Applied Soil Ecology 206 (2025) 105823

Contents lists available at ScienceDirect

Applied Soil Ecology

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

Research paper

Seed inoculation of *Hartmannibacter diazotrophicus* does not alter the rhizosphere bacterial microbiome of wheat and barley in a three-year field trial

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ARTICLE INFO

Keywords:
PGPR
Bio-inoculant
Indigenous microbiome
Bacterial community composition
Differential abundance
Longitudinal analysis

ABSTRACT

The effects of plant growth promoting bacteria used for inoculation on native microorganisms remain unexplored under field conditions and, to a lesser extent, in longitudinal studies using different crops. This study, spanning three seasons across two organic fields, examined through 16S rRNA gene sequencing how the seed inoculation of *Hartmannibacter diazotrophicus* influenced the rhizosphere bacterial communities of wheat and barley. In addition to bacterial inoculation, the effects of row spacing and organic fertilizer application were also assessed. Together with previous results, we determined that *H. diazotrophicus* could improve crop yield parameters without altering the bacterial community composition. Alpha and beta diversity indices showed non-significant effects for most of the three factors evaluated. The 19 most prevalent taxa at the genus level were identified in both crop species, which mainly encompassed the phyla *Pseudomonadota*, *Acidobacteriota*, and *Actinomycetota*. Differential abundance analysis showed that the location significantly influenced the recruitment of different bacterial communities by the same crop species. While in one organic farm, 2860 ASVs were affected by crop species, 232 ASVs were impacted at the other location. Further analyses, including longitudinal analysis, linear mixed model effects, and diversity indices, showed a significant effect of location, crop species, and season on the dynamics of bacterial communities. Our results are unusual compared with most of the studies reported and indicate the resilience of rhizosphere bacterial populations after the incorporation of an allochthonous microorganism such as *H. diazotrophicus*.

1. Introduction

The use of living microorganisms has emerged as a strategy to address the unwanted effects of chemical pesticides and mineral fertilizers. By 2023, a market research report valued the global fertilizer market at over 200 billion USD (Global Market Insights, 2024). For biofertilizers, another report projects a market value of 2.70 billion USD by the current year (Market Data Forecast, 2024). As a sustainable agricultural strategy, biofertilizers are expected to grow, with projections indicating a market value of 4.78 billion USD with a compound annual growth rate (CAGR) of 12.09 % by 2029 (Market Data Forecast, 2024). Along with this continuous increase, several concerns have arisen regarding the environmental impacts of using living microorganisms to enhance plant performance. The current debate aims to elucidate the effects of bio-inoculants, broadly referred to beneficial microorganisms, once they are released into the soil. From an analysis of 108 studies, Mawarda et al. (2020) determined that soil microbial communities were altered in 86 % of cases when bio-inoculants were introduced. Only a few studies have shown that shifts in microbial community structure can negatively influence plant growth promotion (Ciccillo et al., 2002;

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https://doi.org/10.1016/j.apsoil.2024.105823
Received 25 April 2024; Received in revised form 21 October 2024; Accepted 9 December 2024

Kozdrój et al., 2004). This bias towards reporting primarily positive results may distort the perceived efficacy of bio-inoculants (Cardinale et al., 2015).

Plant growth promoting rhizobacteria (PGPR), a category within bio-inoculants, enhance plant growth through mechanisms including 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, nitrogen fixation, iron sequestration, or induction of systemic resistance (for review: El-Saadony et al., 2022; Nagrale et al., 2023). Further studies have determined that bio-inoculants can alter soil physicochemical properties such as soil aggregation, soil organic matter decomposition, and nutrient cycling (Dar et al., 2021; Sun et al., 2020; Zhou et al., 2020). Nevertheless, the positive or negative environmental impacts of introducing bio-inoculants are complex and go beyond determining alterations in soil microbial populations. In natural environments, microbial communities shift in different plant compartments during the plant life cycle (Wagner et al., 2016, 2014).

The introduction of PGPRs has been correlated with changes in community composition and abundance of bacteria involved in plant growth promotion (e.g. *Chryseobacterium* and *Bradyrhizobium*) (Zhong et al., 2019). Interestingly, exogenous bacterial inoculation has been related to an increase in the abundance of biocontrol agents (e.g. *Cryptococcus*) during soil pathogen suppression (Fu et al., 2017). However, it is also crucial to consider factors prior to inoculation. Keswani et al. (2019) highlighted the re-categorization of several PGPR as biosafety level 2 microorganisms (moderate individual risk and low community risk). To establish an appropriate environmental and human safety index (EHSI) and adequate regulatory frameworks, whole genome sequencing is highly recommended. In this regard, differentiating a microorganism at species and strain levels can contribute to an accurate assessment of its biosafety level based on phylogenomic information (Vílchez et al., 2016; Keswani et al., 2019).

Field experiments often present complexities such as harsh environmental conditions, competition with native soil microbial communities, and different agronomical practices. Interestingly, these challenges have begun to provide valuable insights into the efficacy of bio-inoculants. For instance, bio-inoculants have shown to be more effective in dry regions compared to other climatic zones (Schütz et al., 2018) and re-inoculating microorganisms from local fields may offer plant growth promotion advantages due to their intrinsic environmental adaptability (Jiang et al., 2023). Furthermore, bacterial community assembly has been shown to be influenced by different crop rotation practices (Zhou et al., 2023). Nevertheless, the effects of PGPR in the field are limited. Of the studies analyzed by Mawarda et al. (2020), only 36 % were conducted under field experimental conditions. Additionally, 25 % used surface-sterilized seeds prior to inoculation under greenhouse conditions (Appendix A, Supplementary Table S1). This may give the bio-inoculant a partial advantage that does not accurately reflect in situ conditions and may manifest as part of the reported inconsistencies observed when bio-inoculants are applied in the field (Mayer et al., 2010; Jansson et al., 2023; de-Bashan and Nannipieri, 2024). Therefore, understanding the dynamics of bio-inoculants with indigenous soil microbial communities and their surrounding environment is essential for improving their efficacy. Current efforts to standardize the application of bio-inoculants in field studies may enhance the validity and reproducibility of these experiments (Neuhoff et al., 2024).

Hartmannibacter diazotrophicus strain E19^T is a PGPR, originally isolated from a natural salt meadow. In vitro and greenhouse experiments with barley have demonstrated its capability to solubilize phosphate, fix nitrogen, and enhance plant growth through ACC deaminase activity (Suarez et al., 2014, 2015). Later on, these PGPR abilities were corroborated by the identification of several relevant genes in its genome (Suarez et al., 2019). Recently, *H. diazotrophicus* strain E19^T was monitored on the roots of spring barley and winter wheat under field conditions. The bacterium was detected on the roots of both crops up to 273 and 119 days after seeding, mainly in one of the two organic farms evaluated. Additionally, its abundance was correlated with several yield parameters (Quiroga et al., 2024). Nevertheless, its impact on rhizosphere bacterial communities has not been addressed. Current molecular and omics approaches can help to disentangle the interactions between native microbial communities and allochthonous microorganisms, as well as the effects of abiotic and biotic factors (for review: Rai et al., 2023). Therefore, in this study, metabarcode sequencing was performed on soil bacterial communities at two organic farms across three seasons to examine how these populations were affected by strain E19^T inoculation, row spacing, and fertilizer application.

2. Materials and methods

2.1. Experimental site, seed coating, and field experiments

Two organic experimental stations were considered for the experiments: Kleinhohenheim (KH) (48° 44′ N, 9° 11′ E) located in southwest Germany and Gladbacherhof (GH) (50° 23′ N, 8° 15′ E) located in central Germany. The organic fields belong to the Hohenheim and Giessen universities, respectively. The experiments were conducted during three consecutive seasons: season I (2020−2021), season II (2021−2022), and season III (2022−2023). The soil type at each location was classified as Haplic Luvisol (IUSS Working Group WRB, 2015). The farms have been organically managed since 1994 (KH) and 1989 (GH). Detailed information regarding the experimental sites, soil properties, and crop rotation is provided in Appendix A (Supplementary Tables S2 and S4,) and Appendix B (Supplementary Table S6). Seed-coating of winter wheat (WW) and spring barley (SB) seeds was adapted from Kloepper (1981). Briefly, a resuspension in 0.03 M $MgSO_4$ of *Hartmannibacter diazotrophicus* strain E19^T (LMG 27460^T), cultured in a half-concentrated marine bouillon (Carl Roth GmbH, Germany), was mixed in a ratio 1:1 with gum Arabic 25 % and pH-value adjusted to 7.0 ± 0.2 ($\bar{x} = 1.73 \times 10^9 \pm 7.86 \times 10^8$ bacteria ml^{-1}). The mixture was spread over WW (*Triticum aestivum*, cv. Aristaro), and SB (*Hordeum vulgare* L., cv. Odilia/RGT-planet) seeds, properly mixed, and coated with talcum powder (Carl Roth GmbH, Germany). Colony forming unit (CFU) from the seed coated powder for all the seasons was estimated in $\bar{x} = 1.78 \times 10^8 \pm 1.05 \times 10^8$ CFU g^{-1} powder. To assess bacterial-specific effects, control treatments included only 0.03 M $MgSO_4$ without bacteria, following the previously described steps.

