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Research article

Effects of *Bacillus subtilis* and *Pseudomonas fluorescens* as the soil amendment

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ABSTRACT

The application of soil beneficial bacteria (SBB) in agriculture is steadily increasing as it provides a promising way to replace chemical fertilisers and other supplements. Although the role of SBB as a biofertiliser is well understood, little is known about the response of soil physiochemical properties via the change in soil enzymatic activities with SBB growth. In this study, sterilised bulk soil was inoculated with *Bacillus subtilis* (BS) and *Pseudomonas fluorescens* (PF), which exhibit excellent characteristics *in vitro* for potentially improving soil quality. It is found that the contents of bioavailable nitrogen and ammonium in soil inoculated with SBB increased significantly, up to 34% and 57% relative to a control. This resulted from the enhancement of soil urease activity with BS and PF treatments by approximately 90% and 70%, respectively. The increased soil urease activity can be explained by the increased microorganism activity evident from the larger population size of BS (0.78–0.97 CFU mL^{-1}/CFU mL^{-1}) than PF (0.55–0.79 CFU mL^{-1}/CFU mL^{-1}) ($p < 0.05$). Results of principal component analysis also reinforce the interaction apparent in the significant relationship between soil urease activity and microbial biomass carbon ($p < 0.05$). Therefore, it can be concluded that the enhancement of soil enzymatic activities induced bulk soil fertility upregulation because of bacterial growth. These results demonstrate the application of SBB to be a promising strategy for bulk soil amendment, particularly nutrient restoration.

1. Introduction

Soil beneficial bacteria (SBB), as an ecologically significant soil amendment, have been used in agroecosystems for decades (Ahmed et al., 2022; Hayat et al., 2010). When applied to substitute for chemical fertiliser, they can reduce the nutrient loss from farmland soil by more than 50% (Sun et al., 2020). Although the effects of biofertilisers on rhizosphere soil are well studied, less is known about how the bulk soil quality responds to SBB growth (Sun et al., 2022; Zhao et al., 2022). One crucial aspect of soil-SBB interactions is the recognition of soil substance transformation (Richardson et al., 2009). More precisely, SBB affect soil physicochemical properties through fertility improvement and aggregation formation and further contribute to the growth of plants and microorganisms (Haskett et al., 2021). However, whether these benefits of SBB are only observed in rhizosphere soil is still ambiguous because their effects are buried in multiple bacteria-soil-plant reciprocities. Identifying the separate functions of SBB would offer insights into their broader application in sustainable soil restoration practices (Coban et al., 2022).

SBB, which mainly belong to *Bacillus* spp. and *Pseudomonas* spp., show excellent traits for promoting soil quality (Haas and Défago, 2005; Kalam et al., 2020; Khan et al., 2022). A good example is their outstanding ability to secrete auxin indole-3-acetic acid (IAA), one of the most physiologically active phytohormones in soil, contributing to plant growth via metabolisation (Spaepen et al., 2007). Another important mechanism through which SBB promotes fertility in the rhizosphere is by providing nutrients. The functions of an agroecosystem are significantly constrained by the limitation of phosphorus and nitrogen (Xu et al., 2020), so increasing the supply of phosphate and nitrogen fertility would be expected to improve the productivity of terrestrial ecosystems. However, organisms cannot utilise more than half of the nutrients stored in chemical fertilisers in fields (Modolo et al., 2015). Thus, improving the efficiency of soil substrate recycling becomes critical.

Soil nutrient bioavailability is governed by soil physicochemical properties, environmental conditions, and biological interactions driven by microorganisms and enzymes (Richardson et al., 2009). In particular, bacterial activity (e.g., acid secretion) and soil phosphatase activity strongly affect inorganic phosphate mobilisation. Higher phosphatase

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Table 1. The physicochemical properties of the bulk soil.

Parameters	Values
pH	5.84 ± 0.08
EC (dS/m)	7.18 ± 0.45
Dry density (g/cm^3)	1.46
Soil moisture (w/w)	31%
TOC (μg/g)	107.82 ± 8.22
MBC (μg/g)	18.33 ± 3.68
$SUVA_{254}$	1.68 ± 0.36
AN (μg/g)	33.37 ± 1.36
AM (μg/g)	37.06 ± 2.61
MBN (μg/g)	5.70 ± 0.27
AP (μg/g)	12.08 ± 2.35

Note: the results were measured from control soil (CK). The control soil in all figures and tables represents the samples without bacteria inoculation. EC: electrical conductivity. TOC: total organic carbon. MBC: microbial mass carbon. SUVA254: specific ultraviolet absorbance at 254 nm. AN: bioavailable nitrogen. AM: ammonium. MBN: microbial biomass nitrogen. AP: bioavailable phosphate. Each result is presented as the mean ± S.D., calculated from three independent samples.

activity increases rhizosphere soil bioavailable phosphate (AP) (Hassan, 2017; Khajeeyan et al., 2019). As a result, the dynamic equilibrium between inorganic and bioavailable forms of phosphorus in the rhizosphere depends on soil phosphatase activity (Li et al., 2020). Additionally, bacterial activity influences nitrogen fertility because of the stimulating effect on the conversion of soil bioavailable nitrogen (AN) and soil ammonium (AM) (Lisuma et al., 2020). Rhizosphere soil shows an increased bacteria-mediated nitrogen transformation efficacy owing to functional soil urease (SURE) activities (Liu et al., 2022; Sun et al., 2020). Previous research has primarily focused on changes in enzymatic activities to explain the effects of SBB on rhizosphere soil (Li et al., 2020; Wei et al., 2020). However, to what extent such changes occur in bulk soil and how they might happen remain unclear.

The deconstructed microcosm is a small-scale soil system which permits the assessment of the interaction of microorganisms and soil (Höss et al., 2021). This microcosm setup is an appropriate tool for investigating the activities of soil microorganisms at a laboratory scale (White et al., 2000). In the current study, the deconstructed microcosm was built with sterilised bulk soil and two additional beneficial bacteria species, *Bacillus subtilis* (BS) and *Pseudomonas fluorescence* (PF), in a plastic container. To study the soil quality-promoting traits of these SBBs, their capabilities were first quantified *in vitro*. Then they were respectively cultivated in soil. Analyses of the soil physicochemical properties and enzymatic activities revealed the interaction between bulk soil quality and SBB growth.

