



## Article

# Rhizosphere Microbiota Shifts Correlate with Nutrient Composition of Soils and Fruit Metabolite Content in Highbush Blueberry (*Vaccinium corymbosum* L.) Under Different Cultivation Systems

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## Abstract

This study investigated highbush blueberry (*Vaccinium corymbosum* L.) plants cultivated in distinct cultivation systems (greenhouse vs. open field) to determine if they exhibited significant differences in rhizosphere microbiota, soil nutrient profiles, and fruit metabolites. A clear metabolic trade-off was observed: open-field cultivation significantly enhanced fruit secondary metabolites, including anthocyanins (9.5% higher), flavonoids (56.0% higher), and ascorbic acid (15.6% higher). In contrast, greenhouse fruits were enriched in primary metabolites such as water-soluble sugars (28.3% higher) and total organic acids (30.2% higher) ( $p < 0.01$  for all comparisons). These divergent metabolite profiles were correlated with distinct rhizosphere microenvironments. The open field soil exhibited higher organic carbon and microbial  $\alpha$ -diversity, while the greenhouse soil was characterized by a niche with high availability of cations, lower pH, higher electrical conductivity, and elevated levels of exchangeable  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and available potassium. These contrasting niches were correlated with shifts in the rhizosphere microbiota assembly. Notably, the greenhouse soil was associated with a higher relative abundance of copiotrophic bacterial taxa such as *Streptomyces* and *Bacillus*, whose abundances showed strong positive correlations with cation availability (e.g., *Streptomyces* vs.  $\text{Ca}^{2+}$ , correlation coefficient  $r = 0.827$ ,  $p < 0.01$ ). Multivariate analysis integrated these patterns, revealing that soil cations were negatively correlated with fruit antioxidants but positively linked to sugars and acids. This correlative study suggests that cultivation systems are strongly associated with fruit quality, potentially through their association with functionally specific rhizosphere microbiota that covaries with a shift in the plant's resource allocation between growth (primary metabolism) and defense (secondary metabolism). Our findings provide an integrative framework for understanding how agricultural practices are associated with the soil–plant–microbe continuum to correlate with crop quality in perennial systems and generate testable hypotheses for future mechanistic research.



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**Keywords:** cultivation system; agroecosystem; habitat filtering; plant-microbe interactions; primary and secondary metabolism; perennial fruit crops; agro-cultivation systems management

## 1. Introduction

Highbush blueberry (*Vaccinium corymbosum* L.), renowned for its flavorful and nutrient-rich fruits, thrives in specific acidic soil conditions facilitated by its fine, fibrous root system [1]. In perennial fruit crops like blueberry, the rhizosphere microbiota is not merely a transient community but a stable interface critical for long-term nutrient acquisition and stress resilience [2]. The assembly of this microbiota is a product of both the soil physicochemical matrix, which acts as a primary “habitat filter”, and host plant selection [3]. Consequently, agricultural practices that alter the soil environment are potent drivers of rhizosphere community restructuring, with potential cascading effects on crop performance and quality [4].

In perennial cropping systems, the contrast between protected (e.g., greenhouse) and open-field cultivation represents a profound cultivation systems-level intervention. Evidence from perennial fruits demonstrates that such management shifts consistently are associated with changes in the re-engineer the rhizosphere microenvironment. Protected cultivation is often associated with soil acidification, salinization, and the accumulation of soluble cations (e.g.,  $K^+$ ,  $Ca^{2+}$ ,  $Mg^{2+}$ ), creating a high-availability, cation-rich nutrient niche [5]. This niche is associated with a higher relative abundance of copiotrophic bacterial taxa such as *Bacillus* and *Streptomyces*, which thrive in resource-rich conditions and possess plant growth-promoting traits [6,7]. Experimental studies have suggested associations between these community shifts and altered soil properties under protected environments [8]. Conversely, open-field systems typically maintain higher soil organic matter, greater physicochemical heterogeneity, and exposure to environmental fluctuations, fostering more diverse microbial communities with a higher abundance of oligotrophic taxa like Acidobacteriota, which are adapted to slower nutrient mineralization cycles [9,10].

The metabolic profile of a plant represents a functional readout of its physiological state and adaptive strategy. Primary metabolites, such as sugars, organic acids, and amino acids, are directly involved in fundamental processes like growth, development, and energy metabolism. In contrast, secondary metabolites, including phenolic compounds (e.g., anthocyanins, flavonoids) and ascorbic acid, are primarily synthesized for plant defense against abiotic (e.g., UV radiation, temperature extremes) and biotic (e.g., pathogens, herbivores) stresses. These compounds are crucial for plant adaptation and resilience in fluctuating environments. Importantly, in fruit crops like blueberry, these secondary metabolites are also key determinants of fruit nutritional quality and health benefits for consumers, making their accumulation a critical target for quality-oriented cultivation. The biosynthesis of secondary metabolites is energetically costly and is theorized to trade off with resources allocated for growth and primary metabolism—a central tenet of the plant growth–defense trade-off theory.

Building upon this metabolic framework, these shifts in microbial communities associated with cultivation systems are not merely compositional but are hypothesized to be functional. The enrichment of copiotrophic, plant growth-promoting rhizobacteria (PGPR) in stabilized environments is associated with enhanced nutrient solubilization and auxin production, potentially correlating with a shift in plant resource allocation toward growth and primary metabolism [11,12]. In contrast, the diverse and stress-associated microbiota of open fields, along with abiotic factors like UV exposure, may act as mild biotic/abiotic elicitors, priming jasmonic acid and salicylic acid pathways and upregulating plant defense and secondary metabolism [13,14]. This aligns with the central ecological theory of a plant growth–defense trade-off [15]. While correlations between soil microbes and blueberry fruit quality exist [16], and the separate links between cultivation→microbiota and microbiota→plant physiology are established in model systems, an integrated understanding of how the cultivation systems are associated with a functionally specific

microbiota that in turn correlates with a systematic fruit metabolite trade-off in a perennial crop remains elusive.

Therefore, we hypothesize that the greenhouse and open-field cultivation systems are associated with distinct rhizosphere physicochemical niches distinct rhizosphere physicochemical niches (e.g., a cation-rich niche vs. a higher-organic-carbon niche) that may act as habitat filters, which are associated with divergent microbial communities (copiotrophic-dominated vs. diverse/oligotrophic-enriched). Furthermore, these distinct microbiotas, coupled with the prevailing soil nutrient availability, may be associated with a shift in the host plant's metabolic strategy, correlating with a shift in measurable trade-off in blueberry fruit profiles—favoring primary metabolites (sugars, organic acids) in greenhouses and secondary metabolites (anthocyanins, flavonoids, ascorbic acid) in open fields.

This study integrates time-series rhizosphere microbiota analysis, soil nutrient profiling, and fruit metabolite analysis in blueberry. While our approach is observational and treats each cultivation system as an integrated 'management package', it is designed to document whether a coherent, system-level association exists along the cultivation–soil–microbiota–fruit metabolite continuum. The resulting framework aims to provide a framework for developing testable mechanistic hypotheses for how agricultural practices are associated with crop quality through the rhizosphere microbiota.

