

Effects of probiotic consortia on plant metabolites are associated with soil indigenous microbiota and fertilization regimes

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ABSTRACT

Introducing probiotics to soil is a sustainable way to stimulate the production of plant metabolites. However, the soil-resident microbes may compromise the efficiency of probiotics. To date, it remains challenging to integrate the effects of probiotics on plant performance with soil microbiome changes. Using *Cyclocarya paliurus* (Batal.) Iljinsk as a model medicinal plant and two types of probiotic consortia combined with organic fertilizer at three levels (low: 0.5, medium: 1.0, and high: 1.5 kg·plant⁻¹), we examined the impacts of three fertilization regimes (O: organic fertilizer, OMF: O coupled with *Bacillus megaterium* and *Pseudomonas fluorescens*, OCB: O coupled with *Azotobacter chroococcum* and *Azospirillum brasilense*) on plant metabolites and nutrient stoichiometry after three-year applications and identified the key soil microbes relating to the accumulation of plant metabolites via generalized joint attribute model (GJAM) analysis. Our results indicated that the concentration of flavonoids reached 36.9 mg·g⁻¹ in OCB treatment at a low level, and 30.0 mg·g⁻¹ in OMF treatment at a medium level, both were significantly higher than that in O treatment (25.8 mg·g⁻¹ on average). Furthermore, the accumulations of metabolites were associated with plant nutrient acquisition and C: N: P stoichiometry. GJAM analysis showed that higher fertilizer levels restricted the influence of probiotic consortia on the variance of plant-soil-microbe system, with fewer differences observed between fertilizer types. Specific soil microbes were predicted as potential indicators that may assist or impede the effects of probiotics on plant metabolite production. The predictions were further tested in a comparative pot experiment, and the effects of common indicators in both pot and field experiments were consistently associated with probiotics' addition. This study reveals that the effects of probiotics on plant metabolites are associated with fertilization regimes and soil-indigenous microbes. Identifying microbial indicators will help to understand the probiotics' effects and further improve plant productivity.

1. Introduction

Bioactive compounds extracted from medicinal plants' leaves, fruits, or roots are a major source of natural pharmaceuticals for developing medicinal drug products (Hussain et al., 2012). Chemical fertilizers have been used to improve the yield of bioactive compounds but have considerable negative impacts on the environment (Deng et al., 2019a). Recent research revealed that plant growth and metabolite content are dependent on complex interactions with the biotic environment, including a close metabolic interplay with associated microorganisms in the rhizosphere, phyllosphere, and endosphere (Etalo et al., 2018;

Huang et al., 2018; Ravanbakhsh et al., 2018; Wang et al., 2021b). Thus, reshaping the phytobiome could be a sustainable pathway to manipulate plant metabolic performance, while reducing the use of chemical fertilizers (Xiong et al., 2017; Berg et al., 2020).

Until now, reshaping the phytobiome to stimulate plant growth and metabolism remains challenging. Firstly, soil microorganisms have shared a long evolutionary history with plants (van der Heijden et al., 2015). Plants naturally assemble specific communities of microorganisms that colonize plant surfaces and the endosphere and intimately relate to the changes in plant performance (van de Mortel et al., 2012; Huang et al., 2014; Etalo et al., 2018). Thus, reshaping the phytobiome

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may lead to unknown and undesired changes in the plant-soil system. Secondly, due to the multiple players involved, elucidating how microorganisms elicit a specific plant phenotype requires an in-depth understanding of those complex interactions. An incomplete analysis that fails to integrate plant and soil parameters and other environmental factors with microbiome data may mislead our management strategies (Sohn and Li, 2018; Leite and Kuramae, 2020). Thus, selecting a sustainable management strategy for promoting plant performance requires comparative analytical frameworks and experimental validation.

Firstly, to select the sustainable management strategy, it is important to use the natural plant-microbe interactions by inoculating beneficial microbes as biostimulants in plant-soil systems to reshape plant metabolic activities. For instance, phyto-stimulatory strains like *Azospirillum* sp. and *Pseudomonas* sp. participate in synergistic metabolic activities with the host plant to increase the biosynthesis of certain secondary metabolites (Walker et al., 2012; del Rosario Cappellari et al., 2013). Nutrient-enhancing strains like *Bacillus* sp. and *Azotobacter* sp. have been used to modulate plant metabolite production by improving soil nutrient availability and plant nutrient acquisition and balance (Deng et al., 2019b; Latef et al., 2020; Liu et al., 2020b). Although these probiotics have been increasingly used for improving plant performance, the resident microbes are sensitive to external biotic disturbances and may comprise the effect of probiotics on plants. Hence, their impacts on the resident soil community and how the inoculant-resaped microbiome affect the use of microbial inoculation should be investigated when evaluating the effects of probiotics (Xiong et al., 2017; Mawarda et al., 2020; Pagnani et al., 2020).

Secondly, to unravel the tripartite relationships among introduced beneficial microorganisms, the resident soil microbiome, and plant metabolic performance, an appropriate analysis method must handle different sources of bias and integrate all available information. Several studies established a correlation between microbial inoculants and plant metabolites and emphasized the importance of the soil microbiota (Schmidt et al., 2014; Huang et al., 2018; Liu et al., 2020b). However, incomplete analysis, such as the linear correlation analysis, fails to integrate plant and soil parameters with microbiome data, thus may lead to biased results (Sohn and Li, 2018). In particular, our previous study (Leite and Kuramae, 2020) and Gloor et al. (2017) caution that the interactions between microbes and other variables should be represented within a model-based approach. Because of the compositional peculiarity of microbial community data, correlations between the relative abundance of specific taxa and environmental factors might induce false significant relationships (Gloor et al., 2017). Therefore, a model-based approach should be the choice when identifying which microorganisms played crucial roles in regulating plant performance.

Here we choose the *Cyclocarya paliurus* (Batal.) Iljinsk, a woody medicinal plant belonging to the *Juglandaceae* family (Fang et al., 2011), as the model medicinal plant. Bioactive compounds extracted from *C. paliurus* leaves were mainly flavonoids, triterpenoids, and polysaccharides with antidiabetic, antioxidant, and antimicrobial effects (Fu et al., 2015; Wu et al., 2017). Our preliminary studies have showed that co-inoculation of phyto-stimulatory and nutrient-improving microbial strains survived in the *C. paliurus* plantation soil and better stimulated the production of plant metabolites than the application of a single inoculant (Wang et al., 2019a, 2019b, 2021b). The diversity of effects suggests that co-inoculating multiple microbial strains provide an increased range of benefits to the host plant (Bharti et al., 2016), but our study only investigated for one year and did not include the role of the soil microbiome. Additionally, the continuous introductions of phyto-stimulatory and nutrient-enhancing probiotics may affect the resident microbiome and their interactions can further regulate host performance, especially metabolite production, which remains largely unexplored.

