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Full Length Article

Determination of the Community Structure and Diversity of Endophytic Bacteria from *Alpinia zerumbet* Seeds

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Abstract

Alpinia zerumbet (Pers.) Burtt et Smith is an important aromatic plant in China. The goal of this study was to apply culture-independent methods based on high-throughput sequencing technology to determine the endophytic microbial community structure, diversity, and its dominant microbes from the five growth stages of *A. zerumbet* seeds from Southern Sichuan. The number of operational taxonomic units (OTUs) of endophytic bacteria was found to increase first and then decrease during the five periods. A greater number of bacteria were found in the middle maturity period, which included 58 bacterial OTUs. From fruit formation to late maturity, the first population of dominant bacteria from *A. zerumbet* seeds belonged to *Curtobacterium* (25.0%), *Pseudomonas* (34.0%), *Kosakonia* (37.5%), *Curtobacterium* (14.0%), and *Sphingobacterium* (28.5%). These results provided a scientific basis for the reasonable implementation of microbiological control and strengthened the natural fermentation of *A. zerumbet* products, which is necessary to ensure the quality of products. © 2020 Friends Science Publishers

Keywords: *Alpinia zerumbe*t; Endophytic bacteria; Bacterial diversity; High-throughput sequencing

Introduction

Endophytic bacteria refer to such types of bacteria during a certain stage in their life cycle or their entire life, wherein they occur in healthy growing plant tissues or cells. The host plant does not exhibit obvious disease symptoms because of this class of microbes, but they can play an important part in the micro-ecosystem of the plant and play an important role during the process of long-term cooperative coevolution. Furthermore, the plants may form a mutually beneficial relationship with these bacteria (Santoyo *et al*. 2015; Donoso *et al*. 2016).

As an important reproductive organ of plants, seeds carry abundant microbial populations on the surface and inside (Verma *et al*. 2017). Compared with other plant organs, the plant seed endophyte has received relatively less attention. Because seeds are of significance to plant reproduction and agricultural development, studies focused on endophytic bacteria of seeds should receive more attention (Chowdhary and Kaushik 2015; Fouda *et al*. 2015; Shade *et al*. 2017). Therefore, it is necessary to conduct research related to endophytes of plant seeds, especially that of medicinal and spice plant seed microorganisms for which their endogenous community structure and diversity in the seed should be understood.

Alpinia zerumbet (Pers.) Burtt et Smith is an important aromatic plant in China, which has been widely planted in Southern Sichuan in recent years. Studies have shown that it is rich in volatile oils and has antibacterial, antioxidant, analgesic, and anti-inflammatory properties. Furthermore, it regulates the cardiovascular system and various biological activities, such as those of the nervous system (Indrayan *et al*. 2009; Tao *et al*. 2009; Araujo *et al*. 2010; Shen *et al*. 2010). In the southern part of Sichuan, *A. zerumbet* is widely distributed and there are special climatic conditions. Growing *A. zerumbet* may have potential endophytic species resources that are completely different from those from other areas and in other species. At present, the research on *A. zerumbet* has mainly focused on the function of chemical and active ingredients, and research on endophytes that may affect its growth health and active ingredients is lacking (Elzaawely *et al*. 2007; Chompoo *et al*. 2012; Chen *et al*. 2017). Although researchers have studied endophytes in more than 100 plants for over 20 years, there are still a large number of plants in nature with unknown endophytic resources, especially highly utilized and functional plant species. Thus, research on endophytes is of great significance. This study was aimed to establish the relationship between endophytic bacterial communities and their plant developmental stages. High-throughput

sequencing was used to explore the diversity of the endophytic communities of *A. zerumbet* seeds at five different developmental stages. The results presented in this paper provide a scientific basis for the reasonable implementation of microbiological control and strengthen the natural fermentation of *A. zerumbet* products, which is necessary to ensure the quality of products.

Materials and Methods

Experimental materials

The samples of *A. zerumbet* seeds were in five different growth periods, consisting of the fruit formation period, young fruit period, early mature period, middle mature period, and late mature period. A 5.0 g sample of seeds was collected in Yibin, Sichuan Province (28°53′17″N, 104°43′7″E), and stored at 4°C. After the samples were sterilized, they were incubated at 28°C for 3 d to test the effect of surface disinfection. Uncontaminated samples were used in the next experiment.

DNA extraction and PCR amplification

The OMEGA kit was used to extract DNA. For PCR amplification, bacteria with a length of approximately 450 bp were selected as the target amplified fragments for subsequent high-throughput sequencing. The primer sequences were the forward primer ACTCCTACGGGAGGCAGCA and the reverse primer GGACTACHVGGGTWTCTAAT. The PCR reaction system (25 μL) consisted of 5 μL 5 × reaction buffer, 5 μL 5 × GC buffer, 2 μL dNTP (100 mmol·L^{-1}), forward primer (10 μmol·L^{-1}) 1 μL, 1 μL reverse primer (10 μmol·L^{-1}), 0.25 μL Q5 high-fidelity DNA polymerase, 2 μL DNA template, and 8.75 μL ddH_2O. The PCR reaction conditions were denaturation for 2 min at 98°C, denaturation for 15 s at 98°C, annealing for 30 s at 55°C, extension for 30 s at 72°C, and expansion for 25–30 cycles, with a final extension at 72°C for 5 min. After cutting the target fragment, it was recovered by the Axygen gel recovery kit.

Construction of gene clone library

The purified PCR products were ligated to the T3 vector and transformed into *Escherichia coli* DH5α competent cells (Xiang *et al*. 2018). The transformation product was spread on Luria–Bertani (LB) agar plates containing ampicillin (100 mg/L), white clones were randomly selected for streaking, and a cloning library was constructed.

