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Influence of Plant Genotype on Nodule-Associated Bacterial Community Composition and Function in Seasonal Field Pea Varieties

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ABSTRACT

Legume root nodules host symbiotic rhizobia that are essential for nitrogen fixation but also harbor diverse non-rhizobial taxa that remain poorly characterized. Field pea (*Pisum sativum*) cultivars adapted to distinct seasonal growth (spring and winter) offer an opportunity to explore whether host genotype influences nodule-associated bacterial composition and function. This study investigates the taxonomic and functional profiles of nodule bacterial communities in seasonal field pea varieties. A field experiment with 6 field pea cultivars (three spring and three winter types) was conducted in South Dakota. Surface-sterilized root nodules were subjected to full-length 16S rRNA gene sequencing using Oxford Nanopore technology. Reads were quality filtered, organellar sequences removed, and taxonomic classification performed with the Emu pipeline. Microbial diversity, community structure, and core taxa were analyzed using R, with predicted functions inferred by FAPROTAX. The nodule bacterial community was dominated by *Rhizobium*, accounting for up to 98% of classified reads. After excluding *Rhizobium*, non-rhizobial diversity revealed a conserved core microbiome shared across cultivars, including cyanobacteria with potential phototrophic and diazotrophic traits. Minor seasonal differences were observed, with winter cultivars exhibiting higher evenness and specific associations. Field pea nodules harbor a stable, cyanobacteria-enriched core microbiome, largely consistent across seasonal cultivars. Season-specific microbial patterns suggest potential host-genotype influences, warranting further validation.

1 | Introduction

Plants have evolved close associations with soil microbes that contribute to nutrient uptake, enhance resistance to pathogens and abiotic stresses, and support expansion into new environments (Bever et al. 2012). Recent studies highlight the role of the plant microbiome in enhancing plant adaptability to changing environments by modulating phenotypic plasticity (Camargo et al. 2023). In this context, roots are the main point of contact between plants and soil, and in the rhizosphere, they support a diverse and dynamic microbial community. These communities are shaped by constant interactions between plants and microbes, as well as among microbes themselves. Many of these interactions are mediated by molecular signals, such as secondary metabolites, which can influence microbial composition, support beneficial microbes, or suppress harmful ones (Hacquard and Martin 2024). Understanding what drives the assembly of these microbial communities and how plants and

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microbes adapt together can help guide efforts to improve crops and promote more sustainable agricultural practices (Zhang et al. 2017).

As an example of this, legume root nodules have traditionally been studied as specialized organs for the fixation of symbiotic nitrogen, inhabited primarily by rhizobia such as *Bradyrhizobium*, *Rhizobium*, and *Mesorhizobium*. These symbiotic relationships have been shown to contribute significantly to reducing the dependence on synthetic fertilizers (Zhu et al. 2023). In addition, high-resolution microbiome profiling has revealed that nodules contain more than rhizobia; they are microbiome-rich environments colonized by various non-rhizobial taxa (Han et al. 2020; Hossain et al. 2023). Genera such as *Pseudomonas*, *Enterobacter*, and *Bacillus* have been identified in nodules, with roles that extend beyond nitrogen fixation, including the promotion of plant growth, suppression of disease, and improved resilience under environmental stress (Martínez-Hidalgo and Hirsch 2017; Yin et al. 2021; Carter et al. 2023).

Furthermore, the microbiome associated with the nodule is highly dynamic and strongly affected by the genotype of the host plant (Burghardt et al. 2022). Even under uniform environmental conditions, different legume cultivars recruit different microbial communities. For instance, *Glycine max* cultivars exhibit variation in the abundance of non-rhizobial taxa such as Pseudomonadaceae and Enterobacteriaceae (Yang et al. 2023). This characteristic suggests that host plants act as selective filters, possibly through root exudates (Zhalnina et al. 2018), immune responses (Teixeira et al. 2021) and signaling pathways that shape microbial compatibility (Reid et al. 2018). Moreover, some recent studies suggest that host genotypes not only influence microbial recruitment (Ishida et al. 2022; Dilla-Ermita et al. 2021) but also affect community function, with consequences for nitrogen fixation efficiency, community stability, and stress responses (Wendlandt et al. 2019; Crosbie et al. 2022).

Like other legumes, field pea (*Pisum sativum*) can form root nodules that fix atmospheric nitrogen through interactions with host-specific rhizobia in cultivated soils (Sujkowska et al. 2011). This crop is particularly valued for its protein-rich seeds and has attracted increasing interest due to its dual utility as both a cover crop and a source of high-quality protein for food applications (USDA 2010). Historically, winter-sown and spring-sown cultivars were selected based on their seasonal adaptability and agronomic functions. Winter peas offer early soil cover and nitrogen enrichment, while spring peas have been favored for seed production and food quality traits (USDA 2022). These groups also exhibit contrasting physiological traits, including cold tolerance, growth duration, and flowering time, which can influence root development and rhizosphere interactions (Vocanson and Jeuffroy 2008; Vann et al. 2018). Such differences raise compelling questions about whether divergent domestication pathways have shaped distinct rhizobial recruitment strategies or microbiome assemblies between field pea cultivars (Yurgel and McGee 2025).

In this study, we focus on commercially available, food-grade field peas, with cultivars adapted to different seasonal growth conditions (spring and winter). Given the increasing recognition that non-rhizobial microbes can shape legume performance, we hypothesize that pea varieties recruit distinct nodule-associated bacteria that contribute to functional variation in symbiosis based on their growth habit. Using 16S rRNA metabarcoding, we characterized the taxonomic and predicted functional profiles of nodule-associated bacterial communities in six varieties of field pea. We identified both core and variable bacterial taxa associated with each seasonal group, offering new insights into host-microbiome interactions in legumes.

2 | Materials and Methods

2.1 | Field and Experimental Design

The field experiment was conducted at the West River Research Farm of South Dakota State University in Sturgis, South Dakota, located at 44°25′28.53″ N, 103°22′22.41″ W. The site is characterized by a semi-arid climate (DBsk) with an average annual precipitation of 497 mm and a temperature of 8.4°C (Daly and Bryant 2013). The soil at the experimental site is classified as Nunn clay loam (NcA), with a pH in the top 30 cm of 6.4 and organic matter content of 27 g kg^{-1}. This trial was established concurrently with annual field pea variety trials. Field peas were planted on October 3, 2022, for winter cultivars and April 27, 2023, for spring cultivars at 25-cm row spacing with a no-till grain drill (Model 750, John Deere Co., Moline, IL, USA) at a population of 86 pure live seed m^{-2}. A commercial granular inoculant (Exceed, Visjon Biologics, Henrietta, TX, USA) was applied with the seed at manufacturers' recommended rates. No additional fertility was applied. For weed control, a premix herbicide of carfentrazone ethyl and sulfentrazone was applied as a postemerge application at a rate of approximately 365 mL ha^{-1}. The experiment was carried out on plots that have not previously had field peas grown on them using a completely randomized block design with three replications, consisting of three cultivars of two seasonal groups of field pea varieties: spring (cv "Carver," "Durwood," and "Salamanca") and winter (cv "Keystone," "MS-20W3," and "Vail"). Individual plots measured 1.5 m × 9.1 m. accommodating six rows per plot.

2.2 | Sampling, Surface Sterilization, and DNA Extraction

At anthesis, root nodules were carefully collected from the center two rows of each plot, targeting the crown region of five individual plants, resulting in five distinct clusters of nodules per replicate. Each cultivar was represented by three biological replicates, except for 'Carver,' for which only two replicates were obtained due to limited nodule formation. To remove adhering soil particles, the nodules were gently washed with sterile water. Following the sterilization protocol from Sharaf et al. (2019), surface sterilization was performed by rinsing the nodules three times with a 0.9% NaCl solution, followed by rolling them on lint-free wipes to remove any remaining soil. The nodules were then soaked twice for 5 min in a 1.65% (v/v) sodium hypochlorite solution, with gentle inversion during each soak, and rinsed with sterile water between treatments. Finally, the nodules were rinsed three additional times with sterile water to ensure

complete removal of the sterilizing agent. After sterilization, the nodules were stored at −80°C until DNA extraction. DNA was extracted from pooled nodule samples for each cultivar using the DNeasy Plant Pro Kit (Qiagen) according to the manufacturer's instructions.

2.3 | 16S rRNA Full-Length Amplification, Library, and Sequencing

The composition of the microbial community was assessed by amplifying the full-length 16S rRNA gene from 10 ng of DNA using the Bact26F forward primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and the Univ1492R reverse primer (5′-GGTTACCTTGTTACGACTT-3′) (Galkiewicz and Kellogg 2008). Library preparation, including PCR, indexing, quantification, normalization, pooling, and loading, was performed following the 16S Rapid Sequencing Barcoding Protocol (Oxford Nanopore Technologies, SQK-16S024). Sequencing was performed on the PromethION platform (Oxford Nanopore Technologies) at the South Dakota State University (SDSU) Genomics Core Sequencing Facility. PCR reactions were performed in a total volume of 40 μL, containing 25 μL of LongAmp Hot Start Taq 2X Master Mix (NEB, M0533), 10 μL of nuclease-free water, and 5 μL of template DNA (25 ng). The thermal cycling conditions were as follows: initial denaturation at 95°C for 1 min; 25 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s, extension at 65°C for 2 min; and final extension at 65°C for 5 min. Following amplification, the PCR products were purified using AMPure XP beads (Beckman Coulter, A63881) according to the manufacturer's instructions and SQK-16S024 kit recommendations.

