Science of the Total Environment 947 (2024) 174333

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Metabolites of blueberry roots at different developmental stages strongly shape microbial community structure and intra-kingdom interactions at the root-soil interface

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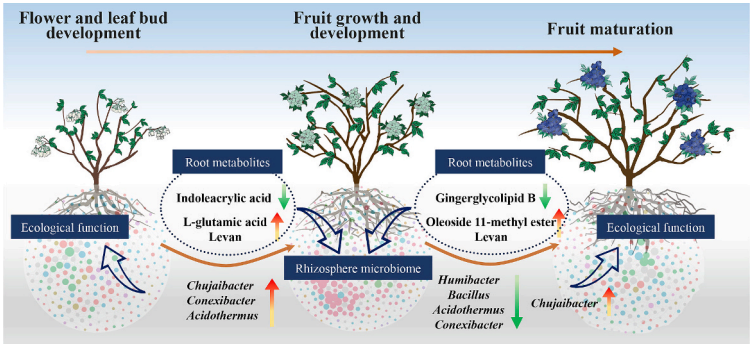
HIGHLIGHTS

- Diversity and composition of rhizosphere bacterial communities varied at different developmental stages of blueberry.
- Proteobacteria and Actinobacteria as dominant phyla showed significant changes in relative abundance.
- Root metabolites of levan and L-glutamic acid highly correlated with altered dominant bacterial taxa.
- Bacterial community niche breadth was highest at the fruit growth and development stage.
- Niche overlap of bacterial communities increased with developmental stage.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Abasiofiok Mark Ibekwe

Keywords:
Blueberry
Developmental stages
Rhizosphere bacterial community
Niche breadth and overlap
Root metabolites

ABSTRACT

The rhizosphere microorganisms of blueberry plants have long coexisted with their hosts under distinctively acidic soil conditions, exerting a profound influence on host performance through mutualistic symbiotic interactions. Meanwhile, plants can regulate rhizosphere microorganisms by exerting host effects to meet the functional requirements of plant growth and development. However, it remains unknown how the developmental stages of blueberry plants affect the structure, function, and interactions of the rhizosphere microbial communities. Here, we examined bacterial communities and root metabolites at three developmental stages (flower and leaf bud development stage, fruit growth and development stage, and fruit maturation stage) of blueberry plants. The results revealed that the Shannon and Chao 1 indices as well as community composition varied significantly across all three developmental stages. The relative abundance of Actinobacteria significantly increased by 10 % ($p < 0.05$) from stage 1 to stage 2, whereas that of Proteobacteria decreased significantly. The

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https://doi.org/10.1016/j.scitotenv.2024.174333
Received 25 April 2024; Received in revised form 24 June 2024; Accepted 25 June 2024
Available online 28 June 2024

co-occurrence network analysis revealed a relatively complex network with 1179 edges and 365 nodes in the stage 2. Niche breadth was highest at stage 2, while niche overlap tended to increase as the plant developed. Furthermore, the untargeted metabolome analysis revealed that the number of differential metabolites of vitamins, nucleic acids, steroids, and lipids increased between stage 1 to stage2 and stage 2 to stage 3, while those for differential metabolites of carbohydrates and peptides decreased. Significant changes in expression levels of levan, L-glutamic acid, indoleacrylic acid, oleoside 11-methyl ester, threo-syringoylglycerol, gingerglycolipid B, and bovinic acid were highly correlated with the bacterial community structure. Collectively, our study reveals that significant alterations in dominant bacterial taxa are strongly correlated with the dynamics of root metabolites. These findings lay the groundwork for developing prebiotic products to enhance the beneficial effects of root microorganisms and boosting blueberry productivity via a sustainable approach.

1. Introduction

Blueberry rhizosphere microbes interact with their hosts on ecological and evolutionary timescales forming species assemblages as an essential part of the holobiont (Trivedi et al., 2020; Vandenkoornhuyse et al., 2015). Selective pressures on this component of the holobiont influence the structure of the plant-associated microbiome and preferentially select microbes that improve the fitness of the plant (Hassani et al., 2018). These plant-associated microbial communities contribute to multiple aspects of plant health, such as acquisition of soil nutrients, resistance to pathogens, and improved tolerance to stressful environments (Poppeliers et al., 2023; Santos and Olivares, 2021; Trivedi et al., 2020). On the other hand, diseased plants are typically characterized by microbial imbalance (dysbiosis) and an altered role of specific microorganisms as antagonists or synergists of pathogens. This ultimately leads to the healthy holobiont pattern falling out of balance (Berg et al., 2017). Thus, mutually beneficial symbiotic patterns between the host plant and its microbiota are key to sustaining the health of the holobiont. A better understanding of the homeostatic mechanisms by which microorganisms promote plant health is essential for exploring the utilization of the plant microbiome for sustainable agricultural production.

The rhizosphere provides a unique ecological niche for complex interactions between plant roots and microbes. Such interactions consist mainly of the effects of biotic/abiotic factors on microorganisms, such as root exudates, soil types and plant developmental stages, as well as on plant growth and disease resistance through the provision of bioavailable nutrients and phytohormones (Qu et al., 2020). Plants can regulate its own rhizosphere microbiome to meet the functional needs in different developmental stages (Chaparro et al., 2014; Moroenyane et al., 2021; Xiong et al., 2021). They can transport carbon fixed by photosynthesis to the rhizosphere in the form of root exudates, which serve as a source of carbon and nitrogen for rhizosphere microorganisms (Chagas et al., 2018; Haichar et al., 2014). These root exudates are complex mixtures of organic and inorganic compounds, which are mainly categorized into a wider variety of small molecular weight compounds such as organic acids, amino acids, sugars, and other secondary metabolites, and a lesser variety of high molecular weight compounds (Feng et al., 2023). The composition and diversity of root exudates vary considerably, even at different developmental stages of the same individual plant (Steinauer et al., 2023; Vives-Peris et al., 2020). Previous studies have shown that beneficial genera in the rhizosphere increased with plant growth, such as *Bacillus*, *Bradyrhizobium*, *Mesorhizobium*, *Agrobacterium*, *Rhizobium*, *Burkholderia*, and *Pseudomonas*. It was observed that these beneficial microorganisms for plant growth were most abundant at the latest developmental stage (reproductive stage) in peanut at 120 days and vegetative growth stage in maize at 100 days, respectively (Anzuay et al., 2021). Therefore, the functional requirements of plants at different developmental stages can actively regulate the community structure of rhizosphere microorganisms through root exudates, leading to the functional expression of microorganisms that can exert beneficial effects on the host plant.

Apart from the structural regulation of the rhizosphere microbial community by root exudates of the host plant, the intense interactions occurring between microorganisms are also play an important role. These microbe-microbe interactions can be unraveled thought the co-occurrence network analysis (Shi et al., 2016; van der Heijden and Hartmann, 2016). This network allows for a more intuitive exploration of microbial hub taxa with significant roles and the identification of microbial mutualistic and antagonistic effects through positive or negative interactions, thus revealing associations between microbial interactions and plant phenotypes (Banerjee et al., 2019; Barberán et al., 2012; Hassani et al., 2018). Compared to the diseased plant, the topological characteristics of co-occurrence network in healthy plants is much larger, with more nodes and edges, longer average path lengths, and a higher degree of modularity. Therefore, based on microbial interactions in healthy and diseased plant, potential key driver taxa behind pathogen inhibition are identifiable (Wei et al., 2019). Interactions between microorganisms within the rhizosphere community are also influenced by the capacity of the species to utilizing the resources, which can be reflected by niche breadth (Xu et al., 2021). Greater niche breadth implies broader resource metabolism, leading to improved resource utilization efficiency and greater potential to promote plant growth and health in low-fertility soils (Jiang et al., 2023; Xu et al., 2022). However, the distribution of microbial taxa with narrower niche breadths is potentially more susceptible to the impacts of dispersal limitation or ecological drift, thus posing a threat to the colonization and stability of the community (Zhang et al., 2018a). Particularly at lower resource levels, resource-specific individuals will be subject to growing niche overlap and more intensive competition for resources, which will affect the growth of their populations (Lesser et al., 2020; Pastore et al., 2021). Thus, how these complex interactions vary with different developmental stages of blueberry plants, the intensity of their modulatory effects on the microbial community, and the extent to which they affect host growth and development remain to be studied in depth.