We hypothesized that introduced probiotic consortia could change the plant metabolites and growth performance by affecting the plant nutrient uptake. At the same time, the effects are dependent on the

probiotic type and levels. We also hypothesized that several crucial microorganisms in the resident microbial community are associated with the introduced probiotic consortia to stimulate the production of plant metabolites. To test these hypotheses, we used integrated analysis, which allowed us to identify the most relevant factors and microbes. Once we have that, we can move to more controlled conditions and test the analysis. Following these principles, we performed two different experiments. First, a field experiment was conducted on a *C. paliurus* plantation for three years to assess the effects of co-inoculating phyto-stimulatory and nutrient-improving probiotics in combination with organic fertilizer at three different doses. Then, we integrated all the data with the help of a model-based approach to identify the key factors and microbes relating to the accumulation of secondary metabolites in *C. paliurus*. After that, a comparative analytical pot experiment was conducted to test our findings of key microbes from the field experiment.

2. Material and methods

2.1. Preparation of plant probiotics and organic fertilizer

Plant probiotics, *Bacillus megaterium* W17 (M), *Azotobacter chroococcum* HKN-5 (C), *Azospirillum brasilense* CW903 (B), and *Pseudomonas fluorescens* W12 (F), were used as dual combinations (M and F; C and B) and applied with organic fertilizer in this study. M and C have been categorized as nutrient-enhancing bacteria; B and F have been categorized as phyto-stimulatory bacteria (see Supplement Fig. S1). Our previous studies selected these combinations where these strains increased soil nutrient availability and plant growth without antagonistic effects (Wang et al., 2019b, 2021a, 2021b). The preparation of each inoculant was as described previously (Wang et al., 2021a). The organic fertilizer is mainly composed of chicken manure, straw, tea residue and mushroom residue; the chemical properties of the fertilizer material are provided in Supplement Table S1.

2.2. Field experimental design

The field site, soil properties, and experimental design were described in a previous study (Wang et al., 2021b). Briefly, the field plantation site is a typical subtropical land where the soil is classified as yellowish-brown clay soil with heavy texture and a serious shortage of organic matter. We set three types of fertilizer (O: organic fertilizer; OCB: organic fertilizer applied with *A. chroococcum* and *A. brasilense*; OMF: organic fertilizer applied with *B. megaterium* and *P. fluorescens*) and three levels for fertilization (low, medium, high: 10^7 , 10^8 , 10^9 cells per plant and, 0.5, 1.0, 1.5 kg organic fertilizer per plant, respectively), totaled nine fertilization regimes (O1, O2, O3, OCB1, OCB2, OCB3, OMF1, OMF2, OMF3) and one control (non-fertilized) in the field. The levels of organic fertilizer and probiotic consortia were set according to our previous results (Wang et al., 2021b). We found distinct patterns among three levels after one-year-old application. The factorial completely randomized block design was used for the field experiment. At least 60 seedlings of *C. paliurus* were used for each fertilization regime and were equally divided into three blocks. Detailed information is provided in Table S2 and a conceptual map of the field experiment is provided in Supplement Fig. S1.

All organic fertilizers were only applied in March 2016 to establish a better soil environment for subsequent applications of probiotic inoculations. The inoculations were conducted over a period of three years: May and July 2016, June and August 2017, and April, May, July, and August 2018. In the first two years, the inoculations of probiotics aimed to increase the population of probiotics during the plants' growing season. The inoculations in the last year were applied to be consistent with the inoculation date in the pot experiment (see 2.9.2 for detailed information), which is used to validate the results obtained in the field. An interval of 45 days between inoculations in the same year was selected based on bacterial growth curve data and the effects of the

inoculations on soil properties (Wang et al., 2019b). The detailed information on organic fertilization and microbial inoculation procedure were presented in our previous study (Wang et al., 2021b). Briefly, a 20-cm-deep circle was dug around the plant's vertical canopy projection as the fertilizer zone to better access the lateral roots. The same procedure was carried out for the control plants without fertilization. Then, the organic fertilizer was spread and the inoculants were sprayed in this zone. The resident microbiome in the non-fertilized soil collected at the end of the experiment is presented in Supplement Fig. S2.

2.3. Net growth of plant height and stem basal diameter

Plant height and stem basal diameter of all healthy seedlings (60 plants for each replicate) were measured in March 2016 and September 2018. The differences between the initial and final measured values were calculated as the net growth of plant height and stem basal diameter.

2.4. Soil sampling

In September 2018, soils were vertically sampled at a 0–20 cm depth in the fertilizer zone using the hole-sampling method (Wang et al., 2019a). Briefly, five vertical soil holes (diameter: 5 cm; depth: 20 cm) were conducted by a sampling tube for the same three seedlings selected for leaf sampling in each treatment. Plant residues and stones in the soil samples were carefully removed and all samples for each treatment in each block were pooled equally and mixed thoroughly to form a composite sample. This resulted in three replicate samples for each treatment. A portion of each sample (about 100 g) was stored at 4 °C until the analysis of biochemical properties (soil total C, N and S contents, soil available N and P contents, acid phosphatase and nitrogenase activities), and another portion (about 20 g) was stored at – 20 °C prior to DNA extraction.

2.5. Leaf sampling and C:N:P stoichiometry in leaves

Plant leaf sampling method and the determination of C:N:P stoichiometry in leaves have been described before (Wang et al., 2021b). Briefly, in September 2018, fresh and fully developed leaf materials were harvested in the same direction of the upper-middle part for three healthy trees in each treatment of each block. All samples were dried, grounded, and stored at room temperature to analyze total C, N, and P contents and bioactive compounds. Then, we used an elemental analyzer (vario MAX CN, Elementar, Hanau, Germany) to determine the total C and N content. The molybdenum-blue method was used to measure total P content. The C/N, C/P, and N/P ratios were subsequently calculated.

2.6. Bioactive compounds in *C. paliurus* leaves

The concentrations of flavonoids, triterpenoids, and polysaccharides were extracted from *C. paliurus* leaves with the methods described in our previous study (Wang et al., 2021b). Briefly, the flavonoids and triterpenoids were extracted from leaves with ethanol (75%) and determined using a colorimetric method at 415 nm for flavonoids (Bao et al., 2005) and 548 nm for triterpenoids (Fan and He, 2006), respectively. Water-soluble polysaccharides were extracted from *C. paliurus* leaves with water and determined by the phenol-sulfuric acid method (Fu et al., 2015).

The total yields of these bioactive components in leaves were calculated by multiplying the concentration by the biomass of leaves.

2.7. Soil DNA extraction, PCR and Illumina MiSeq sequencing

Total soil DNA extraction method and PCR procedures were described before (Wang et al., 2021a). Briefly, the primers 515 F (5'-GTGCCAGCMGCCGCGG-3') and 907 R (5'-CCGTCATTCMTT

TRAGTTT –3') were used to amplify the V4 regions. PCR was performed in triplicate in a 20-μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The PCR products were extracted from a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The purified amplicons were pooled in equimolar ratios and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, California, USA).

