Phyton-
International Journal of
Experimental Botany

Tech Science Press

http://doi.org/10.32604/phyton.2026.084016

ARTICLE

Effect of Nitrogen-Fixing Bacteria on Rhizosphere Nitrogen Forms and Bulb Medicinal Components of *Fritillaria taipaiensis* P. Y. Li

Zhenyu Liu[1], Panpan Wang[1], Yanjing Liu[1], Mingyan Ye[1,2], You Zhou[1], Dongqin Guo[3,*] and Nong Zhou[1,*]

[1]College of Chongqing of Biology and Food Engineering, Chongqing Sanxia University of Science and Technology, Chongqing, China
[2]College of Agriculture and Forestry Science and Technology, Chongqing Three Gorges Vocational College, Chongqing, China
[3]School of Pharmacy, Chongqing Three Gorges Medical College, Chongqing, China
*Corresponding Authors: Dongqin Guo. Email: guodongqin1997@163.com; Nong Zhou. Email: erhaizn@126.com

Received: 15 April 2026; Accepted: 15 July 2026; Published: 28 August 2026

ABSTRACT: Purpose: This study aimed to investigate the effects of nitrogen-fixing bacteria (NFB) on the nitrogen forms in rhizosphere soil and the biomass and medicinal components in the bulbs of *Fritillaria taipaiensis*. **Methods:** Three NFB strains were isolated and inoculated into the rhizosphere of *F. taipaiensis* through a pot experiment. Eight treatment groups were established: control (CK), *Rahnella aquatilis* (N1), *Pseudomonas chlororaphis* (N2), *Paenibacillus stellifer* (N3), *R. aquatilis* + *P. chlororaphis* (N4), *R. aquatilis* + *P. stellifer* (N5), *P. chlororaphis* + *P. stellifer* (N6), and *R. aquatilis* + *P. chlororaphis* + *P. stellifer* (N7). After 8 months of inoculation, the contents of different nitrogen forms in rhizosphere soil and peimine, peiminine, peimisine, sipeimine, and sipeimine glycosides in bulbs were measured. The variations in medicinal components and nitrogen forms were analyzed, and the correlations between bulb alkaloids and nitrogen forms were investigated through principal component and correlation analyses. **Results:** The contents of ammonium nitrogen, nitrate nitrogen, and amide nitrogen significantly increased in the rhizosphere in seven treatment groups compared with the CK group. The bulb biomass increased to varying degrees under all treatments, with the most pronounced effects observed in the N5 group. Similarly, the total alkaloid content in the bulbs was significantly enhanced by NFB inoculation. The total alkaloid content in the bulbs increased by 22.92%, 40.45%, 43.42%, 58.51%, 61.36%, 46.47%, and 54.42%, respectively, in the seven treatment groups compared with the CK group. Principal component analysis indicated that the combination of *R. aquatilis* and *P. stellifer* exhibited the strongest enhancement effect on alkaloid content. Correlation analysis revealed that the content of ammonium nitrogen was negatively correlated with the content of nitrate nitrogen but positively correlated with amide nitrogen content, fresh weight, dry weight, and total alkaloid content. Nitrate nitrogen content showed negative correlations with the contents of total alkaloids, sipeimine, and sipeimine glycosides, and positive correlations with the contents of amide nitrogen, peimine, peiminine, and peimisine. Amide nitrogen content was negatively correlated with sipeimine content. Fresh weight was positively correlated with total alkaloid content. Peiminine content exhibited a negative correlation with sipeimine content but positive correlations with the contents of peimisine and sipeimine glycosides. **Conclusions:** Inoculation with NFB effectively enhanced the contents of nitrogen forms in the rhizosphere soil of *F. taipaiensis*, as well as the biomass and medicinal components in the bulbs. The most pronounced effects were observed in the treatment group inoculated with *R. aquatilis* and *P. stellifer*.

KEYWORDS: Alkaloids; biomass; *Fritillaria taipaiensis* P. Y. Li; microbial fertilizer; nitrogen-fixing bacteria; soil nitrogen form

1 Introduction

Fritillaria taipaiensis is a perennial herb belonging to the family Liliaceae. Its bulbs contain multiple alkaloids, which can cool down body temperature, relieve cough and asthma, and resolve phlegm [1]. As a nationally protected Grade II endangered medicinal plant and an important botanical source of traditional Chinese medicine Chuan Bei Mu, the bulb alkaloid content of *F. taipaiensis* serves as the core indicator for evaluating its medicinal quality [2,3]. Nitrogen application can significantly increase the mass fractions of alkaloids, such as peimine, peiminine, and peimisine, in *Fritillaria* bulbs, effectively promoting the accumulation of total alkaloids while also markedly improving yields [4,5].

Nitrogen is a core macronutrient essential for plant growth and development. It also serves as a key component of important cellular structures such as proteins, nucleic acids, and chlorophyll in plants, as well as an important constituent of numerous plant secondary metabolites [6–9]. Soil nitrogen status is an important marker of soil health and crop nutrition [10], with soil inorganic nitrogen content being the core evaluation index of farmland nutrient management, directly affecting crop growth, yield formation, and quality improvement [11]. Ammonium nitrogen, nitrate nitrogen, and amide nitrogen are the main forms of plant-available nitrogen in soil, and the nitrogen form is a vital factor affecting plant growth [12]. Different nitrogen forms exhibit distinct biological functions in the plant–soil system due to the differences in their chemical properties and plant absorption mechanisms. Amide nitrogen, which is mainly formed via the conversion of ammonium nitrogen, serves as a key intermediate linking inorganic and organic nitrogen pools. It is a relatively stable nitrogen form for storage and transport in plants, which can effectively reduce nitrogen loss and improve nitrogen use efficiency [13]. Ammonium nitrogen can be directly absorbed and used by plants without prior reduction [14]. Its supply can promote auxin accumulation in plant roots and regulate root branching and elongation, thereby enhancing the plant's uptake efficiency of mineral elements [15]. Moreover, ammonium nitrogen can regulate the expression of genes associated with nitrogen metabolism in plants, influence the biosynthesis of plant secondary metabolites, and directly regulate the accumulation of active components in medicinal plants [13,16]. However, ammonium nitrogen is prone to volatilization loss in alkaline soils, which limits its utilization efficiency in soil [13]. Nitrate nitrogen is the most readily absorbed form of nitrogen for most plants [17]. It is mainly transported to aboveground plant tissues via the xylem to participate in nitrogen metabolism and protein synthesis; it can also serve as a signaling molecule to regulate various plant growth and development processes, including flowering and fruiting [18]. Nevertheless, excessive accumulation of nitrate nitrogen in soil leads to leaching losses, thereby posing potential risks to the aquatic environment [13]. However, the excessive and widespread application of nitrogen fertilizers in medicinal plant cultivation driven by market demand has not only resulted in severe nitrogen loss and soil and water pollution but also led to a significant decline in nitrogen use efficiency during the late stages of crop growth [19].

