




## Article

# *Daucus carota* L. Seed Inoculation with a Consortium of Bacteria Improves Plant Growth, Soil Fertility Status and Microbial Community

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**Featured Application:** We analyzed the production of different auxins and the presence of the ability to solubilize phosphates and the 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme in a consortium of four bacteria. The consortium was inoculated in an open field experiment on carrots, with very positive impacts on the development of the plant and on the soil microbial community.



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**Abstract:** The present work aimed to study suitability of a consortium of *Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, and *Burkholderia ambifaria* as biofertilizers. Strains were assayed for plant growth-promoting characteristics (i.e., auxins production, phosphate solubilizing capability, and 1-aminocyclopropane-1-carboxylate deaminase activity). The consortium of four bacteria was then inoculated on carrot seeds and tested in an open field experiment. During the open field experiment, plant growth (morphological parameters, chlorophylls, and carotenoids), soil chemical analysis, and molecular and physiological profiles of soils were investigated. Each strain produced different amounts of indole-3acetic acid and several indole-derivates molecules. All strains showed phosphate solubilization capability, while 1-aminocyclopropane-1-carboxylate deaminase activity was only detected in *H. seropedicae* and *B. ambifaria*. The bacterial consortium of the four strains gave interesting results in the open field cultivation of carrot. Plant development was positively affected by the presence of the consortium, as was soil fertility and microbial community structure and diversity. The present work allowed for deepening our knowledge on four bacteria, already known for years for having several interesting characteristics, but whose interactions were almost unknown, particularly in view of their use as a consortium in a valid fertilization strategy, in substitution of agrochemicals for a sustainable agriculture.

**Keywords:** *Azospirillum brasilense*; *Gluconacetobacter diazotrophicus*; *Herbaspirillum seropedicae*; *Burkholderia ambifaria*; *Daucus carota*; microbial inoculation; soil biodiversity; auxins; phosphate solubilization; 1-aminocyclopropane-1-carboxylate deaminase activity

## 1. Introduction

The development of low consumption energy technologies is of growing relevance in sustainable agriculture to allow sustainable food production while supporting an increasing world population. One possible low energy and environmentally friendly technology is the utilization of plant growth-promoting bacteria (PGPB), which are microorganisms

that live in association with plants, covering practically all their surfaces and colonizing their interior, the xylem vessels in particular [1]. Among PGPB strains inoculated to increase agricultural productions, *Azospirillum* is one of the most well-known. *Azospirillum brasilense*, together with *Gluconacetobacter diazotrophicus* [2], *Herbaspirillum seropedicae* [3], and *Burkholderia ambifaria* [4] strains live in association with crop plants.

Carrots host a specific microbial biomass, composed of Gram positive and negative bacteria, actinomycetes, and fungi, that change according to agronomic management [5]. Furthermore, as reported by Nithya and Babu, cocci are the predominant endophytic bacterial community compared to other salad vegetables, namely cucumber, onion, and tomato. This crop is particularly suitable for Fucino plateau, the main agricultural productive area of the Abruzzo region where the greatest carrots production is obtained [6]. This consortium has already demonstrated to have positive effects on the growth of *Allium cepa* L. [7], *Artemisia eriantha* Ten [8], *Cannabis sativa* L. [9], *Lycopersicon esculentum* L. [10], and ancient *Triticum* [11]. Based on these positive effects, we hypothesized that a *A. brasilense*, *G. diazotrophicus*, *H. seropedicae*, and *B. ambifaria* consortium could be a valid biostimulant tool for *Daucus carota* L.

Strains belonging to PGPB can promote plant growth and development through different mechanisms. Among them, the production of auxins plays an important role in plant growth stimulation [12]. The best-known molecule belonging to this class is indole-3-acetic acid (IAA). Soil bacteria differ in their IAA synthesizing capability depending on soil fertility status and organic matter content [13]. Bacteria use this phytohormone to interact with plants as part of their colonization strategy. Furthermore, this IAA can also be a signalling molecule in bacterial communication [14].

Plant growth and development promotion ascribed to PGPB is also due to their ability to solubilize soil nutrients, such as phosphorus [15]. The plant growth-promoting (PGP) strains with this capability are known as phosphate-solubilizing bacteria (PSB) and can convert inorganic and inaccessible forms for the absorption of phosphate ( $\text{PO}_4^{3-}$ ) into available ones (e.g.,  $\text{HPO}_4^{2-}$ ,  $\text{H}_4\text{PO}_4^-$ ) [16]. This conversion is usually mediated by the organic acids produced by PSB, which induce the release of phosphates from insoluble complexes by using various approaches (i.e., lowering soil pH, chelation, and mineralization) [17].

The bacteria belonging to PGPB also help plants to counteract stress-related to biotic and abiotic factors that play an essential role in diminishing crop productivity [18,19]. This stress affects plant–water relation at cellular and whole plant level, causing specific and unspecific reactions and damage [18]. However, PGPB possess different mechanisms counteracting them. The decrease of the deleterious synthesis of “stress ethylene”, through 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme, is one of these principal mechanisms [18]. The direct precursor of ethylene in the biosynthetic pathway is ACC [20]. The enzyme ACC deaminase catalyzes the cleavage of ACC to ammonia and  $\alpha$ -ketobutyrate, decreasing the synthesis of stress ethylene [21]. The presence and activity of this enzyme have been detected in several PGPB species [22,23].

Several techniques can be applied to investigate changes in the microbial community. Among these, 16S rRNA analysis by Next Generation Sequencing (NGS) allows us to study the genomes of culturable and unculturable bacteria in soil [24]. Furthermore, changes in metabolic activity provide information on any changes in the soil microbiome. The Community Level Physiological Profiling (CLPP) by Biolog® EcoPlates™ is widely applied to study the differences in soil metabolic activities [25].

In the present work, we hypothesized that the consortium of *A. brasilense*, *G. diazotrophicus*, *H. seropedicae*, and *B. ambifaria* can positively affect crops of *D. carota* through several direct and indirect mechanisms. To test this hypothesis, we investigated the PGP traits of these strains (i.e., auxin's production, ACC deaminase activity detection, and phosphate solubilizing capability). Bacteria were also combined in a consortium and inoculated on carrot seeds to evaluate their effects on this valuable species in a field experiment where plant growth and development traits were investigated. We also assessed the bacterial con-

sortium's possible influence on the soil microbial community by CLPP and DNA extraction and NGS and soil chemical composition, after the crop.

