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High-throughput sequencing analysis of bacterial community composition and quality characteristics in refrigerated pork during storage

Ning Li[a,b], Youxiong Zhang[a], Qingping Wu[a], Qihui Gu[a], Moutong Chen[a], Yinzhi Zhang[b], Xiulan Sun[b,**], Jumei Zhang[a,*]

[a] State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangdong Institute of Microbiology, Guangzhou 510070, PR China

[b] State Key Laboratory of Food Science and Technology, National Engineering Research Center for Functional Foods, Synergetic Innovation Center of Food Safety, Joint International Research Laboratory on Food Safety, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, PR China

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ABSTRACT

Spoilage bacteria seriously influence meat quality. In this study, the bacterial community, sensory scores, pH, and total volatile basic nitrogen (TVB-N) in refrigerated (4 °C) pork, the most commonly consumed meat in China, were investigated. In a high-throughput sequencing analysis of the V3–V4 region of the 16S rDNA gene, 259 bacterial genera were belonging to 21 phyla were identified. With the passage of time, the bacterial community diversity decreased. After 5 days, *Pseudomonas*, *Acinetobacter* and *Photobacterium* were dominant in refrigerated pork, especially *Photobacterium*, which rarely associated with meat spoilage. Our results suggest that these taxa contribute to refrigerated pork spoilage. During storage, pH and TVB-N showed similar trends. Additionally, total viable counts (TVC) increased steadily and sensory score decreased. On day 5, TVC, pH, TVB-N and sensory scores changed dramatically, and sensory scores indicating that the shelf life of refrigerated pork was less than 5 days. The predicted metabolic pathways, based o the data of 16S rDNA, indicated an abundant carbohydrate metabolism and amino metabolism in refrigerated pork. This study provides insight into the determinants of shelf life. Furthermore, it provides insight into the process involved in refrigerated pork spoilage.

1. Introduction

Meat spoilage caused by the growth and metabolic activities of specific microorganisms has become a major concern in the food industry (Belak et al., 2011; McMullen and Stiles, 1996). Refrigerated (4 °C) pork is the most commonly consumed fresh meat in China owing to its taste and high nutritional value (Zhang, 2018). According to the Food Industry Development Report, pork accounted for up to 60% of all meat production in 2016. The rich nutrients in meat, including water (71–76%), proteins (20–22%), lipids (3–8%) and carbohydrates (~2%) (Lambert et al., 1991), provide abundant substrates for the growth of microorganisms, leading to fresh pork spoilage. Microbial metabolic activities lead to changes in color, odor, texture, and other spoilage characteristics (Gram et al., 2002; Jaffres et al., 2011; Nychas et al., 2008), resulting in issues with food quality problems and even human heath (De Buyser et al., 2001). Previous study has demonstrated that the spoilage status is related to the type and amount of microorganisms in particular storage conditions and food matrices (Gill, 1983). Therefore, monitoring the development of the bacterial community in refrigerated pork during storage is important for identifying the predominant spoilage bacteria, preventing meat putrefaction, and extending the shelf life.

Traditional cultivation methods and molecular techniques, such as polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE), used to investigate the composition of microbial communities in meat (Carrizosa et al., 2017; Jiang et al., 2011; Nieminen et al., 2011). However, traditional cultivation method could not culture the viable but non-culturable microorganisms (99%) (Cocolin et al., 2013). Though a larger array of microorganisms could be identified by PCR-DGGE, this method has many limitations, such as low resolution and high background interference (Polka et al., 2015). Recently, high-throughput sequencing (HTS) based on the 16S rDNA gene has been used to analyze the composition of microorganisms, but this method has not been widely applied to fresh meat under aerobic storage. Many different bacteria have been identified as specific spoiler organisms by HTS (Remenant et al., 2015). In addition, HTS provides a reliable

* Corresponding author. Guangdong Institute of Microbiology, No. 100 Xianlie Central Road, Guangzhou, 510070, China.
** Corresponding author. Jiangnan University, No. 1800 Lihu Avenue, Wuxi, Jiangsu, 214122, China.
E-mail addresses: sxlzzz@jiangnan.edu.cn (X. Sun), Zhangjm128@126.com (J. Zhang).

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method to comprehensively describe the dynamics of bacterial communities and analyze the correlations between microbial metabolic pathways and spoilage bacteria (Jung et al., 2011; Polka et al., 2015; Yang et al., 2018).

In this study, bacterial community dynamics were investigated in refrigerated pork during storage by HTS. Spoilage indicators, including sensory scores, total volatile basic nitrogen (TVB-N), pH and microbial counts, were analysed to predict shelf life. Dominant spoilage bacteria were identified in analyses of bacterial community dynamics and changes in spoilage indicators. Additionally, functional analyses were performed to understand the underlying mechanisms of microbial spoilage. This study provides basis for detecting spoilage bacteria and controlling the putrefaction of fresh meat.

2. Materials and methods

2.1. Sample collection and storage

A total of 18 fresh pork-pieces samples (different brands) were collected from six local supermarkets in different locations in Guangzhou, China, and were packed in sterile sampling bags (Guangdong Huankai Microbial Sci. & Tech. Co.,Ltd). For each supermarket, three replicate samples were collected from different parts of undivided refrigerated pork. The pork samples were collected on 24 h after slaughter. The fresh pork samples were transported to the laboratory in insulated boxes with melted ice within 1 h. At the laboratory (day 0), samples were separately placed on a white porcelain tray and packed in polyethylene cling wrap (oxygen transmission rate: 18,500 $cm^3/(m^2 \cdot 24\ h \cdot atm)$, carbon dioxide transmission rate: 89,500 $cm^3/(m^2 \cdot 24\ h \cdot atm)$, moisture permeability: 34.6/($m^2 \cdot 24$ h)). Then they were stored at 4 °C for up to 9 days and analysed at days 1, 3, 5, 7, and 9. Samples collected from the six local supermarkets were named PAN, PTT, PWH, PWT, PVL, and PVS.