2.8. Sequence data processing

Raw DNA sequences were processed with the DADA2 (Divisive Amplicon Denoising Algorithm 2) pipeline (Callahan et al., 2016) using the "dada2" package (v.1.7) in R 4.0.1 (R Core Team 2019). DADA2 infers true biological sequences of reads (i.e., amplicon sequence variants [ASVs]) from Illumina sequence data and does not involve sequence clustering (Callahan et al., 2017). Briefly, raw sequences were first demultiplexed by comparing index reads with a key and then quality-filtered using the filterAndTrim function. Primers and adapters were screened and removed using the "cutadapt" package (v.2.10). Error rates were subsequently derived from subsampled reads (i.e., 1 million random reads). Sequences were dereplicated, and unique sequence pairs were denoised using the "dada" function. Finally, paired-end sequences were merged, and chimeras were removed. After filtering and trimming the sequence data, 8796 denoised ASVs were recovered from the 30 experimental samples. Taxonomy group information was then assigned using the SILVA databases (v.138) (Quast et al., 2013).

2.9. Statistical analyses

The effects of the different fertilization regimes on plant growth and nutrient acquisition were tested by the two-way ANOVA for significance (Assaad et al., 2015). Duncan's test was used to compare if the effects between treatments were significantly different ($p < 0.05$). The median concentrations and yields of bioactive compounds in *C. paliurus* leaves were calculated within each fertilizer type to compare the differences between fertilizer levels and within each fertilizer level to compare the differences between fertilizer types, to make it more visualized in the plot than significance letters. Polynomial regression analysis was used to examine the relationship between bioactive compounds and C/N and C/P ratios, and the results were plotted using the "ggplot2" package. The fitness of the regression curve was determined by R^2 values. To test the significance of a polynomial model, we used the F-test to compare if a significantly better fit was found in the polynomial model than in the intercept-only model.

To analyze the responses of soil bacterial alpha diversity to different fertilization regimes, "observed", "Shannon", "InvSimpson", and "Fisher" were selected and plotted by the plot_richness function in the "phyloseq" package (McMurdie and Holmes, 2013). Beta diversity was visualized by non-metric multidimensional scaling (NMDS) analysis based on Bray-Curtis distance calculated by the ordinate function in the "phyloseq" package. The resident microbiome in soil that did not receive any fertilizer was analyzed after Centered Log-Transformation (CLR). The whole community composition was plotted using the plot_composition function in the "phyloseq" package. Linear discriminant analysis (LDA) was subsequently applied to identify potential bioindicator taxa that differentiate the effects of different fertilization regimes.

2.9.1. Integrated analysis with the joint modeling approach

To examine the impacts of the different fertilization regimes on the whole plant-soil-microbe system and to identify the underlying contributors to the observed patterns, we carried out generalized joint attribute model (GJAM) analysis in the "gjam" package (v.2.3.2) developed by Clark et al. (2017). This method has been described in detail before (Clark et al., 2017; Leite and Kuramae, 2020) and is widely used

for microbiome analysis (Bossolani et al., 2021) and species prediction (Zhang et al., 2018). From this model, we extracted the effects of the different factors and obtained the regression coefficients that described the impact of the fertilization regime on all plant-soil variables. Detailed information about the modeling process and model fit are provided in the [supplementary method](#).

Principle component analysis (PCA) was applied to examine how the different fertilization regimes affected the whole system and identify the top variables contributing to the variance of the regression coefficients from the model analysis. As a supplement, a Sankey plot was created using the SankeyDiagram function in the "flipPlots" package based on the coefficients to assess how fertilizer type and level affected the plant and soil variables and microbial communities in the system. We compared the coefficients between different fertilizer types and levels to estimate the effects of different fertilization regimes on the top 10 microbial contributors identified by GJAM. The results were visualized using the ggplot function.

2.9.2. Comparative analytical framework for identification and test of microbial indicators

Biotic interactions have been rarely included in traditional species distribution models, where the joint species attribute model provides a new approach to integrating environmental factors and interspecific interactions simultaneously (Clark et al., 2017; Zhang et al., 2018; Leite and Kuramae, 2020). Hence, the GJAM model was further used to predict the key microbial indicators that affect the production of plant metabolites. Briefly, we set the flavonoid or triterpenoid condition as the maximum concentration. We then used the gjamPredict function to calculate the difference between the predicted and observed soil microbes' values. Those microbes with a positive value of difference were deemed as microbial assistants, which means they may assist the probiotics in improving plant metabolite production. Those microbes with a negative value of difference were deemed as microbial competitors, which may impede the probiotics from improving plant metabolite production. In addition, the impacts of fertilization regimes on these indicators are evaluated.

A comparative pot experiment was conducted to identify the microbial indicators held in common between field and pot experiment. We collected soils from the field, conducted a pot experiment using the same materials, and harvested the soil and plant samples for further comparative analysis. More detailed information concerning the pot experiment's design and sampling method of this was described before (Wang et al., 2021a) and summarized in the [supplementary material](#) (Supplement Fig. S3). We further tested the potential correlations between the common microbial indicators and plant flavonoids via GJAM analysis and evaluated the impact magnitude (quantified by coefficients in the model) of microbial indicators in the pot experiment. Then, we examined how probiotics affected the relative abundances of microbial assistants and microbial competitors, which may link to the use of probiotics in stimulating the accumulation of plant flavonoids.

3. Results and discussion

3.1. Plant growth and nutrient acquisition in response to probiotic inoculation

Inoculation with plant probiotics increased net plant growth in height and stem diameter, but this effect was highly variable across the different fertilizer types and levels (Supplement Table S3). For instance, O2 (O treatment at medium fertilizer level), OCB2 (OCB treatment at medium fertilizer level), and OMF2 treatments (OMF treatment at medium fertilizer level) all significantly improved net growth as measured by plant height compared to the control, and OCB2 and OMF2 exhibited significant advantages over O2 ($p < 0.05$). However, increasing the fertilizer did not promote plant growth. For example, the net growth of plants in treatment OCB3 and OMF3 were lower than that in OCB2 and

OMF2, respectively.

A similar pattern has been reported before (Kumar et al., 2020) in which a medium dose of microbial inoculants caused the maximum plant height, but the highest level reduced plant growth. Plants might lessen reliance on the beneficial microbes when a rich-nutrient environment established with the increasing fertilizing level (Altieri and Nicholls, 2003). An appropriate level of organic matter can establish a better growth environment for probiotics by changing the soil's C/N ratio and related enzyme activity (Wang et al., 2021b). This is also supported by the results of nutrient acquisition, in which OCB2 and OMF2 treatments increased leaf nitrogen (N) content compared to the control, and most of the fertilization regimes increased leaf phosphorus (P) content ($p < 0.05$, Supplement Table S3). As a result, changes in the C/N and C/P ratios were also observed, with lower ratios in the treatments with higher leaf contents of N and P, such as OCB2, OCB3, and OMF2. No significant effects of the treatments were found for N/P ratios.

