 8%-20% of the library size, very close to the nominal ratio (value=2). In contrast, spike-in BI12-4 took 48.9%~57.0% in E05 libraries and only detect on average 1.55 fold abundance difference. For indistinctive abundance detection (G1 vs G3), spike-in-based calibration agreed well with the nominal ratio (value=1) at all dilution gradients, from 1.0 to 1.2 fold (see **Supplementary Fig. 3)**.

We used similar spike-in based quantitative procedure to determine the abundance of each OTUs in fungal community. For ITS-seq data, spike-in reads ranged from 5.7% (E05/20) to 28.5% (E05) in the G2 dataset. Strong linear relationships between spike-in read counts and input amounts were also observed based on varying input spike-in concentrations in G2 (*r* = , **Fig. 3a**). As shown in **Fig 3b and Supplementary Fig. 4**, spike-in also did not affect detection efficiencies of three representative fungal members. Two-sided Dunn’s adjusted test verified no significant differences between E00 and other spiked communities (*P* > 0.05). Compared to promising measurements in bacterial experiment, the diagnostic performance of spike-in BI12-4 for quantitative fungi profile is a little weaker, deviating on average 0.5-fold from the expected ratio. We observed 1.3~ 1.5 fold differences in the microbial load between G1 and G2 (expected ratio = 2) (**Fig. 3c and Supplementary Fig. 5**). This bias was affected a bit due to the coverage of spike-in in library. Even so, the qualitative accuracy of spike-in were still robust for strains which existed indistinctive abundance shift, reflecting little bias (<1.06 fold, **Fig. 3d** and **Supplementary Fig. 6**). The ratio between QA induced error and RA induced error were calculated and plotted in **Fig. 3e.** For the fungal mock community experiment, all dots fell below that line (ratio < 1), indicating the calibrated performance of spike-in is still better than RA approach, even though a slightly lower than in bacterial pools.

**4. Synthetic spike-in allows calibrate the total microbial loads in Semi-synthetic community**

To evaluate the spike-in BI-12-4 performance in environmental microbiota, emphatically demonstrate the utility of spike-in for absolute quantification of total microbial load relative to plant host, we next designed **two groups of** environmental samples (designated as **sample ER1 and ER2**) with serially diluted spike-in, spanning at least 24 dynamic range (E55, E04, E03). In short, ER1 samples were natural rice root DNA extracts. ER2 samples were prepared by diluting ER1 with defined amounts of germ-free rice root DNA extracts. The microbial abundance ratio (relative to plant) between ER1 and ER2 can be calculated from the dilution factor (abundance ratio=1.8:1). BI-12-4 was kept constant within each dilution gradient to measure microbial loads.

Read counts from 16S-seq libraries were clustered into OTUs based on *de novo* at 97% sequence identity. Spike-in BI-12-4 amounts varied at different dilution experiments and accounted for 1.8~69% of total reads in different samples (**See Supplementary Table?**). As shown by one illustrative example (E04) in Fig 4a, relative abundances of individual OTUs in **unamended** and spiked samples were highly comparable (Pearson’s *r* *>* 0.99), suggesting spike-in did not affect measuring bacterial community structure and composition. Correlations for other two dilution experiments are also higher than 99%, shown in Supplementary **Fig.？**. Shannon index estimates showed no significant difference between unamended and spiked samples (**Fig. 4b**, *P* >0.05).

To assess the effects of spike-in based calibration on the outcome of microbiome analyses, we compare the influence of two profiling methods (relative method and quantitative method) on family abundance measurement. As expected, it was not impossible to obtain the total microbial/bacterial load or absolute growths or declines of particular taxa using relative approach. ER1 and ER2 produced similar relative abundance profiles at the family level (**Fig. 4c**). Nevertheless, the evident abundance shift between ER1 and ER2 could be detected using spike-in based calibration. As shown in **Fig. 4d**, when spike-in taking up 20% of the library size (E04), the total microbial load in ER2 displayed a 1.7 fold decline, in response to 1.8-fold increase in host copy number. However, even at 60%~70% coverage (E55) or lower than 5% (E03), the calibration based on spike-in were still reliable, reflecting 2.1 fold and 1.5 fold decline, respectively. We also found the absolute abundance (relative to plant host) for each bacterial family decreased on average 1.78 (±0.28)-fold in ER2. These results demonstrated the utility of the spike-in BI12-4 for quantifying total microbial loads as well as abundances of individual taxa. Ratios for all comparison are provided in **Supplementary Table?**