Sequencing and phylogenetic analysis

Two-hundred clones from each sample were randomly selected for partial sequencing of the 16S rRNA gene. BLASTN was used to compare approximately 700 base nucleotide sequences to the NCBI (https://blast.ncbi.nlm.nih.gov/Blast) database (Naveed *et al*. 2014). The sequences were aligned using CLUSTALW (Hung and Weng 2016), and the neighbor-joining method was used to construct the tree with the MEGA 4 program package (Sudhir *et al*. 2018). Finally, the extent of the cloned library was evaluated by rarefaction analysis.

Results

A 16S rRNA gene clone library of endophytic bacteria from *A. zerumbet* was constructed using the purified PCR products. Two-hundred clones were randomly selected for sequencing and submitted to GenBank (accession no. MF508571—MF508602, MF508535—MF508570, MF541320—MF541368, MF803088—MF803146, and MG346174—MG346221). The endophytic bacteria were detected during the five different seed growth periods in *A. zerumbet*. These were the fruit formation period, young fruit period, early mature period, middle mature period, and late mature period, which included 31, 36, 49, 58, and 46 OTUs. The coverage was calculated as 94.70, 93.55, 91.40, 94.45 and 93.75%, respectively.

Among the endophytic bacteria during the five seed growth periods in *A. zerumbet*, the *Curtobacterium* played a role in four growth periods, which made up the largest fraction of the first four growth periods. At the fruit formation period, 200 clones were analyzed, of which 43 clones (21.50%) belonged to α-proteobacteria, 3 clones (1.50%) belonged to β-proteobacteria, 140 clones (70.00%) belonged to γ-proteobacteria, 6 clones (3.00%) belonged to Bacteroidetes, and 8 clones (4.00%) belonged to Actinobacteria, Actinobacteria, Bacteroidetes, α-proteobacteria, β-proteobacteria, and γ-proteobacteria were made up of 1, 2, 8, 2 and 18 bacterial OTU, respectively. In the clone library, *Curtobacterium* (25.0%), *Pantoea* (25.0%), and *Aureimonas* (10.0%) were the dominant genera (Table 1).

During the young fruit period, among 200 clones analyzed, 31 clones (15.50%) belonged to α-proteobacteria, 67 clones (33.50%) belonged to β-proteobacteria, 99 clones (49.50%) belonged to γ-proteobacteria, and three clones (1.50%) belonged to Bacteroidetes. Bacteroidetes, α-proteobacteria, β-proteobacteria and γ-proteobacteria were made up of 2, 9, 7 and 18 bacterial OTU, respectively. *Pseudomonas* (34.00%), *Acidovorax* (31.00%), *Curtobacterium* (7.50%), and *Sphingomonas* (7.00%) were the dominant genera (Table 2).

Of the 200 clones analyzed during the early mature growth period, 6 clones (3%) belonged to α-proteobacteria, 15 clones (7.50%) belonged to β-proteobacteria, 158 clones (79%) belonged to γ-proteobacteria, 19 clones (9.50%) belonged to Firmicutes, and 2 clones (1%) belonged to Actinobacteria. α-proteobacteria, β-proteobacteria, γ-proteobacteria, Firmicutes, and Actinobacteria were made up of 6, 11, 20, 10, and 2 bacterial OTU, respectively.

Table 1: Distribution of 16S rRNA clones detected from endophytes in the fruit formation period of *Alpinia zerumbet*

Group	OTUs	clones	% total clones	Closest NCBI match	% identity
α-proteobacteria	8	1	0.5	*Roseomonas aerophila* 7515T-07(T)	98.74
		3	1.5	*Sphingomonas pseudosanguinis* G1-2(T)	99.57
		3	1.5	*S. aeria* R1-3(T)	99.10
		2	1.0	*S. parapaucimobilis* NBRC 15100(T)	99.72
		5	2.5	*S. sanguinis* NBRC 13937(T)	100
		1	0.5	*Methylobacterium gossipiicola* Gh-105(T)	99.72
		8	4.0	*Neokomagataea tanensis* AH13(T)	98.87
		20	10.0	*Aureimonas ureilytica* NBRC 106430(T)	99.29
β-proteobacteria	2	1	0.5	*Comamonas kerstersii* LMG 3475(T)	97.37
		2	1.0	*Acidovorax wautersii* DSM 27981(T)	100
γ-proteobacteria	18	1	0.5	*Pantoea conspicua* LMG 24534(T)	99.25
		2	1.0	*P. vagans* LMG 24199(T)	99.57
		1	0.5	*P. rodasii* LMG 26273(T)	99.55
		1	0.5	*P. eucalypti* LMG 24198(T)	99.41
		4	2.0	*P. rwandensis* LMG 26275(T)	98.96
		20	10.0	*P. ananatis* LMG 2665(T)	100
		15	7.5	*P. anthophila* LMG 2558(T)	99.72
		1	0.5	*Tatumella citrea* LMG 22049(T)	99.10
		1	0.5	*Pseudomonas seleniipraecipitans* CA5(T)	98.52
		4	2.0	*P. psychrotolerans* DSM 15758(T)	100
		1	0.5	*P. oleovorans* subsp. *lubricantis* RS1(T)	95.94
		1	0.5	*Rouxiella chamberiensis* 130333(T)	99.44
		2	1.0	*Erwinia rhapontici* ATCC 29283(T)	99.44
		1	0.5	*Rosenbergiella nectarea* 8N4(T)	99.86
		15	7.5	*Acinetobacter nectaris* SAP 763.2(T)	99.86
		8	4.0	*Flavobacterium acidificum* LMG 8364(T)	99.57
		50	25.0	*Curtobacterium plantarum* CIP 108988(T)	99.58
		12	6.0	*Tatumella saanichensis* NML 06-3099(T)	99.30
Bacteroidetes	2	5	2.5	*Chryseobacterium hagamense* RHA2-9(T)	99.25
		1	0.5	*C. lineare* XC0022(T)	99.00
Actinobacteria	1	8	4.0	*Microbacterium testaceum* DSM 20166(T)	99.30

Kosakonia (37.50%), *Curtobacterium* (10.50%), *Erwinia* (7.50%), *Pantoea* (7.50%), and *Luteimonas* (7.00%) were the dominant genera (Table 3).