## 2. Materials and Methods

### 2.1. Collection of Soil Samples

Rhizosphere soil samples were collected from the roots of southern highbush blueberry (*Vaccinium corymbosum* L.) plants cultivated under open field and greenhouse conditions at distinct growth stages according to Wang et al. [9]. Specifically, soil was collected from a depth of 5–20 cm around the base of blueberry plant trunks, following the same protocol. The sampling site was located at 33°12'01" N–33°12'10" N, 107°34'29" E–107°34'49" E, with an elevation of 623.5–624.2 masl. All sampled plants were mature, 3-year-old southern highbush blueberry bushes that had been established under their respective cultivation systems (greenhouse or open field) since planting. Sampling times and corresponding sample designations as well as the monthly average temperature at the time of sample collection are provided in Table 1. Relative humidity in greenhouse system was maintained at a level of 60–70%, whereas, in open-field system, irrigation regimes were modulated according to actual weather conditions to maintain appropriate soil moisture levels around the rhizosphere. For each cultivation method and at each sampling time, three biological replicate soil samples were collected. Sampling points were distributed throughout the cultivation area to ensure that replicates were spatially independent. Three replicate soil samples were collected from three individual blueberry plants spaced at least 5 m. The soil from each sampling point was thoroughly homogenized to form a composite sample and stored at 4 °C prior to analysis.

**Soil characteristics:** The experimental site featured a typical sandy loam soil. The key physicochemical properties of the bulk soil (0–20 cm depth), determined prior to the establishment of the cultivation systems, were as follows: pH (H<sub>2</sub>O, 1:2.5), 5.2 ± 0.1; organic matter content, 1.85 ± 0.15%; total nitrogen (TN), 0.92 ± 0.08 g kg<sup>-1</sup>; available phosphorus (AP, Olsen-P), 15.2 ± 1.5 mg kg<sup>-1</sup>; and available potassium (AK), 85 ± 7 mg kg<sup>-1</sup> (values are mean ± standard deviation, *n* = 3). These baseline properties indicate an acidic, moderately fertile soil representative of the local conditions and are within the suitable range for southern highbush blueberry cultivation. These baseline properties were determined from composite soil samples collected across the entire experimental field prior to the establishment of the greenhouse and open field plots, confirming that the initial soil conditions were homogeneous between the two cultivation systems.

**Table 1.** The sampling time and sample names.

Sampling Time	Sample Names		The Monthly Average Temperature	
	Blueberry Plants Grown in Open Fields	Blueberry Plants Grown in Greenhouses	Blueberry Plants Grown in Open Fields (°C)	Blueberry Plants Grown in Greenhouses (°C)
September, 2024	L09	D09	26.50 ± 6.35	25.07 ± 0.96
November, 2024	L11	D11	11.06 ± 5.96	20.71 ± 2.14
January, 2025	L01	D01	3.76 ± 6.51	18.28 ± 3.15
March, 2025	L03	D03	10.76 ± 7.40	19.90 ± 2.81
May, 2025	L05	D05	22.84 ± 7.76	22.55 ± 2.56
July, 2025	L07	D07	29.05 ± 5.59	25.77 ± 1.34

Light regime: The open-field system relied solely on natural sunlight under ambient photoperiod conditions. In the greenhouse system, plants received natural sunlight transmitted through the polyethylene covering. No supplemental artificial lighting was used in the greenhouse throughout the experiment.

During the experiment, divergent fertilization and irrigation regimes were applied according to standard practices for each system. The greenhouse system received integrated drip irrigation and fertilization, with a compound fertilizer (N-P<sub>2</sub>O<sub>5</sub>-K<sub>2</sub>O: 20-20-20) applied at a rate of approximately 25 kg ha<sup>-1</sup> every 4–6 weeks during the growing season. The open-field system relied on a combination of overhead irrigation and controlled-release fertilizer applied biannually in spring and early summer, with an annual total equivalent to the greenhouse input. These management differences are intrinsic to the cultivation systems and are the primary drivers of the divergent rhizosphere microenvironments investigated in this study.

## 2.2. Collection and Nutritional Components Analysis of Blueberry Sample

Harvest times were different between the two cultivation methods. The greenhouse-grown blueberries were harvested in March 2025, while the open field blueberries were harvested in May 2025. Fruits were harvested at commercial maturity, determined by full blue skin coloration and uniform firmness. Three independent biological replicates were established for each cultivation system, with each replicate comprising a composite sample of approximately 100 fresh fruits randomly collected from 10 individual plants across the cultivation area.

Total anthocyanin (TA) content was determined using the pH differential method [10]. Briefly, homogenized fruit tissue was extracted with acidified methanol (1% HCl, *v/v*) at 4 °C in the dark for 24 h. The absorbance of the supernatant was measured at 520 nm and 700 nm in buffers at pH 1.0 (potassium chloride) and pH 4.5 (sodium acetate). The concentration was calculated using a molar extinction coefficient of cyanidin-3-glucoside ( $\epsilon = 26,900 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ ) and expressed as cyanidin-3-glucoside equivalents. Total flavonoid (TF) content was measured by the aluminum chloride colorimetric assay [11]. An aliquot of the extract was mixed with an equal volume of 2% AlCl<sub>3</sub> (in methanol). After incubation for 30 min at room temperature, the absorbance was measured at 415 nm. A standard curve was prepared using rutin, and results were expressed as mg rutin equivalent per gram fresh weight. Reducing sugar (RS) content was quantified spectrophotometrically [12]. The reaction mixture was heated in a boiling water bath for 5 min, cooled, and the absorbance was read at 540 nm. D-Glucose was used as the standard. Total amino acid (TAA) content was determined spectrophotometrically [13]. The extract was reacted with ninhydrin reagent in a boiling water bath for 15 min. After cooling, the absorbance was measured at 570 nm. Glycine was used to generate the standard curve.

Water-soluble sugar (WSS) and total organic acid (TOA) contents were analyzed using high-performance liquid chromatography [14]. For sugars, separation was performed on a carbohydrate analysis column (Waters XBridge Amide, 3.5  $\mu\text{m}$ , 4.6  $\times$  150 mm) with a refractive index detector. The mobile phase was acetonitrile–water (75:25, *v/v*) at a flow rate of 1.0 mL/min. Sucrose, D-glucose, and D-fructose were used as standards. For organic acids, separation was achieved on a C18 reverse-phase column (Agilent ZORBAX SB-Aq, 5  $\mu\text{m}$ , 4.6  $\times$  250 mm) with a UV detector set at 210 nm. The mobile phase was 0.01 M potassium phosphate buffer (pH 2.8) at a flow rate of 0.8 mL/min. Citric, malic, and quinic acids were used as primary standards. Soluble solids (SS) content was determined using near-infrared spectroscopy [15]. The instrument was calibrated using standard sucrose solutions prior to use. Homogenized fruit puree was scanned directly, and the soluble solids content was derived from the built-in prediction model. Reduced ascorbic acid (RAA) content was quantified by a spectrophotometric colorimetric method [12]. The fruit extract in 2% metaphosphoric acid was reacted with the dye, and the decrease in absorbance at 518 nm was measured. L-Ascorbic acid was used as the standard.

### 2.3. Analysis of Microbial Community

Soil microbial communities were characterized using high-throughput amplicon sequencing. A total of 36 rhizosphere soil samples were analyzed (2 cultivation systems  $\times$  6 time points  $\times$  3 biological replicates).

Total genomic DNA was extracted from 0.5 g of soil using the FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA) following the manufacturer's instructions. The V4 region of the bacterial 16S rRNA gene was amplified using the primer pair 515F (5'-GTGYCAGCMGCCGCGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3'). For fungal community analysis, the ITS2 region was targeted using the primer pair ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3').