## 2. Materials and Methods

### 2.1. Bacterial Strains and Media

The investigated PGPB strains were: *Azospirillum brasilense* Cd, provided by Y. Okon (Hebrew University of Jerusalem, Israel) and isolated from *Cynodon dactylon* root; *Burkholderia ambifaria* PHP7, provided by T. Heulin (C.P.B., CNRS, France) and isolated from *Zea mays* root, and *G. diazotrophicus* Pal5 and *Herbaspirillum seropedicae* Z67, both provided by the late J. Döbereiner (Embrapa Agrobiologia, Seropédica, Brasil) isolated from *Saccharum officinarum* stem and *Sorgum bicolor* root, respectively [10]. The selective media used for their cultivation were: OK [26] for *A. brasilense*; LGI [27] for *G. diazotrophicus*; J-NFb [28] for *H. seropedicae* and KB [29] for *B. ambifaria*. The common medium T4 was also used [10]. Broth cultures were grown in flasks incubated at 30 °C in a rotary shaker (150 rpm).

### 2.2. IAA Production and HPLC-FL Analysis

First tests were performed on bacteria grown on their specific cultural medium with three different amounts of tryptophan (Trp) (0, 200, 400  $\mu\text{g mL}^{-1}$ ), sterilized by filtration, and added after cooling to the specific medium. To grow all the bacteria together, the common medium T4 was used. To test auxins production, bacterial cultures in different combinations (i.e., single, couples, and four together) were grown in T4 medium with and without (control) the addition of Trp (200  $\mu\text{g mL}^{-1}$ ). Colony forming units and optical densities of each strain were recorded up to the stationary phase. Dry weight of bacterial biomass was obtained by keeping 10 mL cultures at 80 °C up to constant weight.

Supernatants were collected after centrifugation at  $5000 \times g$  for 30 min, were adjusted to pH 2, and the resultant solution was eluted through a C-18 Sep-Pak cartridge [30]. The cartridge was washed with distilled water and IAA was eluted with methanol containing 10  $\mu\text{g mL}^{-1}$  butylated hydroxytoluene (Sigma, St. Louis, MO, USA). The methanol solution with IAA was evaporated under vacuum and auxin was dissolved in 400  $\mu\text{L}$  methanol for HPLC analysis according to the method of Forni et al. [30] modified as follows. Reverse-phase HPLC analyses (Pharmacia biotech P-900) were performed using a column C-18 (5  $\mu\text{m}$ ) (4.6  $\times$  250 mm, Alltech). The gradient phase was 25 min, with 10–35% (v/v) acetonitrile containing 1% (v/v) acetic acid and a flow rate of 1 mL min<sup>-1</sup>.

A fluorescence detector (Jasco FP-1520) was set up with an excitation wavelength of 280 nm and an emission wavelength of 350 nm. The standard stock solutions (purchased from Sigma) were prepared in methanol. Standards were injected as a single solution as well as a mixture of all standards, and corresponding peak areas were recorded. The detection limit was determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected (LOD = 8 pmol; LOQ = 28 pmol) [31]. The concentration of the compound was determined using external standards. IAA concentration was calculated according to a calibration curve made with the standard ( $R^2 = 0.999$ ;  $y = (x - 0.5804)/0.0893$ ) and results were expressed as nmol mg dry weight<sup>-1</sup>.

### 2.3. ACC Deaminase Activity

The evaluation of ACC deaminase (EC: 4.1.99.4) activity was performed following the protocol reported by Brígido et al. [32], utilizing fructose instead of glucose for *A. brasilense* in DF salt minimal medium. A calibration curve was calculated with different concentration of the standard  $\alpha$ -ketobutyrate (Sigma) in 0.1 M TRIS-HCl (pH 8.0) ( $R^2 = 0.9945$ ;  $y = 0.004x + 0.0063$ ) and detecting the absorbance of the reaction mixture at 540 nm (Spectrophotometer VARIAN Cary 50 Bio). The protein amount was determined by Bradford method [33]. ACC deaminase activity of the strains was expressed as  $\mu\text{mol } \alpha\text{-ketobutyrate h}^{-1} \text{ mg protein}^{-1}$ .

#### 2.4. Phosphate Solubilization Capability

The bacterial strains were screened for phosphate solubilization in Pikovskaya's agar plates [34]. 10 µL of *A. brasilense*, *G. diazotrophicus*, *H. seropedicae*, and *B. ambifaria* were spotted on Pikovskaya's agar plates and incubated at 30 °C for 7 days. Positive phosphate solubilization was estimated through the presence of clearing zones around the colonies. The assay was repeated five times.

Quantitative estimation of phosphate solubilization was carried out on broth cultures, utilizing NBRIP medium and supernatant recovery procedure as described by Nautyal et al. [35], and a colorimetric estimation procedure proposed by Olsen and Sommers [36]. For *A. brasilense* assays, cultivations were also carried out in modified Pikovskaya's agar and NBRIP liquid media, with the addition of fructose instead of glucose (F-Pikovskaya and F-NBRIP).

#### 2.5. Inoculum Preparation and Seed Treatment

Maestro F1 carrot cultivar was used in this study (Vilmorin, Paris, France). Bacteria were prepared in liquid T4 medium [10] and kept with constant shaking at 30 °C for 24 h. Cell density of each strain was determined spectrophotometrically by comparing the obtained 600 nm optical densities with growth calibration curves. The consortium was obtained by mixing the four strains to a final density of  $10^{10}$  cells mL<sup>-1</sup> and seeds were treated with this solution by 20 min dipping, followed by overnight oven drying at 30 °C (final density on seeds  $10^6$  cells g<sup>-1</sup> determined by plate counting method). Control seeds were treated with the same procedure but using autoclaved working inoculum as a dipping solution.

#### 2.6. Field Experiments and Plant Sampling

A one-year field experiment was carried out during the May–October 2018 growing season at the Agricola Scipioni field (Avezzano, Italy, 42°02'26.31" N, 13°46'45.02" E, 650 m a.s.l.). The climate is relatively continental, with mean annual precipitation ranges of 650–800 mm. The experiments were arranged on a randomized complete block designed with four replicates. The treatments under comparison were represented by seeds inoculated with bacteria (SIB) and autoclaved bacterial application (control). Carrots were sown on June 2018 with a pneumatic seeder (Serie SNT, Agricola Italiana SNC, Massanzago, PD, Italy) at a rate of 250 seeds m<sup>2</sup>. The previous crop was potatoes (*Solanum tuberosum* L.), which were harvested in the middle of October 2017. The soil was previously fallow and was ploughed and harrowed twice before sowing.

The experimental units consisted of a 30 m<sup>2</sup> (5.0 m × 6.0 m) plots with 5 plant tandem rows spaced 5 cm apart. The crop was only protected against fungal disease through the application of Azoxystrobin (Amistar, Syngenta Italia, Milano, MI, USA) at the dose of 0.8 L ha<sup>-1</sup> at development stage 45 [37]. Starting from the development stage 41 (roots beginning to expand, diameter > 0.5 cm), 30 carrot plant samples within each experimental unit were randomly collected. In total, 3 sampling dates, corresponding to development stage 41, development stage 45 (50% of the expected root diameter reached), and harvest (development stage 49 [37], middle October 2018) were considered. Plants were separated into the aerial part and carrots root (referred as 'roots') and their dry matter (%) were determined after oven drying at 80 °C, until constant weight was obtained. The estimation of carotenoids (roots) and chlorophylls (aerial parts) contents were carried out on 80% acetone extracts and obtained following the methods and calculations proposed by Tavarini et al. [38] and Porra et al. [39], respectively. Results were expressed as mg 100 g<sup>-1</sup> FW.

