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Microbial changes in vacuum-packed chilled pork during storage

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ABSTRACT

Microbial composition in vacuum-packed chilled pork was investigated. The number of microbial counts increased during the period of 21 day storage with the deterioration of meat. A total of 28,216 bacterial sequences were obtained for the assessment of microbial diversity from vacuum packed pork during chilled storage. More than 200 bacterial genera belonging to eighteen phyla were observed, and most of them are likely to be associated with contamination via fecal, air and/or water during slaughtering and subsequent meat handling. Microbial populations changed greatly during storage, of which the seventh day was a critical time point for microbial diversity. *Micrococcaceae*, *Flavobacteriaceae*, *Enterobacteriaceae*, *Lactobacillaceae* and *Carnobacteriaceae* were the major components that may be associated with the spoilage of meat. Although the potential impact of detected microbes on meat hygiene and/or safety is unknown, effective decontamination of the whole chain is always important for meat industry to guarantee meat safety and to improve shelf-life of fresh meat.

1. Introduction

Meat hygiene is determined by numerous environmental factors, which could result in meat spoilage and food safety problems. For chilled meat, the growth of bacteria is the main cause of the reduction of freshness and the progress of spoilage (Belák et al., 2011; Ercolini, Russo, Nasi, Ferranti, & Villani, 2009; Horvath, Andrassy, Korbasz, & Farkas, 2007). The microbial diversity and main flora in fresh meat have been widely investigated using traditional cultivation methods. In recent years, cloning and sequencing techniques such as polymerase chain reaction and denaturing gradient gel electrophoresis (PCR–DGGE) have been applied to explore microbial populations in meats (Ercolini et al., 2010; Jiang, Gao, Xu, Ye, & Zhou, 2011; Li, Zhou, Xu, Li, & Zhu, 2006; Osés et al., 2013). The PCR–DGGE overcomes the limitations of traditional cultivation methods. For example, some microorganisms cannot be cultivated by traditional methods; and it takes a long time to identify the composition from a pool of microorganisms. Li et al. (2006) found that the bacterial diversity of chilled pork decreased with storage time and *Arthrobacter* sp., *Enterococcus* sp., *Staphylococcus* sp., *Moraxella* sp., *Pseudomonas* sp., *Lactobacillus* sp., *Aeromonas* sp., *Acinetobacter* sp. and *Brochothrix thermosphacta* were the main flora during a 7-day storage period.

However, PCR–DGGE is still time-consuming and can be only used to detect predominant members of the microbial communities. More recently, the bacterial diversity in beef steaks and a Chinese meat product (Zhenjiang Yao Meat) was examined with high-throughput barcoded parallel 454 pyrosequencing and their data showed that the bacterial phylotypes were more complex than previous studies have suggested (De Filippis, La Storia, Villani, & Ercolini, 2013; Xiao, Dong, Zhu, & Cui, 2013). In these two studies, high-throughput sequencing was shown to be a powerful means of giving insight and understanding of the changes in the microbial populations in meat and meat products during production or storage.

In the present study, parallel pyrosequencing with 16S rDNA and 18S rDNA was applied to characterize bacterial and fungal changes in vacuum-packed chilled pork. The results were expected to provide insights into the understanding of microbial changes in fresh pork during production and during subsequent chill storage in terms of any precautions for meat safety and for the improvement of meat hygiene.

2. Material and methods

2.1. Sampling

A total of 20 pig carcasses from the same herd were selected from a single slaughtering line in a commercial slaughterhouse (Henan, a capacity of 3000 carcasses per day). After 20 h chilling in a 0 °C chiller, carcasses were commercially fabricated. The cut "hind leg" was vacuum-packed in heat-shrink bags (oxygen transmission rates: 18.54×10^{-6} $cm^3/m^3 \cdot d \cdot Pa$). Vacuum packed cuts were stored at 0 °C for up to 21 days and were sampled after 1 h (day 0), 7 days, 14 days, and 21 days after vacuum packaging (5 bags per time point). On each sampling occasion, 100 g of muscle on the exposed surface was cut to a thickness of up to 2 cm and was removed for color and pH measurement, total volatile basic nitrogen (TVB-N) determination, microbial enumeration and DNA extraction.

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2.2. Color measurement

Color parameters were determined as previously described (Li et al., 2012) using a Minolta colorimeter (CR-300; Minolta Camera Co., Osaka, Japan) with illuminant D65, a 0° viewing angle and an 8 mm port/viewing area. Before measurement, the colorimeter was calibrated with a white tile (mod CR-A43). Color coordinates (L*, a*, b*) were recorded. Three measurements were performed on each of five samples at each time point.

2.3. pH measurement

Meat pH was measured as previously described (Li et al., 2012). Briefly, 1 g of sample was homogenized (Ultra Turrax T25, IKA, Germany) at 6000 rpm for 2 × 15 s with a 5 s break in 10 mL of ice-cold buffer containing sodium iodoacetate (5 mM) and potassium chloride (150 mM), pH 7.0. The pH of the homogenate was recorded with a Hanna 211 pH meter (Hanna, Italy). Three measurements were performed on each of five samples at each time point.

2.4. TVBN determination

TVBN concentration (milligrams per 100 g of meat) was determined according to the method of Malle and Poumeyrol (1989). Briefly, 100 g sample was homogenized in 6% trichloroacetic acid at 5000 rpm for 30 s with two bursts. The homogenate was filtered through Whatman filter paper and TVBN in the filtrate was released by adding 1% K_2CO_3 and diffusing with 1% boric acid and then titrated with 0.02 N HCl. Results of TVBN contents were expressed as mg/100 g meat. Three measurements were performed on each of five samples at each time point.