PCR amplifications were performed in a Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The bacterial 16S rRNA gene amplification protocol consisted of initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The fungal ITS amplification utilized the same thermal cycling conditions.

Amplicons from all samples were purified, quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and pooled in equimolar ratios. Sequencing libraries were constructed using the NEBNext Ultra DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). Paired-end sequencing (2  $\times$  250 bp) was performed on an Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA).

Raw demultiplexed sequences were quality-filtered, denoised, and merged into amplicon sequence variants (ASVs) using the DADA2 pipeline [17] within QIIME2 (version 2021.11) [18]. Taxonomic classification was performed against the SILVA 138 database [19] for bacteria and the UNITE 8.0 database [20] for fungi. Alpha diversity indices (Chao1, Shannon, and Simpson) were calculated using QIIME2 [18]. Beta diversity was assessed through principal coordinates analysis (PCoA) based on Bray–Curtis distances. Differential abundance analysis was performed using linear discriminant analysis effect size (LEfSe) with an LDA score threshold of 2.0 [21].

### 2.4. Determination of Nutritional Elements in Rhizosphere Soil

Organic carbon (OC) content, total nitrogen (TN) content, total phosphorus (TPH) content, total potassium (TPO) content, hydrolysable nitrogen (HN) content, available phosphorus (APH) content, and available potassium (APO) content in the samples collected on

day 10 after root irrigation were determined as the methods described by Wang et al. [9]. Exchangeable cations ( $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ ) were extracted using 0.1 M  $\text{BaCl}_2$  and measured by flame photometry [16]. After soil sample was mixed with water at a soil–water ratio of 1:2.5, then pH and electrical conductivity (EC) measurements were performed using a Hanna Instruments HI9813-6 (Woonsocket, RI, USA) portable pH/EC meter [22].

### 2.5. Statistical Analysis

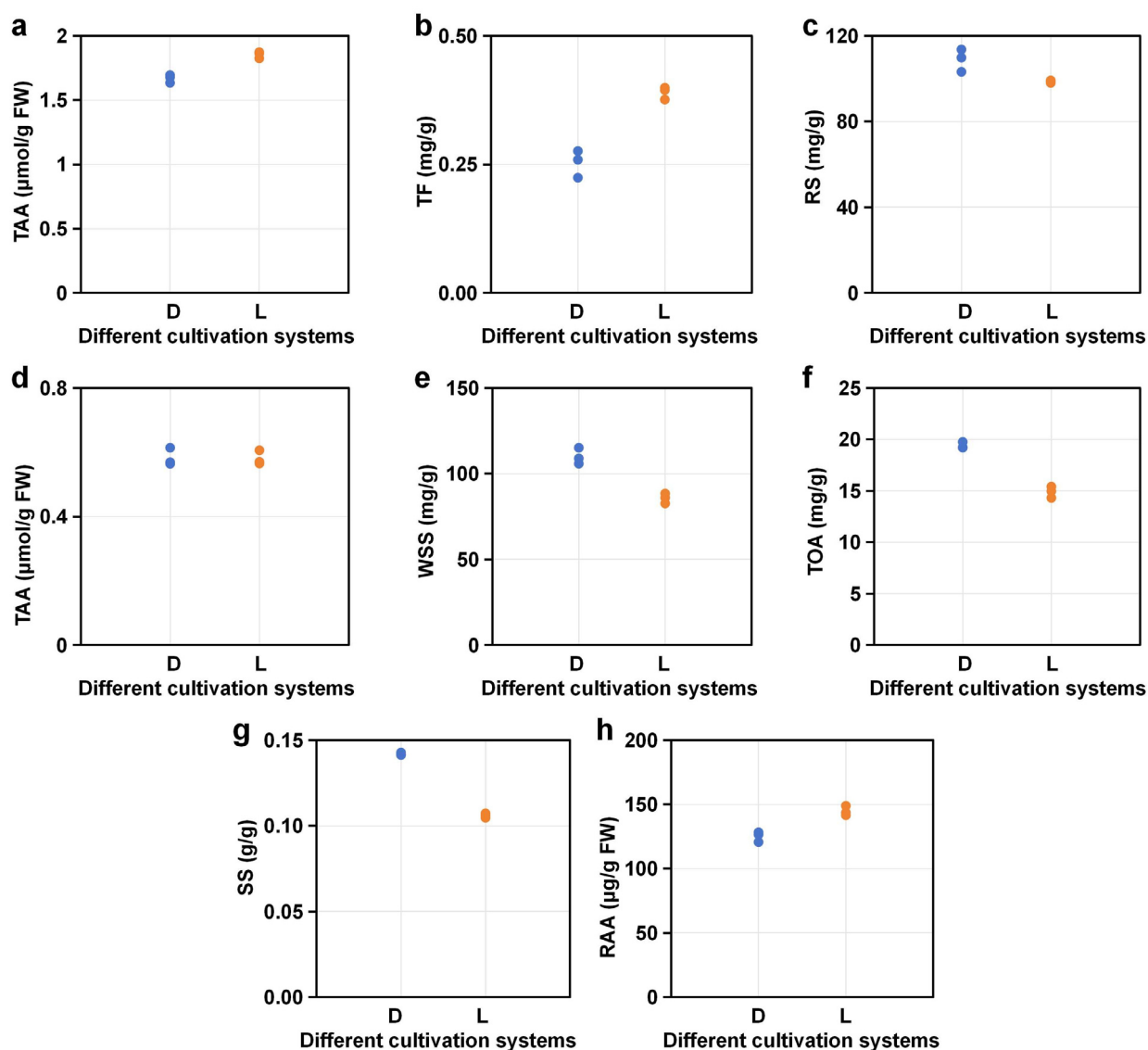
All experiments were conducted with three biological replicates. Data are presented as mean  $\pm$  standard deviation (SD). To assess the significant differences in soil physicochemical properties, fruit metabolites, and microbial alpha diversity indices between the two cultivation systems (greenhouse vs. open field), independent two-sample Student's *t*-tests were performed using SPSS Statistics 23.0 (IBM Corp., Armonk, NY, USA). A *p*-value of less than 0.05 was considered statistically significant. The statistical methodology for microbial beta diversity and differential abundance analysis (e.g., PCoA, LEfSe) is described in Section 2.3. Multivariate correlations (e.g., Spearman's) and PCA were conducted using R (version 4.1.0).

For the integrated correlation analysis, fruit metabolite data (collected at harvest maturity: March 2025 for greenhouse, May 2025 for open field) were matched with corresponding rhizosphere soil physicochemical and microbial data from the sampling time point immediately preceding each harvest. Specifically, greenhouse fruit data were paired with soil data from March 2025 (D03), and open field fruit data were paired with soil data from May 2025 (L05). This temporal alignment ensures that soil conditions reflect the nutrient availability during the critical period of fruit development. The statistical unit for all correlation and PCA analyses was the biological replicate, with three replicates per cultivation system (total  $n = 6$ ). Pearson correlation coefficients were calculated to assess linear relationships between variables.