Of the 200 clones analyzed during the middle mature period, 72 clones (36%) belonged to α-proteobacteria, 33 clones (16.50%) belonged to β-proteobacteria, 66 clones (33%) belonged to γ-proteobacteria, 8 clones (4%) belonged to δ-proteobacteria, 7 clones (3.50%) belonged to Firmicutes, 7 clones (3.50%) belonged to Actinobacteria, and 7 clones (3.50%) belonged to Bacteroidetes. Firmicutes, Actinobacteria, Bacteroidetes, α-proteobacteria, β-proteobacteria, γ-proteobacteria, and δ-proteobacteria were made up of 6, 3, 3, 26, 9, 10 and 1 bacterial OTU, respectively. *Curtobacterium* (14%), *Methylobacterium* (10.50%), *Paraburkholderia* (9%), *Rhizobium* (8.50%), and *Caulobacter* (8%) were the dominant genera (Table 4).

During the late mature period, among 200 clones, 35 clones (17.5%) belonged to α-proteobacteria, 10 clones (5%) belonged to β-proteobacteria, 80 clones (40%) belonged to γ-proteobacteria, 4 clones (2%) belonged to δ-proteobacteria, 9 clones (4.50%) belonged to Firmicutes, 62 clones (31%) belonged to Actinobacteria. α-proteobacteria, β-proteobacteria, γ-proteobacteria, δ-proteobacteria, Firmicutes, and Actinobacteria were made up of 17, 6, 10, 2, 4, and 7 bacterial OTU, respectively. *Sphingobacterium* (28.50%), *Stenotrophomonas* (13.50%), *Luteimonas* (12.50%), and *Methylobacterium* (8.00%) were the dominant genera (Table 5).

A consistent succession of community structures could be observed in endophytic bacteria of *A. zerumbet* seeds. At the growth period one, *Curtobacterium* was the dominant genera, with 25%, and the sec and third genera were *Pantoea* and *Acinetobacter*, with 22 and 7.5%, respectively. At growth period two, *Pseudomonas* was the dominant genera, with 34%, and the sec and third genera were *Acidovorax* and *Curtobacterium*, with 31 and 7.5%, respectively. At growth period three, *Kosakonia* was the dominant genera, with 37.5%, and the sec and third genera were *Curtobacterium*, *Erwinia*, and *Pantoea*, with 10.5, 7.5, and 7.5%, respectively. At growth period four, *Curtobacterium* was the dominant genera, with 14%, and the sec and third genera were *Methylobacterium* and *Paraburkholderia*, with 10.5 and 9%, respectively. At growth period five, *Sphingobacterium* was the dominant genera, with 28.5%, and the sec and third genera were *Stenotrophomonas* and *Luteimonas*, with 13.5 and 12.5%, respectively. It is clear that *Curtobacterium* appeared during the first four growth periods, and reached 25, 7.5, 10.5 and 14%, respectively. The tendency for the occurrence of *Curtobacterium* first decreased and then increased. Similarly, *Pantoea* appeared during growth periods one and three, and reached 22 and 7.5%, respectively, dipping gradually. Noticeably, during growth period three, the clone of *Kosakonia* reached its peak at 37.5%. In addition, both

Table 2: Distribution of 16S rRNA clones detected from endophytes in the young fruit period of *Alpinia zerumbet*

Group	OTUs	clones	% total clones	Closest NCBI match	% identity
α-proteobacteria	9	2	1.0	*Allorhizobium oryzae* Alt505 (T)	97.18
		1	0.5	*Sphingomonas yabuuchiae* GTC 868 (T)	99.72
		1	0.5	*S. pseudosanguinis* G1-2 (T)	99.86
		10	5.0	*S. aeria* R1-3 (T)	99.40
		1	0.5	*S. sanguinis* NBRC 13937 (T)	99.86
		1	0.5	*S. parapaucimobilis* NBRC 15100 (T)	99.72
		1	0.5	*Rhizobium larrymoorei* ATCC 51759 (T)	99.44
		10	5.0	*R. qilianshanense* CCNWQLS01 (T)	97.11
		4	2.0	*Aureimonas ureilytica* NBRC 106430 (T)	99.01
β-proteobacteria	7	1	0.5	*Delftia lacustris* LMG 24775 (T)	99.01
		1	0.5	*Comamonas kerstersii* LMG 3475 (T)	97.54
		1	0.5	*Curvibacter gracilis* 7-1 (T)	98.87
		1	0.5	*Methylovorus menthalis* MM (T)	99.13
		2	1.0	*Acidovorax oryzae* ATCC 19882 (T)	93.32
		60	30.0	*A. wautersii* DSM 27981 (T)	100
		1	0.5	*Herbaspirillum chlorophenolicum* CPW301 (T)	98.30
γ-proteobacteria	18	1	0.5	*Pseudomonas caricapapayae* ATCC 33615 (T)	99.86
		1	0.5	*P. rhodesiae* CIP 104664 (T)	98.88
		1	0.5	*P. hibiscicola* ATCC 19867 (T)	97.85
		6	3.0	*P. psychrotolerans* DSM 15758 (T)	99.16
		1	0.5	*P. libanensis* CIP 105460 (T)	99.86
		1	0.5	*P. trivialis* DSM 14937 (T)	99.16
		3	1.5	*P. paralactis* WS4992 (T)	99.55
		6	3.0	*P. cerasi* 58 (T)	99.72
		12	6.0	*P. tolaasii* ATCC 33618 (T)	99.44
		30	15.0	*P. azotoformans* DSM 18862 (T)	99.86
		6	3.0	*P. simiae* OLi (T)	99.69
		2	1.0	*Flavobacterium acidificum* LMG 8364 (T)	98.30
		5	2.5	*Pantoea anthophila* LMG 2558 (T)	99.44
		6	3.0	*P. ananatis* LMG 2665 (T)	100
		1	0.5	*Kosakonia oryziphila* REICA_142 (T)	97.79
		1	0.5	*Xanthomonas codiaei* LMG 8678 (T)	99.58
		15	7.5	*Curtobacterium plantarum* CIP 108988 (T)	99.86
		1	0.5	*Acinetobacter lwoffii* NCTC 5866 (T)	99.86
Bacteroidetes	2	1	0.5	*Pedobacter sandarakinus* DS-27 (T)	98.14
		2	1.0	*Chryseobacterium hagamense* RHA2-9 (T)	99.25

Erwinia and *Pantoea* reached their lowest point of 7.5%. In addition, the genera in Acinetobacter during growth period one, *Curtobacterium* during growth period two, Erwinia and *Pantoea* during growth period three, all reached their lowest occurrence at 7.5% (Table 6).

