**Bacillus amyloliquefaciens FH-1 significantly affects cucumber seedlings and the rhizosphere bacterial community but not soil**

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Plant growth-promoting bacteria (PGPB) inoculants have been applied worldwide. However, the ecological roles of PGPB under different soil conditions are still not well understood. The present study aimed to explore the ecological roles of *Bacillus amyloliquefaciens* FH-1 (FH) on cucumber seedlings, rhizosphere soil properties, and the bacterial community in pot experiments. The results showed that FH had significant effects on cucumber seedlings and the rhizosphere bacterial community but not on soil properties. The FH promoted cucumber seedlings growth, reduced the rhizosphere bacterial diversity, increased Proteobacteria, and decreased Acidobacteria. Linear discriminant analysis (LDA) effect size (LEfSe) revealed that FH enriched two taxa (GKS2_174 and Nannocystaceae) and inhibited 18 taxa (mainly Acidobacteria, Actinobacteria, BRC1, Chloroflexi, Plantctomycetes, and Verrucomicrobia). Co-occurrence network analysis demonstrated that FH increased bacteria-bacteria interactions and that *Bacillus* (genus of FH) had few interactions with the enriched and inhibited taxa. This might indicate that FH does not directly affect the enriched and inhibited taxa. Correlation analysis results displayed that cucumber seedlings’ weight and height/length (except root length) were significantly correlated with the 18 inhibited taxa and the enriched taxa Nannocystaceae. It was speculated that FH might promote cucumber seedling growth by indirectly enriching Nannocystaceae and inhibiting some taxa from Acidobacteria, Actinobacteria, BRC1, Chloroflexi, Plantctomycetes, and Verrucomicrobia.

Cucumber is an important vegetable in many countries, including China. Due to the higher requirements, higher productivity of cucumbers relies heavily on chemical fertilizers and pesticides1. With increasing pollution and costs of chemical fertilizers and pesticides, plant growth-promoting bacteria (PGPB) inoculants are advantageous for the development of sustainable agriculture2. A substantial number of PGPB inoculants have been applied and commercialized for various crops worldwide4–6. PGPB mainly promote the growth of plants by providing nutrients, secreting hormones, antagonizing pathogens, and resisting stress7,8. However, poor productivity and stability impede the large-scale application of microbial inoculants in mainstream agriculture9,10. Understanding the ecological roles of the PGPB in the complex soil system may guide the development and application of PGPB inoculants in future.

*Bacillus amyloliquefaciens* is known for its ability to suppress plant pathogens and promote plant growth11,12. It has been widely applied on rice, tomato, cucumber, and lettuce, among others13–15. Many studies have demonstrated that *B. amyloliquefaciens* can reduce the incidence or severity of various diseases on a diversity of hosts13,16,17. This might be related to the secretion of antimicrobial lipopeptides, antibiotics, and hydrolases and might also be related to the regulation of the rhizosphere microbiome12,13. Many reports have shown that *B. amyloliquefaciens* can promote the growth of crops and improve the yield and quality of crops. This might be related to the secretion of indoleacetic acid (IAA), the improvement of available nutrients in soil through nitrogen fixation, phosphorus removal, and potassium dissolving, and the regulation of the rhizosphere microbiome18–20.

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In recent years, with the recognition of the importance of the rhizosphere microbiome, research on the effect of *B. amyloliquefaciens* on the rhizosphere microbiome has increased. Rhizosphere microbiomes play key roles in the disease, health, growth, and development of their host. Many reports have indicated that the application of microbial inoculants could influence resident microbial communities. The effects of *B. amyloliquefaciens* on the rhizosphere microbial communities of tomato, rice, lettuce, banana, tobacco, and cucumber were investigated. However, most studies focus on community composition and diversity, while only a few focus on co-occurrence network analysis. Co-occurrence network analysis of taxon co-occurrence patterns might help identify potential biotic interactions between inoculants and soil indigenous microorganisms and increase the understanding of how inoculants affect microbial communities. In addition, many studies on cucumbers are based on peat and vermiculite. This may be different from the results based on soil. Moreover, the ecological roles of *B. amyloliquefaciens* under soil conditions are not well understood. The comprehensive effects of *B. amyloliquefaciens* on crops, soil, and microorganisms still lack systematic and in-depth study.