## 3. Results

### 3.1. Divergent Fruit Metabolic Profiles in Blueberries Cultivated in the Two Systems

Metabolic profiling revealed a clear trade-off between primary and secondary metabolism that was statistically significant and aligned with the cultivation system (Figure 1). As shown in Figure 1a, open-field cultivation resulted in significantly higher total anthocyanin content ( $1.85 \pm 0.03 \mu\text{mol/g}$ ) compared to greenhouse cultivation ( $1.69 \pm 0.03 \mu\text{mol/g}$ ;  $p = 0.002$ ). Similarly, total flavonoids (Figure 1b) were higher in open-field fruits ( $0.39 \pm 0.01 \text{ mg/g}$ ) than in greenhouse fruits ( $0.25 \pm 0.03 \text{ mg/g}$ ;  $p = 0.007$ ). In contrast, reducing sugars (Figure 1c) were significantly higher in greenhouse blueberries ( $108.87 \pm 5.28 \text{ mg/g}$ ) than in open-field blueberries ( $98.64 \pm 0.59 \text{ mg/g}$ ;  $p = 0.003$ ). Total amino acid content (Figure 1d) did not differ between the two cultivation systems (both  $0.58 \mu\text{mol/g FW}$ ;  $p = 0.07$ ). Water-soluble sugars (Figure 1e) were also higher in greenhouse fruits ( $109.96 \pm 4.73 \text{ mg/g}$ ) than in open-field fruits ( $85.70 \pm 2.91 \text{ mg/g}$ ;  $p = 0.006$ ). Total organic acids (Figure 1f) were elevated in greenhouse fruits ( $19.39 \pm 0.31 \text{ mg/g}$ ) compared to open-field fruits ( $14.89 \pm 0.55 \text{ mg/g}$ ;  $p < 0.001$ ). Soluble solids content (Figure 1g) was higher in greenhouse ( $0.14 \pm 0.00 \text{ g/g}$ ) than in open-field ( $0.11 \pm 0.00 \text{ g/g}$ ;  $p < 0.001$ ). Finally, reduced ascorbic acid (Figure 1h) was significantly higher in open-field fruits ( $144.71 \pm 3.80 \mu\text{g/g}$ ) than in greenhouse fruits ( $125.19 \pm 3.99 \mu\text{g/g}$ ;  $p = 0.003$ ). This metabolic divergence indicates a system-level reprogramming of blueberry plant metabolism.



**Figure 1.** Nutritional composition comparison of highbush blueberry (*V. corymbosum*) fruits grown in greenhouse (D) and open field (L) cultivations. Blue bars represent fruits from greenhouse (D), orange bars represent fruits from open field (L). (a) Total anthocyanin (TA) content; (b) Total flavonoids (TF); (c) Reducing sugars (RS); (d) Total amino acids (TAA); (e) Water-soluble sugars (WSS); (f) Total organic acids (TOA); (g) Soluble solids (SS); (h) Reduced ascorbic acid (RAA). Notes: Values are presented as mean  $\pm$  standard deviation. Different letters on bars in each group indicate that the data are significantly different between cultivation methods, independent two-sample *t*-test).

### 3.2. Cultivation Systems Are Associated with Rhizosphere Microbiota Assembly and Diversity

Microbial community analysis revealed distinct differences in the composition and diversity of rhizosphere microorganisms between greenhouse-cultivated (D) and open field-grown (L) blueberry plants. The raw amplicon sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession numbers PRJNA1427202 for 16S and PRJNA1428922 for ITS. At the phylum level, the bacterial communities in both cultivation systems were dominated by Proteobacteria, Actinobacteriota, Acidobacteriota, Chloroflexi, and Bacillota (Figure 2a). A total of 810 genera were also identified in the rhizosphere soils. Among them, 25 bacterial genera with relative abundance greater than 1% were identified as *Acidothermus*, *Streptomyces*, *Bacillus*, *Occallatibacter*, *Acidibacter*, *Rhodanobacter*, *Nakamurella*, *Pseudolabrys*, *Conexibacter*, *Amycolatopsis*, *Devosia*, *Flavobacterium*, *Massilia*, *Nocardioides*, *Pseudomonas*, *Mycobacterium*, *Bradyrhizobium*, *Lysinibacillus*,

*Hyphomicrobium*, *Sphingomonas*, *Paenibacillus*, *Chujaibacter*, *Gaiella*, *Bryobacter*, and *Nitrolancea* (Figure 2b). Fungal communities were primarily composed of Ascomycota, Basidiomycota, Rozellomycota, and Mortierellomycota (Figure 2c). Heatmaps at the genus level further illustrated structural differences between the two groups, with clearly distinctive separation in both bacterial and fungal compositions (Figure 2b,d). There were 331 fungal genera were also identified, and 14 genera with relative abundance greater than 1% were identified as *Fusarium*, *Trichoderma*, *Scytalidium*, *Sistotrema*, *Penicillium*, *Chaetosphaeria*, *Leuconeurospora*, *Talaromyces*, *Mortierella*, *Pseudeurotium*, *Coniochaeta*, *Cladophialophora*, *Solicoccozyma*, *Knufia* (Figure 2d).

Alpha diversity analysis indicated that open field-grown blueberries harbored significantly higher bacterial diversity, as reflected by the Shannon index (community diversity; Figure 2e), and greater species richness, as indicated by the Chao1 index (Figure 2f). Similarly, fungal communities in the open-field system also showed higher diversity (Shannon index; Figure 2g) and richness (Chao1 index; Figure 2h) compared to the greenhouse system.

Beta diversity analysis based on unweighted UNIFRAC distances revealed significant phylogenetic separation between the bacterial communities of the two cultivation methods (Figure 2i). Non-metric Multidimensional Scaling (NMDS) plots further confirmed structural differences in both bacterial (Figure 2k) and fungal (Figure 2l) communities, indicating that cultivation environment was strongly associated with microbial assembly in the blueberry rhizosphere.