2.2. Sensory analysis

Sensory properties, including appearance, odor, slime, and resilience, were assessed according to the previous methods (Small et al., 2012), with some modifications. Five postgraduate students majoring in food science formed the assessment group. They had participated in sensory training courses and were familiar with the sensory characteristics of fresh pork. The rating scale score of each aspect ranged from 1 to 10, representing the limit of acceptability to the best quality (Table 1). According to China National Food Safety Standard methods (Fresh (frozen) livestock and poultry products; GB 2707-2016), the score of any properties, averaged among the five evaluators, below 8 represented the chilled pork begin to spoilage.

2.3. Measurements of TVB-N and pH

TVB-N concentration was measured according to China National Food Safety Standard methods (Determination of Total Volatile Basic Nitrogen in Food; GB/T 5009.228–2016). The results are expressed as mg of TVB-N per 100 g of pork. The pH was determined according to the previous method (Zhao et al., 2015) and China National Food Safety Standard methods (Determination of Food pH; GB/T 5009.237–2016), with some modifications. Briefly, 10 g of chilled pork were minced (SD-JR08D; SANDE, Shunde, China) and homogenized (Pul 100 E (230VAC); Pulsifier, Ontario, Canada) in 90 ml sterile diluent solution (0.85% NaCl) for 2 × 30 s with a 5 s break. The pH of the homogenate was determined with a digital pH-meter (S210 SevenCompact™; Mettler Toledo, Greifensee, Switzerland) with an automatic temperature compensation electrode. After pH determination, the TVB-N content was measured using the Automatic Kjeldahl Analyser (K9840; Hanon Instruments, Jinan, China). Each sample was measured in triplicate.

2.4. Microbial enumeration

The total viable counts (TVC) of each sample were obtained using the pour plate technique according to China National Food Safety Standard methods (Food Microbiology Examination- Aerobic Plate Count; GB 4789.2–2016). Briefly, 25 g of each chilled pork sample was minced and homogenized in 225 ml sterile diluent solution (0.85% NaCl) for 2 × 30 s with a 5 s break. Then, a series of 10-fold dilutions were prepared and mixed with Plate Count Agar (PCA, Guangdong Huankai Microbial Sci. & Tech. Co.,Ltd., Guangzhou, China). The plates were incubated at 37 ± 1 °C for 48 h. The results are expressed as decimal logarithms of colony forming units per gram (Log_{10} CFU/g). Each sample and each dilution were measured in triplicate.

2.5. DNA extraction

Total bacterial genomic DNA extraction was conducted according to previous method (Zhang et al., 2018), with some modifications. Ten millilitres of homogenates (obtained in section 2.4) were centrifuged at 13,000 × g for 10 min. Then, the sediments were harvested to extract total bacterial genomic DNA using HiPure Soil DNA Kits (Magen, Guangzhou, China). The DNA concentration was measured using BioSpec-nano (Shimadzu, Japan) and the DNA quality was confirmed by 1% agarose gel electrophoresis. Each sample was analysed in triplicate.

2.6. Amplification and sequencing of 16S rDNA

The V3 and V4 variable regions of bacterial 16S ribosomal DNA gene were amplified with the primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACHVGGGTATCTAAT) using KOD PCR Master Mix (Aidlab, Beijing, China) with the following protocol: 95 °C for 2 min, followed by 27 cycles at 98 °C for 10 s, 62°Cfor 30 s, and 68 °C for 30 s and a final extension at 68 °C for 10 min. The PCR products were detected by 2% agarose gel electrophoresis and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). The PCR products were subsequently quantified using QuantiFluor-ST (Promega, Madison, WI, USA) and sequenced using the Illumina HiSeq2500 system (Illumina, San Diego, CA, USA). The sequence data obtained from high-throughput analysis in this study were deposited in the NCBI BioProject PRJNA505869.

2.7. Date processing and bioinformatics analysis

Paired and clean reads were merged using FLASH (http://ccb.jhu.edu/software/FLASH/) (Magoc and Salzberg, 2011) with a minimum

Table 1
Sensory analysis of pork during chilled storage.

Scoring standard	10	8	6	4
Appearance	Very bright, red	Bright, slightly dark	Slightly dull, dark red	Dull, dark red
Odor	Desirable	Slightly off-odor	Off-odor	Unacceptable
Slime	None	A little	Some	Much
Resilience	Very tight	Tight	Slightly loose	Loose

overlap of 10bp and mismatch error rates of 2% and filtered by QIIME v1.9.1 (Caporaso et al., 2010) to obtain the high quality sequencing data according to previously methods (Bokulich et al., 2013). These sequencing data were searched against Gold database (http://drive5.com/uchime/uchime_download.html) using UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) (Edgar et al., 2011), and chimeras (Haas et al., 2011) were eliminated to obtain final effective tags.

Effective tags with 97% similarity or higher were clustered into operational taxonomic units (OTUs) cluster using mothur v1.39.1 (https://www.mothur.org/) and UPARSE (Edgar, 2013) pipeline. Based on SILVA Database (https://www.arb-silva.de/) (Quast et al., 2013), the representative sequences from each OTU were assigned to taxa by a naive Bayesian model using RDP classifier v2.2 (Wang et al., 2007). To evaluate bacterial richness and diversity, alpha-diversity indexes, including Good's coverage, Chao 1, ACE, Shannon index, and Simpson's index (Kemp and Aller, 2004) was analysed by QIIME v1.9.1. Beta diversity analysis was performed according to the weighted unifrac distance calculation method, and Principal co-ordinates analysis (PCoA) was used to analyze the difference in bacterial community composition between samples. Based on the species annotation and abundance of effective OTUs, functional annotations were obtained based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway using Tax4Fun v1.0 (Asshauer et al., 2015).