2.4 | Amplicon Sequence Analysis

Raw sequencing reads in FASTQ format were first demultiplexed using the DORADO Fastq Barcoding workflow v3.10.4 (Oxford Nanopore Technologies). The quality assessment of the raw reads was performed with NanoStat v1.6.0, followed by quality filtering using NanoFilt v2.8.0. Reads with a Phred quality score below 12 and sequences shorter than 1000 bp and longer than 1800 bp were discarded to ensure high-confidence data for downstream analysis. The reads were then screened for chloroplast and mitochondrial sequences using Kraken2 v2.1.3 (Wood et al. 2019) with 0.6 of confidence using a custom Viridiplantae organelle database (RefSeq, May 5, 2025). Organellar reads were excluded prior to taxonomic classification. Subsequently, reads were processed through the Emu (expectation–maximization) pipeline (Curry et al. 2022) using default parameters, for taxonomic classification and estimation of relative microbial abundances. The taxonomic assignment was performed using an Emu pre-built database that integrates NCBI 16S RefSeq database (February 1, 2023). Taxonomic annotations were retained at the species, genus, and family levels to capture a broad, yet detailed, overview of the composition of the microbial community. Only taxa with a relative abundance ≥ 0.001 and that were detected in at least two independent replicates were retained for all downstream analyses. The functional profiles of bacteria were predicted using FAPROTAX (Functional Annotation of Prokaryotic Taxa) v1.2.11 (Louca et al. 2016) according to taxonomic assignments at the species level. The predicted functions were grouped as core, spring-specific, and winter-specific for comparative analyses.

2.5 | Data Analysis

All bioinformatic and statistical analyses were performed in R (v4.1; R Core Team 2021). Sequencing depth normalization was tailored to each analysis type. Rarefaction was applied only to assess library completeness and to compute the Chao1 richness estimator from raw counts, while other alpha and beta diversity metrics were based on normalized or relative abundance data. Alpha diversity indices, including Shannon diversity, Simpson diversity, and Pielou's evenness, were calculated from relative abundance data using the vegan package (v2.5-7) (Oksanen et al. 2013). Beta diversity was assessed from variance-stabilized counts obtained using DESeq2's variance-stabilizing transformation (VST) (v1.34.0) (Love et al. 2014), which corrects for library size differences without discarding reads. Bray–Curtis dissimilarity matrices were used to quantify community dissimilarities, followed by principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS), with ordination plots visualized using 95% confidence ellipses. Differences in community composition were tested using PERMANOVA (Adonis2 function, vegan package) with 999 permutations. Taxonomic composition was analyzed at the species and family levels and grouped by seasonal cultivar (spring and winter). The core microbiome was defined as taxa present in at least >2 cultivars within each seasonal group, with a minimum of 500 reads and detection in at least two of three replicates (Neu et al. 2021). To minimize bias, only taxa present in all biological replicates of each cultivar were considered for the classification of the core microbiome. Venn diagrams and Euler plots were generated to compare taxa unique to winter and spring cultivars and those shared between groups. Pairwise differences in *Rhizobium* relative abundance were assessed using Wilcoxon rank-sum tests. Differential abundance analyses at the genus level were performed using DESeq2, with the Benjamini-Hochberg false discovery rate (FDR) correction applied to control for multiple tests ($p < 0.05$ threshold).

3 | Results

3.1 | Sequencing Output and Taxonomic Classification

A total of 11,453,755 full-length 16S rRNA reads were generated averaging 673,750 reads per sample, with a median read length (N50) of 1549 base pairs (bp). After basecalling, reads were filtered for a minimum quality score of 12 and lengths between 1000 and 1800 bp, retaining 10,714,816 high-quality reads. Host-derived organellar contamination represented 0.1% of reads and was removed prior to downstream analysis. Emu-based classification then resolved dominant taxa to the species level, with unmapped and unclassified reads comprising no more than 0.0005% of the dataset (Table S1). In total, Emu identified 197 unique species in 111 genera, 65 families, and six phyla (Table S2).

3.2 | Rhizobium Dominance in Field Pea Nodules

To compare the composition of the bacterial community between spring and winter pea cultivars, we analyzed the relative abundance profiles at different taxonomic levels. At the phylum level, the nodule-associated bacteria were dominated by Proteobacteria (approximately 96% of classified reads), followed by Cyanobacteria (approximately 3%). Other phyla, including Actinobacteria, Bacteroidetes, Firmicutes, and Synergistetes, together contributed less than 1% (Figure 1A). Having established the broad taxonomic landscape, we next assessed seasonal differences at the genus level. As expected from the nodular origin of the samples, *Rhizobium* was the most abundant genus, with a relative abundance ranging from 0.84 to 0.98 (Figure 1B,C). Although its overall dominance was consistent, hierarchical clustering based on *Rhizobium* abundance revealed intra-seasonal variability across cultivars (Figure 1C). For instance, while some winter cultivars such as Keystone and Vail clustered at the high end of the abundance spectrum (> 0.96), spring cultivars showed higher variability. Given the incomplete season-based segregation observed in clustering, *Rhizobium* abundance appears to reflect a combination of cultivar-specific and other ecological/structural factors rather than the season alone.

