Drip irrigation suppresses methane emissions by reshaping the rhizosphere microbiomes in rice.

Authors:

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ABSTRACT

The rhizosphere microbiomes of rice plants consist of a complex consortium of methanogens, methanotrophs, nitrogen- and/or sulphur-assimilating microorganisms. Controlled irrigation has been purported to be the cultivation method of choice over continuous flooding as it reduces water usage and aerates the soil to minimize the emission of the potent greenhouse gases. However, a systemic understanding of the assembly and function of microbiota in the rhizosphere under drip and flood irrigation remains unclear. Here, we report a significant reduction in methane emissions in drip irrigation compared to flooded paddies. Genotypic or varietal differences did not influence such increased methane emissions from flooded soil cultivation of rice. Using metagenomic sequencing and computational analyses, we provide a deeper understanding of how drip irrigation or continuous flooding affect the root-associated microbiomes in rice. Rhizosphere soil from two different rice varieties, Huanghuazhan and Temasek rice, grown under drip or flood conditions in a greenhouse was collected 2 months post-transplantation for metagenomic analysis. Our results reveal that drip irrigation favours microbes involved in the nitrifying-denitrifying processes while continuous flooding enriches for methanotrophs and methanogenic archaea. Syntrophic microbial clusters associated with methanogenesis were significantly reduced in drip irrigation. We identified several keystone taxa in the co-occurrence network models related to methanogenic and methanotrophic activities, as well as beneficial microbes from the soil rhizosphere, and *Actinomycetes* from floodwater. Lastly, oxygen availability and redox potential were identified as key drivers that reshape rhizosphere microbiota leading to the functional differences observed between the two irrigation regimes.

INTRODUCTION

Climate change is a significant threat to the rising global temperatures and adverse weather events, such as typhoons, droughts, heatwaves and floods around the world affecting livelihoods of farmers and impacting crop yields [1]. One of the potent greenhouse gases that is responsible for climate change arising from agriculture is methane. In traditional cultivation, rice is grown in flooded paddies to prevent weed growth and such flood irrigated methods leads to emission of high levels of methane as the standing water provides an anaerobic environment that favours the growth of methanogens which feed on the decaying organic matter in soil [2]. Therefore, to combat climate change, reducing methane emissions from rice fields has become one of the important endeavours in agricultural settings to achieve net zero by 2050.

Soil is rich in microorganisms due to the availability of diverse nutrient and carbon sources and the diversity of microbes is thought to be important for maintaining soil health [3]. The rhizosphere refers to the soil region around the plant roots and is a microenvironment where microorganisms live and interact with the host plants in a variety of ways that may be neutral, beneficial, or detrimental [4]. Therefore, studying plant-soil microbiome interactions can provide further insights into how microorganisms can affect the health and productivity of a crop [5].

Rice (*Oryza sativa* L.) is an important staple food crop in Asia, Africa, and Latin America [6]. Interestingly, rice plants acquire a distinct set of root microbiota as compared to other plants such as *Heteranthera limosa*, *Cyperus iria* and *Ammania coccinea* when grown under similar flooding and nutrient rich conditions [7]. The researchers observed that the rice plants select for methanogenic archaea, *Methanocella*, *Methanosarcina* and *Anaerolinea* whereas the other native plants enrich for methanotrophs with little methanogenic archaea. Their findings

suggest that the rice root microbiome is associated with methane production in rice paddies. Another study has shown that rice paddies in China harbor keystone taxa belonging to the families *Methanosarcinaceae*, *Methanobacteriaceae* and *Methanocellaceae* and are correlated with temperature [8].

Different irrigation regimes such as continuous flooding and drip irrigation may have diverse effects on the microbiomes present in rice paddies. Traditionally, continuous flooding has been used for cultivation, wherein rice plants are grown in water at a depth of at least 10 cm to control weed growth [9]. While continuous flooding is an effective method for weed management, it requires large amounts of water and is harmful to the environment. This is because continuous flooding creates an anaerobic environment that promotes the growth of methanogenic archaea producing greenhouse gas methane. Therefore, recent studies have proposed alternative means such as drip irrigation to save water and to mitigate methane production and emission levels [10]. Drip irrigation methodology reduces water usage and aerates the soil to minimize anoxic conditions and are effective in reducing methane production.

In this study, we assessed and characterized the soil microbiomes of two indica rice varieties, Huanghuazhan (HHZ, China) and Temasek rice (Singapore) under drip irrigation and continuous flooding conditions in a greenhouse. The microbiomes would give us further insight on the possible microbial activities in the soil. In addition, potential interactions among different microbes can be inferred using microbial co-occurrence network [11]. Important keystone members can be identified to predict their activities in the soil and their relation to methane emissions [12]. HHZ is an elite indica rice variety from South China that is known for its high yield [13]. Temasek rice is a variety bred using marker-assisted breeding technique performed in Singapore [14]. Temasek rice also has high yield, disease resistance and submergence-tolerance traits that is adapted for cultivation in tropical urban farms. Using

shotgun metagenomics, we aim to (i) describe the taxonomic and functional shifts in the root microbiomes under flood and drip-irrigated conditions and (ii) identify syntrophic microbial communities that may be involved in methane production. Lastly, we also compared the phenotypic changes in root morphology under flood and drip-irrigated rice systems.

RESULTS

Methane emission is significantly reduced in drip-irrigated rice

Methane emissions were measured using the static closed chamber method with gas emissions measured in the range of 0 to 500 mg/m^2 per day. Based on our findings, methane emissions from rice soils under continuous flooding generally showed two emission peaks throughout the life stages of the rice plants (Fig 1). The first peak occurred at week 4 post transplanting during the seedling to tillering stage while the second peak coincided with the transition from tillering to flowering stage at week 8. In contrast, drip-irrigated rice soils/plants seem to emit little to no methane as compared to their continuously flooded counterparts and, on average, we observed a significant 96-98% reduction in methane emissions in such controlled irrigation regime. No significant differences were observed between HHZ and Temasek rice varieties, suggesting a lack of the genotype-based contribution to methane emissions.

Four hypothetical temporal patterns of relative methane emissions based on 232 *in-situ* rice field sites have been proposed by Qian et al. 2023 to help inform mitigation priorities in either irrigation or organic matter or fertilizer management [15]. Out of the four models, our methane emission data from HHZ and Temasek rice in Fig 1 seems to match the continuous emission pattern model, where methane gas is continuously produced under continuous flooding conditions.

Drip irrigation affects the root-associated microbiomes

An average of 51,176,651 mapped reads were obtained. The metagenomic profiles of the soil microbial samples were normalized and analysed as relative abundances. The results showed that drip and flood-irrigated soils have different microbial community structures and diversity. Principal Coordinate Analysis (PCoA) of Bray-Curtis distance showed two distinct

clusters of drip and flood-irrigated soils (Fig S1). The Bray-Curtis distance measure was bounded between 0 (the sample units are identical) and 1 (the sample units are completely different), and was semi metric. Based on Chao1 index, drip-irrigated soils showed lesser number of microbial species than the ones observed in flooded soils (Table S1). In terms of differences in species diversity between drip and flooded soils, the drip soil is less diverse than flooded soil. Both HHZ and Temasek rice showed similar scores for Chao1, Shannon and Simpson indices and their associated microbiomes are not statistically different from each other (Table S2).

At the class level, *Gammaproteobacteria*, *Cytophagia* and *Nitrospira* are higher in abundance in drip-irrigated soil (Fig 2). On the other hand, *Methanobacteria* and *Methanomicrobia* are higher in abundance in the flooded soil. At the genus level, specifically within the Archaea domain, methanogens such as *Methanothrix*, *Methanosarcina, Methanocella* and *Methanobacterium* are higher in abundance in flooded soil, while *Candidatus Nitrosocosmicus* is higher in abundance in the drip soil (Fig 3 and 4). Canonical correspondence analysis (CCA) analysis showed a significant separation between drip and flood samples along the first CCA axis with 58.6% of variance explained where *Nitrospira*, *Hydrogenophaga*, *Bradyrhizobium* and *Pseudomonas* are driving the change in drip-irrigated soil (Fig 5). *Methylocaldum*, *Streptomyces*, *Nocardiodes* and *Agromyces* are key drivers shaping the continuous flooded soil microbiomes. The drip-irrigated soil seems to enrich for nitrifiers *Nitrospira* and denitrifiers *Hydrogenophaga* when rice plants are grown aerobically while the continuous flooding environment selects for methanotrophs such as *Methylocaldum* and *Nocardiodes* and *Actinomycetes* like *Streptomyces* and *Agromyces*. The drip-irrigated soil tended to be more heterogeneous as compared to the flooded soil where flooded soil samples were found to cluster closely together along CCA2 axis.

Differential taxa analysis was performed in MaAsLin2. Taxa that are statistically significantly and enriched in flood-irrigated rice soil include *Methanothrix*, *Nitrosomonas*, *Thermonospora*, *Fimbriiglobus* and *Methanocella* (Fig S2). Drip-irrigated soil, on the other hand, selects for *Dongia*, *Alcanivorax*, *Flavobacterium*, *Povalibacter* and *Enhydrobacter*. The results suggest an enrichment of methanogens in the flooded anaerobic environment.

Syntrophic microbial clusters are significantly reduced in drip irrigation

Molecular ecological networks were created to unravel the changes in microbial interactions. The analysis reveals the presence of at least one active microbial cluster in drip and six clusters in flood-irrigated soil (Fig 6). Each cluster represents a microbial hub, and we were able to identify taxa with related functions in each hub.

A significant reduction in the number of syntrophic microbial communities was observed when rice was subjected to drip irrigation. The lilac cluster consists of *Methanosarcina*, *Micromonospora*, *Candidatus Nitrosocosmiscus*, *Rhodococcus*, *Dactylosporangium*, *Actinomadura* and *Mycolicibacterium*. Within this cluster, there is the presence of ammonia oxidizer, *Candidatus Nitrosocosmiscus* and methanogens, *Methanosarcina* along with obligate aerobes from the Actinomycetia class like *Micromonospora*, *Rhodococcus* and *Dactylosporangium*. We inferred that the reduced number of clusters in drip irrigation likely contributes to reduction in the overall methanogenesis process, thus resulting in lower methane production (Fig 1) as lesser precursors such as acetate are produced by other members of the associated microbial community.