As a member of the Ericaceae family, blueberry is highly adapted to acidic soils. Owing to the unique flavor of the fruits and the associated health benefits, blueberry plants are now widely cultivated around the world (Silva et al., 2020; Yang et al., 2023). The growth and health of the blueberry plants, to a large extent, determines the productivity and quality of its fruit in such harsh soil conditions (Morvan et al., 2020). Several studies have shown that the blueberry plants have established a symbiotic relationship with ericoid mycorrhizal fungi, which can benefit plants by decomposing complex organic compounds and activating soil nutrients (Grelet et al., 2009; Martino et al., 2018; Wei et al., 2016; Yurgel et al., 2018). However, despite of this stable symbiotic relationship between the blueberry plants and fungi, previous studies showed that blueberry plants had a greater host effect on the bacterial community, which resulted in a similarly intimate association of bacteria with exerting beneficial effects on the host plant (Yurgel et al., 2017). Recently more clues have been found regarding the beneficial bacteria in blueberry rhizosphere, which are responsible in promoting the growth and health of the host plant. In blueberry plantation soils, eight different plant growth-promoting rhizobacteria were found to be present, with relative abundances of *Bradyrhizobium*, *Bacillus*, and *Paenibacillus* all exceeding 0.1 % (Lee et al., 2021). It was reported that a synthetic bacterial consortium comprised of three *Bacillus* strains inoculated into the rhizosphere of blueberry plants improved both yield and quality of blueberry fruits (Yu et al., 2020). In addition, higher

germination rates were observed for blueberry seeds incubated with *Buttiauxella* strains isolated from blueberry rhizosphere, which exhibited strong phosphorus solubilization and auxin-producing ability (Wang et al., 2022). The beneficial role of rhizosphere bacteria in the growth and development of the blueberry has been increasingly revealed. Thus, further insights into host effects in rhizosphere hotspots and dynamic regulation of bacterial communities for plant health will provide an essential foundation for utilizing prebiotics for sustainable agricultural development.

In our study, we investigated the variation of blueberry root metabolites and rhizosphere microbial communities along three developmental stages (flower and leaf bud development stage, fruit growth and development stage, and fruit maturation stage). Our objectives were to (1) reveal the dynamics of rhizosphere bacterial community diversity and composition with plant developmental stages, (2) the complex patterns of interactions between rhizosphere microorganisms during the dynamics, and (3) the host effects of root metabolism associated with the dynamics of rhizosphere bacterial communities. We hypothesized that rhizosphere bacterial community structure and complexity interactions changes during blueberry growth and development and are influenced by the dynamic metabolic characteristics of the blueberry roots.

2. Materials and methods

2.1. Pot experiment and sampling description

We conducted the glasshouse experiment (31°60′ N, 119°20′ E) located in Lishui, Nanjing, Jiangsu Province (31°60′ N, 119°20′ E), which has a humid subtropical monsoon climate with an average annual temperature, sunshine, and precipitation of 16.4 °C, 1980 h, and 1204 mm, respectively. On January 10, 2022, two-year-old cutting seedlings of blueberry (*Vaccinium ashei* Reade) "Brightwell" cultivar were selected and transplanted into the soil of 25 cm diameter plastic pots with trays. One seedling was planted in each pot, for a total of 30 soil-filled pots, 25 of which were planted with seedlings and 5 of which were not planted with any plants to serve as the control. Each pot of seedlings was irrigated weekly with 500 ml of the same concentration of nutrient solution throughout the experiment. The nutrient content of the fertilizer was 20 % N, 20 % P, and 20 % K, which was applied by diluting it one thousand times. And consistent cultivation and management practices were applied, including irrigation, sunlight, and regular weeding. Soil samples for bacterial community analyses and root samples for metabolite analyses were collected at flower and leaf bud development stage (stage 1), fruit growth and development stage (stage 2), and fruit maturation stage (stage 3), according to the characteristics of the growth and development stages of blueberry (Gao et al., 2021; Pescie et al., 2011; Spiers, 1978; Williamson et al., 2002). Samples for analysis were collected on April 18, May 24, and June 21, 2023 for analysis, with five replicates collected at each developmental stage. Bulk soil samples were collected with a sterilized spade from pots where seedlings were not planted, removing the surface soil and collecting mixed samples from each pot. Rhizosphere soil samples were collected by removing the entire root from the pot and then using a sterilized brush to collect the soil tightly attached to the roots to form a mixed sample (Che et al., 2023). After removing impurities from the soil samples with a 2-mm sieve, the samples were placed on ice and immediately transported to the lab for storage in an ultra-low-temperature refrigerator for subsequent microbiome analysis. Root samples were rinsed repeatedly with water and wiped clean, wrapped in tin foil, transferred frozen on dry ice to the laboratory, and stored in ultralow-temperature refrigerator for subsequent metabolite analysis.

2.2. DNA extraction and Illumina sequencing

Total DNA was extracted from 0.5 g of soil samples with FastDNA SPIN Kits (MP Biomedicals, Santa Ana, CA, USA) according to the provided manufacturer's instructions. The V3-V4 region of the bacterial 16S rRNA gene was amplified with specific primers of 338F and 806R. High-throughput sequencing was achieved by the Illumina MiSeq PE300 platform (Illumina, San Diego, USA), with 300 bp sequenced per paired-end. The detailed description of the DNA extraction and amplification methods are described in the Supplementary Material. The raw sequences were filtered using fastp software (v0.20.0, https://github.com/OpenGene/fastp) and merged by using FLASH (v1.2.7, http://www.cbcb.umd.edu/software/flash) software (Chen et al., 2018; Magoč and Salzberg, 2011). The sequences were then denoised, chimeras removed, and aligned by using DADA2, eventually amplicon sequence variants (ASVs) were obtained (Callahan et al., 2016). After quality filtering, a total of 2,435,971 sequence reads were obtained with an average of 81,199 sequence reads per sample. Each sequence was taxonomically categorized according to the SILVA (v13.8, http://www.arb-silva.de) database, resulting in a total of 34,540 bacterial ASVs.

2.3. Metabolite extraction and untargeted metabolomics analysis

Metabolites were extracted from each 50 mg fresh frozen root samples using 400 μL of 80 % methanol extraction solution containing 0.02 mg/ml of internal standard (L-2-chlorophenylalanine). Then the frozen root samples were grinded with a frozen tissue grinder for 6 min, followed by low-temperature ultrasonic extraction for 30 min at 4 °C with 40 kHz. After leaving the samples at −20 °C for 30 min, centrifugation was performed for 15 min (4 °C, 13,000 g), and the supernatant was shifted to the injection bottle for LC-MS/MS analysis (Lin et al., 2024). Quality control (QC) samples were prepared by mixing aliquots of all samples and injecting at intervals of 5–15 samples. LC-MS/MS analysis of the samples were performed on a Thermo UHPLC-Q Exactive HF-X system equipped with an ACQUITY HSS T3 column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, USA). Raw LC/MS data were preprocessed using Progenesis QI (Waters Corporation, Milford, USA) software and then matched using the Kyoto Encylopaedia of Genes and Genomics (KEGG) database (http://www.genome.jp/kegg/) to annotate metabolite biological functions and metabolic pathways (Wang et al., 2023).

2.4. Statistical analysis

The Shannon and Chao1 index of alpha diversity were assessed by Mothur software (v1.30.2). A nonparametric Kruskal-Wallis test for alpha diversity was performed, followed by a false discovery rate (FDR) correction for *p*-values and a post hoc test using the Tukey-kramer test to determine if there were any significant differences between developmental stage groups (Liu et al., 2020). Beta diversity was assessed by nonmetric multidimensional scaling (NMDS) using QIIME software (v1.9.1) based on weighted UniFrac distance matrices, and the analysis of similarity (ANOSIM) was conducted to assess significance of bacterial community composition shifts across the different developmental stages (Voges et al., 2019). Bacterial taxa that exhibited significant shifts in relative abundance between different developmental stage groups were tested by using the Wilcoxon rank-sum test. The bacterial community co-occurrence networks were constructed using ASVs with >50 sequences per ASV and observed in each sample (Bazany et al., 2022; Pérez-Jaramillo et al., 2019). The co-occurrence networks were calculated using the "*SpiecEasi*" package in R to perform SparCC (Friedman and Alm, 2012; Kurtz et al., 2015). Valid co-occurrence correlations between ASVs were determined to be statistically significant (SparCC correlation $|r| > 0.6$ and $p < 0.01$) (Friedman and Alm, 2012; Kurtz et al., 2015; Mendes et al., 2018). The co-occurrence network was conducted by "*igraph*" in R (Gabor and Nepusz, 2006; Harrell and Frank, 2008) and visualized in Gephi software (*v0.10.1*). Topological characteristics describe the interaction patterns within bacterial community (Bastian et al., 2009), among which the metrics of nodes, degrees, edges, modularity, closeness centrality, clustering coefficient, and path distance were used in our study. The hub nodes were identified as a node