Nitrogen-fixing bacteria (NFB), as representative rhizosphere plant growth–promoting rhizobacteria (PGPR) and key functional microorganisms responsible for biological nitrogen fixation, are diazotrophic microorganisms capable of colonizing the interior of plant roots and the surrounding rhizosphere [20]. As an important component of microbial fertilizers, NFB play a pivotal role in regulating the soil environment and nutrient structure. On one hand, they effectively increase the content of available nitrogen in rhizosphere soil, optimize the soil nitrogen structural forms by mediating the transformation of soil nitrogen fractions, and improve the overall nitrogen use efficiency of the soil–plant system [21]. On the other hand, they participate in establishing the soil–plant nutrient cycle. Their metabolic activities can modulate the microecology of rhizosphere soil, shape the diversity and structure of nitrogen-fixing microbial communities in the

rhizosphere, and form a benign interactive cycle of "microbial nitrogen transformation–plant nitrogen uptake–rhizosphere microecological regulation" [22].

NFB strains isolated from plant seeds were used in a pot experiment to investigate the microbial mechanisms by which NFB enhance biomass accumulation and medicinal compound production in *F. taipaiensis*. Moreover, the effects of single-strain and consortium NFB inoculations on rhizospheric soil nitrogen content and plant growth parameters were systematically evaluated. This study was designed to achieve three objectives: (1) to analyze the variations in soil nitrogen availability and vegetative growth of *F. taipaiensis* following inoculation with various NFB strains; (2) to explore the responses of bulb biomass accumulation and alkaloid content to NFB treatments; and (3) to elucidate the mechanistic pathways through which NFB inoculation promotes the biosynthesis of medicinal compounds in *F. taipaiensis*. The findings of this study may provide practical references for the application of NFB in *F. taipaiensis* cultivation, lay a theoretical foundation for sustainable herbal medicine production, and offer novel insights into strategies for improving the quality of traditional Chinese medicinal materials.

2 Materials and Methods

2.1 Site Description

The present study was conducted in the Hongchiba Scenic Area (31°38′8″ N, 108°56′30″ E), Wuxi, Chongqing, China, at an altitude of 1800–2500 m. The site features an annual average temperature below 17°C (with an actual annual average temperature of approximately 7.4°C), a temperate climate, a flat dam within a karst trough valley, an average snow-cover period of 80 days throughout the year, and an annual average precipitation of about 1340 mm. The soil used for the pot experiment consisted of yellow soil, river sand, and organic fertilizer (2:1:1), taken from Chongqing Three Gorges University (108°27′23″ N, 30°45′22″ E). The basic physical and chemical properties of soil are shown in Table 1. The soil used in the experiment was autoclaved at 121°C for 30 min, allowed to cool, and then stored for subsequent use.

Table 1: Basic physical and chemical properties of the soil used in this study.

pH	Organic Matter ($g \cdot kg^{-1}$)	Total Nitrogen ($g \cdot kg^{-1}$)	Readily Available Nitrogen ($mg \cdot kg^{-1}$)	Readily Available Phosphorus ($mg \cdot kg^{-1}$)	Readily Available Potassium ($mg \cdot kg^{-1}$)
7.52	43.36	0.33	37.88	22.29	288.34

2.2 Experimental Design

F. taipaiensis bulbs provided by Green Planting and Deep Processing of Genuine Medicinal Materials in Three Gorges Reservoir Area, Chongqing Engineering Lab., Chongqing Sanxia University of Science and Technology, were cultivated in 1.5-gallon plastic pots with an outer diameter of 19.8 cm, a base diameter of 18 cm, and a depth of 20 cm. Prior to use, the pots were disinfected with 75% ethanol and air-dried naturally. In October 2022, uniformly sized, high-quality 3-year-old *F. taipaiensis* bulbs were selected and transplanted into the prepared pots for the experiment, with five bulbs per pot (total fresh weight of approximately 1.5 g).

The strains used in this experiment were isolated in the early stage [4]. Three dominant nitrogen-fixing strains, namely *Rahnella aquatilis*, *Pseudomonas chlororaphis*, and *Paenibacillus stellifer*, were screened through preliminary evaluation of nitrogen fixation potential, growth-promoting traits, and comprehensive assessment of NFB. In March 2023, each bacterial strain was individually inoculated into a sterile beef extract–peptone liquid medium and cultured at 180 rpm for 2 days. The bacterial cultures were then diluted with sterile water to adjust the bacterial concentration to approximately 1×10^8 $CFU \cdot mL^{-1}$. Subsequently,

the bacterial fertilizer was applied directly to the root zone of *F. taipaiensis* plants via a drenching method under controlled conditions at 18°C. After a 40-day incubation period, the bulb samples of *F. taipaiensis* were harvested for subsequent analysis. A total of eight distinct treatments were established in this experiment (Table 2).

Table 2: Experimental design.

Treatments	Strain	Inoculation Measurement
N1	*Rahnella aquatilis*	120 mL/strain
N2	*Pseudomonas chlororaphis*	120 mL/strain
N3	*Paenibacillus stellifer*	120 mL/strain
N4	*R. aquatilis* + *P. chlororaphis*	60 mL/strain
N5	*R. aquatilis* + *P. stellifer*	60 mL/strain
N6	*P. chlororaphis* + *P. stellifer*	60 mL/strain
N7	*R. aquatilis* + *P. chlororaphis* + *P. stellifer*	40 mL/strain
CK	sterile water	120 mL

2.3 Index Measurement

2.3.1 Determination of Biomass in the Bulbs of F. taipaiensis

On 15 June 2023, the bulbs of treated *F. taipaiensis* were harvested and placed in an insulated box at 4°C for temporary storage. The fresh bulbs of *F. taipaiensis* were washed, dried for epidermal moisture, and weighed. The weight of the initially sown bulbs was subtracted and counted as the fresh weight of the herb. The washed *F. taipaiensis* bulbs were dried in an oven at 35°C to a constant mass. The dry weight was calculated as the dry weight of the herbs, with five plants as a treatment group. Each treatment group was repeated three times to calculate the drying rate. The dried bulbs were ground to a powder, sieved through a 100-mesh sieve, and set aside for later use.

$$W_{\text{fresh}} = W_{\text{harvest}} - W_{\text{sowing}} \tag{1}$$

$$R_{\text{drying}} = W_{\text{dry}} / W_{\text{fresh}} \tag{2}$$

where W_{fresh} is the fresh weight of bulb herbs, W_{harvest} is the weight of bulb herbs at harvest, W_{sowing} is the weight of bulb herbs at sowing, R_{drying} is the drying rate, and W_{dry} is the dry weight of bulb herbs.

2.3.2 Determination of the Contents of Different Nitrogen Forms in the Rhizosphere Soil of F. taipaiensis

Referring to the determination methods of soil nitrogen forms described in Bao's *Soil Agrochemical Analysis* [23], the phenol-disulfonic acid colorimetric method was used to measure nitrate nitrogen content: 5.00 g of sieved rhizosphere soil was weighed and extracted with saturated calcium sulfate solution. The supernatant was collected after filtration, and phenol-disulfonic acid was added to make up to a constant volume. The absorbance was measured at a wavelength of 420 nm, and the nitrate nitrogen content was calculated using the standard curve. The indophenol blue colorimetric method was adopted to determine ammonium nitrogen content: The soil samples were extracted and filtered with a potassium chloride solution. Then, phenol and alkaline sodium hypochlorite solutions were added sequentially for color development, and ammonium nitrogen content was quantified via colorimetry at 625 nm. The urease

colorimetric method was applied to measure amide nitrogen content: Urease was used to specifically hydrolyze amide nitrogen, and quantitative detection was accomplished through a color reaction combined with spectrophotometry.