In contrast, the flood-irrigated soil was rich in microbial clusters. The orange cluster consisted of microbes associated with anaerobic methanogenesis such as *Methanocella*, *Methylococcus*, *Optitutus* and *Flavobacterium*. Methanotrophs can feed on the methane produced by methanogens in this process. The lilac cluster seems to contain a mixture of

sulphur-reducer like *Desulfuromonas*, iron-oxidiser like *Ferrovibrio* and *Pseudomonadota*-related taxa like *Arenimonas*, *Cupriavidus* and *Pseudoxanthomonas* alongside plant growth promoting bacteria like *Variovorax* and *Rhizobium*. Members from the lilac cluster seem to interact closely with the lime cluster. Lime cluster consisted of members that can degrade complex sugars and lignin like *Chryseolinea* and freshwater or marine-related methanotrophs like *Methyloceanibacter* alongside Nitrogen-fixing bacteria like *Mesorhizobium*. These microbes from the lilac and lime clusters are mostly facultative aerobes and are likely found in floodwater too. The cyan cluster consists of members from the *Actinomycetia* classes such as *Micromonospora*, *Actinoplanes*, *Actinotalea* and *Streptomyces* and are of higher abundance in flood-irrigated soil as seen previously in Fig 2.

Function profiles of root-associated microbiomes may be influenced by oxygen availability and redox potential

Oxidation reduction potential (ORP) and pH measurements were captured over the entire course of the growth stages of the rice plants (Fig 7a,b). Drip-irrigated soils tended to be more heterogeneous and showed higher variance in the ORP readings with a median value of 111.5 mV. Conversely, the flood-irrigated soils were reducing in nature with a median ORP of -205.5 mV. Soil redox potential of between -100 and 200 mV has been associated with methane emissions in flooded rice soils [16]. In fact, another study has shown that oxygen availability in the soil controls the soil redox potential [17]. Our experiment showed that drip irrigation aerates the soil and increases the redox potential significantly. Therefore, the reduction in methane emission in drip irrigation is likely due to the increased oxygen availability, which also acts as an environmental filter to select for aerobic microorganisms. pH measurements were kept constant within the range of pH 7. There is no statistically significant difference in pH between drip and flood-irrigated soil.

To investigate the metabolic functions of microbes that are present in drip and flooded soils, the metagenomes were mapped to putative functions in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database. The reads were expressed as RPKM (Reads Per Kilobase per Million mapped reads) and were normalized as Z-scores before plotting them in heatmaps. Predicted gene functions relating to methane and nitrogen would highlight the microbial activities involved in the methanogenesis, denitrification-nitrification processes. The heatmaps show higher Z-scores for methane-related genes such as tetrahydromethanopterin-S-methyltransferase and formylmethanofuran dehydrogenase, which seems to be more abundant in flooded soils (Fig S3). These genes are predominantly found in *Methanobacterium* and *Methanosarcina* and plays significant role as part of the methane biosynthesis metabolic pathway. In addition, we also find reads with putative methanol dehydrogenase gene that are usually found in methanotrophs. Nitrogen metabolism was found to be higher in flood irrigated soil (Fig S4). Nonetheless, *glnB*, *glnK* and *nifU* genes are found equally abundant in both flood and drip-irrigated soil. *glnB* and *glnK* genes are related to ammonium assimilation in phototrophic bacterium like *Rhodobacter* [18]. *nifU* gene is required as part of the assembly component of the iron-sulphur (Fe-S) cluster of nitrogenase needed for nitrogen fixation [19]. Our result suggests that the drip-irrigated soil, unlike the flood-irrigated soil, only exhibits minimal basal levels of nitrogen fixation and ammonium-related activities. Drip soil also has significant reductions in both methanogenic archaeal and methanotrophic bacterial populations.

Drip-irrigated rice plants tend to have shorter roots

Our results showed that drip-irrigated rice plants have shorter roots as compared to the continuously flood-irrigated plants (Fig S5, Table S3). This observation was consistent and similar in both HHZ and Temasek rice. Drip irrigation supplies water directly to the plant roots. It is likely that the plants invest in less metabolic resources by keeping their root

biomass smaller as the roots are constantly receiving water from the drip pipe outlets at the soil surface. Water becomes scarce as it goes deeper into the soil. On the other hand, the flood-irrigated rice plants seem to develop bigger and deeper roots. It is likely that the plant invests in a deeper root system to anchor/stabilize effectively and to absorb more nutrients via the active microbial communities found in both the floodwater and the soil.

DISCUSSION

Potential mechanisms to explain the shift in microbiomes

It is likely that the shift in microbiome is associated with the change in redox potential of electron donors and acceptors in the drip and flood environments respectively. Notably, the two groups of redox reaction pairs that are likely involved in this process are (1) aerobic in the positive ORP range: $O_2/H_2O$and NO_3^-/N_2 and (2) anaerobic in the negative ORP range: SO_4^{2-}/H_2S and CO_2/CH_4. In the drip environment, since oxygen is readily available, therefore microbes would use O_2 as electron acceptor and the dominant redox pair is (1) O_2/H_2O and NO_3^-/N_2. However, in the continuous flooded environment, since oxygen is now limiting or absent at greater soil depth of at least 15 cm and below, sulphate becomes the next best electron acceptor and now the dominant redox pair is shifted to (2) SO_4^{2-}/H_2S and CO_2/CH_4. There is also an increase in the relative abundances of methanogens in flooded soil as compared to the drip soil (Fig 2 to 4). The flood environment also enriches for a more diverse active syntrophic microbial communities that make more precursors of acetate, methanol and formic acid available for methanogens from the breakdown of complex sugars such as cellulose, hemicellulose and lignin.

Co-occurrence network reveals functionally distinct hubs of microbes

The co-occurrence network reveals one functionally distinct hub in the drip-irrigated soil while there are at least six distinct hubs in flood-irrigated soil. The microbes in the orange

cluster corresponds to methanogens and are closely associated with irrigation management practices. Continuous flooding promotes the growth of methanogens as it provides a conducive anaerobic environment for them to thrive.

The cyan cluster observed in Fig 6 is mostly made up of Actinomycetia bacteria present in the flood water. *Agromyces*, *Actinoplanes*, *Micromonospora* and *Streptomyces* are Actinomycetes that helped break down complex biopolymers like cellulose, chitin, keratin and lignocellulose [20]. The presence of Actinomycetes help plants absorb essential elements like, C, H, N, O and P, promoting plant growth [21]. Notably they are higher in abundance in the flood-irrigated soil than the drip-irrigated soil.

Methanotrophs like *Methylomonas*, *Methyloceanibacter*, *Methylomicrobium* have methane monooxygenase to oxidize methane and are present as member of the orange, lime and cyan clusters [22]. It is not surprising that they are present in higher abundance in flood-irrigated soil since methanogens emits methane and are higher in abundance in the anaerobic flooded soil. The lilac cluster consist of rhizobacterium and a group of growth-promoting beneficial bacteria and are part of the rhizosphere-related biome. They are tightly related to the health of the crop plant. *Pseudomonas*, *Bradyrhizobium* and *Rhizobium* have been found in other agricultural crops such as sugarcane (*Saccharum*) [23] and soybean (*Glycine max*) [24]. For example, *Variovorax* has been shown to possess an enzyme to convert indole-3-acetonitrile to plant hormone indole-3-acetic acid (IAA) to promote plant growth [25].

CONCLUSIONS

In summary, there are benefits and trade-offs for continuous flooding and drip irrigation. Traditional continuous flooding is effective against weeds in rice cultivation. While flooding consumes large volumes of water, it enriches for diverse groups of syntrophic microorganisms that could help promote better plant growth and degrade complex sugars to

acetate and methanol precursors favourable for methanogens. The anaerobic environment in floodwater further promotes growth of methanogens that in turn produce more methane. In contrast, drip irrigation saves water and due to the shift in redox potential and oxygen availability, it becomes less favourable for methanogens to thrive in. Drip irrigation remains to be a potential candidate for future sustainable climate-friendly rice farming practices.

MATERIALS AND METHODS

Methane emission measurements

Methane (CH_4) emissions were captured across the growth stages of rice growth. The closed chamber method was used to measure methane where transparent chambers of (30 cm x 30 cm x 100 cm) equipped with an electric fan at the top were placed over the rice trays such that four rice plants are captured in one chamber [26]. A chamber base was installed in the soil and gas samples were collected using a 50 mL syringe at 0, 10, 20 and 30 minutes. CH_4 measurements were then analyzed using a gas chromatograph equipped with a flame ionization detector. Then the CH_4 flux is determined using the following formula:

$$F = \rho \times (\frac{V}{A} \times \frac{\Delta c}{\Delta t} \times \frac{273}{K})$$

F is CH_4 flux expressed in (mg CH_4 m^{-2} h^{-1}), ρ is the gas density of CH_4 gas (0.174 mg cm^{-3}), V is the volume of the chamber in (m^{-3}), A is the surface area of the chamber in (m^{-2}), $\frac{\Delta c}{\Delta t}$ is the rate of gas concentration increase in the chamber (mg m^{-3} day^{-1}) and K is air temperature in the chamber measured in Kelvin.

Experimental design, soil sampling and DNA extraction

HHZ and Temasek rice plants were planted in container trays in a greenhouse facility at Temasek Life Sciences Laboratory, Singapore (103º46’36” E and 1º17’36” N). Each tray was then subjected to different treatment, drip and flood. Drip irrigated trays were laid with drip

irrigation pipes that were connected to a water source where water was only allowed to trickle drop by drop into the tray of soil. In contrast, in the continuous flooded trays, the rice plants were submerged in water to a depth of 10 cm over the entire course of study from March 2024 to June 2024. The soil samples were collected for metagenomic analysis during the last week of harvest.

