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High-Throughput Sequencing Analysis of Endophytic Bacteria Diversity in Fruits of White and Red Pitayas from Three Different Origins

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Abstract

Pitaya contains various types of polyphenols, flavonoid and vitamins which are beneficial for health and it is among the most important commercial tropical fruits worldwide. Endophytic bacteria might be beneficial for plant growth and yield. However, bacterial diversity in pitaya is poorly characterized. In this study, fruits of white and red pitayas from three different origins (Thailand, Vietnam and China) were chosen for endophytic bacteria diversity investigation by using Illumina HiSeq second-generation high-throughput sequencing technology. Large number of endophytic bacteria were detected and 22 phyla, 56 classes, 81 orders, 122 families and 159 genera were identified. Endophytic bacteria diversity was uneven among pitaya fruits from different origins and bacteria structure was different between white pitaya group and red pitaya group. Phylum Bacteroidetes, classes Bacteroidia and Coriobacteriia, orders Bacteroidales and Coriobacteriales, families Prevotellaceae, Bacteroidaceae, Ruminococcaceae, Paraprevotellaceae, Rikenellaceae, Alcaligenaceae and Coriobacteriaceae, genera *Prevotella*, *Bacteroides*, *Roseburia*, *Faecalibacterium* and *Sutterella* were statistically significant different species ($P < 0.05$) between white and red pitayas. These findings might be useful for growth improvement, fruit preservation and processing of different pitaya species from different origins.

Key words: endophytic bacteria diversity, high-throughput sequencing, pitaya from three different origins

Introduction

Pitayas (dragon fruits) are originally native to Latin America and the West Indies and are cultivated in tropical and subtropical regions all over the world. Pitaya belong to the genus *Hylocereus*, three major species of pitaya are *Hylocereus undatus* (white pitaya), *Hylocereus polyrhizus* (red pitaya) and *Hylocereus megalanthus* (yellow pitaya). The white and red pitayas are the most widely cultivated because of their economic values and health benefits (Ortiz and Takahashi, 2015; Suh *et al.*, 2014). Both white and red pitayas contain various types of polyphenols, flavonoid and vitamins which are beneficial for health (Esquivel *et al.*, 2007). The antidiabetic effect of white and red pitayas has been recently demonstrated. It is reported that the consumption of white pitaya attenuates insulin resistance and hepatic steatosis in diet-induced obese mice. Consumption of red pitaya could decrease total cholesterol, triglyceride and low-density lipoprotein cholesterol levels and increase the high-density lipoprotein cholesterol levels in type 2 diabetic subjects (Song *et al.*, 2016). In addition, red pitaya may serve as a therapy for attenuating some signs of high-carbohydrate and high-fat diet-induced metabolic syndrome (Ramli *et al.*, 2014). However, the active components in the flesh of white and red pitayas are different. Most of the betalain-related metabolites, the main contributors to antioxidant activity, were significantly higher in the flesh of red pitaya than in white pitaya (Ortiz and Takahashi, 2015; Suh *et al.*, 2014).

Endophytic bacteria live in almost all studied plant species. Plants provide nutrients for the growth of endophytes, meantime, many endophytes give beneficial feedback for their host by different ways, including growth promotion, pathogens suppression, contaminants remove, phosphate solubilization, nitrogen fixation, *etc*. It has been reported that endophytic bacteria and host plants are in an obligate mutualism through an evolutionary process (Assmus *et al.*, 1995; Hardoim *et al.*, 2008; Long *et al.*, 2008; Sessitsch *et al.*, 2002). Studies of endophytic bacteria diversity could improve knowledge about bacteria-plant interactions and this knowledge

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could be used for improving production and quality of the host plants (Berg, 2009; Compant *et al.*, 2005; Compant *et al.*, 2010). In the case of pitaya, the endophytic bacterial diversity is still poorly characterized.