We also constructed fungal libraries responding to 16S-seq libraries at the same dilution conditions. Even though the amounts of the spike-in we used could be controlled at an appropriate proportion of the total ITS1 reads in the mock microbial community (3%~23%), it was difficult to determine the appropriate amounts of spike-in to add to natural fungal communities. Spike-in reads accounted for more than 75% of the total reads in E55 and E04 ITS libraries, indicating fungi only occupied a small fraction of the microbial community. Excessive proportion might compromise the accuracy of spike-in normalization. Therefore, we restricted our analyses to E03\_fungi library, in which the coverage of spike-in was at 20~30%. To assess the performance of BI-12-4 in calibrating the fungal data, we investigated the fungal community composition at the order level. We observed that ER2 fungal samples displayed 2.3- fold decline in total fungal load, and the ‘absolute abundance’ for top 10 fungal orders decreased on average 2.1±0.16 fold in ER2 **(Supplementary Fig. ?).** These results confirmed that quantitative fungal profile calibrated by spike-in BI-12-4 can truly reflected the abundance shifts than those generated by relative approaches.

**5. Microbial load as a key feature of the microbiome alterations associated with drought stress.** The drought effect has been observed across some plant species, including grass15, rice 16and sorghum17, as well as soil types [文献]. These studies have shown that drought significantly altered the bacterial or fungal communities. Consistent with these published studies, the results of Principal coordinate analyses (PCoA) of Bray Curtis distances from Hainan filed showed that drought treatment impacted the rice root-associated bacterial communities rather than bulk soil communities (**Fig. 5a**). The first two axes explained 58.03 % of the variance, with the primary axis (37.4%) primarily distinguishing samples by compartment and the secondary axis (20.63%) by treatment. Similar effect of drought treatment on the bacterial community were also observed in Anhui filed (**Supplementary Fig. 10**). Higher levels of moisture in bulk soil is likely to weaken the drought stress on bacterial communities16.

The total microbial load effect has been observed in the gut microbiome2,18. Vandeputte et al. found absolute microbial counts were three times lower in individuals with Crohn’s disease through quantifying microbial abundances in faecal material2. We hypothesized that the root microbial load might be sensitive to drought stress. To test this, we calculated the total microbial load and compared them between drought treatment and control group. We observed that the total bacterial load relative to host plant showed approximately 1.6~4.3 fold change (*P* < 0.05) in abundance within drought-treated roots compared to control roots, with an exception on cultivar WYJ in Anhui filed (WYJ-AH, *P* > 0.05) (**Fig. 5b; Supplementary Fig. 11**). However, the major load trends for WYJ-AH were still consistent: the bacterial load is more abundant in drought-treated roots than in control samples. Contract to root samples, no significant variation within microbial loads were shown for bulk soil samples. In general, total bacterial biomass has been observed to go down under drought19【Hueso S, García C, Hernández T (2012) Severe drought conditions modify the microbial community structure, size and activity in amended and unamended soils. Soil Biol Biochem 50:167–173；2013. Microbial enzymatic responses to drought and to nitrogen addition in a southern California grassland】, only few studies have observed bacterial biomass remains stable20 or increase21. However, these cases are soil samples which usually used phospholipid fatty acid (PLFA) content to assess the bacterial biomass. In contrast to prior published results, root samples from our field experiment exhibited an absolute increase in bacterial abundance following drought treatment in both cultivars and both filed sites. Detection methods、compartments and intensity of drought treatment may be responsible for the inconsistence.

