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Microbial community dynamics analysis by high-throughput sequencing in chilled beef longissimus steaks packaged under modified atmospheres

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ABSTRACT

The microbial community dynamics were investigated in this study to understand the spoilage of beef steaks packaged in both modified atmosphere packaging (MAP): 80%O_2-MAP (80% O_2/20% CO_2) and CO-MAP (0.4% CO/30% CO_2/69.6% N_2). Steaks were stored at 2 °C for 20 days. Meat physicochemical changes and microbial counts were monitored, and the microbial communities were evaluated by high throughput-sequencing. The microbial diversity for both MAP decreased over time and it was more complex in CO-MAP steaks than that in 80%O_2-MAP steaks. *B. thermosphacta* and *Pseudomonas* spp. were dominant bacteria of 80%O_2-MAP steaks but *Pseudomonas* spp. gradually outcompeted the former from day 10. *Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Vagococcus* and *Serratia* dominated alternately in CO-MAP steaks during storage, and *Lactococcus* eventually became the most common bacteria. Predicted metagenomes indicated a higher microbial amino acid and lipid metabolism level in 80%O_2-MAP compared with CO-MAP at day 20, which may contribute to the dramatic physicochemical deterioration of 80%O_2-MAP steaks.

1. Introduction

Microbial contamination is the major concern and the most important factor in the deterioration of meat during chilled distribution and retail display (McMullen & Stiles, 1996). The deteriorative effects caused by microbial growth are discoloration, off-odors, and slime formation that make meat unacceptable to consumers (Benson et al., 2014). Microbial spoilage of meat is a complex process affected by competition among different microbial groups and their biotic and abiotic interactions (Doulgeraki, Ercolini, Villani, & Nychas, 2012). Development of this process depends primarily on meat types, hygienic practices during the slaughter and processing, and storage conditions (Chaillou et al., 2015). In particular, the different storage conditions, such as packaging atmospheres, temperature and the application of antibacterial compounds, were found to have a major effect on microbial growth and succession (Doulgeraki & Nychas, 2013; Doulgeraki, Paramithiotis, Kagkli, & Nychas, 2010). Even though bacterial spoilage species bring considerable economic loss to the meat industry, it is sometimes difficult to establish a direct link between a given spoilage phenomenon and one specific species, or the interaction of several species (Zagorec & Champomier-Vergès, 2017).

Commercially, high-oxygen modified atmosphere packaging (MAP) containing 80% O_2 and 20% CO_2 (80%O_2-MAP), or MAP with 0.4% carbon monoxide (CO), 30% CO_2, and 69.6% N_2 (CO-MAP) are two commonly used MAP methods to extend red meat shelf-life and maintain its quality. The US FDA has recognized CO concentration not exceeding 0.4% as generally safe, and permitted its use in case-ready meats (FDA, 2004). Although the EU still has not allowed the use of CO in meat packaging, the appeal of re-evaluation of CO as a packaging gas within the EU is now rising, due to its color stability and prolongation of meat shelf-life (Van Rooyen, Allen, & O'Connor, 2017). Meat under modified atmospheres undergoes an ecological succession of bacteria that tolerate the altered package atmosphere. Previous identification studies illustrated that different gas compositions exerted different selection effects on bacterial communities (Carrizosa et al., 2017; Doulgeraki et al., 2012). Meanwhile, shifts in the community structure under different packaging systems also lead to different types of spoilage defects (Jääskeläinen, Hultman, Parshintsev, Riekkola, & Björkroth, 2016). The summation of all of these effects will determine the final microbial shelf-life of meat.

In recent years, the high-throughput DNA sequencing technology has been proved to be more powerful than traditional cultivation methods or polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to analyze bacterial community dynamics in

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meat. Moreover, it helps us better understand the subsequent spoilage process associated with varying microbial metabolic pathways (Zagorec & Champomier-Vergès, 2017). However, to our knowledge, few have compared the microbial diversity of beef steaks packaged under both 80%O_2-MAP and CO-MAP by amplicon sequencing. Only limited studies assessed the bacterial succession of meat in high-oxygen MAP and the conclusions were not consistent. Ercolini et al. (2011) reported that *Brochothrix thermosphacta* and *Pseudomonas* dominated the communities in 60% O_2/40% CO_2 MAP beef during storage. By contrast, Jääskeläinen et al. (2016) found that *Leuconostoc* were the most prevalent species in 80% O_2/20% CO_2 MAP beef. With respect to anaerobic packaging, Wang et al. (2016) explored the bacterial diversity of lamb in vacuum packs and MAP with different CO_2/N_2 ratios, and found that *Lactococcus* and *Carnobacterium* outcompeted at the end of 28-day storage, whereas the microbial community dynamics in CO-MAP are less well researched.

In this work, the microbial community dynamics from CO-MAP and 80%O_2-MAP beef steaks were investigated to study the effects of different atmospheres on the bacterial community composition and structure. Additionally, the functional analysis of metagenomes was combined with the physicochemical changes of beef to understand the underlying mechanisms for meat microbiological deterioration under MAP preservation.

2. Materials and methods

2.1. Sample preparation

The M. *longissimus lumborum* were collected from four Chinese *Luxi* yellow cattle (24–26 months old, 293–337 kg carcass weights) in a commercial abattoir. Carcasses were conventionally chilled in a chilling room at 2 °C for 48 h. Both the left and right loins were obtained and vacuum packaged, and then transported to the laboratory on ice within 2 h. In the laboratory, muscles were cut into 2.54 cm thick steaks.