#### 2.7. Soil Analyses

Before sowing and at the head emergence phenological phase (development stage 49), 5 soil sub-samples for each experimental unit were randomly collected on the row spacing at 0–10 cm depth. The sub-samples were thoroughly mixed to obtain a composite

and homogenized soil sample. Soil chemical analyses were carried out according to the methods described by the Italian Ministry of Agricultural, Food and Forestry Policies [40].

Homogenized soil samples were sieved (<2 mm) to remove plant roots, fauna, and debris and were subjected to community level physiological profiles (CLPP) and rRNA 16S analysis. CLPP was assessed with Biolog EcoPlate™ (Biolog Inc., Hayward, CA, USA), following the method described by Weber and Legge [41] and the calculations of AWCD and the Shannon–Weaver index (H) proposed by Garland [42]. For each sample, three independent plates were set up.

From each sample, genomic DNA was extracted three times utilizing the NucleoSpin® Soil kit (Macherey Nagel, Düren, Germany), following the manufacturer's protocol. DNA quantification of the samples was obtained through Qubit dsDNA HS (High Sensitivity) Assay Kit and Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA, USA). The three replications of each sample were pooled in equimolar mixtures and sent to Bio-Fab Research Srl (Roma, Italy) for further processing. DNA was amplified with a specific 16S protocol for the amplification of Bacteria and Archaea, using 300 bp Paired-End 16S community sequencing on the Mi-Seq Illumina platform (Bio-Fab Research, Roma, Italy) [43]. The 16S V3 and V4 region were targeted by gene-specific sequences [44], adding Illumina adapter overhang nucleotide sequences (Forward Primer = 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; Reverse Primer = 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). The bioinformatic workflow was as follow: control of the quality of the reads; count after filtering; assembly of the amplicons; construction of the classifier on the database; taxonomic assignment. The Amplicon Sequence Variant (ASV) clustering was performed with DADA2 plugin by qiime2-2020.2 version [45]. The classifier was trained by the fit-classifier-naive-bayes plugin on the specific region of V3-V4 extracted from 16S file of database SILVA 132 release (<https://www.arb-silva.de/> accessed on 6 March 2021). The similitude chosen for Taxa assignment was performed with a similitude of 97%. Taxonomic assignments were checked with LPSN service (<https://lpsn.dsmz.de> accessed on 6 March 2021). The Quality Check report is shown in Table S1 of Supplementary material. Diversity indices were calculated using R (R Foundation for Statistical Computing, Vienna, Austria) with the statistical package vegan v2.5-7 [46].

## 2.8. Statistical Analysis

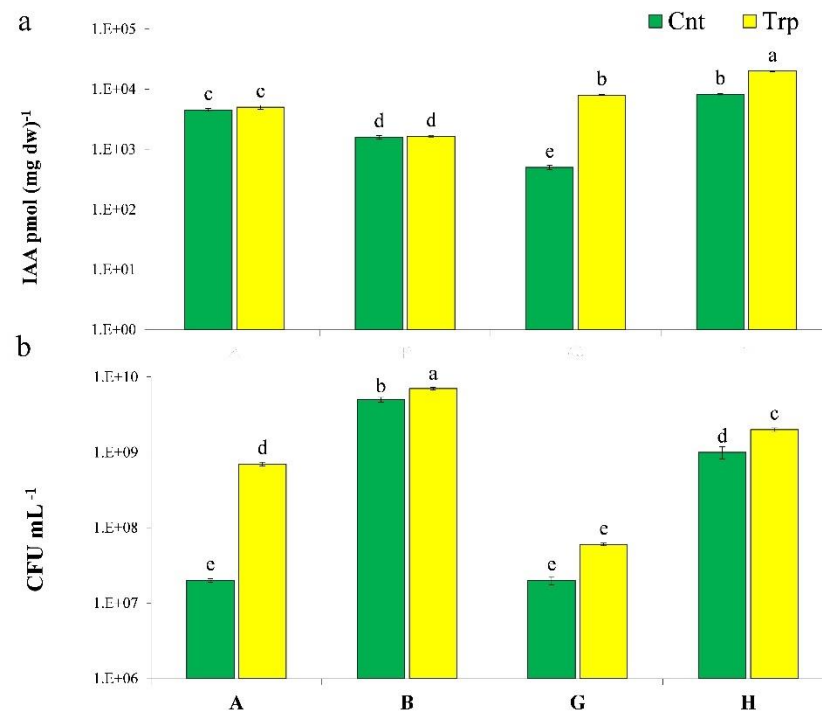
Data of IAA and microbiological analysis on broth cultures and soil chemical analysis were analyzed by one-way or two-way analysis of variance (ANOVA) based on the number of factors present using XLSTAT 2016 software (Addinsoft, Paris, France). One-way ANOVA was also applied on experimental field results according to a randomized block design with four replications, to test (*F*-test) the effects of bacterial application on the selected variables (R software—R Foundation for Statistical Computing, Vienna, Austria). Separation of the means was set at 1% and 5% ( $p < 0.01$  and  $p < 0.05$ , respectively) level of significance by LSD test. Prior to ANOVA, data were tested for normality and homoscedasticity assumptions.