Soil samples were collected using 25 mL serological pipette strips that were modified as soil probes. The serological pipettes were cut using a heat-sterilized knife at the top and between the 15- and 25-mL mark to create an opening at the top and the side. A new 10 mL serological pipette strip was then inserted from the top of the 25 mL pipette strips to enclose the 25 mL strip. The soil probes were then plunged into the soil at a depth of 15 to 20 cm and the 10 mL strip was then released so that soil can enter the 25 mL strip chamber from the side opening. Soil samples were then collected in a 50 mL tube, transported to the laboratory, and kept frozen at -20 ºC freezer until DNA extraction. 2 g of soil was weighed and used for DNA extraction using the DNeasy Power Soil kit (Qiagen, USA) following manufacturer's standard protocols. 100 µL DNA were eluted and stored at -20 ºC freezer for sequencing.

Library preparation, sequencing, and bioinformatics analysis

Extracted DNA samples were quantitated using Nanodrop spectrophotometer (Thermofisher Scientific, USA). High through-put sequencing libraries were prepared with the VAHTS Universal Plus DNA library preparation kit for Illumina V2 ND627 (Vazyme, China) following manufacturer's protocol. The samples were sequenced at 1st BASE, Singapore, using the NovaSeq 6000 sequencer (Illumina, USA). Sequence adapters were trimmed from all paired end reads using bbduk of the BBTools packages [27]. MultiQC [28] was performed and reads with at least a quality Phred score of 20 were filtered. The filtered reads were then assembled *de novo* using Megahit [29] into contigs of at least 500 bp. Ribosomal RNAs were predicted using Barrnap [30] and classified using RDP classifier [31]. Open reading frames

on the assembled contigs were predicted using prodigal [32] and then queried across the NCBI nr database (2 September 2023) using Diamond v2.0.8.146 [33] and HMMER3 [34]. The output is then visualized in Krona [35] and Metagenome Analyzer v6.24.22 [36].

The taxonomic tables at genus and class levels were exported for subsequent statistical analysis in R using *vegan* [37] and *phyloseq* [38] packages. Alpha and beta-diversity were both assessed using the Bray-Curtis dissimilarity metric. Alpha-diversity was analysed using Chao-1, Shannon (H) and Simpson (D) diversity indices. Taxa were collapsed at the Class level and imported into R. The microbiome of top 10 classes was plotted as stacked bar charts using *plotly* package [39] and the similarities between HHZ and Temasek rice were plotted in Venn diagrams using the *Venn Diagram* [40] package in R. Canonical correspondence analysis (CCA) plots were created to observe clustering patterns in relation to drip and flood irrigation. Differential taxa analysis was performed using the *MaAsLin2* package [41] with drip and flood as fixed factors using default parameters in R.

Total number of reads for gene and taxa across different datasets were rarefied and expressed as reads per kilobase per million mapped reads (RPKM). Heatmaps were generated from RPKM counts and converted to normalized Z-scores before plotting using *ggplot2* [42] package in R. The co-occurrence network analysis was performed using a R script adapted from Li, et al. [43]. The co-occurrence network was visualised using Gephi v0.9.2 [44]. To simplify the network graph, the top 100 genera were filtered and used. Using a cut-off score of Spearman Rho 0.6, p-value of 0.01 and clustering algorithm in Gephi, a network model consisting of 30 nodes and 25 edges in drip whereas 69 nodes and 147 edges topology in flood were created.

Soil redox potential measurements

A portable pH, ORP and temperature meter HI991003 (Hanna Instruments, Italy) was used to measure the soil redox potential. A hole of 15-20 cm depth was made using our modified soil

probes before inserting the ORP meter probe. The ORP probe was then left in the soil for 5 minutes to allow readings to become stabilized before recording it.

Analysis of root morphology

HHZ and Temasek rice plants were uprooted first by loosening the soil with running tap water and followed by a spade at the end of the growing season. Triplicates of rice plants of each species were chosen at random from drip and flood irrigation trays. Plants were laid side by side on a black canvas, with drip irrigated plants on the left and continuous flooded plants on the right and a 30 cm ruler in the middle. The images of the plant roots were captured using a Samsung S22 smartphone and imported into ImageJ [45]. 30 cm length on the ruler was used as the reference and measurements were converted to pixels to derive the actual root length of each plant root subject.

Data availability statement

Raw metagenomic sequencing data have been submitted to NCBI with the BioProject ID of PRJNA1193822 and accession numbers of SRX26959100 to SRX26959107. For more details, please refer to: https://www.ncbi.nlm.nih.gov/sra/PRJNA1193822.

The list of differential taxa in drip and flood-irrigated soil is available in the supplementary material as an excel spreadsheet.

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sequencing and computational analyses. This research was funded by grants from the Philanthropy Asia Alliance, the Bill and Melinda Gates Foundation, and the Temasek Life Sciences Laboratory, Singapore.

Author contributions

NIN, SR and KJXL designed the experiments and analysis framework. KJXL performed the soil collection, root imaging, DNA extraction, microbiome data analysis, bioinformatics, statistical analyses. Methane emissions analysis was performed by AM and CB. AM and TMS cultivated and maintained the rice plants. NIN supervised KJXL on both the research work, experimental design, data analyses and manuscript writing. NIN and KJXL analysed the microbiome data and co-wrote the manuscript. NIN, SR and Zhong Chao Yin were responsible for project funding. All listed authors have read and approved the manuscript.

Compliance with ethical standards

The authors declare that they have no conflict of interest.

FIGURES

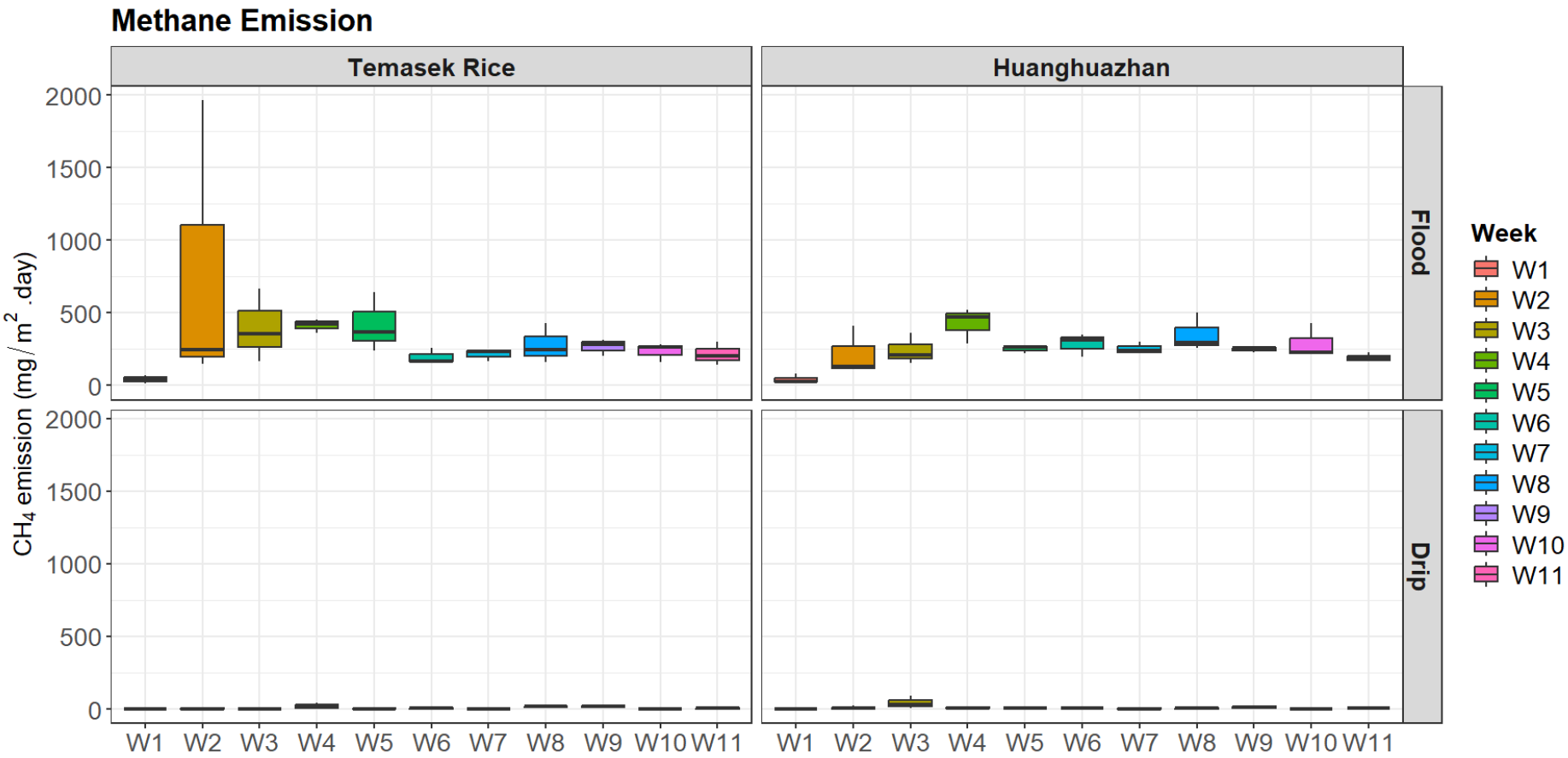

Fig 1 | Weekly methane emission profiles of Huanghuazhan and Temasek Rice. Methane gas measurements showed two peaks at Week 4 and Week 8 post-transplantation. There is a significant reduction in methane emissions when drip irrigation method was implemented.