All field experiments were carried out similarly in a randomized complete block design (RCBD). Four replicates for the combination of the following factors were considered: fertilizer management (with fertilizer, without fertilizer, only for WW), row spacing (15 cm, 50 cm), and bacterial inoculation (strain E19^T, ctrl without bacteria). About 400 seeds m^{-2} were sown in plots of 7.5 m^2. Winter wheat was sown between October and November, whereas spring barley was sown between March and May (Appendix A, Supplementary Table S2). Only WW was fertilized with organic liquid manure by the soil drenching method with an average of 97.6 ± 11 kg N ha^{-1} (<110 kg N ha^{-1}, Appendix A, Supplementary Table S3). Fertilization of SB was not carried out because brewery standards require that protein concentration must be <11 % (Díaz et al., 2022).

2.2. Rhizosphere soil sampling, DNA extraction, and soil analysis

Roots of field plants and corresponding soil were collected from two distinct points in each plot (~1 m from the edge of the plot) at two plant growth stages: flowering (BBCH-60) and ripe (BBCH-75/BBCH-89), according to the Zadoks growth scale (Zadoks et al., 1974). During transportation, the samples were stored at 4 °C. The soil directly in contact with the roots was sieved into a glass bowl next to a Bunsen burner flame, using a metal sieve of 1.5 mm pore size and an ethanol-sterilized spoon. To avoid cross-contamination, newly sterilized glass dishes and sieves (180 °C for 5 h) were used for each sample. The tables were cleaned, disinfected, and covered with sterile covers for each sample. Additionally, bacterial treatments and controls were processed in separate areas within the same room while maintaining aseptic conditions. The obtained rhizosphere soil was stored in sterile plastic centrifuge tubes at −80 °C for DNA extraction and in plastic bags at −20 °C for chemical analyses. DNA extraction and chemical analyses were performed within three months and five months after storage, respectively. To ensure cell disruption, approximately 700 mg of heat-sterilized zirconia beads (0.1 mm) were placed inside two ml screw-cap microcentrifuge tubes, prior to the addition of 400–500 mg of rhizosphere soil. DNA extraction procedure was described in detail by Quiroga et al. (2024). For this study, we modified the protocol by adjusting the amount of rhizosphere soil used, as well as the volumes of reagents: 0.7 ml of extraction buffer (0.2 M sodium phosphate buffer, 0.1 M NaCl, 0.05 M EDTA, 25 g l^{-1} SDS, pH 8), 0.5 ml of phenol/chloroform/isoamyl alcohol (25:24:1), and 0.5 ml of chloroform. Dried DNA pellets were dissolved in 50 µl of nuclease-free water.

Ammonium (NH_4^+) and soil microbial biomass (SMB) were determined from rhizosphere soil samples from seasons I and II, as described by Kandeler and Gerber (1988) and Turner et al. (2001), respectively. For NH_4^+, two grams of soil were used, and colorimetrically determined after extraction with 1 M KCl at 660 nm. For SMB, 12.5 g of soil was employed for each fumigated and unfumigated samples, and the UV absorbance was measured at 280 nm with 0.5 M K_2SO_4. Additional chemical parameters that showed no significant differences, including the carbon/nitrogen ratio, nitrate, and soil microbial respiration, were measured as described by Rosado-Porto et al. (2023).

2.3. Metabarcoding characterization

Soil-extracted DNA was diluted (1:50) and the V4-V5 regions of the 16S rRNA gene were amplified by polymerase chain reaction (PCR) using Kapa High Fidelity (KAPA/Hifi) polymerase (Roche diagnostics GmbH, Mannheim, Germany). Universal 16S rRNA gene primers 520F (AYTGGGYDTAAAGNG) and 926R (CCGTCAATTCMTTTRAGTTT) (Integrated DNA Technologies, Belgium) were used for amplification (Claesson et al., 2009; Engelbrektson et al., 2010). Detailed information on PCR conditions, barcode and adapter addition, as well as elution, purification, quantification, and sequencing of the PCR products via Ion Torrent PGM is provided in the Supplementary section of Kaplan et al. (2019). To avoid contamination, all sensitive steps were carried out under a class II biosafety cabinet, and the corresponding negative controls were included to detect possible contamination.

2.4. Bioinformatics and statistical analysis

Sequencing data were analyzed using QIIME2–2022.11 version (Bolyen et al., 2019) and R studio software, v4.3.2 (R Core Team, 2023). In QIIME2, sequences were demultiplexed using the cutadapt QIIME2 plugin (Martin, 2011). Quality control, denoising, dereplicate of single-end sequences, and chimera filtering were performed using the QIIME2 plugin of the software package DADA2 (Callahan et al., 2016). Sequences for all seasons were trimmed at 15 bp and truncated at 325 bp. Amplicon Sequence Variants (ASVs) produced with DADA2 were taxonomically affiliated with a trained fitted classifier (Pedregosa et al., 2011; Bokulich et al., 2018b) based on the SILVA database (release 138) (Quast et al., 2012). After taxonomic affiliation unassigned, mitochondrial and chloroplast sequences were filtered out.

The R package microViz v0.12.1 (Barnett et al., 2021) was used for data curation. Subsequently, the 50 most prevalent taxa at genus level were considered to create a heatmap with Kendall correlation and compositional data based on centered log-ratio (clr) transformation. In addition, the categorical variables grouped as location, bacterial inoculation, fertilizer management, crop species, row spacing, and plant growth stage were transformed into a binary format. A value of 1 was assigned to GH, strain E19^T, with fertilizer, WW, 15 cm row spacing, and flowering stage, respectively. Similarly, prevalent taxa at the genus level were filtered and plotted based on relative abundance considering ASVs with a detection threshold of 0.20 % and a prevalence of 75 % through R package Microbiome v1.24.0 (Lahti and Shetty, 2019). Differential abundance of compositional data was conducted using the R package ALDEx2 v1.34.0 (Fernandes, 2014) at different grouping levels, including bacterial inoculation, row spacing, fertilizer, plant growth stage, crop species, location, and season. Previously, ASV counts lower than 99 were filtered out through the R package Phyloseq v1.46.0 (McMurdie and Holmes, 2013). To ensure the identification of taxa that were substantially affected, effect size values (≥ 1 or ≤ -1) and adjusted p-values ($p < 0.05$) obtained from the Benjamini-Hochberg correction of Welch's t-test (we.eBH) were utilized, as recommended by the authors.

Alpha diversity analyses were carried out using the R package Vegan v2.6.4 (Oksanen et al., 2019). The samples were rarefied and the diversity indices Observed ASVs, Shannon-Wiener, and Gini-Simpson were estimated and plotted. Pairwise comparisons of each alpha diversity index were obtained through Wilcoxon rank-sum test with Holm adjustment. The QIIME2 Gemelli plugin with Robust Aitchison PCR (RPCA) was used for beta diversity analysis. Subsequently, the significant differences were identified through permutational multivariate analysis of variance (PERMANOVA, 999 permutations) with Benjamini-Hochberg correction (Martino et al., 2021). Similarly, to visualize which taxa were related to changes over time, the q2-longitudinal plugin based on machine learning regressors was used ("longitudinal feature-volatility" action) (Bokulich et al., 2018a). Thereafter, the significant effects across time were assessed using linear mixed-effects (LME) models based on Shannon entropy included within the q2-longitudinal plugin ("linear-mixed-effects" action). For the LME, location, crop species, and season were treated as fixed effects. Finally, to evaluate the effect of bacterial inoculation and fertilizer management on NH_4^+ and SMB concentrations, linear mixed models (LMM) were applied using the R packages lme4 v1.1.35.1 (Bates et al., 2015) and lmerTest v3.1.3 (Kuznetsova et al., 2017). Bacterial inoculation and fertilizer management (only for WW) were considered as fixed effects. Whereas season, location, and experimental block were set as hierarchical random effects, and three models were developed using the following formulas:

Model 1:

$$Y_i = \beta_0 + \alpha_{T(i)} + b_{0i} + e_i$$

$$b_{0i} \sim NID\left(0, \sigma^2\right) \text{ and } e_i \sim NID\left(0, \sigma^{2.}\right)$$

Model 2:

$$Y_{ijk} = \beta_0 + \beta 1_j + \beta 2_k + \alpha_{T(i)} + b_{0i} + b_{1ij} + e_{ijk}$$

$$\begin{bmatrix} b_{0i} \\ b_{1i} \end{bmatrix} \sim NID\left(\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma_0^2 & \sigma_{01} \\ \sigma_{01} & \sigma_1^2 \end{bmatrix}\right) \text{ and } e_{ijk} \sim NID\left(0, \sigma^2\right)$$

Model 3:

$$Y_{ijk} = \beta_0 + \beta 1_j + \beta 2_k + (\beta 1 \beta 2)_{jk} + \alpha_{T(i)} + b_{0i} + b_{1ip} + e_{ijk}$$

$$\begin{bmatrix} b_{0i} \\ b_{1i} \end{bmatrix} \sim NID\left(\begin{bmatrix} 0 \\ 0 \end{bmatrix}, \begin{bmatrix} \sigma_0^2 & \sigma_{01} \\ \sigma_{01} & \sigma_1^2 \end{bmatrix}\right) \text{ and } e_{ijk} \sim NID\left(0, \sigma^2\right)$$

Where $\boldsymbol{Y_i}$ = chemical parameter concentration or response of the $\boldsymbol{i}$-th crop species, $\boldsymbol{j}$ = bacterial inoculation, $\boldsymbol{k}$ = fertilizer management, $\boldsymbol{\beta_0}$ = overall fixed intercept, $\boldsymbol{\beta 1_j}$ = intercept for bacterial inoculation, $\boldsymbol{\beta 2_k}$ = intercept for fertilizer management, $\boldsymbol{(\beta 1 \beta 2)_{jk}}$ = intercept of the interaction between both fixed effects, and $\boldsymbol{\alpha_T}$ = intercept of hierarchical random effects for the $\boldsymbol{i}$-th crop species. Finally, $\boldsymbol{b_{0i}}$ = random intercept, $\boldsymbol{b_{1i}}$ = random bacterial inoculation slope effects for the $\boldsymbol{i}$-th crop species, $\boldsymbol{e}$ = random error, and **NID** = normally and identically distributed effects. Model 1 illustrates the dependent variable (chemical parameter concentration) with only random effects and without fixed effects. Model 2 shows fixed effects but does not infer any interaction between them. Model 3 considers fixed effects and assumes the interactions between them. Finally, the models assumed mutual independence among the variability originated from differences between groups (random effects) and the unexplained variability within groups (random error).

