



## Article

# Characterization and Antagonistic Effect of Culturable Apple-Phyllosphere Endophytic Bacteria from the Cold Plateau in Yunnan, China

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## Highlights:

- The endophytic community in apple leaves relates to geographic locations, apple varieties, and environment, providing the basis to explain the mechanisms underlying the establishment of apple endophytic communities and may help to devise apple disease management strategies.
- The dominant culturable endophytic bacteria *Bacillus velezensis* and *B. subtilis* successfully inhibit not only apple pathogens *Alternaria alternata*, *Botryosphaeria dothidea*, *Valsa mali*, *Fusarium oxysporum*, *F. solani*, and *Rosellinia necatrix*, but also *F. oxysporum* f. sp. *cubense* (banana Fusarium wilt) and *Phytophthora nicotianae* (tobacco black shank).

**Abstract:** The endophytic bacteria in apple leaves from apple-producing areas of Yunnan, China were isolated and identified on the basis of bacterial colony morphology and nucleotide sequences of 16S *rRNA* and *rpoB* genes. The endophytic bacterial isolates with nitrogen, phosphorus, and potassium utilization abilities were screened by culturing on functional media. A total of 5709 isolates of culturable endophytic bacteria (CEB) were isolated from 30 apple leaf samples collected from different regions. A total of 39 CEB representative isolates were identified as *Bacillus velezensis*, *B. subtilis*, *B. licheniformis*, *B. safensis*, *B. pumilus*, and *Priestia megaterium*. Among them, *B. velezensis* and *B. subtilis* were the main CEB, accounting for 55.00% and 34.37%, respectively, which exhibited potential inhibition on not only the main apple disease pathogens of *Alternaria alternata*, *Valsa mali*, *Fusarium oxysporum*, and *Rosellinia necatrix*, but also some important and uncontrollable phytopathogens, including *F. oxysporum* f. sp. *cubense* that causes banana Fusarium wilt, and *Phytophthora nicotianae* that causes tobacco black shank. Among these isolated endophytic bacteria species, a total of 10 strains, including b3, b4, b16, b17, b20, and b23 of *B. subtilis*, b7, b24, and b28 of *B. licheniformis*, and b38 of *B. velezensis*, can fix nitrogen; 8 strains, including b7 and b28 of *B. licheniformis*, b5, b10, and b23 of *B. subtilis*, b8 of *B. safensis*, and b6 of *Priestia megaterium*, could dissolve inorganic phosphorus; 11 strains, including b9, b12, b14, b30, b34, and b43 of *B. velezensis*, b6 of *Priestia megaterium*, and b17, b18, b20, and b26 of *B. subtilis*, could degrade organic phosphorus; and 5 strains, including b4, b5, and b26 of *B. subtilis*, and b7 and b28 of *B. licheniformis* could dissolve potassium. These strains are valuable resources of endophytic bacteria that have adapted to the ecological environment of the Cold Plateau apple-production area and could be used as plant disease biocontrol agents and biofertilizers of crops. The culturable phyllosphere endophytes in apple leaves relate to geographic locations, apple varieties, and environment, providing the basis to explain the mechanisms underlying the establishment of apple endophyte diversity and may help to devise apple disease management strategies.



**Citation:** Pan, X.; He, P.; Zhou, C.; Cun, H.; He, P.; Munir, S.; Wu, Y.; Ahmed, A.; Asad, S.; Ma, J.; et al. Characterization and Antagonistic Effect of Culturable Apple-Phyllosphere Endophytic Bacteria from the Cold Plateau in Yunnan, China. *Horticulturae* **2022**, *8*, 991. <https://doi.org/10.3390/horticulturae8110991>

Academic Editors: Rytis Rugienius, Vidmantas Stanys and Birutė Frercks

Received: 22 August 2022

Accepted: 4 October 2022

Published: 26 October 2022

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**Keywords:** apple; endophytic bacteria; *Bacillus*; low-latitude plateau

## 1. Introduction

The low-latitude plateau of Yunnan is the main apple-producing area in the southern region of China besides the Loess Plateau, Bohai Bay, and the old course of the Yellow River [1]. The large temperature difference between day and night in the Cold Plateau climate and the availability of sufficient sunshine give Yunnan apples unique characteristics such as a high sugar content, their red color, and a high commercial quality. Because of its geographical advantage, being adjacent to Southeast Asia, Yunnan has become an important bridgehead for apple exports to Southeast Asia; for instance, Zhaotong, Lijiang, Malong, and Tuanjie Township located in Yunnan Province are also famous for high-quality apples [2]. However, in recent decades, various apple tree diseases have been reported, and the disease incidence has been alarmingly increasing with the increase in apple planting areas and the change in the planting environment [3,4]. Apple early defoliation, apple canker (*Valsa mali*), rot (*Rosellinia necatrix*, *Fusarium solani*, and *F. oxysporum*), stem and branch ring rot (*Botryosphaeria dothidea*), and other major diseases have caused unprecedented damage, resulting in the decline in apple yield and industry [3]. At present, prevention and control rely upon excessive and unrestrained applications of chemical reagents. The extensive use of pesticides and fertilizers in apple orchards disturbs the soil composition and microbial communities, making the environment conducive to disease. Today, high-quality, contamination-free food is increasingly in demand; therefore, it is extremely important to adopt safe and ecofriendly options for disease control that ensure the protection of orchard microflora and the sustainability of the apple industry [5,6].

Endophytes are microbes, mostly bacteria and fungi, that live in host plants without producing any apparent disease symptoms. The role of endophytic bacteria is to assist the plant in uptaking nutrients, improve stress tolerance, and provide disease and pest resistance. Endophytic bacterial communities have been found to be beneficial for agriculture sustainability, including plant-growth promotion and disease resistance induction [7–9]. Detailed endophytic studies on healthy apple tree roots displayed high phosphorus-solubilizing and nitrogen-fixing activities and also produced indole acetic acid (IAA). The plant height, root length, and dry weight of tomato were significantly increased in a growth-promotion experiment. The growth-promoting microbial community inside and outside the fruit tree and the fruit tree itself form a large-scale micro-ecosystem that is linked to each part. The normal operation of this system is related to the vigorous growth of the apple tree. In contemplating global climate change and the elevated temperature in the present study areas, the bacterial microbiota have a larger role to play in future biological remedies for phytopathogens. To date, endophytic bacteria have been isolated from cotton, rice, potato, tomato, pepper, citrus, banana, pseudo-ginseng, and other plants [10]. Previously, several studies were published that described endophytic micro-organisms of apples, including fungi and bacteria. A culture-independent analysis showed the presence of Proteobacteria, Acidobacteria, Bacteroidetes, Actinobacteria, Chlamydiae, and Firmicutes bacterial classes, including 24 and 17 taxonomic units in healthy and infected roots. A culture-dependent analysis identified firmicutes of the genus *Bacillus*, *Lysinibacillus*, and *Paenibacillus*, and gammaproteobacteria of the genus *Pseudomonas*. There are some studies involving endophytes in reducing apple diseases, such as apple rot and European canker caused by *Valsa mali* and *Neonectria ditissima*, respectively, and the apple scab caused by *Venturia inaequalis* [11–14]. However, the ecological environment and cultivated varieties of apple-producing areas in Yunnan, China are different from those in northern China [2,15] and other apple-production areas in the world. Different varieties and ecological environments are bound to breed different communities of endophytic bacteria [12,16]. The endophytic bacteria in Yunnan are bound to be more suitable for the ecological environment of Yunnan than endophytic bacteria from other places. There have been few reports on

endophytic bacteria from the Cold Plateau apple-production area in Southwest China. It is extremely important to explore the potential of apple endophytic bacteria adapted to this ecological region to increase apple health and mitigate apple diseases. Therefore, this study will focus on the isolation and identification of apple-phyllosphere endophytic bacteria from the Cold Plateau in Yunnan, screening for effective biocontrol and plant-growth-promoting agents and analyzing the impact of endophytic bacteria diversity in this region, so that we can obtain valuable information to explain how endophytic bacteria adapt to this ecological environment and lay the foundation to adjust the cultivation strategies to improve endophytic bacterium communities.