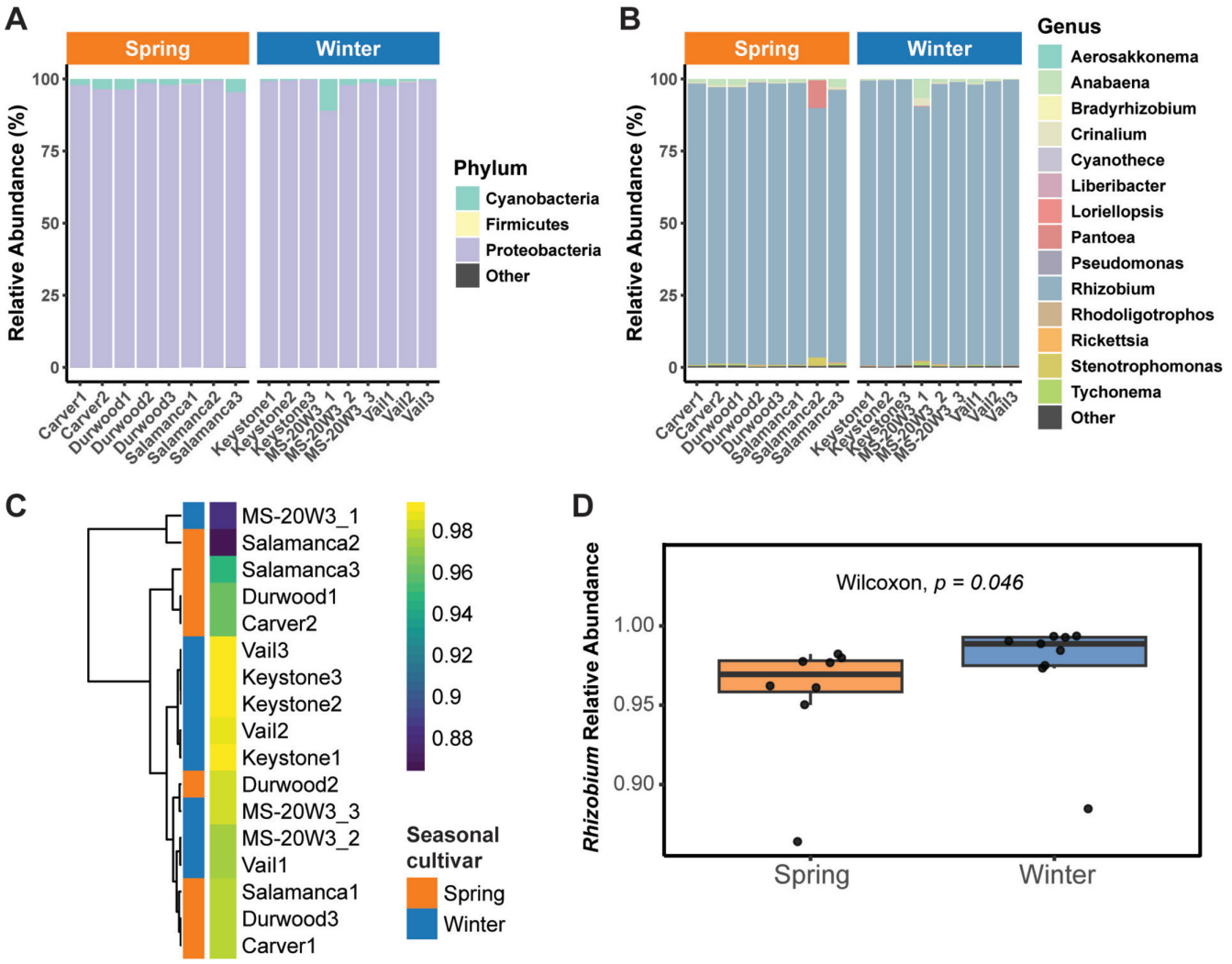


FIGURE 1 | Seasonal differences in the microbial community structure and *Rhizobium* dominance in field pea nodules. (A) Phylum-level composition of nodule-associated bacteria across seasonal cultivars. Stacked bar plots show the relative abundance (%) of bacterial phyla detected in individual nodule samples from spring (orange) and winter (blue) cultivars. Proteobacteria dominated the microbiome in all samples, while Cyanobacteria and Firmicutes were present at lower abundances. Sample names are labeled along the x-axis by cultivar and replicate. Bars are grouped by seasonal cultivar. (B) Genus-level taxonomic composition of nodule-associated bacteria across seasonal cultivars. Relative abundance (%) of bacterial genera detected in each nodule sample, grouped by seasonal cultivar. *Rhizobium* is the dominant genus in both groups. Genera are color-coded as indicated in the legend. Each bar represents one replicate. (C) Heatmap of genus-level relative abundances across samples. A heatmap displays the relative abundance of bacterial genera across spring and winter nodule samples. Rows correspond to individual samples ($n = 18$), and columns represent bacterial genera. The color gradient (purple to yellow) indicates an abundance of values. A dendrogram based on hierarchical clustering of genus profiles reveals patterns of similarity among samples. Colored side bars denote seasonal cultivars (orange = spring, blue = winter). (D) Comparison of *Rhizobium* abundance among seasonal cultivars. Boxplots depict the relative abundance of *Rhizobium* in spring ($n = 8$) and winter ($n = 9$) samples. *Rhizobium* was significantly more abundant in winter nodules (Wilcoxon rank-sum test, $p = 0.046$). Boxes represent the interquartile range (IQR), and individual data points are plotted.

Finally, we formally tested whether the small seasonal difference in *Rhizobium* abundance was significant. A Wilcoxon test showed that winter cultivars harbored a modest but statistically higher median relative abundance of *Rhizobium* compared to spring cultivars (Figure 1D, $p = 0.046$), consistent with the clustering result yet underscoring that the season only explains a small fraction of the overall variation. These results confirm *Rhizobium* as the dominant nodule taxon, with minor but potentially meaningful differences between individual cultivars that could reflect genotype-driven modulation of symbiotic recruitment, but this requires larger sample sizes for confirmation.

3.3 | Microbiome Comparison Between Spring and Winter Cultivars

To evaluate potential seasonal effects on bacterial diversity, we first compared alpha diversity between spring and winter pea cultivars using Shannon and Simpson diversity indices, Pielou's evenness, and Chao1 species richness. While Chao1 richness did not differ significantly between seasonal cultivars, Shannon diversity was marginally higher in spring ($p = 0.0543$), and both Simpson's diversity and Pielou's evenness were significantly greater in spring compared to winter ($p = 0.0433$; Figure 2A). Next, we assessed beta diversity using Bray–Curtis dissimilarity, visualized through PCoA and NMDS. The spring and winter cultivar samples did not form distinct groups in either ordination method, and PERMANOVA analysis confirmed no significant difference in community composition between groups ($p > 0.05$) (Figure 2B,C).

To explore cultivar-level patterns in more detail, we analyzed the alpha diversity indices (Shannon, Simpson, Pielou, Chao1) with 95% confidence intervals for each cultivar (Figure 2D). Although the confidence intervals for spring and winter cultivars broadly overlap, consistent with the marginal significance observed in group-level comparisons, cultivar-specific patterns emerge. Salamanca (spring) exhibits notably high Pielou's evenness (mean approximately 0.22) and Shannon diversity (mean approximately 0.85), while Vail (winter) shows the highest Chao1 richness (mean approximately 70). Keystone (winter) demonstrates high Simpson's diversity (mean approximately 0.45) and Shannon diversity comparable to Salamanca. The remaining cultivars, Durwood and Carver (spring) and MS-20 W3 (winter), cluster at intermediate values across metrics. These cultivar-level variations, though not individually tested due to limited replication ($n = 2$–3 per cultivar), suggest substantial within-season diversity in nodule bacterial community structure, with the overall marginal trend toward higher diversity in spring driven primarily by Salamanca.

Finally, we evaluated differential abundance at the genus level using both DESeq2 and Wilcoxon rank sum tests. After multiple testing corrections, no genera showed statistically significant differences between spring and winter samples (Table S3). Although our hypothesis predicted seasonal cultivar-driven changes in the nodule microbiome, the lack of significant differences between diversity metrics and taxonomic profiles suggests a robust and conserved bacterial community structure, largely dominated by *Rhizobium*, with any genotype-specific effects potentially masked by high variability between samples. In addition, the dominance of *Rhizobium* in all samples may have reduced the sensitivity of diversity metrics to detect differences between taxa of lower abundance. This effect likely contributes to the uniformity observed in the alpha and beta diversity indices and highlights the importance of complementary analyses that account for dominant taxa.

3.4 | Unmasking Microbial Diversity Beyond *Rhizobium*

In all nodule samples, *Rhizobium* was consistently the most dominant bacterial genus, accounting for 84% to 98% of the total classified sequences. This dominance significantly compressed the diversity space, lowering the alpha and beta diversity estimates, which are highly sensitive to the evenness and distribution of taxa (Jousset et al. 2017; Willis 2019). To address this, we filtered the *Rhizobium* genus from the dataset and reanalyzed the bacterial profiles. This filtering step enabled a more detailed examination of the less abundant, non-rhizobial taxa associated with nodules.

At the genus level, several taxa that were previously masked by *Rhizobium*'s dominance became more prominent, including *Crinalium*, *Tychonema*, *Rhodoligotrophos*, *Rickettsia*, and *Liberibacter* (Figure 3A). These genera were present in spring and winter cultivars and contributed more significantly to the overall structure of the community in the filtered dataset. Next, we recalculated species richness (Chao1), overall diversity (Shannon, Simpson), and community differences (Bray–Curtis) after *Rhizobium* removal. No significant effect of *Rhizobium* removal was observed on the total bacterial structure ($p > 0.05$; Figure S1). The only difference was in evenness (Pielou index; $p < 0.05$; Figure 3B), with winter-type cultivars showing a more balanced read distribution between species.

3.5 | Core- and Specific-Associated Bacteria in Seasonal Field Pea Cultivars

To explore group-specific variation among bacteria, we characterized the core and specific bacterial composition of field pea nodules from spring and winter cultivars at the species level after the removal of *Rhizobium* sequences. Core species were defined by absolute abundance–occurrence (see Section 2). We used raw read counts rather than relative abundance to minimize the impact of variable sequencing depths across samples, which can mask rare taxa and disproportionately emphasize dominant taxa. This classification revealed a structured bacterial composition between winter and spring cultivars (Figure 4A and Table S4). A total of 19 species were classified as part of the core bacteria shared between both seasonal groups, suggesting a conserved bacteria community baseline in field pea nodules. Although these 19 taxa represented only 9.7% of the total species richness, they accounted for 83% of the total bacterial community abundance across all samples (Figure 4B). Most of these taxa belonged to cyanobacterial lineages with known epibiotic lifestyles, including members of the Oscillatoriaceae, Nostocaceae, Hapalosiphonaceae, and Chroococcaceae families. Genera such as *Anabaena*, *Fischerella*, *Tychonema*, and *Gloeocapsa* are frequently associated with photosynthetic activity and have been observed in plant-associated

