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# Probiotic *Pseudomonas* communities enhance plant growth and nutrient assimilation via diversity-mediated ecosystem functioning

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ABSTRACT

Plant-associated microbes play an important role in plant growth and development. While the introduction of beneficial microbes into the soil could improve plant production in low-input agricultural systems, real-world applications are still held back by poor survival and activity of the probiotic microbes. In this study, we used a biodiversity-ecosystem functioning (BEF) framework to specifically test how *Pseudomonas* community richness shapes the bacterial inoculant survival and functioning in terms of plant growth. To this end, we manipulated the richness of a probiotic *Pseudomonas* spp. bacterial community inoculant (1, 2, 4 or 8 strains per community) and compared diversity and strain identity effects on plant biomass production and nutrient assimilation *in vivo* with tomato. We found that increasing the richness of the bacterial inoculant enhanced the survival and abundance of *Pseudomonas* communities leading to higher accumulation of plant biomass and more efficient assimilation of nutrients into the plant tissue. Diversity effects were clearly stronger than the *Pseudomonas* strain identity effects and diversity-mediated plant growth promotion could be linked with increased production of plant hormones, siderophores and solubilization of phosphorus *in vitro*. Together these results suggest that multi-strain microbial inoculants can promote plant growth more reliably and effectively compared to single-strain inoculants.



## 1. Introduction

Rapid growth of the human population has created an increasing need for novel low-input and high-yield agricultural practices that do not harm natural ecosystems (Tilman, Cassman, Matson, Naylor and Polasky, 2002). Application of beneficial microbes that promote plant growth is thought to hold potential for reducing the extensive use of chemical fertilizers and pesticides in modern agriculture (Vessey, 2003). Plant growth-promoting microbial communities can provide various services to the plant such as protection from pathogen invasion (Hu et al., 2016; Wei et al., 2015), production of plant growth hormones (Muhammad Arshad, 1991) and mobilization of soil nutrients that would be otherwise inaccessible for plants (Lugtenberg and Kamilova, 2009). Unfortunately, current intensive agricultural practices that depend on high inputs of chemical fertilizers and pesticides are often detrimental to the plant-associated beneficial rhizosphere bacteria and microbial ecosystem functioning (Tsiafouli et al., 2015). While the loss of functionality can be restored by inoculating beneficial microbes into the microbe-poor rhizosphere (Wubs, van der Putten, Bosch and Bezemer, 2016), this approach is often limited by poor survival of microbial inoculants. In particular, direct antagonism and competition for space and resources with the indigenous microbes can severely limit inoculant establishment and functioning in the rhizosphere (Kadam and Chuan, 2016). In this study, we applied the biodiversity-ecosystem functioning (BEF) framework to test how the richness of a probiotic bacterial community affects its survival and plant growth-promotion activity in the tomato rhizosphere *in vivo*.

Biodiversity has consistently been shown to enhance the productivity and stability of ecosystems (Hautier et al., 2015; Hector et al., 1999; Reich et al., 2001). In the context of plant growth-

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promotion, high probiotic bacterial inoculant richness has been linked to increased pathogen suppression via enhanced resource and interference competition (e.g. antimicrobial activity) (Hu et al., 2016; Wei et al., 2015) and improved long-term probiotic survival in the rhizosphere (Hu et al., 2016). While the exact mechanisms are still unknown, these findings suggest that the performance of probiotic microbes could be enhanced by applying the strains as multi-species communities that survive and function better in the rhizosphere.

Probiotic bacterial community richness could improve the survival and subsequent functioning of the inoculated strains in several ways. For example, diverse probiotic communities occupy a broader resource niche together versus alone (M. Loreau and Hector, 2001), which helps them compete more efficiently with already existing microbes in the rhizosphere (Richardson, 2014). Multispecies communities are also likely to harbor at least one species that can perform well under a given set of environmental conditions thereby stabilizing community functioning across different environments (S. Y. a. M. Loreau, 1999; Yang et al., 2017). Bacterial diversity also affects the level of microbial communication and cooperation in the rhizosphere. For example, diversity induces more intensive microbial communication and potentially trigger the expression of traits, such as secondary metabolite production (Garbeva, Silby, Raaijmakers, Levy and Boer, 2011; Jousset et al., 2014; Tyc et al., 2014), which are not expressed in less diverse communities (Fujiwara et al., 2016). Diverse bacterial communities also improve plant growth by maintaining high community-level enzymatic activity, which could for example increase nitrogen mineralization (Weidner et al., 2015). Diversity also has negative effects on bacterial survival and functioning if it increases antagonism between the members of inoculant community (Mehrabi et al., 2016). However, as the level of antagonism often increases with increasing ecological similarity, the members of inoculant communities should be different enough to show complementary effects in terms of ecosystem functioning (Freilich et al., 2011).