Culture dependent methods were used to detect the composition of food associated microbiotas for decades, but these methods are difficult to reveal the real composition of microbiota due to some limitations, such as selective isolation, uncultivable state and outcompeted of low numbers of some microorganisms (Amann *et al.*, 1995; Ercolini *et al.*, 2001). Culture independent methods were developed then to overcome these problems partly. These methods could determine the composition and diversity of complex microbial communities with high speed and sensitivity (Giraffa and Neviani, 2001; Jany and Barbier, 2008). One of the most powerful methods is next generation sequencing (NGS) technology, and it has become more and more important in food quality and safety studies nowadays. NGS could produce millions of sequences in a single run and partly avoid some inherent biases in culture dependent methodology. Different microbial taxa, including uncultivable groups and some small groups which are difficult to detect by cultivated methods, could be identified based on sequence information (Ercolini, 2013; Mayo *et al.*, 2014). In addition, some cryptobiotic, dormant, moribund or latent bacteria, which might affect food quality and safety, could be identified using NGS (Davey, 2011; Mayo *et al.*, 2014). Studies of the microbiota of milk, fermented dairy products, plant, meat and fermented foods using NGS technologies had been reported more and more recently (Mayo *et al.*, 2014). Microaerophilic and anaerobic specific spoilage organisms in vacuum-packed ham had been characterized using culture-plating techniques and MiSeq NGS technologies also some microorganisms which facilitated changes in the pH value and organoleptic characteristics of the product were found (Piotrowska-Cyplik *et al.*, 2017). Comparative analysis of the metagenome mined from four diverse naturally fermented foods (bamboo shoot, milk, fish, soybean) were carried out to study the biases caused by different DNA extraction methods, Illumina MiSeq NGS showed the recovery of different bacteria varied by different DNA extraction methods (Keisam *et al.*, 2016). Assisted with traditional culture dependent and independent methods, NGS technologies have played key roles in studies on food quality and safety (Piotrowska-Cyplik *et al.*, 2017).

In this study, fruits of white and red pitayas from three different origins (Sing Buri, Thailand, Ho Chi Minh City, Vietnam and Jinghong, China) were chosen for endophytic bacteria diversity investigation by using Illumina HiSeq next-generation high-throughput sequencing technology. The aim of this work was to explore endophytic bacteria diversity in white and red pitayas from different origins. Identification of endophytic bacterial composition and diversity in pitayas might be useful for improving their production and quality.

Experimental

Materials and Methods

Sample collection. Fresh fruits of white and red pitayas were collected from tropical and subtropical regions of Thailand, Vietnam and China. Ten white pitaya and ten red pitaya fresh mature fruits were randomly chosen from each site, 60 fruits were chosen in total. All the samples were stored at 4°C and processed within 12 h.

Surface decontamination of the fruits. Sixty fruits were washed by tap water and distilled water several times, then immersed three times in 70% ethanol (v/v) for 1 min. The flesh of surface decontaminated fruits was sampled in a laminar flow cabinet at room temperature.

Genomic DNA extraction. All ten flesh samples from the same site were pooled as one sample and mixed thoroughly. Finally, six samples (white pitayas from Thailand, Vietnam and China and red pitayas from Thailand, Vietnam and China) were generated for genomic DNA extraction. Genomic DNA was extracted by DNA quick plant system kit (tiangen, China) after maceration in liquid nitrogen following the manufacturer's instructions. DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μl using sterile water.

PCR amplification of 16S rDNA-V4 region. PCR experiments were performed with Phusion® High-Fidelity PCR master mix with GC buffer (New England Biolabs) to ensure amplification efficiency and accuracy and run in an Eppendorf Gradient Thermocycler (Brinkman Instruments, Westbury, NY). With diluted genomic DNA as template, 16S rDNA-V4 region was amplified with specific primers 515F (5'-GTT TCG GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GCC AAT GGA CTA CHV GGG TWT CTA AT-3') with the barcode (Berry *et al.*, 2011; Klindworth *et al.*, 2013).

Libraries construction and sequencing. The PCR products were mixed with the same volume of 1× loading buffer (contained SYB green) and checked by electrophoresis on 2% agarose gel. Samples with bright main strip between 400–450 bp were chosen for further experiments. PCR products were mixed in equidensity ratios. Then, the mixture of PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illu-

mina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated.