with high degree (>15) and closeness centrality (>0.25) in co-occurrence networks (Agler et al., 2016; van der Heijden and Hartmann, 2016). Microbial community assembly processes were analyzed by calculating the β-nearest taxon index (βNTI) based on the null modeling approach (Stegen et al., 2012, 2013). The AVSs with >10 sequences per ASV and observed at least one sample were used to calculate the βNTI by using "*picante*" package in R (Kembel et al., 2010). The assembly processes were identified with $|\beta NTI| \geq 2$ were expected to the deterministic processes, while $|\beta NTI| < 2$ indicated a less selection pressure that their assembly processes are governed by stochastic processes (Stegen et al., 2012). Niche breadth based on levins method was calculated by using "*niche width*" function of R package "*spaa*". And niche overlap (levins' niche overlap) was calculated with the AVSs that were observed at each sample by using "*spaa*" package in R (Jiang et al., 2023). The functional annotation of prokaryotic taxa (FAPROTAX) database was used for predicting the functional profile of the bacterial communities.

Metabolic differences between developmental stage groups were determined by using partial least squares discriminant analysis (PLS-DA) with 95 % confidence intervals and 200 permutations. The variable importance in projection (VIP) values were calculated in the PLS-DA model to show the contribution of differences. Differential metabolites that were up-regulated or down-regulated between groups were determined by Student's *t*-text (Unpaired), for differential metabolites with *p* values below 0.05 and with VIP values above 1. We then performed KEGG compound classification to identify metabolites that differed between groups. Similarities and variability between the root differential metabolites and bacterial community structure were examined using Procrustes analysis. Monte Carlo *p* values were used to test the correlations between microbiome and root metabolome, and the correlation was considered significant at *p* values below 0.05. The extent of correlation was characterized by M^2 (proportion of random error to total error), with lower values indicating higher degree of correlation. We then performed the analysis based on Spearman's correlation to determine the correlation between the 15 most abundant root metabolites and the 15 most abundant bacterial taxa.

3. Results

3.1. Plant developmental stages shift bacterial community diversity and network patterns

The alpha diversity in the bulk soils and rhizosphere soils varied with the stage of plant development. A significant decreasing trend of the Shannon index and Chao 1 index were observed in the bulk soils, whereas a significant decreasing and then increasing trend was observed in the rhizosphere soils (Fig. 1A and B). Different patterns of co-occurrence network patterns were observed at the three developmental stages, with average node degrees of 5.51, 6.46, and 4.58 in the rhizosphere soils from stage 1 to stage 3, respectively (Fig. 1D; Table 1).

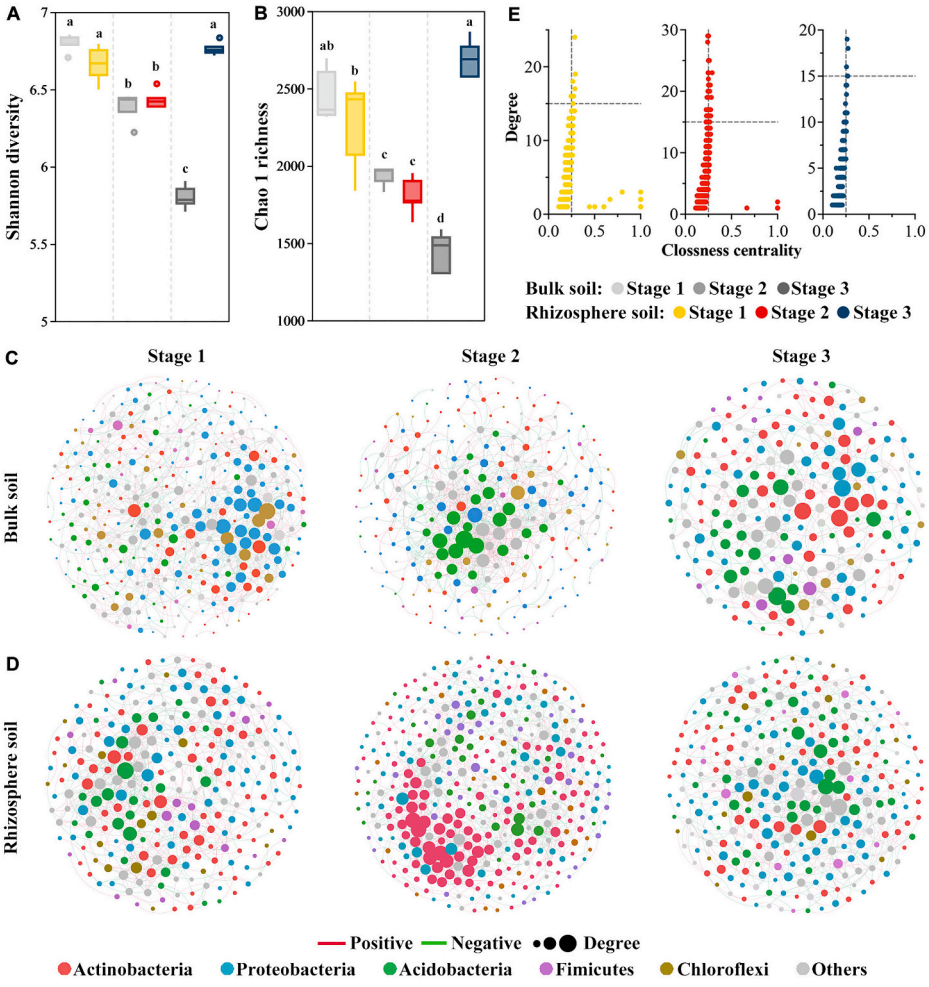

Fig. 1. Alpha diversity of Shannon (A) and Chao1(B) index across three developmental stages of blueberry. Different letters above the boxes indicate a significant difference determined by nonparametric Kruskal-Wallis test ($p < 0.01$). Co-occurrence network analysis of the bulk soil (C) and the rhizosphere soil (D) based on the phylum taxonomy depicting the dynamic patterns within bacterial community and the distribution patterns hub nodes (E) of blueberry rhizosphere bacterial community among different developmental stages.

Table 1
Topological characteristics of co-occurrence network analysis of blueberry bulk soil and rhizosphere soil bacterial community at different developmental stage.

Network metrics	Stage 1		Stage 2		Stage 3	
	Bulk	Rhizosphere	Bulk	Rhizosphere	Bulk	Rhizosphere
Node	320	257	232	365	210	284
Average node degree	5.57	5.51	6.72	6.46	3.85	4.58
Hub node	12	8	27	15	0	3
Edge	891	708	779	1179	404	650
Positive edge	500	421	462	761	242	334
Negative edge	391	287	317	418	162	316
Average clustering coefficient	0.261	0.301	0.314	0.261	0.303	0.222
Average path distance	4.912	4.908	4.128	4.899	6.23	5.134
Modularity	0.66	0.662	0.488	0.628	0.756	0.659

Note: Hub node is defined as a node with high values of degree above 15 and closeness centrality above 0.25 in the network. Path distance is the length of the shortest path between two nodes within the network. Clustering coefficient is the degree of nodes tending to cluster together.

Although the network patterns in the bulk soils showed similar trends to those in the rhizosphere soils in all three stages, it was found that the number of nodes, the number of edges, and the average node degrees were much lower in stage 3 than those in the rhizosphere soil (Fig. 1C and D; Table 1). In addition, we identified nodes with high values of degree (>15) and closeness centrality (<0.25) as network hubs. It was found that 8, 15, and 3 network hubs from stage 1 to stage 3, respectively (Fig. 1E; Table S1), which mainly belonged to Actinobacteria (12 ASVs) and Acidobacteria (7 ASVs).