2.3.3 Detection of Total Alkaloids in the Bulbs of F. taipaiensis

Referring to the methods specified in the Chinese Pharmacopoeia [24] for "Chuan Bei Mu" and considering the properties of alkaloids, the powder of *F. taipaiensis* was soaked in a concentrated ammonia test solution for 1 h. Subsequently, a chloroform–methanol mixture was added, and the solution was refluxed in a water bath for 2 h. After refluxing, the solution was filtered, rinsed with a small amount of the same chloroform–methanol mixture, and then shaken thoroughly to prepare the test solution. Three parallel experiments were performed on samples from different treatment groups, and the content was quantified using ultraviolet spectrophotometry.

2.3.4 Detection of Monomeric Alkaloids in the Bulbs of F. taipaiensis

The tissue powder of *F. taipaiensis* was immersed in a concentrated ammonia test solution for 1 h, followed by the introduction of a chloroform–methanol mixture and subsequent heating to reflux in a water bath for 2 h. Subsequently, the chloroform–methanol mixture was added again, and the solution was evaporated to dryness. The residue was diluted to the desired volume with methanol, and the resulting sample was tested using liquid chromatography–mass spectrometry. The concentration ranges of each component in the mixed standard solution were set as follows: peimine 10–1000 $ng \cdot mL^{-1}$, peiminine 20–1200 $ng \cdot mL^{-1}$, peimisine 50–5000 $ng \cdot mL^{-1}$, sipeimine 20–1200 $ng \cdot mL^{-1}$, and sipeimine-3β-D-glucoside 20–1000 $ng \cdot mL^{-1}$. Ultra performance liquid chromatography (UPLC) detection conditions were as follows: Xbridge BEH C18 chromatographic column (3.0 × 100 mm^2, 2.5 μm, Waters Corporation, Milford, MA, USA) (Thermo Fisher Scientific Inc., Shanghai, China); mobile phase A was a 0.1% formic acid solution (containing 5 $mmol \cdot L^{-1}$ ammonium acetate), and mobile phase B was acetonitrile, with gradient elution (0–3 min: 90%–80% A, 3–16 min: 80%–78% A, 16–18 min: 78%–50% A, 18–19 min: 50%–40% A, 19–25 min: 40%–5% A, 25–35 min: 5%–90% A); flow rate: 0.3 $mL \cdot min^{-1}$; injection volume: 1 μL; and column temperature: 40°C. The MS detection conditions were as follows: Electrospray ionization (ESI) source was used as ion source, with a spray voltage of 3 kV; ionization mode, positive (negative) ion scanning; detection method, first-level full scan mass spectrum (Full MS); scanning range, 300–700 $m \cdot z^{-1}$; secondary selection ion scanning; first-level resolution, 70,000; second-level resolution, 15,000; sheath gas flow rate, 35 $L \cdot min^{-1}$; auxiliary gas flow rate, 10 $L \cdot min^{-1}$; and capillary temperature, 320°C.

2.4 Data Analysis

Data processing and organization were conducted using Microsoft Excel 2010 (Microsoft Corp., WA, USA), followed by statistical analyses performed in R Studio (Posit PBC, MA, USA). Graphical visualizations were generated with Origin 2021 (OriginLab Corp., MA, USA). Pearson correlation coefficients were calculated to assess linear correlations between variables. Single-factor analysis of variance was applied with a 95% confidence interval (α = 0.05) to determine statistical significance.

3 Results

3.1 Effects of Various Treatments on Biomass in F. taipaiensis Bulbs

Inoculation with NFB resulted in significant changes in the fresh and dry weights of *F. taipaiensis* bulbs (Table 3). Specifically, compared with the CK group, all NFB treatments significantly increased the

fresh and dry weights of bulbs, with the increases ranging from 8.08% (*Rahnella aquatilis*; N1 treatment) to 27.61% (*R. aquatilis* + *P. stellifer*; N5 treatment) and from 7.78% (*P. chlororaphis*; N2 treatment) to 37.55% (N5 treatment), respectively. Additionally, the drying rates of bulbs across different treatment groups ranged from 29.67% (*P. chlororaphis* + *P. stellifer*; N6 treatment) to 32.37% (*R. aquatilis* + *P. chlororaphis* + *P. stellifer*; N7 treatment), indicating minimal effects of different NFB treatments on the bulb drying rate.

Table 3: *F. taipaiensis* bulb biomass under different treatments ($\overline{x} \pm s$, $n = 3$).

Treatments	Fresh Weight (g/pot)	Dry Weight (g/pot)	Drying Rate (%)
N1	2.916 ± 0.002^{c}	0.893 ± 0.046^{c}	30.612 ± 1.554^{abc}
N2	2.810 ± 0.008^{c}	0.887 ± 0.042^{c}	31.402 ± 0.911^{abc}
N3	3.086 ± 0.209^{bc}	0.938 ± 0.027^{bc}	30.334 ± 0.391^{bc}
N4	3.354 ± 0.203^{a}	1.077 ± 0.011^{b}	32.164 ± 1.609^{ab}
N5	3.443 ± 0.098^{a}	1.132 ± 0.010^{a}	32.892 ± 0.652^{a}
N6	3.120 ± 0.220^{b}	0.924 ± 0.063^{bc}	29.670 ± 0.662^{c}
N7	2.984 ± 0.097^{c}	0.967 ± 0.083^{b}	32.368 ± 0.640^{ab}
CK	2.698 ± 0.064^{d}	0.823 ± 0.045^{d}	30.492 ± 0.203^{bc}

Note: Different lowercase letters in the same column indicate significant differences at $p < 0.05$.

3.2 Effects of Different Treatments on Nitrogen Forms in F. taipaiensis Rhizosphere Soil

Inoculation with NFB induced significant changes in the contents of ammonium nitrogen, nitrate nitrogen, and amide nitrogen in the rhizosphere soil of *F. taipaiensis* (Table 4). Specifically, compared with the CK group, only the *Paenibacillus stellifer* (N3) treatment significantly decreased the ammonium nitrogen content by 5.21% whereas the N1, *R. aquatilis* + *P. chlororaphis* (N4), N5, N6, and N7 treatments significantly increased it by 0.41%, 1.71%, 12.12%, 5.49%, and 11.65%, respectively. Regarding nitrate nitrogen content, only the N4 treatment significantly decreased (31.49%) whereas the N1, N2, N3, N5, N6, and N7 treatments significantly increased it by 325.83%, 265.45%, 43.40%, 79.60%, 39.74%, and 61.56%, respectively. All NFB treatments significantly increased the amide nitrogen content, with the increase ranging from 34.16% (N4) to 741.46% (N5).

Table 4: Contents of ammonium nitrogen, nitrate nitrogen, and amide nitrogen in *F. taipaiensis* rhizosphere soil under different treatments ($\overline{x} \pm s$, $n = 3$).