2.8. Statistical analysis

Sensory scores were processed using Origin 8.5.1. Correlation between TVB-N and TVC was analysed by IBM SPSS Statistics 20. Differences in mean values for TVB-N, pH, and TVC were analysed using one-way ANOVA implemented in IBM SPSS Statistics 20. Significance was defined as $P < 0.05$.

3. Results and discussion

3.1. Meat quality and shelf life

To monitor changes in meat quality, the sensory scores of refrigerated pork samples during storage were evaluated (Table 2). The scores of appearance, odor, slime, and resilience steadily declined for all samples during storage. There was a change in color from red to dark red and a change in odor from desirable to a slight off-odor on day 5. In addition, slime were observed on the surface of the six samples and the resilience changed from tight to not tight on day 5 of refrigerated storage. Furthermore, pH and TVB-N values for the six samples were determined over the entire storage period. As shown in Fig. 1A, although the pH values of PAN, PWH, and PWT exhibited fluctuations during the first 5 days, the pH values of all samples increased significantly after 7 days of storage and showed an increasing trend, indicating intense protein degradation by microorganisms (Pogacic et al., 2015). As a direct indicator of meat quality, the TVB-N values (Fig. 1B) for all samples displayed an obvious upward trend ($P < 0.05$) due to the evident degradation of amino acids by microorganisms after 5 days (Casaburi et al., 2015). According to the China national food safety standard (GB/T 9959.2–2008), the upper tolerable limit of TVB-N in fresh and frozen pork is 15 mg/100 g. The formation of TVB-N is mainly due to the enzymatic decarboxylation of specific amino acids, which is related to *Pseudomonas* and *Enterobacteriaceae* (Balamatsia et al., 2007). *Enterobacteriaceae* decomposes amino acids into malodorous diamine and sulfide (Samelis, 2006). 3-methylindole produced by the hypoxia metabolism of tryptophan in *Pseudomonas* causes unpleasant odors in meat (Ercolini et al., 2010). In addition, hexanal, which causes rancid odors, is mainly associated with *Brochothrix thermosphact* (Ramirez and Cava, 2007). The changes in slime and color are also associated with volatile compounds produced by microorganisms. Ropiness and brown diffusible pigments were actively produced by *Acinetobacter* (Vanderzant et al., 1987). *Enterococcus faecalis* participate in the greening of meat products (Moreno et al., 2006). Additionally, formation of slime is associated with *Mycelium* spp. (Lyhs et al., 2004). In summary, the three indexes could be used as indicators of food

Table 2
Changes in sensory scores of refrigerated pork samples during storage (4 °C).

Sensory index	Sample	Storage time (day)				
		1	3	5	7	9
Appearance	PAN	9.6 ± 0.55	8.8 ± 0.45	7.4 ± 0.55	6.4 ± 0.55	5.4 ± 0.55
	PWH	9.8 ± 0.45	8.4 ± 0.55	7.2 ± 0.45	5.6 ± 0.55	3.8 ± 0.45
	PTT	9.6 ± 0.55	8.0 ± 0.71	7.2 ± 0.84	5.8 ± 0.45	4.4 ± 0.55
	PVS	9.4 ± 0.55	8.2 ± 0.45	7.2 ± 0.45	6.2 ± 0.55	3.8 ± 0.45
	PWT	9.8 ± 0.45	8.8 ± 0.45	7.6 ± 0.55	5.8 ± 0.45	4.6 ± 0.55
	PVL	9.8 ± 0.45	8.8 ± 0.45	7.6 ± 0.55	6.4 ± 0.55	5.2 ± 0.45
Odor	PAN	9.6 ± 0.55	8.6 ± 0.55	7.6 ± 0.55	6.4 ± 0.55	5.6 ± 0.55
	PWH	9.8 ± 0.45	8.6 ± 0.55	7.4 ± 0.55	6.4 ± 0.55	4.4 ± 0.55
	PTT	9.8 ± 0.45	8.8 ± 0.45	7.8 ± 0.45	6.6 ± 0.55	5.4 ± 0.55
	PVS	8.8 ± 045	7.8 ± 0.45	6.8 ± 0.45	5.4 ± 0.89	4.0 ± 0.71
	PWT	8.8 ± 0.45	7.8 ± 0.45	6.6 ± 0.55	5.6 ± 0.45	4.4 ± 0.55
	PVL	9.6 ± 0.55	8.6 ± 0.55	7.6 ± 0.55	6.4 ± 0.55	4.8 ± 0.45
Slime	PAN	9.4 ± 0.55	8.4 ± 0.55	7.4 ± 0.55	6.4 ± 0.55	5.6 ± 0.55
	PWH	9.4 ± 0.55	8.6 ± 0.55	7.6 ± 0.55	6.6 ± 0.55	4.6 ± 0.55
	PTT	9.6 ± 0.55	8.2 ± 0.45	6.8 ± 0.45	5.6 ± 0.55	4.4 ± 0.55
	PVS	9.2 ± 0.45	8.4 ± 0.55	7.2 ± 0.45	6.4 ± 0.55	4.4 ± 0.55
	PWT	9.8 ± 0.45	8.6 ± 0.55	7.8 ± 0.45	6.6 ± 0.55	5.4 ± 0.55
	PVL	9.8 ± 0.45	9.0 ± 0.00	7.8 ± 0.45	6.6 ± 0.55	5.4 ± 0.55
Resilience	PAN	9.8 ± 0.45	8.6 ± 0.55	7.8 ± 0.45	6.8 ± 0.45	5.6 ± 0.55
	PWH	9.6 ± 0.55	8.4 ± 0.55	7.4 ± 0.55	6.2 ± 0.45	4.4 ± 0.55
	PTT	9.6 ± 0.55	8.6 ± 0.55	7.4 ± 0.55	6.2 ± 0.45	4.2 ± 0.45
	PVS	9.2 ± 0.45	7.8 ± 0.45	7.0 ± 0.00	6.2 ± 0.45	4.6 ± 0.55
	PWT	8.6 ± 0.55	7.2 ± 0.45	6.4 ± 0.55	5.4 ± 0.55	3.8 ± 0.45
	PVL	9.6 ± 0.55	8.8 ± 0.45	7.6 ± 0.55	6.4 ± 0.55	4.2 ± 0.45