## 2. Materials and Methods

### 2.1. Sample Collection

Fresh and healthy apple leaves were collected from the following cultivars between May and August 2019: Red Fuji, Red General, Gala, Golden Handsome, Asus, Red Dew, Longga, Red Love, Variety 2001, Huarui, Dounan Hongguoguang, Sansa, Columnar, Venus, America No. 8, Red Ai, Yantai Fuji No. 3, Richest Man No. 1, and Jinqun from Luxi County, Honghe Prefecture, and Stone Forest County, Kunming City (low latitude in southern Yunnan); Red General, Longga, Longwei, Red Love, Nagafu No. 2, Jinshiji, and Huadan from Malong County, Qujing (central Yunnan); Red Fuji, Gala, Asus, Jonagold, and Benishogun from Tuanjie Township, Kunming City (central Yunnan); Red Fuji, Golden Handsome, Red Dew, Green Banana, Variety 108, and Soft Red from Zhaotong (northeast Yunnan); and Red Fuji, Red General, Gala, Golden Handsome, Nagafu No. 2, and Jonagold from Ninglang County, Lijiang (northwest Yunnan). Three trees were chosen for each variety and considered as a single pool for endophyte isolation. Six leaves were collected from each tree. The samples were stored in a 4 °C refrigerator for endophytic bacteria isolation.

The main geographical locations of the different sampling sites are as follows:

The northeast Yunnan Zhaotong production area, the largest apple-production base in the Yunnan Zhaotong production area, is located in the northeast of Yunnan Province at 26–29° N latitude and 103–105° E longitude; it is about 1900–2200 m above sea level, the annual average temperature is 11.7 °C, the summer is warm and cool, and the winter is cold. Due to the great difference in altitude, the three-dimensional vertical climate is obvious. The Malong production area in Qujing, eastern Yunnan, is about 1900–2300 m above sea level, where the average annual temperature is 12.7 °C and the annual climate is cool. There are long periods of sunshine, the rainfall is moderate, and the temperature difference between day and night is large. The production area of Tuanjie Township, Kunming, central Yunnan, is about 1900–2200 m above sea level, with an average annual temperature of 14.5 °C. The temperature in winter is low, and the temperature difference between day and night is large.

### 2.2. Pathogen Strains

The tested pathogens used in this study were *Alternaria alternata*, *Botryosphaeria dothidea*, *Valsa mali*, *Rosellinia necatrix*, *Fusarium solani*, and *F. oxysporum* from apple, *Botrytis cinerea* from tomato, *A. alternata* and *Phytophthora nicotianae* from tobacco, *Rhizoctonia solani* from corn and *F. oxysporum* f. sp. *cubense* from banana, all which were provided by the Biopesticide Laboratory, Yunnan Agricultural University, Kunming, China [17].

### 2.3. Isolation of Endophytic Bacteria from Apple Leaves

The bacterial endophytes were isolated from healthy apple leaves and cultured in Luria–Bertani medium (LB) based on the following isolation procedure. About 1.0 g of leaves was soaked in 75% alcohol for 30 s, followed by being soaked in 2.5% sodium hypochlorite for 1 min for leaf surface sterilization; then, the leaves were washed with sterile water 5 times and grinded with 9 mL of sterile water using a mortar and pestle for 2 min for homogenization. Through gradual 10-gradient dilutions, the homogenates were spread on LB plates and cultured in a constant temperature incubator at 37 °C for

24 h [18,19]. The single and pure colony was further cultured in 100 mL of LB liquid medium and incubated in a shaker at 37 °C and 160 × g/min for 48 h. Then, 40% aseptic glycerol was added with the same amount of bacterial culture solution and stored in a –80 °C refrigerator [7].

#### 2.4. Morphological Characterization of Endophytic Bacteria

The isolates were cultured in LB agar medium and incubated at 35 °C for 24–48 h. Then, the colony morphology was observed and described according to the Manual for Systematic Identification of Common Bacteria [20].

#### 2.5. Molecular Identification of Endophytic Bacteria

In total, the DNA of 39 endophytic bacterial strains was extracted following Cun (2019) and amplified with PCR using *rpoB* gene and 16S *rRNA* gene sequences as marker genes [18,19,21] for molecular identification.

The PCR amplicons were sent to TSINGKE® Co. Ltd., Beijing, China for sequencing. The obtained sequences were aligned using DNAMAN software (version 6.0.3.99, Lynnon Biosoft, San Ramon, CA, USA) and analyzed using the BlastN program (<http://www.ncbi.nlm.nih.gov/BLAST>, accessed on 13 December 2021). The neighbor-joining (NJ) method in Mega software (version 5.0, MEGA, Richlandtown, PA, USA) was used to construct phylogenetic trees, and the bootstrap value was set to 500.

#### 2.6. Antagonistic Potential of Endophytic Bacteria

The dual-culture assay was used to screen for antagonistic activity of endophytic bacteria. The indicator fungus disc (diameter = 0.8 cm) was placed in the center of the media plate, and then, the endophytic bacterial strains were inoculated with a sterile pipette tip on the plate, 2.5 cm around the disc. When the control plate without the endophyte was full of indicator fungus hyphae, the inhibition zone and colony diameters were measured and recorded, and the inhibition rate of colony growth was calculated. The antagonistic effect was expressed by the inhibition rate and inhibition bandwidth [11,19,22,23]. The antagonistic strains screened in the first dual-culture assay were confirmed in the second dual-culture assay and the inhibition rate was calculated via the following formula:

$$\text{Inhibition rate (\%)} = [(\text{Fungal colony diameter in control group} - \text{Fungal colony diameter in treatment group}) / \text{Fungal colony diameter in control group}] \times 100$$

#### 2.7. In Vitro Assay for Traits Related to Plant-Growth Promotion of the Endophytic Bacteria

##### 2.7.1. Phosphate Solubilization

The Pikovskaya (PVK) medium (glucose 10.0 g/L, Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 5.0 g/L, NaCl 0.2 g/L, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g/L, KCl 0.2 g/L, Yeast extract 0.5 g/L, MnSO<sub>4</sub>·1H<sub>2</sub>O 2.0 mg/L, FeSO<sub>4</sub>·7H<sub>2</sub>O 2.0 mg/L, bromophenol blue 25.0 mg/L, agar 20.0 g/L, pH 7.0), described by YaÑez-Ocampo et al. (2020), and calcium phytate solid medium (glucose 10.0 g/L, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.2 g/L, MgCl<sub>2</sub>·6H<sub>2</sub>O 5.0 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g/L, KCl 0.1 g/L, calcium phytate 2.0 g/L, agar 20.0 g/L, pH 7.0), described by Li et al. (2021), were used to determine the inorganic and organic phosphate-solubilizing activity of 39 endophytic bacteria by incubation at 28 °C for seven days [24,25]. The growth was associated with the uptake of phosphate in the form of Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> or C<sub>6</sub>H<sub>16</sub>CaO<sub>24</sub>P<sub>6</sub> as a sole phosphate source, which was determined as a clear zone around the bacterial colony.

##### 2.7.2. Potassium Solubilization

The potassium feldspar (PF) solid medium (sucrose 10.0 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g/L, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.2 g/L, NaCl 0.1 g/L, CaCO<sub>3</sub> 0.1 g/L, potassium feldspar 5.0 g/L, bromophenol blue 25.0 mg/L, agar 20.0 g/L, pH 7.2) was used to assess the potassium-solubilizing capability of 39 endophytic bacterial strains [26]. These strains were cultured on PF medium

plates and incubated at 28 °C for seven days, and a clear zone around the bacterial colonies was observed.

### 2.7.3. Nitrogen Fixation

To determine the nitrogen fixation ability, 39 endophytic strains were grown on the nitrogen-free (NF) Ashby medium (glucose 5.0 g/L, mannitol 5.0 g/L, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.1 g/L, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.1 g/L, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 5.0 mg/L, K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O 0.9 g/L, KH<sub>2</sub>PO<sub>4</sub> 0.1 g/L, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.01 g/L, CaCO<sub>3</sub> 5.0 g/L, agar 20.0 g/L, pH 7.3) [4,27] and incubated at 28 °C for seven days, and a circle was observed around the bacterial colonies.

### 2.8. Data Processing and Analysis

The data were statistically analyzed using an analysis of variance (ANOVA) in IBM SPSS Statistics 23, and the means were subjected to Duncan's multiple-range test at  $p \leq 0.05$ . The phylogenetic tree of different lines was jointly constructed by the neighbor-joining (NJ) method in MEGA software (Version 5.0, MEGA, Richlandtown, PA, USA).

## 3. Results

### 3.1. Morphological Characteristics of Endophytic Bacteria

A total of 5709 colonies of endophytic bacteria were isolated from leaves of 30 apple varieties collected from the main apple-producing areas in Yunnan Province. Among them, 39 isolates were further selected according to their morphological characters, including colony size, shape, color, edge feature, protrusion shape, surface appearance, transparency, and spores (Figure 1).