To better understand the ecological roles of *B. amyloliquefaciens* under soil conditions, we investigated the effects of *B. amyloliquefaciens* FH–1 (FH), which could significantly promote rice growth in field experiments, on cucumber seedlings, rhizosphere soil properties, and the bacterial community in soil by using high-throughput sequencing technology, network analysis, and multivariate statistical methods. This will provide theoretical guidance for the development and application of PGPB inoculants in future.

### Results

#### FH had significant effects on cucumber seedlings.

The cucumber seedlings' weight and height were significantly affected by FH (Table 1). FH significantly increased the fresh weight of plants, shoots, and roots and increased the plant dry weight and shoot height of cucumber seedlings compared to those drenched with sterile deionized water (CK).

#### FH had no significant effect on rhizosphere soil properties.

FH had no significant effect on soil pH, total organic carbon, total nitrogen, total phosphorus, nitrate nitrogen, or available phosphorus (Table 2). However, the soil total nitrogen, total phosphorus, nitrate nitrogen, and available phosphorus in FH were generally higher than that in CK.

| Cucumber seedlings | CK             | FH             |
|--------------------|----------------|----------------|
| Fresh weight (g)   |                |                |
| Plant              | 1.90 ± 0.20b   | 3.25 ± 1.04a   |
| Shoot              | 1.71 ± 0.19b   | 2.82 ± 0.90a   |
| Root               | 0.19 ± 0.4b    | 0.43 ± 0.17a   |
| Dry weight (g)     |                |                |
| Plant              | 0.15 ± 0.06b   | 0.37 ± 0.17a   |
| Shoot              | 0.12 ± 0.06a   | 0.25 ± 0.11a   |
| Root               | 0.03 ± 0.01a   | 0.12 ± 0.09a   |
| Height/length (cm) |                |                |
| Plant              | 14.22 ± 1.09a  | 16.23 ± 2.00a  |
| Shoot              | 9.73 ± 0.33b   | 11.65 ± 1.55a  |
| Root               | 4.49 ± 1.00a   | 4.58 ± 0.65a   |

Table 1. Effects of *Bacillus amyloliquefaciens* FH–1 inoculation on cucumber seedlings. Values (means ± SD, n = 5) within the same row followed by different letters are significantly different at *P* < 0.05 according to Independent-Samples t Test. CK non-inoculated, FH inoculated with *Bacillus amyloliquefaciens* FH–1.

|                        | CK               | FH               |
|------------------------|------------------|------------------|
| pH                     | 8.48 ± 0.07a     | 8.46 ± 0.09a     |
| TOC (g/kg)             | 6.44 ± 3.40a     | 4.08 ± 0.61a     |
| TN (mg/kg)             | 733.20 ± 199.73a | 765.60 ± 151.03a |
| TP (mg/kg)             | 311.86 ± 20.96a  | 342.35 ± 66.18a  |
| NO3–N (mg/kg)          | 107.07 ± 16.23a  | 117.51 ± 20.82a  |
| AP (mg/kg)             | 88.11 ± 9.5a     | 89.65 ± 1.62a    |

Table 2. Effects of *Bacillus amyloliquefaciens* FH–1 inoculation on rhizosphere soil properties. Values (means ± SD, n = 5) within the same row followed by different letters are significantly different at *P* < 0.05 according to Independent-Samples t Test. TOC total organic carbon, TN total nitrogen, TP total phosphate, NO3–N nitrate nitrogen, AP available phosphate, CK non-inoculated, FH inoculated with *Bacillus amyloliquefaciens* FH–1.
FH significantly affects rhizosphere bacterial community composition. Across all samples, a total of 634,513 high-quality sequences and 57,039–68,492 sequences per sample (mean = 63,451) were obtained. After being rarefied to 57,000 sequences per sample, Alphaproteobacteria, Actinobacteria, Acidobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Gemmatimonadetes, Bacteroidetes, Chloroflexi, Planctomycetes, Firmicutes, Verrucomicrobia, Nitrospirae, Armatimonadetes, Cyanobacteria, TM7, Fibrobacteres, and Chlorobi were found to be the dominant phyla (> 1%) across all treatments (Fig. 1). These dominant phyla accounted for more than 94% of the bacterial sequences from each soil sample. Deltaproteobacteria (\( P = 0.01 \)) was significantly increased, while Acidobacteria (\( P = 0.00 \)) was significantly decreased by FH (Table S1).