Values are mean ± standard deviation of 3 replicates.

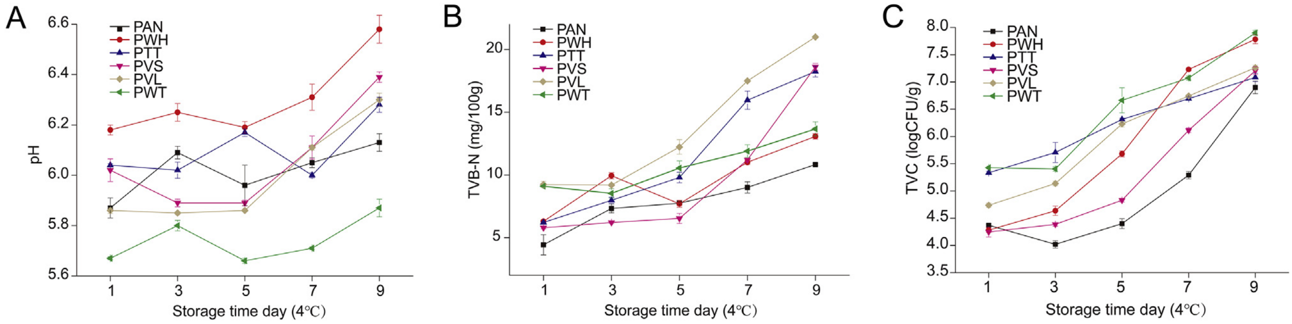

Fig. 1. Changes in pH, TVB-N and TVC of refrigerated pork samples during storage. A: pH changes; B: TVB-N changes; C: TVC changes. The error bars derived from the standard deviation between replicates (n = 3).

spoilage. In addition, according to the description of Bruckner et al. (Bruckner et al., 2012b; Li et al., 2018; Parlapani et al., 2015), it is estimated by sensory analysis that the shelf life of pork is less than 5 days at 4 °C.

3.2. Bacterial enumeration

The TVC of each sample increased during refrigerated storage ($P < 0.05$) (Fig. 1C). According to the China national food safety standard (GB/T 9959.2–2008), the upper tolerable limit of microorganism in fresh and frozen pork is 6 $\log_{10}$ CFU/g. On day 3, the TVC for PWT and PVL exceeded 6 $\log_{10}$ CFU/g, and this was associated with relatively high initial content and more rapid changes in TVB-N. The TVC values for PVS and PWH increased suddenly and exhibited the same trend as that for TVB-N after 5 days. The TVC for PAN exceeded 6 $\log_{10}$ CFU/g after 9 days, which resulted in a lower content and slower change in TVB-N in PAN than those of the other samples. For all samples, TVC and TVB-N both showed increasing trends and were correlated (Table 3), consistent with previous results (Gui et al., 2014; Hernandez-Macedo et al., 2012). Except PAN and PWH samples, the correlation coefficients of other samples were significant ($P < 0.05$) (Table 3). At the end of storage, the TVC values for all samples were up to 7–8 $\log_{10}$ CFU/g, which was far beyond the upper tolerable limit for fresh and frozen pork. The TVC value differed between spoiled samples, and this can probably be explained by the difference in the glucose content. When glucose was insufficient in food substrates, spoilage occurred with a low TVC (Gill, 1983; Gill and Newton, 1980). The activity of spoilage bacteria contributed to the accumulation of TVB-N. Therefore, we can determine the degree of food spoilage by detecting the TVC of microorganisms when food substrates are known.

3.3. Bacterial richness and diversity

A total of 12,531,544 high quality effective sequences were obtained by merging and filtering raw sequence from each sample, with an effective ratio of 79.18%–92.06% and sequence length from 301 bp to 478 bp (Table S1). Effective sequences were clustered into 3691 OTUs using a 97% similarity threshold (Table S1). The alpha-diversity indexes are shown in Table S2. Good's coverage was at least 99% for all samples, suggesting that almost all bacteria in pork samples could be detected. At the end of the storage time, the values of Chao 1 and ACE in PAN, PTT, PVS, and PVL samples were lower than the initial values (day 1). For the Simpson and Shannon indexes, the endpoint values were lower than the initial values for all samples. The changes in the four indexes indicated that the richness and diversity decreased during storage, suggesting that a subset of bacteria became dominant in samples.

Based on the relative abundance of OTUs sequenced at different storage times, PCoA was used to analyze the differences in bacterial community composition between samples (Fig. 2). On day 1, all samples were clustered together, indicating that the difference in bacterial community composition was not significant. PTT and PWT samples were clustered together on day 9, while bacterial community composition of the other samples was similar, which indicated that the difference between the different samples was not significant. The samples stored on the first day were clearly separated from the samples stored on the ninth day, suggesting that the bacterial community composition changed greatly and the effect of time for the bacterial community composition was significant.