Discussion

Plant seeds are infected by microorganisms, which are necessary for their normal germination (Stöckel *et al*. 2014; Mehra *et al*. 2017). Zhang *et al*. (2017) found that many microbial communities occurred in the seeds and on the surface of the seeds. The structure of the microbial community was affected by their physiological state, had a significant effect on the health of plant seeds. However, compared to the extensive research conducted on plant rhizosphere microorganisms, less research has been devoted to seed-related endophytes (Stroheker *et al.* 2018). Therefore, the research on the endophytic community structure of *A. zerumbet* seeds provides new information regarding the influence of endophytic bacteria. The bacteria present on the surface of *A. zerumbet* seeds were washed and disinfected. These bacteria were ignored in the subsequent analysis because it is difficult to prevent pollution from the environment and the sources of such bacteria are highly diverse.

To understand the correlation between seed endophytic bacteria and community diversity in different growth periods, *A. zerumbet* seeds were selected that were in five critical growth periods (fruit formation period, young fruit period, early mature period, middle mature period, and late mature period). Some studies have found that *Bacillus*, *Agrobacterium*, *Burkholderia*, and *Enterobacter* groups do not change during different growth stages of rice, although their numbers gradually increased from the seedling stage to the booting stage and decreased from the filling stage to the milk ripening stage. The changes in the number of endophytic bacterial groups tended to be similar to their overall change (Zhang *et al*. 2015). Wang *et al.* (2015) analyzed the diversity of endophytic bacteria in the seedling stage, tillering stage, flowering stage, and seed setting stage in rice. Among the *Burkholderia*, *Herbaspirillum,* and *Flavobacterium* isolated, *Burkholderia* was dominant. Lin *et al*. (2018) studied *Pennisetum* spp., and their endophytic bacterial diversity was analyzed. *Cyanobacteria* and *Proteobacteria* were the main bacterial genera. In this study,

Table 3: Distribution of 16S rRNA clones detected from endophytes in the early mature period of *Alpinia zerumbet*

Group	OTUs	clones	% total clones	Closest NCBI match	% identity
α-proteobacteria	6	1	0.5	*Sphingomonas sanguinis* NBRC 13937 (T)	99.72
		1	0.5	*S. aquatilis* JSS7 (T)	99.86
		1	0.5	*S. hankookensis* ODN7 (T)	99.16
		1	0.5	*S. herbicidovorans* NBRC 16415 (T)	100.00
		1	0.5	*Croceicoccus naphthovorans* PQ-2 (T)	97.62
		1	0.5	*Azospirillum massiliensis* URAM1	98.45
β-proteobacteria	11	1	0.5	*Pelomonas saccharophila* DSM 654 (T)	99.86
		3	1.5	*Duganella ginsengisoli* DCY83 (T)	99.15
		1	0.5	*Burkholderia thailandensis* E264 (T)	99.29
		1	0.5	*Paraburkholderia bannensis* NBRC 103871 (T)	99.58
		1	0.5	*P. susongensis* L226 (T)	98.44
		1	0.5	*P. oxyphila* NBRC 105797 (T)	99.58
		1	0.5	*Ralstonia pickettii* ATCC 27511 (T)	99.86
		2	1.0	*Massilia varians* CCUG 35299 (T)	99.70
		1	0.5	*Piscinibacter defluvii* SH-1 (T)	99.58
		2	1.0	*P. aquaticus* IMCC1728 (T)	99.58
		1	0.5	*Delftia lacustris* LMG 24775 (T)	99.72
γ-proteobacteria	20	21	10.5	*Curtobacterium plantarum* CIP 108988 (T)	99.86
		4	2.0	*Pantoea ananatis* LMG 2665 (T)	100.00
		1	0.5	*P. conspicua* LMG 24534 (T)	99.40
		8	4.0	*P. anthophila* LMG 2558 (T)	99.72
		1	0.5	*P. beijingensis* LMG 27579 (T)	99.27
		1	0.5	*P. brenneri* LMG 5343 (T)	99.84
		2	1.0	*Klebsiella pneumoniae* subspp. *pneumoniae* DSM 30104 (T)	99.30
		2	1.0	*Acinetobacter lwoffii* NCTC 5866 (T)	100.00
		1	0.5	*A. nectaris* SAP 763.2 (T)	99.86
		1	0.5	*A. junii* CIP 64.5 (T)	99.86
		1	0.5	*A. bouvetii* DSM 14964 (T)	99.72
		1	0.5	*Luteibacter anthropi* CCUG 25036 (T)	100.00
		2	1.0	*Erwinia persicina* NBRC 102418 (T)	99.86
		13	6.5	*E. gerundensis* EM595 (T)	99.85
		7	3.5	*Stenotrophomonas humi* DSM 18929 (T)	100.00
		1	0.5	*Dyella koreensis* BB4 (T)	99.58
		1	0.5	*Enhydrobacter aerosaccus* LMG 21877 (T)	99.15
		1	0.5	*Moraxella osloensis* CCUG 350 (T)	99.86
		14	7.0	*Luteimonas terrae* THG-MD21 (T)	99.86
		75	37.5	*Kosakonia cowanii* JCM 10956 (T)	99.30
Firmicutes	10	3	1.5	*Bacillus tequilensis* KCTC 13622 (T)	99.58
		5	2.5	*B. nakamurai* NRRL B-41091 (T)	100.00
		2	1.0	*B. solani* FJAT-18043 (T)	99.86
		1	0.5	*Lactobacillus fermentum* CECT 562 (T)	99.58
		1	0.5	*L. kefiri* LMG 9480 (T)	99.71
		1	0.5	*Leuconostoc mesenteroides* subspp. *suionicum* DSM 20241 (T)	99.72
		1	0.5	*Pediococcus pentosaceus* DSM 20336 (T)	99.58
		1	0.5	*Thermoactinomyces daqus* H-18 (T)	99.44
		2	1.0	*Clostridium akagii* CK58 (T)	100.00
		2	1.0	*C. saccharoperbutylacetonicum* N1-4 (HMT)(T)	99.44
Actinobacteria	2	1	0.5	*Nocardia ninae* OFN 02.72 (T)	99.02
		1	0.5	*Cutibacterium acnes* DSM 1897 (T)	99.44