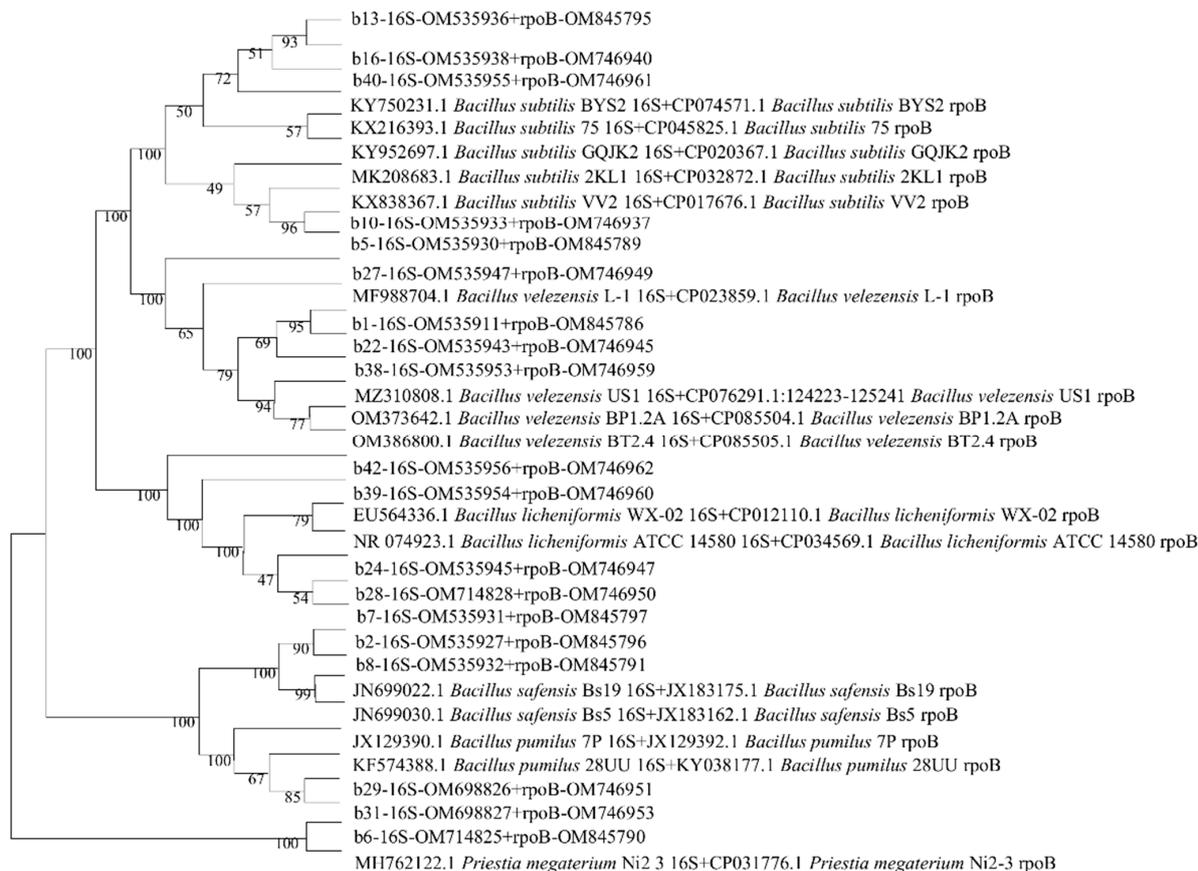


**Figure 1.** Morphological characteristics of 39 endophytic bacteria. (A): b1, (B): b2, (C): b3, (D): b4, (E): b5, (F): b6, (G): b7, (H): b8, (I): b9, (J): b10, (K): b11, (L): b12, (M): b13, (N): b14, (O): b15, (P): b16, (Q): b17, (R): b18, (S): b20, (T): b21, (U): b22, (V): b23, (W): b24, (X): b26, (Y): b27, (Z): b28, (AA): b29, (AB): b30, (AC): b31, (AD): b32, (AE): b33, (AF): b34, (AG): b36, (AH): b37, (AI): b38, (AJ): b39, (AK): b40, (AL): b42, (AM): b43.

### 3.2. Molecular Characterization of Endophytic Bacteria

After amplification, single and bright bands were obtained and sequenced. The BLAST alignment was performed for all the sequences obtained that have known sequences in the NCBI database. The phylogenetic trees of different strains were constructed by the neighbor-joining (NJ) method. The endophytic bacteria isolated from apple leaves mainly belonged to the genus *Bacillus*, including six species: *B. subtilis*, *B. velezensis*, *B. licheniformis*,

*B. safensis*, *B. pumilus*, and *Priestia megaterium*, based on the combination analyses of *rpoB* with 16S *rRNA* gene sequences. Among them, b6 and Ni2-3 are clustered together with high similarity to form *P. megaterium*; b29, b31, 7P, and 28UU are clustered with high similarity to form *Bacillus pumilus* (*B. pumilus*); b2, b8, Bs5, and Bs19 are clustered with high similarity to form *B. safensis*; b7, b24, b28, b39, b42, ATCC, and WX-02 are clustered with high similarity to form *Bacillus licheniformis* (*B. licheniformis*); b1, b9, b12, b14, b15, b22, b27, b30, b34, b38, b43, L-1, LABIM22, BT2.4, and other strains are clustered together with high similarity to form *Bacillus* (*B. velezensis*); and b3, b4, b5, b10, b11, b13, b16, b17, b18, b20, b21, b23, b26, b32, b33, b36, b37, b40, VV2, S-16, BY2, and other strains are clustered with high similarity to form *B. subtilis* (*B. subtilis*). The relationship is expressed as *P. megaterium* > *B. pumilus* > *B. safensis* > *B. licheniformis* > *B. velezensis* > *Bacillus subtilis* (*B. subtilis*), of which *P. megaterium* and *B. subtilis* are the most distantly related and have the greatest difference, and *B. pumilus* and *Bacillus safensis* (*B. safensis*) have the closest relationship and the least difference. All *B. subtilis* (*B. subtilis*) are divided into two branches, among which the b3, b4, b5, b10, b11, b18, b20, b21, b23, b32, b36, and b37 strains are grouped together, and the differences between them are greater. In addition, the b13, b16, b17, b26, b33, and b40 strains are grouped into a branch, and b3 and other strains are quite different and have a farther relationship than that between b13 and other strains. All *B. velezensis* strains are also divided into two branches; b1, b9, b12, b14, b15, b22, b30, b34, b38, and b43 strains are clustered into one branch, the differences between them are smaller, and thus, the relationship is closer. The b27 strain is a separate branch, where the difference between the b27 strain and the b1 strain is relatively large and the relationship is farther away (Figure 2). All the sequences of these strains of endophytic bacteria are available on the website <https://www.ncbi.nlm.nih.gov/>, accessed on 9 January 2022), with their *rpoB* and 16S *rRNA* gene sequence accession numbers OM845786-OM746963.



**Figure 2.** Neighbor-joining phylogenetic tree based on *rpoB* and 16S *rRNA* gene sequences of endophytic bacterial strains. Bootstrap values of 1000 replications are shown next to the branches.

### 3.3. Diversity of Apple CEB in Yunnan Province

#### 3.3.1. Geographical Distribution of CEB

The number and distribution frequency of bacteria were different in the apple-producing areas of Yunnan Province, China. The most abundant number of endophytic bacteria were found in the mornity apple-production area in Ninglang County, Lijiang, Yunnan and in the early- and medium-ripening area in Malong, Qujing, eastern Yunnan with  $2.17 \times 10^4 \pm 6.58 \times 10^3$  cfu/g and  $2.91 \times 10^4 \pm 2.140 \times 10^4$  cfu/g fresh leaf (FL), and the distribution frequency was 33.86% and 45.26%, respectively (Tables 1 and S1); these were followed by the low-altitude Luxi County rocky desertification apple-production area, where the population of endophytic bacteria was  $9.88 \times 10^3 \pm 3.32 \times 10^3$  cfu/g FL, and the distribution frequency was 15.38%. The population and distribution frequency of endophytic bacteria in other ecological regions were very low. In the same apple-production area, the content and distribution frequency of these endophytic bacteria, *B. velezensis*, *B. subtilis*, *B. licheniformis*, *B. safensis*, *B. pumilus*, and *Priestia megaterium*, were different. In Malong, Qujing, in the early- and medium-ripening area in eastern Yunnan, the highest abundance of endophytic bacteria was recorded; among those, *B. velezensis* was the most abundant with  $1.49 \times 10^4 \pm 1.07 \times 10^4$  cfu/g LF. The results proved that *B. subtilis* and *B. velezensis* were the dominant endophytic bacteria in apple leaves and distributed most widely in the Yunnan Cold Plateau apple-production area in China. In the different apple-production areas, *B. subtilis*, *B. licheniformis*, *B. safensis*, *B. pumilus*, and *Priestia megaterium* had different distribution frequencies.