Table 3
Correlation between TVC and TVB-N in each sample.

Correlation parameter	PAN	PWH	PTT	PVS	PWT	PVL
Correlation coefficient	0.797	0.830	0.962	0.975	0.986	0.949
Significance	0.107	0.082	0.009	0.005	0.002	0.014

3.4. Composition of bacterial community

A total of 21 phyla were identified in the sequencing analysis, including *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Deinococcus-Thermus* (Fig. 3A). *Proteobacteria* and *Firmicutes* were the major phyla during storage, accounting for ~90% of all OTUs. As the storage time increased, the proportion of *Proteobacteria* increased from 50% to 90% and the percentage of *Firmicutes* decreased from 40% to 5% in all samples (Table S3).

Bacterial community dynamics were evaluated based on changes in relative abundance at genus level (Fig. 3B) during storage. A total of 259 bacteria genera were identified by HTS. At the beginning of storage, *Exiguobacterium* was the major genus in the PAN (38.26%), PVS (34.76%), PWH (35.93%), PWT (31.96%) and PTT (21.08%) samples, and *Acinetobacter* was the dominant genus in the PVL sample (48.32%) (Table S3). In previous studies, *Exiguobacterium* has been found in aquatic products, vegetables and salted meat but not in fresh meat (Cui et al., 2014; Liu et al., 2010; Lopez-Cortes et al., 2006; Minervini et al., 2015). The abundance of *Pseudomonas* significantly changed after 5 days in the PAN, PVL, and PVS samples, and changed significantly after 3 days in the PWH sample. At the end of storage, *Pseudomonas* was the dominated genus in the PAN and PWH samples. *Photobacterium* was the major genus in the PTT and PWT samples. *Acinetobacter* and *Pseudomonas* were the main genera in both the PVL and PVS samples. These results indicated that the microbial composition changed dynamically in refrigerated pork during storage. In this study, *Pseudomonas*, *Photobacterium* and *Acinetobacter* were the dominant spoilage bacteria at the end of storage, of which *Pseudomonas* and *Acinetobacter* are frequently observed in fresh meat (Doulgeraki and Nychas, 2013; Ercolini et al., 2010; Huang et al., 2013; Lee et al., 2017; Nychas et al., 2008). *Photobacterium* is often reported in sea food (Mace et al., 2013; Remenant et al., 2015), but reported less in meat (Delhalle et al., 2016; Hilgarth et al., 2018; Koo et al., 2016). *Photobacterium* is heat labile and unable

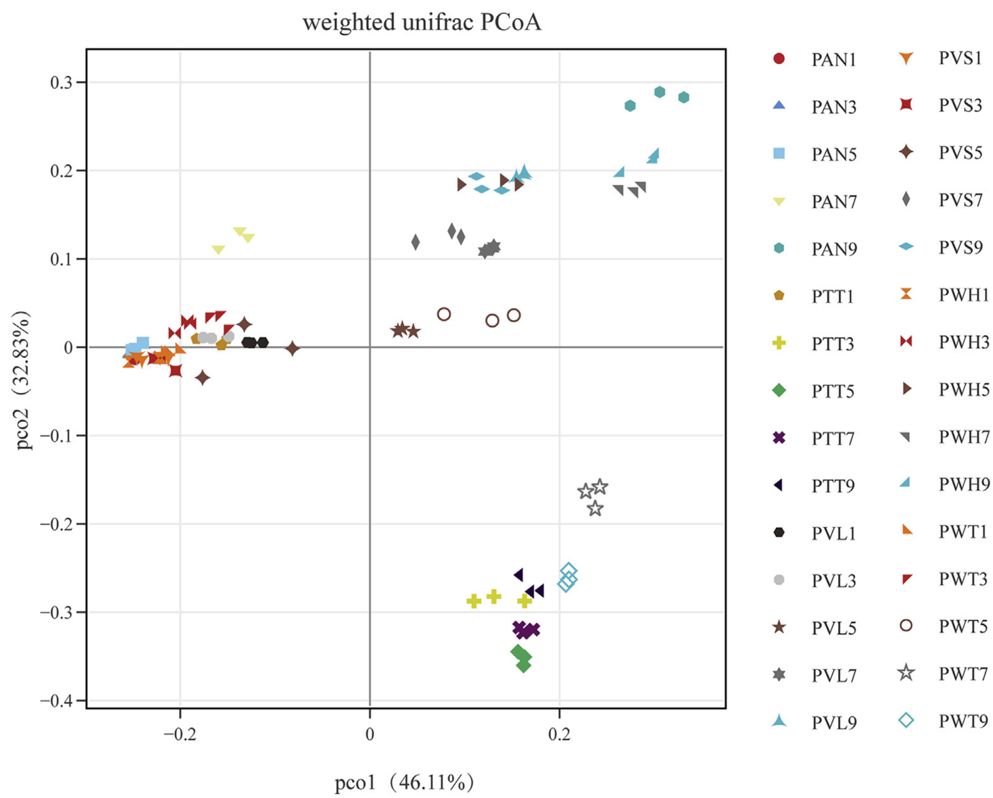

Fig. 2. Principal co-ordinates analysis (PCoA) of bacterial community of refrigerated pork during storage (4 °C). Analysis was assessed by Unifrac method.