## 3. Results

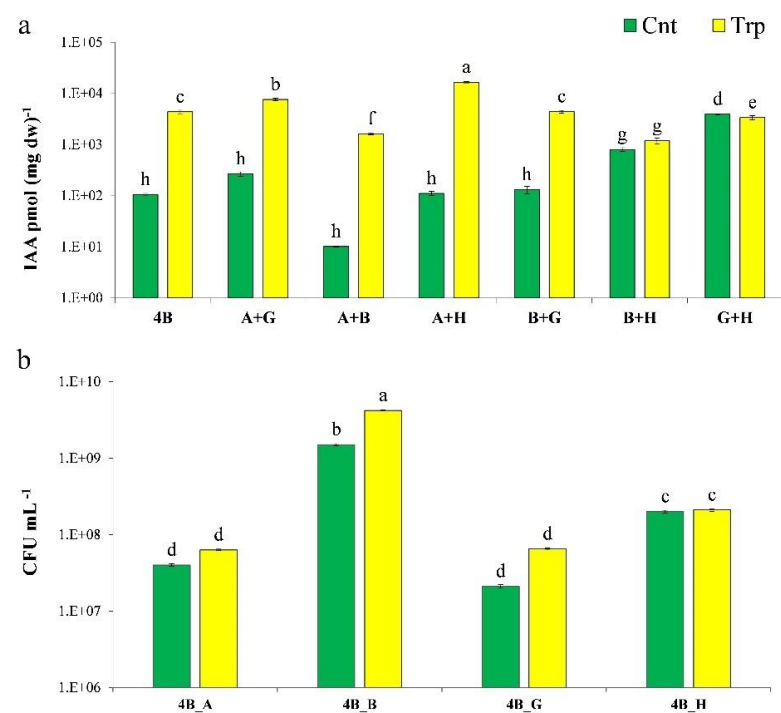
### 3.1. IAA Production

In Figure 1A, IAA concentrations produced by single strains with and without Trp are reported. For *G. diazotrophicus* and *H. seropedicae*, IAA concentrations were higher than respective controls ( $p < 0.05$ ) while for *A. brasilense* and *B. ambifaria* no significant difference were shown ( $p > 0.05$ ). *H. seropedicae* was the best IAA producer; however, if we consider the number of cells present in the medium (Figure 1B) *G. diazotrophicus* showed the highest production, followed by *A. brasilense*. Lowest IAA production was shown by *B. ambifaria*. Average values of IAA concentrations were recorded when the four strains were grown together (Figure 2A). As shown in Figure 2B, the CFU mL<sup>-1</sup> obtained for each strain from mixed broth cultures were similar to those obtained by single cultures.



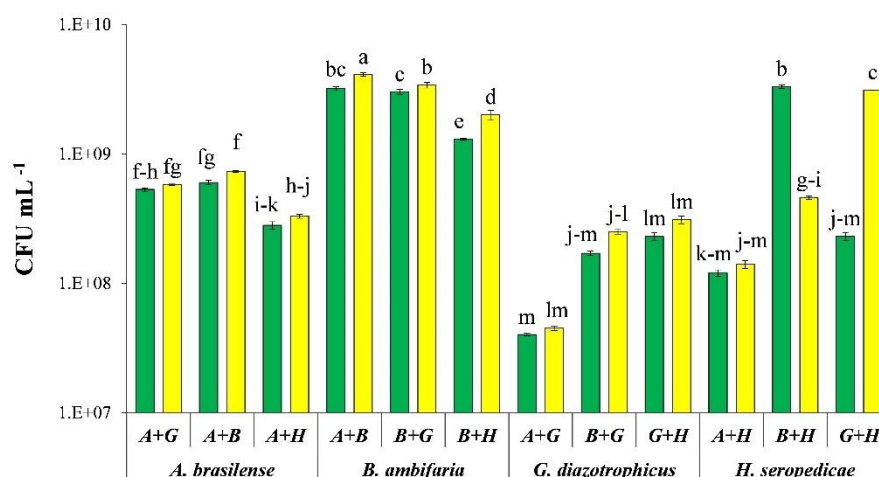


**Figure 1.** (a) Indole-3-acetic acid (IAA) production of the strains grown in single cultures ( $n = 3$ ); (b) CFU mL<sup>-1</sup> of the strains grown in single cultures. In the Figure: Cnt, broth culture without Tryptophan; Trp, broth culture added with Tryptophan; A, *A. brasilense*; B, *B. ambifaria*; G, *G. diazotrophicus*; H, *H. seropedicae*. Error bars are standard error of the means. Means followed by different letters are significantly different according to Fisher's LSD test ( $p < 0.05$ ).



**Figure 2.** (a) IAA production in binary and mixed cultures of strains ( $n = 3$ ); (b) CFU mL<sup>-1</sup> of the single strains grown in a mixed broth culture of all strains ( $n = 5$ ). In the Figure: Cnt, broth culture without Tryptophan; Trp, broth culture added with Tryptophan; A, *A. brasilense*; B, *B. ambifaria*; G, *G. diazotrophicus*; H, *H. seropedicae*. Error bars are standard error of the means. Means followed by different letters are significantly different according to Fisher's LSD test ( $p < 0.05$ ).

In Figure 2A, IAA concentrations found in binary broth cultures in the presence and in the absence of Trp are shown. Except for binary culture of *B. ambifaria* + *H. seropedicae*, the IAA concentrations in Trp-added medium were higher than control ( $p < 0.05$ ). Generally, IAA range concentrations were similar to those obtained by single cultures. However, in the absence of Trp, the presence of *A. brasilense* negatively influenced the synthesis of auxins. In Figure 3, the CFU mL<sup>-1</sup> obtained for each strain from each binary culture are shown. Slight differences in their loads were recorded for *A. brasilense*, *B. ambifaria*, and *G. diazotrophicus* in the different binary cultures. Instead, *H. seropedicae* loads recorded strong decreases when cultured with *B. ambifaria* and *G. diazotrophicus* in the presence and in the absence of Trp, respectively.



**Figure 3.** CFU mL<sup>-1</sup> of strains grown in binary broth cultures ( $n = 5$ ); In the Figure: Cnt, broth culture without Tryptophan; Trp, broth culture added with Tryptophan; A, *A. brasilense*; B, *B. ambifaria*; G, *G. diazotrophicus*; H, *H. seropedicae*. Error bars are standard error of the means. Means followed by different letters are significantly different according to Fisher's LSD test ( $p < 0.05$ ).

### 3.2. Trp and Indole Derivates Characterization

The Trp and indole derivates (IAC and IAM) concentrations in single and combined strains broth cultures, with and without Trp addition, were assessed by HPLC-FL (Table 1). Trp was detected in almost all broth cultures. For the binary culture of *G. diazotrophicus* + *H. seropedicae* with Trp addition, there was no detection while for *H. seropedicae*, the quantification was not allowed due to the presence of numerous and overlapped peaks (data not shown). The highest Trp concentration was shown by *A. brasilense* + *B. ambifaria* culture with Trp addition. For *A. brasilense* + *G. diazotrophicus* combination, lowest concentrations were recorded when Trp was not provided. This behavior was common for almost all cultures. Only in *A. brasilense* and *B. ambifaria* + *H. seropedicae* cultures, the control showed higher concentrations of Trp than those with Trp added. IAC was produced in a relevant amount by most of the strains while IAM was produced only in a low amount, and only without the addition of Trp. Once again, the quantification of *H. seropedicae* Trp-added culture was not possible due to the presence of numerous and overlapped peaks (data not shown). Best IAC concentrations were recorded in *H. seropedicae* control, while the highest IAM concentrations were recorded in *A. brasilense* + *B. ambifaria* binary culture with Trp addition.

### 3.3. ACC Deaminase Activity

Concerning ACC deaminase, the activity was present only in *H. seropedicae* ( $1.83 \pm 0.04 \mu\text{mol } \alpha\text{-KB h}^{-1} \text{ mg proteins}^{-1}$ ) and *B. ambifaria* ( $3.7 \pm 0.07 \mu\text{mol } \alpha\text{-KB h}^{-1} \text{ mg proteins}^{-1}$ ). No ACC deaminase activity was detected in *A. brasilense*, in *G. diazotrophicus* cultures, and when the four strains were grown together.