Statistical analysis. Paired-end reads obtained by sequencing were divided into six groups according to the unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged to generate raw tags (Magoc and Salzberg, 2011); quality filtering on the raw tags was performed to obtain the high-quality clean tags (Bokulich *et al.*, 2013; Caporaso *et al.*, 2010). Clean tags were compared with the reference database to detect and remove chimera sequences to generate effective Tags (Edgar *et al.*, 2011; Haas *et al.*, 2011). Sequences were analyzed by Uparse software and were assigned to the same OTUs with ≥97% similarity (Edgar, 2013). Representative sequence for each OTU was annotated by GreenGene database based on RDP classifier and multiple sequence alignment was performed by MUSCLE software (DeSantis *et al.*, 2006; Edgar, 2004; Wang *et al.*, 2007). Alpha diversity and beta diversity analysis, including chao1, shannon, simpson, ACE, good-coverage, rarefaction analysis, rank abundance analysis, principal component analysis (PCA), principal coordinate analysis (PCoA), unweighted pair-group method with arithmetic means (UPGMA), non-metric multi-dimensional scaling (NMDS) analysis and T-test analysis were performed by QIIME and displayed with R software (Caporaso *et al.*, 2010).

Nucleotide sequence accession number. All the raw sequences after assembling and filtering were deposited in the NCBI SRA database under accession number SRP079944.

Results

OTU annotation and analysis. After quality filtering and chimera sequences removal, 417,519 effective sequences of six groups were obtained in total (Table I). White pitayas from China (HLGW.3) containing more effective tags than from Thailand (HLGW.1) and Vietnam (HLGW.2), while red pitayas from three different origins (HLGR.1, Thailand; HLGR.2, Vietnam; HLGR.3, China) containing a similar amount of effective tags. Red pitayas contained 21.95% more effective tags on average than white pitayas. Nonetheless, white pitayas contained 43.8% more OTUs than red pitayas. OTUs from different origins varied from 313 to 603.

Top three microorganism populations from six samples were enumerated at phylum, class, order, family and genus level. respectively. The relative abundances of bacterial populations were different in each pitaya sample (Table II). Cyanobacteria, Proteobacteria and

Table I
Sequence result from six samples.

Sample name	Sample origin	Raw Tags (#)	Clean Tags (#)	Effective Tags (#)	AvgLen (nt)	GC%	OTUs
HLGW.1	White Thailand	62449	62025	61785	253	53.93	485
HLGW.2	White Vietnam	61102	60691	60442	253	53.90	603
HLGW.3	White China	66586	66139	65885	253	53.82	576
HLGR.1	Red Thailand	76822	76347	76213	252	54.02	313
HLGR.2	Red Vietnam	78232	77729	77519	252	54.05	422
HLGR.3	Red China	76394	75811	75675	252	54.08	423

Table II
Relative abundance of the top three bacteria at different levels of taxonomy.

Taxonomy levels	Top 1 (%)	Top 2 (%)	Top 3 (%)
Phylum	Cyanobacteria (64.6–72.7)	*Proteobacteria* (19.3–25.2)	*Bacteroidetes* (0.5–8.5)
Class	*Alphaproteobacteria* (18.2–24.5)	*Bacteroidia* (0.5–8.5)	*Clostridia* (1.2–4.5)
Order	*Rickettsiales* (18.0–24.2)	*Bacteroidales* (0.5–8.5)	*Clostridiales* (1.2–4.5)
Family	Mitochondria (18.0–24.2)	*Prevotellaceae* (0.2–4.0)	*Bacteroidaceae* (0.2–1.8)
Genus	Prevotella (0.2–3.9)	*Bacteroides* (0.1–1.6)	*Acutodesmus* (1.1–1.2)

Table III
Alpha indices table.

Sample name	Shannon	Simpson	Chao1	ACE	Goods coverage
HLGW.1	2.275	0.556	484.583	490.011	0.999
HLGW.2	2.129	0.497	583.867	593.464	0.998
HLGW.3	2.231	0.533	550.647	569.752	0.998
HLGR.1	1.358	0.444	274.5	292.031	0.999
HLGR.2	1.584	0.462	414.337	429.591	0.998
HLGR.3	1.457	0.451	436.4	454.141	0.998

Bacteroidetes were found to be the most dominant phyla; *Alphaproteobacteria*, *Bacteroidia* and *Clostridia* were found to be the most dominant classes; *Rickettsiales*, *Bacteroidales* and *Clostridiales* were found to be the most dominant orders; Mitochondria, *Prevotellaceae* and *Bacteroidaceae* were found to be the most dominant families; *Prevotella*, *Bacteroides* and *Acutodesmus* were found to be the most dominant genera.