**Table 1.** Distribution frequency of CEB types in apple-producing areas of Yunnan.

Ecological Type of Production Area	Region	<i>B. subtilis</i>	<i>B. velezensis</i>	<i>B. licheniformis</i>	<i>B. safensis</i>	<i>B. pumilus</i>	<i>Priestia megaterium</i>
Northeast main apple-production area in Yunnan	Zhaotong	4/6	3/6	4/6	2/6	1/6	0
Rocky desertification apple-production area in Yunnan	Honghe, Luxi, and Kunming Stone Forest	15/21	18/21	12/21	9/21	6/21	3/21
Northwest mornity apple production area in Yunnan	Lijiang, Ninglang	6/6	6/6	6/6	1/6	2/6	0
Early- and medium-ripening in Yunnan	Qujing, Malong	5/7	6/7	5/7	6/7	3/7	0
Central Kunming apple-production area in Yunnan	Kunming, Tuanjie Township	5/5	5/5	4/5	2/5	1/5	0

The Malong variety in Qujing reported *B. velezensis* as the most abundant, with  $1.49 \times 10^4 \pm 1.07 \times 10^4$  cfu/g FL, and a distribution frequency of 6/7; this was followed by *B. subtilis* with  $1.18 \times 10^4 \pm 1.10 \times 10^4$  cfu/g FL and a distribution frequency of 5/7. The number of colonies and the distribution frequency, however, were very low in Tuanjie Township, Kunming City (Table 1).

#### 3.3.2. Diversity of Endophytic Bacteria in Different Varieties

The most dominant strain isolated in this study belongs to *Bacillus*. The amount and proportion of main endophytes of *B. subtilis*, *B. velezensis*, *B. licheniformis*, *B. safensis*, *B. pumilus*, and *Priestia megaterium* varied among the apple varieties of Red Fuji, Red General, Gala, and Golden Handsome. *B. subtilis* and *B. velezensis* showed the highest abundance and proportion among the four test varieties (Table 2). The average population and proportion of *B. subtilis* were  $1.81 \times 10^4 \pm 6.77 \times 10^3$  cfu/g FL and 39.90%, respectively; *B. velezensis* was  $1.94 \times 10^4 \pm 6.52 \times 10^3$  cfu/g FL and 42.78%; and *B. licheniformis* was  $6.38 \times 10^3 \pm 3.80 \times 10^3$  cfu/g FL and 14.08%. However, *B. safensis*, *B. pumilus*, and *Priestia megaterium* were lower. The average population number was  $1.57 \times 10^4 \pm 6.17 \times 10^3$  cfu/g FL, accounting for 37.18% for Red General,  $1.46 \times 10^4 \pm 1.30 \times 10^4$  cfu/g FL (34.61%) for Golden Handsome,  $8.92 \times 10^3 \pm 7.08 \times 10^3$  cfu/g FL (21.10%) for Red Fuji, and  $3.00 \times 10^3 \pm 2.88 \times 10^3$  cfu/g FL (7.10%) for Gala. The highest number,

$9.21 \times 10^3 \pm 3.25 \times 10^3$  cfu/g of *B. velezensis* was from the Red General cultivar,  $8.82 \times 10^3 \pm 8.50 \times 10^3$  cfu/g FL of *B. subtilis* from the Golden Handsome cultivar,  $4.96 \times 10^3 \pm 4.87 \times 10^3$  cfu/g FL of *B. velezensis* from Golden Handsome,  $4.67 \times 10^3 \pm 3.63 \times 10^3$  cfu/g FL of *B. subtilis* from Red Fuji,  $4.40 \times 10^3 \pm 3.06 \times 10^3$  cfu/g FL of *B. licheniformis* from Red General, and  $2.01 \times 10^3 \pm 1.15 \times 10^3$  cfu/g FL of *B. subtilis* from Red General. The number of other endophytic bacteria from other cultivars was relatively low. The study proved that the number and colony of species of endophytic bacteria varied with the different varieties.

**Table 2.** Distribution frequency of CEB types in apple varieties.

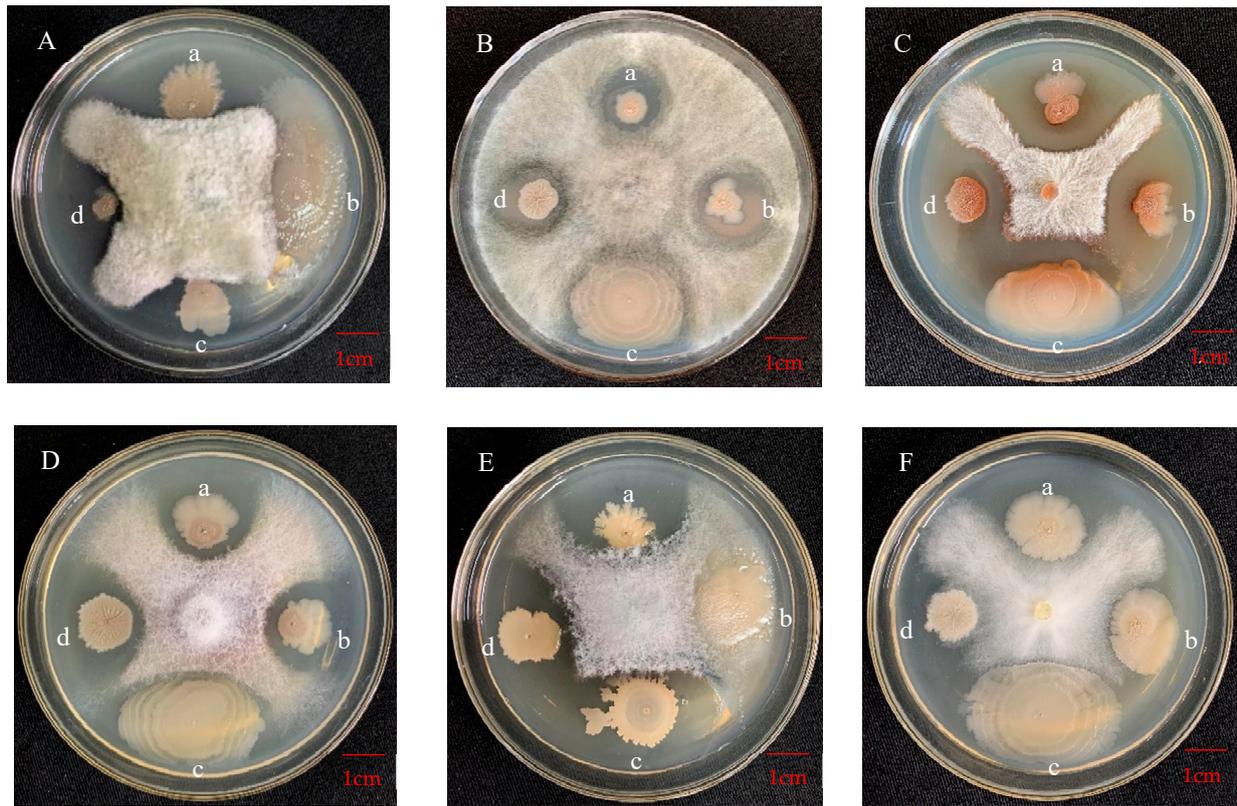
Varieties	<i>B. subtilis</i>	<i>B. velezensis</i>	<i>B. licheniformis</i>	<i>B. safensis</i>	<i>B. pumilus</i>	<i>Priestia megaterium</i>
Red Fuji	3/4	3/4	4/4	2/4	3/4	1/4
Red General	3/3	3/3	3/3	3/3	1/3	0
Gala	2/3	2/3	1/3	0	0	0
Golden Handsome	3/3	2/3	3/3	1/3	1/3	0

### 3.4. Antagonistic Effect of Endophytic Bacteria on Plant Pathogens

#### 3.4.1. Antagonistic Effect on Apple Pathogens

The antagonistic activities of potential endophytic strains of endophytic bacteria against six kinds of apple pathogens, *A. alternata*, *B. dothidea*, *F. oxysporum*, *F. solani*, *R. necatrix*, and *V. mali*, were determined (Table 3). The endophytic strains with reduced activities or no activities were not considered in further experiments.