3. Results

3.1. Nineteen prevalent bacterial taxa at genus level identified in winter wheat and spring barley rhizosphere over a three-year field trial

A total of 13,052,032 sequences were obtained across all seasons, with a minimum of 5037 sequences and a maximum of 65,316 sequences per sample obtained after demultiplexing and quality control using QIIME2. After performing taxonomic cleaning in R, the taxa bar plots were grouped according to crop species, location, and season. ASVs with a relative abundance lower than 0.20 % were excluded. This filtering enabled the identification of the 19 most prevalent taxa at the genus level, along with their respective phyla, for each crop at GH and KH, and over three consecutive seasons (Fig. 1). The results revealed that the rhizosphere of WW and SB shared 19 taxa at the genus level, encompassing five phyla (*Pseudomonadota*, *Acidobacteriota*, *Actinomycetota*, *Bacillota*, and *Candidatus* Methylomirabilota). Of the most prevalent taxa, 47 % were within the phylum *Pseudomonadota*, whereas 89 % belonged to the phyla *Pseudomonadota*, *Acidobacteriota*, and *Actinomycetota.* Differences in the percentage of relative abundance were observed across crop species, location, and seasons in genera considered as PGPR (e.g. *Bradyrhizobium*, *Lysobacter*, and *Microvirga*) or involved in nutrient cycling (e.g. *Arenimonas*, *Luteimonas*).

3.2. Alpha diversity indices significantly varied between locations

After conducting an exploratory analysis, these differences were evaluated using different species diversity measurements. No significant effects of bacterial inoculation, fertilizer, or row spacing were observed on any of the alpha diversity indices evaluated over the three seasons ($p > 0.05$). Instead, the treatments were categorized according to location (GH, KH), crop species (WW, SB), and plant growth stage (flowering/ripe) for each individual season. In addition, the species richness for those indices was estimated (rarefaction, 95 % confidence intervals, Fig. 2). A significant influence of location on all alpha diversity indices was determined across all three seasons for both crops. This was observed in comparisons between GH (WW/SB at the flowering stage) and KH (WW/SB at the flowering/ripe stage). In the case of WW, the Shannon-Wiener and Gini-Simpson indices at KH were significantly higher than those at GH for all alpha diversity indices. In contrast, this trend was not completely followed by SB, where the indices were significantly higher at KH than at GH during seasons II and III, but not in season I (Fig. 2A). Interestingly, a significant variation between the flowering and ripe stages was observed only for WW across all seasons in the three alpha diversity indices (comparison only at GH), with the exception of Simpson index in season II (Fig. 2B). In the case of SB, this trend could not be observed beyond season I (Fig. 2A), when no significant differences were detected for any of the indices during seasons II (Fig. 2B) and III (Fig. 2C). Conversely, crop species showed significant differences mainly in all alpha diversity indices at the same location and stage across seasons. However, exceptions occurred in season I (Simpson index for KH, Fig. 2A) and season II (Observed ASVs for KH, and Simpson index for GH, Fig. 2B), when WW and SB were not significantly different.

3.3. Strain $E19^T$ did not alter the rhizosphere bacterial microbiome, whereas bacterial communities were significantly different between locations

Rhizosphere bacterial communities in WW and SB were assessed by RPCA through the Gemelli plugin. The findings showed that bacterial inoculation, fertilizer management, and row spacing did not significantly alter the bacterial community structure. This was consistent across all seasons, as confirmed by the PERMANOVA analysis, showing no significant differences ($p > 0.05$, 999 permutations). The only exception was observed in the last season, when a significant effect of fertilizer on WW was found at GH during the ripe stage (Table 1). Additionally, significant differences were detected in season III, when fertilizer and bacterial inoculation were combined, attributing the main effect to the fertilizer (Table 1). This trend was not observed in the other two seasons or locations ($p > 0.05$). Similar to the alpha diversity, we

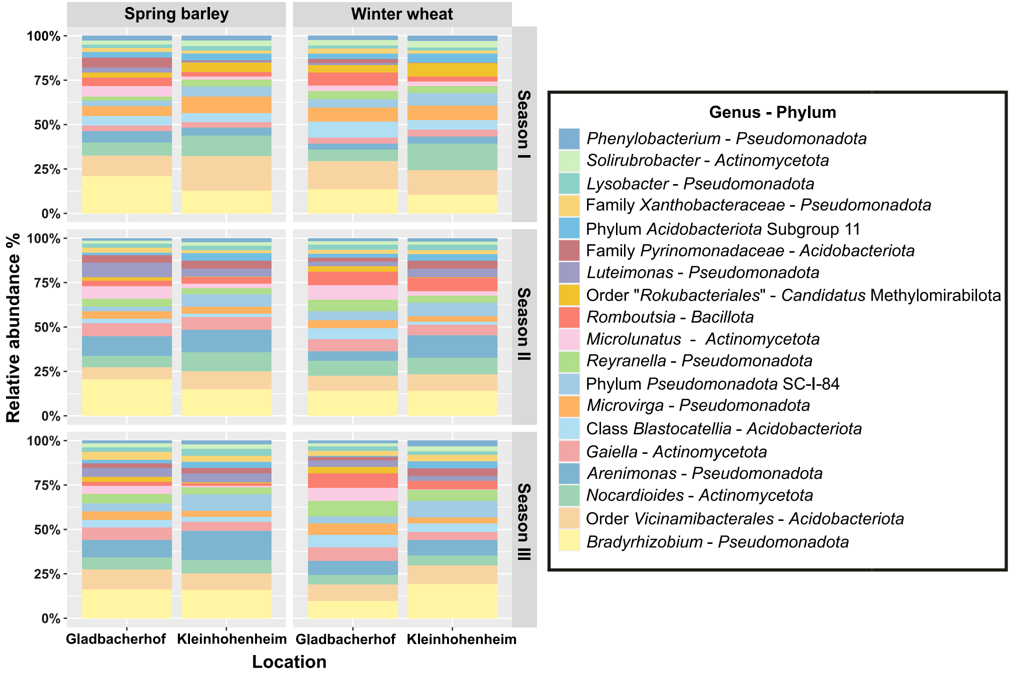

Fig. 1. Rhizosphere bacterial composition based on the relative abundance (%) of winter wheat and spring barley at different locations over three seasons. Taxa were collapsed at the genus level after removing ASVs with a relative abundance lower than 0.20 %.