**Table 1.** Trp, IAC (indole-3-acetaldehyde) and IAM (indole-3-acetamide) concentrations detected in single strains and combined strains broth cultures with and without Trp addition by HPLC-FL.

Bacteria	Trp nmol (mg dry wt cells) <sup>−1</sup>	IAC nmol (mg dry wt cells) <sup>−1</sup>	IAM nmol (mg dry wt cells) <sup>−1</sup>
A_C	2.23 f	0.93 de	-
A_T	1.05 hi	0.50 fg	<LOQ/LOD
B_C	0.44 j–m	0.40 gh	-
B_T	0.80 ij	0.29 h	-
G_C	1.20 g–h	<LOQ	-
G_T	4.30 d	0.82 e	-
H_C	3.68 e	8.10 a	-
H_T	Nq	Nq	Nq
4B_C	0.52 j–l	0.37 gh	0.09 c
4B_T	4.86 c	1.02 cd	-
A+G_C	0.05 mn	0.32 h	-
A+G_T	12.97 b	2.04 b	-
A+B_C	-	-	-
A+B_T	18.02 a	-	2.66 a
A+H_C	-	0.10 i	-
A+H_T	0.59 jk	0.62 f	0.91 b
B+G_C	0.22 k–n	<LOQ	-
B+G_T	2.16 f	0.33 h	0.10 c
B+H_C	1.50 g	1.10 c	-
B+H_T	0.13 l–n	0.33 h	-
G+H_C	-	-	-
G+H_T	-	-	0.06 c
F-test	*	**	**
LSD	0.40	0.15	0.16

In the table: A, *A. brasilense*; B, *B. ambifaria*; G, *G. diazotrophicus*; H, *H. seropedicae*; C, broth culture without Tryptophan; T, broth culture added with Tryptophan; LOQ = limit of quantification; LOD, limit of detection; Nq, not quantifiable, the production was very high and overlapped with other peaks. Error bars are standard error of the means. Results followed by different letters are significantly different according to Fisher's LSD test ( $p < 0.05$ ) following one-way ANOVA. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

### 3.4. Phosphate Solubilization Capability

The strain growth on agar plates of Pikovaska medium allowed underlining that in the presence of glucose, phosphate solubilization is a capability shared by *G. diazotrophicus*, *H. seropedicae*, and *B. ambifaria*. For these strains, clearing zones around the colonies were observed, especially for *G. diazotrophicus*. In contrast, for the *A. brasilense* strain clearing zones were observed only when the cultures were grown in modified Pikovaska medium (F- Pikovaska), with fructose instead of glucose.

Similar results were obtained for NBRIP liquid medium: the colorimetric assay carried out on supernatants of broth cultures showed that the strain with the highest concentration of phosphate was *G. diazotrophicus* ( $20.84 \pm 2.65 \mu\text{g PO}_4^{3-} \text{ mL}^{-1}$ ), followed by *H. seropedicae* ( $15.64 \pm 0.72 \mu\text{g PO}_4^{3-} \text{ mL}^{-1}$ ) and *B. ambifaria* ( $9.18 \pm 1.65 \mu\text{g PO}_4^{3-} \text{ mL}^{-1}$ ). *A. brasilense* in this estimation also showed positive results only in F-NBRIP broth cultures, with a phosphate solubilization capability of  $12.40 \pm 1.34 \mu\text{g PO}_4^{3-} \text{ mL}^{-1}$ .

### 3.5. Field Experiment

Results of chemical analyses of pre-sowing, untreated, and treated soil samples collected at DS (Development Stage [37]) 49, are shown in Table 2.

Among the different parameters analyzed, the initial soil pH—before sowing—(PS) recorded a slight decrease ( $p < 0.05$ ;  $n = 3$ ) in the presence of the bacterial inoculation (SIB), while for the control no significant changes were recorded ( $p > 0.05$ ;  $n = 3$ ). Concerning the pre-sowing soil sample, SIB recorded significant increases of total N, total organic C and organic matter ( $p < 0.01$ ;  $n = 3$ ), while the control recorded significant decreases of the same parameters ( $p < 0.01$ ;  $n = 3$ ). Moreover, the bacterial presence in SIB was able to decrease electrical conductivity ( $p < 0.01$ ;  $n = 3$ ) and to positively influence soil



nutrients. In comparison to pre-sowing soil,  $\text{Na}^+$  and  $\text{K}^+$  showed a decrease ( $p < 0.01$ ;  $n = 3$ ) and  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentrations were statistically similar ( $p > 0.05$ ;  $n = 3$ ). Best concentrations of available  $p$  were recorded for SIB ( $p < 0.01$ ). For the control soil, except for  $\text{Na}^+$  (statistically similar to PS,  $p > 0.05$ ;  $n = 3$ ), lower concentrations of all nutrients were recorded than PS and SIB soils ( $p < 0.01$ ;  $n = 3$ ). The ecological indexes calculated on soil community level physiological profiles are shown in Table 3. Bacterial inoculum positively affected soil microbial communities' physiological profile, improving the overall carbon source metabolic activity of soil. The SIB AWCD (average well color development, index of carbon source utilization) and  $H$  (diversity) indexes, determined at DS 49, were significantly higher than the control ones ( $p < 0.05$ ;  $n = 3$ ), while for the  $R$  index (richness), no significant differences were shown.

**Table 2.** Soil chemical analysis results recorded in the development stage (DS) 49 during the growing season (May–October). The table shows the effects of bacterial application on soil chemical parameters. Means followed by different letters are significantly different according to Fisher's LSD test.

Parameter	Treatment			F Test	LSD
	PS	Control	SIB		
pH	7.67 a	7.65 a	7.52 b	*	0.09
Total N ( $\text{g Kg}^{-1}$ )	2.05 b	1.95 c	2.15 a	**	0.09
TOC ( $\text{g Kg}^{-1}$ )	16.67 b	14.33 c	18.67 a	**	1.15
OM ( $\text{g Kg}^{-1}$ )	28.67 b	24.65 c	32.11 a	**	1.99
EC ( $\mu\text{s cm}^{-1}$ )	0.44 a	0.38 b	0.33 c	**	0.03
Na ( $\text{mg Kg}^{-1}$ )	31.50 a	30.50 a	17.25 b	**	3.07
Ca ( $\text{mg Kg}^{-1}$ )	3055.50 a	2997.75 b	3082.25 a	**	31.61
Mg ( $\text{mg Kg}^{-1}$ )	150.15 a	127.25 b	145.25 a	**	7.91
K ( $\text{mg Kg}^{-1}$ )	421.00 a	284.00 c	311.50 b	**	18.46
Available P ( $\text{mg P}_2\text{O}_5 \text{ Kg}^{-1}$ )	300.25 b	155.00 c	350.75 a	**	9.42

PS, pre-sowing; SIB, seed inoculated with bacteria. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

In Table 3 the diversity indexes calculated on NGS results are shown. Calculated diversity indexes are in line with findings obtained for CLPP analysis. For the SIB condition, Diversity and Richness values were higher than the control.