Alpha diversity analysis. Alpha diversity indices were calculated to analyze bacterial richness and diversity within communities (Table III). All of the alpha indices of white pitaya group (HLGW) were higher than in red pitaya group (HLGR). Rarefaction analysis was carried out to determine the sequence coverage of six samples (Fig. 1). All of the rarefaction curves are almost approaching their asymptotes indicated the sequence amount is adequate. Rank abundance curves indicated the species richness and species evenness of each sample were different (Fig. 2). Venn graphs were constructed to show the number of shared and unique OTUs in different groups (Fig. 3). Each sample contained a certain number of unique OTUs and white

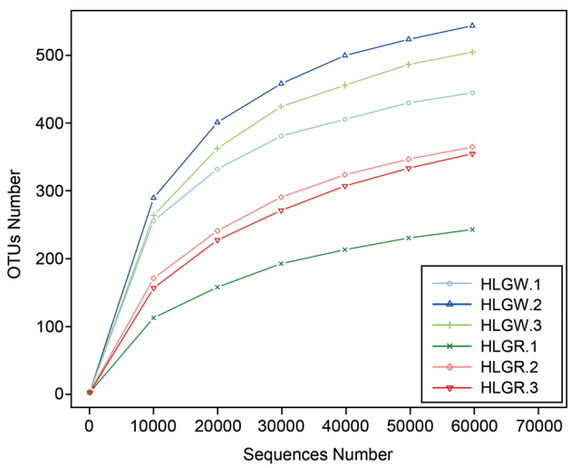

Fig. 1. Rarefaction curves of six pitaya samples. OTUs are shown at the 97% similarity.

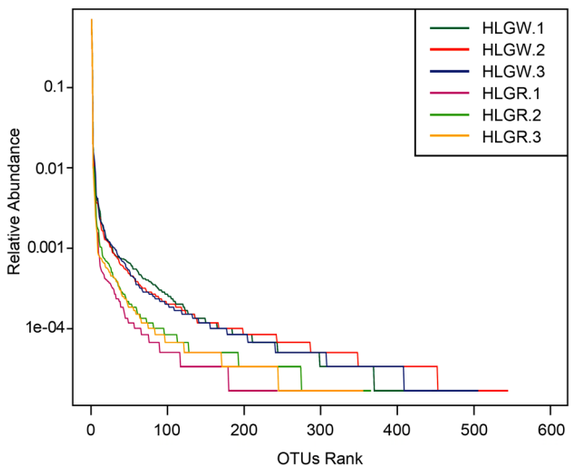

Fig. 2. Rank abundance curves of six pitaya samples.

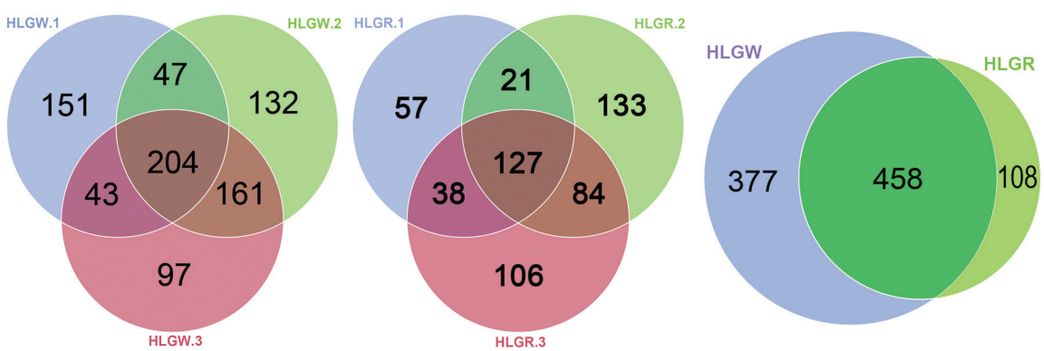

Fig. 3. Venn graphs of six pitaya samples.