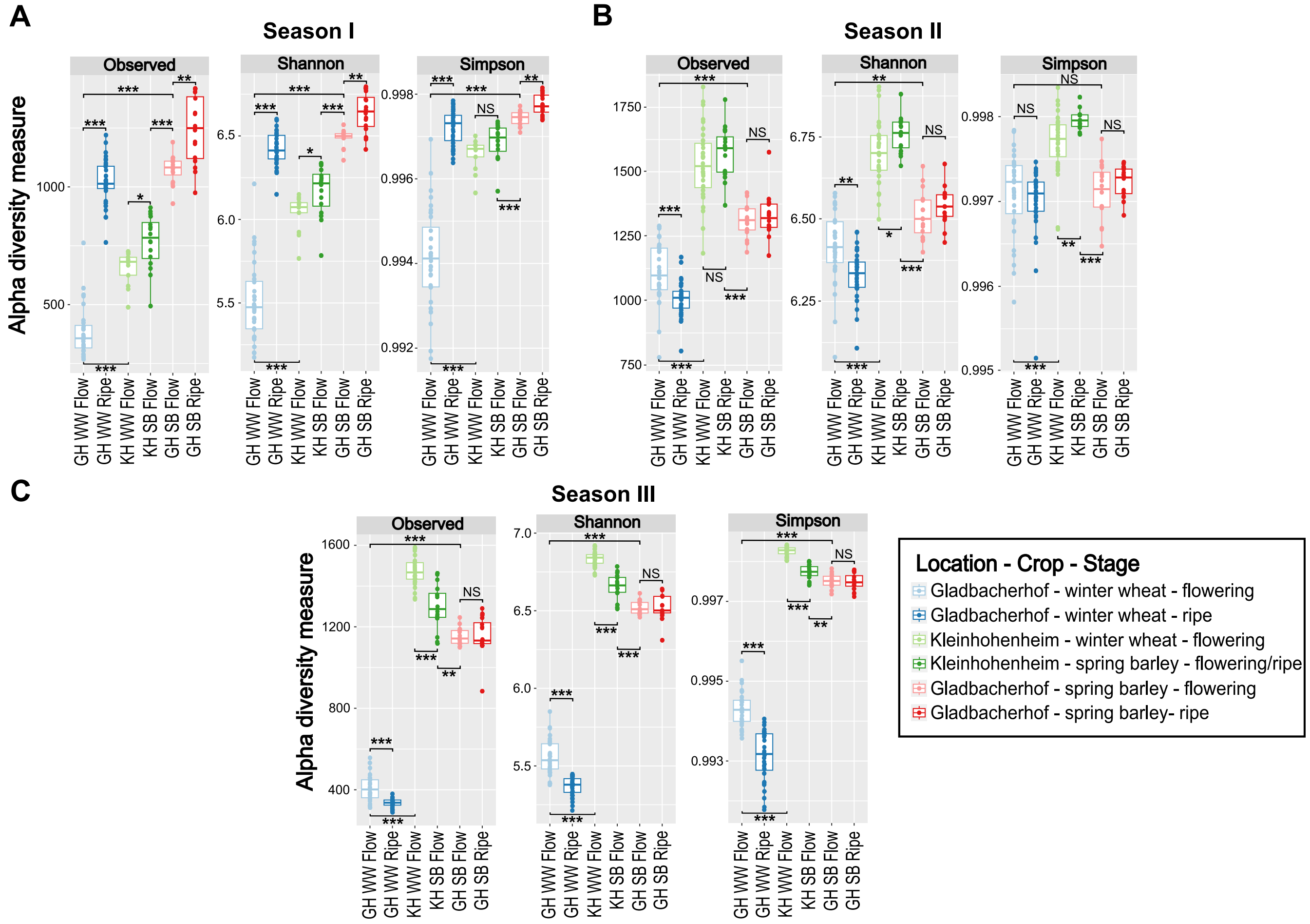

Fig. 2. Wheat and barley rhizosphere bacterial alpha diversity indices across seasons. The observed ASVs, Shannon-Wiener, and Gini-Simpson indices were grouped according to location, crop species, and plant growth stage during **(A)** season I (2020–2021), **(B)** season II (2021–2022), and **(C)** season III (2022–2023). Normalization was performed using rarefaction. NS, not significant. Significance codes: ≤0.001 '***', ≤0.01 '**', ≤0.05, '*'.

determined how the rhizosphere bacterial communities were affected by location, crop species, and plant growth stage in each season (Fig. 3). The RPCA showed clear distinctions between location and crop species across seasons. These findings were corroborated by the PERMANOVA analysis (Table 1), indicating that the rhizosphere bacterial communities at GH differed significantly from those at KH. Additionally, the results revealed that, within the same location, crop species were able to recruit different bacterial communities. However, these differences varied depending on the location. At GH, distinct clusters were consistently found between WW and SB during the three seasons. Conversely, at KH although these clusters were closer together in the RPCA, PERMANOVA analysis showed significant differences only during seasons I and III (Fig. 3B-C).

When the plant growth stage was evaluated, the same consistent trend observed in the alpha diversity was found. WW bacterial communities (only at GH) significantly changed from the flowering to the ripe stage in all seasons (Table 1). In contrast, this pattern was not observed in SB, in which the bacterial communities differed between stages only during season I (Fig. 3A).

3.4. Differential abundance showed the effect of location on the recruitment of different bacterial communities by the same crop species

Following the analysis of ecological diversity indices in terms of bacterial diversity, we delved deeper to determine the taxa that were influenced by fertilizer, bacterial inoculation, and row spacing. Additionally, the variation of these taxa across location, plant growth stage, crop species, and season was also examined. This issue was addressed in two ways: first, through a correlation heatmap using Kendall correlation, and second, by analyzing the differential abundance employing ALDEx2 and considering effect size values ≥1 or ≤ −1.

Most correlations determined in the heatmap were given by location, crop species, season, and, to a lesser extent, by fertilizer and plant growth stage (Fig. 4A). The correlation heatmap mainly showed negative correlations for location and crop species, indicating that the 50 most prevalent taxa at the genus level were negatively correlated with GH and WW (Fig. 4A). Interestingly, several taxa were positively correlated as time progressed from season I to III. In contrast to bacterial inoculation and row spacing, in which almost no correlations were found, fertilizer application showed negative correlations. Based on these findings, we transitioned our approach to conduct a more specific analysis using ALDEx2 differential abundance. To corroborate whether the three factors evaluated had an effect on taxa abundance, each location, crop species, and season were analyzed separately at the flowering stage for comparison purposes. This approach was based on the observation that most of the variation in taxa abundance (Fig. 4A) was explained by these variables. ALDEx2 differential abundance showed no significant differences for the we.eBH ($p > 0.05$) with effect size values≥ -1 and ≤ 1 for any of the factors evaluated. These findings indicate that fertilizer management, bacterial inoculation, and row spacing had no significant effect on taxa abundance. Thereafter, the effect of crop species on differential abundance was examined at both locations for each season. The results showed that depending on the location, crop species were significantly influenced by different taxa

Table 1
PERMANOVA pairwise comparisons of beta diversity. Treatments were grouped according to location, crop species, and plant growth stage as well as by fertilizer and bacterial inoculation plus fertilizer.

Comparison	Evaluated factor	Season I	Season II	Season III
GH WW Flowering vs GH WW Ripe	stage	0.001***	0.0012**	0.0011**
GH WW Flowering vs KH WW Flowering	location	0.001***	0.0012**	0.0011**
GH SB Flowering vs GH SB Ripe	stage	0.001***	0.361	0.903
GH SB Flowering vs KH SB Flowering	location	0.001***	0.0012**	0.0011**
GH WW Flowering vs GH SB Flowering	crop species	0.001***	0.0012**	0.0011**
KH WW Flowering vs KH SB Flowering	crop species	0.001***	0.953	0.0011**
GH WW Ripe with fertilizer vs GH WW Ripe without fertilizer	fertilizer	0.758	0.250	0.0023**
GH WW Ripe Ctrl with fertilizer vs GH WW Ripe E19 without fertilizer	fertilizer + bacterial inoculation	0.763	0.699	0.028*
GH WW Ripe E19 with fertilizer vs GH WW Ripe Ctrl without fertilizer	fertilizer + bacterial inoculation	0.906	0.344	0.0073**
GH WW Ripe E19 with fertilizer vs GH WW Ripe E19 without fertilizer	fertilizer + bacterial inoculation	0.917	0.459	0.0015**

Gladbacherhof (GH), Kleinhohenheim (KH), winter wheat (WW), spring barley (SB). The adjusted *p*-value is shown, considering 999 permutations. Significance codes: ≤0.001 '***', ≤0.01 '**', ≤0.05, '*'.

($p_{\text{we.eBH}} < 0.05$). In fact, it was originally intended to plot all taxa at the genus level with effect size values ≥ 1 and ≤ -1. Nonetheless, due to the large number of taxa significantly affected between SB and WW, higher effect size thresholds were set for plotting at GH (≥ 2.3 to 2.6 and ≤ -2.3 to −2.6) and at KH (≥ 1.2 to 1.6 and ≤ -1.2 to −1.6) (Fig. 4B). At GH, 2860 ASVs were significantly affected by crop species in at least one season, including 15 of the 19 most prevalent taxa. While at KH, 12 times fewer ASVs were found (232 ASVs), including ten of the 19 most prevalent taxa (Appendix B, Table S5). Despite the difference between locations, the taxa in Fig. 4B clearly illustrate that identifying a consistent trend in differential abundance for a specific taxon was difficult. For instance, while the prevalent genus *Microlunatus* was negatively affected by WW at GH across all seasons, the genera *Luteimonas*, *Arenimonas*, and *Bradyrhizobium* were only negatively influenced by WW in one of the three seasons. In contrast, at KH, *Nocardioides* was negatively influenced by WW compared to SB in seasons I and III, but positively affected during season II. These findings show, first, the pivotal role of location in the recruitment of different taxa by the same crop species, and second, the positive or negative influence of season on the same taxa.

3.5. Longitudinal analysis revealed the effects of location, crop species, and season on bacterial community dynamics

To shed more light on how these taxa fluctuated over time, longitudinal feature volatility analysis was performed using the q2-longitudinal plugin. The 35 most potentially relevant taxa were identified (Table 2). These results were in accordance with the 13 prevalent taxa observed in Fig. 1, and were also detected in the correlation heatmap and ALDEx2 analysis (Fig. 4). The feature volatility plot showed how the relative frequency varied according to different factors over time for each taxon. From all of them (Table 2), four prevalent taxa were selected: *Luteimonas* (top one, Table 2), *Reyranella* (top two, Table 2), *Arenimonas*, and *Lysobacter* (observed in both fields after differential abundance analysis, Fig. 4B). Thereafter, their fluctuations were monitored according to crop species and location (Fig. 5).