2. Material and methods

2.1. Characterization of bacteria traits in vitro

The BS strain used in this study is *Bacillus subtilis subsp. subtilis* (GDMCC 1.372, Guangdong Microbial Culture Collection Centre), and the PF strain is *Pseudomonas fluorescens Migula* (GDMCC 1.782, Guangdong Microbial Culture Collection Centre). The IAA secretion capability, inorganic phosphate solubilisation capability, and exopolysaccharides production capability of the bacteria were tested *in vitro*. The bacterial IAA secretion capability was evaluated in King's liquid medium (Nomura et al., 2012). Firstly, the single colony of BS and PF were inoculated in the liquid medium, respectively. The inoculated culture medium was incubated at 30 °C (BS group) and 37 °C (PF group), 120 rpm overnight. Then, the bacteria concentrations were adjusted with sterilised buffer to OD_{595} = 0.13 of BS and 0.10 of PF. After that, the suspensions were inoculated in the King's medium and incubated at 30 °C and 37 °C for BS and PF, respectively, for three days (Glickmann and Dessaux, 1995). In one setup, the King's medium was supplemented with 2.5 mM L-tryptophan (L-Trp) and in another with no supplementation because the bacterial biosynthesis pathways of IAA can be differentiated with and without L-Trp (Glickmann and Dessaux, 1995). After incubation, each sample was centrifuged at 4 °C at 10000 rpm for 20 min. The 100 μL orthophosphoric acid was added to 2 mL of supernatant, and then 4 mL of Salkowski's reagent and 1 mL of 0.5 M $FeCl_3$ solution were added. To prepare the Salkowski's reagent, 0.811 g of anhydrous $FeCl_3$ was dissolved in 10 mL H_2O to gain the 0.5 M solution. A solution of 1 ml of $FeCl_3$ (0.5 M) was mixed with 50 mL $HClO_4$ (35%). Then, the mixture was incubated at room temperature (23 ± 3 °C) without sunlight for 30 min. The intensity of the red colour was measured spectrophotometrically at 530 nm with the uninoculated medium as a reference (Brick et al., 1991). The concentration of IAA produced was calculated from the calibration curve of the IAA standard (Sigma Aldrich, USA).

The ability of the bacteria to dissolve inorganic phosphorus was measured through the molybdenum blue colourimetric method (Murphy and Riley, 1962). BS and PF were incubated in Pikovskaya's broth medium in a shaking incubator at 180 rpm for 12 days at 30 °C and 37 °C, respectively. The 250 mg of tricalcium phosphate was added to the medium and used as an inorganic phosphate source for the bacteria (Maheswar and Sathiyavani, 2012). A 1 ml quantity of each strain was inoculated into Pikovskaya's broth medium. Over the incubation period, the pH value of the culture medium was measured after SBB treatment from 1 to 12 days (Agri et al., 2021; Navprabhjot and Poonam, 2013). The phosphate concentrations in the culture solutions were measured with the molybdenum blue colourimetric method. Phosphorous quantification requires converting the phosphorus to a dissolved orthophosphate. The culture solutions were first taken from the flasks and centrifuged at 4 °C at 5000 rpm for 10 min. Then, to measure phosphorus concentration, 0.2 mL of antimony potassium tartrate and 0.4 mL of ammonium molybdate were added to the acidified supernatants (pH = 4.4) and reacted with the orthophosphate. After that, the concentration of phosphorus present in the suspension was determined against the phosphorus standard curve at 700 nm with a spectrophotometer (PerkinElmer, USA, Lambda 950).

To test for exopolysaccharides production, bacteria were cultivated aerobically in Luria broth (LB) medium to the exponential and stationary growth phases in 250 mL flasks (Dubois and Gilles, 1956). The protocol reported by De Brouwer et al. (2002) was used for exopolysaccharides extraction, except that the extracting solvent was 100 mM NaCl (pH = 7.0) instead of distilled water. Carbohydrates of exopolysaccharides were assayed by the phenol-sulfuric acid method (Dubios and Gilles, 1956).

2.2. Characterization of soil physicochemical properties and enzymatic activities

Soil for the study was collected from Guizhou province (2913′N; 10336′E), China. The depth of the soil sample was 15 mm. The basic properties of the soil are summarised in Table 1. Before being placed in an incubator, 50 g samples of sterilised soil sieved to less than or equal to 2 mm were put into containers. Each soil sample was inoculated with either BS or PF suspension or remained uninoculated as the control group (CK). To prepare the SBB suspensions, the single colony of BS and PF were inoculated in the liquid medium, respectively. The inoculated culture medium was incubated at 30 °C (BS group) and 37 °C (PF group), 120 rpm overnight. The concentration of SBB inoculated was 10^8 CFU mL^{-1}. The volume of SBB suspension was 150 μL, according to the previous study (Reischke et al., 2014). It was determined by serial-dilution cell-plate counts. Each treatment consisted of three replicates.

Before and after the SBB inoculation, IAA contents in the soil were extracted and then measured using Salkowski's assay (Brick et al., 1991). AP was extracted and then measured using the molybdenum blue method (Murphy and Riley, 1962), as mentioned in Section 2.1. Soil total organic

carbon (TOC) and AN were determined using a TOC analyser (TOC-VCPH, Shimadzu, Japan). Next, 5 g air-dried soil was mixed with 25 mL 0.5 M K_2SO_4 solution in a centrifuge tube, and the mixture was shaken in a shaker at 180 rpm and centrifuged at 4 °C at 5000 rpm for 10 min (Schumacher, 2002). The supernatant was filtered with a membrane (0.45 μm) and stored at 4 °C.

UV-visible absorption spectra were measured for the soil extractions using a UV-visible spectrophotometer (Lambda 950, PerkinElmer, USA) with a 1 cm quartz cuvette (Yan et al., 2022). In the spectrophotometer analysis, reference blanks were subtracted from the measured absorption values, and the specific coefficient a_{254} (m^{-1}) was calculated by Eq. (1) (Jamieson et al., 2014):

$$a_{254} = 2.303\frac{A_{254}}{l_L} \quad \text{(Eq. 1)}$$

where 254 is the wavelength, A_{254} is the absorbance value, l_L is the path length of the optical cell in meters (l_L = 0.01 m), and 2.303 is the correction factor. $SUVA_{254}$ (L $mg^{-1}m^{-1}$) was calculated by Eq. (2) (Jamieson et al., 2014):

$$SUVA_{254} = \frac{a_{254}}{TOC} \quad \text{(Eq. 2)}$$

where a_{254} is the absorbance coefficient (m^{-1}) calculated according to Eq. (1), and TOC is the corresponding total organic carbon concentration (mg L^{-1}).