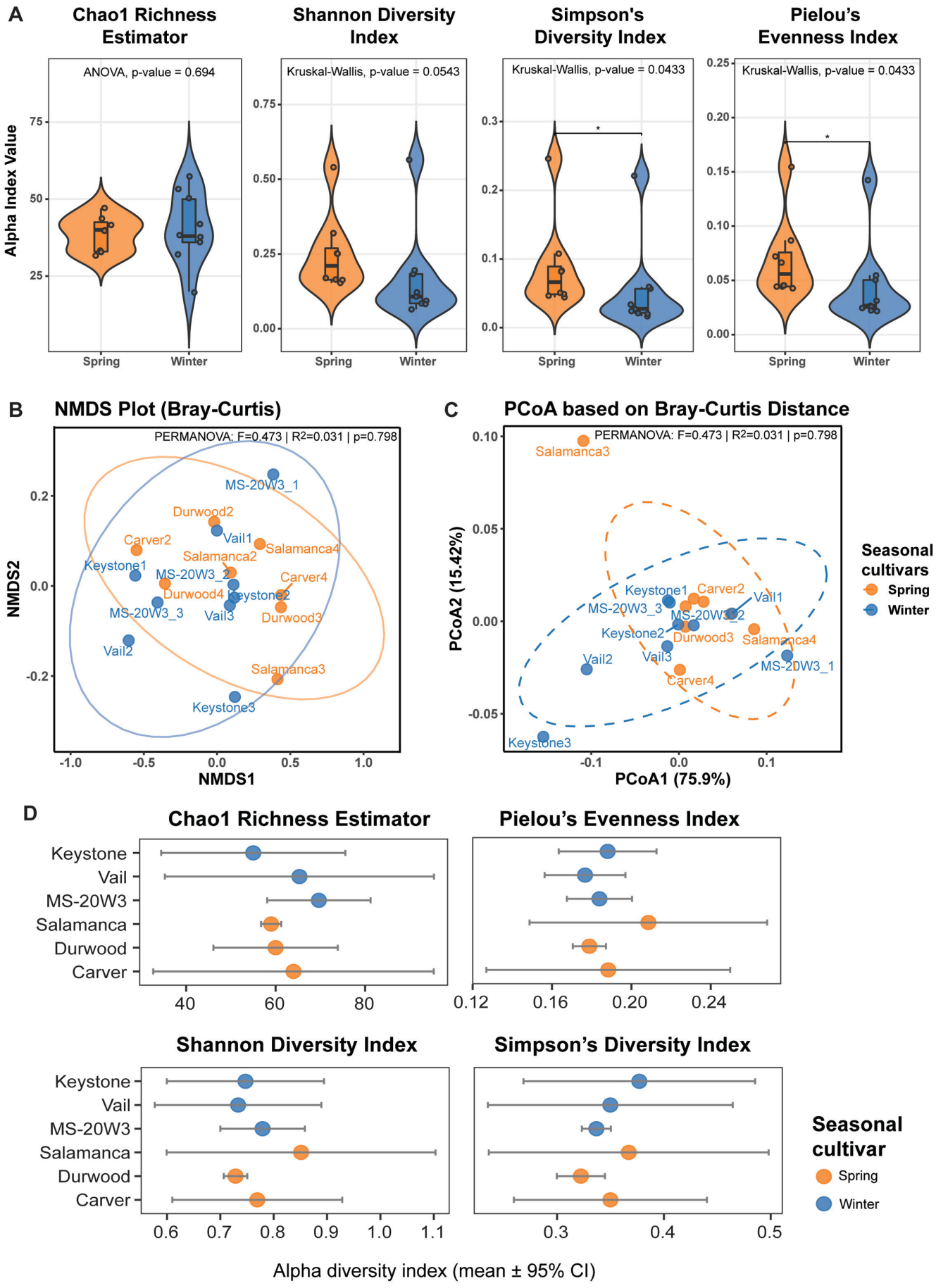


FIGURE 2 | Legend on next page.

FIGURE 2 | Seasonal cultivar effects on alpha and beta diversity of nodule-associated bacterial communities. (A) Alpha diversity indices of spring and winter cultivars at genus-level. Violin plots show distributions of four alpha diversity metrics across field pea nodule microbiomes from spring (orange) and winter (blue) cultivars: Chao1 richness, Shannon diversity, Simpson's diversity, and Pielou's evenness. Center lines denote medians; box widths indicate interquartile ranges. (B) NMDS ordination of microbial community composition. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity shows the similarity of genus-level community profiles among individual samples. Samples are colored by seasonal cultivar group (orange = spring; blue = winter), with ellipses representing 95% confidence intervals. (C) Principal Coordinates Analysis (PCoA) based on Bray-Curtis distance. PCoA illustrates variance in microbial composition across spring and winter samples. The first two principal coordinates explain 75.9% and 15.4% of variation, respectively. Dashed ellipses represent 95% confidence intervals for each group ($n > 8$ per seasonal cultivar). Differences in community composition were tested using PERMANOVA (Adonis2 function, vegan package) with 999 permutations. (D) Alpha diversity comparisons by cultivar. Mean ± 95% confidence intervals of four alpha diversity metrics (Chao1 richness, Shannon diversity, Simpson diversity, and Pielou's evenness) across individual cultivars. Each point represents the cultivar's mean index value, colored by season (orange = spring; blue = winter).

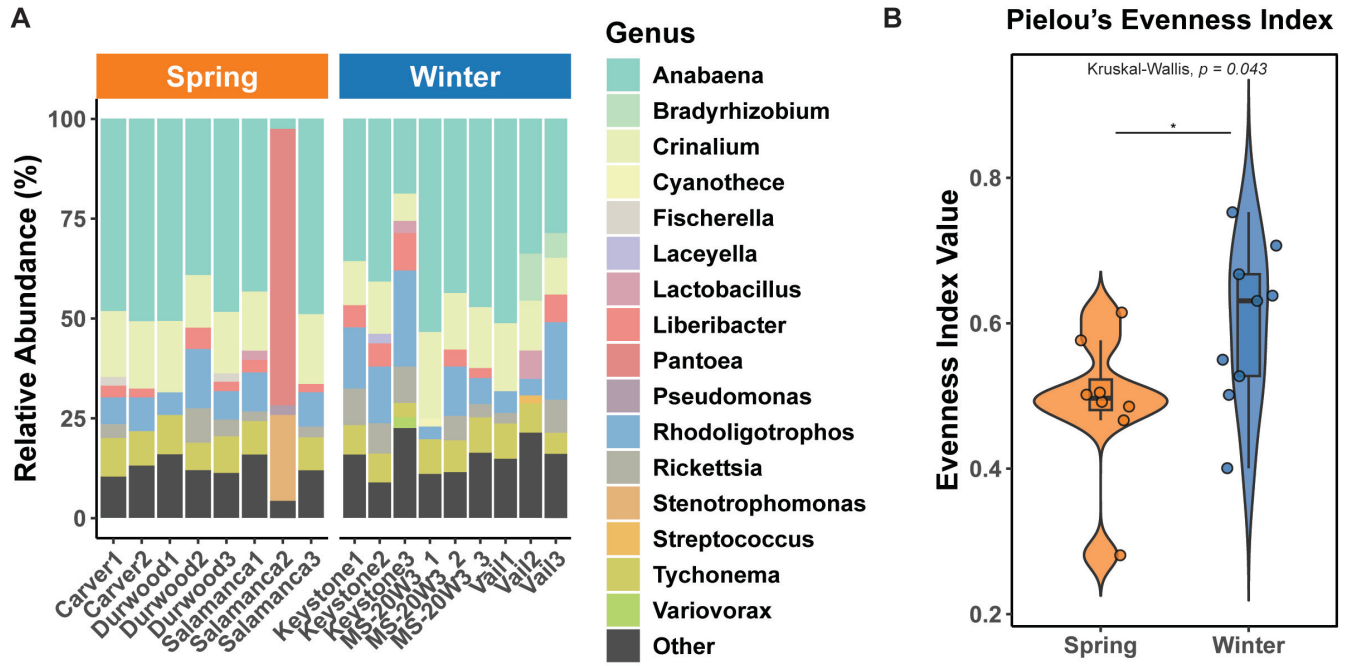


FIGURE 3 | Revealing microbial community composition and evenness after *Rhizobium* sequence removal from field pea nodules. (A) Genus-level composition of non-rhizobial nodule microbiomes. Stacked bar plots show the relative abundance (%) of bacterial genera in field pea nodules after computational removal of *Rhizobium* sequences. Samples are grouped by seasonal cultivar: spring (orange) and winter (blue). Remaining taxa span diverse genera, including *Anabaena*, *Crinalium*, *Liberibacter*, *Pantoea*, and *Rickettsia*, with notable shifts in relative contributions across cultivars. Bars represent individual biological replicates ($n = 18$ total), with each color corresponding to a specific genus as indicated in the legend. (B) Increase in community evenness after *Rhizobium* removal. Violin plots display Pielou's evenness index values for spring ($n = 8$) and winter ($n = 9$) samples following removal of *Rhizobium*. Evenness reflects how evenly taxa are distributed within each sample. Winter cultivars exhibited significantly greater evenness than spring cultivars (Kruskal–Wallis test, $p = 0.043$). Each dot represents one replicate; center lines indicate median values, and boxes reflect interquartile ranges.

environments (Table 1). The consistent presence of these taxa across cultivars suggests stable niche associations that can support or interact with nitrogen-fixing symbioses.