to grow at above 25 °C. In addition, it requires a certain amount of sodium for growth (Nieminen et al., 2016). Thus, *Photobacterium* may be not cultured in traditional culture media and incubation temperature (37 °C). However, in this study, the proportions of *Photobacterium* in PTT and PWT samples reached 65.85% and 64.54%, respectively, on day 9. Dalgaard et al. (2006) reported that *Photobacterium* was associated with biogenic amine formation. However, the mechanism underlying spoilage by *Photobacterium* has not been clarified. Owing to the mesophilic, psychrotolerant nature and the short generation time (less than 4 h) in 0–7 °C (Andreani and Fasolato, 2017), *Pseudomonas* was regarded as a Specific Spoilage Organism for a variety of foods, such as fresh pork, poultry, ready-to-eat vegetables, tofu, sea foods, and beef (Andreani et al., 2014; Bruckner et al., 2012a; Gram et al., 1990; Stoops et al., 2012). Furthermore, *Pseudomonas* has been identified in different environment conditions, such as in air or modified atmosphere packages (Gram and Huss, 1996; Parlapani et al., 2015). In this study, the population of *Pseudomonas* was initially 12%–14% and increased continuously to 70%–82% at the end of storage (Fig. 2B), consistent with previous studies (Li et al., 2018; Yang et al., 2018). Additionally, on day 9, the proportion of *Acinetobacter* and *Pseudomonas* in PVL and PVS samples increased up to 70% (Fig. 2B). Only *Pseudomonas* in PAN and PWH samples reached 70% (Fig. 2B). In addition, TVB-N values for PVL (21 mg/100 g) and PVS (18.62 mg/100 g) samples were higher than those of PAN (10.83 mg/100 g) and PWH (13.07 mg/100 g) samples. Chung et al. reported that *Acinetobacter* sp. with greater bacterial growth resulted in fewer volatile compounds than *Pseudomonas putrifaciens* when they were separately in beef (Chung et al., 2002). Thus we inferred whether *Acinetobacter* and *Pseudomonas* in mixed culture contributed substantially to the production of TVB-N.

3.5. Functional properties of the bacterial community

As the abundance of metabolic pathways in microorganisms determines the status of food spoilage, microbial metabolism can provide further insight into the pork spoilage process. For the PAN sample (Fig. 4A), the abundance of genes associated with carbohydrate metabolism began to decline after 5 days, and amino acid metabolism began to increase. For other five samples (Fig. S1), on day 3, carbohydrate

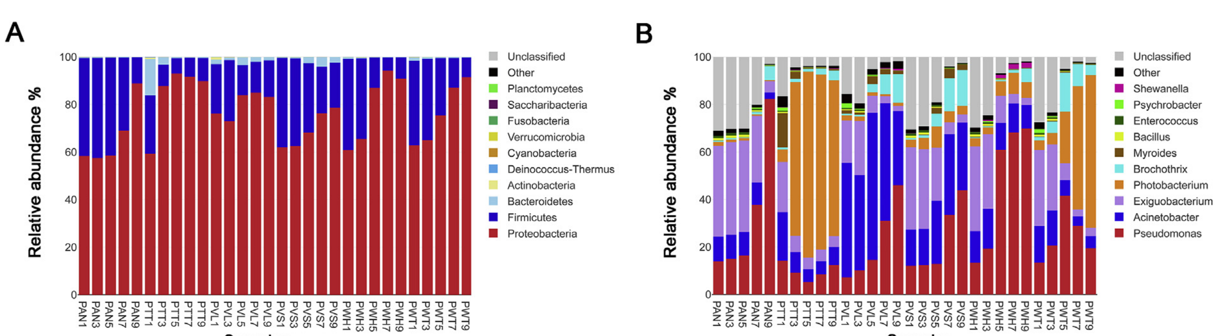

Fig. 3. Dynamics in relative abundance (%) of bacterial taxa based on 16S rDNA sequencing at phylum (A) and genus (B) level in fresh pork samples during refrigerated storage. The number 1 to 9 represents the refrigerated storage time (day).

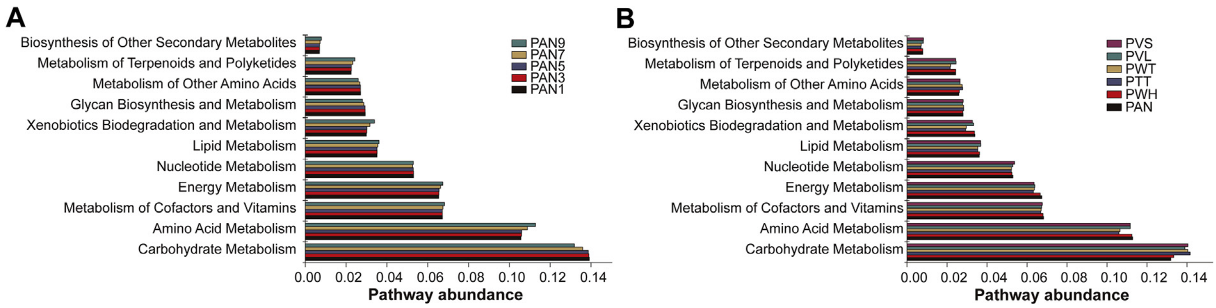

Fig. 4. Abundance of functional properties that related to microbial metabolism in samples. A: Changes of metabolic pathways in PAN sample during storage. The number 1 to 9 represent the refrigerated storage time (day). B: Compare of functional properties that related to microbial metabolism in the six refrigerated pork samples at ninth day.