To assess the influence of detection methods on the patterns of differential taxa abundance between drought and control treatments, we compare the enrichment and depletion profiles based on the RA and AA datasets at the phylum and OTUs levels. For bulk soil samples, no significant abundance shifts of dominant phyla or proteobacterial classes were detected between drought stress and control samples using either RA or AA methods, which was in consistent with previous reports16. For root-associated communities, soil type, rice cultivar and quantitative methods seemed to affect the outcome of differential phyla (**Fig. 5c; Supplementary Fig. 12**). Considering the results from RA methods, increase or decrease in relative abundance after the drought treatment were roughly similar regardless of cultivar or soil type.In contrast to prior published results15,16, root samples showed no significant increase in relative abundanceof *Actinobacteria* or *Chloroflexi*. As expected, method changes affected the detections of differential phyla. Taking MH63 as illustrated examples, most of indistinctive phylum (eg., *Actinobacteria*) and dominant classes (eg., *Alphaproteobacteria* and *Betaproteobacteria*) were detected as significantly enriched groups under drought in both filed sites. On the other hand, *Chloroflexi*, *Gammaproteobacteria* and *Verrucomicrobia* identifiedas depleted groups under drought using RA was found indistinctive in Hainan filed, or even enriched in abundance relative to host in Anhui filed when using AA method. To a great extent, this alternation signal is mainly driven by an overall increasement in total bacterial load. However, some differential phyla identified by RA and AA also existed overlaps within the same filed or cultivar, eg., *Nitrospirae* and *Spirochaetes*, both significantly depleted under drought in Hainan filed regardless of cultivar.

Similar trends were also observed in WYJ. Notably, we even observed the inconsistent directions of the responsed, potentially resulting from the supreme compositionality effects. Phylum *Actinobacteria* was detected as depleted group in root samples of WYJ in both fields when applying RA method, which may be a result of an absolute decrease in their abundance, or an absolute increase in other taxa. After calibrating the data through AA methods, the abundance of *Actinobacteria* did not significantly decrease; Conversely, *Actinobacteria* in drought samples significantly enriched (*P*<0.05) at the phylum level in Hainan filed. More than that, specific classes and families within *Actinobacteria* also responded in the opposite direction when comparing two testing results. This observation highlights the influence of the total microbial load, which have been further responsed at the OTU level.

To demonstrate the potential of AA approach on higher taxonomic rank, we compare the differential taxa confirmed by RA and AA at the OTUs level. Detailed comparison of drought-associated microbiome showed that the major taxonomic trends were roughly consistent in both methods: *Deltaproteobacteria* and *Betaproteobacteria* were depleted under drought stress, whereas *Actinobacteria* and *Alphaproteobacteria* were enriched (**Supplemental Fig. 13**). The similar trend resulted from a considerable overlap between differential OTUs identified by RA and AA methods. According to the venn results (**Fig. 5d; Supplemental Fig. 14**), more significantly depleted OTUs were detected using RA or more enriched OTUs were detected using AA, indicating a potentially misjudgment of the drought-responsive OTUs numbers without considering microbial load. Although RA method did therefore permit discrimination between drought treatment and controls, AA method still detected noteworthy discrepancies which may be associated to the total microbial load relative to host. As shown by three illustrative examples inMH63 from Hainan filed **(Fig. 5e),** OTU11 is significantly depleted in dry root samples only using RA method, which is missing when using AA. Conversely, OTU16 is detected as enriched group responsive to drought only using AA. We even observed the opposite response using two methods: OTU13 is significantly enriched in drought root samples using RA but depleted using AA. Considering the discrepancy resulted from RA and AA method, we should cautiously interpret the microbial community dynamics or design further experiments based on solely the differential taxa produced by relative abundance, as it could be misleading due to compositionality. 1,2,9.

5.3 真菌数据和细菌数据合表，计算真菌和细菌比 （细菌：真菌= 3:1）

We amplified the internal transcribed spacer 1 (ITS1) to characterize the influence of drought stress on the fungal communities associated with rice roots. Due to poor taxonomic annotation for ITS sequences, we restricted our analyses to exploit the effect of drought treatment on the fungal community structure and the associations between bacteria and fungi.

