# Bacterial Diversity Analysis of Zhenjiang Yao Meat During Refrigerated and Vacuum-Packed Storage by 454 Pyrosequencing

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**Abstract** Zhenjiang Yao meat is a traditional variety of cooked marinated and jellied pork food in China. It is usually stored refrigerated to prevent gelatine liquefaction. Our understanding of the bacterial populations found in Zhenjiang Yao meat is limited. This study was designed to explore both the bacterial diversity and the main bacterial flora of Zhenjiang Yao meat using pyrosequencing of tagged amplicons from the V3 and V9 regions of the 16S rRNA gene. A total of 53,363 bacterial sequences from five samples and an average 10,672 reads of each sample were acquired and used in the analysis of microbial diversity. The bacterial diversity was observed to increase weekly, and the main bacterial flora changed significantly under refrigerated vacuum-packaged storage. The predominant bacteria were *Vibrio* during the first 7 days of storage, whereas *Shewanella*, *Lactobacillus*, *Lactococcus*, *Yersinia*, and other *Enterobacteriaceae* members were the main groups after 15 days in refrigerated storage. The bacterial population changed significantly after 15 days, and the bacterial communities in the 30 days sample were significantly different from all other samples. In comparison with culture-dependant method, pyrosequencing provides a more comprehensive estimate of bacterial diversity and more close to the real bacterial composition in Zhenjiang Yao meat.

## Introduction

Zhenjiang Yao meat is a traditional Chinese preparation of meat, dating back to more than 300 years ago. In the traditional Zhenjiang Yao meat process (Fig. 1), a boned pork hoof was marinated with salt and spices and usually stored at 4 °C to prevent the liquefaction of gelatine. Due to its rich nourishment and special attractive flavour, Zhenjiang Yao meat is very popular in China, with a consumption of 20,000 tons each year (http://www.jsw.com.cn/zj news/2010-02/10/content_1965291.htm). However, vacuum-packed Zhenjiang Yao meat is a highly perishable meat product. Its pH of approximately 6.5 and its water activity (higher than 0.98) cannot inhibit the usual types of microorganisms associated with post-processing contamination [7]. Typically, the shelf-life of vacuum-packed Yao meat is less than 30 days under refrigerated storage at 4 °C during retail. Spoilage of Yao meat often results in quality defects such as sour flavour, oxidation, gas production, ropy slime formation, and discoloration [18]. Apart from oxidation and discolouring, the other spoilage symptoms are ascribable to the undesired growth of microorganisms to unacceptable levels that make the product undesirable for human consumption [21].

It is critical for the meat industry to investigate diversity of these contaminating bacteria, identify the bacterial species and control them. Knowledge about bacterial diversity and main flora of meat products may eventually be useful in microbiological inspections, shelf-life predictions and the development of new preservation or production methods [19]. Bacterial diversity and main flora of

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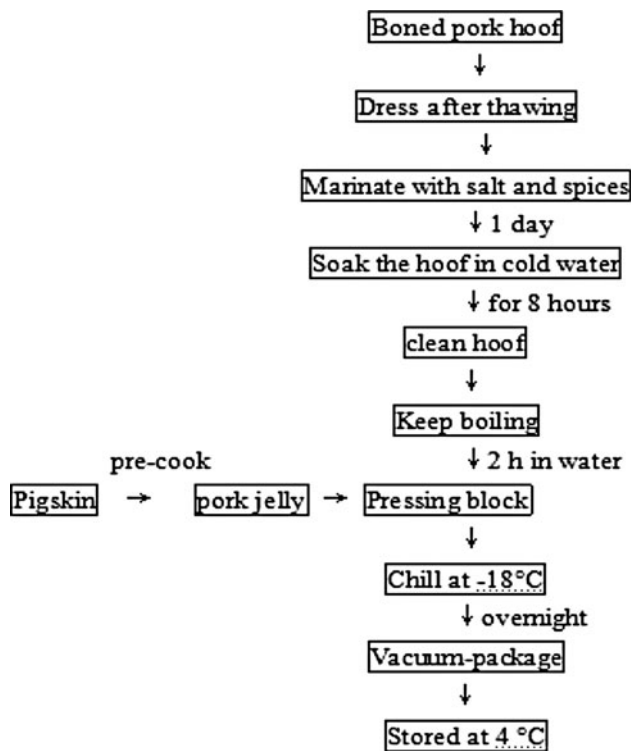
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Fig. 1 Flowchart of manufacturing and ripening stages of the Yao meat