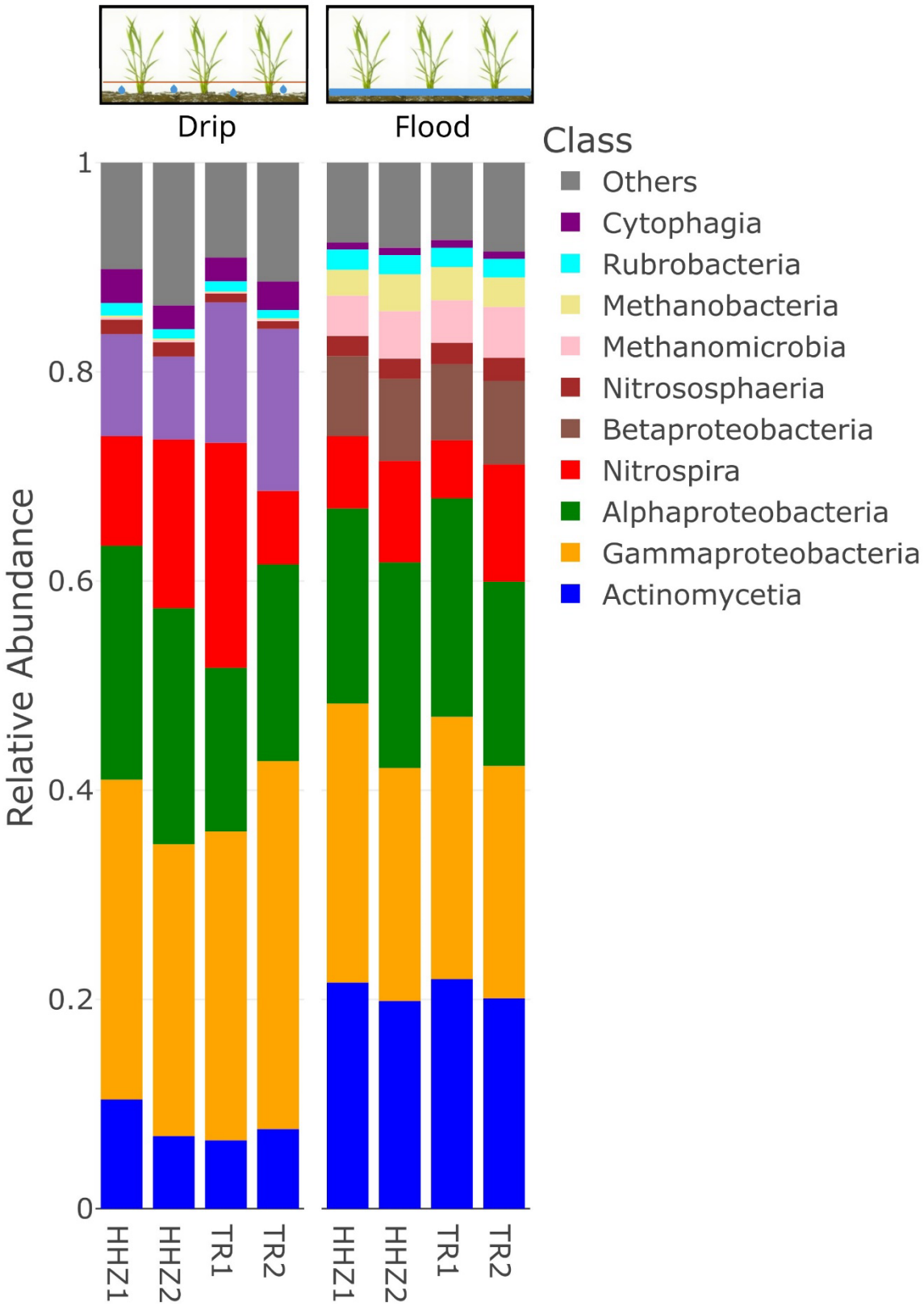

Fig 2 | Taxonomic stacked bar chart of top 10 taxa at the class level. Relative abundance of *Gammaproteobacteria*, *Nitrospira*, *Betaproteobacteria* and *Cytophagia* are higher in drip-irrigated rice soil whereas *Actinomycetia*, *Nitrososphaeria*, *Methanomicrobia* and *Methanobacteria* are higher in flooded soil.

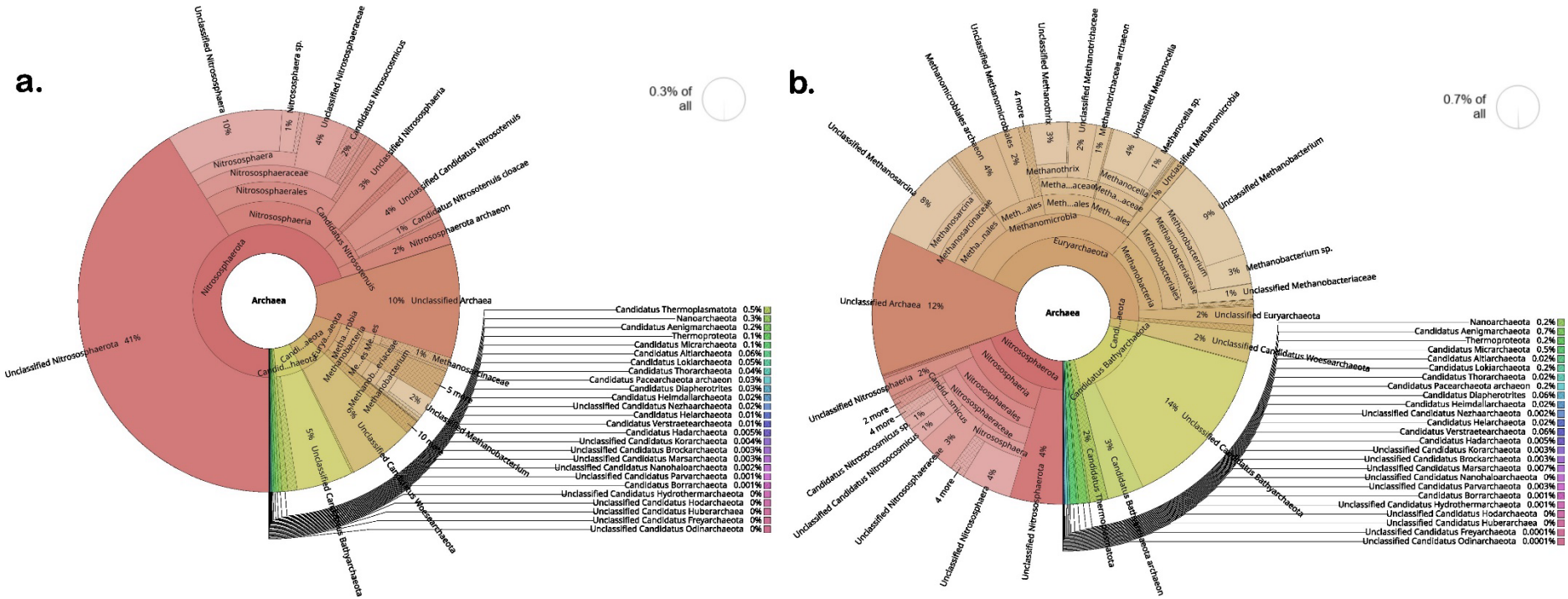

Fig 3 | Krona pie charts of relative abundance of archaea in HHZ rice soil microbiomes. a. Drip-irrigated soil has a lower abundance of methanogens and archaeal members only account for up to 0.3% of the microbiome. b. Flood-irrigated soil enriches for *Methanosarcina*, *Methanothrix*, *Methanobacterium* and *Nitrososphaeria*.

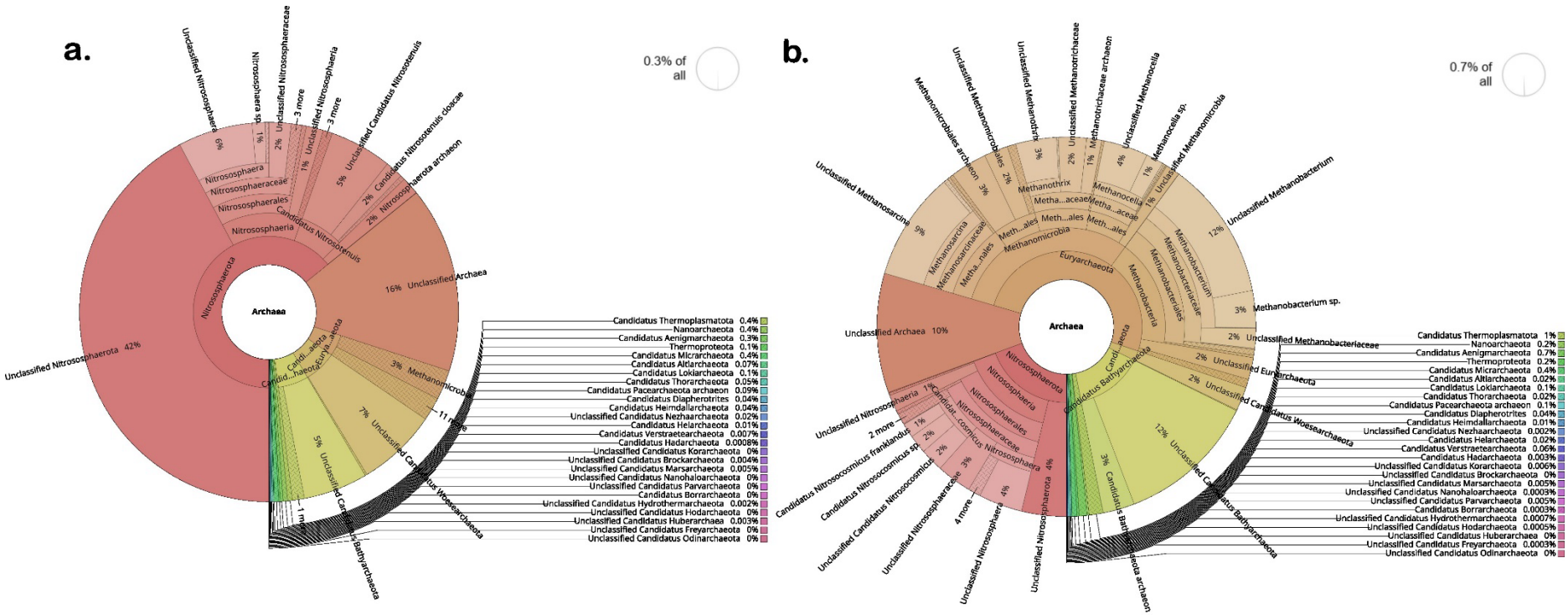

Fig 4 | Krona pie charts of relative abundance of archaea in Temasek rice soil microbiomes. a. Drip-irrigated soil has a lower abundance of methanogens and archaeal members only account for up to 0.3% of the microbiome. b. Flood-irrigated soil enriches for *Methanosarcina*, *Methanothrix*, *Methanobacterium* and *Nitrososphaeria* and archaea accounts for up to 0.7% of the microbiome. There is no significant difference between the microbiomes observed between HHZ and Temasek rice.