PCoA with Bray-Curtis dissimilarities revealed that the fungal community in Hainan was significantly affected by drought treatment (P值？), but not evidently in Anhui (**Supplemental Fig 15**), which is probably due to high spike-in coverage in Anhui fungal library. For spike-in reads accounted for more than 80% of total reads in bulk soil and MH63 root samples from Anhui filed and possibly introduced wrong assessment, we had to remove the related data from our analysis and only restricted our further analyses to samples from Hainan field and WYJ root samples from Anhui, in which the coverage of spike-in was below 70%.

Similar to the calculation of the bacterial load, we quantified the fungal load for each cultivar in Hainan and for WYJ in Anhui. The fungal load is significantly more abundant in drought-treated roots than in control samples in both cultivars in Hainan filed (*P* < 0.05), as well as WYJ in Anhui (**Supplemental Fig 16)**. The ratio of bacterial to fungal ribosomal content was determined thought merging both calibrated bacterial and fungal OTUs tables using AA approach. As shown in **Fig. 5f**, two cultivars MH63 and WYJ in Hainan filed have similar ratio of bacteria to fungi in root regardless of drought treatment: Bacteria contributed to 75.8% (max. %, min %) and fungi to 24.2 % (max. %, min %) of the root-associated microbiota without considering archaeal and other non- fungal eukaryotes. This ratio is far below those detected in Hainan filed soil, which is approximately 28:1 (control) and 44:1 (drought treatment), respectively (**see Supplemental Table ?**). The soil value can be compared with published results, which showed that the ratio of bacteria to fungi ranged from 12:110 to 32:122. The elevated ratio of bacteria to fungi in root 说明了什么问题? 如果样品中存在很多低丰度的序列，提高OTUs聚类标准（本研究用的是30条序列聚成一个OTUs）可能会过滤掉很多序列，无形中降低细菌/真菌的比例。

**Quantitative microbial profiling method reveals absolute increase of total microbial load in the wheat roots infected by *Bipolaris sorokiniana***

Common root rot is a disease of winter wheat mainly attacked by *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*)*,* which caused a series of symptoms include dark brown to black lesions on roots, subcrown internodes and stem bases, finally resulting in yield losses23 [2018. Spatial Distribution of Root and Crown Rot Fungi Associated With Winter Wheat in the North China Plain and Its Relationship With Climate Variables; 1998. Common root rot and Fusarium food rot of wheat]. To demonstrate the utility of the spike-in for quantitative assessment of microbiota variation which is associated with diseased or healthy host plant, we performed spike-in based quantitative method on wheat root samples including ten healthy samples and ten infected samples with Common root rot.

According to traditional PCoA on the basis of microbiome，we found both bacterial and fungal communities associated with root rot disease significantly different from healthy communities (**Fig 6a，6b)**. For bacterial communities, a clear separation of root samples distinguished by healthy state were observed on the PC2 coordinate, explaining 13.5% of the variance. For fungal communities, healthy samples and infected samples were obviously separated on the first two ordination axes, totally explaining 44.6% of the variance. These results indicated fungal community were more affected by root rot disease than bacterial community. Although PCoA could permit distinguish samples based on the RA dataset, it might omitted the microbial abundance as potential feature of the microbiome alterations associated with disease, which has been identified in Crohn’s disease2【2018.Nature 文献】. Quantitative microbial profiling method reveals bacterial load were approximate two times higher in the infected root samples. Compared to bacterial community, fungal load increased four times higher in samples with root rot disease (**Fig 6c**, *P* < 0.05), resulting in decreased ratio of bacteria to fungi. Notably, the disease-associated pathogen *Bipolaris sorokiniana* (OTU45) abundance increased two-fold when applying RA, but increased almost seven fold relative to plant when using AA based calibration. Taken together, these observations…….