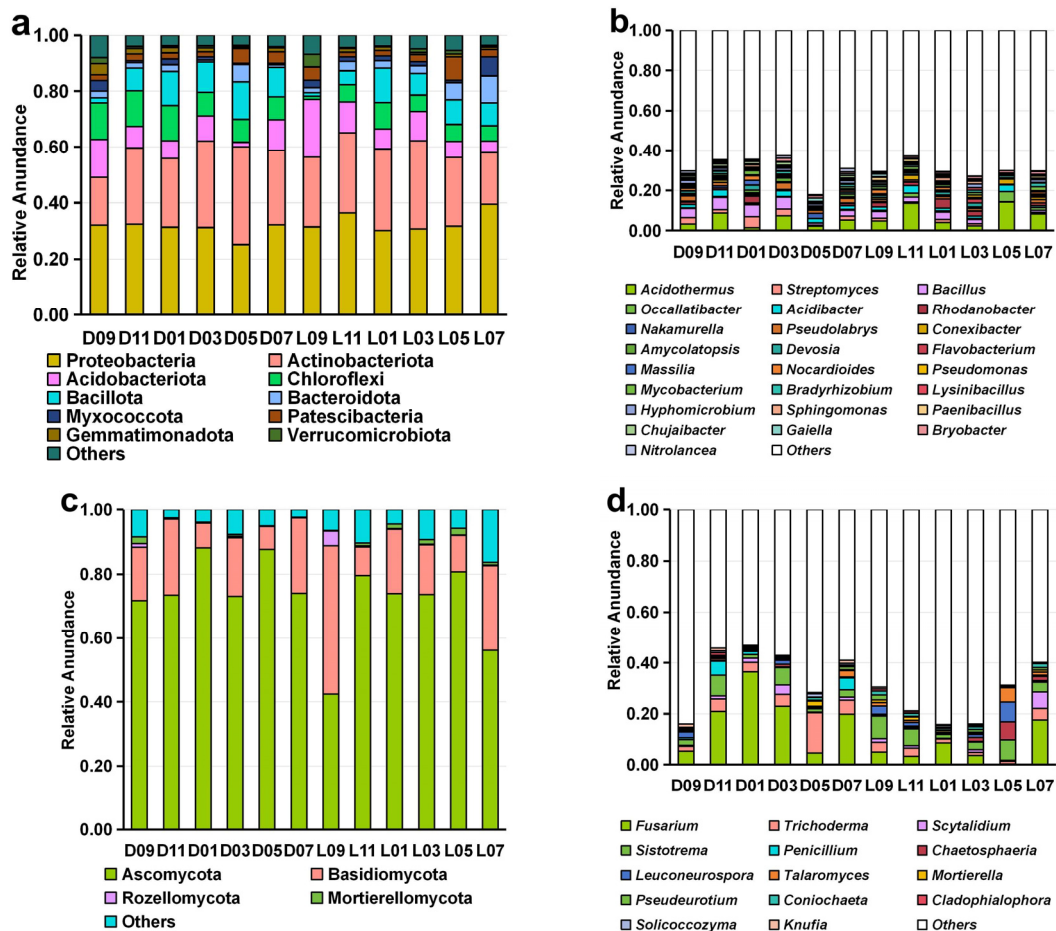
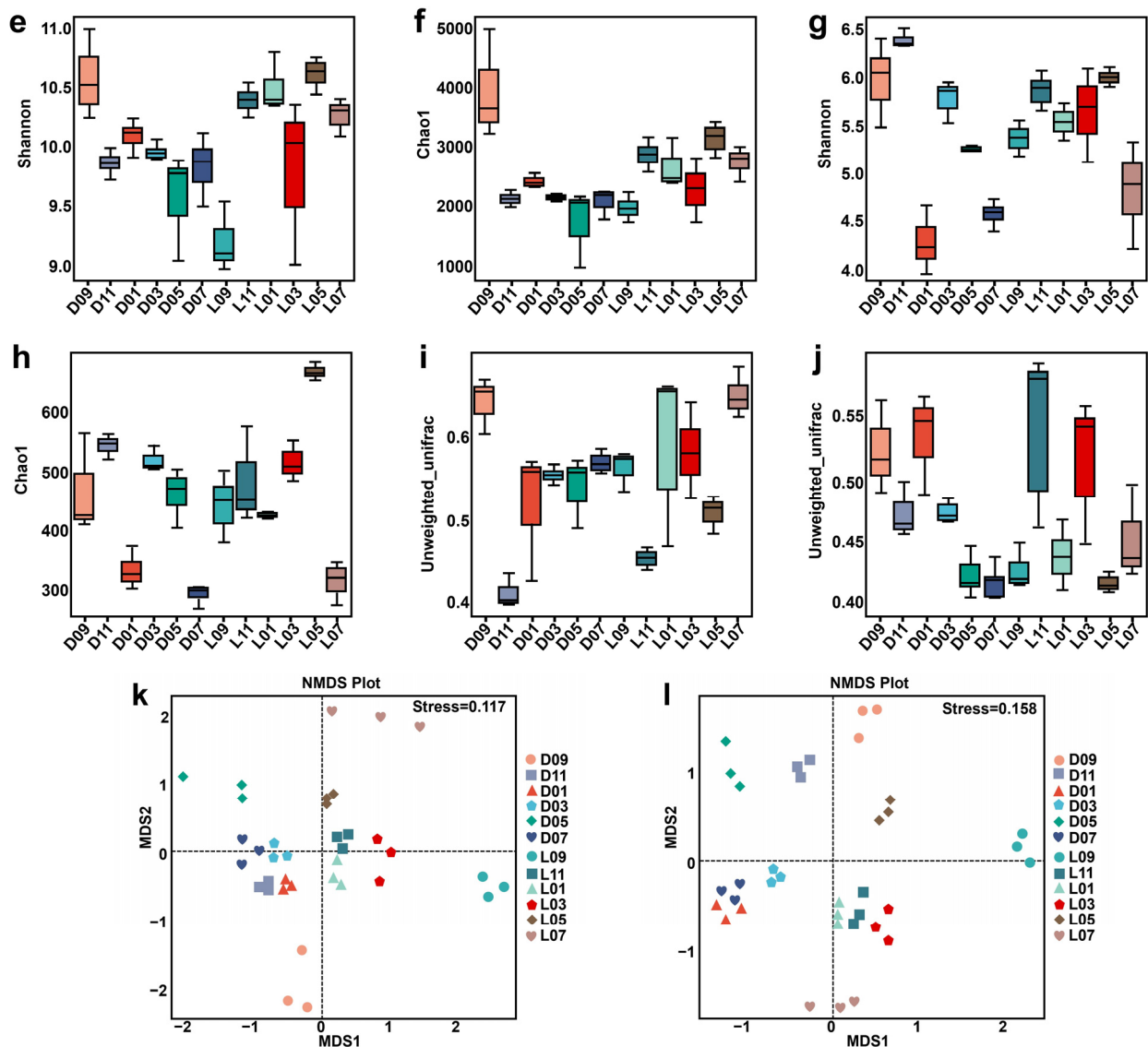


Figure 2. Cont.



**Figure 2.** Microbial community in the rhizosphere of greenhouse-cultivated (D) and open field-grown (L) blueberry plants. (a) Composition of bacterial phyla with a relative abundance  $\geq 1\%$ , and (b) composition of bacterial genera. (c) Composition of fungal phyla and (d) fungal genera. Bacterial alpha-diversity is shown using the (e) Shannon index and (f) Chao1 index, while fungal alpha-diversity is shown using the (g) Shannon and (h) Chao1 indices. Phylogenetic beta-diversity based on unweighted UNIFRAC distances is presented for (i) bacterial and (j) fungal communities. Finally, (k,l) Non-metric Multidimensional Scaling (NMDS) plots illustrate community structure differences for bacterial and fungal communities, respectively.

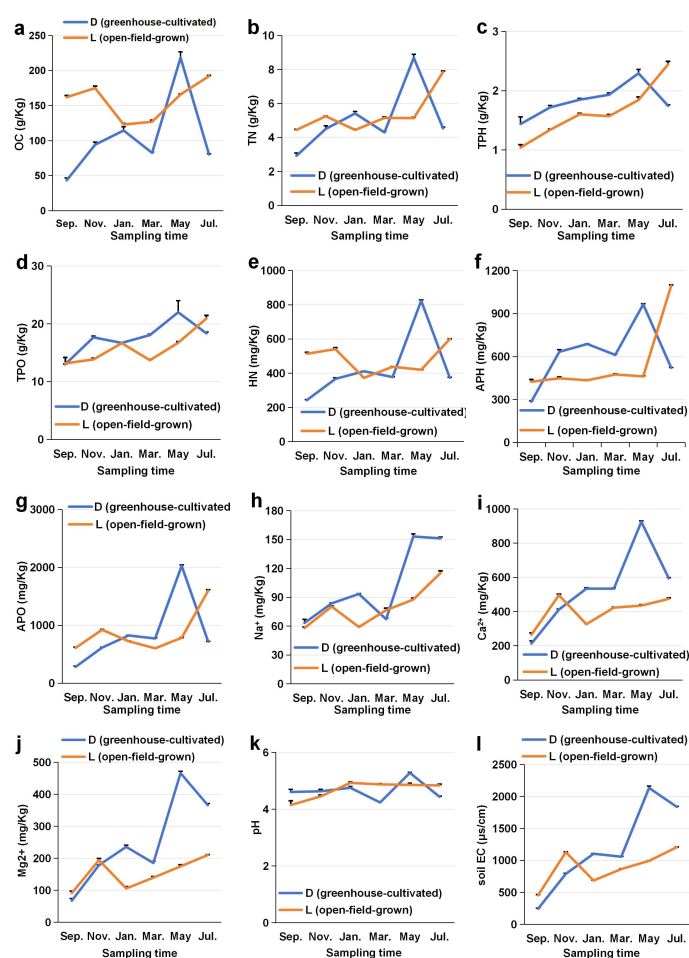
In the Supplementary Materials (Figure S1), Venn diagrams highlight a greater number of unique bacterial and fungal species in the open-field system, suggesting a more diverse and specialized microbial niche under traditional cultivation. LEfSe analysis ( $LDA \geq 4.0$ ) identified several differentially abundant taxa at phylum, class, order, family, genus, and species levels in both bacterial and fungal communities, underscoring the microenvironment-specific microbial recruitment under greenhouse and open field conditions.