3.2. Dynamics of rhizosphere bacterial community composition and potential functional traits in relation to plant developmental stages

Bacterial community composition varied significantly across all three developmental stages (Fig. 2A). There was a significant enrichment of Actinobacteria in the rhizosphere as compared to the bulk soil

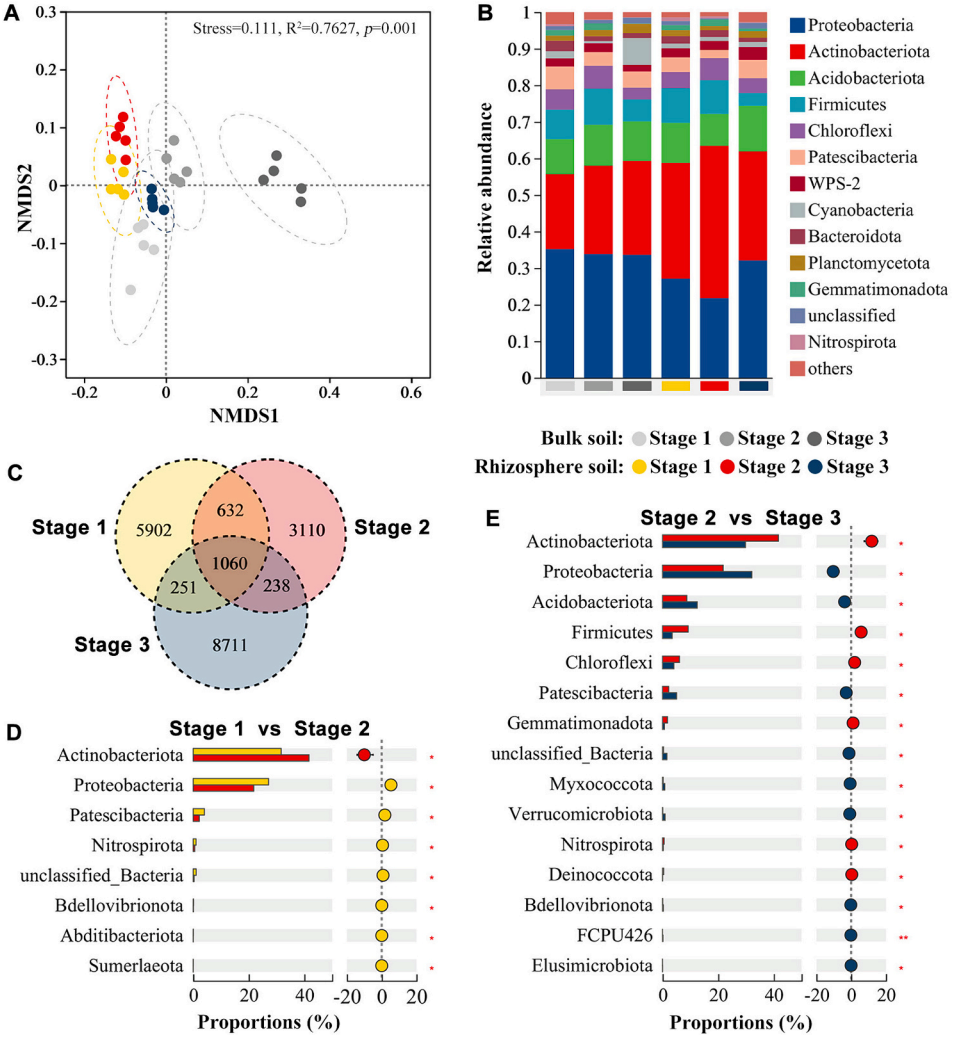

Fig. 2. Nonmetric multidimensional scaling (NMDS) analysis of bacterial community based on weighted UniFrac distance matrices (A), and the taxonomic composition of the bacterial community at the different developmental stages of blueberry (B). Venn diagrams showing the shared and specific bacterial ASVs in different developmental stages (C), significant shifted taxa on phylum level (*$p < 0.05$, **$p < 0.01$) were identified between developmental stage groups (D, E).

(Fig. S2). Although the Proteobacteria and Actinobacteria were the dominant phyla in the both bulk soils and rhizosphere, their relative abundance did not change as much in the bulk soils as in the rhizosphere at different stages (Fig. 2B). It was observed that the relative abundance of Proteobacteria was 35.2 %, 33.8 %, and 33.6 % and that of Actinobacteria was 20.5 %, 24.2 %, and 25.7 % for the three stages of bulk soil, respectively. In the rhizosphere, the relative abundance of Proteobacteria was 27.1 %, 21.8 %, and 32.1 % and that of Actinobacteria was 31.7 %, 41.7 %, and 29.8 % for the three stages, respectively. Among them, *Acidothermus* and *Conexibacter* increased significantly in stage 2, while *Actinospica* decreased significantly in stage 2, and *Chujaibacter* continuously increased significantly from stage 1 to stage 3 (Fig. S2). We then found that 5902, 3110, and 8711 ASVs were specific to stages 1 through stage 3, respectively, and with only 1060 ASVs that were shared by all the stages (Fig. 2C). From developmental stage 1 to stage 2, Actinobacteria significantly increased while the other seven phyla decreased (Fig. 2D). A total of 15 phyla were significantly altered from developmental stage 2 to stage 3, which is considerably more compared to the number of bacterial phyla that significantly changed from stage 1 to stage 2 (Fig. 2E). Among them, Actinobacteria decreased significantly, while Proteobacteria and Acidobacteria were significantly increased. At all developmental stages, the ecological functions of rhizosphere communities were primarily the chemoheterotrophy and aerobic chemoheterotrophy (Fig. 3A). The ecological function analysis showed that more functional changes were observed from stage 2 to stage 3 (Fig. 3B and C). The highest function of celluloysis related to carbon cycling was observed at stage 2, and the function related to nitrogen cycling increased significantly at stage 3.

3.3. Bacterial community assembly and dynamics of niche structure

We then performed null model analysis to determine the assembly processes of the bacterial community. The results indicate that deterministic processes ($|\beta NTI| \geq 2$) dominated the assembly of bulk soil and rhizosphere bacterial communities (Fig. 4A). The effects of deterministic processes on the rhizosphere community were relatively stronger than on the bacterial community in the bulk soil, where deterministic processes accounting for 85.7 % and 70.5 %, respectively. In addition, a significant relationship was observed between the differences in community alpha diversity and the βNTI values, with stochastic processes gradually dominating as the differences in Chao 1 index increased (Fig. 4B).

The niche breadth analysis indicated significant differences across the three developmental stages (Fig. 4C). The niche breadth of the bacterial community was markedly increased in the stage 2 suggested that broader niche breadth enables the bacterial community to become more competitive. From stage 1 to stage 2, 12.2 % of ASVs showed an increase in niche breadth, and more ASVs (27.3 %) increased from stage 2 to stage 3 (Fig. 4D and E). Despite the apparent decline in niche breadth of the bacterial community, the proportion of ASVs with enhanced niche breadth showed an increase from stage 2 to stage 3. For the four dominant bacterial phyla, Proteobacteria, Actinobacteria, Acidobacteria, and Firmicutes, the niche breadth has increased from stage 2 to stage 3 (Fig. 4F). We then calculated the niche overlap for ASVs occurring in all three stages. The proportion of ASVs increasing in niche overlap from stage 1 to stage 2, and from stage 2 to stage 3, was 55.66 % and 61.30 %, respectively (Fig. 4G and H). In comparison of