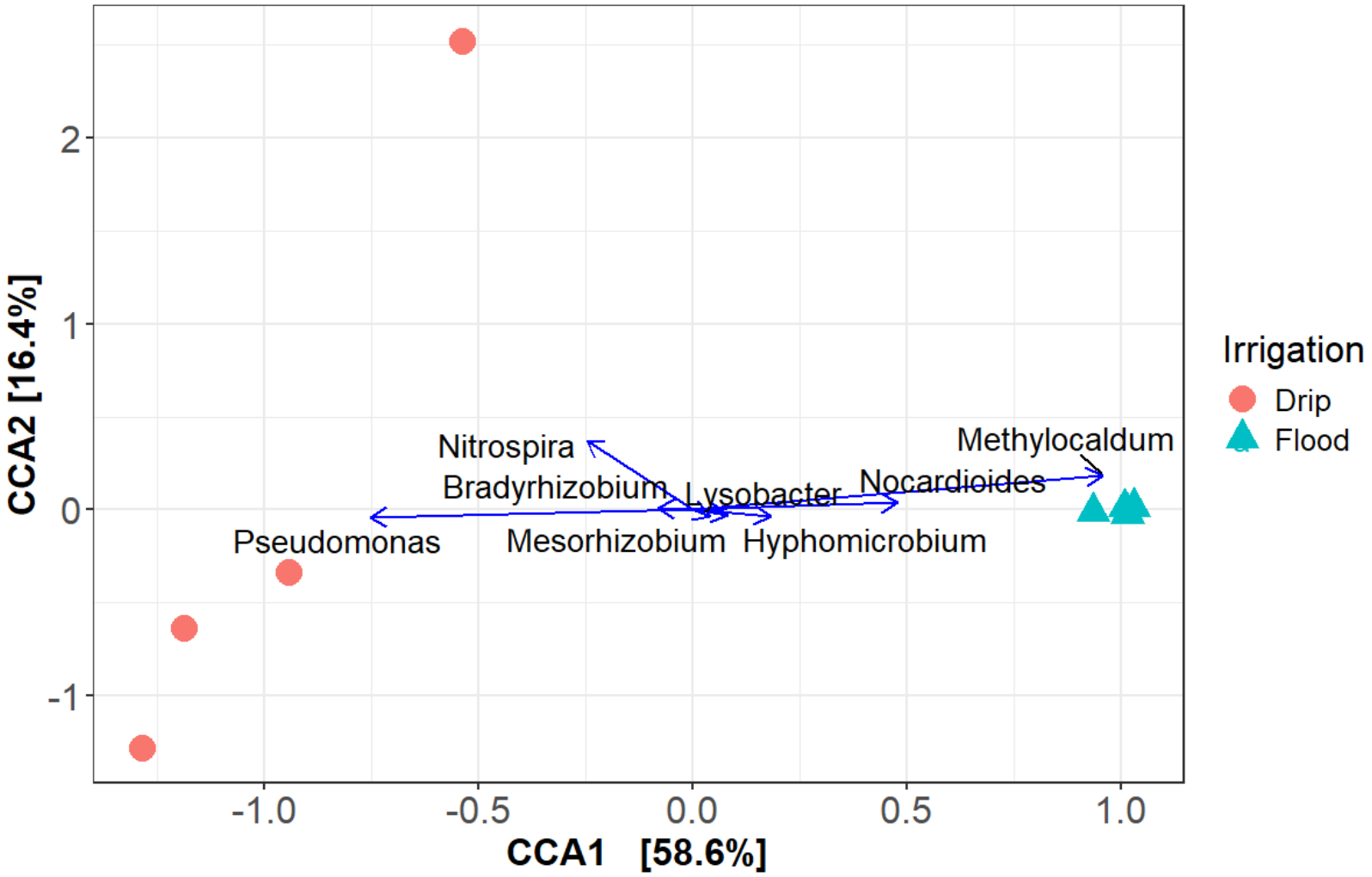

Fig 5 | Canonical Correspondence Analysis plot of rhizosphere microbiomes under drip and flood-irrigated conditions. *Nitrospira*, *Hydrogenophaga*, *Pseudomonas* and *Bradyrhizobium* are key driving taxa in the drip-irrigated soil while *Methylocaldum*, *Nocardiodes* and *Agromyces* are key taxa in the flood-irrigated soil.

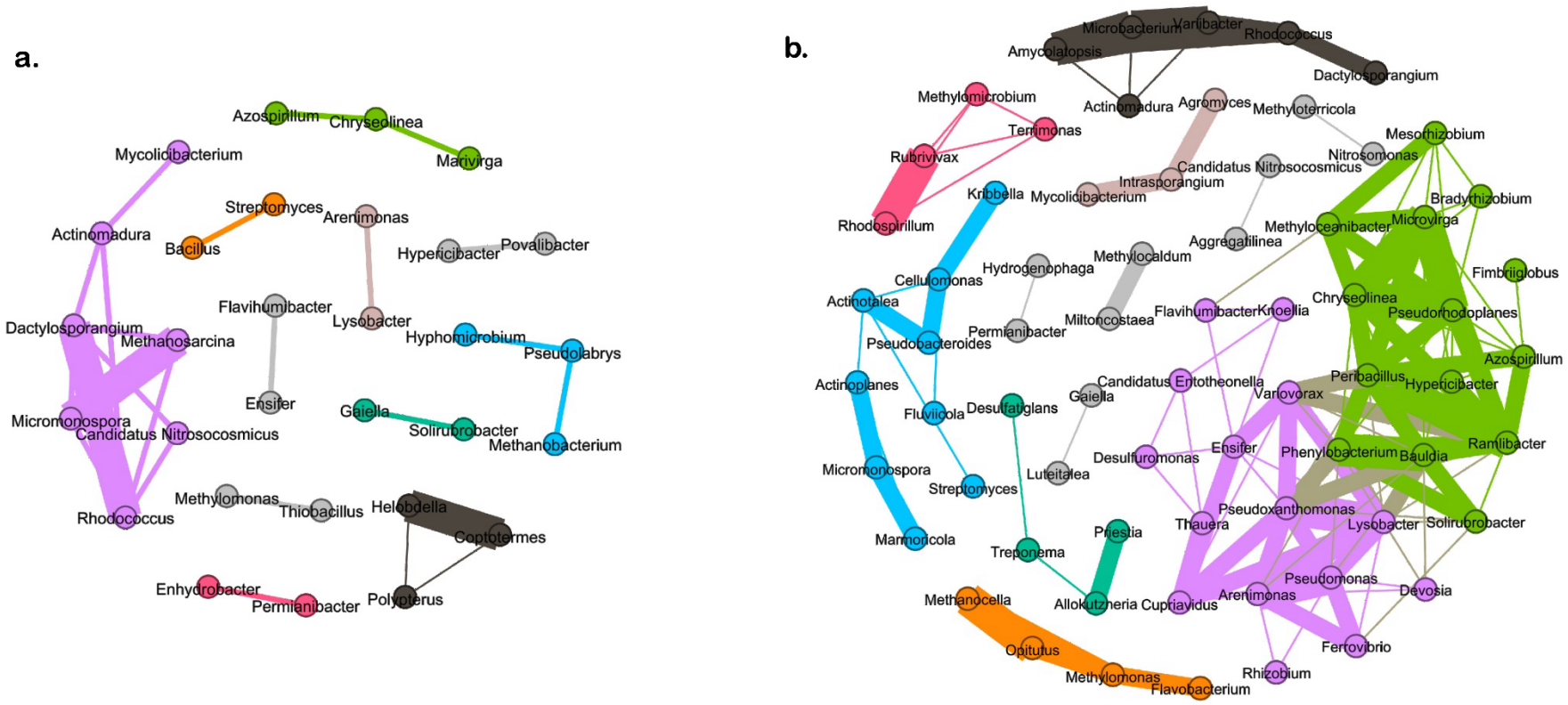

Fig 6 | Cooccurrence network graph of keystone taxa in the top 100 genera. a. Drip-irrigated soil shows an active lilac cluster consisting of *Micromonospora*, *Rhodococcus*, *Candidatus Nitrosocosmicus*, *Methanosarcina*, *Dactylosporangium*, *Actinomadura* and *Mycolicibacterium* b. Flood-irrigated soil shows more active microbial communities consisting of the lilac, lime, orange, cyan, pink and black clusters, where the orange corresponds to the methanogenesis-associated taxa and lilac, lime clusters are likely rhizosphere microbial taxa.