In general, the relative frequency changed depending on the location and crop species, differentiating GH and KH, as well as WW and SB, mainly in all taxa. This differentiation was not observed when the other previously analyzed factors were considered (e.g. bacterial inoculation). The fluctuations in *Arenimonas*, *Lysobacter*, and *Luteimonas* (Fig. 5A-C) correctly fit the results obtained after differential abundance analyses, at least with high effect size values (Fig. 4B, Appendix B, Table S5). For instance, the relative frequency of *Arenimonas* increased during season II (sample point 2.0, Fig. 5B) for SB at GH, whereas the differential abundance showed a negative correlation with WW during this season (Fig. 4B). At KH, the relative frequency was higher in season III (sample point 3.0) for SB than for WW, which was in accordance with the ALDEx2 negative correlation between WW and SB. Similarly, the positive WW correlation obtained for the differential abundance of *Lysobacter* at GH during season II and *Reyranella* for all seasons (Appendix B, Table S5) was related to higher relative frequencies in WW at those sample points (Fig. 5C-D). Nevertheless, several taxa, including some of the taxa analyzed, did not fully match with the differential abundance results (Appendix B, Table S5).

The inconsistent trend observed after differential abundance analyses was better understood with the longitudinal feature volatility plot visualization. A taxon that initially did not show any difference at one sampling point, could increase/decrease at the next point, or converged at another (e.g. *Arenimonas*). These findings indicate that plant-microbe interactions are affected over time by different variables. To corroborate this, LME models based on Shannon entropy within the q2-longitudinal plugin were used to determine the effects of location, crop species, and season (fixed effects) on microbial communities. None of the fixed variables showed any significant effects (Table 3). Nevertheless, Shannon entropy was significantly associated with the interaction of sample point (season) and location ($p = 0.001$), sample point and crop species ($p < 0.001$), and sample point, location, and crop species ($p = 0.001$, Table 3). The findings revealed that the Shannon entropy of the bacterial rhizosphere microbiome significantly increased by a factor of 0.24 at KH as the season progressed. In contrast, the bacterial microbiome decreased by a factor of 0.18 in WW across all seasons. Finally, LME also showed that the bacterial rhizosphere microbiome increased over time by a factor of 0.29 in KH and WW. These results corroborated and complemented the alpha and beta diversity results, unveiling how the rhizosphere microbiome is influenced across seasons, which could not be clearly determined by previous analyses.

3.6. Strain E19^T plus fertilizer showed a positive trend increasing ammonium and microbial biomass concentrations

The last approach attempted to correlate the differential abundance with several soil chemical parameters, including ammonium, microbial biomass, carbon/nitrogen ratio, nitrate, and respiration activity, using different carbon sources (glucose, galactose, *N*-acetylglucosamine, and arginine). Nevertheless, ALDEx2 analysis did not show any relevant correlation for any of the parameters previously mentioned (data

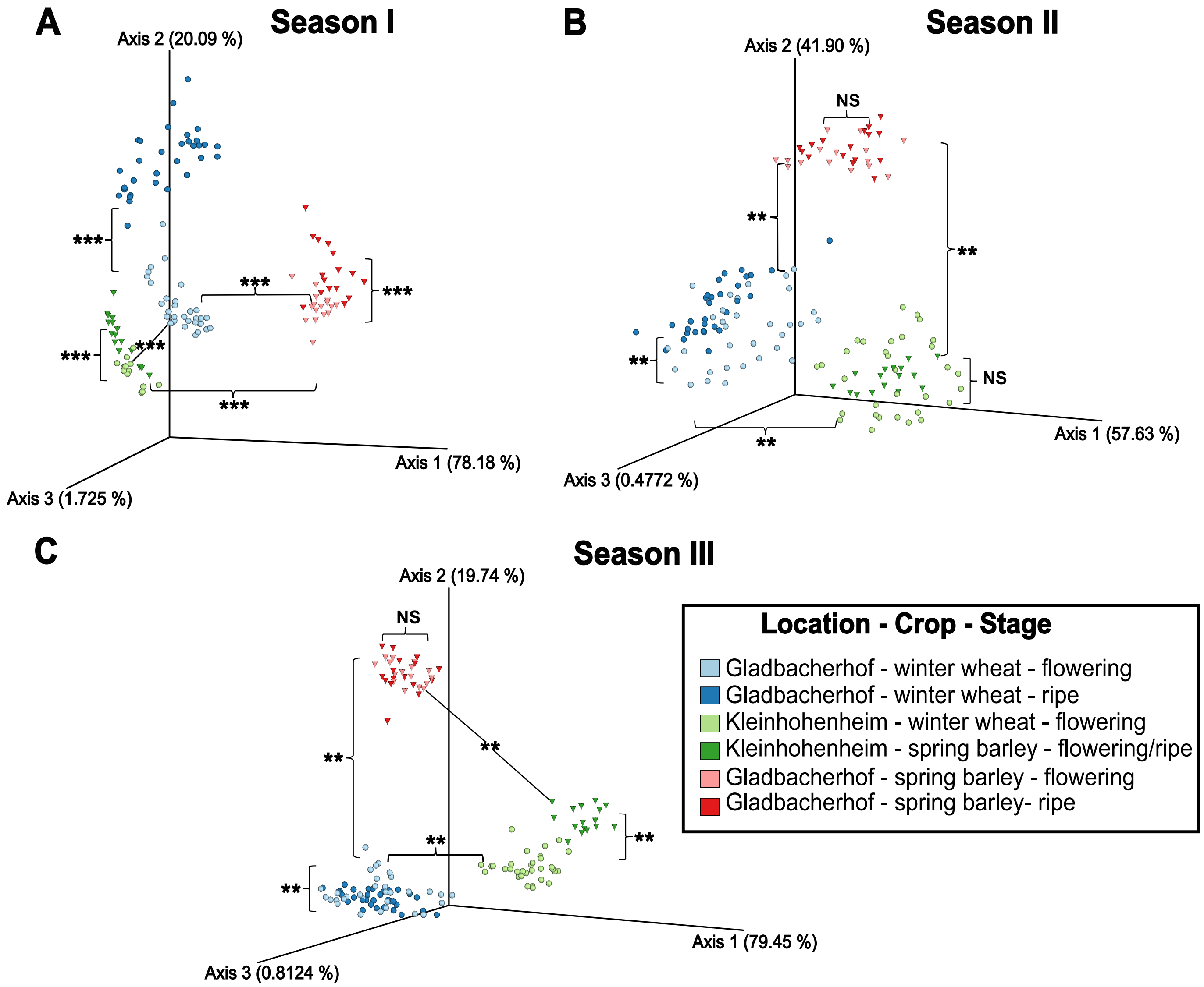

Fig. 3. Beta diversity analysis using robust Aitchison RPCA for winter wheat and spring barley at different locations and plant growth stages. **(A)** Season I (2020–2021), **(B)** season II (2021–2022), and **(C)** season III (2022–2023). NS, not significant. Significance codes: ≤ 0.001 '***', ≤ 0.01 '**'.

available at the BONARES repository). Instead, we decided to evaluate through LMMs whether the combination of fertilizer management and bacterial inoculation (fixed effects) had a significant effect on the soil chemical parameters. For this purpose, location, season, and block were modelled as hierarchical random effects. Significant effects on ammonium and microbial biomass were found in WW (Fig. 6), but not in SB ($p > 0.05$). A *t*-test using Satterthwaite's method showed significant differences only in the variable fertilizer management for microbial biomass (Table 4). However, type III analysis of variance revealed significant effects of fertilizer management on both parameters, ammonium ($p = 0.0021$) and microbial biomass ($p = 0.0004$). The results indicated a significant decrease when fertilizer was not applied by 39.4 µg SMB C g^{-1} DW soil compared to the mean ($\overline{x} = 415.5$ µg SMB C g^{-1} DW soil, Table 4). Even though a significant effect of bacterial inoculation or its combination with fertilizer could not be found (Table 4), a positive trend was observed for E19^T plus the addition of fertilizer to increase ammonium and microbial biomass concentrations (Fig. 6).

4. Discussion

4.1. Resilience of rhizosphere bacterial communities after non-native inoculation of H. diazotrophicus under field conditions

Few studies have focused on the effects of bio-inoculants under field conditions, and to a lesser extent, over an extended period (Fu et al., 2017; Gui et al., 2017; Xu et al., 2018). This study evaluated whether *H. diazotrophicus* strain E19^T could alter the rhizosphere bacterial community structure and how the interactions with these communities differed depending on crop species and location. Remarkably, strain E19^T did not alter the rhizosphere bacterial communities across all seasons (alpha and beta diversity indices). Resident microbial populations can exhibit resistance, resilience, or functional redundancy against disturbances (Allison and Martiny, 2008). It has been stated that an exogenous microorganism can be strongly buffered by native soil microbial communities (Björklöf et al., 2003). However, through mechanisms such as niche competition or antagonism, an external microorganism can be incorporated into native soil microbial communities (Russ et al., 2023). The findings in this study showed the resilience of the resident soil bacterial populations to restructuring themselves after probable disruptions in early plant growth stages due to the high