LEfSe analysis showed that a total of 20 bacterial groups were distinct between FH and CK treatments using the logarithmic (LDA) value of 2 (Fig. 2). The bacterial taxa enriched in FH were GKS2-174 and Nannocystaceae. Acidobacteria-6 (the class and its order CCU21 and iii1-15, the order and its family mb2424), MB-A2-108 (the class and its order 0319-7L14), Rubrobacteria (the class and its order Rubrobacterales, the order and its family Rubrobacteraceae), PRR-11, C0119, Gitt-GS-136, Gemmataceae (the family and its genus Gemmata), Pseudonocardaceae, Leucobacter, and Prosthecobacter were enriched in CK, which also could be regarded as inhibited taxa in FH.

**Figure 1.** Relative abundance of the dominant rhizosphere bacterial phyla (proteobacterial classes) under different treatments. CK non-inoculated, FH inoculated with Bacillus amyloliquefaciens FH-1.

**Figure 2.** Cladogram (A) and linear discriminant analysis (LDA) score (B) of LEfSe analysis of the rhizosphere bacterial community between CK (red) and FH (green) treatments. CK non-inoculated, FH inoculated with Bacillus amyloliquefaciens FH-1.
The relative abundances of both *B. amyloliquefaciens* and *Bacillus* spp. were slightly higher in FH than in CK (Fig. S1). This suggested that *B. amyloliquefaciens* FH-1 might slightly colonize cucumber rhizosphere soil.

**FH had negative effects on rhizosphere bacterial diversity.** The rhizosphere bacterial α-diversity was negatively affected by FH (Table 3). FH significantly decreased Observed_{otus} (P = 0.01) and PD\_whole\_tree (P = 0.02), Chao1 (P = 0.07) and the Shannon index (P = 0.10) were lower in FH than in CK.

Principal coordinate analysis (PCoA) revealed that the rhizosphere bacterial communities of FH were distinct from those of CK (Fig. 3). ANOSIM analysis (global R = 0.488, P = 0.008) and PERMANOVA analysis (R^2 = 0.326, P = 0.009) demonstrated that the structure of bacterial communities was significantly changed by FH.

**FH modified rhizosphere bacterial networks.** Whether FH affected the interaction of bacterial communities and whether FH interacted with enriched or inhibited taxa at the genus level were determined using co-occurrence network analysis based on a strong (Spearman’s r > 0.6) and significant (P < 0.05) correlation. The calculated modularity index was larger than 0.4, and the random modularity index (Table 4) indicated a typical module structure. Overall, the FH showed a remarkable influence on the co-occurrence networks in bacterial communities (Fig. 4). The number of positive correlations was higher than that of the negative correlations in both networks. FH had higher edges, negative correlations, and an average degree and modularity but lower positive correlations than CK (Table 4). There were more species interacting with *Bacillus* in FH than that in CK. There were 19 genera that interacted with *Bacillus*, and seven of them had positive interactions in FH. In CK, only seven genera interacted with *Bacillus*, and six of them had positive interactions (Fig. 4 and Table S2). *Bacillus* only had positive interactions with the inhibited taxa *Leucobacter* in CK and the inhibited taxa MB-A2-108 in FH.

**Cucumber seedling characteristics were significantly correlated with the bacteria inhibited and enriched by FH.** Correlation analysis showed that cucumber seedlings’ weight and height/length (except root length) had a significant correlation with the bacteria taxa inhibited and enriched by FH (Fig. 5). All 18 inhibited taxa (mainly Acidobacteria, Actinobacteria, BRC1, Chloroflexi, Plantctomycetes, and Verrucomicrobia) were significantly and negatively correlated with some cucumber seedlings’ characteristics. These inhibited taxa had a closer relationship with cucumber shoots than roots. Enriched taxa Nannocystaceae had a significant positive correlation with cucumber shoot height.