Soil microbial mass carbon (MBC) and microbial mass nitrogen were estimated by the chloroform fumigation-extraction method using alcohol-free $CHCl_3$ (Brookes et al., 1985; Wu et al., 1990). The AM was determined calorimetrically utilising Nessler's reagent (Mcmullan, 1971). The pH and electrical conductivity of the soil were measured using a digital pH-conductivity meter (OAKTON, Singapore, 510) at a mass-to-volume ratio of 1:2.5 and 1:5, respectively (Thomas, 1996). Water loss was calculated based on the weight difference calculations of every container (Barnard et al., 2013). Soil acid phosphatase (SACP) activity was measured through the sodium phenylene phosphate colourimetric method (Hou et al., 2020). The activity of the enzymes was quantified in units of nmol p-nitrophenol produced per gram of soil (U/g). The activity of SURE was measured based on the colourimetric determination of ammonium in soil (Kandeler and Gerber, 1988), and the result was expressed in units of μg ammonium per gram of soil (U/g). Activities of soil catalase and soil dehydrogenase were also measured as the biological indicators of soil health. The spectrophotometric method determined the soil catalase activity (Johansson and Borg, 1988), while Tripheyltetrazolium chloride and iodonitrotetrazolium chloride were used to determine the soil dehydrogenase activity (Friedel et al., 1994). All data were determined in triplicate.

2.3. Bacterial growth in soil

In the experiment, the growth room was set to a 16-h light/8-h dark cycle under LED lamps, with a total light intensity of 79.198 mW/nm, a temperature of 23.5 ± 3 °C and relative humidity of 40 ± 5% (Ng et al., 2021). Soil samples were collected randomly in each replication and air-dried for further analysis.

The different phases of bacterial growth in soil are described as follows. The initial phase is regarded as an adaptation stage, while the following phase is modelled as the exponential phase, lasting until peak activity, based on the Eq. (3) modified by Reischke et al. (2014):

$$Y = Ae^{\mu t} \quad \text{(Eq. 3)}$$

where Y is bacterial growth, A is the biomass growing on the substrate, μ is the intrinsic growth rate, and t is the time (Blagodatsky et al., 2000). The symbol μ can be calculated from the linear slope of the log-transformed data during the exponential phase ("linear approach"). The "linear approach" regression slope of log bacteria growth during the period of increase can be used to represent the bacterial growth rate (log CFU h^{-1}) (Broughall et al., 1983; Nicola and Bååth, 2019). The last growth stage is described as the lag phase (Reischke et al., 2014). However, in this study, without model fitting insufficient data were available to fit the equation with sufficient precision.

2.4. Statistical analysis

The data were processed using Microsoft Excel 16.57 and SPSS 20.0. One-way analysis of variance (ANOVA) with a 95% confidence interval ($p < 0.05$) was used for data analysis (Sun et al., 2020). Principal component analysis (PCA) and the Pearson correlation coefficient analysis were used to analyse the relationship between soil quality and bacterial growth (Harris et al., 2021; Li et al., 2020).

3. Results

3.1. Bacterial characteristics in vitro

IAA secretion, inorganic phosphate solubilisation, and exopolysaccharides production of BS and PF were measured to assess their efficiency in promoting soil quality (Figure 1 and Table S1). It was found that

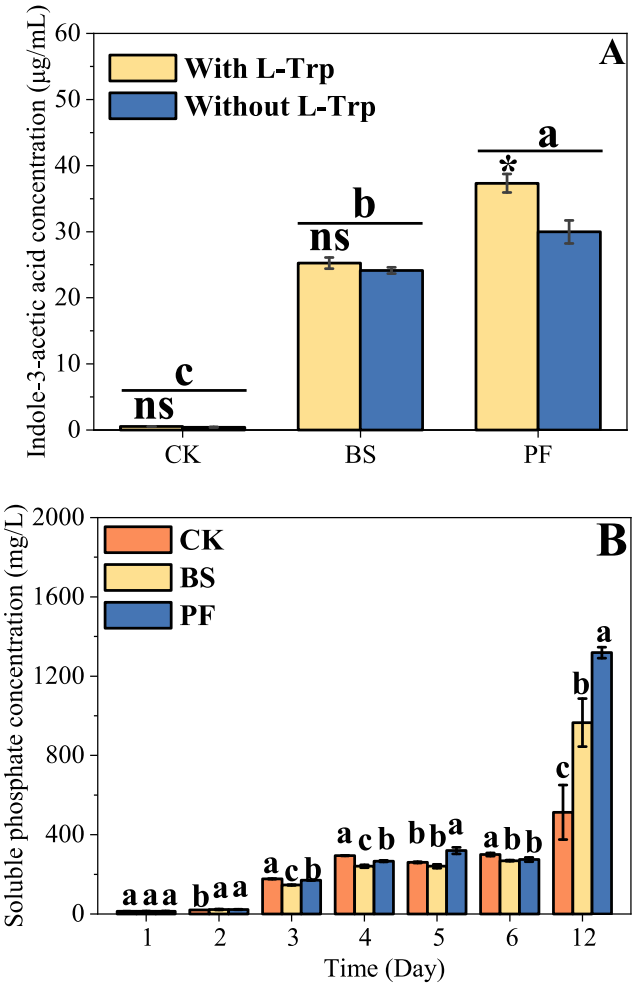

Figure 1. Characterisation of bacterial traits *in vitro* (**A**) bacterial indole-3-acetic acid (IAA) production capability (**B**) bacterial inorganic phosphorus (P) solubilisation capability. L-Trp: L-tryptophan. CK: control. BS: *Bacillus subtilis*. PF: *Pseudomonas fluorescens*. Each result is presented as the mean ± S.D., calculated from three independent samples. * represents a significant difference ($p < 0.05$), and ns represents no significant difference within the group. Letters in each column mean a significant difference ($p < 0.05$) among treatments.

both strains produced auxin IAA and solubilised inorganic phosphate under test conditions (Figure 1). As Figure 1A shows, BS and PF produced the IAA with and without L-Trp, indicating that both bacteria strains possess the L-Trp dependent and L-Trp independent IAA biosynthesis pathways (Spaepen et al., 2007). The IAA levels produced by BS were 23 μg/mL with L-Trp and 22 μg/mL without L-Trp. No significant differences were observed within the BS treatment group, which suggests that L-Trp has no significant influence on IAA production in BS (Koua et al., 2020). In contrast, the IAA secreted by PF was 25% higher in the group with L-Trp than without L-Trp ($p < 0.05$), suggesting the addition of L-Trp contributes to the IAA production of PF significantly. Therefore, these results may imply that the biosynthesis pathways of IAA in BS and PF probably differ during the stationary growth phase, and the presence of L-Trp potentially enhanced IAA secretion in PF (Chaiham et al., 2011).