Treatments	Ammonium Nitrogen ($mg \cdot kg^{-1}$)	Nitrate Nitrogen ($mg \cdot kg^{-1}$)	Amide Nitrogen ($mg \cdot kg^{-1}$)
N1	25.515 ± 0.206^{c}	3.611 ± 0.061^{a}	0.451 ± 0.015^{c}
N2	25.392 ± 0.275^{c}	3.099 ± 0.128^{b}	0.599 ± 0.009^{b}
N3	24.088 ± 0.108^{d}	1.216 ± 0.026^{d}	0.354 ± 0.032^{d}
N4	25.847 ± 0.277^{c}	0.581 ± 0.024^{f}	0.165 ± 0.013^{f}
N5	28.493 ± 0.061^{a}	1.523 ± 0.074^{c}	1.035 ± 0.044^{a}
N6	26.807 ± 0.438^{b}	1.185 ± 0.043^{d}	0.278 ± 0.007^{e}
N7	28.373 ± 0.060^{a}	1.370 ± 0.130^{cd}	1.074 ± 0.031^{a}
CK	25.412 ± 0.454^{c}	0.848 ± 0.037^{e}	0.123 ± 0.011^{f}

Note: Different lowercase letters in the same column indicate significant differences at $p < 0.05$.

3.3 Effects of Different Treatments on Total Alkaloid Content in F. taipaiensis Bulbs

Total alkaloid content is one of the key indicators for evaluating the medicinal components of *F. taipaiensis*. Inoculation with NFB induced significant changes in the total alkaloid content of *F. taipaiensis* bulbs (Fig. 1). Specifically, compared with the CK group, all NFB treatments significantly increased the total alkaloid content, with the increases ranging from 22.92% (N1) to 61.36% (N5).

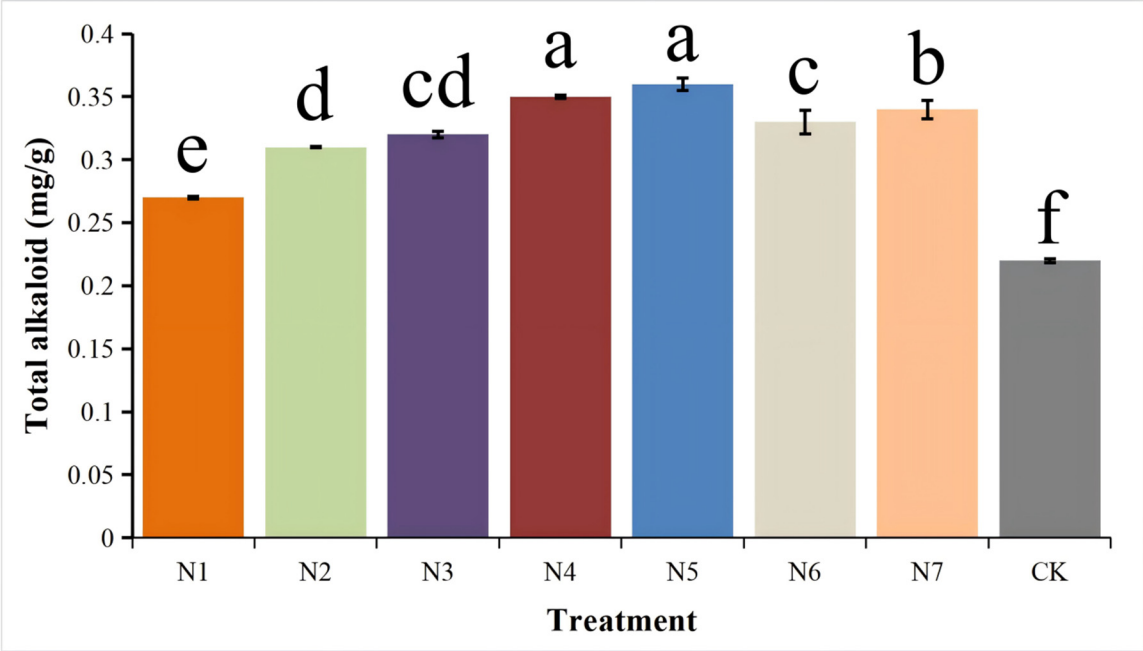

Figure 1: Total alkaloid contents in *F. taipaiensis* bulbs under different treatments. N1, *Rahnella aquatilis*; N2, *Pseudomonas chlororaphis*; N3, *Paenibacillus stellifer*; N4, *R. aquatilis* + *P. chlororaphis*; N5, *R. aquatilis* + *P. stellifer*; N6, *P. chlororaphis* + *P. stellifer*; N7, *R. aquatilis* + *P. chlororaphis* + *P. stellifer*; CK, sterile water. Different lowercase letters indicate significant differences at $p < 0.05$.

3.4 Effects of Different Treatments on Monomeric Alkaloid Content in F. taipaiensis Bulbs

Inoculation with NFB induced significant changes in the alkaloid contents of *F. taipaiensis* bulbs (Table 5). Specifically, compared with the CK group, only the N3, N4, and N7 treatments significantly decreased the peimine content by 0.96%, 6.45%, and 10.04%, respectively, whereas the N1, N2, N5, and N6 treatments significantly increased it by 2.00%, 6.24%, 21.64%, and 9.40%, respectively. Regarding peiminine content, only the N3, N4, and N6 treatments caused significant decreases, with reductions of 2.98%, 30.77%, and 23.90%, respectively, whereas the N1, N2, N5, and N7 treatments significantly increased it by 25.23%, 5.43%, 15.33%, and 32.93%, respectively. Compared with the CK group, only the N2 and N5 treatments significantly increased the peimisine content by 1.34% and 0.20%, respectively, whereas the N1, N3, N4, N6, and N7 treatments significantly decreased it by 9.56%, 8.64%, 3.58%, 18.66%, and 7.43%, respectively.

Compared with the CK group, only the N2, N4, and N6 treatments significantly increased the sipeimine content by 4.79%, 6.67%, and 29.41%, respectively, whereas the N1, N3, N5, and N7 treatments significantly decreased it by 42.67%, 24.11%, 6.72%, and 16.91%, respectively. For sipeimine glycoside content, only the N3, N5, and N7 treatments significantly increased it by 13.32%, 5.81%, and 10.37%, respectively, whereas the N1, N2, N4, and N6 treatments significantly decreased it by 32.35%, 21.90%, 5.90%, and 20.91%, respectively.

The results of monomeric alkaloid contents in *F. taipaiensis* bulbs indicated a better inoculation effect of the N5 treatment on the medicinal components of *F. taipaiensis* bulbs.

Table 5: Monomeric alkaloid contents in *F. taipaiensis* bulbs under different treatments ($\overline{x} \pm s$, $n = 3$).