These findings indicated that the bacterial and fungal community structures were profoundly different between greenhouse and open field conditions (Figure 2a–d). Open-field cultivation supported significantly higher bacterial and fungal alpha diversity, as indicated by the Shannon and Chao1 indices (Figure 2e–h), and hosted a greater number of

unique OTUs (Figure S1), suggesting a more complex and niche-differentiated microbial community. Beta diversity analysis (PCoA and NMDS) confirmed significant phylogenetic separation, suggesting that the cultivation system was strongly associated with microbial community assembly (Figure 2i–l). LefSe analysis identified multiple taxa differentially abundant between systems, highlighting microenvironment-specific microbial selection.

### 3.3. System-Specific Rhizosphere Soil Physicochemical Niches

The physicochemical properties of blueberry rhizosphere soil differed between cultivation methods (Figure 3). The mean concentrations of exchangeable calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ) were higher in the greenhouse cultivation rhizosphere than in the open-field cultivation (Figure 3i,j). Similarly, the mean content of available potassium (APO) was elevated under greenhouse conditions (Figure 3g). In contrast, the mean organic carbon (OC) content was lower in the greenhouse cultivation rhizosphere (Figure 3a). The mean values for total nitrogen (TN), total phosphorus (TPH), total potassium (TPO), hydrolysable nitrogen (HN), available phosphorus (APH), and sodium ion ( $\text{Na}^+$ ) concentrations showed less pronounced differences between the two methods (Figure 3b–f,h).

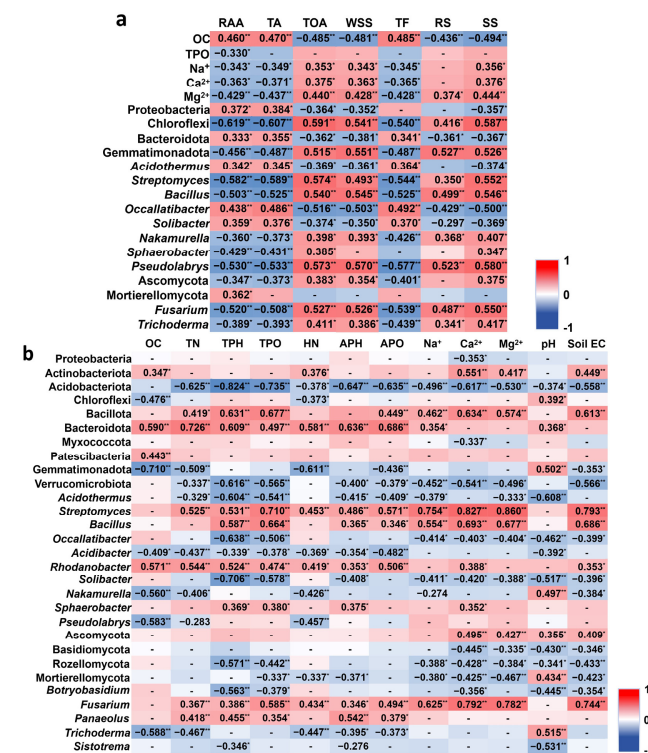


**Figure 3.** Temporal dynamics of rhizosphere soil properties in greenhouse-cultivated (D) and open-field-grown (L) blueberry plants. All subplots (a–l) share the same x-axis representing the sampling time (months from September 2024 to July 2025). Soil parameters measured include: (a) organic carbon (OC), (b) total nitrogen (TN), (c) total phosphorus (TPH), (d) total potassium (TPO), (e) hydrolysable nitrogen (HN), (f) available phosphorus (APH), (g) available potassium (APO), (h) sodium ion concentration ( $\text{Na}^+$ ), (i) calcium ion concentration ( $\text{Ca}^{2+}$ ), (j) magnesium ion concentration ( $\text{Mg}^{2+}$ ), (k) pH, and (l) electrical conductivity (EC). In each subplot, data points marked with “D” (greenhouse-cultivated) and “L” (open-field-grown) correspond to their respective cultivation systems.

Regarding soil properties, the mean pH of the rhizosphere soil under greenhouse cultivation was lower than that under open-field cultivation, while the mean soil electrical conductivity (EC) was higher in the greenhouse environment (Figure 3k,l). These patterns suggest that the greenhouse system was characterized by a niche with higher levels of soluble cations and salts, alongside lower pH and OC, whereas the open-field system maintained a higher OC environment.

### 3.4. Integrated Analysis for the Links of Rhizosphere Microenvironment to Fruit Quality Through Microbial Associations

Integrated correlation and principal component analyses were carried out to assess the associations among rhizosphere soil nutrients, microbial communities, and fruit quality parameters under the two cultivation systems. The correlation heatmap (Figure 4) and statistical analysis (Tables S1 and S2) revealed a network of significant associations. Notably, soil exchangeable cations (Ca<sup>2+</sup> and Mg<sup>2+</sup>) and available potassium (APO) showed consistent and significantly negative correlations with key antioxidant compounds, including total anthocyanins (TA) and total flavonoids (TF). Conversely, these soil nutrients were positively correlated with sugar and acid components, such as water-soluble sugars (WSS) and total organic acids (TOA). This pattern underscored a fundamental trade-off between primary and secondary metabolism in fruit which linked to soil nutrient availability.



**Figure 4.** Integrated correlation heatmap showing associations among blueberry fruit quality parameters, rhizosphere microbial community structure, and key rhizosphere nutrient element contents. (a) Correlation between soil nutrient factors and fruit quality parameters; (b) Correlation between soil nutrient factors and the relative abundance of major bacterial phyla. The color scale represents the Pearson correlation coefficient, ranging from −1 (negative correlation, blue) to +1 (positive correlation, red). Data are presented from three biological replicates (n = 3). \* and \*\* indicate significant correlations at p < 0.05 and p < 0.01, respectively (based on two-tailed t-tests). Note: TA: Total anthocyanin content, TF: Total flavonoids, RS: Reducing sugars, WSS: Water-soluble sugars, TOA: Total organic acids, SS: Soluble solids, RAA: Reduced ascorbic acid; OC: Organic carbon contents, TN: Total nitrogen contents, HN: Hydrolysable nitrogen contents, TP: Total phosphorus contents, AP: Available phosphorus contents, TK: Total potassium contents, and AK: Available potassium contents.