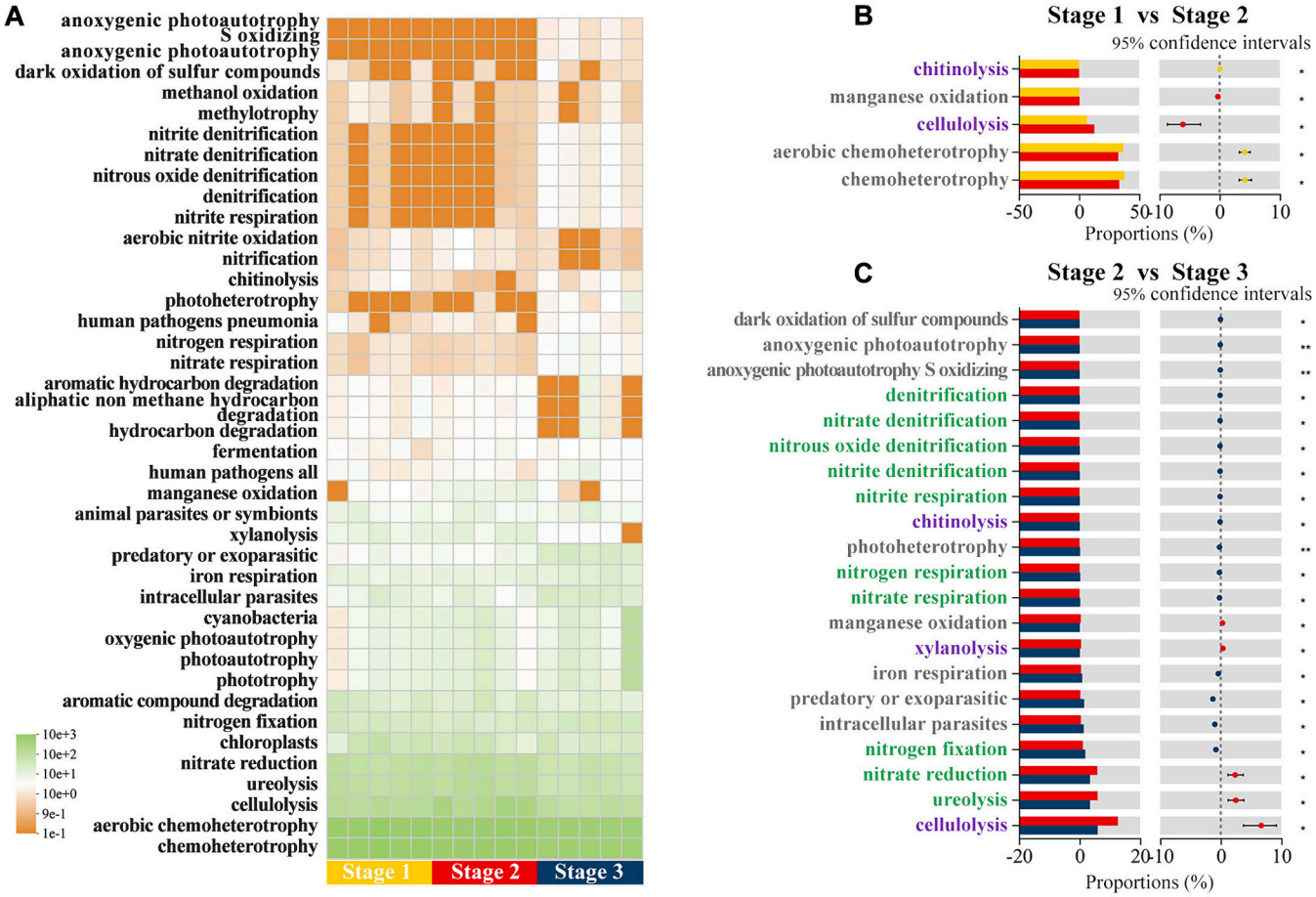

Fig. 3. Heatmap characterizing the relative abundance of ecological functions of rhizosphere bacterial community at different developmental stages (A). The significant variations (*$p < 0.05$, **$p < 0.01$) in the relative abundance of ecological functions among developmental stage groups (B, C). Purple font represents the ecological function of the carbon cycle, green font represents the ecological function of the nitrogen cycle, and gray font represents the others.

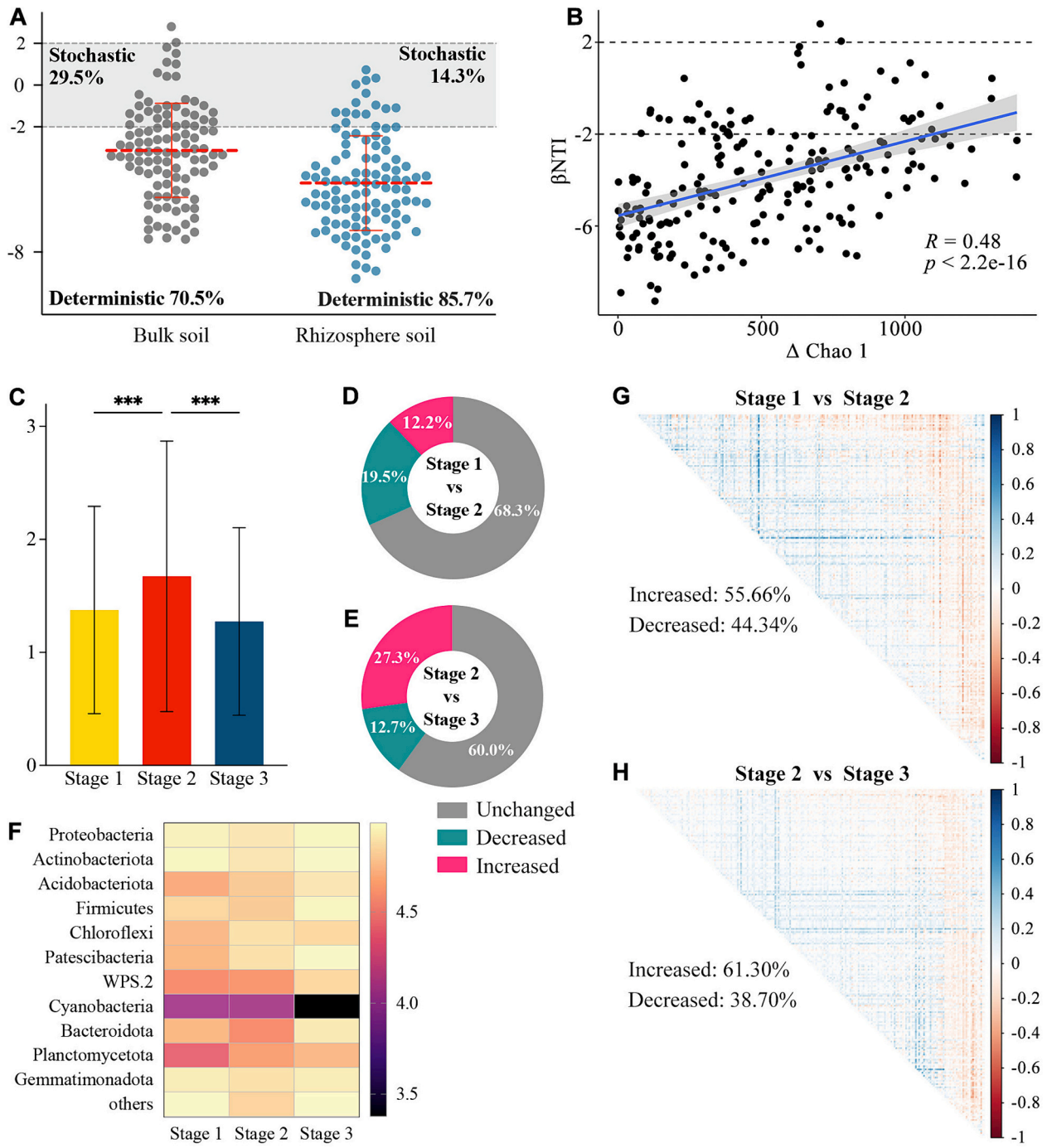

Fig. 4. Deterministic and stochastic processes in bacterial community assembly in bulk soil and rhizosphere soil (A). The β-Nearest Taxon Index (βNTI) values were calculated by using null model, and $|\beta NTI| \geq 2$ and $|\beta NTI| < 2$ represent deterministic processes and stochastic processes in driving bacterial community assembly, respectively. The relationship between βNTI and differences in bacterial community alpha diversity (△ Chao 1) for all data (B). Niche breadth analysis of rhizosphere bacterial community in different developmental stages (C), and the proportion of ASVs that varied in niche breadth across three developmental stages at the community level (D, E). Heatmap depicting the changes in niche breadth of dominant bacterial phyla (F). Niche overlap analysis of rhizosphere bacterial community across different developmental stages (G, H).

stage 1 to stage 2 and stage 2 to stage 3, the proportion of ASVs with increased niche overlap was higher, reflecting that the competition within the rhizosphere bacterial community was progressively strong. Collectively, the rhizosphere bacterial community in stage 2 had the highest niche breadth, and the proportion of ASVs with increased niche breadth and overlap followed an increasing trend with developmental stage of the plant.

3.4. Metabolic profiles of roots and its association with dynamic bacterial communities

To explore the influence of the host on the dynamics of the rhizosphere bacterial community, we proceeded to analyze the root metabolism at three different developmental stages. In total, 1500 known metabolites were identified in all samples by untargeted metabolomics approach, with 774 metabolites and 726 metabolites in positive and negative ion modes, respectively. PLS-DA results showed that distinct

variations in metabolites existed between the different stage groups (Fig. 5A and B). The results of the volcano plot indicated that 115 and 109 metabolites were significantly up- and down-regulated between stage 1 and stage 2, respectively. While 114 and 255 metabolites were markedly up- and down-regulated between stage 2 and stage 3, respectively (Fig. 5C and D). These metabolites with significant differences mainly belong to three KEGG classifications, which are compounds with biological roles, phytochemical compounds and lipids (Fig. 5E and F; Fig. S3). Among these, the number of differential compounds belonging to vitamins, nucleic acids, steroids, and lipids increased between stage 1 to stage2 and stage 2 to stage 3, while the number of differential compounds belonging to carbohydrates and peptides decreased (Fig. 5E and F).