The BS and PF also showed inorganic phosphate solubilisation capabilities (Figure 1B), with a steady but significant rise seen in the concentrations of soluble phosphate in the BS and PF *in vitro* tests over 12 days. From 6 to 12 days, in particular, there was a sharp rise in phosphate concentrations in the BS and PF treatments, and the maximum inorganic phosphate solubility of PF (1318 μg/mL) was 37% higher than that of the BS (965 μg/mL). It is also noteworthy that the current study exhibited much greater phosphate solubilisation capabilities for BS and PF than previous studies. Shakeel et al. (2015) and Zaidi et al. (2006) assessed various *Bacillus* species and found that the phosphorus nutrients solubilised by these strains only ranged from 38 to 90 μg/mL. Moreover, Mukhtar et al. (2017) found that the maximum phosphate solubilisation capability of *Bacillus* strains in Pikovskaya's broth medium could reach 306 μg/mL, which is much lower than the values in this study.

With the increase of soluble phosphate concentrations, the pH values would be expected to decrease due to the release of hydrogen ions. The variation in pH did indeed show lower pH levels in the SBB treatments than in CK after 1-day treatment (Fig. S1). Among all treatments, the BS group showed the lowest pH values after 1-day treatment. The bacterial activity of BS is likely to be inhibited greater than that of PF (Ni et al., 2017; Yang et al., 2016). Consequently, the capability of acid production in BS-treated samples may become weaker, which probably causes a larger decline in the total amount of acid than in PF-treated samples. Thus, the pH in BS treatment was higher than that in PF treatment after 2-day incubation (Fig. S1). Furthermore, the total amount of the acid is less important for P-solubilization than the quality of acids produced by phosphate-solubilising microorganisms (Scervino et al., 2010a). The inorganic phosphate may be solubilised continuously even with larger increased pH in BS-treated samples (Figure 1B). Additionally, the inorganic phosphate in the BS-treated group may be solubilised by chelation and redox reduction other than the acidification process (Altomare et al., 1999; Tian et al., 2021; Khan et al., 2014). Therefore, the BS group showed a similar phosphate concentration to the PF group and a higher pH value than the PF group after 2-day incubation. With the incubation time increased to 12 days, the pH of the BS and PF groups increased. It may be due to the inhabitation of the bacterial capabilities to produce organic acid. It is widely accepted that bacteria secreted organic acids to solubilise the inorganic phosphate (Khan et al., 2014; Hakim et al., 2021). However, with the pH decreasing to a low level at the beginning of incubation, bacterial activity is likely to be suppressed (Ni et al., 2017). Therefore, the capability of organic acid secretion probably is inhibited with the incubation time increased to 12 days, which may cause a decrease in the total amount of acid and the pH increase in the culture medium. This is consistent with a previous study reported by Yang et al. (2016). They also observed an increase in pH values in two different culture mediums after 12 days of incubation. During the solubilisation process, the inorganic phosphate can be solubilised by mechanisms besides the acidification process, such as the chelation process and the reduction process (Altomare et al., 1999; Tian et al., 2021; Khan et al., 2014). Therefore, although the amount of acid produced by SBB is decreased, there still have a great possibility of an increase in the concentration of soluble phosphate.

In contrast to the excellent IAA secretion and inorganic phosphate solubilisation capabilities shown by BS and PF, the concentration of EPS decreased by 23% and 56% in BS and PF treatments, respectively (Table S1). The decrease in the concentration of EPS may result from the weak EPS production capability of SBB in the current study and the biodegradation of EPS by bacteria. The production of EPS is affected by many factors, including bacteria species, growth phase, roles of nutrients, and other abiotic elements (More et al., 2014). The dominant components of EPS are carbohydrates and can be used by bacteria as a source of carbon and energy (Mehta et al., 2021). Therefore, EPS in the current study may be biodegraded by bacteria for their metabolic activity (Zhang and Bishop, 2003). *Bacillus* spp. and *Pseudomonas* spp. have been reported as excellent EPS-producing hosts, but the characterisation and determination of specific strains are still limited (Jautzus et al., 2022; Lian et al., 2022). Thus, the screening of the *Bacillus* strain and *Pseudomonas* strain to produce EPS needs further attention.

3.2. Change of soil physicochemical properties with SBB amendment

To explore how the bulk soil quality responds to SBB, soil properties (pH, electrical conductivity, and water content) and fertility indicators (IAA, AP, TOC, MBC, $SUVA_{254}$, AN, and AM, and microbial biomass nitrogen) were measured (Figure 2 and Table S2). The content of IAA decreased slightly after SBB addition, from 0.09 to 0.08 μg/g (Figure 2A). The AP in the SBB-amended soil showed a very small increase with insignificant differences among treatments (Figure 2B). The variations of pH and electrical conductivity are closely related to the AP transformation. They showed a slight increase without significant difference compared with the previous study (Table S2), whereas TOC values of over six times those reported by Ju et al. (2020) were observed. This suggests that relatively higher bioavailable carbon contents were present in the bulk soil than in rhizosphere soil (Figure 2C). After inoculating BS and PF, TOC in the bulk soil showed a slight variation, averaging 101 μg/g. Still, there were no differences between CK and the inoculated treatments. Not only were the soil carbon contents affected, but their components were also altered, which was evident from the increases in MBC and $SUVA_{254}$. The MBC contents were significantly higher in the BS and PF treatments, at an average of 26 μg/g and 34 μg/g ($p < 0.05$), which were 40% and 88% higher than those of CK, respectively (Figure 2C). The soil inoculated with BS and PF also exhibited $SUVA_{254}$ values 73% and 78% larger than those of CK, respectively ($p < 0.01$) (Figure 2C). Moreover, significant increases were observed for AN and AM (Figure 2D). The upregulation of AN was evident in the BS and PF treatments relative to CK, averaging an increase of 34% and 17%, respectively. Compared with the results of rhizosphere soil (125 mg/kg) (Li et al., 2020), the AN observed in the BS and PF treatments was lower by 64% and 69%, respectively. Significant differences were also found for AM in both the BS and PF treatments ($p < 0.05$). As shown in Figure 2D, the inoculation of BS and PF increased AM by 57% and 46% relative to that of CK. Thus, bulk soil nutrients were altered significantly with the SBB amendment.