In this experiment, the inhibition rate > 35% and the inhibition bandwidth > 0.45 cm were used to evaluate the pathogens and endophytes [23]. The strains with antagonistic activity were mainly concentrated on *B. subtilis* and *B. velezensis*. The endophytes had the highest inhibitory potential on *A. alternata*, *V. mali*, and *R. necatrix*. Among them, a total of 21 strains had good antagonistic activity against *A. alternata*; a total of 10 strains, 31 strains, 18 strains, 14 strains, and 26 strains had good antagonistic activity against *B. dothidea*, *V. Mali*, *F. oxysporum*, *F. solani*, and *R. necatrix*, respectively, and the inhibition rate was more than 35%. A total of 19 strains, 2 strains, 23 strains, 11 strains, 10 strains, and 17 strains of endophytic bacteria had an antifungal bandwidth of more than 0.45 cm against *A. alternata*, *B. dothidea*, *V. mali*, *F. oxysporum*, *F. solani*, and *R. necatrix*, respectively. Inhibiting the pathogen of *A. alternata*, the highest inhibition rate was  $39.55 \pm 1.32\%$  from *B. velezensis* (b27), followed by  $39.09 \pm 0.40\%$  from *B. subtilis* (b36) (Figure 3). For inhibiting the *V. mali* pathogen, the highest inhibition rate is  $46.31 \pm 0.29\%$  from b4 of *B. subtilis*, and it is also the highest inhibition rate in the antagonistic experiment of 39 endophytic bacteria against six kinds of apple pathogens; it was followed by  $45.74 \pm 0.85\%$  from AB b22 of *B. velezensis*. When inhibiting *R. necatrix* pathogens, the highest inhibition rate was  $44.04 \pm 0.29\%$  from b27 of *B. velezensis*, followed by  $43.47 \pm 0.85\%$  from b17 of *B. subtilis*.



**Figure 3.** Inhibition of apple endophytic bacteria to apple disease pathogens. **(A):** *Alternaria alternata*. a: *B. subtilis* b13; b: *B. velezensis* b14; c: *B. velezensis* b15; d: *B. subtilis* b16. **(B):** *Botryosphaeria dothidea*. a: *B. velezensis* b9; b: *B. subtilis* b10; c: *B. subtilis* b11; d: *B. velezensis* b12. **(C):** *Valsa mali*. a: *B. velezensis* b9; b: *B. subtilis* b10; c: *B. subtilis* b11; d: *B. velezensis* b12. **(D):** *Fusarium oxysporum*. a: *B. velezensis* b9; b: *B. subtilis* b10; c: *B. subtilis* b11; d: *B. velezensis* b12. **(E):** *Fusarium solani*. a: *B. subtilis* b21; b: *B. velezensis* b22; c: *B. subtilis* b23; d: *B. licheniformis* b24. **(F):** *Rosellinia necatrix*. a: *B. velezensis* b9; b: *B. subtilis* b10; c: *B. subtilis* b11; d: *B. velezensis* b12.

Inhibiting *B. dothidea*, *F. oxysporum*, and *F. solani*, the highest inhibition rates were  $39.32 \pm 0.58\%$  from b1 of *B. velezensis*,  $38.64 \pm 0.46\%$  from b1 of *B. velezensis*, and  $38.18 \pm 3.34\%$  from b33 of *B. subtilis*, and the maximum inhibition bandwidths were  $0.50 \pm 0.0633$  cm from b30 of *B. velezensis*,  $0.60 \pm 0.0817$  cm from b1 of *B. velezensis*, and  $0.64 \pm 0.1166$  cm from b13 of *B. subtilis*.

The results proved that endophytic bacteria dominated by *B. subtilis* and *B. velezensis* have the highest inhibitory potential on *A. alternata*, *V. mali*, and *R. necatrix*, and have the potential to be developed into biological agents.

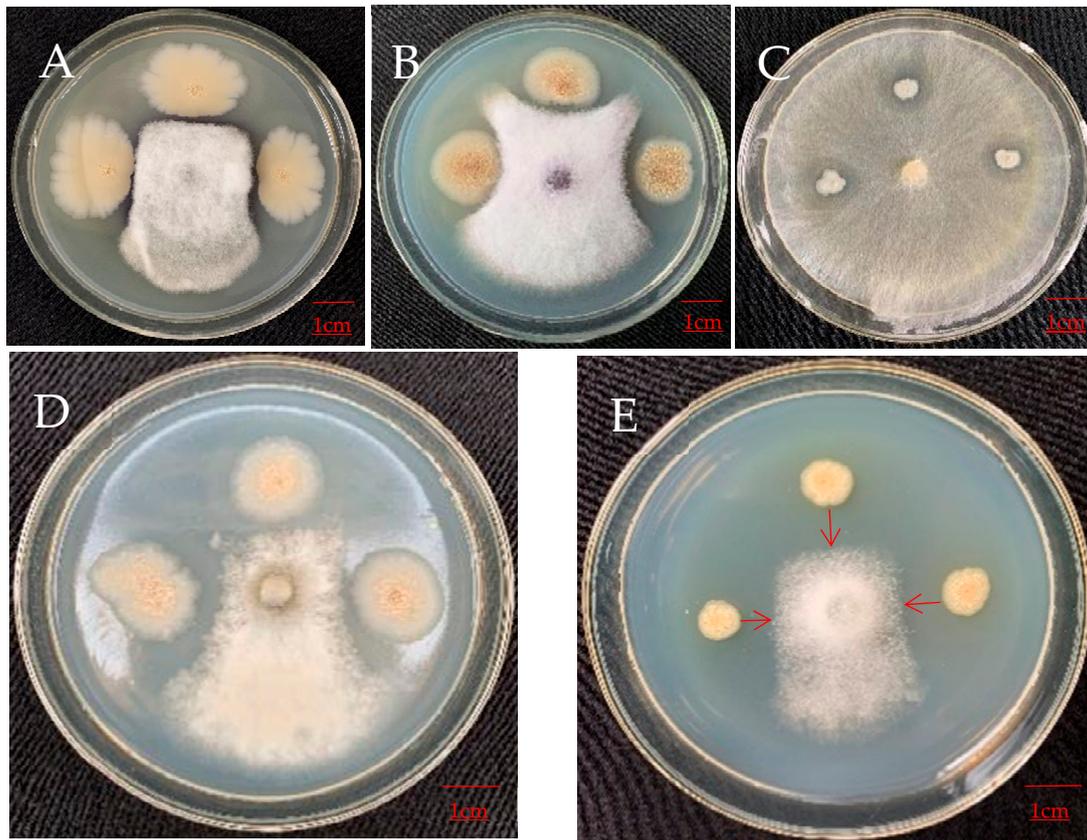
**Table 3.** Antagonism of apple endophytic bacteria against apple fungal pathogens.

Endophyte	<i>Alternaria alternata</i>		<i>Botryosphaeria dothidea</i>		<i>Valsa mali</i>		<i>Fusarium oxysporum</i>		<i>Fusarium solani</i>		<i>Rosellinia necatrix</i>	
Species	IB/cm	IR/%	IB/cm	IR/%	IB/cm	IR/%	IB/cm	IR/%	IB/cm	IR/%	IB/cm	IR/%
<i>B. subtilis</i>	0.48 ± 0.0160 a	35.85 ± 0.38 a	0.26 ± 0.0153 a	34.10 ± 0.37 a	0.60 ± 0.0478 a	40.04 ± 0.53 a	0.37 ± 0.0265 a	34.00 ± 0.74 a	0.39 ± 0.0322 a	34.87 ± 0.42 a	0.39 ± 0.0494 a	37.83 ± 0.69 a
<i>B. velezensis</i>	0.55 ± 0.0170 a	32.03 ± 0.56 a	0.32 ± 0.0252 a	33.58 ± 0.62 a	0.59 ± 0.0571 a	40.88 ± 0.65 a	0.42 ± 0.0308 a	34.07 ± 0.50 a	0.31 ± 0.0303 ab	32.91 ± 0.41 a	0.34 ± 0.0533 a	38.49 ± 0.59 a
<i>B. licheniformis</i>	0.25 ± 0.0486 b	24.40 ± 3.95 b	0.06 ± 0.0205 bc	18.42 ± 4.14 b	0.11 ± 0.0340 b	24.50 ± 5.04 c	0.03 ± 0.0188 b	18.96 ± 4.25 b	0.17 ± 0.0478 abc	19.20 ± 4.31 b	0.12 ± 0.0292 a	29.12 ± 3.79 b
<i>B. safensis</i>	0.10 ± 0.0447 bc	15.34 ± 6.86 c	0.17 ± 0.0644 ab	11.45 ± 4.19 c	0.38 ± 0.0559 ab	28.98 ± 2.29 bc	0.00 ± 0.0000 b	0.00 ± 0.00 c	0.00 ± 0.0000 c	0.00 ± 0.00 c	0.09 ± 0.0404 a	25.29 ± 3.08 b
<i>B. pumilus</i>	0.26 ± 0.1017 b	23.15 ± 6.79 b	0.26 ± 0.0563 a	31.46 ± 1.42 a	0.59 ± 0.1353 a	35.55 ± 2.96 ab	0.20 ± 0.0906 ab	21.88 ± 6.43 b	0.11 ± 0.0581 bc	21.59 ± 6.44 b	0.31 ± 0.1503 a	23.05 ± 8.17 b
<i>Priestia megaterium</i>	0.00 ± 0.0000 d	0.00 ± 0.00 d	0.00 ± 0.0000 c	0.00 ± 0.00 d	0.00 ± 0.0000 b	0.00 ± 0.00 d	0.00 ± 0.0000 b	0.00 ± 0.00 c	0.00 ± 0.0000 c	0.00 ± 0.00 c	0.00 ± 0.0000 a	0.00 ± 0.00 c