### Table 3. Effects of *Bacillus amyloliquefaciens* FH-1 inoculation on rhizosphere bacterial alpha diversity. Values (means ± SD, n = 5) within the same row followed by different letters are significantly different at P < 0.05 according to Independent-Samples t Test. Chao1 richness of the Chao1 estimator, Observed_{otus} observed operational taxonomic units, Shannon index nonparametric Shannon diversity index, CK non-inoculated, FH inoculated with *Bacillus amyloliquefaciens* FH-1.

|                   | CK             | FH             |
|-------------------|----------------|----------------|
| Chao1             | 7176.53 ± 117.44a | 6971.29 ± 159.56a |
| Observed_{otus}   | 4789.86 ± 98.59a  | 4599.68 ± 64.80b |
| PD\_whole\_tree  | 253.21 ± 3.46a    | 246.76 ± 2.38b  |
| Shannon index     | 9.74 ± 0.11a     | 9.63 ± 0.07a    |

**Figure 3.** Principal coordinate analysis (PCoA) of weighted UniFrac distances of the rhizosphere bacterial community under different treatments. CK non-inoculated, FH inoculated with *Bacillus amyloliquefaciens* FH-1.
In this study, the ecological roles of inoculant *B. amyloliquefaciens* FH-1 on cucumber seedlings, rhizosphere soil, and the bacterial community were investigated. The results illustrated that FH had a significant effect on cucumber seedlings and the rhizosphere bacterial community but not on soil. Rhizosphere bacterial communities play a key role in the disease, health, growth, and development of plants. The effect of PGPB on the bacterial community is still unclear. As a well-known PGPB, the effect of *B. amyloliquefaciens* on the bacterial community has been widely studied. Some studies have shown that *B. amyloliquefaciens* has no influence on rhizosphere bacteria, while some have a significant influence. Some increased diversity, while some decreased diversity, and some improved Proteobacteria, while some improved Firmicutes. In this study, we found that FH inoculation significantly reduced bacterial diversity, increased Proteobacteria that may belong to r-strategies, and decreased Acidobacteria that may belong to k-strategies. The influence of *B. amyloliquefaciens* on the bacterial community may be attributed to different strains, plant species, soil types, and environmental factors. Therefore, it is necessary to investigate the influence of *B. amyloliquefaciens*.

### Table 4. Topological properties of rhizosphere bacterial networks obtained from different treatments. CK non-inoculated, FH inoculated with *Bacillus amyloliquefaciens* FH-1.

|                          | CK                  | FH                  |
|--------------------------|---------------------|---------------------|
| **Empirical networks**   |                     |                     |
| Number of nodes          | 817                 | 817                 |
| Number of edges          | 3963                | 4107                |
| Number of positive correlations | 2743 (69.22%)     | 2635 (64.16%)      |
| Number of negative correlations | 1220 (30.78%)  | 1472 (35.84%)      |
| Average degree           | 4.851               | 10.054              |
| Average clustering coefficient | 1                   | 1                   |
| Average path length      | 1                   | 1                   |
| Network diameter         | 1                   | 1                   |
| Graph density            | 0.012               | 0.012               |
| Modularity               | 0.967               | 0.970               |
| **Random networks**      |                     |                     |
| Average clustering coefficient | 0.012 ± 0.001   | 0.012 ± 0.001      |
| Average path length      | 3.202 ± 0.003       | 3.157 ± 0.002      |
| Modularity               | 0.284 ± 0.004       | 0.278 ± 0.004      |

**Figure 4.** Networks of co-occurring rhizosphere bacterial genera in non-inoculated (CK) and *Bacillus amyloliquefaciens* FH-1 inoculated (FH) soil based on correlation analysis. A connection stands for a strong (Spearman’s r > 0.6) and significant (P < 0.05) correlation. A blue edge indicates a negative interaction between two individual nodes, while a red edge indicates a positive interaction. The thickness of each connection between two nodes (i.e., edge) is proportional to the value of Spearman’s correlation coefficient. The co-occurring networks are colored by modularity class. The size of each node is proportional to the number of connections (i.e., degree). *Bacillus* is labeled n65.
Figure 5. Heatmap of Spearman’s correlation coefficients between cucumber seedlings and bacteria inhibited and enriched by FH. The colors represent the correlation, with red being more positive and blue being more negative. Significance is given as *(P < 0.05) and **(P < 0.01).
B. amyloliquefaciens FH-1 might promote cucumber seedling growth by regulating the bacterial community and indirectly enriching Nannocystaceae and inhibiting some taxa from Acidobacteria, Actinobacteria, BRC1, Chloroflexi, Plantctomycetes, and Verrucomicrobia. This result was roughly supported by many previously published studies. Regulating the rhizosphere microbiome is an important mechanism for PGPB to promote plant growth. Whole genome data showed that B. amyloliquefaciens FH-1 had no complete pathway for nitrogen fixation or secretion of IAA, gibberellin (GA), abscisic acid (ABA), or ethylene but had a complete pathway to secrete organic acids (malic acid, acetic acid, succinic acid, and gluconic acid), phytase, zeatin, and siderophore (data not shown). This study showed that FH had no significant effect on soil properties, suggesting that the ability of B. amyloliquefaciens FH-1 to dissolve phosphorus and potassium did not play a role in soil characteristics. In our next work, we will verify whether FH promotes cucumber seedling growth by secreting zeatin and siderophore.