cooked cured meat under different storage conditions have been extensively studied according to traditional cultivation methods [7], but these cultivation methods have proven to be unreliable for the complete characterisation of bacterial diversity [1]. Therefore, culture-independent microbial techniques such as denaturant gradient gel electrophoresis (DGGE) and the construction and analysis of libraries of conserved genes such as the 16S rRNA gene are used to study the microbiology of meat [14, 20, 26]. Currently, metagenomic studies relying on the utilisation and analysis of reads obtained using 454 pyrosequencing have changed the way to analysis the bacterial diversity by permitting the analysis of hundreds of thousands of nucleotide sequences at one time [8]. This technology has been successfully used to identify the microbial communities of various foods such as soybean pastes [29], grains [25], meats [30], and cheeses [12]. This technique can expose more hidden microbial groups than traditional culture-independent microbial techniques [23]. However, there are few data characterising the main flora and changes in bacterial diversity in cooked marinated-jellied pork, including Chinese Yao meat, using DGGE or 454 pyrosequencing. Therefore, the objective of this study was to explore the changes in bacterial diversity of vacuum-packed Yao meat during refrigerated storage by 454 pyrosequencing and culture-dependant methods.

## Materials and Methods

### Sampling

All Yao meat samples were collected on the same day in July 2011 from the Zhenjiang Yuanchun Meat Products Company, which is the largest factory producing Yao meat in Jiangsu Province, China. Day 0 samples were analysed immediately on the day of packaging, whereas other packages were analysed 7, 15, 22, and 30 days after packaging, respectively. Each day's sample was taken from four packages.

### Colony Counts, Total Volatile Basic Nitrogen (TVB-N) and pH

Twenty-five grams of each sample was homogenised with 225 mL of peptone saline solution (0.85 % NaCl and 0.1 % peptone in distilled water) for 30 min in a stomacher blender. Bacterial colony counts were measured as described previously [32], using plate count agar (PCA, pH 7.2) for the total aerobic count, Man–Rogosa–Sharpe agar (MRS, pH 6.2) for lactic acid bacteria (LAB), Violet red bile glucose agar (VRBG, pH 7.4) for *Enterobacteriaceae* members, streptomycin sulphate thallium acetate actidione agar (STAA, pH 7.0) for *Brochothrix thermosphacta* and cetrimide–fucidin–cephaloridine agar (CFC, pH 7.2) for *Pseudomonads*. All plates were incubated 48 h at 30 °C, with the exception of the CFC and VRBG plates, which were incubated at 25 °C. The MRS plates were incubated in jars made anaerobic by MGC AnaeroPack (Anaerogen, Oxoid, Basingstoke, Mitsubishi gas chemical company, JAPAN).

TVB-N was determined according to Farber and Ferro [15].pH was measured with a pH-electrode (InLab Solids Pro, Mettler Toledo GmbH, Schwerzenbach, Switzerland) connected to a pH meter (SevenEasy, Mettler Toledo GmbH). The product temperature during pH measurements was 4.0 ± 2.0 °C.

### DNA Extraction and PCR Reaction

Twenty-five grams of each sample was homogenised with 225 mL of peptone saline solution (0.85 % NaCl and 0.1 % peptone in distilled water) for 30 min in a stomacher blender. 20 mL quantities of the solution were centrifuged for 10 min at 9,000 g, and the pellet was subsequently analysed. Bacterial DNA was extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and then resuspended in 100 μL of TE buffer (containing 10 mmol/L Tris–HCl and 1 mmol/L EDTA). The DNA solution was subjected to agarose gel (1 %, containing DNA green) electrophoresis.