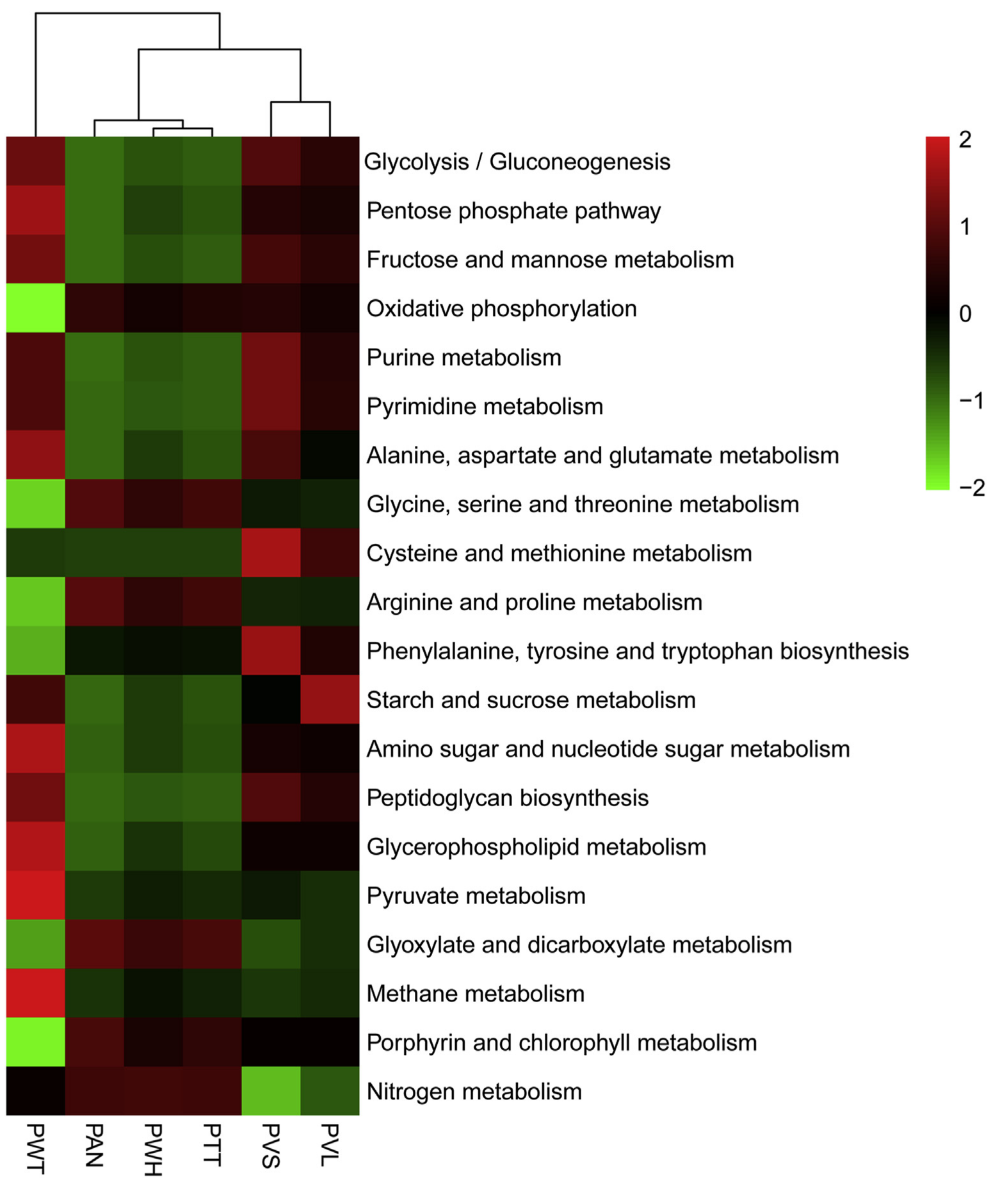

Fig. 5. Heatmap of bacterial metabolic pathways in refrigerated pork during stored on the ninth day (4 °C). Metabolic inference based on the 16S rDNA sequence. The heatmap only showed the relative abundance of metabolic pathways were in top 20.

metabolism began to increase and amino acid metabolism began to decrease in PTT sample, but these two metabolisms were just the opposite in PWH sample. Additional, these two metabolisms began to increase in PVS sample, but only carbohydrate metabolism began to increase in PWT sample. During storage, this two metabolisms are quite stable in PVL sample. In addition, the abundance of metabolic pathways in the six samples was compared on day 9. As shown in Fig. 4B, carbohydrate metabolism and amino metabolism were the main metabolic pathways for all samples, consistent with previous studies (Gill, 1983; Reis et al., 2016). After comparing of metabolic pathways and