3.2. Effects of different fertilization regimes on the accumulation of bioactive compounds in *C. paliurus* leaves

Compared to organic fertilization alone (O), the introduction of probiotics at specific fertilizer levels increased both the concentration and yield of flavonoids and triterpenoids (Fig. 1a, b, d, e), but no significant effects on polysaccharides were observed (Fig. 1c, f). For instance, OCB1 and OMF2 treatments increased flavonoid levels and OCB1 and OCB2 treatments increased triterpenoid levels. The concentration of total flavonoids reached $36.9 \text{ mg}\cdot\text{g}^{-1}$ in OCB1 and $30.0 \text{ mg}\cdot\text{g}^{-1}$ in OMF2, which was significantly higher than that in O treatment ($25.8 \text{ mg}\cdot\text{g}^{-1}$ on average).

Co-inoculation with phyto-stimulatory and nutrient-enhancing probiotics can induce the plant's physiological responses and change secondary metabolisms (Karthikeyan et al., 2009; Bharti et al., 2016; Wang et al., 2016). On the other hand, the effect of organic fertilizer on soil C/N ratio may also influence microbial activities and plant metabolism (Deng et al., 2019b; Jasso-Flores et al., 2020; Latef et al., 2020). Although the fertilizer level is important, the optimal densities of beneficial microbes to achieve plant metabolites' productivity may differ depending on the probiotic type (Bashan, 1986; Haas and Defago, 2005). This explains the results in Fig. 1 that fertilizer type and level influenced the accumulation of total flavonoids, and triterpenoids in *C. paliurus* leaves.

However, plants are more likely to accumulate secondary metabolites under stressed conditions, and a higher fertilizer level may enrich the nutrient environment (Wang et al., 2021b) and thus reduce the allocation of nutritional resources to the secondary metabolism (Deng et al., 2019b). As supported in the present study, the concentration and yield of flavonoids significantly decreased in the OMF3 treatment compared to OCB1 and OMF2. Hence, to increase the plant's medicinal value, it is important to select appropriate fertilization strategies to enhance the yield of target metabolites in the leaves.

In addition to the effect of fertilizer type and level, a strong relationship between leaf nutrient stoichiometry and bioactive compound accumulation was observed in our study: the highest production of flavonoids and triterpenoids was observed at medium C/N (approximately 24) and C/P (approximately 280) ratios (Fig. S4). However, no clear patterns were observed for relating polysaccharide accumulation with *C. paliurus* leaf nutrient stoichiometry, nor were there significant correlations between the N/P ratio and bioactive compounds. Effects of the internal nutrient balance on plant secondary metabolite production have been reported previously (Lillo et al., 2008). C, N and P are central to nearly all biochemical pathways in plants, so plant nutrient stoichiometry somehow determines both primary plant growth and secondary metabolism (Gigolashvili and Kopriva, 2014; Canovas et al., 2018). Our findings partially agree with the predictions of the growth-differentiation balance (GDB) hypothesis (Stamp, 2003, 2004), which states that plants allocate more resources to secondary

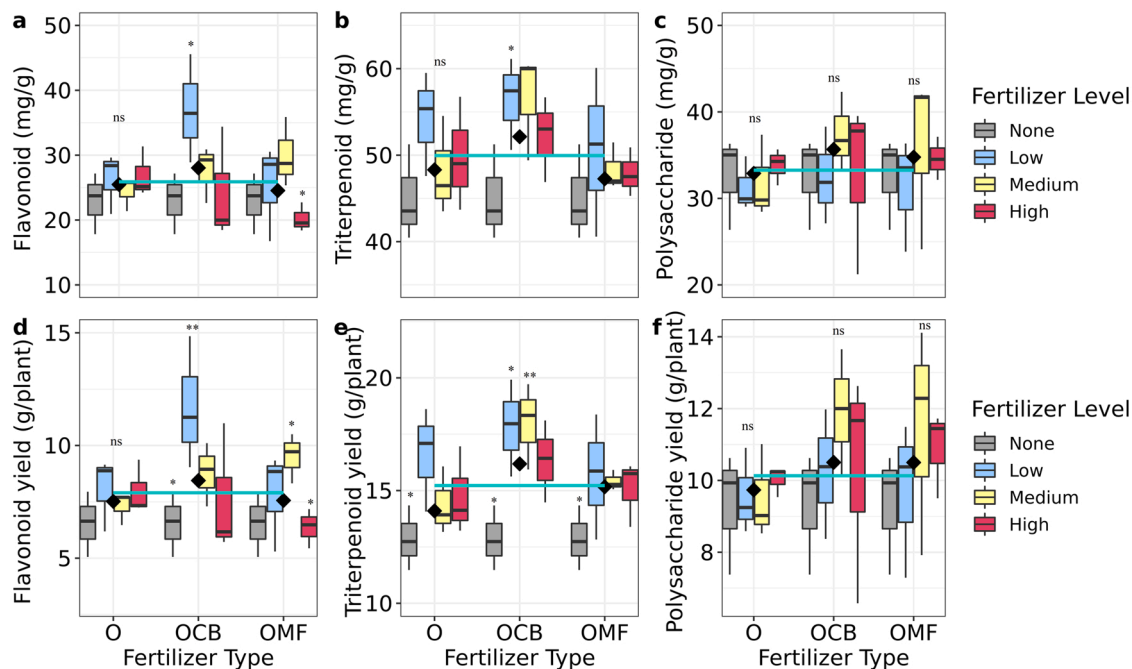


Fig. 1. Effects of different fertilization regimes (type and level) on the total concentrations of flavonoids (a), triterpenoids (b), and polysaccharides (c) and the total yield of each metabolite (d, e, f). The black rhombus indicates the median value for each fertilizer type and serves to compare the variation among different fertilizer levels in the same fertilizer type. The blue line was calculated using treatment O as a reference group to compare the variation among different fertilizer types. Fertilizer type: O = only organic fertilizer, OMF = organic fertilizer and inoculant containing *Bacillus megaterium* and *Pseudomonas fluorescens*, OCB = organic fertilizer and inoculant containing *Azotobacter chroococcum* and *Azospirillum brasilense*. Fertilizer level: Low = 10^7 cells + 0.5 kg organic fertilizer per plant, Medium = 10^8 cells + 1.0 kg organic fertilizer per plant, High = 10^9 cells + 1.5 kg organic fertilizer per plant.

metabolism when experiencing an intermediate resource level. However, it should be noted that not all treatments that produced medium C/N and C/P levels resulted in the highest accumulation of metabolites. Hence, other factors, such as plant-associated microorganisms, may regulate plant metabolism (Badri et al., 2013; Etalo et al., 2018).