there were relatively few types of endophytic bacteria because the seeds were in the process of rapid growth and required more nutrients during the fruit formation period. From the young fruit stage to the middle maturity stage, the number and variety of bacteria in the seeds increased significantly because of the softness of the seeds, which reached a peak during the middle maturity period, explaining the relative endophytic bacterial diversity during this period. Because the internal environment during the early stages of seed development was similar, the same dominant species can be expected and were found in previous samples.

Zhang *et al*. (2018) suggested that the contents and concentrations of water and dry matter in seeds vary greatly during the maturation process, which affects the types of endophytic bacteria that can survive in seeds. If endophytic bacteria are more adapted to the new environment of the seeds, they can accumulate as dominant bacteria (Shahzad *et al*. 2017). This conclusion was proven by the results obtained in this study. For example, *Kosakonia* became the dominant genus in the early maturity period after accumulating during the first two periods, whereas *Sphingobacterium* became the dominant bacterium in the late maturity period after a

Table 4: Distribution of 16S rRNA clones detected from endophytes in the middle mature period of *Alpinia zerumbet*

Group	OTUs	clones	% total clones	Closest NCBI match	% identity
α-proteobacteria	26	1	0.5	*Sphingomonas kyungheensis* THG-B283 (T)	99.86
		1	0.5	*S. aquatilis* JSS7 (T)	100.00
		1	0.5	*S. aeria* R1-3 (T)	98.21
		1	0.5	*S. abaci* C42 (T)	99.86
		1	0.5	*S. yunnanensis* YIM 003 (T)	99.58
		1	0.5	*Sphingobium abikonense* NBRC 16140 (T)	99.15
		1	0.5	*Rhizobium yantingense* H66 (T)	99.15
		10	5.0	*R. nepotum* 39/7 (T)	99.86
		2	1.0	*R. qilianshanense* CCNWQLS01 (T)	99.71
		4	2.0	*R. larrymoorei* ATCC 51759 (T)	99.58
		2	1..0	*Devosia subaequoris* HST3-14 (T)	98.59
		1	0.5	*Mesorhizobium plurifarium* LMG 11892 (T)	100.00
		2	1.0	*Roseomonas aerophila* 7515T-07 (T)	98.60
		1	0.5	*Aureimonas ureilytica* NBRC 106430 (T)	99.29
		1	0.5	*A. frigidaqua*e JCM 14755 (T)	99.86
		5	2.5	*Shinella yambaruensis* MS4 (T)	99.44
		15	7.5	*Caulobacter segnis* ATCC 21756 (T)	99.01
		1	0.5	*C. mirabilis* FWC38 (T)	97.74
		10	5.0	*Methylobacterium komagatae* 002-079 (T)	97.75
		3	1.5	*M. brachiatum* B0021 (T)	100.00
		2	1.0	*M. extorquens* IAM 12631 (T)	100.00
		1	0.5	*M. aerolatum* 5413S-11 (T)	99.27
		2	1.0	*M. phyllostachyos* BL47 (T)	99.58
		1	0.5	*M. rhodesianum* DSM 5687 (T)	99.29
		1	0.5	*M. phyllosphaerae* CBMB27 (T)	99.86
		1	0.5	*M. suomiense* NCIMB 13778 (T)	99.44
β-proteobacteria	9	16	8.0	*Paraburkholderia bannensis* NBRC 103871 (T)	99.43
		1	0.5	*P. tropica* Ppe8 (T)	99.55
		1	0.5	*P. phytofirmans* PsJN (T)	99.86
		1	0.5	*Herbaspirillum aquaticum* IEH 4430 (T)	100.00
		8	4.0	*Limnobacter thiooxidans* CS-K2 (T)	99.72
		1	0.5	*Duganella zoogloeoides* IAM 12670 (T)	99.86
		2	1.0	*Burkholderia thailandensis* E264 (T)	99.15
		1	0.5	*Pelomonas saccharophila* DSM 654 (T)	99.86
		1	0.5	*Aquabacterium commune* B8 (T)	98.51
		1	0.5	*Xylophilus ampelinus* ATCC 33914 (T)	99.44
γ-proteobacteria	10	8	4.0	*Xanthomonas cucurbitae* LMG 690 (T)	99.86
		12	6.0	*Luteimonas terrae* THG-MD21 (T)	100.00
		1	0.5	*Pseudomonas tolaasii* ATCC 33618 (T)	99.72
		1	0.5	*Acinetobacter junii* CIP 64.5 (T)	100.00
		28	14.0	*Curtobacterium plantarum* CIP 108988 (T)	99.44
		7	3.5	*Pantoea anthophila* LMG 2558 (T)	99.86
		5	2.5	*Luteibacter anthropi* CCUG 25036 (T)	100.00
		1	0.5	*Stenotrophomonas humi* DSM 18929 (T)	99.72
		1	0.5	*Acinetobacter guillouiae* CIP 63.46 (T)	100.00
		2	1.0	*Escherichia hermannii* GTC 347 (T)	99.72
δ-proteobacteria	1	8	4.0	*Cystobacter miniatus* DSM 14712 (T)	99.86
Firmicutes	6	1	0.5	*Leuconostoc mesenteroides* subspp. *suionicum*	99.72
		1	0.5	*Bacillus maritimus* KS16-9 (T)	99.44
		2	1.0	*Lactococcus lactis* subspp. *tructae* L105 (T)	100.00
		1	0.5	*Paenibacillus kyungheensis* DCY88 (T)	99.86
		1	0.5	*Nocardia jinanensis* NBRC 108249 (T)	99.72
		1	0.5	*Moraxella osloensis* CCUG 350 (T)	98.87
Actinobacteria	3	5	2.5	*Micrococcus yunnanensis* YIM 65004 (T)	99.86
		1	0.5	*Geodermatophilus brasiliensis* Tu 6233 (T)	99.72
		1	0.5	*Leifsonia soli* TG-S248 (T)	98.94
Bacteroidetes	3	5	2.5	*Chryseobacterium hagamense* RHA2-9 (T)	99.25
		1	0.5	*Flavobacterium akiainvivens* DSM 25510 (T)	99.00
		1	0.5	*Pedobacter ureilyticus* THG-T11 (T)	99.41