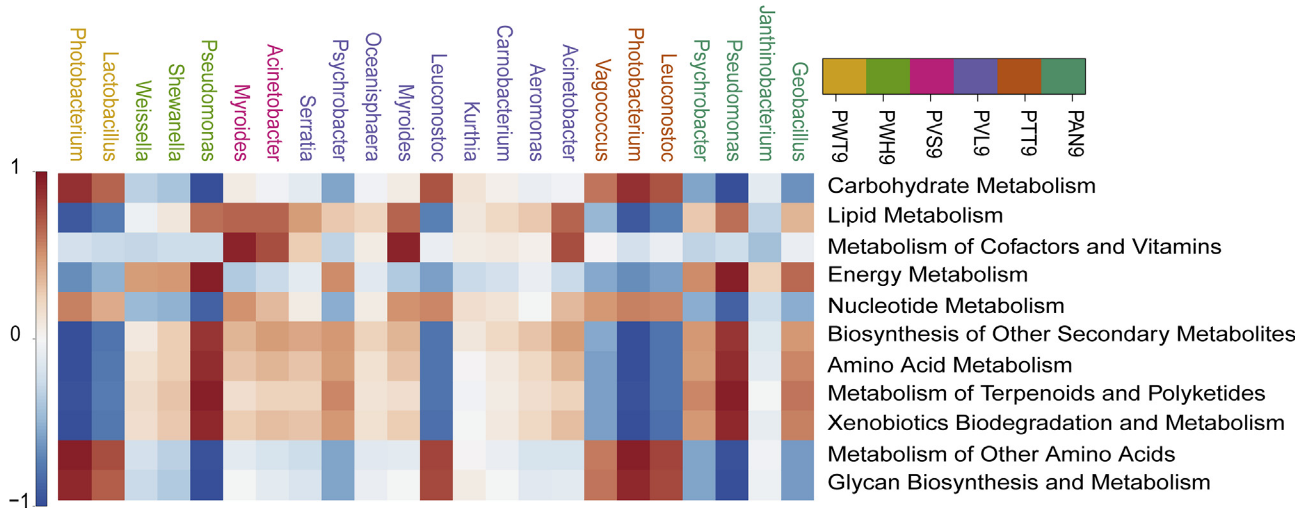

Fig. 6. Heat map of the correlation between the differential abundant genus and metabolic pathway. The number 9 represents the refrigerated storage time (day). The differential genus and their abundance between the six samples were obtained by Lefse analysis, and the abundance of the metabolism-related secondary metabolic pathways in the six samples was analysed by Tax4Fun. The Pearson correlation coefficient between the differential genus and the metabolic pathway was calculated, and the correlation coefficient matrix of the differential genus-metabolic pathway was obtained, and the matrix heat map was drawing by R language. Red represents a positive correlation, blue represents a negative correlation, and the darker the color, the greater the correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

dominated bacteria in each sample, it was found that there was no regular change. Therefore, it is necessary to find out the correlation between the interaction of bacteria and metabolism.

On the level of the thirdly metabolic pathway, the microbial metabolism in each sample on the ninth day of storage were inferred (Fig. 5). Cluster analysis revealed that the six samples were clustered into four groups. The metabolic pathways in PWH and PTT samples were similar, which were also semblable in PVS and PVL samples. The metabolic pathway of PWT samples was the minimal similarity to the other five samples. The abundance of metabolic pathways associated with biogenic amines and sulfide formation in PVS and PVL samples was high, such as phenylalanine, tyrosine and tryptophan biosynthesis metabolism, which were associated with *Enterobacteriaceae* and *Pseudomonas* (Curiel et al., 2011). In PWT sample, the metabolic pathways related to the formation of volatile compounds, such as aldehydes, ketones, acids, and esters, were relatively abundant, such as pyruvate metabolism and glycerophospholipid metabolism. The abundance of glyoxylate and dicarboxylate metabolism, nitrogen metabolism and arginine and proline metabolism, which were related to the formation of biogenic amines and volatile amines, were higher in PAN, PWH and PTT samples. The dominant microorganisms in the PAN and PWH samples were identical, and the metabolic pathway abundance was similar. The same was true for PVS and PVL samples. However, *Photobacterium* was the major bacteria in both PWT and PTT samples, but the metabolic pathways in both samples were the most distant. In combination with Fig. 3B, there was a difference in the abundance of *Pseudomonas* and *Acinetobacter* in PWT and PTT samples, and it was speculated whether it affected the metabolism of *Photobacterium*.

Furthermore, correlation between differential genus and metabolic pathways were analysed, as shown in Fig. 6. In the PTT and PWT samples, *Photobacterium, Leuconostoc, Vagococcus* and *Lactobacillus* were associated with high levels of carbohydrate metabolism, consistent with previous studies showing that these four bacteria have a strong capacity of utilizing carbohydrate (Casaburi et al., 2015; Nieminen et al., 2016; Samelis, 2006). *Pseudomonas* in PAN and PWH samples showed the most pronounced positive correlation with amino metabolism, which consistent with the increase of amino acid metabolism in microorganisms shown in Fig. 4A. In PVL and PVS samples, *Acinetobacter* exhibited high levels of lipid metabolism and metabolism of cofactors and vitamins. However, the abundance of reads associated with these metabolic processes were relatively low on day 9 (Fig. 4B). Thus, further studies of the spoilage mechanism of pork meat caused by *Pseudomonas* and *Acinetobacter* when they are simultaneously dominant are needed.

The metabolic pathways might provide insight into the bacterial metabolic activity in the certain food and the processes involved in spoilage. However, little is known about the enzymes responsible for spoilage (Remenant et al., 2015), so the enzymes related to spoilage in the main metabolic pathways need further research.

4. Conclusions

In this study, bacterial community dynamics of and quality changes in refrigerated pork during storage at 4 °C were evaluated. Using HTS, 259 bacteria genera were identified and microbial community dynamics were analysed. At the genus level, *Pseudomonas*, *Acinetobacter* and *Photobacterium* were the dominated microorganisms at the end of storage; *Photobacterium* is not typically reported as a major bacteria taxon during storage. The pH, TVB-N, TVC, and sensory score indexes could be used as indexes of food spoilage, and the sensory scores suggested that the shelf life of refrigerated pork is less than 5 days at 4 °C. The data of 16S rDNA sequenced by HTS predicted that the carbohydrate metabolism and amino metabolism were the major metabolism in refrigerated pork. Accordingly, we can monitor whether food has been spoiled by the total viable counts and the abundance of spoilage bacteria. Our study improves our understanding of the microbial-induced spoilage of refrigerated pork and offers a basis for controlling the deterioration of fresh meat during aerobic storage. Owing to an insufficient understanding of bacterial metabolism, it is necessary to combine genomics with other omics approaches to analyze the relationship between spoilage characteristics and metabolic pathways for identification of specific spoilage enzymes.

Conflicts of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2019.04.013.

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