The DNA samples from four packages produced at the same day were mixed together and measured by a Nano-drop 1000 spectrophotometer (Thermo Fisher Scientific, Waltman MA). The 3F (5′-CCATCTCATCCCTGCGTG TCTCGACGACT-3′) and 1492R (5′-CCTATCCCCTG TGTGCCTTGGCAGTCTCAG-3′) primers were used for PCR amplification of the V3–V9 variable regions of the prokaryotic 16S rDNA. Eight base-sample-specific barcode sequences (ACACTATA) were followed by the 3′ end of the forward primer, and 454-adaptors were included in both the forward (5′-TACGGRAGGAGCAG-3′) and reverse (5′-ACCTTGTTACGACTT-3′) primers. PCR was performed in 50 μL reaction volumes using a Tag-DNA polymerase master mix (Ampliqon, Denmark) with 20 ng of DNA from each sample as a template and 0.2 mM of each primer. Thermocycling was conducted in a ABI9700 Thermal Cycler (ABI, USA) under the following conditions: initial denaturation at 95 °C for 5 min; 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min 30 s; and a final extension at 72 °C for 10 min. The quality of the amplified PCR products was verified by electrophoresis in a 1 % agarose gel and purified using the AP-GX DNA Gel Extraction Kit (Axygen, USA). The DNA was determined by a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltman, MA).

Pyrosequencing and Data Analysis

Prior to sequencing, PCR amplicons were diluted to the same concentration, and equal quantities (100 ng) of all samples (tagged with sample-specific barcode sequences) were pooled. The pooled DNA was amplified in PCR mixture oil emulsions before sequencing by synthesis using a massive parallel pyrosequencing protocol [28]. Sequencing was performed by the Genome Sequencer FLX454 Titanium System (454 Life Sciences CT, USA) according to the manufacturer's instructions. The sequence data were sorted into each sample batch using the barcode tag in the Pipeline Initial Process at the RDP. The sequences generated from pyrosequencing were mainly analysed with MOTHUR software (version 1.14.0) (http://schloss.micro.umass.edu/mothur/Main_Page) for pre-processing, identification of operational taxonomic units (OTUs), taxonomic assignment, community comparison, and statistical analysis [34]. The sequences were filtered to minimise the effects of poor sequence quality and sequencing errors by removing sequences with more than one ambiguous base call and retaining only sequences that were 200 bp or longer. Sample-specific sequences were collected according to their barcode sequences tagged to each sample, and the barcode, forward and reverse primer sequences were trimmed from the initial sequences. In the first step, the reads were trimmed to analyse only regions with average scores greater than 50 bp and a

window of at least 35 bp. Then, sequences were excluded from the analysis if they were of low quality, if the read length was less than 200 bp, if one of the primer sequences was missing, if a sequence had one or more ambiguous base calls or if it had multiple barcode or primer motifs.

The clusters were constructed at a 3 % dissimilarity cut-off and served as OTUs for determining richness and diversity indices, ACE, Chao1, and Good's coverage ($G = 1 - n/N$, where $n$ is the number of phylotypes that have been sampled once, and $N$ is the total number of individuals in the sample) using MOTHUR [9, 27, 34, 35]. The Ribosomal Database Project (RDP) Classifier program (http://rdp.cme.msu.edu/classifier/classifier.jsp) was used to convert the sample sequence data to bacterial population data in hierarchical levels [33, 37], and the confidence threshold was set at 80 %. If the similarity was below the 3 % dissimilarity cut off value, the read was assigned to an "unclassified" group. The MOTHUR program was also used to perform the Fast UniFrac test, which was employed to compare the phylogenetic structure of the libraries and to generate Venn diagrams. A heat map was generated on the basis of the relative abundance of each bacterial genera using R version 2.13.1 (R Foundation for Statistical Computing). Clustering was performed using the Manhattan distance. To visualise the similarity of bacterial populations among the five samples, we performed a principal component analysis (PCA) on the relative abundance of bacterial species using R version 2.13.1.

## Results

Colony Counts, TVB-N, and pH Values of Yao Meat Samples

Colony counts were measured from Yao meat samples after different durations of storage. As shown in Table 1, No colonies were observed during the first day except in the total aerobic count. The total aerobic counts increased significantly from $10^1$ to $10^8$ cfu/g during the course of storage. *Enterobacteriaceae* also increased significantly from 7 to 30 days, and colonies reached $10^7$ cfu/g at the end of the storage period. *Pseudomonads* grew quickly in the first 7 days and reached their highest level of $10^5$ cfu/g at 15 days. LAB and *Brochothrix thermosphacta* levels reached $10^4$ cfu/g at 30 days. The mean pH values decreased slowly from 6.84 to 6.34 during the refrigerated storage period, while the mean TVB-N values increased from 15.4 to 20.72.