long period of accumulation. Abdallah *et al*. (2016) reported that many endophytic bacteria resistant to high osmotic pressure are present in rice seeds at the late growth stage and the abundance of endophytic bacteria with amylase activity is significantly increased. However, the results obtained in this study were different. In the fourth and fifth periods, the dominant bacteria in the seeds were almost non-exclusive, which may have been caused

Table 5: Distribution of 16S rRNA clones detected from endophytes in the late mature period of *Alpinia zerumbet*

Group	OTUs	clones	% total clones	Closest NCBI match	% identity
α-proteobacteria	17	1	0.5	*Brevundimonas viscosa* CGMCC 1.10683 (T)	98.54
		1	0.5	*B. vesicularis* NBRC 12165 (T)	98.84
		2	1.0	*Rhizobium rubi* NBRC 13261 (T)	99.58
		1	0.5	*R. larrymoorei* ATCC 51759 (T)	99.58
		2	1.0	*R. nepotum* 39/7 (T)	99.86
		1	0.5	*Methylobacterium phyllostachyos* BL47 (T)	99.58
		1	0.5	*M. platani* PMB02 (T)	98.73
		1	0.5	*M. aerolatum* 5413S-11 (T)	98.83
		5	2.5	*M. komagatae* 002-079 (T)	97.75
		5	2.5	*M. brachiatum* B0021 (T)	100
		1	0.5	*M. goesingense* iEII3 (T)	98.87
		2	1.0	*M. suomiense* NCIMB 13778 (T)	98.45
		1	0.5	*Caulobacter mirabilis* FWC38 (T)	97.88
		5	2.5	*C. fusiformis* ATCC 15257 (T)	97.88
		1	0.5	*Sphingomonas aquatilis* JSS7 (T)	99.58
		1	0.5	*S. asaccharolytica* NBRC 15499 (T)	98.74
		4	2.0	*Aureimonas frigidaquae* JCM 14755 (T)	99.44
β-proteobacteria	6	1	0.5	*Xenophilus aerolatus* 5516S-2 (T)	99.71
		2	1.0	*Paraburkholderia bannensis* NBRC 103871 (T)	99.58
		2	1.0	*P. nodosa* R-25485 (T)	99.57
		1	0.5	*Herbaspirillum aquaticum* IEH 4430 (T)	99.58
		2	1.0	*Delftia lacustris* LMG 24775 (T)	99.72
		2	1.0	*Ideonella sakaiensis* 201-F6 (T)	98.44
γ-proteobacteria	10	1	0.5	*Xanthomonas cucurbitae* LMG 690 (T)	99.30
		1	0.5	*Acinetobacter bouvetii* DSM 14964 (T)	99.86
		1	0.5	*Pantoea anthophila* LMG 2558 (T)	99.72
		3	1.5	*Luteibacter anthropi* CCUG 25036 (T)	100
		7	3.5	*Stenotrophomonas humi* DSM 18929 (T)	99.72
		20	10.0	*S. rhizophila* DSM 14405 (T)	99.44
		2	1.0	*Escherichia coli* NCTC9001 (T)	99.30
		12	6.0	*Erwinia persicina* NBRC 102418 (T)	99.86
		25	12.5	*Luteimonas terrae* THG-MD21 (T)	99.58
		8	4.0	*Curtobacterium plantarum* CIP 108988 (T)	98.73
δ-proteobacteria	2	2	1.0	*Melittangium lichenicola* ATCC 25946 (T)	99.71
		2	1.0	*Cystobacter miniatus* DSM 14712 (T)	99.71
Firmicutes	4	2	1.0	*Leuconostoc suionicum* DSM 20241 (T)	99.58
		5	2.5	*Bacillus solani* FJAT-18043 (T)	99.44
		1	0.5	*B. maritimus* KS16-9 (T)	99.58
		1	0.5	*Planomicrobium soli* XN13 (T)	99.44
Actinobacteria	7	2	1.0	*Microbacterium testaceum* DSM 20166 (T)	99.58
		1	0.5	*M. proteolyticum* RZ36 (T)	99.30
		1	0.5	*Sphingobacterium lactis* DSM 22361 (T)	97.99
		56	28.0	*Sphingobacterium hotanense* XH_4 (T)	99.85
		1	0.5	*Hymenobacter fastidiosus* VUG-A124 (T)	95.44
		1	0.5	*Pedobacter humi* THG S15-2 (T)	99.56

by the different selectivity of the species in different regions.