The analysis further identified specific microbial linkages within this network. The bacterial phyla Firmicutes and Bacteroidota, which were enriched in the greenhouse cultivation (D), demonstrated significant positive correlations with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and APO. Specific genera within these phyla, such as *Streptomyces* and *Bacillus*, also showed strong positive correlations with these nutrients (e.g., *Streptomyces* vs.  $\text{Ca}^{2+}$ ,  $r = 0.827$ ,  $p < 0.01$ ). In contrast, the phylum Acidobacteriota, which was more abundant in the open-field cultivation (L), was generally negatively correlated with the same set of nutrients.

Principal component analysis (PCA) provided an integrated visualization of these associations (Table S3). The first two principal components effectively captured the covariance structure among the variables. Loadings analysis indicated that soil factors (especially  $\text{Mg}^{2+}$ , pH, and OC) and specific microbial taxa (e.g., *Bacillus*, Chloroflexi) were the major contributors to the separation along the principal components.

## 4. Discussion

### 4.1. Observed Metabolic Trade-Offs Between Cultivation Systems

Our data indicate a clear divergence in the metabolite profiles of blueberries from the two cultivation systems. Greenhouse-grown fruits accumulated significantly higher levels of primary metabolites, including reducing sugars, water-soluble sugars, total organic acids, and soluble solids. In contrast, open-field-grown fruits exhibited higher concentrations of secondary metabolites, specifically anthocyanins, flavonoids, and ascorbic acid.

This pattern aligns with the well-established plant growth–defense trade-off theory [7,15], which posits that plants face an evolutionary and physiological dilemma in allocating limited resources between growth (primary metabolism) and defense (secondary metabolism). Under resource-rich and low-stress conditions, plants tend to prioritize growth; conversely, under stress conditions, they allocate more resources to defense.

The controlled greenhouse environment, characterized by reduced abiotic stress (e.g., stable temperature, lower UV exposure) and optimized nutrient availability, likely favors carbon allocation toward growth and primary metabolism—which is consistent with the observed accumulation of sugars and organic acids in greenhouse fruits. This represents the “growth” end of the trade-off.

In contrast, the open-field environment exposes plants to natural fluctuations in light intensity, UV radiation, and temperature—factors known to stimulate the biosynthesis of phenolic compounds and antioxidants as part of the plant’s stress response [23,24]. This stress-associated shift toward defense metabolism may account for the higher levels of anthocyanins, flavonoids, and ascorbic acid in open-field fruits, representing the “defense” end of the trade-off.

Our findings are consistent with studies on grapevine [25] and other perennial fruits [1], where environmental variability was associated with increased accumulation of secondary metabolites. Notably, total amino acid content did not differ between the two systems (Figure 1d). At first glance, this observation might appear inconsistent with the growth–defense trade-off theory, which would predict higher levels of all primary metabolites, including amino acids, under growth-promoting greenhouse conditions. However, this apparent discrepancy can be explained by the multifunctional roles and tight homeostatic regulation of amino acids in plants.

Unlike sugars and organic acids, which are primarily carbon-based energy and structural metabolites, amino acids serve dual functions: they are not only building blocks for protein synthesis (supporting growth) but also precursors for numerous nitrogen-containing defense compounds (e.g., alkaloids, certain phenolic amines). Furthermore, free amino acid pools in plants are subject to stringent homeostatic control to maintain nitrogen balance and prevent toxicity, making them less responsive to short-term metabolic shifts

compared to carbon metabolites. Therefore, the absence of significant differences in total amino acids suggests that the observed metabolic trade-off between greenhouse and open-field cultivation is primarily driven by carbon allocation rather than nitrogen allocation. The growth–defense trade-off manifests in the partitioning of photosynthetically fixed carbon: under low-stress greenhouse conditions, carbon is preferentially channeled into sugars and organic acids (growth), whereas under stress-prone open-field conditions, carbon is redirected toward phenolic compounds and antioxidants (defense). Nitrogen-containing metabolites, including amino acids, appear to be more conserved or differentially regulated, possibly reflecting the plant’s need to maintain protein synthesis capacity even under stress.

This interpretation is consistent with previous work on apples and pears grown under contrasting cultivation conditions [26], which also reported stable amino acid levels despite marked differences in sugars and phenolic compounds. Thus, rather than contradicting the growth–defense trade-off theory, our findings refine it by highlighting that the trade-off in this system is predominantly carbon-based, with nitrogen metabolism remaining relatively buffered.

#### 4.2. Distinct Rhizosphere Microbial Communities Associate with Cultivation Systems

Microbial community analysis showed distinct compositional and diversity patterns between the two cultivation systems (Figure 2). Open-field soils contained significantly higher bacterial and fungal alpha diversity (Shannon and Chao1 indices) compared to greenhouse soils (Figure 2e–h). This pattern is consistent with ecological theory, where heterogeneous environments typically support greater biodiversity [27], while controlled settings like greenhouses can lead to biotic homogenization [28].

Beta diversity analyses based on PCoA and NMDS showed separated clustering between the communities of the two systems (Figure 2i–l). This indicates that cultivation environment is strongly associated with microbial assembly in the blueberry rhizosphere, consistent with previous studies on perennial fruit crops [8,16].

Taxon-specific shifts were particularly notable and suggested a functional divergence in the rhizosphere. The greenhouse rhizosphere was significantly enriched in copiotrophic bacterial genera such as *Streptomyces* and *Bacillus* (Figure 2b). Ecologically, these taxa are not merely indicators of resource-rich conditions; they are involved in nutrient mobilization and plant hormonal regulation. For instance, many *Streptomyces* species are prolific producers of siderophores and organic acids, which can solubilize mineral cations (e.g.,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^{+}$ ) [29]. This functional trait provides a plausible mechanism that could explain the correlation between their high abundance in the greenhouse and the elevated levels of exchangeable cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , APO) observed in those soils (Figure 3). Concurrently, *Bacillus* species are well-documented PGPR that produce auxins (e.g., IAA) and ACC deaminase, potentially stimulating root development and enhancing the plant’s capacity for nutrient and water uptake. One hypothesis is that the enrichment of these functional guilds in the greenhouse creates a positive feedback loop: the stabilized, nutrient-rich environment is associated with these copiotrophs, whose metabolic activities may contribute to cation availability and plant growth. This, in turn, could bias the host plant’s resource allocation toward primary metabolism (e.g., synthesis of sugars and organic acids, as seen in Figure 1), consistent with the growth end of the growth–defense trade-off.

In contrast, open-field soils were characterized by a more diverse community with a higher relative abundance of oligotrophic taxa, particularly from the phylum Acidobacteriota (Figure 2a). Unlike the fast-growing copiotrophs, Acidobacteriota are typically adapted to nutrient-limited conditions and are frequently linked to the decomposition of complex, recalcitrant organic matter [30]. Their prevalence in the open-field soil aligns with the higher organic carbon (OC) content observed in this system (Figure 3a), suggesting a

microenvironment where nutrient mineralization is slower and more dependent on microbial processing of organic pools. This oligotrophic microbial milieu, combined with the less buffered abiotic stresses of the open field (e.g., UV fluctuations), could act as a mild, chronic elicitor of plant defense pathways. Previous studies have shown that specific microbial metabolites from complex organic matter decomposition or a general state of “nutrient competition” can prime the plant’s jasmonic acid (JA) and salicylic acid (SA) signaling pathways [31,32]. We therefore propose that this microbially associated stress signature in the open field rhizosphere is correlated with the observed shift in plant metabolism toward the defense end of the trade-off, manifesting as the enhanced accumulation of secondary metabolites (anthocyanins, flavonoids, ascorbic acid) in the fruits (Figure 1).