Phylotypes and Diversity Estimates of Bacterial Populations

With barcoded parallel pyrosequencing, we achieved a view of the diversity from the vacuum-packed Yao meat

**Table 1** Colony counts, TVB-N and pH values of Yao meat samples at different times of refrigerated storage (immediately (IM), after 7 , 15 , 22 and 30 d)

|        | Colony count (log cfu/g) | | | | TVB-N[a] | pH |
|--------|-------|------|----------------|----------------|---------|---------|
|        | Total | LAB  | Enterobacteria | B. thermosphacta | | |
| IM      | 1.92 ± 0.01 | ND[b] | ND           | ND           | 15.4 ± 0.03  | 6.84 ± 0.09 |
| 7 days  | 3.16 ± 0.02 | ND    | 1.81 ± 0.01  | ND           | 16.43 ± 0.02 | 6.78 ± 0.07 |
| 15 days | 6.65 ± 0.05 | 1.74 ± 0.00 | 5.39 ± 0.10 | 4.29 ± 0.03 | 17.46 ± 0.01 | 6.75 ± 0.06 |
| 22 days | 7.50 ± 0.08 | 2.00 ± 0.02 | 7.25 ± 0.09 | 5.68 ± 0.05 | 18.60 ± 0.02 | 6.51 ± 0.04 |
| 30 days | 8.09 ± 0.16 | 4.74 ± 0.03 | 7.01 ± 0.12 | 5.77 ± 0.11 | 20.72 ± 0.04 | 6.34 ± 0.02 |

Table showed a range of four parallel meat packages

[a] Total volatile basic nitrogen

[b] Not determined

**Table 2** Comparison of phylotype coverage and diversity estimation of the 16S rRNA gene libraries at 3 % dissimilarity from the pyrosequencing analysis at different times of refrigerated storage (immediately (IM), after 7, 15, 22, and 30 days)

| Samples | Read number | Average length[a] | OTUs[b] | ACE | Chao[b] | Shannon[b] | Coverage (%)[b] |
|---------|-------------|-------------------|---------|-----|---------|------------|-----------------|
| IM      | 9396  | 434 | 840  | 1115.45 | 1183.57 | 5.45 | 97.84 |
| 7 days  | 10114 | 439 | 1249 | 2411.52 | 1919.59 | 5.94 | 94.62 |
| 15 days | 12095 | 440 | 3169 | 7253.07 | 5463.64 | 7.09 | 86.50 |
| 22 days | 9685  | 415 | 3318 | 7440.28 | 5607    | 7.41 | 82.75 |
| 30 days | 12073 | 413 | 2838 | 6598.46 | 4970.53 | 7.22 | 84.20 |

[a] Average length of the sequences

[b] Calculated with MOTHUR at the 3 % distance level. Values in *brackets* represent 95 % confidence intervals

during the refrigerated storage period at a much deeper level. We obtained 53,363 pyrosequencing tags that passed our quality control methods. Each Yao meat sample was covered by an average of 10,672 reads, and the average length of the sequences was 428 bp after trimming the primers. The diversity richness, coverage, and evenness estimations calculated for each data set are presented in Table 2. In the five Yao meat samples, ACE and Chao1 indices were considerably higher than the observed number of OTUs, which suggests that there could be more additional bacterial phylotypes. Good's coverage [17] of the IM and 7 days samples was approximately 95 %, indicating that approximately five additional phylotypes would be expected for every 100 additional sequenced reads. However, the Good's coverage values of the samples of 15, 22, and 30 days were approximately 85 %, suggesting that a large number of unseen OTUs still existed in the original samples and that more sequencing efforts may be required to detect additional phylotypes.