Note: IB, inhibitory bandwidth; IR, inhibition rate. Different letters indicate the statistic difference among the column ( $p \leq 0.05$ ).

### 3.4.2. Antagonistic Effect on Pathogens of Other Plants

The inhibition spectrum of the total 39 endophytic bacteria strains against 5 indicator pathogens (*Alternaria alternata*, *P. nicotianae*, *F. oxysporum*, *B. cinerea*, and *R. solani*) was determined by the antagonistic experiment (Figure 4). The antagonistic strains were preliminarily screened through the preliminary screening of antagonism, and then, the endophytic bacteria with a strong antagonistic effect were rescreened to verify the results (Table 4).



**Figure 4.** Profile of apple endophytic bacteria against plant fungal pathogens. (A): *Alternaria alternata*–*B. velezensis* b12; (B): *Fusarium oxysporum*–*B. velezensis* b9; (C): *Rhizoctonia solani*–*B. velezensis* b34; (D): *Botrytis cinerea*–*B. velezensis* b9; (E): *Phytophthora nicotianae*–*B. safensis* b8.

From the data, the strains of endophytic bacteria with a wide antibacterial spectrum are mainly concentrated on strains b9, b12, b30, b34, and b43 of *B. velezensis*, b8 of *B. safensis*, and b33 of *B. subtilis*, and the endophytic bacteria have the highest inhibitory potential on *A. alternata*. Among them, there were five strains of endophytic bacteria that displayed an inhibition rate with more than 30% to *A. alternata*, and there were four strains of endophytic bacteria that the inhibition bandwidth of *A. alternata* was more than 0.30 cm. The highest inhibition rate was  $37.54 \pm 3.92\%$  from *B. velezensis* b9, followed by  $36.83 \pm 5.10\%$  from *B. velezensis* b30.

Inhibiting *F. oxysporum*, *R. solani*, *B. cinerea*, and *P. nicotianae*, the highest inhibition rates were  $26.68 \pm 0.43\%$  from b30 of *B. velezensis*,  $20.07 \pm 0.38\%$  from b33 of *B. subtilis*,  $31.53 \pm 0.45\%$  from b33 of *B. subtilis*, and  $34.82 \pm 4.48\%$  from b43 of *B. velezensis*. The maximum inhibition bandwidths were  $0.40 \pm 0.0471$  cm from b30 of *B. velezensis*,  $0.43 \pm 0.0333$  cm from b8 of *B. safensis*,  $0.23 \pm 0.0882$  cm from b9 of *B. velezensis*, and  $0.53 \pm 0.1054$  cm from b8 of *B. safensis*.

**Table 4.** Inhibiting effects of apple endophytic bacteria on fungal pathogens of plants.

Endophyte		<i>Alternaria alternata</i>		<i>Fusarium oxysporum</i>		<i>Rhizoctonia solani</i>		<i>Botrytis cinerea</i>		<i>Phytophthora nicotianae</i>	
Species	Strain	IB/cm	IR/%	IB/cm	IR/%	IB/cm	IR/%	IB/cm	IR/%	IB/cm	IR/%
<i>B. velezensis</i>	b9	0.30 ± 0.0258 ab	37.54 ± 3.92 a	0.23 ± 0.0882 abc	23.96 ± 0.52 ab	0.17 ± 0.0333 c	10.98 ± 0.38 d	0.23 ± 0.0882 a	28.83 ± 1.19 ab	0.30 ± 0.0408 b	33.11 ± 3.24 a
	b12	0.35 ± 0.0342 a	33.37 ± 5.74 a	0.33 ± 0.0500 ab	26.29 ± 1.06 a	0.30 ± 0.0577 abc	15.15 ± 0.76 b	0.10 ± 0.0000 a	25.67 ± 2.81 bc	0.17 ± 0.0882 bc	23.90 ± 0.63 ab
	b30	0.33 ± 0.0211 a	36.83 ± 5.10 a	0.40 ± 0.0471 a	26.68 ± 0.43 a	0.17 ± 0.0333 c	11.36 ± 0.66 cd	0.20 ± 0.0577 a	23.09 ± 1.09 cd	0.20 ± 0.0000 bc	28.30 ± 0.00 a
	b34	0.20 ± 0.0000 bc	28.33 ± 1.67 a	0.15 ± 0.0289 bc	26.17 ± 1.33 a	0.33 ± 0.0667 ab	14.39 ± 1.65 bc	0.10 ± 0.0000 a	19.40 ± 0.47 de	0.00 ± 0.0000 c	12.93 ± 0.81 b
<i>B. safensis</i>	b43	0.32 ± 0.0543 a	32.14 ± 2.46 a	0.13 ± 0.0333 bc	19.79 ± 0.52 b	0.27 ± 0.0333 bc	11.74 ± 1.00 cd	0.10 ± 0.0000 a	18.85 ± 0.80 e	0.38 ± 0.0364 ab	34.82 ± 4.48 a
<i>B. subtilis</i>	b8	0.10 ± 0.0000 c	24.29 ± 1.43 a	0.21 ± 0.0423 abc	5.83 ± 1.18 c	0.43 ± 0.0333 a	14.02 ± 1.00 bc	0.20 ± 0.0577 a	24.51 ± 0.50 c	0.53 ± 0.1054 a	27.38 ± 1.79 a
	b33	0.10 ± 0.0000 c	31.67 ± 0.96 a	0.10 ± 0.0000 c	22.66 ± 3.91 ab	0.17 ± 0.0333 c	20.07 ± 0.38 a	0.17 ± 0.0333 a	31.53 ± 0.45 a	0.00 ± 0.0000 c	13.04 ± 1.23 b

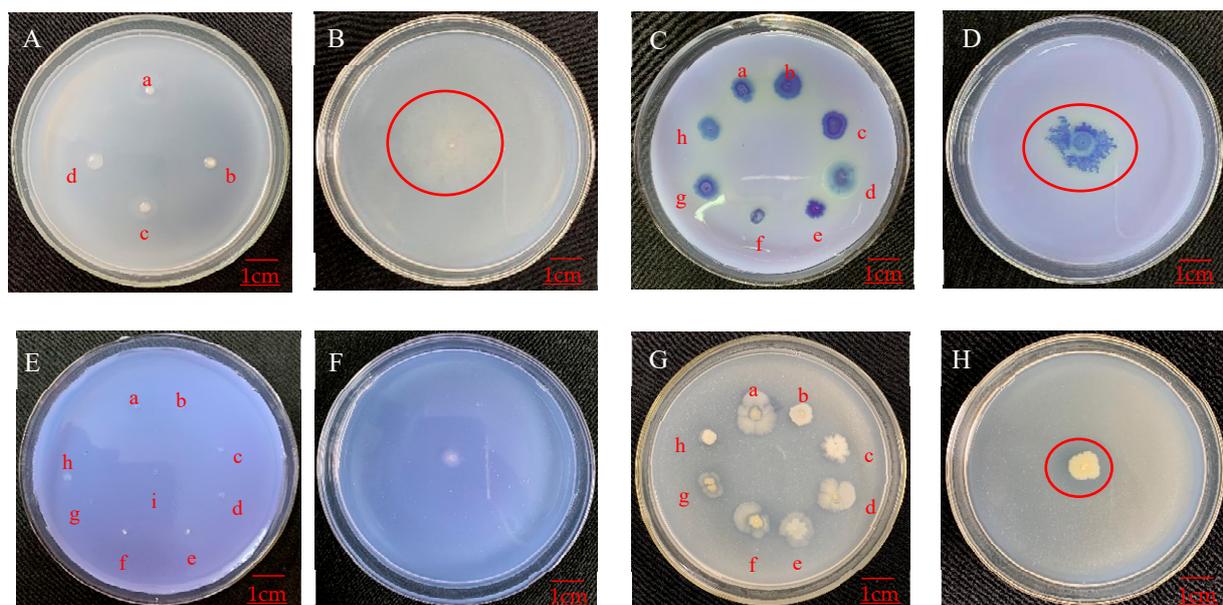
Notes: IB, inhibitory bandwidth; IR, inhibition rate. Different letters indicate the statistic difference among the column ( $p \leq 0.05$ ).