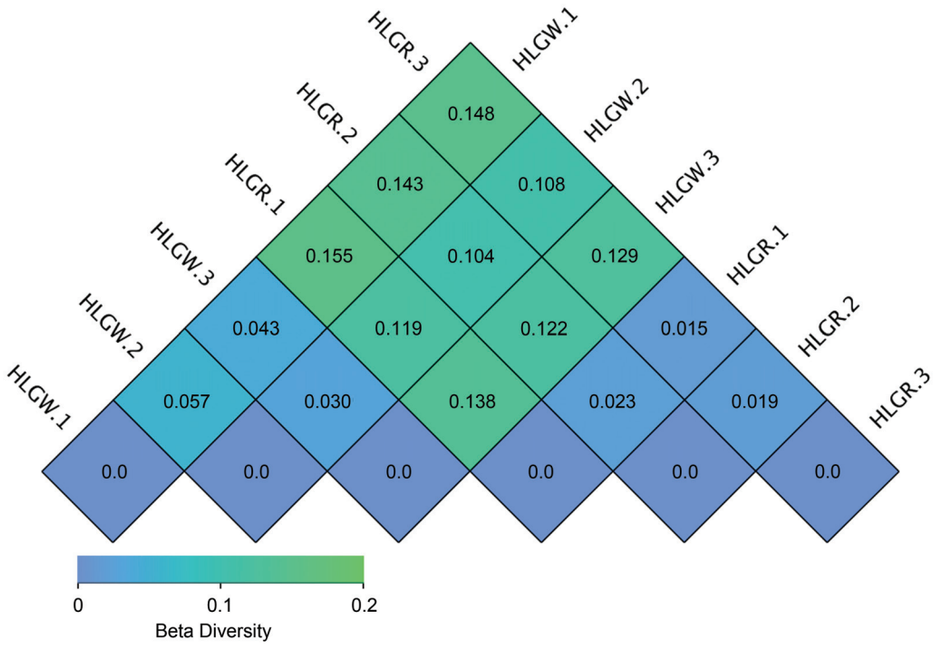

Fig. 4. Heat map of Beta diversity index.

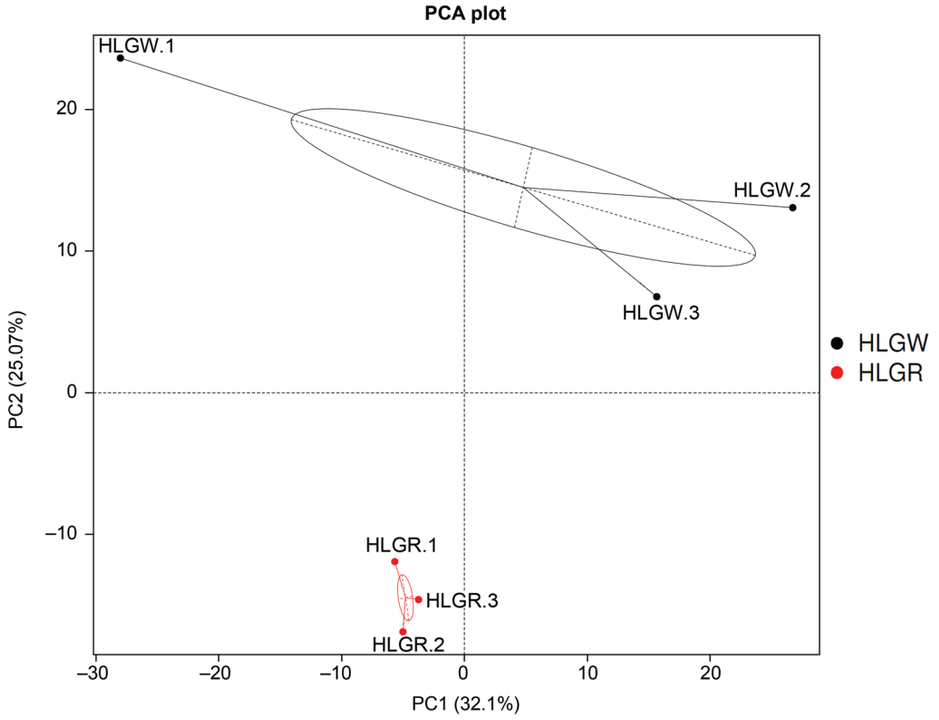

Fig. 5. Principal component analysis (PCA) plot.

pitaya group contained much more unique OTUs than the red pitaya group.