Bacterial Communities of Yao Meat Samples

The phylogenetic classification of sequences from Yao meat samples by phylum is summarised in Fig. 2. As shown in Fig. 2a, all samples were dominated by nine phyla. Of the nine major phyla, *Proteobacteria* was the

predominant microbiota in Yao meat samples, but the relative abundance of phylum *Proteobacteria* changed greatly during the storage period (approximately 68.75–98.82 % of total sequences in each group). *Firmicutes* was the second-most predominant bacteria in Yao meat samples, with a relative abundance that ranged from 1.12 to 30.22 % and an abundance that was negatively correlated with that of the *Proteobacteria*. The other seven phyla were only found with low relative abundances in the total sequences. As shown in Fig. 2b, nine families comprised more than 85.75 % of the bacterial population, but the most abundant family differed in each sample. For example, the IM, 7 and 30 days samples exhibited the highest abundance of *Vibrionaceae* members; the 14 and 22 days samples displayed the highest abundance of *Enterobacteriaceae* members. During the first 7 days of storage, the relative abundance of *Vibrionaceae* members occupied the overwhelming majority (97.71 and 91.15 % for samples IM and 7 days). Seven days later, the relative abundance of *Vibrionaceae* members decreased, whereas that of other families increased, and the entire bacterial population became more diverse. To analyse the bacterial community dynamics associated with Yao meat at different times of storage in detail, a heat-map representing the relative abundance of each genus level phylotype was constructed (Fig. 3). One hundred and sixty-nine genera level phylotypes were
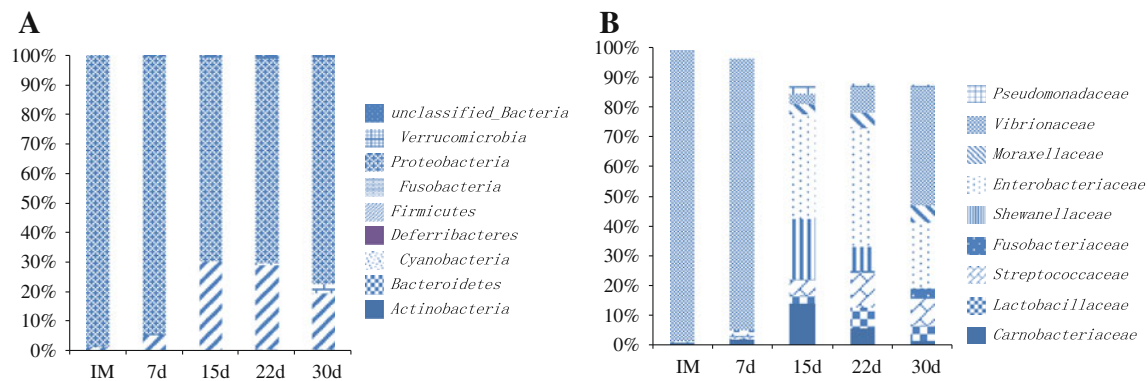
**A**



**B**



**Fig. 2** Relative abundance at the phylum level (**a**) and family level (**b**) based on the classification of partial 16S rDNA sequences of bacteria from Yao meat samples at different times of refrigerated storage (immediately (IM), after 7, 15, 22, and 30 days) using Ribosomal Database Project (RDP) Classifier

identified, and bacterial genera were classified into three different population groups depending on the relative abundance, where 1 % were considered rare, 1–10 % were labelled as subdominant, and greater than 10 % were considered to be a predominant group.

To compare the bacterial composition of each Yao meat sample clearly, phylotype- and phylogeny-based comparisons were carried out. A UPGMA tree constructed by the dissimilarity of phylotypes, concatenated with 97 % sequence identity in each sample, revealed two different clusters (Fig. 4a): group 1—IM and 7 days; group 2—15, 22, and 30 days. This means that the bacterial communities from the IM and 7 days samples were more similar than those of the 15, 22, and 30 days samples, and the 15 and 22 days samples were more similar to each other than to the 30 days sample. The community structures of all five samples were also compared using principal component analysis based on the nine main families (Fig. 4b). The maximum variations in the bacterial communities of the Yao meat samples were found to be 46.6 % (PC1) and 22.8 % (PC2), with a strong separation by region. PCoA also revealed three different groups: group 1—IM and 7 days; group 2—15 and 22 days; and group 3—30 days. The results were similar to the results of the UPGMA analysis, but the bacterial populations at 30 days differed significantly from those of the other samples.