2.5. Enumeration and isolation of microorganisms

Twenty five grams of each meat sample was homogenized in 225 mL of peptone saline (0.85% NaCl and 0.1% peptone in distilled water) for 30 s in a blender. After that, triplicate serial dilutions were prepared and plated onto Plate Count Agar (PCA, Land Bridge Company, China) plates. The plates were incubated for 48 h at 37 °C. The number of microbes was expressed as $\log_{10}$ (counts). Three measurements were performed on each of five samples at each time point.

2.6. Total bacterial genomic DNA extraction

Meat subsamples (25 g) were homogenized in 225 mL of peptone saline (0.85% NaCl and 0.1% peptone in distilled water) for 30 s in a blender. Twenty milliliters of the homogenate was centrifuged at a speed of 10,000 ×*g* for 10 min (Avanti J-HC, Berkman, USA). The pellets were resuspended in 200 μL of lysis buffer and broken with 100 mg of zirconium beads (0.1 mm) in Mini-beadbeater (FastPrep, Thermo Electron Corporation, USA) for 2 min. Microbial DNA was extracted with DNeasy tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and resuspended in 100 μL of TE buffer (containing 10 mmol/L Tris–HCl, pH = 8.0 and 1 mmol/L EDTA, pH = 8.0). The DNA concentration was measured using a Nano-drop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

2.7. Pyrosequencing for 16S rDNA

The bacterial diversity was analyzed by pyrosequencing of the amplified 16S rDNA V4–V5 variable region (Richard et al., 2013). The forward primers included adaptor A (CCATCTCATCCCTGCGTGTCTCCG ACGACT), barcodes, and gene specific primer F (16S 515F: GTGCCAGC MGCCGCGGTAA). The reverse primers included adaptor B (CCTATCCC CTGTGTGCCTTGGCAGTCTCAG), barcodes, and gene specific primer R (16S 926R: CCGTCAATTYYTTTRAGTTT). Four barcodes, i.e., ATGCACGT, ATGTGACT, CAGAGTCT and CAGATAGT were used for 0 day, 7 day, 14 day and 21 day samples, respectively. The reaction mixture (20 μL) included 0.25 μL of TaKaRa Ex Taq®(5 U/μL), 2 μL of 10× Ex Taq buffer (25 mM Mg^{2+} Plus), 1.6 μL of dNTP, 0.4 μL of primer F, 0.4 μL of primer R, 1.0 μL of DNA template, and 14.35 μL of ddH_2O. The amplification was performed using GeneAmp® 9700 PCR system (ABI, USA) as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR products were separated by 2% agarose gel electrophoresis under 100 V for 40 min (Tanon EPS-100 system), purified using AxyPrep DNA gel extraction kit (Axygen, USA) and fluorescently quantified by Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen, USA) and an equimolar pool was obtained prior to further analysis.

Amplicon pyrosequencing was performed using a 454/Roche GS-FLX sequencer (Roche, Germany). The purified pool was amplified using GS FLX Titanium LV emPCR Kit (Lib-L, Qiagen, USA) using GeneAmp® 9700 PCR system (ABI, USA) under the following conditions: initial denaturation at 94 °C for 4 min, followed by 50 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 4.5 min and extension at 68 °C for 30 s, and finally being held at 10 °C. The resulting solution was treated with GS FLX Titanium emPCR Breaking Kit and the final DNA was sequenced using 454 GS FLX platform (Roche, Germany).

2.8. Data analysis

After pyrosequencing, all readings were screened and filtered using QIIME 1.6.0 software (Caporaso et al., 2010). The readings were excluded if their quality scores were lower than 25 and/or their length was shorter than 200 base pairs. Operational taxonomic units (OTUs) were picked only if they had similarity values of 97% or higher. Alpha diversity was evaluated by community richness (rarefaction curves, Chao1 and ACE) and diversity (Shannon indices) using MOTHUR (version 1.32.1) (http://www.mothur.org/) (Schloss et al., 2009).

The representative sequences were compared to the RDP classification (Ribosomal Database Project, http://rdp.cme.msu.edu/wiki/index.php/Main_Page) to obtain the taxonomy assignment (Wang, Garrity, Tiedje, & Cole, 2007). Beta diversity was evaluated by phylogenetic tree analysis and similarity analysis. The Fast UniFrac test in the MOTHUR program was performed to compare the phylogenetic structure and to generate Venn diagrams.

The differences in meat quality data with storage time were evaluated by one-way analysis of variance. Least squares means of 4 time points were compared by Duncan's multiple comparison method at the significance level of 0.05 with the program SAS 9.12 (SAS Institute Inc., Cary, NC, USA, 2003).

3. Results

3.1. Bacterial enumeration and meat quality

Total bacterial counts increased during the storage ($P < 0.05$, Table 1). Although there was a large increase by day 14, the bacterial counts were still at an acceptable level (smaller than 10^6 CFU g^{-1}). At day 21, the natural logarithm of the bacterial count was over 6 and the meat appeared slightly sticky, indicating that meat had began to be deteriorated and would not be suitable for consumption. Meat pH and TVBN values showed a similar trend whereby they both increased with storage time ($P < 0.05$, Table 1). Meat color measurements (L*, a*, b*) did not show significant changes with storage time ($P > 0.05$, Table 1).

3.2. Sequencing data

For 16S rDNA analysis, a total of 28,216 pyrosequencing tags with the length of 173 to 557 base pairs were obtained, of which the majority