3.3. The resident microbial community diversely responded to different fertilization regimes

The application of biofertilizer enhanced bacterial alpha diversity (Supplement Fig. S5a), and both fertilizer type and level affected the dissimilarities in the soil bacterial community (Supplement Fig. S5b) and the abundances of specific phyla (Supplement Fig. S5c). For instance, OCB2, OCB3, OMF2, and OMF3 treatments increased the abundances of the top 10 phyla compared to other treatments ($p < 0.05$). Hence, biofertilization with probiotics and organic fertilizer can increase microbial diversity and change community composition, and the effects are associated with fertilization regimes. The addition of microbes to the soil can alter the native microbial community via resource competition, antagonism, and synergism (Hu et al., 2016; Wei et al., 2018; Mawarda et al., 2020), thus changing microbial diversity and composition. Linear discriminant analysis (LDA) identified the key phyla *Actinobacteriota* and *Gemmatimonadota* that differentiate treatments OCB2 and OCB3 from others, and phyla *Proteobacteria* and *Chloroflexi* differentiated treatment OMF2 and OMF3 from others (Supplement Fig. S6). These phyla can act as biomarkers for the variations between different fertilization regimes.

3.4. GJAM revealed the effects of fertilization regime on the plant-soil-microbe system and the underlying contributors

In contrast to previous studies limited to only microbial parameters, we used GJAM analysis to build a full plant-soil-microbe model incorporating multiple variables to examine the impacts of different

fertilization regimes on the whole system and identify underlying contributors. GJAM showed that the influence of fertilization on the plant-soil-microbe system was highly dependent on fertilizer level and type (Fig. 2a). When the fertilizer level was low, the probiotic treatments (OMF, OCB) performed differently than to the single organic fertilizer application (O). The higher the fertilizer level, the lower influences on the whole system were observed, and the distance among the fertilizer types decreased in the PCA plot (Fig. 2a). This finding is in accordance with the results and discussion in 3.3. In addition, specific plant, soil, and microbial variables appeared to be important traits in influencing the whole system (Fig. 2b). The Sankey plot showed that (Fig. 2c), in comparison with O treatment, OMF and OCB treatment significantly affected the plant and soil variables and microbial communities, namely both significantly positive correlations and significantly negative correlations were found at three fertilizer levels. However, the number of significant correlations decreased along with increasing fertilizer levels.

Furthermore, in this model, we focused on the top 10 microbial taxa contributing to whole-system variance and three fertilizer types exerted different effects on these taxa. Probiotic consortia, especially OMF, exhibited more influence on those taxa (Supplement Fig. S7). In summary, the impacts of OCB and OMF on the microbial community were larger than those of O but decreased with increasing fertilizer levels.

Resident soil microbial communities are frequently subjected to (a) biotic disturbances due to agricultural management practices (fertilization and application of biocontrol and microbial inoculants) as well as naturally occurring disturbances (drought, flooding, and frost) (van Elsas et al., 2012; Mallon et al., 2015). High amounts of fertilizer would be expected to elicit large changes in the microbial community, and nutrient-induced shifts in copiotrophic vs. oligotrophic microbial lifestyles could also affect soil functioning and plant performance (Wieder et al., 2013; Leff et al., 2015). A previously developed experimental framework has suggested that soil microbes are preferentially recruited by host plants under (a)biotic stress, whereas an improved soil environment for the host plant leads to lower dependence on beneficial