To better understand seasonal specificity, we further examined the non-*Rhizobium* core taxa unique to each cultivar group. The spring-specific core bacteria included six species, mainly epibiotic cyanobacteria such as *Calothrix* sp. *336/3*, *Cylindrospermum pellucidum*, and *Chroococcidiopsis thermalis*. These taxa are known for their resilience in variable environments and potential roles in nitrogen dynamics (Johansen et al. 2014; Baldanta et al. 2023; Uesaka et al. 2024). An additional spring-specific species, *Pleomorphomonas koreensis*, is a nitrogen-associated free-living bacterium previously isolated from plant rhizospheres, supporting its relevance in plant-microbe interactions (Im et al. 2006) (Table 1). The winter-specific core microbiome included five taxa, of which *Emcibacter* sp. (Meier et al. 2019), *Insolitispirillum peregrinum* (Balogová et al. 2025), and *Rhodothalassium salexigens* (Madigan et al. 2025) have been identified in soil or aquatic environments and can play roles in carbon or sulfur cycling (Table 1). Although their specific functions in nodules remain unclear, their consistent presence suggests a possible adaptation to winter conditions and a contribution to the seasonal microbial structure. Together,

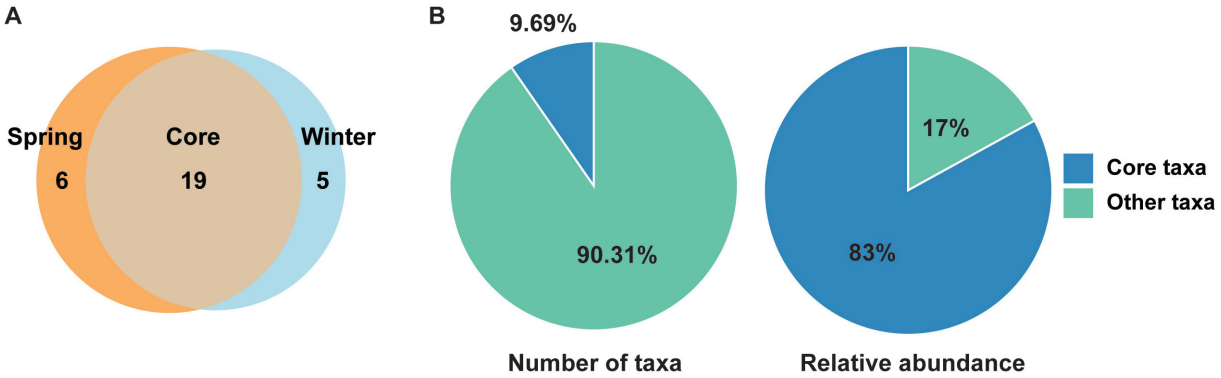


FIGURE 4 | Core and group-specific non-rhizobial microbiome composition in seasonal field pea cultivars. (A) Venn diagram summarizing the number of core taxa shared between spring and winter cultivars, as well as those exclusive to each seasonal group. (B) Pie charts showing the proportion of core versus non-core ("other") taxa by number of species (left) and total relative abundance (right). Although core taxa represent only 9.69% of species, they account for 83% of the total microbial abundance. *Notes:* Core taxa classification was based on prevalence (≥ 90% of samples in a group with ≥ 500 reads per taxon, with $n > 2$ replicates); relative abundances were averaged across all samples.

these results reveal the presence of a dominant and stable non-*Rhizobium* core microbiome in field pea cultivars, accompanied by seasonal differences in cyanobacterial and free-living taxa.

3.6 | Functional Insights From the Core and Group-Specific Bacterial Community

To investigate the functional potential of bacterial communities in field pea nodules, we applied FAPROTAX to species-level taxonomic profiles and grouped the predictions by microbial classification: (1) shared core bacteria, (2) spring-specific taxa, and (3) winter-specific taxa. The shared core bacteria exhibited a clear dominance of phototrophic functions, including general phototrophy, photoautotrophy, and oxygenic photoautotrophy, which showed the highest mean abundances among all predicted traits (Figure 5 and Table S5). The functions associated with photosynthetic cyanobacteria and nitrogen fixation were also enriched in the core group, consistent with the presence of phototrophic and diazotrophic taxa, whereas traits such as intracellular parasitism were detected at lower abundance.

In contrast, spring-specific taxa displayed a broader range of predicted functions, such as photoheterotrophy, methanotrophy, methylotrophy, and hydrocarbon degradation, but these occurred at lower relative abundance, suggesting a more functionally diverse but less dominant microbial contribution. Meanwhile, winter-specific taxa were primarily associated with aerobic chemoheterotrophy and photoheterotrophy, also at low abundance, indicating a more constrained metabolic repertoire. Altogether, these results highlight that the functional potential in field pea nodules is largely driven by a conserved core community, while season-specific groups contribute additional, but limited, functional variability.

4 | Discussion

The advent of long-read sequencing technologies has substantially enhanced our ability to resolve complex microbial communities, particularly by improving taxonomic resolution in 16S rRNA-based studies (Tedersoo et al. 2021). However, each microbiome study presents unique characteristics that often require customized analytical workflows. This is especially relevant when considering the expected variability in microbial diversity between plant compartments. For example, the high microbial diversity typically found in the rhizosphere, phyllosphere, or floral tissues (Trivedi et al. 2020) contrasts with the lower diversity observed in specialized organs such as root nodules (Martínez-Hidalgo and Hirsch 2017; Sharaf et al. 2019; Yurgel and McGee 2025), which are often dominated by a single symbiotic taxon. In this study, we combined stringent filtering of host organellar reads and removal of *Rhizobium* sequences to disentangle the hidden diversity of the field pea nodule-associated bacteria. This approach confirmed *Rhizobium leguminosarum* as the overwhelmingly dominant symbiont, while also exposing a structured assemblage of non-rhizobia taxa, including cyanobacteria and proteobacterial lineages, that form a reproducible core across cultivars.

4.1 | Dominant *Rhizobium* and Implications for Diversity Patterns

We leveraged species- and genus-level resolution to enhance the detection of rare microbial taxa. As expected, the bacterial profiles of the field pea nodules were overwhelmingly dominated by *Rhizobium*, consistent with its central role as the primary nitrogen-fixing symbiont in legumes (Mathesius 2022). The high abundance of *Rhizobium* (84%–98% of reads) reflects successful nodule colonization by compatible symbionts and is consistent with previous reports in peas and other legumes (Ji et al. 2017; Zhang et al. 2018; Yurgel and McGee 2025). This dominance explains why richness (Chao1) remained stable between cultivars, whereas indices sensitive to abundance distribution (Simpson, Pielou) captured differences in evenness. In spring cultivars, higher evenness suggests that secondary taxa achieve more balanced representation, while winter cultivars appear more strongly structured by *Rhizobium* dominance. Similar findings in mung bean indicate that host genotype often influences not

TABLE 1 | Taxonomic composition and relative abundance of core and seasonal-specific nodule-associated taxa.

Species	Family	Phylum	Relative abundance	Classification	Number of cultivars
Aerosakkonema funiforme	Oscillatoriaceae	Cyanobacteria	0.011 (±0.004)	Core	6
Anabaena cylindrica	Nostocaceae	Cyanobacteria	0.403 (±0.130)	Core	6
Calothrix sp. *PCC 7507*	Calotrichaceae	Cyanobacteria	0.002 (±0.001)	Core	6
Candidatus Liberibacter africanus	Rhizobiaceae	Proteobacteria	0.033 (±0.020)	Core	6
Crinalium epipsammum	Gomontiellaceae	Cyanobacteria	0.138 (±0.048)	Core	6
Cyanothece sp. *PCC 7425*	Cyanothecaceae	Cyanobacteria	0.012 (±0.004)	Core	6
Fischerella muscicola	Hapalosiphonaceae	Cyanobacteria	0.006 (±0.003)	Core	6
Fischerella thermalis	Hapalosiphonaceae	Cyanobacteria	0.009 (±0.003)	Core	6
Gloeocapsa sp. *PCC 7428*	Chroococcaceae	Cyanobacteria	0.003 (±0.001)	Core	6
Laceyella sacchari	Thermoactinomycetaceae	Firmicutes	0.010 (±0.006)	Core	6
Loriellopsis cavernicola	Symphyonemataceae	Cyanobacteria	0.008 (±0.002)	Core	6
Oscillatoria nigro-viridis	Oscillatoriaceae	Cyanobacteria	0.004 (±0.001)	Core	6
Rhodoligotrophos appendicifer	Rhodobiaceae	Proteobacteria	0.019 (±0.018)	Core	6
Rhodoligotrophos jinshengii	Rhodobiaceae	Proteobacteria	0.079 (±0.044)	Core	6
Rickettsia akari	Rickettsiaceae	Proteobacteria	0.004 (±0.003)	Core	6
Rickettsia bellii	Rickettsiaceae	Proteobacteria	0.007 (±0.005)	Core	6
Rickettsia endosymbiont of Bemisia tabaci	Rickettsiaceae	Proteobacteria	0.030 (±0.021)	Core	6
Sneathiella chinensis	Sneathiellaceae	Proteobacteria	0.005 (±0.004)	Core	6
Tychonema bourrellyi	Microcoleaceae	Cyanobacteria	0.074 (±0.024)	Core	6
Caldovatus sediminis	Acetobacteraceae	Proteobacteria	0.004 (±0.003)	Only spring	3
Calothrix sp. *336/3*	Calotrichaceae	Cyanobacteria	0.002 (±0.001)	Only spring	3
Chroococcidiopsis thermalis	Chroococcidiopsidaceae	Cyanobacteria	0.002 (±0.001)	Only spring	3
Cylindrospermum pellucidum	Nostocaceae	Cyanobacteria	0.002 (±0.001)	Only spring	3
Kryptousia microlepis	Tolypothrichaceae	Cyanobacteria	0.002 (±0.001)	Only spring	3
Pleomorphomonas koreensis	Methylocystaceae	Proteobacteria	0.002 (±0.001)	Only spring	3
Emcibacter sp. *MEBiC09520*	Emcibacteraceae	Proteobacteria	0.002 (±0.002)	Only winter	3
Insolitispirillum peregrinum	Rhodospirillaceae	Proteobacteria	0.003 (±0.003)	Only winter	3
Oceanibacterium hippocampi	Sneathiellaceae	Proteobacteria	0.002 (±0.001)	Only winter	3
Rhodothalassium salexigens	Rhodothalassiaceae	Proteobacteria	0.006 (±0.004)	Only winter	3
Rickettsia japonica	Rickettsiaceae	Proteobacteria	0.003 (± 0.002)	Only winter	3

the absolute number of taxa but their relative balance, highlighting the selective filtering role of legumes (Favero et al. 2021). Such patterns underscore that diversity metrics must be interpreted considering symbiont dominance and the ecological specialization of nodules.