Sphingomonas, *Methylobacterium*, *Microbacterium*, *Pseudomonas* and *Rhizobium* are common dominant bacteria in plant seeds. (Chaudhry *et al*. 2016; Antunes *et al*. 2017; Durand *et al*. 2017; Tavares *et al*. 2018; Verma and White 2018; Zhang *et al*. 2018). The first dominant genera in the five growth stages were *Curtobacterium*, *Pseudomonas*, *Kosakonia*, *Curtobacterium*, and *Sphingobacterium*, respectively. *Curtobacterium* was the dominant bacteria in the first four stages of the fruit formation process in *A. zerumbet*, indicating that it was closely related to seed development. Therefore, it is speculated that endophytic bacteria are able to adapt and survive in new internal environments in the seeds, and permanently become one of the dominant bacteria. Some researchers have studied the growth-promoting characteristics of rice endophytic *Curtobacterium citreum* and found that it capable of producing IAA, dissolving phosphorus, and fixing nitrogen (Xu *et al*. 2014). Studies have also found that *Kosakonia* has good nitrogen-fixing effects; *Pseudomonas* has a certain bacteriostatic activity, and *Sphingobacterium* is an antagonistic bacterium (Li *et al*. 2016; Shcherbakov *et al*. 2017; Yang *et al*. 2017). In addition, *Pantoea* was considered to be the major dominant genus in rice seeds and common bacteria that promote plant growth (Campestre *et al*. 2016; Megías *et al*. 2017). *Pantoea* was the dominant genus in the first and third growth stages in this study. Therefore, it can be inferred that the results of this study will be of great significance for future microbial regulation of *A. zerumbet*-related products using the endophytes with special functions.

Table 6: Comparison of dominant genera of samples at five different growth stages

Growth stages	Genera
P1 (fruit formation period)	*Curtobacterium* (25.0%)
	Pantoea (22.0%)
	Acinetobacter (7.5%)
P2 (young fruit period)	*Pseudomonas* (34.0%)
	Acidovorax (31.0%)
	Curtobacterium (7.5%)
P3 (early mature period)	*Kosakonia* (37.5%)
	Curtobacterium (10.5%)
	Erwinia (7.5%)
	Pantoea (7.5%)
P4 (middle mature period)	*Curtobacterium* (14.0%)
	Methylobacterium (10.5%)
	Paraburkholderia (9.0%)
P5 (late mature period)	*Sphingobacterium* (28.5%)
	Stenotrophomonas (13.5%)
	Luteimonas (12.5%)

Conclusion

The first population of dominant bacteria from *A. zerumbet* seeds belonged to *Curtobacterium* (25.0%), *Pseudomonas* (34.0%), *Kosakonia* (37.5%), *Curtobacterium* (14.0%), and *Sphingobacterium* (28.5%) during different growth stages, which may have been caused by the complex environmental conditions in the area where *A. zerumbet* plants are located. Related research from the direction of endophytic bacterial communities may bring new ideas for the exploitation of plant resources. The results are of great significance to the development of the theory of plant microbial ecology.

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References

Abdallah RAB, SM Tlili, A Nefzi, HJ Khiareddine, MD Remadi (2016). Biocontrol of fusarium wilt and growth promotion of tomato plants using endophytic bacteria isolated from *Nicotiana glauca* organs. *Biol Cont* 97:811–824

Antunes JEL, MCCP Lyra, FJ Ollero, ADS Freitas, LMS Oliveira, ASF Araújo, MVB Figueiredo (2017). Diversity of plant growth promoting bacteria associated with sugarcane. *Genet Mol Res* 16:1–10

Araujo FYRD, MIG Silva, BA Moura (2010). Central nervous system effects of the essential oil of the leaves of *Alpinia zerumbet* in mice. *J Chin Chem Soc* 57:758–763

Campestre MP, LN Castagno, MJ Estrella, OA Ruiz (2016). *Lotus japonicus* plants of the Gifu B-129 ecotype subjected to alkaline stress improve their Fe^{2+} bio-availability through inoculation with *Pantoea eucalypti* M91. *J Plant Physiol* 192:47–55

Chaudhry V, P Baindara, VK Pal, N Chawla, PB Patil, S Korpole (2016). *Methylobacterium indicum* spp. nov., a facultative methylotrophic bacterium isolated from rice seed. *Syst Appl Microbiol* 39:25–32

Chen JJ, HR Liao, KS Chen, MJ Cheng, CW Shu, PJ Sung, WL Kuo (2017). A new 2*H*-pyran-2-one derivative and anti-inflammatory constituents of *Alpinia zerumbet*. *Chem Nat Compd* 53:40–43

Chompoo J, A Upadhyay, M Fukuta, S Twata (2012). Effect of *Alpinia zerumbet* components on antioxidant and skin diseases-related enzymes. *BMC Complement Altern Med* 12:106–115

Chowdhary K, N Kaushik (2015). Fungal endophyte diversity and bioactivity in the Indian medicinal plant *Ocimum sanctum* Linn. *PLoS One* 10; Article e0141444

Donoso R, P Leiva-Novoa, A Zúñiga, T Timmermann, G Recabarren-Gajardo, B González (2016). Biochemical and genetic basis of indole-3-acetic acid (auxin phytohormone) degradation by the plant growth promoting rhizobacterium *Paraburkholderia phytofirmans* PsJN. *Appl Environ Microbiol* 83:1–20

Durand A, F Maillard, V Alvarez-Lopez, S Guinchard, C Bertheau, B Valot, D Blaudez, M Chalot (2017). Bacterial diversity associated with poplar trees grown on a Hg-contaminated site: community characterization and isolation of Hg-resistant plant growth promoting bacteria. *Sci Total Environ* 622–623:1165–1177

Elzaawely AA, TD Xuan, S Tawata (2007). Essential oils, kava pyrones and phenolic compounds from leaves and rhizomes of *Alpinia zerumbet* (Pers.) B.L. Burtt. & R.M. Sm. and their antioxidant activity. *Food Chem* 103:486–494

Fouda AH, SD Hassan, AM Eid, ED Ewais (2015). Biotechnological applications of fungal endophytes associated with medicinal plant *Asclepias sinaica* (Bioss.). *Ann Agric Sci* 60:95–104