Beta diversity analysis. A heatmap of Beta diversity index was constructed (Fig. 4). Principal component analysis (PCA), principal coordinate analysis (PCoA), non-Metric multi-dimensional scaling (NMDS) and unweighted pair-group method with arithmetic mean (UPGMA) were carried out to evaluate differences of samples in species complexity (Fig. 5–8). As a result, all analysis showed that the microbial communities

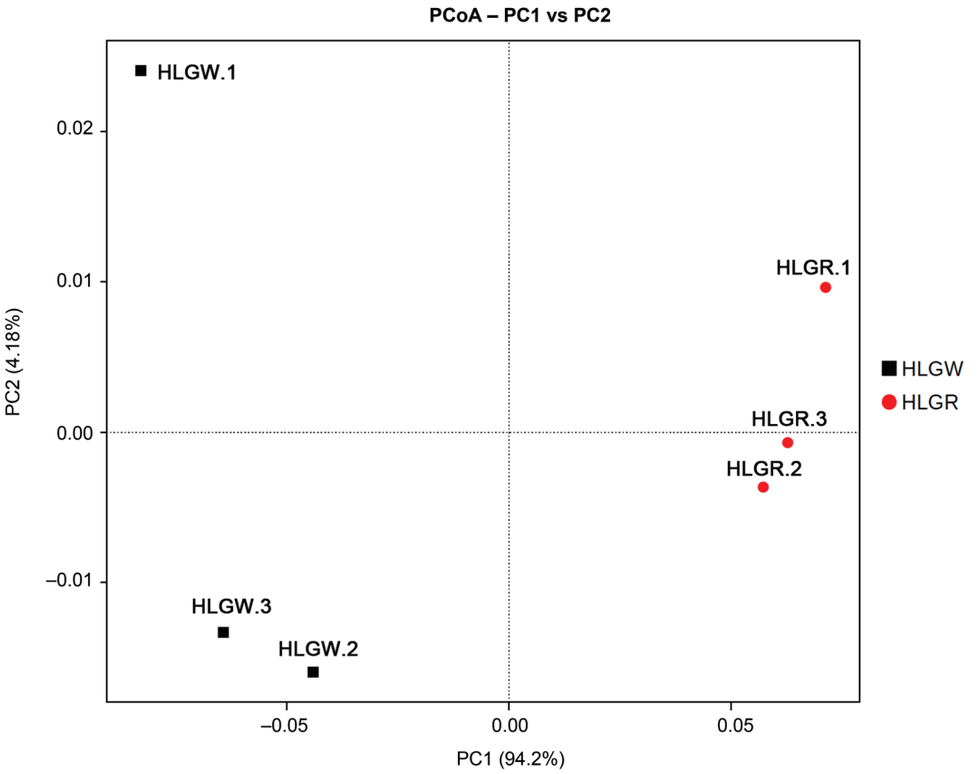

Fig. 6. Principal coordinate analysis (PCoA) plot.