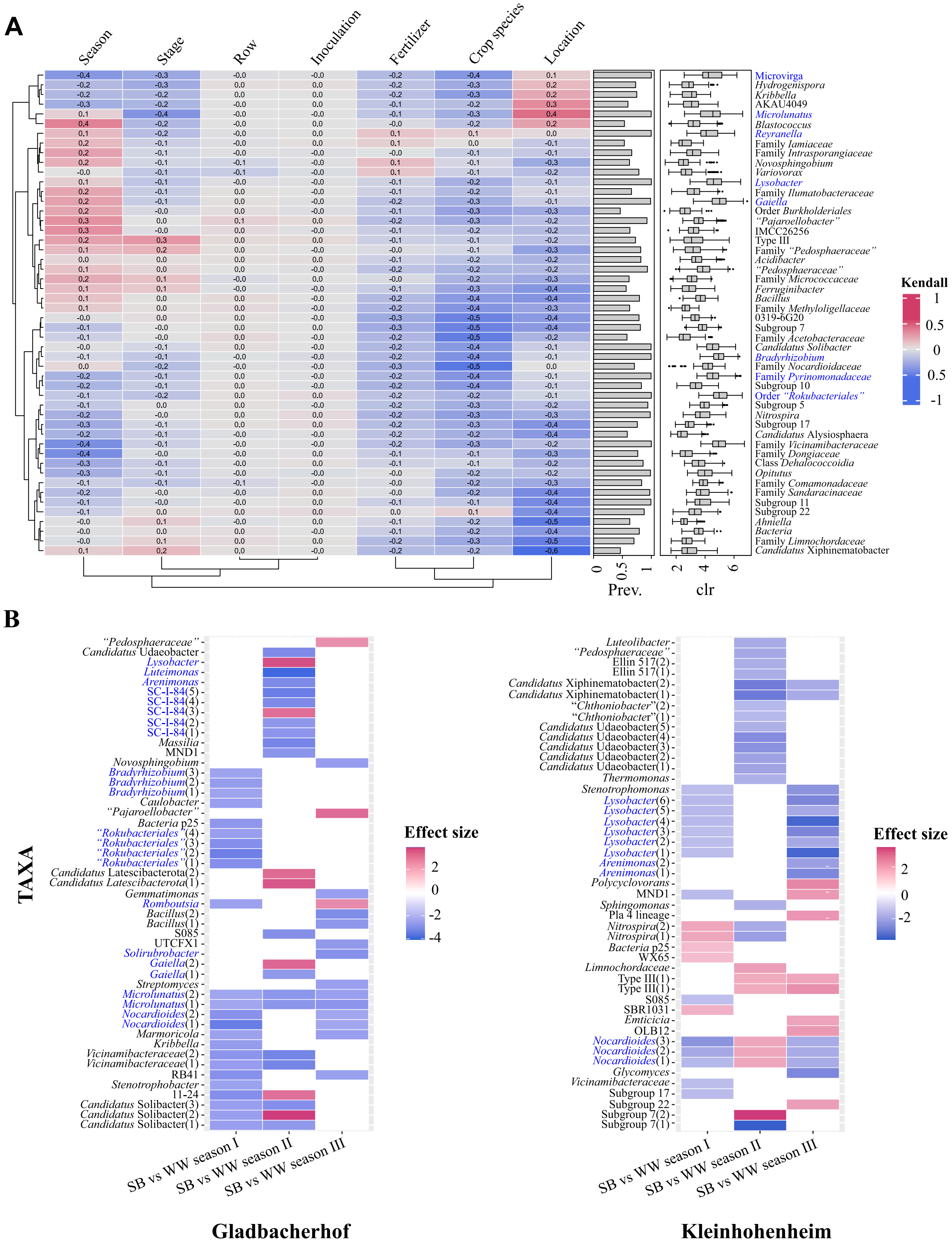

Fig. 4. Heatmaps showing the effects of different variables on taxa at the genus level across all seasons. **(A)** Correlation heatmap with Kendall correlation for the 50 most prevalent taxa **(B)** Heatmap between winter wheat (WW) and spring barley (SB) at two locations based on ALDEx2 differential abundance after removing ASVs with relative abundances <99 ASV counts. Due to the excessive number of taxa that were significantly affected, the effect size threshold varied depending on the location and season. At Gladbacherhof, effect sizes for seasons I and III: ≥ 2.3 and ≤ -2.3, for season II: ≥ 2.6 and ≤ -2.6. At Kleinhohenheim, effect size for season I: ≥ 1.2 and ≤ -1.2, for season II: ≥ 1.4 and ≤ -1.4, and for season III: ≥ 1.6 and ≤ -1.6.

Table 2
Top 35 most potentially relevant taxa at the genus level obtained from longitudinal feature volatility analysis. The taxa were organized according to their importance. Prevalent taxa are highlighted in bold.

Taxa	Importance	Cumulative average change	Global variance	Global mean
Luteimonas	0.192	0.012	5.5×10^{-5}	0.010
Reyranella	0.161	0.014	6.4×10^{-5}	0.019
Vicinamibacteraceae	0.128	−0.037	2.3×10^{-4}	0.038
Tepidisphaerales WD2101	0.098	0.008	6.0×10^{-5}	0.010
Uncultured *Gemmatimonadaceae*	0.071	−0.025	1.7×10^{-4}	0.046
Gaiella	0.061	0.036	3.0×10^{-4}	0.040
Opitutus	0.052	−0.012	3.0×10^{-5}	0.014
Nitrospira	0.044	−0.006	1.7×10^{-5}	0.011
Candidatus Latescibacterota	0.041	−0.017	1.6×10^{-4}	0.033
Entomoplasmatales type III	0.024	0.000	4.7×10^{-5}	0.008
Vicinamibacterales	0.020	−0.002	3.4×10^{-5}	0.018
Microvirga	0.018	−0.004	7.2×10^{-5}	0.021
Pyrinomonadaceae RB41	0.010	−0.018	1.0×10^{-4}	0.025
Anaerolineaceae UTCFX1	0.008	0.001	3.6×10^{-5}	0.013
Uncultured *Gaiellales*	0.007	0.020	2.2×10^{-4}	0.032
Candidatus Solibacter	0.007	−0.002	5.7×10^{-5}	0.024
Holophagae Subgroup 7	0.006	−0.012	6.4×10^{-5}	0.013
Burkholderiales SC-I-84	0.006	0.032	2.1×10^{-4}	0.057
"*Pedosphaeraceae*"	0.005	−0.001	4.0×10^{-5}	0.013
Candidatus Entotheonellaceae	0.005	0.010	1.6×10^{-4}	0.020
Microlunatus	0.003	0.030	2.0×10^{-4}	0.025
Bacillus	0.003	0.002	5.3×10^{-5}	0.012
Solirubrobacter	0.003	0.007	1.3×10^{-5}	0.010
"*Pajaroellobacter*"	0.003	0.007	1.6×10^{-5}	0.011
Xanthomonadaceae	0.003	0.002	3.5×10^{-5}	0.010
Myxococcota bacteria p25	0.002	−0.011	5.5×10^{-5}	0.019
Uncultured *Sandaracinaceae*	0.002	−0.005	2.3×10^{-5}	0.012
Bradyrhizobium	0.002	0.000	1.4×10^{-4}	0.036
Lysobacter	0.002	0.017	9.1×10^{-5}	0.027
Arenimonas	0.002	0.039	3.6×10^{-4}	0.036
***Blastocatellia* 11–24**	0.002	−0.002	1.1×10^{-4}	0.022
Nocardioidaceae	0.001	0.004	7.7×10^{-5}	0.012
***Acidobacteriota* Subgroup 11**	0.001	−0.004	1.3×10^{-5}	0.012
Acidobacteriota Subgroup 5	0.001	−0.006	2.3×10^{-5}	0.014
Romboutsia	0.001	0.008	3.3×10^{-4}	0.020

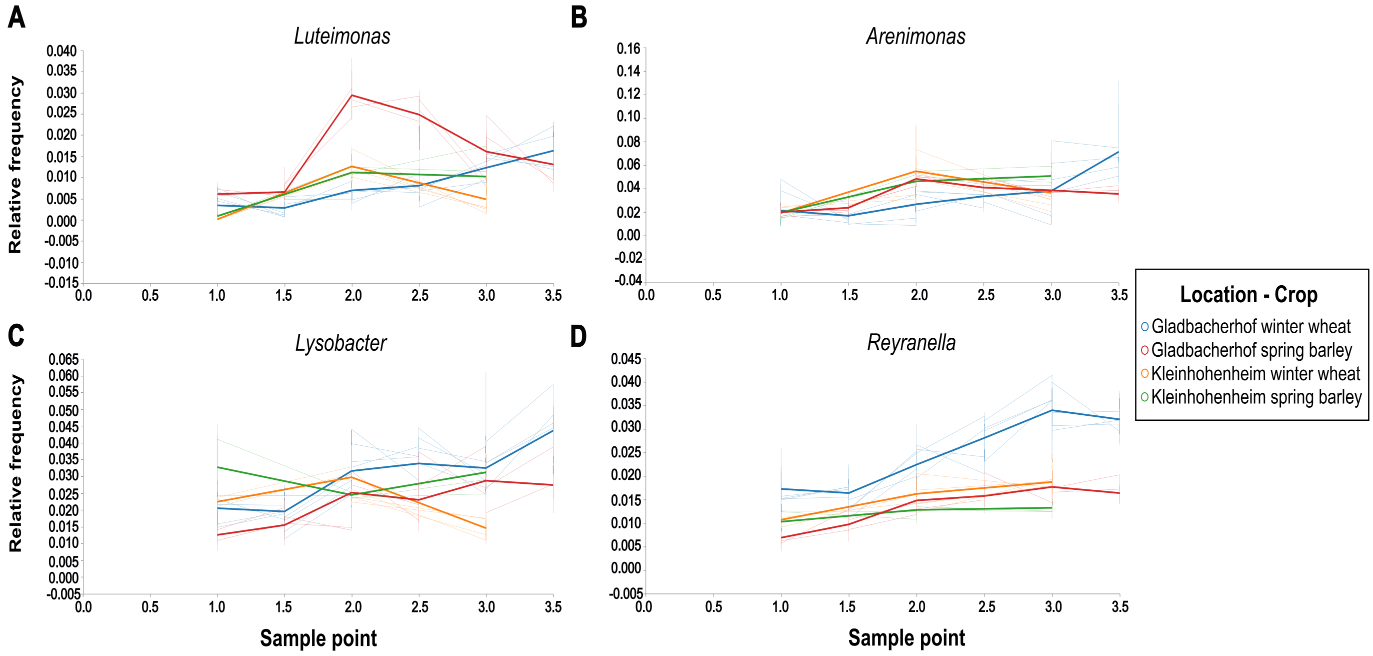

Fig. 5. Longitudinal feature volatility plot for the rhizosphere bacterial microbiome of WW and SB at the two locations. Relative frequencies of the genera **(A)** *Luteimonas*, **(B)** *Arenimonas*, **(C)** *Lysobacter*, and **(D)** *Reyranella* were plotted from the 35 most potentially relevant taxa identified in Table 2. Sample point 1, season I flowering; sample point 1.5, season I ripe; sample point 2, season II flowering (ripe only for KH-SB); sample point 2.5, season II ripe; sample point 3, season III flowering; sample point 3.5, season III ripe.