Treatments	Peimine ($\mu g \cdot g^{-1}$)	Peiminine ($\mu g \cdot g^{-1}$)	Peimisine ($\mu g \cdot g^{-1}$)	Sipeimine ($\mu g \cdot g^{-1}$)	Sipeimine Glycoside ($\mu g \cdot g^{-1}$)	Total Monomeric Alkaloids ($\mu g \cdot g^{-1}$)
N1	30.304 ± 0.599^{bcd}	54.454 ± 1.645^{ab}	387.326 ± 5.424^{ab}	3.234 ± 0.175^{e}	0.757 ± 0.041^{d}	0.274 ± 0.001^{e}
N2	31.562 ± 3.093^{bc}	45.846 ± 3.613^{cd}	433.998 ± 33.744^{a}	5.668 ± 0.385^{a}	0.874 ± 0.116^{cd}	0.314 ± 0.00^{d}
N3	29.424 ± 1.989^{bcd}	42.188 ± 3.523^{d}	391.281 ± 19.34^{ab}	4.281 ± 0.296^{d}	1.268 ± 0.103^{a}	0.320 ± 0.002^{cd}
N4	27.792 ± 3.121^{cd}	30.103 ± 3.571^{e}	412.924 ± 36.89^{a}	6.017 ± 0.476^{a}	1.053 ± 0.143^{bc}	0.354 ± 0.001^{a}
N5	36.137 ± 3.687^{a}	50.151 ± 4.117^{bc}	429.123 ± 29.799^{a}	5.262 ± 0.559^{bc}	1.184 ± 0.141^{ab}	0.360 ± 0.004^{a}
N6	32.502 ± 0.455^{ab}	33.091 ± 0.557^{e}	348.337 ± 9.937^{d}	7.300 ± 0.106^{a}	0.885 ± 0.059^{cd}	0.327 ± 0.008^{c}
N7	26.726 ± 0.712^{d}	57.801 ± 0.993^{a}	396.443 ± 25.634^{a}	4.687 ± 0.237^{cd}	1.235 ± 0.119^{ab}	0.345 ± 0.006^{b}
CK	29.709 ± 2.011^{bcd}	43.483 ± 1.985^{d}	428.262 ± 11.356^{a}	5.641 ± 0.249^{a}	1.119 ± 0.112^{ab}	0.223 ± 0.001^{f}

Note: Different lowercase letters in the same column indicate significant differences at $p < 0.05$.

3.5 Principal Component Analysis of Monomeric Alkaloid in F. taipaiensis Bulbs under Different Treatments

Principal component matrix data were obtained by performing principal component analysis (PCA) on the five monomeric alkaloids in *F. taipaiensis* under different treatments. As shown in Table 6, two principal components greater than 1.000 were extracted accordingly. The eigenvalues of the first principal component (PC1) and the second principal component (PC2) were 1.79 and 1.61, respectively. The cumulative contribution rate of these two principal components was calculated to be 67.99%, indicating that these two components represented most of the information regarding the monomeric alkaloid contents of *F. taipaiensis*. The information of PC1 was mainly contributed by peimisine, peiminine, and sipeimine glycoside, whereas PC2 primarily comprised sipeimine and peimine.

Table 6: Principal component matrix of the five monomeric alkaloids.

Monomericicic Alkaloids	Principal Component 1	Principal Component 2
Peimisine	0.776	0.326
Peiminine	0.741	−0.526
Sipeimine glycosides	0.598	0.232
Sipeimine	−0.296	0.897
Peimine	0.326	0.605
Eigenvalue	1.795	1.605
Variance contribution rate (%)	35.894	32.099
Cumulative contribution rate (%)	35.894	67.993

As shown in Fig. 2, peimine, peiminine, peimisine, and sipeimine glycoside exhibited more prominent projections on the x-axis and weaker projections on the y-axis, indicating that these four alkaloids were associated with the PC1 axis. In contrast, sipeimine showed a more significant projection on the y-axis and a weaker projection on the x-axis, suggesting that it was linked to the PC2 axis. A small vector angle was

observed between peimisine and sipeimine glycoside, demonstrating a close correlation between the two. Also, the vector angles between peimine and both peimisine and sipeimine glycoside were relatively small, indicating a strong correlation of peimine with peimisine and sipeimine glycoside.

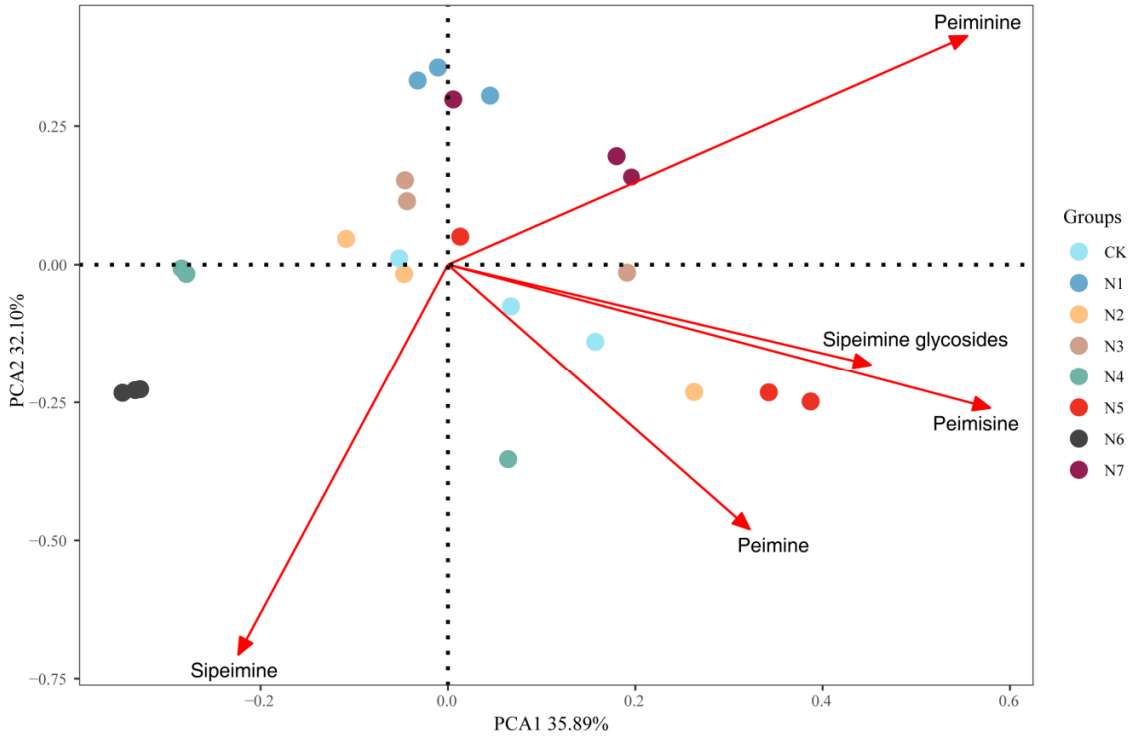

Figure 2: Principal component analysis of the five monomeric alkaloids.

The average contents of the five monomeric alkaloids in each treatment group were used for data standardization, followed by PCA, to address the issue where excessive dispersion of individual data points within each treatment group impaired the expressive effect and analytical reliability of PCA results. The score coefficient matrix of each principal component was constructed by dividing the principal component matrix factors of *F. taipaiensis* by the square root of the corresponding principal component eigenvalues, and the corresponding expression functions were derived as follows:

$$F_1 = 0.580ZX_1 + 0.554ZX_2 + 0.447ZX_3 - 0.221ZX_4 + 0.326ZX_5 \quad (3)$$

$$F_2 = 0.257ZX_1 - 0.415ZX_2 + 0.183ZX_3 + 0.708ZX_4 + 0.447ZX_5 \quad (4)$$

$$F_S = 0.511F_1 + 0.488F_2 \quad (5)$$

where ZX_1, ZX_2, ZX_3, ZX_4, and ZX_5 represent the standardized average contents of peimisine, peiminine, sipeimine glycoside, sipeimine, and peimine in each treatment group, respectively. F_1 denotes the score of Principal Component 1, F_2 denotes the score of Principal Component 2, and F_s denotes the comprehensive principal component score.