## Discussion and Conclusions

In this study, we were able to detect a number of changes introduced over time on bacterial populations using a combination of culture-independent and -dependent methods. Within the period of storage, the quantity of bacteria and TVB-N values increased while pH values decreased. During the first 7 days of storage, the relative abundance of Vibrionaceae was significantly higher than any other fresh

meat samples analyzed before, especially Vibrios occupied more than 65.96 % the overwhelming bacterial population [30, 31]. However, the relative abundance of Vibrio decreased greatly while other genera proliferated, and all bacterial communities became diversified from day 15 to the end of storage. This probably occurred because most aerobic Vibrio gradually died at the beginning of storage, while other types of bacteria or spores grew quickly during this period. It was interesting that Vibrio exhibited rapid growth at the last period of storage and became the predominant bacterium in Yao meat products again, possibly because the facultative anaerobic Vibrio demonstrated delayed growth. Because many Vibrio require NaCl for growth [5], the frequent occurrence of Vibrio bacteria in cooked and marinated meat may be due to the living habits of pigs and the procedure of meat preparation.

Enterobacteriaceae, including the genera Yersinia, Buttiauxella and unclassified Enterobacteriaceae, appeared to be the predominant group from the intermediate stage to the end of storage. Yersinia and other Enterobacteriaceae can be detected in meat on different packaging temperature conditions [10, 11]. But there was no available study before showed that Enterobacteriaceae were the predominant flora in cooked meat during the storage. Plate counts also indicated that Enterobacteria constituted a large fraction of the total population. As most Enterobacteria can be destroyed through heat treatment, whether the high occurrence of Enterobacteria here was caused by the presence of a contaminating flora at the production site or the microbial flora of the meat before processing remains an open question. Further studies are needed to understand how processing conditions affect the presence and growth of potential spoilage bacteria in Yao meat products.

Although there were a great proportion of Lactobacillaceae (including the genera Carnobacterium, Lactobacillus, Weissella, and Lactococcus) and Shewanellaceae (including Shewanella) at the intermediate stage of storage,
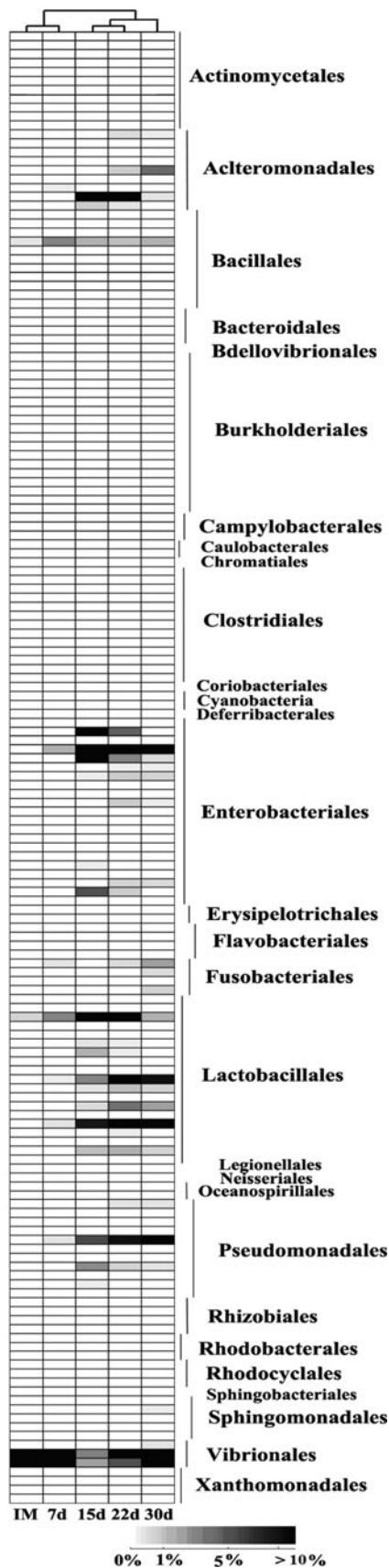
◀ **Fig. 3** Relative abundance of genus level taxa. Each column in the heat-map represents one Yao meat sample, while each *row* represents a genus level phylotype. The colour intensity of the panel is proportional to the abundance of OTUs. The *staggered bars* on the right indicate the specific family groups