4.2 | Cultivar Effects on Nodule-Associated Bacteria Assemblage

Plant genetic variation exerts a significant influence on the assembly of microbiomes across different plant compartments,

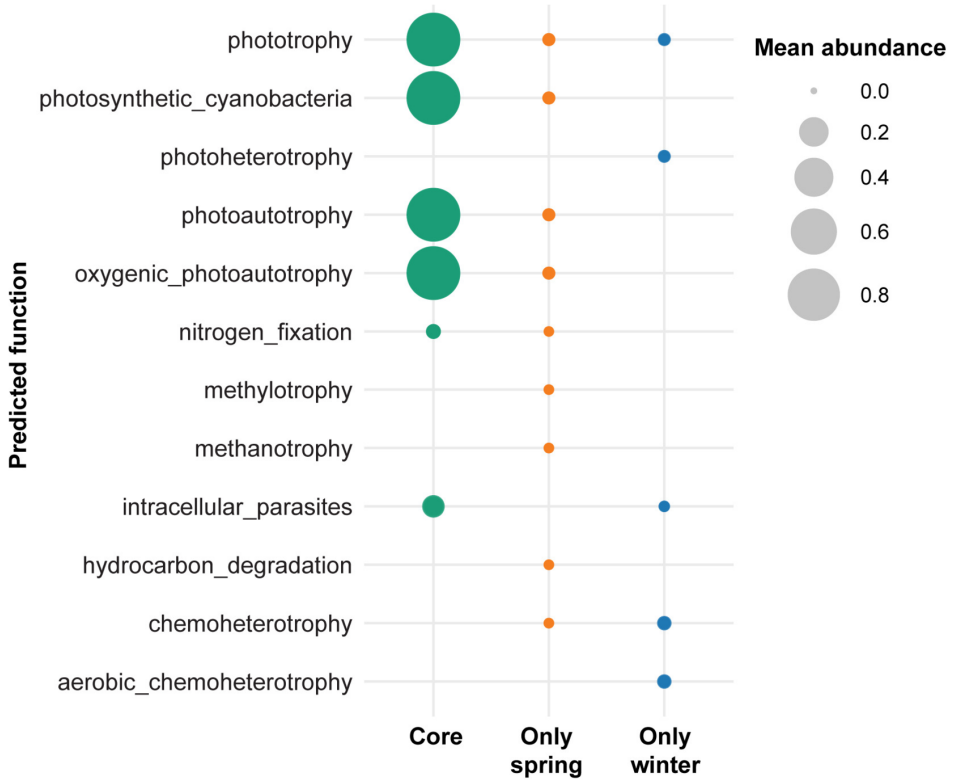


FIGURE 5 | Predicted functional profiles of the nodule-associated microbiome across seasonal field pea cultivars. Bubble plot shows the mean relative abundance of predicted microbial functions for three groups of taxa: Core (detected in all cultivars), Only spring (exclusive to spring cultivars), and Only winter (exclusive to winter cultivars). Functional predictions were inferred using FAPROTAX based on taxonomic profiles. Bubble size reflects mean abundance per group, with larger bubbles indicating higher relative abundance.

such as roots, nodules, and leaves (Singer et al. 2019; Brown et al. 2020; Ishida et al. 2022). Distinct genotypes create unique biochemical and physiological environments through differences in root exudation, tissue chemistry, or immune signaling, which in turn shape the recruitment, abundance, and persistence of specific microbial taxa (Zhang et al. 2023). This host-driven filtering effect helps explain why plants of the same species but with different genetic backgrounds often harbor contrasting microbial communities, even when grown under similar environmental conditions.

In our dataset, differences in *Rhizobium* abundance between cultivars were modest, but the consistent trend toward higher values in winter peas suggests genotype-associated recruitment. Parallel shifts also appeared in the non-rhizobial bacteria, plausibly reflecting subtle variation in exudate chemistry, immune tone, or nodule developmental timing (Zhalnina et al. 2018; Reid et al. 2018; Teixeira et al. 2021). Similar to reports in *Medicago truncatula*, our results reinforce that genotype effects are generally stronger in the rhizosphere and roots than in nodules (Brown et al. 2020). Soybean studies (Han et al. 2025) likewise demonstrate significant genotype influences in the rhizosphere, highlighting that the strength of this effect depends on plant compartment (Brown et al. 2020).

Although the magnitude of cultivar effects in our study was statistically modest, they point to a degree of host-driven filtering even within a single legume species, consistent with patterns observed across broader legume groups (Mutch and Young 2004; Hartman et al. 2017; Zhong et al. 2019). However, genotype-driven effects remain poorly documented in field pea, which crucially, nodules constitute a highly selective environment (Han et al. 2020), which likely compresses variation and makes cultivar effects more subtle.

4.3 | Non-Rhizobial Core: Cyanobacteria and Functional Potential

We identified a conserved non-rhizobial bacterial core comprising primarily cyanobacteria (e.g., *Anabaena*, *Tychonema*, and *Fischerella*), which are well documented in soil, biofilms, and plant-associated environments (Peng and Bruns 2019; Evseev et al. 2023). Their recurrent presence across cultivars suggests stable associations rather than sporadic colonization. Cyanobacteria possess traits such as oxygenic photosynthesis and diazotrophy that could complement nodule function, potentially buffering microaerobic conditions or supplying additional fixed nitrogen (Llamas et al. 2023). Whether they act as true endosymbionts or persist as epibionts on inner surfaces remains unresolved, but their consistent detection following rigorous sterilization indicates at least a close and reproducible association. Functional predictions reinforce this, with phototrophy and nitrogen fixation enriched in the shared core, while seasonal-specific taxa contributed additional but less abundant functions such as methylotrophy or sulfur cycling.

Interestingly, several studies have demonstrated that cyanobacteria can directly interact with rhizobia within legume nodules through co-inoculation approaches. In chickpea, formulations combining *Anabaena* spp. and *Mesorhizobium ciceri* enhanced nodulation, leghemoglobin content, nitrogenase activity, and yield relative to rhizobial inoculation alone (Bidyarani et al. 2016; Prasanna et al. 2017). These effects were linked to higher microbial diversity within nodules, improved nutrient mobilization, and elevated soil enzymatic activity, suggesting synergistic interactions between phototrophic cyanobacteria and diazotrophic rhizobia. More recently, Andrade et al. (2024) reported that co-inoculation of *Bradyrhizobium arachidis* with *Arthrospira platensis* or *Synechocystis* sp. increased groundnut productivity by up to 19% under field conditions. These findings indicate that cyanobacteria-rhizobium partnerships may enhance nodule functionality and plant performance, aligning with our observation of a cyanobacterial core in pea nodules.

Similarly, the identification of non-rhizobial core and group-specific taxa with distinct predicted functional signatures, such as photoheterotrophy, methylotrophy, and hydrocarbon degradation, indicates that nodule-associated bacterial communities may harbor functional diversity beyond canonical nitrogen fixation (see review by Bulgarelli et al. 2013). These observations parallel reports in peanut and soybean nodules, where non-rhizobial taxa extend the functional landscape beyond canonical symbiosis (Hossain et al. 2023; Han et al. 2020).

5 | Considerations and Implications

While filtering strategies were effective in unmasking hidden diversity, limitations remain. Predictive functional profiling relies on taxonomic inference and cannot confirm the associated traits. Moreover, distinguishing between internalized bacteria and persistent epiphytes is challenging without microscopy or culture-based validation. In addition, the modest sample size constrains power to detect fine-scale genotype effects. Addressing these issues will require integrating amplicon sequencing with metatranscriptomics, imaging, and targeted inoculation assays to validate functional contributions of non-rhizobial taxa. Furthermore, the discovery of a stable, cyanobacteria-enriched core suggests that nodule microbiomes may provide ecological functions complementary to nitrogen fixation, with potential implications for crop resilience. Understanding whether these taxa enhance stress tolerance, modulate oxygen availability, or contribute to nutrient turnover could open avenues for microbiome-informed breeding or inoculant design. Future work should test whether core cyanobacteria confer measurable benefits to host performance and whether these associations are conserved across environments. Expanding comparative studies across legumes and seasonal ecotypes will clarify whether the observed patterns in field pea reflect a generalizable feature of legume symbioses.