The variance contribution rates of the two principal components were adopted as weighting coefficients to calculate and rank the comprehensive PCA scores of *F. taipaiensis* under different treatments. Based on

the comprehensive evaluation scores, the ranking of all treatment groups in terms of the comprehensive accumulation level of the five monomeric alkaloids was determined and is presented in Table 7. The ranking results indicated that the N5 group ranked first, followed by the N6, N4, N1, N2, N3, and N7 groups, with the control (CK) group ranking last.

This PCA analysis, based on group average values, effectively eliminated the interference caused by the large dispersion of individual data points within each treatment group, thereby enhancing the stability and reliability of the analytical results. The revised analysis further confirmed that the combined inoculation of *R. aquatilis* and *P. stellifer* (N5 group) exerted the most significant promoting effect on the alkaloid content of *F. taipaiensis*, making it the optimal nitrogen-fixing bacterial combination for improving the accumulation of medicinal alkaloid components in *F. taipaiensis* bulbs.

A typographical error was noted in the original formula of F_2, where the ZX_3 term was repeated. This error has been corrected to ZX_2 (corresponding to peiminine) in this revision, which is consistent with the monomeric alkaloid indicators and the characteristics of the principal component matrix.

Table 7: Principal component scores of monomeric alkaloids in *F. taipaiensis* bulbs.

Treatments	Principal Component 1	Sort	Principal Component 2	Sort	Synthesis Score	Comprehensive Sort
N1	0.368	3	0.410	4	0.270	4
N2	0.233	4	0.403	5	0.247	5
N3	0.212	5	−0507	6	−0.247	6
N4	−1.069	7	0.767	3	0.273	3
N5	1.598	1	0.865	2	0.680	1
N6	−2.148	8	1.401	1	0.473	2
N7	0.805	2	−1.320	7	−0.607	7
CK	0.001	6	−2.018	8	−1.090	8

3.6 Correlation Analysis

The correlations among nitrogen forms, biomass, and alkaloid content of *F. taipaiensis* are illustrated in Fig. 3. Ammonium nitrogen content was negatively correlated with nitrate nitrogen content, positively correlated with amide nitrogen content (r = 0.756), and also showed a positive correlation with bulb fresh weight, dry weight, and total alkaloid content. Nitrate nitrogen content was negatively correlated with the contents of total alkaloids, sipeimine, and sipeimine glycoside, and was positively correlated with the contents of amide nitrogen, peimine, peiminine, and peimisine. Amide nitrogen content was negatively associated with sipeimine content, and bulb fresh weight was positively correlated with total alkaloid content (r = 0.815). Moreover, peiminine content was negatively correlated with sipeimine content (r = −0.721), and a positive correlation was observed between peimisine and sipeimine glycoside contents.

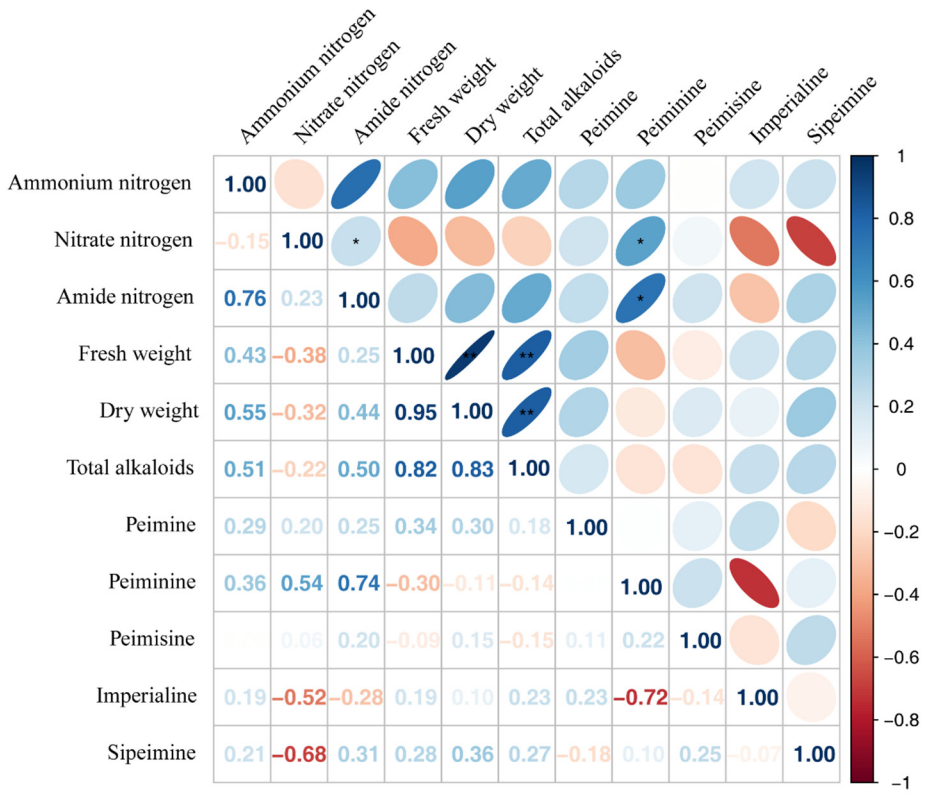

Figure 3: Pearson correlation analysis. *: significant correlation ($p < 0.05$); **: extremely significant correlation ($p < 0.01$).

4 Discussion

4.1 Effects of NFB Inoculation on Nitrogen Nutrition in Rhizosphere Soil

Soil nitrogen content, particularly inorganic nitrogen, is a key indicator of soil fertility, playing a crucial role in regulating the diversity and metabolic activity of rhizosphere microbial communities [25,26]. In this study, the ammonium nitrogen content in the *F. taipaiensis* rhizosphere soil was significantly positively correlated with bulb fresh weight, dry weight, and total alkaloid content. This result indicated a key role of ammonium nitrogen in promoting the growth and synthesis of *F. taipaiensis*, which was consistent with previous findings showing that ammonium nitrogen could regulate the expression of genes related to plant nitrogen metabolism, influence the biosynthesis of plant secondary metabolites, and directly regulate the accumulation of active components in medicinal plants [13]. In the present study, nitrate nitrogen content was positively correlated with peimine, peiminine, and peimisine contents, yet negatively correlated with the contents of total alkaloids and sipeimine, suggesting that it exerted a selective regulatory effect on the biosynthesis of monomeric alkaloids in *F. taipaiensis* [5]. Moreover, previous studies have demonstrated that nitrate nitrogen regulates the translocation of alkaloids across distinct plant tissues, facilitating the transport of certain alkaloids from underground organs to aerial parts [27]. In follow-up experiments, we will simultaneously quantify the contents of various alkaloids in the aerial parts of *F. taipaiensis*, so as to verify whether nitrate nitrogen participates in regulating the tissue-specific translocation of different alkaloid species in this medicinal plant.