【如果有网络数据就可以比较得病和健康，RA和AA的数据 4个网络图4个结果】

**5.4\* 网络分析（简单说下，不得出solid 结论）**

目的：因为网络分析是最能体现组成型数据性质的分析，所以比较下RA和AA的结果

**Methods**

**Design and construction of the spike-in plasmids**

Spike-in BI12-4 sequence was designed comprising selected plant marker gene RID1 fragments and conserved 16S rRNA gene (799F/1193R) and ITS (ITS1F/ITS2) primer regions. Considering the different amplicon sizes between bacteria and fungi, the ITS primer conserved regions were designed to be adjacent to the plant marker gene fragment, and the 16S rRNA primer regions were designed in the two sides of the ITS primer fragments. Design of synthetic spike-in was based on the following two criteria: (i) plant marker gene are universally present in host and not normally found in environmental microbiome; (ii) The amplicon length targeting the bacteria and fungi was close to the most common size of the real microorganism. For spike-in BI12-4, the amplicon fragment is 378 bp for 16S-seq and 336 bp for ITS-seq, respectively. , based on the results of the most common size from our previous sequencing; (iii) GC content was design to close to the real range of microorganisms (48~60%) as far as possible, reducing the bias induced by PCR amplification efficiency.

Assessment of spike-in BI12-4 sequence designed in this work by Blast search against a range of NCBI databases verified that the artificial spike-in shared no identity with known microorganisms. The conserved primer regions were added into two sides of the selected plant marker gene fragment via PCR amplification and the resulted spike-in was checked by gel electrophoresis and Sanger sequencing. The artificial spike-in DNA fragment was cloned into a pGEM®-T Easy Vector by using pGEM®-T Easy Vector System I (Promega, Madison, USA) and was transformed into Competent *Escherichia coli* DH5α. Plasmid DNA was extracted from overnight liquid cultures using Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, USA). Then linearized using ScaI (New England BioLabs (NEB), Hitchin, UK). Linearized plasmid DNA was purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and was confirmed its integrity by amplifying 799F/1193R and ITS1F/ITS2. Spike-in DNA concentrations were measured with Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen, Life Technologies, Grand Island, NY, USA) and plasmid copy number were calculated according to Lee et al (2006) . The spike-in was ~~prepared based on estimated copy numbers and~~ stored at -80℃ for further processing.

**Synthetic communities preparation**

For verify the utility of spike-in BI12-4, two synthetic communities (SynComs) were employed to mimic the taxonomic diversity of root microbiota in natural environments (i) Whole-synthetic community (W-SynComs or mock community) consisting of genomic DNA of germ-free rice roots and 12 different microorganisms. Germ-free plant DNA was extracted from sterilized rice Zhonghua 11 WT roots. Nine different bacteria belonged to four typical phyla found in root microbiomes, contributing to *Bacteroidetes* (Bac186), *Firmicutes* (Fir11), *Actinobacteria* (Act101, Act135, Act140) and *Proteobacteria* (Pro1203, Pro670, Pro1204, Pro672), respectively. Three fungal isolates mainly belonged to *Ascomycota* (AF1, AF105) and *Basidiomycota* (AF78). All the DNA concentrations of microbe and plant were quantified through PicoGreen dsDNA Assay Kit (Invitrogen) and subsequently diluted to 3.5 ng µl-1 for subsequent mixing. Details on the designed mock DNA mixtures were provided in the Supplementary Table S?. Linearized plasmid BI12-4 was spiked into samples with five different concentrations: 7.96\*105 (E05), 1.59\*105 (E05/5), 7.96\*104 (E05/10), 3.98\*104(E05/20), and 0 (E00) copies /PCR; (ii) Semi-synthetic community (S-SynComs or disturbed community) comprising natural wild Zhonghua 11 roots DNA with four different concentrations of spike-in: 1.97\*105(E05/5), 9.86\*104(E05/10), 9.86\*103(E05/100) and 0 (E00) copies /PCR. Then these samples were split into two aliquots: one was used as a normal natural sample, the other was diluted by a defined amount of germ-free plant DNA, responding to the total microbial load change. Details on the disturbed DNA pools were provided in the Supplementary Table S?. For all defined communities we examined 3~5 independent SynCom preparations.