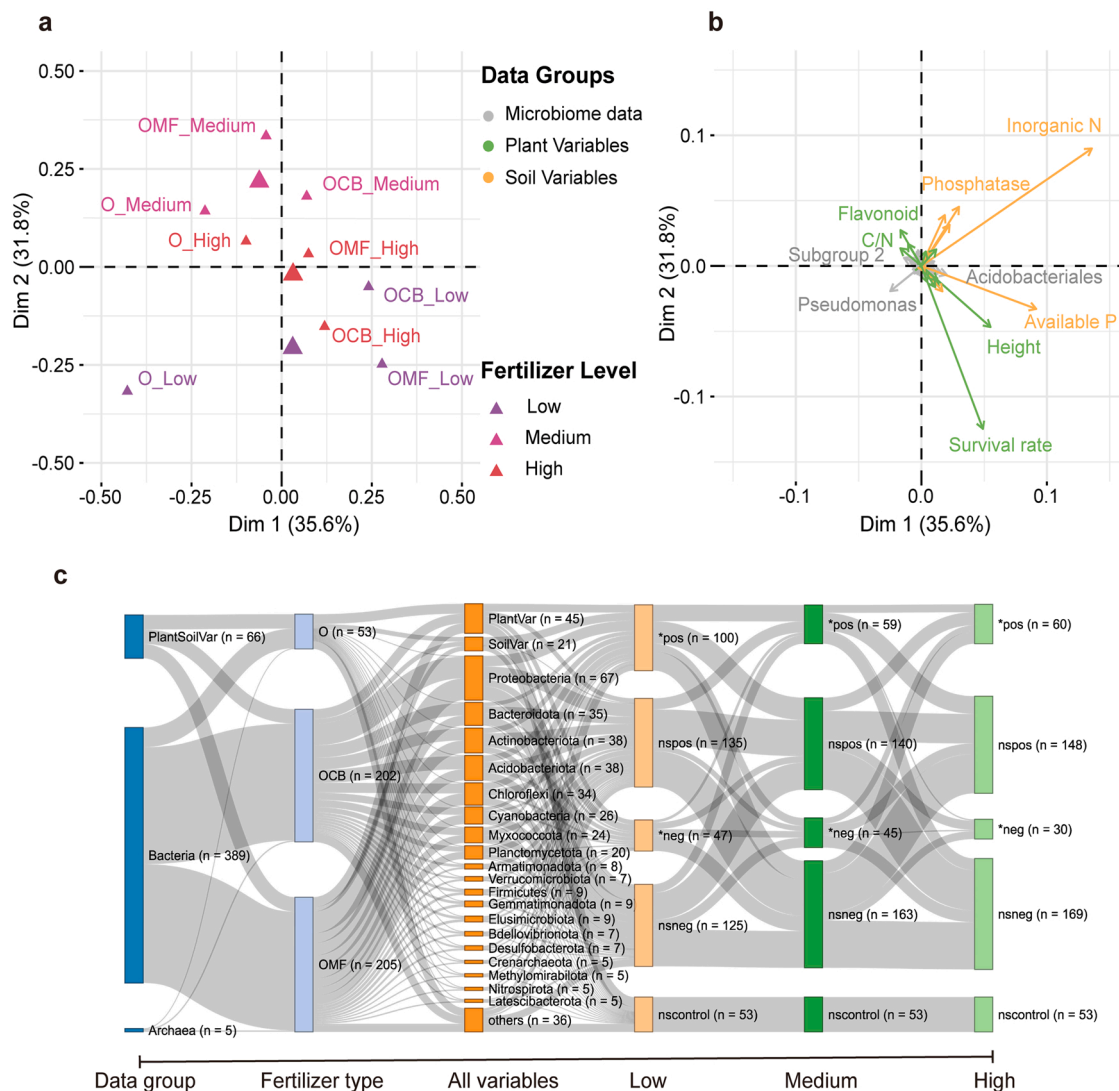


Fig. 2. Differentiation of fertilization regimes and underlying plant, soil and microbial contributors by generalized joint attribute modeling (GJAM). (a) Principle component analysis (PCA) plot reveals the various effects of all fertilization regimes on the plant-soil-microbe system. (b) Identification of the top 10 contributors in the whole system. (c) Within the same fertilizer level, the differences among the fertilizer types were examined by comparison against the reference treatment O. PlantSoilVar: plant and soil variables; *pos: the effects were significantly positive; nspos: the effects were non-significantly positive; *neg: the effects were significantly negative; nsneg: the effects were non-significantly negative; nscontrol: the reference (treatment O) used in comparisons with other treatments.

microbes from the soil (Rashid et al., 2016; da Costa et al., 2020; Liu et al., 2020a), as supported by our study that the effects of probiotics decreased at higher fertilizer levels.

3.5. Linking soil microbial indicators to the accumulation of bioactive compounds under different fertilization regimes via GJAM model

The interplay between the plant and environmental conditions (water stress, light, soil nutrient) has been generally examined via reductionist approaches (Etalo et al., 2018), lacking integration of soil microbiome data. We used GJAM analysis to predict microbial abundances relating to the maximum concentration of flavonoids, and the predicted and observed values were compared to identify the potential microbial indicators (Fig. 3a). For instance, the predicted abundances of microbes belonging to the phylum *Proteobacteria* were higher than the observed values, indicating that increasing the abundances of such microbes might facilitate the accumulation of flavonoids, which made them “microbial assistants” to the probiotics. The opposite pattern was observed for microbes belonging to the phyla *Actinobacteriota* and *Chloroflexi*, which made them act as “microbial competitors”. Previous

studies have highlighted that the indigenous soil microbiota is intimately related to the plant secondary metabolism (Schmidt et al., 2014; Huang et al., 2018), but the distinct roles of various microbes in the resident community have not been classified. Thus, the identification of microbial indicators provides a reference for utilizing nature bio-resources incorporated with native microbes to manipulate plant metabolite production.

Similarly, the predicted and observed effects of the fertilization regimes on microbial indicators are compared in Fig. 3b. The mean values of the predicted microbial abundances were higher than the observed values for treatments OCB1 and OMF2, whereas the opposite pattern was found for treatment OMF3. These patterns were in accordance with the examined results of flavonoid concentration and yield (Fig. 1). These findings indicated that the prediction of key soil microbiota might be used to identify indicators for devising appropriate soil management strategies.

To support this conclusion, we checked the impacts of different fertilization regimes on the selected top-10 microbial assistants (Fig. 3c) and microbial competitors (Fig. 3d). The results showed that, O treatment at the low fertilizer level significantly increased the abundance of

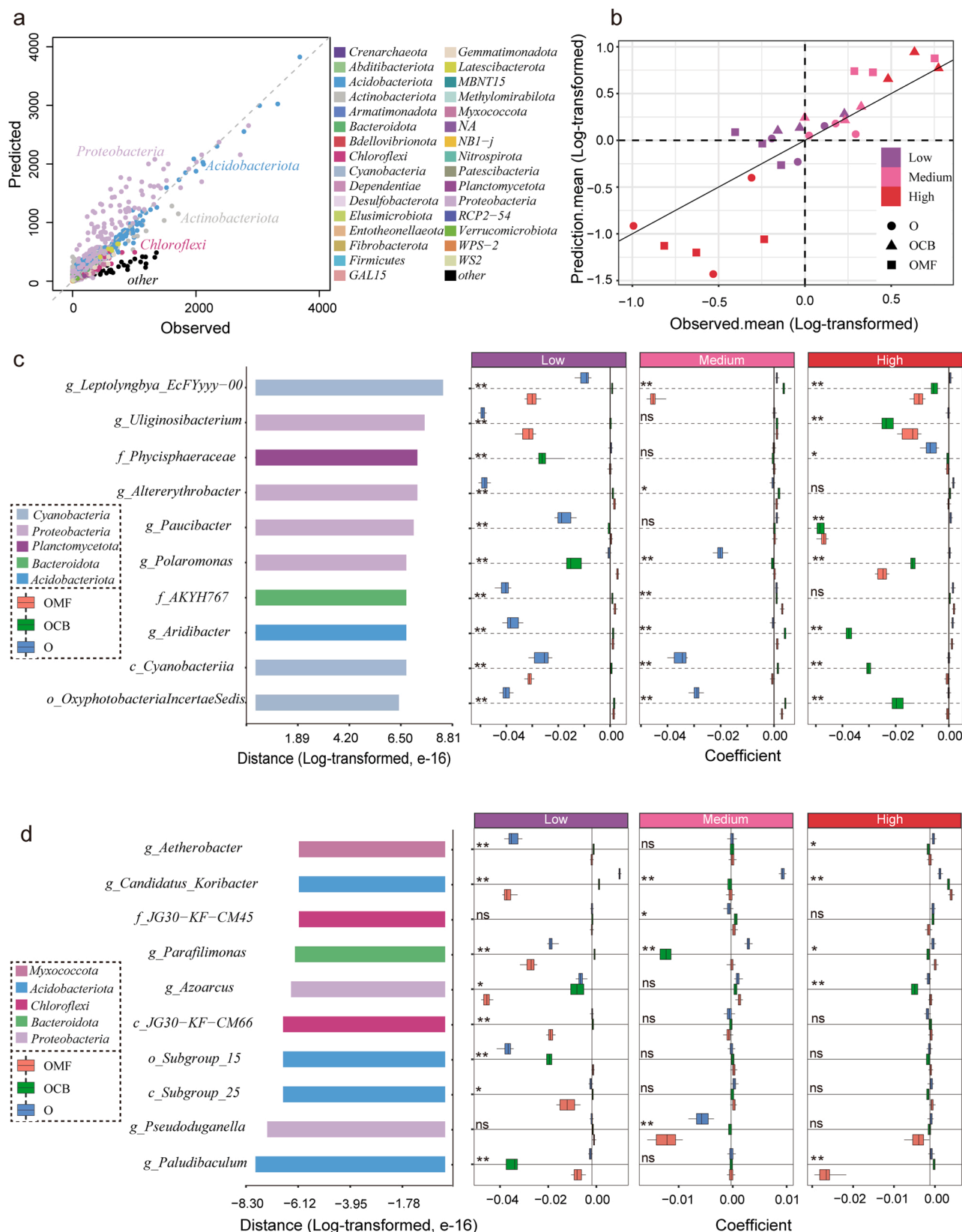


Fig. 3. Prediction of optimal flavonoid accumulation based on microbiome data in association with different fertilization regimes in GJAM. (a) Predicted and observed abundances of all phyla in stimulating the accumulation of flavonoids. To achieve the highest accumulation of flavonoids in this model, the abundance of the microbes above the dashed line should be increased, while the abundance of the microbes below the dashed line should be decreased. (b) Effects of all tested fertilization regimes on the accumulation of flavonoids with attribution of microbes. The treatments above the solid line resulted in a positive effect on the accumulation of flavonoids. (c and d) Identification of the top 10 microbial taxa (c.: class; O.: order; g.: genus; f.: family) that improved (c) or hampered (d) the accumulation of flavonoids and their responses to the different fertilization regimes. *: $p < 0.05$; **: $p < 0.01$; ns: no significance.