3.3. Change of soil enzymatic activity with SBB amendment

The activity of SACP, soil catalase, SURE, and soil dehydrogenase was measured, as shown in Figure 3 and Table S2. The changes resulting from SACP were distinctly different between the BS and PF treatments (Figure 3A). BS amendment decreased the SACP activity to 5904 U/g. In contrast, the PF amendment showed no difference to the CK, averaging 6416 U/g. In contrast, the activity of soil catalase varied with SBB growth (Figure 3B). Although the values in the bulk soil showed no significant change with or without SBB, the levels of soil catalase activity were consistent with a previous study of rhizosphere soil (Cao et al., 2016). Meanwhile, the SURE activity increased from an average value of 59 U/g for CK to 112 U/g with the BS and 101 U/g with the PF treatment ($p < 0.01$) (Figure 3C), nearly 90% and 70% higher than CK, respectively. The

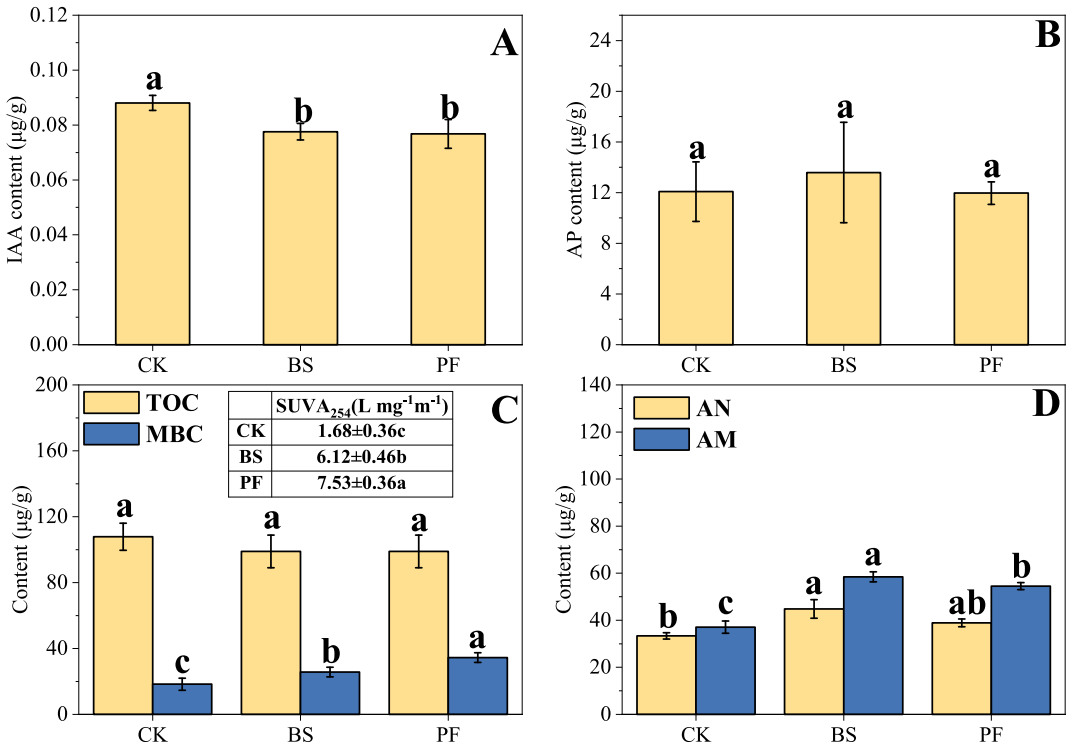

Figure 2. Effects of beneficial bacteria on soil physicochemical properties (**A**) indole-3-acetic acid (IAA) content (**B**) soil bioavailable phosphate (AP) content (**C**) carbon content (**D**) nitrogen content. TOC: total organic carbon. MBC: microbial biomass carbon. $SUVA_{254}$: specific ultraviolet absorbance at 254 nm. AN: soil bioavailable nitrogen. AM: soil ammonium. CK: control. BS: *Bacillus subtilis* treatment. PF: *Pseudomonas fluorescens* treatment. Each result is presented as the mean ± S.D., calculated from three independent samples. Letters in each column mean a significant difference ($p < 0.05$) among treatments.

increased SURE activity here means that the cultivation of BS and PF helped the catalysation produce AM. As shown in Figure 2D, the soil inoculated with BS and PF exhibited 57% and 46% greater AM contents relative to CK. The average SURE activities were similar to that in rhizosphere soil (Wei et al., 2020) (Figure 3C). Finally, the soil dehydrogenase activity showed no significant differences from CK (Table S2), indicating the negative disturbances in the current deconstructed soil systems were minor. These results suggest that the SURE activity was most affected by the SBB amendment.

3.4. Bacterial growth in soil

Whether the change of nutrients in the bulk soil would be affected by bacterial growth during incubation was further studied. Bacterial growth in this work showed three unique phases (decrease phase, exponential growth phase, and steady-state phase (Ansari and Ahmad, 2019)) (Figure 4), and the population sizes of both the BS and PF strains were larger than those in rhizosphere soil (Samaras et al., 2021). The trends for both species were similar, but the responses of the bacterial population were significantly different during all three phases. For PF, the first phase began in the first 18 h, while it took longer for BS to reach the first phase, between 0 to 45 h. The log relative bacterial growth of PF decreased by 45% to 0.55 log CFU. In comparison, the log relative bacterial growth of BS was only reduced by 22% to 0.78 log CFU, suggesting more abundant BS colonies than PF colonies survived in the first phase. The more robust adaptability of BS was likely due to spore formation, a typical resistant cell structure, causing them to be more adaptable to a new environment (Augé, 2001; Meisner et al., 2018). These differences were also evident in the change of MBC in the bulk soil (Figure 2C). BS consumed more carbon resources than PF. Thus, fewer carbon compounds were conserved in the BS colonies.

Following the initial decrease phase, bacterial growth moved to the exponential growth phase (EGP). Based on the log-transformed data, exponential growth could be observed until peak activity at 90 h (PF) and

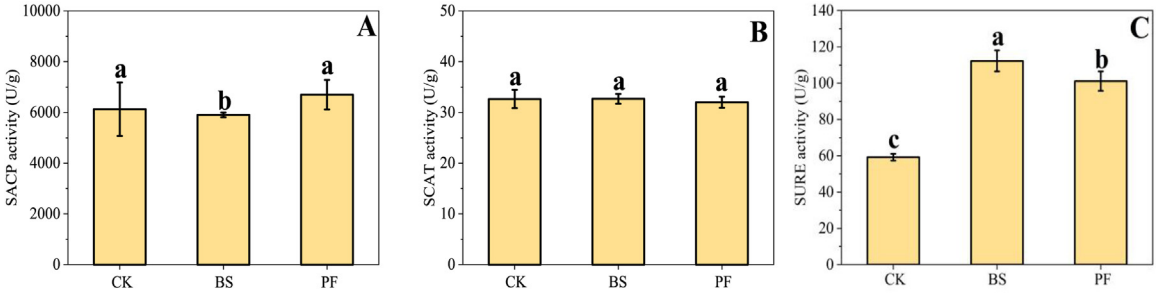

Figure 3. Effects of beneficial bacteria on soil enzymatic activities (**A**) soil acid phosphatase (SACP) activity (**B**) soil catalase (SCAT) activity (**C**) soil urease (SURE) activity. CK: control. BS: *Bacillus subtilis* treatment. PF: *Pseudomonas fluorescens* treatment. Each result is presented as the mean ± S.D., calculated from three independent samples. Letters in each column mean a significant difference ($p < 0.05$) among treatments.