The three NFB employed in this study exhibited significant differences in their regulatory effects on the content and composition of rhizosphere soil nitrogen, owing to their distinct metabolic traits

and nitrogen fixation mechanisms. *R. aquatilis* is a common plant growth–promoting rhizobacterium possessing nitrogen-fixing activity. It can convert atmospheric nitrogen into forms available for plant uptake via nitrogenase-mediated nitrogen fixation while also secreting organic acids that enhance soil nitrogen availability [28]. In this study, the inoculation with *R. aquatilis* significantly increased the nitrate nitrogen content in N1-treated rhizosphere soil by 325.83% compared with that in the CK group, whereas ammonium nitrogen content increased only slightly (by 0.41%). These results were consistent with the findings reported by a previous study demonstrating that *R. aquatilis* promoted the soil nitrification process and accelerated the conversion of ammonium nitrogen into nitrate nitrogen; the nitrification-promoting metabolites secreted by this strain were the primary cause of massive nitrate nitrogen accumulation [29]. *P. chlororaphis* possesses multiple functions including nitrogen fixation, disease prevention, and plant growth promotion. It affects soil nitrogen levels mainly through its own nitrogen fixation and regulation of the rhizosphere nitrogen-cycling microbial community [30]. In this study, the N2 treatment significantly increased nitrate nitrogen content in the rhizosphere by 265.45% and amide nitrogen content by 549.59%, while exerting no significant effect on ammonium nitrogen content. This strain can secrete siderophores [31] and antibiotics to regulate the rhizosphere microbial community, facilitating the release and transformation of soil nitrogen [32]. The pronounced increase in amide nitrogen content under the N2 treatment may be associated with the ability of this strain to promote the synthesis of amide compounds in both plants and soil microorganisms [30]. *P. stellifer* is a nitrogen-fixing bacterium widely distributed in soil, with moderate nitrogen-fixing efficiency and a relatively mild regulatory effect on soil nitrogen [4]. In the present study, the nitrogen content in N3 rhizosphere increased by 43.40% and amide nitrogen content increased by 282.11%, but ammonium nitrogen content significantly reduced by 5.21% compared with that in the CK group. This might be attributed to the high ammonium nitrogen demand of *P. stellifer* during growth and reproduction, such that its uptake and utilization of soil ammonium nitrogen exceeded its nitrogen-fixing capacity [4]. Meanwhile, *Paenibacillus* can facilitate the transformation of ammonium nitrogen into organic nitrogen, further reducing the content of ammonium nitrogen in soil [33].

Among all the mixed inoculation treatments, the rhizosphere in the N4 group exhibited the lowest contents of various nitrogen forms: ammonium nitrogen content increased by only 1.71%, nitrate nitrogen content decreased by 31.49%, and amide nitrogen content increased by merely 34.16%, all of which were significantly lower than those in the other mixed inoculation groups (N5, N6, and N7). This phenomenon was mainly attributed to the antagonistic interaction between *R. aquatilis* and *P. chlororaphis*, which inhibited their respective capabilities for nitrogen fixation and transformation. Existing studies have confirmed obvious interspecific antagonism between *Rahnella* and *Pseudomonas* [4], We speculate that this phenomenon may be attributed to the fact that *P. chlororaphis* secretes antibacterial pyrrolnitrin and phenazine [34] compounds, which damage the cell membrane of *R. aquatilis*, reduce its nitrogenase activity, and thereby suppress its nitrogen-fixing capacity. Meanwhile, the two strains compete for rhizosphere carbon sources, nitrogen sources, and ecological niches, and the limited nutrients in the potting substrate further intensify interspecific competition [35]. The mutual inhibition between these two strains can also disrupt the balance of the rhizosphere microbial community involved in nitrogen cycling and reduce the overall nitrogen transformation efficiency of the soil, which is consistent with the conclusion that the antagonism among strains in compound microbial agents reduces fertilizer efficiency [4].

Except for group N4, the multi-strain combined treatment groups, including N5, N6, and N7, generally exhibited a pattern where the efficacy of combined inoculation was inferior to that of the corresponding single-strain inoculation. Comprehensive analysis indicates that this common phenomenon arises from the combined interactions of multiple strains within the rhizosphere microecosystem. First, the soil ecological

niches, as well as carbon and nitrogen resources, are limited. After mixed inoculation of multiple strains, the interspecific competition for nutrients and living space widely occurs among different strains even in the absence of strong antagonism between strains, which impairs the inherent nitrogen and nitrogen fixation capacities of individual strains [36]. Second, the simultaneous introduction of exogenous multiple strains into the rhizosphere drastically disturbs the structural composition of indigenous microbial communities, disrupts the cooperative balance of native nitrogen-cycling microorganisms, and indirectly reduces the efficiency of soil nitrogen transformation [4]. Third, the metabolic products of different strains exert cross-influences; certain metabolites alter microenvironmental conditions such as rhizosphere pH and redox potential, thereby inhibiting the activity of the strains themselves and other functional microorganisms [37]. The synergistic effect of these multiple factors ultimately leads to the observation that most combined bacterial treatments achieve less improvement in nitrogen content compared with single-strain inoculation.

In comparison, N5 and N6 treatments exhibited synergistic effects. A plausible underlying mechanism lies in the complementary nutrient utilization patterns and metabolic pathways between *P. stellifer* and the other two strains, which alleviates direct resource competition. Follow-up studies can integrate *in vitro* co-culture assays, high-throughput sequencing of rhizosphere microorganisms, and dynamic monitoring of rhizosphere microenvironmental indices to verify antagonistic/symbiotic interactions among strains, shifts in microbial community composition, and disparities in physicochemical conditions, thereby validating the aforementioned hypothesis.

4.2 Effects of NFB Inoculation on the Biomass and Alkaloid Contents in F. taipaiensis Bulbs

As core rhizosphere plant growth–promoting microorganisms, NFB exert remarkable effects in boosting plant growth and regulating the accumulation of active ingredients in medicinal plants, thereby boasting enormous application potential in the green cultivation of Chinese medicinal materials. Numerous field and pot experiments have verified that inoculation with NFB not only promotes bulb biomass expansion but also significantly elevates the accumulation of alkaloids, the primary bioactive constituents responsible for their medicinal efficacy, in bulbous medicinal plants such as *Fritillaria* [4]. In this study, inoculation with single and complex strains both significantly increased the bulb biomass of *F. taipaiensis*. The dry weight in most complex strain groups was higher than that in single-strain groups, which was consistent with the findings of previous studies on the growth-promoting effects of complex NFB [4,5]. The growth-promoting advantages of compound bacteria stem from multiple aspects including functional complementarity of strains, enhanced stability of rhizosphere microbial communities, and superposition of growth-promoting metabolites. Among all groups, the N5 group exhibited the highest bulb dry weight, which increased by 37.55% compared with that in the CK group, demonstrating the optimal synergistic growth-promoting effect of the two strains.

Besides enhancing biomass accumulation, NFB inoculation also significantly elevated the contents of total alkaloid and individual alkaloids in *F. taipaiensis* bulbs, which is crucial for improving the medicinal quality of this species. As nitrogen serves as a fundamental structural element in alkaloid molecules, an appropriate nitrogen supply is an important prerequisite for alkaloid biosynthesis and accumulation in *Fritillaria* plants [38]. The total alkaloid contents in all inoculated groups of this study were significantly higher than those in the CK group, with increases ranging from 22.92% to 61.36%. The N5 group achieved the highest comprehensive alkaloid score, verifying the positive regulatory effect of the combination of *R. aquatilis* and *P. stellifer* on the accumulation of medicinal components. The two strains synergistically regulate soil nitrogen forms and plant nitrogen metabolism: *R. aquatilis* increased the contents of ammonium nitrogen and nitrate nitrogen in the rhizosphere, secreted organic acids to activate soil mineral nutrients,

and provided a material basis for alkaloid synthesis [29]; *P. stellifer* facilitated the conversion of inorganic nitrogen into amide nitrogen [33]. As a stable nitrogen storage form, amide nitrogen can continuously supply nitrogen sources for alkaloid synthesis in bulbs. Subsequent single-inoculation and co-inoculation comparative tests of strains, enzyme activity assays, and integrated transcriptomic and metabolomic analyses can be adopted to clarify the variation patterns of key enzyme activities for alkaloid synthesis and expression of related genes and metabolic pathways under the synergistic effect of the two strains. Meanwhile, the colonization and distribution characteristics of the strains in the rhizosphere can be tracked, so as to verify the mechanism by which the two strains synergistically boost alkaloid accumulation in *F. taipaiensis* from multiple dimensions including microbial interaction, molecular regulation, and substance metabolism.