Hung JH, Z Weng (2016). Sequence alignment and homology search with BLAST and clustalW. *Cold Spr Harb Protoc* 2016; doi 10.1101pdb.prot093088

Indrayan AK, P Agrawal, AK Rathi, A Shatru, DK Tyage (2009). Nutritive value of some indigenous plant rhizomes resembling Ginger. *Nat Prod Rad* 8:507–513

Li QJ, JJ Cheng, SX Sun, YP Chen (2016). Isolation, identification and characterization of associative nitrogen-fixing endophytic bacterium *Kosakonia radicincitans* GXGL-4A in maize. *Microbiol Chin* 43:2456−2463

Lin BS, ZZ Song, LL Zhang, JL Fan, ZX Lin (2018). Composition diversity and differences of endophytic bacteria in root, stem and leaf at different growth stages of *Pennisetum* spp. *J Fujan Agric For Univ* 3:2885–2893

Megías E, FBR Junior, RA Ribeiro, M Megías, FJ Ollero, M Hungria (2017). Genome sequence of *Pantoea* spp. strain 1.19, isolated from rice rhizosphere, with the capacity to promote growth of legumes and nonlegumes. *Genome Announc* 5; Article e00707-17

Mehra S, PD Morrison, F Coates, AC Lawrie (2017). Differences in carbon source utilisation by orchid mycorrhizal fungi from common and endangered species of *Caladenia* (Orchidaceae). *Mycorrhiza* 27:95–108

Naveed M, S Mubeen, S khan, I Ahmed, N Khalid, HAR Suleria (2014). Identification and characterization of rhizospheric microbial diversity by 16s ribosomal RNA gene sequencing. *Braz J Microbiol* 45:985–993

Santoyo G, GM Hagelsieb, MDCO Mosqueda, BR Glick (2015). Plant growth-promoting bacterial endophytes. *Microbiol Res* 183:92–99

Shade A, MA Jacques, M Barret (2017). Ecological patterns of seed microbiome diversity, transmission, and assembly. *Curr Opin Microbiol* 37:15–22

Shahzad R, M Waqas, AL Khan, K Al-Hosni, IJ Lee (2017). Indoleacetic acid production and plant growth promoting potential of bacterial endophytes isolated from rice (*Oryza sativa* L.) seeds. *Acta Biol Hung* 68:175–186

Shcherbakov AV, EN Shcherbakova, SA Mulina, PY Rots, RF Daryu, EI Kiprushkina, LN Gonchar, VK Chebotar (2017). Psychrophilic endophytic *Pseudomonas* as potential agents in biocontrol of phytopathogenic and putrefactive microorganisms during potato storage. *Agric Biol* 52:116–128

Shen XC, HS Hu, HT Xiao (2010). GC-MS analysis of chemical constituents of the essential oil from different parts of *Alpinia zerumbet* (Pers.) Burtt et Smith. *Chin J Pharm Anal* 30:1399–1403

Stöckel M, T Těšitelová, J Jersáková, MI Bidartondo, G Gebauer (2014). Carbon and nitrogen gain during the growth of orchid seedlings in nature. *New Phytol* 202:606–615

Stroheker S, V Dubach, TN Sieber (2018). Competitiveness of endophytic, *phialocephala fortinii*, s.l. –, *acephala applanata*, strains in Norway spruce roots. *Fungal Biol* 122:345–352

Sudhir K, S Glen, L Michael, K Christina, T Koichiro (2018). Mega x: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 35:1547–1549

Tao L, XC Shen, J Peng, N Zhi (2009). Analysis on volatile oils from different parts of *Alpinia zerumbet* by GC-MS. *Chin Trad Patent Med* 31:909–911

Tavares MJ, FX Nascimento, BR Glick, MJ Rossi (2018). The expression of an exogenous ACC deaminase by the endophyte *Serratia grimesii* BXF1 promotes the early nodulation and growth of common bean. *Lett Appl Microbiol* 66:252–259

Verma SK, JF White (2018). Indigenous endophytic seed bacteria promote seedling development and defend against fungal disease in brown top millet (*Urochloa ramosa* L.). *J Appl Microbiol* 124:764–778

Verma SK, K Kingsley, I Irizarry, M Bergen, RN Kharwar, JF White (2017). Seed-vectored endophytic bacteria modulate development of rice seedlings. *J Appl Microbiol* 122:1680–1691

Wang XJ, RZ Jia, YL Guo, L Xu, J Zuo, H Kong, AP Guo (2015). Diversity of Culturable Endobacterial Communities in Rice (*Oryza sativa* L.) Stem at Different Growth Stages. *Chin J Trop Crops* 6:71–78

Xiang HP, TW Guan, SX Zhao, XC Zhang, MY Ou, YJ Lin, PH Wang (2018). Actinobacterial community and ionic composition in sediment of Xinjiang saline lakes: Barkol, Qijiaojing and Taitema. *Microbiol Chin* 45:1228–1236

Xu MS, JP Sheng, ST Guo, L Shen (2014). Identification of rice endophytic strain K12G2 and research on plant growth promoting trait. *Chin Agric Sci Bull* 30:66–70

Yang JY, XL Chen, LL Gao, Q Huang (2017). Isolation of Endophytic Bacteria from *Xanthomonas oryzae* pv. *oryzae* and Its antagonistic function. *Jiangsu Agric Sci* 45:100–105

Zhang J, CW Zhang, J Yang, RJ Zhang, JS Gao, X Zhao, JJ Zhao, DF Zhao, XX Zhang (2018). Insights into endophytic bacterial community structures of seeds among various *Oryza sativa* L. rice genotypes. *J Plant Growth Regul* 38:1–10

Zhang QQ, XR Huang, XY Guo (2015). Advance in Endophytic Bacterial Diversity of Wetland Plants. *Wetl Sci* 13:101–111

Zhang Z, D Ci, G Zhang, H Ding, D Zhang (2017). Diversity of microbial community structure in the spermosphere of saline-alkali soil in Shandong area. *Acta Microbiol Sin* 57:582–596