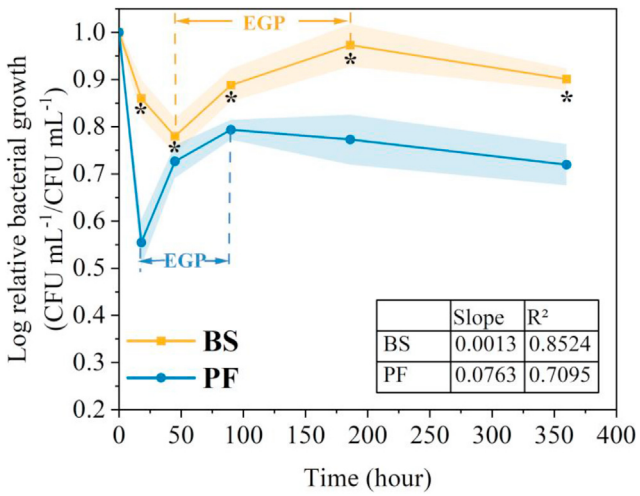

Figure 4. Log relative growth of two bacteria strains (as a colony-forming unit at each time point/colony-forming unit at the beginning) during cultivation in soil. The parameters in the table represent the linear fitting for the exponential growth phase (EGP) (BS) *Bacillus subtilis* (PF) *Pseudomonas fluorescens*. Each result is presented as the mean ± S.D., calculated from three independent samples. * represents a significant difference between BS and FP treatments at each time point ($p < 0.05$).

186 h (BS). PF showed larger slope values of 0.0763 log CFU h^{-1}, $R^2 = 0.71$ than BS (0.0013 log CFU h^{-1}, $R^2 = 0.85$), revealing that PF grew faster than BS during this phase.

Finally, the third phase, steady-state, was observed. Although bacterial activity was limited, showing less significant variations in population sizes, BS still reached 0.90 log CFU, significantly larger than PF (0.72 log CFU) (Figure 4). This result corresponds with the survival ability of the bacteria strains during the first phase (0–45 h). Overall, BS grew better than PF as it had a higher population, and PF grew faster during the EGP.

4. Discussion

4.1. Characterization of bacterial traits in vitro

Bacteria utilise the L-Trp released in a natural soil environment via degrading microbial cells (Duca et al., 2013). The catalysation of L-Trp by decarboxylase produces tyramine which is further converted to indole-3-acetaldehyde, a process mediated in IAA biosynthesis (Spaepen et al., 2007). Biosynthesised IAA is a signalling molecule involved in bacterial communication and directly affects physiology (Lin et al., 2022). In bacteria, the biosynthesis of IAA during the stationary growth phase is divided into different pathways based on the participation/non-participation of L-Trp (Spaepen et al., 2007). The presence of L-Trp correlates with the higher IAA secretion for PF (Figure 1A), indicating PF used L-Trp as the precursor, leading to higher IAA productivity (Islam et al., 2013)(I.

Different to PF, L-Trp was not a precursor in the IAA biosynthesis of BS, as was evident from the lack of significant differences observed between the groups with and without L-Trp *in vitro* (Figure 1A). This is inconsistent with the 5 mM addition of tryptophan contributing to a fivefold increase in IAA production for *Bacillus* strains found in the study by Idris et al. (2007). These differences are partly due to the intrinsic diversity of regulations across IAA biosynthesis pathways and bacterial species (Lin et al., 2022).

Apart from auxin IAA production capabilities, inorganic phosphate solubilisation of BS and PF was also observed *in vitro*. The phosphate concentrations were in the order of PF > BS > CK (Figure 1B) after 12-day incubation, implying that SBB activities had mobilised inorganic phosphate in the substrate after 12-day treatment. Thus, PF and BS are promising candidates for improving soil phosphorus transformation efficiency. The more efficient inorganic phosphate solubilisation with SBB in the rhizosphere soil can be explained by the phosphorus regulons, such as phosphatases (e.g., SACP), functional proteins, and phosphate depletion (Wei et al., 2019). The above observations indicate that L-Trp and phosphate could be the critical determinants in differentiating the application of BS and PF for improving IAA contents and phosphate bioavailability. This provides a foundation for selecting bacteria strains based on initial soil L-Trp and inorganic phosphate conditions.

4.2. Effects of SBB growth on soil physicochemical properties

As discussed in the previous Section 4.1, BS and PF have excellent inorganic phosphate solubilisation ability *in vitro* (Figure 1). It was expected that the AP contents in the bulk soil with SBB treatments would show significant increases, but the results were not different between CK and the SBB treatments (Figure 2B). Few previous studies have observed this phenomenon in rhizosphere soil with SBB amendment. The difference in soil available phosphate contents between this work and Li et al. (2020) may be due to the low transform efficiency of soil phosphate in the deconstructed microcosm. The concentration of soil available phosphate is controlled by chemical and biological processes, which fix and release orthophosphate ions through complex catalysation within soil substrates (Toro et al., 1997). Soil soluble phosphatase secreted by bacteria plays a fundamental role in inorganic phosphate recycling due to soil organisms only assimilating dissolved phosphate (Grillo-Puertas et al., 2021). Thus, the lower response of soil phosphatase to SBB in the bulk soil is assumed to result in the inorganic phosphate solubilisation processes being unobservable. While in the soil system used by Li et al. (2020), the phosphate consumption is expected to be greater than the bulk soil. Therefore, the response of soil available phosphate contents might be more obvious than this work.