Procrustes analyses showed a significant correlation ($p = 0.002$) between root metabolites and bacterial communities at different developmental stages (Fig. 6A). We therefore explored the correlation between differential metabolites and the abundant bacterial genera at different plant developmental stages. The relative expression levels of L-glutamic acid were positively correlated with the relative abundance of *Chujaibacter*, *Conexibacter*, and *Acidothermus* from stage 1 to stage 2, whereas indoleacrylic acid, threo-syringoylglycerol, and ferulic acid were negatively correlated with these bacterial genera (Fig. 6B). Among these, the significant increase in the abundance of *Chujaibacter* and *Conexibacter* was strongly correlated with the increase in the expression levels of L-glutamic acid and the decrease in the expression levels of indoleacrylic acid. Although there were no significant differences in the relative abundance of *Mycobacterium*, *Humibacter* and *Bacillus*, they were still observed to be associated with a few metabolites, with slightly more metabolites associated with *Bacillus* than with the other two bacterial genera. From stage 2 to stage 3, the relative abundance of *Humibacter*, *Bacillus*, *Acidothermus* and *Conexibacter* was positively correlated with the relative expression levels of (*S*)-nerolidol 3-O-[a-L-rhamnopyranosyl-(1->4)-a-L-rhamnopyranosyl-(1->2)-b-D-glucopyranoside] and gingerglycolipid B, and negatively correlated with relative expression levels of oleoside 11-methyl ester and threo-syringoylglycerol (Fig. 6C). In addition to these clearly deceased bacterial genera, significantly increased *Chujaibacter* was observed to be positively correlated with levan, oleoside 11-methyl ester, threo-syringoylglycerol, elenaic acid, psilocybine, ferulic acid, caryoptosidic acid, and vanillactic acid, while negatively correlated with isochorismate, bovinic acid, and scopoletin. In general, the significant increase in the expression levels of levan and L-glutamic acid and the significant decrease in the expression level of indoleacrylic acid were closely related to the variation of the bacterial community from stage 1 to stage 2. Moreover, the significant increase in the expression levels of levan, oleoside 11-methyl ester, and threo-syringoylglycerol and significant decrease in the expression levels of gingerglycolipid B and bovinic acid were clearly correlated with the changes in the bacterial community from stage 2 to stage 3. We further

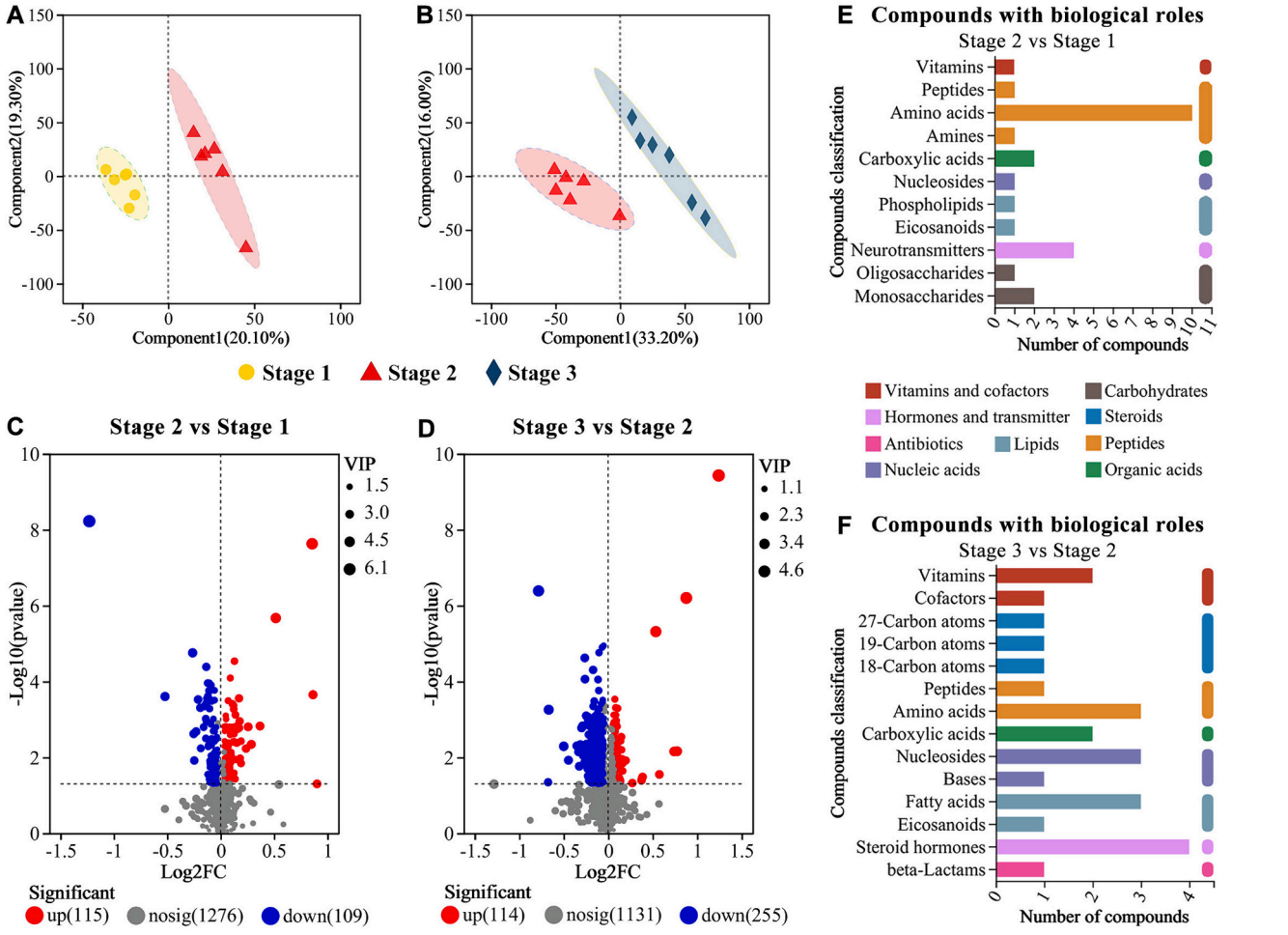

Fig. 5. Partial least squares discriminant analysis (PLS-DA) of root metabolic profiles between different developmental stages (A, B). Volcanic plot depicting the significant root metabolites among different groups (C, D). The horizontal axis represents the fold change value of the difference in the expression of metabolites between the two groups, and the vertical axis represents the statistical test value of the difference in the expression of metabolites. Each point represents a specific metabolite with the variable importance in projection (VIP) value. KEGG compound classification of differential metabolites with biological roles at different developmental stages (E, F).