Materials and methods

Bacterial inoculum preparation. B. amyloliquefaciens FH-1 was grown at 30 °C for 48 h in Luria–Bertani (LB) broth on a rotary shaker (180 rpm). The cells were harvested by centrifugation (5000 × g for 10 min), and the bacterial pellet was washed three times with 0.9% NaCl and finally resuspended in sterile deionized water at 1 × 10^8 CFU/ml.

Pot experiment for the cultivation of cucumber seedlings. For future applications in coastal saline-alkali land, soil (pH 8.14, 4.1 g/kg organic matter, 655 mg/kg total N, 18 mg/kg available N, 250 mg/kg total P, 155 mg/kg available P, 4893 mg/kg total K, and 124 mg/kg available K) was obtained from a weed field in an airport economic area in Tianjin, China. The sampled soil was air dried and mixed thoroughly, followed by a sieving step (0.5-cm mesh) to remove plant debris. Cucumber seeds (Jin you NO.1, Tianjin Kerun Agricultural Science Technologies Inc., Tianjin, China) were procured from the local market (Fig. S2). Two cucumber seeds were sown in each plastic pot (diameter 8 cm; height 10 cm) containing 300 g of soil. Pot soils were drenched with 300 ml of the prepared inoculums or equivalent sterile deionized water. In total, there were two treatments: (1) soil drenched with B. amyloliquefaciens FH-1 (FH), and (2) soil drenched with sterile deionized water (CK). Five replications of each treatment were set up during the entire experimental period. Pots were placed randomly in a growth chamber at 28 °C day/17 °C night, 75% relative humidity, and 9 h light, and watered weekly. All methods were carried out in accordance with relevant guidelines and regulations.

Plant characteristics and soil chemical properties. At 35 days after sowing, plants of each pot were harvested and carefully separated into roots and shoots to determine the growth parameters, including length, fresh weight, and dry weight, using rulers and balances. Meanwhile, rhizosphere soil was collected and stored at 4 °C and − 80 °C, respectively.

The rhizosphere soil pH, total organic carbon, total nitrogen, total phosphorus, nitrate nitrogen, and available phosphorus were determined using commercial chemical assay kits (Suzhou Comin Biotechnology Co. Ltd., Suzhou, China) following the manufacturer’s instructions.

DNA extraction, PCR amplification, and Hiseq sequencing. Soil metagenomic DNA was isolated from 10 soil samples by the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. DNA purity and concentration were monitored by 1% agarose gels and NanoDrop ND-2000 spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA), respectively. The bacterial hypervariable regions (V4) of the 16S rRNA genes were amplified using primer 515F-806R with a barcode. PCR products were purified and sequenced using the Miseq platform at Novogene Co. Ltd (Tianjin, China). The raw sequence data were deposited in the NCBI Sequence Read Archive as accession PRJNA544608 for bacteria. Raw data were processed and analyzed as previously described using the QIIME. The relative abundance of B. amyloliquefaciens was determined by local BLAST.

Data analyses. All statistical analyses were performed using R (version 3.1.1). The cucumber seedlings’ characteristics, soil properties, bacterial α-diversity indices, and relative abundance of taxa in different treatments were compared using Independent Sample t tests. Principal coordinate analysis (PCoA), analysis of similarity (ANOSIM), and permutational multivariate analysis of variance (PERMANOVA) with the ADONIS function based on weighted UniFrac distance were performed to evaluate the overall differences in the bacterial community. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to identify taxa that were function based on weighted UniFrac distance. Heatmaps that illustrate correlation data were generated using the ‘pheatmap’ package in R.

Received: 26 October 2020; Accepted: 21 May 2021
Published online: 08 June 2021
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Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-91399-6.

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