**Drought stress associated rice root samples collection, processing and DNA extraction.**

Two rice (*Oryza sativa*) cultivars MH63(subsp. *indica*) and WYJ (subsp. *japonica*) were grown in two geographically distant locations in China: an agricultural field in Hainan(经纬度), and a filed in Anhui (经纬度). Both fields had only been used for rice cultivation for several years. Dehulled seeds were surface sterilized (75% ethanol for 30s, sodium hypochlorite three times for 15 min, followed by washes with autoclaved water), then germinated on MS agar media. After germination, 2-week-old rice seedlings in MS agar were transferred to fields.

**Drought Treatment (干湿处理):???**

Bulk soil and root samples collection and processing were done following Zhang *et al* (2018). DNA were extracted with the FastDNA SPIN Kit for Soil (MP Biomedicals) according to the manufacturer’s instructions. Extracted DNA was stored in nuclease-free H2O at -80℃ until use. According to the experiment design, spike-in BI12-4 with predefined amounts (? cp/reaction) was added into each DNA extracts.

**16S and ITS amplicon library construction and Illumina sequencing**

Samples were split into two aliquots and each amplified either with 799F/1193R for bacteria (文献引用) or with ITS1F/ITS2 for fungi (文献引用). 16S rRNA gene libraries construction was followed a previously described two-step PCR strategy (Zhang et al., 2018). For fungal libraries, the ITS1 region was amplified according to a modified two-step PCR program: after an initial denaturation step at 98°C for 30 s, the targeted region was amplified by 30 cycles of 98°C for 10 s, 50°C for 15 s and 72°C for 60 s, followed by a final elongation step of 5 min at 72°C. The first PCR products were cleaned with AmPure XP kit (Beckman Coulter) and diluted to 10 ng µl-1 as templates for the second-step PCR. All samples were amplified in triplicate for ten cycles under identical conditions to those of the first step PCR. PCR products were verified by a 1.2% (w/v) agarose gel and then purified with AmPure magnetic beads, quantified and pooled in equimolar concentrations. Final pooled libraries were sent for sequencing by an Illumina Hiseq 2500 platform (BGI-Shenzhen, Shenzhen, China).

**Quantification of plant marker gene by quantitative real-time PCR**

To calibrate the quantitative data, RID1 gene copies in each rice root sample were determined by qPCR on a LightCycler 480 II Instrument (Roche). The DNA concentrations were adjusted to 3.5 ng µl-1 for the subsequent qPCR. Primers were used by Wu *et al* (2008). PCR assay mixtures consisted of 10μl SYBR Green I Master Mix (Roche), two times 1μl primer solution (10 nM), 6μl nuclease-free water, and 2μl template DNA. Quantification PCR conditions were as follow: 5 min at 95 °C, then 40 cycles of 10s at 95 °C, 15s at 60 °C and 15s at 72 °C. The standard curve was generated using 10-fold dilution of a plasmid containing the PCR fragments of the RID1 gene. All qPCR reactions were conducted in duplicate. The copy numbers of marker gene per ng root DNA were calculated based on Ct values and standard curves.

**Illumina Sequencing data processing and analysis for the gene encoding the 16S rRNA.**

**（1）样品质控（2）RA数据处理（3）AA数据校正和处理**

The 16S rRNA gene sequences were processed using QIIME 1.8.1, ***usearch8,*** USEARCH 10.0, vsearch v2.7.1 and with custom scripts. Paired-end Illumina reads were filtered by FastQC, and split libraries with ***usearch10 -fastx\_getseqs*** and joined by ***usearch10 -fastq\_mergepairs*** script, Cut primers and quality filter by ***usearch10 -fastx\_truncate*** and ***usearch10 -fastq\_filter***.