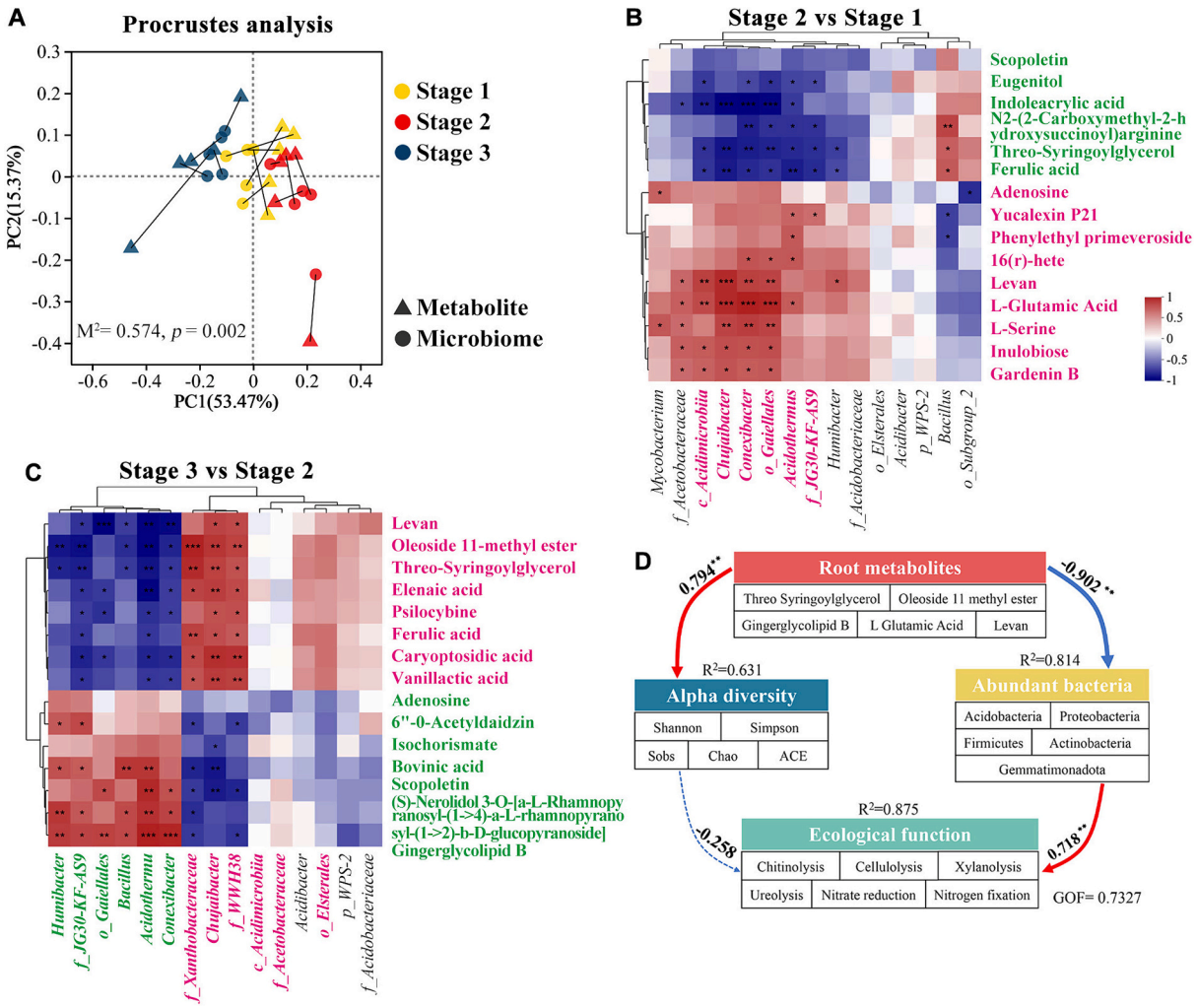

Fig. 6. Procrustes analysis between root metabolites and microbial community structure (A). Heatmap depicting the correlation between top 15 metabolites and abundant bacterial genera (B, C). The color of the grids in the heatmap indicate correlations and the symbols in the grids indicate significance (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Purple font and green font represents significant increase ($p < 0.05$) and significant decrease ($p < 0.05$) in metabolite or bacteria between two stages, respectively. Partial least squares path modeling (PLS-PM) of the correlations between root metabolites, alpha diversity, abundant bacterial phyla, and ecological functions (D). Arrow thickness indicates the strength of the effect of the path coefficient, solid and dashed lines indicating significant and non-significant correlations (*$p < 0.01$, **$p < 0.001$), and R^2 indicates the variance of the dependent variables explained by the model.

selected these metabolites with significantly increased and decreased expression levels, as well as taxa with significant changes in abundance in the bacterial community, and constructed partial least squares pathway model (Fig. 6D). The results revealed significant correlations between root metabolites and bacterial alpha diversity and abundant bacterial taxa. And these bacterial taxa, which altered significantly at different developmental stages, had a great influence on the ecological functions of the rhizosphere community associated with carbon and nitrogen cycling.

4. Discussion

4.1. Plant developmental stages influences rhizosphere bacterial community structure

The diversity and composition of rhizosphere bacterial communities changed dynamically during blueberry growth and development. Similar results were found in the other plants such as wheat, potato, peanut, maize and soybean (Anzuay et al., 2021; Chen et al., 2019; Moroenyane et al., 2021; Pfeiffer et al., 2017). Microbial diversity and balance are key factors in plant health (Berg et al., 2017). Changes in root metabolic activity may drive specific functional microbiota to colonize the rhizosphere (Rolfe et al., 2019; Vives-Peris et al., 2020; Zhong et al., 2022), leading to changes in diversity. Consequently, the ecological function of the rhizosphere microbiome may change as well. Our study found that the ecological functions of the rhizosphere bacterial communities related to carbon and nitrogen cycling changed at different blueberry developmental stages (Fig. 3B and C). Therefore, changes in microbial diversity induced by the root activities may positively contribute to beneficial effects during plant growth and development. In addition, we found the alpha diversity of Shannon and Chao 1 indices decreased from the flower and leaf bud development stage to the flower bloom stage, whereas it increased significantly at the fruit maturity stage. This trend of variation has also been observed in several studies, but there are differences between these trends. It was observed that Shannon index of *Arabidopsis* fluctuated with its growth and development, showed an increasing trend from seedling to vegetative stage and then a decreasing trend from vegetative to flowering stage, with significant lower diversity in the seedling stage (Chaparro et al., 2014). Significant shifts in the abundance and evenness of the rhizosphere bacterial community were observed as the plants matured, along with significant changes in the structure of the rhizosphere bacterial community at the flowering stage, suggesting a stage-dependent effect in the rhizosphere bacterial community of two broomcorn millet

cultivars (Na et al., 2019). Moreover, such differences exist not only between different plants, but even between different species of the same plant. It was observed that bacterial diversity in the rhizosphere of common grassland species showed distinct patterns. During ten weeks of cultivation, a significant increase in diversity within the rhizosphere of *Holcus lanatus* was found at week 9, whereas no change was observed for *Jacobaea vulgaris* (Steinauer et al., 2023). All these findings suggest that such differences may be due to the diverse characteristics of the metabolic activities of the roots in terms of plant species and growth and development, which in turn lead to variations in its specific microbiome.

Plant secretes different compounds and particular phytochemicals in root exudates at distinct stages of growth and development, which contribute to the colonization of rhizosphere microorganisms (Chaparro et al., 2014). It was found that each plant was able to alter the specialized bacterial community in its rhizosphere faster than the fungal community, despite detectable effects from the former plant (Hannula et al., 2021). Meanwhile, the quantity, quality and exudation rate of root exudates varied with plant growth and development, thus affecting the growth of specific microorganisms colonized in the rhizosphere, particularly fast-growing microorganisms, and potentially leading to structural differentiation (Hannula et al., 2021; Philippot et al., 2013). Our results showed that the composition of the rhizosphere bacterial community differed significantly among the different developmental stages of blueberry. In this regard, Proteobacteria and Actinobacteria were the most dominant phylum of the rhizosphere and varied markedly across developmental stages, likely as a consequence of the substrate-driven community responsible for root secretion of photoassimilates (Bulgarelli et al., 2013). Though Proteobacteria are typically fast-growing and able to utilize a wide range of root-derived carbon substrates (Philippot et al., 2013; Quijia Pillajo et al., 2024), it was found significant enriched in the bulk soil (Fig. S2). As a commonly represented phylum, Proteobacteria are involved in the cycling of soil carbon, nitrogen and sulfur (Spain et al., 2009), and are therefore important for plant utilization of the bioavailable soil nutrients. In contrast, Actinobacteria was significant abundant in the rhizosphere (Fig. S2). It has been reported that Actinobacteria are able to promote crop growth and yield, which makes them eco-friendly alternatives not only for agriculture but also for humankind (Boubekri et al., 2022).