Although strain antagonism existed in group N4, it still exerted a certain promoting effect on alkaloid accumulation, yet the effect was weaker than that in group N5. For group N7 with a three-strain combination, resource competition among strains offset part of the synergistic effect, leading to a slightly lower improvement in alkaloid content compared with that in group N5. The results of this study revealed a complex correlation pattern between the contents of nitrate nitrogen and alkaloids: Group N1 had the highest nitrate nitrogen content yet a low total alkaloid content, which intuitively indicated that nitrate nitrogen inhibited the accumulation of total alkaloids. However, a substantial disparity existed in nitrate nitrogen content between group N4 and group N5, while their total alkaloid levels remained similarly high [4,5]. The internal mechanism based on the biochemical pathways of alkaloid synthesis can be explained as follows: Alkaloids are nitrogen-containing secondary metabolites in plants, and their synthesis is a complex process synergistically regulated by multiple enzymes and pathways, which is not directly determined by a single nitrogen form [38]. On the one hand, a dynamic equilibrium of interconversion among nitrate nitrogen, ammonium nitrogen, and amide nitrogen exists, so a single nitrate nitrogen index cannot fully reflect the overall nitrogen nutrient supply level of plants [13]. On the other hand, different nitrogen forms selectively regulate the activities of key enzymes in the alkaloid synthesis pathway: Nitrate nitrogen mainly regulates the synthesis of some monomeric alkaloids such as sipeimine, whereas ammonium nitrogen and amide nitrogen dominate the synthesis of peimine, peiminine, and total alkaloids [27]. Massive accumulation of nitrate nitrogen in group N1 inhibited the total alkaloid synthesis pathway. However, the contents of ammonium nitrogen and amide nitrogen in groups N4 and N5 increased sharply simultaneously, offsetting the impacts caused by the differences in nitrate nitrogen content and ultimately bringing the total alkaloid contents of the two groups to a similar level [4]. In addition, the rhizosphere microbial community structure and endogenous hormone levels of plants also participate in the regulation of alkaloid synthesis, further increasing the complexity of the correlation between nitrogen forms and alkaloid contents [13,26]. Subsequent gradient nitrogen-form proportions can be established to further elucidate the underlying regulatory mechanisms. Combined with the determination of the activities of key enzymes involved in alkaloid synthesis, quantitative analysis of endogenous hormones and transcriptome sequencing can be used to elucidate the differential regulatory effects of different nitrogen forms on the alkaloid synthesis pathway. Meanwhile, fluorescence labeling and quantitative polymerase chain reaction can be adopted to track the colonization quantity of exogenous strains. Combined with metagenomic analysis of structural changes in the rhizosphere microbial community, the internal mechanisms through which strain antagonism and interspecific competition influence nitrogen transformation and alkaloid accumulation can be systematically clarified.

This study combined physiological indicators, nitrogen forms, alkaloid contents, and strain interaction characteristics to preliminarily deduce the mechanism by which NFB improve the cultivation performance of *F. taipaiensis*; this mechanism is merely a hypothesis proposed based on available experimental data.

At present, this study has only completed the determination of phenotypic indicators and routine physicochemical indicators. The profound effects of exogenous strains on rhizosphere microbial community structure and the whole metabolic pathways of plants have not been analyzed. Multi-omics technologies such as metagenomics and metabolomics should be adopted in follow-up research to systematically explore the colonization dynamics of inoculated strains in the rhizosphere, their impacts on the composition and functions of indigenous microbial communities, and their regulatory patterns over plant nutrient cycling and secondary metabolic networks, so as to fully clarify the molecular interaction mechanism among NFB, rhizosphere microorganisms, and *F. taipaiensis*. In addition, not all inoculated strains can successfully colonize the rhizosphere, and exogenous strains vary in their ability to colonize and integrate with indigenous microbial communities. Therefore, further research will be conducted to clarify whether the shifts in plant metabolic indicators arise from the direct effects of successfully colonized exogenous strains, or the indirect effects induced by disturbances to the structural composition of soil microbial communities following inoculation. The specific experimental schemes are as follows: 1. Establish single-strain inoculation groups with sterile substrates to eliminate interference from indigenous microorganisms, so as to independently determine the direct regulatory capacity of colonized strains on nitrogen metabolism and alkaloid biosynthesis of *F. taipaiensis*; 2. Set up two treatment groups, namely sterilized soil supplemented with indigenous microbial suspension plus exogenous strains, and a control group containing only indigenous microorganisms. The rhizosphere microbial community structure, soil nitrogen transformation efficiency and plant metabolic profiles of the two groups will be compared to quantify the indirect impacts caused by exogenous strains disturbing indigenous microflora; 3. Employ fluorescence-labeled strains combined with absolute quantification qPCR to track the colonization abundance of exogenous strains in the rhizosphere at multiple time points. The detected alkaloid and nitrogen-related indicators will be correlated to construct a correlation chain of "strain colonization abundance-microbial community variation-plant metabolism", which can precisely discriminate whether phenotypic changes in plants are attributed to direct functions of colonized exogenous strains or indirect regulatory effects derived from reshaping rhizosphere microbial communities by exogenous inoculants.

5 Conclusions

NFB inoculation significantly increased the available nitrogen content in the rhizospheric soil and promoted the growth of *F. taipaiensis*, thereby enhancing the biosynthesis of its medicinal compounds. Compared with the CK group, NFB treatments exhibited substantially elevated concentrations of nitrate nitrogen and amide nitrogen in the rhizospheric soil of *F. taipaiensis*, which was accompanied by increased biomass accumulation, total alkaloid content, and monomeric alkaloid contents. Particularly under the treatments with *Rahnella aquatilis* and *Paenibacillus stellifer*, the most prominent enhancements were observed in ammonium nitrogen content, biomass production, total alkaloid content, and peimisine content. These findings indicated that NFB application effectively improved both the yield and medicinal quality of *F. taipaiensis*. The mechanisms underlying NFB-enhanced cultivation were elucidated in this study, providing novel insights and practical approaches for sustainable traditional Chinese medicine production systems.

Acknowledgement: Not applicable.

Funding Statement: This research was supported in part by project of Natural Science Foundation of Chongqing (CSTB2023NSCQ-LMX0010).

Author Contributions: Conceptualization, Zhenyu Liu; methodology, Panpan Wang; investigation, You Zhou, Yanjing Liu and Mingyan Ye; writing—original draft preparation, Zhenyu Liu; writing—review and editing, Dongqin Guo and Nong Zhou; supervision, Dongqin Guo. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: All the data supporting the findings of this study are included in this manuscript.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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