In the bacteriostatic spectrum experiment, b9 and b43 of *B. velezensis* met the corresponding indexes with two bacteriostatic bandwidths and two inhibition rates, and b12 of *B. velezensis* met the requirements of corresponding indexes with three antifungal bandwidths and one inhibition rate, occupying the first place, followed by b30 of *B. velezensis*, which had two antifungal bandwidths and one inhibition rate.

The above results proved that endophytic bacteria dominated by *B. velezensis* (b9, b12, b30, b43) have a wide antifungal spectrum and the highest inhibitory potential to *A. alternata*, which has the potential to be developed into biological agents.

### 3.5. Biological Functions of Endophytic Bacteria from Apple Leaves

In this study, some strains of endophytic bacterial were found to possess one or more activities related to nitrogen fixation, phosphorus and potassium solubilization, or the degradation of calcium phytate (Figure 5). The results indicated that a total of 10 strains, including b3, b4, b16, b17, b20, and b23 of *B. subtilis*, b7, b24, and b38 of *B. velezensis*, and b28 of *B. licheniformis* of endophytic bacteria could fix nitrogen. A total of 8 strains, including b5, b10, b11, and b23 of *B. subtilis*, b6 of *Priestia megaterium*, b7 of *B. velezensis*, b8 of *B. safensis*, and b28 of *B. licheniformis*, were able to solubilize inorganic phosphorus, whereas 5 strains, including b4, b5, and b26 of *B. subtilis*, b7 of *B. velezensis*, and b28 of *B. licheniformis*, could solubilize potassium, and 11 strains, including b6 of *Priestia megaterium*, b9, b17, b18, b20, and b26 of *B. subtilis*, and b12, b14, b30, b34, and b43 of *B. velezensis* could solubilize organic phosphorus (Table 5). Some strains of endophytic bacteria did not show any hollow halo on the functional medium with bigger colonies.



**Figure 5.** Growth performance of endophytic bacteria on functional media. Ashby nitrogen-free medium, (A): a—*B. subtilis* b21, b—*B. velezensis* b22, c—b23 (*B. subtilis*), d—b24 (*B. licheniformis*); (B): b17 (*B. subtilis*). Pikovskaya medium, (C): a—b26 (*B. subtilis*), b—b27 (*B. velezensis*), c—b28 (*B. licheniformis*), d—b29 (*B. pumilus*), e—b30 (*B. velezensis*), f—b31 (*B. pumilus*), g—b32 (*B. subtilis*), h—b33 (*B. subtilis*); (D): b28 (*B. licheniformis*). Potassium-dissolving medium, (E): a—b34 (*B. velezensis*), b—b36 (*B. subtilis*), c—b37 (*B. subtilis*), d,e—b38 (*B. velezensis*), f,g—b40 (*B. subtilis*), h—b42 (*B. licheniformis*), i—b43 (*B. velezensis*); (F): b7 (*B. licheniformis*). Calcium phytate solid medium, (G): a—b9 (*B. velezensis*), b—b10 (*B. subtilis*), c—b11 (*B. subtilis*), d—b12 (*B. velezensis*), e—b13 (*B. subtilis*), f—b14 (*B. velezensis*), g—b15 (*B. velezensis*), h—b16 (*B. subtilis*), (H): b34 (*B. velezensis*).

The study showed that b3, b4, b16, b17, b20, and b23 of *B. subtilis*, b7, b24, and b28 of *B. licheniformis*, and b38 of *B. velezensis* have a significant activity of nitrogen fixation and their colony diameters were measured up to >1.0 cm. b5, b10, b11, and b23 of *B. subtilis*,

b6 of *Priestia megaterium*, b7 and b28 of *B. safensis*, and b8 of *B. licheniformis* were able to solubilize inorganic phosphorus. b4 and b5 of *B. subtilis*, b7 and b26 of *B. licheniformis*, and b28 of *B. licheniformis* have maximum potassium-solubilizing activity with a colony diameter of >0.5 cm. Moreover, b6 of *Priestia megaterium*, b9 and b12 of *B. velezensis*, b17, b18, b20 and b26 of *B. subtilis*, and b14, b30, b34 and b43 of *B. velezensis* displayed a maximum degradation of organic phosphorus with over 1.0 cm colony diameters. Finally, b4, b5, b20, b23, and b26 of *B. subtilis*, b6 of *Priestia megaterium*, and b7 and b28 of *B. licheniformis* had the highest probiotic potential (Table 5).

**Table 5.** Characters of endophytic bacteria on functional culture media.

Species	Strain	Nitrogen Fixation	Inorganic Phosphorus Solubilization	Potassium Solubilization	Organic Phosphorus Solubilization
<i>B. subtilis</i>	b3	+++	+	-	++
	b4	+++	++	++	++
	b5	++	+++	++	+
	b10	++	+++	-	++
	b11	++	+++	-	+
	b13	+	+	-	++
	b16	+++	+	+	+
	b17	+++	-	+	+++
	b18	++	++	-	+++
	b20	+++	++	+	+++
	b21	+	++	-	++
	b23	+++	+++	-	++
	b26	+	++	++	+++
	b32	+	+	-	++
	b33	+	+	-	++
	b36	++	+	-	+
b37	+	+	-	+	
b40	++	+	-	-	
<i>B. velezensis</i>	b1	++	+	-	+
	b9	++	-	-	+++
	b12	++	+	-	+++
	b14	++	+	-	+++
	b15	+	-	-	+
	b22	-	-	-	-
	b27	++	++	-	+
	b30	+	++	+	+++
	b34	++	-	-	+++
b38	+++	++	-	+	
b43	+	+	-	+++	
<i>B. licheniformis</i>	b7	+++	+++	++	+
	b24	+++	-	-	+
	b28	+++	+++	++	+
	b39	++	++	-	-
b42	++	+	-	-	
<i>B. safensis</i>	b2	-	-	-	-
	b8	-	+++	-	++
<i>B. pumilus</i>	b29	-	+	-	-
	b31	++	-	-	+
<i>Priestia megaterium</i>	b6	+	+++	+	+++

Notes: +++ colony diameter > 1.0 cm, 0.5 cm < ++ colony diameter ≤ 1.0 cm, 0.2 cm < + colony diameter ≤ 0.5 cm; - colony diameter ≤ 0.2 cm; initial colony diameter 0.1 cm.

#### 4. Discussion

The present study was carried in the Yunnan apple-producing areas, which are the Cold Plateau apple-production areas in China that are different from the other main north Chinese apple-production areas due to diverse ecological conditions. A large number of pesticides and chemical fertilizers are used every year; however, there are still many disease problems that are difficult to solve, such as rot disease caused by *Valsa mali*, early defoliation disease by *Alternaria mali*, and root rot by *Fusarium oxysporum* [28,29].