4.2. Dynamic interactions of bacteria in the rhizosphere microbial communities

Host effects generated by plants can modulate the structure of microbial communities that utilize root exudates, and microbe-microbe interactions in such microhabitats affected by root exudates can also shape their holistic community structure to some extent (Feng et al., 2023). Our results suggested that the complexity of the rhizosphere bacterial network was strongly influenced by the developmental stage of the blueberry plant, which is in line with previous findings (Ma et al., 2021; Xiong et al., 2021). Both macrobiological and microbiological studies suggested that resource and food availability are critical drivers of social network structure, which was observed in rhizosphere potentially fostering more direct and indirect interactions (Shi et al., 2016). In our study, it was found that the number of nodes and edges, average node degrees, and hub nodes were higher at stage 2. This may indicate that rhizosphere deposits regulate more complex and intense interactions of bacterial communities at this stage of blueberry development. Lower average path distances and modularity were also observed, suggesting that there were more external than internal linkages of network modules, and thus possibly related to lower niche differentiation in the stage 2 (Zhang et al., 2018b). It was found that root exudate β-1,4-glucosidase was negatively correlated with network topological characteristics such as modularity, average path length, and network diameter (Hao et al., 2022). Acidobacteria was able to supply energy to other microorganisms by degrading polysaccharides such as cellulose (Lladó et al., 2016), which was found to be essential in each of the co-occurrence network modules in the presence of β-1,4-glucosidase (Hao et al., 2022). This suggests that root exudates have the potential to influence the portion of microorganisms that can superiorly consume particular exudates, thereby altering microbe-microbe interactions in the rhizosphere. Therefore, the changes in the co-occurrence network of bacterial communities observed at different stages of blueberry growth and development may be due to the fact that some bacterial communities with specific utilization preferences were affected by root metabolites, which may trigger the changes in microbe-microbe interactions. To this end, we also demonstrated that the mutually beneficial symbiotic and competitive relationships of the rhizosphere bacterial community altered during the growth and development of blueberry plants by refining the ecological niche breadth and overlap. Modern coexistence theory characterizes coexistence between two species by identifying the relative degree of ecological niche overlap and differences in competitive ability, where species may coexist due to differences in their utilization of resources or the same competitiveness under partial overlap of resources utilization (Pastore et al., 2021). The degree of ecological niche overlap is therefore regulated by the resources that can regulate the growth of their populations. Despite the increased proportion of microorganisms with improved niche breadth as blueberry plants grow and develop, our study observed a narrowing of niche breadth and a growing niche overlap. This suggests that, on the one hand, blueberry root metabolites dynamically regulate rhizosphere bacterial populations as a resource for microbial growth, and on the other hand, the regulated microbiomes are increasingly interacting with each other and becoming more tightly connected.

4.3. Root metabolites mediate complex plant-microbe interactions in the rhizosphere

Our study showed root metabolites of levan, L-glutamic acid, indoleacrylic acid, oleoside 11-methyl ester, threo-syringoylglycerol, gingerglycolipid B, and bovinic acid were closely related to the variation of the bacterial community during blueberry growth. Our results showed the relative abundance of *Chujaibacter* was significant increased from stage 1 to stage 3 (Fig. S2). From stage 1 to stage 2, significantly increased *Chujaibacter* was positively correlated with L-glutamic acid, levan, L-serine, inulobiose, and gardenin B, while from stage 2 to stage 3, it was positively correlated with levan, oleoside 11-methyl ester, threo-syringoylglycerol, elenaic acid, psilocybine, ferulic acid, caryoptosidic acid, and vanillactic acid (Fig. 6). Of these, levan increased significantly from stage 1 to stage 3, which may have contributed more to the significant increase in the relative abundance of *Chujaibacter* compared to the other metabolites. Levan are well known to play an essential role in the establishment of symbiotic interactions between beneficial bacteria of *Bacillus subtilis* and host plants (Dogsa et al., 2013). The effective colonization is the key to promoting plant growth and protecting plants from pathogens. It has been reported that levan as one of the chemical signals in the signaling cascade leading to the efficient colonization of rhizosphere by *Bacillus subtilis* (Tian et al., 2021). In addition, they contribute to the stabilization of microhabitats in the rhizosphere and assist in the establishment of a microaerobic habitat conducive to nitrogen fixation by offering barriers to oxygen diffusion, thus achieving their prebiotic potential (Nasir et al., 2022). It has been reported that inoculation with plant beneficial bacteria significantly increased the relative abundance of *Chujaibacter* (Chen et al., 2021), and *Chujaibacter* was found significantly positively correlated with leaf length and width at the fruiting stage of cucumber (Zhang et al., 2023). Therefore, levan may contribute to the abundance variation of *Chujaibacter*, positively affecting the blueberry plant during its growth and development. L-glutamic acid plays a critical role in abiotic stress by alleviating the oxidative stress induced by hydrogen peroxide in *Streptococcus pentosporus* R1 through direct antioxidant effects and increasing the activity of bacterial antioxidant enzymes (Zhang et al., 2022). It also markedly increased the colonization capacity of *Streptomyces globisporus* in tomato

rhizosphere, providing tolerance to salt stress and facilitating plant growth (Kim and Kwak, 2023). However, for pathogenic bacteria, L-glutamic acid triggers the pathogen to develop negative effects on the host plant. *Ralstonia solanacearum* is a widespread bacterial pathogen, and studies have shown that L-glutamic acid promotes its colonization of roots and stems of tomato plants, thus accelerating the development of the disease (Shen et al., 2020). Previous studies have shown that under various grazing stresses, root metabolites dynamically regulate beneficial microbes, which may be secreted as a source of nutrients and energy for particular microbes, thus triggering the enrichment of beneficial rhizobacteria (Yuan et al., 2023b). Among them, indoleacrylic acid was significantly associated with changes in part of the rhizosphere bacterial community, which is consistent with our findings. Gingerglycolipid B has been observed to be a regulator of sugarcane growth autoregulation factors and microbial interspecies interactions, thereby affecting the availability of fertilizers in the soil (Yuan et al., 2023a). Despite the lack of studies on the interaction of bovinic acid with microorganisms in plant microbiomes, it has been noted that bovinic acid interacts with *Sphaerochaeta* spp. to provide beneficial effects for the host animal (Wang et al., 2023). Taken together, our study shows that specific blueberry root metabolites are closely associated with changes in rhizosphere bacterial communities during blueberry growth and development. However, the functions of specific root metabolites in regulating rhizosphere bacterial communities need to be further explored.

5. Conclusions

During blueberry growth and development, root metabolic activities play a crucial role in the dynamics of rhizosphere bacterial community structure. It was observed that the diversity and composition of rhizosphere communities varied significantly at different developmental stages. Such variations in diversity and composition may potentially affect their ecological functions related to carbon and nitrogen cycling, which in turn influence the uptake of soil nutrients by the roots. Meanwhile, the co-occurrence networks showed that the complexity of microbial interactions varied at different developmental stages. In stage 2, the rhizosphere bacterial community had the highest niche breadth, and the proportion of ASVs with increased niche breadth and overlap tended to increase with plant development. Untargeted metabolome revealed significant up- and down-regulation of metabolites at different developmental stages, which belonged to the three KEGG classifications of compounds with biological roles, phytochemical compounds, and lipids, respectively. Of these, the number of differential compounds for vitamins, nucleic acids, steroids, and lipids increased between stage 1 to stage 2 and stage 2 to stage 3, while those for carbohydrates and peptides decreased. It was found that significant changes in expression levels of levan, L-glutamic acid, indoleacrylic acid, oleoside 11-methyl ester, threo-syringoylglycerol, gingerglycolipid B, and bovinic acid were strongly correlated with changes in the bacterial community at different stages. Since they are closely associated with changes in bacterial communities, some of them can be considered as candidates for regulating the structure of beneficial bacterial communities. These findings deepen our fundamental understanding of plant-microbe interactions during the blueberry growth and development, and provide new insights into the utilization of root metabolites exert beneficial microbial effects to improve plant health and productivity.

CRediT authorship contribution statement

Jilu Che: Writing – original draft, Visualization, Investigation, Data curation, Conceptualization. **Yaqiong Wu:** Writing – review & editing, Supervision, Conceptualization. **Hao Yang:** Writing – review & editing, Investigation, Data curation. **Ying Chang:** Writing – review & editing, Investigation, Data curation. **Wenlong Wu:** Investigation, Data curation. **Lianfei Lyu:** Investigation, Data curation. **Xiaomin Wang:** Investigation, Data curation. **Fuliang Cao:** Writing – review & editing, Supervision, Conceptualization. **Weilin Li:** Writing – review & editing, Supervision, Conceptualization.

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 sequencing reads supporting this study have been submitted to the National Center for Biotechnology Information Sequence Read Archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/) under the accession number PRJNA1102520.

Acknowledgement

This research was supported by the “JBGS” Project of Seed Industry Revitalization in Jiangsu Province (JBGS [2021]021), the Key Project of Modern Agriculture of Science and Technology Department of Jiangsu Province (BE2022372), the Earmarked Fund for Jiangsu Agricultural Industry Technology System (JATS [2023]466), the Chinese Central Financial Project for Cooperative Extension of Major Agricultural Technology (2022-ZYXT-06), the Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX23_1248), and the China Scholarship Council University Postgraduate Programme (202308320316).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.174333.

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