most microbial assistants compared to OCB and OMF treatments, whereas the opposite pattern was observed at the high fertilizer level. By contrast, at the low fertilizer level of fertilization, OMF and OCB treatments significantly decreased the abundance of the microbial competitors compared to O treatment, whereas few differences among these treatments were observed at the medium and high fertilization levels. Similar patterns were observed for triterpenoids (Supplement Fig. S8): the abundances of most microbial assistants were higher under OCB than the other fertilizer types at low and medium levels of fertilization, and both OCB and OMF significantly decreased the abundances of microbial competitors. Thus, probiotic addition can facilitate the relative abundance of microbial assistants and inhibit the relative abundance of microbial competitors in the soil, ultimately changing in plant metabolites.

3.6. Effects of probiotics on microbial indicators in a comparative pot experiment

As shown in Fig. 4a, 168 and 149 microbial assistants (positive) were

identified in the field and pot experiments, respectively. A total of 36 microbial assistants distributed over nine phyla were found in common between the two experiments. The impact magnitudes of specific indicators, including *g. Rhodoplanes*, *g. Stenotrophobacter*, *g. Bryobacter*, and *g. Pajaroellobacter*, were nearly identical in the accumulation of flavonoids between the two experiments. We identified 140 and 139 microbial competitors (negative) in the field and pot experiments (Fig. 4b), respectively. Of these indicators, 29 distributed over 10 phyla were common between the two experiments. The impact magnitudes of specific indicators like *g. Steroidobacter*, *g. Opitutis*, *f. Fimbriimonadaceae*, and *p. Latescibacterota* on flavonoid accumulation were nearly identical between the two experiments.

Moreover, the potential role of these microbial indicators in association with plant metabolic performance and the effects of probiotic addition on their relative abundances were tested in the pot experiment (Fig. 5). Compared to the single application of organic fertilizer (O), the introduction of probiotic consortia (OCB, OMF) significantly increased the relative abundance of these microbial assistants and significantly decreased the relative abundance of microbial competitors (Fig. 5a, b).

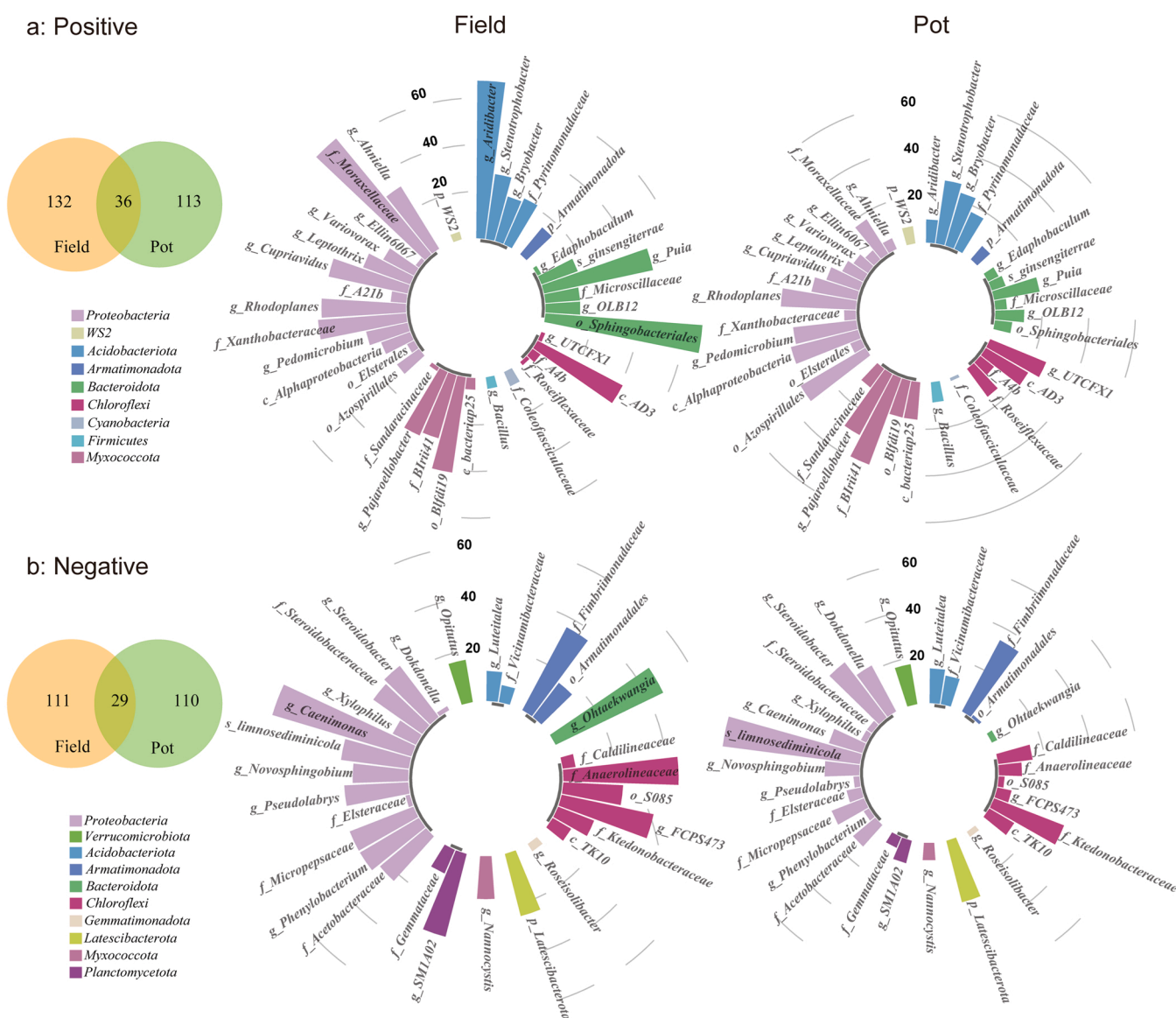


Fig. 4. Identification of the common potential microbial indicators in field and pot experiments to improve plant flavonoids' accumulation. (a) Microbial assistants (positive) for stimulating the accumulation of flavonoids and their contributions in the field and pot experiments. (b) Microbial competitors (negative) for inhibiting the accumulation of flavonoids and their contributions in the field and pot experiments.