Table 3

Linear mixed-effects model for the rhizosphere microbiome based on Shannon entropy across three consecutive seasons (2020−2023). Location, crop species, and sample point were considered as fixed effects.

Variables	Estimate	Z-score	*P* > |z|
Intercept	8.77	26.03	<0.001***
Location [Kleinhohenheim]	−0.49	−1.02	0.31
Crop species [winter wheat]	−0.18	−0.38	0.701
Location[Kleinhohenheim]:crop species [winter wheat]	−0.01	−0.02	0.985
Sample point	−0.03	−0.77	0.442
Sample point:Location [Kleinhohenheim]	0.24	3.41	0.001***
Sample point:crop species [winter wheat]	−0.18	−3.88	<0.001***
Sample point:Location [Kleinhohenheim]:Crop species [Winter wheat]	0.29	3.21	0.001***

Significance codes: $\leq$0.001 '***', $\leq$0.01 '**', $\leq$0.05 '*'.

bacterial concentration (Coniglio et al., 2022). A few studies conducted under field conditions have shown that resilient microbial populations can completely or partially recover their original state after several days or weeks (Kröber et al., 2014; Liu et al., 2018; Yin et al., 2013). The observed plasticity in microbial communities is unusual. In 80 % of the extended studies outlined by Mawarda et al. (2020), these communities failed to reestablish their original state. Nevertheless, it is important to emphasize that these studies represent a minor portion of the studies analyzed, as most of them were performed in the short term under greenhouse conditions (Appendix A, Supplementary Table S1). Further research is required to elucidate whether these alterations are transient or permanent.

4.2. *Different rhizosphere bacterial communities could affect the persistence of H. diazotrophicus strain E19^T*

Previously, strain E19^T showed its adaptability to colonize WW and SB roots, as well as its persistence over time in both crops, but was only detected through qPCR at GH (Quiroga et al., 2024). Our findings demonstrated that the rhizosphere bacterial communities varied significantly between the two locations in both diversity indices, indicating that soil bacterial communities play a fundamental role in the survival of strain E19^T. In addition, the higher values in alpha diversity indices (seasons II and III) and the different bacterial communities assembled at KH compared to GH suggest stronger competition of strain E19^T for a niche. It has been reported that high species richness is inversely correlated with successful microbial invasion due to resource competition (Van Elsas et al., 2012). This may result in niche overlap with native communities at KH, impacting its persistence over time. However, species richness alone is insufficient to explain microbial invasion success, as specific network configurations have also been shown to play a role (Wei et al., 2015). At GH, a possible lower niche overlap could lead to dynamic character displacement, which eventually led to competition but also coexist with the resident rhizosphere bacterial communities (Hemmerle et al., 2022; Russ et al., 2023).

Equally important, environmental factors like pH-value and precipitation are important drivers of soil bacterial communities (Xue et al., 2018; Zhou et al., 2018). In this study, both locations had similar soil characteristics, including soil type, pH-value, total N, and C/N ratio (Appendix A, Supplementary Table S4). Lower precipitation at GH and higher levels at KH during season II (Appendix B, Supplementary Table S6) could be related to higher alpha diversity indices observed at KH in this season, but not in the other two. Li et al. (2022) found that the alpha diversity indices were not significantly affected by precipitation in alpine grasslands. Nonetheless, precipitation significantly altered bacterial community composition and may contribute to the substantial changes observed between stages of WW at GH, where precipitation varied considerably between sampling points (June and July). In contrast, this might partially explain the stage shifts in SB only during season I, but not in the other seasons. Although environmental factors are key drivers of microbial communities, our results align more closely with several studies indicating the role of plant species in recruiting different microbial communities (Abedini et al., 2021; Burns et al., 2015; Kuzmicheva et al., 2017). These populations can vary throughout plant development, primarily due to differences in root exudation over time (Chaparro et al., 2013; Zhalnina et al., 2018). Plant developmental stage has been reported as an important driver of microbial communities (Chaparro et al., 2014; Xiong et al., 2021). Marked changes have been identified at initial stages (e.g. seedling) compared to advanced stages (e.g. reproductive stage), where microbial communities tend to stabilize

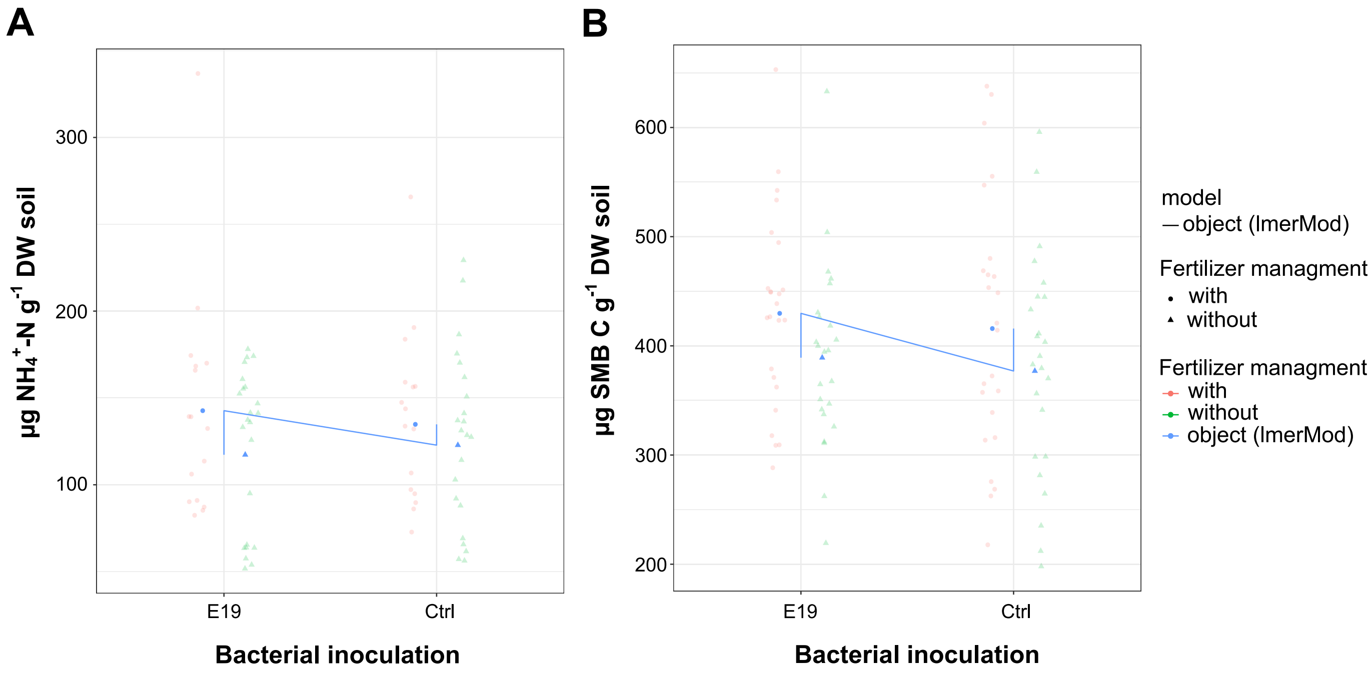

Fig. 6. Linear mixed models fit by REML for WW related to **(A)** ammonium and **(B)** microbial biomass concentrations.

Table 4

Linear mixed models fitted by REML for WW related to ammonium and microbial biomass concentrations. Fertilizer management and bacterial inoculation were considered as fixed effects.

Soil parameter	Variables	Estimate	t value	$P > \|t\|$
Ammonium	Intercept	134.8	6.42	0.005**
	Bacterial inoculation [E19]	7.9	0.92	0.360
	Fertilizer managment [without]	−12.0	−1.46	0.149
	Bacterial inoculation [E19]: Fertilizer managment [without]	−13.4	−1.19	0.239
Microbial biomass	Intercept	415.5	16.97	1.06×10^{-13}***
	Bacterial inoculation [E19]	14.0	0.81	0.418
	Fertilizer managment [without]	−39.4	−2.54	0.013*
	Bacterial inoculation [E19]: Fertilizer managment [without]	−0.9	−0.042	0.967

Significance codes: ≤0.001 '***', ≤0.01 '**', ≤0.05 '*'.