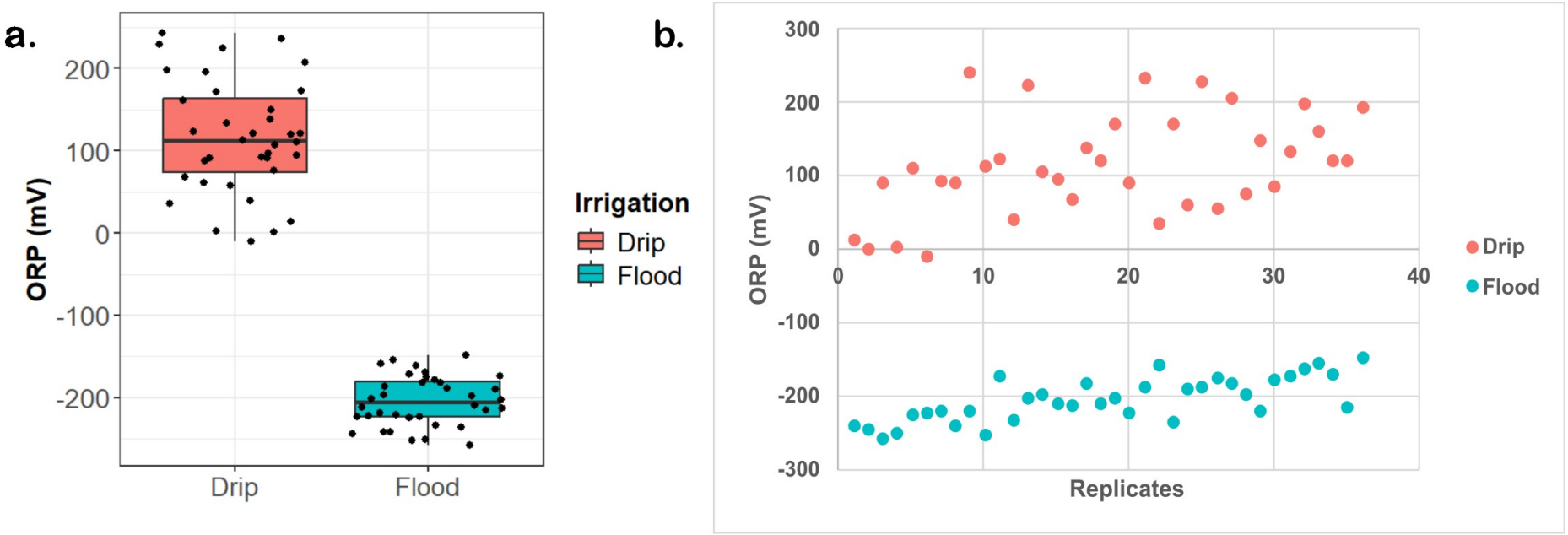

Fig 7 | Oxidation-Reduction Potential (ORP) measurements associated with Drip and Flood. a. Drip-irrigated soil typically yields positive ORP readings with a median of 111.5 mV whereas flood-irrigated soil typically shows readings in the negative range with a median of -205.5 mV. b. Drip and Flood ORP readings shown and plotted in a scatterplot.

SUPPLEMENTARY FIGURES

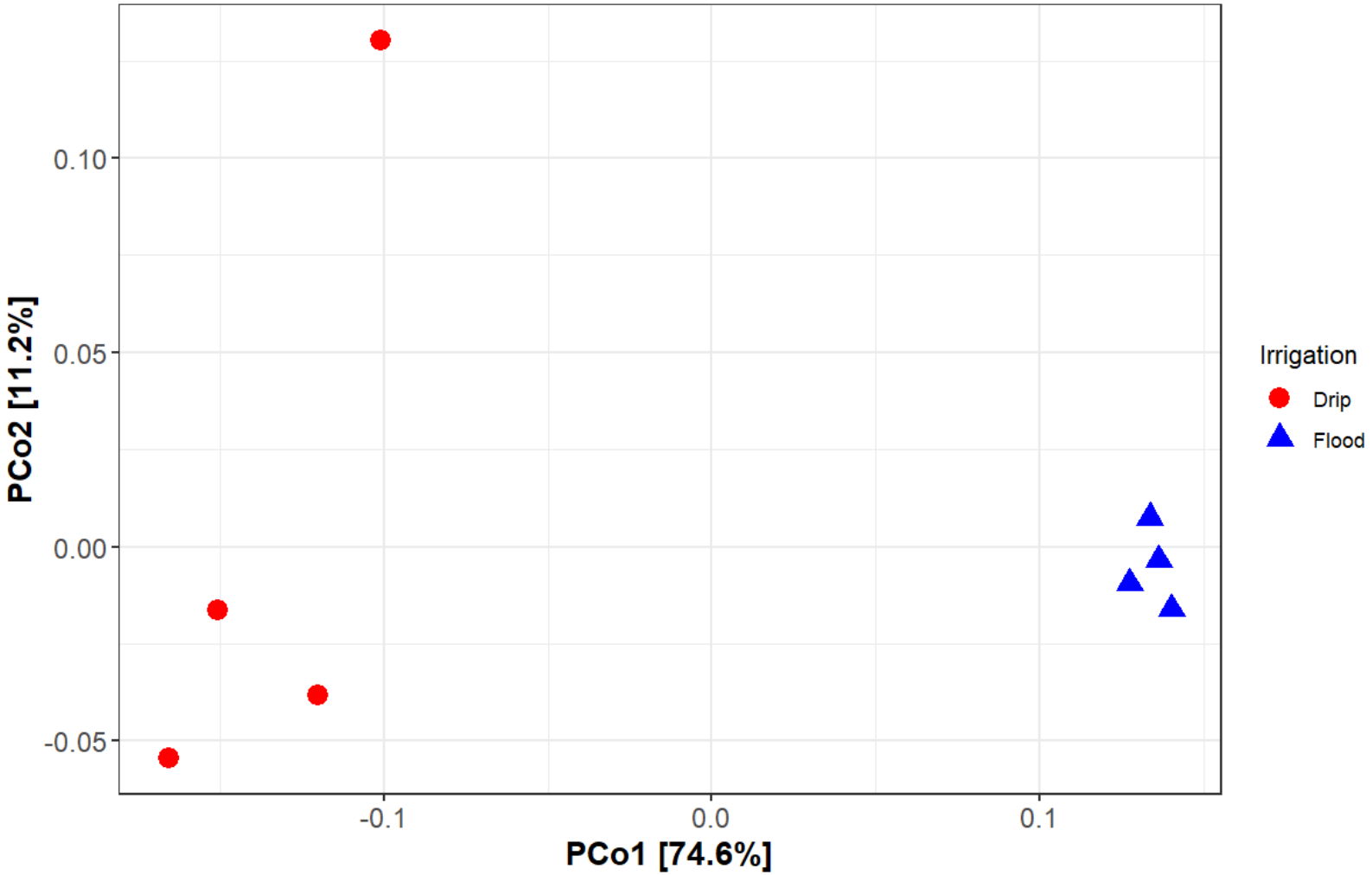

Fig S1 | Principal Coordinate Axis plot of drip and flood-irrigated soil microbiomes. Flood-irrigated soil samples are separated from the drip-irrigated soil along the first principal coordinate axis with 74.6% of variance explained.

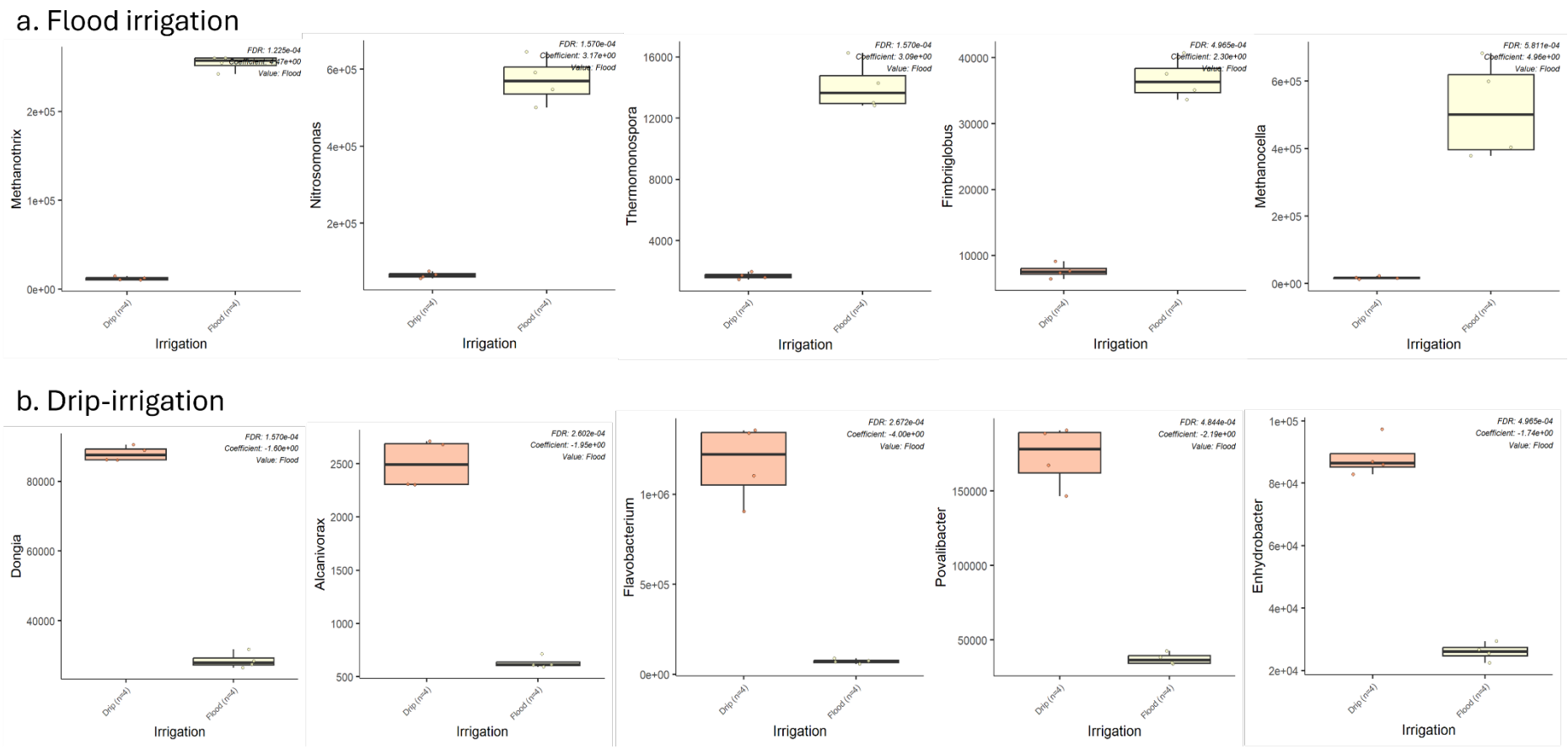

Fig S2 | Boxplot of top 5 taxa enriched in flood and drip-irrigated soil. *Methanothrix, Nitrosomonas, Thermomonospora, Fimbriiglobus* and *Methanocella* are differentially enriched in floodwater rice fields while *Dongia, Alcanivorax, Flavobacterium, Povalibacter* and *Enhydrobacter* are enriched in aerated drip soil.