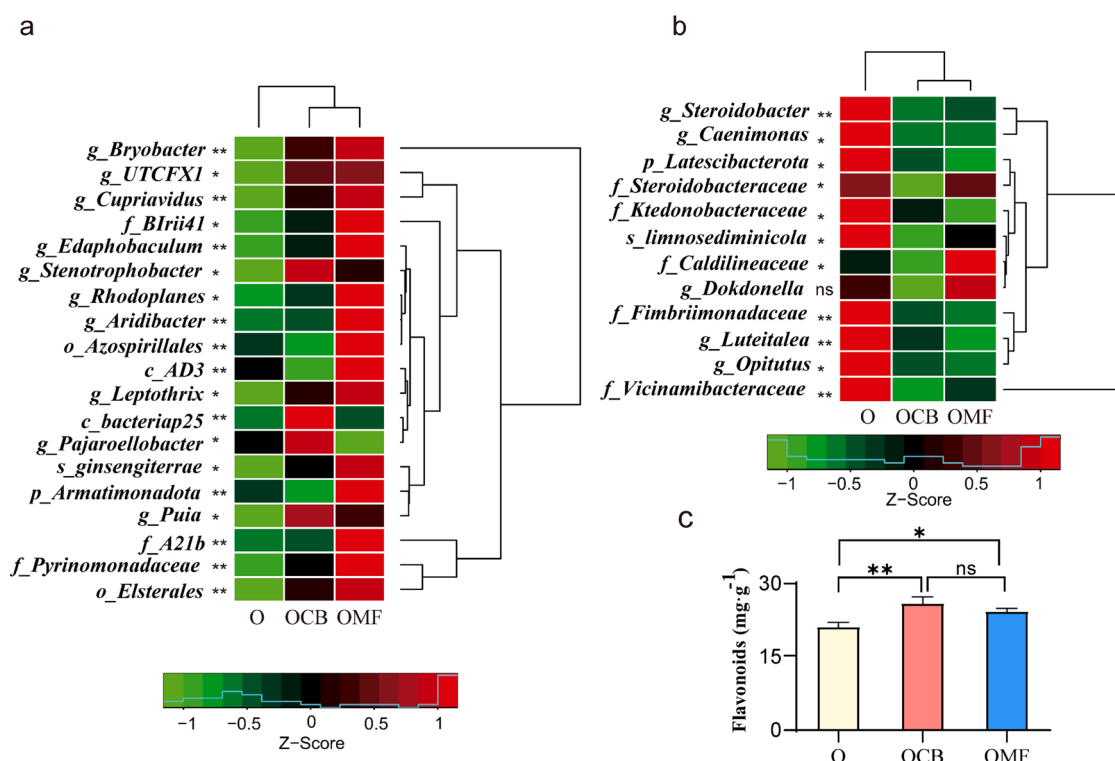


Fig. 5. Impacts of probiotic consortia on the relative abundance of microbial indicators (indicators that have statistical significance) relating to the accumulation of flavonoids in a pot experiment. (a) The relative abundance of microbial assistants in treatments O, OCB and OMF. (b) The relative abundance of microbial competitors in treatments O, OCB and OMF. (c) The effects of different treatments on the concentrations of flavonoids in *C. paliurus*.

This is consistent with the variation of flavonoids concentrations observed in different treatments. The flavonoid concentrations were significantly enhanced in the treatments OCB and OMF, but ANOVA test showed no significant difference between OCB and OMF (Fig. 5c).

Hence, microbial indicators could assist or impede the beneficial effects of probiotics on plant metabolites. The approach adopted in this study provides a holistic perspective for understanding the relationship between soil microbiota and plant metabolites compared with the commonly used linear correlation (Pineda et al., 2020) or principal component analyses (Badri et al., 2013; Chaparro et al., 2013). For instance, using traditional correlation analyses, soil microbiome cannot explain plant physiological features because soil microbiome and plant physiological features might respond to the same variates. GJAM approach can integrate variables from the plants and soil microbiome, and the variations of other environmental variables were controlled. Thus, it accounts for potential interactions, and better understands of the relationship between plant traits and the associated phytobiome. However, it should be noted that it is infeasible to isolate each potential indicator from the resident community and test their functional traits due to the unculturable features of most microorganisms. Future studies should verify the persistence of the beneficial impacts derived from soil microbiota and manipulate the resident microbiota by changing the resources that those microbial indicators prefer (Costa et al., 2020).

4. Conclusions

In summary, our findings emphasize that the impacts of fertilization on the growth and the accumulation of metabolites in *C. paliurus* are part of a joint contribution that includes leaf stoichiometric traits and specific changes within the soil microbiome. The effects of probiotics on the plant-soil-microbes system are associated with fertilizer levels, and a higher fertilizer level is more likely to decrease the impact of probiotics. Furthermore, our state-of-art modeling approach successfully identified potential soil-indigenous microbial indicators that may assist or impede

the production of plant flavonoids and triterpenoids, which brings a new concept of microbial assistant and microbial competitors relating to the use of probiotics. The comparative pot experiment reveals that it is possible to apply the plant probiotics as a sustainable strategy to modulate the resident microbiota (i.e. modulate the relative abundances of microbial indicators), which is further associated with the impacts of probiotics on the production of flavonoids in plants. The identification of microbial indicators provides a novel avenue for understanding the below-aboveground interactions and can be further developed to decipher other biological interactions in plant-soil-microbes systems.

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CRediT authorship contribution statement

Zhikang Wang: Writing – original draft, Conceptualization, Investigation. **Ziyun Chen:** Investigation, Data curation, Conceptualization. **Ziheng Xu:** Investigation, Data curation, Conceptualization. **Quan Lin:** Investigation, Data curation, Conceptualization. **Xiangxiang Fu:** Supervision, Funding acquisition, Writing – review & editing. **Marcio F.A. Leite:** Methodology, Writing – review & editing. **Eiko E. Kuramae:** Writing – review & editing, Supervision. **George A. Kowalchuk:** Writing – review & editing, Supervision.

Ethics approval and consent to participate

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Raw sequences of the field experiment were submitted to the NCBI Sequence Read Archive (SRA) under the accession number from SRR13756033 to SRR13756062 in a BioProject PRJNA703386: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA703386?reviewer=jg9035qgj2srrq1dglr5caft0f>. Raw sequences of the pot experiment were submitted to SRA in a BioProject (PRJNA630558). Plant growth, bioactive compounds, and nutrient stoichiometry data were deposited in Zenodo at <https://doi.org/10.5281/zenodo.4553411>.

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Code availability

Not applicable.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2022.115138.

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