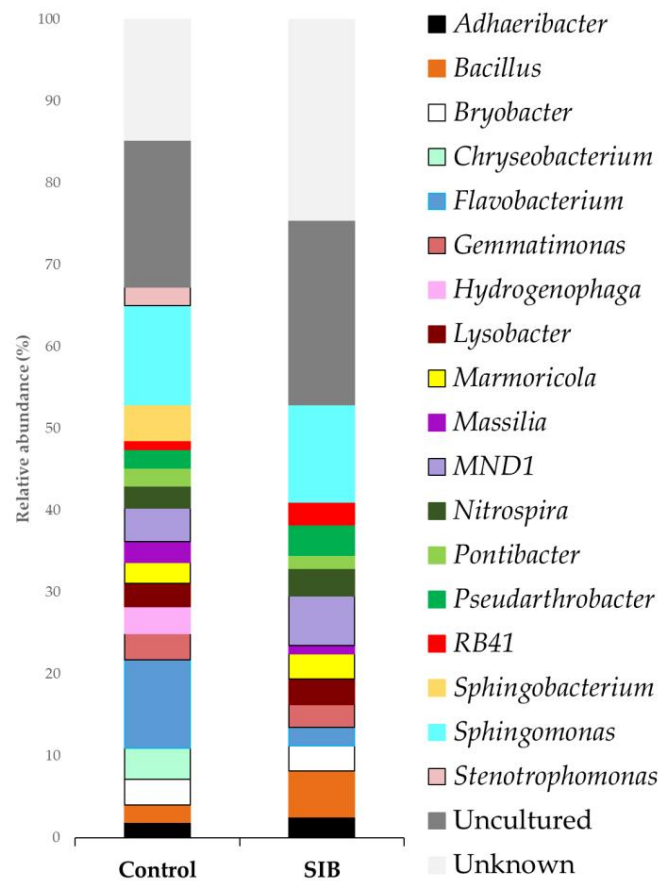
**Table 3.** Indexes calculated on community level physiological profile results recorded in development stage (DS) 49 during the growing season (May–October). The table shows the effects of bacterial application on AWCD (average well color development),  $H$  (Shannon), and  $R$  (Richness) indexes. Means followed by different letters are significantly different according to Fisher's LSD test.

	Parameter	Treatment		F Test	LSD
		Control	SIB		
CLPP	AWCD	1.35	1.47	*	6.03
	H	3.28	3.31	*	1.91
	R	29	30	n.s.	-
NGS	H	6.07	6.11	-	-
	Simpson 1-D	0.9968	0.9971	-	-
	Chao-1	571.6	587	-	-

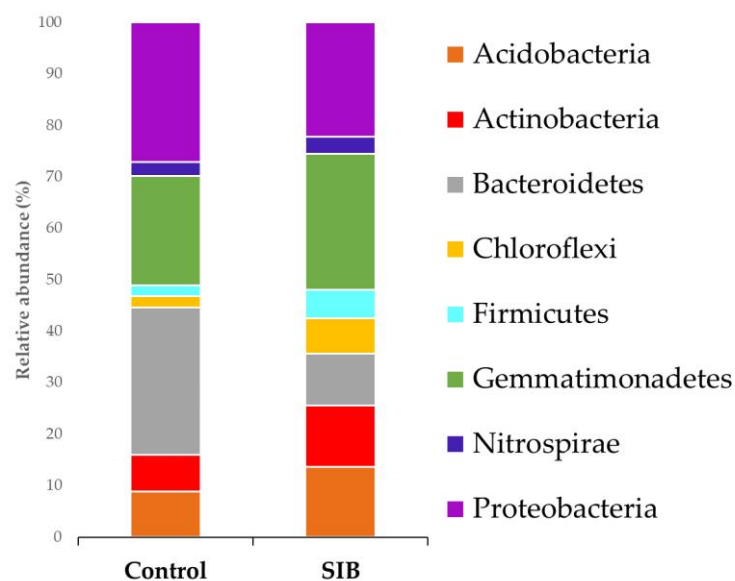
SIB: seed inoculated with bacteria. \*  $p < 0.05$ ; n.s. = not significant. Degrees of freedom: Blocks, 3; Treatment, 1; Residual, 3.

Figure 4 reports the relative abundances of common genera found in control and SIB samples. The relative abundance of *Adhaeribacter*, *Bacillus*, *Lysobacter*, *Marmoricola*, *MND1*, *Nitrospira*, *Pseudarthrobacter*, and *RB41* was higher in SIB soil samples than in the control. Instead, *Chryseobacterium*, *Flavobacterium*, *Gemmatimonas*, *Hydrogenophaga*, *Massilia*, *Pontibacter*, *Sphingobacterium*, *Sphingomonas*, and *Stenotrophomonas* were more numerous in the control. Unknown ASVs (amplicon sequence variants) included Acidobacteria,

Actinobacteria, Bacteroidetes, Chloroflexi, and Gemmatimonadetes, while Uncultured ASVs included Bacteroidetes and Gemmatimonadetes (Table S2). In general, there was a more balanced microbial community in the SIB sample, in which the Phyla Bacteroidetes and Proteobacteria did not predominate in the same way as in the control (Figure 5).



**Figure 4.** Relative abundances of major bacterial Genera (abundances > 1%) found in the bacterial communities of control and seed inoculated with bacteria (SIB) conditions.



**Figure 5.** Relative abundances of major bacterial Phyla (abundances > 1%) found in the bacterial communities of control and seed inoculated with bacteria (SIB) conditions.

Plant growth and development parameters recorded in the open field experiment are shown in Table 4. Root and aerial part length were significantly affected by seed bacterial treatment only in DS 49 (5.4% and 7%, respectively). However, seed inoculation with our PGPB consortium significantly improved total plant growth, as well as dry weights of roots and shoots in all developmental stages. For these parameters, SIB treatment shows a significant difference from the control plants in all stages. Pigments' amount was also influenced by the inoculum at late maturation. For both chlorophyll and carotenoids contents, SIB treatment exhibited the best values than the control in 45 and 49 DS, while there were no significant differences in 41 DS.

**Table 4.** Plant growth and development parameters recorded in different development stage (DS) during the growing season (May–October). The table shows the effects of bacterial application on Root Length, A.P. Length and Total Length (root + a.p.), Dry Weight (DW) of Root and AP, Carotenoids (Car), and Chlorophyll (Chl a + b). Means followed by different letters significantly different according to Fisher's LSD test.