The chemical-based management strategies currently employed across the globe have raised public concerns over pesticide residue in foods. The microbiome is considered as the second genome of the plant host. Members of the microbiome could benefit the host plant by promoting growth and improve defense against disease and abiotic stress [30]. Endophytes have the potential to control all the phytopathogen management strategies and because they are native candidates in plant niches, they are known for not disturbing the existing microflora equilibrium. Higher fungal diversity has been found to be associated with higher antagonistic activity against several apple pathogens [30]. Thus far, studies have focused on exploring endophytic fungi and bacteria for biocontrol of the main apple pathogens. The potential resources of endophytic bacteria from apple trees have been reported from many countries, such as Canada, India, the United States, Lithuania, Japan, and China [13,31–35]. Bacterial endophytes from domestic apples are common inhabitants of plant tissues, which play an important role in the regulation of plant growth and the prevention against pathogens [13]. However, till now, less attention has been paid to studying the community of bacterial endophytes from apple and their role against apple pathogens. Presently, we only have fragmented knowledge about endophytes that reside in the phyllosphere of cultivated tree plants such as domestic apple, especial with regard to the inhibition of *Venturia inaequalis* (causing apple scab) [12–14]. To date, there has been no study on the isolation and identification of apple endophytes in Cold Plateau apple production in China. The biological control of plant diseases with endophytic bacteria brings us hope for solving the above diseases without chemical contamination. In our study, we collected 30 samples from four apple varieties and from five apple-production areas in Yunnan and isolated the 5709 isolates of culturable endophytic bacteria (CEB). The total 39 CEB strains were identified as *Bacillus*. Most of the potential endophytic species could degrade organic phosphorus on functional media, showing the potential to promote plant growth. We tested the ability of those endophytic bacteria to inhibit the main apple pathogens. A total of 33 endophytic bacteria strains mainly belonging to *B. subtilis* and *B. velezensis* were found to have antagonistic effects on the main pathogens of apple, *A. alternata*, *Bo. dothidea*, *F. oxysporum*, *F. solani*, *R. necatrix*, and *V. mali*. Among them, 21 strains were against *A. alternata*. A total of 10, 31, 18, 14, and 26 strains were antagonistic to *B. dothidea*, *V. mali*, *F. oxysporum*, *F. solani*, and *R. necatrix*, respectively, with an over 35% inhibition rate.

*Bacillus* strains have gained much attention as biocontrol agents because they could produce broad spectrum antibiotics, toxins, enzymes, and endospores; therefore, some strains have already been incorporated into commercially available biocontrol products with long-term shelf lives. In addition, the endophytic bacteria from different sources have different levels of adaptability and the community of endophytes is shaped by host variety [12,36,37]. All these endophytic bacteria strains from the Cold Plateau Yunnan apple-production areas may adapt to this ecological area due to isolation from these ecological regions and can be valuable resources as potential biocontrol agents. *B. velezensis* and *B. subtilis* not only show good antagonistic activities against pathogens, but also have the potential to promote plant growth as biofertilizers [38]. In our study, it is found that some strains of endophytic bacteria have at least one or more activities related to nitrogen fixation, phosphorus dissolution (inorganic phosphorus), the degradation of calcium phytate (organic phosphorus) or potassium hydrolysis; thus, we can infer that these endophytic bacteria promote plant growth by helping with host nitrogen fixation, phosphorus dissolution, the degradation of calcium phytate, potassium hydrolysis, etc.

However, the endophytes from apple just confirmed antagonisms and characteristics related to growth-promotion potentials in the laboratory, and their actual application potentials must be tested in the field in the future.

In our study, we analyzed the relationship between the endophytic bacteria community and apple varieties and locations. We found that the number and species of CEB isolated from different apple varieties were different. Those in Red General, Golden Handsome, and Red Fuji were highest. However, the lowest was in Gala. Red Fuji had the most abundance among the four varieties. The species of CEB from apple varieties were different, as either *B. velezensis* or *B. subtilis* was dominant. *B. subtilis*, *B. velezensis*, and *B. licheniformis* were found in four varieties, whereas *Priestia megaterium* was only isolated from Red Fuji. Liu et al. (2020) reported that endophyte communities in apple shoots are determined by tissue type, cultivar, and site [12]. Miliute et al. (2016) isolated 38 endophytic bacteria from apple buds of the cultivars Gala, Golden Delicious, and Orlovim grown under field conditions and 13 strains were assigned to *Curtobacterium*, *Pantoea*, and *Pseudomonas* species [13]. In China, Li et al. (2020) isolated and identified four strains of *B. subtilis*, two strains of *B. velezensis*, and one strain of *B. amyloliquefaciens* from a wild apple (*Malus sieversii*) [39]. Therefore, it is suggested that differences in the distribution of endophytic bacteria communities in apple leaves may depend upon the external environmental conditions, or upon different cultivation methods such as the use of pesticides [12,40,41]. Liu et al. (2020) and our study proved that the site was the main driver shaping the endophytic community in apple, but not the region [12]. Leone Olivieri et al. (2021) studied and found that the apple endophyte community in relation to location, and scion and rootstock genotypes was susceptible to European canker [40]. Liu Jia et al. (2018) stated that the mechanism by which an apple genotype, either rootstock or scion, has a determinant effect on the composition of a microbial community is not known [41]. In this study, CEB were abundant in different areas.

It is worth studying the relationship between apple endophytic bacteria communities and the apple orchard micro-ecology [12]. Wang et al. (2017) demonstrated that the application of bio-organic fertilizer significantly influenced the bacterial community's structure and composition [42]. Plant varieties with the ability to enrich endophytic micro-organisms improve the foliar endophytic bacteria community. Saunders et al. (2010) suggest that the most direct route to understand the mechanisms underlying community assembly is through the study of functional trait variation in the host and its fungal consortium [43]. Carper et al. (2018) studied and found that bacterial endophyte communities in *Pinus flexilis* are structured by host age, tissue type, and environmental factors [16].

*Bacillus*, *Pseudomonas*, *Enterobacter*, and *Paenibacillus* genera of the endophytic bacteria have been widely reported in many crops [44,45]. Jia Liu reported the main endophytic bacterium genera were Xanthomonaceae (30.4%), *Bacteroides* (11.4%), *Propionibacterium* (5.4%), and *Bacillus* (5.2%) from apple endophytic microbiota in different rootstock/scion genotypes in all samples, and the top 20 leaf and root abundances include *Bacillus* (0.01%, 0.0006%), indicating that *Bacillus* is also the dominant strain in apple leaf, although the content will increase or decrease due to varieties and regions. In our study, the results also show that *Bacillus* is the dominant strain, which is consistent with the results of Liu Jia [41]. We suggested that *Bacillus* bacteria in most of the samples could reveal the highest pesticide tolerance among the culturable endophytic bacteria that survived. Therefore, the research on the endophytic bacterial population and pesticide tolerance is worthy of further research in the future. Another reason is the setting of the disinfection time. Our experiment is different from Lucia's (2017), mainly due to the slight difference in the concentration and time of sodium hypochlorite, but in fact, Lucia does not explain the concentration of CEB in apple leaves in their study, and thus, our population size cannot be compared with Lucia's results [7].

## 5. Conclusions

This is the first study of the isolation and identification of culturable apple endophytic bacteria in the Cold Plateau of apple-growing areas in China. The strain effectiveness of the culturable endophytic bacteria was tested against apple disease pathogens, and candidate strains that promoted plant growth and adapted to the Cold Plateau ecological environment were used in the further experiments. Diverse endophytes with vast antagonistic effects against several pathogens displayed a marked potential against apple plant pathogens. The endophytic community in apple leaves possibly relates to geographic locations, apple varieties, and environment. All in all, the study of communities of phyllosphere endophytic bacteria provides the basis to explain the mechanisms underlying the establishment of apple endophytic communities, and may help to devise apple disease management strategies.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae8110991/s1>, Table S1: Number of CEB colonies in apple producing areas of Yunnan.

**Author Contributions:** Conceptualization, J.M. and Y.H.; data curation, H.C., P.H. (Pengfei He) and J.M.; formal analysis, X.P., H.C., Y.Z., K.C. and B.K.; funding acquisition, Y.H.; investigation, X.P., P.H. (Pengbo He), C.Z., H.C., P.H. (Pengfei He), S.M., S.A., Y.M. and Y.Z.; methodology, X.P., P.H. (Pengbo He), C.Z., H.C., P.H. (Pengfei He), J.M. and Y.M.; project administration, B.K. and Y.H.; resources, Y.W., Y.M., Y.Z., K.C., B.K. and Y.H.; software, S.M. and S.A.; supervision, B.K. and Y.H.; validation, A.A. and K.C.; visualization, Y.W.; writing—original draft, X.P., S.M., A.A. and S.A.; writing—review and editing, S.M., A.A., B.K. and Y.H. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was financially supported by the National Apple Industry Technology System Project CARS-27 and the Project of Root Rot Management Technology of Apple in Tuanjie, Xishan District in Kunming City (T2001).

**Data Availability Statement:** All the data is present in the manuscript file.

**Acknowledgments:** We are thankful to Yunguo Li at the Zhaotong Development Center for Apple Industry, Yunnan; Xuelin Yang at the Lijiang Research and Extension Station for Horticultural Crops, Yunnan; and Jinquan Ma at the Fruit Tree Science and Technology Studies and Extension Station of Luxi County, Yunnan for their assistance in apple leaf sampling.

**Conflicts of Interest:** The authors declare no conflict of interest.

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