the relative abundance of these genera dramatically decreased by the end of storage. The most frequently isolated organisms from cooked vacuum-packaged meats were *Lactobacillus* [3, 4]. In our study, *Lactobacillus and Lactococcus* were also the main flora of Yao meat products 15 days after refrigerated storage. However, they were not the dominant populations found in Yao meat because the growth of them can be inhibited under vacuum conditions [6]. Plate counts indicated that LAB constituted only a small fraction (less than 1 %) of the total population. It has been reported that *Carnobacterium* was difficult to isolate from fish because they do not grow well on MRS agar [16], which may be the reason that they are under-represented here compared to results of the culture-independent method. *Shewanella* grew quickly from days 7 to 15 and became the dominant flora of Yao meat. Fifteen days later, the relative abundance of *Shewanella* decreased quickly, possibly because *Shewanella* can grow anaerobically and dies as a result of competitive inhibition in the last period of storage.

*Pseudomonads* were also a very important group in the meat spoilage [2, 13, 24]. But in vacuum-packaged Yao meat, *Pseudomonads* only play a small amount of total bacteria at the last period of storage. This may be because of packaging under vacuum delayed the growth of the *Pseudomonas* [22]. It has been also reported that certain members of the family *Enterobacteriaceae* may grow on the medium CFC [36], which may be the reason the relative abundance here higher than the results of culture-independent method. *Brochothrix thermosphacta,* which has been reported to play an important role in meat, grew slowly during the first 7 days and maintained a stable level of 1.09–1.15 % for the rest of the storage period, similar to the results obtained by the culture-dependent method. *Psychrobacter* grew slowly (but stably overall) during storage.

In conclusion, pyrosequencing using barcoded primers was used for the first time to assess the microbial community of Zhenjiang Yao meat and the overall microbial diversity of Zhenjiang Yao meat was determined. Pyrosequencing provide a more comprehensive estimate of microbial diversity and more close to the real bacterial composition in this kind of meat compared with using culture-dependent techniques. The results of the study had added new information to existing knowledge on microbial communities in meat. The knowledge of understanding variability and change in these communities can assist in
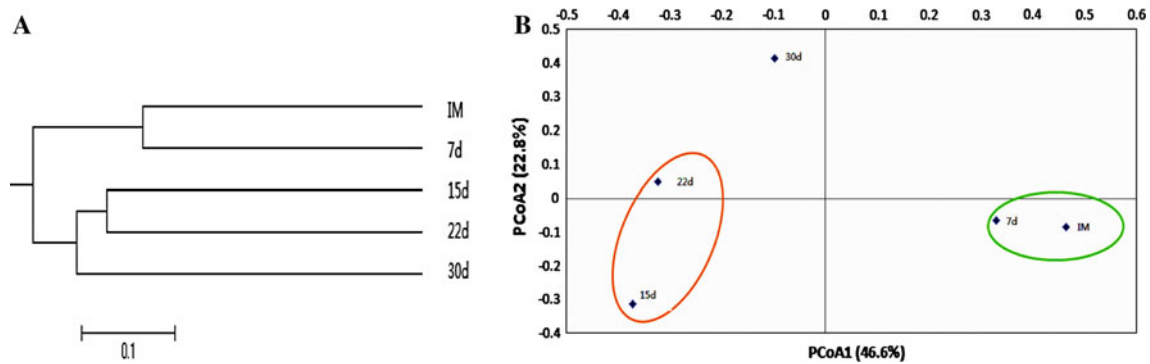
**Fig. 4** Principal component analysis of differentiation bacterial communities in Yao meat samples at different times of storage (immediately (IM), after 7, 15, 22, and 30 days). Each community was clustered by the phylotype-based UPGMA method (**a**) and phylogeny-based weighted unifrac method (**b**)

understanding the spoilage process. Hence, the assessment of microbial species diversity occurring in Zhenjiang Yao meat during storage will be fundamental for improving and implementing processing technology aimed at prolonging the shelf life of products. Based on our study, it was important for the Yao meat company to improve food handling and environmental hygiene, and reheat treatment after vacuum-package might be very useful for prolonging the shelf life of Yao meat.

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