Parameter	D.S.	Treatment		F Test	LSD
		Control	SIB		
Root Length (cm)	41	15.1	15.9	n.s.	-
	45	18.9	20.6	n.s.	-
	49	16.6 b	17.7 a	*	1.05
A.P. Length (cm)	41	27.0	30.7	n.s.	-
	45	54.1	59.3	n.s.	-
	49	56.7 b	60.7 a	*	2.94
Total (cm)	41	42.1 b	46.6 a	*	3.88
	45	73.0 b	79.9 a	*	4.16
	49	73.4 b	78.4 a	*	3.98
DW Root (%)	41	9.0 b	9.8 a	*	0.71
	45	10.9 b	11.7 a	*	0.60
	49	12.9 b	13.6 a	**	0.23
DW A.P. (%)	41	4.6 b	5.1 a	*	0.35
	45	6.1 b	6.6 a	*	0.45
	49	8.2 b	8.8 a	*	0.54
Car (mg 100 g <sup>-1</sup> FW)	41	2.4	2.6	n.s.	-
	45	9.5 b	9.8 a	*	0.15
	49	12.2 b	12.7 a	*	0.41
Chl a + b (mg 100 g <sup>-1</sup> FW)	41	373.6	376.1	n.s.	-
	45	393.6 b	395.3 a	*	2.13
	49	401.4 b	403.6 a	*	1.40

SIB: seed inoculated with bacteria. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; n.s. = not significant. Degrees of freedom: Blocks, 3; Treatment, 1; Residual, 3.

#### 4. Discussion

Among plant growth-promoting substances, IAA has positive effects on root elongation, cell division, and proliferation of root hairs [47]. Our strains demonstrated good production rates in vitro, both in single and mixed culture. The presence of IAA in the control cultures is probably due to the production of Trp or other precursors by some bacteria (in T4 medium no Trp or auxins were detected). The IAA production has been already reported by different authors for *H. seropedicae* [48], *B. ambifaria* [49], and *G. diazotrophicus* [50]. The high level of IAA synthesized by *A. brasilense* is in line with the literature. This strain is considered an excellent producer through several metabolic pathways [51–53].

The indole-derivates molecules were detected in all samples, suggesting the presence of different auxins pathways in all bacterial species. IAC was detected in all samples grown in single and mixed cultures, both in the presence and in the absence of Trp. IAC, in some cases, has been produced in higher concentrations than IAA. The IAM pathway was

detected in *A. brasilense* but only in the presence of Trp. Our data suggest that in *B. ambifaria* this pathway is not active, while in *G. diazotrophicus* is not always active. In *H. seropedicae*, it was difficult to discriminate because of the numerous close and/or overlapping peaks in the chromatograms (data not shown). Pedraza [54] also found that in *G. diazotrophicus*, IAM is not always active. Also, the recent work by Rodrigues et al. [55] demonstrated that *G. diazotrophicus* cultures supplemented with Trp produce IAA via the indole-3-pyruvic acid (IPyA) pathway ( $\text{Trp} \rightarrow \text{IPyA} \rightarrow \text{indoleacetaldehyde (IAAld)} \rightarrow \text{IAA}$ ); in this study, the involvement of an L-amino acid oxidase gene cluster in the biosynthesis of IAA was also shown. In *H. seropedicae* the difficult discrimination is similar to the case of *Arthrobacter* spp. [30]. However, according to genomic studies of Pedrosa et al. [56], there are four possible pathways in this strain to produce IAA from Trp. The most probable is via IPyA to IAA catalyzed by tryptophan transaminase and indole pyruvate ferredoxin oxidoreductase. Genes of the other possible metabolic pathways have been mapped in the genome: (1) Trp to IAA via IAM; (2) from IAM to IAC via indole-acetonitrile and (3) Trp to IAA via tryptamine and IAAld [56].

Considering that many bacteria require Trp supplement to synthesize significant amounts of IAA, the question arises as to whether enough levels of this hormone are available inside the plant. The outcome of the interaction between the plant and PGPB includes stimulation of plant growth and yield, modification of the plant's pool of growth regulators—resulting in stimulation of plant growth [51], scavenging of plant metabolites—particularly the toxic ones—for their growth and, last but not least, nitrogen fixation. Auxin's synthesis reaches its maximum at stationary phase, indicating a cell density-dependent production [53] generally associated with quorum sensing auto-inductor production. The auto-inductor molecules have a community regulatory function which can be closely related to plant colonization. However, further studies are necessary, particularly at a community level, to understand the role played by IAA and auxins in the communications between bacteria and bacteria and host plant.

The competitive behavior showed by *B. ambifaria* for Trp uptake, could be also expressed in field conditions where this bacterium is very common, particularly in Europe in maize rhizosphere [57]. This competitiveness can explain the failure of some strain's inoculation in field trials. However, even if a lower amount of IAA was obtained by mixing the four bacterial strains, a positive effect on plant growth and yield has still been recorded for this consortium [9–11,19,58]. This situation is possibly related to the establishment of a community inside the host-plant, which confers to the consortium the capability of colonization and competitiveness toward the autochthonous microbial community [10].

Several *Burkholderia* species showed ACC deaminase activity; except for *B. ambifaria*—for which enzymatic activity was never detected previously—18 species of the genus exhibited ACC deaminase activities in the range from 2 to 15  $\mu\text{mol}$  of ketobutyrate  $\text{h}^{-1} \text{mg}$  protein $^{-1}$  [59]. The presence of the ACC deaminase gene (*acdS*) has been reported in *H. seropedicae* SmR1 and species and strains belonging to the same genus [23]. Moreover, the presence of *acdR* (ACC deaminase regulatory protein), which is necessary for optimum ACC deaminase expression in the presence of ACC, has been reported in this genus as well. The lack of ACC deaminase activity in *A. brasilense* and *G. diazotrophicus* may be related to the  $\text{N}_2$ -fixing activity of these bacteria. In some Rhizobia, the expression of *acdS* is under the regulation of the nitrogen fixation (*nifH*) promotor, linking ACC deaminase with nodule formation [60]. In *Mesorhizobium loti* MAFF303099, the NifA protein has been reported to play a regulating role of ACC deaminase expression, which occurs only inside formed nodules [60]. However, since no records are present in literature about *G. diazotrophicus*, we cannot exclude that the behavior of ACC deaminase is similar to *M. loti*. Instead, findings so far, suggest that *acdS* gene is not present in *Azospirillum* genus and absence of activity in some strains has been already reported in the literature [61]. Moreover, the absence of ACC deaminase activity when the four strains were cultured together might be due to the  $\text{N}_2$ -fixing activity of *G. diazotrophicus* that inhibits, in single culture as well as in mixed cultures, the use of ACC as a nitrogen source.