#### 4.3. Cultivation Systems Are Associated with Distinct Soil Physicochemical Niches

Soil physicochemical properties differed markedly between the two cultivation systems (Figure 3). Greenhouse soils exhibited lower pH, higher electrical conductivity (EC), and elevated levels of exchangeable  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and available potassium (APO). In contrast, open-field soils maintained higher organic carbon (OC) content.

These contrasting soil properties are likely associated with differences in management practices. The greenhouse system received regular fertigation with compound fertilizer, which can lead to cation accumulation and soil acidification over time [5]. Similar patterns have been observed in protected cultivation of other fruit crops [8,33]. The open-field system, with its reliance on controlled-release fertilizer and organic matter inputs from plant litter, likely supports higher OC content and slower nutrient mineralization cycles [34].

The distinct soil niches observed are consistent with the concept of “habitat filtering” [3], where physicochemical conditions are associated with the presence of specific microbial taxa. The high cation availability in greenhouse soils may favor copiotrophic bacteria such as *Bacillus* and *Streptomyces*, while the higher OC and more neutral pH in open-field soils support a broader range of oligotrophic organisms [35].

#### 4.4. Integrating Soil, Microbiota, and Fruit Metabolite Associations

Integrated correlation analysis revealed consistent associations among soil nutrients, microbial taxa, and fruit quality parameters (Figure 4). Soil exchangeable cations ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) and available potassium (APO) showed strong positive correlations with sugar and organic acid content, and negative correlations with antioxidant compounds. This pattern underscores the fundamental trade-off between primary and secondary metabolism observed at the fruit level.

Specific microbial taxa were strongly correlated with these soil nutrients. *Streptomyces* and *Bacillus*, enriched in greenhouse soils, showed significant positive correlations with  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and APO (e.g., *Streptomyces* vs.  $\text{Ca}^{2+}$ ,  $r = 0.827$ ,  $p < 0.01$ ). These genera are known to be involved in nutrient solubilization and plant growth promotion [23,24], and their enrichment is correlated with the enhanced primary metabolism observed in greenhouse fruits. This strong correlation may reflect the functional activities of these genera in situ. Many *Streptomyces* species actively solubilize mineral-bound cations through the secretion of organic acids and siderophores, a process that may be associated with the elevated pools of exchangeable  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{K}^+$  observed in greenhouse soils. Concurrently, *Bacillus* species are known to produce auxins and ACC deaminase, which can influence root development and enhance nutrient uptake efficiency—processes that may be associated with a shift in plant carbon allocation toward growth and primary metabolite synthesis (e.g., sugars, organic acids) in the fruits.

Conversely, Acidobacteriota, more abundant in open-field soils, showed negative correlations with the same nutrients. This phylum is associated with oligotrophic conditions

and complex organic matter decomposition, which may indirectly influence plant secondary metabolism through slow-release nutrient dynamics and microbial metabolite signaling [33]. Unlike the fast-growing copiotrophs, Acidobacteriota are typically slow-growing oligotrophs adapted to nutrient-limited conditions where organic matter turnover is gradual. Their dominance in open-field soils aligns with the higher organic carbon but lower available cation content (Figure 3), reflecting a system where nutrients are cycled more slowly through organic pools. This nutrient-sparse microenvironment, combined with the metabolic activities of a more diverse microbial community, could act as a mild but persistent physiological cue. Such conditions have been associated with the priming of plant defense pathways, including the phenylpropanoid pathway responsible for anthocyanin and flavonoid biosynthesis [33]. Thus, the negative correlation of *Acidobacteriota* with cations and its indirect association with higher fruit antioxidants could reflect a broader “oligotrophic microenvironment effect” that favors defensive secondary metabolism.

These correlative patterns are consistent with findings from other perennial fruit systems. Studies on apple [32] have similarly reported associations between specific microbial taxa and fruit quality parameters under different management regimes.

#### 4.5. Limitations and Future Perspectives

This study provides a comprehensive description of correlations between cultivation systems, rhizosphere properties, and fruit metabolite profiles, but several limitations should be acknowledged. First, our observational design cannot establish causality. The observed associations between soil properties, microbiota, and fruit metabolites may reflect direct effects, indirect effects through plant physiology, or feedbacks among multiple factors. Second, we did not measure plant physiological parameters (e.g., leaf nutrient status, photosynthetic rates), which are critical to validate the proposed “soil–plant–fruit” pathway. Third, aerial environmental factors (light, temperature, humidity) were not controlled and likely contributed to the observed metabolic differences [23,24].

Future studies should employ controlled experiments to isolate the specific roles of soil nutrients and microbiota. Soil transplant studies or microbial inoculation trials using synthetic communities containing key taxa (*Bacillus*, *Streptomyces*) under shared environmental conditions would help establish causality [35,36]. Additionally, integrating plant physiological measurements, metatranscriptomics, and hormone profiling could elucidate the functional mechanisms underlying the proposed microbiota-mediated metabolic reprogramming [37].

## 5. Conclusions

This comparative study demonstrates that blueberry plants grown in greenhouse and open-field cultivation systems exhibit distinct rhizosphere microenvironments and fruit metabolite profiles. We observed a systematic pattern: the greenhouse system was associated with a rhizosphere niche higher in available cations and enriched in copiotrophic bacterial taxa, and this was correlated with fruits higher in primary metabolites (sugars, organic acids). Conversely, the open-field system was associated with higher soil organic carbon, greater microbial diversity, and fruits enriched in antioxidant secondary metabolites.

The integrated correlations suggest that the rhizosphere microbiota may be an important component linking the cultivation environment to fruit quality, although this remains a hypothesis to be tested. The major limitation of this work is the lack of plant physiological data and the confounding effects of aerial environmental factors, which preclude causal inference.

Therefore, our primary contribution is the description of this robust system-level association and the generation of a clear, mechanistic hypothesis. Future studies employing

soil transplants, microbial inoculations, and comprehensive plant physiological monitoring under controlled conditions are essential to isolate the role of the rhizosphere microbiota and validate the proposed links in the soil–plant–fruit continuum.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16060652/s1>, Figure S1: Microbial community analysis in the rhizosphere of blueberry plants; Table S1: Kendall’s correlation analysis of blueberry fruit quality parameters, rhizosphere microbial community structure and key rhizosphere nutrient element contents; Table S2: Spearman’s correlation analysis of blueberry fruit quality parameters, rhizosphere microbial community structure and key rhizosphere nutrient element contents; Table S3: Eigenvector of nine principal components of blueberry fruit quality parameters, rhizosphere microbial community structure and key rhizosphere nutrient element contents.

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## Abbreviations

APH	available phosphorus
APO	available potassium
EC	electric conductivity
HN	hydrolysable nitrogen
OC	Organic carbon
RAA	reduced ascorbic acid
RS	reducing sugar
SS	soluble solids
TA	total anthocyanin
TAA	total amino acid
TF	total flavonoid
TN	total nitrogen
TOA	total organic acid
TPH	total phosphorus
TPO	total potassium
WSS	water-soluble sugar
OTU	operational taxonomic unit
PCoA	principal coordinates analysis
PCA	principal component analysis

NMDS	non-metric multidimensional scaling
LEfSe	linear discriminant analysis effect size
ASV	amplicon sequence variant
PGPR	plant growth-promoting rhizobacteria
masl	meters above sea level

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