Author Contributions

Henrique M. Dias: conceptualization, investigation, writing – original draft, validation, methodology, visualization, formal analysis, data curation, writing – review and editing. **Shyam Solanki:** methodology, writing – review and editing. **Jose L. Gonzalez-Hernandez:** investigation, writing – review and editing, methodology, data curation. **Ravi V. Mural:** methodology, writing – review and editing. **Svetlana N. Yurgel:** methodology, writing – original draft, writing – review and editing. **Christopher Graham:** investigation, funding acquisition, conceptualization, writing – review and editing, methodology, data curation, supervision, resources, writing – original draft.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Full length 16S rRNA sequences were deposited in NCBI's SRA under BioProject accession number PRJNA1310262.

References

Andrade, D. S., G. M. Lovato, G. Kaschuk, and M. Hungria. 2024. "Does Coinoculation With *Bradyrhizobium* and Cyanobacteria Improve Groundnut Growth and Yield?" *Experimental Agriculture* 60: e9.

Baldanta, S., R. Arnal, A. Blanco-Rivero, G. Guevara, and J. M. Navarro Llorens. 2023. "First Characterization of Cultivable Extremophile *Chroococcidiopsis* Isolates From a Solar Panel." *Frontiers in Microbiology* 14: 982422.

Balogová, M., A. Šimonovičová, H. Vojtková, and O. Novák. 2025. "The Metabolic Fingerprint of Soil Microbiota in an Acidified Environment Using Biolog Microstation^{tm}." *Biologia* 80: 1–15.

Bever, J. D., T. G. Platt, and E. R. Morton. 2012. "Microbial Population and Community Dynamics on Plant Roots and Their Feedbacks on Plant Communities." *Annual Review of Microbiology* 66: 265–283.

Bidyarani, N., R. Prasanna, S. Babu, F. Hossain, and A. K. Saxena. 2016. "Enhancement of Plant Growth and Yields in *Chickpea (Cicer arietinum L.)* Through Novel Cyanobacterial and Biofilmed Inoculants." *Microbiological Research* 188–189: 97–105.

Brown, S. P., M. A. Grillo, J. C. Podowski, and K. D. Heath. 2020. "Soil Origin and Plant Genotype Structure Distinct Microbiome Compartments in the Model Legume *Medicago Truncatula*." *Microbiome* 8: 139.

Bulgarelli, D., K. Schlaeppi, S. Spaepen, E. V. L. Van Themaat, and P. Schulze-Lefert. 2013. "Structure and Functions of the Bacterial Microbiota of Plants." *Annual Review of Plant Biology* 64: 807–838.

Burghardt, L. T., B. Epstein, M. Hoge, D. I. Trujillo, and P. Tiffin. 2022. "Host-Associated Rhizobial Fitness: Dependence on Nitrogen, Density, Community Complexity, and Legume Genotype." *Applied and Environmental Microbiology* 88: e00526-22.

Camargo, A. P., R. S. de Souza, J. Jose, et al. 2023. "Plant Microbiomes Harbor Potential to Promote Nutrient Turnover in Impoverished Substrates of a Brazilian Biodiversity Hotspot." *ISME Journal* 17: 354–370.

Carter, K. R., A. C. Nachtsheim, L. T. Dickman, et al. 2023. "Drought Conditioning of Rhizosphere Microbiome Influences Maize Water Use Traits." *Plant and Soil* 492: 587–604.

Crosbie, D. B., M. Mahmoudi, V. Radl, et al. 2022. "Microbiome Profiling Reveals That *Pseudomonas* Antagonises Parasitic Nodule Colonisation of Cheater Rhizobia in *Lotus*." *New Phytologist* 234: 242–255.

Curry, K. D., Q. Wang, M. G. Nute, et al. 2022. "Emu: Species-Level Microbial Community Profiling of Full-Length 16S rRNA Oxford Nanopore Sequencing Data." *Nature Methods* 19: 845–853.

Daly, C., and K. Bryant. 2013. *The PRISM Climate and Weather System—An Introduction*. PRISM Climate Group.

Dilla-Ermita, C. J., R. W. Lewis, T. S. Sullivan, and S. H. Hulbert. 2021. "Wheat Genotype-Specific Recruitment of Rhizosphere Bacterial Microbiota Under Controlled Environments." *Frontiers in Plant Science* 12: 718264.

Evseev, P., I. Tikhonova, A. Krasnopeev, et al. 2023. "Tychonema sp. BBK16 Characterisation: Lifestyle, Phylogeny and Related Phages." *Viruses* 15: 442.

Favero, V. O., R. H. Carvalho, V. M. Motta, et al. 2021. "*Bradyrhizobium* as the Only Rhizobial Inhabitant of Mung Bean (*Vigna radiata*) Nodules in Tropical Soils: A Strategy Based on Microbiome for Improving Biological Nitrogen Fixation Using Bio-Products." *Frontiers in Plant Science* 11: 602645.

Galkiewicz, J. P., and C. A. Kellogg. 2008. "Cross-Kingdom Amplification Using Bacteria-Specific Primers: Complications for Studies of Coral Microbial Ecology." *Applied and Environmental Microbiology* 74: 7828–7831.

Hacquard, S., and F. M. Martin. 2024. "The Chemical Language of Plant–Microbe–Microbe Associations: An Introduction to a Virtual Issue." *New Phytologist* 244: 739–742.

Han, Q., Q. Ma, Y. Chen, et al. 2020. "Variation in Rhizosphere Microbial Communities and Its Association With the Symbiotic Efficiency of Rhizobia in Soybean." *ISME Journal* 14: 1915–1928.

Han, X., X. Zhang, M. Su, et al. 2025. "Genotype-Specific Rhizosphere Microbiome Assembly and Its Impact on Soybean (*Glycine max*) Yield Traits." *Frontiers in Sustainable Food Systems* 9: 1518597.

Hartman, K., M. G. A. van der Heijden, V. Roussely-Provent, J.-C. Walser, and K. Schlaeppi. 2017. "Deciphering Composition and Function of the Root Microbiome of a Legume Plant." *Microbiome* 5: 2.

Hossain, M. S., P. B. DeLaune, and T. J. Gentry. 2023. "Microbiome Analysis Revealed Distinct Microbial Communities Occupying Different Sized Nodules in Field-Grown Peanut." *Frontiers in Microbiology* 14: 1075575.

Im, W.-T., S.-H. Kim, M. K. Kim, L. N. Ten, and S.-T. Lee. 2006. "Pleomorphomonas Koreensis Sp. Nov., a Nitrogen-Fixing Species in the Order Rhizobiales." *International Journal of Systematic and Evolutionary Microbiology* 56, no. 7: 1663–1666. https://doi.org/10.1099/ijs.0.63499-0.

Ishida, J. K., A. P. Bini, S. Creste, and M. A. Van Sluys. 2022. "Towards Defining the Core *Saccharum* Microbiome: Input From Five Genotypes." *BMC Microbiology* 22: 193.

Ji, Z. J., H. Yan, Q. G. Cui, E. T. Wang, W. F. Chen, and W. X. Chen. 2017. "Competition Between Rhizobia Under Different Environmental Conditions Affects the Nodulation of a Legume." *Systematic and Applied Microbiology* 40: 114–119.

Johansen, J. R., M. Bohunická, A. Lukesová, and K. Hrčková. 2014. "Morphological and Molecular Characterization Within 26 Strains of the Genus *Cylindrospermum* (Nostocaceae, Cyanobacteria), With Descriptions of Three New Species." *Journal of Phycology* 50, no. 1: 187–202.

Jousset, A., C. Bienhold, A. Chatzinotas, et al. 2017. "Where Less May Be More: How the Rare Biosphere Pulls Ecosystems Strings." *ISME Journal* 11: 853–862.

Llamas, A., E. Leon-Miranda, and M. Tejada-Jimenez. 2023. "Microalgal and Nitrogen-Fixing Bacterial Consortia: From Interaction to Biotechnological Potential." *Plants* 12: 2476.

Louca, S., L. W. Parfrey, and M. Doebeli. 2016. "Decoupling Function and Taxonomy in the Global Ocean Microbiome." *Science* 353: 1272–1277.

Love, M. I., W. Huber, and S. Anders. 2014. "Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data With DESeq2." *Genome Biology* 15: 550.

Madigan, M. T., K. S. Bender, M. N. Parenteau, Y. Kimura, Z. Y. Wang-Otomo, and W. M. Sattley. 2025. "Genomic Highlights of the Phylogenetically Unique Halophilic Purple Nonsulfur Bacterium, *Rhodothalassium salexigens*." *Extremophiles* 29: 12.