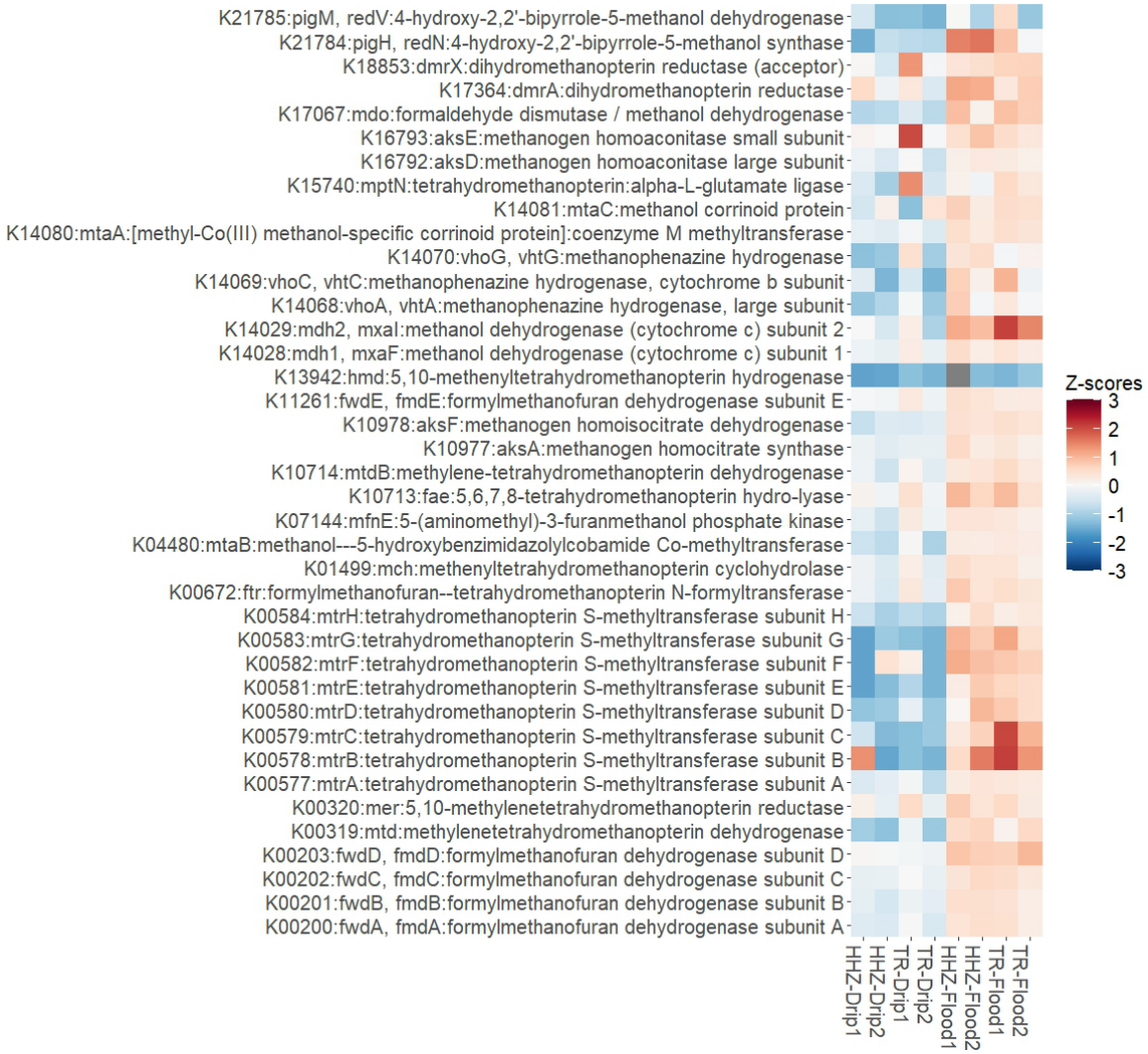

Fig S3 | Heatmap of methane-related metabolic pathway genes. Flood-irrigated soils have higher abundance of methane-related genes when reads were mapped to the KEGG database. These genes can be found in *Methanobacterium* and *Methanosarcina* as part of methane biosynthesis pathways.

Fig S4 | Heatmap of Nitrogen-related metabolic pathway genes. Flood-irrigated soils also tend to have higher abundance of nitrogen-related genes when reads were mapped to the KEGG database. This observation suggests that there could be higher levels of ammonia-utilization and nitrification microbial activities in the flooded environment.

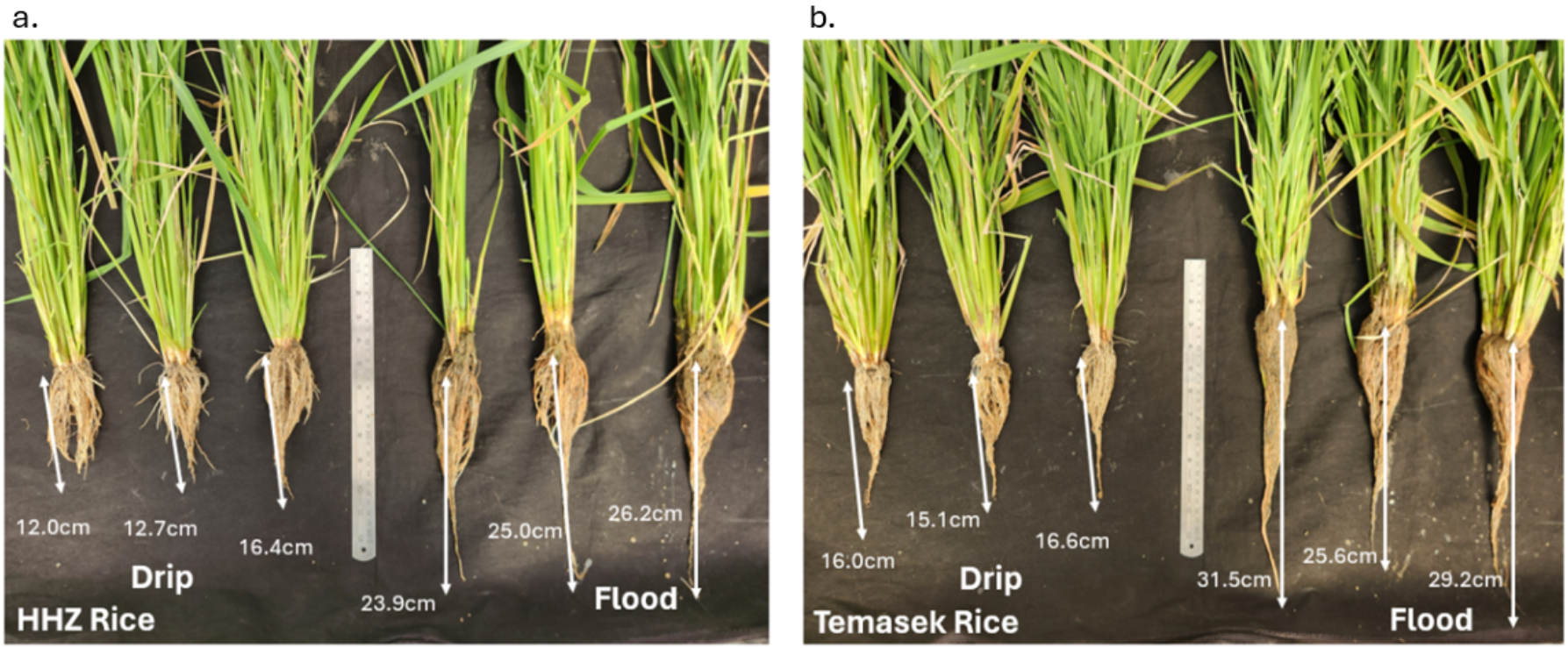

Fig S5 | Root length of drip and flood-irrigated rice plants. a. HHZ and **b.** Temasek rice arranged in triplicates with drip-irrigated plants on the left and flood-irrigated plants on the right. Drip-irrigated rice plants tend to have shorter roots than their flood counterparts.

Table S1 | Alpha diversity of soil microbiomes. Chao1 index indicated that there are higher number of species observed in flooded than drip soil. Additionally, Shannon and Simpson indices suggest that there is no difference in diversity between flooded and drip soil.

Variety	Irrigation	Replicate	Chao1	Shannon (H)	Simpson (D)
HHZ	Drip	1	1938.356	3.817144	0.941835
HHZ	Drip	2	1939.054	3.723193	0.935281
HHZ	Flood	1	2289.52	4.5047	0.973987
HHZ	Flood	2	2253.539	4.434425	0.972119
TR	Drip	1	1886.874	3.617447	0.929599
TR	Drip	2	1888.023	3.752185	0.933789
TR	Flood	1	2217.34	4.539406	0.975476
TR	Flood	2	2187.991	4.401419	0.970731

Table S2 | t-test statistics performed on the richness and diversity indices. Welch t-test were performed on Chao1, Shannon and Simpson indices for a. drip versus flood and b. HHZ versus Temasek rice.

a.

Group	Mean	t-statistic	df	p-value
Drip (Chao1)	1913.077	-12.212	5.2504	4.698e-05***
Flood (Chao1)	2237.097			
Drip (Shannon)	3.727492	-14.209	5.5976	1.323e-05***
Flood (Shannon)	4.469988			
Drip (Simpson)	0.9351260	-13.829	3.9808	0.0001633***
Flood (Simpson)	0.9730783			

b.

Species	Mean	t-statistic	df	p-value
HHZ (Chao1)	2105.117	0.45271	5.9819	0.6667
Temasek Rice (Chao1)	2045.057			
HHZ (Shannon)	4.119865	0.13758	5.9099	0.8951
Temasek Rice (Shannon)	4.077614			
HHZ (Simpson)	0.9558055	0.21736	5.8178	0.8354
Temasek Rice (Simpson)	0.9523987			

Table S3 | Root length measurements. The plant roots were traced using the line drawing tool in ImageJ to estimate the root length of a. HHZ and b. Temasek rice.

a.