Additionally, this study showed that the SBB significantly increased the bulk soil MBC, indicating that the microbial activities were higher relative to CK. This significant result was particularly evident in the PF treatments (Figure 2C). The lack of significant variation of TOC after SBB inoculation implied that the carbon source was not a limiting factor for microbial activity in the separated bulk soil. Instead, microbial activity might be nitrogen-limited (Schimel and Weintraub, 2003). According to the results for MBC and TOC, the bacteria consumed carbohydrate substances with an increase in microbial activity. Still, there was a sufficient supply of carbon compounds left. It is speculated that the readily biodegradable organic matter was the first to be assimilated by the bacteria, and reluctant components accumulated in the environment. As a result, the aromaticity of the organic matter increased (Hu et al., 2021; McLeod et al., 2021). The $SUVA_{254}$ index can describe the aromaticity of dissolved organic carbon compounds in the soil (Hu et al., 2021). Consistent with the assumption about the organic matter, the $SUVA_{254}$ of the SBB treatments increased significantly relative to the CK (Figure 2C), indicating that the aromatic compounds accumulated gradually. These observations appear to support the interpretation that the components of the soil, primarily the biodegradable organic matter, were changed by the SBB. Additionally, the upregulation of nitrogen nutrients with SBB amendment (Figure 2D) suggests enhanced nitrogen transformation. SBB can promote the fixation of free atmospheric nitrogen or conversion of urea with associated enzymes (Nadeem et al., 2014), highlighting the promise of using SBB.

4.3. Effects of SBB growth on soil enzymatic activities

This study further investigated whether soil enzymatic activities contributed to the nutrient change in the bulk soil. As assumed in Section 4.2, the soil phosphatase activity was not different between the CK and PF groups. But it was lower for the BS groups (Figure 3A). It is known that the production of SACP depends on the combination of AP demand, the content of available inorganic phosphate substrate, and the phosphorus limitation in the soil (Toro et al., 1997). An extensive set of regulators

encodes this regulatory system in the bacteria, and a sufficient supply of AP in the environment will result in a lack of physiological responses (Grillo-Puertas et al., 2021). One of the most likely reasons for the insignificant change of SACP would be that less phosphate was consumed in the current soil microcosm than in rhizosphere soil. Therefore, the demand for AP here may have been inadequate to motivate the SBB to produce SACP in the bulk soil. Another possibility is that the inorganic phosphate substrate may have been insufficient. As a result, no significant variations in SACP activity were observed. Furthermore, the variations of AP in the bulk soil are consistent with SACP regulating the production of AP (Figure 2B).

The SURE activities also increased significantly in the BS and PF treatments relative to CK (Figure 3C). The rise of soil enzymatic activities can indicate enhanced diverse metabolism based on catalytic biochemical reactions, leading to efficient substrate transformation. For example, soil urease (urea amidohydrolase) is closely related to the conversion, natural turnover, and bioavailability of nitrogen. Further evidence supporting this specific role of SURE in nitrogen fertility improvement is that AM contents in the SBB-treated soil showed an evident increase (Figure 2D), and BS treatment facilitated the release of AM to a greater degree than PF treatment. This is because the population size of BS was larger (Figure 4), leading to higher transformation efficiency. Thus, the SBB amendment impacted soil carbon and nitrogen properties more than phosphate since bacterial growth promoted associated enzymatic activities.

Generally, SURE participates in catalysation that producing AM. This process changes pH due to the net release of hydroxyl ions (Liang et al., 2003). Once the efficiency of SURE decreases, soil acidification caused by free protons released during nitrification worsens (Steven, 2019; Xu et al., 2020). Even for soil with higher pH values, the neutralisation of protons causes hydroxide consumption from general soil alkaline compounds. At the level of the whole soil environment, these changes are almost adverse. Therefore, balanced soil pH conditions are essential. The SBB treatments showed no significant difference in soil pH values over CK (Table S2). However, their advantage in balancing soil pH becomes apparent in their improvement of nitrogen fertility. Thus, it is demonstrated that SBB amendment is a way to enhance bulk soil nitrogen fertility by improving SURE activity whilst having only a slightly adverse effect on pH conditions. To protect the natural biochemical cycles of soil, applying BS and PF could thus be a more sustainable approach than chemical fertiliser.

4.4. Relationship of soil physicochemical properties and enzymatic activities to SBB growth

To reveal the interaction between bulk soil and SBB amendment, PCA (Figure 5 and Fig. S2) and Pearson correlation coefficient analysis (Figure 6) were conducted. Remarkable differences were observed between CK and the BS and PF treatments, as shown by the 95% confidence ellipse shadow in Figure 5. The results of the BS treatment are clustered in the blue circle, with those of the PF treatment in the orange circle. The results of both are far away from the results of CK in the yellow ellipse shadow. The first two components in the PCA results (PC1 and PC2) can explain 50% and 22% of the variance. SBB, AM, MBC, $SUVA_{254}$, and SURE show a strong positive relationship along PC1, and SBB growth is the dominant factor influencing soil quality, accounting for half the variability. This verifies the significant contribution of SBB evident from the measurements of the soil AM and MBC contents, $SUVA_{254}$ index, and SURE activity (Figures 2 and 3). It is also consistent with the significant correlation demonstrated by the Pearson correlation coefficient analysis (Figure 6). In contrast, soil-dissolved organic matter and bacterial growth were negatively correlated, which is evident from the negative relationship between SBB and TOC.

SURE was the only enzyme with a significant positive correlation with soil carbon and nitrogen contents (MBC, $SUVA_{254}$, and AM) among all measured soil enzyme categories. Yet almost no previous studies have

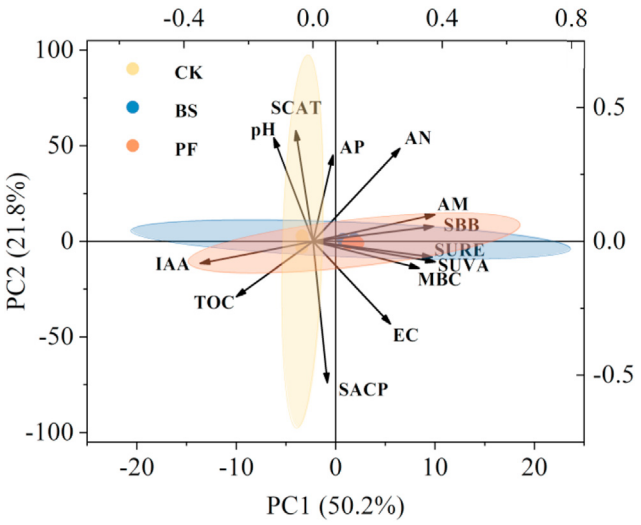

Figure 5. Principal component analysis describing the relationship between soil quality and beneficial bacterial growth. Yellow, blue, and orange shadows are 95% confidence ellipses for CK, BS, and PF treatments, respectively. EC: electrical conductivity. IAA: indole-3-acetic acid. AP: soil bioavailable phosphate. TOC: total organic carbon. MBC: microbial biomass carbon. SUVA: specific ultraviolet absorbance. AN: soil bioavailable nitrogen. AM: soil ammonium. SACP: soil acid phosphatase. SCAT: soil catalase. SURE: soil urease. SBB: soil beneficial bacteria. CK: control. BS: *Bacillus subtilis* treatment. PF: *Pseudomonas fluorescens* treatment.