(Zhang et al., 2018). Nevertheless, a recent study has shown that time influences microbial communities to a greater extent than plant developmental stage per se (Dibner et al., 2021).

The evaluation of different fertilizers has been shown to influence the bacterial communities of WW, increasing the Shannon index, as well as the relative abundance of the phylum *Pseudomonadota* (Liang et al., 2020). The non-significant effects of organic fertilizer application in two of the three seasons could be related to the low concentration used during the experiments ($<$110 kg N ha^{-1}) compared with that used in the aforementioned study (200 kg N ha^{-1}). To enhance robustness of the results, simplified experimental designs with four to six replicates are recommended for field experiments involving PGPR (Neuhoff et al., 2024). Our study considered four replicates for the combination of the three factors, resulting in a complex design.

Differential abundance analysis, including chemical parameters, corroborated the non-significant effects observed in the diversity indices, showing that no relevant taxa were affected by the factors evaluated. Based on this, questions arise about the mechanisms employed by strain E19^{T} to improve yield parameters without altering chemical parameters, or shifting bacterial community structure, or being undetected in advanced developmental stages at one organic farm (Quiroga et al., 2024). In recent years, new mechanisms have begun to shed light on how PGPR can promote plant growth (for review: Kong and Liu, 2022). Kong et al. (2019) found that PGPR inoculation increased co-occurrence interactions among bacterial communities. Although the bacterial community composition changed minimally a few days after inoculation, the more complex co-occurrence associations were linked to plant growth promoting effects. Additionally, Chen et al. (2022) associated changes in DNA methylation in roots during the early phase with lasting PGPR effects observed in later phases, even after the removal of the inoculant from the microbiome. Although genes related to nitrogen fixation have been identified in the genome of *H. diazotrophicus* strain E19^{T} (Suarez et al., 2019), ACC-deaminase activity appears to be one of the mechanisms by which strain E19^{T} promotes plant growth (Suarez et al., 2015). Additionally, these new insights into microbiome improvement may be related to strain E19^{T} plant growth promotion and the positive but not significant trend observed in ammonium and microbial biomass concentrations obtained from LMM analyses.

Several authors have emphasized the importance of investigating plant-microbial dynamics over time (Horton et al., 2014; Wagner et al., 2016; de-Bashan and Nannipieri, 2024). Of the four prevalent genera included in the longitudinal analysis, *Reyranella* and *Arenimonas* have been identified as denitrifiers (Duan et al., 2023; Xing et al., 2018), while *Luteimonas* and *Lysobacter* have been associated with disease suppression (Liu et al., 2020). *Arenimonas* has shown to be more abundant in early stages and decreased as the plant grew. Similarly, *Lysobacter* has been negatively associated with root growth response but has also shown opposite results depending on the plant species (Li et al., 2014; Zhalnina et al., 2018). As aforementioned, our alpha and beta diversity results are consistent with the role of plant species in modulating microbial communities between stages. Nevertheless, the differential abundance, linear mixed models, and longitudinal analyses conducted in this study over three consecutive seasons at two locations revealed that bacterial community dynamics can be not only plant-driven but also multifactorial (Becker et al., 2022; Wei et al., 2022). Zhang et al. (2020) determined that geographic distance had a greater influence on modulating microbial communities than seasonal variations. Nonetheless, rapidly changing environmental conditions were associated with the seasonal variability of microbial community structure.

4.3. Different crop rotation management strategies could result in different microbial communities

The results, based on alpha and beta diversity indices at different locations, were consistent with the extended concept that soil is an important force modulating microbial populations (Bulgarelli et al., 2012; Oberholster et al., 2018; Schreiter et al., 2014). Intriguingly, although both organic fields contained Haplic Luvisol soil types and similar soil properties, crop rotation was different and could be related to the differences observed in the diversity indices. In fact, agricultural management practices have shown to influence microbial populations (Schmidt et al., 2019; Zhou et al., 2023). Interestingly, at GH, the previous crops for WW and SB were potato and spelt, respectively. While at KH, clover grass was commonly used as an intercrop before sowing both cereals (Appendix A, Supplementary Table S2). Several of the most prevalent taxa observed in our study have been reported as part of the rhizosphere microbiome of these crops. The phyla *Pseudomonadota* and *Actinomycetota* have shown to dominate the rhizosphere and root microbiome of the clover grass species *Trifolium pratense* (Wahdan et al., 2021). Similarly, genera such as *Bradyrhizobium*, *Arenimonas*, *Lysobacter*, *Microvirga*, *Nocardioides*, and *Gaiella* have been associated with the co-occurrence network of several potato varieties (Martins et al., 2024). While no reports exist on the rhizosphere bacterial microbiome of spelt, the endophytic microbiome in different plant compartments has been documented (Kuźniar et al., 2020). These different crop management strategies could be related not only to the differences already described between both locations, but also to the marked difference between the bacterial community composition of WW and SB at GH (p < 0.01, Table 1). In contrast to KH, where WW and SB were not significantly different during season II (p = 0.953, Fig. 3).

4.4. Recruitment of different bacterial communities by the same crop species is affected by location

Our findings are in agreement with previous reports indicating that microbial communities are recruiting and shaping in a plant-dependent manner, even varying at different plant growth stages (Oberholster et al., 2018; Smalla et al., 2001; Wagner et al., 2016). Nevertheless, bacterial communities between flowering and ripe stages were more stable for SB than for WW (Table 1). These results suggest that fluctuations across stages could also be plant-dependent. Further research is required to confirm this hypothesis. Additionally, differential abundance analysis corroborated how bacterial communities were significantly influenced by crop species, but also varied depending on location and season (ALDEx2 analysis, Appendix B, Supplementary Table S5). Despite these fluctuations, the 19 most prevalent taxa at the genus level were identified in both crops across seasons. The phyla *Pseudomonadota*, *Acidobacteriota*, and *Actinomycetota*, which encompassed 89 % of the most prevalent taxa, were consistent with the rhizosphere core microbiome phyla previously identified in wheat and barley (Escudero-Martinez et al., 2022; Simonin et al., 2020). Nevertheless, there is still controversy regarding the use of the term 'core microbiome' (Risely, 2020). It has become challenging to identify, for instance, a common genus in all studies due to the different methods and tools employed (Kavamura et al., 2021).

4.5. Limitations of 16S rRNA gene sequencing

H. diazotrophicus was only detected in one sample belonging to WW with fertilizer plus E19^T during season III at flowering stage. The limitations of 16S rRNA gene sequencing include short-length reading, intragenomic gene redundancy, sequencing errors, and other factors that can lead to difficulties during sequence assembly (Poretsky et al., 2014). Considering these constraints, *H. diazotrophicus* was detected through qPCR on the roots of WW and SB under field conditions up to a concentration of 5×10^5 copies g^{-1} DW (Quiroga et al., 2024). Nevertheless, metagenomic analyses are still limited to microbial composition. Therefore, for future research, we suggest a metatranscriptomic approach to better understand the associations of functional genes and metabolic pathways in these communities. Even though there are certain challenges to be addressed regarding metatranscriptomics, such as sample collection and preservation, mRNA enrichment, or restricted transcriptome databases (Bikel et al., 2015; Bashiardes et al., 2016; Rosado-Porto et al., 2023).

5. Conclusion

Seed inoculation of *H. diazotrophicus* strain E19^T in a three-year field trial showed no significant alteration in the diversity indices of the rhizosphere bacterial microbiome of wheat and barley. Differential abundance and linear mixed models corroborated the non-effect of bacterial inoculation and row spacing. Although fertilizer management significantly affected the bacterial microbiome during one season (beta diversity), a significant trend over time was not identified, probably because of the low nitrogen application rate. Furthermore, although both organic fields share the same Haplic Luvisol soil type, the rhizosphere bacterial communities were different, possibly due to different crop management practices. These findings showed not only how the same crop species recruited, assembled, and influenced different bacterial communities depending on the location but also how this influence was affected to different degrees over time. Finally, these results highlight the importance of longitudinal studies in understanding microbial dynamics and the long-lasting effects of introducing bioinoculants into the soil.

CRediT authorship contribution statement

Santiago Quiroga: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Stefan Ratering:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Data curation, Conceptualization. **David Rosado-Porto:** Writing – review & editing, Methodology, Data curation. **Azin Rekowski:** Writing – review & editing, Investigation. **Franz Schulz:** Writing – review & editing, Investigation. **Christian Zörb:** Writing – review & editing, Funding acquisition, Conceptualization. **Sylvia Schnell:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Funding

This work was fully funded by the Federal Ministry of Education and Research (BMBF) of Germany under the BONARES program (Rhizo4bio).

Declaration of competing interest

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

Acknowledgments

We are warmly acknowledged to Rita Geissler-Plaum and Bellinda Schneider for their technical support. We are also grateful to Simon Schäfer for his support during field experiments at Kleinhohenheim.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105823.

Data availability

All DNA sequences are available on the NCBI/GenBank database repository under the bioproject accession numbers PRJNA1030754 (season I), PRJNA1030767 (season II), and PRJNA1095458 (season III). Moreover, all the chemical parameters analyzed during seasons I and II can be found at the BONARES repository under the name "Production of wheat and barley under reduced input in organic farming". The graphical abstract was created using Inkscape v.1.3.2.

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