Variety	**Replicate**	**Drip (cm)**	**Flood (cm)**
HHZ	1	12.0	23.9
HHZ	2	12.7	25.0
HHZ	3	16.4	26.2
Average		13.7	25.0
p-value (significant) $p < 0.05$			0.005607***

b.

Variety	**Replicate**	**Drip (cm)**	**Flood (cm)**
Temasek	1	16.0	31.5
Temasek	2	15.1	25.6
Temasek	3	16.6	30.4
Average		15.9	29.2
p-value (significant) $p < 0.05$			0.01417**

REFERENCES

1. Rezaei, E.E., Webber, H., Asseng, S., Boote, K., Durand, J.L., Ewert, F., Martre, P. and MacCarthy, D.S., 2023. Climate change impacts on crop yields. *Nature Reviews Earth & Environment*, *4*(12), pp.831-846.
2. Qian, H., Zhu, X., Huang, S., Linquist, B., Kuzyakov, Y., Wassmann, R., Minamikawa, K., Martinez-Eixarch, M., Yan, X., Zhou, F. and Sander, B.O., 2023. Greenhouse gas emissions and mitigation in rice agriculture. *Nature Reviews Earth & Environment*, *4*(10), pp.716-732.
3. Chauhan P, Sharma N, Tapwal A, Kumar A, Verma GS, Meena M, et al. Soil microbiome: Diversity, benefits and interactions with plants. Sustainability. 2023;15:14643.
4. Pathan SI, Ceccherini MT, Sunseri F, Lupini A. Rhizosphere as Hotspot for Plant-Soil-Microbe Interaction. In: Datta R, Meena RS, Pathan SI, Ceccherini MT, editors. Carbon and Nitrogen Cycling in Soil. Singapore: Springer Singapore; 2020. p. 17–43.
5. Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. Going back to the roots: the microbial ecology of the rhizosphere. Nat Rev Microbiol. 2013;11:789–99.
6. Ladha JK, Tirol-Padre A, Reddy K, Ventura W. Prospects and Problems of Biological Nitrogen Fixation in Rice Production: A Critical Assessment. In: Palacios R, Mora J, Newton WE, editors. New Horizons in Nitrogen Fixation: Proceedings of the 9th International Congress on Nitrogen Fixation, Cancún, Mexico, December 6–12, 1992. Dordrecht: Springer Netherlands; 1993. p. 677–82.
7. Edwards J, Santos-Medellín C, Nguyen B, Kilmer J, Liechty Z, Veliz E, et al. Soil domestication by rice cultivation results in plant-soil feedback through shifts in soil microbiota. Genome Biol. 2019;20:221.

8. Li D, Ni H, Jiao S, Lu Y, Zhou J, Sun B, et al. Coexistence patterns of soil methanogens are closely tied to methane generation and community assembly in rice paddies. Microbiome. 2021;9:20.
9. Ismaila U, Wada AC, Daniya E, Gbanguba AU. Meeting the local rice needs in Nigeria through effective weed management. Sustainable Agriculture Research. 2013;2.
10. Parthasarathi T, Vanitha K, Mohandass S, Vered E. Mitigation of methane gas emission in rice by drip irrigation. F1000Res. 2019;8.
11. Feng, K., Zhang, Y., He, Z., Ning, D. and Deng, Y. Interdomain ecological networks between plants and microbes. Molecular ecology resources. 2019;19(6):1565-1577.
12. Hu, Q., Tan, L., Gu, S., Xiao, Y., Xiong, X., Zeng, W.A., Feng, K., Wei, Z. and Deng, Y. Network analysis infers the wilt pathogen invasion associated with non-detrimental bacteria. npj Biofilms and Microbiomes, 2020;6(1):8.
13. Zhou S, Li H, Huang D, Lu D, Lai S, Zhou D, et al. Analysis on good characteristics and breeding achievements of Huanghuazhan—the core germplasm be planted as early, middle or late rice. J Agric Sci Technol. 2008;3:017.
14. Luo Y, Ma T, Zhang A, Ong KH, Li Z, Yang J, et al. Marker-assisted breeding of the rice restorer line Wanhui 6725 for disease resistance, submergence tolerance and aromatic fragrance. Rice. 2016;9:1–13.
15. Qian, H., Zhu, X., Huang, S., Linquist, B., Kuzyakov, Y., Wassmann, R., Minamikawa, K., Martinez-Eixarch, M., Yan, X., Zhou, F. and Sander, B.O. Greenhouse gas emissions and mitigation in rice agriculture. Nature Reviews Earth & Environment, 2023;4(10), pp.716-732.

16. Wang, Z.P., Lindau, C.W., Delaune, R.D. and Patrick, W.H., 1993. Methane emission and entrapment in flooded rice soils as affected by soil properties. *Biology and Fertility of Soils*, *16*, pp.163-168.
17. Zhang Z, Furman A. Soil redox dynamics under dynamic hydrologic regimes-A review. Science of the Total Environment. 2021;763:143026.
18. Drepper, T., Groß, S., Yakunin, A.F., Hallenbeck, P.C., Masepohl, B. and Klipp, W. Role of GlnB and GlnK in ammonium control of both nitrogenase systems in the phototrophic bacterium *Rhodobacter capsulatus*. Microbiology, 2003;149(8), pp.2203-2212.
19. Zhao, D., Curatti, L. and Rubio, L.M. Evidence for nifU and nifS participation in the biosynthesis of the iron-molybdenum cofactor of nitrogenase. *Journal of* Biological Chemistry, 2007;282(51), pp.37016-37025.
20. Salwan, R. and Sharma, V. The role of actinobacteria in the production of industrial enzymes. In New and future developments in microbial biotechnology and bioengineering. Elsevier. 2018;165-177.
21. Bhatti, A.A., Haq, S. and Bhat, R.A. Actinomycetes benefaction role in soil and plant health. Microbial pathogenesis. 2017;111:458-467.
22. Hanson, R.S. and Hanson, T.E. Methanotrophic bacteria. Microbiological reviews. 1996;60(2):439-471.
23. Gao X, Wu Z, Liu R, Wu J, Zeng Q, Qi Y. Rhizosphere Bacterial Community Characteristics over Different Years of Sugarcane Ratooning in Consecutive Monoculture. Biomed Res Int. 2019;2019:4943150.
24. Mathews A, Bauer WD, Brockwell J, Carroll BJ, Gault RR, Gresshoff PM. Rhizosphere colonization by Bradyrhizobium japonicum is related to extent of

nodulation of Glycine max CV. Bragg and its supernodulating mutants. Soil Biol Biochem. 1993;25:613–9.

25. Shi-Lei S, Wen-Long Y, Wen-Wan F, Yun-Xiu Z, Ling G, Yi-Jun D. The Plant Growth-Promoting Rhizobacterium Variovorax boronicumulans CGMCC 4969 Regulates the Level of Indole-3-Acetic Acid Synthesized from Indole-3-Acetonitrile. Appl Environ Microbiol. 2018;84:e00298-18.
26. Chaichana, N., Bellingrath-Kimura, S.D., Komiya, S., Fujii, Y., Noborio, K., Dietrich, O. and Pakoktom, T. Comparison of closed chamber and eddy covariance methods to improve the understanding of methane fluxes from rice paddy fields in Japan. *Atmosphere*. 2018; 9(9):356.
27. Whitham JM, Grunden AM. Trimming and decontamination of metagenomic data can significantly impact assembly and binning metrics, phylogenomic and functional analysis. Curr Bioinform. 2023;18:428–39.
28. Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016;32:3047–8.
29. Li D, Liu C-M, Luo R, Sadakane K, Lam T-W. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics. 2015;31:1674–6.
30. Seemann T. barrnap 0.9: rapid ribosomal RNA prediction. Google Scholar. 2013.
31. Lan Y, Wang Q, Cole JR, Rosen GL. Using the RDP Classifier to Predict Taxonomic Novelty and Reduce the Search Space for Finding Novel Organisms. PLoS One. 2012;7:e32491-.
32. Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics. 2010;11:119.

33. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015;12:59–60.

34. Prakash A, Jeffryes M, Bateman A, Finn RD. The HMMER Web Server for Protein Sequence Similarity Search. Curr Protoc Bioinformatics. 2017;60:3.15.1-3.15.23.

35. Ondov BD, Bergman NH, Phillippy AM. Interactive metagenomic visualization in a Web browser. BMC Bioinformatics. 2011;12:385.

36. Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. Genome Res. 2007;17:377–86.

37. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara RB, et al. Community ecology package. R package version. 2013;2:321–6.

38. McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One. 2013;8:e61217-.

39. Sievert, C., Parmer, C., Hocking, T., Chamberlain, S., Ram, K., Corvellec, M., Despouy, P. and Sievert, M.C. Package 'plotly'. R Foundation for Statistical Computing, Vienna. 2021.

40. Chen H, Boutros PC. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinformatics. 2011;12:35.

41. Mallick, H., Rahnavard, A., McIver, L.J., Ma, S., Zhang, Y., Nguyen, L.H., Tickle, T.L., Weingart, G., Ren, B., Schwager, E.H. and Chatterjee, S. Multivariable association discovery in population-scale meta-omics studies. PLoS computational biology, 2021;17(11), p.e1009442.

42. Villanueva RAM, Chen ZJ. ggplot2: Elegant Graphics for Data Analysis (2nd ed.). Measurement (Mahwah N J). 2019;17:160–7.

43. Li B, Yang Y, Ma L, Ju F, Guo F, Tiedje JM, et al. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. ISME J. 2015;9:2490–502.
44. Bastian M, Heymann S, Jacomy M. Gephi: an open source software for exploring and manipulating networks. In: Proceedings of the international AAAI conference on web and social media. 2009. p. 361–2.
45. Collins TJ. ImageJ for microscopy. Biotechniques. 2018;43.