After data preprocessing, we used the UPARSE pipeline with default settings in natural community OTU discovery, or reads were mapped into reference sequences with qiime command map\_reads\_to\_reference.py with perfect match in mock synthetic community unless stated otherwise. After dereplicating with command ***usearch10 -fastx\_uniques*** and option ***-minuniquesize*** 30 and chimeric filtering in reference mode against the silva reference database with the command ***usearch10 -uchime2\_ref***; then, in order to filter the host sequence we make the taxa assignments with ***usearch –sintax*** method to filter the mitochondria, chloroplast or eukaryota sequence and finally, mapping of all reads against the OTU centroids by ***vsearch –usearch\_global*** with default parameters, at 97% sequence identity we fetch the OTUs table and manually to add the spike-in sequence number into the OTU table. Main command lines for read processing and analyses are provided in the github repository https://github.com/TankMermaid/spike-in

**Sequence processing and analysis for ITS**

Like mentioned above, paired-end Illumina reads were filtered by FastQC, joined by qiime script ***join\_paired\_ends.py*** (fastq-join). Dual barcodes were extracted from the index fastq files and concatenated using the QIIME script ***extract\_barcodes.py*** and ***split\_libraries\_fastq.py,*** no errors were allowed in the barcodes and quality trimming and filtering were suppressed by passing the options(--max\_bad\_run\_length 3 --min\_per\_read\_length\_fraction 0.75 --max\_barcode\_err 0) . after demultiplexing was performed, primer regions were removed with cutadapt, with options of `***-e 0.15 --discard-untrimmed***`, a minimum final sequence length of 170.

Reads were then mapped into reference sequences with qiime command map\_reads\_to\_reference.py with perfect match in mock synthetic community and UPARSE pipeline for OTU clustering in natural community, respectively. Briefly, this included dereplication of the reads; abundance-based sorting of the dereplicated reads, with removal of threshold less than 30 reads if applicable; generation of OTUs with a similarity threshold of 97% ***(usearch8 -cluster\_otus***); Chimeric sequences were detected and discarded using UCHIME in reference against version 7 of the UNITE database using an open reference strategy (***usearch8 -uchime\_ref***); and finally, mapping of all reads against the OTU centroids by usearch global with default parameters, at 97% sequence identity and qiime script uc2otutab.py and biom convert to make otus tables (***usearch8 -usearch\_global***).

Taxonomy assignment was performed with the qiime command ***assign\_taxonomy.py*** at a defualt similarity threshold using the BLAST algorithm, OTUs classified not as k\_\_Fungi in taxa kingdom level were filtered from the OTU table before download analysis. Spike-in sequence hit was detected and added into the OTUs table manually

**Statistical analysis**

No statistical methods were used to predetermine sample size and all analyses were conducted in the R Environment version 3.4.3. Statistical analyses and plotting were performed in R using the packages dplyr\_0.7.4, reshape2\_1.4.3, RColorBrewer\_1.1-2, ggsignif\_0.4.0, vegan\_2.5-2, permute\_0.9-4, ggpubr\_0.1.6, ggplot2\_2.2.1. All statistical tests used were two-sided. Before RA and AA analysis, random resampling was performed using the function ‘rrarefy’ (threshold used as the minimal sample reads ), and OTU counts were normalized using the scaling normalization in RA and spike-centered data normalization in AA. microbiome variation between individuals was calculated as Bray-Curtis dissimilarities using the qiime command beta\_diversity.py and Shannon alpha index was performed with function diversity() in R. based on rarefied OTU counts, unconstrained ordination was performed by principle coordinates analysis (PCoA) using the function ‘capscale’, all as implemented in the R package vegan. Dose response curves for individual spike-in standards were fitted with a linear model, using the function ‘lm()’ of the R package MASS, Differential abundance analyses were performed in two-level categorical of OTUs and phyla with Wilcoxon rank-sum tests. For phylum-level analyses, except for Proteobacteria by class, OTU counts were collapsed by phylum. Corrections for multiple testing (Benjamini–Hochberg, FDR, adjusted P value< 0.05) were performed, multi-group comparation was performed using non-parametric Kruskal–Wallis and post hoc Dunn’s test for all pairs of comparisons between groups. All plots were generated with the ggplot2 package, and the Venn diagrams, which were plotted with the VennDiagram package. All scripts and intermediate files have been deposited in GitHub https://github.com/TankMermaid/spike-in

Code availability. An open source R-script and Python-script is available on https://github.com/TankMermaid/spike-in

Data availability. All specimens and associated sequence data were assigned a de-identified code and stored in controlled-access repositories.

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