Martínez-Hidalgo, P., and A. M. Hirsch. 2017. "The Nodule Microbiome: N_2-Fixing Rhizobia Do Not Live Alone." *Phytobiomes Journal* 1: 70–82.

Mathesius, U. 2022. "Are Legumes Different? Origins and Consequences of Evolving Nitrogen Fixing Symbioses." *Journal of Plant Physiology* 276: 153765.

Meier, D. V., P. Pjevac, W. Bach, et al. 2019. "Microbial Metal-Sulfide Oxidation in Inactive Hydrothermal Vent Chimneys Suggested by Metagenomic and Metaproteomic Analyses." *Environmental Microbiology* 21: 682–701.

Mutch, L. A., and J. P. W. Young. 2004. "Diversity and Specificity of *Rhizobium Leguminosarum* Biovar Viciae on Wild and Cultivated Legumes." *Molecular Ecology* 13: 2435–2444.

Neu, A. T., E. E. Allen, and K. Roy. 2021. "Defining and Quantifying the Core Microbiome: Challenges and Prospects." *Proceedings of the National Academy of Sciences of the USA* 118: e2104429118.

Oksanen J., F. G. Blanchet, R. Kindt, et al., 2013. "Package 'Vegan': Community Ecology Package, Version 2.5-7".

Peng, X., and M. A. Bruns. 2019. "Development of a Nitrogen-Fixing Cyanobacterial Consortium for Surface Stabilization of Agricultural Soils." *Journal of Applied Phycology* 31: 1047–1056.

Prasanna, R., B. Ramakrishnan, K. Simranjit, et al. 2017. "Cyanobacterial and Rhizobial Inoculation Modulates the Plant Physiological Attributes and Nodule Microbial Communities of Chickpea." *Archives of Microbiology* 199: 1311–1323.

R Core Team. 2021. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing https://www.R-project.org/.

Reid, D., H. Liu, S. Kelly, et al. 2018. "Dynamics of Ethylene Production in Response to Compatible Nod Factor." *Plant Physiology* 176: 1764–1772.

Sharaf, H., R. R. Rodrigues, J. Moon, B. Zhang, K. Mills, and M. A. Williams. 2019. "Unprecedented Bacterial Community Richness in Soybean Nodules Vary With Cultivar and Water Status." *Microbiome* 7: 63.

Singer, E., J. Bonnette, S. C. Kenaley, T. Woyke, and T. E. Juenger. 2019. "Plant Compartment and Genetic Variation Drive Microbiome Composition in Switchgrass Roots." *Environmental Microbiology Reports* 11: 185–195.

Sujkowska, M., M. Górska-Czekaj, M. Bederska, and W. Borucki. 2011. "Vacuolar Organization in the Nodule Parenchyma Is Important for the Functioning of Pea Root Nodules." *Symbiosis* 54: 1–16.

Tedersoo, L., M. Albertsen, S. Anslan, and B. Callahan. 2021. "Perspectives and Benefits of High-Throughput Long-Read Sequencing in Microbial Ecology." *Applied and Environmental Microbiology* 87: e00626-21.

Teixeira, P. J., N. R. Colaianni, T. F. Law, et al. 2021. "Specific Modulation of the Root Immune System by a Community of Commensal Bacteria." *Proceedings of the National Academy of Sciences of the USA* 118: e2100678118.

Trivedi, P., J. E. Leach, S. G. Tringe, T. Sa, and B. K. Singh. 2020. "Plant–Microbiome Interactions: From Community Assembly to Plant Health." *Nature Reviews. Microbiology* 18: 607–621.

Uesaka, K., N. Fukuda, K. Itoh, F. Maruyama, and T. Hoshino. 2024. "Restoration of the Functional Nif Gene Cluster by Complex Interspecies Recombination in Filamentous Cyanobacteria." *PLoS Computational Biology* 20, no. 3: e11249958.

USDA. 2022. "Winter Pea Cover Crop Fact Sheet." Accessed April 22, 2025. https://www.nass.usda.gov/sites/default/files/2024-07/Winter%20Pea%20Cover%20Crop%20Fact%20Sheet.pdf.

USDA-GIPSA. 2010. "Official United States Standards for Grain: Field Peas." Accessed April 22, 2025. https://www.ams.usda.gov/sites/default/files/media/2010GIPSANR.pdf.

Vann, R., S. Reberg-Horton, M. Castillo, S. Mirsky, and R. McGee. 2018. "Winter Pea Cultivar/Breeding Line Screening for Grain Crop Potential in the Southeastern United States." *Agronomy Journal* 110: 1217–1225.

Vocanson, A., and M. H. Jeuffroy. 2008. "Agronomic Performance of Different Pea Cultivars Under Various Sowing Periods and Contrasting Soil Structures." *Agronomy Journal* 100: 748–759.

Wendlandt, C. E., J. U. Regus, K. A. Gano-Cohen, et al. 2019. "Host Investment Into Symbiosis Varies Among Genotypes of the Legume *Acmispon Strigosus*, but Host Sanctions Are Uniform." *New Phytologist* 221: 446–458.

Willis, A. D. 2019. "Rarefaction, Alpha Diversity, and Statistics." *Frontiers in Microbiology* 10: 2407.

Wood, D. E., J. Lu, and B. Langmead. 2019. "Improved Metagenomic Analysis With Kraken 2." *Genome Biology* 20: 257.

Yang, H. J., W. W. Ye, Y. Ze, et al. 2023. "Host Niche, Genotype, and Field Location Shape the Diversity and Composition of the Soybean Microbiome." *Journal of Integrative Agriculture* 22: 2412–2425.

Yin, C., J. M. Casa Vargas, D. C. Schlatter, C. H. Hagerty, S. H. Hulbert, and T. C. Paulitz. 2021. "Rhizosphere Community Selection Reveals Bacteria Associated With Reduced Root Disease." *Microbiome* 9: 86.

Yurgel, S. N., and R. McGee. 2025. "Bacterial Communities Associated With Food-Quality Winter Pea Cultivars Grown in Pacific Northwest Soils." *Plant and Soil* 513: 2343–2361.

Zhalnina, K., K. B. Louie, Z. Hao, et al. 2018. "Dynamic Root Exudate Chemistry and Microbial Substrate Preferences Drive Patterns in Rhizosphere Microbial Community Assembly." *Nature Microbiology* 3: 470–480.

Zhang, B., N. Du, Y. Li, P. Shi, and G. Wei. 2018. "Distinct Biogeographic Patterns of Rhizobia and Non-Rhizobial Endophytes Associated With Soybean Nodules Across China." *Science of the Total Environment* 643: 569–578.

Zhang, J., W. Liu, J. Bu, Y. Lin, and Y. Bai. 2023. "Host Genetics Regulate the Plant Microbiome." *Current Opinion in Microbiology* 72: 102268. https://doi.org/10.1016/j.mib.2023.102268.

Zhang, R., J. M. Vivanco, and Q. Shen. 2017. "The Unseen Rhizosphere Root–Soil–Microbe Interactions for Crop Production." *Current Opinion in Microbiology* 37: 8–14.

Zhong, Y., Y. Yang, P. Liu, et al. 2019. "Genotype and Rhizobium Inoculation Modulate the Assembly of Soybean Rhizobacterial Communities." *Plant, Cell & Environment* 42, no. 6: 2028–2044.

Zhu, Y. G., J. Peng, C. Chen, et al. 2023. "Harnessing Biological Nitrogen Fixation in Plant Leaves." *Trends in Plant Science* 28: 1391–1405.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** leg370069-sup-0001-Figure_S1.tif. **Seasonal cultivar effects on alpha and beta diversity of non-*Rhizobium* bacterial communities.** Alpha diversity indices of spring and winter cultivars at genus-level. Violin plots show distributions of four alpha diversity metrics across field pea nodule microbiomes from spring (orange) and winter (blue) cultivars: Chao1 richness, Shannon diversity, and Simpson's diversity. Center lines denote medians; box widths indicate interquartile ranges. No significant differences were observed between seasonal groups for any index (ANOVA and Kruskal–Wallis $p > 0.05$; $n = 8$ per group). **Table S1:** Summary of sequencing quality metrics generated by NanoStat before and after NanoFilt processing, including mean read length, mean read quality, number of reads, and N50 read length for each barcode. **Table S2:** Relative abundance of bacterial taxa summarized by phylum across all barcode-filtered samples, showing taxonomic profiles at higher classification levels. **Table S3:** Differential abundance analysis results generated with DESeq2, showing $\log_2$ fold changes, significance values, and taxonomic assignments for microbial taxa across cultivar groups. **Table S4:** Core microbiome classification of bacterial species identified across samples, categorized as core, accessory, or specific to seasonal cultivar groups. **Table S5:** FAPROTAX-based functional predictions of microbial communities, showing the relative contribution of functional groups across all samples.