The synthesis of IAA and the presence of ACC deaminase activity [62], suggest that bacteria utilize the tryptophan precursor—exuded by roots—and synthesize and excrete IAA. The latter, in turn, can be taken up by the plants and, together with the pool of endogenous plant-synthesized hormone, can stimulate plant cell proliferation and/or cell elongation. In the meantime, IAA stimulates the synthesis of ethylene in the plant, through the transcription of the enzyme ACC synthase that catalyzes the formation of ACC, which can be released by the plants, and then taken up by bacteria, acting as an ACC sink [62]. A further step forward, is when bacteria possessing the *acdS* gene can cleaved ACC by ACC deaminase. Bacterial ACC deaminase, which is generally present at a relatively low level, is induced by the increasing amounts of ACC deriving from the stress induction by ACC synthase in the plant [62]. The net result is the lowering of plant ethylene synthesis, which is particularly helpful in reducing stress ethylene, which is deleterious for the plants. Comprehension of the competitiveness of PGPB, based on their characteristics, is rather complex, though several studies are consistent with the possession of ACC deaminase activity facilitating bacterial competitiveness or persistence in the environment.

The phosphate solubilizing capability underlined for the four strains has important environmental and economic consequences. Nowadays, the phosphate supplementation in agriculture is commonly obtained by means of synthetic chemical fertilizers. The application of these products not only represents a huge cost for the farmers but also contributes to environmental pollution. The phosphate supplementation is obtained only through continuous fertilization campaigns and the chemical compounds applied, which lead to a stratification of inaccessible forms of phosphates in soils. Inoculation of PSB, instead of synthetic chemical compounds, could support plant nourishment whilst mobilizing immobilized phosphorous in lightly productive and unproductive agricultural soils [17] and preserving soils from losses of this essential chemical element [63]. Inoculation of PSB—belonging mainly to *Rhizobium*, *Pseudomonas*, *Azotobacter*, and *Bacillus* genera—has been already used as an alternative fertilization strategy, obtaining increasing phosphorous availability, without changing the soil composition [17].

Most of the literature concerning interactions of PGPB and plants, describe in vitro or greenhouse growth experiments; only a few articles contain field experiment and consider the PGPB effects on plant growth promotion and soil health. In our case, we obtained a positive influence of PGPB treatment on all investigated plant-growth and development parameters and, notably, in soil fertility status. Thus, the PGPB stimulant properties—underlined with the broth cultures assays—were confirmed by the field experiment results.

Indeed, soil health was positively influenced in terms of fertility. The application of PGPB consortium increased total organic carbon, organic matter, and available P and allowed to keep good concentrations of the other nutrients until harvest. Soil resources have been exploited to the fullest, allowing the plants to access resources not otherwise available. This phenomenon must be ascribed to the ability of PGPB strains to mobilize nutrients and to fix atmospheric nitrogen [64]. Similar results are already reported by other authors [65,66] and are mandatory for an effective plant growth promotion.

Bacterial inoculum presence also promoted positive changes in soil microbial community physiological profile. PGPB are already known to increase exudation from roots of host plants, which may, in turn, support the growth of microorganisms and/or influence their metabolic activity [67]. Amplitude and variability of substrate spectra metabolized by soil microbial populations are considered an indirect index of community biodiversity [68], including in the case of ectomycorrhizal [69] and endophytic fungi [70].

Based on NGS results, seed bacterization was able to alter the soil bacterial community composition. Diversity and Richness values were higher in the presence of the bacterial treatment. Also, the comparison of the abundances among the common genera found in the control and SIB samples underlined an alteration of the community structure. The effects of PGPR on soil microbial community has also been underlined by other authors [71–74] with transient or long-term effects [75]. In contrast, other authors reported no changes in microbial community structure [76] or only slight changes [77] after inocula application. In



any case, the ways that PGPB perform modifications have not yet been clarified [78]. A major number of developmental stages should be taken into account to study soil microbial community changes during crop growth. A recently published article by Wang et al. [79] reports the resilience of the autochthonous soil microbial community. In any case, the improvement of soil microbial diversity provides stability and support to ecosystem functions linked to biogeochemical cycling [80]. The microbial composition and richness of the soil support various ecosystem functions (e.g., crops' productivity and diversity, carbon assimilation, and cycling of nutrients) [80–82].

This positive effect on soil health and PGP properties were made evident by inoculated plants thriving, especially at harvest. Among PGP properties that induced this status, one key feature was certainly the ability to synthesize IAA described previously for all investigated strains. This hormone, in fact, like other auxins stimulates cell elongation—with consequent better development of plants—and is recognized to have a positive influence on chlorophyll a and b synthesis [83]. Besides, the phosphate solubilizing activity detected in all strains also contributed to this growth stimulation. *A. brasilense*, *B. ambifaria*, *G. diazotrophicus*, and *H. seropedicae* metabolites solubilized phosphorus and made this essential element available for absorption. Therefore, inoculated plants had better nourishment for their growth with respect to the control ones. Moreover, *B. ambifaria* and *H. seropedicae* may have supplied a contribution in lowering stress ethylene, thus giving the possibility for inoculated plants to thrive.

The increase of plant dry matter in response to PGPB inoculation is commonly reported in the literature [9,84]. This increase has to be ascribed mostly to N<sub>2</sub>-fixation [85]. N<sub>2</sub>-fixation promotes plant N uptake [86] and the synthesis of essential elements—modulating hormones, enzymes, and siderophores [87] implicated in plant growth and development. Furthermore, the positive influence of PGPB on all pigment fractions was previously described by different authors and, for plant growth, also under stress conditions [88].

## 5. Conclusions

In summary, the results obtained allowed us to assess the positive influence on carrots growth and development in an open field-experiment, exerted by all PGPB strains investigated. The positive effect can be ascribed to the different pathways of auxins production, phosphate solubilizing capability and, for *H. seropedicae* and *B. ambifaria*, also to enable ACC deaminase activity. All plant-growth and development parameters were positively affected by bacterial inoculation. Seed inoculation also affected soil fertility status and microbial communities' physiological and molecular profiles, underlying that these strains could be useful in improving agricultural productions whilst safeguarding health and microbial diversity of soils. Further analysis is needed to confirm the effects of bacterial inoculation on the soil microbial community, including other microbial techniques. The latter can produce some biases (e.g., NGS includes DNA from dead cells and culturable techniques exclude uncultured taxa); therefore, a study with complementary advanced methods could help to assess the validity of the results (e.g., fluorescence in situ hybridization to ascertain the presence of whole active cells). Future studies should also include the presence of and the connections among other microbial groups relevant to the soil biome, such as fungi, microfauna, and viruses.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/app11073274/s1>, Figure S1: Alpha diversity rarefaction curves of the microbial richness of Control and SIB samples according to their respective Sample Size. Alpha diversity rarefaction curve was obtained by PAST 4.03, Table S1: Filtered Read Paired-End(PE), raw and post-QualityCheck(QC) amplicons (non-chimeric) obtained for Control and SIB (seed inoculated with bacteria) samples, Table 2: Distribution of microbial taxa (%) within Control and SIB (seed inoculated with bacteria) soils. Data were processed by excluding the abundances of ASVs < 1%.

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S.D. formal analysis and investigation. All authors have read and agreed to the published version of the manuscript.

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