explicitly illustrated a strong positive correlation between SURE and MBC. Generally, soil MBC can represent soil microbial activity since the such activity requires a good soil carbon supply (Gan et al., 2021). Thus, the more vital growth ability of BS made a greater contribution to SURE activity enhancement at the expense of more significant assimilation in the bulk soil MBC (Figures 2C and 4). In comparison, the activities of SACP and soil catalase were much less related to soil properties, and their variations in response to SBB were even minor. A negative correlation between SACP and soil catalase was also observed (Figure 5). Previous reports have shown this is most likely because soil catalase is an exoenzyme. Thus, it can be more stable due to its sorption on soil mineral

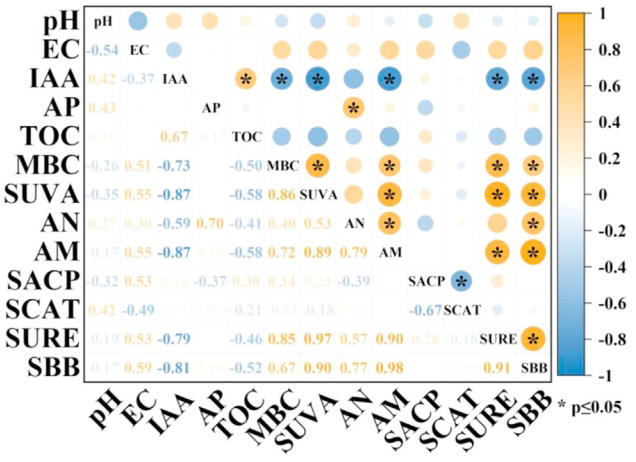

Figure 6. Pearson correlation coefficient analysis plot describing the relationship between soil quality and soil beneficial bacteria. The colour bar shows that the correlation ranges from -1 to 1. Values closer to zero mean a less linear trend between the two variables. EC: electrical conductivity. IAA: indole-3-acetic acid. AP: soil bioavailable phosphate. TOC: total organic carbon. MBC: microbial biomass carbon. SUVA: specific ultraviolet absorbance. AN: soil bioavailable nitrogen. AM: soil ammonium. SACP: soil acid phosphatase. SCAT: soil catalase. SURE: soil urease. SBB: soil beneficial bacteria.

phosphate components, the substrate for the biochemical reactions involved in SACP (Li et al., 2020). Except for the catalysation function during phosphate mobilisation, the SACP activity can act as a soil pH adjustment indicator (Dick et al., 2000). Even though no correlation between SACP and pH from Pearson correlation coefficient analysis is observed (Figure 6), nor are there significant differences in pH values among treatments before and after SBB inoculation (Table S2), these slight variations in soil pH can still benefit soil health by contributing to nutrient recycling and slowing down soil acidification.

The PCA loading plots in Fig. S2 clearly show an SACP and AP relationship change. Without the SBB amendment, nearly no correlation between SACP and AP can be observed. However, with BS or PF growth, SACP and AP show a strong positive relationship along PC1 (Fig. S2B and S2C), implying the potential impact of SBB on soil AP content through mediating SACP activities (Li et al., 2020). As expected, the components of the soil carbon were changed by the SBB due to the strong positive relationship between $SUVA_{254}$ and MBC, in line with the discussion in Section 4.2. PC2 reveals that a higher proportion of SACP and soil catalase variation was associated with pH, electrical conductivity, and TOC changes. According to Brucker et al. (2020), the microbial solubilisation of phosphorus from apatite is limited by the availability of easily decomposable carbon, indicating that SACP is most likely affected by TOC.

Overall, SURE activity depends primarily on microbial activity and affects nitrogen recycling. Previous research regarding linkages between enzymatic activities and rhizosphere soil nutrients has only focused on enzyme effects (Cao et al., 2016; Li et al., 2020; Wei et al., 2020) and the appreciation of the relationship between soil enzymatic activities and SBB growth, particularly bacterial population size, is strikingly limited. It is understood that soil enzymatic activities primarily affect their corresponding soil physicochemical properties (e.g., phosphatase and AP). However, the results in this study, such as the SURE and MBC relationship, show that soil enzymatic activities can be closely related to the biological process of bacterial growth.

5. Conclusions

This work reveals the effects of SBB growth on bulk soil quality. Specifically, these effects are reflected in nitrogen fertility changes regulated by soil enzymatic activities. For bulk soil in a reconstructed microcosm, BS treatment corresponded to an increase in AN and AM contents of 34% and 57% and PF treatment to an increase of 17% and 46%, relative to CK. This was driven by an enhancement in SURE activity, with an increase of 90% and 71% from the BS and PF treatments. The difference in SURE activity between the BS and PF treatments can be ascribed to the greater bacterial abundance of BS than PF. Meanwhile, variations in soil pH conditions showed no significant differences over CK, indicating little adverse effect on soil health from the treatments. The relationship of SBB and soil quality revealed by PCA and Pearson correlation coefficient analysis also confirmed that the contribution of bacterial growth was significant. This was evident in the significant positive correlation between MBC and SURE ($p < 0.05$). The ecological observations and statistical analysis results highlighted that enhancing soil enzymatic activities due to a larger bacterial population is critical in an SBB-soil communication network. These results provide a promising ecological strategy for developing the application of SBB on land types other than rhizosphere soil. Soil samples used in this study were collected from a traditional Chinese medicinal planting site in Guizhou province, Southwest China. Therefore, the results suggest that the SBB amendment has a large potential to improve the quality of agricultural soil planting with herbal plants. For other soil types, there is still a need for further investigation. Nowadays, the SBB amendment can be produced as powder and is rehydrated before spraying using farm-scale technologies (Okon et al., 2015). The dose of bacteria power was applied at 4 g/ha (1×10^{10} CFU g^{-1}) in the crop field (Dal Cortivo et al., 2017). For the bulk soil in this study, the quantity of bacterial amendment is larger (~65.7 g/ha). Actually, the dosage of the SBB amendment needed depends on the field site situation (e.g., soil dry density, soil depth, abiotic and biotic factors and so on) (Renoud et al., 2022) Thus, the restoration of bulk soil in the field with the SBB amendment still needs further investigation.

Declarations

Author contribution statement

Charles Wang Wai NG; Karl Wah Keung TSIM: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Wen Hui YAN: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pui San SO; Yi Teng XIA: Conceived and designed the experiments; Analyzed and interpreted the data.

Chun Ting TO: Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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