Here we studied the impact of probiotic inoculant community richness on its survival and plant-growth promotion activity by using defined communities of eight fluorescent pseudomonads - a common bacterial taxon known for its plant growth promotion capability (Hol, Bezemer and Biere, 2013). We have previously shown that these strains show synergistic antimicrobial activity against *R. solanacearum* (Hu et al., 2016) and could thus exert complementarity with respect to other plant growth-promoting traits. To study this, we created an inoculant richness gradient ranging from 1, 2, 4 or 8 *Pseudomonas* strains per community and tested the community survival and functioning in terms of plant biomass production and nutrient assimilation *in vivo* with tomato. The potential mechanisms underlying bacterial community functioning were also determined *in vitro* in terms of the production of plant hormones auxin (stimulates root growth) and gibberellin (affects elongation and expansion of stem and leaves) (Davies, 2010), iron-scavenging siderophores (positively affect iron availability) and phosphate rock solubilization (positively affect phosphorus availability). We hypothesized that increasing the richness of probiotic *Pseudomonas* communities has positive effects on plant growth via improved survival and expression of plant growth promoting traits in the rhizosphere.

## 2. Materials and methods

### 2.1. Bacterial strains

We included eight probiotic *Pseudomonas* strains (*P. fluorescens* 1m1-96, F113, mvp1-4, Phl1c2 and Q2-87; *P. protegens* Pf-5 and CHA0; *P. brassicacearum* Q8r1-96) in our study system. All strains have been extensively investigated in relation to their ability to promote plant growth (Loper et al., 2012) and to affect biodiversity-ecosystem functioning in microbial communities (Hu et al., 2016; Jousset, Schmid, Scheu and Eisenhauer, 2011) (for more details see Table S1). All strains were routinely stored at −80 °C. Prior to experiments, one single colony of each strain was selected, grown overnight in lysogenic broth (LB), washed three times in 0.85% NaCl buffer and adjusted to an optical density of 0.5 (600 nm) before use.

### 2.2. Establishing a probiotic community richness gradient

A probiotic *Pseudomonas* community richness gradient was created by establishing four richness levels (1, 2, 4 and 8 strain communities in a total of 48 different combinations, Table S2) as described in a previous study (Becker, Eisenhauer, Scheu and Jousset, 2012). Briefly, all *Pseudomonas* monocultures were replicated twice and 8-strain communities were replicated four times. For other richness levels, each probiotic strain was included equally often in the fully assembled communities, which allows to disentangle the effects of diversity and strain identity (Hu et al., 2016). Following assembly, communities were immediately used for subsequent experiments. We used a substitutive design where the total probiotic community biomass was kept constant at all richness level. The same experimental design was followed in both the greenhouse and *in vitro* experiments.

### 2.3. Setting up the greenhouse experiment

We assessed the effect of the inoculated *Pseudomonas* communities on plant growth in a 50-day long greenhouse experiment. We used natural soil that was collected from a tomato field in Qilin (118°57′E, 32°03′N): a town near the city of Nanjing, China. Soil was first sieved through 5 mm mesh to remove stones and roots and then homogenized thoroughly. Tomato seeds (*Lycopersicon esculentum,* cultivar "*Jiangshu*") were surface-sterilized by soaking in 70% ethyl alcohol for 1 min, washed with sterile water, immersed in 3% NaClO for 5 min, and finally rinsed six times with sterile water. Surface-sterilized seeds were then germinated on water-agar plates (three days) before sowing into seedling plates containing Cobalt$^{60}$-sterilized seedling substrate (Huainong, Huaian soil and fertilizer Institute, Huaian, China). At the three-leaf stage (12 days after sowing), tomato plants were transplanted to seedling trays that contained natural, non-sterile soil. The tray dimensions were 370 mm × 272 mm × 83 mm, each tray contained 8 separated sub boxes and each sub box contained 600 g of soil and two tomato plants. After 10 days of transplantation, sub boxes were inoculated with probiotic *Pseudomonas* communities using a root drenching method (Wei et al., 2013). Briefly, 5 mL of *Pseudomonas* probiotic communities were pipetted on to the surface soil at an initial concentration of $5 \times 10^7$ inoculated *Pseudomonas* cells $g^{-1}$ soil. In total, we used 52 seeding trays that contained all 48 different probiotic *Pseudomonas* communities (Table S2) and 4 control treatments that contained only the naturally occurring *Pseudomonas* species present in the non-sterile soil. All tomato plants were watered daily with sterile water. Seedling trays were arranged in arbitrary order and rearranged randomly every two days.

### 2.4. Extracting rhizosphere soil DNA and quantifying Pseudomonas densities with real-time PCR

After 20 and 40 days of *Pseudomonas* probiotic community inoculation, two plants per each *Pseudomonas* community (52 in total including the control treatment) were harvested. Rhizosphere soil was collected by gently removing plants from the sub boxes, shaking off the excess soil then the soil attached to the root system