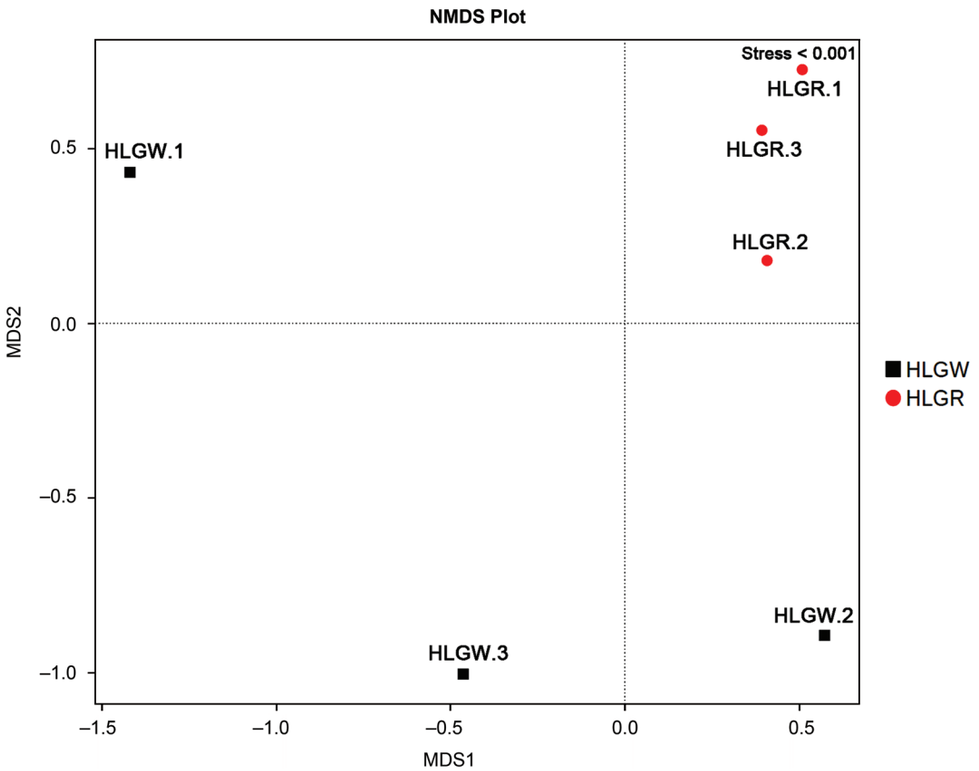

Fig. 7. Non-Metric multi-dimensional scaling (NMDS) plot.

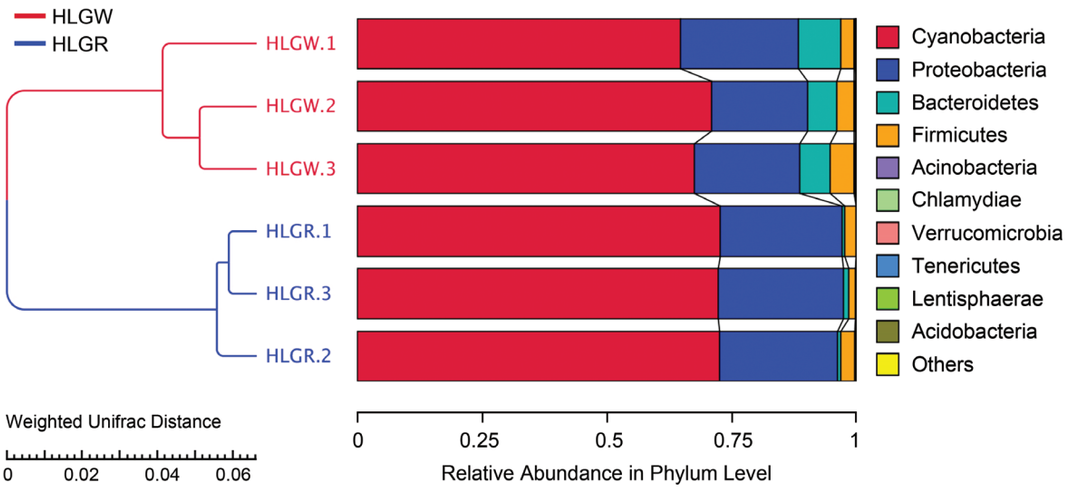

Fig. 8. Unweighted pair-group method with arithmetic mean (UPGMA) analysis.

were different between white and red pitaya groups. Samples from white pitaya and red pitaya were clustered together respectively. The bacterial community structure of each sample in red pitaya group showed higher similarity.