2.2. Packaging and storage

In the experiment, two steaks from both loins of each animal were randomly packaged in 80%O_2-MAP (80% O_2/20% CO_2), or CO-MAP (0.4% CO/30% CO_2/69.6% N_2) at 4 storage times (5, 10, 15, and 20 days). Two steaks from each animal were analyzed prior to packaging for initial data at day 0. A total of eight steaks was chosen for each treatment at each storage time interval. Steaks were individually placed in polypropylene trays (oxygen transmission rate: 10 cm^3/m^2/24 h at 23 °C/0% relative humidity, water vapor transmission rate: 15 g/m^2/24 h at 38 °C/90% relative humidity; TQBC-0775, Sealed Air Corp., Danbury, USA) containing Dri-Loc® soak pads (DLS-25, Sealed Air Corp., Danbury, USA). Trays were then flushed with the desired gas mixture and sealed with oxygen-barrier film (oxygen transmission rate: 25 cm^3/m^2/24 h at 23 °C/0% relative humidity, water vapor transmission rate: 10 g/m^2/24 h at 4 °C/100% relative humidity; Lid 1050, Sealed Air Corp., Danbury, USA) using a DT-6D packaging machine (Dajiang Machinery Equipment Co., Ltd., Wenzhou, China). Carbon monoxide MAP was flushed with a certified gas blend of 0.4% CO, 30% CO_2 and 69.6% N_2 (Xieli Special Gas Co., Ltd., Jining, China). The gas headspace to meat ratio was 3:1. All packages were placed in a walk-in cooler at 2 ± 1 °C for 20 days in the dark. Steaks were removed from packages at each analysis day for microbial and physicochemical evaluation.

2.3. Gas composition

A gas analyzer (CheckPoint O_2/CO_2, PBI-Dansensor A/S, Ringsted, Denmark) was applied to verify gas composition (%O_2 and %CO_2) inside packages on each sampling day. The instrument needle was inserted through a rubber septum attached to the covered material.

2.4. Physicochemical analysis

pH values of steaks were measured using a portable pH meter (SenvenGo, Mettler-Toledo, Switzerland). The pH electrode was calibrated with buffers of pH 4.00/7.00 and inserted into the center of steaks. Each steak was determined in triplicate for further statistical analysis.

The surface color of beef steaks was measured on each analysis day (after blooming for 30 min at 2 °C on day 0) by using a X-Rite spectrophotometer (Model SP62, 8 mm diameter aperture, Illuminant A, 10° observer, X-Rite, Inc., Grand Rapids, USA) to determine the CIE lightness (L^*), redness (a^*) and yellowness (b^*). Chroma was also calculated using the following equation: $(a^{*2} + b^{*2})^{1/2}$. Hue was obtained from *arc* tan (b^*/a^*). In addition, the instrument recorded reflectance values in the range of 400 nm to 700 nm at 10-nm intervals. The ratio of reflectance at 630 nm and 580 nm (R630/580) was calculated. At least three scans were taken per steak immediately after opening packages.

2.5. Plate counts

Bacterial colony counts were measured as described earlier (Yang et al., 2016). Meat samples (10 g) were aseptically removed as a thin slice of tissue from the top surface of each steak after spectral scans were collected (the contact face of aperture was disinfected by alcohol wipes), chopped, and transferred aseptically into stomacher bags containing 90 ml of sterile peptone-saline water (0.1% peptone, 0.85% NaCl). Samples were mixed in a stomacher for 2 min. A 10-fold dilution series was carried out for microbiological analysis. For total viable counts, diluted samples were cultured in Plate Count Agar (PCA); Cephalothin-Sodium Fusidate-Cetrimide (CFC) Agar with CFC selective supplement was used to determine *Pseudomonas* spp. counts; Streptomycin Thallous Acetate Agar (STAA) with STAA selective supplement was used for *Brochothrix* spp. counts; and lactic acid bacterial numbers were determined on De Man, Rogosa, Sharpe Agar (MRS). *Enterobacteriaceae* numbers were determined on Violet Red Bile Glucose Agar (VRBGA). CFC and STAA plates were incubated at 25 °C for 48 h, respectively. PCA and MRS plates were incubated for 48 h, and VRBGA plates for 24 h at 37 °C. Results were expressed as log CFU/g sample.

2.6. DNA extraction, PCR reaction, and sequencing

10 g of meat sample was blended in 90 ml of sterile peptone-saline water (0.1% peptone, 0.85% NaCl) for 2 min in a stomacher. Subsequently an aliquot of 30 ml of the solution was centrifuged at ×800 g and 4 °C for 5 min. Next, 15 ml of the supernatant was collected and centrifuged at ×12,000 g and 4 °C for 5 min. The pellet containing the DNA was suspended in 1.5 ml of 0.15 M NaCl and centrifuged at ×12,000 g and 4 °C for 5 min. DNA was extracted from the pellet obtained using a GenElute™ Bacterial DNA Kit (Sigma Aldrich, St. Louis, MO). DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

PCR amplifications of the V4 region of bacterial 16S rRNA gene were implemented with the primer pairs 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). PCR was performed using Phusion® High-Fidelity PCR MasterMix (New England Biolabs, Ipswich, MA, USA) with the following conditions: 98 °C for 3 min; followed by 30 cycles of 98 °C for 45 s/55 °C for 45 s/72 °C for 45 s, and a final extension at 72 °C for 7 min. The PCR products were detected by 2% agarose gel electrophoresis and purified with the QIAquick PCR Purification Kit (QIAGEN, Germany).

Sequencing was conducted on an Illumina MiSeq PE250 platform. The raw data of each sample were merged using Fast Length Adjustment of SHort reads (FLASH) to obtain raw tags (Magoc & Salzberg, 2011). Then, the raw tags were screened using the Quantitative Insights Into Microbial Ecology (QIIME) to obtain clean tags (Caporaso et al., 2010). After chimeric sequences were identified and