T-test analysis. T-test was carried out to find statistically significant different species ($P < 0.05$) between white and red pitaya groups at different taxonomy levels. As a result, the differences in the relative abundance of phylum Bacteroidetes ($p = 0.017$), classes *Bacteroidia* ($p = 0.017$) and *Coriobacteria* ($p = 0.023$), orders *Bacteroidales* ($p = 0.017$) and *Coriobacteriales* (0.023), families *Prevotellaceae* ($p = 0.001$), *Bacteroidaceae* ($p = 0.006$), *Ruminococcaceae* ($p = 0.026$), *Paraprevotellaceae* ($p = 0.015$), *Rikenellaceae* ($p = 0.043$), *Alcaligenaceae* ($p = 0.023$) and *Coriobacteriaceae* ($p = 0.023$), genera Prevotella ($p = 0.001$), *Bacteroides* ($p = 0.003$), *Roseburia* ($p = 0.041$), *Faecalibacterium* ($p = 0.034$) and *Sutterella* ($p = 0.023$) showed statistically significant between white and red pitaya groups. All of these species in white pitaya group are much more than in red pitaya group.

Discussion

In this study, we demonstrated the endophytic bacteria diversity in fruits of pitaya by using Illumina HiSeq second-generation high-throughput sequencing technology which can provide larger amount information than ever before. Large number of endophytic bacteria was found to colonize in pitaya flesh. A total of 417,519 effective sequences and 2822 OTUs with 97% similarity were obtained from six samples. Among them, 22 phyla, 56 classes, 81 orders, 122 families and 159 genera were identified.

Endophytic bacteria diversity was uneven among pitaya fruits from different origins. Rank abundance and venn graphs reflected the structure of each sample. In white pitaya group, samples from Vietnam and China shared more OTUs than other combinations. Samples from Vietnam contained most OTUs and samples from Thailand possessed most unique OTUs. Genera *Ruminococcus*, *RFN20*, *02d06*, *BF311* in samples from Thailand, genera *Escherichia*, *Bulleidia* and *Anaerostipes* in samples from Vietnam and genera *Oscillospira*, *Chlamydia* and *Succinivibrio* in samples from China showed higher relative frequencies respectively. In red pitaya group, samples from Vietnam and China shared more OTUs than other combinations. Samples from China contained most OTUs and samples from Vietnam possessed most unique OTUs. Genus *Clostridium* in samples from Thailand, genera *Bradyrhizobium* and *Mesorhizobium* in samples from Vietnam and genera *Halomonas* and *Lactobacillus* in samples from China showed higher relative frequencies, respectively.

Endophytic bacteria structure was different between the white pitaya group and red pitaya group. Although the white pitaya group contained a lower number of effective tags, it contained 43.8% OTUs more than the red pitaya group, which might be caused by different environmental factors in different pitaya fruits. All the alpha indices of the white pitaya group were higher than for the red pitaya group which indicates the bacterial richness and diversity were higher in the white pitaya group. Venn graph of the two groups also supported this conclusion. White pitaya group contains over three times more unique OTUs than the red pitaya group. To further investigate diversity differences between white and red pitaya, beta diversity analysis was carried out. The heatmap of beta diversity index clearly showed the difference between the two groups. PCA, PCoA, NMDS

and UPGMA analysis evidently demonstrated the bacterial diversity between white and pitaya groups was different. To explore the significant different species which might be used as biomarkers for white and red groups respectively, T-test was carried out. As a result, phylum *Bacteroidetes*, classes *Bacteroidia* and *Coriobacteria*, orders *Bacteroidales* and *Coriobacteriales*, families *Prevotellaceae*, *Bacteroidaceae*, *Ruminococcaceae*, *Paraprevotellaceae*, *Rikenellaceae*, *Alcaligenaceae* and *Coriobacteriaceae*, genera *Prevotella*, *Bacteroides*, *Roseburia*, *Faecalibacterium* and *Sutterella* were significantly much more in white pitaya group. Since the study showed the active components in flesh of white and red pitayas were different and the main contributors to antioxidant activity were significantly higher in red pitaya than in white pitaya (Suh *et al.*, 2014), the relationship between endophytic bacterial diversity and their living environment needs further study.

In conclusion, a large number of endophytic bacteria is found in fruits of white and red pitaya from Thailand, Vietnam and China. The bacterial diversity was different among pitayas from different origins and between white and red species. Further studies of the roles of these endophytic bacteria are needed. This study might be useful for growth improvement, fruits preservation and processing of different pitaya species from different origins.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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